DISSERTATION

PSEUDOTYPING OF LENTIVIRAL VECTOR WITH NOVEL VESICULOVIRUS ENVELOPE GLYCOPROTEINS DERIVED FROM CHANDIPURA AND PIRY VIRUSES

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ABSTRACT

PSEUDOTYPING OF LENTIVRAL VECTOR WITH NOVEL VESICULOVIRUS ENVELOPE GLYCOPROTEINS DERIVED FROM CHANDIPURA AND PIRY VIRUSES

Lentiviral vector system is widely used in gene therapy. Although the envelope glycoprotein of vesicular stomatitis virus (VSV-G) has been mostly used to pseudotype lentiviral vectors, its disadvantages such as low transduction levels in certain cell types and sensitivity to inactivation by human complement hinders the usage of VSV-G pseudotyped lentiviral vectors in some cells or its direct in vivo clinical application. Aiming at overcoming some of these drawbacks of VSV-G, we evaluated two novel vesiculovirus envelope glycoproteins from Chandipura virus and Piry virus (CNV-G and PRV-G), as alternatives to VSV-G. Our results showed that pseudotyped lentiviral vectors could be generated with both these envelopes with high titers and stabilities similar to VSV-G. While displaying a more selective tropism than VSV-G, both CNV-G and PRV-G pseudotypes were found to be efficient in transducing a variety of cell types that include neuronal, fibroblastic and epithelial cells from across different species in addition to a number of human T-lymphocyte cell lines in vitro. Additionally, both the novel pseudotypes were found to be more resistant to human sera inactivation than the VSV-G pseudotype, thus providing better candidates for systemic administration. These data, taken together, establish that both Chandipura and Piry viral glycoproteins are suitable alternative candidates for lentiviral vector pseudotyping with an additional advantage for potential in vivo use in various gene therapy-based applications.

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Chapter 1

Overview of the Literature

1.1 Gene therapy

Gene therapy is a novel experimental technique that repairs dysfunctional genes and restores normal function [1]. Gene therapy has made great medical advances over the past two decades, and has evolved from only a concept to clinical trials for a variety of life threatening diseases. Although still in an "infancy" stage, gene therapy is a promising approach to treat, control and cure diseases that were once lethal and/or incurable.

Ever since Watson and Crick identified the double-helix molecular structure of human DNA [2], scientists have been acquiring pertinent knowledge on decoding genetic information of humans, animals and microbes. The genes or combinations of genes formed by nucleotide base pairs ultimately direct an organism's growth and characteristics through the production of certain proteins, which carry out most of the body's chemical functions and biological reactions. Genes control heredity and provide the basic biological code for determining specific functions. Scientists started to think whether it is possible to manually modify genes with advanced technique, so that a cell's destiny can be changed. With the development of advanced techniques, scientists started to manipulate genes and subsequent products (RNAs and proteins), and the idea of gene modification or therapy emerged and conceptualized in 1972 [1]. Since then, researchers have been investing considerable effort to shed light on this area. With the application of gene therapy, lethal diseases caused as a result of malfunctional genes, including cancers and inherited diseases, are possible to be finally cured [3-6].

Recent work on gene therapy has been mostly in research stage. Gene therapy seeks to provide therapeutic genes that correct or replace the malfunctional ones, in order to restore cellular function and to eventually cure the disease eventually (Figure 1.1). The delivery of transgenes into target cells by viral vector is known as transduction. In general, there are two types of gene therapy based on different target cells, including differentiated cells and stem cells [7]. Somatic gene therapy inserts genes at the tissue or cellular level to produce a naturally occurring protein or substance that is lacking or not functioning correctly in an individual, whereas germ-line therapy introduces therapeutic genes into reproductive cells (stem cells) or possibly into embryos in hopes of correcting genetic defects that could be passed on to future generations [7, 8]. Overall, gene therapy is a rapidly growing field of medicine in which genes are introduced into the body to treat, control or prevent life threatening diseases, such as genetic diseases, AIDS (Acquired Immuno-deficiency Syndrome), cancers, neurological diseases and others [9, 10].

1.2 Applications of gene therapy

1.2.1 Genetic diseases

Genetic diseases are a type of inherited medical conditions caused by DNA abnormalities [11]. Genes determine the behavior of cells, and are composed of specifically arranged nucleotides to encode information. The genetic information conveyed would eventually be interpreted by expressing a variety of different proteins. Proteins perform different functions in the cell, including biochemical, structural contractile, defensive, hormonal and others [12]. However, if DNA contains an error, it may contribute to an abnormal protein product that does

not function in the usual manner, resulting in diseases known collectively as genetic disorders [13]. Clinically, severe inherited disorders can result from as few as only one nucleotide mutation, indicating the importance of the stability of genetic material [11]. For example, sickle cell disease is caused by one mutation in DNA nucleotides of the hemoglobin gene, resulting in a less functional protein and abnormal red blood cells [14]. With the inefficiency or total loss of a function, patients usually suffer from severe syndromes and probably death [14, 15].

Gene therapy aims at modifying, deleting, or replacing abnormal genes in host cells. The therapeutic genes are usually delivered by retroviral and adenoviral vectors to abnormal cells, where they correct the underlying gene defect, express functional protein and restore cellular activity [8]. Gene therapy is known as the only cure for genetic disorders, and has achieved successful results in the past thirty years [8, 16].

Gene therapy experiment was first time approved by FDA in the United State for a patient with inherited severe combined immunodeficiency disorder in 1990 [4]. Adenosine deaminase deficiency- Severe Combined Immune Deficiency (ADA-SCID) is also known as the bubble boy disease, in which affected children are born without an effective immune system and will succumb to infections, outside of a protective bubble, or without bone marrow transplantation from HLA-matched donors [17, 18]. In one successful study, the therapeutic gene called ADA was introduced into the bone marrow cells of such patients in the laboratory, followed by transplantation of the genetically corrected cells back to the same patients [19]. Surprisingly, the immune system was reconstituted in treated patients without noticeable side effects, who lived normal lives with their families without the need for further treatment [19]. Later on, numerous successful treatments have been developed for various types of genetic disorder diseases. In a clinical trial conducted in the United States, gene therapy was used to treat

patients born with Hemophilia, who were not able to induce blood clots and suffered from external and internal bleeding that could be life threatening [20, 21]. For the treatment, the therapeutic gene was introduced into the liver of patients, who then acquired the ability to have normal blood clotting time [20, 22]. Additionally, gene therapy was also used to treat Cystic fibrosis (CF), which is one of the most common genetic defects that damage the lungs and digestive system [23, 24]. In related studies, lung tropic viral vectors managed to deliver a normal version of the CF gene into target tissues and express functional proteins [25, 26]. There are studies in gene therapy of almost all common genetic disorders, indicating that gene therapy is widely used and can target all types of tissues, including bone marrow, liver, lung and others [8, 11]. This property is actually determined by the tropism of viral vectors, which will be discussed later in this chapter.

Gene therapy is definitely the most effective treatment for genetic disorders. With the direct delivery of normal genes to the defective sites, normal function can be restored in a short period. After many years of laboratory and preclinical research in appropriate animal models of disease, a number of clinical trials were launched for various genetic disorders that include congenital blindness, lysosomal storage disease and muscular dystrophy, among others [27-29].

1.2.2 AIDS

The human Acquired Immuno-deficiency Syndrome is a severe disease caused by Human Immunodeficiency Virus (HIV) [30]. HIV is a retrovirus and has two strains, HIV-1 and HIV-2, both of which can eventually lead to AIDS in humans, with HIV-1 being the leading cause [9]. HIV is thought to be derived from SIV (Simian Immunodeficiency Virus) and was found in

humans in the early 1980s, for the first time [31, 32]. Since then, HIV has been widely spread in the human population and caused devastating disasters, resulting in about 25 million deaths in 30 years [33-35]. In 2014, there were 36.9 million people living with HIV/AIDS in the world [36].

HIV-1 is the dominating strain in human infections; it primarily infects CD4 T helper cells and some macrophages, through the binding to co-receptors (CCR5 and CXCR4) on these cells (Figure 1.2) [37-41]. After infection, HIV virus manages to reverse transcribe its RNA genome into DNA and integrate it into the host genome, hijacking the host machinery to express viral genes (Figure 1.2) [41]. During the onset of infection, patients would experience fever-like symptoms known as the Acute Retrovirus Syndrome (ARS), in which stage the virus goes through tremendous replication and circulates robustly in patients (Figure 1.3) [42, 43]. Since the symptom of the ARS is so similar to fever, most patients are not able to realize that they are actually infected with HIV [44]. After the acute infection stage, viral activities are suppressed by human immune system, but the provirus gets incorporated latently in human bone marrow and lymphoid tissues (Figure 1.3) [42, 45]. However, the integration of HIV genome into human cells has already happened immediately after infection and cannot be reversed, and the outcome of AIDS will eventually be developed after five to ten years of latency (Figure 1.3) [42, 46]. Without proper treatment, the CD4 T helper cells will be destroyed by active HIV replication [47]. The AIDS patients will suffer from severe immunodeficiency and eventually die from subsequent opportunistic infections [48].

The highly active antiretroviral therapy (HAART) with HIV drugs is currently the most efficient treatment for AIDS, managing to suppress the viral replication to an undetectable level [49, 50]. Although the HAART has alleviated AIDS from a death sentence to a chronic disease, patients still have to take drugs continuously and carry HIV throughout their lives [9]. Multiple

drugs that interfere with essential steps in HIV life cycle (HIV entry, reverse transcription, integration, etc) are used in the HAART, which is known as the “cocktail treatment” [51, 52]. The drugs can suppress HIV replication dramatically and reduce the viral load to an undetectable level, in a short term [49, 51, 53]. However, HIV is a single-strand RNA virus with a high mutation rate, and would eventually evolve through mutations to overcome the barrier provided by various drugs in the “cocktail treatment” [54-56]. In this case, a new cocktail of specimens has to be selected from the HIV drug pool and create a stronger barrier, at the time point that a patient’s viral load rebounds in the treatment with the old combination [53]. Therefore, a repeated suppression and rebound of HIV viral load is a common phenomenon throughout the patient’s life. To our relief, there are abundant drug candidates with decent efficiencies in the HIV drug arsenal, and more of them are under development by pharmaceutical companies such as Merck and Shionogi. Albeit as the only practical treatment in AIDS so far, the life-time HAART can be expensive and still cannot manage to eliminate the virus in patients. Moreover, drug related severe side-effects were seen in patients including diarrhea, headache, insomnia and fatigue, which create obstacles for regular drug administration [50, 52]. Nevertheless, the HAART has done excellently in controlling HIV/AIDS and saved millions of lives.

With the progress in science and research, we have managed to control AIDS into a chronic disease and relieved patients’ suffering. However, it is not ambitious to think about how to cure and put an end to this stubborn disease. Indeed, there is one and only example of cure in an AIDS patient, who is known as the “Berlin Patient” [57]. This specific individual suffering from both AIDS and leukemia received bone marrow transplantation and got cured from both diseases [57]. This miracle was rendered by the transplanted bone marrow cells which had a rare mutation on the surface known as CCR5Δ32 that blocks HIV-1 entry and subsequent infection

[57]. As a result, the transplanted cells repopulated functional lymphoid cells that were immune to HIV-1 infection [57]. Replicating this scenario is challenging, since it is rare to find an HLA-identical donor with the CCR5Δ32 mutation. However, this successful case of HIV treatment gives hope to researchers to find a curing method that can be universally applied.

The concept of gene therapy thrived in the mind of AIDS researchers who were enthusiastic to put an end to this persistent disease, not only because it is a technique that modifies cellular behavior at a genetic and molecular level, but also due to its potential to terminally shut down the expression of integrated HIV genome (Figure 1.4) [58]. Actually, the concept of gene delivery to express anti-HIV products was described by Dr. David Baltimore in 1988 [59]. Through the introduction of anti-HIV genes into infected cells, viral genes are possible to be permanently shut down. Over the past 27 years, multiple potent anti-HIV transgenes were developed [60, 61]. As a result of identifying various aspects of the intrinsic HIV viral functions and cellular functions, we are able to design gene therapy strategies to interrupt and shut down HIV replication cycle at many different points [62-64]. These artificial transgenes were designed specifically to inhibit different HIV replication steps at a genetic level. The strategies of HIV gene therapy can be divided into protein and RNA strategies, based on the final products of transgenes in infected cells [62].

In the protein approach, transgenes usually synthesize inhibitive protein structures against viral cycle, including transdominant negative mutants, toxins, intrakines, antibodies and DNA-based vaccines [62]. There are various essential proteins that dominate HIV-1 viral replication, such as Gag, Tat, Rev and others. In recent studies, the introduction of defective derivative of these HIV proteins by transgene delivery managed to inhibit HIV-1 replication at multiple steps, such as viral entry, reverse transcription, integration and others [61, 65-67]. Besides, intracellular

toxins or conditionally toxic proteins were found to be also effective in suppressing HIV-1 activity [68-71]. These HIV-1 toxins were derived from Herpes Simplex Virus thymidine kinase (HSV-tk), diphtheria toxin and modified lytic viruses [68-71]. In addition, ligands for HIV-1 co-receptors CCR5 and CXCR4 (RANTES or MIP-1α and SDF-1, respectively) were introduced to infected cells for competitive inhibition of viral entry in some studies [65, 66, 72]. In one of these studies, researchers suggested that the overproduction of a CCR5 ligand had potential to be a natural means of protection from HIV-1 [65]. At last, scientists managed to design anti-viral genes that expressed intracellular HIV-1-specific single-chain Fv antibodies (SFv) that targeted essential HIV-1 proteins as gp120, Rev, Gag, reverse transcriptase (RT), integrase (IN), thus redirecting these proteins away from required subcellular compartments and blocking their function or processing [73-77].

In the RNA strategies, transgenes render the expression of RNA structures impaired, which include antisense, ribozymes, RNA aptamers and decoys, and RNA interference (RNAi) [60]. More studies have been conducted in RNA approaches than that of protein, possibly because the RNA strategies managed to inhibit HIV-1 viral activity at the transcriptional level for better efficiencies and higher escaping barriers [9, 78]. Similar to the protein approaches, critical HIV-1 genes such as Tat, Rev and integrase were usually targeted by antisense molecules to inhibit viral activity in recent studies [79-85]. In other studies, RNA decoys homologous to HIV-1 TAR and RRE were introduced to bind to viral proteins, resulting in competitive inhibition with native ligands that are necessary for viral cycle [86-89]. Additionally, ribozymes encoded by a second-generation RNA-based antisense transgenes were designed to cleave viral RNAs at specific sequences [90-93]. Furthermore, RNAi strategies showed promising progress in inhibiting HIV-1 viral RNA translation in recent studies [94-97]. Similar to the combinatorial

"cocktail treatment" with HIV drugs, a combination of different RNA structures and proteins is also used in the gene therapy strategy that is ideal and difficult to be circumvented by HIV-1 escaping mutations [60, 61, 97].

AIDS is a devastating condition that is detrimental to humans both physically and mentally, causing millions of deaths and enormous financial burdens. Although a vast amount of efforts has been devoted into HIV research, we still have not found a cure to eradicate it. Currently, HIV gene therapy is a solid alternative to HAART in HIV/AIDS treatment. Compared with drug treatment, HIV gene therapy has more sustainable effects. While patients need to receive life-long treatment with drugs, gene therapy has the potential to completely shut down viral activity at a genetic level and thus the patients only need to receive several injections for a possible cure [89, 98, 99]. Although HIV gene therapy efforts are still in an early stage, tremendous progress has been made to shed insights that this goal is attainable in the near future. After intensive studies on HIV gene therapy, we have seen fascinating results and glimpses for its potential to contribute to a final cure.

1.2.3 Other applications

Gene therapy is thriving as a powerful approach with the potential to treat and even cure some other stubborn diseases, such as cancers, neurological diseases and chronic diseases [6, 100, 101]. Similar to genetic disorders, these diseases are also caused by mutated DNA resulting in malfunctional proteins, which include oncoproteins in cancers and defective enzymes in other diseases [6, 100, 101]. Different strategies were specifically designed to these diseases and their sites of pathogenesis, through the delivery of therapeutic genes by viral vectors [6, 101, 102]. Although most of these studies were performed on lab animals pre-clinically, some gene therapy

strategies have reached clinical trials and shown promising results [6, 100, 101]. After overcoming some technical limitations (low transduction efficiency, toxicity, in vivo delivery, etc), gene therapy approach has the potential to make significant contributions to the therapeutic interventions of these life-threatening diseases.

1.3 Vectors for gene therapy

Although direct application or transfection of naked DNA to host cells has been achieved in some short-term studies, there are several major drawbacks hindering the usage of this gene delivery method [103]. Firstly, mammalian cell membranes are naturally hydrophobic and are not preferably incorporated by charged macromolecules, such as nucleic acids [103]. Secondly, the complexity of in vivo environment would reduce the efficiency of this form of gene delivery, possibly by digesting naked nucleic acids by extracellular nucleases [103]. Moreover, the naked DNA can be easily degraded by the immune system as a foreign invader [103]. Fortunately, these concerns can be relieved by encapsulating the transgene DNA in a vehicle, which is known as the vector [103, 104]. The advances in gene therapy can be attributed to the improvements in vectors for different gene delivery purposes. Both viral and bacterial transport vehicles were developed for better efficiency of transduction [103, 104]. Bacterial vectors were only used in a limited number of studies of cancer gene therapy, all of which have showed considerable results [104, 105]. As the most frequently used vehicle for gene delivery, the viral vectors are derived from viruses from various families, which are infectious to different hosts [103, 106].

The concept of viral vector was introduced in 1972, when the Jackson group created recombinant DNA from the SV40 virus by genetic engineering [107]. The viral vectors can be

utilized for delivering gene product, not only because they can physically harbor and protect transgenes in them, but also due to their natural infectivity to cells [108, 109]. However, for safety concerns, these viral vectors have to be first engineered to delete most genes coding for viral proteins from the viral genome, and in particular of those that are potentially pathogenic [110]. The deleted regions should not affect the viral infectivity, and can be substituted by therapeutic nucleic acids of interest [110]. Therefore, efficient viral vectors possess at least two advantages: firstly, they are able to stably package genetic product. Secondly, they manage to deliver genetic material to target cells. During the past two decades, various types of viral vectors have been developed, each with its own set of advantages [103, 106]. Specific vector is chosen for application based on the size limitation of packaging genes and the preference of target cells. Until now, the viral vectors have been derived from adenovirus, adeno-associated virus, retrovirus, lentivirus and others (Herpesvirus, Poxvirus, etc) [103, 106].

1.3.1 Adenoviral vectors

Adenoviruses are double-stranded DNA viruses belonging to the Group I family in Baltimore classification [103]. Adenoviruses are named after the human adenoids in 1953, from which they were firstly discovered [111]. They are non-enveloped icosahedral viruses with a size of 70-90 nm [112]. They carry a medium-sized genome of 35 kb, harboring various essential genes that determine their distinguishable serotypes and biological activities [113]. So far, there are seven species of adenoviruses (A to G), which can be further classified into 57 different serotypes (HAdV-1 to 57) based on their haemagglutination properties in humans [113]. It is no surprise that these different adenovirus strains are able to infect a wide range of cells, causing

different kinds of illnesses from being asymptomatic or mild respiratory infections to life-threatening multi-organ failure in animals and humans [113]. Adenoviruses are relatively resistant to chemical or physical agents and have prolonged survival rate in vitro [113]. They can be transmitted primarily via respiratory droplets, and also by fecal routes [113].

To the interests of gene therapy researchers, the medium-size DNA genome of adenoviruses is likely to be compatible for genomic manipulation and trans-gene insertions [103]. Besides, the broad tropism of adenoviruses is also an attractive property to deliver therapeutic genes to different host cells [103]. Therefore, in the early development of adenoviral vectors, scientists selected candidates of adenoviruses that were well studied and did not cause severe diseases in humans, among which were the adenovirus type 2 and 5 from the C subgroup [114]. For the purpose of safety, adenoviral vectors were firstly engineered with a whole deletion of the early gene 1 (E1A) to eradicate their ability to replicate and create enough space for gene insertions, since E1A gene is essential for viral replication but dispensable for viral survival and infectivity (Figure 1.5) [113]. Secondly, in order to provide more space for gene insertions, more partial deletions were introduced to E1B and E3 regions in the genome, which were also done under the same conditions that the adenoviruses maintain their infectivity (Figure 1.5) [113]. With these many deletions, a packaging capacity of as big as 7-8 kb was created between E1 and E3 regions of the adenoviral genome, allowing sufficient space for single to multiple trans-gene insertions at the same time [113].

In order to generate these non-replicative yet infective adenoviral vectors, helper cells are needed to be transfected with vector-constructing plasmids and express essential proteins for efficient vector production [103]. Until now, the human embryonic kidney 293 (HEK293T) cells have been found as the most prominent helper cells [115]. HEK293T cells are a specific lab-

adapted immortalized cell line derived from human embryonic kidney cells [115]. They can be easily transfected with vector-packaging plasmids and correspondingly have robust protein output, resulting in ideal titered adenoviral vectors [103]. Furthermore, HEK293T cells are very easy to culture and grow, leading to relatively prolonged proteins or vectors production [115]. With these properties, the adenoviral vector genes for viral proteins that would provoke host immune system could be further deleted, including E1, E2 and E4, which can be compensated and expressed by HEK293T cells in vector packaging stage [103]. By using this vector-constructing system, functional, replication-non-competent and less immunogenic adenoviral vectors can be successfully generated to as high as 10^{10} cfu/ml in titer, ensuring efficient subsequent ex vivo or in vivo gene transfer [103].

After the adenoviral vectors production from HEK293T cells, they show high titer and are ready to infect a wide range of cells, attributable to the natural broad tropism property of adenoviruses [103]. As an advantage of adenoviral vectors, they are capable to express therapeutic genes in episomal status in the host cells right after transduction, leading to rapid and robust foreign protein synthesis that modify or restore cellular function [103]. However, the expression of trans-genes in this state is only transient and can be aborted or silenced by host cellular mechanism in long term [103]. Moreover, adenoviral vectors inevitably carry or synthesize some viral proteins (adenoviral vector envelope and some early genes) along with trans-proteins, provoking host immune system reactions and toxicities [103]. Evidences of this have been seen such that T and B lymphocytes dependent immune responses were activated to reduce the duration of therapeutic genes' expression in vivo [116, 117]. Furthermore, the host immunological memory can even recognize and antagonize adenoviral vectors in successive administrations [103]. However, these drawbacks can be relieved by immune-suppression

treatment, with decent results being achieved in previous studies [118-120]. In these regards, researchers usually utilize adenoviral vector system as a gene therapy strategy to restore normal host function in inherited diseases and cancers [103].

Adenoviral vector system is frequently used in clinical gene therapy applications to treat hereditary disorders and cancers [120-131]. Adenoviral vector has a preference to infect epithelial cells and tissues, therefore it has been heavily used to deliver therapeutic genes to these sites to restore function. As the first step using adenoviral vector system to treat diseases, scientists managed to deliver therapeutic genes to correct a life-threatening Cystic fibrosis disorder in the CFTR (Cystic fibrosis trans-membrane conductance regulator) gene, which can lead to lethal dysfunction in the chloride and/or thiocyanate ions trans-membrane transportation without effective treatment [121]. In this protocol, adenovirus type 2 and 5 vectors efficiently and rapidly delivered the normal CFTR gene into the patients, without detectable immune rejections [122]. The functional CFTR protein reconstituted the normal ion transport in nasal and bronchial epithelium [121, 122]. However, the expression of therapeutic protein was only transient, indicated by gradual reduced output of normal CFTR protein and eventual loss of therapeutic gene expression after 21 days [122]. Later on, numerous clinical studies have been conducted to optimize adenoviral vector gene transfer for CFTR gene therapy, but the relatively short-term expression of trans-genes remained as the major drawback of this vector system [113]. In cancer gene therapy, adenoviral vectors are widely used to deliver anti-tumor factors to suppress carcinogenesis [132]. For example, numerous clinical protocols use adenoviral vectors to deliver the HSV-tk, a suicide gene, for the treatment of various types of cancer, including head and neck cancer, non-small cell lung cancer, prostate cancer, ovarian cancer, malignant glioma and brain tumors [123, 124]. Besides, adenoviral vectors are able to enhance immune activities

by transferring stimulating cytokines (IL-2 and GM-CSF) into tumors [125, 126]. In addition, the p53 tumor suppressor gene can also be introduced to induce growth arrest, by using adenoviral vector system [130, 131].

Different from the aforementioned replication-incompetent adenoviral vector systems, replication-competent adenoviruses are usually used in cancer gene therapy [127, 128, 133]. These vectors are designed to only replicate and spread in tumor cells but do not affect normal cells [127, 133]. They have mutation in the E1B gene that can not actively replicate in normal cells with functional p53 [127, 133]. However, they are able to replicate in tumor cells possessing defective p53 and exploit the cytopathic effect of viral replication and mediate tumor cell lysis [127, 133]. Therefore, these adenoviral vectors can not only lyse tumor cells, but also grow and spread to neighboring tumor cells, whereas normal cells are not affected [127, 133]. In other studies, such replication-competent adenoviruses were combined with suicide gene (HSV thymidine kinase) expression to mediate lysis in malignant cells for more efficient tumor eradication [129].

As a novel application, adenoviral vector system can also be utilized to create animal models to study infectious diseases. By introducing receptors for novel infectious agents to cells, lab animals are able to cross the species barrier and become susceptible animal models to study life-threatening diseases. One such example is the genetic modification of a BALB/c mouse model that acquired long-term hDPP4 (human receptor dipeptidyl peptidase 4) expression by adenoviral vector transduction and thus became susceptible to MERS-CoV (Middle East Respiratory Syndrome Coronavirus), which is a lethal virus that infects bats, camels and human [134]. These vectors deliver human DPP4 receptors to the mouse epithelial cells to overcome species barrier and render susceptibility [134].

Overall, adenoviral vectors are highly compatible with foreign genes for delivery and are easily synthesized on packaging cells to high titers. Besides, adenoviral vectors are very efficient gene transfer vehicles, mediating transient but high level expression of the delivered genes. These vectors are commonly used in gene therapy studies of inherited diseases and cancers, and have achieved decent results [113]. In addition, adenoviral vectors are also used in the vaccine development, as discussed previously in Section 1.2.5. Alternatively, adenoviral vectors have the potential to create new lab animal models to study infectious diseases [134]. Adenovirus vectors have their limitations in long term expression with their epichromosomal status, causing the lost of vector DNA during cell divisions of transduced cells [113]. However, adenoviruses are of great value for gene therapy since these vectors achieve high gene transfer efficacy combined with temporally high level of transgene expression.

1.3.2 Adeno-associated virus vectors

The AAV belongs to the genus of Dependoparvovirus in the Parvoviradae family and has a single stranded 5 kb DNA genome, belonging to the Group II in virus classification (Figure 1.8 A) [110]. This virus is small in size (20 nm) and does not have an envelope [135]. The replication of AAV is highly dependent on the co-infection by a helper virus (adenovirus) [136]. AAV is not known to cause any known diseases and only induces very mild to no immune response [137]. The AAV contains rep and cap genes, encoding for essential polypeptides for the viral replication and encapsidation [103].

The property of low pathogenicity makes AAV a great candidate as a viral vector. With the deletion in parts of the rep and cap genes, the AAV vectors allow insertion of foreign genes

ranging from 4.1 to 4.9 kb (Figure 1.8 B) [138]. AAV vector can infect both dividing and non-dividing cells, providing suitable gene delivery vehicle for in vitro and in vivo gene transfer into muscle, brain, hematopoietic progenitor cells, neurons, photoreceptor cells and liver cells [139-142]. Although wild-type AAV integrates into a specific site of the human chromosome 19 (between q13.3 and qter) after infection [143], the modified AAV vector persists in an extrachromosomal state without genome integration, possibly because of the deletion of the rep gene [103]. With low immunogenicity and good infectivity, AAV was shown to rapidly express transgenes in transduced cells and maintain therapeutic effects for up to two years [144, 145].

However, the disadvantages of AAV vectors are also obvious. The robust production of AAV vector requires adenoviral E1B and E4 proteins from co-infection, reducing the quality and purity in viral stocks. Researchers have optimized the titer by replacing actual adenoviral co-infection by helper plasmids, expressing compensated proteins by adenoviral authentic promoters [146, 147]. Thus, this AAV production system circumvents the problems associated with adenovirus dependent generation of AAV vector, resulting in more purified and high titer stock from its packing HEK293T cells [148, 149]. In addition, the cloning capacity of the AAV vector is relatively limited and is not suitable for insertion with large genes [138]. Therefore, AAV vector is more likely to be used in the gene therapy of monogenic disorders to fix single abnormal gene, while gene therapy strategies involving delivery of multiple genes delivery would not be effective with AAV vector.

AAV vectors have been used in various clinical trials for a number of diseases, including Cystic fibrosis, Hemophilla, Leber’s Congenital Amaurosis, Parkinson’s disease and others [150-154]. In some completed Phase I and II clinical trials, AAV vectors have shown promising results, especially in the treatment of Cystic fibrosis [155]. After the delivery of functional CFTR

gene into epithelial cells of the maxillary sinus, patients evidenced persistent transgene expression for up to ten weeks, with no detectable AAV vector specific immune responses [155]. In another Phase I study on Parkinson's disease, the GAD (glutamic acid decarboxylase) gene deficiency was repaired by AAV vector in the subthalamic nucleus of patients, with persistent therapeutic effects for up to twelve months with no adverse effects [156].

AAV is by far the smallest viral vector [110]. Its advantages include low immunogenicity, rapid and persistent transgene expression, and a wide range of tropism [110]. However, the small insertional size hinders its usage in more complex gene therapies, where multiple functions need to be restored by several therapeutic genes at the same time.

1.3.3 Retroviral vectors

Retroviruses are a family of enveloped viruses whose genome is composed of single-stranded positive-sense RNAs [110]. This virus family was categorized into Group VI by Dr. David Baltimore [110]. As a unique replication mechanism that is different from other virus families, the retrovirus uses its own reverse transcriptase enzyme to produce DNA from its RNA genome, and then incorporate this proviral DNA into the host cell genome by an integrase enzyme [110]. Later on, the host cell treats this integrated viral DNA as part of its own genome, transcribing and translating the viral genes under host cellular machinery [110]. Eventually, viral protein components are synthesized and assembled into new virions, that are released later on for more infections [110].

Retroviral vectors are derived from retroviruses, with tailored RNA genome that has minimum harmful viral genes [103]. The unique and biggest advantage of these vectors is

characterized by the stable integration of viral genome into the host genome, leading to persistent expression of the transgenes [103]. Along with other advantages, retroviral vectors are extensively used for virus vector engineering to achieve long-term effect [103]. The most commonly used retroviral vectors include MMLV (Moloney murine leukaemia virus) vectors and lentivral vectors [103].

1.3.3a MMLV vectors

MMLV (Moloney Murine Leukemia Virus) is a type C retrovirus (gammaretrovirus) and is able to infect murine cells to cause cancer [110]. Besides, MMLV also manages to infect a variety of other species including human cells [110]. MMLV vectors are a type of classic retroviral vectors that possess common retroviral properties. In the earlier development of viral vectors, MMLV vectors stood out with its unique integration mechanism for long lasting transgene expression [110]. However, some of their disadvantages were also obvious and challenging to overcome.

As a common procedure for the development of viral vectors, MMLV was engineered with the removal of the structural genes and other non-essential genes, creating spaces for transgene insertion [103]. Later on, transgene is inserted into the tailored spot, with a capacity of 7-8kb [103]. With the other viral structural proteins compensated by helper cells, packaging cells (usually HEK293T cells) provide infectious but non-replicable viral vectors for safe and efficient production of recombinant virus particles for infection of the desired target cells [103].

With narrow target specificity, MMLV particles cannot infect a wide range of cell populations, limiting its usage in gene therapy [103]. Two mechanisms have been used to

overcome this drawback: modification directly on MMLV envelope and altering its envelope glycoproteins to redirect retroviral tropism [157-164]. As an example of direct modification on envelope, specific single chain antibody-derived (scFv) was linked to the MMLV envelope that have been directed against major histocompatibility complex (MHC) class I molecules, low density lipoprotein (LDL) receptors and others [157-161]. Such chimera indeed permitted specific binding of viral particles to the desired target cells but poor infection efficiencies were also seen to limit its feasibility for future use [159]. In addition, limited success was seen in other efforts in altering the tropism by manipulating the viral Env protein, since such modifications induced reduced fusogenicity of the envelope protein, and provoked rapid sequestration and destruction in endosomes leading to reduced infectivity [159, 165]. As a better approach, the replacement of MMLV envelope by a foreign G protein of vesicular stomatitis virus (VSV-G) was performed to produce stable and infectious particles, making infection of primary hepatocytes, fibroblasts, peripheral blood leucocytes or human stem cells possible [162-164]. This approach was termed as pseudotyping [163].

The possible insertional mutagenesis caused by MMLV vectors hindered their clinical use, with two severe cases reported that were caused by activation of the LMO2 oncogene due to nearby integration of the vector, in a cohort study on ten SCID-X1 patients [166]. In addition, MMLV vectors are only able to infect fast dividing cells, thus have limited therapeutic effect on dormant cells and non-dividing cells, such as neurons [103]. Therefore, much work needs to be done to optimize MMLV retroviral vector system before it can contribute efficiently in gene therapy applications.

1.3.3b Lentiviral vectors

Lentiviruses are a genus of retrovirus, including HIV (Human Immunodeficiency Virus), SIV (Simian Immunodeficiency Virus), FIV (Feline Immunodeficiency Virus) and others [110]. Most of these viruses cause immunodeficiency diseases in different species, such as AIDS (Acquired Immunodeficiency Syndrome) in human [110]. Lentiviral vector is usually referred to a specific vector derived from the HIV-1 virus but has deletions of harmful viral genes, including vif, vpr, vpu nef, et al (Figure 1.6) [167]. There has been a gradual evolution in the production of lentiviral vectors, from the first generation to the third generation, leading to less chance for recombination events, deletion of virulent genes and improved safety [103, 110, 168]. In the production of third-generation lentiviral vectors, a set of four plasmids are co-transfected into vector producing cell line (HEK293T) (Figure 1.6) [97]. Among the four plasmids, one plasmid encodes the envelope protein for the viral vector, two plasmids encode the three HIV-1 packaging proteins (Gag, Pol and Rev) that are essential for the assembly of functional vectors, the fourth plasmid encodes the genome of the vector and harbors the inserted foreign genomic material under a universal promoter (usually CMV promoter) (Figure 1.6) [97]. Each plasmid is then expressed and translated in the packaging cells to synthesize protein components [97]. The components then assemble into a functional “virus” that contains target genes [97]. This synthesized “virus” is infective but non-replicable, it provides efficient and safe gene delivery into target cells (Figure 1.7) [97].

As an advantage over the other retroviral vectors, lentiviruses infect both non-dividing and dividing cells, without the requirement of cellular mitosis for transduction [169, 170]. It is capable of packaging up to 9 kb genetic material [110]. Lentiviral vector was developed as a novel vector by Dr. JC Burns [171]. Compared to the other viral vector systems, lentiviral vector

has the advantage of being easily engineered for gene deletions and insertions [171]. It is highly compatible for genetic insertions, thus managing to deliver genes of interest for different purposes [103, 110]. Alternatively, lentiviral vector can be easily modified and pseudotyped with different envelopes from other viruses, inheriting their tropisms to infect specific cell populations [102]. The envelopes developed by researchers are from various viruses, such as Vesicular Stomatitis Virus, Measles virus, Baboon Retrovirus, Filovirus, Baculovirus, Ross River virus, Cocal virus and others have been used to pseudotype lentiviral vectors [16, 62, 102, 167, 172-184]. Lentiviral vectors usually maintain high titer after pseudotyping with different envelopes, achieving high infectivity and more accurate targeting at the same time [102]. VSV-G is by far the most often used pseudotype with its wide infectivity in various cell populations, but can sometimes be inefficient in certain cell types, such as neuron cells and stem cells [173, 174]. In addition, unlike other oncoretroviral vectors, lentiviral vectors are not known to be associated with tumor development, offering increased safety in terms of insertional mutagenesis [185]. This is probably due to that the region where lentiviral integration occurs corresponds to the whole gene transcription unit as a "benign integration", whereas gammaretroviruses preferentially integrate in correspondence with the transcription start site, including the gene promoter and first intron, thus induce potential oncogenesis [186]. With these elite properties, lentiviral vector system is widely used in all gene therapy fields, such as the treatments of inherited genetic disorders, neurological diseases, cancers, AIDS, and the application of vaccine [173, 187-189].

Lentiviral vectors have shown potential in the gene therapy of neurological disorders, such as Parkinson's disease (PD), Huntington's disease (HD), motor neuron diseases (MNDs) and others [173, 187, 188, 190]. Lentiviral vectors managed to deliver multiple therapeutic genes

to both peripheral neurons and central nervous system (CNS), including neurotrophic factors, anti-apoptotic genes, knocking down expression of dominant genes and restoring protein levels by gene replacement [189]. Their efficiency in transducing non-dividing neurons opened up new approaches for novel treatments. In addition, lentiviral vectors integrate transgenes permanently into host chromosome, leading to stable long-term expression [191, 192]. Moreover, lentiviral vectors were shown to be safe, have minimal immune rejection and did not compromise the viability of transduced cells [110]. The Mazarakis group managed to pseudotype lentiviral vectors with Rabies virus glycoprotein, so that the vector pseudotypes could transport therapeutic factors retrogradely from muscle to spinal motor neurons, with undetected immune response in the CNS [173]. With respect to neuronal tropism and sustained transduction, lentiviral vector is the most attractive gene delivery system used in gene therapy for neurological disorders [102].

Lentiviral vectors might be particularly suitable as recombinant viral vaccines that are promising candidates for individuals who have no pre-existing immune response to animal lentiviruses [106]. In addition, the lentiviral vectors are persistent and non-cytopathic, do not express viral proteins, and can be potentially targeted to DCs [106]. Furthermore, lentiviral vectors can infect both dividing and non-dividing cells, and transduce human or animal peripheral blood-derived dendritic cells and stimulate specific cytotoxic T-lymphocytes [193-195]. Researchers have reported the successful applications of dendritic cell based anti-cancer lentiviral vector vaccines that delivered cancer-specific receptors to T-cells, enhancing immune activity against tumor cells [196, 197].

Lentiviral vector is the best system and has been actively used for gene therapy for HIV/AIDS. Firstly, since all available antiretroviral drug treatments (highly active antiretroviral therapy, HAART) for HIV-infected patients are not likely to eliminate HIV-1 from the body, the

persistence of therapeutic anti-HIV genes is critical for long-term treatment solutions, which can only be achieved by lentiviral vector [9]. Secondly, the ability of lentiviral vector to genetically modify nondividing cells makes them of particular interest to target quiescent stem cells, leading to potential re-establishment from genetically modified stem cells and a possible cure for AIDS [9]. Additional advantage of lentiviral vector over other vectors for AIDS treatment is the competitive inhibition with wild-type HIV virus by cis-acting elements within the vector, enhancing efficacy [9]. With the development of anti-HIV products, lentiviral vector was always chosen as the vehicle to deliver them into infected cells. By using this delivery system, the Akkina and Rossi groups have achieved long-term inhibition of HIV-1 infection in cells by the delivery of a triple combination of anti-HIV genes, encoding HIV-1 shRNA, anti-CCRs ribozyme and a nucleolar-localizing TAR decoy, in a similar mechanism as the “cocktail” treatment with drugs [97]. Other anti-HIV constructs were also delivered by lentiviral vectors and achieved satisfactory results, including RNAi, ribozymes, RNA decoys and aptamers, transgenic T cell receptors, ZFN (Zinc finger nucleases), TALENS (transcription activator-like effector nucleases), CRISPR (clustered regulatory interspaced short palindromic repeats) nucleases, and others, each with its unique mechanism in inhibiting HIV replication [90, 92-95, 198-201]. Moreover, replication-competent lentiviral vector was introduced several years ago for more sustained therapeutic effect [202, 203]. Taking advantage of the viral activity in HIV infected cells, these vector particles managed to propagate with endogenously encoded viral components and transduce more cells, known as “vector mobilization” [202]. By this method, lentiviral vectors were able to deliver transgenes into adjacent cells continuously, with the lasting of viral infection [202]. However, these recombinant lentiviral vectors might become pathogenic or transmissible, causing safety concern in most studies [202-205]. With the great potential of

infinite transductions after limited vector input, mobilized lentiviral vectors need to be well studied and optimized before going to clinical use.

In conclusion, lentiviral vector system stands out compared to other viral vector systems with a set of fascinating properties. With comparable or higher titer and transduction efficiencies as other viral vectors, lentiviral vectors can also infect non-dividing cells such as neurons and stem cells, integrate transgenes into host genome for long-term expression, and switch tropism by different pseudotyping candidates for targeting a broad range of cell populations across different species [9, 102, 110]. With these advantages, lentiviral vector system is becoming popular for various applications in all gene therapy fields.

1.3.4 Other viral vectors

In addition to the viral vectors discussed above, numerous other vectors have been developed for their specific features for different fields in gene therapy. These viral vectors include Herpes virus vectors, Poxvirus vectors, alphavirus vectors, influenza virus vectors, human cytomegalovirus vectors, foamy virus vectors and others [206-215]. With feasible insertion capacities and transduction efficiencies in target cells, these viral vectors have been used in the studies of different gene therapy strategies [103, 216]. Many attempts have been made to improve these viral vector systems for further application in gene therapy of cancers, inherited human diseases, neurological diseases and vaccines [216].

1.3.5 Discussion

Alternative to classic drug treatment and chemotherapy, gene therapy is a novel strategy to treat and cure a disease by restoring normal cellular function at the genetic level [1]. It can be applied to a variety of life-threatening diseases caused by defected gene expression, including cancers, inherited disorders, neurological diseases and AIDS [6, 9-11, 101]. Additionally, the techniques developed in gene therapies have also been used to create a new route for vaccination with high efficiency [217]. Over the past two decades, the great potential of gene therapy has prompted researchers to develop efficient treatment and cure for many diseases, with improved safety and efficient transfer of the therapeutic genes in both preclinical and clinical studies.

Viral vectors have been found as the best vehicles for gene therapy, with their viral ancestors' infectivity but was genetically manipulated to deliver therapeutic genes to target cells [110]. With much effort, researchers developed numerous viral vectors derived from multiple virus families, each with its own unique features and specific application in different gene therapy areas [103, 216]. However, all these viral vectors share some common properties in the appropriate expression levels associated with sufficient duration of gene expression, and the specificity of gene transfer to achieve therapeutic effects in the patient [103, 216].

As discussed previously, different viral vectors have been developed for different purposes of application and have their own advantages and drawbacks in their usage. Availability of many different vectors helped researchers to consider and choose the most competent vehicle for the purpose of gene therapy (Table 1.1) [103, 216]. Among these, lentiviral vectors derived from the HIV-1 seem to be the most effective gene delivery vehicles with some advantages. Lentiviral vectors have good cargo capacity and integrate transgene into

host genome for long-term expression [110]. Especially, lentiviral vectors are easily manipulated to switch tropism, through the pseudotyping with viral envelopes from other viruses [102]. With the acquisition of tropism from various viruses, the targeted cell populations by lentiviral vectors can be unlimited [102].

1.4 Modification of lentiviral vector tropism through pseudotyping

Lentiviral vectors are one of the most popular vector types that researchers use to deliver various genes of interest, not only because of their good performance in numerous studies but also due to their well established, optimized and safe usage protocols [110, 216]. With the progress in research on gene therapy, lentiviral vector is required to meet the needs to efficiently transduce different target cells. However, the wild type HIV-1 with its natural envelope only infects CD4 expressing cells (T helper cells and macrophages), limiting its application in other cell types [30]. To overcome this limitation, the cellular tropism of lentiviral vectors has been expanded through the substitution of lentiviral envelope with that of other heterologous viral glycoproteins possessing broader cell tropism by a process called pseudotyping, which occurs during the vector assembly in packaging cells [102]. Such vector particles possess the tropism of the virus from which the pseudotyping glycoprotein was originally derived, allowing an extension of the host range beyond those cell types that express CD4 receptors [102].

Among the first and most widely used glycoproteins for lentiviral vector pseudotyping is the vesicular stomatitis virus glycoprotein (VSV-G), due to its wide range of vertebrate hosts and infectivity to various cell types [191, 218, 219]. This method of pseudotyping was developed by Dr. Burns on retroviral vector (MLV) and then successfully applied to pseudotyping lentiviral

vector by the Akkina and Naldini groups [171, 191, 218]. VSV-G pseudotyping has proved to confer high vector stability and titer, and has been widely used as the “gold standard” in the lentiviral vector construction with decent performance in most gene delivery studies [102]. Nevertheless, its poor performance has also been addressed in some studies, indicating the need for more efficient pseudotyping envelopes in less-transducible cells including human neuron cells and quiescent B lymphocytes [173, 220]. Moreover, although most current gene therapy studies focused on ex vivo gene transductions with lentiviral vectors, the next desirable step is to be able to inject them directly for in vivo systemic delivery [221, 222]. In this regard, the systemic injection with VSV-G pseudotyped lentiviral vector would induce antibody responses that can neutralize the ability of subsequent injections to infect cells in vivo [223]. Hence, for the purpose of repeated administrations, there are needs for additional non-crossreactive envelope proteins that have comparable value in pseudotyping [221]. Furthermore, VSV-G can be inactivated by human serum complement, which limits its efficiency for systemic administration [176, 223]. In this regard, it is necessary to introduce alternative envelope substitutes for pseudotyping that possess comparable or better transduction efficiencies and better resistance to human complement and IgM-mediated inactivation of the pseudotyped vectors than that of VSV-G [224].

Over the past two decades, multiple pseudotyping envelopes have been developed from various viruses [102]. These viral envelopes usually possess properties and preferences to infect specific cell populations and organs, in which VSV-G might have low infection rate [102]. In addition, alternative pseudotypes with comparable efficiencies to VSV-G can provide substitutes in successive in vivo injections [102]. With such flexibility in pseudotyping, lentiviral vectors

acquire potential to inherit infectivity from various viruses, theoretically allowing for universal targeting [102].

There is a growing list of envelopes for lentiviral vector pseudotyping, each with specific advantages and disadvantages (Table 1.2) [102]. Among the first to develop new envelopes for pseudotyping, the Mazarakis group pseudotyped lentiviral vectors with Rabies virus glycoprotein (RV-G) and showed more efficient gene delivery to neuronal cells, compared with VSV-G pseudotypes [173]. More excitingly, RV-G pseudotypes gained access to the CNS (Central Nervous System) by retrograde transport, providing a featured property for gene therapy design in neurological diseases [173]. Following this study, envelopes from several other viruses were found to be comparable or more neurotropic than VSV-G that could be potentially used as VSV-G substitutes, including that from Mokola and Lymphocytic choriomeningitis viruses (LCMV) [16, 167, 175]. For the transduction of lung cells, the Kobinger group carried out a systemic analysis of lentiviral vector pseudotypes to stably transduce airway epithelial in vivo, whereby they found that Ebola-G pseudotypes were able to efficiently transduce such cells after apical application at levels similar to that obtained with VSV-G [180]. Other pseudotypes were also found to transduce lung cells efficiently, such as Marburg virus and lymphocytic choriomeningtis virus (LCMV) [179-181]. For the transduction of liver cells, Ross River virus (RRV) pseudotypes were found to transduce Kupffer cells (specialized macrophages in liver) at high efficiency by the Kang lab [182]. For the transduction of hematopoietic system, the Sandrin group has developed Feline endogenous retrovirus (RD114) pseudotypes for more efficiency and less toxicity than VSV-G [183]. For the transduction of quiescent lymphocytes, Baboon retrovirus and Cocal Virus pseudotypes were shown to have higher efficiencies than VSV-G by the studies of two different labs [174, 176]. Moreover, envelope proteins derived from various

viruses (Measles Virus, Semliki Forest virus, Influenza virus, Baculovirus GP64, Respiratory syncytial virus, Hepatitis C virus and others) were also developed as potential candidates for lentiviral vector pseudotyping [62, 180, 183, 184, 225].

Alternative to VSV-G, there are about 30 different lentiviral vector pseudotypes that have shown promising results [102]. Although with a more restricted tropism, these pseudotypes have provided similar or better transduction efficiencies on specific cell populations compared to VSV-G [102]. As a common limitation, the titers of lentiviral vectors pseudotyped with these glycoproteins were usually significantly lower than those observed with VSV-G, creating impediment in large scale production of high titer vector stocks for gene therapy [102]. Additionally, since the technique of gene therapy is becoming promising and commonly utilized in many research fields, this limited number of envelope candidates still may not meet all the needs, as researchers are working on a variety of different species, diseases and cell types that require specific pseudotyping methods for their specific purposes. Therefore, alternative novel pseudotyping candidates are needed to possibly overcome the limitation of lower titers for many of the existing pseudotypes and provide better substitute candidates to VSV-G for gene delivery into different cell types.

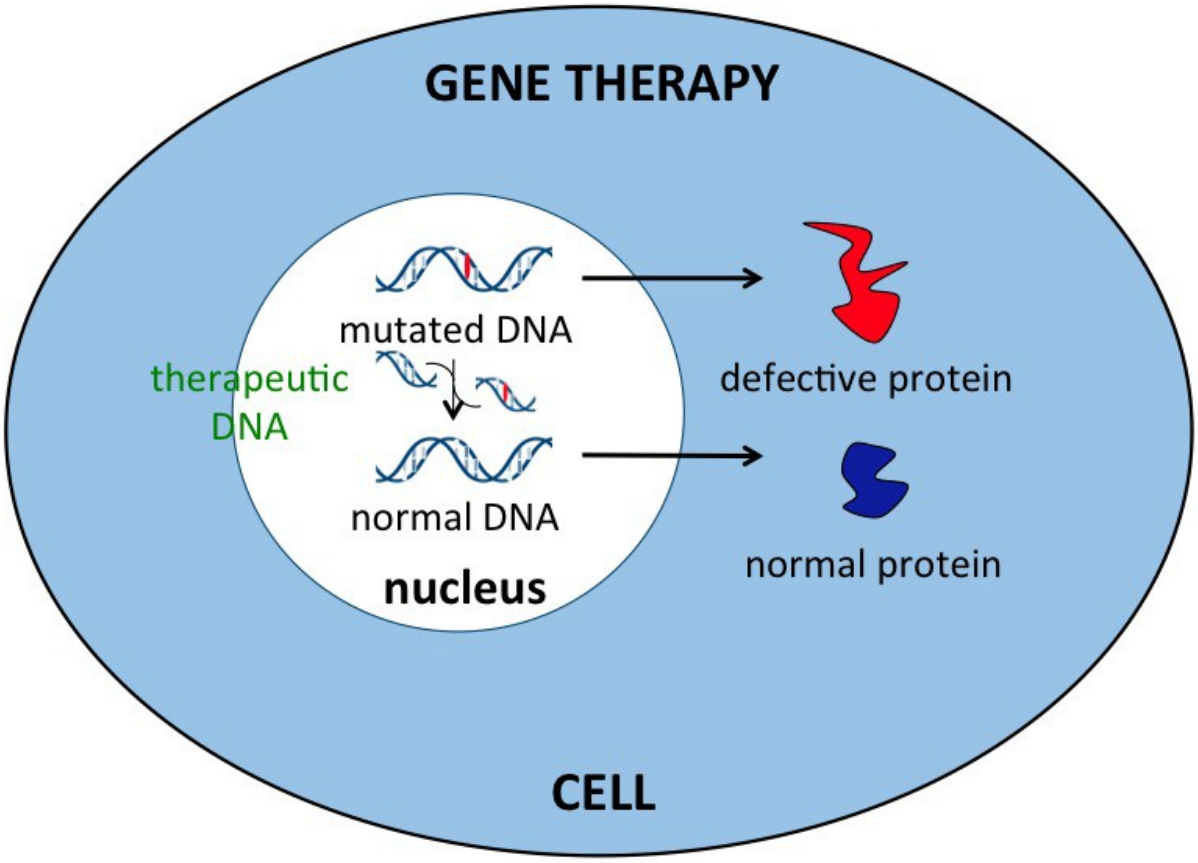


Figure 1.1 Schematic representation of the mechanism of classic gene therapy. The therapeutic DNA is delivered into the host cell to correct mutated DNA, leading to the synthesis of normal protein and restored function. [Figure obtained from Engage-Science (http://www.engage-science.com/gene-therapy-the-future-of-medicine/)]

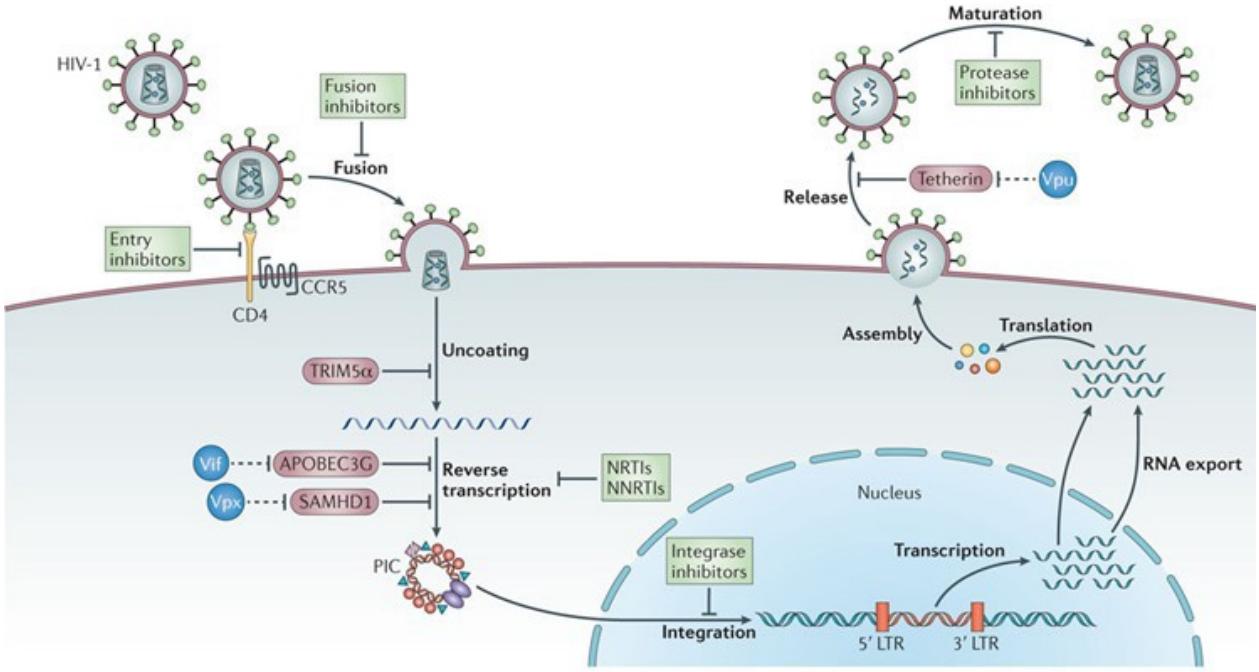


Figure 1.2 HIV-1 replication cycle, host restriction factors and drug antagonists. HIV-1 binds to the CD4 receptor through the help of co-receptor (usually CCR5). After fusion of the viral envelope with the cell membrane, the virus uncoats the capsid and releases the RNA genome into the cytoplasm. The genome is then reverse transcribed to a complementary DNA and form the pre-integration complex (PIC). Later on, the PIC translocates to the nucleus where the viral DNA is integrated into the host DNA to form provirus and harness the host synthetic machinery to form new viral RNA and proteins. Afterwards, newly synthesized viral proteins translocate to the cell surface, viral genome gets packaged to assemble into new virions that finally bud off of the plasma membrane. Also shown are the key HIV restriction factors TRIM5α (tripartite motif-containing 5α), APOBEC3G, SAMHD1 and tetherin and their corresponding viral antagonists (Vif, Vpx and Vpu). In addition, the drugs for HIV-1 include NRTIs (nucleoside reverse transcriptase inhibitors), NNRTIs (non-nucleoside reverse transcriptase inhibitors) and integrase inhibitors, suppressing HIV-1 replication at critical steps as indicated. [Figure adapted from Barre-Sinoussi, Francoise, et al. (2013)] [41]

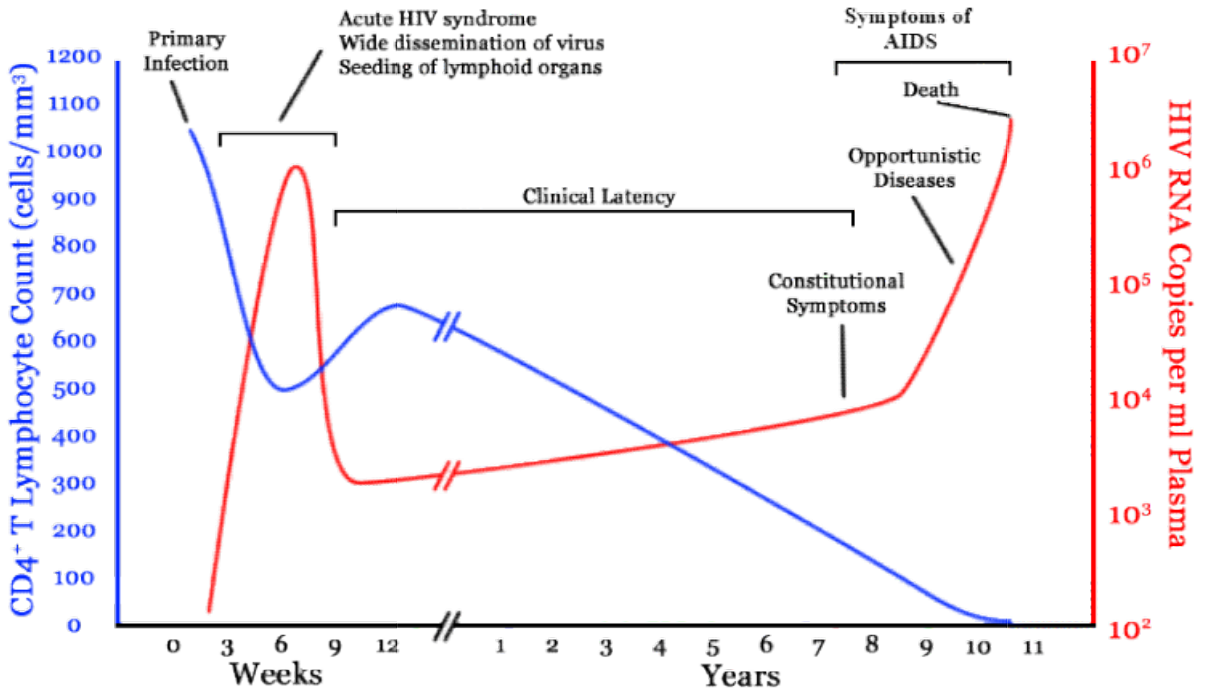


Figure 1.3 HIV time course: represented by CD4 T lymphocyte counts and R A copies per ml plasma at the stages of primary infection, latency and the onset of AIDS. In primary infection, virus replicates rapidly, with the resultant fast decline of CD4 T-cell count, which is known as the acute HIV syndrome. Later on, immune responses kick-in to suppress the viral progression, with temporary recovery of CD4 level and undetectable viremia, resulting in clinical latency. However, pro-virus DNA has already integrated into host genome and resides in bone marrow and lymphoid tissues that would eventually rebound for the synthesis of new viruses. With the destruction of CD4 T lymphocyte population and generalized immune suppression, symptoms of clinical AIDS develop and patients are likely to contract and succumb to deadly opportunistic infections.[Figure adapted from Pantaleo, G, et al. (February 1993)] [42]

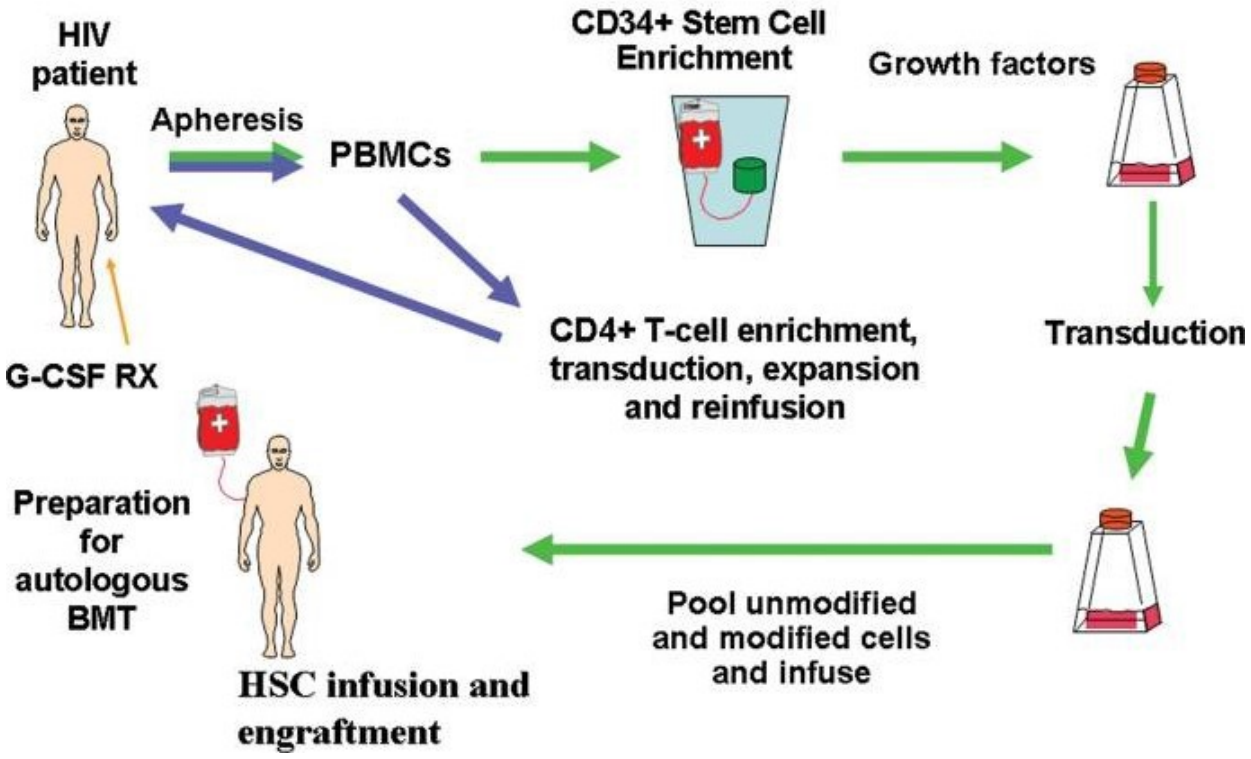


Figure 1.4 Schema for two common ways of ex vivo autologous hematopoietic gene therapy for the treatment of HIV-1 infection. In the first method, peripheral blood mononuclear cells are obtained from patients. The CD4 T lymphocytes are then enriched after CD8 T lymphocytes depletion, followed by transduction with the vector harboring the therapeutic genes. Transduced CD4 cells are stimulated to expand and re-infused back to patients for function restoration. In the second approach, CD34 hematopoietic stem cells (HSC) are collected by apheresis from patients after cytokine treatment. Several rounds of GCSF (granulocyte colony stimulation factor) injection are required for mobilizing CD34 hematopoietic stem cells from the patient. These cells are kept frozen until the patient is ready for the stem cell transplant. At that time, the cells are thawed and manipulated in vitro by transduction with vector delivering the desired therapeutic genes. The transduced autologous cells are then infused back into the patient and allowed for engraftment. [Figure adapted from Scherer L J, et al. (2011)] [58]

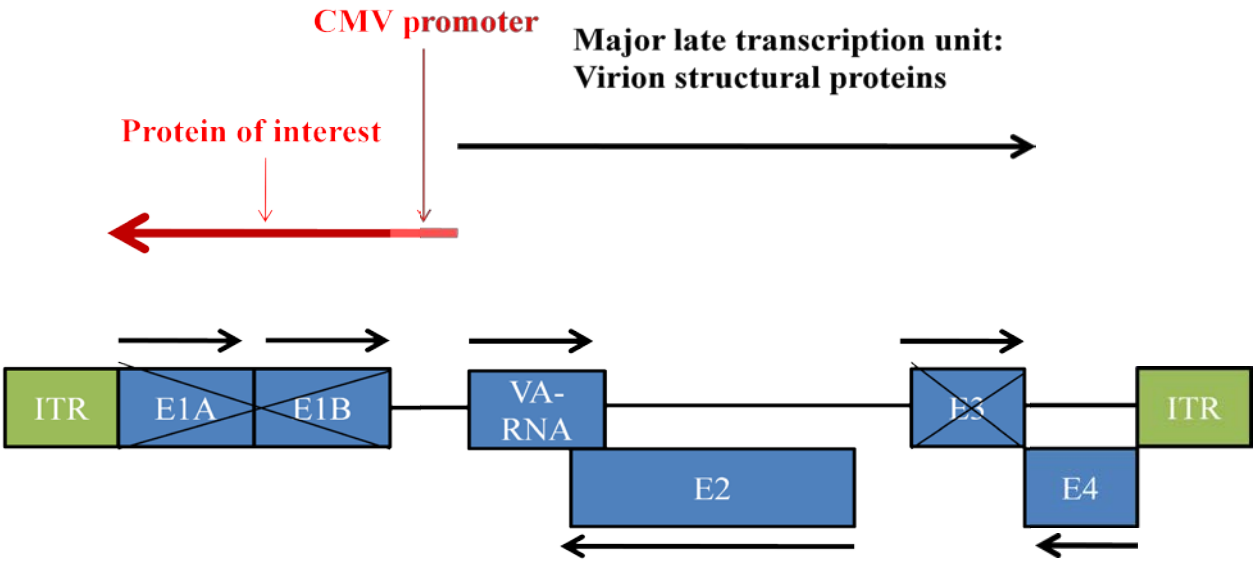


Figure 1.5 Schematic diagram of the adenoviral vector genome. Important viral genes are indicated in the figure. Each gene has specific functions such as E1A-drives cells from G0 to S-phase, induces adenovirus gene transcription, blocks IFN-mediated responses; E1B-inhibits apoptosis; E2-regulates viral DNA replication; VA-RNA-Inhibits apoptosis; E4-regulates viral transcription, mRNA export from nucleus and apoptosis; E3-protects apoptosis, ITR-inverted terminal repeats). Adenoviral vector is engineered by deletions of E1A, E1B and E3 genes and insertion of transgene encoding protein of interest under the regulation of a CMV promoter.

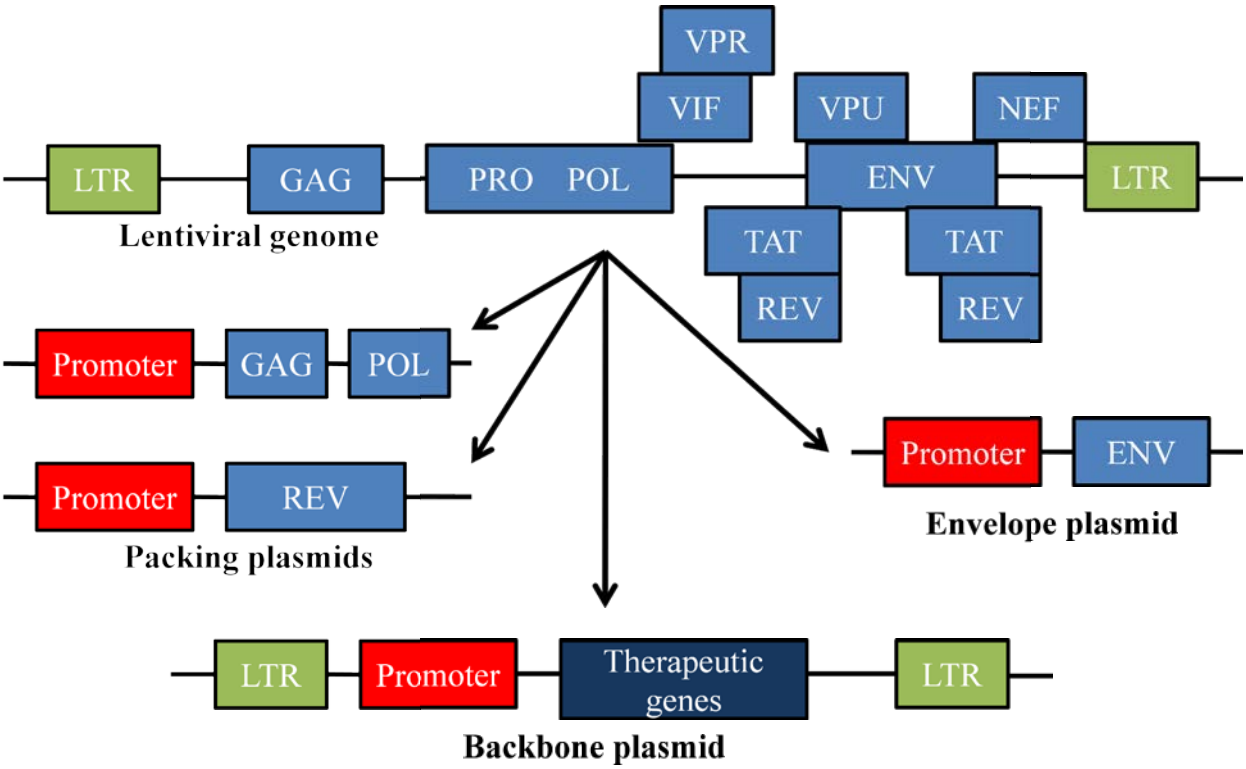


Figure 1.6 Schematic diagram of the lentiviral vector genome and the third-generation packaging system. Important viral genes are indicated in the lentiviral genome (gag, pol, tat, rev-function in reverse transcription, integration, nuclear export and packaging; vif, vpr, vpu-regulate viral replication, and are viral antagonists to host restriction factors; env-encodes viral envelope). In the third-generation system for the production of lentiviral vector, four individual plasmids are used for co-infection of the packaging cell line to generate the whole vector and this approach offers better safety. The backbone plasmid delivers the therapeutic genes and forms the genome of the viral vector. Another two packing plasmids encode the accessory Gag, Pol and Rev proteins. The fourth envelope plasmid encodes the glycoprotein that encapsulates the virion. This separate envelope plasmid confers the ability to pseudotype the third-generation lentiviral vector system with novel glycoproteins that offer a broad tropism or to suit a specific therapeutic application to target specific sets of cells.

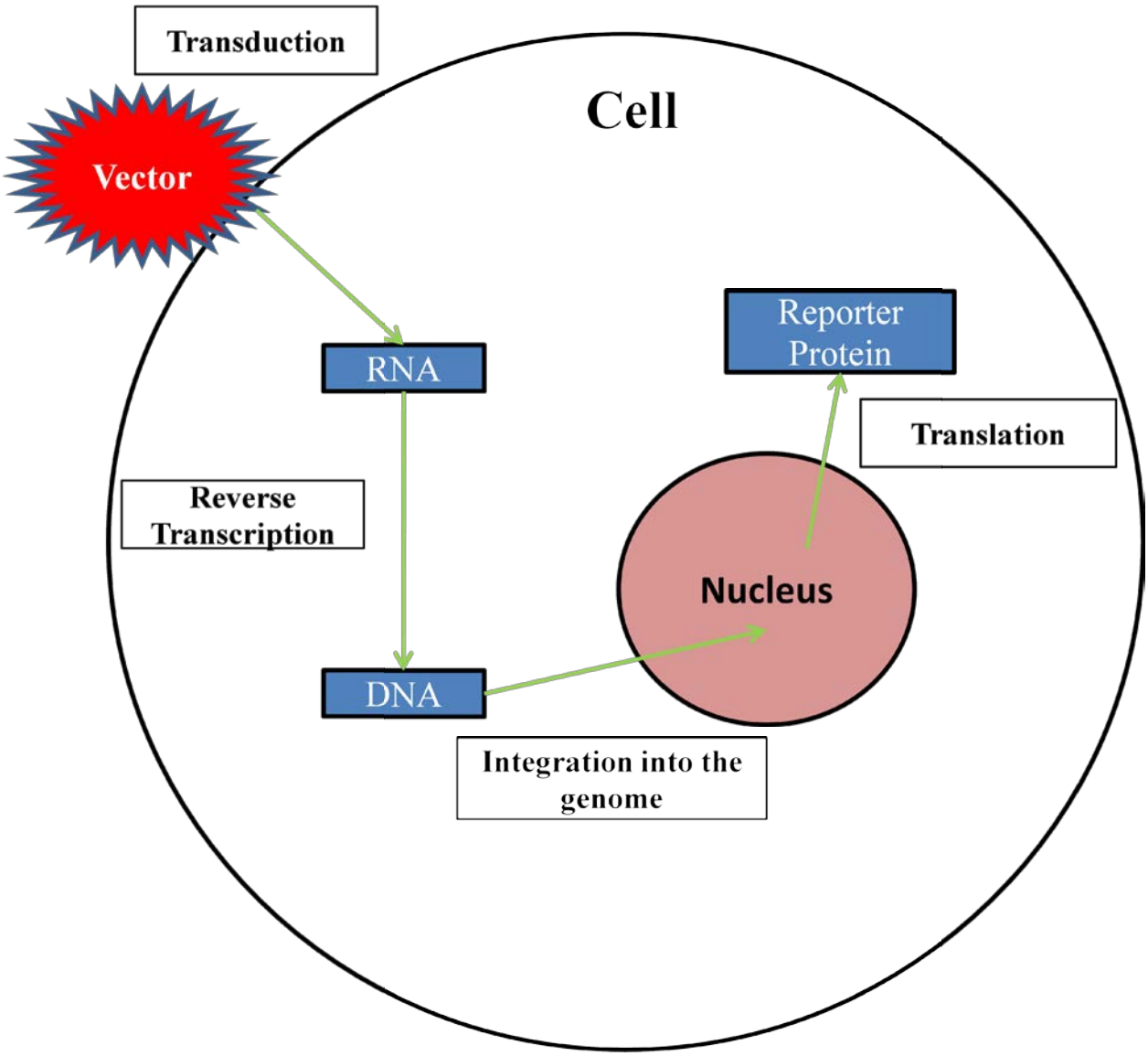


Figure 1.7 Schematic diagram showing lentiviral vector-based gene delivery into target host cells. Recombinant lentiviral vector harboring transgenes is used to infect or transduce host cell. After infection, the lentiviral vector releases its RNA genome that is reverse transcribed and integrated into host genome, in a method similar to wild-type lentiviral infection. The integrated transgene then utilizes host transcriptional and translational machinery for therapeutic protein synthesis and to exert its beneficial effects.

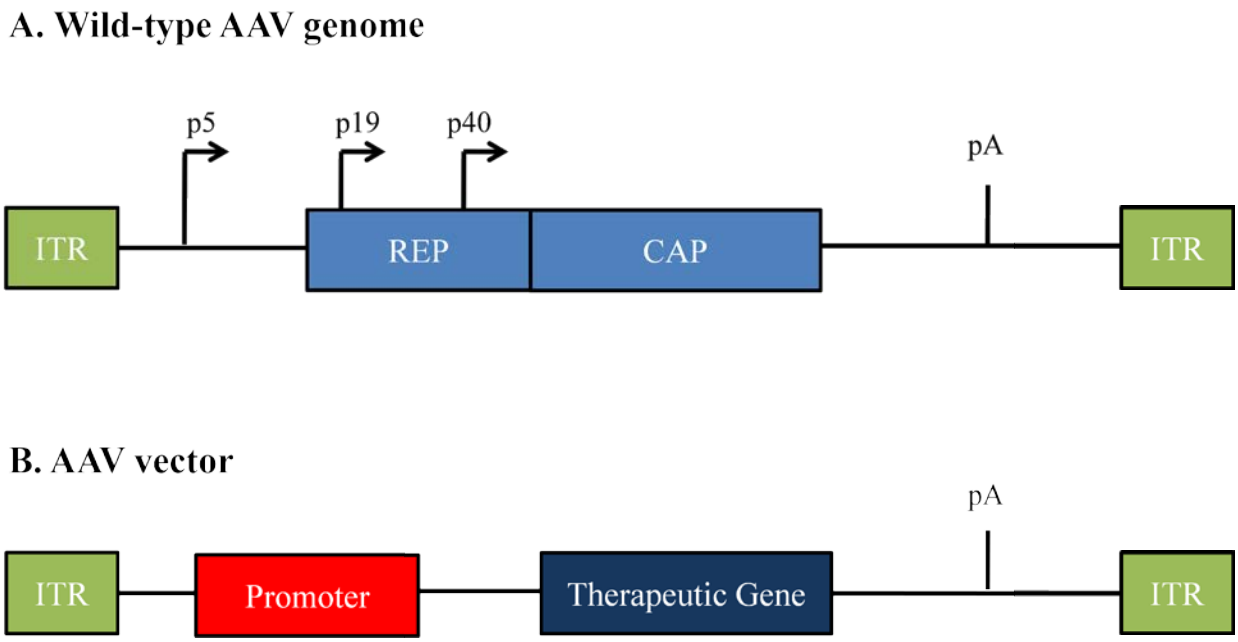


Figure 1.8 Schematic representation of wild-type AAV genome and recombinant AAV vector genome. A. The wild-type AAV consists of the viral genes rep and cap coding for viral proteins, the AAV porters (p5, p19, p40), the polyadenylation site (pA) and the inverted terminal repeats (ITR). B. In recombinant AAV vector, the rep and cap genes are replaced by a transgene cassette harboring the promoter, the therapeutic gene and the pA-site.

Table 1.1 Advantages and disadvantages of the most commonly used viral vector systems.

Viral vectors	Advantages	Disadvantages
Adenoviral vectors	High- titer vector stock, rapid and high level of gene expression, moderate insertion capacity (8 kb), infect both dividing and non-dividing cells	Frequent immune-mediated clearing of adenoviral proteins due to pre-existing antibodies from previous exposures in humans, transient gene expression
Retroviral vectors	High-titer vector stock, integration into host genome, broad infectivity, relatively easy to engineer viral genome and envelope	Random insertional mutagenesis, no infection on non-dividing cells, vector instability
Lentiviral vectors	High-titer vector stock, integration into host genome, broad infectivity, relatively easy to engineer viral genome and envelope, large insertion capacity (9-10 kb), infect both dividing and non-dividing cells, no reported cases of insertional mutagenesis	Potential cytopathic effects contributed by heterologous pseudotyping viral envelopes
AAV vectors	Low immunogenicity, non-pathogenic, broad tropism, infect dividing and non-dividing cells, broad cell tropism	Production is dependent on co-infection, limited insertion capacity, low purity of vector stock
Herpesvirus vectors	High-titer vector stock, high insertion capacity (50 kb), neuronal tropism	Potential toxicities, risk of recombination, transient gene expression, complexity of the viral genome
Poxvirus vectors	Large insertion capacity (25 kb), high gene expression, suited for vaccine development	Cytopathic effects, complexity of the viral genome

Table 2.2 Overview of popular viral envelopes used for lentiviral vector pseudotyping and their cell/organ tropism preferences.

Family	Genus	Virus/glycoprotein	Target cell/organ	References
Rhabdoviridae	Vesiculovirus	VSV-G	Liver, Pancreatic islet cells, CNS, Retina	[191, 218]
Rhabdoviridae	Vesiculovirus	Cocal virus	CD34 cells, lymphocytes	[176]
Rhabdoviridae	Lyssavirus	Rabies virus	Neurons/CNS	[173]
Rhabdoviridae	Lyssavirus	Mokola	Pancreatic islet, CNS, retina, Myocytes/muscle	
Arenaviridae	Arenavirus	LCMV (Lymphocytic choriomeningitis virus)	Liver, Pancreatic islet, CNS, Cancer cells	[226]
Togaviridae	Alphavirus	Ross River virus	Liver and spleen cells, Kupffer cells, neuroglial cells	[182]
Filoviridae	Filovirus	Ebola virus	Lung/apical surface airway, epithelium	[180]
Retrovirus	Lentivirus	Baboon retrovirus	Resting CD34 cells	[174]
Retrovirus	Gammaretrovirus	Feline endogenous retrovirus (RD114)	Human blood cord-derived CD34 cells, clonogenic progenitors	[183]
Retrovirus	Gammaretrovirus	Murine leukemia virus (MLV)	Neuroblastoma cells, human blood cord-derived CD34 cells	[227]
Paramyxoviridae	Morbillivirus	Measles virus	Quiescent T cells	[177]

Chapter 2

Large Scale Production of Lentiviral Vectors Pseudotyped with CNV-G, PRV-G and VSV-G

2.1 Summary

HIV-1-derived lentiviral vector is commonly used as the vehicle to deliver transgenes into target cells. In order to treat a specific disease, successive doses of vector preparation need to be administered to patients for sustainable expression of therapeutic genes, thus requiring an enormous amount of lentiviral vectors. Therefore, an optimum method for large scale production of lentiviral vector preparation is urgently needed. To generate high-quality lentiviral vector for clinical use, packaging plasmids are utilized to transiently transfect HEK293T cells for large scale vector expression. With additional downstream concentration and purification, lentiviral vectors can be prepared to high titer for gene delivery into target cells. Here we evaluated the abilities of two novel envelope glycoproteins from Chandipura and Piry viruses (CNV-G and PRV-G) in the large scale production of lentiviral vectors, in a direct comparison with Vesicular Stomatitis Virus envelope Glycoprotein (VSV-G).

2.2 Introduction

Gene therapy is a promising approach to treat and cure many life-threatening diseases, including cancers, AIDS, inherited disorders and neurological diseases [9, 10]. Through the delivery of therapeutic genes, it fixes the mutated DNA and restores function in the cell [1]. HIV-1-derived lentiviral vector is an efficient vehicle to deliver transgenes into target cells [191, 218]. Lentiviral vector has a naturally narrow tropism but has the ability to be pseudotyped,

which means its envelope can be easily exchanged with that of other viruses with an inherent broad tropism [102]. The most commonly used candidate for lentiviral vector pseudotyping is the glycoprotein of the vesicular stomatitis virus (VSV-G), which is considered as the “gold standard” owning to its wide tropism in nature that allows transduction of a wide range of target cells [191, 218]. Spike glycoprotein of VSV-G plays a major role in viral entry and upon interaction with its cognate cell surface receptor, induces clathrin-mediated endocytosis to enter the cell [228, 229]. VSV uses cell surface LDL (Low-Density Lipoprotein) receptor as its major entry receptor and the wide distribution of LDL receptors in many types of cells explains the broad tropism of VSV [230]. Although regarded as the “gold standard”, pseudotyping of lentiviral vectors with VSV-G still possess some technical challenges and drawbacks. Firstly, the production of VSV-G pseudotyped lentiviral vector can be cytotoxic to the packaging cell lines, due to the fusogenic properties of VSV-G and its high-level expression causes cell fusion and eventually death [231]. Secondly, the transduction efficiency of this pseudotype was found to be not ideal on some cell types, including cells of neuronal and B lymphocytic origins [173, 174]. Thirdly, VSV-G pseudotype was found to be highly sensitive to human serum inactivation, due to the natural IgM and complement effects, thus reducing its efficiency through direct injection [224]. Furthermore, with a non-specific broad infectivity, VSV-G pseudotype is likely to transduce any types of cells that it encounters, resulting in a dramatic loss of functional units when it finally reaches the target cells after systemic injection. Importantly, VSV-G pseudotype induces antibody responses upon in vivo administration that considerably reduces its efficiency in subsequent injections due to immune-clearing and is thus unsuitable for multiple dosage regimens.

In order to overcome some of these drawbacks, we introduced glycoproteins from Chandipura and Piry viruses (CNV-G and PRV-G) which are also vesiculoviruses like VSV,

belonging to the family Rhabdoviridae, but serologically distinct (Figure 2.1) [232, 233]. Chandipura virus is an emerging pathogen in the tropical areas of India that usually causes severe encephalitis in children [234]. Piry virus is also a potential human pathogen which was originally discovered in Brazil [233, 235, 236]. Both of these viruses are phylogenetically related to VSV, but there are no serological relationships between them [232, 233]. The AP2A1 and AP2A2 protein subunits on the cell surface, which are adaptins that constitute the multimeric AP2 adaptor protein complex present on growing clathrin-coated pits, may be important for the entry of Chandipura virus [237]. The exact receptor used by Piry virus is not yet identified. Differences in the specific receptor usage by these different vesiculoviruses might be crucial for the differences in their tropisms and thus their transduction ability into different cell types. The glycoproteins of CNV and PRV are poorly studied, but both possess potential for pseudotyping lentiviral vectors and overcoming the aforementioned limitations of VSV-G for use as non-crossreactive substitutes.

Here we evaluated the abilities of two novel envelopes (CNV-G and PRV-G) in pseudotyping lentiviral vector, in a direct comparison with VSV-G.

2.3 Material and methods

2.3.1 Packaging plasmids for lentiviral vector generation

A third-generation lentiviral vector production system was used, in which a total of four packaging plasmids were required for the generation of each lentiviral vector pseudotype (Figure 2.2) [97]. Firstly, the HIV-1-derived lentiviral vector backbone plasmid containing an EGFP reporter gene (pHIV-7-GFP) was used in this study [97, 238]. This vector backbone contains two

cis-acting elements to enhance vector performance, namely the central DNA flap consisting of a central polypurine track and central termination sequence, to facilitate the nuclear import of the viral pre-integration complex, and the woodchuck hepatitis virus post-transcriptional regulatory element to promote nuclear export of transcripts and/or increase the efficiency of polyadenylation of transcripts [97]. Secondly, a plasmid expressing the envelope protein was needed. For this purpose, VSV-G encoding pCMV-VSVG was cloned into the pTARGET expression plasmid containing a CMV promoter and obtained from our collaborators, Dr. John Rossi and Dr. Mingjie Li from the Division of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, CA, USA. For plasmids encoding PRV-G and CNV-G envelopes, Piry and Chandipura viruses were obtained from Dr. R. E. Shope, University of Texas, Galveston. Both viral G protein genes were amplified by RT-PCR and cloned into the pTARGET expression plasmid containing the CMV promoter. Overall, the heterologous envelope genes, VSV-G, CNV-G and PRV-G were cloned into the pTARGET expression plasmid containing a CMV promoter to produce pseudotyped lentiviral vectors, and the resultant plasmids were named pCMV-VSVG, pCMV-CNV-G and pCMV-PRVG. In addition, the other two packaging plasmids for the synthesis of Rev and Gag-Pol proteins (pCMV-Rev and pCHGP-2) were also obtained from Dr. John Rossi and Dr. Mingjie Li, and were prepared by RT-PCR in a similar way.

2.3.2 Expansion and purification of packaging plasmids

Each plasmid was used to transform electrocompetent E. coli culture (DH5α), followed by expansion and purification by the Maxiprep (Qiagen, Cat. #12191). For transformation, 10 ng

of plasmid in a volume of 2 µl was mixed with 20 µl of DH5α Escherichia coli (Thermo Fisher Scientific, Cat. #18263012) in a 0.2 gene MicroPulser cuvette (BIO-RAD, Cat. #1652081) kept on ice. Later on, the cuvette was slowly placed onto the electroporator (BIO-RAD) for electroporation (2 kV voltage, 25 µF capacitor and 200 Ω pulse controller setting), a molecular biology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing plasmid DNA to be introduced into DH5α E. coli. After transformation, bacteria containing target plasmid was recovered in 1 ml SOC (Thermo Fisher Scientific, Cat. #15544-034) medium and cultured in a tube shaker (300 RPM, 37 ^{o}C). After 45 minutes, transformed DH5α E. coli was streaked onto an LB agar plate and cultured at 37 ^{o}C overnight. The next day, several isolated colonies with regular shapes were picked by pipette tips, individually, and cultured in 5 ml of LB broth for 8 hours. The bacteria culture was sedimented by centrifugation (2,000 RPM, 5 minutes) and the cell pellet was then used for Miniprep (Qiagen, Cat. #27104). The plasmid DNA from it was checked for purity by agarose gel electrophoresis. Recombinant DH5α E. coli transformed with pure plasmid DNA was then expanded and cultured in a large volume of LB medium (1 L in total) for 16 to 20 hours, 800 µl of each recombinant E. coli culture was resuspended in 200 µl glycerol as stock for longer storage at -80 ^{o}C. The bacterial pellet was collected from the rest of the LB bacterial culture by centrifugation (2,000 RPM, 5 minutes). Maxiprep was then performed on this pellet according to manufacturer's protocol. Purified plasmid was eventually diluted in water to a concentration between 2 µg/µl to 4 µg/µl and stored at -20 ^{o}C.

2.3.3 Generation of pseudotyped lentiviral vectors

Four packaging plasmids were used for the production of lentiviral vector, including pHIV-7-GFP, pCMV-Rev, pCHGP-2 and an envelope plasmid from pCMV-VSVG, pCMV-CNV-G or pCMV-PRVG for envelope synthesis (Figure 2.3 A and B) [97]. Transient transfection with packaging plasmids was performed on HEK293T cells (Human Embryonic Kidney 293 cells; ATCC, Cat. #CRL-11268). Two reagents were used in the calcium-phosphate transfection: 2X HBS (HEPES buffered saline) prepared with 3.28 g NaCl, 0.0424 g Na_2HPO_4 and 2.382 HEPES in 200 ml mili-Q water, adjusted to pH of 7.05 and filtered before use; and 0.3 M $CaCl_2$ prepared with 8.82 g $CaCl_2$ powder and 200 ml mili-Q water, filtered before use. In all, 15 µg of the packaging construct pCHGP-2, 15 µg of pHIV-7-GFP transfer vector, 5 µg of pseudotyping envelope (pCMV-VSVG, pCMV-CNVG or pCMV-PRVG), and 5 µg of pCMV-Rev were used to co-transfect 90% confluent HEK293T cells cultured on 100-mm plates by calcium phosphate transfection [97, 239]. The cocktail of four plasmids were first added to the $CaCl_2$ reagent and mixed gently. Afterwards, the $CaCl_2$ reagent with plasmids was poured slowly into 2X HBS reagent at a dilution of 1 to 1, with constant agitation until precipitation was observed. Subsequently, this precipitate was carefully applied to cells for transfection. After a 4-hour incubation at 37 ^{o}C with 5% CO_2, the cells were replenished with fresh DMEM media with 40 µl butyric acid (transcription activator). At 24 hours post transfection, supernatant was collected and stored at -80 ^{o}C, and fresh DMEM media with 50 µl butyric acid was added back to plate. Similarly, two more collections were performed at 48 and 72 hours post transfection, with one media change in between with DMEM containing 60 µl butyric acid. Overall, a volume of 24 ml of vector preparation was collected from each plate and stored at -80 ^{o}C for further modification.

2.3.4 Concentration of lentiviral vectors by ultra-centrifugation

Vector preparations were concentrated by ultracentrifugation (Figure 2.3 B) [97]. First, vector supernatant was thawed partially in a 37 ^{o}C water bath until 90% of the culture medium was thawed and was then placed on ice to thaw completely. The vector supernatant was then filtered through a 0.2 µm filter, while still on ice, to remove cell debris from vector collections. 38 ml of flow-through was then transferred into each of six sterile plastic centrifuge tubes and placed into SW28 ultracentrifuge steel tubes (Beckman Coulter) individually. Later on, these steel tubes were tightly secured in SW28 rotor and transferred into the ultracentrifugation machine (Beckman Coulter). After 2 hours of centrifugation at 24,000 RPM, the supernatant was poured off slowly and the resultant pellet left intact. More filtered vector preparation was added back to the same tubes for additional 3 to 4 rounds of centrifugation, depending on the volume of vector preparation. After the final centrifugation, the pellet that contains the concentrated vector was resuspended in DMEM, aliquoted into small volume and stored at -80 ^{o}C for future use.

2.3.5 Determination of titer

Functional vector titers were obtained using serial dilutions from vector stocks to transduce HEK293T cells and measured for subsequent GFP expression by FACS (Figure 2.3 B) [97]. Briefly, serial dilutions with 0.2 µl, 0.5 µl, 1 µl, 2 µl, 5 µl and 10 µl of vector stock in 1 ml of DMEM media were used to transduce 500,000 HEK293T cells in the presence of polybrene (8 µg/ml). After 72 hours, cells were harvested and estimated for GFP expression percentage by FACS. After analysis in FlowJo software, the functional titer in TU/ml was calculated with the equation (TU/ml = GFP% x Dilution Factor x 500,000). The p24 content of vector preparation

was determined using the Lenti-X p24 Rapid Titer Kit from Clontech per the manufacturer's instructions [97]. Vector p24 titer was then converted to ng/ml. The vector RNA copy number was determined by qRT-PCR (quantitative real-time-PCR) with primers and probe for the HIV-1 LTR region as described in our previous publication [240]. The titer of vector RNA was indicated as RNA copies/ml in this study.

2.3.6 Statistical analysis

To assess the significance of differences seen among the three pseudotypes, statistical analysis was used to evaluate data from multiple experiments using GraphPad Prism version 6 (GraphPad Software, USA). Student's unpaired t-test was used to compare the titers. P value less than 0.05 was considered to be significant.

2.4 Results

All packaging plasmids were successfully purified to high concentration by Maxiprep and were sufficient for the large scale production of lentiviral vector production. Subsequently, 40 to 60 plates of low-passaged HEK293T cells with 90% confluency and greater than 95% viability were transfected with four lentiviral vector packaging plasmids. At 12 hours post transfection, HEK293T cells became rounded and showed robust GFP expression under the fluorescent microscope, indicating intrinsic cellular activity for the expression of the packaging plasmids. At 24 hours post transfection, the first collection was performed when cells still looked healthy and remained attached to the plate. However, a certain number of cells became detached during subsequent collections, due to the transfection-associated toxicity and the accumulating

butyric acid's effect. As a result, there was noticeable cell debris in the second and third collections. At 72 hours, more than half of the transfected cells detached from the plate and no further collection could be achieved. Infectivity of these raw vector preparations was measured in HEK293T cells, with the second collection (48 hour post transfection) showing the highest titer among all three collections. At the onset of this project, transfection efficiency was not ideal because of inconsistency with calcium-phosphate precipitation (Figure 2.4 A and B). Later on, the efficiency was improved dramatically with high-quality transfection buffers, improved timing in precipitation and better techniques for collecting vector supernatant (Figure 2.4 C). Eventually, high-quality lentiviral vectors were able to be produced consistently for further experiments.

Cell culture supernatants (titers around 1×10^6) were concentrated approximately 500-1000 fold by ultra-centrifugation. Afterwards, several batches of lentiviral vector particles were prepared from different time points and titered by three methods. . The student's unpaired t-test was used to analyze the different titers of lentiviral vector pseudotypes, the values of which followed normal distribution [241]. In the HEK293T titrations as interpreted by GFP expression (Figure 2.5), the titers of VSV-G pseudotypes ranged from 1.1×10^8 to 1.1×10^9 TU/ml, the titers of CNV-G pseudotypes ranged from 7.8×10^7 to 6.8×10^8 TU/ml, and the titers of PRV-G pseudotypes ranged from 4.6×10^7 to 5×10^8 TU/ml (Figure 2.6 A). In the p24 titrations, the titers of VSV-G pseudotypes ranged from 1.27×10^4 to 6.47×10^4 ng p24/ml, the titers of CNV-G pseudotypes ranged from 2.2×10^4 to 1.2×10^5 ng p24/ml, and the titers of PRV-G psudotypes ranged from 4.1×10^3 to 2.8×10^4 ng p24/ml (Figure 2.6 B). By qRT-PCR, the titers of VSV-G pseudotypes ranged from 4.8×10^9 to 1.9×10^{10} RNA copies/ml, the titers of CNV-G pseudotypes ranged from 8.0×10^9 to 3.2×10^{10} RNA copies/ml, and the titers of PRV-G

pseudotypes ranged from 2.1 x 10^9 to 8.7 x 10^9 RNA copies/ml (Figure 2.6 C). After statistical analysis, no significant differences were seen among three pseudotypes in three titer methods ($p>0.5$).

2.5 Discussion

Several groups have reported multiple envelope proteins that could be utilized to pseudotype HIV-1 based lentiviral vectors for specific purposes, tailored to suit their fields of study [173, 174, 176, 177, 180, 183]. Novel pseudotypes, such as Baculovirus GP64 and Arenavirus LCMV, were developed with tropisms intended to target specific cells [226, 242]. However, the titers of these pseudotypes were usually greater than 1 log lower than VSV-G [226, 242]. In addition, the stability of most pseudotypes is not ideal for ultra-centrifugation concentration, a procedure required to obtain a high viral titer, but that could potentially damage recombinant viral particles before their preparation for transduction. In our lab, we were interested in the broad tropism of vesiculoviruses, which include Chandipura virus (CNV) and Piry virus (PRV) that cause generic diseases in humans [243]. Both viruses are serologically distinct from VSV [232, 233], thus providing pseudotype substitutes for successive vector administrations for cases in which patients have mounted an antibody response against a previous VSV-G pseudotype. Although poorly characterized in previous studies, CNV and PRV have the potential to infect certain cell types at high efficiencies, an ideal property for pseudotyped vectors. Moreover, the neurotropic potential of CNV may make it a better candidate for transduction of neuron cells than VSV-G pseudotype [234, 237]. Therefore, we pseudotyped lentiviral vector with these novel envelopes. We used the third-generation lentiviral vector production system, in which the vector only requires three out of nine HIV genes (gag, pol, rev)

to form mature virions, which are compensated by helper plasmids [110]. Compared to the previous two generations, the third-generation lentiviral vector production system has additional deletions of several HIV genes (tat, nef and vpu) for safer application, and is generated through expression of the Rev protein in trans [110]. Additionally, the vector backbone used in this project harbors a deletion of the 3' U3 region in the LTR [244]. The deletion is then transferred to the 5' U3 after reverse transcription, resulting in a non-functional LTR in the proviral DNA following integration into host genome [244]. These vectors, which lack necessary cis-acting elements and viral promoter in the LTR region, are known as replication non-competent or self-inactivating (SIN) vectors, and were designed for safer gene therapy applications [244]. For lentiviral vector generation, VSV-G, CNV-G and PRV-G pseudotyped lentiviral vectors carrying the GFP transgene were produced by transient transfection in HEK293T cells with four plasmids (pHIV-7-GFP backbone plasmid, gag/pol plasmid, rev plasmid, and env plasmid expressing VSV-G, CNV-G or PRVG), and concentrated by ultracentrifugation [97, 238]. To confirm that CNV-G and PRV-G could efficiently pseudotype lentiviral vectors, pseudotyped lentiviral vector particles were functionally titered by transducing HEK293T cells to measure transduction units, non-functionally titered by p24 ELISA and qRT-PCR to measure vector particle quantity and genome copies, respectively. VSV-G pseudotyped lentiviral vectors were also titered in the same manner for comparison.

The differences in the titer range between batches of pseudotypes could be due to the varying levels of vector output from the transient transfections. These differences were not statistically significant and the titers of the three pseudotypes were very similar overall. In the functional titration, titers of CNV-G and PRV-G pseudotypes were slightly lower than those for VSV-G pseudotypes, but were still higher than several previously developed envelope

pseudotypes [62, 180, 183, 222, 245]. In the p24 ELISA titration, titers for CNV-G pseudotypes were higher than VSV-G, indicating a better expression of lentiviral vector proteins but possibly a lower overall packing efficiency in the transient transfection as shown by the functional titration studies. Titers for PRV-G pseudotypes were 2-3 fold lower compared with VSV-G pseudotypes in both titrations. Measured RNA copies/ml from the qRT-PCR titration were about 20 to 50 fold higher than the TU/ml from the HEK293T titration, indicating a greater number of genome copies than infective units in vector preparations. One possible reason for this is that there was an inevitable margin of error in the lentiviral vector packaging, during which a certain amount of non-functional lentiviral vector was synthesized that contained backbone genomes but were not infective. Additionally, some decrease in infectivity is unavoidable during the process of vector collection and concentration. Finally, although the vector preparation was filtered before ultra-centrifugation, cell debris from the vector packaging cell line was likely collected and concentrated in the vector stock. Upon vector administration, this cellular debris could activate an immune response in human patients and cause unwanted side effects. One way to mitigate this problem is by filtering the concentrated vector stock through a sucrose cushion to eliminate cellular debris, though this additional purification step could potentially also result in the loss of a significant number of vector particles [246]. Therefore, for future studies, an alternative protocol is necessary which will eliminate cellular debris yet maintain vector infectivity.

Overall, the titers of the novel CNV-G and PRV-G pseudotypes were found to be surprisingly more efficient than many of the previously developed and documented envelopes [62, 180, 183, 222, 245]. In fact, the titers from all three lentiviral vector pseudotypes were among the best of all viral vectors and pseudotypes that have ever been developed [102], which

maybe because these glycoproteins are highly compatible with lentiviral vector virion structure. Both CNV-G and PRV-G envelopes showed promising efficiencies in pseudotyping lentiviral vector particles. PRV-G pseudotype was found to be slightly less efficient than VSV-G pseudotype but was still comparable, whereas CNV-G pseudotype was very similar to VSV-G and resulted in even higher p24 levels and genome copies in lentiviral vector pseudotypes. For successful clinical gene therapy, large quantities of viral vectors are usually necessary for several rounds of boosting transduction. Therefore, the production of high titers of pseudotyped lentiviral vector is crucial for CNV-G and PRV-G pseudotypes to be used in further gene therapy applications.

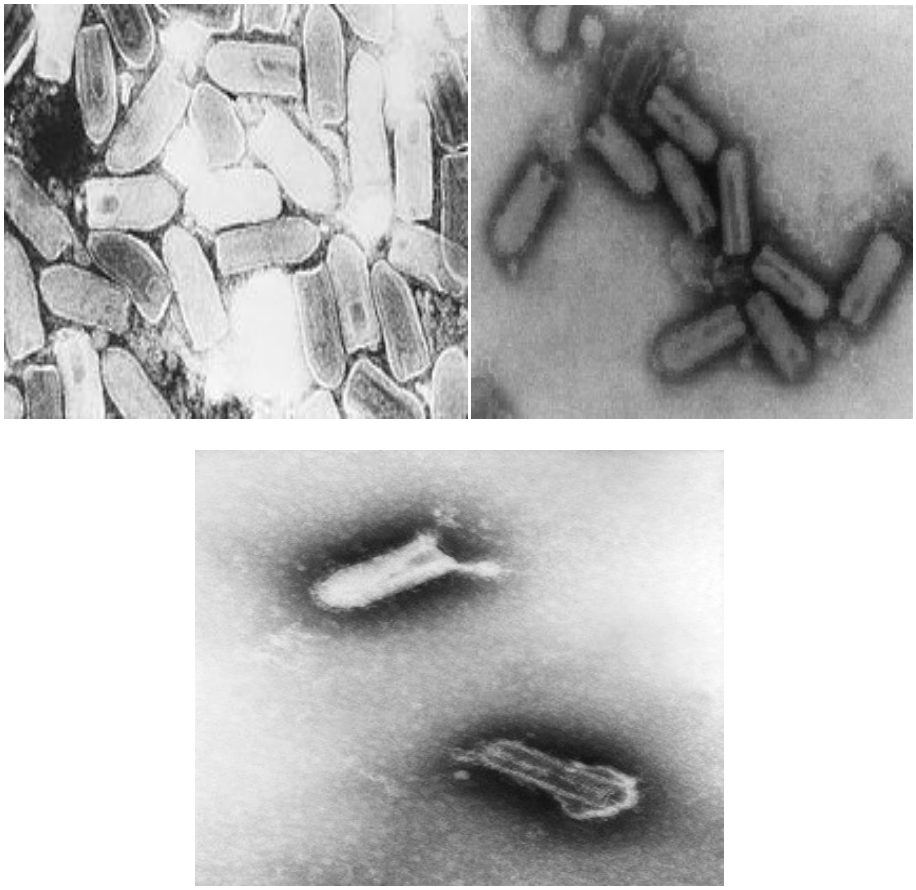


Figure 2.1 Electron micrographs of the Vesicular Stomatitis virus (top left), the Chandipura virus (top right) and the Piry virus (bottom). All three viruses belong to the genus Vesiculovirus in the family Rhabdoviridae and share a similar bullet-shaped morphology. Although they share many similarities, these three viruses are serologically distinct from each other. [Pictures adapted from online]

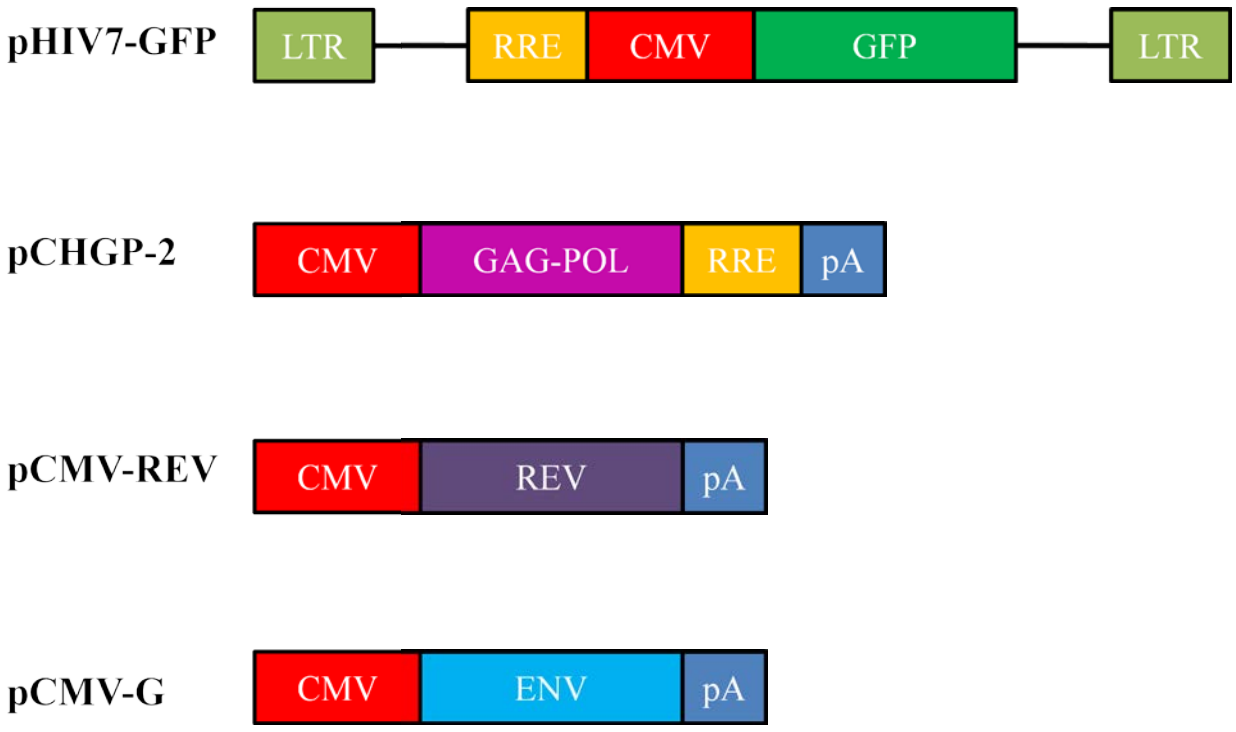


Figure 2.2 The four plasmids used in the production of third-generation lentiviral vectors. The pHIV7-GFP backbone plasmid is derived from the pHIV7 plasmid and encodes a GFP reporter protein placed under the transcriptional regulation of a CMV promoter. The pCHGP-2 and pCMV-REV encode accessory proteins for the synthesis of functional vectors. The pCMV-G is the envelope plasmid, which encodes the surface glycoprotein that encapsulates the virion. In this project, we used each of the three different envelope plasmids encoding VSV-G, CNV-G and PRV-G for the production of three different lentiviral vector pseudotypes, respectively.

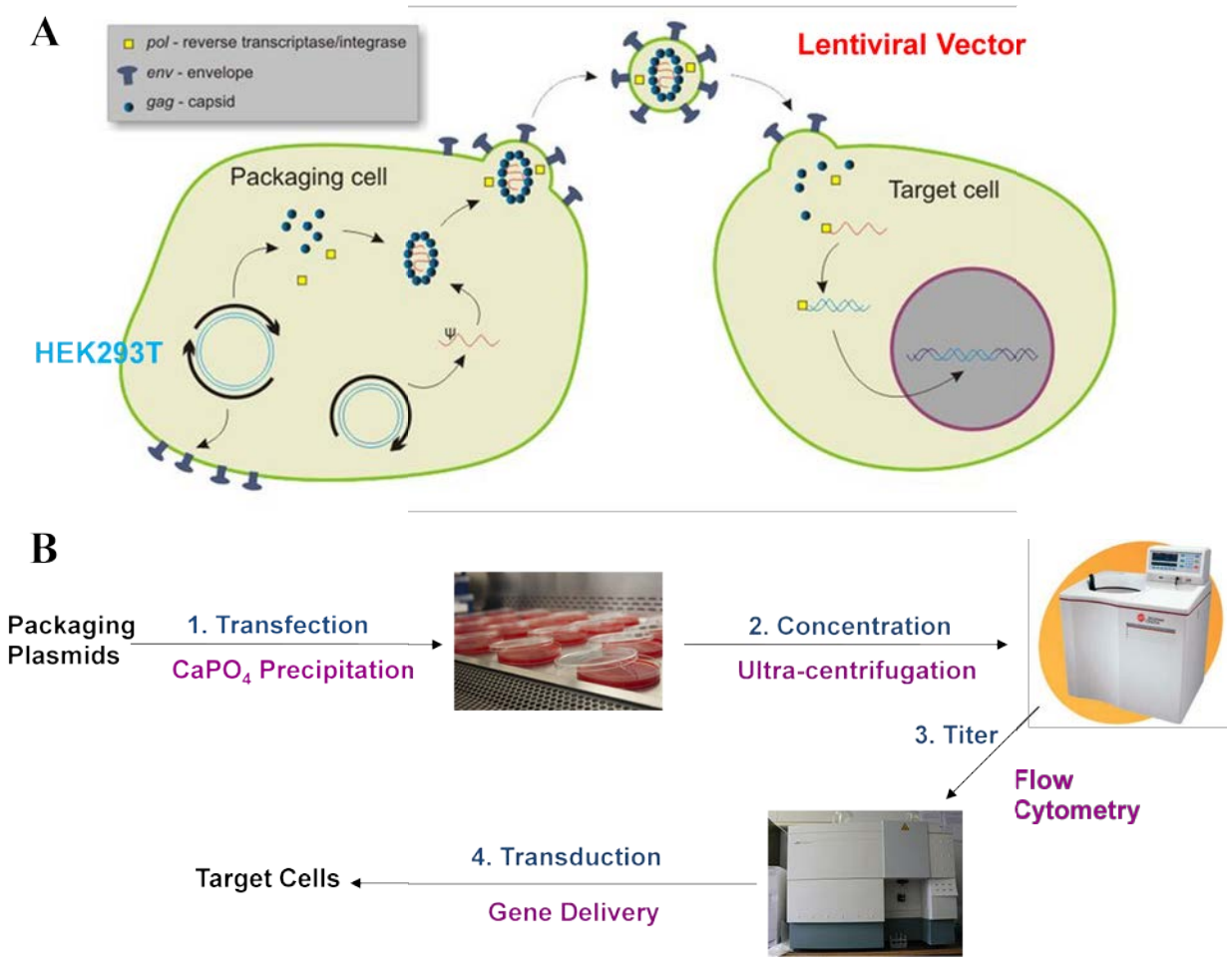


Figure 2.3 Schema for the production and usage of lentiviral vectors. A. Functional lentiviral vector is synthesized in the packaging cells (HEK293T) and is then used to deliver transgenes to the target cells by integrating into host chromosome eventually. [Figure adapted from online] B. The four packaging plasmids (as mentioned in Figure 2.2) are co-transfected by calcium-phosphate precipitation into packaging cells. Later on, several batches of vector supernatant are collected and concentrated by ultra-centrifugation. Vector stock is then titered on HEK293T by flow cytometry. Aliquots are frozen down for future use or are readily for gene delivery into specific target cells.

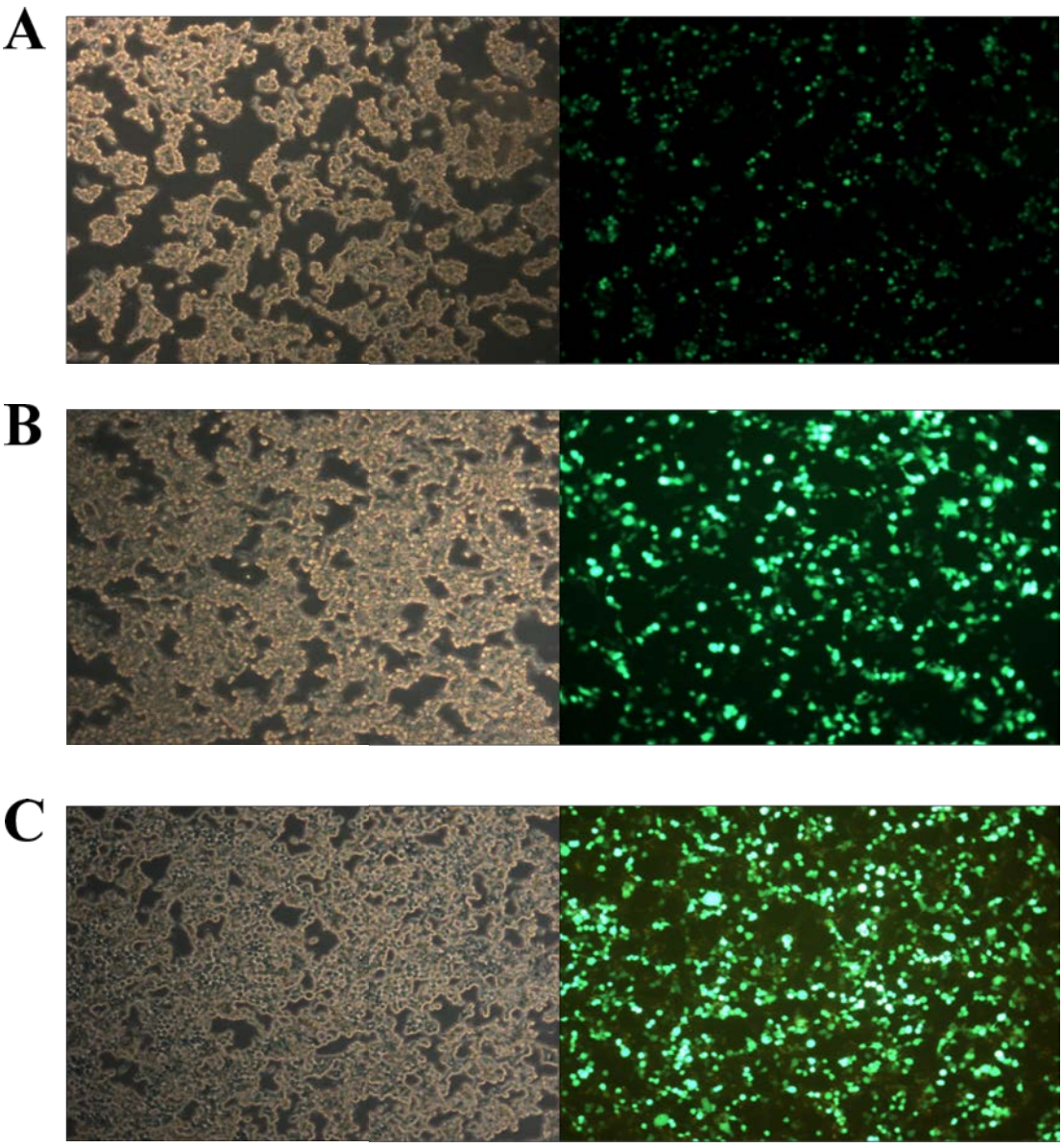


Figure 2.4 Optimization of calcium-phosphate transfection on HEK293T cells and cell maintenance. Figure A, B and C represent phase contrast image (left panel) and fluorescence image (right panel) of cells 24 hours post-transfection, at three different trial points with A being the earlier low-efficiency trial period to C being the later high-efficiency trial p int in the project timeline, respectively. The photos on the left are phase contrast images of HEK293T cells under 10× magnification showing the overall cell density and health, while the photos on the right are fluorescence micrograph showing GFP expressing cells after transduction under fluorescent microscope. As shown in the figures, there were significantly more cells attached to the plate after transfection, from A to C. In addition, the percentage of GFP expressing cells have increased dramatically, indicating more robust synthesis of lentiviral vector. In conclusion, the transfection efficiency and cell maintenance have improved dramatically.

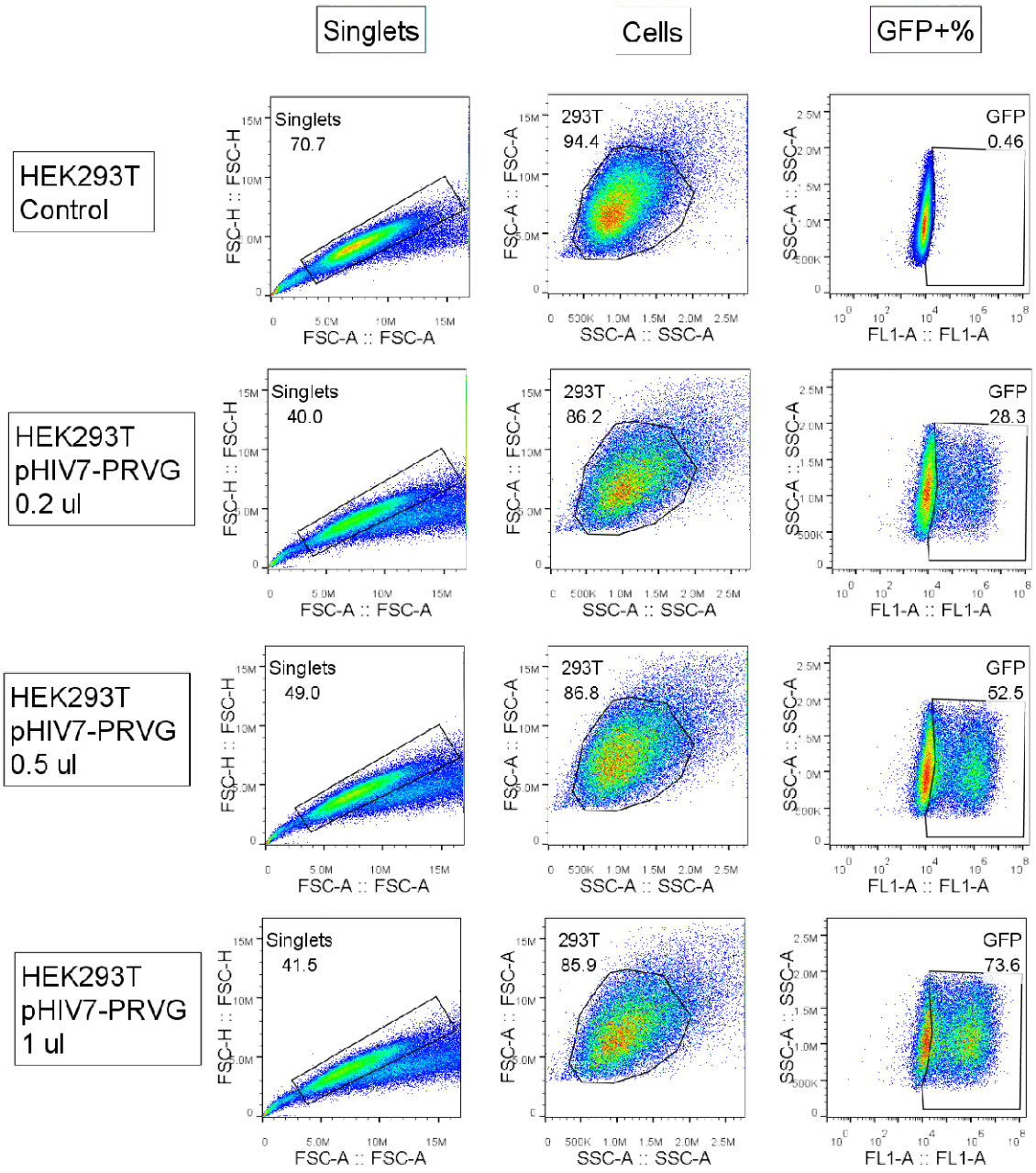


Figure 2.5 Determination of titer on HEK293T cells. This figure shows a representative gating tree on cells, using FlowJo analysis software for non-transduced control cells and cells transduced with 0.2 µl, 0.5 µl and 1 µl PRV-G pseudotyped vector, from top to bottom, respectively. The same gating tree was applied to all samples, sorting out singlets, cells and GFP expressing cells, successively. Increasing values of percentage of GFP expressing cells were seen along with increasing amount of vector added. The titer value was calculated eventually based on the percentage GFP and cell density. The titer thus obtained with HEK293T cells were used in all further experiments to calculate the vector to cell MOI, irrespectively of the cell.

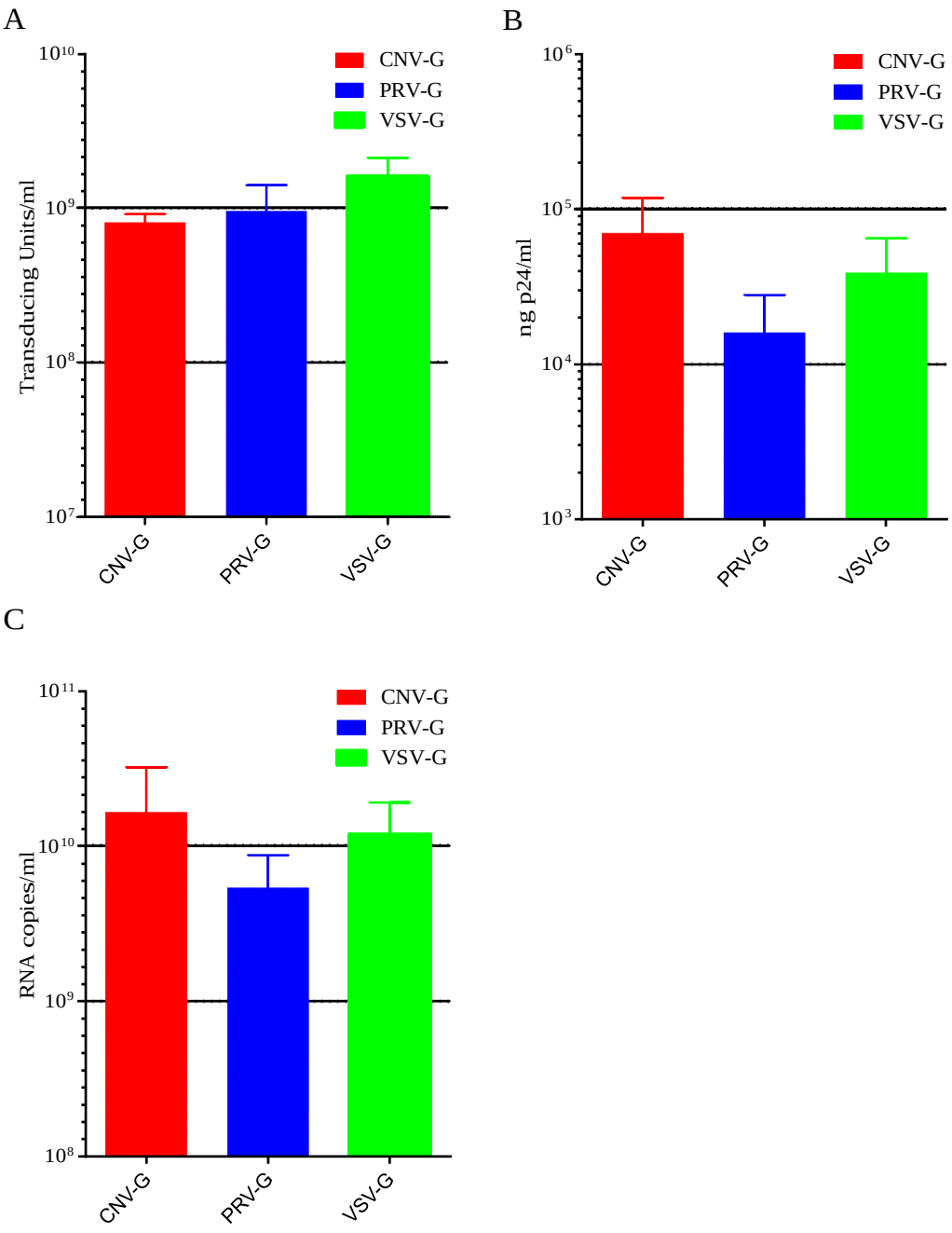


Figure 2.6 Determination of vector titers. A. Functional vector titers (TU/ml) were assayed on HEK293T cells. Dilutions of the vector preparations were used to infect $5x10^5$ cells/well and the cells were later assayed for GFP expression at 72 hours post transduction to determine the titer. B. Non-functional vector titers (ng p24/ml) were determined by the p24 ELISA assay. C. RNA copy numbers were evaluated by qRT-PCR. Results presented here are the mean values obtained from 3 to 4 independent repeated experiments on different batches of vector preparations.

Chapter 3

Transduction Efficiencies of VSV-G, CNV-G and PRV-G Pseudotyped Lentiviral Vectors in Different Cell Lines

3.1 Summary

In previous study, we were able to produce Chandipura glycoprotein (CNV-G) and Piry virus glycoprotein (PRV-G)-pseudotyped lentiviral vectors to high titers, which is crucial for large-scale applications of lentiviral vectors [10, 247]. As an initial step to determine the gene-delivery efficiencies of these two novel lentiviral vector pseudotypes, we tested their levels of infectivity on several commonly used adherent and non-adherent cell lines of different origins in vitro, in direct comparison with that of VSV-G pseudotyped vector. The cell lines included six adherent cell lines of epithelial, fibroblast and neuroblast origins (HEK293T, GHOST, HeLa, BHK, MDCK and N2a), three non-adherent cell lines derived from human lymphocytes (Sup-T1, CEM and JURKAT) and H9 human embryonic stem cell line (hESC). On all cell lines, we performed transductions with the three pseudotypes at different series of MOIs (Multiplicity of Infection, as determined by functional titration on HEK293T cells). For the transduction of HEK293T, BHK, HeLa, MDCK, GHOST cells, MOI of 0.2, 0.5 and 1 were applied due to their relative ease of transduction. For the transduction of N2a cells, MOI of 0.5, 1 and 5 were used for optimized efficiencies. For the transduction of Sup-T1, CEM and JURKAT cells, MOI of 1, 5 and 10 were used due to their relatively low transducibility. For the transduction of hESC cells, MOI of 10 was used. Transductions at each condition were repeated at least four times at different time points, and unpaired t-test was used to analyze the comparisons between different pseudotypes. Overall we found CNV-G pseudotype had comparable or even better transduction

efficiency than VSV-G pseudotype in certain adherent cell types, especially in those of neuronal origin, whereas PRV-G pseudotype showed mildly lower efficiency in these cells. However, VSV-G pseudotype was significant better than the other two pseudotypes in transducing human lymphotropic cell lines. By contrast, VSV-G pseudotype transduced hESC colonies at a higher efficiency and sustained more persistent transgene expression for up to three months. In general, the transduction efficiencies were improved with increasing MOI.

3.2 Introduction

As work on gene therapy strategies progresses and lentiviral vectors continue to play a major role in gene transduction of diverse sets of target cells, there is a growing need to identify and employ alternative viral envelopes for pseudotyping these vectors. Since VSV-G is still the most commonly used heterologous envelope for pseudotyping lentiviral vectors due to its broad host tropism and stability, here we sought to identify and develop related viral envelopes and evaluate their ability to generate high titer vector stocks and transduce cells of different lines of origin and different species. For this reason we chose viral envelopes from the Chandipura and Piry viruses that belong to the same vesiculovirus genus in Rhabdoviridae family [248]. Piry virus was originally isolated from an opossum in Brazil and is known to cause generalized human infection [233]. Chandipura virus is an emerging pathogen in the tropical areas of India that usually causes severe encephalitis in children [234, 249]. In contrast, VSV is primarily a livestock pathogen. Based on these viruses' natural history, different cell tropisms are to be expected, with Chandipura virus presumably being most neurotropic. Another advantage to the use of these new envelopes is that they are immunologically non-cross reactive with VSV-G,

permitting their use in vivo in a sequential manner as an alternative to VSV-G pseudotyped vectors, for boosting gene delivery and repetitious dosage as becomes necessary [232, 233].

We have already shown that lentiviral vectors can be efficiently pseudotyped with the envelopes from both these viruses, but their preferences to deliver genes into target cells remain unknown. In order to determine their tropisms to different cell types, multiple lab-adapted and immortalized cell lines were utilized for efficiency tests. Various cell types from different species were intentionally selected to give us a broad spectrum of analysis. HEK293T, HeLa and GHOST cell lines are of human origin whereas the BHK, MDCK and N2a cells are of hamster, canine and murine origin, respectively. These cell lines are adherent and are derived of epithelial, fibroblast or neuroblast origins. These cells are known to be generally permissive to transduction and would represent some characteristic tropism for novel pseudotypes. In addition, Sup-T1, CEM and JURKAT cell lines are derived from T lymphocytes from human patients suffering from lymphoma or leukemia. Therefore, the tropism to T lymphocytes was specially evaluated on these certain cell lines. Alternatively, the hESC cell line is derived from the inner cell mass of blastocysts formed five days post-fertilization, which is in the earliest cellular state [250]. These cells behave like stem cells that are self-renewing, multipotent and hematopoietic, meaning that they are capable of giving rise to indefinitely more cells of the same types, as well as differentiating into specific cell lineages when exposed to specific stimuli and cytokines. The potential tropism of the hESC cell line would give us insights into potential applications for human stem cells transduced with novel pseudotypes.

3.3 Material and methods

3.3.1 Cell culture

We obtained HEK293T cells (Human Embryonic Kidney 293 cells; ATCC Cat. #CRL-11268), HeLa cells (human cervical epithelial cells; ATCC Cat. #CCL-2), BHK cells (Baby Hamster Kidney fibroblast cells; ATCC Cat. #CCL-10), MDCK cells (Madin-Darby Canine Kidney epithelial cells; ATCC Cat. #CCL-34), and N2a cells (mouse neuroblastoma cells; ATCC Cat. #CCL-131), from the American Type Culture Collection (ATCC, Manassas, VA), and GHOST cells (genetically modified cells from Human Osteosarcoma cells; NIH AIDS Reagent program, Cat. #3942) from the NIH AIDS Reagent program. Cells were cultured according to the protocols from the providers. Briefly, HEK293T cells were culture in Dulbecco's Modified Eagle's Medium (DMEM; ATCC Cat. #30-2002) supplemented with 10% fetal bovine serum (FBS). HeLa cells, BHK cells, MDCK and N2a cells were cultured in ATCC-formulated Eagle's Minimum Essential medium (EMEM; ATCC Cat. #30-2003) supplemented with 10% FBS. GHOST cells were cultured in 10% FBS supplemented DMEM, in the presence of 100 µg/ml hygromycin B, 1 µg/ml puromycin and 0.5 mg/ml G418. All cell lines were cultured with sufficient media in T75 tissue culture flasks in 37 ^{o}C incubator supplemented with 5% CO_2. After reaching confluency every 3 to 4 days, cells were washed with PBS followed by trypsin (10%) treatment for 1 to 5 minutes, depending on cell types. Once the cells were lifted-off, the trypsin activity was neutralized with fresh media and cells collected by centrifugation at 1,500 RPM/min for 5 minutes. Cell pellet was resuspended in 10 ml of fresh media, and 1 ml out of that was seeded to cell culture flask containing fresh media for propagation.

We obtained Sup-T1 cells (human T lymphocyte cells; ATCC Cat. #CRL-1942), CEM cells (human T lymphocytes; ATCC Cat. #CCL-119) and JURKAT cells (human T lymphocytes;

ATCC Cat. #TIB-152) from the American Type Culture Collection (ATCC, Manassas, VA). As instructed by the supplier's protocol, Sup-T1, CEM and JURKAT cells were cultured in ATCC-formulated RPMI-1640 medium with 10% FBS. Cell passage was performed every 3 to 4 days, where cells were directly harvested from the flask and centrifuged at 1,500 RPM/min for 5 minutes. The cell pellet was resuspended in 10 ml of media, and 1 ml out of that was seeded back to cell culture flask containing fresh media.

We obtained H9 hESC cells from the NIH Human Embryonic Stem Cell Registry. Cell colonies were cultured in hESC medium (DMEM-F12, 20% knockout serum replacer, 100 mM L-Glut + BME solution, non-essential amino acids, 2 µg/ml basic FGF solution) on mouse embryonic fibroblast (MEF) feeder layer in a 6-well plate. Culture medium was changed daily and colonies were allowed to grow for 6-7 days. Cells were passaged using a collagenase protocol. Briefly, one day before passaging hESCs, a new plate covered with MEF was prepared by aliquoting thawed MEF onto each well. During the passage, 1 ml of collagenase solution (50 mg collagenase type IV plus 50 ml DNEM-F12) was added to each well and incubated for five to ten minutes at 37 ^{o}C. Collagenase was then removed and hESCs were lifted off mechanically in 1 ml of culture medium with a pipette, spun down at 1,500 RPM for five minutes. Next, additional culture medium was removed, and hESCs were resuspended carefully in fresh hESC medium and plated onto new plate with MEF layers.

3.3.2 Cell transduction

Cell culture conditions and cell health were frequently checked under the microscope. For the transduction of adherent cells, cells with viability above 90% were trypsinized, collected and washed with fresh media. After centrifugation, cells were resuspended and aliquoted such

that 100,000 cells from each cell type were seeded to each well of a 12-well tissue culture plate. Seeded plates were incubated at 37 ^{o}C for 4 hours to allow for cell attachment. For transduction, media supernatant was replaced with 200 µl vector preparation mix. The vector mix consisted of fresh media, 1.6 µl polybrene (for a final concentration of 8 µg/ml) and different amount of vector stocks, as per the MOI [97]. After 4 hours of transduction, cells were incubated in the medium for an additional 72 hours before preparing the cells for FACS analysis for ascertaining the rate of GFP expression [97].

For the transduction on all T-lymphocytic cell lines, cells with viability above 90% were harvested and washed with fresh media. After centrifugation, cells were resuspended in a calculated volume of fresh media to achieve a concentration of 1 million cells in 1 ml of media. Later on, 100 µl of cell suspension was added onto each well of a 24-well plate. Afterwards, 100 µl of vector preparation mix was added to each well, with the mix consisting of fresh media, 1.6 µg polybrene (for a final concentration of 8 µg/ml) and differing amounts of vector stock according to the target MOI. We used MOI of 1, 5 and 10, and the MOI depicted for input vector here were based on titers in HEK293T cells. After 4 hours of transduction in the incubator at 37 ^{o}C, cells were cultured in medium for 72 hours before the FACS analysis for GFP expression rate [97].

For the transduction of hESC, cells were transferred onto Matrigel and cultured for two passages in MEF-conditioned hESC media, in order to remove MEF cells for more efficient transduction. MEF conditioning was performed by collecting hESC media from non-manipulated MEF cultured in the incubator at 37 ^{o}C every 24 hours, thus the filtered conditioned media contained growth factors from MEF. A Matrigel aliquot of 1 mg was removed from the -80 ^{o}C freezer and 1 ml of cold DMEM-F12 was added to the aliquot dissolving the frozen Matrigel.

Immediately following, Matrigel was transferred to a 15 ml conical and an additional 11 ml of DMEN-F12 was added. Lastly, 1 ml per well was plated onto two 6-well plates and the plates were allowed to incubate for one hour at room temperature. Non-manipulated hESCs on the MEF feeder layer were passaged using collagenase and placed onto Matrigel plus an additional 2 ml of MEF-conditioned hESC media. Cells were allowed to grow for 5 days and passaged an additional time onto Matrigel to remove residual MEFs. After 24 hours of recovery, cells were transduced with lentiviral vector at an MOI of 10 using a similar method to the other cell lines.

For infecting different cell types at a certain MOI, the HEK 293T cell titers were used to determine the input MOI in all our experiments.

3.3.3 Determination of transduction efficiency by FACS and fluorescent microscope

The transduction efficiency on all cell lines (except hESC) was determined by flow cytometry. 72 hours post transduction, cells in each well were trypsinized (for certain adherent cells), harvested, and eventually resuspended in 300 µl PBS containing 1.4% paraformaldehyde for fixing. Samples were then analyzed by FACS (Fluorescence-activated cell sorting) in Accuri C6 flow cytometer (BD Bioscences, USA). Raw data was then exported from the machine and analyzed in FlowJo for GFP percentage evaluation (Figure 3.1). Finally, GFP percentage values were obtained, representing transduction efficiencies of the three pseudotyped vectors in each respective cell line.

The transduction efficiency on hESC colony was evaluated using the fluorescent microscope. After 72 hours, GFP expression of cells was observed by fluorescence microscopy imaging. At 5 days post-transduction, transduced hESCs were transferred back to MEF feeder

layer and cultured as detailed above. Every 3 to 4 days, GFP expression was checked under fluorescence electronic microscope.

3.3.4 Statistical analysis

To assess the significance of differences seen among the three pseudotypes, statistical analysis was used to evaluate data from multiple experiments using GraphPad Prism version 6 (GraphPad Software, USA). Student's unpaired t-test was used to compare the transduction efficiencies of HEK293T, GHOST, HeLa, BHK, MDCK, N2a, Sup-T1, CEM and JURKAT cell lines. P value less than 0.05 was considered to be significant.

3.4 Results

In this project, we evaluated the gene transfer efficiencies with novel pseudotypes into various cell types for the first time. Although minor disparities were seen, probably due to marginal differences in vector preparations and the variation of the titer, we were able to combine data from repeated experiments and analyze the differences between the three pseudotypes with statistical tools. The student's unpaired t-test was used to analyze the different transduction levels of cell lines, the values of which followed normal distribution [241].

To determine the gene-delivery efficiencies of the novel CNV and PRV glycoprotein pseudotyped lentiviral vectors, we started with various lab adapted adherent cell lines of distinctive origins from different species. VSV-G pseudotyped vector was used in parallel for direct comparison under the same conditions. The adherent cells consisted of epithelial, fibroblast or neuroblast origin (HEK293T, GHOST, HeLa, BHK, MDCK and N2a cells). MOI

equivalents, as based on HEK293T titers, of 0.2, 0.5, 1 or 5 were used to transduce these adherent cells, based on prior knowledge on the different transducibility of these cell types (Figure 3.2). First of all, varying efficacies of transduction with different viral envelopes could be seen with a general trend showing increased transduction with increased MOI. Relative to the VSV-G pseudotype, CNV-G pseudotype was found to be significantly more efficient in transducing GHOST (88% transduction) and MDCK cells (60% transduction) with 1.2 to 2 fold increase in efficiency ($p<0.5$). Besides, superior efficiency rendered by CNV-G pseudotype was also seen in HEK293T, HeLa and BHK cell lines (75 to 95% transduction) at an MOI of 1 (Figure 2A-D) but this was not statistically significant (possibly due to the limited experimental replicates and repeats, $p>0.5$). By comparison, the PRV-G was less efficient in transducing all adherent cells tested than VSV-G and CNV-G (30 to 80% transduction efficiency, depending on cell type). For transducing neuroblastoma cells, CNV-G pseudotype was found to be better than VSV-G pseudotype (85% for CNV-G pseudotype compared to 75% for VSV-G pseudotype, at an MOI of 5), whereas PRV-G pseudotype showed only moderate transduction ability (45% transduction at an MOI of 5) (Figure 2E). Although PRV-G pseudotype showed the lowest transduction efficiency among the three pseudotypes, the gene-delivery efficiencies achieved were still decent given that the PRV-G pseudotype registered only 2-fold or lower efficiency than the other two pseudotyped lentiviral vectors. We did not observe any overt transduction-associated toxicity with any of these pseudotypes in all the cell lines tested as the cells were all relatively healthy after transductions, indicated more than 80% of normal cells gated as the main population in FACS (live/dead stain). This scenario could be because that lab-adapted cell lines are robust and able to recover quickly from transduction reagent-associated toxic effects. Additionally, although statistical analysis did not show significance due to the limited

experimental replicates and repeats, CNV-G pseudotype appeared to have similar or better efficiencies than VSV-G pseudotype in cell lines of epithelial, fibroblast and neuronblast origins, such as HEK293T, BHK and HeLa cells.

For the transductions on human T lymphocytic cell lines, the overall transduction efficiencies showed an increase with increased MOI of transduction in general (Figure 3.4). In Sup-T1 cells, VSV-G pseudotype managed to transduce 75% of cells even at a lower MOI of 1, and reached almost the saturation level for gene delivery at MOI of 5 and 10 with more than 95% GFP expression, showing excellent gene delivery efficiency into these cells (Figure 3.4A). CNV-G pseudotype was also found to impart reliable gene transduction in Sup-T1 cells, with 23%, 60% and 75% GFP expression at MOI of 1, 5 and 10 (Figure 3.4A). PRV-G pseudotype seemed to be the least efficient at transducing Sup-T1 cells, but still achieved 40% transduction at MOI of 10 (Figure 3.4A). Similar levels of efficiencies rendered by these three pseudotypes were seen in the JURKAT cell line, in which VSV-G, CNV-G and PRV-G transduced 83%, 38% and 20% cells at MOI of 10, respectively (Figure 3.4C). The differences in the three pseudotypes in transducing CEM cell line appeared to be not as great as with the previous two cell lines, showing 60%, 37% and 30% GFP percentages with VSV-G, CNV-G and PRV-G pseudotypes at MOI of 10, respectively (Figure 3.4B). No obvious transduction associated cell death was observed under the microscope or in FACS analysis, as no significant difference in the percentages of live cells was observed between non-transduced control samples and transduced experimental samples (Figure 3.3).

Transduction at MOI of 10 was performed on hESC and the efficiency was characterized by fluorescence microscopy because of a limited access to flow cytometry when the experiment was performed (Figure 3.5). At 3 days post transduction, hESC transduced with all three

pseudotypes showed GFP expression under the microscope. Although no statistical data was collected, VSV-G pseudotype transduced hESC showed the best transduction efficiency among all pseudotypes, indicated by noticeablely more GFP expressing cells under the UV microscope. Cells transduced by CNV-G and PRV-G pseudotypes also showed GFP expression, but with obviously lower percentage of expression. In addition, the maintenance of GFP expression was seen on hESC cells transduced with VSV-G pseudotype, interpreted by sustained GFP expression up to 3 months (Figure 3.6). However, the persistent GFP expression was not seen on CNV-G and PRV-G pseudotypes transduced hESC cells due to lower transduction efficiency.

3.5 Discussion

In the past decade, gene therapy has become popular as a promising approach for treatment of many types of diseases that were largely considered to be incurable. This has resulted in a rising demand for novel tools that may be used in a broader range of cells, increasing the potential applications of this form of therapy. Regarded as the gold standard for lentiviral pseudotyping, VSV-G pseudotype shows decent to good gene delivery efficiency in most cell types but has less than optimal efficiency for transgene delivery in certain cell types (such as neuronal cells), resulting in low therapeutic value for gene therapy of respective diseases. Here we introduced two novel pseudotyping candidates, CNV-G and PRV-G, with an expected affinity for a wide vary of cell types, based on the natural history of their parental viruses. Although these viruses are poorly characterized, we hypothesized that both of these viruses should possess broad tropism similar to VSV, based on the observation that they cause generic diseases in humans and animals. Furthermore, CNV is known to cause encephalitis in

humans, therefore CNV-G might be a better candidate than VSV-G for transducing cell types of neuronal origin. Several rounds of treatment are usually required to attain full therapeutic value from gene therapy, necessitating successive rounds of lentiviral vector injections into patients in clinical trials. Since VSV-G readily induces antibody responses that reduce its efficiency in subsequent injections by augmented immune-clearance, novel, serologically distinct pseudotypes with comparable or higher efficiency are necessary to circumvent this immune response in successive deliveries of the therapeutic gene. For these reasons, comparable or better lentiviral pseudotyping candidates need to be identified and evaluated.

After introducing these two novel pseudotypes, we attempted to determine their tropism range by testing them on different types of adherent and non-adherent cell lines of different origin species. Transduction evaluation of six adherent cell lines demonstrated that CNV-G pseudotype efficiency was similar or better to that of VSV-G in transducing adherent cells of epithelial, fibroblast and neuronal origins of different species, suggesting that CNV-G pseudotype might be a promising candidate for gene delivery into related tissues. This superior efficiency might improve the gene therapy for diseases such as Parkinson's disease, epilepsy, cystic fibrosis and others in which epithelial, fibroblast and neuronal cells are the main targets. Additionally, PRV-G pseudotype was also able to transduce all attached cells at a comparable level, with only 1.2-2 fold reduction in efficiency compared to the other two pseudotyped vectors. This level of efficiency can easily be optimized at higher MOI and is adequate for PRV-G pseudotype to be a feasible alternative to VSV-G and CNV-G, especially as a later candidate during successive injections in patients that have already mounted an immune rejection to previous pseudotypes.

In contrast to adherent cells, the overall gene delivery efficiency achieved with non-adherent cells, as determined by GFP expression percentage, was considerably lower at the same MOI. VSV-G pseudotype excelled in transducing cells of T lymphocytic origin, including Sup-T1, CEM and JURKAT cell lines, with transduction efficiencies of 95%, 83% and 60% at MOI of 10 in these three cell lines, respectively. CNV-G pseudotypes were not as efficient as VSV-G pseudotypes, but still showed comparable efficiencies in transducing these cells with only 1.5 to 2 fold less efficiency, resulting in 70%, 38% and 37% transduction at MOI of 10 in corresponding cell lines. In general, PRV-G pseudotypes displayed lower transducing efficiencies in all the three tested non-adherent cell lines. However, PRV-G pseudotype only showed 2 to 4 fold lower efficiency compared to the VSV-G pseudotype, and could potentially transduce more cells at higher MOI.

For the transduction of hESC cell colonies, collected data was based on florescent microscopy imaging. We observed that VSV-G pseudotype and the novel pseudotypes could transduce human stem cell lines. However, higher and more persistent GFP expression from VSV-G pseudotype transduction was seen compared with the novel pseudotypes. Human stem cells represent an attractive cell target for gene therapy for AIDS, as they produce all the cells involved in HIV-1 pathogenesis, including CD4 T cells, macrophages, dendritic cells and microglia. Theoretically, the genetic modification of these stem cells could protect the entire spectrum of susceptible cells. After gene therapy, modified stem cells may function for years and could therefore serve as an enduring source of HIV-1 resistant cells, including cells generated by de novo lymphopoiesis to replenish central and mucosal lymphoid organs [99]. Therefore, the ability to transduce hESC by CNV-G and PRV-G pseudotypes showed their potential to be used in gene modification in human primary stem cells. Although displaying lower transduction

efficiencies than the VSV-G pseudotype, CNV-G and PRV-G pseudotypes could potentially be used as alternatives to the VSV-G pseudotype. Continued evaluation with novel pseudotypes on human primary stem cells is required for further verification.

Overall, we utilized cell lines from various species and tissue origins to characterize the potential tropisms exhibited by novel pseudotypes, in direct comparison with the VSV-G pseudotype that exhibits a known global tropism. Different cell types display varying sets of receptors on the cell surface for viruses to bind. Testing novel pseudotypes on a wide range of cell types is a common method of evaluating the tropism range of viral glycoprotein pseudotypes. The novel pseudotypes were not expected to perform significantly better than VSV-G pseudotype in transducing any tested cell lines, since the protocol of using VSV-G pseudotype has been established for decades and is efficient in transducing most cells. Of all pseudotypes that have been developed, only 4 to 5 pseudotypes showed significantly better transduction efficiency than the VSV-G pseudotype in certain cell types, including Rabies virus (human neuronal cells), Cocal virus and Baboon retrovirus (resting human T cells) [173, 174, 176]. Thus, it was surprising when we noted significantly better transduction efficiencies of CNV-G pseudotype in epithelial, fibroblastic and neuronal cell lines than the VSV-G pseudotype. Although immortalized cell lines cannot be equated to primary cells, our data suggest that the CNV-G pseudotype might potentially be a better candidate than the standard VSV-G pseudotype for gene therapy of neurological diseases, lung diseases and fibroblast related diseases. Rationally, the next step to prove this hypothesis would be to acquire specific primary cells to verify the efficiency of CNV-G pseudotype, followed by in vivo experimentation for further verification. However, due to limited access to these cells, related experiments have not been performed to date. In addition, we have successfully transduced hESC with CNV-G and PRV-G

pseudotypes, demonstrating that these novel pseudotypes might be also competent in transducing other primary hematopoietic stem cells (CD34) or iPSC (induced pluripotent stem cells). In other experiments with human lymphotropic cell lines, although VSV-G pseudotype was found to have significantly better gene delivery efficiency, CNV-G and PRV-G pseudotypes still provided decent transduction efficiencies of these cells which were comparable to those of the VSV-G pseudotype. Nevertheless, the ability of CNV-G and PRV-G to transduce human lymphocytes requires further verification on primary cells. The slightly lower gene delivery efficiency of these novel envelopes can be easily compensated by higher MOI. Alternatively, transduction efficiency might also be improved by transducing cells in a smaller volume so that cells could come into contact with concentrated vector virions at a higher frequency. Moreover, double transductions on the same cells might also improve the transduction efficiency. T lymphocytes are a common target for gene therapy in AIDS and other related inherited lymphocytic disorders. Therefore, with lower but comparable transduction efficiencies, CNV-G and PRV-G pseudotype may be used as substitutes for VSV-G pseudotype to transduce lymphocytic cells, especially in patients that have already been exposed to a VSV-G pseudotyped vector and thus have mounted an immune response against VSV-G pseudotype but still require boosting from successive injections.

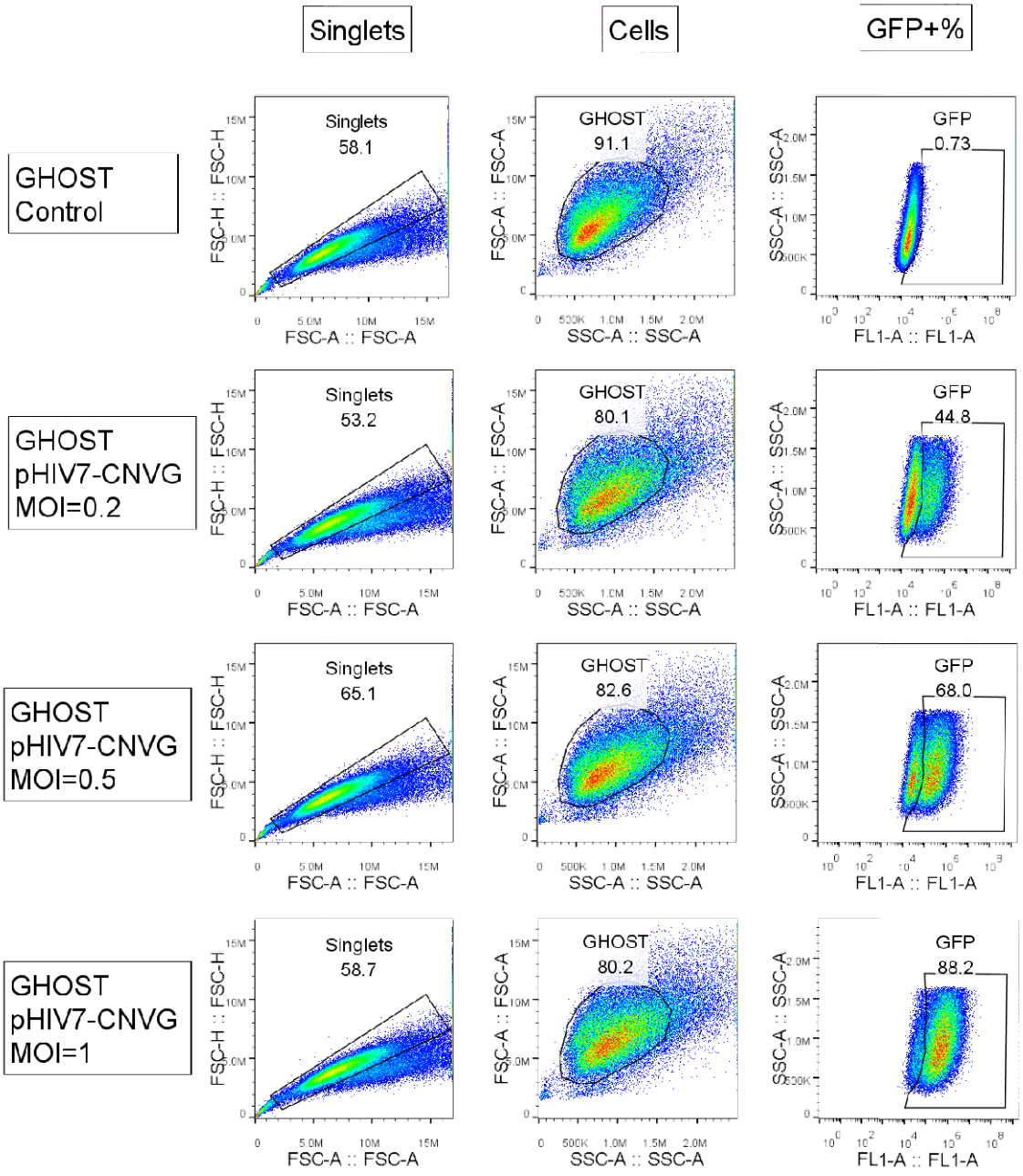


Figure 3.1 Gating tree for the determination of GFP expression at different MOI using FlowJo. Shown in the figure are examples of FlowJo analysis on transduced GHOST cells. The non-transduced control and the transduced samples at MOI of 0.2, 0.5 and 1 are indicated from top to bottom. The same gating tree was applied to all samples, sorting out singlets, cells and GFP expressing cells, successively. GFP percentage at different MOI was determined in the end.

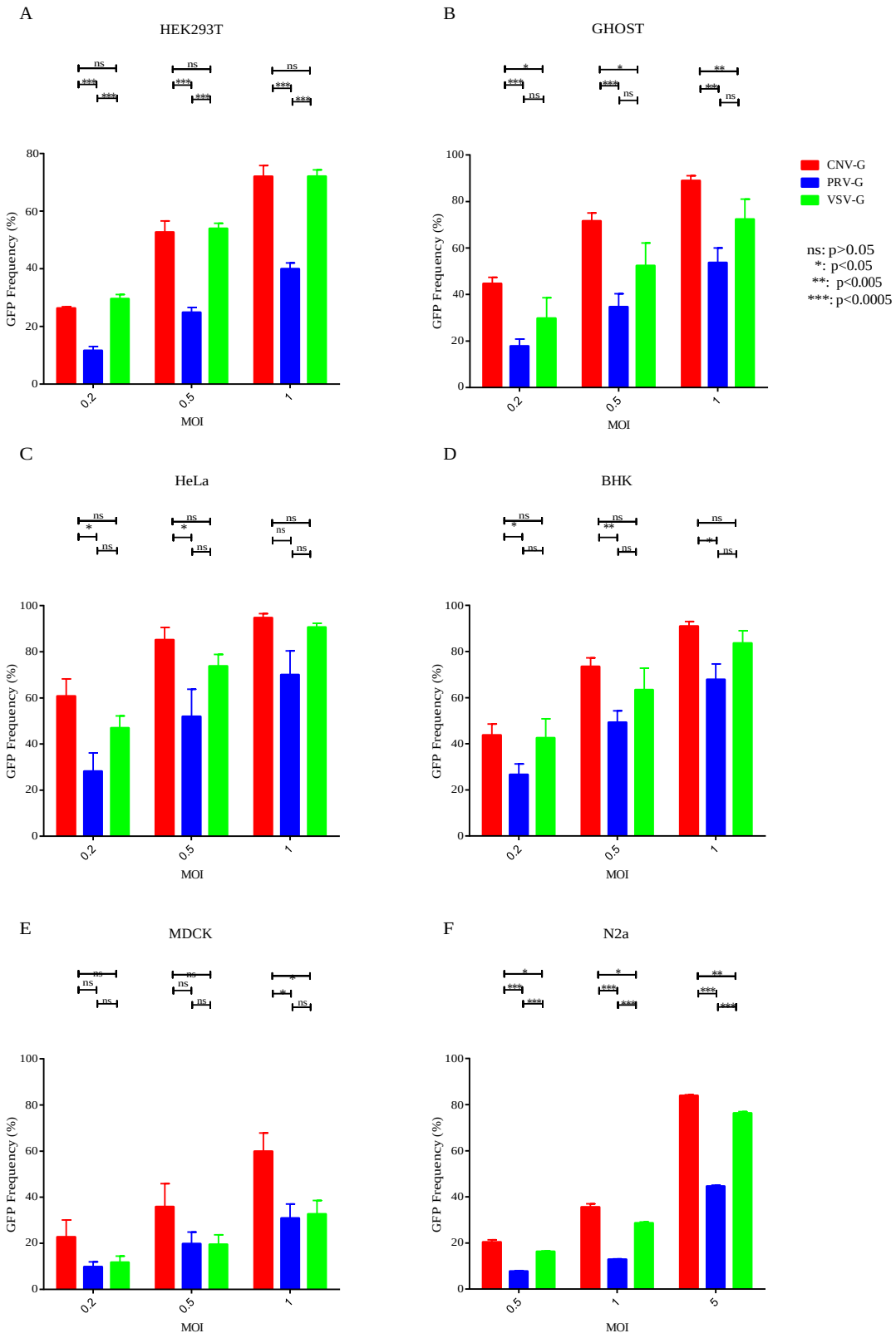

A
HEK293T
B
GHOST
C
HeLa
D
BHK
E
MDCK
F
N2a
GFP Frequency (%)
MOI
0.2
0.5
1
5
CNV-G
PRV-G
VSV-G
ns: p>0.05
*: p<0.05
**: p<0.005
***: p<0.0005

Figure 3.2 Transduction efficiencies of CNV-G, PRV-G and VSV-G pseudotyped lentiviral vectors in different adherent cells. (A-F) Transduction of adherent cells HEK293T, GHOST, HeLa, BHK, MDCK and N2a. Cells were exposed to MOI of 0.2, 0.5, 1 or 5 for 4 hours and cultured for 72 hours before assaying for GFP expression. Percent transduction of different cell types are indicated. For each cell line, the difference in transduction efficiency among the vector pseudotypes is indicated at the same MOI, the corresponding p values are represented by ns/*/**/*** and $p<0.05$ was considered significantly different. Transduction efficiency of CNV-G was significantly higher than that of VSV-G in GHOST, MDCK and N2a cells ($p<0.05$). Results presented are mean plus SEM values obtained from 3 to 4 independently repeated experiments.

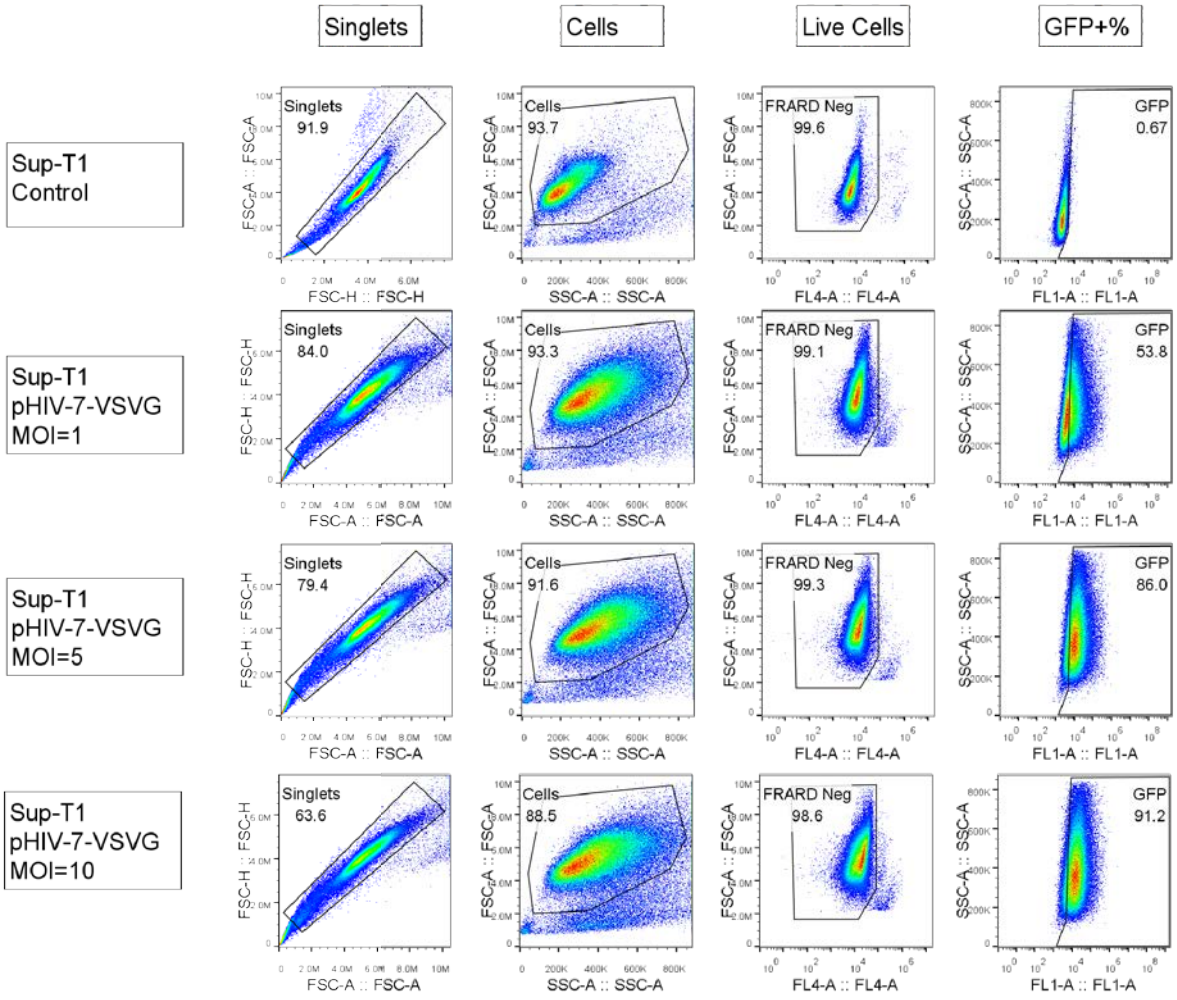


Figure 3.3 Gating tree for the determination of GFP expression at different MOI in FlowJo. Shown in the figure are examples of FlowJo analysis on transduced Sup-T1 cells. The non-transduced control and the transduced samples at MOI of 1, 5 and 10 are indicated from top to bottom. The same gating tree was applied to all samples in the same batch of transductions. Firstly, singlets were gated from the entire population. Secondly, Sup-T1 cell population was gated on all singlets, based on its size and granularity. Finally, the live population was gated (FRARD Negative) and their GFP expression percentage was determined.

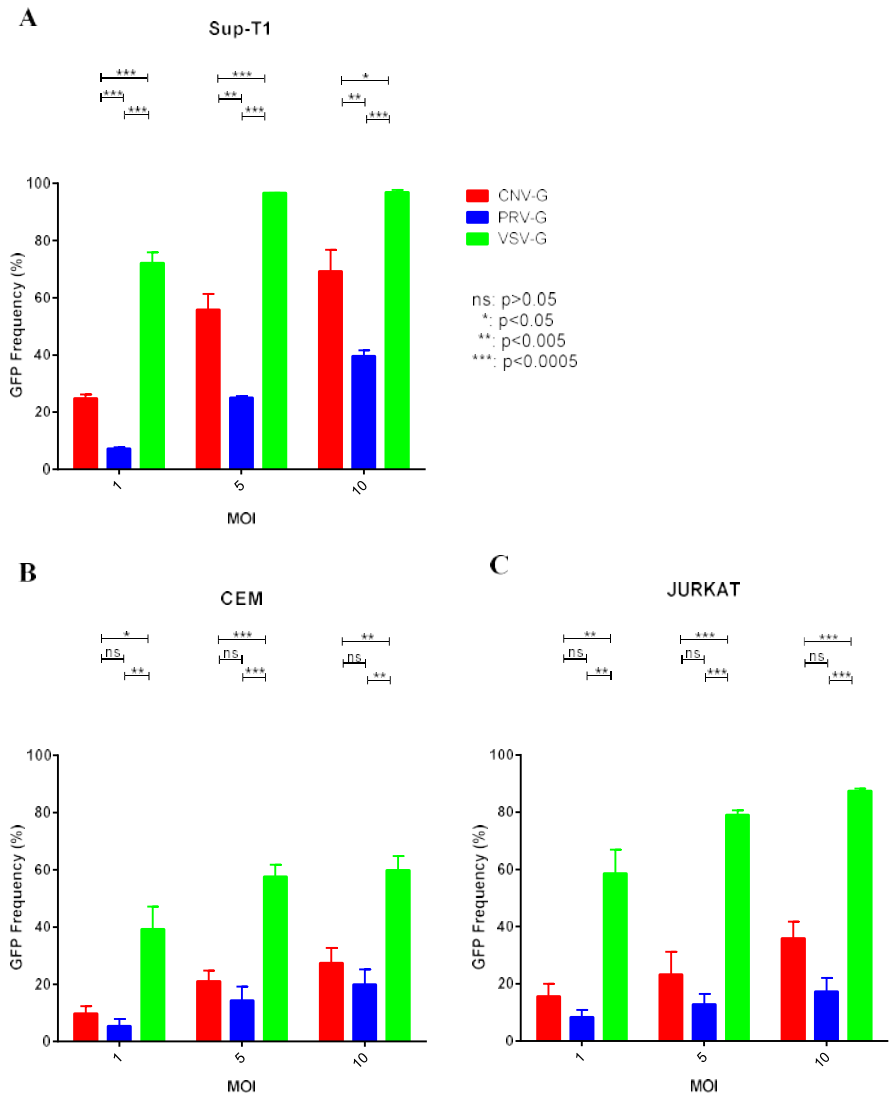


Figure 3.4 Transduction efficiencies of CNV-G, PRV-G and VSV-G pseudotyped lentiviral vectors in different non-adherent cells. (A-C) Transduction of cell lines Sup-T1, CEM and JURKAT. Cells were exposed to MOI of 1, 5 and 10 for 4 hours and cultured for 72 hours before assaying for GFP expression. Percent transduction of different cell types are indicated. For each cell line, the difference in transduction efficiency among the vector pseudotypes is indicated at the same MOI, the corresponding p values are represented by ns/*/**/*** and $p<0.05$ was considered significantly different. Results presented are mean plus SEM values obtained from 3 to 4 independently repeated experiments.

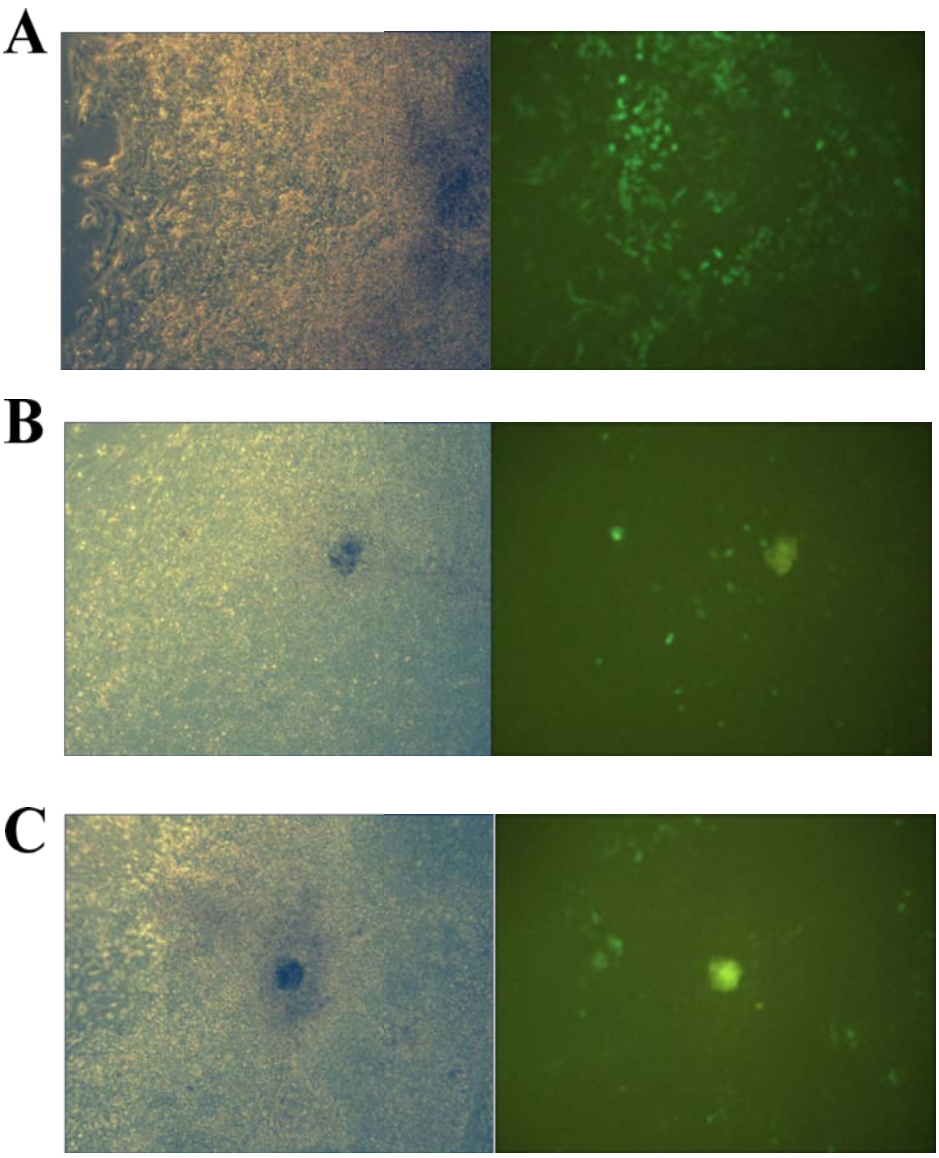


Figure 3.5 Phase contrast (left) and fluorescence (right) micrographs of transduced hESC cells 72 hours post transduction (10× magnificence). Figure A, B and C represent hESC transduced by VSV-G, CNV-G and PRV-G pseudotypes, respectively.

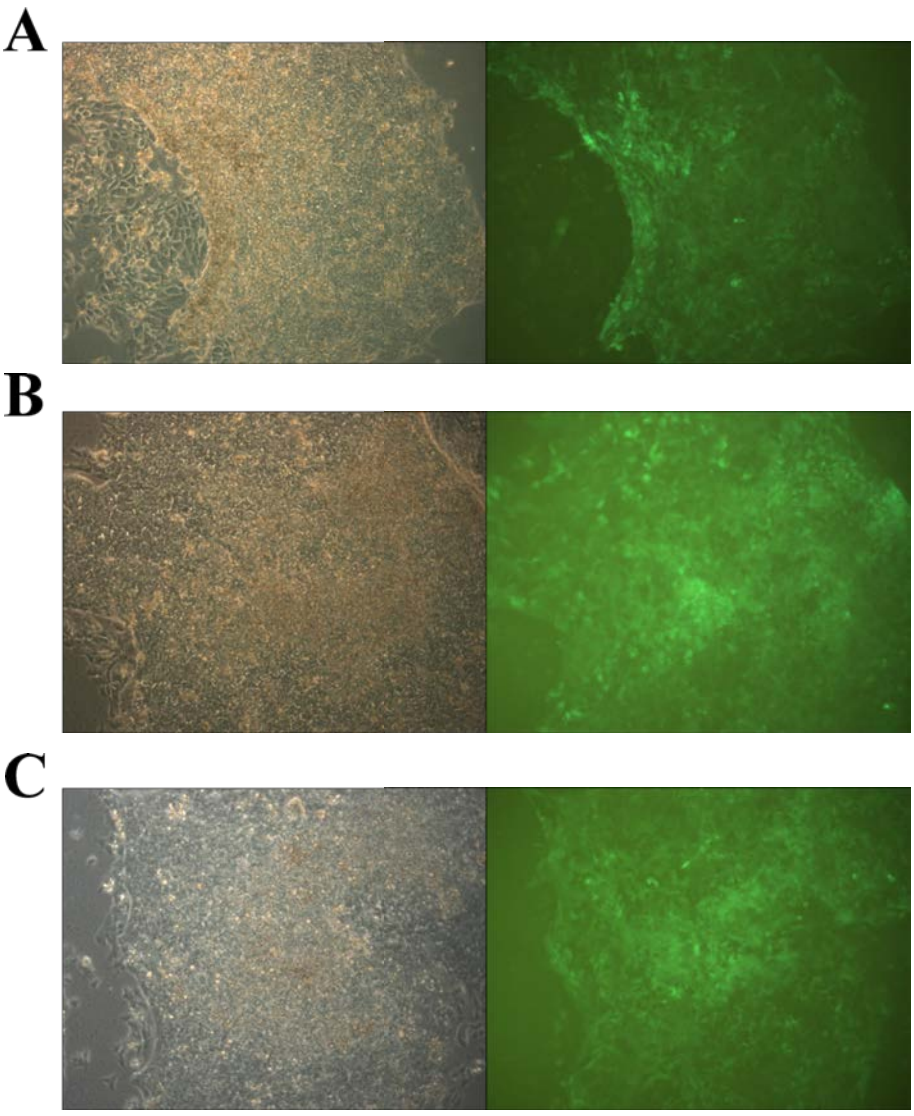


Figure 3.6 The maintenance of GFP expression in transduced hESC. Phase contrast (left) and fluorescence (right) micrographs of transduced hESC cells at different time points post transduction (10× magnificence). Figure A, B and C represent hESC transduced by VSV-G pseudotype after 1, 2 and 3 months, respectively.

Chapter 4

Transduction Efficiencies of VSV-G, CNV-G and PRV-G Pseudotyped Vectors in Human Primary Cells

4.1 Summary

Human peripheral blood-derived mononuclear cells (PBMC) are defined as any blood cell with a round nucleus normally seen in the peripheral blood, including T and B lymphocytes, macrophage and/or monocyte, nature killer (NK) cell, dendritic cell, etc. This mixed population of blood cells circulate in the blood stream and play critical roles in the immune system, fighting infection and warding off intruders. PBMCs are easily obtained and purified from patient's blood and cultured in vitro for a short period of time. In addition, administration of genetically modified autologous PBMCs would not cause any immune rejection. Therefore, PBMCs are the most popular human primary cell target for ex vivo gene delivery for many diseases. In our project, we determined the efficiencies of three lentiviral vector pseudotypes to transduce a mix population of PBMCs at MOI of 1, 5 and 10. Additionally, we also evaluated the preference of different lentiviral vector pseudotypes to transduce specific cell types falling in the PBMC category, including B lymphocytes, T helper lymphocytes, T cytotoxic lymphocytes and Nature Killer cells. VSV-G pseudotyped registered the highest transduction efficiency followed by CNV-G and PRV-G pseudotypes. Significant differences in the susceptibility of specific cell types to transduction by all the three pseudotypes were evident, with T cells being the most susceptible and B cells being the most resistant.

Human bone marrow stem cells (CD34) are another type of primary cells. Since stem cells have the potential to derive and differentiate into new cell lineages, they are also excellent targets for gene modification to eradicate and replace malfunctional genes or cell types, which can ultimately lead to complete restoration of normal function and result in cure. Therefore, we evaluated the efficiencies of three lentiviral vector pseudotypes in transducing CD34 cells at MOI of 1, 5 and 10. We observed successful transductions with all three pseudotypes, with VSV-G pseudotype having the highest efficiency in transducing CD34.

4.2 Introduction

Human PBMCs and stem cells are the two most common targets in human gene therapy [10, 58]. PBMCs are the main target for gene therapy to correct mutations in white blood cells, or to restore CD4 T cell function in HIV infected patients. In these strategies, lentiviral vector is injected into the blood for systemic delivery, in which the PBMCs would be some of the first cells to come into contact with these lentiviral vectors [251]. Since PBMCs are a mixed population of different types of cells, including T and B lymphocytes, macrophages, NK cells, monocytes, neutrophils, eosinophils, dendritic cells, etc., one of the biggest challenges encountered with this delivery method would arise from the difficulty for the lentiviral vectors to find the right targets out of this varied mixed population of cells. The VSV-G pseudotype, which exhibits a broad tropism for multiple cell types, is especially likely to transduce most of these cells non-specifically. This would potentially lower efficiencies for VSV-G pseudotyped lentiviral vectors to deliver transgenes into the specific target cell types of interest, posing a considerable block in targeted therapy. In this experiment, we wanted to address the preferences

of VSV-G pseudotypes to transduce various terminally differentiated cell types in a mixed population of PBMCs in vitro. In addition, we also wanted to evaluate the efficiencies of CNV-G and PRV-G pseudotypes in transducing PBMCs in direct comparison with VSV-G. The CD34 cells are isolated stem cells from adult bone marrow and/or fetal liver. CD34 cells are pluripotent cells and precursors for progeny cells which give rise to all myeloid and lymphoid lineages in human [252]. They are characterized by their remarkable multi-potential and self-renewal abilities and are common targets in gene therapy to correct various hematopoietic disorders [218, 252].

From what we have observed from previous experiments on various cell lines, we anticipated to characterize the ability of novel CNV-G and PRV-G pseudotypes to transduce human primary cells, including PBMCs and CD34 bone marrow cells, in direct comparison to VSV-G pseudotype.

4.3 Material and methods

4.3.1 Purification of PBMC from human blood

The usage of human PBMCs was under the approval of the CSU RICRO parf #06-107B. Fresh LeukoPak blood samples from individual human donors were obtained from the Farth Englund Blood Donor Centers, University of Colorado Health. PBMCs were purified by Ficoll separation (GE Healthcare Life Sciences, Cat. #17-1440-02). Briefly, blood specimens were transferred individually from LeukoPaks and slowly layered on top of the Ficoll solution. After centrifugation (1000 X g for 20 minutes), different layers containing different types of cells were formed based on cell density. The bottom layer was made up of red blood cells (erythrocytes) that are much heavier than Ficoll medium and hence collect at the bottom. The next layer is

primarily comprised of granulocytes, which are denser and thus migrated down through the Ficoll-Paque solution. The layer of cells immediately on top of the Ficoll medium is the PBMCs or lymphocytes, which are typically found at the interface between the plasma and the Ficoll solution, along with monocytes and platelets. To recover the lymphocytes, this layer was carefully recovered, washed with a salt solution to remove platelets, Ficoll, and plasma followed by centrifugation (1000 X g for 5 minutes). PBMCs with more than 99% purity were cryopreserved in liquid nitrogen for storage.

4.3.2 Culture, activation and transduction of PBMC

The base media for PBMC culture was RPMI with 10% FBS. PBMCs were thawed from cryopreservation, stimulated with PHA (2 μg/ml) and cultured in the presence of IL-2 (3.2 ng/ml) for 72 hours [239]. For transduction experiments, 0.5×10^6 PBMCs were incubated with respective vectors at MOI of 1, 5 and 10, for 4 hours in the presence of 8 μg/ml polybrene, as mentioned before. After transduction, cells were incubated in media supplemented with IL-2 (3.2 ng/ml) for 72 hours before FACS analysis.

4.3.3 Human hematopoietic stem cells (CD34) culture and transduction

The usage of human CD34 cells was under the approval of the CSU RICRO parf #06-107B. CD34 cells were purified from human fetal liver using monoclonal antibody-conjugated immunomagnetic beads (Miltenyi Biotech, Auburn, CA) [97]. The purity of CD34 cells obtained was routinely >97% (data not shown). Cells were cultured in Iscove's medium containing 10% fetal bovine serum and 10 ng/ml of each of the cytokines IL-3, IL-6, and stem cell factor.

For the transductions, 100,000 CD34 cells were incubated with respective vectors at an MOI of 1, 5 and 10, for four hours in the presence of 8 μg/ml polybrene [97]. After transduction, cells were washed with fresh media and cultured in the incubator at 37 ^{o}C. A second transduction was performed on several batches of cells, in which the cells received another dose of lentiviral vector 24 hours after the first transduction. Cells were subsequently washed and cultured.

4.3.4 Determination of transduction efficiency by FACS

For transduced PBMCs, at 72 hours post transduction, cells in each well were collected and washed with PBS. To determine the transduction efficiencies of different PBMC cell types, various cell surface markers were analyzed. Briefly, cells were stained with CD3 Alexa Fluor (BD Pharmingen Cat. #557917), CD4 PECy5 (BD Pharmingen Cat. #555348), CD8 Pacific Blue (BD Pharmingen Cat. #558207), CD14 APC (BD Pharmingen Cat. #555399), CD19 PE (BD Pharmingen Cat. #555413), CD56 (BD Pharmingen Cat. #557747) and live/dead marker (Fixable Aqua Dead Cell Stain, Life Technologies, Cat. #L34957). Before experimentation, all antibodies were titrated and identified with FMO (fluorescence minus one) for optimized staining and fluorescence separation. In the experiment, stained samples were fixed in 1.4% formaldehyde and analyzed by the 8-color BD FACSCANTOII machine. For the purpose of FACS compensation, beads stained with individual antibodies were also analyzed by flow cytometry. After compensation and gating in FlowJo, GFP expression percentages in specific cell types constituting PBMCs were interpreted (Figure 4.1).

For transduced CD34 cells, at 72 hours post transduction, cells were harvested and resuspended in 300 μl PBS containing 1.4% paraformaldehyde for fixing. Samples were then

analyzed by FACS (Fluorescence-activated cell sorting) in the Accuri C6 flow cytometer (BD Bioscences, USA). Raw data was then exported from the machine and analyzed in FlowJo for GFP percentage evaluation.

4.3.5 Statistical analysis

To assess the significance of differences in transduction efficiency seen among the three pseudotypes, statistical analysis was used to evaluate data from multiple experiments using GraphPad Prism version 6 (GraphPad Software, USA). Student's unpaired t-test was used to compare the transduction efficiencies in different cell types in PBMCs and CD34 cells. P values less than 0.05 were considered to be significant.

4.4 Results

We performed transduction experiments with three lentiviral vector pseudotypes at MOI of 1, 5 and 10 on four batches of PBMCs (Figure 4.2). The student's unpaired t-test was used to analyze the different transduction levels of primary cells, the values of which followed normal distribution [241]. In order to identify specific PBMC cell types, we used different antibodies targeting specific cell surface markers to stain corresponding cell types, including CD3 T lymphocyte, CD4 helper T cell, CD8 cytotoxic T cell, CD14 monocyte, CD19 B lymphocyte and CD56 NK cells. We then analyzed these samples using the 8-color BD FACSCANTOII FACS machine. After applying compensation parameters and a gating tree, transduction efficiencies interpreted by GFP percentages for the various cell subsets of PBMCs were obtained. However, we noticed that CD14 was never detectable in our samples, which might be due to the fact that

monocytes were not sufficiently proliferative after PHA stimulation (PHA is mainly stimulatory to lymphocytes) and were eventually lost during culture [253]. Similar numbers of viable cells were observed by live/dead stain in the flow cytometer, indicating similar transduction associated toxicity rendered by different lentiviral vector pseudotypes. When the total PBMCs were evaluated for gene transduction by FACS for GFP expression, VSV-G pseudotype was found to be more efficient than either CNV-G or PRV-G pseudotypes (42% for VSV-G pseudotype, compared to 5 to 7% for PRV-G and CNV-G pseudotypes at an MOI of 10- 6 to 8 fold greater transduction with VSV-G pseudotype) (Figure 4.2A). When individual cell types were analyzed (CD4 T, CD8 T, CD56 NK and CD19 B cells), all three pseudotypes had the highest efficiency in transducing CD8 T cells, whereas CD4 T and NK cells displayed lower transducibility (Figure 4.2B-E). However, all the three lentiviral vector pseudotypes were found to transduce CD19 B cells at extremely low levels (below 5%) compared to the other cell types detected (Figure 4.2D). VSV-G pseudotype was again found to be more efficient in transducing all cell types analyzed (Figure 4.2B-E). In general, higher MOI resulted in higher transduction levels.

Transductions at each MOI were repeated on CD34 cells from different human donors at different time points. Increasing efficiencies were seen with increasing MOI with all three pseudotypes (Figure 4.3). In addition, we observed that VSV-G pseudotypes out-performed the other two novel pseudotypes in transducing CD34 cells, with a great than 5-fold increase in GFP expression compared to CNV-G and PRV-G pseudotypes, at MOI of 10. This demonstrates that CNV-G and PRV-G pseudotypes have a narrower set of target cell types compared to VSV-G pseudotype, as we have seen so far in the study. Transduction efficiency of CD34 cells with

CNV-G and PRV-G pseudotypes improved marginally with double transductions at an MOI of 10 (data not shown).

4.5 Discussion

PBMCs are a popular target for gene therapy, and constitute a mixed population of cell types that include T and B lymphocytes, monocytes/macrophages and NK cells. Here we evaluated the transduction efficiency of the new pseudotyped vectors on the mixed population of PBMCs to determine the overall transduction efficiency as well as to determine the cell types that are most efficiently transduced. PBMC from four independent and unrelated donors were tested to prevent any spurious result that could be introduced from an individual donor. In our experiment, despite minor batch-to-batch differences, VSV-G pseudotype showed significantly higher efficiency over the other two pseudotypes in transducing the various PBMC cell types. Moreover, CD4 and CD8 T lymphocytes were found to be the most transducible cell types over all the other cell populations that constitute PBMCs. Furthermore, CD19 B lymphocytes were found to be the most resistant cell type for transduction by any of the three tested pseudotypes. This could also imply that a direct injection of lentiviral vectors may not be ideal to deliver transgenes into B cells. PBMCs were stimulated with PHA in this current study before transduction experiments. Since PHA is a T-lymphocyte specific mitogen rather than a B-cell stimulator, this observed low transduction efficiency for B cells might be due to the fact that the B cell population was not as activated and proliferative upon transduction as T cells in our experiments. Re-evaluation of the transduction efficiencies achieved by these pseudotyped vectors in B cells after activation of the cells with a B-cell specific stimulator such as the

pokeweed mitogen (PWM) warrants further investigations [254]. In fact, a previously developed MV (measles virus) glycoprotein pseudotyped lentiviral vector was found to transduce B cells at a higher efficiency than VSV-G pseudotype [220]. In addition, the low gene delivery efficiency into B cells could also potentially be optimized by another novel pseudotype with envelope glycoprotein gHgLgp42 from EBV (Epstein-Barr virus), taking advantage of its ability to fuse with B-cell membrane and a natural tropism to infect B cells [255]. Although no studies have been carried out on this topic, EBV lentiviral vector pseudotype has the potential to achieve high level transduction on B cells. In our experiments, CD14 staining was originally designed to track macrophages in PBMCs. However, they were likely to sink down and attach to the bottom of the culture plate during incubation, and were not obtained for flow analysis. In future experiments, lifting off the adherent cells either mechanically with a cell scraper or chemically with a cell-stripping solution might be needed to recover these cells for more accurate evaluation. Overall, the novel lentiviral vector pseudotypes were not found to be superior to the gold standard VSV-G pseudotype in transducing the different PBMC cell types. However, CNV-G and PRV-G pseudotypes still managed to transduce all PBMC cell types, albeit at lower efficiencies, and thus may be adequate substitutes for VSV-G in subsequent lentiviral vector injections in vivo.

While most gene therapy strategies currently involve ex vivo gene transduction with lentiviral vectors on purified target cells, future directions are focused on direct systemic injection of vectors for in vivo applications. In this scenario, the PBMCs would be the initial cells to come in contact with these lentiviral vectors. Ironically, in this case, the broad tropism of VSV-G pseudotype might be a disadvantage since it encounters and transduces cell populations non-specifically in the blood diluting out the effect and thereby dramatically reducing the infective titer that is available to transduce the target cell type. Besides, the off-target

transduction could also result in undesirable and severe side effects. However, with very low transduction efficiency in all cell types in PBMCs, CNV-G and PRV-G pseudotypes are likely to maintain their infectivity until they reach the target site. As we observed previously, the CNV-G pseudotype has potentially better transduction efficiencies than the VSV-G pseudotype on epithelial, fibroblastic and neuronal cells. Therefore systemic delivery using the CNV-G pseudotype might outperform the VSV-G pseudotype in gene therapy of Cystic fibrosis, a genetic disorder affecting lung epithelial cells.

Although it is highly desirable to transduce genes into stem cells at a high level of efficiency, it is challenging to do so and methods other than transduction with VSV-G pseudotyped vectors have not shown promising results so far [218]. Here, we estimated the gene delivery efficiencies of the three lentiviral vector pseudotypes in CD34 human bone marrow stem cells. We noticed that the VSV-G pseudotype had the best transduction efficiency in CD34 cells compared to the CNV-G and PRV-G pseudotypes. However, the low efficiencies of CNV-G and PRV-G pseudotypes could be improved by increasing MOI. In hESC cells also, we observed better GFP expression following transduction with VSV-G pseudotype, although a decent number of cells expressing GFP were also observed after transduction with CNV-G and PRV-G pseudotyped lentiviral vectors. Therefore, for the purpose of gene modification of human stem cells, CNV-G and PRV-G pseudotypes are not likely to outperform VSV-G pseudotype and may only be used as modest substitutes when necessary.

In summary, we were able to evaluate the transduction efficiencies achieved by the three pseudotypes on various cell lines and human primary cells. In an effort to better characterize and assess the merits of the novel pseudotypes in a direct comparison with the “gold standard”, we observed that the CNV-G pseudotype rendered better transduction efficiencies than VSV-G

pseudotype in neuronal, epithelial and fibroblastic cell lines. However, the transduction efficiencies of these novel pseudotypes were mediocre in other cell lines tested as well as for the primary cells. Despite this drawback, novel pseudotypes provide serologically distinct viral vehicles to deliver therapeutic genes to these cells at a reasonable level of efficiency and could be acceptable substitutes to the VSV-G pseudotype when neccessary.

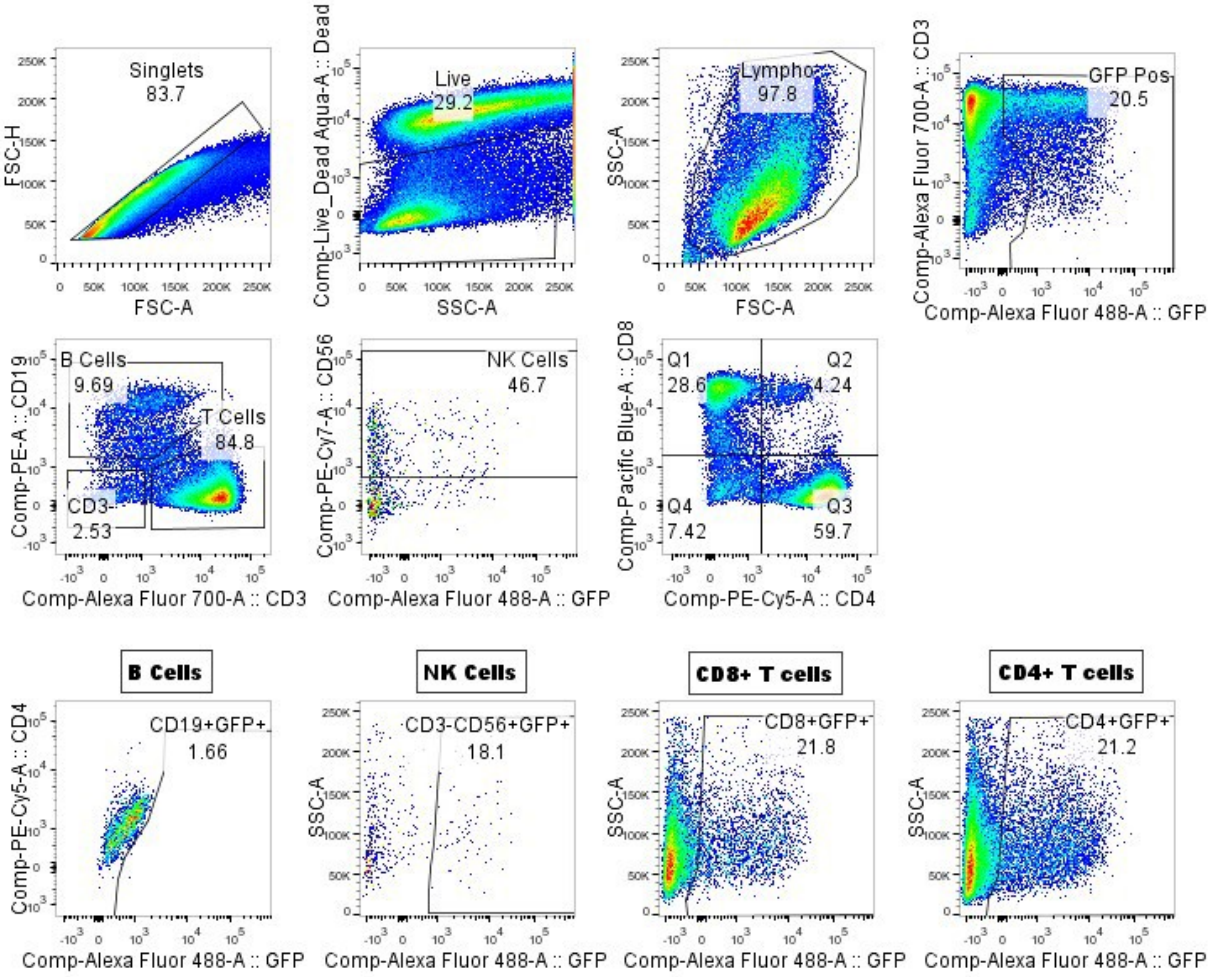
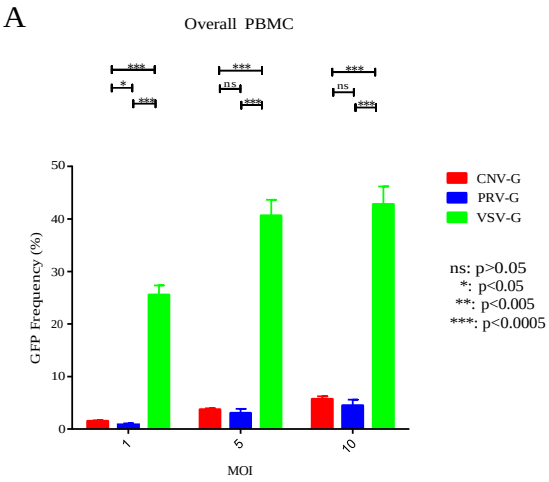
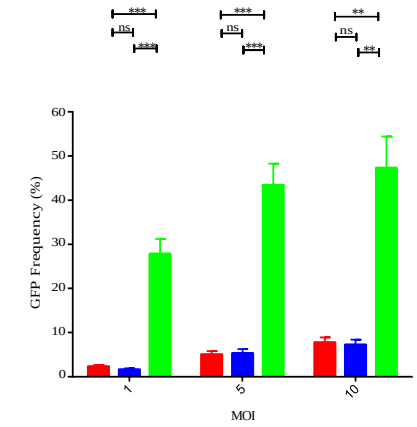
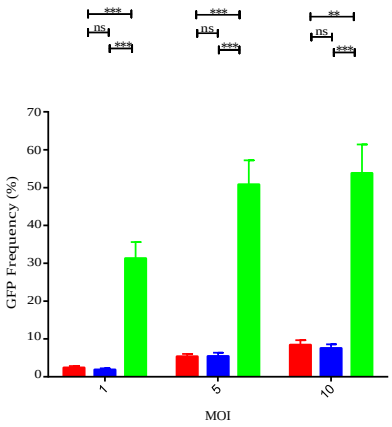
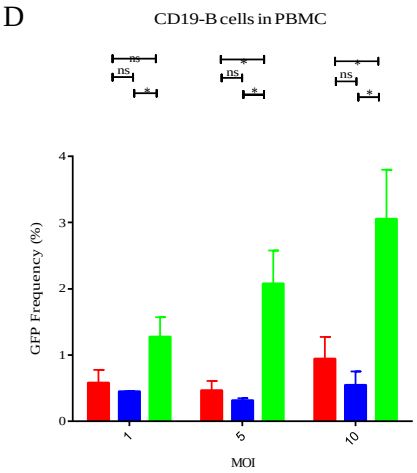


Figure 4.1 Gating tree for the determination of GFP expression in distinct cell populations that constitute human PBMC (peripheral blood-derived mononuclear cells). Firstly, lymphocyte population was sorted out from live singlet cells, in which overall GFP positive population was determined based on non-transduced control (control not shown). Later on, B cells, NK cells, CD8 T cells and CD4 cells were sorted out from the lymphocyte population, based on their specific staining markers (CD19, CD56, CD8 and CD4, respectively). Finally, the percentage of GFP expressing cells was determined in specific cell type of human PBMC, based on non-transduced control.


A
Overall PBMC
CNV-G
PRV-G
VSV-G
ns: p>0.05
*: p<0.05
**: p<0.005
***: p<0.0005
GFP Frequency (%)
MOI


B
CD4-T cells in PBMC
GFP Frequency (%)
MOI


C
CD8-T cells in PBMC
GFP Frequency (%)
MOI


D
CD19-B cells in PBMC
GFP Frequency (%)
MOI

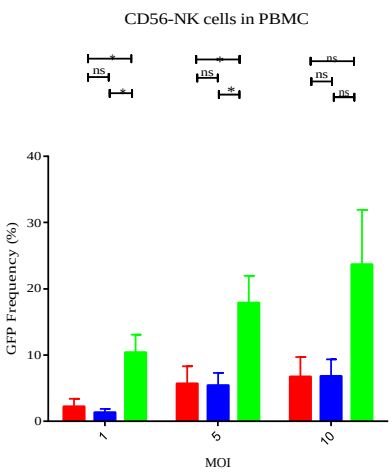

E
CD56-NK cells in PBMC
GFP Frequency (%)
MOI

Figure 4.2 Transduction efficiencies of CNV-G, PRV-G and VSV-G vector pseudotypes in human PBMC (peripheral blood-derived mononuclear cells). (A-E) PBMC were transduced with different vector pseudotypes at MOI of 1, 5 and 10 in the presence of polybrene for 4 hours. Culture medium was replenished after 4 hours and the cells were cultured for 72 hours. Cells were collected, washed and stained for CD4, CD8, D19, CD56 and GFP expression assayed by FACS to determine the levels of overall transduction and that of in the respective cell types. Percent transduction for each pseudotype are indicated. For each cell line, the difference in transduction efficiency among the vector pseudotypes is indicated at the same MOI, the corresponding p values are represented by ns/*/**/*** and $p<0.05$ was considered significantly different. Results presented are mean plus SEM values obtained from 3 to 4 independently repeated experiments.

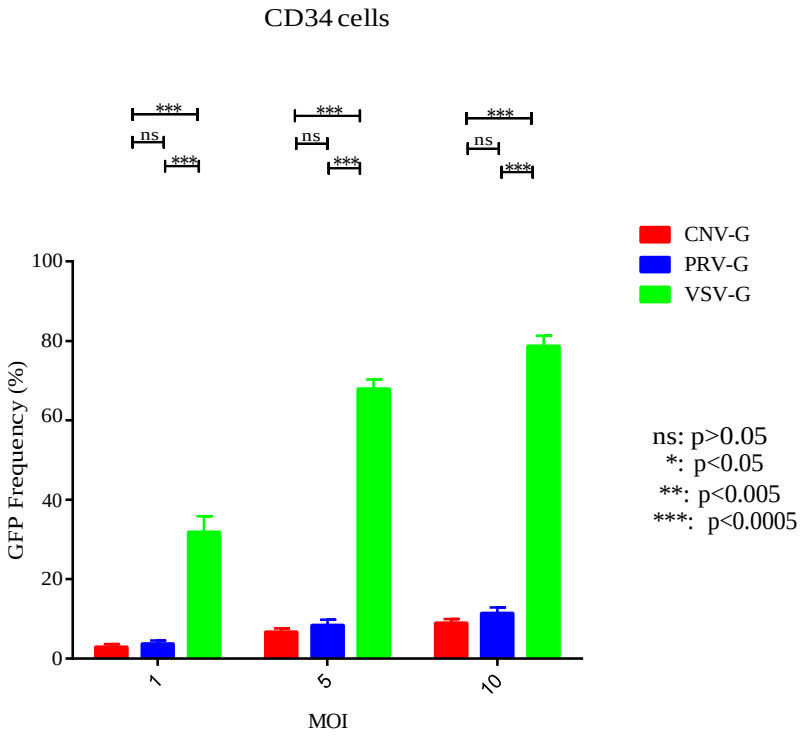


Figure 4.3 Transduction efficiencies of CNV-G, PRV-G and VSV-G vector pseudotypes in human CD34 hematopoietic stem cells. CD34 cells were transduced with different vector pseudotypes at MOI of 1, 5 and 10 in the presence of polybrene for 4 hours. Percent transduction at each MOI is shown, and the difference in transduction efficiency among the vector pseudotypes is indicated at the same MOI, the corresponding p values are represented by ns/*/**/*** and $p<0.05$ was considered significantly different. Results presented are mean plus SEM values obtained from 3 to 4 independently repeated experiments.

Chapter 5

Envelope Toxicity Assays with VSV-G, CNV-G and PRV-G Pseudotypes

5.1 Summary

Lentiviral vector transduction of cells mimic viral infection, during which the viral envelope attaches to cellular membrane, followed by fusion, viral nucleocapsid delivery into the cell, transcriptional and translational activities, and replication in the host cell. This process disrupts the normal activity of the cell and can inevitably cause some level of transduction associated toxicity. A robust population of live transduced cells is necessary to maintain and express the transgenes delivered by lentiviral vector. In this project, we evaluated the toxicity associated with individual lentiviral vector pseudotypes on different cell types by the MTS cell viability assay. Although the overall transduction induced toxicities of all three pseudotypes were within acceptable levels, cell death associated with VSV-G pseudotype transduction was found to be greater compared to that with CNV-G or PRV-G pseudotypes.

5.2 Introduction

An ideal lentiviral vector should have a minimal effect and/or low toxicity on the normal physiology and function of the cell it infects. As the gold standard for lentiviral vector pseudotyping, VSV-G pseudotype has broad infectivity, but also possesses a fusogenic property when presented in high concentrations at a high MOI that induces cell fusion and eventually cell death. Researchers have conducted a number of studies on VSV-G pseudotype mediated toxicity,

most of which did not find dramatic cell death after VSV-G pseudotype transduction both in vitro and in vivo [102, 256]. However, toxicity remains an important concern for VSV-G pseudotyped lentiviral vector when high MOI is required. Therefore, researchers are attempting to identify new pseudotypes that have lower cytotoxicity than VSV-G.

In this project, we wanted to directly compare the toxicity of VSV-G, CNV-G and PRV-G pseudotyped lentiviral vectors in various cell types by MTS assay, which is a common method to evaluate cell viability after transduction [257].

5.3 Material and methods

5.3.1 Evaluation of CNV-G, PRV-G and VSV-G pseudotyped vector-associated toxicity by MTS assay

On day 3 and/or day 4 post transduction, transduction associated cell death rate was analyzed by MTS assay using a kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Cat. #G3580; Promega, Madison, WI), following the manufacturer's instructions. The MTS assay is a colorimetric method to determine the number of viable cells in proliferation or cytotoxicity assays, in which the MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture media [257]. This conversion is presumably accomplished by NADPH or NADH produced by dehyrogenase enzymes in metabolically active cells [257]. The number of cells used for MTS assay is optimized according to the readable signal range, thus 5,000 attached cells or 10,000 suspension cells were used in the assay. Briefly, in each transduction experiment, the cells were harvested and equally distributed onto flat-bottom 96-well plate (5,000 attached cells or 10,000 suspension cells). MTS reagent

was added, which was then bioreduced by active cells to produce a colored formazan that is soluble in tissue culture media. The screen was performed in triplicate and the plate was read on a spectrophotometer at 490 nm OD after 4 hours of incubation at 37 ^{o}C. Viability loss after transduction was evaluated by the differences in absorbance values between non-transduced controls and transduced samples. Cell types evaluated include five adherent cell lines (HEK293T, GHOST, HeLa, BHK and MDCK), two non-adherent cell lines (Sup-T1 and JURKAT) and human primary CD34 cells (Hematopoietic stem cells).

5.4 Results

In lab adherent cell lines, no obvious viability loss was seen in cells transduced with all three pseudotypes at MOI of 1, with only slight drop in absorbance value compared to non-transduced controls, maintaining more than 95% live cells after transductions (Figure 5.1). Similarly, we observed satisfactory cell viability in non-adherent cell lines after transduction at MOI of 10, with 95% or more viable cells compared to non-transduced controls (Figure 5.2). In addition, transduced cells continued to replicate at similar level to non-transduced controls, resulting in similar numbers of cells at 3 days post transduction in all tested cell lines. However, some level of transduction associated cell death was noticed in primary CD34 cells with all three pseudotypes, which displayed increasing cell death along with increasing MOI of 1, 5 and 10, indicated by decreasing absorbance values (Figure 5.3 A) and percentage recovery (Figure 5.3 B). Among the three pseudotypes, VSV-G pseudotype displayed slightly less toxicity, indicated by 90% viable cells compared to 85% of CNV-G pseudotype and 75% of PRV-G pseudotype at MOI of 5.

5.5 Discussion

In our experiments, lab-adapted adherent and non-adherent cells transduced with VSV-G, CNV-G and PRV-G maintained more than 90% viability and similar cell number at 3 days post-transduction compared to the respective non-transduced control cells, indicating only a minimal transduction-associated toxicity imparted by these pseudotyped vectors on cell viability. This might be because these cell lines are highly proliferative and able to quickly recover from transduction. The vectors were engineered with deletions of most HIV-1 virulent genes and 3'-U3 region in LTR. The deletion in the 3'-U3 region is then transferred to the 5' region after reverse transcription, which results in a non-functional 5' LTR in proviral DNA after integration into the host genome [244]. Such vectors, without the necessary cis-acting elements and viral promoter are known as replication non-competent or self-inactivating (SIN) vectors, for the purpose of safer gene therapy applications [244]. In addition, these deletions eliminate severe toxicity in host cells following transduction. However, vectors need to hijack the host transcriptional and translational machinery for the production of transgenes, which might disrupt normal cell activity and cause cell death by induction of apoptotic signals [256]. The cell disruption associated with transgene expression appeared minimal on the tested cell lines in our experiments, and was caused mainly by the intrinsic properties of the vectors rather than their pseudotyping envelope glycoproteins. In some other studies of HIV-1 gene therapy, a type of replication competent vectors with the retention of normal viral LTR was used [204]. These vectors were able to replicate in HIV infected cells with the compensation of packaging proteins from an active HIV infection, and transduce and deliver anti-HIV genes to more cells, the procedure of which is known as "vector mobilization" [204]. Safety is the major concern hindering further usage of these replicable vectors, since the vectors need to maintain viral

replication properties in the host cells, which might cause severe interruption in host cellular activity. The safety issue might be addressed by performing similar experiments as in this project.

However, we have noticed an increasing rate of cell death corresponding to an increase in MOI in CD34 cells, probably due to a lower recovery rate for this cell type following transduction as compared to lab-adapted immortal cell lines, indicating some level of transduction associated toxicity in primary cells. This might be because primary cells can not replicate as efficiently as lab-adapted cell lines following transduction and thus recovery was affected. In addition, CD34 cell death induced by transduction of PRV-G pseudotype was found to be higher compared to the other two pseudotypes. However, CD34 cultures transduced by all the three pseudotyped vectors still maintained decent number of viable cells 3 days post transduction, indicated by more than 65% recovery of viability compared to non-transduced controls. The reason why primary cells like CD34 cells are more sensitive to transduction associated interference than cell lines might be that the cellular activity in primary cells is intricate and easily interrupted, thus they are not able to multiply and repopulate as efficiently. This sensitivity to transduction is a major concern in gene therapy application, since the target cells are usually primary cells rather than cell lines. Especially, a more severe issue might be encountered in transduced primary cells that would not be able to further proliferate, differentiate or be functionally compensated following transduction. Although we have not seen dramatic cell damage after transduction of CD34 cells, more primary cells should be tested particularly for in vivo application in future studies.

In summary, all the three pseudotypes showed low transduction-associated toxicity on lab adapted immortal cell lines. All pseudotypes induced an escalating level of toxicity on CD34 cells with increased MOIs, but optimal viable cell populations were still maintained at 3 days

post transduction. This less tolerance to vector transduction may be exhibited by other primary cells, especially for those that are non-proliferative such as neurons, which require more detailed toxicity study before in vivo application. The novel pseudotypes and VSV-G pseudotype displayed only relatively low toxicity and can thus be considered as safe vehicles for transgene delivery, according to the data we have obtained from this study. While the cell death caused by vector transduction in vitro is mainly attributed to the disruption of host cellular machinery, the toxicity associated with vector delivery in vivo is much more complex with the involvement of the host immune system and cellular interactions. As a result, vector-associated toxicity studies need to be carried out in suitable animal models in order to verify the in vitro results obtained in this study with the pseudotyped vectors.

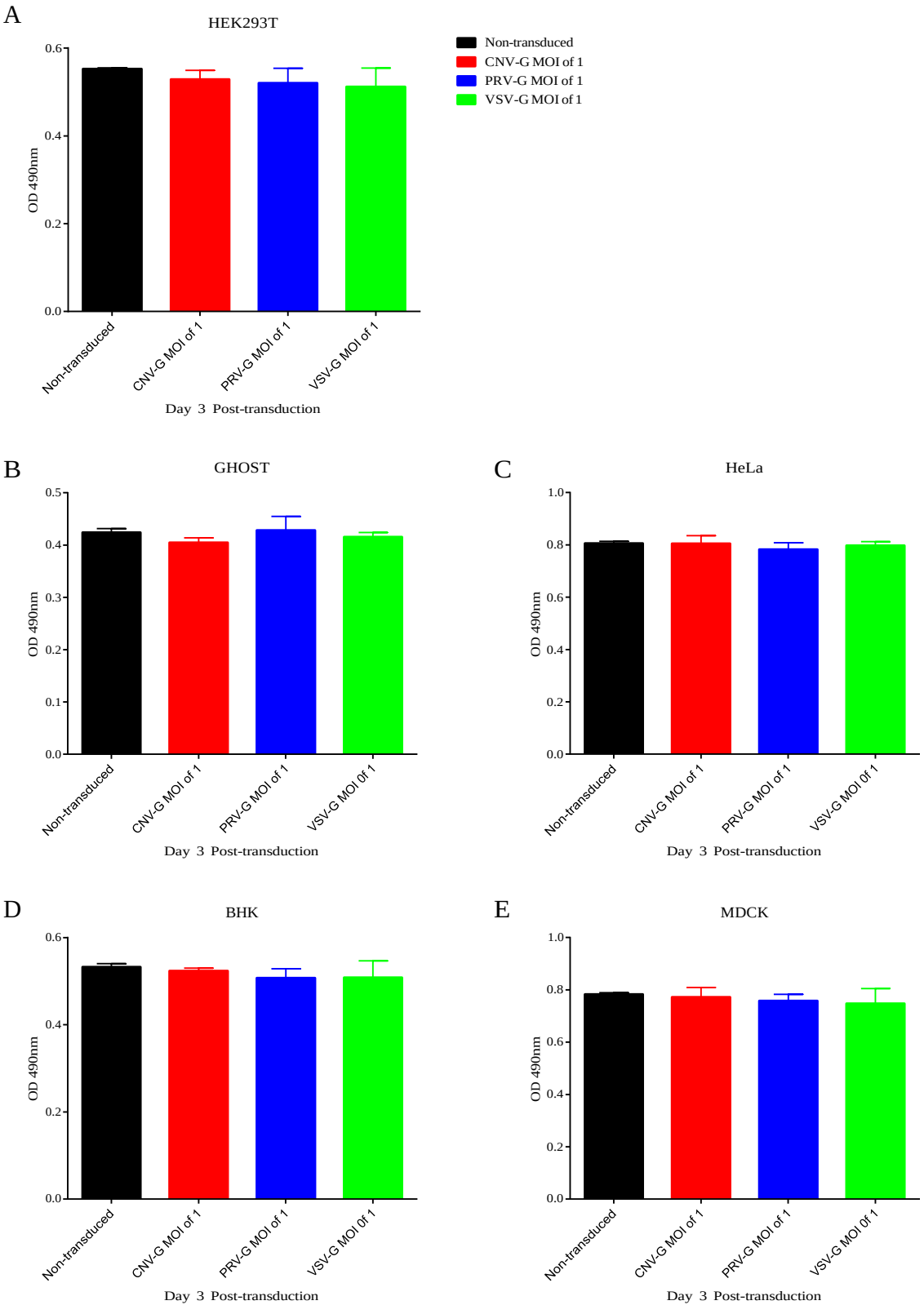

A
HEK293T
Non-transduced
CNV-G MOI of 1
PRV-G MOI of 1
VSV-G MOI of 1
OD 490nm
0.0
0.2
0.4
0.6
Non-transduced
CNV-G MOI of 1
PRV-G MOI of 1
VSV-G MOI of 1
Day 3 Post-transduction
B
GHOST
OD 490nm
0.0
0.1
0.2
0.3
0.4
0.5
Non-transduced
CNV-G MOI of 1
PRV-G MOI of 1
VSV-G MOI of 1
Day 3 Post-transduction
C
HeLa
OD 490nm
0.0
0.2
0.4
0.6
0.8
1.0
Non-transduced
CNV-G MOI of 1
PRV-G MOI of 1
VSV-G MOI of 1
Day 3 Post-transduction
D
BHK
OD 490nm
0.0
0.2
0.4
0.6
Non-transduced
CNV-G MOI of 1
PRV-G MOI of 1
VSV-G MOI of 1
Day 3 Post-transduction
E
MDCK
OD 490nm
0.0
0.2
0.4
0.6
0.8
1.0
Non-transduced
CNV-G MOI of 1
PRV-G MOI of 1
VSV-G MOI of 1
Day 3 Post-transduction

Figure 5.1 MTS assays on adherent cell lines. Assays were performed on 5,000 cells at 72 hours post transduction at MOI of 1. Assayed plates were read in a spectrophotometer at 490 nm for absorbance reading. Cell lines evaluated include (A) HEK293T, (B) GHOST, (C) HeLa, (D) BHK and (E) MDCK.

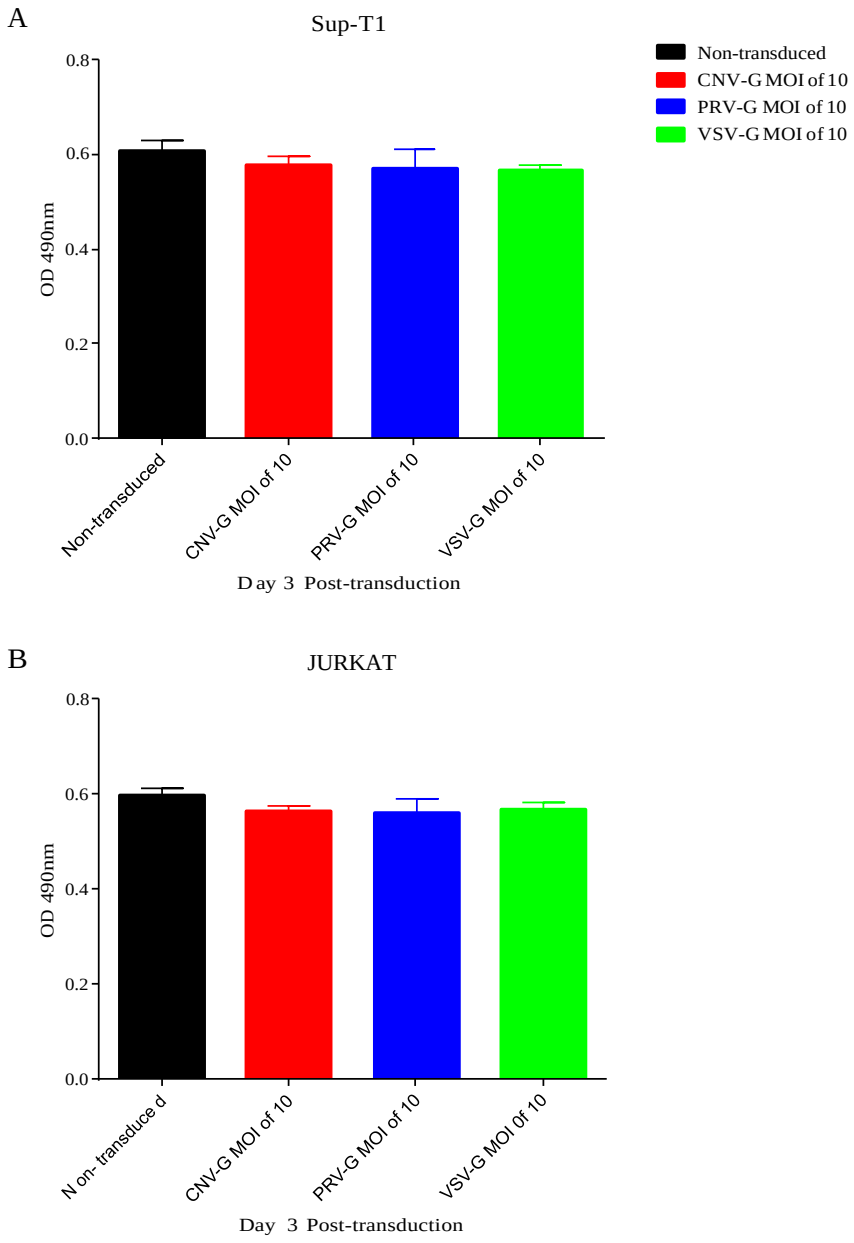
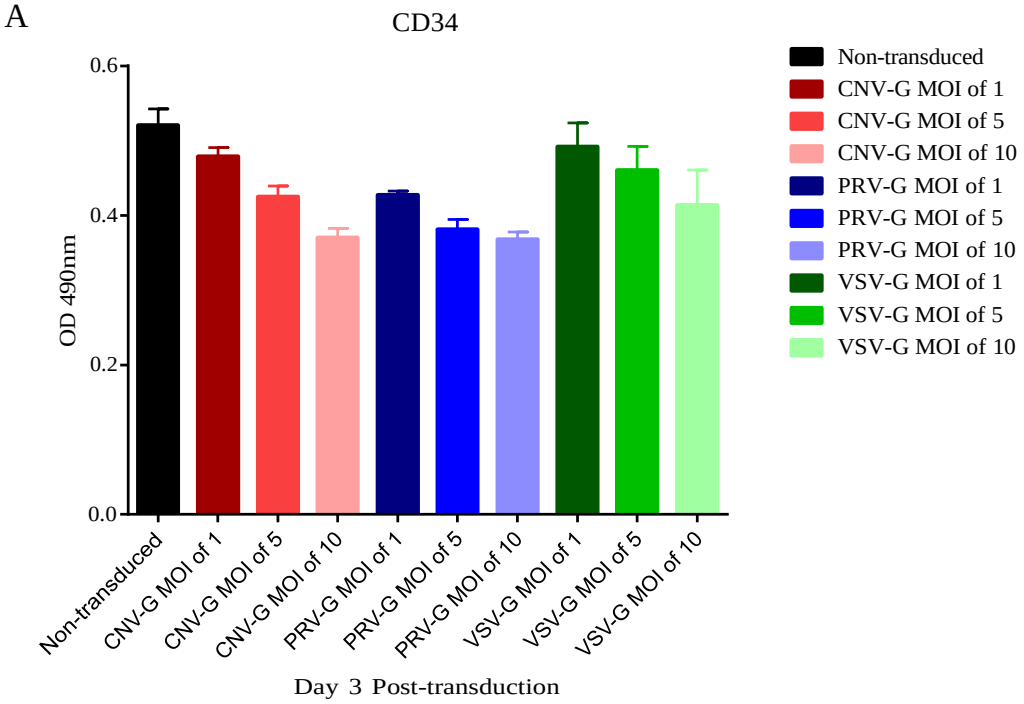


Figure 5.2 MTS assays on non-adherent cell lines. Assays were performed on 10,000 cells at 72 hours post transduction at MOI of 10. Assayed plates were read in a spectrophotometer at 490 nm for absorbance reading. Cell lines evaluated include (A) Sup-T1 and (B) JURKAT.

A


CD34
OD 490nm
0.0
0.2
0.4
0.6
Non-transduced
CNV-G MOI of 1
CNV-G MOI of 5
CNV-G MOI of 10
PRV-G MOI of 1
PRV-G MOI of 5
PRV-G MOI of 10
VSV-G MOI of 1
VSV-G MOI of 5
VSV-G MOI of 10
Day 3 Post-transduction

B

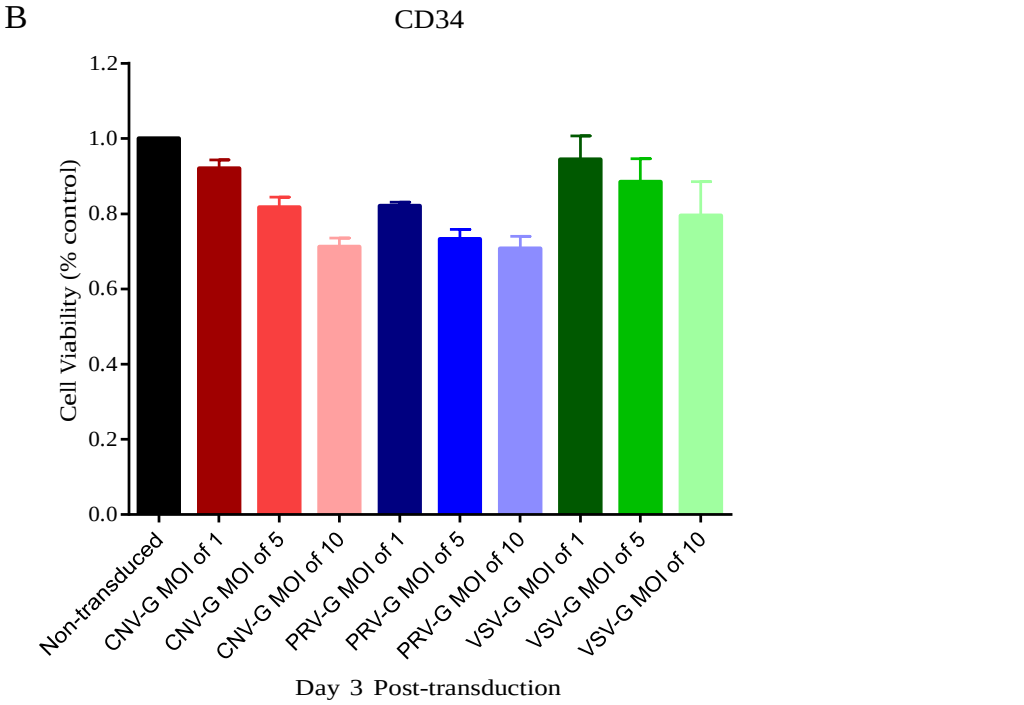

CD34
Cell Viability (% control)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Non-transduced
CNV-G MOI of 1
CNV-G MOI of 5
CNV-G MOI of 10
PRV-G MOI of 1
PRV-G MOI of 5
PRV-G MOI of 10
VSV-G MOI of 1
VSV-G MOI of 5
VSV-G MOI of 10
Day 3 Post-transduction

Figure 5.3 MTS assays on CD34 cells. Assays were performed on 10,000 cells at 72 hours post transduction at MOI of 1, 5 and 10. Assayed plates were read in a spectrophotometer at 490 nm for absorbance reading. A. The absorbance value for each transduced sample compared to non-transduced control. B. The absorbance value for non-transduced control was set as the baseline, and the result was interpreted as the percentage recovery of viability compared to this control.

Chapter 6

Determination of the Stability of VSV-G, CNV-G and PRV-G Pseudotyped Vectors to Freeze-Thaw Cycles and Human Serum Complement

6.1 Summary

In general, lentiviral vector stocks are bulk-produced and stored at -80 ^{o}C freezer as aliquots for future use in transduction. As a practical concern, the vector stock may need to be thawed for use at different time points for multiple transductions. However, during the conversion of lentiviral vector stock from a deeply frozen state to a fully thawed state may contribute to loss of infectivity, thus reducing the efficiency in transgene delivery. Here we evaluated the amount of infectivity lost during freeze/thaw conditions for VSV-G, CNV-G and PRV-G pseudotyped lentiviral vectors in an effort to determine the overall stability of these vectors. All three vectors behaved similarly and were moderately stable to five successive freeze/thaw cycles with around 10% infectivity loss observed with each cycle.

Although novel CNV-G and PRV-G pseudotyped vectors have been evaluated in in vitro experiments so far, they are intended for in vivo applications eventually. Injection of lentiviral vector preparation into the human body (intravenously) is a common method of direct delivery, through which lentiviral vector is carried by the blood stream to transduce target cells. However, the first obstacle in this route is the inactivation of the pseudotyped vectors by human serum complement. Human serum tends to neutralize foreign invaders and it is mediated by the concerted actions of natural IgM and complement components [224]. Such inactivation of vectors by human serum can dramatically reduce the infectivity of lentiviral vector preparations

and hinder the transduction efficiency on target sites. Resistance of vectors to inactivation by human complement, which is determined by Env, is an important characteristic for in vivo application. Here we evaluated the resistance to human serum complement by CNV-G, PRV-G and VSV-G pseudotyped lentiviral vectors in a direct comparison. PRV-G pseudotyped vector was found to be the most resistant to inactivation by human serum complement followed by CNV-G and VSV-G pseudotypes.

6.2 Introduction

After large scale production of lentiviral vectors, long-term storage is usually required to perform successive transductions at different time points. Although lentiviral vector stock is aliquoted into small volumes in multiple vials for storage, it is inevitable that some vials will be subjected to multiple freeze and thaw cycles to achieve the full use of stock. For the gold standard VSV-G pseudotyped lentiviral vector, relatively stable infectivity levels were seen after freeze/thaw cycles, as documented by other researchers [183, 242]. However, some other pseudotypes, such as MLV-A (amphotropic murine leukemia virus) and GALV (gibbon ape leukemia virus), had dramatic infectivity loss after freeze/thaw [183]. Although these pseudotypes may have better gene delivery efficiency in neuroblastoma cells and human blood cord-derived CD34 cells than VSV-G in some studies, they may not be ideal for practical usage, as their stability can be easily disrupted by freeze/thaw [183, 227, 258]. In this experiment, we wanted to evaluate the stability of our novel CNV-G and PRV-G lentiviral vector pseudotypes in a direct comparison with VSV-G pseudotype, to determine and optimize storage conditions.

As another important aspect of stability, lentiviral vectors need to be stable in in vivo environments. Systemic injection of lentiviral vector into humans is a simple delivery method, and vector products can be carried to the target site through the blood stream. Therefore, resistance to human serum inactivation is a critical requirement for lentiviral vector pseudotype suitability for systemic injection delivery. However, despite being considered the gold standard, VSV-G pseudotype is prone to easy neutralization by human serum complement, thus limiting its effectiveness in vivo [223]. Additionally, the VSV-G pseudotype has broad infectivity and is likely to transduce cells in peripheral blood non-specifically, which is its major drawback. Therefore, it is challenging to maintain a sufficiently high dose of VSV-G pseudotype in the actual target site, after dramatic infectivity loss by human serum inactivation and non-specific transduction. However, other pseudotypes might possess a better resistance to serum complement than VSV-G pseudotype [102, 176]. The Trobridge group developed a pseudotype from Cocal virus envelope that had much better resistance to human serum complement, thus providing a better candidate vector than VSV-G in this aspect [176]. In our project, we also wanted to characterize the resistance to human serum complement of the novel envelopes pseudotyped lentiviral vector, directly comparing to VSV-G pseudotype.

6.3 Material and methods

6.3.1 Freeze and thaw of lentiviral vector

Small aliquots (20 µl) of each of the three lentiviral vectors were prepared from fresh vector preparations of the corresponding glycoprotein pseudotyped vector. These aliquots were stored at -80 ^{o}C until fully frozen. Next, vector preparations were thawed in a 37 ^{o}C water bath

for 3 to 5 minutes, until the preparations were fully thawed. Vector aliquots were successively cycled between -80 ^{o}C and 37 ^{o}C in the same manner for up to 5 times to assess the effect of multiple cycles of freeze/thaw on their infectivity.

6.3.2 Serum separation from human whole blood and processing

Serum samples were obtained from 5 human donors. In brief, blood was collected in clotting tubes and allowed to sit for 45 minutes to clot. Afterwards, the tubes were spun at 1,000 X g for 10 minutes, and the serum supernatant was removed, aliquoted and stored at -80 ^{o}C for future use. For the serum treatment, serum samples from each individual human donors were thawed and half of each serum sample was heated to 56 ^{o}C for 1 hour to inactivate the complement components. The other half was kept as normal serum.

6.3.3 Determination of infectivity loss on HEK293T cells

Following freeze and thaw cycles as detailed above, dilutions of individual vector preparations were used for titration on HEK293T cells as mentioned previously. The titer after each freeze/thaw cycle was presented as a percentage of the original titer measured before commencing the first cycle.

6.3.4 Determination of the complement sensitivity of CNV-G, PRV-G and VSV-G pseudotyped vectors

Before the assay, CNV-G, PRV-G and VSV-G pseudotyped vector preparations were diluted to around 10^7 TU/ml. In the experiment, a volume of 5-20 µl of the vector was diluted 1 to 5 with normal or heat-inactivated serum (or DMEM medium as the no-serum control) and the mixture was incubated at 37 ^{o}C for 1 hour. Following the incubation, vector suspensions were titered on HEK293T cells in the presence of 8 µg/ml polybrene as mentioned earlier [97]. The titer values obtained with DMEM medium were set as the no-serum control baseline values, and the results were reported as the percentage recovery of titer compared to this control.

6.3.5 Statistical analysis

To assess the significance of differences seen among the three pseudotypes, statistical analysis was used to evaluate data from multiple experiments using GraphPad Prism version 6 (GraphPad Software, USA). Two-way ANOVA grouped test was used to evaluate the stability to freeze/thaw cycles. Mann-Whitney U test was used to analyze the resistance to human serum complement. P values less than 0.05 were considered to be significant. P values less than 0.05 were considered to be significant.

6.4 Results

As an important practical consideration, stability of pseudotyped HIV-1 vectors during freeze/thaw cycles determine the storage conditions and utility of the vector [242]. In this study,

vector stability during freeze/thaw cycles was examined by vector titration on HEK293T cells, after cycling between -80 ^{o}C and 37 ^{o}C successively for five times, to ascertain the levels of intact vector following exposure to each of these freeze/thaw cycle. The relative GFP titer after each freeze/thaw cycle was compared to the control titer (prior to any freeze/thaw cycling) for each vector pseudotype is presented in Figure 6.1. The two-way ANOVA grouped test was used to analyze the data, indicating the interaction between two independent variables (different pseudotypes) on the dependent variable (freeze/thaw cycles) [241]. After statistical analysis, there was no substantial difference in the stability between each of the three pseudotypes ($p>0.05$). All pseudotypes showed similarly moderate stability to freeze/thaw cycles, with about 10% drop in infectivity after each cycle. After five freeze/thaw cycles, all pseudotypes still maintained around 50% of their original titers, indicating their relative stability to the damage from freeze/thaw cycling.

In the serum sensitivity assay, we compared the ability of serum from five unrelated human donors to inactivate each of the pseudotyped lentiviral vectors (Figure 6.2). The pseudotyped lentiviral vectors were incubated separately with normal serum, heat-inactivated serum or DMEM as a no-serum control. After 1 hour incubation at 37 ^{o}C, the vector suspensions were titered on HEK293T cells to ascertain the levels of intact vectors following the treatment. The titer value of samples exposed to normal or heat-inactivated serum is reported as a fraction of the titer determined for the no-serum control sample for the same vector. The Mann-Whitney U Test was used to analyze the data since it comes from the same population (sera) against an alternative hypothesis (different pseudotypes) [241]. For all the five donors, VSV-G pseudotypes were neutralized significantly more than CNV-G and PRV-G pseudotypes ($p<0.01$), causing a 70% to 80% reduction in the overall titer. Interestingly, PRV-G pseudotyped lentiviral vectors

exhibited the highest resistance to serum complement ($p<0.01$), showing only 10% to 45% drop in its titer. CNV-G was moderately resistant to serum complement inactivation with 45% to 65% decline in titer.

6.5 Discussion

In our experiments to ascertain the effect of successive freeze-thaw cycles on the infectivity of the three pseudotyped vectors, we observed that there was some infectivity loss with each cycle but the effect was minimal and all the pseudotyped vectors behaved similarly. The infectivity loss after freeze/thaw cycling might be due to the formation of intracellular ice crystals during the freezing stage that can disrupt the envelope integrity during the thawing stage. These damaged and defective vector particles would impact the titer and thus the gene delivery to target cells. From our data, we concluded that CNV-G, PRV-G and VSV-G pseudotypes were able to withstand the freeze-thaw induced disruption to a great extent and maintained high infectivity. Similar properties in maintaining stability after freeze/thaw cycles were also observed with other pseudotypes, such as the murine leukemia virus (MLV-A) and gibbon ape leukemia virus (GALV) pseudotypes, with 5% to 10% drop in infectivity after each cycle [242]. This might be due to that the envelope lipid layer is stable to ice-crystal damage. In addition, the way that the preparation of vector is frozen might also contribute to different properties in further freeze-thaw sensitivity. As of now, the snap-freezing is a common protocol for vector storage, but a different or better preservation might be achieved by cooling down slowly at -1 ^{o}C per minute using Mr. Frosty freezing container. This slower process of freezing might minimize the ice-crystal damage to vector envelope, and needs to be evaluated in future studies. Overall, the novel lentiviral vector pseudotypes were relatively stable to freeze/thaw cycling, at a level

similar to that of VSV-G pseudotype. Given the minimal loss of titer, all the three pseudotypes could be considered ideal for storage for use at a later time point, adding value to their practical advantage. As of now, the ideal method to preserve vector preparation is to aliquot it into small volume to circumvent freeze/thaw issues, but this might not be practical in large-scale vector production and transduction [247]. These data provide us with a general idea regarding the stability of these pseudotyped vectors during freeze/thaw, and will help the optimization of the storage conditions necessary for freezing lentiviral vector pseudotypes for scientific applications.

A common drawback with the VSV-G pseudotyped vector, for in vivo applications, is its sensitivity to inactivation by human serum complement. In our experiments, we have observed that PRV-G pseudotype was highly resistant to human sera complement components, registering only a slight decline in its titer after incubation with sera from five different and unrelated donors. CNV-G pseudotype showed some level of inactivation by human sera, but still retained much higher titer than VSV-G pseudotype. A recent study determined that VSV-G neutralization or inactivation by human sera is mediated by the concerted actions of natural IgM and complement components, as the classical complement pathway [224]. Natural IgM is likely to recognize highly conserved antigens on the surface of viral vectors, and activate a cascade of complement proteins (C1 to C9) to coat and drill holes on the vector's envelope membrane, thus blocking their further transduction and a resultant reduction in infectivity [224]. Among the five different sera that were tested in the experiments, VSV-G pseudotype was recognized globally by their antibodies and its titer diminished by complement cascade. However, the clinical histories of the serum donors were not identified with regard to their previous exposure to VSV infection or not. Since VSV infection is pretty common in humans, the individuals that have been infected with it earlier in their lives would develop memory immune cells and high-affinity antibodies that

recognize VSV in subsequent infection, which might be the case in some of our serum donors. Besides, for more statistical power, more human sera need to be obtained to perform additional complement inactivation assays to confirm our findings, since five donors might not be enough sample size. Similar in vitro complement assays have been performed with other lentiviral vector pseudotypes, among which Bacolovirus GP64 pseudotype was sensitive to complement inactivation at a level similar to VSV-G pseudotype [222], while the envelope glycoproteins from related vesiculoviruses, Maraba virus and Cocal virus G proteins were found to be relatively resistant to this phenomenon [176, 224]. The marked resistance shown by PRV-G and CNV-G pseudotypes to human serum exposure might be similar to that of Maraba virus and Cocal G proteins but warrants further studies. Nevertheless, due to the relative higher resistance exhibited by the novel pseudotypes against neutralization, they possess a definite advantage over VSV-G pseudotyped vectors for their future use in in vivo applications. Overall, these data suggest that CNV-G and PRV-G pseudotypes are more effective than VSV-G pseudotype for direct in vivo applications, as they appear to be inherently more resistant to human serum inactivation. Although VSV-G pseudotype was shown to have much better transduction efficiency in various cell types than CNV-G and PRV-G pseudotypes, its high sensitivity to complement inactivation is likely to reduce its infectivity dramatically in systemic delivery. In such a scenario, CNV-G and PRV-G pseudotypes might be better viral vehicles to transduce these specific cells, although possessing slightly less efficiency than VSV-G pseudotype. During the actual administration of vector to a patient, the individual has the ability to mount humoral response and generate antibodies in a few days to eliminate the foreign intruder, the action of which is specifically through the recognition to the envelope glycoproteins, such as VSV-G, CNV-G and PRV-G. This might not be much of a problem in the first administration, but during

the second treatment, the memory lymphocytes rapidly generate effective antibodies to bind to the glycoprotein spikes and result in the vector clearance by opsonization induced phagocytosis. For such scenario, CNV-G and PRV-G pseudotypes provide two serologically distinct substitutes to VSV-G pseudotype to circumvent the immune recognition in subsequent administrations.

Overall, VSV-G pseudotype has shown superior transduction in certain cell types in our previous experiments. However, CNV-G and PRV-G pseudotypes might be better candidates for systemic delivery to target cells, despite their lower transduction efficiencies in vitro, due to their ability to withstand degradation by human complement system in vivo. The low transduction efficiency can usually be compensated by using higher MOI for transduction. In addition, these novel pseudotypes have narrow tropism and lower ability than VSV-G to transduce cells of hematopoietic and lymphoid lineages. This might be advantageous during intravenous administration when targeting the other cell types to avoid any off-target transduction, thus maintaining adequate infectivity until they reach the target site. Therefore, regardless of slightly lower efficiencies of CNV-G and PRV-G pseudotypes than that of VSV-G pseudotype in vitro, further experiments are warranted to examine the efficiency of novel pseudotypes to deliver therapeutic genes into target tissue through systemic administration.

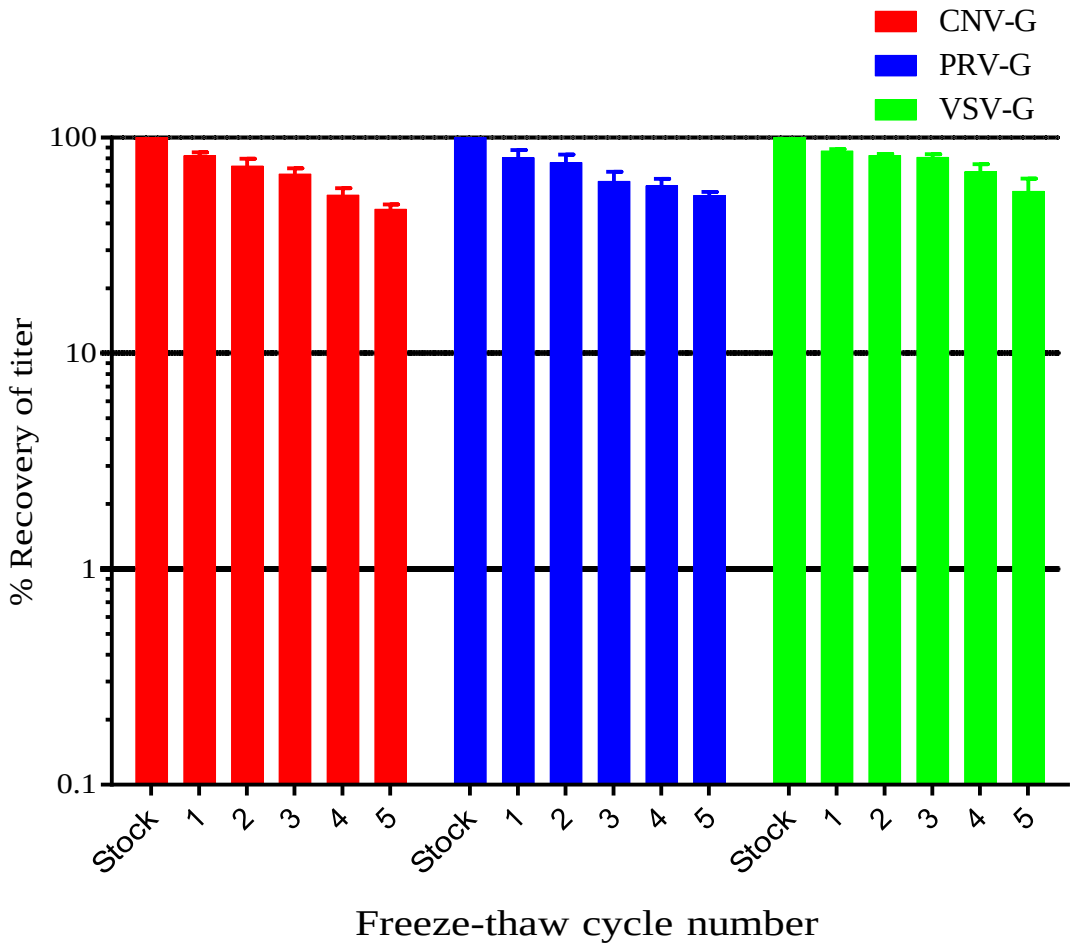


Figure 6.1 Stability of CNV-G, PRV-G and VSV-G pseudotyped lentiviral vectors to freeze/thaw cycles. To determine the stability of different pseudotyped vectors during freeze/thaw cycles, the vector stocks were subjected to 5 successive cycles of freeze/thaw and later titered on HEK293T cells as described in Methods. Results presented are from three different experiments.

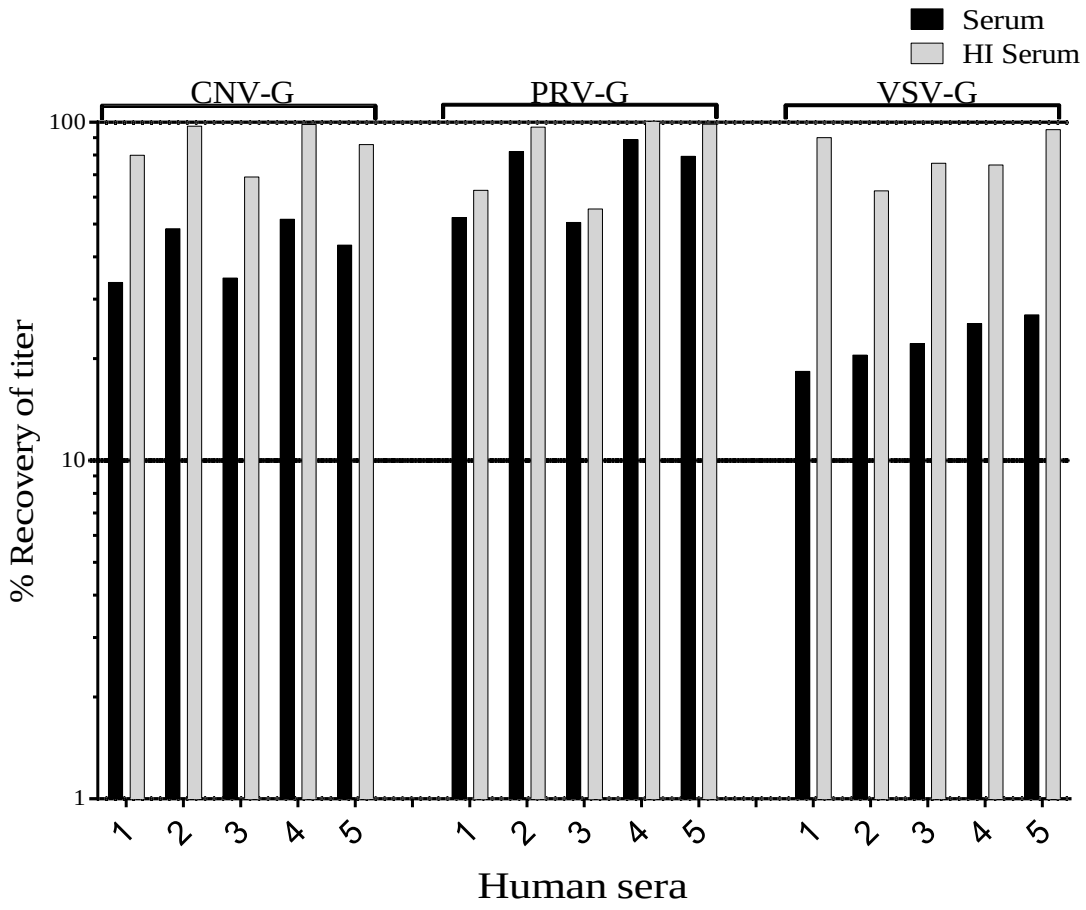


Figure 6.2 Sensitivity of CNV-G, PRV-G and VSV-G pseudotyped lentiviral vectors to human serum complement inactivation. To determine the vector sensitivity to human serum complement, vector preparations were exposed to control and heat inactivated human sera for 1 hour at 37 ^{0}C. Later, the exposed vector suspensions were titered on HEK293T cells. Titer values obtained from DMEM media exposure alone were set as baseline and compared with those of exposure to heat inactivated and non-inactivated sera treatment.

Chapter 7

Project Summary and Future Considerations

Gene delivery using lentiviral vectors is among the most efficient methods for gene therapy, due to the capacity of these vectors to transduce a wide variety of both dividing and non-dividing cells [10, 259]. Low toxicity, stable integration into the genome and minimal triggering of inflammatory responses render this method an attractive tool for efficient gene delivery [10, 260]. However, challenges with lentiviral-based gene therapy have been identified, including its varying tropism to specific target cells as well as its susceptibility to inactivation by the human innate immune system or during long-term storage of vectors [223, 242]. Pseudotyping the lentiviral vectors with glycoprotein envelopes from unrelated viruses have helped overcome some of these challenges [102, 171]. Expression of the heterologous envelope glycoprotein G from the Rhabdovirus Vesicular Stomatitis Virus has considerably improved the tropism and range of cell types that the lentiviral-based system can target [191, 218]. However, the relative susceptibility of VSV-G to serum complement inactivation and other drawbacks hamper the practical utility of this lentiviral vector pseudotype for in vivo applications [223]. Here in an effort to identify alternative heterologous viral envelopes for vector pseudotyping, we evaluated viral envelope glycoproteins from two related viruses, Chandipura and Piry viruses that belong to the same Vesiculovirus genus as VSV. While both of these viruses are phylogenetically related to VSV, they are serologically distinct and hence do not cross-react [232, 233].

In this study, we report efficient pseudotyping of lentiviral vectors using these two novel envelope glycoproteins from Chandipura and Piry Vesiculoviruses. In comparison to Vesicular

Stomatitis Virus glycoprotein (VSV-G)-pseudotyped lentiviral vector, Chandipura glycoprotein (CNV-G) and Piry virus glycoprotein (PRV-G)-pseudotyping resulted in similarly high titers. The ability to produce high titered lentiviral vector is crucial for large-scale applications [10, 247]. Besides, both CNV-G and PRV-G pseudotypes showed comparable and moderate stability to successive freeze/thaw cycles. This relative stability against damage from freeze/thaw and maintenance of the structural integrity of the vector is an important practical consideration, given that the vector preparation might be subjected to multiple freeze/thaw cycles during gene delivery applications. Moreover, both CNV-G and PRV-G pseudotypes were relatively more resistant to inactivation by human serum complement components than VSV-G. Furthermore, lentiviral vectors pseudotyped with these novel glycoproteins were able to efficiently transduce fibroblast and epithelial cells derived from different tissues across different species, cells of neuronal origin and some human T-lymphocyte cell lines. However, novel pseudotypes were less efficient in transducing human primary hematopoietic stem cells (CD34) and human peripheral blood mononuclear cells (PBMCs). The restricted tropism of CNV-G and PRV-G can be improved by multiple rounds of transduction for gene therapy applications targeting the cells of the hematopoietic or immune system. Nevertheless, a narrow yet specific tropism might be necessary for systemic treatment to achieve targeted gene delivery. Examples of this may be the gene therapy of disseminated metastases or a large number of specific population of somatic cells, both of which require specific targeting [261]. Furthermore, this selected tropism may contribute to reducing unwanted off-target side effects, which are considered a potential drawback to VSV-G pseudotype. Finally, these two novel pseudotypes may be highly effective for ex vivo and in vivo delivery of transgenes for many applications, such as gene therapies and gene-based vaccines of various targets including neuronal and epithelial cells.

Chandipura envelope glycoprotein exhibited high efficiencies in lentiviral vector production, with a similar infectivity to VSV-G on HEK293T cells and a comparable or even higher concentration than VSV-G in p24 titration. Chandipura and Piry virus envelope glycoproteins mediated efficient transduction of cells from several important therapeutic target cell types including fibroblast and epithelial cells from liver, kidney, cervix and bone marrow of humans and other species. Importantly, lentiviral vectors pseudotyped with CNV-G consistently transduced these cells at a similar or even improved level of efficiency than VSV-G pseudotypes at all MOIs evaluated ($p<0.05$ in GHOST, MDCK, Mann-Whitney Test). These data indicate that CNV-G may be a better pseudotyping glycoprotein than VSV-G for gene therapy studies in these cell types and other related cells. On the other hand, PRV-G showed adequate pseudotyping efficiencies as indicated by HEK293T infectivity and p24 titrations. PRV-G also exhibited good, albeit decreased, gene delivery efficiencies on tested cell lines and may be an ideal vector substitute when necessary. Therefore, in future studies, it will be exciting to evaluate the in vivo delivery of novel gene targets with these envelope pseudotypes for the treatment of hepatic, renal and cervical diseases [262-264]. Moreover, it will also be interesting to determine the efficacy of novel envelopes in treating other diseases, such as neurological, pulmonary diseases and others, whose cell types were not tested in this study but which have the potential to be effectively transduced by these novel envelope pseudotypes rather than VSV-G [10, 101, 265-267].

Vector virions pseudotyped with CNV-G and PRV-G outperformed VSV-G pseudotype in the human sera sensitivity assay, with much higher resistance to complement inactivation ($p<0.01$, Mann-Whitney U Test). The VSV-G pseudotype was dramatically inactivated by complement-mediated degradation in sera from five human donors, which was consistent with

results from previous publications [176, 222, 223]. Surprisingly, the PRV-G pseudotype was highly resistant to human sera complement components, registering only a slight decline in its titer after incubation with sera from five individual donors. The CNV-G pseudotype showed some level of inactivation by human sera, but still had much higher titer post-treatment than the VSV-G pseudotype. This difference in susceptibility to complement components between these three pseudotyped-lentiviral vectors may be due to specific characteristics of the individual envelope glycoproteins. It is known that glycoproteins of certain viruses such as Paramyxoviridae can activate the classical or alternative pathway, resulting in neutralization of these viruses by human serum [268-270], whereas certain other viruses such as Herpes simplex virus type 1 and 2, whose glycoprotein C binds to and sequesters complement C3b, is protected from serum complement-mediated inactivation [271]. Therefore, these novel envelopes seem to be superior to VSV-G as pseudotyping candidates for direct in vivo delivery. In addition, in cases of gene therapy and vaccine studies where successive vector administrations are necessary, the undesirable priming of the immune system (and thus its activation) with continued administration can be problematic [272]. In such a scenario, using CNV-G and PRV-G pseudotyped vectors sequentially may be beneficial to overcome the immune response mounted against VSV-G pseudotyped lentiviral vectors. In future preclinical animal studies, it will be interesting to determine whether the immune response mounted in animals injected with one pseudotype will be cross-reactive to other pseudotypes. Such screening can help determine a pool of available pseudotypes that could be used for consecutive vector dosing.

Additional future studies should include the testing of primary cells from neurons, epithelial cells and fibroblasts for transduction assays in vitro, in order to verify the observations made on various cell lines in this study. In addition, bio-distribution studies should be conducted

after systemic delivery of vector pseudotypes to determine their tropism to organs and tissues, using immunohistochemistry and in situ PCR hybridization techniques to track the presence of viral vectors [273]. Although CNV-G and PRV-G had lower transduction efficiencies in certain cell types than VSV-G pseudotype in our in vitro experiments, they may not exhibit such a significant difference in vivo, since they possess better resistance to human serum complement inactivation and more specific tropism to target cells. After further verification of tropism, gene therapy strategies for disease treatment may be evaluated with the CNV-G and PRV-G pseudotypes. These vectors may be ideal for specific gene delivery treatments due to their preferences for neuronal, epithelial and fibroblastic cell types, since defects in these cells can lead to numerous human diseases. For gene therapy of Parkinson's Disease, the neurotropic CNV-G pseudotype could be employed to deliver therapeutic genes to restore dopaminergic synthesis in substantia nigra neurons, through systemic injection or intraputaminal infusion [101]. For gene therapy of Cystic fibrosis, CNV-G and PRV-G pseudotypes could be delivered intravenously or intranasally into lung epithelial related tissues to restore normal chloride transportation [11]. In gene therapy strategy for Chronic Ischemic Heart Disease, which is caused by abnormal fibroblasts, the CNV-G and PRV-G pseudotypes could be utilized as vehicles to deliver therapeutic genes (growth factors) through intracoronary or direct myocardial injections [274]. Potential gene therapy strategies for which these novel pseudotypes could be used are not limited to the few listed here, but all therapies require testing in animal models before entering clinical trials. Humanized mice would be the optimal animal model for most of these in vivo pilot studies [275], and experiments could be performed in direct comparison to the VSV-G pseudotype, as has been done in this project.

In conclusion, Chandipura and Piry virus envelope glycoproteins have been developed that are effective in lentiviral vector pseudotyping and both demonstrated efficient transductions of certain cell types that are important targets for gene therapy, including neuronal, fibroblastic and epithelial cells from different species. CNV-G pseudotyped vectors transduced these cells at similar or better efficiencies than VSV-G pseudotypes. While relatively less efficient, PRV-G pseudotyped vectors also showed gene delivery efficiencies at levels adequate for experimentation and thus provide a good alternative vector pseudotype when necessary. Moreover, both CNV-G and PRV-G envelope glycoproteins are inherently more resistant to human serum complement inactivation. With these superior properties, these novel envelope glycoproteins may be a better choice or a useful alternative to VSV-G for lentiviral vector pseudotyping for many gene therapies requiring successive in vivo gene delivery, depending on the target cell types and delivery route. Furthermore, the CNV-G and PRV-G pseudotyped vectors are expected to fill a gap when alternative envelope proteins are needed to transduce a particular cell type and when neutralizing immune responses preclude the use of the standard VSV-G pseudotyped vectors for in vivo gene therapy.

References

1. Gene therapy for human genetic disease? Science, 1972. 178(4061): p. 648-9.
2. Watson, J.D. and F.H.C. Crick, Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. Nature, 1953. 171(4356): p. 737-738.
3. Fischer, A., S. Hacein-Bey-Abina, and M. Cavazzana-Calvo, 20 years of gene therapy for SCID. Nat Immunol, 2010. 11(6): p. 457-60.
4. Sheridan, C., Gene therapy finds its niche. Nat Biotechnol, 2011. 29(2): p. 121-8.
5. Ferrua, F., I. Brigida, and A. Aiuti, Update on gene therapy for adenosine deaminase-deficient severe combined immunodeficiency. Curr Opin Allergy Clin Immunol, 2010. 10(6): p. 551-6.
6. Cross, D. and J.K. Burmester, Gene therapy for cancer treatment: past, present and future. Clin Med Res, 2006. 4(3): p. 218-27.
7. Barnett, S.W., et al., An AIDS-like condition induced in baboons by HIV-2. Science, 1994. 266(5185): p. 642-6.
8. Naldini, L., Gene therapy returns to centre stage. Nature, 2015. 526(7573): p. 351-360.
9. Strayer, D.S., et al., Current status of gene therapy strategies to treat HIV/AIDS. Mol Ther, 2005. 11(6): p. 823-42.
10. Amer, M.H., Gene therapy for cancer: present status and future perspective. Mol Cell Ther, 2014. 2: p. 27.
11. Pickler, R.H. and C.L. Munro, Gene therapy for inherited disorders. Journal of Pediatric Nursing, 1995. 10(1): p. 40-47.
12. Radivojac, P., et al., A large-scale evaluation of computational protein function prediction. Nat Methods, 2013. 10(3): p. 221-7.

13. Blaese, R.M., Progress toward gene therapy. Clin Immunol Immunopathol, 1991. 61(2 Pt 2): p. S47-55.

14. Gravitz, L. and S. Pincock, Sickle-cell disease. Nature, 2014. 515(7526): p. S1.

15. Vichinsky, E. and B.H. Lubin, Suggested guidelines for the treatment of children with sickle cell anemia. Hematol Oncol Clin North Am, 1987. 1(3): p. 483-501.

16. Azzouz, M., et al., VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. Nature, 2004. 429(6990): p. 413-7.

17. Cournoyer, D. and C.T. Caskey, Gene therapy of the immune system. Annu Rev Immunol, 1993. 11: p. 297-329.

18. Hirschhorn, R., Overview of biochemical abnormalities and molecular genetics of adenosine deaminase deficiency. Pediatr Res, 1993. 33(1 Suppl): p. S35-41.

19. Blaese, R.M., Development of gene therapy for immunodeficiency: adenosine deaminase deficiency. Pediatr Res, 1993. 33(1 Suppl): p. S49-53; discussion S53-5.

20. Nathwani, A.C., et al., Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med, 2014. 371(21): p. 1994-2004.

21. Ponder, K.P., Hemophilia gene therapy: a Holy Grail found. Mol Ther, 2011. 19(3): p. 427-8.

22. Murphy, S.L. and K.A. High, Gene therapy for haemophilia. Br J Haematol, 2008. 140(5): p. 479-87.

23. Collins, F.S., Cystic fibrosis: molecular biology and therapeutic implications. Science, 1992. 256(5058): p. 774-9.

24. Tizzano, E.F. and M. Buchwald, Recent advances in cystic fibrosis research. J Pediatr, 1993. 122(6): p. 985-8.

25. Conese, M., et al., Gene and cell therapy for cystic fibrosis: from bench to bedside. J Cyst Fibros, 2011. 10 Suppl 2: p. S114-28.

26. Zeitlin, P.L., Cystic fibrosis gene therapy trials and tribulations. Mol Ther, 2000. 1(1): p. 5-6.

27. Bennett, J., et al., AAV2 gene therapy readministration in three adults with congenital blindness. Sci Transl Med, 2012. 4(120): p. 120ra15.

28. Aronovich, E.L. and P.B. Hackett, Lysosomal storage disease: gene therapy on both sides of the blood-brain barrier. Mol Genet Metab, 2015. 114(2): p. 83-93.

29. Bowles, D.E., et al., Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. Mol Ther, 2012. 20(2): p. 443-55.

30. Embretson, J., et al., Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature, 1993. 362(6418): p. 359-62.

31. Safai, B., et al., Seroepidemiological studies of human T-lymphotropic retrovirus type III in acquired immunodeficiency syndrome. Lancet, 1984. 1(8392): p. 1438-40.

32. Barre-Sinoussi, F., et al., Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science, 1983. 220(4599): p. 868-71.

33. Landesman, S.H., H.M. Ginzburg, and S.H. Weiss, The AIDS epidemic. N Engl J Med, 1985. 312(8): p. 521-5.

34. Peterman, T.A., D.P. Drotman, and J.W. Curran, Epidemiology of the acquired immunodeficiency syndrome (AIDS). Epidemiol Rev, 1985. 7: p. 1-21.

35. Lou, L.X., et al., National HIV/AIDS mortality, prevalence, and incidence rates are associated with the Human Development Index. Am J Infect Control, 2014. 42(10): p. 1044-8.

36. Maartens, G., C. Celum, and S.R. Lewin, HIV infection: epidemiology, pathogenesis, treatment, and prevention. Lancet, 2014. 384(9939): p. 258-71.

37. Dalgleish, A.G., et al., The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature, 1984. 312(5996): p. 763-7.

38. Klatzmann, D., et al., T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature, 1984. 312(5996): p. 767-8.

39. Allan, J.S., et al., Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. Science, 1985. 228(4703): p. 1091-4.

40. Wain-Hobson, S., et al., Nucleotide sequence of the AIDS virus, LAV. Cell, 1985. 40(1): p. 9-17.

41. Barre-Sinoussi, F., A.L. Ross, and J.F. Delfraissy, Past, present and future: 30 years of HIV research. Nat Rev Microbiol, 2013. 11(12): p. 877-83.

42. Pantaleo, G., C. Graziosi, and A.S. Fauci, New concepts in the immunopathogenesis of human immunodeficiency virus infection. N Engl J Med, 1993. 328(5): p. 327-35.

43. Little, S.J., et al., Viral dynamics of acute HIV-1 infection. J Exp Med, 1999. 190(6): p. 841-50.

44. Mellors, J.W., et al., Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science, 1996. 272(5265): p. 1167-70.

45. Ruelas, D.S. and W.C. Greene, An integrated overview of HIV-1 latency. Cell, 2013. 155(3): p. 519-29.

46. Hwang, S.S., et al., Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science, 1991. 253(5015): p. 71-4.

47. Coffin, J. and R. Swanstrom, HIV pathogenesis: dynamics and genetics of viral populations and infected cells. Cold Spring Harb Perspect Med, 2013. 3(1): p. a012526.

48. Fauci, A.S., Pathogenesis of HIV disease: opportunities for new prevention interventions. Clin Infect Dis, 2007. 45 Suppl 4: p. S206-12.

49. Arts, E.J. and D.J. Hazuda, HIV-1 antiretroviral drug therapy. Cold Spring Harb Perspect Med, 2012. 2(4): p. a007161.

50. Stolbach, A., et al., A Review of the Toxicity of HIV Medications II: Interactions with Drugs and Complementary and Alternative Medicine Products. J Med Toxicol, 2015. 11(3): p. 326-41.

51. Flexner, C. and M. Saag, The antiretroviral drug pipeline: prospects and implications for future treatment research. Curr Opin HIV AIDS, 2013. 8(6): p. 572-8.

52. Margolis, A.M., et al., A review of the toxicity of HIV medications. J Med Toxicol, 2014. 10(1): p. 26-39.

53. WHO Guidelines Approved by the Guidelines Review Committee, in Consolidated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing HIV Infection: Recommendations for a Public Health Approach. 2013, World Health Organization

54. Johnson, V.A., et al., Update of the drug resistance mutations in HIV-1: March 2013. Top Antivir Med, 2013. 21(1): p. 6-14.

55. Walker, B.D. and M.S. Hirsch, Antiretroviral therapy in early HIV infection. N Engl J Med, 2013. 368(3): p. 279-81.

56. Wensing, A.M., et al., 2015 Update of the Drug Resistance Mutations in HIV-1. Top Antivir Med, 2015. 23(4): p. 132-41.

57. Hutter, G., et al., Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N Engl J Med, 2009. 360(7): p. 692-8.

58. Scherer, L.J. and J.J. Rossi, Ex vivo gene therapy for HIV-1 treatment. Hum Mol Genet, 2011. 20(R1): p. R100-7.

59. Baltimore, D., Gene therapy. Intracellular immunization. Nature, 1988. 335(6189): p. 395-6.

60. Chung, J., D.L. DiGiusto, and J.J. Rossi, Combinatorial RNA-based gene therapy for the treatment of HIV/AIDS. Expert Opin Biol Ther, 2013. 13(3): p. 437-45.

61. Peterson, C.W., et al., Combinatorial anti-HIV gene therapy: using a multipronged approach to reach beyond HAART. Gene Ther, 2013. 20(7): p. 695-702.

62. Strang, B.L., et al., Human immunodeficiency virus type 1 vectors with alphavirus envelope glycoproteins produced from stable packaging cells. J Virol, 2005. 79(3): p. 1765-71.

63. Wang, C. and Q. Zhou, Systemic delivery of siRNA: challenging but promising. Recent Pat Drug Deliv Formul, 2012. 6(1): p. 19-30.

64. Tebas, P., et al., Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med, 2014. 370(10): p. 901-10.

65. Yang, A.G., et al., Phenotypic knockout of HIV type 1 chemokine coreceptor CCR-5 by intrakines as potential therapeutic approach for HIV-1 infection. Proc Natl Acad Sci U S A, 1997. 94(21): p. 11567-72.

66. Yang, A.G., et al., Anti-HIV type 1 activity of wild-type and functional defective RANTES intrakine in primary human lymphocytes. Hum Gene Ther, 1998. 9(14): p. 2005-18.

67. Gonzalez, E., et al., The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science, 2005. 307(5714): p. 1434-40.

68. Curiel, T.J., et al., Long-term inhibition of clinical and laboratory human immunodeficiency virus strains in human T-cell lines containing an HIV-regulated diphtheria toxin A chain gene. Hum Gene Ther, 1993. 4(6): p. 741-7.

69. Smith, S.M., R.B. Markham, and K.T. Jeang, Conditional reduction of human immunodeficiency virus type 1 replication by a gain-of-herpes simplex virus 1 thymidine kinase function. Proc Natl Acad Sci U S A, 1996. 93(15): p. 7955-60.

70. Mebatsion, T., et al., A CXCR4/CD4 pseudotype rhabdovirus that selectively infects HIV-1 envelope protein-expressing cells. Cell, 1997. 90(5): p. 841-7.

71. Schnell, M.J., et al., Construction of a novel virus that targets HIV-1-infected cells and controls HIV-1 infection. Cell, 1997. 90(5): p. 849-57.

72. Chen, J.D., et al., Inactivation of HIV-1 chemokine co-receptor CXCR-4 by a novel intrakine strategy. Nat Med, 1997. 3(10): p. 1110-6.

73. Marasco, W.A., W.A. Haseltine, and S.Y. Chen, Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody. Proc Natl Acad Sci U S A, 1993. 90(16): p. 7889-93.

74. Levy-Mintz, P., et al., Intracellular expression of single-chain variable fragments to inhibit early stages of the viral life cycle by targeting human immunodeficiency virus type 1 integrase. J Virol, 1996. 70(12): p. 8821-32.

75. Levin, R., et al., Inhibition of early and late events of the HIV-1 replication cycle by cytoplasmic Fab intrabodies against the matrix protein, p17. Mol Med, 1997. 3(2): p. 96-110.

76. Rondon, I.J. and W.A. Marasco, Intracellular antibodies (intrabodies) for gene therapy of infectious diseases. Annu Rev Microbiol, 1997. 51: p. 257-83.

77. Duan, L., et al., Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-Rev single-chain antibody. Proc Natl Acad Sci U S A, 1998. 95(17): p. 10344.

78. Durand, C.M. and R.F. Ambinder, Hematopoietic stem cell transplantation in HIV-1-infected individuals: clinical challenges and the potential for viral eradication. Curr Opin Oncol, 2013. 25(2): p. 180-6.

79. Goodchild, J., et al., Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides. Proc Natl Acad Sci U S A, 1988. 85(15): p. 5507-11.

80. Buck, H.M., et al., Phosphate-methylated DNA aimed at HIV-1 RNA loops and integrated DNA inhibits viral infectivity. Science, 1990. 248(4952): p. 208-12.

81. Lisziewicz, J., et al., Specific inhibition of human immunodeficiency virus type 1 replication by antisense oligonucleotides: an in vitro model for treatment. Proc Natl Acad Sci U S A, 1992. 89(23): p. 11209-13.

82. Chang, H.K., et al., Block of HIV-1 infection by a combination of antisense tat RNA and TAR decoys: a strategy for control of HIV-1. Gene Ther, 1994. 1(3): p. 208-16.

83. Kim, J.H., et al., Inhibition of HIV replication by sense and antisense rev response elements in HIV-based retroviral vectors. J Acquir Immune Defic Syndr Hum Retrovirol, 1996. 12(4): p. 343-51.

84. Browning, C.M., et al., Potent inhibition of human immunodeficiency virus type 1 (HIV-1) gene expression and virus production by an HIV-2 tat activation-response RNA decoy. J Virol, 1999. 73(6): p. 5191-5.

85. Coburn, G.A. and B.R. Cullen, Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J Virol, 2002. 76(18): p. 9225-31.

86. Sullenger, B.A., et al., Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. Cell, 1990. 63(3): p. 601-8.

87. Morgan, R.A. and R. Walker, Gene therapy for AIDS using retroviral mediated gene transfer to deliver HIV-1 antisense TAR and transdominant Rev protein genes to syngeneic lymphocytes in HIV-1 infected identical twins. Hum Gene Ther, 1996. 7(10): p. 1281-306.

88. Li, M.J., et al., Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. Mol Ther, 2005. 12(5): p. 900-9.

89. Dey, R. and B. Pillai, Cell-based gene therapy against HIV. Gene Ther, 2015. 22(11): p. 851-5.

90. Rossi, J.J., et al., Ribozymes as therapies for AIDS. Ann N Y Acad Sci, 1990. 616: p. 184-200.

91. Sarver, N., et al., Ribozymes as potential anti-HIV-1 therapeutic agents. Science, 1990. 247(4947): p. 1222-5.

92. Ojwang, J.O., et al., Inhibition of human immunodeficiency virus type 1 expression by a hairpin ribozyme. Proc Natl Acad Sci U S A, 1992. 89(22): p. 10802-6.

93. Bai, J., et al., Characterization of anti-CCR5 ribozyme-transduced CD34+ hematopoietic progenitor cells in vitro and in a SCID-hu mouse model in vivo. Mol Ther, 2000. 1(3): p. 244-54.

94. Anderson, J., et al., Potent suppression of HIV type 1 infection by a short hairpin anti-CXCR4 siRNA. AIDS Res Hum Retroviruses, 2003. 19(8): p. 699-706.

95. Li, M.J., et al., Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. Mol Ther, 2003. 8(2): p. 196-206.

96. Das, A.T., et al., Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virol, 2004. 78(5): p. 2601-5.

97. Anderson, J., et al., Safety and efficacy of a lentiviral vector containing three anti-HIV genes--CCR5 ribozyme, tat-rev siRNA, and TAR decoy--in SCID-hu mouse-derived T cells. Mol Ther, 2007. 15(6): p. 1182-8.

98. Zou, S., et al., Hematopoietic cell transplantation and HIV cure: where we are and what next? Blood, 2013. 122(18): p. 3111-5.

99. Rossi, J.J., C.H. June, and D.B. Kohn, Genetic therapies against HIV. Nat Biotechnol, 2007. 25(12): p. 1444-54.

100. Xu, R., et al., Diabetes gene therapy: potential and challenges. Curr Gene Ther, 2003. 3(1): p. 65-82.

101. Simonato, M., et al., Progress in gene therapy for neurological disorders. Nat Rev Neurol, 2013. 9(5): p. 277-91.

102. Cronin, J., X.Y. Zhang, and J. Reiser, Altering the tropism of lentiviral vectors through pseudotyping. Curr Gene Ther, 2005. 5(4): p. 387-98.

103. Walther, W. and U. Stein, Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs, 2000. 60(2): p. 249-71.

104. Palffy, R., et al., Bacteria in gene therapy: bactofection versus alternative gene therapy. Gene Ther, 2006. 13(2): p. 101-5.

105. Baban, C.K., et al., Bacteria as vectors for gene therapy of cancer. Bioeng Bugs, 2010. 1(6): p. 385-94.

106. Ura, T., K. Okuda, and M. Shimada, Developments in Viral Vector-Based Vaccines. Vaccines, 2014. 2(3): p. 624.

107. Jackson, D.A., R.H. Symons, and P. Berg, Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. Proc Natl Acad Sci U S A, 1972. 69(10): p. 2904-9.

108. Gardlik, R., et al., Vectors and delivery systems in gene therapy. Med Sci Monit, 2005. 11(4): p. Ra110-21.

109. Katz, M.G., et al., Gene therapy delivery systems for enhancing viral and nonviral vectors for cardiac diseases: current concepts and future applications. Hum Gene Ther, 2013. 24(11): p. 914-27.

110. Giacca, M. and S. Zacchigna, Virus-mediated gene delivery for human gene therapy. J Control Release, 2012. 161(2): p. 377-88.

111. Enders, J.F., et al., Adenoviruses: group name proposed for new respiratory-tract viruses. Science, 1956. 124(3212): p. 119-20.

112. McConnell, M.J. and M.J. Imperiale, Biology of adenovirus and its use as a vector for gene therapy. Hum Gene Ther, 2004. 15(11): p. 1022-33.

113. Appaiahgari, M.B. and S. Vrati, Adenoviruses as gene/vaccine delivery vectors: promises and pitfalls. Expert Opin Biol Ther, 2015. 15(3): p. 337-51.

114. Russell, W.C., Adenoviruses: update on structure and function. J Gen Virol, 2009. 90(Pt 1): p. 1-20.

115. Graham, F.L., et al., Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol, 1977. 36(1): p. 59-74.

116. Yang, Y. and J.M. Wilson, Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. J Immunol, 1995. 155(5): p. 2564-70.

117. Yang, Y., Q. Su, and J.M. Wilson, Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. J Virol, 1996. 70(10): p. 7209-12.

118. Vilquin, J.T., et al., FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. Hum Gene Ther, 1995. 6(11): p. 1391-401.

119. Lei, D., et al., Nondepleting anti-CD4 antibody treatment prolongs lung-directed E1-deleted adenovirus-mediated gene expression in rats. Hum Gene Ther, 1996. 7(18): p. 2273-9.

120. Lochmuller, H., et al., Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice. Gene Ther, 1996. 3(8): p. 706-16.

121. Knowles, M.R., et al., A controlled study of adenoviral-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. N Engl J Med, 1995. 333(13): p. 823-31.

122. Bellon, G., et al., Aerosol administration of a recombinant adenovirus expressing CFTR to cystic fibrosis patients: a phase I clinical trial. Hum Gene Ther, 1997. 8(1): p. 15-25.

123. Freeman, S.M., et al., The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res, 1993. 53(21): p. 5274-83.

124. Roth, J.A., Modification of tumor suppressor gene expression and induction of apoptosis in non-small cell lung cancer (NSCLC) with an adenovirus vector expressing wildtype p53 and cisplatin. Hum Gene Ther, 1996. 7(8): p. 1013-30.

125. Tenbusch, M., et al., Coexpression of GM-CSF and antigen in DNA prime-adenoviral vector boost immunization enhances polyfunctional CD8+ T cell responses, whereas expression of GM-CSF antigen fusion protein induces autoimmunity. BMC Immunol, 2008. 9: p. 13.

126. Alekseenko, I.V., et al., Therapeutic properties of a vector carrying the HSV thymidine kinase and GM-CSF genes and delivered as a complex with a cationic copolymer. J Transl Med, 2015. 13: p. 78.

127. Heise, C., et al., ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. Nat Med, 1997. 3(6): p. 639-45.

128. Dix, B.R., S.J. Edwards, and A.W. Braithwaite, Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway? J Virol, 2001. 75(12): p. 5443-7.

129. Doronin, K., et al., Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. J Virol, 2000. 74(13): p. 6147-55.

130. Abe, T., et al., Intra-arterial delivery of p53-containing adenoviral vector into experimental brain tumors. Cancer Gene Ther, 2002. 9(3): p. 228-35.

131. Tazawa, H., S. Kagawa, and T. Fujiwara, Advances in adenovirus-mediated p53 cancer gene therapy. Expert Opin Biol Ther, 2013. 13(11): p. 1569-83.

132. Alemany, R., Cancer selective adenoviruses. Mol Aspects Med, 2007. 28(1): p. 42-58.

133. Bischoff, J.R., et al., An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science, 1996. 274(5286): p. 373-6.

134. Zhao, J., et al., Rapid generation of a mouse model for Middle East respiratory syndrome. Proc Natl Acad Sci U S A, 2014. 111(13): p. 4970-5.

135. Wilson, J.M., Adeno-associated virus and lentivirus pseudotypes for lung-directed gene therapy. Proc Am Thorac Soc, 2004. 1(4): p. 309-14.

136. Fisher, K.J., et al., Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. J Virol, 1996. 70(1): p. 520-32.

137. Blacklow, N.R., et al., Epidemiology of adenovirus-associated virus infection in a nursery population. Am J Epidemiol, 1968. 88(3): p. 368-78.

138. Dong, J.Y., P.D. Fan, and R.A. Frizzell, Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. Hum Gene Ther, 1996. 7(17): p. 2101-12.

139. Kaplitt, M.G., et al., Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat Genet, 1994. 8(2): p. 148-54.

140. Ali, R.R., et al., Gene transfer into the mouse retina mediated by an adeno-associated viral vector. Hum Mol Genet, 1996. 5(5): p. 591-4.

141. Nakai, H., et al., Adeno-associated viral vector-mediated gene transfer of human blood coagulation factor IX into mouse liver. Blood, 1998. 91(12): p. 4600-7.

142. Srivastava, A., Adeno-associated virus-mediated gene transfer. Journal of cellular biochemistry, 2008. 105(1): p. 17-24.

143. Kotin, R.M., R.M. Linden, and K.I. Berns, Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. Embo j, 1992. 11(13): p. 5071-8.

144. Fisher, K.J., et al., Recombinant adeno-associated virus for muscle directed gene therapy. Nat Med, 1997. 3(3): p. 306-12.

145. Koeberl, D.D., et al., Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. Proc Natl Acad Sci U S A, 1997. 94(4): p. 1426-31.

146. Gao, G.P., et al., High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. Hum Gene Ther, 1998. 9(16): p. 2353-62.

147. Inoue, N. and D.W. Russell, Packaging cells based on inducible gene amplification for the production of adeno-associated virus vectors. J Virol, 1998. 72(9): p. 7024-31.

148. Matsushita, T., et al., Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther, 1998. 5(7): p. 938-45.

149. Xiao, X., J. Li, and R.J. Samulski, Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J Virol, 1998. 72(3): p. 2224-32.

150. Ozawa, K., et al., Gene therapy of Parkinson's disease using adeno-associated virus (AAV) vectors. J Neural Transm Suppl, 2000(58): p. 181-91.

151. Moss, R.B., et al., Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. Chest, 2004. 125(2): p. 509-21.

152. Daya, S. and K.I. Berns, Gene Therapy Using Adeno-Associated Virus Vectors. Clinical Microbiology Reviews, 2008. 21(4): p. 583-593.

153. Simonelli, F., et al., Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Mol Ther, 2010. 18(3): p. 643-50.

154. Nathwani, A.C., et al., Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med, 2011. 365(25): p. 2357-65.

155. Griesenbach, U., D.M. Geddes, and E.W. Alton, Gene therapy progress and prospects: cystic fibrosis. Gene Ther, 2006. 13(14): p. 1061-7.

156. Kaplitt, M.G., et al., Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. Lancet, 2007. 369(9579): p. 2097-105.

157. Cosset, F.L., et al., Retroviral retargeting by envelopes expressing an N-terminal binding domain. J Virol, 1995. 69(10): p. 6314-22.

158. Somia, N.V., M. Zoppe, and I.M. Verma, Generation of targeted retroviral vectors by using single-chain variable fragment: an approach to in vivo gene delivery. Proc Natl Acad Sci U S A, 1995. 92(16): p. 7570-4.

159. Ager, S., et al., Retroviral display of antibody fragments; interdomain spacing strongly influences vector infectivity. Hum Gene Ther, 1996. 7(17): p. 2157-64.

160. Marin, M., et al., Targeted infection of human cells via major histocompatibility complex class I molecules by Moloney murine leukemia virus-derived viruses displaying single-chain antibody fragment-envelope fusion proteins. J Virol, 1996. 70(5): p. 2957-62.

161. Schnierle, B.S. and B. Groner, Retroviral targeted delivery. Gene Ther, 1996. 3(12): p. 1069-73.

162. Yee, J.K., et al., A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. Proc Natl Acad Sci U S A, 1994. 91(20): p. 9564-8.

163. Sharma, S., et al., Efficient infection of a human T-cell line and of human primary peripheral blood leukocytes with a pseudotyped retrovirus vector. Proc Natl Acad Sci U S A, 1996. 93(21): p. 11842-7.

164. Sinclair, A.M., et al., Interaction of vesicular stomatitis virus-G pseudotyped retrovirus with CD34+ and CD34+ CD38- hematopoietic progenitor cells. Gene Ther, 1997. 4(9): p. 918-27.

165. Cosset, F.L. and S.J. Russell, Targeting retrovirus entry. Gene Ther, 1996. 3(11): p. 946-56.

166. Hacein-Bey-Abina, S., et al., LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science, 2003. 302(5644): p. 415-9.

167. Azzouz, M., et al., Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. J Clin Invest, 2004. 114(12): p. 1726-31.

168. Markowitz, D., S. Goff, and A. Bank, A safe packaging line for gene transfer: separating viral genes on two different plasmids. J Virol, 1988. 62(4): p. 1120-4.

169. Weinberg, J.B., et al., Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human monocytes. J Exp Med, 1991. 174(6): p. 1477-82.

170. Lewis, P., M. Hensel, and M. Emerman, Human immunodeficiency virus infection of cells arrested in the cell cycle. Embo j, 1992. 11(8): p. 3053-8.

171. Burns, J.C., et al., Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc Natl Acad Sci U S A, 1993. 90(17): p. 8033-7.

172. Hu, S., et al., Pseudotyping of lentiviral vector with novel vesiculovirus envelope glycoproteins derived from Chandipura and Piry viruses. Virology, 2015. 488: p. 162-168.

173. Mazarakis, N.D., et al., Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. Hum Mol Genet, 2001. 10(19): p. 2109-21.

174. Girard-Gagnepain, A., et al., Baboon envelope pseudotyped LVs outperform VSV-G-LVs for gene transfer into early-cytokine-stimulated and resting HSCs. Blood, 2014. 124(8): p. 1221-31.

175. Stein, C.S., I. Martins, and B.L. Davidson, The lymphocytic choriomeningitis virus envelope glycoprotein targets lentiviral gene transfer vector to neural progenitors in the murine brain. Mol Ther, 2005. 11(3): p. 382-9.

176. Trobridge, G.D., et al., Cocal-pseudotyped lentiviral vectors resist inactivation by human serum and efficiently transduce primate hematopoietic repopulating cells. Mol Ther, 2010. 18(4): p. 725-33.

177. Frecha, C., et al., Measles virus glycoprotein-pseudotyped lentiviral vector-mediated gene transfer into quiescent lymphocytes requires binding to both SLAM and CD46 entry receptors. J Virol, 2011. 85(12): p. 5975-85.

178. Witting, S.R., P. Vallanda, and A.L. Gamble, Characterization of a third generation lentiviral vector pseudotyped with Nipah virus envelope proteins for endothelial cell transduction. Gene Ther, 2013. 20(10): p. 997-1005.

179. Miletic, H., et al., Retroviral vectors pseudotyped with lymphocytic choriomeningitis virus. J Virol, 1999. 73(7): p. 6114-6.

180. Kobinger, G.P., et al., Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. Nat Biotechnol, 2001. 19(3): p. 225-30.

181. Sinn, P.L., et al., Lentivirus vectors pseudotyped with filoviral envelope glycoproteins transduce airway epithelia from the apical surface independently of folate receptor alpha. J Virol, 2003. 77(10): p. 5902-10.

182. Kang, Y., et al., In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. J Virol, 2002. 76(18): p. 9378-88.

183. Sandrin, V., et al., Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. Blood, 2002. 100(3): p. 823-32.

184. Frecha, C., et al., Stable transduction of quiescent T cells without induction of cycle progression by a novel lentiviral vector pseudotyped with measles virus glycoproteins. Blood, 2008. 112(13): p. 4843-52.

185. Winkler, T., et al., No evidence for clonal selection due to lentiviral integration sites in human induced pluripotent stem cells. Stem Cells, 2010. 28(4): p. 687-94.

186. Daniel, R. and J.A. Smith, Integration site selection by retroviral vectors: molecular mechanism and clinical consequences. Hum Gene Ther, 2008. 19(6): p. 557-68.

187. Deglon, N., et al., Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. Hum Gene Ther, 2000. 11(1): p. 179-90.

188. Azzouz, M., et al., Multicistronic lentiviral vector-mediated striatal gene transfer of aromatic L-amino acid decarboxylase, tyrosine hydroxylase, and GTP cyclohydrolase I induces sustained transgene expression, dopamine production, and functional improvement in a rat model of Parkinson's disease. J Neurosci, 2002. 22(23): p. 10302-12.

189. Davidson, B.L. and X.O. Breakefield, Viral vectors for gene delivery to the nervous system. Nat Rev Neurosci, 2003. 4(5): p. 353-64.

190. Bensadoun, J.C., et al., Lentiviral vectors as a gene delivery system in the mouse midbrain: cellular and behavioral improvements in a 6-OHDA model of Parkinson's disease using GDNF. Exp Neurol, 2000. 164(1): p. 15-24.

191. Naldini, L., et al., In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science, 1996. 272(5259): p. 263-7.

192. Blomer, U., et al., Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. J Virol, 1997. 71(9): p. 6641-9.

193. Dyall, J., et al., Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. Blood, 2001. 97(1): p. 114-21.

194. Esslinger, C., P. Romero, and H.R. MacDonald, Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors. Hum Gene Ther, 2002. 13(9): p. 1091-100.

195. Palmowski, M.J., et al., Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. J Immunol, 2004. 172(3): p. 1582-7.

196. Breckpot, K., J.L. Aerts, and K. Thielemans, Lentiviral vectors for cancer immunotherapy: transforming infectious particles into therapeutics. Gene Ther, 2007. 14(11): p. 847-62.

197. Hu, B., A. Tai, and P. Wang, Immunization delivered by lentiviral vectors for cancer and infectious diseases. Immunol Rev, 2011. 239(1): p. 45-61.

198. Konopka, K., et al., Rev-binding aptamer and CMV promoter act as decoys to inhibit HIV replication. Gene, 2000. 255(2): p. 235-44.

199. Yuan, J., et al., Zinc-finger nuclease editing of human cxcr4 promotes HIV-1 CD4(+) T cell resistance and enrichment. Mol Ther, 2012. 20(4): p. 849-59.

200. Ebina, H., et al., Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci Rep, 2013. 3: p. 2510.

201. Manjunath, N., et al., Newer gene editing technologies toward HIV gene therapy. Viruses, 2013. 5(11): p. 2748-66.

202. Turner, A.M., J. De La Cruz, and K.V. Morris, Mobilization-competent Lentiviral Vector-mediated Sustained Transcriptional Modulation of HIV-1 Expression. Mol Ther, 2009. 17(2): p. 360-8.

203. Levine, B.L., et al., Gene transfer in humans using a conditionally replicating lentiviral vector. Proc Natl Acad Sci U S A, 2006. 103(46): p. 17372-7.

204. Evans, J.T. and J.V. Garcia, Lentivirus vector mobilization and spread by human immunodeficiency virus. Hum Gene Ther, 2000. 11(17): p. 2331-9.

205. Hanawa, H., D.A. Persons, and A.W. Nienhuis, Mobilization and mechanism of transcription of integrated self-inactivating lentiviral vectors. J Virol, 2005. 79(13): p. 8410-21.

206. Xiong, C., et al., Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. Science, 1989. 243(4895): p. 1188-91.

207. Davis, N.L., K.W. Brown, and R.E. Johnston, A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. J Virol, 1996. 70(6): p. 3781-7.

208. Lei, D.C., et al., Episomal expression of wild-type CFTR corrects cAMP-dependent chloride transport in respiratory epithelial cells. Gene Ther, 1996. 3(5): p. 427-36.

209. Izumo, T., et al., Epstein-Barr virus-based vector improves the tumor cell killing effect of pituitary tumor in HVJ-liposome-mediated transcriptional targeting suicide gene therapy. Int J Oncol, 2007. 31(2): p. 379-87.

210. Liljestrom, P. and H. Garoff, A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Biotechnology (N Y), 1991. 9(12): p. 1356-61.

211. Mocarski, E.S., et al., A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. Proc Natl Acad Sci U S A, 1996. 93(21): p. 11321-6.

212. Palese, P., et al., Negative-strand RNA viruses: genetic engineering and applications. Proc Natl Acad Sci U S A, 1996. 93(21): p. 11354-8.

213. Caley, I.J., et al., Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. J Virol, 1997. 71(4): p. 3031-8.

214. Duboise, S.M., et al., Use of virion DNA as a cloning vector for the construction of mutant and recombinant herpesviruses. Proc Natl Acad Sci U S A, 1996. 93(21): p. 11389-94.

215. Medin, J.A. and S. Karlsson, Viral vectors for gene therapy of hematopoietic cells. Immunotechnology, 1997. 3(1): p. 3-19.

216. Walther, W. and U. Stein, Viral Vectors for Gene Transfer. Drugs, 2012. 60(2): p. 249-271.

217. Collins, M.K. and V. Cerundolo, Gene therapy meets vaccine development. Trends Biotechnol, 2004. 22(12): p. 623-6.

218. Akkina, R.K., et al., High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. J Virol, 1996. 70(4): p. 2581-5.

219. Reiser, J., et al., Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc Natl Acad Sci U S A, 1996. 93(26): p. 15266-71.

220. Kneissl, S., et al., CD19 and CD20 targeted vectors induce minimal activation of resting B lymphocytes. PLoS One, 2013. 8(11): p. e79047.

221. Banerjea, A. and R. Akkina, 731. Pseudotyping of Lentiviral Vectors with New Envelope G Proteins from Vesiculoviruses VSV-New Jersey, Chandipura and Piry. Mol Ther, 2004. 9(S1): p. S278-S278.

222. Schauber, C.A., et al., Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. Gene Ther, 2004. 11(3): p. 266-75.

223. DePolo, N.J., et al., VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. Mol Ther, 2000. 2(3): p. 218-22.

224. Tesfay, M.Z., et al., Vesiculovirus neutralization by natural IgM and complement. J Virol, 2014. 88(11): p. 6148-57.

225. Humbert, J.M., et al., Measles virus glycoprotein-pseudotyped lentiviral vectors are highly superior to vesicular stomatitis virus G pseudotypes for genetic modification of monocyte-derived dendritic cells. J Virol, 2012. 86(9): p. 5192-203.

226. Park, F., Correction of bleeding diathesis without liver toxicity using arenaviral-pseudotyped HIV-1-based vectors in hemophilia A mice. Hum Gene Ther, 2003. 14(15): p. 1489-94.

227. Stitz, J., et al., Lentiviral vectors pseudotyped with envelope glycoproteins derived from gibbon ape leukemia virus and murine leukemia virus 10A1. Virology, 2000. 273(1): p. 16-20.

228. Le Blanc, I., et al., Endosome-to-cytosol transport of viral nucleocapsids. Nat Cell Biol, 2005. 7(7): p. 653-64.

229. Cherian, S.S., et al., Whole genomes of Chandipura virus isolates and comparative analysis with other rhabdoviruses. PLoS One, 2012. 7(1): p. e30315.

230. Finkelshtein, D., et al., LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proc Natl Acad Sci U S A, 2013. 110(18): p. 7306-11.

231. Yao, Y., et al., Membrane fusion activity of vesicular stomatitis virus glycoprotein G is induced by low pH but not by heat or denaturant. Virology, 2003. 310(2): p. 319-32.

232. Cartwright, B. and F. Brown, Serological relationships between different strains of vesicular stomatis virus. J Gen Virol, 1972. 16(3): p. 391-8.

233. Brun, G., X. Bao, and L. Prevec, The relationship of Piry virus to other vesiculoviruses: a re-evaluation based on the glycoprotein gene sequence. Intervirology, 1995. 38(5): p. 274-82.

234. Baquero, E., et al., Structure of the low pH conformation of Chandipura virus G reveals important features in the evolution of the vesiculovirus glycoprotein. PLoS Pathog, 2015. 11(3): p. e1004756.

235. Bergold, G.H. and K. Munz, Characterization of Piry virus. Arch Gesamte Virusforsch, 1970. 31(1): p. 152-67.

236. Bell, J.C. and L. Prevec, Proteins of vesicular stomatitis virus. V. Identification of a precursor to the phosphoprotein of Piry virus. J Virol, 1979. 30(1): p. 56-63.

237. Rajasekharan, S., et al., Predicting the host protein interactors of Chandipura virus using a structural similarity-based approach. Pathog Dis, 2013.

238. Dull, T., et al., A third-generation lentivirus vector with a conditional packaging system. J Virol, 1998. 72(11): p. 8463-71.

239. Anderson, J. and R. Akkina, HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. AIDS Res Ther, 2005. 2(1): p. 1.

240. Berges, B.K., et al., Humanized Rag2−/−γc−/− (RAG-hu) mice can sustain long-term chronic HIV-1 infection lasting more than a year. Virology, 2010. 397(1): p. 100-103.

241. Fay, D.S. and K. Gerow, A biologist's guide to statistical thinking and analysis. WormBook, 2013: p. 1-54.

242. Strang, B.L., et al., Characterization of HIV-1 vectors with gammaretrovirus envelope glycoproteins produced from stable packaging cells. Gene Ther, 2004. 11(7): p. 591-8.

243. Guleria, A., et al., Reviewing host proteins of Rhabdoviridae: possible leads for lesser studied viruses. J Biosci, 2011. 36(5): p. 929-37.

244. Zufferey, R., et al., Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol, 1998. 72(12): p. 9873-80.

245. Kolokoltsov, A.A., S.C. Weaver, and R.A. Davey, Efficient functional pseudotyping of oncoretroviral and lentiviral vectors by Venezuelan equine encephalitis virus envelope proteins. J Virol, 2005. 79(2): p. 756-63.

246. Tiscornia, G., O. Singer, and I.M. Verma, Production and purification of lentiviral vectors. Nat Protoc, 2006. 1(1): p. 241-5.

247. Sheu, J., et al., Large-scale production of lentiviral vector in a closed system hollow fiber bioreactor. Mol Ther Methods Clin Dev, 2015. 2: p. 15020.

248. Douglas S. Lyles, I.V.K., and Charles E. Rupprecht, FIELDS VIROLOGY. 6th ed. Rhabdoviridae, ed. P.M.H. David M. Knipe. Vol. LIPPINCOTT WILLIAMS & WILKINS. 2013. 885-922.

249. Dhanda, V., F.M. Rodrigues, and S.N. Ghosh, Isolation of Chandipura virus from sandflies in Aurangabad. Indian J Med Res, 1970. 58(2): p. 179-80.

250. Thomson, J.A., et al., Embryonic stem cell lines derived from human blastocysts. Science, 1998. 282(5391): p. 1145-7.

251. Burtner, C.R., et al., Intravenous injection of a foamy virus vector to correct canine SCID-X1. Blood, 2014. 123(23): p. 3578-84.

252. Seita, J. and I.L. Weissman, Hematopoietic stem cell: self-renewal versus differentiation. Wiley Interdiscip Rev Syst Biol Med, 2010. 2(6): p. 640-53.

253. Coutts, M.C., Human gene therapy. Kennedy Inst Ethics J, 1994. 4(1): p. 63-83.

254. Hammarstrom, L., et al., Pokeweed mitogen induced differentiation of human B cells: evaluation by a protein A haemolytic plaque assay. Immunology, 1979. 38(1): p. 181-9.

255. Hellebrand, E., et al., Epstein-Barr virus vector-mediated gene transfer into human B cells: potential for antitumor vaccination. Gene Ther, 2006. 13(2): p. 150-62.

256. Pan, D., et al., Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of in vivo transduction of bone marrow. Mol Ther, 2002. 6(1): p. 19-29.

257. Koya, R.C., et al., Making dendritic cells from the inside out: lentiviral vector-mediated gene delivery of granulocyte-macrophage colony-stimulating factor and interleukin 4 into CD14+ monocytes generates dendritic cells in vitro. Hum Gene Ther, 2004. 15(8): p. 733-48.

258. Hanawa, H., et al., Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. Mol Ther, 2002. 5(3): p. 242-51.

259. Matrai, J., M.K. Chuah, and T. VandenDriessche, Recent advances in lentiviral vector development and applications. Mol Ther, 2010. 18(3): p. 477-90.

260. Hélio, A.T., et al., Lentiviral Gene Therapy Vectors: Challenges and Future Directions. 2013.

261. Waehler, R., S.J. Russell, and D.T. Curiel, Engineering targeted viral vectors for gene therapy. Nat Rev Genet, 2007. 8(8): p. 573-587.

262. Kim, H.L., A.S. Belldegrun, and R.A. Figlin, Immune gene therapy for kidney cancer: the search for a magic trigger. Mol Ther, 2003. 7(2): p. 153-4.

263. Sangro, B. and J. Prieto, Gene therapy for liver cancer: clinical experience and future prospects. Curr Opin Mol Ther, 2010. 12(5): p. 561-9.

264. Peralta-Zaragoza, O., et al., Targeted treatments for cervical cancer: a review. Onco Targets Ther, 2012. 5: p. 315-28.

265. Kumar, M.S., et al., Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc Natl Acad Sci U S A, 2008. 105(10): p. 3903-8.

266. DuPage, M., A.L. Dooley, and T. Jacks, Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. Nat Protoc, 2009. 4(7): p. 1064-72.

267. Coune, P.G., B.L. Schneider, and P. Aebischer, Parkinson's disease: gene therapies. Cold Spring Harb Perspect Med, 2012. 2(4): p. a009431.

268. Johnson, J.B., G.A. Capraro, and G.D. Parks, Differential mechanisms of complement-mediated neutralization of the closely related paramyxoviruses simian virus 5 and mumps virus. Virology, 2008. 376(1): p. 112-23.

269. Lambris, J.D., D. Ricklin, and B.V. Geisbrecht, Complement evasion by human pathogens. Nat Rev Micro, 2008. 6(2): p. 132-142.

270. Johnson, J.B., K. Grant, and G.D. Parks, The paramyxoviruses simian virus 5 and mumps virus recruit host cell CD46 to evade complement-mediated neutralization. J Virol, 2009. 83(15): p. 7602-11.

271. Hook, L.M., et al., Herpes simplex virus type 1 and 2 glycoprotein C prevents complement-mediated neutralization induced by natural immunoglobulin M antibody. J Virol, 2006. 80(8): p. 4038-46.

272. Croyle, M.A., et al., PEGylation of a vesicular stomatitis virus G pseudotyped lentivirus vector prevents inactivation in serum. J Virol, 2004. 78(2): p. 912-21.

273. Berges, B.K., et al., HIV-1 infection and CD4 T cell depletion in the humanized Rag2-/- gamma c-/- (RAG-hu) mouse model. Retrovirology, 2006. 3: p. 76.

274. Kapur, N.K. and J.J. Rade, Fibroblast growth factor 4 gene therapy for chronic ischemic heart disease. Trends Cardiovasc Med, 2008. 18(4): p. 133-41.

275. Akkina, R., New generation humanized mice for virus research: comparative aspects and future prospects. Virology, 2013. 435(1): p. 14-28.