Development of Potent Foamy Retroviral Vectors for AIDS Gene Therapy

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Anti-HIV gene therapy

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Submitted on: *08.05.2015*

Table of Contents

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8th of May 2015 ***Rahaf Qamou***

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Abstract

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Abstract for Bachelor thesis II, Submitted on May 8th 2015

Development of potent foamy retroviral vectors for AIDS gene therapy.

Acquired immune deficiency syndrome (AIDS) is a major international health problem for which there is currently no vaccine. It is caused by the human immunodeficiency virus (HIV), which infects immune cells. The current therapy for AIDS has prolonged the life of many AIDS patients, but it requires the daily administration of a cocktail of drugs and can have severe side effects. A better AIDS therapy is needed.

Gene therapy inserts genes into the DNA genome of cells to treat disease. Retroviruses, a family of viruses that insert their DNA into a cell's DNA during their normal course of infection, are commonly used as vectors, or DNA-delivering agents, after some of their genes are replaced with therapeutic genes. In AIDS gene therapy, blood stem cells, which can become any immune cell, are taken from the AIDS patient, infected with a retrovirus vector containing genes that combat HIV, and transplanted back into the patient. These modified blood stem cells can then populate the immune system with cells resistant to infection by HIV.

Foamy viruses are a type of retrovirus, named because infected cells look "foamy" under a microscope. Gene therapy vectors developed from foamy viruses have several advantages over vectors developed from other kinds of retroviruses, including potentially increased safety. However, HIV is able to mutate rapidly and escape inhibition by anti-HIV genes, so it is desirable to include multiple potent anti-HIV genes in AIDS gene therapy vectors.

The work for this thesis addresses these problems by creating four new foamy virus vectors. Two of the vectors contain the strong SFFV promoter, and two contain a housekeeping gene promoter called the elongation factor 1-alpha (EF1α) promoter. One vector with each promoter contains the anti-HIV gene humanized TRIM5αCypA fusion, while the other SFFV- and EF1α-containing vectors have the new anti-HIV gene MxB. The first step in testing new gene therapy vectors is to measure the vector titer, which is the number of virus particles that can be generated for each vector. This is important because some anti-HIV genes can reduce vector titer, making that vector impractical for use in gene therapy. The titer of each of the new foamy virus vectors was measured, and all of the titers were found to be high enough to continue with future gene therapy experiments.

These vectors will be used in multiple future experiments. Since they contain each combination of the two promoters and the two anti-HIV genes, they will enable direct comparison of the level of expression provided by both promoters, testing the EF1α against SFFV. Direct comparison between the ability of humanized TRIM5αCypA and MxB to inhibit HIV replication will also be measured through a cell culture assay to see if they are similarly potent to the previously tested C46 transgene.

Finally, if the results of these experiments are promising, the vectors can be tested in mouse and primate models and prepared for use in human patients. This thesis represents the first step in this process, with the final goal of developing safer, more potent, and clinically relevant vectors for AIDS gene therapy to reduce the burden on AIDS patients resulting from the disease and its current therapies.

Abbreviation list:

aa	amino acid
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APOBEC3	Apolipoprotein B mRNA-editing catalytic polypeptide 1-like protein 3
bp	base pair
CA	Capsid
cDNA	Complementary DNA
Cyp	Cyclophilin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphide DNA Deoxyribonucleic Acid
DNA	Deoxyribonucleic acid
EF1α	Elongation Factor 1 type α
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbant assay
env	Envelope
FACS	Fluorescence activated cell sorting
FCS	Fetal bovine serum
FIV	Feline immunodeficiency virus
FV	Foamy virus
HAART	Highly active retroviral therapy
HIV-1	Human immunodeficiency virus-1
HSC	Haematopoietic stem cell
IFN	Interferon
IL	Interleukin
IN	Integrase
IRES	Internal ribosomal entry site

kb	Kilobase
kDa	Kilodaltons
LTR	Long terminal repeat
LV	Lentivirus
MA	Matrix
min	Minutes
ml	Millilitre
MLV	Murine Leukaemia virus
MOI	Multiplicity of infection
Mx	Myxovirus
Mx1	mice Myxovirus resistance 1
Mx2	mice Myxovirus resistance 2
MxA	human Myxovirus resistance A
MxB	human Myxovirus resistance B
NC	Nucleocapsid
p24	HIV-1 p24 gag capsid protein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PGK	Phosphoglycerate kinase
polyA	Polyadenylation
PR	Protease
PPT	Polypurine tract
Rev	Regulator of expression of virion proteins
rhTRIM5α	TRIM5α of Rhesus macaque
RING	Really interesting new gene
RNA	Ribonucleic Acid

RPMI	Roswell Park Memorial Institute-1640
RRE	Rev responsive element
RT	Reverse transcriptase
s	Seconds
SCID	Severe combined immunodeficiency
SFFV	Spleen focus-forming virus
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
TAR	Transactivation response element
Tat	Transactivator of Transcription
TRIM5αCypA	TRIM5α-CyclophilinA fusion protein
TRIM	TRIpartite motif
TU	Transducing unit
WPRE	Woodchuck hepatitis virus post transcriptional regulatory element

1 Introduction

Over the past three decades, HIV and AIDS have swept across the globe, affecting communities on every continent. The human immune deficiency disease (HIV) is one of the most challenging retroviral infections with no functional scientifically proven treatment to reliably eradicate the virus from patients or reverse the damage to the immune system (Mamo et al., 2010). The virus was first found and described in humans in 1983 (Gallo et al., 1983), while initial transmission occurred by zoonosis of the simian immunodeficiency virus (SIV) from chimpanzees to humans in the early 20th century (Korber, 2000). Since then, HIV continued to be a serious health issue worldwide with an estimated number of 2.1 million people became newly infected in 2013 and about 35 million people are still living with HIV around the world. Easily transmitted through sexual contact, by blood/blood products or vertically from mother to child, more than 39 million deaths were caused by AIDS worldwide since the epidemic began (Levy et al., 1984).

Since attempts to develop a preventive vaccine failed till now, research has refocused in the last years on unravelling HIV's biology and developing new therapeutic intervention strategies to improve existing antiretroviral therapy. With the introduction of highly active antiretroviral therapy (HAART) there has been a significant decline in morbidity and mortality (Palella et al., 1998). The treatment uses a cocktail of anti-retroviral drugs which attack the virus at different points in its life cycle (Jones, 2002). Antiretroviral drugs are divided into different classes by the way the individual drug interrupts the viral replication. The classes currently available include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors and entry/fusion inhibitors. However, there are numerous downsides associated with this treatment regimen including cost, toxicity and long-term complications (Lewden et al., 2005). In addition, this mode of treatment does not offer the possibility of curative therapy. It was incapable of targeting latent proviruses residing in long-lived reservoirs (Dinoso et al., 2009), even when the virus replication was suppressed for many years, viral load rebound reappears indicating

that patients are not cured. All of these issues and limitations have driven research into finding a longer term alternative approach to drug treatment based on gene therapy, which would theoretically provide a one-off treatment against HIV virus.

Under this strategy, numerous anti-HIV genes could be engaged in gene therapy, targeting both viral and cellular molecules at different crucial steps of the viral life cycle. These inhibitory genes for restriction can be categorized into two main groups: RNA based and protein based. There are also different method of delivery to consider, including: adenoviral and retroviral vectors, with either lentiviral vectors derived from HIV-1, HIV-2, simian immunodeficiency (SIV) and feline immunodeficiency (FIV) or foamy retroviral vectors (FV) which are becoming increasingly attractive.

One basic strategy for treating HIV infection by gene therapy would be to genetically modify a population of susceptible cells such as T-cells with an anti-HIV gene to confer resistance to the infection or to specifically target the infection (Digiusto and Kiem, 2012). If these HIV resistant cells have a survival advantage in vivo, they would replicate and repopulate the immune system, providing the patient with a long-term stable resistance against the virus. Some anti-HIV gene therapies have reached clinical trials testing for both safety and efficacy of various strategies.

1.1 HIV genome and life cycle:

1.1.1 HIV-1 structure:

In the family of Retroviridae, HIV-1 together with HIV-2, the simian, bovine and the feline immunodeficiency viruses belong to the complex genus of the lentiviruses which are all characterized by persistent infection with slow progression course of the disease. All of these retroviruses share three major structural genes: Gag (group specific antigen) which is the genetic material that codes for the core structural proteins, Pol (polymerase) the genomic region encoding the viral enzymes and Env (envelop) that serves to form the viral envelop. In addition to these, HIV-1 carries other auxiliary proteins: both Tat (transactivator of transcription) and Rev (regulator

of viral expression) are crucial for viral replication (Gramberg et al., 2009). Furthermore, other genes like Vif (viral infectivity factor), Vpr (viral protein R), Vpu (viral protein unique) and Nef (negative factor) are involved in multiple roles in the viral lifecycle and immune evasion (Malim and Emerman, 2008). The HIV-1 genome composed of two identical single- strand RNA copies is packaged into the center of the spherical Capsid (CA) core protein (Figure 1). This core domain contains the cleavage products of Pol encoding the Protease (PR), Integrase (IN) and Reverse Transcriptase (RT) enzymes which are essential for virus maturation and early infectivity steps (Sundquist and Kräusslich, 2012). Wrapped with a second layer of Matrix (MA) proteins, a cell-derived lipid membrane surrounds the viral spikes consisting of trimers of Env, the 160 kDa glycoprotein (gp160) which is eventually cleaved into the gp120 surface protein (SU) and the transmembrane protein (TM) gp41 (Pancera et al., 2014).

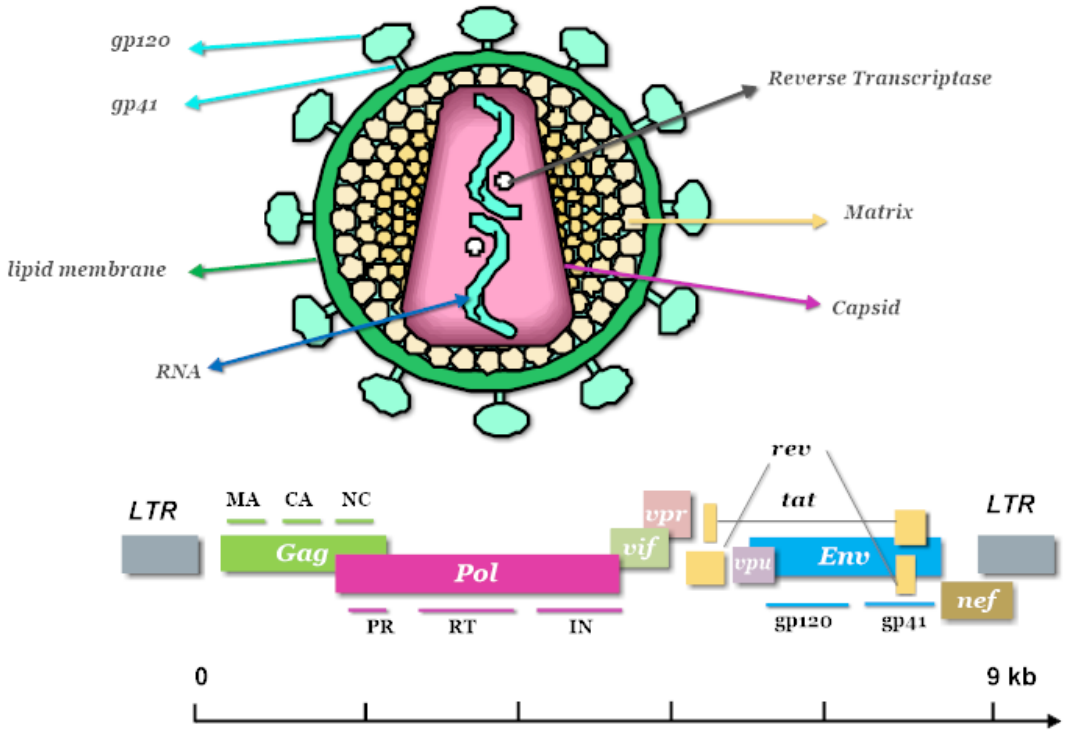

Figure 1 HIV-1 structure and molecular organization. Upper panel: the individual components of an HIV-1 particle, as gp120 and gp41 correspond to full length SU and TM proteins respectively. Lower panel: the localization of HIV-1 encoded proteins within the virus genome. Illustration by Rahaf Qamou using Smart Draw ci. Software.

1.1.2 HIV-1 life cycle:

The first step of HIV-1 lifecycle is attachment to target cells. Although virus entry can be mediated by endocytosis (Miyauchi et al., 2009), the main route of infection uses sequential binding of Env SU protein to CD4 cell receptors and CCR5 or CXCR4 chemokine receptors which are present in T-helper lymphocytes (Maddon et al., 1985), monocyte-macrophages (Filion et al., 1990), follicular dendritic cells (O'Doherty et al., 1993), Langerhans cells in the skin and microglia in the nervous system (Clapham and McKnight, 2001) (Jordan et al., 1991). Additional target cells like NK and epithelial cells might be entered by using an alternative co-receptors or the galactosyl ceramide receptor binding domain located in the gp41 protein (Yu et al., 2008). This binding induces a conformational change in the gp120-gp41 complex inserting the hydrophobic fusion peptide (FP) into the target cell membrane and promoting membrane fusion. Subsequently, these conformational changes brings the viral and cellular membrane into close proximity to allow fusion.

Following fusion, the uncoating process allows the release of HIV-1 RNA and enzymes into the host cytoplasm, where a DNA copy of the virus genome is produce by RT. The linear DNA associated with the additional proteins forms the pre-integration complex which is then actively transported into the nucleus for integration into the host genome with the help of IN. Once integrated into the host genome, the provirus is driven by flanking long terminal repeats (LTRs) which are further divided into unique U3, repeat R and U5 region. Viral transcription can be produced from the provirus using host machinery, including RNA polymerase II (Harrich and Hooker). Transcription begins at the U3-R region of the 5' LTR, and terminates at the polyadenylation signal just after the R region of the 3' LTR. Transcription of the provirus results in spliced viral RNAs which are grouped into three main categories: 9kb full length RNAs which are translated into Gag and Gag/Pol (Freed, 1998), 4kb partially spliced mRNAs encode Env, Vif, Vpu and Vpr, and finally 2kb mRNAs encoding Rev, Tat and Nef (Zolla-Pazner and Cardozo, 2010). These mRNAs would be exported from the nucleus to the cytoplasm by host mRNA export machineries. Once expressed, Tat and Rev are imported back into the nucleus where they assist in transcription and export of unspliced and partially spliced

transcripts respectively (Malim et al., 1989). Rev is able to transport longer transcripts by binding with its N-terminal domain to a conserved cis-acting RNA sequence called rev responsive element (RRE). The C-terminal leucine rich domain of Rev, functions as a nuclear export signal (NES) that promotes the transition between the viral life cycle early phase to the late phase where structural proteins are produced (Freed, 1998).

In the cytoplasm, host ribosomes synthesize viral proteins. Ribosomal frameshifting occurs at the gag-pol junction where the ribosome translates the full length Gag-pol polyprotein which encodes the viral enzymes RT, PR, and IN (Jacks et al., 1988). Gag protein is essential for virion assembly and its expression, it's sufficient alone to promote viral assembly, budding and the release of immature virus particles. The MA domain of Gag is primarily responsible for the localization of Gag at the plasma membrane through myristoylation of an N-terminal glycine and an N-terminal basic region providing a signal for trafficking to the membrane (Gheysen et al., 1989). When Gag proteins accumulate and multimerise at the plasma membrane, they start forming a roughly spherical shaper with the N terminal associated with the membrane and C terminal at the sphere's center (Derdowski et al., 2004).

Full length RNA transcripts include a 5' packaging signal located at the 5' UTR region and extends to the Gag coding sequence (Lever et al., 1989). This sequence interacts with Gag to mediate efficient packaging into virions (Mann and Baltimore, 1985). Specifically, it is the nucleocapsid (NC) region of Gag that binds the packaging signal leading to virions budding. This signal also enables the dimerization of RNA resulting in the packaging of two strands of RNA per virions. Then, the packaging signal is removed by splicing to ensure that only full length RNA transcripts are packaged into virions. In addition, a collection of viral proteins are also incorporated into the budding virion. These include Vpr, Vif and Nef (Sundquist and Kräusslich, 2012). Following that, the Env glycoprotein precursor gp160 is synthesized in the rough endoplasmic reticulum (RER). In the RER gp160 forms trimers that are transported to Golgi apparatus where they are a subject to mannose trimming and cleavage into gp120 and gp41 subunits by furin (Stein and Engleman, 1990). The gp41 anchors gp120 to the membrane surface in a trimeric

manner forming viral spikes. Then, these complexes are transported to the cell surface and incorporated into the virus particles.

As the virion is forming and budding from the host cell, it is coated with the host cell plasma membrane, which must be excised to allow virion release. Shortly after budding, viral maturation occurs and involves structural rearrangements from a sphere core to a cone shaped structure. Gag polypeptide is cleaved by protease to form MA, CA and NC monomers. Gag-Pol structure cleavage results in viral enzymes RT and IN. These molecules gather to form the mature virus particle which consists of an outer layer of lipid membrane associated MA with a CA conical core surrounding the NC containing the RNA genome and IN and RT enzymes (Figure 2).

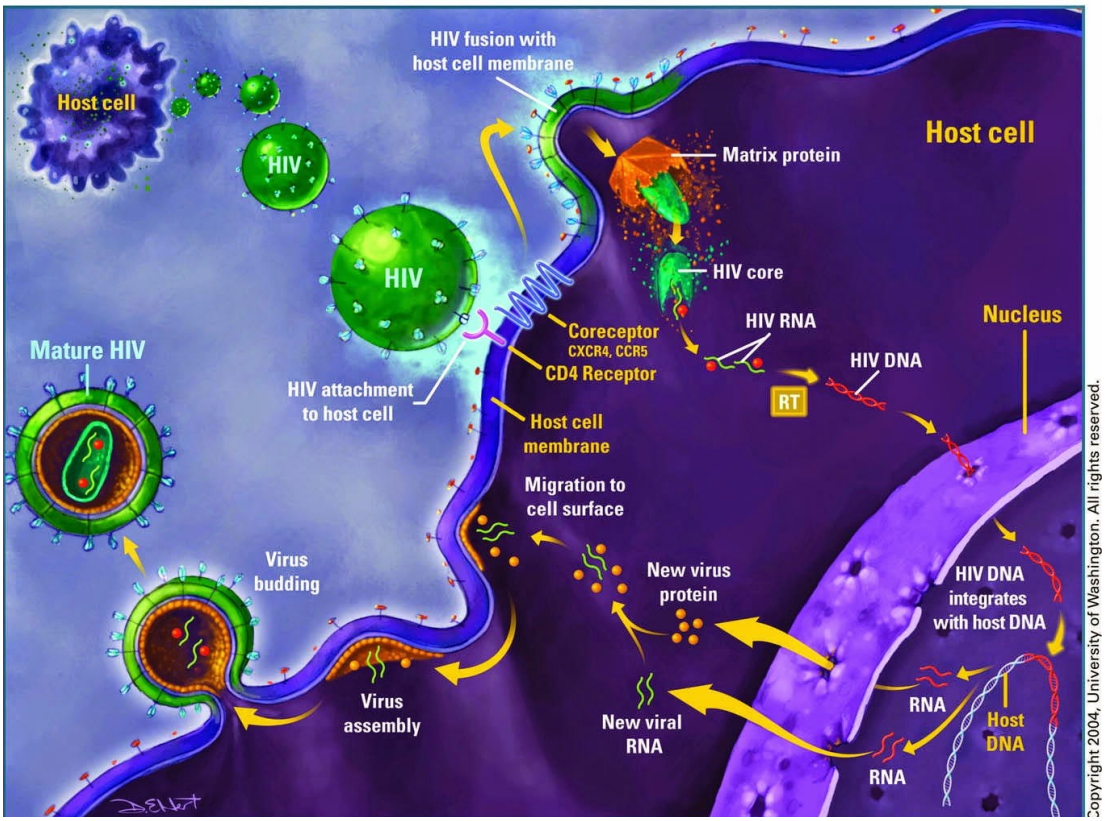

Figure 2 The replication cycle of HIV-1 virus. After attachment of the virus particles to CD4 and co-receptors, virus and cellular membrane fuse and the virion content is released into the cytoplasm where cDNA synthesis is accomplished by the Reverse Transcriptase (RT). Associated with Integrase (IN), Matrix protein (MA) and Viral protein R (VPR) the pre-integration complex translocates into the nucleus where the viral DNA is integrated into host genome by IN. Expression of viral genes is achieved by binding of Tat and transcription by RNAPII. Viral structural and accessory proteins along with two copies of unspliced ssRNA then assemble at the plasma membrane to bud into a new particle. All copyrights are reserved for University of Washington (David H. Spach, 2015)

1.2 Gene therapy:

The basic principle of gene therapy is the insertion of therapeutic transgene into target cells (Figure 3). Depending on the disease being treated, the vector used for transgene delivery could carry a wild type gene to replace endogenous, mutated copy in a genetic disease. Alternatively, gene therapy could be used to express novel proteins that would either confer a resistance to particular pathogens or enable modified cells to target and destroy abnormal cancer cells (Poluri et al., 2003).

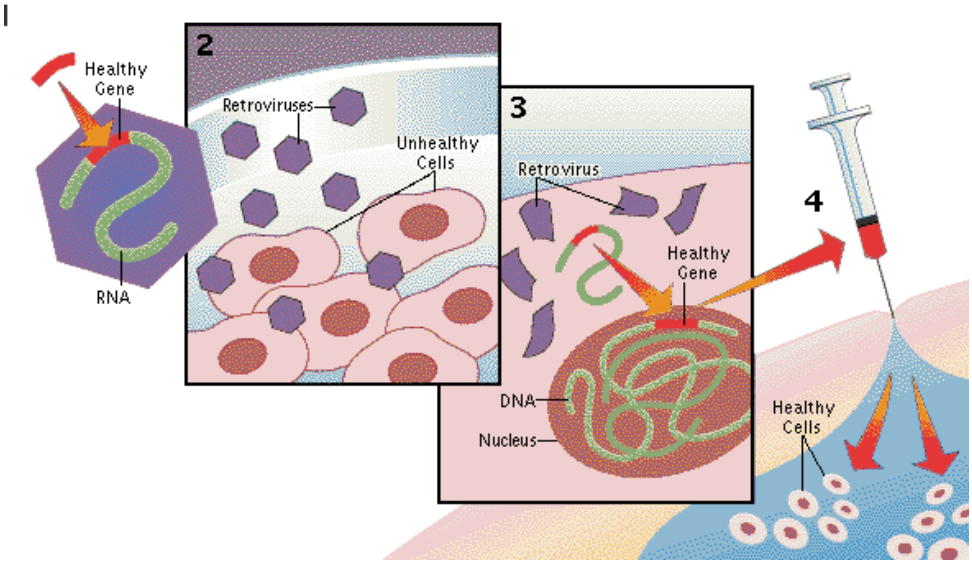

Figure 3 Retroviral gene therapy. Genetically modified retroviruses are used to deliver a therapeutic gene into target cells in vitro. Modified cells are then injected back into the patients to repopulate and provide protection. Figure taken from (2004 et al.)

1.2.1 Retroviral gene therapy development:

Different mechanisms can be employed to deliver therapeutic transgenes into target cells, both viral and non-viral. Retroviruses have characteristics that make them highly desirable as vectors for gene therapy, including their integration profile into the host genome to mediate long term expression, susceptibility to genome

modification to enable the insertion of therapeutic genes and efficient infection of a wide range of target cells (Piacentini and Kroemer, 2005). These vectors have been vastly used for research and are continuously being developed for more efficient potent gene therapy.

One of the first gene therapy clinical trials using retroviral vectors began to treat severe combined immunodeficiency (SCID) by the delivery of gamma retroviral vectors expressing adenosine deaminase (ADA) gene to $CD34^+$ cells. Although normalized levels of blood T cells was obtained, effects were short lived with only transient gene expression (Blaese et al., 1993)(Blaese et al., 1995). Despite of the rise in the gene therapy clinical trials throughout the 90s, most studies failed to show maintained efficacy and potency. At the beginning of the 21^{st} century, human stem cell gene therapy trial took place for the treatment of X-linked SCID (SCID-X1) using gamma-retroviral vector carrying the interleukin (IL) γ chain gene (Hacein-Bey-Abina et al., 2003). Even though there was an improved immunological function and a sustained benefit, an unexpected adverse events were uncovered later when the trial participants developed leukemia due to insertional mutagenesis (Hacein-Bey-Abina et al., 2008).

1.2.2 Retroviral gene therapy against HIV-1:

Since the first appearance of AIDS in the beginning of the eighties, the development of a safe and effective HIV treatment was essential to avoid further spread of the epidemic. Even though using HAART greatly improved the out comes of patients living with HIV, the urge to develop an alternative approch that can preferably cure HIV infection was the main focus in research. For this purpose and due to all the advancement in gene therapy, the field of anti-HIV gene therapy research is currently in its full extent. Many transgenes are being generated and testes *in vivo,* targeting both viral and host machinaries at different point of the viral lifecycle. Blocking the very early stages of the virus lifecycle preferbly at the point of viral entry would be the most powerful and effective strategy that would protect cells and make them resistable to the infection. This would reduce the opportunity of the HIV virus to mutate as reverse transcription is not occuring, and it would prevents the

establishment of latent reservoirs. HIV gene therapy has progressed into clinical trials, alongside with anti-HIV transgenes analysis, vector development and transduction protocols are still being improved.

Major retrovirus system being used for the efficient delivery of anti-HIV transgenes into target cells by ex vivo transduction. The right choice of the vector system has a huge impact on the effectivness of anti-HIV gene therapy approach. Early work primarly involved the use of gamma retrovirus vector as anti-HIV delivery vehicles targeting either stem cells or normal T cells. These vectors have been tested extensively due to their ability to efficiently transduce hematopoietic stem cells (HSC) (Bunnell et al., 1997). Typically, these vector carried transgenes that inhibit late stages of the HIV lifecycle, such as inhibiting Rev, Tat and RNA interference (RNAi) to prevent translation of viral genes. Some of these transgenes have reached clinical trials. However, more effective therapy using transgenes targeting early stages prior to integration have been developed.

Although these retrovirus vectors initially appeared to be safe, several limitation of this system limited its use and created a move towards the use of lentiviral vectors. Lentiviruses has an improved integration safety profile and also have the ability to transduce both dividing and non-dividing cells which are considered to be important reservoirs for HIV infection (Finzi, 1997). Advanced research is being performed testing lentiviral vectors in mouse models, and some lentiviral vectors are now in clinical trials (Escors and Breckpot, 2010). Results from these trials have provided safety data and important information about the clonal expression of modified cells *in vivo*. However, with all the promising results relating to the efficacy of the treatments, potential risks are still associated with lentivirus gene therapy. Due to the genome similarity between lentivirus (LV) and the HIV virus itself, LV titers can be severely suppressed by the expression of anti-HIV genes that target functions shared by LV and HIV (Mautino and Morgan, 2000). Another safety concern is the recombination and mobilization of the therapeutically modified integrated lentiviral vectors with the wild type virus of HIV (Evans and Garcia, 2000). One approach to overcome this problem is the use of other types of LV vectors such as HIV-2 based vectors. Even though much work has been done using LV vectors that are mobilized

by HIV-1 (Turner et al., 2009), extensive research is carried out testing feline immunodeficiency virus (FIV) (Poeschla et al., 1998) and feline immunodeficiency virus (FIV) (Siapati et al., 2005) vector systems. These two vector systems had their limitations, as EIAV vectors do not transduce human HSCs as efficiently as second generation HIV-1-based vectors (Siapati et al., 2005) and FIV vectors showed inefficient transgene expression in human hematopoietic cells (Price et al., 2002) .

Moreover, to minimize the risk of these systems, advanced research focused on using engineered self-inactivating (SIN) retrovirus vectors (Yu et al., 1986). The virus particles of these replication incompetent vectors are infectious for only one replication cycle due to the deletion of the viral promoter in the U3 region of their 3' LTR. As the vector sequence is reverse transcribed, this deletion is copied into the 5' LTR region, causing the deletion in both LTRS of the integrated vector and consequently preventing it from replication. These SIN systems are less likely to stimulate nearby cellular genes, thus less likely to induce genotoxicity (Bokhoven et al., 2009).

Although SIN vectors were considered one of the most promising strategies for a safer treatment, other safety concerns were raised regarding the integration profile (Haviernik and Bunting, 2004). Since retroviral vectors insert their genome into the target cell DNA, they can disrupt cellular genes either by direct integration into the coding sequence or by dysregulation. Dysregulation of proto-oncogenes in hematopoietic stem cells has the potential to transform cells and develop into leukemia (Kohn et al., 2003). While gamma-retroviruses usually integrate near transcriptional start sites, lentiviruses integrate in transcribed regions (Persons, 2010). Consequently, the use of these vectors is always associated with a risk of transformation due to insertional mutagenesis (Escors and Breckpot, 2010).

Consequently, all the sustained efforts in anti-HIV gene therapy to design safer and more potent gene delivery systems shifted towards testing recently identified foamy virus (FV) vectors that have shown great promise in HSC gene therapy due to their ability to efficiently deliver genes to multiple progenitors cell lines (Trobridge, 2009).

1.3 Foamy virus vectors for gene therapy:

1.3.1 Foamy viruses:

In the group of retroviruses, vectors derived from foamy viruses (FV) are members of the *Spumaviriniae* sub-family, which contain FVs derived from various species including primates, non-human primates, cats, cattle, horses and sheep (Kehl et al., 2013). Initially described in the mid 50's and later isolated in the 70's from a human nasopharyngal carcinoma cell tissue of a patient from African origin, they were named according to their foamy-like appearance of infected cells in culture (RUSTIGIAN et al., 1955) (Achong et al., 1971). FVs unique virus biology and replication strategy sets them apart from other members of *Retroviridae* (Rethwilm, 1996). As all retroviruses they encode the Gag, Pol and Env genes as well as two accessory bel genes which give raise to Bel-1/Tas (transactivator of spumaviruses) and Bet genes as shown in (Figure 4). However, differences comprise the translation of Pol from a spliced mRNA transcript instead of the synthesis and the cleavage of Gag-Pol fusion protein (Bodem et al., 1996) (Rethwilm, 2003), also the incomplete cleavage of Pol into individual enzymes (Flügel and Pfrepper, 2003) and the presence of an internal promoter located in the C terminal of Env gene (Löchelt et al., 1994). In addition, reverse transcription of FV RNA occurs late during replication results in DNA containing viral particles (Moebes et al., 1997). Furthermore, since FV capsid protein does not contain a membrane-targeting signal, FV particles budding depends only on the presence and interaction with the Env protein (Baldwin and Linial, 1998). This specific Gag/Env interaction prevents the use of heterologous envelops for pseudotyping FV (Hütter et al., 2013). Where FV basic research has been conducted for more than two decades, attempts to test their potential for clinical application has just recently advanced into practice (Taylor et al., 2008). FV several unique characteristics make them interesting vectors for gene therapy as will be outlined in the next section.

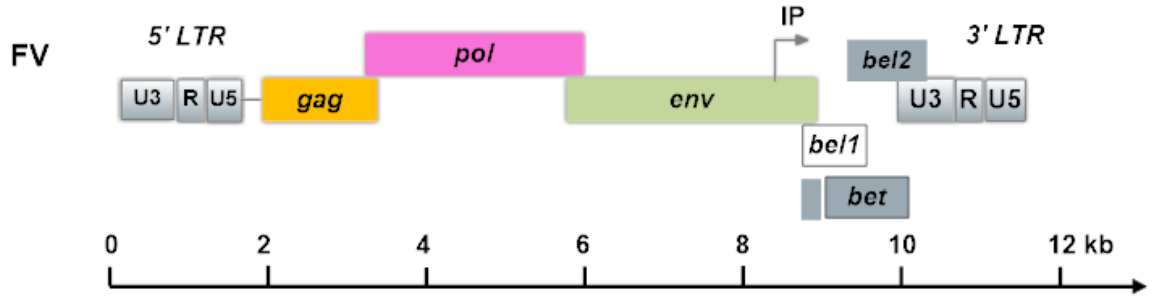

Figure 4 The foamy virus genome. Foamy viruses contain the canonical retroviral *gag*, *pol*, and *env* genes, as well as long terminal repeats (LTR) containing the U3, R, and U5 regions. Foamy viruses also express two accessory proteins, Tas and Bet. Low-level basal transcription occurs from the internal promoter (IP) in the *env* gene, and Tas binds to the IP and 5' LTR to activate high-level transcription. Arrows indicate sites of transcription initiation, and the dotted line indicates splicing of the *bet* mRNA. The scale bar gives length in kilobases (kb).

1.3.2 The development of foamy virus vectors for gene transfer:

Vectors developed from foamy viruses are promising AIDS gene therapy vectors. Unlike other retroviruses, foamy viruses reverse transcribe their genome from RNA to dsDNA at late stage in the replication cycle, before leaving the host cells, so most infectious virion particles contain DNA, on the contrary to other retroviruses (Moebes et al., 1997). After host cell entry and uncoating, foamy viruses stably integrate their genome into the host's genome, forming provirus. Once integrated, a low level of basal transcription takes place from the internal promoter (IP) in the Env gene, driving Bel1/tas and Bet transcription (Trobridge, 2009). Tas then activates high level of transcription from both the IP and the LTR, which drives transcription of essential genes Gag, Pol, Env (Meiering and Linial, 2001).

When used as vectors for gene therapy, foamy viruses are modified in several ways to make them safer by making them replication incompetent. Because, using replication competent vectors can cause a great damage to modified cells that would produce infectious FV virion that can infect other non-target cells in the patient. Since Gag, Pol and Env are necessary to make infectious virions, they are not essential for gene expression or maintenance of integrated provirus. Instead, the

three structural genes Gag, Pol and Env can each be produced in trans to create virion stocks that are able to infect cells and integrate the host genome, but are not able to synthesize new Gag, Pol and Env to create more infectious particles (Trobridge, 2009). This is done by simultaneously transfecting cells with four plasmids that each separately code for vector genome, Gag, Pol and Env. These plasmids that code the three structural proteins Gag, Pol and Env are termed helper plasmids (Trobridge, 2009). Consequently, targeted cells will receive all four plasmids and Gag, Pol and Env will be synthesized to package the vector genome into infectious, replication-incompetent virions.

While unnecessary components of the viral genome for efficient gene transfer can be removed, only essential cis-acting regions (CAR) should be retained for successful gene delivery. Foamy virus vectors retain their CAR in their genome, but prevent the expression of Gag, Pol and Env by introducing stop codons into these genes sequence in the vector (Kiem et al., 2010). However, chances of identical sequences between the vector CAR and helper plasmids could allow recombination to occur by introducing functional Gag, Pol and Env into the vector (Kiem et al., 2010). This chance of homology can be greatly reduced by the incorporation of silent mutations without changing the protein sequence into the helper plasmids and by codon optimizing the helper plasmids which will increase the efficiency of expression in the target cells (Kiem et al., 2010).

The Tas and Bet genes can be deleted as well from the vector, and the absence of Tas means that transcription will not occur from the LTR promoter and drive the expression of CAR, making the vector and self-inactivating vector (Trobridge, 2009). In addition, the U3 region of the 3' LTR is deleted, so that following reverse transcription, the 5' LTR will be truncated (Kiem et al., 2010). Subsequently, transcription of the delivered genes is accomplished by promoters added between the LTRs.

1.3.3 The use of foamy virus vectors in anti-HIV gene therapy:

Foamy virus vectors have several advantages over other retrovirus vectors for gene therapy. Foamy viruses are the only retroviruses to transcribe gag and pol on

separate mRNAs, allowing gag and pol to be placed on different helper plasmids, reducing the chance of creating replication-competent virus further than with other retroviral vectors (Trobridge et al., 2012). Since foamy viruses are the largest retroviruses, they can carry the largest transgene cassettes, efficiently delivering cassettes as large as 9.2 kilobases (kb) (Olszko and Trobridge, 2013). Foamy viruses also have a very wide tropism, infecting humans and all large and small mammal models for gene therapy (Trobridge, 2009). Unlike lentiviral vectors, foamy virus vectors do not need to be pseudotyped (Trobridge, 2009). Since the transgenes that AIDS gene therapy vectors deliver are anti-HIV genes, they can inhibit the titers of HIV-derived lentivirus vectors (Kiem et al., 2010). Foamy virus is not closely related to HIV, so it is much less likely that anti-HIV genes will inhibit foamy virus vector titers (Kiem et al., 2010).

Different retroviral vectors have different profiles of integration into host cell genomes, and the apparently safer integration profile of foamy virus vectors provides another advantage over other retroviral vectors (Rethwilm, 2007). While murine leukemia virus-based vectors integrate preferentially near transcription start sites and HIV-based vectors prefer to integrate in transcribed genes, foamy virus vectors do not integrate preferentially in genes and have only a modest preference for integrating near transcription start sites (Trobridge et al., 2006). A reporter gene assay also showed foamy viruses to be less likely to transactivate nearby genes than gamma retrovirus or HIV-based vectors (Hendrie et al., 2008).

1.4 Restriction factors of the innate immune system

1.4.1 Antiretroviral restriction factors:

Since one of the major characteristics of HIV infection is the failure of the immune system to recognize and defeat the virus, much research focus has been on the early events following virus entry. The Innate immunity constitutes the line defense against any foreign pathogens, it acts quickly after infection to provide protection and allowing time for the adaptive immunity to mount a response (Mogensen et al., 2010). This defensive systems provides a mechanism by which cells can detect the presence of pathogens and employ certain a series of local and systemic defense

measures that could enhance host protection and anti-viral defense. This includes interferons (IFN), pattern recognition receptors (PRR) and secreted soluble molecules such as toll-like receptors or complement (Towers and Noursadeghi, 2014). Adaptive immunity is more complex and can take day to come into effect. It involves the activation of T and B lymphocytes and production of pathogen specific antibodies. Since innate immunity begins controlling the infection much sooner than the adaptive response, PRR stimulation leads to activation of signaling cascades that triggers consequent transactivation innate immunity response genes (Stark and Darnell, 2012). This is primarily dominated by the induction and the secretion of soluble type 1 interferon (IFN). IFN signaling induces the expression of many effector genes, making the system rather complicated to defeat (Benecke et al., 2012). These genes are called restriction factors and their expression is now far more widespread that initially thought, many mammals have now been shown to express these anti-viral proteins (Neil and Bieniasz, 2009).

However, retroviruses have evolved systems to avoid inhibition by these restriction factors in species in specific manner. Typically, HIV-1, HIV-2 and SIV are not significantly inhibited by restriction factors of their target host species (Ortiz and Silvestri, 2009). Due to the extensive research that has been performed on retroviruses, most of the knowledge obtained on restriction factors is related to inhibition of these viruses. Such restriction factors that are considered to be promising candidates for anti-HIV gene therapy are APOBEC3G (Sheehy et al., 2002), Trim5α (Stremlau et al., 2004), Tetherin (Neil et al., 2008), SAMHD1 (Hrecka et al., 2011) and most recently MxB (Goujon et al., 2013). Later, we will discuss the role of both Trim5α and MxB proteins in HIV-1 restriction.

1.5 TRIM proteins:

1.5.1 Identification of TRIM5α as an antiretroviral restriction factor:

At the beginning of the 21st century, tripartite motif-containing protein 5 isoform - alpha (TRIM5α) was identified as the restriction factor responsible for the resistance of Old World monkeys to HIV-1 (Stremlau et al., 2004). The naturally occurring protein from rhesus monkey damages HIV-1 life cycle at a post-entry, preintegration

stage by blocking the uncoating process in human cells (Nakayama and Shioda, 2010). Human TRIM5α (hTRIM5α) mediates mild restriction of HIV-2 (Ylinen et al., 2005), but does not cause a significant inhibition to HIV-1. However, human/rhesus macaque chimeric TRIM5a isoform, carries better restrictive ability to impair HIV-1 infection (Li et al., 2006). Since the method of rhTRIM5α delivery into human cells using a lentivirus led to problems with immune rejection as it is a foreign protein (Anderson and Akkina, 2005), hTRIM5α was modified to provide specificity to HIV-1. Modification with a single amino acid change at 332 in the human protein was sufficient to allow restriction of HIV-1 by hTRIM5α (Yap et al., 2005). This advantage of using restriction factor of human origin with few amino acid substitutions guarantees a low risk of immune rejection in vivo. Although HIV-1 is not susceptible to restriction by TRIM5α of some species, unlike for APOBEC and tetherin, the virus has not been able to evolve a mechanism to avoid the general restrictive effects of TRIM5α, making it a good therapeutic possibility (Chan et al., 2014).

TRIM5α is a member of the large family of TRIM proteins, which has around 70 human proteins with diverse roles (Ozato et al., 2008). This family is characterized by its Triple partite Motif (TRIM), which consists of a RING, B-box and coiled coil domains (RBCC). In addition to the RBCC domains, TRIM5α also includes a C terminal SPRY domain (Li et al., 2014). SPRY is the domain that is responsible for binding of restricted virus CA and therefore the proteins specificity (Perez-Caballero et al., 2005a). Numerous other TRIM5 isoforms code for the proteins in the RBCC domains, but in which the SPRY domain is replaced by alternative sequences. These include TRIM5β, TRIM5δ and TRIM5γ (Reymond et al., 2001) . The TRIM5δ and TRIM5γ isoforms, which lack the SPRY domain, can act to inhibit TRIM5α by heterodimerization of these isoforms with TRIM5α, restricting its antiretroviral activity (Perron et al., 2004). The ability of these isoforms to inhibit TRIM5α suggests that the activity of TRIM5α in cells depends on the relative proportion of the isoforms expressed (Battivelli et al., 2011).

TRIM5 is expressed in all tissues throughout the human body, including peripheral blood lymphocytes which are the target of HIV (Reymond et al., 2001). Expression levels in many of these tissues are low, but TRIM5 expression is upregulated by IFN

through interferon-stimulated response element (ISRE). As IFN expression has a key role in the innate immunity system, it supports the role of TRIM 5 as a restriction factor in response to viral infection.

1.5.2 Mechanism of TRIM5α restriction of HIV-1

Although the exact process by which TRIM5α restricts retroviruses has not been fully determined, it is likely that TRIM5α mediates restrictive effects through several different mechanisms. TRIM5α recognizes retroviruses when they enter cells via its SPRY domain (Sebastian and Luban, 2005). It's clear that the interaction between TRIM5α and the viral CA indicate a complex interaction and that recognition between two monomers is weak (Luban, 2007). TRIM5 recognizes the complex surface of the CA and spontaneously forms a hexameric protein lattice that is greatly enhanced in the presence of incoming viral CA (Stremlau et al., 2006). This mediates more efficient capsid binding that is required for efficient restriction. It is suggested that the hexameric structure of TRIM5α multimers allows multiple SPRY domains to cover and interact with the incoming viral core with high avidity (Ganser-Pornillos et al., 2011).

Like the other family members, human TRIM5 contains an N-terminal RING domain with a B-box and a coiled-coil domain. The B box and the coiled coil domains promote the multimerization of the TRIM5 protein which is required for its restriction activity (Diaz-Griffero et al., 2009). The TRIM5 RING domain possesses an E3 ubiquitin ligase activity which can in cooperation with certain E2 enzymes allow TRIM5 self-ubiquitination that is essential for its viral restriction activity (Lienlaf et al., 2011). Mutations on the E2 interacting face can disrupt this TRIM5 autocatalytic activity and consequently blocking the restriction activity (Lienlaf et al., 2011). However, the restriction activity has been reported in the absence of the TRIM5 RING domain as well as in the absence of ubiquitination (O'Connor et al., 2010). One possible explanation for this is that aside from the capsid binding and multimerization, TRIM5α is capable of inhibiting infection in multiple steps in the restriction pathway (Berthoux et al., 2004).

In addition to CA recognition and interaction with incoming virus, TRIM5α has been identified as playing a role in cell signaling in innate immunity. TRIM5α affects NFκB signaling through two different pathways. Human TRIM5α and the mouse paralog TRIM30 downregulate NFκB signaling through proteasome independent degradation of TAB2, an adaptor protein upstream of NFκB. In contrast, human and Rhesus TRIM5α are able to activate NFκB expression. The relative activity of these two opposing effects upon NFκB by the human protein are thought to depend upon TRIM5α levels (Tareen and Emerman, 2011). TRIM5α activates NFκB signaling in conjunction with UBC13/UEV1A. Together they synthesize the K63-linked ubiquitin chains that multimerize and activate the TAK1 kinase complex (Pertel et al., 2011). TAK1 subsequently stimulates AP-1 and NFκB transcription factors involved in innate immune signaling (Deng et al., 2000). Through this function, TRIM5α and TRIM5Cyp act as a pattern recognition receptors as recognition of a restricted retroviral CA enhances the activation of NFκB signaling and innate immune response (Uchil et al., 2008).

1.6 Cyclophilins:

1.6.1 Role of Cyclophilins in the HIV-1 lifecycle:

Another protein that interacts with HIV-1 and plays an important role in its lifecycle is the peptidyl-prolyl isomerase (PPIase), cyclophilin A (CypA). Humans express at least 17 Cyps, including proteins that contain cyclophilin-like domains. CypA is a widely expressed, highly conserved protein found in both the cytosol (Harding et al., 1986)and nucleus (Arévalo-Rodríguez and Heitman, 2005). It is a globular protein consisting of a β barrel made up of eight anti-parallel β sheets, capped at both ends by an α helix.

CypA is probably the most extensively studied PPIase due to its ability to interact with HIV-1, and the key role that it plays in the virus lifecycle (Luban et al., 1993). CypA and B were found to bind to HIV-1 Gag protein (Luban et al., 1993), although only the interaction with CypA has been recorded in vivo. CypB is localised to the endoplasmic reticulum and is not thought to have the opportunity to interact with either incoming or outgoing Gag (Price et al., 1991). The active site of CypA binds

to the G89-P90 peptide bond located on a nine amino acid flexible loop (P85-P93) in the N terminus of the CA, and catalyses cis/trans isomerisation of the bond (Bosco et al., 2002) . The interaction between CA and CypA results in its incorporation into newly synthesised HIV-1 virions at a ratio of ten CA molecules to one CypA (Gamble et al., 1996). Despite its inclusion in virions, the presence of CypA in the target cell, rather than in the virus producing cell, is required for infectivity (Sokolskaja et al., 2004). Binding of CypA is not a conserved ability between all lentiviruses, but HIV-1, SIV and FIV have been shown to be targeted by CypA.

CypA acts upon incoming HIV-1 particles soon after entry and before reverse transcription, at the same time as TRIM5α restriction in non-human primates occurs (Braaten et al., 1996). It is thought that CypA binding to incoming HIV-1 CA protects the virus from restriction and that altering the isomerization state of the proline bond may make the CA more recognizable to restriction factors such as TRIM5α (Neagu et al., 2009). This is possibly by CypA mediated isomerisation that may make the viral CA more efficiently bound by TRIM5α (Berthoux et al., 2005) or by stabilizing the CA core and increasing the time for recognition by TRIM5α (Towers et al., 2003). However, even in the absence of CypA or when disrupting the CypA-CA interaction, TRIM5α is still able to restrict HIV-1 in Old World monkey cells (Nisole et al., 2004).

1.6.2 TRIMCyp fusion proteins anti-viral effect:

The species specific antiviral activity of TRIM5α confers resistance to HIV-1 in Old World monkeys. However, most New World monkey cells are susceptible to infection by HIV-1 virus (Hofmann et al., 1999). An exception is the expression of fusion protein between TRIM5 and CypA in the Aeotus genus of New World owl monkey. This has arisen from the LINE-1 (L1) mediated retrotransposition of a CypA cDNA into the TRIM5 intron 7 resulting in an in-frame fusion between exons 2 to 7 of TRIM5 and an entire CypA cDNA (Berthoux, 2004). CypA replaces the SPRY domain that is encoded by exon 8, and is linked to the RBCC domains of TRIM5 via 11 amino acids encoded by the CypA 5' UTR. Owl monkeys are homozygous for this altered gene and do not have any other TRIM5 alleles (Nisole et al., 2004).

Similarly to TRIM5α, TRIMCypA mechanism of antiviral restriction has not been fully confirmed, but it is also likely to function through multiple restriction steps during the viral life cycle. Restriction occurs rapidly after viral entry into the cell before reverse transcription, and does not require ubiquitin mediated proteasomal degradation or cytoplasmic body formation (Perez-Caballero et al., 2005b). Deletion of the RING domain causes a reduction in restriction, and deletion of the RING and B-Box2 domains eliminates restriction (Diaz-Griffero et al., 2006) implying how essential they are for efficient viral inhibition.

Like TRIM5, TRIM5Cyp forms multimers, and dimers, trimers and hexamers. Multimerization is mediated by the coiled coil domain, and this domain alone is sufficient to interact with full length TRIM5Cyp (Diaz-Griffero et al., 2006). This interaction means that mutated TRIM5Cyp proteins can elicit a dominant negative effect on native TRIM5Cyp in owl monkey cells (Nepveu-Traversy et al., 2009). The L2 region, which is critical for the higher order multimerization and therefore antiviral activity of TRIM5α (Sastri et al., 2010), is present in all naturally occurring primate TRIM5Cyp proteins.

In addition, like TRIM5α, TRIM5Cyp has been shown to disrupt CA cylinders in vitro, suggesting that at least one mechanism of antiviral activity is through interference with uncoating (Black and Aiken, 2010). Human and feline TRIM5Cyp fusion proteins have been generated and are able to restrict HIV and both HIV and FIV respectively (Neagu et al., 2009)(Dietrich et al., 2010). Human TRIM5Cyp was able to restrict HIV-1 at levels comparable to the owl monkey fusion protein when expressed in cell lines and primary T cells and macrophages (Neagu et al., 2009). It was also able to provide a potent restriction of HIV-1 in a humanized mouse model of HIV-1 (Neagu et al., 2009). Also TRIMCyp fusion proteins have been generated using alternative TRIM proteins to TRIM5, but that have the same domain structure (Yap et al., 2006) and Cyp has been fused to Fv1 to produce an inhibitor of HIV-1 (Schaller et al., 2007). These data suggest that fusion of CypA to the effector domain of restriction factors mediates efficient viral CA binding and is an effective method for the generation of more potent HIV-1 restriction factors.

1.7 The MX proteins family:

1.7.1 Identification of MX proteins as an antiretroviral restriction factor:

Previously we discussed how IFN is the key component of the innate immunity response to exogenous pathogens, by inducing the transcription of many IFN-stimulated genes (ISGs). The MX proteins are among the most potent antiviral effector proteins. These proteins belong to dynamin-like large GTPase protein family and they differ from the other IFN-inducible GTPases by their stronger induction by type I and type III IFNs as compared to IFNγ (Holzinger et al., 2007). The MX family comprises Mx1 and Mx2 in mice, and MXA and MxB in humans. MX1 localizes at the nucleus, whereas MxA and Mx2 are located near the smooth ER. MxB is found in the intranuclear and the cytoplasmic face of nuclear pores. Their discovery dates back on the genetic studies on inborn resistance of mice to influenza viruses about 60 years ago (LINDENMANN, 1962). Mx1 protein was cloned from influenza virus resistance mice and was shown to mediate cell intrinsic antiviral activity (Dreiding et al., 1985)(Staeheli et al., 1986). Human MX proteins were identified from cross interactions of a monoclonal antibody against mouse MX1 in human cells (Staeheli and Haller, 1985). Subsequently, MxA and MxB were found to be encoded by closely linked genes on human chromosome 21(Reeves et al., 1988).

Like most dynamin-like GTPases, Mx proteins are composed of an amino-terminal (N-terminal) GTPase (G) domain, a central middle domain (MD), and a carboxy-terminal (C-terminal) GTPase effector domain (GED). MX proteins inhibit replication of a board range of RNA viruses and some DNA viruses. The mechanism of MX action has been extensively studied, but is still not entirely clear. Dependent on their localization MX proteins can recognize and trap essential viral structures, the main targets appear to be the viral CA protein. Cytoplasmic human MXA is able to suppress a broad range of different virus classes, including members of the bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, toga-viruses, picornaviruses, reoviruses and hepatitis B virus (HBV) (Haller and Kochs, 2002) (Haller et al., 2007). Recently, the antiviral potential of human MxB was identifined against multiple HIV virus strains (Goujon et al., 2013), which makes this gene a new promising aspect in HIV treatments.

In an early stage study, scientists have identified MxB as a potent anti-viral IFN-induced gene and a key point of vulnerability in HIV life cycle (Kane et al., 2013). They released the HIV virus into two distinct cell lines. One cell line had the MxB gene “switched on,” while the other cell line had no MxB gene expression. On monitoring the results, the scientists identified that in the cells in which the MxB gene was expressed, the HIV virus was not able to duplicate, consequently preventing new viruses from being generated. On the other hand, in the cell line in which the MxB gene was turned off, the HIV virus has propagated (Goujon et al., 2013). Even though the mechanistic details of how MxB inhibits HIV-1 are currently unclear, the MxB gene seems to play an essential role to initiate viral control in HIV patients which can be highly promising in the AIDS gene therapy field.

1.7.2 Mechanism of MxB restriction of HIV-1:

Recent research has pointed out that HIV of IFN-induced myxovirus resistance B (MxB) is an important gene against AIDS (Goujon et al., 2013). More specifically, human MxB efficiently restrict infection by various HIV-1 strains, however it’s less active against HIV-2 and SIVs and it has no significant inhibition of feline immunodeficiency virus (FIV) or murine leukemia virus (MLV) (Goujon et al., 2013; Kane et al., 2013). MxB restrict HIV-1 infection at a late post-entry stage, however recent findings raise many interesting regarding its mechanism of action. One study demonstrated that MxB blocks specifically the accumulation of integrated proviral DNA, it impairs the chromosomal integration rather than the nuclear entry of HIV-1 DNA (Liu et al., 2013). In a series of well-controlled experiments, genetic evidence suggested that HIV-1 capsid is the determinant for the MxB antiviral ability (Goujon et al., 2013; Kane et al., 2013; Liu et al., 2013), as mutations in HIV-1 virus capsid protein such as P90A, G89V, and N57S caused the escape from MxB restriction. These experiments implied that MxB is directly interacting with HIV-1 core, however the ability of MxB to associate with HIV-1 cores has not been explored yet.

Recent research evidence suggested that MxB might be interfering with more than one process during the viral life cycle. It revealed that the 20 N-terminal amino acids are responsible for the ability of MxB to bind to HIV-1 Capsid protein during infection

(Fricke et al., 2014). Interestingly, the binding of MxB to HIV-1 core proteins inhibits the virus uncoating process (Fricke et al., 2014). The evidence behind the avidity of interaction between the MxB protein and HIV-1 capsid was traced back to the C-terminal leucine zipper of MxB (Fricke et al., 2014). In addition to its prevention of the viral capsid uncoating process, evidence suggested that MxB plays an important key role during nuclear import and nuclear maturation of the HIV-1 preintegration complex (Fricke et al., 2014; Kane et al., 2013; Liu et al., 2013). Many open questions remain and are expected to stimulate research on MxB gene. A great hope we suggest in this thesis as an initial step to control HIV infections and AIDS.

1.8 Thesis activity:

Gene therapy offers potential for a single long term treatment of HIV-1, eliminating the requirement of intensive drug regimens. Many anti-HIV transgenes have been proposed for modification of T cells to inhibit HIV-1 infection. Creating two new foamy virus vectors for AIDS gene therapy using SFFV and EF1α promoters and the humanized TRIM5αCypA and human MxB anti-HIV genes, testing their HIV restriction potency and whether each vector can be produced at high titers. In future, these vectors can be tested for high transgene expression and safety with the goal of obtaining clinically relevant vectors for AIDS gene therapy.

2 Material and Methods

2.1 Material

Unless otherwise stated, all enzymes for molecular cloning were supplied by New England Biolabs.Inc and sequencing primers by Integrated DNA technologies (IDT.Inc). Moreover, DNA sequencing reactions were performed by Macrogen Korea using HiSeq sequencing system.

2.1.1 General reagents:

λ DNA-HindIII Digest marker	New England Biolabs
Agarose	Genepure LE
Ampicillin	Fisher
Kanamycin Monosulfate	Fisher
Poly-L-lysine	Sigma-Aldrich
Live/Dead fixable blue cell stain	Sigma-Aldrich
Quick load purple 2-log DNA ladder	New England Biolabs
TRIzol	Life Technologies
Ethidium Bromide Solution	EMD Millipore
Ethanol 200 Proof	Decon Labs Inc.
2-propanol	Fisher
Chloroform	Avantor J. T Baker

2.1.2 Buffers:

Tris-Base Molecular biology Grade	Fisher
Tris-HCL	Fisher
50x TAE	Fisher
D-PBS	Fisher
Gel loading dye Blue (6x)	New England Biolabs
10x buffer for T4 DNA Ligase	New England Biolabs

*2.1.3 **Antibodies:***

G418 Sulfate	EMD Millipore
Puromycin Dihydrochloride	VWR
Hygromycin B solution	Fisher
Penicillin-Streptomycin solution	Sigma-Aldrich

*2.1.4 **Kits:***

Wizard Plus SV Minipreps DNA purification	Promega Scientific
Purelink HiPure Plasmid Midiprep kit	Invitrogen, Life Technologies
Purelink HiPure Plasmid Maxiprep kit	Invitrogen, Life Technologies
Zymoclean Gel DNA recovery kit	Genesee
Puregene Cell & Tissue (DNA extraction)	QIAGEN
Zero-blunt Topo PCR cloning kit	Invitrogen, Life Technologies
Transcriptor First Strand cDNA synthesis kit	Roche

*2.1.5 **Cells:***

- *Bacteria*

E. cloni Competent Cells, Lucigen - 10G ELITE Electrocompetent > 2 × 10^{10} cfu/µg pUC DNA

E. cloni 10G Electrocompetent Cells are E. coli strains optimized for high efficiency transformation. They give high yield and high quality plasmid DNA due to the endA1 mutation. E. cloni 10G contain the inactive mcr and mrr mutations, allowing methylated genomic DNA that has been isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements. The rpsL mutation confers resistance to streptomycin.

E. cloni 10G genotype:

F - mcrA Δ(mrr-hsdRMS-mcrBC) endA1 recA1 ϕ80dlacZΔM15 ΔlacX74 araD139 Δ(ara,leu)7697 galU galK rpsL (StrR) nupG λ– tonA

- *Mammalian cell lines*

HEK293T	Human embryonic kidney cell line
HT1080	Human fibrosarcoma cell line
$CD4^+$CEMx174	Human somatic hybrid culture between human T cell line CEM and human B cell line 721.174
MAGI-CCR5 cells	HeLa cell clones expressing human CD4, CXCR4 and CCR5 receptors.

*2.1.6 **Media:***

- *Bacteria*

LB broth, Miller	Sigma-Aldrich
LB Agar, Bacto	VWR

- *Mammalian cells*

Fetal bovine Serum	Atlanta Biologicals
DMEM/ high glucose	Fisher

Iscove's Modified Dulbecco's Medium	Lonza
Trypsin-versene mixture	Lonza
Dimethyl sulfoxide	MP biomedicals, LLC

2.1.7 PCR Primers:

GoTaq DNA polymerase master mix	Fisher
Primers	IDT.Inc

Table 1 Table of primers used in the study

Primer name	Sequence
Trim5αCypA Fwd	AGCTCCTGATCTGAAAGGAATG
Trim5αCypA Rev	CCTGACACATAAACCCTGGAATA
MxB Fwd	CCCCTGAAAAAGGGCTACAT
MxB Rev	ACACCGGCCTTATTCCAAG

2.1.8 Devices:

Table 2 Table of devices used in the study

Name	Vendor
Leica DM4000 B LED	Leica Microsystems
BD Accuri™ C6	BD biosciences
S3e™ Cell sorter	BIORAD
PCR icycler	BIORAD
2720 Thermal Cycler	Applied Biosystems® LifeTech
Beckman I7 65 Ultracentrifuge	Beckman Coulter
Avanti J-26 XP centrifuge	Beckman Coulter
CR312 Refrigerated Centrifuge	Jouan Inc.
ChemiDoc™ MP System	BIORAD
NanoDrop 2000 Spectometer	Fisher

2.1.9 Software:

Table 3 Table of software used in the study

Name	Used for
NanoDrop 2000/2000c	Spectrophotometer
Image Lab™ software	Biorad gel documentation system
ProSort™ software	S3e Cell sorter instrumental control and analysis
BD Accuri™ C6	Flow cytometry sample analysis
LAS AF software	Fluorescence multichannel imaging documentation system

2.1.10 Genes:

- *MxB gene*

Provided by GeneArt® Gene Synthesis, Life Technologies
MxB Codon optimized sequence:

Sbf1 Kozak Seq.

ATCTGCCGCCACCTGCAGGGCCACCATGAGCAAGGCCCACAAGCCCTGGCCCTACAGAAGGCGGAGCCAGTTCA
GCAGCCGGAAGTACCTGAAAAAAGAGATGAACAGCTTCCAGCAGCAGCCCCCTCCCTTCGGAACTGTGCCCCCCC
AGATGATGTTCCCCCCAAATTGGCAGGGCGCCGAGAAGGATGCCGCCTTCCTGGCCAAGGACTTCAACTTCCTGA
CCCTGAACAACCAGCCCCCACCCGGCAACAGATCCCAGCCTAGAGCCATGGGCCCCGAGAACAACCTGTACAGCC
AGTACGAGCAGAAAGTGCGGCCCTGCATCGACCTGATCGACAGCCTGAGAGCACTGGGCGTGGAACAGGATCTG
GCCCTGCCTGCCATTGCCGTGATCGGCGATCAGAGCAGCGGCAAGAGCAGCGTGCTGGAAGCACTGAGCGGAGT
GGCTCTGCCTAGAGGCAGCGGCATCGTGACCAGATGCCCCCTGGTGCTGAAGCTGAAGAAGCAGCCTTGCGAGG
CCTGGGCCGGCAGAATCAGCTACAGAAACACCGAGCTGGAACTGCAGGACCCCGGCCAGGTGGAAAAAGAGATC
CACAAGGCCCAGAACGTGATGGCCGGCAACGGCAGAGGCATCAGCCACGAGCTGATCTCCCTGGAAATCACCAG
CCCCGAGGTGCCCGACCTGACCATCATCGATCTGCCCGGCATCACCAGAGTGGCCGTGGACAATCAGCCCCGGGA
TATCGGCCTGCAGATCAAGGCCCTGATCAAGAAGTACATCCAGCGGCAGCAGACCATCAACCTGGTGGTGGTGCC
CTGCAACGTGGACATTGCCACCACAGAGGCCCTGTCCATGGCCCACGAAGTGGACCCTGAGGGCGACAGAACCAT
CGGCATCCTGACAAAGCCCGATCTGATGGACCGGGGCACCGAGAAGTCCGTGATGAACGTCGTGCGGAACCTGA
CCTACCCCCTGAAAAAGGGCTACATGATCGTGAAGTGCAGAGGCCAGCAGGAAATCACAAACCGGCTGAGCCTG
GCCGAGGCCACCAAAAAAGAGATCACCTTCTTCCAGACCCACCCCTACTTCCGGGTGCTGCTGGAAGAGGGCTCT
GCCACCGTGCCTAGACTGGCCGAGAGACTGACCACCGAACTGATCATGCACATCCAGAAGTCCCTGCCCCTGCTG
GAAGGCCAGATCAGAGAGAGCCACCAGAAGGCCACCGAGGAACTGAGAAGATGCGGAGCCGACATCCCCAGCC
AGGAAGCCGACAAGATGTTCTTCCTGATCGAGAAGATCAAGATGTTTAACCAGGACATCGAGAAGCTGGTGGAA
GGCGAGGAAGTCGTGCGCGAGAACGAGACACGGCTGTACAACAAGATTCGCGAGGATTTCAAGAACTGGGTGG
GAATCCTGGCCACCAACACCCAGAAAGTGAAGAATATCATCCACGAGGAAGTGGAGAAGTACGAGAAGCAGTAC
CGGGGCAAAGAACTGCTGGGCTTCGTGAACTACAAGACCTTCGAGATCATCGTGCACCAGTATATCCAGCAGCTG
GTGGAACCCGCCCTGAGCATGCTGCAGAAAGCCATGGAAATCATTCAGCAGGCCTTCATCAACGTGGCCAAGAAG
CACTTCGGCGAGTTCTTCAACCTGAACCAGACCGTGCAGAGCACCATCGAGGACATCAAAGTGAAGCACACCGCC
AAGGCCGAGAATATGATCCAGCTGCAGTTCAGGATGGAACAGATGGTGTTCTGCCAGGACCAAATCTACAGCGTG
GTGCTGAAAAAAGTGCGCGAGGAAATCTTCAACCCCCTGGGCACCCCTAGCCAGAACATGAAGCTGAACAGCCAC
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AAGCTATGATGCAGATTCTGCAGGAAAAGAACCGGTACAGCTGGCTGCTGCAGGAACAGAGCGAGACAGCCACC
AAGCGGCGGATCCTGAAAGAGCGGATCTACCGGCTGACCCAGGCCAGACATGCCCTGTGCCAGTTCTCCAGCAAA
GAGATTCACTGACCGCGGATCCGCCCCTCTCCCT

SacII

- *Humanized TRIM5α-Cyp A fusion gene:*

Provided by gBlock, IDT Inc.

Trim5a-CypA Sequence

ATGGCTTCTGGAATCCTGGTTAATGTAAAGGAGGAGGTGACCTGCCCCATCTGCCTGGAACTCCTGACACAACCCC
TGAGCCTGGACTGCGGCCACAGCTTCTGCCAAGCATGCCTCACTGCAAACCACAAGAAGTCCATGCTAGACAAAG
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TCAACCCCACCGTGTTCTTCGACATTGCCGTCGACGGCGAGCCCTTGGGCCGCGTCTCCTTTGAGCTGTTTGCAGA
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2.1.11 Parental plasmids:

pFV-EC46-IMPG-EGFP-W2: self-inactivating foamy vector backbone containing codon optimized C46 anti-HIV gene, MGMT (O^6-methylguanine-DNA methyl-transferase) coding gene, and an enhanced green fluorescent protein (EGFP) coding gene. These genes are driven by human elongation factor 1 type α (EF1α), an internal ribosomal entry site (IRES) and phosphoglycerate kinase (PGK) promoters, respectively. Ending with Woodchunk post transcriptional regulatory element (WPRE).

pFV-SFFV-hTCypA-IMPG-EGFP-W2: a SIN foamy vector backbone containing a codon optimized humanized TRIM5αCypA fusion anti-HIV gene driven by spleen focus forming virus (SFFV) promoter. Followed by MGMT driven by IRES and EGFP gene driven by PGK.

pFV-SFFV-IMPG-EGFP-W2: previously created control vector. A SIN foamy vector with same backbone of the previously described vectors, but without any anti-HIV gene.

PCR-BluntII-TOPO: linearized and topo isomerase 1 activated PCR bluntII topo vector used for subcloning. Provided by Invitrogen, Life Technologies Inc. The vector contains the ccdB gene fused to C-terminus of LacZα fragment which expression is disrupted by the ligation of desired gene.

pFV-SFFV-C46-IMPG-EGFP-Neo-W /pFV-EF1a-C46-IMPG-EGFP-Neo-W:

SIN foamy vector backbone containing a codon optimized humanized C46 anti-HIV gene driven by SFFV/EF1α promoter. Followed by MGMT driven by IRES and EGFP gene with Neomycin resistance site driven by PGK.

2.1.12 Generated plasmids:

pFV-EF1α-hTCypA-IMPG-EGFP-W: TRIM5αCypA insert fragment was extracted from pFV-SFFV-hTCypA-IMPG-EGFP-W vector and ligated into the recipient foamy virus backbone driven by EF1α promoter. Cloning using SacII and Sbf1 restriction enzymes.

pCR-TOPO-MxB: Codon optimized MxB gene fragment was cloned into PCR-BluntII Topo vector backbone by Topo cloning.

pFV-SFFV-MxB-IMPG-EGFP-W / pFV-EF1a-MxB-IMPG-EGFP-W:

MxB insert fragment was extracted from pCR-TOPO-MxB vector and ligated into the recipient foamy virus backbone driven by SFFV /EF1α promoter. Cloning using SacII and Sbf1 restriction enzymes.

pFV-SFFV-hTCypA-IMPG-EGFP-NeoW/pFV-EF1a-hTCypA-IMPG-EGFP-NeoW:

TRIM5αCypA insert fragment was extracted from pFV-SFFV-hTCypA-IMPG-EGFP-W vector and ligated into the recipient foamy virus backbone with Neomycin

resistance domain driven by SFFV/EF1α promoter. Cloning using SacII and Sbf1 restriction enzymes.

2.2 Methods:

2.2.1 TOPO cloning:

Gel extracted and purified DNA fragments was ligated pCR-BluntII-TOPO plasmid following manufacturers guidelines, which involved the incubation of DNA with salt solution, water and vector for 5 minutes at room temperature. The resultant plasmid was used to transform electrocompetent *E.cloni* cells by electroporation, which were then plated onto kanamycin LB agar plates. The generated plasmid was extracted from colonies.

2.2.2 Restriction endonuclease digest:

DNA plasmids were digested in a final volume of 20µl containing NEB buffer (provided by BioLabs) and one or more desired restriction enzymes. The reaction is incubated at 37ºC overnight.

2.2.3 Agarose gel electrophoresis:

Agarose gels were made by dissolving 0.8% agarose in 1×TAE buffer and boiling it until homogenous solution developed. Then, the solution is poured in a horizontal gel chamber and cooled to room temperature. After the gel is solidified, the diluted samples with 6× loading buffer are loaded and separated under an applied voltage of 110V. When the samples run is done, the gel is stained with ethidium bromide diluted solution (5µl EtBr in 200 ml 1×TAE). Then, Images are documented using UV transilluminator Chemidoc™ system.

2.2.4 Purification of desired DNA fragments:

Desired DNA bands were cut out of the agarose gel using a razor blade. Then, DNA fragments were purified using Zymoclean gel DNA recovery kit, as recommended by the manufacture.

2.2.5 DNA ligation:

Digested DNA insert was ligated into plasmid backbone in approximately 1:3 molar ratio using 0.5µl of T4 DNA ligase and 1µl 10×T4 DNA ligase buffer (supplied by BioLabs) in a final volume of 10 µl. The mixture was incubated for 2 hours at room temperature.

2.2.6 Transformation of electrocompetent cells E.cloni:

In order to remove excess salts and decrease the chance of arching during electroporation we dialyze ~ 6µl of the DNA ligation product. We transfer 10 µl E.cloni thawed cells into a tube containing the dialyzed sample. Then, we place the mix into the Electroporator with the voltage of 180V for 4 (msec). Following that, we transfer the transformed cells into 1ml of SOC-recovery media and let them grow for one hour at 37ºC and then spread them on agar plates containing appropriate antibiotic. For an optimal colony growth, the agar plates were incubated at 37ºC overnight.

2.2.7 Plasmid DNA preparation:

E.cloni transformed with plasmid was grown overnight in LB broth with the appropriate antibiotic. Plasmid was extracted by Wizard Plus Minipreps DNA purification kit (provided by Promega Scientific) or HiPure Plasmid Maxiprep kits (provided by Invitrogen, Life Technologies) according to manufacturer's instructions. DNA concentrations was measured using a Nanodrop 2000 spectrophotometer at wavelength 260nm.

2.2.8 Preparation of glycerol stocks:

To assure expression under identical conditions in future experiments and create backups of generated expression constructs. Therefore, glycerol stocks were prepared by mixing equal amounts of grown culture with 50% glycerol and direct freezing in -80ºC.

2.2.9 Cell cultures:

CEM×174 and MAGI-CCR5 cell lines were obtained from the National Institute of Health acquired immunodeficiency syndrome Research & Reagent Program.

CEM×174 cells were cultured in IMDM medium with 10% (vol/vol) FBS and MAGI-CCR5 cells were cultured in Iscove's Modified Dulbecco's medium with 10% FBS, 1% penicillin/streptomycin, 0.2 mg/ml G418, 0.1 mg/ml hygromycin B and 1µg/ml puromycin.

2.2.10 Construction and production of anti-HIV foamy virus vectors:

FV vector stocks will be produced by polyethylenimine (PEI) mediated transfection of HEK293T cells with helper packaging plasmids and the anti-HIV gene transfer foamy virus vector plasmids. 14µg of the gene transfer vector plasmid, 14 µg of the foamy Gag helper, 5.6µg of the Pol helper and 1.4µg of the Env helper were mixed in 2ml of serum free DMEM medium. 105 µl of 1 µg/µl PEI was added to the plasmid DNA mixture and incubated at room temperature for 15 minutes. The DNA/PEI complex was added to the cells grown in 10cm culture plates. Approximately 16 hours after transfection the medium was replaced with complete DMEM medium supplemented with 10% FBS. Culture supernatant containing virons are harvested, concentrated to 100x by centrifugation at 19000 rpm for 2 hr at 20°C and frozen in 5% DMSO at -80ºC.

2.2.11 Titration of foamy virus vectors:

DMSO was removed from FV frozen stocks by dialysis with IMDM using a Microcon Ultracel YM-50 Centrifugal Filter (provided by Millipore). FV titers will be determined by transducing HT1080 cells with serial dilutions of thawed dialyzed concentrated and unconcentrated vector stocks and evaluated after 72hr of transduction for GFP expression using C6 Accuri™ flow cytometer and C6 software. The vector titers in transducing units per ml (TU/ml) was calculated from GFP positive cells.

2.2.12 Spin-inoculation transduction of anti-HIV foamy virus vectors:

1×10^5 CEM×174 cells were transduced with FV stocks at a MOI of 0.5 (based on titers measured by HT1080 cells) in IMDM media by spin-inoculation. Transduced cells in TC culture plates were sealed with parafilm and centrifuged at 1200×g for 2 hours at 25ºC. The essence of this method is to increase transduction efficiency and to monitor GFP expression at the end of transduction (the following 3-4 days).

2.2.13 G418 Selection:

1×10^5 CEM×174 cells were transduced with FV vectors containing a neomycin resistant domain (at MOI of 0.5) by spin-inoculation. After 2 days of transduction, cells were grown in media supplemented with 600 µg/ml of G418 until we attained >95% GFP marking by transduced cells.

2.2.14 HIV infections of transduced CEM×174 cells:

For in vitro HIV challenge assay, 1×10^5 G418 selected/GFP sorted FV-transduced 174xCEM cells were plated in a 12-well plate and incubated at 37°C in 5% CO2. Next day, cells were infected with HIV89.6 at a MOI of 0.05. Every 4 days, the cells were reloaded with fresh culture media. Culture supernatant containing infectious particles was collected at 7, 14 and 21 days post infection and centrifuged to remove the cell debris. The titer of HIV-1 in the culture supernatants of was determined by MAGI assay.

2.2.15 MAGI assay:

8×10^4 MAGI-CCR5 cells/well in 12 well plates will be exposed to the supernatant containing virus in complete DMEM supplemented with 1 µg/ml puromycin and 20 µg/ml DEAE-Dextran. Two hours after exposure, the media is replaced with complete DMEM containing G418, hygromycin and puromycin. After 48 hr of incubation, cells will be fixed with 3.7% formaldehyde and stained for β-galactosidase expression. In this assay, HIV-1 infection results in the inducing the expression of a stable, integrated bacterial β-galactosidase (β-Gal) gene which is under the transcriptional control of HIV-1 long terminal repeat (LTR). The expressed β-Gal enzyme can be detected colorimetrically where the cells turn blue in presence of X-gal substrate. The number of blue color forming cells indicates the number of infectious HIV-1 particles present in the supernatant used in the MAGI assay. This will be used to determine the efficacy of the therapeutic FV vector to inhibit HIV.

2.2.16 Genomic DNA extraction:

DNA was purified from transduced CEM×174 cultured cells in 10 cm plate using the Puregene Cell & Tissue DNA extraction kit (provided by QIAGEN) under the

instructions provided by the manufacturer. The supernatant containing genomic DNA was removed and stored at -20ºC.

2.2.17 Total RNA isolation and reverse transcription:

RNA isolation and purification is one of the key factors for the RT-PCR assays and other related molecular biology detections. TRIzol Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. During sample homogenization, TRIzol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol.

Reverse transcription was performed on RNA samples using Transcriptor First Strand cDNA synthesis kit provided by Roche as recommended by the manufacturer with random hexamer primers. Followed by denaturation of the template-primer mixture by heating at 65ºC in a thermal block cycler. After the addition of all reagent, we start the RT reaction with incubation for 10 min at 25ºC followed by 30 min at 55ºC. Inactivation of the transcriptase is done by heating to 85ºC for 5 min then storing the obtained cDNA containing samples at -20ºC, to be used later for PCR analysis.

2.2.18 PCR:

100 ng of DNA was used as template and mixed on ice with GoTaq PCR Master Mix Buffer, 1µM of each primer in 20 µl volume. Initial denaturation was performed at 95ºC for 5 minutes, then 30 amplification cycles were carried out, denaturation at 95ºC for 45s, primer annealing 58.5ºC for 45s, extension at 72ºC for 45s, and final extension step for 10 minutes at 72ºC, followed by incubation at 4ºC till the PCR products are loaded on a gel.

3 Results

3.1 Evaluation of humanized TRIM5α-Cyclophilin A fusion effect on foamy virus vector transduction titers:

TRIM5α-CyclophilinA fusion gene was previously shown to inhibit HIV-1 replication in primary CD4+ T cells and macrophages and confers a survival advantage to these cells without disrupting their function (Neagu et al., 2009). In this study, a foamyviral vector expressing this anti-HIV transgene was tested for its ability in effective transgene delivery at detectable levels and its potency in blocking HIV replication. Furthermore, to test the efficacy of housekeeping gene promotors in driving anti-HIV transgenes and inhibiting anti-HIV replication in human cells, we used EF1α promoter which has also been shown that it provides high levels of transgene expression in transduced hematopoietic cell and less likely to cause any aberrant activation (Zychlinski et al., 2008). For this, pFV-EF1α-C46-IMPGKW was digested with SbfI and SacII to release C46 transgene and then ligated with SbfI, SacII digested TRIM5αCypA gene fragment (provided by gBlock) released from pFV-SFFV-TRIM5αCypA-IMPGKW foamyviral plasmid. A foamyviral plasmid without an anti-HIV transgene pFV-SFFV-IMPGKW was used as a control plasmid in our experiments. For selection of cells transduced with these foamyviral vectors we replaced EGFP with EGFP-Neomycin fusion gene by subcloning TRIM5αCypA transgene into BglII, MluI digested foamy viral plasmid backbones pFV-SFFV-C46-IMPGK-N-W and pFV-EF1a-C46-IMPGK-N-W. Foamyviral stocks were produced by polyethylenimine mediated transfection of HEK293 cells along with foamyviral packaging plasmids. Images of HEK293 cells transfected with FV plasmids were visualized using fluorescence microscopy (Figure 5). Culture supernatant containing vector particles was harvested at 72 hours after transfection, concentrated 100 folds by ultracentrifuge at 19,000 rpm at 20ºC. FV titers were determined by transducing HT1080 cells with serial dilutions of foamy vector stock. Transduced HT1080 cells were evaluated for GFP expression by flow cytometry as shown in Table (4).

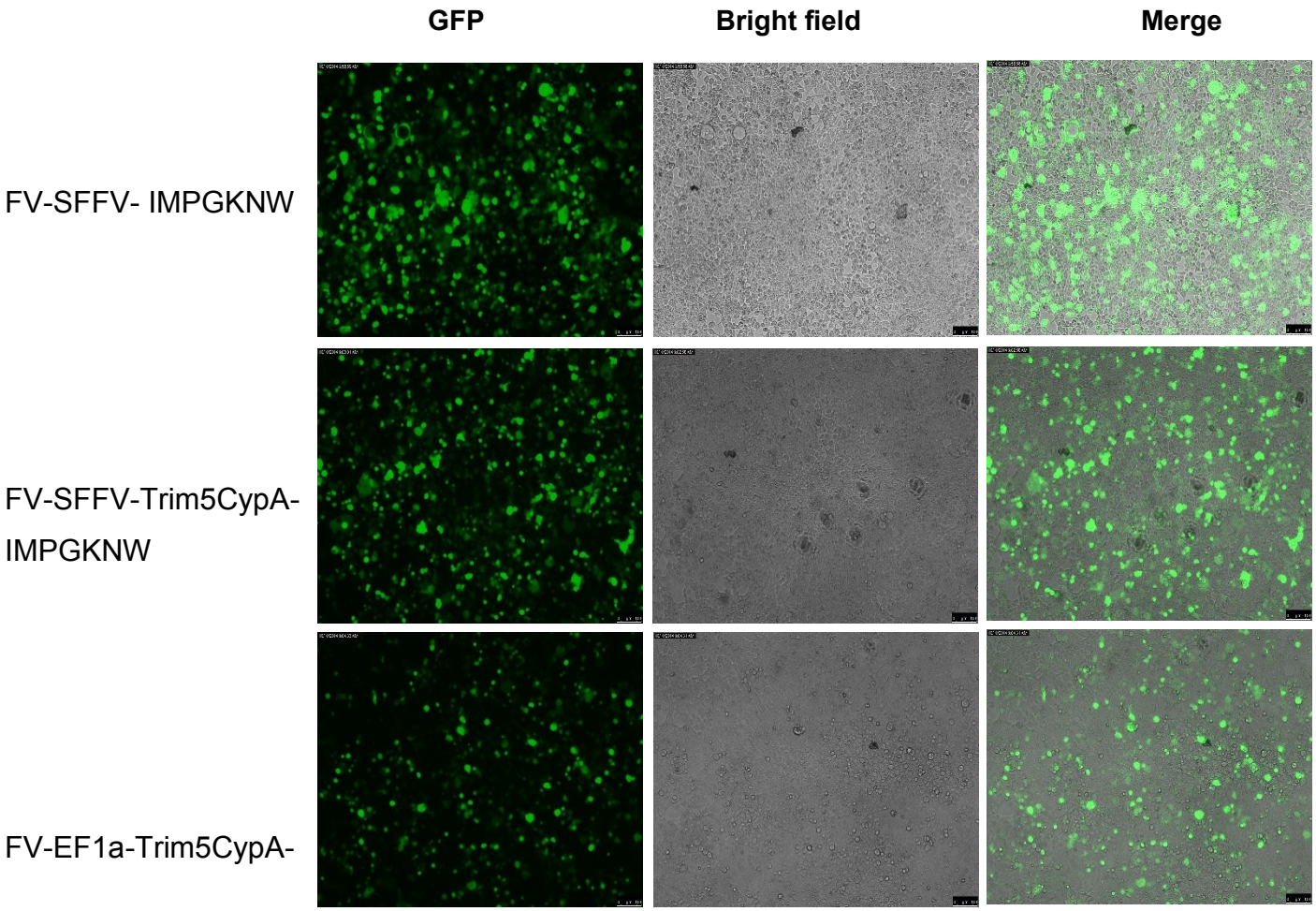

Figure 5 Foamyviral vector transfections efficiencies in HEK293 cell cultures. Transfections of foamy viral vectors expressing Trim5CypA transgene compared with the control vector FV-SFFV-IMPGKNW. Using fluorescence microscopy we were able to check cells with GFP encoding plasmid as they fluorescent in green. Scale bar 100 µm.

Vector Name	Promoter	Anti-HIV gene	Estimated Transfection efficiency	Concentrated vector titer (TU/ml)
FV-SFFV- IMPGKNW	SFFV	None	~60%	6.25×10^{6}
FV-SFFV-TRIM5αCypA-IMPGKNW	SFFV	TRIM5αCypA	~50%	1.78×10^{7}
FV-EF1α-TRIM5αCypA-IMPGKNW	EF1a	TRIM5αCypA	~40%	6.00×10^{6}

Table 4 **Summary of foamy viral vectors expressing TRIM5αCypA fusion compared to the control vector with their transfection efficiencies and titers indicated in TU/ml.** For each vector, the type of promoter, anti-HIV gene, the estimated transfection efficiency, and concentrated titer are given. A viral promoter indicates the strong spleen focus-forming virus promoter (SFFV), and a housekeeping promoter indicates the weaker cellular elongation factor 1-alpha (EF1α) promoter.

3.2 Evaluation of MxB (Myxovirus resistance protein 2) effect on foamy virus vector transduction titers:

MxB Myxovirus resistance B was identified recently as an ISG (IFN-stimulated genes) that contributes to the inhibition of HIV-1 replication by type I IFN at an early stage post infection, after reverse transcription but prior to proviral integration into host DNA (Kane et al., 2013). Moreover, recent studies have demonstrated that MxB expression in human osteoblast cell line (HOS) inhibited infection by GFP reporter viruses based on a variety of primate lentiviruses, including simian immunodeficiency viruses SIVMAC, SIVAGMTan and SIVAGMSab (Goujon et al., 2013)(Kane et al., 2013), which led to lowering the viral titers of these vectors. Toward this end, we tested MxB expression effects on foamyviral vector titers in order to obtain more efficient transgene delivery. For this, we optimized the MxB protein sequence to improve the protein expression in living organism by increasing the translational efficiency of our gene using GeneArt® codon optimization tool.

Following the codon optimization procedure we checked for the presence of any polyadenylation signals, because any insertion of a premature polyadenylation signal between the LTRs will reduce full length vector transcription which will interfere with the production of vector expressing the transgene. After obtaining the codon optimized MxB (provided by Lifetechnologies.Inc), we cloned MxB gene fragment in our previously constructed potent foamy viral vectors plasmids pFV SFFV-C46-IMPGKW and pFV EF1α-C46IMPGKW after releasing the C46 fragment using SbfI and SacII restriction enzymes. Then, FV vector stocks were produced by polyethylenimine (PEI) mediated transfection of HEK293 cells with helper packaging plasmids and the MxB anti-HIV gene transfer foamy virus vector plasmids. After transfection, culture supernatant containing concentrated virons were harvested and frozen in 5% DMSO -80ºC. FV titers were determined by transducing 10^5 HT1080 cells in triplicate with unconcentrated vector stocks of our MxB expressing foamy vector FV-SFFV-MxB-IMPGKW and a control vector with the same backbone but without an anti-HIV transgene FV-SFFV-IMPGKW. Titers were evaluated three days after vector exposure for GFP expression using flow cytometry. The percentage of EGFP positive cells was determined by a gate set using untransduced cells to define the negative population.

Means for the triplicate titers were calculated for each vector with the standard deviations shown by the error bars in Figure 6 below.

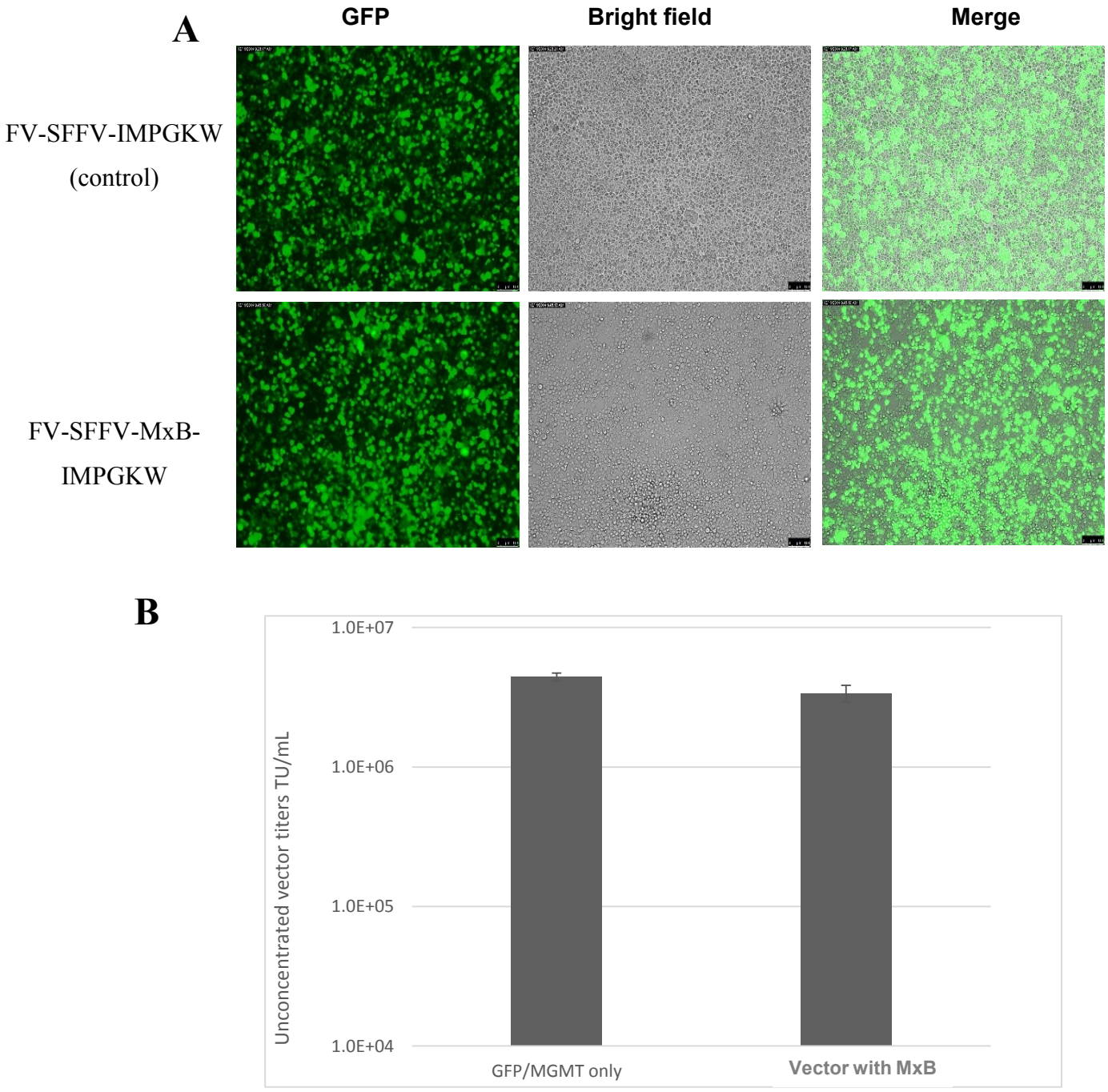

Figure 6 Analysis of MxB expression effects on foamyviral vector gene delivery system. A) Comparison between the GFP expression efficiencies in HEK293 cells of two foamy vectors, one expressing MxB anti-HIV gene and another control vector with same backbone but without an anti-HIV gene effect. Both transfection efficiencies were similar (~75% GFP positive cells) which indicate that MxB2 expression did not impact foamy viral vector transfections in HEK293 cells. Scale bar 100µm**. B)** Foamy vector titers comparison between MxB expressing vector and a control foamy vector. Unconcentrated titers (in TU/ml) represent the mean titers of triplicate transductions from a single vector stock preparation for each vector. Error bars address any variation in the transduction triplicates experiment (n=3).

3.3 Comparision of foamy virus vectors constructs transfection and transduction efficiencies:

Using polyethylenimine mediated transfection method with the proper ratios of Gag, Pol and Env helper packaging plasmids, virions for each of the constructed vectors (Figure 7) were produced by transfecting HEK293 cells along with three controls, the previously developed potent foamy vector FV-EF1a-C46-IMPGW and a previously created vector with the same backbone but without an anti-HIV gene, FV-SFFV-IMPGW. Three days following the transfections, EGFP fluorescent cells were visualized using Leica Microsystems Fluorescence Microscopy and the transfection efficiencies were estimated as shown in (Figure 8).

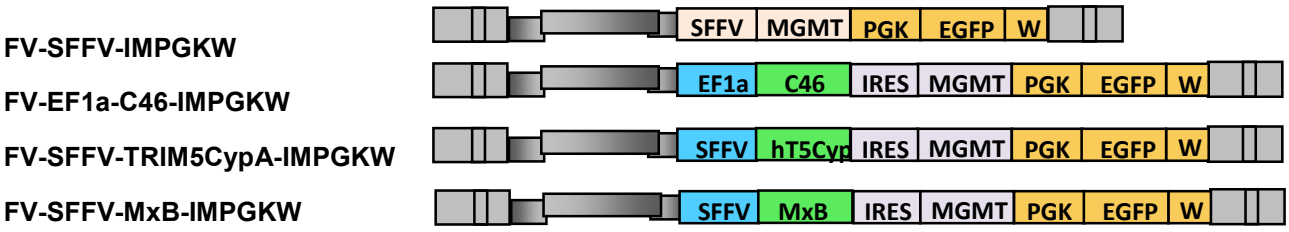

Figure 7 Foamy virus vector genomes. The FV-SFFV-IMPGKW foamy vector is a previously created control vector without an anti-HIV gene. Another control, FV-EF1a-C46-IMPGKW vector was used as a backbone to construct the other new generated vectors, and it has previously shown to provide potent inhibition of HIV replication *in vitro* with high titers. The bottom two vectors containing each an anti-HIV gene TRIM5CypA and MxB, respectively, are used to show the effects of those genes on foamy virus vector transduction titers and efficiencies.

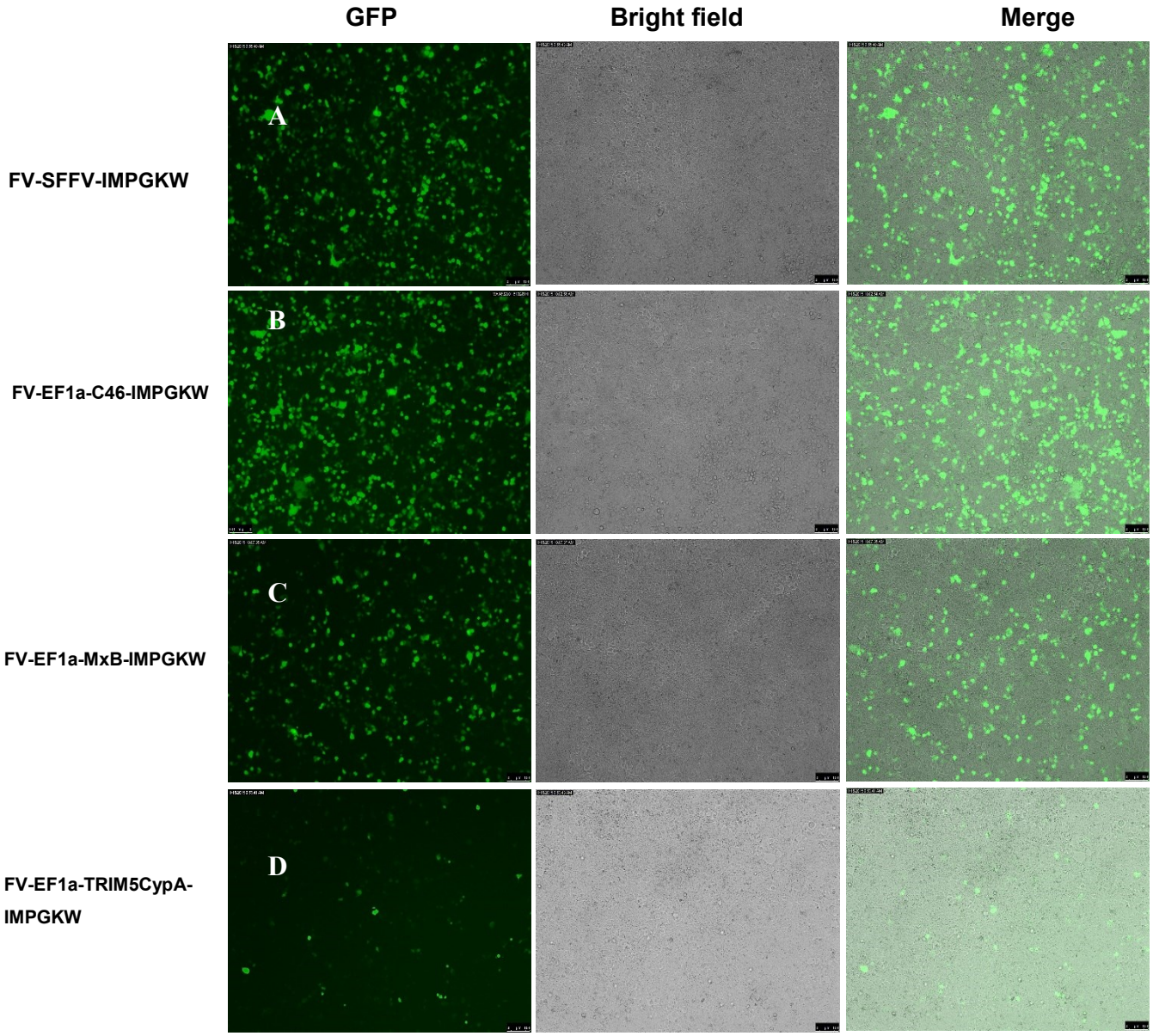

Figure 8 **Comparison of foamy virus vector transfection efficiencies in HEK293 cells.** Green fluorescent infected cells express EGFP delivered by the foamy vectors, and the transfection efficiencies of these vectors were estimated visually using fluorescence microscopy. A) HEK293 cells transfected with FV-SFFV-IMPGKW control vector without an anti-HIV gene with the transfection efficiency of ~60%. B) Transfected HEK293 with previously constructed potent foamy vector FV-EF1a-C46-IMPGKW with the efficacy of ~70%. C) HEK293 cells transfected with FV-EF1a-MxB-IMPGKW vector, with the transfection efficiency of ~50%. D) A very low transfection efficiency ~10% was observed with HEK293 cells transfected with FV-EF1a-TRIM5CypA-IMPGKW. The percentage of EGFP positive cells was later determined by flow cytometry Figure (9). Scale bar 100μm.

- In order to determine the percentage of EGFP expressing cells supernatants were collected from the cell cultures, and 250 µl of each unconcentrated virion stock were filtered and added onto 10^5 HT1080 cells in triplicates in 12 well TC plates. Three days later, the cells were collected and the percentage of EGFP positive cells was determined by flow cytometry. Data was analyzed by gating the cell population on the forward scatter versus side scatter plot. Gated cells were displayed on a histogram measuring EGFP fluorescence, and the percentage of EGFP positive cells was determined by a gate set using untransduced cells to define the negative population. Titers in transformation unit/ml were determined by multiplying the start cell number by the percentage of EGFP positive cells and the volume of the vector stock added. Means were calculated for the triplicate titers for each vector and the standard deviations were determined as shown in (Figure 9). The data show, that none of the newly created vectors have titers that were inhibited by the anti-HIV genes that they carry. More importantly, all vectors were produced at high enough titers to be used with further experiments.

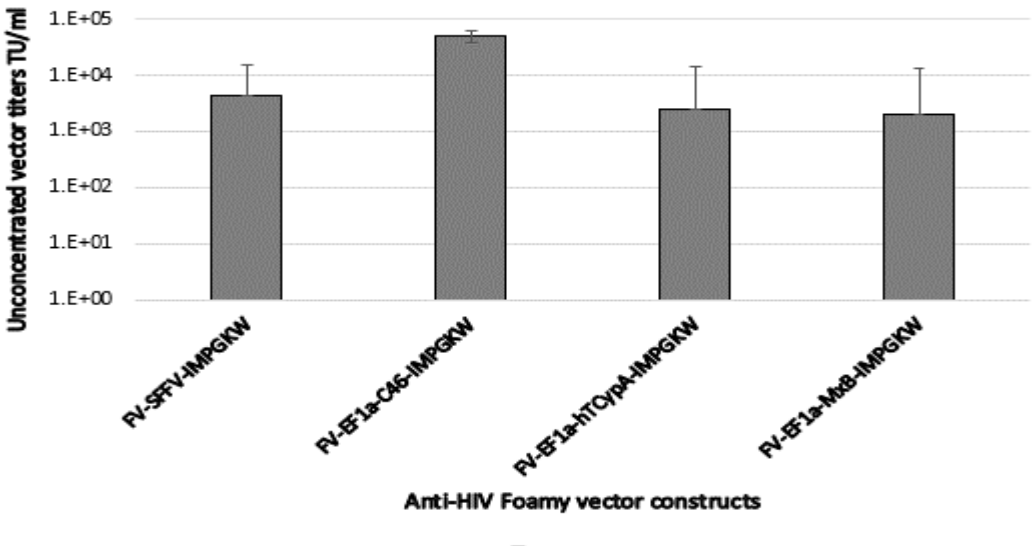

Figure 9 Comparison of foamy virus vector titers. Unconcentrated titers in (TU/ml) indicating the mean titers of HT1080 cells triplicate transductions from a single viral stock preparation for each foamy vector with FV-SFFV-IMPGKW and FV-EF1a-C46-IMPGKW as controls. Error bars represent the standard deviations of the titers between the triplicate transductions.

3.4 TRIM5αCypA fusion and MxB mediated FV vectors HIV-1 restriction challenge:

For the in vitro HIV challenge assay, the CEMx174 cell line will be used to model CD4+ T cells, a novel HIV-1 virus target cell line. 10^5 CEMx174 cells were transduced with FV stocks at a MOI of 0.5 (based on the viral titers of HT1080 cells) by spin inoculation transduction method. Transduced CEM×174 cell cultures were continuously sorted using fluorescence activated cell sorting technique and maintained to yield the highest GFP expression possible. Then, they were challenged with HIV-1 virus strain 89.6 in a multiple-cycle infection assay to measure HIV replication efficiency. HIV was allowed to replicate in the cell culture for a period 7, 14 and 21 days, and supernatant were periodically collected to measure the HIV titer. HIV titer was measured by the number of MAGI-CCR5 cells producing a blue color in cells infected by HIV (BCFU). The results of this assay will show the ability of each foamy virus vector to inhibit HIV replication in vitro and will allow comparison of the efficacy of the SFFV and EF1α promoters, as well as comparison of the potency of the TRIM5αCypA fusion and MxB anti-HIV proteins (Figure 10,11)

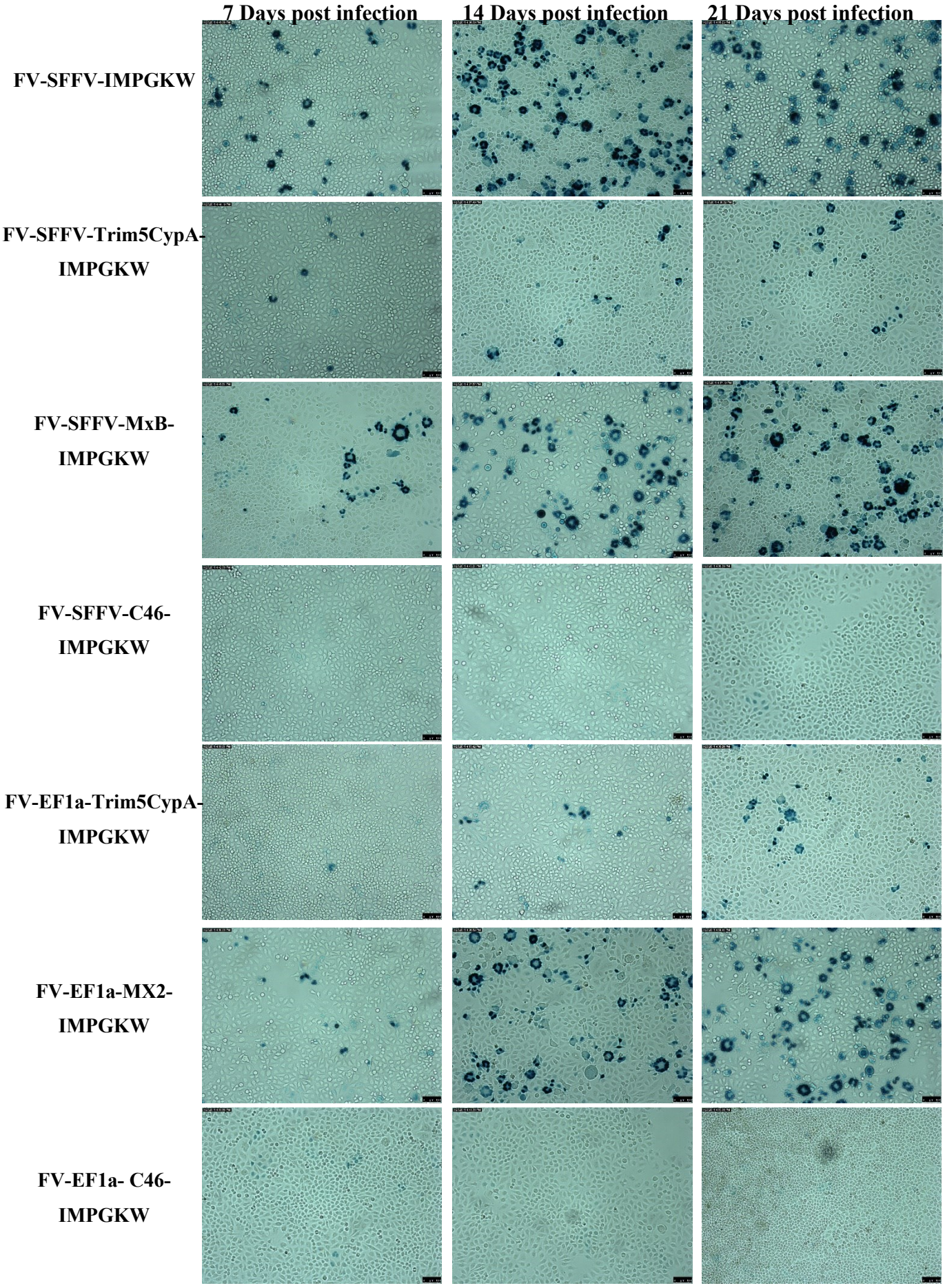

Figure 10 MAGI assay indicating HIV replication efficacy during 3 weeks period. The expressed cellular β-Gal enzyme expression was detected visually using fluorescence microscopy. The ability of the foamy vectors to inhibit HIV replication in vitro was determined by number of blue-infected cells. Scale 100μm

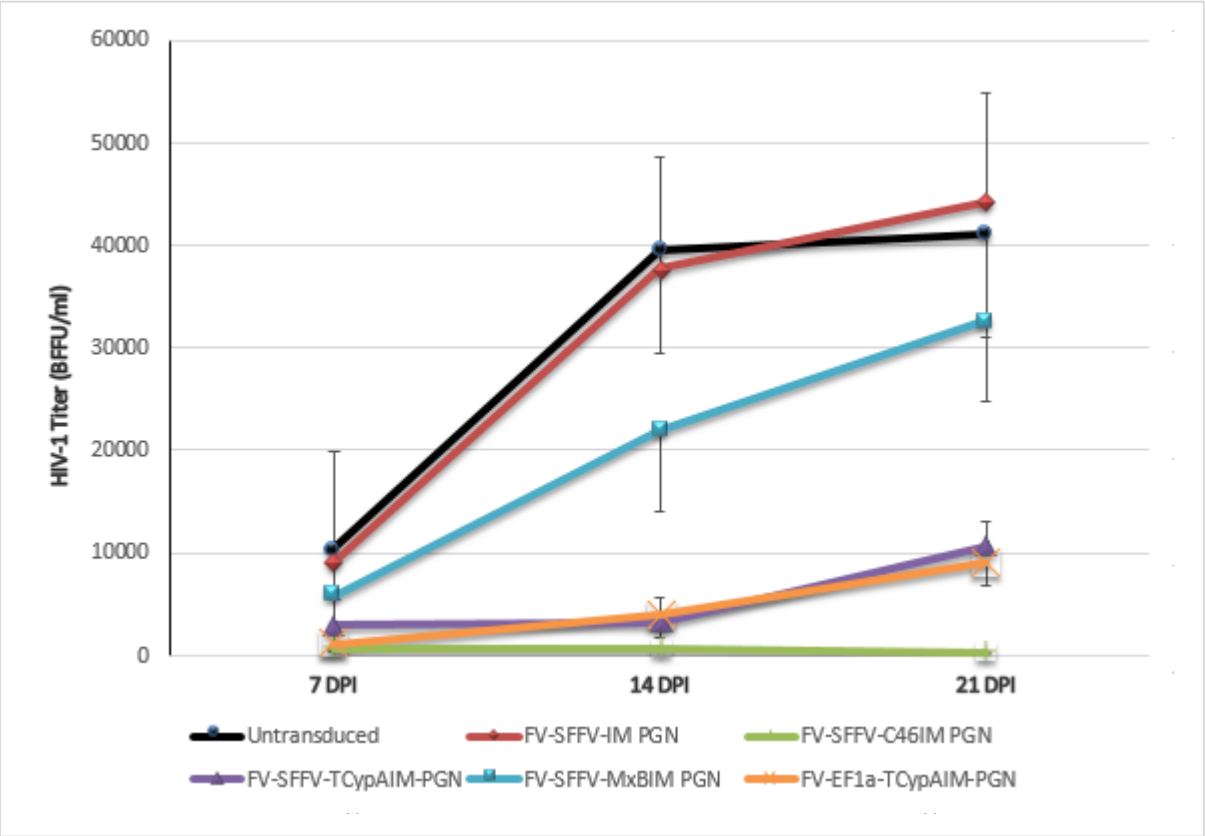

Figure 11 Comparison of anti-HIV efficacy of FV using MAGI assay. Infected blue MAGI cells were counted and the HIV-1 titers (BFFU/ml) were calculated by multiplying the mean number of infected MAGI cells triplicate of each vector stock preparation with the Dilution factor. HIV-1 Titers are shown during three weeks period in order to indicate potent inhibition or virus inhibition escape. Titers were compared with three controls of untransduced stocks and a previously tested potent inhibitor FV-SFFV-C46-IMPGKN and foamy vector backbone without anti-HIV transgene FV-SFFV-IMPGKN. Newly synthesized foamy viral vectors expressing Trim5αCypA lowered the HIV-1 virus titers by four folds showing a potent impact of Trim5αCypA fusion gene on HIV-1 replication in primary cells. However, MxB expressing foamy vectors seemed to lack any potent inhibition impact on HIV-1 titers since the virus was able to escape any inhibition effect by 14 days post-infection (n=3).

The data show that, C46 efficiently inhibited HIV-1 entry and replication more than the other anti-HIV genes tested. However, based on the long term ability to block HIV-1 replication, TRIM5αCypA transduced cells infected with HIV-1 initially restricted HIV-1 but a viral escape caused the infection of more cells by the third

week after the challenge. On the other hand, initially MxB transduced cells reduced HIV-1 particle release but at later time point breakdown was observed.

3.5 Validation of the correct integration and expression of the delivered anti-HIV genes and promoters:

To ensure if successful transduction of cells with the constructed anti-HIV foamy vectors confer the correct integration and expression of these anti-HIV genes with their promoters, transduced CEM×174 cells were analyzed by semi-quantitative PCR method. Once our therapeutic foamy virus infects a cell, the RNA is reversed-transcribed into the DNA form which integrates into the genome of the infected cell. In order to determine the correct integration, total genomic DNA was extracted from transduced cells and analyzed by PCR with specific primers for the respective vector transgene and the driving promoter (either SFFV or Ef1α) (Figure 12,13) . Following integration, transcription begins as the first step for gene expression in which the information in DNA is copied into messenger RNA (mRNA) for protein production. For this reason, we isolate the intracellular RNA from transduced cells and reverse transcribe it into cDNA. First strand cDNA synthesis was generated using Transcriptor First Strand cDNA synthesis kit provided by Roche. Then, another PCR was performed using GoTaq Master Mix with specific primers, to more accurately verify the expression of the integrated anti-HIV transgenes and their respective promoters (Figure 12, 13). GAPDH was used as internal control. We found that our newly created vectors efficiently delivered the anti-HIV transgenes into the target cell genome. More importantly, transduced cells are expressing these transgenes with their respective promoters which can be further analyzed by Western blotting or MTT assay.

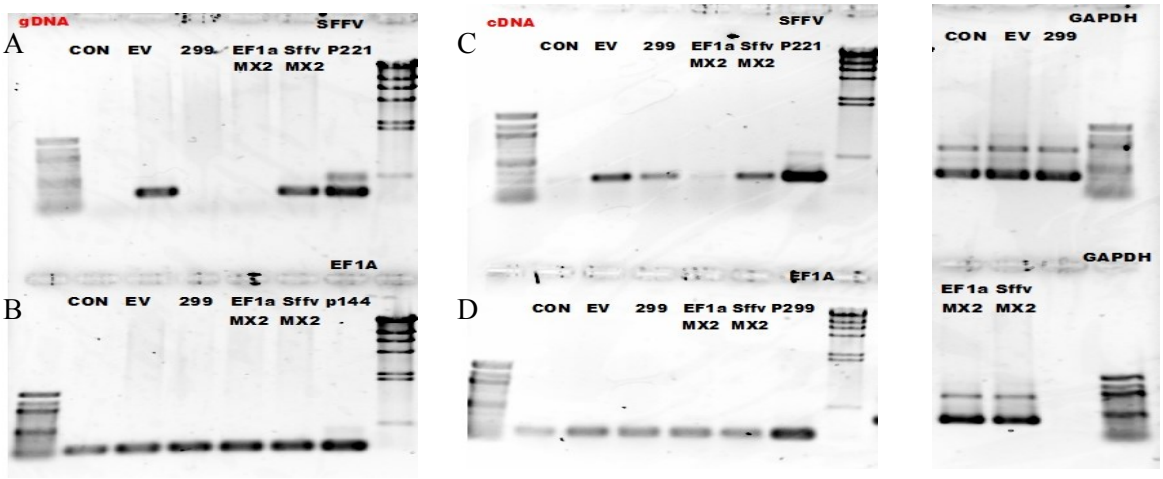

Figure 12 Semi-quantitative PCR to determine the correct integration and expression of FV vectors promoters. Genomic DNA was isolated from transduced CEM174 cells. A) PCR analysis was carried with SFFV specific primers (fwd & rev) with a negative control of nontransduced cells (CON) and a positive control of plasmid (p221) which already expresses SFFV promoter. EV is plasmid from cells transduced with empty vector (FV-SFFV-IMPGW), 299 is plasmid from cells transduced with EF1α-TRIMCyp expressing vector, we notice correct integration of SFFV promoter in cells transduced with EV and SFFV-MX2 as desired. B) PCR analysis was carried with EF1a specific primers (fwd & rev), with positive control (p144 and p299) that already express EF1a promoter. Since EF1a is a housekeeping promoter, it will always be present and expressed in CEM174 cells. For gene expression analysis we extracted mRNA and reverse transcribed it into cDNA that was analyzed in PCR. C &D) specific primers show specific expression of integrated promoters as expected. GAPDH is a control which is expressed by all cells Markers used are λDNA-HindII and 100bp marker.

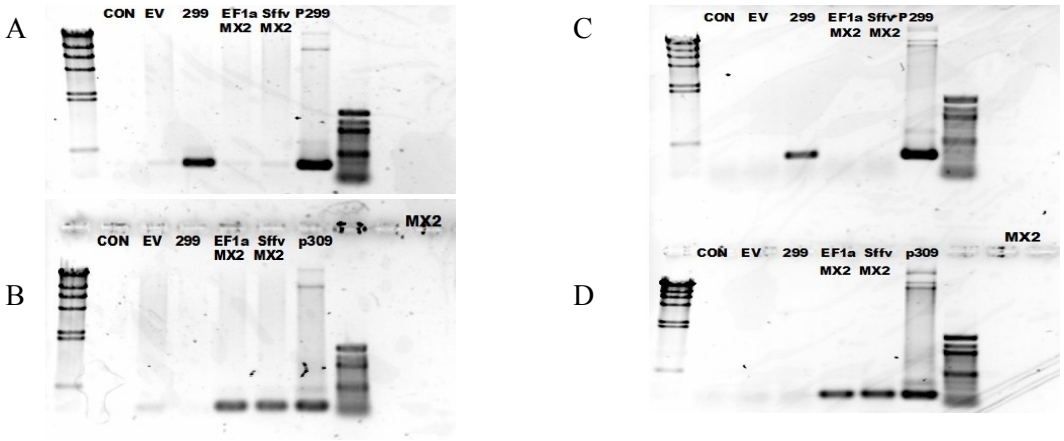

Figure 16 Semi-quantitative PCR to determine the correct integration and expression of FV vectors transgenes. Genomic DNA was isolated from transduced CEM174 cells. A) PCR analysis was carried with TRIM5Cyp specific primers (fwd & rev) with a negative control of nontransduced cells (CON) and a positive control of plasmid (p299) which already expresses TRIMCyp protein. EV is cells transduced with empty vector (FV-SFFV-IMPGW), 299 plasmid from cells transduced with EF1α-TRIMCyp expressing vector, we notice correct integration of SFFV promoter in cells transduced with EV and SFFV-MX2 as desired. B) PCR analysis was carried with MxB specific primers (fwd & rev), the correct integration is confirmed by the bands observed. For gene expression analysis we extracted mRNA and reverse transcribed it into cDNA that was analyzed in PCR. C &D) specific primers show specific expression of integrated promoters as expected. GAPDH is a control which is expressed by all cells. Markers used are λDNA-HindII and 100bp marker.

4 Discussion:

This thesis is a continuation of the research efforts by Dr. Grant Trobridge laboratory. The Trobridge laboratory has shown that the use of specific combinations of anti-HIV genes in this vector system can potently inhibit the replication of HIV in vitro (Kiem et al., 2010). Here we designed new foamy virus vectors constructs, using the SFFV and EF1α promoters and the humanized TRIM5αCypA fusion and MxB anti-HIV genes to test the efficacy of these genes against HIV infection. In addition, creating these constructs with each combination of the above promoter and anti-HIV genes will allow the comparison of the safety and expression provided by the EF1α promoter over the previously used SFFV promoter.

4.1 FV vectors mediated by human TRIM5αCypA produce high titer virions and restrict HIV:

For use in gene therapy, TRIM5αCypA (hTCypA) vectors would be used to transduce patient cells ex vivo, producing a resistant population to HIV-1, before reintroducing them into the patient. The primary target cells of HIV-1 are $CD4^+$ T cells, making $CD4^+$ CEM×174 which are hybrid culture between human T cell line (CEM) and human B cell line (721.174) an excellent choice for vector testing. It is necessary to show that hTCypA foamy vectors are capable of transducing these cells and driving efficient transgene expression.

Titers of two foamy vectors (FV-SFFV-hTCypA-IMPGNW, FV-EF1α-hTCypA-IMPGNW) along with a control (FV-SFFV-IMPGNW) has the same backbone but it does not include any anti-HIV gene, were determined by the percentage of GFP positive transduced cells. Transfection efficiencies for each three vectors are given in Table 4 and Figure 5. For TRIM5αCypA vectors the percentage of GFP positive cells after transfection ranged from 40-50%. In all experiments the transfection efficiency with the GFP vector (without transgene) was higher than those carrying the TRIM5αCypA transgene, reaching up to 60-70%. Importatntly, titers produced by TRIM5αCypA did not have much significant variation from the GFP control vector.

Additionally possibly not significant, vectors containing TRIM5αCypA driven by the SFFV promoter tend to have higher titers than vectors using EF1α promoter.

A later titering comparison experiment tested the titers and the transfection efficiencies of four foamy vectors constructs shown. Unconcentrated titers were determined in triplicates with two controls, a previously developed potent FV-EF1α-C46-IMPGKW and an empty vector with the same backbone but without an anti-HIV transgene. The genome of the four vectors used shown in Figure 7, and a flow chart of the titering in Figure 9. Since the virion stocks were tittered on HT1080 in triplicate, the error bars given address variation in the concentration efficiencies of the different vector virions. Thus, this titer data can be viewed as useful preliminary data for indicating whether certain promoter/anti-HIV gene combinations may affect vector titer, but it is not suitable for robust statistical analysis.

FV-EF1α-C46-IMPGKW the previously created and tested vector was shown to be produced at very high titers. As expected, the difference between the TRIM5αCypA vector (FV-EF1α-hTCypA-IMPGKW) and the GFP control vector titers was found to be within the margin of error. On the contrary, transfection efficiencies in Figure 8 were significantly low for the TRIM5αCypA vector, hardly any cells expressing green fluorescent protein. These two experiments demonstrated that TRIM5αCypA did not have any noticeable effect on foamy virus vector titers and the constructed vectors mediated by this anti-HIV transgene were able to provide high levels of transgene expression.

The next step in testing these new foamy virus vectors to determine their ability to inhibit HIV replication in vitro. For this assay, the $CD4^+$ CEM×174 cell line was used to model CD4+ T cells. Cells were transduced with virions from each of the vectors described above, and then they were challenged with HIV-1 in a multiple-cycle infection assay. HIV was allowed to replicate in the cell culture for a period of three weeks, and supernatant were periodically removed from the culture to measure the HIV titer. HIV titer were measured by adding the supernatant to the MAGI-CCR5 cell line, an indicator cell line that expresses β-galactosidase in the presence of HIV Tat, producing a blue color in cells infected by HIV. This is shown in both Figure 10

and Figure 11. HIV virus titer were quantified by the number of blue cell focus-forming units (BCFU) in the indicator cell culture. Multiple-cycle infection assays such as this more closely replicate the in vivo environment of HIV replication than single-cycle assays, and they allow time to observe escape from inhibition or continued potent inhibition. The assay was carried out in triplicate with two controls, the previously tested potent FV vector mediated by C46 transgene and a GFP control vector without any anti-HIV transgene. As expected, the previously tested FV vector expressing C46 transgene showed a relative potent inhibition of HIV-1 infection along 21 days post-infection without any blue infected MAGI-CCR5 cells. This is because C46 transgene codes for a membrane-anchored protein that binds to the HIV surface protein gp41, preventing the HIV from entering and infecting the cell. On the other hand, both TRIM5αCypA constructs (FV-SFFV-hTCypA-IMPGNW, FV-EF1α-hTCypA-IMPGNW) showed a good restriction of the HIV-1 virus. However, the restriction failed in few cells that got already infected during the first 7 days post-infection. This explains the virus escape during the next 2 weeks after the HIV challenge and the increase in the HIV-1 titers. Even though TRIM5αCypA was not capable of inhibiting the HIV virus completely, it was potent enough to lower the HIV virus titers by 4 fold. This can be confirmed with further studies, by analyzing p24 levels/ELISA based detection method.

Furthermore, for validation purposes, we confirmed the correct integration of our TRIM5αCypA FV vector constructs into the genome of the CEM×174 cells. This was done by isolating the whole genomic DNA and analyzing the samples by PCR using specific primers including TRIM5αCypA, SFFV and EF1α forward and reverse primers. The correct bands appeared in the desired samples, which means that our construct transgene and promoter were successfully integrated in transduced cells. In addition, TRIM5αCypA and promoter gene expression were also confirmed with semi-quantification of the cDNA by PCR analysis.

4.2 MxB has no effect on FV titers, making foamy viruses an ideal candidate for gene delivery.

MxB Myxovirus resistance B was identified recently as an ISG (IFN-stimulated genes) that contributes to the inhibition of HIV-1 replication by type I IFN at an early stage post-infection. Both (Goujon et al., 2013; Kane et al., 2013) studies indicated the ability of human MxB to block infection by all HIV-1 strains as well as suppressing the titers of other lentiviruses that are commonly used as gene delivery systems. One explanation is that MxB transgene targets properties that are shared between both lentiviruses and HIV. This issue makes testing of foamy viruses, a promising strategy for more efficient and potent transgene delivery. Also, due to its human origin, this natural process in the body's defense system against HIV infection represent a safer less immunogenic therapeutic approach for HIV treatment. Toward this, we constructed a foamy vector expressing MxB gene and tested its capability of effective transgene delivery and inhibition of HIV replication.

A codon optimized MxB protein was provided by LifeTechnologies, GeneArt®Tool. After foamy vector production and transduction, testing for MxB effects on foamy vector titers was carried out. Where two vectors, FV expressing MxB transgene and a control vector with the same backbone but without any anti-HIV transgene, were transfected in to HEK293T cells in triplicates, and unconcentrated virions from each plate were tittered onto HT1080 cells. First, we notice that the transfection efficiencies of both vectors is relatively similar. Which indicate that MxB expression had no impact on FV transfection in HEK293 cells Figure 6. As desired as well, the difference between MxB vector and the control vector titers was found to be within the margin of error. This experiment provided a rigorous answer to our hypothesis that MxB does not affect foamy vector titers as it does with lentiviruses, which makes foamy viruses a better candidate for the delivery of this gene into target cells.

In comparison with the previous titering experiment, which included the testing of TRIM5αCypA and MxB constructs with two controls, the previously developed C46 construct and an empty GFP control vector shown in Figures 7, 8 & 9. Transfection efficiency of MxB expressing FV vector was way better than TRIM5αCypA expressing FV vector. On the other hand, when comparing the titers of both vectors

following HT1080 transduction, we notice there is no significant difference between both titers since it is found to be between the margins of error bars. The standard deviation of the resulting unconcentrated titers address the variation across multiple preparations for each vector. Since both titers of MxB expressing vector and the control GFP vector are relatively similar and differences are in the margins of error. This support our previous finding that MxB had no effect on foamy vectors titers or transfection efficiencies.

Surprisingly, in the HIV-1 infection challenge assay, MxB had a little effect in HIV-1 restriction. Many cells were already infected during the first week following infection, which allowed the virus escape and the infection of other cells Figure 10 & 11. This finding contradict with the other publications that indicated the tremendous potential of MxB transgene for blocking HIV-1 infection (Goujon et al., 2013; Kane et al., 2013). One possible explanation of our results, we used a highly cytopathic HIV-189.6 strain that utilized both CCR5 and CXCR4 co-receptors and this strain was not previously tested in these publication. However, upon sequencing the FV plasmid expressing MxB by Macrogen HiSeq Sequencing system we found many point mutations and nucleotide deletion that definitely caused a frameshift mutation in the MxB genome, that we believe altered the whole project outcome. Another experiment is currently running with new non-mutant MxB gene, to verify whether these mutations altered its antiviral restriction function.

Furthermore, we confirmed the correct integration of our MxB FV vector constructs into the genome of the transduced CEM×174 cells. As well as MxB and promoter gene expression were also confirmed with semi-quantification of the cDNA by PCR analysis. This means our foamy vector was capable of therapeutic gene delivery and expression in transduced cells.

Once the ability of the vectors to inhibit HIV replication in vitro has been established, they will be tested for safety by two complementary approaches. The first method will utilize a functional transformation assay (Li et al., 2009). This assay uses the 32D myeloid cell line, which is dependent upon supplemental interleukin-3 (IL-3) for growth, but has been shown to be transformed to IL-3 independence by transfection

with oncogenes (Li et al., 2009). 32D cells will be transduced with each of the vectors described above, and they will be grown in semisolid methylcellulose media with and without IL-3 to identify colonies transformed to IL-3 independence. Transduced cells can also be expanded in the presence of IL-3 and transplanted into mice, and the frequency and rapidity of tumor development in mice can be compared for each vector. Vectors that cause a higher frequency of IL-3 independent colony formation in culture and tumor formation in mice have a higher risk of causing leukemia in human patients if used for AIDS gene therapy.

The second approach will determine the frequency of integration near proto-oncogenes for each vector using a shuttle vector rescue strategy (Trobridge et al., 2006). Briefly, a bacterial origin of replication and a bacterial promoter driving an antibiotic resistance gene will be added to the vectors, and these vectors will be used to transduce cells. Genomic DNA will then be extracted from the cells, digested with restriction enzymes that form compatible sticky ends, where a site for one of the two enzymes is within the vector, and the fragments will be circularized with DNA ligase. These will be transformed into bacteria, and the LTR-chromosome junctions will be sequenced in bacterial colonies surviving antibiotic selection. The relative frequency of integration near proto-oncogenes for each vector will then be compared. The integration profile of each of these foamy virus vectors should be similar, but integration near a proto-oncogene that leads to its activation can give that cell clone a selective advantage, increasing the observed frequency of the integration site relative to the rest of integration sites. So, an observed increased frequency of integration near proto-oncogenes indicates that a given vector has a higher probability of aberrantly activating proto-oncogenes and a greater danger of causing leukemia if used for AIDS gene therapy in human patients.

Foamy virus vectors that are found to provide potent inhibition of HIV replication in vitro and have a low risk for dysregulating proto-oncogenes and leading to leukemia can be further tested for their clinical relevance. Human CD_{34}^{+} hematopoietic stem cells can be transduced and evaluated for their engraftment efficiency in NOD/SCID IL2Rγnull mice, a xenotransplantation model, by a competitive repopulation assay (Kiem et al., 2010). The ability to increase the gene-modified cell population in vivo

for each vector can also be tested by treating the mice with O6BG and BCNU and evaluating the change in EGFP positive cell levels (Kiem et al., 2010). Additionally, the ability of these foamy virus vectors to inhibit HIV infection in vivo and ex vivo can be evaluated using a macaque model (Trobridge et al., 2009). If these studies are successful, there is great potential to move these vectors into the clinic.

5 Conclusion:

The results described above represent a step forward in the process of designing and testing foamy virus vectors for AIDS gene therapy, in an effort to meet the long term goal of providing safer, more potent, and clinically relevant vectors for AIDS gene therapy. TRIM5αCypA and MxB new foamy virus AIDS gene therapy vectors have been created successfully, and a preliminary analysis of their titers suggests that they will all be able to be produced at high enough titers to be practical for use in gene therapy applications. These vectors will enable direct testing of the potency of the hTRIM5αCypA fusion and MxB anti-HIV genes, the anti-HIV gene expression level provided by the SFFV and EF1α promoters. The results of these tests can inform the design of future combinatorial vectors, which can be tested against the vectors created here. There is a clear future experimental progression for these vectors, from the tittering assay conducted here to the clinic, if they are found to be effective and safe. Two of these future experiments are already in progress, and the assays for the others have been previously established and used by the Trobridge lab and its collaborators. This thesis describes the first step in this process, which may one day lead to improved AIDS gene therapy vectors that help alleviate the hardships associated with AIDS and AIDS treatment.

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List of Figures:

List of Tables: