Vesiculovirus G protein-based stable cell lines for continuous lentiviral vector production

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Thesis submitted for the degree of Doctor of Philosophy

Infection and Immunity University College London

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Declaration

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

Lentiviral vectors (LV) are often pseudotyped with the envelope G protein from vesicular stomatitis virus Indiana strain (VSVind.G). However, VSVind.G based continuous LV producer cell lines have not been reported; it has been assumed that VSVind.G is fusogenic and cytotoxic. To find alternative G proteins for LV production, we investigated other vesiculovirus G proteins (VesG) from VSV New Jersey strain (VSVnj), Cocal virus (COCV), Piry virus (PIRYV), VSV Alagoas virus (VSVala), and Maraba virus (MARAV). All these VesG envelopes were used in transient transfection to produce infectious particles that were robust during concentration and freeze-thawing. We then found, surprisingly, that VSVind.G and all the other VesG proteins could be constitutively expressed in 293T cells, and showed no cytotoxicity when compared to a retroviral Env protein. These VesG expressing cells could support LV production when other components were transiently supplied. However, we showed that VesG expressing cells do not show receptor interference hence LV can superinfect their producer cells, resulting in vector genome accumulation and possible toxicity. We attempted to knock-out the low-density lipoprotein receptor (LDLR) gene on producer cells, which was reported to be the primary cell entry receptor for VSVind.G. However, only a slight reduction in LV transduction was observed in LDLR-KO cells. Hence, other methods such as using anti-retroviral drugs to block superinfection may be necessary to allow construction of stable producer cell lines.
Impact Statement

Lentiviral vectors (LVs) are usually pseudotyped with the G protein of vesicular stomatitis virus Indiana strain (VSVind.G) due to its high physical stability. However, this G protein has been believed to be toxic when expressed constitutively in cells owing to its high fusogenicity. Therefore, well-studied alternative G proteins can provide crucial insights related to their use in clinical grade LV production. In this study, I demonstrate that other tested vesiculovirus G proteins are as stable, both physically and thermally, as the gold standard VSVind.G. Also, I show that all tested G proteins, including VSVind.G, show similar cytotoxicity to that of the negative control. Also, I demonstrate that, against the commonly accepted concept that VSVind.G cannot be expressed in cells continuously, this G protein could be expressed stably in cells for up to five months.

Moreover, these stable VSVind.G expressing cells supported LV production when were supplemented with other vector components transiently in this period. Similarly, other tested G proteins could also be stably expressed in cells for at least five months and generated transient LVs during this time. These G proteins can be employed to generate stable packaging cell lines for lentiviral vector production.
Acknowledgements

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<td>A3G</td>
<td>APOBEC3G</td>
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<td>AA</td>
<td>Amino acid</td>
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<td>AAV</td>
<td>Adeno-associated virus</td>
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<td>ADA-SCID</td>
<td>Adenosine deaminase deficiency</td>
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<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>ALD</td>
<td>Adrenoleukodystrophy</td>
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<td>APC</td>
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<td>APOBEC3</td>
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<td>Arginine</td>
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<td>Aspartate</td>
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<td>BIV</td>
<td>Bovine immunodeficiency virus</td>
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<tr>
<td>BLV</td>
<td>Bovine leukaemia virus</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CA</td>
<td>Capsid</td>
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<td>CAR</td>
<td>Chimeric antigen receptors</td>
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<td>Cas9</td>
<td>CRISPR-associated 9</td>
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<td>Core catalytic domain</td>
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<td>CHR</td>
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<td>cPPT</td>
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<td>DMEM</td>
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<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGFPH</td>
<td>Epidermal growth factor precursor homology</td>
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<td>EIAV</td>
<td>Equine infectious anaemia virus</td>
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<td>Env</td>
<td>Envelope glycoprotein</td>
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<td>ESCRT</td>
<td>Endosomal sorting complexes required for the transport</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FIV</td>
<td>Feline immunodeficiency virus</td>
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<td>FLV</td>
<td>Feline leukaemia virus</td>
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<td>FMDV</td>
<td>Foot-and-mouth disease virus</td>
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<td>FSC</td>
<td>Forward-scattered light</td>
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<td>Glycoprotein</td>
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<tr>
<td>Glu</td>
<td>Glutamate</td>
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<td>GMP</td>
<td>Good Manufacturing Practice</td>
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<td>Guide-RNA</td>
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<td>GRV</td>
<td>Gammaretroviral vector</td>
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<td>GVHD</td>
<td>Graft-versus-host disease</td>
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<td>Homology-directed repair</td>
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<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>HSCT</td>
<td>Haematopoietic stem cell transplantation</td>
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<td>Herpes simplex virus-thymidine kinase</td>
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<td>Common interleukin receptor γ-chain</td>
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<td>Ligand-binding domain</td>
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<td>Low-density lipoprotein receptor</td>
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<td>Lens epithelium-derived growth factor</td>
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<td>LDLR-related protein</td>
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<td>Matrix protein</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MARAV</td>
<td>Maraba virus</td>
</tr>
<tr>
<td>MARAV.G</td>
<td>Maraba virus G protein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>MHR</td>
<td>Middle-heptad repeat</td>
</tr>
<tr>
<td>MLD</td>
<td>Metachromatic leukodystrophy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MLV</td>
<td>Murine leukaemia virus</td>
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<tr>
<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
</tr>
<tr>
<td>MX2</td>
<td>Myxovirus resistance 2</td>
</tr>
<tr>
<td>N</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end-joining</td>
</tr>
<tr>
<td>NHR</td>
<td>N-heptad repeat</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>P</td>
<td>Phosphoprotein</td>
</tr>
<tr>
<td>PAM</td>
<td>Protospacer adjacent motif</td>
</tr>
<tr>
<td>PBS</td>
<td>Primer binding site</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Packaging cell line</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHD</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>PID</td>
<td>Primary immunodeficiency</td>
</tr>
<tr>
<td>PIRYV</td>
<td>Piry virus</td>
</tr>
<tr>
<td>PIRYV.G</td>
<td>Piry virus G protein</td>
</tr>
<tr>
<td>PPT</td>
<td>Polypurine tract</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>P-TEF-b</td>
<td>Positive transcription elongation factor b</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<tr>
<td>RAP</td>
<td>Receptor-associated protein</td>
</tr>
<tr>
<td>RCV</td>
<td>Replication-competent viruses</td>
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<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RMCE</td>
<td>Recombinant mediated cassette exchange</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
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<tr>
<td>RRE</td>
<td>Rev-response element</td>
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<td>RSV</td>
<td>Rous sarcoma virus</td>
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<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
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<tr>
<td>RTC</td>
<td>Reverse transcription complex</td>
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<tr>
<td>RV</td>
<td>Retroviral vector</td>
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<tr>
<td>SAMHD1</td>
<td>Sterile α motif domain – and histidine-aspartate domain-containing protein</td>
</tr>
<tr>
<td>scFv</td>
<td>Single-chain variable fragment</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SERINC</td>
<td>Serine incorporator</td>
</tr>
<tr>
<td>SFFV</td>
<td>Spleen focus-forming virus</td>
</tr>
<tr>
<td>SFV</td>
<td>Simian foamy virus</td>
</tr>
<tr>
<td>Sh ble</td>
<td>Streptococcicidium hindustanus phleomycin inhibitor</td>
</tr>
<tr>
<td>SIN</td>
<td>Self-inactivating</td>
</tr>
<tr>
<td>6HB</td>
<td>Six-helical bundle</td>
</tr>
<tr>
<td>SIR</td>
<td>Superinfection resistance</td>
</tr>
<tr>
<td>sLDLR</td>
<td>Soluble low-density lipoprotein receptor</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scattered light</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>SU</td>
<td>Surface</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour-associated antigens</td>
</tr>
<tr>
<td>TALENs</td>
<td>Transcription activator-like effectors nucleases</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
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<tr>
<td>TALES</td>
<td>Transcription activator-like effectors</td>
</tr>
<tr>
<td>TAR</td>
<td>Transactivation response</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator protein</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline-responsive promoter element</td>
</tr>
<tr>
<td>TRIM</td>
<td>Tripartite motif</td>
</tr>
<tr>
<td>tRNA&lt;sup&gt;Ly3&lt;/sup&gt;</td>
<td>Transfer RNA Lys3</td>
</tr>
<tr>
<td>TU</td>
<td>Transducing unit</td>
</tr>
<tr>
<td>U5</td>
<td>Unique 5</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VLDDL</td>
<td>Very low-density lipoprotein receptor</td>
</tr>
<tr>
<td>VesG</td>
<td>Vesiculovirus G protein</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<tr>
<td>VSVa</td>
<td>Vesicular stomatitis virus Alagoas virus</td>
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<td>VSVa.G</td>
<td>Vesicular stomatitis Alagoas virus G protein</td>
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<tr>
<td>VSVind</td>
<td>Vesicular stomatitis virus Indiana strain</td>
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<tr>
<td>VSVind.G</td>
<td>Vesicular stomatitis virus Indiana G protein</td>
</tr>
<tr>
<td>VSvn</td>
<td>Vesicular stomatitis virus New Jersey strain</td>
</tr>
<tr>
<td>VSvn.G</td>
<td>Vesicular stomatitis virus New Jersey G protein</td>
</tr>
<tr>
<td>WAS</td>
<td>Wiskott-Aldrich Syndrome</td>
</tr>
<tr>
<td>WASP</td>
<td>WAS protein</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>WP</td>
<td>WinPac</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>X-CGD</td>
<td>X-linked Chronic Granulomatous Disorder</td>
</tr>
<tr>
<td>X-SCID</td>
<td>X-linked severe combined immunodeficiency</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc finger nucleases</td>
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</tbody>
</table>
Introduction
1.1 Gene therapy- From concept to reality

Gene therapy has the potential to treat a disease at its genetic roots, in both hereditary and acquired conditions [7]. Gene therapy can be simply defined as a treatment that involves the introduction of a new gene into a patient's cells in an attempt to cure a disease or improve a condition. Recent advances in molecular biology and the sequencing of the human genome helped to transform gene therapy from a revolutionary concept to an important tool in the treatment of genetic diseases [8].

In the 20th century, it was already clear that some conditions are passed on from parents to offspring [9]. The idea of using viruses to deliver genes was first started in the mid-1970s when Varmus and Bishop reported that gamma-retrovirus could obtain cellular genes naturally [10, 11]. In the late 1970s, recombinants of both non-integrating viruses (e.g. adenovirus, bovine papilloma, vaccinia and herpes simplex viruses) and integrating retroviruses were being studied by several laboratories [12].

Although gene therapy was still controversial, by 1980s retrovirus-based gene transfer to mammalian cells was routinely performed [13-16]. In the early 1990s, the first approved clinical trial was performed in patients with severe combined immunodeficiency (SCID) using gammaretrovirus based vectors. Temporary improvement of the immune system was observed in the patients, and although they still required enzyme replacement therapy, this can be considered as the first successful human gene therapy trial [16-18].

In the mid-1990s, long-term expression of vectors based on adeno-associated virus (AAV) was demonstrated in mice which led to the first AAV gene therapy
clinical trial for haemophilia B in the late 1990s [19, 20]. While these early trials were relatively safe, AAVs were cleared as a result of pre-existing neutralising antibodies in the body [21].

In early 2000, Cavazzana-Calvo and colleagues introduced the common interleukin receptor γ-chain (IL2RG) in the bone marrow of patients with X-linked severe combined immunodeficiency (X-SCID) using a murine leukaemia virus (MLV)-based vector. Although this therapy restored the immune system function, several cases of leukaemia (including the death of one patient) that were induced due to the insertional mutagenesis caused by the vectors were reported [22]. Similar results were observed by Thrasher group (University College London, UK) in 2004 [23].

The first effective gene therapy trial for cancer treatment was reported in 2006. In this trial, the T cells of the patients were engineered using a retroviral vector to express TCR against a melanoma antigen ex vivo. Cells were then re-administrated into the patients. This treatment resulted in regression in 2 out of fifteen patients [24].

Currently, many human gene therapy clinical trials have proved to be effective. All the scientific efforts in the past decades, both successful and unsuccessful, helped to improve gene therapy through the development of better vectors regarding safety, and efficacy.

### 1.2 Vectors used for gene delivery

Vectors are vehicles that are used to transfer genetic materials into target cells and tissues. The ideal vector for gene delivery should be specific to target cells
efficiently, stable (e.g. thermostable at body temperature), safe, and to have high and long-term expression levels for as long as required.

Naked DNA injection into tissues locally or into systemic circulation might seem to be the simplest way of gene delivery to the body, however, injected naked DNA can be rapidly degraded by nucleases or cleared by the immune system, which can limit the efficiency of gene transfer into target cells [25, 26]. Therefore, other methods were developed to increase the efficiency of gene delivery. These methods include viral and non-viral based gene delivery systems. Viral vectors are effective tools for gene transfer. However, there are limitations associated with their use including immunogenicity [27, 28], carcinogenesis [29], difficulty in production and scaling up [30, 31], and limited capacity for the transgene [32]. Non-viral delivery systems were developed as an alternative to virus-based delivery systems owing to several advantages such as lower immunogenicity and toxicity, larger carrying capacity, and simpler production and scale-up [33]. Non-viral methods are used for gene delivery in around 20% of all current clinical trials [34, 35] and are categorised into two types, physical and chemical. In physical methods, physical force is used to permeabilise the cellular membrane. The most common physical methods are microinjection, electroporation, ultrasound, and gene gun. Chemical methods, on the other hand, use carriers such as liposomes, cationic lipids, and polymers to deliver genetic material into target cells [36-38]. Nevertheless, despite all the advances so far, the ideal vector has yet to be constructed.

On the other hand, viruses have evolved naturally to transfer their nucleic acid into target cells for replication. This feature makes viruses to be attractive as
gene delivery vehicles. Over the years they have been engineered to make suitable vehicles for gene transfer into humans. Specific viruses have been chosen based on their characteristics to develop viral vector systems. DNA viruses such as adenoviruses, adeno-associated viruses, and herpesviruses have been widely used in clinical trials [39].

Amongst RNA viruses, retroviral vectors are the most commonly used due to their high gene transfer efficiency and expression of therapeutic genes. These vectors have a relatively large carrying capacity of ~9kb, can integrate into the host cell genome, and therefore, can induce long-term expression of the transgene in target cells [40]. Over the past decades, a number of patients with various immunodeficiencies have been treated using MLV-based gammaretroviral vectors to transduce autologous haematopoietic cells with the desired transgene [41-43] (Table 1-2). In recent years, lentiviral vectors that are derived from human immunodeficiency virus type 1 (HIV-1) have been utilised due to their unique ability to infect both dividing and non-dividing cells [44-46] (Table 1-3). This is a desirable characteristic in gene therapy applications that slowly-dividing cells such as haematopoietic stem cells or T lymphocytes are the gene delivery target. The focus of this thesis will be on HIV-1- based lentiviral vectors.

1.3 Retroviruses

The Rous sarcoma virus (RSV) was the first retrovirus to be discovered when it was isolated from tumours in chickens [47]. Other retroviruses then were isolated from a range of animals [48-50]. The first human retrovirus was isolated in the 1980s in human T-cell leukaemia virus type 1 (HTLV-1) affected
patients [51]. Shortly after, the human immunodeficiency virus (HIV) which is now known as the cause of acquired immune deficiency syndrome (AIDS), was discovered in 1984 [52, 53].

Retroviruses belong to the family of Retroviridae which consists of a large number of enveloped RNA viruses. This family is divided into two main sub-families; Orthoretrovirinae and Spumaretrovirinae. Orthoretrovirinae is further divided into two groups: simple and complex viruses. Simple viruses are alpharetroviruses, betaretroviruses, and gammaretroviruses; complex viruses are deltaretroviruses, epsilonretroviruses, and lentiviruses. Spumaretrovirinae sub-family has one known genus, spumavirus (Table 1-1).

The RNA genome of the Retroviridae family is positive sense, linear, single-stranded, and 7-12 kb in size. The replication method of retroviruses is considered the hallmark of this family as they employ a reverse transcriptase enzyme, encoded by the viral pol gene, to transcribe the viral single-stranded RNA (ssRNA) into linear double-stranded DNA (dsDNA) which after entering the nucleus integrates into the target cell genome [54, 55]. Retroviruses are surrounded by an envelope consisting of a host cell-derived lipid bilayer and virus-encoded envelope glycoprotein.

The genome of both simple and complex retroviruses contain three main coding domains: gag, which codes for the viral structure including capsid (CA), matrix (MA) and nucleocapsid (NC) proteins; pol, that codes for the viral enzymes necessary for replication such as reverse transcriptase (RT), protease (PR), and integrase (IN); and env, that codes for the viral envelope glycoprotein that consists of the transmembrane (TM) and surface (SU)
subunits [56]. Complex retroviruses such as lentiviruses contain additional open reading frames that code for regulatory genes, for example the regulatory genes tat and rev in HIV-1, which are essential for the viral genome expression and nuclear export, respectively, in addition to a set of accessory genes (e.g. HIV-1 vpu, vif, vpr and nef) that are essential for viral pathogenicity. The retrovirus genome has the long terminal repeat (LTR) at both 5’ and 3’ ends. Sequences within the viral LTR contain cis-acting regulatory sequences that regulate viral gene expression and genome replication [55].

The RNA genome of HIV contains R and U5 sequences at the 5’ end, followed by a packaging signal (ψ) which is responsible for incorporating the RNA genome into viral particles [57]. Another R sequence is repeated at the 3’ end of the genome following U3. The R sequences on both ends of the viral genome have a pivotal role in reverse transcription (Figure 1-1A). What makes retroviruses unique is the reverse transcription and integration into the cell genome, which makes the retroviral infection permanent [54] as it is inherited by daughter cells.

The attachment of retroviral surface glycoprotein to specific receptors on target cells induces conformational changes in the glycoprotein exposing the fusion domain which leads to the fusion of the viral membrane to that of the target cell. This membrane fusion results in the release of the viral core, including the RNA genome, into the cell’s cytoplasm. Reverse transcriptase then transcribes this RNA into DNA that enters the nucleus and integrates into the host cell genome via the viral integrase [54, 58]. With integration, the viral genome becomes part of the cellular genome and can employ the cellular systems such as RNA polymerase II and ribosomes to express the viral genes.
In simple retroviruses, from the integrated provirus two different mRNAs are synthesised; spliced mRNAs that encode the viral proteins, and un-spliced mRNAs that are destined for incorporation into progeny virions, and for gag/pol expression. After viral protein synthesis from the mRNAs by the cellular machinery in the cytoplasm, in most retroviruses, the viral proteins are transferred to the plasma membrane where viral particle assembly takes place. The envelope is acquired during the budding process where the viruses are released from the plasma membrane.

As retroviruses efficiently deliver their genome into the host cell, they have been converted to vehicles for gene delivery. In addition to the ability to integrate into the target cell genome, the simple genetic organisation is the primary factor in choosing gammaretroviral vectors (GRVs) for delivering genetic materials [54]. Most gammaretroviral vectors have been developed based on MLV (murine leukaemia virus) for gene transfer applications where they have been used widely over the past decades [43, 56]. However, there are some adverse effects caused by gammaretroviral vector integration into the host cell genome; the integrase and LTRs of these vectors seem to promote preferential interaction with active host cell promoter and enhancer regions that are enriched for transcription factor binding sites [59]. Therefore, transcriptional activation of nearby proto-oncogenes by the vector has been occurring in some clinical trials (e.g. X-SCID) leading to myelodysplasia or lymphoid leukaemia [41, 60].

Moreover, GRVs enter the nucleus only when the cells are undergoing mitosis while the nuclear membrane degenerates. Therefore, they can only infect dividing cells [61, 62]. This can be a major limitation when the target cells are
non-dividing and/or slow-dividing cells such as haematopoietic stem cells (targeted in immunodeficiency disorders and hereditary anaemia clinical trials), quiescent lymphocytes (targeted in cancer clinical trials), hepatocytes, neurons, and muscle fibres.

On the other hand, as lentiviruses use nuclear pores to enter the nucleus, they can infect both dividing and non-dividing cells [63]. This unique characteristic makes lentiviruses to be even more attractive to be used as viral vectors. Additionally, these vectors have been developed without the viral LTR enhancer elements (SIN or self-inactivating vectors) to reduce the chances of transcriptional activation of nearby proto-oncogenes. Furthermore, the overall insertion site of lentiviral vectors (LVs) seems to be different from that of gamma-retroviral vectors, possibly due to the cellular factors associated with IN or the alterations of the LTR in LVs as well as the differences between the integrases of these two vectors [41, 59, 64]. Owing to these advantages, more studies have been conducted in an attempt to develop safer and more efficient HIV-1 derived vectors over the past few years.
<table>
<thead>
<tr>
<th>Family</th>
<th>Sub-family</th>
<th>Class</th>
<th>Genus</th>
<th>Prototype virus</th>
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</thead>
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<tr>
<td>Retroviridae</td>
<td>Orthoretrovirinae</td>
<td>Simple</td>
<td>Alpharetrovirus</td>
<td>RSV</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Betaretrovirus</td>
<td>Mouse mammary tumour virus</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Gammaretrovirus</td>
<td>MLV, FLV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complex</td>
<td>Deltaretrovirus</td>
<td>BLV, HTLV</td>
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<td></td>
<td></td>
<td>Epsilonretrovirus</td>
<td>Walleye dermal sarcoma virus</td>
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<td>Lentivirus</td>
<td>HIV, BIV, FIV</td>
</tr>
<tr>
<td></td>
<td>Spumaretrovirinae</td>
<td></td>
<td>Spumavirus</td>
<td>HFV, SFV</td>
</tr>
</tbody>
</table>

Table 1-1. Retroviridae family classification.  
BIV: Bovine immunodeficiency virus; BLV: Bovine leukaemia virus; FIV: Feline immunodeficiency virus; FLV: Feline leukaemia virus; HFV: Human foamy virus; HIV: Human immunodeficiency virus; HTLV: Human T-lymphotropic virus; MLV: Murine leukaemia virus; RSV: Rous sarcoma virus; SFV: Simian foamy virus.
1.3.1 Gamma-retroviral vectors in clinical trials

Stable integration into the host cell genome, ease of manipulation and production of therapeutic-containing vectors are some of the reasons behind retroviral vectors (RVs) becoming the most frequently used vectors for gene therapy applications [65]. Haematopoietic stem cells (HSCs) (CD34+ cells), usually autologous, are obtained from the patient, expanded and then transduced with therapeutic-containing RVs ex vivo before administration back into the patient. Allogeneic haematopoietic stem cell transplantation (HSCT) is considered to be the gold standard for treating primary immunodeficiencies (PID) [66-68]. This method not only minimises the risk of immunologic complications including graft-versus-host disease (GVHD) and graft rejection but also is more accessible to patients especially those without HLA-matched donors [69]. MLV-based gammaretroviral vectors (GRVs) have been used for the treatment of some PIDs including X-linked severe combined Immunodeficiency (SCID-X1), adenosine deaminase deficiency (ADA-SCID), X-linked Chronic Granulomatous Disorder (X-CGD), and Wiskott - Aldrich syndrome (WAS). Although some of these trials resulted in clinical benefits comparable to that of the allogeneic HSCT [67, 70, 71], the occurrence of insertional mutagenesis (IM) caused by GRV integration was one of the complications reported in these clinical trials. Consequently, studies have been done to improve all aspects of ex vivo transduction to minimise the risk of IM as well as to optimise HSC culture and transduction conditions [60, 72]. Furthermore, to eliminate the off-target effects and to guarantee adequate transgene expression, the use of specific promoters have been studied in the past few years [73].
Third generation self-inactivating (SIN) LVs have been developed in an attempt to eliminate the risk of IM caused by GRVs [69]. These SIN LVs have shown encouraging results in recent trials including adrenoleukodystrophy (ALD) [74], metachromatic leukodystrophy (MLD) [75], β-thalassemia [76], and WAS [77, 78].

Some selected gamma-retroviral vector-mediated gene therapy clinical trials are listed in Table 1-2.
<table>
<thead>
<tr>
<th>Target cells</th>
<th>Disease group</th>
<th>Disease/Transgene</th>
<th>Env</th>
<th>Production method</th>
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<td>PIDs ADA-SCID</td>
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<td>MLV</td>
<td>Gp+Am12</td>
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<tr>
<td></td>
<td></td>
<td>ADA</td>
<td>GALV</td>
<td>PG13</td>
<td>[67]</td>
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<td></td>
<td>IL2RG</td>
<td>GALV</td>
<td>PG13</td>
<td>[80]</td>
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<tr>
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<td></td>
<td>X-CGD gp91(phox)</td>
<td>MLV</td>
<td>ψCRIP</td>
<td>[68]</td>
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<td>PG13</td>
<td>[81]</td>
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<td>GALV</td>
<td>PG13</td>
<td>[82]</td>
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<td>WAS WASP</td>
<td>GALV</td>
<td>PG13</td>
<td>[83]</td>
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<td>T cells</td>
<td>Cancer Neuroblastoma</td>
<td>GALV</td>
<td>PG13</td>
<td>[88, 89]</td>
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<td>Lymphoma/Leukaemia</td>
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<td>NS</td>
<td>[90, 91]</td>
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<td></td>
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<td>[92]</td>
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<td>NS</td>
<td>NS</td>
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<td>PG13</td>
<td>[94]</td>
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<td>GALV</td>
<td>PG13</td>
<td>[24]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synovial sarcoma</td>
<td>GALV</td>
<td>PG13</td>
<td>[97]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectal cancer</td>
<td>RD114</td>
<td>Transient</td>
<td>[98]</td>
<td></td>
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</table>
1.4 Lentiviruses

Lentiviruses are a subclass of retroviruses (Table 1-1). They consist of two copies of positive-sense single-stranded RNA genome. They are characterised by a long incubation period (Lente means slow in Latin) in which the virus pathogenicity is at a low level. Lentiviruses include a number of viruses such as human immunodeficiency virus-1 (HIV-1), HIV-2 and simian immunodeficiency virus (SIV). Amongst these viruses, HIV-1, which causes acquired immunodeficiency syndrome or AIDS in humans, has been the interest of many researchers.

1.4.1 HIV-1 life cycle

HIV-1 wild-type genome contains nine open reading frames: \textit{gag}, \textit{pol}, \textit{env}, \textit{rev}, \textit{tat}, \textit{vpr}, \textit{vpu}, \textit{vif}, and \textit{nef} (Figure 1-3A). The HIV-1 replication cycle [99] includes two stages: early and late. Early phase begins with viral attachment/entry to the cell and ends in proviral integration. The late phase includes viral gene expression, viral particle assembly, budding, release, and maturation [58].
1.4.1.1 Cell attachment and viral entry

Viral entry to the target cell is the first stage of the life cycle. This stage requires the attachment of the virus Env to the host cell receptor followed by the fusion of virus and host cell membranes [100, 101].

Following the initial attachment to the cell surface, the envelope engages with the specific receptors to enter the cells. The CD4 membrane glycoprotein and a G-protein-coupled chemokine receptor act as cellular receptor and co-receptor for HIV entry. The chemokine receptors include cysteine-cysteine chemokine receptor 5 (CCR5) [102] and chemokine (C-X-C motif) receptor 4 (CXCR4) (Figure 1-2, step 1) [103]. CD4 is mainly expressed on helper T cells (Th) and engages with the antigens presented by the major histocompatibility complex class II (MHC II) inducing immune response against the specific antigen [104].

The envelope glycoprotein of HIV is a homotrimer of non-covalently linked heterodimers composed of a receptor-binding surface unit (gp120) and a fusogenic transmembrane unit (gp41). In the early stage of replication cycle, attachment of gp120 to CD4 receptor leads to conformational changes in gp120 and gp41 subunits. Conformational changes in the gp120 lead to the attachment of this protein to a co-receptor, CCR5 or CXCR4 [105]. Conformational rearrangement in gp41 occurs due to folding at the hinge regions of the trimer resulting in an amino-terminal helical region (HR-N) and a carboxy-terminal helical region (HR-C) to form a six-helical bundle (6HB). This results in the exposure of the hydrophobic fusion peptide leading to its insertion into the target cell membrane (Figure 1-2, step 2) [106-108]. The HIV attachment and entry into the cells can happen through pH-independent
endosomal uptake [109]. Next, the virion core is released into the cytoplasm. This core, which is now called the reverse transcription complex (RTC), in simple viruses such as Molony MLV consists of the viral genome, RT, IN, and CA, however, the HIV RTC contains Nef and Vpr in addition [110].

1.4.1.2 Reverse transcription

Before 1970, the accepted concept of the information flow or the ‘central dogma’ described that the information in genes flows from DNA to protein through RNA; DNA is required for RNA transcription and RNA for protein synthesis. In 1970 reverse transcription was first reported by two independent groups [40, 111] which changed the concept of information flow in molecular biology.

Retroviruses package two copies of ssRNA. These two copies are used in reverse transcription to make the viral dsDNA (Figure 1-2, step 3). In addition, the HIV RT generates the LTRs on both ends of the DNA genome which will aid in the integration process [112].

To initiate reverse transcription [110], a transfer RNA Lys3 (tRNA\textsuperscript{Lys3}) binds to the primer binding site (PBS) of the HIV genome which is located downstream of the unique 5 (U5) in RNA [113, 114]. This binding mediates the synthesis of the minus cDNA including the U5 and R of the 5’ end which is termed as strong stop cDNA (Figure 1-1A). RT then degrades the RNA associated with the newly synthesised cDNA via its RNase H function (Figure 1-1B) [115]. As the R sequence is present in both 5’ and 3’ ends of the genome, the strong stop cDNA jumps to the 3’ end to bind the R sequence. This jump is known as the first strand transfer which leads to the minus strand cDNA extension to the 5’
end of the RNA genome (Figure 1-1C). Apart from the 3' polypurine tract (PPT) and the central PPT (cPPT) which are resistant to RNase H degradation, the rest of the template RNA is digested by the RNase H function of RT (Figure 1-1D). The purine-rich sequences then act as primers to extend the complementary strand, or the positive strand, of DNA (Figure 1-1E). Having multiple transcription initiation sites results in the faster synthesis of the positive strand DNA compared to that of the minus strand [116]. The second strand transfer occurs as the complementary PBS on both strands bind together which results in the development of a circular DNA (Figure 1-1F/G). Strand displacement synthesis follows which results in copying the LTR sequence on both ends of the viral DNA which produces a longer DNA genome compared to the viral RNA [117]. By completing the reverse transcription and DNA synthesis, RTC develops into the pre-integration complex (PIC) [118].

RT is a complicated process with two template exchange. Moreover, RT is error-prone (approximately one substitution per 10,000 bases) as it lacks proofreading activity [119, 120]. These factors contribute to recombination and high diversity of the HIV genome which contributes to the challenges of vaccine development against this virus.
Figure 1-1. Steps of Reverse Transcription. Schematic of HIV-1 reverse transcription illustrating the conversion of ssRNA viral genome into double stranded DNA. RNA is shown in black and DNA in grey. (A) Initiation of reverse transcription by RT following tRNA binding with the primer binding site downstream of U5. (B) Synthesis of the minus-strand starts from the U5 and R sequences in the 5’ end of the genome. The RNase H activity of RT degrades the viral RNA in the generated RNA-DNA complex (dotted line). (C) The first strand transfer: the minus strand is transferred to the identical R sequences at the 3’. (D) cDNA extension while viral RNA gets degraded except for RNase H resistant cPPT and PPT sequences. (E) cPPT and PPT sequences act as primers for the plus strand DNA synthesis. (F) The second strand transfer: the tRNA primer is cleaved by RNase H domain and plus strand DNA elongated from the PPT sequence is transferred to the 3’ side of the minus strand DNA. DNA synthesis of the plus strand is resumed from the cPPT. (G) Following elongation of both strands of DNA, a double stranded provirus is produced with a central DNA flap caused by the cPPT. Adapted from [6].
1.4.1.3 Viral uncoating

HIV capsid encloses the viral RNA genome, viral proteins such as CA, NC, RT, IN, and Vpr. RT are present within the capsid [121]. The shape and stability of the capsid have a direct effect on RT efficiency and hence viral infectivity [122]. The loss of capsid prior to entry of the viral genome to the cell nucleus is known as uncoating [123, 124]. This process is essential for viral genome entry to the nucleus as the diameters of the nuclear pore is smaller than that of the viral capsid.

Although uncoating is known to take place during the transition between RTC and pre-integration complex (PIC) [118], the exact time and location of uncoating are yet to be elucidated. A study demonstrated that some CA stays in association with the PIC in the nucleus [125]. In a different study, complete cores were observed at the nuclear pore complex (NPC) suggesting that uncoating occurs when RTC arrives at the NPC. This might be the viral strategy to protect its genome against the host immune system [126, 127].

1.4.1.4 Nuclear transport of the viral genome

While GRVs require nuclear membrane breakdown during mitosis in order to enter the nucleus (hence infecting only dividing cells) [61], HIV PIC contains MA, IN, and Vpr in association with the viral genome [128] allowing the complex to access the host cell chromatin in non-dividing cells (Figure 1-2, step 4) [129].

NPCs are vessels cross the nuclear membrane that allows selected molecules and proteins such as ribonucleoproteins to pass through to or from the nucleus [130]. While molecules smaller than 9 nm in diameters can pass through NPCs
through diffusion, as HIV PIC is considerably larger, other mechanisms of nuclear transport are in place [131].

One mechanism is by the interaction of the remaining capsid in the PIC with some nuclear transport factors (e.g. NUP358, NUP153) in the NPCs [132]. The other mechanism of PIC nuclear transport is mediated via energy-dependent transport machinery through the interaction of the IN of the PIC with members of an importin-α protein family that exist in the NPCs [133].

1.4.1.5 Integration of viral DNA into the host genome

Upon entering the nucleus, the proviral DNA integrates into the host cell genome to maintain stable infection (Figure 1-2, step 5). This integration is mediated by the viral IN [134, 135]. The retroviral IN structure was analysed through the crystal structure of prototype foamy virus IN revealing that IN exists in dimers in the integration complex. While each monomer plays a distinct role in this complex, contact of the viral LTR is allowed via an extended conformation of the inner monomer. In HIV, IN was shown to have a few distinct structural and functional domains: The N-terminal domain (NTD), the N-terminal extension domain, the core catalytic domain (CCD), and the C-terminal domain (CTD) [136].

Structural analysis of integration intermediates revealed three steps in the integration process. The first step takes place prior to PIC entering the nucleus where two nucleotides are removed from both ends of the viral dsDNA (from the U3 of the upstream 5’ LTR and the U5 of the downstream of 3’ LTR). This process is known as 3’ end processing [137]. Upon nuclear entry of the PIC, the exposed oxygen of the hydroxyl group on the processed 3’ ends of each
strand attack and join phosphodiester bonds on the host cell DNA strand. This step is known as the strand transfer during which a few nucleotides (four for MLV, five for HIV) on each side of the provirus remain single-stranded. In the final step of integration which is known as the gap repair process, the DNA repair system of the host cell fills in the gaps flanking the integrated viral genome [138, 139].

Lentiviral integration in host cells depends upon the interaction between the viral IN and the host cell protein lens epithelium-derived growth factor (LEDGF/p75). LEDGF/p75 protein contains a nuclear localisation signal and chromatin binding elements on its N-terminal region while the C-terminal region attaches to the IN via the integrase-binding domain (IBD) [140]. It has been shown that the absence of LEDGF/p75 protein affects HIV integration [141] and reduces viral infectivity [142, 143].

While the elucidation of the complete human genome helped with identifying retroviral integration sites [144, 145], advancement in next-generation sequencing enhanced the quantitative analysis of larger number of integration sites [135]. Various studies revealed that retroviral integration is not random [146]. For instance, at the chromosomal level, while MLV preferentially integrates near transcription start sites, HIV favours integrating within active transcriptional units [147, 148], which may promote efficient viral gene expression after integration. Accordingly, HIV-based vectors are considered safer vectors compared to MLV-based ones. Other retroviruses show different specificity to integrate into the host cell genome. For instance, avian sarcoma-leukosis virus (ASLV) is less likely to integrate into gene-dense regions [148],
and mouse mammary tumour virus (MMTV) has shown the most random integration profile so far [149].

1.4.1.6 Viral gene expression

After integration, the late phase of HIV infection occurs in which the viral genome is transcribed. This process is controlled by the DNA regulatory elements in the viral LTR that employ the cellular RNA polymerase II complex. One of the first products to be transcribed is the transactivator protein (Tat). This protein then binds to the transactivation response (TAR) element at the 5′ LTR on the HIV genome to stimulate transcription initiation of the viral genome. This mediates the recruitment of the cellular positive transcription elongation factor b (P-TEF-b) which includes proteins that are necessary for elongation (e.g. Cyclin-dependent kinases). This recruitment results in increased affinity of Tat protein to TAR which triggers hyper-phosphorylation of the C-terminal domain of RNA polymerase II stimulating efficient transcriptional elongation [150].

1.4.1.7 Viral RNA export from the nucleus

In early stages of transcription, while unspliced transcripts are retained in the nucleus, fully spliced mRNAs coding for Tat, Rev, and Nef are exported to the cytoplasm via the nuclear export factor NXF1 (Figure 1-2, step 6) [151]. The nuclear export of unspliced transcripts in HIV is controlled by Rev. Rev is imported to the nucleus via cellular importin β [152] where it binds to its associate cis-acting element the Rev-response element (RRE) within env on the mRNA transcripts [153]. This complex then interacts with cellular export proteins and GTP-bound form of the Ran GTPase to promote nuclear export of viral transcripts [154, 155].
1.4.1.8 Viral assembly, budding, and release

Gag which is the main viral structural protein is synthesised in the cytoplasm from the full-length RNA as a polyprotein precursor, containing the domains for MA, CA, NC, p6, and two spacer peptides. This RNA then is translated into Gag and GagPol precursors. Gag-Pol which codes for the viral enzymes as well as the Gag proteins is synthesised by programmed ribosomal frameshift and its expressed level is at approximately 5% of that of Gag (Figure 1-2, step 7) [156].

After transport to the cytoplasm, HIV RNAs are either translated into viral proteins or packaged into the newly assembled particles [157]. Two copies of RNA are packaged into each virion. This allows the recombination during reverse transcription. Moreover, this might help the virus to carry on with reverse transcription even if one RNA copy is functional [157, 158]. After the RNA is dimerised, it is encapsidated by NC of Gag via the packaging signal (ψ) [159]. This complex then is anchored to the plasma membrane via MA. It has been shown that defective MA results in mistargeting the complex to the late endosomes [160].

While Gag and Pol proteins are translated in the cytoplasm then transported to the cell membrane, Env is trafficked via the secretory pathway in the rough endoplasmic reticulum (RER) where it is glycosylated and trimerised. The cellular protease furin then cleaves Env into the surface unit (SU; gp120) and transmembrane unit (TM; gp41) and is delivered to the cell membrane via vesicular transport [161]. These glycoproteins are then transported to the cell membrane where the assembly of the virions takes place. Gag and Env are localised to common sites, the inner leaflet of the plasma membrane where
HIV-1 particles assemble. Env is recruited by Gag, either directly or via a cellular bridging protein, to be incorporated to the viral particle [162]. The N-terminal of Gag is known as MA, which directly binds to Env. The central domain of Gag is known as CA which creates the shell that encapsidates the viral core. Mutations in Gag’s MA has been reported to block Env incorporation highlighting the role of Env-MA interaction in viral assembly [163]. As the virions bud out, they acquire the lipid bilayer from the plasma membrane and the Env spikes.

Progeny viruses are released from the infected cells through membrane splitting. This process is mediated by the cellular endosomal sorting complexes required for the transport (ESCRT) pathway which is involved in intracellular membrane fission processes required for particle release such as cytokinesis and budding of enveloped viruses away from the membrane [164]. This process is recruited by Gag after which non-infectious immature viral particles are released from the cells (Figure 1-2, step 8).

**1.4.1.9 Viral maturation**

For the viral particles to be infectious, they need to go through maturation (Figure 1-2, step 9). This requires the protease protein which is encoded by the Gag-Pol precursor. As soon as the viral particles are released from the cells, to trigger maturation, Gag and Gag-Pol polyproteins are cleaved into MA, CA, NC, and p6 proteins by protease encoded by pol [165, 166].
1.4.2 Restriction factors influencing HIV-1 infection

Like other viruses, HIV-1 utilises various host cellular proteins for its replication in the host cells. However, some cellular proteins, known as restriction factors, have evolved as part of the host defence mechanism to block the HIV-1 lifecycle [167]. In turn, HIV-1 has evolved to evade the anti-viral defence mechanism, via accessory proteins such as Vif and Vpu, in order to infect and replicate in human cells [168]. As these factors tend to be species-specific, the anti-viral activity of these proteins is considered as a barrier preventing cross-species transmission [168].

Some factors such as Fv4 in mice can prevent viral infection by blocking cell entry while some other proteins act as post-entry restriction factors. Fv4 is a
mutant form of retroviral provirus that expresses a mutant env. This Env prevents the spleen focus-forming virus (SFFV) from entering the cell by interacting with the viral receptor on the cell surface [169, 170]. This process is known as receptor interference.

1.4.2.1 APOBEC3

Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like-3 (APOBEC3) is a family of cytidine deaminases that includes APOBEC3B, APOBEC3DE, APOBEC3F, APOBEC3G (A3G), and APOBEC3H proteins that inhibit HIV-1 replication [171, 172]. A3G is one of the most studied HIV-1 restriction factors and has been shown to be the most potent anti-HIV-1 in this family [167, 173]. During reverse transcription, this protein causes deamination of the DNA minus strand resulting in multiple hypermutations in the plus strand. This leads to the production of abnormal viral transcripts and thus degradation [167]. In the presence of Vif however, this protein prevents A3G to be packaged in the viral particle to retain viral infectivity [174].

1.4.2.2 TRIM5α

TRIM5α belongs to the family of tripartite motif (TRIM)-containing protein [175]. This protein has been shown to bind to the viral capsid. It has been proposed that the interaction with the capsid can either mediate degradation or can induce an immune reaction against the virus. Either way, this interaction with the capsid is suggested to inhibit or block life-cycle before reverse transcription [176-179].

1.4.2.3 SAMHD1
The sterile α motif domain – and histidine-aspartate domain-containing protein 1 (SAMHD1) is found in myeloid cells and resting T cells [180-182]. It was believed that this protein functions as an anti-HIV-1 restriction factor by reducing the nucleotide pool and thus inhibiting reverse transcription [183]. Yet, recently it was reported that this protein degrades the viral RNA via its RNase activity [184]. Nevertheless, HIV accessory protein Vpx inhibits SAMHD1 anti-viral activity [180, 181].

1.4.2.4 MX2

Myxovirus resistance 2 protein (MX2) is a restriction factor that is induced by IFN. This protein inhibits nuclear transport of the viral cDNA or prevents its integration into the genome through interaction with the viral capsid [185, 186]. HIV-1 however, averts MX2 anti-viral activity by reducing cellular cyclophilin A (CypA) which has a role in stabilising the HIV-1 capsid and hence facilitating viral infection [186, 187].

1.4.2.5 Tetherin

Tetherin which is also known as BST2 or CD317 is an IFN-inducible protein that is expressed on the cell surface of different cell types including myeloid and lymphoid cells [188]. It functions as an anti-HIV-1 restriction factor by blocking viral release from the infected cells [189]. HIV-1 accessory gene vpu (or nef in lentiviruses that do not express vpu) has been reported to antagonise tetherin's anti-viral function [190].

1.4.2.6 SERINC

Serine incorporator (SERINC) family consists of multi-pass membrane transporters [191]. While there is still no evidence of direct interaction between
SERINC5 and viral envelope, it has been recently reported that SERINC5 might destabilise the trimer conformation of the envelope, therefore, blocking fusion [167]. The absence of Nef antagonises the activity of SERINC5 by mediating its relocalisation from the plasma membrane to the reticulum compartment [192, 193].

1.4.3 Development of HIV-1 derived lentiviral vectors

Lentiviral vectors have been developed from various lentiviruses such as HIV-2 [194], the simian [195], feline [196], bovine immunodeficiency [197] or the caprine arthritis-encephalitis virus [198], and equine infectious anaemia virus (EIAV) [199] however, LVs derived from HIV-1 are most widely used for gene transfer applications.

LVs are produced by cells that are transfected to express the necessary viral components including gag/pol, and env genes [200]. The first generation LV production plasmids included three plasmids: a plasmid coding for the packaging (gag/pol), accessory (vif, vpu, vpr, and nef), and regulatory (tat and rev) genes; a plasmid transferring the gene of interest, known as transfer vector, flanked by the LTRs and including the packaging signal (Ψ); a plasmid coding for env. In order to widen the tropism of these vectors, the endogenous HIV-1 envelope glycoprotein (gp41/120) is often replaced with that of the vesicular stomatitis virus Indiana strain (VSVind.G) [201]. This process is known as vector pseudotyping. Other envelope glycoproteins such the feline endogenous retrovirus RD114, Cocal virus, and the Gibbon Ape leukaemia virus (GALV) were also used to pseudotype LVs. Also, transduction of neuronal cells was accomplished by using LVs pseudotyped with Venezuelan Equine Encephalitis and Rabies virus. Moreover, efficient transduction of
CD34+ HSC was achieved by using chimeric envelopes such as RD114/LTR (contains the cytoplasmic domain of the MLV) and RDpro (contains the HIV-1 cleavage site) [202]. Nevertheless, as so far VSVind.G pseudotyped LVs demonstrated higher titres compared to other envelope glycoproteins, VSVind.G is considered to be the gold standard for LV pseudotyping [203].

In the second generation of LVs, while utilising the three plasmid system, the safety was improved by eliminating the viral accessory genes (*vif, vpu, vpr,* and *nef*), from the packaging plasmid (Figure 1-3B) [204]. As the likelihood of generating replication competent viruses (RCV) remained a major concern for clinical application of these vectors [205], the third generation of LV production system was developed (Figure 1-3C). This system improved the biosafety of the vectors over 2nd generation system firstly by using self-inactivating (SIN) vectors in which the promoter and enhancer are deleted (e.g. deletion of a 399bp DNA fragment including the TATA box which is necessary for transcription initiation) from the U3 in 3’LTR [206]. This deletion in the 3’LTR is transferred to the 5’LTR after the first round of infection. Furthermore, tat is eliminated in this system and is replaced by a constitutive promoter, such as the Rous sarcoma virus [205] or the cytomegalovirus (CMV) [207] promoters (as used in the vector constructs pRRL and pCCL, respectively), is fused to the 5’LTR. Consequently, the expression of the transfer vector genome is no longer dependent on tat activation [208]. Moreover, the packaging plasmid is split into two plasmids; one encoding the *rev* gene and the other encoding a codon-optimised *gag/pol* gene [208-210]. These improvements had major roles in reducing the risk of developing RCV or insertional mutagenesis in target cells [207].
Figure 1-3. Lentiviral vector packaging systems. (A) Lentiviral packaging system is based on the HIV-1 wild type genome which consists of nine open reading frames. To increase safety, this genome is edited and split across multiple plasmids. (B) Second generation system that contains a single packaging plasmid encoding the Gag, Pol, Rev, and Tat. The transfer plasmid contains the viral LTRs and psi (ψ) packaging signal. The envelope is encoded by a third, separate plasmid. (C) In third generation systems the packaging plasmid is further split into two plasmids, one encoding rev and one encoding gag and pol. In this system the transgene is encoded by a SIN vector containing a mutated 3′ LTR and a foreign promoter fused to the 5′ LTR. RRE: rev response element; LTR: long terminal repeats; ψ: Psi or packaging element.
1.4.3.1 VSVind.G-pseudotyped lentiviral vectors

Retroviruses have limited natural host range and cell tropism. For gene therapy, one might either want to limit or expand the range of cells susceptible to transduction by a gene therapy vector. So far, many vectors have been developed in which the endogenous viral envelope proteins have been replaced either by envelope proteins from other viruses or by chimeric proteins. Viruses in which the envelope proteins have been replaced are referred to as pseudotyped viruses [211]. Depending on the envelope used for pseudotyping, some of the virus particle characteristics such as tropism and stability might be altered. Due to mentioned restrictions in using lentiviral vectors with the HIV-1 envelope such as lower particle stability as well as the limited cell tropism (the CD4 receptor is usually expressed in T-lymphocytes, monocytes, macrophages and dendritic cells, the primary target cells for primate immunodeficiency viruses in vivo [212]), LVs are usually pseudotyped with VSV-G Indiana strain glycoprotein as it has an extremely broad tropism. Furthermore, VSVind-G seems to increase the vector particle stability that can provide purification advantage by allowing the vector concentration to a high titre using ultracentrifugation [213].

Nevertheless, this pseudotyping has several drawbacks; first, VSVind.G is highly fusogenic, which can lead to syncytia formation and cell death at mildly basic pH levels, and highest levels of fusogenic activity have been seen at pH around 7.2 [214]. Syncytium formation by VSV G protein at neutral pH might be considered as an unusual characteristic as this protein triggers viral entry through pH-triggered fusion [215]. Nevertheless, VSV G protein was reported to be an atypical fusion protein that undergoes reversible conformational
changes [216]. Upon its transport to the plasma membrane via the secretory pathway, it goes through the acidic environment of the Golgi apparatus which triggers conformational changes. Therefore, G protein reaches the plasma membrane in a fusion-active form and thus is able to perform cell-cell fusion. The resulting syncytia are inevitably destined for apoptosis, as shown for other fusogenic viruses [217-219].

1.4.4 Lentiviral vector production methods

The main LV production method is by delivering the packaging plasmid, envelope protein, and transfer vector into a cell line such as HEK 293T cells that is able to package all the viral components into a viral particle [220]. The size and quality of transgene, the employed method of transfection, and the target cells are amongst factors that can influence the efficiency of transfection [221].

1.4.4.1 Transient production of lentiviral vectors

Transient production of LVs is easy and less time consuming compared to stable cell line development. In addition, this method enables the expression of some cytotoxic viral proteins. Although transient method is the most efficient technique for research, the high cost and low adaptability of this method to large-scale Good Manufacturing Practice (GMP) vector production [222], potential contamination with transfection plasmids [223], batch-to-batch variability, and difficulty in optimisation of transfection conditions limit the use of transient method for clinical purposes. The yield of current methods which are set in cell factories ($10^9$-$10^{11}$ TU), is sufficient to treat only a few numbers of patients [74, 76, 222].
Recent work optimising the different aspects of production such as cell type, culturing method (adherent vs suspension) and density, culture media supplementation (e.g. with or without serum), and plasmid delivery systems (e.g. calcium phosphate [224] or polyethyleneimine (PEI) [225]), has led to production of larger scale industrial batches of LVs [226].

HEK 293 cells have been commonly used for vector production, lab-based protein production, and gene expression studies. These cells have been modified to different derivatives such as HEK 293T and HEK 293FT (traceable HEK 293T cells) [227]. HEK 293T cells express the SV40 (simian virus 40) T-Antigen which is compatible with vectors carrying the SV40 origin of replication resulting in higher expression levels. Moreover, these cells have a high growth rate and transfection efficiency [228]. While these cells are heavily used for small-scale vector production, they are as desirable for larger scale applications. One method used for larger-scale LV production is the direct scale-up, which refers to expanding the small-scale production culture surface by adding production units such as Cell Factory™ (Nunk) and CellSTACK® systems (Corning) [229].

Although using adherent cells for LV production is the common gold standard method, the optimal technique for industrial-scale vector production at the moment is using cell suspension in bioreactors. Therefore, HEK 293T cells have been recently adapted to grow in suspension culture. Serum or other animal-originated components are not required for maintaining these cells in culture. Thus, these cells are more suitable for clinical applications due to reduced risk of contamination. Moreover, these suspension cells can be readily expanded into different types of vessels (e.g. wave bags, shake flasks,
stainless steel bioreactors) which aids with the scaling up of the production. Recently, a HEK 293 clone, HEK 293SF-3F6, was established for suspension culture [230, 231]. Culturing this clone in serum-free media in 3 litres stirred tank bioreactors yielded in titre up to $10^8$ transduction units (TU)/mL of LV [232].

Still, the optimisation of these large-scale production methods is needed for the development of an industrial-friendly method of LV production where lower amounts of DNA is used, and the cost of production is reduced. Consequently, development of stable producer cells seems to be a more affordable way of production of LVs to be used to treat various diseases [229].

1.4.4.2 Stable production of lentiviral vectors

Stable vector production can potentially overcome the limitations of the transient method such as batch-to-batch variability and cost of production, as well as can reduce the risk of DNA recombination and generating RCVs [233]. A packaging cell line (PCL) stably expresses gag-pol, rev, and/or env. When the transgene is provided, the PCL becomes a producer cell line. Even though a stable cell line should ideally be consistent in growth and to be able to produce large volumes of infectious LVs, PCLs developed so far not only achieved low titres of $10^5$ - $10^7$ TU/mL, but also demonstrated less stability over generations due to the cytotoxic effects of some viral components [201, 234, 235]. It has been reported that VSVind.G and some viral elements such as protease are toxic to cells when expressed continuously [201]. To overcome the toxicity of the envelope glycoprotein in stable cell line production, VSVind.G has been replaced with other envelope glycoproteins such as
RDpro which is derived from the feline endogenous virus envelope RD114, and GALV [236].

1.4.4.2.1 Established packaging cell lines

Several stable packaging cell lines have been developed over the past years [237-239]. The STAR packaging cell line was developed via a novel approach of transfecting HEK 293T cells with second-generation MLV vectors [240]. While titres were higher than $10^7$ TU/mL, as non-SIN vectors were utilised, the clinical application of these cells was limited. RD2-MolPack is another packaging cell line that was developed that continuously expresses RD114-RT (a chimeric RD114 containing the cytoplasmic domain of MLV [241]). This cell line was also developed using a non-SIN vector. Next, RD3-MolPack was then developed using a SIN vector instead. Nevertheless, in both cell lines, the titres were around $10^6$ TU/mL which is not ideal for clinical use [239]. Recently, Humbert et al. reported a third-generation SIN LV producer cell line using Cocal G protein. These cells were adapted to grow in serum-free suspension culture and achieved concentrated titres of $10^8$ IU/mL [237].

The Collins/Takeuchi group developed the WinPac packaging cell line that can support the continuous production of LV. A non-toxic gammaretroviral envelope from the feline endogenous virus RD114 with an altered cytoplasmic tail to allow LV incorporation, RDpro, was adopted [240]. Due to its lack of cytotoxicity, also as the receptor of this envelope is widely expressed on hematopoietic stem cells [242], RDpro was used in the STAR packaging cells [240] as well as WinPac-RD packaging cell line [238]. The WinPac (WP) cell line was developed by stably transfecting a codon-optimised gag-pol into HEK 293FT cells using recombinant mediated cassette exchange (RMCE). In this
study, MLV vectors introduced recombinase recognition sites in actively transcriptional sites. *Gag-pol* genes were then introduced via RMCE into this active locus, and the *rev* and *env* genes were finally transfected into the cells stably. Although isolated single clones produced titres for several months, the titres of $10^5$ TU/mL were sub-optimal for clinical purposes. Furthermore, due to the limited host tropism and reduced physical stability, compared to that of VSVind.G, which lead to suboptimal titres and virus recovery, the usage of the RDpro packaging cell line is limited.

In 2010, Trobridge et al. published a paper in which lentiviral vectors were pseudotyped with the envelope G protein of Cocal virus (COCV). It has been reported that, in transient production systems, G protein of COCV (COCV.G) can efficiently pseudotype LVs producing relatively high titres ($10^7$ TU/mL). COCV.G LVs also demonstrated broad tropism as they could transduce a variety of cells of different origins including human, primate, and dog-hematopoietic progenitor cells. Moreover, compared to VSVind.G-pseudotyped LVs, COCV.G- pseudotyped LVs showed more resistance to inactivation by human and dog serum, which shows promise for *in vivo* applications [243]. Lastly, some preliminary data suggest that COCV.G might be less cytotoxic compared to VSVind.G when expressed continuously [244]. These favourable characteristics would facilitate affordable large-scale vector production if COCV.G also demonstrates high vector stability like VSVind.G. This would greatly improve the range of clinical applications of lentivectors and would help propel the field of LV-mediated gene therapy forward [238]. Consequently, our laboratory decided to replace the RDpro envelope in WinPac-RD packaging cell line with that of Cocal virus to generate WinPac-
COCV.G cell line. From the WinPac-COCV.G transfected cells, single clones were isolated and vectors were produced by transiently transfecting them with SIN pHV plasmid which codes for GFP gene. The titres of these vectors then were tested by measuring the percentage of GFP using flow cytometry. The initial results of the test were promising, as COCV.G seemed to show similar properties to its close phylogenetic relative VSVind.G [244].

Although it has been reported that the VSVind.G glycoprotein is cytotoxic, due to its advantages including high tropism, the stability of the viral particle, and higher vector titres, other strategies to express this glycoprotein in packaging cell lines were investigated. One of these strategies was the introduction of the inducible expression systems. Systems commonly use tetracycline or doxycycline [245-248] as inducible agents were developed to regulate the gene expression of cytotoxic viral components like VSVind.G. The Tet-off system was first introduced by Gossen and Bujard [249] where the expression of a Tet-inducible promoter was lessened when tetracycline was added into the system. In this system, a tetracycline-controlled transactivator protein and a tetracycline-responsive promoter element (TRE) are used to regulate the expression of the transgene.

The tet-off system allows for the silencing of a gene as long as it remains in the system outside the expression period. In addition, the tetracycline has to be removed entirely from the system which might be easy in smaller scale cultures yet; it can be challenging in larger scale and suspension cell culture systems. Furthermore, as several days are needed for the production of the protein to reach its peak, as well as the risk of leakiness of expression in the
off-state, transcriptional instability was reported a few months after cultivating the cells [250].

A few years after the development of the tet-off system, the Tet-on system was introduced in which, instead of removal, the addition of tetracycline allows the expression of the gene. In this system, the expression of the gene is switched on by tetracycline binding to the tetracycline transactivator and TRE [251, 252]. While this system is better for transient gene expression, the background activity of the inducible gene in the absence of the inducer agent is a limiting factor [253, 254]. Although this promoter has been through lots of modification, more improvement is needed to optimise the final product [255].

1.4.5 Lentiviral vectors in clinical gene therapy

While both GRVs and LVs have the advantage of integration into the host cell genome, LVs seem to be less mutagenic and hence safer vectors compared to GRVs, as they often do not show preferential integration near oncogenes or cell cycle genes [9, 256]. LVs have shown high efficacy in recent clinical trials for the treatment of HIV, cancer, and genetic diseases such as X-linked Adrenoleukodystrophy (X-ALD) and β-thalassaemia. The ability of LVs to stably infect non-dividing cells makes them better candidates compared to other integrating vectors [257].

1.4.5.1 Cancer immunotherapy

Tumour cells have the ability to evade the immune system. Cancer immunotherapy includes antibody and cytokine therapies, cancer vaccines, and adoptive cell transfer [258]. These methods attempt to improve the targeting ability and cytotoxicity of the immune response in order to eliminate
tumour cells [257]. In anti-cancer vaccine design, effective delivery of tumour-associated antigens (TAA) as well as avoiding tumour-induced immune tolerance are the major challenges [259].

In cancer immunotherapy, ongoing CD8 T cell (cytotoxic T cell) immunity is required. Viral vectors have been shown to be very effective in inducing T cell response. Accordingly, LV-based vaccines seem to be suitable options due to their integration and therefore a prolonged expression of TAA which can be processed by the antigen-presenting cells (APCs), especially dendritic cells (DCs) as key T cell activators [260]. LV-based anti-cancer vaccines are also designed to target the tumour cells by specific antibodies incorporated in their envelope [9, 261].

To induce an anti-cancer immune response, autologous DCs are usually transduced with LVs carrying TAA [262] or co-stimulatory signals [263] ex vivo, then re-administrated into the patients [257]. LV-transduced DCs have been reported to induce expression of CD4+ T cells and a higher concentration of IFN-γ. Despite the potential advantages of this method, some challenges remain. One is the low numbers of DCs in the peripheral blood. To overcome this issue, CD34+ blood or bone marrow DC precursors and CD14+ monocyte DC precursors have been stimulated by cytokines to differentiate into mature to DCs [264, 265]. Another obstacle is the short lifespan of antigen-carrying DCs [266]. This issue has been tackled by transducing DCs with LVs carrying survival genes such as c-FLIPS, c-FLIPL, and Bcl-XL resulting in prolonged DC survival time [267].
As production of mature DCs can be demanding, an alternative method of turning cancer cells themselves into APCs to stimulate the immune response has been developed. This method is based on the fact that cancer cells already express TAAs. Therefore, autologous cancer cells are transduced by co-stimulatory factors and cytokines which can induce Th1 and hence cytotoxic T lymphocyte (CTL) response against TAAs upon administration back to the patient [257].

Moreover, B-cell lymphoma cells transduced with LVs expressing IL-12 (which is usually expressed by APCs in response to tumour antigens) have been tested in mouse models in comparison to systemic delivery of IL-12. Long-term immunity and survival were reported in mice injected by modified cells [268]. Other LV-based cancer immunotherapies have been developed such as acute myeloid leukaemia (AML) cells transduced with CD80 and GM-CSF (inflammatory cytokine stimulating macrophage and DC infiltration) [269] or CD80 and IL-2 [270]. Both these cells induced an anti-tumour response. This method is believed to offer an improved immunotherapy response compared to that of the systemic delivery of IL-12 and IL-2 due to toxic effects [271].

1.4.5.1.1 T-cell therapy

In T-cell therapy, expressing engineered antigen-specific T-cell receptors (TCRs) or chimeric antigen receptors (CARs) aid in conveying cytotoxic T-cells against tumour-associated antigens [272, 273]. This approach has been used in treating several malignancies including neuroblastoma, lymphoma/leukaemia, colorectal cancer [98], melanoma, and synovial sarcoma. So far, no IM was reported despite the use of GRVs suggesting that
differentiated T-cells are resistant against malignant transformations [274, 275].

T cell therapy is based on selecting the autologous T cells that robustly express antigen-specific T cell receptors, activating and expanding them, then administrating them back to the patient. However, due to low numbers of Ag-specific T cells and the challenges in their sufficient expansion, other strategies had to be developed. One of these strategies is based on the TCRs’ ability to be transferred from one T cell population to another [276]. Accordingly, a method has been developed in which TAA-specific TCR genes from highly active T cells are transferred to other isolated autologous T cells. LVs are the vector of choice for stable and efficient delivery of TCR cDNA; especially bicistronic LVs that express both the α and β genes of TCR [277]. Moreover, autologous T cells transduced with LV-TCRs targeting NY-ESO-1 [278] and MART-1 [279] have been used in clinical trials for treating oesophageal cancer or metastatic melanoma, respectively.

A possible disadvantage in using TCR is the risk of recombination between the introduced TCR with the endogenous one resulting in off-target genotoxicity [280]. This can be dealt with by using endonuclease to suppress the expression of the endogenous TRC. Zinc finger nucleases (ZFN)-edited T cells expressing LV-transferred TCRs against Wilms tumour-1 antigen have demonstrated higher purity and lower off-target effect compared to unedited T cells [281].

In the past few years, chimeric antigen receptors or CARs against CD19+ have become very popular for treating different B cell malignancies. CARs consist
of an extracellular and intracellular domain. The extracellular domain contains a single-chain variable fragment (scFv) of the light and heavy chains of a TAA-specific antibody that activates the immune system by directly recognising these tumour antigens. The intracellular region of CARs contains 1-3 domains that activate and enhance the cytotoxicity of T cells through increased cytokine secretion and proliferation [282, 283].

Despite recent success using CARs against CD19+ B cell malignancies, they show some limitations. Since the extracellular scFv is of mouse origin, they can be immunogenic. Even antibodies derived from humanised mice have the potential of provoking the immune system as novel proteins resulting in their clearance from the body. This immune response can range from fever to organ failure and death and usually occurs due to uncontrolled overexpression of various cytokines which is known as cytokine storm or cytokine release syndrome (CRS). In addition, CARs are designed to recognise TAA independent of MHC, giving them an advantage over TCRs which are MHC-dependant. This allows CARs only to identify extracellularly expressed antigens which limits the targets for this therapy.

A possible approach to overcome CRS exploits suicide gene or cell-fate control system. In this system, a cDNA sequence such as an enzyme [e.g. herpes simplex virus-thymidine kinase (HSV-TK)], which can be activated upon administration of its associated prodrug (ganciclovir or GCV for HSV-TK), is transduced into cells leading in the elimination of target cells [284, 285]. Moreover, universal CARs have been introduced recently. These T cells are engineered to have disrupted HLA-I [286], or to be devoid of TCR, β-2 m, and PD1 triple loci [287]. Anti-CD19 CAR-T cells were also recently developed by
TALENs which are less likely to develop GVHD. Tumour regression was reported after administration of genome-edited cells into two patients with refractory B cell acute leukaemia [288]. These universal CARs have been reported to demonstrate high anti-tumour activity while reduced allogeneic activity [289]. Nevertheless, as TAAs are usually self-antigens (e.g. CD19 which is a marker on healthy B cells is overexpressed in B cell malignancies), on-target off-tumour response in T cell therapy is of concern. Consequently, further research to identify novel TAAs and to optimise CARs’ affinity is required in order to improve T cell treatment efficacy and off-target effects [284, 290, 291].

A number of selected clinical trials utilising LVs are listed in Table 1-3.
<table>
<thead>
<tr>
<th>Target cells</th>
<th>Disease group</th>
<th>Disease</th>
<th>Env</th>
<th>Production method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSCs</td>
<td>PIDs</td>
<td>WAS</td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[77]</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[78]</td>
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<tr>
<td></td>
<td></td>
<td>X-SCID</td>
<td>VSVind.G</td>
<td>Tet-off Inducible</td>
<td>[292]</td>
</tr>
<tr>
<td></td>
<td>Hereditary anaemia</td>
<td>B-thalassemia</td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[76]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sickle cell anaemia</td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[293]</td>
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<tr>
<td></td>
<td>Storage disease</td>
<td>ALD</td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[74, 294]</td>
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<td></td>
<td>MLD</td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[75, 295]</td>
</tr>
<tr>
<td>T cells</td>
<td>Cancer</td>
<td>Lymphoma/Leukaemia</td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[296, 297]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VSVind.G</td>
<td>Transient</td>
<td>[298, 299]</td>
</tr>
</tbody>
</table>

Table 1-3. List of selected recent LV-mediated gene therapy clinical trials. HSCs: hematopoietic stem cells; PID: primary immunodeficiency; WAS: Wiskott Aldrich syndrome; WASP: WAS protein; ALD: Adrenoleukodystrophy; MLD: metachromatic leukodystrophy; ARSA: arylsulfatase-A; LV: Lentiviral vector; Env: envelope; VSVind.G: vesicular stomatitis virus (Indiana strain). HEK 293T cells were used for transient vector production; X-SCID: X-linked severe combined immunodeficiency.
1.4.6 LV delivery for genome editing

Genome editing technologies based on endonucleases have been the centre of attention in recent years as they have the advantage of genome modification over the viral vector which can only mediate gene addition [300]. Endonucleases mediate double-strand break (DSB) resulting in recombination in mammalian cells through non-homologous end-joining (NHEJ) and homology-directed repair (HDR) systems. At the site of DSB, NHEJ can inactivate a gene by creating insertion or deletion mutations while HDR, in the presence of a donor DNA, can modify specific sequences to correct a mutation or to insert a new sequence in a site-specific fashion [301].

Genome editing systems offer the possibility to tackle the problems of viral vector gene transfer such as genotoxicity due to integration and activation of proto-oncogenes. In addition, this method is desirable for the treatment of diseases with dominant detrimental mutations [300]. Moreover, the regulation of the modified gene expression is improved as its expression is controlled by the endogenous promoter [302].

Zinc finger nucleases (ZFN) [303] or meganucleases [304] were amongst the early genome editing molecules to be used. Transcription activator-like effectors or TALES are bacterial DNA binding domains that were used to create TALE nucleases (TALENs) which can be engineered to target DNA sequence of interest. Although these nucleases opened new doors to genome manipulation, the design of specific nuclease for each DNA target is required which can limit their use [305-307].
More recently, the clustered regulatory interspaced short palindromic repeats (CRISPR)-associated 9 (Cas9) gene-editing system has become popular as it simplified the process of site-specific gene editing. The CRISPR-Cas9 nucleases are based on a prokaryotic adaptive immune system by which they silence the nucleic acids of the invading viruses and plasmids [308]. This system uses a guide-RNA (gRNA) to target the specific target sequence and a Cas nuclease to cut [257, 308].

As TALENs and CRISPR-Cas9 systems can target specific DNA sequences, they have been heavily studied and used in basic research applications in recent years [309, 310]. LVs delivering the CRISPR system used in mouse embryonic stem cells for site-directed mutagenesis showed higher gene suppression efficiency compared to RNAi [311]. Targeted gene suppression or activation using LVs delivering CRISPR was also achieved in human cells where the CRISPR-Cas transgene maintained stable expression for two weeks [312].

Genome editing as a therapeutic agent has made its way into the clinic. For instance, ZFN against CCR5 has been engineered to block HIV infection in T-cells and HSCs [313, 314]. Several CRISPR-Cas nuclease clinical trials targeting PD1 expression had also been recently approved in China [300].

Despite all the advances in this field, further preclinical studies are required for safer and more feasible designs, to minimise off-target cutting and mutations due to homologous DNA sequences. Also, the immunogenicity of nucleases in *in vivo* applications and targeted delivery need further investigations [300].
1.5 Rhabdoviruses

The Rhabdoviridae family (the Greek word “rhabdos” meaning rod or wand) is one of the most ecologically diverse families of enveloped, bullet-shaped RNA viruses. This family includes two main prototype viruses: vesicular stomatitis virus (VSV) which belongs to the genera of vesiculoviruses, and rabies virus (RABV) which belongs to lyssaviruses. While the primary hosts of lyssaviruses are bats by which these viruses are transmitted, vesiculoviruses are usually transferred via insects to a wide host range of mammals. For many decades, VSV and RABV have been studied extensively as model systems for investigating the molecular mechanisms of rhabdovirus life cycle, infectivity, and antigenic properties [315].

1.5.1 Vesiculoviruses

Vesiculoviruses consist of 16 viruses classified based on their host range, serological properties, and genome organisation [316, 317]. Vesicular stomatitis virus (VSV) was the first vesiculovirus to be described and is the most studied virus. VSV includes four serotypes: VSV Indiana (VSVind), VSV New Jersey (VSVnj), VSV Alagoas (VSVala), and Cocal virus (COCV). Two other major vesiculoviruses are Chandipura virus (CHAV) and Piry virus (PIRYV).

VSV causes vesicular disease (initially known as ‘sore mouth/ tongue’) in farm animals such as horses, pigs, and cattle [214]. They were first seen in livestock in the USA and South America. Both VSVind and VSVnj are endemic especially in central and South America. VSV’s natural hosts are their insect vectors as well as cattle, horses, pigs, and other mammals [318-320]. VSV
infection in human, however, can result in flu-like symptoms. PIRYV was first isolated in northern Brazil, and only five cases of non-fatal lab-related human infection have been reported so far. CHAV was first known as the cause of febrile disease and recently has been related to outbreaks of fatal encephalitis which is becoming a health concern in India [321, 322].

1.5.1.1 Genome organisation of vesiculoviruses

Vesiculovirus' genome is composed of a non-segmented, single-strand, negative-sense RNA encoding five structural proteins; nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large protein or the viral polymerase (L). The RNA genome combines with N, P, and the viral polymerase to form the helical ribonucleoprotein (RNP) complex [215, 323]. This complex is connected to the envelope via the matrix protein (M), which binds to both the N protein and the inner leaflet of the lipid-bilayer membrane [324]. The matrix protein partly regulates the replication of VSV. This protein codes for the RNA polymerase as well as aids in viral budding. The matrix protein also has a pivotal role in the virus early infection by blocking the expression of antiviral genes (e.g. IFN), which aids the virus to replicate freely. The VSV envelope is a single-spanning transmembrane glycoprotein with receptor-binding and fusion activities. It contains a small transmembrane and C-terminal regions while 90% of G protein is its N-terminal region that protrude of the viral envelope [1, 325-327].

Full-length genome analysis of VSVind, VSVnj, and COCV structural proteins indicates that while these viruses are genetically closely related to each other, they all are serologically different [328, 329]. N, M, and L are the most conserved proteins while G and P are more variable [330].
1.5.1.2 Vesiculovirus glycoprotein

In enveloped viruses, the envelope glycoproteins play an essential role in viral attachment to the host cell receptor, fusion and viral infectivity [326]. VSVind (the prototype of vesiculoviruses) contains one glycosylated protein, G protein (VSVind.G) [214, 331]. The mature VSVind.G protein is a 65-kDa type I transmembrane protein (single-pass trans-membrane protein, N-terminal is exposed to the exterior of the cell while the C-terminal is located on the cytoplasmic side) containing 511 amino acids that oligomerises into a homotrimer during transport to the cell surface, where the trimer is then assembled into the viral particle [325].

While the glycoprotein of VSVind, VSVnj, and CHAV are well studied, there is little known about other vesiculovirus G proteins (VesGs) such as Maraba virus G protein (MARAV.G), COCV.G, VSVala.G, or PIRYV.G. Sequence analysis of VSVind.G indicates that this protein shares some characteristics with both class I and class II membrane-associated glycoproteins, however, due to reversibility of its conformational changes, this G protein is classified in a separate group of fusion proteins, class III [306, 332]. The structure of these classes of proteins consists of long extracellular ends, a hydrophobic transmembrane section, and a C-terminal domain within the cell membrane [317, 333].

While CHAV.G and PIRYV.G share a similar number of amino acid residues (530 and 529, respectively), the G protein in other VesGs ranges around 511-517 amino acid residues long [334-338] (Figure 1-4). Nevertheless, a high degree of homology on the amino acid level suggests high functional and structural similarities amongst these seven VesGs [323, 327, 333, 339, 340].
Amino acid alignment of the G protein of MARAV.G and COCV.G shows the highest homology to VSVind.G of around 78% and 72%, respectively. VSVala.G and VSVnj.G follow with 64% and 50% homology, respectively while PIRYV.G shows the lowest levels of homology (of around 40%) to VSVind.G (Figure 1-5A). Moreover, the phylogenetic analysis of the amino acid sequences of these G proteins highlights that MARAV and COCV are closely related to VSVind while PIRYV and CHAV are close to each other and distant from VSVind (Figure 1-5B).
Figure 1-4. Multiple amino acid sequence alignment of the G proteins of Vesiculoviruses. Dashed lines represent gaps introduced to maximise matching of amino acid residues. Blue shading indicates percent identity; dark blue: 80-100%, medium blue: 60-80%, light blue: 40-60%, and no colour indicating <40% identity. The sequences were aligned using Clustal Omega online multiple sequence alignment tool (EMBL-EPI) and the alignments were visualised using JalView software. The arrow indicates the signal peptide cleavage site.
Figure 1-5. Phylogenetic relationship of vesiculoviruses based on G protein amino acid sequences.

(A) The percent amino acid identities of the G proteins compared to VSVind.G are summarised in the table. (B) G proteins of the major vesiculoviruses, as well as the G protein of the rabies virus (another prototype rhabdovirus, utilised as the base for the phylogenetic tree), were included in the analysis. The amino acid sequences were aligned using Clustal Omega online multiple sequence alignment tool (EMBL-EPI). The evolutionary analyses were conducted in MEGA7 [3]. The evolutionary history was inferred by using the maximum likelihood method based on the Jones-Taylor-Thornton matrix-based model [5]. The tree with the highest likelihood is shown with the bootstrap confidence values (out of 100) indicated at the nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, depicted in the linear scale. CJSV: Carajas virus, ISFV: Isfahan virus. Vesiculoviruses that this thesis focuses on are highlighted in coloured boxes.
Although the signal peptide shows different lengths amongst the seven vesiculoviruses, it shares similar characteristics in the four VSV serotypes; a charged residue near the N-terminal end, a hydrophobic central area, and a less hydrophobic C-terminal end [341, 342]. The signal peptide is 21 residues long in PIRYV.G and CHAV.G while it is 16 residues in VSVind.G, VSVnj.G, and MARAVA.G, and 17 residues in COCV.G [333, 338].

Crystal structure of VSVind.G indicates four distinct domains (Figure 1-6C): Domain I (DI) which is called the lateral domain is rich in β-sheets. Domain II (DII) or the trimerisation domain consists of α-helices that are involved in the trimeric pre-fusion formation of the protein. This domain is further divided into three sections: the N-heptad repeat (NHR), the middle-heptad repeat (MHR), and the C-heptad repeat (CHR). Domain III (DIII) contains the pleckstrin homology domain (PHD) which consists of 2 α-helices and two β-sheets. This domain is inserted into domain II, and as it is the most exposed domain in the pre-fusion conformation, it has been suggested that might play a role in receptor recognition. Domain IV (DIV) is inserted into domain III and contains six β-sheets with highly preserved amino acid residues. Two hydrophobic fusion loops in this domain interact with the target cell membrane stabilising the structure of the G protein [1]. During conformational changes from pre- to post-fusion (Figure 1-6A/B), while domains I, III, and IV retain tertiary structure, their relative orientation is rearranged due to refolding of domain II [1, 2, 332]. These structural reformation has been suggested to move the fusion domain from the viral surface towards the target cell membrane.

The low-pH conformation of CHAV.G has been recently revealed its high structural similarity to the post-fusion structure of VSVind.G within the central,
fusion, and PHD domains [2, 327, 343, 344]. Although the central and fusion domains are almost indistinguishable, PHD seems to be more diverse. Overall, PHD is the most diverse domain in VesGs, and it has been suggested to be the target for the neutralising antibodies [327, 345].

Figure 1-6. Structure and domain organisation of VSVind.G. Mature VSVind.G protein (AA 17-429) generated by thermolysin-mediated limited proteolysis of virions [1, 2] in (A) prefusion and (B) postfusion structures. 3D structures were retrieved from RCSB Protein Data Bank, visualised, and coloured using JalView software. (C) Domain architecture of VSVind.G’s amino acid structure in a linear diagram. The C-terminus is not shown in the structure, indicated here in grey. Domain boundaries are numbered according to the G protein precursor which includes the signal peptide. Domains are indicated in colours: Lateral domain DI: red; Trimerisation domain DII: blue; Pleckstrin Homology domain (PHD) DIII: orange; Fusion domain DIV: yellow. Adapted from [1].
1.5.1.3 Receptor-mediated viral entry

VSV.G protein is responsible for entry to the target cell [332]. This includes attachment to the receptor on the cell surface followed by pH-dependant fusion to the endosomal membrane. After attachment to a cell receptor, VSV particles go through endocytosis in a clathrin-based manner [346].

Current studies suggest that low-density lipoprotein receptor (LDLR) and its family members are the receptors for VSVind.G cell entry [347]. It has also been suggested that LDLR serves as the primary receptor for VSV and VSV.G pseudotyped LV infection, while other LDLR family members act as alternative receptors. This is based on a study where the VSVind infection was reduced in a dose-dependent manner by soluble LDLR (sLDLR). This infection was fully blocked when receptor-associated protein (RAP), which blocks all other LDLR family members except for LDLR itself, was added [348]. Similar data have been reported for COCV infection [244]. However, the receptor usage of other vesiculoviruses is yet to be known [349].

1.5.1.3.1 The low-density lipoprotein receptor family

The LDLR family is a class of single-transmembrane glycoproteins that are known as cell-surface receptors [350]. The LDLR family members are widely expressed on different cell types and variety of species which explains the broad tropism of VSVind and VSVind.G based vectors. In mammals, this family consists of different receptors: the LDLR itself, apolipoprotein E receptor 2 (APOER2), very low-density lipoprotein receptor (VLDLR), the LDLR-related protein (LRP), LRP1B, megalin (LRP2), LRP3, LRP4, LRP5, and LRP6 [351].
The extracellular domains are structurally conserved amongst these receptors. This domain contains ligand binding repeats and epidermal growth factor (EGF) precursor homology domains which bind to and internalise the ligand, respectively. After endocytosis, these sites are involved in the pH-dependent release of the ligand. The cytoplasmic tails are involved in regulation of ligand-internalisation through clathrin-mediated endocytosis [352-356].

Other than acting as an entry point for some viruses, the LDLR family members are involved in a range of cellular activities such as binding to signalling molecules and carrying various components like vitamins, toxins, and antibiotics. VLDL receptor interacts with the serine protease urokinase-type plasminogen activator (uPA) to modulate proteolysis processes [357]. In addition, it is an entry port for the hepatitis C virus, independent of CD81 [358, 359]. On the other hand, LRP and megalin are known as scavenger receptors as they bind to more than 20 ligands due to having 31 and 36 ligand recognition sites, respectively [360, 361].

1.5.1.3.2 The low-density lipoprotein receptor

The LDL receptor was first identified in association with the genetic disease familial hypercholesterolemia (FH) in which the receptor is dysfunctional due to mutations in the LDLR gene [362]. The primary role of the LDLR is to transport cholesterol-carrying lipoproteins to cells. The main lipoprotein ligand is LDLR which carries approximately 70% of the plasma cholesterol. LDLR molecules usually cluster on the cell surface in clathrin-coated pits. After binding to the ligand, this complex is endocytosed. Upon interaction with the
low pH of the endosome, the ligand is released, and LDLR is recycled back to the cell surface [350].

The mature LDLR is a type I transmembrane protein consisting of 839 amino acids [363-366]. The ectodomain of LDLR contains two main regions: the ligand-binding domain (LBD) which consists of seven cysteine-rich repeats (CR1-CR7), each of about 40 amino acid residues (Figure 1-7). Each CR contains six cysteine residues that form three disulfide bridges, and an acidic residues cluster that coordinates the Ca\(^{2+}\) ion [349]. The ligand-binding domain is followed by the epidermal growth factor precursor homology (EGFPH) domain which has a role in the pH-dependant release of LDL in the endosome. The EGFPH contains two EGF-like repeats followed by six repeats of YWTD (β-propeller) which includes tyrosine, tryptophan, threonine, and aspartic acid residues, followed by another EGF-like repeat [367, 368]. Right after this domain, there is a serine/threonine-rich sequence that gets highly glycosylated which is then followed by the transmembrane and the cytosolic end of the protein enriched in O-linked oligosaccharides [364, 369-371].

It has been shown that calcium ions have a crucial role in the binding of LDLR to LDL as the removal of these ions blocked the LDLR-LDL interaction [372]. These ions are necessary for the disulfide bonds between the conserved cysteine residues in the CR repeats as well as for the structure and binding ability of these residues [373, 374]. LDLR is highly flexible in binding various ligands. This characteristic has been linked to the lack of inter-domain connectivity between its CR repeats which results in an independent ligand-binding structure in the receptor allowing the ligand-binding repeats to adjust
their position to be able to bind to various ligands in a variety of shapes and
diameters [375].

Electron microscopy and crystal structure studies suggest two different pH-
dependent structural conformations for LDLR. In neutral pH, this receptor has
an elongated structure which is considered to be the binding-active or the open
structure of LDLR. However, in acidic pH, this receptor adopts a closed
conformation as the CR4 and CR5 interact with the β-propeller of the EGFPH
domain resulting in ligand release [376]. The receptor is then recycled back to
the cell surface.

1.5.1.3.3 LDLR as the main receptor for VSVind.G cellular entry

In the endosome, the acidic pH triggers conformational changes in G protein
resulting in viral membrane fusion to that of the endosome releasing the viral
genome into the cell cytoplasm. LDLR and other members of the LDLR family
have been reported to be the primary receptors for VSVind.G [347, 349, 377].
VSV infection was successfully inhibited in a dose-dependent manner via
soluble LDLR molecules and was entirely blocked by the addition of RAP
[347].

It has been shown that CR2 and CR3 domains directly bind to VSV G protein
in neutral pH (Figure 1-7). Binding was not detected in pH 6, suggesting that
this interaction occurs only in the pre-fusion structure of VSV. Also, mAbs
against the LDLR-CR3 were shown to block VSV infection [347, 349].
Moreover, VSV infection was blocked after incubation with soluble forms of
CR2 and CR3 [349].
Crystal structures of soluble G protein interaction with soluble CR2 and CR3 indicate that VSVind.G interacts with both domains using the same recognition epitope [349]. The binding site on the G is organised by sections from residues 8 to 10 and 350 to 354 in the lateral domain, 180 to 184 in the PHD domain, and 47 to 50 in the S2 segment. These sections undergo reorganisation in the post-fusion state hence losing the ability to interact with these binding sites [2]. Some of these residues seem to play a critical role in this interaction. For instance, the basic residues H8 and K47 are positioned closer to the acidic residues on the CR domains. K47 also binds to the amide groups of Q71 on CR2 via H-bond and forms a salt bridge with the acidic group of D110 on CR3. R354 also binds to the C=O groups of the main chain in both CRs. Furthermore, the mutated forms of K47 and R354 blocked the interaction between CR domain and G protein [349].
Figure 1-7. Interactions between VSVind.G and CR2-CR3 domains of LDLR. Diagram demonstrates the bound structure of VSVind.G to both CR2 (right) and CR3 (left) on LDLR at neutral pH while both proteins are anchored to the viral and cell membranes, respectively. EGFL domain: epidermal growth factor-like domain.
1.5.1.4 pH sensing

The envelope of enveloped viruses is derived from the phospholipid bilayers of the host cells [378]. For the viruses to deliver their genetic material to the target cell, they need to overcome the physical barrier of the phospholipid bilayers; the electrostatic repulsion of the polar groups of the phospholipids between the cell and the envelope. This is achieved through the viral glycoproteins in the viral envelope [106, 379, 380]. Upon exposure to the mildly acidic pH of the endosome, the viral glycoprotein goes through conformational changes that induce pore formation allowing proton penetration. This virion acidification reduces the affinity of the viral M protein to the viral ribonucleoprotein releasing it into the cytosol to initiate a successful infection [381].

Crystallography studies report three distinct conformational states in the glycoproteins; the prefusion state (Figure 1-4A), the extended intermediate state, and the postfusion state (Figure 1-4B). Structural analysis of the G protein indicates conformational changes take place due to side-chain protonation on Asp, Glu, and His. Three histidine residues H76, H178, and H423 have been shown to act as pH sensors on the pre-fusion structure of VSVind.G [2, 332, 382]. These residues have been shown to be conserved amongst all seven vesiculoviruses studied in this project yet, their role in pH sensing has not been confirmed in other G proteins [2, 332].

In contrast, in the post-fusion state of VSVind.G, deprotonation of four acidic residues D268, D274, D393, and D395 have been reported to act as pH sensors for the transition back to the prefusion state [383]. While D274, D393, and D395 are conserved amongst all VesGs, it appears that vesiculoviruses
other than VSVind.G, recruit alternative residues as pH sensors. For instance, it has been reported that in CHAV.G, deprotonation of D269, E234, and H209 destabilises the post-fusion structure triggering the transition back to pre-fusion structure [327].

VSVind.G is a class III glycoprotein and is responsible for both receptor recognition and membrane fusion. This glycoprotein undergoes reversible conformational changes. It has been demonstrated that while acidic pH inactivates the viral infectivity, the infectivity was recovered when the pH was changed to the neutral range [384].

The prefusion form of the VSVind.G is compact where many interactions exist between all four domains. Histidine residues act as pH sensors in the prefusion state [382, 385-387]. Two distinct pH-dependant residue interaction has been identified in the G protein, the Histidine/Cationic (HisCat) pairing where histidine (His) exists in cluster forms when pairing with other cationic residues such as lysine (Lys), arginine (Arg), or His. These clusters are mainly localised in the interfaces between DIV and DI and DII (Figure 1-4C). Various studies have investigated these clusters for pH sensing. His132/Lys15 and His162/His407 have been identified to mediate the pH-dependant conformational rearrangements in VSVind.G [388]. The second residue interaction is Anion-Anion (AniAni) pairing. The AniAni pairing refers to the interaction between two anionic residues such as aspartate (Asp) and glutamate (Glu). At neutral pH, these two amino acids are negatively charged due to their acidic side chains. Upon pH acidification and therefore protonation, they form hydrogen bonds which stabilise the postfusion form of the molecule [389, 390]. All AniAni pairings are formed in DII at the trimer interface [339].
In neutral pH, DI usually exists in the interior while DIII occupies the base of the molecule. DII however, is mainly unstructured in the neutral pH. Upon contact with the acidic pH of the endosome, DI moves to the exterior side while DII folds into a six α-helical structure which is highly stable [2]. The NHR and MHR of DII create the postfusion trimer by forming the three-helix core of the molecule enveloping the CHR section. DIII then folds beside DII while DIV extends towards the target cell membrane. While HisCat pairs are reduced, the number of AniAni interactions are increased in the postfusion formation [339, 390]. However, His residues that were involved in HisCat pairing in the prefusion state can form salt bridges with D145 of DIV in acidic pH stabilising the postfusion conformation. All these mechanisms that result in pH-dependent stabilising of the G protein conformational changes, in both pre and postfusion states, are suggested to be the reason behind reversibility of the conformational changes in VSVind.G [390].
1.5.1.5 Fusogenic activity

In the acidic pH of the endosome, the G protein undergoes conformational changes from the prefusion state to postfusion reformation where the fusion loops are exposed to merge the viral membrane to that of the endosome [2, 106, 332, 339, 391, 392]. It has been reported that the highest levels of VSVInd infectivity occur around the physiological pH (pH 7.2) and that these titres drop by 30-folds when pH is reduced to 7.0 and drop even further when pH value is at 6.8 [214, 327, 393].

To investigate which part of the G protein is responsible for the viral fusogenic activity, after incubation of wild-type (WT) VSVind virus in acidic pH and comparing the amino acid sequence to that of the neutral pH, four mutations were identified in low-pH adapted VSVind virus: F18L, Q301R, K462R, and H65R. Three out of four changes in the above mutations are to arginine residues, suggesting that positive charges might play a role in pH-induced conformational changes of the G protein [214].

Similar studies on VSVnj.G and CHAV.G reported that both these G proteins have lower pH threshold compared to VSVind.G [214, 327]. Moreover, cell-to-cell fusion assays at mildly acidic pH demonstrated that while VSVind infectivity was completely stopped, VSVnj and CHAV were still able to replicate in infected cells causing them to fuse together, a process known as syncytia formation. This lower pH threshold and higher stability of VSVnj and CHAV in slightly acidic pH have been suggested to be related to higher pathogenicity and more severe outbreaks compared to VSVind, in animals and humans, respectively [214, 327]. Amino acid sequence alignment reveals that VSVnj.G contains the K462R amino acid exchange and CHAV.G contains the F18L.
Interestingly, while no similar data is reported for PIRYV.G, the G protein also contains the K462R amino acid replacement which suggests that PIRYV.G could also have lower pH threshold compared to VSVind.G.

Martinez and Wertz also compared the G proteins of VSV Indiana and New Jersey strains. They reported that while VSVnj.G was still infectious in mildly acidic pH (6.8), the infectivity of VSVind.G was severely reduced. Moreover, VSVnj.G was shown to have a lower pH threshold compared to VSVind.G as its infection was inhibited in the presence of ammonium chloride. These biological differences between VSVind.G and VSVnj.G were linked to the higher pathogenicity of New Jersey serotyped compared to Indiana serotype. Sequence analysis of VSVind.G after repeated culture in pH 6.8 revealed a single amino acid substitution (F18L) to be responsible for adaptation to pH 6.8. Repeated cultures of VSVind.G in lower acidic pH (6.6 and 6.4) exposed additional amino acid substitutions. Moreover, VSVind.G infectivity could be recovered after incubation at neutral pH indicating the reversibility of the conformational changes of the G protein in Indiana serotype [214].
1.5.1.6 Cell tropism of vesicular stomatitis virus

VSV is small, replicates rapidly, can easily be manipulated, and has broad cell tropism, most human cell types and organisms as distant as Drosophila or zebrafish [394-396]. These advantages make this virus a popular model for basic research applications and studies such as vaccine development and pseudotyping other viruses. Although vaccine vectors have been originally developed based on human DNA viruses, in recent years the use of RNA viruses, especially animal RNA viruses like VSV, has been considered. The lack of a pre-existing neutralising antibody is one of the advantages the animal viruses have over the human viruses. Moreover, RNA viruses have rapid growth compared to DNA viruses which could be beneficial in vaccine development and dose amplification [397].

The glycoprotein of VSVind (VSVind.G) is the most widely used envelope as a model in many studies [194, 211]. Besides broad tropism, VSVind.G pseudotyped viral particles demonstrate high physical stability and can produce high titres under ultracentrifugation. Nonetheless, VSVind envelope is highly sensitive to human serum limits their potential for in vivo applications [213]. In addition, VSVind.G is reported to be cytotoxic [201]. Accordingly, studies investigating alternative G proteins have been conducted to replace VSVind.G [237, 238].

1.5.1.7 VSV as a vaccine vector and an oncolytic virus

VSV can be engineered to stimulate the immune system against disease such as influenza and AIDS [397]. Studies have demonstrated that without the need of injection, mucosal vaccination of a live modified VSV has a protective effect against infection: intranasal administration of the live attenuated recombinant
VSV expressing hemagglutinin of influenza or the measles virus, protected the rodent subjects after a challenge with influenza [398] or measles viruses [399], respectively.

The fact that many tumours are either non-responsive to IFN treatment or can develop resistance to the immune system [400] led to studies investigating the ability of viruses to infect tumour cells selectively. This was based on the hypothesis that tumour cells infected by the virus can be cleared by the IFN that is expressed against the virus infection [401]. VSV can be modified to target cancer cells [397]. A recombinant strain of VSVind has been reported to show oncolytic ability where it infected and killed cancer cells in both in vitro and in vivo studies, while did not affect normal cells. While this oncolysis effect was improved massively in the presence of IFN [401-403], VSV was able to infect malignant primary cells with defective IFN response [404]. The efficiency of VSV against different malignancies [405] as well as recombinant VSV coding for the IFN-β gene against hepatocellular carcinomas [406] has been reported. These strains of IFN-inducing VSVs are not only efficient in promoting oncolysis, but they might also be utilised as vaccine vectors [397].

Investigations have been performed to alter the broad tropism of VSV to target specific cells. For this, a single-chain variable fragment (scFv) antibody against the human major histocompatibility complex class I (MHC-1) was linked to the N-terminus of VSV.G. Although LVs pseudotyped with this chimeric VSV.G specifically bound to the MHC-1, their infectivity was reduced due to the loss of fusogenic ability of the altered VSV.G [407].
VSV is neurotropic and can cause lethal CNS infections in rodents and non-human primates [408] and encephalitis in humans [409]. While post intranasal (i.n.) injection of WT VSV into non-human primates, no trace of the virus in the brain or the spinal cord was found, intrathalmic (i.t.) injection caused severe neurological disease [408, 410]. Although the mechanisms of this neurotropism are not clear, they need to be addressed for VSV to be safe for gene therapy and clinical applications [409].

Improved knowledge of the molecular biology and epidemiology of VSV in recent years, enabled scientists to work on engineering new strains of this virus that would be able to function not only as a safe vaccine but also as a killer of malignant cells. Nevertheless, extensive work is needed to investigate the safety and the optimal way of delivery of this virus to human. Moreover, comprehensive investigations are required to study the interaction between the virus and the human immune system as well as the impact of virus shedding on the environment [397].
1.6 Aims of the thesis

VSVind.G is commonly used to pseudotype LVs. LVs pseudotyped with this G protein exhibit high physical stability. This characteristic enables vector concentration via ultracentrifugation yielding high vector titres. Nevertheless, when this protein was expressed continuously in cells, cytotoxicity due to high fusogenic activity as well as superinfection was reported. These have detrimental effects on the cell’s integrity and eventually vector titres.


In the first part of this project, these G proteins were tested for functional lentiviral vector generation as well as for their physical and thermal stability in comparison to VSVind.G, to assess their suitability for high titre vector production.

Chapter 4 of this thesis explored vesiculovirus G proteins’ cytotoxicity via comparing the fusogenicity amongst these G proteins including VSVind.G. Adaptation of all these G proteins to be stably expressed in cells was also examined.

The final part of this project explores the occurrence of superinfection in other vesiculovirus G proteins as well as their receptor usage. Furthermore, the effect of receptor knock out was examined in an attempt to block superinfection.
Materials and Methods
2.1 Cell culture

2.1.1 HEK 293T cells

The human embryonic kidney (HEK) 293T cells are widely used in cell biology experiments and are used throughout this project. These cells are derived from the original 293 cells that were first isolated from the kidney of an aborted human embryo in 1973 [411]. HEK 293T cells stably express the SV40 large T antigen which allows them to be transfected with high efficiency and grow in much higher rate than their parental cell line making them a good candidate for LV production [411, 412].

These cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated Foetal Calf Serum (Gibco), 2mM L-Glutamine (Gibco), 100 units/ml Penicillin and 100μg/ml Streptomycin (Gibco). All cells were kept in cell culture incubators at 37ºC and 5% CO2.

2.1.2 TE671 cells

TE671 are human rhabdomyosarcoma cells. In this project, they were mainly used for the pH-sensitivity assay as they showed robustness, especially when incubated at atmospheric CO2, compared to HEK 293T cells. These cells were also maintained in DMEM supplemented with 10% heat-inactivated Foetal Calf Serum, 2mM L-Glutamine, 100 units/ml Penicillin and 100μg/ml Streptomycin. All cells were kept in cell culture incubators at 37ºC and 5% CO2.
2.1.3 WinPac cells

WinPac (WP) is a lentiviral vector packaging cell line derived from HEK 293FT cells. HEK 293FT cells are a traceable variant derived from HEK 293 cells [412]. The WinPac cells were produced by transfecting a codon optimised gag/pol cassette via recombinase-mediated cassette exchange (RMCE) as well as stable transfection of a plasmid encoding HIV rev protein. Stable transfection of these cells with plasmids encoding RDpro and COCV-G envelope proteins generated WinPac-RD and WinPac-CVG cell lines, respectively [238, 244].

WinPac cell lines were cultured in DMEM containing Glutamax (Gibco) supplemented with 100U/mL Penicillin/Streptomycin (Sigma-Aldrich), and 10% heat-inactivated Foetal Calf Serum (Gibco) at 37°C and 5% CO2. When indicated, antibiotics (Invivogen) were added to the culture medium. Antibiotics and their working concentration are listed in table 2-1.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Mechanism of action</th>
<th>Working concentration (µg/mL)</th>
<th>Vector component selected for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puromycin</td>
<td>Inhibition of protein synthesis</td>
<td>1</td>
<td>Gag/pol</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>Inhibition of protein synthesis</td>
<td>100</td>
<td>Rev</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>DNA cleavage</td>
<td>30</td>
<td>Envelope</td>
</tr>
<tr>
<td>Blasticidin</td>
<td>Inhibition of protein synthesis</td>
<td>10</td>
<td>CRISPR-Cas9</td>
</tr>
</tbody>
</table>

Table 2-1. Antibiotics used in mammalian cell lines.
2.2 Gene transfer to mammalian cells

2.2.1 Transfection of Cells for G Protein Expression

To express VesG on HEK293T cell surface, these were seeded one day prior to transfection at 3x10^6 cell per 10cm plate density. These cells were transfected by lipofection using FuGENE6 (Promega). 5μg/plate of G protein-encoding plasmid was mixed with 20μl/plate of dH2O. When co-transfecting with pHV-Luc, 2.5μg/10cm plate of each plasmid was added. This DNA mixture was then added to a solution of OptiMEM (Gibco) and FuGENE6 (1:5 μg of DNA: μl of FuGENE6), incubated at room temperature for 20-30 minutes and then added to each 10cm plate of HEK293T cells dropwise. 48 hours later, cells were harvested to be used in different assays.

2.2.1.1 Plasmid details

VesG envelopes were cloned into the high-expression plasmid backbone pMD2, driven by a CMV promoter (Figure 3-1A). Amino acid sequences for VSVnj.G (UniProt Accession Number: P04882), PIRYV.G (UniProt Accession Number: Q85213), MARAV.G (UniProt Accession Number: F8SPF4), VSVVala.G (UniProt Accession Number: B3FRL4) were retrieved from UniProt. Codon-optimised genes were ordered from GENEWIZ (NJ, USA) and subcloned into the backbone.

2.2.2 Transient lentiviral vector production and concentration

Three-plasmid co-transfection of 293Ts was used to make lentiviral vectors. 2x10^7 HEK 293T cells were seeded per 15cm plate a day prior to transfection. Cells were then transfected using FuGENE6 with the following plasmids: 2.5μg p8.91 (Gag-Pol and Rev expression plasmid), 2.5μg of envelope expression
plasmids, and 3.75μg of SIN pHV-GFP [green fluorescent protein (GFP)] expressing vector plasmid (Table 2-2).

24 hours post-transfection, the medium is replaced with fresh complete DMEM. 48 hours post-transfection, the vector-containing media is collected for 2-3 days in 24-hour periods. Following collection, the supernatant was filtered through Whatman Puradisc 0.45μm cellulose acetate filters (SLS) and concentrated ~100X using ultracentrifugation [Beckmann Optima LK-90 ultracentrifuge, SW-28 swinging bucket rotor (radius 16.1cm)] at 22,000 rpm (87,119 g) for 2 hours at 4°C. The vectors are resuspended in cold DMEM and incubated on ice for 1 hour before aliquoting and storage at -80°C.

2.3 Plasmid amplification

2.3.1 Transformation of competent cells with plasmid DNA

5-alpha high-efficiency competent E.coli (NEB) was thawed on ice. 1-5µl of 100ng of plasmid DNA/ligation mix was added to 50µl of competent cells, incubated on ice for 30 mins and heat shocked at 42°C for 30 seconds. The cells were then incubated on ice for 5 minutes before adding 950µl of SOC medium (provided by the manufacturer). The mixture was incubated at 37°C for 60 minutes in a shaker at 250 rpm. Following incubation, 100µl of the mixture was spread on LB (Luria-Bertani) agar plates containing 100μg/ml ampicillin (Sigma-Aldrich). The plates were incubated at 37°C overnight to allow colony formation.
2.3.2 Colony screening by PCR

Ampicillin-resistant colonies were screened for the correct construct using Polymerase chain reaction (PCR). GoTaq HotStart Green Master Mix (Promega) was prepared in a final volume of 25μl per reaction. Colonies were picked with a pipette tip, streaked on an agar plate containing 100μg/ml ampicillin (Sigma-Aldrich), and then transferred to PCR mastermix. PCR cycling conditions were as follows: 98°C for 5 minutes; 25-35 cycles (denaturation at 98°C for 30 seconds; annealing for 30 seconds at 65°C; and elongation at 72°C for 30 seconds/kb of the insert); 72°C for 7 minutes. Melting temperatures were calculated using the NEB Tm Calculator: (http://tmcalculator.neb.com/#/).

2.3.3 Plasmid purification

Mini and midi-preps were used for plasmid DNA purification. Single positive colonies were picked from LB agar plates and transferred to 5ml (for mini-
preps) or 100ml (for midi-preps) of LB broth containing 100µg/ml ampicillin. Cultures were grown overnight at 37°C, shaking at 250 rpm.

All plasmids were purified from bacterial cultures using the QIAGEN Plasmid Mini or Midi-prep kits (QIAGEN) according to the manufacturer’s instructions. In this system, LB cultures were pelleted by centrifugation at 4,000 rpm for 30 minutes. Bacteria were resuspended in Buffer P1 and exposed to a detergent in high pH conditions with the addition of Buffer P2 which causes denaturation of plasmid and genomic DNA as well as proteins. While the hydrogen bonds of the base pairs are completely disrupted, the circular structure of the plasmid prevents two DNA strands from separating in the short duration of exposure to these conditions. The addition of Buffer N3 neutralises the aforementioned conditions and enables the reformation of the base pairings in the plasmid DNA. The addition of Buffer N3 also helps co-precipitation of genomic DNA and proteins leaving plasmid DNA in the supernatant. Then plasmids are bound to an anion-exchange resin provided by the kit, washed with Buffer PB and PE once each, and finally eluted via Buffer EB under low salt conditions. After elution, DNA concentrations were determined using a NanoDrop (Spectrophotometer, ND-1000).

2.4 Cloning

2.4.1 Polymerase chain reaction (PCR)

Phusion PCR Kit (NEB) was used to perform PCR reactions. Phusion polymerase was provided in an inactive form requiring activation before the polymerase reaction. This was achieved by incubating the reaction tubes at 98°C for 5 minutes after the samples were mixed with the freshly prepared
master mix and Phusion polymerase. Since the enzyme was inactive at room temperature any endonuclease activity that would degrade primers were avoided as well as making it possible for the reaction to be set up at room temperatures. The reactions were carried out in a PCR thermal cycler (Applied Biosystems, GenAmp PCR system 2700).

2.4.2 Overlap extension PCR

As in most PCR reactions, two flanking primers, one for each end, are used per sequence. To link two DNA molecules, special primers are used at the ends that are to be joined. For each molecule, the first of two PCRs creates a linear insert such that it has a 5' overhang complementary to the end of the other molecule. Following annealing when replication occurs, these extensions allow the strands of the PCR product to act as a pair of oversized primers and the two sequences will be fused. Once both DNA molecules are extended, a second PCR is carried out with only the flanking primers to amplify the newly created double-stranded DNA.
Figure 2-1. Overlap extension PCR. Schematic of overlapping extension PCR. Four primers were designed for the creation of each chimeric protein: flanking primers (a and d) and overlapping primers (b and c) which contain sequences from both templates. Primers b and c create overlapping sequences at each end after the first step PCR. In the second step PCR, the overlapping sequences, which are complementary to each other, bind together then the rest of the sequence is generated. The newly synthesised sequence is then amplified via a and d flanking primers.
2.4.3 Gel electrophoresis, DNA extraction, and purification

Agarose gel electrophoresis was used to separate different sizes of DNA, to analyse PCR and colony screen PCR products as well as to separate and purify restriction endonuclease reaction fragments. 1% agarose gels were prepared by dissolving agarose (Sigma-Aldrich) in Tris/Borate/EDTA (TBE) Buffer. Depending on the final volume of the solution the mixture was heated up in a microwave for 1.5-2 minutes shaking approximately every 30 seconds until the agarose was fully dissolved. The solution was then cooled before adding 0.1µl/mL SyberSafe DNA gel stain (Invitrogen). Gels were set in various casting trays using different sizes of combs depending on need and were left to set at room temperature for 30-45 minutes.

To separate DNA fragments the set gels were placed in electrophoresis tanks containing TBE buffer in orientation in which wells closest and parallel to the cathode. A 1 kb GeneRuler DNA ladder (Thermo-Fisher Scientific) was added to 1 of the wells while samples, positive and negative controls containing DNA loading dye (Thermo-Fisher Scientific) to the other wells. The gels were run using a constant voltage of 100mW for an hour. Lastly, the DNA bands were visualised using a UV transilluminator (UltraBright Blue, Maestrogen), and necessary bands were extracted using a scalpel (Swann-Morton).

The extracted DNA fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN) according to the provided protocol. In short, pieces of gel extracted using a scalpel were dissolved at 60°C in Buffer QG and then centrifuged through an anion exchange resin to which DNA binds to under high salt and in acidic pH conditions created by the buffer. After a wash with Buffer
PE with ethanol, the DNA fragments were eluted with Buffer EB under low salt and high pH conditions.

2.4.4 DNA digestion

Various restriction endonucleases were used in the production of several plasmid constructs. All endonucleases were obtained from New England Biolabs. In a sample reaction, 1µg of the DNA sample was digested with ten units of enzyme/µg DNA, 1X of the buffer with bovine serum albumin (BSA) (according to manufacturer’s instructions) and nuclease-free water (MEB) with the final reaction volume of 50µl. The reaction mix was incubated at 37°C for 10 minutes and was stopped by the DNA loading dye provided by the manufacturer.

2.4.5 DNA ligation

The quick ligation kits used were obtained from NEB and Thermo-Fisher Scientific and T4 DNA ligase was used according to the manufacturer’s instructions for cloning DNA plasmids. In a typical reaction, 50µg of backbone was mixed with the DNA insert in 1:3 molar ratio in a final concentration of 1X. Ligase buffer (supplied by the manufacturer) made up to a final volume of 10 µl with nuclease-free water. The reaction was incubated at room temperature for 5 minutes and chilled on ice until transformation or stored at -20°C.

2.5 Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was used to determine the G protein or GFP expression, on the cell membrane and titrate lentiviral vectors, respectively. FACS is a specialised type of flow cytometry in which
cells are carried by liquid flow in a way so that they pass through laser light beams one cell at a time for sensing. Then a detector measures the forward-scattered light (FSC) and side scattered light (SSC), as well as dye-specific fluorescence signals. FSC is proportional to the cell size while SSC is a measure of cell granularity. All flow cytometry experiments were carried out using FACSCanto II (BD Biosciences) machine and FACS Diva analysis software (BD Biosciences).

To analyse the data, FlowJo single cell analysis software (BD Biosciences) was utilised. This analysis was based on the negative HEK293T cells, which were gated for single cell population based on FSC and SSC data to eliminate any dead cells and debris. These gated cells represent the working population for the assay analysis.

2.5.1 Titration of lentiviral vectors

Vector titres were determined via flow cytometry analysis of GFP expression in vector-transduced HEK 293T cells. Titres are reported as TU/ml indicating how many cells can be transduced with a given volume of the vector. 3x10^5 of HEK 293T cells/well of 12-well plate are seeded in 1mL medium containing 8µg/mL polybrene (Sigma-Aldrich). Cells are challenged with a serial dilution of vectors 3-4 hours after seeding. GFP expression (5-30%) is measured using flow cytometry and the formula below:

Titre (transfection unit/mL) = number of cells seeded*%GFP of positive cells*dilution factor/volume of vector added (mL)
2.5.2 Extracellular antibody staining

HEK 293T cells were transfected based on section 2.2.1. 48 hours after, cells were harvested, washed twice with phosphate-buffered saline (PBS), and plated in U-bottom 96-well plates (Thermo-Fisher Scientific). Cells were then incubated with extracellular antibodies in 1% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS (Sigma-Aldrich) in a total reaction volume of 200μl for 30 minutes at 4°C. After washing twice with PBS to remove unbound antibodies, the cells were incubated for another 30 minutes at 4°C with their respective fluorophore-conjugated secondary antibodies in 1% BSA in PBS in a total reaction volume of 200μl. Cells were then washed twice, fixed in 1% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS, and analysed by flow cytometry.

2.5.3 Intracellular antibody staining

HEK 293T cells were transfected based on section 2.2.1. 48 hours after, cells were harvested, washed twice with phosphate-buffered saline (PBS), and plated in U-bottom 96-well plates (Thermo-Fisher Scientific). Cells were then fixed with 1% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature. After washing twice with PBS, cells were permeabilised by incubation in 0.05% saponin (Sigma-Aldrich) in PBS for 10 minutes at room temperature. The cells were then washed twice in PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 60 minutes at room temperature. These cells were then stained with an optimised dilution of the primary antibody in 1% BSA in PBS for 60 minutes at room temperature in a total reaction volume of 200μl. After washing twice with PBS to remove unbound antibodies, the cells were incubated with the respective secondary antibody in
the same buffer composition for another hour at room temperature. Following two washes with PBS, stained cells were analysed by flow cytometry.

2.6 CRISPR-Cas9 gene editing system

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a nuclease system utilised to mediate DNA cleavage (Figure 2-2A). To improve genome editing in mammalian cells, a codon-optimised Cas9 (CRISPR-associated) gene in association with a specific single guide RNA (gRNA) has been adapted to be delivered to cells using lentivectors. To create individual lentiCRISPR targeting a gene (in this study the LDLR gene) three gRNAs were designed, based on the human LDLR gene sequence (Ensembl genome browser). These gRNAs were 20 nucleotides each and were flanked on their 3’ end by the NGG PAM sequence (Protospacer adjacent motif) which is essential in target binding [411]. While two of the designed gRNA could not be sub-cloned into the plasmid backbone, the three oligo, which targeted the beginning of the open reading frame, was sub-cloned successfully. This oligo was phosphorylated (Table 2.3) and then annealed: 37 °C for 30 minutes, 95 °C for 5 minutes, then ramped down to 25 °C.

The lentiCRISPR plasmid (AddGene) contains two expression cassettes, one for the chimeric gRNA and one for the Cas9 nuclease (Figure 5-5A) [413]. This vector was digested by BsmBI restriction enzyme (NEB) (see section 2.4.4 for DNA digestion). After gel purification of the digested backbone (see section 2.4.3), 1:200 oligos diluted in ddH2O, were ligated into the backbone (Table 2-4) at room temperature. Competent cells were then transformed by this plasmid (see section 2.3.1). To produce LVs, HEK 293T cells were co-
transfected transiently with lentiCRISPR, packaging, and VSVind.G plasmids (see section 2.2.2). Transfected cells were selected with blasticidin for 10 days then were lysed and checked for LDLR gene expression via western blot (Figure 2-2B).

Figure 2-2. CRISPR-Cas9 gene editing system schematic. (A) CRISPR-Cas9 schematic exhibiting complex formation and target binding via sequence-specific gRNA. The Cas9 cleaves both strands of DNA. The cellular repair system attempts to repair the cleaved DNA where it can introduce mutation in the sequence leading to gene disruption. (B) CRISPR-Cas9 workflow for generating gene knock out.
Table 2-3. Phosphorylation and annealing reaction of the oligos.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted oligo</td>
<td>1</td>
</tr>
<tr>
<td>Digested plasmid (50 ng)</td>
<td>X</td>
</tr>
<tr>
<td>2X Quick Ligase Buffer (NEB)</td>
<td>5</td>
</tr>
<tr>
<td>Quick Ligase (NEB)</td>
<td>1</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Up to 11</td>
</tr>
</tbody>
</table>

Table 2-4. Ligation reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo 1 (100 μM)</td>
<td>1</td>
</tr>
<tr>
<td>Oligo 2 (100 μM)</td>
<td>1</td>
</tr>
<tr>
<td>10X T4 Ligation Buffer (NEB)</td>
<td>1</td>
</tr>
<tr>
<td>T4 PNK (NEB)</td>
<td>0.5</td>
</tr>
<tr>
<td>ddH2O</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Table 2-5. Guide RNA sequences used for against the *LDLR* gene.

<table>
<thead>
<tr>
<th>gRNA</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo 1</td>
<td>Oligo1-FW</td>
<td>CCAGCCTCATCCCCCAACCTG</td>
</tr>
<tr>
<td></td>
<td>Oligo1-RS</td>
<td>CAGGTTGGGGATGAGGCTGG</td>
</tr>
<tr>
<td>Oligo 2</td>
<td>Oligo2-FW</td>
<td>GACAACGACTAGACGACGAGCA</td>
</tr>
<tr>
<td></td>
<td>Oligo2-RS</td>
<td>TGCTCGTCTGAGCGCAGTTGTC</td>
</tr>
<tr>
<td>Oligo 3</td>
<td>Oligo3-FW</td>
<td>CGTCGCCTTGCTCTCGCGCG</td>
</tr>
<tr>
<td></td>
<td>Oligo3-RS</td>
<td>CGGCAGGAGCAAGGCCGACG</td>
</tr>
</tbody>
</table>
2.7 SDS-PAGE and immunoblotting

HEK 293T cells (LDLR-wild-type and LDLR-KO; G protein expressing in 10 cm plates were washed with PBS, dissociated with trypsin (Sigma-Aldrich). After spinning and removing the media, the cell pellets were lysed with 0.40 ml per plate of lysis buffer [(25mM Tris-HCl pH7.5, 150mM NaCl, 1% v/v Triton X-100) supplemented with Roche cOmplete™ protease inhibitor cocktail (Sigma-Aldrich)]. The lysates were centrifuged (17,000xg, 20 minutes) and the supernatants containing the cellular proteins were collected. Protein concentrations were determined using a Pierce BCA Protein assay reagent kit (Thermo-Fisher Scientific) using BSA as the standard. After normalisation of protein concentrations, samples were boiled at 95°C for 5 minutes in 5X Laemmli buffer [0.5M Tris-HCl pH6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.2mg/ml Bromophenol blue, 5% β-Mercaptoethanol, and 0.1M dithiothreitol (DTT)] and were resolved by electrophoresis in a 4% (wt/vol) SDS-polyacrylamide staking gel, followed by 10% (wt/vol) resolving gel for 1.5-2 hours at 120 volts. Proteins were then transferred onto a nitrocellulose membrane (GE Healthcare), blocked with 5% (wt/vol) skimmed milk in PBS-T (washing buffer; PBS with 0.1% (v/v) Tween20), and incubated with the indicated antibodies in the blocking buffer. Secondary antibodies conjugated to horse radish peroxidase were visualised by ECL™ Prime Western Blotting Reagent (GE Healthcare) using X-ray machine and CL-XPosure Film (Thermo Scientific).
2.8 Luminescence Assay

After trypsinisation, transfected cells were washed with PBS and plated on white 96-well plates (Thermo-Fisher Scientific) in triplicates at a volume of 50-100μl/well following resuspension in PBS. Bright-Glo™ luciferase assay system (Promega) was utilised to quantify relative protein expression levels. For this, isovolume of the Bright-Glo substrate was added onto the cell suspensions, incubated at room temperature for 5 minutes and luminescence was measured using MicroBeta2 2450 Microplate Counter (PerkinElmer).

2.9 Quantitative real-time PCR (qRT-PCR)

To investigate for the presence/levels of expression of various genes qRT-PCR was utilised. For all qPCR reactions, QuantiTect SYBR Green PCR Kit (QIAGEN) was employed. The SYBR Green dye is an asymmetrical cyanine dye used as a nucleic acid stain in molecular biology. The dye preferentially binds to double-stranded DNA, resulting in a DNA-dye complex that absorbs blue light and emits green light. As the specific DNA sequence is amplified by the polymerase (e.g. Taq DNA polymerase) the amount of double-stranded DNA thus bound SYBR Green amount and the fluorescence of the sample increase. The number of PCR cycles taken to reach a threshold fluorescence (Ct) gives a measure of how much specific DNA sequence was present in the sample at the beginning of the qPCR program. Meanwhile, the presence of ROX, a passive reference dye, compensates for non-PCR-related variations in fluorescence detection. As fluorescence from ROX dye does not change during the qPCR, PCR-related fluorescent signals can be normalised using the ROX signal. Furthermore, to control for non-specific DNA amplification,
which is a potential drawback of SYBR Green-based detection, a melting curve is run, whereby the thermocycler detects the melting point of the PCR product. Detection of a single melting point is indicative of a single PCR product.

Total RNA was isolated from cells using the RNeasy kit according to manufacturer’s protocol (QIAGEN). Using the extracted RNAs, cDNA was synthesised using the First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific) according to the manufacturer’s information. Fold-expression level differences compared to the utilised house-keeping gene (β-actin) were calculated using the Ct values obtained. Beta-actin gene was used as endogenous gene control.

To convert the Ct value into a useful measure of DNA amount, absolute quantification was used throughout this thesis. Therefore, standards containing known quantities of the DNA sequence of interest were included in the qPCR, and Ct values from these were used to calculate a standard curve. Calculations for standards made for qPCR using HIV leader primers to detect reverse-transcribed LV provirus copies. The size of the plasmid was used to calculate the molecular weight of the molecule which was then used to calculate the weight of $10^{10}$ plasmid copies in nanograms (ng). $10^{10}$ plasmid copies/2 μl aliquot of the plasmid was prepared in nuclease-free water which was then serially diluted to obtain $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, $10^1$ plasmids/2μl. Linear regression was later utilised to calculate the number of copies of the DNA sequence of interest in the reactions containing genomic DNA according to the standard curve.
A master mix was prepared according to the manufacturer’s protocol. 23μl of the master mix was added into each well of a 96-well qPCR plate (Agilent). Next, 2μl of the standard, sample or water per reaction was added to corresponding wells to a final volume of 25μl per each well. qPCR plates were sealed with an optically clear adhesive tape (Thermo-Fischer Scientific) and centrifuged briefly. A Stratagene Mx3005P qPCR System (Agilent) was used to run qPCR reactions and the data were analysed using the MxPro Software (Agilent).

2.10 Microscopy

2.10.1 Bright field microscopy

Wide-field light microscopy (Leica Microsystems) was used to visualise cells after Giemsa staining. HEK 293T cells were transfected with various VesG. One hour post incubation in various pH values, cells were washed with PBS, fixed in 100% methanol for 10 minutes, stained with Giemsa stain for 30-60 minutes, and washed with tap water. Stained samples were then visualised using wide-field light microscopy via 10X or 20X objective lens.

2.10.2 Confocal microscopy

HEK 293T cells were transfected with various VesG. One hour post incubation in various pH values, cells were washed with PBS, fixed in 100% methanol for 10 minutes, and stained with WGA (green) and SYTO® 61 (red) for cytoplasm and nuclei, respectively. Stained samples were visualised using SP8 X
confocal Laser Scanning Microscope (Leica Microsystems) via 20X objective lens.

2.11 Appendix

2.11.1 Antibodies

<table>
<thead>
<tr>
<th>Against</th>
<th>Antibody</th>
<th>Host</th>
<th>Manufacturer</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSVind.G</td>
<td>8G5F11 VSV-Poly P5D4</td>
<td>Mouse</td>
<td>Kerafast Dr Hoshino (Japan) Sigma-Aldrich</td>
<td>1:1000 1:500 1:500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Goat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>GAPDH</td>
<td>Mouse</td>
<td>Sigma-Aldrich</td>
<td>1:5,000</td>
</tr>
<tr>
<td>RDpro</td>
<td>Anti-RDpro antiserum</td>
<td>Mouse</td>
<td>Dr Pule (UCL)</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 2-6. List of primary antibodies used.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Manufacturer</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 647 AffiniPure Goat Anti-Mouse IgG</td>
<td>Goat</td>
<td>Jackson Immunoresearch</td>
<td>1:500</td>
</tr>
<tr>
<td>(subclasses 1+2a+2b+3), Fcy Fragment Specific</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 647 AffiniPure Rabbit Anti-Goat IgG, Fc Fragment Specific</td>
<td>Rabbit</td>
<td>Jackson Immunoresearch</td>
<td>1:500</td>
</tr>
<tr>
<td>Amersham ECL Mouse IgG, HRP-linked whole Antibody</td>
<td>Sheep</td>
<td>GE Healthcare</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 2-7. List of secondary antibodies used.
### 2.11.2 Primers

<table>
<thead>
<tr>
<th>Vector</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMD-2A-1A.G</td>
<td>COCV-OL-FW</td>
<td>GCAAAATCAGTGCCACCATGAATTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTTCTCTTGACC</td>
</tr>
<tr>
<td>pMD-2A-2A.G</td>
<td>VSVind-OL-RS</td>
<td>GAGATGAACCGACTTGGAAAAATGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAAATCATTTGC</td>
</tr>
<tr>
<td>pMD-2A-3A.G</td>
<td>VSVind-OL-FW</td>
<td>GAAATTCACGTGGCCACCATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAGTGCTTTTG</td>
</tr>
<tr>
<td>pMD-2A-4A.G</td>
<td>COCV-OL-RS</td>
<td>GCCGCTTTCAAGGAAGTGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCTTTTCC</td>
</tr>
</tbody>
</table>

Table 2-8. Primers used for cloning A and B chimerae.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV-flanking RS</td>
<td>GAATTCTTTAATTTGCCAGTGGTTCACTCTC</td>
<td></td>
</tr>
<tr>
<td>PIRY-flanking FW</td>
<td>ATCAACACGTGTCTAGAGCCACCATGATC</td>
<td></td>
</tr>
<tr>
<td>NJ-flanking FW</td>
<td>ATCAACACGTGTCTAGAGCCACCATGCTG</td>
<td></td>
</tr>
<tr>
<td>COCV-flanking FW</td>
<td>ATCAACACGTGGCCACCATGAT</td>
<td></td>
</tr>
<tr>
<td>PIRY-inner FW</td>
<td>CCAGGGATGGTTCTAGTAGTGAAAGGC</td>
<td></td>
</tr>
<tr>
<td>PIRY-inner RS</td>
<td>GCTTTTCCAATCTACTGAACCATCCCTGATC</td>
<td></td>
</tr>
<tr>
<td>NJ-inner FW</td>
<td>GAGGGCTGGTTCACTAGTGGAAAAGGC</td>
<td></td>
</tr>
<tr>
<td>NJ-inner RS</td>
<td>GCTTTTCCAATCTACTGAACCAGCCCTCCAC</td>
<td></td>
</tr>
<tr>
<td>COCV-inner FW</td>
<td>GAAGGTTGTGTTAGTAGTGGAAAAGCTCT</td>
<td></td>
</tr>
<tr>
<td>COCV-inner RS</td>
<td>CCAACTACTAAACCAACCCTCGATAAGCTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-9. Primers used for cloning the Tail-VesG.
### Gene Primer Sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tr>
<td>LDLR</td>
<td>LDLR-FW</td>
<td>CCAAGTGTCCAGGGAGATGT</td>
</tr>
<tr>
<td></td>
<td>LDLR-RS</td>
<td>TCTCATTTCCTCTGCGAGCA</td>
</tr>
<tr>
<td>VLDLR</td>
<td>VLDLR-FW</td>
<td>GCCCGATTTGCTTAGGACAG</td>
</tr>
<tr>
<td></td>
<td>VLDLR-RS</td>
<td>TGCTAATTTCCTCTGGGGCA</td>
</tr>
<tr>
<td>APOER2</td>
<td>APOER2-FW</td>
<td>CAGTCAGTGCGCTTTATC</td>
</tr>
<tr>
<td></td>
<td>APOER2-RS</td>
<td>AAATCAGGACTGGAGGTG</td>
</tr>
<tr>
<td>Lgr4</td>
<td>Lgr4-FW</td>
<td>TGACTTAAGGACACTGAGGCT</td>
</tr>
<tr>
<td></td>
<td>Lgr4-RS</td>
<td>GGGTTAAGGACACTGAGGCT</td>
</tr>
</tbody>
</table>

Table 2-10. Primers used to quantify gene expression in WT vs LDLR-KO cells.

### 2.11.3 Proteins

<table>
<thead>
<tr>
<th>Virus</th>
<th>UniProtKB/Swiss-Prot Accession Number</th>
<th>Genbank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicular stomatitis Indiana virus (VSVind) – San Juan strain</td>
<td>P03522</td>
<td>M35219</td>
</tr>
<tr>
<td>Maraba virus (MARAV)</td>
<td>F8SPF4</td>
<td>HQ660076</td>
</tr>
<tr>
<td>Cocal virus (COCV)</td>
<td>O56677</td>
<td>AF045556</td>
</tr>
<tr>
<td>Vesicular stomatitis Alagoas virus (VSVala)</td>
<td>B3FRL4</td>
<td>ACB47442</td>
</tr>
<tr>
<td>Vesicular stomatitis New Jersey virus (VSVnj) – Ogden strain</td>
<td>P04882</td>
<td>V01214</td>
</tr>
<tr>
<td>Piry virus (PIRYV)</td>
<td>Q85213</td>
<td>D26175</td>
</tr>
<tr>
<td>Chandipura virus (CHAV) - I653514 strain</td>
<td>P13180</td>
<td>AY614717.1</td>
</tr>
</tbody>
</table>

Table 2-11. Accession numbers of vesiculovirus G proteins.
2.11.4 Cloning and sub-cloning steps

Figure 2-3. Gel electrophoresis displaying various steps of constructs cloning. (A) Gel electrophoresis showing cloning various genes for the pMD.2A plasmid backbone and, (B) various Vesiculovirus G proteins.

Figure 2-4. Gel electrophoresis displaying various steps of cloning the chimeric G protein via overlap extension PCR. Gel electrophoresis showing cloning various segments of each chimerae (upper panel) and the final step of PCR ligating the two overlapping segments to create each of the chimeric G proteins (lower panel).
CHAPTER 3

Physical stability of transiently produced LVs pseudotyped with various vesiculovirus G protein
3.1 Overview

Lentiviral vectors (LVs) are important tools for gene delivery into cells for gene therapy purposes. As LVs use in clinical gene therapy requires highly concentrated and high-purity vectors produced by large-scale preparations, the stability of LVs during production, concentration, and transduction is a vital practical consideration [227, 414]. Overall stability of the vector, mediated by its envelope glycoprotein, is dependent on several factors including temperature, pH, freeze-thaw frequency, and incubation and production conditions (i.e. growth media, CO2 levels).

However, the stability of HIV-1 based vectors has not been well-characterised [415]. Previous studies have demonstrated that when incubated at 37°C half-life of retroviral vectors lies between 3.5-7 hours dependent on the envelope utilised [416-418]. The envelope glycoprotein of vesicular stomatitis virus Indiana strain (VSVind.G) is the most commonly used envelope to pseudotype LVs as it provides the vector particles with high physical stability enabling vector concentration to higher titres using high-speed ultracentrifugation [201, 419].

Stability of the prototype VSVind virus has been investigated from a health and safety perspective. The wild-type virus was remarkably stable in long-term incubation at 4°C demonstrating minimal loss of infection after over a month; however, upon incubation at 37°C complete degradation of the virus and loss of infectivity was observed within 21 days [420]. However, a similar inactivation pattern was not observed when short-term incubation (i.e. up to 30 mins) on viral inactivation was tested. No significant decrease in viral infectivity was
detected when the virus was incubated in the temperature range of 20-46°C, but it was rapidly (i.e. in less than four mins) degraded when incubated at temperatures above 50°C. All in all, the data acquired from this study corroborated previous studies performed on rabies virus and other rhabdoviruses (e.g. mokolo virus, spring viremia of carp virus, and pike fry rhabdovirus) [421]. Therefore, it can be inferred that thermal stability characteristics are shared amongst the rhabdovirus subfamily.

Through directed evolution studies, several key amino acid residues have been identified which confer VSVind.G thermostability [422]. Following several rounds of plaque rescue, several advantageous mutations including K66T, T368A, and E380K were identified which enhanced the G protein’s existing thermostability. This increased thermostability may be the result of formation of new disulfide bond and electrostatic interactions [423]. Furthermore, an increased thermostability may lead to better physical stability and improved vector recovery following freeze-thaw cycles. All in all, the generation or usage of more stable envelope G proteins will aid in the enhancement of vector production and potentially reducing the dosage needed in vivo.

The LVs used in the clinic are produced transiently by DNA transfection of HEK 293T cells with three or four plasmids [229, 424], while a stable producer cell line will have the advantages of being cheaper, easily scalable with less batch to batch variation.

The Collins/Takeuchi group developed packaging cell lines that can support the continuous production of LV, STAR packaging cells [240] as well as WinPac-RD packaging cell line [238]. They initially adopted a non-toxic
gammaretroviral envelope, from the feline endogenous virus RD114, with an altered cytoplasmic tail to allow LV incorporation, RDpro [240]. This was preferred to VSVind.G due to its reported high cytotoxicity and inactivation by human serum complement [211, 213]. Due to limited host tropism and reduced physical stability, which lead to suboptimal titres and virus recovery, the usage of the RDpro packaging cell line is limited.

In 2010, Trobridge et al. published a paper in which lentiviral vectors were pseudotyped with the envelope G protein of Cocal virus (COCV). It has been reported that, in transient production systems, G protein of COCV (COCV.G) can efficiently pseudotype LVs producing relatively high titres ($10^7$ transducing units/mL). COCV.G LVs also demonstrated broad tropism as they could transduce a variety of cells of different origins including human, primate, and dog-hematopoietic progenitor cells. Moreover, compared to VSVind.G-pseudotyped LVs, COCV.G-pseudotyped LVs showed more resistance to inactivation by human and dog serum, which shows promise for in vivo applications [243]. Lastly, some preliminary data suggest that COCV.G might be less cytotoxic compared to VSVind.G when expressed continuously [244]. These favourable characteristics would facilitate affordable large-scale vector production if COCV.G also demonstrates high vector stability like VSVind.G. This would greatly improve the range of clinical applications of LVs and would help propel the field of LV-mediated gene therapy forward [238]. Consequently, our laboratory decided to replace the RDpro envelope in WinPac-RD packaging cell line with that of Cocal virus to generate WinPac-COCV.G cell line. The initial results of the test were rather promising, as
COCV.G seemed to show similar properties to its close phylogenetic relative VSVind.G [244].

Therefore, well-structured research investigating COCV.G and other members of the vesiculovirus genus exploring their ability to pseudotype HIV-1 based LVs and the beneficial characteristics they confer upon the vector particles will be beneficial in the development of gene therapy products more suitable to the clinic.
3.2 Aims

Several vesiculovirus G proteins have been reported to have beneficial characteristics over VSVind.G (e.g. reduced toxicity and nonimmune serum resistance). Therefore, their adaptation to LV producer cells may lead to the generation of promising pseudotypes increasing the therapeutic potential of LVs in gene therapy. The work presented in this chapter was aimed towards evaluating various vesiculovirus G proteins for their ability to pseudotype HIV-1 based LVs and the stability of the pseudotyped particles:

- Investigate whether lentiviral vectors can be pseudotyped with other vesiculovirus G proteins including COCV.G, VSVnj.G, PIRYV.G, CHAV.G, VSVala.G, and MARAV.G.
- Inspect whether other vesiculovirus G proteins are as robust as VSVind.G during vector production, concentration, and storage.
3.3 Results

3.3.1 Development of pMD-2A-VesG constructs

In order to examine the pseudotyping ability of the vesiculovirus G proteins, expression vectors were constructed. To allow for selection of G protein expressing cells a selectable marker, the *Streptococcaceae* *hindustanus* phleomycin inhibitor (Sh ble) was expressed as a fusion protein with the G proteins, separated by a foot-and-mouth disease virus (FMDV) 2A-like self-cleaving peptide [425, 426] (Figure 3-1A). 2A is an oligopeptide (usually 19–22 amino acids) with a cleavage site located between the glycine and the last proline residues on its C-terminal end (indicated by an arrow in below sequence). Successful cleavage during translation results in two cleaved proteins: the protein upstream of the 2A which is attached to the complete 2A sequence apart from the C-terminal proline, and the protein downstream of the 2A which is attached to the proline at the N-terminus [427]. The use of such peptides allows for expression of two (or more) proteins in a single open reading frame controlled by the same promoter. The 2A-like peptide chosen for the construct was derived from the insect virus *Thosea asigna* virus [426] and coded for twenty amino acids: RAEGRGSLLTCGVEENPG↓P.

Codon-optimised 2A peptide sequence as well as sequences of seven different vesiculoviruses, VSVind.G, COCV.G, VSVnj.G, MARAV.G, VSVala.G, and PIRYV.G, were subcloned into high expression plasmid backbone pMD2. As a control envelope RDpro, derived from the feline endogenous gammaretrovirus RD114 with a modifies cytoplasmic tail, was utilised [240], which has previously been adopted to two LV packaging cell
lines in the Collins/Takeuchi group [238, 240]. The G protein sequences were introduced into the C terminal end of the 2A peptide while Sh ble in the N-terminal end. G proteins were subcloned either from previously constructed plasmids or from codon-optimised sequences obtained commercially (see section 2.2.2) generating the pMD-2A-VesG plasmids (Figure 3-1A).

As the first step, pMD-2A-VesG constructs were examined for their functionality to ensure that the presence of the 2A peptide had no adverse effect on the G protein expression. To investigate this, HEK 293T cells were transfected with these constructs. 48 hours later, the cells were stained with goat anti-VSVind.G antiserum (VSV-Poly), and the envelope expression was determined via flow cytometry (Figure 3-1B). Although this antibody was produced against VSVind.G, cross-reaction with other G proteins was observed in earlier investigations (accepted manuscript [428]). Expression of all G proteins was detected on the cell surface. The median fluorescence intensities of the signals detected, except that of CHAV.G, were at comparable levels to that of the saturated signal determined in affinity analyses [428, 429]. The fluorescent signal detected from cells expressing CHAV.G were comparable to that of mock-transfected HEK293T cells (Figure 3-1B). This may be due to lack of cross-reaction of the polyclonal antibody against the CHAV.G protein, or lower CHAV.G expression.

Following detection of expression, the functional LV titres pseudotyped with these G proteins were investigated. LVs were generated via transient 3-plasmid co-transfection. The vector containing media were harvested 48h after transfection and were titrated on HEK 293T cells to determine infectious titres. Titres indicated that all VesG were able to pseudotype HIV-1-based LVs.
yielding functional vectors (Figure 3-1C). Moreover, all VesG except for CHAV.G (6.1x10^4), achieved high titres comparable (COCV- 7.5x10^6; VSVnj- 4.3x10^6; PIRYV- 2.0x10^6; VSVala- 1.0x10^6; MARAV- 1.5x10^6) to that of the widely used VSVind.G (1.1x10^7).
Figure 3-1. Construction of vesiculovirus G protein envelope expression plasmids.

(A) Vesiculovirus G proteins were sub-cloned into the pMD2 plasmid backbone in which the marker gene, Sh ble, and the G protein are linked by the TAV 2A- self-cleaving peptide and therefore are controlled by a single CMV promoter. The Sh ble-G-protein fusion protein is flanked by an upstream bovine beta-globin intron and a downstream bovine growth hormone poly-A site. The restriction endonuclease enzyme sites used for subcloning are marked.

(B) G proteins were expressed in HEK 293T cells and stained with VSV-Poly and analysed via flow cytometry to deduce transient envelope expression levels.

(C) Functional titres of LVs pseudotyped with pMD-2A-VesG were produced by transient 3-plasmid transfection. LV containing media were collected and titrated on HEK 293T cells to determine the infectious titres. Data shown represent relative titres +/- SD for three experiments performed in duplicates. One-way ANOVA with Dunnett’s post-test was performed to compare all VesG to the gold standard VSVind.G (*** p<0.001).
3.3.2 Development of pMD-2A-Chimerae constructs

Mapping key determinant dictating the envelope stability may be critical to tailor these G proteins to improve G protein-containing advanced therapy products as well as improve packaging cell line’s health and LV titres.

Phylogenetic analysis of amino acid sequences of the G proteins, shows that COCV.G is closely related to VSVind.G [335]. The sequence alignment of the amino acid sequences of COCV.G and VSVind.G highlights the structural similarities between these two G proteins. These include N-terminal signal peptide [333] and two N-linked glycosylation sites at amino acids 180 and 337. As well as having the similar 4-domain structure, VSVind.G and COCV.G have 72.5% sequence homology on the amino acid level (see Figure 1-2) [335].

Two sets of chimeric G proteins were generated between COCV.G and VSVind.G (Figure 3-2A). They were produced, based on amino acid alignment, by having the protein junctions in regions where the G proteins are conserved. Eight chimeric G proteins were created: series A (COCV.G N-terminal, VSVind.G C-terminal) and series B (VSVind.G N-terminal, COCV.G C-terminal). These chimerae were sub-cloned into the pMD-2A backbone as the G proteins. The functionality of these constructs was investigated by producing transient LVs pseudotyped with pMD-2A-Chimerae constructs via 3-plasmid LV production system. The unconcentrated titres were then determined by measuring GFP expression 48 post-transduction of HEK 293T cells via flow cytometry (Figure 3-2B). While all pMD-2A-Chimerae produced reasonable crude titres, 3-A titres were around 100-folds lower. To investigate the envelope expression of pMD-2A-Chimerae, HEK 293T cells were
transfected with these constructs. 48 post-transfection, the cells were stained with VSV-Poly, and the envelope expression was measured using flow cytometry. Analysed histograms indicate that all pMD-2A-Chimerae except 3-A transfected cells express envelope G protein on their surface (Figure 3-2C).

To further investigate the low envelope expression in 3-A construct, the transfected cells were also stained intracellularly using a monoclonal anti VSVind.G antibody (P5D4, Sigma). Analysed histograms show higher expression of 3-A in intracellular compared to extracellular staining (Figure 3-2D). This data suggests that the mix-and-match of VSVind.G and COCV.G might have introduced some modifications in 3-A construct which might be affecting the trafficking of this protein to the cytoplasm.
Figure 3-2. Construction of chimeric G protein envelope expression plasmids.
(A) The design and the cross-over points of the chimeric G-proteins are represented in linear diagrams, in which black bars stand for wild-type (WT) VSVind.G sequences and WT COCV.G sequences are represented by white bars. The cross-over points between the two WT sequences is indicated by amino acid number above the bar. (B) A- and B-chimerae were sub-cloned into the pMD-2A backbone. LVs pseudotyped with pMD-2A-Chimerae were produced using 3-plasmid transient production. The titres of these vectors were calculated by measuring GFP percentage via flow cytometry. Data shown represent relative titres +/- SD for three experiments performed in duplicates. (C) Extracellular staining of A-chimerae (left panel) and B-chimera (right panel) with VSV-Poly 48 hours post-transfection. (D) Intracellular staining of A-chimerae (left panel) and B-chimera (right panel) with anti-VSVind.G antibody, P5D4, 48 hours post-transfection. These graphs are representative of one of two independent experiments performed in duplicates. One-way ANOVA with Dunnett's post-test was performed to compare 3-A to VSVind.G and COCV.G (** p<0.001).
3.3.3 Physical stability of VesG-pseudotyped LVs

To establish which vesiculovirus G proteins (VesG) are most promising to generate a stable packaging cell line, we decided to test the physical stability of the VesG-pseudotyped LVs. HEK 293T cells were co-transfected with HIV packaging, VesG or RDpro, and transfer vector (GFP) plasmids. Starting at 48 hours post-transfection, the vector containing supernatant was collected, filtered and kept at 4°C. This was repeated for three consecutive days with 24 hours intervals. On the day of the third collection, the pooled supernatant of each vector was concentrated using high-speed (87,119 g) ultracentrifugation and resuspended in a 1:300 of the original volume. To check the titres of the produced vectors, HEK 293T cells were transduced with both crude and concentrated vectors. The titres were determined by measuring GFP expression using flow cytometry. Figure 3-3 shows that LV with all vesiculovirus envelopes were readily concentrated to around hundred-fold higher titres by centrifugation (COCV- from 3.4x10⁶ to 2.1x10⁸; VSVnj- from 1.9x10⁶ to 2.2x10⁸; PIRYV- from 1.0x10⁶ to 2.2x10⁸; VSVala- from 6.5x10⁵ to 3.3x10⁷; MARAV- from 1.5x10⁶ to 9.1x10⁷), as was reported for VSVind.G (from 3.1x10⁶ to 1.6x10⁸). Moreover, all VesG-pseudotyped LVs could be recovered with high efficiency (overall average approximately 75%) following concentration indicating minimal envelope shedding due to shearing forces of the centrifugation process (Figure 3-3; right Y axis). On the other hand, although CHAV.G could also be concentrated to 100-fold higher (3.5x10⁴ to 1.7x10⁶) implying high physical stability like other VesG, its titres remained low.
Figure 3. High physical stability in VesG pseudotyped LVs. Transient LVs pseudotyped with VesG were concentrated using high speed ultracentrifugation. The titres before and after centrifugation indicate successful concentration of these vectors. For all pseudotypes, the percentage of recovery was higher than 50%, indicating minimal envelope shedding and robust physical stability. The graph represents three repeats from two separate transient LV production; error bars indicate +/- SD for three experiments performed in duplicates. One-way ANOVA with Dunnett's post-test was performed to compare all VesG/RD titres to that of VSVind.G (*** p<0.001; ** p<0.01; * p<0.05).#
3.3.4 Thermal stability of VesG-pseudotyped LVs

To further examine VesG-LVs, the transient vectors were also tested for their thermostability by several cycles of freezing-thawing, incubation at 37°C (heat block) for 2 and 6 hours, and incubation at 4°C (refrigerator) for 24 and 48 hours.

Figure 3-4 demonstrates the crude titres after the vectors went through multiple cycles of freeze-thawing. Data show that titres of most LVs remained stable throughout freeze-thaw cycles. RDpro LV titres were less stable and dropped considerably (~10-fold) after each freeze-thaw cycle compared to VesG LVs while CHAV.G-LV significantly lost infectivity after the second cycle.

Figure 3-5 shows the percentage of recovery of LVs after going through freeze-thaw cycles or incubation at 37°C and 4°C. VSVind.G, COCV.G, VSVnj.G, and PIRYV.G did not lose infectivity after freeze-thaw cycles, also, COCV.G recovery seemed to exceed that of VSVind.G (90% vs 85%, respectively (p<0.001). VSVala.G and MARAV.G infection dropped from around 80% to approximately 70%, and RDpro infection dropped from around 60-50% compared to their respective original titres.

VSVind.G and COCV.G demonstrated similar sensitivity to incubation at 4°C. Although a slight decrease in infectivity was observed, 10% after 24 hours and a further 10% after 48 hours, overall both pseudotypes were stable. Similarly, VSVnj.G and MARAV.G were robust and retained infection levels at more than 80% for up to 48 hours of incubation. VSVala.G infection dropped slightly from around 90-70%. In contrast, PIRYV.G seemed to be less stable at 4°C compared to other VesG as the percentage of infectivity dropped from around
70% after 24 hours incubation to around 60% at 48 hours. CHAV.G LVs were substantially sensitive to incubation at 4°C losing more than half of their original titres within 24 hours. However, following this initial loss of titre, the infectivity remained constant (around 30-40%). RDpro LVs were also highly sensitive to incubation at 4°C; their infectivity dropped from around 60% after 24 hours to around 20% after 48 hours.

Furthermore, COCV.G, VSVala.G, and MARAV.G seemed to be following a similar pattern of retaining around 90% infectivity for up to 6 hours at 37°C. VSVind.G and VSVnj.G LVs infectivity remained stable (around 100%) for up to two hours of incubation at 37°C however, infectivity dropped to around 80% after additional 4 hours of incubation. RDpro-LVs retained stable infectivity (around 70%) for up to 6 hours of incubation while CHAV.G infectivity dropped from around 70% after 2 hours of incubation to around 60% up to 6 hours of incubation at 37°C.
Figure 3-4. Sensitivity of VesG-LV to repeated cycles of freezing and thawing. VesG and RDpro LVs went through three cycles of freeze-thawing (x-axis). HEK 293T cells were then transduced with these vectors and 48 hours later the titres were measured by GFP expression using flow cytometry. Error bars indicate mean titres +/- SD of three repeats performed in duplicates, from two separate batches of transient LV productions. One-way ANOVA with Dunnett's post-test was performed comparing 1x, 2x, 3x to the control column of 0 (* p<0.05).
Figure 3-5. Thermostability of VesG pseudotyped LVs.
The relative titres of VesG-LV were calculated after LVs underwent several different thermostability assays (i.e. freeze-thaw, incubation at 4°C and 37°C). Data represent the mean value of three repeats performed in duplicates, from two separate batches of transient LV productions. The titres are presented as a percentage of titre before commencing the thermostability test. One-way ANOVA with Dunnett's post-test was performed to compare all VesG to the gold standard VSVind.G (*** p<0.001; ** p<0.01; * p<0.05). The significance of a selection of comparisons are indicated on the graph.
3.4 Discussion

Maintaining virus viability over long periods with little or no change of infectivity is valuable for research and clinical applications. The manufacture of gene therapy products comprises several steps: vector production and purification, storage, and application of the vectors for gene transfer. Throughout these steps, vectors can get inactivated which can affect the final quality and efficacy of the vector preparations as well as vector transduced cells [430]. In addition, the efficiency of transduction into primary cells using viral vectors depends on the initial viral titres applied and on the stability of vector during the transduction period [431, 432]. Moreover, compounds of cellular origin released into the medium might affect gene transfer results negatively [433].

For ex vivo transduction, cells are isolated from a patient and externally propagated. In that case, the stability in growth medium is predominant. For in vivo applications, vectors are mostly delivered to the bloodstream and therefore the stability in the human blood is of interest. Both procedures require stable particles. Additionally, in the in vivo approach, the biological stability of the vectors in the presence of the immune system is of great importance [434].

Lentiviral vectors are used commonly for gene transfer protocols while their use is expanding rapidly in gene therapy clinical trials. These vectors present the main advantage of being able to insert the gene of interest into the host cell genome in both dividing and non-dividing cells [435, 436]. Nevertheless, the production of lentiviral vectors presents several problems. One of the most critical is the low stability of these vectors. For in vitro cell transduction, crude (unconcentrated) vector stocks are often sufficient, whereas concentrated
vector stocks are needed for in vivo applications. Concentrating lentiviral vector stocks are typically performed using ultracentrifugation approaches [437, 438]. This is a rapid and robust method for vector concentration at a small scale. However, the low stability of the vectors affects their production, storage, and efficacy in preclinical and clinical settings, making high-quality clinical preparations a challenging goal to achieve [430]. At 37°C, these vectors are less stable, which is a critical issue concerning the efficacy of viral preparations in clinical studies. During purification and short-term storage, LVs are usually kept at 4°C while for long-term storage; they are kept at -80°C however, freeze-thaw has a detrimental effect on viral titres [439].

Amongst other advantages, to increase the overall vector particle stability, LVs are commonly pseudotyped with VSVind.G proteins. Nevertheless, due to some drawbacks pertaining to VSVind.G, alternative envelopes have been studied for LV pseudotyping. The work presented in this chapter aimed to investigate the stability of lentiviral vectors at temperatures relevant for production and usage: 37°C and 4°C, in freeze-thaw cycles as well as to check the physical stability of the vectors by ultracentrifugation.

### 3.4.1 Development of vesiculovirus G protein-pseudotyped lentiviral vectors and their physical and thermal stability

Transient LVs pseudotyped with VSVind.G, COCV.G, VSVnj.G, PIRYV.G, CHAV.G, VSVala.G, MARAV.G, and RDpro were produced to establish which vesiculovirus are most promising to generate a stable packing cell line with. As there are only specific commercial antibodies available against VSVind.G, All VesG were stained with goat anti-VSVind.G antiserum, VSV-Poly, which
can cross-react with VesG other than VSVind.G (accepted manuscript [428]) in order to examine the expression of the G proteins on the cell surface following transfection of HEK 293T cells.

Figure 3-1 demonstrates that all VesG except CHAV.G were able to pseudotype LVs with titres comparable to that of VSVind.G and exceeding that of RDpro. Furthermore, they could readily be concentrated by ultracentrifugation at 22,000 rpm (87,119) for 2 hours at 4°C. As VSVind.G pseudotyped LVs are stable, these vectors were expected to produce high titres before and after concentration. All VesG pseudotyped LVs except for CHAV.G, produced crude titres of around $10^6$ TU/mL and concentrated titres of around $10^8$ TU/mL or higher (Figure 3-2). This shows that these VesG can produce LVs with high vector particle stability. CHAV.G produced LVs with crude titres of around $10^4$ TU/mL and concentrated titres of around $10^6$ TU/mL, which are considered too low titres to be useful for gene delivery purposes.

VesG vectors were also stable during freeze-thawing achieving recovery levels of minimum 80% on average (CHAV.G excluded). Under different incubation conditions, the infectivity recovery varied amongst different G proteins (Figure 3-4). Overall, COCV.G, VSVnj.G, VSVala.G, and MARAV.G, similar to VSVind.G, seemed to be stable under all three conditions. PIRYV.G was stable during freeze-thaw cycles and at 37°C, yet the percentage of recovery after 48 hours incubation at 4°C dropped to around 60%.

In contrast, CHAV.G and RDpro vectors were less stable after freeze-thawing and incubation at 4°C however, short-term (<6 hours) incubation at 37°C did not decrease their infectivity dramatically ($p<0.001$). Due to CHAV.G
pseudotyped LVs demonstrating low titres even after concentration (p<0.001), as well as the low performance in thermostability experiments, we decided to exclude this G protein from our further experiments.

In summary, we have compared, by identical technique, the effect of temperatures, centrifugation and freeze-thawing upon the viability of HIV-1 based LVs pseudotyped with VesG. All G proteins except for CHAV.G were overall stable in all conditions demonstrating decay rates comparable to that of VSVind.G. Their resistance to 37°C incubation is promising as it will allow systemic and local delivery of LVs in vivo without the concern of body-temperature dependent loss of titre. Furthermore, robust vector stability demonstrated at 4°C and during freeze-thaw cycles is useful for short and long-term storage of the vectors. Taken together, these results support further consideration of COCV.G, VSVnj.G, PIRYV.G, VSVala.G and MARAV.G LV for their use in stable packaging cell lines for preclinical and clinical applications.

### 3.4.2 Development of chimeric G proteins and their functionality

To investigate the key elements affecting the envelope stability, based on G proteins of VSVind and COCV, I created eight mix-and-match chimeric G proteins. Four of these chimerae (A-chimerae) have COCV.G N-terminal and VSVind.G C-terminal; while the other four (B-chimerae) have VSVind.G N-terminal and COCV.G C-terminal. These chimeric G proteins were sub-cloned into the pMD-2A backbone as the G proteins.
Overall, all chimeric G proteins were successfully produced, except 3-A, demonstrated similar transduction ability and protein display characteristics compared to their wild-type counterparts (figure 3-1). Although highly conserved amino acid sequences were targeted in the generation of the chimerae, 3-A’s LV titre was significantly lower than the other seven G proteins (p<0.001). Antibody binding assays revealed that 3-A faced membrane trafficking issues and was trapped in the intracellular compartments of the cell (figure 3-5C/D). The cross-over point of 3A corresponded to the lateral domain of VSVind.G and is close to one of the N-linked glycosylation sites of the WT protein [2, 332]. Therefore, the switch of this domain with that of COCV.G might be hindering post-translational modifications preventing the protein to be transported to the cell membrane. In addition, although there were no substantial stability differences between the chimerae they were later utilised to map serum sensitive determinants on VSVind.G (in press [440]). Thus, generation of such chimerae with PIRYV.G or CHAV.G could provide insights on other biochemical characteristics of VesG including thermal stability. Partly because no substantial difference in stability between VSVind.G and COCV.G was observed, these chimeric constructs were not studied for stability.

Furthermore, understanding the destabilising mechanism enforced on VesG pseudotyped vectors in variable conditions will provide valuable insights regarding approaches to improve vector stability further. In addition, the effect of thermal and physical conditions on HIV-1 capsid and matrix proteins which have essential roles in LV structure should also be investigated. Combined, this will allow modification of LVs and G protein-containing advanced therapy
medicinal products to generate more stable regents with better longevity under critical manufacturing conditions.
CHAPTER 4

Investigating the fusogenic activity and cytotoxicity of the vesiculovirus G proteins
4.1 Overview

Vesicular stomatitis virus (VSV) is a well-studied virus with a small genome that can be easily manipulated. In addition to its broad tropism and ability to infect target cells with high efficiency, VSV has intrinsic oncolytic properties [403]. These characteristics of VSV, as well as its short replication cycle, makes it a promising platform for oncolytic virotherapy [441].

VSV pathogenesis in natural hosts depends on the virus serotype. The two major serotypes of VSV, Indiana and New Jersey, are endemic in Central and South America and some regions of the USA. Their natural hosts include cattle, pigs, horses, and their insect vectors. However, VSVind and VSVnj do not have the same pathogenicity; VSVnj causes more frequent and severe outbreaks compared to VSVind strain [214, 405].

The high toxicity of VSV was mainly attributed to its matrix protein (M). This protein promotes viral pathogenesis by inhibiting host gene expression, acting on both nuclear transcription and RNA transport, and inducing apoptosis [442]. Nevertheless, M-mutant VSVs were found to trigger apoptosis via a distinct pathway compared to wild-type VSV. This has been shown to be due to fusion caused by the viral G protein.

It was reported that VSV infection could lead to cell fusion when G protein accumulates on the cell surface [443]. Cell fusion was shown to be dependent on the interaction between positively charged amino acid residues in G proteins and the negatively charged membrane surface [444]. In addition, protonation of histidine residues (His) in acidic pH has been reported to promote fusion [382]. VSV enters the cells through receptor-mediated
endocytosis. The acidic environment of the endosome induces a fusion-competent form of the G protein allowing the viral envelope to fuse with the endosomal membrane releasing the viral RNA genome into the cytoplasm [445]. This fusogenic activity is claimed to be a sign of toxicity for VSV G protein [215]. After infection, the adjacent infected cells are fused together to form large multinucleated syncytia. A similar syncytia formation mediated by viral envelope protein has been reported for other viruses such as Gibbon Ape leukaemia virus and respiratory syncytial virus where the resulting syncytia are destined to apoptosis [217, 218].

To improve the vector particle stability as well as to increase its tropism, LVs are commonly pseudotyped with vesicular stomatitis virus Indiana strain G protein (VSVind.G) [129, 211, 446]. To generate clinically suitable LVs however, stable production of these vectors is advantageous as this will aid to improve biosafety by standardising the production process and reducing the risk of DNA recombination. In addition, a stable producer cell line will reduce the cost of producing large batches of vectors required for clinical and some preclinical use in large animals [233, 238]. The development of a stable packaging cell line for LVs proved to be challenging due to cytotoxicity of some viral components such as the Gag-Pol proteins. Moreover, VSVind.G has been reported to be toxic to cells when expressed continuously [211, 238] which was attributed to its high fusogenic activity causing syncytia formation and apoptosis. This affects the integrity of the stable producer cells utilising this G protein affecting vector titres. Several cell lines have been developed so far either by using alternative G proteins or inducible expression, however; they demonstrated either low titres or limited tropism [447-450].
To overcome the toxicity issue of VSVind.G, other vesiculovirus G proteins have been proposed as alternative proteins. However, no studies have been done to investigate whether the cytotoxicity issue is shared amongst these family members. Therefore, a detailed analysis of their cytotoxicity and pH-dependent fusogenicity will be instrumental in their adaptation into PCLs.
4.2 Aims

The work presented in this chapter aimed at investigating the fusogenic activity and relative cytotoxicity of the vesiculovirus G proteins. Initially, the effects of pH on VesG mediated syncytium formation and VesG-LV were explored. The difference in G protein-mediated cytotoxicity was also examined, and the generation of cell lines stably expressing VesG was attempted for LV production.
4.3 Results

4.3.1 Fusogenicity in vesiculovirus G protein expressing cells

Cocal virus envelope G protein (COCV.G) has been utilised to generate a stable producer cell line for LV production [237]. VSVind.G and COCV.G have a high level of homology on the amino acid level (~72%) conferring them some shared advantageous characteristics including stability and broad tropism. Yet, it has been suggested that COCV.G is less cytotoxic compared to VSVind.G [237, 243]. Consequently, the fusogenicity of COCV.G under mildly acidic pH in comparison to VSVind.G was investigated, as a reference for toxicity.

First attempts were carried out using HEK 293T cells. However, they proved to be pH sensitive and easily detached from the plate after incubation at acidic pH and atmospheric CO₂ conditions. Therefore, TE671 cells (human rhabdomyosarcoma cell line) were utilised instead as they are less pH sensitive compared to HEK 293T cells. TE671 cells were co-transfected transiently by a luciferase expressing plasmid, pHV-Luc (as transfection efficiency control), and VSVind.G or COCV.G. 72 hours post-transfection, media was replaced with fresh Minimum Essential Medium (MEM) adjusted to pH 6.0 and 5.0. Cells were incubated at 37°C in atmospheric CO₂ for one hour, were washed and fixed in methanol. The cells were then stained with Wheat germ agglutinin (WGA) and SYTO® 61 for cytoplasmic membrane and nucleus staining, respectively. The cells were then visualised using a Leica SP8 X confocal Laser Scanning Microscope (Figure 4-1).
Interestingly, at neutral pH, both VSVind.G and COCV.G formed a few syncytia compared to un-transfected cells (approximately 1-2 per field of view). When the pH value was reduced to 6.0, an expanded cytoplasm was observed in un-transfected, yet no fusion was observed in these cells. This change in the shape might be due to the acidic extracellular condition affecting the intracellular pH and Ca\(^{2+}\) [451, 452]. On the other hand, both VSVind.G and COCV.G expressing cells demonstrated a higher number of syncytia per field of view compared to that of neutral pH. While both G proteins induced cell fusion at this pH, the syncytia formed by VSVind.G expressing cells were visibly larger and contained more nuclei (>10 nuclei) compared to that of COCV.G.

At pH 5.0, while un-transfected cells retained their elongated shape, no fusion was observed. In transfected cells, however, cells seemed very unhealthy; expanded cytoplasms of cells were fused to generate large syncytia. Multinuclear syncytia of at least eight nuclei were observed and in places, especially in VSVind.G expressing cells, all remaining cells seem to have fused together. The morphology of fused cells in both transfected cells were rather different from un-transfected cells.
Figure 4-1. Fusogenicity in VSVind.G and COCV.G expressing cells. TE671 cells were co-transfected with pHV-Luc and plasmids expressing VesG. (A) Luciferase activity was checked with Bright-Glo™ Luciferase Assay system as a reporter for transfection efficiency. Data shown is the mean value of one experiment performed in duplicates. (B) One-hour post incubation in various pH values, cells were washed, fixed, and stained with WGA (green) and SYTO® 61 (red) for cytoplasm and nuclei, respectively. Stained samples were visualised using a Leica SP8 X confocal Laser Scanning Microscope. Shown images are magnified by 20X objective lens.
The fusogenic activity has been related to cytotoxicity in VSVind.G [215] while COCV.G was reported to be less cytotoxic. Yet, VSVind.G and COCV.G displayed similar characteristics regarding fusion and syncytia formation in acidic pH. This suggests that COCV.G might be as toxic to cells as VSVind.G. To further investigate the syncytia formation of other VesG, in addition to VSVind.G and COCV.G, VSVnj.G and PIRYV.G were also incubated in pH 7.0, 6.0, and 5.0. A similar protocol to above was followed by cell staining with Giemsa stain to check for syncytia formation. The cells were then checked using bright-field microscopy (Figure 4-2).

At pH 7.0, 6.0, and 5.0, as expected, cell fusion was observed in both VSVind.G and COCV.G expressing cells. For both, the syncytia looked smaller in neutral pH while in acidic pH they seemed larger and included a higher number of fused cells (i.e. higher numbers of nuclei per syncytia). At pH 5.0, cells seemed to be very unhealthy compared to those of un-transfected cells, as they were lifted off the plate in both VSVind.G and COCV.G but looked more severe in VSVind.G.

On the other hand, expression of VSVnj.G and PIRYV.G did not lead to cell fusion in neutral pH. However, upon acidification of the environment, syncytia were formed in both G protein-transfected cells. At both pH 6.0 and 5.0, VSVnj.G induced smaller syncytia containing less number of cells, and the morphology of the cells was similar in both these pH values. PIRYV.G however, induced the formation of larger syncytia in both pH 6.0 and 5.0. Moreover, the number of syncytia formed seemed higher in PIRYV.G compared to VSVnj.G (approximately double). The level of fusogenicity of this
G protein seemed to be higher in pH 6.0 compared to pH 5.0 as most cells seemed to either be involved in fusion or very unhealthy.

Overall, VSVind.G and COCV.G demonstrated similar characteristics in terms of cell fusion and syncytia formation that was distinct from that of VSVnj.G and PIRYV.G infected cells. This might be due to differences in fusogenicity, lower pH threshold for fusion, or other characteristics of the G protein such as the nature of the conformational changes the VesG go through. Next, we decided to investigate the effect of pH on vector infectivity in more detail.
Figure 4-2. Fusogenicity vesiculovirus G protein expressing cells. TE671 cells were co-transfected with pHV-Luc and VesG. (A) Luciferase activity was checked with Bright-Glo™ Luciferase Assay system as a measure of transfection efficiency. (B) Envelope expression in transfected cells stained with VSV-Poly antiserum. (C) One hour post incubation in various pH values, cells were washed, fixed, and stained with Giemsa stain. Stained samples were visualised using wide-field light microscopy. Shown images are magnified by 10X objective lens.
4.3.2 Vesiculovirus infectivity levels in various pH values

In infections by enveloped viruses, the fusion of the viral membrane with the host cell follows the initial step of receptor attachment. In VSV, the G protein is the sole viral protein that mediates both attachment and fusion. A three-stage model has been proposed for VSV.G fusion: first, the G protein undergoes pH-dependent conformational changes from the pre-fusion to an extended open form. This open conformation results from protonation of each G monomer. The extended form of G protein directs the fusion loops positioned in the Plekstrin homology domain (PHD) towards the target membrane. The adjacent extended monomers are then trimerised and fold back to bring the two membranes together [2, 332, 453].

While pH-dependent conformational changes of VSVind.G has been well studied, no data have been reported for other vesiculovirus G proteins. Hence, we planned to investigate the effect of pH changes on VSVind.G, COCV.G, VSVnj.G, and PIRYV.G pseudotyped LVs. VesG pseudotyped LVs were incubated at 37°C for 30 minutes in pH values ranging from 2.0 to 10.0. These LVs were then plated on HEK 293T cells in neutral pH at MOI 1.0. 48 hours post-transduction, the infectivity of VesG-LVs was investigated by measuring GFP expression in challenged cells (Figure 4-3).

Figure 4-3A demonstrates that all LVs remained infectious after incubation at pH values between 10.0 and 5.0. In pH ranges lower than 5.0 (i.e. 4.0 and 2.0), the infectivity of all LVs reduced gradually, then was completely abrogated (i.e. dropped to 0% GFP expression) at pH 2.0. These results were in line with previous studies reporting that VSV is more sensitive to acidic
conditions compared to alkaline conditions [454]. Moreover, all four LVs followed a similar pattern of infectivity at various pH values to that of VSVind.G. Accordingly, we decided to investigate the effects of lower pH ranges more in-depth. Therefore, the experiment was repeated where LVs were incubated in pH ranges from 7.3 to 2.0, with 0.3-0.4 intervals (Figure 4-4B). The normalisation of the GFP expression to that of pH 7.0 revealed that at pH between 3.6 and 3.3, the infectivity of VSVind.G, VSVnj.G, and PIRYV.G drop drastically. At pH 3.3, while VSVind.G-LV infectivity reduced by more than half, GFP expression was almost undetectable in cells challenged with VSVnj.G- and PIRYV.G-LV. COCV.G infectivity seemed to reduce by 50% when the pH was decreased from 4.0 to 3.6. COCV.G-LV infection was completely blocked by reducing the pH further to 3.3. These results may suggest that the pH threshold of COCV.G conformational change is slightly higher than the other G proteins tested. All VesG infectivity demonstrated similar trends between pH 6.0 and 5.0, infectivity remaining stable compared to neutral pH.

Overall, the data show that the inactivation point for all LV’s infectivity lies between pH 3.0-3.6 with pH 3.0 being the endpoint of infectivity for all VesG. This contradicts previously published data reporting pH 2 as the point of deactivation for recombinant VSVind virus [454]. Furthermore, at pH levels below 5, VSVind.G and VSVnj.G seem to be more infectious compared to COCV.G and PIRYV.G (COCV.G/PIRYV.G infectivity approximately around 40-50% while NJ/IND around 70-80%). However, until pH 3.6 and below, overall infectivity of all VesG was retained at >50%. This implies that all VesG tested can go through a wide range of pH changes during which they can
remain infective. In addition, no striking difference was observed between strains, and all VesG demonstrated a similar overall response to pH changes.
Figure 4-3. pH-dependent inactivation of VesG-LV.
VSVind.G, COCV.G, VSVnj.G, and PIRYV.G pseudotyped LVs were incubated at 37°C for 30 minutes in pH values ranging from 2.0 to 10.0. These LVs were then added on HEK 293T cells in neutral pH at an MOI of 1 TU per cell. (A) The percentage of GFP expression indicative of LV infectivity, was measured by flow cytometry 48 hours post-transduction. The percentage of live cells is indicated in dotted lines (right axis). (B) The experiment was repeated with more in-depth investigation of pH range 2-7.3. Measured GFP expression was normalised to that of at neutral pH. Data represent mean of three repeats done in duplicates.
Following the establishment of pH-dependent VesG-LV inactivation by testing the infectivity of VesG-LV pre-treated at different PH, we decided to investigate the effects of pH on viral attachment and following steps, by conducting the infection while cells are incubated at different pH range. For this, cells were challenged with LVs via spinoculation in media adjusted to a range of pH. After the spin (2,000 g, for 30 minutes at 4ºC), the LV containing media with different pH were removed from the cells and was replaced with media with neutral pH. 48 hours post-infection, the GFP expression was measured in cells. This experiment was based on the hypothesis that at different pH range, G proteins go through conformational changes resulting in pre- or post-fusion forms. While in the pre-fusion form, the G protein will be able to interact with its putative receptor and enter the cells, the change to the post-fusion structure will block this interaction.

LV infectivity was completely abrogated between pH 3.3 to 3.6, while cell viability was more than 50% (Figure 4-4A). This profile is similar to that shown in Fig 4-3B, suggesting that the observation resulted, at least partly, from pH-dependent VesG-LV inactivation. A maximum level of infection was observed at pH 6.0 to 6.6 implying the highest level of interactions and attachment of G proteins to receptors and internalisation at this pH range. In some cases even direct fusion might have been achieved as the pH range in early and late endosomes, where VSVind.G-LV fuses, are around 6.5 and 5.5, respectively [455]. In line with this, Figure 4-4B shows the relative infection rates at different pH compared to that of neutral pH. It can be observed that the infectivity levels gradually increase as pH decreases from 7.0 to 6.0, reaching a maximum around pH 6.0-6.3.
Furthermore, compared to VSVind.G and VSVnj.G, COCV.G and PIRYV.G lost infectivity much faster, especially for COCV.G where the infectivity dropped suddenly when the pH was reduced from 6.0 by only 0.4 points to 5.6. On the other hand, VSVind.G and VSVnj.G still had higher infectivity at pH 5.0-5.3 compared to pH 7.0 suggesting a lower pH threshold for shifting to postfusion trimerisation for these G proteins compared to COCV.G and PIRYV.G.
Figure 4-4. Effect on LV infection of pH at LV attachment to the cell. HEK 293T cells were challenged with VesG LVs via spinoculation in the presence of media adjusted to various pH. Following spinoculation the media was removed, cells were washed, and complete media in neutral pH was added. GFP expression and live cell percentage was measured 48 hours post-challenge via flow cytometry. (A) Measured GFP expression was normalised to that off pH7 control. Dotted lines represent the percentage of live cells (right axis). (B) The relative infection change compared to neutral pH 7.0. Data represent mean of three repeats done in duplicates.
4.3.3 Investigating the cytotoxicity of vesiculovirus G proteins

As discussed in section 3.3.1, to allow for the selection of G protein expressing cells, the phleomycin resistance gene was linked to the G protein via the 2A self-cleaving peptide in the pMD-2A-VesG constructs. The use of 2A peptide allows for both the G protein and the marker gene to be controlled by a single promoter. This allows the co-expression of both genes at a more similar ratio compared to genes expressed under two separate promoters [427]. This feature was essential for this assay enabling us to have a more reliable comparison of expression of different VesG after phleomycin selection.

The high fusogenic activity of VSVind.G is linked to high cytotoxicity through syncytia formation and apoptosis [201]. On the other hand, COCV.G, a phylogenetically close vesiculovirus G, was reported to be less cytotoxic compared to VSVind.G [237]. However, the data presented thus far through investigating syncytia formation and the effect of pH changes on various VesG, demonstrated that the VesG tested do not exhibit major differences. This suggested that all VesG might be equally cytotoxic.

Accordingly, to compare relative toxicity levels amongst different VesG, HEK 293T cells were co-transfected with pHV-Luc and VesG including VSVind.G, COCV.G, VSVnj.G, PIRYV.G, VSVala.G, and MARAV.G. 48 hours later, the luciferase activity of the cells was checked to determine relative transfection levels (Fig 4-5A) and 2.5x10^4, 5.0x10^3, and 1.0x10^3 of transfected cells per well were seeded in 6-well plates. These cells were selected with phleomycin for up to one week to ensure the selection of G protein expression. After seven
days of selection, G protein expression was measured on cells by staining with VSV-Poly antiserum (Figure 4-5B). Cells were then washed with PBS, fixed in methanol and stained with Giemsa stain. The number of colonies grown in cells transfected with various G proteins was investigated (Figure 4-5C). While higher levels of cytotoxicity would lead to reduced numbers of colonies, higher numbers of colonies indicate less cytotoxicity. In this assay, VSVind.G was used as the positive control for cytotoxicity [215] while RDpro envelope, a non-toxic feline retrovirus, was used as negative control [238].

Interestingly, compared to RDpro, the negative control (in LFA backbone which was adapted to WinPac-RD cell line [238]), all envelopes yielded a similar number of phleomycin resistant colonies, with approximately one colony per one hundred transfected cells. Colony numbers in VSVind.G, the positive control, seem to be higher in all cell dilutions compared to that of COCV.G and RDpro. This data challenges the well-accepted concept of VSVind.G being highly toxic to cells [201]. The similar number of colonies formed by all VesG in all dilutions were comparable to that of non-toxic RDpro indicating that all tested G proteins demonstrated similar levels of toxicity.
Figure 4-5. Colony formation of various vesiculovirus G proteins in the presence of the selection marker.
To compare the cytotoxicity in various VesG, HEK 293T cells were co-transfected with pHV-Luc and pMD2-2A-VSVind.G, -COCV.G, -VSVnj.G, -PIRYV.G, and RDpro. 48h post-transfection; (A) Luciferase expression was checked as a measure of transfection efficiency. Data shown mean of two repeats performed in duplicates/triplicates. (B) After 7 days in selection, cells were stained for G protein expression by VSV-Poly antiserum. (C) Cells were seeded in different dilutions (seeding density is indicated) and were selected with phleomycin for one week, then fixed and stained with Giemsa. Similar number of colonies were formed for each construct suggesting that all these G proteins have similar levels of cytotoxicity. Data shown represent one of the two separate repeats performed.
4.3.4 Long-term G protein expression and support for LV production

Based on obtained data highlighting that all VesG share similar level of cytotoxicity, comparable to that of non-toxic RDpro, we decided to investigate the long-term expression of G proteins and the effect this might have on viral titres. For the first attempt, HEK 293T cells were transfected with VSVind.G, COCV.G, VSVnj.G, and PIRYV.G plasmids. These cells were kept in phleomycin selection as bulk populations for up to 14 weeks. During this period, the envelope expression was monitored every two weeks using the antiserum VSV-Poly. VSV-Poly binds to VSVind.G with high affinity, while it has an intermediate affinity to COCV.G and weak affinity to VSVnj.G and PIRYV.G (Figure 4-6A). The flow cytometry histograms indicated that although G protein expression gradually decreased over time in all stable VesG expressing cells (Figure 4-6B), VSVind.G and COCV.G could still be detected for up to 22 weeks (Figure 4-6C). Cells were also transiently transfected with $gag/pol$, $rev$, and $SIN-pHV$ (coding for GFP transgene) over time to produce transient LVs. For all VesG, the transient LV titres were around $10^6$ TU/ml after six weeks of phleomycin selection (Figure 4-6D). A ten-fold reduction was observed after ten weeks of selection in all VesG. After this time point, while VSVind.G and COCV.G still produced LVs, vectors titres were undetectable in VSVnj.G, and PIRYV.G so was G protein expression via flow cytometry.

VSVind.G and COCV.G stable expressing cells were kept in selection for further ten weeks. The G protein expression fell gradually in VSVind.G expressing cells over this period (Figure 4-6C). Moreover, while transient LV titres were stable around $10^5$ TU/ml for up to 14 weeks in selection, they also
fell gradually over the next four weeks. The LV titres could not be detected at week 22 post-selection. On the other hand, COCV.G envelope expression and transient LV titres remained detectable for up to 22 weeks of selection. While the titres reduced slowly over time, they were still around $10^4$ TU/ml at week 22, when this experiment was terminated (Figure 4-6D).
Figure 4-6. Long-term G protein expressing cells and long-term vector production. HEK 293T cells were transfected with VSVind.G, COCV.G, VSVnj.G, and PIRYV.G. (A) Transient G protein expression was measured by VSV-Poly antiserum 72 hours post-transfection via flow cytometry. (B) Stable G protein expression in transfected cells kept in phleomycin selection was measured for up to 14 weeks. (C) Stable G protein expression levels in VSVind.G and COCV.G was measured in cells kept in selection for up to 22 weeks. (D) Transient VesG-LV titres were measured over the course of selection; Data shown represent one experiment performed in duplicates.
Achieving stable envelope expression in cells for around 5 months, especially for VSVind.G that is considered to be cytotoxic, was a promising result which prompted us to try a similar approach with two additional VesG: VSVala.G, and MARAV.G. Consequently, HEK 293T cells were co-transfected with pHV-Luc and VSVind.G, COCV.G, VSVnj.G, PIRYV.G, VSVala.G, and MARAV.G. Luciferase activity was measured 48 hours post-transfection (Figure 4-7A). Cells were kept in phleomycin selection for up to 14 weeks, during which envelope expression and transient LV titres were measured every two weeks.

Envelope expression was measured by staining with VSV-Poly antiserum. All VesG-transfected cells demonstrated G protein expression for up to 14 weeks (Figure 4-7B). Although the expression levels decreased gradually over time, they were still detectable at week 14, when this experiment was terminated. G protein expression levels in these stable cells seemed to be higher compared to the ones from the first attempt.

As per the previous experiment, to investigate LV production, these cells were transfected transiently with *gag-pol, rev*, and *SIN-pHV* once every two weeks. LV titres were then measured in the harvested supernatant. COCV.G demonstrated initial titres of $10^6$ TU/ml for up to eight weeks (Figure 4-7C). The titres dropped to around $10^5$ TU/ml at week ten where they remained stable until week 14. Although VSVala.G initial titres started at around $10^5$ TU/ml, similar to COCV.G, they remained stable throughout the experiment. VSVind.G, VSVnj.G, and PIRYV.G titres started at slightly lower than $10^6$ TU/ml at week four and six, then fell gradually to around $10^4$ TU/ml over the next two months. MARAV.G expressing cells, on the other hand, produced titres at around $10^5$ TU/ml at week four and six. These titres then dropped to
$10^4$ TU/ml at week eight. From week 12 onwards, despite measurable G protein expression in these cells, the transient titres were undetectable (Figure 4-7C, arrows).

Overall, both experiments highlighted the possibility of expressing VesG, including VSVind.G, stably in HEK 293T cells for at least 3-4 months. In addition, transient LVs produced by COCV.G and VSVala.G expressing cells persisted at $10^5$ TU/ml for over 14 weeks in phleomycin selection.
Figure 4-7. Establishment of stable VesG expressing cells and long-term vector production. HEK 293T cells were transfected with pHV-Luc and VSVind.G, COCV.G, VSVnj.G, and PIRYV.G, VSVala.G, and MARAV.G. (A) Luciferase expression was checked as a measure of transfection efficiency. (B) Stable G protein expression in transfected cells kept in phleomycin selection was measured for up to 14 weeks. (C) Transient VesG-LV titres was measured for up to 14 weeks. Data shown represent one experiment performed in duplicates.
4.4 Discussion

This chapter aimed to investigate the fusogenic activity and relative cytotoxicity of the vesiculovirus G proteins. Therefore, the effects of pH on VesG mediated syncytium formation and VesG-LV infectivity were explored. Also, the differences in pH dependence observed were further studied as a cause of G protein-mediated cytotoxicity and the generation of cell lines stably expressing VesG was attempted.

4.4.1 Fusogenic activity amongst various vesiculovirus G proteins in a range of pH

Cocal envelope G protein was reported to be less cytotoxic compared to that of VSVind.G [237, 243]. Thus, the first attempt was set to compare the fusogenic activity of these two G proteins. Both G proteins induced cell fusion at neutral pH. The formed syncytia were larger and in higher quantities per field of view when transfected cells were pre-incubated at pH 6.0 and 5.0 (Figure 4-1B). This experiment was then repeated to include VSVnj.G and PIRYV.G as well to investigate fusogenic activity amongst other vesiculovirus family members.

At mildly acidic pH of 6.0 and 5.0, all four VesG induced cell fusion and syncytia formation. However, at neutral pH 7.0, while as expected, both VSVind.G and COCV.G induced cell fusion, VSVnj.G and PIRYV.G did not trigger syncytia formation (Figure 4-2C). This suggests that VSVnj.G and PIRYV.G are not fusion-competent at neutral pH. This may be indicative of differences in pH dependence amongst the G proteins. For VSVnj.G and PIRYV.G, the population of prefusion trimer at neutral pH might not be enough.
to trigger fusion at this pH. Another explanation might be that the level of G protein expression might be lower compared to that of VSVind.G and COCV.G. Due to this the critical number of post-fusion trimers might not be reached hence not resulting in cell-cell fusion. It was not possible to control the VesG expression level without universal quantitative binding assay for all VesG.

Moreover, based on VSVind.G structure, the acidic residues D268, D274, D393 and D395, have been shown to play a role as pH-sensitive switches. While the acidic character of D274, D393 and D395 is conserved among vesiculovirus genus, D268 is not. As these pH-sensitive switches are essential for the function of G protein, in other VesG they are located in different positions (e.g. in CHAV.G in D269-E234-H209 cluster) [456]. It has been shown that clustering of protonatable residues can affect the acidification of these residues which in turn can affect the conformational changes of the G protein [457]. This might affect the stability of the prefusion trimer conformation creating the different fusogenic profiles observed (Figure 4-2).

4.4.2 Effect of pH on the infectivity of vesiculovirus G protein-pseudotyped lentiviral vectors

We further investigated the pH-sensitivity of VesG pseudotyped LVs by pre-incubating the vectors in media adjusted to a wide range of pH. The data revealed that all VesG are more sensitive to acidic than alkaline conditions; infection remained stable up to pH 10.0 (Figure 4-3A), in line with previous studies which elucidated the stability of the conformational changes up until pH 11.0 [454].
While all investigated G proteins seem to be more stable at alkaline pH, in highly acidic conditions the G protein may undergo irreversible denaturation. While other studies reported that VSV inactivation occurred at pH 2, here we show that G protein’s infectivity in all VesG was abolished between pH 3.0-3.6 (Figures 4-3 and 4-4). It has been reported that low pH treatment can induce viral aggregation [458]. This can be another reason, besides viral denaturation, behind reduced viral infectivity in acidic conditions.

A maximum level of infection for all VesG was observed at pH 6.0 to 6.6 (Figure 4-4B). This data is in line with previously published studies reporting a high pH threshold of 6.0 for VSV G protein-induced fusion [443]. In addition, COCV.G and PIRYV.G seem to have higher pH threshold as they lose infectivity at higher pH than VSVind.G and VSVnj.G. VSVind.G and VSVnj.G had higher infectivity levels at pH 5.0-5.3, higher than neutral pH, implying a lower pH threshold for going to postfusion trimer than the other two. Further analyses of pH-dependence of LV infectivity may allow optimisation of culturing conditions to improve LV titres.

4.4.3 Colony formation in the presence of selection marker in vesiculovirus G protein-transfected cells

Overall, pH-induced syncytia formation appeared similar amongst VesG investigated, indicating similar cytotoxicity. We tested colony formation of these G proteins in the presence of the selectable marker using the pMD-2A-VesG constructs in which the VesG ORF is linked to the phleomycin resistance gene via a 2A peptide, in comparison to RDpro, a non-toxic G protein. These results also suggested that there is no major difference in VesG-induced
syncytia formation among various VesG as a comparable number of phleomycin resistant colonies were produced in all VesG (Figure 4-5).

4.4.4 Generation of stable vesiculovirus G protein expressing cells

Consequently, we inspected the G protein expression over time in stable VesG expressing cells. Surprisingly, we found that VSVind.G could be stably expressed in cells for up to at least five months. Moreover, these cells could produce transient LVs with titres of more than $10^4$ TU/ml for up to 20 weeks post-selection (figure 4-6). These are striking results as it was believed that VSVind.G could not be stably expressed in cells due to high toxicity [201]. These results were in line with a recently published report by Humbert et al. [237].

Moreover, we were able to generate stable cell lines with other VesG (Figure 4-7). While these cell lines performed adequately with regards to G protein expression and LV titres, COCV.G and VSVala.G produced transient titres of $10^5$ TU/ml for at least approximately four months. This performance was followed by VSVind.G, VSVnj.G, and PIRYV.G which produced LV titres of $10^4$ TU/ml for almost four months.

We have demonstrated that all VesG tested can be stably expressed in HEK293T cells without demonstrable cytotoxicity for up to 5 months with satisfactory LV titres following transient supply of gag-pol, rev, and the transgene. The longevity of the stable cells, when adapted into packaging cell lines, will allow for sufficient cell expansion for clinical LV characterisation and production. For instance, the best performing VesG constructs can be stably
transfected into the WinPac cell line. This may improve the low LV titres produced by the WinPac-RD cell line [238]. Furthermore, we have proposed an alternative continuous LV production method: admixing. In this method, unenveloped bald LVs and G proteins secreted from HEK 293T cells in vesicles are produced in trans via their respective stable cell lines and later admixed to produce infectious LV particles. This method not only circumvents the superinfection problem identified (see chapter 5) but also can support continuous LV production. Therefore, these stable G expressing cells can be used for such methods as well (in press [440]).

Taken together, the work presented in this chapter suggest that overall all VesG tested demonstrated similar properties with regards to toxicity, pH-dependent fusion, and infectivity, with modest differences which may be dictating key structural and functional changes. However, the most striking conclusion is that VSVind.G, as well as other VesG, are not demonstrably cytotoxic when expressed constitutively in cells. Furthermore, most G proteins could be stably expressed and supported LV production for at least 3-4 months making them promising candidates for clinical grade PCL formation (in press [440]).
CHAPTER 5

Lack of Superinfection Interference by Vesiculovirus G Proteins
5.1. Overview

The cellular entry is the first step in viral infections and involves multiple viral and host proteins [459]. Attachment of viruses to the cellular receptor initiates endocytosis and fusion resulting in virus genetic material to be delivered to the cytoplasm. Several host cell mechanisms have been developed as immune mechanisms to interfere with viral infections [460]. However, there is also a viral interference mechanism preventing secondary infections called superinfection resistance (SIR) [459, 461]. SIR is observed mainly between serologically related viruses and usually dependent on differences in time or multiplicity of infection [462-466]. SIR can proceed through several different mechanisms including attachment and replication hindrance, or competition for viral polymerase and host factors [467-469]. Although not exclusive to retroviruses, the occurrence of SIR in retroviruses has been extensively studied.

One of the most common types of SIR observed is receptor occupancy via virus-encoded envelope proteins on the plasma membrane blocking the attachment of other viruses or, in short, receptor interference [459, 470]. This was first observed in avian leukosis virus in chicken embryos [471]. Since then receptor interference has been widely utilised to characterise and group many serotypes of retroviruses including Rous sarcoma virus [472, 473], feline, and murine leukaemia viruses [474-476]. It is postulated that the interference happens on the cell surface and functions through interactions of the viral envelope proteins already expressed on the cell surface with its receptor. This theory was later supported as it was demonstrated that when envelope
glycoproteins pre-adsorbed on the cell surfaces fully blocked infection and fusion by viruses utilising the same receptor [477, 478].

As alternative mechanisms, HIV, a lentivirus, achieves SIR via downregulating its putative receptor CD4 in infected cells [479-481]. It has been demonstrated that HIV proteins Vpu, Env, and Nef are involved in this down-regulation process through distinct mechanisms [482, 483]. While Nef down-regulates CD4 via direct interactions with its C-terminal end, Env and Vpu mediate this process by preventing intracellular transport of the newly synthesised receptor molecules.

SIR can pose an issue with regards to the use of retroviral envelopes to pseudotype GRVs and LVs [484]. There have been studies reporting the occurrence of superinfection limiting transduction efficacy and levels of transgene expression [484, 485]. On the other hand, it becomes an advantage in the construction of stable packaging cell lines for clinical grade GRV and LV production. Without SIR, produced viral particles can re-enter and re-infect the producer cells. This results in accumulation of vector genome and many random genome integrations which might lead to loss of titres and possible cell death [486]. As retroviruses display a robust SIR against viruses which utilise the same receptor [470], several envelopes derived from retroviruses, for example, amphotropic MLV [487] and modified RD114 envelope RDpro have been successfully adapted to packaging cell lines (PCLs) [238, 240].

It has been suggested that VSVind.G’s toxicity occurs through superinfection [487]. Vogt and colleagues have reported a lack of superinfection interference with VSVind.G pseudotyped LVs (Figure 5-1). They suggest that this might be
due to the abundance of the target receptors on the cells which are less likely to be saturated with the expressed glycoprotein [486]. VSVind.G’s primary receptor for cell entry has been identified as the low-density lipoprotein receptor (LDLR) and other LDLR family members [347, 349]. This family of proteins are involved in the metabolism and transport of cholesterol and are abundantly expressed in several different tissues and cell types [488].

Figure 5-1. Receptor interference of expressed G protein. Receptor occupancy via virus-encoded RDpro envelope proteins on the plasma membrane blocking the attachment of viruses that use the same receptor for cell entry (left panel). Lack of superinfection interference in VSVind.G expressing cells allows viruses to enter the cells (right panel).
Furthermore, it has been found that even virus-like particles which lack envelope glycoproteins can efficiently transduce VSVind.G expressing target cells. The phenomenon of VSVind.G converting non-infectious unenveloped particles into functioning pseudotypes has been previously demonstrated [486, 489]. It has been hypothesised that the LDLR expressed on Env negative particles act as fusion targets for the VSVind.G expressed on the cell surface leading to fusion into the target cells [487]. This may become a substantial issue because immortalised cell lines widely utilised in LV production, HEK 293 or HEK 293T cells, express LDLR on their surface.

Therefore, lack of superinfection interference has limited the use of VSVind.G envelope in generating stable producer cell lines for clinical use and clinical trials using VSVind.G pseudotyped LVs thus far. While most trials have employed transient production methods, some groups have been experimenting with inducible G protein systems [486]. In inducible systems, G protein expression is induced by addition of induction/suppression reagents to the culture media [233, 246, 490]. Another alternative to overcome the superinfection problem is to study alternative G proteins to replace VSVind.G. Meanwhile, it is essential not only to investigate these G proteins for superinfection but also to explore alternative ways to overcome this issue as well.

Identifying potential solutions to superinfection would help to catapult lentiviral gene therapy as not only it will enable the establishment of packaging/producer cells with the current gold-standard envelope VSVind.G but also possibly lengthen the longevity of vector producer cells and cut down on production costs.
5.2. Aims

The primary aim of this chapter was to investigate superinfection in other VesG both in stable packaging cells and transient infection, and to explore whether VSVind.G ability to superinfect could be curtailed. To achieve this, the following points of investigation were utilised:

- Confirm superinfection in previously established WinPac-COCV.G packaging cells and stable VesG expressing cell lines.
- Examine the main pathway for cell entry utilised by other VesG.
- Explore the effects of knocking out the LDL receptor to superinfection and G protein expression.
5.3 Results

5.3.1 Superinfection in WinPac stable packaging cell line

The Collins/Takeuchi group has previously developed the WinPac (WP) stable packaging cell line which stably expresses HIV Gag-Pol and Rev proteins [238]. To avoid VSVind.G- related cytotoxicity [215], WP cells were stably transfected with RDpro to develop WinPac-RD cell line. These cells can continuously produce third generation SIN LV at titres of 10^6 TU/ml. Moreover, vector titres were relatively stable over a period of 4-5 months. Compared to inducible packaging cells developed for clinical LV production, stable production using WP cells is easier to scale up and avoids the rapid decline in titres following induction [238].

Following this, a phylogenetically close relative of VSVind.G, COCV.G, was introduced into WP cells owing to its advantageous characteristics over RDpro (higher stability and titres, Chapter 3) and VSVind.G [243].

To investigate whether Cocal envelope glycoprotein blocks superinfection, WP-RD and WP-COCV.G cell lines were challenged with GFP-LVs pseudotyped with VSVind.G, COCV.G, and RDpro at MOIs 0.5 and 3.0. RDpro and COCV.G expression levels in WP cells were determined by extracellular antibody staining (Figure 5-2A). To detect RDpro, WP-RDpro cells were incubated with mouse anti-RDpro polyclonal antibody (Pule group- UCL Cancer Institute). WP-COCV.G cells were stained with VSV-Poly antiserum. After being selected for gag-pol, rev and env for 3-4 passages, these cells were checked for envelope expression (Figure 5-2A). After infection with GFP-LVs, GFP expression in the producer cells demonstrated that, as expected,
WP-RDpro cells blocked RDpro-LV infection (Figure 5-2B) while they were permissive to VSVind.G- and COCV.G-LVs. The level of GFP positive WP-RD cells was significantly less than that of WP-COCV following challenge with RDpro-LV (p<0.0001). In contrast, WP-COCV.G cells were infected by all LVs, including COCV.G-LVs, at similar levels (Figure 5-2B) suggesting that COCV.G lacks the ability of receptor interference.
Figure 5-2. WinPac-COCV.G cells lack superinfection resistance through receptor interference.
(A) Env expression levels for WinPac-RD and WinPac-COCV.G cells of their respective envelope glycoproteins. WinPac-RD cells were stained with rat anti-RD antiserum. WinPac-COCV.G cells were stained with goat VSV-Poly antiserum (see Table 2-4 and 2-5 for primary and secondary antibodies, respectively). Data shown represent one of the three repeats performed. Envelope negative WinPac cells were utilised as negative control.
(B) WP-COCV.G and WP-RDpro packaging cell lines were challenged with COCV-G-VSVind.G- and RDpro-LV at two MOIs: 0.5 and 3.0. GFP expression of the cells was measured 48h after transduction via flow cytometry. Data shown represent mean +/- SD of three repeats performed in triplicates. One-way ANOVA analysis with Tukey’s post-test was performed to compare percentage GFP positive cells (**** p<0.0001).
5.3.2 Superinfection in stable VesG expressing cells

The lack of superinfection interference of COCV.G, similar to VSVind.G, led us to hypothesise that this may be a shared characteristic amongst VesG. Therefore, we decided to test this using the stable envelope expressing cell lines generated (section 4-3-4). As a superinfection negative control, stably transfected HEK 293T cells with RDpro were used. We tested the bulk cell populations for superinfection permissiveness similarly as done in 5.3.1, after four weeks of antibiotic selection. The infection rates were determined by measuring the GFP expression via flow cytometry while the envelope expression was monitored by VSV-Poly antiserum and anti-RDpro. VSV-Poly antiserum cross-reacts with all VesG with various levels of affinity. After confirming that G proteins were expressed in all stable VesG and RDpro cells (Figure 5-3A), they were challenged with VesG- and RDpro-LV. As previously demonstrated RDpro-LV transduced RDpro expressing cells at a significantly lower level than that of naïve HEK 293Ts (Figure 5-3) (p<0.001). Furthermore, corroborating previously published data and results from section 5.3.1 VSVind.G and COCV.G-LVs could superinfect their respective Env expressing cells (Figure 5-3B). Interestingly, the same was observed for the other VesG tested: no statistically significant differences were observed between the infectivity of VesG expressing cell and naïve HEK 293Ts.
Figure 5-3. All VesG expressing cells are susceptible to superinfection by LVs pseudotyped with their relative envelopes. (A) Env expression levels for stable VesG (left) and RDpro (right) expressing cells. Data shown represent one of the three repeats performed. VesG expressing cells were stained with goat VSV-Poly and WinPac-RD cells were stained with rat anti-RD antisera (see Table 2-4 and 2-5 for list of antibodies). (B) Stable Env expressing cells were challenged with VesG and RDpro-LV at MOI 1. GFP expression was measured 48h later via flow cytometry. Data shown represent mean +/- SD of three repeats performed in triplicates. One-way ANOVA analysis with Tukey’s post-test was performed to compare percentage GFP positive cells (*** p<0.001).
5.3.3. Investigating the role of LDLR family members in VesG-LV entry

LDLR has been reported to be the primary cellular receptor responsible for VSVind.G entry while other LDLR family members have been demonstrated as alternative receptors [347, 491]. Soluble LDLR (sLDLR) has been reported to bind VSVind.G at high affinity preventing its binding to cells thus inhibiting transduction by VSVind.G pseudotyped LVs. Furthermore, this viral infection is successfully inhibited in a dose-dependent manner via soluble LDLR molecules and was entirely blocked by the addition of receptor-associated protein (RAP). RAP blocks all LDLR family members, except for LDLR itself [315]. Similar dose-dependent partial inhibition of COCV infection by soluble LDLR has also been demonstrated [243].

Combined with this information, the data presented in section 5.3.2 implied that the various VesG might be sharing their main path for cell entry and hence sharing the apparent lack of superinfection resistance. To test whether LDLR is responsible for the VesG-LV infection, an infection assay in the presence and absence of sLDLR and RAP proteins was performed. HEK 293T cells were challenged with VesG- and RDpro-LV with and without prior treatment with sLDLR, RAP, or both (Figure 5-4).
As expected RDpro-LV infection was unaffected by the presence of both LDLR and RAP as it uses ASCT-2, a neutral amino acid transporter, as its primary receptor. On the other hand, all VesG-LV infections except that of PIRYV.G-LV, was inhibited in the presence of sLDLR. The results implied that while VSVind.G, COCV.G, VSVnj.G, VSVala.G, and MARAV.G utilised LDLR for cell entry, PIRYV.G did not interact with sLDLR and therefore most probably does not utilise this protein as a receptor. RAP effect was not observed in this study using 293T cells as the infection target, unlike previous studies on WT FS-11 fibroblasts and LDLR-deficient GM701 fibroblasts cells [347].
5.3.4. Effects of LDLR-Knock Out to VSVind.G infection

Results from the experiments in section 5.3.3 suggested that all VesG except for PIRYV.G use LDLR to infect HEK 293T cells. Consequently, we decided to knock out (KO) the LDLR gene on the producer cell lines to obtain further evidence that VesG-LVs enter cells via LDLR and to investigate whether LDLR-deficient cells are still permissive to superinfection.

To KO the LDLR gene, the CRISPR-Cas9 genome editing technology was utilised. The CRISPR-Cas9 system was initially developed a few years ago based on a bacterial defence mechanism against invading phages and plasmids. In this system, a guide RNA (gRNA) complementary to the target DNA is designed which will guide the nuclease to induce double-strand break (DSB) in the target sequence. For this project, the LentiCRISPR system was used. This system contains the blasticidin resistance gene as the selectable marker as well as the Cas9 enzyme to induce DSB at the target sequence, which is encoded into the vector plasmid of an LV. Exploiting LV ability to target a broad range of cells and to integrate its genome into the host cell, a mammalian codon-optimised Cas9 along with the specific gRNA is delivered into the cells, and the genomic cut is carried out (Figure 5-5A).

For this, I designed three different guide RNAs (gRNA) targeting different parts of the gene, checked functionality and proceeded with diagnostic processes including sequencing and plasmid digestion with restriction enzymes after inserting into the lentiCRISPR construct (section 2-6). One gRNA that targeted the LDLR open reading frame was selected to subclone into the LentiCRISPR plasmid (Figure 5-5B). LDLR-KO LVs were produced via transient production
method. Briefly, HEK293T cells were transfected with VSVind.G encoding env plasmid, p8.91, and LentiCRISPR plasmids to produce transient VSVind.G pseudotyped LVs encoding the gRNA and Cas9 protein to KO the LDLR gene (αLDLR-LVs). LVs were harvested 48 hours post-transfection while the LV producing HEK293T cells were put under blasticidin selection.

WT HEK 293T cells were transduced with 1.5 mL, 1mL, and 500 µL of αLDLR-LVs and kept under selection for two weeks before being investigated for LDLR expression via immunoblotting. For this 8x10⁶ cells were lysed and immunoblotted for LDLR using anti-LDLR mAb, 5E6. After infection with αLDLR-LVs, LDLR gene was successfully knocked out from 293T cells at all three doses, as the receptor protein was not detected (Figure 5-5C).
Figure 5-5. KO of LDLR from HEK 293T cells utilising the LentiCRISPRv2 system. (A) LV vector backbone encoding the SpCas9 and the selectable marker blasticidin resistance gene. The designed gRNAs were subcloned into the backbone utilising the BsmBI cloning site. (B) The diagram for the designed gRNA. This oligo was digested using BsmBI restriction enzyme, then subcloned into the lentiCRISPR backbone. (C) Western blot analysis of LDLR expression in naïve HEK 293T cells, αLDLR-LV producer cells, and αLDLR-LV transduced cells two weeks post-antibiotic selection. GAPDH was used as protein input control. Data shown represent one of the three repeats performed. Psi packaging signal (psi); rev response element (RRE); central polypurine tract (cPPT); elongation factor-1α short promoter (EFS); FLAG octapeptide tag (FLAG); 2A self-cleaving peptide (P2A); blasticidin selection marker (Blast); Woodchuck posttranscriptional regulatory element (WPRE); blasticidin selection marker (blast); gRNA: Guide RNA.
After the LDLR knock out confirmation in the cells, the infection levels of VesG-LV in WT and KO HEK 293T cells were examined by flow cytometry (Figure 5-6). RDpro uses ASCT-2, a neutral amino acid transporter, as its primary cell entry pathway [492, 493]. Therefore, RDpro-LVs were used as a control where no changes in infection were expected. On average, a modest reduction in infectivity was detected in KO cells compared to WT when the cells were challenged with all VesG-LV except for PIRY (Figure 5-6A). This data corroborated our previous findings that sLDLR blocks infection of LV with G proteins of VSVind, COCV, VSVnj, VSVala and MARAV and suggest that these viruses are sharing LDLR as a cell entry receptor. However, the reduction of infection effect was subtler than expected as just the addition of 0.5 μg/ml sLDLR to the culture media had blocked on average 80% of infection (Figure 5-4). These two findings both pointed towards the involvement of other receptors, possibly other LDLR family members as previously described.
Figure 5-6. Investigation of VesG-LV infection of LDLR-KO HEK 293T cells. 
(A) VesG-LV were produced using transient production protocol. LVs were titrated on LDLR-KO and naïve HEK 293T (WT) cells using equimolar mounts. Cells were harvested 48h later, and GFP expression was measured via flow cytometry. Data presented represent mean +/- SD of three experiment performed in duplicates. One-way ANOVA analysis with Dunnett’s post-test was performed to compare VesG infection decrease to that of RDpro, the negative control with an unrelated primary receptor. (B) LDLR-KO HEK293T cells were challenged with GFP expressing VesG and RDpro pseudotyped LV at MOI 0.5 in the absence and presence of 0.5 μg/ml sLDLR and 2.5 μg/ml RAP. The infection rates were analysed 48h later via flow cytometry. Data shown represent relative infection +/- SD from three experiments performed in duplicates. One-way ANOVA analysis with Dunnett’s post-test was performed to compare percentage GFP positive cells to that of virus only samples (** p<0.01; * p<0.05; ns = no significant difference).
One hypothesis behind the lack of infection inhibition observed by RAP and the modest reduction of transduction efficiencies in LDLR-KO cells was the upregulation of the other family members’ expression in order to compensate for the lack of LDLR in endogenous lipid/cholesterol pathways. Therefore, qPCR analyses on both naïve and LDLR-KO cells were performed to compare expression levels of LDLR, and two other LDLR family members: very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor (APOER). In addition, we also checked for the Leucine-Rich Repeat Containing G Protein-Coupled Receptor 4 (Lgr4) as it has been reported to be essential for VSVind.G entry to target cells [494].

Total RNA from WT and KO cells were extracted, and expression levels of different LDLR family members were measured by quantitative PCR. Figure 5-7A demonstrates the data obtained from the qPCR indicating that LDLR gene was still detectable in KO cells. This suggests that complete LDLR KO might not have been achieved. As bulk population of LDL-KO cells was used, to ensure that LDLR is still absent from the population in use, we decided to check the LDLR expression in KO HEK 293T cells from an earlier passage (i.e. two weeks in selection) compared to the batch that was currently in use that was in culture under blasticidin selection for around 12 weeks (indicated here as late passage). qPCR result for LDLR-KO early passage shows that LDLR expression, while still detectable, is considerably lower compared to LDLR-KO late passage. Moreover, it seems that as well as LDLR, VLDLR expression was lower in KO cells compared to WT. When the gRNA was aligned against VLDLR sequence to check for any off-target effect, the gRNA recognised 5-6 nucleotide long sequences in VLDLR exons 1 and 2 (6
determinants in total) suggesting that \textit{VLDLR} gene might have also been knocked out through off-target effect. Also, a slight increase in expression in \textit{Lgr4} (\textit{p}<0.05) and \textit{APOER} genes can be observed in KO cells compared to WT suggesting these genes might have been elevated in order to compensate for the absence of \textit{LDLR}.

To investigate \textit{LDLR} expression by immunoblotting, cells from LDLR-WT, LDLR-KO late passage, and LDLR-KO early passage were lysed and blotted using anti-LDLR antibody 5E6. The immunoblotting data indicates that two weeks post-selection transduced HEK 293T cells were negative for LDLR expression when compared to WT HEK 293T cells, (Figure 5-7B- early pass). However, in 12-weeks post knockout cells, an LDLR band was detected, similar to the WT control (Figure 5-7B- late pass). These results implied that the LDLR expression returned in bulk LDLR-KO population although they were kept under blasticidin selection.
Figure 5-7. Exploring the expression Levels of LDLR and other LDLR family members in early and late passages of LDLR-KO cells. qPCR assay performed on genomic DNAs extracted from WT and LDLR-KO cells: (A) Late-passage cells; (B) early passage cells. Copy numbers measured for each gene was normalised to that of WT HEK 293T. Data shown represent means +/- SD of three experiment performed in duplicates. (C) Western blot analysis of LDLR expression in early passage KO cells (week 2) and late passage KO cells (week 12). Data shown represent one of the three repeats performed. One-way ANOVA with Tukey’s post-test was performed to compare each receptor to its wild type expression (*** p<0.001; ** p<0.01; * p<0.05). ns: not significant.
5.3.5 Effect of the LDLR-KO to VesG expression on the cell surface and shed in the supernatant

To investigate whether LDLR-KO influences envelope expression, this receptor was knocked out on the stable VesG expressing cells (section 4-3-4) and checked the envelope expression in WT compared to KO cells. VSVind.G, COCV.G, VSVnj.G, PIRYV.G, VSVala.G, and MARAV.G expressing cells that were in phleomycin selection for two weeks were transduced with 500µL of αLDLR-LVs and were selected with blasticidin in addition to phleomycin for another two weeks. WT and KO Env expressing cells were lysed and blotted with VSV-Poly antiserum and P5D4 to detect VesG, and with anti-LDLR 5E6 to confirm the LDLR KO (Figure 5-8). As expected, the LDLR-KO cells did not express the LDLR gene while bands were detected in the WT ones. When blotted with anti-VSVind.G mAb P5D4 C-terminally, a modest increase in the VSVind.G expression was observed in the KO cells in comparison to the WT ones, however, as P5D4 did not cross-react with other VesG on a western blot the expression levels of other G proteins could not be deduced. For this, the samples were blotted with anti-VSVind.G polyclonal VSV-Poly which was able to react with all VesG tested in flow cytometry (section 3-3-1). Although an increase in the signal was observed in the KO cell in comparison to WT ones, the signals were deemed unspecific, and no conclusive results could be deduced with regards to VesG expression. The data suggest that while VSV-Poly is suitable for flow cytometry staining and can cross-react with all VesG, is not suitable for western blot staining. Moreover, as other available Abs only detect VSVind.G and do not cross-react with other VesG, using western blot for G protein detection is not feasible.
Figure 5-8. Western blot analysis of VesG expression on stable G protein expressing cell surface following LDLR KO. 8x10^6 cells were lysed, and 50 μg of total protein/ well were loaded. The samples were blotted for LDLR expression (5E6), VSVind.G C-terminal (P5D4), and extracellular domain polyclonal VSV-Poly for VesG proteins expression. The data shown represent one of the three repeats performed.
To observe the VesG expression level differences upon LDLR KO, chimeric VesG proteins with the transmembrane and C-terminal domains of VSVind.G, produced in our laboratory by Altar Munis, were used; these allowed for G protein detection by the mAb P5D4 (Figure 5-9A).

WT and LDLR-KO cells were seeded at $3 \times 10^5$ cells/well density in 6 well plates and transiently transfected with 2.5μg of plasmids expressing VesG-tail chimerae. 24 hours later the media was changed with 2 ml of Opti-MEM supplemented with L-glutamine and Pen/Strep. 48 hours post-transfection, supernatants were harvested, and cells were lysed. Supernatants were concentrated via centrifugation at 17,000 g for 2 hours, and total protein amounts were normalised using the Pierce BCA Kit.

The samples were blotted with 5E6 anti-LDLR antibody to confirm LDLR-KO and P5D4 to investigate VesG expression and secretion (Figure 5-9B). The western blot analysis demonstrated that even by transient transfection a modest difference between VesG expression on cells and secretion could be observed (depicted by the thickness and density of the bands). All VesG-tail chimerae could be detected using P5D4 and comparison of the bands between WT and KO cells highlighted that in KO cells the G protein expression and secretion were more prominent compared to that of WT indicating that the absence of the primary receptor does indeed increase the level of G protein expression. However, as this experiment was performed following transient transfection all expression levels were relatively high, therefore the preliminary results acquired should be further investigated following stable transfection of the VesG-tail chimerae.
Figure 5-9. Western blot analysis of VesG-tail expression on stable G cell surface following LDLR KO.

(A) Diagram of VesG-tail chimeric proteins in which the transmembrane and C-terminal domains of the G proteins were switched with that of VSVind.G (White Square). (B) 1x10^6 cells were lysed, and 40 μg of total protein/well were loaded on SDS-page gel. The samples were blotted for LDLR expression (5E6, Sigma) and C-terminal P5D4 (Sigma) for G expression. Data shown represent one of the three repeats performed.
5.4 Discussion

Cells infected by retroviruses display resistance to superinfection by viruses that utilise the same receptor as the pre-infecting virus. This superinfection interference is most likely due to saturation of the virus receptors with the retroviral envelope glycoprotein expressed in the infected target cells [486].

The host range of retroviral vectors including lentiviral vectors can be expanded or altered by pseudotyping. VSVind.G is among the first, and still one of the most widely, used glycoproteins for pseudotyping LVs, due to its advantages including broad tropism and stability [211]. A major issue that has been reported in stable VSVind.G pseudotyped LV producer cell lines is that these cells are prone to be re-infected by the produced LVs. This might be because VSV receptors, i.e. LDLR and LDLR family members, are abundant components of plasma membranes and receptor saturation most likely is not achieved easily to mediate superinfection resistance [486, 495]. The lack of superinfection resistance in the producer cells can lead to the accumulation of the vector genome in the cells causing genotoxic effects, cell death, and loss of titres [486].

5.4.1 Superinfection in stable cell lines

The work presented in this chapter aimed to investigate whether other VesG pseudotyped LVs, superinfect producer cells similarly to VSVind.G-LVs. This was explored in two separate sets of cell lines; in WinPac (WP) stable packaging cell lines as well as the stable VesG expressing cells (chapter 4). Both WP-derived cells lines, WP-RD and WP-COCV.G, and stable VesG expressing cells were challenged with VesG pseudotyped LVs expressing
GFP. Both sets of cells were checked for envelope expression prior to challenge as well as for GFP expression after the challenge (Figures 5-2A and 5-2B). While stable RDpro expressing cells reduced the superinfection by RDpro pseudotyped LVs significantly, complete inhibition like that of WP-RD cell line was not observed. This can be explained by that the stable RDpro expressing cells were a mixed population and not clonally selected ones. Therefore, the bulk population might be made up of cells expressing RDpro at low levels and/or not expressing RDpro. This will lead to receptors not being saturated permitting RDpro LVs entry to the cells.

On the other hand, in both approaches, VesG-LV infection was unaffected pointing to a lack of superinfection resistance in the genus. This might pose some challenges such as genotoxicity and loss of titres of VesG based LV producer lines, which need to be addressed. A possible way that has been suggested to block superinfection is the use of antiretroviral drugs such as azidothymidine (AZT) [486]. AZT is widely used as an antiretroviral medicine in patients suffering from HIV-1 infection/AIDS. AZT mechanism of action is by inhibiting the HIV-1 reverse transcriptase activity by its triphosphate form competing with its natural nucleotide counterpart thymidine triphosphate for incorporation into newly synthesised viral DNA. Once incorporated, it leads to DNA chain termination stopping further DNA synthesis. However, if used, AZT would need to be included in the culture media and later filtered out of the final LV preparation, a similar issue currently is present in inducible systems which utilise induction/suppression chemicals [233, 496]. This will complicate the simple continuous stable production process which currently is an advantage of constitutive PCLs over inducible systems.
Another approach to block superinfection is the blocking of the primary receptor which the pseudotypes use to enter the cells. It has been reported that VSVind.G employs the low-density lipoprotein receptor (LDLR) as the main receptor for cell entry [347, 377], while other LDLR family members act as alternative receptors for this G protein. Furthermore, published studies reported that while knockdown of LDLR in a human fibroblast cell line (FS-11) reduced VSVind.G infection, overexpression of this gene in LDLR-deficient GM701 cells increase VSVind.G infection by 6-fold. It was also shown that following stimulation of T cells and haematopoietic stem cells with a cytokine cocktail, the surface expression of LDLR was elevated on these cells resulting in increased VSVind.G pseudotyped LV transduction efficiency [377].

However, little is known about the receptor for other vesiculoviruses. As these viruses show some degrees of homology on the amino acid level to VSVind.G, from higher levels in MARAV.G and COCV.G to lower homology levels in PIRYV.G, we hypothesised that they might be sharing the same receptors for cell entry. Preliminary experiments were performed in our lab investigating the putative receptor for COCV.G using sLDLR. These data suggested that VSVind.G and COCV.G LVs infectivity was partially blocked in a dose-dependent manner by sLDLR [244]. Similar results were also reported by another research group [237]. When other VesG were investigated for their use of LDLR and other family members for infection, a hindrance of infectivity by sLDLR was observed for all VesG but PIRYV.G (Figure 5-4).
5.4.2 Development LDLR knock out cell line

Accordingly, we tried to knock-out LDLR on HEK 293T cells in an attempt to block superinfection in VesG cells. Although PIRYV.G does not interact with LDLR, it still demonstrated a lack of superinfection resistance (Figure 5-3). Therefore, LDLR KO was expected not to affect PIRY-LV infection, and it was utilised as a negative control in the LDLR KO experiments. Hence, a gRNA targeting the LDLR gene was designed and cloned into a LentiCRISPR plasmid containing a blasticidin resistance gene. After producing transient αLDLR-LVs, HEK 293T cells were transduced and kept in blasticidin selection. After confirming LDLR KO via immunoblotting (Figure 5-5C), WT and LDLR-KO HEK 293T cells were transduced with various volumes of VesG pseudotyped LVs to investigate the infectivity levels in LDLR-deficient cells compared to WT ones. A slight decrease of infectivity was observed in all VesG LVs except for PIRYV.G (Figure 5-6) which was in agreement with our previous results suggesting that PIRYV.G LVs likely employ receptors other than LDLR (Figure 5-4). While in higher volumes added of LV the infection seemed to get saturated, in lower volumes yielding 1-30% GFP expression, which is the window utilised to calculate LV titres, statistically significant (p<0.001) reduction of infectivity in LDLR-KO cells compared to WT ones could be observed.

However, as LDLR was reported to be the main entry port for VSVind.G, a greater effect on infectivity level in the absence of LDLR was expected. We hypothesised that a possible explanation could be that other LDLR family members are elevated to balance out the lack of LDLR.
When investigating the levels of mRNA of the other LDLR family members by qPCR analysis, it was revealed that the LDLR-KO cells were not homogenously and completely knocked out as LDLR could be detected, albeit in substantially lower levels (approximately $10^3$ times less compared to WT) in the KO cells. The differences in gene expressions also translated into differences in protein expression levels (Figure 5-7B-C). Results from immunoblotting analysis demonstrated that while there was no detection of LDLR protein in early passages, its expression reappeared in later passages.

This phenomenon can be explained by the karyotypic phenotype of the initial KO population; first, the HEK 293T cells have a complex karyotype of hypotriploid DNA, with different mean chromosome numbers in cells from different sources. The diversity has been suggested to be due to the long-term cultivation of these cells causing different clones of 293 cells to undergo karyotype diversification [411, 497]. Having a complex karyotype can make having a homologous knock out of genes in all alleles to be challenging. Furthermore, as the LDLR-KO cells did not undergo clonal selection, we utilised a mixed/bulk population of homologous and heterologous KO cells in my experiments. After a few weeks of cultivation and selection, the population of cells with heterologous LDLR KO might have dominated the homologous one via spontaneous clonal selection. Having a mixed population of KO cells could also be the reason behind the unconvincing results we attained from WT vs KO cells' transduction (Figure 5-6). Accordingly, clonal selection and isolating single clones with higher performance is of high importance in order to achieve a reliable knock out HEK 293T cell line.
5.4.3 Investigating other LDLR family members as entry receptors for vesiculovirus G proteins

We then investigated whether the absence of LDLR influenced the expression of G protein by checking its levels in the collected supernatant and expressed on the cells from WT or KO HEK293T cells. One issue we faced was the lack of available antibodies against VesGs other than that of VSVind.G. For flow cytometry, we mainly used the VSV-Poly Ab owing to its cross-reacting with VesG other than VSVind.G; however, due to it being an antiserum with a collection of different immunoglobulins, using it in immunoblotting might result in unspecific interactions with lysate proteins. As other available monoclonal antibodies do not cross-react with other VesG, comparing G protein expression amongst various constructs has proven challenging (Figure 5-8). Therefore, we used VesG-tail chimerae which were synthesised by switching the endogenous transmembrane and C-terminal tails of VesG with that of VSVind.G provided by Altar Munis at NIBSC. This enabled the use of anti-VSVind.G mAb P5D4 for G protein expression and secretion detection. Following transient transfection of these new chimeric G proteins into KO and WT HEK 293T cells, western blot analysis was carried out to determine G protein expression (Figure 5-9). A modest increase of G protein expression was detected for all VesG in LDLR-KO cells in comparison to the WT cells. This may be because when expressed on cells, LDLR interacts with VesG and other plasma proteins on the secreted vesicles containing VesG to mediate their endocytosis. It has been demonstrated that LDLR and LDLR family members are continuously recycled at the plasma membrane approximately every ten mins [347]. Therefore, when expressed on the cell surface they may
interact with the G proteins, incorporate them in the endocytosis cycle and reduce their secretion to the supernatant. It is of value to explore the use of LDLR-KO cells in the production of LVs hence the preliminary results obtained via stable and transient G expressing cells imply that the lack of LDLR on producer cells may boost G protein expression and secretion and thus LV titres.

5.4.4 Summary

Taken together, the work presented in this chapter demonstrates that all VesG tested display a lack of superinfection interference (regardless of their primary receptor use) and therefore their adaptation to PCLs may lead to instability through vector genome accumulation (in press [440]). As a potential approach to address this LDLR-KO HEK 293T cells were generated in order to prevent produced LVs from re-entering the PCLs. Preliminary data demonstrated a modest superinfection block, however, due to incomplete LDLR KO in the cell population the results were inconclusive. Therefore, this approach should be further investigated following clonal selection of LDLR-KO cells and/or generation of LDLR-KO utilising a more stable and homogenous cell line.
CHAPTER 6

General Discussion and Future Directions
LVs are promising gene therapy tools. Their translation to the clinic has proven successful considering recent clinical trials have demonstrated curative therapies using LVs for several acquired and heritable diseases [300]. Maintaining virus viability over several steps of vector manufacture, production, purification, storage, and application, is vital for the efficacy of the therapies developed as well as the quality of the transduced cells [430]. Moreover, the vector titres and cellular contaminants in the vector containing medium are also of concern in achieving successful gene therapies [431-433].

Vesicular stomatitis virus Indiana strain G protein (VSVind.G) is the most widely used glycoprotein to pseudotype LVs for both in vivo and ex vivo studies. Despite conferring high stability and titres and broad tropism, VSVind.G is claimed to be cytotoxic and cannot be expressed constitutively in producer cells [201, 238]. Therefore, recently, G proteins derived from other members of the vesiculovirus genus (VesG), namely Cocal [237, 243] and Piry virus [498], have been proposed as alternative LV envelopes with possible advantages over VSVind.G. Therefore, detailed investigation of VSVind.G and other VesGs is of interest for their adaptations of clinical LV production.

In this work, we investigated VSVind.G and other VesG, namely Cocal virus G protein (COCV.G), VSV New Jersey strain G protein (VSVnj.G), VSV Alagoas virus G protein (VSVala.G), Maraba virus G protein (MARAV.G), and Piry virus G protein (PIRYV.G), for their use in LV producer cell lines. Therefore, in chapter 3, we undertook detailed characterisation of these VesG with regards to their thermal and physical stability because vector production, storage, and efficacy in use, can be affected greatly by the stability of the vector particle. Transient LVs pseudotyped with VSVind.G, COCV.G, VSVnj.G, PIRYV.G,
VSVala.G, MARAV.G, and RDpro were produced to establish which vesiculovirus are most promising to generate a stable packaging cell line with. They were explored for their ability to be readily concentrated via ultracentrifugation and resistance to repeated freeze-thaw cycles and incubation at 4°C and 37°C. While unconcentrated vectors are often sufficient for *in vitro* studies, for *in vivo* applications higher titres and hence concentrated vector preparations are required. LV concentration is usually achieved by ultracentrifugation as it is a fast and robust method [437, 438]. LVs are usually kept at 4°C during purification and short-term storage. For long-term storage, however, they are kept at -80°C, this implies freeze-thaw of the vector preparation and has a detrimental effect on viral titres. Also, at 37°C, these vectors are less stable, which is a critical issue concerning the efficacy of viral preparations in clinical studies. In this project, all tested G proteins were stable after cycles of freeze-thaw and incubation at 4 and 37°C exhibiting similar characteristics to that of VSVind.G. These G proteins’ stability at 37°C is promising as it will allow systemic and local delivery of LVs *in vivo* without the concern of body-temperature dependent loss of titre. Furthermore, robust vector stability demonstrated at 4°C and during freeze-thaw cycles is useful for short and long-term storage of the vectors.

Although VSVind.G has often been the choice of the envelope for LVs, VSVind.G-based continuous producer cell lines have not been reported. This has been believed to be owing to its highly fusogenic characteristic and cytotoxicity due to fusogenicity [201]. Therefore, less or non-toxic envelopes such as RD114 derived envelope, RDpro, has been used in the generation of
stable cell lines [238, 240]. On the other hand, Cocal envelope G protein was reported to be less cytotoxic compared to that of VSVind.G [237, 243].

In chapter 4, we attempted to compare VesG’s fusogenic activity and pH-dependence through pH-induced syncytia formation and LV inactivation. All VesG demonstrated similar pH-dependent fusogenicity overall, implying similar toxicity levels. This was later confirmed via a colony formation assay. Consequently, all VesG were stably expressed in HEK 293T cells and kept under selection for the marker gene. Possibly the most striking finding of this study was that VSVind.G could be stably expressed in cells for up to at least five months. Moreover, these cells could produce transient LVs with titres of more than $10^4$ TU/ml for up to 20 weeks post-selection. This challenged the widely-accepted notion that VSVind.G cannot be stably expressed in cells due to high toxicity [201]. Furthermore, all tested VesG could stably be expressed in cells for up to 5 months, without any apparent cytotoxicity. In addition, following transient supply of *gag-pol*, *rev*, and the transgene to these stable VesG expressing cells, reasonable LV titres were achieved. This durability of the stable cells is of high importance when adapted into packaging cell lines, allowing for sufficient cell expansion for clinical LV production. For instance, the best performing VesG constructs could replace the RDpro G protein in WinPac-RD cell line to improve the produced LV titres and stability (Sanber, Knight et al. 2015). These stable VesG expressing cells are also suitable for cell-free in trans LV production (in press [440]).

A major issue in stable VSVind.G pseudotyped LV producer cell lines is the lack of superinfection interference which can lead to accumulation of vector genome in the cells resulting in genotoxic effects, cell death, and loss of titres.
Lack of superinfection interference might be because VSV receptors, LDLR and LDLR family members, are not saturated by the expressed G protein as these receptors are abundantly expressed on most cell lines where they are recycled back to the cell membrane after internalisation every 10 minutes [347, 486, 495].

Consequently, in chapter 5, we explored the adaptation of VesG, starting with COCV.G into WinPac packaging cell line, WinPac-COCV.G, and investigated the occurrence of superinfection. Experiments performed demonstrated that VesG-LV infection was unaffected by the expression of the G proteins on the cell surface indicating a lack of superinfection resistance. This might pose some challenges such as genotoxicity and loss of titres of VesG based LV producer lines, which needs to be addressed. A possible approach to block superinfection can be the use of antiretroviral drugs such as azidothymidine (AZT) [486] or Nevirapine (in press [440]).

We explored an alternative option by knocking out the primary receptor utilised by the G proteins to block superinfection. Previously, transient knockdowns of LDLR in human cell lines have proven to reduce VSVind infection modestly [347]. Preliminary experiments performed previously in our group and later by me revealed that all the tested VesG but PIRYV.G, shared the binding ability to LDLR suggesting that they use LDLR as the primary cell entry receptor. CRISPR-Cas9 mediated knock out of LDLR in HEK 293T cells resulted in a modest reduction of VesG-LV infectivity while PIRYV.G transduction was unchanged. mRNA analysis of the knock out cells revealed that an initial reduction of about 1000 times in LDLR mRNA expression compared to the wild-type cells was achieved; however, although the KO cells were kept in
selection media, the level of LDLR mRNA increased in time. The differences in gene expressions also translated into differences in protein expression levels in later passages of LDLR-KO 293T cells.

6.1 Future Directions

The work presented in this thesis has provided insights related to VesGs’ use in clinical grade LV production. Studies focusing on the stability of VesG may help researchers understand the destabilising mechanisms enforced on VesG-LV in variable conditions they face during the LV manufacturing process. Although most G proteins tested demonstrated both high thermal and physical stability, the effect of thermal and physical pressure on HIV-1 capsid and matrix proteins which have essential roles in LV structure should also be investigated. Combined, this will allow modification of LVs and G protein containing advanced therapy medicinal products to generate more stable reagents with better longevity under critical manufacturing conditions.

In addition, our experiments revealed that, against commonly accepted notion, all VesG including VSVind.G can be expressed constitutively in cells and support LV production up to 5 months following transient supply of gag-pol, rev, and the transgene. Taken together, this work revealed that all VesG tested demonstrated similar properties with regards to toxicity, pH-dependent fusion, and infectivity, with modest differences. These G proteins should be further utilised in clinical grade PCL formation and closely monitored for their ability to produce functional LVs constitutively.

Our preliminary work in attempting to adapt COCV.G into the clinical grade WinPac LV producer cell line revealed that similar to VSVind.G, COCV.G and
the other VesG lack superinfection interference. Although the use of anti-
retroviral drugs in cell culture have been suggested to prevent this, removal of
these substances from the culture media poses another step in the complex
manufacture of LVs and may result in further titre losses. Therefore, the option
of knocking out the putative receptor utilised by VesG was explored. The
complex karyotype of HEK 293T cells amongst a couple of other issues
prevented the achievement of a complete LDLR-KO cell line, however, despite
this, a modest reduction in infection permissiveness and increase in G protein
expression have been observed. Further work is essential to follow up on
these promising data. Clonal selection and isolating single clones with higher
performance or the use of alternative cell lines with more stable karyotypes
are essential in achieving a true complete LDLR-KO cell line. This cell line may
later be utilised in the generation of PCLs with less of the problem of
superinfection and thus vector genome accumulation and genotoxicity.
Although, the role of other LDLR family members as alternative receptors for
viral entry has to be investigated. Lastly, we were able to demonstrate that
PIRYV.G’s, unlike the other VesG, does not bind to LDLR. Therefore, the
identification of this G protein’s main receptor may reveal vital information on
vesiculovirus infection, evolution and tropism as well as provide the
opportunity to tailor and re-target LVs and G protein containing medicinal
products.

Overall, generation of stable packaging cell lines is of great importance to both
basic and clinical research. This method reduces the cost of production and
batch-to-batch variability resulting from transient plasmid transfection which is
the method of use for lentiviral vector production.
As well as low toxicity, vesiculovirus G proteins exhibited high vector particle stability. Implementing a physically stable G protein in stable packaging cell lines will aid in achieving high vector titres suitable for clinical use. Investigating the receptor usage of these G proteins is of pivotal importance. This will aid to develop strategies in order to block superinfection to generate a reliable packaging cell line with reduced risk of decline in vector titres as well as to produce useful batches of therapeutic LVs.
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