Lentiviral Vectors for Treatment of Haemophilia

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Declaration

I, Natalie Ward, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Haemophilia A and B are X-linked recessive disorders caused by defects in coagulation factors (F) VIII and IX, respectively. Severe cases of haemophilia are characterised by episodes of spontaneous bleeding, predominantly into the joints and muscles, and can result in permanent disability and even mortality if left untreated. The haemophilias are compelling candidates for treatment with gene therapy as therapeutic benefit only requires a modest increase in the endogenous coagulation factor level, response to treatment can be easily monitored, and factor expression can be mediated by many cell types *in vivo*. Integration deficient lentiviral vectors (IDLVs) offer marked advantages over currently used integrating lentiviral vectors (ILVs) as side effects caused by insertional mutagenesis are potentially minimised. Previous work has shown that efficient and sustained transgene expression in non-dividing cells, such as brain and muscle tissue, using IDLVs can be achieved. ILVs have previously been used to mediate long term expression of coagulation factors *in vivo*. In this study, we investigated the use of IDLVs as treatment for haemophilia with muscle and liver tissue (primarily non-dividing hepatocytes) as principal targets. Transduction efficiency and relative transgene expression *in vivo* from ILVs and IDLVs were assessed in both tissues, and a number of strategies, including pseudotyping and tissue specific promoters, were utilised to improve targeted expression. Overall, despite achieving sustained transgene expression from IDLVs, in comparison to ILVs, the levels obtained were significantly lower and IDLVs were unable to mediate expression of human FIX at therapeutic levels in liver. Finally, the expression of bioengineered forms of human factor VIII (hFVIII) was assessed using ILVs *in vivo* after neonatal delivery. Long-term expression was achieved and a 20-fold increase in expression was observed after codon optimisation of the hFVIII cDNA sequence. In conclusion, IDLVs can mediate sustained transgene expression *in vivo,* however, vectors may need to be further optimised for increased expression to achieve clinical benefit for haemophilia patients.

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Abbreviations

α	Alpha
AAV	Adeno-associated virus
ADA	Adenosine deaminase
APC	Antigen presenting cell
β	Beta
bp	Base pair
BSA	Bovine serum albumin
CA	Capsid
CAI	Codon adaptation index
CCCD	Cooled charged-coupled device camera
cDNA	Complimentary DNA
CMV	Cytomegalovirus
CpG	Cytosine and guanine separated by a phosphate
cPPT	Central polypurine tract
Ci	Curie
CO_2	Carbon dioxide
cpm	Counts per minute
dH_20	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation Factor 1α
EFS	Short form of elongation factor 1α promoter
eGFP	Enhanced green fluorescent protein
EIAV	Equine Infectious Anaemia Virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay

Env	Envelope
FACs	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FIV	Feline Immunodeficiency Virus
FIX	Coagulation Factor IX
FVIII	Coagulation Factor VIII
γ	Gamma
γc	Common cytokine receptor gamma chain
Gag	Group specific antigens
GALV	Gibbon ape leukaemia virus
GFP	Green fluorescent protein
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human Immunodeficiency Virus-1
HSC	Haematopoietic stem cell
HSV	Herpes simplex virus
IDLV	Integration deficient lentiviral vector
Ig	Immunoglobulin
ILV	Integrating lentiviral vector
IN	Integrase
kb	Kilobases
kDa	Kilo Dalton
LB	Luria-Bertani
LCL	Lymphoblastoid cell line
LMO2	LIM domain only 2
LTR	Long terminal repeat
M	Molar
m	milli (10^{-3})
µ	micro (10^{-6})
MA	Matrix
MFI	Mean fluorescence intensity

MHC	Major histocompatibility complex
MIU	Molecular Immunology Unit
MLV	Murine leukaemia virus
MLV-A	Amphotropic 4070A Murine Leukaemia Virus
MLV-E	Ecotropic Moloney Murine Leukaemia Virus
MOI	Multiplicity of infection
n	nano
NC	Nucleocapsid
OD	Optical density
Ψ	Packaging signal
$p24^{Gag}$	HIV-1 p24 gag capsid protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PR	Protease
qPCR	Quantitative Real Time PCR
RCR	Replication competent retrovirus
RD114	Envelope of the endogenous feline type C virus
RRE	Rev-response element
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RSV	Rous sarcoma virus
RT	Reverse Transcriptase
SCID	Severe combined immunodeficiency
SCID-X1	X-linked severe combined immunodeficiency
SIN	Self-inactivating
SFFV	Spleen focus forming virus
SU	Surface glycoprotein
TAE	Tris-acetate-EDTA
T cell	Thymus derived lymphocyte

TCR	T cell receptor
TM	Transmembrane glycoprotein
VSVg	Vesicular stomatitis virus glycoprotein
v/v	Volume per volume
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
w/v	weight per volume
w/w	weight per weight

Chapter One

Introduction

1.0 Gene Therapy

Gene therapy is a form of molecular medicine broadly considered as the repair, addition, or replacement of a defective gene responsible for disease. During the last four decades the potential for treating patients using gene therapy has been realised in an exponential fashion and has moved from preclinical studies for monogenic diseases, such as haemophilia, to more complex diseases such as cancer, cardiovascular disease, and HIV [14-16].

1.1 Overview and History of Gene Therapy

The first viral system for gene transfer was developed in the early 1980's by the Verma group. They successfully transferred the human gene for hypoxanthine phoribosyltransferase (HPRT) into *hprt* deficient mouse and human cells using a retrovirus [17-19]. This was followed in 1985 by the first *in vivo* expression of a transgene in an animal model: Here, the retroviral expression vector N2 was used to transfer the gene coding for resistance to neomycin (NeoR) into hematopoietic progenitor cells taken from the bone marrow of immune competent mice. Transduced cells were then engrafted into lethally irradiated mice and vector DNA able to be detected 10 days post injection [20]. In 1989 Rosenberg and colleagues initiated the first human gene transfer clinical trial to treat patients with advanced metastatic melanoma. Human tumour infiltrating lymphocytes (TIL) were modified by retroviral-mediated gene transfer to introduce NeoR. These cells were then infused into patients and the tracking of modified cells was made possible using the neomycin gene as a marker; the study therefore demonstrating the successful introduction of a gene by retroviral transduction [21]. The Anderson group also started the first approved gene therapy clinical trial in 1990 treating two children with adenosine deaminase-deficiency (ADA)-severe combined immunodeficiency (SCID) with their own T-cells engineered *ex vivo* with a retroviral vector carrying the normal adenosine deaminase gene. Treatment was not optimal due to only transient transgene expression, but the study demonstrated

that gene therapy could be an additional treatment for some patients with this disease [22].

During the 1990's, however, many gene therapy trials had only limited success [23]; major obstacles included inadequate gene delivery systems, poor transgene expression, and induction of immune response to both vectors and modified cells [24]. A major setback in the field was the death of an 18 year old patient Jesse Gelsinger in 1999 who a suffered massive immunological response after systemic delivery of an adenoviral gene transfer vector whilst enrolled in a pilot gene therapy safety study for ornithine transcarbamylase deficiency [25]. Success of gene therapy has also been tempered by toxic effects seen in patients treated for X-linked SCID, a disease caused by mutations in the gene encoding the common γ chain (γc). Worldwide, 20 patients have been treated for this disease using a gammaretroviral vector to introduce the corrected γc gene *ex vivo* into patient derived $CD34^+$ bone marrow stem cells. Increase in T- and B-cell population number and improvement of immune function has been observed for nearly all patients treated. However, despite this, five patients developed acute lymphoblastic leukemia almost three years after treatment, causing the death of one, as the retrovirus carrying the γc gene inserted itself near, and caused aberrant expression of, proto-oncogenes including *LMO2* [26-28]. As aberrant cell populations were a direct result of therapeutic intervention, the ability to prevent adverse effects will depend on the availability of vectors with improved safety, a thorough assessment of the oncogenic capacity of genes which are incorporated into them, and the findings of additional studies in appropriate models.

More recently however, early hype, failures, and tragic events in gene therapy have now largely been replaced by necessary stepwise progress to realise clinical benefits. Since 1989 more than 1400 gene therapy clinical trials have been completed in 28 countries worldwide utilising over 36 types of vectors. Over 65% of trials to date have been for the treatment of cancer, reflecting the critical need for novel therapies in this field (Journal of Gene Medicine clinical trials database; http://www.wiley.co.uk/genetherapy/clinical/).

Research for treatment of monogenic disease remains foremost. When considering gene therapy for monogenic disorders, parameters for treatment include whether the gene mutation leads to a loss or gain of function, whether the gene product's function affects survival of the cell or development, and if the disease gene has cellular specificity [29]. There are four different gene therapy strategies for monogenic diseases:

(1) Addition of a normal copy of the mutated gene; best suited to loss-of function mutations and the focus of most gene therapy strategies so far [30].
(2) Modification of messenger RNA to avoid the consequences of mutation; a possible option when the mutated exon is not indispensable. mRNA modifications can be brought about by small nuclear RNAs (snRNAs) [31].
(3) Inhibition of expression of the mutated gene; used to prevent the expression of a protein in a gain-of-function mutation or to inhibit cryptic splice sites which lead to expression of an abnormally spliced protein. Small interfering RNAs (siRNAs) have been used in this approach [32].
(4) Repair of the mutated gene in the host DNA sequence and reversal of the disease. This strategy utilises a DNA-sequence-specific binding domain and an endonuclease capable of inducing site-specific double-strand breaks in DNA. Simultaneously, a template encompassing the wild-type sequence that corresponds to the mutated stretch of DNA is introduced into the cell and acts as a substrate for repair. Zinc finger nucleases are a promising tool for this strategy, however, many technical concerns and low efficiency keep them far from clinical application at present [33].

In order to achieve clinical benefit two approaches for delivering genetic material exist; *in vivo* and *ex vivo*. The *in vivo* strategy involves the direct delivery of DNA to resident cells of the target tissue, whereas *ex vivo* methods involve the transfer of a therapeutic gene to cells *in vitro* followed by transplantation of these modified cells to the target tissue (Figure 1.1). Ideally for an *in vivo* gene therapy strategy: (i) the target cells should be easily accessible for infusion or injection by a vector; (ii) the vector should be able to readily and specifically infect the target cells; (iii) expression of the transgene of

interest should occur in the target cells only at effective levels for the specific time period necessary [2]. One of the most difficult challenges in gene therapy is the development of vectors and delivery systems that are safe, efficient and targeted. Success requires understanding of the strengths and weaknesses of different gene transfer systems and the ability to choose a vector appropriate for each individual disease [34].

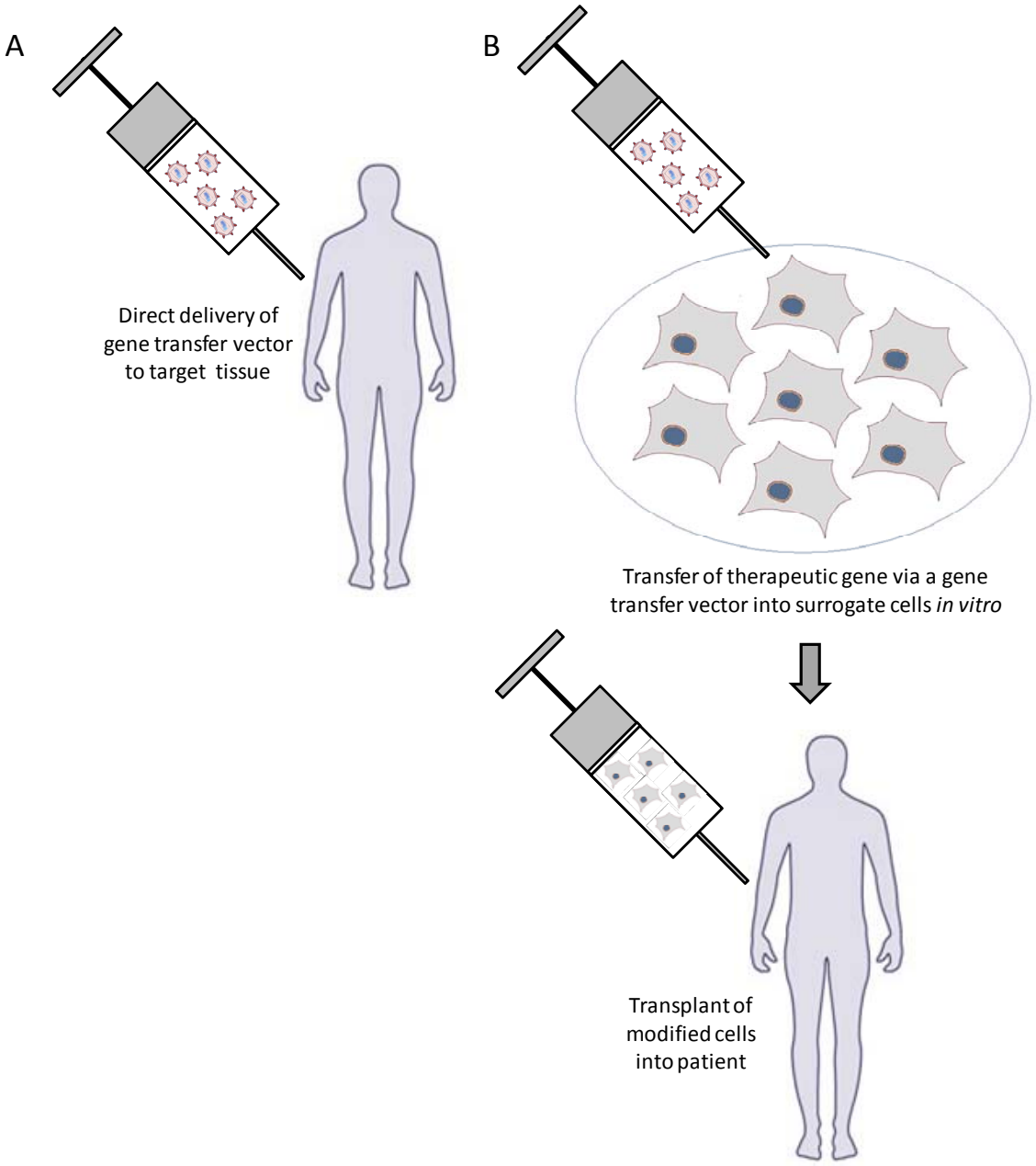


Figure 1.1 Schematic of *in vivo* and *ex vivo* gene therapy strategies. (A) *In vivo* gene transfer. The *in vivo* strategy is based on directly injecting or infusing a gene transfer vector to cells of the target tissue. The therapeutic gene will then be expressed in target cells. **(B)** *Ex vivo* gene transfer. The *ex vivo* strategy is based on transfer of the therapeutic gene to selected surrogate cells *in vitro* and subsequent transfer of the cells into the target tissue to express the gene. Figure modified from (Selkirk 2004) [2].

1.2 Viral Vectors for Gene Therapy

Many different types of vector, both viral and non-viral, have been explored as a means of transporting corrective DNA into target cells [35,36]. However, it is viral vectors which currently represent the most effective means of gene delivery and have been used in the majority of gene therapy clinical trials (over 70%), with adenoviral and retroviral vectors used in 24% and 21% of all clinical trials to date, respectively (Journal of Gene Medicine clinical trials database http://www.wiley.co.uk/genetherapy/clinical/). The choice of which viral vector to use for a particular application depends mainly on the cell type to be targeted and duration of expression required [16]. However, desirable properties of all viral vectors are the ability to be reproducibly propagated, purified to high titres, and to mediate transgene delivery and expression to the target cell without substantial toxicity [37]. Many viruses having different properties have been exploited to produce gene therapy vectors; some of the most commonly used are derived from gammaretroviruses, lentiviruses, adenoviruses, adeno-associated viruses (AAVs) and herpes simplex viruses (HSVs) (Table 1. 1).

	Genetic Material	Packaging Capacity	Vector Yield (TU/mL)	Tropism re-targeting	Duration of Transgene Expression	Vector Genome Forms	Genotoxicity	Inflammatory Potential	Transduction of quiescent cells
Gammaretrovirus	RNA	~7kb	1×10^{10}	Broad	Long-term (years)	Integrated	Integration may induce oncogenesis	Low	No
Lentivirus	RNA	~8kb	1×10^{10}	Broad	Long-term (years)	Integrated	Integration may induce oncogenesis	Low	Yes
Integration Deficient Lentivirus	RNA	~8kb	1×10^{10}	Broad	Transient	Episomal	Low	Low	Yes
Herpes Simplex Virus	dsDNA	>30kb	1×10^{12}	Broad (strong for neurons)	Transient	Episomal	Low	High	Yes
Adenovirus	ssDNA	~30kb	1×10^{12}	Broad	Short-term (weeks)	Episomal	Low	High	Yes
Adeno Associated Virus	ssDNA	~5kb	1×10^{12}	Broad except for HSCs	Medium to long-term (year)	Episomal (90%) Integrated (10%)	Needs further investigation	Low	Yes

Table 1.1 The properties of commonly used viral gene transfer vectors. Table adapted from (Bouard *et al.,* 2009) [1].

TU; Transducing Units.

1.3 Retroviruses

Retroviruses are a large group of enveloped RNA viruses characterised by their diploid, single stranded RNA genomes, reverse transcription of the RNA genome into a double stranded DNA intermediate (the provirus), and integration of the provirus into the host cell chromatin. Virions are on average approximately 100nm in size and contain an RNA genome of 7-12kb in size. The hallmark of retroviruses is their replicative strategy which involves reverse transcription of the viral RNA into linear DNA and the subsequent integration of this DNA into the host cell genome. Within the family *Retroviridae* there are seven genera; *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, and *Epsilonretrovirus* (containing simple retroviruses with oncogenic potential), and *Lentivirus* and *Spumavirus* (containing complex retroviruses). Each genus is characterised by differences in genetic structure, genome complexity, site of particle assembly, and virion morphology. For the development of vectors for gene therapy the focus has mainly been on utilising *Gammaretroviruses*, *Lentiviruses*, and *Spumaviruses* [38]. The retroviral vector used in this study was an HIV-1-based lentiviral vector, so the following summary is focused principally on this virus. However, where important differences exist between lentiviruses and other retroviruses, these are described.

1.3.1 Retrovirus Biology

During the course of their life cycle, retroviruses alternate between two principal forms: the provirus and the virion. The provirus consists of double-stranded DNA integrated into a host cell chromosome. Viral RNA and proteins are then expressed from the provirus using the host transcription and translation apparatus, and are subsequently packaged at the host plasma membrane into a virion. After reverse transcription the integrated proviral genome comprises both coding genes and non-coding *cis* acting gene regulatory sequences.

1.3.1.1 Coding Sequences

Retroviruses are broadly divided into two categories; simple and complex. The basic retroviral genome contains four genes: *gag, pro, pol,* and *env* which encode proteins found in all retroviruses [39]. Simple retroviruses contain only these four basic genes, while complex retroviruses possess additional regulatory and accessory genes (Figure 1.2). The *gag, pro, pol* and *env* genes are expressed as polyproteins which are processed into mature forms during assembly of the virion and maturation. The major structural protein of the virion is encoded by *gag* and is cleaved during maturation to form the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. The *pol* gene codes for the viral enzymes reverse transcriptase (RT) and integrase (IN), essential for reverse transcription of the viral RNA genome to double stranded DNA and integration of the DNA genome into a host cell chromosome, respectively. *Env* codes for the surface (SU) and transmembrane (TM) components of the viral envelope protein which are targeted to the virion lipid bilayer. Finally, *pro* encodes the viral protease (PR) which acts to process the viral polypeptides formed by *gag, pro, pol,* and in some strains *env* [35]. HIV-1 also contains genes coding for the regulatory proteins Tat (trans-activator of transcription) and Rev (regulator of virion) and the accessory proteins Vif, Vpr, Vpu, and Nef which can act to protect the virus from host restriction factors [40].

1.3.1.2 Non-Coding Sequences

The provirus is flanked by long terminal repeats (LTRs), subdivided into U3, R, and U5 regions [41]. U3 (unique in 3') is approximately 450 nucleotides long in both murine leukaemia virus (MLV) and HIV-1 and acts as a promoter of viral transcripts. The R (repeat) region is approximately 100 nucleotides long in HIV-1 and contains a polyadenylation signal, typically AAUAAA, which allows efficient processing of viral RNA. Homology between the two R regions is also essential for reverse transcription of the genome. U5 (unique in 5') consists of a GU-rich stretch of roughly 80 nucleotides which enhances the recognition and processing of the polyadenylation signal contained within R [42]. Other *cis* acting regulatory sequences sites include the primer binding site

(PBS). This site is complementary to an 18bp region of a host-derived tRNA which unwinds and binds to this region to act as a primer for minus strand DNA synthesis during reverse transcription [43,44]. The region downstream of the primer binding site forms the encapsidation signal (Ψ) which allows efficient, preferential encapsidation of viral genomic RNA into virions. It is removed during splicing, so only full length (unspliced) transcripts can be packaged into virions [45]. The polypurine tract (PPT), just 16 nucleotides long in HIV-1, is located immediately upstream of the 3' U3 and acts as a primer for plus strand DNA synthesis during reverse transcription [46,47]. Lentiviruses such as HIV-1 also possess a second origin for plus strand DNA synthesis called the central polypurine tract (cPPT) located within the integrase component of the *pol* [48].

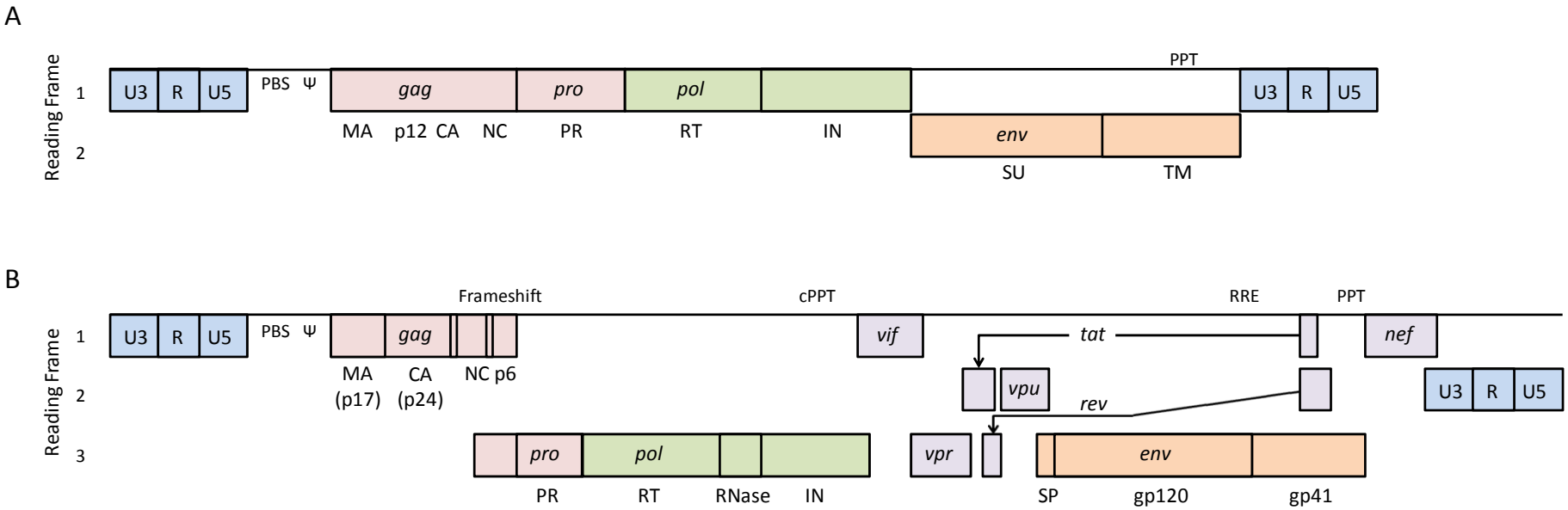


Figure 1.2 Proviral genome organisation of (A) a simple wildtype murine leukaemia virus and (B) a complex wildtype human immunodeficiency virus type I genome. Both genomes contain the basic structural genes: *gag* which encodes matrix (MA), capsid (CA) and nucleocapsid (NC) proteins, *env* which encodes surface (SU) and transmembrane (TM) glycoproteins, *pol* and *pro* which encode the enzymes protease (PR), reverse transcriptase (RT), integrase (IN) and RNase. HIV-1 also contains the regulatory genes *tat* and *rev*, and the accessory genes *vif, vpr, vpu* and *nef*; Primer binding site (PBS); encapsidation signal (Ψ); Polypurine tract (PPT); Central Polypurine tract (cPPT); Rev response element (RRE), spacer peptide (p6). Figure modified from (Coffin *et al.,* 2007), (Watts *et al.,* 2009) and (Sinn *et al.,* 2005) [10-13].

1.3.2 Retrovirus Structure

In mature virions of retroviruses the matrix (MA) protein is bound to the envelope obtained from the host plasma membrane during the budding process. The envelope contains the viral glycoprotein where the surface (SU) receptor protrudes from the virion and is anchored by the transmembrane (TM) region which spans the lipid bilayer [49]. A dense virion core is contained inside the matrix and can be cone shaped (as for HIV-1) or round (as for MLV). Around 2000-4000 copies of capsid (CA) protein form the outer shell of the core inside which is a nucleocapsid (NC)-RNA complex. Viral proteins including reverse transcriptase (RT) and integrase (IN), and some host-derived proteins including the host tRNA used during minus strand DNA synthesis are packaged inside the virion [50] (Figure 1.3).

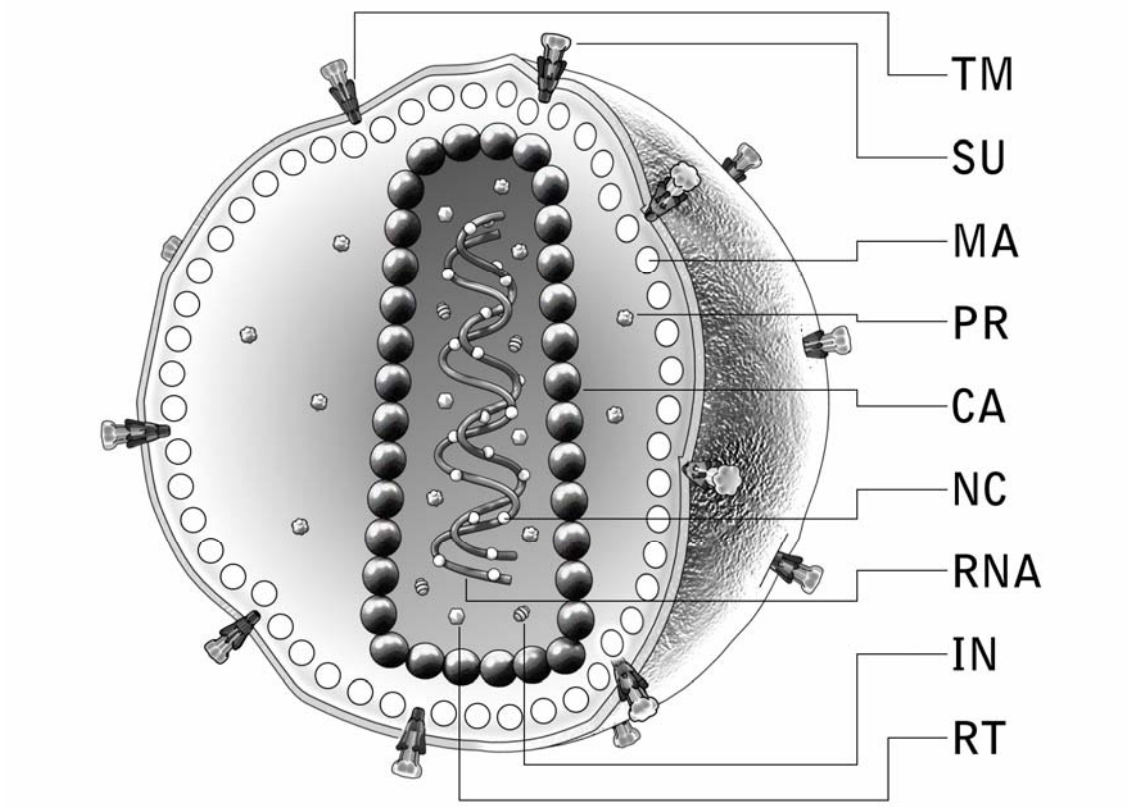


Figure 1.3 Schematic of a mature HIV-1 virion. Viral proteins depicted in this picture: transmembrane glycoprotein (TM); surface glycoprotein (SU); matrix (MA); protease (PR); capsid (CA); nucleocapsid (NC); single stranded RNA genome (RNA); integrase (IN); reverse transcriptase (RT).

1.3.3 Lentiviral Life Cycle

The HIV-1 viral life cycle can be divided into two temporarily distinct phases: infection and replication. Infection results in the introduction of the viral genome into the cell. Lentiviruses differ from other retroviruses due to their long latent period during infection. This leads to an early phase of gene expression characterised by the appearance of viral regulatory products involved in replication, followed by a late phase when structural genes are expressed and assembly of new viral particles occurs [35]. A schematic view of the lentiviral life cycle is shown in Figure 1.4.

Entry and Uncoating

The first stage of the viral life cycle is entry of the virion into a target cell. This is mediated by recognition of a host cell surface receptor by Env; the major determinant of viral tropism. The primary receptor for HIV-1 is CD4 [51], along with coreceptors CCR5 [52-56], and CXCR4 [57]. SU and CD4 binding induces a conformational change in SU revealing a conserved, high affinity, coreceptor binding domain. Coreceptor binding leads to exposure of the TM fusion peptide, a hydrophobic region which interacts with the cell membrane, allowing fusion of the cell and viral membranes and internalisation of capsid [58]. Once inside the cell, the protein composition of the HIV-1 core changes in a process known as uncoating, firstly producing a complex called the reverse transcription complex (RTC) in which DNA synthesis occurs, and then a preintegration complex (PIC) which is transported to the nucleus for integration into the host genome.

Reverse Transcription

During uncoating, the capsid protein dissociates and the core forms the RTC which contain the proteins RT, IN, NC, phosphorylated MA, and Vpr [59,60]. Reverse transcription of the viral RNA takes place prior to nuclear entry mediated by RT and is one of the defining features of retroviruses. It occurs in a series of steps:

1. A specific cellular tRNA ($tRNA^{lys3}$) acts as a primer and hybridizes to the complementary primer binding site (PBS) on the viral RNA initiating DNA synthesis.
2. Synthesis proceeds to the 5' end of the genome so the U5 and R regions are encoded on this short (-)ssDNA.
3. The synthesis results in an RNA:DNA duplex and the RNA template strand is degraded by the RNase H activity of RT. The (-)ssDNA is then transferred to the RNA 3' end, a process made possible by the homology between the 5' and 3' R elements, this is known as minus strand transfer.
4. The first strand of complementary DNA is extended and the majority of viral RNA is degraded by RNase H. Once the strand is completed, second strand synthesis is initiated at the PPT.
5. Additionally, other RNA sequences such as the central polypurine tract (cPPT) are not degraded and act as primers for positive strand synthesis. This proceeds from the PPT to the end of the tRNA primer.
6. This creates an area of homology at the PBS (18 nucleotides) between the plus and minus DNA strands which enables plus strand transfer.
7. Synthesis continues from the transferred section of the plus strand but stops at the cPPT, where plus strand DNA initiated at the PPT displaces plus strand DNA initiated at the cPPT. This creates a discontinuous 'DNA flap' of 99 nucleotides between the cPPT and central termination sequence (CTS) thought to aid nuclear entry [48].
8. Following plus strand transfer, DNA synthesis continues in both directions to the ends of the LTRs and can be incorporated into the host genome.

While reverse transcription takes place, the complex migrates to the nucleus. RT then dissociates forming the PIC and translocation occurs through the nuclear pore. Gammaretroviruses are unable to cross the nuclear envelope, therefore they are only able to integrate their genetic material when the envelope breaks down during mitosis. As lentiviruses, and the vectors based upon them, can cross the nuclear envelope they

have the ability to integrate into non-dividing cells this makes them very useful tools in gene therapy.

Integration

The second defining feature of retroviruses is integration of proviral DNA into the host chromosome. This is mediated by the integrase (IN) encoded by *pol.* IN binds to attachment (att) sites in the U3 and U5 LTRs and integration occurs in two catalytic steps. The first step, known as 3'-processing, occurs in the cytoplasm within the pre-integration complex (PIC) and involves cleavage of the 3' terminal dinucleotide (pGpT) at both ends of the viral DNA. The resulting recessed 3'-OH groups provide the sites of viral genome attachment to the host DNA [60]. The second step, strand transfer, occurs in the nucleus. Here, IN mediates a nucleophillic attack via the viral 3'-OH groups against phosphodiester bonds on opposite strands of the host target DNA. During this step, the energy of the broken phosphodiester bonds in the chromosome is used for the formation of new bonds joining the viral 3' ends to the target DNA [61]. In the final step of integration, the unpaired dinucleotides from the 5' ends of the viral DNA are removed and the single-stranded gaps created between the 5' end and the target DNA are filled causing the integrated DNA to be flanked by the same five base pairs [62]. DNA episomes are also generated as by-products of integration. Aside from the linear DNA precursor, two types of circular episome with intact viral coding regions are produced. These molecules can have one LTR - formed by homologous recombination between the two LTRs, or two LTRs – formed by non-homologous end-joining of the double stranded blunt viral DNA [63]. Non-integrated viral DNA can support transcription [64], and it is on this basis that integration defective lentiviral vectors mediate expression [65-68] (see section 1.4.1).

Transcription

Once integrated into the host genome, the provirus relies on cellular machinery for transcription and translation. Transcription of the integrated proviral DNA involves the interaction of of both *cis*-acting regulatory sequences in the LTR and trans-acting proteins made by the cell and the virus. The HIV-1 promoter does not assemble a particularly efficient RNA polymerase complex; the initiation rate is slow and the elongation rate is poor. The transcriptional activator protein Tat binds a stem loop structure (TAR) present at the immediate 5' end of all mRNA transcripts recruiting host cellular elements, such as cyclin T and Cdk9 to the transcriptional complex increasing transcription 100-fold [69]. Rev then binds to unspliced mRNA at a Rev responsive element (RRE) and this complex is then exported from the nucleus into the cytoplasm, where Rev stabilizes viral transcripts [70,71].

Translation

The Gag and Gag-Pro-Pol polyproteins are translated from unspliced mRNA in a ratio of 20:1. The reading frame of Pol is -1 with respect to Gag therefore translation of Gag-Pol-Pro requires a translational frameshift by the ribosome. After translation the Gag and Gag-Pol-Pro polyproteins associate to the cellular membrane where Gag multimerisation occurs and the viral genome is bound by NC through an encapsidation signal (Ψ) which is present only on unspliced mRNA, allowing it to be packaged. The *env* gene is transcribed as one full mRNA product and is then translated to produce a precursor protein to the viral glycoproteins gp120 and gp41. During translation Env is inserted into the rough endoplasmic reticulum membrane through the signal recognition particle (SRP) [49] and subsequently recruited to the virion.

Virion Assembly and Budding

Virions are packaged with a number of host proteins and are then released from the cell surface as immature, non-infectious particles. The Gag and Gag-Pol-Pro polyproteins are then cleaved by protease (PR) to produce the IN and RT as well as the structural proteins MA, CA, and NC. This proteolytic processing is required for condensation of immature spherical Gag to the mature cone-shaped core for the development of HIV-1 infectivity and gives rise to a mature HIV-1 virion [72].

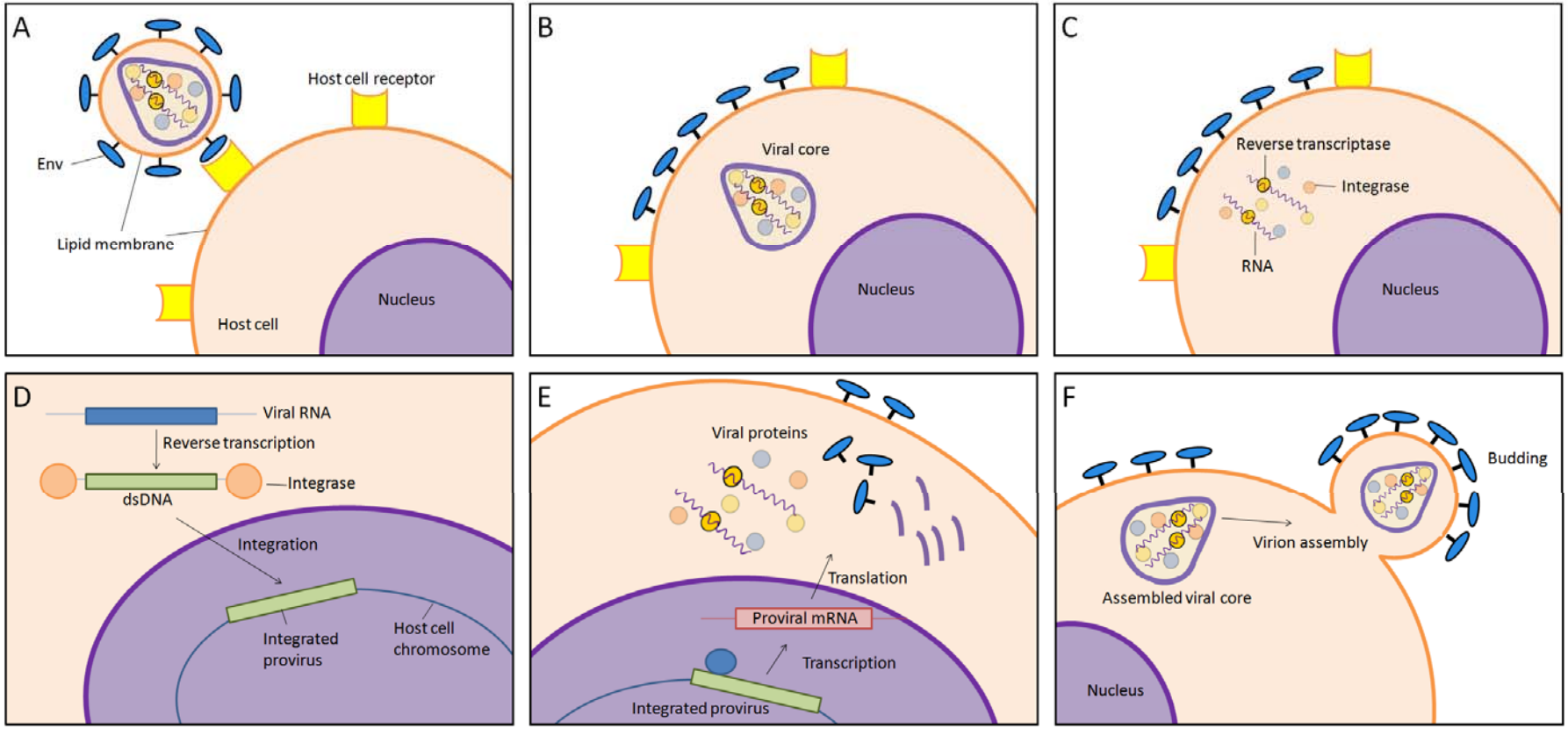


Figure 1.4 A schematic of the lentiviral life cycle showing (A) attachment, (B) entry, (C) uncoating, (D) reverse transcription and integration, (E) transcription and translation (F) assembly and budding. Figure based on animation by

http://www.sumanasinc.com/webcontent/animations/content/hiv.html

1.4 Lentiviral Vectors

Retroviral vectors are derivatives of Retroviruses modified to carry a therapeutic gene for delivery into target cells. Gammaretroviral vectors, especially those based on MLV, have been frequently used in research as well as clinical trials. However, lentiviral vectors, such as those based upon HIV are now more common as they have the added advantage that they are able to integrate into non-proliferating cells [73]. Lentiviral vectors have many other advantageous features; (i) the proteins for reverse transcription and integration are carried within the virion allowing the viral genes that code for them to be deleted from vector constructs, (ii) a large coding capacity of 8-10kb of transgene cassette, in addition to all the required *cis*-acting sequences [74], (iii) reduced *in vivo* immunogenicity in comparison to other gene therapy vectors (Table 1.1) [75], (iv) stable transmission of the transgene to the target cell and subsequent generations through integration of the provirus into the host cell genome.

The first lentiviral vectors to be developed were derived from HIV-1 [76]. Both coding and *cis*-acting sequences are required for lentivector construction, however, these sequences are separated to prevent the generation of replication-competent retroviruses (RCRs). Consequently, the *cis*-acting sequences (LTRs, RRE, splice donor and acceptor, Ψ, PBS, PPT and cPPT) are contained within the transfer vector construct, and *trans*-acting genes (*gag*, *pol*, and env) are located on separate plasmids. The transfer vector consists of modified LTRs, a transgene driven by a suitable promoter, and the packaging signal. The 3' sequence of the U3 element has been modified so that only 18bp remains, this eliminates promoter activity and means that proviruses resulting from this vector cannot transcribe their full genome and are therefore said to be self-inactivating (SIN) [74]. Further features of these vectors include an extended packaging signal [77], the cPPT/CTS (central termination sequence) element which enhances transduction in non-dividing cells [78], and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) which is known to enhance viral titre [79] (Figure 1.5).

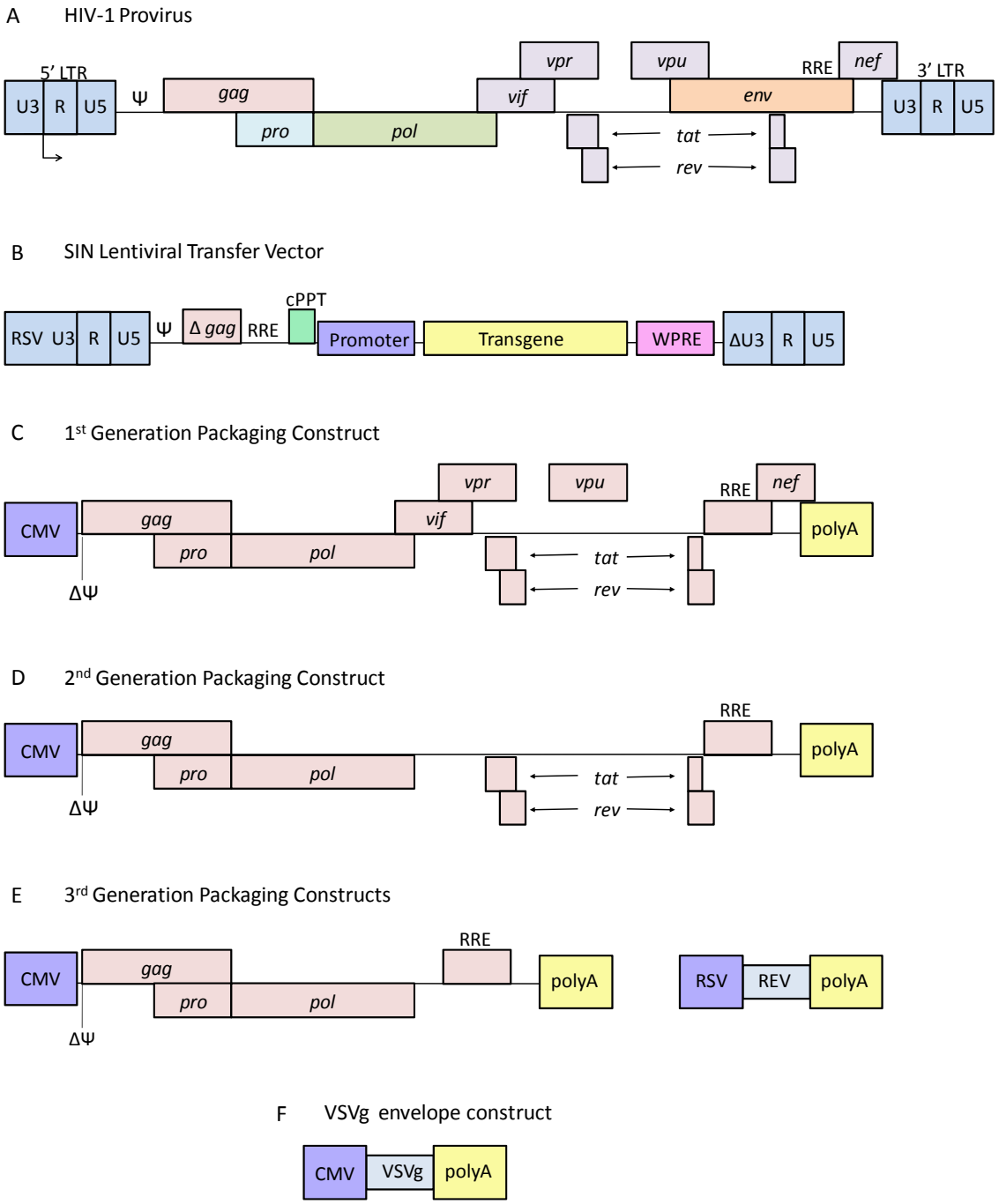


Figure 1.5 Lentiviral vector transfer and packaging constructs. (A) HIV Provirus (B) Self-inactivating (SIN) lentiviral transfer vector; U3 is replaced by the Tat-independent Rous sarcoma virus (RSV) promoter. The vector also contains a central polypurine tract (cPPT) and Woodchuck Hepatitis virus posttranscriptional element (WPRE) to enhance vector potency and transgene expression. (C, D and E) First, second and third generation packaging constructs. (F) Envelope Construct, vesicular stomatitis virus glycoprotein (VSVg) gene driven by the CMV promoter. (Ψ - packaging signal).

The *gag-pol* packaging plasmid contains sequences for the Gag and Gag-Pro-Pol polyproteins required to assemble virions. Initial packaging constructs (1st generation) maintained the HIV accessory genes *vpu, vpr, vif* and *nef*, however, they were found to be dispensable for vector generation, so were deleted in second generation packaging constructs [77]. This deletion also increased the biosafety of vectors, since any RCR generated during vector production will lack the essential factors for HIV-1 virulence *in vivo*. Both first and second generation packaging plasmids contain *tat* to enable transcription in the vector and to produce high vector titres [78], and *rev* to mediate nuclear export. However, in third generation vectors *tat* has been removed as the U3 region in the 5'LTR has been replaced with a Tat independent promoter, and *rev*, which is still required, is supplied on a separate plasmid (Figure 1.5). A final plasmid codes for an envelope glycoprotein (*env*), most commonly the envelope glycoprotein from Vesicular Stomatitis Virus (VSV) is used due to its broad tropism, however, other envelope proteins can be used to confer different tropism [80]. There is now an additional fourth generation system where the *gag-pol* cassette has been split onto two plasmids with a Vpr-Pol fusion to ensure transport of RT/IN to the viral particle and a Tat-IRES-Rev fusion (Clontech, La Jolla, CA).

Upon co-transfection of all plasmids coding for all constructs into HEK-293T cells, these cells produce the vector components and release infectious vector particles into cell culture medium which are capable of only one round of infection [35] (Figure 1.6).

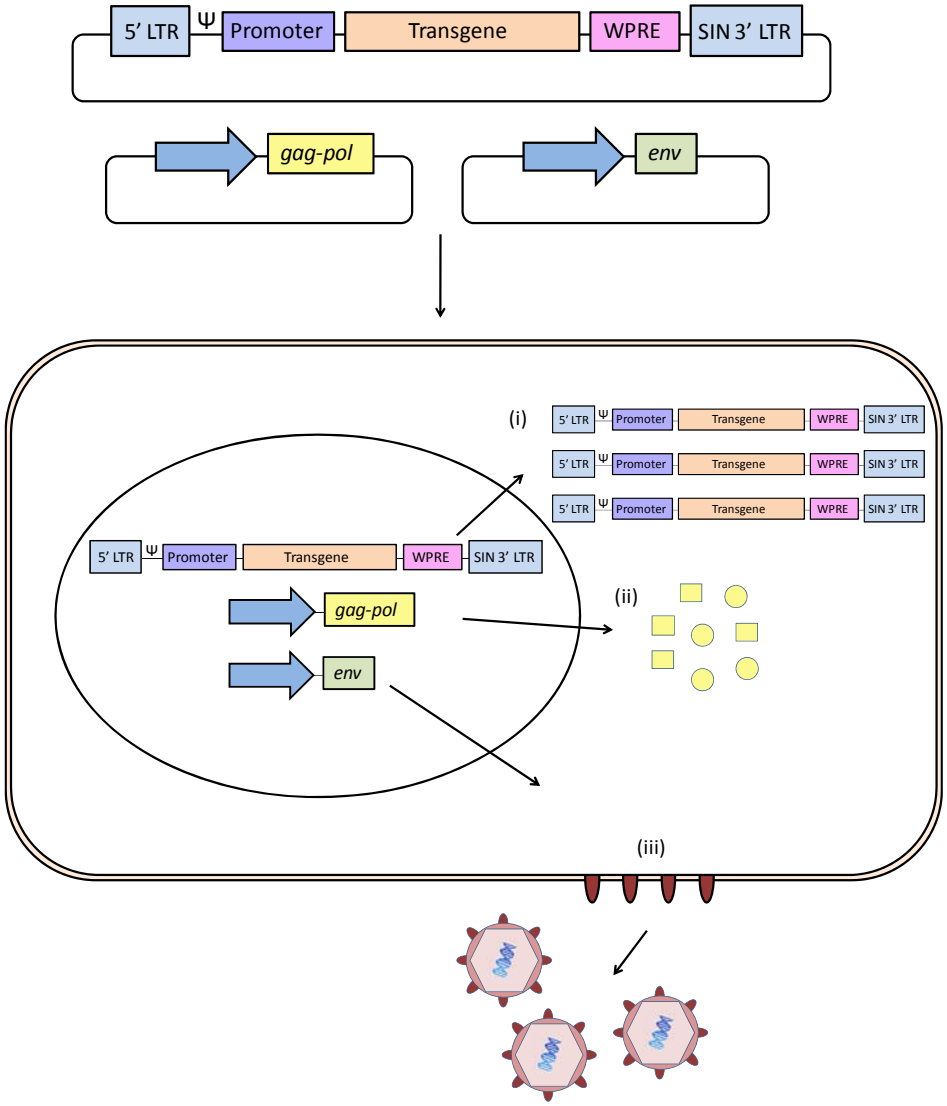


Figure 1.6 Second generation lentiviral vector production. 2nd generation lentiviral vectors are produced by transient transfection of HEK-293T cells with: (i) transfer vector construct containing the transgene driven by an internal promoter; (ii) the *gag-pol* packaging construct; (iii) the *env* envelope plasmid, usually VSVg. The host cell machinery is then utilised to produce the vector.

1.4.1 Integration Deficient Lentiviral Vectors

Lentiviral vectors are efficient at transducing both dividing and quiescent cells and stable integration of the transgene allows long term expression in the target and subsequent daughter cells. However, the recent leukaemia observed in clinical trials for SCID-X1 caused by insertional mutagenesis has highlighted the need for safer vectors. Integration deficient lentiviral vectors (IDLVs) can be produced through the use of integrase mutations that specifically prevent proviral integration, resulting in the generation of increased levels of circular vector episomes in transduced cells. Compared to integrating lentivectors (ILVs), IDLVs have a greatly reduced risk of causing insertional mutagenesis and a lower risk of generating replication competent retroviruses (RCRs).

Although proviral integration is generally described as the end point of lentiviral gene transfer it is also a relatively inefficient process. A significant amount of DNA is processed into episomal forms by lentiviruses and their derived vectors, among them: (i) the linear episome which is the precursor to integrated DNA, and two types of circular episome; (ii) 1-LTR circles are circular episomes with a single LTR, formed by homologous recombination of the LTRs, and also to a much lesser extent by ligation of nicks in circular DNA intermediates during reverse transcription [81] and (iii) 2-LTR circles are circular episomes with two adjacent LTRs, formed by non-homologous end joining of the linear episome [82]. Generally 1-LTR circles are more prevalent than 2-LTR circles, however, ratios can vary [83-85] (Figure 1.7). Both 1- and 2-LTR episomal forms have intact viral coding regions allowing them to function for many applications including transgene expression, recombination and transposition. However, as they lack an origin of replication (ORI) they do not replicate in dividing cells and are diluted out over time by cell division. This can make IDLVs ideal for mediating transient gene expression in proliferating cells. The episomal DNA will, however, persist in non-dividing cells allowing stable transgene expression.

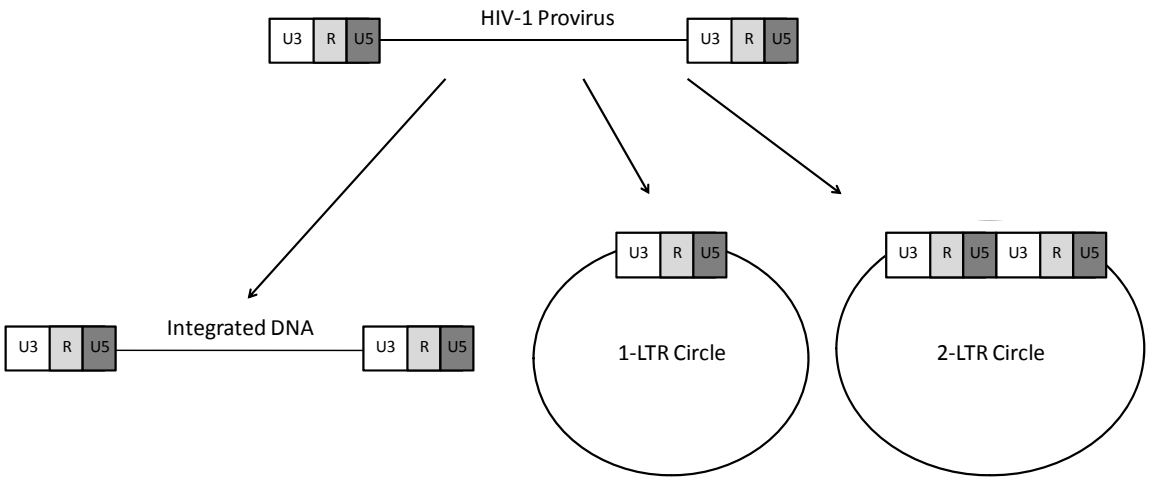


Figure 1.7 Formation of lentiviral vector episomes. The product of reverse transcription is a linear double-stranded DNA (dsDNA) with LTRs at both ends. This DNA is imported into the nucleus as part of the viral preintegration complex. Conventional lentiviral vectors, with a functional IN, can be integrated into the host genome as proviruses. However, the linear DNA can also be circularized by nonhomologous end-joining to produce 2-LTR circles. Homologous recombination between the LTRs in linear DNA or 2-LTR episomes in intermediate products of reverse transcription, lead to 1-LTR circles.

1.4.1.1 IDLV Biology

Given that viral episomes can be used for various applications, preventing integration to favour the formation of 1- and 2-LTR circles can be a desirable goal [37]. There are several ways to do this, the most efficient being to create a mutated virus strain with impaired integration. Viral integrase is pleiotropic, being involved in virion morphogenesis, reverse transcription, PIC nuclear translocation, and integration; therefore it cannot be deleted entirely to produce a non-integrating vector. However, the introduction of specific point mutations is sufficient to disrupt its normal function (Figure 1.8). Mutations are broadly divided into two classes: Class I mutations result in the vector being defective specifically for integration, whereas class II mutations cause additional defects in assembly or reverse transcription resulting in a lack of integration. Class II mutations, such as changing the conserved histidine and cysteine residues in the integrase N-terminal domain, also result in a lack of vector transgene expression [86],

and are therefore inappropriate for IDLV development. Class I mutations normally include substituting any one of the three amino acids of the catalytic triad (D64, D116 and E152) in the catalytic core of IN.

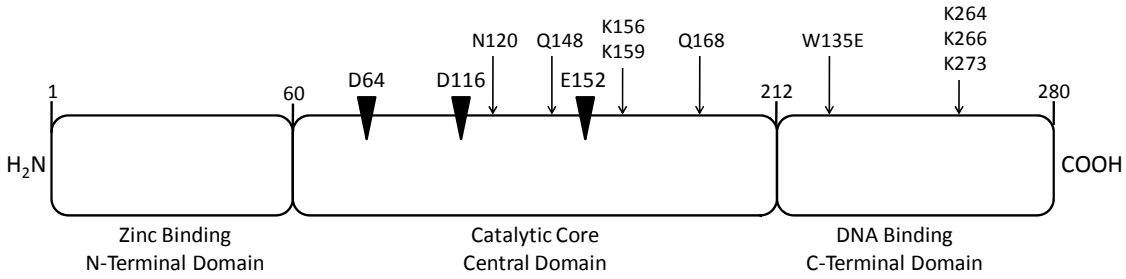


Figure 1.8 The structure of HIV-1 integrase. The HIV-1 integrase protein showing the three functional domains; N-terminal zinc-binding domain, the central catalytic core, and the C-terminal DNA binding domain. The DDE catalytic triad is shown with bold arrows. Smaller arrows denote amino acids that give rise to class I integrase mutants. Figure modified from (Philpott and Thrasher 2007) [3].

Mutating or deleting both the U3 and U5 attachment (*att*) sites on the viral genome involved in recognition by IN has also been shown to impede provirus formation. However, these mutations are less effective in comparison to IN mutations, and do not lead to further reduction of integration frequencies when used in conjunction with class I IN mutations in IDLVs [67,87]. It is also possible to impair integrase function using specific inhibitors of strand-transfer, such as raltegravir [88] and elvitegravir [89]. Also, compounds which interfere with the interaction of IN and cellular cofactors (such as LEDGF/p75, integrase interactor-1 and gemin2) are being explored as targets for intervention [90].

IDLVs have been efficiently produced with first, second and third generation packaging plasmids with the most commonly used integration specific mutation D64V in the *gag-pol* packaging plasmid. IDLVs are defined as integration deficient; however, in reality IDLVs have some residual integration. Studies carried out with human colon adenocarcinoma (HT-29), human fibrosarcoma (HT1080), and human cervical cancer (HeLa) cell lines *in vitro* comparing SIN HIV-1 based vectors have estimated that residual integration is around 3 to 4-logs lower for the IDLV D64V mutant in comparison to wild type vector [65,67,87]. However, results of residual integration *in vivo* are limited to studies carried out by Yanez-Munoz and colleagues in 2006 [66]. Here, linear amplification mediated (LAM)-PCR based strategies on genomic DNA taken from eyecups of rats and mice injected subretinally with D64V vectors showed only a single integration event, out of 815 amplicons sequenced, in a rat eyecup 2.5 months post-injection [66]. They also observed, using the same technique, that IDLV injected mice had 8-fold higher levels of 2-LTR-junction containing molecules in comparison to ILV injected mice; corresponding to viral episomes being present at much higher levels. IDLVs are also much less likely to generate RCRs in comparison to integrating vectors; however, they are not exempt from the risk of mobilization by superinfection with replicating HIV-1 because they can generate full-length, encapsidation competent transcripts [91].

1.4.1.2 Transgene Expression from IDLVs

Unintegrated lentiviral DNA can be maintained as transcriptionally competent templates both in cell culture and also *in vivo*. Saenz and colleagues in 2004 showed that growth-arrested fibroblasts transduced with a lentivector based upon feline immunodeficiency virus (FIV) containing a double class I mutation in integrase (D64N and D116N), showed efficient and persistent expression of a β-galactosidase transgene [92]. However, expression levels and patterns of expression from IDLVs *in vitro* can differ significantly from ILVs. For measurement of transgene expression, it is important not only to calculate the percentage of transduced cells, but also the expression level per cell i.e. mean fluorescent intensity (MFI) if using an eGFP transgene for example.

Experiments using HeLa cells have demonstrated that the level of gene expression per cell in a similar percentage of transduced cells is consistently lower with IDLVs, and that the pattern of expression is dependent upon the internal promoter element [37]. In 2007, Cornu and Cathomen estimated that, based on experiments in HEK-293T cells transduced with either an ILV or IDLV expressing eGFP, a 10-fold higher dose of IDLV would need to be administered to achieve the same expression of an equivalent integrating vector [93].

1.4.1.3 Applications of IDLVs

IDLVs have been shown to successfully transduce a variety of post-mitotic tissues *in vivo* with sustained transgene expression [3]. These tissues include: (i) retina; a rat model of Retinitis pigmentosa showed visual improvement after injection of an IDLV expressing RPE65 [66], (ii) brain; efficient marker gene delivery to areas of rodent brain and spinal cord has been shown following stereotatic injection [65,68], and (iii) muscle; efficient transduction of muscle fibres with eGFP occurs following intramuscular injection of IDLV [67]. IDLVs have also been used for transient expression in dividing cell types, including transduction of dendritic cells for vaccination strategies [94], and haematopoetic stem cells (HSCs) for strategies to express genes to stimulate cell division or induce differentiation [87].

IDLVs can be used as platforms for alternative transgene integration systems, including: (i) Delivery and expression of a DNA transposon system; two recent papers describe a Sleeping Beauty (SB) transposase-mediated transposition which was mediated using an IDLV [95,96]. Unlike retroviral integration, SB transposition shows little preference for specific genomic regions and therefore a reduced risk of insertional mutagenesis. (ii) Site specific integration platforms such as zing-finger nuclease systems; Lombardo *et al.*, in 2007 [97] used a three IDLV system where vectors carried either one of the two necessary zinc-finger nucleases or the donor template. They observed successful gene correction at the IL2RG locus and site-specific integration into the CCR5 locus in 50% of transduced cultured cells and 5% of human ES cells [97]. Initial success is however still

hampered by a very low efficiency in both systems [98]. IDLVs represent a class of novel viral vectors with emerging applications, however further *in vivo* testing is required to prove the value of these techniques.

1.5 Achieving Cell Specific Expression of Therapeutic Genes

One of the benefits of gene therapy is the ability to specifically target the expression of a therapeutic gene in desired cells or tissues. This ability gives a huge advantage over other areas of medicine where systemic treatments, such as delivery of small molecule drugs, can cause unknown and potentially serious side-effects. For example, for the treatment of cancer, the ability to target the viral tropism and/or gene expression of oncolytic viruses, or vectors expressing suicide genes, specifically to tumour cells should prevent gene expression and subsequent death of normal patient cells. Selective gene expression can also be important in treatment of many haematologic disorders where only selective lineages of cells are affected and expression of a functional protein is not required, or is detrimental, in other cell types.

For *ex vivo* gene therapy, the use of viral vectors which have a broad tropism is not a problem as the required cell type is isolated from the organism and then transduced; the risk of vector dissemination and off target expression is therefore very low. However, for *in vivo* gene delivery, cell targeting is critical to enhance therapeutic effect, reduce side effects, and lower the amount of vector required [1]. To achieve this goal a number of methods can be employed: (i) Pseudotyping; this involves the manipulation of viral tropism through the use of alternative or surface engineered envelope glycoproteins (outlined in Figure 1.9) [1,99]. (ii) Tissue-specific regulatory elements; using a gene's endogenous promoter, or a known tissue specific promoter, to drive a transgene will allow expression in the required cell type, both at the correct level and at the correct developmental stage. This gives tighter regulation of expression over a ubiquitous promoter; however, the site of integration and silencing will still influence expression. (iii) Detargeting expression; preventing transgene expression in off-target cell types has been achieved using cell specific microRNA (miRNA) target sequences incorporated into vectors. These sequences allow endogenous miRNA binding to vector mRNA transcripts in certain cell types, blocking transcription and therefore inhibiting protein expression in these cells [100].

Strategies including cell surface, transcriptional and post-transcriptional targeting allow the design of safer, better controlled, and high performance expression vectors for gene therapy. Some of the approaches and elements used in this project for cell specific targeting will be discussed in further detail.

1.5.1 Pseudotyping

Pseudotyping, in its original sense, means that one or more structural proteins of a virion are not encoded by the nucleic acid carried in the virus [101]. This can occur during co-infection of a host cell by two unrelated enveloped viruses; virions are then created which contain the genome of one virus packaged within a membrane that contains the envelope glycoproteins of either the other virus, or both viruses. These viruses are known as pseudotypes and the consequences of 'sharing' these envelope proteins is an increased breadth in the virus host range [102]. This has been described as the 'pseudotypic paradox' because the assembly of surface glycoproteins appears to be both specific and non-specific [103]. It is non-specific in the sense that during dual infection, viruses non-selectively incorporate both their own glycoprotein as well as those from viruses belonging to other families. However, in the main cellular proteins are not recognised and are excluded from incorporation into virions during budding [103]. Viruses must therefore share a common, highly specific mechanism of assembly.

In research, a pseudotyped virus is one in which the outer shell of the vector (the envelope glycoproteins of an enveloped virus or the capsid proteins of a non-enveloped virus) originates from a different virus than that which is the source of the genome and genome replication apparatus [101]. Pseudotyped vectors have several clinical and experimental applications; the study of a pseudotyped vector may have safety advantages so that viral entry and envelope proteins can be studied without the risk of using the infectious virus. A pseudotype may also be manipulated with greater ease, allowing mutations in the outer shell to be introduced and tested, leading to identification of the cellular receptors and attachment factors more easily [101]. For gene therapy, the main use of pseudotyping has been to exploit the natural cellular tropism

of other viruses for gene transfer vectors, expanding or restricting cell type. Using pseudotyped vectors may also modulate the physiochemical properties of the vector and change its interaction with the host immune system [104].

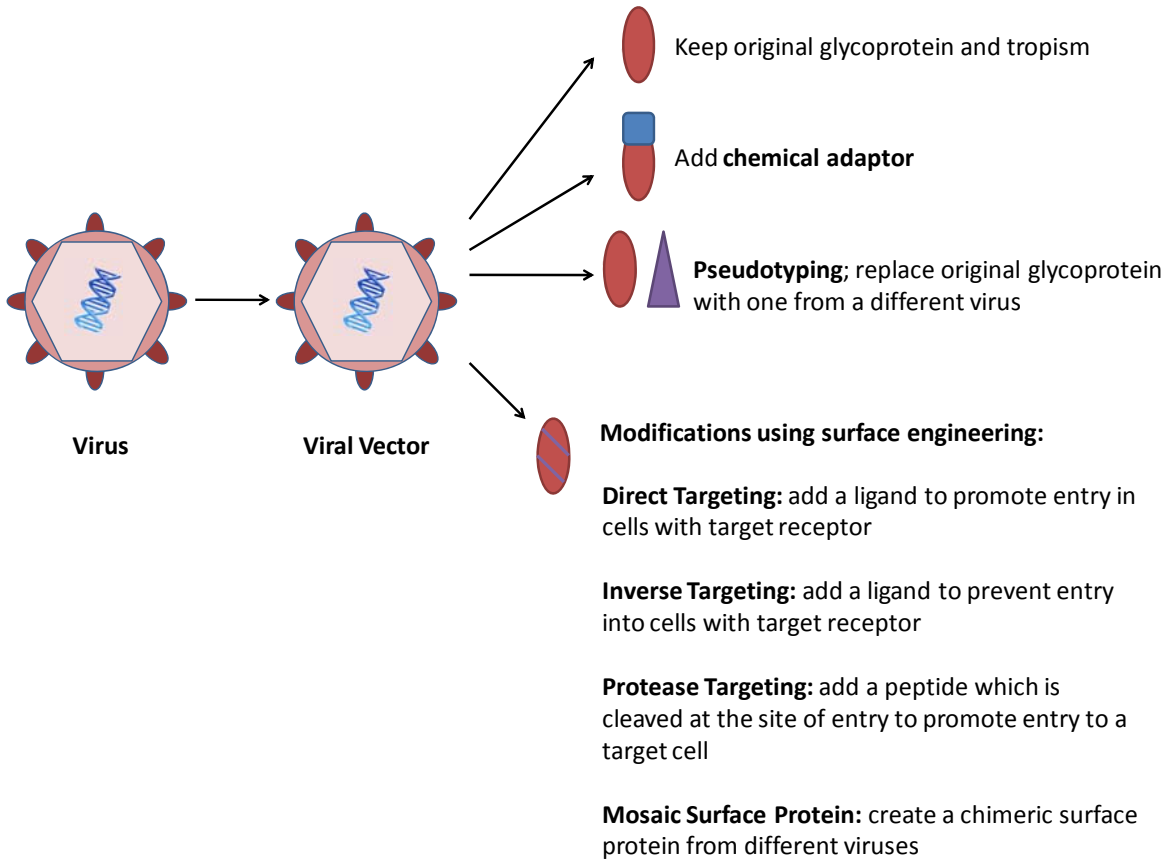


Figure 1.9 Cell specific targeting for viral vectors. Options for pseudotyping and surface engineering modifications to target viral vectors to specific cell types. Figure modified from (Bouard *et al.,* 2009) [1].

The first group to produce and test HIV-1 based vectors pseudotyped with an heterologous glycoprotein was Page *et al.* in 1990 who pseudotyped a replication defective HIV-1 vector with an MLV-A glycoprotein [105]. After which, in 1996, three groups independently showed that the glycoprotein from VSV was also efficiently incorporated into HIV-1 virions [76,106,107]. Today VSVg remains the most popular glycoprotein for pseudotyping lentiviral vectors and has effectively become the standard to which all alternative pseudotypes are measured [99]. Advantages of VSVg include that the glycoprotein confers a very broad tropism on vectors through its interaction with an unknown cellular 'receptor' on host cells. It also produces a very high vector particle stability, allowing for concentration to a high titre by ultracentrifugation [108]. However, VSVg has a number of shortcomings, most importantly that the glycoprotein cannot be constitutively expressed by cells as it is cytotoxic, therefore it is difficult to set up packaging cell lines (although the use of tetracycline-regulated promoters has relieved this to an extent [109]). Observations of pseudotransduction (protein transfer rather than stable gene transfer) with VSVg pseudotypes viruses have also been recorded [110,111]. VSVg pseudotypes are inactivated by human serum and VSVg virus preparations may harbour tubulovesicular structures which can stimulate the host antiviral immune system [112]. Five alternative pseudotypes are investigated during the course of this project, a description and the reported tropism of each vector is outlined below.

1.5.1.1 Baculovirus (gp64)

Baculoviruses are insect viruses which primarily infect members of the Lepidopteran family, and are used mainly as biopesticides [113]. Gp64 is the envelope glycoprotein from the insect fusion virus *Autographica californica* and was first used to pseudotype a lentiviral vector by Kumar *et al.* in 2003. Gp64 pseudotypes tolerate ultracentrifugation well and expression of gp64 is not toxic to cells allowing the production of packaging cell lines [114]. Gp64 pseudotyped HIV-1 based vectors efficiently transduced a number of different cell types and had a more restricted tropism than VSVg, with an especially

poor ability to transduce hematopoietic cell types such as dendritic cells [115], but a good ability to transduce hepatocytes [116].

1.5.1.2 Ebola Zaire (EboZ)

Ebola is a highly pathogenic filovirus which induces hemorrhagic fevers in humans and non-human primates. Of the four human strains of Ebola virus; Zaire, Ivory Coast, Sudan and Reston, the Zaire strain induces the highest death rates in humans. The glycoprotein for Ebola Zaire (EboZ) uses folate receptor-alpha as a cofactor for cellular entry [117] and mononuclear phagocytic cells form the primary target for filoviral replication, while endothelial cells serve as secondary targets [99]. MacKenzie *et al.* 2002 [118] compared the *in vivo* tropism of lentiviral vectors pseudotyped with EboZ to VSVg by *in utero* intramuscular and intrahepatic injection. In these experiments the EboZ pseudotype was seen to efficiently transduce monocytes after intrahepatic injection, cardiomyocytes after both intrahepatic and intramuscular injection, and predominantly muscle satellite or stem cells following intramuscular injection [118]. EboZ pseudotypes have not been seen to transduce cells after direct injection into adult brain, this may be because folate receptor-alpha expression is low in the CNS [119]. However, they have been seen to transduce airway epithelial cells after apical application *in vitro,* and have been shown to be more effective than VSVg pseudotypes in an *in vivo* study where virus was administered intratracheally to mice [120].

1.5.1.3 Ross River Virus (RRV)

Ross River Virus (RRV) is an alphavirus belonging to the family *Togaviridae.* Kang *et al.,* in 2002, were the first to describe a lentiviral vector pseudotyped with RRV. Here, they showed that RRV pseudotyped FIV predominantly transduced the liver, both hepatocytes and Kupffer cells (hepatic macrophages), after systemic administration at a level 20-fold higher than VSVg pseudotyped FIV [121]. It was also shown that RRV/FIV preferentially transduced neuroglial cells (astrocytes and oligodendrocytes) in the

brain, this was in comparison to VSVg/FIV which targeted predominantly neurons. RRV is also non-toxic when expressed *in vitro* allowing stable packaging cell lines to be produced [122]. The glycoprotein structre of RRV is known, however, it's host cellular receptor is still unknown [123].

1.5.1.4 Murine Leukaemia Virus – Amphotropic and Ecotropic Strains (MLV-A and MLV-E)

A variety of envelope glycoproteins from gammaretroviruses have been shown to pseudotype lentiviral vectors including those from ecotropic MLV, amphotropic 4070A MLV, gibbon ape leukaemia virus (GALV) and RD114. Ecotropic MLV (MLV-E) has numerous strains including Moloney, Friend, and AKV, all of which infect only murine cell types. The glycoprotein itself is processed from the protein Pr85 which is then processed into a surface (SU) subunit which mediates viral binding, and a transmembrane (TM) subunit which mediates viral fusion [124]. The glycoprotein for Amphotropic 4070A MLV (MLV-A) is also made up of the SU and TM subunits and in this case the host cellular receptor - Pit2 (a sodium phosphate transporter) – is known. MLV-A will transduce both murine and human cells and has been shown to have a particular tropism towards hematopoietic cell types. In particular Hanawa *et al.* in 2002 showed that an HIV-1 vector pseudotyped with MLV-A was more effective than VSVg at transducing human cord blood-derived CD34+ cells and clonogenic progenitors [125].

1.5.2 Promoter Elements

Gene promoter regions are DNA sequences typically located upstream of genes and are the key *cis*-acting regulatory regions that control transcription. Promoters contain specific sequences which act as binding sites for RNA polymerase II, which transcribes DNA to mRNA, and *trans*-acting factors (transcription factors) which can help activate or suppress transcription by binding the DNA and help recruit or block binding of the polymerase enzyme. Promoters also work in conjunction with other regulatory elements, such as enhancers, silencers, and insulators, to direct the level and cell specificity of transcription of a given gene.

There are two basic classifications of promoter which can be categorised according to the type or degree of control of gene expression; control in all tissues, or control depending on the tissue and/or stage of development. Additionally, promoters may operate in response to external or controllable stimuli and are therefore classified as follows: (i) Constitutive promoters; these induce the expression of downstream coding regions in all tissues irrespective of environmental or developmental factors. (ii) Synthetic promoters; comprising of consensus DNA sequences of common elements from natural promoter regions. (iii) Inducible promoters; functional only in the presence of chemical (compounds not normally found within the cell) or physical (abiotic and external factors such as light, heat, and injury) stimulus. (iv) Tissue-specific promoters; operate only in particular tissues and at certain developmental stages which are induced by endogenous and exogenous cellular factors.

In vivo gene therapy strategies are largely dependent upon the therapeutic gene being expressed at an appropriate amount in the target cells, during the correct developmental stage, without developing toxicity, and without being eliminated by the immune system. Depending upon the disease, different profiles of expression are also required, for example, constant or regulated. Without this control, off target, over- and under-expression can cause unwanted effects. For example, off target expression of the growth factor HOXB4 in haematopoetic stem cells (HSCs) enhances the primitive

growth activity of HSCs, whereas high expression of HOXB4 impedes myeloid and lymphoid differentiation [126,127].

Within the SIN lentiviral construct, an internal promoter is required to regulate transgene expression. Several constitutive mammalian promoter elements have been employed including the phosphoglycerate kinase (PGK-1) [74,128] and elongation factor 1α (EF1α) [129] gene promoters. In addition, strong, ubiquitous viral elements, such as the immediate/early promoter/enhancer from Cytomegalovirus (CMV) and the 3' LTR from Spleen Focus Forming Virus (SFFV) have also been extensively studied. The aim with these constructs is to achieve high transgene expression in all cells transduced in order to produce therapeutic levels of transgene expression. However, strong expression conferred by constitutive mammalian and viral promoters can be detrimental to transduced cells due to potential effects of over-expression of the transgene product. Further to this, the viral promoters CMV and SFFV have also been shown to undergo silencing *in vivo* as a result of *de novo* CpG methylation which prevents transcription [130,131]. Therefore, the use of tissue-specific promoters in this context is a good alternative to achieve; (i) restrictive transgene expression in target cells, (ii) controlled expression in the right cells, reducing the risk of toxic effects related to aberrant expression profiles, (iii) reduced risk of a transgene-induced immune response, (iv) the potential to avoid silencing [132].

Promoter targeting can also be combined with suicide gene therapy for the treatment of cancer, or to control graft-versus-host disease (GvHD) [133]. This can be achieved by gene transfer of a suicide gene, such as herpes simplex virus thymidine kinase (HSV-TK), into the cells that need to be eliminated. Following this, administration of the drug ganciclovir in this case, will then induce apoptotic death of HSV-TK transduced cells. This strategy reduces the loss of normal transduced cells. However, the genome environment in which the provirus will be inserted will also modify vector expression. Preferential insertion of lentivirus proviral DNA in transcriptionally-active sites of the genome [134,135] may mean that off-target expression takes place despite the presence of a tissue-specific promoter. Inclusion of insulator sequences, such as the beta-globin

intron, flanking the ILV expression cassette has been used to avoid this unwanted expression [136].

A number of promoter elements have been explored during this project in the context of a SIN lentiviral vector including the strong viral promoter/enhancer SFFV, the mammalian ubiquitously-acting chromatin opening element (A2UCOE), and the hepatocyte specific promoter elements, human alpha-1 antitrypsin (hAAT) and LP1.

1.5.2.1 Spleen Focus Forming Virus LTR (SFFV) Promoter

The polycythemic strain of spleen focus-forming virus is a replication-incompetent virus related to Friend mink cell focus-forming viruses [137]. The retroviral enhancer/promoter is contained in the U3 region of the long terminal repeat (LTR) and consists of an array of *cis*-acting elements condensed into two direct repeats. Flanking this region, at the 5' end is a high affinity binding site for the ubiquitous transcription factor Sp1, and at the 3' end, a binding site for Friend Virus factor c (FVc) [7]. The core motif in the direct repeat contains targets for transcription factors including: (i) CAAT/enhancer binding protein (C/EBP): This family of proteins contribute to tissue-specific gene expression in the liver, adipocytes, and myeloid cell. (ii) Core binding factor (CBF) also known as polyomavirus enhancer binding protein (PEBP) involved in the regulation of both retroviral and endogenous genes in lymphoid and myeloid precursor cells (Figure 1.10) [7,138]. Within a retroviral vector, the SFFV LTR was shown to provide extremely high transgene expression in haematopoetic cells [139,140], spermatogenic cells [141], and hepatocytes *in vivo* [142], and has been used a number of times within the context of SIN lentiviral vectors to confer high transgene expression in a number of cell types .

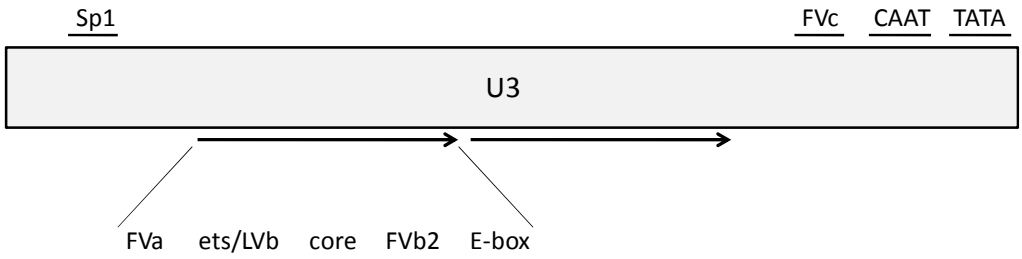


Figure 1.10 Schematic of the spleen focus forming virus (SFFV) promoter element. The U3 region of the SFFV promoter element contains a number of transcription factor binding sites including; the ubiquitous factor Sp1, two direct repeats containing binding sites for factors Friend Virus a and b2, and Leukaemia Virus b. The 3' Friend Virus c, CAAT and TATA box make up the promoter element of U3. Figure modified from (Baum *et al.,* 1997) [7].

1.5.2.2 Ubiquitously Acting Opening Chromatin Element (UCOE)

Ubiquitously acting chromatin opening elements are genetic elements responsible for establishing a transcriptionally competent, open chromatin structure at a region of the genome where housekeeping genes are transcribed. They consist of a methylation-free CpG island spanning closely spaced, dual, divergently transcribed promoters, derived from housekeeping gene loci. The 2.2kb UCOE from the human *HNRPA2B1-CBX3* gene loci (A2UCOE) allows stable transgene expression and prevents transcriptional silencing in stably transfected tissue culture cells in the absence of drug selection when integrated within centromeric heterochromatin [143]. A2UCOE can also been used in conjunction with other promoters; it was shown to reduce silencing and enhance CMV promoter expression of a transgene *in vitro* [144]. A2UCOE has been used in the context of a SIN lentiviral vector both *in vitro* and *in vivo* where it rescued a model of SCID-X1 as it was able to prevent transcriptional variegation and silencing, both alone and in conjunction with the SFFV and CMV promoters [8] (Figure 1.11).

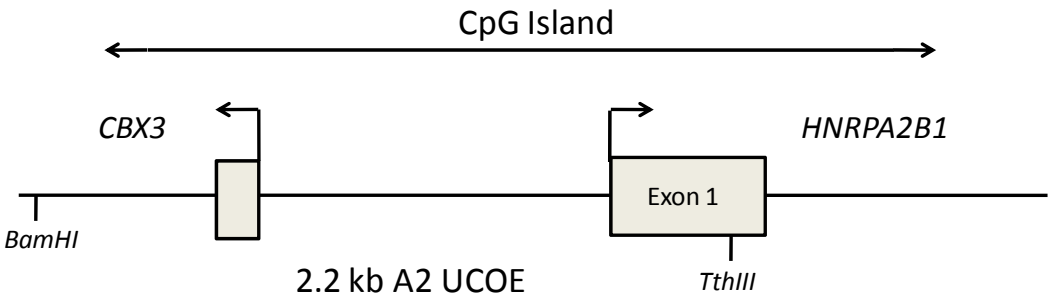


Figure 1.11 Schematic of the A2 ubiquitous chromatin opening element (UCOE). Expanded view of the 3kb methylation free CpG island region encompassing the divergent *CBX3* and *HNRPA2B1* promoters. The 2.2kb A2UCOE promoter extends from the *Bam*HI restriction site within the first intron of *CBX3* and the *Tth*III site within exon 1 of *HNRPA2B1*. Arrows denote the direction of transcription, grey boxes denote exons. Figure modified from (Zhang *et al.,* 2007) [8].

1.5.2.3 Human Alpha-1 Antitrypsin and LP1 Hepatocyte Specific Promoter Elements

The gene encoding the protease alpha-1 antitrypsin (AAT) is expressed in both macrophages and hepatocytes. The transcription occurs from two different promoters, a macrophage specific promoter approximately 7kb upstream and a hepatocyte specific promoter approximately 2kb upstream, from the coding region [145]. These promoters produce alternatively spliced mRNA products which differ in the 5'UTR region. The hepatocyte specific promoter consists of two non-specific distal control elements which function to increase transcription efficiency overall, and a proximal, hepatocyte-specific, control element which binds hepatocyte nuclear factors (HNF) 1 and 4 which are enriched in liver cells [9]. The 305bp human AAT promoter inserted into vectors in this project contains the 3' non-specific distal element and the proximal hepatocyte specific element (Figure 1.12) [10]. The LP1 promoter is a truncated liver specific promoter consisting of a 192bp sequence containing core segments from the human apolipoprotein apoE/C1 hepatic control region (HCR-1), and a 255bp truncated human alpha-1–antitrypsin gene promoter containing only the proximal control elements. It has been previously described within AAV vectors where strong liver specific transgene expression has been conferred [146-148].

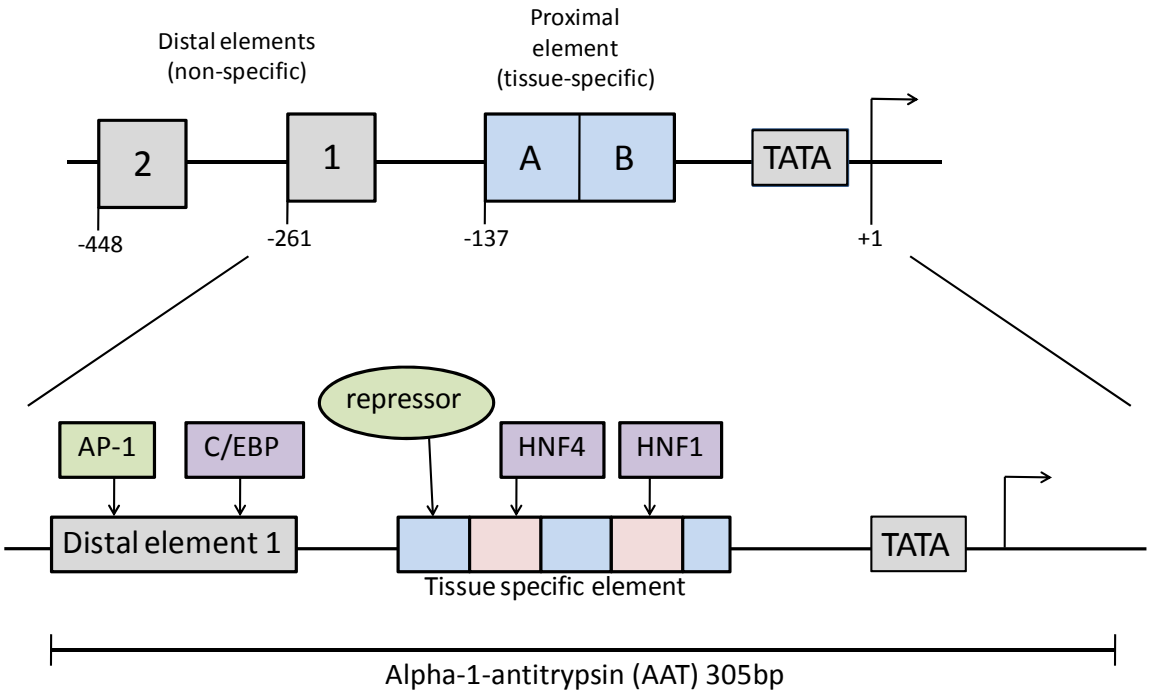


Figure 1.12 Schematic of the human Alpha-1-antitrypsin (AAT) promoter. The hepatocyte-specific control region consists of tissue specific proximal element and two non-specific distal elements. A and B represent factor binding sites essential for activity. Numbering is relative to the transcriptional start site in hepatocytes. The 305bp AAT promoter contains distal element 1, the tissue specific element and TATA box. Activator and repressor proteins are indicated, green shapes are ubiquitous factors, and purple shapes liver-enriched factors. Activator protein 1 (AP-1); CCAAT enhancer binding protein α or β (C/EBP); hepatocyte nuclear factor (HNF). Figure modified from (Kramer *et al.,* 2003) and (De Simone *et al.,* 1987) [9,10].

1.5.3 Exploiting microRNA Regulation for Vector Targeting

The use of promoters to confer tissue specificity for lentiviral vectors still contains drawbacks including; (i) the ability of enhancers or other neighbouring promoters to affect transgene expression once the vector is integrated, (ii) read through into other tissues from tissue specific promoters, (iii) the difficulty in reconstituting an endogenous tissue-specific promoter within vector context.

Recently, incorporation of microRNA (miRNA) target sequences into vectors has added a further level of tissue specificity at the post-transcriptional level. MicroRNAs are small, non-coding RNA sequences which work by binding to complementary target sites in an mRNA, thereby preventing translation of the transcript or accelerating its decay [100]. In mammals, over 400 different miRNAs have been identified, most of which are well conserved among species. Some are ubiquitously expressed; however, others have an expression pattern that depends upon the developmental stage or cell type. A large study in 2004, for example, identified 5 out of 340 miRNAs which were highly specific for haematopoetic cells; miR-142, miR-144, miR-150, miR-155 and miR-233 [149]. Incorporation of an miRNA target sequence into a vector construct will then exploit endogenous miRNA regulation and cause the transcript to be degraded in a cell type where, or at a developmental stage when, this miRNA is expressed; subsequently detargeting expression of the transgene. The overall success of this strategy has been surprising as the change in expression mediated by miRNAs on their natural targets is modest in comparison to the level of regulation required to degrade vector-encoded transcripts. However, a number of studies have shown that expression of a transgene can be suppressed up to 100-fold in a specific cell type by utilising properly engineered target sites for a highly expressed miRNA.

In gene therapy, the most notable use of this strategy was by Brown *et al.,* in 2006 [150]; four copies of a miR-142 miRNA target sequence (miR-142) were incorporated into a lentiviral vector construct expressing a GFP reporter gene. They showed a striking

reduction in GFP expression in monocytes and dendritic cells, whilst retaining a high level of expression in non-haematopoetic cells [150].

In addition, this strategy was also shown to be successful *in vivo*; intravenous injection of immune competent mice with a lentiviral vector expressing human Factor IX (hFIX) allows expression in antigen presenting cells (APCs) even under regulation of a liver specific promoter. This causes an anti-hFIX cellular immune response and clearance of transduced hepatocytes [151]. To combat this, four tandem copies of a sequence complementary to miR-142-3p, an miRNA expressed highly in APCs but not in hepatocytes, were incorporated into the 3'UTR of the vector. After *in vivo* administration of the miRNA modified vector into immune competent factor IX-knockout mice, an immune response was not initiated and long-lasting expression of hFIX was established, rescuing the disease [152]. Interestingly, by detargeting transgene expression from APCs, hFIX tolerance is also achieved and this is thought to be induced by a population of specific regulatory T cells [153]. Furthermore, the vector did not cause saturation of the miRNA or a loss of natural function of the miR-142-3p sequence. Emerging data on miRNA expression levels and profiles, both from normal and disease tissues will be paramount in designing miRNA regulated vectors with a high specificity, and would allow controlled therapeutic transgene expression in certain tissues, cell lineages and even according to cell differentiation state.

1.6 Haemophilia A

Haemophilia A is a serious bleeding disorder caused by a deficiency in or complete absence of the blood coagulation factor VIII (FVIII). It is the most common hereditary coagulation disorder with an incidence approaching around 1 in 5000 males. Inherited as an X-linked disorder, males are predominantly affected with females as carriers. However, in around a third of cases there is no family history of a bleeding disorder as patients have a recent, spontaneous mutation [154]. Haemophilia B is clinically indistinguishable from haemophilia A and is also transmitted by X-chromosome inheritance. It is a result of coagulation factor IX (FIX) deficiency and affects around 1 in 25,000 males. The treatment of the two conditions is quite different however, and the diagnosis of either disease must be established by coagulation-factor assays [4]. A very low concentration of circulating FVIII, just 200 ng/mL, is needed to ensure adequate procoagulant function in healthy subjects; in comparison, FIX circulates at approximately 5000 ng/mL. The clinical severity of haemophilia A largely correlates with the measured level of endogenous factor; less than 1% of normal factor levels are categorised as severe, 1-5% as moderate, and >5% as mild. Severe haemophilia can result in serious morbidity and mortality, with episodes of spontaneous bleeding, predominantly into the joints and muscles, resulting in permanent disability if left untreated. In moderate or mild haemophilia, bleeding episodes are rare and are usually in conjunction with trauma or surgery.

1.6.1 Factor VIII Treatment and Risks

In the past the only available treatment for haemophilia A patients was whole blood or plasma transfusions which were often not sufficient to achieve proper haemostasis. In the 1950s however, plasma-derived FVIII concentrates were developed which could be used for treatment of acute bleeding and prophylaxis. This revolutionised haemophilia care and had a dramatic improvement on the life expectancy of severe haemophilia patients. However, treatment with blood products is connected with a risk of viral transmission such as Hepatitis B and C, and HIV. In the early 1980's it was estimated

that 63% of the 15,500 patients living with haemophilia in the United States contracted HIV through contaminated plasma-derived FVIII concentrates [155]. Extensive heat and solvent-detergent treatment of plasma concentrates limited cases of new viral infections of plasma derived FVIII. However, after FVIII was cloned in 1984, recombinant FVIII (rFVIII) concentrates were developed. Remarkably, rFVIII was infused into a patient just 3 years later [156], and rFVIII concentrate was available for widespread clinical use in 1992. Recombinant factor VIII concentrates used in clinics today include Advate™ (Baxter Healthcare, USA), Kogenate™ (Bayer, USA) and ReFacto™ (Wyeth, USA).

Despite these advances however, prophylactic treatment is not a phenotypic cure and is extremely costly; it is estimated that the annual cost for treatment for a severe haemophilia A patient with no inhibitor development is US$150,000, and for a patient who develops antibody inhibitors to FVIII this can be four-fold higher [157]. Of the approximately 400,000 people worldwide living with haemophilia, 75% live predominantly in developing countries and receive only sporadic factor replacement therapy, or none at all (The World Federation of Haemophilia, 2009). Replacement therapy also requires patients to adhere to a challenging routine of intravenous injections which can bring about long-term problems with both intravenous access and infection [158] and can have negative psychological and social impacts [159]. A high proportion of patients also develop antibody inhibitors against FVIII. This occurs in 5-15% of patients with mild to moderate severity and about 25% of patients with severe haemophilia A, after treatment with factor VIII concentrates [160]. The FVIII inhibitor titre is expressed in Bethesda units which may vary from 0.6-50,000 units [161]. Patients are classified as high or low responders; high being a patient that presents with a titre above 5 units at any point after treatment, and low being a titre below 5 units despite repeated treatments with FVIII concentrate. The type of gene mutation often affects the risk of developing inhibitors; patients with large deletions and nonsense mutations or gene inversions are at higher risk than those with frameshift or missense mutations [162]. There is also a higher risk of raising antibodies at the beginning of treatment, usually within the first 50 days of exposure. The desired goal of tolerance has been

achieved in some high responding patients by using immunosuppression in combination with IgG and administration of FVIII concentrate, and also in low responding patients using high doses of FVIII concentrate alone [163]. Despite possible complications with inhibitory antibodies, haemophilia A is considered a good candidate for gene therapy which could potentially be a cost-effective and safe method of treatment accessible to a greater proportion of the world's haemophiliacs.

1.6.2 Haemostasis

Haemostasis is the physiological response for the prevention and stopping of bleeding. It results in the blocking of any vascular breach and most of the time includes the changing of blood from a fluid to a solid state. Abnormalities in haemostasis can result in bleeding (haemorrhage) or blood clots (thrombosis). Maintenance of haemostasis is therefore a complex and delicate balance between procoagulant and anticoagulant events and their regulation. In mammalian blood coagulation, 6 proteases (factor VII, factor IX, factor X, factor XII, protein C, and prothrombin) act with five cofactors (tissue factor, thrombomodulin, protein S, factor V and factor VIII) to control the generation of fibrin. Mammalian blood coagulation is initiated and regulated by a complex network of interactions controlled by positive and negative feedback loops. Blood coagulation is initiated in two ways: (i) The extrinsic pathway, and (ii) the intrinsic pathway (Figure 1.13)

When damage occurs, tissue factor (TF), a lipoprotein normally found on the outside of blood vessels, is exposed at the surfaces of the endothelium, smooth muscle and other cells inside the blood vessel. TF binds to circulating Factor VII, activating it to Factor VIIa (FVIIa) with the help of other proteases including prothrombin, Factor Xa (FXa) and the FVIIa-TF complex itself. FVII is a serine protease produced in the liver and is vitamin K dependant. The FVIIa-TF complex in turn binds and activates circulating Factor X (a serine endopeptidase produced in the liver which is also vitamin K dependant). FXa then binds to its cofactor, activated factor V (FVa), and calcium to catalyse the conversion of prothrombin to thrombin. Because the source of tissue factor is external

to the blood this cascade initiation mechanism is called the extrinsic pathway. Triggering the intrinsic pathway is linked to alterations in blood vessel lining which expose blood to collagen and other subendothelial elements. Exposure to these proteins activate factor XII, which leads to activation of factor XI and factor IX. FIXa binds to its cofactor, activated factor VIII (FVIIIa), calcium and membrane phospholipids to activate factor X resulting in the formation of fibrin. Thrombin then regulates coagulation by proteolytically activating an anticoagulation pathway. It binds to the cellular receptor thrombomodulin (TM) and alters its substrate specificity. The thrombin-TM complex acts to catalyse protein C to activated protein C (APC) which then rapidly inactivates cofactors FVa and FVIIIa. Further negative regulation also occurs through the activity of antithrombin (AT) which inhibits the activity of FIXa, FXa and thrombin [164] . A simplified diagram of the coagulation cascade is shown in Figure 1.13.

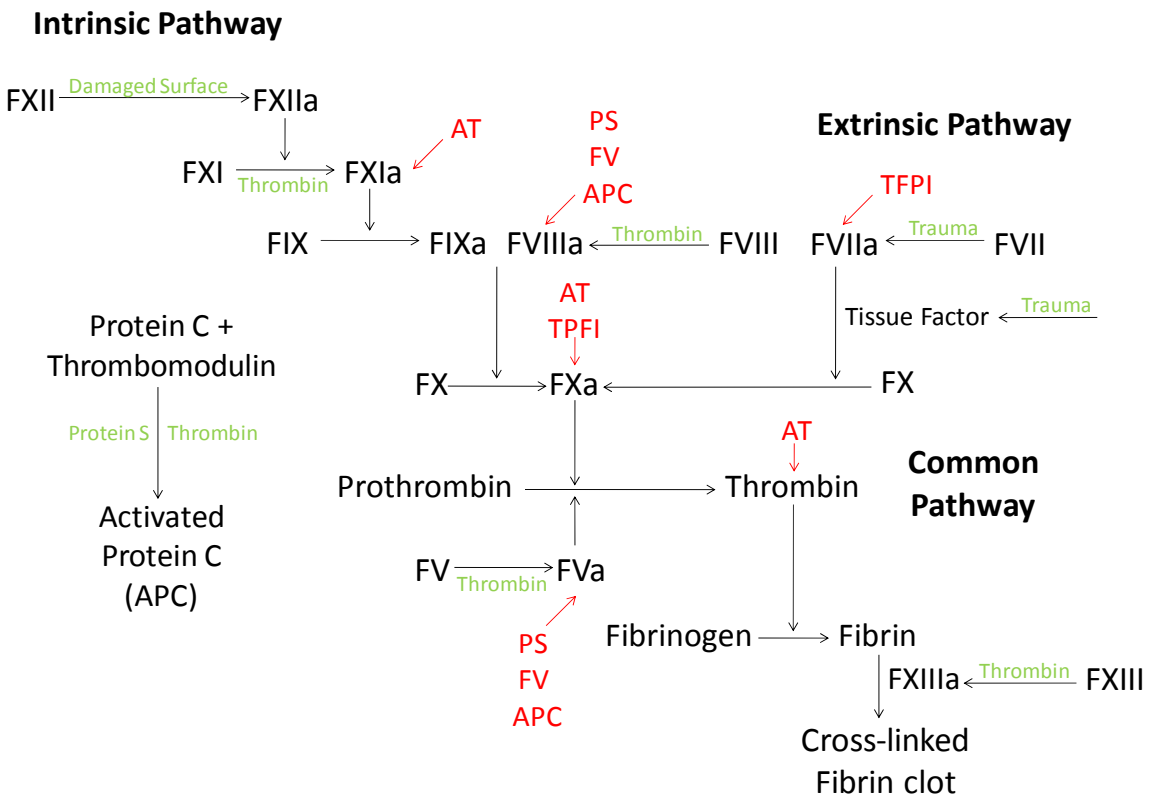


Figure 1.13 The coagulation cascade. Blood coagulation can be described as a cascade system of proteolytic reactions initiated in response to tissue damage. In each reaction, an inactive zymogen is converted to its active enzyme counterpart, which then participates in the subsequent step of the coagulation cascade. Thrombin activates factors V, VIII and XI, thereby promoting its own generation. The protein C anticoagulant pathway down-regulates blood coagulation by inactivating factors VIIIa and Va. Tissue factor pathway inhibitor (TFPI), protein S (PS), and antithrombin (AT) (shown in red) inhibit the generated enzymes factor VIIa, IXa, Xa and thrombin.

1.6.3 Factor VIII Protein Structure and Activation

Factor VIII is a large 330 kDa glycoprotein which acts as an essential cofactor for blood coagulation. Its primary structure was identified in the early 1980's when two groups simultaneously cloned the FVIII cDNA and the protein was purified to homogeneity [165,166]. The FVIII gene is located at the tip of the long arm of the X chromosome and comprises 26 exons over a length of 186 kb; constituting nearly 0.1% of the total chromosome. The coding DNA is approximately 9 kb in total, producing a single 2332 amino acid single polypeptide chain preceded by a 19 amino acid hydrophobic signal sequence. FVIII has the discrete domain structure A1-a1-A2-a2-B-a3-A3-C1-C2 [167,168]. Domains a1 (residues 337–372), a2 (residues 711–740), and a3 (1649–1689) are acidic amino acid rich regions, containing sulphated tyrosine residues, located between the major structural domains [169]. FVIII A domains share approximately 40% amino acid sequence identity with each other and to the A domains of factor V (FV) which shares the same discrete domain structure [170,171]. The A domains also share homology with the copper-binding plasma protein ceruloplasmin and the enterocyte transmembrane protein hephastin, which have the structure A1-A2-A3 [164]. The C domains again share 40% homology with each other and the C domains of FV, and are homologous to phospholipid-binding proteins, suggesting a role in phospholipid interaction [172]. The B domain of factor VIII is encoded by one unusually large uninterrupted exon, exon 14, and spans amino acids 741-1648 of the FVIII polyprotein, constituting 38% of the total sequence. The B domain shares no amino acid homology with other known proteins, including the B domain of FV. They do however share the property of having extensive glycosylation of asparagine, serine and threonine residues; the B domain of FVIII containing 19 of the 26 asparagine (N)-linked glycosylation sites on the whole molecule [5].

FVIII is believed to be produced primarily in hepatocytes and endothelial cells [168,173]. Upon translocation of the FVIII polypeptide into the endoplasmic reticulum (ER) the amino terminal signal peptide is removed. Within the ER, post-translation modifications of the mature protein occur including N-linked glycosylation, which helps to stabilize

folded domains, prevent aggregation of folding intermediates, and allow newly synthesized polypeptide chains to interact with ER chaperones and enzymes [5]. The importance of these moieties on protein secretion has been demonstrated by treatment of cells with tunicamycin, which blocks N-glycosylation and dramatically reduced FVIII secretion [174]. After correct folding, FVIII proceeds towards the golgi. For efficient trafficking from the ER to the golgi FVIII binds the proteins mannose-binding protein lectin 1 (LMAN1) and multiple coagulation factor deficiency protein 2 (MCFD2) within the ER-golgi intermediate compartment (ERGIC). The binding sites for these proteins are thought to be within the FVIII B domain [175]. Once within the golgi FVIII undergoes intracellular cleavage by furin, a subtilisin-like protease which recognises the motif Arg-X-X-Arg. The sites of cleavage are in the middle and C-terminal regions of the B domain after residues 1313 and 1648 generating a 210 kDa heavy chain (A1-a1-A2-a2-B) and an 80 kDa light chain (a3-A3-C1-C2). Factor VIII is secreted from the cell as a heterodimer of the 80 kDa light chain linked by the divalent metal ion Cu^{2+} to a heavy chain between 90-210 kDa; the heterogeneity of the heavy chain arises due to limited proteolysis of the C-terminal of the B domain causing variable lengths to remain [176]. However, due to the cleavage at Arg^{1313} the predominant form of inactivated FVIII circulating after secretion contains 572 residues of the B domain [177]. In circulation, the FVIII heterodimer binds to its carrier protein, von Willebrand factor (vWF), at two sites within the A3 and C2 domains to form a tight non-covalent complex. Clearance of FVIII and in activation by interaction with FIXa and platelets are blocked by binding to vWF and FVIII half-life is increased to around 12 hours several fold over that of unbound FVIII. Activation of FVIII occurs by thrombin which cleaves at three points; first at residue Arg^{740}, then Arg^{372}, and finally Arg^{1689} [169]. This creates a heterotrimer (A1-a1/ A2-a2/ A3-C1-C2) which participates as a cofactor for FIXa in the formation of FXa in the tenase complex on the surface of platelets.

Cofactor activity is rapidly lost either through dissociation of the A2-a2 subunit, or through proteolytic degredation. The main physiological inactivator of FVIIIa is activated protein C (APC) which cleaves in the A1 domain at Arg^{336} and in the A2 domain at Arg^{562}. Degradation is also mediated to a lesser extent by thrombin, FIXa and

FXa which cleaves FVIIIa in the A1 domain at Lys^{36} and Arg^{336}. Inactivated FVIII is then cleared from circulation. A schematic of FVIII structure and activation is shown in Figure 1.14.

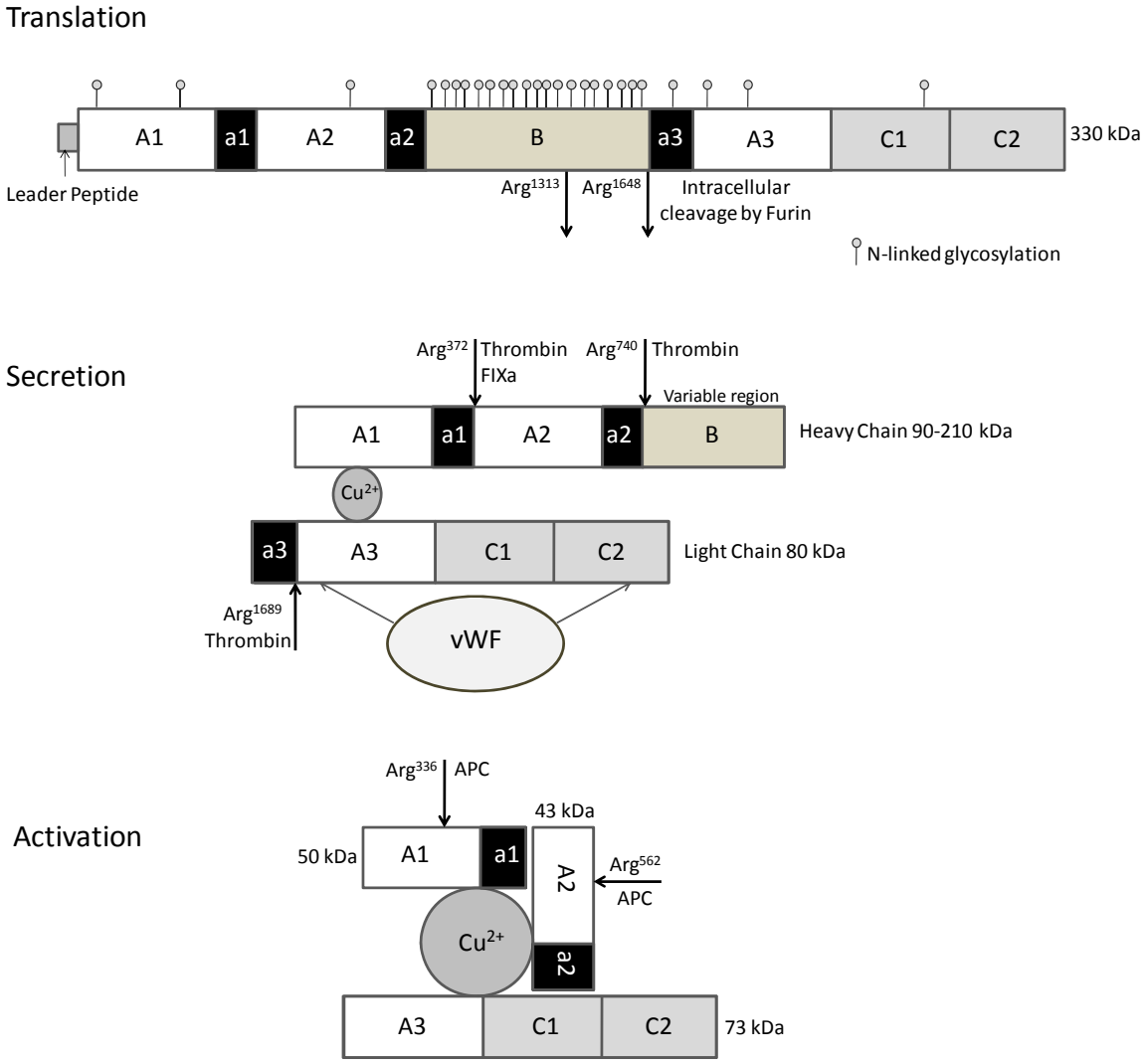


Figure 1.14 Domain structure and processing of factor VIII. The translation product of the factor VIII gene is a 2332aa polypeptide with a leader peptide sequence of 19aa. FVIII has the distinct domain structure A1-a1-A2-a2-B-a3-A3-C1-C1 where both the A and C domains have sequence homology. The molecule is heavily glycosylated containing 26 N-asparagine linked glycosylation sites, 19 of which are found in the B domain. Upon secretion the FVIII is a heterodimer of heavy and light chains bound to each other by a divalent metal ion, and in complex with the carrier protein von Willebrand Factor (vWF). Activation is mediated by cleavage by thrombin and FIXa at specific residues to form a heterotrimer. Inactivation is mainly mediated by activated protein C (APC). Figure modified from (Pipe, 2009) and (Mannucci and Tuddenham, 2001) [5,6]

1.6.4 Gene Therapy for Haemophilia A

Haemophilia A is considered a good candidate for gene therapy for many reasons; (i) it is a well characterised monogenic disorder of which the genes cDNA has been successfully identified and cloned, (ii) although FVIII is normally secreted by hepatocytes and endothelial cells, as the gene product is a systemic plasma protein, the transgene can be expressed in other cell types including adipocytes, myocytes and fibroblasts, (iii) only a modest increase in FVIII plasma concentration is needed for therapeutic benefit with levels of >1% able to achieve markedly reduced rates of spontaneous bleeding and long term arthropathy. Therefore the long term goal would not be to normalise the level of endogenous FVIII, only to replace enough factor to improve bleeding symptoms, (iv) the effects of gene therapy can be readily monitored by changes in phenotype and obtaining peripheral blood to measure FVIII antigen levels and clotting factor activity, (v) there are well characterised murine and large animal (canine) models available for preclinical experiments.

Gene therapy may offer hope of solving many of the issues associated with today's haemophilia treatment including the high cost, intravenous injection regimes, and availability of treatment in the developing world. Both *in vivo* and *ex vivo* strategies are appropriate for factor VIII gene therapy; many approaches have been tested preclinically including systemic injections of both viral and non-viral vectors expressing FVIII cDNA, and modification of cells such as haematopoietic stem cells (HSCs) or fibroblasts *ex vivo*. However, for over 20 years although preclinical results in animal models have been impressive, no approach as yet has been successful for long-term treatment in human trials [4]. Gene therapy clinical trials for haemophilia A are summarised in Table 1.2; the first was reported by Roth *et al.* in 2001 where autologous fibroblasts from skin biopsies of six severe haemophilia A patients were transfected *ex vivo* with a plasmid containing a B domain deleted (BDD) FVIII cDNA transgene. Modified clones were propagated and implanted back into the patient into the omentum. The results were disappointing; two patients failed to respond to treatment, and the four which did respond showed only marginal benefit [178]. Systemic injections of

a retroviral vector expressing BDD-FVIII, and a mini adenoviral vector expressing full length factor VIII cDNA have also been trialled. Unfortunately expression was very low and was unable to be maintained long term in both cases [4,179].

Institute	Number of Patients	Treatment	Maximum Expression of Transgene	Duration of Expression	Ref
Transkaryotic Therapies (1998) Trial: US-247	6	*Ex vivo* skin fibroblast transfection with BDD-FVIII plasmid	0.8 to 4% in 4 patients	<6 months	[178]
Chiron Corporation (1999) Trial: US-284	13	Systemic infusion of retroviral vector (MoMLV) with BDD-FVIII	1 to 2%	<13 weeks	[179]
Genstar Therapeutics (2002) Trial: FR-038	1	Max Adenoviral vector carrying full length FVIII	3%	Transient	N/A

Table 1.2 Human gene therapy clinical trials for Haemophilia A.
Table adapted from (Viiala *et al.* 2009) [4] and The Gene Therapy Clinical Trials Database, 2009. N/A; not available as trial was not reported.

1.6.5 Bioengineering of Factor VIII

Insights over the past two decades into the secretion pathway, FVIII structure and function, and mechanisms of inhibitor development have led to bioengineered forms of factor VIII to further improve haemophilia A therapy. Bioengineering recombinant factor VIII (rFVIII) aims to improve properties such as biosynthesis, secretion efficiency, functional activity, plasma half-life, and to reduce antigenicity/immunogenicity [180]. These improvements aim to overcome some of the limitations in current haemophilia A treatment for better production of rFVIII concentrates, and also in gene therapy which is hampered by inadequate gene expression *in vivo*.

rFVIII for commercial production of concentrates is derived through heterologous transfection of rFVIII plasmid into a nonhuman mammalian cell line such as Chinese Hamster Ovary (CHO) or Baby Hamster Kidney (BHK) cells. These cell lines are able to synthesise rFVIII protein that includes all of the complex ER and golgi post-translational modifications such as addition of asparagine (N)-linked oligosaccharides, and heavy and light chain cleavage. rFVIII is then secreted into the culture media, stabilised by the addition of human or bovine proteins, and subsequently purified using chromatographic techniques. Expression of rFVIII in this system is 2 to 3 fold lower than that of other comparably sized proteins [181], which is also true for gene therapy applications. This drop in expression occurs because FVIII mRNA is very inefficiently expressed [181-183], and a significant proportion of the primary translation product is misfolded and ultimately degraded [174,184]. Therefore, most research in FVIII bioengineering has so far been directed at overcoming the inherent limitations of low expression.

It was shown several years ago that the B domain of FVIII could be removed without any loss of procoagulant function [185]. Removal of the B domain also significantly improved the yield of rFVIII protein as a result of increased mRNA levels and translation. The reduced size of the cDNA, now just 4.4kb, also facilitated improved packaging in viral vectors. B domain deleted (BDD) FVIII has a biochemical profile

similar to full length FVIII, and the shortened protein is less prone to proteolytic degradation, therefore no addition of plasma derived albumin is needed to stabilise the final product when producing concentrates. BDD-rFVIII was adopted as a commercial product marketed as ReFacto^{TM} (Wyeth, USA). This molecule has a deletion of the B domain between residues 744 and 1637; thereby retaining a 14 amino acid bridge which contains no asparagine (N)-linked glycoslation sites, but does retain the furin intracellular cleavage site [186]. ReFacto^{TM} remains the first and only modified human rFVIII on the commercial market. Clinical studies using BDD-rFVIII have shown that it provides safe, well-tolerated treatment for haemophilia A and, most significantly, that the rates of inhibitor formation in previously untreated patients were similar to that observed with full length equivalents; proving that despite such a major modification it is not more immunogenic *in vivo*. However, some biological differences between BDD-FVIII and full length FVIII remain, (i) BDD-FVIII binds to activated platelets with a higher affinity, (ii) it has an increased sensitivity to thrombin cleavage causing consistent discrepancies in one-stage clotting assays, and (iii) it has approximately a four-fold increase in specific activity compared to full length FVIII. Biochemical analysis *in vitro* also revealed that despite a 17-fold increase in mRNA production within cells transfected with BDD-FVIII in comapsion to wild-type FVIII [187], only a 30% increase in the levels of secreted protein were observed, suggesting that the rate of ER-Golgi transport was actually reduced [172]. To challenge this problem and build on the advantages of BDD-FVIII, Miao *et al.,* in 2004 [188] added back a short B domain sequence to the BDD-FVIII, optimally 226 amino acids and retaining 6 sites for *N*-linked glycosylation. This resulted in a 10-fold increase in secretion *in vitro* from transfected COS-1 cells [188]. The B domain has also been shown to have further functions and interactions which may be important to consider when designing bioengineered rFVIII molecules, these include, (i) interactions with chaperone proteins within the ER and golgi which allow correct folding and stabilisation of it's tertiary structure, (ii) interactions within the ERGIC which facilitates efficient ER to golgi transport and therefore increases secretion efficiency, (iii) shielding thrombin activation sites, preventing premature proteolysis, (iv) reducing the affinity of unactivated FVIII to activated platelets, and (v) reducing proteolysis by activated protein C [5]. Therefore

when designing bioengineered molecules it may be important to retain some, or all, of these B domain function.

Further functional modifications to improve secretion efficiency have also included substituting the B domain with the intron from human factor IX [189]; successful at increasing mRNA levels. Reducing interactions with chaperone proteins which can retain FVIII within the ER also improve secretion. FVIII contains an 11-residue hydrophobic beta-sheet within the A1 domain which actively binds to the ER chaperone immunoglobulin-binding protein (BiP). The single mutation Phe309Ser within this hydrophobic region has been shown to increase secretion by 3-fold [190]. Improved functional ability has been achieved by the use of inactivation resistant FVIII molecules.

The FVIII variant IR8 was engineered to have enhanced stability due to resistance of subunit dissociation and proteolytic inactivation. This was achieved by deletion of residues 794 – 1689 so that the A2 domain was covalently linked to the light chain, in combination with the missense mutations at thrombin and APC cleavage sites (Arg336Ile, Arg562Lys, and Arg740Ala) which provided resistance to proteolytic cleavage. These changes resulted in a single chain product which, despite having a lower affinity to vWF, had a five-fold higher specific activity and a retained 3% of peak activity after 4 hours [191]. In 2003, a similar approach was also used by Gale and Pellequer, who produced a FVIII variant with the mutations Cys664 and Cys1826 which created a disulphide link between the A2 and A3 domains increasing its stability *in vitro* [192].

Increasing the plasma half-life of FVIII by mutating areas which bind to clearance proteins is a further option. FVIII may interact with the low-density lipoprotein receptor-related protein (LRP) at the A2 domain residues 484-509 and within the C2 domains. It has been shown that when LRP binding is blocked by an antagonist, the half-life of FVIII was significantly prolonged in a mouse model [193,194]. FVIII is also thought to bind to heparin sulphate proteoglycans (HSPGs) within the A2 domain. Targeted mutations within the binding sites for these clearance proteins, whilst

avoiding the binding sites for FIXa and vWF, may have a profound effect upon plasma half life *in vivo* [180]. Many of the modifications described involve different steps of FVIII secretion, activation and inactivation therefore it is possible they may be used in combination to provide an additive effect. A reduction in immunogenicity may also be achieved; within the A2 domain there is a 25 residue epitope which is frequently the target of anti-A2 domain antibodies. FVIII knock-out mice injected with a construct containing mutations within this domain (Arg484Ala / Arg489Ala / Phe492Ala) produced significantly lower inhibitory antibody titres in comparison to those injected with BDD-FVIII [195]. It may be possible to identify further epitopes and modify them to become less antigenic.

One of the major concerns of bioengineering is that changing the tertiary structure of the FVIII molecule may expose neoantigens or that modifications may cause neoantigenicity *in vivo*. With rFVIII molecules tested so far this has not been the case, however, with the addition of more and more functional mutations this may become likely.

1.7 Project Aims

Integrating lentiviral vectors have been used extensively to mediate expression of factor VIII and IX *in vivo* for treatment of haemophilias A and B, respectively. They are excellent candidates for gene therapy as only a modest increase in the endogenous factor level is required for therapeutic benefit, response to treatment can be easily monitored, and factor expression can be mediated by many cell types *in vivo* [116,196-207]. In this study we aim to investigate the use of integration deficient lentiviral vectors as treatment for haemophilia with long term expression of factor VIII or IX mediated by a non-dividing cell type with muscle and liver tissue (primarily non-dividing hepatocytes) as principal targets. Further strategies including pseudotyping, tissue specific promoters and miRNA target sequences will be used to further target vectors to specific tissues and for tissue specific expression to avoid immune response.

To achieve this, the reporter gene luciferase will be used to assess relative transduction efficiency and relative transgene expression in muscle and liver *in vivo* from both integrating and non-integrating vectors. Finally, the expression of B domain bioengineered forms of a human factor VIII construct will be investigated both *in vitro* and *in vivo* from integrating vectors from a codon-optimised and a non-codon optimised cDNA sequence.

Chapter Two

Materials and Methods

2.0 Materials

Unless otherwise stated, all tissue culture reagents were supplied by Gibco BRL (Invitrogen) and all general chemicals by Sigma Aldrich. Buffers and solutions are listed in Table 2.1, bacterial media and agar solutions in Table 2.2, and qPCR primers and probes in Table 2.3.

Molecular Cloning

1kb Plus DNA Ladder	Invitrogen
Agarose	Invitrogen
Restriction Enzymes	New England Biolabs
Primers	Invitrogen
Taq DNA polymerase	Promega
Pfu DNA polymerase	Promega
dNTPs	Promega
Calf intestinal alkaline phosphatase (CIAP)	Promega
Mung Bean Nuclease	New England Biolabs
T4 DNA Ligase	Promega
Bovine Serum Albumin (BSA)	Promega
Plasmid miniprep/maxiprep/megaprep kit	Qiagen
QIAquick gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
One Shot® Stbl3™ chemically competent *E. coli*	Invitrogen
Agar	MERCK
Ampicillin	Stratagene
TOPO-TA® cloning vector pCR4®	Invitrogen
pSL301 cloning vector	Invitrogen
pcDNA 3.1(+) cloning vector	Invitrogen
Sequencing	Eurofins MWG Operon

Site Directed Mutagenesis (for production of factor VIII construct pLNT/SFFV-SQ FVIII Fugu B)	Eurofins MWG Operon
Codon Optimisation of factor VIII constructs	GeneArt

Quantitative Real Time PCR

Primers	Invitrogen
Probes	Eurofins MWG Operon
Platinum® qPCR SuperMix-UDG with ROX (11743-500)	Invitrogen

Antibodies

Mouse anti-luciferase (IgG_1) (35-6700)	Invitrogen
Polyclonal anti-factor VIII Capture Antibody (F8C-EIA-C)	Affinity Biologicals
Monoclonal anti-factor VIII (C7F7), biotinylated in house	Bayer
In house rhesus anti-human factor IX capture antibody	Kindly provided by Dr. Amit Nathwani, UCL
HRP conjugated Goat anti-human factor IX (GAFIX-APHRP)	Affinity Biologicals
HRP conjugated Rabbit anti-mouse (IgG) (W4021)	Promega

Assay Standards and Kits

QuantiLum® Recombinant Luciferase Protein (E1701)	Promega
Biophen Normal Control Plasma (223201)	Quadratech Diagnostics
Biophen Abnormal Control Plasma (223301)	Quadratech Diagnostics
Biophen Factor VIII:C Chromagenic Assay (221402)	Quadratech Diagnostics

Factor VIII ELISA (F8C:EIA)	Affinity Biologicals
In vitro and *Ex vivo* Luciferase Assay Pack (E1501)	Promega
MethylCode^{TM} Bisulfite Conversion Kit (MECOV-50)	Invitrogen
Calcium phosphate transfection kit (125K4075)	Sigma Aldrich
HIV-1 p24 Antigen ELISA Kit	Beckman Coulter
Reverse Transcriptase Assay, colorimetric (11468120910)	Roche Applied Science
Human factor IX protein concentrate (F-0650)	Sigma
Murine plasma, obtained in house from normal mice	Kindly provided by Dr. Amit Nathwani, UCL
Bradford Protein Assay Kit (500-0001)	BioRad
D-Luciferin (Potassium salt) (LUCK-1G-OPT2)	Gold Biotechnology Inc.
MethylCode™ Bisulfite Conversion Kit (MECOV-50)	Invitrogen

General Reagents and Equipment

Proteinase K, PCR grade	Roche
Sodium Dodecyl Sulfate (SDS)	Sigma Aldrich
OPD peroxidase substrate tablet set (P-9187)	Sigma Aldrich
(methyl-^{3}H) Thymidine	Amersham
Paraformaldehyde	Sigma Aldrich
TMB (3,3',5,5'-tetramethylbenzidine) Substrate kit for peroxidase (SK-4400)	Vector Laboratories
NUNC MaxiSorp™ ELISA plates (44-2404)	NUNC, eBioscience
96-well, black/clear bottom, flat bottom, tissue culture treated, sterile plates (4939)	Thermo Scientific
96-well, white, clear bottom, flat bottom plate (4924)	Thermo Scientific
TRI reagent (T9424)	Sigma Aldrich
Phytohemagglutinin (PHA) (11 082 132 001)	Roche
50KDa Polyethyleneimine (PEI)	Sigma Aldrich
5X Reporter Gene Assay Lysis Buffer (11 897 675 001)	Roche

FLUOstar OPTIMA Microplate Fluorometer	BMG Labtech, GmbH
Isoflurane	Abbott Laboratories
Halothane	Concord Pharmaceuticals
Hypnorm™	Janssen Pharmaceuticals
Ketaset®	Wyeth
Rompun®	Bayer
Boyle's apparatus	British Oxygen Company
IVIS cooled charge-coupled device (CCCD) camera	Caliper Life Sciences
Microcentrifuge	Heraeus Biofuge Fresco
Tabletop centrifuge	Sorvall Legend RT
Superspeed centrifuge	Sorvall Evolution RC
Ultracentrifuge	Sorvall *Discovery* SE

Cell Lines

HEK 293T	Human embryonic kidney cell line, based on the HEK 293 cells (ATCC# CRL-1573); the 293T cell line expresses the SV40 large T-antigen that allows for episomal replication of transfected plasmids containing the SV40 origin of replication
C2C12	Murine myoblast cell line (ATCC# CRL-1772)
NIH/3T3	Murine fibroblast cell line (ATCC# CRL-1658)
A549	Human lung carcinoma alveolar basal epithelia cell line (ATCC# CCL-185)
Neuro-2a (N2A)	Murine neuroblastoma cell line (ATCC# CCL-131)
HT1080	Human fibrosarcoma cell line (ATCC# CCL-121)
HeLa	Human adenocarcinoma epithelial cell line (ATCC# CCL-2)
THP-1	Human acute monocytic leukemia cell line (ATCC# TIB-202)
Hep-G2	Human hepatocellular carcinoma cell line (ATCC# HB-8065)
Huh7	Human hepatoma cell line [208] (Nakabayashi *et al.* 1982)
ARPE-19	Human retinal pigment epithelial cell line (ATCC# CRL-2302)

Buffers and Solutions (Table 2.1)

1X Phosphate-buffered saline (PBS)	For 1L: 8.0g NaCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCl, up to 1L with dH_2O.
50X TAE buffer	For 1L: 242g Trisamine (Tris), 57.1 mL Glacial Acetic Acid, 100 mL 0.5M EDTA.
5x DNA loading dye (Orange G)	For 25mL: 6.75g Ficoll (15%), 0.25g Orange G, up to 25mL with dH_2O.
Nuclei lysis Buffer	For 1L: 5mLs Tris (2M), 80mL NaCl (5M), 4mL Na_2EDTA (0.5M)
Proteinase K Buffer	For 500mLs: 50mLs 10% SDS, 2mLs Na_2EDTA (0.5M)
hFIX ELISA Coating Buffer	For 500mLs: 4.201g $NaHCO_3$, pH 9-9.2, up to 500mLs with dH_2O.
hFIX ELISA Wash Buffer	PBS with 0.05% (v/v) Tween 20
hFIX ELISA Blocking Buffer	6% bovine serum albumin (BSA) in PBS with 0.05% (v/v) Tween 20
hFIX ELISA Dilution Buffer	2% bovine serum albumin (BSA) in PBS with 0.05% (v/v) Tween 20
hFVIII ELISA Coating Buffer	50mM Carbonate buffer. For 1L: 1.59g Na_2CO_3, 2.93g $NAHCO_3$, up to 1L with dH_2O, pH9.6
hFVIII ELISA Wash Buffer	PBS with 0.1% (v/v) Tween 20, pH 7.4
OPD Substrate Buffer (Citrate-phosphate buffer)	For 500mLs: 2.6g citric acid, 6.9g Na_2HPO_4, up to a volume of 500mLs with dH_2O, pH 5

OPD substrate solution	5mg of o-Phenylenediamine. 2HCl added to 12mL of citrate-phosphate buffer containing 0.03% H_2O_2
0.1M Carbonate Buffer	For 1L: 3.18g of Na_2CO_3, 5.86g of $NaHCO_3$ up to 1 litre with dH_2O,pH 9.6.
Anti-luciferase ELISA wash buffer	PBS with 0.05% (v/v) Tween 20
Anti-luciferase ELISA blocking buffer	PBS + 1% milk protein (Marvel)

Bacterial Media and Agar Solutions (Table 2.2)

LB (Luria-Bertani) Broth	For 1L: 10g NaCl, 5g Yeast extract, 10g tryptone peptone. Autoclave.
LB (Luria-Bertani) Agar	For 1L: 10g NaCl, 5g Yeast extract, 10g tryptone peptone, 15g bacto agar. Autoclave.
SOC Media	For 250mLs: 5g Tryptone Peptone, 1.25g Yeast extract, 0.15g NaCl, 0.05g KCl, 0.51g $MgCl_2$, 2.5mLs $MgSO_4$. Autoclave. Add 10mLs 20% glucode after autoclaving.

qPCR Primers and Probes (Table 2.3)

Total viral DNA (WPRE) Forward	5’ – TGGATTCTGCGCGGGA – 3’
Total viral DNA (WPRE) Reverse	5’ – GAAGGAAGGTCCGCTGGATT – 3’

Total viral DNA (WPRE) Probe	(FAM) 5' – CTTCTGCTACGTCCCTTCGGCCCT – 3' (TAMRA)
2-LTR circles Forward	5' – AACTAGAGATCCCTCAGACCCTTTT – 3'
2-LTR circles Reverse	5' – CTTGTCTTCGTTGGGAGTGAATT – 3'
2-LTR circles Probe	(FAM) 5' – CTAGAGATTTTCCACACTGAC – 3' (TAMRA)
Titin Forward	5' – AAAACGAGCAGTGACGTGAGC – 3'
Titin Reverse	5' – TTCAGTCATGCTGCTAGCGC – 3'
Titin Probe	(FAM) 5' – TGCACGGAAGCGTCTCGTCTCAGTC – 3' (TAMRA)
Beta-actin Forward	5' – TCACCCACAAGTTGCCCATCTACGA – 3'
Beta-actin Reverse	5' – CAGCGGAACCGCTCATTGCCAATGG – 3'
Beta-actin Probe	(FAM) 5' – ATGCCCTCCCCCATGCCATCCTGCGT – 3' (TAMRA)

2.1 Methods

2.1.1 Bacterial Manipulation

2.1.1.1 Growth and Maintenance of *E. coli*

Escherichia coli (*E. coli*) were grown in liquid LB media at 37°C with agitation at 250 rpm or streaked out on solid LB plates containing 1.5% bacto agar. *E.coli* transformed with plasmid was grown on the same media supplemented with ampicillin (50 µg/mL). For long-term storage, bacterial cultures were stored in 15% volume for volume (v/v) glycerol at -80°C.

2.1.1.2 Transformation of One Shot® Stbl3™ Bacterial Cells

One Shot® Stbl3 chemically competent *E. coli* cells are transformed by heat shock; one 50µL vial of cells is thawed on ice for each transformation. 10 pg to 100 ng of DNA is added to the vial, gently mixed and incubated on ice for 30 minutes. Cells are then heat-shocked for 45 seconds at 42°C without shaking. The vial is then placed on ice for 2 minutes. 250µL of pre-warmed S.O.C. media is added and cells shaken at 250 rpm at 37°C for 1 hour, after which they were diluted in LB media and spread on LB agar plates containing ampicillin (50 µg/mL). The plates were incubated overnight at 37°C, after which colonies were picked using sterile 20 µl pipette tips and grown overnight in 5 ml liquid cultures.

2.1.2 Cloning

2.1.2.1 Restriction Enzyme Digest

1µg plasmid DNA was routinely digested in a final volume of 20µL containing 1x buffer (supplied by the manufacturer), 1x BSA (0.1mg/mL) and restriction enzyme. The volume of enzyme used varied depending of the concentration of the enzyme stock but did not exceeded 10% (v/v) of the total reaction volume. Reactions were carried as per manufacturers' instructions at the recommended temperature for 1-2 hours and DNA digestion was verified by agarose gel electrophoresis. Double or triple digestions were performed either in compatible buffers or sequentially, after clean-up of DNA by ethanol precipitation using the QiaQuick PCR purification kit as per manufacturers' instructions.

2.1.2.2 Isolation of DNA Fragments by Gel Electrophoresis

DNA fragments were resolved by electrophoresis through 0.8-2% agarose gels in 1x TAE buffer. To prepare the gels, agarose was dissolved in 1x TAE buffer by boiling in a microwave oven. After cooling, ethidium bromide was added to obtain a final concentration of 0.5g/ml for visualisation of DNA. DNA samples were mixed with DNA loading buffer before loading onto agarose gels. A 1 kb plus DNA ladder was included in each gel to enable size determination of DNA fragments. Gels were electrophoresed using a voltage of 50-100V (up to 150mA) and the separated DNA fragments subsequently visualised by exposure to ultra-violet light using an UVIdoc gel documentation system. Following electrophoresis, DNA fragments were excised from agarose gels using a clean scalpel blade under ultra-violet light. The DNA was then extracted from the agarose using a QIAquick gel extraction kit as per the manufacturer's instructions.

2.1.2.3 Polymerase Chain Reaction (PCR)

PCR reactions were performed in a total volume of 50µL containing 100ng plasmid template DNA, 0.5µM of both forward and reverse primers, 200µM of each dNTP, 1x PCR buffer and 1U of *Pfu* DNA polymerase. Cycling conditions depended upon the annealing temperatures of the primers used and whether a restriction enzyme recognition site was added into a primer, however, typical cycling conditions included: 30 cycles of 94°C for 1min, 60°C for 1min, 72°C for 1min.

2.1.2.4 DNA Ligation

Digested vector DNA was treated with calf intestinal alkaline phosphatase (CIAP) to dephosphorylate DNA ends prior to ligation. Dephosphorylation reactions were performed directly in restriction endonuclease buffers, immediately following digestion by adding 10 units of CIAP enzyme to the reaction mixture and incubating at 37°C for one hour. Ligation of DNA fragments were performed using vector to insert ratio of 1:3 (molar) and carried out in a final volume of 10µL containing; 1x T4 DNA Ligase buffer and 1 unit of T4 DNA Ligase. The vector DNA concentration used was always 100ng of DNA. Ligation reactions were incubated for 3 hours at room temperature or overnight at 16°C, after which they were immediately transformed into One Shot® Stbl3™ *E.coli* cells.

2.1.3 Genomic DNA Isolation

Genomic DNA was isolated by using the salting out method [209]. For 100mLs stock lysis solution: 80mLs nuclei lysis buffer, 5.3mLs 10% SDS, 13.3mLs proteinase K buffer, 1.4mLs proteinase K. For *in vitro* experiments 1 x 10^5 cells in a 24-well plate were washed with PBS and 750µL of stock lysis solution added directly to the well of the plate and gently mixed. Cells were digested overnight at 37°C or for 3 hours at 56°C. The mixture was transferred to a 1.5mL microfuge tube and incubated on ice for 10

minutes. 250µL of saturated NaCl (6M) was added and the tubes were shaken vigorously for at least fifteen seconds. Tubes were centrifuged at 16000xg in a microcentrifuge ten minutes and the supernatant transferred to a clean 15mL Falcon tube. Two volumes of absolute ethanol were added and the tubes were inverted several times until the DNA precipitated. The DNA was then removed, passed through ethanol (70%), transferred to a clean micro-centrifuge and resuspended in 100µL of H2O. For *in vivo* experiments tissue samples (~20mg) were homogenised in 1.5mL Micro-centrifuge tubes in 100µL lysis stock solution. A further 900µL of lysis mix was added to each tube and cells digested overnight at 37°C or for 3 hours at 56°C. The protocol then continues as above. The final concentration of DNA was accessed by quantification the absorbance at 260nm using a NanoDrop ND-1000 spectrophotometer.

2.1.4 Total RNA Isolation

1-10 x 10^6 cells were well resuspended in 1mL of TRI reagent and incubated at room temperature for ten minutes. 0.2mL of chloroform was then added to each sample after which tubes were shaken vigorously and then left resting at room temperature for fifteen minutes. The tubes were then centrifuged at 13000rpm for fifteen minutes. The upper aqueous phase was transferred to a new tube and 0.5mL of isopropanol added to each sample to precipitate the RNA. The samples were incubated for one hour at –20°C and then RNA pelleted by centrifugation at 12000rpm for ten minutes. The supernatant was discarded and RNA washed with 1mL of ethanol. The ethanol was removed by centrifugation (16000xg, ten minutes) and the RNA resuspended in H_2O. RNA was quantified by measuring the absorbance at 260nm using a NanoDrop ND-1000 spectrophotometer, at this wavelength 40µg/mL of RNA has an absorbance of 1.

2.1.5 Plasmid DNA Preparation

Plasmid DNA was prepared using Qiagen Mini-Prep kits as per the manufacturer's instructions from 5 ml overnight cultures. For large-scale plasmid DNA preparation 1L LB media containing ampicillin (50µg/mL) was inoculated with 500 µl of a fresh 5 ml culture and incubated over night at 37°C with agitation (250 rpm). Plasmid DNA was subsequently prepared using Qiagen Mega-Prep kits as per the manufacturer's instructions.

2.1.6 Measurement of DNA Concentration

Plasmid DNA concentration was calculated by measuring the absorbance of light with a wavelength of 260 nm (A_{260}) using a NanoDrop ND-1000 spectrophotometer with a 0.2 mm pathlength; at this wavelength 50µg/mL of double-stranded DNA has an absorbance of 1.

2.1.7 Cell Culture

2.1.7.1 Propagation of Adherent Cell Lines

All adherent cell lines were maintained in Dulbecco's modified eagle medium (DMEM) containing GlutaMAX supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin (referred to as complete DMEM). Cells were grown in 80 cm^2 or 175 cm^2 tissue culture flasks in 37°C incubators in a 5% CO_2 atmosphere. Cells were passaged when 80-90% confluent; monolayers were first washed with Dulbecco's phosphate buffered saline (1x) (PBS) and then incubated for 5 minutes at 37°C with trypsin/EDTA. Cells were subsequently diluted into fresh complete DMEM and transferred to new tissue culture flasks.

2.1.7.2 Propagation of Non-Adherent Cell Lines

Non-adherent cell lines were maintained in RPMI 1640 media containing GlutaMAX supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin (referred to as complete RPMI). Cells were grown in 25 cm^2, 80 cm^2 or 175 cm^2 tissue culture flasks (standing upright) in 37ºC incubators in a 5% CO_2 atmosphere. Cells were passaged following media colour-change; the cells were transferred to 15 ml centrifuge tubes and centrifuged at 1200 rpm in a tabletop centrifuge for 5 minutes, washed with PBS, diluted 1:10 or 1:20 in fresh complete RPMI and transferred to new tissue culture flasks.

2.1.7.3 Long Term Storage of Cell Lines

For long term storage of cell lines a 90% confluent monolayer from a 80 cm^2 tissue culture flask or 2-5 x 10^6 cells were pelleted by centrifugation at 1200 rpm in a tabletop centrifuge for 5 minutes, resuspended in 1 ml freezing medium; 90% FCS, 10% dimethylsulfoxide (DMSO), and transferred to a cryovial. Cells were frozen slowly overnight to -70°C in an isopropanol freezing box and then transferred to liquid nitrogen. To revive frozen cells aliquots were thawed rapidly in a 37°C waterbath and slowly transferred to 9 ml cold growth medium. The cells were pelleted at 1200 rpm in a tabletop centrifuge to remove the DMSO and then resuspended in 5 ml growth medium and transferred to a 25 cm^2 tissue culture flask.

2.1.8 Lentiviral Vector Production

The VSVg plasmid pMD.G2 and the gag-pol plasmids pCMVΔR8.74 was obtained from Professor Luigi Naldini, Istituto Scientifico H San Raffaele. The MLV-A plasmid, the mutated gag-pol plasmid pCMVΔR8.74D64V, and the basic vector backbone were all obtained in-house. The gp64 envelope plasmid pgp64 was a kind gift from Dr. Paul McCray Jr., University of Iowa. The EboZ envelope plasmid pEboZ was a kind gift from

Dr. François-Loïc Cosset, Université de Lyon. The RRV envelope plasmid pRRV was a kind gift from Dr. David Sanders, Purdue University. The MLV-E envelope plasmid pCEE was kindly provided by Dr. Michael Green, University of Massachusetts.

2.1.8.1 Production of VSVg, gp64, RRV and EboZ Pseudotyped Lentivectors

20 x 10^6 293T cells were seeded in complete DMEM media in a T175cm^2 flask and incubated at 37˚C, 5% CO_2, overnight. A DNA solution was made containing 50µg vector backbone construct, 32.5µg gag-pol packaging plasmid, and 17.5µg envelope plasmid for VSVg or 32.5µg for gp64, RRV and EboZ pseudotypes in 6mLs OptiMEM and sterilised by filtration (0.22µm). This was then added to 6mL of OptiMEM containing 2nM of Polyethylenimine (PEI) which had been sterilised by filtration (0.22µm), gently mixed, and incubated for twenty minutes at room temperature prior to addition onto cells. Cells were washed with PBS and the transfection media was added to the cells for 3-4 hours at 37˚C and 5% CO2. The transfection media was removed and 15mLs fresh complete DMEM was added to the cells. On the following day, the media was removed and 15mL of fresh complete DMEM was added to each flask. 48 and 72 hours after transfection, the media was collected and cell debris were removed by filtration through a 0.22µm filter. For VSVg, gp64 and EboZ pseudotypes media was then centrifuged at 100,000xg in a Sorvall *Discovery* SE ultracentrifuge for 2 hours at 4˚C. For RRV pseudotyped vector media was centrifuged at 10,000rpm in a Sorvall *Discovery* SE ultracentrifuge overnight at 4˚C. Supernatant was removed and 100µL of cold OptiMEM added to each tube on ice. The pellet was resuspended and aliquots were prepared and rapidly frozen at –80˚C.

2.1.8.2 Production of MLV-A and MLV-E Pseudotyped Lentivectors

20 x 10^6 293T cells were seeded in complete DMEM media in a T175cm^2 flask and incubated at 37˚C, 5% CO_2, overnight. A DNA solution was made by adding 19.2µg

vector backbone construct, 28.8µg gag-pol packaging plasmid, and 28.8µg MLV-A or MLV-E envelope plasmid to a total volume of 1080µL sterile dH_2O provided by the calcium phosphate transfection kit (Sigma). 120µL $CaCl_2$ is then added to the DNA solution. A 15mL Falcon tube is pre-loaded with 1200µL 2X HBS solution, the 1200µL of DNA mix is added drop by drop whilst bubbling the 2X HBS using a pipetor, and then incubated at room temperature for 20 minutes. The media on the cells is changed for 8mLs transfection media (DMEM + antibiotics + 10% FCS + 20mM HEPES buffer) + 25µM Chloroquine, and 2.4mLs transfection reagent added to each flask. Cells are incubated at 37˚C, 5% CO_2 for 6 hours after which the media is changed to 18mLs transfection media. 48 and 72 hours after transfection, media is collected and cell debris removed by filtration through a 0.22µm filter. 48mLs of supernatant is then added to a 50mL Falcon tube and centrifuged at 4000rpm in a Sorvall Legend RT centrifuge for 20 hours at 4˚C. Supernatant is then removed and 150µL of cold OptiMEM added to each tube on ice, the pellet is resuspended and aliquots prepared and rapidly frozen at –80˚C.

2.1.9 Flow Cytometry

To calculate the titre of a vector batch expressing a GFP marker the percentage of GFP transduced cells was calculated by flow cytometry. 1 x 10^5 cells per well in a 24-well plate were transduced with serial dilutions of LV in a final volume of 300µL. The cells were harvested 48 hours after transduction, washed with PBS and pelleted in a FACS tube by centrifugation at 1500rpm for five minutes. The samples were then resuspended in 300µL of 1% PFA in PBS. All samples were analysed on a Beckman Coulter XL flow cytometer using Expo2 software. A live gate was enabled to eliminate debris and 10,000 events were collected, identified by forward scatter side scatter analysis. Samples were analysed using a CyAn ADP flow cytometry analyser and Summit version 4.1 software (DakoCytomation) to show the percentage of GFP positive cells. The titre of the vector in transducing units per mL (TU/mL) was calculated by multiplying the percentage of GFP^+ cells minus the percentage of positive cells in an

untransduced well, by 1 x 10^5 and dividing by the volume of vector (mLs) used in that well. Only wells that had between 5 and 40% of fluorescent positive cells were used to quantify the titre.

2.1.10 Titration of Lentiviral Vectors by p24 Antigen ELISA

The quantity of p24 antigen in concentrated lentiviral vector preparations was measured using a Beckman Coulter HIV-1 p24 antigen assay as per the manufacturer's instructions. Vector was diluted 1:1 x 10^6, 1:5 x 10^6 and 1:1 x 10^7 and added to p24 antibody-coated wells together with diluted calibration standards. Samples were lysed, washed and then incubated with biotinylated anti-HIV-1. The samples were washed again and subsequently incubated with streptavidin-HRPO followed by the addition of tetramethybenzidine (TMB) and Coulter stop reagent (4N H_2SO_4). The absorbance of each plate was then read at 450 nm and the quantity of p24 reagent calculated from a standard curve generated from the diluted calibration standards.

2.1.11 Titration of Lentiviral Vectors by Reverse Transcriptase Activity

The quantity of reverse transcriptase (RT) protein in concentrated lentiviral vector preparations was measured using the colorimetric reverse transcriptase assay (Roche) as per the manufacturer's instructions. Vector was diluted 1:200, 1:250, and 1:300, lysed for 30 mins, then added to a template/primer hybrid, dNTPs, digoxigenin (DIG)- and biotin-labeled dUTP, in a 500µL Micro-centrifuge tube, and incubated for 3 hours at 37˚C. The reverse transcriptase present in the sample synthesises a new DNA strand which incorporates the labelled dUTP. The mixture is then transferred to a microplate coated with streptavidin, and is incubated for 1 hour at 37˚C. This allows binding of the biotin-labelled DNA onto the plate. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate ABTS is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of

the samples can be determined using a microplate (ELISA) reader at wavelength 405nm and is directly correlated to the level of RT activity in the sample.

2.1.12 Quantitative Real-Time PCR (qPCR) for the Determination of Proviral Copy Number

Quantitative real-time PCR (qPCR) was used to determine proviral copy number both in transduced cell lines and transduced tissue. Genomic DNA was isolated as described in 2.1.3 and approximately 100 ng of DNA used as a template for each PCR. Reactions were performed in triplicate in a final volume of 25µL containing forward and reverse primers at 0.9 µM, a fluorescently-labelled probe at 0.2 µM and a pre-mixed mastermix (Platinum qPCR SuperMix-UDG with ROX) diluted to 1 x with sterile distilled water. DNA samples were analysed using primers and probe to the viral WPRE sequence for detection of integrated proviral copies, and using primers and probe to exon 5 of the murine *titin* gene (*Ttn*) (a gift from A Galy, Genethon, Paris, France) to enable quantitation of the number of cells in murine samples assuming 2 copies are present in a diploid cell. For human cells primers and probe to the housekeeping gene β-actin was used to quantify the number of cells per sample. The probes are fluorescently labelled on the 5' end and have a quencher on the 3' terminus. The endonuclease activity of *Taq* releases the 5' fluorescent label from the fret activity of the quencher increasing the fluorescence in the well with each cycle of the PCR. By comparing the cycle threshold values, an arbitrary value where all the samples are in log phase expansion and are increasing without converging, obtained against a standard curve for the lentiviral vector, *titin* or β-actin, the number of copies of integrated virus could be calculated per cell. Reactions were performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the default cycling parameters – 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

2.1.13 Coagulation Assays

For factor IX experiments conducted at UCL approximately 100µL of peripheral blood was collected by tail vein bleed from adult mice and mixed immediately in a ratio of 1:9 with sodium citrate. Samples were then centrifuged at 13000rpm in a micro-centrifuge for ten minutes and the plasma transferred to fresh micro-centrifuge tubes and stored at -20°C before assaying. For factor VIII experiments conducted at Imperial College approximately 50µL and 100µL of peripheral blood was collected from anaesthetised neonate and adult FVIII knock-out mice, respectively, by tail vein bleed. Samples were then mixed in a ratio of 1:9 with sodium citrate, centrifuged, and stored as above.

2.1.13.1 Factor IX Antigen ELISA

This assay was developed in-house by Dr. Amit Nathwani at the UCL Department of Haemotology. Maxisorp^{TM} 96-well NUNC-Immuno ELISA plates (NUNC) were coated with 50µL/well with FIX capture antibody diluted 1:500 in coating buffer, sealed, and incubated overnight at 4°C. The coated plates were washed five times with 200µL wash buffer, then blocked for one hour at 37°C with 200µL blocking buffer. The plates were washed twice and 50µL of sample diluted 1:50 in dilution buffer added in duplicate, 50µL of standards ranging from 2500-39ng/mL FIX protein, made by diluting FIX protein concentrate (Sigma) in FIX knock-out mouse plasma, were also added in duplicate. Plates were incubated at room temperature for two hours and subsequently washed five times times. 100µL/well of goat anti-human FIX-HRP labelled detection antibody (Affinity Biologicals) diluted 1:2000 in dilution buffer was added to each well and plates incubated at room temperature for one hour. Plates were then washed three times and 200µL/well of OPD substrate solution was added. The plates were incubated for exactly 2.5 minutes at room temperature in the dark, at which time the reaction was stopped by adding 50µL stop solution (3M HCl). The absorbance of each well was then read at 490nm on a spectrophotometer. The FIX concentrate standard gives a standard curve used to quantify the percentage of human FIX contained in each sample.

2.1.13.2 Factor VIII Antigen ELISA

The levels of factor VIII antigen in cell media and blood plasma were determined using the FVIII ELISA (F8C:EIA, Affinity Biologicals) as per manufacturers instructions. Maxisorp^{TM} 96-well NUNC-Immuno ELISA plates (NUNC) were coated with 100µL/well with FVIII capture antibody diluted 1:100 in coating buffer, sealed, and incubated for two hours at 22°C. The plates were washed three times and 100µL of sample diluted 1:20 to 1:40 in dilution buffer added in duplicate. A standard curve in % FVIII protein was constructed by diluting biophen normal control plasma (Quadratech Diagnostics) 1:40, carrying out ten 1:2 serial dilutions, and running in duplicate on the plate. Biophen abnormal control plasma was also used as a further quality control for the assay. Plates were incubated at room temperature for two hours at 22°C and subsequently washed three times times with wash buffer. 100µL/well of pre-diluted FVIII detection antibody was added to each well and plates incubated at 22°C for one hour. Plates were then washed three times and 200µL/well of OPD substrate solution was added. The plates were incubated for exactly approximately 15 minutes at 22°C in the dark, at which time the reaction was stopped by adding 50µL stop solution (2.5M H_2SO_4). The absorbance of each well was then read at 490nm on a spectrophotometer.

2.1.13.3 Factor VIII Chromagenic Activity Assay

The cofactor activity of blood plasma samples and *in vitro* cell culture media samples was assessed using the Biophen Factor VIII:C Chromagenic Assay (Quadratech Diagnostics) as per manufacturer's instructions. When activated by thrombin, activated Factor VIII forms an enzymatic complex with Factor IXa, phospholipids, and calcium, which activates Factor X to Factor Xa. In this assay factor IXa, phospholipids, calcium, and factor X are all supplied at a constant concentration and in excess to FXa, meaning the generation of FIXa is rate limited by, and directly proportional to, FVIIIa. Factor Xa generation is measured by its activity on a specific Factor Xa chromogenic substrate (SXa-11), cleaving the substrate and releasing pNA. The amount of pNA generated is

directly proportional to the Factor Xa activity and can be read by colour development at 405nm. Samples were diluted 1:20 and 1:40 in sample diluent provided and run in duplicate on plates. A standard curve in % FVIII cofactor activity was constructed by diluting biophen normal control plasma (Quadratech Diagnostics) 1:20, carrying out four 1:2 serial dilutions, and running in duplicate on the plate. Biophen abnormal control plasma was also used as a further quality control for the assay.

2.1.14 Splenocyte Proliferation Assay

A crude splenocyte proliferation assay was carried out to assess cellular immunogenicity against luciferase protein based on that used by Brown *et al.,* in 2007 [151] for human Factor IX. Spleens collected from all treated mice and one naive mouse were processed into single cell suspensions and splenocytes plated at 2 x 10^5 cells/well, in triplicate, in 96-well plates. Cells were then stimulated with recombinant luciferase protein (Promega) at 10µg/mL, phytohemagglutinin (PHA) (Sigma), a known mitogen and positive control, at 5µg/mL, or left naïve. After 96 hours incubated at 37°C, 5% CO_2, each well of cells was pulsed with 1µCi/well methyl-^{3}H-Thymidine and incubated at 37°C, 5% CO_2 for a further 20 hours. Plates were then harvested onto filter paper and uptake determined using a beta-counter (MicroBeta TAILUX, Wallac) measuring the ionizing radiation measured for each well. Results were displayed as mean counts per minute (CPM).

2.1.15 Humoral Antibody Response Assay

A sandwich ELISA based upon that used by Bates *et al.* (2006) [210] was used to measure anti-luciferase antibody levels in sera of mice injected intramuscularly with lentivector expressing a luciferase transgene. Maxisorp™ 96-well NUNC-Immuno ELISA plates (NUNC) were coated with 200µg/mL recombinant luciferase protein (Promega) in 0.1M carbonate buffer, in 100µL/mL, and incubated overnight at 4°C. The plates were washed 3 times with wash buffer and blocked in for 1.5 hours at room temperature. Blood was obtained from mice by tail vein bleed or terminal cardiac puncture (at the

end of the experiment) all under anaesthesia. Primary antisera were collected by allowing the blood to clot at room temperature for 30-60 minutes, centrifuging for 10 mins at 13000rpm in a microcentrifuge and collecting supernatant into a fresh Micro-centrifuge tube. Sera were then aliquoted and stored at -20°C until needed. For the assay sera were diluted 1:10000, 1:100,000 and, 1:1,000,000 in blocking buffer and 100µL added to microplates in triplicate. Standards were generated by diluting a mouse anti-luciferase IgG antibody (Invitrogen) to 100ng/µL, carrying out seven serial 1:2 dilutions, and adding to plates in duplicate. Samples and standards were incubated for 2 hours at room temperature. Plates were washed 3 times, and a secondary HRP-conjugated rabbit anti-mouse antibody (Promega) was added. The plates were then washed five times and detected with the TMB (Vector Labs). The reaction was stopped by the addition of 50µL 3M H_2SO_4 (0.5 M final concentration). Plates were then read at a wavelength of 450 nm using a spectrophotometer.

2.1.16 *In Vitro* Luciferase Assay

Quantification of intracellular luciferase for *in vitro* cell line experiments was carried out using the Luciferase Assay System (Promega) as per manufacturers instructions. Cells were plated at a density of 15,000 cells/well into a 96-well, black, clear bottom, flat bottom, tissue culture treated, sterile plates (Thermo Scientific) in 100µL complete DMEM media, and incubated overnight at 37°C, 5% CO_2. The following day media was removed, 100µL of fresh complete DMEM containing a known volume or MOI of vector added and plates incubated for a further 48 hours at 37°C, 5% CO_2. All media was then removed and each well washed with 100µL 1X PBS. 20µL of 1X reporter gene assay lysis buffer (Roche) was then added to each well, plates covered with cling film and incubated overnight at -80°C. Plates were subsequently thawed at room temperature for 1 hour before assaying. Luciferase mix was made up by adding one bottle of luciferase assay buffer to one bottle of luciferase assay substrate, both provided in the kit, and kept in the dark. 100µL of luciferase assay mix was then added to each well and

the resulting light emission measured using a luminometer (FLUOstar OPTIMA plate reader) for 30 seconds and reported in relative luciferase untis (RLU).

2.1.17 *Ex Vivo* Luciferase Assay

Quantification of luciferase protein for *ex vivo* tissue homogenates was carried out using the Luciferase Assay System (Promega) as per manufacturers instructions. Tissue was freeze-thawed in 1X reporter gene assay lysis buffer (Roche), homogenized, and cell debris removed by centrifugation. 20µL of homogenate is added to a 96-well, white, clear bottom, flat bottom plate (Thermo Scientific). 100µL of luciferase mix is then added to each well and the resulting light emission measured using a luminometer (FLUOstar OPTIMA plate reader) for 30 seconds and reported in relative luciferase untis (RLU). Homogenate is then diluted 1:250 and a Bradford protein assay (BioRad) carried out as per the manufacturers instructions in a clear 96 well microplate to determine the protein concentration of each homogenate in µg/mL. Luciferase quantification in tissue can then reported as relative luciferase units per µg protein (RLU/ µg protein).

2.1.18 Bioluminescent Imaging for Intravenous Injection

Mice were treated according to approved UK Home Office and institutional guidelines at Imperial College London. Mice were anesthetised with isoflurane (Abbott Laboratories) using Boyle's apparatus (British Oxygen Company), and injected intraperitoneally with 300µL 15mg/mL D-luciferin salt (Gold Biotechnology). Mice were left for 5 minutes exactly and then imaged with an IVIS cooled charge-coupled device (CCCD) camera (Caliper Life Sciences) for 1, 10, 60 or 300 seconds. After acquiring a gray-scale photograph a bioluminescence image was obtained with a 12-cm field of view, a binning (resolution) factor of 1 or 8, and a 1/*f* stop and open filter. Regions of interest (ROIs) were defined manually, and signal intensities were calculated with Living Image software (Caliper Life Sciences) and expressed as photons per second per centimetre squared per steradian (photons/second/cm^2/sr). Background photon flux

was defined from an ROI drawn over a control mouse, or control region, into which no vector had been administered.

2.1.19 Bioluminescent Imaging for Intramuscular Injection

Mice were treated according to approved UK Home Office and institutional guidelines. at Queen Mary's University London. Mice were injected intraperitoneally of 200µL 30mg/mL D-luciferin salt. After 5 minutes animals were anaesthetised by intraperitoneal injection of 50µL of a 2:1 mixture of Ketaset® (Wyeth) and Rompun® (Bayer) and imaged with an IVIS cooled charge-coupled device (CCCD) camera (Caliper Life Sciences) for 60 seconds. After acquiring a gray-scale photograph a bioluminescence image was obtained with a 12-cm field of view, a binning (resolution) factor of 8, and a 1/*f* stop and open filter. Regions of interest (ROIs) were defined manually, and signal intensities were calculated with Living Image software (Caliper Life Sciences) and expressed as photons per second per per centimetre squared per steradian (photons/second/cm^2/sr). Background photon flux was defined from an ROI drawn over a control mouse, or control region, into which no vector had been administered.

2.1.20 Promoter Methylation Analysis Using Bisulfite Conversion

De novo methylation of promoter sequences from tissue of transduced mice was analysed using the MethylCode™ Bisulfite Conversion Kit (MECOV-50) (Invitrogen) as per manufacturer's instructions. DNA was extracted from transduced tissue and treated with bisulfite, causing unmethylated cytosines to be converted into uracil while methylated cytosines remain unchanged. Bisulfite-modified DNA was then amplified by two rounds of PCR using *Taq* and the resulting product cloned into the TOPO-TA® cloning vector pCR4® and transformed into One Shot® Stbl3™ cells. Five clones for each test group were then analyzed by DNA sequencing and the methylation profile of the promoter region determined by comparing the sequence of the bisulfite-treated DNA

to that of the untreated DNA. Primers were designed using MethPrimer software [211] and were as follows for each promoter:

Promoter Element	Primer Sequence
SFFV	FWD: 5' AAATTGTTGAGGGTTGGGTTATAT 3'
	REV: 5' ACCAAACCAAAAATAAAAAAATTCA 3'
LP1	FWD: 5' TGGAAGTTGTATATTGTTTAGGTAAAG 3'
	REV: 5' ACTAAAAAAACAAAACCCTATCCTC 3'
hAAT	FWD: 5' TGGAAGTTGTATATTGTTTAGGTAAAG 3'
	REV: 5' ACTAAAAAAACAAAACCCTATCCTC 3'
UCOE	FWD: 5' GAGAGTTAGAGAGAAGGAAAGTTAT 3'
	REV: 5' CTAAAACCCCAATTCCCAAC 3'

2.1.21 *In Vivo* Procedures

2.1.21.1 Adult Intramuscular Injection

Adult intramuscular injections were performed by Dr. David Gould (Bone and Joint Research Unit, Queen Mary's University). Mice were treated according to approved UK Home Office and institutional guidelines. Naïve adult (6-8 weeks) DBA/1 mice were injected intraperitoneally with the muscle relaxant Hypnorm™ (Janssen Pharmaceuticals) and were anaesthetised with halothane (Concord Pharmaceuticals) using Boyle's apparatus (British Oxygen Company). The fur covering the right quadracep was shaved and the exposed skin sprayed with disinfectant. 20µL of concentrated lentivector or OptiMEM was then injected intramuscularly in the right quadricep.

2.1.21.2 Adult Intravenous Injection

Adult intravenous injections of luciferase or factor VIII expressing lentivector into MF1 (outbred), or factor VIII knockout mice were performed by Dr. Simon Waddington (University College London) according to approved UK Home Office and institutional guidelines at Imperial College London. 7 to 10 week old mice were anaesthetised with isoflurane (Abbott Laboratories) using Boyle's apparatus (British Oxygen Company) and injected via the tail vein with up to 150µL of concentrated lentiviral vector. For hydrodynamic injection vector was diluted up to 2mLs in 1X PBS and injected via the tail vein in 5-7 seconds. Adult intravenous injections of luciferase or factor IX expressing lentivector into BALB/c or C57BL/6 mice was carried out at University College London via the tail vein using no anaesthetic.

2.1.21.3 Neonatal Intravenous Injection

This procedure was performed by Dr Simon Waddington (University College London). Mice were treated according to approved UK Home Office and institutional guidelines at Imperial College London. 0-1 day old neonatal mice were subject to brief (<5 minutes) hypothermic anaesthesia. The heads were subsequently illuminated and 40µL of concentrated lentiviral vector injected into the superficial temporal vein (a prominent vessel located on either side of the head, visible just below the eye) using a 33 G Hamilton needle. Following the procedure, the mice were allowed to return to normal temperature on a warming mat before being returned to their mothers.

2.1.21.4 Statistical Analysis

Statistical analyses were performed using general linear model (GLIM) based on two-way analysis of variance (ANOVA) with groupwise and individual pairwise comparisons performed using Bonferroni simultaneous tests (Minitab software, Myerstown, PA).

Chapter Three

Analysis of Transgene Expression from Lentiviral Vector Pseudotypes in Muscle

3.0 Aims

- To evaluate the tropism of lentiviral vectors pseudotyped with glycoproteins from VSV, baculovirus (gp64), EboZ, RRV, MLV-A or MLV-E in target *in vitro* cell lines.
- To assess the kinetics of GFP expression *in vitro* from both integrating and non-integrating lentiviral vector pseudotypes in the mouse muscle cell line C2C12.
- To compare expression of luciferase *in vivo* from adult DBA/1 mice injected intramuscularly with integration proficient and deficient lentiviral vectors pseudotyped with glycoproteins from VSV, baculovirus (gp64), EboZ, RRV, MLV-A or MLV-E.

3.1 Introduction

IDLV's have relatively low immunogenicity and are ideally suited for long term transgene expression in differentiated non-dividing cells. Effective, long-term (>8 months) transgene expression of GFP has been observed from integration deficient lentiviral vectors (IDLVs) in murine muscle *in vivo* by transduction of non-dividing myoblasts after intramuscular injection of vector into neonate mice [67]. Transgene expression from muscle cells can be effective for gene therapy of many monogenic diseases, such as Duchenne muscular dystrophy (DMD) and haemophilia. Genetic modification of muscle cells to produce plasma proteins has been established [212], and despite concerns that a protein not usually produced by muscle fibres will not be active due to a lack of, or incorrect, post-translational modifications, factor IX (FIX) produced by myotubes or in liver (natural producer) had similar post-translational modifications and specific activity was not affected [213]. The haemophilias are good candidates for expression from non-integrating lentiviral vectors in muscle as low levels of transgene expression (1-5%) of normal levels are sufficient for disease amelioration. Intramuscular injection of immunodeficient mice with AAV vectors expressing FIX was shown to generate 5-7% of normal FIX levels in plasma [214], the same vectors were also

studied in a canine model of haemophilia where levels reached a 1.4% normal [215]. Intramuscular injection of AAV vectors were also used in a FIX clinical trial, however, expression recorded was low [216]. The use of non-integrating lentiviral vectors may increase expression levels of FIX or FVIII *in vivo* and vectors may be less immunogenic.

The aim of this chapter is to assess whether pseudotyping lentiviral vectors with alternative envelope glycoproteins can achieve specific cell tropism and more efficient delivery to differentiated muscle cells *in vivo* in comparison to VSVg. Using alternative envelope glycoproteins may allow increased tropism and transduction of target tissue, reduce cellular toxicity associated with the VSV glycoprotein and moderate immune response to vectors *in vivo.* Integrating vectors pseudotyped with envelope glycoproteins from Baculovirus (gp64), Ebola Zaire (EboZ), Ross River Virus (RRV), MLV-Amphotropic (MLV-A) or MLV-Ecotropic (MLV-E) expressing GFP were tested *in vitro* on a variety of cell lines to assess tropism. Subsequently, expression from all pseudotypes was assessed *in vivo* using ILVs expressing the reporter gene luciferase. Expression from the most efficient pseudotypes was then tested using IDLVs expressing luciferase.

3.2 Tropism of Pseudotyped Lentiviral Vectors *In Vitro*

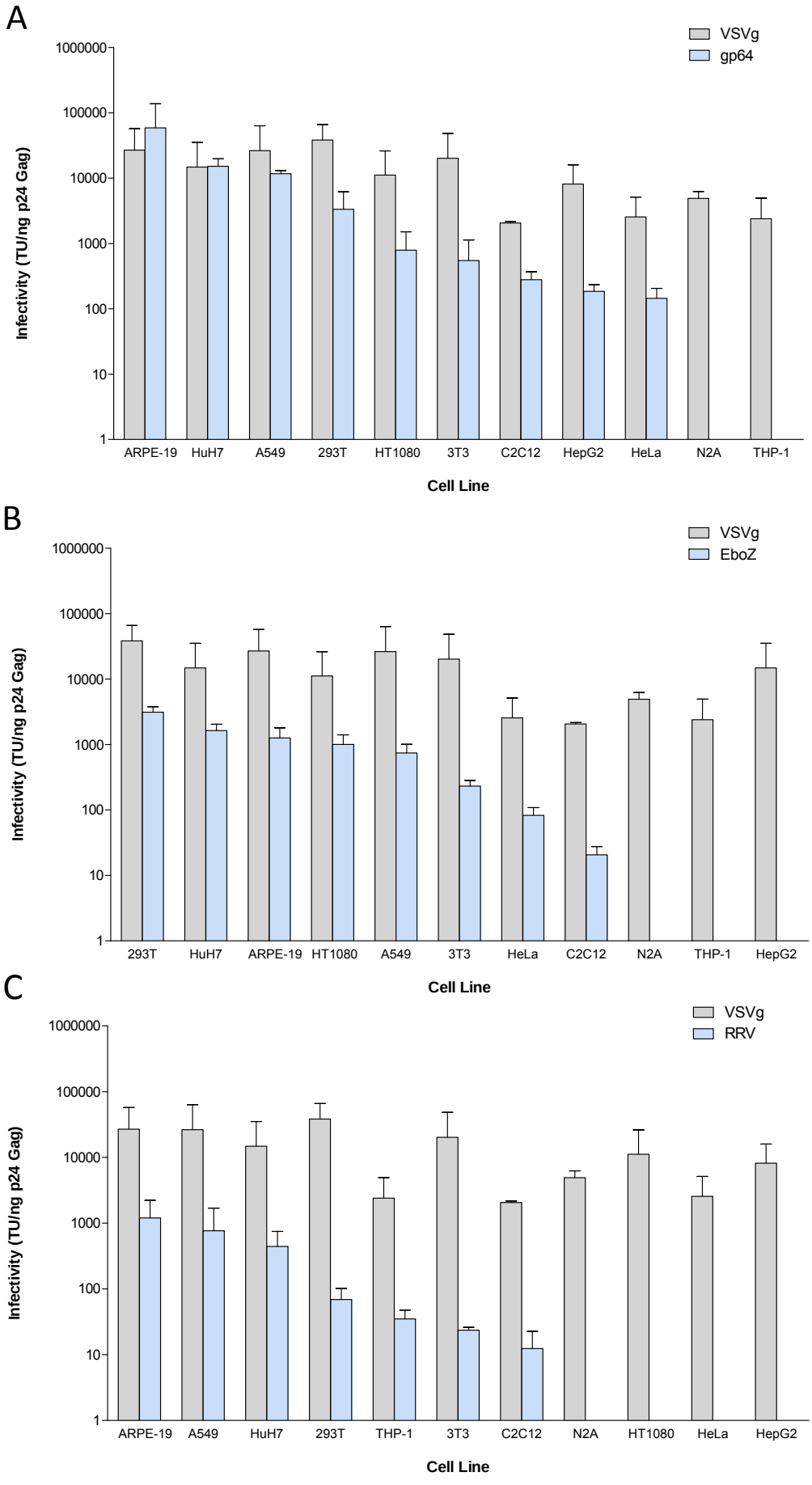
To assess the tropism of lentiviral vectors (LVs) pseudotyped with glycoproteins from Baculovirus (gp64), EboZ, RRV, MLV-A or MLV-E vectors expressing a GFP transgene driven by the Spleen Focus Forming Virus (SFFV) promoter were tested for transduction efficiency *in vitro* in eleven target cell lines. Vectors were produced by transient transfection of 293T cells and concentrated by ultracentrifugation. As VSVg is known to have a broad tropism of infection and a high transduction rate both *in vitro* and *in vivo* it was used as the standard against which all other pseudotypes were compared. Table 3.1 shows cell lines tested.

Cell Type	**Description**
HEK 293T	Human embryonic kidney cell line
C2C12	Murine myoblast cell line
NIH/3T3	Murine fibroblast cell line
A549	Human alveolar basal epithelial cell line
Neuro-2a (N2A)	Murine neuroblastoma cell line
HT1080	Human fibrosarcoma cell line
HeLa	Human adenocarcinoma epithelial cell line
THP-1	Human acute monocytic leukemia cell line
Hep-G2	Human hepatocellular liver carcinoma cell line
Huh7	Human hepatoma cell line
ARPE-19	Human retinal pigment epithelial cells

Table 3.1 Target cell lines tested for viral infectivity of pseudotyped vectors. Eleven cell lines were tested for viral infectivity of lentiviral vectors pseudotyped with envelope glycoproteins from Baculovirus (gp64), Ross River Virus, Ebola Zaire, MLV-Ampho and MLV-Ecotropic in comparison to VSVg. All cell types shown are cultured *in vitro* cell lines, no primary cells were tested.

Viral tropism was calculated using a measure of 'infectivity': 1 x 10^5 cells were transduced with 10, 2, 0.4, 0.08 or 0.016µL concentrated integrating vector for each pseudotype and the viral titre calculated in transducing units per mL (TU/mL) based on the percentage GFP positive cells as determined by flow cytometry. Each individual titre was then normalized against total viral particles in each vector preparation using $p24^{Gag}$ concentrations in ng/mL, as determined by ELISA. 'Infectivity' values were therefore calculated by dividing the titre values in TU/mL by the $p24^{Gag}$ concentrations in ng/mL giving a measure in TU/ng $p24^{Gag}$. This method of investigating infectivity was based upon a method used by [115]. Each vector pseudotype was tested twice using two separate vector preparations and results collated (Figure 3.1).

Figure 3.1 shows the results for integrating vectors pseudotyped with glycoproteins from baculovirus (gp64), EboZ, RRV, MLV-A and MLV-E, respectively, in comparison to VSVg. All pseudotyped vectors were produced efficiently and were capable of transducing cell lines. All alternative pseudotyped vectors showed narrower tropic ranges in comparison to VSVg and reduced transduction efficiency with the notable exception of gp64, where ARPE-19 (retinal pigment epithelial) and Huh7 (human hepatoma) cells were more efficiently transduced (Figure 3.1A). Vector pseudotyped with MLV-E, a mouse specific envelope protein, was only seen to transduce cells of murine origin.


A
VSVg
gp64
Infectivity (TU/ng p24 Gag)
1000000
100000
10000
1000
100
10
1
ARPE-19
HuH7
A549
293T
HT1080
3T3
C2C12
HepG2
HeLa
N2A
THP-1
Cell Line
B
VSVg
EboZ
Infectivity (TU/ng p24 Gag)
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HuH7
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HepG2
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VSVg
RRV
Infectivity (TU/ng p24 Gag)
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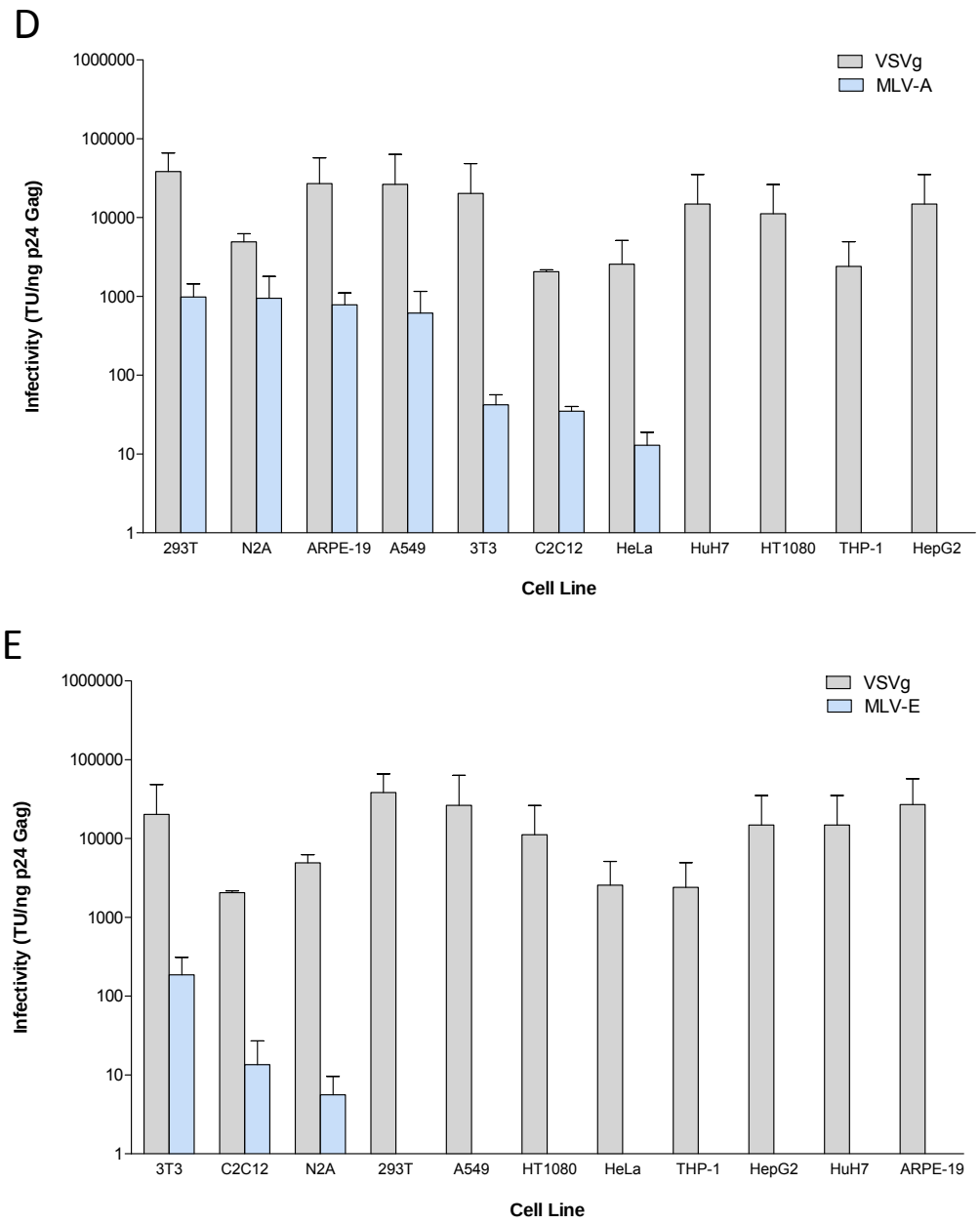


Figure 3.1 Analysis of the infectivity of VSVg, gp64, EboZ, RRV, MLV-A and MLV-E pseudotyped lentivectors on eleven target cell lines. Lentiviral vectors expressing GFP were pseudotyped with envelope glycoproteins from either VSVg, **(A)** baculovirus (gp64), **(B)** EboZ, **(C)** RRV, **(D)** MLV-A or **(E)** MLV-E. These preparations were titred by FACs on eleven target cell lines and titre values in TU/mL were normalized against total particles in each vector preparation via dividing by $p24^{Gag}$ concentrations in ng/mL to give infectivity values (TU/ng $p24^{Gag}$). Each vector was tested twice using two vector preparations.

3.3 Kinetics of Transgene Expression by Integration Proficient and Deficient Vectors *In Vitro*

VSVg pseudotyped IDLVs have previously been shown to mediate stable expression of GFP in murine muscle both *in vitro* in C2C12 cells and *in vivo* in neonate MF1 mice [67]. C2C12 cells provide a convenient *in vitro* model of mouse muscle cells as they can remain undifferentiated and divide under normal cell culture conditions or can be terminally differentiated into myoblasts which form contractile myotubes and express muscle specific proteins when media is supplemented with 2% horse serum [217]. In this experiment we assessed the kinetics of transgene expression of vectors pseudotyped with glycoproteins from Baculovirus (gp64) and MLV-A as these were identified from the experiment in section 3.2 as having a relatively high infectivity of C2C12 cells. Both integration proficient and deficient vectors were produced which expressed GFP and 1 x 10^5 C2C12 cells transduced at an MOI of 10 for VSVg, and 25 for gp64 and MLV-A. Post-transduction, dividing cells were grown and split 1:6 in complete DMEM every 3 days. To produce non dividing cells addition of 2% horse serum to the media differentiated cells, arresting cell division at day 3. Transgene expression was assessed over time using flow cytometry.

Results reported in Figure 3.2 show that dividing C2C12 cells transduced with all integration proficient vectors had stable expression of GFP over time due to integration of the provirus into the cell genome. Expression was maximum at 6 days post-transduction for all pseudotypes tested. Due to cell division, by day 8 IDLV expression had dropped significantly in dividing cells, and by day 12 there were only 0-5% GFP positive cells remaining for each group. For non-dividing C2C12 cells a similar pattern of expression was seen for both integration proficient and deficient vectors over the 12 day duration of the experiment for each vector group. A loss of expression was seen over time with all vectors and this likely due to cell death. GFP expression was similar between all vector groups however VSVg pseudotyped vectors had a higher percentage of GFP positive cells after transduction of cells at an MOI of 10, in comparison gp64 and

MLV-A pseudotyped vectors where GFP expression was around 20% lower despite transduction of cells at an MOI of 25 (Figure 3.2).

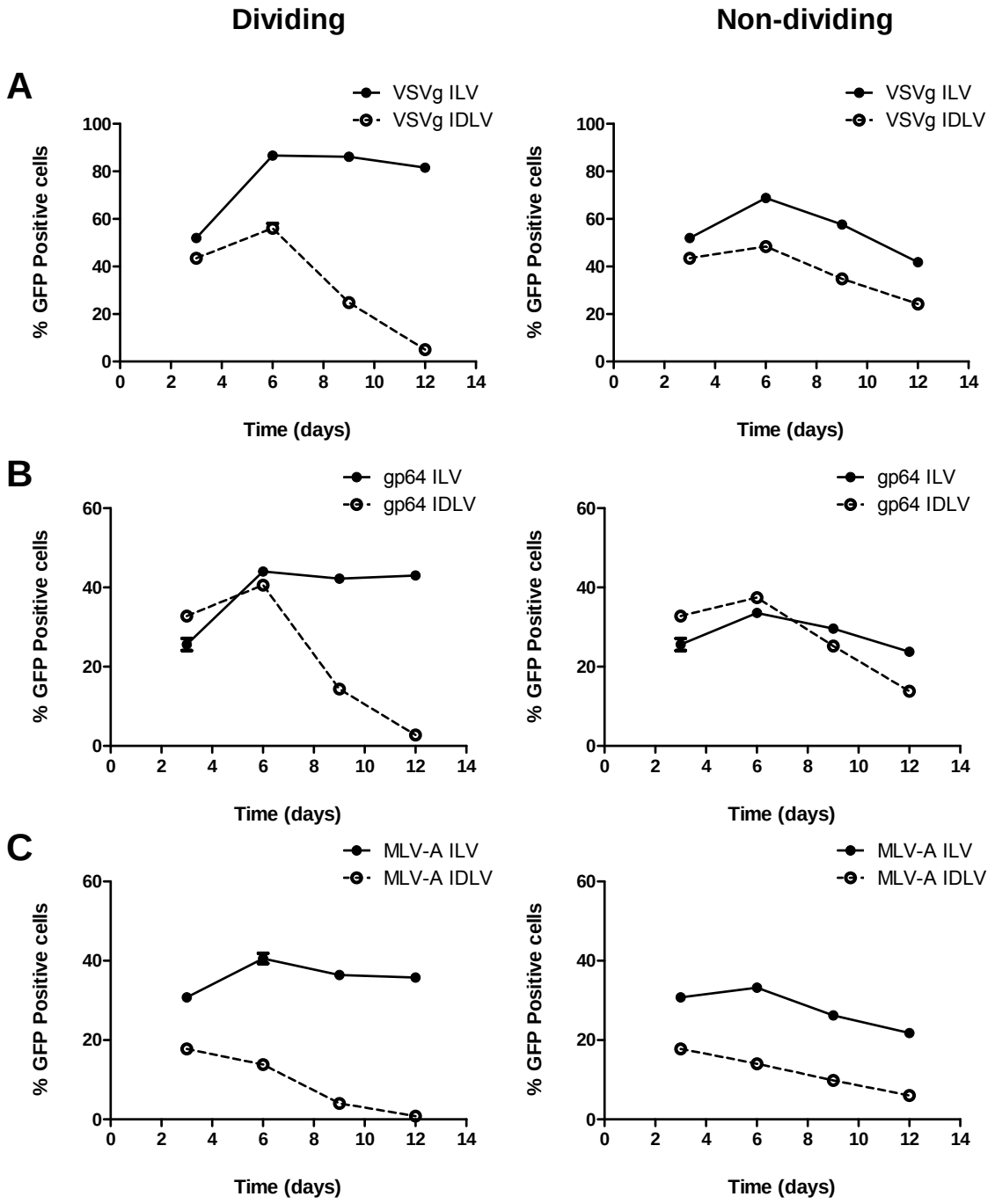


Figure 3.2 Expression kinetics profile of pseudotyped lentiviral vectors in dividing and non-dividing C2C12 cells. Five wells of cultured C2C12 myoblast cells were infected with vectors expressing GFP at an MOI of 10 for VSVg and 25 for both gp64 and MLV-A. Transgene expression was measured over time by flow cytometry. Differentiation was induced using medium containing 2% horse serum, arresting cell division at 3 days after transduction. Graphs on the left represent dividing C2C12 cells and those on the right represent differentiated non-dividing C2C12 cells. **(A)** Cells transduced with VSVg pseudotyped vectors **(B)** Cells transduced with gp64 pseudotyped vectors **(C)** Cells transduced with MLV-A pseudotyped vectors. Mean and SEM plotted for five values for each point. (n=5).

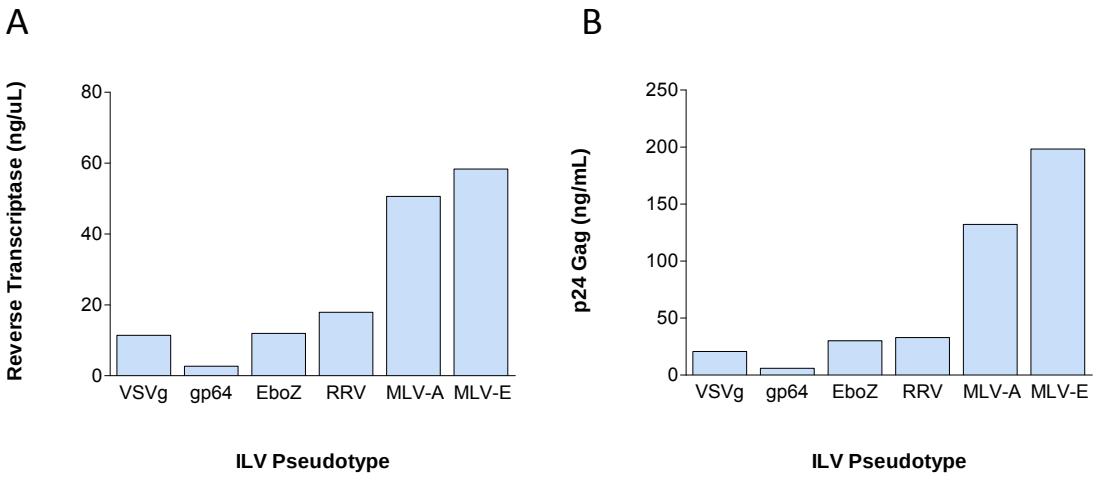
3.4 Transduction Efficiency of Pseudotyped Lentiviral Vectors *In Vivo* in Murine Muscle

To investigate the transduction of murine muscle by pseudotyped lentivectors *in vivo* vectors were produced expressing a firefly luciferase transgene. Luciferase is an enzyme which catalyses the oxidation of the pigment luciferin, in this reaction light is produced: Luciferin + O_2 → Oxyluciferin + Light. Luciferase is a commonly used tool in biology, and is especially useful in gene therapy for investigating the biodistribution and efficiency of transgene expression *in vivo* as light emission can be detected after the administration of luciferin into transduced animals. Luciferase reporter gene expression in anaesthetised animals in this experiment is measured in photons using an IVIS cooled charge-coupled device (CCCD) camera. The low background of luminescence from normal tissue, the rapid turnover of luciferase enzyme, and the non-immunogenic characteristics of luciferin make this method ideally suited for temporal *in vivo* imaging of gene expression. It also allows consecutive bioluminescent images to be acquired from the same animals, providing temporal and spatial information throughout an entire experiment, and a reduction in the number of mice needed for experiments.

3.4.1 Physical Titre and *In Vitro* Expression from Integrating Lentivector Pseudotypes

ILVs expressing luciferase under control of the SFFV promoter and pseudotyped with either VSV, gp64, EboZ, RRV, MLV-A or MLV-E glycoproteins were produced by transient transfection methods. Vector preparations were tested for physical titre using both a reverse transcriptase (RT) colorimetric assay and p24 ELISA to calculate the RT and $p24^{Gag}$ protein concentration, respectively, in each vector stock, allowing an estimation of the number of physical transducing units (TU). Figure 3.3 shows the protein concentration and estimated titres for each vector preparation calculated from **(A)** RT protein (ng/µL) and **(B)** $p24^{Gag}$ (ng/mL). The RT and $p24^{Gag}$ titre estimations were calculated using the methods outlined in Buckley *et al.* (2008) [218]. Transgene expression

was then quantified *in vitro* in three cell lines; 293T, C2C12 and 3T3 to assess the relative expression levels of luciferase for each pseudotype. 1.5 x 10^3 cultured cells were transduced with serial dilutions of vector and luciferase expression quantified 72h later as relative luciferase units (RLU) using an *in vitro* luminescence assay. RLU values were then normalized against total particles in each vector preparation via dividing by RT concentration in ng to give expression values as RLU/ng RT (Figure 3.4). Surprisingly, all pseudotypes showed higher relative luciferase expression in C2C12 cells than in 293T cells, including VSVg, contradicting the previous experiment (Figure 3.1) where GFP expression was normalised by $p24^{Gag}$ protein in ng. The reason for such a large difference in expression between these experiments is unknown, but could possibly be due to differences in assay conditions or vector preparations.



Virus	RT protein (ng/µL)	Estimated Titre (TU/mL)
VSVg	11.4	3.88×10^{9}
gp64	2.7	9.03×10^{8}
EboZ	11.9	4.05×10^{9}
RRV	17.9	6.10×10^{9}
MLV-A	50.6	1.72×10^{10}
MLV-E	58.3	1.98×10^{10}

Virus	$p24^{Gag}$ protein (ng/mL)	Estimated Titre (TU/mL)
VSVg	20.7	1.24×10^{9}
gp64	5.9	3.54×10^{8}
EboZ	30.1	1.80×10^{9}
RRV	32.9	1.98×10^{9}
MLV-A	132.1	7.93×10^{9}
MLV-E	198.3	1.19×10^{10}

Figure 3.3 Quantification of physical vector titres for ILV pseudotypes expressing luciferase. (A) Quantification of Reverse Transcriptase (RT) protein concentration in viral stocks, measured by performing a RT colorimetric assay, quantified in ng/µL and estimated vector titres from this assay. **(B)** Quantification of $p24^{Gag}$ protein concentration in viral stocks, measured by performing a $p24^{Gag}$ ELISA, quantified in ng/mL and estimated vector titres from this assay. Mean of two values plotted.

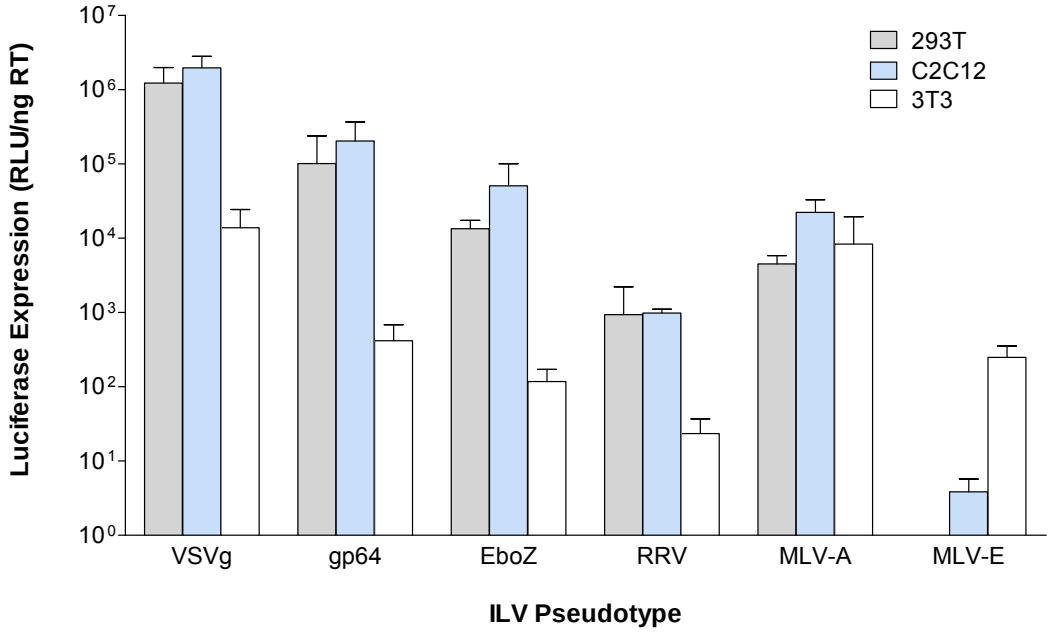


Figure 3.4 Relative luciferase expression from ILV pseudotypes *in vitro*. Concentrated preparations of lentiviral vectors carrying a luciferase transgene were produced by transient transfection and pseudotyped with envelope glycoproteins from either VSVg, gp64, EboZ, RRV, MLV-A or MLV-E. 1.5 x 10^3 cultured cells were transduced with serial dilutions of vector and luciferase expression quantified 72h later as relative luciferase units (RLU) using an *in vitro* luminescence assay. RLU values were then normalized against total particles in each vector preparation via dividing by RT concentration in ng. Expression for 293T; human embryonic kidney cells, C2C12; murine myoblasts and 3T3; murine fibroblasts was calculated. Mean and SEM plotted for 6 values.

3.4.2 Expression of Luciferase *In Vivo* after Intramuscular Injection of ILV Pseudotypes

Integrating vectors tested *in vitro* in section 3.4.1 were injected intramuscularly into adult (6-8 week old) DBA/1 mice to assess transduction *in vivo*. Each vector was injected based upon volume and not upon titre with 20µl of each preparation injected into the right quadricep for four mice per vector pseudotype. The estimated number of transducing units (TU) injected into each mouse, based upon the reverse transcriptase protein assay, was:

Vector Pseudotype Group	Estimated Transducing Units injected per mouse
VSVg	7.76×10^7
gp64	1.81×10^7
EboZ	8.10×10^7
RRV	3.96×10^7
MLV-A	3.44×10^8
MLV-E	3.96×10^8

Two control mice were also injected with 20µL OptiMEM as negative controls. Quantification of *in vivo* luciferase expression was assessed using an IVIS cooled charge-coupled device (CCCD) camera which measures bioluminescence in photons after intraperitoneal injection of luciferin. Imaging was carried out for all animals at 7, 30 and 60 days post-injection at a binning of 8 for 60 seconds. Representative images from day 7 post-injection are shown in Figure 3.5. Light emission was quantified in a defined region of interest (ROI) in photons/second/cm^2/sr over the right quadricep; shown as a circle on the representative images. Quantification of light emission in the defined ROI for consecutive images allowed expression levels to be monitored over time in each animal, the results shown in Figure 3.6. Fatalities occurred during the experiment with the deaths of two mice injected with VSVg pseudotyped vector; one at day 0 and another at day 30 post-injection. All mice injected with MLV-A vector died day 30 post-injection. Fatalities were due anaesthetic toxicity.

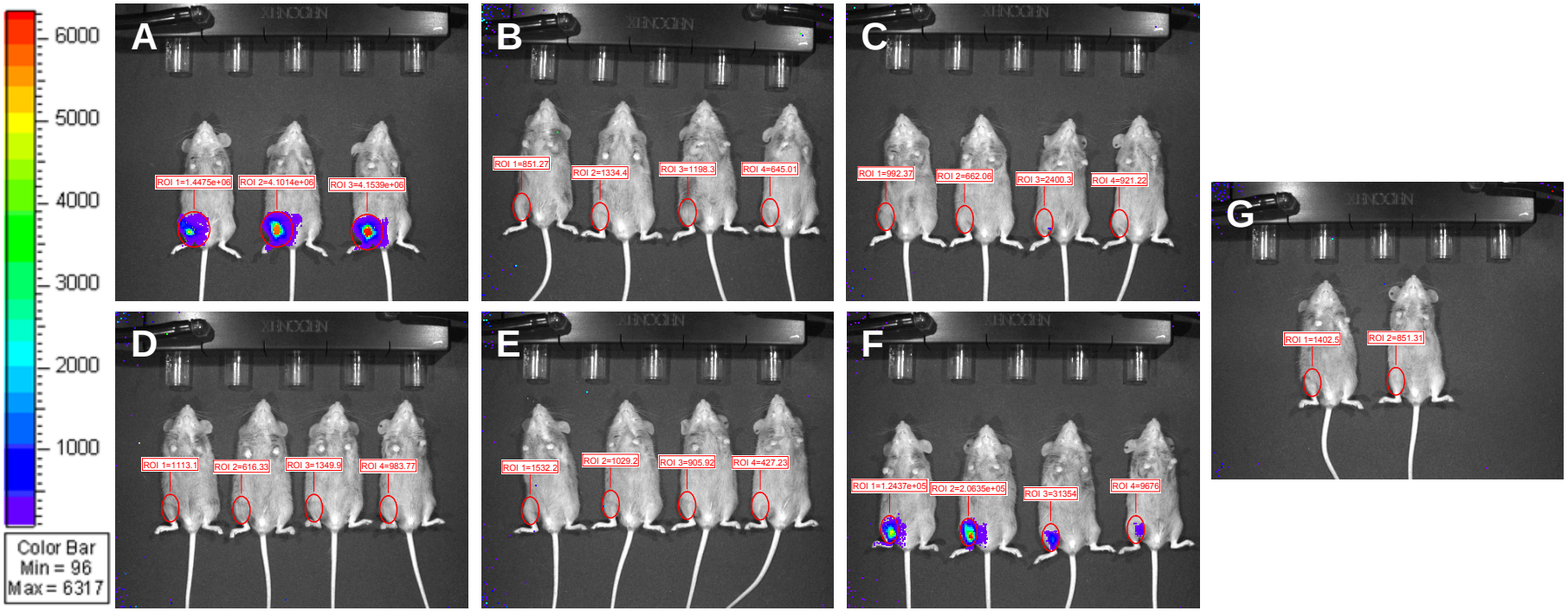


Figure 3.5 Representative images of luciferase expression 7 days post-intramuscular injection of pseudotyped lentiviral vectors. Representative images are shown of mice 7 days post-injection of 20µL **(A)** VSVg, **(B)** gp64, **(C)** EboZ, **(D)** RRV, **(E)** MLV-A, **(F)** MLV-E pseudotyped integrating lentiviral vector expressing a luciferase transgene into the right quadricep. **(G)** Control mice were injected with 20µL OptiMEM into the right quadricep. Light emission was measured using an IVIS CCCD camera in photons at a binning of 8 for 60 seconds. A defined region of interest (ROI) over the injected area is shown as a circular region over each mouse. Luminescence within this ROI is quantified in photons/second/cm^2/sr.

All test mice had expression in the area of injection above background. In particular, VSVg and MLV-E vector groups showed expression substantially above that recorded for control mice at day 7 post-injection. However, luciferase expression in these groups was then seen to fall from this point: At day 7 post-injection expression from VSVg injected mice was an average of 3.23 x 10^6 photons/second/cm^2/sr, by day 30 this had dropped 50-fold to 6.04 x 10^4, and by day 60 a further 2-fold drop to 2.41 x 10^4. Loss of expression for MLV-E injected mice was equally as pronounced with an average of 1.63 x 10^5 photons/second/cm^2/sr at day 7 dropping 8-fold to 2.07 x 10^4 by day 30, and a further loss by 2-fold to 9.80 x 10^3 at day 60.

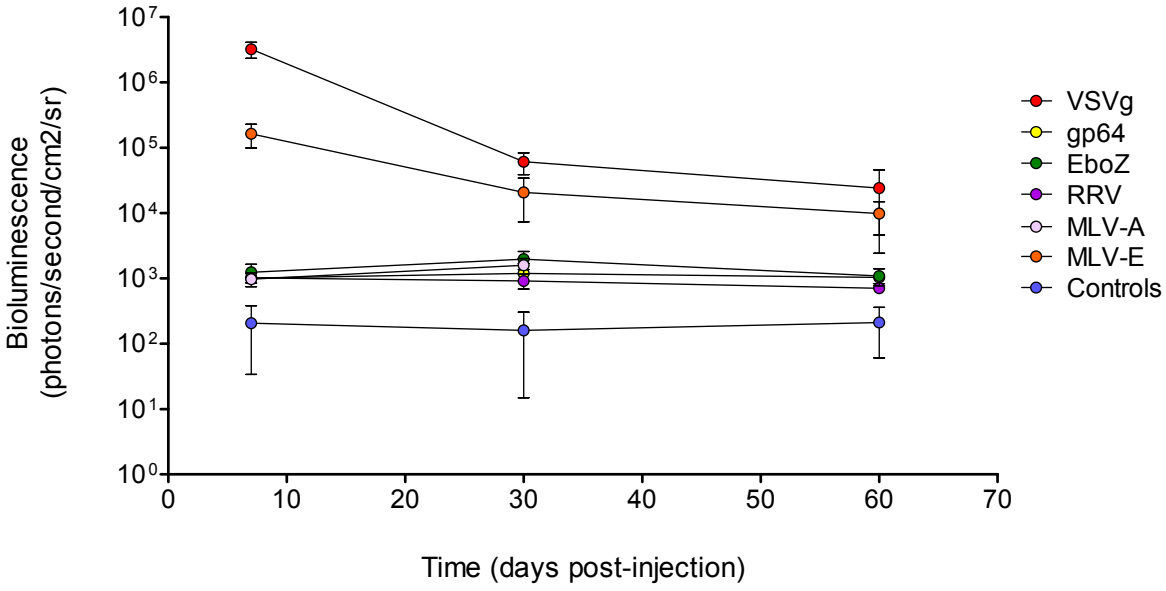


Figure 3.6 Quantification of luciferase expression after intramuscular injection of pseudotyped lentiviral vectors. Light emission was quantified in a defined region of interest (ROI) over the right quadricep in photons/second/cm^2/sr for adult DBA/1 mice injected intramuscularly with 20µL pseudotyped lentiviral vector expressing a luciferase transgene or 20µL OptiMEM for control mice. Pseudotypes tested were; VSVg, gp64, EboZ, RRV, MLV-A and MLV-E. Mean and SEM plotted for each point. (VSVg; n=3 for days 7 and 30 and n=2 for day 60, gp64 n=4, EboZ n=4, RRV n=4, MLV-A n=4 and MLV-E; n=4, controls n=2).

3.4.3 Proviral Copy Number and Quantification of Luciferase Expression in Injected Tissue

All remaining mice were sacrificed at 60 days post-injection. The right quadricep, spleen, and blood by cardiac puncture were taken from each mouse for analysis (see section 3.4.4). Injected muscle tissue was analysed for luciferase expression using the Luciferase Assay System (Promega) and a Bradford protein assay (BioRad) to give relative luciferase units (RLU) per μg protein. The number of integrated proviral copies was also assessed using quantitative real-time PCR (qPCR). Primer and probe sets to the murine housekeeping gene *titin* and the viral WPRE sequence allows determination of integrated proviral copy number per cell.

Proviral copy in muscle tissue from all injected animals was very low, <0.1 copies/cell for all injected groups (Figure 3.7A). VSVg and MLV-E injected mice had the highest results with an average of 0.09 and 0.02 copies/cell for 2 and 3 mice, respectively. All other mice, test and control, had undetectable levels of WPRE DNA and therefore were seen to have zero viral copies per cell. Luciferase protein was detected in all samples, including control tissue which had a basal expression level of ~1500 RLU/ μg protein. Muscle tissue from VSVg and MLV-E injected mice had average values of 3.0×10^4 and 1.4×10^4 RLU/μg protein, respectively. All other vector groups expressed average levels between 2400-4400 RLU/μg protein; marginally higher than the control tissue, yet not significantly different.

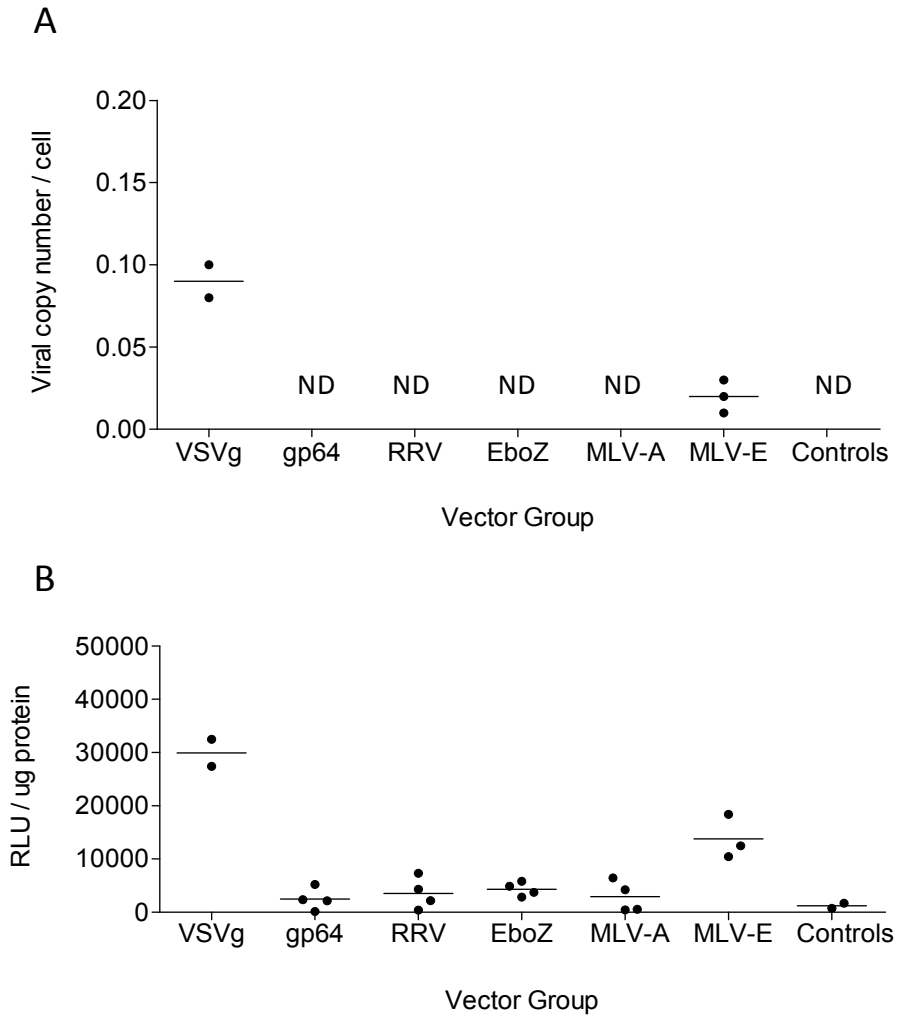


Figure 3.7 Quantification of viral copy number and luciferase protein in muscle tissue taken from mice injected intramuscularly with pseudotyped lentiviral vectors. Injected muscle tissue (right quadricep) was taken from each mouse at 60 days post-transduction, except MLV-A injected mice where tissue was taken at day 30 due to fatality. **(A)** Genomic DNA was extracted and qPCR carried out for each sample for both the murine housekeeping gene *titin* and the viral WPRE sequence to determine integrated proviral copy number per cell. ND = Not-detected; WPRE copy was not detected in these tissues. **(B)** The *ex vivo* Luciferase Assay System (Promega) and a Bradford protein assay (BioRad) were used to quantify the amount of luciferase protein in relative luciferase units (RLU) per µg protein in tissue homogenates. Mean plotted for each pseudotype. (VSVg n=2, gp64 n=4, RRV n=4, EboZ n=4, MLV-A n=4, MLV-E n=3, controls n=2).

3.4.4 Analysis of the Loss of Expression Observed in VSVg and MLV-E Vector Groups

Mice injected with VSVg or MLV-E pseudotyped ILV suffered a significant loss of transgene expression from 7 days post-injection (see Figure 3.6). To assess why this loss occurred two rationales were investigated; firstly whether an immune response had been mounted against the transgene product, and secondly if *de novo* methylation of the SFFV promoter caused expression to be down regulated.

3.4.4.1 Analysis of Immune Response to Luciferase

To see if an anti-transgene immune response was involved in the loss of gene expression we monitored both the animals' cellular and humoral immunologic responsiveness to luciferase protein.

To test for cellular immunogenicity to luciferase protein a splenocyte proliferation assay was conducted. The method devised was based on that used by Brown *et al.* (2007) [151] for human factor IX who reported that after intravenous injection of lentivector a cellular immune response resulted in clearance of transduced cells and a loss of transgene expression. Spleens collected from control, VSVg and MLV-E vector groups were processed into single cell suspensions and splenocytes plated at 2 x 10^5 cells/well in triplicate in 96-well plates. Cells were then stimulated with recombinant luciferase protein (Promega) at 10µg/mL, phytohemagglutinin (PHA) (Sigma), a known mitogen and positive control, at 5µg/mL, or left naïve. After 96 hours cells were pulsed with 1µCi/well methyl-^{3}H-Thymidine and after a further 20 hour incubation plates harvested onto filter paper and uptake determined using a beta-counter. Results are displayed as mean counts per minute (CPM) (Figure 3.8). The proliferation assay demonstrates that when splenocytes were grown in the presence of PHA cell populations were viable and able to be stimulated; average counts for all groups were between 15,000 – 30,000 CPM. However, cells grown in the presence or absence of

luciferase showed no significant difference in CPM for control, VSVg or MLV-E injected mice, indicating no cellular immune response to luciferase protein with this assay.

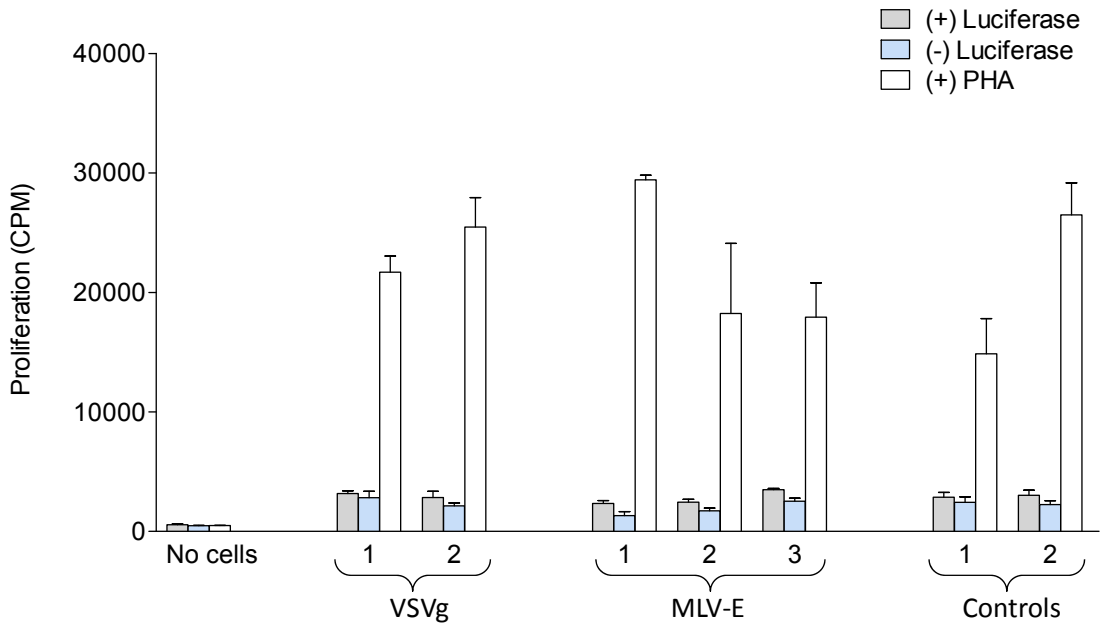


Figure 3.8 Evaluation of cellular immune response to luciferase protein. A splenocyte proliferation assay was carried out to determine response to luciferase protein. Splenocyte populations were collected from VSVg (2 mice), MLV-E (3 mice) and control (2 mice) and plated at $1x10^5$ cells/well in triplicate in 96-well plates in the presence or absence of luciferase protein (10µg/mL) or in the presence of PHA (5µg/mL). After 96 hours 1µCi/well methyl-^{3}H-Thymidine was added and after 20 hours further incubation plates were harvested and read. Results presented as counts per minute (CPM). Mean and SEM of two separate experiments. (n=6 microplate wells)

To measure humoral immune response to luciferase protein a sandwich ELISA based on that used by Bates *et al.* (2006) [210] was used to detect anti-luciferase antibody levels in the sera of treated and untreated mice. Immunoplates were coated with 200µg/mL recombinant luciferase protein, blocked, washed, and primary antisera from control, VSVg and MLV-E treated mice were diluted and incubated on plates. Plates were then measured using a peroxidase-conjugated anti-mouse IgG and TMB substrate and read at an OD of 450nm. Absorbance values were converted to antibody levels in µg/mL by comparison to a monoclonal antibody of known concentration (Figure 3.9). No difference was seen between values obtained for control, VSVg or MLV-E injected groups using this assay, with all antibody levels calculated between 3-8µg/mL (Figure 3.9).

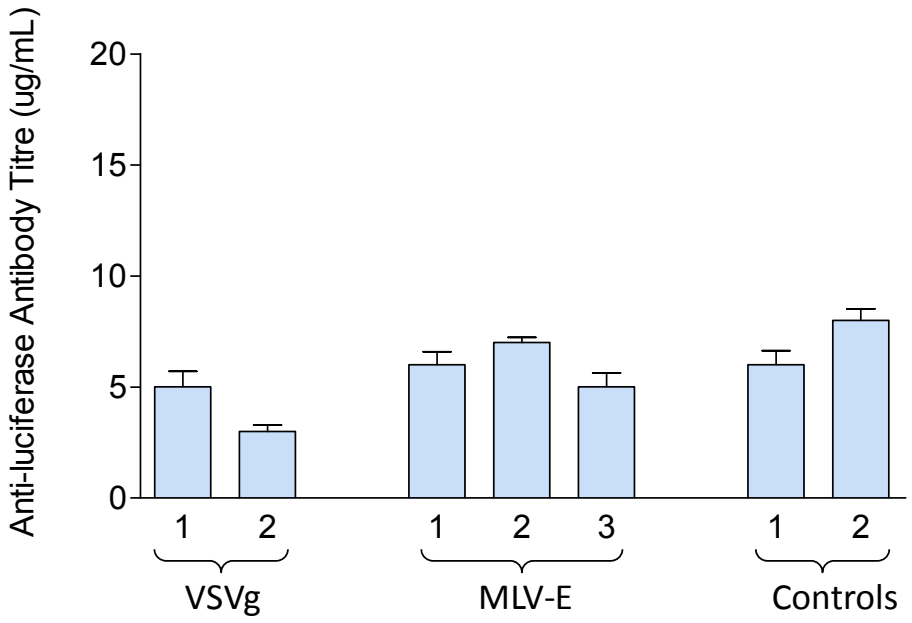


Figure 3.9 Evaluation of a humoral immune response to luciferase protein. Levels of anti-luciferase antibodies were determined in the blood sera of VSVg (2 mice) and MLV-E (3 mice) injected and control mice (2 mice). Blood sera were collected from mice at day 60 post-injection via cardiac puncture. Antibody levels were measured by ELISA and converted to µg/mL by comparison to a monoclonal antibody of known concentration. Results presented as the mean and SEM of two separate experiments. (n=6 microplate wells)

3.4.4.2 Methylation Analysis of the SFFV Promoter

To study if *de novo* methylation of the SFFV promoter has occurred causing inhibition of transgene transcription, and therefore a drop in luciferase expression, methylation analysis of a 194bp region of the SFFV promoter containing 10 potential CpG methylation sites was carried out using the MethylCode™ Bisulfite Conversion Kit (Invitrogen). Genomic DNA from injected muscle from one mouse in the VSVg and MLV-E test groups was extracted and treated with bisulfite, causing unmethylated cytosines to be converted into uracil while methylated cytosines remained unchanged. Bisulfite-modified DNA was then amplified by PCR and the resulting product cloned into the vector pCR4® and transformed into Stbl3 cells. Five clones for each test group were then analyzed by DNA sequencing and the methylation profile of the promoter region determined by comparing the sequence of the bisulfite-treated DNA to that of the untreated DNA.

Figures 3.10 shows analysis for 5 different clones for DNA cloned from injected muscle of one mouse in VSVg or MLV-E test groups where shaded boxes indicate the presence of a methylated CpG site. For both the VSVg and MLV-E injected mice sequence analysis of five clones showed that of the ten sites contained in the 194bp region of the SFFV promoter tested on average only one CpG site was methylated in each clone indicating that the entire promoter region is unlikely to be extensively methylated.

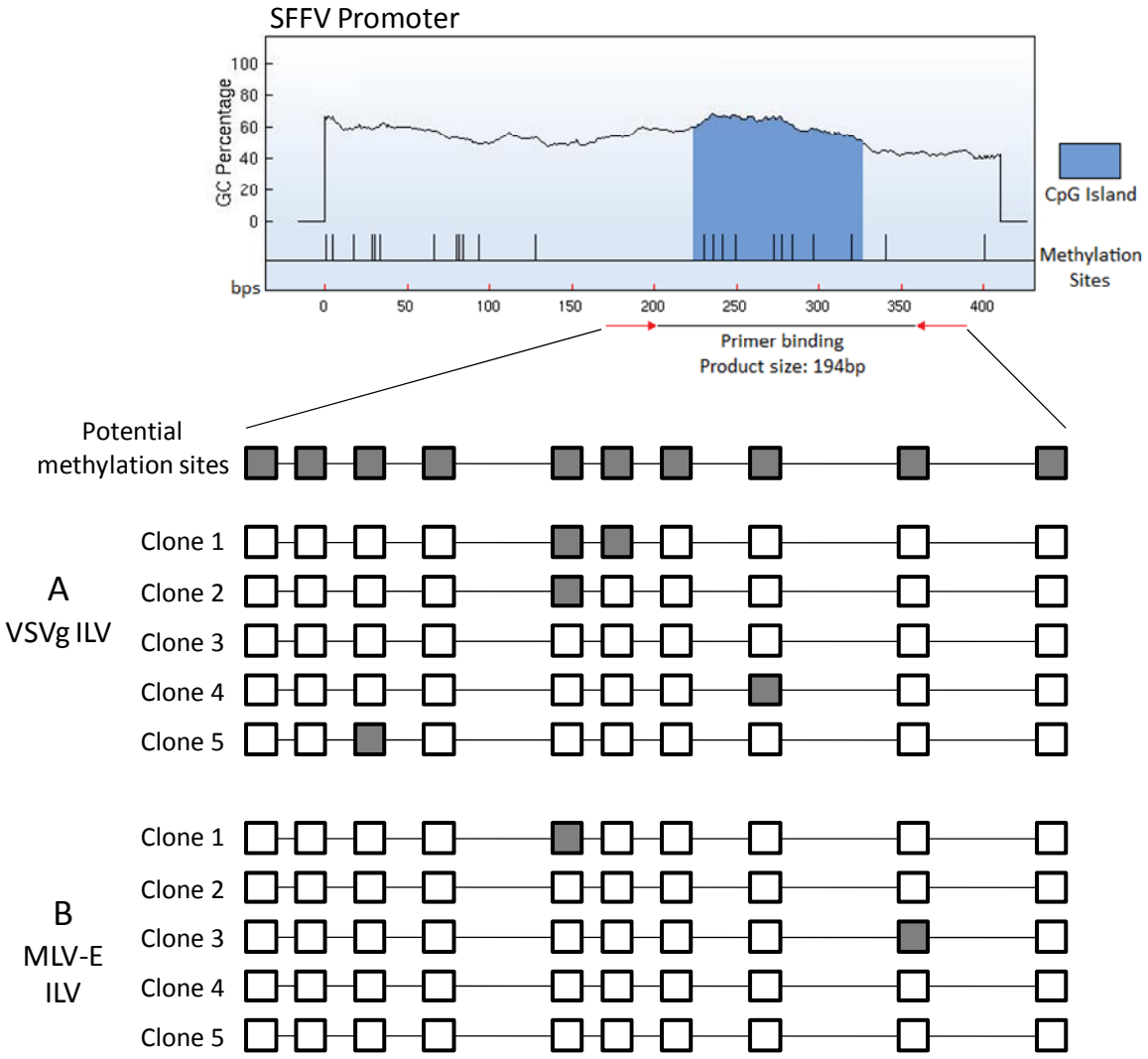


Figure 3.10 Methylation analysis of the SFFV promoter from integrated proviral DNA taken from muscle injected with ILV pseudotypes. A 194bp region of the SFFV promoter containing 10 potential CpG methylation sites was analysed from integrated proviral DNA taken from ILV injected muscle. DNA was treated with bisulfite, causing unmethylated cytosines to be converted into uracil while methylated cytosines remain unchanged. Bisulfite-modified DNA was then amplified by PCR using *Taq* and the resulting product cloned into the TOPO-TA® cloning vector pCR4®. Five clones were then analyzed by DNA sequencing and the methylation profile of the region determined by comparing the sequence of the bisulfite-treated DNA to that of the untreated DNA. Potential CpG methylation sites are represented as squares and are shaded if determined to be methylated. **(A)** Analysis of muscle DNA from VSVg ILV injected mouse **(B)** Analysis of muscle DNA from MLV-E injected mouse.

3.5 Transduction Efficiency of Integration Deficient Lentiviral Vectors *In Vivo* in Murine Muscle

It has previously been reported that sustained expression of a GFP transgene can be mediated from a non-integrating vector *in vivo* in neonate muscle [67]. In this section we investigate if non-integrating vectors pseudotyped with envelope glycoproteins from VSVg or MLV-E can achieve sustained expression of a luciferase transgene *in vivo* in adult muscle.

3.5.1 *In Vitro* Expression from Integration Proficient and Deficient Lentiviral vectors

Both integrating and non-integrating vectors pseudotyped with VSVg or MLV-E glycoproteins were produced by transient transfection of 293T cells. Vector preparations were tested using a reverse transcriptase (RT) protein assay to calculate physical titre and estimate transducing units per mL. Luciferase transgene expression was then quantified *in vitro* for all vectors in 293T, 3T3 and C2C12 cell lines in RLU and then normalized against total viral particles to give expression values as RLU/ng RT (Figure 3.11).

The reverse transcriptase assay shows that all vectors were able to be made effectively at titres of ~2 x 10^9 TU/mL for VSVg pseudotyped vectors and ~1 x 10^{10} TU/mL for MLV-E pseudotyped vectors. The *in vitro* assay reported very high levels of luciferase expression per ng RT protein for all pseudotypes tested in all cells lines. However, all vectors showed higher relative luciferase expression in 293T and 3T3 cells than in C2C12 cells, contradicting the previous experiment shown in section 3.4.1.

Vector Group	RT protein (ng/µL)	Estimated Titre (TU/mL)
VSVg ILV	7.50	2.55×10^9
VSVg IDLV	6.20	2.11×10^9
MLV-E ILV	32.5	1.11×10^{10}
MLV-E IDLV	40.5	1.38×10^{10}

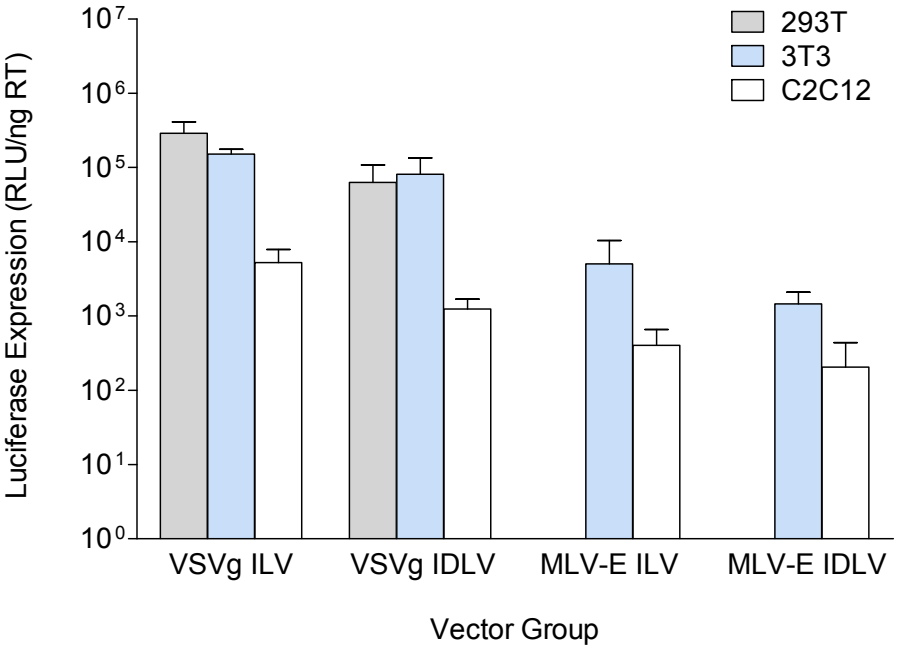


Figure 3.11 Quantification of vector titre and relative luciferase expression *in vitro* of VSVg or MLV-E pseudotyped ILV or IDLVs. Quantification of Reverse Transcriptase (RT) protein concentration in viral stocks, measured by performing a RT colorimetric assay, quantified in ng/µL and estimated vector titres from this assay. 1.5×10^3 cultured cells were transduced with serial dilutions of vector and luciferase expression quantified as relative luciferase units (RLU) which were then normalized against virus physical titre via dividing by RT in ng. Expression is shown for 293T, 3T3 and C2C12 cells. Mean and SEM plotted for n=6.

3.5.2 Expression of Luciferase *In Vivo* after Intramuscular Injection of ILV and IDLV Pseudotypes

All vectors tested *in vitro* in section 3.5.1 were injected intramuscularly into adult (6-8 week old) DBA/1 mice to assess transduction efficiency of integration deficient vectors in murine muscle. Vector was injected based upon volume and not upon titre; 20µl of each preparation was injected into the right quadricep for three mice per for integrating vector groups, and four mice for non-integrating vector groups. The estimated number of transducing units (TU) injected into each mouse, based upon the reverse transcriptase protein assay, was:

Vector Pseudotype Group	**Estimated Transducing Units injected per mouse**
VSVg ILV	5.1×10^7
VSVg IDLV	4.22×10^7
MLV-E ILV	2.22×10^8
MLV-E IDLV	2.76×10^8

Quantification of *in vivo* luciferase expression was assessed using an IVIS cooled charge-coupled device (CCCD) camera after intraperitoneal injection of luciferin. Imaging was carried out for all animals at 8, 30 and 80 days post-injection at a binning of 8 for 60 seconds. Representative images from day 8 post-injection are shown in Figure 3.12 (please note the different scales for each image). Light emission was quantified in a defined region of interest (ROI) in photons/second/cm^2/sr over the right quadricep; shown as a circle on the representative images. As this experiment has no control mice a control ROI of the same size and shape was also measured in the abdominal area of each mouse at each time point. The value from the control region is then subtracted from the test value and this level plotted as a quantification of luciferase expression over time (Figure 3.13).

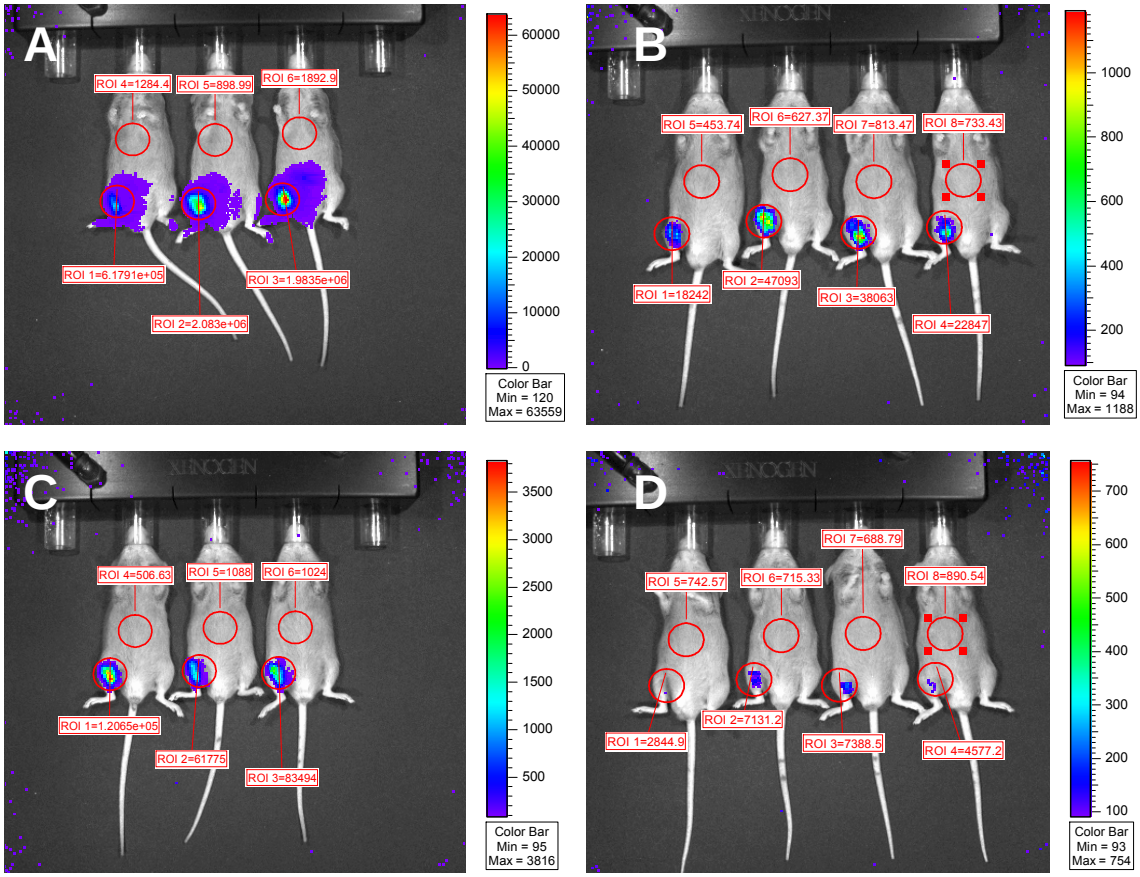


Figure 3.12 Representative images of luciferase expression 8 days post-intramuscular injection of integrating and non-integrating pseudotyped lentiviral vectors. Representative images are shown of mice 8 days post-injection of 20µL **(A)** VSVg ILV, **(B)** VSVg IDLV, **(C)** MLV-E ILV, **(D)** MLV-E IDLV expressing a luciferase transgene into the right quadricep. Light emission was measured using an IVIS CCCD camera in photons at a binning of 8 for 60 seconds. Defined regions of interest (ROIs) over (i) the injected area, and (ii) a control region in the abdomen are shown as a circular regions over each mouse. Luminescence within ROIs are quantified in photons/second/cm^2/sr and to avoid bias all areas measured are equal size and shape. (Please note the difference in scale for each image).

All vectors displayed expression in the injected area which was above background levels and both VSVg and MLV-E integrating vectors had similar average levels of expression to the experiment in section 3.4.2 at 8 days post injection of 1.56 x 10^6 and 8.78 x 10^4 photons/second/cm^2/sr, respectively. Integration deficient vectors had significantly lower levels of expression at day 8 post-injection at an average of 3.53 x 10^4 and 5.60 x 10^3 photons/second/cm^2/sr for VSVg and MLV-E pseudotypes, respectively.

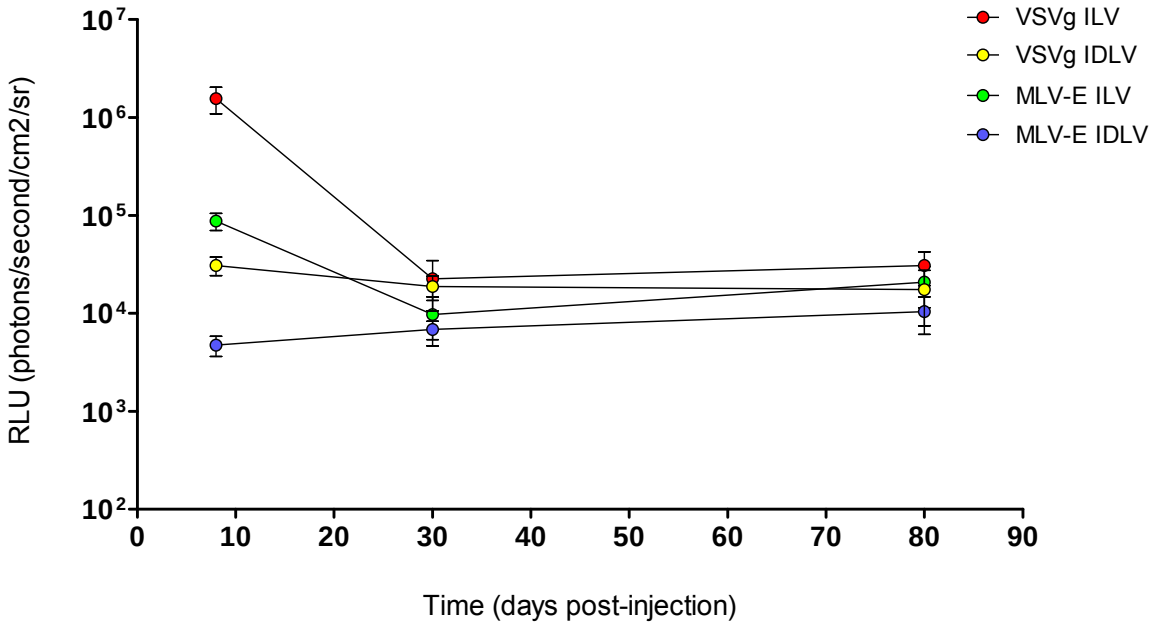


Figure 3.13 Quantification of luciferase expression post-intramuscular injection of integrating and non-integrating pseudotyped lentiviral vectors. Light emission was quantified as photons/second/cm^2/sr in defined regions of interest (ROIs) over both the area of injection and a control region for adult DBA/1 mice injected intramuscularly with 20µL VSVg or MLV-E pseudotyped ILV or IDLV expressing a luciferase transgene. Values measured inside the control region were subtracted from the injected region and plotted as relative luciferase units (RLU) in photons/second/cm^2/sr. Mean and SEM plotted for each point. (VSVg ILV and MLV-E ILV; n=3, VSVg IDLV and MLV-E IDLV; n=4 for all time points).

A loss of luciferase expression occurred over time for integrating vector groups with a 70-fold reduction in average expression for the VSVg ILV group from 8 to 30 days at 1.56 x 10^6 to 2.26 x 10^4 photons/second/cm^2/sr, respectively. A 9-fold reduction in expression was observed for MLV-E ILV from 8 to 30 days at 8.78 x 10^4 to 9.72 x 10^3 photons/second/cm^2/sr, respectively. The loss in expression was less pronounced for non-integrating vectors with only a 2-fold drop in expression for VSVg IDLV injected mice from an average at 8 days post-injection of 3.53 x 10^4 photons/second/cm^2/sr, to 1.41 x 10^4 photons/second/cm^2/sr at day 30 post-injection. For MLV-E IDLV expression appeared to be maintained over 80 days at average of 5.6 x 10^3, 8.29 x 10^3 and 1.26 x 10^4 for days 8, 30 and 80 post-injection, respectively. However, levels most likely

remained constant due to being extremely low. Mice were sacrificed at day 80 post-injection and both injected and uninjected muscle tissue, spleen and blood sera was taken from all animals for analysis.

3.5.3 Proviral Copy Number and Quantification of Luciferase Protein in Injected Tissue

Mice were sacrificed at 80 days post-injection and muscle tissue, spleen, and blood sera were taken for analysis. Muscle tissue was assayed for viral copy number and luciferase expression. Genomic DNA was extracted and qPCR carried out to determine integrated proviral copy number per cell. Tissue from mice injected with integrating vector was determined an average of 0.057 and 0.027 viral copies per cell for VSVg and MLV-E vector groups, respectively (Figure 3.14A). Viral copy for tissue from mice injected with non-integrating vector was also extremely low at only 0.019 and 0.014 copies per cell detected for VSVg and MLV-E vector groups, respectively.

Luciferase expression was determined in relative luciferase units (RLU)/µg protein using the Luciferase Assay System (Promega) and a Bradford protein assay (BioRad). Quadricep tissue taken from mice injected with integrating vector showed average expression in muscle at 2.4 x 10^4 and 1.2 x 10^4 RLU/µg protein for VSVg and MLV-E pseudotypes, respectively (Figure 3.14B). Luciferase expression for mice injected with non-integrating vector was significantly lower at an average of 2646.1 and 3446.4 RLU/µg protein for VSVg and MLV-E pseudotypes, respectively, reflecting both the lower expression observed with bioimaging and viral copy number (Figure 3.14B). Left, uninjected, quadricep tissue from two mice chosen at random was also tested in both assays and had an average background copy number of 0.001 proviral copies per cell, and background luciferase expression at 1100 RLU/µg protein.

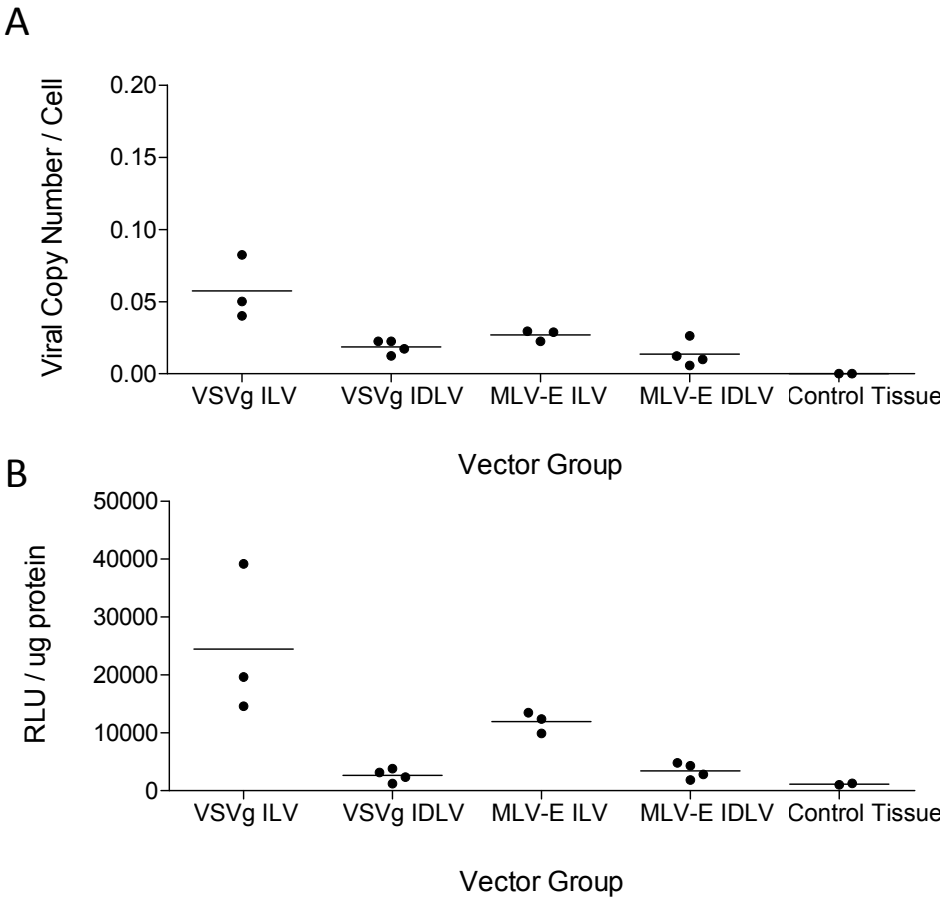


Figure 3.14 Quantification of viral copy number and luciferase protein expression in muscle tissue after intramuscular injection with ILV and IDLV pseudotypes. Injected muscle tissue in all mice and uninjected muscle from VSVg IDLV injected mice was taken at 80 days post-transduction. **(A)** Genomic DNA was extracted and qPCR carried out for each sample for both the murine housekeeping gene *titin* and the viral WPRE sequence allowing determination of viral copy number per cell. **(B)** Muscle tissue was homogenised and an *ex vivo* luciferase protein assay carried out to determine Relative Luciferase Units (RLU) per µg protein. Mean plotted for each pseudotype. (VSVg ILV and MLV-E ILV; n=3, VSVg IDLV and MLV-E IDLV; n=4).

3.5.4 Analysis of the Loss of Expression Observed in VSVg and MLV-E ILV and IDLV Groups

Mice injected with VSVg or MLV-E pseudotyped integrating vector suffered a 70- and 9-fold reduction in luciferase expression 8 days post-injection, respectively. As in section 3.4.4 to assess why this loss occurred immune response against luciferase and *de novo* methylation of the SFFV promoter were investigated.

3.5.4.1 Analysis of Immune Response to Luciferase for ILV and IDLV Groups

To assess cellular anti-luciferase immune response a splenocyte proliferation assay was conducted using splenocytes collected from mice in all vector groups and also from one naïve DBA/1 adult mouse using the same method outlined in section 3.4.4.1. Figure 3.15 displays the results in counts per minute (CPM) which demonstrate that when splenocytes were grown in the presence of PHA cell populations were viable and able to be stimulated; average counts for all groups were between 12,000 – 25,000 CPM. However, cells grown in the presence or absence of luciferase showed no significant difference in CPM for all injection groups with average counts between 500-3000 CPM for both luciferase treated and untreated groups.

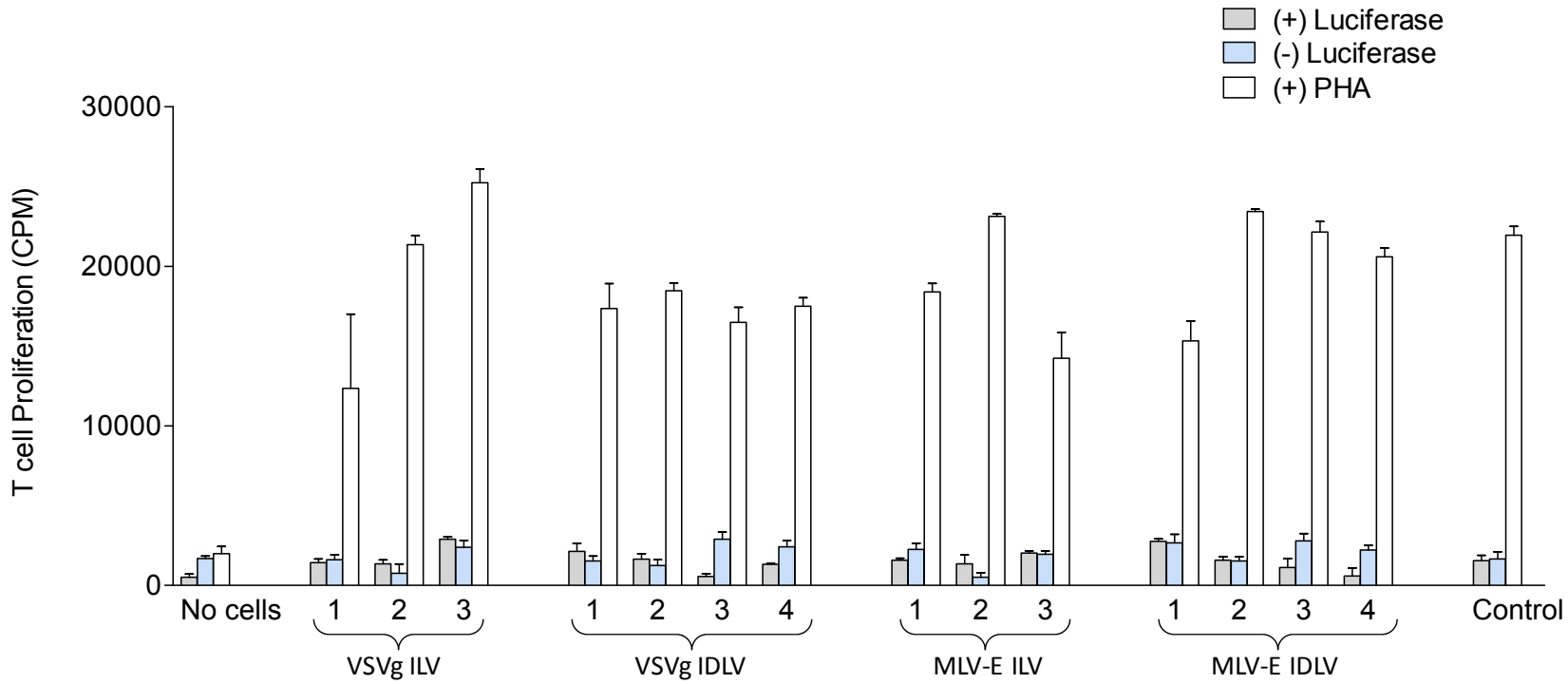


Figure 3.15 Evaluation of cellular immune response to luciferase protein. Splenocyte proliferation assay showing the proliferation response of cells to luciferase protein. Splenocyte populations were collected from mice injected with VSVg ILV (2 mice), VSVg IDLV (4 mice), MLV-E ILV (3 mice), MLV-E IDLV (4 mice) and one naïve control mouse and plated at $2x10^5$ cells/well in triplicate in 96-well plates in the presence or absence of luciferase protein (10µg/mL) or in the presence of PHA (5µg/mL). After 96 hours cells were pulsed with 1µCi/well methyl-^{3}H-Thymidine and after a further 20 hour incubation plates harvested onto filter paper and uptake determined using a beta-counter. Results presented as counts per minute (CPM) and are the mean and SEM of two separate experiments. (n=6)

To measure humoral immune response to luciferase protein a sandwich ELISA based was used to detect anti-luciferase antibody levels in the sera of treated mice and the blood from one naive DBA/1 adult mouse as described in section 3.4.4.1. No difference was seen between values obtained for control, VSVg or MLV-E injected groups using this assay, with only low levels (0-3µg/mL) of serum antibody levels detected in blood sera of injected mice (Figure 3.16).

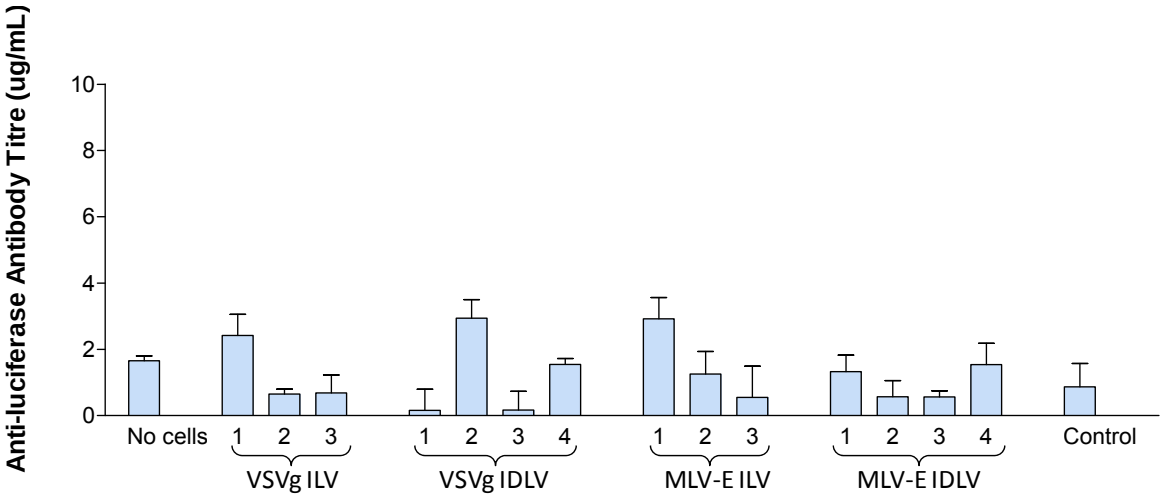


Figure 3.16 Evaluation of a humoral immune response to luciferase protein. Levels of anti-luciferase antibodies were determined in the blood sera of mice injected with VSVg ILV, VSVg IDLV, MLV-E ILV, MLV-E IDLV and one naïve control mouse. Blood sera were collected at 80 days post-injection via cardiac puncture. Antibody levels were measured by ELISA and converted to µg/mL by comparison to a monoclonal antibody of known concentration. Results presented as the mean and SEM of two separate experiments. (n=6)

3.5.4.2 Methylation Analysis of the SFFV Promoter

To study if *de novo* methylation of the SFFV promoter has occurred causing inhibition of transgene transcription, and therefore a drop in luciferase expression, methylation analysis of a 194bp region of the SFFV promoter containing 10 potential CpG methylation sites was carried out using the MethylCode™ Bisulfite Conversion Kit (Invitrogen) as described in section 3.4.4.2.

Figure 3.17 shows analysis for 5 different clones for DNA cloned from transduced muscle of one mouse from either VSVg ILV or MLV-E ILV vector groups. Shaded boxes indicate the presence of a methylated CpG site. PCR products were unable to be obtained for mice injected with non-integrating vector; this is likely due to the low amount of template DNA obtained from injected muscles in these mice as determined by qPCR in section 3.5.3. For both the VSVg ILV and MLV-E ILV injected mice sequence analysis of five clones showed that of the ten sites contained in the 194bp region of the SFFV promoter tested on average that only one CpG site was methylated in any clone, indicating that the whole SFFV promoter is unlikely to be extensively methylated.

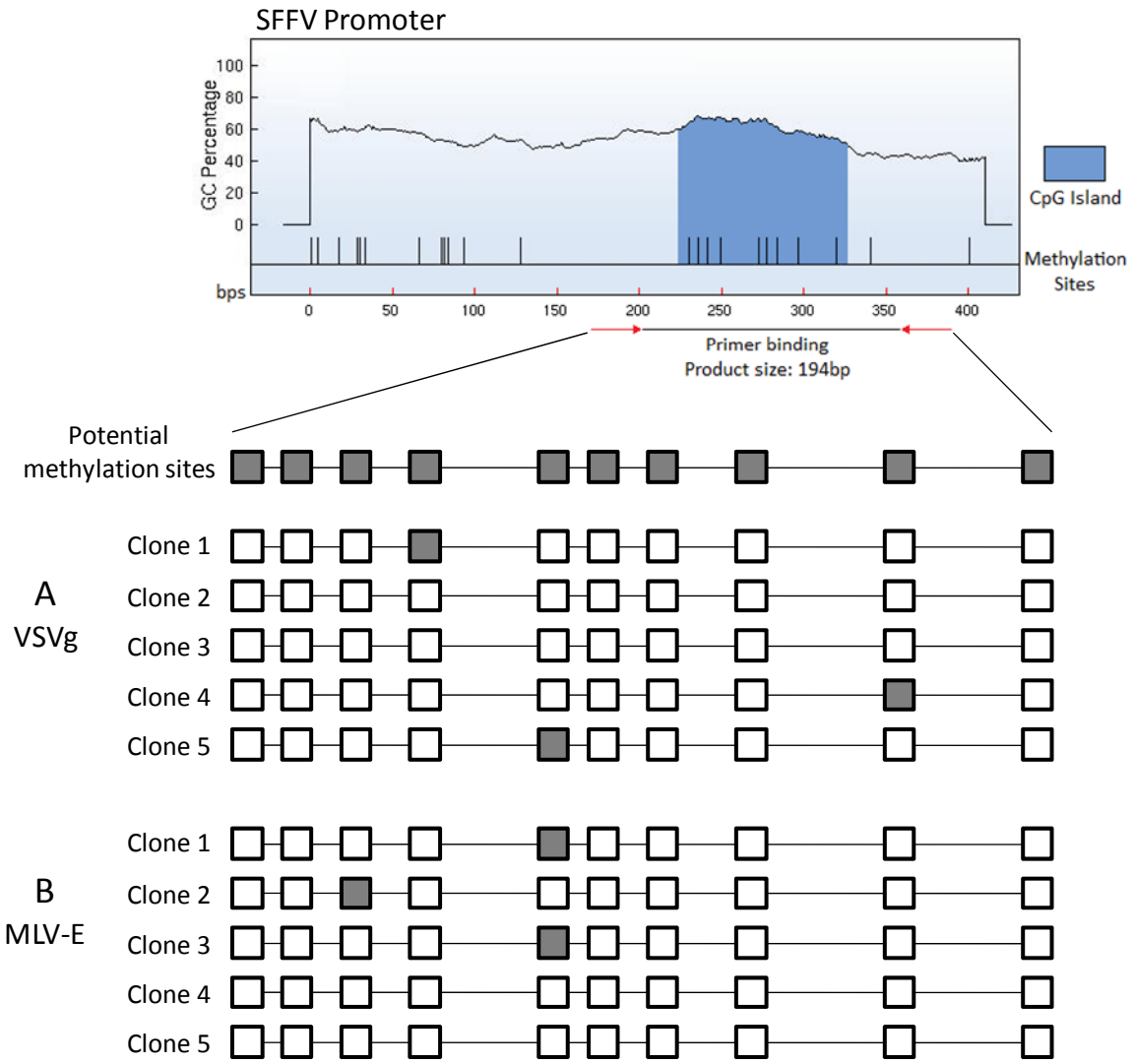


Figure 3.17 Methylation analysis of the SFFV promoter from integrated proviral DNA taken from muscle injected with ILV pseudotyped with VSVg or MLV-E. A 194bp region of the SFFV promoter containing 10 potential CpG methylation sites was analysed from integrated proviral DNA taken from transduced muscle. DNA was treated with bisulfite, causing unmethylated cytosines to be converted into uracil while methylated cytosines remain unchanged. Bisulfite-modified DNA was then amplified by PCR using *Taq* and the resulting product cloned into the TOPO-TA® cloning vector pCR4®. Five clones were then analyzed by DNA sequencing and the methylation profile of the region determined by comparing the sequence of the bisulfite-treated DNA to that of the untreated DNA. Potential CpG methylation sites are represented as squares and are shaded if determined to be methylated. **(A)** Analysis of muscle DNA from VSVg ILV injected mouse **(B)** Analysis of muscle DNA from MLV-E injected mouse.

3.6 Summary

- The tropism of integrating lentiviral vectors pseudotyped with alternative viral glycoproteins to VSVg was investigated *in vitro*. Alternative pseudotypes were found to have a narrower tropic range and their transduction efficiency was, in most cases, lower (10-fold plus) compared to VSVg.
- The kinetics of transgene expression in dividing and non-dividing C2C12 cells from ILV and IDLV pseudotypes was assessed. There was no significant difference between pseudotypes tested and in non-dividing cells a similar percentage of GFP^+ cells were observed over time for ILV and IDLVs.
- All pseudotypes were tested *in vivo* by direct intramuscular injection of ILV expressing luciferase into DBA/1 mice. Only VSVg and MLV-E pseudotypes showed expression which was significantly above background.
- A large drop in luciferase expression was observed *in vivo* for ILV and IDLV VSVg and MLV-E pseudotyped vector groups, the reason for this drop in expression was investigated and no immune response to luciferase protein or extensive CpG methylation of the SFFV promoter was observed.
- Expression of luciferase from IDLVs *in vivo* was very poor, approximately 15 to 44-fold lower in comparison to ILVs after injection of a similar number of transducing units.

In this chapter the tropsim of vectors pseudotyped with glycoproteins from Baculovirus (gp64), Ebola Zaire (EboZ), Ross river virus (RRV), Amphotropic 4070A murine leukaemia virus (MLV-A), or Ecotropic Moloney murine leukaemia Virus (MLV-E) were investigated in comparison to the standard VSVg envelope. All vectors were efficiently produced and the physical titre determined using both the $p24^{Gag}$ ELISA and reverse transcriptase (RT) assays. Where both assays were used to titre the same viral preparation the results for the number of transducing units were comparable. Gp64, EboZ, and RRV pseudotyped vectors were produced at a similar physical titre to VSVg throughout the experiments, however, MLV-A and MLV-E pseudotyped vectors were

consistently made at 5- to 10-fold higher titres. This may be due to reduced toxicity in producer cells, the difference in transfection method used (calcium phosphate in comparison to PEI), or that virus particles were not put under stress through ultracentrifugation (see section 2.1.8.2).

Vectors expressing GFP were first tested *in vitro* upon a variety of cell lines to study the tissue tropism of each virus through a measure of 'infectivity' where percentage of GFP positive cells was normalised by physical titre. All envelopes had a narrower tropic range to VSVg, which was able to efficiently transduce all cell lines, and a lower infectivity of all cell lines, in most cases a one to five fold decrease. The notable exception to this was gp64 pseudotyped vector which showed a higher infectivity of both ARPE-19 (retinal pigment epithelial) and Huh7 (human hepatoma) cells in comparison to VSVg. Gp64 pseudotyped vectors have previously been shown to transduce cells of the CNS, with a particular tropism towards astrocytes *in vivo* and no transduction of neurons after vector injection into the corpus striatum of rats [68], in this assay gp64 pseudotyped vector was unable to transduce Neuro-2a (murine neuroblastoma) cells. Gp64 vector has also been shown to efficiently transduce hepatocytes *in vivo* [116,151]. Therefore, it was encouraging to confirm these results using this *in vitro* assay. However, many results in this experiment contradicted reported findings. For example, we saw relatively low infectivity of A549 (human alveolar basal epithelial) cells in this experiment by EboZ pseudotyped, in comparison to the increased transduction *in vitro* and *in vivo* shown by [120]. Also, lentivector (FIV) pseudotyped with RRV glycoproteins was reported to have a 20-fold increase in transduction of hepatocytes *in vivo* compared to VSVg [121], whereas in this experiment RRV pseudotyped vector had an infectivity of Huh7 (human hepatoma) cells 60-fold lower than VSVg.

The three most efficient pseudotypes to transduce C2C12 (murine myoblast) cells, VSVg, gp64 and MLV-E, were then used to study the kinetics of integration proficient and deficient vectors in a model of dividing and non-dividing muscle cells. This experiment has been previously described by Apolonia *et al.,* in 2007 [67] using VSVg

pseudotyped ILVs and IDLVs, however, it was important to establish that this could be carried out sucessully using vectors alternatively pseudotyped. This experiment proved that a difference in pseudotype did not change vector kinetics, however, an increased MOI (determined by p24 titre) was required for gp64 and MLV-A vectors to obtain a similar percentage transduction of cells as with VSVg.

All pseudotypes were then tested *in vivo* by intramuscular injection of vector expressing a luciferase reporter gene into the right quadricep with significant expression only observed from VSVg and MLV-E vectors. The amount of vector injected was not normalised on transducing units (TU), and this may reflect the results obtained; a ten-fold increase in TU was injected for MLV-E in comparison to VSVg. Therefore, if expression achieved is normalised upon TU injected MLV-E performs significantly lower. The results obtained *in vivo* reflect the *in vitro* results in that VSVg consistently proves to be the most efficient pseudotype to transduce murine muscle, however, there is disparity between *in vitro* and *in vivo* results obtained for the ability and efficiency of each pseudotype to transduce cells. Therefore, although the *in vitro* methods used in this chapter provided much data in a timely fashion, in practice accurate discovery on the ability of different pseudotypes to transduce particular cell types will need to be obtained by injection *in vivo*. Further to this, after injection of approximately equivalent TU of integrating and non-integrating vectors into adult murine muscle relative expression of luciferase from IDLVs *in vivo* was 15- to 40-fold lower. This is a much more pronounced difference than the 1-6 fold lower luciferase expression observed *in vitro* (section 3.5.1). This may be due to differences in processing of episomal DNA in cells *in vivo*, however, again this highlights the need for *in vivo* testing to obtain accurate results for expression from different vectors.

We were also not able to determine if sustained expression of IDLVs occured in transduced muscle as a significant loss in luciferase expression was observed for both ILVs and IDLVs between 7 to 30 days post-injection which levelled to a point above background. Kafri *et al.,* in 1997 [219] described long term expression of GFP (>8 weeks) after direct muscle injection of VSVg pseudotyped lentiviral vectors in to adult female

rats, furthermore they reported no inflammation or recruitment of lymphocytes into the area or around the site of injection [219]. Similary, Seppen *et al.,* in 2001 [220] described expression of erythropoietin sustained for 14 months after a single intramuscular injection of 6 x 10^7 TU VSVg pseudotyped HIV-1 based lentiviral particles into adult rats [220]. Finally, O'Rourke *et al.* in 2003 [221] observed expression of eGFP for 3 months after intramuscular injection of an EIAV based SIN lentiviral vector into adult mice [221]. The loss in expression was therefore unexpected and both an anti-luciferase immune response and promoter silencing was investigated.

To investigate an anti-luciferase immune response a splenocyte proliferation assay was performed to determine if luciferase specific T cells were activated in response to lentivirus delivery. Antigen presenting cells (APCs) are present in skin and muscle tissue which can become transduced processing and presenting luciferase protein on class I MHC molecules to $CD8^+$ T cells. Two reports have shown that intramuscular injection of lentivectors stimulates anti-HIV envelope T-cell and antibody responses [222,223]. Further to this, $CD8^+$ T-cell and antibody responses were also observed in mice to the secreted hepatitis B virus (HBV) surface antigen expressed from a lentivector injected intramuscularly [94]. Finally, Limberis *et al.,* in 2009 [224] showed that mice injected intramusculary with VSVg pseudotyped lentivectors expressing luciferase produced transgene specfic splenocytes as a result of transgene expression in APCs [224]. Therefore, it is likely that an immune response to luciferase occurred. The proliferation assay used was adapted from that designed by Brown *et al.,* in 2007 [151] where it had been used to confirm the prescence of factor IX specific T cells. The assay showed that cells were viable and responded well to the positive control of PHA; high counts suggest they proliferated quickly. However, cells did not respond to the prescence of luciferase in media. In this case a more sensitive assay may have been more appropriate to identify an anti-luciferase cellular response. In our assay whole luciferase protein was used to try and stimulate splenocytes. An alternative way of presenting luciferase protein to cells could have been achieved by stimulating splenocytes with APCs, such as murine dendritic cells, which have been transduced with a vector expressing luciferase. These APCs would correctly process and present luciferase protein to the cells. A more

sensitive way to assess the prescence of specific T cells would also be to measure cytokine release by stimulated cells using an ELISA rather than look at cellular proliferation. For example, an IFNγ enzyme-linked immunosorbent spot (ELISPOT) assay, such as that used by Limberis *et al.* (2009) [224], could be used to identify both vector and transgene specific T cells by stimulation of cells with specific luciferase or p24 (gag) peptide libraries, respectively.

A humoral immune response to luciferase was also investigated, measuring anti-luciferase antibodies in the blood sera of transduced mice at the end of each experiment in comparison to a monoclonal antibody of known concentration. No evidence of anti-luciferase antibodies were detected, however, positive control sera containing a known quantity of anti-luciferase was not available which would have been a useful positive control. Again, a more sensitive B-cell ELISPOT could have been used. CpG methylation of a section of the SFFV promoter was also investigated to determine if transcriptional silencing caused loss of expression. Very little methylation was observed in all samples tested from mice injected with integrating vector suggesting that the whole promoter element was not significantly methylated. However, only a small section of the SFFV promoter (194bp) was analysed due to the difficulties in obtaining a larger PCR products from genomic DNA extracted, therefore these results may not be representative of the whole promoter. IDLV injected mice were also unable to be analysed for methyation due as PCR fragments from genomic DNA could not be obtained, this likely due to lack of template DNA.

Overall, the tropism of pseudotyped lentiviral vectors was assessed both *in vitro* on various cell lines and *in vivo* after intramuscular injection of DBA-1 mice. VSVg was found to have the highest infectivity *in vitro* in comparison to all other pseudotypes tested and that pseudotype did not affect the kinetics of vector expression in a model of murine muscle cells. However, low expression from both ILVs and IDLVs *in vivo* in adult murine muscle and the drop in expression possibly caused by an immune response to the transgene product suggests that neither will be suitable for expression of FVIII or FIX *in vivo* in adult muscle in order to achieve therapeutic levels.

Chapter Four

Analysis of Transgene Expression from Integration Proficient and Deficient Lentiviral Vectors in Liver

4.0 Aims

- To better characterise expression from IDLVs in liver with a view to express human FIX protein for treatment of haemophilia B.
- To compare expression kinetics of GFP *in vitro* from both integration proficient and deficient vectors in dividing and non-dividing Huh7 (human hepatoma) cells.
- To assess expression of human factor IX from integration proficient and deficient vectors *in vivo* via intravenous injection of adult C57BL/6 and BALB/c mice.
- To compare biodistribution and expression of luciferase from integration proficient and deficient vectors *in vivo* via intravenous injection of adult NOD/SCID mice.

4.1 Introduction

Lentiviral vectors are effective tools for transducing both dividing and quiescent cells, making them useful for both genetic modification and analysis of a wide range of tissues [98]. IDLVs can be produced through the use of integrase mutations that specifically prevent proviral integration. This results in the generation of increased levels of circular vector episomes in transduced cells which lack replication signals and which are gradually lost by dilution in dividing cells, but are stable in non-dividing cells [225]. Cells in the liver are primarily quiescent, with the turnover of normal adult liver very low [226], therefore hepatocyes, which normally produce both FVIII and FIX [168,173], are ideal targets for long-term expression of coagulation factors by IDLVs. Expression of human factor IX from integrating lentiviral vectors in liver has been well characterised [116,200,227-229]. Park *et al.,* in 2000 [200] reported levels of 1-2% normal expression of FIX in C57BL/6 mice after portal vein injection of HIV-1 based vectors, with a 5-fold increase in expression after partial hepatectomy (likely caused by a stress response and

activation of NFκB). Levels of 15% were observed in the same paper for FVIII expression, however, a humoral immune response meant expression was transient [200]. Tsui *et al.* (2002) [229], report sustained expression of FIX at 4% of normal human expression and transduction of 4% of hepatocytes after intravenous injection of HIV-1 based vectors [229], and finally, Follenzi *et al.,* in 2002 [228] showed sustained expression (> 1 year) of FIX at approximately 2% of normal human expression after a single injection of lentivector into SCID mice [228].

In this chapter we aimed to study expression of human factor IX from both integration proficient and deficient vectors both *in vitro* and *in vivo*, with quiescent liver cells, specifically non dividing hepatocytes, as the primary cell target. The haemophilias are good candidates for expression from non-integrating lentiviral vectors in liver as low levels of transgene expression (1-5%) of normal levels are sufficient for disease amelioration. To study expression from IDLVs in liver a GFP transgene was used for *in vitro* studies in dividing and non-dividing hepatocyte cells (Huh7 cell line). Expression of FIX from ILV and IDLVs was then studied in vivo using both C57BL/6 and BALB/c mice. Finally, the distribution and expression of ILV and IDLVs by both intravenous and hydrodynamic delivery *in vivo* was studied using vectors expressing a luciferase reporter gene into immune deficient NOD/SCID mice.

4.2 Kinetics of Expression from Integration Proficient and Deficient Vectors *In Vitro* in a Liver Cell Line

The kinetics of transgene expression from integration proficient and deficient lentiviral vectors were assessed *in vitro* in the liver cell line Huh7 (human hepatoma). These cells were used as a crude model of adult non-dividing hepatocytes by removing serum from the cell growth media thereby arresting cell division. Integration proficient and deficient vectors expressing a GFP transgene were tested upon both dividing and serum starved Huh7 cells to determine if transgene expression from IDLVs could be maintained over time in arrested cells. Cell viability was continually monitored throughout the experiment and when assessing results using flow cytometry only viable cells were gated upon. However, unlike the terminal differentiation of C2C12 cells serum starvation of Huh7 cells causes much higher levels of cell death and apoptosis in cultures [230].

Transgene expression kinetics of vectors pseudotyped with the envelope glycoproteins from Baculovirus (gp64) or VSVg were investigated. Integrating vector pseudotyped with gp64 was identified as having a higher 'infectivity' of Huh7 cells compared to VSVg pseudotyped vectors (see section 3.2). The use of gp64 pseudotyped vectors for transduction of hepatocytes *in vivo* after intravenous injection of lentivector would be advantageous. Effective transduction of hepatocyte cells and a narrower tropic range *in vivo* would cause less off target expression reducing the risk, compared to VSVg, of transducing cells of the immune system such as APCs which would cause transduced cells to be cleared. It may also prevent transduction of dividing cell types, such as Kupffer cells in the liver, in which IDLV episomes would be lost over time. Both integration proficient and deficient vectors expressing a GFP transgene were produced pseudotyped with either VSVg or gp64 envelope glycoproteins. 1 x 10^5 Huh7 cells then were transduced at an MOI of 1 based on p24 titre for all vectors. Post-transduction, dividing cells were grown and split 1:6 in complete DMEM every 3 days. To produce

non dividing cells serum was removed from the media arresting cell division at day 3. Transgene expression was assessed over time using flow cytometry. (Figure 4.1).

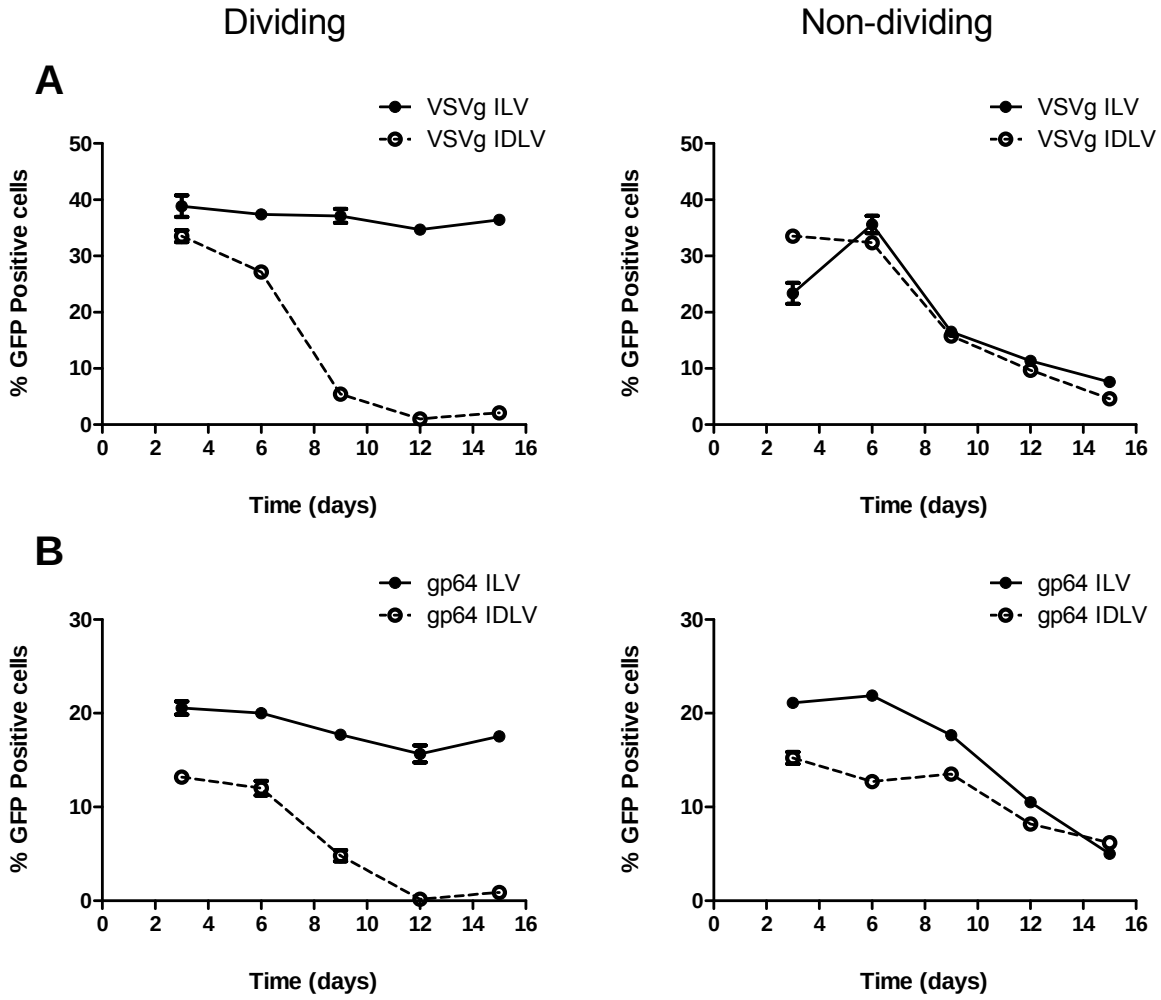


Figure 4.1 Expression kinetics profile of VSVg and gp64 pseudotyped vectors in dividing and serum starved, non-dividing, Huh7 cells. Five wells of cultured Huh7 cells were infected with vectors expressing GFP at an MOI of 1. Transgene expression was measured over time by flow cytometry. Arrest of Huh7 cells was induced by serum starvation 3 days post-transduction. Graphs on the left represent dividing Huh7 cells and those on the right represent non-dividing Huh7 cells. **(A)** Cells transduced with VSVg pseudotyped vectors **(B)** Cells transduced with gp64 pseudotyped vectors. Mean and SEM plotted for five values for each point. (n=5).

All vectors were able to transduce both dividing and serum starved non-dividing Huh7 cells. A similar pattern of expression was also observed for vectors pseudotyped with VSVg or gp64. Integration proficient vectors had sustained GFP expression in dividing cells, whilst expression of GFP from IDLVs was transient due to loss of vector episomes through cell division. In non-dividing cells both ILVs and IDLVs had similar expression levels over time. However, expression is seen to decrease likely due to a loss of cell viability.

The mean fluorescence intensity (MFI) and viral copy number per cell was also measured for each well of serum starved, non-dividing, Huh7 cells. The calculated copy number was determined using qPCR for the human housekeeping gene β-actin and the viral WPRE sequence allowing determination of proviral copies per cell. Figure 4.2 shows the results for both VSVg and gp64 vectors at each time point. Copy number for integrating vectors of both pseudotypes is around 0.3 to 1 proviral copies per cell for all wells over time. This is in contrast to non-integrating vectors which had higher copy numbers at 2 to 3 copies per cell. Calculated MFI, on average, was around five times higher for integrating vectors compared to non-integrating. Integrating vectors had a population MFI ranging from 175-225 for VSVg and 267-324 for gp64, at all time points. For non-integrating vectors, population MFI was between 60-100 for VSVg, and 57-82 for gp64. Taking into account both the copy number and the number of GFP positive cells expression from episomal vectors is significantly lower compared to integrated proviral DNA.

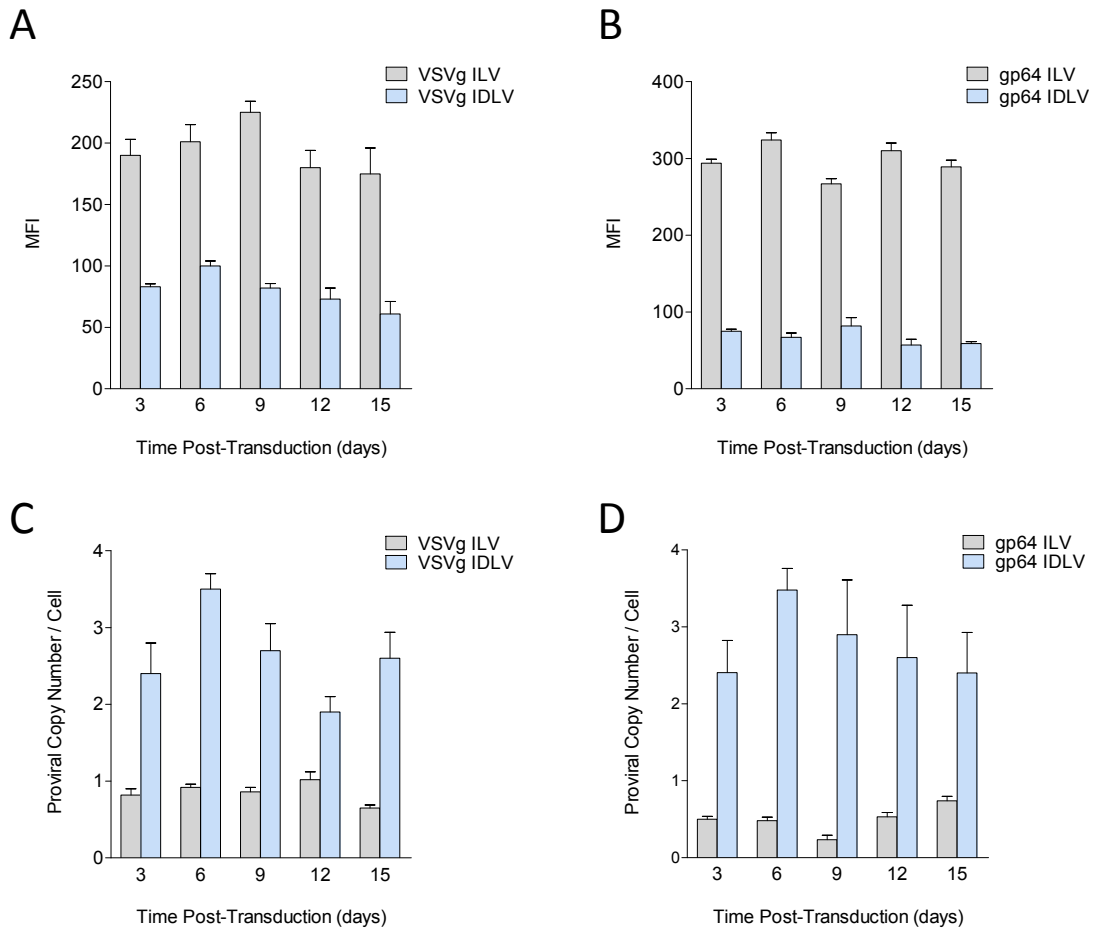


Figure 4.2 MFI and proviral copy number in non-dividing, serum starved, Huh7 cells transduced with VSVg or gp64 ILV or IDLVs. Five wells of cultured Huh7 cells were infected with vectors expressing GFP at an MOI of 1 and cells were arrested by serum starvation 3 days post-transduction. Population MFI was measured by flow cytometry, and viral copy number using qPCR. **(A)** MFI of serum starved Huh7 cells transduced with integrating or non-integrating VSVg pseudotyped vectors **(B)** MFI of serum starved Huh7 cells transduced with integrating or non-integrating gp64 pseudotyped vectors **(C)** Proviral copy number per cell of serum starved Huh7 cells transduced with integrating or non-integrating VSVg pseudotyped vectors. **(D)** Proviral copy number per cell of serum starved Huh7 cells transduced with integrating or non-integrating gp64 pseudotyped vectors. Mean and SEM plotted for five values for each bar. (n=5).

4.3 Expression of Human Factor IX from Integration Proficient and Deficient Lentiviral Vectors in Liver

Haemophilia B is a bleeding disorder resulting from mutations in the gene for human blood clotting factor IX (hFIX). It is a good candidate for gene therapy as low plasma concentrations are sufficient for disease amelioration [200]. The liver is the natural producer of hFIX with hepatocytes the primary producing cells [231]. Hepatocytes make up approximately 70-80% of the livers mass and are quiescent, turning over approximately once a year [226], therefore in this section it is our aim to use IDLVs targeted to hepatocyte cells to produce therapeutic levels of hFIX protein *in vivo*.

4.3.1 *In Vitro* Expression of hFIX from Integration Proficient and Deficient Lentiviral vectors

Both VSVg and gp64 integration proficient and deficient vectors were produced expressing a hFIX therapeutic transgene driven by the SFFV promoter. Vector titre was quantified by $p24^{Gag}$ and was estimated at 8 x 10^8 TU/mL for VSVg ILV and 1 x 10^9 TU/mL for VSVg IDLV. These values were around 1-log higher than titres obtained for gp64 vectors which were estimated at 4 x 10^7 TU/mL for gp64 ILV and 6 x 10^7 TU/mL gp64 IDLV. Expression of hFIX protein from vectors was then assessed *in vitro* by transducing the liver and non-liver cell lines 293T, 3T3 and Huh7 at an MOI of 1 as determined by p24 titres. 1 x 10^5 cells were transduced in a total volume of 300µL complete DMEM. 48 hours later media was removed and 500µL of OptiMEM added. At 72 hours post-transduction media was assayed for hFIX protein using an ELISA. Figure 4.3 shows that all vectors expressed hFIX protein effectively in all cells lines and that there was little difference in expression between cell type, vector pseudotype, or integration proficiency.

Virus	p24 Gag (pg/mL)	Estimated Titre (TU/mL)
VSVg hFIX ILV	2.82×10^7	8.46×10^8
VSVg hFIX IDLV	3.55×10^7	1.07×10^9
gp64 hFIX ILV	1.48×10^6	4.43×10^7
gp64 hFIX IDLV	2.26×10^6	6.77×10^7

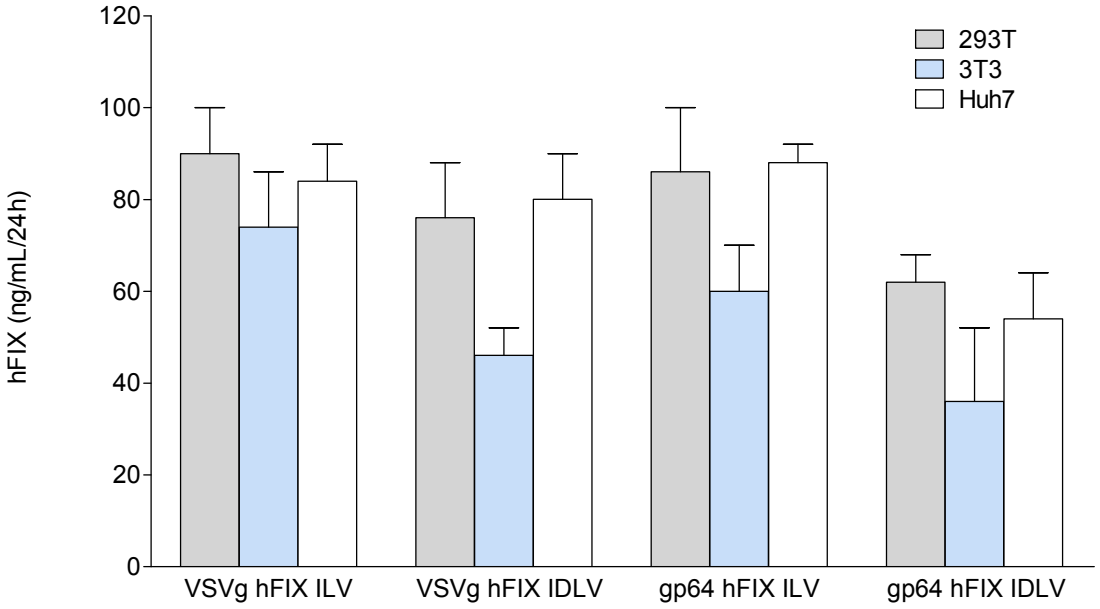


Figure 4.3 Quantification of physical vector titres and *in vitro* testing of lentivectors expressing human FIX. (A) Quantification of $p24^{Gag}$ protein concentration in viral stocks, measured by performing an HIV-1 p24 ELISA. Protein concentration was then used to estimate transducing units/mL (TU/mL). Mean of two values plotted. (n=2). **(B)** VSVg or gp64 ILV or IDLVs were produced and tested against 293T, 3T3 and Huh7 cells. 1×10^5 cultured cells were transduced at an MOI of 1 based on p24 titres, culture media was changed 48h after transduction and hFIX expression quantified by ELISA at 72h post transduction. Units are expressed as ng of hFIX/mL/24hrs (n=5).

4.3.2 *In Vivo* Expression of hFIX from Integration Proficient and Deficient Lentiviral vectors

All vectors were then tested *in vivo* by direct intravenous injection via the tail vein into adult C57BL/6 mice (injections performed by Dr. Amit Nathwani, UCL). Intravenous injection was chosen as the method of delivery previous reports have shown that high levels of liver cells are transduced using this method. Follenzi *et* al., in 2002 [228] reported high levels (up to 30%) transduction of liver cells, both parenchymal (hepatocytes) and non-parenchymal after intravenous injection of integrating lentivectors, and subsequently sustained expression of hFIX. 100µL vector was injected into four adult mice aged between 7 and 10 weeks. The amount of vector injected into each mouse was normalized on volume; therefore the estimated number of transducing units (TU) injected was different between each vector group as shown below:

Vector Group	Estimated Transducing Units injected per mouse
VSVg hFIX ILV	8×10^7
VSVg hFIX IDLV	1×10^8
gp64 hFIX ILV	4×10^7
gp64 hFIX IDLV	6×10^6

Two mice were also injected with 100µL OptiMEM as controls. Blood was taken via tail vein bleed at four time-points post injection; 3, 5, 30 and 90 days and plasma analysed for hFIX protein using ELISA. Figure 4.4 shows the results for blood plasma concentration of hFIX in ng/mL at all time points. At 3 and 5 days post-injection levels of hFIX in all test groups were undetectable. At 30 days post-injection VSVg hFIX ILV transduced mice had an average blood plasma concentration of 641ng/mL (13% normal human physiological levels), however, it is noted that one mouse in this vector group did not respond to vector injection. Expression was maintained in this group for the duration of the experiment and at 90 days mice had an average expression of 615ng/mL. At 30 days post-injection mice injected with integration proficient gp64 pseudotyped vector had no detectable levels of hFIX, but at 90 days protein was

detected at 58ng/mL (~2% of normal). The ten-fold lower expression in comparison to VSVg hFIX ILV may correlate with the ten-fold reduction in estimated TU injected. Mice injected with integration deficient vectors, both VSVg and gp64 pseudotypes, had undetectable levels of hFIX in blood plasma at all time points.

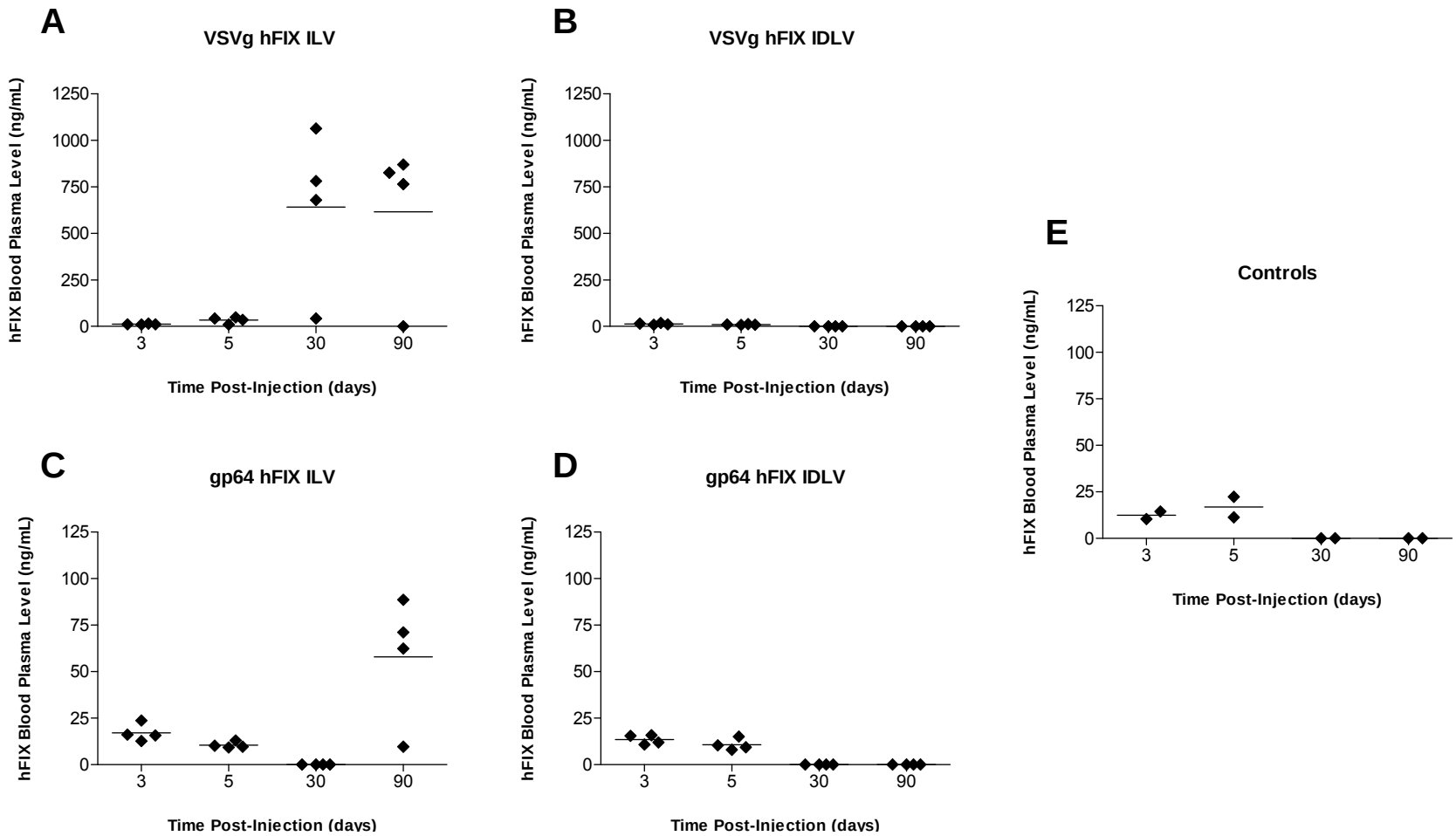


Figure 4.4 Blood plasma hFIX protein concentration in adult C57BL/6 mice injected intravenously with VSVg or gp64 pseudotyped, ILV or IDLVs expressing hFIX. Plasma concentrations of human factor IX (hFIX) protein in blood taken at 3, 5, 30 and 90 days post-injection from four normal adult C57BL/6 mice injected with 100µL vector preparation containing **(A)** 8.46 x 10^7 TU VSVg hFIX ILV, **(B)** 1.07 x 10^8 TU VSVg hFIX IDLV **(C)** 4.43 x 10^6 gp64 hFIX ILV, **(D)** 6.77 x 10^6 gp64 hFIX IDLV **(E)** two uninjected C57BL/6 control mice. Blood was collected by tail vein bleed and blood plasma analysed for hFIX protein concentration using an in house ELISA.

4.3.3 Proviral Copy Number Determination

Mice were sacrificed at 90 days post-injection and liver, spleen, kidney, heart and lung tissue taken for viral copy number analysis using qPCR. Figure 4.5 shows the copy number calculated for all tissues. Viral copy was highest for mice injected with VSVg hFIX ILV and predominantly found in liver and spleen tissues at an average of 1.8 copies/cell and 0.9 copies/cell, respectively. All other tissues tested in this group had <0.01 copies/cell. Proviral copy was above background in liver tissue for gp64 hFIX ILV injected mice at 0.2 copies/cell, and <0.001 copies/cell for all other tissues. Mice injected with integration deficient vectors had no detectable vector copy in all tissue analysed.

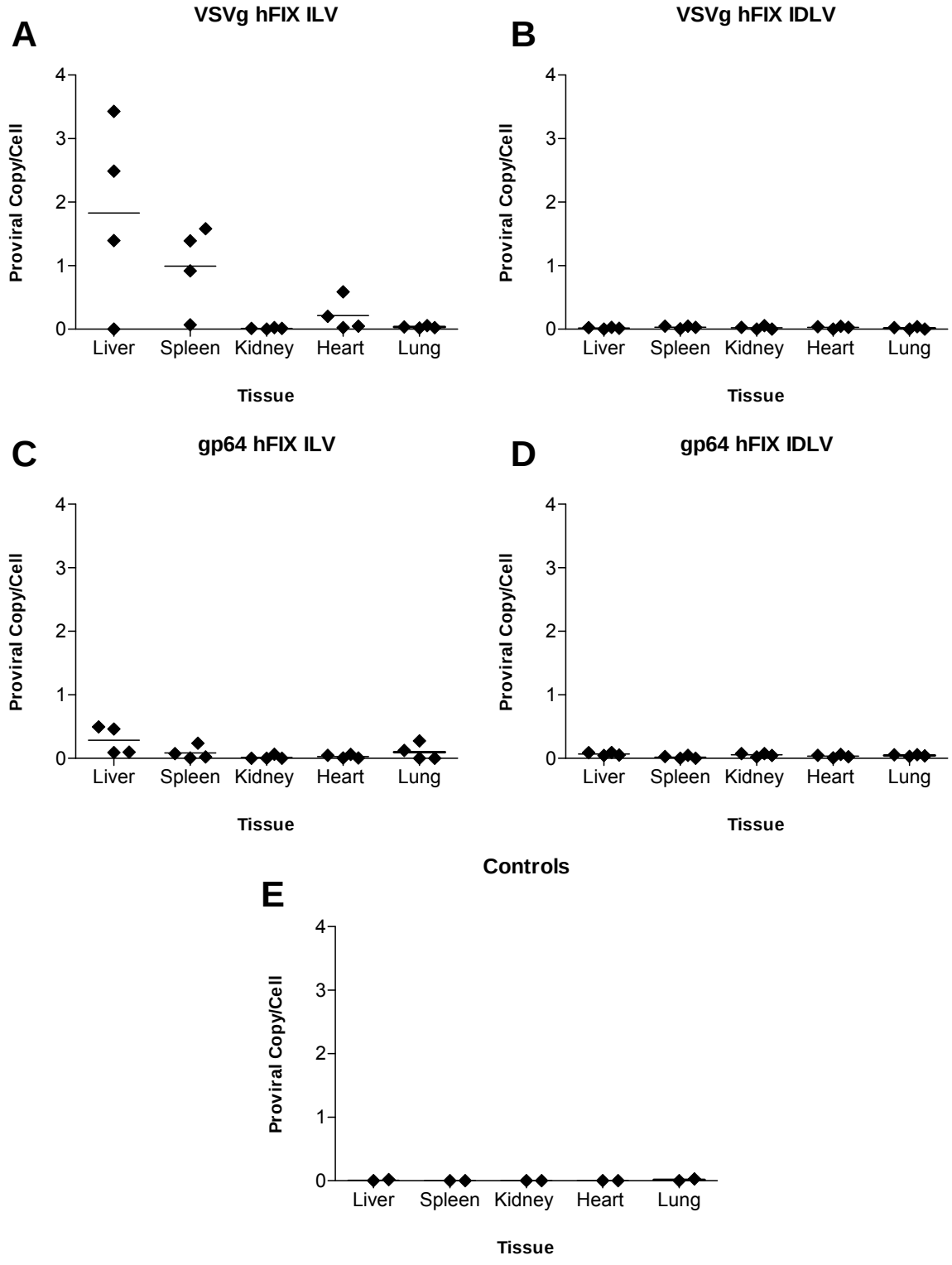


Figure 4.5 Proviral copy number in tissue from mice injected lentivector expressing hFIX. C57BL/6 mice injected with VSVg or gp64 integration proficient or deficient vectors expressing hFIX were sacrificed at 90 days post injection and liver, spleen, kidney, heart and lung tissue taken for viral copy analysis using qPCR. Vector groups are: **(A)** VSVg hFIX ILV, **(B)** VSVg hFIX IDLV, **(C)** gp64 hFIX ILV, **(D)** gp64 hFIX IDLV, **(E)** Control mice.

4.4 Repeat hFIX Experiment

To determine why hFIX expression could not be mediated from integration deficient vectors *in vivo* a repeat experiment was carried out. In this experiment only VSVg pseudotyped vector was investigated and injected into two strains of mice, BALB/c and C57BL/6. Both strains were tested to observe if a type I interferon response and clearance of transduced cells would occur in BALB/c mice after intravenous injection of vector as described by Brown *et al.,* in 2007 [151]. A ten-fold increase in the number of transducing units was also used in this repeat experiment. Brown *et al.* (2007) report that efficient gene transfer to the liver can be achieved but that hepatocytes are transduced at a relatively low efficiency compared with nonparenchymal cells and that at a low vector dose, this effect is particularly pronounced [151]. They found a high frequency of Kupffer cells to be vector positive and only a small fraction of hepatocytes transduced by ILVs. They state that by increasing the concentration of injected vector a threshold is reached in which hepatocyte transduction becomes dose responsive, and improved hepatocyte gene transfer is achieved and that this may be due to the requirement for saturating the particle-clearance systems of the sinusoid-lining cells in blood-filtering organs [151,232].

A 'ghost vector' control group was also included in this study as a control for pseudo-transduction. Ghost vector is a lentiviral vector containing no genome and is produced by co-transfection of 293T cells with:

(i) The expression vector pcDNA 3.1(+) (Invitrogen, UK) containing the human FIX (hFIX) transgene driven by the CMV promoter; transfection with this plasmid allows hFIX protein to be produced by the 293T cells and secreted in the cell media.
(ii) The pCMVΔR8.74 gag-pol packaging plasmid
(iii) The pMD.G2 VSVg envelope plasmid.

Cells will then produce empty VSVg pseudotyped virions containing no vector genome. This vector serves as a control for pseudo-transduction as hFIX protein present in the cell media will be contained in the concentrated viral prep. Therefore, any human hFIX detected in the plasma of mice injected intravenously with this vector will be solely from pseudo-transduction (production represented in Figure 4.6).

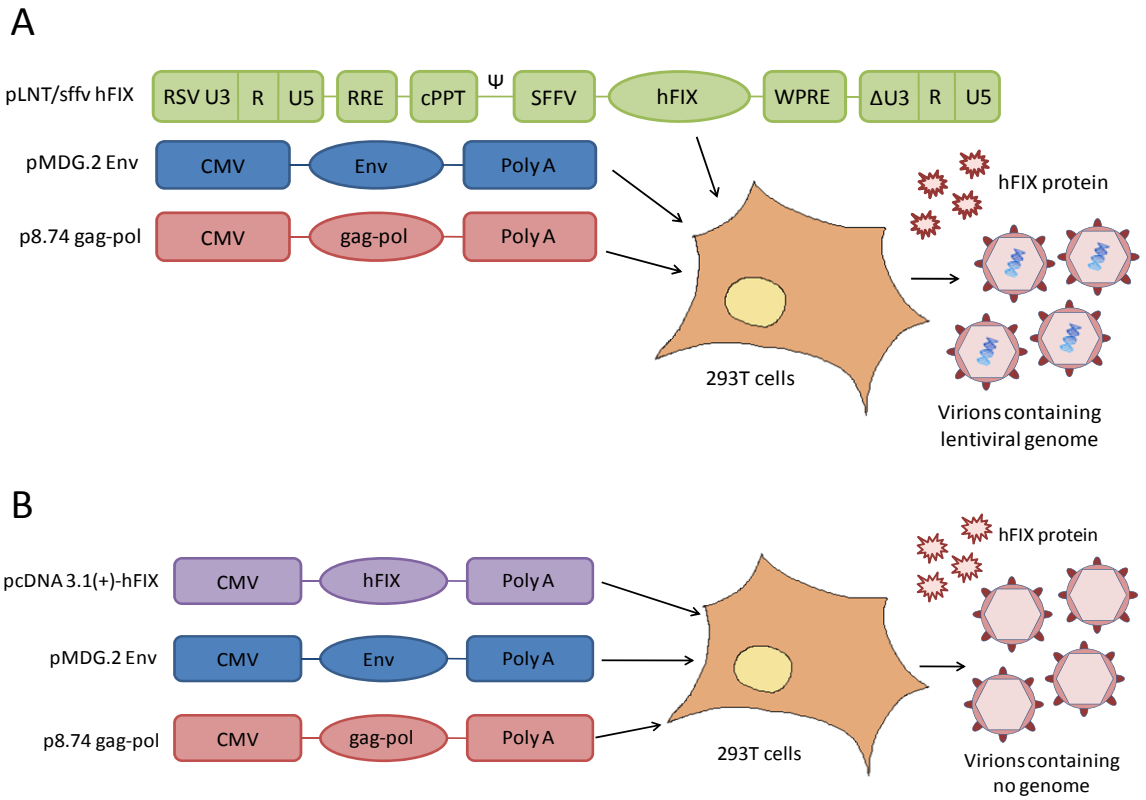


Figure 4.6 Schematic representation showing production of empty vector particles or 'ghost vector'. (A) Normal lentiviral vector production in 293T cells of a lentivirus expressing hFIX. Normal virus will be produced by cotransfection of lentiviral backbone LNT/sffv-hFIX, pCMVΔR8.74 gag-pol plasmid, and pMD.G2 VSVg envelope plasmid. hFIX protein will also be secreted into cell media will be concentrated with vector particles. **(B)** Ghost vector is produced by cotransfecting 293T cells with the expression vector pcDNA3.1(+)-hFIX, pCMVΔR8.74 gag-pol plasmid, and pMD.G2 VSVg envelope plasmid. This produces empty virions with no lentivector genome, however, hFIX protein will be secreted into the media and concentrated with virus when the media is ultracentrifuged, therefore any hFIX protein detected from this vector will be pseudotransduction.

4.4.1 *In Vitro* Expression of hFIX from Integration Proficient and Deficient Lentiviral vectors

All vectors were produced by transient transfection, tested for reverse transcriptase activity as a measure of physical viral titre, and subsequently *in vitro* for expression of hFIX in 293T, Huh7 and 3T3 cells. Results show that integration proficient, deficient and ghost vectors were all produced at a high titre and that integrating and non-integrating vectors were able to express hFIX protein *in vitro.* Ghost vector did not deliver hFIX protein (Figure 4.7).

Virus	RT (ng/µL)	Estimated Titre (TU/mL)
VSVg hFIX ILV	40.6	1.38×10^{10}
VSVg hFIX IDLV	29.2	9.92×10^{9}
hFIX Ghost Vector	22.9	7.79×10^{9}

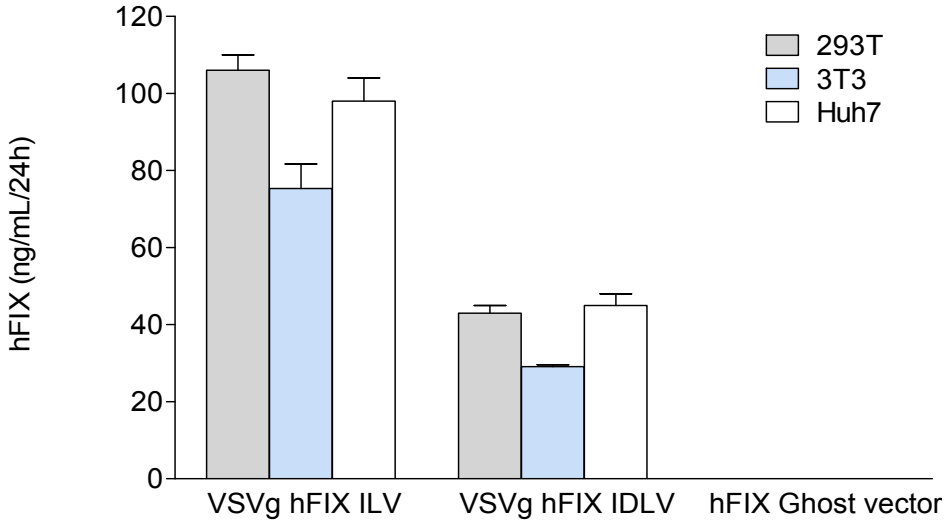


Figure 4.7 Quantification of physical vector titre and *in vitro* hFIX expression. Quantification of reverse transcriptase (RT) protein concentration in viral stocks quantified in ng/µL. RT concentration was used to estimate transducing units/mL (TU/mL) (n=3). VSVg ILV, VSVg IDLV, and Ghost vector groups were produced and tested against 293T, 3T3 and Huh7 cells. 1×10^5 cultured cells were transduced at an MOI of 1 based on estimated RT titres, culture media was changed 48h after transduction and hFIX expression quantified by ELISA at 72h post transduction as ng/mL/24hrs (n=5).

4.4.2 *In Vivo* Expression of hFIX from Integration Proficient and Deficient Lentiviral vectors

Vectors were tested *in vivo* by intravenous injection via the tail vein of each vector into adult (7-10 weeks of age) C57BL/6 or BALB/c mice. The amount of vector injected into each mouse was normalised on the reverse transcriptase (RT) titre with an estimated 7.8 x 10^8 TU (2.3µg RT protein) of each vector injected into five adult mice per lentivector group, and 2 mice for ghost control vector, in a total volume of 100µL. This was a ten-fold increase in the number of transducing units injected compared to the experiment in section 4.3.2. Blood was taken via tail vein bleed from 2 mice in each group 2 days post-injection and then from all mice at 4, 16, 30 and 80 days post-injection. Blood plasma was analysed for hFIX protein using ELISA.

Figures 4.8A-C show the results for C57BL/6 mice. Mice injected with integrating vector have detectable hFIX protein in blood plasma after just four days, and the average level of hFIX expression from 16-80 days post-injection was approximately 1500ng/mL (~30% normal expression levels). C57BL/6 mice injected with non-integrating vector, however, had no expression of hFIX protein at any time point throughout the experiment. Figures 4.8D-F show the results for BALB/c mice. No expression was seen for integrating vector at 2 days post injection, at 4 days all mice show expression of hFIX at an average level of 564.2ng/mL, however, at 16 days post-injection the hFIX blood plasma concentration dropped to zero, likely due to the immune response described previously [151]. BALB/c mice injected with non-integrating vector had no detectable hFIX protein in their blood plasma for the duration of the experiment. All mice injected with ghost vector had no detectable hFIX blood plasma level for the duration of the experiment.

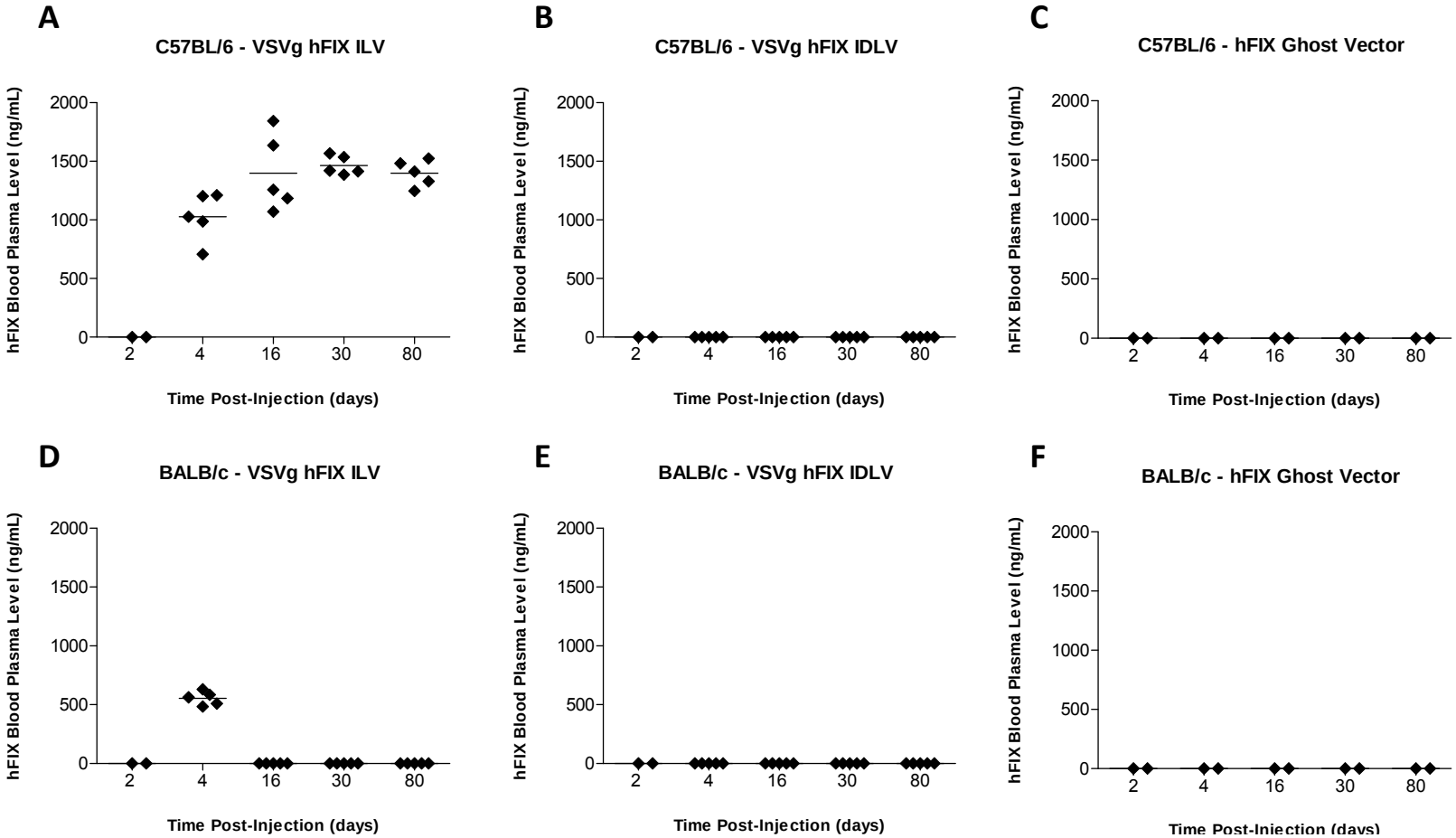


Figure 4.8 Blood plasma hFIX protein concentration in five adult C57BL/6 and BALB/c mice injected intravenously with VSVg pseudotyped ILV or IDLV expressing hFIX. Plasma concentrations of human factor IX (hFIX) protein in blood taken at 2, 4, 16, 30 and 80 days post-injection from five normal adult C57BL/6 or BALB/c mice injected with 7.8 x 10^8 TU of lentivector preparation based on RT titre. Vector groups: **(A)** VSVg hFIX ILV in C57BL/6, **(B)** VSVg hFIX IDLV in C57BL/6, **(C)** Ghost vector in C57BL/6, **(D)** VSVg hFIX ILV in BALB/c, **(E)** VSVg hFIX IDLV in BALB/c, **(F)** Ghost vector in BALB/c. Blood was collected via tail vein bleed and blood plasma analysed for hFIX protein concentration using an ELISA. Line represents the mean of all points.

4.4.3 Proviral Copy Number Determination

Mice were sacrificed at 80 days post-injection and liver, spleen, kidney, heart and lung tissue taken for analysis of viral copy number. Figures 4.9A-C show copy number for C57BL/6 mice. Mice injected with integrating vector had an average viral copy number in liver of 3.64 copies/cell, and spleen of 5.02 copies/cell. <1 copies per cell were also seen in heart, lung and kidney tissue. For the non-integrating vector group copy number was detected in liver of 0.13 copies/cell, and 0.01 copies/cell in spleen. Copy per cell in other tissues was <0.10 copies per cell for heart, lung and kidney.

Figures 4.9D-F show copy number for BALB/c mice. Mice injected with integrating vector lost expression of hFIX protein after 4 days post-injection, however, tissue taken from mice at 80 days post-injection showed detectable levels of viral copy of 1.69 copies/cell in liver and 0.43 copies/cell in spleen. All other tissues were <0.01 copies per cell. BALB/c mice injected with non-integrating vector showed negligible levels of viral copy number in liver at an average of 0.008 copies/cell, this is regarded as background. Copy in spleen tissue was also negligible at <0.01 copies per cell for all mice except one which was 1.08 copies/cell. All mice injected with ghost vector had no detectable WPRE sequence in any tissues.

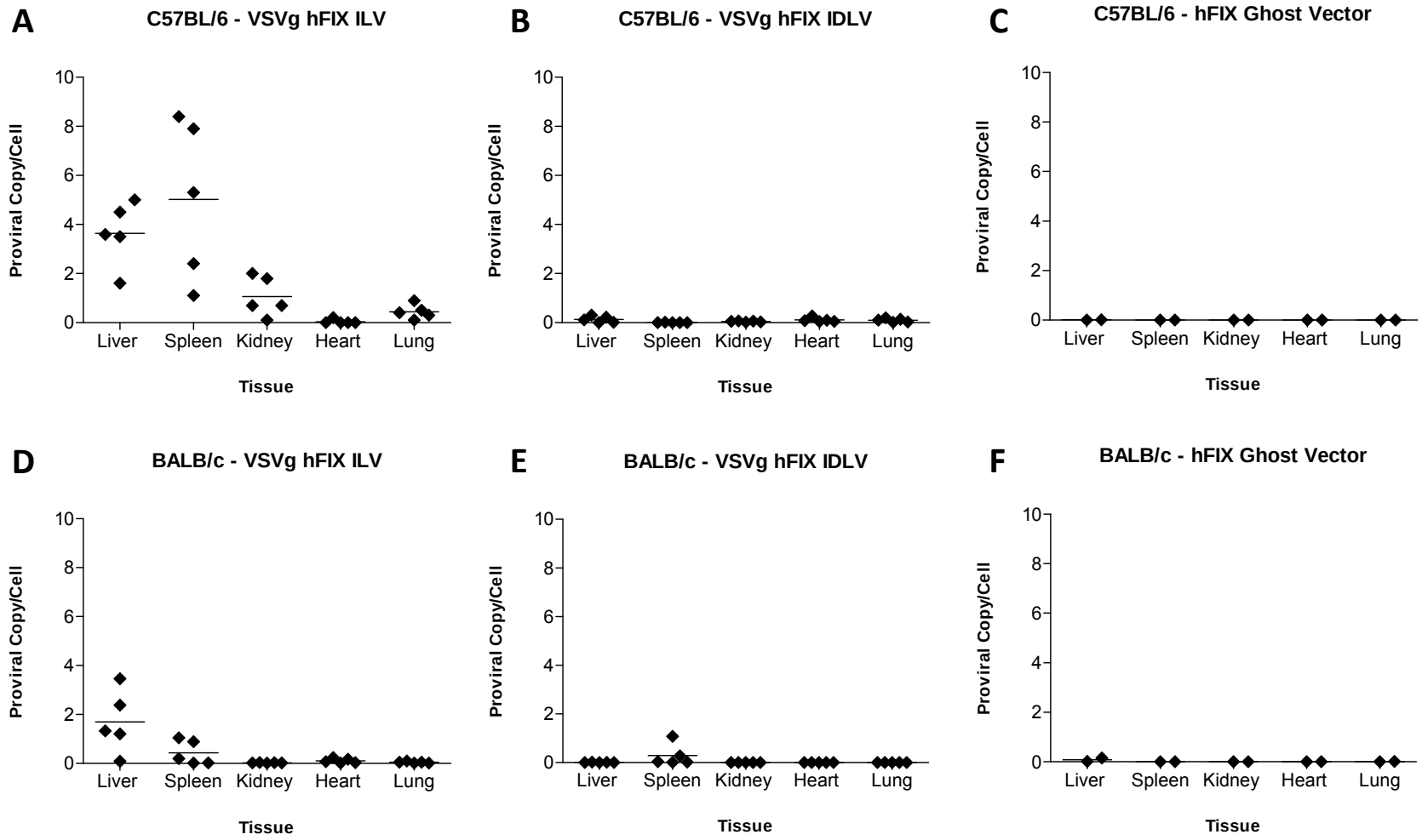


Figure 4.9 Quantification of proviral copy number in tissues from mice injected with lentivector expressing hFIX. C57BL/6 and BALB/c mice injected with vector expressing hFIX were sacrificed at 80 days post injection and liver, spleen, kidney, heart and lung tissue taken for proviral copy analysis using qPCR. Vector groups are: **(A)** VSVg hFIX ILV in C57BL/6, **(B)** VSVg hFIX IDLV in C57BL/6, **(C)** Ghost vector in C57BL/6, **(D)** VSVg hFIX ILV in BALB/c, **(E)** VSVg hFIX IDLV in BALB/c, **(F)** Ghost vector in BALB/c. Line represents mean of all points

4.5 Expression of Luciferase in Liver from Integration Proficient and Deficient Lentiviral Vectors

hFIX expression from IDLV injected intravenously was not detected and viral copy number in liver tissue was approximately 30-fold lower in comparison to ILV injected at the same TU. To assess why, vectors containing a luciferase transgene were produced and tested *in vitro* and *in vivo*. Using vectors expressing luciferase allows consecutive bioluminescent images to be taken of expression in the same animals after intravenous delivery of vector. This will help to assess biodistribution and track transgene expression over time.

4.5.1 Expression of Luciferase *In Vitro*

VSVg pseudotyped integrating and non-integrating lentiviral vectors expressing luciferase were produced by transient transfection. They were first tested for physical titre by a reverse transcriptase protein assay which showed that both vectors could be produced efficiently at an equivalent to 1.23 x 10^{10} TU/mL and 1.40 x 10^{10} TU/mL, for integrating and non-integrating vectors, respectively. Vectors were then tested *in vitro* by transducing 293T, 3T3 and Huh7 cells with a serial dilution of each vector. Luciferase expression was calculated 72 hours post-transduction as relative luciferase units (RLU) and values normalised by dividing by ng of reverse transcriptase (RT) protein (Figure 4.10).

Luciferase expression in RLU/ng RT for integrating vector in 293T, 3T3 and Huh7 cells were 4.23 x 10^4, 1.85 x 10^3 and 7.48 x 10^4 RLU/ng RT, respectively. Non-integrating vector had a similar pattern of expression between cell lines, however, expression levels were around 100-fold lower compared to integrating vector for 293T, 3T3 and Huh7 cells at 3.29 x 10^3, 1.0 x 10^2 and 1.80 x 10^3 RLU/ng RT, respectively (Figure 4.10).

Virus	RT (ng/µL)	Estimated Titre (TU/mL)
VSVg Luc ILV	36.2	1.23×10^{10}
VSVg Luc IDLV	41.3	1.40×10^{10}

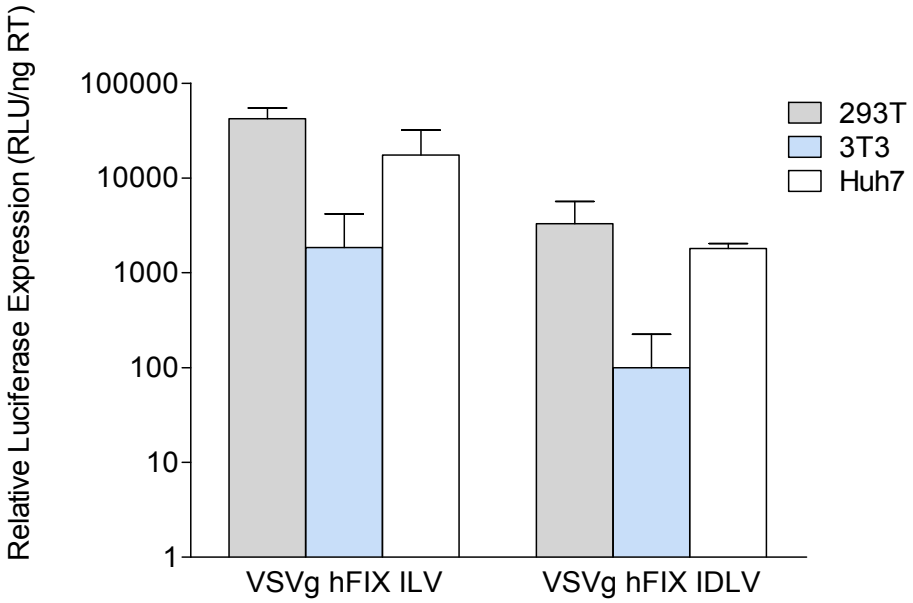


Figure 4.10 Quantification of physical vector titre and relative luciferase expression *in vitro.* Quantification of reverse transcriptase (RT) protein concentration in viral stocks, measured by performing a RT colorimetric assay, quantified in ng/µL and estimated titre calculated from this (n=3). 1.5×10^3 cultured cells were transduced with serial dilutions of ILV or IDLV expressing luciferase. Expression was quantified as relative luciferase units (RLU) using an *in vitro* luminescence assay. RLU values were then normalized against total particles in each vector preparation via dividing by RT protein in ng. Expression for 293T; human embryonic kidney cells, 3T3; murine fibroblasts, and Huh7; human hepatocyte cells were calculated. Mean and SEM plotted for 7 values. (n=7)

4.5.2 Expression of Luciferase *In Vivo*

Vectors were then tested *in vivo* by intravenous injection into adult (7 to 10 weeks of age) NOD/SCID mice. NOD/SCID mice are immune deficient and were chosen for this experiment to avoid any issue of vector clearance by a cellular immune response to transgene product or transduced cells. Three mice were injected intravenously via the tail vein per vector group (ILV and IDLV) with 7.8 x 10^8 transducing units (TU) as determined by the reverse transcriptase assay (2.3µg RT protein) in a total volume of 100µL. One mouse for each vector group was also injected via hydrodynamic delivery with the same vector concentration.

Hydrodynamic injection was developed as an alternative method for DNA or RNA delivery *in vivo*. Delivery is achieved by rapid intravenous injection of a large aqueous volume of nucleic acid solution [233]. Delivery via the tail vein primarily targets the liver, however, other organs such as kidneys, spleen, heart and lungs are also amenable to transfection [234]. The most successful application of hydrodynamic delivery is seen in gene delivery to hepatocytes in rodents; this procedure involves a tail vein injection, in 5-7 seconds, of a physiological solution, for example naked DNA in PBS, equivalent to 8-10% of body weight [235]. The injection of such a large volume of liquid entered directly into the inferior vena cava stretches the myocardial fibres beyond the optimal length for contraction, inducing cardiac congestion and driving the injected solution into the liver in retrograde [236-239].

Hydrodynamic delivery has also been used for enhancing the transduction efficiency of viral vectors *in vivo*; reports suggest that both adenoviral and lentiviral vectors injected by hydrodynamic delivery via the tail vein have increased transduction efficiency [240-245]. To assess if hydrodynamic delivery method could increase hepatocyte transduction levels and luciferase expression for integrating or non-integrating vectors one mouse for ILV and IDLV vectors was injected hydrodynamically with 7.8 x 10^8 TU vector, based on RT titre, in a total volume of 2mLs PBS via the tail vein. Three uninjected mice were also present as a control group.

Quantification of *in vivo* luciferase expression was assessed using an IVIS cooled charge-coupled device (CCCD) camera at 2, 4, 12, 16, 23, 47, 84, 135 and 201 days post-injection at a binning of 8 for 300 seconds. Representative images from days various time points post-injection are shown in Figure 4.11. Light emission was quantified in a defined region of interest (ROI) in photons/second/cm^2/sr over the liver area; shown as a circle on the representative images.

Fatalities occurred throughout the 201 day experiment: Per vector group: ILV intravenous; one mouse died at day 47 and one at day 135. IDLV intravenous; one mouse died at day 135. ILV hydrodynamic; mouse died at day 4. IDLV hydrodynamic; mouse died at day 47. Unfortunately, it was not possible to harvest tissues for analysis from mice which died before the end of the experiment.

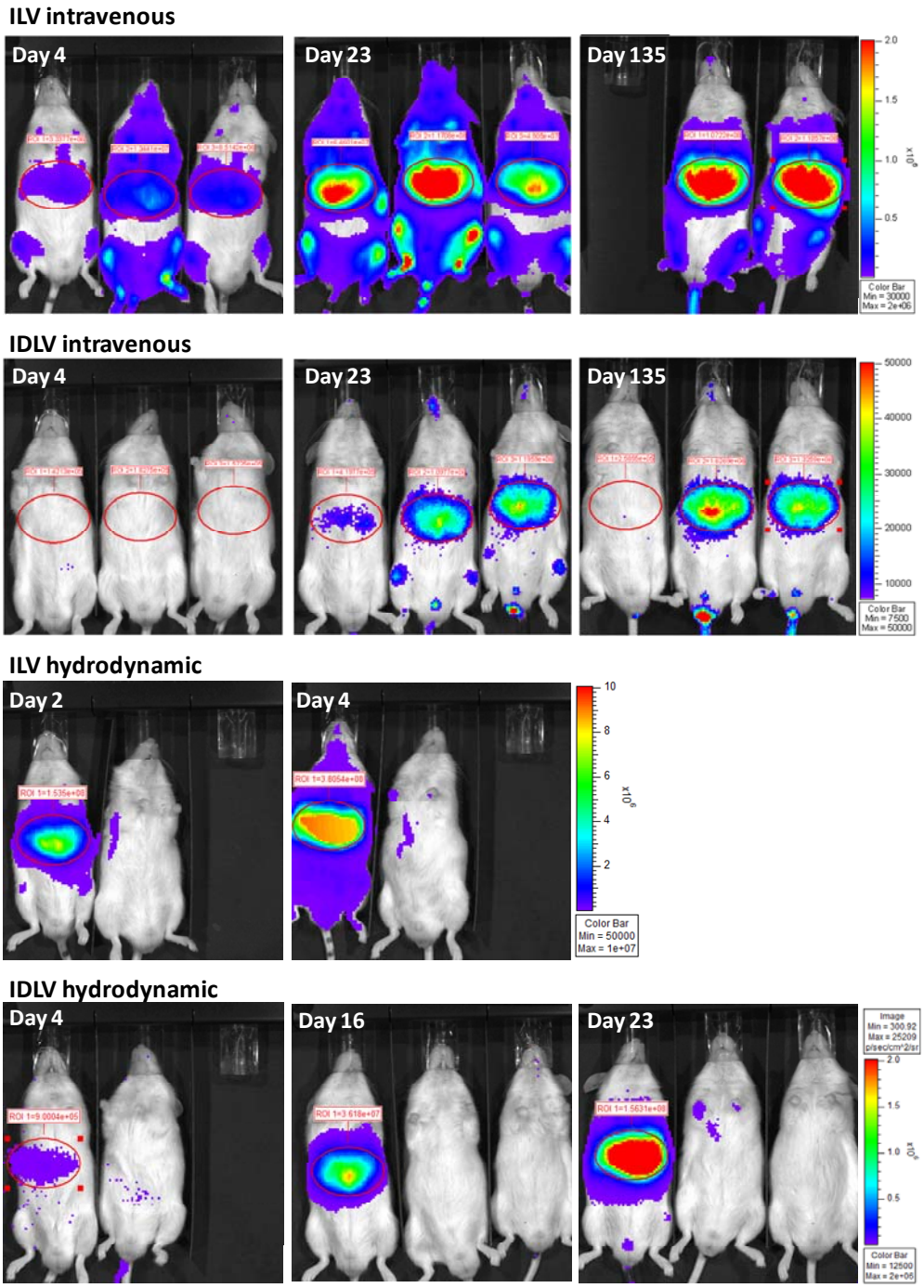


Figure 4.11 Representative images of luciferase expression after injection of integrating and non-integrating VSVg pseudotyped vectors expressing luciferase. Representative images of mice post intravenous injection of vector of 7.7 x 10^8 TU lentivector by intravenous or hydrodynamic delivery. Vector groups: ILV intravenous, IDLV intravenous, ILV hydrodynamic, IDLV hydrodynamic. Bioluminescence was measured in photons using an IVIS CCCD camera at a binning of 8 for 300 seconds. The area inside the circular region of interest (ROI) shown over each mouse was quantified in photons/second/cm^2/sr. For hydrodynamic groups where only one mouse was present imaging was always carried out with control mice present; the injected test mouse is therefore always shown on the far left of the image. Control group imaged separately but not shown.

Quantification of light emission in the defined ROI for consecutive images allowed expression to be monitored over time in each animal, the results shown in Figure 4.12. Quantification in these regions shows that intravenous injection of both integrating and non-integrating vector allowed sustained expression of luciferase above for the duration of the 201 day experiment. Expression for ILV after intravenous injection rose sharply over the first 2 weeks post-injection from an average of 2.26 x 10^6 photons/sec/cm^2/sr in the defined ROI on day 2 to 4.77 x 10^7 photons/sec/cm^2/sr by day 12. After this time point expression levelled and was sustained from 6.19 x 10^7 at day 16 rising slightly to 1.55 x 10^8 photons/sec/cm^2/sr by day 201. Expression of luciferase was seen to be strongest in the liver region however, it was not restricted to this area and luminescence is evident in many other tissues including, interestingly, the thigh and calf region in all mice (Figure 4.11).

IDLV injected intravenously encouragingly showed sustained expression above background for 201 days. There was an initial rise in expression in the first 2 weeks post-injection from an average of 1.61 x 10^5 photons/sec/cm^2/sr in the defined ROI on day 2 rising to 6.51 x 10^5 photons/sec/cm^2/sr by day 16. Expression then became stable at 1.37 x 10^6 photons/sec/cm^2/sr on day 47 rising slightly to 1.88 x 10^6 photons/sec/cm^2/sr by day 201. The expression levels observed were approximately 100-fold less compared to the ILV intravenous vector group and was mainly concentrated in the liver region; however, some expression can be seen in other tissues including joints (Figure 4.11).

One mouse was injected hydrodynamically with ILV. Expression observed in the liver region after hydrodynamic injection was extremely high at 1.54 x 10^8 photons/sec/cm^2/sr at day 2 and increased at day 4 to 3.81 x 10^8 photons/sec/cm^2/sr. Luminescence was mainly detected in the liver region and did not show a similar pattern of expression as observed for vector injected intravenously. Unfortunately, this mouse died at day 4, it is unknown if this is related to vector expression, however, extremely high expression of luciferase has been linked to toxicity (Dr. Simon

Waddington, personal communication). It was unfortunately not possible to obtain tissue for analysis from this mouse after death.

One mouse was also injected hydrodynamically with IDLV. High expression in the liver region was observed and levels rose quickly over the first two weeks from 2.64 x 10^6 photons/sec/cm^2/sr up to 8.13 x 10^7 photons/sec/cm^2/sr by day 12. Expression was then steady at 3.62 x 10^7 - 1.56 x 10^8 photons/sec/cm^2/sr from days 16 to 47, at which point the mouse died (Figures 4.11). The level of expression in the defined ROI was equivalent to mice injected with ILV intravenously, but expression was restricted to the liver area (Figure 4.11). It was not possible to take tissues for analysis.

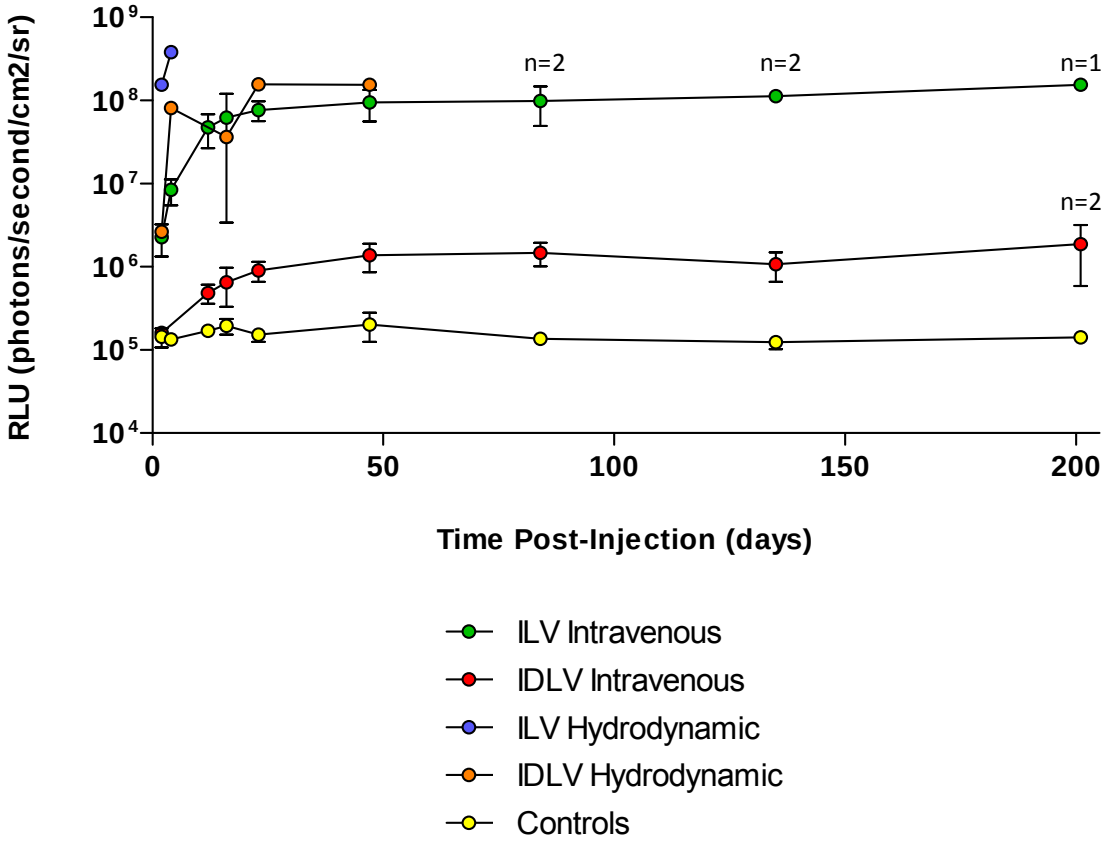


Figure 4.12 Quantification of luciferase expression. Light emission was quantified as photons/second/cm^2/sr in a defined ROI over the liver area (shown in Figure 4.11) for adult NOD/SCID mice injected intravenously or hydrodynamically with integrating or non-integrating vector expressing luciferase at a concentration of 7.8 x 10^8 TU. Control mice were uninjected. Vector groups: ILV intravenous, IDLV intravenous, ILV hydrodynamic, IDLV hydrodynamic. Luciferase expression inside the defined region is plotted as relative luciferase units (RLU) in photons/second/cm^2/sr. Mean and SEM plotted for each point. (n=3 for IV injected vectors and controls, n=1 for hydrodynamic injected vectors).

4.5.3 Determination of Proviral Copy Number and Quantification of Luciferase Protein in Tissues from Transduced Mice

All remaining mice were sacrificed at 201 days post-injection and liver, spleen, heart, lung, kidney, testes and bone marrow taken for analysis. Tissue was analysed for luciferase expression using the Luciferase Assay System (Promega) and a Bradford protein assay (BioRad) to give relative luciferase units (RLU) per µg protein. The number of integrated proviral copies was also assessed using quantitative real-time PCR (qPCR).

One mouse survived in the ILV intravenous vector group. Proviral copy in liver and spleen tissue was detected at 13.6 and 5.2 copies/cell, respectively. Bone marrow also contained copy number at 2.0 copies/cell, and all other tissues had low levels at <0.2 copies per cell. Two mice survived in the IDLV intravenous vector group. Viral copy was present predominantly in liver and spleen tissue at an average of 2.5 and 2.1 copies/cell, respectively. All other tissues were <0.02 copies per cell for heart, lung, kidney, bone marrow and testes. Copy number in liver was 5-fold lower for IDLV when compared to copy in the liver of the remaining ILV injected mouse. Two mice survived from the control group of three and all tissues taken showed a viral copy number <0.01 copies per cell (Figure 4.13A).

Luciferase expression was observed in liver and spleen tissue from the mouse injected with ILV intravenously at 1.8×10^6 and 1.0×10^6 RLU/µg protein, respectively. 9.9×10^5 RLU/µg protein was also detected in the bone marrow. All other tissues showed detectable levels of expression; heart at 9.1×10^3 RLU/µg protein, and lung, kidney and testes all at levels of ~2000 RLU/µg protein. Luciferase expression in the liver tissue of the two mice which survived injected intravenously with IDLV was 6.0×10^4 and 1.6×10^4 RLU/µg protein, approximately 50-fold lower in comparison to liver tissue from the ILV mouse. Levels were also detected in the spleen at 873 and 465 RLU/µg protein. Background luciferase protein was quantified in all tissues from control mice at ~300 RLU/µg protein (Figure 4.13B)

This experiment demonstrates that long term expression of luciferase can be mediated by a non-integrating lentiviral vector *in vivo* after intravenous injection. However, expression from an IDLV in comparison to an ILV when the same number of TU based on reverse transcriptase titre is ~100-fold lower, and copy number in the target liver tissue is approximately 5-fold lower at 201 days post-injection.

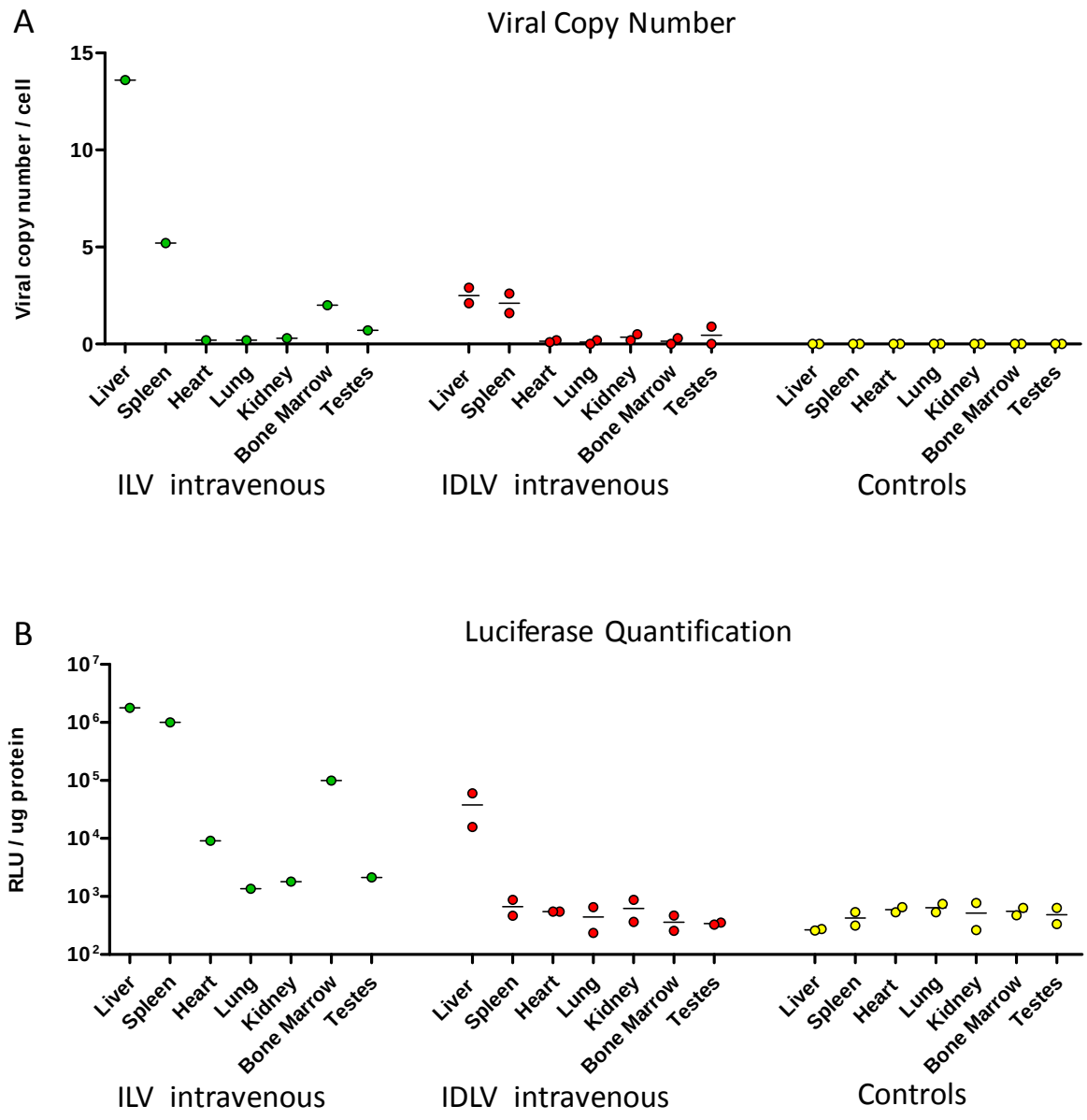


Figure 4.13 Quantification of viral copy number and luciferase protein in tissue from transduced mice. Liver, spleen, heart, lung, kidney, bone marrow and testes were harvested at 201 days post-injection. One mouse remained in the ILV intravenous vector group, two in IDLV intravenous, and two control mice. **(A)** Genomic DNA was extracted and qPCR carried out to determine proviral copy number per cell. **(B)** *Ex vivo* luciferase and Bradford protein assays were carried out to determine Relative Luciferase Units (RLU) per µg protein.

4.6 Repeat Luciferase Experiment

In the previous experiment 7.8 x 10^8 TU, based on reverse transcriptase titre, of ILV or IDLV expressing a luciferase transgene was injected into NOD/SCID mice. Two methods of vector delivery were investigated; intravenous injection via the tail vein, or hydrodynamic delivery in 2mLs PBS and both methods were found to successfully mediate expression of luciferase in liver, for up to 47 days for hydrodynamic and 201 days for intravenous injection. However, proviral copy number in liver from animals injected intravenously with non-integrating vector was 5-fold lower than that observed for integrating vector, and expression of luciferase, as determined by both an *ex vivo* luciferase assay and bioimaging, was aproximately 100-fold lower.

Due to low numbers of animals injected and fatalities during the experiment there was little tissue to analyse. A repeat experiment was carried out: 12 NOD/SCID mice were injected with vector expressing luciferase based on a titre determined on qPCR viral copy number determined *in* vitro per vector group: (i) ILV intravenous; (ii) IDLV intravenous; (iii) IDLV hydrodynamic; (iv) Uninjected control mice. Mice were imaged using a CCCD camera, and four mice were culled at four time points to allow tracking of both expression and copy number.

4.6.1 Expression of Luciferase *In Vitro*

To avoid possible problems with using the RT assay to determine titre integrating and non-integrating vectors expressing a luciferase transgene were produced and titred using qPCR. Titres for vectors were calculated at 2.38 x 10^9 and 1.18 x 10^7 vector genomes per mL, for integrating and non-integrating vectors, respectively. Vectors were then tested *in vitro* on liver and non-liver cell lines. 1.5 x 10^3 293T, 3T3 and Huh7 cells were transduced with a serial dilution of each vector. Luciferase expression was then measured 72 hours post-transduction. Values were then normalised via dividing by vector genomes to give units of RLU/ vector genomes (vg). Both integration

proficient and deficient vectors showed luciferase expression in all cell lines with a similar pattern between cell lines for both. However, expression values in RLU/vg for non-integrating vector was seen to be 280 fold lower than for integrating vector (Figure 4.14). This difference in expression is much more extreme than that observed when vector expression was normalised against titre based on reverse transcriptase protein.

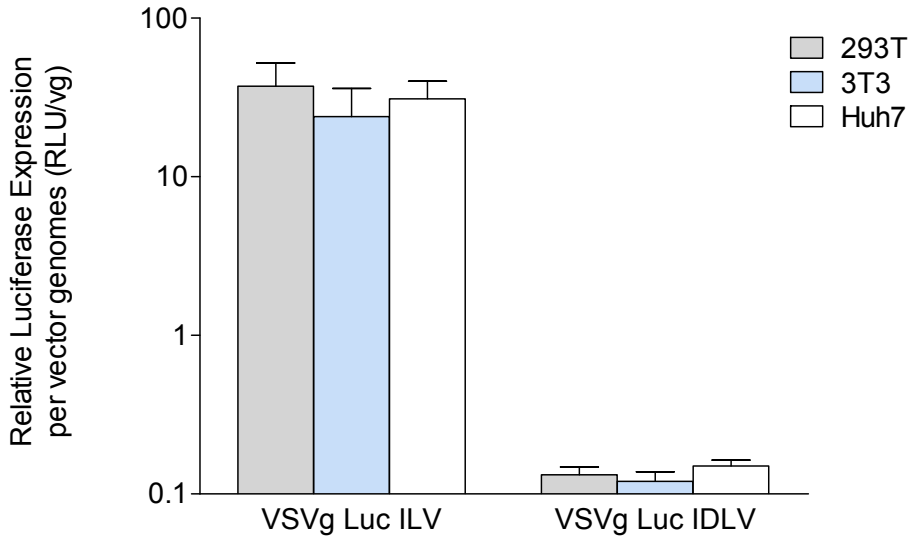


Figure 4.14 Relative luciferase expression of vectors expressing a luciferase transgene *in vitro*. 1.5 x 10^3 cultured cells were transduced with serial dilutions of integrating or non integrating vector. Luciferase expression was quantified as relative luciferase units (RLU) using the Luciferase Assay System (Invitrogen) at 72h post-transduction. RLU values were then normalized against titre via dividing by vector genomes per mL. Expression for 293T; human embryonic kidney cells, 3T3; murine fibroblasts, and Huh7; human hepatocyte cells were calculated. Mean and SEM plotted for n=7.

4.6.2 Expression of Luciferase *In Vivo*

Vectors were tested *in vivo* via injection into adult (7 to 10 weeks of age) NOD/SCID mice. Four vector groups were tested: (i) ILV intravenous; (ii) IDLV intravenous; (iii) IDLV hydrodynamic; (iv) Uninjected control mice. 12 mice were injected per group with a titre of 1.18 x 10^6 vector genomes, as determined by qPCR. Injections carried out intravenously were done so via the tail vein in a total volume of 100µL. Hydrodynamic injections were carried out via the tail vein in a total volume of 2mLs PBS injected over 5-7 seconds.

In vivo luciferase expression was assessed using an IVIS cooled charge-coupled device (CCCD) camera carried out for all animals at 2, 21 and 50 days post-injection at a binning of 8 for 10, 60, or 300 seconds. Representative images of mice at each time point for each vector group are shown in Figure 4.15. Light emission was quantified in a defined region of interest (ROI) in photons/second/cm^2/sr over the liver area; shown as a circle on the representative images. At each time point four mice in each group were sacrificed and tissues taken for proviral copy analysis and quantification of luciferase expression *ex vivo*. This allowed tracking of viral copy and protein expression over time to determine if vector genomes and protein expression in tissues were stable over time or transient within different vector groups.

Luciferase expression quantified in the defined ROI was plotted. However, as the imaging time varied between vector groups appropriate controls were always measured and these values subtracted from test values. Therefore, only test values are plotted in Figure 4.16.

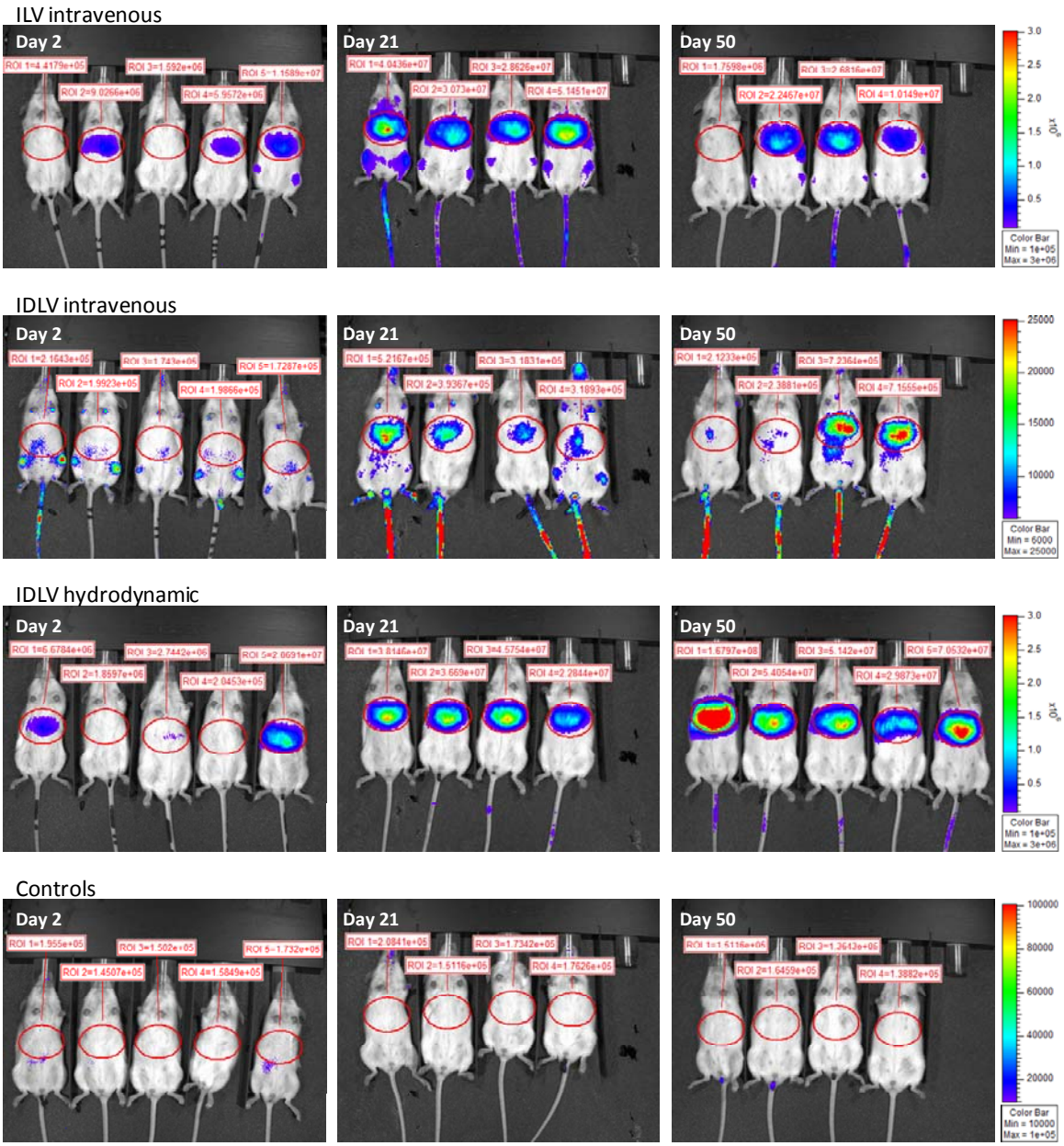


Figure 4.15 Representative images of luminescence imaging after injection of integrating or non-integrating vectors expressing luciferase. Representative images at days 2, 21 and 50 post-injection of mice injected with integrating or non-integrating vector at a titre of 7.8 x 10^6 vector genomes. Vectors groups were: (i) ILV intravenous; (ii) IDLV intravenous; (iii) IDLV hydrodynamic; (iv) Uninjected control mice. Luminescence was measured using an IVIS CCCD camera at a binning of 8 for 10, 60 or 300 seconds. The area inside the circular region shown over each mouse was quantified in photons/second/cm^2/sr. Please note the different scale for each set of images.

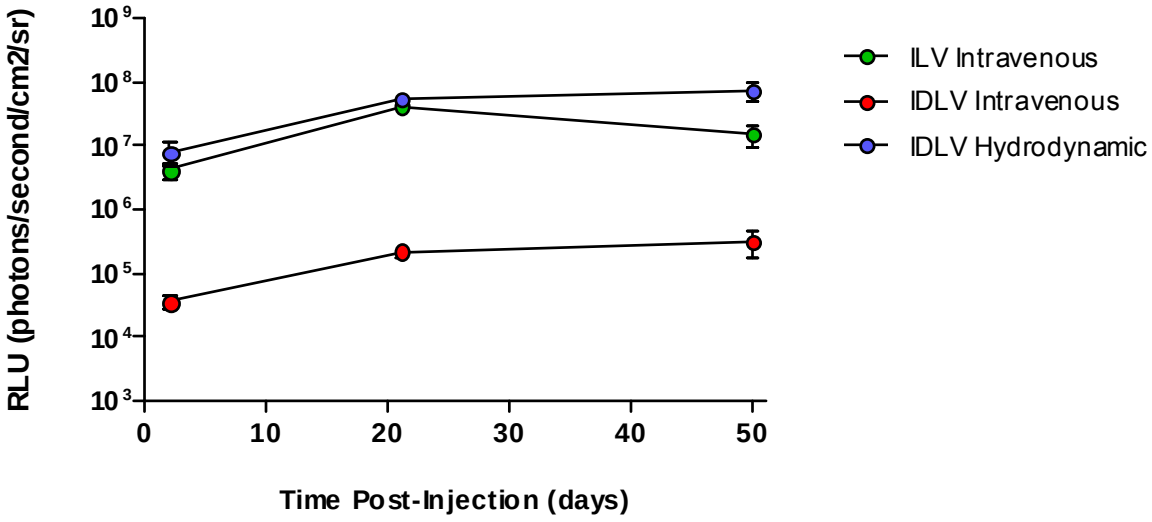


Figure 4.16 Quantification of luciferase expression. Luminescence was quantified as photons/second/cm^2/sr in a defined region of interest (ROI) over the liver area for adult NOD/SCID mice injected intravenously or hydrodynamically with integrating or non-integrating vector expressing luciferase at a titre of 7.8 x 10^6 vector genomes. Control mice were uninjected. Vector groups tested were: ILV intravenous, IDLV intravenous, IDLV hydrodynamic and controls. Values inside a defined region were measured and plotted as relative luciferase units (RLU) however, as the image time and binning varied between each group control values of the same time/binning were calculated subtracted for each test value. Mean and SEM plotted for 12, 8 and 4 mice in each vector group for days 2, 21 and 50, respectively.

Figure 4.16 shows each vector group had sustained expression over 50 days post-injection and there were no fatalities for the duration of the experiment. The ILV intravenous vector group had expression in the defined liver ROI from day 2 at an average of 4.18 x 10^6 photons/sec/cm^2/sr for twelve mice imaged rising to an average of 4.30 x 10^7 p/s/cm^2/sr by day 21 for eight mice imaged and dropping slightly at 50 days to an average of 1.51 x 10^7 p/s/cm^2/sr for the four remaining mice. Distribution of integrating vector is mainly in the liver area throughout the experiment, high expression is also seen at the site of injection in the tail, interesting as this was not observed in the previous *in vivo* experiment (see section 4.5.2). Luminescence observed in the femur and tibia areas, found to be expression in bone marrow in the previous experiment was not so pronounced this time, possibly due to the decrease in vector

injected. The IDLV intravenous vector group had expression for the duration of the experiment, at day 2 post-injection ten mice imaged had an average expression of 3.59 x 10^4 p/s/cm^2/sr in the defined ROI, this rose slightly at day 21 to 2.21 x 10^5 p/s/cm^2/sr for an average of eight mice imaged and levelled at 3.25 x 10^5 p/s/cm^2/sr at day 50 for the four remaining mice. Vector distribution was mainly in the liver area and also higher expression in the tail at the site of injection.

Unexpectedly, non-integrating vector injected hydrodynamically gave the highest expression of luciferase, concentrated in the liver area. At day 2 expression was 8.04 x 10^6 p/s/cm^2/sr in the defined ROI, rising at day 21, at a similar rate to integrating vector, to 5.44 x 10^7 p/s/cm^2/sr and then again 7.46 x 10^7 p/s/cm^2/sr at day 50.

4.6.3 Determination of Proviral Copy Number and Quantification of Luciferase Protein in Tissues from Injected Mice

Four mice were sacrificed at three time points for the experiment at 2, 21 and 50 days post injection. Liver, spleen and bone marrow tissue were taken for both proviral copy analysis and luciferase protein quantification. For the ILV intravenous vector group copy number was primarily located in the liver and spleen tissue and a negligible amount in the bone marrow. Viral copy in the liver tissue of ILV injected mice at 2, 21 and 50 days post-injection was calculated at 1.62, 1.98 and 2.56 copies per cell, respectively. Copy number is seen to increase slightly over time, however, this is mainly due to one mouse at 50 days post-injection with 5.57 copies/cell. Generally, copy is seen to be stable at ~2 copies per cell throughout the experiment. Copy number in the spleen is lower at an average of 0.84, 0.86 and 1.71 copies per cell, and bone marrow at 0.36, 0.54 and 0.23 copies per cell for 2, 21 and 50 days post-injection, respectively.

For the IDLV intravenous vector group viral copy number was mainly seen in the liver and at only negligible levels in spleen and bone marrow. Viral copy in liver did not increase or decrease substantially over time and was calculated at an average of 0.22,

0.22 and 0.63 copies per cell for 2, 21, and 50 days post-injection, respectively. For the IDLV hydrodynamic vector group the majority of viral copy was found in the liver at high levels of 2.04, 2.61 and 2.68 copies per cell for 2, 21 and 50 days post-injection, respectively. Copy number in spleen and bone marrow of these injected mice was found to be negligible at <0.3 copies per cell. Copy number in control mice was negligible at <0.4 copies per cell for all tissues at all time points.

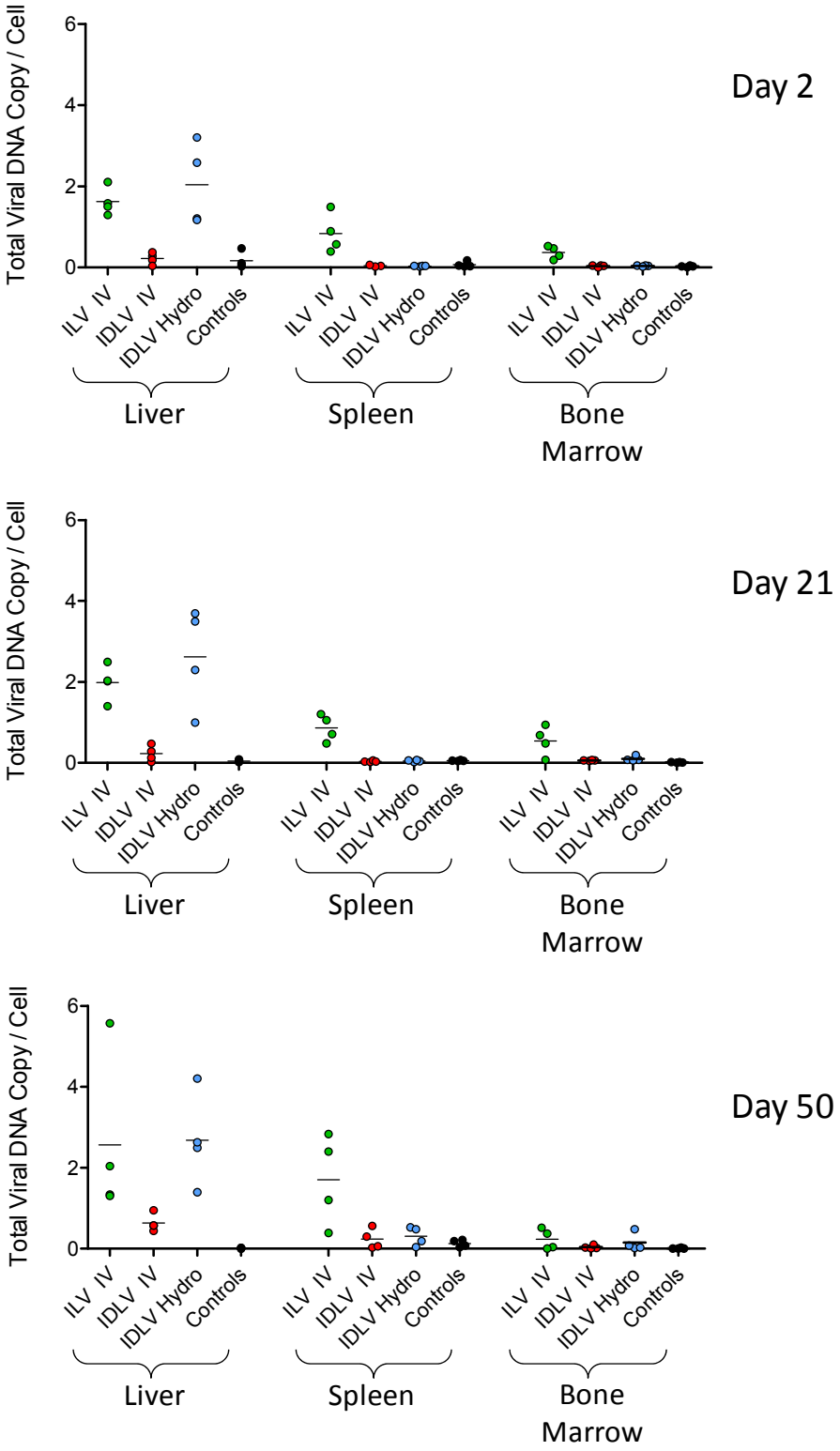


Figure 4.17 Quantification of Total viral DNA per cell in tissue taken from mice injected with ILV or IDLV. Liver, spleen and bone marrow tissue was taken from four mice sacrificed at three time points of 2, 21 and 50 days post-injection. Genomic DNA was extracted and qPCR carried out for each sample using primer and probe sets for the murine housekeeping gene *titin* and the viral WPRE sequence allowing determination of total viral copy per cell for vector groups: ILV intravenous, IDLV intravenous, IDLV hydrodynamic and control mice. Line represents the mean for n=4.

Hydrodynamic delivery of IDLV gave expression of luciferase equivalent to ILV injected intravenously. To eliminate the possibility of plasmid contamination in viral preparations causing the expression qPCR was carried out for the 2-LTR virus episome using a primer and probe set to the join between the two LTRs. The results were then normalised against *titin* copy number for viral episomes per cell.

2-LTR copy number was detected in all tissues tested, for the ILV intravenous vector group copy was primarily located in the liver at an average of 0.4, 0.2 and 0.1 2-LTR copies per cell at 2, 21 and 50 days post-injection, respectively, dropping slightly over time. Levels in the spleen and bone marrow are negligible at all time points at <0.1 copies per cell. For the IDLV intravenous vector group viral copy was mainly seen in the liver at 0.7, 0.5 and 0.2 2-LTR copies per cell at 2, 21 and 50 days post-injection, respectively. This is also seen to drop over time. Negligible levels of <0.1 2-LTR copies per cell are again seen in spleen and bone marrow tissue at all time points. For the IDLV hydrodynamic vector 2-LTR viral copy liver was found at higher levels than for the same amount of vector delivered by intravenous delivery. Levels of 1.2, 0.6 and 0.3 2-LTR copies per cell were seen in liver at 2, 21 and 50 days post-injection, respectively, again levels dropping over time. Copy number in control mice tissue was negligible at <0.1 copies per cell for all tissues at all time points.

There is a disparity between the total viral DNA copy and the 2-LTR copy; this could be due to inaccuracy of the qPCR assays used, the presence of 1-LTR circles which cannot be detected using this assay, or integrated DNA. A relatively low increase in copy number for the IDLV hydrodynamic groups appears to have a large increase in expression. Again, this disparity between copy number and expression may be due to inaccuracy of qPCR assays, plasmid DNA contamination, or pseudo-transduction.

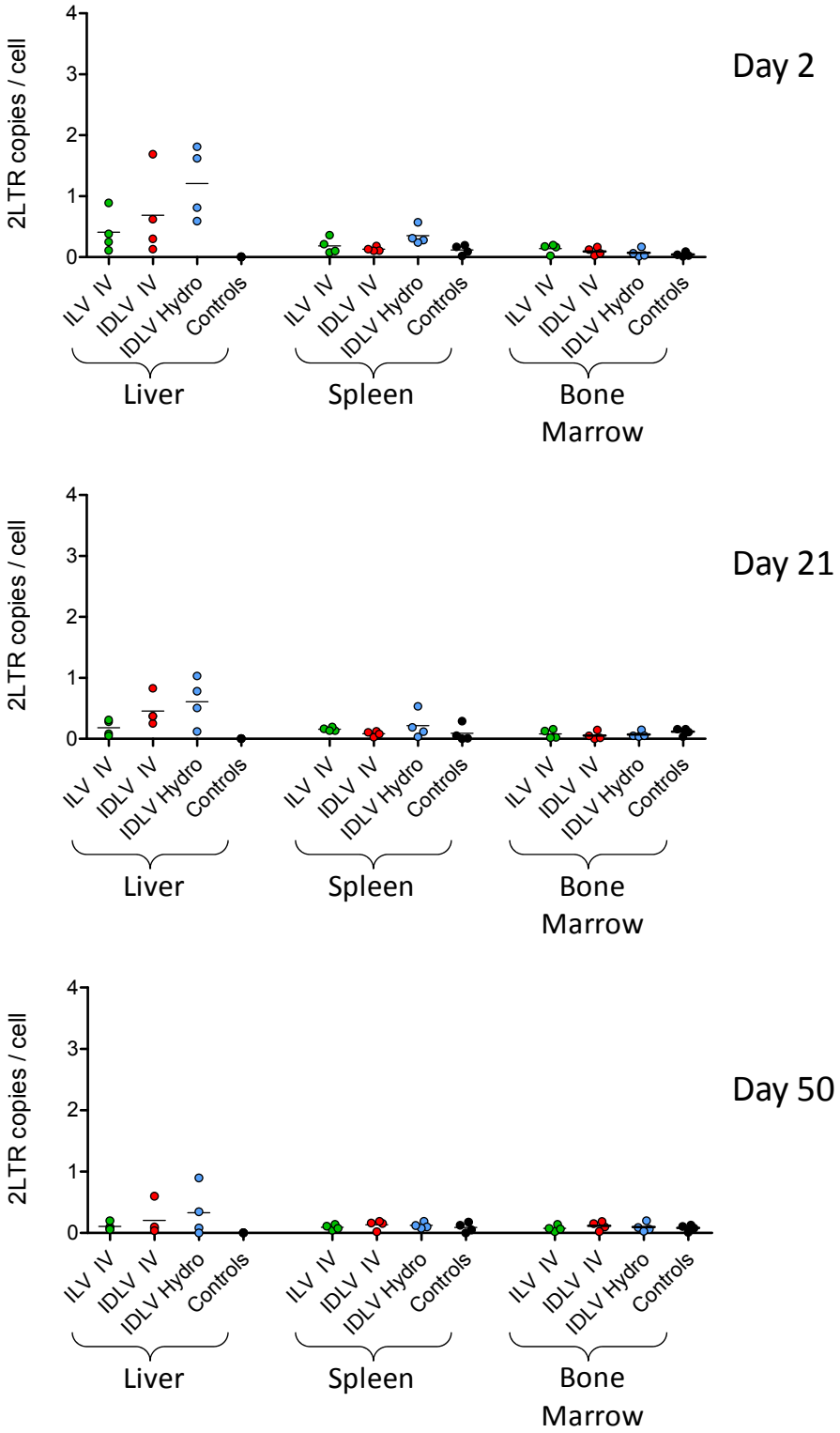


Figure 4.18 Quantification of 2-LTR episomal DNA per cell in tissue taken from mice injected with ILV or IDLV. Liver, spleen and bone marrow tissue was taken from four mice sacrificed at three time points of 2, 21 and 50 days post-injection. Genomic DNA was extracted and qPCR carried out for each sample using primer and probe sets for the murine housekeeping gene *titin* and the join between viral LTRs allowing determination of 2-LTR copies per cell for vector groups: ILV intravenous, IDLV intravenous, IDLV hydrodynamic and control mice. Line represents the mean for n=4.

Luciferase expression was quantified *ex vivo* in relative luciferase units (RLU) per µg protein. For the ILV intravenous vector group luciferase expression in liver tissue at day 2 was an average of 3.5×10^4 RLU/µg protein, rising 14-fold to 5.1×10^5 RLU/µg protein by day 21 and remaining at 3.4×10^5 RLU/µg protein at day 50 post-injection. Similarly, average levels in spleen taken at day 2 was 3.4×10^3 RLU/µg protein, rising to 9.3×10^3 and 1.1×10^4 at days 21 and 50 post-injection, respectively. Luciferase expression was detected in bone marrow at averages of 810, 375 and 1375 RLU/µg protein for days 2, 21 and 50 post-injection, respectively. These levels correlate to both the imaging data and viral copy number collected.

For IDLV injected intravenously luciferase expression was only detected in liver tissue, average levels were recorded at 1.2×10^3, 7.2×10^3 and 9.0×10^3 RLU/µg protein from tissue taken at days 2, 21 and 50 post-injection. Protein was not detected above that of control values in spleen and bone marrow tissue at all time points. For IDLV injected hydrodynamically luciferase protein was again only detected at significant levels in liver tissue at averages of 5.5×10^5 RLU/µg protein at day 2, rising 2-fold on day 21 to 1.3×10^6 RLU/µg protein and remaining at 8.4×10^5 RLU/µg protein at day 50. Spleen and bone marrow tissue did not have luciferase expression above that detected in control tissues. Background luciferase protein of 400-800 RLU/µg protein was detected in all control tissues.

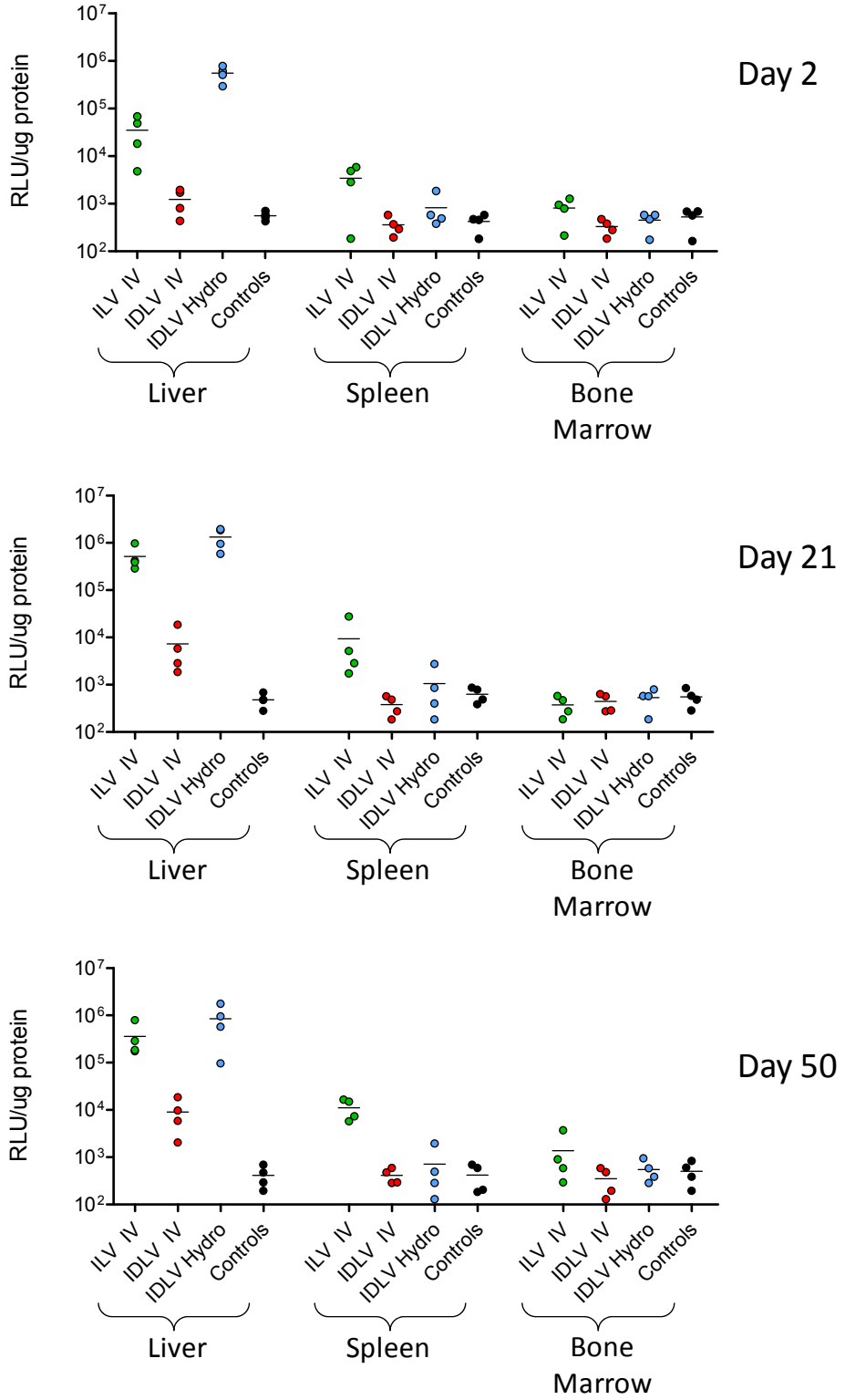


Figure 4.19 Quantification of expression in tissue taken from mice injected with ILV or IDLV expressing luciferase. Liver, spleen and bone marrow tissue was taken from four mice sacrificed at three time points of 2, 21 and 50 days post-injection. Relative Luciferase Units (RLU) per µg protein was determined by using the Luciferase Assay System (Promega) and a Bradford protein assay (BioRad) for vector groups: ILV intravenous, IDLV intravenous, IDLV hydrodynamic and control mice. Line represents the mean for n=4.

4.7 Summary

- The kinetics of transgene expression of ILVs and IDLVs in dividing and non-dividing Huh7 cells was assessed. For IDLVs expression was lost over time in dividing cells, however, expression was maintained in non-dividing cells over time at a similar percentage of GFP^+ cells to ILV.
- IDLVs were unable to mediate expression of human FIX *in vivo* after intravenous delivery, copy number in the liver tissue of transduced animals was 30-fold lower compared to ILV.
- IDLVs were able to mediate long-term expression of luciferase *in vivo* after intravenous delivery. However, expression was approximately 200-fold lower in comparison to ILVs.
- Hydrodynamic delivery of IDLV vector allows sustained expression of vector and increased expression over 200-fold *in vivo* in comparison to IDLV injected intravenously.

In this chapter expression of both FIX and luciferase from ILV and IDLV in liver were investigated *in vivo* in adult mice after intravenous delivery of vector. Firstly, the kinetics of VSVg and gp64 pseudotyped ILV and IDLV were assessed in dividing and non-dividing Huh7 (human hepatoma) cells as a model of dividing and non-dividing liver cells. The cells were sucessfully growth arrested through serum starving but there was a loss in cell viability. For dividing cells transduced with IDLV GFP expression was lost over time, in contrast, in non-dividing cells IDLV episomes were maintained and percentage of GFP cells observed at a similar rate to integrating vector for the duration of the experiment. No difference in expression kinetics was observed between VSVg or gp64 pseudotyped vectors. Viral copy number and MFI for transduced non-dividing Huh7 cells was also determined for all vector groups tested. IDLV epsiomes have previously been reported to be less transcriptionally active, Cornu and Cathomen (2007) [93] showed that a ten-fold increase in IDLV was required *in vitro* to achieve the same expression of GFP compared to an equivalent ILV.

Vectors expressing a factor IX transgene were produced and tested *in vitro* and IDLVs were able to mediate expression of FIX in cell lines at similar levels to ILV. Adult mice were then injected intravenously with ILV or IDLV expressing FIX and blood plasma assayed over time for FIX protein using ELISA. Both BALB/c and C57BL/6 strains of mice were investigated and a loss of expression was observed in BALB/c mice after 4 days, likely due to the immune reponse as reported by Brown *et al.,* in 2007 [151]. C57BL/6 mice injected with ILV expressed FIX protein at 1500ng/mL (30% normal human levels) after injection of 7.8 x 10^8 TU. In contrast, mice injected with the same TU of IDLV did not produce any detectable FIX protein. Viral copy number for ILV transduced C57BL/6 mice was investigated and copy was highest in liver and spleen tissue; similar to results reported by Follenzi *et al.* (2002) [228]. Viral copy number for IDLV transduced C57BL/6 mice was on average 30-fold lower in comparison. This, coupled with lower expression found from IDLV episomes, may explain the lack of hFIX detected in blood plasma of transduced mice.

It was unknown at this point if viral episomes were lost over time due to cell proliferation or if target cells were not being transduced upon delivery of vector. To investigate biodistribution and expression from IDLVs in liver vectors expressing the more sensitive marker gene luciferase were produced and injected intravenously into adult immune deficient NOD/SCID mice. Long-term expression of luciferase was maintained in mice injected with IDLV, primarily in the liver, but expression was ~200-fold lower compared to mice injected with ILV of the same number of vector genomes. IDLV copy number determined in liver at time-points throughout the experiment indicated that viral episomes were not lost over time. However, levels were 100-fold lower in comparison to ILV injected mice throughout the experiment. As there was no substantial increase over time in integrated viral copy in liver for ILV injected mice, or a decrease of vector episomes in liver for IDLV injected mice, these results suggest that there is no significant cellular proliferation. This may therefore indicate that cells are inefficiently transduced by IDLV, that vector episomes may be inefficiently produced, or that episomes are lost due to cell processes *in vivo* which are not evident *in vitro*. Further work studying the formation of viral DNA episomes in liver tissue, mRNA copies

transcribed from both integrated provirus and episomal DNA, and the amount of protein produced from each viral copy would be interesting to study.

Interestingly, hydrodynamic injection via the tail vein of the same number of TU of IDLV gave expression of luciferase equal to that achieved by ILV injected intravenously. The copy number determined in liver was also higher, and was shown to be predominantly 2-LTR viral episomes. However, a control for pseudotransduction was not included in this experiment. Hydrodynamic injection allows targeting of the liver to a certain extent, this suggests that changing the route of vector administration to overcome *in vivo* barriers to allow better liver transduction may increase expression from IDLV. This may be achieved by techniques such as intraportal injection [200] or surgical approaches [246]. Removal of cellular barriers to hepatocyte transfer in the liver may also be achieved: van Til *et al.,* in 2005 [232] showed that inhibition of Kupffer cell function *in vivo* using gadolinium chloride treatment before vector injection allowed a seven-fold increase in hepatocyte transduction. Similarly, Chuah *et al.,* in 2003 [247] showed that pre-treatment of mice with an intraportal injection of clodronate liposomes transiently depleted macrophages and that subsequent gene transfer allowed significantly higher transduction and expression from an adenoviral vector. Avoiding off target expression, and more specific hepatocyte expression for IDLVs may also avoid vector episome loss in proliferating cell types, increasing transgene expression while minimising interaction of vectors with cells of the immune system.

A further approach to study distribution of lentivector in more detail would be to create a vector which directly delivered a hetrologous protein such as GFP or luciferase which could be visualised within the cell hours after transduction. This would minimise the delay between cell entry and development of viral episomes, during which time the viral genome may be lost to processes within the cell.

HIV-1-mediated delivery of heterologous proteins has been previously reported. The general approach is to fuse the heterologous protein to an HIV-1 protein so that it is incorporated into vector particles in producer cells, and then to transduce target cells and investigate the intracellular distribution of protein. Fusions are most commonly

made with the Vpr protein, a Vpr-eGFP fusion has previously been used to investigate the biology of HIV-1 assembly [248] and cell entry and trafficking [249,250]. Vpr is a good candidate for protein transduction fusions as it remains associated with the viral DNA in the cytoplasm, demonstrating its ability to escape from endosomes after cell entry [251], however, as Vpr is not present in third generation vector genomes an alternative such as gag-eGFP could be investigated.

Overall this chapter demonstrated that IDLVs are not able to mediate expression of factor IX upon direct intravenous injection at therapeutic levels. However, IDLVs can mediate long term expression of luciferase in liver for >200 days, and hydrodynamic delivery of IDLV expressing FIX may produce therapeutic levels.

Chapter Five

Analysis of Reporter Gene Expression from Mammalian and Viral Promoters in Liver

5.0 Aims

- To investigate luciferase reporter gene expression under control of various mammalian promoter elements versus viral promoter elements in a lentiviral vector construct both *in vitro* in liver and non-liver cell lines and *in vivo* in neonate MF1 mice.

5.1 Introduction

In SIN lentiviral vector constructs internal promoter elements are essential for transgene expression. Viral promoter elements such as the 5' LTR of the spleen focus forming virus (SFFV), or the immediate-early enhancer and promoter of human cytomegalovirus (CMV) are both commonly used. However, in studies involving both viral and plasmid vectors targeting adult liver cells *in vivo*, viral promoters elements are seen to drive very high initial transgene expression but subsequent expression is seen to decline sharply [252]. This decline can often be attributed to transcriptional silencing of the promoter, either by DNA methylation and/or because of the lack of transcriptional factors in the liver that can support continuous expression [9,252,253]. Cytokines such as α- or γ-interferon have also been shown to actively inhibit the CMV promoter [254]. In comparison many mammalian promoter elements do not undergo transcriptional silencing and are able to promote long-term transgene expression *in vivo* in liver [255]. Along with promoting long term expression mammalian promoter elements may also confer tissue specificity preventing off target expression in immune cells such as APCs. Hepatocyte specific transduction would be advantageous for intravenous delivery of lentivectors expressing coagulation factors as this is the normal site production, and also for integration deficient vectors to avoid loss of vector episomes in proliferating cell types.

In this chapter we compare expression from a lentiviral vector containing the reporter gene luciferase driven by either mammalian or viral promoter elements. The mammalian promoter elements used are the cellular non-specific ubiquitous chromatin opening element (UCOE), the liver-specific promoter human alpha-1-antitrypsin (hAAT), and a liver-specific regulatory element LP1 that consists of the core domains from the human apolipoprotein hepatic control region and the human alpha-1-antitrypsin gene promoter. The viral promoter element used was the 5' LTR from the Spleen Focus Forming Virus (SFFV) which has been used as standard throughout this project.

5.2 Expression of Luciferase from Lentiviral Vector Constructs Containing Mammalian or Viral Promoter Elements

5.2.1 Cloning of LP1, hAAT and UCOE Promoter Elements

The liver specific promoters LP1 and human alpha-1-antitrypsin (hAAT), and the non-specific mammalian promoter UCOE have all been previously been described (see section 1.5.2) and the following literature; [8,9,143,144,146,255].

The 448bp LP1 promoter was obtained from the plasmid psc-AAV-LP1-hFIXco, a kind gift from Dr. Amit Nathwani, UCL. The LP1 promoter was amplified by PCR from this plasmid using primers that created *EcoR*I and *Bcl*I restriction sites at the 5' and 3' ends of the PCR product, respectively. Digestion of the plasmid pLNT/SFFV-Luc-WPRE with *Eco*RI and *Bam*HI allowed removal of the SFFV promoter and ligation of the LP1 PCR product to create the construct pLNT/LP1-Luc-WPRE shown in Figure 5.1B. The hAAT promoter was obtained from the plasmid pLucA1, a kind gift from Dr. Richard Harbottle, Imperial College London. The 255bp hAAT promoter and a 374bp region upstream of the promoter containing alpha-1-globin were amplified by PCR using primers that created *Eco*RI and *Bcl*I restriction sites at the 5' and 3' ends of the PCR product, respectively. Digestion of the plasmid pLNT/SFFV-Luc-WPRE with *Eco*RI and *Bam*HI allowed removal of the SFFV promoter and ligation of the hAAT PCR product to create the construct pLNT/hAAT-Luc-WPRE shown in Figure 5.1C. The 2.5kb UCOE promoter was obtained from the in house plasmid pLNT/UCOE-eGFP by digestion with *Eco*RI and *Sal*I. Digestion of the plasmid pLNT/SFFV-MCS-WPRE with *Eco*RI and *Xho*I allowed removal of the SFFV promoter and creation of pLNT/UCOE-MCS-WPRE. The 1.6kb luciferase cDNA was then amplified by PCR from pLNT/SFFV-Luc-WPRE using primers that created *Sbf*I restriction sites at both the 5' and 3' ends of the PCR product. Digestion of the plasmid pLNT/UCOE-MCS-WPRE with *Sbf*I allowed the ligation of luciferase and creation of the pLNT/UCOE-Luc-WPRE construct shown in Figure 5.1D. All constructs were confirmed to be correct by sequencing.

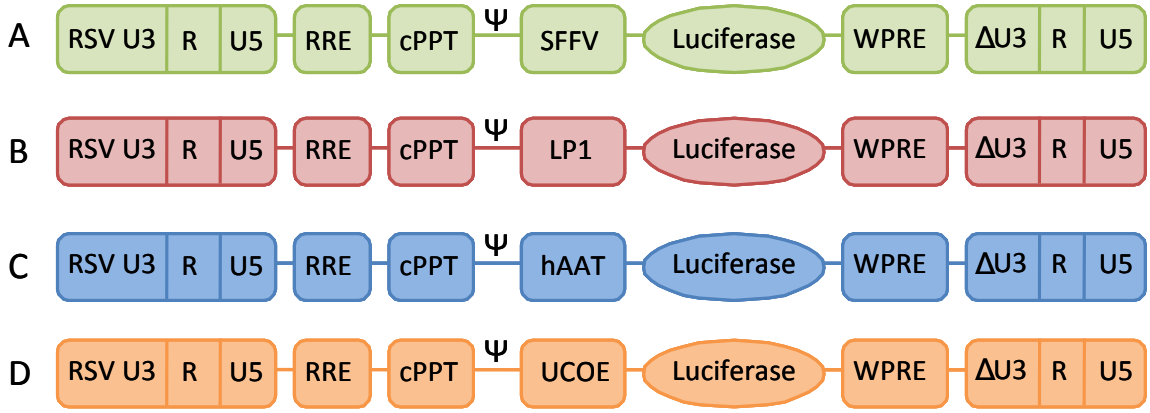


Figure 5.1 Schematic of SIN lentiviral vector constructs expressing a luciferase transgene. Lentiviral vector constructs were cloned containing mammalian or viral promoter elements driving a luciferase transgene. Promoter elements cloned were: **(A)** the 5' LTR of the Spleen Focus Forming (SFFV) **(B)** Liver-specific regulatory element LP1 containing the human apolipoprotein hepatic control region and the human alpha-1-antitrypsin gene promoter **(C)** Human alpha-1-antitrypsin gene promoter (hAAT) **(D)** Human ubiquitous chromatin opening element (UCOE).

5.2.2 Analysis of Tissue Specificity *In Vitro*

Integrating, VSVg pseudotyped vectors were produced by transient transfection methods and physical titre estimated using the reverse transcriptase assay. Vectors were also titred using qPCR; 1 x 10^5 293T cells were transduced with a serial dilution of each vector, after 72 hours genomic DNA was extracted from cells and qPCR carried out using primer and probe sets to both viral WPRE sequence and the human housekeeping gene β-actin. From these results, the copy number per cell can be determined for each dilution and an approximate titre in vector genomes per mL (vg/mL) calculated (Table 5.1).

Vector Group	Average Reverse Transcriptase (ng/µL)	Estimated Titre (TU/mL)	qPCR Titre (vg/mL)
LV.SFFV.Luc.WPRE	2.33	7.92×10^8	1.40×10^6
LV.LP1.Luc.WPRE	1.24	4.22×10^8	2.59×10^5
LV.hAAT.Luc.WPRE	3.43	1.17×10^9	2.04×10^6
LV.UCOE.Luc.WPRE	2.98	1.01×10^9	7.74×10^5

Table 5.1 Physical titre of vectors as determined by reverse transcriptase assay and qPCR. Quantification of reverse transcriptase (RT) protein concentration in viral stocks, measured by performing a RT colorimetric assay, quantified in ng/µL and estimated titre calculated from this. Mean shown of n=3. Quantification of titre in vector genomes per mL determined using qPCR. 1×10^5 293T cells were transduced with a serial dilution of vector, after 72 hours genomic DNA was extracted from cells and qPCR carried out using primer and probe sets for both the viral WPRE sequence and the human housekeeping gene β-actin. Mean shown of n=5.

To assess the tissue-specificity and relative expression of each promoter *in vitro* a variety of liver and non-liver cell lines were transduced with vector and analysed for transgene expression. 293T, Huh7, Hep-G2 and 3T3 cells were transduced with a serial dilution of each vector. Luciferase expression was calculated 72 hours post-transduction as relative luciferase units (RLU) using the Luciferase Assay System (Promega) and values normalised via dividing by the vector titre in vector genomes (Figure 5.2).

Relative expression for vector containing luciferase driven by the SFFV promoter was high in all cell lines tested with no tissue specificity evident. Similarly, expression driven by the UCOE promoter was also high but had no discernable cell preference. The two liver specific promoters LP1 and hAAT, however, showed higher levels of gene expression in the liver cell lines Huh7 and Hep-G2, with LP1 showing the most dramatic difference with a 425 and 260-fold increase in expression in Huh7 and Hep-G2 cells, respectively, compared to expression in 293T cells. The hAAT promoter showed more modest expression level in all cell lines, however a 9 and 7-fold increase of expression was seen in Huh7 and Hep-G2 cells, respectively, in comparison to expression in 293T cells.

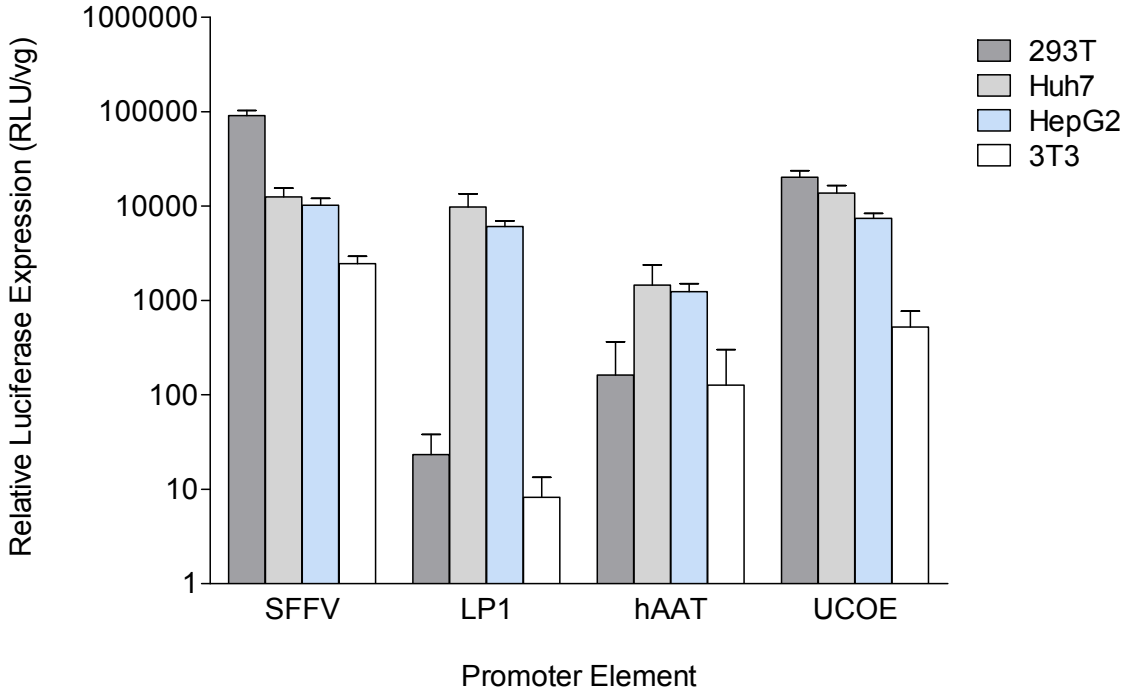


Figure 5.2 Relative luciferase expression from integrating vectors containing viral or mammalian promoter elements *in vitro*. 1.5 x 10^3 cultured cells were transduced with serial dilutions of vector containing a luciferase transgene driven by promoter element SFFV, LP1, hAAT or UCOE. Luciferase expression quantified as relative luciferase units (RLU) using the Luciferase Assay System (Promega) 72h post-transduction. RLU values were then normalized against titre via dividing by vector genomes. Expression for 293T; human embryonic kidney cells, Hep-G2; human hepatocellular carcinoma cells, Huh7; human hepatocyte cells and 3T3; murine fibroblasts, were calculated. Mean and SEM plotted for n=7.

5.2.3 Analysis of Expression *In Vivo*

Tissue specificity and relative expression was also tested *in vivo* via direct injection of concentrated lentiviral vector into the superficial temporal vein of neonatal (1-2 day old) MF1 mice. Injection of neonate mice via this method has been shown to permit transduction of a variety of different organs and cell types *in vivo* [256-258] and analysis of luciferase expression from each vector can be monitored over time using consecutive bioluminescent imaging. 40 µL of VSVg pseudotyped LV.SFFV.Luc.WPRE, LV.LP1.Luc.WPRE, LV.hAAT.Luc.WPRE or LV.UCOE.Luc.WPRE vector was intravenously injected into the superficial temporal vein of one-day old neonatal mice (injections

performed by Simon Waddington) with estimated titres calculated in vector genomes of each vector group is as follows:

Vector Group	**Estimated Transducing Units Injected Per Mouse (vg)**
LV.SFFV.Luc.WPRE	5.60×10^4
LV.LP1.Luc.WPRE	1.04×10^4
LV.hAAT.Luc.WPRE	8.16×10^4
LV.UCOE.Luc.WPRE	3.08×10^4

A total of six neonates were injected per vector group and three mice served as uninjected controls, however, one mouse died post-injection in the LV.UCOE.Luc.WPRE vector group.

Quantification of *in vivo* luciferase expression was assessed using an IVIS cooled charge-coupled device (CCCD) camera at 24, 37, 52, 66, and 80 days post-injection at a binning of 1 or 8, for 1 or 10 seconds. The earliest time at which mice can be reliably anaesthetised is 21 days old. Representative images from day 37 post-injection are shown in Figure 5.3. Light emission was quantified in a defined region of interest (ROI) in photons/second/cm^2/sr over the liver area; shown as a circle on the representative images. To avoid bias areas measured are the same size and shape for all mice at all time points (Figure 5.3).

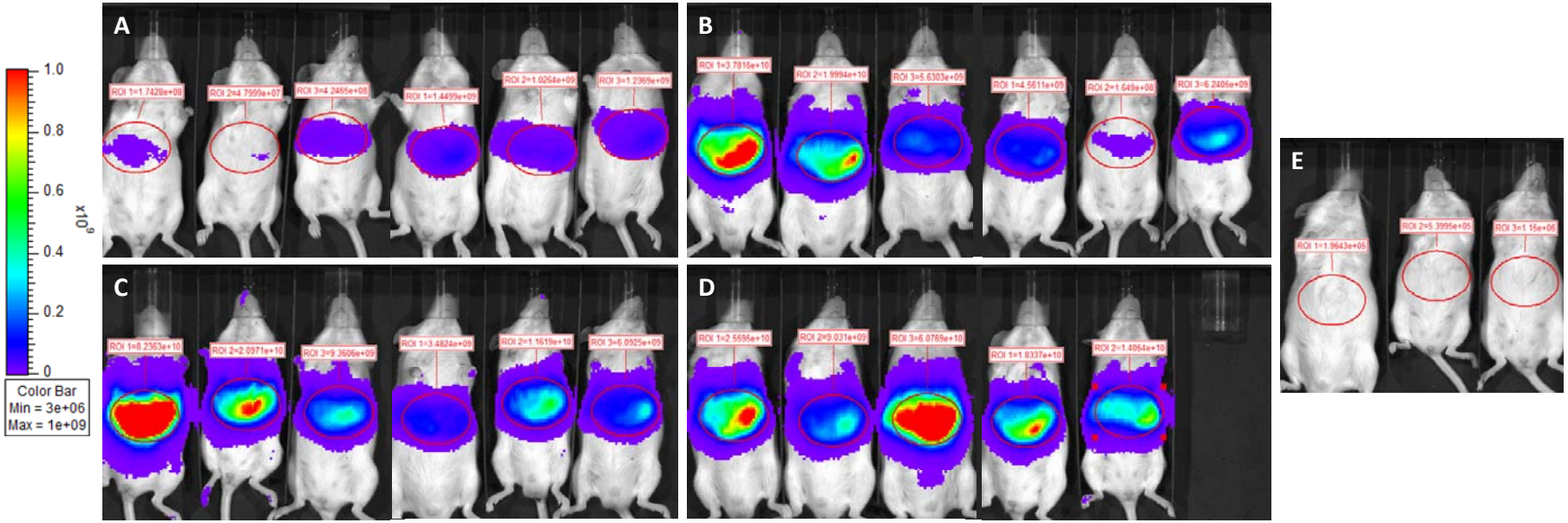


Figure 5.3 Representative images of luciferase expression 37-days post-intravenous injection of VSVg pseudotyped, integrating vectors expressing luciferase under control of various promoter regions. Representative images of mice 37 days post-injection after neonatal injection via the superficial temporal vein of vector groups: **(A)** LV.SFFV.Luc.WPRE **(B)** LV.LP1.Luc.WPRE **(C)** LV.hAAT.Luc.WPRE **(D)** LV.UCOE.Luc.WPRE **(E)** Uninjected control mice. Bioluminescence was measured in photons using an IVIS CCCD camera at a binning of 8 for 1 second. Light emission was quantified in a defined region of interest (ROI) in photons/second/cm^2/sr over the liver area; shown as a circle on the representative images. To avoid bias areas measured are the same size and shape for all mice at all time points

Quantification of light emission in the defined ROI for consecutive images allowed expression to be monitored over time in each animal. As the imaging time and binning varied between vector groups appropriate controls were also measured and these values subtracted from test values. Therefore, only test values are plotted in Figure 5.4.

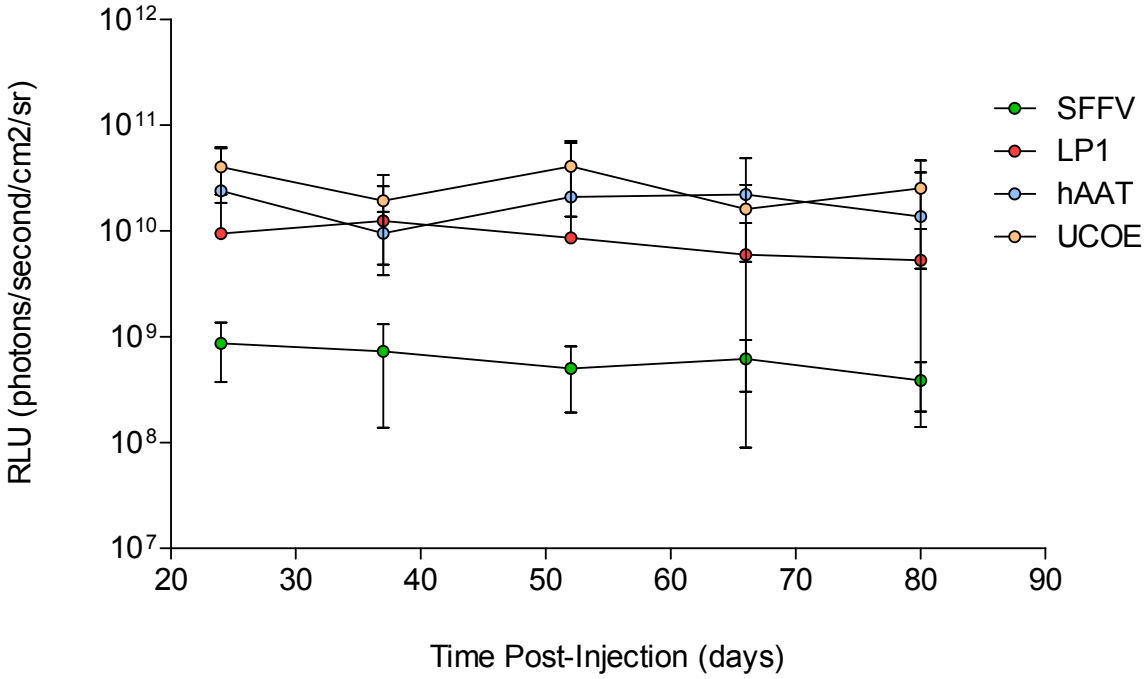


Figure 5.4 Quantification of expression after neonatal injection of lentiviral vectors expressing luciferase from various promoter elements. Light emission was quantified as photons/second/cm^2/sr in a defined region of interest (ROI) over the liver area for MF1 mice injected neonatally via the superficial temporal vein with integrating VSVg pseudotyped vector expressing luciferase under control of SFFV, LP1, hAAT or UCOE promoter elements. ROI values were measured and plotted as relative luciferase units (RLU), however, as the imaging time and binning varied between each group values from control mice imaged in the same conditions were calculated and subtracted from each test value. Therefore only test values are plotted upon this graph. Mean and SEM plotted for 6 mice for SFFV, LP1 and hAAT vector groups, and 5 mice for the UCOE vector group.

Figures 5.3 and 5.4 show that injection of lentiviral vector via the superficial temporal vein in neonate mice mediates expression mainly in the liver area. No expression was observed in joints or bone marrow as seen after injection of vector into adult mice. Similar levels of expression were observed for LP1, hAAT and UCOE promoter elements with average levels defined in the liver ROI measured at 5×10^9 to 5×10^{10} photons/second/cm^2/sr throughout the duration of the experiment. However, expression observed from the viral SFFV promoter in the liver ROI was ten-fold lower with average values between 3×10^9 to 9×10^9 photons/second/cm^2/sr throughout the duration of the experiment.

5.2.4 Proviral Copy Number and Luciferase Quantification in Tissue from Mice Post Neonatal Injection

Mice were sacrificed at 80 days post injection and liver, spleen, heart, lung, kidney and brain tissue taken for analysis. Tissue was analysed for luciferase expression using the Luciferase Assay System (Promega) and a Bradford protein assay (BioRad) to give relative luciferase units (RLU) per µg protein. The number of integrated proviral copies was also assessed using quantitative real-time PCR (qPCR).

Viral copy number was restricted mainly to liver and spleen tissue where average values in these tissues were high for all vector groups tested. In liver average values for LP1, hAAT and UCOE groups were similar at 9.58, 8.27 and 10.24 viral copies per cell, respectively. Average copy in liver for the SFFV vector group was lower at just 3.42, however two mice have very low copy at just 0.53 and 1.09 copies per cell, along with expression data this suggests tissues in these mice were not efficiently transduced after injection. Provirus is also seen in spleen tissue with higher average values for LP1, hAAT and UCOE vector groups at 3.21, 3.41 and 4.98 copies per cell, respectively. Average copy number for SFFV mice was again lower at 1.77 copies per cell. All other tissues

showed negligible copy number at <0.8 copies per cell for tissues from all mice in all vector groups (Figure 5.5).

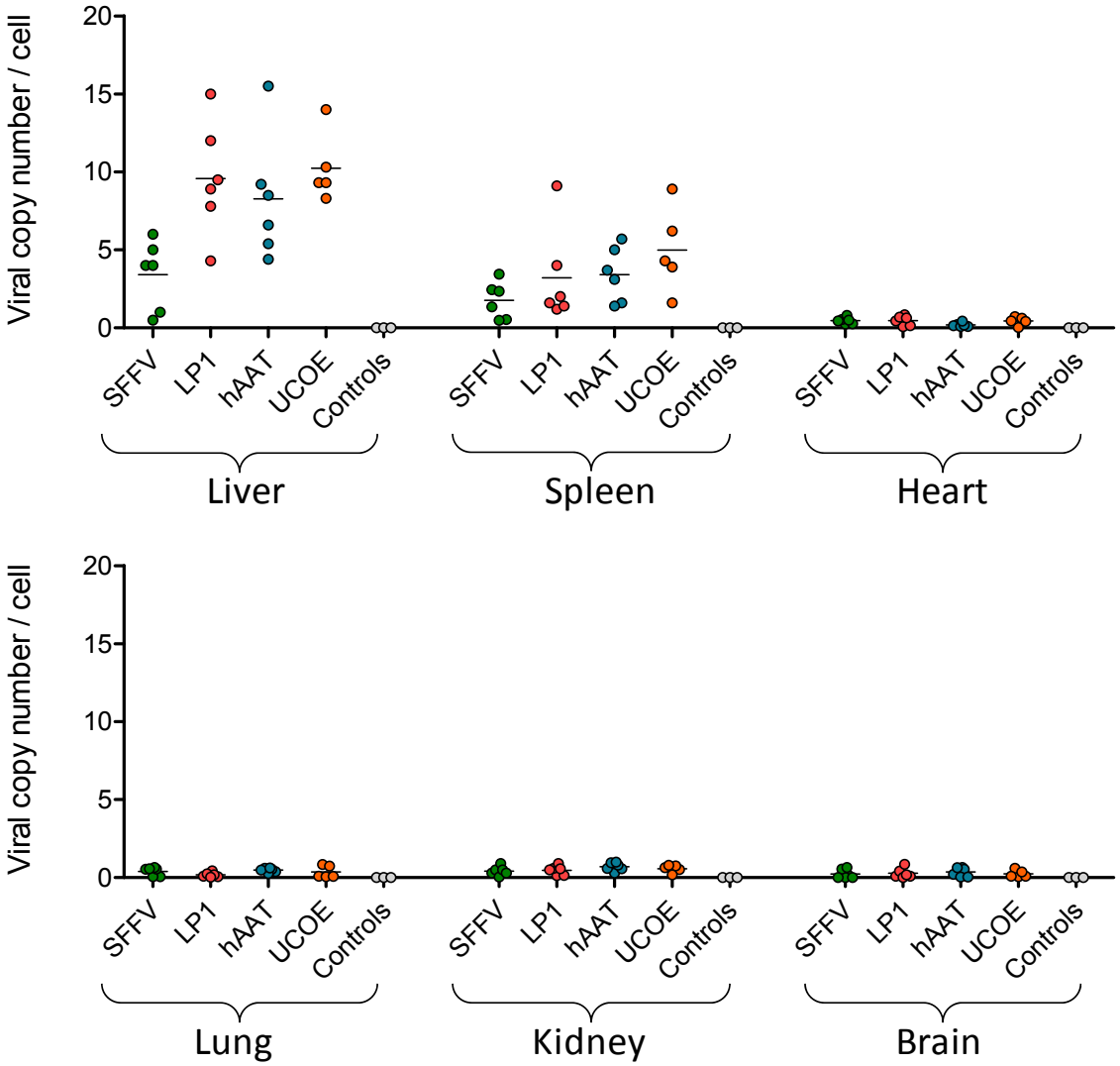


Figure 5.5 Quantification of viral copy number after neonatal injection of lentiviral vectors expressing luciferase from various promoter elements. Liver, spleen, heart, lung, kidney and brain tissue was taken from mice sacrificed at 80 days post neonatal injection of lentivectors expressing luciferase from SFFV, LP1, hAAT or UCOE promoters. Genomic DNA was extracted and qPCR carried out for each sample for both the murine housekeeping gene *titin* and the viral WPRE sequence allowing determination of viral copy number per cell. Line represents the mean of all points.

Luciferase expression in liver was detected at average levels of 5.2×10^5, 6.5×10^6, 3.3×10^6, 1.3×10^7 RLU/µg protein for SFFV, LP1, hAAT and UCOE promoter groups, respectively. Values for SFFV injected mice were around 10-fold lower than for other promoter groups, reflecting imaging results and to a lesser extent, viral copy number. Values for control tissue in this experiment were higher than normally observed, with expression in control liver tissue detected at an average of 4110 RLU/µg protein compared to a normal background value of ~1500 RLU/µg protein. Average levels in spleen for LP1, hAAT and UCOE injected mice were 2.9×10^5, 1.9×10^5, and 7.3×10^4 RLU/µg protein, respectively. For spleen tissue for SFFV injected mice expression was around 100-fold lower at 2189 RLU/µg protein, and control values were an average of 1.2×10^5 RLU/µg protein, however, of this average two values were 363 and 243 and one outlier value of 3.5×10^5 RLU/µg protein. Expression detected in heart, lung kidney and brain tissue for all groups was not significantly different to controls. It is not clear why luciferase protein was detected at such high levels in control tissues.

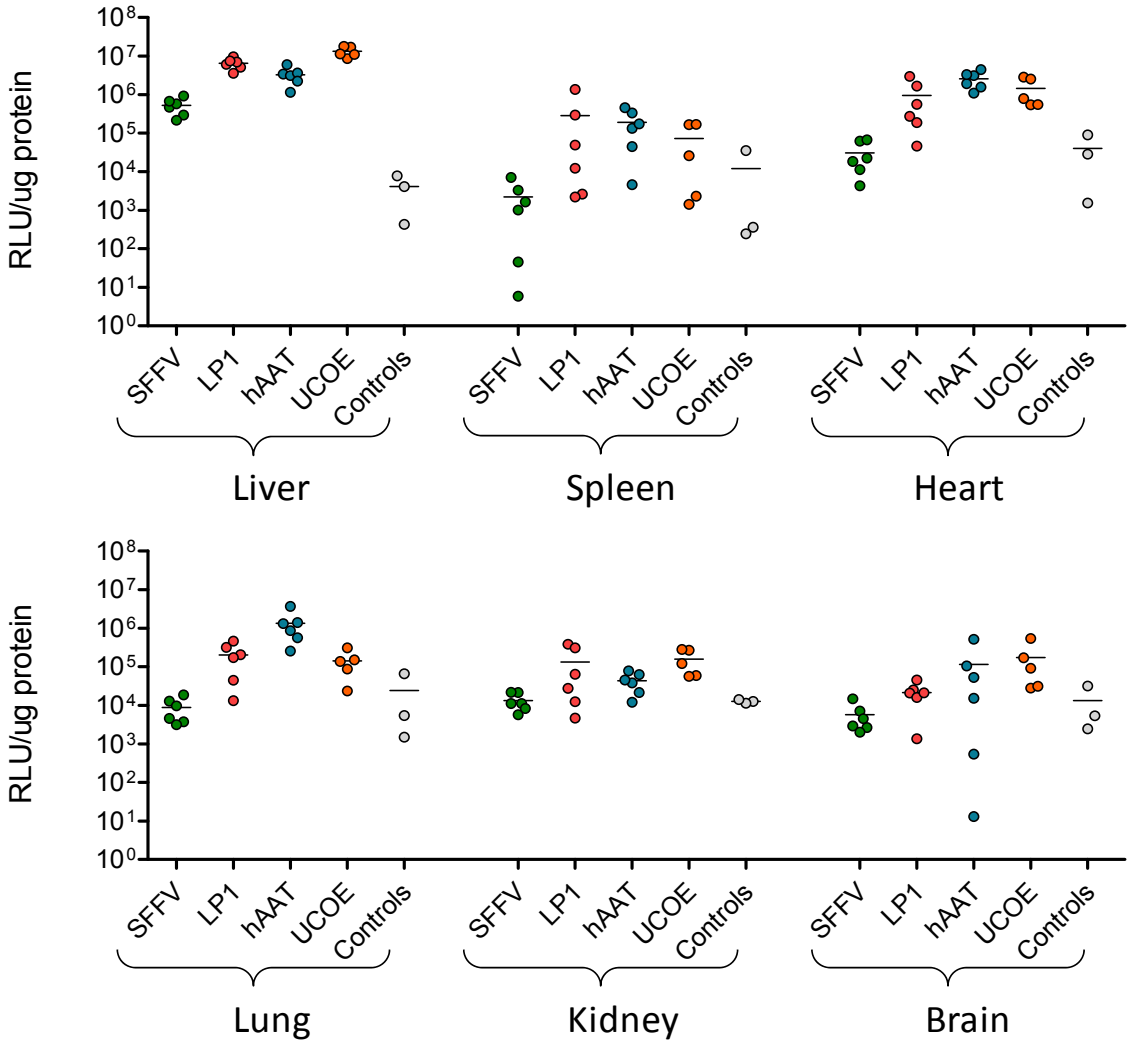
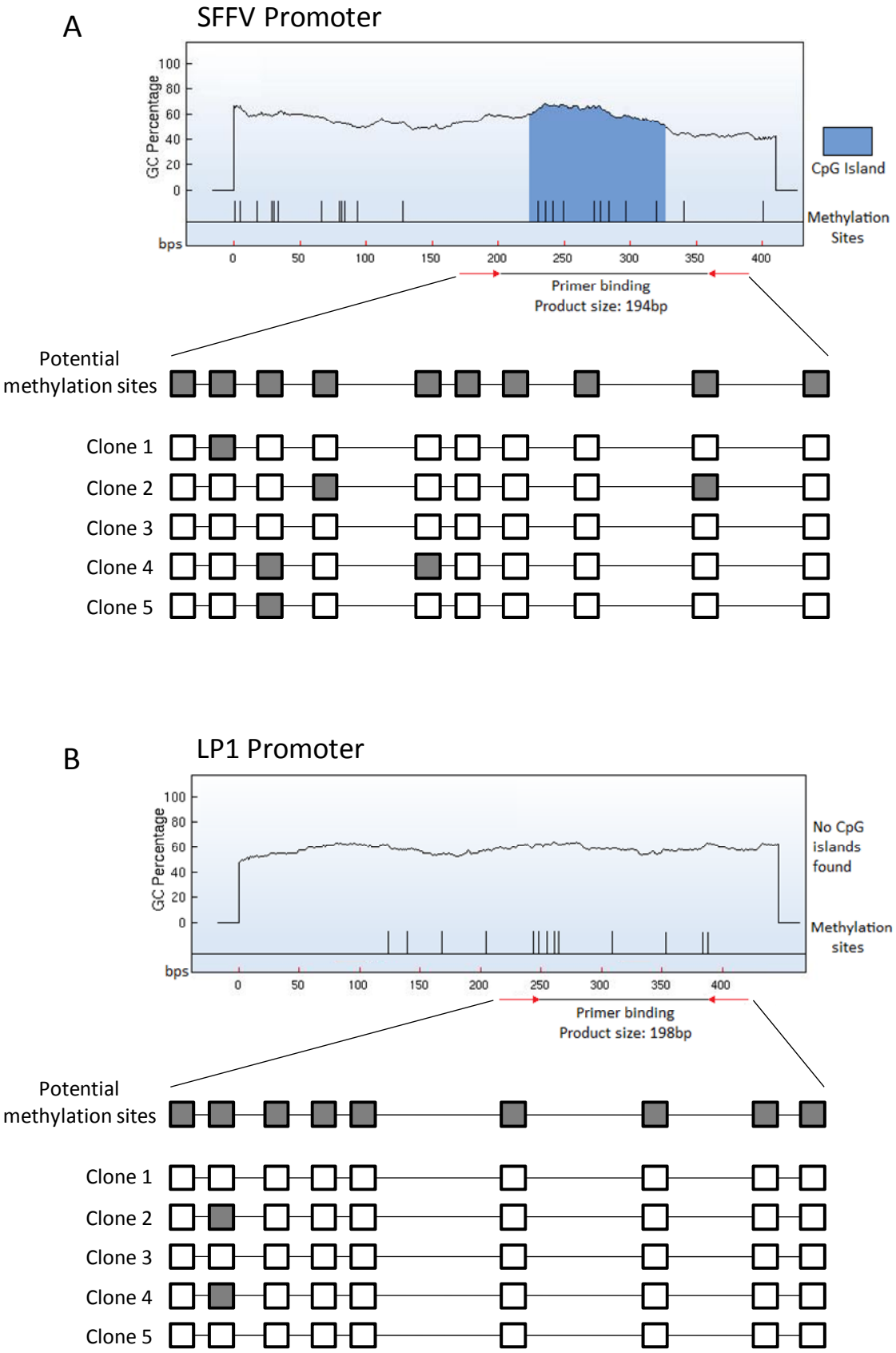


Figure 5.6 Quantification of luciferase expression in tissue taken from mice after neonatal injection of lentiviral vectors expressing luciferase from various promoter elements. Liver, spleen, heart, lung, kidney and brain tissue were taken from mice sacrificed at 80 days post-neonatal injection of lentivectors expressing luciferase from SFFV, LP1, hAAT or UCOE promoters. Relative Luciferase Units (RLU) per µg protein was determined using the Luciferase Assay System (Promega) and a Bradford protein assay (BioRad). Line represents the mean of all points.

5.2.5 Methylation Analysis of Promoter Elements After Neonatal Injection of Lentivector

Viral promoters such as CMV and SFFV are prone to silencing *in vivo* as a result of *de novo* CpG methylation which prevents transcription [130,131], whereas the use of tissue specific promoters and chromatin opening elements have been shown to avoid this issue [132]. Therefore, to explore CpG methylation in this experiment DNA from transduced liver tissue was extracted and methylation analysis of promoter regions investigated.

A 194bp region of the SFFV promoter containing 10 potential CpG methylation sites, a 198bp region of the LP1 promoter containing 9 potential CpG methylation sites, a 195bp region of the hAAT promoter containing 8 potential CpG methylation sites, and a 257bp region of the UCOE promoter containing 35 potential CpG methylation sites were isolated and methylation investigated using the MethylCode™ Bisulfite Conversion Kit (Invitrogen). DNA from the liver from one mouse in each test group was extracted and treated with bisulfite, causing unmethylated cytosines to be converted into uracil while methylated cytosines remained unchanged. Bisulfite-modified DNA was then amplified by PCR and the resulting product cloned into the vector pCR4® and transformed into Stbl3 cells. Five clones for each test group were then analyzed by DNA sequencing and the methylation profile of the promoter region determined by comparing the sequence of the bisulfite-treated DNA to that of the untreated DNA. The results of five clones for each promoter are shown in Figures 5.7A-D.


A
SFFV Promoter
GC Percentage
100
80
60
40
20
0
bps
0
50
100
150
200
250
300
350
400
CpG Island
Methylation Sites
Primer binding
Product size: 194bp
Potential methylation sites
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
B
LP1 Promoter
GC Percentage
100
80
60
40
20
0
bps
0
50
100
150
200
250
300
350
400
No CpG islands found
Methylation sites
Primer binding
Product size: 198bp
Potential methylation sites
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5

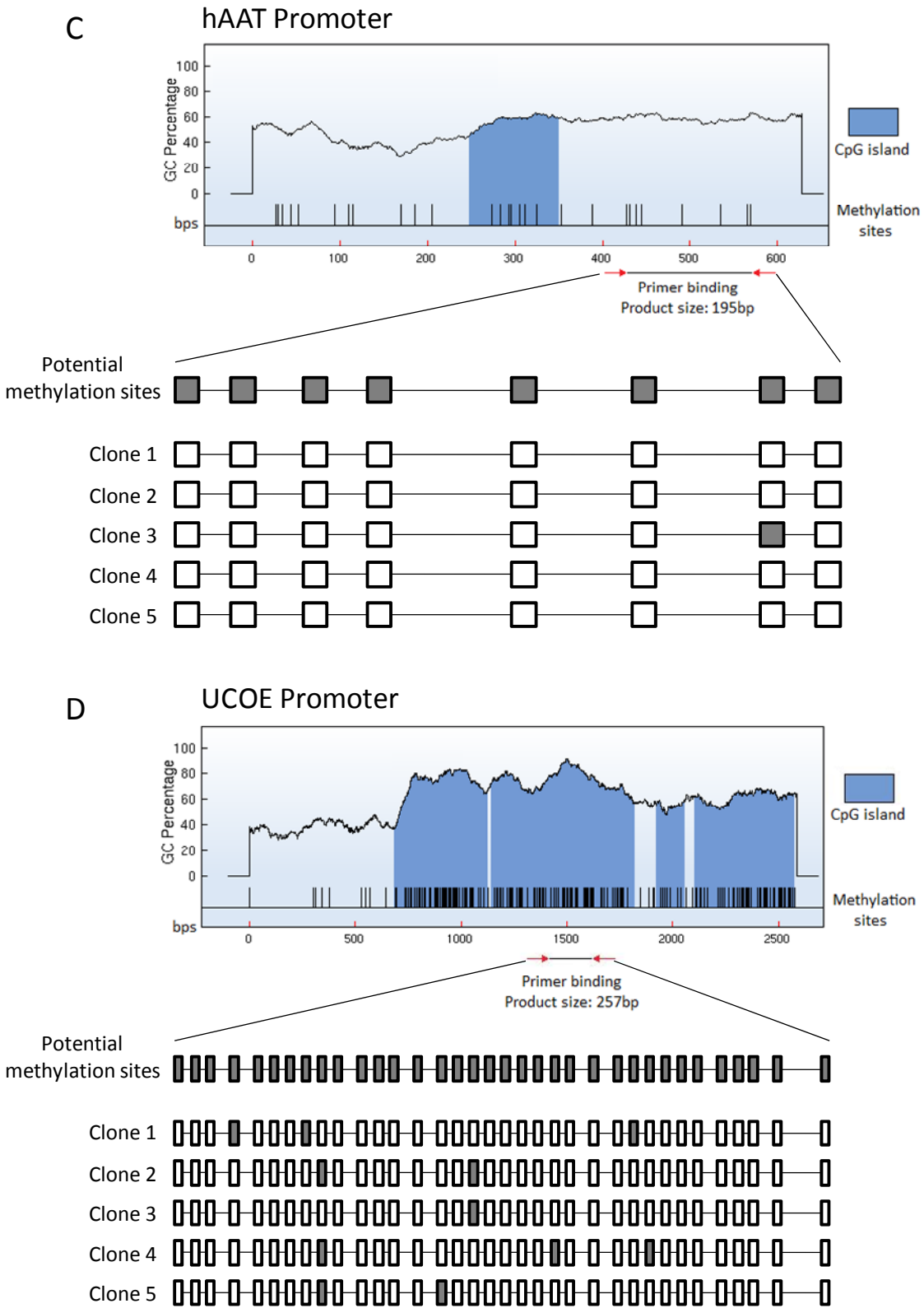


Figure 5.7 Methylation analysis of promoter regions in integrated proviral regions of intravenously injected mice. *De novo* CpG methylation for 5 clones from one mouse in each vector group were analysed using the MethylCode™ Bisulfite Conversion Kit (Invitrogen). Promoter regions: **(A)** SFFV, **(B)** LP1, **(C)** hAAT, **(D)** UCOE.

Methylation analysis for the SFFV promoter in liver shows that for the 5 clones tested of the 10 possible CpG sites available on average 15% were methylated (1-2 sites) in each clone. For LP1 only 1 site in 2 separate clones was seen to be methylated out of 9 possible sites, 11% of those tested, and hAAT had only 1 site in 1 clone methylated. For UCOE the number of possible CpG methylation sites was greatly increased compared to the other promoter regions. For the five clones tested on average around 1-3 sites were methylated out of a possible 35 for each clone, 6% of the total tested.

5.3 Summary

- Within a SIN lentiviral vector context the promoter elements SFFV and UCOE allowed good expression *in vitro* in all cell lines tested. Promoter elements LP1 and hAAT provided high expression and tissue specificity *in vitro* in liver cell lines.
- All promoters provided strong expression *in vivo* in liver after intravenous delivery into neonate mice, however, expression from the SFFV promoter was around 10-fold lower than that from LP1, hAAT, and UCOE after injection of a similar number of TU.
- Analysis showed limited CpG methylation of each promoter sequence *in vivo* in liver tissue, with no substantial differences between the elements tested.

In this chapter the mammalian and viral promoters in the context of a SIN lentiviral vector were investigated with a view to provide long term expression in hepatocytes after intravenous injection into neonate mice. The SFFV promoter is used as standard throughout this project, however, although it provides strong ubiquitous expression *in vivo* it is also suceptible to silencing by *de novo* CpG methylation with loss of transgene expression over time. Limiting expression of a therapeutic transgene exclusively to the target tissue would also provide many benefits: Off-target expression of some transgenes may result in unforeseen complications or toxicity, and high levels of systemic expression may also induce or intensify host immune responses to both the transgene and the gene delivery vector. In addition, tissue-specific promoters may be free from the cytokine-mediated transcriptional silencing that limits the use of some viral promoters.

The liver specific promoters LP1 and hAAT were investigated with a view to provide hepatocyte specific expression of coagulation factors, and the ubiquitous chromatin opening element (UCOE) was also studied to provide ubiquitous expression which is resistant to silencing.

All promoters were cloned into a vector backbone containing a luciferase transgene and vectors were efficiently produced and tested *in vitro* in liver and non-liver cell lines. SFFV and UCOE showed strong expression in all cell lines, whereas LP1 and hAAT showed significantly higher expression in the liver cell lines Huh7 (human hepatoma) and Hep-G2 (human hepatocellular carcinoma). Vectors were then tested by intravenous injection via the superficial temporal vein of a similar number of TU per vector group into neonatal (1-2 day old) MF1 mice. Luciferase imaging, the *ex vivo* luciferase assay, and viral copy number all showed that liver and spleen tissue were primarily transduced by all vectors. All promoters conferred high luciferase expression in the liver, however, expression from SFFV vector was approximately ten-fold lower in comparison to other promoters. As mice can only be anaesthetised reliably from 21 days old the first imaging time point was taken at 24 days post-injection, therefore it was unknown if expression from the SFFV vector had decreased from the time of injection. QPCR analysis showed that provirus copy number in liver for mice injected with SFFV vector to be approximately half of that observed for other promoters. This may indicate that fewer cells were transduced and explain the lower expression. As mice were injected neonatally it is unlikely that there was an immune response which would clear transduced cells after transduction. The methylation state of each promoter in liver tissue was investigated at the end of the experiment. Methylation analysis showed minimal CpG sites methylated for each promoter. SFFV had the highest methylation state of all promoters tested with 15% of possible sites assayed methylated. However, due to problems obtaining large PCR fragments from genomic DNA only small (~200bp) regions could be analysed in each tissue; these regions were considered to be representative of the whole promoter, however, this may not be the case.

The cellular specificity of each promoter could not be investigated. Sections were taken of liver tissue from each mouse at the end of the experiment and immunohistochemical staining was carried out for luciferase. Unfortunately, the staining was unsuccessful and a very high background was observed in all tissues including controls. This would have provided information on the pattern of expression

for all promoters and may have confirmed *in vivo* tissue specificity for the LP1 and hAAT elements. A possible solution to this problem would be to repeat the experiment using vectors containing a GFP transgene. Transgene expression can then be visualised easily using UV microscopy of liver sections without the need for staining.

Overall, this chapter showed that high expression of luciferase can be mediated by integrating SIN lentivectors containing mammalian and viral internal promoter elements. However, the cellular pattern of expression *in vivo* is unknown at this time.

Chapter Six

Analysis of Human Factor VIII Expression from a Lentiviral Vector

6.0 Aims

- To investigate the effects of codon optimisation of the human Factor VIII (hFVIII) cDNA sequence upon expression of hFVIII protein from a lentiviral vector utilizing the ubiquitous promoter SFFV both *in vitro* in a non-liver cell line and *in vivo* in a haemophilia mouse model.
- To investigate the role of the B domain in expression of human Factor VIII (hFVIII) protein from a lentiviral construct both *in vitro* in a non-liver cell line and *in vivo* in a mouse model of haemophilia.
- To assess expression of an optimal human factor VIII gene construct under the control of the liver specific promoter LP1 in a SIN lentiviral vector.

6.1 Introduction

Factor VIII is a non-enzymatic plasma protein that is essential for normal blood coagulation. The deficiency of factor VIII activity in humans is associated with the congenital bleeding disorder haemophilia A, which affects around 1 in 5000 males. Haemophilia A patients are treated with factor VIII concentrate for maintenance of haemostasis but regrettably prophylactic treatment is not in general use worldwide [4]. Cost is a prime issue in the treatment of haemophilia and is the main obstacle in providing proper treatment worldwide. The yearly cost of providing care for a severe haemophilia A patient with no inhibitor development is roughly US$150,000, for patients with inhibitor development the cost is approximately four-fold higher [259,260]. Since only minute amounts of factor VIII have to be present in plasma to ameliorate symptoms of haemophilia A great efforts are made in gene therapy research. In this chapter the role of the human factor VIII B domain for effective expression of factor VIII protein from an integrating lentiviral vector construct is investigated, as is the effect of

codon optimising the FVIII cDNA sequences. Studies were carried out both *in vitro* and *in vivo* in a haemophilia mouse model.

6.2 The Factor VIII B domain

Encoded by one large uninterrupted exon, exon 14, the B domain spans amino acids 741 to 1648 of the 2332 amino acid full length factor VIII protein, over 38% of the total structure. The human B domain is without homology to other known proteins and is not directly necessary for central procoagulant activity of factor VIII. However, it is extensively glycosylated at asparagine, serine and threonine sites throughout its length and contains 19 of the potential 26 asparagine *(N)*-linked glycosylation attachment sites on the entire FVIII molecule [165,166]. B domain deleted (BDD) constructs are effective at producing fully functional factor VIII, and actually increase the amount of mRNA production over 17-fold, however the increase in secreted FVIII protein from cells is only 30% due to inefficient trafficking of the protein from the endoplasmic reticulum (ER) to the golgi [185,261]. It is thought to be this glycosylation that enables functional trafficking [188]. Insights into the functional role of the factor VIII B domain are important for improving our understanding of the molecular mechanisms of haemophilia A and the development for replacement therapy. This chapter investigates the use of various alternative B domains to a full length human B domain for secretion of human factor VIII protein from a lentiviral vector both *in vitro* and *in vivo.*

6.2.1 Varying B Domains

6.2.1.1 226/N6 Human B Domain Fragment

The importance of glycosylation of the human B domain has previously been investigated *in vitro*. In 2004 Miao *et al.,* [188] created and tested factor VIII expression plasmids containing human B domain fragments with varying numbers of glycosylation

sites from 1-8 [188]. Tested in both a simian (COS-1) and Chinese Hamster Ovary (CHO) cell line they showed that the number of glycosylation sites which gave optimal expression of factor VIII protein was six, contained in the first 226 amino acids of the B domain. This construct was referenced as 226/N6.

6.2.1.2 *Fugu rubripes* B domain

The teleost puffer fish *Fugu rubripes* is a commonly used organism for investigation of genetics. Fugu has a basic vertebrate genome and contains a similar repertoire of genes to humans, however, in 1993 it was shown that the Fugu genome is only 390 Mb, about one-eighth the size of the human genome [262]. This makes Fugu an extremely useful model for annotating the human genome and a valuable 'reference' genome for identifying genes and other functional elements such as regulatory elements in the human and other vertebrate genomes, and for understanding the structure and evolution of vertebrate genomes.

Sequence analysis of genes in the blood coagulation system showed that Fugu amino acid sequences are highly conserved relative to their human orthologues. For factor VIII cDNA sequences the Fugu A1, A2, A3, C1 and C2 domains show 46, 43, 47, 52 and 50 percent sequence identity to human orthologues, respectively. Conversely, the Fugu factor VIII B domain shares only 6% sequence homology to its human orthologue. However, although there is no apparent sequence conservation between B domains the Fugu B domain is also highly glycosylated with 11 asparagine *(N)*-linked glycosylation attachment sites across its length of 224 amino acids [164]. In 2005 Dr. Steven W. Pipe's laboratory prepared a factor VIII expression plasmid construct in which the human B domain was replaced with the factor VIII B domain from *F. rubripes*. They reported that in COS-1 and CHO cell lines that this construct was secreted as efficiently as the 226/N6 construct (Dr. Steven Pipe, personal communication). This construct is referenced as 'Fugu B'.

6.2.1.3 r-VIII SQ : ReFacto™

Human factor VIII is secreted into the blood as a heavy chain of 90-200 kDa (A1-A2-B) and a light chain of 80 kDa (A3-C1-C2) associated by a metal ion. The smallest of these molecules, a 90 kDa heavy chain in complex with an 80 kDa light chain is devoid of any B domain (between amino acids Arg740 – Glu1649) and is known as low molecular weight factor VIII (p-VIII-LMW). Recombinant factor VIII SQ (r-VIII SQ), is a therapeutic factor VIII product marketed as ReFacto® (Wyeth), designed to mimic p-VIII-LMW. It is produced using a single gene encoding the 170 kDa protein comprising the A1-a1-A2-a2-a3-C1-C2 domains. In this structure Ser743 in the N-terminus of the B domain was fused to Gln1638 in the C-terminus of the B domain, creating the SQ link of 14 amino acids (SFSQNPPVLKRHQR) between the a2 and a3 domains. The presence of the SQ link in the complex promotes efficient intracellular cleavage of the primary single chain translation product of 170 kDa due to the basic arginine residues found at positions -1 and -4 relative to the cleavage site at Glu1649, a recognition motif for proteolytic cleavage by the membrane bound subtilisin-like protease furin [186,263]. A schematic of the SQ sequence is shown in Figure 6.2A.

6.2.2 Design of Factor VIII Constructs

Nine factor VIII constructs were created and investigated as part of this project; their names and structures are described in Table 6.1 and are represented schematically in Figure 6.1. The sequences SQ, SQ^m, SQ^a and SQ^b and their associated features are also shown in Figures 6.2 A-D, respectively.

Factor VIII Construct Name	**Protein Description**
BDD FVIII	hFVIII with a B domain deletion between amino acids 740-1649 and site directed mutagenesis used to engineer an *Mlu*I restriction site ACG'CGT at positions 739-740 causing the missense mutation Pro739 to Thr739 in the a2 domain.
FVIII Fugu B	hFVIII containing the 201aa Fugu B domain, added using flanking *Mlu*I restriction sites into the 'BDD FVIII' construct between aa's 740 and 1649.
FVIII N6	hFVIII containing the 226/N6 B domain, added using flanking *Mlu*I restriction sites into the 'BDD FVIII' construct between aa's 740 and 1649.
SQ FVIII	hFVIII with a B domain deletion between amino acids 740-1649 with a modified SQ sequence (SQ^m) of 14 amino acids -SFSQNPPVLKTRHQR- inserted. The modified sequence contains an *Mlu*I restriction site for insertion of the Fugu and N6 B domains giving a missense mutation Lys1644 to Thr1644.
SQ FVIII Fugu B	hFVIII containing the Fugu B domain with flanking *Mlu*I restriction sites inserted into the 'SQ FVIII' construct. This causes the SQ^m sequence to be split either side of the B domain insert: The N-terminal sequence

	(SFSQNPPVLTR) is referred to as SQ[a], and the C-terminal sequence containing the furin recognition site RHQR as SQ[b] (TRHQR).
SQ FVIII N6	hFVIII containing the 226/N6 B domain with flanking *Mlu*I restriction sites inserted into the 'SQ FVIII' construct creating SQa and SQb sequences on the N- and C-terminal sides of the B domain, respectively.
SQ FVIII (co)	The amino acid sequence is identical to SQ FVIII but the sequence is translated from a codon optimised cDNA sequence
SQ FVIII Fugu B (co)	The amino acid sequence is identical to SQ FVIII Fugu B but the sequence is translated from a codon optimised cDNA sequence
SQ FVIII N6 (co)	The amino acid sequence is identical to SQ FVIII N6 but the sequence is translated from a codon optimised cDNA sequence

Table 6.1 Table of factor VIII constructs designed and cloned into a lentiviral vector backbone for this project. Nine constructs were designed and cloned into a lentiviral backbone plasmid as part of this project. This table describes their designated names and a description of the structure of each gene. *Mlu*I restriction sites are shown underlined and the missense mutation Lys1644 to Thr1644 caused by the insertion of this sequence is shown in red. The FVIII Fugu B gene was a kind gift from Dr. John McVey. A graphical representation of each construct is shown in Figure 6.1, and the structure of SQ, SQ[m], SQ[a] and SQ[b] sequences in Figure 6.2. The cloning of all constructs is described in section 6.2.3.

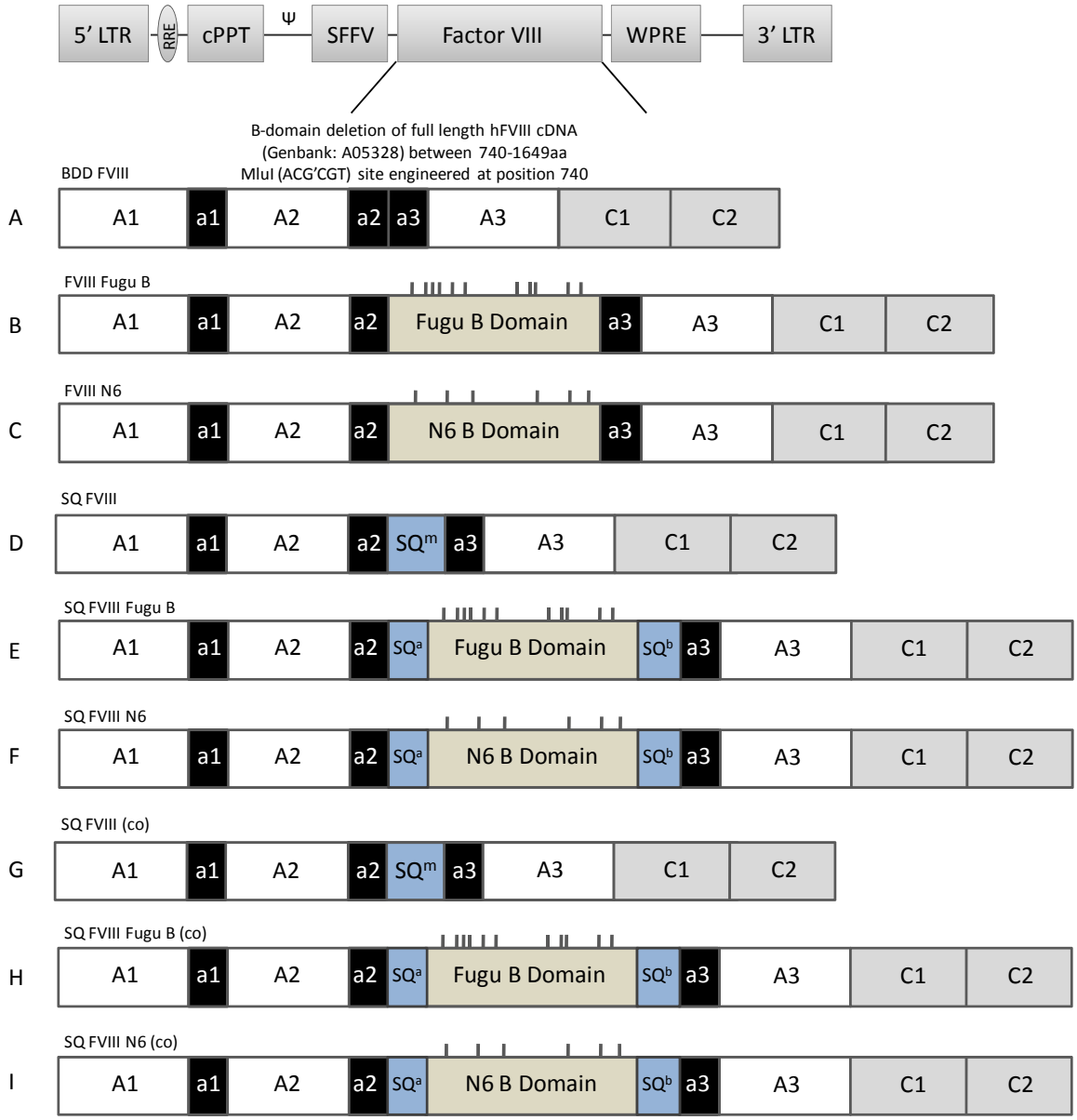


Figure 6.1 Schematic of human Factor VIII variants designed and cloned into a basic lentiviral vector backbone. Nine different human Factor VIII variants were designed and cloned into a lentiviral vector backbone plasmid. **(A)** BDD FVIII; B domain deleted hFVIII. **(B)** FVIII Fugu B; hFVIII containing the Fugu B domain. **(C)** FVIII N6; hFVIII containing the human N6 B domain **(D)** SQ FVIII; B domain deleted hFVIII containing a modified version of the SQ amino acid sequence SQ^m. **(E)** SQ FVIII Fugu B; SQ^m hFVIII containing the Fugu B domain. **(F)** SQ FVIII N6; SQ^m hFVIII containing the human N6 B domain. Constructs **(G**) SQ FVIII (co), **(H)** SQ FVIII Fugu B (co), **(I)** SQ FVIII N6 (co) are the same amino acid structure as constructs **(D)**, **(E)** and **(F)**, respectively, but are produced from a codon optimised cDNA sequence. Relative domain size is not accurate. Dashes on constructs mark asparagine *(N)*-linked glycosylation sites within the B domain only.

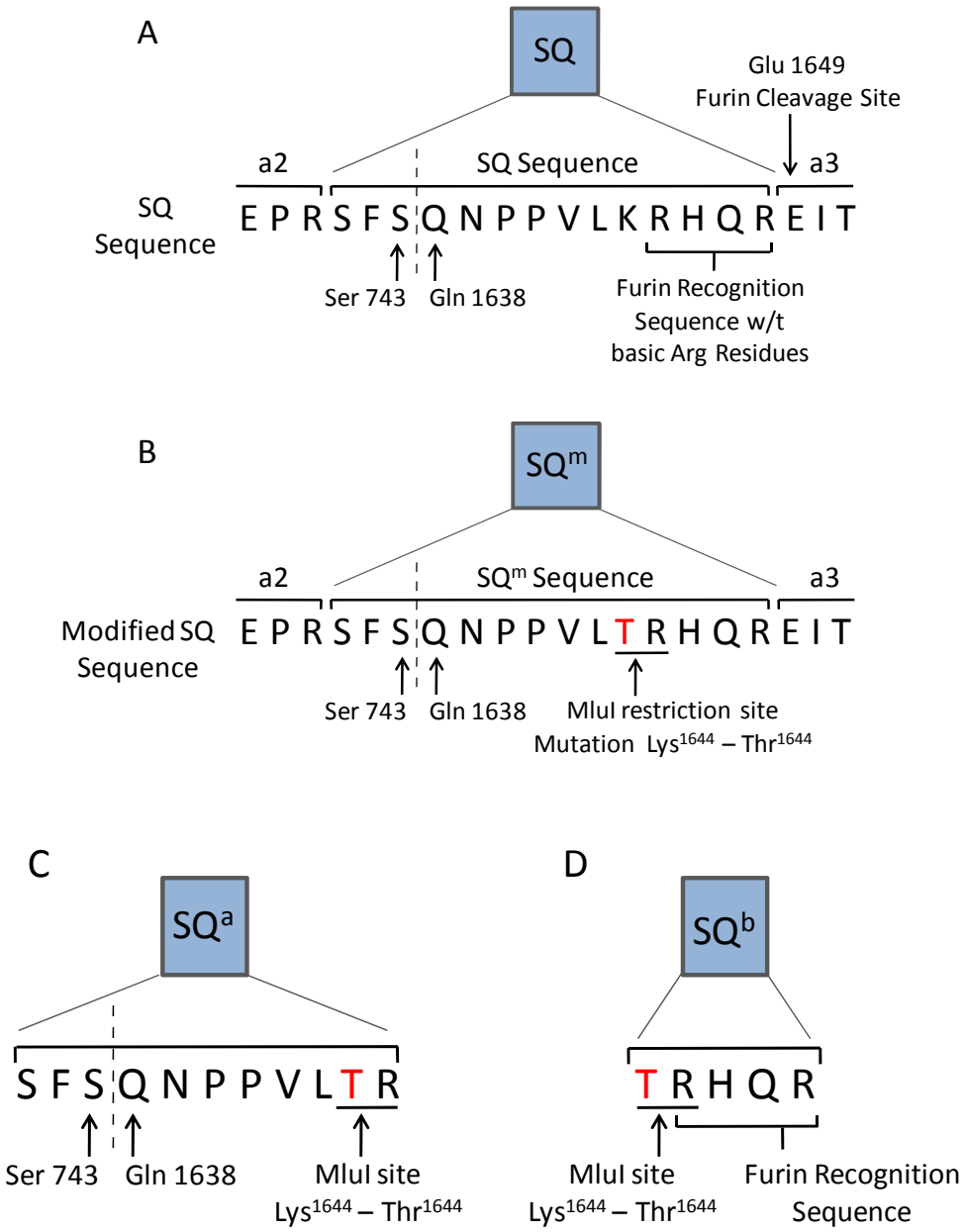


Figure 6.2 Schematics of SQ and modified SQ sequences. (A) The SQ sequence is a 14 amino acid bridge between the a2 and a3 domains of factor VIII created by fusing Ser743 and Gln1638 in the B domain. The sequence promotes efficient intracellular cleavage by containing the 4 amino acid protease recognition site -RHQR-. **(B)** A modified SQ sequence SQ^m was created containing a missense mutation from Lys1644 – Thr1644 caused by the creation of an *Mlu*I restriction enzyme site within the cDNA sequence for insertion of the Fugu and N6 B domains. **(C)** SQ^a is the 11aa sequence created at the N-terminal of the B domain after insertion of the N6 or Fugu B domain sequences into the SQ FVIII construct. **(D)** SQ^b is the 5aa sequence created at the C-terminal of the B domain after insertion of the N6 or Fugu B domain sequences into the SQ FVIII construct, this sequence retains the 4aa protease recognition site. *Mlu*I restriction sites are shown underlined and the K → T missense mutation shown in red.

6.2.3 Cloning Factor VIII Constructs

The expression plasmid pMT2-FVIII was obtained as a kind gift from Dr. Steven W. Pipe at the University of Michigan, this plasmid contained the human factor VIII gene with a Fugu B domain. The hFVIII gene had a B domain deletion from amino acids 740-1649 and an *Mlu*I restriction site (ACG'CGT) engineered by site directed mutagenesis at amino acid positions 739-740 causing the missense mutation Pro739 to Thr739 in the a2 domain. The Fugu B domain had been cloned in using flanking *Mlu*I restriction sites on 5' and 3' creating a 4935bp FVIII Fugu B gene. FVIII Fugu B was flanked by *Xho*I and *Sal*I restriction sites in this plasmid and although it could be removed successfully from pMT2 the ligation into the *Xho*I site of the lentiviral backbone pLNT/SFFV-MCS was unsuccessful after a number of attempts. Therefore, the FVIII Fugu B gene was removed in three parts (shown in Figure 6.3) using a digest with *Xho*I and *Kpn*I to remove a 1.83 kb fragment, a partial digest with *Kpn*I and *Mlu*I to remove a 1.06kb fragment, and PCR amplification of the last 2.066kb section using primers that created *Mlu*I and *Sbf*I sites on the 5' and 3' ends, respectively. Each section was sequentially cloned into pLNT/SFFV-MCS using the same enzymes to create pLNT/SFFV-FVIII Fugu B. The construct was fully sequenced upon completion.

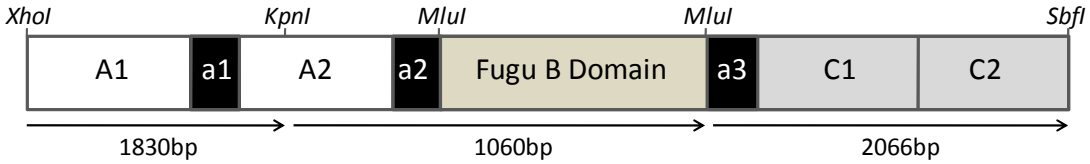


Figure 6.3 Schematic of strategy to clone the FVIII-Fugu B gene into a lentiviral backbone. The 4935bp FVIII Fugu B gene was removed from plasmid pMT2-FVIII in three parts (indicated by arrows): Enzyme digest of pMT2-FVIII with *Xho*I and *Kpn*I created a 1830bp fragment. Partial digest of pMT2-FVIII with *Kpn*I and *Mlu*I created a 1060bp fragment. PCR amplification of a 2066bp fragment using primers which created *Mlu*I and *Sbf*I restriction sites on the 5' and 3' ends, respectively. Each fragment was sequentially cloned into pLNT/SFFV-MCS to create pLNT/SFFV-FVIII Fugu B which was fully sequenced upon completion.

pLNT/SFFV-BDD FVIII was produced by digest of pLNT/SFFV-FVIII Fugu B with *Mlu*I to remove the Fugu B domain and religation. The 226/N6 B domain sequence was manufactured by GeneArt (Regensburg, Germany) to produce a standard GeneArt plasmid containing 226/N6; pGA_N6_nonopt, the sequence was obtained by taking the first 678bp of the human factor VIII B domain (cDNA found at Genbank: A05328), 5' and 3' flanking *Mlu*I sites were then added. N6 was then removed from pGA_N6_nonopt and ligated into pLNT/SFFV-BDD FVIII using *Mlu*I to create pLNT/SFFV-FVIII N6. The SQ cDNA sequence was obtained from [186] and was modified to contain an *Mlu*I site (underlined) to give the SQ^{m} cDNA sequence: 5'-AGC'TTC'AGC'CAG'AAC'CCC'CCC'GTG'CTG'ACG'CGT'CAC'CAG'CGG-3'. pLNT/SFFV-SQ FVIII Fugu B was produced by site directed mutagenesis performed by Eurofins MWG Operon (Ebersberg, Germany) to add the flanking SQ^{a} and SQ^{b} sequences into the plasmid pLNT/SFFV-FVIII Fugu B to produce pLNT/SFFV-SQ FVIII Fugu B. pLNT/SFFV-SQ FVIII was then produced by removal of the Fugu B domain from pLNT/SFFV-SQ FVIII Fugu B by digest with *Mlu*I and religation. pLNT/SFFV-SQ FVIII N6 was produced by removal of the 226/N6 B domain from pGA_N6_nonopt by digestion with *Mlu*I and ligation into pLNT/SFFV-SQ FVIII. In this construct there is a repeat of the 11aa SQ^{a} sequence caused by the insertion of the N6 B domain into the SQ^{m} sequence. It is unknown the effect that this repeat will have upon factor VIII secretion and function, or inhibitor formation *in vivo*.

6.2.4 Codon Usage and Optimisation of Factor VIII cDNA Sequences

The degeneracy found in the genetic code allows each amino acid to be encoded by between one and six synonymous codons allowing many alternative nucleic acid sequences to encode the same protein [264]. The frequencies with which different codons are used within genes is referred to codon usage and can vary significantly between different organisms, between proteins expressed at high or low levels within the same organism, and sometimes even within the same operon [265].

Several indices can be used to estimate the extent to which the codon usage of a gene of an organism has been altered towards preferential usage of optimal codons. These include: Codon Bias Index (CBI) a measure of the directional codon bias towards a subset of optimal codons [266]. F_{OP}; a species specific measure of the frequency of optimum codons (F_{OP}) in a gene, expressed as a ratio between the frequency of optimal codons and the total number of synonymous codons [267]. The codon adaptation index (CAI) is the most widely used measure of codon bias for both prokaryotic and eukaryotic genes [268]. Here each of the 61 codons (stop codons excluded) is assigned a measure of 'relative adaptiveness' defined as its frequency relative to the most often used synonymous codon in a set of genes known to be highly expressed in a particular organism i.e. *E. Coli* or *H. Sapiens* [269]. Finally, the 'codon usage model' also uses the occurrence of codons in a gene sequence to predict whether genes are likely to be highly expressed, although the formalism is quite different from the one used for the CAI [270].

To create codon optimised sequences analysis of SQ FVIII Fugu B an adaption of the codon usage to the bias of *Homo sapiens* using CAI was carried out by GeneArt (Regensburg, Germany) using their in-house proprietary software GeneOptimizer®. Optimisation also removes *cis*-acting sequence motifs including; internal TATA-boxes, chi-sites and ribosomal entry sites, AT- or GC-rich sequence stretches, AU-rich elements, inhibitory and *cis*-acting repressor sequence elements, repeat sequences, RNA secondary structures, and all cryptic splice sites (GeneArt http://www.geneart.com/). The analysis of the optimisation of SQ FVIII Fugu B is shown in Figure 6.4. It included the removal of 14 splice sites, an increase in GC-content from ~45% to ~60% to prolong mRNA half-life, and an increase in CAI from 0.74 to 0.97. A Kozak sequence was introduced to increase translation initiation, and two stop codons were added to ensure efficient termination. The optimised gene retained the B domain flanking *Mlu*I restriction sites on the Fugu B domain and has 75.8% sequence similarity to the original non-optimised sequence. The optimised gene was cloned into pLNT/SFFV-MCS by GeneArt to give the plasmid pLNT/SFFV-SQ FVIII Fugu B (co). The plasmid pLNT/SFFV-SQ FVIII (co) was created by digestion of pLNT/SFFV-SQ FVIII Fugu B

(co) with *Mlu*I and religation. The 226/N6 B domain sequence from pGA_N6_nonopt was codon optimised and manufactured by GeneArt. It was received in the plasmid pGA_N6_opt and as the *Mlu*I restriction sites were maintained cloned directly into the pLNT/SFFV-SQ FVIII (co) plasmid to obtain the construct pLNT/SFFV-SQ FVIII N6 (co), again, this construct will contain an 11aa SQ^a repeat sequence caused by the insertion of the B domain into the SQ^m sequence. Each construct was fully sequenced before testing.

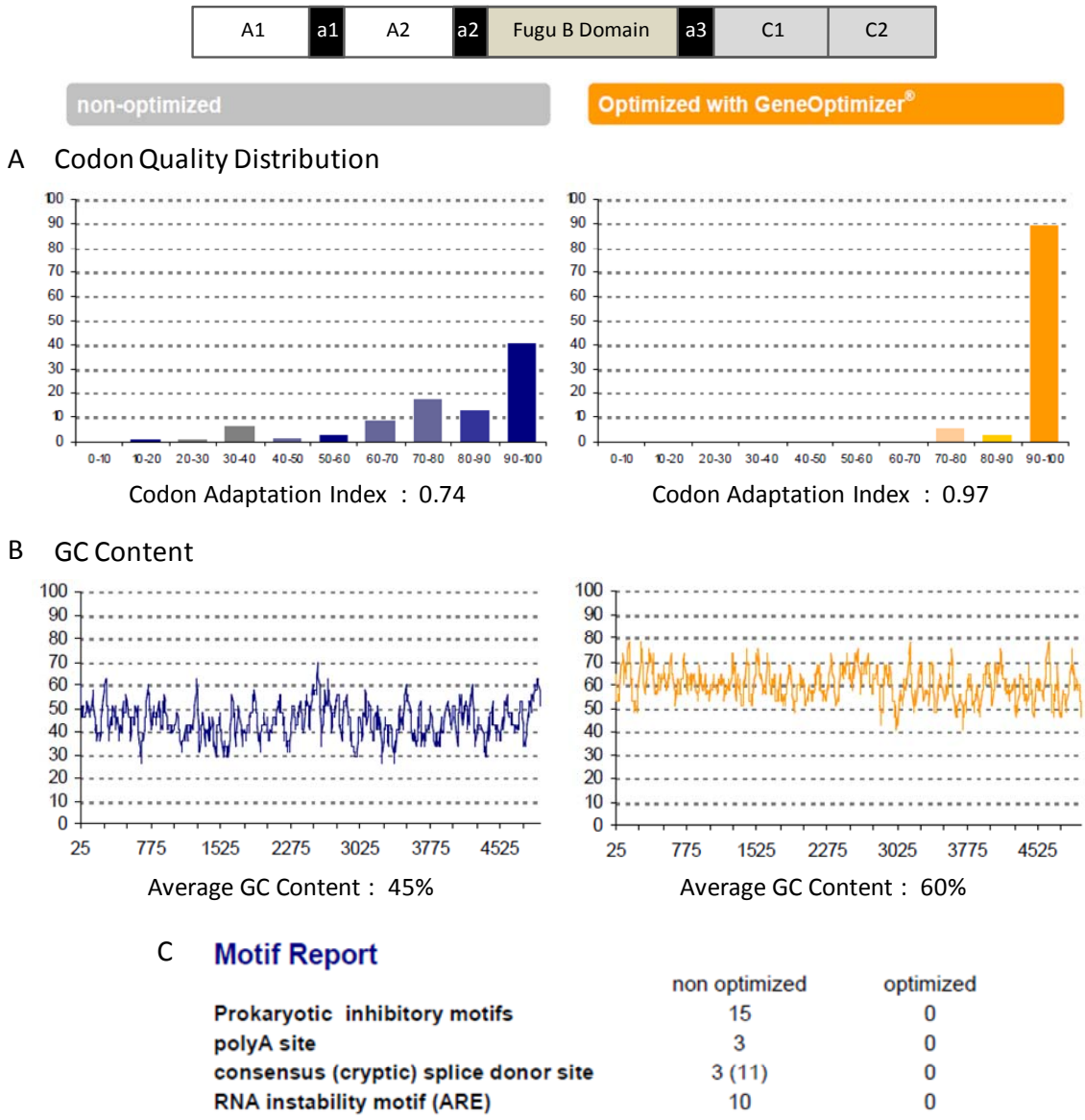


Figure 6.4 Analysis of codon optimisation of the 4935bp gene sequence 'FVIII Fugu B'. Codon optimisation of the FVIII Fugu B gene was performed to the bias of *Homo sapiens* using CAI was carried out by GeneArt (Regensburg, Germany) using their in-house proprietary software GeneOptimizer®. **(A)** Histograms showing the codon quality distribution of non-optimised and optimised sequences and their relative CAI indexes of 0.74 and 0.97, respectively. **(B)** A plot showing the GC content of non-optimised and optimised sequences. The optimised sequence was increased to prolong mRNA half-life. **(C)** A report of detrimental sequence motifs found in the non-optimised and their deletion in the optimised gene sequence. All figures reproduced from GeneArt information sheets provided with plasmids.

6.3 Testing of Lentiviral Factor VIII Constructs

6.3.1 Expression of Factor VIII *In Vitro*

All nine factor VIII constructs were used to produce integrating VSVg pseudotyped lentiviral vectors by transient transfection of 293T cells. They were first tested for physical titre using the reverse transcriptase protein assay, and were then titred using qPCR; 1 x 10^5 293T cells were transduced with a serial dilution of each vector, after 72 hours genomic DNA was extracted from cells and qPCR carried out using primer and probe sets for both WPRE and β-actin. From these results, the copy number per cell can be determined for each dilution and an approximate titre in vector genomes per mL (vg/mL) calculated (Table 6.2).

Virus	Average Reverse Transcriptase (ng/µL)	Estimated Titre (TU/mL)	Titre (vg/mL)
BDD FVIII	10.9	3.71 x 10^9	1.14 x 10^8
FVIII Fugu B	46.5	1.58 x 10^{10}	1.58 x 10^9
FVIII N6	30.7	1.04 x 10^{10}	1.07 x 10^9
SQ FVIII	68.3	2.32 x 10^{10}	2.91 x 10^9
SQ FVIII Fugu B	44.8	1.52 x 10^{10}	1.18 x 10^9
SQ FVIII N6	78.0	2.65 x 10^{10}	2.0 x 10^9
SQ FVIII (co)	69.6	2.37 x 10^{10}	4.45 x 10^9
SQ FVIII Fugu B (co)	71.8	2.40 x 10^{10}	2.65 x 10^9
SQ FVIII N6 (co)	87.9	2.99 x 10^{10}	3.39 x 10^9

Table 6.2 Physical titre of Factor VIII vectors as determined by reverse transcriptase assay and qPCR. Quantification of reverse transcriptase (RT) protein concentration in viral stocks, measured by performing a RT colorimetric assay, quantified in ng/µL and estimated titre calculated from this. Mean shown of n=3. Quantification of titre in vector genomes per mL was determined using qPCR. 1 x 10^5 293T cells were transduced with a serial dilution of vector, after 72 hours genomic DNA was extracted from cells and qPCR carried out for both WPRE and the human housekeeping gene beta-actin. Mean shown of n=5.

Relative factor VIII protein expression for each vector was first tested *in vitro.* Five wells of 1 x 10^5 293T cells were transduced with serial dilutions of vector: 10, 2, 0.4, 0.08 and 0.016µL in a total volume of 300µL DMEM + 10% FCS. Plates were incubated for 48 hours at which point cell media was changed for 500µL OptiMEM. After a further 24 hours incubation media was collected from all wells and assayed for factor VIII expression using a sandwich ELISA (Affinity Biologicals) to measure FVIII antigen (Figure 6.5) and a chromogenic assay (Quadratech Diagnostics) to determine FVIII cofactor activity (Figure 6.6).

In both assays factor VIII units were expressed in % normal human levels as determined using human control plasmas in which reported amounts of all coagulation factors are accurately determined by reference to the corresponding NIBSC (National Institute for Biological Standards and Controls). Two human control plasmas were used for standard curves and assay quality control. Normal control plasma (Biophen, Quadratech Diagnostics) contained on average a hFVIII level >60% of normal, and abnormal control plasma (Biophen, Quadratech Diagnostics) contained around 25-65% of normal hFVIII. Genomic DNA was extracted from 293T cells so viral copy number could be determined using qPCR for each well tested. Expression values were then normalised against copy number allowing accurate values for protein expression per factor VIII gene copy to be determined.

Figure 6.5 shows the results for measurement of factor VIII protein expression using ELISA. Results indicate that factor VIII expression could be accurately detected for all vectors. Average expression levels for non-codon optimised constructs were 25.8, 7.4, and 45.5% hFVIII protein/mL/24hr/copy number for BDD FVIII, FVIII Fugu B and FVIII N6 constructs, respectively. Non-codon optimised constructs containing the associated SQ peptide sequences showed similar levels of expression with 18.8, 5.2, and 13.5% hFVIII protein/mL/24hr/copy number for SQ FVIII, SQ FVIII Fugu B and SQ FVIII N6 constructs, respectively, indicating that the presence of the SQ sequences makes no substantial difference to the secretion of factor VIII protein.

Expression of factor VIII protein from codon optimised sequences was substantially higher. Levels of 590, 425.8, and 750.1% hFVIII protein/mL/24hr/copy number were recorded for SQ FVIII (co), SQ FVIII Fugu B (co) and SQ FVIII N6 (co) constructs, a 31-, 82- and 56-fold increase in expression in comparison to equivalent non-codon optimised sequences, respectively.

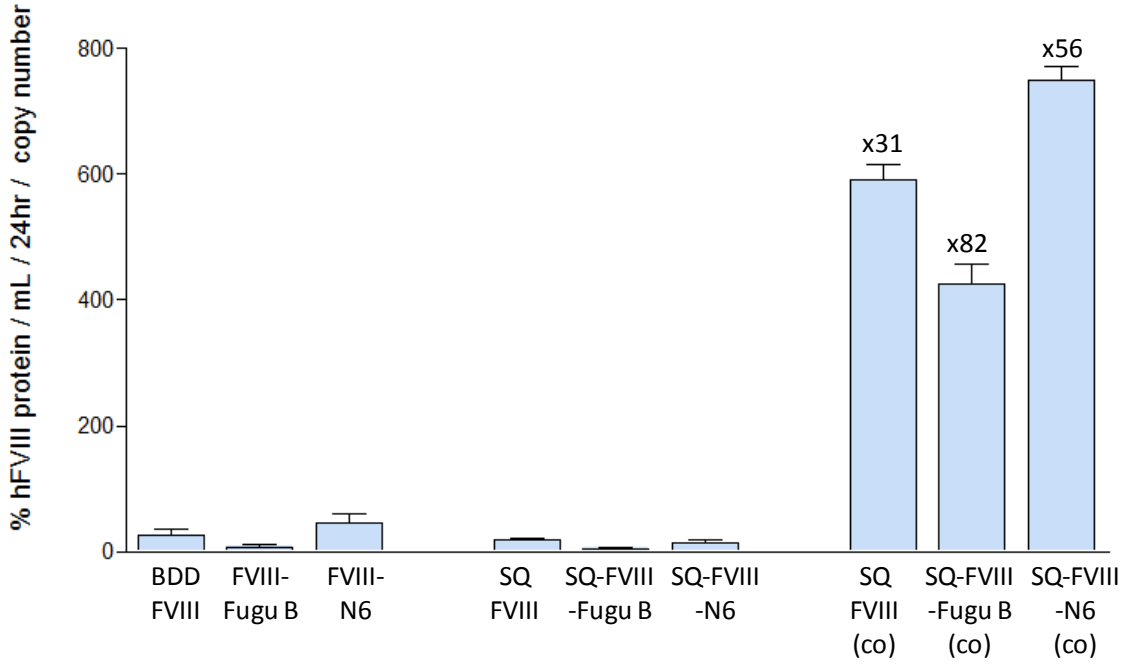


Figure 6.5 Relative human FVIII protein expression from integrating lentiviral vectors *in vitro* determined by ELISA. 1 x 10^5 293T cells were transduced with serial dilutions of BDD FVIII, FVIII Fugu B, FVIII N6, SQ FVIII, SQ FVIII Fugu B, SQ FVIII N6, SQ FVIII (co), SQ FVIII Fugu B (co), or SQ FVIII N6 (co) vector. Plates were incubated for 48 hours at which point cell media was changed for 500µL OptiMEM. After a further 24 hours incubation media was collected from all wells and assayed for factor VIII expression using a sandwich-ELISA (Affinity Biologicals) to measure factor VIII protein. Results were then normalised on copy number per cell determined by qPCR. Mean and SD shown for n=5. Values above bars represent the fold increase in FVIII expression from codon optimised constructs in comparison to the equivalent non-codon optimised.

Figure 6.6 shows the results for measurement of factor VIII cofactor activity using a chromogenic assay. Average expression levels for non-codon optimised constructs were recorded at 1.4, 2.4 and 2.7% hFVIII activity/mL/24hr/copy number for BDD FVIII, FVIII Fugu B and FVIII N6, respectively. Non-codon optimised constructs containing the associated SQ peptide sequences showed similar levels of expression with 1.4, 2.9 and 2.8% hFVIII activity/mL/24hr/copy number for SQ FVIII, SQ FVIII Fugu B and SQ FVIII N6 constructs, respectively, indicating that the presence of the SQ sequences has no substantial influence on the secretion and cofactor activity of factor VIII protein. However, expression of factor VIII protein from codon optimised sequences was substantially higher, with average expression levels recorded at 22.9, 48.2, and 35.8% hFVIII activity/mL/24hr/copy number for SQ FVIII (co), SQ FVIII Fugu B (co) and SQ FVIII N6 (co) constructs, a 16-, 16- and 13-fold increase in comparison to equivalent non-codon optimised sequences, respectively.

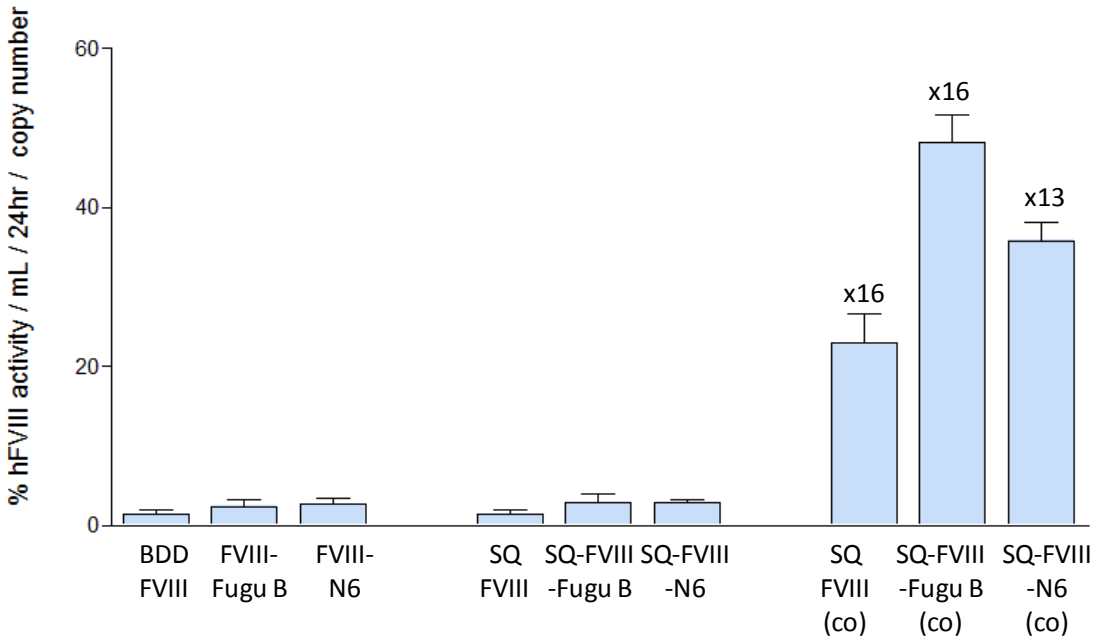


Figure 6.6 Relative human FVIII cofactor activity from integrating lentiviral vectors *in vitro* as determined by a chromogenic assay. 1 x 10^5 293T cells were transduced with serial dilutions of BDD FVIII, FVIII Fugu B, FVIII N6, SQ FVIII, SQ FVIII Fugu B, SQ FVIII N6, SQ FVIII (co), SQ FVIII Fugu B (co), or SQ FVIII N6 (co) plates were incubated for 48 hours at which point cell media was changed for 500µL OptiMEM. After a further 24 hours incubation media was collected from all wells and assayed for factor VIII expression using a chromogenic based assay to measure factor VIII cofactor activity. Results were then normalised on copy number per cell determined by qPCR. Mean and SD shown for n=5. Values above bars represent the fold increase in FVIII expression from codon optimised constructs in comparison to the equivalent non-codon optimised.

6.3.2 Expression of Factor VIII *In Vivo*

Long term expression of hFVIII was tested *in vivo* via intravenous injection of vector in a murine model of haemophilia A. Only vectors containing modified SQ sequences were tested *in vivo* as *in vitro* results indicated that the presence of SQ^m sequences in non-codon optimised sequences did not make a substantial difference to either protein expression or activity of factor VIII *in vitro* (Figures 6.5 and 6.6). 40 µL of concentrated VSVg pseudotyped integrating lentiviral vector containing SQ FVIII, SQ FVIII Fugu B, SQ FVIII N6, SQ FVIII (co), SQ FVIII Fugu B (co), or SQ FVIII N6 (co) constructs under control of the SFFV promoter was directly injected via the superficial temporal vein of neonatal (0-1 day old) haemophilia A ($F8^{tm2Kaz}$ generated by deletion of exon 17 [271] and maintained on a 129SV background) mice (injections performed by Simon Waddington). The estimated titre of vectors calculated in vector genomes (vg) injected per mouse and the numbers of successfully injected mice are shown in Table 6.3.

Factor VIII Construct	Estimated Titre in Vector Genomes (vg)	No. Injected Mice	Bleed Time-points (days post-injection)
SQ FVIII	1.16×10^8	6	35, 63, 93, 126, 170, 197, 240
SQ FVIII Fugu B	4.72×10^7	6	29, 57, 87, 120, 164, 191, 234
SQ FVIII N6	8.00×10^7	8	38, 47, 91, 118, 161, 187
SQ FVIII (co)	1.78×10^8	7	43, 73, 106, 150, 177, 220, 246
SQ FVIII Fugu B (co)	1.06×10^8	7	36, 66, 99, 143, 170, 213, 239
SQ FVIII N6 (co)	1.35×10^8	10	28, 58, 91, 135, 162, 205, 231

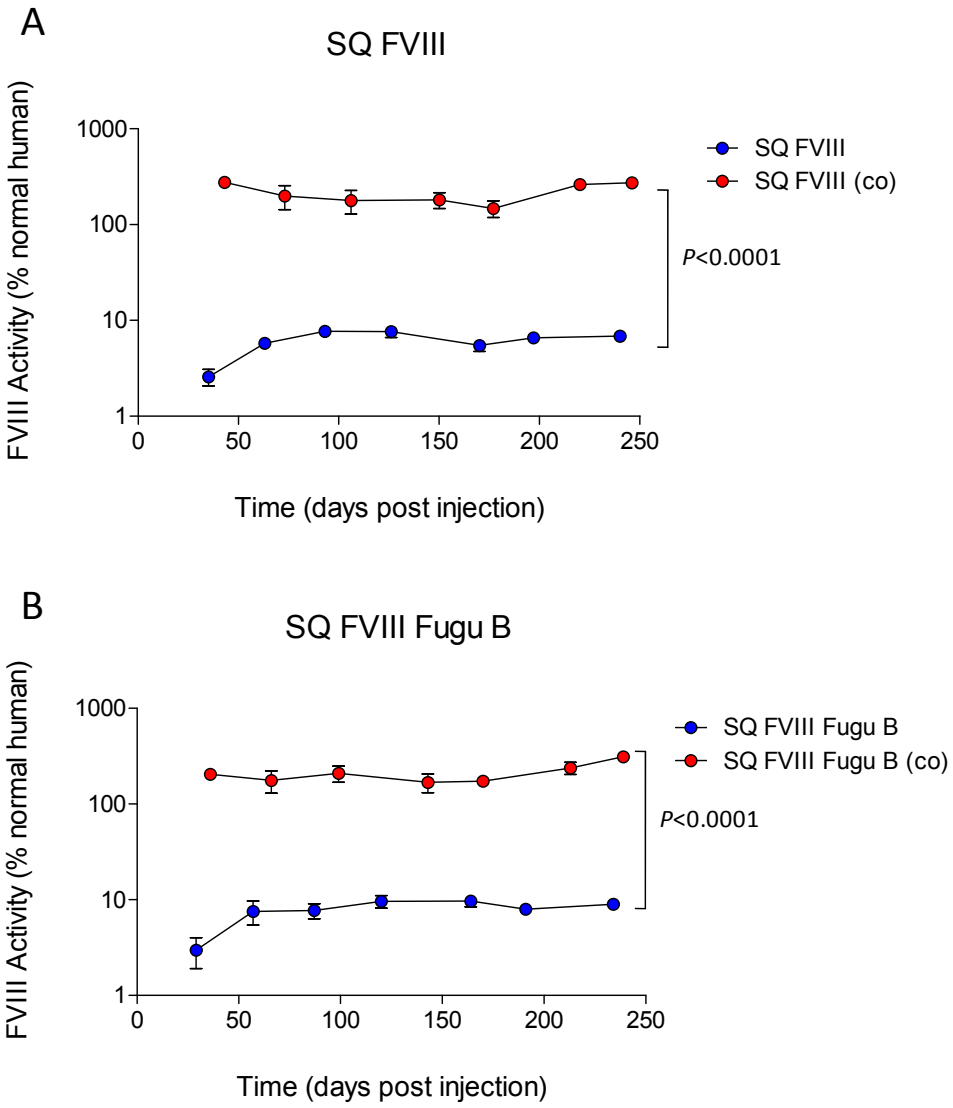
Table 6.3 Estimated titres in vector genomes (vg) injected per mouse for factor VIII *in vivo* study. The number of successfully injected neonate $F8^{tm2Kaz}$ mice, bleed time-points in days post-injection and the estimated vg titre injected per mouse for each FVIII vector tested determined using qPCR.

Six to ten neonates were injected per vector group, and vector injection and tail vein bleeds were staggered (Table 6.3). The amount of vector injected into each mouse in each test group was approximately equivalent with each mouse receiving between 2 and 9 x 10^7 vg/mL, the titre as determined by qPCR. Each vector group was bled approximately every 30 days over 250 days post-injection, with the first bleed time-point around 30 days post-injection due to the high risk of mortality when bleeding and anaesthetising neonate haemophiliac animals. Analysis of factor VIII was monitored by analysing blood plasma samples for cofactor activity using the chromogenic assay (Quadratech Diagnostics).

The factor VIII ELISA (Quadratech Diagnostics) was not used to analyse the amount of FVIII antigen in blood samples. The peroxidise-conjugated detection antibody used in this kit is polyclonal and a new batch was unable to detect either the SQ FVIII or SQ FVIII Fugu B constructs, likely due to conformational changes within the factor VIII structure. This was confirmed by using Surface Plasmon Resonance (SPR) analysis (data not shown) to assess binding of factor VIII protein to the antibody, carried out by Dr. John McVey, Thrombosis Research Institute (see reference [272] for an overview of SPR analysis) and unfortunately, a suitable replacement antibody was not found.

Figures 6.7A-C show the mean hFVIII cofactor activity level in the blood plasma of mice injected with vector containing non-codon optimised vs. codon optimised for SQ FVIII, SQ FVIII Fugu B and SQ FVIII N6, respectively. Functional FVIII was detected in the plasma of all transduced mice at all time points. Plasma from mice transduced with vector containing non-codon optimised FVIII sequences; SQ FVIII, SQ FVIII Fugu B, or SQ FVIII N6 contained on average 5.72% ± 2.31%, 7.79% ± 3.66%, and 9.53% ± 2.24% normal human FVIII activity, respectively, for the duration of the experiment. The ability to clot rapidly following tail vein bleeds indicated that the mice treated with sequences SQ FVIII Fugu B, or SQ FVIII N6 were able to achieve adequate haemostasis, however 4 of the 6 mice injected in the SQ FVIII vector group did not survive indicating that the levels of FVIII were insufficient to correct the murine haemophilia A phenotype. None of the other vector groups showed morbidity associated with low

FVIII expression. For mice transduced with vector containing codon optimised FVIII cDNA sequences; SQ FVIII (co), SQ FVIII Fugu B (co), or SQ FVIII N6 (co), average FVIII levels were detected at 256.1% ± 63.4%, 232.2% ± 74.1%, and 283.7% ± 56.2% normal human FVIII activity, respectively, for the duration of the experiment. This is a 44-, 29-, and 29-fold increase in expression for SQ FVIII (co), SQ FVIII Fugu B (co), and SQ FVIII N6 (co), respectively, in comparison to expression from equivalent non-codon optimised sequences (P<0.0001, Bonferroni simultaneous test). Furthermore, no substantial loss in FVIII expression was observed in any vector groups. Importantly, no significant difference in expression was observed for constructs containing different B-domain elements for vectors containing codon optimised or non-codon optimised cDNA sequences (Figure 6.8).



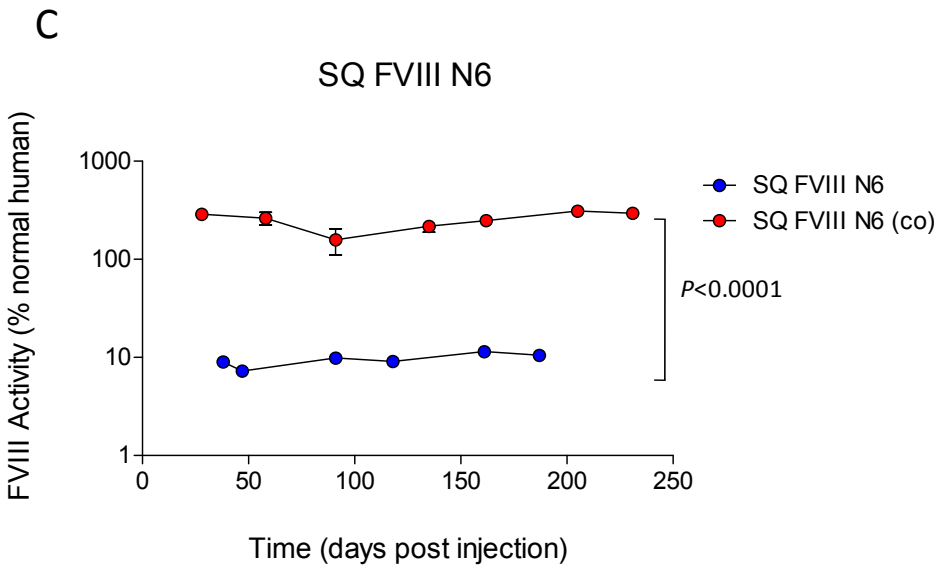


Figure 6.7 Percentage expression of human FVIII cofactor activity *in vivo* after neonatal injection of lentiviral vectors. Six to ten $F8^{tm2Kaz}$ neonate mice were injected intravenously via the superficial temporal vein with integrating lentiviral vectors expressing factor VIII constructs. Mice were bled at various time-points over approximately 250 days and a chromogenic assay (Quadratech Diagnostics) used to calculate the activity of human factor VIII in blood plasma taken from each mouse. **(A)** Codon optimised vs. non-codon optimised SQ FVIII. **(B)** Codon optimised vs. non-codon optimised SQ FVIII Fugu B. **(C)** Codon optimised vs. non-codon optimised SQ FVIII N6. Significance determined by Bonferroni simultaneous test.

These results indicate that codon optimisation of factor VIII cDNA sequences using the codon adaptation index (CAI) for *H. sapiens* and removing detrimental sequence motifs significantly increases factor VIII expression *in vivo* as determined by chromogenic assay. However, the effect of varying B domain within the codon optimised SQ Factor VIII molecule did not appear have a considerable difference. Figure 6.8 shows collated results for the factor VIII cofactor activity *in vivo* from blood samples taken from mice injected with codon optimised vectors.

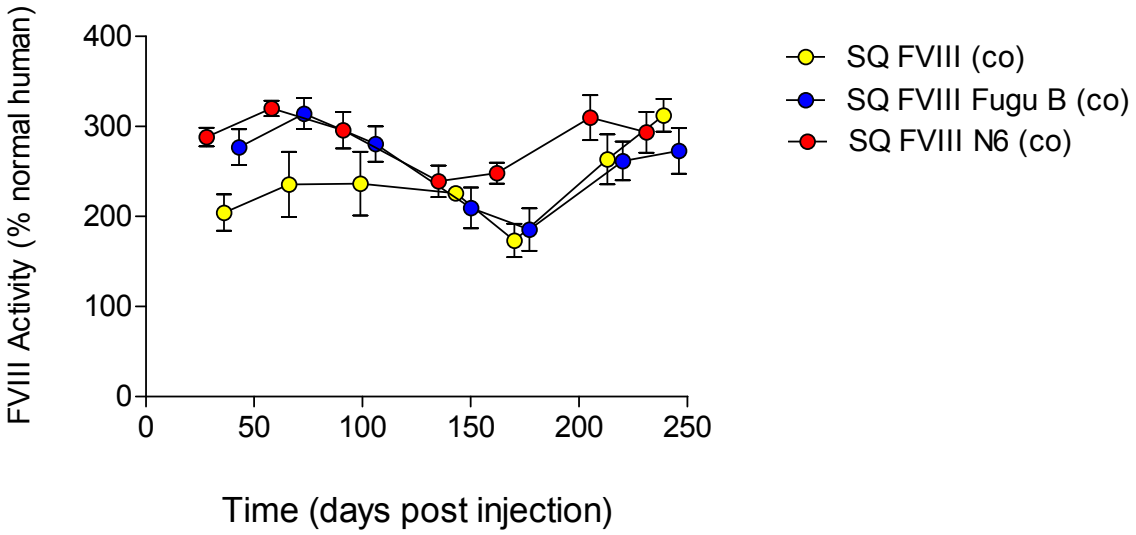


Figure 6.8 Collated data for hFVIII activity levels *in vivo* in plasma taken from mice injected with codon optimised vectors. Percentage activity data from plasma samples from mice injected with lentiviral vector expressing SQ FVIII (co), SQ FVIII Fugu B (co), and SQ FVIII N6 (co) collated.

6.3.3 Proviral Copy Number in Tissue Taken from Mice Injected with Lentiviral Vectors containing hFVIII Constructs

Mice were sacrificed at ~250 days post injection and liver, spleen, heart, lung and kidney tissue taken for viral copy number analysis using qPCR. Figure 6.9 shows viral copy number was restricted mainly to liver and spleen tissue. In liver tissue average values for non-codon optimised groups SQ FVIII, SQ FVIII Fugu B and SQ FVIII N6 were 5.8, 7.0 and 5.3 viral copies per cell, respectively. Average copy number in liver for the codon optimised vector groups was marginally higher at 5.0, 9.2 and 8.8 viral copies per cell for SQ FVIII (co), SQ FVIII Fugu B (co) and SQ FVIII N6 (co), respectively. Viral copy number is also seen in spleen tissue with similar values detected in both non-codon optimised and codon optimised groups. Average values for SQ FVIII, SQ FVIII Fugu B and SQ FVIII N6 were recorded at 1.5, 3.1 and 2.8 viral copies per cell, respectively, and average values for SQ FVIII (co), SQ FVIII Fugu B (co) and SQ FVIII N6 (co) at 2.3, 2.6 and 2.6 viral copies per cell, respectively. Copy number was also detected in heart tissue for

all groups at average values of 0.55, 1.03, 1.09, 0.55, 1.00 and 0.82 copies per cell for SQ FVIII, SQ FVIII Fugu B, SQ FVIII N6, SQ FVIII (co), SQ FVIII Fugu B (co) and SQ FVIII N6 (co), respectively. All other tissues showed negligible copy number at <0.9 copies per cell for tissues from all mice in all vector groups (Figure 6.9).

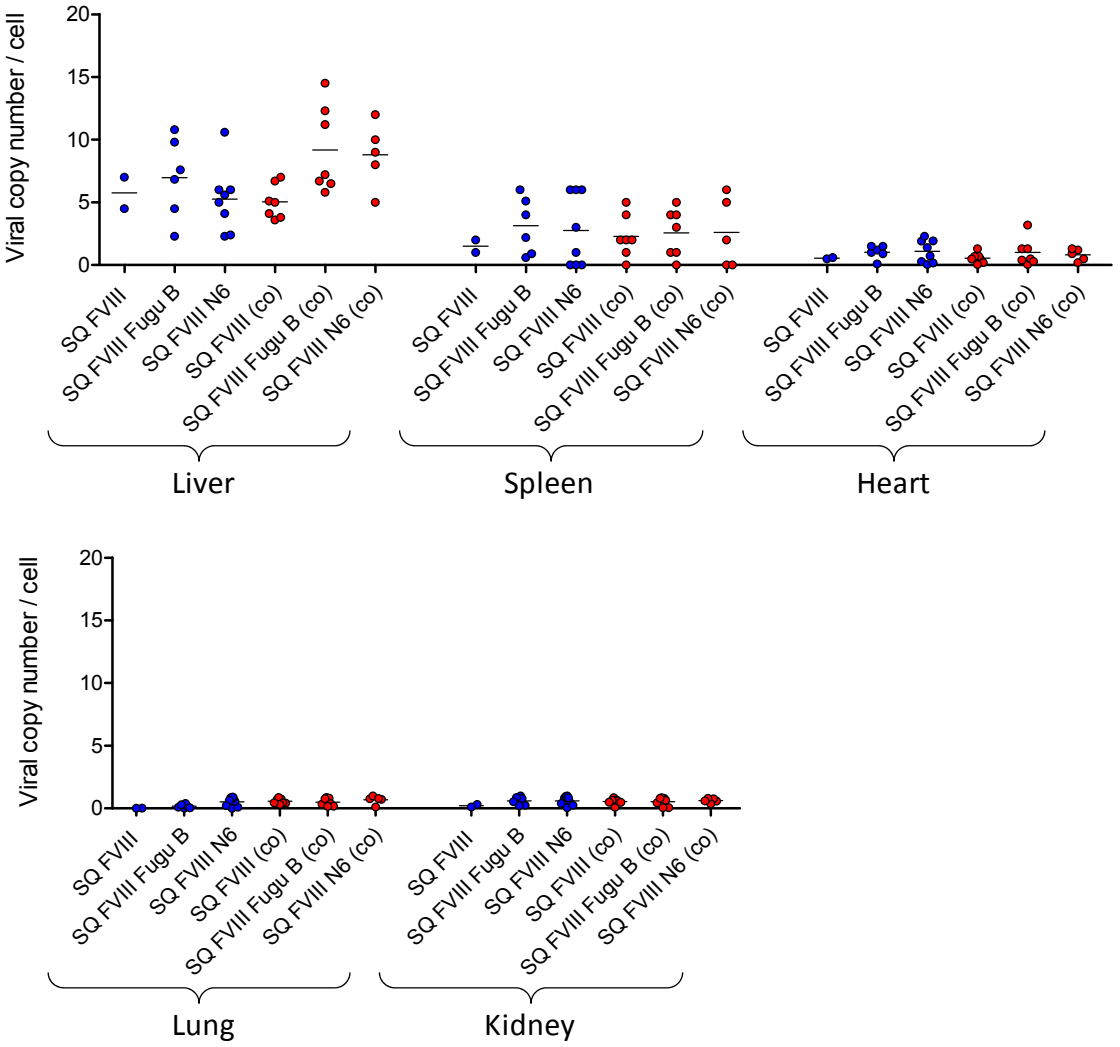


Figure 6.9 Quantification of viral copy number. Liver, spleen, heart, lung and kidney tissue were taken from mice sacrificed at ~250 days post neonatal injection of lentivectors expressing factor VIII constructs. Genomic DNA was extracted and viral copy number determined using qPCR. Line represents the mean of all points.

6.4 Summary

- Within an integrating SIN lentiviral vector construct inclusion of the 14 amino acid SQ peptide region containing the intracellular furin recognition and cleavage site made no difference to the expression of factor VIII *in vitro*.
- Expression of bioengineered forms of factor VIII from a lentiviral vector were increased over 13-fold per integrated copy *in vitro* and over 29-fold *in vivo* when cDNA sequences were codon optimised using CAI for *Homo sapiens*
- Intravenous injection of integrating lentivector containing codon optimised factor VIII cDNA sequences into neonate factor VIII knockout mice successfully rescued the disease phenotype and mice showed expression of factor VIII in excess of 200% normal human levels.
- Changing the B domain of factor VIII to the 14 amino acid SQ region, the B domain of the teleost puffer fish *Fugu rubripes,* or the first 226 amino acids of the human B domain (226/N6) did not make a significant difference to expression of factor VIII *in vivo*.

In this chapter we describe the expression of bioengineered forms of factor VIII from non-optimised and codon optimised cDNA sequences by an integrating lentiviral vector. mRNA instability, interactions with resident endoplasmic reticulum (ER) chaperone proteins, and the requirement for carbohydrate-facilitated transport from the ER to the Golgi apparatus means that FVIII is expressed at much lower levels from mammalian cells than other proteins of similar size and complexity [174,273]. A number of bioengineered forms of human FVIII have been incorporated into gene transfer systems and have been shown to have enhanced expression both *in vitro* and *in vivo*. Lentiviral vectors have been used to mediate expression of bioengineered forms of FVIII both *in vitro* and *in vivo*, and studies are reported using FIV [116,196,201], EIAV [274], SIV [275,276] and HIV-1 [198,200,202] based vectors. Previous *in vivo* studies have demonstrated expression of therapeutic levels of factor VIII *in vivo* in adult haemophilia A mice after systemic injection of vector [116,196,200,202], transplant of transduced bone marrow cells [199,198],

transplant of transduced bone marrow cells with targeted platelet-specific expression [276,277], and transplant of transduced blood outgrowth endothelial cells (BOECs) [278]. However, FVIII expression levels mediated from these approaches have been low (1-5% normal human) and in the majority of studies expression has been transient due to formation of neutralising antibodies. Two exceptions where sustained expression of FVIII has been mediated are: A study carried out by Sinn *et al.,* in 2007 [196] where intravenous delivery into adult mice of an FIV vector expressing a hFVIII-226/N6 construct mediated expression levels of ~25% normal human levels. Also a recent study by Ramezani and Hawley in 2009 [279] showed sustained expression 24 weeks post-injection of both BDD hFVIII and an enhanced FVIII containing the 226/N6 B domain and a series of mutations: F309S/L303E for enhanced secretion and R484A/R489A/ P492A for reduced immunogenicity. In this study both constructs produced around 30% normal human FVIII levels in blood plasma of after transplantation of bone marrow cells transduced with a SIN gammaretroviral vector [279].

B domain deleted (BDD) factor VIII constructs are used widely in gene transfer experiments as there is no loss of FVIII procoagulant function and its smaller size is more easily incorporated into vectors. A variation of this construct is a BDD FVIII containing the 14 amino acid link SQ between the A2 and A3 domains, currently produced as a recombinant product and marketed as ReFacto™ (Wyeth) [186]. The SQ link promotes efficient intracellular cleavage of the primary single chain translation product of FVIII as it contains the intracellular furin recognition and cleavage site. [186,263]. This construct has been incorporated into plasmid vectors where it has conferred therapeutic levels of expression [275,280,281]. Miao *et al.,* in 2004 [188] showed that after plasmid transfection of COS-1 cells a human FVIII construct containing the first 226 amino acids of the B domain including 6 N-linked asparagine glycosylation sites was secreted 4 fold more efficiently in comparison to BDD FVIII [188]. This construct has now been incorporated into many gene transfer vectors including plasmid [198], lentiviral vectors [196], and oncoretroviral vectors [279] and is more efficiently secreted *in vitro*, however, there are no reports *in vivo* where this construct has produced significantly higher levels of FVIII protein in blood plasma in comparison to BDD FVIII. A further B

domain engineered construct is hFVIII containing the B domain of *Fugu rubripes.* In 2005 Dr. Steven Pipe reported that this construct was secreted as efficiently as the 226/N6 construct *in vitro* in COS-1 and CHO cell lines (Dr. Steven Pipe, personal communication).

In this study we investigated expression of BDD FVIII, SQ FVIII, FVIII Fugu B, and FVIII N6 constructs using integrating SIN lentiviral vectors. Measuring factor VIII activity *in vitro* using the chromogenic assay no difference was found between constructs containing the SQ sequence or not. However, incorporation of B domain regions into constructs allowed a 1.5-fold increase in secretion for SQ FVIII N6 compared to SQ FVIII, and a 2-fold increase for SQ FVIII Fugu B in comparison to SQ. This is likely due to more efficient ER-Golgi transport by protein constructs which retain *N*-linked oligosaccharides within the short B domain spacer [188], however, the difference is small. *In vivo*, the presence of a B domain did not substantially affect the amount of factor VIII produced.

The expression of factor VIII from a codon optimised cDNA sequence in a lentiviral vector was also investigated. We observed a 13- to 16-fold increase in expression of functional factor VIII per integrated gene copy *in vitro* from a sequence optimised for *H. sapiens* expression. A 29- to 44-fold increase in blood plasma activity levels of functional factor VIII were also observed in knockout mice from the codon optimised cDNA sequences to >200% normal human expression, significantly higher than from a non-codon optimised sequence. Multiple transcriptional silencers and inhibitory motifs which are widely distributed throughout the FVIII cDNA [181,183,282,283], and these sequences act as potent inhibitors of RNA production and protein formation which hamper expression *in vivo.* It is likely that the increase in expression observed from codon optimised cDNAs is due to the elimination of these sequences. Radcliffe *et al.,* in 2007 [274] have previously codon optimised both full length and BDD factor VIII cDNA sequences and tested *in vitro* in an EIAV based vector. They observed a 2-fold increase vector titre when incorporating the codon optimsed BDD FVIII cDNA, but only a 3-fold increase in expression of factor VIII per integrated copy number *in vitro* in HepG2 cells

[274]. The modest increase in FVIII expression in comparison to that observed by our constructs may be due to differences in cDNA sequence, cell type, or vector type.

Overall, this chapter showed that a high level of factor VIII expression can be mediated from a lentiviral vector containing a codon optimised cDNA sequence *in vivo*, rescuing a mouse model of haemophilia A.

Chapter Seven

Discussion

7.0 Final Discussion

The increasing number of gene therapy clinical trials resulting in patient clinical benefit highlight the rising use of gene therapy to treat a variety of disease conditions [284-291]. Many trials have used retroviral vectors which are successful as they integrate their genetic material into patient chromosomes. This allows therapeutic DNA to be passed to daughter cells during mitosis, enabling stable gene expression in dividing tissues. However, five cases of acute lymphoblastic leukemia in two SCID-X1 clinical trials caused by integration of the retroviral genome near proto-oncogenes including *LMO2* have raised concerns about the safety of integrating vectors [26-28]. To address the problem of insertional mutagenesis whilst retaining attractive properties of lentiviral vectors, such as the ability to transduce both dividing and quiescent cells, integration deficient lentiviral vectors (IDLVs) have been developed. IDLVs can be produced through the use of integrase mutations that specifically prevent proviral integration, resulting in the generation of increased levels of 1- and 2-LTR circular episomes in transduced cells. Compared to integrating lentivectors IDLVs have a significantly lower risk of causing insertional mutagenesis and a lower risk of generating replication competent retroviruses (RCRs) [3,66,98]. In this project IDLVs were investigated as treatment for haemophilia, with quiescent muscle and liver cells as a primary target.

Haemophilia A and B are serious X-linked bleeding disorders caused by a deficiency in, or complete absence of, coagulation factors VIII or IX, respectively. The clinical severity of haemophilia mainly correlates with the measured level of endogenous factor. Current therapy for both inherited disorders includes factor replacement, however, this is not a phenotypic cure, can be costly, and the development of inhibitors remains a serious cause of morbidity. The World Federation of Haemophilia also estimate that the majority of sufferers (up to 75%), predominately living in developing countries, do not receive any, or only sporadic, factor replacement therapy (www.wfh.org). Only a modest increase in blood plasma levels of coagulation factor is needed for therapeutic benefit of haemophilia; above 1% of normal levels can markedly reduce the rate of

spontaneous bleeding. The cDNA sequences have been defined and cloned, and there are well characterised murine and large animal models available for preclinical experiments, therefore haemophilia A and B are considered to be appropriate candidates for gene therapy. Many successful preclinical studies have been carried out using a wide variety of viral and non-viral vectors, however gene therapy for inherited coagulation disorders still faces many hurdles before establishing a role in patient care.

In this project I investigated the use of IDLVs for gene therapy treatment of haemophilia, our first approach was to deliver IDLVs to terminally differentiated muscle cells. Although FVIII and FIX are secreted normally by endothelial cells and/or hepatocytes, respectively, they are also successfully secreted by many other cell types including myocytes, adipocytes and fibroblasts. Arruda *et al.,* in 2001 [213] showed that FIX was successfully expressed from myotubes and that post-translational modifications were the same as FIX protein expressed from liver tissue [213]. Administration of non-integrating AAV vectors expressing factor IX via multiple intramuscular injection has successfully demonstrated long-term transgene expression in both mouse and large animal models. AAV-1 and AAV-2 vectors expressing canine factor IX injected intramuscularly into both immunodeficient (SCID) and immunocompetent (FIX knock-out) mice have been successful [292]. However, all immunocompetent mice treated with AAV-2-canine FIX developed inhibitors. Direct muscle injection of rAAV-human FIX in the Chapel Hill strain of a haemophilia B dog resulted in expression of FIX in myocytes, but consequently, the animal developed an anti-human FIX inhibitor [293]. However, Herzog *et al.,* in 1999 [215] showed sustained expression of canine FIX and partial correction of the bleeding phenotype after administering AAV-canine FIX into the haemophilia B dog model [215]. Arruda *et al.,* in 2005 [294] also achieved long-term expression of FIX in haemophilia dogs by regional intravenous delivery to skeletal muscle [294]. One clinical trial has also examined the use of AAV vectors injected intramuscularly into human subjects with haemophilia B. Here, Manno *et al.*, in 2003 [216] administered an AAV-2 vector containing a modified FIX gene via 10 to 90 intramuscular injections into 8 patients with severe haemophilia B. There was evidence of gene transfer and expression in all patients, however, the efficacy was

low and only 2 of 8 patients achieved levels which were >1% of normal levels. This trial however, did demonstrate that intramuscular administration of vector was not complicated by systemic illness or inhibitory antibodies to FIX, and that long-term expression could be achieved [216]. IDLVs have previously been shown to mediate long-term expression of GFP in neonate muscle in MF1 mice after a single intramuscular injection [67], therefore we hoped to obtain long-term expression of a luciferase transgene *in vivo* in adult muscle using IDLVs and further target differentiated myocyte cells by using alternative envelope glycoproteins to pseudotype vectors. Unfortunately, none of the alternative pseudotypes tested *in vivo* in this study showed better transduction than the most commonly used VSVg envelope after a single intramuscular injection of integrating vector. A significant loss in luciferase expression was also observed for all vectors tested, both integrating and non-integrating. The cause of the loss was not determined, however, it is likely due to a transgene specific immune response as observed by Limberis *et al.* (2009) [224] after intramuscular injection of a VSVg pseudotyped lentivector expressing luciferase [224]. Direct presentation of antigen epitopes can occur when novel genes are expressed within professional APCs such as dendritic cells. The expressed proteins are processed and presented upon class I MHC molecules, the APC then migrates to the lymph nodes for interaction with naive T cells. An antigen-specific, class I-restricted response can also occur through cross-presentation from a somatic cell, such as a myocyte or myoblast, to a professional APC. Both of these pathways for elicitation of a cellular immune response may function more efficiently in damaged muscle tissue. In the clinical trial reported in 2003 up to 90 intramuscular injections were needed to effectively transduce tissue [216]. Repeat injections may cause damage to cells and an immune response may become more likely simply because more resident APCs will be available for infection by vector. Increased cross-presentation may also occur due to the presence of damaged cells. This can also be a problem treating muscle disorders such as Duchene Muscular Dystrophy with gene therapy vectors as tissue is inherently damaged due to the disease [295].

Challenges also remain in achieving efficient delivery into muscle tissue due to its large size. Intramuscular injection of viral vectors results in transduction of the immediate

area with only limited diffusion into the surrounding area, therefore, a large number of injections are required to treat large muscles. Methods for systemic administration of vectors to transduce both skeletal and cardiomyocyte muscle tissue are promising; transduction of myocardium and skeletal muscle was achieved after a single intravenous administration of AAV-6 vector into mice treated with VEGF by Gregorevic *et al.,* in 2004 [296]. Enhancing vascular permeabilisation to achieve increased viral transduction of muscle by perfusing tissue with histamine has also been investigated [297]. However, all of these methods will allow off-target expression in non-muscle cells. To achieve muscle specific expression tissue specific regulatory elements such as the CK6 promoter element can be incorporated into vectors. CK6 is a 600bp regulatory element from the muscle creatine kinase gene which when incorporated into adenoviral vectors decreased transduced cell clearance by cytotoxic T cells. However, the CK6 promoter is only around 12% as active as the CMV promoter/enhancer which may not be sufficient for effective gene expression from IDLVs [295].

Our second approach in this project was to deliver IDLVs to quiescent hepatocytes to achieve transgene expression in liver. Stable expression of therapeutic genes in the liver is beneficial for patients with a wide variety of hepatic and systemic diseases, including metabolic and infectious disorders, haemophilias, hypercholesterolemias, and lipid storage diseases [228]. Lentiviral vectors have been previously shown to transduce hepatocytes and mediate expression of FIX in liver [116,200,227-229]. We confirmed these results and achieved high expression (~30% of normal levels) of human FIX after injection of an integrating lentivector into C57BL/6 mice. However, FIX was not detected *in vivo* after intravenous delivery of a comparative number of transducing units of IDLV expressing FIX. 200-fold lower expression of a luciferase transgene expressed from IDLV in liver in comparison to ILV was also observed in a similar experiment when a comparative number of transducing units were injected intravenously. Viral copy number in the liver of IDLV transduced animals was also 100-fold lower in comparison to ILV, suggesting not only lower expression from viral episomes in cells but also a problem with loss of vector, or inefficient viral entry. In this

study we also found that hydrodynamic delivery of IDLV via the tail vein increased episome copy number in the liver, which in turn increased expression of luciferase.

Dr. Thierry Vandendriessche has reported detectable levels of human FIX *in vivo* after intraportal delivery of VSVg pseudotyped IDLV. Levels were approximately 100-fold lower in comparison to ILV at around 0.3-1% normal human levels. They also confirmed that expression was generated from vector episomes as levels of FIX protein in blood plasma decreased after a partial hepatectomy of transduced mice (Dr. Thierry Vandendriessche, personal communication). Professor Luigi Naldini also reported detectable levels of GFP in the liver of transduced mice after intravenous delivery of IDLVs. However, both expression and copy number were determined to be significantly lower in comparison to ILVs when the same number vector genomes were injected (Dr. Alessio Cantore, personal communication).

The disparity between transgene expression from ILVs and IDLVs in liver tissue is poorly understood, therefore these results suggest that further investigation into viral entry, processing, and epigenetic effects on vector episomes would be advantageous. Transgene expression by lentiviral vectors integrated into host DNA can be subject to transcriptional silencing or insertion site-dependent positional effects. Epigenetic silencing can occur in a number of ways including DNA methylation and chromatin modification [298-300]. IDLV episomes may be more susceptible to this type of modification, or cell processes in general, as extrachromosomal DNA. Analysis of epigenetic modifications can be achieved using bisulfite conversion of episomal DNA to study *de novo* CpG methylation, or chromatin immunoprecipitation (ChIP) to study protein-DNA interactions *in vivo*, respectively. Studying viral entry may be achieved by fusing a reporter protein such as GFP to a viral structural protein such as Gag, similar studies having been carried out using an eGFP-Vpr fusion protein to study HIV entry [249,250]. Further work to deliver an IDLV expressing FIX by hydrodynamic delivery would also be interesting to discover if therapeutic levels of FIX could be sustained.

Limiting expression of a therapeutic transgene exclusively to the target tissue would provide many benefits in a gene therapy setting. Off-target expression of some transgenes can result in complications and toxicity. High levels of systemic expression can induce or intensify host immune responses to both the transgene and the gene delivery vector. Ectopic expression could be reduced or eliminated by use of highly active, tissue-specific promoters. In addition, tissue-specific promoters may be free from the cytokine-mediated transcriptional silencing that limits the use of some viral promoters. In the majority of recent preclinical studies in which the liver is the target organ a tissue-specific promoter, such as the human alpha-1-antitrypsin (AAT) promoter, has been used to direct expression of coagulation factors VIII an IX. Although viral promoters such as CMV and SFFV direct high-level expression in hepatocytes *in vitro* they have been reported to be shut down in liver *in vivo* [252]. This may be due to transduction and subsequent expression in APCs which trigger immune-mediated destruction of transduced hepatocytes and the transgene product, and also through epigenetic silencing. Restricting expression to the liver by using a tissue-specific promoter appears to alleviate this in some [301], but not all cases [204,302].

In this project we investigated luciferase reporter gene expression under control of the strong viral promoter SFFV, the mammalian ubiquitous chromatin opening element (UCOE) and the liver specific promoters LP1 and hAAT, for expression in the liver after intravenous delivery into neonate mice. High expression of luciferase was observed in liver *in vivo* for all promoters, and there was limited methylation in each region. High tissue specificity for the liver specific promoters was also observed *in vitro,* however, this could not be confirmed *in vivo* as immunohistochemical staining of liver sections to see if hepatocyte cells were specifically transduced was unsuccessful. Further investigation using a GFP or beta-galactosidase (β-gal) transgene would be a good alternative to more easily observe transduced cells in liver sections. Investigation into the pattern of expression driven by these promoters in adult mouse liver would also be interesting to carry out, as would the use of specific promoters within the context of an IDLV.

Finally, we investigated the expression of bioengineered forms of factor VIII from non-optimised and codon optimised cDNA sequences by an integrating lentiviral vector. One of the challenges of successful gene therapy for haemophilia A concerns the biochemical characteristics of FVIII that result in difficulties in its production. Expression of FVIII is 2 to 3 fold lower than that of comparably sized proteins, and misfolding of newly synthesised FVIII in the endoplasmic reticulum lumen causes oxidative stress and induces apoptosis both *in vitro* and *in vivo* [181,303]. However, as our knowledge of factor VIII expression and secretion increases we are able to design more efficient proteins that can overcome the problems of factor VIII production. The incorporation of the short 14 amino acid SQ sequence between the a2 and a3 domains of a B domain deleted FVIII construct was found to promote efficient intracellular cleavage and have similar activity to full length plasma derived FVIII [186,263]. Similarly, addition of a 226 aa segment of B domain containing 6 *N*-linked glycosylation sites (FVIII N6) has also been shown to significantly increase secretion compared to BDD FVIII [188,279,303,304]. Human factor VIII containing the B domain from *Fugu rubripes,* which contains 11 *N*-linked glycosylation sites, is also reported to be secreted as efficiently as FVIII N6 (Dr. Steven Pipe, personal communication).

We investigated the expression of BDD factor VIII constructs containing various B domain elements including SQ, Fugu B, and 226/N6 from both codon optimised and non codon optimised cDNA sequences from a SIN lentiviral vector. *In vitro* in 293T cells there was no significant difference between constructs containing the SQ sequence or different B domains expressed from non-codon optimised cDNA sequences. However, when constructs were expressed from codon optimised cDNA sequence we saw a 13- to 16-fold increase in FVIII activity *in vitro*. Six constructs were then expressed *in vivo* by intravenous injection into neonate haemophiliac mice. Again, there was no significant difference between constructs containing different B domains from either codon optimised or non-codon optimised cDNA sequences, however, we observed a 29- to 44-fold increase in blood plasma activity levels of functional factor VIII in mice from the codon optimised cDNA sequences in comparison to non-codon optimised sequences to >200% normal human expression. Multiple transcriptional silencers and inhibitory

sequences are widely distributed throughout the FVIII cDNA [181,183,282,283] and the increased expression following codon optimisation may be in part due to the elimination of such sequences. However, deletion of the entire B-domain which led to a 17-fold increase in mRNA and primary translation product only resulted in a 30% increase in the levels of secreted protein, suggesting that the rate of ER-Golgi transport was reduced [172] and that levels of FVIII mRNA were not limiting expression. The introduction of multiple N-linked glycosylation sites known to be important in ER-Golgi transport of FVIII increased levels of secreted FVIII, suggesting that the rate of ER-Golgi transport may be a rate limiting step [188]. However, a significant amount of FVIII within the ER never transits to the Golgi compartment due to a failure to fold correctly and misfolded FVIII accumulation in the ER can result in oxidative damage and apoptosis, perhaps suggesting that FVIII folding is the rate limiting step in FVIII expression [303]. Although protein secondary structure is determined primarily by the amino acid sequence, protein folding within the cell is affected by a range of factors: these include interaction with other proteins (chaperones) and ligands, translocation through the ER membrane and redox conditions. The rate of translation can also affect protein folding and it has been suggested that codon usage may be a mechanism to regulate translation speed and thus allow stepwise folding of individual protein domains [305,306]. FVIII is a complex multi-domain protein in which nonsequential segments of the nascent polypeptide chain may interact in the three dimensional fold. Ribosome stalling at 'rare' codons may therefore lead to alternative folding pathways generating altered conformations and potentially misfolded protein. A potential explanation for the observed effect of codon optimised sequences utilised in this study may be that they allow effcient translation and transport across the ER membrane allowing the nascent FVIII polypeptide chain to fold correctly leading to the increased levels of secreted FVIII observed *in vitro* and *in vivo*.

Expression of >200% is not required in haemophilia patients, and production of such high levels of FVIII may be detrimental to producer cells [181,303], a major advantage of the optimised sequence is the ability to minimize the number of genetically modified cells needed to produce therapeutic levels, thereby reducing the risk of insertional

mutagenesis and insertion site-dependent positional effects. Also, the use of strong, ubiquitous promoter elements such as SFFV needed before to drive high expression of FVIII constructs could be replaced by weaker, tissue specific promoters which are less prone to transcriptional silencing [198].

The development of neutralising antibodies to FVIII is one of the most serious complications of FVIII protein replacement therapy. As injections were carried out in neonate mice the development of antibodies to FVIII was not observed as mice were tolerised. Nonetheless, intravenous injection of adult mice with integrating lentivector expressing FVIII is likely to induce production of inhibitory antibodies. In addition there may be an increased risk of mounting an immune response to bioengineered constructs, particularly if xenogeneic protein sequences such as the Fugu B domain are incorporated into FVIII gene therapy vectors. However, as a main goal of haemophilia therapy is to treat adult patients, we are currently investigating the expression of construct SQFVIII N6 (co) plus four copies of the microRNA target sequence miR-142 *in vivo* in haemophiliac mice to observe if tolerance can be induced (data not shown). Most FVIII inhibitory antibodies recognize epitopes localized to the A2 and C2 domains of the molecule [307]. Therefore, incorporation of the R484A/R489A/P492A mutations within the A2 epitope, which are reported to produce lower inhibitory antibody titres in FVIII knockout mice while retaining full functionality [195], may be useful to incorporate into our constructs. Further approaches to reduce the development of FVIII inhibitors could be to target hematopoietic stem cells (HSCs) which are readily accessible for *ex vivo* genetic modification. HSCs are an attractive target cell population for haemophilia A gene therapy as after transduction they will allow sustained expression of FVIII in circulating peripheral blood cells for the lifetime of the patient following transduction [308]. The number of vector copies per cell and the dose of transduced cells transplanted into a patient is also minimised [279], and there is the possibility of inducing immune hyporesponsiveness and, ideally, stable long-term tolerance to the transgene product [309,310]. It would therefore be of great interest to extend these studies to incorporate this strategy.

At present, it seems likely that future gene therapy vectors for haemophilia will employ numerous methods to improve the efficacy and safety of gene therapy treatment used to replace coagulation factors. These may include the use of integration deficient vectors to decrease the risk of insertional mutagenesis, targeting and de-targeting strategies to improve specific transduction and expression in target cells, transgene cDNA codon optimisation to improve expression, and manipulation of protein structure to produce improved coagulation function. This thesis has utilised many of these approaches and the data found gives a base for future work and optimisation to provide safe, efficient gene therapy for haemophilia.

8.0 References

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