Elisa Thomé Cabral

Degree in Applied Chemistry - Biotechnology

Chimeric lentiviral vectors for gene therapy- Improving transduction efficiency

Dissertation to obtain Master Degree in Biochemistry for Health

Supervisor: Dr. Ana Sofia Coroadinha, Cell Line Development and Molecular Biotechnology Laboratory, (IBET/ITQB-UNL, Oeiras).

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Abstract

Lentiviral vectors (LV) are excellent gene transfer tools enabling the transduction of proliferative and non-proliferative cells and the long-term transgene expression. However, LV manufacture is a high cost process characterized by low transducing competent particles yields. This work aimed to address these challenges by 1) enhancing LV transduction efficiencies, and by 2) assisting the development of constitutive LV producer cell lines for continuous, reproducible, and low-cost LV manufacture.

To this end, first the enhancing capacity of several commercially available LV transduction adjuvants was evaluated using HEK 293T, Jurkat E6-1 and SUP-T1 target cell lines and VSV-G, 4070A, RD114 and GaLV pseudotyped LV. VSV-G LV showed the higher and wider target cells transduction capacity. However, vectofusin-1 was able to increase up to 56%, 70% and 61% of transduced cells with 4070A, RD114 and GaLV pseudotyped LV, respectively, therefore, opening the possibility to replace VSV-G in the clinic by these non-toxic LV pseudotypes, which are compatible with constitutive manufacture systems. Additionally, vectofusin-1 usage eliminates the need of the centrifugation step during transduction procedure, allowing increased cells viabilities after transduction.

Secondly, the adaptation of a high-throughput method to assess constitutive LV high producer cells using vectofusin-1, was performed. Nevertheless, further studies are required since the use of antibiotics and the formation of fibrils affected cell viability.

In sum, this work describes how LV pseudotypes and adjuvant compounds influence vector transduction efficiencies in several target cells. Furthermore, it provides insights that LV preparation quality is not only a consequence of defective particles production but also of low particles transduction capacity. Overall, this work contributes to the academic and clinical sectors by demonstrating the potential of current LV preparations and by providing improved transduction procedures, expanding LV gene therapy boundaries to more cost-effective and available treatments.

Keywords: Gene therapy; Lentiviral vectors; Transduction efficiency; Transduction adjuvants; Vectofusin-1.

Resumo

Os vetores lentivirais (LV) são excelentes veículos de transferência de genes, permitindo entregá-los a células proliferativas e não proliferativas e posteriormente possibilitando a sua expressão permanente. Contudo, o processo de fabrico de LV torna-se caro e caracteriza-se por preparados virais de baixa qualidade. Assim, este trabalho teve como objetivos: 1) melhorar a eficiência de transdução dos LV, aumentando a qualidade dos preparados virais e 2) auxiliar o desenvolvimento de linhas celulares para produção constitutiva de LV, reduzindo os custos de fabricação.

Para este fim, a utilização de adjuvantes no processo de transdução viral foi avaliada utilizando as linhas celulares HEK 293T, Jurkat E6-1 e SUP-T1 e LV pseudotipados com VSV-G, 4070A, RD114 e GaLV. Os LV que demonstraram maior capacidade de transduzir as linhas celulares foram os pseudotipados com VSV-G. Contudo, na presença de vectofusin-1 as eficiências de transdução destas células com os pseudotipos 4070A, RD114 e GaLV aumentaram para 56%, 70% e 61%, respetivamente. Adicionalmente, a utilização de vectofusin-1 tornou possível abdicar do uso de centrifugação no processo de transdução viral, aumentando a viabilidade celular. O aumento de eficiência de transdução com estes invólucros não tóxicos possibilita a sua utilização clínica.

A otimização de um método rápido de isolamento de clones, assistindo o desenvolvimento de células produtoras, foi realizada para o adaptar à utilização de vectofusin-1 e produção de LV. Contudo, não se conseguiu identificar e isolar um clone produtor, exigindo otimizações futuras.

Os aumentos da capacidade de transdução dos LV, aqui reportados, indicam que a baixa qualidade das preparações virais não de deve apenas à existência de partículas defeituosas, mas também à baixa eficiência de transdução das partículas. Este trabalho gerou conhecimento para os setores académico e clínico, contribuindo para a expansão da terapia genética e para tratamentos mais económicos e disponíveis.

Termos-chave: Terapia génica; Vetores lentivirais; Eficiência de transdução; Adjuvantes de transdução; Vectofusin-1.

List of Contents

Figures Index

Table Index

Abbreviations List

AAV	*Adeno-associated vectors*
ADA-SCID	Adenosine deaminase deficiency-SCID
AIDS	Acquired immunodeficiency syndrome
ASCT2	Neutral amino acid transporter-type 2
BSA	Bovine serum albumin
CA	Capsid protein
CAR	Chimeric antigen receptor
CCR5	CC chemokine receptor type 5
CMV	*Cytomegalovirus*
cPPT	Central polypurine tract
CXCR4	CXC chemokine receptor type 4
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EMA	European medicine agency
Env	Envelope glycoprotein
EO	Ethylene oxide
FBS	Fetal bovine serum
FDA	Food and drug administration
GaLV	*Gibbon ape leukemia virus*
GFP	Green fluorescent protein
GMP	Good manufacturing practices
Gp	Glycoprotein
HEK	Human embryonic kidney
HIV-1	*Human immunodeficiency virus type 1*
hPGK	Human phosphoglycerate kinase 1
IN	Integrase
LDL	Low-density lipoprotein
LTR	Long terminal repeat
LV	Lentiviral vector
MA	Matrix protein
MLV	*Murine leukaemia virus*
MOI	Multiplicity of infection
NC	Nucleocapsid
Nef	Negative factor
NK	Natural killer cells
ORF	Open reading frame

PBS	Primer binding site
PEI	Polyethylenimine
PIC	Pre-integration complex
PiT-1	Inorganic phosphate transporter 1
PiT-2	Inorganic phosphate transporter 2
PO	Propylene oxide
PR	Protease
qPCR	Quantitative polymerase chain reaction
RCL	Replicative-competent lentiviral vector
RD114	*Feline endogenous virus*
RNA	Ribonucleic acid
Rev	Regulator of expression of viral proteins
RRE	Rev responsive element
RSV	*Rous sarcoma virus*
RT	Reverse transcriptase
RV	Retroviral vector
SCID	Severe combined immunodeficiency disease
SEVI	Semen-derived enhancer of viral infection
SIN	Self-inactivating transfer vector
SIV	*Simian immunodeficiency virus*
SSCS	Single-step cloning-screening
ssRNA	Single-stranded RNA
SU	Surface subunit
SV40	*Simian vacuolating virus 40*
TAR	Transactivation response element
Tat	Viral transactivator
TCR	T cell receptor
TM	Transmembrane
T.U.	Transducing units
V.G.	Viral genomes
VIF	Viral infectivity factor
VLA-4	Very late antigen-4
VLA-5	Very late antigen-5
VPR	Viral protein R
VPU	Viral protein U
VSV-G	*Vesicular stomatitis virus* envelope glycoprotein G
WPRE	*Woodchuck hepatitis virus* post-transcriptional regulatory element
X-SCID	X-linked severe combined immunodeficiency disease
RV	γ-retroviral vector

1

Introduction

1.1 Gene therapy

Gene therapy is the treatment or prevention of a disease by introducing new genetic material into patient cells in order to add, replace, or inactivate gene functions. The rise of gene therapy is a consequence of its prolonged treatment action or ability to cure diseases that current therapies or drugs can only alleviate the symptoms.[1]

Gene therapy treatments where the delivery of genetic material into target cells is done by specific vehicles, generally called vectors, can be performed *ex vivo* or *in vivo*. In the *ex vivo* settlement, cells are first removed from the patient, cultured and modified in the laboratory to transfer the therapeutic gene, and are afterwards reintroduced into the patient. Due to the use of patient-derived cells, this set-up demands expensive clinical grade cell manipulation. However, the amount of vector needed is lower than *in vivo*, consequently decreasing production costs.[1, 2]

The *in vivo* gene therapy approach is characterized by a direct administration of a vector containing the therapeutic gene into patients. This procedure is less patient-specific, thus it presents reduced costs and fewer infrastructure requirements. Nevertheless, it requires high vector doses to circumvent the low gene delivery efficiency to a specific organ or tissue, and more strict quality demands on the vector.[1, 3, 4]

Worldwide, almost 2930 gene therapy clinical trials have been completed, are ongoing or have been approved.[5] During the last decades, gene therapy has moved from the initial concept of treating monogenic diseases caused by a single gene defect, such as cystic fibrosis, hemophilia, muscular dystrophy, sickle cell anaemia, severe combined immunodeficiency (ADA-SCID / X-SCID) and Duchenne muscular dystrophy, to more complex diseases, dependent on environmental factors, lifestyle or a polygenic background, such as cancer, diabetes and cardiovascular diseases (Figure 1.1).[6]

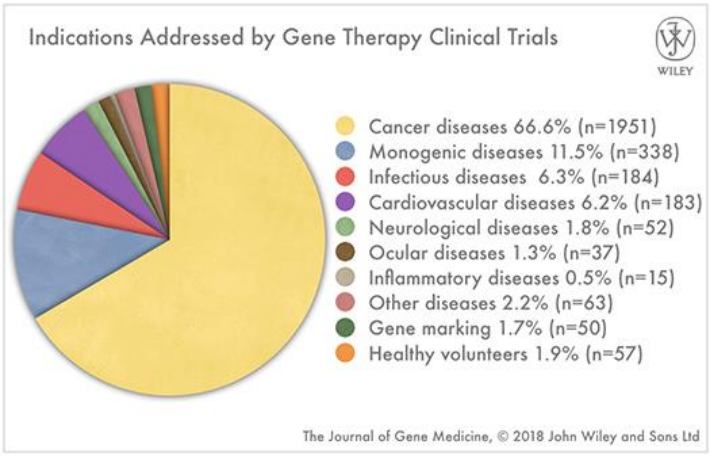

Figure 1.1 - Targeted diseases of worldwide gene therapy clinical trials. Adapted from [5].

Advances in cancer immunotherapy make cancer the disease best represented and targeted by gene therapy. The overwhelming majority of cancer clinical trials deliver genes into tumor cells to directly kill them or into patient immune cells to enhance recognition and killing of tumor cells. Adoptive T cell transfer is a fine example of the latter case, where autologous T-lymphocytes are genetically modified to express either T cell receptor (TCRs) or chimeric antigen receptor (CAR) to expand or redirect their antigen profile towards malignant cells.[6-9] Immunotherapies with this sort of technology lead to the Food Drug Administration (FDA) (2017) approval of the first CAR-T therapy products Kymriah™, developed by Novartis and Yescarta™, and marketed by Kite Pharma.

A variety of different vectors and delivery techniques have been applied in gene therapy clinical trials. These vectors can be viral or non-viral, although viral vector approaches remain by far the most popular.[6] Currently, several gene therapy products have already been approved and commercialized (Table 1.1), of which half is for cancer indications.[8-11]

Table 1.1 - Approved gene therapy products for commercialization, using viral vectors.

Product	Viral Vector	Target Disease	Regulatory Agency approval	Year	Company	
Gendicine®	Adenoviral vector	Head/neck squamous cell carcinoma	CFDA, China	2003	SiBiono GeneTech	[12]
Oncorine®	Adenoviral vector	Nasopharyngeal carcinoma	CFDA, China	2005	SiBiono GeneTech	[13]
Rexin-G®	Gamma-retroviral vector	All solid tumors	BFAD, Philippines	2007	Epeius Biotechnologies	[14]
Glybera®	Adeno-associated viral vector	Lipoprotein lipase deficiency	EMA, Europe	2012	uniQure biopharma	[15]
Imlygic®	Herpes simplex viral vector	Advanced melanoma	FDA, US	2015	Amgen	[16]
Strimvelis®	Gamma-retroviral vector	Severe Combined Immunodeficiency due to Adenosine Deaminase deficiency	EMA, Europe	2016	GlaxoSmithKline	[17]
Zalmoxis®	Gamma-retroviral vector	Host vs graft disease in haploidentical hematopoietic stem cell transplantation	EMA, Europe	2016	MolMed SpA	[18]
Kymriah®	Lentiviral vector	B-cell precursor acute lymphoblastic leukemia	FDA, USA	2017	Novartis	[19]
Yescarta®	Gamma-retroviral vector	Diffuse large B-cell lymphoma	FDA, USA	2017	Kite Pharma	[20]
Luxturna®	Adeno-associated virus	Bi-allelic RPE65 mutation-associated retinal dystrophy	FDA, USA	2017	Spark Therapeutics Inc	[21]
Zolgensma®	Adeno-associated virus	Bi-allelic SMN1 gene mutations-associated spinal muscular atrophy	FDA, USA	2019	AveXis/Novartis	[22]
Zynteglo®	Lentiviral vector	Transfusion-dependent β-thalassemia	EMA, Europe	2019	Bluebird bio	[23]

1.2 Viral vectors

Gene transfer systems, so-called vectors, can be viral or non-viral. Their use depends on the characteristics of each disorder and the respective gene therapy treatment. Therefore, its selection needs to be well-thought-out before deciding the delivery system that would be most appropriate.
Non-viral gene delivery system approaches essentially use naked DNA or DNA complexed with cationic lipids or polymers. The capacity of therapeutic DNA has no insert size limitation, they are less immunogenic and easier to manufacture, but lead to short-term gene expression and have a less efficient transfection.[1, 24]

On the other hand, viral vectors are recombinant viruses derived from different viral species which have naturally evolved to become highly efficient at genetic material delivery to host cells, while exploiting the cellular machinery and avoiding immunosurveillance. Thus, non-pathogenic viral vectors are the most used gene delivery systems due to their high gene transfer and gene expression efficiency. However, each recombinant virus has its strengths as well as weaknesses, factors that need to be well weighted for gene therapy applications.[24, 25] From all the viral vectors, the most used are the ones derived from adenoviruses, *γ-retroviruses,* adenovirus-associated viruses, and *lentiviruses* (Table 1.2).

Table 1.2 - Properties of the mainly used viral vectors. Adapted from [25].

Vector	Genetic material	Packaging capacity	Tropism	Inflammatory potential	Vector genome forms	Main limitations	Main advantages
Enveloped							
Retrovirus	RNA	8 kb	Dividing cells only	Low	Integrated	Only transduces dividing cells; integration might induce oncogenesis in some applications	Persistent gene transfer in dividing cells
Lentivirus	RNA	8 kb	Broad	Low	Integrated	Integration might induce oncogenesis in some applications	Persistent gene transfer in most tissues
Non-enveloped							
AAV	ssDNA	<5 kb	Broad, with the possible exception of haematopoietic cells	Low	Episomal (>90%) Integrated (<10%)	Small packaging capacity	Non-inflammatory; non-pathogenic
Adenovirus	dsDNA	30 kb	Broad	High	Episomal	Capsid mediates a potent inflammatory response	Extremely efficient transduction of most tissues

Up to date, adenovirus derived vectors compose the majority of clinical trials in gene therapy. They are nonenveloped, double-stranded DNA viral vectors and can accommodate approximately 30 kb of DNA inserts. In addition, they are able to efficiently deliver genes into both proliferating cells and resting cells of most tissues. However, they can trigger unwanted immune responses and do not integrate into the host genome, limiting the persistence of transgene expression.[6, 9, 25]

Other frequently used viral gene delivery options include recombinant adeno-associated viruses (AAV). These vectors have single-stranded DNA, are non-pathogenic, with a 4.6-kb cargo hold, showing high targeting infection rates in human cells with minimal immune responses. Their unique life cycle requires the helper functions from adenovirus or herpes simplex virus.[9, 26, 27]

γ-retroviral vectors (RV) are recombinant retrovirus that compose the second most type of vectors used in gene therapy clinical trials. These recombinant viruses carry a RNA genome and are able to accommodate up to 8 kb of therapeutic genetic material which is inserted in the patient cells genome, thereby providing a stable expression of the desired genes. However, they can only infect dividing cells which is suited for many cancer gene therapy trials because tumor cells are actively dividing. Although much has already been learned about the basic science of retrovirus, their genotoxicity due to insertional mutagenesis events, which raise the possibility of causing oncogenesis, have highlighted the desire to develop other options for gene delivery. To circumvent this problem, lentiviral vectors were developed, and their use in gene therapy is growing exponentially, as can be seen in Figure 1.2.[1, 9, 28, 29]

Lentivirus derived vectors (LV) are single-stranded RNA vectors with a capacity to accommodate up to 8 kb of genetic material. They have been widely used in gene therapy clinical trials due to its ability to transduce dividing and non-dividing cells and stably integrate the therapeutic gene in the patient cells genome, with less risk of insertional mutagenesis. Such characteristics have facilitated their usage in targeting cells of the nervous system, muscles, lungs, and liver for gene therapy applications of prolonged expression and little immunogenicity.[28-30] Overall, the unique features of LV have made this vector an attractive option in clinical trials.

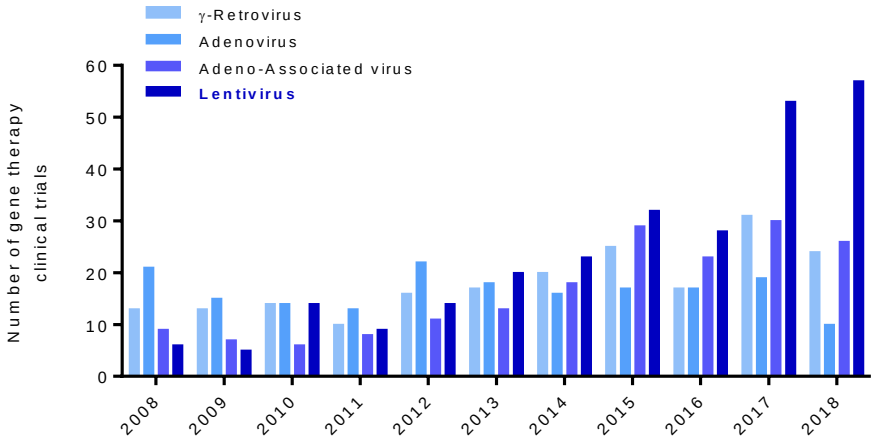

Figure 1.2 - Clinical trials incidence from 2008 to 2018 using viral vectors. Adapted from [5].

1.3 Biology of Lentiviruses

Lentivirus genus from the family *Retroviridae*, are more complex than the above described *γ-retroviruses*. Their name “Lenti-,” from the Latin “slow,” is derived from its long incubation periods (months or years), causing slowly progressive diseases such as immunodeficiency, pneumonitis, anemia, and encephalitis in their specific hosts (human, monkey, cow, sheep cat, horse and goat).[2, 3, 28, 31] In addition, there are numerous types of *Lentiviruses* that can be classified according to the animal species from which they are derived, e.g. the *Human immunodeficiency virus 1* and *2* (HIV-1, HIV-2) and *Simian immunodeficiency virus* (SIV).

Scientists have focused their attention on *Lentiviruses* since 1981, when the first cases of the acquired immunodeficiency syndrome (AIDS) epidemic were observed. Eventually, HIV-1 was isolated and identified as the cause of AIDS. Since then, the biology of HIV-1 has been intensively studied and

nowadays it is one of the best-understood viruses.[32, 33] Hence, HIV-1 derived vectors were the first to be developed due to their greater potential for use in human therapy and their high-titer vector stocks.[28]

The HIV-1 virion (Figure 1.3) is a spherical structure, and the inner part is composed of two positive single-stranded copies of genomic RNA (ssRNA) associated with nucleocapsid proteins (NC) and enzymes required for viral infection, such as integrase (IN), protease (PR), reverse transcriptase (RT) and accessory proteins, which are all enclosed within a capsid protein (CA) core. Matrix proteins (MA) form a spherical shell outside the core, which in turn is surrounded by the lipid bilayer derived from the host cellular membrane, where envelope glycoproteins (Env) are attached. The envelope is formed by a surface (SU, gp120) subunit which binds to the host cell surface receptors, and the transmembrane (TM, gp41) subunit, which together originate the membrane envelope glycoprotein (gp160), responsible for the anchorage in the lipidic membrane. The TM subunit harbors an ectodomain, a transmembrane domain, and a cytoplasmatic tail domain.[3, 33, 34]

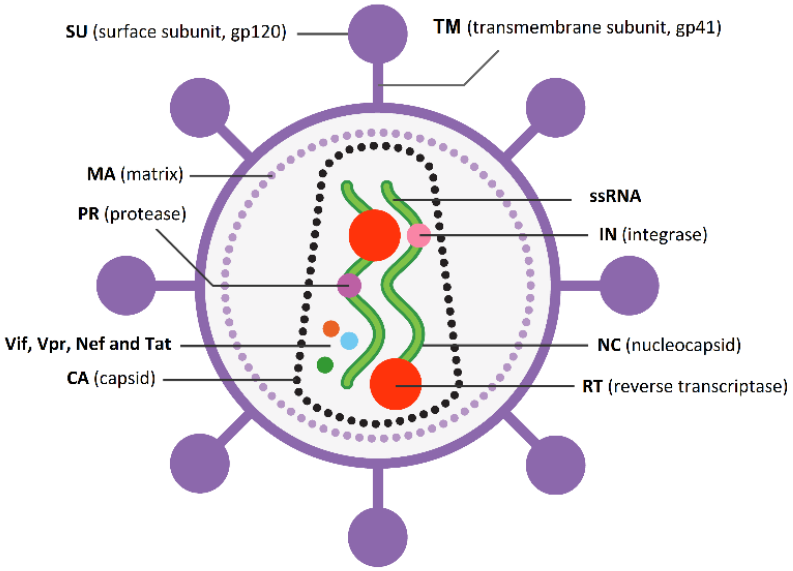

Figure 1.3 - Schematic representation of a mature HIV-1 particle. Adapted from [35].
CA-capsid protein; Env-envelope glycoprotein; MA-matrix protein; NC-nucleoprotein; PR-protease; RT-reverse transcriptase; ssRNA-single-stranded RNA; SU-surface subunit; Tat-viral transactivator; TM-transmembrane subunit; Nef-Negative factor; Vif-Viral infectivity factor; Vpr-Viral protein R.

The HIV-1 genome (Figure 1.4) is approximately 9 kb in length and can be divided into two categories of *cis* and *trans* components.

The *trans*-acting viral elements encode three groups of proteins: structural, regulatory and accessory, composed by nine open reading frames. Three of these contain the conserved genes encoding the polyproteins Gag, Pol, and Env. The *gag* gene encodes a polyprotein which is cleaved by the viral protease, during and after the release of the newly formed virion, resulting in the main structural proteins MA, CA and NC. The *gag-pol* gene encodes another polyprotein (Gag-Pol), that will give rise to PR, RT, and IN after protease cleavage also during virus maturation. These enzymes are responsible for proteolytic cleavage of most viral polyprotein precursors, the reverse transcription of viral RNA genome to double-stranded DNA (provirus) and the integration of the provirus into the cellular host genome.

The *env* gene encodes the viral glycoprotein (SU and TM subunits) that will interact with receptors of the host cell allowing for virus entrance.[3, 31, 34]

The remaining six genes of HIV-1 genome code for two regulatory proteins (Tat and Rev) and four accessory proteins (Vif, Nef, Vpr, and Vpu).
Tat protein plays a critical role in producing large quantities of mRNA transcripts from the integrated proviral genome by acting as a transactivator of transcription, whereas, Rev controls the export of unspliced mRNA from the nucleus, by multimerizing along the Rev responsive element sequence (RRE), which is localized in the middle of the *env* gene. Finally, the accessory proteins (Vif, Vpr, Vpu, and Nef) are responsible for disease pathogenesis in man but are not strictly necessary for viral replication *in vitro*.[3, 31, 34]

Additionally to the coding sequences, the HIV-1 genome also has several non-coding *cis*-acting sequences that play important roles in viral replication. The viral genome is flanked by LTRs (long terminal repeats) sequences, composed of 3' untranslated region (U3), repeat elements (R) and 5' untranslated region (U5). These regulatory sequences of DNA act as promoters of transcription for the viral genome and help stabilize the newly synthesized transcripts by regulating their polyadenylation. Within the 5'LTR, there is the transactivator response element (TAR) that interacts with the complex formed by Tat and transcriptional factors, functioning as a promoter. The 5'LTR is followed by the primer binding site (PBS) where the reverse transcription starts, and by the packaging signal (Ψ) for the viral genome incorporation into the new particle. Finally, within the *pol* sequence, there is also the central polypurine tract (cPPT), which contributes for an efficient reverse transcription. In the beginning of the 3'LTR, there is a purine-rich region responsible for the start of plus-strand DNA synthesis during reverse transcription (PPT).[3, 31, 34]

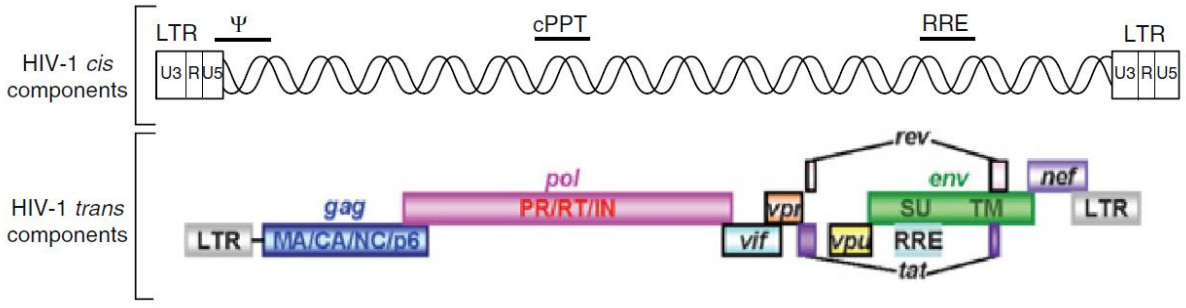

Figure 1.4 - Schematic representation of the HIV-1 viral genome cis- and trans-acting components. Adapted from [3, 31].
HIV-1: Human immunodeficiency virus 1; LTR-long terminal repeat; MA-matrix protein; CA-capsid protein; NC-nucleoprotein; ψ-packaging signal; cPPT-central polypurine tract; RRE-Rev Responsive Element; PR-protease; RT-reverse transcriptase; IN-integrase; Env-envelope glycoprotein; SU-surface subunit; TM-transmembrane subunit;

The viral life cycle begins when the natural HIV-1 particle envelope gp120 binds to the CD4 cell receptor and its co-receptor CXCR4 (CXC chemokine receptor 4), expressed on T-lymphocytes or CCR5 (CC chemokine receptor type 5) on macrophages and dendritic cells. Following binding to the host cell receptors, the viral lipid membrane fuses with the host cell membrane. Once inside the cell, virus

uncoating allows the release of the viral core components into the cytoplasm. The single-stranded RNA genome is converted to DNA by the viral RT, forming a complex with viral and cellular proteins which is called the pre-integration complex (PIC).

The PIC is assembled and the double-stranded DNA product is translocated into the nucleus and integrated into the chromosome by IN. The integrated viral DNA is subsequently transcribed and translated to form new viral RNA and viral proteins. Viral components are transported to the cell surface to assemble into virus particles that will be released. After leaving the cells, during maturation, infectious virions are generated by proteolysis by the viral PR of Gag and Gag-Pol polyproteins.[3, 34]

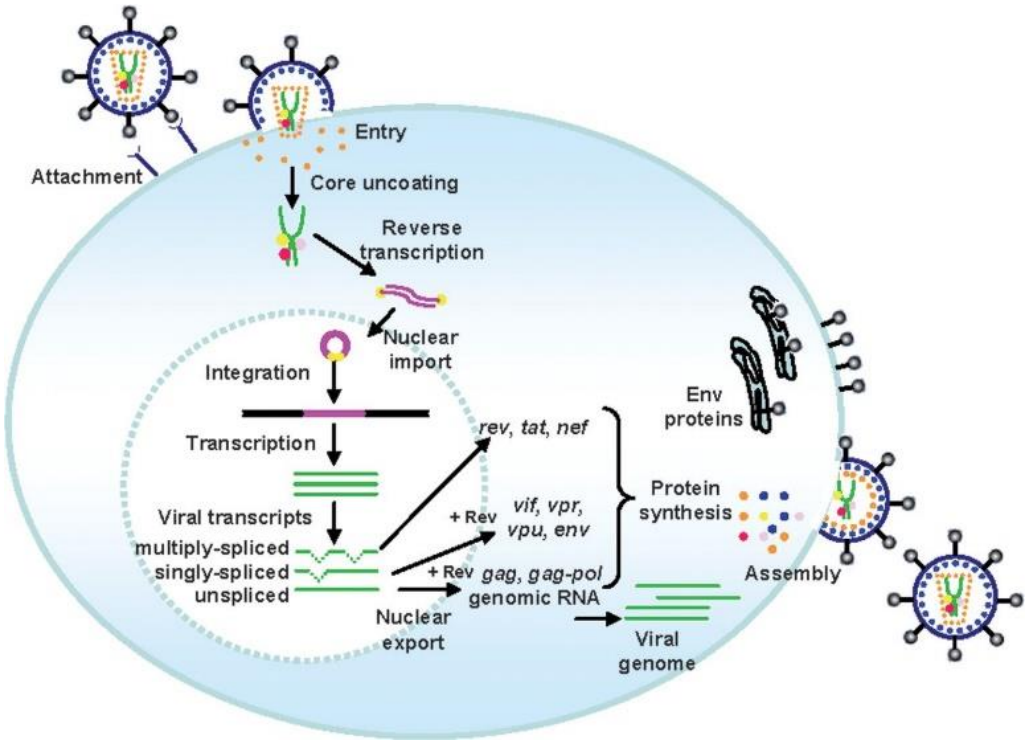

Figure 1.5 - Schematic representation of the HIV-1 life cycle. Adapted from [31].

1.3.1 From Virus to Vector

Safety concerns regarding the use of LV arise because the main recombinant virus used for this purpose is derived from HIV-1, a well-known and potentially lethal human pathogen. Additionally, another important safety issue is the possibility of generating replication-competent (RCL) virus. Therefore, the design of LV has progressed towards the minimization of RCL and elimination of pathogenicity. The steps taken by Naldini *et al.,* in 1996 originated an era of intense interest in the use of LV for gene therapy which attracted many new researchers to the field.

The system for HIV-1-based LV was achieved by splitting vector components into three plasmids, creating the 1st generation of LV (Figure 1.6). The first plasmid is a packaging vector encoding all necessary *trans*-acting genes with its LTRs replaced by a heterologous CMV (*Cytomegalovirus*) promoter. The second plasmid contains an envelope construct expressing a heterologous viral glycoprotein, VSV-G (vesicular stomatitis virus envelope glycoprotein G). Finally, the third plasmid is a

transfer vector containing the transgene of interest combined with all *cis*-acting sequences necessary for expression, packaging, reverse transcription, and integration.[3, 31]

2nd generation vectors have been developed by removing the accessory genes from the system. Although these accessory genes are important for HIV-1 as a pathogen, they can be deleted without negatively effecting vector titer. Still, *tat* and *rev* regulatory genes were maintained: Tat to activate transgene expression through the 5'LTR and Rev to allow the export of the packaging and transgene mRNA nucleus export.[3, 31]

The 3rd generation system, and the most currently used, improved the 2nd generation by deleting *tat* from the packaging construct, and splitting this plasmid in two, one encoding *gag* and *pol* genes and another for Rev. Moreover, Tat-independence was achieved by replacing the U3 promoter region of the 5'LTR, in the transfer vector, for a strong viral heterologous promoter from CMV or RSV (*Rous Sarcoma virus*). With four constructs, the number of homologous recombination events required for RCL formation is increased. Although safer, this system resulted in lower viral titers due to the presence of one additional plasmid.[34, 36]

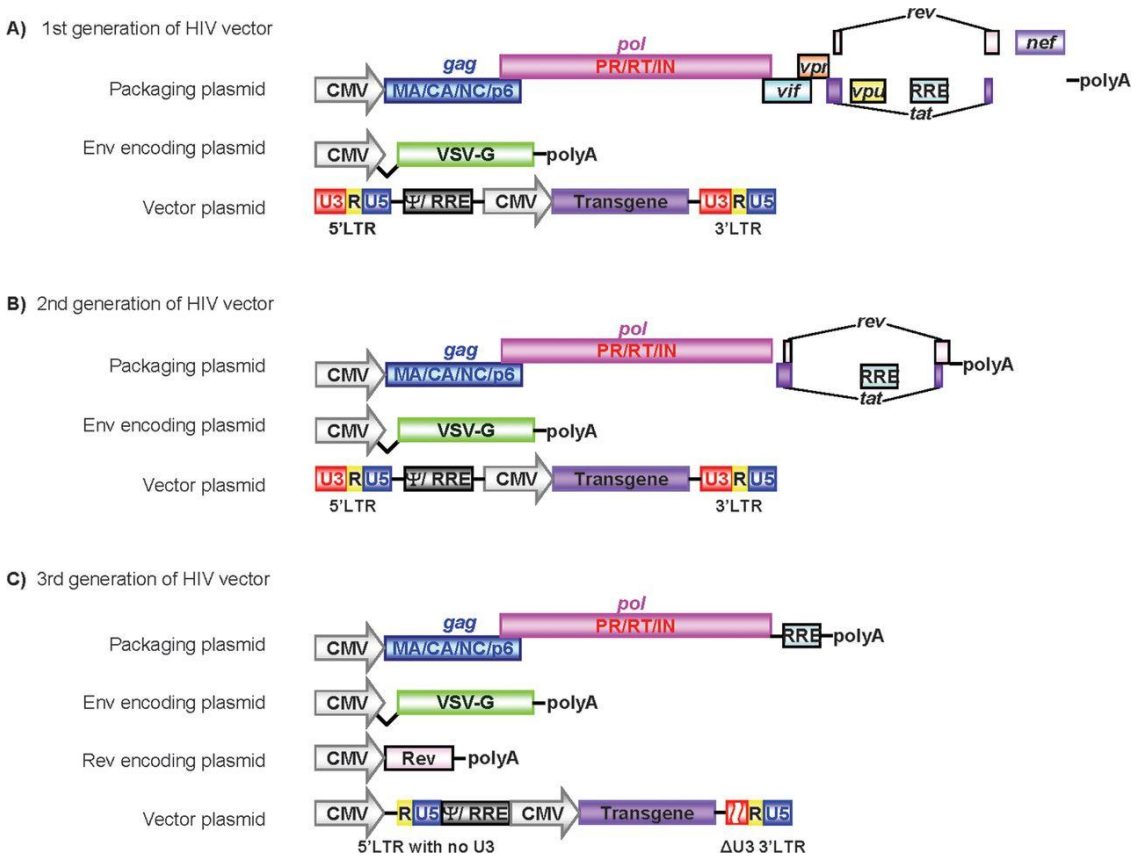

Figure 1.6 - First, second, and third generations of HIV-1 derived LV plasmids constructs. Adapted from [31].

Further safety alterations were made to the transfer vector construct, including the development of a self-inactivating (SIN) transfer vector (Figure 1.6, c). SIN constructs contain a deletion in the U3 region of 3'LTR that, upon reverse transcription of the vector genome, results in the transcriptional inactivation of the 5'LTR U3 and substantially diminishes the risk of vector mobilization and recombination after transduction. This inactivation also reduces concerns related to insertional mutagenesis in the neighboring sequences that can lead to the transactivation or up-regulation of neighboring host genome sequences, such as oncogenes. This SIN design is mostly used in the 3rd generation systems, but can also be used with the 1st and 2nd generations. [8, 9, 37]

Efforts to develop a Rev independent 4th generation system were also made. One approach was the replacement of the RRE sequence by heterologous viral sequences known to enhance the export and stability of unspliced RNAs by interacting with cellular factors. Another approach was by codon-optimization of the packaging plasmid, which resulted in the elimination of *cis*-acting inhibitory sequences. Nonetheless, due to the low titers of both approaches, the 3rd generation is still the most used for clinical applications.

1.3.2 Pseudotyping

In the context of gene therapy viral vector development, pseudotyping is the exchange of the natural envelope glycoproteins of a given virus for another of interest to manipulate the vector tropism and bioprocess resistance. Due to the natural tropism of HIV-1 envelope glycoprotein, the use of HIV-1 derived LV in gene therapy applications would be restricted to $CD4^+$ cells, e.g. macrophages or T cells. The absence of this specific receptor in other target cell types would result in a dramatically attenuated or undetectable viral transduction.[3, 33, 38, 39] However, viral envelopes derived from retroviruses offer a variety of possible associations with heterologous viral surface glycoproteins. Thus, customized LV with heterologous glycoproteins broaden the range of target cells and tissues and improve gene transfer efficiency.[28, 38, 40] The most common envelope glycoproteins used for LV pseudotyping are mostly derived from other retrovirus, namely from the amphotropic Murine leukemia virus (MLV), the Gibbon ape leukemia virus (GaLV), the Feline endogenous virus (RD114) and the non-retroviral vesicular stomatitis virus (VSV-G).[41]

Since lipid membranes do not mix spontaneously, it is described that viral fusion proteins suffer complex conformational changes, firstly anchoring themselves to the host membrane and then folding back, bringing the viral and cellular membranes together, forcing their merger in an energy-dependent process that results in the formation of fusion pores through which the viral content is released (Figure 1.7).[42, 43]

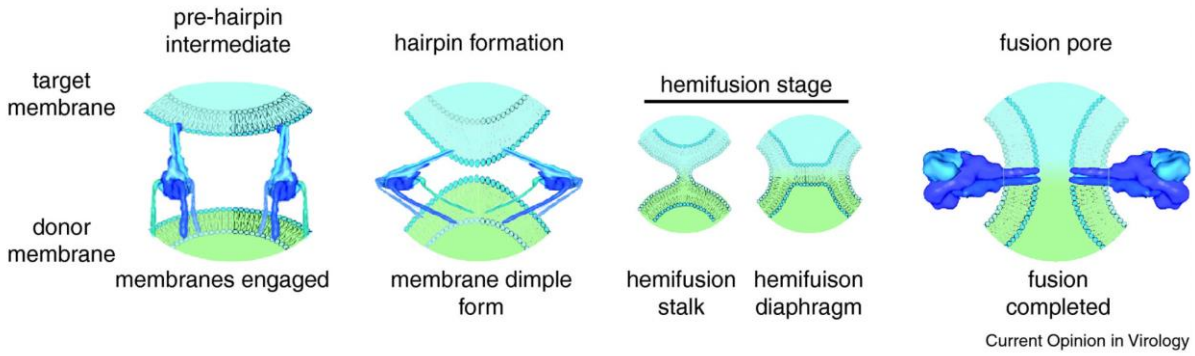

Figure 1.7 - Scheme of the viral membrane merger of class I fusion proteins. Adapted from [43].
Upon fusion, conformational changes in the glycoprotein result in viral envelope and target membrane engagement, creating a hemifusion stage, finally leading to pore formation.

Two different pathways by which the interaction between virus envelope glycoprotein and the host cell receptor leads to membrane fusion are known, pH-dependent and pH-independent.
In retroviruses (MLV, GalV, and RD114), the envelope glycoprotein is expressed as a precursor protein. During maturation, SU subunit is functionalized through viral protease cleavage of a short sequence (R-peptide) from the cytoplasmic tail domain of the TM subunit. This cleavage activates the fusogenic competence of the envelope glycoprotein, which, after recognition and attachment to host cell receptors, leads to lipid membrane fusions. This viral entry is not dependent on exposure to an acidic environment, and fusion with cell surface membranes occurs with equal efficiency over a pH range of 5–8.[11, 42]
In contrast, VSV-G envelope glycoprotein does not need to be cleaved to become fusogenic. Instead, it binds to the host cell, and after clathrin-mediated endocytosis of the virion, membrane fusion with endosome vesicles is triggered via a low pH-induced structural rearrangement, releasing the virion into the cytoplasm.[11, 42, 44, 45] This envelope has several advantages since it is very stable and allows for concentration of viral particles by high-speed ultracentrifugation.[46-48]

Based on the structure, viral fusion proteins are currently categorized into three classes based on their common structural motifs (Figure 1.8). Class I fusion proteins comprise *Retroviruses* glycoproteins, which are characterized by their trimeric pre- and post-fusion conformation, and are synthesized as an inactive precursor requiring proteolytic cleavage to become active. Class II fusion proteins are known to transit from a pre-fusion dimer to a post-fusion trimer through an intermediate monomer. Class III fusion proteins combine structural elements found in the two other classes being trimeric in both the pre- and post-fusion conformations, comprising the G protein of VSV.[42, 43, 49]

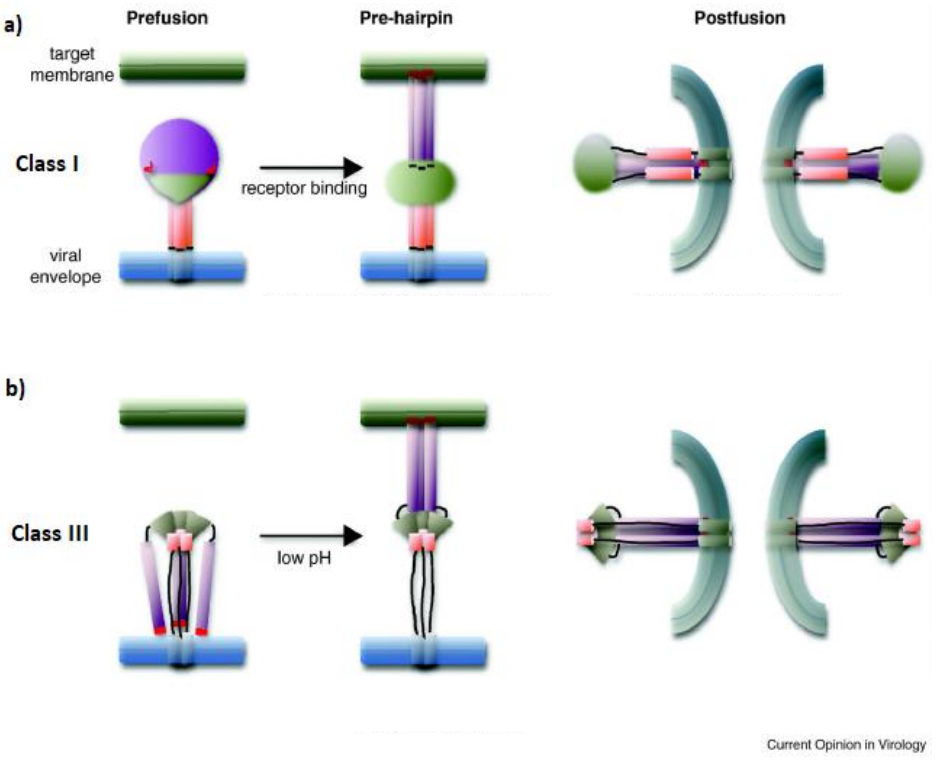

Figure 1.8 - Schematic representation of the conformational changes of viral fusion protein classes I and III. Adapted from [43].
Class I fusion proteins in a) where fusion peptides are highlighted in red, in purple the trimeric coils and in orange TMD domains. Class III fusion protein in b) where the fusion domains are highlighted in purple and the trimerization domains in orange.

Identification of virus receptors has frequently provided insights into disease pathogenesis, virus life cycle and has offered fruitful areas for therapeutic interventions. Each envelope glycoprotein confers a different set of properties to the LV and so, each pseudotype may have its potential niche.[3, 50]

The most widely used LV pseudotype is the VSV protein G, due to its ability to infect a broad range of tissues. However, due to the wide distribution of its receptor, the recently discovered low-density lipoprotein (LDL), the use of VSV-G pseudotypes in *ex-vivo* therapy may target undesired cell types, posing a safety concern for their use in clinical settings. Also, systemic administration in humans is hampered by complement and antibody immune responses directed to this envelope.[31, 41, 51, 52] Finally and unfortunately, this protein is toxic to cells which constrains its expression in LV constitutive production systems.

In response to these obstacles, the envelopes from MLV, GaLV, and RD114 have been explored.[3, 50]

The amphotropic MLV (4070A) envelope glycoproteins targets an inorganic phosphate transporter, known as PiT-2, whereas for GaLV pseudotype, its host cell receptor is also an inorganic phosphate transporter, known as PiT-1. These target receptors share 62% amino acid identity, yet they have divergent host ranges. Furthermore, they are expressed in $CD34^+$ hematopoietic precursor cells, one of the main targeted cell types in genetic pathologies treatment by gene therapy, therefore making 4070A and GaLV pseudotyped vectors optimal for the treatment of monogenic diseases.[3, 48, 50, 53]

The receptor for RD114 has been identified as sodium-dependent neutral amino acid transporter-type (ASCT2) which is an acronym standing for alanine, serine, cysteine transporter 2. Interestingly, ASCT2

expression increases in highly proliferative cells, as in many human cancers. This receptor is widely expressed in human tissues and cell lines, including hematopoietic cells, making it also particularly interesting to pseudotype LV.[41, 54-56]

1.4 Transient and stable production of LV

As gene therapy is on its way of becoming a routine treatment, not only for rare genetic disorders, but also for acquired and infectious diseases, the implementation of robust and scalable LV production protocols is urgent to satisfy the clinical and market demands.[57]

To ensure production of high-quality and yield vectors preparations for clinical application, the use of Good Manufacturing Practices (GMP) is required. Large-scale productions are needed since the treatment of a single patient may require up to 10^{12} total functional particles, which at the average production titer (10^6-10^8 per milliliter) represents hundreds or thousands of viral supernatant liters, leading to an enormous high cost and rendering this process unfeasible.[58]

LV can be produced by transient transfection or stable producer cell lines (Figure 1.9). Traditionally, LV production has relied on a transient co-transfection of cells with the expression cassettes (Figure 1.9, a), undoubtedly a faster process when compared to the time frame necessary to develop a stable cell line. Nevertheless, transient productions are not the ideal system when moving to large scale productions since a large amount of highly pure DNA and transfection reagents is required. Moreover, substantial batch-to-batch variations and the short time-window of LV production exponentially increases the costs.[2, 57, 59, 60]

Alternatively, stable production relies on the separate and sequential integration of the viral constructs into the cell genome, thus allowing their constitutive and continuous expression.[61]

The establishment of stable LV cell lines has remained a challenge due to the intrinsic cytotoxicity of the LV components. Lentipro26 is an example of a LV stable producer cell line developed in-house by Tomás *et al*., (2018), which uses a less active mutated viral protease.[59] Either way, compared with transient productions, stable LV production for gene therapy product commercialization still represents a milestone to reduce the manufacturing costs.[59] Only ~1% of LV particles are functional, and its use in either *in vivo* or *ex vivo* settings in suboptimal conditions lead to an expensive process.[1-3] Considering this, there is an urgent need to develop methodologies for a faster manufacture of LV stable cell lines, and to improve LV transduction efficiencies in order to alleviate LV production costs per patient.

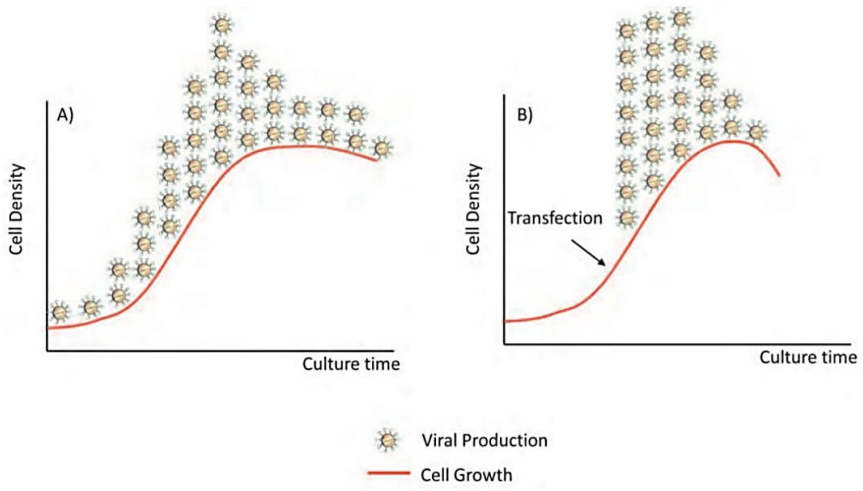

Figure 1.9 - LV production scheme showing the proportion of viral particles produced throughout cell culture time. Adapted from [61].
A) Stable systems showing a continuous production throughout cell culture time, until the end of the exponential phase of cell growth. In b) a transient production system, where high viral titers are obtained after 24 to 72 hours of transfection.

1.4.1 Accelerating cell line development

The time spent in the insertion of viral expression cassettes, selection of the producer cell population and screening the high titer producer clones makes the process of cell line development very laborious and time-consuming. In order to accelerate this process, Rodrigues *et al*., (2015) developed a fast and high-throughput cloning and titration method. Using the single-step cloning-screening (SSCS) method, in only two weeks it is possible to assess clone growth and productivity at earlier stages, shortening the process of cloning and screening of viral vector producer cells.[62]

The method is based on the split GFP, composed of two non-fluorescent fragments, GFP S10 coding for the first 214 amino acids and GFP S11 for the last 15.[62, 63] Briefly, cells producing RV carrying a GFP S11 transgene are co-cultured with target cells which stably express GFP S10 fragment (Figure 1.10). During the period of clone expansion, recombinant GFP S11 virus transduces GFP S10 target cells, and GFP fluorescent signal is activated. The amount of green fluorescence obtained at the end of the protocol will directly estimate each clone productivity, allowing a fast identification of high-producing clones. Only the clones yielding the highest GFP signal will be isolated for posterior studies.[62]

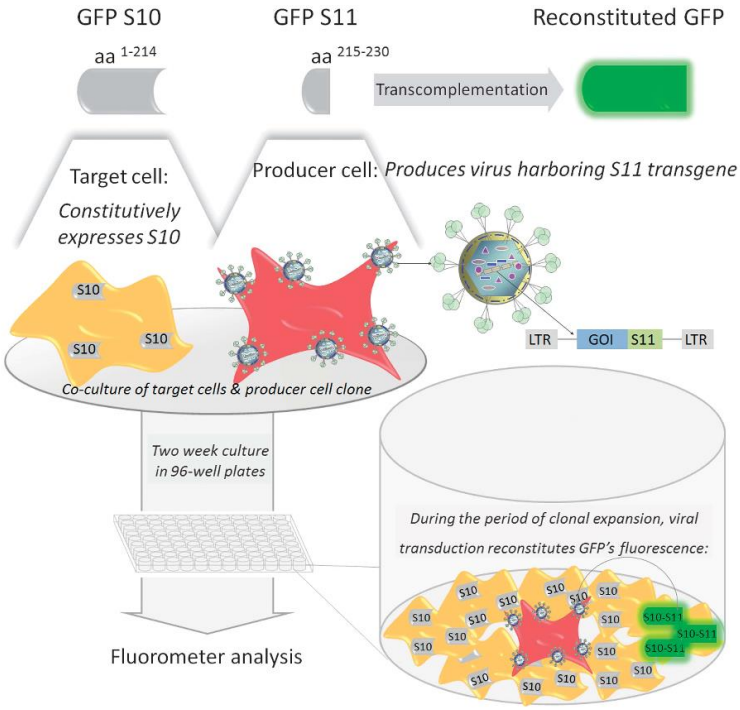

Figure 1.10 - Schematic representation of the single-step cloning-screening titration protocol. Adapted from [62].

Initially, this method was developed for the establishment of RV producer cell lines. However, due to the aforementioned urge to develop constitutive LV producer cells lines, the adaptation of this methodology to LV would be of great interest and importance.

1.5 Transduction adjuvants to improve LV transduction efficiency

Considering that only 1% of produced LV particles are able to efficiently deliver the therapeutic gene, it is mandatory to functionalize the remaining 99% to efficiently decrease costs and increase product availability. This low LV transduction efficiency results partially from strong electrostatic repulsions between the negatively charged cell membrane and the enveloped virus, limiting clinical success. Therefore, the identification of safe, soluble, clinical-grade, easy to manipulate additives with a potent capacity to promote target cell transduction is highly necessary. These transduction enhancing reagents are also called boosters, or adjuvants.[64-67]

These are mostly cationic additives with the capacity to neutralize the repulsive membrane charges, mediating viral adhesion, virus aggregation and/or virus pull-down.[37] Importantly, most of these adjuvant treatments have toxic effects, limiting their use especially in sensitive target cells of primary origin.[68]

For low molecular weight cationic polymers, the mechanism of action is through charge shielding. Yet, large molecular weight polymers can affect the electrostatics of the virus-cell interaction by resulting in virus aggregation and sedimentation (Figure 1.11).[64]

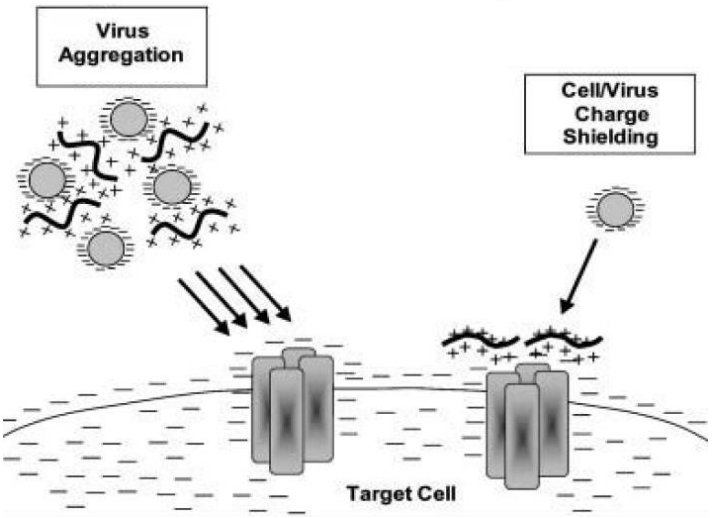

Figure 1.11 - Mechanism of action regarding electrostatic interactions between charged polymers, virus, and target cell membrane. Adapted from [64].

Several commercialized products are available for viral transduction enhancement, some of them being compatible with clinical trials, and others commonly used in research academic settings. Among several classes of chemicals, there are polycations (polybrene-like substances) and pluronic block copolymers (termed poloxamers).

Polybrene

Hexadimethrine bromide (Polybrene), is a linear polycationic polymer described as a positively charged chemical adjuvant used for LV transduction. It is the main compound used in laboratory research and is able to improve gene transduction rates for a broad range of target cells. Unfortunately, polybrene can only be used over short application times and at low concentrations to avoid cellular toxicity through

disruption of the transmembrane potential, therefore severely limiting its use in sensitive cells, such as those of the hematopoietic lineage targeted in gene therapy.[31, 64, 66, 68]

Rectronectin

Retronectin reagent (also referred to as CH-296) is a recombinant human fibronectin of 63 kD that contains three domains: the cell-binding domain (C-domain), the heparin-binding domain (H-domain), and the RGD in segment-1 (CS-1) sequence (Figure 1.12). Retronectin reagent enhances viral vectors transduction by assisting the colocalization of target cells and viral particles. This reagent interacts with virus particles and with target cells through the interaction of cell surface integrin receptors VLA-5 and/or VLA-4 with the fibronectin C-domain and/or CS-1 sites, respectively.[46, 69]

For high gene delivery efficiency, target cells must express the integrin receptors VLA-4 and/or VLA-5 to mediate physical proximity. VL-4 integrin is expressed in T cells, B cells, NK cells, monocytes, bone marrow monocytic cells, eosinophils, and lymphoid progenitors, while VLA-5 is mainly expressed in thymocytes, activated T cells and hematopoietic cells.[70]

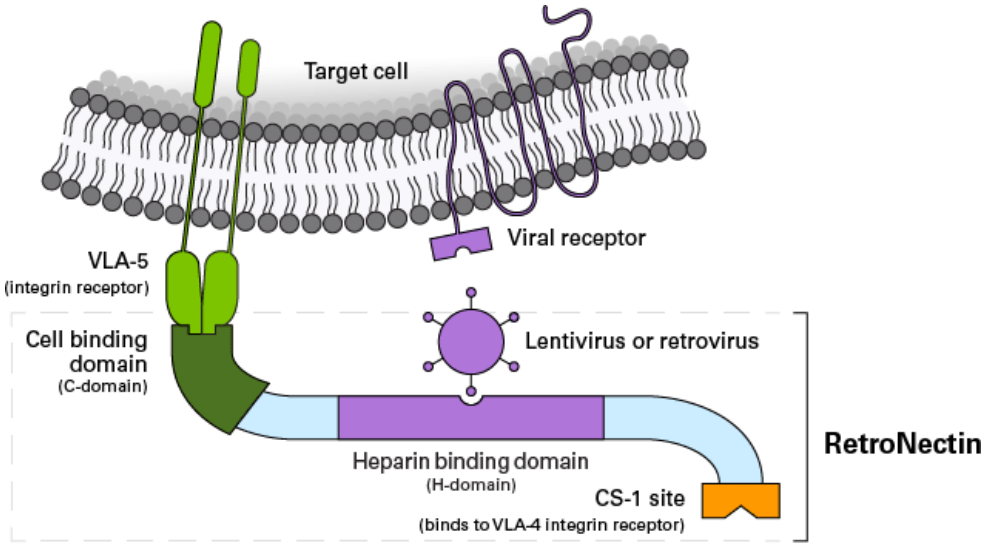

Figure 1.12 - Ilustrative figure of retronectin mode of action and binding sites. Adapted from [69].

In addition, retronectin is manufactured according to GMP guidelines and thus can be used in clinical settings of *ex vivo* transduction of human $CD34^+$ hematopoietic stem cells and T lymphocytes. Consequently, this reagent is now the gold standard transduction enhancer for gene transfer to hematopoietic cells and holds great promise in clinical applications, having been already used in over 40 clinical trials.[31, 55] Unfortunately, although this peptide is very efficient in enhancing vector transduction, it is also expensive and requires pre-coating of cell culture dishes before cell seeding, lacking high-throughput and making the transduction process cumbersome and tedious.[55]

Vectofusin-1

Vectofusin-1, also known as LAH4-A4, is a member of the LAH4 family of DNA transfection agents. This compound is a fully synthetic, short, non-toxic, histidine-rich amphipathic peptide (Figure 1.13) which can exert functions of viral transduction enhancer. The cationic charges of this peptide are conferred by the presence of four lysine and four histidine residues which make an overall charge of +9 at pH below 6.[46, 66]

In contact with culture medium, vectofusin-1 spontaneous forms supramolecular complexes in the 10 nm diameter range that further assemble into annular and extended nanofibrils. These associate with viral particles leading to their sedimentation, and thus increasing local concentration at the cell surface, allowing optimal virus-cell interaction.[66] Vectofusin-1 also promotes the entry of several RV and LV pseudotypes into several target cells acting at the entry step by improving adhesion and fusion between vector and cellular membranes. One hypothesis is that vectofusin-1 is able to insert itself in the viral and/or cellular membranes, leading to a modulation of the latter steps.[55] However, the critical determinants of vectofusin-1 that play a crucial role in LV transduction enhancement are still unknown.[46]

Peptides are interesting for their biodegradable property, reduced size, simplicity of characterization, and large-scale production, when compared with polymers. In addition, its reversible fibril formation capacity makes it easier to handle in GMP conditions guaranteeing high purity for clinical use.[66, 67]

The enhancing effects of vectofusin-1 are analogous to other viral transduction enhancers such as retronectin or the semen-derived enhancer of viral infection (SEVI) peptide which are currently used in clinical settings.[46]

In conclusion, some examples of using short cationic peptides for intracellular delivery *in vivo* and *in vitro* have already been reported, making the LAH4-A4 peptide a considerable promise for LV transduction in clinical settings.[71]

Figure 1.13 - Vectofusin-1 peptide sequence composed of 26 amino acids. Adapted from [46].

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LentiBOOST™

LentiBOOST™ is a transduction enhancer for preclinical and clinical application of LV. It is a non-ionic, amphiphilic poloxamer, which consists of two hydrophilic ethylene oxide (EO) branches flanking a hydrophobic propylene oxide (PO) core region. In aqueous solution, these kinds of molecules are able to self-assemble as micelles (Figure 1.14).[68, 72]

Independently of the entry mechanism, due to their hydrophobic core, poloxamer monomers can interact with lipid membranes, leading to a lower membrane microviscosity and a higher lipid exchange. Such characteristics may increase membrane permeability and therefore facilitate the membrane transport of viral particles, which are expected to attach to the EO corona.[68, 72]

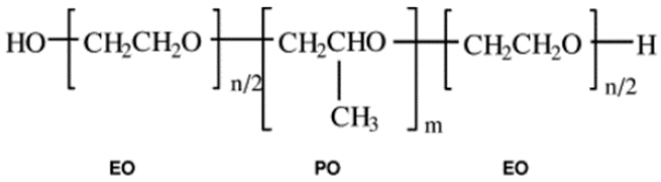

Figure 1.14 - Illustration of a pluronic copolymer composed of two hydrophilic EO blocks and a hydrophobic PO block. Adapted from [72].

The core-shell architecture of these micelles has also been already used in animal model studies as a 'cargo hold' for therapeutic drug delivery. Moreover, the distribution of this compound showed low toxicity when injected intravenously, intraperitoneal or subcutaneously, suggesting that lentiBOOST™ could be used in therapeutic applications.[51]

Another advantage is its good aqueous solubility, which eases its use in the transduction process under GMP conditions. Therefore, it can be useful for a wide range of clinically relevant cell types hard to transduce, including $CD34^+$ hematopoietic stem cells, primary T cells, and NK cells.[73, 74] Considering this compound to be non-cytotoxic, highly effective, cost-effective and clinically safe, it can eventually lead to its straightforward use in the optimization of large-scale clinical LV transduction protocols.[51, 68]

LentiBlast™

LentiBlast™ is based on a patent-protected chemical composition, composed of two reagents (A and B) that permits the neutralization of electrostatic repulsions between the cellular membrane and the viral particles, allowing improved virus fusion with the target cell membrane. It is known to bypass some obstacles that avoid successful transduction, such as cell density, LV purity, and low MOI. In addition, it is non-toxic and maintains cell viability even at high concentrations, making it ideal for increasing transduction of lentivirus in primary human cells, $CD34^+$ hematopoietic stem cells, T lymphocytes, and suspension cell lines.[75]

DEAE-Dextran

Diethylaminoethyl (DEAE)-dextran is a polycationic derivative of the carbohydrate polymer dextran produced at an average molecular weight of 500 kDa. It positive charge derives from the presence of the amine group at physiological pH (Figure 1.15).[76, 77]

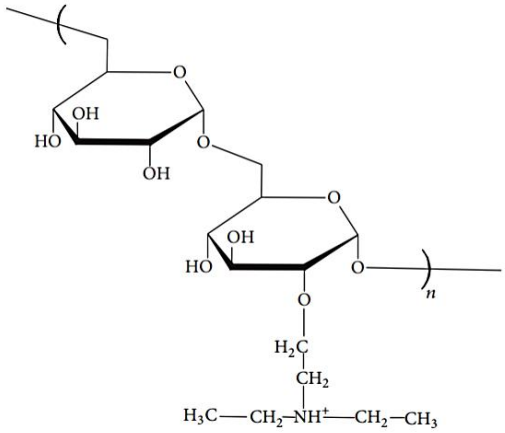

Figure 1.15 - DEAE-Dextran molecule. Adapted from [77].

It was one of the first chemical reagents used for DNA delivery into cultured mammalian cells and to be reported as an enhancer of the viral infectivity of cells.[78] Currently, it has a wide variety of applications including in medicinal products, chromatography and as a polycationic stabilizing molecule.[37, 79]
The use of this compound in the laboratory setting is relative simple, reproducible, and low cost, while its disadvantages include high cytotoxicity *in vivo* for a range of cell types and compound concentrations.[77, 79]

Transplus™

TransPlus™ is composed of a patent-protected mixture of polymers optimized for the infection of LV or RV, and more specific details regarding its composition or action are unknown.[80]

1.6 Aims and strategy

Currently, transient production is the standard bioprocess for LV production. However, its problematic scalability and poor viral preparation quality, impairs LV use and accessibility for clinical contexts. One way to improve current LV products, is to increase the functionality of the existing viral preparations, contributing to reduce production volumes and consequently lowering the costs.

Considering this, we first aimed to study the LV transduction efficiency impact of four different viral envelopes - VSV-G, 4070A, GaLV and RD114 - in three target cell lines - HEK 293T, Jurkat E6-1 and SUP-T1. HEK 293T target cells were chosen since they are commonly used in laboratory context for research purposes. Jurkat E6-1 and SUP-T1 are cell lines acting as T cell models, important gene therapy cell targets of emerging immunotherapies.

Several strategies to promote LV cell contact were also studied, including spin-inoculation and different infection volumes aiming to force the physical contact between LV and cell membranes. Furthermore, seven different viral entry adjuvants, including clinical grade and laboratory use compounds were also screened in order to study their transduction enhancing capabilities.

With the knowledge generated, the second aim of this work was to benefit from the viral adjuvant that better enhanced LV transduction efficiency to adapt the SSCS method. By adapting this method to a LV stable producer cell line, Lentipro26, we aimed to facilitate the development of new stable producer cell lines, another major challenge faced by the industry.

2

Materials and Methods

2.1 Plasmids

pMDLg/RRE is a third generation LV packaging plasmid encoding for HIV-1 structural and enzymatic proteins codified in *gag-pol* under the control of a CMV promoter. Additionally, it has the RRE RNA sequence, a binding site for Rev protein, enabling the export of unspliced or partially spliced viral RNA from the nucleus to the cytoplasm. Addgene plasmid repository (Cambridge, MA, USA) plasmid #12251.

pRSV-Rev is a third generation LV packaging plasmid containing the second and third exons of HIV-1 Rev protein under the control of RSV U3 promoter. Addgene plasmid repository #12253.

pRRLSIN.cPPT.hPGK-GFP.WPRE is SIN LV transgene plasmid, carrying a green fluorescent protein (GFP) gene, expressed through an internal human phosphoglycerate kinase 1 (hPGK) promoter and a *Woodchuck hepatitis virus* post-transcriptional regulatory element (WPRE) which enhances transgene expression. Addgene plasmid repository #12252.
All above described plasmids were kindly provided by Dr. Didier Trono through Addgene plasmid repository, and used to produce LV particles.

The following plasmids enabled the expression of viral envelope glycoproteins and were used to pseudotype the LV particles.

pMD2.G encodes for the envelope glycoprotein G (VSV-G) under the control of a CMV promoter, kindly provided by Dr. Didier Trono through Addgene plasmid repository #12259.

pCMV-4070A encodes the amphotropic MLV envelope glycoprotein 4070A, expressed through CMV promoter control. This plasmid was developed by Tomás *et al*., (2019).[81]

pCMV-RD114pro, drives the expression of the chimeric RD114 envelope glycoprotein, through the CMV promoter. The cytoplasmic tail was replaced by the one from 4070A and the natural R-peptide cleavage site sequence was previously replaced by the cleavage site of HIV-1 MA/CA (SQNY↓PIVQ). This plasmid was developed by Tomás *et al*., (2019).[81]

pCMV-GaLV10A1giflet, drives the expression of a chimeric GaLV envelope glycoprotein, under the control of a CMV promoter. The cytoplasmic tail domain was substituted by the one from 10A1 murine leukemia virus, and R-peptide cleavage site by a synthetic HIV-1 protease (GSGIF↓LETSL) cleavage sequence. This plasmid was developed by Tomás *et al*., (2019).[81]

2.2 Plasmid production, purification and quality control

Escherichia coli Stellar™ (Clontech Laboratories, Mountain View, CA, USA) and Library Efficiency® DH5α™ (Invitrogen, Carlsbad, CA, USA) competent cells were used for the production of DNA plasmids. Plasmid production was performed using working cell bacteria banks of each plasmid, previously established in-house and stored at -80ºC with 15% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA). Liquid and agar cultures were performed with Luria Broth (Fast-Media® LB; InvivoGen, San Diego, CA, USA), already supplemented with 100 mg/mL of ampicillin and prepared using ultrapure water (Milli-Q, Merck Millipore, Burlington, MA, USA).

GeneJET Plasmid Miniprep Kit (Thermo Scientific™, Waltham, MA, USA) was used for small-scale DNA purification (yields up to 20 µg of DNA). Genopure Plasmid Maxi Kit (Roche Applied Science, Penzberg, Germany) for larger scale DNA purification (yields up to 500 µg of DNA), was used according with manufacturer instructions.
Finally, DNA concentration of each plasmid solution, was determined using Nanodrop™ 2000C Spectrophotometer (Thermo Scientific™). Plasmid purity and integrity was assessed by the Abs_{260nm}/Abs_{280nm} and Abs_{260nm}/Abs_{230nm} ratios and with 1% (w/v) agarose gels (NZYtech, Lisbon, Portugal) prepared in TAE buffer.

2.3 Cell lines and culture conditions

HEK 293T (ATCC CRL-3216) is a Human Embryonic Kidney (HEK) 293 derived cell line that constitutively expresses the SV40 (*Simian vacuolating virus 40*) large T antigen. This cell line was used for the production and titration of LVs.

Te671 S10 is a Human rhabdomyosarcoma derived cell line (ATCC CCL-136) stably expressing a truncated GFP fragment, S10, and used as target cell line for titration of LV and RV by a Split-GFP system, as described in Rodrigues *et al*., (2015) [62].

293 FLEX S11 is a HEK 293 derived cell line developed by Rodrigues *et al*., (2015), stably producing infectious recombinant RV pseudotyped with GaLV envelope and harboring a LacZ-S11 transgene. This cell line was used in this work as a positive control in the SSCS method.

Lentipro26 4070A S11 mCherry, is a HEK 293T derived cell line previously developed in-house from Lentipro26 4070A cell line (Tomás *et al*., (2018)). This cell line constitutively produces infectious LV pseudotyped with 4070A amphotropic envelope, harboring a S11 mCherry fusion transgene. It was used for the adaptation of the SSCS method to LV producer cells. Cells were cultured under antibiotic selection pressure (zeomicin 150 µg/mL, hygromycin 150 µg/mL, blasticidin 20 µg/mL, puromycin 0.5 µg/mL) (InvivoGen).

All of the above cell lines were cultured under adherent conditions (T-flask), maintained in Dulbecco's modified Eagle's medium (Corning Inc, Corning, NY, USA) supplemented with 10% (v/v) Fetal bovine serum (Gibco™, Thermo Scientific™) at 37°C in a humidified atmosphere containing 8% CO_2.

Jurkat Clone E6-1 (ATCC TIB-152) is a suspension T lymphocyte cell line used for LV titration.
SUP-T1 (ATCC CRL-1942) is a suspension T lymphoblast cell line, also used for the titration of LV.
Both cell lines were cultured under suspension conditions in a non-treated tissue cultured flask (VWR, Pennsylvania, PA, USA), in an ATCC-formulated RPMI-1640 medium (Gibco™), supplemented with 10% (v/v) FBS at 37°C in a humidified atmosphere containing 5% CO_2.

2.4 Determination of cell concentration and viability

Cell concentration and viability were determined by the trypan blue exclusion assay, using a 0.1% (v/v) Trypan Blue (Sigma-Aldrich) in Dulbecco's Phosphate Buffer Saline (DPBS) solution (Gibco™). Cell counting was performed in a Fuchs-Rosenthal counting chamber (Marienfeld-Superior, Lauda-Königshofen, Germany) using an inverted microscope (Olympus, Tokyo, Japan).

2.5 Production of LV

Transient production of replication-defective LV was performed using a third-generation LV packaging system. HEK 293T cells were co-transfected with plasmids providing the packaging, envelope, and transgene vector genome functions. Transfection was performed with polyethyleneimine PEI (Polysciences Inc., Warrington, PA, USA) at 1:1.5 (w/w) ratio of DNA:PEI either in cells seeded 24 hours before ($0.8x10^5$ cell/cm^2) or at the time of seeding ($1.6x10^5$ cell/cm^2).

The plasmids were provided making a total of 4.65 or 3.33 µg of plasmid *per* million cells using a DNA ratio of 1:4:3.6:10 (pRSV-Rev: pMDLg/pRRE: appropriate envelope plasmid: pRRLSIN.cPPT.PGK-GFP WPRE).
Nude LV particles were produced using the same cotransfection procedures, using three, instead of four plasmids, providing the packaging and transgene vector genome functions (without the envelope plasmid).

PEI solution and plasmid mix solution were prepared separately in serum-free DMEM. Plasmid mix solution was filtered through 0.22 µm pore-size cellulose acetate filter and added on top of the PEI transfection solution. This transfection solution was incubated for 10 minutes at room temperature and added afterwards to the cells either cultured for 24 h or to the cells seeding solution. The medium was replaced by fresh RPMI/DMEM 10%(v/v) FBS (0.1 mL/cm^2) 24 h and 48 h after transfection. Supernatants containing LV were harvested 48 h and 72 h post-transfection, filtered through 0.45 µm pore-size cellulose acetate filters for clarification, aliquoted and stored at -80°C until further use.
At the end of production, cells were harvested and subjected to flow cytometry analysis to evaluate the transfection efficiency (CyFlow-space (Sysmex Partec GmbH, Görlitz, Germany) or Gallios (Beckman Coulter, Brea, CA, USA)).

2.6 LV Quantification

2.6.1 Titration of infectious particles

Viral supernatants of four different LV pseudotypes (VSV-G, 4070A, RD114 and GaLV), or without envelope (Nude LV), either in DMEM or RPMI medium supplemented with 10% (v/v) FBS, were analyzed for their functional titer. LV infectious particles titers were quantified using different target cells: HEK 293T, Te671 S10, Jurkat E6-1 and SUP-T1.

Adherent cell lines (HEK 293T and Te671 S10) were seeded at $1x10^5$ cells/cm^2 in 24 well flat-bottom tissue treated culture plates with low-evaporation lid (Corning Inc), one day before infection. Cell concentration was determined at the time of infection. Transduction was performed in duplicates by removing the cell supernatant and infecting with 0.2 mL of viral suspension diluted in fresh DMEM with 10% (v/v) FBS and 8 ug/mL polybrene (Sigma-Aldrich). Spin-inoculation step was performed by centrifugation at 1200xg for 2 hours at 25ºC, and then 0.5 mL of warm medium was added. Static incubation was performed at 37ºC 5% CO_2 for 4 hours and after 0.5 mL of warm medium was added. Cells were incubated at 37ºC for an additional 48 hours before being harvested and analyzed for GFP fluorescence by flow cytometry.

Suspension cell lines (Jurkat E6-1 and SUP-T1) were seeded at $2.5x10^5$ cells/cm^2, to a final volume of 0.1 mL, 0.25 mL or 0.5 mL per well, in 24 well flat-bottom non-tissue treated culture plates with a low-evaporation lid (Corning Inc), at time of infection. Transduction was performed in duplicates, by adding the same volume of viral suspension as the cell seeding. Viral suspension was diluted in fresh RPMI with 10% (v/v) FBS and 8 ug/mL of polybrene. Spin-inoculation step was performed at 938xg for 90 minutes at 25ºC. Static incubation was performed at 37ºC for 4 hours, and RPMI 10% (v/v) FBS was added to a final culture volume of 1 mL/well. Upon spin-inoculation and static incubation, 48 h post-infection cells were analyzed by flow cytometry to measure GFP fluorescence.

The infectious particles titer was determined by taking into account the percentage of GFP positive cells, the number of cells determined at infection time and the dilution factor of the viral supernatant. Only infections between 2-20% of positive GFP cells were considered for titer calculations.

Viral titers, defined as transducing units (T.U./mL) were calculated using the following equation:

$$\frac{\mathbf{T.U.}}{\mathbf{mL}} = \frac{\%\mathbf{GFP} \times \mathbf{number\ of\ transduced\ cells} \times \mathbf{dilution\ factor}}{\mathbf{transduced\ volume\ (mL)}}$$

2.6.2 Titration of viral genomes

To quantify genome containing LV particles, viral supernatants were analysed by real-time quantitative PCR based on the SYBR Green I detection system, targeting WPRE LV genome sequence, as described and adapted from Carmo *et al.,* (2004).[82]

Briefly, LV RNA genome extraction was performed by viral capsid disruption at 75ºC for 10 minutes. In order to remove traces of plasmid DNA contamination from the transfection procedure, samples were treated with 1U/µL RNase-free DNase I (Roche Diagnostics, Basel, Switzerland) in the presence of the respective incubation buffer for 30 minutes at room temperature. DNase treatment was stopped by enzyme inactivation at 75°C for 10 minutes.
For cDNA synthesis, Transcriptor High Fidelity cDNA Synthesis Kit AMV (Roche Applied Science) was used, according to manufacturer instructions. The reverse transcribed product was aliquoted and stored at -20°C until further use.

The number of LV vector genomes was calculated with the following formula:

$$\textbf{Titer}\left(\frac{\textbf{V. G.}}{\textbf{mL}}\right) = \textbf{nrº copies}\ (\mu\textbf{L}) \times \textbf{dilution} \times \textbf{1000} \times \textbf{30/100} \times \textbf{0.5}$$

where, 30/100 is the estimated percentage of the reverse transcription effectiveness, and the 0.5 factor normalizes the two copies of viral RNA genome carried per capsid.

2.7 Cell transduction using adjuvant supplementation

Transduction was performed as described in section 2.6.1, where other transduction adjuvants compounds (Table 2.1) were used, instead of polybrene.

Table 2.1 - Adjuvant compounds used for LV transduction.

Adjuvant	Concentration/Dilution	Stock Solution	Company
Polybrene	8 µg/mL	1 mg/mL	Sigma-Aldrich
LentiBoost	1:20,1:100,1:1000	patent protected	SIRION Biotech, Martinsried, Germany
LentiBlast	1:100, 1:000	patent protected	OZ Biociences Inc, San Diego, CA, USA
Transplus	1:500	patent protected	ALSTEM Inc, California, CA, USA
DEAE-Dextran	8 µg/mL, 20 µg/mL, 50 µg/mL	1 mg/mL	Sigma-Aldrich
Vectofusin-1	10 µg/mL, 12 µg/mL	1 mg/mL	MACS Miltenyi Biotec, Bergisch Gladbach, Germany
RetroNectin	20 µg/cm^2	1 mg/mL	Takara Bio Inc, Nojihigashi, Japan

The LV transduction procedure was adapted to cope with vectofusin-1 and retronectin manufacturer instructions.
Vectofusin-1 solution was prepared and separately added in equal volume to the viral suspension dilutions, in plain culture medium (DMEM/RPMI 0% (v/v) FBS). This final mixture was added to the cells after 10 minutes incubation at room temperature, as described by manufacturer instructions.
Retronectin was used to precoat 24-well non-tissue treated plate (Corning Inc), at 4ºC overnight, with 0.5 mL corresponding to 20 µg/cm^2. After retronectin solution removal, wells were blocked with 0.5 mL DPBS 2% (v/v) BSA (Bovine serum albumin) at room temperature for 30 minutes and then washed twice with 0.5 mL of DPBS. Finally, the plates were ready to use.

2.8 Single-step cloning-screening titration method protocol

This method uses a split GFP protein, GFP S10 and GFP S11 fragments, that only emit fluorescence upon transcomplementation. GFP S11 is coded in the viral vector genome and GFP S10 in the target cells.[62] Two producer cell lines were used in this work: 293 Flex S11 (positive control) and LentiPro26 4070A S11 mCherry. The Te671 S10 were used as target cells.

The protocol takes 15 days to allow clone expansion, virus production and subsequent infection of the target cells. During this time cells were maintained in an incubator with a humidified atmosphere at 37°C and 8% CO_2.

In the first day, 293 Flex S11 and LentiPro26 4070A S11 mCherry were seeded in 96 well clear-bottom tissue treated black culture well plates (Corning Inc.) at concentration of 0.5 cells/well, in a final volume of 200 µL. In order to minimize medium evaporation from the cloning wells, plate borders were filled with 300 µL of DPBS (-/-) (Gibco™). Only LentiPro26 cells were cultured under antibiotic selection pressure (zeomicin 75 µg/mL, hygromycin 75 µg/mL, blasticidin 10 µg/mL puromycin 0.25 µg/mL) (InvivoGen). At day 7 (Figure 3.15), culture medium was replaced by 200 uL of target cells (Te671 S10) at 250 cells/well. Three infection rounds were performed at days 8/10/12, by the addition of polybrene to a final concentration of 8 µg/mL or vectofusin-1 to a final concentration of 10 µg/mL proceeded by 30 minutes of agitation, with 48 hours interval in-between rounds.

At day 15, GFP and mCherry signal was quantified using a microplate fluorescence reader (Tecan Infinite 200pro, Mannedorf, Switzerland).

3

Results

3.1 Transduction efficiency assessment using LV adjuvants

3.1.1 Optimizing LV production

The main objective of this work was to systematically evaluate the enhancing effects of available LV transduction adjuvants. To this end, LV production conditions and LV transduction methods using several target cell lines (HEK 293T, Jurkat E6-1, SUP-T1) had to be optimized.

The preparation of a high yield LV stock to be commonly used in all cell transduction procedures was a priority. For that, transfection procedures, 24 h post cell seeding or at time of seeding, providing the best LV transient yields were assessed (Figure 3.1). Transfection at time of cell seeding rendered higher LV titers and was consequently chosen as production method for all the pseudotyped LV stocks later produced in this work. Also, 48 h post-transfection supernatants presented higher LV titers are than at 72 h.

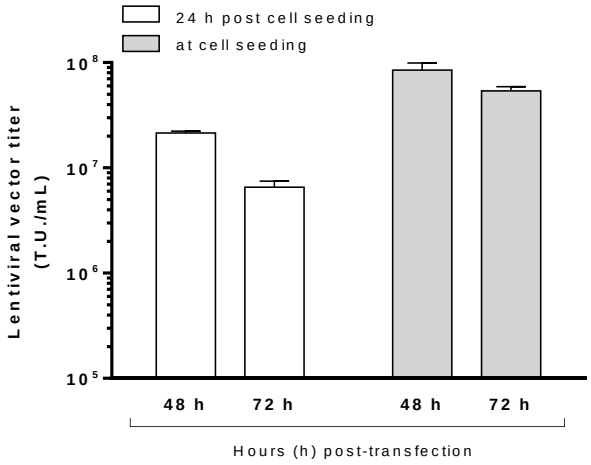

Figure 3.1 - Transient productivities using different transfection procedures.
White and grey bars represent transfection protocols performed one day after cell seeding and at cell seeding time, respectively. Volumetric yields of transducing competent LV particles supernatant recoveries at 48 h and 72 h after transfection, obtained with spin-inoculation titration protocol and using polybrene as infection adjuvant in HEK 293T target cells. Values are shown as average ± standard deviation of n=2 technical replicates. T.U., transducing units.

VSV-G has been the envelope glycoprotein of choice to pseudotype LV particles due to its broad tropism and stability. However, its cytotoxicity has hampered its use in stable and constitutive productions. To overcome this challenge, other LV pseudotypes have been explored, namely, 4070A, RD114, and GaLV, which are being used in gene therapy clinical trials and therefore were also studied in this work. The four pseudotyped LV stocks were produced and characterized for their transduction competent particle yields using polybrene (8 µg/mL) as adjuvant compound. Spin-inoculation (centrifugation) procedure, described to potentiate the contact of viral particles with target cells was compared to static

conditions (Figure 3.2).[83] Indeed, the process of spin-inoculation increased lentiviral vector titers from 2.1 to 6.6 in all cases with fold changes by more than 2 fold.

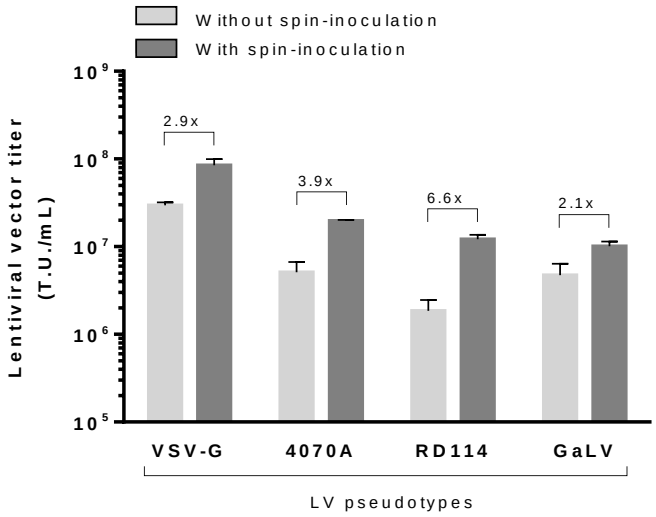

Figure 3.2 - Viral vector titer of LV particles pseudotyped with VSV-G, 4070A, RD114, and GaLV envelopes.
Titration protocol performed without and with spin-inoculation, using polybrene as infection adjuvant in HEK 293T target cells. The numbers on the top of the bars indicate fold change of infectious titer between without and with spin-inoculation condition. Values are shown as average ± standard deviation of n=2 technical replicates. T.U., transducing units.

In order, to confirm that LV transduction ability is envelope-mediated, LV particles without the incorporation of any viral envelope glycoproteins (nude LV) were produced. Several strategies exist to determine titer and transduction efficiency of LV. This work is based on functional assays that provide a measure of transduction competence by assessing viral genome entrance and expression in the target cell.[84] However, assuming that nude LV could not infect target cells, and with the interest of quantifying these particles, a non-functional assay based on the quantification of viral RNA genomes present in LV particles stock was performed. Nude LVs were produced with a titer $1.4x10^8$ V.G./mL (data not shown).

The last optimization performed within this section evaluated two different culture media, DMEM and RPMI. HEK 293T cells are cultured in DMEM media, however T cell model cell lines are cultured in RPMI media. In addition, LV stocks in RPMI cell culture medium were described to significantly enhance the transduction efficiency and the survival of transduced T cells when compared to LV stocks generated in DMEM cell culture medium.[8] Therefore, LV stocks, each pseudotyped with a different envelope glycoproteins were produced in both RPMI and DMEM cell culture medium. Figure 3.3 shows a similarity in the transducing units titer between LV stocks produced in RPMI or DMEM. Nude LV did not transduce HEK 293T target cells as expected.

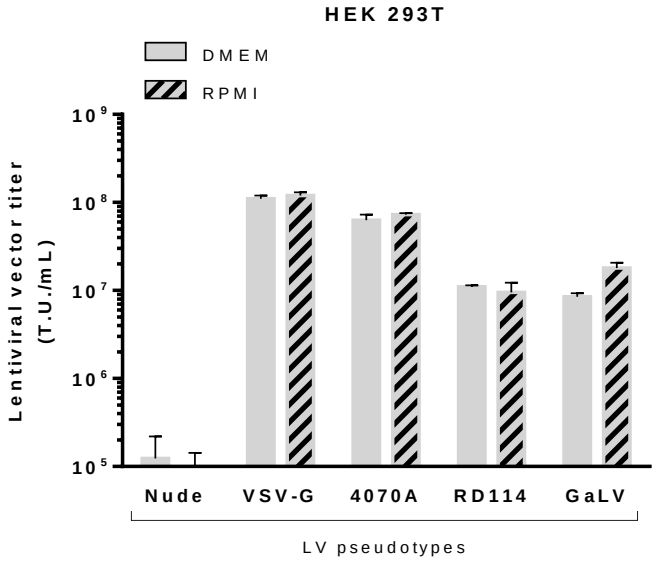

Figure 3.3 - Lentiviral vector titer of pseudotyped LV produced using DMEM or RPMI cell culture medium.
Transient LV production, of nude and four different pseudotyped LV particles. Titration protocol performed in HEK 293T target cells, with spin-inoculation, using polybrene as infection adjuvant and supernatants recovered 48 h post-transfection. Values shown as average ± standard deviation of n=2 technical replicates. T.U., transducing units.

3.1.2 Optimizing transduction protocol for suspension cells

After LV stocks production and quantification optimization, the next step was to optimize the protocol conditions for other target cell lines. Previous titration protocols were established in adherent cells to achieve optimal efficiency, but not in suspension cell lines. Therefore, using Jurkat E6-1 as target cells, three infection volumes were tested, 200 µL, 500 µL, and 1000 µL (Figure 3.4). Results showed a vector titer increase of 6.1 fold for the 200 µL infection volume in 4070A pseudotyped LV compared to 1000 µL of infection volume. Small increases are also observed in RD114 and GaLV pseudotyped LV, except for VSV-G pseudotype, which maintained the yield in all conditions. The volume of 200 µL was considered optimal and was chosen for the subsequently experiments.

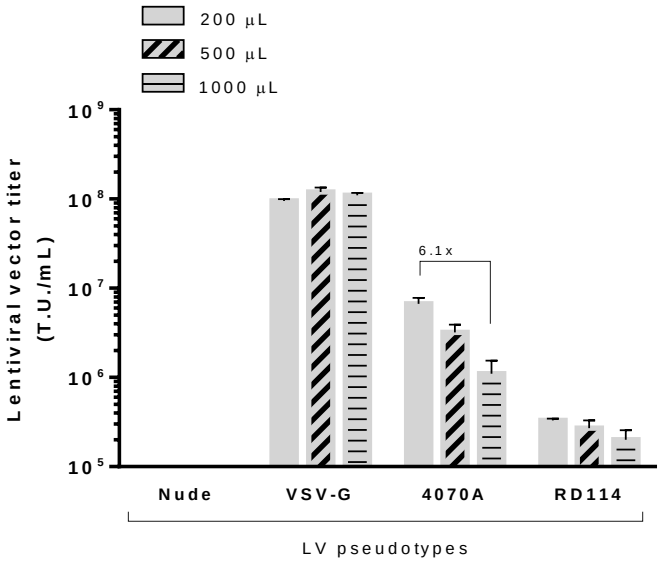

Figure 3.4 - Infection volume optimization for titration of suspension Jurkat E6-1 target cells.
Titration of pseudotyped LV with spin-inoculation and using polybrene as viral entry helper. LV stocks were produced in RPMI medium harvested 48 h after transfection. Viral infection volumes of 200 µL, 500 µL, and 1000 µL were evaluated. Values shown as average ± standard deviation of n=2 technical replicates. T.U., transducing units.

3.1.3 Evaluating transduction enhancers

With all optimization procedures concluded, pseudotyped LV stocks were characterized for their transduction behavior in target cells of standard laboratory use (HEK 293T), and in cell lines representative of T cells (Jurkat E6-1, SUP-T1). Since each cell line has a specific expression pattern of each envelope glycoprotein receptor, transduction efficiency may vary and therefore LV stock titer will be cell line specific. When comparing the results between these three cell lines (Figure 3.5), LV pseudotyped with VSV-G could efficiently transduce all cell lines equally rendering a similar LV stock titer (~$1.3x10^8$ T.U./mL). Whereas 4070A, RD114, and GaLV has different titers in all cell lines, the highest being always in HEK 293T target cells and the lowest in Jurkat E6-1 target cells. The higher titers obtained with HEK 293T target cells were $7.3x10^7$ T.U./mL, $9.5x10^6$ T.U./mL and $1.8x10^7$ T.U./mL for 4070A, RD114, and GaLV pseudotyped LV, respectively. Additionally, and as expected from previous results, nude LV cannot transduce HEK 293T, Jurkat E6-1 and SUP-T1 cell lines.

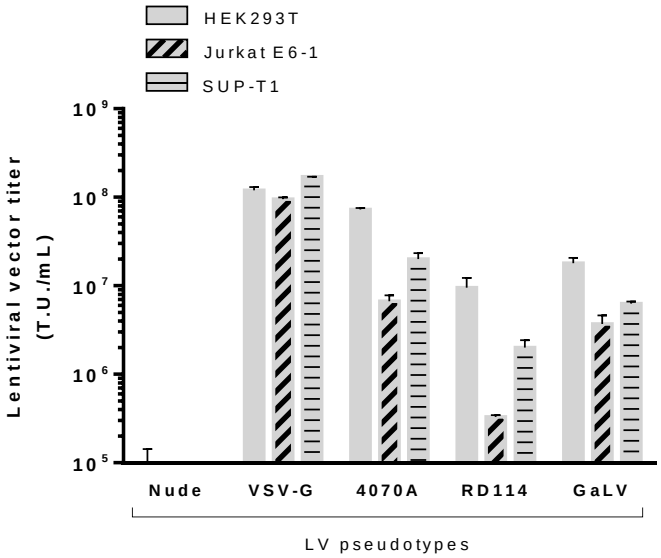

Figure 3.5 - Infectious LV titer using different target cell substrates: HEK 293T, Jurkat E6-1, and SUP-T1 cell lines.
Transduction of nude LV and viral vectors pseudotyped with VSV-G, 4070A, RD114, and GaLV glycoproteins, with spin-inoculation, polybrene. RPMI medium supernatants recovered 48 h after transfection. Values shown as average ± standard deviation of technical replicates. T.U., transducing units.

Here, we carried out a methodical comparison between five commercialized and clinical compatible transduction adjuvants, using LV pseudotyped with VSV-G, 4070A, RD114, GaLV, and nude LV.
Before conducting these experiments, and as required by several manufacturer instructions, the used concentration of each adjuvant had to be optimized, and such studies are included in the “Annexes” section as figures A.1, A.2, A.3, and A.4. In addition to these compounds, polybrene (a compound commonly used in academia) and DEAE-dextran (the most economical viable compound, still not clinical grade), were also included. Furthermore, a negative control where nothing was added to enhance viral transduction was also tested. For these assays, the highest possible multiplicity of infection (MOI) was used, depending on the LV stocks yield. Therefore, MOI superior to 1 was used in VSV-G, RD114, and GaLV LV pseudotypes (MOI of 2.40, 1.69 and 1.23) and inferior to 1 in 4070A LV pseudotypes (MOI 0.75).
For the choice of the cell line, the gene therapy interest in mostly targeting the immature T cell phenotype was taken into account. Thus, we started the screening of the transduction adjuvants using SUP-T1 cell as the T cell line representative, since SUP-T1 cell line is a model of immature T cells, whereas Jurkat E6-1 is a cell line model for committed and mature T cells.

The systematic screening of adjuvants was first addressed using nude LV to exclude envelope unspecific transduction. A small transduction with these particles was only observed in HEK 293T target cells. Approximately 2% of cells were unspecifically transduced in the presence of vectofusin-1, when spin-inoculation was performed. (Figure 3.6). In the remaining conditions, the percentage of transduction was among the negative control values (non-transduced cells). In figure 3.6 a) it is possible to observe that 2% of transduction are indeed positively transduced cells with high intensities of GFP expression and not observed in the negative control.

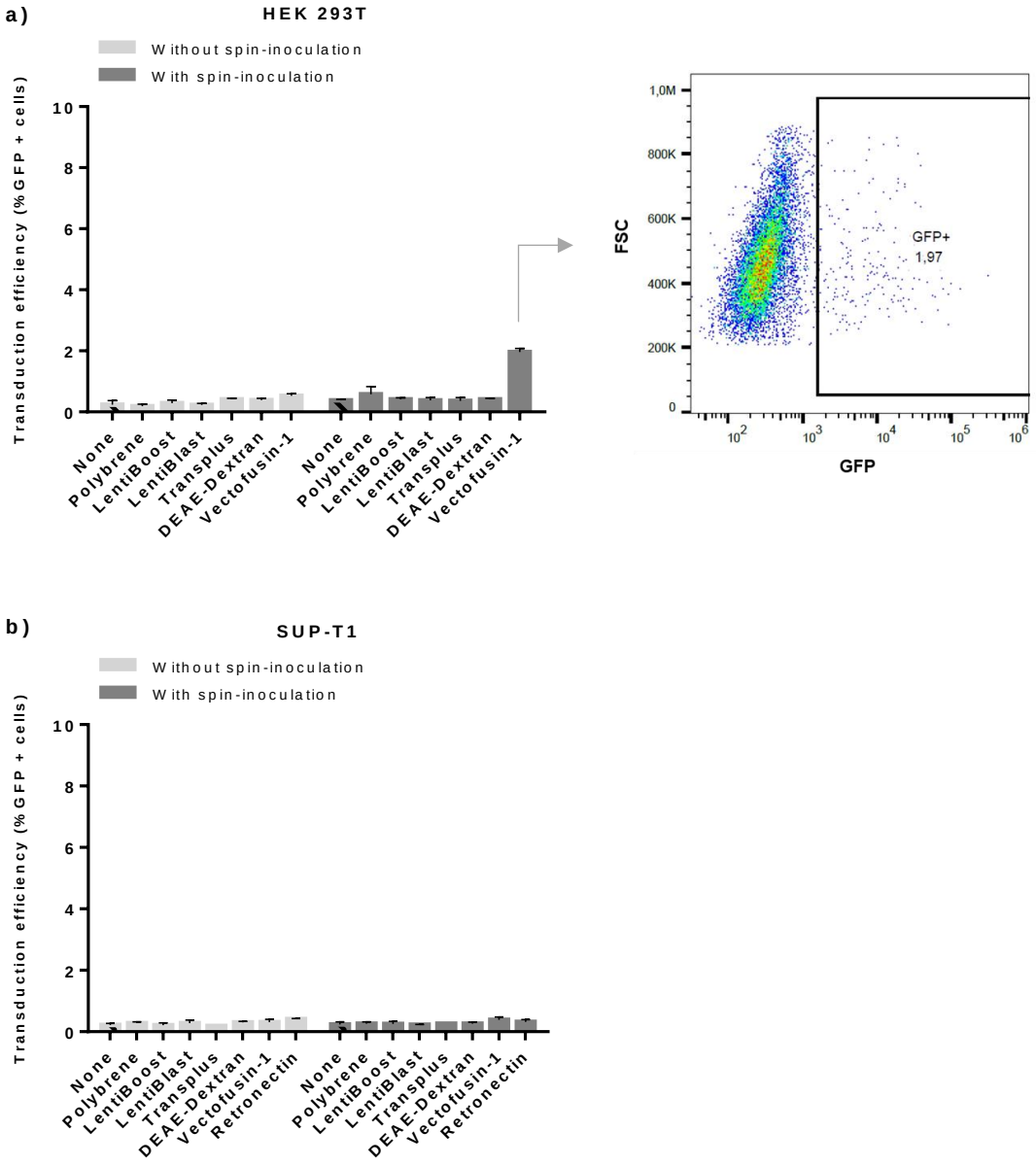

Figure 3.6 - Transduction efficiency using nude LV in HEK 293T and SUP-T1 target cell lines.
LV transduction efficiency in a) HEK 293T cells and in b) SUP-T1 cells with nude LV particles, in the absence (none) or in the presence of polybrene (8 µg/mL), lentiboost (1:100), lentiblast (1:100), transplus (1:500), DEAE-dextran (20 µg/mL), vectofusin-1 (12 µg/mL) and retronectin (20 µg/cm^2). Dashed bars represent the experiment negative controls. Flow cytometry dot plots of HEK 293T target cells 48 h after infection with nude LV and vectofusin-1 12 µg/mL. FSC, forward scatter. GFP, green fluorescent protein. Data was treated using FlowJo software. Values shown as average ± standard deviation of n=2 biological replicates.

Regarding VSV-G pseudotyped LV (Figure 3.7), it is possible to observe that the transduction efficiency is higher in SUP-T1 cell line than in HEK 293T cells, confirming what was previously detected in figure 3.5. Also, spin-inoculation did not cause a high impact on the target cells transduction efficiency, HEK 293T or SUP-T1. In HEK 293T target cells (Figure 3.7, a), lentiboost adjuvant was able to increase the transduced cells by more 17% and 26% of without and with spin-inoculation, respectively. On the other hand, the best performance in SUP-T1 target cells was with spin-inoculation in the presence of DEAE-dextran, enhancing transduced cells by 17%. In contrast, vectofusin-1 had a considerable negative

impact in the transduction efficiency of SUP-T1 target cells when spin-inoculation is not performed (-56% of transduced cells).

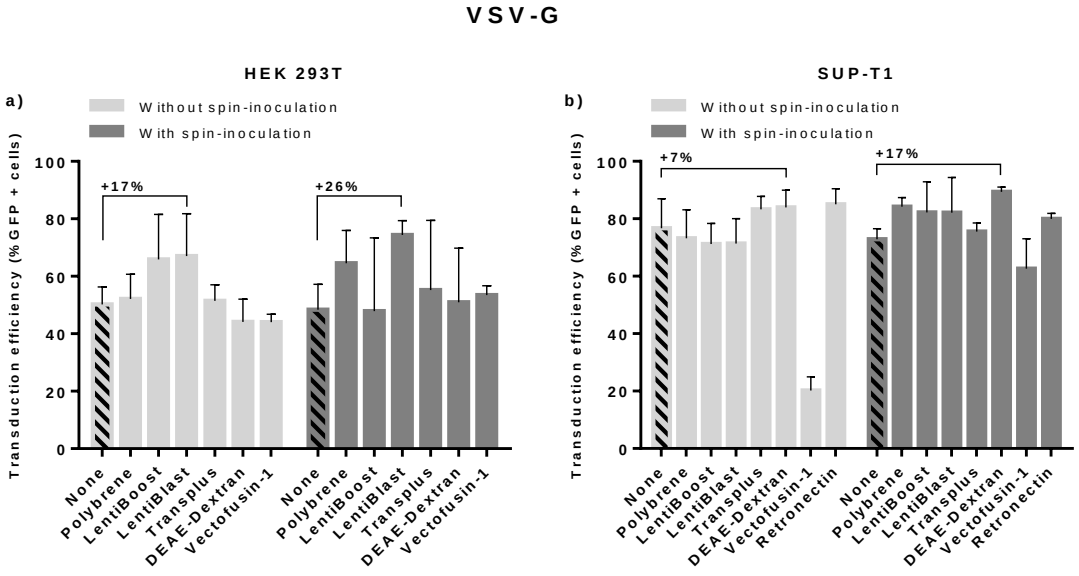

Figure 3.7 - Transduction efficiency of VSV-G pseudotyped LV in HEK 293T and SUP-T1 target cell lines.
LV transduction efficiency with VSV-G pseudotyped LV at MOI 2.40, in the absence (none) or in the presence of polybrene (8 µg/mL), lentiboost (1:100), lentiblast (1:100), transplus (1:500), DEAE-dextran (20 µg/mL), vectofusin-1 (12 µg/mL) and retronectin (20 µg/cm^2). Dashed bars represent the experiment negative controls. Values shown as average ± standard deviation of n≥2 biological replicates.

In contrast with the previous results, vectofusin-1 was the transduction enhancing compound displaying higher transduction efficiency when using 4070A pseudotyped LV (Figure 3.8). It was able to enhance the transduced cells (+45%) from 7% to 52% without spin-inoculation, and from 12% to 56% of transduced cells (+44%) with spin inoculation in HEK 293T target cells (Figure 3.8, a). In SUP-T1 target cells there was an improvement from 2% to 39% of transduced cells (+37%) without spin-inoculation and more 31% transduced cells were obtained with spin-inoculation (Figure 3.8, b). Such increase observed without spin-inoculation in the presence of vectofusin-1 allowed transduction efficiencies to get as high as what was observed for spin-inoculation, meaning that it is possible to withdraw the centrifugation step in the transduction protocol and keep the high levels of transduction efficiency.
Lentiboost adjuvant was also able to increase HEK 293T transduced cells by 13% (Figure 3.8, a) with spin-inoculation, whereas in the SUP-T1 target cell line (Figure 3.8, b), DEAE-dextran allowed to transduce 17% more cells without spin-inoculation. On other hand, polybrene, transplus, or retronectin only exerted minor enhancing effects.

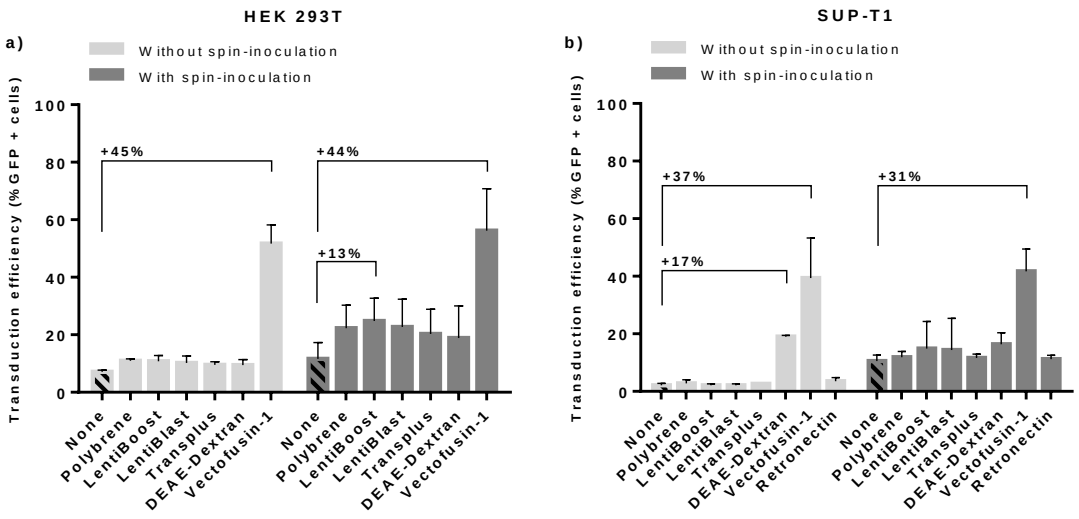

Figure 3.8 - Transduction efficiency using 4070A pseudotyped LV in HEK 293T and SUP-T1 target cell lines.
LV transduction efficiency with 4070A pseudotyped LV at MOI 0.75, in the absence (none) or in the presence of polybrene (8 µg/mL), lentiboost (1:100), lentiblast (1:100), transplus (1:500), DEAE-dextran (20 µg/mL), vectofusin-1 (12 µg/mL) and retronectin (20 µg/cm^2). Dashed bars represent the experiment negative controls. Values shown as average ± standard deviation of n≥2 biological replicates.

RD114 pseudotyped LV, likewise, also experiences a remarkable transduction efficiency enhancing effect from vectofusin-1 (Figure 3.9). Similarly, in HEK 293T cells (Figure 9, a), transduction efficiency without spin-inoculation in the presence of vectofusin-1 also resulted in 51% more transduced cells, (from 12% to 63%). Vectofusin-1 also mediated LV transduction in SUP-T1 target cells (Figure 3.9, b) increasing the transduced cells by 65% and retronectin, being the second-best, yielded 20% more of transduced cells. However, and overall, without spin-inoculation, transduction efficiencies are higher in HEK 293T target cells when comparing to SUP-T1 target cells, as also seen in figure 3.8, with 4070A pseudotyped LV.

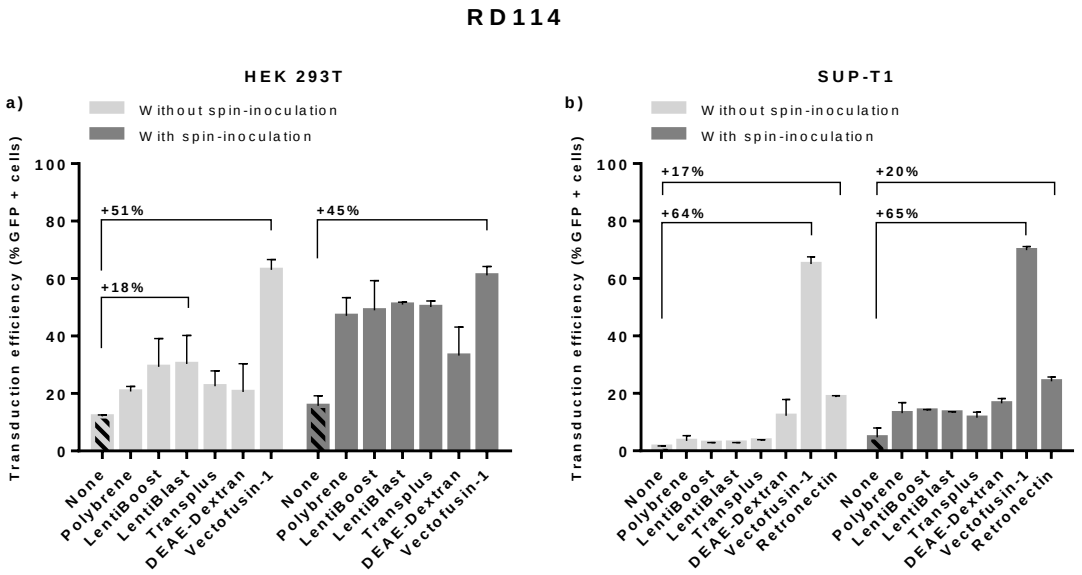

Figure 3.9 - Transduction efficiency using RD114 pseudotyped LV in HEK 293T and SUP-T1 target cell lines.
LV transduction efficiency with RD114 pseudotyped LV at MOI 1.69, in the absence (none) or in the presence of polybrene (8 µg/mL), lentiboost (1:100), lentiblast (1:100), transplus (1:500), DEAE-dextran (20 µg/mL), vectofusin-1 (12 µg/mL) and retronectin (20 µg/cm^2). Dashed bars represent the experiment negative controls. Values shown as average ± standard deviation of n≥2 biological replicates.

GaLV pseudotyped LV showed higher transduction efficiencies when the step of spin-inoculation was carried out in both cell lines (Figure 3.10). In addition, vectofusin-1 adjuvant compound, as previously described in figures 3.8 and 3.9, remained the top transduction adjuvant, enhancing transduction by more than 34%, from 6% to 40% of transduced cells with spin-inoculation in HEK 293T target cells (Figure 10, a). In the SUP-T1 target cell line, without spin-inoculation, there was 45% more transduced cells, from an increase from 5% to 50% of cells transduced in the experiment (Figure 10, b). Enhanced transduction efficiency using DEAE-dextran in SUP-T1 target cells was also observed. Nevertheless, when comparing the transduction efficiencies obtained with GaLV pseudotyped LV with the remaining viral envelopes, less transduced HEK 293T target cells without spin-inoculation were observed, and the percentage of transduced SUP-T1 cells with spin-inoculation is much higher.

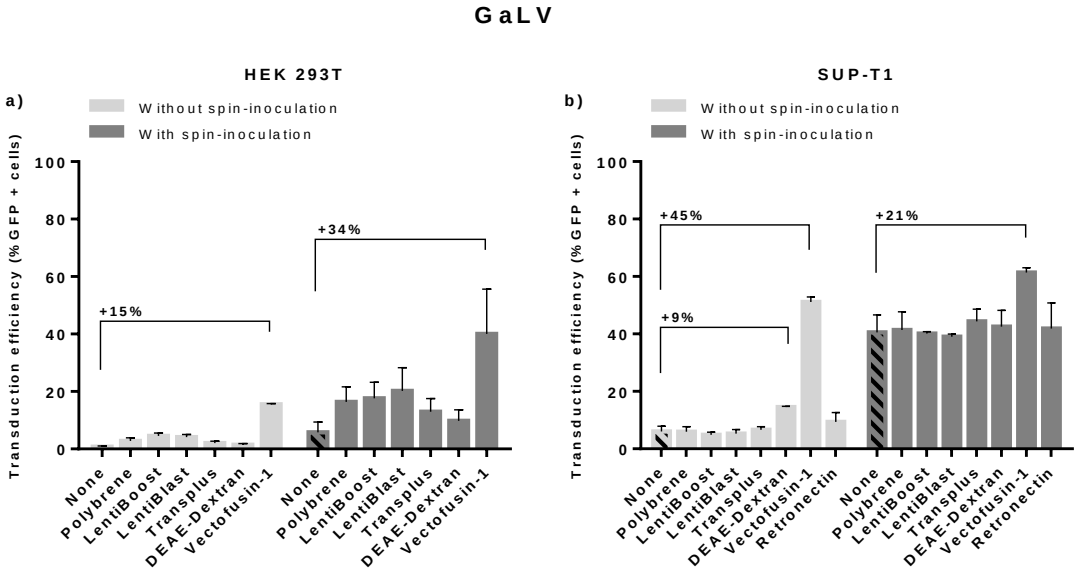

Figure 3.10 - Transduction efficiency using GaLV pseudotyped LV in HEK 293T and SUP-T1 target cell lines.
LV transduction efficiency with GaLV pseudotyped LV at MOI 1.23, in the absence (none) or in the presence of polybrene (8 µg/mL), lentiboost (1:100), lentiblast (1:100), transplus (1:500), DEAE-dextran (20 µg/mL), vectofusin-1 (12 µg/mL) and retronectin (20 µg/cm^2). Dashed bars represent the experiment controls. Values shown as average ± standard deviation of n≥2 biological replicates.

A similar comparison between transduction adjuvants and both cell lines, for all pseudotyped LV, was performed using the same MOI (0.4) (Figure 3.11). For the same MOI, VSV-G pseudotyped LV provide the highest LV transduction efficiencies in both target cell lines, except when vectofusin-1 was used, which dramatically decreased its capacity to transduce cells. This effect is different in 4070A, RD114, and GaLV pseudotypes, which had higher percentages of transduction with this compound, surpassing VSV-G transduction efficiency in HEK 293T target cell line (Figure 3.11, a). In the presence of vectofusin-1, 4070A pseudotyped LV achieved the highest transduction efficiencies when comparing

with all transduction adjuvants used and the other LV pseudotypes, enabling more 35% and 29% transduced HEK 293T target cells without and with spin-inoculation, than the control. In SUP-T1 target cell line (Figure 3.11, b), the higher transduction efficiencies were achieved using DEAE-dextran with VSV-G pseudotyped LV, 47% of transduced cells with spin-inoculation. Thus, 4070A, RD114 and GaLV pseudotyped LV almost reached VSV-G pseudotyped LV transduction efficiency in the presence of vectofusin-1, being able to transduce 23%, 31% and 28% of SUP-T1 target cells. Additionally, spin-inoculation protocol in HEK 293T cells can increase the overall transduction efficiencies, whereas in the SUP-T1 target cell line there is only a mild enhancement.

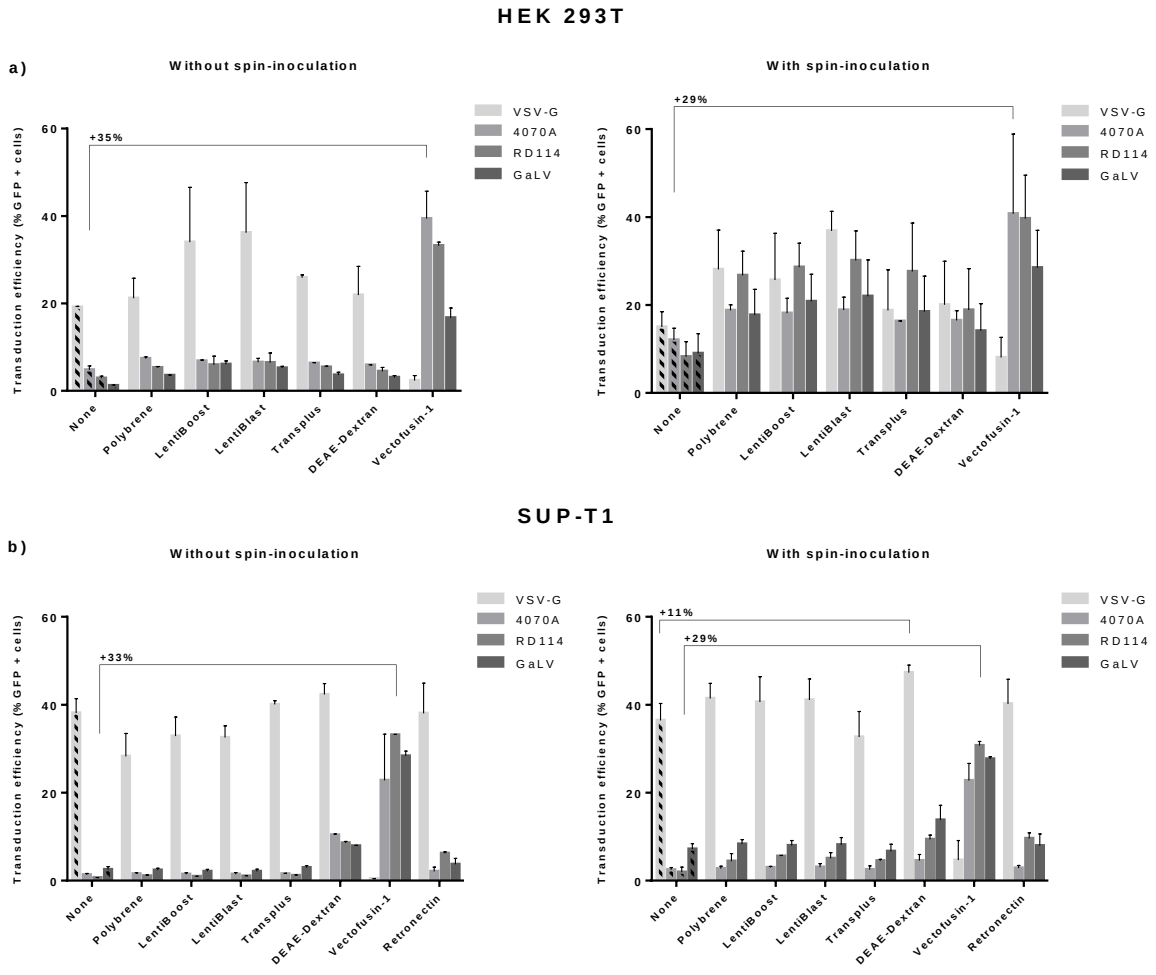

Figure 3.11 - Adjuvants influence in transduction efficiency using VSV-G, 4070A, RD114, and GaLV pseudotyped LV, in HEK 293T and SUP-T1 target cells at MOI 0.4.
LV transduction efficiency with pseudotyped LV at MOI 0.4, in the absence (none) or in the presence of polybrene (8 µg/mL), lentiboost (1:100), lentiblast (1:100), transplus (1:500), DEAE-dextran (20 µg/mL), vectofusin-1 (12 µg/mL) and retronectin (20 µg/cm^2). Values shown as average ± standard deviation of n≥2 biological replicates

3.2 Single Step Cloning Screening method improvement

The second purpose of this work was the adaptation of the SSCS method to LV (pseudotyped with 4070A and carrying a GFP-S11 transgene) producer cells. Since vectofusin-1 showed to be the best transduction enhancer, it was chosen to assist transduction instead of polybrene. To achieve this, we had to first evaluate if 1) vectofusin-1 transduction enhancement effect was observed in Te671 S10 target cells and, 2) if this effect also happened in 4070A pseudotyped LV derived from a stable constitutive production (Figure 3.12), which are characterized by lower yields than transient system used in section 3.1.

In Te672 S10 target cells, vectofusin-1 was able to enhance cell transduction from 20% to 88% of transduced cells, by more 68%, with 4070A pseudotyped LV stocks transiently produced when compared to polybrene (Figure 3.12, a). When using LV stocks stably produced (Figure 3.12, b), there was also an increase from 1% to 4% of transduced Te671 S10 cells, with the presence of vectofusin-1 when compared to polybrene (Figure 3.12, b). The transduction enhancement with this booster is in agreement with our previous findings, where vectofusin-1 action without spin-inoculation promotes a higher percentage of transduced cells than with spin-inoculation.

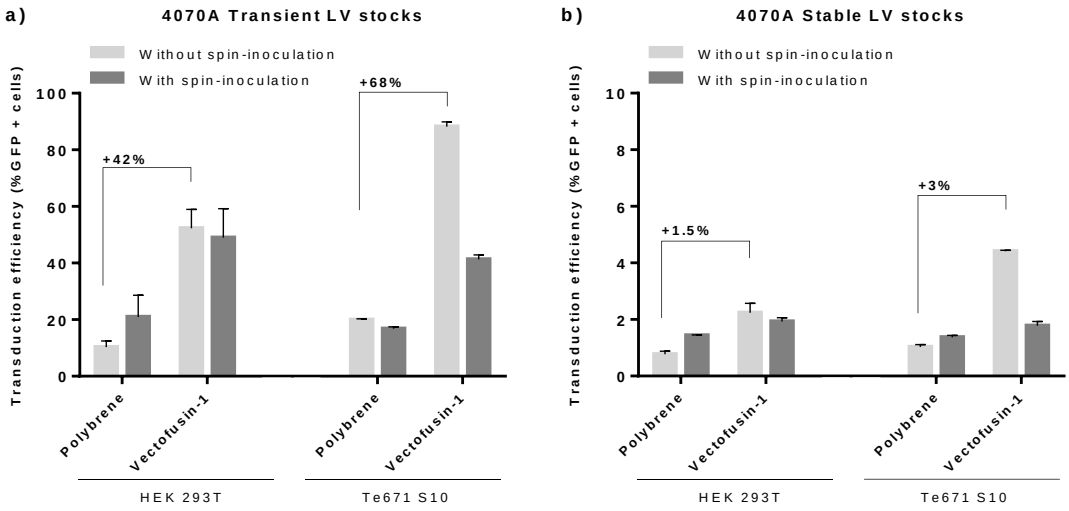

Figure 3.12 - Transduction efficiency with transient or stable produced 4070A pseudotyped LV, in HEK 293T and Te671 S10 target cell lines.

Transduction efficiency with 4070A pseudotyped LV produced a) transiently or b) constitutively, comparing the use of polybrene (8 µg/mL) and vectofusin-1 (12 µg/mL) viral adjuvants in HEK 293T and Te671 S10 cell lines. HEK 293T values in a) are shown as average ± standard deviation of n≥2 biological replicates. Remaining values are shown as average ± standard deviation of n=2 technical replicates.

Since the action of vectofusin-1 rendered higher transduction percentages than polybrene in Te671 S10 cells with stable produced 4070A pseudotyped LV, the next step was to optimize the amount of FBS supplementation to the cell culture medium. This step was crucial as, according to the manufacturer, vectofusin-1 only worked with 0%FBS, which is not enough for clone growth in the SSCS method. Expectedly, cell transduction efficiency with vectofusin-1 adjuvant decreased with FBS addition, however, kept higher values were obtained with polybrene at the same condition (10% FBS) (Figure 3.13).

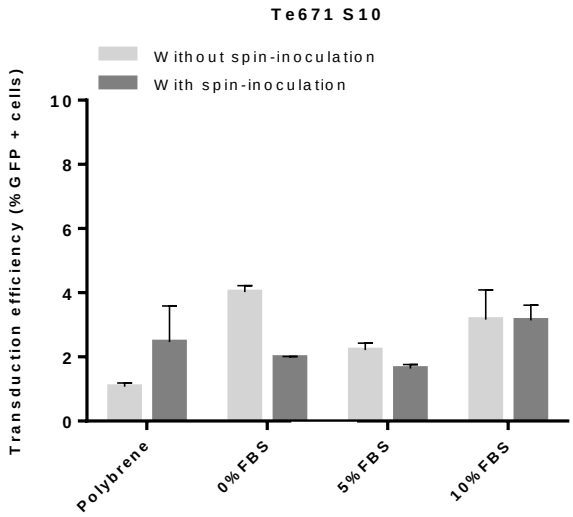

Figure 3.13 - LV transduction efficiency with 12 µg/mL vectofusin-1 supplementation in different concentrations of FBS in cell culture medium.
Cell culture medium FBS supplementation (0%, 5%, 10% FBS), in Te671 S10 target cell lines, using stable produced 4070A pseudotyped LV, with and without spin-inoculation. Values shown as average ± standard deviation of n≥2 biological replicates.

Due to the high cost of vectofusin-1, the last optimization step before proceeding to SSCS was to reduce the amount of compound used (Figure 3.14). Transduction efficiency at 5 µg/mL of vectofusin-1 decreased to values lower than using polybrene. We therefore chose to reduce the amount of used compound to 10 µg/mL since no difference to 12 µg/mL was observed.

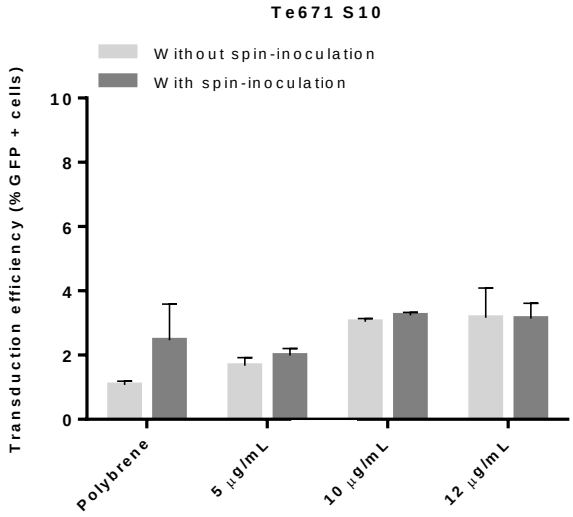

Figure 3.14 - LV transduction efficiency, in 10% FBS cell culture medium using different vectofusin-1 concentrations.
Vectofusin-1 concentrations (5 µg/mL, 10 µg/mL, 12 µg/mL), in Te671 S10 target cell lines, using stable produced 4070A pseudotyped LV. Values shown as average ± standard deviation of n≥2 biological replicates.

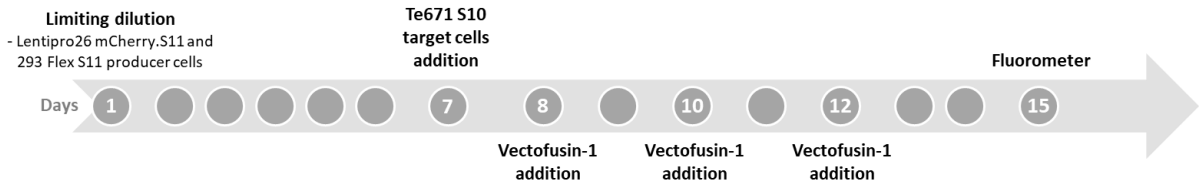

Figure 3.15 - Illustrative figure of SSCS method timeline during 15 days.
Producer and target cells seeding at day 1 and 7. Vectofusin-1 infection rounds at day 8, 10 and 12. Fluorescence analysis with a fluorometer plate reader at day 15.

Finally, after gathering the optimal conditions for vectofusin-1 use, the SSCS method was performed using Lentipro26 mCherry S11 producer cells and Te671 S10 target cells (Figure 3.15, 3.16). As a positive control, 293 Flex S11 producer cells were used since their ability to transduce Te671 S10 target cells has already been studied when the SSCS method was developed.[62]

The first unexpected results were observed after vectofusin-1 addition, when a mesh appeared in the bottom of the well culture plate (Figure 3.16, b). In addition to this macroscopic “net”, altered cell morphology was also observed in Te671 S10 target cells when in contact with this booster.

At day 15, it was clear that the positive control (293 Flex S11 + Te671 S10 cells) was able to transcomplement GFP fragments, meaning that the method was performed correctly (Figure 3.16, a). However, with Lentipro26 mCherry S11, no GFP transcomplementation signal was detected and only mCherry expression was observed (Figure 3.16, a).

Additionally, almost at the end of the protocol, Te671 S10 target cells co-cultured only with Lentipro26 mCherry S11 producer cells, either in the presence of vectofusin-1 or polybrene, started to detach and die. This death was not detected in Te671 S10 target cells co-cultured with the positive control. Instead, a healthy confluent layer of target cells was observed when in the presence of polybrene (Figure 3.16, a).

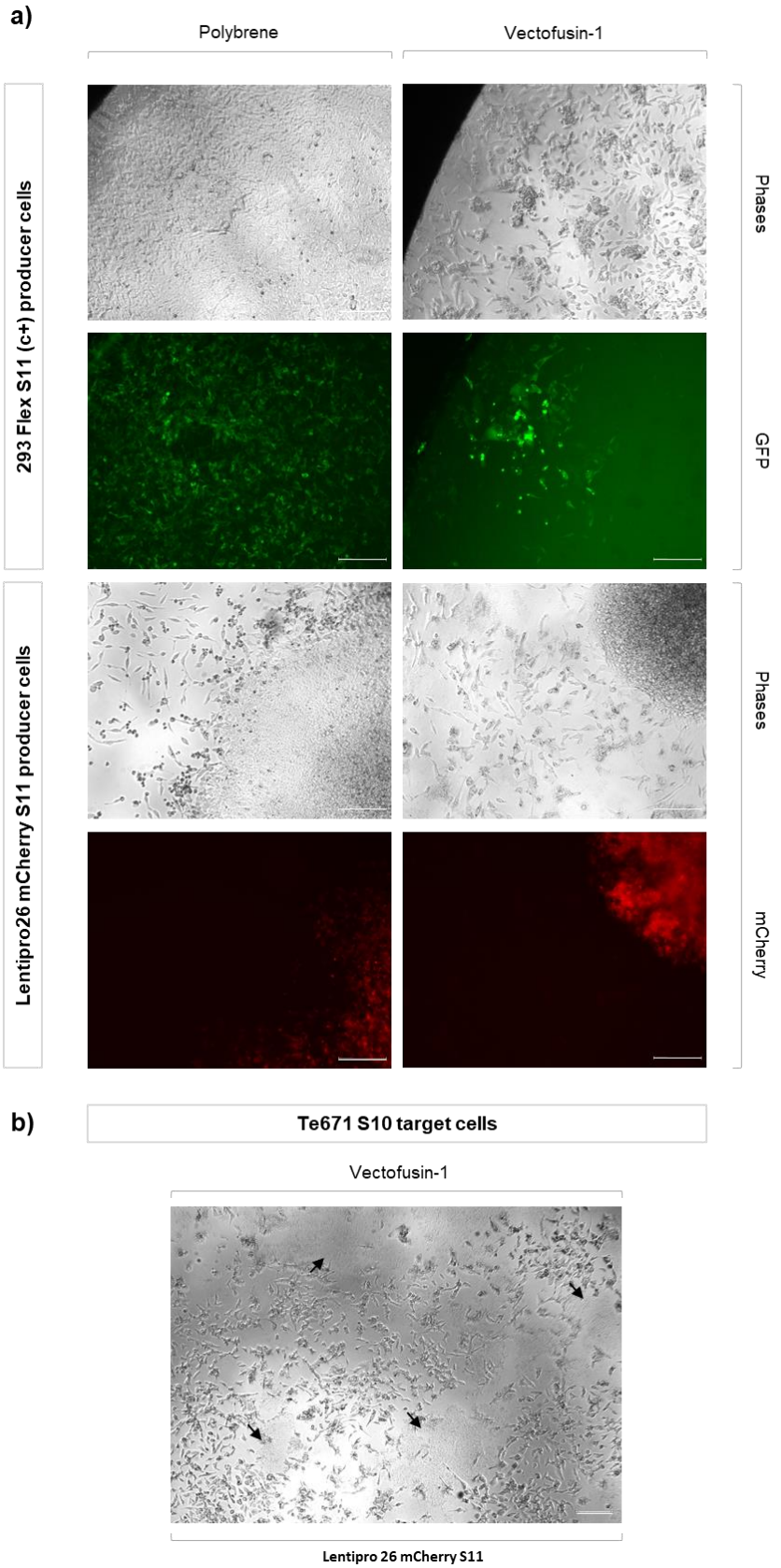

Figure 3.16 - SSCS method adaptation to the use of vectofusin-1 as transduction adjuvant at day 15. Fluorescence and phase contrast images of a) 293 Flex S11 and Lentipro26 mCherry S11 producer clones co-cultured with Te671 S10 target cells and b) only Te671 S10 target cells. Transduction performed in the presence of adjuvant compounds polybrene (8 µg/mL) and vectofusin-1 (10 µg/mL). Black arrows point to vectofusin-1 mesh. Scale bar represents 200 µm.

4

Discussion and Conclusion

LV have been extensively studied over the past decades and became particularly attractive for clinical applications due to their capacity to efficiently transfer, integrate and permanently express the genetic material to slowly or non-proliferating cells.[85]

To apply LVs in a clinical setting, they need to be produced with high quality and purity using GMP. For current LV manufacturer yields, the treatment of a single patient might require several liters of viral supernatant since the LV preparations are characterized by low functional titers, where only <1% of the produced particles are able to deliver the genetic material. Moreover, the majority of the manufacture processes are based on transient systems, where the need to co-transfect four different plasmids introduces undesirable variations in batch to batch yield/quality and increases in production costs.[23]

As a more cost-effective alternative to transient productions, stable and constitutive LV producer cell lines allow robust, characterized, and predictable production processes. However, the development of these cell lines has been more challenging because of the cytotoxicity of some viral proteins and the standard envelope of choice for LV production (VSV-G). One approach to enable stable LV production was the replacement of VSV-G envelope by other non-toxic retroviruses envelopes such as RD114, 4070A, and GaLV.[86] Unfortunately, the development of these stable producer cell lines is extremely time-consuming since it requires the insertion of each expression cassette one by one, followed by exhaustive screening to find the best producer clone.[23]

Therefore, two main challenges are hampering gene therapy feasibility using LV: 1) insufficient LV quality yields, and 2) development of constitutive producer cell lines.

This work addressed these challenges by 1) screening viral adjuvants to improve LV transduction efficiency for a range of target cell types using LV stocks generated by current manufacturer processes and by 2) applying the generated knowledge in 1) to a previously developed high-throughput method (SSCS) that reduces the time of stable cell lines development by implementing a fast detection of the higher producing clones.

Transferring genetic material, using LV, into suspension cells such as T cells, has several therapeutic applications ranging from cancer, monogenic, and infectious diseases.[71] For this purpose, we studied LV transduction in Jurkat E6-1 and SUP-T1 cell lines derived from suspension T lymphocyte and T lymphoblast leukemias, acting as T cell models. In addition, the HEK 293T cell line, one of the most common cell line used for research purposes, was also used due to their properties such as easy maintenance, fast-growing, ease of transfection, transduction and high titers production of viral vectors.[87]

Several factors influence the LV transduction efficiency, being the availability of the known cell receptors in target tissues and cell types, one of them.

The cell receptors responsible for the LV pseudotypes entry herein studied are known: LDL for VSV-G, Pit-1 for GaLV, Pit-2 for 4070A, and ASCT2 for RD114. Although it is also hypothesized that other cell surface receptors could help the virus binding process. Other limitations that might influence LV transduction efficiency can be the physical and chemical constraints. Therefore, to potentiate cell receptor binding, spin-inoculation and chemical compounds may be used.

LV particles without a viral envelope (Nude LV) were used to address the questions of: envelope glycoproteins dependency for viral entry mediated by cell receptor recognition; the impact of spin-inoculation process; and the influence of using viral adjuvants. In figures 3.5 and 3.6, we can conclude that nude LV cannot transduce any of the used target cell lines, proving the envelope dependence for this process. Only in the presence of vectofusin-1, and when spin-inoculation is performed in HEK 293T target cells, we observed a mild positive signal of approximately 2% of LV transduced cells (Figure 3.6, a). This value is minimal when considering transduction efficiencies obtained with LV pseudotyped 4070A, RD114 and GaLV (56%, 61% and 40% respectively) in the presence of this compound (Figure 3.9, 3.9, 3.10). However, this was not expected since Fenard *et al.,* (2013), described that in the absence of any envelope glycoproteins these particles were unable to fuse with the plasma membrane when vectofusin-1 was used.[88]

T lymphoblasts maturation in thymus results in T lymphocytes which eventually leave to the lymphatic system.[89] It is hypothesized that this immunophenotypic alteration in cell surface receptors could influence LV entry and therefore justify the decrease in viral titer for Jurkat E6-1 cell line, for 4070A, RD114 and GaLV pseudotyped LV, when compared to SUP-T1 cell line. On the other hand, a different expression pattern of viral glycoprotein receptors on the cell surface between HEK 293T cells and T cell derivative cell lines is probably the reason behind the observed variations (Figure 3.5).
In contrast, VSV-G pseudotyped LV titer differences between cell lines is minimal, suggesting that the amount of its receptor might be equally expressed. The high transduction efficiency in all cell lines with this pseudotype is probably explained by its interaction with a ubiquitously expressed cell receptor (LDL) (Figures 3.5, 3.7, 3.11), also explaining why 4070A, RD114 and GaLV pseudotyped LV transduction efficiencies are lower compared to VSV-G (Figure 3.11).[90]
4070A pseudotyped LV transduction efficiency in HEK 293T is higher than in SUP-T1 (Figure 3.8), and GaLV transduction efficiencies are higher in SUP-T1 than in HEK 293T cell lines (Figure 3.10). However, as seen by Uckert *et al.,* (1998), 4070A PiT-2 and GaLV PiT-1 receptors have higher RNA expression in the thymus than in the kidney tissue. We only saw a higher transduction efficiency in the SUP-T1 cell line (cell line derived from T lymphoblasts present in the thymus), with GaLV pseudotyped LV and not with 4070A (Figures 3.8, 3.10).[91] In addition, the RD114 pseudotype LV showed lower titers in Jurkat E6-1 than in HEK 293T cells, which is consistent with a low expression of the ASCT2 receptor in cells of the lymph node and high expression in the kidneys (Figures 3.5).[92] Further analysis of receptor expression of these tissues could help to better understand this variation in transduction efficiencies with different pseudotyped LV.

Quantification of the LV stocks was performed with a step of spin-inoculation, which enhanced transduction efficiency and consequently LV viral titers in HEK 293T and SUP-T1 cells (Figure 3.2, 3.7, 3.8, 3.9, 3.10). It is suggested that, besides contributing to virus sedimentation on target cells by increasing cellular permissiveness this process also induces alterations in cell physiology as a consequence of cellular responses to centrifugal stress. Specifically, the altered cytoskeletal dynamics could promote receptor mobilization, viral entry, and post-entry processes.[93, 94] However, spin-inoculation is also likely to dramatically decrease the survival of sensitive T cells, which might explain why spin-inoculation transduction efficiency in SUP-T1 cells with VSV-G pseudotype (Figures 3.7), the most toxic, was maintained when compared to its absence condition.[51]

In addition to receptor availability and spin-inoculation, several factors are described to influence viral vector quantification, such as the volume of inoculum, the number of target cells, or even the cell exposure time to viral vectors.[95] Considering a constant virus amount, the volume of inoculum plays a vital role in the determination of transduction efficiency.[95] Results showed that a lower volume in the infection step (200 µL) results in higher transfection efficiency. This might happen due to higher proximity between LV and the target cells in lower volumes than when higher volumes are used (500 and 1000 µL) (Figure 3.4).

Despite this capacity of LVs to be pseudotyped with numerous viral envelope glycoproteins, a highly efficient transduction process frequently requires the addition of culture additives to promote viral entry.[46] Transduction enhancers such as cationic polymers or peptides are commonly used to boost LV cell entry in laboratory settings. However, the need to discover more advantageous compounds led to the intensive comparison between new developed and standard compounds. Polycationic adjuvants, such as polybrene, lentiboost, lentiblast, transplus and DEAE-dextran, are described to enhance viral vectors infectivity by acting as electrostatic bridges between the negative membrane charges of both viral vectors and target cells. Unfortunately, some such as polybrene, are toxic to primary cells and, as an alternative, the scientific community developed retronectin.[96] Although it has some technical drawbacks making it very difficult to handle in gene therapy, it is still the most used in the clinic. Retronectin requires a plate precoating step, which leads to unprecise amounts of compound, difficult to standardize. Also, after transduction retronectin coated surfaces may reduce the yield of cells recovery and might accelerate cell differentiation.[67, 71]

Herein, seven transduction adjuvant candidates –polybrene, lentiboost, lentiblast, transplus, DEAE-dextran, vectofusin-1, and retronectin– were screened aiming to improve LV transduction. All the tested transduction adjuvants increased LV transduction efficiencies in comparison with the negative control and behaved differently depending on the cell line and the LV pseudotype. In these transduction efficiency assays, MOI was maximized taking into account LV stock titer. It was possible to achieve MOI>1 with almost all pseudotypes, 4070A being the exception (MOI 0.75). Theoretically, by using a MOI of 1 every single cell should experience one viral mediated gene transfer event. However, the measured transduction efficiencies were lower than 100%. This may be explained due to the presence of defective particles or even loose envelope glycoproteins in the LV supernatant, which occupy target cells receptors and consequently block the binding of functional particles. Nevertheless, LV studies

showed that the higher the MOI, the higher the efficiency of gene transfer, and the level of gene expression.[95] It is also of interest to perform further studies with higher MOI to mimic clinical settings. When comparing viral envelopes side by side at the same MOI conditions (0.4) (Figure 3.11), we can conclude that VSV-G indeed allowed LV mediated gene delivery in HEK 293T and SUP-T1 better than 4070A, RD114 and GaLV LV in the control with polybrene. However, in the presence of vectofusin-1, 4070A, RD114, and GaLV pseudotyped LV transduction efficiencies surpass VSV-G. Such rise in transduction efficiencies without spin-inoculation makes possible the elimination of centrifugation step during infection, which is time-consuming and laborious. Furthermore, these high transduction efficiencies with these non-toxic LV pseudotypes makes them competitive enough to replace VSV-G in the clinic.

Although some compounds such as lentiboost, lentiblast and DEAE-dextran, led to enthusiastic results, vectofusin-1 was the most promising one for 4070A, RD114 and GaLV pseudotypes but not for VSV-G (Figures 3.7, 3.8, 3.9, 3.10). The vectofusin-1 impact in HEK 293T and SUP-T1 cells for 4070A, RD114 and GaLV pseudotypes (Figures 3.8, 3.9, 3.10, 3.11) herein presented is in agreement with reports describing it to enhance gene delivery of $CD34^+$ hematopoietic stem progenitor cells and T cells without cell toxicity and with higher efficacy than retronectin.[11, 55, 66]

Interestingly, while the enhancing effects of this adjuvant with GaLV, RD114, and 4070A are frequently mentioned, its effect in VSV-G pseudotyped LV is more contradictory. Our observations show a clear decrease in transduction efficiency with VSV-G LV in SUP-T1 (Figures 3.7, 3.11). Jamali *et al.*, (2019) transduction experiments also revealed that vectofusin-1 was detrimental for VSV-G transduction.[11] The only reports mentioning that vectofusin-1 action was negligible on transduction of T cells (Piovan *et al.,* (2017)) or had enhancing effects (Fenard *et al.,* (2013)) used highly purified particles, suggesting the existence of inhibitory particles in the VSV-G medium supernatant.[55, 67]

It seems that the entry pathways of VSV-G LV might be conditioned by vectofusin-1 action since this envelope cell entry process is the only one mediated through endocytosis and pH-dependent fusion. Therefore, we hypothesize that vectofusin-1 might be hampering VSV-G mediated LV entry either by affecting the cell process of endocytosis or the pH-dependent membrane fusion in the endosome. The mechanism of viral and cell membrane fusion enhancement was described to be unique to vectofusin-1 and was not observed with other polycationic transduction enhancers.[67] Briefly, it was speculated that its cationic properties would neutralize lipids, leading to increased adhesion, followed by a subsequent destabilization of the plasma membrane driving to fusion.[67] Considering that vectofusin-1 mediated high transduction efficiencies for 4070A, RD114, and GaLV in both target cells used (Figures 3.8, 3.9, 3.10, 3.11), we can hypothesize that what is limiting the functional LV yields of current manufacturer bulks is the LV ability to fuse with the cell membrane.

To take advantage of the LV full potential for gene therapy and aid a faster academic to clinical transition, the establishment of robust and high-throughput analytical means for stable producer cell lines development would help speed up their implementation. For this reason, the adaptation of the SSCS method to LV producer cell lines would be of great importance. Previous work in-house was performed trying to achieve this, however viral transduction with polybrene did not result in high enough

transduction signals. Therefore, SSCS adaptation to vectofusin-1 use was performed. After optimizing compound concentration and FBS culture medium supplementation (Figures 3.13, 3.14), vectofusin-1 transduction enhancing properties were tested in this method (Figure 3.15). Subsequently to the first vectofusin-1 addition, a subtle mesh appeared in the bottom of the cell culture well (more visible in figure 3.16, b). It is important to remember that vectofusin-1 activity is dependent on fibrils formation that result in supramolecular structures, firstly assembled with viral vectors supernatant and only later are added to the cell culture medium.[66] However, when this compound is used in SSCS, it is directly added to cell culture wells where few cells are growing (producing clone and target cells). Consequently, we hypothesize that vectofusin-1 fibril formation starts independently of the LV amount present and sediment by gravity in the bottom of the well. This assumption is in agreement with not having seen this macroscopic effect in earlier LV quantifications, since vectofusin-1 is mixed beforehand with LV, which are in higher amounts than in SSCS. The presence of this mesh on the bottom seems to block cell growth in those areas and subsequently does not allow a confluent cell growth, which is highly necessary for the SSCS proper transduction signal.

Another issue appeared around the 12th day, when Te671 S10 target cells, co-cultured with Lentipro26 mCherry S11 producing clones, started to detach and die. This behavior did not happen with Te671 S10 target cells cultured with our positive control (293 Flex S11). In the first six days of the protocol, the Lentipro26 mCherry S11 culture medium had antibiotic supplementation to ensure LV cassettes expression. Since Te671 S10 target cells are not antibiotic-resistant, at the 7th day of experience, the culture medium was replaced by an antibiotic-free one. Bearing in mind that the only difference in cell culture between Lentipro26 mCherry S11 and 293 Flex S11 cell lines, was the presence of antibiotic supplemented culture medium, it is possible to assume that an inefficient culture medium removal contributed to kill the non-antibiotic resistant target cells. At the end of the experiment, no GFP transcomplementation signal between Lentipro26 mCherry S11 producing clones and the few live Te671 S10 target cells (Figure 3.15, a) was observed either in the presence of polybrene or vectofusin-1. Therefore, although the SSCS adaptation to a stable LV producer cell line is of great interest, further tests should be performed, namely the complete elimination of antibiotics from the assay.

Concluding, the present data demonstrates that the choice of viral entry adjuvant, LV pseudotype, and target cells, undoubtedly affects the resulting transduction efficiency and should facilitate more rational decisions. The use of vectofusin-1, which has numerous advantages over conventional additives, could help to simplify clinical cell manipulation allowing the use of lower amounts of LV and thereby reducing production costs.[67] Based on these results, there is interest for further testing this additive in clinical applications. Although very promising, vectofusin-1 adaptation to high-throughput screening LV titer methods needs further studies.

The knowledge generated by this work contributed to understand that LV functional yields can be improved by the addition of transduction enhancers to the cell transduction procedure allowing to achieve higher transduction efficiencies in different target cells with pseudotyped LV.

4.1 Future work

LV have already won its place as flexible and valuable tools for gene therapy being used in several applications.[34] However, lowering gene therapy treatment costs is still a goal for the upcoming years. Future lines of work could include a screening of these adjuvant candidates in higher MOI doses to better mimic clinical trials conditions. Posterior studies on adjuvant enhancing mechanism behavior, namely, its action in the adhesion or fusion viral entry steps in transduction, should be pursued. Also, gene therapy using LV is becoming a promising clinical strategy for the treatment of most inherited blood cell diseases, such as immune deficiencies and stem cell defects, which can be treated by transplantation of allogeneic hematopoietic stem cells.[97] In this sense, extending the screening of viral entry adjuvants to $CD34^+$ representative cell lines, such as K652, would be of great importance and one of the next steps to take in this work.[98] Finally, further tests on primary cells would also enable to validate the value of these adjuvants for clinical use.

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Annexes

A.1 Primers

Table A.1 - Primers for RT-qPCR

Target/Gene	Orientation	5’ to 3’ sequence
WPRE	Forward	ACTGTGTTTGCTGACGCAAC
	Reverse	ACAACACCACGGAATTGTCA

A.2 Boosters concentration optimization

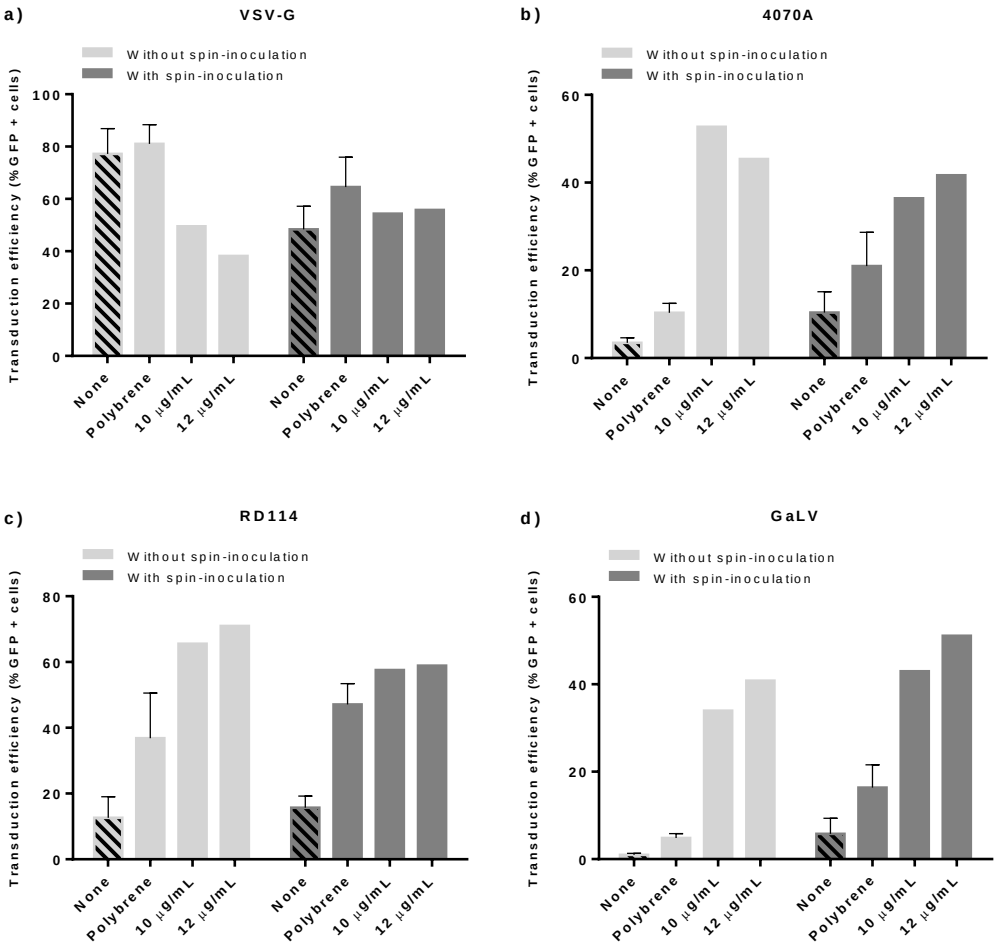

Figure A.1 - Optimization of assisted lentiviral transduction with vectofusin-1 adjuvant.
Two recommended concentrations of vectofusin-1, 10 µg/mL and 12 µg/mL, were tested without and with spin-inoculation in HEK 293T target cell line, using LV particles pseudotyped with a) VSV-G, b) 4070A, c) RD114 and d) GaLV envelope glycoproteins. Values shown as average ± standard deviation of n≥2 biological replicates (none and polybrene conditions represent biologic variability).

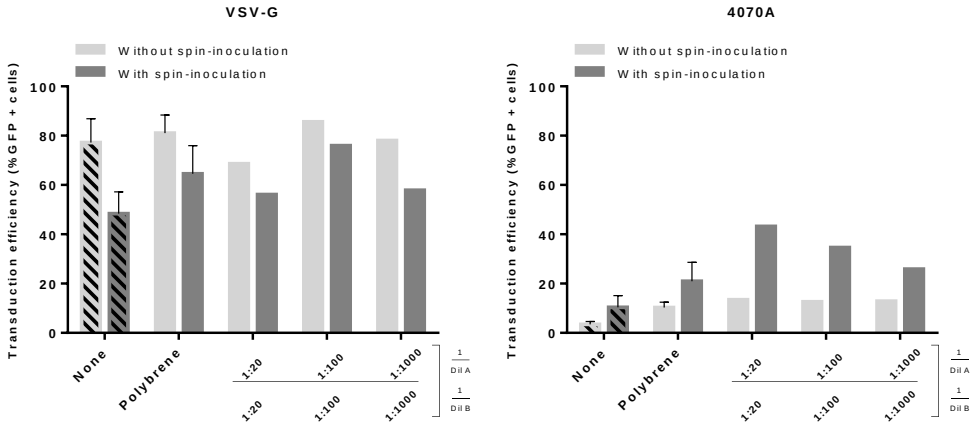

Figure A.2 - Optimization of assisted lentiviral transduction with lentiboost adjuvant.
Transduction efficiency in HEK 293T target cell line without and with spin-inoculation, at different combinations of LentiBoost solutions (A and B) recommended dilutions (from 1:20 to 1:1000), for a) VSV-G and b) 4070A pseudotypes. Values shown as average ± standard deviation of n≥2 biological replicates (none and polybrene conditions represent biologic variability).

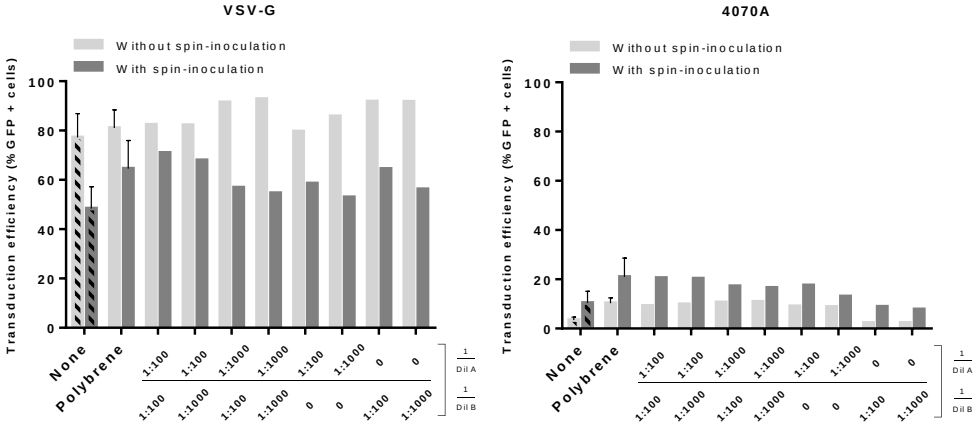

Figure A.3 - Optimization of assisted lentiviral transduction with lentiblast adjuvant.
LV transduction efficiency in HEK 293T target cell line without and with spin-inoculation, at different combinations of LentiBlast solutions (A and B) dilutions, as suggested by the manufacturer, in a) VSV-G and b) 4070A pseudotypes. Values shown as average ± standard deviation of n≥2 biological replicates (none and polybrene conditions represent biologic variability).

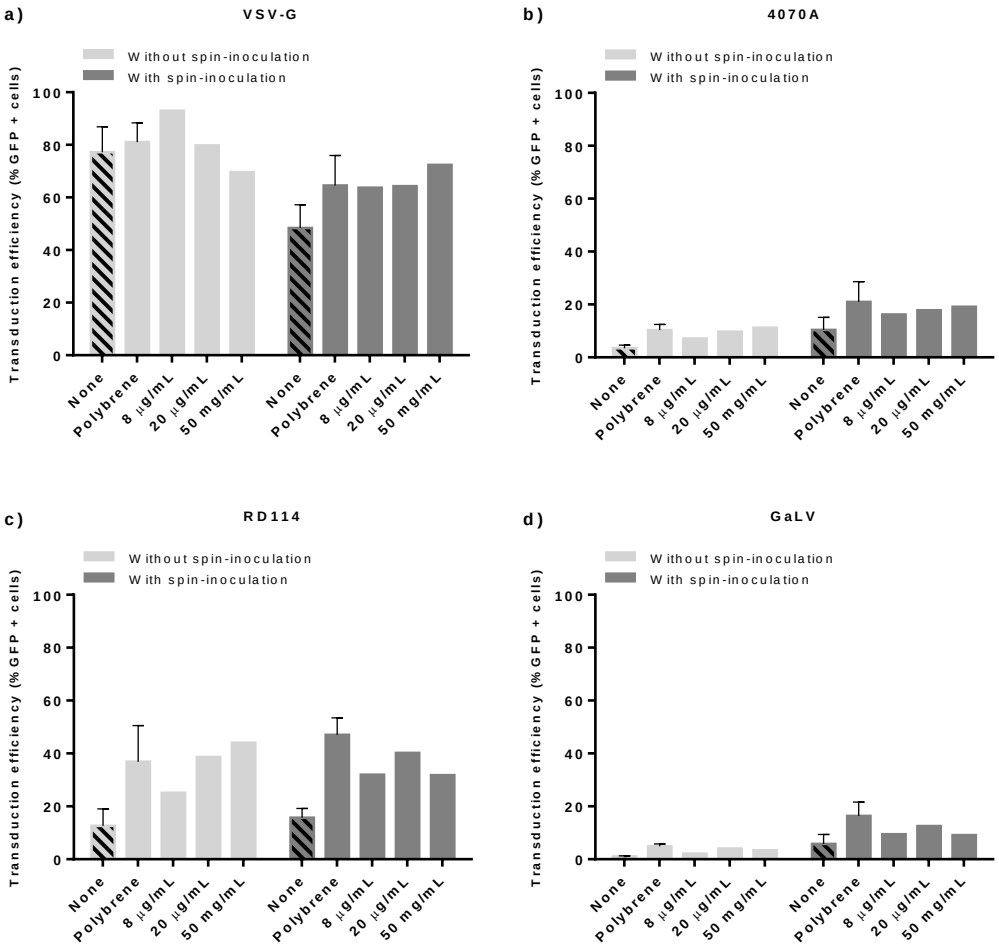

Figure A.4 - Optimization of assisted lentiviral transduction with DEAE-dextran adjuvant.
Transduction experiments performed with a) VSV-G, b) 4070A, c) RD114 and d) GaLV LV in the presence of DEAE-Dextran at 8 μg/mL, 20 μg/mL and 50 μg/mL. Values shown as average ± standard deviation of n≥2 biological replicates (none and polybrene conditions represent biologic variability).