

Lentiviral Gene Therapy for the Production of Systemic Proteins

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Abstract

Lentiviral vectors are highly effective gene transfer agents (GTAs) used to restore expression of deficient proteins in target cells. In pursuit of a GTA with efficient pulmonary transduction, the UK Respiratory Gene Therapy Consortium developed a lentivirus pseudotyped with the envelope proteins, F and HN from Sendai virus (rSIV.F/HN). In contrary to other viral vectors, pulmonary delivery of rSIV.F/HN achieves sustained gene expression (~2 years in mice) in the lungs and the systemic circulation following a single dose. Building upon previous data, the project objective was to investigate the application of the rSIV.F/HN vector-platform for wider indications, including systemic disorders and diseases that require regulated expression of a therapeutic protein.

To investigate rSIV.F/HN therapy for systemic diseases, intravenous vector delivery was characterised and compared against intranasal administration, the canonical delivery route. Intranasal and intravenous vector administration achieved sustained (at least 1 year) systemic expression of secreted reporter protein, Gaussia luciferase, with one-log higher expression observed following intravenous delivery of the highest vector dose. Systemic rSIV.F/HN delivery resulted in widespread biodistribution, with the generation of significant anti-vector neutralising antibodies limiting vector re-administration. In contrast, localised airway transduction was observed following intranasal administration and maintained transduction efficiency following re-administration demonstrated previously (Chapter 3).

Thrombotic thrombocytopenic purpura (TTP), caused by deficiency of protease ADAMTS13 (a-disintegrin-and-metalloproteinase-with-a-thrombospondin-type-1-motif-13), was selected as an exemplar disease to investigate rSIV.F/HN therapy for systemic disorders. rSIV.F/HN-ADAMTS13 transduction generated dose-dependent levels of protease activity *in vitro*. Limited plasma expression of ADAMTS13 was achieved following delivery of rSIV.F/HN-ADAMTS13 to deficient mice, however restoration of wildtype activity levels was demonstrated in the lungs following intranasal administration (Chapter 4).

To permit the application of rSIV.F/HN vectors towards indications with narrow efficacy/toxicity windows, temporal control of gene expression was investigated. Based

upon a commercial yeast-progesterone system, proof-of-concept that lentivirus-mediated gene expression could be regulated was demonstrated in a human airway model and a pulmonary cell therapy model. Humanised regulated expression cassettes with yeast elements substituted for zinc finger sequences were constructed, with further investigation required to assess efficacy of humanised constructs in lentiviral vectors (Chapter 5).

Data presented here supports the application of rSIV.F/HN towards systemic disorders whilst regulated expression vectors have the potential to broaden the rSIV.F/HN technology platform for therapeutic indications that require controlled gene expression.

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Statement of Originality

I declare that the content presented in this thesis, submitted for consideration by examiners, embodies my own work which was carried out during the course of my PhD degree at Imperial College London. Any work performed in collaboration with others has been appropriately referenced.

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Abbreviations

1V	One vector
2V	Two vector
A549	Adenocarcinomic human alveolar basal epithelial
AATD	Alpha-1-antitrypsin deficiency
AAV	Adeno-associated virus
ACD	Acid-citrate-dextrose
ADA	Adenosine deaminase
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
AI	Auto-inducible
ALD	X-linked adrenoleukodystrophy
ALI	Air-liquid interface
APC	Antigen-presenting cells
B2M	Beta-2-microglobulin
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
C'	C-terminal
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disorder
cPPT	Central polypurine tract
CRISPR	Clustered regularly interspaced short palindromic repeats
ddPCR	Droplet digital PCR
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide triphosphate
DTI	Defined trypsin inhibitor
dUTP	2'-Deoxyuridine, 5'-Triphosphate
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EF1 α	Elongation factor 1 α
EGFP	Enhanced green fluorescent protein
EGFP _{Lux}	Firefly luciferase and EGFP fusion
EGTA	Ethylene glycol tetraacetic acid
EIAV	Equine infectious anaemia virus

ELISA	Enzyme-linked immunosorbent assay
F	Fusion protein
FBS	Fetal bovine serum
Fct4	Cytoplasmic domain of the SeV F glycoprotein truncated to 4 nucleotides
FIV	Feline immunodeficiency virus
FIX	Factor nine
FKB12	FK506-binding protein 12
FL	Full-length
FL1	Fluorescence channel 1 (FITC)
FL2	Fluorescence channel 2 (PE)
FL3	Fluorescence channel 3 (PerCP)
FL4	Fluorescence channel 4 (APC)
FRAP	FKB12-associated protein
FRET	Fluorescence resonance energy transfer
FVIII	Factor eight
GFP	Green fluorescent protein
GLux	Gaussia luciferase
GMCSF	Granulocyte-macrophage colony-stimulating factor
GOI	Gene of interest
GP	Glycoprotein
GP1b α	Glycoprotein Ib-IX-V transmembrane protein α
GST	Glutathione S-transferases
GTA	Gene transfer agent
GusB	Glucuronidase beta
hADAMTS13	Human ADAMTS13
HBEC	Human bronchial epithelial cells
hCEF	Chimeric human CMV enhancer coupled to the eukaryotic EF1 α promoter fusion
HEK	Human embryonic kidney cells
HEK293	Human embryonic kidney 293 cells
HEK293T	Human embryonic kidney 293 cells expressing the simian virus 40 large T antigen
His	Histidine
HIV	Human immunodeficiency virus HIV
HN	Hemagglutinin-neuraminidase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cells
HSV	Herpes simplex virus
hVWF	Human VWF
IC50	Inverse dilution of serum required for 50% virus neutralisation

IL	Interleukin
IN	Intranasal
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRES	Internal ribosome entry site
IU	International units
IV	Intravenous
KB	Kilobase
kDa	Kilo Daltons
KO	Knockout
KRAB	Krüppel associated box
LB	Luria broth
LDH	Lactate dehydrogenase
LMO2	LIM domain-only 2
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LTR	Long terminal repeats
mADAMTS13	Murine ADAMTS13
MDTCS	Metalloproteinase, disintegrin-like, TSP1 repeat 1, cysteine-rich and the spacer domains
MEM	Modified Eagle Medium
MFD	Maximum feasible dose
MHC	Major histocompatibility complex
miRNA	Micro RNA
MLV	Murine leukaemia virus
MND	Murine leukaemia virus-derived
MOI	Multiplicity of infection
mRNA	Messenger RNA
mTORC1	Rapamycin complex 1
mVWF73	Murine VWF 73 amino acid fragment
mVWF	Murine VWF
N'	N-terminal
NHP	Normal human plasma
NMP	Normal mouse plasma
nm	Nanometer
NPC	Neuronal progenitor cell
NTA	Nitrilotriacetic acid
NTC	Non-template control
OD600	Optical density measured at 600 nanometres
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAP	Pulmonary alveolar proteinosis
PBS	Phosphate buffered saline

PCD	Primary ciliary dyskinesia
PCR	Polymerase chain reaction
PEG	Monomethoxypoly(ethylene) glycol
PEI	Polyethylenimine
PFA	Paraformaldehyde
PRT	Protein replacement therapy
qPCR	Quantitative PCR
rADAMTS13	Recombinant ADAMTS13
RBC	Red blood cell
rhVWF	Recombinant human VWF
RLU	Relative light units
rmVWF	Recombinant murine VWF
RO	Reverse orientation
RPMI	Roswell Park Memorial Institute
RSV	Rous sarcoma virus long terminal repeat
RT	Room temperature
RT-ddPCR	Reverse transcriptase ddPCR
RT-PCR	Reverse transcriptase PCR
rVWF	Recombinant VWF
SCID	Severe combined immunodeficiency disease
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SERPINA	Serpin Family A Member 1
SeV	Sendai virus
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
SIVagm	African green monkey SIV
SIVct+HN	SeV HN glycoprotein fused with the SIV Env cytoplasmic domain
TAE	Tris-acetate-EDTA
TAR	Transactivation-response elements
TBS	Tris-buffered saline
TERT	Telomerase reverse transcriptase
tet	Tetracycline
tetO	Tetracycline operator
tetR	Tetracycline repressor
Tk	Thymidine kinase
TLR	Toll-like receptor
TNF α	Tumor necrosis factor
TRAP	Tryptophan RNA-binding attenuation protein
TTP	Thrombotic thrombocytopaenic purpura
TU	Transduction units

ULVWF	Ultra-large VWF
UT	Untransduced
UTx	Untransfected
UV	Ultraviolet
VCN	Vector copy number
vVWF	Voncento plasma-purified VWF
VWD	von Willebrand Disease
VWF	von Willebrand Factor
VWF:RCo	VWF ristocetin cofactor
VWF73	VWF 73 amino acid fragment
WAS	Wiskott-Aldrich Syndrome
WBC	White blood cell
WPBs	Weibel-Palade bodies
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory elements
WT	Wildtype
ZF	Zinc finger
ZFHD1	Zinc finger homeodomain 1

Chapter 1- General Introduction

1.1 Lentivirus to lentiviral vector

Lentiviruses are enveloped, single-stranded RNA viruses which belong to the *Retroviridae* family. Common to all viruses in this family, lentiviruses have an RNA genome which is converted to double-stranded DNA by viral enzyme reverse transcriptase, then integrated into the host cell genome by viral enzyme, integrase (1). Key characteristics inherent to their nature make lentiviruses desirable as vectors for the delivery of therapeutic genes, encouraging their conversion from lentiviruses into lentiviral vectors. These characteristics include the integration of genes into the host cell's genome, their capacity to package 9kb of genetic material and their ability to transduce post-mitotic cells, such as neurons and differentiated airway epithelial cells (2). Particularly, the capability of lentiviruses to infect non-dividing cells was one of the defining factors that converted researchers from utilising other *Retroviridae* family members such as γ -retroviruses to lentiviruses as gene delivery vectors.

The first versions of lentiviral vectors were based upon human immunodeficiency virus-1 (HIV-1). The wildtype tropism of HIV-1 is mediated by the expression of CD4 receptors that bind a complex of glycoproteins on the virus envelope, gp120 and gp41 (3). In order to transform HIV-1 from lentivirus into a lentiviral vector, modifications were made to reduce the possibility of recombination events generating a replication competent virus. In the first instance, this entailed encoding the HIV-1 envelope glycoproteins (Env) onto a separate plasmid and disrupting the *Env* gene on the HIV-1 genome plasmid by introducing deletions (4). Akkina *et al.* (1996) first substituted the endogenous HIV-1 envelope proteins for the vesicular stomatitis virus envelope glycoprotein (VSV-G), an approach referred to as pseudotyping, which had already proven successful with murine leukaemia virus-based retroviral vectors (5,6). VSV-G pseudotyped HIV-1-based vectors transduce broader cell types owing to ubiquitous expression of VSV-G receptors, low-density lipoprotein receptors, in contrast to the restricted number of CD4⁺ cells that HIV-1 infects. A further advantage was that VSV-G pseudotyped vectors could be produced at high titres due to their improved stability over endogenous HIV-1 Env proteins during vector concentration (5).

Further improving upon the safety of VSV-G pseudotyped HIV-1-based vectors, Naldini *et al.* (1996) introduced a three-plasmid system for the manufacturing of replication incompetent lentiviral vectors, referred to as first generation lentiviral vectors (7). The VSV-G envelope protein, transgene and all remaining viral proteins were encoded on three separate plasmids, known as the envelope, transfer and packaging plasmids, respectively (Figure 1.1C) (7). All HIV-1 proteins (Figure 1.1 A-B) are supplied in trans expressed only in producer cells, while the transgene expression cassette and cis-elements (rev-responsive element (RRE) essential for nuclear export of transcripts, and genome packaging signal ψ) flanked by HIV-1 long terminal repeats (LTRs) are integrated into the host-genome (2). The progression towards eliminating all genes encoding viral proteins from the vector genome and instead supplying viral proteins in trans can be seen in Figure 1.1 C-E with sequential generations of lentiviral vectors (2).

Second generation vectors removed accessory genes *vif*, *vpr*, *nef* and *vpu* which are important for preventing anti-viral responses in host cells but are not essential for vector production (Figure 1.1D) (8). Third generation vectors include the three previously used plasmids with *rev* separated to a fourth plasmid (Figure 1.1E). The gene which encodes Tat and its corresponding transactivation-response element (TAR) in the 5'LTR U3 domain were deleted, with expression of the vector genome instead driven by a strong promoter such as the cytomegalovirus (CMV) promoter (2). Further deletion of enhancer and promoter sequences within the 3'LTR U3 region ensures that neighbouring genes upon integration of pro-viral DNA do not become activated, hence third generation vectors are referred to as self-inactivating (SIN) (9). Although predominantly based upon HIV-1, vectors have been developed from other species, including simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV) and equine infectious anaemia virus (EIAV) (7,10–12).

To date, lentiviral vectors have been investigated in over 45 clinical trials for monogenetic diseases, demonstrating great success in *ex vivo* applications for diseases such as primary immunodeficiencies, metabolic diseases and haemoglobinopathies (13). VSV-G pseudotyped lentiviral vectors are highly efficient at transducing haematopoietic stem cells (HSC), highlighting advantages over previously used γ -retroviral vectors. Children suffering from

the fatal disease, X-linked adrenoleukodystrophy (ALD) were amongst the first to receive HSCs modified with lentiviral vectors (14). Remarkably, disease progression has been prevented in these patients for over 10 years. Further example diseases where lentiviral-modified HSCs have been delivered to patients, include β -thalassemia (15), metachromatic leukodystrophy (16) and Wiskott-Aldrich Syndrome (WAS) (17). Of note, WAS patients treated with modified HSCs demonstrated long-term (median follow-up 7.6 years after infusion in 8 patients) immune reconstitution and reduction in bleeding events without treatment-related adverse events (18). Investigating the *in vivo* application of lentiviral vectors, a phase I study was conducted delivering EIAV vectors to patients suffering from age-related macular degeneration (19). Sub-retinal injections of lentiviral vectors carrying dual transgenes, *endostatin* and *angiostatin*, were well tolerated and demonstrated stable and prolonged expression in the long-term follow up of patients (last observation at 4.5 years post-treatment) (19).

In contrast to γ -retroviral vectors, no adverse events related to insertional mutagenesis have been observed in patients receiving lentiviral gene therapy (20). Follow-up of a clinical trial carried out in 2000 investigating the use of γ -retroviral vectors for the *ex vivo* treatment of X-linked severe combined immunodeficiency disease (X-SCID) revealed the first evidence of genotoxicity in humans (21). Integration of the γ -retroviral vector in proximity to multiple proto-oncogenes, including *LIM domain-only 2* (*LMO2*), caused their activation and resulting leukaemia in 4/10 of treated patients (22,23). γ -retroviral vectors have the propensity to integrate near transcriptional start sites. Consequently the strong enhancer activity conferred by the intact LTR sequences included in early γ -retroviral trials, caused transcriptional activation of oncogenes and downstream T-cell malignancy (24).

The preferred integration site of lentiviral vectors is similarly within actively expressed genes, however in contrast to γ -retroviral vectors, integration is observed anywhere within the transcriptional unit without preferential integration upstream of the transcription start site (25). Analysis of integration sites following infusion of HSC therapies transduced with lentiviral vectors is now performed routinely in the follow-up to clinical trials, thus far without clonal expansion of integration sites near proto-oncogenes being identified (26–28). With the aim to improve the safety of integrating vectors, Miyoshi *et al.* (1998) generated

novel lentiviral vectors with transcriptionally inactive LTR sequences, termed self-inactivating (SIN) vectors (29). Replacement of the 5'LTR U3 region with an internal promoter to drive transgene expression and a 133-bp deletion in the 3'LTR U3 domain (Figure 1.1E) eliminated the enhancer activity of LTR sequences within integrated SIN lentiviral vectors, improving their safety. However, the inclusion of internal promoters within SIN vectors still have the potential to elicit genotoxic effects, thus the selection of promoters with limited capacity to activate neighbouring sequences is required, the elongation factor 1 α (EF1 α) promoter being one example (30).

An additional area where lentiviral vectors are used widely is cancer immunotherapy for the development of chimeric antigen receptor (CAR) T cell therapies. The principle of CAR T cell therapies involves the *ex vivo* modification of patient's own (autologous) CD4⁺/CD8⁺ T cells or more recently donor (allogenic) T cells to recognise specific cancer antigens and elicit a T cell response against cancer cells upon re-infusion into patients (31). Most commonly, lentiviral vectors are employed in the manufacturing of CAR T cells due to their capacity to efficiently transduce and integrate into the genome of mature T cells (32). Since the original conception of engineered CAR T cells, the design of CARs has progressed significantly. First generation structures included an extracellular single-chain variable fragment of an antibody specific to the tumour-specific antigen, a transmembrane domain and cluster of differentiation 3 ζ - chain (CD3 ζ) intracellular signalling domain for T cell activation (33). Further iterations of CARs (second to fourth generation) incorporated various co-stimulatory domains, such as CD28, as well as other functions to significantly enhance anti-tumour activity and T-cell proliferation upon tumour-specific antigen recognition *in vivo* (34).

The majority of clinical studies investigating CAR T cell therapies have been targeted towards haematological cancers, largely due to the enhanced accessibility of surface tumour-specific antigens (35). Specifically, many of these therapies target CD19⁺ B cell malignancies with three CD19 T cell therapies engineered by lentiviral vectors that are licenced in the UK, Kymriah (Novartis), Yescarta and Tecartus (Kite Pharma/Gilead Sciences) (36). Despite encouraging efficacy against B cell malignancies, significant challenges are faced by CAR T cell therapies, particularly in their application towards solid tumours where

CAR T cell infiltration can be limited (37). Hyper-activation of T cells and subsequent excessive secretion of cytokines following infusion of engineered T cells, known as cytokine release syndrome, is a potential fatal outcome that can occur in recipient patients (37). Strategies to remove engineered T cell populations in the event of toxicity as well as cell trafficking approaches to improve tumour infiltration will continue to improve the safety and efficacy of CAR T cell therapies in the future (35).

Despite great clinical progression *ex vivo*, the *in vivo* application of lentiviral vectors has been much slower, with only a handful of clinical trials investigating intracerebral and subretinal delivery for diseases such as macular degeneration and Parkinson's disease (19,38,39). The transduction of terminally differentiated cells by lentiviral vectors may in fact be safer than actively dividing HSC, with a greater frequency of integrations into non-protein coding regions observed in post-mitotic cells (40). However, barriers faced by the field of somatic *in vivo* gene therapy, largely efficacy and vector production based, have required significant innovations over the last 20 years to reach the promising stage it is now. Particularly, the advancements of lentiviral vectors as suitable candidates for *in vivo* lung delivery can be exemplified by pulmonary gene therapy for cystic fibrosis (CF).

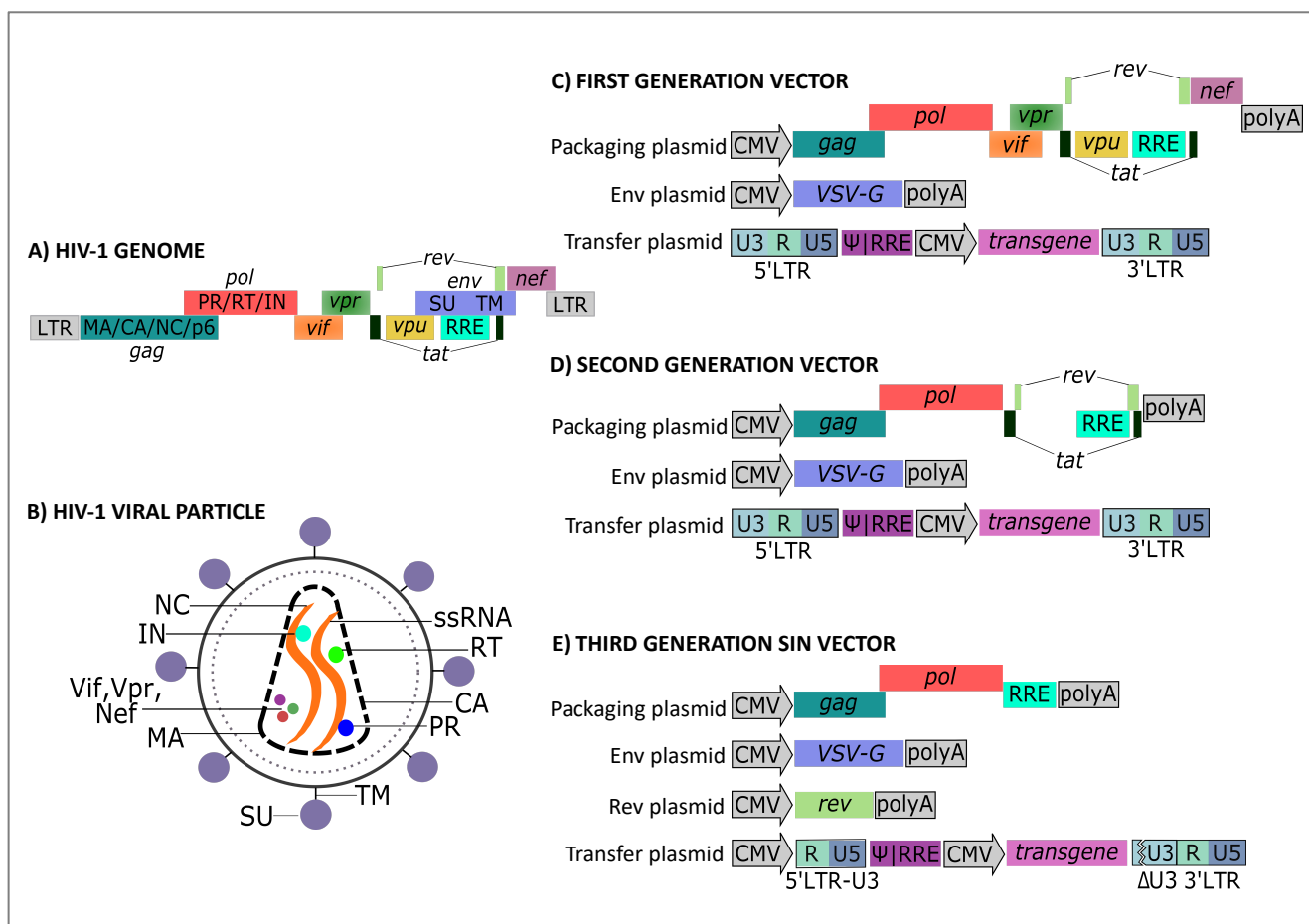


Figure 1.1- Schematic of the HIV-1 genome, viral particle structure and generations of lentiviral vector genomes derived from HIV-1.

A+B) The HIV-1 genome within flanking long terminal repeats (LTRs) contains structural gene, *gag* which encodes matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins, *pol* which encodes replication enzymes protease (PR), reverse transcriptase (RT) and integrase (IN), and *env* which encodes the precursor gp160 which is cleaved by PR into surface (SU) protein gp120 and transmembrane (TM) protein gp41. Accessory proteins are encoded by genes *vpr*, *vif*, *vpu* and *nef*. C) First generation lentiviral vectors where the genome is split across three plasmids including a packaging plasmid, envelope plasmid and transfer plasmid encoding the transgene. HIV-1 Env protein is substituted for the VSV-G envelope glycoprotein. D) Second generation vectors removed accessory genes *vif*, *vpr*, *nef* and *vpu*. E) Third generation self-inactivating (SIN) vectors due to deletion of the *tat* gene and the 5' LTR U3 region. The *rev* gene is separated to a fourth plasmid. Replicated from Sakuma et al. (2012).

1.2 Gene therapy for respiratory diseases

The lungs were originally conceived as a relatively simple organ for gene therapy as gene transfer agents (GTAs) could be delivered to the lungs non-invasively through inhalation, bronchoscopy or through the blood stream. However, success in the clinic thus far has been limited due to significant challenges associated with delivery of GTAs to the lungs (41). Alpha-1-antitrypsin deficiency (AATD) caused by mutations within the *Serpin Family A Member 1* (*SERPINA1*) gene is one of the disease indications where a pulmonary gene therapy approach has been investigated (42). Mutations within *SERPINA1* can lead to deficient levels of AAT, a serine protease inhibitor that protects the elastin fibres of the lungs from degradation by neutrophil elastase (43). In patients with AATD, reduced AAT levels fail to protect the elastin fibres of the lungs from degradation, resulting in chronic obstructive pulmonary disease (COPD) and early onset emphysema (43). In some patients, gain-of-function mutations cause the accumulation of AAT aggregates in the liver, which can lead to liver cirrhosis and ultimately liver failure (43).

Regular infusions of plasma-purified AAT has been shown to slow the progression of emphysema in patients by raising AAT levels within the circulation (44). However high treatment cost associated with regular infusions and dependency on donor plasma has encouraged the investigation of gene therapies aiming to reconstitute AAT expression. A phase II clinical trial investigating intramuscular injection of adeno-associated virus (AAV) vectors encoding AAT reported stable serum levels in AATD patients 5 year after a single injection (45,46). In an alternative approach, lung targeted expression of AAT was investigated following intrapleural injection of AAV vectors. Initial studies demonstrated long-term lung and serum AAT expression in mice whilst persistent expression in non-human primates generated supportive data for the initiation of a phase I/II clinical trial (47–50).

Another disease where pulmonary gene therapy has been investigated is primary ciliary dyskinesia (PCD). As opposed to AATD, PCD has primary lung pathology whereby heterogenous mutations within multiple gene leads to dysfunctional ciliary kinetics and consequently impaired mucociliary clearance, causing chronic lung disease (51). Lentiviral

vectors pseudotyped with avian influenza hemagglutinin and encoding the mouse ortholog of *DNAI1* (mutated in 10% PCD patients) were delivered to mice by nasal inhalation. Low level and transient transgene expression was observed in the PCD animal model compared to wildtype mice, with gene transfer severely limited by mucus accumulation, characteristic of the mouse model (51). More recently, a gene editing approach to correct a non-sense mutation within the *DNAH11* gene (alternative gene mutated in PCD patients) showed proof-of-concept in primary cells derived from two PCD patients (52).

Although pulmonary gene transfer approaches for the treatment of rare congenital diseases such as AATD and PCD have been explored in a handful of studies, by far the most frequently investigated disease in the field of lung gene therapy is CF. At first, CF was believed to be an ideal disease for gene therapy; it is a life-limiting monogenic disorder where lung manifestations are poorly managed by current treatments. Four years after the cloning of the *CF transmembrane conductance regulator (CFTR)* gene in 1989, the first clinical trial was conducted in CF patients (53). The early trials used adenoviral vectors (AdV), which have a natural tropism for the lungs and a large packaging capacity, easily managing the 4.7kb *CFTR* coding sequence compared to smaller adeno-associated virus (AAV). In the following years, AdV clinical trials established proof-of-principle for *CFTR* gene transfer into human airways (54). However, transfer efficiency was generally low and expression transient, lasting only a few days to several weeks following induction of immune responses without possibility of repeat administration (55).

Non-viral GTAs, consisting of plasmid DNA complexed to materials such as cationic lipids, peptides, and synthetic polymers are generally less efficient than viral vectors. However non-viral vectors are considered safer, cheaper to manufacture and are less immunogenic, raising the opportunity for repeat administration (56). In 1999, delivery of *CFTR* plasmid DNA complexed with cationic liposome, GL67A, was first administered to the lungs of CF patients (57). Electrophysiological improvements were observed in the active group however transient pulmonary inflammation was observed in 7/8 of patients receiving the lipid-DNA complexes (57). Plasmid DNA from bacterial origin contains a high frequency of unmethylated CpG dinucleotides which have immunostimulatory effects through recognition by Toll-like receptor 9 (TLR9) (58). Inclusion of mammalian promoters and

depletion of CpG dinucleotides through codon optimisation is a common strategy employed in the design of non-viral GTAs to reduce immune recognition and consequently extend transgene expression (59–61). As part of a translational programme established by the UK Respiratory Gene Therapy Consortium, Hyde *et al.* (2008) systematically removed all CpG nucleotides from the CFTR plasmid, extending duration of gene expression in mice (62). In 2015 the UK Respiratory Gene Therapy Consortium completed the first multi-dose, phase IIb CF gene therapy clinical trial (63). Nebulisation of GL67A-pCFTR was well tolerated and led to a stabilisation in lung function of CF patients, providing proof-of-concept that non-viral gene therapy could at least slow disease progression in CF patients. To improve upon moderate treatment effect, continued development of non-viral GTAs is underway as well as research into viral vectors which demonstrate considerably higher efficacy in animal models.

Lentiviral vectors were investigated for CF pulmonary gene therapy since HIV-1 based vectors were pseudotyped with VSV-G. Goldman *et al.* (1997) established that transduction of CF patient bronchial xenografts with VSV-G pseudotyped lentiviral vectors led to CFTR expression and functional correction of electrochemical properties (64). Despite early optimism, significant hurdles have been faced by researchers to achieve efficient lung transduction and translation into CF clinical studies.

1.3 Challenges of *in vivo* pulmonary gene therapy

As part of the natural lung physiology there is mucus lining the conducting airways, referring to the trachea, bronchi and bronchioles, which sits atop a thin electrolyte layer on the bronchial surface called the periciliary layer (65). The bronchial surface is populated by ciliated columnar cells, known as the airway epithelium, whose coordinated beating contributes to the movement of mucus up the airways to the pharynx to be swallowed. This mechanism of ‘mucociliary clearance’ is essential to remove any inhaled pathogens or harmful substances that have entered the airways (66). GTAs can also become trapped in the mucus through electrostatic forces and subsequently be cleared by mucociliary transport or cough (67). Use of viscoelastic gels which decrease cilia movement, such as carboxymethylcellulose and methylcellulose, was investigated by Sinn *et al.* (2005) as an

adjunct for delivery of Ad5, AAV-5 and FIV vectors. For each vector, formulation with viscoelastic gels significantly increased airway gene transfer in mice. The suggested mechanism of action was increased efficiency of transduction due to greater residency of vectors within the airway epithelium before clearance (68).

Entry into the desired cell type raises another challenge for pulmonary gene therapy. Airway epithelial cells are the major target for PCD and CF, however the polarised nature of the epithelium mean that only receptors present on the apical membrane are usually accessible to GTAs via the airway lumen (41). VSV-G pseudotyped lentiviral vectors were able to transduce undifferentiated cells harvested from CF bronchial xenografts, however vectors failed to transduce fully differentiated pseudostratified epithelium (64). Johnson *et al.* (2000) further identified that transgene expression was 30x greater when VSV-G pseudotyped vectors were applied to differentiated airway epithelial cells via the basolateral surface rather than apically (69). Preference for basolateral infection is due to the presence of their receptors, low-density lipoprotein receptors, on the basal surface of airway epithelial cells (70,71). Similarly receptors for the AdV fibers, coxsackie and adenovirus receptor and major histocompatibility complex (MHC)-1 receptors, are located on the basolateral surface of airway epithelia (72).

To overcome this limitation, multiple strategies have been developed to disrupt the tight junctions between epithelial cells including the use of Ca^{2+} chelator (ethylene glycol tetraacetic acid) EGTA (72), surfactant and perfluorochemical vehicles (73), and lysophosphatidylcholine (LPC) (74). Pre-treatment with agents capable of disrupting tight junctions allows access of VSV-G pseudotyped lentiviral vectors and AdV vectors to receptors on the basolateral membrane, increasing gene transfer efficiency dramatically (69,75). However, there are concerns regarding the translation of these pre-treatments into clinical applications where opening of tight junctions may create an opportunistic environment for bacterial infection, of particular risk for diseases with chronic infection such as PCD and CF (76).

1.4 Wildtype Sendai virus

To identify more suitable gene transfer agent for the airways, researchers have looked to viruses that naturally infect the respiratory system, both in humans and across different species. One example is the paramyxoviridae family of viruses, such as the negative-strand RNA Sendai virus, which demonstrates exclusive pulmonary tropism and pneumonia-like pathology in mice (77). The Sendai virus envelope protein, hemagglutinin-neuraminidase (HN), is responsible for binding of virus to cellular receptors, while the fusion protein (F) is required for fusion of the viral envelope with the cell membrane (78). Within the F protein is a single arginine residue that during the infection cycle is cleaved by an arginine-specific protease, trypsinase Clara, produced in limited cell types including Clara cells in the lungs (79). In order for activation of the F protein to occur, the precursor F₀ protein is cleaved into F₁ and F₂ subunits, imperative for the production of infectious virus progeny (80). Interestingly, the receptors used for binding of Sendai virus to host cells, specific sialic acid containing gangliosides, are widely distributed across many cell types and organs (81–83). Thus the tropism of the wildtype virus is not solely determined by receptor expression but rather is dictated by the presence of the specific proteases that activate the F envelope protein (84). Tashiro *et al.* (1990) demonstrated the presence of a lung-specific activator by characterising a Sendai virus F protein mutant, F1-R, which had mutations near the cleavage site predicted to enhance susceptibility to ubiquitous proteases (85). Organ blocks infected with wildtype Sendai virus produced activated and infectious viral progeny only in lung cultures, whereas F1-R infection produced activated progeny in multiple organs, due to ubiquitous proteases (85).

Tashiro and Homma (1983) established that wildtype Sendai virus activated by trypsin *in vitro* and delivered intranasally to mice exhibited multiple replication cycles, yet uncleaved virus delivered intranasally was not converted to the active form. Their hypothesis was that the F protein protease that activates viral progeny is absent from the airway lumen and instead exists within the respiratory epithelium, thus non-activated virions cannot access the protease (84). However it was later shown by Tashiro *et al.* (1992) that activation of Sendai virus occurred partly extracellularly in the lumen of the respiratory tract, with secreted trypsinase Clara identified in bronchoalveolar lavage fluid (86).

1.5 Recombinant Sendai virus vectors

Sendai virus was first engineered into recombinant vectors carrying an exogenous cDNA sequence, firefly luciferase, by Hasan *et al.* (1997). These first generation, replication-competent vectors demonstrated proof-of-concept that a foreign gene could be inserted into the Sendai virus genome and efficiently expressed *in vitro* (87). Given the known tropism of wildtype Sendai virus for murine airways and the abundant expression of sialic acid receptors on airway epithelial cells, the recombinant vector was investigated as a pulmonary gene transfer agent in mice. Yonemitsu *et al.* (2000) demonstrated highly efficient luciferase expression in the lungs of mice and additionally in ferrets delivered Sendai vectors carrying β -galactosidase, where as high as 70-80% of epithelial cells across the conducting airways were transduced (88).

To explore the capacity of Sendai vectors to deliver a therapeutic transgene to the airways, the 4.7kb *CFTR* cDNA sequence was packaged and delivered topically to CF knockout mice (89). Although the *CFTR* cDNA was successfully packaged and a partial correction in chloride channel function observed 7 days post-transduction, functional improvements were lost by 14 days post-transduction due to inflammatory responses in the lungs (89). The use of replication-defective 'second-generation' vectors and Sendai vectors carrying temperature sensitive mutations within the viral genome were explored in an attempt to reduce the immunogenicity of vectors (90). However transient gene expression and reduction in transduction efficiency following repeat administration caused by generation of neutralising anti-vector antibodies, limited their application as therapeutics for chronic diseases and has since encouraged their investigation as a vector for vaccination (91)

1.6 Pseudotyping lentiviral vectors with alternative envelope proteins

An alternative approach in search of a pulmonary GTA is to pseudotype lentiviral vectors with envelope proteins from a range of lung pathogens, given their efficiency at transducing the airway epithelium via the apical surface. These include lentiviral vectors pseudotyped with baculovirus, filovirus, influenza and parainfluenza envelope proteins (92–95). Given the ubiquitous expression of Sendai virus receptors across many tissues, however considering the poor expression profile of recombinant Sendai vector, Kobayashi *et al.* (2003) attempted

to pseudotype a lentiviral vector with the Sendai virus glycoproteins, F and HN. Specifically, the lentiviral vector selected was the simian immunodeficiency virus (SIV) of the African green monkey (SIVagm). Kobayashi *et al.* (2003) successfully incorporated the Sendai virus envelope proteins into lentiviral vectors by fusing the wildtype Sendai virus HN protein with the cytoplasmic tail from the SIVagm envelope protein, referred to as SIVct+HN (Figure 1.2A). In contrast, the F protein was incorporated more efficiently following truncation of its endogenous cytoplasmic tail to 4 nucleotides, termed Fct4 (Figure 1.2B) (94). Using a polarised epithelial culture derived from primary rat tracheal epithelial cells, transduction efficiency of the novel pseudotyped vectors was assessed. SIVct+HN Fct4 pseudotyped SIV vectors (abbreviated to rSIV.F/HN, Figure 1.2C) achieved successful transduction of the apical and basolateral surface. In contrast, VSV-G pseudotyped SIV vectors only resulted in basolateral transduction, although at much higher efficiency than the F/HN pseudotype (94). The production of a novel lentiviral pseudotype that was able to efficiently transduce airway epithelial cells from the apical surface was an important discovery towards developing a pulmonary GTA.

1.7 Investigating rSIV.F/HN-mediated gene transfer

The UK Respiratory Gene Therapy Consortium investigated the use of rSIV.F/HN vectors for cystic fibrosis and later for broader disease applications. First the production of the pseudotyped vectors was optimised to enhance viral titre. The incorporation of the central polypurine tract (cPPT) and the Woodchuck hepatitis virus posttranscriptional regulatory elements (WPRE) increased titre from 1e5 transduction units (TU)/ml to 5e7 TU/ml. In addition, inclusion of cPPT and WPRE within the vector genome enhanced the proportion of infectious particles from 1/100 to 1/20 vector particles (96). Proof-of-concept was established in the nasal epithelium of mice, demonstrating that rSIV.F/HN vectors can efficiently transduce multiple cell types *in vivo*, with the majority of positive cells being ciliated respiratory epithelial cells. Expression was persistent for the study length (12 months) and repeat administration was feasible (96). Importantly, expression levels of a firefly luciferase reporter gene was ~500 fold higher following vector delivery than the best-in-class non-viral formulation at that time, lipid GL67A, which the consortium progressed to

a Phase IIb clinical trial for CF patients (97). Expression was also demonstrated in differentiated human airway epithelial cells grown as air-liquid interface (ALI) cultures (96).

Following the generation of positive data, the consortium began a translational programme to develop rSIV.F/HN towards the clinic and human trials. This included the assessment of rSIV.F/HN toxicity over the lifetime of mice and the evaluation of lung transduction efficiency, including maintained transgene expression following monthly repeat administrations (98). In more relevant models, human/sheep *ex vivo* precision-cut lung slices, human nasal brushings and ALI cultures all demonstrated persistent gene expression. In the first application, rSIV.F/HN was investigated for the treatment of cystic fibrosis and has progressed the furthest towards this indication with a partnership between Boehringer Ingelheim and the UK Respiratory Gene Therapy Consortium announced to develop a first-in-class gene therapy product for CF. A first-in-man phase 1 clinical trial investigating the delivery of rSIV.F/HN-CFTR vectors to CF patients is expected to begin in the coming years to first assess safety of the gene therapy product.

1.8 Broadening the application of rSIV.F/HN

Beyond using the rSIV.F/HN vector for the expression of membrane bound proteins, such as CFTR, the generation of secreted proteins in the lungs has been investigated. As a potential therapy for AATD, Paul-Smith *et al.* (2018) demonstrated long-term expression of therapeutic levels of AAT in the lungs of mice following a single dose of rSIV.F/HN (99). Alternatively rSIV.F/HN was investigated for the treatment of rare lung disorder, pulmonary alveolar proteinosis (PAP) characterised by the alveolar build-up of surfactant caused by acquired or genetic deficiency in cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) signalling in the lungs (100). Intranasal delivery of vectors generated significant expression of GM-CSF in the lungs, leading to a correction in disease phenotype of a mouse model of PAP (Lund-Palau, Molecular Therapy-manuscript under review).

Broadening the application of lung gene transfer further, a strategy to use the respiratory epithelium as a factory for secretion of proteins to treat systemic diseases was explored by Griesenbach *et al.* (2002). Proof-of-concept that the lungs could act as an efficient factory

for the production and secretion of interleukin 10 into the circulation was demonstrated using recombinant Sendai vectors (101). With the superior expression profile and reduced immunogenicity of rSIV.F/HN vectors, Paul-Smith *et al.* (2018) investigated using the murine lungs as factories for systemic production of alpha-1-antitrypsin for the treatment of AATD and clotting factor, Factor VIII (FVIII) towards the treatment of haemophilia B. Haemophilia B was selected as a prototype disease to investigate lung-targeted gene transfer for a systemic disorder. Stable expression of a reporter protein and both therapeutic proteins were measured in the blood for extended periods (19 months for alpha-1-antitrypsin), with FVIII expression (~1% normal plasma levels) approaching therapeutic levels required by patients (99).

Utilising the lungs as a factory for the production of systemic proteins may present a more efficient and less immunogenic route of administration compared to intravenous delivery (102). The vast majority of clinical trials that assess gene therapy treatments for systemic congenital disorders use intravenous administration. This includes the prominent example of systemic AAV gene therapy for haemophilia A and B which has demonstrated significant amelioration of bleeding phenotype in clinical trials thus far (103). The maintained expression of FXI in haemophilia B patients for >10 years following a single AAV dose has been reported (104). However the sustained expression of therapeutic levels of FIX is unlikely to persist for the lifetime of the patient and thus a second or third dose of gene therapy will be required, which is not currently feasible for AAV vectors (105). On the other hand rSIV.F/HN can be repeatedly administered to mice without loss of transduction efficiency (98). Thus for the treatment of chronic disorders, intranasal delivery of rSIV.F/HN may represent an attractive alternative to systemic delivery of AAV vectors.

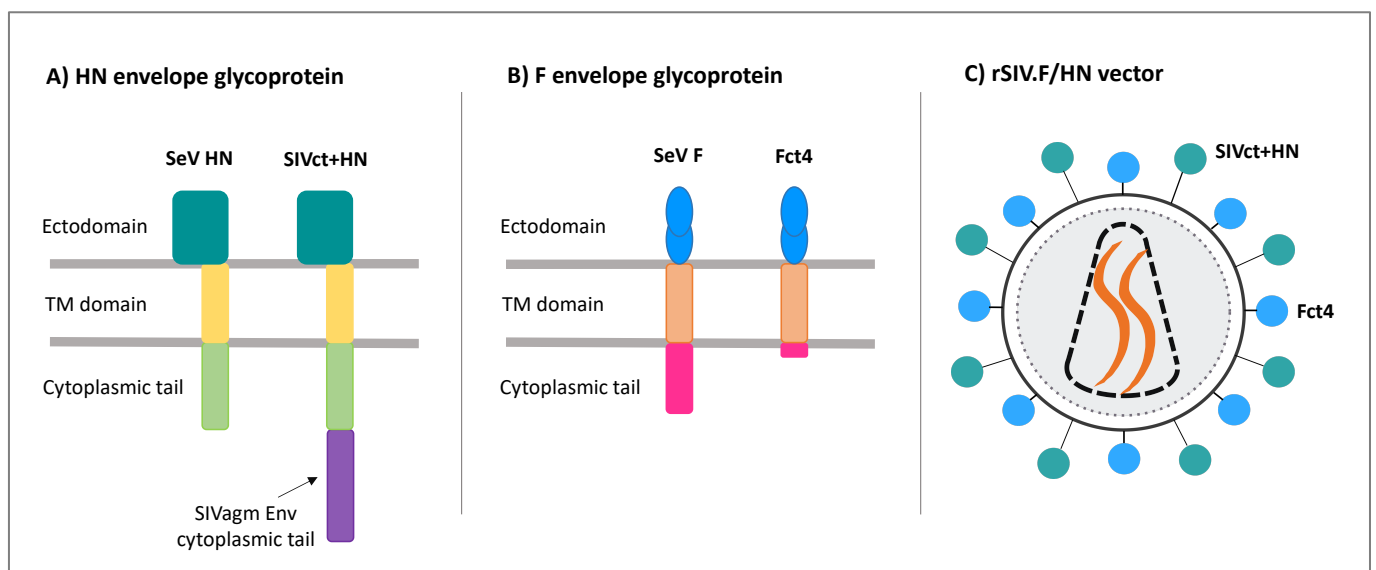


Figure 1.2- The incorporation of Sendai virus envelope proteins into simian immunodeficiency virus to generate pseudotyped lentiviral vectors.

In the pursuit of an efficient pulmonary gene transfer agent, the African green monkey simian immunodeficiency virus (SIVagm) was pseudotyped with envelope glycoproteins F and HN from Sendai virus (SeV). A) The Sendai virus envelope protein was incorporated into lentiviral vectors by fusing the wildtype SeV HN protein with the cytoplasmic tail from the SIVagm envelope protein (Env), referred to as SIVct+HN. B) The F protein was incorporated following truncation of the SeV F cytoplasmic tail to 4 nucleotides, termed Fct4. C) Recombinant lentiviral vectors were generated with SIVct+HN and Fct4 envelope proteins incorporated, termed rSIV.F/HN.

1.9 Project aims & objectives

Previously it was shown that rSIV.F/HN achieves efficient pulmonary transduction and the murine lungs can act as factories for the generation of secreted protein. The overall project aim is to develop the rSIV.F/HN vector platform for wider indications including the treatment of systemic disorders and diseases that require regulated transgene expression.

As expected for the development of a lung GTA, all previous studies investigating rSIV.F/HN had targeted the nasal or pulmonary epithelium. However intravenous vector administration for the delivery of systemic proteins has not yet been investigated, which may hold promise for broader diseases. The first specific aim is to investigate the intravenous delivery of rSIV.F/HN and compare against intranasal vector administration. The hypothesis is that gene transfer by systemic delivery of rSIV.F/HN is feasible, leading to sustained gene expression in the circulation. To investigate this, duration of transgene expression and vector biodistribution will be compared following intravenous and intranasal vector delivery. Furthermore, stability of transgene expression following repeat intravenous administration will be assessed (Chapter 3).

The second aim is to investigate rSIV.F/HN gene transfer for the treatment of a systemic disorder. An exemplar disease was selected, haematological disorder thrombotic thrombocytopenic purpura (TTP), due to the unmet clinical need of current treatments and the novelty of an *in vivo* lentiviral gene therapy approach for TTP. The hypothesis is that rSIV.F/HN-mediated transduction will restore gene expression in a TTP mouse model. Vector will be delivered to knockout mice by intranasal delivery, targeting the lungs as factories for production of secreted protein, and by intravenous administration. It will be attempted to restore gene expression in a mouse model of TTP, and if successful investigate the protection against the development of disease symptoms (Chapter 4).

Finally, temporal control of gene expression could be beneficial in order to improve the safety of lentiviral gene therapy and broaden its application towards diseases that require controlled gene expression. Thus, the final aim is to generate a lentiviral vector that can be turned on upon administration of a drug and turned off upon withdrawal of the drug. The

hypothesis is that lentiviral-mediated transgene expression can be regulated following administration of an inducing drug. To achieve this, lentiviral vectors carrying various designs of expression cassettes will be constructed and the induction of gene expression will be assessed in various models (Chapter 5).

All three aims contribute towards the development of the rSIV.F/HN technology platform to broaden its applications beyond the original lung-related pathologies.

Chapter 2- Common Materials and Methods

This chapter will describe the core experimental techniques that are used frequently across the whole project to avoid repetition within each individual chapter. Alterations to the general protocol and specific experimental techniques not covered in this chapter will be given in the chapter specific Materials and Methods.

2.1 Molecular Biology Techniques

2.1.1 Polymerase chain reaction

Polymerase chain reaction (PCR) reactions for the subsequent construction of novel plasmids were performed using the Hot-Start Q5 High-Fidelity DNA Polymerase (NEB, Massachusetts, US) following supplier's instructions. Briefly, 50 μ l PCR reactions consisted of 1X Q5 reaction buffer, 200 μ M deoxyribonucleotide triphosphate (dNTPs), 0.5 μ M forward and reverse primers, 20 ng of plasmid template DNA and 0.02 U/ μ l Q5 High-Fidelity DNA Polymerase. Thermocycling conditions were as follows: 94°C for 5 minutes, 30 cycles of 94°C for 45 seconds, annealing temperature (Table 2.1) for 30 seconds and 72°C for the elongation duration (Table 2.1) followed by 10 minutes at 72°C. Annealing temperatures were calculated using the NEB Tm calculator. For difficult to amplify templates, the Q5 High-Fidelity DNA Polymerase with inclusion of 1X Q5 High GC enhancer (NEB, Massachusetts, US) was used in PCR reactions. Further optimisations included reducing template concentration to 2 ng/reaction and annealing temperatures 5°C lower than the calculated annealing temperature. Alternatively, PCR reactions for the genotyping of mice were conducted using OneTaq DNA Polymerase (NEB, Massachusetts, US) following supplier's instructions.

Table 2.1- Polymerase chain reaction (PCR) conditions and primer/oligo sequences for cloning and sequencing reactions.

Oligos with complementary sequences to the parent plasmid are shown in capital letters whilst novel sequences added to oligos are shown in lower case letters. PCR reactions for the for the construction of novel plasmids were performed using the Hot-Start Q5 High-Fidelity DNA Polymerase (NEB, Massachusetts, US). PCR reactions for the genotyping of mice were conducted using OneTaq DNA Polymerase (NEB, Massachusetts, US).

PCR	Annealing Temperature/ Elongation Time	Primer/oligo	Sequence 5'-3'
1. pIC056-hCEF-mADAMTS13 sequencing	Outsourced (Genewiz, Takeley, UK)	oIC001	GGGAAAGTGATGTGGTGTACTG
		oIC004	AAGCCATACGGGAAGCAATAG
2. pIC040-hCEF-mADAMTS13+Kozak Kozak extension	66°C /5 minutes	oIC049	AGGACCTGGAACCTAAGGA
		oIC050	CAAACCTCACAGGAGGGAGAAG
	N/A	Kozak_insert	CTTCTCCCTCCTGTGAGTTTGCTAGCCCA CCATGAGCCAGCTGTGTCTGTGGCTGAC CTGCCAGCCTTGTTACGCCGTGTCTGTCA GAGGCATCCTGACCGGCGCTATCTTCAT CCTCGGATGTTGGGGCCTGAGCGACTTC CAGAAGTCCCTGCTGCAGGACCTGGAAC CTAAGGAC
3. pIC040-hCEF-mADAMTS13+Kozak sequencing	Genewiz	oIC001	GGGAAAGTGATGTGGTGTACTG
		oIC004	AAGCCATACGGGAAGCAATAG
		oIC014	CAAACCTCACAGGAGGGAGAAG
		oIC068	AAGGCATCTGGCCACGTGATG
		oIC069	CAGGATGATGGCACACTGTCT
		oIC070	CATGTCTAGCGCTTGTGAAGC
4. pIC083-hCEF-hADAMTS13 construction	67°C /3 minutes	oIC121	cttctccctcctgtgagtttGCTAGCCACCATGCACCAGCGTCAC
		oIC122	cagaggttgattgcggccgcTCAGGTTCTTCCTTCCCTTCCAGG
	72°C/3 minutes	oIC123	GCGGCCGCAATCAACCTCTGGATTA
		oIC124	AAACTCACAGGAGGGAGAAGGCAGAAG

5. pIC083-hCEF-hADAMTS13 sequencing	Genewiz	oIC001	GGGAAAGTGATGTGGTGTACTG
		oIC004	AAGCCATACGGGAAGCAATAG
		oIC050	CAAACCTCACAGGAGGGAGAAG
		oIC125	TACAGCGCCAACGAGCAGTG
		oIC126	TCGTGTCGAGTACAGAGTGG
		oIC127	TACAAGCTGGCGGCCTGCAG
6. Genotyping transgenic mice	59°C /15 seconds	wildtype_for	AGCCCCAACTCTTGTCTTTAAT
		mut_for	TGGTTCTAAGTACTGTGGTTTCC
		com_rev	GAGTTGCTAGGTTATCAGGAAG
7. GS-1V-GLux construction	67°C/1 minute	3AGLP65For	CttctccctcctgtgagtttGCTAGCAGTAATACTAACGGTTC
		3AGLP65Rev	CTTAGGAGCTGATCTGACTCAG
	65°C/30 seconds	3APolyAFor	gagtcagatcagctcctaagGATCCGATCAGCCTCGACTGTG
		3APolyARev	gcaccttcactcccatggtgGCTAGCAAACCTCAACGTTACAGG
	N/A	PolyA-GAL4 TATA	GGATCCGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCT GTTGTTTGCCCCCTCCCCTGTGCCTTCCTTGACCCTGGAAGGTGCC ACTCCCACTGTCCTTTCTAATAAAATGAGGAAATTGCATTGCAT TGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGC AGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATG CCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATC CCCGGAGTACTGTCCTCCGAGTTTAAAAGCGGAGTACTGTCCTC CGAGGATATCAGCGGAGTACTGTCCTCCGAGTCGCGAAGCGGA GTACTGTCCTCCGAGGGTATATAATGAAGCTTCTGCCTTCTCCCT CCTGTAACGTTGAGTTTGCTAGC
8. GS-1V-GLux sequencing	Genewiz	oIC001	GGGAAAGTGATGTGGTGTACTG
		oIC020	GCAGATCAGGGCAAACAGCAC
9. GS-2V-GLux construction	59°C/1 minute	pGeneFor	ttaaatttagagccgcggaCCGAGCTCTTACGCGGGT
		pGeneRev	caccttcactcccatggtggGGGATCTATCATCGTTTCGATCTGC
10. GS-2V-pSwitch construction	61°C/2 minutes	pSwitchFor	ttaaatttagagccgcggaCGAGCTTGCATGCCTGCAGG
		pSwitchRev	atccagaggttgattgggccTCGAGGCTGATCAGCGGGTTTC

11. GS-2V-GLux sequencing	Genewiz	oIC019	GGGACAGACTTCAGCAGAGAG
		oIC004	AAGCCATACGGGAAGCAATAG
12. GS-2V-pSwitch sequencing	Genewiz	oIC019	GGGACAGACTTCAGCAGAGAG
		oIC004	AAGCCATACGGGAAGCAATAG
13. GS-1V-GLux RO construction	59°C/3 minutes	BBFor	CTTCTGAGGCGGAAAGAACC
		BBRev	CCGCGGCTCTAAAATTTAAAATTTTTG
	65°C/2 minutes	InsertFor	tttaaatttagagccgaggAGACCGCCTCAGAAGGCATG
		InsertRev	ggttcttccgcctcagaagGTTACATAACTTATGGTAAATGGCCTGC
14. GS-1V-GLux RO sequencing	Genewiz	oIC019	GGGACAGACTTCAGCAGAGAG
		oIC020	GCAGATCAGGGCAAACAGCAC
15. ZF-1V-GLux construction	67°C/2 minutes	1ZFFor	cttctccctctgtgagtttGCTAGCCACCATGGGCTATG
		1ZFRev	gcaccttcactcccatggtgGCTAGCAAACCTCAACGTTACAG
16. ZF-1V-GLux sequencing	Genewiz	oIC001	GGGAAAGTGATGTGGTGTACTG
		oIC020	GCAGATCAGGGCAAACAGCAC
17. AI ZF-1V-GLux construction	67°C/2 minutes	2ZFFor	tttaaatttagagccgaggAGATCTGCTTCGAATTCTGCAG
		1ZFRev	gcaccttcactcccatggtgGCTAGCAAACCTCAACGTTACAG
18. AI ZF-1V-GLux sequencing	Genewiz	oIC019	GGGACAGACTTCAGCAGAGAG
		oIC020	GCAGATCAGGGCAAACAGCAC

2.1.2 Restriction digestion

To isolate fragments of interest or to perform diagnostic restriction digests, 1 µg plasmid DNA was digested with restriction enzymes of choice in 50 µl reactions following supplier's instructions (NEB, Massachusetts, US).

2.1.3 Gel electrophoresis

Gel electrophoresis was performed to separate DNA fragments by size. Agarose powder (Sigma, Missouri, USA) was dissolved in Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific, Massachusetts, US) to a final concentration of 0.5% or 1% weight per volume (w/v) for the separation of DNA fragments less than 1 kilo base (kb) and greater than 1 kb respectively. Finally 1X SYBR Safe DNA Gel Stain (Invitrogen, Massachusetts, US) was added and gels cast. Samples were diluted in 1X gel loading dye (NEB, Massachusetts, US) and loaded alongside a 100 base pair (bp) DNA ladder (NEB, Massachusetts, US) for fragments less than 1 kb or alongside a 1 kb DNA ladder (NEB, Massachusetts, US) for fragments greater than 1 kb. Electrophoresis was performed in TAE buffer at 80 volts for 1 hour. Gels were visualised under ultra-violet (UV) light and images taken using a MultiDoc-It Multi-User Imaging System (UVP, California, US). DNA fragments of interest were isolated with a scalpel and purified using Monarch DNA Gel Extraction Kit (NEB, Massachusetts, US) following supplier's instructions.

2.1.4 Ligation and HiFi DNA assembly

Fragments isolated using restriction enzymes that produced 5' or 3' overhangs were ligated together using Instant Sticky-end Ligase (NEB, Massachusetts, US) following the supplier's protocol. Alternatively, for the ligation of blunt-end fragments or ligation of greater than 2 fragments, NEBuilder HiFi DNA Assembly (NEB, Massachusetts, US) was utilised. NEBuilder Assembly Tool was used to design primers for the amplification of each fragment and conditions for PCR reactions detailed in . The ligation protocol for HiFi reactions was followed as per manufacturer's instructions, ensuring the extended incubation of fragments at 50°C for 1 hour was utilised.

2.1.5 Bacterial transformation and plasmid purification

Transformations of plasmid DNA were performed using standard methodology. Briefly, 50 ng of plasmid DNA was added to 50 µl of NEB 5-alpha Competent *E.coli* cells (NEB, Massachusetts, US), unless otherwise stated, and incubated on ice for 30 minutes. Cells were heat-shocked for 30 seconds at 42°C followed by 5 minutes incubation on ice. 200 µl outgrowth media (NEB, Massachusetts, US) was added and incubated at 37°C for 1 hour with shaking at 225 rotations per minute (rpm). Transformations were spread onto Luria broth (LB) agar plates (Sigma, Missouri, US) supplemented with kanamycin (final concentration 50 µg/ml, Sigma, Missouri, US) or ampicillin (final concentration 100 µg/ml, Sigma, Missouri, US) and incubated at 37°C overnight. To prepare starter cultures, colonies were inoculated under standard conditions (37°C overnight with shaking at 225 rpm) in 5 ml LB broth supplemented with kanamycin/ampicillin. Larger cultures (100 ml/ 500 ml) were generated by transferring 1 ml of starter culture to fresh LB broth supplemented with kanamycin/ampicillin and culturing under standard conditions. Plasmids were purified from cultures using Monarch Plasmid Miniprep Kit (NEB, Massachusetts, US) and Qiagen Plasmid Maxi/Giga kits (Qiagen, Crawley, UK) following manufacturer's instructions.

2.2 Cell Culture Techniques

2.2.1 Cell culture

Immortal mammalian cell lines, human embryonic kidney 293 cells expressing the simian virus 40 large T antigen (HEK293T) and adenocarcinomic human alveolar basal epithelial (A549), were sourced from The European Collection of Authenticated Cell Cultures (UK Health Security Agency). Cell lines were reconstituted from cryopreserved vials into fetal bovine serum (FBS) (Thermo Fisher Scientific, Massachusetts, US) then cells pelleted by centrifugation at 500xg for 5 minutes. Cell pellets were resuspended and subsequently grown in complete media consisting of: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5000 U/ml penicillin and streptomycin, and 10% volume per volume (v/v) FBS, referred to as DMEM complete media. Cell lines were grown in T25 and T75 vessels (Thermo Fisher Scientific, Massachusetts, US) maintained under standard conditions (37°C and 5% CO₂) using aseptic technique. HEK293T and A549 cells have doubling times of

approximately 20-24 hours and thus were seeded approximately 24 hours prior to the beginning of transfection/ transduction experiments.

The seeding protocol included removal of old media from flasks of cells, repeated washing (x2) of cells with phosphate buffered saline (PBS) -/- (Thermo Fisher Scientific, Massachusetts, US) followed by detachment of cells with 1 or 2 ml of trypsin/ ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Massachusetts, US) for T25 and T75 flasks, respectively. Upon sufficient trypsinisation, DMEM complete media was added to neutralise the trypsin and cells pelleted by centrifugation at 500xg for 5 minutes. Cell pellets were resuspended in DMEM complete media and cell counts performed using the Luna-II Automated Cell Counter (Logos Biosystems, Gyeonggi-do, South Korea) seeding culture vessels at densities and media volumes suggested by the vessel manufacturer. Alternatively for cryopreservation, cell pellets from a T75 flask were resuspended in 1 ml of freezing solution (FBS and 10% dimethyl sulphoxide (DMSO)) and transferred to cryovials. Initially cryovials were frozen in cryo-freezing ethanol containers at -80°C then transferred for long-term storage in a liquid nitrogen cell bank.

2.2.2 Transfection with plasmid DNA

HEK293T and A549 cells were transfected with polyethylenimine Pro (PEIPro) transfection reagent (Polyplus Transfection, New York, US) following supplier's instruction. Briefly, a PEIPro:DNA ratio of 2 µl of PEI for every 1 µg of plasmid DNA was used. Transfection reactions made up 10% of the well volume and comprised of equal parts DNA diluted in reduced serum media, Opti-MEM, and PEIPro diluted in Opti-MEM (Thermo Fisher Scientific, Massachusetts, US). Plasmid DNA and PEI were diluted separately then PEIPro-Opti-MEM added dropwise to DNA-Opti-MEM and incubated at room temperature for 15 minutes. Meanwhile, complete media was removed from cells and 90% well volume replaced with Opti-MEM. Following incubation, transfection mixes were added to wells and cells incubated for 24 hours. Subsequently, reduced serum media was replaced with complete media and incubated for 48 hours further.

2.2.3 Transduction with lentiviral vectors

HEK293T and A549 cells were seeded, as described above, 24 hours prior to transduction. Aliquots of virus were defrosted at room temperature and diluted to the appropriate multiplicity of infection (MOI) in Opti-MEM. Media was removed from wells, replaced with diluted virus and incubated for 24 hours. Subsequently, equal volumes of complete media were added to wells and incubated for 48 hours further.

2.2.4 Harvesting cell culture experiments and sample collection

Following completion of transfection/transduction experiments, samples were collected dependent on the downstream analysis carried out. In general, supernatant was collected for analysis of secreted proteins by carefully removing to labelled collection tubes and centrifuging at 300xg for 5 minutes to pellet any cells carried over. Subsequently cells were harvested with Trypsin/EDTA and split into two halves for protein and molecular analysis.

To collect cell lysate, RIPA Lysis Buffer (Thermo Fisher Scientific, Massachusetts, US) supplemented with cOmplete, EDTA-free Protease Inhibitor Tablets (Roche, Basel, Switzerland, 1 tablet/50 ml extraction solution) and Benzonase endonuclease (Merck, Feltham, UK, 25 U/ml) was added to cell pellets, incubated on ice for 5 minutes then centrifuged at 13,000xg for 15 minutes to pellet cellular debris. Total protein was determined using a DC Protein Assay Kit (BioRad, California, US) according to manufacturer's instructions.

For molecular analysis, RNA/DNA was extracted from cell pellets using the AllPrep DNA/RNA Mini Kit (Qiagen, Crawley, UK) following manufacturer's instructions. Nucleic acid concentration was quantified using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Massachusetts, US) with absorbance measured at 260 nanometer (nm). Absorbance at 280 nm was further measured using the spectrophotometer to determine protein content, and the ratio of 260/280 used as a readout of sample purity. All samples were stored at -80°C until further analysis.

2.3 F/HN vector production and downstream molecular analysis

2.3.1 Small-scale F/HN vector manufacturing

Recombinant SIV.F/HN vector was produced by transient transfection of 5 plasmids into suspension HEK293T cells, following previously detailed methodology (106). The HEK293T/17 cell line was utilised for all vector production, adapted for growth in suspension to allow vector production to be scaled from 20 ml to 5 L batches. The five plasmids required to produce third generation SIV-based lentiviral vectors were packaging plasmids encoding *gag/pol* and *rev*, plasmids encoding the *Fct4* and *SIVct+HN* Sendai virus envelope proteins and finally the vector genome. Multiple vector genome plasmids were used for vector production, with codon-optimised cDNA sequences inserted between restriction enzyme sites, *NheI* and *Apal* and expression driven by the chimeric human cytomegalovirus (CMV) enhancer coupled to the eukaryotic EF1 α promoter fusion (hCEF) promoter. These included vector genome plasmids encoding a variety of reporter transgenes: *Gaussia luciferase* (*GLux*), *enhanced green fluorescent protein* (*EGFP*), *firefly luciferase* and *EGFP* fusion (*EGFPLux*), and therapeutic transgene *murine ADAMTS13* (*mADAMTS13*).

To begin vector production, HEK293T/17 cells were seeded at a density of 1e6 cells/ml, grown in Freestyle media (Life Technologies, Paisley, UK) under standard conditions (37°C and 5% CO₂, 130rpm). The vector genome, *gag/pol*, *rev*, *Fct4* and *SIVct+HN* plasmids were diluted in a ratio of 20:9:6:6:6 to a final concentration of 0.33 μ g/ml and mixed with transfection reagent PEIPro (PolyPlus, New York, US) in a ratio of 2:1 PEI:DNA. After 24 hours, Freestyle media supplemented with sodium butyrate (Sigma-Aldrich, Missouri, US), final concentration 5 mM, was added to flasks. Virus supernatant was harvested after 72 hours and activated by adding 25 U/mL Benzonase endonuclease, 1 mM MgCl₂ (Sigma-Aldrich, Missouri, US) and 0.05X TrypLESelect (Thermo Fisher Scientific, Massachusetts, US) at 37°C for 1 hour. To inactivate the TrypLESelect, 0.1X defined trypsin inhibitor (DTI) (Thermo Fisher Scientific, Massachusetts, US) was added to viral supernatant which was filtered through a 0.45 μ m syringe (Aligent, California, US) and stored in aliquots at -80°C.

2.3.2 Calculation of functional titre

To determine the functional titre of recombinant vector, the infectious activity was measured in an attached HEK293T cell line. Serial dilutions of vector diluted in Opti-MEM was applied to cells for 24 hours before cells were washed with PBS +/- then media replaced with DMEM complete media for 48 hours. Subsequently cells were harvested with Trypsin/EDTA and DNA extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, UK) following manufacturer's instructions. Vector genomes per cellular genome, referred to as vector copy number (VCN), was measured using the droplet digital PCR (ddPCR) platform (BioRad, California, US) following manufacturer's protocols. Reactions were performed in multiplex using primer/probe qPCR assays (Integrated DNA Technologies, Iowa, US) against the vector-specific WPRE sequence and human housekeeping gene, *telomerase reverse transcriptase* (*hTERT*), as detailed in Table 2.2. Reactions included 1X ddPCR Supermix for Probes (No 2'-Deoxyuridine, 5'-Triphosphate (dUTP)) Supermix (BioRad, California, US), 900 nM primers and 250 nM probes and 5 ng template DNA. Thermocycling conditions were as follows: 95°C for 10 minutes, 40 cycles of 94°C for 45 seconds and 60°C for 1 minute followed by 10 minutes at 98°C. Once the VCN was established, the infectious titre in transduction units/ml (TU/ml) could be calculated by accounting for the dilution of virus. The average titre was taken from multiple readings.

2.3.3 Optimising detection of viral transcripts by reverse transcriptase-droplet digital PCR

To quantify vector-specific RNA using the ddPCR platform, reaction conditions were first optimised to determine optimal template concentration and primer/probe combinations against WPRE and three different housekeeping genes *glucuronidase beta* (GusB), *beta-2-microglobulin* (B2M) and *hypoxanthine-guanine phosphoribosyltransferase* (HPRT). To determine transcript levels of housekeeping genes, primer/probes were designed against cDNA sequences (span codons). The One-Step reverse transcriptase ddPCR (RT-ddPCR) Advanced kit for Probes (Biorad, California, US) was used following the supplier's instructions, however including 1, 5 and 10 ng of template RNA/reaction (RNA extracted from HEK293T cells transduced with rSIV.F/HN at MOI 50). Primers/probes were used in single-plex (WPRE, GusB, B2M or HPRT only/reaction) or multiplex (WPRE+HPRT and GusB+B2M in multiplex). Reactions included an initial reverse transcriptase step, 50°C for 1

hour, then 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds and 60°C for 1 minute followed by 10 minutes at 98°C. Copy number of housekeeping and viral targets were expressed in single-plex and multiplex.

Table 2.2- qPCR primers/probes for droplet digital PCR.

Woodchuck hepatitis virus posttranscriptional regulatory elements (WPRE), human telomerase reverse transcriptase (hTERT), beta-2-microglobulin (B2M), hypoxanthine-guanine phosphoribosyltransferase (HPRT1), glucuronidase beta (GusB).

Target	Sequence	Dye
WPRE (D/RNA)	Forward primer: TGGCGTGGTGTGCACTGT Reverse primer: CCCGGAAGGAGCTGACA Probe: TTGCTGACGCAACCCCCACTGG	HEX/ZEN/IBFQ
hTERT (DNA)	Forward primer: CCTCAGGAACACCAAGAAGTT Reverse primer: CGCTCATCTTCCACGTCAG Probe: GCGAGAGCTTGGCATGCTTC	6-FAM/ZEN/IBFQ
B2M (DNA)	Forward primer: CCTCAGGTAACCAAAGATTCAG Reverse primer: TGGATGAAACCCAGACACATAG Probe: ACTCACGTCATCCAGCAGAGAATGG	6-FAM/ZEN/IBFQ
HPRT1 (RNA)	Forward primer: TTGTTGTAGGATATGCCCTTGA Reverse primer: GCGATGTCAATAGGACTCCAG Probe: AGCCTAAGATGAGAGTTCAAGTTGAGTTTGG	6-FAM/ZEN/IBFQ
GusB (RNA)	Forward primer: GCCCATTAATTCAGAGCGAGTA Reverse primer: GTTTTTGATCCAGACCCAGATG Probe: TGCAGGGTTTCACCAGGATCCAC	HEX/ZEN/IBFQ
B2M (RNA)	Forward primer: GGACTGGTCTTTCTATCTCTTGT Reverse primer: ACCTCCATGATGCTGCTTAC Probe: CCTGCCGTGTGAACCATGTGACT	6-FAM/ZEN/IBFQ

2.4 *In vivo* techniques

All animal procedures were performed in accordance with the UK Home Office Project (P3B2E3511) and Personal licence (ID0FF549A) regulations under the Animal Scientific Procedure Act (1986). Both female and male C57BL/6 mice were purchased from Charles Rivers Laboratories (Margate, UK) and unless otherwise stated were 8-10 weeks of age at the beginning of experiments. Mice were housed in individually ventilated cages with food and water ad libitum and with 12 hour light/dark cycles.

2.4.1 *In vivo* gene transfer

For intranasal dosing, mice were sufficiently anaesthetised with isoflurane (1 L/minute oxygen and 3% isoflurane) then held in an upright position with their mouth gently shut. Treated mice received vector and control mice were delivered TSSM buffer (tromethamine 20 mM, NaCl 100 mM, sucrose 10 mg/ml, and mannitol 10 mg/ml) (100 µl total volume/dose) applied to the nostrils using a pipette and inhaled by the mouse into their lungs. Mice were monitored until fully recovered. To dose intravenously, mice were pre-warmed for a maximum of 10 minutes in a heating chamber set to 38°C to encourage vasodilation. Mice were then secured in a restriction tunnel with their tail exposed. Using insulin needles, vector or TSSM buffer (100 µl total volume/dose) was injected into either of the lateral tail veins. Mice were removed from the restraint once the puncture site had clotted. At indicated time points post-transduction, blood was sampled from mice by repeat tail venepuncture, as described below.

2.4.2 Repeat tail venepuncture

To measure serum levels of reporter and therapeutic transgenes in the same mouse over time, blood was repeatedly sampled through tail venepuncture. Mice were pre-warmed, as described above, then a puncture made in either of the lateral tail veins using a 25-gauge needle (BD, New Jersey, US). Blood was collected using a pipette and for the preparation of plasma mixed with 3.8% sodium citrate (1/10 dilution, Sigma-Aldrich, Missouri, US) or 0.5 M EDTA (1/20 dilution, Sigma-Aldrich, Missouri, US). Alternatively for the preparation of serum, blood was collected without anticoagulant allowing clots to form at room

temperature for at least 15 minutes. Subsequently both plasma and serum were separated by centrifugation at 3000xg for 10 minutes at 4°C, followed by storage of aliquots at -80°C.

2.4.3 Endpoint sample collection

At indicated time-points, mice were culled by overdose with Euthatal (Merial, Essex, UK) (200 µl of 200 mg/ml pentobarbital by intraperitoneal injection). Once death was confirmed, blood was extracted by cardiac puncture and serum or plasma prepared as detailed above. Subsequently bronchoalveolar lavage fluid (BALF) was collected by securing a 22-gauge canula into the trachea and washing the lungs three times with a syringe containing 200ul of PBS (-/-). BALF was centrifuged at 5000xg for 10 minutes, supernatant removed then stored at -80°C. Organ tissue was collected and split into two parts, with one half snap-frozen in dry ice for downstream homogenisation and protein analysis and the other half stored in RNAlater for downstream molecular analysis.

2.4.4 Organ homogenisation for protein analysis/ molecular analysis

Small amounts of dissected organ (<50mg tissue) were homogenised in Reporter Lysis Buffer (Promega, Southampton, UK) for protein analysis or Buffer RLT supplemented with 1% β-Mercaptoethanol (Qiagen, Crawley, UK) for nucleic acid purification. Samples were loaded into Lysing Matrix D tubes (MP Biomedicals, California, US) and processed using a Fast-Prep homogeniser (Thermo Fisher Scientific, Massachusetts, US) at 4.0 meters/second for 45 seconds. The homogenate was transferred to a Qias shredder column (Qiagen, Crawley, UK) and centrifuged at 13,000xg for 1 minute. Columns were then removed and the homogenate centrifuged for an additional 5 minutes. The supernatant was transferred to a new Eppendorf and total protein determined using a DC Protein Assay according to manufacturer's instructions or taken forward for RNA/DNA extraction using the AllPrep DNA/RNA Mini Kit.

2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. Gaussian distribution and lognormal distribution were assessed using the D'Agostino-Pearson omnibus normality test. Where lognormal distribution was established, particularly datasets measuring luciferase

expression, data was transformed by taking the logarithm of each value. Datasets where Gaussian and lognormal distribution were established ($p > 0.05$) were analysed using parametric statistical tests (individual comparisons and post-hoc tests are stated in the results section) and data plotted as mean $\pm$ standard deviation (SD). Where data was not Gaussian or lognormally distributed, non-parametric tests were utilised and data expressed as median + range. The null hypothesis was rejected at $p < 0.05$.

Chapter 3- Comparing Intravenous and Intranasal Delivery of rSIV.F/HN

3.1 Introduction

3.1.1 Immune responses to systemic delivery of viral vectors

A major impediment that will limit transduction efficiency and consequently the success of gene transfer following *in vivo* administration to humans, is the detection of viral vectors by the immune system. Although initially highlighted as the favourable GTA in early gene therapy studies due to their large packaging capacity and high transduction efficiency, AdV highlighted the dramatic consequences an immune response to *in vivo* vector delivery can have (107). The mounting of inflammatory responses to AdV caused not only transient gene expression in early clinical trials but in the case of Jesse Gelsinger in 1999, caused systemic inflammatory response syndrome and a cytokine cascade that led to multi-organ failure and death (55,108). Due to the strong induction of an immune response, AdV have become candidates for vaccine development and anti-cancer therapies, yet their investigation towards treatment of genetic diseases has largely ceased. Conversely, AAV vectors have become the most popular vector for *in vivo* gene transfer and lentiviral vectors the most widely used *ex vivo* vector.

AAV vectors are considered to have a favourable safety profile because they are helper-dependant and thus replication incompetent viruses, non-pathogenic and less immunogenic as vectors are devoid of viral genes (109). Furthermore, AAV vectors are considered safe because their genomes remain largely episomal upon transduction, although the rare occurrence of random integrations is debated somewhat (109). Numerous different serotypes of AAV have been isolated from multiple species in nature and subsequently an array of recombinant vectors generated using the different capsid proteins (105). As is observed between the natural serotypes, inclusion of different capsid proteins alters transduction properties and particularly cell tropism which allows for the selection of the appropriate serotype based on the clinical objective (110). Due to these characteristics, AAV vectors have been investigated for the treatment of various genetic diseases with two AAV licenced products approved for clinical use, Luxturna for retinal dystrophy and Zolgensma

for the treatment of Spinal Muscular Atrophy, whilst the licencing of an AAV product for treatment of haemophilia is also anticipated shortly (103,111).

Although AAV vectors have demonstrated great success in the clinic and are demonstrably safer than AdV, immune responses against AAV vectors have been observed (109). The greatest barrier in the clinical application of AAV vectors is that humans are commonly infected by AAVs during their lifetime, usually during childhood (105). Neutralising antibodies, developed by individuals previously exposed to AAVs are most prevalent against AAV2, which most closely resembles wildtype AAVs that infect the human population. However prevalence of neutralising antibodies against other serotypes varies from 5-60% with evidence of cross-reactivity between serotypes (109,112). Possessing neutralising antibodies against AAV vectors limits their transduction efficiency and thus screening of pre-existing immunity prior to patient enrolment in clinical trials is required. Evidence of cell-mediated immunity was highlighted following intravenous delivery of AAV2 vectors to patients during a haemophilia B clinical trial. Elevation of liver transaminases were observed transiently with consequential loss in FIX expression in two patients (113). Upon investigation, clonal expansion of CD8⁺ T cells targeting AAV capsid antigens likely lead to the elimination of transduced hepatocytes. In future trials, monitoring of liver enzymes and fast immunosuppressant intervention helped mitigate a T-cell mediated response in some patients (114).

An advantage of using lentiviral vectors over AAV vectors is that previous exposure of humans to lentiviruses in the environment, such as HIV-1, are rare and thus patients are unlikely to have a pre-existing immunity against these vectors. However systemic delivery of lentiviral vectors has shown limited efficacy in the literature. Cantore *et al.* (2015) noted acute inflammatory responses and hepatotoxicity following intra-portal delivery of lentiviral vectors in dogs, an unexpected finding following successful restoration of FIX activity and lack of toxicity in mouse models (115,116). Milani *et al.* (2019) demonstrated that incorporation of an inhibitor of phagocytosis, CD47, can mediate VSV-G vector clearance by liver phagocytes, reduce recognition by the innate immune system, and enhance hepatocyte gene transfer and FIX activity in non-human primates (117). Phagocytosis of

lentiviral vectors by antigen-presenting cells (APCs) in the liver and spleen therefore highlights an impediment of systemic vector delivery.

Innate immune responses to lentiviral vectors can further be induced by the detection of viral DNA genomes upon reverse transcription by TLRs, leading to the secretion of inflammatory cytokines such as interferon α/β which in-turn activate APCs (118). DNA from bacterial origin contains a high frequency of unmethylated CpG dinucleotides which have immunostimulatory effects through TLR9 recognition (58). Conversely, DNA from mammalian origin contain few CpG dinucleotides and methylation of existent CpG dinucleotides prevents TLR9 activation (58). Thus, inclusion of mammalian promoters and depletion of CpG dinucleotides through codon optimisation is a common strategy employed to reduce immune recognition and consequently extend transgene expression (59–61).

Despite efforts to disguise exogenous DNA from the innate immune system, a further challenge faced by lentiviral vectors is their ability to efficiently transduce APCs and consequently the mounting of an adaptive immune response (109). Thus the efficiency with which lentiviral vectors can transduce APCs and the route of administration will greatly influence host immune recognition and duration of gene expression (119). All previous studies investigating rSIV.F/HN had targeted the nasal or pulmonary epithelium by intranasal administration, however intravenous vector administration for the delivery of transgenes encoding systemic proteins has not yet been investigated.

3.1.2 Aims and objectives

The aim of this research is to investigate intravenous delivery of rSIV.F/HN with comparisons made against intranasal vector administration. The hypothesis is that gene transfer by systemic delivery of rSIV.F/HN is feasible, leading to sustained gene expression in the circulation. Specific objectives include:

1. Assess duration of transgene expression in the systemic circulation following a single dose of rSIV.F/HN.
2. Characterise vector biodistribution following intravenous and intranasal rSIV.F/HN administration.

3. Determine the feasibility of repeat intravenous administration by measuring transduction efficiency and production of neutralising antibodies following three monthly doses.

3.2 Materials & Methods

3.2.1 Gaussia luciferase duration expression study

Vector encoding GLux (rSIV.F/HN-GLux) was delivered to wildtype female mice to compare secretion of protein into the blood following intravenous and intranasal delivery. Mice were treated with increasing doses of vector (2.4×10^7 , 7.6×10^7 , 2.4×10^8 TU/mouse, $n=6$ mice/group) or TSSM buffer via intravenous or intranasal delivery and blood sampled via tail venepuncture at regular intervals post-dosing (1, 2, 5, 12 and 24 weeks post-dosing). End-point sample collection took place 52 weeks post-dosing, as described below. GLux expression was quantified in serum using the method described in section 3.2.6 and expressed as RLU.

3.2.2 Pilot biodistribution study

To first establish if bioluminescent imaging could be used as a tool to investigate biodistribution following intravenous administration of rSIV.F/HN vector, a small pilot study was conducted. Vector encoding intracellular reporters, firefly luciferase and EGFP fusion (rSIV.F/HN-EGFPLux) was delivered intravenously to female mice ($n=4$ treated and $n=1$ untransduced, 1.4×10^8 TU/mouse) and *in vivo* bioluminescent imaging of live animals performed 24 hours and 11 days post-dosing, following the protocol outlined in section 3.2.5. Organs were subsequently dissected from each mouse (liver, lung and spleen) and imaged then split into two for downstream processing.

3.2.3 Biodistribution study

Upon completion of the pilot, a larger study was conducted where rSIV.F/HN-EGFPLux was delivered intravenously ($n=4$ females and $n=4$ males, 2.58×10^8 TU/mouse) and intranasally ($n=3$ females and $n=3$ males, 2.58×10^8 TU/mouse) to mice, alongside untransduced mice ($n=3$ females and $n=2$ males). *In vivo* bioluminescent imaging was performed 35 days post-dosing, following the protocol outlined in section 3.2.5. Multiple organs were subsequently dissected from each mouse (liver, lung, spleen, brain, kidney, heart and gonads) and imaged. Organ tissue was collected for analysis as described.

3.2.4 Repeat intravenous administration study

Repeat administration was investigated by comparing transgene expression between female mice receiving two doses of control vector (rSIV.F/HN-mADAMTS13, 1.42×10^8 TU/mouse) followed by a final dose of rSIV.F/HN-EGFPLux (1.52×10^8 TU/mouse, $n=6$ mice) and mice that received two doses of TSSM buffer followed by a single dose of rSIV.F/HN-EGFPLux (1.52×10^8 TU/mouse, $n=6$). Mice received doses (control vector or TSSM) at monthly intervals. One month following the final dose, mice underwent whole-body bioluminescent imaging following the protocol in section 3.2.5. Organs were subsequently dissected from each mouse (liver, lung, spleen, brain, kidney, heart and gonads) and imaged. Organ tissue was collected as described in section 2.4.3.

3.2.5 Bioluminescence imaging

One day prior to imaging, mice were depilated under isoflurane anaesthetic using an electric razor to remove pigmented fur that may interfere with signal output. At indicated time-points post-dosing, each mouse received 150 mg/kg of VivoGlo Luciferin (Promega, Southampton, UK) injected intra-peritoneally. Luciferin is oxidised by firefly luciferase producing light of wavelength 562nm, which 15 minutes later is imaged using the IVIS Spectrum CT (Perkin Elmer, Massachusetts, US) system with mice kept under isoflurane anaesthesia. Organs were dissected from mice immediately after whole-body imaging and placed in petri-dishes for imaging. Analysis of IVIS images was through the image-analysis software platform Fiji (120), a distribution of ImageJ, taking area under curve (AUC) as the combined readout of signal intensity and area.

3.2.6 Gaussia luciferase measurements in serum

GLux levels were quantified in serum (10 μ l of sample/assay) using the Pierce Gaussia Luciferase Flash Assay Kit (Thermo Fisher Scientific, Massachusetts, US) following the supplier's protocol. Coelenterazine substrate is oxidised by GLux enzyme into light of wavelength 470-600 nm, which is detected by the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). GLux expression was expressed as relative light units (RLU). To account for differences in serum volume that may be assayed, standard curves were generated by measuring GLux expression with increasing volumes of serum (volumes

between 1-10 μ l). Serum was pooled from mice demonstrating high GLux expression (RLU>3000), medium expression (RLU>400) and low expression (RLU<400), regardless of which delivery or treatment group the mice were in (n=6 mice/expression group). Figure 3.1 shows linear regressions fitted for each level of GLux expression. However only points up to 5 μ l were included in the high signal linear regression due to a saturation in signal beyond this volume. Using these equations, volume could be accounted for if less serum was available to measure in GLux assays.

3.2.7 Firefly luciferase detection in organ homogenate

Firefly luciferase activity was measured in organ homogenate using the Bio-Glo Luciferase Assay System (Promega, Southampton, UK) following the supplier's protocol. 5'-fluoroluciferin is oxidised by firefly luciferase, emitting light of wavelength 562nm which is detected using the FLUOstar Omega plate reader. Total protein was determined using a DC Protein Assay Kit (BioRad, California, US) according to manufacturer's instructions and normalised bioluminescence signal expressed as relative light units (RLU)/mg total protein.

3.2.8 Measurement of neutralising antibody titre

The measurement of neutralising antibodies was performed in collaboration with PhD student, Nikhil Faulkner from The Francis Crick Institute/Imperial College London. Neutralising antibody measurement in mouse serum was performed by incubation of rSIV.F/HN-EGFP with 1 in 2 serial dilutions of serum from 1/50 up to 1/6400. Serum and vector were incubated for 30 minutes at 37°C then applied to HEK293T cells seeded in 96-well plates (3000 cells/well). The percentage of transduced (GFP+) cells was assessed by flow cytometry 72 hours later using standard methodology. The inverse serum dilution leading to 50% reduction of GFP+ cells was taken as the neutralising titre (IC₅₀).

3.3 Results

We have previously demonstrated that rSIV.F/HN delivered topically to murine lungs successfully achieved therapeutic plasma protein levels of secreted proteins, Factor VIII and AAT (99). To investigate further the suitability of rSIV.F/HN vector for treatment of systemic diseases, intravenous administration to wildtype mice was characterised and compared to intranasal delivery. Specifically, the aim was to compare the lungs and the liver as factories for secretion of systemic proteins.

3.3.1 Systemic expression of Gaussia luciferase is stable for at least 12 months following a single dose

To investigate transgene expression in the systemic circulation, wildtype mice were delivered increasing doses of rSIV.F/HN encoding the secreted reporter transgene, GLux. Mice were repeatedly sampled over a 52-week period and serum expression of GLux quantified, comparing between treatment groups. Across the 52-week study, three mice died prematurely from the intranasal group and none from the intravenous group. One mouse was found dead (intranasal 2.4×10^7 TU/mouse group, 36 weeks post-transduction), one mouse was culled due to an infected wound to the eye (intranasal 7.6×10^7 TU/mouse treatment group, 10 weeks post-transduction) and one mouse were culled due to dermatitis caused by over-grooming (intranasal TSSM group 40 weeks post-transduction). Thus 45/48 mice reached endpoint harvesting. GLux measurements from serum samples collected prior to the mice dying were included in analyses.

To account for differences in serum volume that may be assayed, standard curves were generated by measuring GLux expression with increasing volumes of serum. Serum was pooled from mice demonstrating high GLux expression, medium expression and low expression, regardless of which delivery or treatment group the mice were in. Figure 3.1 shows linear regressions fitted for each level of GLux expression. However only points up to 5 μ l were included in the high signal linear regression due to a saturation in signal beyond this volume. Using these equations, volume could be accounted for if less serum was available to measure in GLux assays.

Figure 3.2A demonstrates that transgene expression is stable and persistent for the duration of the experiment (52 weeks) following a single intranasal dose of rSIV.F/HN. All three treatment groups (2.4e7, 7.6e7 and 2.4e8 TU/mouse) resulted in significantly higher GLux expression than background levels detected in control (TSSM) mice, at every time point sampled (two-way ANOVA, Dunnett's post-hoc analysis, + = $p < 0.05$ compared to TSSM mice, $n = 5/6$ mice/group). GLux expression measured in serum of mice administered rSIV.F/HN by intravenous administration is shown in Figure 3.2B. Similarly to mice dosed topically, persistent expression is observed for the duration of the experiment. In contrast however, significant levels of GLux above background was observed in the two high-dose groups only (two-way ANOVA, Dunnett's post-hoc analysis, * = $p < 0.05$ when 7.6e7 and 2.4e8 TU groups are compared to TSSM mice, $n = 6$ mice/group) with the lowest dose resulting in minimal serum expression above background. An increase in signal was observed between the 24 weeks and 52 weeks data points across all treatment groups that received vector. Overall, all treatment groups across both delivery methods, except the low dose intravenous treatment group, resulted in significant GLux expression above background levels observed in control mice.

3.3.2 Intravenous administration of rSIV.F/HN vector results in superior transduction efficiency at the highest treatment dose

To compare transduction efficiency between delivery methods, Figure 3.3 shows GLux expression observed in each treatment group at the study endpoint (52 weeks). As expected, no significant difference was found between intranasal and intravenous TSSM groups (ns=not significant, two-way ANOVA, Šídák's multiple comparisons test, $n = 5/6$ mice/group). Within the low dose group (2.4e7 TU/mouse), mice administered vector by intranasal administration demonstrated significantly higher serum GLux expression than intravenous mice (** = $p < 0.05$ when IN and IV are compared, two-way ANOVA, Šídák's multiple comparisons test, $n = 5/6$ mice/group), whereas no significant difference was found between delivery methods in the medium dose group (7.6e7 TU/mouse, ns- not significant, two-way ANOVA, Šídák's multiple comparisons test, $n = 5/6$ mice/group). Finally, in the high dose cohort (2.4e8 TU/mouse) serum levels are significantly higher in intravenously dosed mice (** = $p < 0.05$ when IN and IV are compared, two-way ANOVA, Šídák's multiple comparisons test, $n = 6$ mice/group). GLux expression within the intranasal cohort

demonstrated a dose response between TSSM, low dose and high dose groups ($p < 0.0001$ when TSSM vs 2.4×10^7 TU/mouse and $p < 0.001$ when 2.4×10^7 vs 2.4×10^8 TU/mouse, one-way ANOVA with Bonferroni's multiple comparisons test, data not shown). Similarly the intravenous cohort observed an increase in GLux expression with higher doses, however this was not significant between doses ($p < 0.0001$ when TSSM vs 7.6×10^7 TU/mouse and $p > 0.05$ when 7.6×10^7 vs 2.4×10^8 TU/mouse, one-way ANOVA with Bonferroni's multiple comparisons test, data not shown).

3.3.3 Serum GLux levels at week 12 are predictive of endpoint expression levels

To understand how consistent transduction efficiency was in the same mouse over time, the correlation between GLux expression levels at 12 and 52 weeks post-dosing was investigated (Figure 3.4). A positive relationship was found (Pearson's correlation coefficient, $r = 0.6755$ and $p < 0.0001$, $n = 41$) demonstrating a significant relationship between expression levels observed in a mouse at week 12 and 40 weeks later at week 52 post-transduction. A significant correlation suggests that mice demonstrating high serum expression at week 12 were likely to have high expression at week 52 post-transduction, therefore transgene expression was stable over time overall across both delivery methods. Only mice with GLux expression measured at both 12 and 52 weeks were included ($n = 41/48$ mice, including the three mice that died).

Taken together, the results of the duration expression study demonstrate that both intranasal and intravenous delivery of rSIV.F/HN-GLux result in long-term transgene expression in serum. Differences in expression between delivery routes can be observed at different treatment doses, with intranasal delivery demonstrating higher transduction efficiency at the lowest dose (2.4×10^7 TU/mouse) and intravenous delivery showing superior expression levels only at the highest dose (2.4×10^8 TU/mouse).

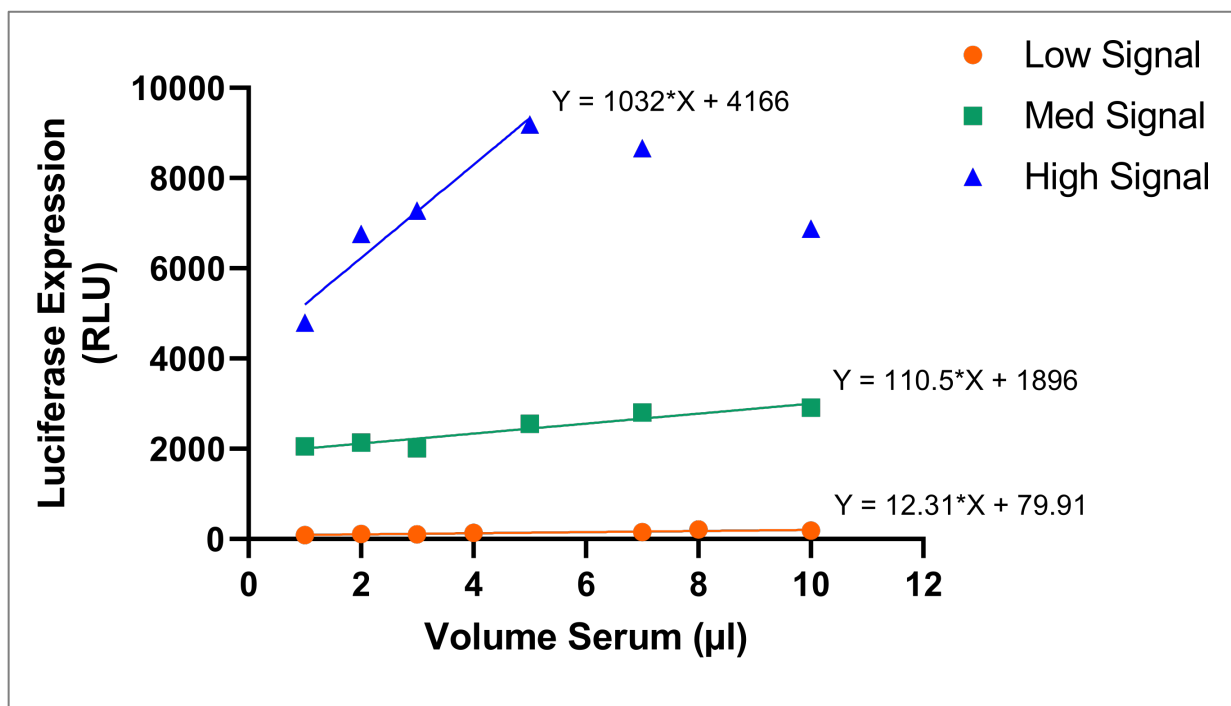


Figure 3.1- Standard curve to determine relationship between serum volume and Gaussia luciferase expression.

Wildtype C57BL/6 mice received increasing doses of rSIV.F/HN-GLux (2.4e7, 7.6e7, 2.4e8 transduction units (TU)/mouse) while control mice received TSSM buffer (n=6 mice/group). At regular intervals post transduction blood was sampled from mice and Gaussia luciferase (GLux) expression quantified in serum, expressed as relative light units (RLU). Serum was pooled from mice that demonstrated high, medium (med) and low GLux signal (n=6 mice/group), regardless of treatment group. GLux expression was then measured in increasing volumes of pooled serum. Linear regression was fitted for each level of GLux signal, with data points up to 5 µl included in the high signal linear regression due to a saturation in signal beyond this point. Equations for each linear regression stated on the graph.

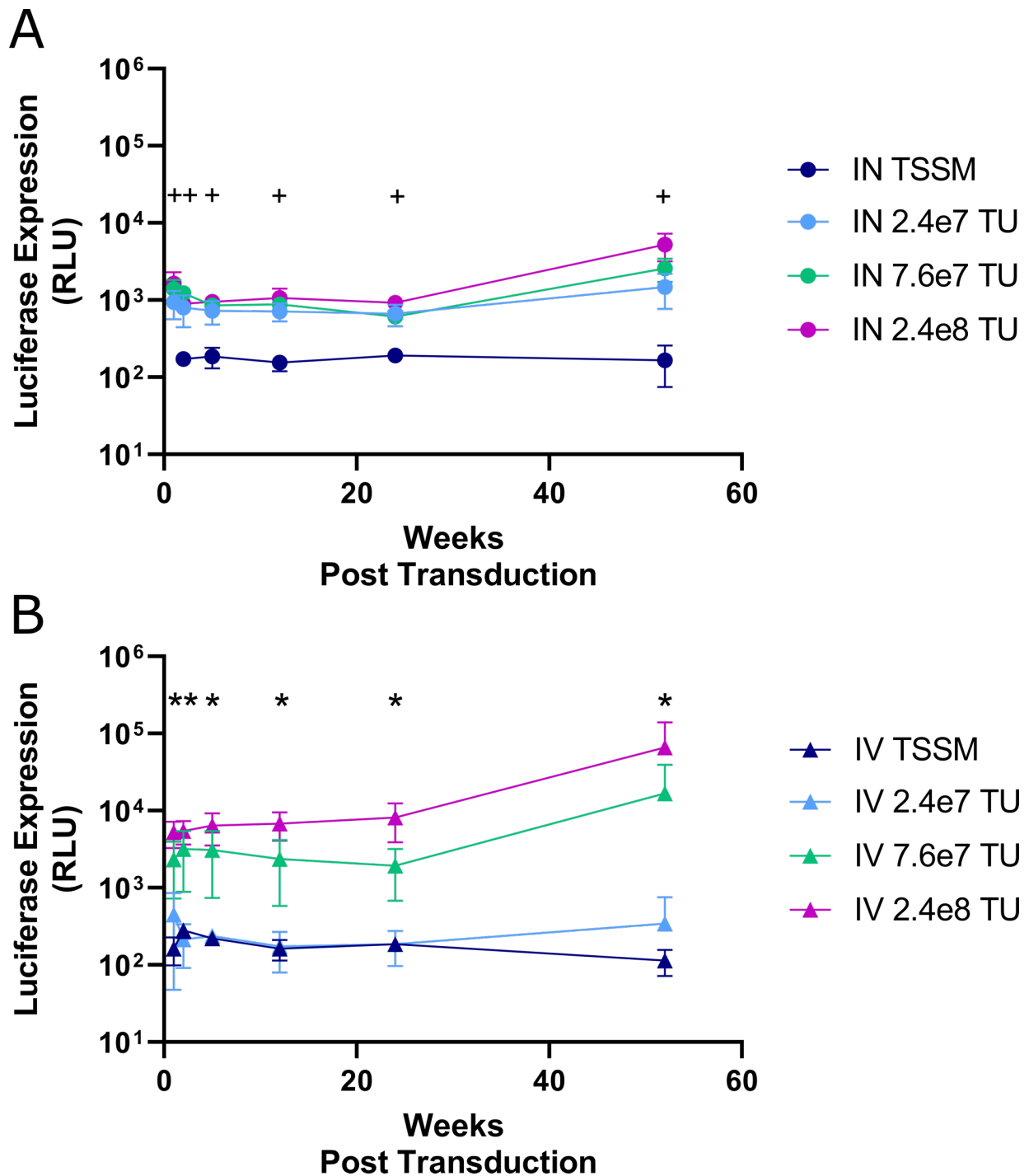


Figure 3.2- Serum Gaussia luciferase (GLux) expression over 12 months following a single dose of rSIV.F/HN.

Wildtype C57BL/6 female mice received increasing doses of rSIV.F/HN-GLux (2.4e7, 7.6e7, 2.4e8 transduction units (TU)/mouse) while control mice received TSSM buffer (n=6 mice/group). At regular intervals post transduction (1, 2, 5, 12, 24 and 52 weeks) blood was sampled from mice and GLux expression quantified in serum, expressed as relative light units (RLU). Data expressed as mean $\pm$ standard deviation. A) Mice delivered vector by intranasal (IN) administration, + = $p < 0.05$ for all treatment groups compared to TSSM control mice, two-way ANOVA, Dunnett's post-hoc analysis. B) Mice dosed intravenously (IV), * = $p < 0.05$ when 7.6e7 and 2.4e8 TU/mouse treatment groups were compared to TSSM control mice, two-way ANOVA, Dunnett's post-hoc analysis.

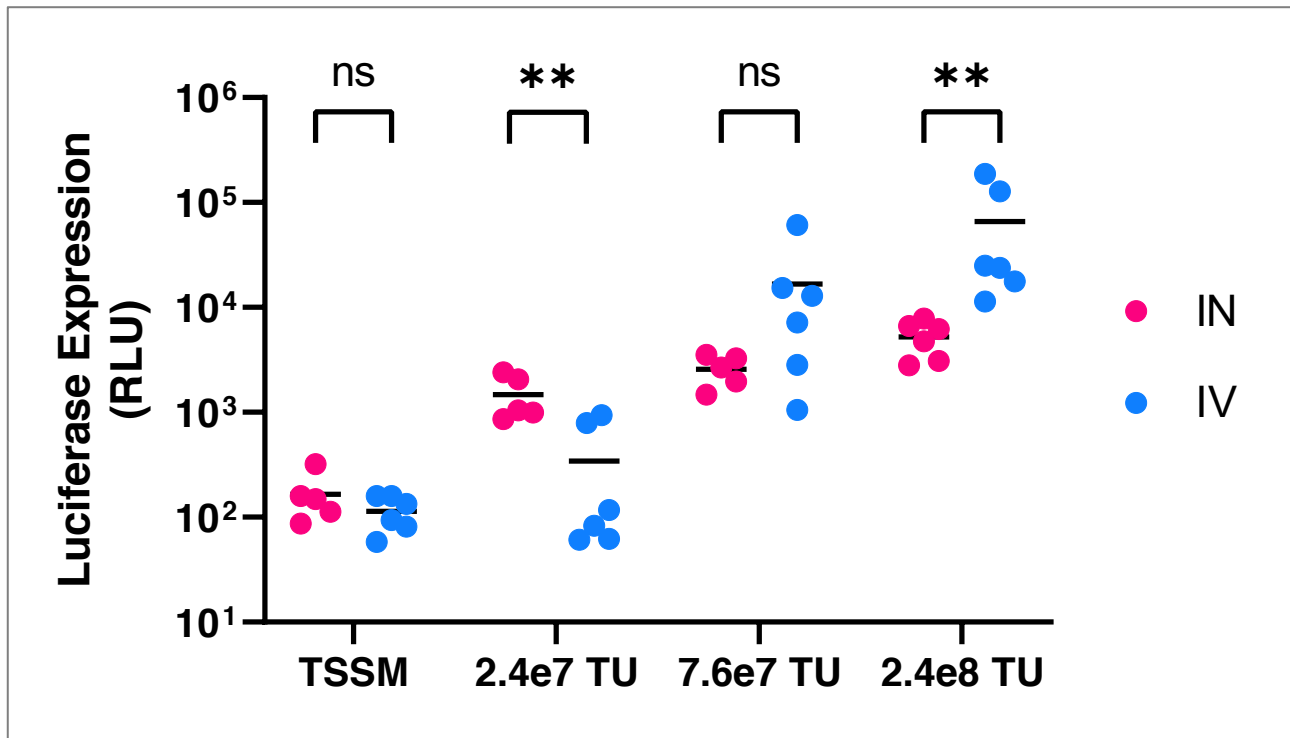


Figure 3.3- Comparison of Gaussia luciferase (GLux) expression following intranasal and intravenous delivery of rSIV.F/HN-GLux.

Wildtype C57BL/6 female mice received increasing doses of rSIV.F/HN-GLux (2.4e7, 7.6e7, 2.4e8 transduction units (TU)/mouse) while control mice received TSSM buffer (n=5/6 mice/group). Blood was sampled from mice and GLux expression quantified in serum, expressed as relative light units (RLU). GLux expression 52 weeks post-transduction (experiment endpoint) was compared between mice dosed by intranasal (IN) and intravenous (IV) administration for each treatment group. Each shape represents a single mouse. Horizontal bars represent mean values, ** = $p < 0.05$ when IN and IV compared, two-way ANOVA, Šídák's multiple comparisons test. Ns= not significant.

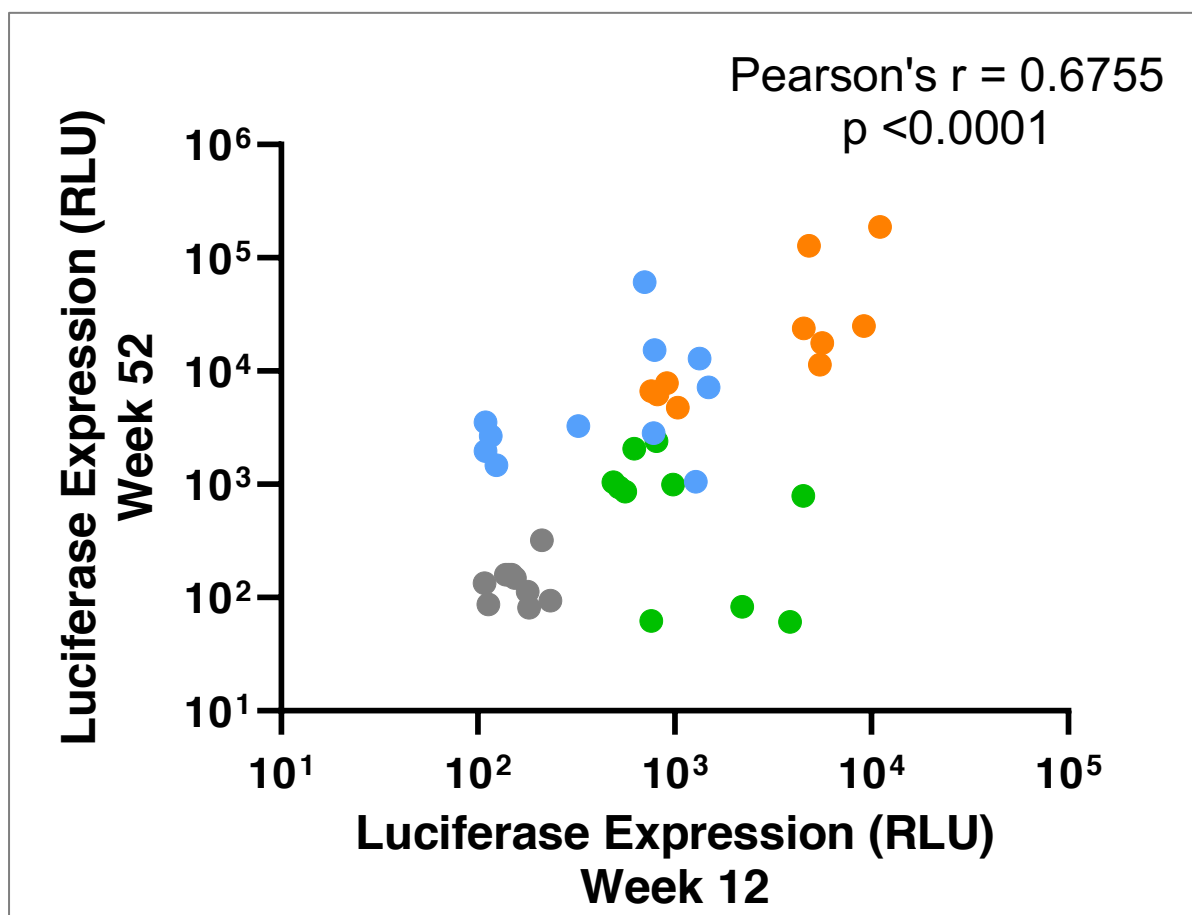


Figure 3.4- Relationship between Gaussia luciferase (GLux) expression levels at 12 and 52 weeks post-transduction across all doses.

Wildtype C57BL/6 female mice received increasing doses of rSIV.F/HN-GLux (2.4×10^7 -green, 7.6×10^7 -blue, 2.4×10^8 -orange transduction units (TU)/mouse) while control mice received TSSM buffer-grey ($n=5/6$ mice/group). Blood was sampled from mice and GLux expression quantified in serum 12 and 52 weeks post-transduction, expressed as relative light units (RLU). A significant positive relationship was found between GLux expression measured at week 12 and 52 weeks post-transduction across all treatment groups (Pearson's correlation coefficient, $r=0.6755$ and $p<0.0001$). Only mice with GLux measured at both 12 and 52 weeks were included in analysis (41/48 mice). Each shape represents a single mouse.

3.3.4 Bioluminescent imaging can detect transgene expression following intravenous administration of rSIV.F/HN-EGFP_{Lux}

A small pilot study was conducted to assess if transgene expression following intravenous delivery of rSIV.F/HN-EGFP_{Lux} could be detected by whole body and organ bioluminescent imaging. Four wildtype mice were treated with rSIV.F/HN-EGFP_{Lux} by intravenous administration and one mouse was left untransduced. Figure 3.5 shows bioluminescent images captured 24 hours post-transduction (Figure 3.5A) and the same mice 11 days post-transduction (Figure 3.5B). As shown by the maximum signal measurements, the overall signal intensity was increased by 65-fold from 24 hours to 11 days post-transduction. Signal can be observed largely in the tail, the nose and what appears to be the liver, with smaller contributions from multiple other sites across the body. After whole body images were taken, the spleen, lungs and liver were dissected and imaged individually (Figure 3.6). Images of the spleen had the strongest signal (maximum signal 31378 counts), followed by the liver (maximum signal 624 counts) then the lungs (maximum signal 174 counts). Organs from the untransduced mouse showed no detectable signal, however organs from all treated mice indicated luciferase activity.

One of the treated mice (IV-1) showed strong bioluminescence at 24 hours (Figure 3.5A), yet at 11 days demonstrated no signal in whole body imaging (Figure 3.5B) and reduced signal in organ images (Figure 3.6). The conflicting imaging results is believed to be a result of poor intraperitoneal injection of the luciferin substrate at day 11 and thus an underestimate of luciferase levels. When measuring luciferase activity in lung and liver homogenate, IV-1 levels surpassed the other treated mice overall (Figure 3.7) supporting the hypothesis that the luciferin substrate was poorly delivered prior to day 11 imaging. At 24 hours post-transduction, signal can be detected on the nose and right paw of the untransduced mouse (Figure 3.5A), which is not present 11 days post-transduction (Figure 3.5B) nor in organs (Figure 3.6, Figure 3.7). This is likely due to co-housing of the untransduced mouse with the other treated mice, which has led to a small transfer of vector to the untransduced mouse as a result of grooming. The results of the pilot study confirmed that bioluminescent imaging can be used to investigate biodistribution in mice dosed intravenously with rSIV.F/HN-EGFP_{Lux}. Furthermore, because of the observed widespread distribution, it was decided to dissect further organs for analysis.

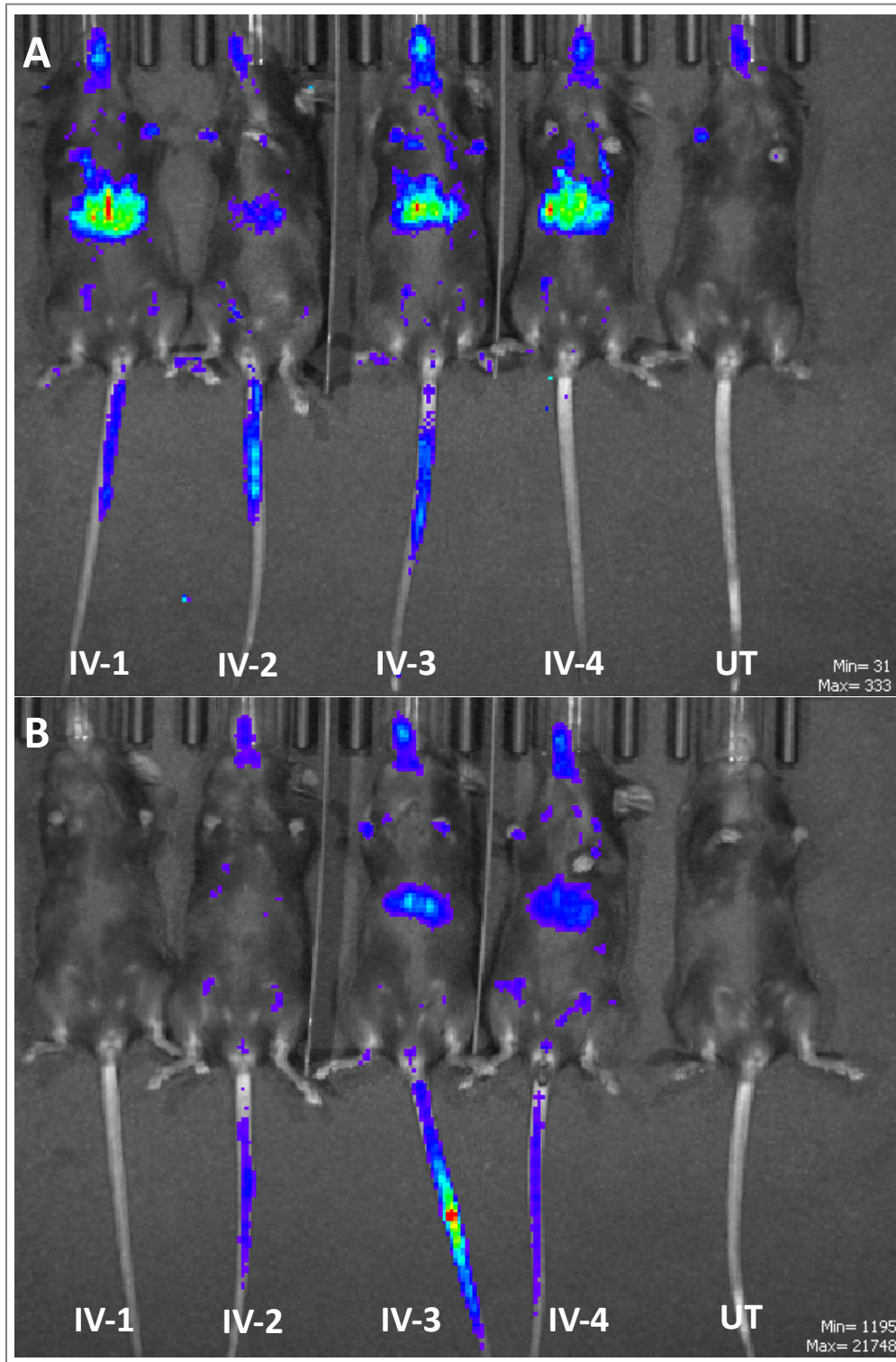


Figure 3.5- Whole-body bioluminescent imaging following intravenous delivery of rSIV.F/HN-EGFP/Lux.

Vector encoding the firefly luciferase and EGFP fusion was delivered intravenously to C57BL/6 wildtype female mice (n=4 mice treated by intravenous administration- IV-1,2,3,4 1.4×10^8 transduction units (TU)/mouse and n=1 untransduced UT) and *in vivo* bioluminescent imaging performed 24 hours (A) and 11 days (B) post-dosing. At indicated time-points post-dosing, each mouse received 150 mg/kg of luciferin substrate intraperitoneally, and 15 minutes later imaged using the IVIS Spectrum CT system under isoflurane anaesthesia. 5-minute exposure per image. Min and Max represent the minimum and maximum signal, measured in counts, per image.

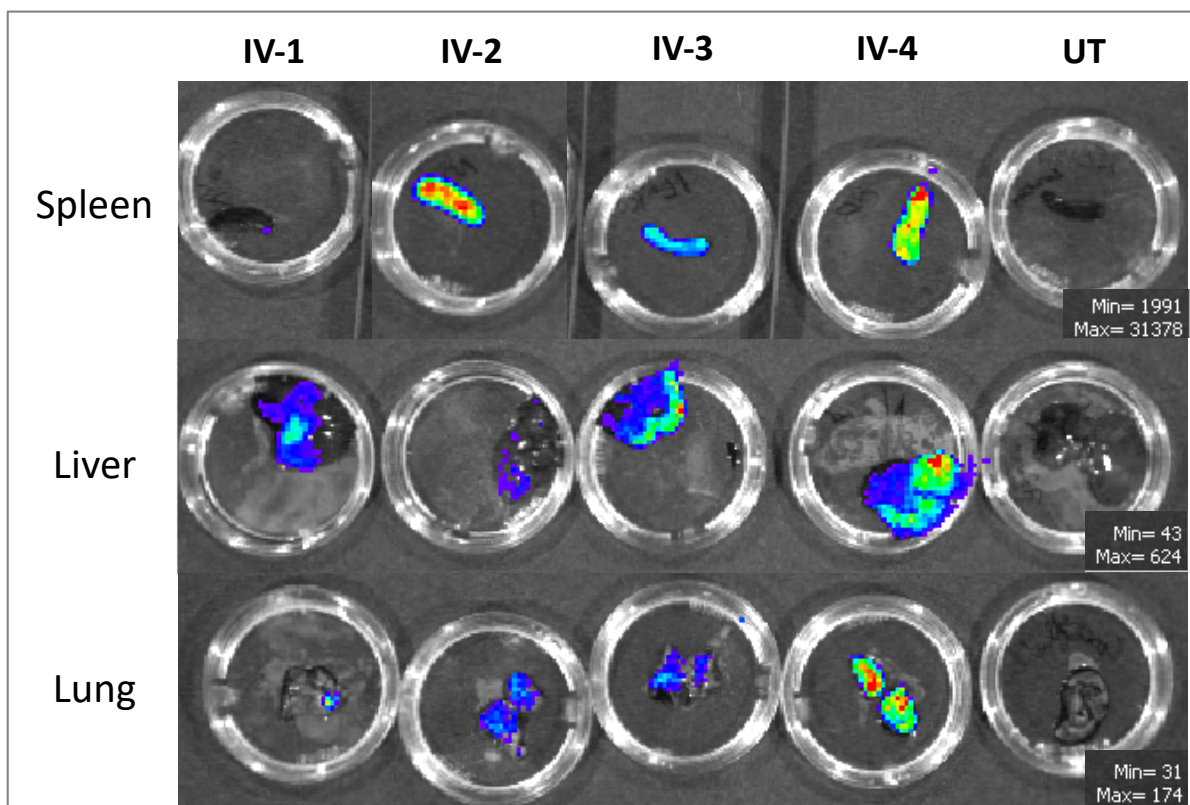


Figure 3.6- Bioluminescent imaging of organs dissected from mice following intravenous delivery of rSIV.F/HN-EGFP_{Luc}.

Vector encoding the firefly luciferase and EGFP fusion (rSIV.F/HN-EGFP_{Luc}) was delivered to C57BL/6 wildtype female mice by intravenous administration (n=4 mice treated by intravenous administration- IV-1,2,3,4 1.4e8 transduction units (TU)/mouse and n=1 untransduced UT) and bioluminescent imaging performed of dissected spleen, liver and lung. 11 days post-transduction each mouse received 150 mg/kg of luciferin substrate by intraperitoneal injection and approximately 30-minutes later organs were dissected and imaged using the IVIS Spectrum CT system. 5-minute exposure per image. Min and Max represent the minimum and maximum signal, measured in counts, per image.

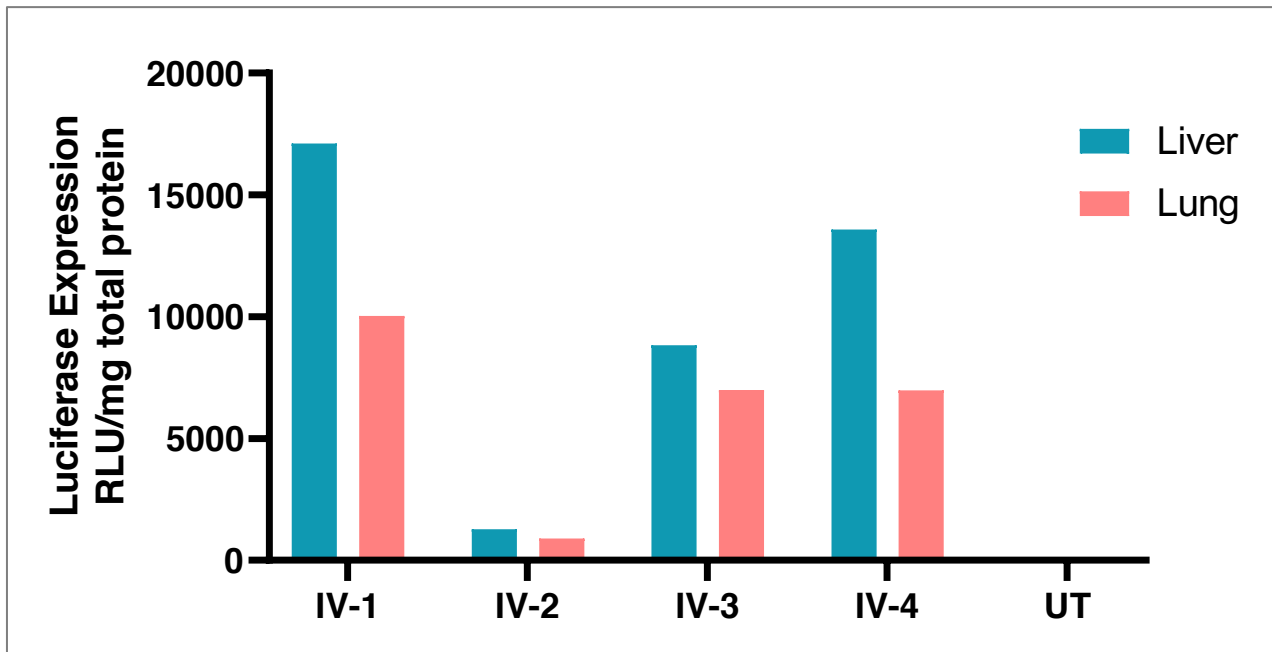


Figure 3.7- Luciferase expression measured in lung and liver homogenate of mice delivered rSIV.F/HN-EGFPLux by intravenous administration.

Vector encoding the firefly luciferase and EGFP fusion (rSIV.F/HN-EGFPLux) was delivered to C57BL/6 wildtype female mice by intravenous administration (n=4 mice treated by intravenous administration- IV-1,2,3,4 and 1.4e8 transduction units (TU)/mouse n=1 untransduced UT) and 11 days post-transduction, liver and lungs dissected and homogenised. Luciferase activity was quantified *in vitro* and normalised for total protein, expressed as relative light units (RLU)/mg total protein. Each bar represents a mean of two measurements from liver (blue bar) and lung (pink bar) homogenate.

3.3.5 Intravenous administration of rSIV.F/HN results in widespread biodistribution

Following on from the pilot study, a biodistribution study was conducted including a greater number of mice and an intranasal arm. rSIV.F/HN-EGFP_{Lux} was delivered by intravenous (n=4 males and 4 females, 2.58e8 TU/mouse) and intranasal administration (n=3 males and 3 females, 2.58e8 TU/mouse) to wildtype mice alongside untransduced mice (n=2 males and 3 females). Figure 3.8 shows representative *in vivo* bioluminescent images taken 35-days post-dosing, with vector distribution patterns following intranasal (Figure 3.8A) and intravenous (Figure 3.8B) administration shown alongside an untransduced mouse (Figure 3.8A). The signal is highly localised to the nose and lungs in mice administered vector by intranasal delivery, however the expression pattern is much more widespread when administered intravenously, with signal distributed across the lower thorax and abdomen, in the nose and in the tail. In addition, the maximum signal counts are significantly reduced in Figure 3.8B (5894 and 2130 counts) compared to Figure 3.8A (55127 and 51914 counts), suggesting the overall bioluminescent signal measured is lower following intravenous delivery despite a longer exposure time to capture images. The untransduced mouse shown in Figure 3.8A shows no evidence of bioluminescence signal.

Following *in vivo* imaging, seven different organs (lung, liver, kidney, heart, brain, gonads, spleen) were dissected from each mouse and imaged, grouped together in petri dishes. Figure 3.9 shows representative images from an intranasally dosed and untransduced mouse (Figure 3.9A) as well as two mice administered vector intravenously (Figure 3.9B). Using a 5-minute exposure to capture each image, the lungs from the intranasal mouse caused a saturation in bioluminescent signal, whilst a much reduced signal was observed from organs dissected from intravenously dosed mice. Figure 3.9B shows bioluminescent signal in the kidneys, lungs, gonads (testes in the representative image) and liver, with the strongest signal detected in the spleen, across all intravenous mice.

To quantify biodistribution more accurately, organ homogenate was prepared from each of the seven organs and luciferase activity measured. Figure 3.10 shows luciferase bioluminescence normalised for total protein, expressed in two graphs to compare mice delivered vector intranasally against untransduced mice (Figure 3.10A) and mice dosed intravenously to untransduced mice (Figure 3.10B). The expression pattern observed in

Figure 3.10 replicates the organ imaging (Figure 3.9), *in vivo* whole-body imaging (Figure 3.8) and furthermore the pilot study data from section 3.3.4, whereby intravenous administration of vector resulted in a diffuse and overall weaker expression pattern compared to the strong, localised pulmonary signal observed following intranasal delivery. Significant expression above background untransduced levels was detected in the lungs, liver, heart and spleen following intravenous administration (** = $p < 0.01$, **** = $p < 0.0001$ when compared to untransduced mice, two-way ANOVA, Šídák's multiple comparisons test, $n = 5/8$ mice/group). Significant luciferase expression above background was identified in the lungs of intranasal administered mice only (**** = $p < 0.0001$ when compared to untransduced mice, two-way ANOVA, Šídák's multiple comparisons test, $n = 5/6$ mice/group).

Finally, biodistribution results gained by software analysis of bioluminescent images and *in vitro* luciferase assays of organ homogenate were compared (Figure 3.11). A strong positive relationship was found (Pearson's correlation coefficient, $r = 0.9066$ and $p < 0.0001$, $n = 98$) demonstrating there is a significant concordance between both methods for quantifying luciferase activity. Similarly, the biodistribution patterns characterised in the pilot study, *in vivo* imaging, organ imaging and *in vitro* luciferase quantification were consistent, demonstrating overall weaker and diffuse biodistribution following intravenous delivery of rSIV.F/HN to mice.

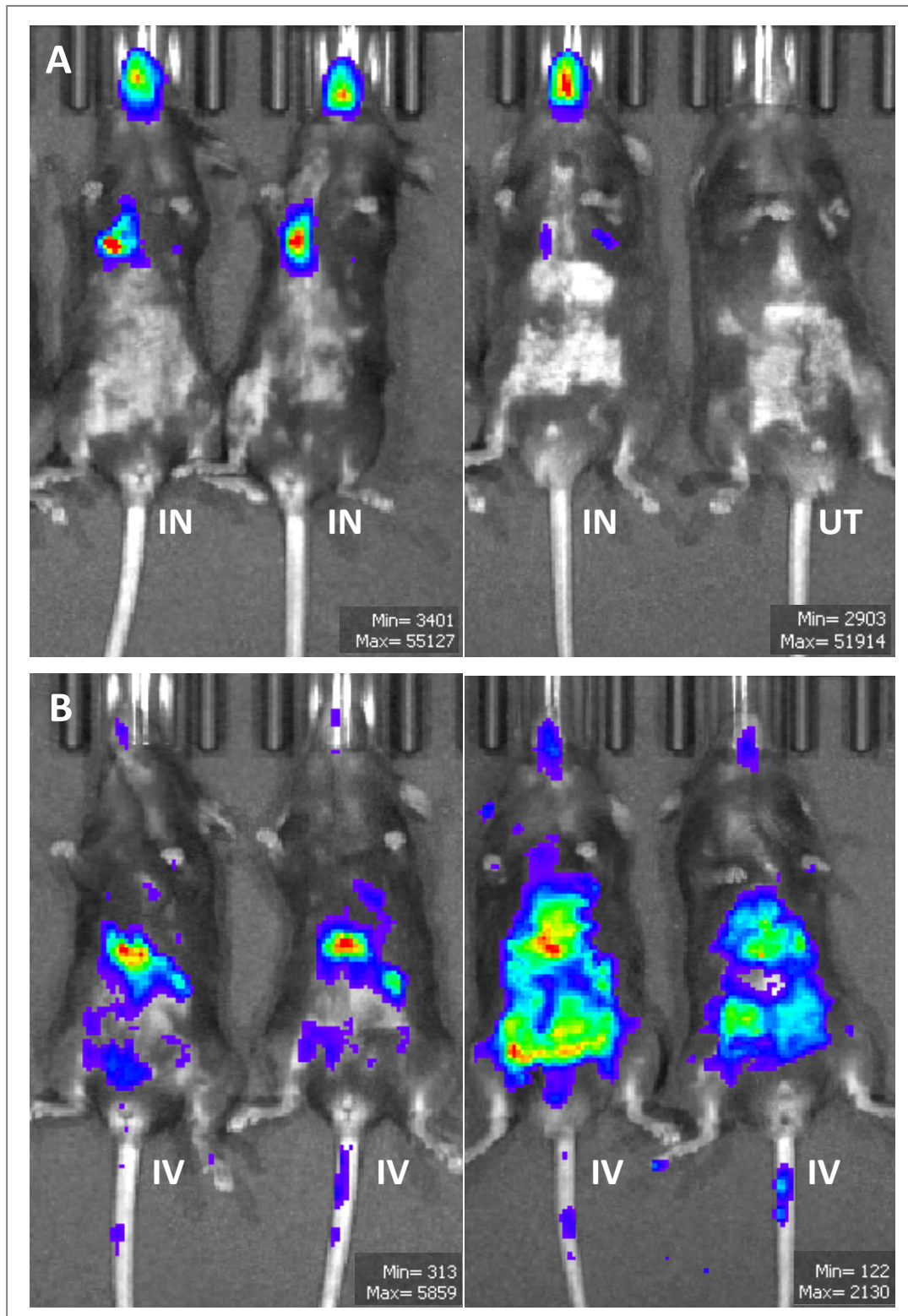


Figure 3.8- In vivo bioluminescent imaging following intranasal and intravenous delivery of rSIV.F/HN-EGFP_{Lux}.

Vector encoding the firefly luciferase and EGFP fusion (rSIV.F/HN-EGFP_{Lux}) was delivered to C57BL/6 wildtype mice by intranasal (n=3 females and n=3 males, 2.58e8 TU/mouse) and intravenous administration (n=4 females and n=4 males, 2.58e8 TU/mouse) alongside untransduced mice (n=3 females and n=2 males). 35-days post-transduction, each mouse received 150 mg/kg of luciferin substrate by intraperitoneal injection, and 15-minutes later imaged using the IVIS Spectrum CT system under isoflurane anaesthesia. Representative images of (A) 2 female mice and 1 male mouse dosed intranasally (IN) and 1 male untransduced mouse (UT) 3-minute exposure per image and (B) 2 female and 2 male mice delivered vector intravenously (IV) 5-minute exposure per image. Min and Max represent the minimum and maximum signal, measured in counts, per image.

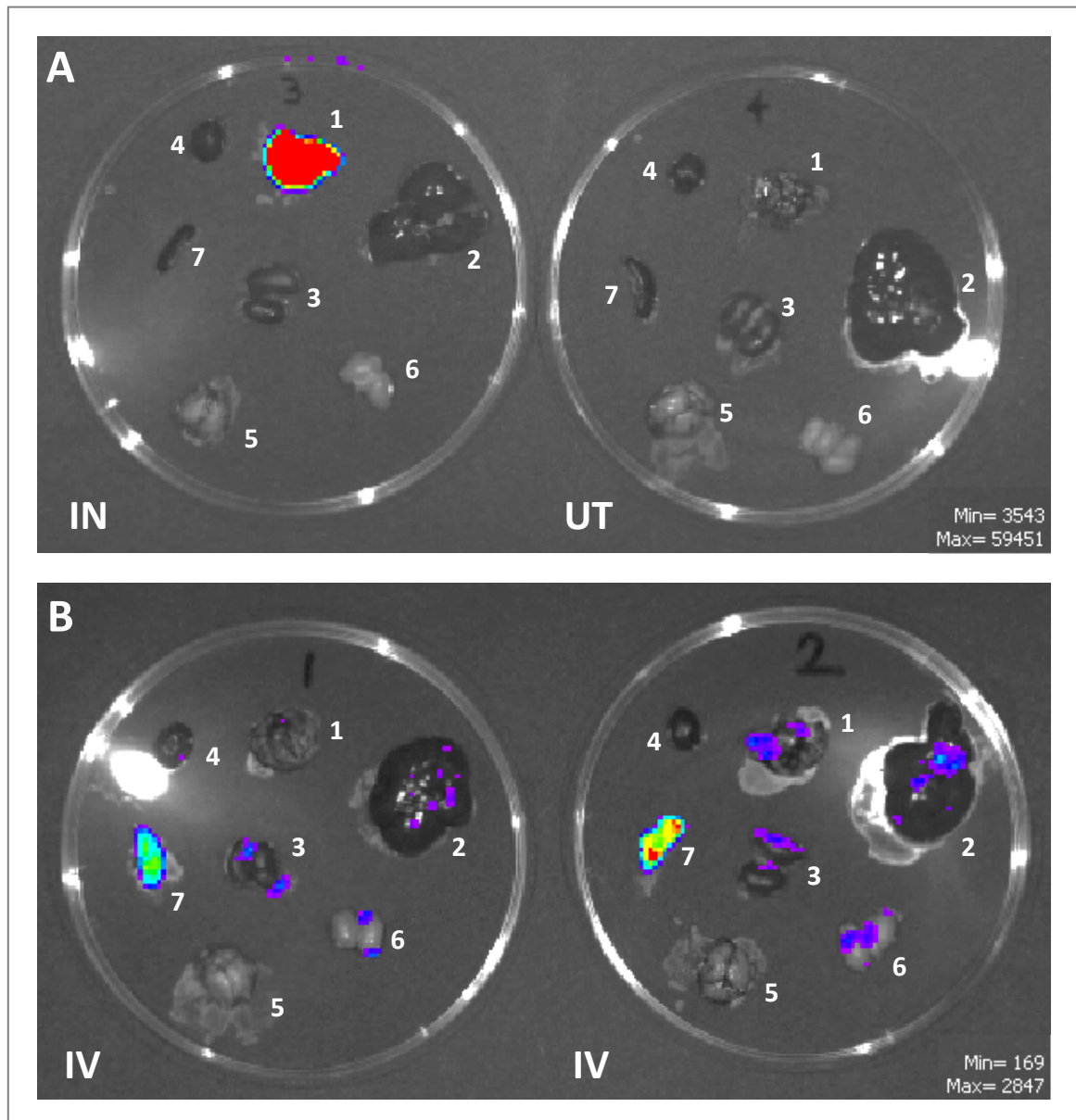


Figure 3.9- Bioluminescent imaging of organs dissected from mice transduced with rSIV.F/HN-EGFP Lux.

Vector encoding the firefly luciferase and EGFP fusion (rSIV.F/HN-EGFP Lux) was delivered to C57BL/6 wildtype mice by intranasal (IN n=3 females and n=3 males, 2.58×10^8 TU/mouse) and intravenous administration (IV n=4 females and n=4 males, 2.58×10^8 TU/mouse) alongside untransduced mice (n=3 females and n=2 males). 35-days post-transduction, each mouse received 150 mg/kg of luciferin substrate by intraperitoneal injection, and approximately 30-minutes later (1) lung (2) liver (3) kidney (4) heart (5) brain (6) gonads and (7) spleen dissected and imaged using the IVIS Spectrum CT system, 5-minute exposure per image. Representative images of (A) a male mouse dosed intranasally (IN) and a male untransduced mouse (UT) and (B) male mice delivered vector intravenously (IV). 5-minute exposure per image. Min and Max represent the minimum and maximum signal, measured in counts, per image.

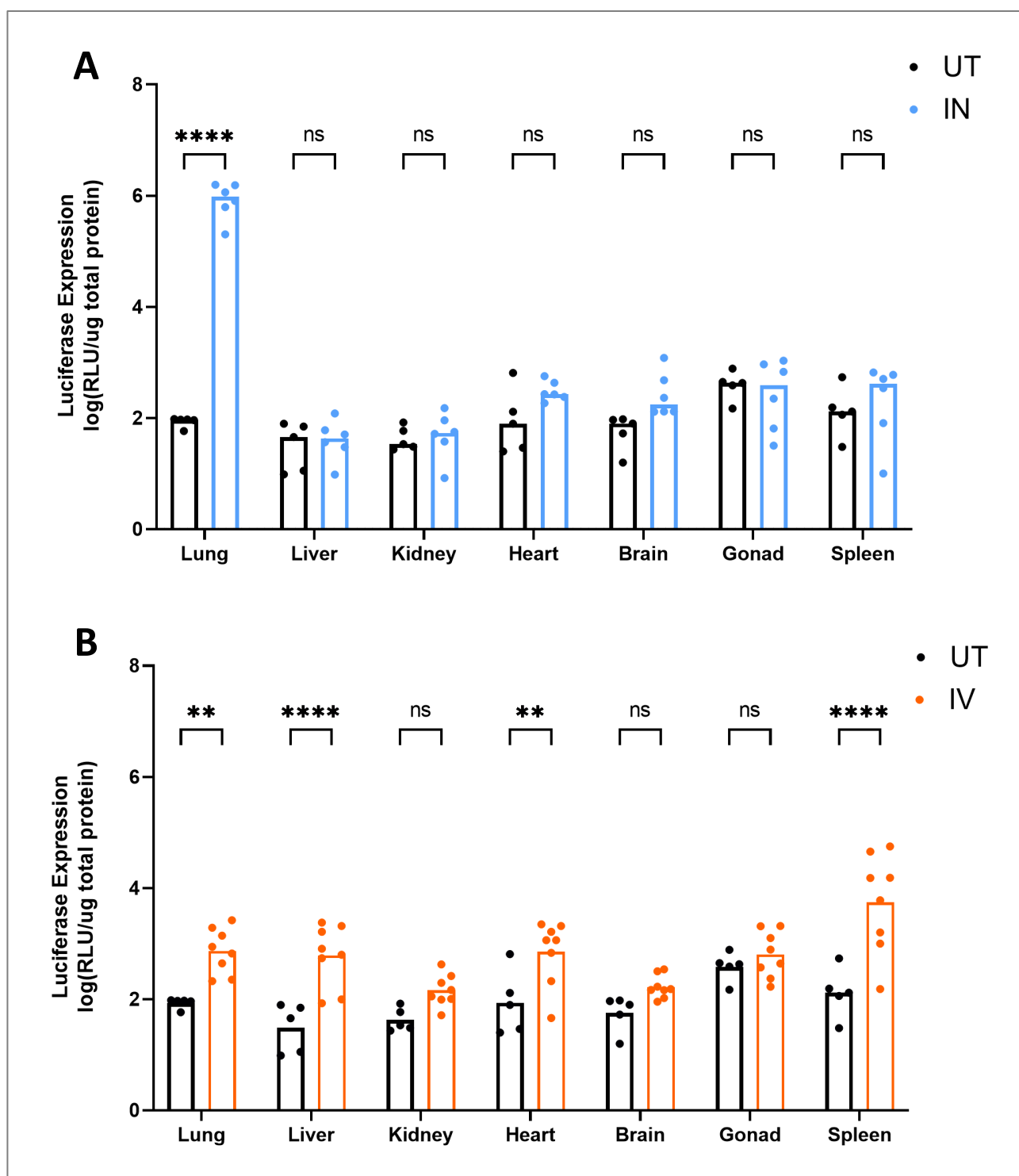


Figure 3.10- Biodistribution of rSIV.F/HN vector following intranasal and intravenous administration.

Vector encoding the firefly luciferase and EGFP fusion (rSIV.F/HN-EGFPLux) was delivered to C57BL/6 wildtype mice by intranasal (n=3 females and n=3 males, 2.58e8 TU/mouse) and intravenous administration (n=4 females and n=4 males, 2.58e8 TU/mouse) alongside untransduced mice (n=3 females and n=2 males). 35-days post-transduction, organs were harvested and homogenate prepared. Luciferase activity was quantified *in vitro* and normalised for total protein, expressed as relative light units (RLU)/mg total protein. Data has been log transformed, each bar represents mean luciferase expression per organ and each shape represents a single mouse. (A) Data from intranasal (IN blue) and untransduced mice (UT black), (B) data from intravenous (IV orange) and untransduced mice (UT black). ** = $p < 0.01$, **** = $p < 0.0001$, ns= non-significant when compared to untransduced mice, two-way ANOVA, Šídák's multiple comparisons test.

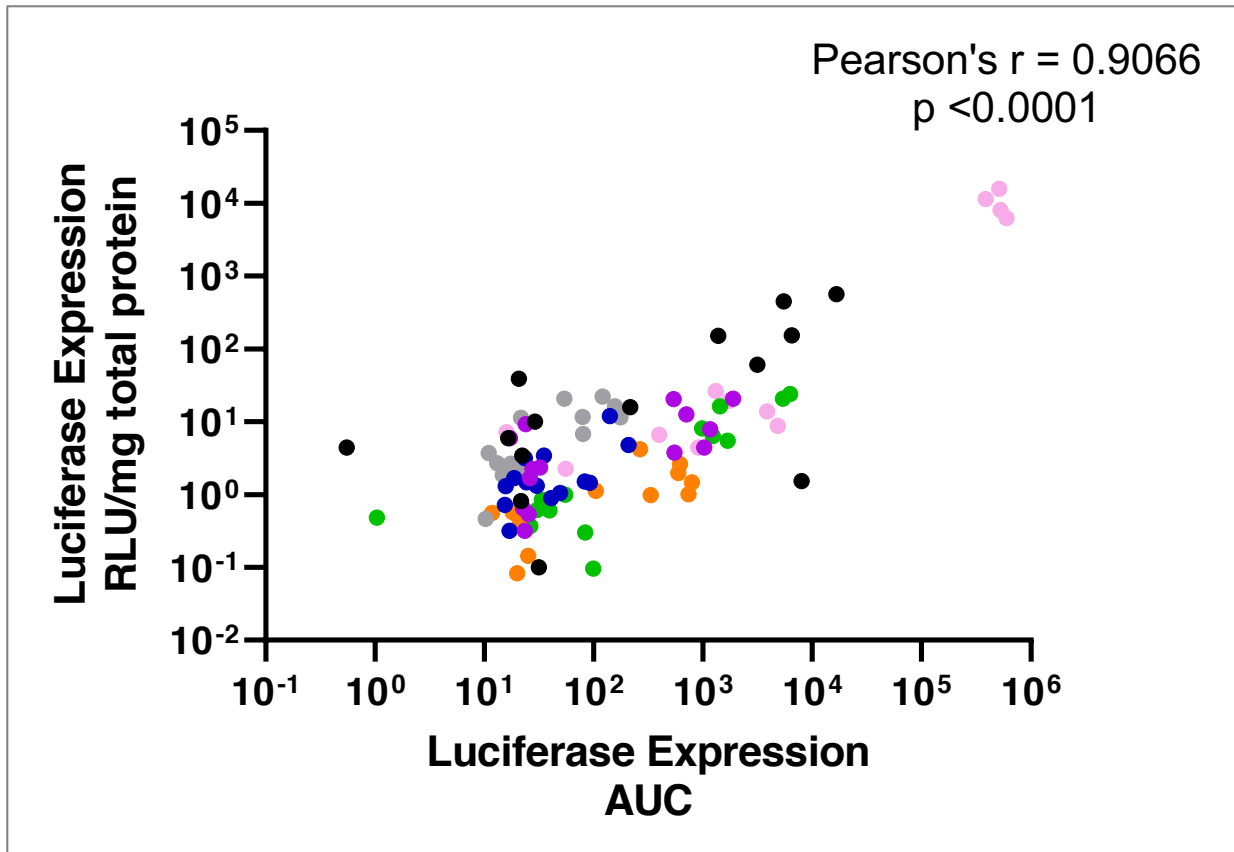


Figure 3.11- Correlation between luciferase expression determined by image analysis software and in vitro assay of organ homogenate.

Vector encoding the firefly luciferase and EGFP fusion (rSIV.F/HN-EGFPLux) was delivered to C57BL/6 wildtype mice by intranasal (IN $n=3$ females and $n=3$ males, 2.58×10^8 TU/mouse) and intravenous administration (IV $n=4$ females and $n=4$ males, 2.58×10^8 TU/mouse). 35-days post-transduction, each mouse received 150 mg/kg of luciferin substrate by intraperitoneal injection, and approximately 30 minutes later lung (pink), liver (green), kidney (orange), heart (grey), brain (blue), gonads (purple) and spleen (black) dissected and imaged using the IVIS Spectrum CT system, 5-minute exposure/image. Analysis of IVIS images was through the image-analysis software platform Fiji, taking area under curve (AUC) as the combined readout of signal intensity and area, as a readout of luciferase expression. Luciferase activity was then quantified in organ homogenate and normalised for total protein, expressed as relative light units (RLU)/mg total protein. A significant positive relationship was found between luciferase quantification from image-analysis and organ homogenate (Pearson's correlation coefficient, $r=0.9066$ and $p<0.0001$, $n=98$). Each shape represents a single organ ($n=98$ organs).

3.3.6 Transduction efficiency is reduced following repeat intravenous administration.

To investigate if repeat administration of rSIV.F/HN is tolerated via intravenous delivery, mice were delivered three doses of rSIV.F/HN vector at monthly intervals, ensuring the final vector encoded a different transgene to the previous two doses to differentiate from possible anti-transgene immune responses. Transgene expression was compared between mice receiving two doses of control vector, rSIV.F/HN-mADAMTS13, followed by a final dose of rSIV.F/HN-EGFP_{Lux} and mice that received two doses of TSSM buffer followed by a single dose of rSIV.F/HN-EGFP_{Lux}. Figure 3.12A and B show representative *in vivo* bioluminescent images of the anterior and posterior view of the mice, respectively. In both images, there is an obvious reduction in signal from the lower thorax and abdomen in the 3X group compared to single dosed mice. Similarly, no bioluminescent signal can be observed from the nose in the triple dose group.

In Figure 3.12C, images of dissected organs (liver, lung, spleen, brain, kidney, heart and gonads) further support that transduction efficiency is diminished upon repeat intravenous administration. No detectable signal can be visualised in any organ dissected from 3X mice, in contrast to signal detected in multiple organs from single dosed mice. To quantify the difference in transgene expression, software analysis of anterior whole-body bioluminescent images was performed and AUC measurements determined using the Fiji platform (Figure 3.12D). A statistically significant reduction in AUC measurements was found between mice that received 1 dose (1X) and 3 doses (3X) of rSIV.F/HN (*= $p < 0.05$, unpaired t-test, $n = 6$ mice/group). The mean AUC value calculated was 77% lower in the mice receiving three doses compared to a single dose (mean $\pm$ standard deviation = 1 dose (767.1 $\pm$ 513.9), 3 doses (179.1 $\pm$ 42.68)).

Following bioluminescent imaging, organ homogenate was prepared from the lungs, liver and spleen and luciferase expression quantified *in vitro*. Figure 3.13 shows luciferase expression, normalised for total protein, in untransduced mice (UT $n = 5$ mice) and mice that received a single intravenous dose (1X $n = 6$ mice) or three doses (3X $n = 6$ mice) of rSIV.F/HN vector. In the lungs, a small increase in luciferase expression above background can be observed in 1X mice, however no significant difference was found between any treatment group. Alternatively, mice administered a single dose of vector showed significant luciferase

expression above untransduced levels in the liver (** = $p < 0.01$, two-way ANOVA, Tukey's multiple comparisons test) and the spleen (**** = $p < 0.0001$) and similarly above 3X mice in the liver and spleen (** = $p < 0.01$, **** = $p < 0.0001$, respectively). Interestingly, luciferase expression in triple dosed mice was not significantly different to untransduced levels in the liver and spleen, suggesting transduction efficiency had been reduced to background levels after repeat rSIV.F/HN intravenous administration.

Across the data presented in Figure 3.12 and Figure 3.13, there is evidence to suggest that repeat administration of rSIV.F/HN via intravenous administration is not feasible and likely an anti-vector response is generated when vector is delivered systemically. The measurement of neutralising antibodies in mouse serum was performed in collaboration with PhD student, Nikhil Faulkner from The Francis Crick Institute/ Imperial College London. To test for neutralising antibodies against F/HN, serial dilutions of mouse serum was incubated with rSIV.F/HN-EGFP vector then applied to HEK293T cells. Antibody titre is the inverse of the dilution of serum required for 50% virus neutralisation compared to no-serum control (IC50). Figure 3.14 show the neutralising antibody titre measured in mice that received one dose (n=5), two doses (n=6) and three doses (n=2) of rSIV.F/HN compared to naïve mice (n=6). Due to the difficulty in collecting enough blood whilst dosing mice, only a subset of mice were sampled. In all cases blood was collected one month after the dose was given. Figure 3.14 demonstrates that even after a single intravenous dose, neutralising antibodies against the vector are detectable and there is an increase in antibody titre with successive administrations. In some cases, the maximum antibody titre was reached following two doses and by three doses both mice demonstrated maximal neutralising antibody titre. Despite samples missing from mice, there is evidence that a strong neutralising antibody response is generated when rSIV.F/HN vector is repeatedly delivered systemically.

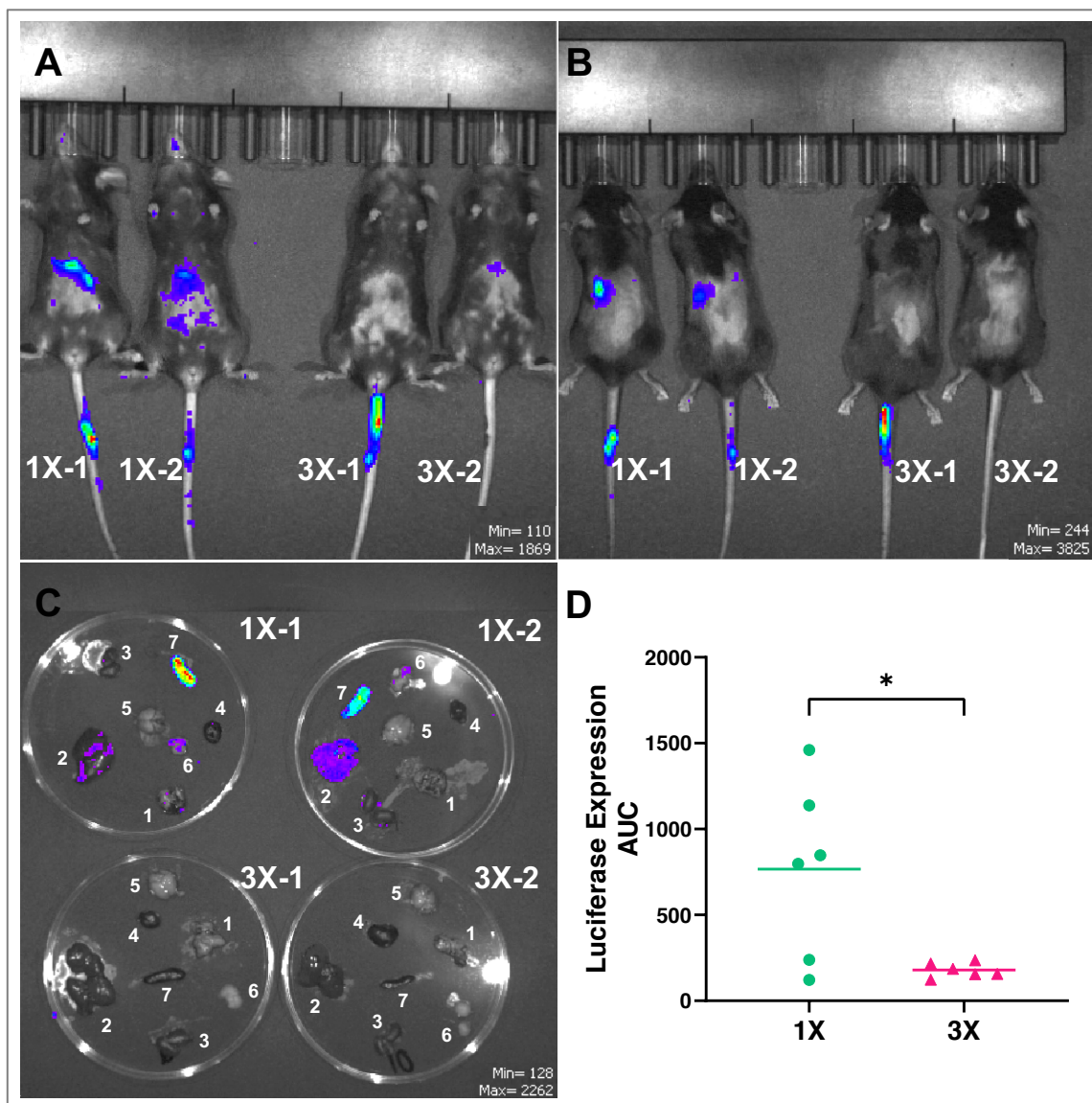


Figure 3.12- Bioluminescent imaging following repeat intravenous administration of rSIV.F/HN.

Wildtype C57BL/6 female mice were split into two groups at random. The single dose group (1X) received two doses of TSSM buffer followed by a single dose of rSIV.F/HN-EGFP_{Lux} (1.52×10^8 TU/mouse, $n=6$ mice). The triple dose group (3X) received two doses of control vector (rSIV.F/HN-mADAMTS13, 1.42×10^8 TU/mouse) followed by a final dose of rSIV.F/HN-EGFP_{Lux} (1.52×10^8 TU/mouse, $n=6$ mice). Mice received intravenous doses (vector or TSSM) at monthly intervals. One month following the final dose, each mouse received 150 mg/kg of luciferin substrate by intraperitoneal injection, and 15 minutes later imaged using the IVIS Spectrum CT system under isoflurane anaesthesia. Representative images showing (A) the anterior view and (B) the posterior view of two mice administered vector once (1X-1,2) and two mice administered three doses (3X-1,2), 3-minute exposure per image. (C) Organs were subsequently dissected from each mouse- (1) lung (2) liver (3) kidney (4) heart (5) brain (6) gonads and (7) spleen and imaged, 5-minute exposure per image. Min and Max represent the minimum and maximum signal, measured in counts, per image. (D) Analysis of anterior bioluminescent images was performed through the image-analysis software platform Fiji, taking area under curve (AUC) as the combined readout of signal intensity and area, as a readout of luciferase expression. AUC values shown for single dose (1X green circles) and triple dose (3X pink triangles) mice. Horizontal bars represent mean AUC values, each shape represents a single mouse. Significant reduction in overall signal between mice that received 1 dose and 3 doses of rSIV.F/HN (*= $p < 0.05$, unpaired t-test $n=6$ mice/group).

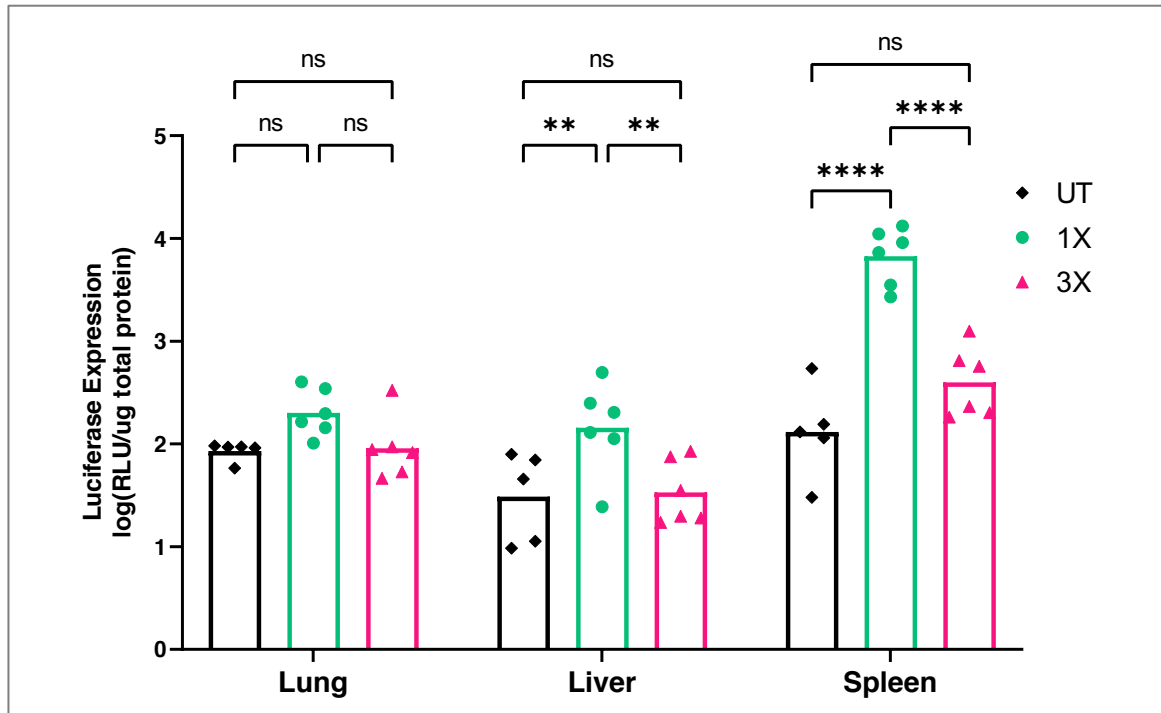


Figure 3.13- Luciferase expression measured in organ homogenate from intravenously dosed mice.

Wildtype C57BL/6 female mice were split into two groups at random. The single dose group (1X) received two doses of TSSM buffer followed by a single dose of rSIV.F/HN-EGFP_{Lux} (1.52e8 TU/mouse, green circles n=6 mice). The triple dose group (3X) received two doses of control vector (rSIV.F/HN-mADAMTS13, 1.42e8 TU/mouse) followed by a final dose of rSIV.F/HN-EGFP_{Lux} (1.52e8 TU/mouse, pink triangles n=6 mice). Mice received intravenous doses (vector or TSSM) at monthly intervals. One month following the final dose, organs were harvested and homogenate prepared from lung, liver and spleen. Luciferase activity was quantified *in vitro* and normalised for total protein, expressed as relative light units (RLU)/mg total protein. Previous data measured in untransduced mice (UT, black diamonds n=5 mice) shown as a comparison. Data has been log transformed, each bar represents mean luciferase expression per organ and each shape represents a single mouse. ** = p<0.01, **** = p<0.0001, ns= non-significant when comparisons between untransduced (UT), single dose (1X) and triple dose (3X) mice made, two-way ANOVA, Tukey's multiple comparisons test.

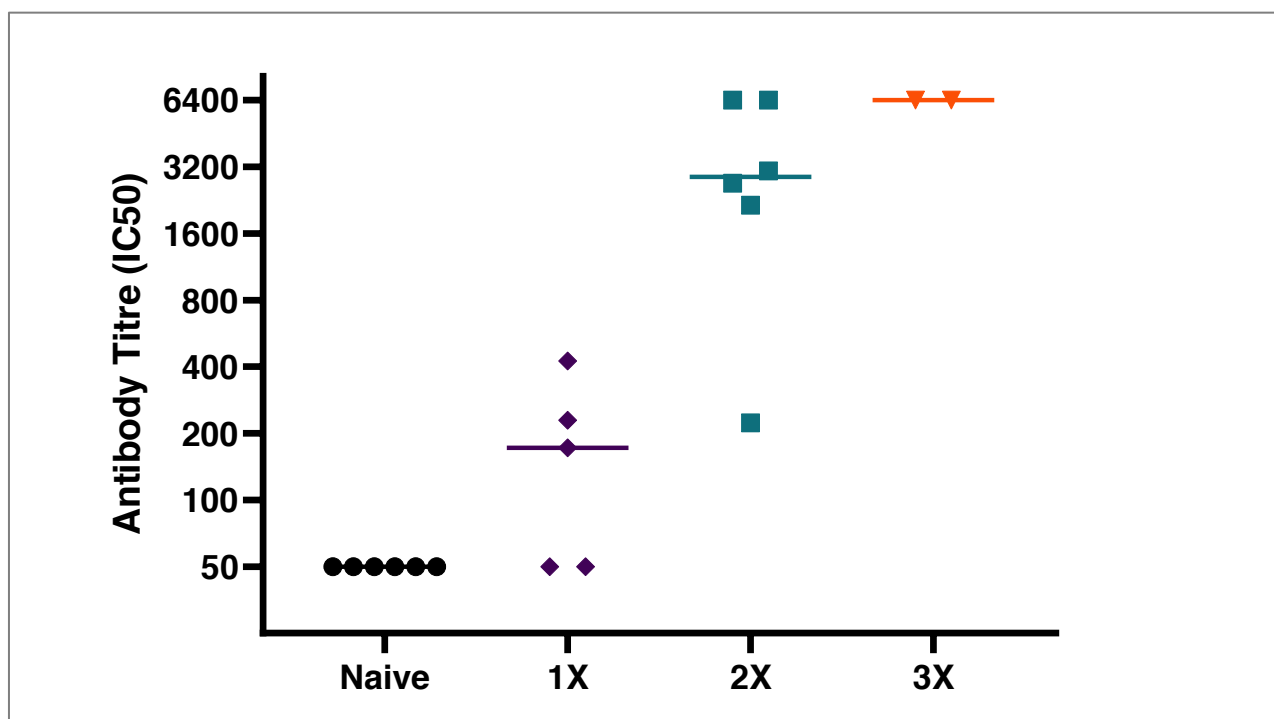


Figure 3.14- Measurement of neutralising antibodies produced in serum following repeat intravenous doses of rSIV.F/HN.

Antibody titre was measured in serum of wildtype C57BL/6 female mice that received three doses of rSIV.F/HN: two doses of control vector (rSIV.F/HN-mADAMTS13, 1.42e8 TU/mouse) and a final dose of rSIV.F/HN-EGFP Lux (1.52e8 TU/mouse), and mice that received a single dose of rSIV.F/HN-EGFP Lux (1.52e8 TU/mouse). Intravenous doses of vector were delivered at monthly intervals. Blood was sampled from mice one month after each rSIV.F/HN dose, just prior to the intravenous infusion of the next dose. Neutralising antibodies were measured by incubating rSIV.F/HN-EGFP virus with serial dilutions of mouse serum, then measuring the percentage GFP positive cells by flow cytometry 72 hours later. Antibody titre is inverse of the dilution of serum required for 50% virus neutralisation compared to no-serum control (IC50). Neutralising antibody titre shown in naïve mice (n=6 mice black circles), mice that received a single dose of rSIV.F/HN (n=5 mice purple diamonds), two doses (n=6 mice green squares) and three doses (n=2 mice orange triangles). Assay is saturated at 1/6400 dilution. Each shape represents a single mouse. Horizontal bars represent the median.

3.4 Discussion

Intranasal delivery of rSIV.F/HN has been characterised previously, with promising features including persistent pulmonary gene transfer (at least 2 years) following a single dose, highly localised and efficient transduction of the respiratory epithelium and feasible repeat administration at daily and monthly intervals without loss of transduction efficacy (96,98). As such, intranasal administration of rSIV.F/HN is progressing towards human trials for disease indications with primary lung pathology, such as cystic fibrosis (106). Importantly the vector may hold promise for broader disease indications. More recently, rSIV.F/HN delivered topically to murine lungs successfully achieved therapeutic plasma protein levels of secreted proteins, Factor VIII and alpha-1-antitrypsin (99). To investigate further the suitability of rSIV.F/HN vector for treatment of systemic diseases, intravenous administration to wildtype mice was investigated and compared to intranasal administration.

Vector encoding Gaussia luciferase (rSIV.F/HN-GLux) was utilised to compare the expression of secreted protein into the blood following intravenous and intranasal vector delivery. GLux was selected as the reporter transgene as it was demonstrated that GLux generated over 1000-fold greater signal intensity in mammalian cells when total bioluminescence was compared to firefly luciferase or Renilla luciferase (121). GLux therefore demonstrates enhanced sensitivity, with fewer positive cells required for detection of transgene expression. Furthermore, GLux is unique among the luciferase reporter genes given that it is secreted. Importantly, this allows for assessment of gene expression by repeated sampling in the same animal over time, as demonstrated by Griesenbach *et al.* (2011) by repeated measurement of GLux expression in BALF and serum of sheep (122). In the study assessing duration of expression following intravenous delivery of rSIV.F/HN-GLux, serum was repeatedly sampled from mice by tail venepuncture at weeks 1, 2, 5, 12, 24 and 52 post-transduction. Measurement of serum GLux levels over time significantly reduced mouse numbers by avoiding the use of separate groups for different experimental end points, contributing to the three R's (refine, reduce, replace). Additionally, repeat sampling allows longitudinal monitoring of transgene expression in the same animal over 52 weeks, better controlling for inter-animal variability and improving the reliability of data.

The long-term expression study demonstrated that rSIV.F/HN administered via intranasal and intravenous delivery leads to stable transgene expression in serum for the duration of the study (at least 12 months). This replicates data shown previously by Paul-Smith *et al.* (2018) whereby a single intranasal dose of rSIV.F/HN-GLux demonstrated stable expression in serum 12 months post-transduction, albeit at $1e7$ TU/mouse and with separate groups for each time point (99). Unfortunately Paul-Smith *et al.* (2018) reported serum GLux levels as % of day 7, thus direct comparisons to the expression levels generated in the present study cannot be made (99). Auricchio *et al.* (2002) demonstrated serum expression of erythropoietin 5 months after a single intranasal dose of AAV serotype 2/5, however expression had significantly declined by 5 months suggesting AAV transduction of the lungs elicits only short-lived serum expression due to gene silencing or immune recognition (123). To investigate systemic protein delivery using recombinant Sendai, Bitzer *et al.* (2003) delivered vectors encoding human factor IX (FIX) intravenously to mice and measured transgene expression in plasma. Human FIX was detected in treated mice however only 0.1% of human plasma levels were reached 5 days post-injection, limiting its therapeutic potential (124).

The overall trend in both the intravenous and intranasal cohorts was a dose-dependent increase in transduction efficiency. However only the intranasal cohort demonstrated a statistically significant increase in serum GLux levels between doses and above background levels. This may suggest that increased doses ($>2.4e8$ TU/mouse) of vector delivered topically may achieve higher GLux expression in serum, however higher doses were not tested and thus this cannot be concluded. The highest dose ($2.4e8$ TU/mouse) was the maximum feasible dose that could be administered to mice while adhering to the dosing volume limitations stated in our project licence. However higher titres of virus have been produced previously, thus a future experiment investigating higher doses may be feasible.

The delivery method with superior transduction efficiency changed dependent on the vector dose. Intranasal delivery was more potent at the lowest dose ($2.4e7$ TU/mouse), both delivery methods performing equally at the medium dose and intravenous delivery demonstrating enhanced efficacy (approximately 10-fold) only at the highest dose tested

(2.4×10^8 TU/mouse). Vector delivered by intravenous administration demonstrated a threshold dose below which GLux expression was at background levels. This may be due to vector being diluted among multiple organs following systemic delivery compared to the highly localised transduction of the nose and lungs following intranasal delivery. Thus higher doses of vector are required to achieve detectable transgene expression in serum following intravenous delivery.

As can be shown by the biodistribution of rSIV.F/HN following intravenous delivery, the original aim to compare the lungs and liver as factories for secretion of systemic protein is an over-simplification due to widespread organ transduction. Instead a comparison in systemic protein production following two delivery methods has been demonstrated. In a similar experiment, Griesenbach *et al.* (2002) compared delivery methods for production of systemic therapeutic protein, IL-10, utilising recombinant Sendai vector. Substantial serum levels of IL-10 were detected, with 2-fold greater levels observed following intranasal administration compared to gold-standard intramuscular injection. Significantly, 30x higher expression of IL-10 was observed following intrapulmonary delivery compared to intranasal, albeit measured in serum two days post-infection rather than the 12 month study shown here (101).

To characterise biodistribution of rSIV.F/HN when delivered systemically to mice, firefly luciferase was selected as the reporter gene. The advantages of firefly luciferase over gaussia or Renilla luciferase for *in vivo* imaging are two-fold, the bioluminescence range of coelenterazine luciferases is within the blue wavelength which can be absorbed by pigmented molecules such as haemoglobin whereas firefly luciferase emits into the red spectra which can penetrate tissue for improved imaging. Secondly due to the kinetics of firefly luciferase the signal decays slower (50% loss of signal in 10 minutes) compared to the much faster kinetics of gaussia luciferase (90% loss in 90 seconds) permitting for images to be taken (121).

Widespread distribution, determined by qPCR analysis of vector copy number, was previously demonstrated by Sarracino *et al.* (2014) following intravenous delivery of different pseudotypes of HIV-1 lentiviral vector (VSV-G and murine leukaemia virus (MLV)-

amphotropic envelope) and γ -retroviral vectors in adenosine deaminase (ADA) deficient mice (125). Previously, it was shown that several cell types within the lungs are transduced by the rSIV.F/HN pseudotyped vector, including airway epithelial cells, goblet and club cells and type I and II pneumocytes (106). However it was not known the diversity of cell types rSIV.F/HN could transduce outside of the airways. The Sendai virus envelope protein HN binds to specific sialic acid-containing gangliosides present on cell surfaces (82). Therefore the tropism of recombinant rSIV.F/HN should be dictated by the expression of receptors, known to be ubiquitous across many cell and tissue types (83). Successful transduction has been demonstrated with recombinant Sendai vectors in the vascular endothelium, skeletal muscle, cardiomyocytes, upper and lower respiratory tract, brain and retina across multiple species including rats, mice, ferrets and sheep (88,126–130). The results presented here by the firefly luciferase biodistribution study revealed that systemic delivery of rSIV.F/HN vector results in widespread organ transduction, reflecting previous literature utilising recombinant Sendai vectors. A limitation of these findings however, is that luciferase expression was quantified per organ and not on a per cell basis. To further support the current data, analysis of luciferase expression intensity per cell could have been determined by flow cytometry. Alternatively, bulk DNA extracted from organs could have been utilised to determine vector copy number per organ.

Luciferase expression determined in lung homogenate following a single intranasal dose of rSIV.F/HN shows comparable levels between the current study and by Griesenbach *et al.* (2012) ($\sim 10^4$ - 10^5 RLU/mg protein) (98). However, a 10-fold greater dose was delivered to mice in the current study (1e8 TU/mouse) compared to previously reported by Griesenbach *et al.* (2012) (1e7 TU/mouse) (98). This difference in transduction efficiency may be related to the use of a fusion transgene in the current study (rSIV.F/HN-EGFP/Lux) compared to the luciferase only vector reported previously (rSIV.F/HN-Lux) (98). Interestingly, enhanced luciferase activity was reported in lung homogenate following intranasal delivery of recombinant Sendai vectors encoding luciferase ($>10^5$ RLU/mg protein, 1e7 TU/mouse), albeit with a short duration of expression (88).

Broad tropism may suggest the potential of rSIV.F/HN vector as a therapeutic for wider disease indications. For example, respiratory diseases of the pulmonary endothelium,

haematological diseases including clotting disorders, infectious diseases and inherited metabolic disorders to name a few. The effective transduction of organs and tissues other than the lungs, may allow greater flexibility in the selection of transgene, including those which encode proteins which may be more efficiently post-translationally modified in organs such as the liver rather than the airways. To investigate rSIV.F/HN as a therapeutic for a systemic disease, intravenous delivery of rSIV.F/HN for the treatment of congenital clotting disorder, TTP, is investigated in Chapter 4.

Widespread organ transduction may however enhance the likelihood of immune recognition and anti-vector/ transgene responses. Many groups use tissue-specific promoters to drive expression away from off-target sites and towards organs such as the liver. Follenzi *et al.* (2004) achieved stable plasma expression of human FIX (25 weeks post-transduction) in mice following systemic delivery of VSV-G lentiviral vectors carrying the endogenous albumin promoter (131). Similarly, Mount *et al.* (2002) utilised AAV vectors carrying the human α 1-antitrypsin promoter and ApoE enhancer to drive expression of canine FIX in a canine model of Haemophilia B. Stable plasma expression was observed in 3/4 dogs (40+ weeks post-transduction) with 1 dog exhibiting anti-FIX antibodies and consequently a loss of transduction efficiency (132).

Despite use of tissue-specific promoters, exclusive expression in target cells is not always achieved. Brown *et al.* (2007) found that despite using the endogenous albumin promoter, significant transgene expression was measured in splenocytes (0.4% as determined by flow cytometry) in addition to hepatocytes following intravenous delivery of VSV-G lentiviral vectors (133). Of greater concern, was that a subset of the transduced splenocytes were CD45 positive cells, a hallmark of cells from the haemopoietic lineage which could develop into antigen presenting cells. Brown *et al.* (2007) developed a strategy to prevent transgene expression in splenocytes by including a micro RNA (miRNA) target sequence in the vector genome, complementary to a haematopoietic-specific miRNA that would lead to selective mRNA degradation in the spleen. The inclusion of the miRNA sequence significantly reduced transgene expression in splenocytes without mitigating hepatocyte expression, resulting in significantly enhanced and stable FIX expression in a haemophilia B mouse model (133).

Utilising the same strategy, Matsui *et al.* (2011) included a miRNA target sequence and hepatocyte-specific promoter to drive liver expression of FVIII following intravenous delivery of VSV-G vectors (134). However the combination of promoter and miRNA sequence was not sufficient to prevent anti-FVIII inhibitory antibody production. A change in pseudotype to the baculovirus envelope glycoprotein, GP64, which changed the vector tropism away from splenocytes was the necessary step to achieve stable expression of FVIII in mice and prevent inhibitory antibody production (134). As significant expression is observed in the spleen following intravenous delivery of rSIV.F/HN, the detargeting strategy may suggest an approach that could be utilised if intravenous delivery of rSIV.F/HN is pursued further. It should be established if cells from the haemopoietic lineage are transduced in the spleen. However, given the expression of GLux is stable for at least 12 months following a single intravenous dose, the development of antibodies against GLux is theoretically minimal or at least insufficient to prevent gene expression. However the contrasting results demonstrated by Brown *et al.* (2007) and Matsui *et al.* (2011) illustrate that the transgene itself influences the level of immune recognition and thus response (118). It is reasonable to assume therefore that encoding other therapeutic transgenes within rSIV.F/HN vectors may elicit an immune response when delivered intravenously, despite GLux data shown here suggesting a level of immune tolerance.

Despite demonstrating sustained long-term expression following a single dose, rSIV.F/HN was repeatedly administered to explore if intravenous delivery of vector provokes an immune response. To separate between an anti-transgene and anti-vector response, the first two doses of vector encoded a different transgene (mADAMTS13 in this instance) than the final dose which encoded firefly luciferase. Differences in luciferase expression between single dose and repeatedly dosed mice was evident by bioluminescent imaging, overall decrease in signal by 77% following repeat administration, and from organ homogenate where a significant reduction in expression towards background levels was observed in the liver and spleen of mice receiving three doses.

A reduction in transduction efficiency is in contrast to data presented by Griesenbach *et al.* (2012) where repeat intranasal administration of rSIV.F/HN was investigated. Although the experimental design was closely matched between studies, Griesenbach *et al.* (2012)

demonstrated no loss in transduction efficiency following repeat intranasal dosing (three doses at monthly intervals). One difference in experimental design was the dose delivered to mice, which was $1\text{e}7$ TU/mouse intranasally and $1.5\text{e}8$ TU/mouse intravenously. Thus the 15-fold difference may have a role in eliciting a stronger immune response due to a greater number of vector particles. However, it was shown in the GLux dose-response/duration study that a dose of $2.4\text{e}7$ TU/mouse was insufficient to produce significant GLux expression above background levels. Perhaps delivering the lower dose would not elicit sufficient transgene expression to compare transduction between single and triple dosed groups. It is plausible that a lower dose of vector is tolerated following repeat intravenous delivery, however this seems unlikely if a moderate neutralising antibody response was generated following just one administration and a strong response produced following two doses.

To next determine if the fall in transduction efficiency was immune-related, neutralising antibodies against rSIV.F/HN were investigated in mouse serum. Antibody titre was calculated as the inverse dilution of serum required for 50% virus neutralisation (IC₅₀). Mice that received a single intravenous dose of vector demonstrated a detectable level of neutralising antibodies (mean IC₅₀ ~200). After two doses, a strong induction of neutralising antibodies was observed (mean IC₅₀ ~3200) which plateaued to the maximum antibody titre (IC₅₀ 6400) following three doses, albeit with limited n numbers. Therefore as early as the second dose of rSIV.F/HN there is likely a significant reduction in transduction efficiency due to the strong antibody response.

Interestingly, Griesenbach *et al.* (2017) performed a similar *in vitro* transduction inhibition assay to quantify neutralisation antibodies in pooled mouse sera following a single intranasal dose of rSIV.F/HN ($1\text{e}8$ TU/mouse). A 50% inhibition of rSIV.F/HN transduction was observed at a 1/32 dilution, equivalent to a IC₅₀ of 32 (106). The antibody titre is higher following a single intravenous administration than intranasal, which could explain why repeat intravenous delivery is not tolerated and a significant reduction in transduction efficiency is observed. However this interpretation is made cautiously as neutralising antibodies were not investigated after successive intranasal doses and differences in protocol such as vector concentration prevents direct comparison between studies. Similarly, Sinn *et al.* (2008) demonstrated that repeat administration (7x weekly doses) of

FIV-GP64-Lux to the nasal epithelium was feasible with a dose-dependent increase in luciferase expression observed without significant detection of neutralising antibodies (equivalent to IC₅₀ of <10) in BALF (135). This was in contrast to a significant neutralising antibody response generated following repeat intranasal administration of AdV serotype 5 vectors, with significant antibodies detected after 2 administrations in BALF and a corresponding fall in luciferase expression (135). Therefore, the generation of significant neutralising antibodies and reduction in transgene expression following repeat intravenous administration of rSIV.F/HN was expected to be an artefact of the delivery method, particularly given repeat intranasal administration of lentiviral vectors was shown to be feasible by Sinn et al. (2008) and Griesenbach et al. (2012) previously (98,136).

Illustrated across all three studies is the large variance in data within intravenous treatment groups, which is in contrast to mice administered vector by intranasal sniffing whereby mice performed similarly with much less variance between groups. This is believed to be an artefact of intravenous delivery in mice, which is significantly more difficult owing to the small size of lateral tail veins that are utilised for dosing. An added layer of variance is the use of needles to deliver vector which can introduce dead volume and are inevitably much harder to deliver the same dose to each mouse. Intranasal delivery requires application of vector to the nose with a pipette, thus requiring less skill and delivering more consistent doses. Overall however, consistency could be improved with greater expertise in intravenous injections.

3.5 Conclusion

In summary, rSIV.F/HN lentiviral vectors successfully achieved persistent (at least 12 months) expression of a reporter protein in serum following a single intravenous dose. Serum expression was 10-fold higher compared to intranasal administration at the highest dose tested, with comparable expression observed at lower doses. In contrast to highly localised pulmonary expression following intranasal administration, rSIV.F/HN transduces several organs when administered systemically, with an overall lower signal intensity detected. A strong neutralising antibody response is detectable after just two doses of

vector via intravenous administration, with a significant reduction in transduction efficiency observed after three doses.

The production of a strong anti-vector response following repeat intravenous administration may suggest that systemic rSIV.F/HN delivery is not suited for chronic diseases that require lifelong transgene expression and thus repeat doses. However, other therapeutic viral vectors such as AAV are equally limited to single administrations due to immune recognition, yet have shown remarkable long-term correction of disease following a single AAV dose (10+ years in haemophilia B patients and 4+ years in haemophilia A patients) (104,137). Moving forward, strategies such as tissue-specific promoters could be investigated to enhance transgene expression in desired organs. Alternatively, utilising the lungs as a factory for production of systemic proteins has previously demonstrated sustained expression of therapeutic transgenes in serum with feasible repeat administration. Intranasal administration might therefore be an efficient yet safer delivery method than systemic delivery moving forward, particularly if the disease application requires long-term gene delivery.

Chapter 4- Correction of ADAMTS13 Deficiency in Knockout Mice

4.1 Introduction

4.1.1 Project background

Previously rSIV.F/HN was used for the delivery of Factor VIII and alpha-1-antitrypsin to the lungs of mice, successfully achieving therapeutic plasma protein levels and providing proof-of-principle that the lung can be used as a factory for the production of systemic proteins (99). Importantly, rSIV.F/HN may have application for broader disease indications. Haematological condition, thrombotic thrombocytopenic purpura (TTP) was selected as an exemplar disease to investigate the application of rSIV.F/HN for systemic disorders.

4.1.2 Thrombotic thrombocytopenic purpura (TTP)

Thrombotic thrombocytopenic purpura (TTP) is a rare (~1 case per 100,000) but life-threatening haematological disease characterised by the widespread dissemination of platelet-rich thrombi within the microvasculature (138). TTP is caused by a severe deficiency (<0.1U/ml plasma levels) of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, ADAMTS13 (139). Congenital TTP (5% of TTP cases) manifests through biallelic mutations within the *ADAMTS13* gene, however in the majority of cases ADAMTS13 deficiency is acquired through the presence of autoantibodies (138).

4.1.3 von Willebrand factor (VWF)

ADAMTS13 has one known substrate; it specifically cleaves a scissile bond (Tyr¹⁶⁰⁵-Met¹⁶⁰⁶) within the A2 domain of von Willebrand factor (VWF), a plasma glycoprotein with an essential role in haemostasis (140). Exclusively produced by endothelial cells and megakaryocytes, VWF is synthesised as multidomain monomers containing multiple binding sites that are inherent to its function (Figure 4.1) (141). Following synthesis, VWF monomers assemble into multimers that are packaged into specialised storage organelles found within endothelial cells called Weibel-Palade bodies (WPBs). Smaller VWF multimers are released constitutively from endothelial cells in addition to ultra-large VWF (ULVWF) multimers released from WPBs in response to secretagogue signals such as thrombin, histamine, epinephrine and vasopressin (142).

When the haemostatic environment is at rest, VWF multimers circulate in the blood in a loosely coiled or globular conformation while platelets circulate in a rested state. Upon insult to the vascular wall, VWF binds to exposed collagen in the extracellular matrix unravelling into a string-like conformation in response to the flow of blood passing the vascular site (141). As the multimers elongate under shear stress, platelet receptor binding sites in VWF A1 domains are exposed and consequently the larger the VWF multimers the more 'sticky' or thrombogenic they become due to their greater capacity to bind platelets (143). Binding of platelets to the VWF A1 domain is mediated by the platelet surface glycoprotein Ib-IX-V transmembrane protein α (GPIb α) (144). Binding of GPIb α to the VWF A1 domain has a rapid attachment and dissociation rate causing platelets to attach transiently as they translocate along VWF multimers, slowing their velocity to 2% of free-flowing platelets (143). GPIb α -VWF binding activates platelets, eliciting binding between additional platelet receptors and VWF, as well as recruitment of VWF from storage bodies and further platelet activation through the production of adenosine diphosphate and thromboxane A₂, culminating in the formation of a platelet plug (145). Individuals with dysfunctional VWF or lower plasma concentrations of the protein consequently form a platelet plug much more slowly or in severe cases not at all.

4.1.4 ADAMTS13

Levy *et al.* (2001) first identified ADAMTS13 as the VWF-cleaving protease following genetic linkage analysis of four families with congenital TTP (139). Linkage analysis mapped to the locus 9q34 containing an unidentified ORF, C9ORF8, which showed homology to and shared characteristics with ADAMTS proteases. Specifically, ADAMTS13 contains thrombospondin-1-like (TSP1) repeats and is dependent upon cations Zn²⁺ and Ca²⁺ for activity, and henceforth was named the 13th member of the ADAMTS family (146). ADAMTS13 contains 29 exons and encodes a multidomain protease of 1427 amino acids (Figure 4.1) (147). ADAMTS13 is synthesized predominantly by hepatic stellate cells however other cell types including renal podocytes, endothelial cells and platelets contribute to circulating plasma levels (140). ADAMTS13 has unique qualities; it is released as an active enzyme following intracellular folding, it specifically cleaves a single peptide within its only known substrate VWF, there are no known inhibitors of ADAMTS13 and knowledge of its mechanism of clearance remains limited (146,148).

4.1.5 ADAMTS13-VWF interaction in haemostasis

During normal haemostasis, large VWF multimers are proteolysed into smaller, less reactive forms. Cleavage by ADAMTS13 is dependent upon a conformational change of VWF in a shear-induced manner. ADAMTS13 is particularly active against the highly thrombogenic, ULVWF multimers released from endothelial cells upon cellular activation by a secretagogue stimulus (138). Once secreted, the multimers become extended and ADAMTS13 cleavage sites buried within VWF A2 domains are exposed and proteolysis occurs successively, releasing smaller VWF multimers into the circulation, regulating VWF's platelet-capturing and thus thrombogenic potential (Figure 4.2) (149).

In contrast, soluble VWF multimers circulate in the plasma in a closed globular conformation under normal physiological conditions. Globular VWF under low shear restricts binding of ADAMTS13 proximal domains (metalloproteinase, disintegrin-like, TSP1 repeat 1, cysteine-rich and the spacer domains (MDTCS)) and access to the cleavage site is shielded by a β -sheet within the VWF A2 domain (150). In fact, ADAMTS13 also circulates in a closed, folded conformation through the interaction of distal TSP8-CUB2 domains and the spacer domain (151). Zanardelli *et al.* (2009) proposed an initial interaction between the constitutively exposed C-terminal (C') domains of VWF, specifically D4-CK, and the distal domains of ADAMTS13 including TSP5-CUB domains, without proteolysis occurring (152). This initial binding to VWF distal domains releases ADAMTS13's autoinhibition mechanism leading to conformational activation. At sites of high shear stress, VWF unravels in a shear-induced manner and the initial ADAMTS13-VWF docking enables subsequent conformational changes in the VWF molecule, eventually allowing high affinity binding between ADAMTS13 cysteine-rich and spacer domains with the VWF A2 domain (153). Further interactions between residues in the disintegrin-like and A2 domain result in the correct positioning of the metalloprotease domain, essential for proteolysis of the Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ peptide bond (146).

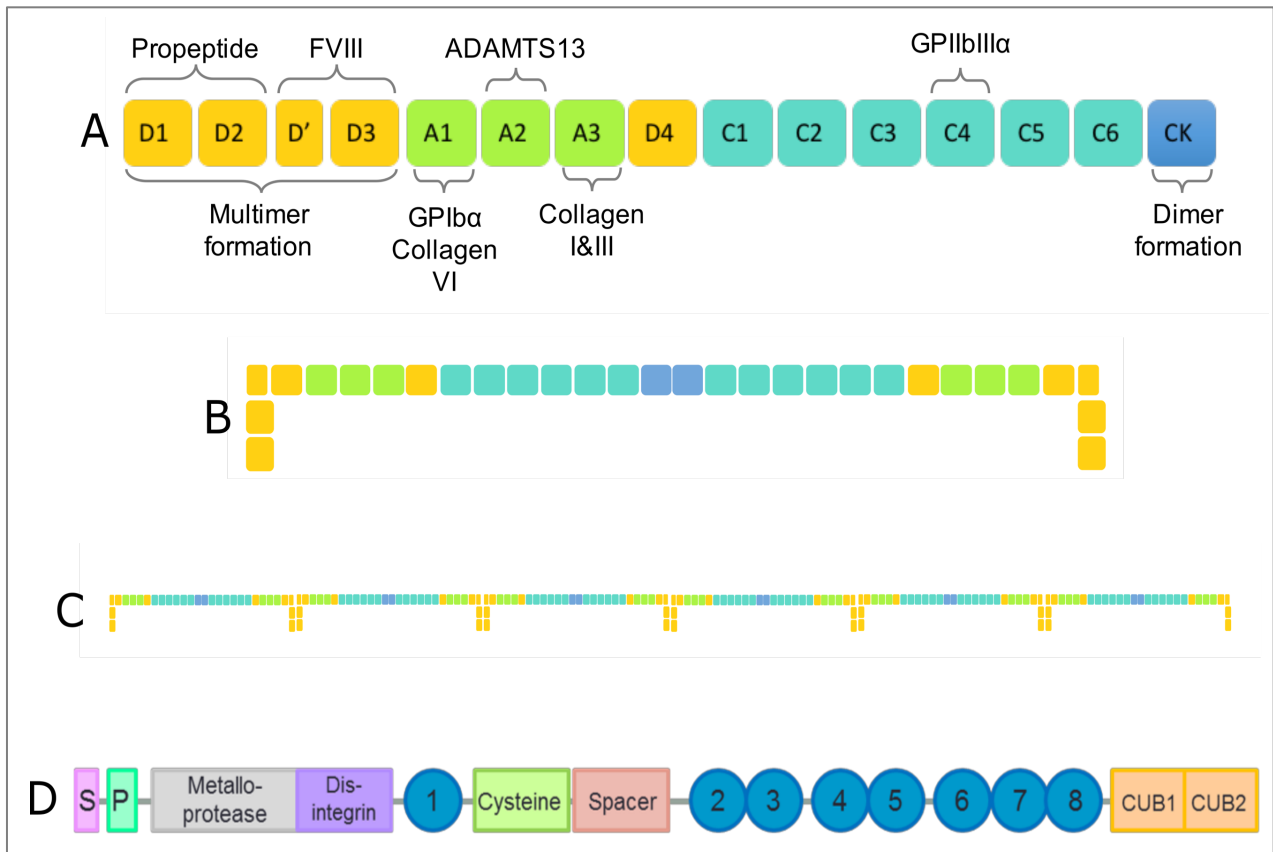


Figure 4.1- Schematic of von Willebrand factor (VWF) and a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, (ADAMTS13) domain organisation.

A) VWF monomer with labelled domains and binding sites for factor VIII, platelet receptors GPIIb/IIIα and GPIIb/IIIα, and collagen. ADAMTS13 cleavage site located in the A2 domain (Tyr¹⁶⁰⁵-Met¹⁶⁰⁶). B) VWF monomers first dimerise through adjacent cysteine knot (CK) domains and subsequently dimers form N-terminal multimers (C) through juxtaposed propeptide (D1, D2) and D', D3 domains. D) Domain organisation of ADAMTS13 include a signal peptide (S), a propeptide (P), a metalloproteinase domain, a disintegrin-like domain, the first TSP1 repeat (1), a cysteine-rich domain, a spacer domain, seven TSP1 repeats (2–8) and two CUB domains (CUB1 and CUB2).

4.1.6 TTP disease pathophysiology

Individuals with TTP have deficient ADAMTS13 levels and thus ULVWF multimers remain uncleaved tethered to endothelial cells in a thrombogenic form, capturing platelets and accumulating in size (Figure 4.2B) (154). This leads to the formation of VWF- and platelet-rich thrombi which when dislodged can disseminate and become trapped in small arterioles and capillaries causing vessel occlusion, multi-organ ischaemia and death if untreated (140). Organs with high arteriole pressure (brain, kidney, heart, and pancreas) may be more susceptible to accumulation of thrombi in acute TTP attacks compared to organs with lower pressure (lung and liver) due to the reduced shear stress acting upon VWF (154). However, ADAMTS13 deficiency is not sufficient for presentation of clinical symptoms and some individuals with a congenital deficiency in ADAMTS13 may not be diagnosed until early adulthood (155). It is now understood that a second genetic or environmental trigger is required to induce the onset of TTP symptoms. This could be an event such as trauma or pregnancy which causes endothelial cell damage or activation and release of ULVWF, triggering sudden widespread thrombi formation (156).

Individuals suffering an acute TTP attack experience severe thrombocytopenia (a fall in platelet levels due to the consumption of platelets in thrombi), microangiopathic haemolytic anaemia recognised by red blood cell fragmentation (schistocytes) in peripheral blood smears, accompanied by a severe deficiency in ADAMTS13 (<10% activity) (148). Presence of anti-ADAMTS13 autoantibodies can further differentiate between acquired and congenital forms (157). Alwan *et al.* (2017) identified high anti-ADAMTS13 IgG antibody and low ADAMTS13 antigen levels as predictive of mortality in individuals presenting with acute TTP in a prospective study in the UK (158). In fact, individuals presenting within the highest quartile for antibodies in combination with the lowest quartile for ADAMTS13 levels increased mortality rates to 27.3% compared to the 10.3% average mortality rate across the 312 episodes (158).

4.1.7 Congenital TTP

Congenital TTP, also known as Upshaw-Schulman syndrome, manifests through mutations in the *ADAMTS13* gene (159). Individuals with a severe deficiency in ADAMTS13 (<10% plasma levels) usually have compound heterozygous or homozygous mutations (biallelic mutations)

whereas carriers are usually asymptomatic of TTP (157). Over 100 disease-causing mutations have been identified across all ADAMTS13 domains, with 65% of mutations being missense and the majority of mutations causing decreased secretion or reduced activity (157). Two recurring mutations c.4143_4144dupA (frameshift) and c.3178 C>T (missense) have been identified, with the latter mutation being the most prevalent amongst British patients (140). Familial TTP has equal prevalence in males and females and manifests 50% of the time in the first 5 years of life, however some individuals may be adults before they experience an acute TTP episode, precipitated by a vascular insult (138). Nearly all individuals with familial TTP exhibit a relapse-remission pattern of symptoms, defined as the recurrence of TTP symptoms 30 days after they entered remission (157). In some cases multiple relapses can cause progressive organ failure due to the repeated accumulation of lesions within organs (147).

4.1.8 Acquired TTP

Acquired TTP is an autoimmune disease which manifests through autoantibodies, predominantly inhibitory, against ADAMTS13. The majority of individuals develop polyclonal IgG autoantibodies with epitopes against multiple protein domains, nearly always possessing inhibitory autoantibodies against the ADAMTS13 spacer domain (157). Patients may have autoantibodies in immune complexes with ADAMTS13, which are thought to induce inflammation and enhance clearance by an unknown mechanism (160). Acquired TTP is more prevalent in females and usually develops from 20-60 years of age, with 20-50% of patients relapsing after the initial acute TTP event (161). Peyvandi *et al.* (2008) confirmed reduced ADAMTS13 activity and the presence of autoantibodies in patient's plasma whilst in remission as predictors of disease reoccurrence (161).

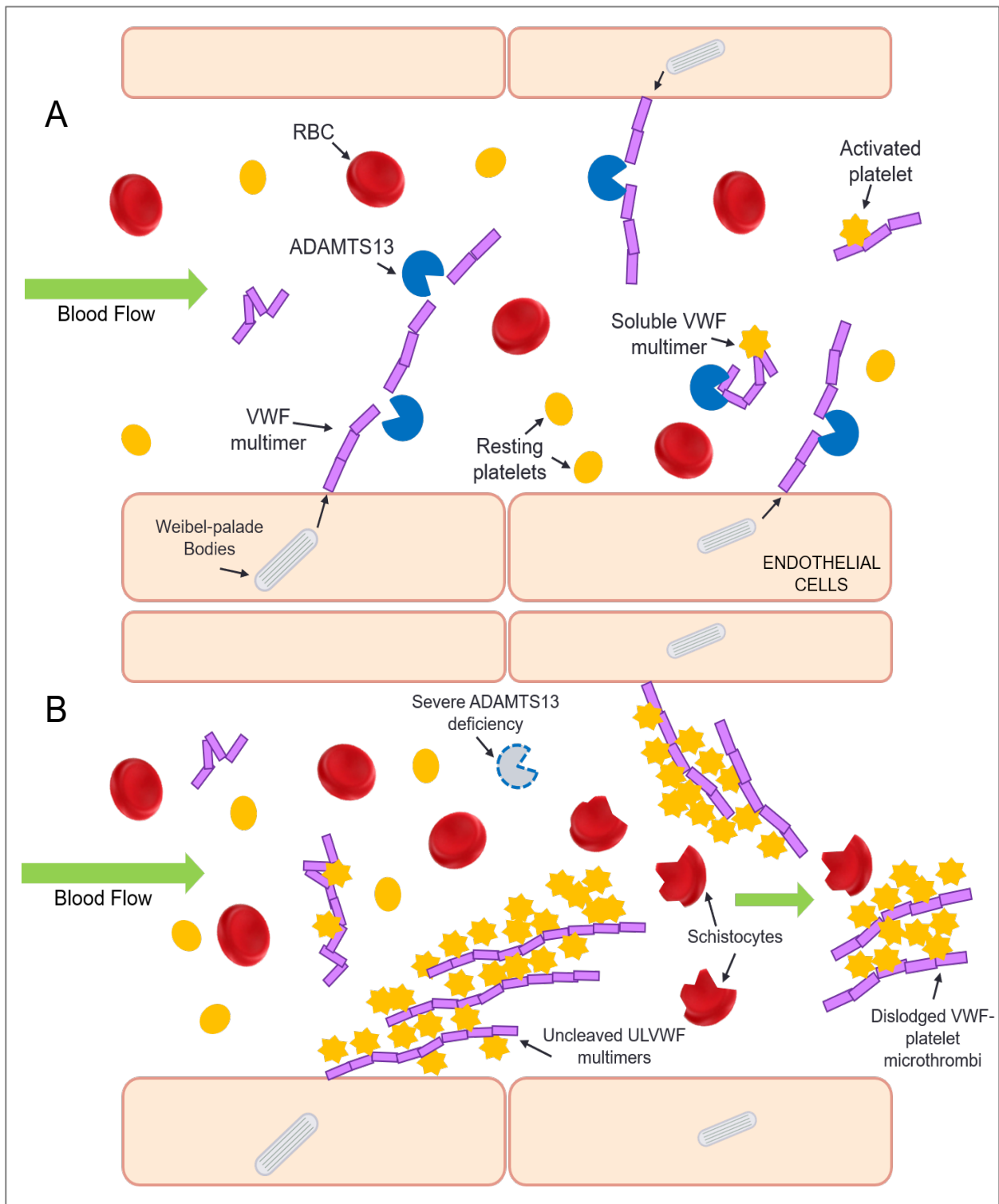


Figure 4.2- Role of ADAMTS13 in regulating von Willebrand factor (VWF)-mediated platelet capture.

A) In normal haemostasis, ultra-large VWF (ULVWF) multimers released from Weibel-Palade bodies (WPBs) in endothelial cells are sequentially cleaved by ADAMTS13. Cleaved VWF multimers released into the plasma are smaller and less thrombogenic. B) Deficient ADAMTS13 causes the accumulation of highly thrombogenic ULVWF multimers which capture platelets, accumulating in size which leads to the formation of VWF- and platelet-rich thrombi. These thrombi can disseminate into the microvasculature where they can cause the fragmentation of red blood cells (RBC), termed schistocytes, vessel occlusion and organ ischaemia.

4.1.9 Current treatments

Patients suffering an acute TTP event receive therapeutic plasma exchange (TPE) which entails the exchange of large volumes of patient plasma (1.5x total plasma volume) with donor supplies to replenish ADAMTS13 plasma levels, remove large VWF multimers and in the case of acquired TTP remove autoantibodies (157). Patients receive TPE once or twice daily until recovery of platelet levels, which has reduced mortality rates substantially from 90 to 20% (159). For acquired TTP, TPE is used in conjunction with immunosuppressants such as corticosteroids or more commonly rituximab, an anti-CD20 antibody which depletes B-cell populations (140). Patients with congenital TTP receive prophylactic plasma infusions ranging in frequency from every 3-4 weeks to only in circumstances of potential endothelial challenge, such as infection or pregnancy (159). Despite this, there are significant morbidities associated with exposure to donor blood products, particular as plasma from multiple donors may be combined to meet the volume demand (156).

More recently, a first-in-man clinical trial administered a single dose of recombinant ADAMTS13 (rADAMTS13) to congenital TTP patients, with no significant adverse events and predicted pharmacokinetics (162,163). Interestingly, rADAMTS13 was able to recover ADAMTS13 activity *in vitro* by overriding TTP patient autoantibodies (164). Jian *et al.* (2012) created a rADAMTS13 variant with residues modified within the spacer domain that are commonly targeted by autoantibodies, creating a variant less susceptible to neutralisation by inhibitory antibodies (165). Protein replacement therapy (PRT) using rADAMTS13 would reduce the dependency on donor plasma, however patients would require frequent re-administrations for the rest of their lifetime. The substantial treatment burden and expense associated with PRT taken together with the significant morbidities associated with donor plasma highlight the need for a novel TTP therapy.

4.1.10 Animal models of TTP

The complex aetiology of TTP has proven difficult to emulate in an animal model (166). To generate transgenic mice, Motto *et al.* (2005) delivered a gene targeting vector to embryonic stem cells derived from mice of the 129/Sv genetic background, replacing exons 1-6 of *Adamts13* with a neomycin resistance cassette (167). Positive clones were injected into C57BL/6 blastocysts then implanted into pseudopregnant females, and agouti offspring

backcrossed consecutively with wildtype C57BL/6 mice, generating a B6/129 mixed strain. Adamts13^{B/129-/-} mice were completely deficient in ADAMTS13 yet interestingly showed no spontaneous symptoms of TTP such as thrombocytopenia, schistocyte formation or reduced survival (167). Unusually, both Adamts13^{B/129-/-} and Adamts13^{B/129+/+} mice displayed bands representing ULVWF multimers upon multimer analysis and both genotypes remained largely unaffected following challenge with bacterial toxin, Shiga toxin, used to stimulate endothelial activation. Therefore Adamts13^{B/129-/-} mice from this genetic background do not develop spontaneous TTP and are non-susceptible to TTP-like symptoms following Shiga toxin challenge.

In contrast Motto *et al.* (2005) found that B/129 mice backcrossed with wildtype CASA/Rk mice (Adamts13^{B/CN2-/-}), a genetic background where mice have 50% higher plasma VWF levels, displayed symptoms of spontaneous TTP including a 40% reduction in survival, thrombocytopenia and schistocyte formation. As opposed to the B/129 cross, Adamts13^{B/CN2+/+} and Adamts13^{B/CN2-/-} littermates showed dissimilar multimer distributions whereby knockout mice displayed ULVWF multimers not observed upon analysis of wildtype littermates. When challenged with Shiga toxin, Adamts13^{B/CN2-/-} mice developed severe thrombocytopenia, anaemia and hyaline thrombi formation with high mortality rates. Therefore the CASA/Rk genetic background, characterized by higher VWF levels, increases spontaneous TTP symptom occurrence and susceptibility following TTP induction with Shiga toxin.

Interestingly upon analysis of strain-specific variants of *Adamts13*, Banno *et al.* (2004) identified that the C57BL/6J strain (and other strains such as BALB/c) contain a 6kb retrotransposon insertion in intron 23 which leads to incorporation of a premature stop codon and C' truncation (168). Analysis of cDNA sequences extracted from the liver of C57BL/6J mice by RT-PCR revealed transcripts 3114 bp in length. In contrast, cDNA sequences from mice of the 129/Sv genetic background were 4281 bp in length, of comparable size to the human cDNA sequence (4284 bp). Banno *et al.* (2004) subsequently compared the proteolytic properties of the 'long' mADAMTS13 and 'short' mADAMTS13 proteins, revealing the C' truncation did not significantly affect ADAMTS13 activity (168). Although the retrotransposon insertion does not affect ADAMTS13 activity, this may

account for the lack of phenotypic differences between wildtype and knockout mice. Furthermore, this strain may have a balancing mutation which reduces the phenotypic severity of knockout mice compared to the CASA/Rk background. (167).

More recently, Schiviz *et al.* (2012) demonstrated a TTP mouse model whereby the Adamts13^{B/129-/-} genetic background is used however TTP symptoms are induced by infusion of recombinant human VWF (rhVWF) protein (169). In response to rhVWF infusion, mice demonstrated a range of TTP-like symptoms, including thrombocytopenia, schistocyte formation, reduction in body weight and behavioural changes. Importantly however, all symptoms were reversed between 3 and 14 days post-infusion and no animals died or required euthanasia, thus demonstrating a milder and more preferential disease model than Shiga toxin challenge in the CASA/Rk background (166,169).

As reported by Turecek *et al.* (2009), rhVWF is separated as an intermediate product in the manufacturing process of recombinant FVIII product, Advate (Takeda, Tokyo, Japan), following co-expression of FVIII and VWF in Chinese hamster ovary (CHO) cells (170). Following column selection of FVIII, the flow-through contains pro-VWF that is processed into mature-VWF by recombinant furin. Recombinant VWF generated in such a way is licenced by Baxter International (Illinois, US) and is being investigated as a treatment for VWD. As a standardised measurement of VWF composition, VWF ristocetin cofactor (VWF:RCo) activity is measured using an automated turbidimetric analyser. The effective dose to induce thrombocytopaenia in ADAMTS13 deficient mice was established in a previous study by Muchitsch *et al.* (2010) to be 2 VWF:RCoU/g with lower doses failing to induce significant effect (171).

4.1.11 Gene therapy approaches for TTP

Previous gene therapy attempts for the treatment of congenital TTP in knockout mice have utilised AAV (172), AdV (173), lentivirus (174,175), hydrodynamic gene transfer (176) and pronuclear injection (177) methods to restore ADAMTS13 expression (summarised in

Table 4.1). Jin *et al.* (2013) and Verheene *et al.* (2017) targeted the liver for expression of ADAMTS13 through intravenous administration and incorporating a human alpha-1-antitrypsin promoter (172,176). Despite recombinant AdV preferentially infecting the liver when administered systemically, Trionfini *et al.* (2009) identified ADAMTS13 expression in the lungs and elsewhere (173). Similarly, Niiya *et al.* (2009) achieved systemic expression following *in utero* gene transfer (174). In contrast, *ex vivo* transduction of haematopoietic stem cells performed by Laje *et al.* (2009) resulted in predominantly ADAMTS13 expression from the haematopoietic lineage, and Pickens *et al.* (2017) demonstrated platelet expression using the GPIIb/IIIa promoter (175,177). The longest duration of expression was achieved by Verheene *et al.* (2017) with ADAMTS13 plasma antigen levels of 200% 25 weeks post-treatment and protection from TTP symptoms following disease induction at 20 weeks (176). Three of six papers utilised B/129 transgenic mice and two of three papers that challenged mice administered recombinant VWF to induce TTP symptoms, both of which will be used to model TTP in this project (see Table 4.1).

4.1.12 Aims and objectives

The aim of this research was to investigate rSIV.F/HN gene transfer for the treatment of a systemic disorder. TTP was selected as an exemplar disease due to the unmet clinical need of current treatments and the novelty of an *in vivo* lentiviral gene therapy approach for TTP. The hypothesis is that rSIV.F/HN-mediated transduction will restore gene expression in a TTP mouse model. The specific objectives of this chapter were to:

1. Characterise the phenotype of ADAMTS13 deficient mice.
2. Establish suitable assays for assessment of transduction efficacy in knockout mice.
3. Establish a TTP model whereby deficient mice exhibit TTP-like symptoms when challenged.
4. Investigate the restoration of plasma ADAMTS13 expression following intranasal and intravenous administration of rSIV.F/HN vectors.
5. If gene transfer has been successful, investigate the protection against TTP-like symptoms in challenged mice.

Table 4.1- Comparison of literature investigating gene therapy approaches for the treatment of congenital TTP.

Adenoviral vector (AdV), Rous sarcoma virus long terminal repeat (RSV) promoter, Vesicular stomatitis virus G (VSV-G) glycoprotein, cytomegalovirus (CMV) promoter, murine leukaemia virus-derived (MND) promoter, white blood cell (WBC), adeno-associated virus (AAV), human alpha-1-antitrypsin (hAAT), glycoprotein Ib-IX-V transmembrane protein α (GPIb α).

Reference and gene transfer agent	Mouse strain	Method of administration and expression profile	Duration of expression	TTP induction
Trionfini et al. (2009)- AdV-RSV-human ADAMTS13	C57BL/6J /129Sv	Intravenous infusion. Systemic expression.	12 weeks	N/A
Niiya et al. (2009)- VSV-G-CMV-human ADAMTS13	C57BL/6J /129Sv	Administered <i>in-utero</i> by intra-amniotic administration (embryonic day 8) and intravascular (embryonic day 14). Systemic expression.	24 weeks	N/A
Laje et al. (2009)- VSV-G-MND-murine ADAMTS13	C57BL/6J /129Sv	Intravenous injection of haematopoietic stem cells transduced <i>ex vivo</i> . Platelet, multi-nucleated WBC expression and sporadic systemic expression.	20 weeks	N/A
Jin et al. (2013)- AAV8-hAAT- <i>mdtcs</i>	CAST/Ei	Intravenous infusion. Liver directed expression.	20 weeks	Shiga toxin
Pickens et al. (2015)- GPIb α -human ADAMTS13	CAST/Ei	Pronuclear injection of human ADAMTS13 cDNA. Platelet specific expression.	N/A	Shiga toxin & rmVWF
Verhenne et al. (2017)- hAAT-murine ADAMTS13 transposon	CASA/Rk	Hydrodynamic gene transfer of transposon and transposase. Liver directed expression.	25 weeks	rhVWF
Liu-Chen et al. (2018)- Human ADAMTS13 mRNA formulated in lipid nanoparticles	C57BL/6J /129Sv	Intravenous infusion. Systemic expression.	72 hours	N/A

4.2 Materials and Methods

4.2.1 Construction of lentiviral expression plasmid encoding murine ADAMTS13

The mADAMTS13 coding sequence was first cloned into a lentiviral expression plasmid containing the hCEF promoter and enhanced green fluorescent protein (EGFP) transgene (pGM357-hCEF-EGFP). All lentiviral expression plasmids used in this project contained the LTR sequences and other essential regulatory sequences for third generation lentiviral production (Figure 4.3A). The mADAMTS13 coding sequence, 4281 bp in length (accessed from GenBank: AB112362.1), was codon-optimised by GeneArt Gene Synthesis (Invitrogen, Massachusetts, US) to enhance expression in mice and the restriction sites NheI and NotI inserted 5' and 3' of the mADAMTS13 sequence, respectively. NheI-mADAMTS13-NotI was synthesised commercially by GeneArt Gene Synthesis (Invitrogen, Massachusetts, US) into transfer vector pMAD13 (Figure 4.3B). Plasmid pGM357-hCEF-EGFP was restriction digested using NheI and NotI to remove the EGFP reporter gene from the lentiviral backbone and similarly pMAD13 was digested to isolate the NheI-mADAMTS13-NotI sequence. Fragments were separated by gel electrophoresis and bands of the appropriate size corresponding to the pGM357-hCEF backbone (6089 bp) and NheI-mADAMTS13-NotI insert (4288 bp) were excised and DNA purified. NheI-mADAMTS13-NotI was ligated into the lentiviral expression plasmid creating pIC056-hCEF-mADAMTS13-hCEF-mADAMTS13 (Figure 4.3C). The molar ratio of vector to insert was 1:2, respectively. Single colonies were cultured and restriction digested with SnaBI and XmnI to confirm correct ligation of pIC056-hCEF-mADAMTS13. Ligations showing expected restriction patterns were sent for sequencing using primers oIC001 and oIC004 (Table 1- Reaction 2).

4.2.2 Construction of modified ADAMTS13 lentiviral expression plasmids

Variants of pIC056-hCEF-mADAMTS13 were produced, firstly by inserting an extended Kozak sequence (CCACC) to the 3' of the NheI site and the 5' of the ATG start codon of the mADAMTS13 cDNA sequence. pIC056-hCEF-mADAMTS13 was used as the template for the PCR amplification of vector backbone with forward primer oIC049 and reverse oIC050 (Table 2.1- Reaction 3). An insert oligo was synthesised, Kozak_insert (Table 2.1- Reaction 3) (Integrated DNA Technologies, Iowa, US) and ligated into the backbone using the HiFi assembly protocol, vector:insert ratio of 1:5. Sequencing of the novel construct, pIC040-

hCEF-mADAMTS13+Kozak, was performed with forward primers oIC001/68/69/70 and reverse primers oIC004/14 (Table 2.1- Reaction 4).

The second variant of pIC056-hCEF-mADAMTS13 exchanged the murine ADAMTS13 cDNA sequence with a human ADAMTS13 (hADAMTS13) cDNA sequence. hADAMTS13 was amplified from the pcDNA3.1-hADAMTS13 coding plasmid (Figure 4.4B) using forward primer oIC121 and reverse primer oIC122 (Table 2.1-Rection 5) which added a 5' NheI and 3' NotI restriction site. The lentiviral backbone was amplified from pIC056-hCEF-mADAMTS13 using forward primer oIC123 and reverse primer oIC124 (Table 2.1-Rection 5). Ligations were performed using the HiFi assembly protocol, vector:insert ratio of 1:2. Sequencing of the novel construct, pIC083-hCEF-hADAMTS13, was performed with forward primers oIC001/125/126/127 and reverse primer oIC004/50 (Table 2.1- Reaction 6). Plasmid maps of pIC056-hCEF-mADAMTS13, pcDNA3.1-hADAMTS13 and pIC083-hCEF-hADAMTS13 are shown in Figure 4.4.

4.2.3 Measurement of ADAMTS13 levels by ELISA

Two commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits were investigated for the quantification of murine ADAMTS13 in mouse plasma and cell supernatant. The first, ADAMTS13 ELISA Kit (Mouse) (AVIVA Systems Biology, California, US, OKEH00676) and the second, Mouse ADAMTS13 ELISA Kit (Abbexa Limited, Cambridge, UK, abx514433) were carried out following manufacturer's instructions. To determine human ADAMTS13 levels in plasma and cell supernatant, the commercial sandwich ELISA kit from R&D Systems (Minnesota, US, DADT130) was utilised following manufacturer's instructions. Upon completion of the ELISA, absorbance was measured at 450 nm and 570 nm using the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Wavelength correction (absorbance at 570 nm) was subtracted from absorbance at 450 nm. Calculated m/hADAMTS13 antigen levels were expressed as concentration (ng/ml).

4.2.4 Detection of ADAMTS13 cleavage activity by western blotting

To confirm mADAMTS13 encoded by pIC056-hCEF-mADAMTS13 was functional, HEK293T cells were co-transfected with pIC056-hCEF-mADAMTS13 and a plasmid encoding human

VWF (hVWF), (pcDNA3.1-FL-VWF, Figure 4.4D). Negative controls included HEK293T cells transfected with PEIPro reagent only or transfected with hVWF only. As a positive control, hVWF was co-transfected with pcDNA3.1-hADAMTS13 encoding human ADAMTS13 (pcDNA3.1-hADAMTS13 Figure 4.4B). Transfections were performed with 0.5 µg/ml plasmid DNA in 12-well plates using standard methodology. After 72 hours, cell lysates were collected and diluted 1:2 with PBS.

Cleavage was visualised by reducing sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), 7.5 µl of reducing SDS loading buffer (200µl SDS loading buffer (Thermo Fisher Scientific, Massachusetts, US) mixed with 15 µl 2-mercaptoethanol (Sigma-Aldrich, Missouri, US)) was added to 30 µl of cell lysate and denatured at 95°C for 5 minutes. Samples were loaded into a 12-well Bolt 4-12% Bis-Tris Plus gel (Thermo Fisher Scientific, Massachusetts, US) along with a protein standard (Invitrogen, Massachusetts, US). The gel was run for 90 minutes at 100 volts then transferred to a nitrocellulose membrane by electroblotting with transfer buffer (Thermo Fisher Scientific, Massachusetts, US) using the high molecular weight programme for 20 minutes. Subsequently, the membrane was blocked with 3% (w/v) semi-skimmed milk powder in PBS (milk-PBS) for 90 minutes, washed with PBS then incubated overnight with rabbit polyclonal anti-VWF horseradish peroxidase (HRP) antibody (diluted 1/1000 in PBS) (Dako, Glostrup, Denmark). The membrane was washed with PBS supplemented with 0.1% Tween 20 (PBS-T) then overlaid with Immobilon Western-HRP substrate (Merck, Feltham, UK) (1 ml luminol and 1 ml peroxidase) for 5 minutes at RT. Chemiluminescence signal was visualised using a ChemiDoc Imaging system (Bio-Rad, California, US).

4.2.5 Production and purification of His-VWF73-FLAG cleavage fragment

To develop a quantitative plate-based assay for measuring ADAMTS13 cleavage activity, a small VWF fragment was designed with tags linked to each end of the fragment for protein purification and detection. The cDNA sequence encoding a murine VWF 73 amino acid fragment (VWF73, D1596-T1668) containing the ADAMTS13 cleavage site (Tyr1605-Met1606) and flanked by N' FLAG-tag and C' 6X Histidine (His)-tag (Figure 4.5A). The cDNA sequence was inserted downstream of a T7 promoter (VectorBuilder, Illinois, US) and the encoding plasmid, named pIC016-FLAG-mVWF73-His (Figure 4.4E), was transformed into

BL21(DE3) competent cells (NEB, Massachusetts, US) and grown in a 400 ml culture until the optical density reached 0.6, as measured using the NanoDrop 2000c Spectrophotometer at 600 nm (OD₆₀₀) (Thermo Fisher Scientific, Massachusetts, US). Once log phase had been reached, a pre-induction sample was removed, then recombinant protein expression induced with 1mM isopropyl β -D-1-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific, Massachusetts, US) and incubated at 37°C for 3 hours with shaking. IPTG releases T7 RNA polymerase from the lac repressor, thus allowing transcription from the T7 promoter. Subsequently, cultures were pelleted at 5000xg for 20 minutes and supernatant discarded. To lyse bacterial cells, CellLytic B reagent (Sigma-Aldrich, Missouri, US) was used following manufacturer's instructions. Briefly, 15 ml of reagent was added per gram of cell paste, resuspended and both cOmplete Protease Inhibitor and 50 U/ml Benzonase endonuclease added to prevent protease degradation and to decrease solution viscosity, respectively. The mixture was incubated at RT for 15 minutes with shaking at 225 rpm, centrifuged at 16000xg for 10 minutes and the soluble fraction carefully removed. The sample was then filtered using a 0.4 μ m Acrodisc low protein binding filter (Pall, New York, US) and stored at -80°C.

To visualise if FLAG-mVWF73-His had been successfully produced, SDS-PAGE was performed as described above, however using protein lysate extracted from bacterial cultures before and after IPTG induction of protein expression. Once the loading dye had reached an appropriate distance, the gel was added to fixation buffer (40% MeOH, 10% HoAc, 50% H₂O) for 1 hour then transferred to Coomassie Blue stain used for the detection of protein (40% MeOH, 10% HoAc, 50% H₂O with 0.25% Coomassie Blue R-250, Bio-Rad, California, US) for 2-4 hours, until the gel was a uniform blue colour. The gel was de-stained for 24 hours (5% MeOH, 7.5% HoAc, 87.5% H₂O) with frequent washes until protein bands appeared and the background cleared.

Affinity liquid chromatography using the ÄKTA chromatography system (GEHealthcare, Illinois, US) and UNICORN 7.0 software (GEHealthcare, Illinois, US) was performed. Within the system is a UV monitor, which is a readout for protein due to the absorbance of UV light at wavelength 280 nm by aromatic rings-containing amino acids. The conductivity of solution passing through the system is also monitored, whereby conductivity is increased

with greater ion concentration, detecting properties such as the salt concentration of buffer. Protein lysate was passed through HisTrap HP columns (GEHealthcare, Illinois, US) packed with nickel sepharose affinity resin to purify the His-tagged recombinant protein. Within the resin of agarose beads, the chelating agent nitrilotriacetic acid has been charged with nickel ions (NTA-Ni²⁺) that will bind poly-histidine chains, including His-tagged proteins. Following the supplier's instructions, the protein lysate was diluted in 9 parts binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole Sigma-Aldrich, Missouri, US), pH 8) to one part lysate and applied to the column. The low imidazole concentration binding buffer prevented non-specific binding of protein to the resin. To displace bound His-tagged protein from the column, elution buffer with high imidazole concentration (20 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, pH 8) was applied with an increasing concentration gradient (0-100% in 20 column volumes). An elution fraction was collected once a peak in UV absorbance was observed and purified protein was stored in aliquots at -80°C. To determine total protein concentration a protein assay was performed followed by SDS-PAGE and Coomassie staining to visualise protein purity.

4.2.6 Development of a plate-based assay to measure ADAMTS13 cleavage activity

Cleavage reactions (total volume 250 µl) were carried out in collection tubes including mouse plasma (plasma from wildtype or knockout mice) diluted 1/20 and recombinant mVWF73 protein diluted 1/10 (final concentration ~100 µg/ml) in reaction buffer (5mM MgCl₂, 5mM Tris HCl, 1 tablet cOmplete, Mini EDTA-free Protease Inhibitor Cocktail, p.H. 5.5). Reactions were incubated at 37°C for 45 minutes and stopped with EDTA (final concentration 5mM) which is an ion chelator thus inhibiting ADAMTS13 protease activity. Wildtype plasma + EDTA and reactions without plasma served as negative controls. Different conditions were attempted during the optimisation of this assay, including mVWF73 and plasma concentrations, reaction buffer composition and reaction duration. The conditions given above generated the data presented in the results.

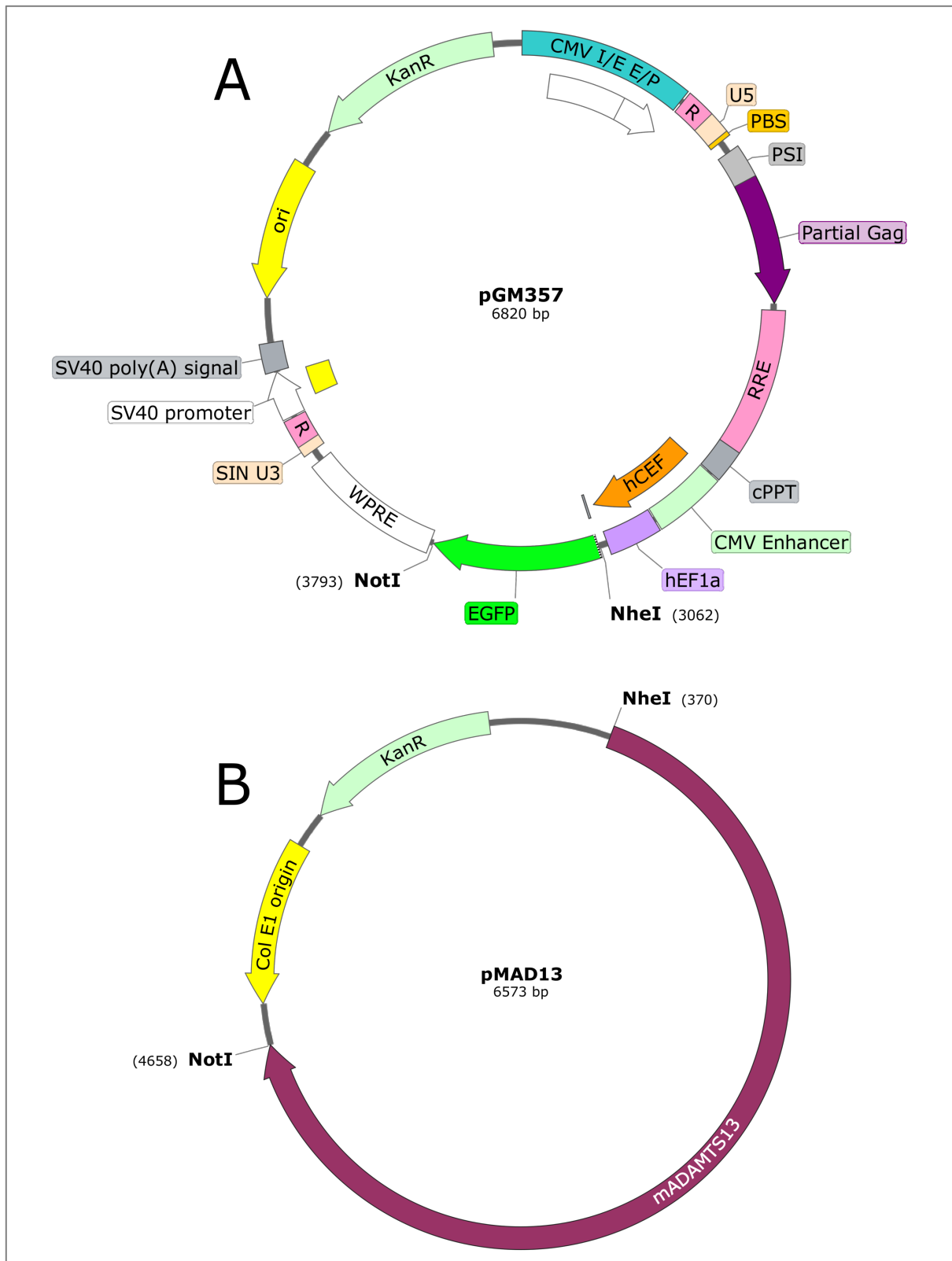
As shown by the illustration in Figure 4.5B, a plate-based assay was developed to detect levels of FLAG-mVWF73-His recombinant protein, an inverse measurement of cleavage activity. The greater levels of ADAMTS13 cleavage results in lower detection of FLAG-mVWF73-His. To capture recombinant protein, corning 96-well Standard Polystyrene

Stripwell Microplates (Corning, New York, US) were coated with 100 µl of Monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich, Missouri, US) diluted 1:500 in Tris Buffered Saline (TBS) supplemented with 3% non-fat milk (pH 8.0) and incubated at 37°C for 1 hour. Wells were washed 5X with 200 µl of PBS-0.05% Tween 20 (PBS/T) as for all further wash steps performed between every incubation step. To reduce non-specific binding, wells were blocked with 100 µl of 3% bovine serum albumin (BSA)-PBS for 2 hours at 37°C. After further washes 100 µl of FLAG-mVWF73-His protein dilutions (1:4 serial dilutions from neat) or cleavage samples were added in triplicate along with 100 µl of reaction buffer acting as a blank, and incubated for 1 hour at 37°C. Following washes, biotinylated 6X-His Tag Monoclonal Antibody (Thermo Fisher Scientific, Massachusetts, US) diluted 1:500 in 1% BSA-PBS was added to wells for 1 hour at 37°C followed by Avidin-HRP conjugate (Merck, Feltham, UK) diluted 1:2000 in 1% PBS-BSA for a further hour at 37°C. Finally 100 µl of TMB Substrate Solution (Thermo Fisher Scientific, Massachusetts, US) was added to each well for 15-30 minutes until the optimal blue colour developed. Each reaction was terminated with 100 µl of Stop Solution (Thermo Fisher Scientific, Massachusetts, US) and absorbance measured at 450 nm and 570 nm using the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Wavelength correction (absorbance at 570 nm) was subtracted from 450 nm. Absorbance was expressed as optical density (O.D.) or percentage cleavage when values were compared to wildtype plasma (% Cleavage Activity).

4.2.7 FRET-VWF73 cleavage assay

The human VWF-73 amino acid fragment (D1596-R1668) containing the ADAMTS13 cleavage site (Tyr¹⁶⁰⁵-Met¹⁶⁰⁶) was chemically modified to include a fluorophore and fluorescence-quencher (Figure 4.5C). Specifically, Q1599 and N1610 were substituted with the fluorophore A2pr (Nma), and quencher A2pr (Dnp) respectively, termed fluorescence resonance energy transfer (FRET)-VWF73 (synthesised by Peptide Institute, Osaka, Japan and sourced through PeptaNova GmbH, Sandhausen, Germany). Reactions were carried out following the manufacturer's protocol (178). Lyophilized FRET-VWF73 substrate was dissolved in 25% DMSO and deionised water to prepare the 100 µmol/l stock solution. Cell supernatant from transfection experiments (10 µl) or mouse plasma (4 µl) was diluted in assay buffer (5 mmol/l Bis-Tris, 25 mmol/l CaCl₂, 0.005% Tween-20, pH 6.0) up to 100 µl then added to wells of a 96-well white microplate (Thermo Fisher Scientific, Massachusetts,

US). To samples, 100 μ l of 4 μ mol/l FRET-VWF73 substrate diluted in assay buffer was added which commenced reactions. Fluorescence was measured at 30°C in a FLUOstar Omega plate reader equipped with a 340 nm excitation filter and a 450 nm emission filter (Figure 4.5D). Fluorescence was measured every 5 minutes for 2 hours. Data was expressed as Fluorescence in counts or as the rate of cleavage expressed as counts per minute.



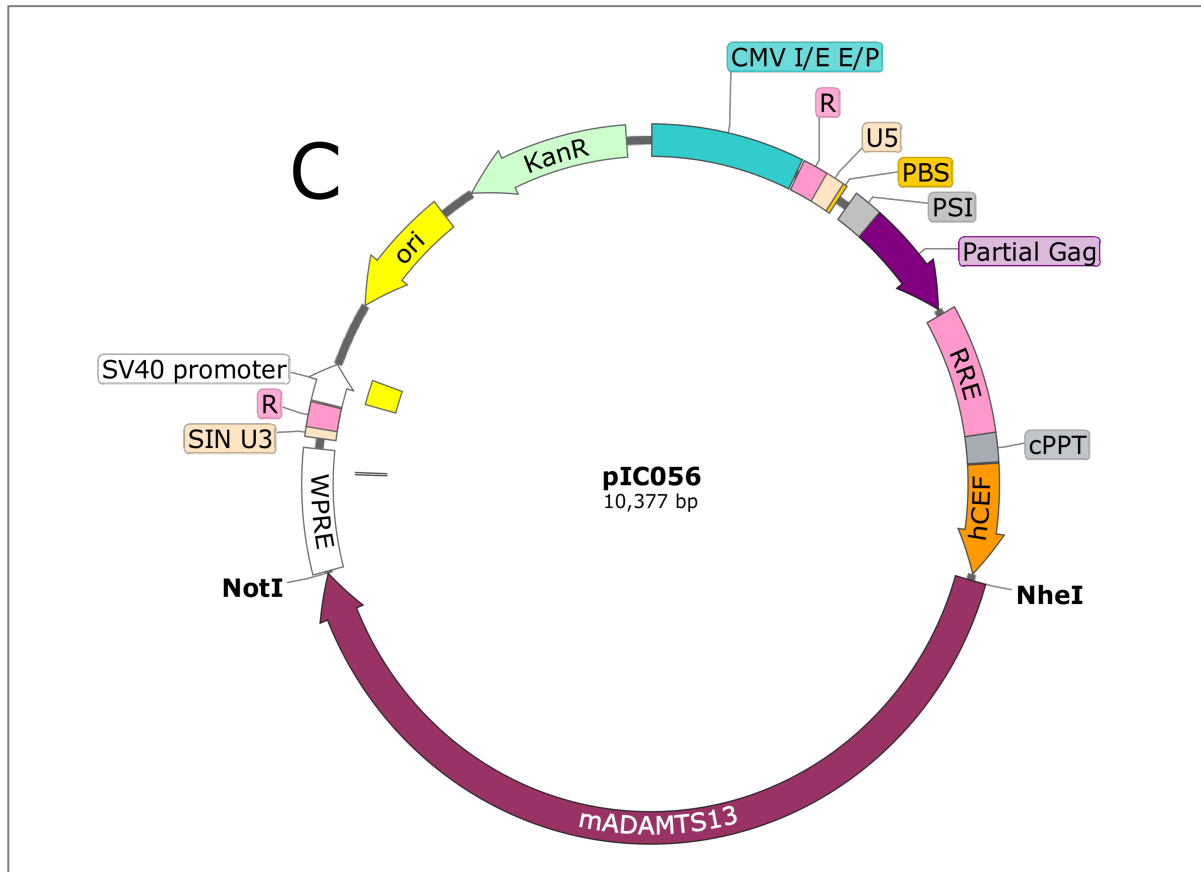
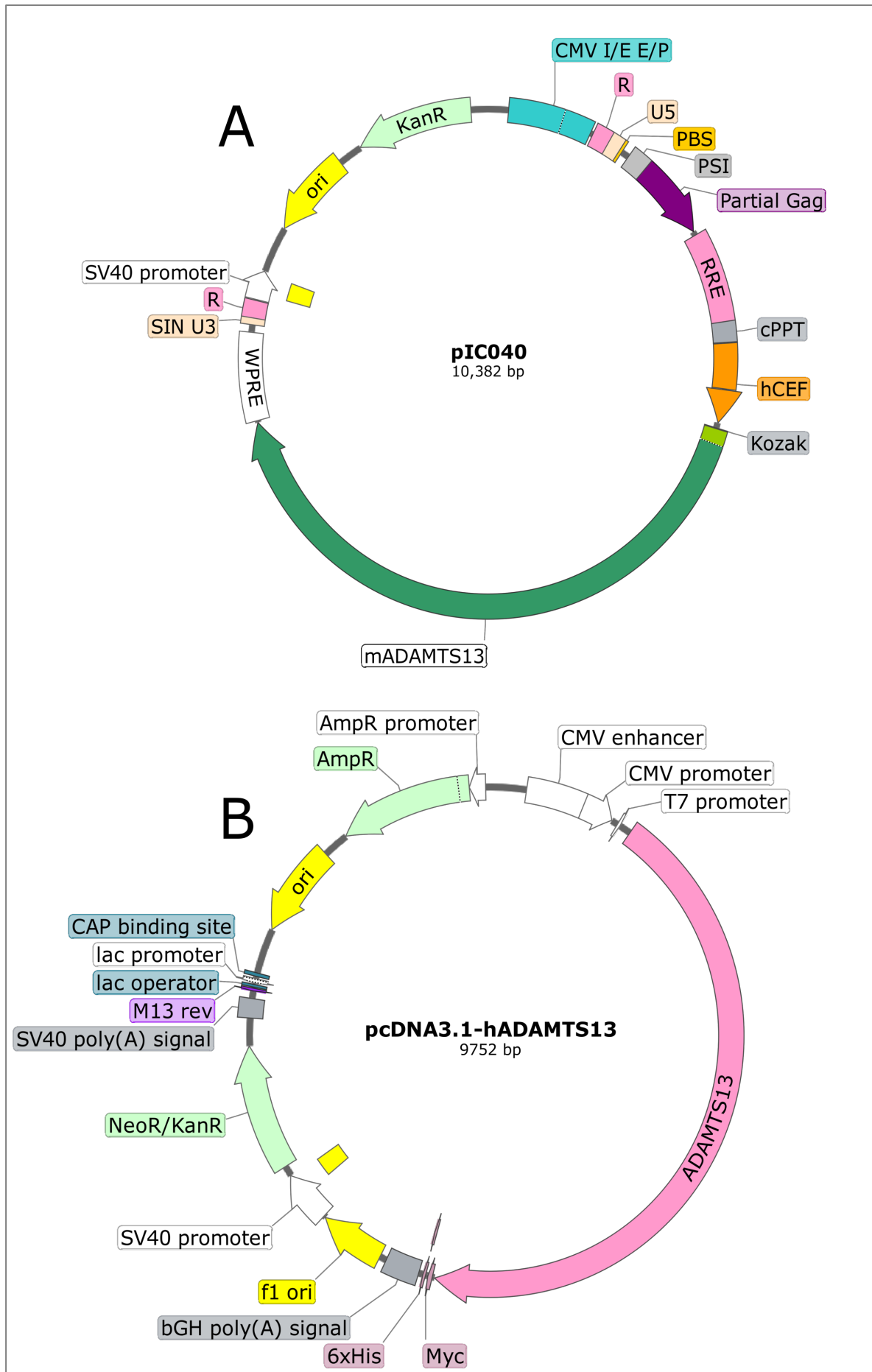
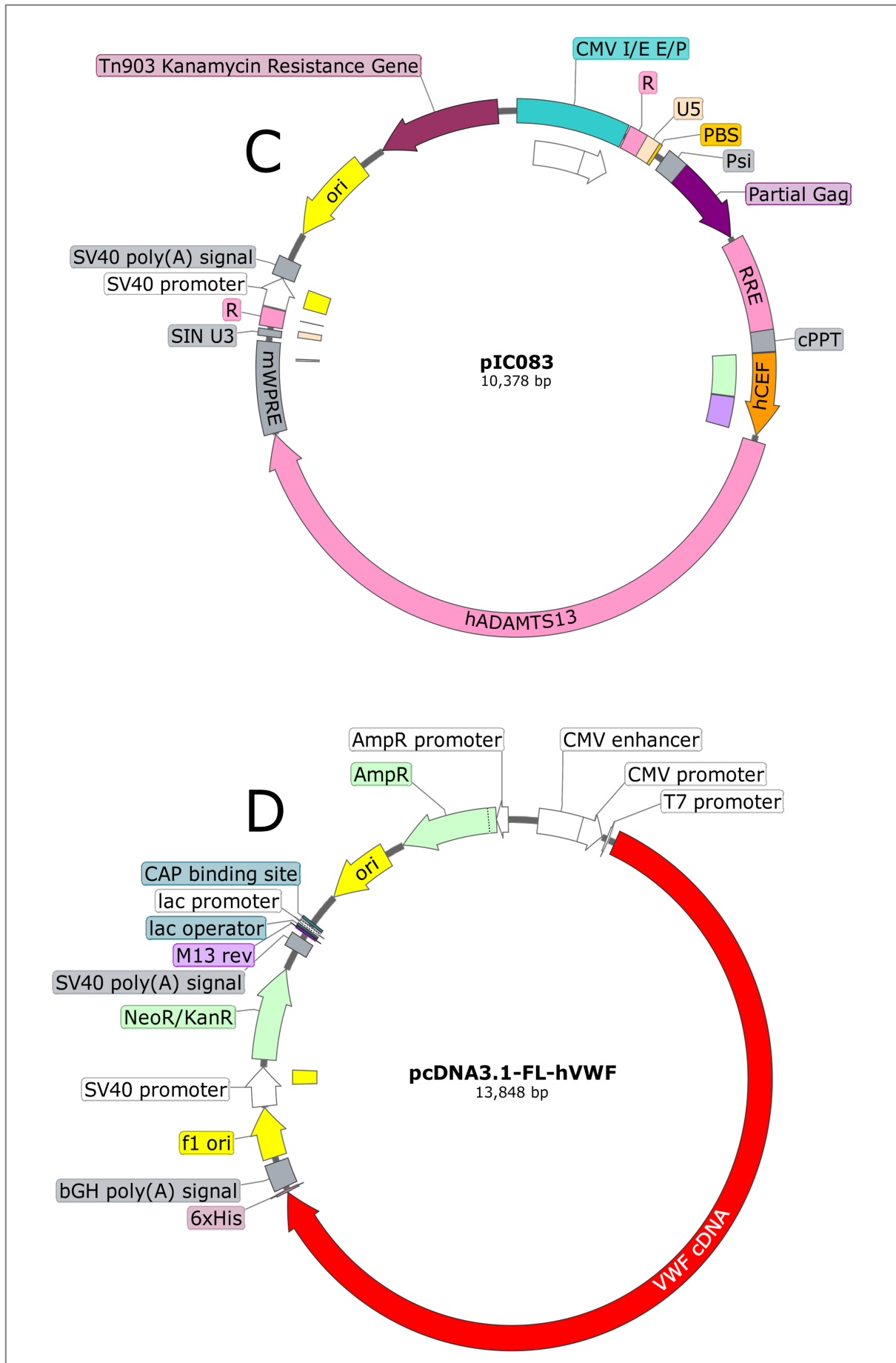


Figure 4.3- Cloning of lentiviral expression plasmid encoding murine ADAMTS13 (pIC056-hCEF-mADAMTS13).

Plasmid maps of (A) parent plasmid pGM357-hCEF-EGFP containing lentiviral LTR sequences and encoding reporter gene enhance green fluorescent protein (EGFP), (B) transfer plasmid pMAD13 encoding the murine ADAMTS13 coding sequence commercially synthesised and codon-optimised for mouse. (C) mADAMTS13 sequence was digested by *NheI* and *NotI* and ligated into the lentiviral backbone, generating pIC056-hCEF-mADAMTS13.





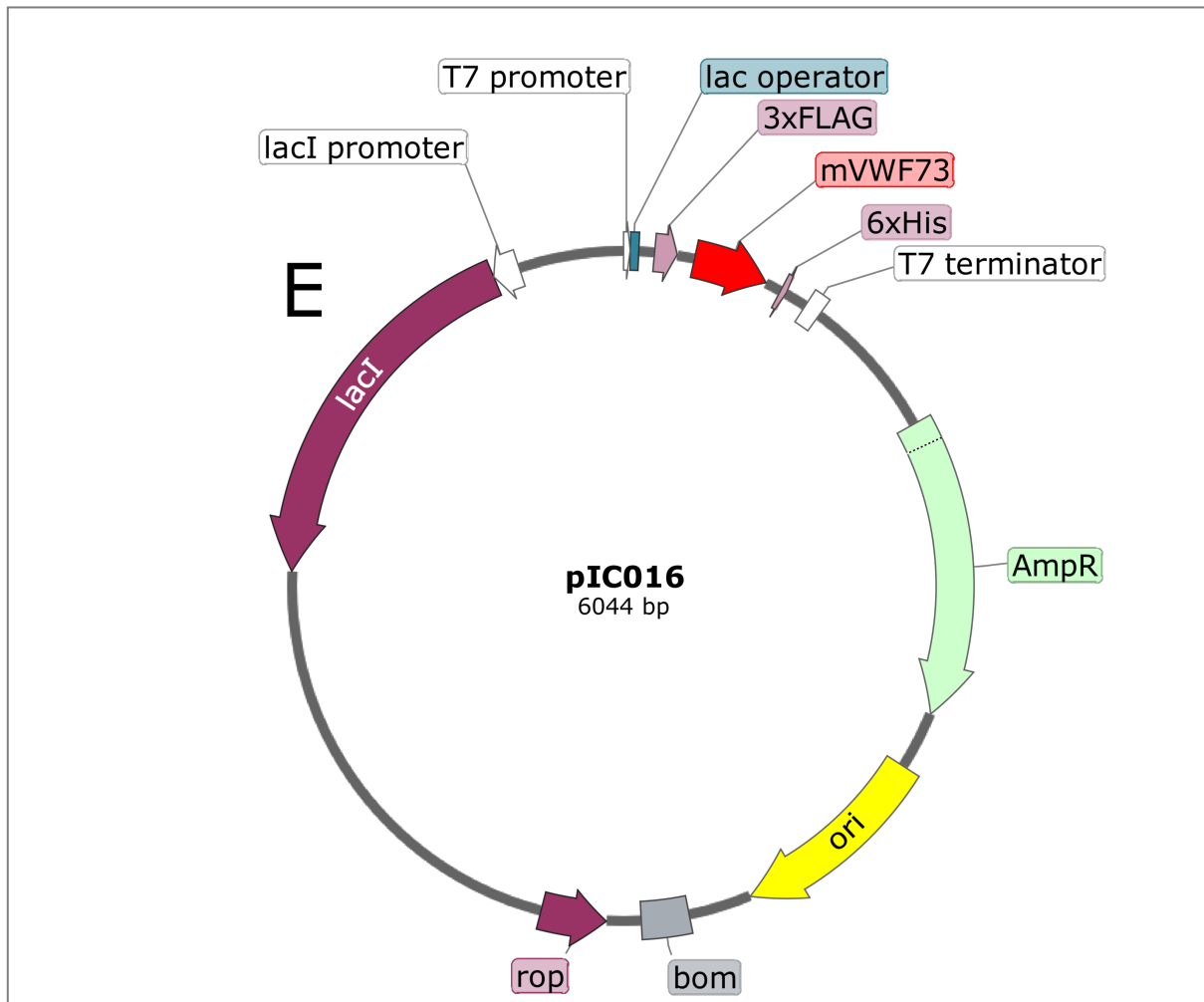


Figure 4.4- Plasmid maps of expression/ lentiviral plasmids encoding murine or human ADAMTS13 and human VWF.

(A) pIC040-hCEF-mADAMTS13+Kozak was constructed to extend the Kozak sequence by inserting 5 nucleotides (CCACC) 3' of the NheI site and 5' of the start codon upstream of the murine ADAMTS13 (mADAMTS13) cDNA encoded by pIC056-hCEF-mADAMTS13. (B) pcDNA3.1-hADAMTS13 expression plasmid which was used as template for the amplification of human ADAMTS13 (hADAMTS13) cDNA. (C) hADAMTS13 cDNA was ligated into the lentiviral backbone amplified from pIC056-hCEF-mADAMTS13, creating novel construct pIC083-hCEF-hADAMTS13. (D) pcDNA3.1-FL-hVWF expression plasmid encoding full-length human von Willebrand Factor (hVWF). (E) pIC016-FLAG-mVWF73-His expression plasmid encoding FLAG-mVWF73-His cleavage fragment with T7 promoter for expression in bacteria.

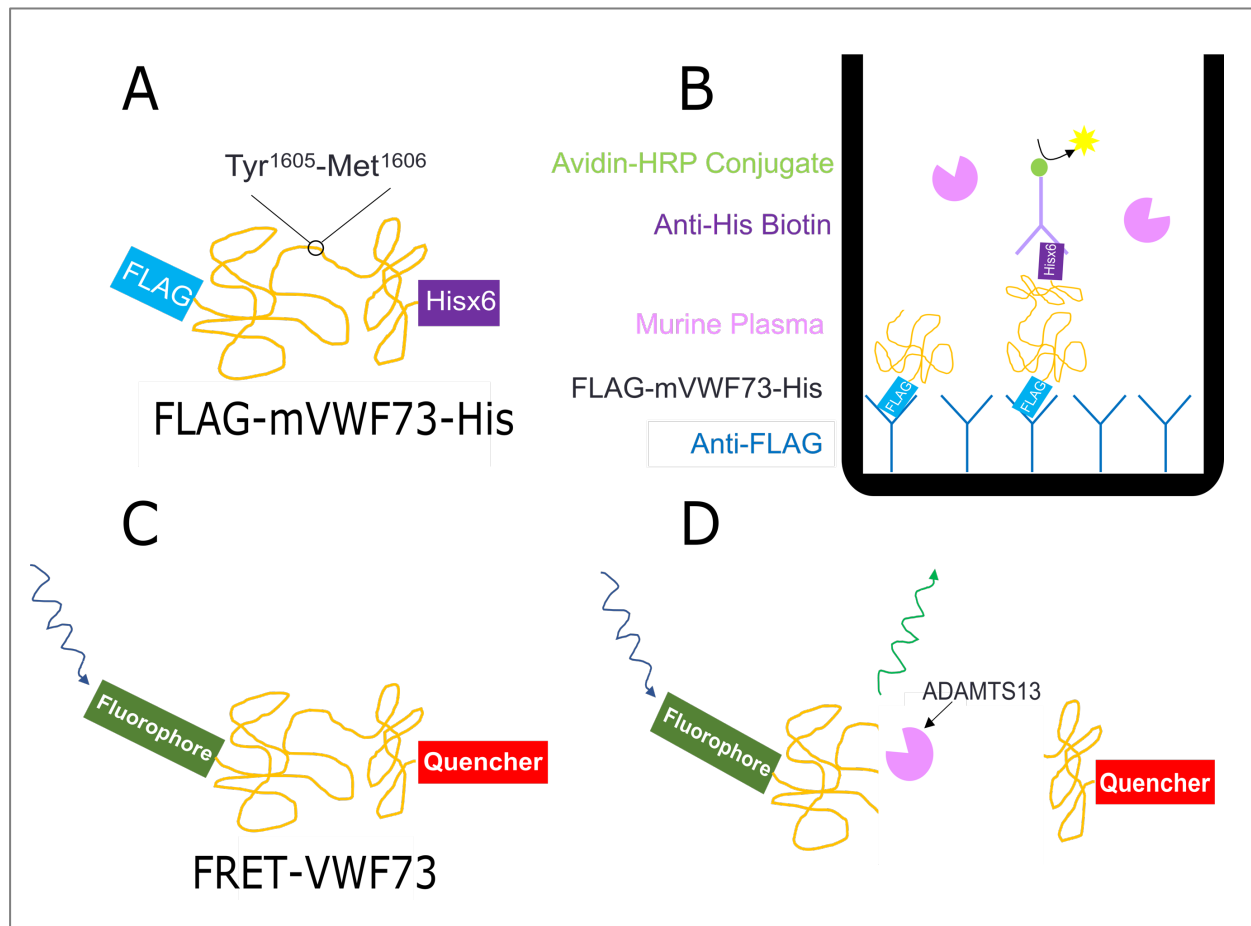


Figure 4.5- Assays for the quantification ADAMTS13 cleavage activity against a VWF73 fragment.

A) To quantify ADAMTS13 activity in murine plasma, the cDNA sequence encoding a murine VWF-73 amino acid fragment (D1596-T1668) containing the ADAMTS13 cleavage site (Tyr¹⁶⁰⁵-Met¹⁶⁰⁶) and flanked by N' FLAG-tag and C' 6X His-tag was synthesised and protein recombinantly expressed. B) A plate-based assay designed to capture mVWF73 fragments with anti-FLAG antibodies and detect with biotinylated anti-His antibodies. The avidin-Horseradish Peroxidase (HRP) conjugate subsequently binds to biotinylated antibodies and catalyses a chromogenic substrate which will emit light of wavelength 450 nm. VWF73 protein pre-incubated with murine plasma may undergo cleavage, whereby proteolysed products are lost during wash steps and a reduction in signal will be measured. Thus, the more ADAMTS13-mediated cleavage that occurs, the lower the detection of mVWF73 in the plate-based assay. C) The human VWF-73 amino acid fragment (D1596-R1668) containing the ADAMTS13 cleavage site (Tyr¹⁶⁰⁵-Met¹⁶⁰⁶) was chemically modified to include a fluorophore and fluorescence-quencher. Specifically, Q1599 and N1610 were substituted with the fluorophore A2pr (Nma), and quencher A2pr (Dnp) respectively. Excitation of the Nma fluorophore at 340 nm (blue line) is transferred to the Dnp quencher. D) Upon cleavage of the Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ peptide bond by ADAMTS13, quenching of the fluorescence transfer is removed and fluorescence is emitted at wavelength 440 nm (green line) from Nma.

4.2.8 Genotyping ADAMTS13 Transgenic mice

Transgenic mice, B6.129-ADAMTS13tm1Dgi sourced from The Jackson Laboratory (Maine, USA), were selected as the disease model for congenital TTP whereby exons 1-6 of the Adamts13 gene were replaced with a neomycin resistance cassette. Genomic DNA was isolated from ear notches using the Extract-N-Amp Tissue PCR Kit (Sigma, Missouri, USA) as per supplier's instructions. A genotype specific region was amplified by PCR, performed using OneTaq DNA Polymerase (NEB, Massachusetts, US), 50 ng of genomic DNA template and using wildtype and mutant specific forward primers (wildtype_for and mut_for) and a common reverse primer (com_rev) in one reaction following conditions given in Table 2.1- Reaction 6. PCR products were separated on a 1% agarose gel by gel electrophoresis using standard methodology.

4.2.9 Determination of murine platelet levels by flow cytometry

Two blood sampling techniques were performed for the measurement of platelet levels in mice. For cardiac puncture, mice were culled by schedule 1 overdose with pentobarbital. Once death had been confirmed, direct extraction of blood from the left ventricle was performed using an insulin needle and mixed immediately with 3.8% w/v sodium citrate anticoagulant (1/10 final volume) prepared in collection tubes. As an alternative method to collect blood with supposed reduction in coagulation, retro-orbital bleeding was performed under non-recovery anaesthesia as recommended by the Named Veterinary Surgeon of the animal facility. Briefly, ketamine (delivered dose 75 mg/kg) and medetomidine (delivered dose 1.0 mg/kg) were diluted then administered by intraperitoneal injection to achieve a surgical anaesthetic duration of 30-40 minutes. Capillary tubes (Hirschmann, Eberstadt Germany) were placed in 3.8% w/v sodium citrate anticoagulant (1/10 final volume) prepared in collection tubes. Once the pedal reflex was lost and deep anaesthesia reached, the capillary tube was inserted into one corner of the eye and blood extracted directly into the collection tube. Once sufficient blood was collected, the mouse was sacrificed by cervical dislocation.

Anti-coagulated blood was diluted 1/20 in HEPES-Tyroses buffer (10 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, 11.9 mM NaHCO₃, 5 mM glucose, pH 7.4). In flow cytometry tubes, PE-conjugated anti-mouse CD41 antibody (BioLegend,

California, US, final concentration 20 µg/ml) was added to diluted blood (total volume 50 µl) and incubated at RT for 15 minutes. Following incubation, 1 ml of filtered PBS -/- and 50 µl of vortexed Precision Count Beads (10 µm diameter, BioLegend, California, US) which emit broad-spectrum fluorescence were added and samples analysed using a BD Accuri flow cytometer (BD, New Jersey, US). Samples were analysed using a forward scatter vs. side scatter dot plot with the threshold reduced to minimum so to identify platelets, erythrocytes and beads. Fluorescent beads were gated using the FL3 channel and murine platelets using the FL2 (PE) channel. To standardise the volume of sample analysed, 500 beads were acquired in each instance, with the counting beads having a known concentration of 100 beads/µl. Platelet counts were recorded in addition to volume of blood collected so that anticoagulant dilutions could be accounted for. Observations such as ease of blood withdrawal and clot formation were recorded for each instance.

4.2.10 Gene transfer to ADAMTS13 mice

To investigate if ADAMTS13 expression can be generated in ADAMTS13 knockout mice, F/HN vector was delivered to male and female knockout mice, between the ages of 8-21 weeks. rSIV.F/HN vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak and TSSM were delivered to mice via intravenous and intranasal delivery (n=3 mice/group). Max feasible dose (MFD) was administered to mice, dictated by the viral titres achieved during manufacturing (vGM213-mADAMTS13=1.42e8 TU/mouse, vGM228-mADAMTS13+Kozak=3.07e6 TU/mouse). Blood was sampled via tail venepuncture at regular intervals post-dosing (1, 2 and 5 weeks). End-point sample collection took place 16 weeks post-dosing, as described above. ADAMTS13 cleavage activity was determined in plasma and BALF using the FRET-VWF73 assay, described in 4.2.7 and in some cases expressed as a percentage of wildtype activity measured in plasma or BALF.

A multidose pilot study was conducted in a small number of mice whereby ADAMTS13 knockout mice were delivered 5x intravenous doses of vGM213-mADAMTS13 (7.5e8 total TU/mouse, n=3 males, 17 weeks old) or TSSM (n=4, 2 males/2 females, 37 weeks old) on five consecutive days. Blood was sampled via tail venepuncture 1 week after the final dose. End-point sample collection took place 14 weeks post-dosing, as described above.

mADAMTS13 cleavage activity was determined in plasma and BALF using the FRET-VWF73 assay, described in 4.2.7.

4.2.11 Induction of TTP

ADAMTS13 knockout mice have no spontaneous symptoms of TTP and therefore mice must be challenged to exhibit disease like phenotypes. This includes VWF infusion, whereby the literature states 2 VWF ristocetin cofactor units per gram of body weight (2 VWF:RCoU/g) of recombinant VWF will cause severe thrombocytopenia (169). Using the sources of VWF available, plasma-derived hVWF (1 U/g dose of Voncento, CSL Behring, Pennsylvania, US) and recombinant VWF expressed by stably transfected HEK293T cells and a fraction containing 'high molecular weight multimers' separated by size exclusion chromatography (1 µg/g dose, sourced from the Laffan Lab, Imperial College London) was delivered to mice by intravenous injection. After 3 hours, blood was sampled by retro-orbital bleeding and platelet counts performed (described in 4.2.9). In an attempt to induce endogenous release of VWF in mice, recombinant mouse TNFα (0.1 µg/g dose, BioLegend, California, US) was delivered to knockout and wildtype mice by intravenous infusion and 3 hours later, blood sampled by retro-orbital bleeding and platelet counts performed (179).

4.2.12 Measurement of hADAMTS13 cleavage against F/HN and VSV-G

To determine if ADAMTS13 expressed by vector producer cells cleaves F/HN envelope proteins, thus reducing the transduction capacity of vector, virus encoding EGFP (rSIV.F/HN-EGFP) was incubated with ADAMTS13 standard from a murine ADAMTS13 ELISA kit (Abbexa, Cambridge, UK) then transduction efficiency measured by flow cytometry. Specifically, rSIV.F/HN-EGFP, and as a comparison VSV-G vector encoding EGFP, was diluted in Opti-Mem (MOI 1) and incubated with ADAMTS13 standard (0, 200, 500, 1000 pg/ml) in collection tubes at 37°C for 1 hour. As a negative control, F/HN and VSV-G vectors were incubated with human AAT standard (Abcam, Cambridge, UK) at 1000 pg/ml. Next, incubated virus was added to 24 well-plates seeded with HEK293T cells, following the usual transduction protocol. Following 72 hours, cells were trypsinised, pelleted then resuspended in 500 µl PBS -/- supplemented with 1% v/v FBS and stored on ice. Cell suspensions were filtered through a 35 µm mesh into flow cytometry tubes (Thermo Fisher Scientific, Massachusetts, US) and TO-PRO-3 Iodine (working solution 1 µM in PBS -/-,

Thermo Fisher Scientific, Massachusetts, US) applied as the live/dead stain just before analysis. Transduced cells were analysed using a BD Accuri flow cytometer, using a forward scatter vs. side scatter dot plot to first identify cells. EGFP signal was detected using the FL1 channel (FITC) and TO-PRO dead stain in the FL4 channel (APC), gating for live cells only. 10,000 live cells were analysed and percentage GFP positive cells recorded for each condition.

4.3 Results

Shown in the present report, intravenous and intranasal administration of rSIV.F/HN achieved long-term GLux expression in serum following a single dose (Chapter 3). To investigate the suitability of rSIV.F/HN vector for treatment of a systemic disease, gene transfer for the correction of ADAMTS13 deficiency in a mouse model of TTP was investigated.

4.3.1 ADAMTS13 transgenic mice were successfully bred and a colony of knockout mice established

Adamts13 transgenic mice (B6.129-ADAMTS13^{tm1Dgi}) were sourced from The Jackson Laboratory whereby exons 1-6 of the murine ADAMTS13 gene had been replaced with a neomycin cassette. Upon their arrival, genomic DNA was isolated from ear notches and their genotype determined by PCR using mutant/wildtype specific primers. Figure 4.6 shows a representative agarose gel where PCR reactions were separated by gel electrophoresis. All three genotypes can be visualised; expected band sizes were wildtype Adamts13^{+/+} mice 361 bp, heterozygous Adamts13^{-/+} mice 361bp and 290bp, and knockout Adamts13^{-/-} mice 290bp. Upon arrival, wildtype and Ht mice were crossed and subsequently Ht offspring bred to generate knockout mice. Knockout mice breeding pairs were established and a colony of knockout mice bred for gene transfer studies. The phenotype of knockout mice was mild, with no premature deaths or breeding abnormalities observed across 3 years (data not shown, observations only).

4.3.2 Commercial ELISA kits do not specifically detect murine ADAMTS13

To determine plasma levels of ADAMTS13 in mice, two commercially available ELISAs were investigated. According to the manufacturers (AVIVA Systems Biology and Abbexa Limited), the ELISAs were validated against 'natural' mADAMTS13 rather than recombinantly expressed protein. Thus to investigate the detection of plasma mADAMTS13, blood was sampled from mice by cardiac puncture and antigen levels measured using the AVIVA Systems Biology ELISA. Figure 4.7 demonstrates the plasma ADAMTS13 levels measured across all three genotypes of transgenic mice (wildtype, heterozygote and knockout) using commercial ELISA kit, AVIVA Systems Biology. Overall differences in plasma ADAMTS13

levels (mean $\pm$ SD) observed in wildtype mice (210.0 ± 96.34 ng/ml), heterozygous mice (127.9 ± 72.71 ng/ml) and knockout mice (133.3 ± 70.10 ng/ml) were not significantly different from each other (one-way ANOVA, using Šídák's multiple comparisons test). A second ELISA kit from Abbexa Limited was used to remeasure mADAMTS13 levels in plasma and similarly a lack of genotype-related difference was found between wildtype, heterozygous and knockout mice (data not shown).

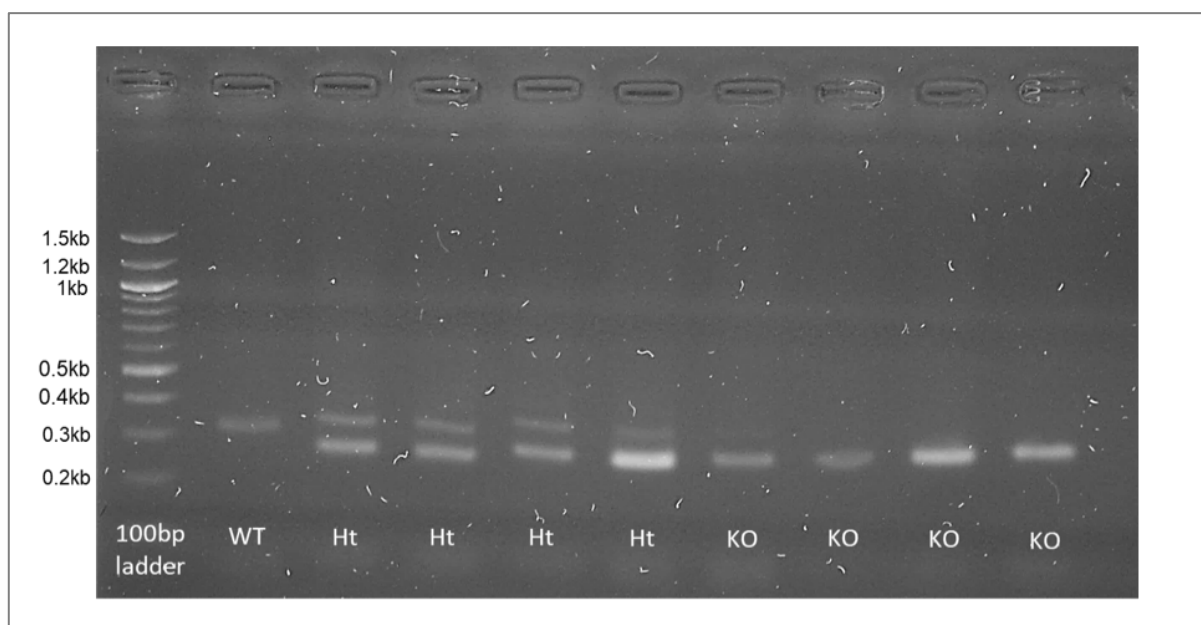


Figure 4.6- PCR reactions to determine the genotype of B6.129-ADAMTS13tm1Dgi transgenic mice.

Genomic DNA was extracted from ear notches and part of the murine *ADAMTS13* gene amplified by PCR using One-Taq DNA Polymerase. Wildtype/mutant forward primers and common reverse primers were used in PCR reactions, using 50 ng genomic DNA template following the conditions given in Table 2.1- Reaction 6. PCR products were separated by gel electrophoresis using a 1% agarose gel. A 100bp ladder is shown with reference band sizes labelled, kb= kilobases. Expected band sizes were: *Adamts13*^{+/+} (WT) = 361 bp, *Adamts13*^{+/-}(Ht) = 361bp and 290bp, and *Adamts13*^{-/-} = 290bp. Representative gel displaying all three genotypes. Wildtype (WT), heterozygote (Ht) and knockout (KO) mice.

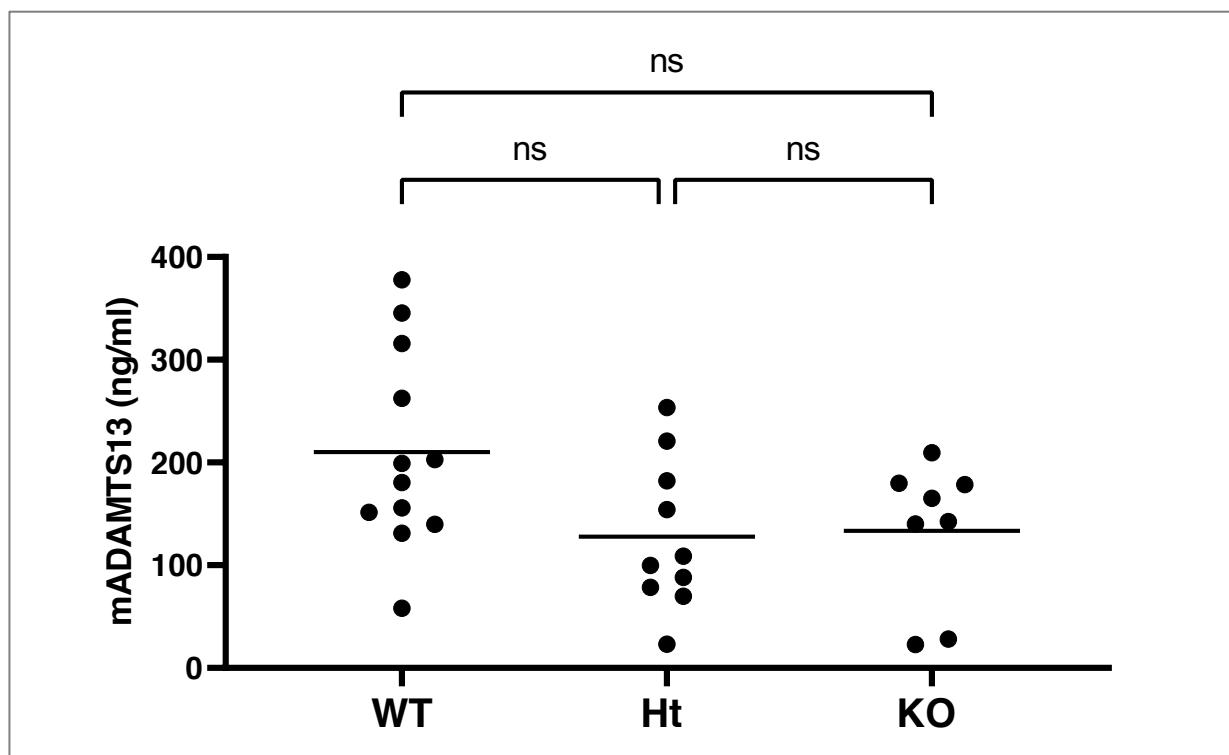


Figure 4.7- Measurement of murine ADAMTS13 (mADAMTS13) levels in plasma using an ELISA.

Blood was collected from ADAMTS13 transgenic mice by cardiac puncture and mixed immediately with 0.5M EDTA, final dilution 1/20. Plasma was prepared by centrifugation then ADAMTS13 levels measured by commercial ELISA kit, AVIVA Systems Biology. ADAMTS13 levels in wildtype (WT, n=12), heterozygous (Ht, n=10) and knockout (KO, n=8) female and male mice measured across five separate occasions. Horizontal bars represent mean values, and each dot represents an individual mouse. Ns = not significant difference between all groups when measured by one-way ANOVA, using Šídák's multiple comparisons test.

4.3.3 A VWF73 cleavage fragment was expressed and successfully purified

Without a suitable ELISA to measure mADAMTS13 antigen levels, a functional assay was required which could determine the level of ADAMTS13 activity in plasma and cell supernatant. Expanding upon various in-house developed functional assays, a cleavage fragment consisting of an N' 3X FLAG-tag, a minimal VWF fragment consisting of 73 amino acids (D1596-T1668) and codon-optimised for mouse, and C' 6X His-tag (FLAG-mVWF73-His) was designed and expressed in bacteria. Recombinant protein was extracted from bacterial cultures and protein separated by SDS-PAGE. Figure 4.8 A+C are SDS-PAGE gels stained with Coomassie Blue, which is a stain for detection of protein. Figure 4.8A shows protein lysate before and after-IPTG induction of FLAG-mVWF73-His expression in bacteria. As can be seen, there is the appearance of a protein band of size ~15-20kDa in the post-IPTG lysate only, which is predicted to be the FLAG-mVWF73-His fragment.

To purify the recombinant protein fragment, affinity liquid chromatography was performed whereby the His-tagged protein within the protein lysate bind to NTA-Ni²⁺ ions and are eluted by high imidazole concentration buffer (500 mM). Figure 4.8B shows the UNICORN trace which is recorded throughout the chromatography process. After an initial equilibration of the column with 100% buffer B (elution buffer) followed by 100% buffer A (binding buffer), the protein lysate diluted in binding buffer is injected through the system 10 ml at a time. The UV absorbance (blue trace) peaks during sample injection, due to detection of flow-through proteins non-specifically bound to the column, and falls between applications. Conversely the conductivity (orange trace) of solution shows a decrease between 100% buffer A and the injection of sample diluted in nine-parts buffer A, with small peaks in conductivity between sample injections. Once all the sample was delivered, a sharp elution peak thought to be FLAG-mVWF73-His can be observed when 100% buffer B was reached. The eluted protein fraction was compared to pre-purification sample by SDS-PAGE followed by Coomassie staining. Figure 4.8C demonstrates that multiple proteins were eliminated during purification, whilst the suspected FLAG-mVWF73-His band was intensified and concentrated when compared to the pre-purification fraction. Despite not reaching 100% purity, the selection for FLAG-tagged proteins during the plate-based assay should eliminate further impurities.

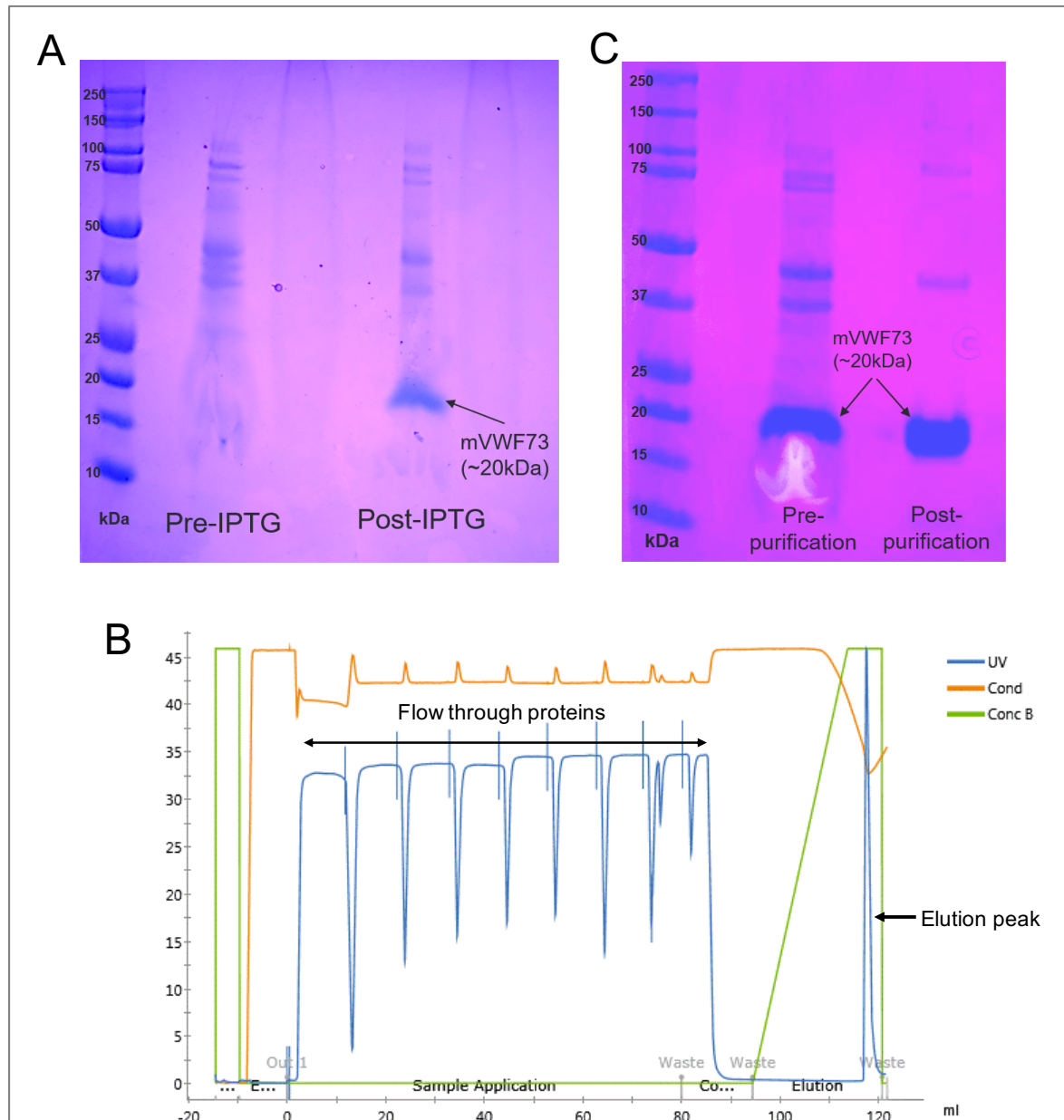


Figure 4.8- Expression and purification of FLAG-mVWF73-His cleavage fragment.

A VWF73 cleavage fragment was expressed in BL21 (DE3) *E.coli* cells, extracted then visualised by reducing sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) 4-12% Bis-Tris Plus gel, followed by Coomassie staining. Expression of recombinant protein was induced by 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Recombinant protein was purified by liquid affinity chromatography using a Ni^{2+} -chelating column to bind His-tagged protein. A) SDS-PAGE gel showing protein lysate extracted from bacterial cultures before and after-IPTG induction. Band of appropriate size (15-20kDa) anticipated to be VWF73 is indicated with an arrow. B) Trace recorded by UNICORN 7.0 software during affinity chromatography. UV absorbance (blue line), conductivity (Cond-orange, y axis measured in mS/cm) and concentration of buffer B (Conc B-green), the elution buffer, were monitored against the volume of solution that had passed through the column (x axis measured in ml). Detection of flow-through proteins eluted with 5 mM imidazole are indicated with an arrow. Anticipated elution peak of FLAG-mVWF73-His recombinant protein eluted with 100% buffer B (500 mM imidazole elution buffer) shown with an arrow. C) SDS-PAGE gel showing protein lysate pre- and post-purification by affinity chromatography using a NTA- Ni^{2+} column. Arrow indicates anticipated VWF73 fragment.

4.3.4 His-mVWF73-FLAG can be quantified by an in-house developed ELISA

Next an assay was developed to quantify levels of FLAG-mVWF73-His recombinant protein, an inverse measurement of ADAMTS13 cleavage activity. As shown by the illustration in Figure 4.5B, cleavage fragments were captured by anti-FLAG antibodies then detected with anti-His antibodies. Figure 4.9A shows the standard curve generated with serial dilutions of FLAG-mVWF73-His (1.12-0.0175 mg/ml) generating a dose-dependent increase in absorbance (optical density) following optimisation of the in-house developed ELISA. A linear regression was fitted, equation $y=0.9026 \cdot x - 0.07489$ with an $R^2=0.9303$ ($n=4$ standard curves, generated on two separate occasions), indicating there is a strong relationship between recombinant protein concentration and absorbance. Once the plate-based assay for detection of FLAG-mVWF73-His was optimised, the addition of cleavage reactions was investigated.

4.3.5 ADAMTS13-mediated cleavage of FLAG-mVWF73-His can be measured in plasma from wildtype and knockout mice

Reactions between murine plasma from ADAMTS13 transgenic plasma and FLAG-mVWF73-His recombinant protein was carried out in collection tubes and reactions stopped with EDTA. Detection of recombinant protein levels was then determined using the in-house developed ELISA. Figure 4.9B shows a representative experiment whereby cleavage reactions comprising knockout mouse plasma (KO #1-3) demonstrated similar absorbance to the wildtype +EDTA (WT+EDTA) negative control reactions (KO #1-3 O.D. = 0.509, 0.5140, 0.5070, compared to WT + EDTA 0.5340, median of $n=3$ wells/condition). Conversely reactions including wildtype plasma gave a significantly lower signal than knockout mice (O.D. = 0.2170). The negative control which included reactions without plasma present gave the highest signal overall (O.D. = 0.7165). Converting absorbance to % cleavage activity, Figure 4.9C demonstrates ADAMTS13-mediated activity in wildtype and knockout plasma. With cleavage activity in wildtype and WT+EDTA reactions representing 100% and 0% respectively, ADAMTS13 activity in plasma from knockout mice was found to be 2.123, 0-18.47% (median + range, $n=4$ mice measured twice/plasma sample over three experiments).

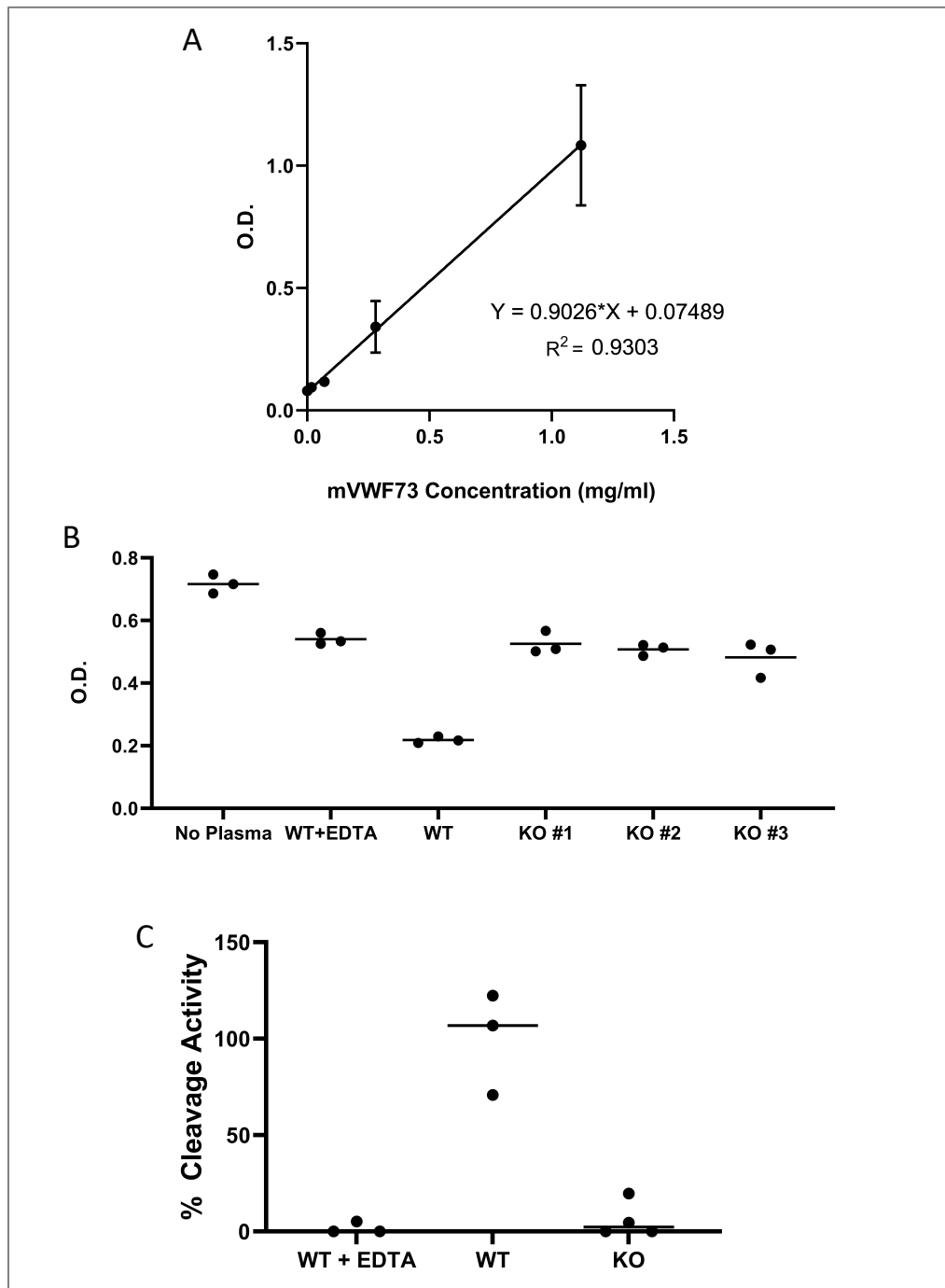


Figure 4.9- Development of an in-house assay to quantify ADAMTS13 proteolytic activity.

A minimal 73-amino acid murine VWF fragment with N-terminal FLAG- and C-terminal Histidine-tags (FLAG-mVWF73-His) was recombinantly expressed in BL21 (DE3) *E.coli* cells and purified by affinity chromatography. An in-house developed ELISA was established to quantify FLAG-mVWF73-His protein levels. Recombinant protein was captured with anti-FLAG antibodies and detected with anti-His antibodies. A) Absorbance at 450 nm (units given as optical density O.D.) was measured with increasing mVWF73 concentrations (mg/ml). A linear regression was fitted, equation $y=0.9026*x-0.07489$, with an $R^2=0.9303$. Data is expressed as mean $\pm$ standard deviation, $n=4$ standard curves measured on two separate occasions. B) Representative experiment with cleavage reactions between FLAG-mVWF73-His and wildtype (WT)/knockout (KO #1-3) murine plasma carried out in separate tubes and reactions stopped with 5mM EDTA. Reaction samples were added to the FLAG-mVWF73-His ELISA in triplicate and absorbance measured, including no plasma and WT+EDTA negative controls. C) Absorbance converted to % Cleavage activity in wildtype and knockout mice plasma, including WT+EDTA negative control. $n=3$ WT mice measured on 3 different occasions and $n=4$ KO mice measured on 3 different occasions, mix of males and females. Horizontal bars are median values, and each dot represents individual mice.

4.3.6 FRET assay can reliably determine ADAMTS13 activity in murine plasma

With the aim to identify a more consistent assay to determine ADAMTS13 proteolytic activity in murine plasma, the fluorogenic FRET-VWF73 substrate used widely in the literature was investigated (180). Upon cleavage of the minimal VWF73 substrate, the quencher is released from proximity to the fluorophore and fluorescence can be detected. Figure 4.10A shows the detection of fluorescence over time when FRET-VWF73 substrate is incubated with plasma from wildtype and knockout ADAMTS13 transgenic mice in one representative experiment. Fluorescence measurements taken at 5-minute intervals were plotted and linear regression fitted for each reaction. There is a clear separation in lines, whereby the lines which are shades of pink/orange, representing knockout plasma, remains flat over time with no significant increase in fluorescence. Conversely linear regressions which are shades of blue, representing wildtype plasma, show a distinct increase in fluorescence signal over time. Linear regression analysis of fluorescence over time was used to calculate the reaction rate (counts/minute) which was expressed as percentage of wildtype cleavage activity. Figure 4.10B shows ADAMTS13 proteolytic levels in plasma from knockout mice ($8.729\% \pm 2.812\%$, $n=18$, mean $\pm$ SD) and wildtype mice ($100\% \pm 18.94\%$, $n=15$) which was found to be a statistically significant difference ($p<0.0001$, unpaired t-test). Overall, using the FRET-VWF73 substrate to determine plasma activity levels showed high consistency between experiments suggesting the suitability of this assay to characterise plasma ADAMTS13 expression following gene transfer experiments.

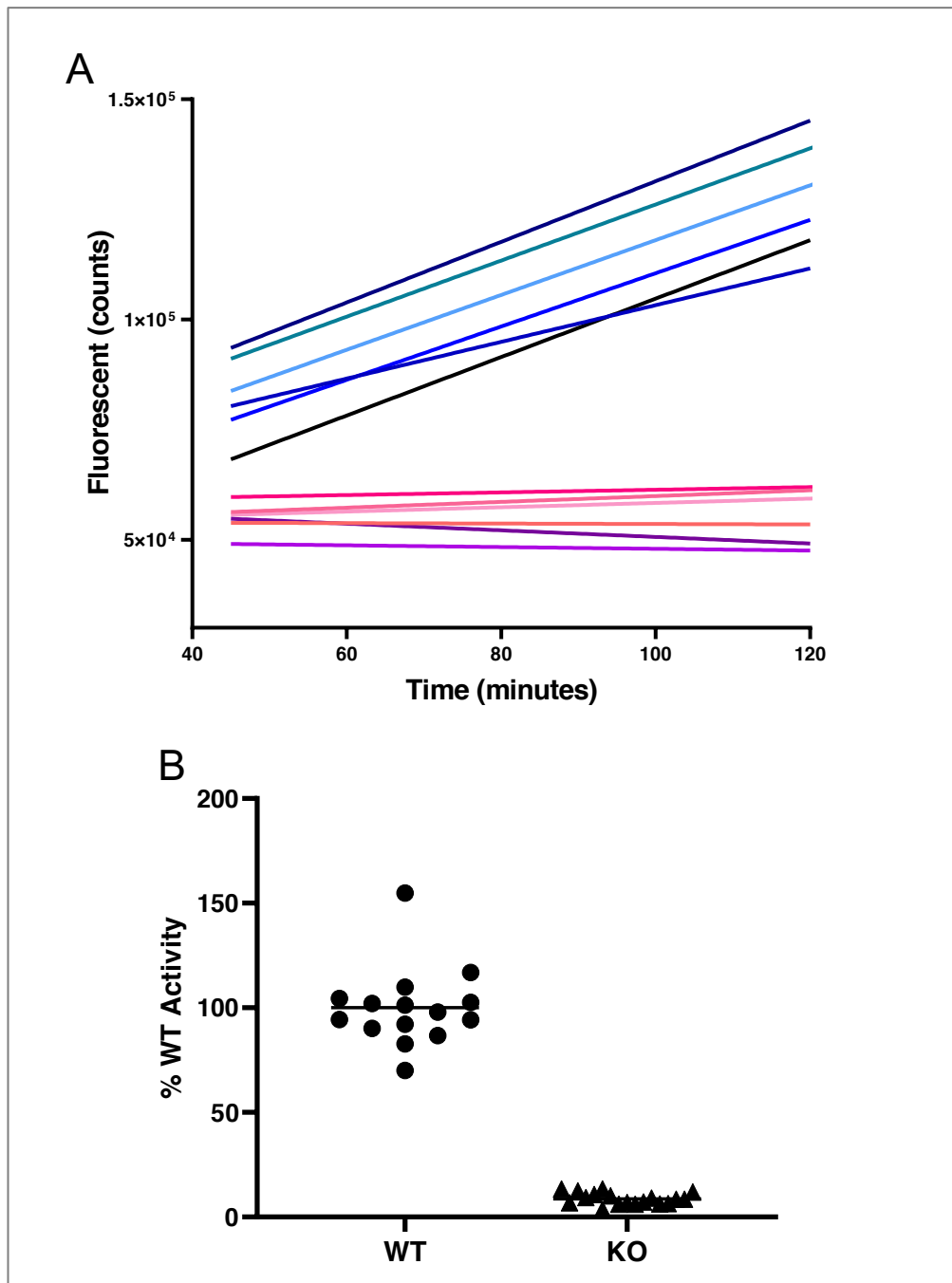


Figure 4.10- Measurement of ADAMTS13 activity in murine plasma using substrate FRET-VWF73.

The fluorescence resonance energy transfer (FRET) assay for determination of ADAMTS13 activity utilises a human VWF-73 amino acid fragment (D1596-R1668) chemically modified to include fluorophore A2pr (Nma) and quencher A2pr (Dnp) at positions Q1599 and N1610, respectively (FRET-VWF73). Proteolysis of VWF73 at the ADAMTS13 cleavage site (Tyr¹⁶⁰⁵-Met¹⁶⁰⁶) releases the quencher from proximity to the fluorophore, and fluorescence signal is emitted. Reactions containing the FRET-VWF73 substrate and murine plasma (WT and KO) were carried out in 96-well plates at 30°C with measurements taken every 5 minutes over 2 hours (excitation wavelength 340 nm, emission wavelength 450 nm). A) Linear regression was fitted for each reaction, whereby lines which are shades of blue represent wildtype mice and lines which are shades of pink/orange are knockout mice, mix of males and females. Linear regression analysis of fluorescence over time was used to calculate the reaction rate (counts/minute) which was expressed as percentage of WT cleavage activity. B) ADAMTS13 activity in plasma from knockout (KO) mice, expressed as the percentage of wildtype (WT) plasma activity. Horizontal bars represent mean values and each shape represents plasma activity from an individual mouse (n=13 WT, n=18 KO).

4.3.7 Retro-orbital bleeding is superior to cardiac puncture for the determination of platelet levels in mice

Once ADAMTS13 plasma levels could be quantified, an assay was required for the determination of platelet levels in transgenic mice. Two blood sampling methods were investigated, cardiac puncture and retro-orbital bleeding. To determine platelet counts by flow cytometry, anticoagulated whole blood was labelled with PE-conjugated antibody against mouse platelet marker, CD41, and broad-spectrum fluorescent beads added as an internal standard. Figure 4.11A illustrates the gating strategy utilised, whereby samples were analysed using a forward scatter vs. side scatter dot plot to initially identify platelets, erythrocytes and beads. Subsequently fluorescent beads were gated using the FL3 channel and platelets using the FL2 (PE) channel. To standardise the volume of sample analysed, 500 beads were acquired in each instance, having a known concentration of beads/ μl . Figure 4.11B shows platelet counts measured in ADAMTS13 knockout mice following cardiac puncture ($n=18$) and retro-orbital bleeding ($n=42$). Although median values of both blood sampling techniques were similar (505.0×10^3 vs 529.4×10^3 platelets/ μl , cardiac puncture vs retro-orbital respectively) the range was $5.05\text{-}873.1 \times 10^3$ platelets/ μl following sampling by cardiac puncture and $202.5\text{-}1095 \times 10^3$ platelets/ μl by retro-orbital bleeding with haemolysis of blood samples and clot formation occurring much less frequently. Thus, retro-orbital sampling of blood and determination of platelet counts by flow cytometry was the protocol utilised moving forward.

4.3.8 Infusion of VWF protein is not sufficient to induce TTP-like symptoms in ADAMTS13 knockout mice

Various strategies were employed to induce TTP-like symptoms, such as thrombocytopenia, in ADAMTS13 knockout mice. Mice were challenged with intravenous infusion of Voncento plasma-purified VWF (vVWF, $n=10$), recombinant VWF (rVWF, $n=5$) and $\text{TNF}\alpha$ then platelet levels determined by flow cytometry following retro-orbital blood sampling. Figure 4.12A shows platelet counts determined in knockout mice challenged with VWF (Voncento and recombinant) compared to unchallenged mice (KO, $n=42$). Similar platelet counts across all conditions can be seen (unchallenged KO = 529.4×10^3 , vVWF = 627.7×10^3 , rVWF = 431.1×10^3 platelets/ μl , median values) suggesting a lack of effect. To induce endogenous release of VWF from endothelial cells, $\text{TNF}\alpha$, a known VWF

secretagogue, was infused into knockout mice (n=7) with the addition of rVWF (n=3) and platelet levels measured. To eliminate non-strain specific effects of TNF α , wildtype mice unchallenged (n=14) and infused with TNF α (n=5) were included for comparison. Figure 4.12B shows that mice challenged with TNF α alone showed a minimal reduction in platelets (unchallenged KO = 529.4×10^3 , KO + TNF α = 477.4×10^3 platelets/ μ l), which was accentuated with the addition of rVWF (KO + TNF α + rVWF = 339.4×10^3 platelets/ μ l) although only demonstrated in few mice. In contrast, wildtype mice showed a more pronounced fall in platelets following TNF α challenge (WT = 425.6×10^3 , WT + TNF α = 237.3×10^3 platelets/ μ l) suggesting the effect of stimulated VWF release on platelet levels was not specific to the ADAMTS13 genetic background and was in fact enhanced in wildtype mice. Overall, attempts to induce thrombocytopenia in knockout mice was unsuccessful, however suitable assays were established to measure success of gene transfer in ADAMTS13 deficient mice.

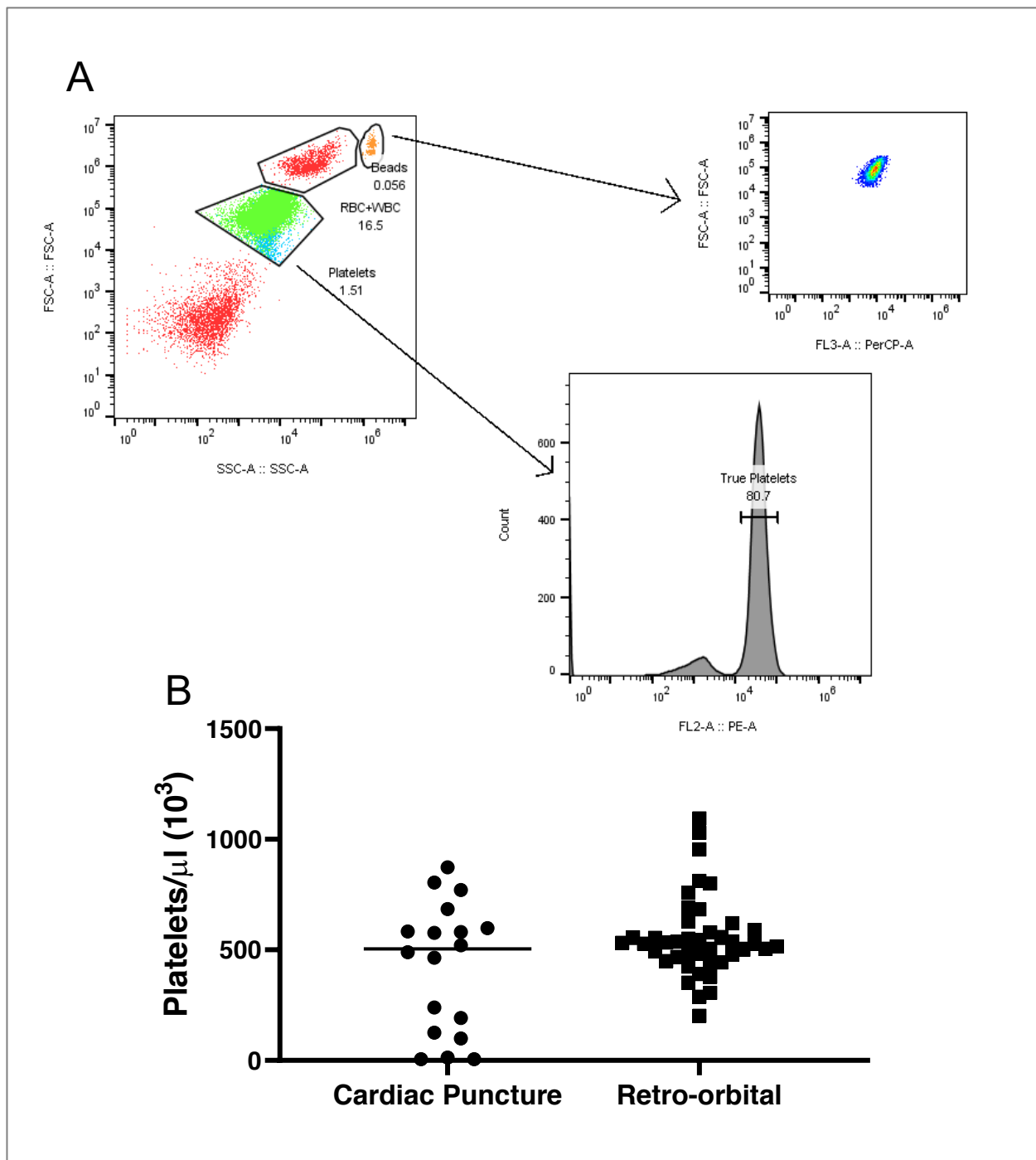


Figure 4.11- Determination of platelet counts in ADAMTS13 knockout mice by flow cytometry.

Whole blood was collected by cardiac puncture or retro-orbital bleeding into sodium citrate anticoagulant. Diluted whole blood was labelled with PE-conjugated anti-mouse CD41 (final concentration 20 $\mu\text{g}/\text{ml}$) and broad-spectrum fluorescent beads (5% final volume) added as an internal standard. A) Representative image of the gating strategy used for the measurement of platelets using a BD Accuri flow cytometer. Samples were analysed using a forward scatter vs. side scatter dot plot with the threshold reduced to minimum to first identify platelets, erythrocytes and beads. Fluorescent beads were further identified and gated using the FL3 (PerCP) channel and platelets using the FL2 (PE) channel. To standardise the volume of sample analysed, 500 beads were acquired in each instance, having a known concentration of 100 beads/ μl . B) Platelet counts determined by flow cytometry were compared following cardiac puncture ($n=18$) and retro-orbital ($n=42$) blood collection from ADAMTS13 KO mice, mix of males and females. Values expressed as platelets per microliter of blood $\times 10^3$. Horizontal bars are median values and individual shapes represent a single mouse.

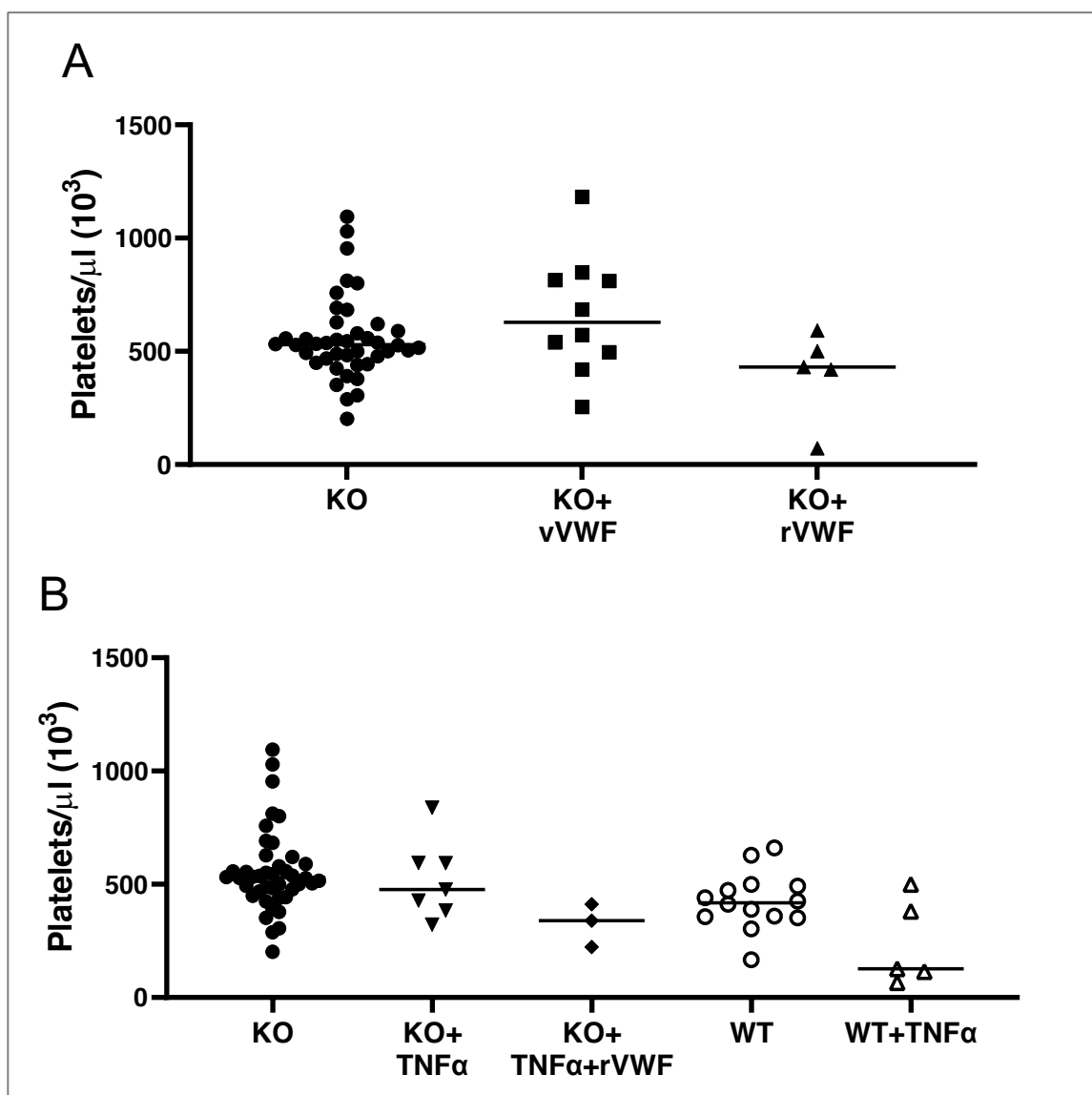


Figure 4.12- Platelet levels measured following challenge of ADAMTS13 knockout mice with von Willebrand Factor (VWF) and Tumour Necrosis Factor alpha (TNF α).

The ability of various substances to induce thrombocytopaenia in ADAMTS13 knockout (KO) mice was investigated. A) Blood was sampled from knockout mice by retro-orbital bleeding 3 hours after intravenous infusion of plasma-purified Voncento von Willebrand Factor (vVWF, n=10) or recombinant VWF (rVWF, n=5). Platelet counts were determined by flow cytometry, expressed as platelets per microliter of blood $\times 10^3$, and compared to unchallenged knockout mice (n=42, mix of males and females). B) KO mice were delivered TNF α (n=7) or TNF α in combination with rVWF (n=3) whilst WT mice received TNF α alone (n=5). Blood was sampled from mice by retro-orbital bleeding after 3 hours and platelet counts determined by flow cytometry. Horizontal bars are median values and individual shapes represent a single mouse.

4.3.9 Packaging of mADAMTS13 within a lentiviral backbone generates functional protein

Next the aim was to investigate gene transfer and restoration of ADAMTS13 expression in deficient mice. First the cDNA sequence encoding mADAMTS13 needed incorporated into the lentiviral backbone for the generation of vector. The lentiviral backbone (pGM357-hCEF-EGFP) and mADAMTS13 coding plasmid (pMAD13) were digested and ligation in the correct orientation confirmed by sequencing, creating the novel expression plasmid, pIC056-hCEF-mADAMTS13. To determine if recombinant protein expressed from the lentiviral backbone was functional against full-length VWF (FL-VWF), plasmids pcDNA3.1-hADAMTS13 or pIC056-hCEF-mADAMTS13 were co-transfected with pcDNA3.1-FL-hVWF expression plasmid into HEK293T cells. Figure 4.13 shows the detection of hVWF by western blotting, with two distinct bands indicating the presence of FL-VWF (~240 kDa) and one of the VWF cleavage products (~140 kDa). The presence of both bands is only seen following co-transfection of ADAMTS13 with VWF, regardless of species, indicating mADAMTS13 recombinant protein is proteolytically active against hVWF. The absence of the smaller cleavage fragment following transfections with VWF encoding plasmid alone, support evidence of ADAMTS13-mediated cleavage.

Due to the qualitative nature of western blots, quantitative comparisons between the densities of FL-VWF and cleaved VWF bands following hADAMTS13 or mADAMTS13 cleavage should be made cautiously. However an observation is that the FL-VWF band is larger after mADAMTS13 cleavage rather than the even densities observed between FL and smaller VWF following cleavage by hADAMTS13. The fainter bands identified underneath the full-length band could be differentially glycosylated/ post-translationally modified VWF proteins. PEI controls demonstrated no visible VWF protein, suggesting that HEK293T cells do not express detectable levels of VWF. When repeated (data not shown) the observation that pIC056-hCEF-mADAMTS13 generates proteolytically active protein was replicated.

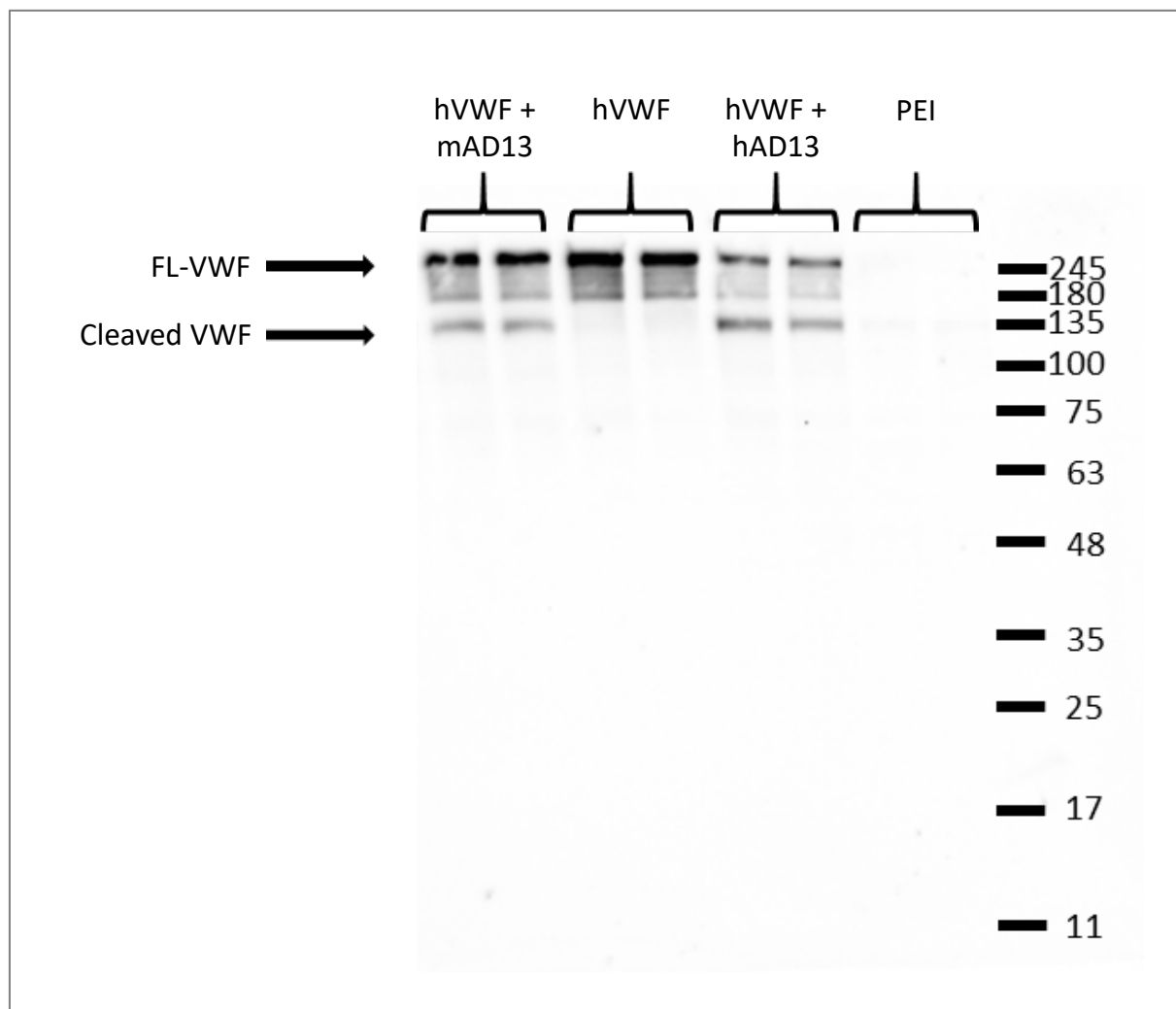


Figure 4.13- Western blot detection of human VWF after co-transfection of HEK293T cells with plasmids encoding ADAMTS13 and VWF.

HEK293T cells were co-transfected with full-length human VWF (pcDNA3.1-FL-hVWF) and either the pIC056-hCEF-mADAMTS13 or pcDNA3.1-hADAMTS13. Cell lysates were separated by reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-12% Bis-Tris Plus gel, blotted to nitrocellulose membrane and incubated with a rabbit anti-VWF HRP antibody (diluted 1/1000 in PBS) overnight at 4°C. Bands corresponding to full-length (FL), uncleaved VWF (~270 kDa) can be visualised in addition to one of the VWF cleavage products (~140 kDa). Simulated protein standard shown on the right hand side. As a negative control, cells were transfected with pcDNA3.1-FL-hVWF only. PEI controls are cells transfected with PEI reagent only. Cell lysate from two wells/condition are shown.

4.3.10 Dose dependent increase in ADAMTS13 activity following HEK293T transfection with mADAMTS13 expression plasmids

Although a quantitative assay had been selected for the measurement of ADAMTS13 activity in murine plasma, the FRET assay was next investigated to determine recombinant protein levels produced by cells *in vitro*. HEK293T cells were transfected with increasing concentrations of pIC056-hCEF-mADAMTS13/pIC040-hCEF-mADAMTS13+Kozak and ADAMTS13 activity measured in cell supernatant, shown in Figure 4.15. The FRET assay successfully detected ADAMTS13 activity in the supernatant of transfected cells, despite protein being recombinantly expressed. Figure 4.15 shows a dose-dependent increase in ADAMTS13 activity following transfections with both pIC056-hCEF-mADAMTS13 and pIC040-hCEF-mADAMTS13+Kozak, with significant levels above background (untransfected cells) detected at 0.2 µg/ml (pIC056-hCEF-mADAMTS13/pIC040-hCEF-mADAMTS13+Kozak $p < 0.01$, Kruskal-Wallis test, Dunn's multiple comparisons test, $n = 4$ wells) and 1.0 µg/ml (pIC056-hCEF-mADAMTS13 = $p < 0.001$ $n = 4$ wells, and pIC040-hCEF-mADAMTS13+Kozak = $p < 0.0001$ $n = 6$ wells, Kruskal-Wallis test, Dunn's multiple comparisons test). Transfections with 0.1 µg/ml pIC056-hCEF-mADAMTS13/pIC040-hCEF-mADAMTS13+Kozak did not demonstrate activity above background levels (no significant difference, Kruskal-Wallis test, Dunn's multiple comparisons test, $n = 4$ wells) suggesting a threshold concentration is required to achieve detectable ADAMTS13 activity by FRET assay. Comparing proteolytic activity between pIC056-hCEF-mADAMTS13 and pIC040-hCEF-mADAMTS13+Kozak transfected cells, comparable levels can be observed at all concentrations (0.1 µg/ml = 306.9 and 304.7, 0.2 µg/ml = 503.3 and 499.9, 1.0 µg/ml = 791.3 and 831.6, median values in counts/minute for pIC056-hCEF-mADAMTS13 and pIC040-hCEF-mADAMTS13+Kozak transfections, respectively). Overall, the FRET assay was shown here to detect dose-dependent increases in ADAMTS13 activity following transfections of HEK293T cells with pIC056-hCEF-mADAMTS13 and pIC040-hCEF-mADAMTS13+Kozak, with no differences in transfection efficiency at all concentrations.

4.3.11 Dose dependant increase in hADAMTS13 expression determined by both FRET and ELISA assays

Next, the suitability of FRET and ELISA assays for the detection of hADAMTS13 was investigated *in vitro*. HEK293T cells were transfected with increasing concentrations of

pIC083-hCEF-hADAMTS13 and activity against the FRET-VWF73 substrate measured in cell supernatant, shown in Figure 4.15A. Significant hADAMTS13 proteolytic activity above background (untransfected cells, n=11) was detected at 0.1 µg/ml (**p<0.01, one-way ANOVA, Tukey's multiple comparisons test, n=9 wells) and 0.2 µg/ml (****p<0.0001, one-way ANOVA, Tukey's multiple comparisons test, n=9 wells) with results combined from two separate transfection experiments (circles and triangles represent separate experiments). Furthermore, a dose response was detected with a significant increase in hADAMTS13 activity between transfection concentrations (****p<0.0001, one-way ANOVA, Tukey's multiple comparisons test).

Utilising a commercially available ELISA kit (R&D Systems) hADAMTS13 was assayed in cell supernatant from the same transfection experiments described above. Figure 4.15B shows that untransfected cells expressed low levels of hADAMTS13 (0.2977 ± 0.2598 ng/ml, mean $\pm$ SD, n=9) demonstrating low endogenous expression in the HEK293T cell line. Transfection of HEK293T cells with 0.1 µg/ml pIC083-hCEF-hADAMTS13 resulted in 10-fold higher levels of protein (2.549 ± 1.137 ng/ml, n=9 wells) yet this difference was not statistically significant (no significant difference, one-way ANOVA, Tukey's multiple comparisons test). However, protein expression measured after transfection with 0.2 µg/ml pIC083-hCEF-hADAMTS13 (30.71 ± 15.21 ng/ml, n=10) was significantly enhanced compared to untransfected cells (****p<0.0001, one-way ANOVA, Tukey's multiple comparisons test) and transfections with 0.1 ng/ml pIC083-hCEF-hADAMTS13 (****p<0.0001, one-way ANOVA, Tukey's multiple comparisons test). The correlation between ADAMTS13 activity and antigen levels was found to be significant (Pearson $r=0.6370$, $p=0.0005$). Thus, two assays are suitable for the quantification of hADAMTS13, with data replicated across two different transfection experiments.

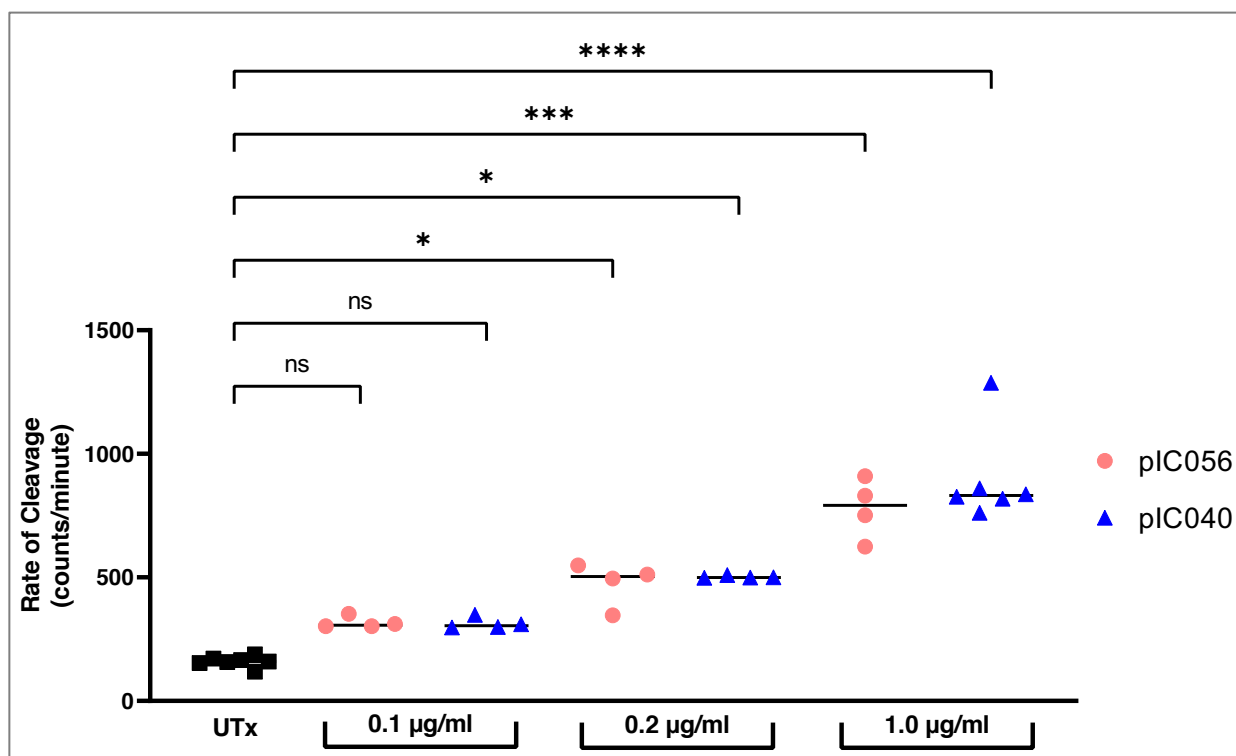


Figure 4.14- FRET assay determination of mADAMTS13 activity following transfection of HEK293T cells.

HEK293T cells were transfected with pIC056-hCEF-hADAMTS13 (pink circles, n=4 wells/condition) and pIC040-hCEF-mADAMTS13+Kozak (blue triangles, n=4 or 6 wells/condition) at three different plasmid concentrations (0.1, 0.2, 1.0 µg/ml) and mADAMTS13 levels measured in cell supernatant 72 hours post-transfection. mADAMTS13 activity was measured using the fluorescence resonance energy transfer (FRET) assay and linear regression analysis of fluorescence over time used to calculate the reaction rate (counts/minute). Cleavage activity was compared between transfection conditions and untransfected (UTx) cells (n=7 wells). Results are grouped from two separate transfection experiments, horizontal bars are median values and each shape represents an individual well. Ns = not significant difference, *p<0.05, ***p<0.001, ****p<0.0001 when compared to untransfected cells, using Kruskal-Wallis with Dunn's multiple comparisons test.

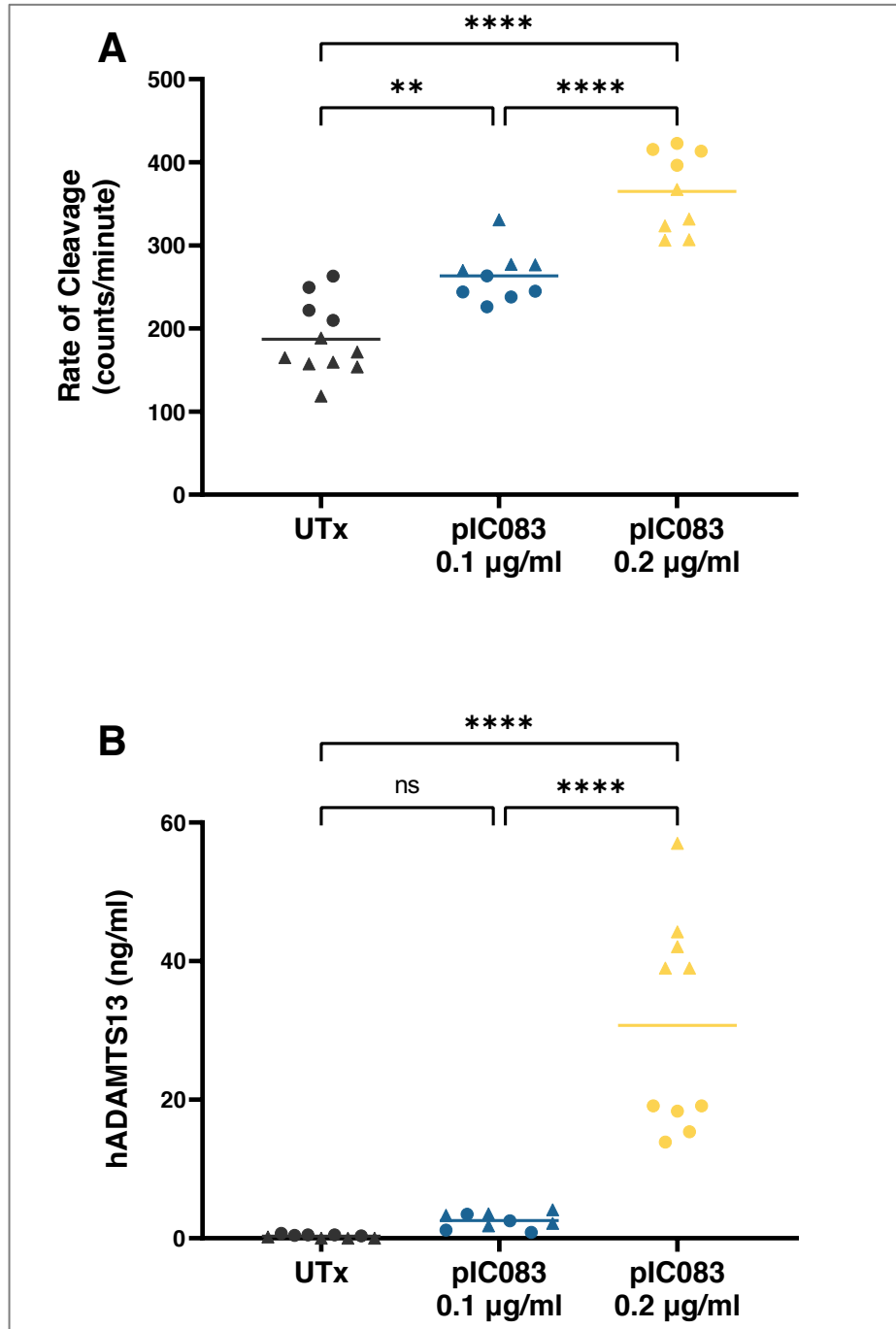


Figure 4.15- Detection of hADAMTS13 by FRET and ELISA assays following *in vitro* transfections of HEK293T cells.

HEK293T cells were transfected with pIC083-hCEF-hADAMTS13 at two different plasmid concentrations (0.1 µg/ml n=9, 0.2 µg/ml n=9). hADAMTS13 antigen and activity levels were measured in cell supernatant 72-hours post-transfection and compared to untransfected cells (UTx n=11). A) hADAMTS13 activity was measured in cell supernatant using the fluorescence resonance energy transfer (FRET) assay and linear regression analysis of fluorescence over time used to calculate the reaction rate (counts/minute). B) hADAMTS13 antigen levels in cell supernatant were measured using a commercial ELISA kit (R&D Systems) with protein levels expressed as ng/ml. Results are grouped from two separate transfection experiments (circles and triangles representing separate experiments), horizontal bars are mean values and each shape represents an individual well. Ns = not significant difference, **p<0.01 and ****p<0.0001 when each condition is compared to each other using one-way ANOVA with Tukey's multiple comparisons test.

4.3.12 Small-scale lentiviral production generates low titres of rSIV.F/HN vector

To generate lentiviral vector encoding mADAMTS13 in-house, suspension HEK293T-S17 cells were co-transfected with the five plasmids required for viral production, including pIC056-hCEF-mADAMTS13. After 72 hours vector was harvested and infectious titre calculated by transducing HEK293T cells with serial dilutions of viral supernatant (vGM213-mADAMTS13). Genomic DNA was extracted and viral integration quantified using ddPCR with primers and probes specific to WPRE. Primers and probes specific to housekeeping gene hTERT were used to determine WPRE copies/genome which allows calculation of infectious titre once vector dilution was taken into account. Figure 4.16 shows the infectious titre calculated following ddPCR analysis of transduced cells. Two flasks of vGM213-mADAMTS13 were produced in parallel, generating comparable vector titres (2.81e6 and 3.96e6 TU/ml, Flask 1 and Flask 2, n=4 and n=5 respectively).

Overall, the vector generated in-house by small-scale production was unlikely to deliver sufficient titres for *in vivo* experiments. Thus, large-scale vector (5 litre) production was outsourced to collaborators at Oxford University to generate vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak. Three smaller batches of vGM213-mADAMTS13 were received (2.41e8, 3.97e8 and 1.42e9 TU/ml) and one larger batch of vGM228-mADAMTS13+Kozak (3.07e7 TU/ml). To align titres calculated in-house with Oxford's, ddPCR determination of infectious titre following titration of HEK293T cells with vGM213-mADAMTS13 3.97e8 TU/ml was calculated as 3.5e8 TU/ml, suggesting an alignment of titre calculations.

4.3.13 Optimised ddPCR protocol for the determination of viral RNA in transduced cells

Having successfully determined viral integrations in transduced cells, a protocol was optimised to quantify vector transcript copy number by RT-ddPCR. Primer/probes specific to the cDNA of three different housekeeping genes and vector-specific WPRE were investigated with reactions performed in single-plex and multiplex at three different RNA concentrations (1, 5, 10 ng/reaction). Figure 4.17 shows the transcript copy number determined for each of the primer/probe targets as single-plex reactions (Figure 4.17A-D), B2M multiplexed with GusB (Figure 4.17 A+B) and WPRE multiplexed with HPRT (Figure 4.17 C+D). With each assay, a linear relationship between copy number and template was

observed. GusB and HPRT reactions had similar levels of transcript copies across all template concentrations, yet B2M had over ten-fold greater levels. B2M transcript numbers are consistent between single-plex and multiplex reactions at 1 and 5 ng/reaction with an anomalous result detected in the 10 ng single-plex B2M reaction (Figure 4.17A). GusB copies were highly consistent between single-plex and multiplex reactions at 1, 5 and 10 ng (Figure 4.17B). Alternatively, the number of HPRT and WPRE transcripts are significantly reduced following multiplexing at 10 ng, with greater consistency observed at 1 and 5 ng (Figure 4.17 C+D). HPRT and WPRE assays were performed together for future RT-ddPCR reactions with an RNA concentration of 5 ng/reaction where minimal interference between HEX and FAM fluorescent channels were detected.

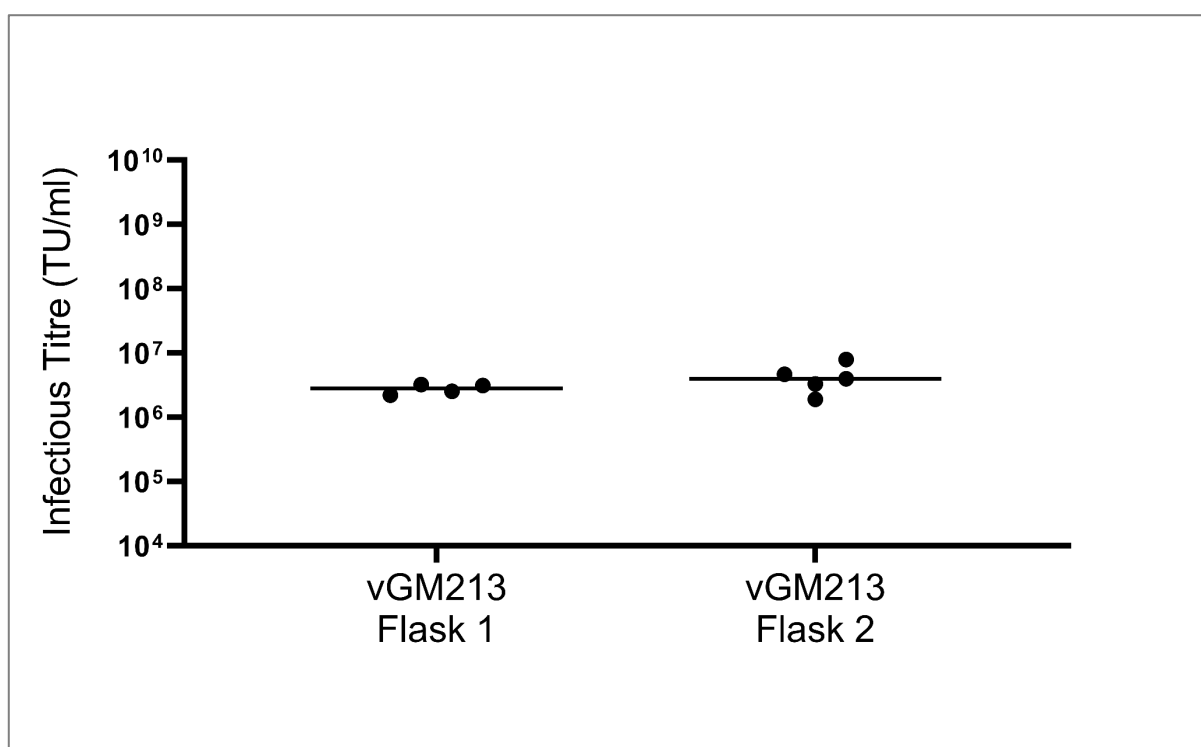


Figure 4.16- Small-scale production of rSIV.F/HN vector and quantification of infectious titre by ddPCR.

Two flasks of suspension HEK293T-S17 cells were co-transfected with the five plasmids required for the production of rSIV.F/HN, including the mADAMTS13 expression plasmid pIC056-hCEF-mADAMTS13. After 72-hours vector was harvested and infectious titre calculated by transducing HEK293T cells with serial dilutions of viral supernatant (vGM213-mADAMTS13). After 48 hours, genomic DNA was extracted from transduced cells and viral integration quantified using droplet digital PCR (ddPCR). WPRE copies normalised to housekeeping gene hTERT (WPRE copies/genome) allows calculation of infectious titre, expressed as transduction units/ml (TU/ml). Infectious titre calculated for two flasks of vGM213-mADAMTS13 produced in parallel (Flask 1 n=4, Flask 2 n=5). Horizontal bars are median values and each shape represents an individual well.

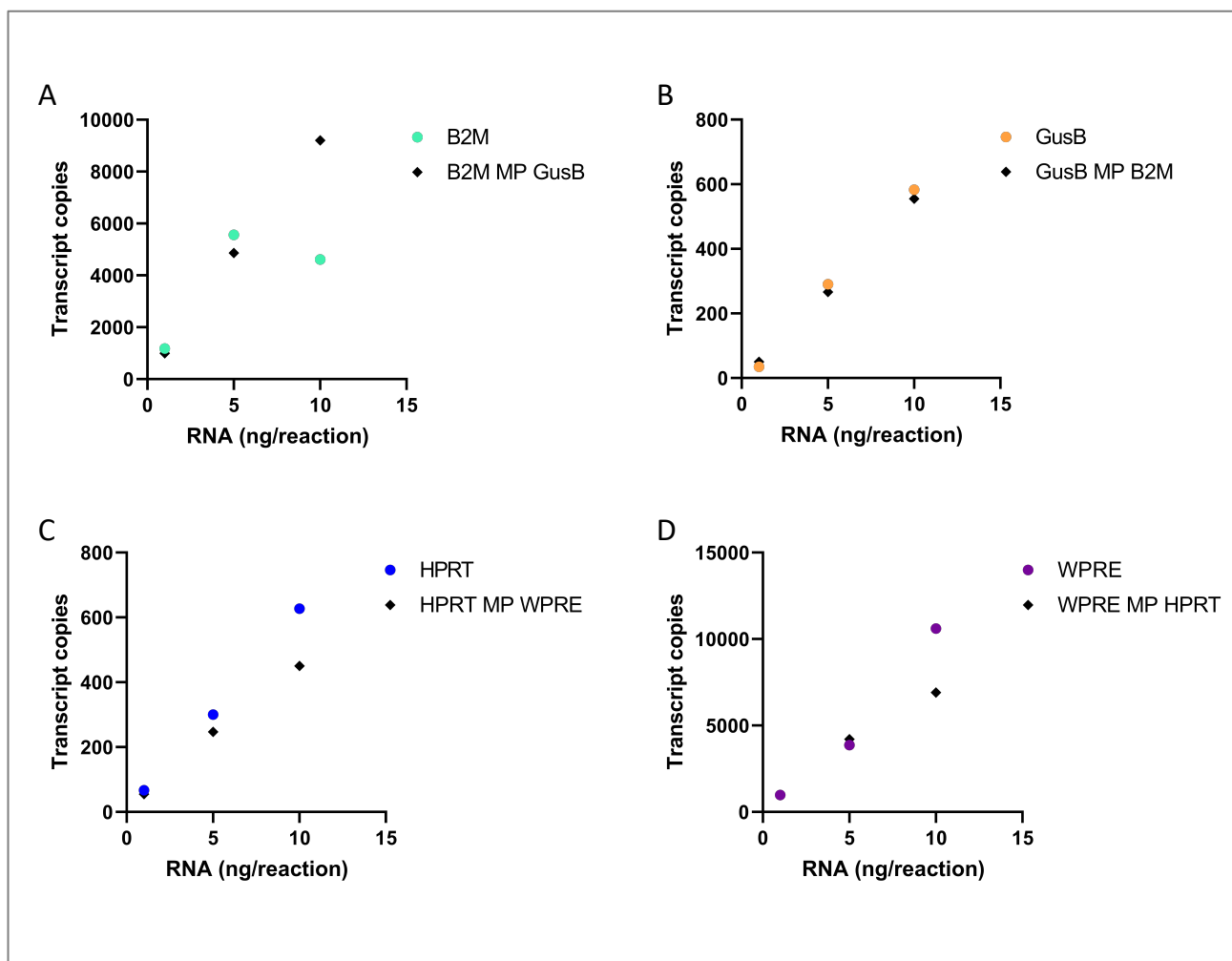


Figure 4.17- Optimisation of reverse transcriptase droplet digital PCR (RT-ddPCR) for the determination of viral transcript copy number.

Optimal template concentration and primer/probe combinations against WPRE and three different housekeeping genes, *GusB*, *B2M* and *HPRT* were investigated. Transcript copy number of housekeeping genes/WPRE was determined in single-plex (A-B2M, B-GusB, C-HPRT and D-WPRE only/reaction) or multiplex (MP) (A+B-GusB+B2M, C+D- WPRE+HPRT in multiplex) reactions at three concentrations of RNA (1, 5 and 10 ng/reaction) using the One-Step RT-ddPCR Advanced kit for Probes. RNA template was the same for each reaction, extracted RNA from transduced HEK293T cells. Each shape represents the average of two readings, black diamonds are multiplexed reactions and coloured circles are the single-plex reactions.

4.3.14 Dose dependant increase in WPRE transcript number detected by RT-ddPCR

To demonstrate the detection of WPRE transcripts, A549 cells were transduced with increasing concentrations of vGM213-mADAMTS13 (MOI 1, 10, 50, 100) and RT-ddPCR performed in a small number of samples. Figure 4.18 shows the number of WPRE transcripts normalised per transcript of housekeeping gene (HPRT) detected in extracted RNA by RT-ddPCR. Cells transduced with the lowest dose of vector (MOI 1) showed minimal WPRE transcripts above background (0.1567 vs 0.04473 normalised WPRE copies, MOI 1 vs untransduced cells UT respectively, median values n=2). An MOI of 10 resulted in an approximately 10-fold increase in WPRE transcripts compared to MOI 1 (15.23 WPRE copies/HPRT, MOI 10, n=2), however an increase in MOI from 10 to 50 did not see a 5-fold enhancement in vector transcripts (22.50 normalised WPRE transcripts, MOI 50, n=4). Interestingly there was almost exactly a two-fold increase in detected vector RNA from MOI 50 to 100 (44.94 WPRE transcripts/HPRT, MOI 100, n=2) yet MOI 10 to 100 did not generate a 10-fold increase in WPRE transcripts. Thus, a dose dependant increase in vector transcript copy number could be detected in transduced A549 cells using the optimised RT-ddPCR protocol.

4.3.15 Increasing MOIs of rSIV.F/HN-mADAMTS13 generates enhanced levels of ADAMTS13 activity

To next compare the transduction efficiency of vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak, HEK293T cells were transduced with two doses of vector (MOI 1 and 10) and ADAMTS13 activity measured in cell supernatant using the FRET assay. Figure 4.19A shows the comparisons in ADAMTS13 levels between vectors at increasing MOIs. All transductions resulted in significant ADAMTS13 activity above background (data not shown on graph, ****p<0.0001, one-way ANOVA, Tukey's multiple comparison test). At lower vector dose (MOI 1) there was no significant difference between cells transduced with vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak (ns= not significant, one-way ANOVA, Tukey's multiple comparison test) yet at higher vector dose (MOI 10) there was significantly higher levels of ADAMTS13 following transduction with vGM213-mADAMTS13 compared to vGM228-mADAMTS13+Kozak (****p<0.0001, One-way ANOVA, Tukey's multiple comparison test). In fact, levels of ADAMTS13 activity were comparable between

the two doses of vGM228-mADAMTS13+Kozak (ns= not significant, one-way ANOVA, Tukey's multiple comparison test).

To compare transduction efficiency on a molecular level with functional cleavage results, WPRE transcript copy number was determined in transduced cells by RT-ddPCR. Figure 4.19B shows the number of WPRE transcripts normalised per transcript of HPRT detected in extracted RNA across the transduction conditions. Despite small n numbers, all transduction conditions generated detectable viral transcript above background levels detected in untransduced cells (0.0003174 median WPRE copies/HPRT). Furthermore, it can be seen that higher doses of vector resulted in greater transcript copies following vGM213-mADAMTS13 transduction (0.08140 and 1.099 median WPRE copies/HPRT, MOI 1 and 10 respectively) and vGM228-mADAMTS13+Kozak transduction (0.09232 and 0.6315 median WPRE copies/HPRT, MOI 1 and 10 respectively). Therefore on a molecular level, dose-dependent increases in transduction can be detected, which is not the case utilising the functional FRET assay. Although, transcript copies are higher at MOI 10 following transduction with vGM213-mADAMTS13 compared to vGM228-mADAMTS13+Kozak (1.099 and 0.6315, median WPRE copies/HPRT vGM213-mADAMTS13 compared to vGM228-mADAMTS13+Kozak respectively) which does reflect the analysis of ADAMTS13 protease activity. Overall, rSIV.F/HN vector encoding mADAMTS13 generates detectable levels of protease activity when used to transduce HEK293T cells *in vitro*. A dose-dependent increase in transduction efficiency is observed after vGM213-mADAMTS13 transduction on a protein level and following vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak transduction on a molecular level.

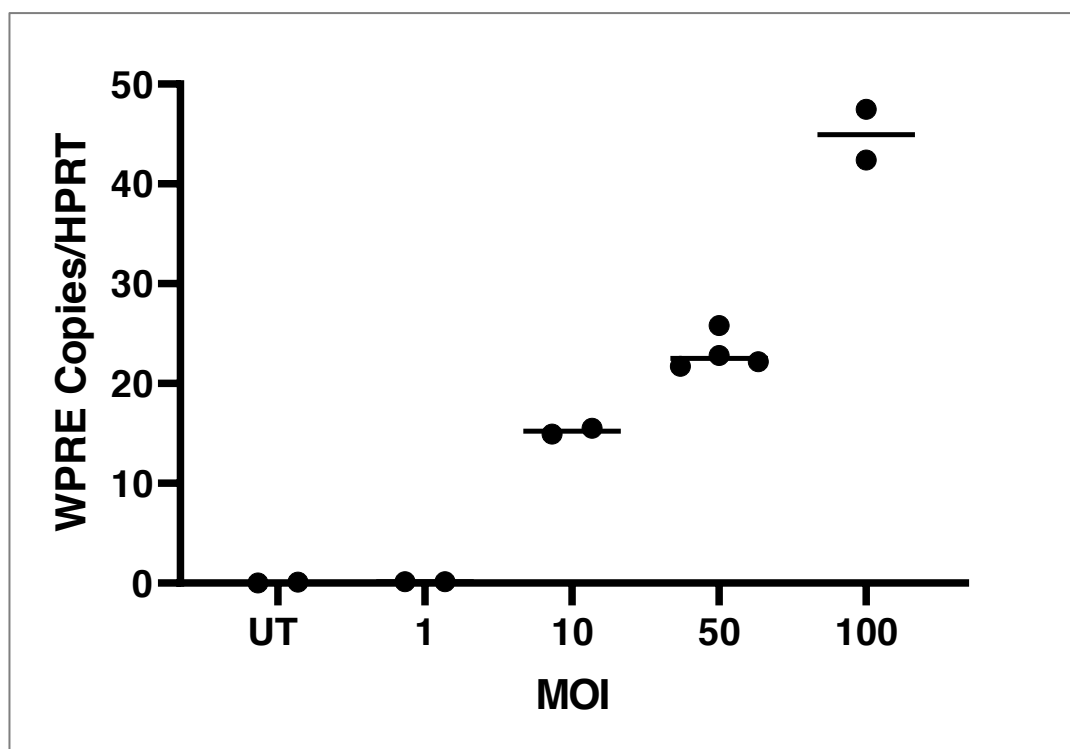


Figure 4.18- Detection of viral transcripts by reverse transcriptase droplet digital PCR (RT-ddPCR).

A549 cells were transduced with rSIV.F/HN vGM213-mADAMTS13 at multiplicity of infections (MOIs) of 1 (n=2), 10 (n=2), 50 (n=4) and 100 (n=2). Viral-specific RNA was quantified by reverse transcriptase droplet digital PCR (RT-ddPCR) using the One-Step RT-ddPCR Advanced kit for Probes and 5 ng of extracted RNA from transduced cells as template/reaction. WPRE (viral-specific sequence) transcript copy number was normalised to transcript copies of housekeeping gene *HPRT* and compared between transduction conditions and untransduced cells (UT n=2). Horizontal bars are median values and each shape represents an individual RT-ddPCR reaction.

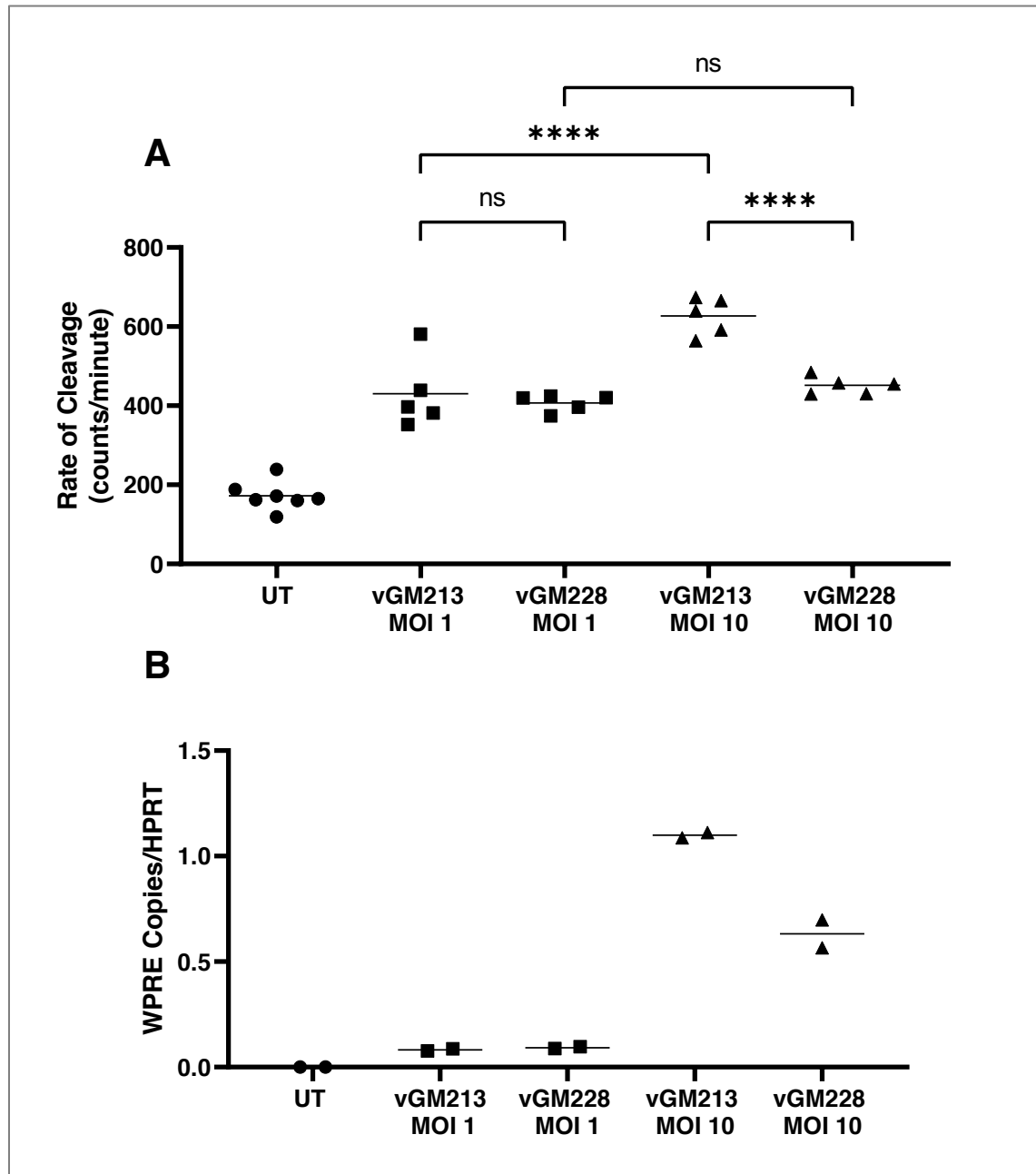


Figure 4.19- rSIV.F/HN mediated expression of mADAMTS13 in HEK293T cells.

HEK293T cells were transduced with rSIV.F/HN vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak at multiplicity of infections (MOIs) of 1 (squares) and 10 (triangles). A) mADAMTS13 activity was measured in supernatant using the fluorescence resonance energy transfer (FRET) assay and linear regression analysis of fluorescence over time used to calculate the reaction rate (counts/minute). Cleavage activity was compared between transduction conditions (n=5 wells/condition) and untransduced cells (UT n=7 wells). Horizontal bars are mean values and each shape represents an individual well. Ns = not significant difference, ****p<0.0001 when comparing groups using one-way ANOVA with Tukey's multiple comparisons test. B) Viral-specific RNA was quantified by reverse transcriptase droplet digital PCR (RT-ddPCR) using RNA extracted from transduced cells. WPRES (viral-specific sequence) transcript copy number was normalised to housekeeping gene *HPRT* and compared between transduction conditions. RT-ddPCR was performed on RNA extracted from two wells/condition. Horizontal bars are median values and each shape represents an individual well.

4.3.16 Gene transfer to deficient mice restores ADAMTS13 expression in BALF

To next investigate if rSIV.F/HN-mediated gene transfer can lead to ADAMTS13 expression in knockout mice, vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak were delivered to mice via intravenous and intranasal delivery. Maximum feasible dose (MFD) in a single administration was delivered to mice (vGM213-mADAMTS13=1.42e8 TU/mouse, vGM228-mADAMTS13+Kozak=3.07e6 TU/mouse, n=3 mice per condition) and blood was sampled via tail venepuncture at regular intervals post-transduction (weeks 1, 2 and 5) and by cardiac puncture 16 weeks post-transduction. Figure 4.20 shows ADAMTS13 cleavage activity measured by the FRET-VWF73 assay in plasma 5 weeks (Figure 4.20A) and 16 weeks (Figure 4.20B) post-transduction (activity at 1 and 2 weeks not shown) expressed as a percentage of wildtype. Mean activity measured in wildtype mice was assigned as 100% and mean background activity measured in knockout plasma as 0% cleavage. Plasma activity in knockout mice 5 weeks after they received a single dose of vGM213-mADAMTS13/vGM228-mADAMTS13+Kozak vector is shown in Figure 4.20A. ADAMTS13 activity was 10-fold greater in mice delivered vGM213-mADAMTS13 by intranasal administration (median 21.15%, 14.67-24% range, n=3) compared to TSSM control mice (median 2.063%, -2.456-19.55% range, n=8), whilst activity in mice delivered vGM228-mADAMTS13+Kozak by intranasal administration was only marginally above background (median 5.868%, 3.005-18.14% range, n=3). Mice delivered vGM213-mADAMTS13 or vGM228-mADAMTS13+Kozak by intravenous administration showed background levels of activity 5 weeks post-transduction.

End-point analysis of plasma ADAMTS13 activity (16 weeks post-transduction) after a single dose of vGM213-mADAMTS13/vGM228-mADAMTS13+Kozak vector is shown in Figure 4.20B. ADAMTS13 activity was negligible across all transduction conditions with background levels shown in the majority of treated mice. ADAMTS13 activity measured in a single mouse that received vGM213-mADAMTS13 by intravenous delivery was 33.5% of wildtype levels. TSSM control mice showed similar background levels of activity at 16 weeks (median 2.727%, -3.462-12.38% range, n=8) compared to 5 weeks post-transduction.

To determine if multiple doses of vector would generate detectable levels of plasma activity, a small number of mice received 5x daily intravenous doses of vGM213-mADAMTS13 (7.5e8 total TU/mouse, n=3 mice) or TSSM (n=4) and plasma ADAMTS13

activity measured in blood sampled by cardiac puncture 14 weeks after the final dose. As shown in Figure 4.21, plasma activity measured in mice that received 5 consecutive doses (median 9.444%, 7.234-20.35% range, n=3) was moderately higher than background levels (median 2.880%, 1.954-9.703% range, n=4).

BALF was collected from transduced mice at endpoint sample collection and ADAMTS13 activity determined by FRET-VWF73 assay. Figure 4.22 shows BALF cleavage activity, expressed as percentage wildtype BALF levels, determined 16 weeks after mice received a single intranasal dose of vector (vGM213-mADAMTS13/vGM228-mADAMTS13+Kozak grouped together, n=6 mice) or single intravenous dose of vector (vGM213-mADAMTS13/vGM228-mADAMTS13+Kozak grouped together, n=6 mice) and compared to TSSM control mice (n=8 mice). Wildtype levels of BALF ADAMTS13 activity was determined in mice administered vector by intranasal administration (median 93.92%, 4.824-285.6% range, n=6), compared to TSSM mice (median -3.395%, -52.91-100.5% range, n=8) found to be statistically significant (*p<0.05 Kruskal-Wallis test, Dunn's multiple comparison test). Mice delivered vector (vGM213-mADAMTS13/vGM228-mADAMTS13+Kozak) by intravenous administration demonstrated detectable ADAMTS13 activity in BALF (mean 34.05%, -51.06-305.2% range, n=6) however levels were not significantly above background (ns=not significant Kruskal-Wallis test, Dunn's multiple comparison test).

BALF cleavage activity measured in mice that received 5x intravenous doses of vGM213-mADAMTS13 (7.5e8 total TU/mouse, n=3 mice) was determined 14 weeks post-transduction and is shown in Figure 4.22 alongside single dosed mice and TSSM control mice. BALF cleavage activity in mice that receive 5x vGM213-mADAMTS13 (median 38.14%, 20.47-58.70% range, n=3) was not significantly above background levels (ns= not significant, Kruskal-Wallis test, Dunn's multiple comparisons test). Interestingly, single and 5x intravenous doses of rSIV.F/HN vector resulted in similar BALF ADAMTS13 activity. In summary, limited plasma expression of ADAMTS13 was detected following gene transfer, although significant cleavage activity was detected in BALF following intranasal administration of rSIV.F/HN mADAMTS13.

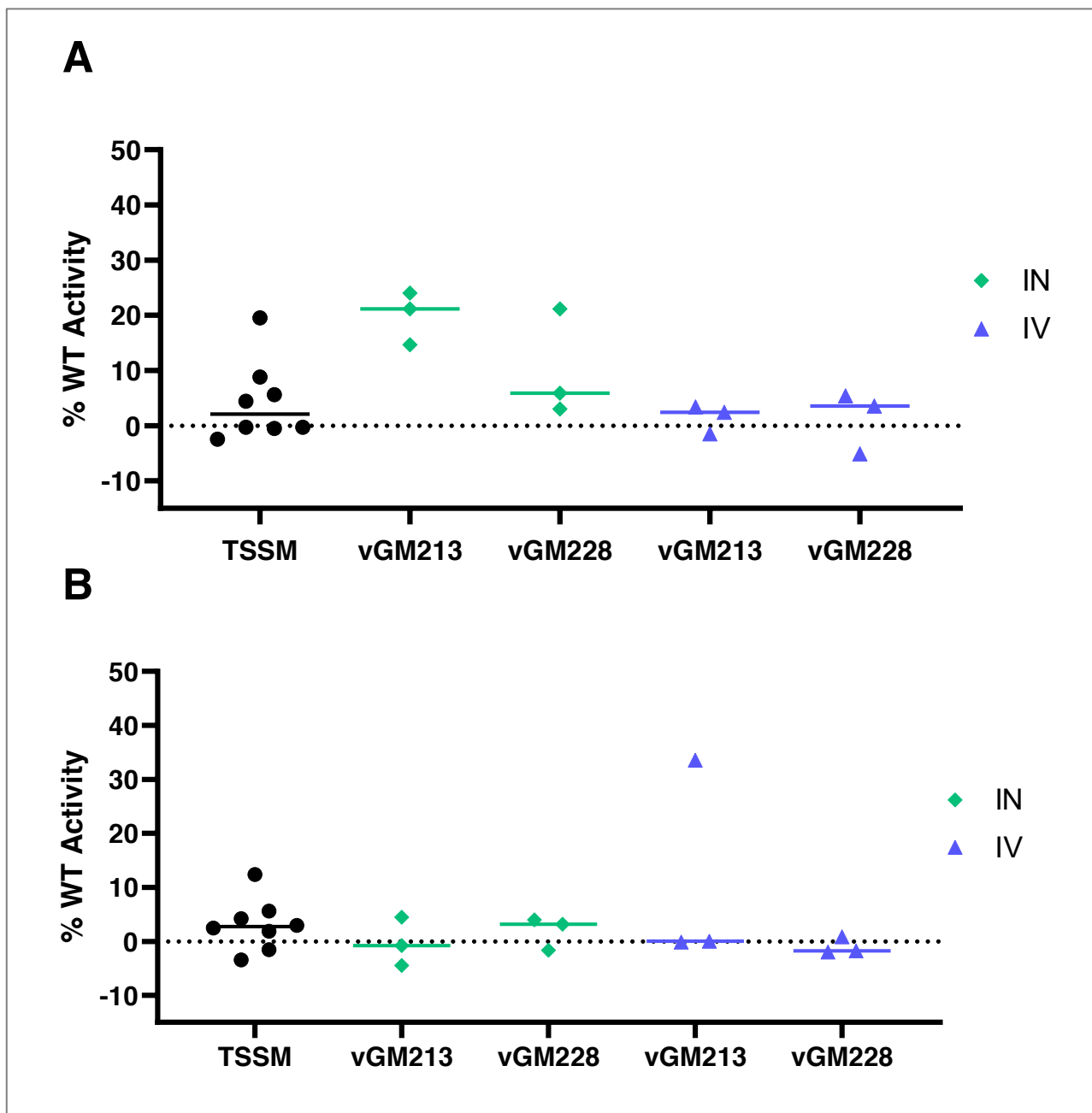


Figure 4.20- ADAMTS13 activity measured in plasma following rSIV.F/HN-mediated gene transfer to deficient mice.

rSIV.F/HN vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak were delivered to ADAMTS13 knockout mice via intranasal (IN) and intravenous (IV) delivery, mix of males and females. Maximum feasible dose (MFD) was administered to mice (vGM213-mADAMTS13=1.42e8 TU/mouse, vGM228-mADAMTS13+Kozak=3.07e6 TU/mouse) and blood was sampled via tail venepuncture at specified intervals post-dosing. mADAMTS13 activity was measured in plasma using the fluorescence resonance energy transfer (FRET) assay and cleavage activity expressed as % WT plasma activity. Cleavage activity in plasma of mice delivered TSSM (black circles, n=8) or vGM213-mADAMTS13/vGM228-mADAMTS13+Kozak vectors by intranasal administration (IN green diamonds) and intravenous administration (IV purple triangles) was measured 5 weeks (A) and 16 weeks (B) post-dosing (n=3 mice/condition). Horizontal bars are median values and each shape represents an individual mouse.

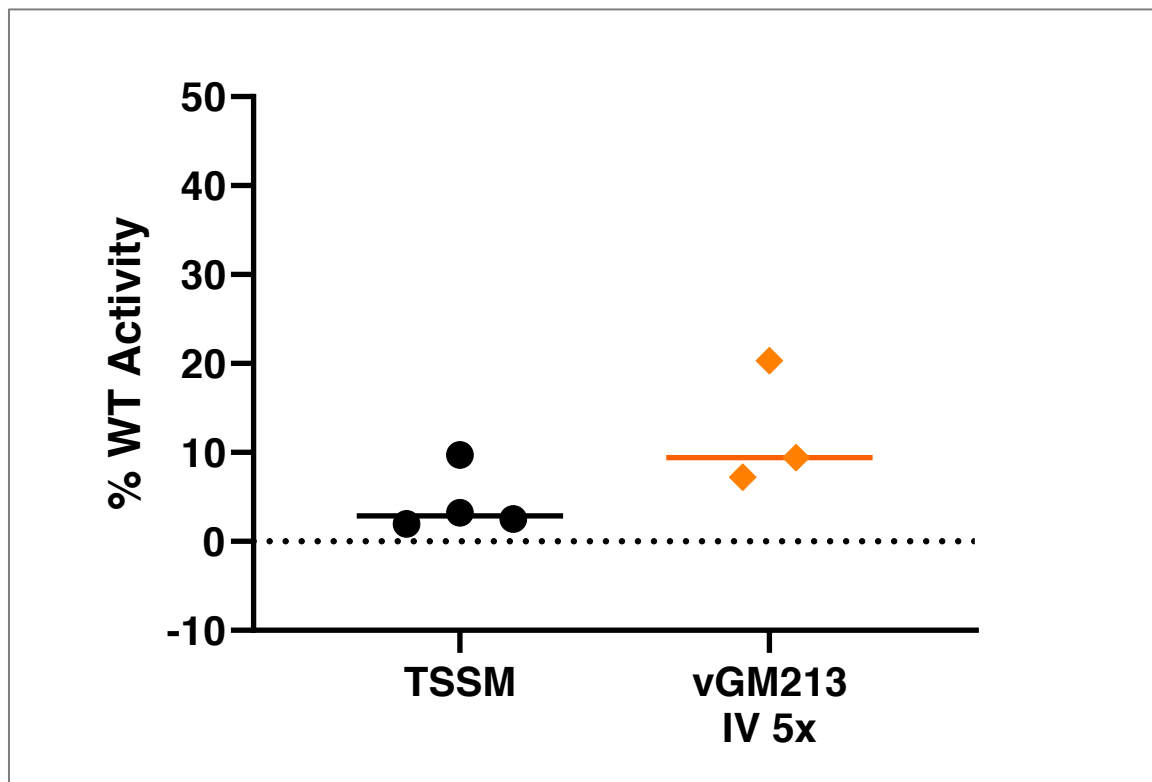


Figure 4.21- Plasma ADAMTS13 activity following multi-dosing of rSIV.F/HN vector

rSIV.F/HN vGM213-mADAMTS13 was delivered to ADAMTS13 knockout mice (mix of males and females) via intravenous (IV) delivery. mADAMTS13 activity was measured in plasma using the fluorescence resonance energy transfer (FRET) assay and cleavage activity expressed as % WT plasma activity. Plasma cleavage activity measured in mice that received 5x daily IV doses of vGM213-mADAMTS13 (7.5×10^8 total TU/mouse, orange diamonds $n=3$ mice, collected 14 weeks post-transduction) compared to TSSM control mice (black circles, $n=4$ mice). Horizontal bars are median values and each shape represents an individual mouse.

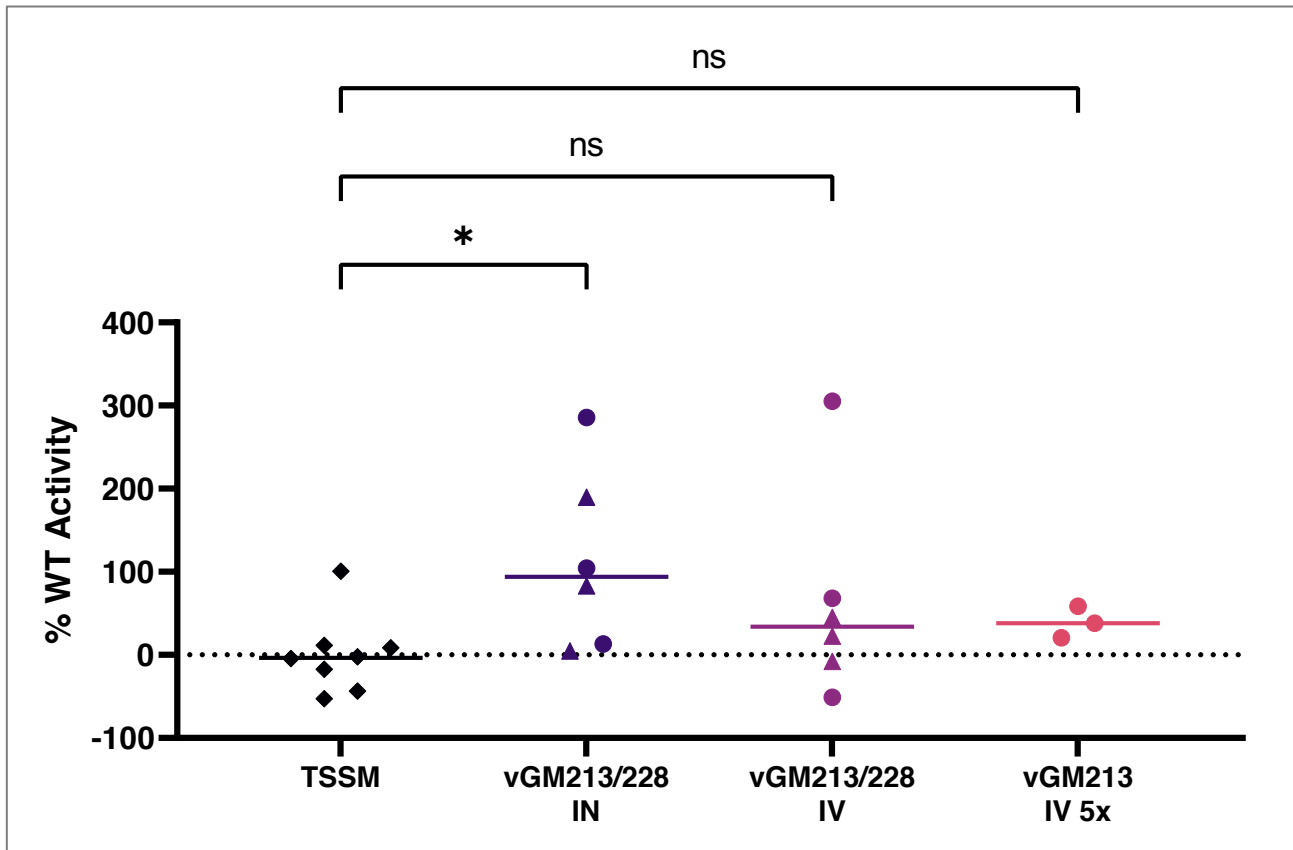


Figure 4.22- Determining mADAMTS13 activity in bronchoalveolar lavage fluid (BALF) following rSIV.F/HN-mediated gene transfer to knockout mice.

rSIV.F/HN vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak were delivered to ADAMTS13 knockout mice (mix of males and females) via intranasal (IN) and intravenous (IV) delivery. Maximum feasible dose (MFD) was administered to mice (vGM213-mADAMTS13=1.42e8 TU/mouse, vGM228-mADAMTS13+Kozak=3.07e6 TU/mouse) and collection of bronchoalveolar lavage fluid (BALF) took place 16 weeks post-dosing. mADAMTS13 activity was measured in BALF using the fluorescence resonance energy transfer (FRET) assay and linear regression analysis of fluorescence over time used to calculate the reaction rate (counts/minute). mADAMTS13 activity measured in BALF of mice delivered vector by IN and IV administration (vGM213-mADAMTS13 circles, vGM228-mADAMTS13+Kozak triangles, n=3 mice/condition) compared to TSSM controls (n=8 mice). Cleavage activity measured in BALF of mice that received 5x daily IV doses of vGM213-mADAMTS13 (7.5e8 total TU/mouse, n=3 mice, collected 14 weeks post-transduction) also shown. mADAMTS13 activity in BALF compared between transduced mice and TSSM controls (* p<0.05, ns not significant, Kruskal-Wallis test, Dunn's multiple comparisons test) Horizontal bars are median values and each shape represents an individual mouse.

4.3.17 ADAMTS13-mediated cleavage of F/HN envelope proteins reduces transduction efficiency of rSIV.F/HN vector

To determine if ADAMTS13 expressed by vector producer cells cleaves F/HN envelope proteins, thus reducing their transduction efficiency, rSIV.F/HN-EGFP and VSV-G EGFP vectors were incubated with increasing concentrations of ADAMTS13 standard (0, 0.2, 0.5, 1.0 ng/ml) or human AAT standard (1.0 ng/ml) in collection tubes at 37°C for 1 hour then transduction efficiency measured. Transduced cells were analysed using a BD Accuri flow cytometer, and percentage GFP positive cells recorded for each condition, expressed as a percentage of the ADAMTS13 negative transduction (% Transduction). Figure 4.23A shows the transduction efficiency of rSIV.F/HN-EGFP is significantly reduced following incubation with all concentrations of ADAMTS13 protein (0.2 µg/ml $p < 0.05$ $n = 4$, 0.5 µg/ml $p < 0.0001$ $n = 8$, and 1.0 µg/ml $p < 0.0001$ $n = 4$, one-way ANOVA, Šídák's multiple comparisons test) compared to the ADAMTS13 negative control.

Figure 4.23B similarly demonstrates an ADAMTS13-mediated effect on VSV-G EGFP transduction efficiency at all tested concentrations (0.5 µg/ml $p < 0.0001$ and 1.0 µg/ml $p < 0.0001$, one-way ANOVA, Holm-Šídák's multiple comparisons test, $n = 4$ wells/condition). Increasing concentrations of ADAMTS13 protein resulted in a dose dependant decrease in transduction efficiency across both vectors, with ~71% and ~67% reduction following incubation of rSIV.F/HN and VSV-G vectors with 1 ng/ml ADAMTS13, respectively. Interestingly incubation of rSIV.F/HN and VSV-G with AAT protein (1.0 ng/ml), matching the highest tested concentration of ADAMTS13 protein, did not have a significant effect on transduction efficiency with both vectors (ns =not significant, one-way ANOVA, Šídák's multiple comparisons test, $n = 4/8$ wells/condition). In summary, a significant effect of ADAMTS13 protein on rSIV.F/HN and VSV-G transduction efficiency was observed in a dose response manner, which was not replicated by incubation with AAT protein.

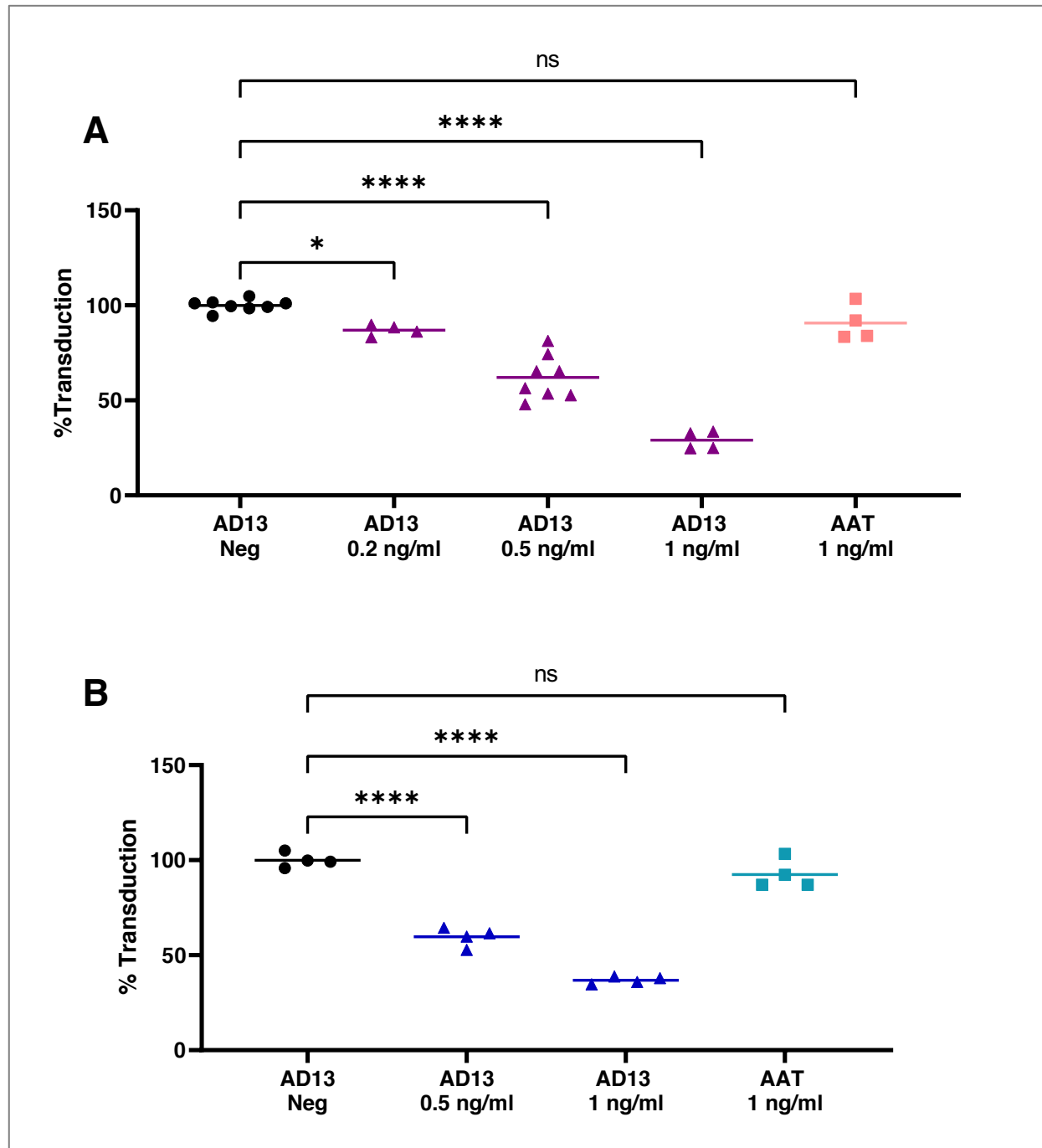


Figure 4.23- Transduction efficiency following incubation of vector with ADAMTS13 protein.

rSIV.F/HN-EGFP (A) and VSV-G EGFP vectors (B) were incubated with increasing concentrations of ADAMTS13 standard (AD13 0.2, 0.5, 1.0 ng/ml, Abcam, Cambridge, UK) or human AAT standard (1.0 ng/ml, Abcam, Cambridge, UK) then transduction efficiency compared to ADAMTS13 negative (AD13 Neg) cells (black circles, n=4/8 wells/condition). Transduced HEK293T cells were quantified using a BD Accuri flow cytometer, using a forward scatter vs. side scatter dot plot to first identify cells. EGFP signal was detected using the FL1 (PE) channel and TO-PRO dead stain in the FL4 (APC) channel, gating for live cells only. 10,000 live cells were analysed and percentage GFP positive cells recorded for each condition, expressed as a percentage of ADAMTS13 negative (% Transduction). Data in (A) has been combined from two separate experiments. Horizontal bars are mean values and individual shapes represent a single well of cells. Ns not significant, * $p < 0.05$, **** $p < 0.0001$ compared to AD13 Neg, one-way ANOVA, Šídák's multiple comparisons test.

4.4 Discussion

The present chapter aimed to investigate the suitability of rSIV.F/HN vector for treatment of a systemic disease, congenital TTP. A mouse model of TTP was first characterised and assays developed to determine efficacy of gene transfer in ADAMTS13 deficient mice. The ADAMTS13 transgenic mice purchased for this project were originally generated by Motto *et al.* (2005) by utilising a gene targeting vector to replace exons 1-6 of *Adamts13* with a neomycin resistance cassette creating a transgenic model on a B6/129 mixed strain (167). The phenotype of knockout mice was found to be very mild, with similar levels of platelets measured in wildtype mice, and normal survival/breeding behaviours observed. This reflects the observations made by Motto *et al.* (2005) who noted that *Adamts13*^{B/129-/-} mice were completely deficient in ADAMTS13 yet showed no spontaneous symptoms of TTP such as thrombocytopenia, schistocyte formation or reduced survival. Banno *et al.* (2006) similarly described no differences in haematological parameters between wildtype and ADAMTS13 deficient mice harbouring a gene disruption between exons 3-6 of *Adamts13* on the B6/129 genetic background (155).

Assessment of murine plasma ADAMTS13 levels was initially attempted using two different commercial ELISA kits, which subsequently failed to detect genotypic differences in plasma expression and measured equal antigen levels between wildtype, heterozygous and knockout mice. Neither ELISA kit had citations from journal publications thus the reliability of the findings were limited. Upon enquiry, it was found that the ELISA detection antibodies were polyclonal against the N-terminal (N') half of murine ADAMTS13, which despite deletion of exons 1-6 and the transcription start site, truncated variants could have been expressed by a downstream cryptic start codon. Alternatively, there may have been cross reactivity of antibodies with other ADAMTS proteins if the epitopes were within conserved protein domains (MDTCS) (181). In the literature, a range of strategies were employed to circumvent the apparent lack of commercial murine ADAMTS13 ELISA kits. Firstly, some research groups undertook immunisation protocols to generate antibodies for the development of in-house ELISAs (172,174–176). Other groups delivered human ADAMTS13 and thus exploited human-reactive commercial ELISA kits (173,182). Alternatively, Schiviz *et al.* (2012) and Tersteeg *et al.* (2015) utilised readouts such as thrombocytopaenia, lactate

dehydrogenase (LDH) levels and cleavage activity against FRET-VWF73 in their studies, as opposed to plasma antigen levels measured by ELISA (169,183). It was decided to follow two of these strategies in this project, using functional readouts of ADAMTS13 activity and clone the hADAMTS13 cDNA sequence into the lentiviral backbone so that commercial ELISA kits could be used following transfection/transduction experiments with pIC083-hCEF-hADAMTS13.

In pursuit of assays to measure ADAMTS13 protease activity in plasma, multiple approaches were investigated. A mVWF73 minimal fragment with N' FLAG and C' His tags was expressed and a plate-based assay designed to quantify uncleaved fragments, thus acting as an inverse measurement of ADAMTS13 activity. Kokame *et al.* (2004) first discovered the minimum VWF region required for ADAMTS13 substrate recognition and cleavage, incorporating the Y1605 and M1606 peptide bond and spanning 73 amino acids of the VWF A2 domain (D1596 to R1668), denoting its name as VWF73 (180). An advantage of using small peptides is that unlike full length VWF they do not require a denaturing agent such as urea to expose ADAMTS13 binding and cleavage sites (184). Kokame *et al.* (2004) developed a cleavage assay, including recombinantly expressed N'-tagged glutathione S-transferases (GST)-VWF73- C'-tagged His fragments, which could be used to semi-quantitatively determine ADAMTS13 activity in patient plasma through densitometry of western blots (180). Advancing upon western blot detection of VWF73 fragments, Kato *et al.* (2006) designed an enzyme-based assay to accurately quantify cleaved GST-VWF73-His fragments, using a monoclonal antibody that specifically detects Y1605 as a C' residue only and not within intact uncleaved VWF73 (185). Performed using routine laboratory equipment, enzyme-based assays represent a sensitive method for measurement of ADAMTS13 activity. Many other groups have developed similar assays, with variations in reaction conditions, capture/detection antibodies and purification tags (186,187).

In this chapter, a plate assay for detection of FLAG-mVWF73-His was optimised. The murine sequence D1596-T1668 (mVWF73) was selected as mADAMTS13 activity against a human substrate was found previously to be significantly less efficient (175,187). Despite demonstrating genotypic-differences in murine plasma ADAMTS13 activity, proof-of concept was generated in a small number of mice without the ability to replicate findings

consistently between experiments. A further consideration was that the assay was time consuming, taking a full day to perform the ELISA. Taken together, it was decided to pursue an alternative assay which could deliver reliable results in a shorter time frame. Kokame *et al.* (2005) developed a fluorogenic substrate to provide fast diagnostics for the determination of ADAMTS13 activity in patient plasma samples (178). The minimal VWF73 fragment was chemically modified, with residues Q1599 and N1610 substituted with a fluorophore (Nma) and quencher (Dnp), respectively. Within one hour, plasma activity levels could be determined using a commercial plate reader. Given the number of papers using this assay and the favourable characteristics, FRET-VWF73 was investigated. Although the commercial FRET-VWF73 sequence was human, previous literature had shown reactivity with mADAMTS13, although reduced compared to mVWF73, thus it was selected to determine protein expression in the current project (172,175,188).

FRET-VWF73 was an essential assay for the measurement of ADAMTS13 expression following transfections/transductions *in vitro* and endogenous/recombinant expression *in vivo*. Knockout mice were characterised as having 8.729% of wildtype levels (mean wildtype activity ascribed as 100%), which is higher than the 0% activity claimed by other reports using FRET-VWF73 in the same transgenic strain of mice (155,172,174). The auto-fluorescent signal observed in plasma without ADAMTS13 activity was likely accounted for by other groups, assigning background noise measured in knockout mice and signal detected in wildtype plasma as 0% and 100%, respectively. TSSM treated knockout mice were included in each gene transfer experiment to compare FRET-activity in treated mice to background levels in control mice. The variance in wildtype ADAMTS13 activity, standard deviation of 18.94%, was found equivalent to levels measured in wildtype mice by Banno *et al.* (2006) and Deforche *et al.* (2016) by FRET assay and likely represents the natural variation found in mice populations, influenced by factors such as sex and age.

Joly *et al.* (2014) compared measurement of ADAMTS13 activity using the Technozym ELISA (Technoclone, Vienna, Austria) containing a chromogenic GST-VWF73 substrate with the routinely used FRET assay (189). The lower limit of detection using the chromogen GST-VWF73 assay was found to be 3% normal human plasma (NHP), supporting previous data by Mackie *et al.* (2013), which is clinically relevant for the diagnosis of TTP patients (<10% NHP)

(190). However both groups identified occasions where detectable levels of ADAMTS13 activity were measured in severely deficient TTP patients, and high variability compared to FRET-VWF73 was observed. The lower limit of detection for the FRET-VWF73 assay was determined as 10% NHP by Joy *et al.* (2014) and 5% by Mackie *et al.* (2013), suggesting FRET-VWF73 may be useful in prescribing ADAMTS13 activity as below or above the diagnosis threshold of 10%, yet with limited capacity to accurately measure within the lower range of activity (189,190). Therefore, 8.729% of wildtype levels that was measured in ADAMTS13 deficient mice in the present study is likely to be below the limit of detection of the FRET assay and can be regarded as <10% activity.

The second readout investigated during this project was the measurement of murine platelets. The consensus method in the literature is using an automated haematological analyser, however veterinary-specific analysers are expensive and were not a facility available for this project (191). Thus platelet counts by flow cytometry using internal control beads was utilised, as performed by Crawley *et al.* (2014) and Alugupalli *et al.* (2001). The formation of large clots and consequently the variability in platelet counts following blood sampling by cardiac puncture was likely due to multiple punctures of the heart leading to platelet activation and haemolysis occurring once blood is ejected from needles (192). Sampling technique was switched to retro-orbital bleeding under non-recovery anaesthesia, by far the most prevalent method used for platelet analysis in the literature (174–176,187,193).

The median platelet count measured in control knockout mice during the present study was 529.4×10^3 platelets/ μl , measured by flow cytometry following retro-orbital blood sampling. This figure is significantly lower than $1,350 \pm 252 \times 10^3$ platelets/ μl (mean $\pm$ SD) measured by Motto *et al.* (2005) and $1,128 \times 10^3$ platelets/ μl (mean) measured by Schiviz *et al.* (2012) in Adamts13^{B/129-/-} control mice using an automated haematological analyser. As part of the Mouse Phenome Project, Peters *et al.* (2003) performed high-throughput measurements of haematological parameters, including platelet levels, across 42 mouse strains (194). The average platelet count was $1,138.70 \pm 287.75 \times 10^3$ platelets/ μl (mean $\pm$ SD) with a range of $\sim 400 \times 10^3$ to $\sim 1,800 \times 10^3$ platelets/ μl observed across strains, highlighting the highly variable levels between different genetic backgrounds. However, the average figure

calculated by Peters *et al.* (2003) strongly reflects platelet counts by Motto *et al.* (2005) and Schiviz *et al.* (2012) in the B6/129 mixed strain, although measured in wildtype mice not knockout, suggesting this genetic background phenotypically has the average number of platelets.

Conversely, Banno *et al.* (2006) describes platelet counts of $532 \pm 62 \times 10^3$ platelets/ μ l (mean $\pm$ SD) in control knockout mice from the B6/129 background, closely matching levels described here. Blood sampling/handling technique and counting method (automated analyser vs flow cytometry) are likely responsible for the differences in platelet counts (192). Furthermore, the incorrect characterisation of red blood cell fragments as platelets using automated analysers could overestimate levels, suggesting a benefit of flow cytometry using platelet-specific markers (195). Overall, blood sampling was improved by retro-orbital bleeding and flow cytometry analysis was suitable for the determination of platelet counts in the current project.

Once a method to assess platelet counts was optimised, it was attempted to induce thrombocytopenia in ADAMTS13 knockout mice as a readout for restoration of ADAMTS13 expression. In the literature, two methods are utilised to induce TTP-like symptoms in knockout mice including Shiga toxin and recombinant VWF infusion. Motto *et al.* (2005) found that B/129 mice backcrossed with the CASA/Rk genetic background demonstrated spontaneous TTP and susceptibility following TTP induction with Shiga toxin, yet the B/129 cross did not (167). It was hypothesised that the increased levels of VWF found in mice from the CASA/Rk background, >5-fold higher than levels in C57BL/6J mice and >2-fold greater than levels in B/129 mice, was responsible for the enhanced susceptibility (167,196). Yet interestingly, no correlations were found between phenotypic severity; mortality and thrombocytopenia, in Shiga toxin-challenged knockout mice (CASA-Rk cross) and plasma VWF levels. Later, Chauhan *et al.* (2008) replicated these findings by again demonstrating a lack of correlation between plasma VWF levels and degree of response to Shiga toxin challenge (197). However, a requirement of VWF for the induction of thrombocytopenia was confirmed by creating transgenic mice on the CASA/Rk background that were deficient in ADAMTS13 and VWF. Mice lacking both proteins showed no response to Shiga toxin challenge, with stable platelet levels measured for 5 days post-infusion. In contrast, mice

deficient in ADAMTS13 yet with normal plasma levels of VWF exhibited the expected thrombocytopaenia (197). Thus, the hypothesis was constructed that a threshold level of plasma VWF is required for Shiga toxin-induced thrombocytopaenia in ADAMTS13 deficient mice, yet increasing levels of VWF does not worsen disease pathology.

As mentioned previously, groups investigating the correction of ADAMTS13 deficiency either utilised the Shiga toxin model on the CASA/Rk or CAST/Ei susceptible backgrounds (172,177) or infused rVWF into mice on the B/129 background (169) or CASA/Rk cross (176). Alternatively, induction of TTP was not performed in some studies and gene transfer assessed by plasma ADAMTS13 antigen/activity levels instead (173–175,182). In the present project B/129 knockout mice were challenged with intravenous infusion of Voncento plasma-purified VWF, recombinant VWF and TNF α then platelet levels determined by flow cytometry following retro-orbital blood sampling. Delivery of 1 μ g/g dose of Voncento VWF did not result in thrombocytopaenia nor any physical symptoms such as piloerection or hunched posture. Voncento is a commercially available VWF-FVIII concentrate, similar to other products such as Haemate (CLS Behring, Pennsylvania, US) and Wilfactin (LFB, Les Ulis, France) which have been purified from human donor plasma and stabilised with albumin. Due to the exposure of endogenous VWF to ADAMTS13 in human plasma, the VWF multimeric pattern of commercial VWF products when separated by non-reducing agarose gel electrophoresis shows an absence of high molecular weight VWF multimers and presence of small satellite bands indicative of proteolysis (198,199). Thus, the absence of ultra-large VWF multimers in plasma-purified products may not be sufficient to challenge ADAMTS13 deficient mice and induce thrombocytopaenia.

The second approach investigated to challenge knockout mice was the delivery of rhVWF expressed by stably transfected HEK293T cells. Recombinant protein was separated into fractions by size-exclusion chromatography and a fraction with 'high molecular weight multimers' used for *in vivo* experiments. Delivery of 1 μ g/g dose of rhVWF resulted in comparable platelet counts to unchallenged knockout mice, although tested in a small number of mice (n=5). Xiao *et al.* (2012) and Pickens *et al.* (2015) expressed rmVWF in house, achieving a >50% fall in platelets in CAST/Ei knockout mice delivered 5 μ g/g rmVWF (177,200). The use of CAST/Ei mice and delivery of higher concentration of VWF protein may

explain the difference in outcome to the present study, despite the similarity in recombinant protein expression and purification method (200). Furthermore, the delivery of murine VWF instead of human may have prolonged the half-life of recombinant protein in the bloodstream, which was found to be just 4.5 hours following infusion of 2 VWF:RCoU/g rhVWF (169).

Alternatively, Schiviz *et al.* (2012) and Kopic *et al.* (2016) achieved severe thrombocytopaenia (~5% of platelets in unchallenged knockout mice) in B/129 knockout mice following infusion of 2 VWF:RCo U/g rhVWF (169,201). Verhenne *et al.* (2018) used the same method to induce TTP-like symptoms in ADAMTS13 deficient mice, demonstrating efficacy in the CASA/Rk genetic background (176). The reason that thrombocytopaenia was not observed in the present study following recombinant VWF infusion was likely the dose of protein delivered and perhaps the multimeric distribution. Muchitsch *et al.* (2010) determined that high doses of rhVWF (>2 VWF:RCo U/g) were required to illicit a significant effect on platelet levels in knockout mice, likely to be substantially greater than 1 µg/g dose of recombinant VWF delivered in this study, despite differences in units. The distribution of multimers in the 'high molecular weight' recombinant VWF was not investigated by agarose gel electrophoresis, thus the exact multimer composition was not guaranteed. Therefore given the sources of VWF available, the required dose of ultra-large VWF multimers to trigger thrombocytopaenia was not reached and an alternative strategy subsequently investigated.

Another method to challenge ADAMTS13 deficient mice could be to induce endothelial damage or release of endogenous UL-VWF from WPBs. The cross-over in symptoms between TTP and Haemolytic uremic syndrome (HUS); thrombocytopaenia and haemolytic anaemia, inspired the investigation of Shiga toxin which is known to cause endothelial damage and induction of VWF secretion in HUS upon infection of Shiga toxin (Stx)-producing *Escherichia coli* (202). Administration of Shiga toxin-2 to ADAMTS13 deficient mice consistently produced thrombocytopaenia in genetically susceptible transgenic mice, CASA/Rk and CAST/Ei, yet the B/129 background investigated in the present study was non-susceptible (167,203).

Substances that cause endothelial damage or VWF release may consequently reproduce thrombocytopaenia in knockout mice. Inflammatory agents such as TNF α and IL-1 β are known to stimulate endothelial cell WPBs to release their contents, including UL-VWF multimers which remain tethered to the endothelial plasma membrane (204). It was hypothesised that using a VWF secretagogue may emulate the effect of rVWF and Shiga toxin challenge leading to a consumption of platelets and thrombocytopaenia. When TNF α was delivered to ADAMTS13 deficient mice in the present study, no significant effect on platelet levels was observed. The co-delivery of recombinant VWF with TNF α demonstrated a reduction in platelets (~30%), although in a small number of mice. Interestingly, wildtype mice administered TNF α also showed significant thrombocytopaenia in 3/5 mice tested, which suggests the effect of TNF α on platelet levels is not specific to ADAMTS13 deficiency. Tacchini-Cottier *et al.* (1998) demonstrated ~40% fall in platelets in wildtype B/129 mice one hour after intravenous infusion of TNF α (179). The lack of response in ADAMTS13 deficient mice and partial response in wildtype mice could be dose related, 10 μ g/mouse infused by Tacchini-Cottier *et al.* (1998) compared to ~2.5 μ g/mouse delivered in the present study.

Similarly, Chauhan *et al.* (2008) investigated lipopolysaccharide (LPS)-induced thrombocytopaenia in ADAMTS13 knockout mice on the C57BL/6J genetic background. As shown previously, the endotoxin LPS caused a fall in platelets in wildtype mice (205). Yet thrombocytopaenia was demonstrated to the same extent in ADAMTS13 deficient mice, indicating the effect on platelet levels was not genotype specific (197). Banno *et al.* (2004) reported a collagen-induced thrombocytopaenia model whereby infusion of collagen mixed with epinephrine to B/129 knockout mice induced a reduction in platelets to ~10% levels of control mice (155). However thrombocytopaenia was also observed in wildtype mice, albeit to a lesser extent. A small number of mice died as a consequence of treatment, with similar mortality rates observed across wildtype and knockout mice, meaning this model would surpass the severity limit of the project licence for the current project (206,207). Therefore it was concluded that although some inflammatory mediators or endotoxins may induce thrombocytopaenia upon infusion to mice, the response is not specific to ADAMTS13 deficient mice and consequently is not useful to assess gene transfer in a TTP model. Given the lack of efficacy of the attempted thrombocytopaenia models, gene transfer was

assessed in the present study by measurement of plasma ADAMTS13 activity against FRET-VWF73 and molecular analysis.

Packaging of the murine ADAMTS13 cDNA into the lentiviral backbone (pIC056-hCEF-mADAMTS13) was carried out so that gene transfer studies could be conducted. Activity of recombinant ADAMTS13 protein expressed from the hCEF promoter was investigated *in vitro* following co-transfection of VWF and ADAMTS13 expression plasmids followed by SDS-PAGE and staining with anti-VWF antibodies. Complete proteolysis of VWF substrate into fragments (~170 and ~140 kDa) was demonstrated by Bruno *et al.* (2005) upon incubation with recombinant ADAMTS13 expressed by HEK293 cells (208). The absence of cleavage product (~140 kDa) following VWF-only transfections, and the presence of a clear band following co-transfection with ADAMTS13 shown here, suggests detection of ADAMTS13-mediated proteolysis. The appearance of faint bands beneath the full length VWF product (~270 kDa) could represent differentially glycosylated VWF monomers or unprocessed pro-VWF present in cell lysate without furin co-expression (170,198). Alternatively, there could be detection of satellite band (~170 kDa) due to proteolysis of VWF by low levels of endogenous ADAMTS13 expression from HEK293T cells, although limited due to the undetectable ~140 kDa product (209).

Complete proteolysis of VWF into fragments was not observed in any condition, likely due to the excess of VWF substrate. However analysis of cell medium may show differential levels of cleavage compared to the cell lysate results shown here (208). Stronger appearance of product at ~140 kDa following co-transfection of human VWF and ADAMTS13 compared to murine ADAMTS13 may infer species-related differences in cleavage activity (210). This reflects results generated by Laje *et al.* (2009) whereby comparison of the ~170 kDa product generated by murine ADAMTS13 was approximately 10% relative to cleavage by human recombinant protein (175).

In addition to pIC056-hCEF-mADAMTS13, a novel plasmid was cloned to include an extended Kozak sequence directly upstream of the mADAMTS13 protein coding sequence. The surrounding sequences of the AUG translation initiation codon, referred to as the Kozak sequence, determines the strength of the start site and thus its recognition by ribosomes

during scanning of mRNA sequences (211). In vertebrates, the consensus Kozak sequence is GCC(A/G)CCA**AUGG** (**AUG** being the translation initiation codon) whereby the most conserved position is a purine at -3 relative to the adenine in AUG, most commonly being an adenine (212). The second position that strongly influences recognition of the initiation site is guanine at +4 relative to the adenine in AUG (211). The pIC056-hCEF-mADAMTS13 plasmid (DNA sequences given) includes a NheI restriction site (*GCTAGC*) directly upstream of the ATG codon, *GCTAGCATGA*. Whilst pIC040-hCEF-mADAMTS13+Kozak includes sequence CCACC between the NheI site and ATG codon *GCTAGCCCACCATGA*, referred to as the extended Kozak. Neither plasmids contain a guanine at +4, however Kozak (1999) demonstrated comparable AUG codon recognition and thus polypeptide expression between Kozak sequences GCCACCA**AUG**(G/A). Comparable proteolytic activity against FRET-VWF73 was demonstrated between pIC056-hCEF-mADAMTS13/40 across increasing transfection concentrations, indicating the CCACC insertion did not affect mADAMTS13 expression greatly at the concentrations investigated. Significant expression levels in a dose dependant manner indicates the adenine at -3 position in both plasmids was sufficient to produce a strong recognition site for the initiation of translation, despite the adenine at +4.

Similarly, a dose dependant increase in activity was observed following transfections with pIC083-hCEF-hADAMTS13. A contributing factor that led to cloning the human cDNA sequence was to allow for more sensitive detection of ADAMTS13 expression by using commercially available ELISAs. Interestingly however, transfection of HEK293T cells with 0.1 ug/ml pIC083-hCEF-hADAMTS13 resulted in detectable cleavage activity, yet hADAMTS13 antigen levels were not significantly above background when cell supernatant from the same samples were analysed by ELISA. Overall however, a significant correlation was found between ADAMTS13 cleavage activity and antigen levels. Interestingly, this observation was not made by Laje *et al.* (2009) who found that proteolytic activity of GST-mVWF73 by mouse plasma following transplantation of transduced haematopoietic progenitor cells, did not correlate well with plasma antigen levels determined by ELISA (175). The hypothesis was that prolonged incubation of GST-VWF73 substrate with murine plasma lead to an overestimation of activity.

To investigate this relationship in human subjects, Rieger *et al.* (2006) analysed ADAMTS13 activity by a range of functional assays and plasma levels by ELISA in healthy donors and found a high degree of correlation between the two measurements (213). It was noted that the polyclonal antibodies used in the ELISA also detected antibody-bound ADAMTS13 in acquired TTP patients which are not functional in cleavage assays, although this is less of a consideration in congenital TTP mouse models. Overall, encoding the human sequence gives the benefit of having two assays, ELISA and FRET-VWF73, for detection of ADAMTS13 plasma levels.

The first rSIV.F/HN vector produced in-house, vGM213-mADAMTS13, was generated from expression plasmid pIC056-hCEF-mADAMTS13. Two flasks were produced in tandem, resulting in similar vector titres measured by ddPCR detection of integrated viral genomes. Calculation of physical titre, for example measurement of viral capsid protein p24 by ELISA or by measurement of pro-viral RNA, is routinely performed for titration of lentiviral vectors. However non-functional or empty vectors cannot be discerned from functional using physical measurements of titre and thus an overestimation of vector titre can occur. In fact, Sastry *et al.* (2002) and Geraerts *et al.* (2006) found that RT-qPCR amplification of pro-virus RNA in concentrated viral preps led to a significant overestimation of titre, up to 1000-fold compared to calculated titre by functional methods (214,215). Reasons for this are the amplification of plasmid DNA transferred from transiently transfected producer cells, which can be mitigated by DNase-treatment of viral supernatant (215). Furthermore, incomplete or rearranged genomes could be incorporated into vector particles that do not result in transgene expression yet contribute to titre calculation. Geraerts *et al.* (2006) illustrated the inaccuracies of p24 and pro-viral RNA titration by measuring comparable titres between vector batches produced with and without the VSV-G envelope plasmid, despite undetectable transduction measured when the envelope plasmid was absent during production (215). The p24 ELISA does however provide a fast, diagnostic method to establish if vector production has been successful in the first instance, with the need for follow-up measurement of infectious titre.

Measurement of titre by determining integration of vector genomes into host cells or GFP positive cells by flow cytometry accounts for only functional vector particles, thus allowing

for the accurate comparison between experiments and vector batches. The protocols include first the transduction of cell lines with serial dilutions of vector then the analysis of transduced cells by flow cytometry or qPCR to generate a reading of transduction units/ml (TU/ml). Titration by flow cytometry relies upon the lentiviral vector encoding a reporter protein, such as GFP, which limits the applications of this method in the titration of therapeutic vectors. Furthermore, analysis by flow cytometry cannot differentiate between single and multiple integrations thus requiring a sufficient dilution range of vector (214). Quantitative PCR (qPCR) assessment of vector genome integrations per cellular genome has been utilised for some time, with the accurate determination of vector copy number (VCN) demonstrated using real-time PCR with primers/probes against WPRE and a housekeeping gene (216,217). More recently, Wang *et al.* (2018) reported the use of ddPCR as a novel qPCR method for the calculation of VCN (217). In comparison of the two methods, superior accuracy and reproducibility were noted for ddPCR, allowing for the absolute quantification of template without dependency on a standard curve, alleviating variability introduced by the quality and dilution of DNA standard (217,218).

The protocol described by Wang *et al.* (2018) was directly replicated in the current study for the determination of vGM213-mADAMTS13 titre produced in-house (217). Similar titres were found between flasks (~3-4e6 TU/ml), however the in house produced vGM213-mADAMTS13 batch would not be sufficient to perform dose-escalation *in vitro* and *in vivo* gene transfer experiments. Thus production of large-batches of rSIV.F/HN vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak was outsourced to collaborators at Oxford to generate higher quality and titre of vector. To align titres calculated in-house with Oxford's, ddPCR determination of infectious titre following titration of HEK293T cells with vGM213-mADAMTS13 3.97e8 TU/ml was calculated as 3.5e8 TU/ml. This suggests a close alignment of titre calculations and therefore Oxford titres were utilised for the remainder of the study.

The measurement of vector RNA by RT-ddPCR was optimised following a similar method to Heredia *et al.* (2013), so that transcript expression could be determined on a molecular level following transduction (219). Assays against three different housekeeping genes and vector-specific WPRE were investigated to determine normalised transcript copies, performing

reactions with increasing concentrations of template RNA and in single-plex versus multiplex (B2M and GusB, HPRT and WPRE). The effect of multiplexing HPRT and WPRE assays on transcript copy number was minimal at both 1 and 5 ng template RNA, yet a reduction in signal was observed in multiplex reactions including 10 ng RNA. Interference can be explained by partition specific competition, which can occur in droplets or partitions where both templates are present (double positive for HPRT and WPRE) and thus two PCR reactions are competing for resources (220). When the PCR reactions have differential efficiencies, amplification of one template may occur at the expense of the other and result in a drop in signal from one probe channel. At higher template concentration, the likelihood of double positive partitions is greater which can lead to the observed reduction in transcript copies. The concentration of 5 ng template was therefore selected for future experiments.

Reactions including B2M and GusB assays performed in single-plex and multiplex showed robust detection of transcript copy number with no evidence of competition across all tested RNA concentrations. Indeed vector transcript number could have been normalised to a geometric mean of all three housekeeping genes, which perhaps would have been more accurate. However, this would have meant twice as many reactions and thus use of consumables which increases the cost and time of assay. Therefore a single reference gene was used to normalise WPRE transcript copies for further investigation of transduction efficiency, as performed by multiple groups (221–223).

Once an optimised protocol was established, transductions were performed in A549 cells with increasing MOIs of vGM213-mADAMTS13 and vector transcripts measured as a readout of transduction efficiency. Increasing MOIs of vector generated dose dependant increases in vector mRNA. An overall positive linear relationship was observed between vector MOI and transcript copies, where a doubling in MOI from 50 to 100 was reflected by a doubling in measured vector transcripts. In a separate experiment two methods were utilised to compare the transduction efficiency of vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak in HEK293T cells. Measurement of functional ADAMTS13 activity and vector mRNA determined a strong agreement between the two methods, 1.4-fold and 1.7-fold increase in ADAMTS13 activity and vector transcripts following transduction with

vGM213-mADAMTS13 MOI 10, respectively. Thus despite the extended Kozak sequence, vGM213-mADAMTS13 demonstrated a small increase in ADAMTS13 expression over vGM228-mADAMTS13+Kozak at higher vector doses although transduction efficiency was largely reduced compared to the previous experiment performed in A549 cells, with less than 10% transcript copies measured at matching MOIs. Numerous variables could contribute towards the difference in transduction efficiency including the disparity in vector titre between vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak (~45 fold difference) and thus volume of virus added per transduction (224). However given the small difference in transduction measured between vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak here and the lack of difference in ADAMTS13 activity measured at a plasmid level previously, both vectors were taken forward to gene transfer experiments.

Chapter 3 characterised the intravenous delivery of rSIV.F/HN to wildtype mice, whereby stable gene expression of GLux was observed in serum for 12 months following a single dose. To investigate gene transfer of mADAMTS13, vGM213-mADAMTS13 and vGM228-mADAMTS13+Kozak were delivered to knockout mice by intravenous and intranasal delivery and plasma ADAMTS13 activity measured by FRET assay. Cleavage rates measured in plasma following gene transfer were subsequently expressed as a percentage between 100% activity measured in normal mouse plasma (NMP) and 0% activity measured as background levels in control knockout mice. No significant expression was measured in plasma 1 and 2 weeks post-transduction across all treatment groups. A small increase in activity (~20% wildtype plasma ADAMTS13 activity) following intranasal delivery of MFD of vGM213-mADAMTS13 (1.42×10^8 TU/mouse) was measured 5 weeks post-transduction. Lower levels of activity, just above the noise of the assay (~6% wildtype activity) were measured in mice delivered MFD of vGM228-mADAMTS13+Kozak (3.07×10^6 TU/mouse) by intranasal administration, which was considerable given the ~45-fold lower dose of vGM228-mADAMTS13+Kozak delivered.

At endpoint analysis 16 weeks post-transduction, no detectable ADAMTS13 activity above background was found across all transduction conditions except for a single mouse delivered vGM213-mADAMTS13 by intravenous administration. Activity 34% of wildtype levels was measured in this single mouse, considerably above all other treated mice. Overall

however, plasma levels of ADAMTS13 are likely at the limit of detection and no conclusive evidence was generated that suggests significant plasma expression was generated by either vGM213-mADAMTS13 or vGM228-mADAMTS13+Kozak by intravenous or intranasal administration. The single mouse that showed elevated plasma expression following intravenous administration of vGM213-mADAMTS13 may be a true result and reflect a mouse in which transduction efficiency was higher, or alternatively this is an anomalous result despite measurements being taken in duplicate. A lack of elevated activity was measured in this mouse at earlier time points, however it should be noted that there was a difference in blood sampling between mid-experiment and endpoint sampling, with cardiac puncture performed as a terminal procedure.

Interestingly, Chan *et al.* (2014) reported a distinct difference in lipid and glucose profile when blood was sampled from mice by tail venepuncture or cardiac puncture, anticipated to be the result of differences between venous and arterial blood composition (225). Therefore it is conceivable that difference in ADAMTS13 levels measured at 5 and 16 weeks may be caused by differences between venous blood sampled by tail venepuncture and a mix of arterial/venous blood when sampled by cardiac puncture. In fact, a similar result was observed in the GLux duration expression study in Chapter 3. Serum GLux expression was higher when blood sampling was performed by cardiac puncture at the final timepoint (12 months post-transduction) compared to expression at earlier timepoints when blood was sampled by tail venepuncture. It is considered that the enhanced expression at 12 months is an artefact of the sampling method and not enhanced gene expression because GLux levels had been very stable at all other time points.

Conversely, mice delivered 5x vGM213-mADAMTS13 by intravenous administration were only sampled at a single timepoint (14 weeks post-transduction) by cardiac puncture. A ~4-fold increase in activity above control mice and mice that receive a single intravenous dose of vGM213-mADAMTS13 was detected which may indicate a summative effect of the ~5x total dose delivered to mice, inducing sufficient ADAMTS13 expression to generate detectable plasma levels by FRET assay. Although greater consistency was observed within the multidose group, median activity of ~10% wildtype levels was still well below the 34% activity observed by the single mouse despite 5x the total dose being administered. Due to

limited availability and cost of vector, repeat administration was only investigated by systemic delivery, demonstrating limited evidence of ADAMTS13 expression in plasma. However repeat intranasal administration may well have generated higher protein levels in plasma given the highly localised and efficient transduction of the lungs observed following intranasal rSIV.F/HN delivery. Sinn et al. (2008) reported that repeat (7x daily doses) intranasal doses of FIV-GP64 encoding erythropoietin achieved a significant increase in systemic haematocrit levels (indirect readout of erythropoietin levels), with undetectable levels generated following just a single administration (136). Thus, repeat intranasal administration of rSIV.F/HN-mADAMTS13 may hold greater potential for the future than intravenous re-administration shown here.

Upon analysis of BALF, significant ADAMTS13 activity was found following intranasal delivery of mADAMTS13 vector, with median activity levels reaching 94% of wildtype endogenous BALF activity. The noise associated with the FRET assay is clearly demonstrated in the TSSM group, however activity observed in the intranasal group is visibly above background. Surprisingly the single mouse with high serum expression generated the lowest level of BALF ADAMTS13 activity across all treated mice, which may support evidence of a spurious finding. BALF ADAMTS13 levels were comparable between mice that received single and multiple doses of vector by intravenous administration, which is in contrast to the summative effect in plasma observed following 5x vGM213-mADAMTS13. Significant luciferase expression was observed in the lungs following intravenous delivery of rSIV.F/HN-GLux (Chapter 1) although orders of magnitude lower than expression observed following intranasal delivery. Thus detectable BALF ADAMTS13 activity following intranasal but not intravenous administration was an expected result.

Overall, administration of a single dose of rSIV.F/HN vector encoding mADAMTS13 failed to generate meaningful levels of ADAMTS13 activity in plasma, with potentially some evidence of expression detected at 5 weeks following intranasal delivery of vector and in a single mouse at 16 weeks following intravenous delivery of vGM213-mADAMTS13. Alternatively, significant expression was detected in BALF of transduced mice 16 weeks after treatment. Five daily administrations of vGM213-mADAMTS13 generated a ~4-fold increase in plasma

ADAMTS13 activity compared to a single dose, however a summative increase in activity was not observed in BALF following multiple doses.

To relate these findings with the literature, plasma ADAMTS13 activity was compared to papers that delivered ADAMTS13 cDNA or mRNA to adult mice but not papers where gene transfer had occurred by pro-nuclear injection or during embryo development. Jin *et al.* (2013) measured stable activity levels in murine plasma 20 weeks following intravenous delivery of 2.6×10^{11} vg/kg AAV8 vectors encoding the C' truncated ADAMTS13 variant, MDTCS, under expression of the liver-specific hAAT promotor (172). Plasma ADAMTS13 activity in treated mice was 0.6 U/ml determined by FRET-VWF73 assay compared to 1.0 U/ml calibrated from pooled NHP (60%) and 0 U/ml in control mice delivered AAV-hAAT-LacZ. Liu-Chen *et al.* (2018) determined peak plasma activity levels, measured by commercial activity ELISA (TECHNOZYM, Diapharma West Chester, Ohio, US), as 0.2 IU/ml and 0.4 IU/ml 24 hours following intravenous infusion of mRNA encoding wildtype ADAMTS13 and an M5 variant of ADAMTS13 respectively, compared to 0 IU/ml in control mice delivered EGFP mRNA (182). Trionfini *et al.* (2009) reported ~12% activity in mice 12 weeks following delivery of 1×10^9 p.f.u AdV-ADAMTS13 vectors measured by collagen-binding assay, expressed as % NHP (173). Overall, Verhenne *et al.* (2017) demonstrated the most significant, long-term expression of ADAMTS13 following gene transfer by Sleeping Beauty transposon system, however only reporting plasma antigen levels determined by ELISA rather than activity (176).

From the relevant papers aforementioned, only Jin *et al.* (2013) utilise the FRET-VWF73 assay whilst Liu-Chen *et al.* (2018) utilised a commercial ELISA to determine ADAMTS13 activity and Trionfini *et al.* (2009) used a collagen-binding assay (172,173,182). In all three papers, control mice demonstrate exactly 0% activity compared to NHP, thus activity levels measured in the present study were expressed in the same format, as a percentage of wildtype, accounting for background levels detected in control knockout mice which were determined as ~8.5% by FRET VWF73. This allows for results to be directly compared to figures reported in the literature. rSIV.F/HN transduction achieved 34% wildtype levels in a single mouse and ~20% wildtype levels in plasma at 5 weeks post-transduction, lower than the 60% NHP levels following AAV8-MDTCS gene transfer, yet similar to activity generated

following intravenous administration of mRNA encoding wildtype ADAMTS13 (20% NHP) and the M5 variant (40% NHP) and superior to ~12% levels achieved following delivery of AdV encoding ADAMTS13 (172,173,182).

Of note are the MDTCS and M5 ADAMTS13 variants utilised in these gene transfer studies, which reportedly exhibit enhanced protease activity compared to wildtype ADAMTS13 against a VWF73 substrate. South *et al.* (2014) reported a ~2.5-fold increase in activity by the ADAMTS13 variant lacking its C' tail (MDTCS) over wildtype protein, whilst Jian *et al.* (2012) described a gain-of-function mutant carrying 5 amino acid substitutions (M5-R660K/F592Y/R568K/Y661F/Y665F) which exhibited ~4-fold greater activity against a VWF73 substrate (186,226,227). Thus given murine wildtype ADAMTS13 was delivered to deficient mice in the present study, widely reported to have significantly reduced activity against FRET-VWF73 compared to human ADAMTS13, and the use of human ADAMTS13 variants with superior activity by others, it was not surprising that limited expression was generated in plasma during this project (175). It is also conceivable that delivery of rSIV.F/HN encoding human ADAMTS13 or the MDTCS and M5 variants in future experiments may well generate superior activity. The M5 mutant was originally generated to mitigate the binding of patient IgG autoantibodies against the ADAMTS13 spacer domain, particularly arginine at amino acid position 660 which is essential for VWF substrate recognition (227). Interestingly, the M5 mutant demonstrated near wildtype proteolytic activity despite incubation with plasma pooled from 12 acquired TTP patients, suggesting the M5 variant had resistance to autoantibody inhibition (227). Thus a potentially straightforward way to generate a GTA suitable for congenital and acquired TTP patients, is to encode the full-length or MDTCS variant harbouring the 5 amino acid substitutions within lentiviral vectors.

A further consideration is the reduced sensitivity associated with the FRET-VWF73 assay. Other research groups that have attempted gene transfer of ADAMTS13 to deficient mice have relied upon multiple assays to demonstrate transduction efficiency and reconstitution of expression. Specifically, this involves the measurement of plasma antigen levels by ELISA and the protection in thrombocytopaenia following challenge with Shiga toxin or recombinant VWF. Failure to find a murine-specific ELISA and unsuccessful attempts to induce thrombocytopaenia in ADAMTS13 deficient mice meant that there was complete

reliance upon one assay, FRET-VWF73, to determine transduction efficiency following gene transfer in this project. Therefore any limitations associated with the FRET assay, including the sensitivity of the assay (10%), noise of the assay and potential interference from plasma proteins were all influential on the only readout of transduction efficiency in this project.

As just mentioned a limitation of the FRET-VWF73 assay is that proteins within the blood, including bilirubin and haemoglobin, can interfere with the FRET substrate due to absorbance and autofluorescence of plasma proteins in a similar spectra of light (400-500 nm) (228,229). Indeed marked haemolysis as a consequence of poor blood sampling may effect ADAMTS13 activity assays. Solutions to this have included the addition of bilirubin oxidase to plasma samples, further dilution of plasma samples and the designing of a novel FRET substrate, rVWF71 labelled with fluorescent dye which absorbs and emits light of wavelength 638 nm and 658 nm, respectively (230,231). Infact, Muia *et al.* (2013) claim that FRET-rVWF71 allowed for the reliable determination of ADAMTS13 activity in plasma, with greater sensitivity than FRET-VWF73 and without interference from plasma proteins (231). The repeat sampling of blood by venepuncture in the present study may have led to haemolysis and consequently underestimation of plasma ADAMTS13 activity levels following gene transfer. This could be an explanation why greater activity was detected in BALF than plasma, particularly following intravenous delivery of vGM213-mADAMTS13/ vGM228-mADAMTS13+Kozak.

It was attempted to enhance expression levels of ADAMTS13 in transgenic mice by repeated administration at daily intervals for 5 consecutive days. A dose response (~4-fold increase vs single dose vGM213-mADAMTS13) in activity was observed in plasma 14 weeks post transduction however increased ADAMTS13 cleavage was not observed in BALF following repeat intravenous administration. rSIV.F/HN gene transfer demonstrated a summative effect previously by Griesenbach *et al.* (2012) when vector was delivered repeatedly (5x or 10x daily doses) by intranasal administration (98). Luciferase expression in lung homogenate was shown to correlate with number of doses, where the cumulative dose administered was 5e6 total TU/mouse or 1e7 total TU/mouse for 5x and 10x doses, respectively. Similarly, Sinn *et al.* (2008) demonstrated a linear increase in luciferase expression when GP64-FIV-

Luc was delivered repeatedly (2x, 4x, 6x or 8x daily doses) by nasal instillation, achieving a total dose of 2.5×10^8 TU/mouse (136).

Despite the total delivered dose in the 5x intravenous group being 75-fold greater than the 10x cumulative dose delivered by Griesenbach *et al.* (2012) only modest ADAMTS13 expression was observed in plasma and only background levels in BALF (98). This finding is likely an artefact of how rSIV.F/HN is administered whereby intravenous delivery leads to lower transgene expression contributed from multiple organs (Chapter 3), compared to the strong and highly localised expression from the respiratory system following intranasal delivery. Similarly, stark differences are noted following repeat administration of rSIV.F/HN by intravenous delivery which generated a significant neutralising antibody response with successive doses, yet no difference in transduction was observed following repeat intranasal delivery by Griesenbach *et al.* (2012) (98). Therefore a level of immune-tolerance or reduced immune surveillance is noted in the lungs compared to the systemic circulation that has contributed to these findings.

Carbonaro-Sarracino *et al.* (2020) demonstrated a significant anti-vector response in ADA deficient mice (ADA^{-/-}) following a single intravenous dose of VSV-G-ADA (1.5×10^{10} TU/kg) (232). Interestingly, this response was not observed in immune competent ADA^{+/-} heterozygotes, and thus was concluded by the author to be a consequence of immune dysregulation observed in ADA^{-/-} deficient mice. In further studies using ADA^{+/-} mice, Carbonaro-Sarracino *et al.* (2020) aimed to investigate repeat administration of VSV-G-EGFP vectors conjugated with monomethoxypoly(ethylene) glycol (PEGylated) which reportedly extends vector half-life in the blood and reduces immune inactivation of VSV-G vectors (233). Comparisons between mice delivered two doses of PEG-VSV-G-EGFP with a 3 day or 3 month interval revealed a significantly enhanced immune response compared to single dosed mice, demonstrating PEGylation fails to protect VSV-G vectors from immune recognition. Comparable detection of anti-vector antibodies was observed between multi-dose groups, however there was a 100-fold reduction in liver VCN detected in mice with a 3 month interval between doses yet comparable levels between mice receiving a single dose and two doses within 3 days. Therefore, it is plausible that repeat intravenous administration of rSIV.F/HN with daily or monthly intervals generates comparable levels of

anti-vector neutralising antibodies, yet monthly intervals may demonstrate a greater reduction in transduction efficiency compared to daily. What is clear is that no summative effect in transduction efficiency was observed following repeat intravenous dosing by Carbonaro-Sarracino *et al.* (2020) although demonstrated in the present study.

Estimations of the minimum plasma levels of ADAMTS13 to ameliorate disease in patients with congenital TTP are between 5-10% (234). Verhenne *et al.* (2017) noted that low levels of ADAMTS13 plasma expression (~10% normal mouse plasma levels) following delivery of the mADAMTS13 encoding transposon (without transposase) was not sufficient to alter VWF multimer distribution nor protect against Shiga toxin challenge (176). Pickens *et al.* (2015) observed no difference in plasma multimer distribution in transgenic mice expressing ADAMTS13 in platelets (~8% NHP activity) yet showed some protection to Shiga toxin and recombinant mVWF-mediated thrombocytopenia (177). In contrast, Niiya *et al.* (2009) found that limited plasma activity of ADAMTS13 (~5-7% NHP activity) somewhat ameliorated the number of high molecular weight multimers in plasma (174). In a phase 1 clinical trial investigating rVWF delivery to congenital TTP patients, Scully *et al.* (2017) observed a reduction in ultra large and large-VWF multimers following infusion of 20 U/kg rVWF (~36% NHP activity) but not 5 U/kg rVWF (~7.5% NHP activity) (163). Although intermediary doses were not tested, this result may suggest that 5% plasma ADAMTS13 levels may not be sufficient to reduce the high molecular weight multimers and thus ameliorate disease in humans.

Given the consensus that a minimum of 10% plasma ADAMTS13 activity is required to protect against Shiga toxin or rVWF-mediated thrombocytopenia, even modest levels of expression following rSIV.F/HN gene transfer may well be sufficient. However the lack of other assays to measure efficacy of gene transfer and the dependency of this project on one functional assay, drove the decision late in the project to clone the human ADAMTS13 cDNA sequence into the lentiviral backbone (pIC083-hCEF-hADAMTS13) to enable detection of plasma antigen levels by commercial ELISA kits. As already described, increasing plasmid concentrations of pIC083-hCEF-hADAMTS13 generated dose dependent levels of ADAMTS13 in cell supernatant, and the next step was to generate rSIV.F/HN vector encoding hADAMTS13. During the manufacturing of rSIV.F/HN vectors encoding murine

ADAMTS13, difficulties were faced by collaborators in Oxford in generating significant titres. Three separate batches of vGM213-mADAMTS13 were required to achieve the quota of vector for the order (2.41e8, 3.97e8 and 1.42e9 TU/ml) while one large batch of vGM228-mADAMTS13+Kozak with low titres (3.07e7 TU/ml) was generated for the second order. This is in contrast to the titres generated for rSIV.F/HN encoding therapeutic gene hAAT, which are consistently >5e9 TU/ml. Consequently, there was a hypothesis that pseudotransduction of producer cells by the expression plasmid encoding mADAMTS13 lead to generation of the protease during vector production which negatively impacted generation of functional rSIV.F/HN vectors.

To test this hypothesis, transduction efficiency was measured following incubation of rSIV.F/HN-EGFP and VSV-G-EGFP with increasing concentrations of ADAMTS13 standard. A dose dependant decrease in GFP positive cells was observed with increasing ADAMTS13 concentrations. Surprisingly, the impact on transduction was comparable between rSIV.F/HN and VSV-G vectors (~70% reduction at 1 ng/ml ADAMTS13) despite different envelopes proteins. Of note, incubation of vectors with a matching concentration of hAAT standard did not replicate this dramatic loss in transduction, with only a ~10% reduction in efficiency observed across both vectors. The similarity in response by both vectors to ADAMTS13 standard was not expected as lentivirus pseudotyped with the VSV fusion protein (G glycoprotein) are notoriously robust and able to withstand concentration by ultracentrifugation (5,235). On the other hand, lentiviral vectors pseudotyped with the Sendai virus F and HN glycoproteins and other envelope proteins are not stable enough to withstand ultracentrifugation without loss in infectivity and thus were anticipated to be more susceptible to protease activity (236). ADAMTS13 may therefore have activity against a common feature of both vectors, although this would be surprising given cleavage activity against only one physiological substrate has been reported previously, VWF (139).

Using a similar protocol, Dautzenberg *et al.* (2021) investigated the stability of various pseudotypes of lentiviral vector by measuring percentage GFP-positive cells following incubation of vectors with trypsin-EDTA for 5 minutes at 37°C (237). VSV-G vectors demonstrated no change in transduction efficiency following trypsin incubation, whereas HIV-1 pseudotyped with the Measles virus hemagglutinin (H) and fusion (F) glycoproteins

were sensitive to inactivation by trypsin demonstrating a ~75% fall in transduction. Measles virus is a paramyxovirus with glycoproteins, H and F, required for cellular attachment and fusion (238). Frecha *et al.* (2008) generated recombinant lentiviral vectors with the Measles virus glycoproteins incorporated into vectors by truncation of the wildtype F and H glycoprotein cytoplasmic tails (239). It may be postulated that given the similarities between lentiviral vectors pseudotyped with Measles virus glycoproteins and rSIV pseudotyped with envelope proteins from fellow paramyxovirus family member, Sendai virus, it is conceivable that rSIV.F/HN vectors may be sensitive to trypsin. However, trypsin activation of F proteins is required during production of rSIV.F/HN vectors and thus it is unlikely to reduce transduction efficiency at the doses tested (0.05% trypsin-EDTA) (86,237).

It is possible that the high concentrations of ADAMTS13 standard may have simply been toxic to cells, reducing cell viability and transduction in a concentration-dependant manner. Although 10,000 live cells were analysed per transduction condition, the time taken for analysis was not recorded and could be investigated in the future. Similarly, the experiment should be repeated utilising recombinant ADAMTS13 which has demonstrated FRET activity rather than an ELISA standard of unknown activity. Therefore, it is suspected that generation of low rSIV.F/HN titres and reduction of VSV-G and rSIV.F/HN transduction efficiency following incubation with ADAMTS13 are caused by two separate phenomena.

Looking to the literature for gene transfer studies that packaged ADAMTS13 within lentiviral vectors, Laje *et al.* (2009) and Niiya *et al.* (2009) generated HIV-1 VSV-G pseudotyped vectors encoding mADAMTS13 and hADAMTS13 respectively, achieving titres of 1e8-1e10 TU/ml after concentration by centrifugation (174,175). It does not seem evident therefore that ADAMTS13 expression negatively affected VSV-G vector production in these papers, although titres from crude cell supernatant prior to centrifugation are not detailed. Many factors could influence the generation of low vector titres, including the size of vector genome and the biological effect of the transgene product on vector packaging or incorporation of envelope proteins (240). In fact, Radcliffe *et al.* (2008) demonstrated a clear negative effect of the FVIII transgene in the incorporation of VSV-G glycoproteins during production of EIAV lentiviral vectors (241). Interestingly, the interference in envelope protein incorporation was not VSV-G nor EIAV specific and was replicated with recombinant

HIV-1 vectors and alternative pseudotypes. Inclusion of a tissue-specific promoter (hAAT) to minimise FVIII expression in producer cells was sufficient to ameliorate poor vector titres. An alternative solution shown by Maunder *et al.* (2017) included the use of a bacterial tryptophan RNA-binding attenuation protein (TRAP) that binds to a consensus RNA sequence upstream of the transgene to repress translation during vector production (242). Thus given the significant cDNA length of ADAMTS13 (4.7kb) and biological protease activity, the use of either a tissue-specific promoter to drive ADAMTS13 expression or a repressor of transgene transcription or translation during vector production should be considered moving forward.

4.5 Conclusion

The data presented in this chapter demonstrates that a single administration of rSIV.F/HN-ADAMTS13 to deficient mice was not sufficient to generate substantial levels of ADAMTS13 in plasma, yet multiple doses of vector achieved detectable levels of plasma ADAMTS13 activity. In contrast, single doses of vector delivered by intranasal administration fully restored ADAMTS13 levels in BALF to wildtype levels. Attempts to generate a TTP model that exhibited symptoms upon VWF-challenge were unsuccessful, likely due to the insufficient multimer composition of recombinant VWF protein. Surprisingly, intravenous administration of vector did not generate higher plasma ADAMTS13 expression compared to intranasal administration, which could reflect a threshold dose required to achieve detectable ADAMTS13 activity, particularly when using the FRET-VWF73 assay for detection. In future experiments, rSIV.F/HN encoding human ADAMTS13 will be investigated which may allow for the more sensitive detection of expression levels due to the use of commercial ELISAs. Overall, this chapter highlights the challenges that are faced when developing a gene therapy treatment for a novel disease, particularly in the establishment of an animal model and in the development of assays to measure efficacy.

Chapter 5- Regulated Expression of Lentiviral Vectors following Administration of an Inducing Molecule

5.1 Introduction

In pursuit of a GTA for pulmonary gene therapy, the UK Respiratory Gene Therapy Consortium have developed a lentiviral vector platform, rSIV.F/HN, which achieves highly efficient lung transduction in mice. Previous data showed that following a single dose of rSIV.F.HN, the pulmonary epithelium can act as a factory for long-lasting (at least 19 months) production of secreted proteins AAT and FVIII (99). Similarly, the pulmonary phenotype in the PAP mouse model was corrected through rSIV.F/HN-mediated expression of GMCSF (Lund-Palau, Molecular Therapy- manuscript under review). However, in PAP mouse models there is evidence that sustained expression of GMCSF can cause toxicity (~9 months post-transduction) and perhaps enhanced temporal control over GMCSF expression may be desired (Lund-Palau, Molecular Therapy- manuscript under review). To enhance the efficacy/toxicity window of lentivirus-mediated gene transfer, it may therefore be beneficial to turn gene expression on and off as required depending on the disease status. TTP is a pertinent example, whereby patients may stay in remission for durations of time without disease manifestation (243). However environmental or biological challenges such as infection can trigger acute TTP events in which enhanced requirements of ADAMTS13 expression are faced (156). Similarly delivery of vectors encoding the hormone erythropoietin for the treatment of anaemia does not require constitutive expression and in fact over production can be toxic and potentially fatal (244). Instead, short intermittent bursts of gene expression may be required for example when a fall of haematocrit levels are detected in the blood. Furthermore, therapies for cancer treatment may require controlled expression of therapeutic genes, particular when using cytokines such as TNF α or chemotherapeutic agents that are cytotoxic (245).

Multiple ligand-inducible gene expression systems exist which have been investigated extensively in the literature since their conception in the early 90's (246). The systems which have been assessed in mammalian cells/models can be broadly categorised as based upon bacterial operon expression systems, chemical-inducers of dimerisation or nuclear receptors

(246). The overall design of these systems, true of eukaryotic transcription activators, includes modular DNA-binding and activation domains which can be exchanged and modified independently (247). The modular nature of gene expression cassettes represents a major advantage in the design of customisable expression systems suited to the specific experimental requirements. Explanations of the three main classes of ligand-inducible gene expression systems will be given below, using one exemplar system from each class to further explain the benefits and disadvantages of each system particularly in their suitability for clinical use. First however, the necessary traits of a ligand-regulated gene expression system will be clarified.

5.1.1 Desirable traits of a regulated expression system

The desirable characteristics looked for in an inducible expression system are numerous however ultimately the decision of which system to use is driven by the research objective and downstream application. Thus the traits listed below are generic and may not represent essential requirements for all experimental scenarios. One of the important requirements is the stringent and reversible control of gene expression, which specifically refers to low 'leakiness' of basal expression from the expression construct and high induction of expression following delivery of a ligand or inducer molecule (246). The difference between the two will be referred to as the 'induction ratio' which in the ideal expression system would be large as a product of low leakiness and high induction. The kinetics with which gene expression is turned 'on' or 'off' should be timely, particularly given the potential application of inducible systems to control expression of transgenes with narrow therapeutic windows (248).

A further requirement is the consistent response to ligand-induced gene expression over time, particularly if the envisaged application is within gene therapy vectors which can persist for many years (249). Factors which may influence the continued response to ligand-induction over time is the immunogenicity of both the gene expression construct and the inducer molecule (250). The inclusion of 'humanised' activators and DNA-binding domains are less likely to be immunogenic to humans when translating these constructs to the clinic, as will using inducer molecules with favourable bioavailability and minimal side-effects in humans. Finally, a consideration when including components derived from human

regulatory systems, is the interference of the engineered inducible construct with endogenous biological pathways. Currently, a ligand-inducible system which incorporates all these requirements does not exist with some but not all desirable traits offered by existing systems as discussed below.

5.1.2 Tetracycline-regulated gene expression

The first and most widely used ligand-inducible expression system is the tetracycline system (tet) based upon the Tn10 tet-resistance operon isolated from *E.coli* (251). It was first used to develop transgenic mice with ligand-dependent expression of a reporter gene by Furth *et al.* (1994). The tet system consists of two components, a tet-responsive transactivator and an inducible promoter upstream of the gene of interest (GOI). Within the transactivator is the tet repressor (*tetR*) DNA-binding domain, derived from the *E.coli* operon, fused to an activation domain sequence taken from the herpes simplex virus (VP16) (252). The *tetR* within the transactivator binds to tet operator (*tetO*) sequences arranged in tandem upstream of a minimal promoter. Upon binding, the transactivator initiates transcription activation of the GOI which occurs only in the absence of tet, or similar antibiotic derivatives including doxycycline. In contrast, tet/doxycycline bound *tetR* renders the transactivator unable to bind *tetO* sequences and thus transcription is not initiated. Consequently this conformation is termed the tet-OFF system due to the prevention of transgene transcription in the presence of tet/doxycycline (253). Since its conception, many novel tet configurations have been generated including the tet-ON system which contains a mutated *tetR* that confers transcriptional activation only in the presence of doxycycline (254). Others include a repressor system that exchanges VP16 for the Krüppel associated box (KRAB) domain of Kox-1, permitting inducible repression of your GOI (255).

To incorporate the system into mice, two separate transgenic lines were produced with one expressing the transactivator/repressor under a tissue- or developmental-specific promoter and the other strain harbouring the *tetO* inducible promoter and GOI. Upon breeding, mice transgenic for both components of the tet system are generated and transgene expression can be turned on/off in certain tissues or during specific developmental stages (252,256). This provided an incredibly useful tool for scientists to study gene function, particularly of wildtype or mutant genes that are embryonic lethal if expressed constitutively during

development. Advancement of the tet system to incorporate transactivator and inducible transgene into a single plasmid, reducing the requirement for two separate transgenic lines, was efficiently demonstrated by Schultze *et al.* (1996), establishing background levels of leaky expression without doxycycline-induction (257). More recently, Sun *et al.* (2019) combined the tet repressor and CRISPR/Cas9 systems for genome-wide functional screening in mice. Doxycycline-inducible gRNA expression in CRISPR/Cas9 transgenic mice allowed for temporal control of high-throughput Cas9-mediated gene knockout for investigation of gene function (258).

Although the tet systems have proven extremely useful for *in vitro* applications, investigation of gene function and generation of disease models in mice, and the ultimate translation of tet-inducible systems to the clinic is not feasible. Ultimately, the immunogenicity of sequences from bacterial origin in humans is the major drawback of the tet system, although some have attempted to produce immunologically inert tet-based vectors (259). Furthermore, the side effects associated with continued use of antibiotic doxycycline include the disturbance of natural microbiota which can have wide-spread manifestations. Impacts on the immune system and off-target effects in mitochondrial function by doxycycline-inducible systems are described by Wüst *et al.* (2020), highlighting the alternative effects that should be considered when using this system in animal models (260). Furthermore, the lag between addition/withdrawal of drug and the subsequent change in protein levels, reported to be 1 week by Schultze *et al.* (1996) is considered a further limitation of this system (248,257). Alternative bacterial systems that are widely used include the IPTG-responsive Lac operon derived from *E.coli* and the cumate operon derived from *Pseudomonas putida* (261). Together with other bacterial operon-based designs, the tet-system represents a powerful tool for temporal control of gene expression in pre-clinical research.

5.1.3 Rapamycin-induced dimerisation system

An example of a chemical-inducer of dimerisation is the rapamycin-inducible system which takes advantage of a human drug-protein interaction. Rapamycin's natural binding partner is human protein FK506-binding protein 12 (FKB12) (262). FKB12-associated protein (FRAP) can subsequently bind FKB12 only when in complex with rapamycin. Thus attaching a DNA

binding domain to FKB12 and an activation domain to FRAP will lead to the formation of a transcription activation complex upon rapamycin-induced dimerization (263).

Transcriptional activation of target genes containing complementary binding sequences to the DNA-binding domain upstream of a minimal promoter, thus occurs following rapamycin-induction. Most often zinc fingers (ZFs), small modular DNA-binding domains that can be engineered to bind target DNA sequences, are included as the DNA-binding element and NF-KB p65 subunit as the activation domain, with many groups showing stringent control of gene expression in multiple disease indications (244,264,265). Advantages of the rapamycin system are potent drug-induced activation of transgene expression with minimal basal expression and inclusion of only human sequences. In a comparison of popular inducible systems, Xu *et al.* (2003) found that rapamycin-induced dimerization demonstrated enhanced temporal control of gene expression over other designs (266).

However an important consideration for the translation of regulated expression cassettes to humans is the safety of the inducer molecule. The drug rapamycin acts upon the endogenous target of rapamycin complex 1 (mTORC1) inhibiting its essential function in cell metabolism and growth (267). The growth-inhibitory effects of rapamycin led to its clinical use as an immunosuppressant following organ transplantation and thus has implications towards human use, particularly in patients with disease and co-morbidities. Non-toxic alternatives to rapamycin have been investigated including the synthetic compound FKCsA, a FK506 and cyclosporin A heterodimer (268). Targeted gene expression following FKCsA induction is achieved by fusing a DNA-binding domain to FKB12 and an activation domain to the cyclosporin A binding partner, cyclophilin. However, the barrier facing these small molecules is that often they have not been tested in humans before and require clinical trials to show their tolerability (269). A further limitation of this system is the slow kinetics after withdrawal of rapamycin before a fall in protein expression (261).

5.1.4 Mifepristone-inducible modified progesterone system

The mifepristone-inducible system is an example of the nuclear receptor class of engineered transcription factors. This system incorporates a modified nuclear progesterone receptor which is one of a large family of ligand-activated transcription regulators that contain conserved functional domains such as DNA- and ligand-binding domains (270). Specifically,

the ligand-binding domain of the progesterone receptor is fused to a heterologous DNA-binding domain and activation domain to create a chimeric transactivator complex which will bind to response elements and activate transcription of the GOI. The mutant progesterone receptor is non-responsive to endogenous progesterone but instead can be activated by the steroid, mifepristone, with high affinity (dissociation constant (K_d) of ~3 nM) (271). Clinically, mifepristone is used as a progesterone antagonist to abort pregnancies in women, however the affinity with which mifepristone can bind to the truncated progesterone receptor is sufficiently high that a sub-therapeutic dose of mifepristone is required for activation of gene expression (250). Use of a sub-therapeutic dose is anticipated to reduce the possibility of mifepristone exerting anti-progesterone effects on endogenous receptors *in vivo* (272).

The first mifepristone-inducible construct was designed by Wang *et al.* (1994) was composed of two parts. The transactivator included the DNA-binding and dimerisation domain from the yeast transcription activator GAL4 (amino acids 1-94), the C' truncated progesterone receptor ligand-binding domain (amino acids 640-891) and the VP16 activation domain (271,273). The second component included the regulated promoter which contained four tandem copies of consensus GAL4-recognition sites (4x 17 nucleotide sequences which each serve as the binding site for two transactivators) upstream of a minimal promoter containing the TATA box sequence from adenovirus *E1B* gene and the GOI (273,274). In the presence of mifepristone, chimeric transactivators dimerise and subsequently scan for GAL4 recognition sites, where transcriptional activation of the inducible transgene will occur upon binding (271). In the absence of mifepristone, low levels of transgene expression (leakiness) were detected due to the incorporation of a minimal promoter upstream of the GOI.

Thermo Fisher Scientific subsequently licenced a commercial mifepristone-inducible system, GeneSwitch, based upon the original construct designed by Wang *et al.* (1994) however with some additional features and modifications. The GeneSwitch product is composed of two plasmids, one encoding the transactivator consisting of the GAL4 DNA-binding domain fused to the truncated progesterone ligand-binding domain and a p65 activation domain (referred to as the pSwitch plasmid, Figure 5.1) and the other plasmid (referred to as the

pGene plasmid, Figure 5.1) encoding the GAL4 binding sites and TATA box minimal promoter upstream of a reporter gene, *LacZ*. The pGene plasmid could be adapted according to the experimental setting, for example easy insertion of alternative transgenes. A further modification was the addition of an auto-regulated promoter driving expression of the transactivator, instead of a strong constitutive promoter. Specifically, GAL4 binding sites were included upstream of a thymidine kinase (TK) minimal promoter to drive auto-inducible expression of the transactivator. In the presence of mifepristone, active transactivator complexes will drive transcription of its own coding sequence through an auto-regulatory feedback loop, quickly amplifying the levels of transactivator and consequently the transgene in the presence of mifepristone (Figure 5.1). Based upon the GeneSwitch system, many have shown effective regulation of gene expression for an array of therapeutic applications in pre-clinical models, with sensitive mifepristone-activation of transcription at low doses (275–282).

A potential limitation associated with the translation of mifepristone-inducible modified progesterone systems to the clinic is the risk mifepristone will exert anti-progesterone effects on endogenous progesterone and glucocorticoid receptors in patients. As described above however, the effective dose with which mifepristone activates transcription in the GeneSwitch system is claimed to be 2-3 orders of magnitude below mifepristone's anti-progesterone therapeutic dose (246,271). A further limitation is the inclusion of yeast elements that will enhance the immunogenicity of GeneSwitch in humans. However as exemplified by the rapamycin- and tet-inducible systems, the modular nature of engineered transcription factors permits the exchange of functional domains for 'humanised' equivalents, such as ZF DNA-binding domains which are abundant in human transcription factors (283).

5.1.5 A humanised mifepristone-inducible gene therapy product

The lack of drug-inducible regulated expression system suitable for translation to the clinic is a major limitation faced by the gene and cell therapy field (250). One strategy could be to 'humanise' an already established expression system, such as the GeneSwitch system which shows favourable control of gene expression following induction by a drug suitable for use in man. Construction of a humanised expression cassette which could be incorporated into a

viral vector would enable the generation of gene therapy products with temporal control of gene expression. The potential applications of GTA with regulated gene expression are numerous, however the main incentive that drives their development is to enhance the safety of viral gene therapies, particularly vectors that integrate into the host cell genome such as lentiviral vectors.

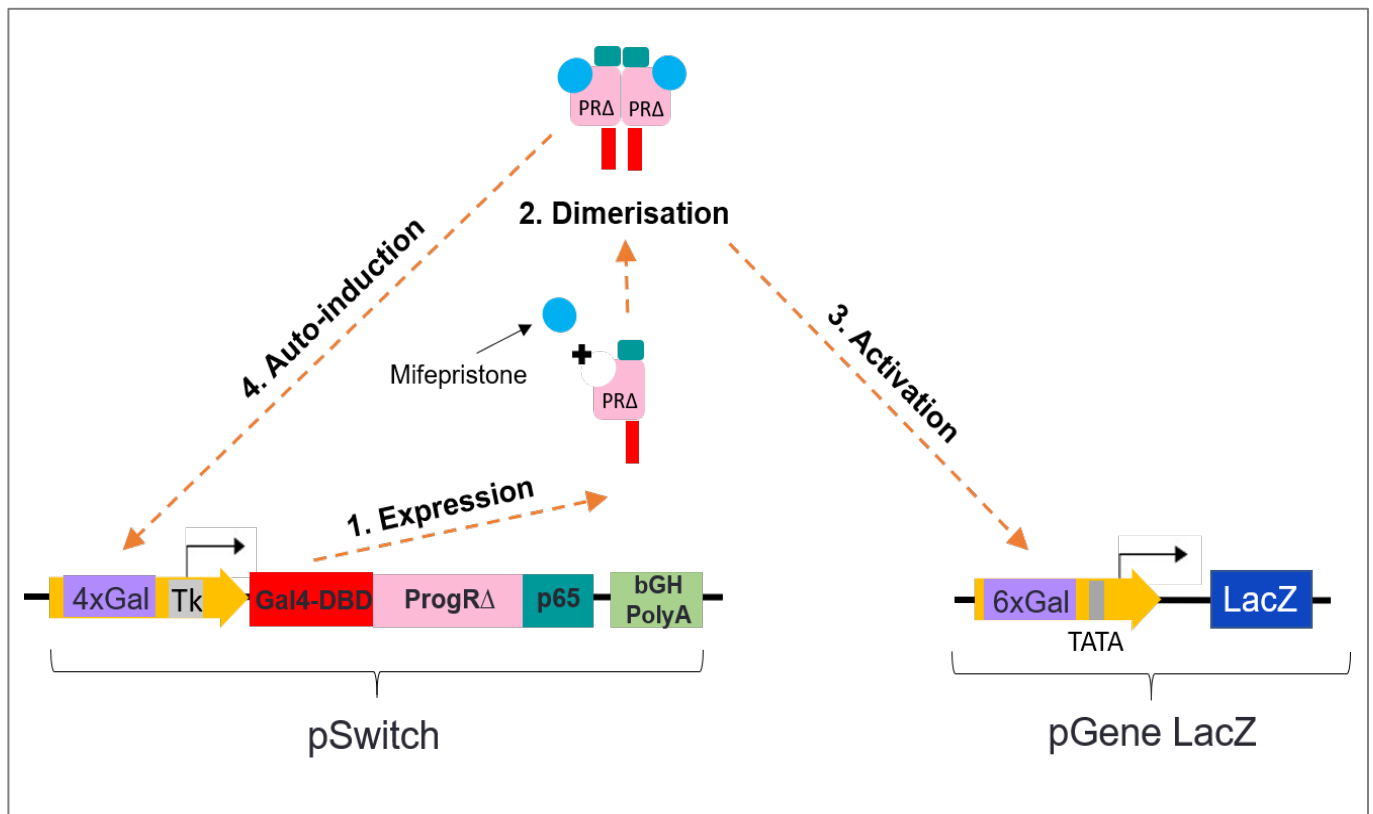


Figure 5.1- Schematic of the auto-inducible GeneSwitch regulated expression system.

Thermo Fisher Scientific licenced a commercial mifepristone-inducible system, GeneSwitch, based upon the original construct designed by Wang et al. (1994). GeneSwitch is composed of two plasmids, the pSwitch plasmid encodes the transactivator consisting of the GAL4 DNA-binding domain (DBD) fused to the truncated progesterone ligand-binding domain (ProgRΔ) and a p65 activation domain. The pGene plasmid encodes the GAL4 binding sites (6xGal) and TATA box minimal promoter sequence upstream of a reporter gene, LacZ. GAL4 binding sites (4xGal) were included upstream of a thymidine kinase (Tk) minimal promoter to drive auto-inducible expression of the transactivator. 1. Transcription of the transactivator is driven by the minimal Tk promoter. 2. In the presence of mifepristone, inactive transactivator monomers will dimerise. Active transactivator complexes will bind to GAL4 binding sites and transcriptional activation of 3. the gene of interest (LacZ) will occur. 4. Induced expression of the transactivator coding sequence is achieved through an auto-regulatory feedback loop.

5.1.6 Aims and objectives

The aim of this chapter is to generate a lentiviral vector carrying a regulated expression cassette, in which gene expression can be turned on upon administration of a drug and turned off upon withdrawal of the drug. Temporal control of gene expression could be beneficial to improve the safety of lentiviral gene therapy and to broaden the application of the rSIV.F/HN vector platform towards diseases that require controlled gene expression. The hypothesis is that lentiviral-mediated transgene expression can be regulated following administration of an inducing drug. Specific objectives include:

1. Generate regulated expression cassettes based upon the commercial mifepristone-inducible system, GeneSwitch (Thermo Fisher Scientific, Massachusetts, US) and assess their function *in vitro*.
2. Construct a novel mifepristone-inducible expression construct suitable for use in man and compare efficacy against the 'gold-standard' commercial system.
3. Establish proof-of-concept that lentivirus-mediated expression can be regulated in a range of models including immortal cell lines, bone-marrow derived macrophages, human primary airway cultures and *in vivo*.

5.2 Materials & Methods

5.2.1 Induction of LacZ expression using the commercial GeneSwitch system

To first investigate the GeneSwitch system in its original conformation encoding reporter gene *LacZ*, the pGene LacZ and pSwitch plasmids (Figure 5.2 A+B) were co-transfected in a ratio of 4:1 into HEK293T cells seeded into 8 well chamber slides (Merck, Feltham, UK) at a density of 1×10^4 cells/ml following standard methodology. To determine background expression, pGene LacZ was transfected alone. As recommended in the GeneSwitch protocol, 24 hours post-transfection Opti-Mem was removed from cells and replaced with complete media for control conditions (uninduced cells) or complete media supplemented with mifepristone (1×10^8 M) for induced cells. After a further 48 hours, media was removed from cells and the physical barrier between wells detached, drawing a hydrophobic perimeter around each well. Cells were washed with PBS-/- twice then fixed with 100 μ l 4% paraformaldehyde (PFA) for 15 minutes before further washes with PBS. Cells were stored in humidified boxes at 4°C with excess PBS -/- until staining. Excess XGal stain was added to cells (0.5 mg/ml XGal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 1 mM $MgCl_2$, 0.02% NP-40 in 1X PBS) overnight at 4°C. After washing with PBS, images of stained cells from each transfection condition were captured using standard light field microscopy. Cells expressing β -galactosidase from the *LacZ* gene cleave XGal into a blue product which can be visualised easily under the microscope.

5.2.2 Construction of lentiviral expression plasmids encoding the GeneSwitch regulated expression cassettes

To establish proof-of-concept that lentivirus-mediated expression can be regulated, the commercial GeneSwitch system was cloned into the pGM358-hCEF-GLux lentiviral backbone (Figure 5.1C) encoding reporter protein GLux. The commercial expression cassette was kept in the original configuration, encoded by two vectors (GeneSwitch (GS)-2V), and additionally the transactivator and inducible gene cloned into a single vector (GS-1V). To clone the single vector construct, the transactivator was amplified from the pSwitch vector by primers 3AGLP65For and 3AGLP65Rev (Table 2.1- Reaction 7) and polyA sequence amplified from the oligo PolyA-GAL4 TATA synthesised commercially by GeneArt Gene Synthesis (Invitrogen, Massachusetts, US) using primers 3BPolyAFor and 3BPolyARev (Table 2.1-

Reaction 7). Following the HiFi Assembly protocol, the two inserts were ligated into the pGM358-hCEF-GLux backbone which had been linearised with restriction enzyme, NheI, creating GS-1V-GLux (Figure 5.3A). Ligations showing expected restriction patterns were sent for sequencing using primers oIC001 and oIC020 (Table 2.1- Reaction 8).

The regulated promoter and transactivator were cloned directly from pGene and pSwitch plasmids respectively, into the pGM358-hCEF-GLux lentiviral backbone to create GS-2V-GLux and GS-2V-pSwitch (Figure 5.3 B+C). Primers pGeneFor and pGeneRev were used to isolate the regulated promoter from parent plasmid, pGene (Table 2.1- Reaction 9) and primers pSwitchFor and pSwitchRev used to isolate the transactivator from pSwitch (Table 2.1- Reaction 10). Fragments were inserted into the NheI linearised pGM358-hCEF-GLux backbone by HiFi assembly and correct ligations confirmed by sequencing with primers oIC019 and oIC004 (Table 2.1- Reactions 11 + 12).

A final configuration of the commercial-based system that was cloned, was a reverse orientation one vector encoding GLux (GS-1V-GLux RO, Figure 5.3D). Primers BBFor and BBRev were used to amplify the lentiviral backbone of the GS-1V-GLux plasmid and primers InsertFor and InsertRev used to amplify the hCEF promoter, transactivator and polyA from GS-1V-GLux parent plasmid (Table 2.1- Reaction 13). The Insert primer sequences included overlapping sequences to the lentiviral backbone yet in the opposite orientation, thus the two fragments would only align with the transactivator inverted so that the plasmid assembles with two open reading frames facing in opposite directions. Sequencing was performed to confirm correct orientation using oligos oIC019 and oIC020 (Table 2.1- Reaction 14).

5.2.3 Construction of lentiviral expression plasmids encoding zinc finger-regulated expression cassettes

In collaboration with Professor Mark Isalan from Imperial College London, humanised ZF inducible constructs were designed. Using the template of the commercial regulated cassette, the GAL4 yeast DNA-binding domain was substituted for a humanised ZF to improve the immunogenicity and translation of the regulated promoter towards humans. The ZF sequence HIV BA', published by Reynolds *et al.* (2003), was encoded instead of the

GAL4 domain and 3x ZF binding sites (GCCTGGGCGGGACTGGGGA) included upstream of the GLux cDNA sequence, replacing the GAL4 binding sites (284). The novel 2887 bp ZF fragment was synthesised by GeneArt Gene Synthesis (Invitrogen, Massachusetts, US, Figure 5.4A) then transactivator and regulated promoter amplified using primers 1ZFFor and ZFRev (Table 2.1- Reaction 15). The ZF fragment was inserted into the NheI linearised pGM358-hCEF-GLux backbone downstream of the hCEF promoter by HiFi assembly, generating construct ZF-1V-GLux (Figure 5.4B). Correct ligations were confirmed by sequencing with primers oIC001 and oIC020 (Table 2.1- Reaction 16).

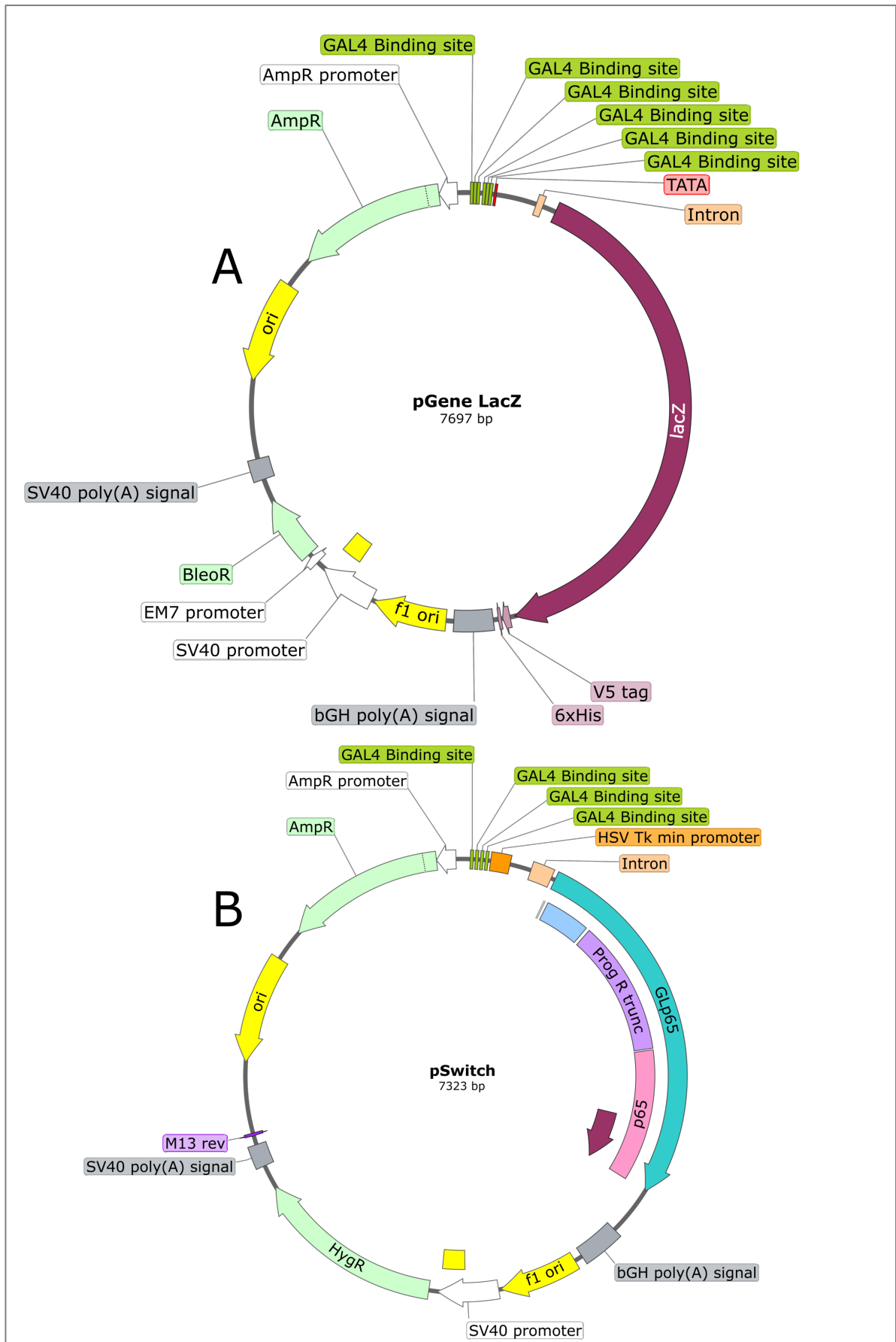
An alternative configuration of ZF vector was produced, including an auto-inducible (AI) TK minimal promoter with 3x ZF binding sites driving expression of the ZF transactivator, called AI ZF-1V-GLux (Figure 5.4C). Amplification of the AI promoter-transactivator-regulated GLux promoter was performed with primers 2ZFFor and ZFRev (Table 2.1- Reaction 17) and inserted into the pGM358-hCEF-GLux backbone which had been digested with BglII and NheI. Ligations were confirmed with sequencing using primers oIC019 and oIC020 (Table 2.1- Reaction 18). The final configuration of ZF construct was the reverse orientation, ZF-1V-GLux RO (Figure 5.4D), which was cloned by VectorBuilder (Illinois, US) using ZF-1V-GLux parent plasmid as template. In this construct, the ZF transactivator was encoded in sense strand orientation and the GLux transgene in reverse orientation.

5.2.4 Generation of VSV-G vectors encoding regulated expression cassettes

To generate proof-of-concept data that lentiviral-mediated transgene expression can be regulated, the 1V and 2V commercial-based expression cassettes were transferred from SIV backbones into a HIV-1 backbone by VectorBuilder. Subsequently, ultra-pure large scale batches of VSV-G vector were generated, encoding GS-1V-GLux RO, GS-2V-GLux and GS-2V-pSwitch. Later substitution of GLux cDNA for therapeutic transgene, murine GMCSF (CCDS 24692.1), was performed by VectorBuilder and VSV-G vectors generated, GS-1V-GMCSF RO and GS-2V-GMCSF (Figure 5.5 A+B). The method used by VectorBuilder for titre calculation was the p24 ELISA, thus to recalculate titre using the in-house protocol, ddPCR assessment of vector genomes was performed as previously described. An average of ddPCR calculated titre and p24 titre was used for each vector moving forward.

5.2.5 Assessment of regulated expression cassettes/vectors in HEK293T cells

Regulated expression cassettes were first investigated in HEK293T cells. Single plasmid constructs GS-1V-GLux, ZF-1V-GLux/GLux RO and AI ZF-1V-GLux were transfected at concentrations from 0.1-1.0 µg/ml. Transfections were carried out using standard methodology. GS-2V-GLux and GS-2V-pSwitch plasmids were co-transfected with a 4:1 or 1:1 molar ratio and GS-1V-GLux/GLux RO transfected at a matching molar ratio to the GS-2V-GLux plasmid. Single vectors, GS-1V-GLux RO and GS-1V-GMCSF RO, were transduced at MOI 10 whilst dual vectors were transduced from MOI 2.5 to 40 using a range of transgene to transactivator ratios. For both transfections and transduction, reduced serum media was replaced after 24 hours with either complete media or complete media supplemented with 10^{-8} M mifepristone. After 48 hours, cell supernatant was removed for analysis and cell lysate harvested to normalise gene expression for total protein, as described previously. GLux assays were performed using standard methodology, expressed as normalised GLux expression (RLU/mg of total protein). GMCSF expression was determined by ELISA (R&D Systems, Minnesota, US, MGM00) and normalised for total protein (GMCSF ng/mg of total protein). Transgene expression was compared between transfection/transduction conditions following induction with mifepristone.



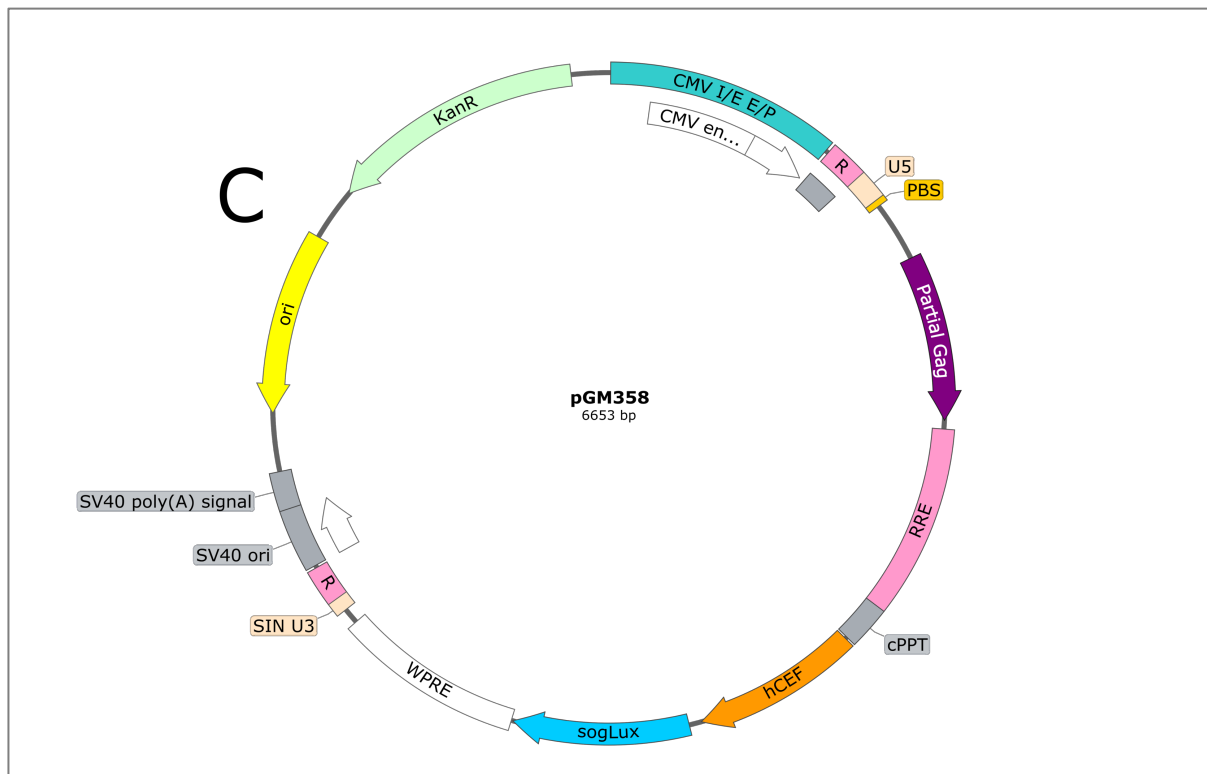
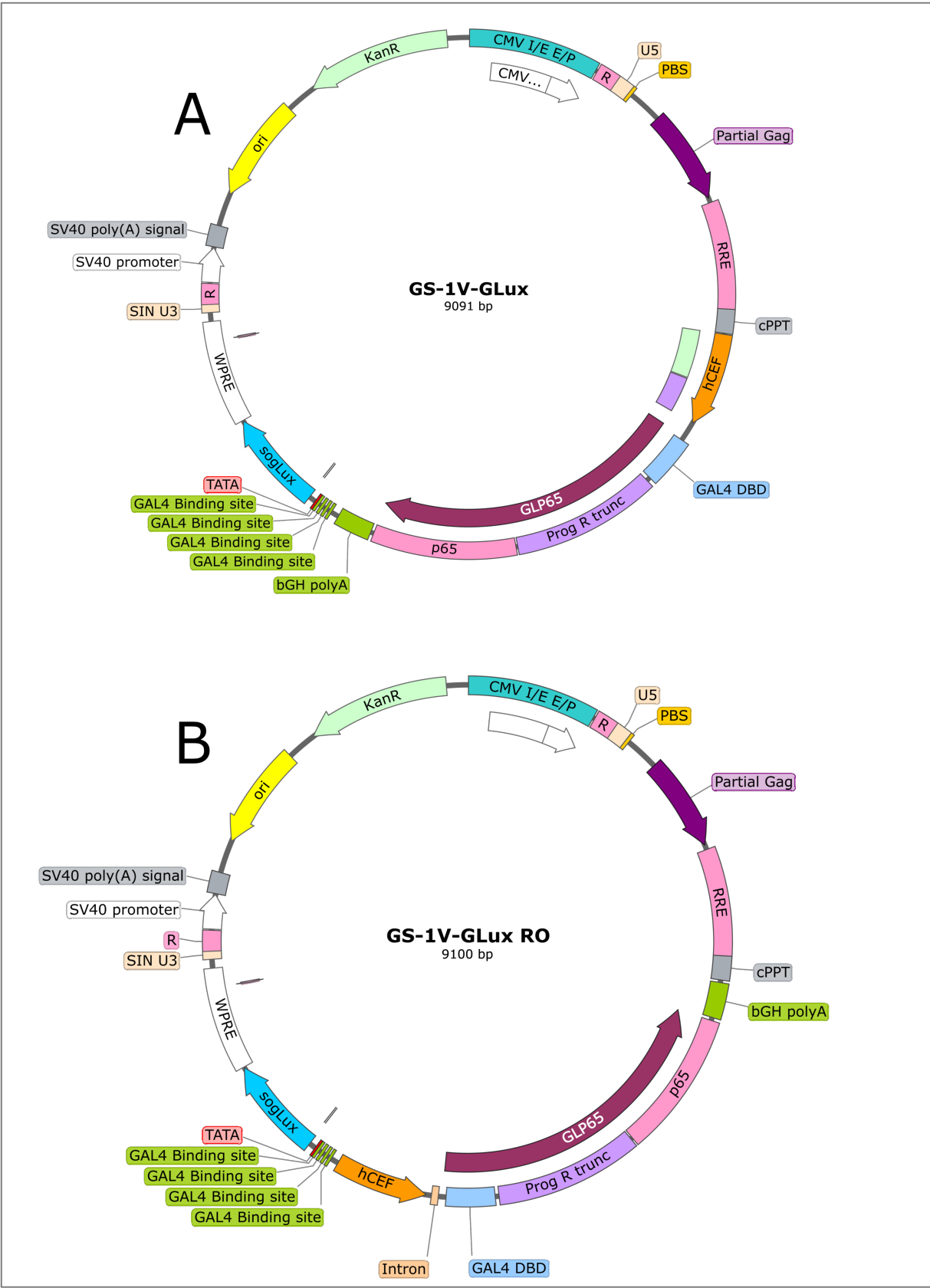


Figure 5.2- Plasmid maps of commercial GeneSwitch two plasmid system, and SIV lentiviral backbone.

The two components of the commercial GeneSwitch system in the original conformation. A) pGene encodes reporter gene LacZ with expression driven by 6x upstream GAL4 binding sites and a TATA box. B) The pSwitch plasmid encodes the transactivator consisting of GAL4 DNA-binding domain, truncated progesterone receptor and p65 activation domain. Expression of transactivator is driven by Herpes simplex virus (HSV) thymidine kinase (Tk) minimal promoter and upstream 4x GAL4 binding sites. C) SIV backbone pGM358-hCEF-GLux encodes the reporter protein Gaussia luciferase (GLux) expressed by the chimeric human CMV enhancer coupled to the eukaryotic EF1 α promoter fusion (hCEF) promoter.



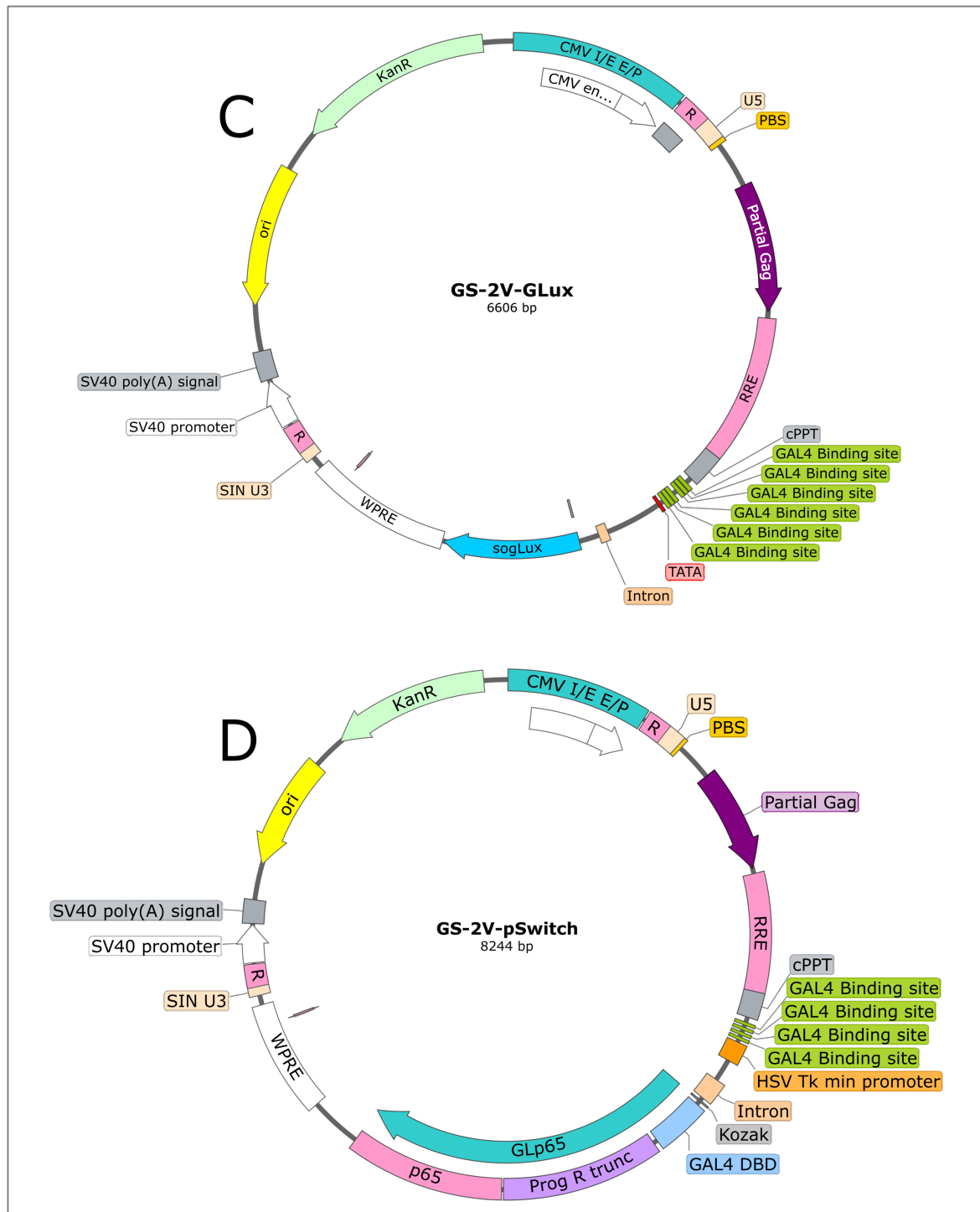
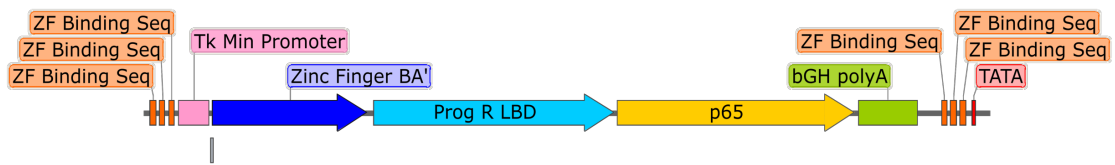


Figure 5.3- One vector and two vector regulated expression cassettes based of the GeneSwitch system.

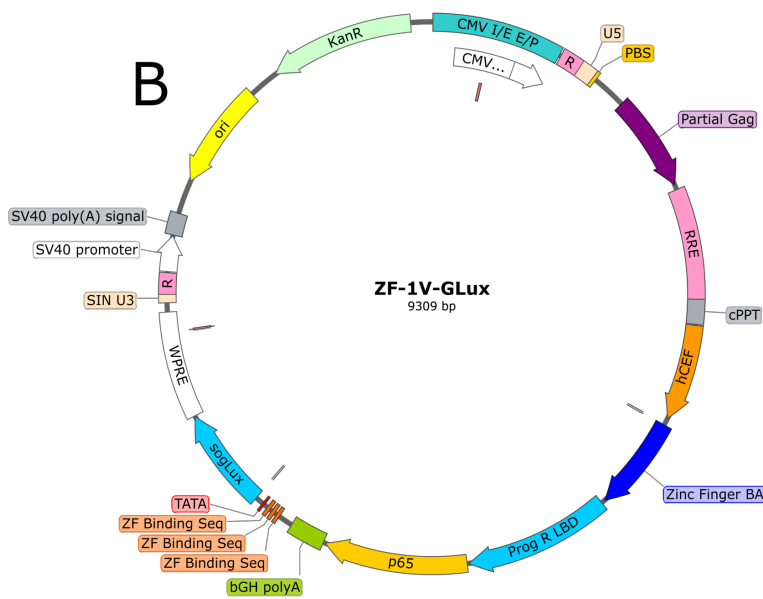
Sequences amplified from the commercial GeneSwitch (GS) system were cloned into the SIV lentiviral backbone (pGM358-hCEF-GLux- Figure 5.2C). A) To generate a single vector based from the GS system (GS-1V-GLux) the transactivator sequence derived from pGene LacZ (Figure 5.2A) was inserted downstream of the hCEF promoter and then polyA sequence and inducible promoter inserted after the transactivator, upstream of the GLux transgene. B) GS-1V-GLux modified so that the transgene remains in sense orientation but the hCEF-transactivator-polyA open reading frame is encoded in the reverse orientation. C) The regulated promoter including 6x GAL4 binding sites and TATA sequence were amplified from pGene LacZ and inserted upstream of GLux transgene, generating GS-2V-GLux. C) Transactivator sequence and auto-inducible promoter amplified from (Figure 5.2B) pSwitch plasmid was inserted into pGM358-hCEF-GLux upstream of WPRE, called GS-2V-pSwitch.

A



Zinc finger regulated expression cassette
2887 bp

B



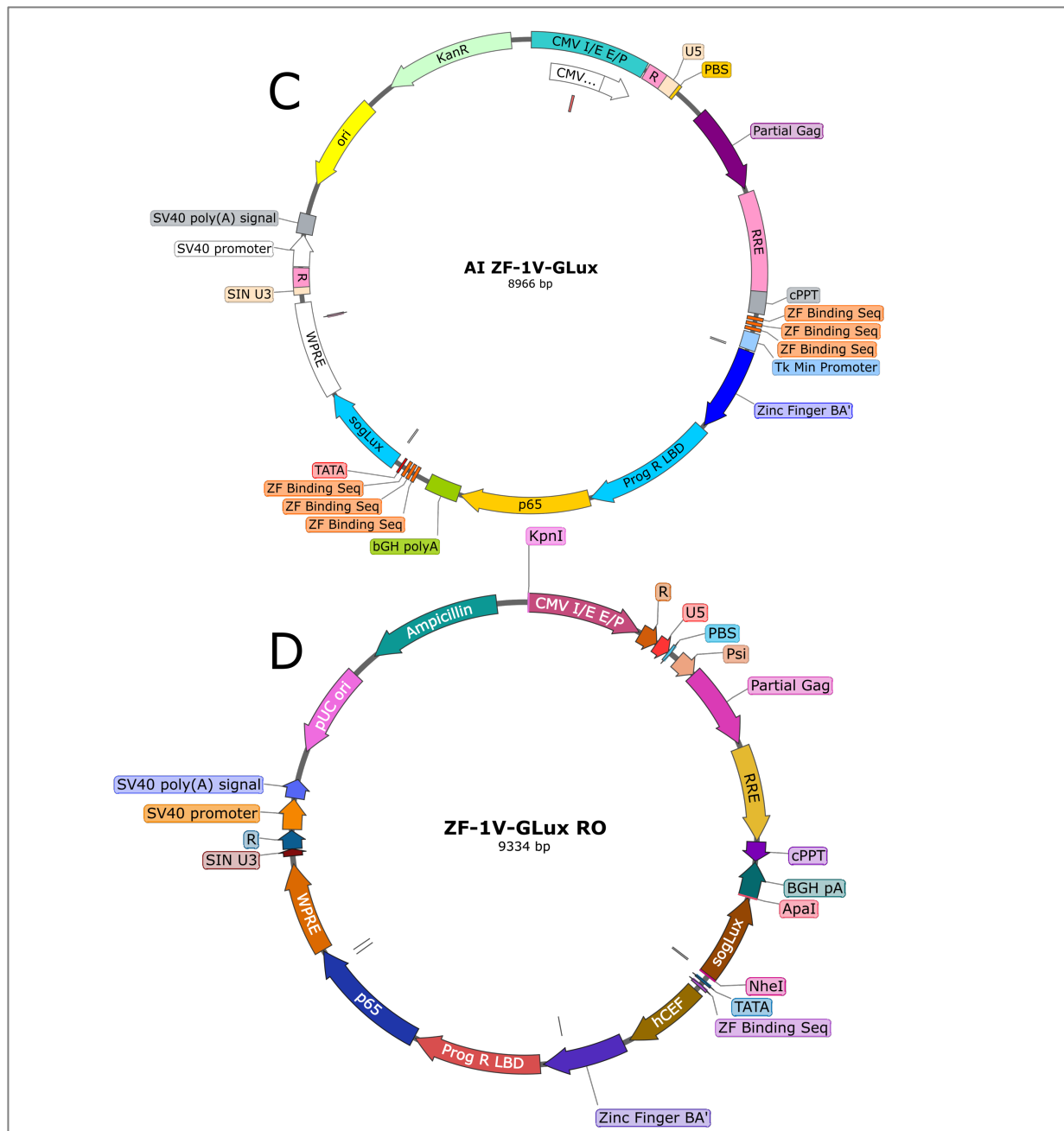


Figure 5.4- Novel regulated expression cassettes including a zinc finger DNA binding domain.

The template of the GeneSwitch system was used however replacing the GAL4 DNA binding domain with a zinc finger (ZF), specifically ZF BA'. GAL4 binding sites were replaced with ZF binding sequences. A) The novel ZF regulated expression cassette synthesised by GeneArt. The full sequence includes 3x ZF binding sites and Herpes simplex virus (HSV) thymidine kinase (Tk) minimal promoter upstream of the ZF transactivator, followed by a polyA sequence and further 3x ZF binding sites plus TATA sequence. B) The ZF transactivator and inducible promoter were amplified from the ZF synthesised fragment and inserted within pGM358-hCEF-GLux, downstream of the hCEF promoter and upstream of GLux transgene (ZF-1V-GLux). C) The auto inducible promoter containing 3x ZF binding sites and Tk minimal promoter was inserted upstream of the ZF transactivator instead of the hCEF constitutive promoter, generating AI ZF-1V-GLux. D) VectorBuilder generated a reverse orientation version of ZF-1V-GLux, by keeping the hCEF-transactivator in the sense orientation yet encoding the regulated promoter-GLux-polyA open reading frame in the opposite orientation (ZF-1V-GLux RO).

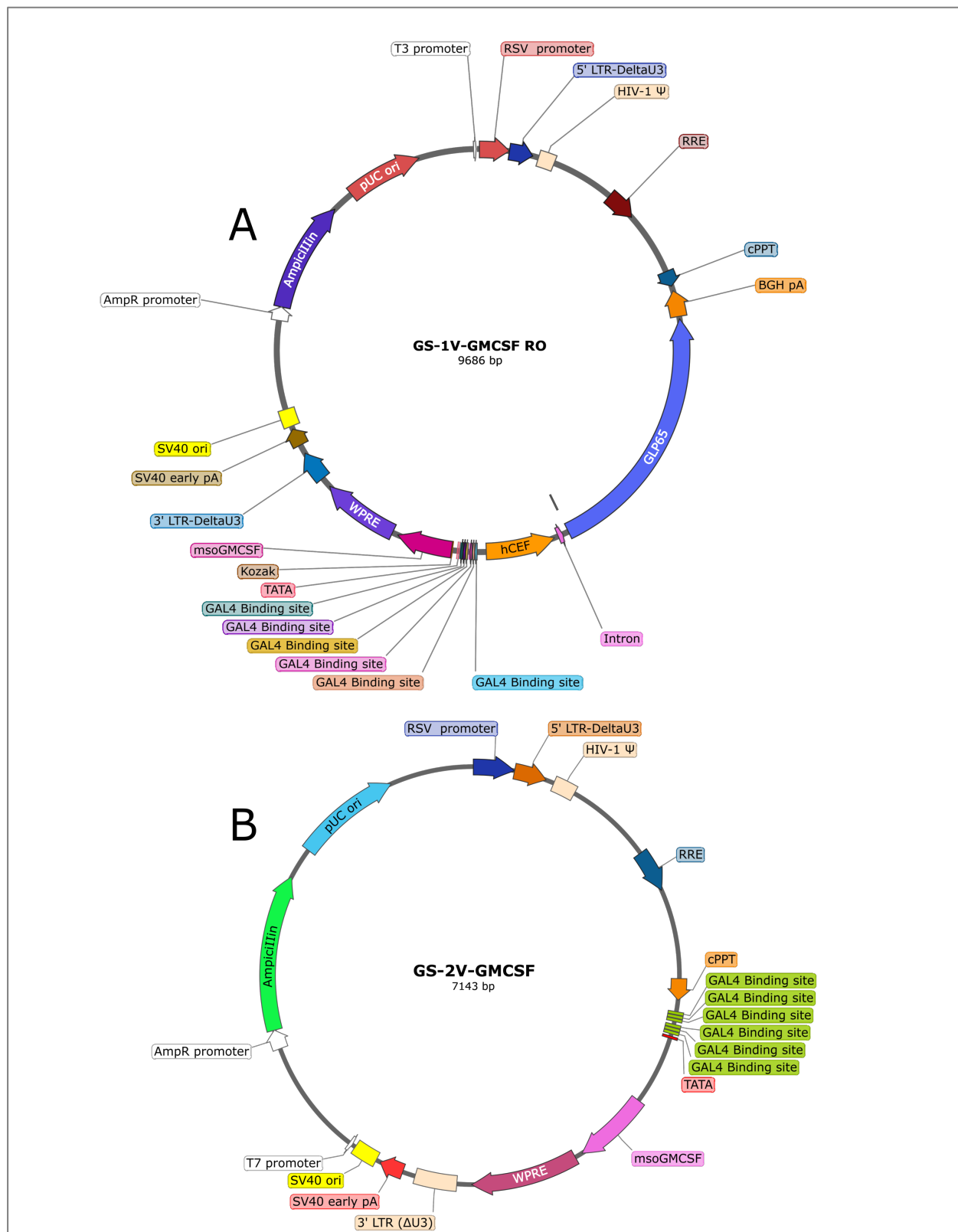


Figure 5.5- One vector and two vector regulated expression cassettes encoding a therapeutic transgene.

The GeneSwitch (GS)-based one vector and two vector configurations were transferred from a SIV backbone into a VectorBuilder HIV-1 backbone for generation of VSV-G vector. The Gaussia Luciferase (GLux) transgene was replaced with the granulocyte-macrophage colony-stimulating factor (GMCSF) cDNA sequence in GS-1V-GLux RO plasmid, creating A) GS-1V-GMCSF RO and GS-2V-GLux, creating B) GS-2V-GMCSF. GAL4 binding sequences increased to 6x GAL4 sites upstream of GMCSF cDNA in GS-1V-GMCSF RO.

5.2.6 Induction of transgene expression in bone marrow-derived macrophages

Macrophages were derived from murine bone marrow and collected per published protocols under sterile conditions (285). Briefly, femurs and tibia were isolated from wildtype C57BL/6 mice and suspended in 70% ethanol to assist muscle removal. Bones were then washed in PBS -/- and carefully scraped to provide access to the bone marrow cavity. Bone marrow cells were flushed from the cavity using Roswell Park Memorial Institute (RPMI) 1640 Medium (Thermo Fisher Scientific, Massachusetts, US) supplemented with 10% FCS and pelleted at 500xg for 15 minutes. Following treatment with 1x RBC lysis buffer (Thermo Fisher Scientific, Massachusetts, US), cells were filtered with a 70 µm cell strainer (Corning, New York, US) and plated into non-treated 24-well plates at 150,000 cells/well. Bone marrow cells were grown in differentiation media (RPMI supplemented with 100 ng/ml macrophage colony stimulating factor (M-CSF) (PreproTech, New Jersey, USA)) for 7 days to differentiate macrophages, exchanging media on day 3 with fresh differentiation media.

Once differentiated, one well was harvested by first washing cells with PBS -/- and then incubating with versene solution (Thermo Fisher Scientific, Massachusetts, US) for no more than 30 minutes at 37°C to determine a cell count prior to transduction. Subsequently, plated macrophages were transduced in 24-well plates with GS-1V-GLux at MOI 10 and GS-2V-GLux/GS-2V-pSwitch at MOI 10 and 40, respectively. As a positive control, macrophages were transduced with rSIV.F/HN-GLux at MOI 10. Transductions were performed following the standard protocol, exchanging reduced serum media 24 hours after transduction with complete RPMI media (RPMI 1640 Medium supplemented with 10% FCS and 1% penicillin/streptomycin) supplemented with 10^8 M mifepristone. Macrophages show reduced proliferative capacity after they are differentiated thus media was exchanged for complete RPMI media daily for 6 days, with the final 24 hours including a second dose of mifepristone (10^8 M). GLux expression from daily media collections was measured by standard GLux assay, normalised for total protein/well as described previously (refer to section 3.2.6).

5.2.7 Transduction of air liquid interface (ALI) cultures with VSV-G vectors

Regulated vectors were further investigated in healthy fully-differentiated human air liquid interface (ALI) cultures (MucilAir Epithelix, Geneva, Switzerland). ALIs are used as a model of the human airway epithelium with long-term survival of cultures allowing for transgene expression to be monitored over time. ALI cultures were grown in PneumaCult ALI media supplemented with 1% penicillin/streptomycin, 0.48 µg/ml hydrocortisone, 4 µg/ml heparin and 1X PneumaCult ALI Supplement (Stemcell Technologies, Vancouver, Canada) with basal media changed every 48 hours. Prior to transductions, mucus was carefully washed from the apical surface of ALIs using PBS containing calcium and magnesium (PBS +/+). Washings were performed twice with 100 µl PBS +/+, incubating at 37°C for 30 minutes each time. ALIs were transduced with VSV-G GS-1V vectors encoding GLux and GMCSF at MOI 100 and GS-2V vectors at MOI 100 (GS-2V-GLux/GMCSF) and MOI 200 (GS-2V-pSwitch). VSV-G vector diluted in Opti-Mem was applied to the apical surface of ALI cultures and incubated for 4 hours at 37°C before removing. One-week post-transduction, ALI cultures were induced by adding 10^8 - 10^9 M mifepristone diluted in PBS +/+ to the apical surface for 2 hours at 37°C or by adding mifepristone directly to the basal media. Daily collection of basal media or apical PBS +/+ washes (100 µl) was performed and GLux/GMCSF expression determined as described previously. Mifepristone induction was performed repeatedly over 3-5 weeks and ALIs harvested after 10 weeks.

5.2.8 Molecular analysis of ALIs

ALIs cultures were harvested approximately 10-weeks after transduction. To break tight junctions, cells were first incubated with PBS -/- added to the apical and basal membrane surface for 10 minutes at 37°C. PBS was removed from ALIs and cells filtered through a 70 µm cell strainer. To dissociate the cells, 1X Accutase (Stemcell Technologies, Vancouver, Canada) was added to the apical and basal surface of ALIs and incubated at 37°C for 10 minutes before removing to the strainer and repeating. The ALI insert was washed thoroughly with PBS -/- to remove all remaining cells then the strainer washed with FACS buffer (PBS -/-, 0.1% BSA, 1mM EDTA). Pelleting of cells by centrifugation at 200xg for 7 minutes at 4°C followed by resuspension in FACS buffer was repeated twice then cells counted to take forward 1×10^4 cells for DNA extraction. Using the automated Maxwell RSC Instrument and Maxwell RSC Cell DNA Purification Kit (Promega, Wisconsin, US), genomic

DNA was extracted from ALI cultures and VCN determined using the ddPCR platform as described previously. Reactions were performed in multiplex using primer/probe qPCR assays against WPRE and human housekeeping gene *B2M*, as detailed in Table 2.2. The calculated VCN for each ALI culture was expressed as VCN/genome.

5.2.9 Mifepristone induced expression in VSV-G transduced mice

To investigate regulated expression *in vivo*, MFD of GS-1V-GLux (2.03e8 TU/mouse) and dual vectors GS-2V-GLux (5.93e7 TU/mouse)/ GS-2V-pSwitch (1.2e8 TU/mouse) mixed together were delivered to wildtype female mice (n=3 mice each for 1V and 2V) by intranasal administration. As a positive control, female wildtype mice (n=3 mice) received rSIV.F/HN-GLux (7.96e7 TU/mouse) by intranasal administration. At 1-week post-transduction mifepristone was delivered by intraperitoneal injection (400 µg/kg) to treated mice and 48 hours later blood sampled by tail venepuncture. End-point sample collection took place 20 weeks post-dosing, 24 hours after intraperitoneal injection of mifepristone (400 µg/kg). GLux expression was determined in serum and BALF as described previously.

5.3 Results

To establish proof-of-concept that lentivirus-mediated expression could be regulated, a commercial mifepristone-inducible system, GeneSwitch, was identified and cloned into a lentiviral backbone. Based upon the design of GeneSwitch, a humanised regulated expression cassette was produced with a ZF in replacement of the yeast GAL4 sequences. The inducible vectors were cloned into various configurations and investigated *in vitro*. VSV-G vectors were generated and expression of reporter and therapeutic transgenes following mifepristone-induction investigated in a range of models, from human airway cultures to mice.

5.3.1 Co-transfection of pGene and pSwitch plasmids generates positively stained cells upon mifepristone induction

In the first instance, the GeneSwitch two plasmid system was tested in its original conformation encoding reporter gene *LacZ* following the protocol optimised by the supplier. The pGene *LacZ* and pSwitch plasmids were co-transfected into HEK293T cells and transgene expression induced with mifepristone. Figure 5.6 shows representative images captured under standard light field microscopy of cells stained with XGal following different transfection conditions. Cells expressing β -galactosidase from the *LacZ* gene cleave XGal into a blue product which can be visualised. Cells transfected with pGene *LacZ* alone showed no evidence of positively stained cells (Figure 5.6 A+B). Overall cells co-transfected with pGene *LacZ* and pSwitch plasmids showed minimal evidence of *LacZ* expression without mifepristone induction (Figure 5.6 C+D), however blue vacuoles were identified in a small number of cells as indicated by the black arrow heads in the magnified section of Figure 5.6D. In contrast, cells co-transfected with pGene *LacZ* and pSwitch plasmid demonstrated clear *LacZ* expression upon mifepristone induction which was evidenced by dark blue staining in numerous cells across Figure 5.6 E+F. Although the percentage of positive cells was not quantified, the GeneSwitch system demonstrated clear efficacy and low levels of leakiness in the original configuration as investigated in HEK293T cells.

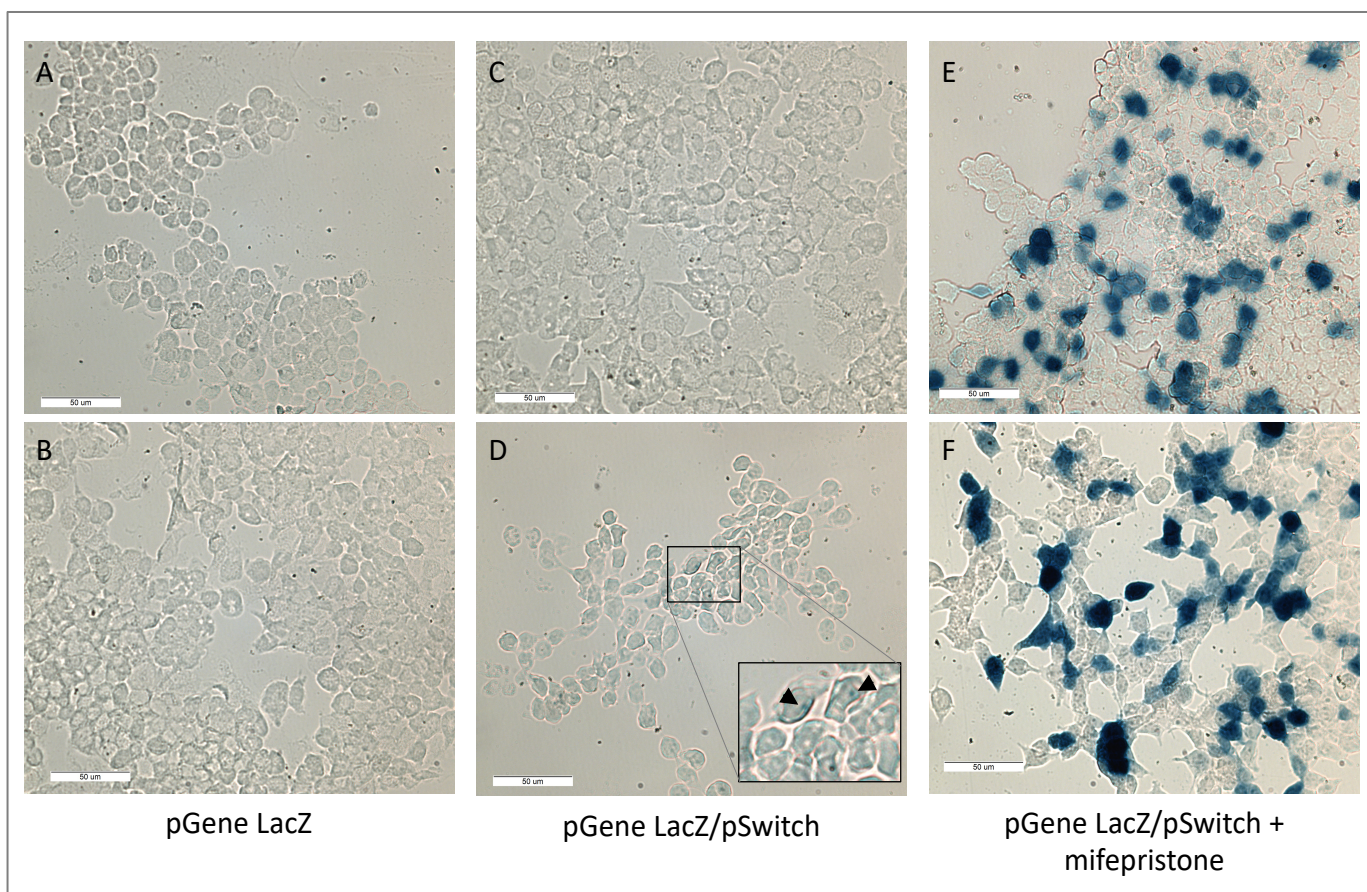


Figure 5.6- XGal staining following mifepristone-induction of HEK293T cells transfected with GeneSwitch inducible plasmids.

HEK293T cells were transfected with pGene LacZ and pSwitch plasmids from the commercial GeneSwitch system (1 $\mu\text{g}/\text{ml}$ plasmid DNA) and transgene expression induced with 10^8 M mifepristone added to cell culture medium 24 hours post-transduction. 24 hours after mifepristone addition, cells were stained with XGal and images captured under standard light field microscopy. Representative images of HEK293T cells transfected with pGene LacZ plasmid (A+B) or cells co-transfected with pGene LacZ and pSwitch plasmids (C+D) without mifepristone. A section of cells that have been magnified is shown in the bottom right corner (D) with black arrowheads identifying blue vacuoles within cells suspected to be evidence of LacZ expression. E+F) Representative images of cells co-transfected with pGene LacZ and pSwitch plasmids and stimulated with mifepristone. Scale bars of 50 μm shown in each image.

5.3.2 Cloning of GeneSwitch and zinc finger constructs into the lentiviral backbone generates modest GLux expression following mifepristone induction

The components of the GeneSwitch system essential for its function, the transactivator and inducible promoter were subsequently cloned into the SIV lentiviral backbone, pGM358-hCEF-GLux. Both components were incorporated into a single plasmid (GS-1V-GLux) and similarly the novel ZF constructs designed in collaboration with Professor Mark Isalan, were inserted into pGM358-hCEF-GLux in the same configuration (ZF-1V-GLux and AI ZF-1V-GLux). To investigate the efficacy of the novel configurations, A549 cells were transfected with the single plasmid constructs alongside a pGM358-hCEF-GLux positive control and GLux expression in cell supernatant compared following mifepristone induction. Figure 5.7A shows data accumulated from two separate experiments. The 'leakiness' of each construct, defined as the basal transgene expression without mifepristone induction, was comparable between regulated expression constructs yet was appreciably above background levels determined in untransfected cells (> 1 log above untransfected). GLux expression following transfection with GeneSwitch-based plasmid GS-1V-GLux, was increased by 4.2 fold following induction by mifepristone (difference in mean GLux expression). In contrast, the ZF constructs generated less than a 2-fold increase in expression after induction (ZF-1V-GLux 1.9-fold and AI ZF-1V-GLux 1.5 fold) termed the induction ratio. Peak GLux expression, achieved by GS-1V-GLux-transfected cells induced with mifepristone (57634 ± 22734 RLU/mg protein, $n=6$), was approximately one log below cells transfected with the positive control, pGM358-hCEF-GLux (582142 ± 260016 RLU/mg protein levels, $n=6$) with constitutive transgene expression.

Given the induction ratio was not improved by inclusion of an auto-inducible promoter upstream of the ZF transactivator, the ZF-1V-GLux design was taken forward along with the GS-1V-GLux construct for further investigation. Figure 5.7B shows GLux expression generated following transfection of A549 cells with increasing concentrations of single plasmid expression constructs. With increasing transfection concentrations GS-1V-GLux demonstrated greater induction ratios upon mifepristone stimulation (0.1 $\mu\text{g/ml}$ 1.48-fold, 0.5 $\mu\text{g/ml}$ 3.04-fold and 1.0 $\mu\text{g/ml}$ 6.81-fold). In contrast, the greatest induction of GLux expression was generated in cells transfected with the lowest concentration of ZF-1V-GLux with no difference in response at higher plasmid concentrations (0.1 $\mu\text{g/ml}$ 2.60-fold, 0.5

µg/ml 0.75-fold and 1.0 µg/ml 1.15-fold). GLux expression following transfection with pGM358-hCEF-GLux (608830 ± 277646 RLU/mg protein, n=4) was exceeded by mifepristone-induced expression following GS-1V-GLux transfection at the two highest doses (0.5 µg/ml 797799 ± 153378 RLU/mg protein and 1.0 µg/ml 973821 ± 309347 RLU/mg protein, n=3). Leakiness of GS-1V-GLux and ZF-1V-GLux were generally comparable across transfection concentrations, with the ZF construct generating only marginal increases in expression upon induction.

5.3.3 The GeneSwitch regulated expression cassette cloned into a single construct outperforms the two-plasmid system *in vitro*

The components of the GeneSwitch system were incorporated into a single plasmid (GS-1V-GLux) and cloned into two separate plasmids (GS-2V-GLux/GS-2V-pSwitch) to compare efficacy between the original two-plasmid configuration and a single construct design. Figure 5.8 shows levels of GLux produced by HEK293T cells following transfection with increasing concentrations (0.05, 0.1 and 0.2 µg/ml) of single and two-plasmid constructs. The leakiness of the dual plasmid construct at all transfection concentrations was high, with co-transfection of GS-2V-GLux and GS-2V-pSwitch generating significantly greater (~50 fold higher) levels of GLux than positive control pGM358-hCEF-GLux, at 0.05 and 0.1 µg/ml. Only at the lowest transfection concentration of GS-2V-GLux/pSwitch was an increase in GLux expression observed following mifepristone induction (0.05 µg/ml 1.8-fold induction). In contrast, the greatest induction ratio was observed at the highest transfection concentration for the GS-1V-GLux single construct (0.2 µg/ml 37-fold induction) with significant induction observed at all transfection concentrations (0.05 µg/ml 18-fold induction and 0.1 µg/ml 8-fold induction). Transfection of cells with the GS-2V-GLux plasmid resulted in comparable expression to pGM358-hCEF-GLux at all transfection concentrations, despite including a TATA box upstream of the transgene. No response to mifepristone induction was observed at all concentrations of GS-2V-GLux transfection. Overall, the GS-1V-GLux single plasmid system demonstrated greater mifepristone induction and reduced leakiness at all tested concentrations.

5.3.4 Reverse orientation constructs are functional and can be efficiently packaged into VSV-G vectors

The inclusion of two consecutive open reading frame (ORFs) within the single plasmid expression cassettes is suitable for non-viral applications, however the premature stop codon included after the transactivator would likely have a negative impact on vector production. Thus the ORFs for transactivator and inducible transgene were encoded in opposite directions, generating GS-1V-GLux RO and ZF-1V-GLux RO. Figure 5.9 shows the comparison of mifepristone-induced transgene expression for single plasmid expression constructs in sense and reverse orientation. The GS-1V-GLux RO plasmid showed greater leakiness (~10x higher) and induction (~20x greater) compared to the original conformation (GS-1V-GLux) whereas the ZF-1V-GLux RO showed reduced leakiness and lower induced expression (~20-25x lower) compared to ZF-1V-GLux. A marginal improvement in induction ratio (ZF-1V-GLux RO 1.63-fold vs ZF-1V-GLux 1.37-fold induction) was observed by the reverse orientation ZF construct. Leakiness of the GS-1V-GLux RO construct was comparable to constitutive GLux expression from pGM358-hCEF-GLux and induced expression 10-fold greater than pGM358-hCEF-GLux. Thus GS-1V-GLux RO was taken forward to vector production by transferring the expression cassette into a backbone containing HIV-1 LTRs and subsequent generation of ultrapure VSV-G vector by VectorBuilder. Significant titres were generated (GS-1V-GLux RO 2.1e9 TU/ml) despite the inclusion of two ORFs within the viral genome. VSV-G vector encoding the transactivator and GLux under expression of the inducible promoter were also produced, achieving similar titres (GS-2V-GLux 1.3e9 TU/ml and GS-2V-pSwitch 3.0e9 TU/ml). An average of vector titre determined by VectorBuilder using the p24 ELISA and titre calculated in-house by ddPCR was used for future experiments.

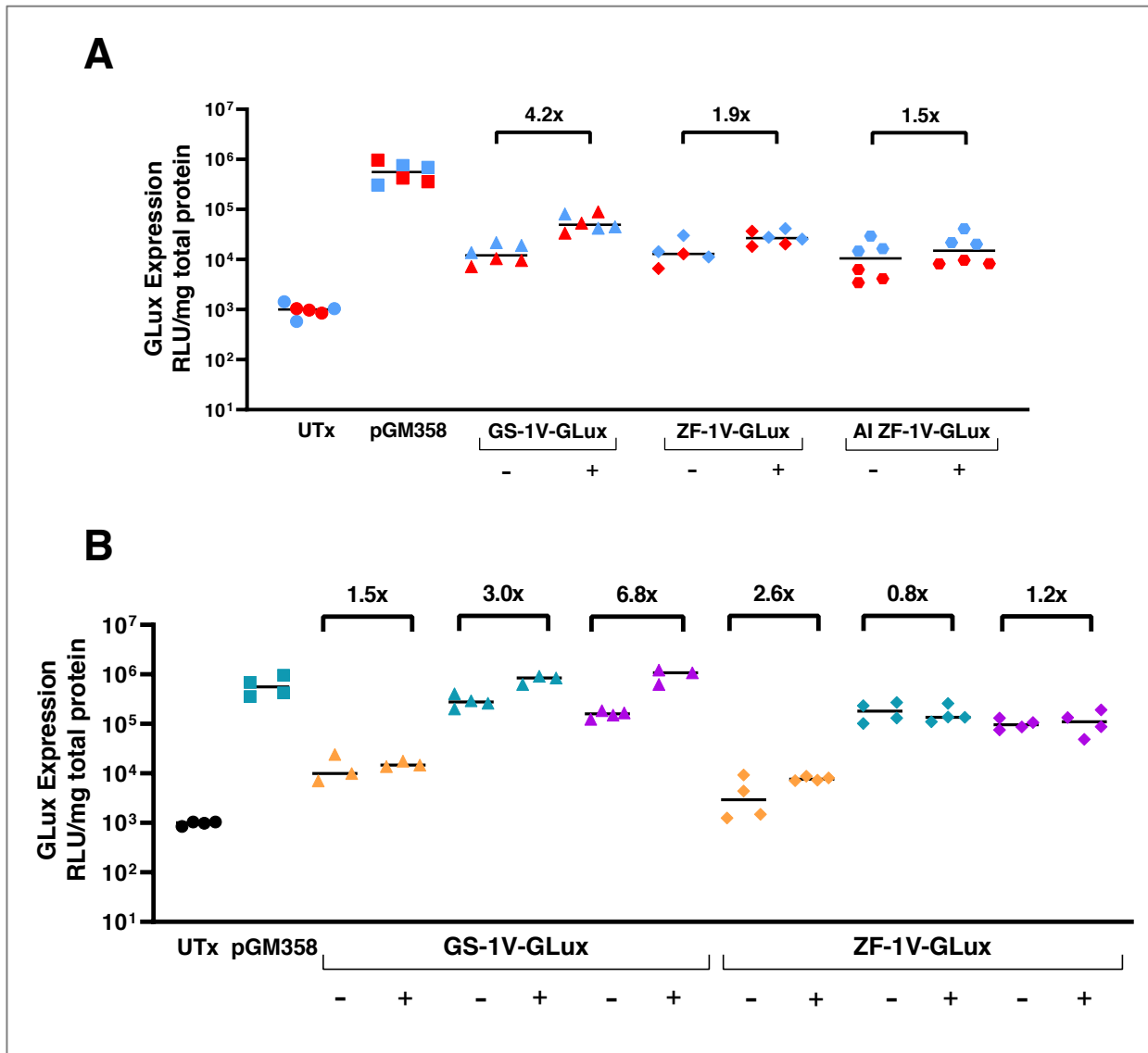


Figure 5.7- Mifepristone-induction of transgene expression in single plasmid constructs.

Single plasmid constructs containing both transactivator and inducible Gaussia luciferase (GLux) transgene were cloned into the pGM358-hCEF-GLux backbone. GS-1V-GLux was derived from the commercial GeneSwitch system and ZF-1V-GLux and AI ZF-1V-GLux were novel ZF designs. Cells pre-seeded into 24-well plates were transfected with single plasmid constructs and transgene expression induced with 10^8 M mifepristone added to cell culture medium. GLux expression was quantified and normalised for total protein, expressed as relative light units (RLU)/ mg total protein and expression compared between cells that were induced with mifepristone (+) and those that were not (-). GLux expression following transfection of cells with positive control GLux plasmid (pGM358-hCEF-GLux) and negative control untransfected cells (UTx) also shown. A) A549 cells were transfected with pGM358, GS-1V-GLux, ZF-1V-GLux and AI ZF-1V-GLux constructs (1 μ g/ml plasmid DNA). Data accumulated from two separate experiments, shown by the blue and red coloured shapes (n=3 wells/condition for each experiment). B) HEK293T cells were transfected with GS-1V-GLux, ZF-1V-GLux (0.1 (yellow), 0.5 (green) and 1.0 μ g/ml (purple) plasmid DNA, n=3/4 wells/experiment). Horizontal bars are median values and each shape represents an individual well. Brackets show the induction ratio between cells uninduced (-) and induced (+) by mifepristone.

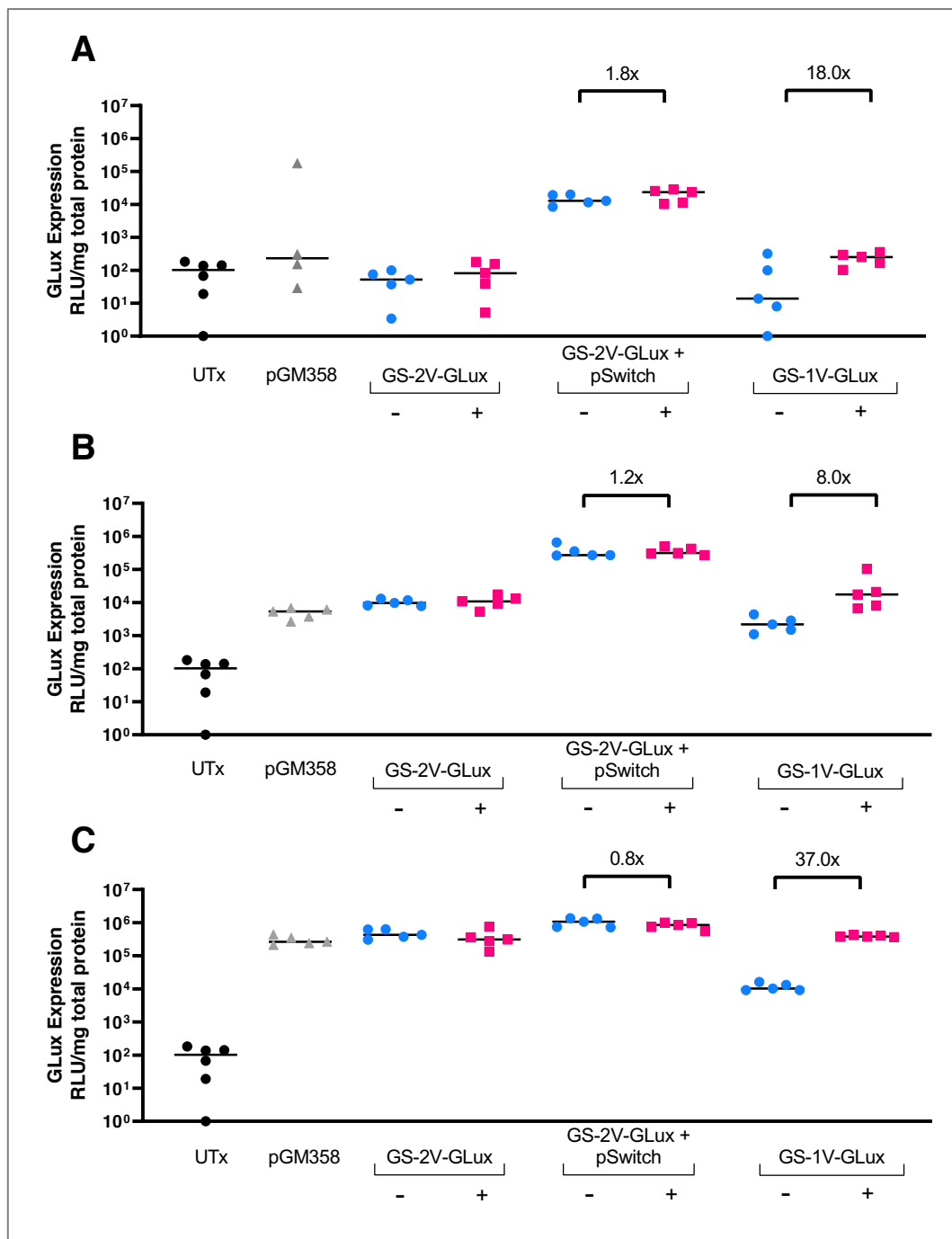


Figure 5.8- Comparison between GeneSwitch-based inducible systems encoded by a single and dual plasmid.

Single plasmid constructs (GS-1V-GLux) containing both transactivator and inducible GLux transgene cloned into the pGM358-hCEF-GLux lentiviral backbone. Dual plasmid constructs with the transactivator (GS-2V-pSwitch) and inducible gene (GS-2V-GLux) cloned into the pGM358-hCEF-GLux lentiviral backbone as two separate constructs. HEK293T cells were transfected with single and dual plasmid constructs (A- 0.05, B- 0.1, C- 0.2 µg/ml plasmid DNA, n=6 wells/condition) and transgene expression induced after 24 hours with 10^8 M mifepristone added to cell culture medium. GLux expression was quantified in supernatant 24-hours after mifepristone addition and normalised for total protein, expressed as relative light units (RLU)/ mg total protein and expression compared between cells that were induced with mifepristone (+ pink) and those that were not (- blue). GLux expression following transfection of cells with positive control GLux plasmid (pGM358-hCEF-GLux, n=4/5 wells), GS-2V-GLux only (n=6 wells) and negative control untransfected cells (UTx, n=6 wells) also shown. Horizontal bars are median values and each shape represents an individual well. Brackets show the induction ratio between cells uninduced (-) and induced (+) by mifepristone.

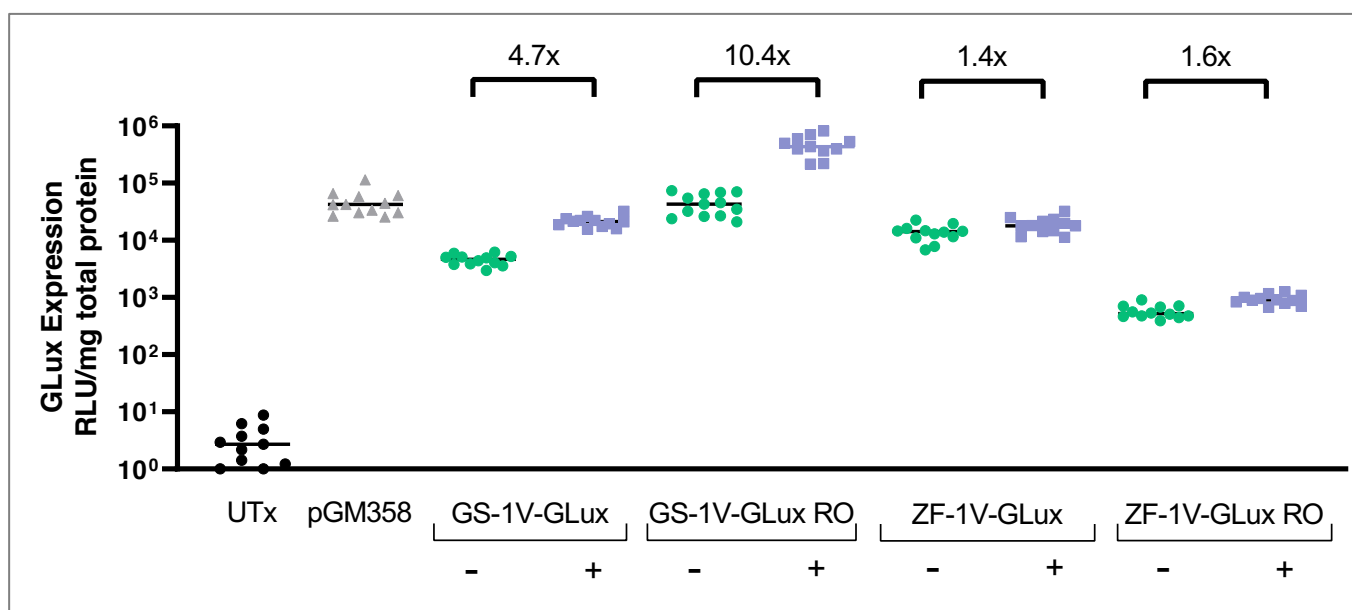


Figure 5.9- Comparison of inducible transgene expression following transfection of single plasmid constructs in sense and reverse orientation (RO).

Single plasmid constructs containing both transactivator and inducible GLux gene were cloned into the SIV lentiviral backbone, pGM358-hCEF-GLux. GS-1V-GLux/GLux RO was derived from the commercial GeneSwitch system and ZF-1V-GLux/GLux RO were novel ZF designs. Reverse orientation constructs contained the transactivator and transgene encoded in opposite orientations. HEK293T cells were transfected with single plasmid constructs (0.2 µg/ml plasmid DNA) and after 24 hours transgene expression induced with 10⁸ M mifepristone added to cell culture medium. GLux expression was quantified in supernatant 24 hours after mifepristone addition and normalised for total protein, expressed as relative light units (RLU)/ mg total protein and expression compared between cells that were induced with mifepristone (+ purple) and those that were not (- green). GLux expression following transfection of cells with positive control GLux plasmid (pGM358-hCEF-GLux) and negative control untransfected cells (UTx) also shown. Data combined from two separate experiments (n=6 wells/condition for each experiment). Horizontal bars are median values and each shape represents an individual well. Brackets show the induction ratio between cells uninduced (-) and induced (+) by mifepristone.

5.3.5 Inducible VSV-G vectors generate a significant fold-induction in transgene expression following mifepristone addition

Single and dual VSV-G vectors were investigated in HEK293T cells to determine lentiviral-mediated expression of GLux following mifepristone induction. Figure 5.10A shows GLux expression measured in cell supernatant following transduction with increasing MOIs (0.1, 1.0 and 10.0) of single and dual VSV-G vectors transduced in a 1:1 ratio. As was the case for both GS-1V-GLux RO and GS-2V-GLux/pSwitch, increasing MOIs of vector results in greater leakiness, with the single vector consistently demonstrating ~5x greater leakiness compared to dual vectors at all MOIs. GS-1V-GLux RO demonstrated a doubling in induction ratios for each log increase in MOI (MOI 0.1 4.9x, MOI 1.0 11.8x and MOI 10.0 22.1x) whereas similar induction ratios were observed following transduction with GS-2V-GLux/pSwitch in a 1:1 ratio at MOI 1.0 and 10.0 (~300 fold induction) which was ~7.5x greater than the induction ratio achieved at MOI 0.1 (40.4 fold induction). Cells transduced with GS-2V-GLux alone (data not shown) showed comparable levels of leakiness to cells transduced with the dual vector system, suggesting co-transduction of the GS-2V-pSwitch vector did not affect leaky expression.

Whereas the data generated in Figure 5.10A was produced following transduction of the dual vectors in a 1:1 ratio, Figure 5.10B investigated the effect of changing the proportion of transactivator to inducible transgene on GLux expression. Reducing the MOI of GS-2V-pSwitch to 2.5 whilst keeping GS-2V-GLux as MOI 10 and vice versa was compared to GLux expression following transduction of GS-2V-GLux and GS-2V-pSwitch at MOI 10. Leakiness was comparable between cells transduced with GS-2V-GLux at MOI 10 (5347, 4671-6809 vs 4224, 3200-5780 RLU/mg protein, GS-2V-GLux/pSwitch MOI 10:2.5 and 10:10 respectively, median + range n=6/transduction) which was approximately 3-4x greater leakiness than observed following transduction of GS-2V-GLux at MOI 2.5. Similarly, transductions including GS-2V-pSwitch at MOI 10 (GS-2V-GLux/pSwitch 10:10) generated 4.7x higher GLux levels following mifepristone induction compared to GS-2V-pSwitch transduced at MOI 2.5. Yet mifepristone induced GLux expression was only 2.6x greater than GS-2V-pSwitch MOI 2.5 when GS-2V-pSwitch was transduced at MOI 10 in combination with GS-2V-GLux at reduced MOI of 2.5.

The greatest induction ratio was observed by reducing the proportion of inducible transgene to transactivator (991.7 fold-induction), followed by equal ratios of GS-2V-GLux to pSwitch (583.0 fold-induction). The proportion of vectors demonstrating the least favourable expression profile, high leakiness and low induced expression, was excess transgene to transactivator (97.7 fold-induction). Expression of dual vectors following mifepristone induction exceeded constitutive GLux expression from rSIV.F/HN-GLux by 6-27x (Figure 5.10B), despite a matching MOI of 10. In summary, the optimal ratio of 1:4 with the transactivator supplied in excess to the inducible transgene achieved the greatest induction ratio following mifepristone addition.

To next evaluate the ability of mifepristone to regulate expression of a therapeutic transgene, the cDNA sequence of murine GMCSF was cloned into lentiviral backbones in replacement of GLux, and VSV-G vectors generated. Titres of GS-1V-GMCSF and GS-2V-GMCSF were 1.89×10^9 and 1.35×10^9 TU/ml, respectively and the same GS-2V-pSwitch vector used in previous experiments was co-transduced alongside GS-2V-GMCSF. Figure 5.11 shows mifepristone-induced GMCSF expression following transduction of HEK293T cells with single and dual VSV-G vectors. Only transductions with the transactivator supplied in excess of the inducible transgene were investigated with a matching MOI of GS-1V-GMCSF and GS-2V-GMCSF (MOI 10) used to compare leakiness of each configuration. Leakiness across all transduction conditions were comparable ($\sim 1-2 \mu\text{g}/\text{mg}$ protein) as was mifepristone-induced expression between single vector GS-1V-GMCSF (1207, 929-1825 $\mu\text{g}/\text{mg}$ protein, median + range $n=6$) and dual vectors (GS-2V-GMCSF/pSwitch 10:20- 1433, 916.1-1619 and GS-2V-GMCSF/pSwitch 10:40- 1002, 912.8-1756, $n=6/\text{condition}$) found to be not-significantly different upon analysis (ns = not significant Kruskal Wallis test, Dunn's multiple comparison test, $n=6$ wells/condition). Induction ratio following mifepristone addition was the greatest following transduction with GS-2V-GMCSF/pSwitch in a 10:20 ratio (1279x), followed by a 10:40 ratio (884x) then lastly by the single vector construct (494x). Overall the dual vector system outperformed the single vector for mifepristone induced expression of both GLux and GMCSF. However the single vector system including GMCSF was far more successful, demonstrating comparable induction of transgene expression compared to dual VSV-G vectors.

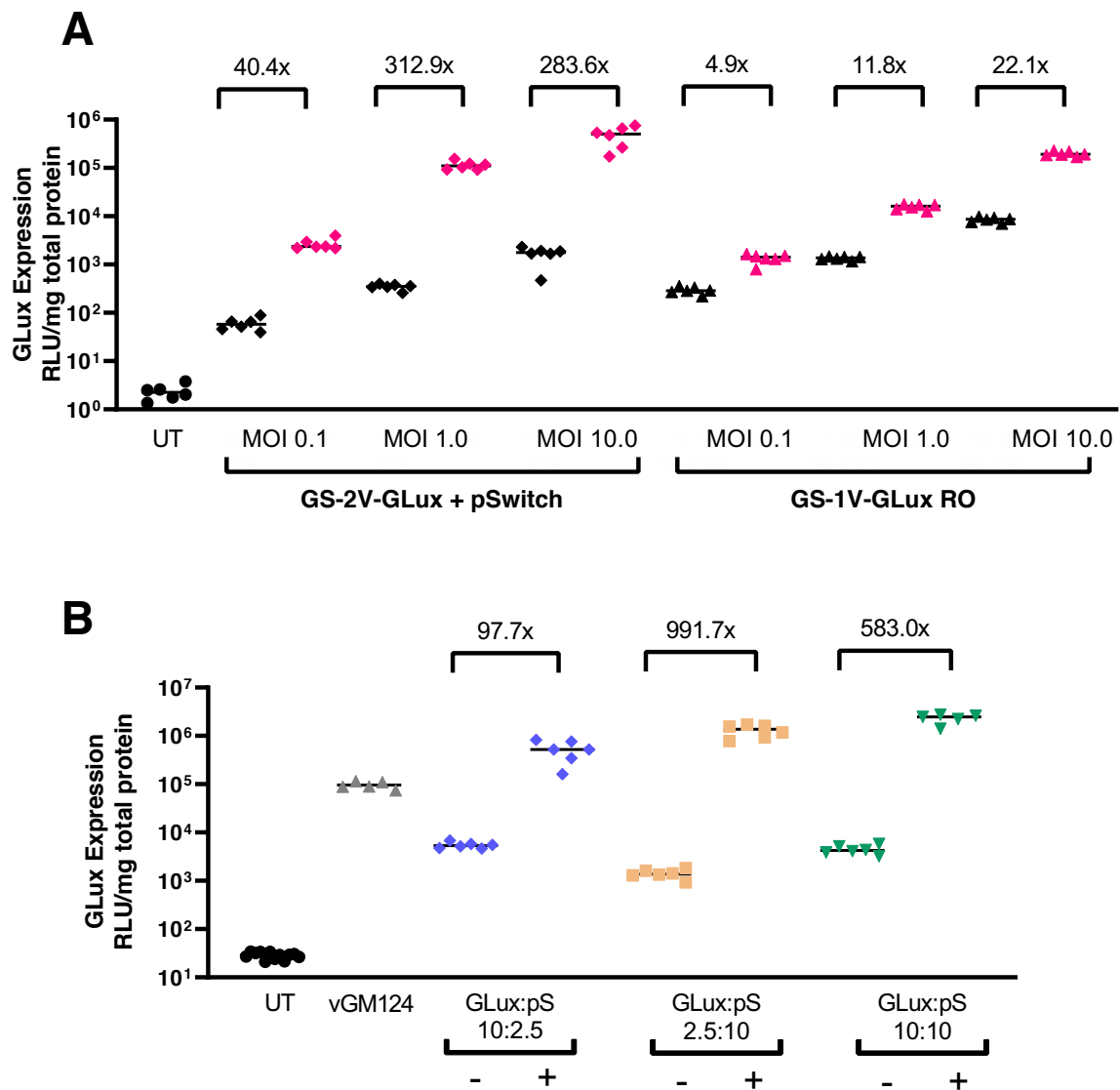


Figure 5.10- Mifepristone induction of GLux expression following transduction of HEK293T cells with single and dual inducible VSV-G vectors.

VSV-G vectors were generated encoding transactivator and inducible GLux transgene within a single vector (GS-1V-GLux RO) and within two separate vectors (GS-2V-GLux/pSwitch). HEK293T cells were transduced with VSV-G vectors and transgene expression induced after 24 hours with 10^8 M mifepristone added to cell culture medium. GLux expression was quantified in supernatant 24 hours after mifepristone addition and normalised for total protein, expressed as relative light units (RLU)/ mg total protein and expression compared between cells that were induced with mifepristone (+) and those that were not (-). GLux expression following transfection of cells with positive control rSIV.F/HN-GLux vector (vGM124-GLux, n=5 wells) and negative control untransduced cells (UT, n=6/12 wells) also shown. A) Single and dual vectors (1:1 ratio GS-2V-GLux:GS-2V-pSwitch) transduced at increasing multiplicity of infection (MOIs 0.1-10.0). B) Dual vectors transduced at different ratios (GS-2V-GLux:GS-2V-pSwitch MOI 10:2.5, 2.5:10, 10:10) and vGM124-GLux at MOI 10. Horizontal bars are median values and each shape represents an individual well. Brackets show the induction ratio between cells uninduced (-) and induced (+) by mifepristone.

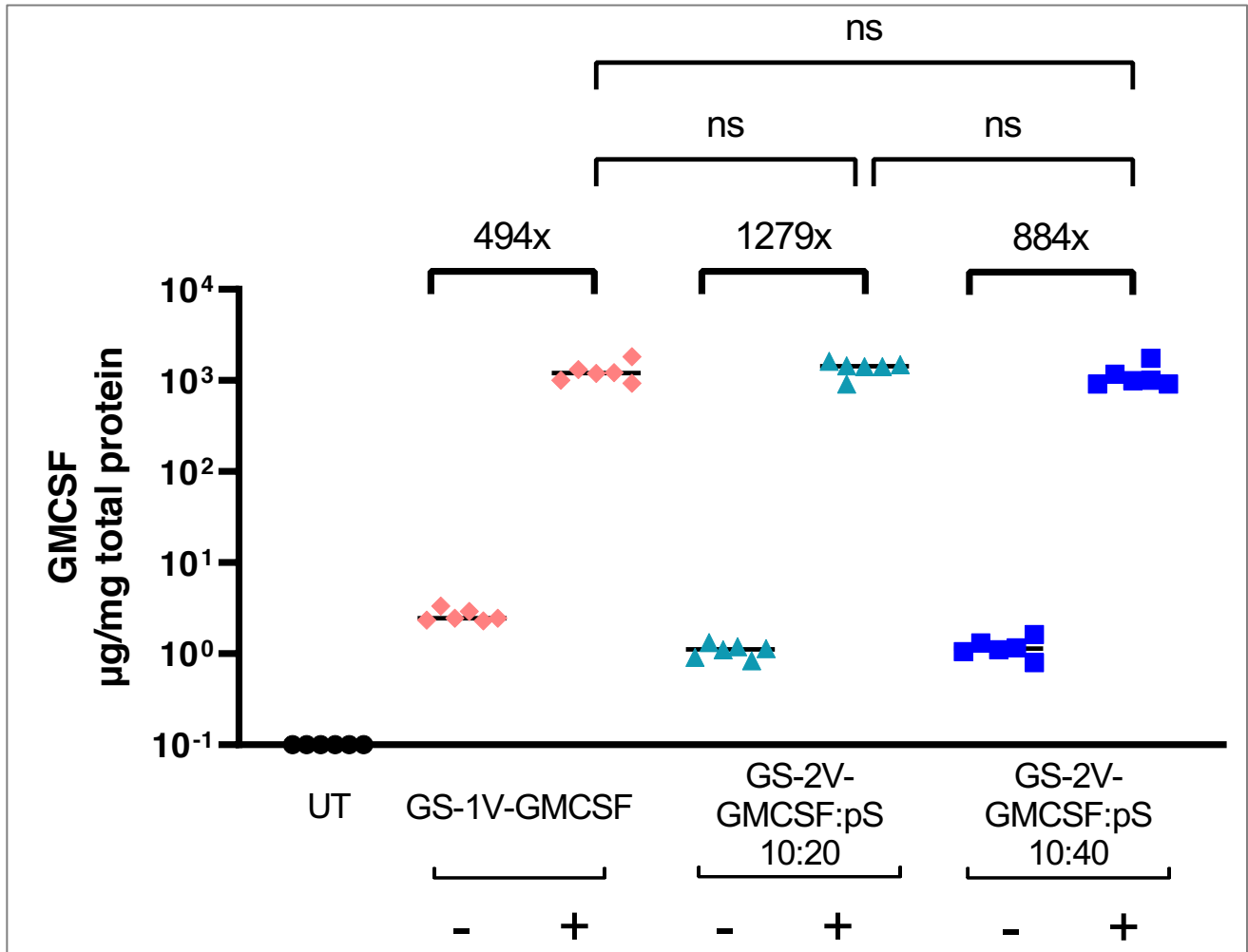


Figure 5.11- Comparison of GMCSF expression following mifepristone-induction of HEK293T cells transduced with single and dual inducible VSV-G vectors.

VSV-G vectors were generated encoding transactivator and inducible GMCSF transgene within a single vector (GS-1V-GMCSF RO) and within two separate vectors (GS-2V-GMCSF/pSwitch). HEK293T cells were transduced with single (multiplicity of infection (MOI) 10) and dual VSV-G vectors (GS-2V-GMCSF:GS-2V-pSwitch MOI 10:20 and 10:40) and transgene expression induced after 24 hours with 10^8 M mifepristone added to cell culture medium. GMCSF expression was quantified in supernatant after 24 hours of mifepristone and normalised for total protein, expressed as GMCSF μ g/ mg total protein and expression compared between cells that were induced with mifepristone (+) and those that were not (-). GMCSF expression measured in negative control untransduced cells (UT, n=6 wells) also shown. Horizontal bars are median values and each shape represents an individual well. ns= not significant, Kruskal Wallis test, Dunn's multiple comparison test, n=6 wells/condition. Brackets show the induction ratio between cells uninduced (-) and induced (+) by mifepristone.

5.3.6 GS-2V-GLux/pSwitch dual vector system demonstrates superior mifepristone induction of GLux expression compared to a single vector system in bone marrow-derived macrophages

To investigate the application of regulated expression vectors towards an *ex vivo* cell therapy being developed in house by PhD student Nora Clarke, a collaborative experiment was performed whereby bone marrow-derived macrophages were transduced with single and dual vectors encoding GLux and expression induced by mifepristone. As opposed to the previous experiments in HEK293T cells where transgene expression was evaluated at one time-point (72 hours post-transfection/transduction), macrophages show reduced proliferative capacity after they are differentiated thus transgene expression could be assessed daily up to one week. Mifepristone was added to macrophages for 24 hours at two time points, 1 day and 5 days post-transduction to investigate the response following repeated mifepristone stimulation.

Figure 5.12A shows GLux expression measured over 6 days following transduction of macrophages with GS-1V-GLux RO MOI 10 and GS-2V-GLux/pSwitch at MOI 10:40 respectively. Leakiness was similar between cells transduced with single and dual vectors which increased marginally across the week. Similarly, macrophages transduced with positive control F/HN vector at MOI 10, demonstrated a small increase in daily GLux expression across the week. In contrast background levels detected in untransduced cells remained steady across the week. The single and dual vector systems demonstrated an induction in GLux expression following the initial mifepristone incubation (between day 1 and 2 post transduction) and the second stimulation (between day 5 and 6 post transduction) with the dual GS-2V-GLux/pSwitch system achieving comparable expression levels to rSIV.F/HN-GLux at day 2,3 and 6 post-transduction. The highest GLux expression achieved by the dual GS-2V-GLux/pSwitch vectors following mifepristone induction (day 2- 509711 ± 110760 RLU/mg protein, day 6- 1098547 ± 245913 RLU/mg protein, mean $\pm$ SD n=6 wells/condition) was 21x and 8x greater than GS-1V-GLux RO at day 2 and 6, respectively.

Figure 5.12B illustrates the fold-induction in GLux expression of mifepristone stimulated macrophages compared to unstimulated cells following transduction with single and dual

VSV-G vectors. The GS-2V-GLux/pSwitch vectors achieved peak induction of expression at day 2 (121.3 ± 21.2 fold-induction, mean $\pm$ SD n=6 wells/condition) with a gradual return to lower induction levels by day 5 (7.0 ± 1.8 fold-induction, mean $\pm$ SD n=6 wells/condition) followed by a second peak in induction following mifepristone stimulation at day 6 (115.1 ± 31.3 fold-induction, mean $\pm$ SD n=6 wells/condition). Levels of GLux expression after the first and second mifepristone induction were comparable. The kinetics of the GS-1V-GLux RO inducible system included a peak in mifepristone induced expression at day 3 post transduction (11.8 ± 5.1 fold-induction, mean $\pm$ SD n=6 wells/condition) with a fall in induction by day 5 (2.5 ± 0.9 fold-induction, mean $\pm$ SD n=6 wells/condition) and a second peak by day 6 after mifepristone stimulation (11.1 ± 3.3 fold-induction, mean $\pm$ SD n=6 wells/condition). As was the case for the dual vector system, peaks in induction were highly comparable between the first and second mifepristone stimulation of GS-1V-GLux RO transduced macrophages. Overall the two vector inducible system outperformed the single vector (~10x greater induction) when comparing mifepristone induced gene expression in macrophages.

5.3.1 Air liquid interface (ALI) cultures transduced with inducible VSV-G vectors demonstrate reproducible induction of transgene expression upon repeat mifepristone stimulation

Building upon data generated in macrophages where mifepristone-induced transgene expression was monitored for a week post transduction, human ALI cultures were utilised to follow transgene expression over greater durations (5 weeks post transduction) due to their ability to survive up to one year. ALIs were transduced with single VSV-G vectors at MOI 100 and with dual vectors at MOI 100:200 transgene:transactivator. Figure 5.13 A+B show GLux expression followed in ALI cultures (n=3 ALIs/condition) from day 7 to day 37 post-transduction with GS-1V-GLux RO and GS-2V-GLux/pSwitch VSV-G vectors. Measurement of GLux expression in washes taken from the apical surface of ALI cultures (Figure 5.13A) and in collections of basal media that submerge the basolateral membrane of ALI cultures (Figure 5.13B) were compared between single and dual vector transductions upon mifepristone induction. Overall, the initial observation is that GLux measured in apical washes and basal media shows a gradual decline in gene expression over time, across all transduction conditions.

The initial mifepristone induction, which lasted 48 hours, demonstrated the greatest induction in GLux expression (day 9 post-transduction) across the whole experiment. A consistent induction was generated by all three ALI cultures transduced with GS-1V-GLux (A1-3.2, A2-8.7 and A3-10.6 fold-induction apical wash, A1-2.8, A2-2.1 and A3-1.7 fold-induction basal media) whereas a more variable response was generated in ALI cultures transduced by GS-2V-GLux/pSwitch (A4-0.3, A5-8.5 and A6-0.9 fold-induction apical wash, A4-1.4, A5-8.4 and A6-2.6 fold-induction basal media). In fact, transduction of ALIs A6 and particularly A4 demonstrated low efficiency with detection of gene expression at background levels in basal media (Figure 5.13B) and expression below the single vector constructs in apical washes (Figure 5.13A). VCN determined in transduced ALIs by ddPCR (Table 5.1) shows that ALIs A4 and A6 had reduced VCN compared to A5 (A4- 0.36, A6- 1.05 vs A5- 1.65 WPRE copies/genome) which reflects the GLux expression data. Furthermore, VCN determined from ALIs transduced with single vector GS-1V-GLux reflected the GLux expression data, being largely matched between all three ALIs (A1- 0.27, A2-0.14, A3-0.46 WPRE copies/genome). At 20 days post-transduction, consecutive mifepristone induction generated modest increases in GLux expression which was greater in ALIs transduced with GS-1V-GLux RO (A1-3.0, A2-2.8 and A3-2.4 fold-induction apical wash) compared to dual vectors (A4-1.13, A5-2.0 and A6-1.4 fold-induction, apical washes). In summary, ALIs transduced with single vector constructs encoding GLux demonstrated a more consistent response to repeated mifepristone induction and transduction efficiency compared to dual vectors.

Assessing the performance of GMCSF regulated expression vectors in ALIs, a similar experimental design was performed whereby ALIs were transduced with GS-1V-GMCSF RO at MOI 100 and with GS-2V-GMCSF/pSwitch at MOI 100:200. Figure 5.13C shows GMCSF expression measured in apical washes of ALIs over 23 days. As demonstrated in ALIs transduced with GLux vectors, overall a decline in transgene expression over time was observed. Similarly, the first mifepristone induction of 48 hours resulted in the greatest induction of GMCSF expression with peak expression at day 7 post-transduction. ALIs transduced with single vectors demonstrated an overall higher induction ratio (B1-27.8, B2-166.9, B3-19.2) compared to dual vectors (B4-19.5, B5-28.0, B6-20.8), although peak GMCSF

expression was appreciably higher for all dual vector ALIs (B4-739.3, B5-265.0, B6-575.9 pg/ml) compared to single vectors (B1-27.8, B2-166.9, B3-19.3 pg/ml). Higher leakiness of dual vectors, an observation across the experiment duration, resulted in a reduced induction ratio after the first mifepristone stimulation compared to ALIs transduced with single vectors.

The second mifepristone induction at 21 days post-transduction resulted in no increase in GMCSF expression across ALIs transduced with GS-1V-GMCSF RO and only modest inductions of expression in dual vector ALIs (B4-1.6, B5-1.7, B6-2.2 fold-induction). VCN of ALIs transduced with GS-1V-GMCSF RO (Table 5.1) reflected the enhanced induction of transgene expression by ALI-B2 (B1-0.08, B2-0.26, B3-0.04 WPRE copies/genome). Similarly, enhanced GMCSF expression by ALI B4 and B6 is reflected by VCN calculated from ALIs transduced with dual vectors (B4-0.18, B5-0.03, B6-0.2 WPRE copies/genome). Overall, ALIs transduced with GS-2V-GMCSF/pSwitch generated greater peaks in GMCSF expression upon mifepristone stimulation, although accompanied by increased leakiness compared to the single vector system.

5.3.2 Inducible vectors show evidence of efficacy when delivered to mice

The final experiment included assessing inducible VSV-G vectors *in vivo*. Mice were delivered GS-1V-GLux RO or GS-2VGLux/pSwitch vectors by intranasal administration (n=3 mice/group) then after 7 days, mice induced with mifepristone delivered by intraperitoneal injection. Figure 5.14A demonstrates GLux expression measured in mouse serum sampled by tail venepuncture prior to mifepristone induction (T0) and two days after induction (T2). Small increases in serum GLux expression following mifepristone injection was observed by mice transduced with GS-1V-GLux RO (M1-2.8, M2-2.5 and M3-1.5 fold-induction) however not by mice transduced with dual vectors (M4-1.32, M5-1.1 and M6-1.0 fold-induction).

After 20 weeks, mice were again dosed with mifepristone then culled after 24 hours and GLux expression determined in serum (Figure 5.14B) and BALF (Figure 5.14C). Serum expression in mice transduced with GS-1V-GLux RO was 2.3x higher than control mice sampled at the same time and 2.2x higher in mice treated with dual vectors. Comparing serum GLux expression at endpoint sampling following mifepristone induction to the T0

expression levels, mice treated with single vectors demonstrate small increases in expression (M1-3.7, M2-3.1, M3-1.7 fold-induction) whilst mice treated with dual vectors showed minimal increases in expression (M4-1.4, M5-1.2, M6-1.1 fold-induction). In contrast, analysis of BALF extracted from mice showed 75.6x greater GLux expression in mice treated with GS-1V-GLux RO compared to control mice and 235.3x greater expression in mice treated with dual vectors compared to control mice. Although fold-induction in GLux expression following mifepristone stimulation is not possible to measure in BALF in the same animal, there is evidence that mifepristone induced transgene expression is detectable in BALF.

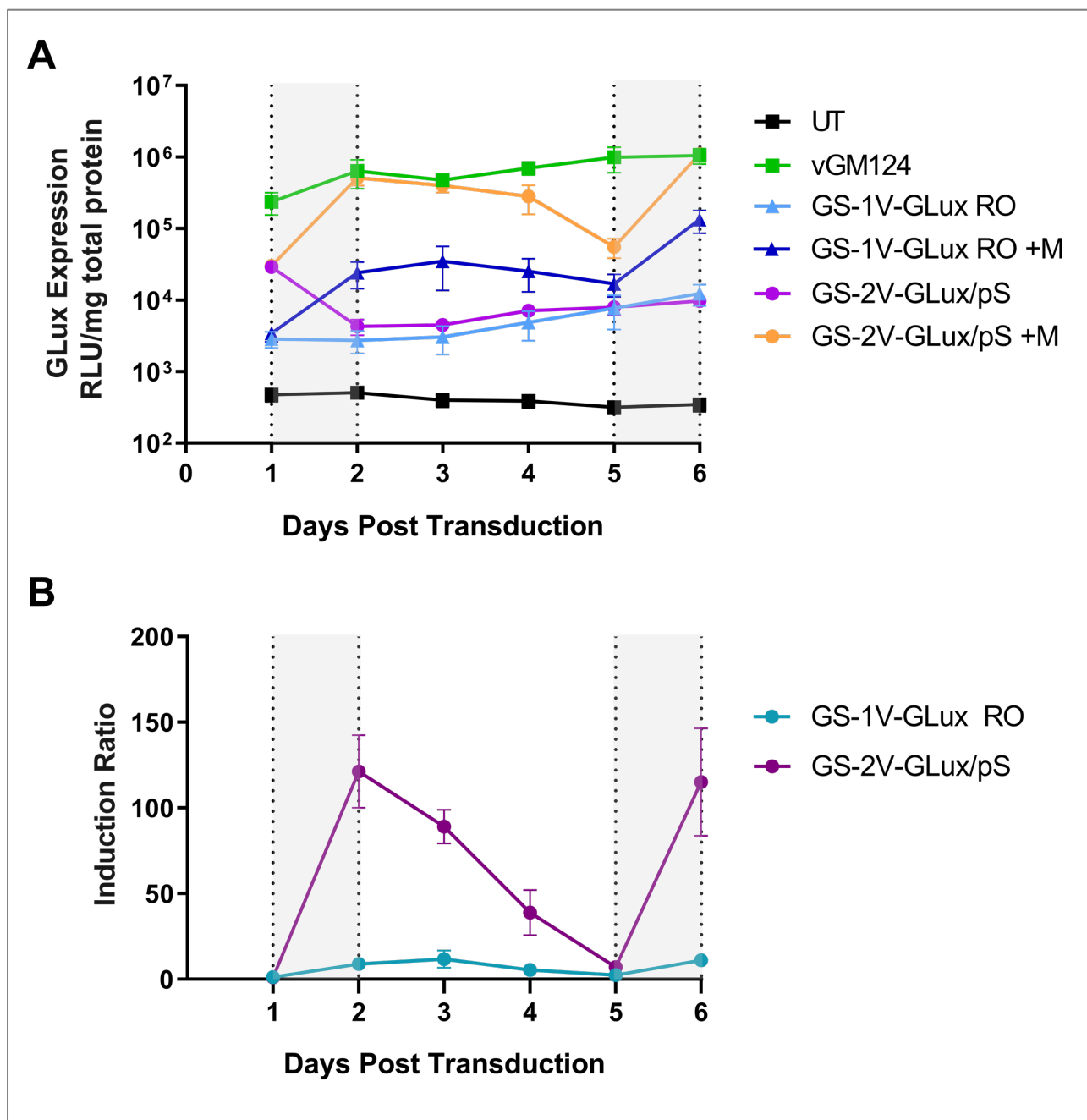


Figure 5.12- Mifepristone-induced transgene expression in bone marrow-derived macrophages.

VSV-G vectors were generated encoding transactivator and inducible Gaussia luciferase (GLux) transgene within a single vector (GS-1V-GLux RO) and within two separate vectors (GS-2V-GLux/pSwitch). Differentiated murine bone marrow-derived macrophages extracted from a single wildtype mouse were transduced with single (multiplicity of infection (MOI) 10) and dual VSV-G vectors (GS-2V-GLux:GS-2V-pSwitch MOI 10:40) and transgene expression induced with 10^8 M mifepristone added to cell culture medium for a 24 hour period (grey shaded region) at day 1 and 5 post-transduction. Media was collected daily and GLux expression quantified and normalised for total protein. A) GLux levels expressed as relative light units (RLU)/ mg total protein or B) difference in GLux expression (induction ratio) between uninduced and induced macrophages transduced with the same vector. GLux expression/calculated induction ratio shown for 6 days post-transduction. GLux expression following transfection of macrophages with positive control rSIV.F/HN-GLux vector (vGM124-GLux, MOI 10) and negative control untransduced macrophages (UT) also shown. Data presented as mean $\pm$ standard deviation, n=6 wells/condition, n=1 mouse.

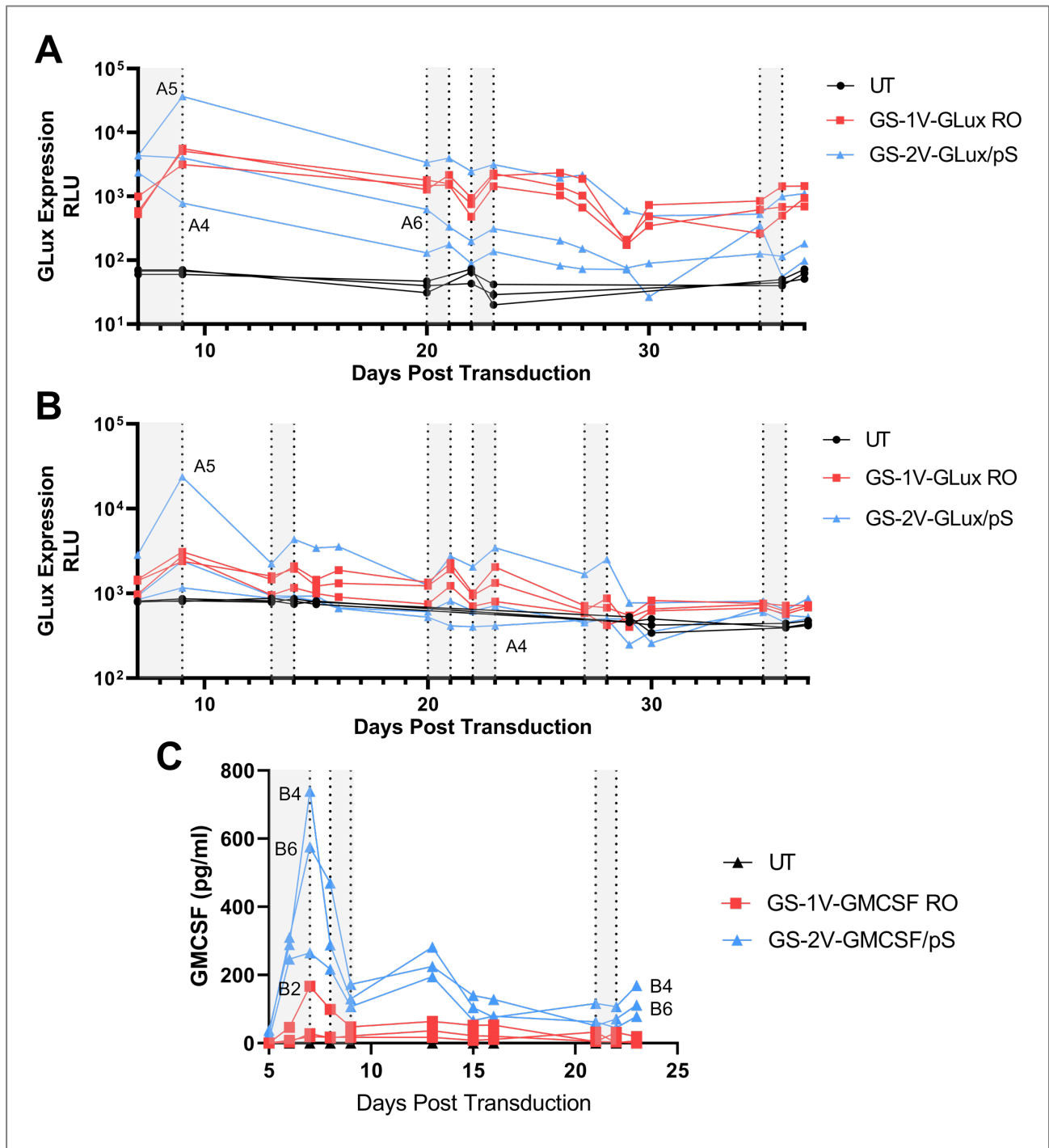


Figure 5.13- Monitoring of transgene expression following repeat mifepristone induction in air liquid interface (ALI) cultures transduced with inducible VSV-G vectors.

VSV-G vectors were generated encoding transactivator and inducible transgene within a single vector (GS-1V-GLux RO/GS-1V-GMCSF RO) and within two separate vectors (GS-2V-GLux/pSwitch and GS-2V-GMCSF/pSwitch). Fully-differentiated human air liquid interface (ALI) cultures were transduced with single (multiplicity of infection (MOI) 100) and dual VSV-G vectors (GS-2V-GLux/GMCSF:GS-2V-pSwitch MOI 100:200) and transgene expression induced with 10^8 M mifepristone for a 24/48 hour period (grey shaded region) at multiple times post-transduction. Collection of basal media or PBS +/- washes of the apical membrane was performed and A) GLux expression assessed in apical washes or B) basal media of ALIs transduced with single (n=3 ALIs, red points/lines) or dual GLux vectors (n=3 ALIs, blue points/lines), expressed as relative light units (RLU). C) Apical washes collected from ALIs transduced with single (n=3 ALIs, red points/lines) and dual GMCSF vectors (n=3 ALIs, blue points/lines) and GMCSF expression determined, expressed as GMCSF μ g/ml. Transgene expression in untransduced ALIs (UT, n=3 ALIs, black points and lines) also shown. Transgene expression over 23/37 days shown for each individual ALI.

Table 5.1- Vector copy number (VCN) determined by droplet digital PCR (ddPCR) of air liquid interface (ALI) cultures transduced with single and dual inducible vectors.

Vector copy number (VCN), untransduced (UT), non-template control (NTC).

Vector	ALI	VCN	Vector	ALI	VCN
GS-1V-GLux RO	A1	0.27	GS-1V-GMCSF RO	B1	0.08
	A2	0.14		B2	0.26
	A3	0.46		B3	0.04
GS-2V-GLux/pS	A4	0.36	GS-2V-GMCSF/pS	B4	0.18
	A5	1.65		B5	0.03
	A6	1.05		B6	0.2
UT	UT1	0	NTC	NTC1	0
	UT2	0		NTC2	0

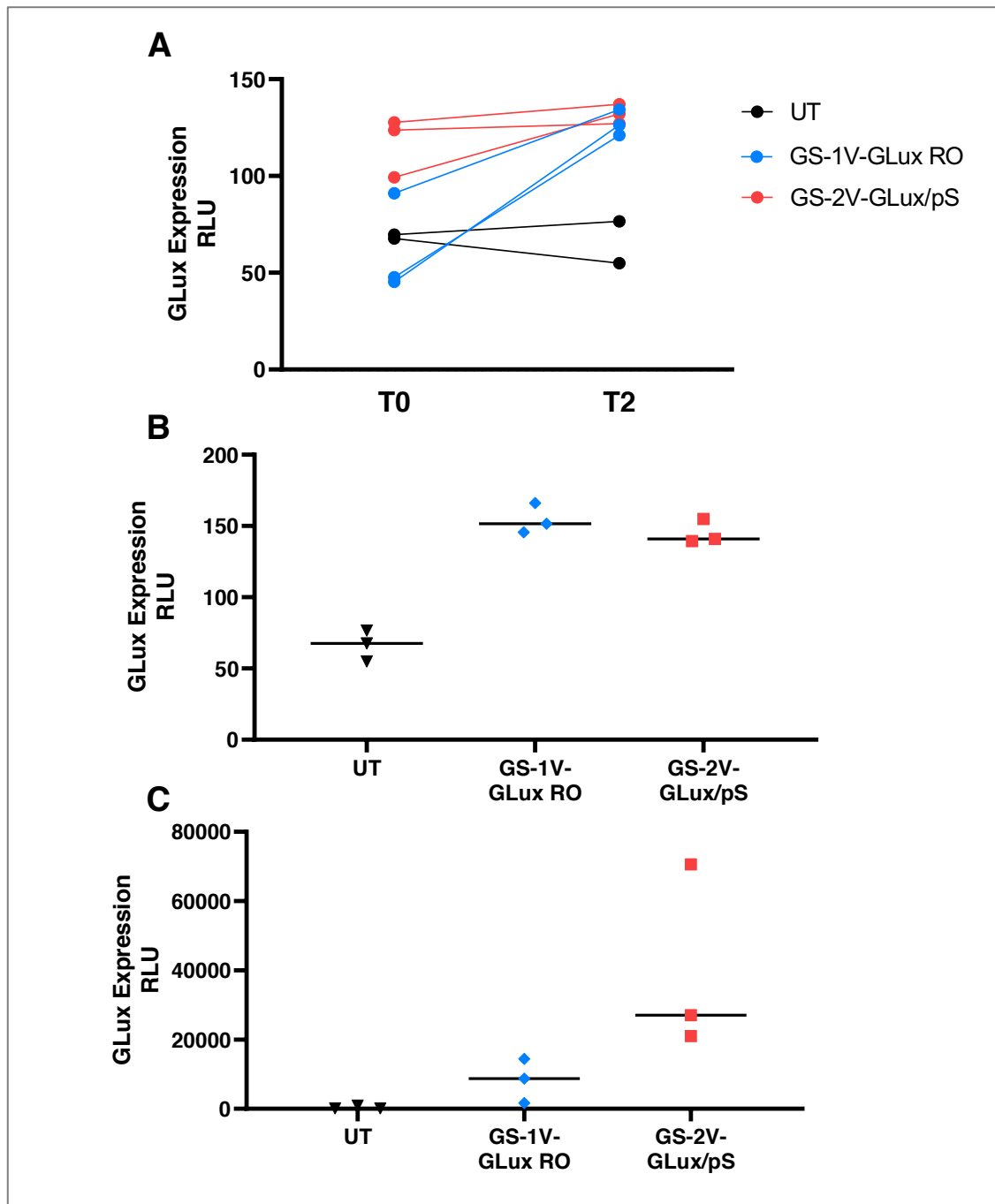


Figure 5.14- Assessment of inducible VSV-G vectors encoding Gaussia luciferase (GLux) *in vivo*.

Female wildtype mice (n=3 mice each for one vector (1V) and two vector (2V)) received maximum feasible dose (MFD) of GS-1V-GLux RO (2.03e8 TU/mouse) and dual vectors GS-2V-GLux (5.93e7 TU/mouse)/ GS-2V-pSwitch (1.2e8 TU/mouse) VSV-G vectors by intranasal administration. A) 1-week post-transduction, blood was sampled from mice by tail venepuncture then mice were induced with mifepristone delivered by intraperitoneal injection (400 µg/kg). 48 hours later blood was sampled again by tail venepuncture and GLux expression determined in serum before (T0) and 48 hours after (T2) mifepristone induction, expressed as relative light units (RLU). GLux expression for each mouse is shown at T0 and T2 joined by a line. Serum expression in untransduced mice also shown (UT black n=2). End-point sample collection from mice treated with single (blue diamond) and dual VSV-G (red squares) vectors took place 20 weeks post-dosing. 24 hours after intraperitoneal injection of mifepristone (400 µg/kg), blood and bronchoalveolar lavage fluid (BALF) were collected from mice and GLux expression measured in B) serum and C) BALF. GLux expression in untransduced mice shown (UT black, n=3). Horizontal bars are median values and each shape represents a single mouse.

5.4 Discussion

The UK Respiratory Gene Therapy Consortium developed a lentiviral vector, rSIV.F/HN, which achieves efficient gene transfer to the lungs and generation of secreted proteins. However, not all target diseases require constitutive expression of the therapeutic transgene and diseases such as PAP may only require short, intermittent bursts of gene expression. To broaden the application of the rSIV.F/HN vector platform for wider disease indications and to enhance the efficacy/toxicity window of GTAs we aimed to generate a lentiviral vector carrying a regulatable expression cassette, which will initiate gene expression following administration of a drug. In pursuit of a 'gold standard' commercial system, GeneSwitch (Thermo Fisher Scientific) was identified consisting of a mifepristone-inducible expression system modified from the human progesterone receptor. The GeneSwitch product could be adapted according to the experimental setting, for example easy insertion of alternative transgenes (271). Developing a regulated expression system based upon a commercial system would establish proof-of-concept that temporal control of gene expression could be achieved in a lentiviral gene therapy context.

To first investigate the GeneSwitch system in its original configuration, the control expression plasmid containing the *LacZ* gene was co-transfected with the transactivator encoded by the pSwitch plasmid. Low levels of leakiness and clear induction of β -galactosidase expression were evident upon mifepristone induction, however the limited ability to easily quantify gene expression using *LacZ* led to the decision to consider alternative report transgenes which could be quantified less subjectively. GLux was selected as a suitable candidate given it is secreted, easily quantifiable and expression can be detected over a large dynamic range (refer to Chapter 3 for further advantages). It was particularly important that a secreted reporter protein was encoded in expression cassettes as the disease applications that regulated lentiviral expression may be most beneficial for involve secreted proteins, such as GMCSF in PAP. In addition, clearance of proteins once they are secreted from cells allows for the therapy to be "turned off" upon withdrawal of mifepristone, which is not possible if membrane-bound proteins such as CFTR were encoded.

In the first configurations, transactivator and inducible promoter were directly cloned from the original pGene and pSwitch GeneSwitch plasmids and incorporated into the pGM358-hCEF-GLux SIV lentiviral backbone with the *LacZ* gene replaced by *GLux*. The two plasmid configuration, denoted as GS-2V-GLux and GS-2V-pSwitch was expected to perform optimally given this configuration was developed for the commercial system. In addition to a regulated expression system that is split across two vectors, the aim was to fit all components necessary for inducible transgene expression within a single lentiviral vector as this will be more desirable for use in man. The reasons that a single vector is advantageous over a dual vector system are numerous, including the cost associated with the manufacturing of two separate viral vectors, the increased immunogenicity associated with delivering twice the quantity of viral particles and significantly the requirement for both vectors to transduce the same target cell in order for the transgene expression to be regulated. The latter consideration is a major limitation of dual regulated vectors and is similarly faced by other fields within gene therapy.

The limit of transgene size that can be efficiently incorporated into a single AAV vector (4.7 kb including promoter, transgene and polyA sequence) largely restricts their application towards disease indications with transgenes over ~3.5 kb (286). Thus the AAV field have investigated various strategies to expand the packing capacity of AAV vectors, including the use of dual vectors whereby the transgene and cis-acting sequences are split across two separate vectors. Upon co-transduction of the same target cell, viral genomes are reconstituted using either the overlapping, cis-activation or trans-splicing techniques (287). Dual AAV vectors have shown modest, but therapeutically relevant levels of transgene expression in pre-clinical models. Chao et al demonstrated 4% human FVIII expression levels four months post-transduction using trans-splicing AAV2 vectors, whilst others have investigated dual AAV vectors encoding mini-dystrophin for the treatment of Duchenne muscular dystrophy and other applications (288–290). Although the use of dual vectors may potentially be efficacious when delivered to confined area such as the retina, delivery to larger organs such as the airways or systemic delivery are far less likely to result in co-transduction in the same target cell (291). Thus the development of a regulated expression cassette encoded by a single lentiviral vector was pursued in the present project.

A single inducible vector based upon the GeneSwitch system was constructed into the SIV pGM358-hCEF-GLux lentiviral backbone. The first configuration included the GAL4 transactivator and polyA sequence downstream of the hCEF promoter, followed by the inducible promoter containing GAL4 binding sites and TATA box upstream of the GLux transgene (GS-1V-GLux). Based upon this conformation, a humanised version was created in collaboration with Professor Mark Isalan from Imperial College London, by exchanging the yeast GAL4 DNA binding domain of the transactivator and GAL4 binding sites with ZF BA' and ZF target sequences, respectively. Reynolds *et al.* (2003) originally engineered the ZF BA' to recognise and bind sites within the HIV-1 integrated pro-viral sequence located in the HIV-1 5'LTR. The 3-finger ZF A' and ZF B were fused together by peptide linker TGGSGERP to create six-finger ZF BA' which demonstrated highly efficient repression of LTR promoter activity when attached to a KOX-1 repression domain (284). The GAL4 element were swapped for a ZF as the immunogenicity against the expression of transactivators containing sequences of yeast origin was anticipated to be stronger than ZFs which are prevalent transcription factors in humans.

The inclusion of ZFs within inducible expression systems is not novel. Rivera *et al.* (1996) first describe the rapamycin-inducible system developed by Ariad Therapeutics which includes fusion protein ZFHD1, designed by Pomerantz *et al.* (1995) (262,292). ZFHD1 acts as a transcription factor within the inducible system, binding complementary binding sites within target genes and successfully achieving temporal control of gene expression following rapamycin-induction in many applications (293–296). Beerli *et al.* (2000) first described the fusion of engineered ZFs to the truncated progesterone receptor and p65 domain, in replacement of GAL4 DNA binding domain, generating a 'humanised' mifepristone-inducible ZF transcriptional regulator (297). Poutou *et al.* (2017) later incorporated the ZFHD1 domain into the mifepristone-inducible system demonstrating sustained response to repeated mifepristone induction over 62 days following delivery of constructs encoded by a single AdV to mice (298). Similarly other groups have incorporated various ZF designs into the mifepristone-inducible system, benefitting from the modular nature of the transactivator complex by swapping yeast GAL4 sequences for 'humanised' ZFs (297,299).

Following this strategy, the present study generated a humanised mifepristone-inducible system by incorporating ZF BA' into the transactivator complex and including complementary binding sites upstream from the reporter transgene, *GLux*. Encoded within a single plasmid, ZF-1V-GLux and AI ZF-1V-GLux were generated with the constitutive, high expression promoter hCEF and autoregulated Tk minimal promoter driving expression of the ZF transactivator, respectively. Induction of GLux expression following mifepristone stimulation was investigated *in vitro* and performance compared to GeneSwitch-based single plasmid construct, GS-1V-GLux. Although matching leakiness was measured across all configurations, the GAL4-containing construct demonstrated the greatest induction ratio, followed by the ZF transactivator expressed constitutively from the hCEF promoter followed by the auto-inducible promoter. Although little difference was observed between promoters of the ZF systems, the constitutive hCEF promoter was pursued only following data presented by Poutou *et al.* (2017) which described a failure of auto-inducible ZF transactivators when constructs were investigated *in vivo* (298).

The ZF and commercial-based single plasmid constructs were investigated further by assessing induction of transgene expression at increasing concentrations of transfection. Whereas GS-1V-GLux demonstrated greater transgene induction with increasing concentrations of transfection, the ZF construct performed optimally at the lowest tested concentration (0.1 µg/ml plasmid DNA). A lack of dose response might indicate that maximal expression of GLux was reached through basal expression of ZF-1V-GLux without mifepristone, thus no induction of expression could be detected. Similarly, when the dual plasmid constructs were investigated (GS-2V-GLux/pSwitch) a saturation in signal from leaky transgene expression was observed even at low transfection concentrations (0.1 and 0.2 µg/ml plasmid DNA). Interestingly, GLux expression measured from cells transfected with GS-2V-GLux alone was lower than basal expression from cells transfected with both GS-2V-GLux and GS-2V-pSwitch. This may indicate that the transactivator complex was attaching to binding sites upstream of GLux and inducing transcription even without mifepristone present, perhaps due to extremely high expression levels of the transactivator. The single plasmid construct however, demonstrated lower leakiness compared to the dual plasmid system and consequently a significant induction ratio observed at the highest transfection concentration (37 fold-induction).

The cause of leaky transgene expression was likely the strong CMV promoter/enhancer upstream of the expression cassettes that is present in the pGM358-hCEF-GLux backbone to drive expression of the viral genome for vector production. This was exemplified when reverse orientation plasmids were generated. Basal GLux expression was significantly reduced in cell transduced with ZF-1V-GLux RO compared to ZF-1V-GLux due to the transgene being encoded in the reverse orientation. Changing the direction of the transgene ORF prevented expression of the transgene from the distant CMV promoter/enhancer in the pGM358-hCEF-GLux backbone, thus expression was solely driven by the inducible promoter, significantly reducing leakiness. In contrast the GS-1V-GLux RO was constructed in a different way, with the GLux ORF in sense orientation and the transactivator encoded in the reverse direction. Consequently, an enhancement of leakiness was observed by GS-1V-GLux RO compared to the GS-1V-GLux plasmid, despite the induction ratio being improved. Given the large influence that promoters within the pGM358-hCEF-GLux backbone can have, lentiviral vectors encoding the reverse orientation expression cassettes were generated for further investigation as only sequences between the 5' and 3' LTRs are packaged into virions as the viral genome.

Reiser *et al.* (2000) evaluated the incorporation of two transcription units within a single HIV-1 vector with transgene expression driven separately by an internal promoter and through activation of the 5'LTR (300). The *tat* gene was incorporated into the viral genome so that *tat* could bind to transactivation-response elements (TAR) in the 5'LTR activating transcription of the second reporter gene. However the *tat* gene was removed in third generation vectors to reduce their ability to activate neighbouring genes upon genome insertion in host cells (8). Therefore, transcriptional activity of LTRs was lost and transgene expression became dependent on internal promoters, making this strategy unsuitable for third generation vectors as used in the present study. Reiser *et al.* (2000) explored an alternative approach to co-express two transgenes from the same HIV-1 based vector, which utilised an internal ribosome entry site (IRES) between the two cDNA sequences (300). Expression of two transcriptional units linked by an IRES from a single promoter, referred to as bicistronic vectors, was shown to be efficacious with 74% cells expression both transgenes *in vitro* as determined by FACs.

However given the different requirements of the promoter driving transactivator expression and inducible promoter driving transgene expression, using a single promoter would not be adequate in the present study. Furthermore, others have reported the limitations associated with the use of IRES elements, namely the influence of cistron arrangement on mRNA expression in bicistronic constructs (301). An alternative approach investigated by Amendola *et al.* (2005) was the inclusion of bidirectional promoters within lentiviral vectors which drive transcriptional activity in opposite directions (302). Of comparable design to the reverse orientation constructs investigated in the present study, the first transcription unit including transgene and polyA sequence was encoded in the antisense orientation, with a bidirectional promoter in the middle followed by the second transgene and WPRE element in sense orientation. Specifically, the bidirectional promoter consisted of a section of the highly efficient ubiquitin C promoter joined to the minimal CMV promoter encoded in the opposite direction (302). The highly efficient promoter enhanced transcription from the minimal promoter with equal gene expression observed when the reporter gene was inserted in the sense or antisense orientation.

Although not investigated by Amendola *et al.* (2005), it is conceivable that two transgenes could be expressed at different efficiencies by perhaps using weaker promoters with minimal enhancer activity on neighbouring sequences or incorporating greater distances between the two promoters. Poutou *et al.* (2017) investigated multiple configurations of ZF and GAL4-based regulated expression cassettes, with transactivator and inducible gene incorporated into a single construct (298). Constructs encoding the transcription unit Tk promoter-ZF transactivator-polyA in antisense orientation and 12x ZF binding sites-interleukin-2 minimal promoter-transgene-polyA in sense orientation demonstrated low basal expression and high induction *in vitro*. When assessed *in vivo* however, configurations with both transcriptional units arranged in sense orientation and with two separate promoters demonstrated much greater efficacy compared to reverse orientation constructs (298). Similarly, Wang *et al.* (2004) describe the generation of Adenoviral vectors encoding the GAL4-mifepristone system within a single vector encoding the transactivator and regulated gene ORFs in consecutive nature (278). Although suitable for viral vectors with a DNA genome, such as Adenovirus, RNA viruses require the vector genome to be transcribed

into mRNA before incorporation into vector particles. Thus inclusion of a polyA sequence in sense orientation between the LTR sequences will prevent generation of full-length transcripts that can be packaged.

The generation of GS-1V-GLux and GS-1V-GMCSF reverse orientation constructs permitted the production of lentiviral vectors with considerable titres ($>1e9$ TU/ml). Despite initially constructing the expression cassettes within the SIV backbone for downstream production of rSIV.F/HN vectors, the cloning of constructs into a HIV-1 backbone and generation of VSV-G vectors was outsourced to VectorBuilder for time and convenience reasons. In addition to single vector constructs encoding transgenes GLux and GMCSF, dual vector designs were included in further analysis as this was considered the 'gold-standard' design derived from the GeneSwitch two plasmid system.

In vitro analysis of inducible VSV-G vectors demonstrated much greater efficacy than the non-viral plasmid constructs. As described earlier, the promoters within the SIV backbone interfered with inducible transgene expression and thus an overestimation of leakiness likely occurred, consequently reducing the induction ratio. However the difference in performance when expression was assessed in a plasmid compared to vector form highlights the importance of assessing inducible designs in the intended downstream format and model as early as possible. Further considerations associated with evaluating inducible systems *in vitro* include the considerably high number of plasmids that enter cells upon PEI transfection. Cohen *et al.* (2009) reported between 75-50000 plasmid copies/nucleus depending on the plasmid dose, following transfection of A549 cells using PEI (303). Thus high leaky expression without mifepristone induction was anticipated given the plasmid copy number per cell. In contrast, transduction of cell lines using viral vectors often achieves less than one integration per cell at low MOIs (304). Transductions using an MOI of 1 theoretically delivers one vector per cell however in reality the number of successful integrations is less than one per genome.

Thus in the present study, inducible vectors were investigated over a range of MOIs (0.1-10.0) and dose-dependant changes in induction ratio observed. Similar induction ratios were observed following transduction of the two vector system in a 1:1 ratio at MOI 1 and

10, yet an increase in induction ratio was observed with increasing MOIs for the one vector construct. This observation could be the result of the ratio of GS-2V-GLux to GS-2V-pSwitch vector used, which would affect both leakiness and levels of mifepristone-induced expression. To investigate the influence of transgene: transactivator ratio further, different proportions of vector were used to transduce cells and GLux expression determined. In contrast to the recommended ratio of 4:1 transgene: transactivator given by the GeneSwitch protocol, transduction of cells with excess of the transactivator was by far the most efficacious ratio (~1000 fold-induction) followed by even ratios of each vector. To narrow down the level of excess transactivator that is beneficial for mifepristone induction, a 1:2 GS-2V-GMCSF:GS-2V-pSwitch ratio achieved ~1300x induction in GMCSF expression, which was taken forward as the optimal ratio for future experiments. Investigation of a wider range of transgene to transactivator ratios could be conducted to further identify the optimal proportion of vectors *in vitro*.

Assessment of regulated expression cassettes had up to this point been in immortal cell lines, which although are convenient for the initial screening of constructs, they are not truly representative of the end application which is generation of a product suitable for use in man. In addition, the optimisation of constructs based upon data gained in one cell line may not reflect the results achieved in another, as evidenced by Pollock *et al.* (2000), Wang *et al.* (2004) and Poutou *et al.* (2017) (278,298,305). Therefore the present study aimed to investigate the inducible system in multiple models, including bone marrow-derived macrophages. The use of macrophages as an *ex vivo* lung cell therapy is being studied by PhD student Nora Clarke, thus working in collaboration the aim was to generate mifepristone-inducible macrophages for transplantation into the lungs. As proof-of-concept, macrophages differentiated from murine bone marrow were transduced with single and dual vectors and GLux expression monitored over 6 days. Macrophages cultured *in vitro* have minimal proliferative activity after being differentiated, thus can be cultured for extended periods (up to one week) allowing transgene expression to be monitored over time (306).

Reproducing the results determined in HEK293T cells, dual vectors demonstrated superior induction of GLux expression in response to mifepristone (>100 fold-induction) compared to

the single vector. Importantly equal activation of transgene expression was observed following the second mifepristone induction, suggesting transduced cells remain responsive to mifepristone upon successive stimulations. Further, gene expression was 'turned off' in-between activations, with baseline GLux expression achieved 72 hours after withdrawal of mifepristone. As an *ex vivo* cell therapy, macrophages would be transduced prior to transplantation, therefore the requirement for a single inducible vector is somewhat alleviated. In a similar approach, Hjelm *et al.* (2016) investigated transduction of neuronal progenitor cell (NPCs) using the GeneSwitch system cloned into two lentiviral vectors (279). Inclusion of selectable markers attached by an IRES sequence downstream of the transgene/transactivator allowed for initial screening of transactivator positive cells, transduction of this sub-population with the second lentiviral vector encoding the inducible transgene, and subsequent screening and expansion of double-positive cells. Transduced cells were then transplanted into the rat striatum and successfully activated *in vivo* by mifepristone stimulation (279). The proportion of macrophages expressing both transactivator and transgene was not determined in the present study, however inclusion of a selectable marker could be considered to further increase the efficacy of the dual vector macrophage system, ensuring the selectable marker is pharmacopoeia compliant.

To next generate proof-of-concept in a human airway model, ALI cultures were transduced with inducible vectors. ALI cultures are primary human bronchial epithelial cells (HBECs) derived from a single healthy donor cultured at the air-liquid interface (ALI cultures). HBECs cultured in this way present many characteristics that resemble the *in vivo* respiratory epithelium, including mucus production and mucociliary clearance, pseudostratified morphology consisting of a heterogeneous population of cells and the formation of tight junctions that generate epithelial barrier properties (307,308). Single and dual VSV-G vectors encoding GLux and GMCSF were delivered to ALI cultures via the apical surface and transgene expression monitored in washes of the apical surface and in basal media over a period of weeks.

Interestingly the results generated with vectors encoding GLux and GMCSF were different. ALIs transduced with GS-1V-GLux RO vectors demonstrated detectable leakiness and only moderate activation following mifepristone induction however performance was highly

consistent across all three ALIs. In contrast, ALIs transduced with the dual GS-2V-GLux/pSwitch demonstrated great variability in performance with only one ALI showing induced expression after stimulation, reflected in the molecular analysis of VCN. In contrast, ALI cultures transduced with dual GMCSF vectors achieved demonstrably higher GMCSF expression than single vectors, although induction ratios were overall higher for single vectors due to the enhanced performance of one ALI culture (167x induction) and lower leaky expression. ALIs within each transduction condition showed largely consistent expression profiles over time. Overall, transduction of ALIs with inducible vectors encoding GMCSF were more efficacious than GLux, however a general decrease in expression with time was evident, likely caused by loss of positively-transduced cells due to frequent apical washes. Higher leakiness observed following transduction of ALIs with vectors encoding GLux may well be an artefact of the assay, measuring enzymatic activity against a substrate rather than antigen levels by ELISA. Interestingly this observation was made in Chapter 4 when comparing the FRET functional assay to ADAMTS13 ELISA.

Transduction efficiency was extremely low in this experiment, with VCN at the limit of detection in some ALI cultures despite an MOI of 100 or 300 being used for single and dual vectors, respectively. Given that the efficacy of the dual vector system relies upon co-transduction of transactivator and transgene into the same cell and given the overall low transduction efficiency, the mixed performance of the dual vector system could be explained by these observations. Similar to challenges faced following *in vivo* delivery of VSV-G vectors, the location of receptors are on the basolateral membrane of airway epithelial cells and thus are not easily accessible by application of vector to the apical surface of ALIs without permeabilization (71,309). Use of agents such as sodium caprate, ethyleneglycol-bis-(β -aminoethyl ether)-N,N'-tetraacetic acid (EGTA) and lysophosphatidylcholine (LPC) have been utilised successfully to enhance AdV transduction of ALIs and thus may represent a strategy moving forward if VSV-G vectors are pursued (310–312). Alternatively, incorporation of inducible constructs into F/HN pseudotyped vectors may permit more thorough investigation of expression dynamics. Griesenbach *et al.* (2012) showed persistent expression of firefly luciferase in ALI cultures for 100 days following rSIV.F/HN transduction, with significant expression levels observed at MOI 250 and detectable levels above background observed at MOI 25 (98). Due to the presence of

sialic acid receptors on the apical membrane of airway epithelial cells, application of rSIV.F/HN vectors to ALI cultures does not require pre-conditioning. Thus future assessment of inducible vectors within ALI cultures could be improved by encoding expression constructs within F/HN vectors.

Moving beyond *in vitro* transduction, inducible VSV-G vectors were investigated *in vivo*, representing the most challenging model of the project. Although inefficient to deliver VSV-G vectors to the murine lungs without conditioning to damage the epithelial barrier, inducible vectors were nonetheless delivered by intranasal administration to wildtype mice (313). Initial delivery of mifepristone by intraperitoneal injection lead to a modest (median 2.5x induction) yet detectable increase in serum GLux expression in mice transduced with GS-1V-GLux RO however not by the dual GS-2V-GLux/pSwitch vectors. After 20 weeks, enhanced serum GLux expression was detected in mice treated with single and dual vectors (~3x higher than control mice). Comparative to serum levels of the same mice prior to initial mifepristone induction, endpoint serum levels were three-fold greater in mice delivered GS-1V-GLux RO and only marginally greater in mice delivered the dual vectors. In BALF significant levels of GLux expression were observed in treated mice, with greater expression observed in mice transduced with dual inducible vectors. Without control unstimulated mice it was not possible to determine basal BALF expression, yet evidence of significant expression above background may indicate efficacy of the inducible system in mice. According to serum data, where leaky expression could be measured in the same mouse, the single vector construct was more efficacious with lower basal expression. Due to the aforementioned advantages associated with encoding regulated expression cassettes within a single lentiviral vector, positive serum data in mice treated with GS-1V-GLux RO was a promising finding. Overall, proof-of-concept showing regulation of lentiviral-mediated expression in mice using a sub-optimal GTA for the lungs was generated. Experiments to establish the optimal ratio of transgene to transactivator in the two vector system could be further investigated to expand upon the preliminary data shown here with a 1:2 ratio.

One envisaged application of a lentiviral vector with regulated expression was for therapies that have a narrow therapeutic window, such as the treatment of PAP. rSIV.F/HN vector encoding GMCSF achieved substantial lung expression and correction of disease phenotype

following intranasal delivery to GMCSF KO mice (Lund-Palau, Molecular Therapy-manuscript under review). However, at approximately 9 months post-transduction, evidence of toxicity in treated mice was observed. Upon histopathological examination, long-term GMCSF expression was found to drive excessive inflammation and tissue damage by the accumulation of alveolar macrophages and neutrophils which caused oedema. Therefore, regulation of rSIV.F/HN-mediated GMCSF expression may provide a solution to the toxicity observed following constitutive GMCSF expression in mice. A similar application is mifepristone-regulated expression of human IL-12 as an anti-tumour treatment for liver cancer. IL-12 has therapeutic potential as a key mediator of cell mediated immunity, however high doses of recombinant IL-12 delivered to humans in a Phase 2 clinical trial caused severe toxicity and death in two patients, highlighting the narrow therapeutic window of IL-12 treatment (314). Thus mifepristone-regulated expression of IL-12 by AdV has been explored by multiple groups for the treatment of liver tumours, either alone or in combination with chemotherapy (278,298,315,316). Intraperitoneal injection of mifepristone was performed at doses between 250-4000 µg/kg, compared to 400 µg/kg delivered in the present study.

Both Gonzalez-Aparicio *et al.* (2011) and Reboredo *et al.* (2008) described the requirement of a dose-escalation of mifepristone to compensate for the gradual silencing of GAL4-based systems 90 days after transduction (315,316). In contrast, maintained responsivity to the same mifepristone dose 20 weeks post-transduction was found in the present study utilising VSV-G lentiviral vectors. The therapeutic dose that exerts abortive effects in women is considered 10 mg/kg, thus the dose found efficacious in the present study is 25 fold lower than the progesterone antagonistic effect (317,318). For the translation of mifepristone-regulated systems into human, efficacy at a sub-threshold dose of mifepristone is imperative to alleviate concerns over side-effects. Ideally a dose-escalation study would be performed to identify the minimum dose of mifepristone that can induce sufficient levels of gene expression for the intended application. It is anticipated that mifepristone induction of rSIV.F/HN-mediated expression in the lungs would be more efficient compared to proof-of-concept data generated with VSV-G vectors. Greater transduction efficiency achieved by F/HN pseudotyped vectors may consequently result in regulated gene expression at lower

doses of mifepristone, which would further improve the safety profile when translating these experimental therapies to humans.

A further strategy to reduce the immunogenicity of regulated expression cassettes was to humanise the construct by including a ZF DNA binding domain. Although initial data was generated with a single plasmid ZF construct, the limited evidence of induced expression led to a decision to pursue the GeneSwitch-based design which was demonstrating more favourable induction. However as was illustrated by the two-plasmid system which showed high leakiness and negligible induction initially, assessment of the dual vector system upon generation of VSV-G vector demonstrated highly efficient induction of gene expression with low levels of leakiness. Thus generation of lentiviral vectors encoding the ZF system may still hold potential if the development of inducible vectors is pursued further.

A major limitation associated with the mifepristone system and other ligand-inducible gene expression systems, is the co-expression of engineered transcription factors in host cells. The production of transactivator complexes may be immunogenic, even if derived from human sequences, whilst their large cDNA size can assert a metabolic burden onto host cells or initiate difficulties in vector packaging (261). An alternative strategy to engineered transcription factors that regulate gene expression by binding to DNA sequences, is employing the diverse RNA regulatory mechanisms that are widespread in bacteria such as riboswitches. Riboswitches are short cis-elements (<100 nucleotides) within the untranslated regions (UTRs) of bacterial mRNA that through the formation of tertiary structures can respond to metabolites or various cellular ligands to regulate expression of the protein coding region within the transcript (319). Kumar, An and Yokobayashi (2009) first described riboswitch inducible expression systems utilised for temporal regulation of RNA interference in mammalian cells (320). Since then, a range of RNA regulatory systems have been harnessed to generate synthetic ligand-inducible riboswitches with diverse functions, including mRNA degradation, exon-skipping and ribosome frameshifting (321). Although RNA-regulatory mechanisms for gene expression have some clear advantages, synthetic riboswitches have thus far showed inferior expression dynamics compared to other gene expression systems (322). Particularly, their incorporation into AAV vectors showed limited induction of expression *in vivo* (323,324). Thus, the mifepristone-inducible

expression systems represent a favourable candidate for incorporation into lentiviral vectors and the continued development towards human application.

5.5 Conclusion

The aim of this chapter was to generate a lentiviral vector carrying a regulatable expression cassette in which gene expression could be ‘turned on’ following administration of a small molecule drug and ‘turned off’ when the drug is withdrawn. The ability to regulate lentiviral-mediated expression will broaden the application of the rSIV.F/HN vector platform for wider disease indications, particularly for the delivery of therapeutic genes with a narrow efficacy/toxicity window. Proof-of-concept was demonstrated in a range of *in vitro* and *in vivo* models with single and dual VSV-G vectors carrying regulated expression cassettes based upon a commercial mifepristone system. The superior induction of gene expression by dual vectors *in vitro* may suggest their suitability for *ex vivo* cell therapy approaches. Alternatively, single vectors showed greater efficacy *in vivo*, although only modest increases in gene expression were observed.

The design and construction of a humanised expression cassette within the lentiviral backbone was performed, however limited induction of gene expression was measured on a plasmid level. The disparity between the performance of regulated expression constructs encoded by a plasmid compared to a viral vector, highlights the importance of investigating novel constructs in the intended form as quickly as possible, albeit costly for the generation of viral vectors. The importance of developing drug-inducible regulated expression systems suitable for use in humans is paramount for enhancing the safety of GTAs. The data generated in this chapter, although based upon the commercial mifepristone system, will be valuable towards the development of a humanised drug-inducible expression system given the modular nature of engineered transcription factors, holding potential for development in the future.

Chapter 6- Conclusions and Future Directions

In this thesis, different strategies have been explored to expand the rSIV.F/HN vector platform for wider therapeutic indications. Specifically, I have investigated intravenous vector delivery and benchmarked expression against intranasal vector administration, the canonical route of rSIV.F/HN delivery. I have investigated rSIV.F/HN-mediated restoration of ADAMTS13 expression in deficient mice for the treatment of systemic disease, TTP. Finally, I have generated proof-of-concept that temporal control of lentiviral-mediated gene expression can be achieved, demonstrating efficacy of a commercial based system in various *in vitro* and *in vivo* models.

Given the above findings, I believe the project aim to investigate wider applications of the rSIV.F/HN vector platform was achieved and important conclusions generated. A prominent finding was that despite systemic vector delivery achieving sustained gene expression, the immunogenicity that prevents reapplication and safety concerns associated with widespread vector biodistribution highlights intranasal administration as a superior route of delivery, even potentially for treatment of systemic disease. The significant levels of reporter protein generated in serum by intranasal administration supports previous data shown by Paul-Smith *et al.* (2018) that the murine lungs act efficiently as factories for the secretion of proteins into the circulation. The surprising finding was that detectable serum expression was achieved at lower doses following delivery of vector to the lungs compared to systemic administration. Thus rSIV.F/HN administration to a restricted localised area may be more efficient than the dilution of vector across multiple organs, which considering the extremely high cost of vector could be economically beneficial when translating therapies into humans.

To generate data with a therapeutic transgene, systemic and intranasal delivery routes were investigated for the treatment of systemic disease, TTP. Overall the results generated were inconclusive, with further data required before the rSIV.F/HN vector can be decisively ruled in or out as a potential therapy for TTP. The major challenges faced during this project was the lack of assays available for detection of murine ADAMTS13 expression and limited readouts of efficacy in the TTP animal model due to the unsuccessful attempts to generate

TTP-like symptoms in deficient mice. Given the sources of VWF available during this project, the required dose of ultra-large VWF multimers to trigger thrombocytopaenia was not reached. In order to progress with the B/129 ADAMTS13 knockout mouse model, higher doses of rhVWF must be obtained (>2 VWF:RCo U/g as established by Muchitsch *et al.* (2010)) to induce thrombocytopaenia (171). Alternatively, knockout mice on the CASA/Rk genetic background could be purchased for future studies although the severity of this mouse model is not permitted on our current project licence (167).

Administration of a single dose of rSIV.F/HN vector encoding mADAMTS13 failed to generate meaningful levels of ADAMTS13 activity in plasma. Daily vector administrations generated a ~4-fold increase in plasma ADAMTS13 activity compared to a single dose, however plasma levels remained modest (~10% wildtype levels). A potentially straightforward way to boost plasma ADAMTS13 levels is incorporating the MDTCS and M5 ADAMTS13 variants into lentiviral vectors, which exhibit enhanced protease activity compared to wildtype ADAMTS13 (186,226,227). Gene transfer of the MDTCS variant to knockout mice by Jin *et al.* (2013) and Niiya *et al.* (2009) achieved superior plasma ADAMTS13 activity compared to full-length ADAMTS13, which could represent a viable solution to the limited expression levels observed in the present study (172,174). The M5 mutant furthermore demonstrates resistance to autoantibody inhibition by acquired TTP patient plasma and thus could broaden the application of a gene therapy approach to both congenital and acquired TTP (227).

Difficulties in rSIV.F/HN vector production caused by pseudotransduction or metabolic burden on producer cells due to large cDNA size represents an additional hurdle that must be overcome if the project is pursued further. As discussed previously, the strategy to use the human ADAMTS13 cDNA sequence so that commercial ELISAs could be used with greater sensitivity than the FRET assay, was initiated late in the project and may hold promise for the future. Generation of rSIV.F/HN vector encoding human ADAMTS13 was delayed due to concerns of pseudotransduction, particularly given the production of each batch of rSIV.F/HN vector by collaborators at Oxford University costs £15,000. If the project is pursued further, repression of transgene expression during vector manufacturing may alleviate detrimental ADAMTS13 protease activity and metabolic burden, permitting higher

titres of vector to be generated. A potentially solution is the co-expression of a clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 mutant which is nuclease-inactive (dCas9) fused to the KRAB repressor and a guide RNA specific to the hCEF promoter which would inhibit transcription of ADAMTS13 in producer cells (325).

A concern that may be faced upon continued research into a lung-targeted gene therapy approach for TTP, is the local effect of high ADAMTS13 expression in the lungs. The majority of endogenous ADAMTS13 expression is contributed from the liver, specifically hepatic stellate cells, with other organs such as the spleen and the lungs demonstrating far lower levels of expression (326). Given the central role of ADAMTS13 cleavage in regulating VWF-multimer size and consequently thrombogenic potential, it is possible that the generation of high levels of ADAMTS13 in the lungs may in fact convert the pro-thrombotic phenotype observed in deficient mice into a bleeding phenotype due to extremely high levels of ADAMTS13 expression. However this is unlikely given the difficulties in generating significant levels of expression thus far. Furthermore, a wide therapeutic window of ADAMTS13 expression was demonstrated by Verhenne *et al.* (2017) with supraphysiological levels (~100-200% NHP) of ADAMTS13 tolerated in mice for 25 weeks without apparent side effects (176).

The generation of regulated expression rSIV.F/HN vectors could be a solution if high ADAMTS13 expression in the lungs was found to be detrimental, as well as having far-reaching benefits in many gene/cell therapy applications. As described, efficient production of GMCSF following rSIV.F/HN-mediated lung gene transfer was previously found to be toxic in mice at the tested vector doses (Lund-Palau, Molecular Therapy-manuscript under review). Thus regulated lentiviral vectors were generated, with mifepristone-inducible expression of GMCSF demonstrated in various models including immortal cell lines, a human airway model and a pulmonary cell therapy model. Proof-of-concept was initially generated using constructs based upon a commercial system yet moving forward, development of ZF alternatives will be pursued to ensure humanised constructs are created with potential for downstream human application. Screening of novel ZF constructs by generating VSV-G vectors is cheaper and faster than generating F/HN pseudotyped vectors. Subsequently however, rSIV.F/HN vector encoding successful designs could be generated downstream.

Given the flexible modular nature of the engineered transcription factor, further elements could be optimised and substituted, including the activation domain, highlighting the potential of drug-inducible expression systems for a wide range of applications.

In conclusion, the current project achieved its objective to investigate the rSIV.F/HN lentiviral platform developed by the UK Respiratory Gene Therapy Consortium in wider therapeutic settings. Beyond applications for the treatment of respiratory diseases such as CF, data generated here supports the use of the rSIV.F/HN technology towards the treatment of systemic disorders. Intranasal administration of rSIV.F/HN was identified as the superior route of vector administration, demonstrating sustained systemic expression (at least 1 year) of a reporter protein and given repeat dosing by intravenous vector delivery was not tolerated. TTP was selected as an exemplar disease to investigate rSIV.F/HN therapy for systemic disorders. Limited plasma expression of ADAMTS13 was achieved following delivery of rSIV.F/HN-mADAMTS13 to deficient mice, however restoration of wildtype activity levels was demonstrated in the secretions lining the lungs following intranasal administration. To improve upon the safety of GTAs, proof-of-concept was demonstrated that mifepristone-induced control of lentiviral-mediated transgene expression could be achieved. Temporal control of gene expression will permit the use of rSIV.F/HN vectors towards therapeutic indications with narrow efficacy/toxicity windows. Taken together, data was generated supporting the application of rSIV.F/HN vectors towards the treatment of systemic disorders, whilst the generation of regulated expression vectors has the potential to broaden the technology platform for still wider therapeutic indications.

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