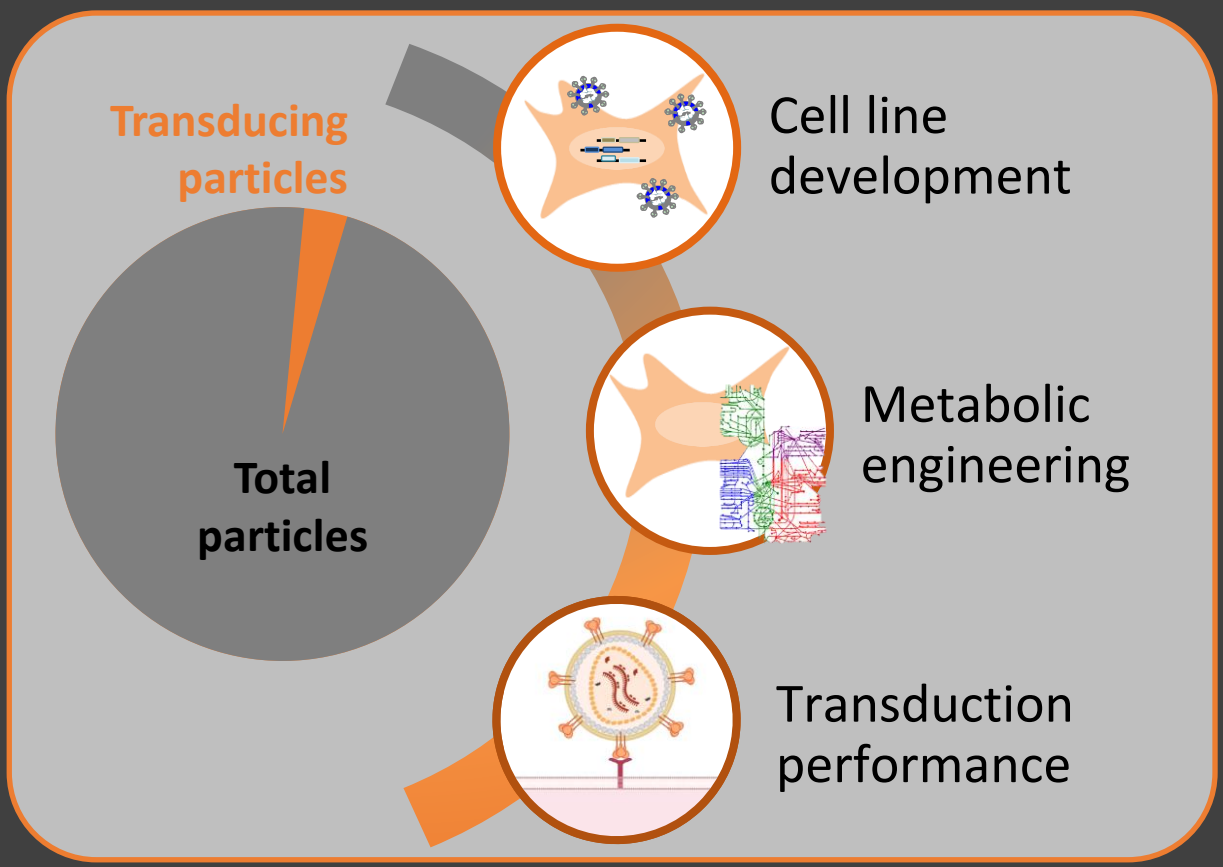
Improving γ-retrovirus and lentivirus gene therapy vectors

From producer cell engineering to transduction performance

Ana Sofia Formas Oliveira



Dissertation presented to obtain the Ph.D degree in Molecular Biosciences - Engineering Sciences and Technology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
July, 2020

UNIVERSIDADE NOVA DE LISBOA

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Supervisor: Dr. Ana Sofia de Sousa Valente Coroadinha

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Instituto de Biologia Experimental e Tecnológica

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Foreword

I declare that the work presented in this doctoral thesis, except where otherwise stated, is based on my own research. This thesis is the result of five years of research at the Animal Cell Technology Unit, in iBET and ITQB NOVA (Oeiras, Portugal), under the supervision of Dr. Ana Sofia Coroadinha.

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Pai, Mãe, Mano, Sofia e Xico
A vós muito devo.
Infinita gratidão e amor
Vos ofereço.

À minha familia,

List of Contents

Abbreviations

4070A	Amphotropic mlv
AAV	Adeno-associated virus
ACTR	Antigen-coupled t-cell receptor
AdV	Adenoviral vector
ADA-SCID	Adenosine deaminase severe combined immune deficiency
ALB	Albumin
ASCT2	Alanine, serine, cysteine transporter 2
Bax	BCL2-associated x protein
BCL2	B-cell lymphoma 2
BET	Bromodomain/extraterminal domain proteins
BFAD	Bureau of food and drugs Philippines
bsr	Blasticidin resistance gene
CAR	Chimeric antigen receptors
CBS	Cystathionine-beta-synthase
CEM	Human acute lymphoblastic leukemia cell line
CFDA	China food and drug administration
CMV	Cytomegalovirus
COS-1	African green monkey kidney fibroblast-like cell line
COS-7	African green monkey kidney fibroblast-like cell line
CRISPR	Clustered regularly interspaced short palindromic repeats
CTH	Cystathionine gamma-lyase
CV-1	African green monkey kidney fibroblast-like cell line
DNA	Deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
EMA	Europe medicine agency
EMCV-IRES	Encephalomyocaerditis virus-internal ribosome entry site
FBS	Fetal bovine serum
FRT	Flippase recombinase target
FRT-F5	Spacer mutant flippase recombinase target
FRT-WT	Wild type flippase recombinase target
G6PD	Glucose-6-phosphate dehydrogenase
GaLV	Gibbon ape leukemia virus
GMP	Good manufacture practice
GOI	Gene of interest
GPX7	Glutathione peroxidase 7
GSH	Reduced glutathione

GSR	Glutathione-disulfide reductase
GSS	Glutathione synthetase
GSTM1	Glutathione s-transferase mu 1
hEF1α	Human elongation factor-1α
Hela	Human adenocarcinoma cell line
HEK	Human embryonic kidney cells
HIV-1	Human immunodeficiency virus type 1
hPGK	Human phosphoglycerate kinase
hph	Hygromycin resistant gene
HSPA5	Heat shock 70kDa protein 5
HSV	Herpes simplex virus
HT 1080	Human fibrosarcoma cell line
HTLV	Human T-cell leukemia virus
IDH1	Isocitrate dehydrogenase
kDa	Kilodaltons
LacZ	B-galactosidase
LDL	Low-density lipoprotein
LTR	Long terminal repeat
LV	Lentiviral vectors
MARs	Matrix attachment regions
MLV	Murine leukemia virus
MOI	Multiplicity of infection
MoMLV	Moloney murine leukemia virus
MPSV	Myelo-proliferative sarcoma virus
neo	Neomycin phosphotransferase gene
OTC	Ornithine transcarbamylase deficiency
pac	Puromycin resistance gene
PBS	Primer binding site
PDIA2	Protein disulfide isomerase family A member 2
PEI	Polyethylenimine
Pit-1	Inorganic phosphate transporter 1
Pit-2	Inorganic phosphate transporter 2
P.P.	Physical particles
PPT	Polypurine track
RCP	Replicative competent particles
RD	Human rhabdomyosarcoma cell line
RD114	Feline endogenous retrovirus envelope

RMCE	Recombinase mediated cassette exchange
RNA	Ribonucleic acid
RNAi	Rna inhibition
RPE65	Retinal pigment epithelium-specific 65 kda protein
RSV	Rous sarcoma virus
Sh ble	Zeocin resistance gene
SIN	Self-inactivating vector
SMN1	Survival of motor neuron, telomeric copy gene
SSCS	Single-step cloning-screening
STARs	Stabilizing anti-repressors
SV40	Simian vacuolating virus 40
TIL	Tumor-infiltrating lymphocytes
Trna	Transfer RNA
T.U.	Transducing units
UBB	Ubiquitin B
UCOEs	Ubiquitously acting chromatin opening elements
US FDA	United States Food and Drug Administration
VACV	Vaccinia virus
V.G.	Viral genomes
VSV-G	Vesicular stomatitis virus glycoprotein G
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
XBP1	X-box binding protein 1
γ-RV	Gamma-retroviral vector
Ψ	Packaging signal

Abstract

In the last decade gene therapy became an effective form of treatment for several disease indications. Successful clinical trials saved the lives of many patients otherwise with untreatable diseases and numerous products were granted market approval. Gammaretroviral and lentiviral vectors (γ-RV and LV) are the gene delivery vehicles with higher representation in clinical trials and commercialized virus-based products. What makes these retroviral vectors so attractive is their unique feature of stable integrating the therapeutic gene into patient cells allowing long-term gene expression. These enveloped viral vectors are manufactured using HEK 293 derived producer cells where crude supernatants are characterized by low ratios of transducing units to total physical particles (T.U./P.P.). This can be a consequence of vector cassettes unbalanced cellular expression stoichiometry, defective viral particle synthesis due to cellular metabolic constrains or, inefficient vector transducing capacity. Therefore, to meet the needs of the increasing number of clinical trials and marketed products it is extremely important to develop robust producer cells and provide efficient cell transduction protocols. To do that it is imperative to understand what is limiting high titers of functional vectors. This thesis aimed to generate knowledge and strategies enabling improved vector transducing functional yields. The challenges on the production of γ-RV and LV were addressed, and herein organized in three sections, by: 1st) studying the impact of vector genetic cassette design, transfection procedure and stable expression levels on cell vector yields; 2nd) engineering cell metabolic pathways, pursuing superior specific cell production performance; and 3rd) studying adjuvant compounds for enhanced vector transduction efficiency of four viral envelope pseudotypes.

The first part of this work focused on constitutive vector production and provides valuable knowledge and methodologies to establish faster and optimized cell line development strategies. In **Chapter II** a comprehensive

analysis of factors influencing γ-RV producer cell line development process was conducted. The study focused on (i) vector genetic cassette design (promoter selection), (ii) transfection procedures (non-viral or viral) and (iii) clone isolation methods (random or relying on productivity screening tools). By merging high throughput clone screening methods and recombinase mediated cassette exchange technology, γ-RV producer cells of clinical relevance and cGMP compliant (10^7 T.U./mL), were successfully obtained in a short timeframe. In this chapter, the development of stable LV producer cells was also addressed. LV transgene cassette was optimized enabling to isolate high producer clones in less than 3 weeks using a high throughput screening methodology. To further elucidate constitutive production systems, in **Chapter III** a detailed study on LV production yields and cassettes copy number and expression levels was conducted. From the four LV expression cassettes, envelope expression seemed to modulate functional LV yields. Moreover, sustained, and high transgene expression levels were associated to high functional LV yields and producer cell stability. Overall, the results of this first part emphasize the importance of thorough clone screening and characterization to find a stable high producing clone to improve bioprocess robustness.

The second part of this work studied how cell metabolic pathways modulate vectors productivity. Genetic engineering was used to stably integrate and overexpress candidate genes from different pathways in the producer cells. Gene overexpression levels were tightly controlled to obtain a cell productivity response as a function of gene dose. The overexpression of genes related to endoplasmic reticulum protein processing, unfolded protein response and anti-apoptosis cellular mechanisms were explored in **Chapter IV**. Glutathione metabolic genes overexpression impact on cells productivity was addressed in **Chapter V**. Functional γ-RV yields were improved in a gene dose dependent manner up to 13-fold when overexpressing genes related to: protein processing and folding, unfolded protein response, cysteine formation through the transsulfuration pathway

and glutathione mediated detoxification. These studies highlight protein building blocks and synthesis and, oxidative stress, as cell metabolic constrains limiting constitutive vector production quality, effectively counteracted through metabolic gene engineering. On the other hand, LV functional yields were mildly improved when producer cells were genetically engineered with the same metabolic genes. Improvements up to 2-fold in LV T.U. titer were obtained when overexpressing a rate limiting enzyme of pentose phosphate pathway or an anti-apoptotic gene. The later improvements resulted in vector preparations with higher quality (T.U./P.P). These results suggest, oxidative stress, nucleotide availability and, cell death (due to transfection procedures and expression of toxic vector components), as the main restrictive cause for LV transient manufacture. In sum, this second part of the work identified and validated engineering targets for the development of robust production platforms.

The third and last part of this work, **Chapter VI**, evaluated the transduction efficiency of 4070A, RD114, GaLV and VSV-G LV pseudotypes. SUP-T1 and HEK 293T were used as target cell models to compare the transduction efficiency of the four LV pseudotypes. Seven transduction enhancers, the majority of clinical grade, were screened. Particles pseudotyped with VSV-G showed the highest cell transduction efficiency in all target cells, independently of adjuvant supplementations. From all the seven transduction enhancers tested, Vectofusin-1 enabled the highest LV transduction efficiency improvements for 4070A, GaLV and RD114 pseudotypes, reaching similar percentage of transduced cells as VSV-G pseudotype. These three *Gammaretrovirus* pseudotypes are of high relevance since they are compatible with constitutive expression for stable producer cell lines development. Also, have a more specific cell tropism and for certain cell types provide higher transduction efficiencies than VSV-G. Overall, these results revealed that the transduction enhancers were able to increase LV transduction efficiency without any pseudotype or bioprocess optimization, an evidence of the intrinsic particles transduction

capacity. Thus, the transduction conditions are an additional constrain in vector functional yields to have in consideration.

In summary, this thesis provides an integrative perspective over multiple factors influencing the manufacture of gene therapy products, including vector design, cell line development, metabolic engineering, and vector transduction efficiency. The strategies herein explored enabled disclosing critical parameters which improved vector functionalization. These strategies can be further combined in rational-driven approaches to develop high-producer hosts and efficient vector transduction protocols for biotechnological and clinical applications.

Resumo

Na última década, o bom desempenho das terapias génicas em ensaios clínicos permitiu salvar a vida a vários pacientes que outrora não teriam tratamento disponível. Actualmente, este tipo de terapias constituem uma opção de tratamento para diversas patologias, existindo vários produtos aprovados para comercialização. Vectores virais, nomeadamente gammaretrovírus e lentivírus recombinantes, estão entre os veículos mais comumente utilizados para entregar o material genético às células do paciente neste tipo de terapias. Uma particularidade destes vectores retrovirais, que os tornam tão atractivos, são a sua capacidade de integrar o material genético terapêutico no genoma das células alvo do paciente. Assim, permitem modificar as células de forma permanente e hereditária (divisão celular), potencialmente contribuindo para curar doenças. Estes vectores retrovirais são produzidos em linhas celulares de origem humana (HEK 293) dos quais os sobrenadantes virais são caracterizados por baixos rácios de partículas funcionais em relação às totais sintetizadas (unidades de transdução por partículas físicas - T.U./P.P.). Vários factores podem contribuir para este baixo rácio, nomeadamente; a estequiometria da expressão das cassetes genéticas virais nas células produtoras; produção de partículas defeituosas devido a constrangimentos metabólicos da plataforma celular; e por último, reduzida eficiência de transdução do vírus. Portanto, de forma a garantir o abastecimento/disponibilidade de productos de terapia génica baseados em retrovírus recombinantes, é de extrema importância perceber o que está a limitar a qualidade da produção viral para desenvolver linhas celulares com rendimentos elevados e estabelecer protocolos de transdução eficientes. Assim, este projecto de doutoramento teve como objectivo gerar conhecimento e estratégias que permitam aumentar a produção de vectores retrovirais funcionais. Para tal, três linhas de investigação foram exploradas, organizadas também em três secções

nesta tese. Na primeira parte estudou-se a produtividade das células em função do desenho, método de transfecção e níveis de expressão das cassetes genéticas virais. Na segunda parte, utilizou-se engenharia metabólica para contornar constrangimentos metabólicos associados à síntese de retrovírus recombinantes e melhorar a produtividade específica celular. Na terceira parte, a eficiência de transdução de vírus pseudotipados com quatro invólucros virais foi estudada e melhorada com suplementação de compostos que actuam como adjuvantes da transdução viral.

O objecto de estudo da primeira parte deste trabalho (**Capítulo II** e **III**) foram os sistemas de produção constitutivos. Estes sistemas são preferíveis aos transientes uma vez que permitem bioprocessos mais simples, reprodutíveis e economicamente mais viáveis. Contudo, o desenvolvimento de linhas celulares de produção constitutiva é um processo bastante demorado e trabalhoso. No **Capítulo II** uma análise sistemática dos factores inerentes ao desenvolvimento de linhas celulares produtoras de vectores retrovirais foi efectuada de modo a optimizar e acelerar este processo. Neste estudo focamo-nos: (i) no desenho das cassetes genéticas virais (escolha dos promotores), (ii) nos métodos de transfecção (não-virais ou virais), e (iii) nos métodos de isolamento de clones (selecção aleatória ou monitorizada). A optimização deste processo permitiu desenvolver num curto espaço de tempo células produtoras de vectores gammaretrovirais terapêuticos (10^7 partículas funcionais por mililitro (T.U./mL)) e em conformidade com as actuais boas práticas de fabricação. A cassete de genoma viral desta linha celular foi desenhada para permitir o uso da tecnologia de troca de cassete, flexibilizando a sua troca por qualquer outra e para qualquer aplicação clínica. Adicionalmente, no **Capítulo II**, também demonstramos a adaptação e aplicação de um método rápido de rastreio de clones para o desenvolvimento de linhas células produtoras de vectores lentivirais. Permitindo assim, reduzir para três semanas o processo de isolar clones com elevada produtividade. No

Capítulo III caracterizou-se a produção constitutiva de vectores lentivirais analisando em vários clones produtores os títulos virais, o número de cópias e o nível de expressão das cassetes virais. Verificou-se não haver relação entre o número de cópias das cassetes virais e os seus níveis de expressão, evidenciando a diversidade genómica dos clones. Das quatro cassetes virais, a expressão do envelope pareceu modular o título funcional destes vectores. Concluiu-se também que os níveis de expressão do genoma viral são importantes para suportar elevados títulos de partículas funcionais e que, a estabilidade da produtividade dos clones está associada à estabilidade da sua expressão. Os resultados desta primeira parte enfatizam a necessidade de uma caracterização detalhada dos melhores clones produtores para assegurar um bioprocesso robusto.

Na segunda parte deste trabalho (**Capítulos IV** e **V**) explorámos o metabolismo celular para modular a produtividade específica das células. Através de engenharia genética das células produtoras, estudamos o aumento dos níveis de expressão de genes associados ao processamento de proteínas no retículo endoplasmático, anti-apoptose (**Capítulo IV**) e metabolismo do glutationo (**Capítulo V**). Os níveis de expressão génica dos genes sob estudo foram cuidadosamente controlados, de forma a estudar a resposta de produtividade celular em função da dosagem génica. Com esta abordagem, os títulos gammaretrovirais foram aumentados até 13 vezes quando se aumentou a expressão de genes associados ao processamento de proteínas no retículo endoplasmático, síntese de cisteína e destoxificação mediada pelo glutationo. Estes resultados identificaram a disponibilidade de aminoácidos, síntese proteíca e stress oxidativo como limitantes na produção constitutiva de vectores gammaretrovirais. No caso dos vectores lentivirais, quando as linhas celulares produtoras foram geneticamente modificadas, os aumentos de produtividade foram mais modestos. Os títulos de vectores lentivirais duplicaram quando se aumentou a expressão de genes associados à via de pentose-fosfato e anti-apoptose. Sugerindo que a produção de vectores

lentivirais transientemente é afectada pela disponibilidade de nucleótidos e pela morte celular. Em suma, esta segunda parte do trabalho identificou e validou genes alvo para a engenharia genética de linhas celulares com a finalidade de melhorar a sua produtividade.

Na terceira e última parte deste trabalho, **Capítulo VI**, avaliámos a eficiência de transdução de quatro invólucros virais diferentes (4070A, RD114, GaLV and VSV-G) utilizados para pseudotipar partículas lentivirais. Duas linhas celulares, SUP-T1 e HEK 293, foram utilizadas como células alvo modelo para comparar a eficiência de transdução. Sete adjuvantes foram individualmente usados no processo de transdução numa tentativa de melhorar a quantidade de células transduzidas. Os vectores lentivírais pseudotipados com VSV-G foram os que transduziram as duas linhas celulares mais eficientemente, independentemente da adição de adjuvantes. De todos os compostos testados, Vectofusin-1 possibilitou o maior aumento de percentagem de células transduzidas por lentivírus pseudotipados com 4070A, GaLV e RD114. Chegando mesmo a atingir percentagens de células transduzidas semelhantes às conseguidas com VSV-G. Estes resultados são de extrema importância uma vez que os três invólucros virais são compatíveis com expressão constitutiva para o desenvolvimento de linhas celulares produtoras. São também envelopes com maior especificidade de tropismo e mais eficientes na transdução de determinadas células primárias. Os melhoramentos conseguidos nesta parte do trabalho indicam também que as condições de transdução viral são um constrangimento nos títulos funcionais deste vector e devem ser optimizadas.

Em conclusão, esta tese fornece uma perspectiva integrada sobre múltiplos factores que podem influenciar a produção de retrovírus recombinantes, incluindo: o desenho das cassetes virais, a estratégia de desenvolvimento de linhas celulares produtoras, estratégias de engenharia metabólica e as condições de transdução dos invólucros virais. As estratégias exploradas nesta tese permitiram elucidar parâmetros

críticos que contribuíram para o melhoramento dos títulos virais funcionais. A combinação destas estratégias pode ser futuramente utilizada em aplicações biotecnológicas e clínicas para desenhar processos baseados no conhecimento aqui gerado de forma a desenvolver rapidamente células super produtoras e protocolos de transdução viral eficientes.

List of publications

Published by the author (1)

Formas-Oliveira AS, Basílio JS, Rodrigues AF, Coroadinha AS. Overexpression of ER protein processing and apoptosis regulator genes in HEK 293 cells improves gene therapy vectors production. *Biotechnol J.* 2020. DOI: 10.1002/biot.201900562.

In preparation by the author (4)

Formas-Oliveira AS, Almeida AI, Tomás HA, Alves PM, Coroadinha AS. Accelerating cell line development for constitutive production of gene therapy gammaretroviral and lentiviral vectors: case study of expression cassette design, transfection procedures, and clone selection. (*in preparation*)

*Formas-Oliveira AS, *Ferreira MV, Coroadinha AS. Lentiviral vector components expression stoichiometry and stability: impact on constitutive producer cell yields and vector quality. (*in preparation*)

Formas-Oliveira AS, Rodrigues AF, Gelder V, Vaz T, Coroadinha AS. Improving gene therapy retroviral vector production by genetic engineering glutathione metabolism. (*in preparation*)

*Formas-Oliveira AS, *Cabral ET, Coroadinha AS. Lentiviral vector cell transduction efficiency: impact of different pseudotypes and transduction enhancers. (*in preparation*)

*Authors with equal contribution

Other publications by the author (2)

Rodrigues AF, Guerreiro MR, Formas-Oliveira AS, Fernandes P, Blechert AK, Genzel Y, Alves PM, Hu WS, Coroadinha AS. Increased titer and reduced lactate accumulation in recombinant retrovirus production through the down-regulation of HIF1 and PDK. *Biotechnol Bioeng*. 2016, 113(1):150-62. DOI: 10.1002/bit.25691.

Rodrigues AF, Formas-Oliveira AS, Guerreiro MR, Tomás HA, Alves PM, Coroadinha AS. Single-step cloning-screening method: a new tool for developing and studying high-titer viral vector producer cells. *Gene Ther*. 2015 (9):685-95. DOI: 10.1038/gt.2015.44.

CHAPTER I

General Introduction

Author contribution

Ana Oliveira wrote this chapter based on the referred bibliography

Chapter I

1.1 Gene therapy

Dysfunctional genes account for 80% of the total rare diseases reported to date, few having available treatment [1]. Gene therapy emerged as a groundbreaking concept to treat, with the potential to cure, these diseases. The approach consists in inserting genetic material into the patient cells to restore, add or suppress gene function.

Since the first human gene transfer experiment (1989), thirty years have passed. During this time, human genome was sequenced, contributing for the understanding of human pathologies, and recombinant DNA technologies developed substantially leading to innovative gene therapy approaches (Fig. 1.1). On the other hand, post gene therapy fatal failures occurred, disclosing constraints and intensifying safety awareness in gene therapy applications. All in all, the built know-how lead to the present success of gene therapy, which is now a real therapy for numerous disease targets and market approved products [2,3].

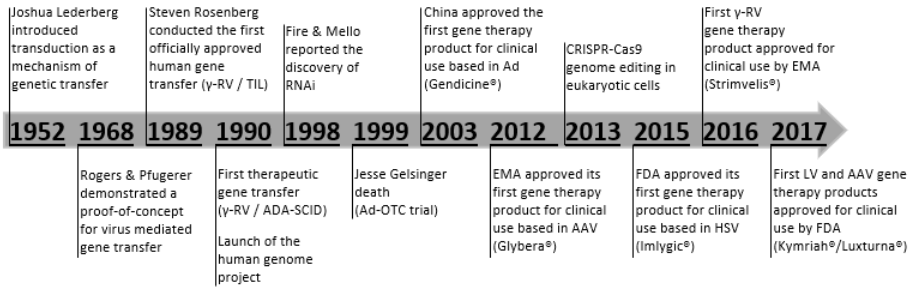

Figure 1.1 - Timeline highlighting some important milestones contributing for the development of human gene therapy. AAV – adeno-associated virus; Ad – adenoviral vector; ADA-SCID – adenosine deaminase severe combined immune deficiency; CRISPR – clustered regularly interspaced short palindromic repeats; HSV – herpes simplex virus; LV – lentiviral vector; OTC – ornithine transcarbamylase deficiency; RNAi – RNA interference; TIL – tumor-infiltrating lymphocytes; γ-RV – gammaretroviral vector. Adapted from [3,4].

Gene therapy can target germline or somatic cells and can be performed *in vivo* or *ex vivo*. The modifications of germline cells allow the transmission of the genetic changes to the offspring and due to ethical issues is not authorized. The somatic cells genetic alterations remain in the patient and, for that reason, are considered safer [1]. In the *in vivo* therapy, the genetic material is delivered systemically or directly to the patient organs/tissue. In this approach, targeted expression of the therapeutic genetic material can be accomplished by tissue specific promoters or using a vehicle with precise tropism. This strategy requires higher amounts of the transfer vector and the patient immune system may clear it before it reaches the target. On the other hand, *ex vivo* therapy involves the isolation of patient cells, followed by its modification with the therapeutic genetic material and the return of these modified cells into the patient. This system presents several advantages concerning safety and efficacy: it is an autologous transplant; less transfer vector is needed; presents low risk of off-target effects; avoids metabolic, renal, and immune clearance; and allows selection, expansion and quality control of the modified cells before reinfusion. However, it is limited to cells and tissue/organs capable of dividing or regenerating, respectively [5].

1.1.1 Clinical trials

Over thirty years of human gene therapy, almost 3000 clinical trials were executed, are ongoing or have been approved worldwide (Fig.1.2a). Although not always consistent, the number of approved clinical trials has been increasing, achieving records in the last two years. The majority is now in early stages, phase I, I/II or II (Fig.1.2b), as a result of joined investment and efforts among industry, scientists and clinicians. What started as a concept to correct monogenetic defects causing primary immunodeficiencies evolved to a therapy with a wide variety of

applications (Fig.1.2c), from cancer to chronic or progressive diseases such as, heart failure, neurodegenerative, vision loss, inflammatory or metabolic disorders, including Parkinson and diabetes [5–7].

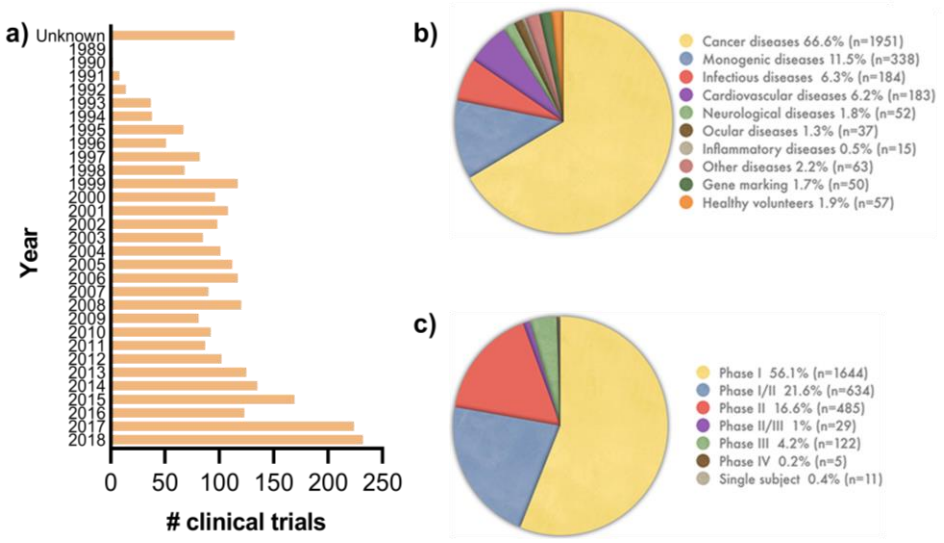

Figure 1.2 – Approved gene therapy clinical trials. a) Number per year; b) Phases; c) Indications. Adapted from [The Journal of Gene Medicine – Gene Therapy Clinical Trials Worldwide, list of websites – 1].

Cancer is the most targeted disease, representing more than 2/3 of all indications (Fig.1.2c), owing it to the advances of immunotherapy approaches. In this field, the therapies with higher demonstrated potential make use of patient modified T cells expressing T cell or chimeric antigen receptors (CAR), specialized in targeting tumor-associated cell-surface antigens to fight malignant cells. Usually, cell modification is performed *ex vivo* using integrative transfer vectors. Non-solid tumors, as lymphoid and myeloid leukemia, showed the best treatment efficacy with hundreds of patients treated in multiple trials presenting unprecedented remission rates. Other strategies tackling cancer, use tumor suppressor genes, oncolytic virotherapy, and gene directed enzyme pro-drug therapy [7].

The second most represented gene therapy indication in clinical trials are monogenic diseases (11.5%). The treatment is generally based on the delivery of the missing functional gene to the patient cells [7]. Within this gene therapy indication category, adenosine deaminase severe combined immunodeficiency (ADA-SCID) was the first and most studied disease, with over 40 treated patients and 70% of disease-free survival rate [8].

1.1.2 Transfer vectors

The delivery of the therapeutic genetic material into the patient cells can be divided into two main categories: non-viral physical-chemical and recombinant viral transfer vectors (Fig.1.3). By far, recombinant viral transfer vectors, derived from a panoply of viruses, are the most employed in clinical trials representing at least 63.9% of the total vectors and constituting four of the top five gene vehicles used: adenoviral vectors (18.5%), gammaretroviral vectors (17.5%), naked/plasmid DNA (15.4%), lentiviral vectors (9.5%), and adeno-associated viral vectors (8.1%) [7].

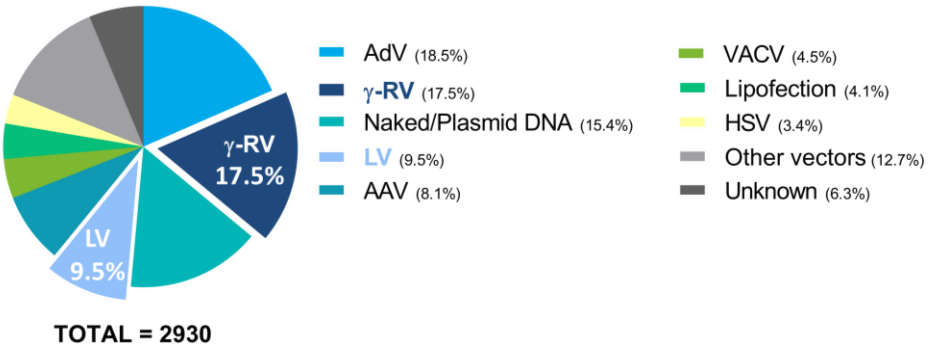

Figure 1.3 – Total gene therapy clinical trials per vector. AAV – adeno-associated viral vector; AdV – adenoviral vector; HSV – herpes simplex viral vector; LV – lentiviral vector; VACV – vaccinia viral vector; γ-RV – gammaretroviral vector. Adapted from [The Journal of Gene Medicine – Gene Therapy Clinical Trials Worldwide, list of websites – 1].

In Figure 1.3, two recombinant viral transfer vectors are highlighted, gammaretroviral and lentiviral vectors (γ-RV and LV). Derived from viruses belonging to the *Retroviridae* family, they both represent important hallmarks of gene therapy clinical trials (Fig.1.4). γ-RV were historically one of the first vectors used in the early clinical trials in gene therapy, maintaining its incidence generally in the top three, and therefore one of the transfer vectors where higher clinical expertise exists. LV gained the attention of the community since the beginning of its clinical trials usage (2001), generally increasing its presence throughout the years, and attained the higher vector occurrence ever in 2018, with 57 approved trials [7, The Journal of Gene Medicine – Gene Therapy Clinical Trials Worldwide, list of websites - 1].

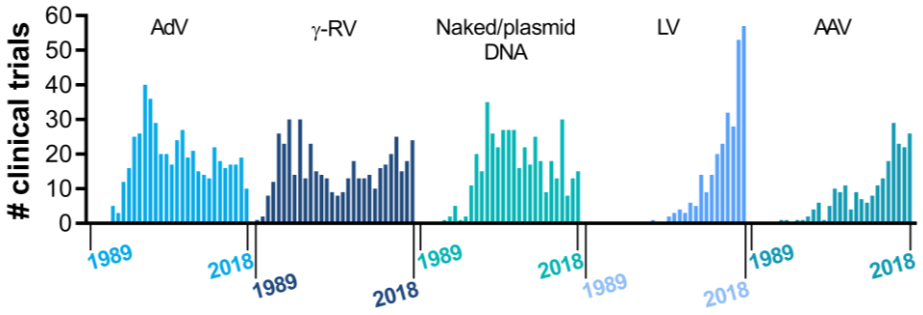

Figure 1.4 – Total gene therapy clinical trials of the top five transfer vectors per year, from 1989 to 2018. AAV – adeno-associated viral vector; AdV – adenoviral vector; LV – lentiviral vector; γ-RV – gammaretroviral vector. Adapted from [The Journal of Gene Medicine – Gene Therapy Clinical Trials Worldwide, list of websites – 1].

Relevant selection criteria are used to choose the most suited transfer vector for a given gene therapy application. Each transfer vector is characterized by specific properties, namely: 1) genetic load capacity, i.e. the size of the therapeutic gene the vector is able to pack and deliver; 2) transgene expression as episomal or integrative; 3) cell tropism, i.e.

transfection of specific or broad cell types; 4) pre-existing immunity leading to antibody-mediated vector inactivation (possible extending to transduced cells exposing vector proteins) affecting the therapy efficacy; and 5) transfection efficiency.

Table 1.1 resumes the top five transfer vectors profiles of utilization in gene therapy clinical trials. Adenoviral vectors (AdV) do not integrate the therapeutic transgene in host cell genome and thus its expression is transient and particularly vulnerable to cell silencing mechanisms. As such, currently they are mostly selected for oncolytic therapies aiming to kill the target cells right after vector transduction or to deliver genes to non-dividing cell tissue [9]. Recombinant retroviruses, γ-RV and LV, integrate their genome in transduced cells, enabling long-term expression of the therapeutic gene. With low immunogenicity, these vectors are preferably employed in gene addition applications, such as immunotherapies (cancer) and monogenic disease treatments [8].

Table 1.1 – Comparison of the top five gene therapy transfer vectors. Adapted from [3,10].

Vector	Genetic load	Integrative	Tropism	Immuno-genicity	Transfection efficiency
AdV	35 kb	No	Broad	High	High
γ-RV	8 kb	Yes only in dividing cells	Flexible by pseudotyping	Low	High *ex vivo* Low *in vivo*
Naked/ Plasmid DNA	No limit	No	Broad	Low	Low
LV	8 kb	Yes	Flexible by pseudotyping	Low	High *ex vivo* Low *in vivo*
AAV	4.5 kb	No	Flexible dependent on capsid serotype	Intermediate	High

AAV – adeno-associated viral vector; AdV – adenoviral vector; LV – lentiviral vector; γ-RV – gammaretroviral vector.

Naked/plasmid DNA is the most simple and popular non-viral system used in clinical trials, the only among the top five vector systems. Although characterized by significant lower transfection efficiency – which can be improved by injecting directly into certain tissues – safety concerns and the relatively small capacity for therapeutic genetic load of viral vectors have encouraged the development and use of these transfer vectors [11]. Adeno-associated viral vectors (AAV) have limited genetic payload capacity, restricting their use to therapies requiring the expression of short coding sequences, which was shown to be through episomal transgene expression and rarely through chromosomal integration [12].

1.1.3 Viral vectors-based gene therapy products and market

Back in 2014, only four viral gene therapy products were approved. Now, this number triplicated and gene therapy market is predicted to growth up to US$11 billion in the next 10 years (Table 1.2) [1]. Still, product approval and commercialization are at initial stages, many issues are under debate and lacking answers to efficiently cope with the high costs and to enable its broad availability to the society.

Each market authorization was a hallmark pioneering the field. The first ever market approval by a governmental agency for a gene therapy product was attributed to Gencidine® (2003) by the China Food and Drug Administration (CFDA). Gencidine® is an oncolytic AdV carrying the *p53* tumor suppressor gene to treat head and neck squamous cell carcinoma. The administration of this oncolytic drug enables *p53* expression in the tumor cells, leading to cell cycle arrest, DNA repair, apoptosis, senescence, and autophagy, ultimately resulting in tumor regression [13]. Oncorine® was the second gene therapy product reaching the market and was approved by the same regulatory agency, CFDA in 2005, for the treatment of nasopharyngeal carcinoma [13]. This product is an AdV

containing a deletion in its genome (E1B 55K region), which restricts its replication in p53-deficient cells (malignant cells) leading to cell host lysis. The first similar oncolytic viral therapy agent approved by the United States Food and Drug Administration (US FDA) and the Europe Medicine Agency (EMA) was IMLYGIC® (2015). In this case, the drug is a recombinant herpes simplex virus (HSV) carrying the immunostimulatory granulocyte-macrophage colony stimulating factor gene for the treatment of melanoma [14].

Rexin-G, a γ-RV expression vector coding a dominant-negative mutant construct of human cyclin gene, was granted market approval in 2007 by the Bureau of Food and Drugs the Philippines (BFAD) for the treatment of cancer. This drug is particularly suited for solid tumors since the vector used selectively targets and accumulates in metastatic lesions upon intravenous infusion [15].

In 2012, EMA approved its first gene therapy product, Glybera®, an AAV able to drive the lipoprotein lipase gene expression in the muscle for the treatment of lipoprotein lipase deficiency. This product was priced 1.1 million euros per treatment, failing to encourage health care agencies to subsidize patients suffering from this rare disease and culminating in its market withdraw in 2016 [16,17]. Other products followed EMA approval, Strimvelis® and Zamoxis® (2016), both using γ-RV targeting different indications, correction of ADA-SCID and prevention of host *vs* graft cell transplant complications using a suicide gene, respectively [18]. Kymriah® and Yescarta®, first approved by the US FDA (2017) and afterwards by EMA (2018), are CD19 CAR T cell immunotherapies targeting B cell related leukemia or lymphoma using LV and γ-RV, respectively [19,20,European Medicines Agency, list of websites - 2].

Table 1.2 – Approved viral vectors-based gene therapy products.

Product	Viral Vector	Target Disease	Regulatory Agency (Year)	Company	Price
Gendicine®	AdV (*in vivo*)	Head/neck squamous cell carcinoma	CFDA (2003)	SiBiono GeneTech	US$3 200
Oncorine®	AdV (*in vivo*)	Nasopharyngeal carcinoma	CFDA (2005)	SiBiono GeneTech	-
Rexin-G®	γ-RV (*in vivo*)	All solid tumors	BFAD (2007)	Epeius Biotechnologies	-
Glybera® ***(alipogene tiparvovec)***	AAV (*in vivo*)	Lipoprotein lipase deficiency	EMA (2012)	uniQure	1 100 000 €
Imlygic® ***(talimogene laherparepvec)***	HSV (*in vivo*)	Advanced melanoma	FDA EMA (2015)	Amgen	US$ 65 000
Strimvelis®	γ-RV (*ex vivo*)	ADA-SCID	EMA (2016)	GlaxoSmithKline	594 000 €
Zalmoxis®	γ-RV (*ex vivo*)	Host vs graft disease in haematopoietic stem cell transplantation	EMA (2016)	MolMed SpA	130 000 €
Kymriah® ***(tisagenlecleucel)***	LV (*ex vivo*)	B-cell precursor acute lymphoblastic leukemia	FDA (2017) EMA (2018)	Novartis	US$ 475 000
Yescarta® ***(axicabtagene ciloleucel)***	γ-RV (*ex vivo*)	Relapsed or refractory large B-cell lymphoma	FDA (2017) EMA (2018)	Kite / Gilead	US$ 373 00
Luxturna® ***(voretigene neoparvovec-rzyl)***	AAV (*in vivo*)	Bi-allelic *RPE65* gene mutation-associated retinal dystrophy	FDA (2017)	Spark Therapeutics	US$ 850 000
Zolgensma® ***(onasemnogene abeparvovec-xioi)***	AAV (*in vivo*)	Bi-allelic *SMN1* gene mutation-associated spinal muscular atrophy	FDA (2019)	AveXis / Novartis	US$ 2 125 000
ZYNTEGLO®	LV (*ex vivo*)	Transfusion-Dependent β-Thalassemia	EMA (2019)	Bluebird bio	1 575 000 €

AAV – Adeno-associated viral vector; AdV – Adenoviral vector; BFAD – Bureau of Food and Drugs the Philippines; CFDA – China Food and Drug Administration; EMA – European Medicine Agency; FDA – United States Food and Drug Administration; LV – Lentiviral vector; RPE65 – Retinal Pigment Epithelium-Specific 65 KDa Protein; *SMN1* - Survival of Motor Neuron, telomeric copy gene; γ-RV – Gammaretroviral vector.

The latest FDA approvals were AAV based gene therapy products, branded as Luxturna® (2017) and Zolgensma® (2019), delivering the human retinal pigment epithelium-specific protein gene to patients with retinal dystrophy [21], or the *SMN1* gene to bi-allelic mutation-associated spinal muscular atrophy pediatric patients [US Food and Drud Administration, list of websites – 3], respectively.

The newest market authorization is ZYNTEGLO® (EMA, 2019), a LV-based gene therapy product to correct the genetic cause of transfusion-dependent β-thalassemia patients. This therapy works by delivering autologous $CD34^+$ cells encoding $\beta^{A\text{-}T87Q}$-globin gene to patients, making them transfusion-independent [22,European Medicines Agency, list of websites - 2].

These approvals are just the beginning of the gene therapy market. In the near future more products are expected to be approved as result industry research intensification in potentially lucrative disease indications [7]. The high costs of the treatments are still a concern to ensure therapy affordability and applicability, being the last two approved products the most expensive ever reaching the market. Nevertheless, most of the indications are characterized by unmet medical needs – orphan diseases, non-treatable, non-curable and/or dependent on life-long conventional therapies – constituting a major burden in health care systems. Gene therapy offers, in certain cases, a single treatment with high success rates of cure, counter-balancing the costs associated with chronic treatments and patient's incapacity and dependency [18].

1.1.4 Retrovirus based viral vectors in gene therapy

The majority of the market approved viral based gene therapy products (Table 1.2) use recombinant retroviruses to permanently modify target cells *ex vivo*. The main feature granting these vectors success is the stable integration of its genome into target cells, which is perpetuated

upon cells division, and allows long-term expression of the therapeutic gene in a single treatment. As such, these vectors are highly sought for monogenic disease treatments and modified T-cell immunotherapies.

The transfer vectors used in the earliest gene therapy clinical trials (1989 - 1993) were γ-RV derived from murine leukemia virus (MLV). They were first employed in the transformation of tumor-infiltrating lymphocytes *ex vivo* with a neomycin resistant gene, enabling to understand the role of these cells in cancer by following its distribution and accumulation sites in patients. Later, γ-RV were used in the first-steps of immune therapies, by modifying T cells with tumor necrosis factor or interleukin-2 genes to treat patients suffering from advanced melanoma [23]. Subsequently, vectors carrying adenosine deaminase gene were used to add gene function to white blood cells of two ADA-SCID patients [24].

The use of γ-RV declined upon development of leukemia in four (out of nine) patients, causing the death of one person enrolled in a clinical trial aiming the treatment of X-linked SCID. This tragic outcome was later attributed to the transcriptional activation of LMO2 proto-oncogene in the transduced $CD34^+$ bone marrow cells by the viral enhancer/promoter sequences present in the long terminal repeat (LTR) of the transfer vector [25–27]. The genotoxicity event observed in the mentioned clinical trial was the first but not isolated, as other cases not restricted to γ-RV were also reported [28]. Moreover, patients transplanted with cell clones presenting insertion sites within and/or near potentially oncogenic loci have been followed over a considerable time frame and never developed related diseases [29].

Human immunodeficiency virus type 1 (HIV-1) derived LV are used in clinical trials since 2001 and, in contrast to γ-RV that only insert their genome in actively dividing cells, are able to transduce slowly-proliferating and non-proliferating cells by actively translocating their genome across

the nuclear membrane. Additionally, LV are considered safer than γ-RV due to different genome insertion pattern, preferably in transcriptionally active genes provoking transcript truncation, over transcriptional start sites leading to the activation of proto-oncogenes (for more details concerning integration patterns of Retroviruses consult Poletti & Mavilio (2018) [30]). Nowadays, LV are one of the most used vectors entering clinical trials, outperforming their γ-RV counterparts and presenting higher representability in later phases (Fig. 1.5).

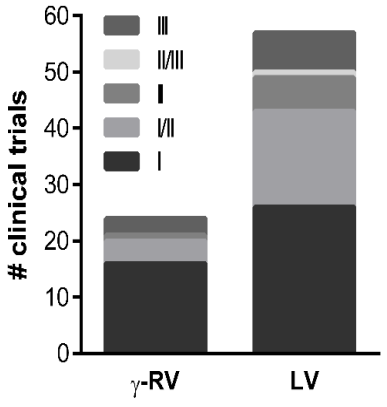

Figure 1.5 –Gene therapy clinical trials phases using recombinant gammaretroviruses (γ-RV) or lentiviruses (LV) in 2018.
Adapted from [The Journal of Gene Medicine – Gene Therapy Clinical Trials Worldwide, list of websites – 1].

Several factors influence the genotoxicity induced by transfer viral vectors, the major safety concern of using these integrative recombinant retroviruses. While some are vector related, as vector design and integrative vector biology, others are associated to the therapeutic transgene, target cell, epigenetic events, patient age or disease state (for more details of viral vectors genotoxicity consult David & Doherty (2017) [28]). The post-experience allowed however to learn several lessons, contributing to improved vector design and safety [31,32]. Although

potentially safer, LV possess far less clinical experience than γ-RV and long-term safety and efficacy assessment is needed.

1.2 Retrovirus

Retrovirus is the common name of all the *Retroviridae* family members, which by definition are characterized by the ability to reverse transcribe the two copies of positive single stranded RNA genome (7 to 12 Kb) into double stranded DNA, followed by its stable integration into the host cell genome [33].

1.2.1 Retrovirus particle and genome organization

The Retroviruses are enveloped, round shaped, measure around 100 nm and the dry weight composition is approximately of 60% proteins, 35% lipids derived from the host cellular membrane, 3% carbohydrates and 2% RNA [34]. The particles structure organization (Fig. 1.6a) from inside out has the RNA viral genome protected by nucleocapsid proteins and enclosed in the capsid together with enzymatic proteins (reverse transcriptase, integrase and protease). The matrix encompasses the capsid and is covered by a lipid membrane which harbors virus glycoproteins responsible for specific host receptor recognition [33].

The *Retroviridae* family is divided in seven genera, differing on the shape of the internal protein core (Fig. 1.6a) and the viral genome type (Fig. 1.6b), which can be classified as simple or complex. Both genome types possess the following coding domains: 1) the *gag* region codifying for the matrix, capsid and nucleocapsid structural proteins; 2) the *pro* gene to express the protease enzymatic protein; 3) the *pol* sequence coding the reverse transcriptase and integrase enzymatic proteins; and 4) the *env* gene encoding the surface and transmembrane viral glycoproteins subunits, which upon expression will be linked by disulfide bonds. The

complex viral genome differs from the simple by having extra coding regions enclosed in multiple spliced mRNAs for accessory proteins which play crucial roles in the regulation of gene expression and virus replication [33].

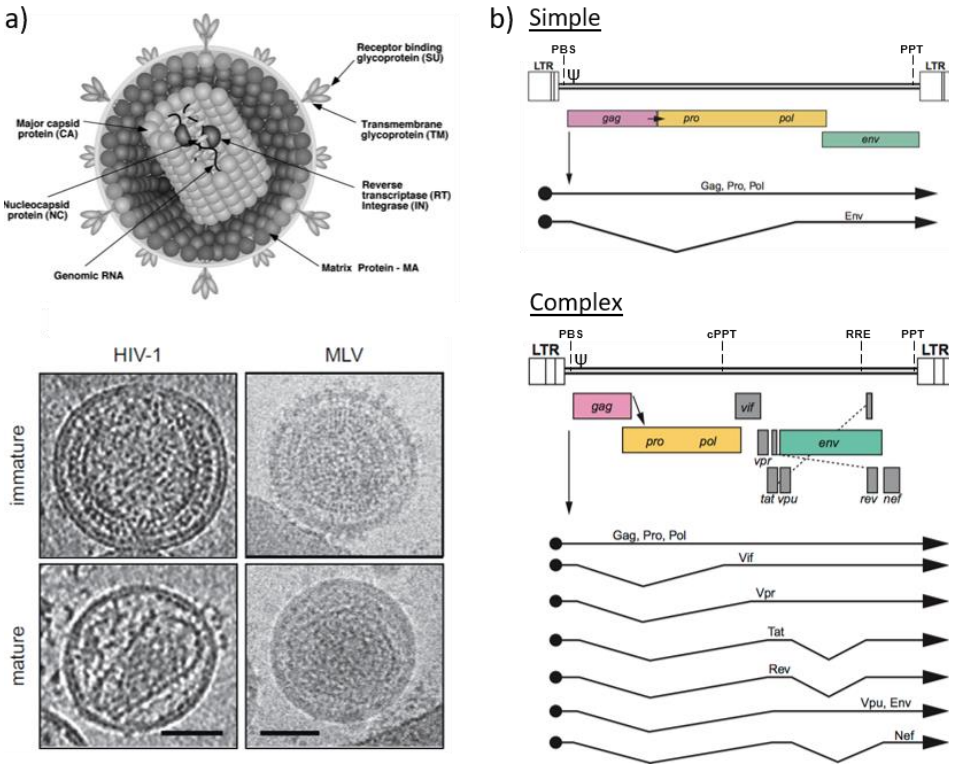

Figure 1.6 – Simplified schematic representation of a retrovirus particle and genome. a) Viral particle structure and Human immunodeficiency virus type 1 (HIV-1; *Lentivirus*) and murine leukemia virus (MLV; *Gammaretrovirus*) internal protein core shape differences. b) Examples of a simple (MLV; *Gammaretrovirus*) and complex (HIV-1; *Lentivirus*) genomes (provirus) organization and derived transcripts. cPPT – central polypurine track; LTRs – long terminal repeat; PBS – primer binding site; PPT – polypurine track; RRE – Rev regulatory element; ψ – packaging signal. Arrow between the *gag-pro* reading frames indicate the read-through (MLV) or ribosomal frameshift (HIV-1) site. Colored rectangles represent the gene protein products. Solid line arrows with protein names represent the transcripts. Adapted from [35, International Committee on Taxonomy of Viruses, list of websites – 4].

In addition to the described coding sequences, retrovirus genome has many *cis*-acting sequences, namely: two LTR sequences composed of U3, R, and U5 elements, each flanking the coding region and displaying promoter/enhancer activity; the primer binding site (PBS), a sequence complementary to a portion of the cellular tRNA used by reverse transcriptase to initiate reverse transcription; the packaging signal (ψ), involved in the viral genome packaging; and the polypurine tract (PPT), a RNA sequence resistant to RNase H degradations enabling it to function as the initiating site for the positive strand DNA synthesis during reverse transcription [33].

The open reading frames and splicing events are fine regulated processes, differing among virus species, to safeguard the proper ratios of each viral component to form a functional particle. For instance, there is a long open reading frame encoding for *gag*, *pro* and *pol*, however, viral particles contain 10 to 20-fold more GAG than GAG-PRO-POL proteins. In the case of MLV, this GAG and GAG-PRO-POL imbalance is caused by a stop codon at the end of *gag* which is misinterpreted by the translation machinery resulting in a read-through. Moreover, only part of this full transcript is spliced to form the *env* gene products [36].

1.2.2 Retrovirus life cycle

Retrovirus replication cycle (Fig. 1.7) begins with the specific binding of viral surface glycoproteins to host cell receptors. This is a critical step as it determines the viral host range, from the animal species to the target cells within the host. Next, virus and host cell membrane fuse, releasing the viral capsid into the cytoplasm of the target cell. At this cellular compartment, reverse transcription of the virus RNA genome into double stranded DNA (provirus) occurs in the reverse transcription complex comprising the dimeric RNA genome, capsid, nucleoproteins, reverse

transcriptase, and integrase. The reverse transcriptase enzyme is responsible not only for the DNA synthesis, through its DNA polymerase functions, but also for the degradation of the RNA viral genome, as it possesses RNAse H activity [33]. Still in the cytoplasm, the provirus is protected from degradation by associating to viral and cellular proteins in a large pre-integration complex. This complex comprises many of the reverse transcription complex elements, being the key component the viral integrase. Among retroviruses genera, different pre-integration complex compositions are formed comprising distinct accessory cellular and viral proteins which are involved in the provirus nuclear import strategy [37].

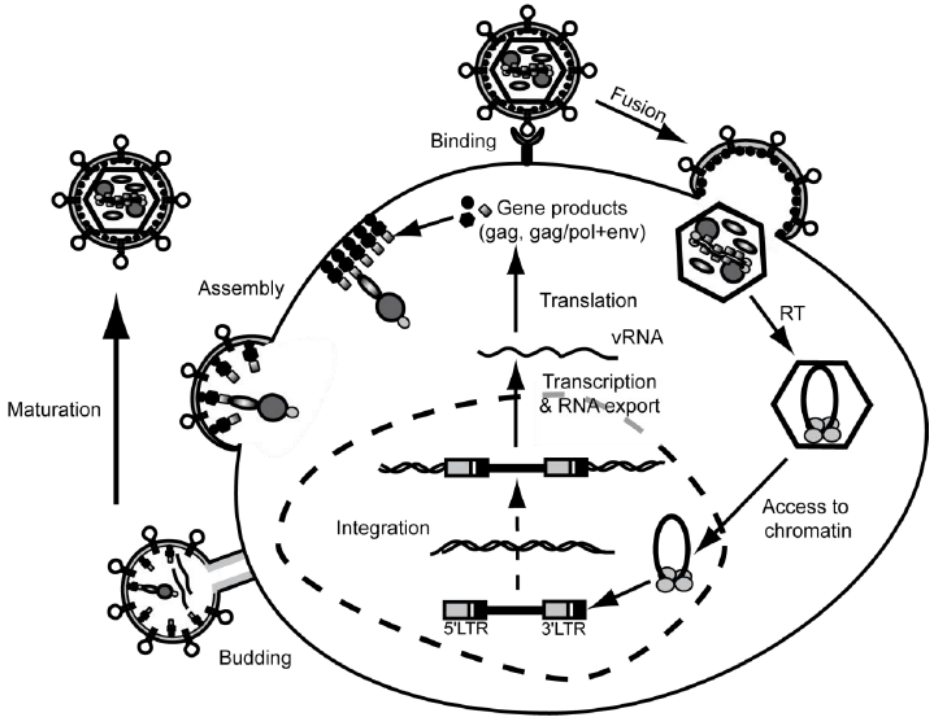

Figure 1.7 – Simplified representation of the retrovirus life cycle. Adapted from [38].
RT – reverse transcriptase. vRNA – viral RNA.

Capsid proteins are present in both reverse transcription and pre-integration complexes, and its resistance to degradation varies among virus species, also depending on each provirus nuclear entry mechanism. Some viruses (e.g. MLV – *Gammaretrovirus* genus) depend on the nuclear membrane collapse, during cell mitosis to access the nucleus, requiring a capsid with high resistance to cellular factors. Others possess nuclear import elements from viral and cellular origin (e.g. HIV-1 – *Lentivirus* genus) assisting the provirus transfer to the nucleus, thus independent of cell cycle phase, and requiring a less stable capsid [36].

Once in the nucleus, the viral integrase mediates the insertion of the linear provirus into the host chromosomal DNA. The sites of integration can be randomly attributed but some viral species show preferences to specific gene/regulatory elements sequences or chromatin state [28,30,39].

Stable insertion into host cell genome allows retrovirus genome propagation during cell division. Provirus genome expression is mediated by cellular RNA polymerase II, generating full length and spliced RNA transcripts. The full-length RNA transcript encodes the precursor proteins of *gag*, *pro* and *pol* gene products and is also amenable of being packed inside the newly assembled viral particles at the host cell membrane. The *gag*, and *gag-pro-pol* gene products are polyproteins that will be assembled in the cellular membrane. The spliced RNA transcript encodes the *env* gene product, a Env precursor protein which is cleaved by furin within the golgi apparatus while being trafficked to the cell surface, generating a bipartite transmembrane protein [33]. At the host cell membrane, the virion molecules co-localize in lipid rafts regions, assemble and bud from the plasma membrane. Later in the virus cycle, a step of maturation occurs which consists on the cleavage of Gag and Gag-Pro-Pol polyproteins and envelope glycoprotein by the viral protease to form functional monomers conferring particle infectivity [33].

1.2.3 From viruses to vectors: recombinant retroviral vectors

The development of recombinant retroviral based particles devoid of virulence and with gene delivery and protein display properties was initiated in the early 80's. The vector development consisted in the physical separation of the virus genome into different expression cassettes. The generated particles can then be classified into replication-competent or replication-defective (ability to propagate after cell transduction or not) and into genome containing or empty [33]. Regarding the later, genome free particles have shown excellent immunogenic properties, well suited for vaccine candidates. The genome containing particles are gene therapy vectors and their application depend greatly on the treatment mechanism. One fine example is the application of replicative-competent particles to treat cancer. In this case, γ-RV carrying a suicide gene, which are only able to transduce actively dividing cells, are used to infect and spread in the tumor site, since the neighborhood healthy cells are non-dividing [40]. This thesis focused on genome containing replication defective γ-RV and LV particles, the type most used in gene therapy, providing diverse gene therapy applications to through different mechanisms (e.g. gene addition).

Gammaretroviral vectors

γ-RV are so called as they derive from virus of the *Gammaretrovirus* genus, mainly from MLV. As previously mentioned, MLV was the first virus-based gene therapy vector developed and the most widely used retroviral vector in gene therapy. Historically, two distinct transfer plasmids designs (Fig. 1.8a), responsible for the expression of the therapeutic gene, and three generations of packaging function plasmids were created (Fig. 1.8b).

The transfer vector was developed by deleting all structural and enzymatic protein genes (*gag*, *pro*, *pol* and *env*) from the virus genome and replacing them with the therapeutic gene. The expression of the therapeutic gene can be driven by the virus 5' LTR or by an internal promoter in the case of self-inactivating (SIN) vectors [41,42]. The SIN vectors were developed to minimize the genotoxic effects of the LTR driven transfer plasmid, which upon integration can activate the transcription of oncogenes surrounding the integration site, which may lead to cancer development in the treated patients. As such, SIN vectors have a deletion on the promoter/enhancer region of the 3' LTR, which upon reverse transcription in the target cell is present in both 3' and 5' LTRs, preventing its activity in neighborhood genes [43]. To further reduce the genotoxicity, *cis*-acting elements such as enhancer blocking chromatin insulators can be added in the transfer vector, inhibiting the activation of cellular genes [28].

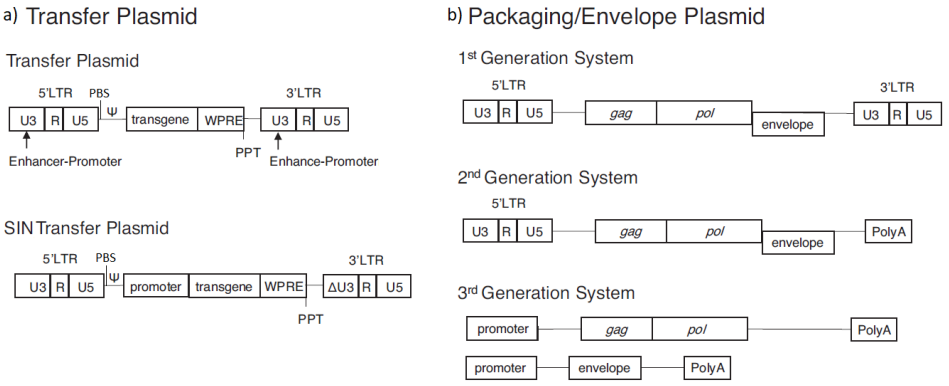

Figure 1.8 – Schematic representation of different γ-RV designs and generations. a) Transfer plasmid design. Transgene expression derived from LTR or from an internal promoter of a self-inactivating (SIN) vector. b) Split genome system, generation development. LTR – long terminal repeat; PBS – primer binding site; PPT – Polypurine tract; WPRE – woodchuck hepatitis virus pos-transcriptional regulatory element; ψ – packaging signal. Adapted from [42].

The packaging functions are given in *trans* in another expression cassette encoding the *gag*, *pro*, *pol* and *env* gene sequences but without the

packaging signal. Thus, the resulting vector can enter and integrate into the target cell genome but lacks replication capacity. In the first generation the virus genome was maintained almost intact, only missing the packaging signal. This design had high probability of one homologous recombination event between the packaging and the transfer plasmids leading to the generation of replicative-competent particles. In the second generation, the 3'LTR was replaced by the Simian vacuolating virus 40 (SV40) polyadenylation site. Therefore, two recombination events were required to generate replicative-competent particles. In the third (and current) generation, the *gag-pro-pol* and *env* coding regions were separated into two expression cassettes under the regulation of heterologous promoters. This last generation not only reduces considerably the recombination probability leading to replicative competent particles, but also enables to pseudotype the particles by choosing a given viral envelope [41].

Other strategies to improve safety of the third packaging generation system include the mutation of the MLV integrase gene in the *gag-pro-pol* expression cassette. A fine example of these strategy is the BET-independent vectors, where the integrase was mutated to lose its natural affinity to bromodomain/extraterminal domain (BET) proteins. These cellular proteins are present in the MLV pre-integration complex and own high affinity to cellular transcription factors, thereby defining the MLV integration profile in active regulatory elements [44,45]. Another recent strategy involved the insertion of DNA-binding zinc-finger domains into an internal position of the MLV integrase, perturbing its structure and reducing the inherent and strong MLV integration preference for genomic regions near transcriptional start sites [46].

Lentiviral vectors

LV can be derived from several species from the *Lentivirus* genus, being the most well studied and used in gene therapy the HIV-1 derived vectors. As its γ-RV counterparts, the viral components are separated into different expression cassettes granting transfer, envelope, and packaging functions to minimize the generation of replicative-competent particles (Fig. 1.9).

The transfer plasmid (Fig. 1.9a) contains the transgene and all the essential *cis*-acting elements needed for reverse transcription, integration and encapsidation ability, without any HIV-1 proteins. Thus 5'LTR promoter activity is minimal after integration, since the Tat protein is provided in a separate plasmid. To overcome this, transfer plasmid uses an internal promoter to express the transgenes in transduced cells. The full-length transcript can be expressed from a wild type LTR or from a chimeric 5'LTR promoter (SIN and LTR1 constructs). SIN and LTR1 transfer vectors present higher safety genotoxic profiles due to the deleted U3 region of the 3'LTR [31] – inactivating the LTR promoter/enhancer activity in the provirus – and lower HIV-1 homology, owing it to the chimeric 5'LTR [32]. Both chimeric 5'LTRs have the U3 promoter/enhancer region replaced by an heterologous one, making the long transcriptional unit independent of Tat protein. Furthermore, LTR1 transfer vector replaced essential signals (primer binding site, ψ and Rev responsive element) downstream of 5' LTR with a functional primer binding site and relocated the original signal to downstream of 3'LTR, which increases biosafety due to absence of the packaging signal in the provirus genome [47]. This innovative design was particularly important since integrative SIN LV were described as able to express full-length genomic transcripts, competent of encapsidation/mobilization, conferred by the remaining U3 sequences present in the SIN vector [48]. As with any other retrovirus

based vector, chromatin insulators can be added to the transfer vector to prevent undesired cellular gene activation [28].

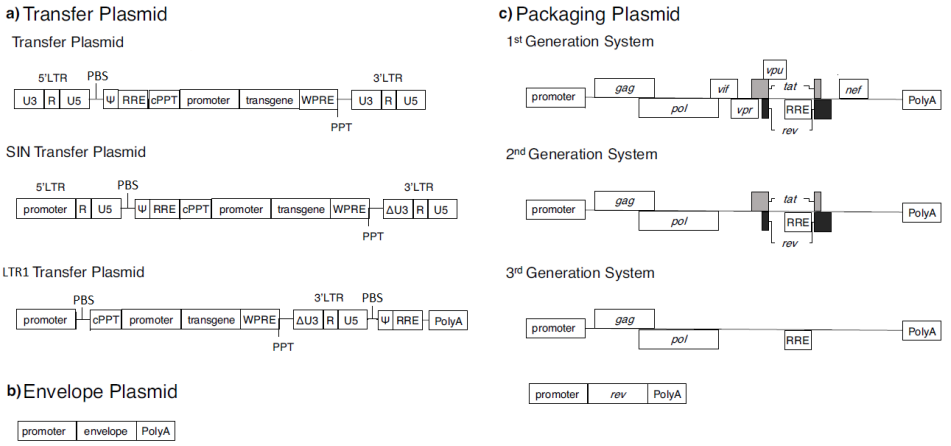

Figure 1.9 – Schematic representation of different LV design and generations. a) Transfer plasmid design. Transgene expression derived from an LTR, SIN, and LTR1 vector design. b) Envelope plasmid design. c) Split genome system, generation development. cPPT – central purine tract; LTR – long terminal repeat; PBS – primer binding site; PPT – Polypurine tract; RRE – Rev responsive element; WPRE – Woodchuck hepatitis virus post-transcriptional regulatory element; ψ – packaging signal. Adapted from [42].

The helper functions are split in different expression cassettes: one plasmid coding the envelope glycoprotein (Fig. 1.9b) rendering pseudotype customization, and packaging plasmids which can be categorized in three generation systems (Fig. 1.9c) [42,49]. The first-generation system had the HIV-1 *gag-pro-pol* and accessory genes – almost a complete HIV-1 genome only lacking the LTR, encapsidation signal and *env* gene – in one expression cassette under the control of a heterologous promoter. The second generation is characterized by the removal of the accessory genes, *vpr*, *vif*, *vpu*, and *nef*, not needed for vector production, reducing HIV-1 homology and replicative competent

particles occurrence upon potential recombination events. Still *tat* and *rev* genes were maintained. For these two generation systems, the production was performed using three plasmids (transfer, envelope and packaging) and *tat* gene was required due to the transcription dependency of the non-SIN transfer vector available at the time. Currently, third generation packaging system is the mostly used and was developed by deleting *tat* and *rev* from the *gag-pro-pol* expression cassette and using a separate plasmid to provide *rev* gene. In this system, *tat* was no longer necessary due to the adoption of SIN transfer vectors and production required four plasmids (reviewed in Sakuma et al. (2012) [49]). Still, Rev - a RRE RNA sequence recognizing protein - is needed for the efficient nuclear exportation of unspliced RNAs, such as those of the packaging construct and transfer vector [50]. Attempts of developing a Rev independent fourth generation system severely decreased vector titer. The most convincing strategy, still with a 5-fold yield reduction, was accomplished by eliminating packaging and transfer plasmids sequence homology by means of codon-optimization. This strategy inactivated the HIV-1 *gag-pol* *cis*-repressive inhibitory sequences present in those plasmids, making them completely independent of Rev [51].

Pseudotyping

The split system allows to express the envelope glycoprotein in an independent plasmid, providing flexibility to insert any compatible viral glycoprotein at the particle surface, thus enabling to customize it for a desired target cell.

Several envelope glycoproteins from foreign virus may be used to pseudotype γ-RV and LV. Proven to be suitable for gene therapy applications are the ones from amphotropic MLV (4070A), the gibbon ape leukemia virus (GaLV), the feline endogenous retrovirus (RD114) and the

vesicular stomatitis virus glycoprotein G (VSV-G) [52,53]. Each of these envelope glycoproteins has specific cell receptors recognition leading to different cell tropisms. Briefly, the interest in amphotropic MLV, GaLV and RD114 is in its human hematopoietic stem cells high transduction efficiency [54–56]. Meanwhile, the wide distribution of VSV-G cell receptor allows to successfully transduce several cell types. Due to the pantropic feature of VSV-G, its clinical application is preferable for *ex vivo* rather than *in vivo* to avoid undesirable transduction of non-target cells [57].

Using heterologous glycoproteins may pose several challenges, namely: i) inefficient envelope fusogenicity activation during vector maturation step, compromising the production of functional particles [58,59], and ii) each envelope have different resistance to the bioprocess steps. Although VSV-G expression is toxic to the producer cells, it is probably the most used envelope since it does not require protease cleavage to reach the fusogenic state, it is resistant to freeze-thaw cycles, and to concentration by ultracentrifugation [52,60].

1.3 Retrovirus-based viral vectors production systems

The demand for robust retroviral vectors bioprocesses is growing to meet clinical trials and market needs. In the last years, sponsored research in the academic sector to develop GMP grade vector stocks increased. Unmet challenges include the assurance of sufficient manufacturing capacity and rigorous product characterization regarding purity, potency, and safety [61].

Retroviral vectors are produced in mammalian cells either in transient platforms – where the transfer, packaging and envelope plasmids are delivered to the cells by means of transfection and expressed momentarily – or in stable systems – where all the vector constructs are integrated in the producer cell genome and stably expressed. Transient production has

the advantage of being more flexible regarding pseudotype and therapeutic transgene, avoiding the cumbersome and time-consuming process of stable producer cell development. The establishment of stable producer cells involves a round of transfection, selection and clone screening steps per each viral component expression cassette. Due to the random chromosomal integration profile, sufficient vector components expression providing high titer are rare, making the process tedious [62]. However, stable production is preferred over transient since it allows extended production periods, easier process scalability for commercial manufacture, reproducible yields, and the product is free of contaminating plasmid and transfection reagents facilitating the downstream vector purification. In all cases, the certification of either the producer cell line and/or the transfection reagents is mandatory in clinical manufacturing processes [61].

1.3.1 Gammaretroviral vectors manufacture

Historically, γ-RV were first produced using packaging cell lines, i.e., cells stably expressing the helper function construct(s) where the transfer vector would be provided either stably or transiently. The first packaging cell lines were of murine origin (NIH/3T3 cell line) [63,64]. However, these cells hold endogenous retroviral sequences posing safety concerns if mobilized to the transfer vector after potential recombination events [65]. Additionally the produced vectors carried a murine glycosylation pattern recognized by the human complement, rendering them highly immunogenic [66]. Thus, human derived cell lines were developed, namely HT 1080 [67], RD [53], CEM [68], HEK 293 [69] and HEK 293T [70] derived. Currently, the industry preference lies on HEK 293 cells stably expressing and constitutively producing the γ-RV, which can be adapted to suspension and serum free culture conditions, facilitating scale up [62].

Transient or semi-transient (where some components are constitutively expressed and others are given in *trans*) productions are used only when the transfer vector carries a cytotoxic or cytostatic cDNA [70].

The split genome approach requires to fine tune the vector components expression stoichiometry in the producer cell to achieve the best product yield and quality. It was described the need for high transfer vector expression and balanced expression between *gag-pro-pol* and *env* cassettes [71–74].

To speed cell line development, producer cell lines with recombinase mediated cassette exchange technology associated to a vector component expression cassette were established, enabling to tag and exchange a highly expressing, open chromatin locus with the desired construct. Either only the transgene could be replaced by any therapeutic gene of interest [75,76], or the transgene and the envelope cassette can be exchanged [77]. These stable producer cells are denominated modular cell lines and present several advantages. They are considered safer because the vector components are integrated in defined producer cell chromosomal loci. Furthermore, they allow to adapt a well characterized and high titer producer cell to the desired vector therapeutic properties without the need to devote time and resources to screen and optimize the culture conditions of the best candidate clone, hence decreasing the production timelines and costs [62].

1.3.2 Lentiviral vectors manufacture

In contrast to γ-RV, LV are mainly produced transiently, as stable production is hampered by the intolerance of packaging cell lines to cope with some toxicity of vector components expression (e.g., VSV-G envelope and HIV-1 protease) [36].

Several cell lines have been used to produce LV, such as HEK 293 derived (e.g. HEK 293T and HEK 293SF), COS-1, COS-7, CV-1, HeLa, HT 1080 and RD. Among all, HEK 293T cells are the most used [78]. This cell line expresses SV40 T antigen and presents higher cell growth and transfection efficiency than its HEK 293 parental cell line. In LV transient production context, the transfer and helper plasmids contain the SV40 origin of replication, enabling its episomal propagation after transfection in HEK 293T dividing cells [79]. Several transfection methods have successfully been optimized to cope with multiple plasmids delivery into producer cells. In large scale settings, DNA precipitation using calcium phosphate and linear 25 kDa polyethylenimine are the most used reagents. In general, the four plasmid system has reduced titer over the three plasmid system because it is less efficiently delivered into the cells [80].

It is highly desirable to implement alternative stable systems to avoid the high costs associated to plasmid production and process variability using transient systems. To overcome constitutive expression toxicity, conditional stable systems were developed with inducible promoters controlling the toxic components expression. For clinical trials applications only the conditional stable production systems using the third LV generation system are used [81–89]. In the latter, production is controlled by adding an inductor or removing suppressor from the culture medium, and similar LV titers to transient production system are achieved. However, those are still not the ideal systems due to medium supplementation dependency and short production periods [81]. As such, constitutive stable systems were developed by stably expressing *gag-pro-pol* and *rev* cassettes and substituting the VSV-G envelope by engineered nontoxic viral glycoproteins [90–94]. However, these systems are characterized by lower p24 Gag production comparing with inducible stable producers [80].

1.3.3 Upstream process

Retroviral vectors production has been mostly based on adherent systems, such as roller bottles, hyperflasks, cell factories, fixed bed bioreactors or wave and stirred tank bioreactors supplemented with cell adhesion substrates (Table 1.3). Suspension serum-free cultures facilitate the upstream bioprocess scale-up. Although producer cells were developed/adapted to suspension cultures, changes in cellular morphology and membrane properties occurring during this process may result in viral productivity loss [78,95,96]. So far, clinical manufacture preference has been towards cell factories and fixed-bed bioreactors systems [17,80]. The later enables higher cell densities per culture volume, and reduces shear stress than for instance microcarrier cultures, reducing vector inactivation [97]. Perfusion mode benefits transducing titer by avoiding viral vector decay at 37 ºC (production temperature), which ranges from 10 to 4 hours. Finally, the supernatant harvesting should be performed at 4 ºC to reduce the vector decay rate [98,99].

Independently of the system, tight control of process parameters is mandatory and showed to be cell line and viral vector dependent. Decreasing culture temperature from 37 ºC to 32 ºC improved vector production yields and quality. Moreover, optimum pH level should be in the range of 6.8 to 7.2 and dissolved oxygen levels between 20% to 80% to not negatively affect titers [98,100].

Table 1.3 – Examples of retrovirus-based vector production systems. Adapted from [61].

	System	Cells	Method	Ref.
Gammaretroviral vector	Stirred Tank reactor (microcarriers and clump cultures) Cell Cube Celligen	TE Fly GALV	Adherent Stable constitutive	[97]
	Spinner Flask	CEMFLYA	Supension Stable contitutive	[68]
	Spinner Flask (microcarriers)	PA317-RCM1	Adherent Stable constitutive	[101]
	CellCube RollerCell 40	Phoenix Frape-1	Adherent Stable constitutive	[102]
	Roller Bottles	PG13 and GPE-Am12 derived	Adherent Stable constitutive	[103]
	Cell Factories	PG13 derived	Adherent Stable constitutive	[104]
	Shake Flask	HEK 293GP-A2/GFP	Supension Stable contitutive	[105]
	Wave Bioreactor (Fibra-Cel)	HEK 293T	Adherent Transient (CaPhos)	[106]
	iCELLIs	PG13 and HEK 293Vec derived	Adherent Stable constitutive	[107]
Lentiviral vector	Chemap CF-2000 bioreactor	HEK 293E	Suspension Transient (PEI)	[79]
	Spinner Flask & Stirred Bioreactor	HEK 293SF-3F6	Suspension Transient (PEI)	[108]
	HYPERFlask	HEK 293T	Adherent Transient (CaPhos)	[109]
	Cell Factories	HEK 293 and HEK 293T	Adherent Transient (CaPhos)	[110]
	Wave Bioreactor (Fibra-Cel)	HEK 293T GPRG-EF1α-hγ$_c$OPT	Adherent Stable inducible	[84]
	iCELLIs fixed-bed bioreactor	HEK 293T	Adherent Transient (CaPhos/PEI)	[111]
	BioBLU® packed-bed bioreactor (Fibra-Cell)	HEK 293T	Adherent Transient (PEI)	[112]

1.4 Producer cells metabolism: impact on productivity

Production processes involve the coordination of several parameters to ensure best manufacture scenario. Producer cells are the main players and to maximize their performance it is critical to ensure the best conditions are provided. Since mammalian cells are affected by nutrient and by-products environment, understanding the metabolic features influencing productivity has been one important topic of study. Among many, Rodrigues et al. (2013) conducted a study on γ-RV producer cells where the transcriptome differences of the transition 'parental-to-producers' and the 'high vs. low producer' from different genetic backgrounds were analyzed [113]. Metabolic pathways were the category showing more significant changes when transitioning to a producer state. The recruited pathways exhibiting changes in high producers were lipid metabolism, energy metabolism, glutathione metabolism, amino acid catabolism, nucleotide metabolism, polyamines biosynthesis and pentose phosphate pathway [113]. It is important to stress that metabolic pathways are redundant, thus difficult to attribute meaningful outcomes to just a single pathway.

1.4.1 Serum, lipids, and cholesterol

Serum is the most common additive of cell culture media; however, it poses safety concerns. In one hand, its use makes product approval by the regulatory agencies more bureaucratic and increases manufacturing and purification costs. On the other hand, serum removal may result in low transducing titers and reduced vector stability. The metabolic hinge between serum and titers was identified has being the lipids and cholesterol content [95,96]. This association is highly correlated with retroviral vector biology. As an enveloped virus, retroviral vectors recruit

host cellular membrane for its composition during budding which occur in lipid rafts, cholesterol and sphingolipids-enriched cell membrane microdomains [114]. Therefore, during production, cells experience a depletion in their lipid and cholesterol content. If not efficiently renewed, the vector quality and stability may be affected as it is highly dependent on viral envelope lipid and cholesterol composition [115,116].

The lipids of the producer cells are mainly acquired directly from the culture media. However, lipids can also be synthesized using the glucose and glutamine present in the culture medium [117]. In serum-free conditions, while some γ-RV producer cells can rescue their lipid and cholesterol synthesis others do not, making the adaptation process unpredictable [95]. Hence, the addition of cholesterol and lipids cocktail to serum-free cell culture medium is a common strategy to improve the production of these viral vectors [96,118]. To circumvent cells dependency on a given substrate, in addition to media supplementation, metabolic engineering offers a long-lasting and preferable strategy. An explored approach to make vector production independent of serum and cholesterol was the expression *de novo* cholesterol synthesis in HEK 293T cells by overexpression of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase isoform 1. This was performed in transient productions by adding the plasmid encoding the referred enzyme to the transfection mix. This strategy not only enabled 2 and 3-fold improvements in total and transducing LV titers, respectively, but also supported high yields in serum-free conditions [119].

1.4.2 Energy metabolism

Commercially supplied media have glucose as the traditional sugar source, which, along with glutamine, represents the major energy and carbon sources for cells in culture. However, immortalized cell lines, such

as the ones used for retroviral vector production, exhibit high glycolytic flux. As a consequence, glucose is rapidly consumed and inefficiently metabolized, with the majority being converted to lactate which in turn can inhibit cell growth [120].

The use of alternative sugar sources, such as fructose (alone or in combination with glucose), had a beneficial effect on γ-RV yield and stability, which was also associated with reduced sugar oxidative metabolism, reduced lactate secretion, and increased lipid synthesis [121].

Metabolism improvements were also performed through genetic engineering. For example, by down-regulating the expression of hypoxia inducible factor 1 (responsible for the activation of glycolytic enzymes and glycolysis-related genes) alone, or in combination with pyruvate dehydrogenase kinase. This metabolic engineering strategy resulted in 20 and 30-fold increase in specific productivity of transducing particles and up to 4-fold reduction in lactate production, accomplishing the pyruvate channeling into the tricarboxylic acid cycle [120].

1.4.3 Oxidative stress

Oxidative stress may be a consequence producer cells face when forced to high protein synthesis rates [122,123]. Supplementation with antioxidants and reduced glutathione could increase the production of γ-RV by 2.1-fold [113]. Over-expressing glutathione synthase and cystathionine β-synthase enzymes, involved in glutathione metabolism, could further improve the yields by up to 15-fold [73]. Overall, these results suggest glutathione metabolism as a potential metabolic engineering target to increase retroviral vector yields.

1.4.4 Polyamines and nucleic acids metabolism

Polyamines have a complex metabolism and several compensatory mechanisms to maintain polyamine homoeostasis. Thus, many reports consider polyamines as critical to cell survival. These molecules can bind to nucleic acids (participating in their stabilization and modulating gene transcription events), play a role in membrane rigidity and present anti-oxidant properties [124]. Addition of these molecules to producer cell culture media rendered 1.8-fold increment of γ-RV titer [113].

Regarding nucleic acids, these are one of the principal viral components and producer cells metabolism must ensure cellular availability as vector vector building blocks. However, cellular machinery might become insufficient, as supplementation with nucleosides increased the production of γ-RV by 1.9-fold [113].

1.4.5 Protein processing

Producer cells need to cope with high protein synthesis, correct protein folding, and post-translational modifications to ensure retroviral vector yield and quality.

Supplementing producer cell culture media with amino-acid cocktails, the protein building-blocks and the most abundant vector component, also has shown to increase the γ-RV titer by 1.3-fold [113]. Furthermore, protein processing and secretion pathways have been targeted by metabolic engineering. For example, engineering the glycosylation pattern of murine derived producer cells improved in 3.5-fold γ-RV particles resistance to human complement [125]. Also, overexpression of Munc18b (vesicle trafficking protein) in HEK 293 cells increased in 2-fold the production of LV [126]. These results demonstrate the importance of engineering producer cell pathways to improve vector properties.

1.4.6 Apoptosis

The production of retroviral vectors may activate cellular apoptotic pathways directly or indirectly. Cell viability can be directly compromised by the toxicity associated to the transfection reagents [127] and/or by the expression of toxic vector components such as the HIV-1 protease [128], VSV-G envelope [36], and cDNAs from the transfer vector [70]. Indirectly, the manufacture of these viral vectors may cause cell death when oxidative and protein synthesis burden activates the unfolded protein response [122,123]. Anti-apoptotic strategies were commonly studied using genetic engineering. Recently, HEK 293T apoptosis-resistant cells were developed by knocking out Caspase3, Caspase6, Caspase7, and AIF1 genes. This strategy improved the yields of LV carrying apoptosis-inducing genes. [129]. This achievement is of extreme relevance to enable superior producer platforms, facilitate retroviral vector production and broaden its gene therapy applications.

1.5 Scope of the thesis

This PhD project aims to further elucidate the parameters influencing producer cells functional retroviral vector yields. To provide novel insights and strategies enabling higher retroviral vector transducing titers, different approaches were followed and divided in three parts in this thesis manuscript: i) analysis of the impact of vector genetic cassettes design, expression, and copy number in constitutive retroviral vector manufacture yields and stability; ii) genetic engineering of cell metabolic pathways and, iii) evaluation of different pseudotypes and transduction enhancers in vector transduction efficiency (Fig. 1.10).

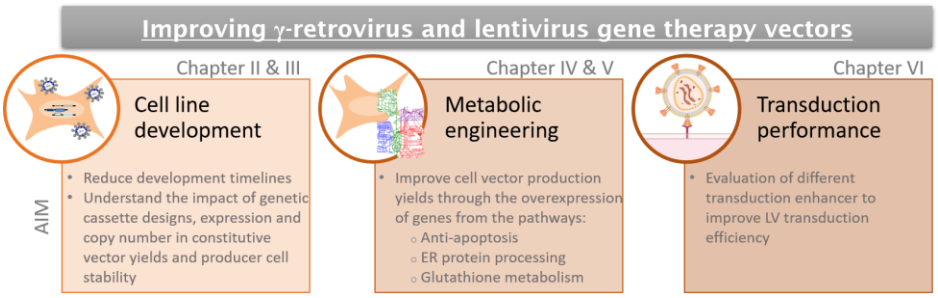

Figure 1.10 – Schematic representation of the different strategies and major aims of this thesis on improving retroviral vectors for gene therapy. 4070A – Amphotropic murine leukemia virus. GaLV - Gibbon ape leukemia virus. LV – Lentiviral vectors. RD114 – Feline endogeneous retrovirus. RMCE – Recombinase mediated cassette exchange. VSV-G – Vesicular stomatitis virus. γ-RV – Gammaretroviral vectors.

The development of stable constitutive producer cell lines requires the optimization of viral vector expression cassettes design and transfection methods and involves intensive and time-consuming screening of producer clones. Hence, robust and predictable methodologies are needed to enable faster bench to bedside. Towards faster cell line development strategies, in the first part of the work systematic

comparisons were performed to elucidate how cassette designs, transfection methods, and clone isolation procedures can contribute for the successful establishment of retroviral vector producer cell lines (**Chapter II**). Regarding constitutive lentiviral vector producer cells, a detailed study on the characterization of clone productivity, vector genetic cassettes copy number and expression level is presented. Cells production stability over long sub-culturing periods in the absence of antibiotic selective pressure was also monitored (**Chapter III**).

Metabolic engineering of producer cell platforms holds great potential to increase vector yields and was explored in the second part. Manipulation of three cell biologic pathways recruited in vector production – protein processing, anti-apoptosis and glutathione metabolism – were explored by gene overexpression (**Chapters IV** and **V)**. Engineered cells presented different production phenotypes depending on the gene overexpression level, the targeted pathway, the retroviral vector type, and the producer cell substrate.

The transduction capacity of LV is dependent on heterologous envelope glycoproteins used for pseudotyping. In the third part, several transduction enhancers were evaluated for their capacity to improve LV cell transduction efficiency (**Chapter VI**).

In the last **Chapter** (**VII**). the thesis major outcomes are summarized and discussed highlighting its contribution to the field and current manufacturing challenges.

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CHAPTER II

Accelerating constitutive producer cell lines development

This chapter was adapted from

Formas-Oliveira AS, Almeida AI, Tomás HA, Alves PM, Coroadinha AS. Accelerating cell line development for constitutive production of gene therapy gammaretroviral and lentiviral vectors: case study of expression cassette design, transfection procedures, and clone selection (*in preparation*)

Author contribution

Ana Oliveira designed and participated in the experimental setup, analyzed the data and wrote the chapter

Abstract

Recombinant retroviruses are among the most used gene delivery vehicles in gene therapy clinical trials and virus-based market approved products. To supply these types of ground-breaking therapies, stable and constitutive manufacture systems are preferable. This work aimed to study the impact of the design of the viral vector expression cassettes, transfection procedure and screening methodology on the titer yields retrieved in stable gammaretroviral and lentiviral vectors (γ-RV and LV) producer cells. A high throughput clone screening method was used to pick high yielding γ-RV and LV producer clones.

Optimizing the promoter elements on γ-RV *gag-pro-pol* expression cassette enabled to substitute the long terminal repeat (LTR) by CMV heterologous promoter thus, reducing sequence homologies with the γ-RV genome transgene cassette. Different LTR sequences tested in the γ-RV genome transgene cassette provided similar transducing titers but different expression levels of the gene of interest (GOI). The optimized designs were used to establish γ-RV producer populations. Transfer vector integration in producer cells genome using electroporation and γ-RV transduction were compared. Although viral transduction generated, in average, higher titer producer clones, when electroporation was coupled with a high throughput method for clone selection, as the single step cloning and screening (SSCS), clones producing up to 10^7 T.U./mL could be isolated. Electroporation presents the benefit of enabling complete removal of the viral vector genome cassette from the producer cell genome by site-specific recombination (i.e. recombinase mediated cassette exchange – RMCE). RMCE allowed to generate a producer cell for therapeutic purposes from a previous optimized producer clone. We demonstrated this concept by expressing an antigen-coupled T-cell receptor.

In the context of LV producer cell line development, we have optimized the vector genome transgene cassette to include a GOI enabling the use of the SSCS high throughput screening methodology. For LV vectors the size of the transgene cassette severely impacted viral transducing titers while the internal promoter affected GOI expression. The final cassette was thus optimized to not surpass 7 Kb and contain a strong retrovirus derived internal promoter. Finally, stable LV producer clones were successfully isolated using SSCS method enabling to reduce clone isolation step to less than 3 weeks.

In summary, the designs and optimizations herein described enabled to accelerate γ-RV and LV cell line development strategies for constitutive viral vector production bioprocesses.

Chapter II

2.1 Introduction

Gene therapy clinical trials started in 1990's and decades of research were necessary for the first gene therapy product achieve market approval (Glybera, 2012) in Europe [1]. Currently, approximately half of the virus-based products with commercial authorization are based on recombinant retroviruses, either derived from *Gammaretrovirus* or *Lentivirus*. Retrovirus-based gene therapy vectors success can be attributed to its most unique characteristic: stable integration of the therapeutic gene into the host genome, enabling long term expression and thus potentially offering a single treatment for a life-long cure. Gammaretroviral vectors (γ-RV) were the first type of virus-based vectors used in gene therapy clinical trials. Nevertheless, in the last years the use of these vectors have declined, while lentiviral vectors (LV) use increased due to its potentially safer genomic integration profile [2]. Combined, both viral vectors represent approximately 25% of the total gene delivery systems used in clinical trials for predominantly cancer and monogenic diseases. In the case of cancer, adoptive T-cell immunotherapy is one of the current breakthrough therapies. The later uses modified patient's T cells, expressing specific T cell receptors or chimeric antigen receptors to destroy cancer cells. The most used strategy to modify T-cells is by retroviral vectors transduction [3].

Production of retroviral vectors can be performed either using transient transfection systems or stable producer cell lines. Stable producer cell lines are preferable over transient manufacture, enabling to reduce batch-to-batch variability and costs. However, the development of these producer cell lines requires laborious and time consuming steps and can be hampered by virus toxic components, compromising bench to bedside transition [4,5]. To develop these stable producer cell lines the integration of three to four expression cassettes is required coding for all

viral vector components: 1) vector genome carrying the therapeutic gene; 2) envelope glycoproteins for vector pseudotyping; 3) vector enzymatic and structural proteins, which for LV is encoded in one or two cassettes. Generally, each cassette is sequentially introduced into the desired cell substrate followed by antibiotic selection and completed by exhaustive screening to find a high expressing clone. Several strategies have been employed to overcome this extensive work. For instance, recombinase mediated cassette exchange (RMCE) technology associated with vector genome and/or envelope glycoproteins genomic cassettes enabled to use the same packaging cell line for the production of different therapeutic particles with predictable productivities [6–8]. Alternatively or in combination, high throughput methods can be employed to isolate high producing clones reducing the time for cell line development, for instance by avoiding viral vectors stoichiometry optimization [4,9].

Research in γ-RV stable manufacture has disclosed crucial parameters for safer high-titer producer cell line development, such as expression cassettes design, stoichiometry of the viral components, and cell substrate [10–17]. On the other hand, LV stable cell line development is more recent and challenging and thus with larger potential to be explored and advanced. Due to the cytotoxicity of HIV-1 protease, only a few cell lines constitutively expressing all LV components were reported [5,18–20]. In all, intensive screening was performed to find a high producer clone. Moreover, only LentiPro26 circumvented protease toxicity by using a mutated and less active version, while avoiding also viral transduction to stably integrate the viral vector expression cassettes [5].

The work here described aimed at disclosing parameters influencing cell line development process for the stable manufacture of recombinant retroviruses to enable faster and successful establishment of robust producer cells. To this end, we studied: 1) the impact of expression

cassettes design in γ-RV production in order to develop new stable producers with less sequence homology among cassettes; and 2) the influence of the transfection method in γ-RV productivity.

Previously we described a novel tool, single-step cloning-screening method (SSCS), for developing and study stable high-titer γ-RV producer cells [4]. Herein we make the proof of concept to therapeutic γ-RV CAR-T transgenes and developed a similar protocol to generate LV vector producer cell clones.

2.2 Materials and methods

Plasmids

Table S2.1 (Supplementary data) summarizes all primers, templates and backbones used in cloning procedures. All reactions were conducted using In-Fusion HD Cloning system (Takara, Mountain View, CA, USA).

pCeB contains moloney murine leukemia virus (MoMLV) *gag-pro-pol* and blasticidin resistance (*bsr*) genes, expressed through the MoMLV 5' long terminal repeat (LTR) as described by Cosset et al. (1995) [21].

pMLV-GP is a plasmid driving the expression of MoMLV *gag-pro-pol* through a composite promoter – human Elongation Factor-1α (hEF1α) promoter and 5' untranslated region of the Human T-Cell Leukemia Virus (HTLV) – derived from pSELECT-blasti-mcs (Invivogen, San Diego, CA, USA).

pSV40-GP, pSV40i-GP, pRSV-GP, pRSVi-GP, phEF1α-GP, phEF1αi-GP, pCMV-GP and pCMVi-GP are MoMLV *gag-pro-pol* and blasticidin resistance expression plasmids, each harboring a different sequence for transcription initiation promoter: SV40 - Simian Vacuolating Virus 40, RSV – Rous Sarcoma Virus, hEF1α or CMV – Cytomegalovirus. For each promoter the introduction of rabbit β globin intron (SV40i, RSVi, CMVi or hEF1αi) was also developed.

pTAR LacZS11 is a pEm MFG derived plasmid for MoMLV based γ-RV transgene genome with a MoMLV LTR driving the expression of the viral vector genome and codifying for a LacZ-S11 fusion protein as GOI, described in Rodrigues et al. (2015) [4].

pUC19 Fw_LTR$_{MoMLV}$ - F5_dNEO is a tagging construct for MoMLV based γ-RV genome expression and its sequence was synthesized by GenScript (Piscataway, NJ, USA).

pLTR$_{MPSV}$ ACTR-S11, pLTR$_{MPSV}$ LacZ-S11, pLTR$_{MoMLV}$ ACTR-S11, and pLTR$_{MoMLV}$ LacZ-S11 are tagging plasmids and γ-RV vector genomes with transgenes coding GFP S11 fusion proteins (ACTR-S11 or LacZ-S11), and contain an EMCV-IRES (encephalomyocaerditis virus-internal ribosome entry site) element driving the expression of hygromycin resistant (*hph*) gene. The above LTR sequences are either derived from myeloproliferative sarcoma virus (MPSV) or MoMLV LTRs. pLTR$_{MPSV}$ ACTR-S11 sequence was synthesized by GenScript. All tagging plasmids contain two FRT sites, a wild type (FRT-WT) and a spacer mutant (FRT-F5) followed by an ATG defective neomycin phosphotransferase gene (*neo*).

pTAR ACTR_IRES is a RMCE targeting plasmid containing one FRT-WT site and a γ-RV genome with MPSV LTR driving the ACTR gene expression followed by an EMCV-IRES element next to an ATG and FRT-F5. FRT sites and ATG sequence complement the *neo* gene in tagging plasmids after targeting and enable positive entrapment selection after cassette exchange. This plasmid sequence was synthesized by GenScript.

pGaLV expresses the gibbon ape leukemia virus (GaLV) envelope protein and a zeocin resistance (*Sh ble*) gene under the control of a CMV promoter.

pSVFLPe codes flipase recombinase enzyme that it is expressed through an SV40 promoter and was used for RMCE procedure.

pRRLSIN Puro LTR_{MoMLV} S11.Cherry is a self-inactivating LV genome transgene – derived from pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene plasmid 12252) kindly provided by Prof. Didier Trono from the Swiss Federal Institute of Technology (EPFL) through Addgene plasmid repository (Cambridge, MA, USA) – coding for puromycin resistant (*pac*) gene and S11-mCherry fusion protein, which are expressed from RSV and MoMLV LTR promoters, respectively.

pRRLSIN GFP S10_zeo is a self-inactivating (SIN) LV transgene vector driving the expression of GFP S1-10 and *Sh ble* under the control of a CMV promoter, developed from pRRLsin S10 described in Rodrigues et al. (2015) [4].

pMDLg/pRRE (Addgene plasmid 12251) and pRSV-REV (Addgene plasmid 12253) are third generation LV packaging constructs containing LV *gag-pro-pol* and the second and third exons of LV *rev* coding sequences, respectively. pMD2.G (Addgene plasmid 12259) expresses the envelope G glycoprotein of the vesicular stomatitis virus under the control of the CMV promoter. The three plasmids were kindly provided by Prof. Didier Trono through Addgene repository.

Cell lines and cell culture

HEK 293, a human embryonic kidney derived cell line (ATCC CRL-1573), was used to develop γ-RV stable producer cell lines.

HEK 293T, a HEK 293 derived cell line expressing large T antigen from SV40 (ATCC CRL-11268), was used to transiently produce γ-RV and LV.

LentiPro26 is a LV packaging HEK 293T derived cell line expressing 4070A envelope, Rev and HIV-1 Gag-Pro-Pol, described in Tomás et al. (2018) [5]. This cell line was used to develop LV stable producer cell lines.

RD S10, a human rhabdomyosarcoma derived cell line (ATCC CCL-136) stably expressing GFP S1-10 fragment, described in Rodrigues et al. (2015) [4]. These cells were used as target cell to titer transducing γ-RV harboring GFP S11 fragment.

HT1080, a human fibrosarcoma derived cell line (ECACC 85111505), was used to develop HT1080 S10 cell line and as target cell to quantify the transducing γ-RV harboring only ACTR.

293 FLEX S11 is a HEK 293 derived cell line producing MoMLV based γ-RV expressing GaLV envelope and harboring a LacZ reporter gene, developed from 293 FLEX by RMCE [4,22]. 293 FLEX GP, clone 22, is the precursor cell line of 293 FLEX before envelope expression [4,22]. These cells were used as single-copy reference cells to estimate transgene cassette genomic copy number in the novel γ-RV producer cells and as target cells to titrate LV harboring GFP S10.

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) with 25 mM of glucose, 4 mM of glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) at 37 ºC inside an incubator with a humidified atmosphere containing 8% CO_2. For SSCS method, DMEM containing 20% (v/v) FBS and (1X) B27 supplement (Gibco) was used. Cell concentration and viability were assessed by trypan blue exclusion method.

Establishment of stable producer cell lines

For the establishment of γ-RV stable packaging cell lines, *gag-pro-pol* and GaLV expression cassettes were chemically transfected separately and sequentially in HEK 293 cells. Calcium phosphate precipitation method (CAPHOS; Sigma, St Louis, MO, USA) and polyethylenimine (jetPRIME; Polyplus, Graffenstaden, France) transfection reagents were used with 5 µg of pGaLV and 4 µg of pCMVi-GP, respectively. To establish γ-RV vector producer cells the transgene cassette – $pLTR_{MPSV}$

ACTR-S11 or pLTR$_{MoMLV}$ LacZ-S11 plasmids – was delivered to cells by electroporation – 0.5 µg DNA/10^6cells, 120 mV, 20 msec and 2 pulses (Neon Transfection System; Invitrogen, Carlsbad, CA, USA) – or by γ-RV transduction at a multiplicity of infection (MOI) of 0.2. These conditions aimed at single copy integration.

LentiPro26 cell line was used to insert the transgene vector genome cassette – pRRLsin Puro LTR$_{MoMLV}$ S11.Cherry – by chemical transfection using linear 25 KDa polyethylenimine (PEI; Polyscience, Hirschberg an der Bergstrasse, Germany), 5 µg of total DNA/10^6cells and a mass ratio of 1:1.5 (DNA:PEI).

For selection, cells were subcultured under antibiotic selective pressure 48 hours post-transfection: 100 µg/mL zeocin (Invivogen, San Diego, CA, USA) for pGaLV, 10 µg/mL blasticidin (Invivogen) for pCMVi-GP, 200 µg/mL hygromycin (Invivogen) for γ-RV transgene cassette and 1.5 µg/mL puromycin (Invivogen) for LV vector genome transgene cassette.

De novo stable producer clones were isolated either by limiting dilution when producing γ-RV ACTR-S11, or by SSCS method, when producing γ-RV LacZ-S11 or LV S11-mCherry.

Establishment of HT1080 S10 target cell line

LV harboring pRRLSIN GFP S10_zeo transgene cassette were used to transduce HT1080 cells at a MOI of 5. A population stably expressing GFP S1-10 was selected using 100 µg/mL zeocin and cloned by limiting dilution. Target cell clones were selected by GFP transcomplementation assays as reported in Rodrigues et al. (2015) [4].

Single-step cloning-screening method procedure

SSCS method was performed as previously described in Rodrigues et al. (2015) [4], with the following modifications: 1) for γ-RV stable producers screening, HT1080 S10 target clone was used and seeded

(175 cells/well) in co-culture nine days after producer cells limiting dilution, fluorometer readings were performed ten days after; 2) for LV stable producers screening, limiting dilution was performed with half of the antibiotic concentration used in the selection step in the cell culture medium, RD S10 target cells were seeded (2.6×10^4 cells/well) in co-culture fourteen days after limiting dilution of producer cells with simultaneous full culture medium replacement supplemented with 8 µg/mL of polybrene (Sigma), 24 hours after the plates were centrifuged at 25 °C for 2 hours at 1200 g (spinoculation)[23] and fluorometer readings were performed two days after.

Relative transgene genomic copy number estimation

To determine the transgene cassette genomic integration copy number in *de novo* generated γ-RV producer cells, genomic DNA was extracted using DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA, USA) according to manufacturer instructions.

To estimate the number of copies *per* cell, the genomic DNA was quantified by qPCR using primers for the *hph* and for *LacZ* gene (Table S2.1, Supplementary data) on a thermocycler LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) and using LightCycler 480 SYBR Green I Master (Roche Applied Science) PCR kit. The number of copies *per* cell was quantified relative to single-copy controls (293 FLEX GP, clone 22) and 293 FLEX S11 after normalization to a reference gene (*RPL22*) [4,22]. Single copy was considered for ratios of 'analysis *vs*. single-copy control' below 1.4 [24].

Recombinase Mediated Cassette Exchange

For site specific RMCE, tagged *de novo* γ-RV producer cells were seeded in 6 well plates and co-transfected using lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer

instructions. A total of 2.5 µg of DNA was used with the mass proportion of 1 targeting plasmid (pFlpIN_IRES) to 4 recombinase expressing plasmids (pSVFlpe). Post-transfection (48 hours), neomycin selection was started with 1000 µg/mL G418 (Invivogen). Medium was regularly exchanged during 21 days after which limiting dilution method was applied. The clones were amplified and analyzed for correct cassette exchange and γ-RV production. PCR with specific primers (Table S1.1, Supplementary data) was performed with extracted clone's genomic DNA. The amplification products were separated in agarose gel. The PCR product size allows to confirm correct cassette exchange.

Viral vector production

For transient retroviral vectors production HEK 293T cells were transfected using linear 25 KDa PEI (Polysciences) at a mass ratio of 1:1.5 (DNA:PEI), with the respective plasmids. To generate γ-RV, a total of 5 µg DNA/10^6 cells was used: 2.5 µg transgene, 0.8 µg envelope and 1.7 µg *gag-pro-pol*. For LV, the third generation LV packaging system was used with a total of 4.65 µg DNA/10^6 cells, composed of 2.5 µg transgene, 1 µg *gag-pro-pol*, 0.25 µg *rev* and 0.9 µg envelope [25].

For semi-stable LV production, LentiPro26 cells were transfected with 2.5 µg of transgene *plus* 2.15 µg of stuffer plasmid to ensure the similar transfection conditions among all LV productions. For all, medium was replaced 24 hours after transfection and supernatant was harvested after another 24 hours period.

For γ-RV and LV produced from stable producer cells, clones and cell populations vector yields were assessed using a 48 *plus* 24 hours production/harvesting procedure. Briefly, producer cells were seeded at 8 x 10^4 cells/cm^2 in 6 well plates (γ-RV) or 25 cm^2 flasks (LV). Medium was exchanged 48 hours after and culture volume was decreased by

half in 25 cm^2 flasks. The supernatant from the following 24 hours production period was harvest.

All supernatants containing viral vectors (from transient or stable productions) were clarified using a 0.45 µm cellulose acetate filter, aliquoted and stored at -85 °C until further use.

Transducing particles quantification

For transduction units (T.U.) viral titer determination, target cells were seeded at 5 x 10^4 cells/cm^2 (for γ-RV) or 1 x 10^5cell/cm^2 (for LV) in 24 well plates one day before transduction. RD S10 cells were used when γ-RV and LV transgenes coded GFP S11, HT1080 cells were used when γ-RV harbored ACTR and 293 Flex S11 cells were used when LV carried GFP S10. Cell concentration was determined at the time of transduction. Transduction was performed in duplicates by removing the cell supernatant and transducing with 0.2 mL of viral suspension diluted in fresh DMEM with 10% (v/v) FBS and 8 µg/ml of polybrene (Sigma). Only for LV produced in LentiPro26 derived cells, plates were centrifuged at 25 °C for 2 hours at 1200 g (spinoculation) and then 1 mL/well of cell culture medium was added [5,23]. For other titration assays, cells were incubated at 37 ºC overnight after which 1 mL of fresh supplemented DMEM was added. Two days after transduction, cells were harvested. For GFP expression, analysis proceeded immediately. For ACTR expression, target cells were stained with CD16 (clone B73.1) APC, or mouse IgG1 κ APC isotype control (BioLegend, San Diego, CA, USA). The percentage of GFP or ACTR positive cells was assessed using CyFlow Space flow cytometer (Sysmex Partec GmbH, Görlitz, Germany). The titer was determined considering only vector dilutions providing between 2 to 20% of transduced cells (GFP or ACTR positive cells), taking into account the cell concentration at transduction time, and the vector dilution factor [5].

2.3 Results

Optimization of γ-RV *gag-pro-pol* and transgene cassettes

Several key parameters should be optimized in the expression cassettes to cope with safety, RMCE, SSCS and attain high titers. We first optimized the *gag-pro-pol* expression cassette design. *Gammaretrovirus* expresses the *gag-pro-pol* genes through the promoter present at its long terminal repeat (LTR). For γ-RV generation many initial *gag-pro-pol* constructs used this strategy, being the LTR of MoMLV the most used [21]. However, the γ-RV genome cassette also requires the use of a γ-RV LTR. Although the later LTR might be substituted by a LTR from a different *Gammaretrovirus* species, strain or even a recombinant modified version, it might present some homologies to MoMLV LTR. Indeed, one of the most used γ-RV genomes to express the transgene cassette is the MFG vector containing a MoMLV promoter (LTR_{MoMLV}). This MFG vector was modified at the LTR 5' upstream sequences improving its expression and is one of the vectors tested in this work [26].
To avoid sequence homology between the *gag-pro-pol* and vector genome transgene cassettes, that increases the possibility of replicative competent retrovirus (RCR) formation, four heterologous promoters, with and without an intron, were tested to drive *gag-pro-pol* expression (Fig. 2.1a). In transient productions (Fig. 2.1b), all promoters supported higher γ-RV titers when complemented with an intron. CMV promoter alone or together with the intron enabled the highest transducing γ-RV yield and was selected to stably express Gag-Pro-Pol.

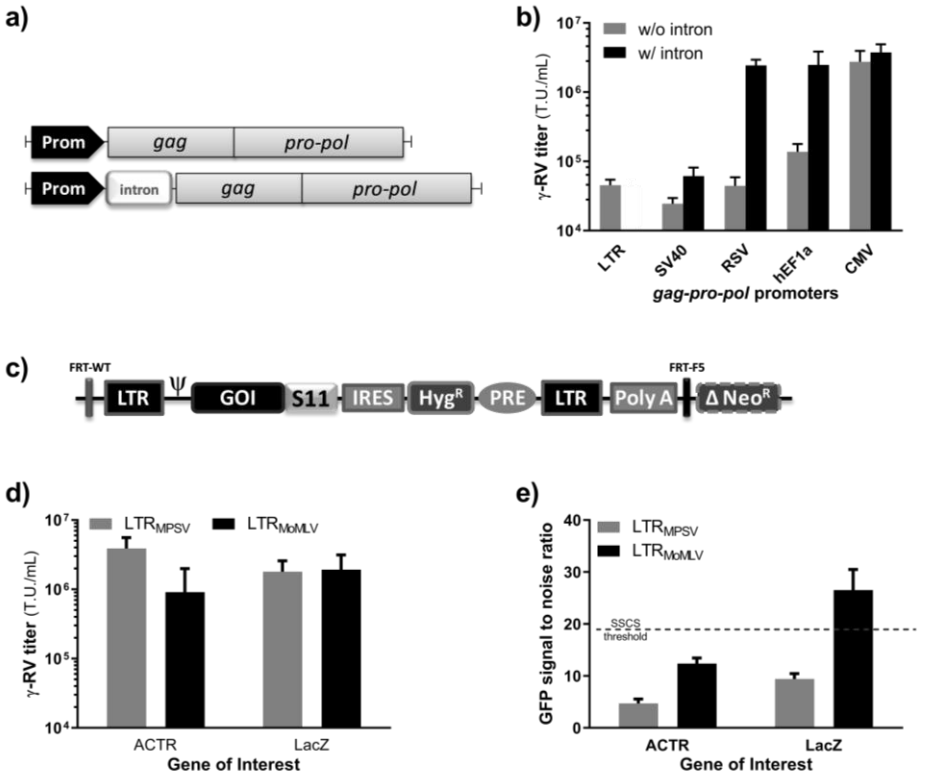


Figure 2.1 - Optimization of *gag-pro-pol* and transgene γ-RV expression cassettes. **a)** Schematic representation of *gag-pro-pol* expression cassettes design. **b)** Volumetric γ-RV transducing titer obtained for the different *gag-pro-pol* expression cassettes. γ-RV were pseudotyped with GaLV (Gibbon Ape Leukemia Virus) and carry a transgene driving the expression of ACTR.S11 through LTR_{MPSV}. **c)** Schematic representation of transgene expression cassettes. **d)** γ-RV volumetric transducing titer obtained for the different transgene cassettes. γ-RV were produced in transient conditions using pMLV-GP and pGaLV. **e)** GFP signal to noise ratio obtained for the different transgene cassettes. Signal to noise was defined as the ratio of mean fluorescence intensity of GFP-positive cells to non-transduced cells, analyzed by flow cytometry. Transduction was performed using the transiently produced vectors described in d). Dotted line marks the minimum GFP signal to noise ratio necessary to use SSCS. Data shown represents mean ± SD from 3 independent experiments. ACTR – Antibody-coupled T-cell receptor; CMV – Cytomegalovirus; FRT-F5 – F5 mutated flipase recombinase target site; FRT-WT - wild type flipase recombinase target site; GFP – Green fluorescent protein; GOI – Gene of interest; hEF1α – Human elongation factor-1α; Hyg^R – Hygromycin resistance gene; IRES – Internal Ribossonal Site; LacZ – β-galactosidase; LTR – Long terminal repeat; MoMLV – Moloney murine leukemia virus (modified); MPSV – Myelo-proliferative sarcoma virus; PolyA – Signal for adenine polynucleotide chain; Prom – promoter; PRE – posttranscriptional regulatory element; RSV – Rous sarcoma virus; S11 – Split-GFP S11 fragment; SV40 – Simian vacuolating virus 40; T.U. – Transducing units; ΔNeo^R – Neomycin resistance gene without ATG start codon; γ-RV – Gammaretroviral Vector; Ψ – Encapsidation signal.

For the γ-RV genome transgene cassette (Fig. 2.1c) we designed a cassette compatible with: 1) flexible expression of any desired transgene, by inserting two non-compatible flipase recombinase target (FRT) sites (*wt* and F5) surrounding the LTRs and a deleted start codon neomycin resistant gene (ΔNeo^R) (allowing positive entrapment selection by RMCE); 2) clone selection using SSCS method, requiring fusion of split-GFP S11 fragment to the GOI (GOI.S11) and; 3) expression of a therapeutic relevant GOI, the antibody-coupled T-cell receptor (ACTR), developed by UNUM therapeutics for cancer therapy.

The transcomplemented GFP signal to noise ratio for fluorometer detection in SSCS method requires a minimum value of 20. As the ACTR is a transmembrane protein, which could mask the GFP signal, a cytoplasmatic β-galactosidase (LacZ) protein was tested in parallel. To sustain enough transgene transcription levels to reach the required GFP signal to noise ratios, two LTR sequences – from Myelo-proliferative sarcoma virus (MPSV) and MoMLV (from MFG) – were evaluated. In transient γ-RV productions (Fig. 2.1d), all transgene cassettes provided similar yields, reaching the highest γ-RV titer with LTR_{MPSV} and ACTR combination. Analyzing the GFP signal to noise ratios (Fig. 2.1e), we observed that only when using a cytoplasmatic protein (LacZ) fused to split-GFP S11 fragment, and expressed through LTR_{MoMLV}, provided sufficient GFP signal to noise ratios to use SSCS method for clone screening.

The cassette with highest γ-RV transient productivity (LTR_{MPSV}, ACTR.S11) and the cassette supporting higher GFP signal to noise ratio (LTR_{MoMLV}, LacZ.S11) were selected to be used for stable expression.

Establishment of γ-RV stable producer cell lines with optimized expression cassettes

To stably express the three genetic cassettes coding for γ-RV components, HEK 293 cells were sequentially transfected with each cassette followed by selection with the respective antibiotic resistant marker (Fig. 2.2a). The transfection methods and reagents used were all compliant with current good manufacture practice (cGMP) in order to provide clinical grade producer cell lines. PEI chemical transfection was the method of choice with exception of the vector genome transgene cassette delivery, because single copy number integration was required. Therefore, we used electroporation which allows to reduce the amount of DNA delivered without compromising transfection efficiency. Two γ-RV stable populations were obtained, each expressing a different transgene cassette (ACTR.S11 or LacZ.S11) with similar γ-RV vector volumetric productivities (Fig. 2.2b).

SSCS method was then evaluated for its applicability to isolate clones from the two developed γ-RV producer cell population by seeding 300 clones of each. However, as previously predicted, ACTR.S11 did not provided sufficient GFP signal to noise ratio and producing clones from this population were not detected (data not shown). Therefore, 46 clones (15% of the seeded clones) expressing ACTR.S11 transgene were randomly isolated from limiting dilution plates. For the γ-RV producer population expressing LacZ.S11 transgene, the 22 clones (7% of the seeded clones) presenting the highest GFP relative fluorescent units in the SSCS protocol, were isolated. Comparing the efficiency of both isolation procedures (Fig. 2.2c), only 20% (9 out of 46) of randomly isolated clones from the limiting dilution plates were γ-RV vector producers, whereas using the SSCS method 100% of the isolated clones were producers. Independently of the transgene design and

clone isolation procedure, the stable expression of optimized cassettes enabled to develop producer cells yielding up to 1 x 10^7 transducing units per milliliter (T.U./mL) (Fig. 2.2d).

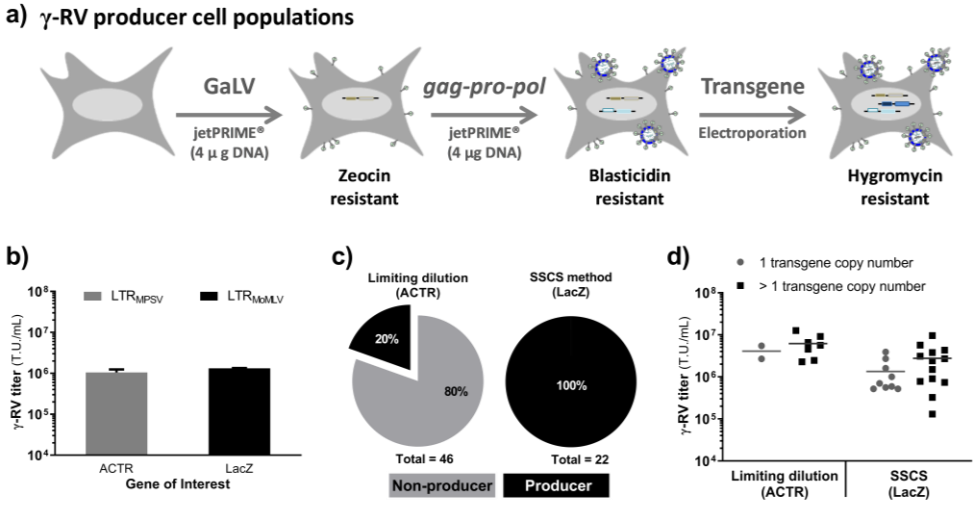


Figure 2.2 - Development of γ-RV stable producer cell lines with optimized expression cassettes. **a)** Cell line development procedure schematic representation. γ-RV genetic cassettes were sequentially transfected into HEK 293 cells. Cells were selected for the respective resistant marker generating a final γ-RV producer population. **b)** Stable γ-RV volumetric productivity of two *de novo* populations, each expressing a different optimized transgene (ACTR.S11 or LacZ.S11). **c)** Percentage of producing clones according to isolation procedure: limiting dilution for ACTR.S11 population and SSCS method for LacZ.S11 population. **d)** Clonal γ-RV volumetric productivity. Lines indicate average titer of isolated clones. Horizontal lines indicate average titer of isolated clones. Data shown represents mean ± SD from at least 2 technical replicates. ACTR – Antibody-coupled T-cell receptor; GaLV – Gibbon ape leukemia virus envelope; LacZ – β-galactosidase; LTR – Long terminal repeat; MoMLV – Moloney murine leukemia virus; MPSV – Myelo-proliferative sarcoma virus; S11 – Split-GFP S11 fragment; SSCS – Single-step clone-screening; T.U. – Transducing Units; γ-RV – Gammaretroviral Vector.

For RMCE procedure it is important to have only one copy of the vector genome transgene cassette integrated in producer cell genome. Single transgene copy number clones supported productivities up to 5 x 10^6 T.U./mL for both transgenes (Fig. 2.2d). On average γ-RV vector

volumetric transducing titer increased when clones contained more than one copy of the vector genome transgene cassette integrated.

Impact of stable integration transfection method in γ-RV clonal productivity

Retroviral transduction of the vector genome transgene cassette is the most common transfection procedure to generate γ-RV vector producer cells from packaging cells. In addition, it allows to control copy number integrations ensuring its incorporation in high expression genome locus [4,8,22,27]. To compare retroviral transduction with electroporation – in terms of resulting γ-RV vector titers obtained with single copy number integration of the vector genome transgene cassette – another *de novo* γ-RV producer population was established where the LacZ.S11 transgene cassette was delivered to the packaging population through transduction (MOI 0.2). Comparing the productivity of each LacZ.S11 cell population (Fig. 2.3a), transduction only generated a slightly higher titers than electroporation (2.3×10^6 *vs*. 1.3×10^6 T.U./mL). SSCS method was used to isolate clones from both LacZ.S11 populations (Fig. 2.3b). Independently of the number of integrated copies of the vector transgene cassette, the average volumetric transducing units titer was higher when retroviral transduction was used. Similarly, multiple copy integrations of the transgene cassette provided in average higher γ-RV vector transducing titers than single copy. Moreover, retroviral transduction showed to be more efficient to establish high producer clones, an effect even more pronounced for single copy transgene cassette integrations where γ-RV vector titers of 1×10^7 T.U./mL are reached. Yet, SSCS protocol showed to be an efficient method to find rare events, indeed multiple and single transgene copy number clones

established by electroporation could reach values of 1 x 10^7 T.U./mL and 4 x 10^6 T.U./mL, respectively.

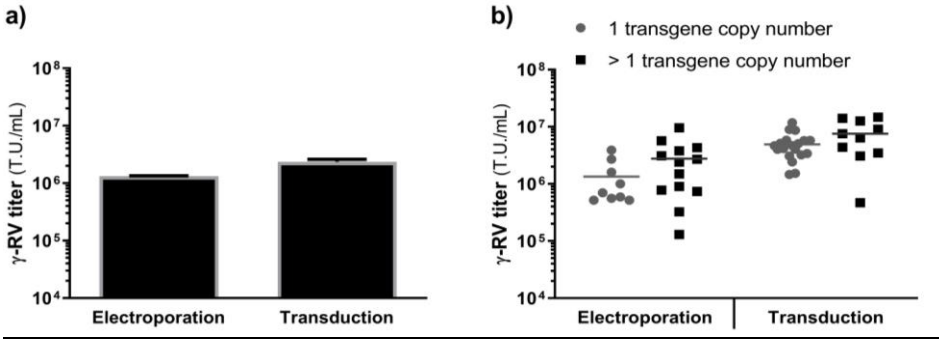


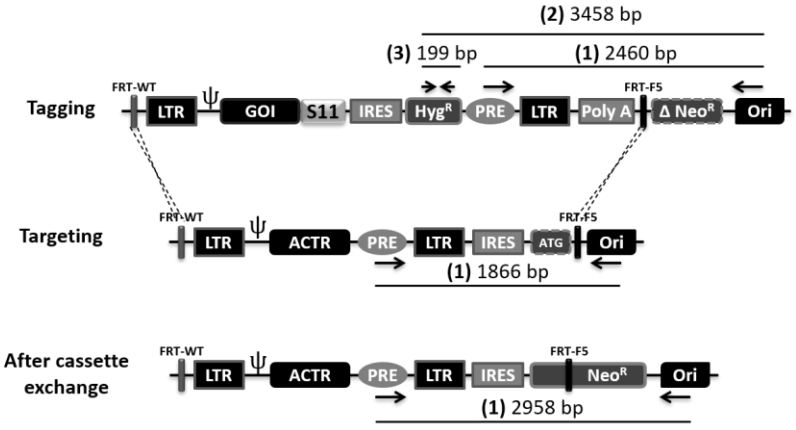
Figure 2.3 - Comparison of transgene cassette delivery method in γ-RV producer cell line establishment: electroporation *vs.* transduction. Stable γ-RV volumetric transducing titer productivity of **a)** populations and **b)** clones isolated using SSCS method. Horizontal lines indicate average titer of isolated clones. Data shown represents mean ± SD from at least 2 technical replicates. LacZ – β-galactosidase; S11 – Split-GFP S11 fragment; SSCS – Single-Step Clone-Screening; T.U. – Transducing Units; γ-RV – Gammaretroviral Vector.

Validation of vector transgene cassette for RMCE

Split-GFP S11 fragment is an excellent reporter gene for cell line development using SSCS method but is not compatible with clinical applications. The same is valid for the antibiotic resistance gene present in viral genome cassette. Both can be removed after cell line establishment through RMCE (Fig. 2.4a).

To validate vector transgene tagging cassette design for RMCE, the single transgene copy number clone established by electroporation and delivering the highest γ-RV volumetric titer was selected. From the several neomycin resistant clones screened, #1 and #2 presented the correct amplicon profile (Fig. 2.4b), indicating accurate RMCE.

a)



b)

Primers pair	Tagging	Cassette exchange	Integration
1	2460 bp	2958 bp	1866 bp
2	3458 bp	—	—
3	199 bp	—	—

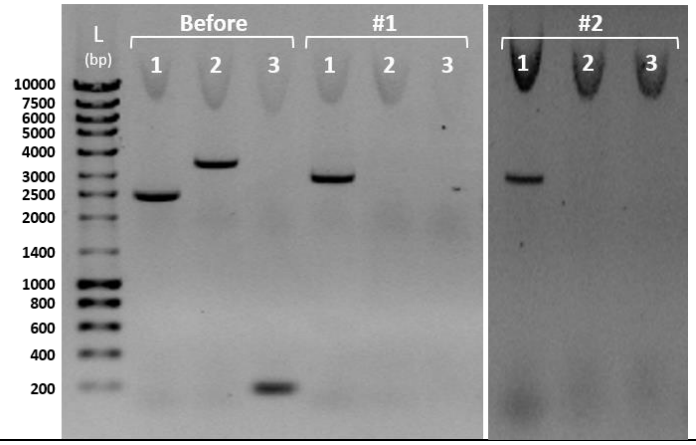


Figure 2.4 - Generation of γ-RV producer cells expressing a therapeutic transgene by RMCE. **a)** Schematic representation of RMCE tagging (integrated) and targeting constructs (before and after integration through recombination). Arrows indicate primer binding sites. Solid lines represent PCR amplicon size of each primer pair (1, 2 or 3). Dashed lines represent recombination event. **b)** PCR analysis of extracted DNA from tagged (before RMCE) and targeted cells (after RMCE from two clones, #1 and #2). FRT-WT - wild type flipase recombinase target site. ACTR – Antibody-coupled T-cell receptor; FRT-F5 – F5 mutated flipase recombinase target site; GOI – Gene of interest; Hyg^R – Hygromycin resistance gene; IRES – Internal ribosomal site; LTR – Long terminal repeat; PolyA – Signal for adenine polynucleotide chain; PRE – Posttranscriptional regulatory element; S11 – GFP S11 fragment; ΔNeo^R – Neomycin resistance gene without ATG start codon; Ψ – Encapsidation signal.

After RMCE, the volumetric transducing titer productivity presented similar average titers (ACTR titration; 2 x 10^6 T.U./mL) to parental cells (Fig. 2.5) validating RMCE cell line development strategy.

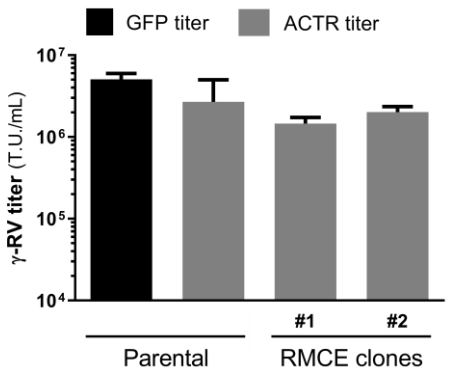


Figure 2.5 - Stable γ-RV transducing units volumetric productivity of clones before and after RMCE procedure. Data shown represents mean ± SD from at least 2 technical replicates. ACTR – Antibody-coupled T-cell receptor; T.U. – Transducing units.

Establishment of LV stable producer cell lines using SSCS method

LV are gaining representation in the clinic; however, the development of stable LV producer cell lines is challenging. Similar to γ-RV, the establishment of LV producer cell lines requires time consuming manual screening of hundreds of clones in order to find a few high producers. To reduce this laborious step the SSCS for LV producer clone screening was herein developed.

The self-inactivating (SIN) LV genome transgene cassette compatible with SSCS method was first optimized (Fig. 2.6a) (optimization of the packaging cassettes was performed in previous work [5]). The optimization aimed at achieving both high LV titers and high GFP transcomplementation signal. In this context, the LV genome transgene size (information provided in Supplementary data, Fig. S2.1) and internal promoters were optimized (data not shown). The optimal final LV genome contained S11.mCherry fusion protein as reporter gene

introduced at downstream of the internal promoter (LTR_{MoMLV}). Additionally, the puromycin antibiotic resistance gene was placed under the control of the external chimeric RSV-LTR promoter enabling the selection of cells expressing packageable viral RNA as described elsewhere [5,28]. To evaluate construct efficiency, transient LV productions using the developed pRRLSIN Puro LTR_{MoMLV} S11.Cherry and pRRLSIN.hPGK.GFP-wPRE (positive control) LV transgene cassettes were performed. The new optimized transgene cassette showed a 2-fold reduction in volumetric titer (0.9×10^7 T.U./mL) when compared to the control (1.7×10^7 T.U./mL) (Fig. 2.6b). Regarding GFP signal to noise ratio, the new optimized LV transgene construct enabled a ratio above the SSCS method threshold value of 20 (Fig. 2.6c). These results validated the developed LV transgene cassette. Therefore, the pRRLSIN Puro LTR_{MoMLV} S11.Cherry construct was used for semi-stable LV production followed by its stable expression in LentiPro26-4070A packaging cell population (Fig. 2.6d) [5]. A *de novo* stable LV producer population was established delivering 0.5×10^6 T.U./mL. This stable LV producer population was subjected to SSCS method after protocol optimization for HEK 293T derived LV producer cells. Methodology adaption to LV producer cells included the following modifications to the protocol: 1) antibiotics supplementation in the limiting dilution step to avoid cassette silencing; 2) culture time-frame modification to adjust and allow clonal growth in the presence of antibiotics; 3) removal of the antibiotics for target cells co-culture; and 4) spinoculation to promote transduction step of 4070A amphotropic envelope [29]. Clones with stable LV volumetric productivities between 0.4×10^5 and 1×10^6 T.U./mL were isolated (Fig. 2.6e).

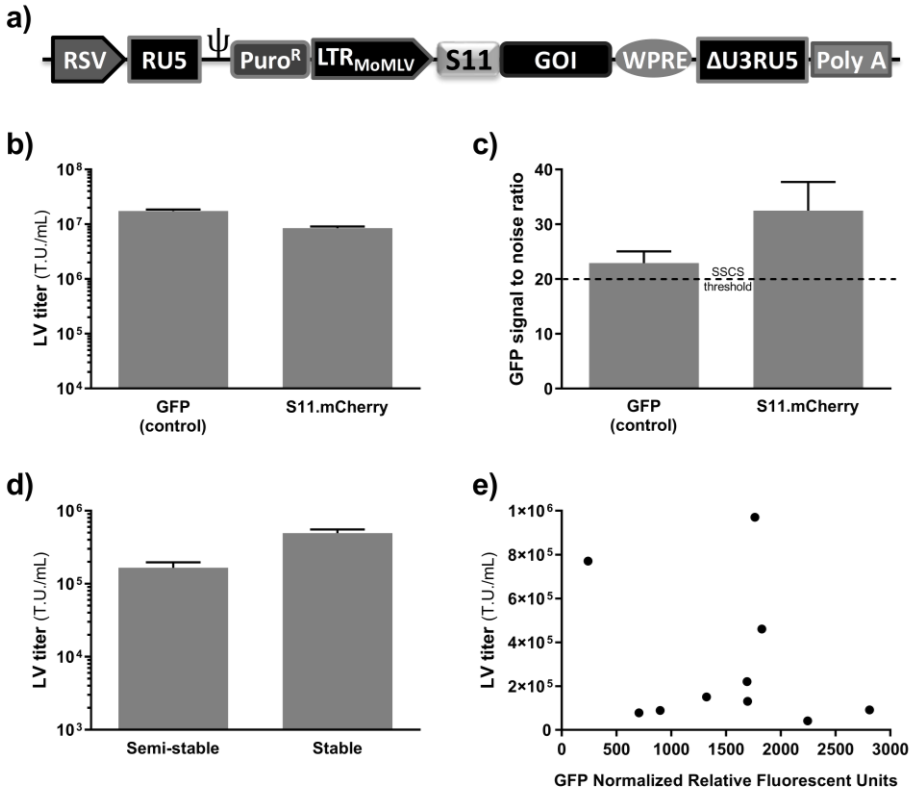


Figure 2.6 - Development of LV stable producer cell lines using SSCS method. **a)** Schematic representation of the optimized vector transgene expression cassette design. **b)** LV transient production. Particles were pseudotyped with VSV-G. **c)** GFP signal to noise ratio of the optimized developed transgene cassette. Signal to noise was defined as the ratio of mean fluorescence intensity of GFP-positive to non-transduced cells, analyzed by flow cytometry. LV transduction was performed using transiently produced LV described in b). Dotted line marks the minimum GFP signal to noise ratio necessary to use SSCS. **d)** Semi-stable and stable LV productivity using the optimized developed transgene LV cassette using a LV packaging cell line population established as described in Tomás, *et al.* (2018) [5]. **e)** Stable LV volumetric titer of the generated clones. Data shown represents mean ± SD from at least 2 technical replicates. GFP – Green fluorescent protein; GOI – Gene of interest, in this case mCherry; LTR_{MoMLV} – Long terminal repeat of moloney murine leukemia virus; PolyA – Signal for adenine polynucleotide chain; $Puro^R$ – Puromycin resistance gene; RSV – Rous sarcoma virus promoter; RU5 – Untranslated 5' region of LTR; S11 – Split-GFP S11 fragment; SSCS – Single-step clone-screening; T.U. – Transducing units; VSV-G – Vesicular stomatitis virus protein G; WPRE – Woodchuck hepatitis virus posttranscriptional regulatory element; ΔU3 – mutated untranslated 3' region of long terminal repeat; Ψ – Encapsidation signal.

2.4 Discussion and conclusions

In this work we studied and optimized key parameters for recombinant retrovirus stable producer cell line development.

The development process of γ-RV producer cells herein studied followed cGMP compliant procedures to mimic clinical requirements. We started by optimizing *gag-pro-pol* cassette expression to reduce sequence homology among viral cassettes. For that, CMV promoter in conjugation with RβG intron efficiently provided high *gag-pro-pol* expression enabling to achieve maximum yields of almost 4 x 10^6 T.U./mL without any upstream bioprocess optimization (Fig. 2.1b). These results are in agreement with previous reports stating that this promoter/intron combination enables high expression in HEK 293 cells [30]. Since CMV promoter was already used to establish producer cell lines and avoid RCR generation, we selected this cassette for stable *gag-pro-pol* expression [31]. Importantly, in all *gag-pro-pol* expression cassettes the addition of the intron sequence increased transducing γ-RV titer (Fig. 2.1b). This productivity improvement might be a consequence of higher mRNA availability for Gag-Pro-Pol translation due to intron known capacity of increasing transcript levels [32].

γ-RV and LV vector genome transgene cassettes were designed aiming to achieve (i) high transgene expression, (ii) high transducing vector titers and (iii) high GFP transcomplementation signal to enable the use of high throughput SSCS methodology [17]. The designed γ-RV transfer vector cassettes and the non-viral transfection methodology used for its stable expression showed to support the generation of high producer clones amenable of RMCE without losing specific γ-RV productivity (Fig. 2.3, Fig. 2.4 and Fig. 2.5) [6,8,27]. Although, electroporation provided single copy clones with lower titers than viral transduction, it poses great advantages over the latter (Fig. 2.3). The most important benefits being

the complete replacement of the vector genome by any expression cassette, including SIN γ-RV genomes, and the complete removal of the transfer vector cassette, including both 5' and 3' LTR, leaving no residual sequences (Fig 2.4a). Thus, the conducted cell line development process allowed to establish γ-RV producer cells suitable for a variety of therapeutic purposes and to accelerate this bioprocess stage [6,8,17,22].

The expression of γ-RV genome cassette is a key parameter determining γ-RV titer [27]. Our results showed that electroporation of the vector genome cassette generated clones with higher range of viral vector titer productivity than using retroviral transduction (Fig. 2.3b). This can be explained by the potential different vector genome expression levels obtained as a result of different cassette genomic integration patterns of each transfection method [33]. Electroporation delivers arbitrarily integration of the expression cassette in the cell genome, from silenced to highly active transcribed *loci* [34]. Whereas, retroviral transduction preferentially integrates the expression cassette in active transcriptional sites less prone to silencing [35]. Moreover, we also observed that clones with multiple γ-RV genome cassette copy number presented higher volumetric productivities than single copy clones (Fig. 2.2d and Fig 2.3b). Which can also be due to the potential higher expression of the vector genome transgene cassette in multiple copy number clones. Nevertheless, the designed vector genome transgene cassette construct requires electroporation to maintain the FRTs intact and single copy clones for successful RMCE, thus imposing γ-RV titer limitations. These limitations were overcome by using SSCS method to identify and isolate rare events of high producer clones (Fig. 2.2d and Fig. 3). Moreover, without any bioprocess optimization, clones yielding up to 1 x 10^7 T.U./mL were successfully established using only chemical and physical transfection methods (Fig. 2.2d).

The two LTR promoters and *GOI.S11* transgenes tested in γ-RV genome cassette enabled similar volumetric titer productivities (Fig. 2.1d and Fig. 2.2b). However, GFP signal to noise ratios (Fig. 2.1e) were affected by the LTR promoter strength and GOI nature. We hypothesize that the cellular localization of the fusion protein derived from the *GOI.S11* expression impacted GFP transcomplementation signal. ACTR is a cellular membrane bound protein, which could mask S11 GFP fragment and impair GFP transcomplementation, leading to lower GFP intensities than LacZ.S11 (cytoplasmatic protein).

To improve and adapt the LV genome cassette to SSCS methodology, we took advantage of the knowledge obtained in the first part of this work and in the recent published work on LV cell line development [5]. In the later we developed a LentiPro26 packaging cell line using a breakthrough approach of expressing a mutated less toxic HIV-1 protease enabling the stable expression of *gag-pro-pol* cassette [5]. Therefore, we made use of this LentiPro26 packaging cell line to develop SSCS method to another recombinant retrovirus, the HIV-1 derived LV. The transgene vector cassette was designed taking in consideration preliminary work (Fig. S2.1) in our group and others [5,28,36]. Three parameters were optimized: the internal promoter driving the expression of the S11 fusion protein; the GOI fused with the S11; and the overall size of the LV genome. The combination of LTR_{MoMLV} derived promoter with mCherry as GOI fused with GFP S11 provided the highest GFP transcomplementation signal while improving LV transduction titers. These were selected for the final LV genome transgene cassette design, also containing an antibiotic selection marker (Fig. 2.6a).

GFP signal obtained at the end of the SSCS method optimization enabled the isolation of clones, however, this signal was less robust when compared with γ-RV producer cells (data not shown). This can be due to the 4070A envelope used to pseudotype LV particles which has

been widely reported to have lower transduction profiles than end-point titer potency [37]. LV transducing titers similar to what was previously obtained by manual screening were achieved but in a significantly reduced timeline [5]. Hence, SSCS method application to other retrovirus derived vectors was validated and enabled faster stable LV producer cells development.

In conclusion, herein we studied and described how different genetic elements, transfection procedures and screening methodologies impact the development and quality (i.e. transducing titer productivity) of γ-RV and LV producer cell lines. A therapeutic gene (ACTR) and cGMP compliant materials and techniques were used to demonstrate the applicability to clinical applications. Moreover, we further advanced LV stable producer cell line development by establishing a SSCS high-throughput method to identify high-titer clones. The knowledge generated in this work can ultimately be used for rational planning master producer cells establishment. Overall, this work contributes to the progress of gene therapy field by accelerating recombinant retroviruses producer cell line development.

2.5 Acknowledgments

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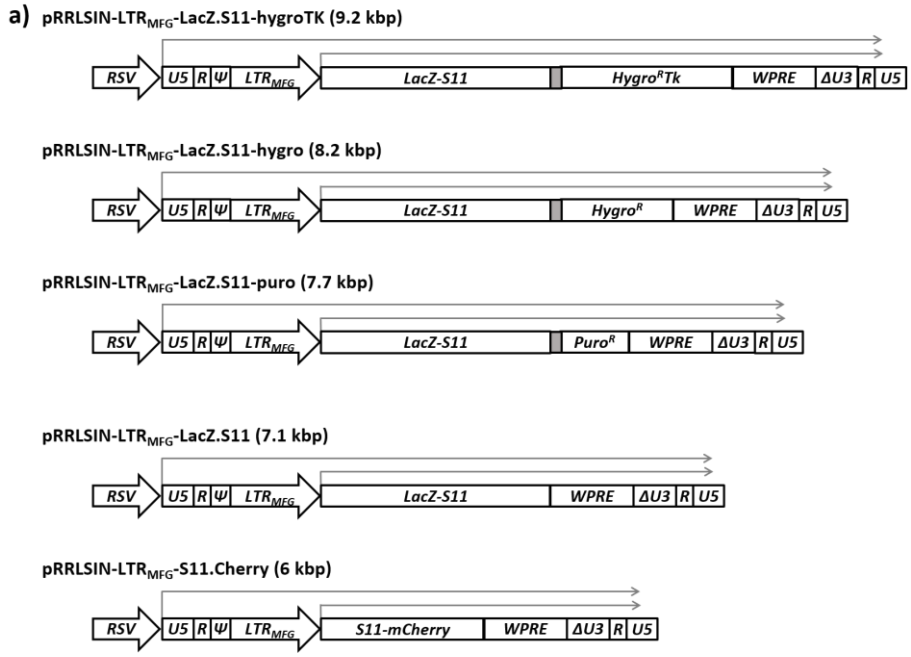
2.6 Supplementary data

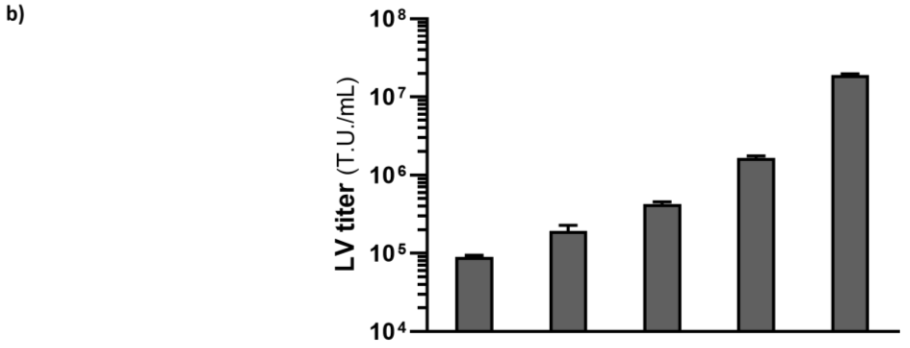
Table S2.1 – Plasmid construction and primer sequences.

	Final Construct	Insert: Fragment	Insert: Source	Backbone	Primers 5' --> 3' sequence
Cloning	pMLV-GP	MoMLV gag-pro.pol	pCEB	pSELECT-blasti-mcs	F - CACCGGCGTGTCGACATGGGCCAGACTGTTACCAC R - GTCTGGCCAGCTAGCTTAAATGTTTTCATGGTGGCC
	pSV40-GP	SV40	pCI-neo E1K9/pSVFLPe?	pGaLV	F - TTCGAGCTCGGTACATCTGTGCGGTATTTCACACC R - TGATTACGCCAAGCTGGTGGCTCTAGCCTTAAGTTCG
		MoMLV gag-pro-pol_bsr	pCEB		F - CTTTTGCAAAAAGCTATATGGGCCAGACTGTTACCACTCC R - CAGAAGAATCAAGCTCCTTTGCCTAATTTAAATGAGGAC
	pSV40i-GP	rabbit β globin intron + MoMLV gag-pro-pol_bsr	pCMVi-GP	pSV40-GP	F - CTTTTGCAAAAAGCTCGACCGATCCTGAGAACTTCAG R - CAGAAGAATCAAGCTCCTTTGCCTAATTTAAATGAGGAC
	pRSV-GP	RSV	pRSV-REV	pGaLV	F - GACGGCCAGTGAATTCACACAGGAAACAGCTATGACATG R - CAGAAGAATCAAGCTTGGCGATCTGACGGTTCACTAA
		MoMLV gag-pro-pol_bsr	pCEB		F - TCAGATCGCCAAGCTATATGGGCCAGACTGTTACCACTCC R - CAGAAGAATCAAGCTCCTTTGCCTAATTTAAATGAGGAC
	pRSVi-GP	rabbit β globin intron + MoMLV gag-pro-pol_bsr	pCMVi-GP	pRSV-GP	F - TCAGATCGCCAAGCTCGACCGATCCTGAGAACTTCAG R - CAGAAGAATCAAGCTCCTTTGCCTAATTTAAATGAGGAC
	phEF1α-GP	hEF1α	phEF1αi-GP	phEF1αi-GP	F - AGCTTGATTCTTCTGACACAACAGTC R - CTGTGTTCTGGCGGCAAACC
		MoMLV gag-pro-pol_bsr	pCEB		F - GCCGCCAGAACACAGATATGGGCCAGACTGTTACCACTCC R - CAGAAGAATCAAGCTCCTTTGCCTAATTTAAATGAGGAC
	phEF1αi-GP	hEF1αi	pU115	pGaLV	F - GACGGCCAGTGAATTCTCTCCAAGCTCACTTACAGGGC R - CAGAAGAATCAAGCTTGCTCACGACACCTGAAATGGAA
		MoMLV gag-pro-pol_bsr	pCEB		F - TGTCGTGAGCAAGCTATATGGGCCAGACTGTTACCACTCC R - CAGAAGAATCAAGCTCCTTTGCCTAATTTAAATGAGGAC
	pCMV-GP	MoMLV gag-pro-pol_bsr	pCEB	pCMVi-GP	F - CCAGCCTCCGGTCGAATATGGGCCAGACTGTTACCACTCC R - TGATTACGCCAAGCTTCCTTTGCCTAATTTAAATGAGGAC
	pCMVi-GP	MoMLV gag-pro-pol_bsr	pCEB	pGaLV	F - ATCATTTTGGCAAAGGATCCATATGGGCCAGACTGTTACCACTCC R - TGATTACGCCAAGCTTCCTTTGCCTAATTTAAATGAGGAC
	$pLTR_{MPSV}$ LacZ-S11	LacZ-S11	pTAR LacZ-S11	$pLTR_{MPSV}$ ACTR-S11	F - AACGACGGCCAGTGCACGAAGTCTGGAGACCTCTGGG R - TCCAGCGCTGCGGCCCCACTAGTTCTAGAGTCGCGGC
	$pLTR_{MFG}$ ACTR-S11	ACTR-S11_IRES-Hygro-PRE	$pLTR_{MPSV}$ ACTR-S11	pUC19 Fw_LTR_{MFG} - F5_dNEO	F - TTACAGCTTCTCGAGCGCCACCATGGCCTTACCAGT R - TTTCATTGCTCTCGAGACAGGTGGGGTCTTTCATTCC
	$pLTR_{MFG}$ LacZ-S11	LacZ-S11_IRES-Hygro-PRE	$pLTR_{MPSV}$ LacZ-S11	pUC19 Fw_LTR_{MFG} - F5_dNEO	F - TTACAGCTTCTCGAGACGAAGTCTGGAGACCTCTGGG R - TTTCATTGCTCTCGAGACAGGTGGGGTCTTTCATTCC
	pRRLsin Puro LTR_{MFG} S11.Cherry	puromycin	pRRLSIN-mCP-GFP	pRRLsin LTR_{MFG} S11.Cherry	F - CATATTAATTACTAGCTATAGGAGGGCCACCATGA R - CAACTAATTGACTAGTTAAGCTCCAGGCTTCCTTG

	Target gene	Primers 5' --> 3' sequence
Real Time quantitative PCR for transgene copy number assessment	LacZ	F - ACTATCCCGACCGCCTTACT R - TAGCGGCTGATGTTGAACTG
	Hyg^R	F - CGCAAGGAATCGGTCAATAC R - ACATTGTTGGAGCCGAAATC
	RPL22	F - CTGCCAATTTTGAGCAGTTT R - CTTTGCTGTTAGCAACTACGC

	Target sequence	Primers 5' --> 3' sequence
Recombinase Mediated Cassette Exchange Evaluation by PCR	PRE	F - GGCACTGATAATTCCGTGGTGTTG
	Ori	R - CGATTTTTGTGATGCTCGTCAGG
	Hyg^R	F - CGCAAGGAATCGGTCAATAC R - ACATTGTTGGAGCCGAAATC





		LV genome size (kbp)	9.2	8.2	7.7	7.1	6.0
Transient LV productions	Cells	HEK 293T	+	+	+	+	+
	Plasmids	pMDLg/pRRE	+	+	+	+	+
		pRSV-REV	+	+	+	+	+
		pMD2.G	+	+	+	+	+
		pRRLSIN-LTR$_{MFG}$-LacZ.S11-hygroTK	+	-	-	-	-
		pRRLSIN-LTR$_{MFG}$-LacZ.S11-hygro	-	+	-	-	-
		pRRLSIN-LTR$_{MFG}$-LacZ.S11-puro	-	-	+	-	-
		pRRLSIN-LTR$_{MFG}$-LacZ.S11	-	-	-	+	-
		pRRLSIN-LTR$_{MFG}$-S11.Cherry	-	-	-	-	+

Figure S2.1 – LV genome cassette size impact in LV productivity. **a)** Schematic representation of transgene cassettes. **b)** Transient LV production titers using transgenes of different sizes described in a).

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CHAPTER III

Lentiviral vector components expression stoichiometry and stability: impact on constitutive producer cell yields and vector quality

This chapter was adapted from

*Formas-Oliveira AS, *Ferreira MV, Coroadinha AS. Lentiviral vector components expression stoichiometry and stability: impact on constitutive producer cell yields and vector quality (*in preparation*)

Author contribution

Ana Oliveira designed and participated in the experimental setup, analyzed the data, and wrote the chapter

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Abstract

Lentiviral vectors (LV) are one of the most used delivery vehicles for the transfer of genetic material in gene therapy. Supported on the recent product approvals to treat primary immunodeficiencies and cancer, LV use in clinical trials increased significantly. For these clinical applications, high quantities of high-quality LV preparations need to be produced/provided and to meet these demands, stable producer cell lines for LV manufacture are desirable. However, in contrast to gammaretroviral vectors, constitutive LV producer cell line development is still immature and knowledge on the impact of vector components expression stoichiometry on vector yield and quality is lacking. This work aimed at studying the impact of vector genetic cassettes copy number, expression levels and stability on LV yields and vector preparation quality on the newly developed LentiPro26 cell lines.

Ten clones displaying heterogeneous productivities and different genomic integration profiles of the vector cassettes were characterized. The genomic copy number of the LV cassettes was not predictive of their gene expression levels. Different vector cassette gene expression levels enabled similar physical particles volumetric productivity (10^9 P.P./mL.day). These results indicated that *rev* and *gag-pro-pol* expressions were not limiting clones production performance. On the other hand, the volumetric yields of viral genomes (10^7 to 10^8 V.G./mL.day) and transducing competent particles (10^5 to 10^6 T.U./mL.day) were very diverse. Long term LV production stability of high titer producers was analyzed for two months. The stability of the LV production yields was clone specific and correlated to vector components expression profiles throughout cell passages. Transfer vector genome availability was the principal component limiting LV titers

quality, followed by envelope balanced expression to maximize particles functionalization.

In summary, this work further elucidates the main bottlenecks in LV manufacture providing further insights on how to optimize constitutive cell line development.

Chapter III

3.1 Introduction

In 2018 recombinant lentivirus registered the highest number of starting viral vector clinical trials, with 57 approved trials [1, The Journal of Gene Medicine – Gene Therapy Clinical Trials Worldwide, list of websites - 1]. Lentiviral vectors (LV) have been replacing gammaretroviral vectors as gene transfer vehicles for therapeutic purposes due to safer genotoxicity profiles [2,3] and capacity of transducing slowly-proliferating and non-proliferating cells [4]. Still, in contrast to gammaretroviral vectors, LV manufacture is a suboptimal and costly bioprocess. It is mainly performed using transient transfection systems, requiring large amounts of plasmid DNA and transfection reagents, which decrease process reproducibility and increase the downstream complexity. Therefore, constitutive, and high yielding LV producer cells are sought to cope with simpler and easier scalable bioprocesses. Such platforms are rare and characterized by lower production yields than transient transfection systems. Different constitutive producer cell lines were established with diverse transfection approaches, LV system generations and expression strategies to cope with LV associated toxicity [5–9]. Impairing the development of standardized procedures to generate robust and high yielding constitutive producer platforms is the limited knowledge on vector genetic cassettes optimal design and stoichiometry.

Currently, the most common and accepted system to produce this recombinant virus is the third LV generation composed of four plasmids providing Rev, Gag-Pro-Pol, envelope, and transfer vector functions in *trans*. However, this split genome approach disregards *Lentivirus* complex and tightly controlled wild type genome expression, which provides the appropriate ratios of splicing and translation events to generate functional viral particles [10]. Indeed, constitutive producer cells expressing the four LV genetic cassettes lack part of those regulatory

mechanisms. For instance, the expression of self-inactivating transfer vector genome is TAT independent [11]. But still, *gag-pro-pol* genetic cassette remains with the ribosomal frameshift translation regulation, providing approximately twenty times more Gag than Gag-Pro-Pol polyprotein [12].

This work aimed at understanding how LV genetic cassettes expression stoichiometry in constitutive producer cells influences vector yields and quality. To this end, the newly developed LentiPro26 cell lines were used as constitutive producer cell models. These producer cell lines were originated by random integration of the LV genetic cassettes in HEK 293T cell genome. Clone isolation was performed only upon the establishment of a population expressing the four cassettes [9]. As a result, each clone is unique, with a specific genomic background. Herein we describe the characterization of ten constitutive LV producer clones regarding viral cassettes genomic copy number, expression level, and the productivity of physical, genome containing and transducing particles.

3.2 Materials and methods

Plasmids

pGP(T26S)P-blast plasmid, codes for HIV-1 Gag-Pro(T26S)-Pol protein under the control of a cytomegalovirus (CMV) promoter. The expression of a blasticidin resistance marker is coupled to HIV-1 Gag-Pro(T26S)-Pol expression by a spacer region, as described elsewhere [9].

pREV-hygro-WPRE plasmid, codes for HIV-1 Rev protein and the hygromycin resistance marker, both under the control of the Rous sarcoma virus (RSV) U3 promoter, as described elsewhere [9].

pMONO-zeo-4070A plasmid, codes for the murine leukemia amphotropic envelope (4070A) under the control of a CMV promoter. The expression of a zeocin antibiotic resistance marker is linked to the expression of the envelope glycoprotein by an internal ribosome entry site of foot and mouth disease virus, as described elsewhere [9].

pRRLSIN-mCP-GFP vector transgene plasmid is a SIN LV vector, described elsewhere [9]. The chimeric 5' LTR-RSV promoter regulates the expression of full-length LV RNA genome, including the expression of the mCherry and green fluorescent protein (GFP) reporter genes and the puromycin selection marker. The expression of puromycin resistance marker is coupled to the expression of mCherry by a spacer region. GFP expression is additionally controlled by human phosphoglycerate kinase 1 (hPGK) internal promoter.
pRRLSIN-mCP-GFP-ALB-REV-POL-4070A plasmid has *mCherry*, *gfp*, *ALB*, *rev*, *pol*, and *4070A* amplicon sequences of qPCR primers, thus, it is used as universal template for calibration curves in gene copy number quantifications. It was generated by cloning a double stranded DNA (gBlock) synthetized by Integrated DNA Technologies (Coralville, Iowa, USA) into the pRRLSIN-mCP-GFP plasmid digested at the XhoI restriction site.

Cell lines and culture conditions

HEK 293T (ATCC CRL-3216), a human embryonic kidney 293 derived cell line expressing SV40 large T antigen, was used for LV transduction units (T.U.) quantification.
LentiPro26-4070A-mCPGFP population and the top 10 producing clones (#6, #10, #15, #30, #42, #54, #59, #68, #88 and #99) [9], are HEK 293T derived cell lines constitutively producing HIV-1 based recombinant LV, pseudotyped with 4070A envelope.

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, NY, USA), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco, Thermo Scientific) and maintained at 37 °C in a humidified atmosphere containing 8% CO_2.

LentiPro26-4070A-mCPGFP were kept under selective pressure supplementing the culture medium with the following antibiotics (all from Invivogen, San Diego, CA, USA): 20 µg/mL blasticidin, 150 µg/mL hygromycin b gold, 150 µg/mL zeocin and 0.5 µg/mL puromycin.

Cell concentration and viability were assessed by trypan blue exclusion method.

Cell growth and lentiviral vector production studies

Producer cells were seeded at 8 x 10^4 cells/cm^2 and 0.1 mL/cm^2 in the presence or absence of selective pressure. Total RNA, genomic DNA, and whole cell protein extracts were isolated at 48 hours post seeding. Medium was exchanged 72 hours post-seeding and the supernatant from the following 24 hours period was harvested, clarified (using a 0.45 µm cellulose acetate filter), aliquoted, and stored at -85 °C until further use. The expression of reporter proteins (GFP and mCherry) in the producer cells was measured by flow cytometry (LSR Fortessa, BD Bioscience- Franklin Lakes, New Jersey, EUA).

Lentiviral vector decay

After harvest, supernatants were immediately diluted in fresh culture medium, incubated at 37 °C in a humidified atmosphere containing 8% CO_2, for 0, 0.5, 1.5, 2.5, 4, 6 or 22 hours and stored at -80 °C until the T.U. titer was assessed. The decay rate constants and vector half-life were calculated by regression according to a first-order exponential decay model [13].

Lentiviral vector particles quantification

Transduction competent LV particles quantification was performed using HEK 293T target cells seeded at 5 x 10^4 cells/cm^2 in 24 well plates. Transduction was performed 24 hours after, in duplicates, by removing the cell supernatant and adding 0.2 mL of viral suspension diluted in fresh DMEM with 10% (v/v) FBS and 8 µg/ml of polybrene (Sigma). Cell concentration was determined at time of transduction. Following spinoculation – centrifugation at 1200 x g for 2 hours at 25 ºC – 1 mL of fresh supplemented DMEM was added [14]. Cells were harvested and analyzed 48 hours post-transduction using CyFlow Space flow cytometer (Sysmex Partec GmbH, Görlitz, Germany). The T.U titer was determined taking into account the percentage GFP positive cells, the cell concentration at the time of transduction and the dilution factor [9].

Viral genome (V.G.) titer was quantified using a method previously described elsewhere [15]. The protocol was adapted for LV by using primers against the WPRE sequence (Table 3.1, end of material and methods section) for cDNA synthesis and qPCR.

For LV total particles quantification, a p24 enzyme-linked immunosorbent assay (ELISA) was conducted using the Lenti-X p24 Rapid Titer kit (Clontech, Takara, CA, USA), according to the recommendations and manufacturer's instructions.

Genomic DNA and RNA extraction and cDNA synthesis

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, USA) following the manufacturer's instruction and stored at -20 °C until further use.

Total RNA extraction was performed using QIAamp RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, eluted in 60 µl H_2O, aliquoted, and stored at -80 °C until further use.

cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany), in agreement with manufacturer's instructions, using 1 µg of total RNA and anchored-oligo(dT) primers. The cDNA products were aliquoted and stored at -20 °C until further use.

Gene expression and copy number

Relative gene expression was quantified by qPCR and normalized to two reference genes: *Ribosomal Protein L22* (*RPL22*) and *Ubiquitin B* (*UBB*) [16,17].

Gene copy number was quantified by qPCR, using human *Albumin* (*ALB*) gene as reference gene, assuming 2 copies per cell (n = 2) in HEK 293T [18]. pRRLSIN-mCP-GFP-ALB-REV-POL-4070A plasmid, harboring all gene target sequences of qPCR primers, was used for the calibration curves. The copy number per cell was calculated based on the following equation [18,19]:

$$\text{Gene copy/cell} = \frac{\text{Copy number of target gene}}{\text{Copy number of reference gene } (\textit{ALB})} \times \text{genomic ALB copies } (n = 2)$$

Quantitative real-time PCR

qPCR was performed in a thermocycler LightCycler 480 Real-Time PCR System (Roche Applied Science) using a LightCycler 480 SYBR Green I Master (Roche Applied Science) PCR kit. Primers sequences for all reactions are listed in Table 3.1 (end of material and methods section).

Western Blotting

Whole cell extracts were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rock- ford, IL, USA) supplemented with cOmplete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Total protein quantification was performed with BCA Protein Assay Kit (Thermo

Scientific) according to the recommendations and manufacture's protocol. Equal amounts of protein (20 µg), in reduced and denatured conditions, were resolved in a 4–12% (w/v) acrylamide NuPAGE gradient precast gel (LifeTechnologies), with NuPAGE MES SDS Running Buffer, and then transferred to poly(vinylidene difluoride) membranes. Anti-HIV1 p24 antibody (1:2000, ab9071 - Abcam, Cambridge, UK) and anti-α-tubulin (1:5000, T6199 - Sigma-Aldrich) were used as primary antibodies. Secondary antibody incubation was performed with horseradish peroxidase-linked ECL Anti-Mouse IgG (1:5000, NA931 – GE Healthcare, Little Chalfont, UK).

Table 3.1 – Sequences of qPCR primers.

Target		5' to 3' sequence
Transgene: *egfp*	Forward	CAGAAGAACGGCATCAAGGT
	Reverse	CTGGGTGCTCAGGTAGTGG
Transgene: *mcherry*	Forward	CCCCGTAATGCAGAAGAAGA
	Reverse	TCTTGGCCTTGTAGGTGGTC
Envelope: *4070A*	Forward	AGACCAGGAACCGTATGTCG
	Reverse	GGGAGATTAGGTCCCACGAT
Gag-Pro-Pol: *pol*	Forward	TTTGCAGGATTCGGGATTAG
	Reverse	CTGATTCCAGCACTGACCAA
rev	Forward	ACCTCCTCAAGGCAGTCAGA
	Reverse	GGTAGCTGAAGAGGCACAGG
RPL22	Forward	CTGCCAATTTTGAGCAGTTT
	Reverse	CTTTGCTGTTAGCAACTACGC
UBB	Forward	ATCACTCTGGAGGTGGAGCC
	Reverse	ACTGCGAATGCCATGACTGA
ALB	Forward	GCTGTCATCTCTTGTGGGCTGT
	Reverse	ACTCATGGGAGCTGCTGGTTC
WPRE	Forward	ACTGTGTTTGCTGACGCAAC
	Reverse	ACAACACCACGGAATTGTCA

3.3 Results

LV productivity and quality

LentiPro26 clones were first characterized regarding LV productivity in terms of physical, genome containing, and transducing particles (Fig. 3.1). LentiPro26 cell population was also analyzed providing an average productivity of the cells before cell cloning. Based on the cell specific transducing LV particles yields (Fig. 3.1a), four groups were distinguished from very low (I) to high producers (IV). The cell growth of the clones was similar therefore these group categories mirrored the volumetric transducing particles titers profile, ranging from 1 x 10^5 T.U. /mL.day (I), 2 x 10^5 T.U. /mL.day (II), 6 x 10^5 T.U. /mL.day (III) and 1 x 10^6 T.U./mL.day (IV) (Fig. 3.1b). Non-functional yields were assessed by quantifying the total physical particles and the viral genomes content of the LV preparations (Fig. 3.1b). The LV volumetric yields of the total physical particles (P.P.) were found to be very similar among all clones, 1 x 10^9 to 3 x 10^9 P.P./mL.day, whereas of the viral genomes (V.G.) varied from 7 x 10^7 to 5 x 10^8 V.G./mL.day. Both total physical and genome containing particle yields did not predict LV transducing titer.

The three types of LV particles volumetric titers can be analyzed in terms of viral preparation quality (Fig. 3.1c). Within the total physical particles produced by the clones, 1% to 30% contained viral genomes. Again, these percentages of viral genome incorporation did not correlate to particles transducing capacity, which did not exceed 0.1% of the total particles. For example, while clones #54 and #59 (group IV) presented the highest percentage of transducing particles (up to 0.06%), the viral genome particles content was less than 20%. On the other hand, clone #42 (group II) showed the highest percentage of viral genome particles (almost 30%) but 6-fold less (0.01%) transducing particles than clone #59. In contrast, clone #88 (group III) presented the lowest viral genome

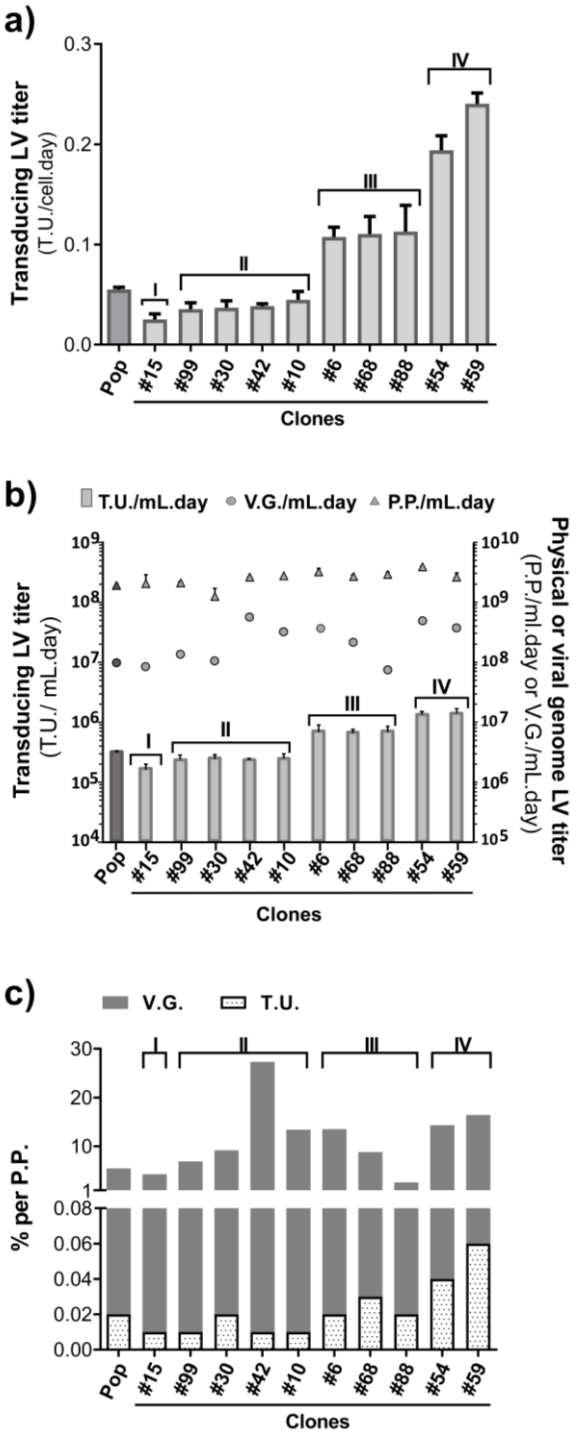

Figure 3.1 – Lentiviral vector production from LentiPro26 population and clones. **a)** Cell specific transducing units titer. **b)** Transducing, genome containing, and total physical particles volumetric titers. **c)** Viral vector preparation quality, percentage of particles within total physical particles. Productions were performed in batch mode for 24 hours. Data shown represents mean ± SD from technical replicates (n = 2). Pop – population; P.P. - physical particles; T.U. **–** transducing units; V.G. **–** viral genomes. Transducing units volumetric titer categories: I) ± 1 x 10^5, II) ± 2 x 10^5, III) ± 6 x 10^5, and IV) > 1 x 10^6.

particles content (10%) but had more transducing particles (0.02%) than clone #42.

LV genetic cassettes: integration copy number and expression level

The genomic copy number and gene expression of the four LV genetic cassettes were assessed for each clone and plotted according to the cell specific transducing particles productivity group (Fig. 3.2). The transfer vector (Fig. 3.2a and Fig 3.2b) and envelope (Fig. 3.2e) cassettes copy number varied among all clones, whereas the *rev* (Fig. 3.2c) and *gag-pro-pol* (Fig. 3.2d) cassettes presented a more homogeneous copy number. In general, each cassette genomic copy number did not correlate with its mRNA levels. As such, for the same number of genomic copies of *rev* and *gag-pro-pol*, mRNA levels were considerably diverse (approximately 3-fold range) but supported similar physical particles productivities (Fig. 3.2c and Fig. 3.2d). Also, higher transfer vector and *env* envelope genomic copy numbers did not always resulted in high gene expression (Fig. 3.2a, Fig. 3.2b and Fig. 3.2e).

The transfer vector cassette contains two open reading frames: one under the control of a chimeric RSV/HIV-1 LTR, driving the transcription of the full viral RNA genome; and another from an internal human promoter driving the expression of the gene of interest. Both mRNA levels were measured, by targeting two genes, *gfp* present in both transcripts (Fig. 3.2a), and *mcherry* (Fig. 3.2b), present only in the full viral RNA genome. The *gfp* mRNA levels were systematically higher than *mcherry*. The genomic copy number of these genes was similar.

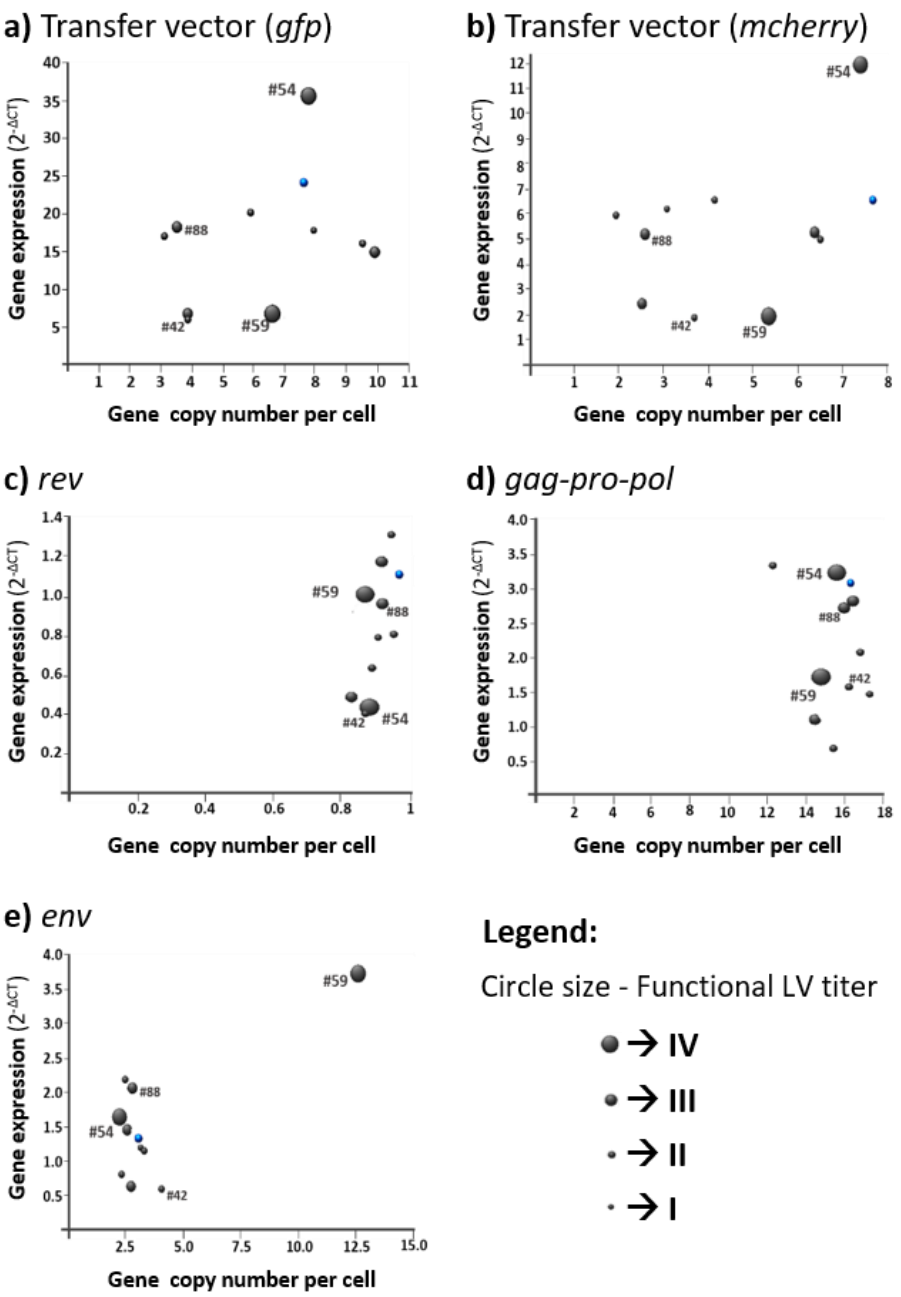

Figure 3.2 – LV genetic cassettes copy number and mRNA levels in LentiPro26 clones. Transfer vector assessed using primers targeting **a)** *gfp* and **b)** *mcherry* reporter genes. **c)** *rev* cassette. **d)** *gag-pro-pol* cassette. **e)** *env* cassette. Gene expression was calculated after normalization to an average of two reference genes (*RPL22* and *UBB*) using the $2^{-\Delta CT}$ method. Blue circle represents the cell population. This figure was performed using XLSTAT statistical and data analysis solution.

In summary, the high transducing unit titers observed for group IV could be sustained with both high or low viral cassettes genomic copies and mRNA expression levels. No correlation between viral genome titers and LV genetic cassettes expression levels was observed. Although clone #42 (group II, 2 x 10^5 T.U./mL.day) exhibited the highest viral genome titer, it was not associated to the higher transfer vector expression. The low levels of functionalized particles should be a consequence of the low *env* expression obtained for this clone. The need to surpass a threshold envelope expression to attain transducing particles is further supported by the fact that clone #88 (group III, 6 x 10^5 T.U./mL.day) showed one of the highest *env* mRNA expressions while presenting a low content of viral genome titers.

Rev recognizes the RRE element present in transfer vector and *gag-pro-pol* mRNA, assisting in its nuclear exportation to the cytoplasm. Therefore, a direct correlation between both gene expressions would be expected. However, clone #54 and # 59 (group IV, 10^6 T.U./mL.day) showed inversed expressions of *rev* and *gag-pol* without visible impact in physical and transducing particles yields.

Protein expression levels arising from the transfer vector cassette were assessed by measuring GFP and mCherry fluorescence intensity (Supplementary data Fig. S3.1). Clones with higher mRNA levels also presented higher fluorescence intensities.

Stability of lentiviral producer clones

A stability study was performed to the clones of group IV (#54 and #59). The cell clones were maintained over two months in culture and the production of LV and the expression of the LV genetic cassettes evaluated. During LV manufacture the stability of the cell clones in culture without the antibiotic selective pressure of LV genetic cassettes,

is of high importance. The cell clones were subcultured for over two months (68 days) with or without antibiotic selective pressure in cell culture. Volumetric productivities of LV transducing, genome containing, and physical particles were assessed once a week (Fig. 3.3).

In the presence of antibiotic selective pressure, both clones maintained the volumetric productivities throughout the 2 months culture period. Removal of the antibiotic selective pressure affected the LV production.

In the third week in culture (day 19) without antibiotics clone #54 presented transducing titers only 2-fold lower than in week 1, maintaining 0.8 x 10^6 T.U./mL.day production titers until the end of the study. The genome containing and physical particles production of clone #54 were not affected by the antibiotic removal, oscillating between 3-5 x 10^8 V.G./mL.day and 2-3x10^9 P.P./mL.day.

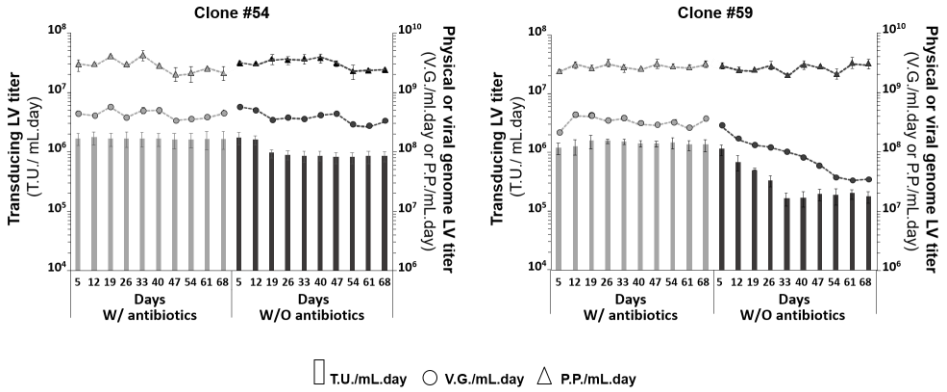

Figure 3.3 – Stability of clones #54 and #59. Producer cells were maintained for two months in culture with (W/) and without (W/O) antibiotic selective pressure. Volumetric titers of batch (24 hours) productions. Data shown represents mean ± SD from technical replicates (n = 2). P.P. - physical particles; T.U. - transducing units; V.G. - viral genomes.

Functional LV titers of clone #59 decreased immediately after antibiotic removal from the culture. An 8-fold reduction in transducing LV particles titer was observed at day 33. From then onwards, productivities of

approximately 0.2 x 10^6 T.U./mL.day were maintained. A simultaneous gradual decrease in viral genome titers, from 2.9 x 10^8 V.G./mL.day (day 5) to 3.7 x 10^7 V.G./mL.day (day 61 and 68) was observed, corresponding to almost 10-fold reduction. Yet, removal of antibiotic selective pressure did not affect physical LV particles yields (2-3x10^9 P.P./mL.day).

The vector half-life at 37 °C of the transducing LV particles was also analyzed. No considerable differences in vectors half-life were observed, with or without antibiotic selective pressure (Supplementary data Table S3.1).

Stability of LV genetic cassettes

To determine the genetic stability of the integrated LV expression cassettes, mRNA levels, genomic copy number and protein expression was analyzed throughout the two months subculture of clones #54 and #59, with or without antibiotic selective pressure. Figure 3.4 resumes the gene expression data.

The first time point (with antibiotic selective pressure) was assumed as the gene expression baseline. Both clones exhibited gene expression variations of 1.5-fold (higher or lower) from the baseline when cultured in the presence of antibiotic selective pressure throughout the 68 days. These mRNA levels were considered the interval of biological variability of LV cassettes expression for each clone. In the case of clone #54, when antibiotics were removed from the cells culture medium, only the *env* mRNA levels decreased below clone expression variability interval (1.7-fold). These results suggested that the loss of transducing LV titers could be a consequence of limited envelope glycoprotein incorporation/availability. On the other hand, clone #59 showed a drastic gene expression reduction of all LV cassettes in the absence of

antibiotic selective pressure in cell culture medium. Specifically, in the last week of the study, gene expression decreased 3.0- (*env*), 3.7- (*rev*), 4.2- (*gag-pro-pol*), 4.4- (*gfp*) and 4.6-fold (*mcherry*). The later affected LV preparation yields and quality.

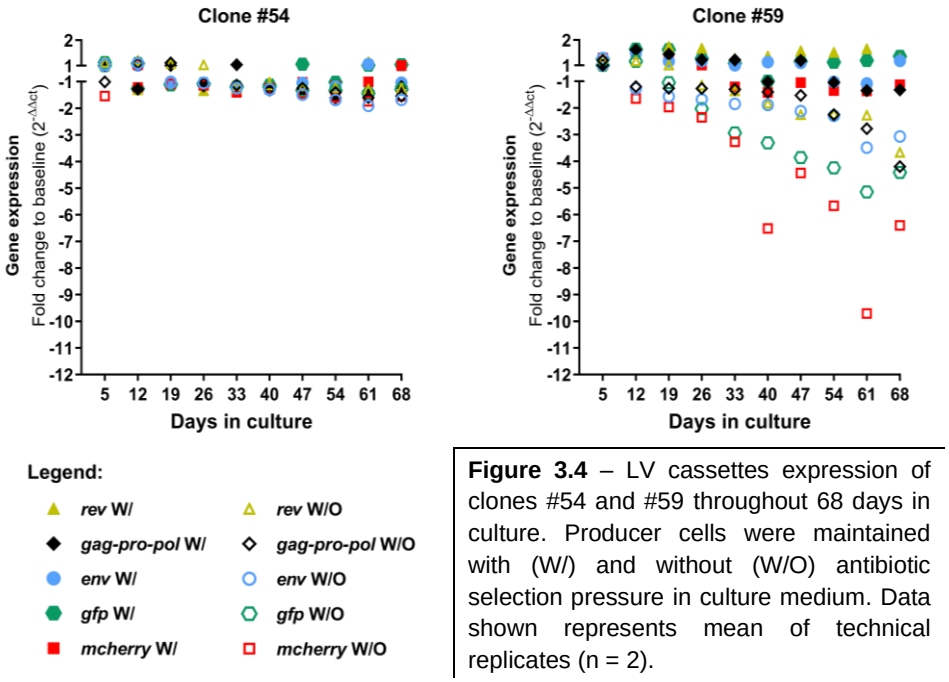

Figure 3.4 – LV cassettes expression of clones #54 and #59 throughout 68 days in culture. Producer cells were maintained with (W/) and without (W/O) antibiotic selection pressure in culture medium. Data shown represents mean of technical replicates (n = 2).

The genomic stability of both clones was assessed by the number of genomic copies of each LV cassette (Supplementary data Fig. S3.2). The copy number of integrated cassettes remained constant throughout the 68 days, with and without antibiotic selection pressure.

Protein expression levels from the reporter genes expressed from the transfer vector cassette (Fig. 3.5) support mRNA data. In the absence of antibiotics, clone #54 decreased reporter protein expression by 40% (GFP) and 34% (mCherry). Still, the absolute fluorescence intensity values of the reporter proteins in clone #54 remained higher than clone

#59. Without antibiotics in the cell culture medium, clone #59 lost 80% of GFP and 70% of mCherry protein expression. These results confirmed previous observations of lower vector genome availability throughout successive subcultures in the absence of antibiotic selective pressure. The reduced LV genome availability could be the main cause for clone #59 viral genome titer loss.

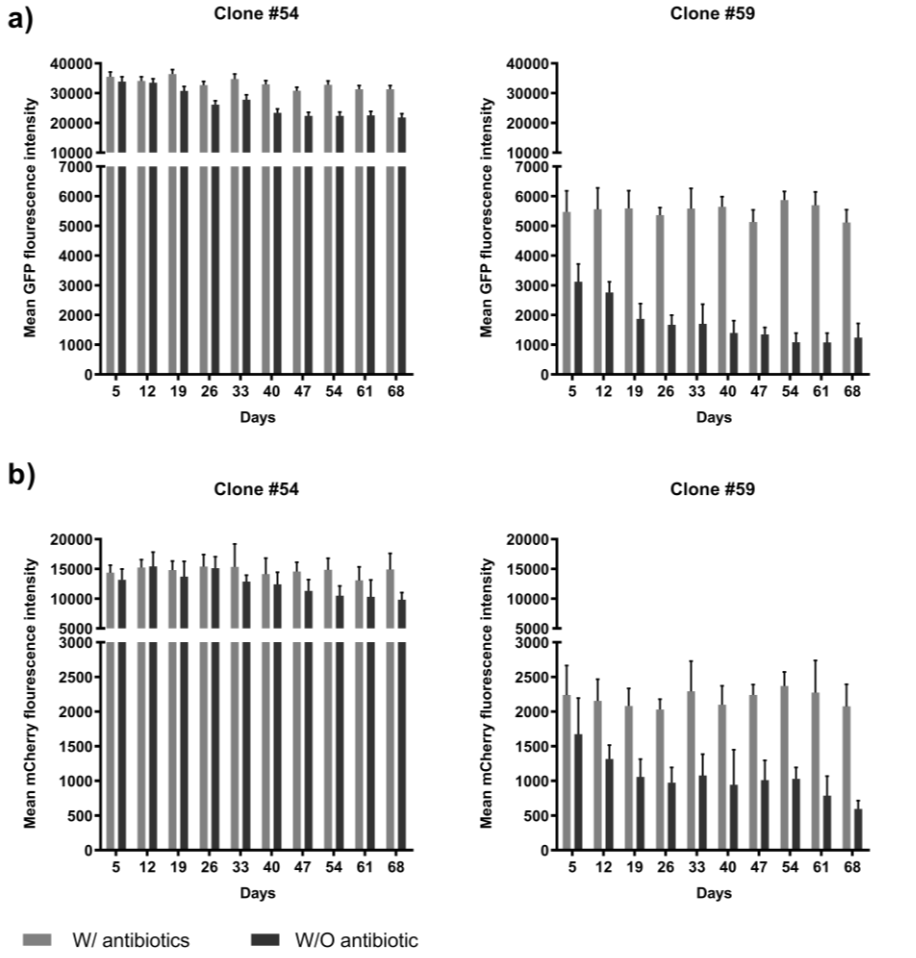

Figure 3.5 – Transfer vector protein expression stability of clones #54 and #59 throughout 68 days in culture. Producer cells were maintained for two months in culture with (W/) and without (W/O) antibiotic selective pressure in culture medium. **a)** GFP and **b)** mCherry fluorescence intensity. Data shown represents mean ± SD of technical replicates (n = 2). Data was analyzed using FlowJo™ software.

As previously mentioned, clone #59 *gag-pro-pol* mRNA levels decreased more than 4-fold throughout the 68 days in culture without antibiotic selective pressure. However, physical particle titer was not affected. To understand how mRNA levels were impacting Gag (p55) and Gag-Pro-Pol (p160) protein synthesis (Fig. 3.6a), a western blot analysis of whole cell extracts was performed (Fig. 3.6b and Fig. 3.6c).

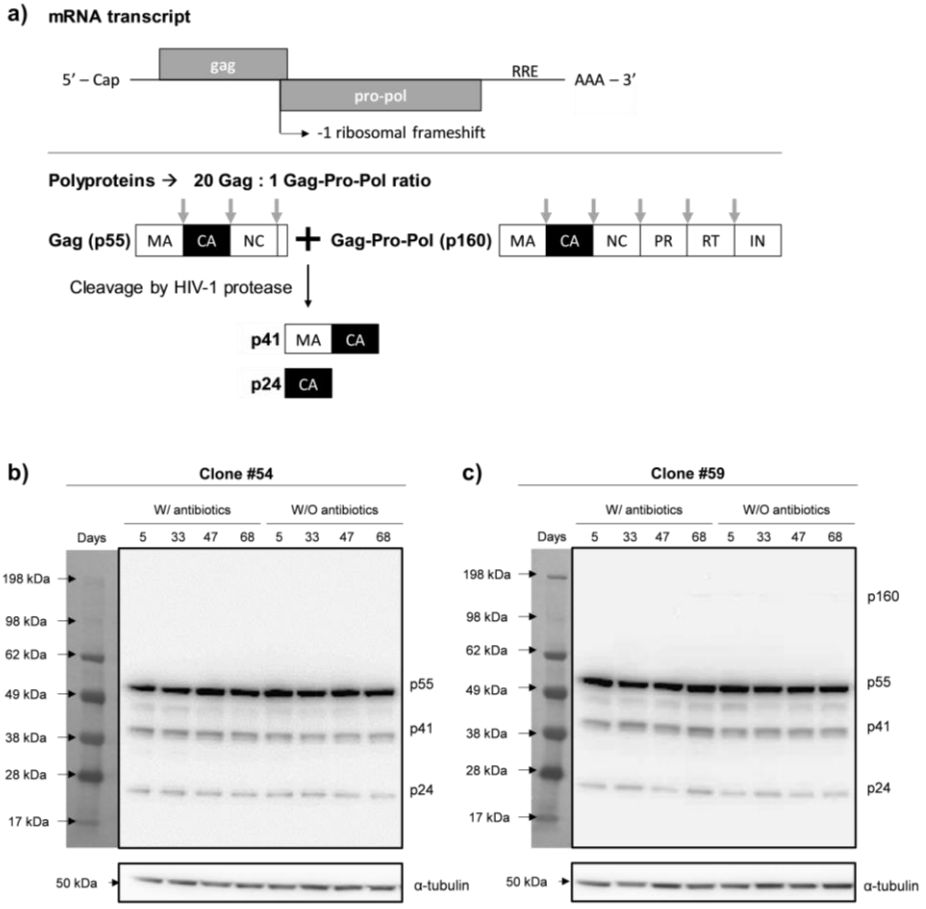

Figure 3.6 – Gag-Pro-Pol protein expression stability of clones #54 and #59. **a)** Precursor polyproteins and cleavage products containing p24 peptide sequence (identified in black). Grey arrows represent the cleavage sites for T26S HIV-1 protease. **b)** Clone #54 and **c)** clone #59 Gag-Pro-Pol and Gag expression during two months in culture with (W/) and without (W/O) antibiotic selective pressure. Molecular weight marker is identified on the left. Precursor proteins and cleavage products are identified on the right. CA – capsid; IN – integrase; MA – matrix; NC – nucleocapsid; PR – protease; RT – reverse transcriptase.

Clones #54 (Fig. 3.6b) and #59 (Fig. 3.6c) presented similar band intensities of Gag precursor polyprotein (p55) and protease cleavage products (p41 and p24) along the 68 days, with or without antibiotics in cell culture media. Therefore, even though gene expression levels decreased, Gag and protease cleavage product levels in both clones remained constant in the absence of antibiotic selective pressure.

3.4 Discussion and conclusions

Vector components expression stoichiometry was studied in the past assisting the development of recombinant gammaretrovirus constitutive high producer cells [13,20–22]. Even though considerable knowledge for gammaretrovirus constitutive production manufacturing exists, few can be applied in LV production systems. Thus, it is of extreme importance to understand the cassettes expression stoichiometries behind LV yields. To the best of our knowledge, this is the first report studying the influence of LV genetic cassettes expression levels in constitutive producer cells productivity.

The ten LentiPro26 clones herein studied showed no correlation between the genomic copy number and the mRNA levels of LV cassettes (Fig. 3.2). This lack of correlation could be a consequence of LentiPro26 development procedure, where LV cassettes were chemically transfected and, thus, randomly integrated into producer cells genome [9]. Hence, depending on chromosomal integration site (low or high expressing *loci*), LV cassette genomic copy number provided different gene expression profiles. Particularly, *gag-pro-pol* and *rev* cassettes exhibited similar genomic copy number values but 7 and 3-fold expression differences, respectively, among all studied clones. These *gag-pro-pol* and *rev* cassettes low genomic variability may be a

consequence of two factors. Firstly, despite the lower cytotoxic profile of T26S viral protease, its activity may have introduced a bias in the resistant cell selection process, where the cells exposed to lower cytotoxic levels survive and are maintained throughout the cell line development process. Secondly, these LV genetic cassettes were the earliest to be transfected and selected in the LentiPro26 cell line development process. Therefore, *gag-pro-pol* and *rev* expressing cells were the ones suffering longer subculture periods and rounds of antibiotic selection. In combination, these two factors may result in the over-representation of a few clones in the population (i.e. the clones presenting higher growth overtaking the cell culture population), leading to an homogenic genotype and low *gag-pro-pol* and *rev* expression variability. In opposition, the transfer vector cassette was the last to be transfected and selected in the LentiPro26 cell line development process. Thus, the inferior rounds of selection and subculture time may have allowed to maintain the heterogenic transfer vector genetic variability of LentiPro26 clones.

Vector genome expression levels in the producer cells were previously associated to the resulting genome containing particle titer [20]. In this work, the range of transfer vector expression levels provided yields of 10^7 to 10^8 V.G./mL.day without a direct correlation (Fig. 3.2). Nevertheless, two important observations could be made in the clones exhibiting the highest (#54) and lowest (#59) vector genome expression levels (Fig. 3.2). Clone #54 maintained the genome containing particles titer and vector genome expression throughout 68 days of subculture without antibiotics (Fig 3.2 and Fig. 3.4). In opposition, clone #59 subculture in the same conditions severely showed a decreased both in genome containing particles titer and vector genome expression in a dependent manner (Fig. 3.3 and Fig. 3.4). Considering that from all clones, #59 was the clone with lower transfer vector expression, it

seems that its expression level is the threshold from which viral genome incorporation could be affected.

Regarding transducing particle yields, the LV genome expression levels of the ten clones herein analyzed allowed LV titers from 10^5 to 10^6 T.U./mL (Fig. 3.1 and Fig. 3.2). The one log difference in functional yields could be a result of each clone *env* expression level, as previously shown for gammaretroviral production [13,20]. This LV component is responsible for the first critical step of host recognition; thus, envelope glycoprotein incorporation is essential for LV functionalization [23]. This hypothesis is supported by several observations in this work. For instance, the clone #42 presenting higher viral genome titer was also the clone with lowest transducing units titer and *env* expression. Moreover, clone #88 with the lowest viral genome titer, presented higher LV transducing unit yields and *env* expression than clone #42. The high producer clones #54 and #59, also showed higher *env* expression than clone #42. In this line, when clone #54 functional LV yields decreased 2-fold (Fig. 3.4), only *env* expression was reduced below clone variability interval (Fig. 3.5).

Functional LV production stability over time, without antibiotic supplementation, is an important feature in manufacturing. The high producer clones herein analyzed behaved differently over 2 months of subculture without antibiotic selective pressure. Clone #54 showed relatively stable LV production whereas clone #59 decreased the transducing units and viral genome titers during the 68 days in culture. The LV production decrease of clone #59 was highly correlated to the expression loss of all LV cassettes (Fig. 3.3 and Fig 3.4). These observations reinforced previous work hypothesis of epigenetic silencing mechanisms regulating LV cassettes expression stability [9]. In addition, the instable LV cassettes expression in clone #59 did not impact LV decay rate. These results are in agreement with previous work in our

group where this vector property also showed to be independent of the vector cassettes expression stoichiometry in the producer cell line [13].

Surprisingly, upon the drastic reduction of *rev* and *gag-pro-pol* expression (Fig.3.4), clone #59 maintained total physical particles titer and Gag content (Fig. 3.3 and Fig. 3.6). These results suggested that constitutive producer cell lines require a certain threshold of *gag-pro-pol* expression to provide enough levels to reach 10^9 P.P./mL.day productivities. In fact, the expression of *gag-pro-pol* has been pointed has the main challenge limiting LV constitutive producer cells development [7,8,24]. However, in this work we showed that LentiPro26 clones presented different *rev* and *gag-pro-pol* expression levels (Fig. 3.2 and Fig. 3.4) enabling similar LV 10^9 P.P. yields (Fig. 3.1 and Fig. 3.3). Therefore, the cell line development process scheme based on T26S protease, chemical transfection and stringent antibiotic selective pressure steps, did not limited *gag-pro-pol* expression and LV physical particles production [9].

A point of concern was the almost undetectable Gag-Pro-Pol content in producer cell extracts (Fig. 3.6b and Fig. 3.6c). Still, protease (Pro in Gag-Pro-Pol polyprotein) activity was detected by the observation of Gag cleavage products, raising two hypotheses. First, due to a ribosomal frameshift event (Fig. 3.6a) during protein translation, Gag-Pro-Pol and Gag are synthetized in a ratio of 1:20 [25], which may difficult the detection of the higher molecular weight polyprotein precursor. Secondly, the constitutive activity of T26S protease in producer cells cytoplasm may be processing Gag-Pro-Pol in such way that its levels are not detectable by western blot. If so, the cytoplasmatic activity of T26S protease could be affecting particles functionalization by limiting the incorporation of viral enzymes present in Gag-Pro-Pol into LV particles [26,27]. Namely, the lack of reverse transcriptase and integrase present in Gag-Pro-Pol might restrain particle transduction cycle since

these enzymes carry out reverse transcription of the viral RNA genome and provirus genomic integration, respectively [28]. Furthermore, the low content of Gag-Pro-Pol unprocessed polyprotein in LV leads to lower T26S protease activity inside the LV, which might dysregulate the particle maturation cycle and consequently affecting the envelope fusogenicity state [10,29].

In summary, two major conclusions can be taken from this work. First, *gag-pro-pol* expression was sufficient to generate 10^9 physical particles volumetric titers. Yet, it remains unanswered if higher expression levels would increase this yield. Second, particle functionalization was highly associated to balanced transfer vector and envelope mRNA levels, as it was previously reported for the case of constitutive cells for the production of gammaretrovirus [13,22]. Even so, the high production yields of non-functional particles are imposing the urgent need to understand how leveraging these components expression contributes to improve the viral preparation quality. Namely, further studies to assess the influence of *env* expression levels on transducing LV titers of producer cells. Exploring *cis*-regulatory elements, such as insulators, would also be worth it to increase LV producer clones stability.

Overall, this work contributes to extend the knowledge on how LV components expression influence cell production yields, enabling to rationally design LV genetic cassettes and clone screening strategies improving the quality of LV constitutive manufacture systems.

3.5 Acknowledgments

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3.6 Supplementary data

Table S3.1 - Clone #59 LV preparations decay at 37 ºC.
Supernatants were obtained in batch (24 hours) productions over two months.
Data is shown as average ± SD of technical replicates (n = 2).

	Viral vector half-life (hours)	
Days in culture	With antibiotic selective pressure	Without antibiotic selective pressure
5	3.24 ± 0.08	3.06 ± 0.02
40	2.92 ± 0.01	4.01 ± 0.14
47	2.81 ± 0.04	3.64 ± 0.01
68	3.02 ± 0.03	4.05 ± 0.07

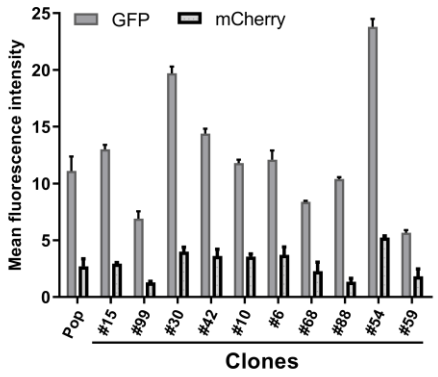

Figure S3.1 – Transfer vector reporter proteins expression. GFP and mCherry fluorescence intensity values shown as the mean ± SD of technical replicates (n = 2). Data was analysed using FlowJo™ software.

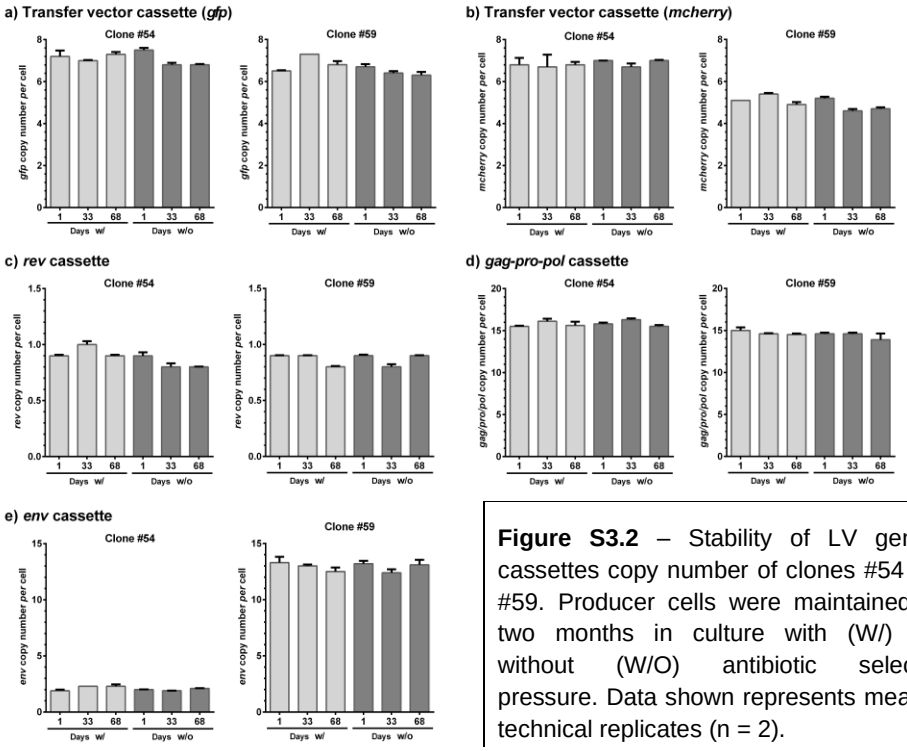

Figure S3.2 – Stability of LV genetic cassettes copy number of clones #54 and #59. Producer cells were maintained for two months in culture with (W/) and without (W/O) antibiotic selective pressure. Data shown represents mean of technical replicates (n = 2).

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CHAPTER IV

ER protein processing and anti-apoptosis genes

This chapter was adapted from

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Author contribution

Ana Oliveira designed and participated in the experimental setup, analyzed the data and wrote the chapter

Abstract

Gammaretroviral and lentiviral vectors (γ-RV and LV) are among the most used vectors in gene therapy clinical trials and market approved products. Currently, HEK 293 cells, the manufacture platform of choice for these vectors, provide low transducing particle yields, challenging their therapeutic applications and commercialization. This work studied metabolic pathways, focusing on endoplasmic reticulum (ER) protein processing and anti-apoptotic mechanisms, influencing vector productivity in HEK 293 cell substrates. To that end, four candidate genes – PDIA2, HSPA5, XBP1 (ER protein processing) and BCL2 (anti-apoptotic) – were individually stably integrated in producer cell genome. How candidate gene overexpression level influence vector yields was analyzed by establishing engineered cell populations with incremental genomic copies of each. γ-RV volumetric productivity raised up to 97% when overexpressing genes associated to ER protein processing. LV volumetric production was increased in 53% when overexpressing the anti-apoptotic gene. Improvements were associated with higher specific cell productivities and dependent on gene overexpression level. Highlighting the importance of fine-tuning gene expression levels to achieve improved cell productivities. ER protein processing and anti-apoptotic mechanisms were validated has pivotal to producer cell performance. Overall, this work disclosed gene engineering targets enabling efficient gene therapy product manufacture.

Chapter IV

4.1 Introduction

Human embryonic kidney (HEK) 293 derived cell lines are the prevalent choice for gene therapy viral vectors manufacturing.[1] The main industrial advantages of these cell lines are the straightforward adaptation to suspension and serum free cultures. As a human cell line, HEK 293 offer the adequate replication environment and essential building blocks for virus assembly.

Gene therapies mediated by recombinant *Gammaretrovirus* and *Lentivirus* derived vectors (γ-RV and LV) comprise a quarter of all clinical trials and half of the virus-based commercially approved gene therapy products.[2] Currently, the yields of unprocessed supernatants from HEK 293 derived producer cell lines are in the order of 10^6 – 10^8 viral particles transducing units per milliliter (T.U./mL).[3–7] The low vector yields may compromise clinical trials feasibility and vector therapeutic accessibility. Hence, improved yields are desired, and, to that end, exploring approaches aiming to improve the production of γ-RV and LV vectors are important.

The manufacture of γ-RV and LV requires several steps occurring at the cellular level, from transcription, translation, glycosylation, and assembly of the viral vector components, to the particle budding. All these processes directly compete with cellular physiologic metabolism and some may become saturated. Furthermore, some of the vector components are toxic, such as the vesicular stomatitis virus envelope G glycoprotein (VSV-G), threatening cell viability.[8] Omics studies have shed the light on HEK 293 cells metabolism and how it may influence viral production performance.[1,9,10] In the context of γ-RV production, endoplasmic reticulum (ER) protein processing and anti-apoptosis regulation pathways were described has being highly recruited when HEK 293 cells are producing viral vectors. Higher levels of *PDIA2*, *HSPA5*, *XBP1* (ER protein

processing) and *BCL2* (anti-apoptotic) genes were identified as expression signatures of high producers.[11]

The *PDIA2* gene encodes an ER resident protein disulfide enzyme acting in disulfide bond formation and as molecular chaperone contributing for proteins three-dimensional structure stabilization.[12] The protein encoded by *HSPA5* gene is a binding immunoglobulin protein (BiP), the member 5 of the heat shock protein 70 family. Localized in the ER lumen, it is involved in several cellular processes, from ER protein folding, import and degradation to the unfolded protein response (UPR) activation.[13] The *XBP1* gene encodes a transcription factor intervening in UPR execution by activating the transcription of several genes involved in ER biogenesis, ER-associated protein degradation and molecular chaperones.[14,15] The *BCL2* gene codes for an apoptosis regulator protein involved in preventing the release of cytochrome c from mitochondria, an important step in the apoptotic cascade leading to caspase activation.[16] Overall, the protein products of these genes operate in essential networks of cellular mechanisms to guarantee protein folding homeostasis and control cell viability. To note that, directly (*HSPA5* and *XBP1*) or indirectly (*PDIA2*, *BCL2*), all of them contribute to the cellular UPR, a signaling cascade activated upon the accumulation of misfolded proteins to restore ER homeostasis.[13,17]

Aiming to improve γ-RV and LV vectors yields, in this work we studied the influence of overexpressing these ER protein processing (*PDIA2*, *HSPA5* and *XBP1*) and anti-apoptotic regulator (*BCL2*) genes in HEK 293 derived producer cells. As producer cell hosts we selected the 293 FLEX S11[5,18] – a stable and constitutive γ-RV producer cell line – and HEK 293 T cells – vastly employed in transient biopharmaceutical production and known to enable higher LV titers compared to its HEK 293 counterpart.[19,20] The employed genetic engineering strategy efficiently targeted gene overexpression and permitted to infer how regulated gene expression

levels must be to boost HEK 293 cells productivity. The overexpression of ER protein processing related genes could increase up to 97% γ-RV production in a dose dependent manner. Whereas LV yields could be enhanced by 53% when overexpressing *BCL2* anti-apoptotic gene.

4.2 Materials and methods

Plasmids

pRRLsin MOCK is a self-inactivating LV transgene vector with a human phosphoglycerate kinase internal promoter and empty of any coding region.[5] This plasmid was used as a backbone to clone the target genes cDNA - human *PDIA2* (GENE ID 64714), *HSPA5* (GENE ID 3309), *XBP1* variant 1 (GENE ID 7494) and *BCL2* variant alpha (GENE ID 596). The target genes cDNAs were amplified by PCR from plasmids obtained from DNASU Plasmid Repository (Biodesign Institute, Arizona State University, Tempe, AZ, USA). Table 4.1 summarizes all primers, templates and backbones used in cloning procedures (end of Materials and methods section). All reactions were conducted using In-Fusion HD Cloning system (Takara, Mountain View, CA, USA). The resulting plasmids - pRRLsin *PDIA2*, pRRLsin *HSPA5*, pRRLsin *XBP1* and pRRLsin *BCL2* - and pRRLsin MOCK were used to produce LV stocks for genetic manipulation. The remaining plasmids required for the transient production of LV were kindly provided by Prof. Didier Trono from the Swiss Federal Institute of Technology (EPFL) through Addgene plasmid repository (Cambridge, MA, USA). pRRLSIN.cPPT.PGK-GFP.WPRE is a self-inactivating LV transgene vector (Addgene plasmid 12252). pMDLg/pRRE (Addgene plasmid 12251) and pRSV-REV (Addgene plasmid 12253) are third generation LV packaging constructs containing HIV-1 *gag-pro-pol* and the second and third exons of HIV-1 *rev*, respectively. pMD2G (Addgene plasmid 12259) expresses the VSV-G envelope.

Cell lines and culture conditions

HEK 293T is a HEK 293 derived cell line expressing the large T antigen from SV40 (ATCC CRL-3216). This cell line was used to i) transiently produce LV stocks for genetic manipulation, ii) titrate LV and as iii) target LV producer cell line for genetic engineering. 293 FLEX S11 is a HEK 293 derived cell line stably producing moloney murine leukemia virus based γ-RV, pseudotyped with gibbon ape leukemia virus envelope and harboring a LacZ reporter gene. These cells were established from HEK 293 FLEX[18] by recombinase mediated cassette exchange as described in [5]. These cells were used as target γ-RV producer cell line for genetic engineering.

RD is human rhabdomyosarcoma-derived cell line (ATCC CCL-136) and was used to quantify transducing units (T.U.) titer of γ-RV. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, VA, USA) with 25 mM of glucose, 4 mM of glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Paisley, UK) at 37 ºC inside an incubator with a humidified atmosphere containing 8% CO_2. Cell concentration and viability were assessed by trypan blue exclusion method.

Genetic engineering

293 FLEX S11 or HEK 293T cells were modified by transducing 1 million cells with LV generated to codify the respective metabolic gene. For that purpose, the plasmids pRRLSIN MOCK, pRRLsin *PDIA2*, pRRLsin *HSPA5*, pRRLsin *XBP1* or pRRLsin *BCL2* were used. Transduction was performed using a multiplicity of infection (MOI) of 5 T.U./cell, in a final volume of 1 ml at seeding time, in DMEM supplemented with 10% (v/v) FBS and 8 μg/mL of polybrene (Sigma-Aldrich, St Louis, MO, USA). Four hours post-transduction, 1 ml of DMEM with 10% (v/v) FBS was added. To obtain a MOI of 10 the cell population established previously with a

MOI of 5 was transduced again with and MOI of 5. This was sequentially repeated to attain cell populations engineered with MOIs up to 25. This procedure guaranteed multiple copies per cell to establish populations with increased gene overexpression levels in a controlled manner, ranging from a MOI 5 up to 25 (Fig. 4.1A).

RNA and genomic DNA extraction

Total RNA was isolated using RNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany), eluted in 60 µl of nuclease-free water (Qiagen) and stored at -85 °C. cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany), using 2 µg of total RNA and oligo dT primer for mRNA reverse transcription. Extraction of genomic DNA was performed using DNeasy® Blood & Tissue kit (Qiagen) and stored at -20 °C. All kits were used according to manufacture instructions.

Real Time quantitative PCR for gene expression and copy number

Real Time quantitative PCR was performed in a thermocycler LightCycler 480 Real-Time PCR System (Roche Applied Science) using the LightCycler 480 SYBR Green I Master (Roche Applied Science) PCR kit. The housekeeping ribosomal protein L22 (RPL22) gene was used as reference gene. Primers sequence for gene expression and genomic integration quantification are listed in Table 4.1 (end of Materials and methods section). For the assessment of gene or WPRE integration copy number a total of 200 ng of genomic DNA was used in the PCR reaction. The number of γ-RV vector transgene copies per cell was quantified relative to a single copy control (293 FLEX S11 - parental cells) after normalization to the reference housekeeping gene. Single copy was considered for ratio of ‘analysis vs single-copy control’ below 1.4.[21]

Viral vector production

For γ-RV, 293 FLEX S11 cells were seeded at 8 x 10^4 cells/cm^2 in 6 well plates. Medium was exchanged 48 hours after seeding and the supernatant from the following 24 hours period was harvested. For LV transient production, 1 x 10^5 cells/cm^2 were transfected at the time of cell seeding in 25 cm^2 T-flasks. Cells were transfected using linear 25 KDa polyethylenimine (PEI; Polyscience, Hirschberg an der Bergstrasse, Germany) with a mass ratio of 1:1.5 (DNA:PEI). Third generation LV vector packaging system[22] was used with a total of 3.3 µg of DNA per million cells: 1.8 µg transgene, 0.7 µg gag-pro-pol, 0.2 µg Rev and 0.6 µg envelope. Culture medium was replaced every day after 24 hours post-transfection and supernatant was harvested every day 48 hours post-transfection. Supernatants containing viral vectors were clarified using a 0.45 µm cellulose acetate filter, aliquoted and stored at - 85 °C until further use.

Viral vector particles quantification

LV particles harboring transgenes coding for *PDIA2*, *HSPA5*, *XBP1* or *BCL2* target genes, as well as MOCK controls, were quantified for T.U. titer using a modified version of the universal real-time quantitative PCR assay described in detail in Barczak et al. (2014).[23] Target cells were substituted by 293 FLEX S11 and albumin reference gene was replaced by LacZ gene which is validated for single copy integration (N=1) in Coroadinha et al. (2006).[18] Primers are listed in Table 1. Quantification of γ-RV T.U. titer was performed as previously described.[24] Titration of LV T.U. harboring a GFP transgene was performed using a functional cell based assay as described elsewere.[6] Physical LV particles titer was assessed by HIV-1 P24 protein quantification in viral supernatants using the INNOTEST HIV Antigen mAb (Fujirebio Diagnostics, Inc., Malvern, PA, USA) enzyme-linked immunosorbent assay (ELISA), according to the

manufacture instructions. It was assumed that 1 pg of P24 corresponds to $1.25x10^4$ physical particles (P.P.).[7]

Statistical analysis

GraphPad Prism 6 was used to calculate data set P values by unpaired two-tailed Student's t-test. P values ≤ 0.05 were considered significant.

Table 4.1 - Primer sequences for cloning, gene expression and genomic copy number assessment

		Insert			
	Final Construct	**Fragment**	***DNASU**	**Backbone**	**Primers 5' → 3' sequence**
Cloning	pRRLsin PDIA2	PDIA2	GENE ID 64714	pRRLsin Mock	F - GACCTCTCTCCCCAGCCACCATGAGCCGCCAGCTTCTG R - TACAGCTCGTGCTAGGGACCCCATAGTGGAGTTGG
	pRRLsin HSPA5	HSPA5	GENE ID 3309		F - CGGGCCCGGGATCCACCATGAAGCTCTCCCTGGTG R - CATGGTGGCGACCGGCAACTCATCTTTTTCTGCTG
	pRRLsin XBP1	XBP1	GENE ID 7494		F - CGGGCCCGGGATCCACCATGGTGGTGGTGGCAGCC R - CATGGTGGCGACCGGCAAGTTCATTAATGGCTTCC
	pRRLsin BCL2	BCL2	GENE ID 596		F - CGGGCCCGGGATCCACCATGGCGCACGCTGGGAGA R - CATGGTGGCGACCGGCTTGTGGCCCAGATAGGCAC

	Target sequence	**Primers 5' → 3' sequence**
Real Time quantitative PCR	RV transgene (LacZ)	F - ACTATCCCGACCGCCTTACT R - TAGCGGCTGATGTTGAACTG
	RV GagPol	F - GTCCACTATCGCCAGTTGCT R - CTGGGTCCTCAGGGTCATAA
	RV envelope	F - GGACCAAAATAGCGAATGGA R - GGTGAACTGTACGCCTGGAT
	WPRE	F - ACTGTGTTTGCTGACGCAAC R - ACAACACCACGGAATTGTCA
	PDIA2	F - CCAGGAGATACCCCCTGATT R - GATGTCCTCGTGGTCTTGGT
	HSPA5	F - CTTCTGGGTACATTTGATCTG R - CGATTCTGGTCATTGGTG
	XBP1	F - GCTGGAACAGCAAGTGGTAG R - CCACTGGCCTCACTTCAT T
	BCL2	F - AGGATTGTGGCCTTCTTTGA R - ACAGTTCCACAAAGGCATCC
	RPL22	F - CTGCCAATTTTGAGCAGTTT R - CTTTGCTGTTAGCAACTACGC

*DNASU Plasmid Repository (Biodesign Institute, Arizona State University, Tempe, AZ, USA).

4.3 Results

Overexpression of ER protein processing and anti-apoptotic genes in HEK 293 γ-RV producer cells

To study the effect of the different ER protein processing (*PDIA2*, *HSPA5*, *XBP1*) and anti-apoptotic (*BCL2*) genes in γ-RV production of HEK 293 derived cells, these genes were stably overexpressed following an increasing gene load strategy as schematized in Figure 4.1A. Candidate genes were delivered to the producer cell line through LV transduction. MOCK controls were created transducing cells with LV lacking any coding gene. A fixed MOI of 5 T.U./cell was used to transduce all cells, bypassing the need of antibiotic selection. Cell populations with incremental MOI's (5, 10, 15, 20 and 25) were established by repeating the transduction step and thus, generating MOCK, *PDIA2*, *HSPA5*, *XBP1* and *BCL2* cell populations with increasing copy number integrations (Fig. 4.1B). For cell populations engineered with MOI 10, 15, 20 and 25, the cassette mRNA levels (Fig. 4.1C) increased on average 3.7, 4.8, 6.1 and 6.6-fold in relation to the corresponding population engineered with MOI 5, respectively. *HSPA5* and *XBP1* engineered populations exhibited lower cassette mRNA levels (Fig. 4.1C), however, this was not due to lower genomic insertions (Fig. 4.1B).

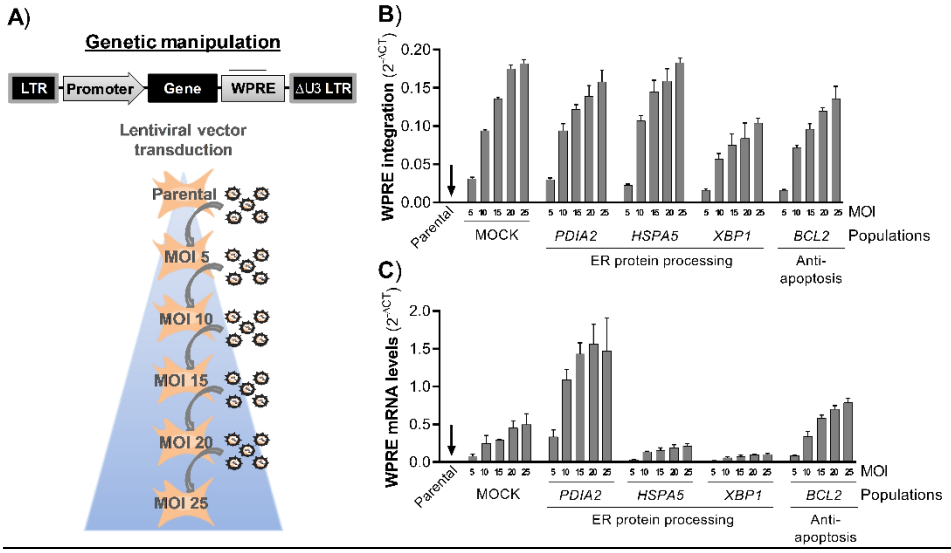

Figure 4.1 – Genetic engineering of HEK 293 γ-RV producer cells with ER protein processing and anti-apoptotic pathway genes. A) Schematic representation of the experimental strategy used to establish cell populations with increasing integrated gene copies. The candidate gene was cloned into a self-inactivating LV transfer vector and stably integrated in producer cell genome by transduction using a MOI of 5 T.U./cell. Repeated and step wise transductions were performed to establish populations with increased relative gene copy number (ranging from a MOI 5 up to 25). The bar in top of WPRE sequence symbolizes the common amplicon used to assess the integration copy number and overexpression level of all engineered populations. B) Relative WPRE genomic DNA copy number in engineered populations for each of the candidate genes (*PDIA2*, *HSPA5*, *XBP1* or *BCL2*) or MOCK control. C) WPRE mRNA levels in cell populations engineered with *PDIA2*, *HSPA5*, *XBP1* or *BCL2* gene (or MOCK control). WPRE copy number and mRNA levels were normalized to RPL22 housekeeping gene. Data shown represents mean +/- SD from 3 independent experiments. Arrows indicate undetected gene expression. WPRE - Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element. PDIA2 - Protein Disulfide Isomerase Family A Member 2. HSPA5 - Heat Shock Protein Family A (Hsp70) Member 5. XBP1 - X-box binding protein 1. BCL2 - B-cell lymphoma 2 protein.

Candidate genes overexpression was also analyzed and confirmed (Fig. 4.2). The latter results also showed the parental candidate genes basal expression level and the low MOCK populations expression variability. *HSPA5* (Fig. 4.2B) and *XBP1* (Fig. 4.2C) were the candidate genes with the highest basal expression levels (parental cells) and those showing the lowest overexpression increment.

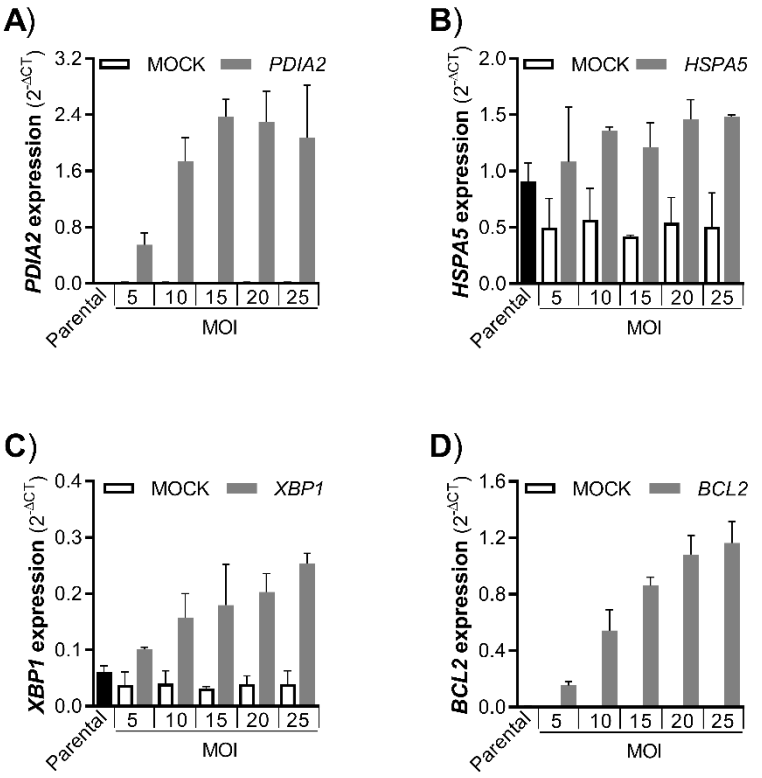

Figure 4.2 – Candidate genes mRNA expression in parental, MOCK controls and engineered HEK 293 γ-RV producer cells with: A) *PDIA2*, B) *HSPA5*, C) *XBP1* or D) *BCL2* gene. Gene expression was normalized to *RPL22* housekeeping gene. Data shown represents mean +/- SD from 3 independent experiments. WPRE - Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element. PDIA2 - Protein Disulfide Isomerase Family A Member 2. HSPA5 - Heat Shock Protein Family A (Hsp70) Member 5. XBP1 - X-box binding protein 1. BCL2 - B-cell lymphoma 2 protein.

Overexpression of ER protein processing pathway genes increase γ-RV production

Engineered HEK 293 γ-RV producer cells overexpressing either *PDIA2*, *HSPA5*, *XBP1* or *BCL2* gene were characterized for T.U. volumetric and cell specific productivity. An upswing pattern was observed for all cell populations engineered with ER protein processing genes (Fig. 4.3A), i.e. T.U. volumetric titer increased with higher target gene insertion level (MOIs). The highest T.U. volumetric titer improvements were of 37%, 45% and 97%, when overexpressing *PDIA2*, *HSPA5* and *XBP1* genes, respectively. These results were associated to a significant increase in cell specific γ-RV productivity (Fig. 4.3B). *BCL2* cell populations (anti-apoptotic gene) presented similar specific γ-RV T.U. productivity levels as parental and MOCK control cells (Fig. 4.3A and Fig. 4.3B). These results suggest ER protein processing genes has debottlenecking targets for enhanced γ-RV production of HEK 293 cells, but not *BCL2* anti-apoptotic gene.

The expression of the γ-RV viral cassettes was assessed through mRNA analysis (Fig. 4.3C). The Gag-Pro-Pol and envelope expression were within the biologic variability range observed for the parental cells. However, the transgene expression showed a trend to increase as the copy number of the candidate genes raised (i.e. higher MOIs). Cell populations engineered with MOI 25 of *HSPA5*, *XBP1* (upswing) and *BCL2* (no change) genes modestly surpassed parental transgene expression variability. The tendency to increase γ-RV transgene expression levels in genetic engineered producer cells had been reported in previous works.[5,25] The γ-RV transgene genomic cassette copy number was quantified (Supplementary data Fig. S4.1) and all engineered cell populations maintained the same copy number as parental cells.

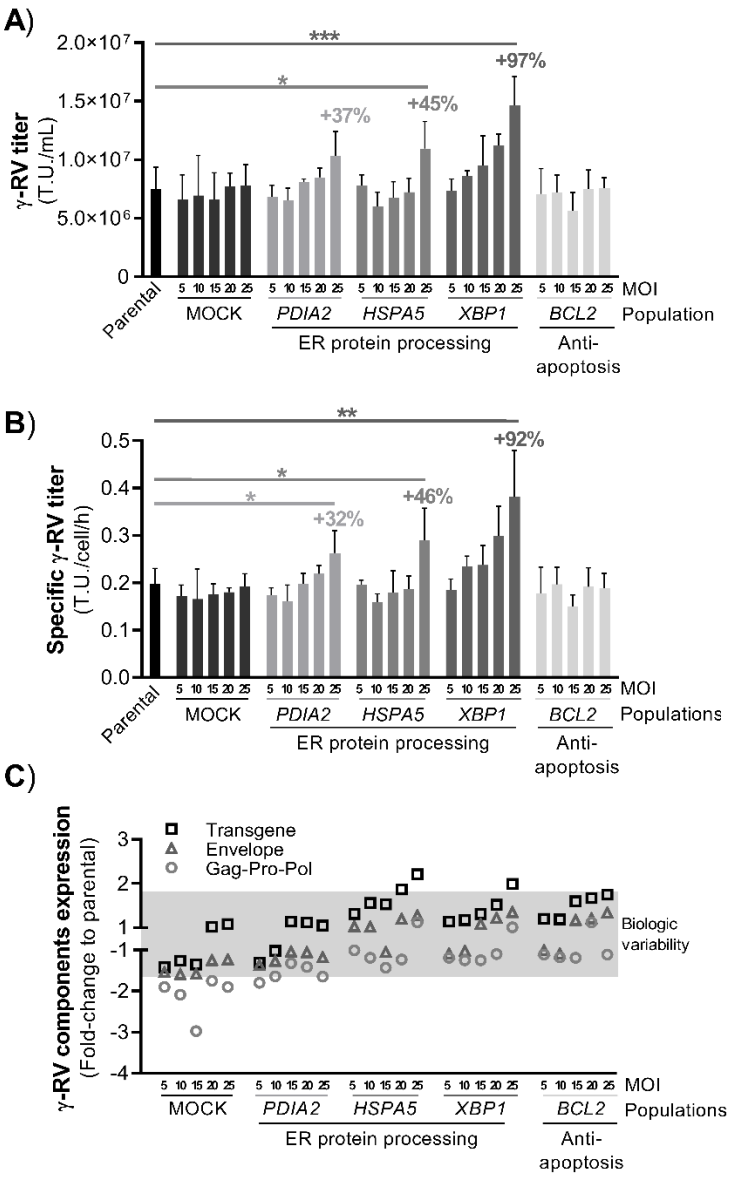

Figure 4.3 – Overexpression of ER protein processing and anti-apoptotic pathway genes in HEK 293 γ-RV producer cells: A) transducing particles volumetric yields; B) transducing particles cell specific production; C) vector components cassette expression; Data shown represents mean +/- SD from 3 independent experiments. The probability of significant differences between two groups (parental vs engineered population MOI 25) was assessed using unpaired two-tailed Student's t-test. P values < 0.05 were considered statistically significant: * P < 0.05; ** P < 0.01; *** P < 0.001. PDIA2 - Protein Disulfide Isomerase Family A Member 2. HSPA5 - Heat Shock Protein Family A (Hsp70) Member 5. XBP1 - X-box binding protein 1. BCL2 - B-cell lymphoma 2 protein. T.U. – Transducing Units.

Overexpression of ER protein processing and anti-apoptotic genes in HEK 293T LV producer cells

To study the potential of ER protein processing and anti-apoptosis related genes in LV production, HEK 293T cell line was engineered using a similar genetic engineering strategy as previously described. Considering i) the cytotoxic nature of LV Gag-Pro-Pol and VSV-G components expression and ii) transient LV manufacture related labor-intensive procedures; target genes were reduced to one from each studied pathway: *BCL2* (anti-apoptosis) and *XBP1* (ER protein processing). Engineered cell populations of MOI 10 and 25 were first characterized.

Engineered HEK 293T cell populations with increased candidate gene load were successfully generated (Fig. 4.4A). On average, the established cell populations of MOI 25 showed 2.5-fold increased cassette mRNA levels (Fig. 4.4B) relatively to the MOI 10 cell population. The expression levels of each candidate gene also increased from MOI 10 to 25 (Fig. 4.4C and Fig. 4.4D). *BCL2* expression in the parental and MOCK cells was undetectable, suggesting absence or very low basal expression levels (Fig. 4.4D).

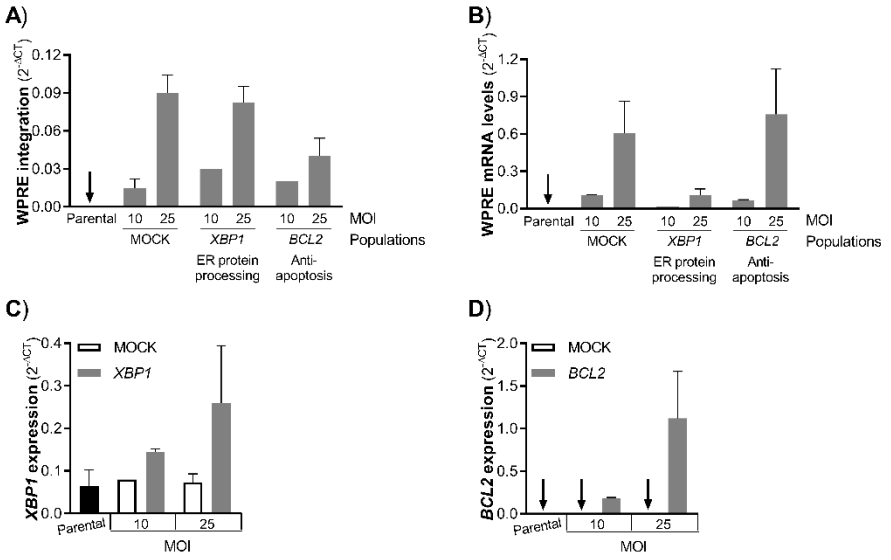

Figure 4.4 – Genetic engineering of HEK 293T LV producer cells with ER protein processing and anti-apoptotic pathway genes. A) Relative WPRE genomic DNA copy number in engineered populations with each of the target genes (*XBP1* or *BCL2*) or MOCK control. B) WPRE mRNA levels of HEK 293T cell populations engineered with *XBP1* or *BCL2* gene (or MOCK control). C) *XBP1* expression in HEK 293T parental cells, MOCK control and populations engineered with *XBP1* gene. D) *BCL2* expression in HEK 293T parental cells, MOCK control and populations engineered with *BCL2* gene. Relative genomic copy number and mRNA levels were normalized to RPL22 housekeeping gene. Data shown represents mean +/- SD from 3 independent experiments except for MOI 10 HEK 293T cell populations (n=1). Arrows indicate undetected gene expression. WPRE - Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element. XBP1 - X-box binding protein 1. BCL2 - B-cell lymphoma 2 protein.

BCL2 overexpression improves LV transient production

Engineered HEK 293T cell populations overexpressing *XBP1* or *BCL2* for the highest MOI (25) were used to transiently produce LV. Engineered and parental cells, transiently transfected or non-transfected controls, presented similar cell growth and viability (Fig. 4.5A). These cells LV productivity was monitored during four days after transfection. The T.U. volumetric production peak occurred two days after transfection. For this

optimal harvesting time, engineered cell populations showed volumetric T.U. increments of 27% for *XBP1* and 53% for *BCL2* overexpressing cells (Fig. 4.5B). Only *BCL2* engineered cell population presented a significant improvement in specific productivity (Fig. 4.5C). Milder improvements could be hindered by the high variability occurring in LV transient transfection productions. Total physical particles content (Fig. 4.5D) was similar for all vector preparations from different producer cells. Thus, the higher T.U. volumetric LV titer, obtained in *BCL2* engineered cell population, represents improved viral vector preparation quality (Fig. 4.5D).

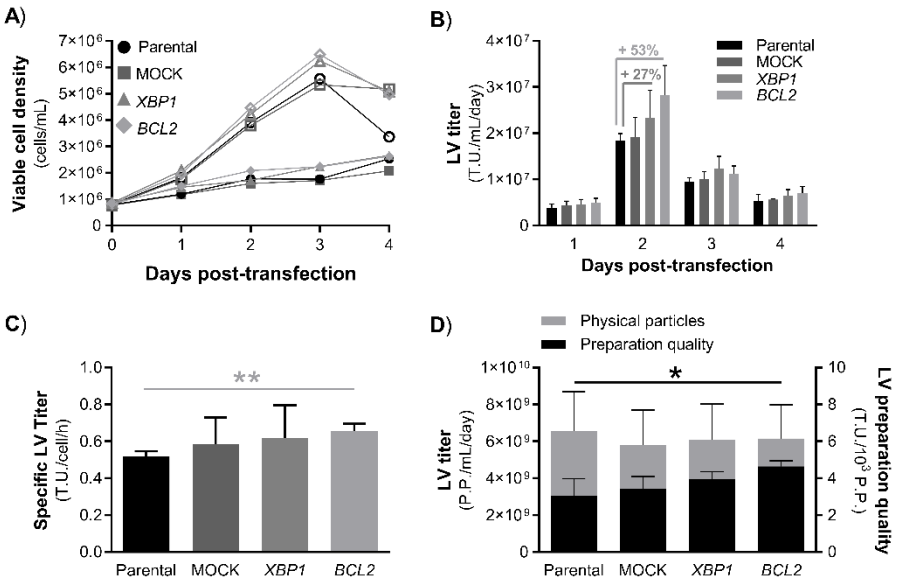

Figure 4.5 – Overexpression of ER protein processing and anti-apoptotic pathway genes in HEK 293T LV producer cells: A) cell growth and viability of transfected (full symbols) and non-transfected cells (empty symbols); B) transducing particles volumetric yields; C) transducing particles cell specific production; D) LV total particles volumetric yield and LV preparation quality ratio. Data shown represents mean or mean +/- SD from 3 independent experiments. The probability of significant differences between two groups (parental vs engineered population MOI 25) was assessed using unpaired two-tailed Student's t-test. P values < 0.05 were considered statistically significant: * P < 0.05; ** P < 0.01. XBP1 - X-box binding protein 1. BCL2 - B-cell lymphoma 2 protein. T.U. – Transducing Units.

4.4 Discussion and conclusions

Gene therapy success is driving the industry to develop high-yielding recombinant virus bioprocesses, namely robust cell substrates for the manufacture of γ-RV and LV. HEK 293 derived cells are the standard platforms to produce those vectors. Thus, understanding how HEK 293 yields can be improved will be important to meet the growing gene therapy clinical demands.

When producing recombinant viral particles, a burden is imposed on the cell machinery, especially when expressing toxic proteins, such as the VSV-G and the HIV-1 Gag-Pro-Pol.[6,26] In previous work, genes related to ER protein processing and anti-apoptotic pathways were identified as being recruited for the cell γ-RV producer state.[11] To understand the impact of those candidate genes in γ-RV and LV production yields of HEK 293 cell, we developed a genetic engineering strategy based on stably increasing the overexpression level of those genes (Fig. 4.1A). Other strategies, such as transient transfection and medium supplementation experiments are useful to obtain preliminary information on how a certain gene and metabolic pathway affects a desired phenotype in a high throughput fashion. However, the stable overexpression of a gene is preferable for manufacturing, assuring long term and reproducible effects without the need to use often expensive supplements. Therefore, the candidate genes (*PDIA2*, *HSPA5*, *XBP1*, *BCL2*) cDNAs were stably integrated in HEK 293 derived producer cells. Rational manipulation of ER protein processing and anti-apoptotic cell metabolic pathways to improve cellular properties, increase product yield and quality and cells life span, has been explored.[27–31] However, none of the gene targets studied in this work were explored in the context γ-RV and LV manufacture. Also, were never applied with controlled gene copy number addition.

Even though cassette integration levels were similar among engineered γ-RV producer cell populations (Fig. 4.1B), the cell populations engineered with *HSPA5* or *XBP1* gene presented the lowest levels of cassette expression for all tested sequences (Fig. 4.1C, Fig. 4.2B, and Fig. 4.2C). These genes were also the ones presenting the highest basal expression levels in parental cells (Fig. 4.2B and Fig. 4.2C). Thus, depending on the gene of interest, cells transduced with the same dose of integration-competent vectors lead to different transgene expression levels. This effect can be attributable to the target cell intrinsic expression regulation.[32] Moreover, all MOCK populations (γ-RV producers and HEK 293T cells) presented comparable expression cassette genomic copy number as the cell populations established with the candidate genes under study (Fig. 4.1B and Fig. 4.4A). As well as similar basal candidate genes expression levels (Fig. 4.2, Fig. 4.4C, and Fig. 4.4D) and vector productivities (Fig. 4.3 and Fig. 4.5) as the parental counterparts. These results suggested that the employed genetic engineering strategy did not interfered with the expression of the genes under study or with the viral vector productivities. Additionally, none of the engineered cell populations decreased cell productivity performance, indicating that the candidate genes herein studied, with the overexpression levels obtained, were not detrimental to vector production.

Transducing competent γ-RV yields were significantly improved, in terms of cell specific productivity, with the overexpression of ER protein processing related genes, but not *BCL2* anti-apoptotic gene (Fig. 4.3A and Fig. 4.3B). Therefore, we suggest ER protein folding as an essential pathway for viral vector synthesis. Also, our results could indicate improved γ-RV preparation quality (higher T.U. per P.P.), since engineering cell metabolism often impacts γ-RV stable production by increasing transducing particles titers without altering total physical particles yields.[5,11,25,33] For instance, this improved γ-RV preparation

quality might result from better envelope glycoprotein formation (complex protein composed of two subunits linked by disulfide bonds) due to *PDIA2* and *HSPA5* gene overexpression.[34]

All overexpressed ER protein processing genes under study, directly or indirectly, participate in the UPR activation. Since both recombinant viral vectors production yields were improved with the overexpression of ER protein processing related genes, we can hypothesize that unfolded proteins are being accumulated, possibly due to the high protein synthesis required. In that context, the vector components mRNA levels - either constitutive (Fig. 4.3C) or transient - may not be limiting the yields. Instead, vector preparations might be affected by the cells insufficient capacity to provide correct protein folding, generating non-functional vectors.[35,36] Moreover, *XBP1* also has a role in lipid biosynthesis and N-glycan structural remodeling.[37,38] Therefore, the increase in transducing competent particles (Fig. 4.3A and Fig. 4.5B) could also result from the improved envelope glycoproteins synthesis, glycosylation pattern and/or improved vector lipid bilayer properties, which are known to play important roles in vectors biologic activity.[24,33,39–41]

BCL2 overexpression did not had any impact on γ-RV productivities (Fig. 4.3A and Fig. 4.3B). Most probably due to the 24 hours batch manufacture process herein analyzed and the low or absent toxicity associated to γ-RV.[42] Although *BCL2* did not altered cell specific productivity herein, it cannot be excluded it may improve bioprocess and volumetric yields on long-term bioreactions, as shown by other authors.[27,43–48]

VSV-G pseudotyped LV particles present a broad tropism, high particle yields and bioprocess resistance, representing the most used LV. However, VSV-G expression is highly toxic[8], shortening manufacture bioreaction campaign due to loss of cell viability. Herein, *BCL2* overexpression improved 53% the T.U. volumetric titer and showed increased cell specific titers. Yet, *BCL2* overexpression did not enabled

bioreaction extension, most probably due to the transient transfection nature of this process and high cytotoxicity of VSV-G. However, HEK 293T *BCL2* engineered population growth, with and without transfection, registered slightly higher cell densities (Fig. 4.5A), which could indicate improved cell robustness. Overall, the work herein conducted point has main limitation for LV production the capacity of HEK 293T cells to remain viable during bioprocess time which has also been supported by others.[49]

In γ-RV producer cells we observed an increment of vector productivity with increasing MOIs. This productivity effect suggests that higher candidate gene expressions could further improve the titers obtained in this study. Thus, it cannot be excluded that even higher vector productivity improvements could be attained through further increase of candidate gene copy number doses. Moreover, it is possible that within these cell populations, clonal cells might present higher γ-RV titer improvements. Indeed, other studies on metabolic engineering reported mild productivity enhancements for engineered populations and higher improvements in derived clones.[5,25]

In summary, through gene overexpression, this work validates ER protein processing and anti-apoptotic cell mechanisms has critical pathways for γ-RV and LV production. Functional γ-RV particles production benefited from ER protein processing engineering while LV functional titer seems to be enhanced through improved cell robustness by anti-apoptotic gene overexpression, highlighting the different viral vector production system requirements. Specifically, γ-RV stable and constitutive production requires protein processing checkpoints to cope with the possible congested protein formation in the ER. On the other hand, LV transient production requires cell viability improvement strategies to cope with the transfection procedure and expression of cytotoxic compounds. Such knowledge elucidates constrains on viral vector production in HEK 293

derived cells and supports the use of metabolic engineering as strategies to enhance gene therapy vector yields and quality.

4.5 Acknowledgments

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4.6 Supplementary data

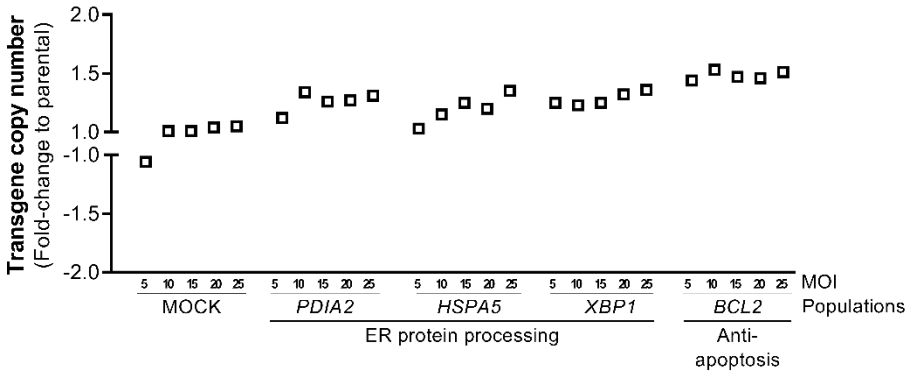

Figure S4.1 – Engineered γ-RV producer cells transgene cassette relative genomic copy number. Data shown represents mean from 3 independent experiments. PDIA2 - Protein Disulfide Isomerase Family A Member 2. HSPA5 - Heat Shock Protein Family A (Hsp70) Member 5. *XBP1* - X-box binding protein 1. BCL2 - B-cell lymphoma 2 protein. T.U. – Transducing Units.

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CHAPTER V

Glutathione metabolic genes

This chapter was adapted from

Formas-Oliveira AS, Rodrigues AF, Gelder V, Vaz T, Coroadinha AS. Improving gene therapy retroviral vector production by genetic engineering glutathione metabolism (*in preparation*)

Author contribution

Ana Oliveira designed and participated in the experimental setup, analyzed the data and wrote the chapter

Abstract

Recombinant retrovirus, namely gammaretroviral and lentiviral vectors (γ-RV and LV), are efficient gene delivery systems in gene therapy applications aiming to permanently express a therapeutic gene. The success of immune therapies clinical trials and the growing approval of gene therapy-based products increased the demand for robust and high-yielding production hosts for these viral vectors. Still, its manufacture is currently characterized by insufficient vector titers in crude producer cell supernatant. Thus, in this work we genetically engineered glutathione metabolism, described to be up-regulated in high γ-RV producers, aiming to improve recombinant retrovirus production.

Eight glutathione metabolic genes were overexpressed, at increasing doses, using two different genetic engineering strategies in HEK 293 derived producer cell substrates. Different dynamics in cells producer state were observed - from downswing to upswing - depending of the metabolic gene and its overexpression level. Cell specific productivity was raised up to 13-fold for γ-RV and 1.2-fold for LV. The γ-RV high-titer yielding manipulations were associated to the overexpression of genes codifying for enzymes of cysteine formation through the transsulfuration pathway (CBS and CTH) and glutathione mediated detoxification (GSTM1). LV titer improvements were achieved by overexpressing a rate limiting enzyme of pentose phosphate pathway, *G6PD*, which feeds the redox cycle with NADPH.

The results herein described confirm glutathione metabolism has a genetic engineering target to improve recombinant retrovirus manufacture and support strategies of controlling host metabolic gene expression to achieve high vector yield phenotypes.

Chapter V

5.1 Introduction

Gammaretroviral and lentiviral vectors (γ-RV and LV) are recombinant retrovirus widely used in gene therapy due to its long-term expression provided by the genome integration into target cells. This characteristic enables permanent expression of a gene of interest, which is extremely useful for numerous gene therapy disease indications, such as, monogenic diseases and cancer. Over the past years, both recombinant retroviral vectors represent more than 25% of the used vectors in gene therapy clinical trials and have also been leading the number of virus-based gene therapy market approved products [1,2]. Nevertheless, the manufacture of these vectors is hampered by low cell supernatant titers, limiting vector availability. Hence, robust and high yielding cell hosts are required to develop high-quality production bioprocesses able to support vector supply and reduce health care costs [3].

Metabolic genetic engineering – gain of cell functions delivered by a set of key genes – offers the means to develop high producer cell hosts by providing an optimized cell environment for product synthesis [4,5]. Although widely studied to improve small molecules production, the use of metabolic genetic engineering was far less explored for complex products manufacture, such as viral vectors [6–15]. In a recent study, a set of genes associated to γ-RV production were identified based on the transcriptome analysis of constitutive producer cells. This study described evident changes in the expression of metabolic genes when cells undergo to the producer state and between high and low producer cell substrates. One of the key metabolic pathways presenting gene expression upregulastion when cells transit to the producer state to high producers was glutathione metabolism [16]. The contribution of glutathione metabolism genes expression to the producer state should be further explored since imbalanced host cellular redox environment is

a known consequence of retrovirus infection, replication and recombinant vectors production [16,17]. Moreover, additional cellular functions related to glutathione metabolism may also contribute to improve producer cell yields, namely: i) cysteine storage, which can be redirected to protein production; ii) correction of protein disulfide bond formation in hyperoxidizing conditions and, iii) assisting the formation of disulfide bonds of secreted protein synthesis in endoplasmic reticulum [18]. Therefore, in this work we conducted a systematic gene engineering study to evaluate the effect of individually overexpressing eight glutathione metabolic genes in recombinant retrovirus producer cell yields.

Previous metabolic engineering studies reported viral vector productivity improvements as a result of gene expression manipulation disregarding the level of up or down regulation, and/or focused on clonal effects, rather than average population changes. We hypothesize that to fully understand how a given gene modulates cells productivity phenotype, the relation between its expression level and the resulting producer cell yield dynamics should be analyzed. To this end, we genetic engineered γ-RV and LV producer cells with each glutathione metabolic gene at different gene doses. Five out of the eight glutathione metabolic genes generated a gene dose response in γ-RV producer cells specific productivity. Transducing γ-RV titers were increased up to 13-fold when overexpressing genes associated with cysteine formation and glutathione mediated detoxification. Regarding LV yields, specific production was improved 1.2-fold in cells overexpressing a glutathione metabolic gene intervening in feeding the redox cycle with NADPH.

5.2 Materials and methods

Plasmids

The plasmids providing the helper functions to transiently produce the LV batches for gene overexpression were kindly provided by Prof. Didier Trono from the Swiss Federal Institute of Technology (EPFL) through Addgene plasmid repository (Cambridge, MA, USA). pRRLSIN.cPPT.PGK-GFP.WPRE is a self-inactivating (SIN) LV transfer vector (Addgene plasmid 12252) with a human phosphoglycerate kinase (hPGK) internal promoter controlling the expression of eGFP. pMDLg/pRRE (Addgene plasmid 12251) and pRSV-REV (Addgene plasmid 12253) are third generation LV packaging constructs containing *gag-pro-pol* expressed from cytomegalovirus (CMV) promoter and the second and third exons of *rev* expressed from Rous sarcoma virus promoter, respectively. pMD2G (Addgene plasmid 12259) expresses the envelope G glycoprotein of the vesicular stomatitis virus (VSV-G) under the control of the CMV promoter.

pRRLsin MOCK is a SIN LV transfer vector derived with a hPGK internal promoter and split-GFP S11 fragment coding region [15]. It was used as backbone to clone the glutathione metabolism genes – human *GSR* (GENE ID 2936), *GPX7* (GENE ID 2882), *GSTM1* (GENE ID 2944), *GSS* (GENE ID 2937), *CBS* (GENE ID 875), *CTH* (GENE ID 1491) and *IDH1* (GENE ID 3417) – which were amplified by PCR from the cDNA carrying plasmids obtained from DNASU Plasmid Repository (Biodesign Institute, Arizona State University, Tempe, AZ, USA). The resulting plasmids - pRRLsin GSR, pRRLsin GPX7, pRRLsin GSTM1, pRRLsin GSS, pRRLsin CBS, pRRLsin CTH and pRRLsin IDH1 - and pRRLsin MOCK were used as transfer vectors to produce LV batches used for gene overexpression in γ-RV constitutive producer cells.

pLenti CMVtight eGFP Puro (w771-1) was a gift from Eric Campeau (Addgene plasmid 26431) and is a SIN LV transfer vector. This plasmid codifies for eGFP under the control of a chimeric CMV/tetracyclin responsive promoter (CMVtight) and puromycin resistant marker expressed from hPGK. This plasmid was used as backbone to clone: i) the simian vacuolating virus 40 promoter and tetracycline-controlled transactivator after removal of hPGK and ii) the mCherry reporter protein or the previously mentioned glutathione metabolisc genes by replacing eGFP. The resulting plasmids - pLenti CMVtight MOCK, pLenti CMVtight GSTM1, pLenti CMVtight GSS, pLenti CMVtight CBS and pLenti CMVtight IDH1 - were used as transfer vectors to produce LV batches used for gene overexpression in transient LV HEK 293T producer cells.
Table S5.1 (Supplementary data) resumes all primers, templates and backbones used in cloning procedures. All reactions were conducted using In-Fusion HD Cloning system (Takara, Mountain View, CA, USA).

Cell lines and cell culture conditions

293 FLEX S11 is a human embryonic kidney (HEK 293) derived cell line stably producing moloney murine leukemia virus based γ-RV pseudotyped with gibbon ape leukemia virus envelope coding a LacZ reporter gene [15]. This cell line was developed from 293 FLEX by recombinase mediated cassette exchange and used as γ-RV producer cell model in gene engineering studies [15,19].
HEK 293T is a HEK 293 derived cell line expressing large T antigen from SV40 (ATCC CRL-3216). This cell line was used as: i) LV producer cell model in gene engineering studies; ii) cell host to produce the LV batches used to overexpress the glutathione metabolism genes in γ-RV and LV producer cell models; and iii) cell target for LV transducing units (T.U.) titration.

RD is human rhabdomyosarcoma-derived cell line (ATCC CCL-136). These cells were used as target cells to titer T.U. of γ-RV.

RD S10, a human rhabdomyosarcoma derived cell line (ATCC CCL-136) stably expressing GFP S1-10 fragment, described in Rodrigues et al. (2015) [15], was used as target cell to titer transducing γ-RV harboring GFP S11 fragment.

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) with 25 mM of glucose, 4 mM of glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco). Cells were maintained at 37 ºC inside an incubator with a humidified atmosphere containing 8% CO_2.

Cell concentration and viability were assessed by trypan blue exclusion method.

Cell specific growth rate (μ) at the exponential phase were calculated by linear regression using the concentration of viable cells (X) and the culture time (*t* in hours) given by: $\mu\ (h^{-1}) = \frac{1}{X} \cdot \frac{dx}{dt}$

Genetic engineering of producer cells

To engineer γ-RV constitutive producer cells, 1 x 10^6 cells/well were transduced in 6 well plates with LV coding for one of the eight glutathione metabolic genes (pRRLsin construct) at a multiplicity of infection (MOI) of 5 T.U./cell. Transduction was performed at seeding time with 1 mL of LV diluted in DMEM supplemented with 10% (v/v) FBS and 8 µg/mL of polybrene (Sigma-Aldrich, St Louis, MO, USA). After overnight incubation, 1 mL of DMEM supplemented with 10% (v/v) FBS was added. To guarantee synchronous infection and multiple copies per cell, step wise transduction phases were performed using a fixed MOI of 5 T.U./cell to establish cell populations with increased gene load, ranging from MOI 5 up to 25 T.U./cell (Fig. 4.1a).

To engineer transient LV producer cells, 0.5 x 10^6 cells/well were seeded in 6 well plates and incubated overnight. Cells were transduced with 1 mL of LV coding for one of the glutathione metabolic genes (pLenti CMVtight construct) using a MOI of 1 T.U./cell. Transduction was performed for 4 hours with LV diluted in DMEM supplemented with 10% (v/v) FBS and 8 µg/mL of polybrene. These cells were subjected to 1.5 µg/mL puromycin (Invivogen, San Diego, CA, USA) antibiotic selective pressure 48 hours post-transduction.

Genomic DNA and RNA extraction, cDNA synthesis and qPCR

Genomic DNA was extracted using DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany) according to manufacturer instructions and stored at -20 °C.

Total RNA was isolated using RNeasy® Blood & Tissue Kit (Qiagen) following the manufacturer instructions, eluted in 60 µl of nuclease-free water (Qiagen) and stored at -85 °C. cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) following manufacturer instructions, using 2 µg of total RNA and oligo dT primer for mRNA reverse transcription. The reverse transcribed product was diluted in nuclease-free water (Qiagen) with a final volume of 120 µl and stored at -20 °C.

Real Time quantitative PCR was performed in a thermocycler LightCycler 480 Real-Time PCR System (Roche Applied Science) using a LightCycler 480 SYBR Green I Master (Roche Applied Science) PCR kit. The housekeeping ribosomal protein L22 (RPL22) gene was used as reference gene. Primers sequence for all gene expression and genomic integration quantification are listed in Table S5.1 (Supplementary data).

For the assessment of the metabolic gene expression cassette integration copy number a total of 200 ng of genomic DNA was used to run the qPCR with primers against LV LTR.

The number of γ-RV transgene copies *per* cell was quantified relatively to a single copy control (293 Flex S11 - parental cells) after normalization to the reference gene. Single copy was considered for ratio of 'analysis vs single-copy control' below 1.4 [20].

Viral vectors production

Transient transfection procedure was used to generate LV to genetic engineer producer cells and to assess productivity changes of modified LV producer cell populations. Briefly, 1 x 10^4 cell/cm^2 of HEK 293T cells or derived engineered cell populations were transfected at time of seeding using 0.2 mL/cm^2 of culture volume. Linear 25 KDa polyethylenimine (PEI; Polyscience, Hirschberg an der Bergstrasse, Germany) was used has transfection agent at a mass ratio of 1:1.5 (DNA:PEI). Third generation LV vector packaging system was used with a total of 3.3 µg of DNA per million cells composed of 1.8 µg transfer vector, 0.7 µg *gag-pro-pol*, 0.2 µg *rev* and 0.6 µg envelope [21,22]. Cell culture medium was replaced using 0.14 mL/cm^2 24 hours post-transfection and supernatant was harvested 24 hours after. When assessing LV productivity of engineered LV producer cell populations, medium replacement at 24 hours post-transfection was also supplemented with 1 µg/mL of doxycycline (Sigma-Aldrich).

Stable and constitutive γ-RV production was used to assess productivity changes of engineered γ-RV producer cell populations. Briefly, producer cells were seeded in 6 well plates at 8 x 10^4 cells/cm^2 in 0.2mL/cm^2 of cell culture medium. Medium was exchanged 48 hours after. The supernatant from the following 24 hours period was harvested.

All cell supernatants containing viral vectors were clarified using a 0.45 µm cellulose acetate filter, aliquoted and stored at -85 °C until further use.

Viral vector transducing particles quantification

To quantify γ-RV T.U. a LacZ staining protocol was used. Briefly, target cells were seeded at 4 x 10^4 cells/cm^2 in 96-well plates and transduced with 80 µl of viral supernatant 24 hours after. Serial dilutions were performed in DMEM supplemented with 10% (v/v) FBS and 8 µg/mL of polybrene. Two days after transduction cells were fixed using a solution of formaldehyde at 0.3% (v/v) and 1.35% (v/v) glutaraldehyde in PBS and stained for 24 hours using a solution of 0.2 mg/mL X-gal (Stratagene, La Jolla, CA, USA), 5 mM $K_3Fe(CN)_6$ (Merck Millipore, Billerica, MA, USA), 5 mM $K_4Fe(CN)_6$ (Merck) and 1 mM $MgCl_2$ (Merck) in PBS. Transducing Units per mililiter (T.U./mL) was determined after counting manually the blue stained cells using the following equation:

$$T.U./mL = \frac{number\ of\ positive\ cells\ x\ dilution\ factor}{transduced\ volume\ (mL)}$$

Titration of LV T.U. was performed using two cell-based assay methods: i) eGFP positive cells quantification by flow cytometry [15,23] and ii) LV genome copy number quantification in target cells by qPCR [24]. Briefly, target cells were seeded at 5 x 10^4 cells/cm2 in 24 well plates. Transduction was performed 24 hours after in duplicates by removing the cell supernatant and transducing with 0.2 mL of viral suspension diluted in fresh DMEM with 10% (v/v) FBS and 8 µg/ml of polybrene (Sigma). Cell concentration was determined at the time of transduction. Following overnight incubation, 1 mL of fresh culture medium was added. Two days after infection, cells were either harvested for eGFP fluorescence analysis (CyFlow Space flow cytometer, Sysmex Partec GmbH, Görlitz, Germany) or for genomic DNA extraction.

The titers of LV harboring eGFP or split-GFP S11 fragment coding region were determined considering the transductions that rendered 2-

20% of GFP positive cells, the cell concentration at transducing time and the dilution factor. Titer was calculated using the following formula:

$$T.U./mL = \frac{\%\ GFP\ positive\ cells\ x\ \text{number of transduced cells}\ x\ dilution\ factor}{transduced\ volume\ (ml)}$$

The titers of LV harboring inducible glutathione metabolism transgenes were quantified by qPCR using 200 ng/reaction of transduced cells genomic DNA with adapted procedures of a methodology reported elsewhere [24,25]. Briefly, LV integration was quantified using primers against the LV LTR sequence and a genomic DNA standard curve prepared from cells transduced with LV with a known titer. RPL22 was used as reference gene. Primer sequences are listed in Table S5.1 (Supplementary data). These titers were calculated based on the following formula: $T.U./mL = 10^{\frac{\Delta CT - intercept}{slope}}$

5.3 Results

To study the effect of expressing glutathione metabolic genes in recombinant retrovirus producer cell yields, eight genes from this pathway (Fig. 5.1) – reported as being recruited when cells are producing γ-RV [16] – were overexpressed at different levels in producer cell models.

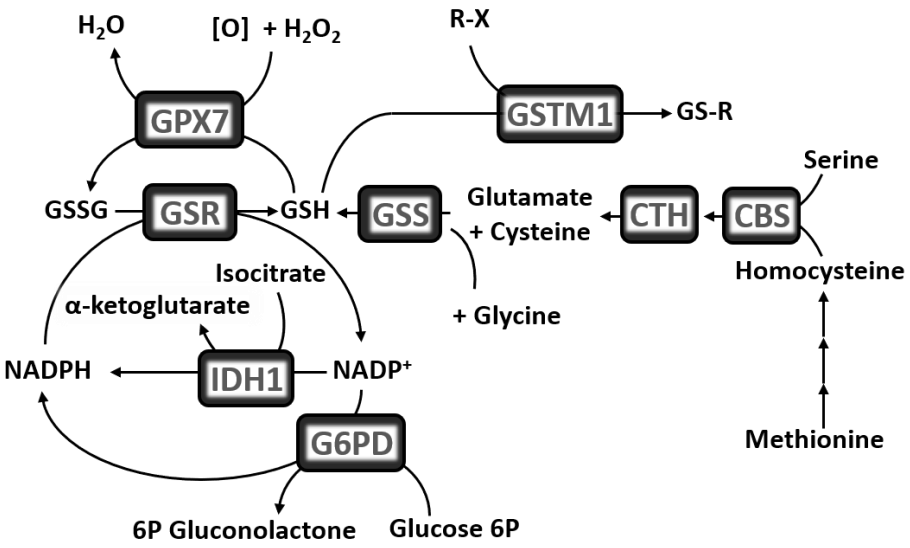

Figure 5.1 – Simplified glutathione metabolism scheme. Biosynthesis of reduced glutathione (GSH) occurs in the cytosol and is dependent on the availability of cysteine, its sulfur amino acid precursor. This amino acid can be synthetized by the cell from homocysteine, derived from methionine, and serine ligation through the transsulfuration pathway by the action of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CTH). γ-glutamyltranspeptidase links glutamate and cysteine which in turn is used by GSH synthase (GSS) to form GSH. The antioxidant GSH function can be used by GSH peroxidase 7 (GPX7) to catalyze hydrogen peroxide reduction reactions or by GSH S-transferase mu 1 (GSTM1) to reduce a variety of products of oxidative stress (R-X), giving origin to oxidized GSH (GSSG). GSSG is reduced back to GSH by GSH reductase (GSR) at the cost of NADPH, forming a redox cycle. NADPH is recycled by isocitrate dehydrogenase 1 (IDH1) or by the conversion of glucose 6-phosphate (6P) to 6P-gluconolactone by glucose-6-phospahte dehydrogenase (G6PD) [18]. Adapted from [16].

Overexpression of glutathione metabolic genes: engineering γ-RV producer cells

Stable overexpression of glutathione metabolic genes was performed using LV as gene delivery vehicles (Fig. 5.2a) to: i) stably integrate the metabolic genes in producer cells and ii) to control gene copy number integrations through the use of a controlled MOI. Cell populations with increasing metabolic gene copy numbers, herein designated as MOI 5, 10, 15, 20, and 25, were successfully established as shown by the quantification of the LV LTR sequence in the engineered cell population genomes (Fig. 5.2b). *CTH* and *CBS* engineered cell populations presented the higher and lower, respectively, metabolic gene cassette integration copy number than the average of the several delivered genes.

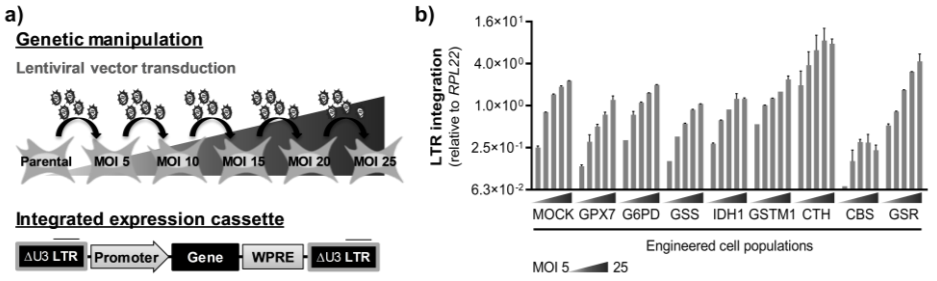

Figure 5.2 – Genetic engineering of constitutive γ-RV producer cells. **a)** Schematic representation of the used genetic engineering strategy. Glutathione metabolic genes were individually cloned into SIN LV transfer vectors. LV harboring each glutathione metabolic gene were used to transduce γ-RV producer cells. Sequential transduction steps using a fixed MOI of 5 were used to establish cell populations with increased gene integration copies, ranging from a MOI 5 up to 25 (triangle). The bar on top of the LTR sequences symbolizes the common amplicon used to assess the LV transgene integration copies in all engineered cell populations. **b)** Expression cassette copy number in cell populations engineered with one of the glutathione metabolic genes (*GPX7*, *G6PD*, *GSS*, *IDH1*, *GSTM1*, *CTH*, *CBS* and *GSR*) or MOCK control. LTR integration was normalized to ribosomal protein L22 (*RPL22*) housekeeping gene. Data shown represents mean +/- SD from technical replicates (n=2). LTR – Long Terminal Repeat. WPRE - Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element.

A step wise glutathione metabolic genes incremental overexpression in engineered cell populations from MOI 5 to MOI 25 was achieved (Fig. 5.3). In general, MOI 25 engineered cell populations showed 5-fold increased gene expression of the glutathione metabolic gene of study, when compared to the respective MOI 5 engineered cell population. The exceptions were *IDH1* and *CBS* engineered cell populations, which showed the lowest overexpression increments (2.4-fold) between populations of MOI 5 and MOI 25 (Fig. 5.3d and Fig. 5.3g). Of notice, *GPX7* and *GSTM1* expression in parental cells was undetectable (Fig. 5.3a and 5.3e, respectively).

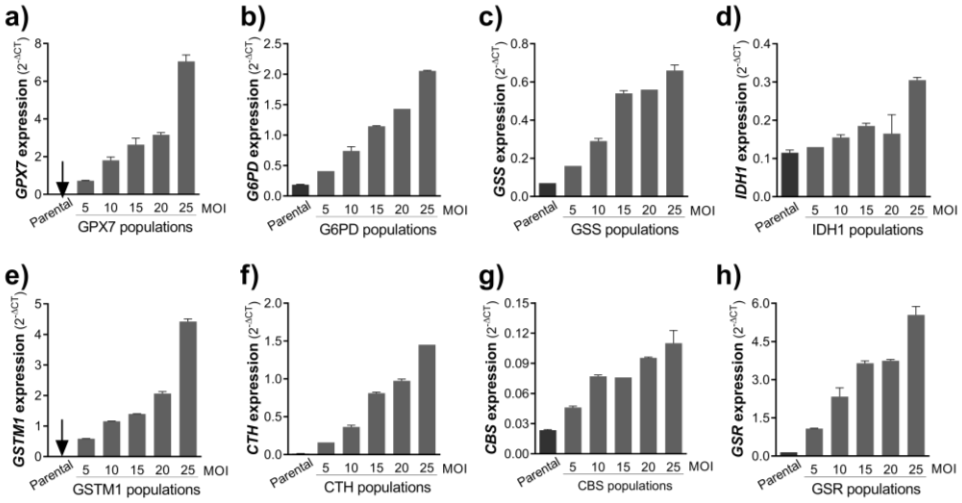

Figure 5.3 – Expression of glutathione metabolic genes in γ-RV producer cell populations. Gene expression levels of parental and respective engineered cell populations: **a)** *GPX7*, **b)** *G6PD*, **c)** *GSS*, **d)** *IDH1*, **e)** *GSTM1*, **f)** *CTH*, **g)** *CBS*, and **h)** *GSR*. Gene expression was normalized to *RPL22* housekeeping gene. Data shown represents mean +/- SD from technical replicates (n=2). Arrows indicate undetected gene expression. MOI – Multiplicity of infection.

The overexpression of the eight glutathione metabolism genes did not affect the growth of γ-RV producer cells, when comparing to parental cells (Fig. 5.4).

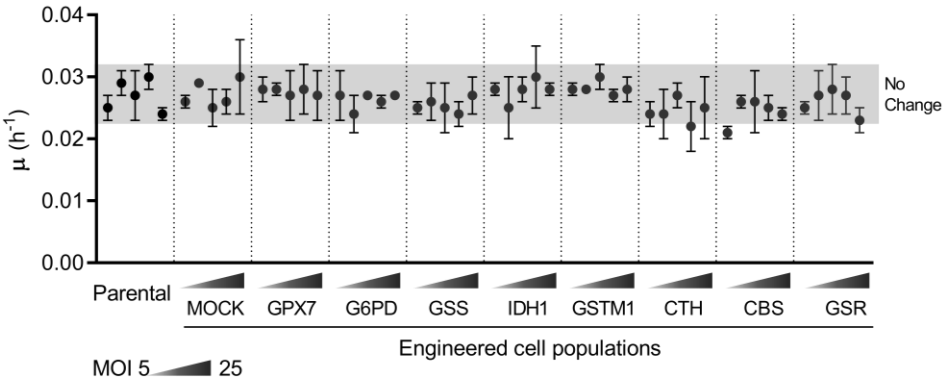

Figure 5.4 – Specific growth rate of parental and engineered γ-RV producer cells. Data represents mean +/- SD from technical replicates (n=2). MOI – Multiplicity of infection.

Overexpression of glutathione metabolism genes: effect in γ-RV yields

The impact of glutathione metabolic genes overexpression in transducing γ-RV volumetric yields was grouped in three distinct effects: no-change, up-swing, and down-swing effects (Fig. 5.5a). The no-change effect – T.U. production yields within parental values variability – was observed in MOCK control, *GPX7*, *G6PD* and *GSS* cell populations. The up-swing effect – T.U. productivity improved with increased glutathione metabolic gene load – was observed in *IDH1*, *GSTM1*, *CTH* and *CBS* cell populations. Lastly, the down-swing effect – T.U. productivity declined with increased glutathione metabolic gene load – was observed in *GSR* cell populations. These transducing γ-RV volumetric yields effects were a result of cell specific productivity

changes (Fig. 5.5b). The used genetic engineering strategy to overexpress glutathione metabolic genes in γ-RV producer cells did not have any effect in the cells, as shown for the MOCK control cell populations.

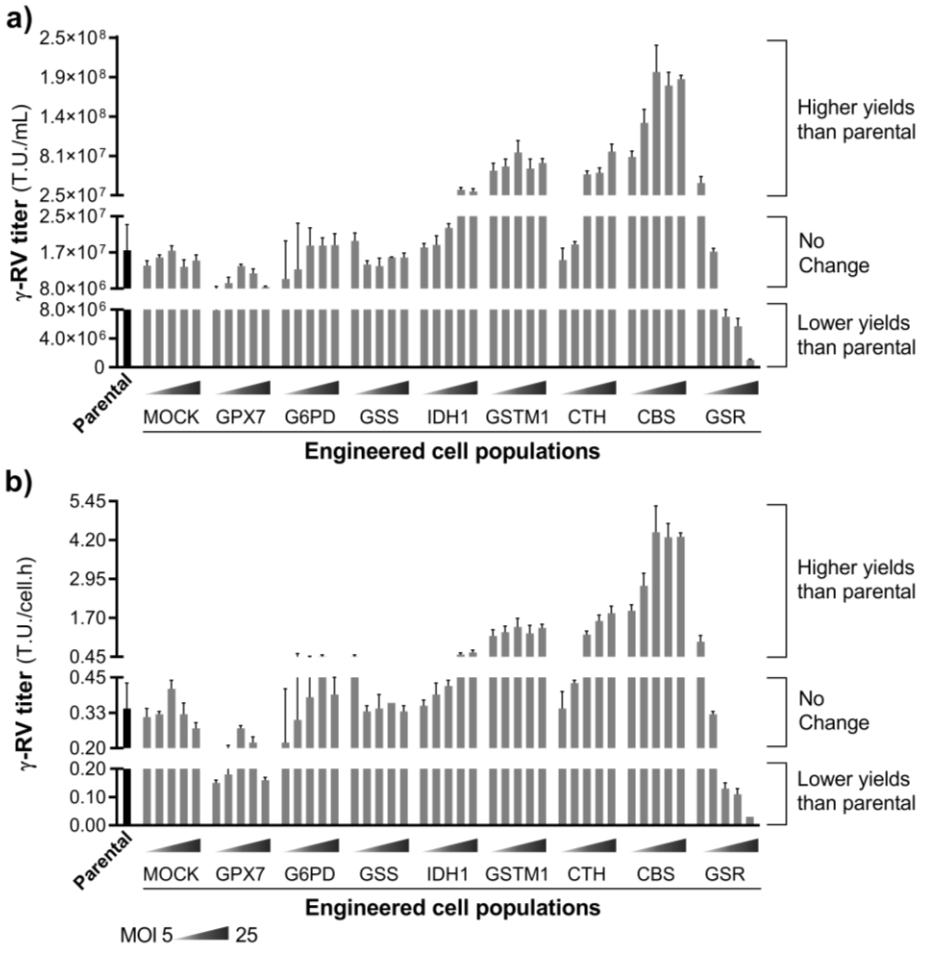

Figure 5.5 - γ-RV yields of parental and engineered producer cells. **a)** Volumetric transducing particles productivity. **b)** Cell specific transducing particles productivity. Parental values represent mean ± SD from independent experiments (n=5). Engineered cell populations data represents the mean +/- SD from technical replicates (n=2). MOI – Multiplicity of infection.

In the up-swing and down-swing effects it was observed that the gene dose (MOI) influenced γ-RV T.U. titers but distinctively. Specifically, *IDH1* and *CTH* cell populations established with lower MOIs presented transducing particles γ-RV productivities similar to parental cell. At higher MOIs, transducing γ-RV productivities of *IDH1* and *CTH* cell populations surpassed parental up to 1.7 and 5.4-fold, respectively. In the case of *GSTM1* overexpression, for all gene doses cell populations showed an average of 4-fold improvement in T.U. cell specific productivity. The highest cell productivity improvements were observed for *CBS* overexpressing cell populations. From MOI 5 to 15, *CBS* cell populations presented 5.6 to 13-fold T.U. titer improvements. Further *CBS* doses (MOI 20 and 25) provided similar γ-RV T.U. yields to cell population of MOI 15. The only gene overexpression leading to higher and lower T.U. titers than parental cells was *GSR*. At the lowest gene dose (MOI 5), *GSR* cell populations showed T.U. specific productivity improvements of 2.8-fold. Increasing the gene load to MOI 15 or above resulted in decreased γ-RV T.U. yields when compared to parental cells. Decreases of 13.1-fold in T.U. titers were observed for GSR cell population established at MOI 25.

The mRNA levels of the three γ-RV expression cassettes in engineered cell populations were quantified (Fig. 5.6a). The results showed that the genetic engineering strategy (MOCK control) did not altered γ-RV components expression stability. This stability was also observed for the cell populations where no changes in the T.U. titer occurred (no-change effect group - *GPX7*, *G6PD*, *GSS*). However, in cell populations overexpressing genes providing an up-swing or down-swing productivity profile, the γ-RV components expression changed. Depending on the MOI, the transgene expression increased in *GSTM1*, *CTH*, *CBS* and

GSR cell populations. Also, *gag-pro-pol* expression diminished in *GSR* cell populations.

The up regulation of transgene expression was further investigated by quantifying the number of copies of this cassette in the genome of engineered cells (Fig. 5.6b). Increased genomic transgene cassette copies were observed for the cell populations with higher transgene gene expression levels (*GSTM1*, *CTH*, *CBS* and *GSR*).

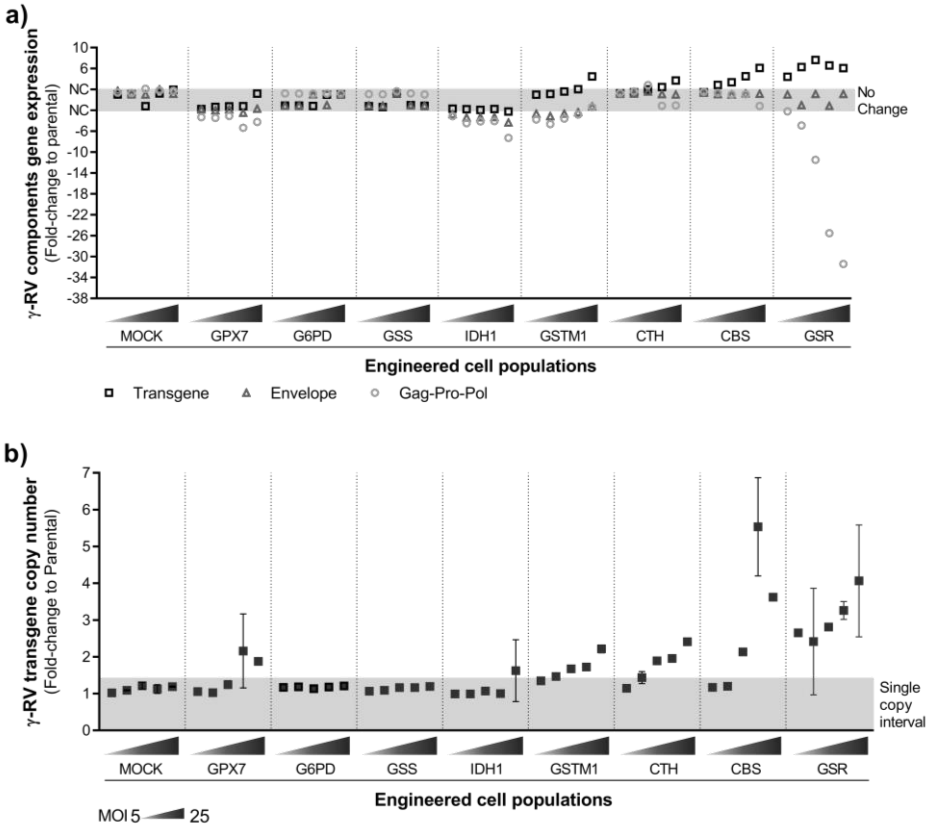

Figure 5.6 - γ-RV genetic cassettes expression and genomic copy number. **a)** γ-RV gene expression. **b)** Transgene cassette genomic integration copy number. Gene expression and copy number were normalized to *RPL22* housekeeping gene. Data shown represents the mean +/- SD from technical replicates (n=2). MOI – Multiplicity of infection.

Overexpression of glutathione metabolism genes: engineering LV producer cells

The most common industrial system for LV production is transient transfection of HEK 293T cells. This manufacture system involves the labor-intensive step of plasmid DNA transfection and is characterized by batch to batch variability. Therefore, the previously described genetic engineering strategy technically challenges the study of the effect of glutathione genes overexpression in LV yields by generating a considerable number of established cell populations. To circumvent the transfection of several cell populations and to avoid reproducibility contrasts, an inducible expression cassette was used to conditionally overexpress the glutathione metabolic genes (Fig. 5.7a). Using this strategy, the same cell population was transfected for LV production and the effect of metabolic gene overexpression level was studied by supplementing cell culture media with doxycycline. Stable insertion of the inducible expression cassette in producer cells was again performed using LV transduction (MOI 1). Engineered cell populations were successfully established using antibiotic selective pressure as shown by the quantification of the LV LTR sequence (Fig. 5.7b). Different integration levels of the inducible expression cassette in engineered cell populations genome was observed. The higher and lower inducible expression cassette integration levels were observed in MOCK and *IDH1* cell populations, respectively.

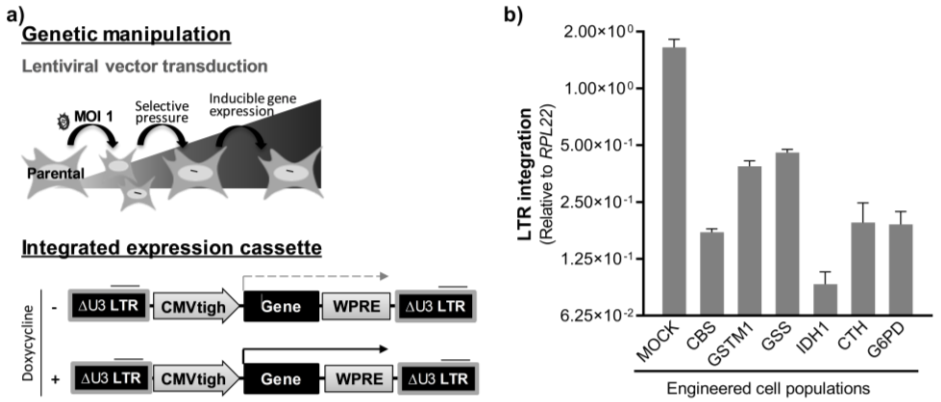

Figure 5.7 – Genetic engineering strategy of HEK 293T cells used to transient produce LV. **a)** Schematic representation of the genetic engineering strategy: glutathione metabolic genes were cloned into SIN LV transfer vector with a tetracycline responsive promoter (CMVtight) driving gene expression by doxycycline addition to control the gene transcription levels. The bar on top of LTR sequences symbolizes the common amplicon used to assess the copy integration levels of all engineered cell populations. Arrows identify expression activation of inserted genetic cassette (dashed line – OFF; full line – ON). **b)** Inducible expression cassette copy number in engineered cell populations (*CBS*, *GSTM1*, *GSS*, *IDH1* and *G6PD*) or MOCK control. LTR integration was normalized to *RPL22* housekeeping gene. Data shown represents mean +/- SD from technical replicates (n=2). LTR – Long Terminal Repeat. MOI – Multiplicity of infection.

The overexpression of glutathione metabolic genes in the established engineered cell populations was detected even in the absence of doxycycline induction (Fig. 5.8). Compared to the engineered cell population basal overexpression, doxycycline supplementation increased glutathione metabolism gene overexpression from 2.3-fold for *CTH* to 9.0-fold for *CBS* (Fig. 5.8e and Fig. 5.8a).

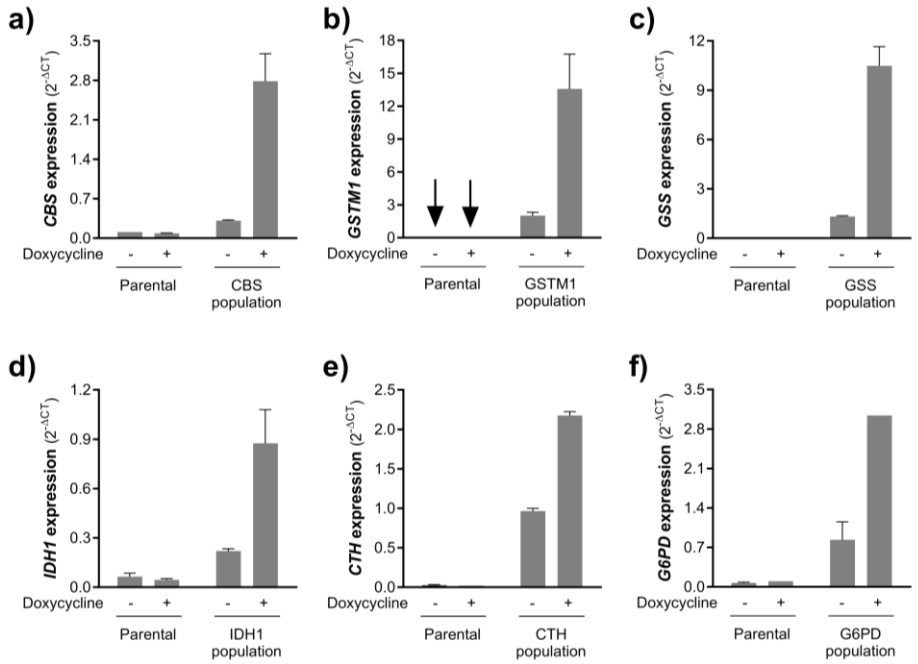

Figure 5.8 – Expression of delivered glutathione metabolic genes in LV producer cell populations. Expression levels of parental and respective engineered cell populations for **a)** *CBS*; **b)** *GSTM1*; **c)** *GSS*; **d)** *IDH1*; **e)** *CTH*; and **f)** *G6PD*. Gene expression was normalized to RPL22 housekeeping gene. Data shown represents mean +/- SD from technical replicates (n=2). housekeeping gene. Data shown represents mean +/- SD from technical replicates (n=2). Arrows indicate undetected gene expression. MOI – Multiplicity of infection.

The gene engineering strategy did not interfere with HEK 293T cell growth (Fig. 5.9).

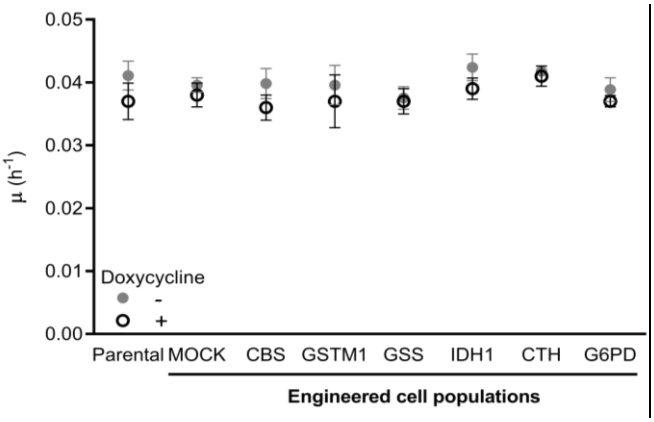

Figure 5.9 – Specific growth rate of engineered LV transient producer cells. Data shown represents mean +/- SD from technical replicates (n=2).

Overexpression of glutathione metabolism genes: effect in LV yields

Since we were not able to completely shut-off gene expression, we used the doxycycline supplementation condition to analyze different gene expression levels effect in LV yields: low and high (without and with supplementation, respectively).

The engineered cell populations were grouped in three categories of T.U. LV volumetric production: higher, no-change or lower than parental cells (Fig. 5.10a). The changes in transducing LV volumetric yields were a result of cell specific productivity changes (Fig. 5.10b). Of notice, in the presence of doxycycline, LV T.U. productivity decreased in parental and MOCK cell population. Moreover, MOCK cell population presented less 1.5-fold LV specific T.U. titer than parental cells. These results may indicate a potential negative impact of the genetic engineering strategy in the producer cell yields.

Transducing LV particles titer improvements up to 1.9-fold were obtained for *G6PD* overexpression. The lower level of *GSS*, *IDH1* and *CTH* overexpression had no impact in LV T.U. production yields. At higher overexpression levels, *GSS* and *IDH1* cell populations decreased LV T.U. titer to similar yields of the MOCK cell population. Independently of gene overexpression level, in *CBS* and *GSTM1* cell populations the LV T.U. specific productivities declined in average 2.3 and 1.6-fold, respectively, when compared to parental cells.

Overall, when comparing to γ-RV production changes, the overexpression of glutathione metabolic genes resulted in minor changes.

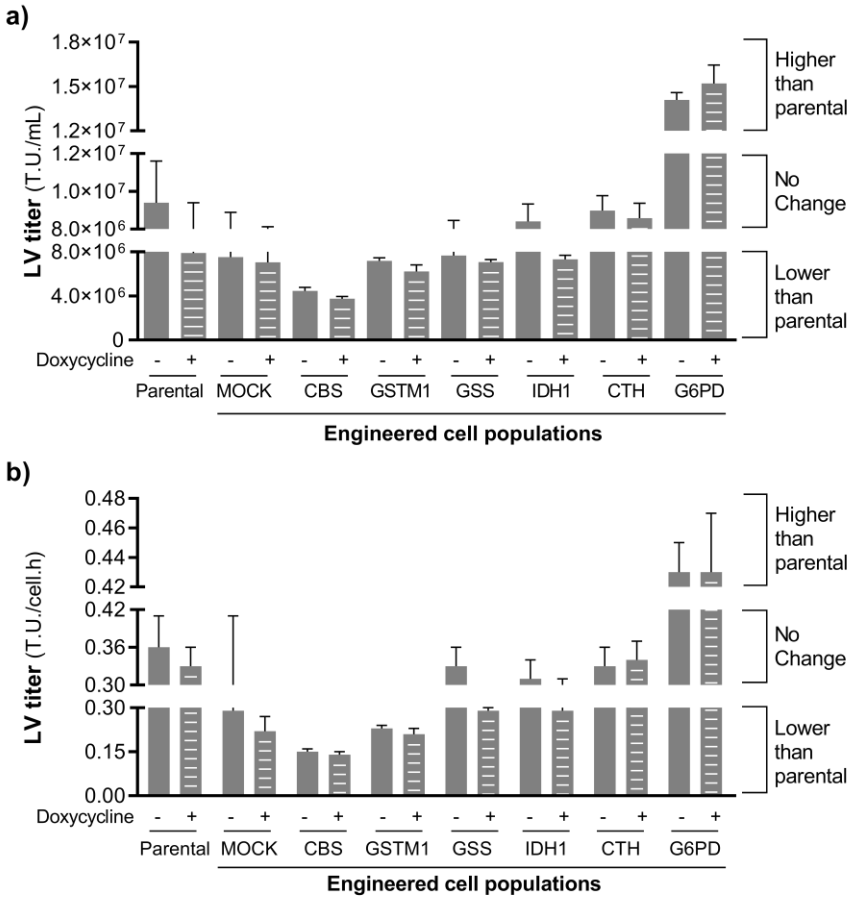

Figure 5.10 - LV transducing particles yields of parental and engineered cells. **a)** Volumetric productivity. **b)** Cell specific productivity. Parental and MOCK values represent mean ± SD from independent experiments (n=5). Engineered cell populations data represents the mean +/- SD from technical replicates (n=2).

5.4 Discussion and conclusions

Metabolic engineering of producer cell substrates could tailor-made robust high yielding and quality manufacture hosts for a wide variety of biopharmaceutical products, including retrovirus derived vectors [4,11,12,15,26]. In this work we studied the overexpression effect of glutathione metabolic genes in the production yields of recombinant retrovirus producer cells. Previously, this metabolic pathway was studied for the same purpose of recombinant retrovirus production improvement using media supplementation strategies or gene (*GSS* and *CBS*) overexpression effects in a clonal environment [15,16]. Herein we further explored glutathione metabolism engineering by i) studying the overexpressing of more genes; ii) controlling the metabolic gene dose, and iii) analyzing the average productivity effect by studying engineered cell populations, rather than clonal cells which may have accumulated other unrelated phenotypes..

Two genetic engineering strategies were explored to overexpress glutathione metabolic genes: constitutive and inducible gene expression (Fig. 5.2 and 5.7). The strategy for constitutive overexpression of glutathione metabolic genes did not interfered with producer cells transducing vector yields, assessed in MOCK cell populations (Fig. 5.5). Whereas the genetic manipulation to inducible express glutathione metabolic genes reduced transducing vector productivity of MOCK cell population (Fig. 5.10). The interference observed in the inducible strategy may have masked glutathione metabolic gene overexpression effect in the engineered cells productivity. Possibly contributing for the latter observed interference are: i) the high trans-activator cellular content in engineered cell populations which was previously associated to cause cytotoxicity [27,28]; and ii) doxycycline supplementation, from tetracyclines class of antibiotics, which was described to target

mitochondrial translation and impair mitochondrial function [29]. Regarding this genetic engineering strategy, the observed leaky expression behavior of Tet-ON inducible systems has also been previously reported (Fig. 5.8) [30].

Two of the constitutive overexpressing cell populations established had altered copy number integrations of the vector expression cassettes (Fig. 5.6). These observations are in agreement with former engineering strategy descriptions, where transgene up-regulation was also observed [11,15]. The mechanisms underlying these observations are not known. However, HEK 293 cells genomic instability upon genetic manipulations was previously described [31,32]. Another hypothesis is based on re-infection events caused by the increased γ-RV transducing titer environment in culture medium due to cells improved productivity. Supporting this latter hypothesis is the description that GaLV receptors masking does not prevent re-infection in chronically infected cells with murine leukemia replicative virus [33]. Another factor contributing for transgene copy number/expression increase could be the existence of an unknown selective pressure in cell populations of the up-swing and down-swing group favoring the duplication of the transgene LTR core enhancer sequence, as previously described for other leukemia retroviruses [34,35].

The higher retroviral vector productivity improvements were observed in γ-RV producer cells overexpressing *CBS* or *CTH* gene (Fig 5.5). These genes provide the enzymes responsible for cysteine cellular synthesis (Fig. 5.1). In humans, cysteine availability is one of the two rate limiting steps for glutathione synthesis [18]. Therefore, *CBS* and *CTH* overexpression may have debottlenecked cysteine cellular availability, contributing for higher transducing γ-RV particle production either by enhancing glutathione synthesis or by increasing protein building blocks availability. These results also suggest that the cysteine present in the

culture medium might be insufficient for optimal γ-RV production. Moreover, the plateau in γ-RV yields observed at higher *CBS* gene doses (MOI 15 to 25, Fig. 5.5) might indicate a threshold of *CBS* expression level (MOI 15) enabling higher cell productivity. Conversely, this plateau could also result from exhaustion of the enzyme co-factor (vitamin B6) and/or the substrates (serine and homocysteine) [36].

The other rate limiting step in glutathione synthesis is described to be the activity of glutamate cysteine ligase which catalyzes the reaction of glutamate and cysteine ligation to be used as substrate for GSS (Fig. 5.1) [18]. Thus, the lack of GSS substrate may explain why cell populations overexpressing *GSS* provided similar retroviral vector yields as parental cells (Fig. 5.5 and Fig. 5.10).

Glutathione peroxidases and glutathione S-transferases are the main enzymes granting glutathione antioxidant functions by using it to reduce hydrogen peroxide, lipid peroxide or oxidized proteins (Fig. 5.1) [18,37]. These antioxidant functions were studied by overexpressing *GPX7* – glutathione peroxidase – and *GSTM1* – glutathione S-transferase. Only γ-RV producer cell populations overexpressing *GSTM1* increased productivity (4.1-fold, Fig. 5.5). This fold improvement was consistent in all MOIs, suggesting that *GSTM1* expression at MOI 5 provided antioxidant functions that ameliorate the known imbalanced host cellular redox environment in γ-RV producer cells [16,17].

γ-RV producer cell populations engineered with *GSR* showed a clear example on how gene overexpression level must be controlled to achieve a desirable effect (Fig. 5.10). Relatively to parental cells, the gene doses herein studied provided three γ-RV production profiles: no-change, increased, and decreased. We hypothesize that these phenotypes could be related to the level of GSR (reduction of oxidized glutathione at the expenses of NADPH, Fig. 5.1). Increased GSR activity in MOI 5 cell population was beneficial to regenerate the reduced

glutathione through the redox cycle (Fig. 5.1). However, the even higher GSR activity in the other cell populations might have created an imbalance in NADPH/NADP+ pool, affecting several other pathways. Such as, the synthesis of Gag-Pro-Pol polyprotein leading to its down-regulation (Fig. 5.6).

Regarding NADPH generation to feed the glutathione redox cycle, *IDH1* and *G6PD* overexpression increased, respectively, γ-RV and LV productivities (Fig. 5.5 and Fig. 5.10). In addition to NADPH generation, *IDH1* may be affecting functional γ-RV by increasing the mitochondrial α-ketoglutarate shuttle, thus, feeding the TCA cycle. Of notice, *G6PD* overexpression was the only gene providing higher transducing LV yields. This gene codes for the first rate-limiting enzyme of pentose phosphate pathway. Therefore, the overexpression of *IDH1* and *G6PD* in recombinant retrovirus producer cell yields may also be due to cumulative functions in metabolic pathways other than glutathione metabolism, namely the energy and the nucleotide biosynthesis pathways [38] .

In summary, the knowledge generated in this work elucidates constrains of different genetic engineering strategies has metabolic engineering tools. The production yields of the two retrovirus derived vectors herein studied benefitted differently from glutathione metabolic gene engineering. Functional γ-RV yields improved with the overexpression of genes involved in cellular cysteine synthesis (*CBS* and *CTH*), glutathione mediated detoxification (*GSTM1*) and NADPH generation (*IDH1*) pathways. In the other hand, functional LV yields were raised by overexpressing *G6PD* which generates NADPH for the glutathione redox cycle in the first step of pentose phosphate pathway. We can further predict energy and pentose phosphate pathway metabolism as potential metabolic engineering targets to improve transient LV producer cell yields.

5.5 Acknowledgments

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5.6 Supplementary data

Table S5.1 – Primers used in cloning and Real-Time qPCR procedures

	Final Construct	Insert	Backbone	Primers 5' → 3' sequence
Cloning	pRRLsin GSR	GSR	pRRLsin Mock	F - CGGGCCCGGGATCCACCATGGCCCTGCTGCCCCG R - CATGGTGGCGACCGGACGAAGTGTGACCAGCTCTTCTG
	pRRLsin GSTM1	GSTM1		F - CGGGCCCGGGATCCACCATGCCCATGATACTGGGGTA R - CATGGTGGCGACCGGCTTGTTGCCCCAGACAGCCA
	pRRLsin GPX7	GPX7		F -CGGGCCCGGGATCCACCATGGTGGCGGCGACGGTGGC R - CATGGTGGCGACCGGTAAGTCTTCTCGCTTCAGTAGGATG
	pRRLsin GSS	GSS		F - CAATGCACCGGTATAATGCCAACTTTGTAC R - GCTGGGACCGGTAGTTTGGCCTGAGCTAG
	pRRLsin CBS	CBS		F - CGGGCCCGGGATCCACCATGCCTTCTGAGACCCCC R - CATGGTGGCGACCGGCTTCTGGTCCCGCTCCTGGG
	pRRLsin CTH	CTH		F - CGGGCCCGGGATCCACCATGGACCTCCAGGGCTGT R - CATGGTGGCGACCGGGGGTGTGCTGCCTTCAAAGC
	pRRLsin G6PD	G6PD		F - CGGGCCCGGGATCCACCATGGCAGAGCAGGTGGCC R - CATGGTGGCGACCGGGAGCTTGTGGGGGTTCACCC
	pRRLsin IDH1	IDH1		F - CAATGCACCGGTATAATGCCAACTTTGTAC R - GCTGGGACCGGTACAGGGTATGGGTTGTC
	pLenti CMVtight GSTM1	GSTM1	pLenti CMVtight eGFP Puro (w771-1)	F - TCGTCGACTAGTCCACCATGCCCATGATAC R - TCGAGCGGCCGCCACTTACTTGTTGCCCCAGAC
	pLenti CMVtight GSS	GSS		F - TCGTCGACTAGTCCACCATGGCCACCAACT R - TCGAGCGGCCGCCACTTATACAGGGTATGGGTT
	pLenti CMVtight G6PD	G6PD		F - TCGTCGACTAGTCCACCATGGCAGAGCAGG R - TCGAGCGGCCGCCACTTAGAGCTTGTGGGGGTT
	pLenti CMVtight CBS	CBS		F - TCGTCGACTAGTCCACCATGCCTTCTGAGA R - TCGAGCGGCCGCCACTTACTTCTGGTCCCGCTC
	pLenti CMVtight IDH1	IDH1		F - TCGTCGACTAGTCCACCATGTCCAAAAAAA R - TCGAGCGGCCGCCACTTACTAAAGTTTGGCCTG

	Target sequence	Primers 5' → 3' sequence
Real Time quantitative PCR	γ-RV transgene (LacZ)	F - ACTATCCCGACCGCCTTACT R - TAGCGGCTGATGTTGAACTG
	γ-RV GagPol	F - GTCCACTATCGCCAGTTGCT R - CTGGGTCCTCAGGGTCATAA
	γ-RV envelope	F - GGACCAAAATAGCGAATGGA R - GGTGAACTGTACGCCTGGAT
	LV LTR	F - ATTGACTGAGTCGCCCGG R - AGCGAGACCACAAGTCGGAT
	GSR	F - CAGTGGGACTCACGGAAGAT R - AAACCCTGCAGCATTTCATC
	GSTM1	F - TCGTGTGGACATTTTGGAGA R - GGGCTCAAATATACGGTGGA
	GPX7	F - TTCACAGACCAGCACTACCG R - GTCTGGGCCAGGTACTTGAA
	GSS	F - CAGCGTGCCATAGAGAATGA R - GACGTGCTTCCCAATTCTGT
	CBS	F - CATCGTGATGCCAGAGAAG R - TTGGGGATTTCGTTCTTCAG
	CTH	F - ATCCACAGCATGAGTTGGTG R - CTCAGCAAGGCTTTCGAATC
	G6PD	F - GAGGCCGTGTACACCAAGAT R - AGCAGTGGGGTGAAAATACG
	IDH1	F - GCTTCATCTGGGCCTGTAAA R - GCTTTGCTCTGTGGGCTAAC
	RPL22	F - CTGCCAATTTTGAGCAGTTT R - CTTTGCTGTTAGCAACTACGC

5.7 References

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CHAPTER VI

Lentiviral vector cell transduction efficiency: impact of different pseudotypes and transduction enhancers

This chapter was adapted from

*Formas-Oliveira AS, *Cabral ET, Coroadinha AS. Lentiviral vector cell transduction efficiency: impact of different pseudotypes and transduction enhancers (*in preparation*)

Author contribution

Ana Oliveira designed and participated in the experimental setup, analyzed the data, and wrote the chapter

*Authors with equal contribution

Abstract

Lentiviral vectors (LV) are widely used as gene delivery tools, either in gene therapy clinical settings or in basic research. Still, LV unprocessed preparations are characterized by low transducing to total particles ratios, impairing their quality and availability in clinical contexts. To improve LV transduction capacity, transduction enhancers based on cationic compounds have been developed to reduce the repulsive interactions between virus and cell lipid membranes. In this work, we screened the transduction efficiency of LV using seven transduction adjuvant compounds aiming to improve LV functionality. To this end, HEK 293T and SUP-T1 target cell lines were transduced with LV pseudotyped with different envelopes: VSV-G, 4070A, RD114, and GaLV.

Vectofusin-1 was the adjuvant compound providing higher transduction efficiency improvements for 4070A, GaLV and RD114 pseudotyped LV, increasing the percentage of transduced cells up to 56%, 61% and 70%, respectively. While VSV-G pseudotyped LV showed the highest transduction capacity in both target cells, the results obtained with Vectofusin-1 open the possibility of clinical LV pseudotyping with glycoproteins alternatives to VSV-G. Additionally, Vectofusin-1 supplementation eliminated the need for spinoculation transduction procedure, thus avoiding the impairment of cell viability after transduction and facilitating clinical procedures.

Overall, we describe how different pseudotypes and adjuvant compounds influence LV transduction efficiency in two target cell lines. This work contributes for the development of knowledge-driven LV transduction protocols to support academic and clinical sectors using LV has gene delivery tools.

Chapter IV

6.1 Introduction

The unique gene transfer properties of lentiviral vectors (LV) enable to deliver and permanently express genetic material into proliferative and non-proliferative cell types. Due to these features, we are observing a fast growth of LV use in the clinic [1]. Additionally, its simple use make LV a powerful gene transfer tool, highly employed in fundamental research to study genetic functions or create cell and animal models [2,3].

LV transduction properties, tropism, and host cell internalization pathway can be modulated to better suit their gene transfer application. Historically, these recombinant vectors have been mainly pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G), a heterologous viral envelope rendering LV broad tropism and physical resistance during manufacture bioprocess [4]. Although less common, viral envelopes from other retroviruses have been also employed, namely from the amphotropic murine leukemia virus (4070A), gibbon ape leukemia virus (GaLV), and feline endogenous retrovirus (RD114) [5]. These latter viral glycoproteins are synthetized as precursor proteins, requiring a two-step fusogenic activation process. The first step consists on furin proteolytic cleavage in producer host cells, giving rise to two (surface and transmembrane) glycoprotein subunits linked by a disulfide bond [6]. The second steps provides fusogenic competent glycoproteins through the cleavage of the R-peptide sequence (transmembrane subunit) by the viral protease during virus maturation step [7]. As such, 4070A, GaLV, and RD114 pseudotypes are particularly suited for constitutive LV manufacture, since the expression of inactive fusogenic viral glycoproteins does not compromise producer cells viability [8–10]. Nevertheless, their complex particle functionalization process may contribute to lower fusogenic active LV content in the final vector preparation. In contrast, VSV-G is synthetized has a complete active fusogenic glycoprotein, which is convenient for

efficient LV particles functionalization. However, VSV-G expression is highly cytotoxic due to induced syncytium formation, limiting its use to transient or inducible LV manufacture systems [11].

Currently, crude LV preparations are characterized by low transduction efficiencies, where only approximately 1% of the produced LV can efficiently deliver the genetic material into target cells. Therefore, it is of outmost importance to functionalize the remaining 99% of the LV particles. In part, this low LV transduction efficiency is a consequence of strong electrostatic repulsions between the negatively charged lipid membranes of LV and target cells. To neutralize these repulsive membrane charges and improve virus and target cell membranes adhesion and fusion, cationic additives have been extensively developed and studied [12–17]. However, the absence of methodic and systematic studies comparing all the available transduction enhancers impairs its rational selection and implementation in LV transduction protocols.

Herein, we aimed to study the LV transduction efficiency of four viral envelopes – VSV-G, 4070A, GaLV and RD114 – in two target cell models – HEK 293T and SUP-T1 – using different transduction enhancers. HEK 293T adherent cells were chosen as they are commonly used in laboratory contexts and research purposes to titrate LV. SUP-T1 non-adherent cells were used as T cell model, an important gene therapy cell target of emerging immunotherapies [18]. Seven commercially available transduction enhancers (some of clinical grade) were screened for their capacity to improve LV cell transduction efficiency. Vectofusin-1 compound showed the highest LV cell transduction efficiency improvements, up to 51-fold for LV pseudotyped with 4070A, GaLV and RD114. These improvements rendered LV pseudotyped with 4070A, GaLV, and RD114 similar transduction efficiencies to VSV-G pseudotyped LV particles without requiring spinoculation.

6.2 Materials and methods

Plasmids

The following plasmids were kindly provided by Dr. Didier Trono through Addgene plasmid repository and were used to produce LV particles.

pMDLg/RRE is a third generation LV packaging plasmid encoding HIV-1 structural and enzymatic proteins under the control of cytomegalovirus (CMV) promoter (Addgene plasmid repository #12251, Cambridge, MA, USA).

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

pRRLSIN.cPPT.PGK-GFP.WPRE is a SIN LV transgene plasmid, carrying enhanced green fluorescent protein (GFP) gene, expressed through an internal human phosphoglycerate kinase 1 promoter (Addgene plasmid repository #12252).

The expression of viral envelope glycoproteins to pseudotype LV particles was provided by the following plasmids:

pMD2.G, encodes for the VSV-G envelope under the control of a CMV promoter, kindly provided by Dr. Didier Trono (Addgene plasmid repository #12259); pCMV-4070A, pCMV-RD114^{pro}, and pCMV-GaLV-TR^{synt}, described in Tomás et al. (2019), have a CMV promoter driving the expression of the 4070A, RD114 and GaLV envelope, respectively [11].

Cell lines and cell culture

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 as: i) cell substrate for LV transient production, ii) target cell for LV titration and LV transduction studies. This cell line was maintained in Dulbecco's modified

Eagle's medium (Corning Inc, Corning, NY, USA) supplemented with 10% (v/v) Fetal bovine serum (FBS) (Gibco, Paisley, UK) at 37 °C in a humidified atmosphere containing 8% CO_2.

SUP-T1 (ATCC CRL-1942) is a non-adherent T lymphoblastic cell line and was used as target cell for LV transduction studies. This cell line was cultured in ATCC-formulated RPMI-1640 medium (Gibco), supplemented with 10% (v/v) FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

Viable cell concentration was determined using trypan blue exclusion method.

Lentiviral vector transient production

HEK 293T cells were transfected at the time of seeding (1 x 10^4 cell/cm^2 and 0.2 mL/cm^2) using linear 25 kDa polyethylenimine (PEI; Polyscience, Hirschberg an der Bergstrasse, Germany) at a mass ratio of 1:1.5 (DNA:PEI), with the respective plasmids. Third generation LV vector packaging system was used in a total of 3.3 µg of DNA per million cells composed of 1.8 µg transgene, 0.7 µg *gag-pro-pol*, 0.2 µg *rev*, and 0.6 µg envelope [19,20]. After 24 hours, cell culture medium was replaced (0.1mL/cm^2) and supernatant was harvested 24 and 48 hours after.

Nude LV particles were produced using the same co-transfection procedures, but in absence of plasmid coding for the envelope.

Lentiviral vector particles quantification

Titration of LV transducing units (T.U.) harboring a GFP transgene was performed using HEK 293T target cells seeded at 1 x 10^5 cells/cm^2 in 24 well plates. Transduction was performed one day after, in duplicates, with 0.2 mL of viral non-adherent diluted in fresh DMEM with 10% (v/v) FBS and 8 µg/ml of polybrene (Sigma, St Louis, MO, USA). Transduction was followed by spinoculation – centrifugation at 1200 x g for 2 hours at 25 ºC

– and then 0.5 mL of warm medium was added [21]. Cell concentration was determined at the time of transduction. Two days after transduction, cells were harvested and analyzed using CyFlow Space flow cytometer (Sysmex Partec GmbH, Görlitz, Germany). The GFP titer was determined taking into account only LV dilutions rendering 2-20% of GFP positive cells, the cell concentration at time of transduction and the dilution factor [10].

LV RNA genomes were determined using the method described in [22]. The protocol was adapted for LV by using primers targeting WPRE sequence (Forward ACTGTGTTTGCTGACGCAAC; Reverse ACAACACCACGGAATTGTCA) for cDNA synthesis and qPCR.

Assessment of lentiviral vector transduction efficiency

Adherent HEK 293T cells were transduced as previously described in LV particles quantification section, with or without transduction adjuvants compounds (Table 6.1). Also, static incubation was performed at 37 ºC, 5% CO_2, for 4 hours. Then, 0.5 mL/well of warm culture medium was added.

Non-adherent SUP-T1 cells were seeded at 2.5 x 10^5 cells/cm^2, in a final volume of 0.1 mL/well, in 24 well flat-bottom non-tissue treated culture plates, at time of infection. Transduction was performed in duplicates, by adding 0.1mL of viral suspension. Viral suspension was diluted in fresh cell culture medium, with or without transduction enhancers (Table 6.1). Spinoculation step was performed at 938 x g, for 90 minutes, at 25ºC. Static incubation was performed at 37 ºC for 4 hours. Then, RPMI 10% (v/v) FBS was added to have a final cell culture volume of 1 mL/well.

GFP fluorescence was measured 48 hours post-transduction by flow cytometry (Gallios, Beckman Coulter Life Sciences, IN, USA).

Transduction enhancers (Table 6.1) were used accordingly to manufacturer's instructions.

Table 6.1 - LV transduction enhancers.

	Concentration/ Dilution	Stock Solution	Company
Polybrene	8 µg/mL	1 mg/mL	Sigma-Aldrich
LentiBoost	1:100	patent protected	SIRION Biotech, Martinsried, Germany
LentiBlast	1:100	Patent protected	OZ Biociences Inc, CA, USA
Transplus	1:500	Patent protected	ALSTEM Inc, CA, USA
DEAE-Dextran	20 µg/mL	1 mg/mL	Sigma-Aldrich
Vectofusin-1	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

6.3 Results

LV cell transduction efficiency without transduction enhancers: static incubation *vs*. spinoculation

Centrifugal inoculation (spinoculation) is widely used to enhance viral vector transduction [21]. To understand the influence of this procedure, we first evaluated the impact of spinoculation *vs.* static incubation (without any adjuvant compound) in the LV cell transduction efficiency of four LV pseudotypes: with VSV-G, 4070A, RD114 and GaLV (Fig. 6.1). Two target cells lines, adherent HEK 293T and non-adherent SUP-T1, were used to be transduced at the multiplicity of infection (MOI) of 0.4.

For 4070A, RD114, and GaLV pseudotypes, spinoculation increased LV cell transduction efficiency from 2 to 8-fold. In the case of VSV-G pseudotyped LV, similar LV cell transduction efficiencies were observed in static incubation and spinoculation transduction conditions.

The highest cell transduction efficiency was obtained by VSV-G pseudotyped LV, which preferentially transduced SUP-T1 cells (38%, Fig.

6.1b) than HEK 293T cells (19%, Fig. 6.1a). Except for GaLV, *Gammaretrovirus* derived envelopes provided higher LV transduction efficiencies in HEK 293T cells than in SUP-T1 cells.

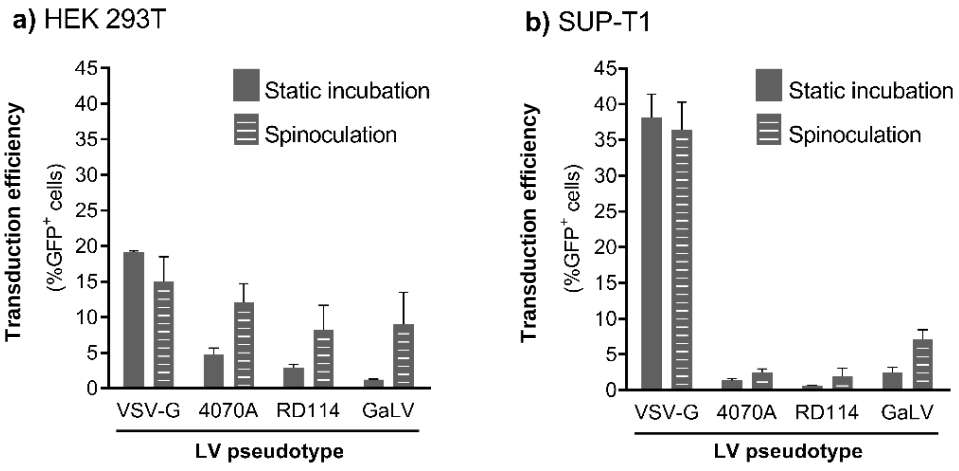

Figure 6.1 – Transduction efficiency of different LV pseudotypes. VSV-G, 4070A, RD114 and GaLV pseudotyped LV coding for green fluorescent protein (GFP) (n = 2 *per* pseudotype) were used to transduce **a)** HEK 293T or **b)** SUP-T1 target cells at same multiplicity of infection (MOI = 0.4). Data shown represents the average percentage of GFP positive cells ± SD from independent experiments (n ≥ 2). Lentiviral vectors (LV) displaying envelope glycoproteins from: VSV-G – vesicular stomatitis virus; 4070A – amphotropic murine leukemia virus; RD114 – feline endogeneous retrovirus; GaLV - gibbon ape leukemia virus.

LV cell transduction efficiency with transduction enhancers

Seven transduction adjuvant compounds were tested in parallel for their ability to improve LV transduction efficiency in HEK 293T or SUP-T1 target cells (Fig. 6.2).

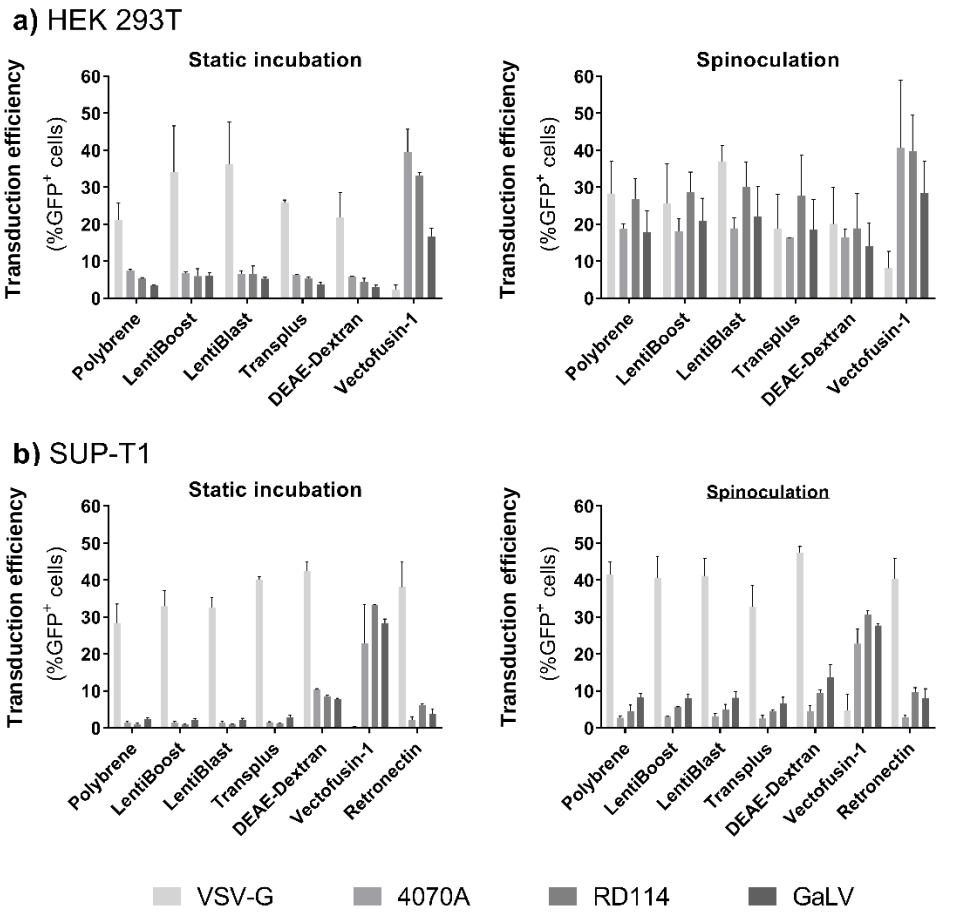

Figure 6.2 – Transduction efficiency of different LV pseudotypes using adjuvant compounds. Green fluorescent protein (GFP) encoding LV (n = 2 per pseudotype) were used to transduce **a)** HEK 293T or **b)** SUP-T1 target cells at same multiplicity of infection (MOI = 0.4). Data shown represents the average percentage of GFP positive cells ± SD from independent experiments (n ≥ 2). Lentiviral vectors (LV) displaying envelope glycoproteins from: VSV-G – vesicular stomatitis virus; 4070A, amphotropic murine leukemia virus; RD114 – feline endogenous retrovirus; GaLV - gibbon ape leukemia virus. Adjuvant compounds were used according to manufacturer instructions.

When compared to previous results without transduction enhancers, HEK 293T cell transduction efficiencies increased for all LV pseudotypes in the presence of adjuvant compounds (Fig. 6.2a). VSV-G pseudotyped LV registered the highest HEK 293T cell transduction efficiency – 36% (static-incubation) and 37% (spinoculation) – when using LentiBlast adjuvant

compound. Spinoculation procedure coupled with adjuvant supplementation enabled increased HEK 293T cell transduction efficiencies by LV pseudotyped with 4070A, RD114, and GaLV, reaching similar percentages of LV transduced cells as VSV-G pseudotype. Vectofusin-1 was the adjuvant compound enabling the highest HEK 293T cell transduction efficiencies by LV with *Gammaretrovirus* derived envelope pseudotypes. Particularly, for LV pseudotyped with 4070A or RD114, Vectofusin-1 supplementation enables to withdraw spinoculation procedure to attain efficient HEK 293T cell transduction.

In SUP-T1 cells, VSV-G pseudotyped LV delivered the higher cell transduction efficiencies – 42% (static-incubation) and 47% (spinoculation) - with DEAE-Dextran supplementation (Fig. 6.2b). DEAE-Dextran also improved the percentage of SUP-T1 transduced cells by LV pseudotyped with 4070A (10% in static incubation), RD114 (9% in spinoculation) and GaLV (14% in spinoculation). For these latter retroviral pseudotypes, Vectofusin-1 compound provided the highest cell transduction improvements and enabled similar percentage of transduced cells in static-incubation and spinoculation conditions (transductions efficiencies between 23-33%).

Although widely used in clinical applications, Retronectin only showed LV cell transduction improvements for RD114 pseudotyped LV: 6.2% (static incubation) and 9.6% (spinoculation) of LV transduced SUP-T1 cells (Fig. 6.2b).

In both cell lines, Vectofusin-1 impaired VSV-G pseudotyped LV cell transduction.

The use of these adjuvants could lead to unspecific, non-envelope mediated, LV cell transduction. Therefore, nude LV, particles without viral envelope glycoproteins, were used as controls of this experimental set up (Supplementary data Fig. S6.1). Only Vectofusin-1 enabled cell transduction by nude LV, delivering less than 2% of unspecific LV

transduced cells. Therefore, compared to the higher levels herein reported we may conclude that unspecific transduction is negligible, and the enhancement effects are specific through viral envelope mediated cell transduction.

These adjuvant compounds were then tested in the context of higher LV MOIs to understand if transduction efficiency could be further improved, as required for clinic applications.

VSV-G

At higher MOI, the addition of adjuvants further increased VSV-G pseudotyped LV transduction efficiency up to 74% in HEK 293T cells (LentiBlast) but not in SUP-T1 cells (Fig. 6.3). This LV pseudotype showed similar cell transduction efficiency levels in static and in spinoculation conditions. Also, lower transduction efficiency of HEK 293T cells (approximately 50%, Fig. 6.3a) than SUP-T1 cells (above 70%, Fig. 6.3b) was confirmed.

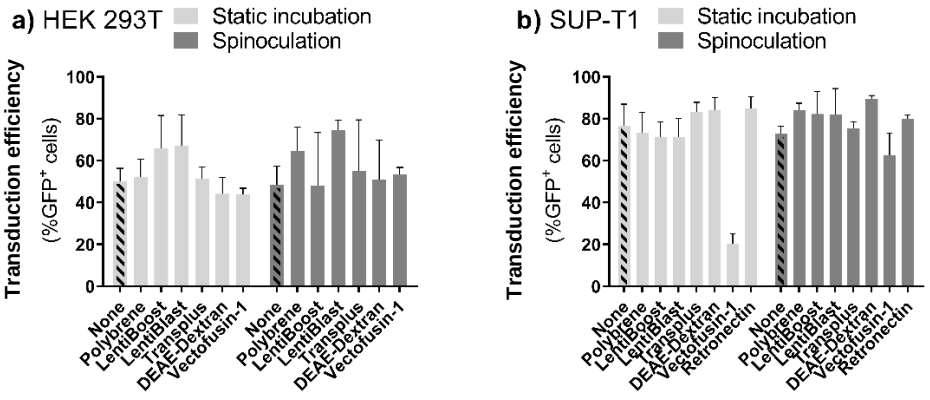

Figure 6.3 – Transduction efficiency of LV pseudotyped with **VSV-G** using adjuvant compounds. Green fluorescent protein (GFP) encoding LV (n = 2) were used to transduce **a)** HEK 293T or **b)** SUP-T1 target cells at same multiplicity of infection (MOI = 2.4). Data shown represents the average percentage of GFP positive cells ± SD from independent experiments (n ≥ 2). Lentiviral vectors (LV) displaying envelope glycoproteins from vesicular stomatitis virus (VSV-G). Adjuvant compounds were used according to manufacturer instructions.

4070A

Increasing the MOI of LV pseudotyped with 4070A coupled with Vectofusin-1 supplementation enabled to increase transduction efficiency to 52% for HEK 293T and to 39% for SUP-T1 cells in static condition. Similar transduction efficiencies were obtained in spinoculation condition. SUP-T1 cells transduction efficiency was also improved 8.7-fold with DEAE-Dextran, reaching 19% of transduced cells in static-incubation.

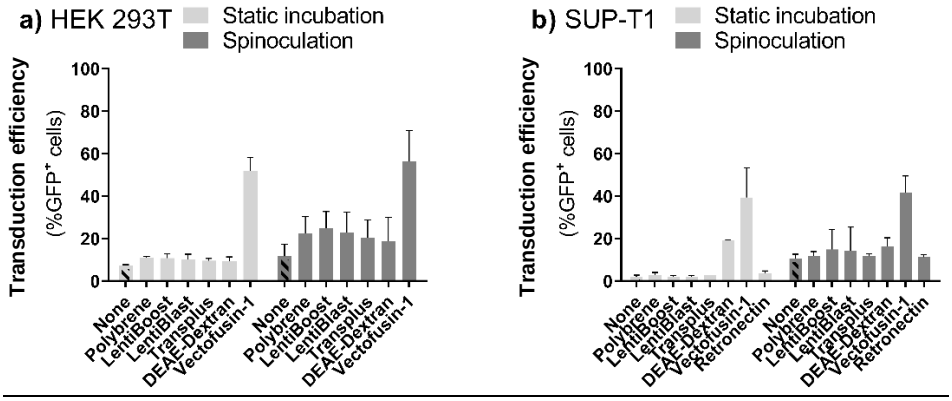

Figure 6.4 – Transduction efficiency of LV pseudotyped with **4070A** using adjuvant compounds. Green fluorescent protein (GFP) encoding LV (n = 2) were used to transduce **a)** HEK 293T or **b)** SUP-T1 target cells at same multiplicity of infection (MOI = 0.8). Data shown represents the average percentage of GFP positive cells ± SD from independent experiments (n ≥ 2). Lentiviral vectors (LV) displaying envelope glycoproteins from amphotropic murine leukemia virus (4070A). Adjuvant compounds were used according to manufacturer instructions.

RD114

As previously observed, RD114 pseudotype preferably transduced HEK 293T than SUP-T1 cells (Fig. 6.5). Importantly, at higher MOI, all adjuvants improved the transduction performance of RD114 pseudotyped LV. Again, Vectofusin-1 provided the highest transduction efficiencies, enabling more than 60% of transduced cells for both cell lines, eliminating the need to perform spinoculation.

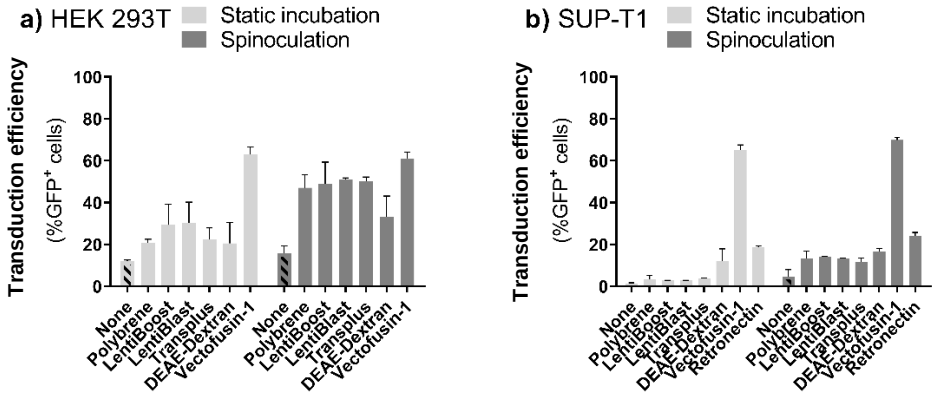

Figure 6.5 – Transduction efficiency of LV pseudotyped with **RD114** using adjuvant compounds. Green fluorescent protein (GFP) encoding LV (n = 2) were used to transduce **a)** HEK 293T or **b)** SUP-T1 target cells at same multiplicity of infection (MOI = 1.7). Data shown represents the average percentage of GFP positive cells ± SD from independent experiments (n ≥ 2). Lentiviral vectors (LV) displaying envelope glycoproteins from feline endogenous retrovirus (RD114). Adjuvant compounds were used according to manufacturer instructions.

GaLV

Increasing the MOI showed GaLV pseudotyped LV preference for SUP-T1 cells transduction than HEK 293T (Fig. 6.6). The need for spinoculation to efficiently transduce both cell lines was again observed. Using Vectofusin-1, this pseudotyped LV increased cell transduction efficiencies up to 40% in HEK 293 T and 61% in SUP-T1 cells (spinoculation). With Vectofusin-1 transduction efficiencies in SUPT-1 (51%) enable the removal of spin inoculation step.

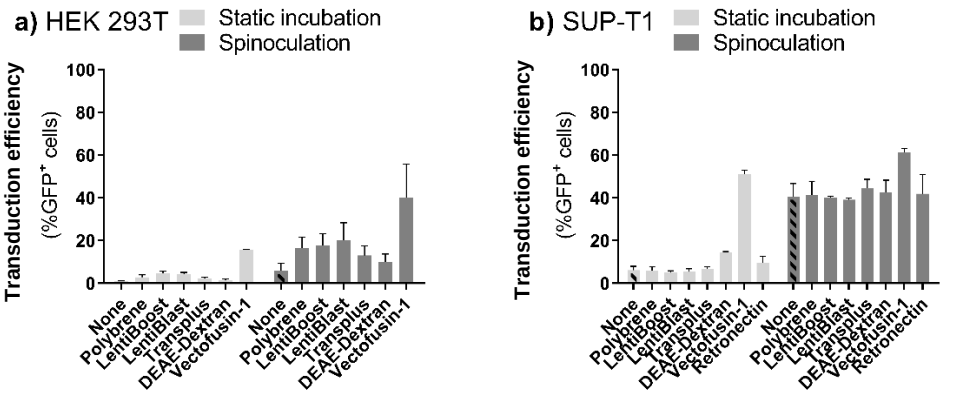

Figure 6.6 – Transduction efficiency of LV pseudotyped with **GaLV** using adjuvant compounds. Green fluorescent protein (GFP) encoding vectors (n = 2) were used to transduce **a)** HEK 293T or **b)** SUP-T1 target cells at same multiplicity of infection (MOI = 1.2). Data shown represents the average percentage of GFP positive cells ± SD from independent experiments (n ≥ 2). Lentiviral vectors (LV) displaying envelope glycoproteins from gibbon ape leukemia virus (GaLV). Adjuvant compounds were used according to manufacturer instructions.

6.4 Discussion and conclusions

LV are the gene delivery vehicles of choice in research and clinical settings for permanent cell modification. This work investigated the transduction performance of four LV pseudotypes in two cell lines representative of academia and clinical cell models. Additionally, a systematic study comparing the action of seven adjuvants was performed, enabling optimized LV transduction procedures.

LV displaying envelopes of *Gammaretrovirus* origin (4070A, RD114, and GaLV) required the spinoculation procedure to increase its transduction efficiency (Fig. 6.1), supporting previous observations [5,23]. This centrifugation step alters cell cytoskeletal dynamics, promoting receptor mobilization, viral entry, and post-entry processes [24]. Therefore, this spinoculation dependency revealed the limitation of 4070A, RD114, and GaLV pseudotypes to trigger the early steps of the transduction process.

LV transduction initiates when particles displaying fusogenic active envelope glycoproteins recognize and attach to host cell receptors. Then, the release of LV content in host cells cytoplasm may occur by direct LV and host cell lipid membrane fusion (pH independent pathway - retrovirus derived pseudotypes) or by endocytosis (pH dependent pathway – VSV-G pseudotype) [25]. As shown, Vectofusin-1 supplementation improved the transduction efficiency of LV pseudotyped with glycoproteins mediating pH independent cell entry pathway, eliminating the need of spinoculation (Fig. 6.2, Fig. 6.4, Fig. 6.5, and Fig. 6.6). Conversely, Vectofusin-1 supplementation was detrimental to the transduction efficiency of LV pseudotyped with VSV-G (pH dependent cell entry pathway, Fig. 6.2, and Fig. 6.3). Vectofusin-1 forms cationic nanofibrils, pelleting LV and potentiating cell contact by neutralizing lipid membrane charges [13,26]. In the case of VSV-G pseudotype, Vectofusin-1 nanofibrils could have hampered the LV endocytosis process or the pH-dependent membrane

fusion in the endosome, as others also described [26,27]. However, for LV pseudotyped with *Gammaretrovirus* envelopes we speculate that this adjuvant improved glycoproteins and cell receptor binding by increasing its proximity, followed by enhanced pH independent membrane fusion. Indeed, using this adjuvant, LV pseudotyped with 4070A, RD114, and GaLV became spinoculation independent (Fig. 6.2, Fig. 6.4, Fig 6.5, and Fig. 6.6). This simplification of the transduction step is particularly important in the case of sensitive cells as T lymphocytes, a major gene therapy cell target, to guarantee high cell survival after modification [15]. Moreover, Vectofusin-1 coupled with 4070A, RD114, and GaLV pseudotyped LV provided similar cell transduction efficiencies as VSV-G vectors (Fig. 6.2). Although the highest cell transduction efficiency without adjuvants was provided by VSV-G (Fig. 6.1), the use of Vectofusin-1 opens the possibility to replace VSV-G in the clinic by non-toxic pseudotypes compatible with constitutive manufacture systems and *in-vivo* gene therapy applications.

The LV pseudotypes used in this work showed transduction preferences for one of the two target cell lines. For instance, VSV-G pseudotyped LV enabled higher percentage of SUP-T1 transduced cells than HEK 293T cells (Fig. 6.1, Fig. 6.2 and Fig. 6.3), as previously described [28]. Two factors may explain this difference. First, internal promoter activity driving the GFP expression could vary between cell lines, altering the measurement of transduced cell percentage [29]. Second and most likely, this behavior could be a consequence of different levels of respective viral envelope receptor expression by each target cell [30]. Still, compared to other viral envelopes, the high transduction of both cell lines by VSV-G pseudotyped LV suggests that its cell receptor (low-density lipoprotein, LDL) is ubiquitously expressed in both targeted cells [31]. Conversely, the low percentage of transduced cells when using the other pseudotyped LVs (4070A, RD114 and GaLV) could result of lower expression of the

respective (Pit-1, Pit-2, and ASCT2) cell receptor (Fig. 6.1) [30]. However, when using Vectofusin-1 this hypothesis is debatable, since higher cell transduction efficiencies of 56% for 4070A (Fig.6.4), 61% for GaLV (Fig. 6.6) and 70% for RD114 (Fig. 6.5) pseudotyped LV were observed.

In summary, the LV cell transduction improvements herein achieved with Vectofusin-1 for 4070A, RD114 and GaLV pseudotypes evidence that low LV preparation quality is not only a problem of defective non-fusogenic particles contamination, but also inability of fusogenic LV particles to transduce efficiently the target cells. This highlights vector and cell membrane fusion has one of the main transduction constraints of LV pseudotyped with *Gammaretrovirus* derived glycoproteins. This further provides indications on the conditions how to perform LV transduction with these pseudotypes in academic and clinical settings without compromising cell viability (i.e. which adjuvant to use without spinoculation). The removal of spinoculation facilitates clinic transduction procedures. From the LV manufacture perspective, the increase in transduction efficiencies of LV pseudotyped with 4070A, RD114 and GaLV glycoproteins allows to decrease the dose and quantity of material to produce. To note that these envelopes can be used in constitutive LV production systems facilitating manufacturing. Therefore, our results contribute not only to efficiently utilize current LV preparations, decreasing production volumes, but also to establish economic and reproducible LV manufacture bioprocesses.

6.5 Acknowledgments

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6.6 Supplementary data

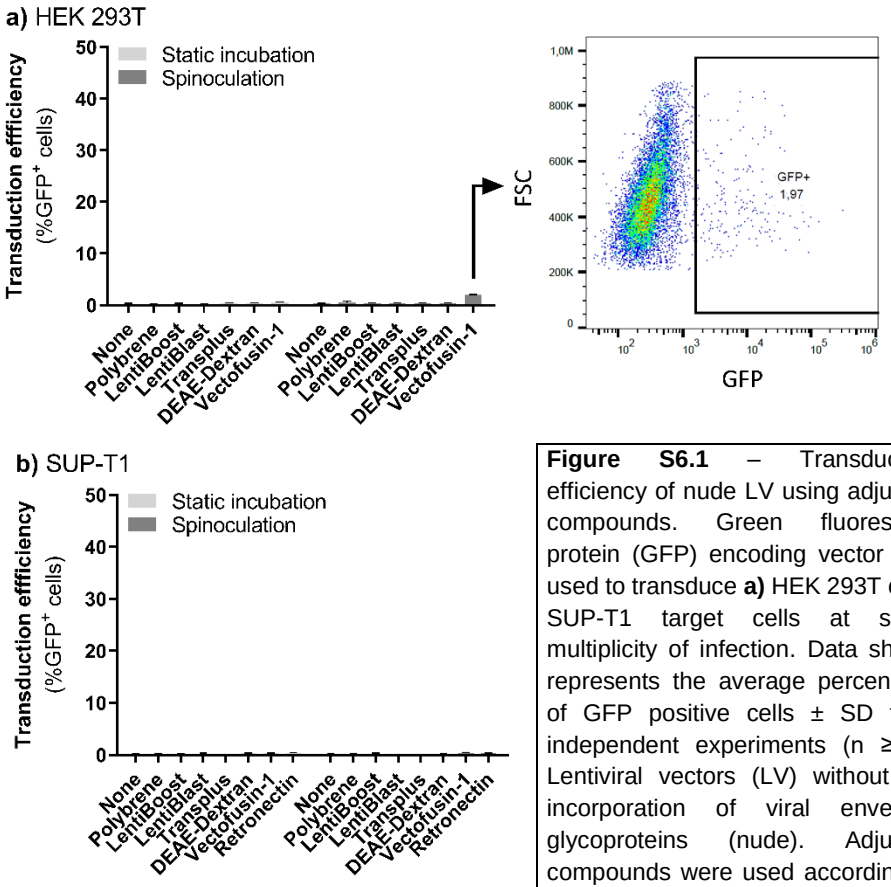

Figure S6.1 – Transduction efficiency of nude LV using adjuvant compounds. Green fluorescent protein (GFP) encoding vector was used to transduce **a)** HEK 293T or **b)** SUP-T1 target cells at same multiplicity of infection. Data shown represents the average percentage of GFP positive cells ± SD from independent experiments (n ≥ 2). Lentiviral vectors (LV) without the incorporation of viral envelope glycoproteins (nude). Adjuvant compounds were used according to manufacturer instructions.

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CHAPTER VII

General discussion, Conclusion, and future perspectives

Author contribution

Ana Oliveira wrote this chapter based on the referred bibliography

Chapter VII

7.1 General discussion

After decades of intensive research in academia and industry, gene therapy is now on its golden age. Every year, new products are granted market approval and a wide array of unmet clinical needs are being addressed. Recombinant retrovirus are particularly suited as permanent gene delivery vehicles of these therapies and therefore highly represented in clinical trials and commercialized products [1]. As such, there is a high demand for rapid and reliable large-scale bioprocesses compatible with GMP manufacturing facilities. However, these enveloped vectors are produced at low ratios of transducing to total particles and have a very low stability, easily loosing titers during bioprocessing [2]. To sustain the growing market needs and to assist the development of emerging therapies, it is critical to understand what limits the quality of recombinant retrovirus preparations. Several approaches have been explored aiming to improve vector quality, namely: optimization of vector genetic cassettes expression [3] and stoichiometry [4,5]; vector engineering [6–8]; metabolic engineering of producer cells [9]; and development of transduction enhancers [10,11]. Motivated on further exploring the advantages of all these strategies, this PhD thesis overarching aim was to improve recombinant gammaretrovirus and lentivirus for gene therapy by: (i) studying constitutive producer cell lines bottlenecks to accelerate the establishment of these production platforms, more specifically to understand the impact of vector genetic cassette design, expression, copy number and transfection method in vector yields; (ii) exploring metabolic engineering to improve functional vector yields; and (iii) screening transduction enhancers to increase the vector transduction efficiency. The major achievements obtained in this thesis, from **Chapter II** to **Chapter VI,** are summarized in Figure 7.1.

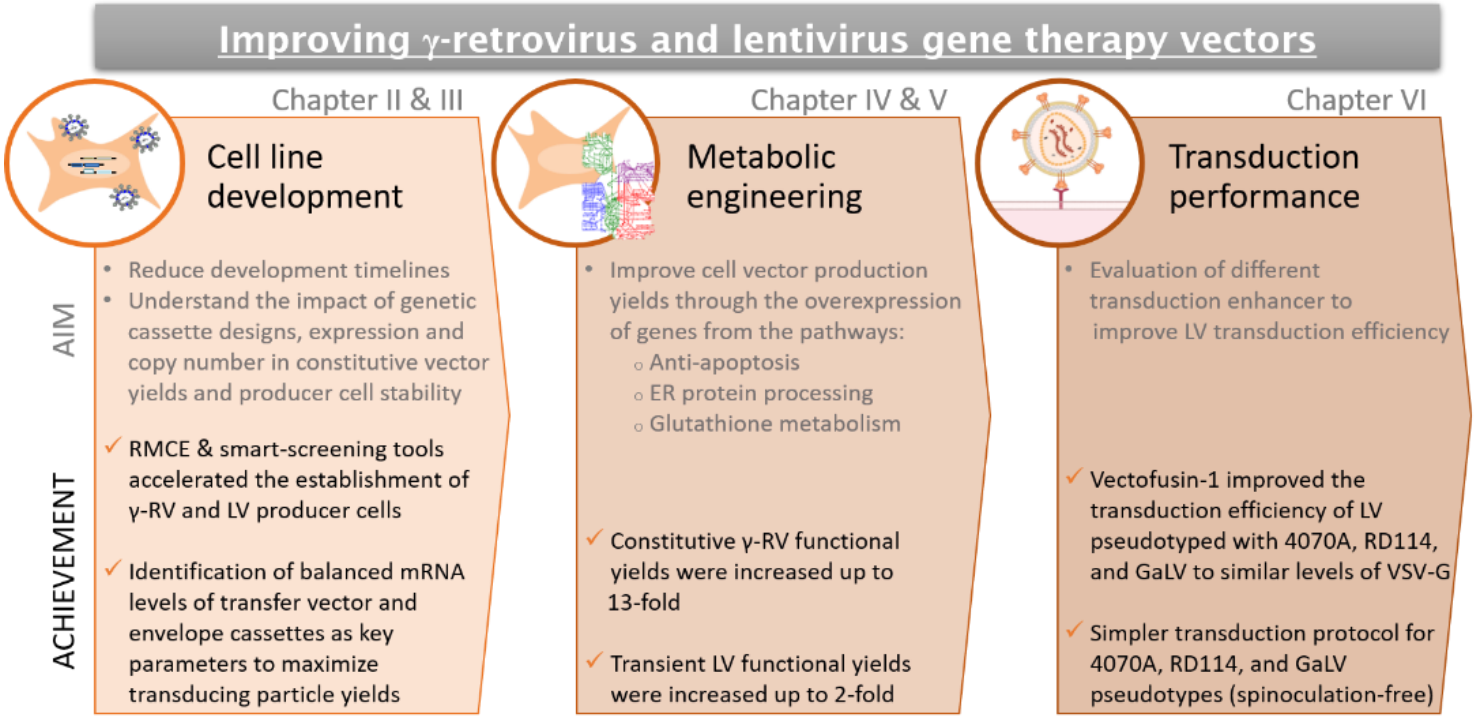

Figure 7.1 – Schematic representation of thesis aims and major achievements improving retroviral vectors for gene therapy.
4070A – Amphotropic murine leukemia virus. GaLV - Gibbon ape leukemia virus. LV – Lentiviral vectors. RD114 – Feline endogenous retrovirus. RMCE – Recombinase mediated cassette exchange. VSV-G – Vesicular stomatitis virus. γ-RVs – Gammaretroviral vectors.

7.1.1 Accelerating constitutive producer cell line development

From the manufacture perspective, constitutive producer cells are the preferred production platform, granting bioprocess scalability and reproducibility at lower costs. Due to the long clinical experience with gammaretroviral vectors (γ-RVs) its bioprocess is more mature than for lentiviral vectors (LV). Currently, LV representation in clinical trials overcomes γ-RV and the same trend is expected in the following years for market approved therapies [1,12]. Therefore, it is necessary to invest in LV manufacture. In the first part of this thesis we extended all previous knowledge and tools implemented for γ-RV to constitutive manufacture of LV (**Chapter II** and **III**).

Transfection and clone screening methods

The development of constitutive production platforms is a laborious and time-consuming process. Therefore, in **Chapter II** we took advantage of previous developed technologies – recombinase mediated cassette exchange (RMCE), split-GFP reporter, and single-step cloning-screening protocol (SSCS) – to accelerate the development of such platforms. A few viral vector producer cells were already developed using RMCE system. However, all of them used viral transduction methods (posing safety concerns) and exhaustive clone screening steps [13–15]. We demonstrated the feasibility of establishing a rapid and standard cell line development protocol for γ-RV and LV (cGMP compliant) based only on chemical and physical transfection methods. The resulting producer cells presented vector yields within the range of current established constitutive systems [16,17]. Nevertheless, using non-viral transfection procedures reduced the overall probability of finding high producer clones and lowered RMCE efficiency. These disadvantages could be a consequence of vector genetic cassette random chromosomal

integration pattern (no preference for transcriptional active sites) [18], but could be successfully overcome by coupling the SSCS method to isolate the rare high producer clones. The random chromosomal integration pattern also occurred in the establishment of LentiPro26 cells [19]. As shown in **Chapter III**, LentiPro26 viral cassettes expression stability throughout subculturing was clone dependent. To overcome the limitations associated with *locus*-specific expression levels and ensure expression stability, the addition of *cis*-acting regulatory sequence elements to all vector cassettes could be beneficial [20], as: insulators [21], ubiquitously acting chromatin opening elements (UCOEs), matrix attachment regions (MARs), stabilizing anti-repressors (STARs) or synthetic promoters [22]. Moreover, promoters should be carefully chosen to safeguard epigenetic silencing, as described for CMV and RSV [23,24].

Planning beforehand the use of smart clone-screening methodologies is of extreme importance since not all make use of unlabelled virus, such as the case of the SSCS method [17] and others [25]. For the SSCS method it is mandatory to use additional genome editing tools to remove the reporter gene for subsequent clinical applications. Indeed, in **Chapter II** we validated for the first time the advantage of using SSCS to establish master producer cells endowed with RMCE technology to exchange the reporter gene by a therapeutic gene, with predictable productivity [17,26]. We also confirmed the applicability of the SSCS protocol to screen LV producer cells. However, the implementation of the SSCS method to any γ-RV transgene or LV was not as straightforward as initially predicted. Reconstitution of the GFP fluorescent signal was low in target cells transduced with 4070A pseudotyped LV. Moreover, GFP intensity was shown to be dependent on the localization of the fusion protein. Thus, not all therapeutic genes can be fused to split GFP. By disclosing the factors that influenced

SSCS parameters, rational decisions were implemented, such as the use of cytosolic slip-GFP fusion proteins and adjusting co-culture timeline of the clones with target cells during SSCS protocol. In addition, other transduction enhancers, such as Vectofusin-1 studied in **Chapter VI**, might improve SSCS method applicability. Further SSCS optimization for the detection of label-free virus could be attained by establishing target cells expressing the recently developed switch-on biosensor methodology [27].

Vector cassettes

As shown throughout this thesis, high expression levels of the viral genome transgene cassette were determinant to achieve high transducing retroviral vector particles yields. The work performed in **Chapter II** evidenced higher γ-RV productivity for clones with multiple genomic transgene integrated copies than single copy clones. In agreement, LV producer clone #54 presented the highest vector genome copy number and expression, which was also associated to high LV functional transducing particles yields (**Chapter III**). Likewise, in **Chapter IV** and **V**, the improved performance of γ-RV producer cells was observed to be strongly associated to increments in transgene expression. All these results are in line with previous reports, confirming the importance of viral RNA genome expression to attain high producer cells [17,18]. Thus, we strongly recommend developing constitutive recombinant retrovirus producer cells based on high vector genome cassette expression, as previously demonstrated for γ-RV [4]. This strategy should be further explored in the future to establish constitutive LV producer cell lines.

The *gag-pro-pol* expression has been proposed as one of the main vector components limiting the development of constitutive LV producer

cells [14,28–31], while also leading to cellular cytotoxicity [32]. To enable sufficient *gag-pro-pol* expression the majority of constitutive LV producer cells – e.g., STAR [28], WinPac [14], RD-MolPack derived [30,31] – inserted this vector component in the producer cells genome by viral transduction (**Chapter I**, section 2.3). To study the vector genetic cassettes expression influence in LV yields (**Chapter III**) we made use of LentiPro26 producer clones previously developed in our group through chemical transfection of third LV generation plasmids and expressing a less active, and potentially less cytotoxic, HIV-1 mutated T26S protease [19]. Our results showed that the *gag-pro-pol* expression levels of these clones (4-fold range) were sufficient to allow total physical particles production in the order of 10^9 P.P./mL and viral genome titers above 10^8 V.G./mL. These results suggest that the LV yields of LentiPro26 clones were not being limited by the *gag-pro-pol* or *rev* components expression. The high *gag-pro-pol* expression in the clones was obtained using a low translation efficiency strategy to express the antibiotic resistant gene incorporated in this LV genetic cassette. The later provided a high stringent cell selection process [19]. The sequential order in which viral genetic cassettes were introduced in the producer cells – first *gag-pro-pol* and then *rev* – could have also enabled the selection of high *gag-pro-pol* expressing cells. Since, it delayed protease toxic activity in the first step of cell line development [19]. Considering that viral genome and transducing LV titers were not correlated, other factors than vector genome content may be influencing particle functionalization, such as particle maturation state and fusogenic envelope content. These latter factors are intrinsically related since both require protease activity to generate mature LV particle [33]. Preliminary results confirmed the presence of active LV protease in the cytoplasm of producer cells. We hypothesise that this cytosolic LV protease activity may have an impact on LV functional titer by decreasing the maturation and the envelope

fusogenic states of genome containing LV particles. Further studies should be conducted to assess the levels of unprocessed Gag and Gag-Pro-Pol proteins, as well as the envelope glycoprotein, in the producer cell cytoplasm and in released LV particles. It is possible, that premature HIV-1 protease activity in producer cell cytoplasm might result in the release of particles lacking viral enzymes [29,32]. If so, engineering the structural and enzymatic unprocessed protein (Gag-Pro-Pol) with heterologous myristoylation signals holds great potential to increase its trafficking and accumulation in producer cell membrane [7].

7.1.2 Metabolic engineering targets for improved recombinant retrovirus production yields

The vision of high producer mammalian cell chassis through multi metabolic engineering strategies has long been proposed [34]. CHO cells have been extensively and successfully targeted by metabolic gene engineering strategies to improve their productivity for several biopharmaceutical molecules, such as antibodies, interferon-γ and erythropoietin [35–47]. In contrast, similar strategies to improve complex virus based biopharmaceuticals manufacture, commonly produced in HEK 293 derived cell lines, have been underexplored albeit harbouring great potential [9,17,48,49]. Previous research performed in our group identified upregulation of protein processing, anti-apoptosis and glutathione metabolic genes as a hallmark of high γ-RV producing cells [50]. These findings were in line with the proposed metabolic engineering targets fundamental for enhanced cell attributes by Seth and colleagues (2007) [34]: energy metabolism, redox balance, growth/death control, and protein processing. In the context of retroviral vectors production using HEK 293 cells, energy metabolism has been previously studied [9]. In

Chapter IV and **V**, we explored the three other proposed functional classes.

The effect of the metabolic gene engineering upon cells specific productivity was dependent on the retroviral vector type and production system. Transducing γ-RV yields of constitutive production, where viral components are continuously synthesized, benefitted from the overexpression of protein processing (**Chapter IV**) and glutathione metabolism (**Chapter V**) related genes. Both pathways are intrinsically associated, as high protein production rates induces oxidative stress, particularly in the endoplasmic reticulum as a consequence of oxidative protein folding associated to disulfide bond formation [51]. This indicates that transducing vector yields of constitutive manufacture systems are affected by protein synthesis crowding in the producer cells. As consequence, we hypothesize that protein (the major retroviral vector building block [52]) quality is being impaired, increasing oxidative stress [50] and compromising cell transducing particles productivity. Further analysis of these engineered constitutive producer cell populations using the SSCS would also be useful to assess clonal productivity distribution [17].

When expressing toxic products, such as the case of VSV-G or transfer vectors carrying death inducer genes, transient production systems are commonly preferable. As shown in **Chapter V**, the improvements in LV transient productivity were associated to the overexpression of an anti-apoptotic gene, highlighting the need for apoptotic-resistant HEK 293 derived cell substrates for industrial manufacture [49].

As shown, metabolic engineering strategies can indeed improve recombinant retrovirus production yields. However, the genetic engineering strategies used may induce other unspecific effects in the producer cell, masking the impact of gene manipulation [9,53]. We

assessed two strategies to overexpress the candidate genes. The first used multiple LV transduction steps to increase the metabolic candidate gene expression level (**Chapter IV** and **V**). In this strategy, gene dose was studied using five engineered cell populations with different metabolic gene expression levels. As a second strategy, inducible gene expression was employed to study the metabolic gene overexpression level, thus making use of only one engineered cell population. This latter genetic engineering strategy showed to interfere in the LV transducing particle yields of HEK 293T cells. Contributing for this effect could be the expression and supplementation of potential cytotoxic factors (trans-activator and doxycycline) [54–56] used to modulate gene expression levels (**Chapter V**). Overall, these results emphasise that, when studying metabolic engineered cells, thoughtful considerations have to be made to ensure the genetic manipulation itself does not impact the vector yields of the producer cells under study [53,57].

7.1.3 Increasing LV transduction efficiency

The viral envelope glycoproteins are responsible for virus attachment to cell host receptors. Several heterologous envelopes (**Chapter I**, section 2.3) have been used to pseudotype recombinant retrovirus, being VSV-G the most widely used due to its increased bioprocess stability. Nevertheless, its broad tropism limits its application exclusively to *ex vivo* therapies. *Gammaretrovirus* derived envelope glycoproteins are the second most used for LV pseudotyping, rendering viral vectors with more restricted tropism [58]. However, as these glycoproteins are a disulfide linked complex of two subunits, they are fragile to manufacturing bioprocesses [59]. Also, these glycoproteins are incorporated in the viral particles in an unprocessed form, requiring cytoplasmic tail cleavage by the virus protease to form fusogenic

competent vectors. This proteolytic cleavage has been the target of extensive engineering efforts, aiming at an optimized sequence for efficient protease processing [8,60,61]. Still, the transduction efficiency of *Gammaretrovirus* derived pseudotypes remains suboptimal. Many compounds and transduction conditions have been developed to counteract the repulsive mechanisms associated to virus and cell membrane fusion [10,11,62–65]. Nonetheless, a systematic comparison of the most used transduction enhancers was never performed. Herein, we compared and disclosed which viral transduction adjuvants have the best performance in two cell line models using four LV pseudotypes (**Chapter VI**).

Vectofusin-1 was by far the clinical grade compound enabling higher transduction efficiencies of *Gammaretrovirus* derived pseudotypes, rendering their efficiencies comparable to VSV-G. These *Gammaretrovirus* derived pseudotypes mediate LV and cell membrane fusion through pH-independent cell entry pathway. In contrast, VSV-G pseudotype enables LV and cell membrane fusion through pH-dependent cell entry pathway. Thus, our results indicate that inefficient pH-independent cell entry pathway is limiting the transduction efficiency of *Gammaretrovirus* derived pseudotypes, ultimately leading to an under estimation of the real functional LV titer. In this part of the work, we provided simpler transduction procedures yielding up to 70% of transduced cells with *Gammaretrovirus* derived pseudotypes, which corresponded to an increase of 3-fold when compared to Retronectin transduction enhancer used in standard clinical protocols [11]. These optimized LV transduction procedures (spinoculation-free) are not only important to increase cell viability in *ex vivo* gene therapy applications but also to extend the use of alternative pseudotyped vectors in *in vivo* settings. In addition, 4070A, RD114 and GaLV envelopes are non-toxic, allowing the establishment of constitutive producer cells. The

improvement of transduction protocols for these envelopes pseudotyped LV further support, their use in clinic and, the development of cost-effective stable constitutive producer cells for their manufacturing.

7.2 Conclusion and future perspectives

This PhD work disclosed multiple strategies delivering improved retroviral vector transduction capacity, either addressed in terms of producer cell yields or vector transduction efficiency.

Different constitutive cell line development strategies were explored, highlighting vector cassettes design, transfection and high-throughput clone screening methods as enablers of rapid development procedures. High and stable expression of all vector cassettes was associated to the maintenance of high producer cell yields, but we found vector genome and envelope expression to be more limiting. Further advance cell line development process optimizations should target these two viral cassettes.

The metabolic gene engineering targets herein studied were validated for improved productivity of HEK 293 derived cells. Specifically, constitutive γ-RV yields increased when overexpressing endoplasmic reticulum protein processing and glutathione metabolism genes. LV yields were raised with the overexpression of anti-apoptotic and pentose phosphate pathway genes. Further improvements to the producer platforms herein developed should include adaptation to serum-free, suspension culture conditions, as well as bioreaction scale-up.

Regarding LV transduction potential, this attribute was associated to the efficiency of envelope glycoprotein mediated cell entry mechanism, being lower for pseudotypes with a pH-independent cell entry (4070A, RD114, and GaLV). Vectofusin-1 was identified has the transduction enhancer providing higher transduction efficiencies for 4070A, RD114,

and GaLV pseudotyped LV, it allowed to achieve comparable transductions levels to VSV-G. This transduction enhancer may be an option to improve the GFP signal of the SSCS, facilitating the identification of constitutive LV producer cells.

The integration of all the strategies explored in this work pursuing synergistic effects will deliver superior transducing retroviral vector yields (Fig. 7.2). We envision that the flexibility of recombinant retrovirus producer cells can be further increased by adding additional non-compatible RMCE sites for recombinases such as Cre, Flipase and φC31, flanking each vector genetic cassette [66–68]. If so, the master flexible cell line would express optimized designs of the vector genetic cassettes allowing optimal expression stoichiometry, resistance to epigenetic silencing and thus provide improved vector producer cell stability. Genetic engineering of this flexible cell line using validated metabolic targets in this thesis adds a further level of improvement. Next, a high producer clone could be rapidly isolated using SSCS methodology, generating a robust producer cell platform for bioprocess. Finally, retroviral vector preparations should be efficiently utilized by applying optimized cell transduction procedures. Ultimately all contributing for decreasing the required vector production volumes and thus, lowering costs.

The knowledge gathered in this thesis contributes to expedite the manufacture and applicability of recombinant retrovirus in basic research, biotechnological applications, and clinical settings.

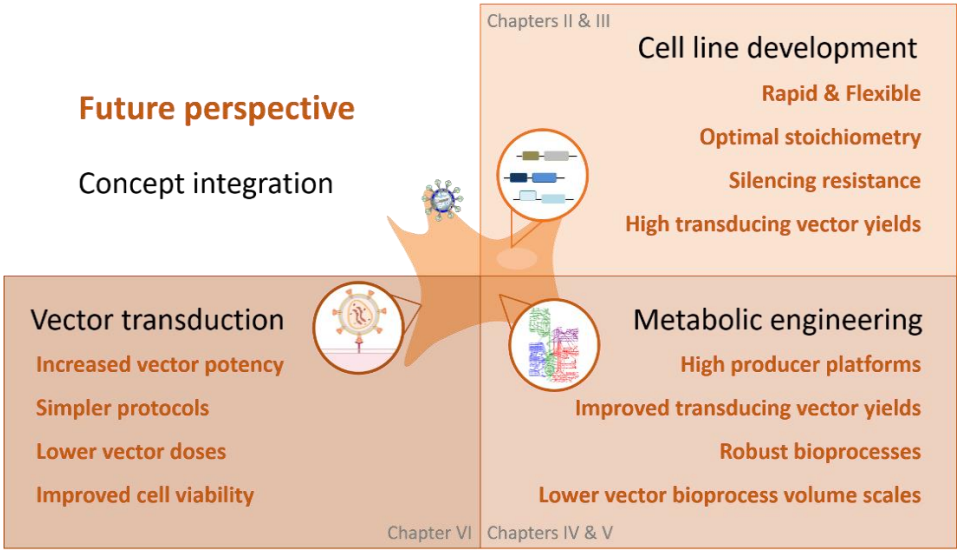

Figure 7.2 – Schematic representation of important milestones in cell line development explored in this thesis and their future synergistic integration towards improved retroviral vectors for gene therapy.

7.3 References

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