Tiago André Gonçalves Vaz

Degree in Biomedical Sciences

Genetic engineering of mammalian cell lines for improved production of gene therapy viral vectors

Dissertation to obtain Master Degree in
Molecular Genetics and Biomedicine

Supervisor: Doutora Ana Sofia Coroadinha, PhD, ITQB/iBET UNL

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September 2017

Tiago André Gonçalves Vaz

Degree in Biomedical Sciences

Genetic engineering of mammalian cell lines for improved production of gene therapy viral vectors

Dissertation to obtain Master Degree in

Molecular Genetics and Biomedicine

Supervisor: Doutora Ana Sofia Coroadinha, PhD, ITQB/iBET UNL

September 2017

Acknowledgements

The first words of acknowledgement go to my supervisor, Dr. Ana Sofia Coroadinha, for giving me the opportunity to work with the Molecular Biotechnology and Cell Line Development group of the Animal Cell Technology Unit at ITQB/iBET.

To Ana Filipa Rodrigues for the constant guidance, advice and support she provided. The professionalism and commitment she puts into everything she does, every single day are for me an inspiration and a role model.

To all my friends and colleagues of the Molecular Biotechnology and Cell Line Development group: Hugo, Hélio, Miguel, Ana Oliveira, Ana Isabel, Sofia, Daniel, Daniela and Victor; Words do not suffice to thank for all the support, encouragement and moments of joy we shared throughout this year.

To all the professors that lecture the Masters in Molecular Genetics and Biomedicine at FCT-UNL for the valuable knowledge they share with their students.

Aos meus pais e irmã pelo apoio incondicional, pelas chamadas demoradas, os fins-de-semana bem passados, os jogos em família e as coisas mais simples e belas que dão valor ao dia-a-dia.

Aos meus tios, Tina e Beto, que me acolheram em sua casa enquanto frequentei o mestrado. É impossível expressar a gratidão que sinto por terem acompanhado todos os dias desta jornada.

Aos restantes familiares que de incontáveis maneiras me ajudaram a avançar a cada dia.

Aos meus amigos de mestrado: Rita, Cláudia, Carolina, Joana, Filipe, João, Sara e Sandra; que transformaram um ciclo de estudos numa fase memorável da minha vida.

Aos meus amigos e colegas da Licenciatura em Ciências Biomédicas na Universidade de Aveiro, o tempo passa, o caminho diverge mas a amizade fica.

Aos Escuteiros do Agrupamento 1282 de São João da Madeira que desde sempre me ensinaram a dar mais do que o que tenho e deixar o mundo um pouco melhor do que o encontrei.

Abstract

Viral vectors are widely used in gene therapy as vehicles to deliver therapeutic gene cargos. Among the different viral vectors, retroviral and lentiviral vectors are of particular interest due to their ability to sustain long-term stable expression of the therapeutic gene. However, current production systems for these vectors face several challenges namely, the low yields and the need of animal blood serum.

This work focused on improving retroviral vector production by genetic engineering targeting glutathione and lipid metabolic pathways. To this end, molecular and analytical tools were also developed, namely, a system for inducible gene expression and a method for universal titration of lentiviral vectors. The inducible system, based on a TET-ON configuration, uses a tetracycline analogue to induce dose-responsive expression of the gene of interest and was used as a molecular tool for genetic manipulation. The lentiviral vector titration method is based on the quantification of lentiviral long terminal repeats (LV-LTR) integrated into the target cells genome. In the context of this work, it allowed to titrate lentiviral vector stocks containing the inducible system, assuring the populations were uniformly established.

Genetic manipulation of glutathione metabolism was able to increase retroviral vector production up to 5-fold. This effect was associated with increased retroviral transgene expression and copy number in the producer cells. Lipid metabolism was studied in two producer cell lines that displayed different phenotypes regarding retroviral vector production under serum deprivation, to guide further genetic engineering.

This work contributes to the state-of-the-art on gene therapy based on improvement of viral vector producer cell lines by means of metabolism manipulation. The novel tools also developed expand the boundaries of genetic engineering and cell line development.

Keywords: Gene therapy; Retroviral vectors; Genetic engineering; Metabolism; Inducible gene expression; Lentiviral titration.

Resumo

Os vetores virais são amplamente usados em terapia génica como veículos de entrega de material genético. Entre os diferentes tipos de vetores virais, os vetores retrovirais e lentivirais são particularmente interessantes pois permitem uma expressão estável e a longo-termo do gene terapêutico. No entanto, os atuais sistemas de produção destes vetores enfrentam dificuldades, nomeadamente a nível dos títulos de produção e a sua dependência de soro animal.

Este trabalho focou-se na melhoria da produção de vetores retrovirais através de engenharia genética nas vias metabólicas da glutationa e dos lípidos. Para isso, foram desenvolvidas ferramentas moleculares e analíticas nomeadamente, um sistema indutível de expressão génica e um método universal para titulação de vetores lentivirais. O sistema indutível, baseado numa configuração TET-ON, é ativado por um análogo da tetraciclina, levando a uma expressão do gene de interesse proporcional à dose aplicada e foi por isso usado como ferramenta molecular para manipulação genética. O método universal para titulação de vetores lentivirais baseia-se na quantificação das long terminal repeats lentivirais (LV-LTR) integradas no genoma das células-alvo. No contexto deste trabalho, permitiu a titulação de preparações de vetores lentivirais usadas para entregar as construções do sistema indutível, de forma a estabelece populações uniformes.

A manipulação genética do metabolismo da glutationa levou ao aumento da produção de vetores retrovirais até 5 vezes. Este efeito foi acompanhado de aumento da expressão e do número de cópias do transgene retroviral nas células produtoras. O metabolismo lipídico foi estudado em duas linhas celulares produtoras que manifestaram diferentes fenótipos no que toca à produção de vetores retrovirais, guiando futuras abordagens de engenharia genética.

Este trabalho contribui para o estado-da-arte da terapia génica através do melhoramento de células produtoras de vetores virais recorrendo à manipulação metabólica. As novas ferramentas desenvolvidas expandem as aplicações da engenharia genética e do desenvolvimento de linhas celulares.

Termos-chave: Terapia Génica; Vetores retrovirais; Engenharia genética; Metabolismo; Expressão génica indutível; Titulação de vetores lentivirais

List of Contents

Figure Index

Table Index

Abbreviations

CA	Capsid protein
CMV	*Cytomegalovirus*
CT	Crossing threshold
DMEM	Dulbecco's modified Eagle medium
GFP	Green fluorescent protein
EMA	European Medicines Agency
Env	Envelope glycoprotein
FBS	Fetal bovine serum
FDA	Food and drug administration
Gag-pol	Group specific antigen – polymerase: structural and enzymatic viral proteins
GaLV	*Gibbon ape leukemia virus*
HEK	Human embryonic kidney
HIV-1	*Human immunodeficiency virus 1*
I.P.	Infectious particle
I.U.	Integrating unit
IN	Integrase
LTR	Long terminal repeat
LV	Lentiviral vector
MA	Matrix protein
MLV	*Murine leukemia virus*
NC	Nucleocapsid protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PGK	Phosphoglycerate kinase 1
pol	Polymerase
PPT	Polypurine tract
PR	Protease
Rev	Regulator of expression of viral proteins
RRE	Rev responsive element
RSV	*Rous sarcoma virus*
RT	Reverse transcriptase
RT-qPCR	Real-time quantitative PCR
rtTA3	Reverse tetracycline-controlled transactivator 3
RV	Retroviral vector

SCID	Severe combined immunodeficiency
SIN	Self-inactivating
SU	Surface
SV40	*Simian virus 40*
TRE	Tetracycline responsive element
TM	Transmembrane
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
VSV-G	Vesicular stomatitis virus G glycoprotein

1. Introduction

1.1. Gene therapy

Gene therapy is the treatment or prevention of diseases by delivering genetic material to the patients' cells or tissues. Since the conception, in the early 80's of the 20th century, gene therapy was considered a revolutionary approach targeting previously unmet medical needs such as severe combined immunodeficiency (SCID) and rare blood disorders (Mountain, 2000).

The delivery of genetic material into target cells is done by specific vehicles, generally called vectors. Gene therapy can be performed *in vivo* and *ex vivo.* In the *in vivo* approach the vector is administrated to the patient where it targets the cells and delivers the genetic material while in the *ex vivo* method, patient cells are collected, modified by vector-mediated gene delivery followed by re-insertion of the modified cells back into the patient (Wirth et al., 2013). Viral vectors are recombinant viruses modified to act as vehicles of therapeutic gene delivery and are the most used type of vectors for gene therapy due to their high efficiency (Thomas et al., 2003). A general representation of *ex vivo* gene therapy using viral vectors is shown in **Figure 1.1**.

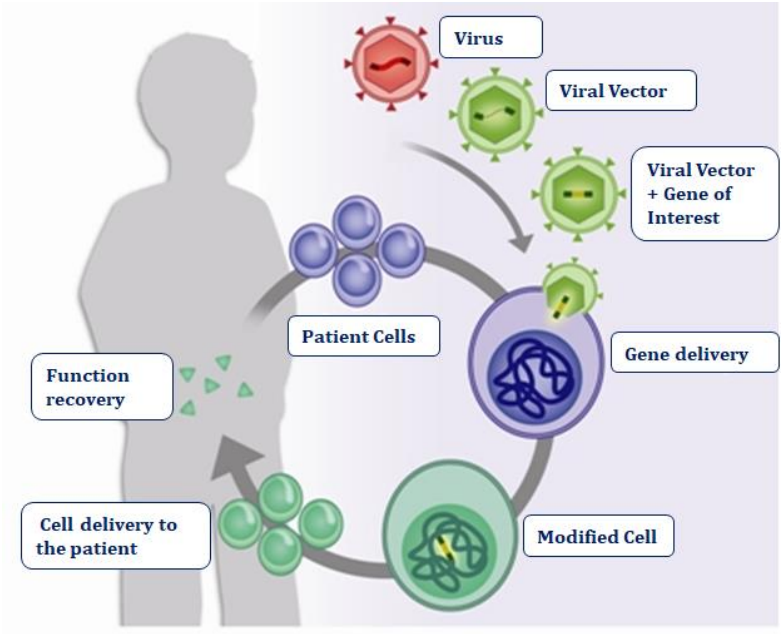


Figure 1.1 - Schematic representation of *ex vivo* viral gene therapy. Main steps of *ex-vivo* gene therapy using viral vectors. Defective cells are collected from the patient and corrected *in vitro* using viral vectors as gene cargo vehicle. The modified cells are then re-inserted into the patient resulting in a therapeutic effect. Adapted from https://vector.childrenshospital.org/2011/05/a-new-start-for-gene-therapy-for-bubble-boy-disease-first-u-s-treated-patient-doing-well/ - accessed: 16th September, 2017 (Fliesler, 2011).

The first gene therapy clinical trials started in 1989 and targeted advanced melanoma (Rosenberg et al., 1990) followed by the more well-known applications of gene therapy to treat patients suffering from SCID (Blaese et al., 1995). As of today, over 2400 clinical trials have been conducted or are still ongoing (Edelstein, 2017). Cancer is the main disease targeted by gene therapy and together with monogenic, infectious and cardiovascular diseases makes up the large majority of the indications addressed (Edelstein, 2017).

Although originally conceived to target rare genetic disorders, the market for gene therapy has been growing due to its potential to treat other conditions with increasing incidence such as cancer and chronic diseases (Wirth et al., 2013). The distribution of clinical trials of gene therapy targeting different diseases is presented in **Figure 1.2**. The revenues from gene therapy reached over 9 million dollars in 2016 but, as more and more products reach the later stages of clinical trials, the predictions to 2020 point to revenues of over 200 million dollars and potential to continue expanding until 2026 (Visiongain, 2016).

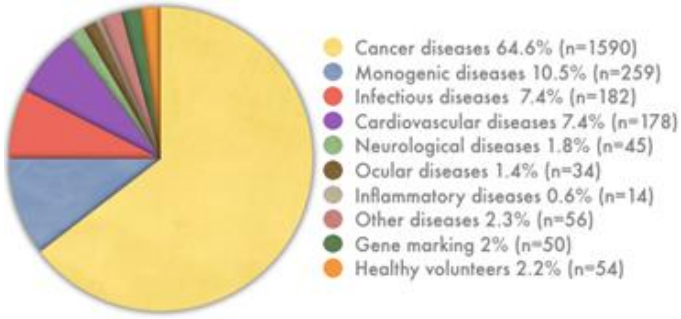


Figure 1.2 - Diseases targeted in gene therapy clinical trials. Distribution of gene therapy clinical trials by disease. Adapted from The Journal of Gene Medicine (Edelstein, 2017) - http://www.wiley.com//legacy/wileychi/genmed/clinical/ accessed: 16th September, 2017)

Currently, several gene therapy products have reached the market. The first to be approved by the European Medicines Agency (EMA) in 2012, Glybera (uniQure, Amsterdam, Netherlands) targets lipoprotein lipase deficiency using adeno-associated viral vectors encoding lipoprotein lipase (Moran, 2012). In 2016, Strimvelis (GSK, London, United Kingdom), was approved by EMA for the treatment of adenosine deaminase deficiency – SCID (ADA-SCID) using retroviral vectors encoding adenosine deaminase (Booth et al., 2016). Gendicine (SiBiono Gene Tech, Shenzhen, China) for head and neck cancer (Pearson et al., 2004) and Oncorine H101 (Sunway Biotech, Shangai, China) for nasopharyngeal carcinoma (Liang, 2012) were approved by China Food and Drug Administration (CFDA) in 2004 and 2005 respectively, using adenoviral vectors. In 2007, Philippines Food and Drug Administration approved Rexin G (Epeius Biotechnologies, San Marino, CA, USA) an oncolytic therapy using retroviral vectors (Gordon & Hall, 2010). Finally in the USA, IMLYGIC (Amgen, Thousand Oaks, CA, USA) targeting various

cancers using *Herpes simplex virus* based vectors (Greig, 2016) and CAR-T therapy using lentiviral vectors to modify T Lymphocytes (Novartis, Basel, Switzerland, Sheridan, 2017) were approved by U.S. FDA in 2015 and 2017, respectively.

1.2 Viral vectors for gene therapy

Viral vectors are widely used in gene therapy due to their natural ability to infect cells and delivering their genetic material. This feature makes viral vectors the most efficient delivery vehicle in gene therapy (Thomas et al., 2003). Viral vectors make up to 70% of the vectors used in gene therapy clinical trials (Edelstein, 2017), as shown in **Figure 1.3**.

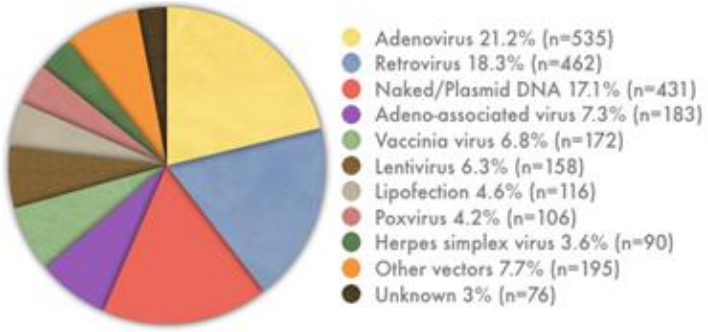


Figure 1.3 - Vectors used in gene therapy clinical trials. Distribution of gene therapy clinical trials by vectors used. Adapted from The Journal of Gene Medicine (Edelstein, 2017) - http://www.wiley.com//legacy/wileychi/genmed/clinical/ accessed: 16th September, 2017)

Viral vectors based on recombinant viruses retain some properties of the virus they derive from, giving each of them a set of properties that can be used in different applications for gene therapy (Thomas et al., 2003) briefly listed in **Table 1.1**.

Table 1.1 - Properties of viral vectors. (Adapted from: Thomas et al., 2003)

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

*Replication defective. ‡Amplicon. §Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDNA, single-stranded DNA.

Adenoviral vectors have been the most widely used vectors in gene therapy. They are able to transduce most tissues, supporting transient expression of the gene delivered without integrating into the host genome. Adenoviral vectors have found most of their use on cancer treatment as vectors for oncolytic therapy (Wold & Toth, 2013) and were the first to be commercialized as a therapeutic product - Gendicine (SiBiono).

Gama-retroviral vectors, generally known as retroviral vectors, are the second most used type of vector for gene therapy. *Gammaretroviruses* are a part of the *retroviridae* family that among other sub-families includes *lentiviruses*. When considering both retroviral and lentiviral vectors together they actually surpass adenoviral vectors in terms of usage. Contrary to *adenoviruses*, the use of *retroviruses* is presently growing. Retroviral vectors are substantially less immunogenic, can accommodate up to 9 kb of gene cargo and they integrate into the target cell genome sustaining long-term expression of the delivered transgene (Coroadinha et al., 2010). Due to their integrative nature, retroviral vectors are particularly suitable for the treatment of monogenic and chronic infectious diseases (Thomas et al., 2003). The main limitation of these vectors is their inability to transduce non-dividing cells and the possibility to cause oncogenesis due to their integration into the targets genome (Thomas et al., 2003).

While retroviral and adenoviral vectors have long been used in gene therapy clinical trials, *Adeno-associated viruses* (AAV) based vectors have recently experienced substantial growth in clinical trials for gene therapy (Edelstein, 2017). They are able to transduce most cell types with minimal immune responses. However, their gene cargo is limited to 5 kb (Thomas et al., 2003). A recombinant AAV based vector - Glybera (uniQure) - was the first gene therapy product to be commercialized in Europe (Moran, 2012).

Lentiviruses are a sub-family of *retroviruses*, they are more complex than *gammaretroviruses* and are particularly interesting due to their ability to also integrate into non-diving cells (Thomas et al., 2003). The use of lentiviral vectors in gene therapy has experienced remarkable growth, second only to AAVs'. Lentiviral vectors serve the same applications as retroviral vectors but with increased safety (Montini et al., 2009) while also being able to transduce non-dividing cells. Because of this, lentiviral vectors are expected to take over retroviral vectors place in gene therapy in the near future (Edelstein, 2017).

1.3. Retroviral and Lentiviral vectors

1.3.1. Retrovirus biology

Gammaretroviruses, include viruses like *Murine Leukemia Virus* (MLV) and *Gibbon ape Leukemia Virus* (GaLV) and are a *genus* of the *retroviridae* family. They are enveloped viruses and their genome consists of two single-stranded positive-sense RNA molecules with sizes ranging from 7 to 12 kb. Their most distinctive feature is the ability to reverse transcribe their RNA genome into DNA followed by stable integration of this DNA into the host cell genome. These functions are supported by the viral reverse transcriptase and integrase respectively (Coffin et al.,1997). The virions are 80 - 100 nm in diameter, delimited by a lipid bilayer (envelope) derived from the host cell membrane in which envelope glycoproteins are anchored. These proteins are composed of two subunits, a transmembrane (TM)

subunit that anchors the complex to the lipid membrane and a surface (SU) component that interacts with the cellular receptor to mediate viral entry. Underneath the envelope, matrix proteins (MA) delimit the interface with the capsid, made of capsid proteins (CA), which encloses the RNA molecules complexed with nucleocapsid proteins (NC). Additionally the virion contains three enzymes, the reverse transcriptase (RT, that reverse transcribes the viral RNA genome into DNA), the integrase (IN, that mediates integration of the reverse transcribed viral genome into the host cell genome) and the protease (PR, that cleaves the products of the transcriptional domains into active peptides or proteins). A schematic representation of a retroviral particle is shown on **Figure 1.4** (Coffin et al., 1997).

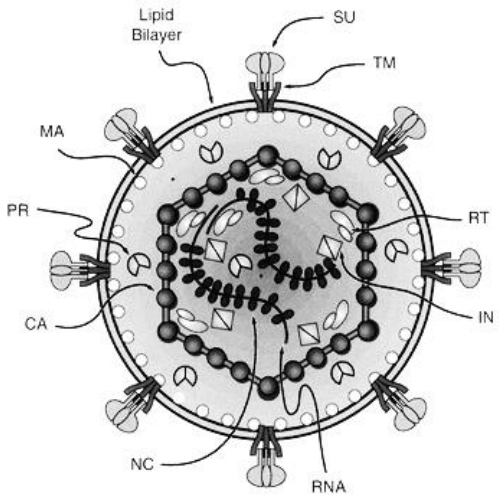


Figure 1.4 - Schematic representation of a retroviral particle. Retroviral virions are enveloped within a cell-derived lipid bilayer that displays complexes of glycoproteins on the surface. A surface subunit that interacts with the host cell receptors is anchored by a transmembrane subunit (TM). The inside of the particle contains the matrix proteins (MA) and the capsid, made of capsid proteins (CA). The capsid encloses the retroviral genome (single-stranded positive RNA), complexed with nucleocapsid proteins (NC) and contains enzymes essential for the *retrovirus* life cycle: reverse transcriptase (RT), protease (PR) and integrase (IN). Adapted from: Coffin et al., 1997.

The retroviral genome codes for three major transcriptional domains: *gag* (group specific antigen), *pol* (polymerase) and *env* (envelope) as well as a smaller transcriptional domain – *pro* (protease). The main structural proteins, MA, CA and NC are encoded in the *gag* transcriptional domain; the glycoproteins of the enveloped are coded by the *env* transcriptional domain (TM and SU); *pol* codes for reverse RT and IN and *pro* codes for PR. The genome also contains non-coding elements such as long terminal repeats (LTRs) that drive gene expression and a packaging signal (ψ) required to pack the specific RNA molecule into the virion during assembly (Coffin et al., 1997). A representation of a retroviral genome is shown in **Figure 1.5.**

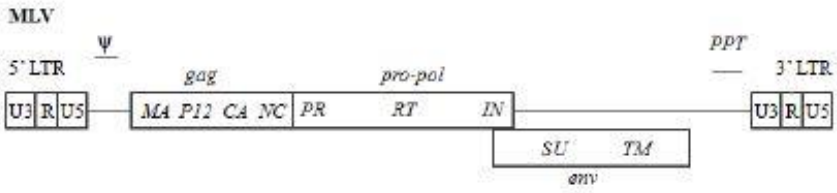


Figure 1.5 - Schematic representation of a retroviral genome. Schematic representation of *Murine Leukemia Virus* (MLV) genome. The single stranded positive RNA molecule contains four transcriptional domains, *gag* and *pro-pol* are within the same open reading frame (ORF) while *env* is coded in a different ORF. Both ends of the RNA molecule contain long terminal repeats (LTRs). A packaging signal (ψ) is located after the 5' LTR. Adapted from: Rodrigues et al., 2011.

The life cycle of *retroviruses* starts with the binding of the SU proteins to the host membrane receptors, promoting the fusion of the viral envelope with the cell membrane and releasing the capsid into the cytoplasm. The viral genome is then converted into DNA by the viral reverse transcriptase and is integrated into the host cell genome by the integrase. Using the cellular transcriptional machinery the different coding domains of the virus are transcribed and translated. The viral peptides resulting from translation and the viral genome, resulting from unspliced RNA molecules that contain the packaging signal, migrate to the inner part of the cell membrane where virion assembly takes place. This structure starts to bud from the cell and is ultimately released taking with it a portion of the membrane that forms the envelope. Outside the cell the particle goes through a process of maturation where the protease cleaves the peptides into functional viral proteins resulting in a new infectious particle (Maetzig et al., 2011). A representation of a *retrovirus* life cycle is shown in **Figure 1.6**.

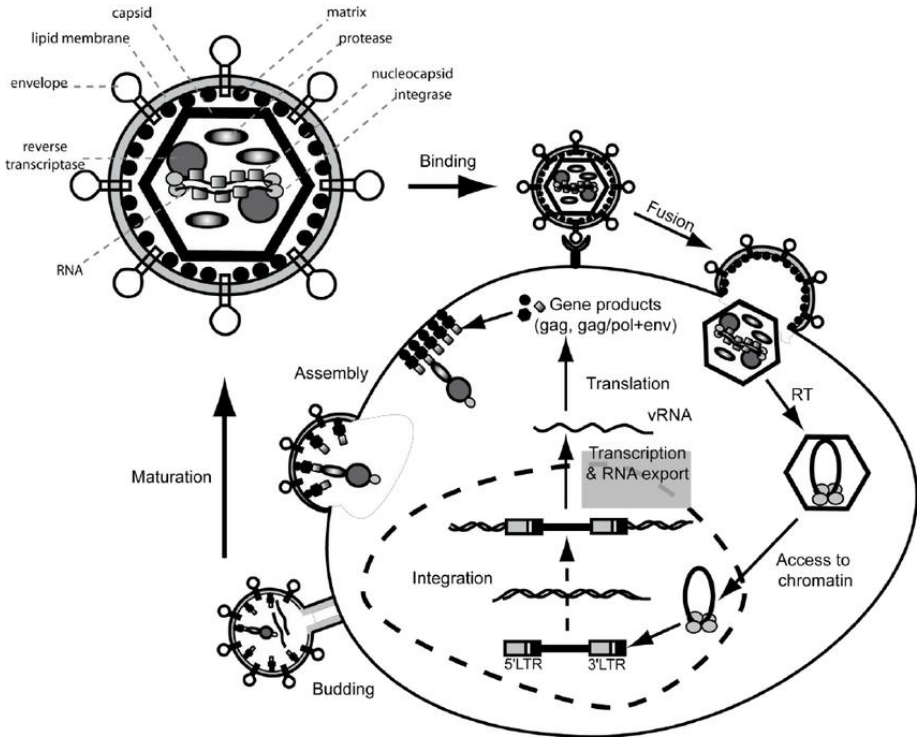


Figure 1.6 - Schematic representation of MLV life-cycle. Main steps of MLV replication cycle. The envelope glycoproteins interact with the cell receptors' resulting in the fusion of the envelope with the cell membrane and the release of content of the retroviral particle. The viral RNA genome is converted into DNA by the reverse transcriptase and stably integrated in the host cell genome leading to the expression of the different proteins coded within the viral genome transcriptional domains. These products and the viral genome migrate to the assembly site at the inner part of the cell membrane and start assembling the new viral particle that buds out of the cell as an immature particle. During the final step of maturation outside of the cell, viral peptides undergo proteolysis by the viral protease resulting in functional proteins that make the virion an infectious particle. Source: (Maetzig et al., 2011)

1.3.2. Retroviral vectors

Retroviral vector production is based on the expression of the different viral transcriptional domains in physically separate units by producer cell lines resulting in a viral particle where the viral genome is replaced by a gene of interest. This production can be transient or stable depending if the producer cell line is transfected with the viral constructs leading to short-term production or if these constructs are stably integrated into the producer cell genome and constitutively expressed (Rodrigues et al., 2011).

Retroviral vectors evolved in different generations. In each generation the safety was increased by dividing the viral transcriptional domains across a higher number of constructs and adding specific elements and sequences (Rodrigues et al., 2011). Currently, the most common approach for retroviral vector production uses an optimized third generation system where the *gag-pro-pol* genes are expressed from a single construct driven by a heterologous promoter, a second construct expresses the transgene

typically driven by the 5´ LTR promoter and contains the packaging signal (ψ) to be encapsidated into the retroviral particle and a third construct expressing *env* genes, usually driven by another heterologous promoter (Rodrigues et al., 2011). A schematic representation of a retroviral vector producer cell and a retroviral vector particle are shown in **Figure 1.7**.

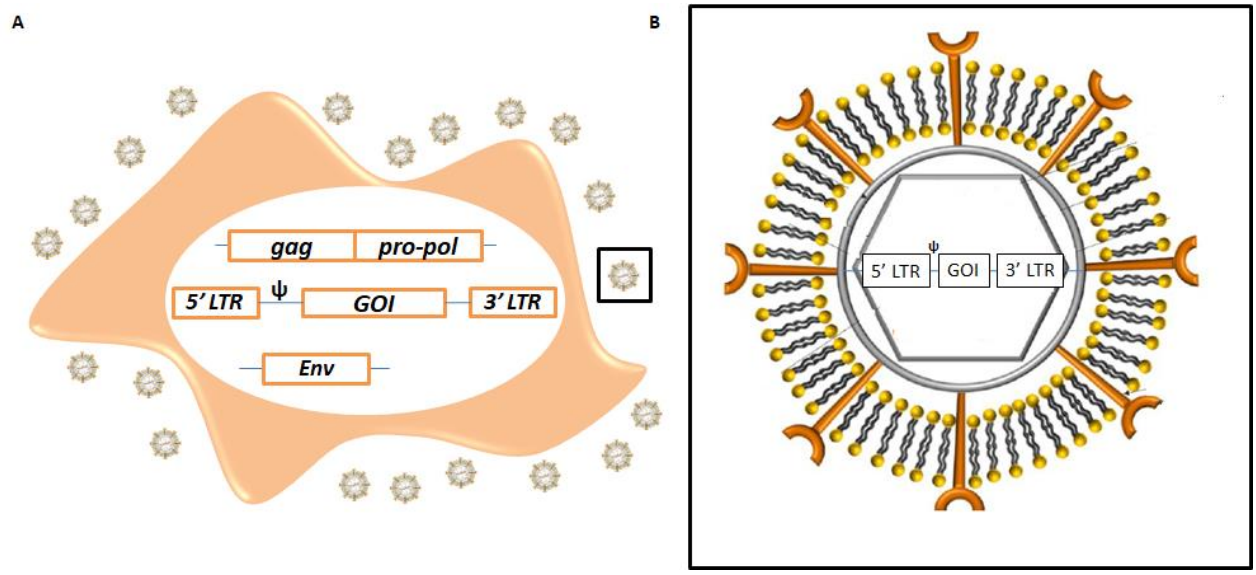


Figure 1.7 - Third generation retroviral vector production system. Schematic representation of a producer cell line **(A)**. Third generation split viral genome constructs for: packaging functions (*gag-pro-pol*), transgene expressing a gene of interest (GOI) and envelope (*env*) are integrated in the genome of the producer cell. Retroviral vector particle carrying the transgene construct **(B)**. Adapted from: Rodrigues et al., 2011.

With the physical separation of the viral genome alone, three homologous recombination events had to occur to form a replicative particle. Although extremely unlikely such events are possible and were previously reported (Chong & Vile, 1996). As so this system has been further optimized with the use of LTR sequences of different species between the transgene, *gag-pro-pol* and *env* constructs (Cosset et al., 1995) or by completely replacing the LTR sequences of *gag-pro-pol* and *env* constructs by heterologous promoters (Rigg et al., 1996).

The envelope glycoproteins of retroviral (and lentiviral) vectors can be exchanged according to the *env* construct introduced in the producer cell line in a process known as pseudotyping. Each species of *retroviruses* expresses their own type of envelope glycoproteins which, in turn, specifically binds to different host cell receptors. In nature, *retrovirus*es are only able to infect cells that present that specific receptor on their membrane making it so these viruses have a particular tropism. Applying this knowledge to retroviral vectors allows selecting which envelope glycoproteins should be presented by the viral particle to specifically and effectively bind and infect the target cell (Rodrigues et al., 2011).

1.3.3. Lentivirus and lentiviral vectors

Lentiviruses are also a *genus* of the *retroviridae* family that have long incubation periods and establish persistent infection. Some examples of *lentiviruses* include *Human immunodeficiency virus 1* and *2* (HIV-1 and HIV-2) and *Simian immunodeficiency virus* (SIV). The genomes of *lentiviruses* share the same structure as *gammaretroviruses* and their size ranges from 7 - 13 kb albeit slightly more complex in terms of transcriptional domains and accessory proteins. Due to their additional components and accessory proteins, *lentiviruses* are able to infect non-dividing cells (Coffin et al., 1997). The exact mechanism through which *lentiviruses* are able to infect non-diving cells is not fully understood. Components like matrix proteins, integrase and some accessory proteins seem to be implicated in this process that ultimately allows the import of the viral genome, through the nuclear pore, into the nucleus, while other *retroviruses* need the nuclear membrane to be disrupted during cell division in order to integrate their viral genome into the host cell genome (Vodicka, 2001).

Vectors based on *lentivirus* are powerful tools for gene delivery, useful not only for gene therapy but also for genetic engineering namely in the establishment of engineered stable cell lines and gene overexpression (Quinonez & Sutton, 2002). Much like retroviral vectors, lentiviral vector production systems evolved over time in generations. Currently the most widely used method for lentiviral vector production is a transient production system based on co-transfection of three or four constructs or plasmids, supporting short-term expression of the lentiviral vector components. The high cytotoxicity of some of the vector components is hindering the development of stable producer cell lines (Schweizer & Merten, 2010).

1.4. Manipulation of cell metabolism for improved viral vector production

Current retroviral and lentiviral vector production systems face limitations that difficult the transition these products from clinical-to-market. These challenges mainly arise from insufficient viral vector titers (Stacey & Merten, 2011), low ratios of infectious-to-total particles hampering the efficiency of infectious particles (Carrondo et al., 2008) and safety concerns related with pathogen contamination due to the reliance on animal serum for viral vectors production (Rodrigues et al., 2011).

Compared with lentiviral vectors, retroviral vectors still represent a larger portion of viral vectors used in gene therapy (Edelstein, 2017) meaning optimization of retroviral vector production systems is still valuable and due to the similarities between these two types of viral vectors, strategies for improved retroviral vector production are, in principle applicable to lentiviral vector manufacturing (A. Rodrigues, PhD thesis).

Metabolic optimization has proven to be one of the best approaches to improve viral vector production in producer cell lines. This optimization is achievable either by culture medium design or genetic manipulation. Genetic engineering of producer cell lines holds great potential to improve the production of viral vectors by targeting metabolic pathways recruited in this process (Rodrigues et al., 2014).

In previous work, the main pathways involved in retroviral vector production were identified by functional genomics studies, comparing metabolic changes in “parental *vs* producer” cell lines (Rodrigues et al., 2013). The results pointed eight metabolic pathways to be recruited by the producer cell line: amino acid catabolism, carbohydrate catabolism and integration of the energy metabolism, nucleotide metabolism, glutathione metabolism, pentose phosphate pathway, polyamines biosynthesis and lipid metabolism. These pathways are, thus, good candidates for genetic engineering approaches towards improved viral vector production. To narrow down the targets from complex pathways to key target genes an alternative to the previous “parental vs. producer” was taken in the form “low vs. high producer” clonal comparison that resulted in a list of potential gene targets for metabolic manipulation by genetic engineering (Rodrigues et al., 2013).

In the scope of this thesis, two of the pathways identified were chosen for further study: glutathione and lipid metabolism.

1.4.1. Lipid metabolism in retroviral vector producer cell lines

Animal serum used to culture producer cell lines is a limitation to the use of the viral vectors produced because it is a source of potential pathogens and represents extra costs for purification and downstream processing to reach clinically approved standards (Rodrigues et al., 2012). On the other hand, serum deprivation aggravates the challenges faced by retroviral vector production systems, because it results in a decrease of retroviral vector titers. In previous work, the lipid fraction of animal serum, particularly cholesterol, was found to be the main component affecting retroviral vector production (Rodrigues et al., 2009). Further studies identified lipid metabolism, particularly cholesterol biosynthesis pathway as a potential target for genetic manipulation to improve retroviral vector production under serum deprivation (Rodrigues et al., 2012).

1.4.2. Glutathione metabolism in retroviral vector production

Glutathione metabolism was found to play a major role in retroviral vector production (Rodrigues et al., 2013). This pathway serves as detoxification of oxygen reactive species and regulate oxidative stress metabolism.

In previous work, glutathione metabolic genes were overexpressed by lentiviral vector delivery and the results suggested that some of the target genes lead to increased retroviral vector production (Oliveira et al., 2016). Herein, further metabolic engineering studies were conduted to investigate the effect of glutathione metabolic genes on retroviral vector production. To this end, an inducible gene expression system was developed and later used with glutathione metabolic genes. Lentiviral vectors were used in this work as tools to deliver the inducible gene expression system. To better control genetic manipulation the system had to be uniformly delivered by the lentiviral vectors to the producer cells. Hence, a universal method for lentiviral vector titration, devoid of reporter genes, was implemented.

1.5 Aim and strategy

The aim of this work was to engineer metabolic pathways previously identified as potential targets to improve viral vector production, namely glutathione and lipid metabolism. To this end, an inducible gene expression system and a method for lentiviral vector titration were developed as means to enable genetic engineering and optimize the experimental set-up.

The first goal was to develop the inducible gene expression system for the ensuing metabolic engineering studies. In concept, this molecular tool allows to fine-tune gene expression and reverse gene expression in manipulated populations allowing to distinguish effects associated with the gene of interest of those caused by manipulation and selection processes. The second goal was to establish a universal method for lentiviral vector titration used in the context of this work to establish uniform manipulated producer cell populations. The final goal of this work was to genetically engineer producer cells, targeting glutathione and lipid metabolism.

2. Materials and methods

2.1. Plasmids

Primers and templates for all plasmids constructed in this work are listed in **Table A.1** in annexes. A schematic representation of constructed plasmids and main transcriptional units is provided in **Figure A.1** in annexes.

2.1.1. Helper plasmids for lentiviral vector production

pMDLg/RRE is a third generation lentiviral packaging plasmid encoding the Human Immunodeficiency Virus (HIV) 1 Gag-Pro-Pol under the control of a CMV (Cytomegalovirus) promoter, as well as RRE, a binding site for Rev protein which facilitates the export of RNA from the nucleus to the cytoplasm.

pRSV-Rev is a third generation lentiviral packaging plasmid coding for Rev protein under the control of RSV (Rous Sarcoma Virus) U3 promoter.

pMD2.G is a plasmid coding for G glycoprotein envelope of Vesicular Stomatitis Virus (VSV-G) under the control of a CMV promoter.

All these plasmids were kindly provided by Dr. Didier Trono through Addgene plasmid repository (Cambridge, MA, USA) (plasmids #12251 #12253 and #12259, respectively) and are described in Dull et al., (1998).

2.1.2. Lentiviral vector transgenes

All lentiviral vector transgenes used in this work are self-inactivating (SIN) third generation vectors, containing HIV-1 long terminal repeats (LTR), HIV-1 packaging signal for encapsidation of RNA into the lentiviral particle, a cPPT (central polypurine tract) to facilitate nuclear import and export upon transduction, a WPRE (Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element) to stabilize the viral RNA, as well as a Rev responsive element (RRE).

Two types of lentiviral vector transgenes were used: for constitutive expression (*pRRLSIN* based vectors) and inducible expression (*pInducible* based vectors) constructions.

pRRLSIN.cPPT.PGK-GFP.WPRE (for short: *pRRLSIN GFP*) is a lentiviral vector transgene driving constitutive expression of enhanced green fluorescent protein (GFP) from an internal human phosphoglycerate kinase 1 (hPGK) promoter and was kindly provided by Dr. Didier Trono through Addgene plasmid repository (plasmid #12252).

pLenti CMV rtTA3 Blast is a lentiviral vector transgene driving constitutive expression of the reverse tetracycline-controlled transactivator 3 (rtTA3) under the control of a CMV promoter and a blasticidin resistance gene, under the control of a Simian Virus 40 (SV40) promoter.

pLenti CMVtight GFP Puro is a lentiviral vector transgene coding for GFP under the control of a chimeric CMV promotor along with a tetracyclin-responsive element (TRE) resulting in a tetracycline responsive composite promoter (CMVtight); an additional murine PGK (mPGK) promoter drives the expression of a puromycin resistance gene. Both *pLenti CMVtight GFP Puro* and *pLenti CMV rtTA3 Blast*

were a gift from Dr. Eric Campeau through Addgene plasmid repository (plasmids #26431 and #26429 respectively).

pLenti CMVtight GFP SV40 Puro is a lentiviral vector transgene obtained from *pLenti CMVtight GFP Puro* by inverted PCR and molecular cloning where the mPGK promoter was replaced by a SV40 promoter obtained from *pLenti CMV rtTA3 Blast*.

pLenti CMVtight GFP SV40 Puro and *pLenti CMV rtTA3 Blast* are two separate components of an inducible gene expression system and were used in this work to construct a single plasmid with both components: *pLenti CMVtight GFP SV40 rtTA3 Puro* (for short: *pInducible GFP*), a lentiviral vector transgene for tetracycline-dependent inducible gene expression. In this vector an additional expression cassette, independent of the CMVtight promoter, drives the constitutive expression of rtTA3 connected to a puromycin resistance gene by a 70-base pair spacer for a re-initiation of translation mechanism (Kozak, 1987; Kozak, 2002). The SV40-puro fragment was PCR amplified from *pLenti CMVtight GFP SV40 Puro* and the spacer was PCR amplified from pCeb (Cosset et al., 1995).

pInducible mCherry, pInducible Luciferase, pInducible CBS - cystathionine-beta-synthase, *pInducible CTH* - cystathionine-gamma-lyase, complete (c) or truncated (t) form, *pInducible IDH1* - Isocitrate dehydrogenase 1, *pInducible IDH2* - Isocitrate dehydrogenase 2, *pInducible GSS* - glutathione synthase, *pInducible GSR* - glutathione-disulfide reductase, *pInducible GSTM1* - glutathione S-transferase mu 1*, pInducible G6PD* - glucose-6-phosphate-dehydrogenase and *pInducible GPX7* - glutathione peroxidase 7 are lentiviral vector transgenes for inducible expression of the respective genes described in the plasmid name. All plasmids were derived from *pInducible GFP* where GFP was removed by *BstXI* enzymatic restriction followed by *In-Fusion HD Enzyme* (Clontech Laboratories, Mountain View, CA, USA) ligation of the respective genes. Luciferase was amplified from *pGL4.13 luc2/SV40* (Promega, Fitchburg, WI, USA), while the remaining genes were cloned from templates originally derived from *pDONR221* plasmids containing the different genes of interest, acquired through DNASU Plasmid Repository (Biodesign Institute, Arizona State University, Tempe, AZ, USA). Template plasmids and primers used to amplify each gene are detailed in **Table A.1.**

2.2 Cloning procedures

All PCR reactions were performed in a *Biometria*® *T3Personal Thermocycler* (Biometria, Göttingen, Germany) using *Phusion*® *High-Fidelity* DNA Polymerase (Finnzymes Oy, Vantaa, Finland), using appropriate PCR conditions for each fragment as suggested by the manufacturer.

All enzymatic restrictions were performed using *NEB*® (New England Biolabs, Ipswich, MA, USA) enzymes and buffers according to the manufacturer's instructions.

PCR products and restriction fragments were isolated on 0.7% (w/v) agarose gels (NZYTech, Lisbon, Portugal), visualized using *GelDoc*^{TM} *XR*$^{+}$ system (BioRad, Hercules, CA, USA) either by adding 0.5 µL/mL *RedSafe*^{TM} *Nucleic Acid Staining Solution* (INtRON Biotechnology, South Korea) to the gel or staining the gel using *GelRed*^{TM} (Biotium, Fremont, CA, USA) and purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) following manufacturer's instructions.

Vector-insert ligations were performed using *In-Fusion® HD Cloning Kit* (Clontech) following manufacturer's instructions.

2.3 Bacteria strains and culture media

Escherichia coli (*E.coli*) Stellar™ (Clontech) competent cells were used for the cloning ligations. NZY5α (NZYTech) and One Shot® Stbl3™ (Life Technologies, Carlsbad, CA, USA) competent cells were used for plasmid amplification: Stbl3 were used for lentiviral vector plasmids and NZY5α for the remaining plasmids.

The agar and liquid cultures were performed in *Luria Broth* media (LB) (Fast-Media® LB from Invivogen, San Diego, CA, USA) and *Terrific Broth* media (TB) (Invivogen), supplemented with the appropriate antibiotic for bacteria selection. Media was prepared using milliQ water (Milli-Q® System, Merck Millipore, Billerica, MA, USA).

2.4 Plasmid purification and quality control

Plasmid purification was performed at small-scale using GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA) and large-scale using Genopure Plasmid maxi Kit (Roche Applied Science, Penzberg, Germany), following the manufacturer's instructions.

DNA concentration was determined using Nanodrop™ 2000 Spectophotometer (Thermo Scientific, USA) and purity was assessed by Abs_{260nm}/Abs_{280nm} and Abs_{260nm}/Abs_{230nm} ratios.

All plasmids constructed in this work were sequenced by Sanger sequencing using GATC Biotech services (Constance, Germany).

2.5 Cell lines and culture condition

HEK 293T (ATCC, American Type Culture Collection, CRL-11268) is a cell line derived from Human Embryonic Kidney 293 (HEK 293) cells, expressing large T antigen of SV40 and were used for lentiviral vector production and to establish stable populations to evaluate the functionality of the inducible system.

293 FLEX S11 (Rodrigues et al., 2015) and 293 FLEX 18 (Coroadinha et al., 2006) cell lines are HEK 293 derived cell lines stably producing murine leukemia virus (MLV) based recombinant retroviral vectors, pseudotyped with Gibbon ape leukemia virus (GaLV) ecotropic envelope and harboring a LacZ-S11 or a LacZ reporter gene, respectively and were used as study models to evaluate the effect of metabolic engineering in retroviral vector production.

Te 671 (ATCC CRL-8805) is a Human rhabdomyosarcoma derived cell line and was used for retroviral vector titration by LacZ staining.

Te 671 S10 is a Te 671 derived cell line stably expressing a truncated GFP fragment, S10 fragment (Cabantous et al., 2005) and was used to titrate retroviral vector productions by Split-GFP system, as described in Rodrigues et al. (2015).

All cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA), supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco) and maintained at 37°C in a humidified atmosphere with 8% CO_2. All cells were cultured under adherent conditions using tissue culture flasks (T-flasks, Starstedt, Numbrecht, Germany).

Advanced DMEM (Gibco) was used in serum deprivation studies supplemented with 10% (v/v) or 1% (v/v) FBS (Gibco) for normal and restricted serum conditions as well as additional supplementation of 4 mM L-Glutamine (Gibco).

Clontech tetracyclin-free FBS (Takara Bio USA, Inc, Mountain View, CA, USA) was used to assess the effect of residual tetracyclin present in Gibco FBS.

For establishing working cell banks, cells line were frozen in a cryopreservation solution of FBS containing 5% (v/v) of Dimethyl sulfoxide (DMSO, Sigma-Aldrich) and stored at -80°C.

2.6 Determination of cell concentration and viability

Cell concentration and viability were assessed by trypan blue exclusion assay using 0.1% (v/v) Trypan Blue (Sigma-Aldrich, St, Louis, MO, USA) solution in Phosphate Buffer Saline, PBS (Gibco). Cell counting was performed in a Fuchs-Rosenthal hemocytometer (Marienfield-Superior, Lauda-Konigshofen Germany) using an inverted microscope (Olympus, Tokyo, Japan).

2.7 Genomic DNA Extraction, RNA Extraction and cDNA synthesis

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

Total RNA was extracted using QIAamp® RNeasy Mini Kit (Qiagen) following the manufacturer's instructions and stored at -80°C until further use.

cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) following manufacturer's instructions, using 2 µg of total RNA and oligo dT primer for total mRNA reverse transcription. The reverse transcribed product was aliquoted and stored at -20°C until further use.

2.8 Real-Time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed using LightCycler® 480 SYBR Green I Master (Roche Applied Science) according to the manufacturer's instructions on a LightCycler® 480 Real Time PCR System (Roche Applied Science). Relative gene expression (mRNA quantification) and relative copy number (genomic DNA) were calculated using the $2^{-\Delta CT}$ method (Livak & Schmittgen, 2001). Ribosomal protein L22 (RPL-22) was used as reference gene. The primers used for RT-qPCR are listed in **Table A.2** in annexes.

2.9 Lentiviral vector production

Lentiviral vectors were produced by transient transfection using third generation lentiviral packaging system and the transfection procedure described in Dull et al. (1998). Briefly, HEK 293T cells

were seeded at $5x10^4$ cells/cm^2. After 24 hours, transfection was performed using polyethylenimine (PEI, linear 25 kDa, Polysciences, Inc., Warrington, PA, USA) at 1:1.5 (w/w) ratio of DNA:PEI, using a total of 4.65 µg DNA *per* 10^6 cells with the following proportions: 1 µg of *pMDLG/RRE*, 0.25 µg *pRSV-Rev*, 0.9 µg of *pMD2.G* and 2.5 µg of vector transgene plasmid. Both PEI solution and plasmid mix solution were prepared in serum-free DMEM. Plasmid mix solution was filtered through 0.22 µm pore-size cellulose acetate filter and added to the PEI transfection solution. After 10-15 minutes of incubation at room temperature the final mix was added to the cells. The medium was replaced by DMEM supplemented with 10% (v/v) FBS, 20 to 24 hours after transfection. The supernatant containing the lentiviral vectors was harvested 24 hours after the previous medium exchange, filtered through 0.45 µm pore-size cellulose acetate filters for clarification, aliquoted in appropriate and convenient volumes (1 - 1.5 mL) and stored at -80°C until further use. When possible, transfection efficiency was assessed by flow cytometry analysis (CyFlow® Space, Sysmex Corporation, Kōbe, Japan).

2.10 Retroviral and lentiviral vector titration

- **By flow cytometry analysis**

For lentiviral vector titration, HEK 293T cells were seeded at $5x10^4$ cells/cm^2 in 24-well plates. Cells were transduced 24 hours after seeding by removing the medium and infecting with 0.2 mL of viral supernatant at several dilutions performed in fresh DMEM supplemented with 10% (v/v) FBS and containing a final concentration of 8 µg/mL of polybrene (Sigma-Aldrich). Two days after infection cells were harvested and analyzed for GFP or mCherry fluorescence by flow cytometry (CyFlow® Space).

For retroviral vector titration the Split-GFP titration method was used (Rodrigues et al., 2015). This method is based on the transcomplementation of GFP in which two non-fluorescent fragments of GFP: S10 – stably expressed in target cells (Te671 S10) and S11 - frament carried by the retroviral vector transgene – assemble to reconstitute the GFP signal. Briefly, Te671 S10 target cells were seeded at $5x10^4$ cells/cm^2 in 24-well plates. Cells were transduced 24 hours after seeding by removing the medium and infecting with 0.2 mL of viral supernatant at several dilutions performed in fresh DMEM supplemented with 10% (v/v) FBS and containing a final concentration of 8 µg/mL of polybrene (Sigma-Aldrich). Two days after infection cells were harvested and analyzed for GFP or mCherry fluorescence by flow cytometry (CyFlow® Space).

For both retroviral and lentiviral vector titration using flow cytometry, the titer (infectious particles *per* mL, I.P./mL) was determined by taking into account the percentage of GFP positive cells, the number of cells at time of infection and the dilution factor of the respective viral supernatant (only dilutions delivering between 2-20% of positive cells where considered to avoid multiple transduction events) according to the following equation:

$$I.P._{/ml} = \frac{\%\ Fluorescent\ Cells\ \times number\ of\ cells\ at\ infection\ \times dilution\ factor}{100\ \times infection\ volume\ (ml)}$$

- **Retroviral vector titration by β-galactosidase (LacZ) staining**

To titrate retroviral vectors that carry LacZ as transgene a LacZ staining protocol was used. Briefly, Te671 cells were seeded at $5x10^4$ cells/cm^2 in 96-well plates. Cells were transduced 24 hours after seeding by removing the medium and infecting cells with 80 µl of viral supernatant. Serial dilutions were performed in fresh DMEM supplemented with 10% (v/v) FBS and containing a final concentration of 8 µg/mL of polybrene (Sigma-Aldrich). Two days after infection cells were fixed using a solution of formaldehyde at 0.3% (v/v) and 1.35% (v/v) glutaraldehyde in PBS for 5 minutes. After a washing step with PBS, staining was performed using a solution of 0.2 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyeanoside, Stratagene, La Jolla, CA, USA), 5 mM $K_3Fe(CN)_6$ (Merck), 5 mM $K_4Fe(CN)_6$ (Merck) and 1 mM $MgCl_2$ (Merck) in PBS. The viral titer was determined by counting the blue stained cells, using an inverted phase contrast microscope, multiplied by the dilution factor.

- **Lentiviral vector titration by RT-qPCR – quantification of LV-LTR**

HEK 293T cells were seeded at $5x10^4$ cells/cm^2 in 6-well plates. After 24 hours cells were transduced by removing the medium and infecting with 1 mL of viral supernatant at several dilutions performed in fresh DMEM supplemented with 10% (v/v) FBS and containing a final concentration of 8 µg/mL of polybrene (Sigma-Aldrich). Two days after infection cells were harvested and processed for genomic DNA extraction followed by RT-qPCR as described above.

This method requires the establishment of calibration curves that correlate LV-LTR copy number with infectious particles titer, allowing to calculate titers from the linear regression. This curve was established with a GFP reporter lentiviral vector stock (presenting the same molecular design of the vector to titrate) previously titrated by flow cytometry as described above. If the calibration curve is not prepared together with the samples to be titrated, the lentiviral vector stock used to establish the curves should be included as an internal standard along with the samples and used to correct the titer calculation from the linear regression.

2.11. Establishment of stable cell lines

HEK 293T cells were used to establish stable cell lines for validation and characterization of the inducible gene expression system. For studies of metabolism manipulation, 293 FLEX S11 cells were

used to establish stable cell lines. In both cases, stable populations were established by lentiviral vector transduction followed by selection using the appropriate antibiotic. Blasticidin (Invivogen) at a concentration of 10 µg/mL was used for selection of *pLenti SV40 rtTA3 Blast* and Puromycin (Invivogen) at a concentration of 3 µg/mL was used for selection of all the different *pInducible* constructions.

2.12. Doxycycline usage

Doxycycline (DOX, 1 µg/µl, Sigma-Aldrich) was used at concentrations of 1, 10, 100 or 1000 ng/mL in DMEM 10% (v/v) FBS to induce gene expression on cells expressing the different *pInducible* constructs. Medium containing doxycycline was refreshed every 24 hours.

When titrating lentiviral vector stocks of *pInducible GFP* and *pInducible mCherry* by flow cytometry, doxycycline (1000 ng/mL) was added to the cells 24 hours after infection to induce the expression of the respective reporter gene.

2.13. Growth studies

- **Lipid metabolism growth study**

293 FLEX S11 and 293 FLEX 18 cells were cultured for 2 passages under normal or under serum deprivation conditions. Normal serum conditions refer to the previously described culture conditions on Advanced DMEM (Gibco) supplemented with 10% (v/v) FBS while cells under serum deprivation were kept on Advanced DMEM (Gibco) supplemented with 1% (v/v) FBS (Rodrigues et al., 2012). Cells were seeded at $2x10^4$ cells/cm^2 under normal and serum deprivation conditions and cultured for one week. During this period viral supernatants were harvested and cell concentration and viability were assessed at 24 hours intervals.

- **Inducible gene expression for metabolic engineering studies**

To evaluate the effect of doxycycline and the impact of the inducible system on cell growth and retroviral vector production, 293 FLEX S11 expressing *pInducible GFP* or *pInducible mCherry* were seeded at $4x10^4$ cells/cm^2 under standard culture conditions as described above. Medium containing DOX at the concentrations of 1, 10, 100 or 1000 ng/mL was added to the cells 24 hours after seeding. Every day over the course of one week, the medium was exchanged, cells were counted and viral supernatant was harvested.

To evaluate the effect of metabolic engineering using the inducible gene expression system on cell growth and retroviral vector production, 293 FLEX S11 stably expressing the different *pInducible* constructs were seeded at $8x10^4$ cells/cm^2 under standard culture conditions as described above. Medium with DOX (1000 ng/mL) or without DOX was added to cells 24 hours after seeding. Every day, during the following three days, the medium was exchanged, cells were counted and viral supernatant was harvested. Additionally, mRNA and genomic DNA were extracted at 72 hours after seeding and stored until further use.

2.14. Fluorescence microscopy

Cells expressing the inducible system or its components individually were seeded at $5x10^4$ cells/cm^2 in 6-well plates. After 24 hours, doxycycline was added at different concentrations (0, 1, 10, 100 or 1000 ng/mL). Cells were analyzed for GFP fluorescence at 48 hours post-induction using Leica DMI6000 B (Leica Microsystems, Wetzlar, Germany) inverted fluorescence microscope. Cells were imaged in phase contrast and exposed to 488 nm laser for GFP imaging using a total amplification of 100x (10x ocular and 10x objective).

3. Results

3.1. An inducible system for metabolic engineering studies

Commonly in genetic engineering, gene overexpression is achieved by delivery of the gene of interest and results in constitutive expression (Khan, 2013). In this work, a molecular system of inducible gene expression was implemented, enabling a new a new approach on metabolic engineering studies on retroviral vector production. In this system, the expression of a gene of interest is driven by a chimeric promoter containing the tetracycline responsive element (TRE). In the same construct, a constitutive promoter drives the expression of the reverse tetracycline-controlled transactivator 3 (rtTA3) which binds to TRE in the presence of tetracycline – or its' analogues such as doxycycline herein used – activating the TRE chimeric promoter. This system configures a TET-ON controlled expression, leading to the expression of the gene of interest in the presence of doxycyline (Urlinger et al., 2000). In the presence of sufficient levels of rtTA3, this system is dose-responsive, i.e. increasing concentrations of doxycycline lead to higher amounts of rtTA3 binding to and activating the chimeric promoter ultimately resulting in higher expression of the gene of interest.

For easy deliver and integration into the cell genome, the system was cloned into a lentiviral vector. To this end, *pLenti CMVtight GFP Puro* (**Figure 3.1 A**) and *pLenti CMVrtTA3 Blast* (**Figure 3.1 B**) were used as starting points and, through a series of molecular cloning steps, a final and single vector for inducible expression of GFP was obtained: *pInducible GFP* (**Figure 3.1 C**) - a lentiviral vector transgene that stably expresses rtTA3 and can be induced in the presence of doxycycline to express the gene of interest, GFP, in this case. The system was design in such way that features like the gene of interest, the constitutive promoter that drives rtTA3 expression and the selection marker can be exchanged by enzymatic restriction and insert ligation. Using this strategy *pInducible GFP* served as backbone for all other vectors developed for studying different genes of interest (**Figure 3.1 D**).

To assess the functionality of the system before proceeding with the cloning of other genes of interest, populations of HEK 293T stably expressing rtTA3, CMVtight GFP or both components of the system were established. These were then subjected to different concentrations of doxycycline and analyzed by fluorescence microscopy (**Figure 3.2**). For cells containing both components of the system, increasing concentrations of doxycycline resulted in increasingly high GFP expression showing the system was not only DOX responsive but also exhibited the dose responsive behavior (**Figure 3.2 A**). Cells expressing only CMVtight GFP displayed small but consistent GFP signal that suggests leaky expression from this promoter (**Figure 3.2 B**). This leakiness was not affected by the concentration of doxycycline. No GFP signal was detected in cells expressing rtTA3 only (**Figure 3.2 D**).

.

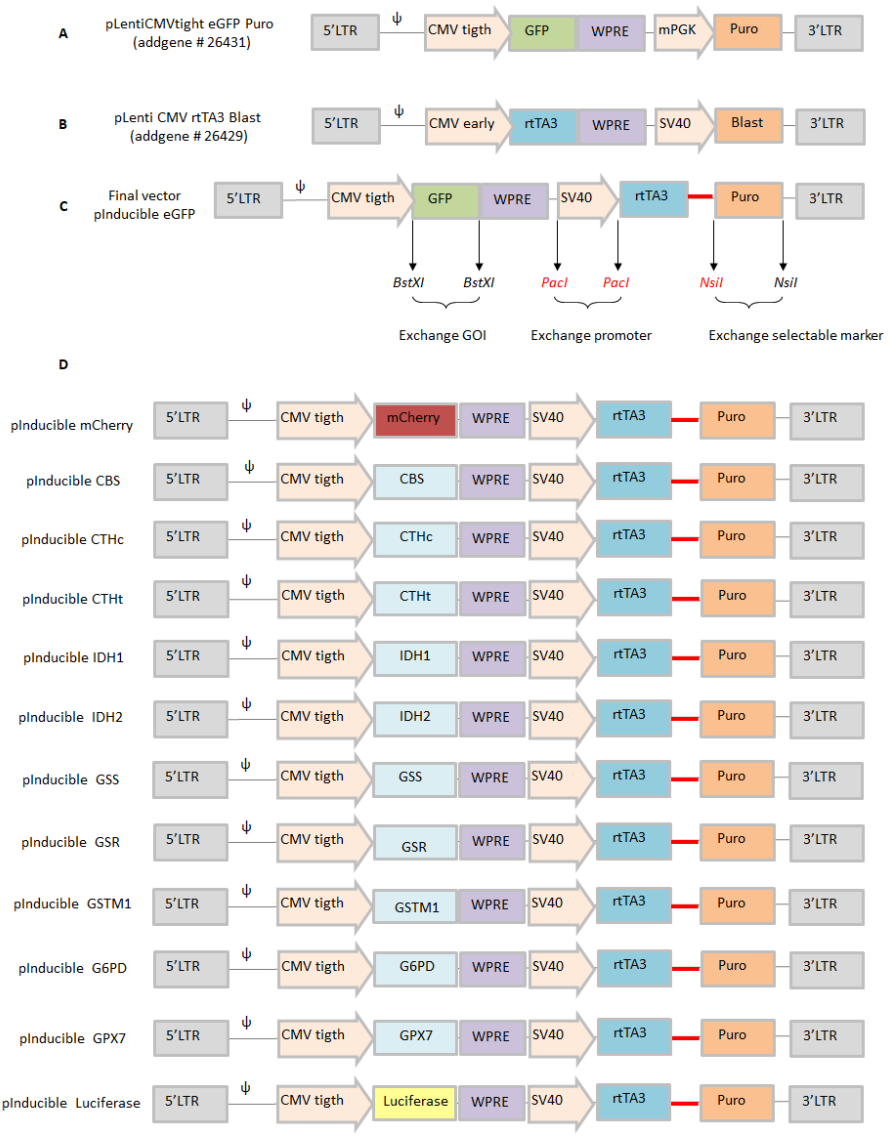


Figure 3.1 - Schematic representation of inducible constructions. *pLentiCMVtight GFP Puro* **(A)** and *pLentiCMV rtTA3 Blast* **(B)** were the starting plasmids used to construct *pInducible GFP* **(C)**. Bellow the schematic representation of the main features, in black are shown the enzymatic restriction sites already present on the starting plasmids while in red are shown the sites added during plasmid construction. *BstXI* sites flank GFP and allow for replacing the gene of interest (GOI). *PacI* sites flank the SV40 Promoter and allow for replacing it for another promoter. *NsiI* sites flank the puromycin resistance gene and allow replacing it for another selection marker. All other *pInducible* constructs **(D)** were derived from *pInducible GFP* by enzymatic restriction and molecular cloning of each gene of interest. CBS - cystathionine-beta synthase, CTH - cystathionine-gamma lyase (CTHc refers to the complete gene while CTHt refers to a truncated version), IDH1 - isocitrate dehydrogenase 1, GSR - glutathione-disulfide reductase, GSS - glutathione synthetase, GSTM1 - glutathione S-transferase mu 1, G6PD - glucose-6-phosphate dehydrogenase, GPX7 - glutathione peroxidase 7.

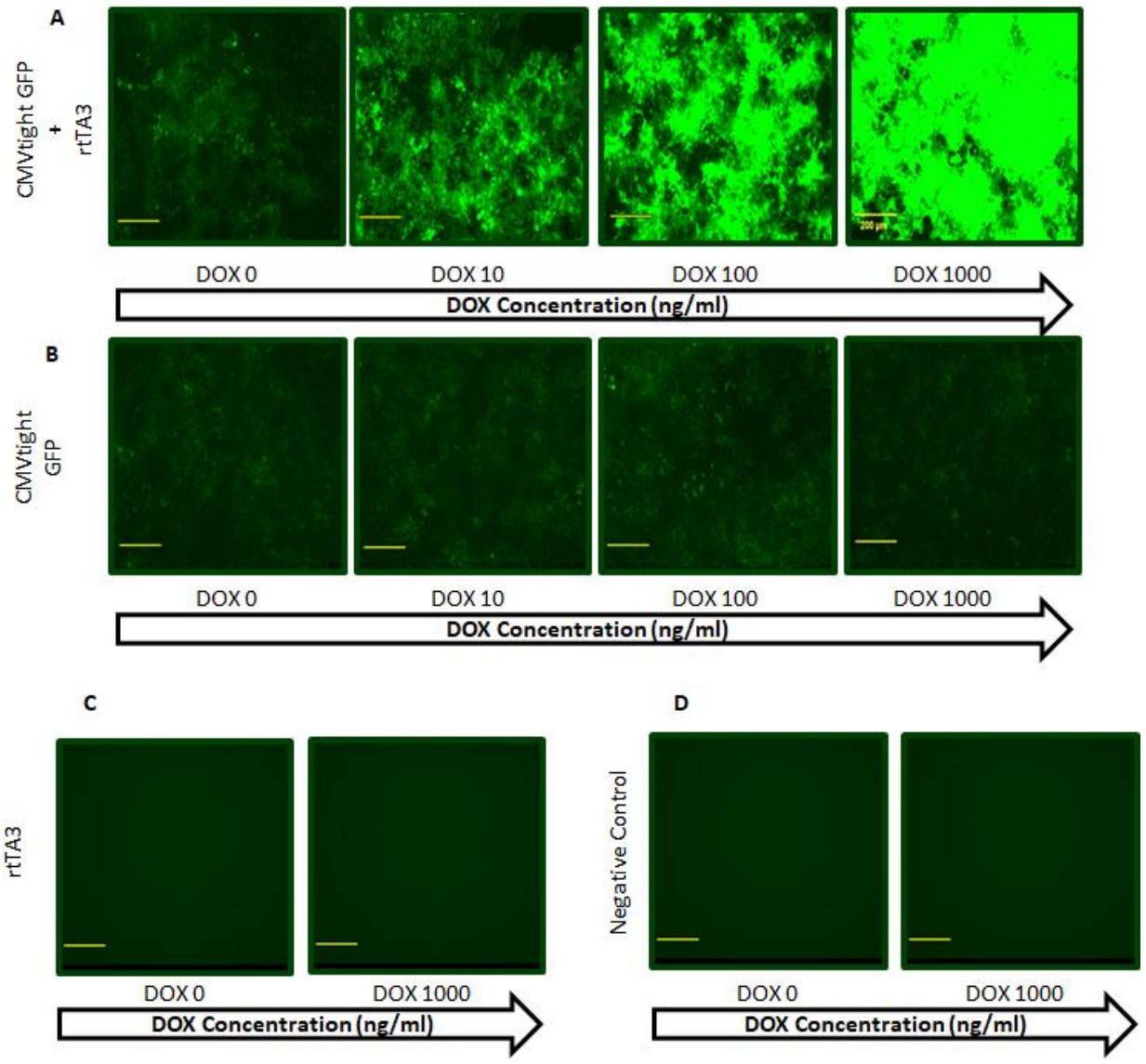


Figure 3.2 - Fluorescence microscopy images of GFP in HEK 293T cells stably expressing the different components of the inducible system. Fluorescence microscopy images of GFP expression in HEK 293T cells expressing both CMVtight GFP and rtTA3 **(A)**, CMVtight GFP only **(B)**, rtTA3 only **(C)** and a negative control HEK 293T **(D)** under different concentrations of doxycycline. Scale bar corresponds to 200 µm.
For visualization purposes these images were digitally enhanced using ImageJ software.

To complement fluorescence microscopy data, expression of GFP at the mRNA level was also assessed by RT-qPCR. HEK 293T cells stably expressing only CMVtight GFP once again displayed a leaky expression of GFP while cells expressing both components of the system presented the same dose-

dependent expression. Due to the quantitative nature of this method it was possible to see that GFP expression was induced up to approximately 40-fold at the highest concentration of doxycycline compared to that of non-induced cells expressing both components (**Figure 3.3**).

While the first generations of TET-inducible systems exhibited substantial leakiness, the most recent constructions, in particular TET-ON Advanced vectors (Eric Campeau Lab, unpublished results) are described as being relatively tight, hence the designation of "CMVtight" promoter. Therefore the leakiness observed for CMVtight was not expected. Alternatively, non-residual levels of tetracycline have been reported in some FBS lots or manufacturers. Although the results in **Figure 3.2** suggested that the leakiness inherently stems from the CMVtight promoter, tetracycline in the serum used for cell culture could still contribute with additional leakiness. To assess this, HEK 293T cells stably expressing *pInducible GFP* were cultured under Gibco FBS (commonly used in the laboratory) or Clontech tetracycline-free FBS (Clontech, special FBS to use in TET experiments). Flow cytometry results showed that GFP expression was similar for both cells cultured under Gibco or Clontech FBS and independent of doxycycline concentration (**Figure 3.4** and **Table 3.1**). These results corroborated that the leakiness observed was not derived from traces of tetracycline in the FBS but instead inherent to CMVtight promoter.

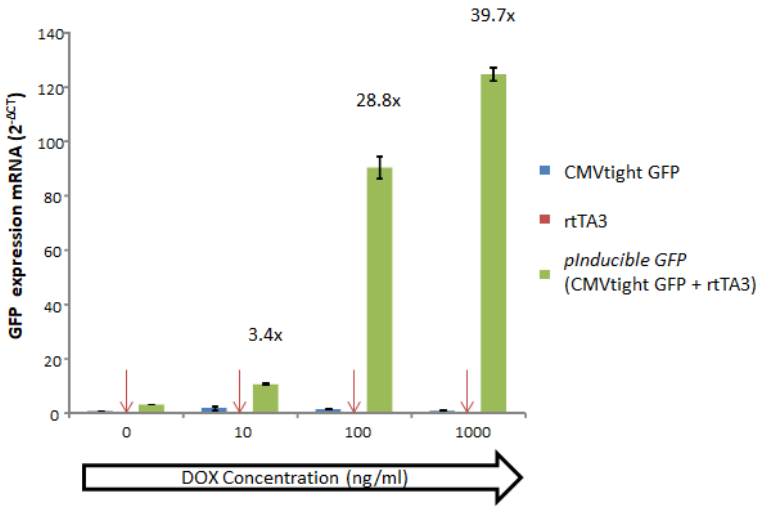


Figure 3.3 - GFP expression in HEK 293T cells stably expressing different components of the inducible system. mRNA expression levels of GFP in cells stably expressing CMVtight GFP (blue), rtTA3 (red, arrows indicate that expression was not detected) or a combination of CMVtight GFP and rtTA3 - *pInducible GFP* (green) - under increasing concentrations of doxycycline. Values are shown as average ± standard deviation (n = 2 technical replicates). The number above green bars indicates fold-change induction of GFP mRNA levels relatively to non-induced *pInducible GFP* cells (0 ng/mL of doxycycline).

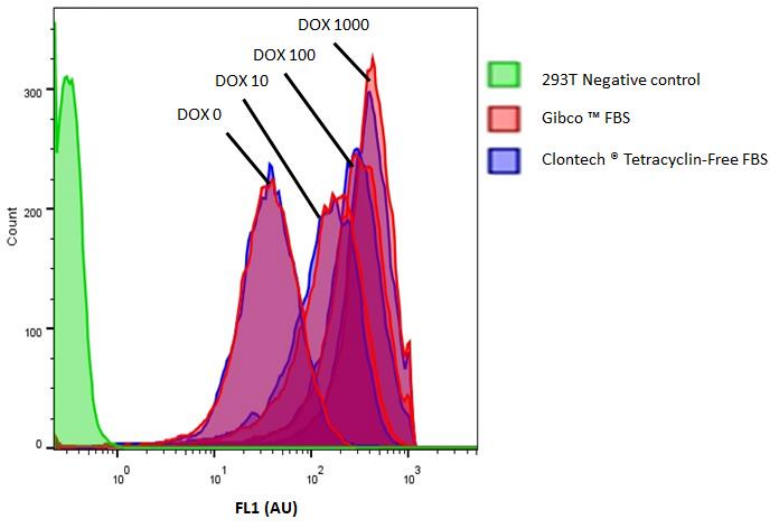


Figure 3.4 - Effect of tetracycline-free FBS on GFP expression. Flow cytometry analysis comparing GFP induction in HEK 293T cells stably expressing *pInducible GFP* cultured in medium supplemented with Gibco FBS or Clontech tetracycline-free FBS under different concentrations of doxycycline (data treated using FlowJo software). Fluorescence intensity levels in **Table 3.1**.

Table 3.1 - GFP fluorescence intensity in standard and tetracycline-free FBS.

Doxycycline concentration (ng/mL)	GFP Intensity	
	Gibco FBS	Clontech FBS
0	43	45
10	185	164
100	323	303
1000	426	404

To further characterize the inducible system and evaluate the reproducibility of doxycycline response, HEK 293T cells stably expressing either *pInducible GFP* or *pInducible mCherry* were induced with different concentrations of doxycycline and the expression of the respective fluorescent protein was assessed by flow cytometry (**Figure 3.5**).

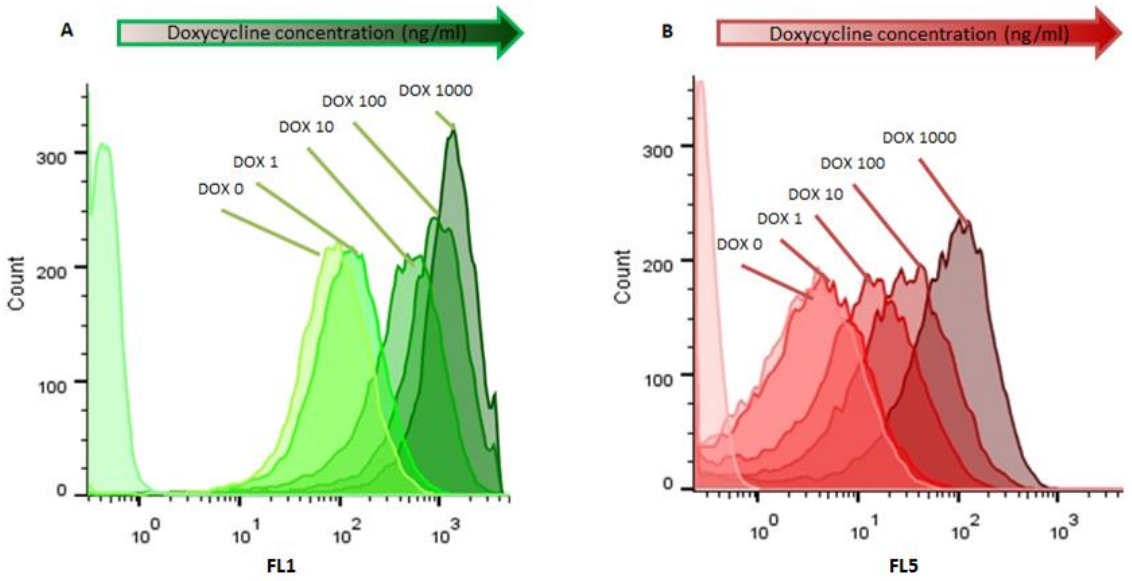


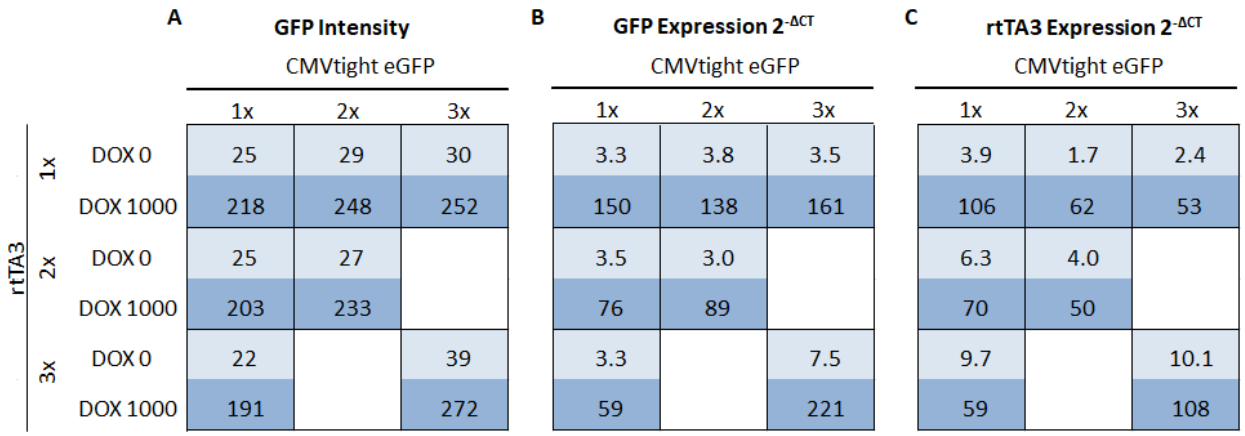
Figure 3.5 - GFP and mCherry induction of *pInducible* system. Flow cytometry analysis of HEK 293T cells stably expressing *pInducible GFP* **(A)** or *pInducible mCherry* **(B)** under different concentrations of doxycycline (0 to 1000 ng/mL, gradient of green and red respectively). Data was treated using FlowJo software. Fluorescence intensity values in **Table 3.2.**

The results from **Figure 3.5** confirm that gene expression was induced in a dose-dependent manner in both reporters. In comparison to non-induced cells, GFP expression was induced up to approximately 10-fold while mCherry was induced up to approximately 23-fold at the highest concentration of doxycycline (**Table 3.2**).

Table 3.2 - Fluorescence intensity of inducible GFP and mCherry

	Doxycycline concentration (ng/mL)	Fluorescence Intensity	Fold Induction
GFP	0	44	-
	1	59	1.3
	10	185	4.2
	100	323	7.3
	1000	426	9.7
mCherry	0	2.4	-
	1	2.8	1.2
	10	7.7	3.2
	100	18	7.4
	1000	56	23.1

To evaluate if any of the inducible system components (rtTA3 or CMVtight) was limiting, populations of HEK 293T cells were established with increasing amounts of each component and evaluated for GFP expression, in the absence or presence of doxycycline (0 or 1000 ng/mL), both by flow cytometry and mRNA quantification (**Figure 3.6**). GFP intensity increased with increasing amounts of CMVtight GFP and decreased with increasing amounts of rtTA3 both in the absence or presence of doxycycline (**Figure 3.6 A**). However the differences are too small and not corroborated by GFP gene expression (**Figure 3.6 B**) which remains mostly unchanged with the exception of the population containing 3 copies of both components (CMVtight and rtTA3). Expression of rtTA3 (**Figure 3.6 C**) increases steadily with increasing amounts of rtTA3 but only in the absence of doxycycline. In the presence of doxycycline rtTA3 expression is substantially increased and although it seems that populations transduced with higher amounts of either rtTA3 or CMVtight have lower levels of rtTA3 expression these values are within a fold-change of two.



A **GFP Intensity**

rtTA3		CMVtight eGFP 1x	CMVtight eGFP 2x	CMVtight eGFP 3x
1x	DOX 0	25	29	30
	DOX 1000	218	248	252
2x	DOX 0	25	27	
	DOX 1000	203	233	
3x	DOX 0	22		39
	DOX 1000	191		272

B **GFP Expression $2^{-\Delta CT}$**

rtTA3		CMVtight eGFP 1x	CMVtight eGFP 2x	CMVtight eGFP 3x
1x	DOX 0	3.3	3.8	3.5
	DOX 1000	150	138	161
2x	DOX 0	3.5	3.0	
	DOX 1000	76	89	
3x	DOX 0	3.3		7.5
	DOX 1000	59		221

C **rtTA3 Expression $2^{-\Delta CT}$**

rtTA3		CMVtight eGFP 1x	CMVtight eGFP 2x	CMVtight eGFP 3x
1x	DOX 0	3.9	1.7	2.4
	DOX 1000	106	62	53
2x	DOX 0	6.3	4.0	
	DOX 1000	70	50	
3x	DOX 0	9.7		10.1
	DOX 1000	59		108

Figure 3.6 - Effect of inducible system components on its functionality. Populations of HEK 293T cells stably expressing increasing amounts of each of the inducible system components (CMVtight GFP and rtTA3) were established and used to assess GFP expression by flow cytometry **(A)** and GFP and rtTA3 expression by RT-qPCR (**B** and **C** respectively) in the absence or presence of doxycycline (0 or 1000 ng/mL). For fluorescence intensity **(A)** values are shown as mean FL1 intensity. For gene expression (**B** and **C**) values are shown as average $2^{-\Delta CT}$ (n= 2 technical replicates, errors were omitted for simplicity purposes).

3.2. A universal method for lentiviral vector titration

Genetic engineering for metabolism manipulation using the inducible gene expression system requires that all populations are established with the same amount of *pInducible* lentiviral vector. To this end, a universal method for lentiviral vector titration was designed to assess the titer of lentiviral vector stocks by quantifying the integration of lentiviral vectors long terminal repeats (LV-LTR) within the genome of the target cells. Since this method directly quantifies LV integration it is able to titrate any lentiviral vector regardless of the transgene and without the need for a reporter gene or tag.

The starting point of this method is a lentiviral vector stock of known titer with the same molecular configuration of those to be titrated. This stock is used to transduce target cells at different multiplicities of infection (MOI), followed by genomic DNA extraction and RT-qPCR that allows establishing a calibration curve correlating infectious particles delivered and LV-LTR integration into the genome (**Figure 3.7 A**). Samples of unknown titer are used to transduce target cells once again followed by genomic DNA extraction and RT-qPCR. The titer of the lentiviral vector stocks can be directly calculated from the linear regression if the samples are processed simultaneously to those used to establish the calibration curve. Alternatively, if the samples of unknown titer are not processed simultaneously to the establishment of the calibration curve, they can be titrated by taking an internal standard of the same stock used to establish the curve. This allows to calculate the titer from the linear regression followed by normalization to the internal standard (**Figure 3.7 B**). This method was first implemented and validated for *pRRLSIN* based vectors using *pRRLSIN GFP* as standard for calibration curve establishment (**Figure 3.7 C**).

In order to apply this titration method to the establishment of *pInducible* engineered producer cell populations, it was characterized and validated using *pInducible GFP* and *pInducible mCherry*. Since these vectors contain a reporter gene they were initially titrated by flow cytometry, each of them in three biological replicates. Knowing the titer of each lentiviral stock allowed establishing individual calibration curves for *pInducible GFP* and *pInducible mCherry* also using three biological replicates. The individual calibration curves of each replicate were combined in a final calibration curve for *pInducible GFP* or *pInducible mCherry* (**Figure 3.8 A and B**). To validate the method, *pInducible GFP* lentiviral vector stock titer was calculated from the *pInducible mCherry* calibration curve and *vice-versa* (**Figure 3.8 C**). The titers calculated using the universal lentiviral vector titration system were similar to those obtained by flow cytometry validating the method for *pInducible* based lentiviral vector stocks.

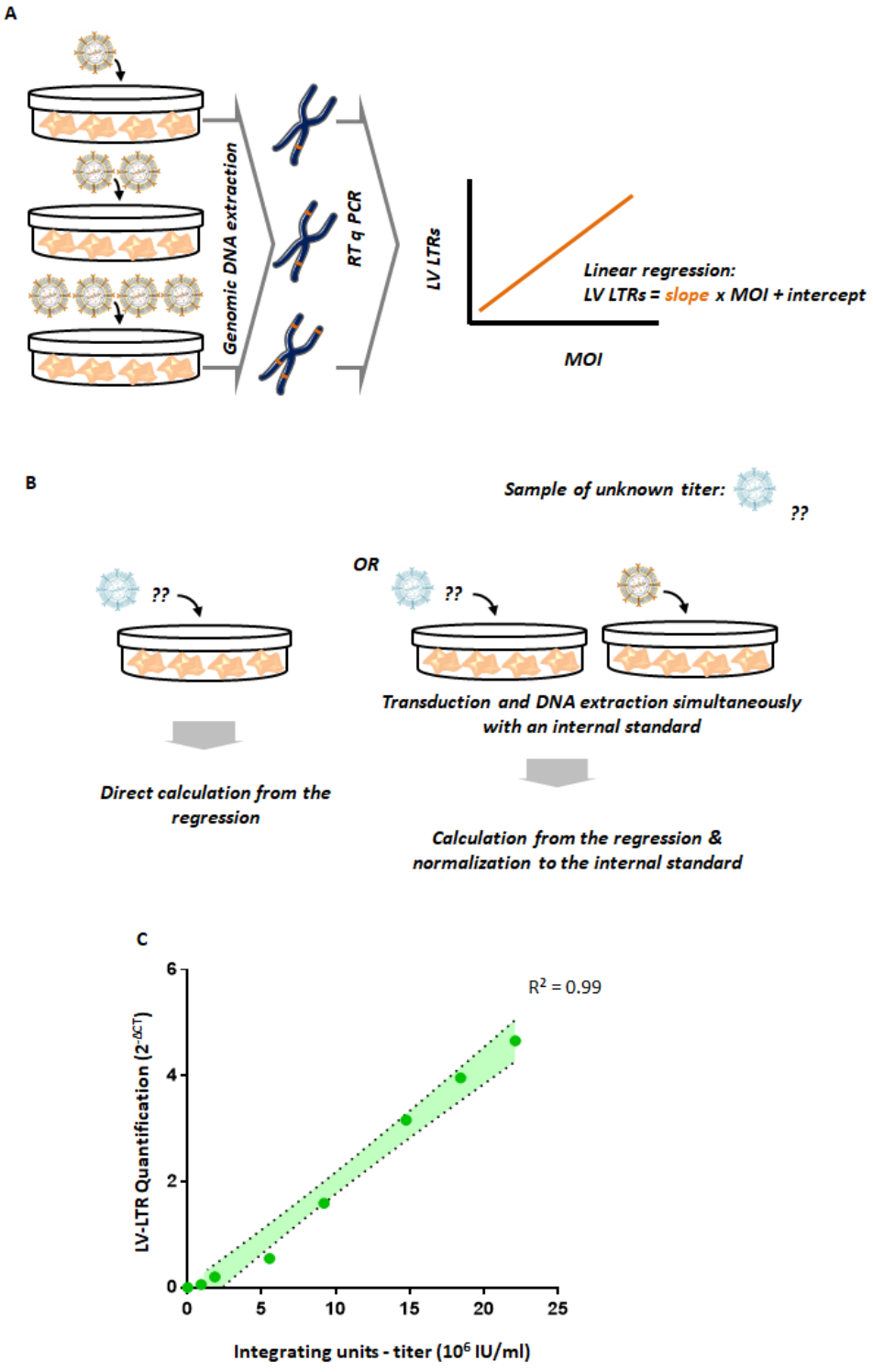


Figure 3.7 - Universal lentiviral vector titration method. Schematic representation of the universal lentiviral vector titration method. In the first part of this method cells are transduced with serial dilutions of a lentiviral vector stock of known titer. Then, quantitative analysis of lentiviral vector long terminal repeats (LV-LTR) integrated into genomic DNA of transduced cells is used to establish a calibration curve **(A)**. Samples of unknown titer can be titrated by direct

calculation from the calibration curve if their transduction and genomic DNA extraction is simultaneous to those of the calibration curve. The calibration curve can be re-used even when samples of unknown titer are not processed together with those used to establish the curve provided that an internal standard is included. This internal standard corresponds to the lentiviral vector stock used to establish the curve **(B)**. The calibration curve is established from linear regression analysis using the least-squares method of relative LV-LTR quantification *versus* LV titer. Calibration curve for a lentiviral vector stock of *pRRLSIN.cPPT.PGK-GFP.WPRE* **(C)**, for this RT-qPCR genomic DNA was used at 40 ng/µL. Values are shown as average $2^{-\Delta CT}$ ± standard deviation (n = 2 technical replicates, error bars are not visible).

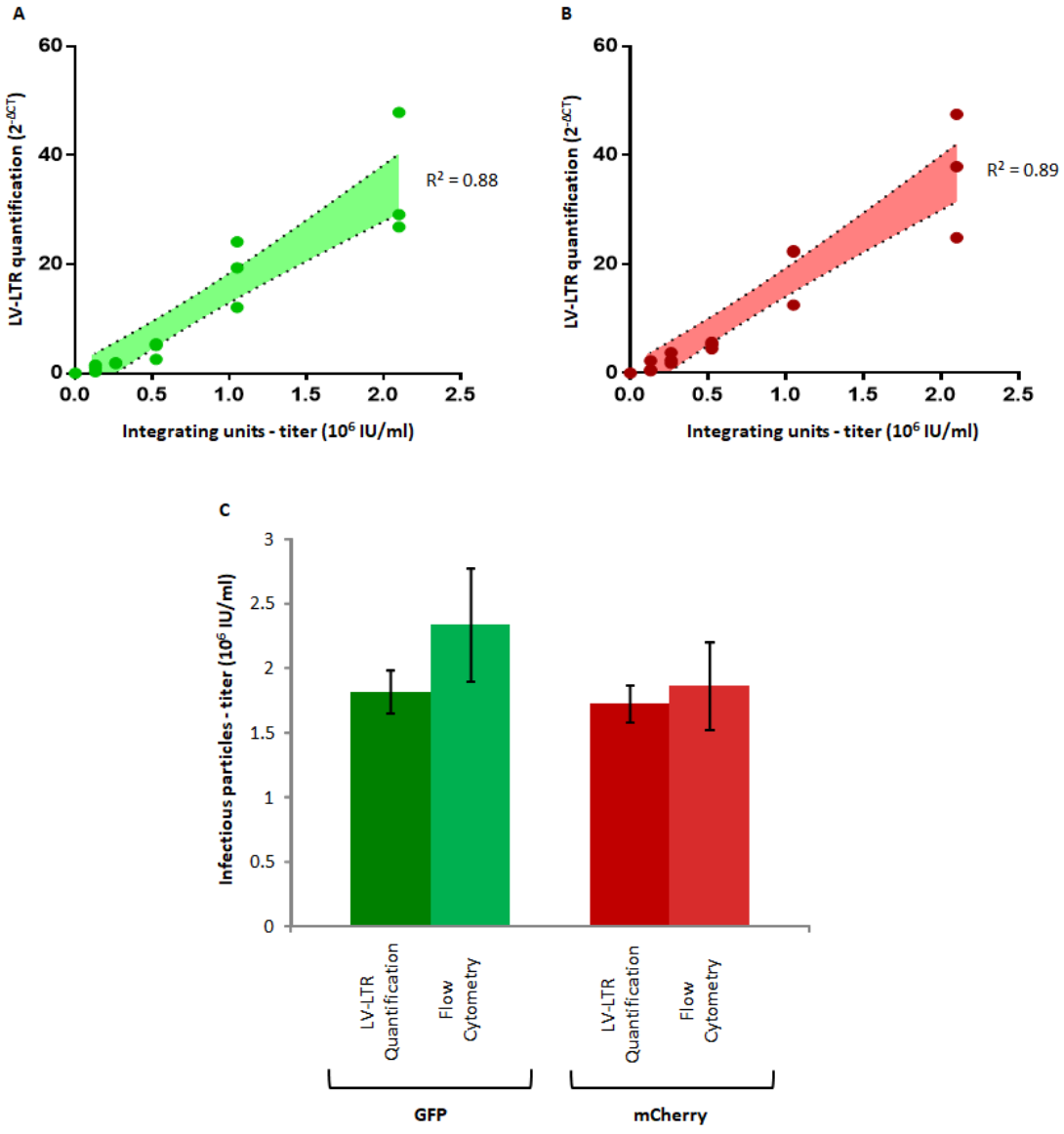


Figure 3.8 - Universal lentiviral vector titration method validation for *pInducible GFP* and *pInducible* mCherry. Linear regression analysis of LV-LTR quantification *versus* lentiviral vector titer using least-squares method for *pInducible GFP* **(A)** and *pInducible mCherry* **(B),** for this RT-qPCR genomic DNA was used at 80ng/µL. The points

correspond to three biological replicates calculated as average $2^{-\Delta CT}$ ± standard deviation (n = 2 technical replicates for each biological replicate, error bars omitted for simplicity) used to establish individual linear regressions combined in a best fit calibration curve, the shading represents the 95% confidence interval (data treated on GraphPad software using linear regression analysis tools). Titers of *pInducible GFP* calculated from *pInducible mCherry* calibration curve and titers of *pInducible mCherry* calculated from *pInducible GFP* calibration curve, error bars correspond to linear regression error (values calculated as average titer ± standard deviation, n = 2 technical replicates for each of the biological replicates) and titers of *pInducible GFP* and *pInducible mCherry* calculated by flow cytometry analysis, values are shown as average titer, n = 3 biological replicates **(C)**.

3.3. Metabolic engineering studies

Genetic engineering of metabolic pathways targeted at improving retroviral vector production has shown promising results (A. Rodrigues, PhD thesis). In this thesis two major metabolic pathways involved in retroviral vector production were studied: glutathione metabolism using an inducible gene expression system, following up on previous work developed on this pathway (Oliveira et al., 2016) and lipid metabolism and its role on retroviral vector production under serum deprivation (Rodrigues et al., 2009). Yet, before proceeding to metabolic engineering studies, the inducible system developed, that was to be used as manipulation tool, was further characterized. In particular, the effects of doxycycline and the system *per se* on retroviral vector production, were evaluated.

3.3.1. Impact of inducible system on retroviral vectors titers

To evaluate the impact of doxycycline on cell growth and retroviral vector production, 293 FLEX S11 cells were cultured under different concentrations of doxycycline. Over the course of five days, cultures were daily monitored to assess cell growth and retroviral vector production (**Figure 3.9**). The results showed no substantial effect on retroviral vector production whereas cell growth was slightly impaired at higher concentrations of doxycycline. Highlighting the fact that the cells in **Figure 3.9** do not express the inducible system these results reflected only the effect of doxycycline itself.

To determine if the inducible gene expression system *per se* had an effect on cell growth and retroviral vector production, 293 FLEX S11 cells stably expressing *pInducible GFP* or *pInducible mCherry* were cultured in the presence or absence of doxycycline. Cell growth (**Table 3.4**) and viral vector production (**Figure 3.10**) were assessed on a daily basis over the course of five days. The results of this study showed that the growth of cells expressing the inducible system is similar to that of non-manipulated 293 FLEX S11 cells (negative control). In terms of retroviral vector productivity, non-manipulated cells generally suffer a small drop in the presence of doxycycline (**Figure 3.10 A**). Compared to the negative control, cells expressing the inducible system generally exhibited a slight increase in retroviral vector productivity, particularly in the presence of doxycycline (**Figure 3.10 B**). However these productivity differences are within the variance of biological replicates associated to this method which can go up to 2-fold.

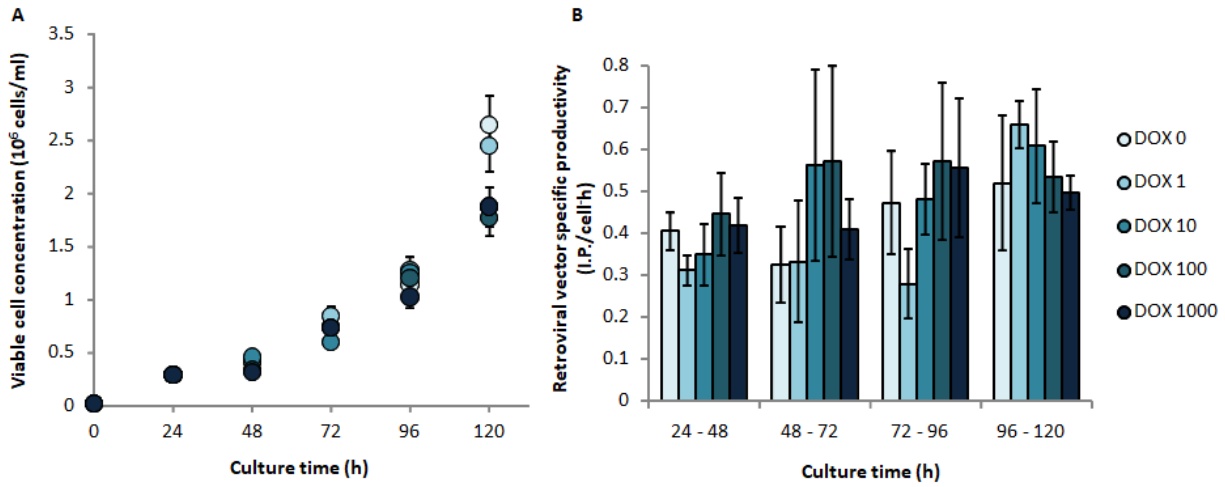


Figure 3.9 - Impact of doxycycline on 293 FLEX S11 cell growth and retroviral vector production. Cell growth of 293 FLEX S11 cells under different concentrations of doxycycline **(A)**. Error bars correspond to hemocytometer counting standard error of 10%. Retroviral vector productivity of 293 FLEX S11 cells under different concentrations of doxycycline **(B)**. Doxycycline was added 24 hours after seeding and medium was exchanged every 24 hours until the end of the culture. Retroviral titer was assessed in the culture supernatant and corresponds to average specific productivity at each of these 24-hour intervals. Values are shown as average ± standard deviation (n = 4 technical replicates).

Table 3.4 - Effect of inducible gene expression system on 293 FLEX S11 cell growth.

	Doxycycline concentration (ng/mL)	Viable cell concentration (10^6 cells/mL)					
		Culture time (h)					
		0	24	48	72	96	120
293 FLEX S11	0	0.17	0.28	0.49	1.0	1.6	1.9
	1000	0.17	0.28	0.56	0.76	1.9	2.2
GFP	0	0.19	0.29	0.58	0.85	1.3	2.3
	1000	0.19	0.29	0.40	0.61	1.4	1.8
mCherry	0	0.21	0.31	0.64	1.0	1.7	2.6
	1000	0.21	0.31	0.43	0.84	1.7	2.6

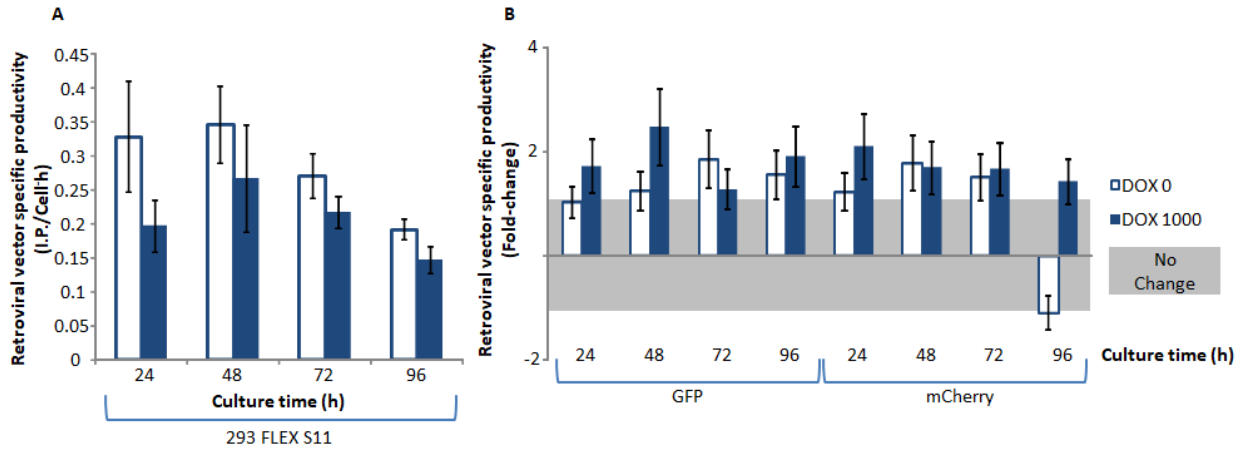


Figure 3.10 - Impact of the inducible gene expression system on 293 FLEX S11 on retroviral vector production. Retroviral vector specific productivity of 293 FLEX S11 cells with or without doxycycline **(A)**. Values are shown as average ± standard deviation (n = 4 technical replicates). Retroviral vector specific productivity of 293 FLEX S11 cells stably expressing *pInducible GFP* or *pInducible mCherry* with or without doxycycline induction (0 or 1000 ng/mL respectively) **(B)**. Doxycycline was added 24 hours after seeding and medium was exchanged every 24 hour until the end of the culture (maintaining the appropriate doxycycline concentration). Retroviral titer was assessed in the culture supernatant and corresponds to average specific productivity at each of these 24-hour intervals. Values are shown as fold-change relatively to 293 FLEX S11 at the correspondent time interval and doxycycline condition. Error bars correspond to maximum error of titration (30%).

In order to assess the optimal time point of gene expression, the kinetics of the inducible system were characterized using 293 FLEX S11 cells stably expressing *pInducible GFP* cultured under different concentrations of doxycycline. Every 24 hours over the course of three days, GFP expression was assessed by flow cytometry (**Figure 3.11**). As expected, cells cultured under higher concentrations of doxycycline showed a higher GFP intensity and thus a higher GFP expression. GFP expression also increased over time and seems to stabilize 48 hours after induction although an extra time-point at 96 hours after induction would be required to assure this conclusion.

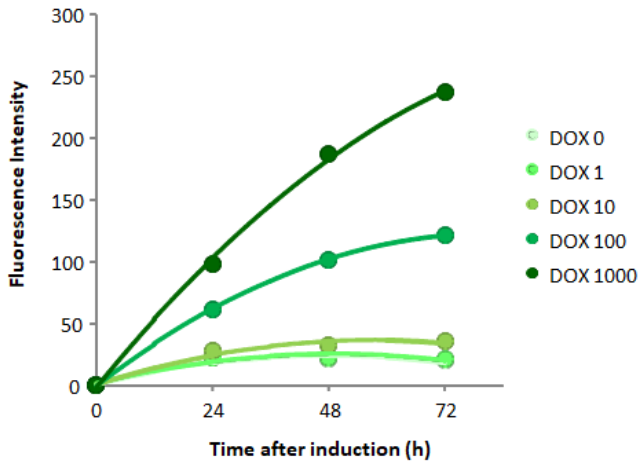


Figure 3.11 - Kinetics of inducible GFP expression. Expression kinetics of inducible GFP expression in 293 FLEX S11 cells stably expressing *pInducible GFP* under different concentrations of doxycycline. Lines shown for visual guidance purposes only.

3.3.2. Manipulation of glutathione metabolism

Glutathione metabolism was found to play a major role in retroviral vector production (Rodrigues et al., 2013). In previous work, the following target genes of this pathway were overexpressed by lentiviral vector delivery at different multiplicities of infection: CBS - cystathionine-beta synthase, CTH - cystathionine-gamma lyase, IDH1 - isocitrate dehydrogenase 1, GSR - glutathione-disulfide reductase, GSS - glutathione synthetase, GSTM1 - glutathione S-transferase mu 1, G6PD - glucose-6-phosphate dehydrogenase, GPX7 - glutathione peroxidase 7 (**Figure 3.12**). The results of previous work showed that the overexpression of some genes, namely GSTM1, CTH and CBS led to increased retroviral vector production. CBS expression resulted in the largest improvement in retroviral vector productivity, up to 13-fold (Oliveira et al., 2016). However these productivity increases were accompanied by increased retroviral vector transgene expression and more surprisingly increase of retroviral vector transgene copy number in the cell genome (A. Oliveira, unpublished data). To clarify these results, this work uses an inducible and reversible gene expression system aiming to investigate a possible cause-effect relation between retroviral vector productivity, metabolic gene overexpression and retroviral transgene expression and copy number.

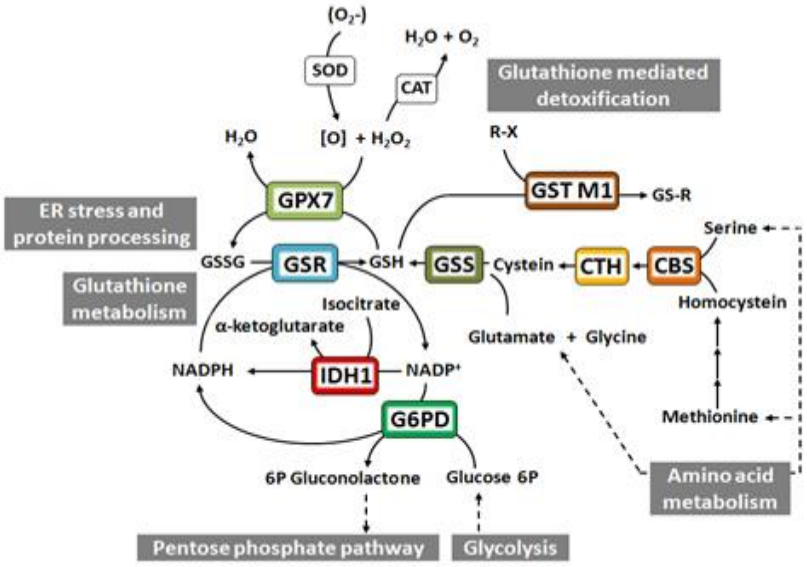


Figure 3.12 - Schematic representation of glutathione metabolism pathway and the genes studied in this work. CBS - cystathionine-beta synthase, CTH - cystathionine-gamma lyase, IDH1 - isocitrate dehydrogenase 1, GSR - glutathione-disulfide reductase, GSS - glutathione synthetase, GSTM1 - glutathione S-transferase mu 1, G6PD - glucose-6-phosphate dehydrogenase, GPX7 - glutathione peroxidase 7. Adapted from Oliveira et al., 2016

After implementing the system for inducible gene expression, as well as a method to universally titrate lentiviral vectors, these two new tools were used to genetically engineer retroviral vector producer cells.

The genes of interest of glutathione metabolic pathway were cloned into the inducible system and used to produce lentiviral vector stocks of each construct. These stocks were titrated using the universal lentiviral vector titration method described above and then used to transduce 293 FLEX S11 cells at a multiplicity of infection (MOI) of one infectious particle *per* cell, meaning each cell contains one copy of the inducible system integrated into the genome.

Successfully transduced cells were selected in the presence of puromycin and the resulting populations were used to assess the expression levels of each of the different target genes by RT-qPCR (**Figure 3.13**). The results showed that in the presence of doxycycline the expression of the delivered genes is greatly increased in the respective population, with the exception of IDH1. These results corroborate the system functionality, previously assessed by reporter genes (GFP and mCherry) but also the leakiness of the system. Indeed, even without doxycycline, the expression values or manipulated genes were already substantially higher than in non-manipulated controls. Besides the gene delivered to each population the gene expression of the remaining genes was mostly unchanged although populations stably expressing *pInducible mCherry* or *pInducible GSS* in the absence of doxycycline and *pInducible*

IDH1 in the presence of doxycycline display a slight decrease of gene expression in some of the glutathione metabolic genes.

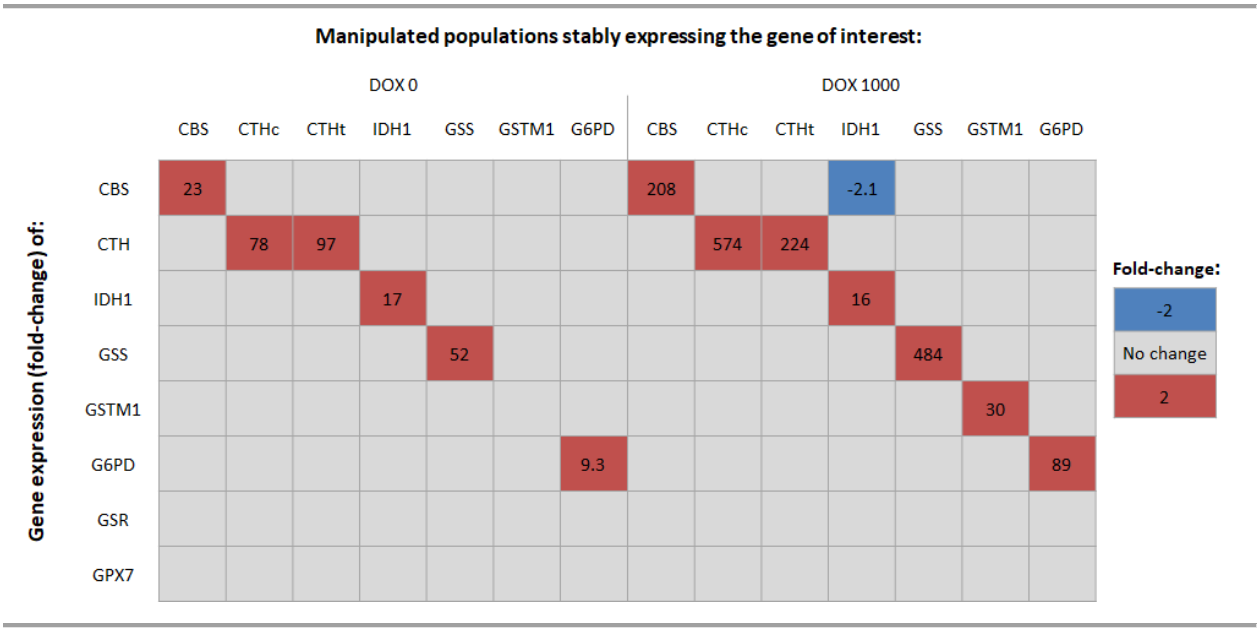


Figure 3.13 - Heat map of gene expression of 293 FLEX S11 cells expressing inducible glutathione metabolism genes. Expression of all genes of interest in 293 FLEX S11 populations stably expressing different *pInducible* constructs without or with doxycycline induction. Values were calculated as average gene expression ($2^{-\Delta CT}$) ± standard variation (error omitted for simplicity), correspond to fold-change relatively to non-manipulated 293 FLEX S11 cells normalized to the respective doxycycline condition and highlighted according to the color code on the right. Cells did not express GPX7 and only cells expressing *pInducible GSTM1* expressed this gene. In this particular case GSTM1 expression in non-induced cells was used as the base line for the induced condition.

The engineered populations were used to study the effect of each gene of interest on cell growth and retroviral vector production (**Table 3.5** and **Figure 3.14**). The results showed that no gene substantially affects cell growth. In terms of retroviral vector productions considerable increases were displayed in producer cells engineered for CBS, GSS, GSTM1 and G6PD. These increases were evident even without induction. Only cells stably expressing *pInducible G6PD* increase retroviral vector productivity in the presence of doxycycline compared to the respective non-induced condition. CBS exhibits the largest retroviral vector productivity increase, reaching up to 5-fold higher than non-manipulated 293 FLEX S11.

Table 3.5 - Effect of inducible expression of glutathione metabolism target genes on cell growth.

	Doxycycline concentration (ng/mL)	Viable cell concentration (10^6 cells/mL)			
		Culture time (h)			
		0	48	72	96
293 FLEX S11	0	0.39	1.3	2.8	3.6
	1000	0.39	1.1	2.1	2.7
GFP	0	0.40	0.9	1.8	2.9
	1000	0.40	0.9	2.1	3.5
mCherry	0	0.42	1.1	2.3	3.3
	1000	0.42	1.3	2.1	3.4
CBS	0	0.39	0.83	1.5	2.8
	1000	0.39	0.81	1.6	2.4
CTHc	0	0.43	1.4	2.5	4.4
	1000	0.43	1.2	2.1	3.0
CTHt	0	0.39	1.1	2.0	3.6
	1000	0.39	1.4	2.0	3.3
IDH1	0	0.36	1.3	2.1	3.6
	1000	0.36	1.2	2.2	3.4
GSS	0	0.41	1.0	2.1	2.8
	1000	0.41	0.88	1.4	2.1
GSTM1	0	0.42	1.1	1.8	3.1
	1000	0.42	0.90	1.9	3.6
G6PD	0	0.44	1.3	2.5	3.7
	1000	0.44	1.1	2.3	3.3

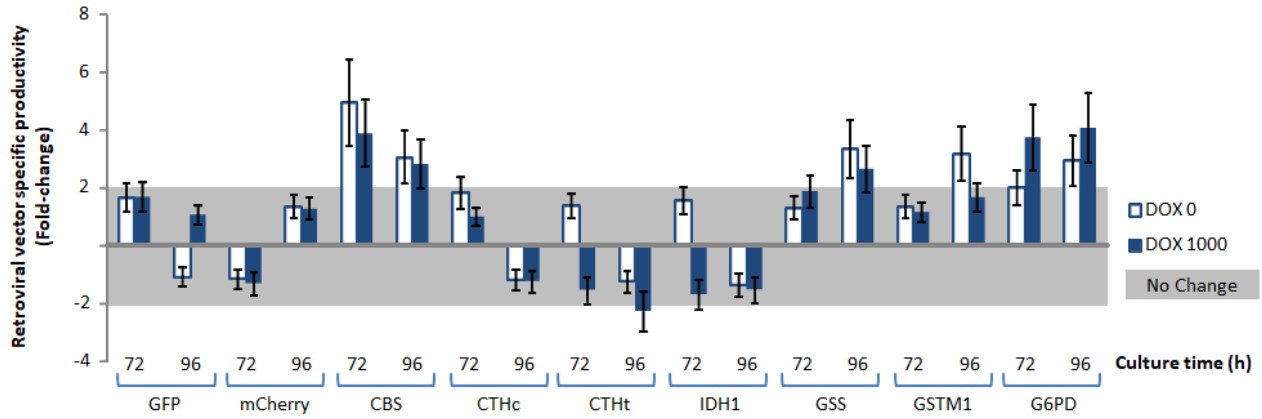


Figure 3.14 - Effect of inducible expression of glutathione metabolism target genes on retroviral vector production. Retroviral vector specific productivity of 293T FLEX S11 cells stably expressing *pInducible* constructs coding for the respective glutathione metabolic gene of interest (CBS - cystathionine-beta synthase, CTH - cystathionine-gamma lyase, CTHc refers to the complete gene while CTHt refers to a truncated version, IDH1 - isocitrate dehydrogenase 1, GSR - glutathione-disulfide reductase, GSS - glutathione synthetase, GSTM1 - glutathione S-transferase mu 1, G6PD - glucose-6-phosphate dehydrogenase) with or without doxycycline induction. Doxycycline was added 24 hours after seeding and medium was exchanged every 24 hours until the end of the culture. Retroviral titer was assessed in the culture supernatant and corresponds to average specific productivity at each of these 24-hour intervals. Values are shown as fold-change relatively to 293 FLEX S11 at the corresponding time interval and doxycycline condition. Error bars correspond to maximum error of titration (30%).

In the previous work, increased productivity of retroviral vectors was associated with increased expression of retroviral components, particularly the transgene (Oliveira et al., 2016). To assess this, mRNA was extracted from 293 FLEX S11 cells stably expressing *pInducible* constructs of the different target genes without or with doxycycline induction (0 or 1000 ng/mL, respectively) and the expression of retroviral vector components was quantified by RT-qPCR (**Figure 3.15**). The results showed that cells stably expressing *pInducible CBS* and *pInducible G6PD* present substantially higher expression levels of transgene (LacZ), up to 4-fold and 5-fold more than non-manipulated 293 FLEX S11 cells, respectively. For G6PD this effect is increased under doxycycline induction. On the other hand cells expressing *pInducible IDH1* under doxycycline induction show a decrease of retroviral vector components expression. The remaining genes had no substantial effect on retroviral components expression levels

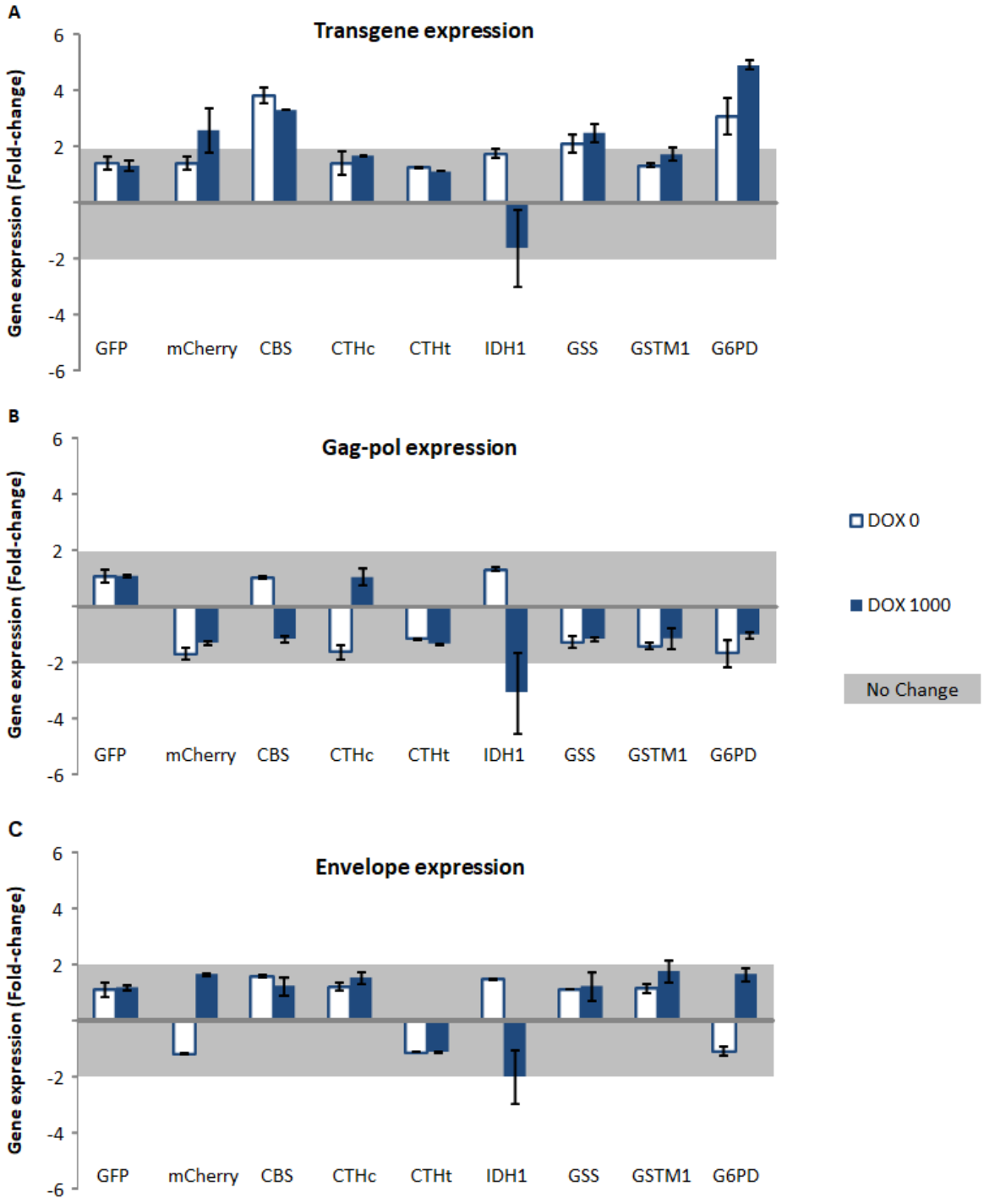


Figure 3.15 - Effect of inducible glutathione metabolism target genes on the expression levels of viral components. Expression levels of viral components: transgene - LacZ - **(A)**, gag-pol **(B)** and envelope **(C)** in 293 FLEX S11 cells stably expressing different *pInducible* constructs coding for the respective glutathione metabolic gene of interest with or without doxycycline induction. Values were calculated as average expression ($2^{-\Delta CT}$) ± standard

deviation (n = 2 technical replicates) and correspond to fold-change relatively to 293 FLEX S11 levels of each viral component normalized to the respective doxycycline condition.

In the previous work, increased expression of retroviral vector components was associated with increased copy number of these components in the producer cells genome (A. Oliveira, unpublished data). To assess this, 293 FLEX S11 populations expressing the different *pInducible* constructs were cultured with or without doxycycline induction followed by genomic DNA extraction and RT-qPCR (**Figure 3.16**). The results show that transgene copy number was slightly increased, particularly in populations stably expressing *pInducible CBS, pInducible GSS* and *pInducible G6PD.* Gag-pol and envelope copy number remains mostly unchanged. Doxycycline does not seem to have a clear effect on viral components copy number.

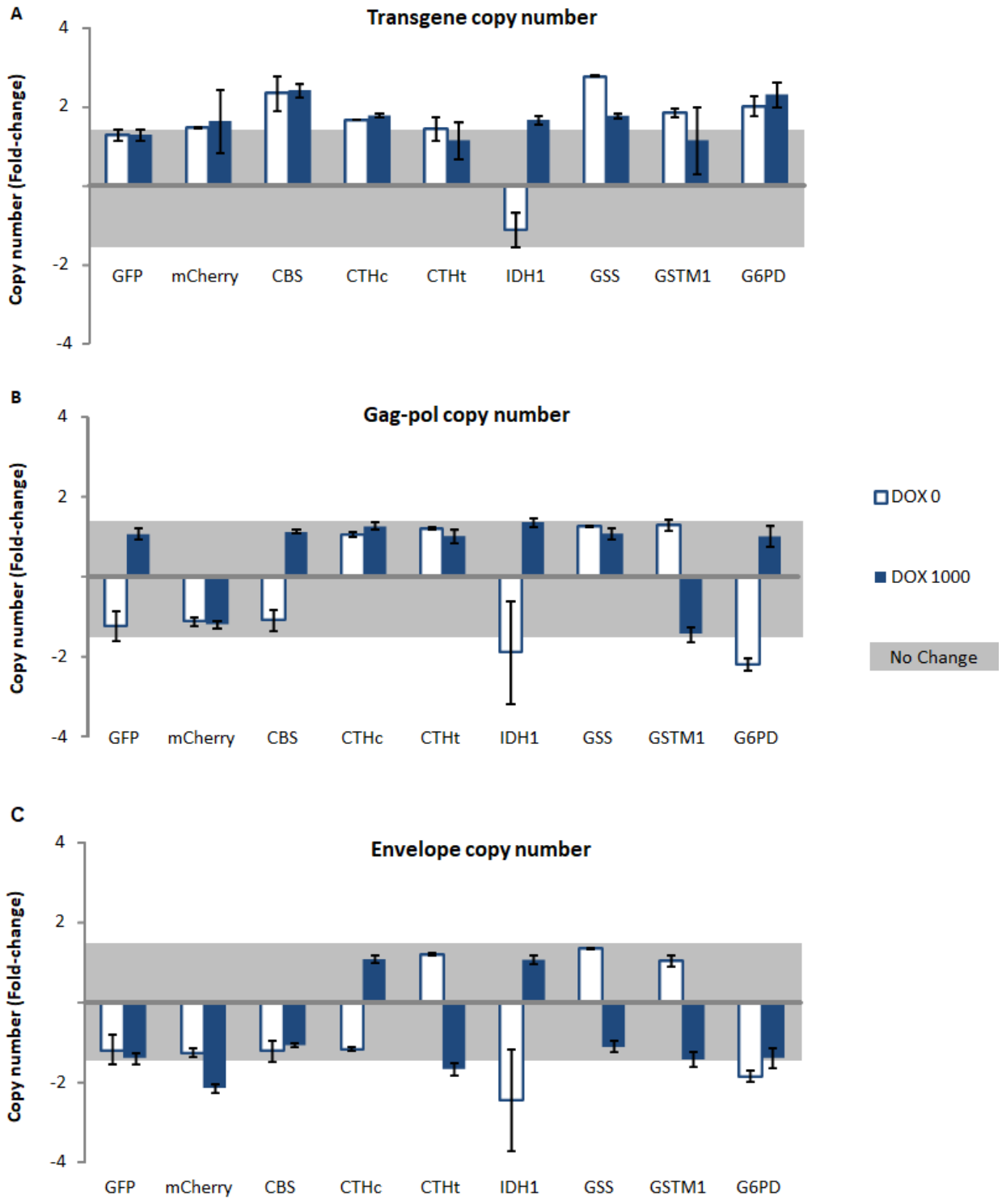


Figure 3.16 - Copy number of viral components in 293 FLEX S11 cells expressing inducible glutathione metabolism genes. Copy number of retroviral vector components: transgene – LacZ **(A)**, gag-pol **(B)** and envelope **(C)** in 293 FLEX S11 populations stably expressing the different *pInducible* constructs. Values were calculated as average copy number integrated in the genome ($2^{-\Delta CT}$) ± standard variation (n = 2 technical replicates) and are

displayed as fold-change relatively to non-manipulated 293 FLEX S11 cells normalized to the corresponding doxycycline condition. No change in copy number is considered between -1.4 and +1.4 fold-change (Bodin et al., 2005)

3.3.3. Manipulation of lipid metabolism

In addition to glutathione metabolism, the role of lipid metabolism in retroviral vector production was also studied. This metabolic study stems from previous work where lipid metabolism was found to play a major role in retroviral vector production under serum deprivation (Rodrigues et al., 2009). Previous work additionally suggested that retroviral vector production in producer cell line 293 FLEX 18 was impaired under serum deprivation while their derivate, 293 FLEX S11, was seemingly not affected (A. Rodrigues, PhD thesis).

To compare the effect of serum deprivation on cell growth and retroviral vector production, these cells were cultured over the course of a week under serum deprivation conditions, 1% (v/v) FBS and normal serum conditions, 10% FBS (v/v), and monitored daily to assess cell growth and retroviral vector production in 293 FLEX S11 and 293 FLEX 18 (**Figure 3.17 B** and **C**, respectively).

The results showed that although 293 FLEX S11 cell growth was slightly lower than that of 293 FLEX 18, serum deprivation does not affect cell growth of these cells (**Figure 3.17 A**). In terms of retroviral vector production, 293 FLEX S11 yielded retroviral vector titers about 10-fold higher than 293 FLEX 18 but more importantly, retroviral vector production in 293 FLEX 18 drops to half under serum deprivation while 293 FLEX S11 were not affected.

To assess the role of lipid metabolism on retroviral vector production in these two producer cell lines, gene expression of target genes was evaluated. These targets, namely: ACYL - ATP citrate lyase, SREBF1 - sterol regulatory element binding transcription factor 1, ACACA - acetyl-CoA carboxylase-alpha, FASN - fatty acid synthase, SREBF2 - sterol regulatory element binding transcription factor 2, ACAT - acetoacetyl-CoA thiolase, HMGCR - HMG-CoA reductase, HMGCS - HMG-CoA synthase, MVK - mevalonate kinase and LSS - lanosterol synthase (**Figure 3.18 A**). In previous work, LSS, MVK and SREBF2 were identified as bottlenecks in retroviral vector production (A. Rodrigues, PhD thesis).

Producer cell lines 293 FLEX S11 and 293 FLEX 18 were cultured under normal serum conditions 10% (v/v) FBS and under serum deprivation conditions 1% (v/v) FBS and their mRNA was extracted for RT-qPCR quantification of the target genes (**Figure 3.18 B**).

The results showed that the lipid metabolism gene expression profile slightly differs between the two cell lines, with 293 FLEX S11 expressing less FASN, SREBF2 and HMGCS than 293 FLEX 18, but more importantly 293 FLEX 18 respond to serum deprivation with a generalized overexpression of all genes studied, with the exception of FASN, while the expression of these genes remains mostly unchanged in 293 FLEX S11.

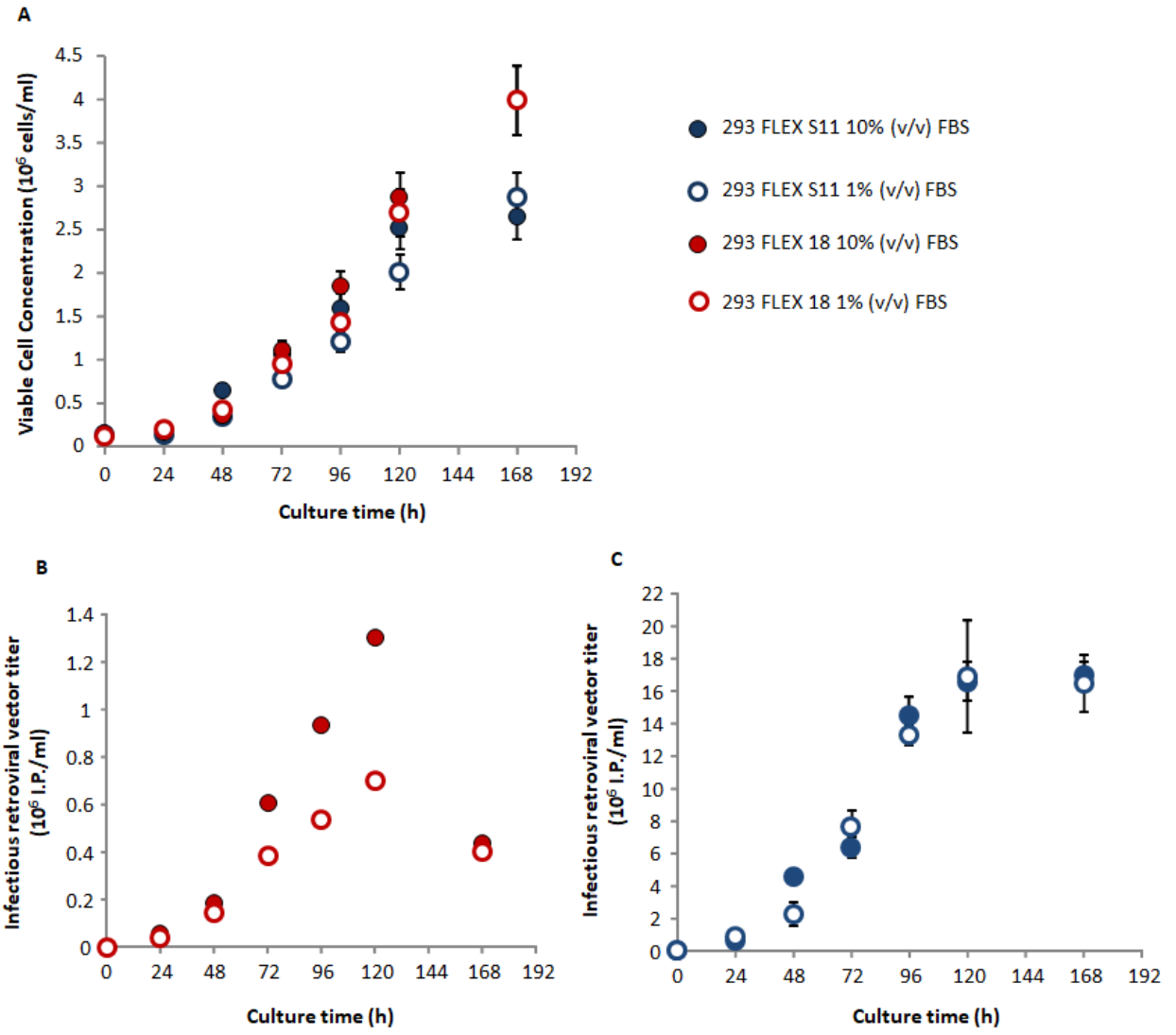


Figure 3.17 - Effect of serum deprivation on cell growth and retroviral vector production of 293 FLEX S11 and 293 FLEX 18 cells. Viable cell concentration of 293 FLEX S11 and 293 FLEX 18 **(A)**. Values are shown as viable cell concentration (10^6 cells *per* mL) and error bars correspond to hemocytometer counting standard error of 10%. Retroviral vector production of 293 FLEX 18 **(B)** or 293 FLEX S11 **(C)** over time under normal serum supplementation 10% (v/v) FBS and serum restriction 1% (v/v) FBS conditions. Values are shown as average retroviral vector titer (10^6 infectious particles *per* mL) ± standard deviation (n = 4 technical replicates, error bars in **B** are not visible).

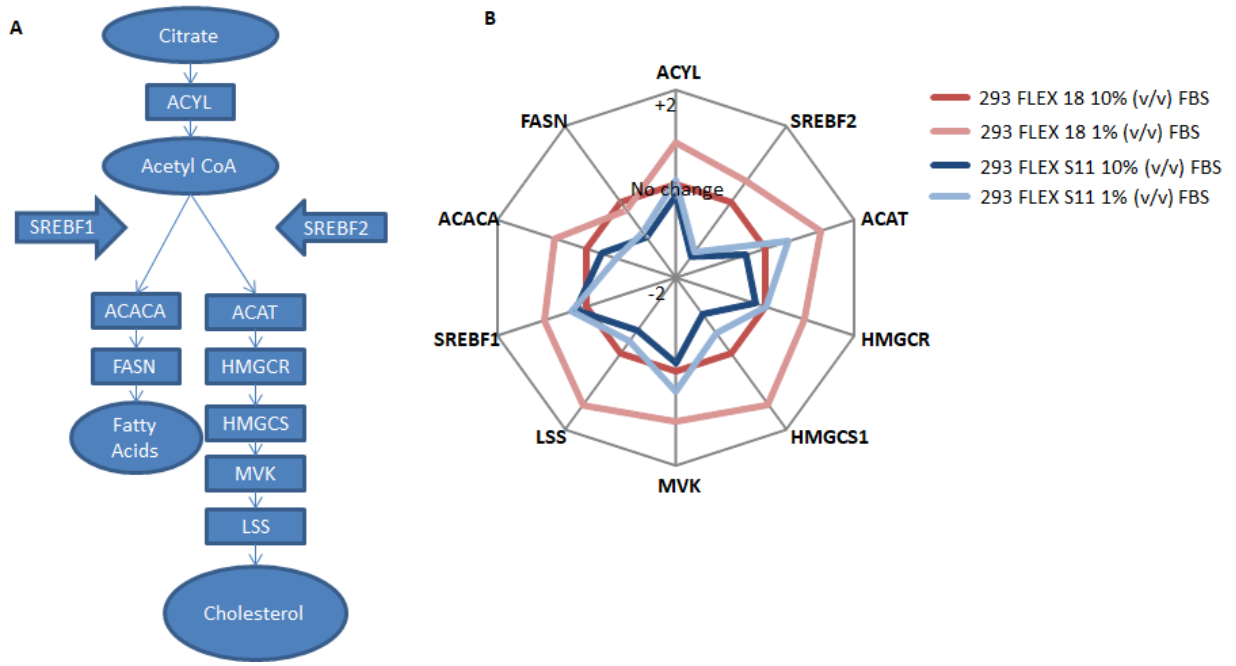


Figure 3.18 - Lipid metabolism gene expression levels under serum deprivation. Simplified schematic representation of the main steps of fatty acids metabolism and cholesterol biosynthesis pathway **(A).** Only the steps corresponding to the analyzed genes are shown. ACYL - ATP citrate lyase, SREBF1 - sterol regulatory element binding transcription factor 1, ACACA - acetyl-CoA carboxylase-alpha, FASN - fatty acid synthase, SREBF2 - sterol regulatory element binding transcription factor 2, ACAT - acetoacetyl-CoA thiolase, HMGCR - HMG-CoA reductase, HMGCS - HMG-CoA synthase, MVK - mevalonate kinase, LSS - lanosterol synthase. Comparison of expression levels of different lipid metabolism genes under normal serum supplementation 10% (v/v) FBS and serum deprivation 1% (v/v) FBS conditions **(B)**. Values were calculated as gene expression ($2^{-\Delta CT}$) ± standard variation (n = 2 technical replicates) and are shown as fold-change relatively to 293 FLEX 18 under 10% (v/v) FBS (red line, no change), error bars were omitted for simplicity.

4. Discussion and Conclusions

Gene therapy has experienced considerable growth over the last years and is expected to continue expanding based on the high number of products going into clinical trials and transitioning into the market (Edelstein, 2017). Viral vectors proved to be extremely efficient tools for gene delivery constituting the vast majority of vectors used in gene therapy (Edelstein, 2017). Among viral vectors, those based on *retroviruses* are of particular interest due to their ability to stably integrate into the genome of target cells, sustaining long-term expression of the therapeutic gene (Thomas et al., 2003). This makes recombinant *retroviruses* a vector of choice to treat monogenic diseases (Thomas et al., 2003).

Current systems for retroviral vector production face several challenges that hinder the transition of products from clinical-to-market, mainly due to low titers, low infectious-to-total particles ratios and the need of animal blood serum during production (Rodrigues et al., 2011). Improving viral vector production systems is necessary for the development of gene therapy and therefore it is a subject of active research. Different approaches have been used to improve viral vector production such as process-optimization and metabolism manipulation. Genetic engineering of producer cells is one of the best approaches to improve viral vector production and holds the potential to overcome some of the limitations that current systems face (Rodrigues et al., 2014).

In previous work, functional genomics studies identified metabolic bottlenecks in stable cell lines producing retroviral vectors and potential target genes for metabolic engineering to improve vector production (Rodrigues et al., 2013). In this work, genetic engineering was used to target the identified genes, particularly focusing on glutathione and lipid metabolism pathways.

Previous efforts on genetic manipulation of glutathione metabolism confirmed the potential of this pathway to improve retroviral vector production (A. Oliveira, unpublished data). However, the strategy used in those manipulations yielded intriguing results and did not allow to distinguish the effect of genetic manipulation from the expression of the delivered gene. The strategy applied in previous work used lentiviral vectors to deliver the gene of interest to producer cell lines leading to continuous and irreversible overexpression. With such experimental design it was difficult to discriminate the effect of gene expression from the manipulation or to correlate gene expression and titer improvement. Additionally it was not possible to titrate the lentiviral vectors used for genetic manipulation because they devoided reporter markers. Because of this, the expression levels of the different genes were not uniform among the different manipulated populations. Finally, the results were intriguing because engineered cells that yielded improved titers also exhibited increased copy number of retroviral transgene in the cell genome. Such genomic instability has never been reported and, more importantly, it remained to elucidate whether this was cause or consequence of titer improvement.

Based on these results, this thesis developed along three work lines: i) the design and construction of an inducible system for controllable and reversible expression of the target genes ii) the implementation of a universal lentiviral titration method for establishment of uniformly manipulated

populations and iii) genetic engineering of producer cell lines, targeting glutathione metabolism using the inducible system.

The inducible system herein developed is based on a TET-ON configuration (Urlinger et al., 2000), consisting of two components: i) a chimeric promoter which contains a tetracycline responsive element (TRE) driving the expression of a gene of interest and ii) a reverse tetracycline-controlled transactivator 3 (rtTA3) which activates the chimeric promoter. In the presence of tetracycline or its analogues (doxycycline, herein used) rtTA3 binds to the TRE in the chimeric promoter resulting in the inducible expression of the gene of interest. The system operates in a dose-responsive manner according to the amount of doxycycline added, leading to increasing amounts of active rtTA3 activating the chimeric promoter. This system is reversible and allows the fine-tuning of gene expression making it a powerful tool for metabolic manipulation studies, overcoming some of the limitations of the previous designs.

The starting points of this system were *pLenti CMVtight GFP Puro* and *pLenti CMV rtTA3 Blast*, plasmids encoding the chimeric promoter driving the expression of GFP and the transactivator (rtTA3), respectively (**Figure 3.1 A and B**). To facilitate the delivery and integration into the target cells genome, the system was cloned into a single lentiviral vector construct (**Figure 3.1 C**). The final plasmid was designed in a way that allowed easy exchange of the main features by enzymatic restriction and insert ligation. This flexibility was used to clone the glutathione metabolic genes (**Figure 3.1 D**).

To characterize the system, populations of cells stably expressing each of the components, individually or in combination, were established. The expression of the reporter gene, GFP, was assessed in the different populations by fluorescence microscopy, flow cytometry and gene expression (mRNA levels). Fluorescence microscopy (**Figure 3.2 A**) showed that GFP expression was successfully induced in a dose-responsive manner, confirming the functionality of the system. However substantial leaky expression was also observed (**Figure 3.2 B**). Moreover, similar levels of leaky expression were exhibited by cells stably expressing CMVtight GFP only, regardless of doxycycline concentration, suggesting that the promoter itself is leaky. These results were corroborated by GFP gene expression levels assessed by RT-qPCR (**Figure 3.3**). The leaky expression observed was not expected. Inducible systems evolved in generations, while 1st generation systems were associated with substantial leakiness, 2nd generation systems like the one used herein, were optimized to minimize this limitation hence even naming the promoter “CMVtight” (Urlinger et al., 2000). Thus, the leaky expression was hypothesized to be due to non-residual levels of tetracycline in the serum used for cell culture. However, this was not the case since GFP expression levels were similar in cells cultured under standard serum, Gibco FBS, and the manufacturer’s approved tetracycline-free serum, Clontech FBS (**Figure 3.4** and **Table 3.1**), indicating that the leaky expression was an intrinsic feature of CMVtight promoter.

The system functionality was further evaluated using a different reporter gene (mCherry) and confirmed the dose-dependent induction. Fold-induction of GFP and mCherry were comparable up to 100 ng/mL of doxycycline (**Figure 3.5** and **Table 3.2**). At 1000 ng/mL of doxycycline GFP intensity was near the maximum limit of detection of the flow cytometer making it difficult to accurately measure the GFP expression at this condition. This might explain the difference in fold-induction compared to mCherry

intensity. In fact, the values obtained for GFP gene expression by RT-qPCR support that GFP fold-induction, at the highest doxycycline concentration, assessed by flow cytometry was not correctly measured resulting in an underestimation (**Figure 3.3**).

Although both components of the system are within the same plasmid construction (**Figure 3.1 C**), each of them is an independent expression cassette, one consisting of the chimeric promoter driving the expression of the gene of interest and the other having a constitutive promoter (SV40 promoter) driving the expression of the transactivator (rtTA3) and puromycin resistance gene, the latter through a mechanism of translation re-initiation (Kozak, 1987; Kozak, 2002). This may result in a stoichiometric imbalance between the two components, ultimately leading to limitations in the expression of the gene of interest. To evaluate this possibility, cells were transduced with increasing amounts of each of the components, individually or in combination, and assessed for GFP expression by flow cytometry as well as GFP and rtTA3 gene expression by RT-qPCR. The results show that the delivery of additional copies of the system did not lead to sufficient levels of expression increase to evaluate the hypothesis (**Figure 3.6**). The populations established for this study derived from a selected population stably expressing the final system construct (*pInducible GFP*). During the selection process cells expressing higher levels of puromycin resistance gene were selected. However, this gene is preceded by a spacer sequence driving a re-initiation of translation mechanism with a reported efficiency of approximately 35% (Kozak, 1987). Hence the ratio of rtTA3 and puromycin resistance gene expression is about 3:1, leading to a stringent selection of the population resulting in the selection of cells expressing extremely high levels of the system. Since the starting population was already resistance to both the resistance markers in the components constructs, there were no other means to select the populations established. Hence, the expression levels of both components in the starting population were so high that probably “diluted” the additional expression provided by the delivery of additional copies of the components.

After developing and characterizing the system for inducible gene expression there was still the need to quantify the lentiviral vectors for its delivery. To this end, a universal method for lentiviral vector titration was implemented. Based on the quantification of the integration of conserved lentiviral sequences (LTR, herein used) into the target cells genome, this method is able to titrate lentiviral vector stocks without reporter genes or tags. In concept, the method requires a lentiviral vector of known titer used to establish calibration curves to correlate LTR integration with lentiviral vector titer from which samples of unknown titer can be titrated (**Figure 3.7 A** and **B**). The lentiviral vector used in the stock to establish the calibration curves should have the same molecular design of the samples to be titrated to account for titer differences arising from transgene size. This issue was evident when using *pRRLSIN* and *pInducible* vectors (**Figure 3.7 C** and **Figure 3.8 A** and **B**). In the first step, a standard of known titer was used to implement the method; a lentiviral vector stock of *pRRLSIN.cPPT.PGK-GFP.WPRE* was produced by transient production, titrated by flow cytometry and used to establish calibration curves to validate the method (**Figure 3.7 C**).

After validation for *pRRLSIN* based lentiviral vector, the method was used for *pInducible* based lentiviral vectors. Lentiviral vector stocks of *pInducible GFP* and *pInducible mCherry* were produced by

transient production and titrated by flow cytometry, yielding titers of approximately $2x10^6$ infectious particles *per* mL (**Figure 3.8 D**) which were about 10-fold lower than those of *pRRLSIN* based lentiviral vectors. Since the titers yielded for *pInducible* lentiviral vectors were substantially lower, the amount of infectious particles used to establish the curves had to be adjusted accordingly which meant that the overall amount of LTR integration was lower as well. To compensate for this, the amount of DNA used when performing RT-qPCR in genomic DNA extracted from cells transduced with *pInducible* constructs was doubled.

The lentiviral vector stocks of *pInducible GFP* and *pInducible mCherry* were used to establish the respective calibration curves in triplicates (**Figure 3.8 A** and **B**). Although the curves showed moderate differences between them, they were within the expected variation from biological replicates and the combination of the triplicates maintained the linear behavior. The method was validated by calculating *pInducible GFP* lentiviral vector titers from the calibration curve established using *pInducible mCherry* and *vice-versa* (**Figure 3.8 C**). The titers calculated by LTR integration were similar to those obtained by flow cytometry, showing the method was able to accurately titrate these samples (**Figure 3.8 C**). After validating this universal method for lentiviral vector titration the conditions were met to uniformly deliver the different constructs of the inducible system to producer cell lines for metabolic engineering.

Before proceeding with metabolic engineering studies, a series of studies were conducted to evaluate the impact of doxycycline and the inducible system on cell growth and retroviral vector production. Doxycycline is a broad-spectrum antibiotic ("Doxycycline - PubChem," accessed Sept. 18, 2017) and has been reported to negatively impact cell growth (Moullan et al., 2015). This cytotoxic effect could potentially affect retroviral vector production. To assess this, non-manipulated cells were cultured under different concentrations of doxycycline. The results showed that at higher doses of doxycycline cell growth was slightly impaired (**Figure 3.9 A**) while retroviral vector production was seemingly not affected (**Figure 3.9 B**). Interestingly, in following studies performed to evaluate the effect of the inducible system *per se* on retroviral vector production, non-manipulated producer cells used as negative control yielded slightly lower retroviral vector titers in the presence of doxycycline compared to those obtained in the absence of doxycycline (**Figure 3.10 A**). The extent of these effects was however very small, suggesting that doxycycline could be used to induce genetically engineered producer cell lines without substantially affecting retroviral vector production. To evaluate the impact of the inducible system *per se*, producer cell lines stably expressing *pInducible GFP* or *pInducible mCherry* were cultured in the presence or absence of doxycycline. Cell growth and retroviral vector productivity in producer cell lines stably expressing *pInducible GFP* or *pInducible mCherry* was similar to those of non-manipulated producer cells in the respective doxycycline condition (**Table 3.4** and **Figure 3.10 B**), demonstrating that the inducible system did not affect either of them.

The metabolic studies conducted during this work aimed for the observation of an "all-or-nothing" effect of the expression of the gene of interest on retroviral vector production. Therefore the kinetics of inducible GFP expression were characterized to assess at which time-point the system reached the maximum expression (**Figure 3.11**). This study showed that higher doses of doxycycline resulted in higher

expression levels. GFP expression seems to start stabilizing between 48 and 72 hours after induction, however an additional later time point would be required to confirm this hypothesis. Additionally, GFP expression increased over time which can be explained by two factors: i) the kinetics of the system itself led to an increase in gene expression due to the doxycycline induction and ii) the fact that GFP is a stable structural protein with a relatively long half-life, approximately 26 hours (Corish & Tyler-Smith, 1999) accumulating inside the cell led to an increase of GFP signal over time. A construct for the inducible expression of Luciferase was cloned (**Figure 3.1 D**) as an additional control that will be used in the follow-up of this thesis, to better understand the kinetics of the system. Unlike GFP, Luciferase is an enzyme with a 2 hours half-life (Ignowski & Schaffer, 2004) and would more closely mimic the kinetics of the target genes which all code for enzymes. Still in the pursuit of an "all-or-nothing" effect of the target genes on retroviral vector production, the doxycycline concentration selected was 1000 ng/mL and the time points selected to assess retroviral vector productivity were 48 and 72 hours after induction, based on the results of the results of the kinetics studies (**Figure 3.11**).

After characterizing the inducible gene expression system, its effects on retroviral vector production and expression kinetics, it was finally used for genetic engineering. In this work it was applied for inducible expression of glutathione metabolism genes.

Glutathione metabolism was found to play a major role in retroviral vector production (Rodrigues et al., 2013). This pathway is mostly associated with detoxification of oxygen reactive species and regulation of oxidative stress. In previous work, glutathione metabolic genes were overexpressed by lentiviral vector delivery following the hypothesis that the overexpression of target genes would reduce oxidative stress of producer cells and result in improved retroviral vector production. The overexpression of some of the genes of glutathione metabolism in retroviral vector producer cells resulted in titer increases up to 13-fold, relatively to non-manipulated cells (Oliveira et al., 2016). Engineered producer cells that exhibited titer increase also displayed increased expression of retroviral vector transgene and, more surprisingly, increased copy number of retroviral vector transgene in the genome (A. Oliveira, unpublished data).

In this thesis, following the same hypothesis, glutathione metabolism was manipulated by genetically engineering producer cell lines using the inducible gene expression system to express the target glutathione metabolic genes. This experimental design has two main advantages: i) the same population can be studied under normal and overexpression conditions of the gene of interest, allowing to associate titer increase with either effect of the gene of interest or effect of genetic manipulation/population selection *per se*; ii) the gene expression is reversible allowing to assess if production yields revert to the previous values in the absence of overexpression of the gene of interest.

To this end, populations of producer cell lines were established, stably expressing *pInducible* construct for the different target genes: CBS - cystathionine-beta synthase, CTH - cystathionine-gamma lyase, IDH1 - isocitrate dehydrogenase 1, GSS - glutathione synthetase, GSTM1 - glutathione S-transferase mu 1, G6PD - glucose-6-phosphate dehydrogenase and using *pInducible GFP* and *pInducible*

mCherry as mock controls. Constructs for inducible expression of GPX7 and GSR have been developed and are a part of on-going metabolic studies.

Gene expression of the different target genes was assessed in the engineered producer cell populations (**Figure 3.13**). Even in the absence of doxycycline, all populations of producer cell lines exhibited substantial increased gene expression of the respective gene delivered which was further increased under doxycycline induction for all manipulated populations except for the one stably expressing *pInducible IDH1*. These results were consistent with the functionality of the system previously assessed for GFP: there was a substantial leaky gene expression in the absence of doxycycline and in the presence of doxycycline gene expression was greatly increased. The high levels of leaky expression however, invalidate the use of the system to test the hypothesis because the non-induced manipulated populations cannot provide the intended no-increase expression of gene of interest, facing then the same issues as previous work. Moreover, even in the non-induced condition, the expression levels of gene of interest in the respective manipulated population were higher than what was achieved even at the highest gene overexpression in previous work.

Cell growth and retroviral vector productivity were assessed in every engineered population. None of the populations showed a noticeable effect on cell growth (**Table 3.5**). However, producer cell populations stably expressing *pInducible CBS, pInducible GSS, pInducible GSTM1* and *pInducible G6PD* exhibited increased retroviral vector specific productivity (**Figure 3.14**). The largest increase in retroviral vector production was achieved with the population stably expressing *pInducible CBS* reaching up to 5-fold increase compared to non-manipulated producer cells. In previous work, overexpression of CBS and GSTM1 also resulted in increased retroviral vector production (Oliveira et al., 2016) corroborating that the overexpression of these genes leads to improved retroviral vector production. With the exception of producer cells stably expressing *pInducible G6PD*, increase of retroviral vector production was more prominent in the absence of doxycycline. This raised the hypothesis that overexpression of the gene of interest up to a certain threshold was able to improve retroviral vector production. Beyond that threshold overexpression was no longer able to improve retroviral vector production and may even have impaired it. Gene expression levels (**Figure 3.13**) and previous data (Oliveira et al., 2016) seem to corroborate this hypothesis.

Increased retroviral vector production was previously associated with increased gene expression of retroviral transgene (A. Oliveira, unpublished data). To evaluate the expression of retroviral vector components (transgene, gag-pol and envelope), RT-qPCR was performed on RNA extracted from the different populations (**Figure 3.15**). The results showed that, retroviral transgene expression was increased in populations stably expressing *pInducible CBS, pInducible GSS* and *pInducible G6PD.* With the exception of the population stably expressing *pInducible GSTM1*, the populations that displayed increased retroviral vector production also exhibited increased retroviral transgene expression which was consistent with the results from previous work (Oliveira et al., 2016).

Increased transgene expression was, in the previous work, associated with increased copy number of retroviral vector transgene in the producer cells genome (A. Oliveira, unpublished data). To

evaluate if this was also happening, copy number of the retroviral vector components integrated into the genome of engineered populations was assessed (**Figure 3.16**). Retroviral transgene copy number was increased across all engineered populations. Albeit very slight, this increase is particularly noticeable in the populations that yielded increased retroviral vector production. Due to the leaky expression it was not possible to evaluate if the transgene copy number increase was associated with the overexpression of the gene of interest because the design did not provide the "all-or-nothing" behavior. Different hypothesis have been raised as to which mechanisms were behind this unexpected genome instability such as re-infection, gene duplication and homologous recombination.

Some results of the metabolic engineering developed in this thesis differ from the ones obtained previously. Overexpression of G6PD and GSS did not result in increased retroviral vector production while herein higher titers were obtained. Conversely, (truncated) CTH overexpression increased retroviral vector production up to 5-fold in previous work although it did not seem to have any effect on the producer cell population stably expressing *pInducible CTH* (either complete or truncated form). Additionally, the increase in retroviral vector production by CBS overexpression achieved in previous work is substantially higher (up to 13-fold increase) than what was achieved using the inducible construct for this gene (up to 5-fold). An hypothesis that could explain these differences is the fact that the expression levels of the gene of interest using the inducible system were substantially higher than those achieved by previously (even without doxycycline induction) resulting in increased retroviral vector production by genes that previously showed no effect. Conversely, the gene expression achieved by the inducible system may be so high that it surpasses the threshold that promotes increased retroviral vector production and led to an attenuated or even cytotoxic effect. Indeed the overexpression of some of these enzymatic genes, for example CBS, is a cause of disease (Ignoul & Eggermont, 2005). This advocates for a possible cytotoxicity that might have been attained in this experimental set-up.

The inducible gene expression system used for metabolic manipulation in this thesis is a powerful and versatile tool for genetic engineering, in concept, able to provide fine-tuning of gene expression. However, it faces a limitation associated with its' substantial leaky expression, possibly deriving from the construct design that inherently selects cells sustaining extremely high expression levels. Optimization of the system could be achieved by de-coupling puromycin resistance gene expression from rtTA3 expression resulting in a less stringent selection. Additionally, inserting a repressor of the chimeric promoter in the construct functional in the absence of doxycycline could also result in lower leakiness (Zhu et al., 2001).

The leaky expression conditioned the results and did not allow to observe the intended reversible or even "all-or-nothing" effects of target gene expression. It invalidated the possibility to test the hypothesis: i) if increased retroviral vector transgene and copy number was a cause or a consequence of retroviral vector production increase and ii) if these effects were caused by the expression of the gene of interest or due to the manipulation and selection process itself.

Nevertheless, inducible expression of CBS, GSS, GSTM1 and G6PD in producer cells populations sustained improved retroviral vector production making them appealing candidates for further

metabolic engineering studies in this context and validated data from previous work. Additionally, manipulation of more than one gene of a metabolic pathway often results in synergistic effects that could make a combination of some of the above mentioned able to extensively improve retroviral vector production.

This thesis also focused on metabolic manipulation of lipid metabolism, to sustain retroviral vector production under serum deprivation. In previous work, lipid metabolism, particularly cholesterol biosynthesis pathway was identified as the main pathway limiting retroviral vector production under serum deprivation (Rodrigues et al., 2009). Bottlenecks within this pathway were identified by transcriptional profiling of a producer cell line, 293 FLEX 18 (A. Rodrigues, PhD thesis). In this work, however, 293 FLEX S11 were used as study model. Therefore, retroviral vector production under serum deprivation of 293 FLEX S11 cells had to be assessed and compared to that of 293 FLEX 18 cells. To this end, a growth study of the two producer cell lines, under serum restriction and normal serum conditions was conducted. The results showed that although 293 FLEX S11 cell growth was lower than 293 FLEX 18, none of the producer cell lines is affected by serum restriction in terms of cell growth (**Figure 3.17 A**). These results were expected because serum deprivation, 1% FBS (v/v), had been previously defined for 293 FLEX 18 as the minimal concentration of serum that still maintained normal cell growth when using a reduced-serum formulation, Advance DMEM (Rodrigues et al., 2009). 293 FLEX S11 yielded retroviral vector titers approximately 10-fold higher than 293 FLEX 18 also described in Rodrigues et al., (2015). However, 293 FLEX S11 displayed a different phenotype of retroviral vector production under serum deprivation, compared to 293 FLEX 18. The titers yielded by 293 FLEX S11 did not exhibit a drop under serum deprivation, while 293 FLEX 18 retroviral vector titers drop to half (**Figure 3.17 B** and **C**). Although unexpected, these differences might be explained by the fact that 293 FLEX S11, derived from 293 FLEX 18 by recombinase-mediated cassette exchange of the retroviral transgene, and have been submitted to clonal selection and amplification, resulting in a producer cell line phenotypically different from 293 FLEX 18 in terms of retroviral vector production yields and response to serum deprivation.

Since previous data showed that lipid metabolism was the main pathway involved on the decrease of retroviral vector production under serum deprivation (Rodrigues et al., 2009) it was hypothesized that differences in the transcriptional profiling of lipid metabolism genes were the cause of the different phenotype displayed by 293 FLEX S11 producer cells. To evaluate this hypothesis gene expression of key lipid metabolic genes was assessed by RT-qPCR in 293 FLEX S11 cells and 293 FLEX 18 cells under normal serum conditions and under serum deprivation conditions. To compare the transcriptional profile of the producer cell lines under the different serum conditions, 293 FLEX 18 cells under normal serum conditions was considered the baseline (**Figure 3.18 B**). The transcriptional profile of 293 FLEX S11 cells under normal serum conditions remained mostly unchanged when these cells were submitted to serum deprivation. Conversely, 293 FLEX 18 cells responded to serum restriction by slightly increasing the expression levels of most of the lipid metabolic genes studied as previously reported (Rodrigues et al., 2012). Comparing the transcriptional profile of lipid metabolic genes in 293 FLEX S11 cells and 293 FLEX 18 cells the most significant differences were the decreased expression of SREBF2 -

sterol regulatory element binding transcription factor 2, FASN - fatty acid synthase and HMGCS - HMG-CoA synthase in 293 FLEX S11 cells. SREBF2 in particular, is a transcription factor that promotes the expression of all genes of cholesterol biosynthesis pathway (Sakakura et al., 2001). In 293 FLEX 18, overexpression of this transcription factor resulted in a dose-responsive response of retroviral vector titer recovery under serum deprivation, even reaching at certain point improved retroviral vector titers. The fact that 293 FLEX S11 showed substantially decreased expression of SREBF2, as well as some other lipid metabolism genes, and yet did not displayed retroviral vector titer drops suggests that there may be other factors to consider when comparing 293 FLEX S11 and 293 FLEX 18 response to serum deprivation. Hence, further studies are required to evaluate the role of the differences between the transcriptional profile of these producer cell lines identified in this work as well as approaches to assess other factors that might cause the different phenotypes observed.

The results of this work allowed to corroborate some of the data from previous work and provide new data for the understanding of the mechanisms underlying viral vector production particularly those related with glutathione metabolism. The insight this thesis provides in these pathways may prove valuable for genetic manipulation of producer cell lines in the future, ideally to overcome the challenges faced by the current production systems of viral vector for gene therapy. In addition, this thesis contributed with the implementation and characterization of two valuable tools – an inducible gene expression system for genetic engineering and a method for universal titration of lentiviral vectors – that can be used in the continuation of this work or in new research projects.

5. References

Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., … Anderson, W. F. (1995). T Lymphocyte-Directed Gene Therapy for ADA− SCID: Initial Trial Results After 4 Years. *Science*, *270*(5235).

Bodin, L., Beaune, P. H., & Loriot, M.-A. (2005). Determination of Cytochrome P450 2D6 (CYP2D6) Gene Copy Number by Real-Time Quantitative PCR. *Journal of Biomedicine and Biotechnology*, *2005*(3), 248–253.

Booth, C., Gaspar, H. B., & Thrasher, A. J. (2016). Treating Immunodeficiency through HSC Gene Therapy. *Trends in Molecular Medicine*, *22*(4), 317–327.

Cabantous, S., Terwilliger, T. C., & Waldo, G. S. (2005). Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nature Biotechnology*, *23*(1), 102–107.

Carrondo, M. J. T., Merten, O.-W., Haury, M., Alves, P. M., & Coroadinha, A. S. (2008). Impact of Retroviral Vector Components Stoichiometry on Packaging Cell Lines: Effects on Productivity and Vector Quality. *Human Gene Therapy*, *19*(2), 199–210.

Chong, H., & Vile, R. G. (1996). Replication-competent retrovirus produced by a “split-function” third generation amphotropic packaging cell line. *Gene Therapy*, *3*(7), 624–9.

Coffin, J. M., Hughes, S. H., & Varmus, H. E. (1997). *Retroviruses*. *Retroviruses*. Cold Spring Harbor Laboratory Press.

Corish, P., & Tyler-Smith, C. (1999). Attenuation of green fluorescent protein half-life in mammalian cells. *Protein Engineering*, *12*(12), 1035–40.

Coroadinha, A. S., Alves, P. M., Santos, S. S., Cruz, P. E., Merten, O.-W., & Carrondo, M. J. T. (2006). Retrovirus producer cell line metabolism: implications on viral productivity. *Applied Microbiology and Biotechnology*, *72*(6), 1125–1135.

Coroadinha, A. S., Gama-Norton, L., Amaral, A. I., Hauser, H., Alves, P. M., & Cruz, P. E. (2010). Production of retroviral vectors: review. *Current Gene Therapy*, *10*(6), 456–73.

Cosset, F. L., Takeuchi, Y., Battini, J. L., Weiss, R. A., & Collins, M. K. (1995). High-titer packaging cells producing recombinant retroviruses resistant to human serum. *Journal of Virology*, *69*(12), 7430–6.

Doxycycline - PubChem. (n.d.). Retrieved September 18, 2017, from https://pubchem.ncbi.nlm.nih.gov/compound/doxycycline#section=Top

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., & Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *Journal of Virology*, *72*(11), 8463–71.

Edelstein, M. (2017). Jounal of Gene Medicine Clinical Trial Site. Retrieved September 10, 2017, from http://www.wiley.com//legacy/wileychi/genmed/clinical/

Fliesler, N. (2011). A new start for gene therapy for “bubble boy” disease: First U.S.-treated patient doing well. Retrieved September 10, 2017, from https://vector.childrenshospital.org/2011/05/a-new-start-for-gene-therapy-for-bubble-boy-disease-first-u-s-treated-patient-doing-well/

Gordon, E. M., & Hall, F. L. (2010). Rexin-G, a targeted genetic medicine for cancer. *Expert Opinion on Biological Therapy*, *10*(5), 819–832.

Greig, S. L. (2016). Talimogene Laherparepvec: First Global Approval. *Drugs*, *76*(1), 147–154.

Ignoul, S., & Eggermont, J. (2005). CBS domains: structure, function, and pathology in human proteins. *American Journal of Physiology - Cell Physiology*, *289*(53), C1369–C1378.

Ignowski, J. M., & Schaffer, D. V. (2004). Kinetic analysis and modeling of firefly luciferase as a quantitative reporter gene in live mammalian cells. *Biotechnology and Bioengineering*, *86*(7), 827–834.

Khan, K. H. (2013). Gene expression in Mammalian cells and its applications. *Advanced Pharmaceutical Bulletin*, *3*(2), 257–63.

Kozak, M. (1987). Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. *Molecular and Cellular Biology*, *7*(10), 3438–45.

Kozak, M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. *Gene*, *299*(1–2), 1–34.

Liang, M. (2012). Clinical development of oncolytic viruses in China. *Current Pharmaceutical Biotechnology*, *13*(9), 1852–7.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408.

Maetzig, T., Galla, M., Baum, C., & Schambach, A. (2011). Gammaretroviral Vectors: Biology, Technology and Application. *Viruses*, *3*(12), 677–713.

Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Bartholomae, C. C., Ranzani, M., … Naldini, L. (2009). The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *The Journal of Clinical Investigation*, *119*(4), 964–75.

Moran, N. (2012). First gene therapy nears landmark European market authorization. *Nature Biotechnology*, *30*(9), 807–809.

Moullan, N., Mouchiroud, L., Wang, X., Ryu, D., Williams, E. G., Mottis, A., … Auwerx, J. (2015). Tetracyclines disturb mitochondrial function across eukaryotic models: A call for caution in biomedical research. *Cell Reports*, *10*(10), 1681–1691.

Mountain, A. (2000). Gene therapy: the first decade. *Trends in Biotechnology*, *18*(3), 119–28.

Oliveira, A., Rodrigues, A., Alves, P., & Coroadinha, A. (2016). Poster presentation *Improving gene therapy vector production by engineering glutathione metabolism*. Florence, Italy: European society of gene and cell therapy.

Pearson, S., Jia, H., & Kandachi, K. (2004). China approves first gene therapy. *Nature Biotechnology*, *22*(1), 3–4.

Quinonez, R., & Sutton, R. E. (2002). Lentiviral Vectors for Gene Delivery into Cells. *DNA and Cell Biology*, *21*(12), 937–951.

Rigg, R. J., Chen, J., Dando, J. S., Forestell, S. P., Plavec, I., & Böhnlein, E. (1996). A Novel Human Amphotropic Packaging Cell Line: High Titer, Complement Resistance, and Improved Safety. *Virology*, *218*(1), 290–295.

Rodrigues, A. F. (2013). PhD thesis: *Integrated functional genomics for improved manufacture of recombinant enveloped virus - Transcriptional profiling and metabolic engineering of retroviral producer cells*. Universidade Nova de Lisboa.

Rodrigues, A. F., Amaral, A. I., Veríssimo, V., Alves, P. M., & Coroadinha, A. S. (2012). Adaptation of retrovirus producer cells to serum deprivation: Implications in lipid biosynthesis and vector production. *Biotechnology and Bioengineering*, *109*(5), 1269–1279.

Rodrigues, A. F., Carmo, M., Alves, P. M., & Coroadinha, A. S. (2009). Retroviral vector production under serum deprivation: The role of lipids. *Biotechnology and Bioengineering*, *104*(6), 1171–1181.

Rodrigues, A. F., Carrondo, M. J. T., Alves, P. M., & Coroadinha, A. S. (2014). Cellular targets for improved manufacturing of virus-based biopharmaceuticals in animal cells. *Trends in Biotechnology*, *32*(12), 602–607.

Rodrigues, A. F., Formas-Oliveira, A. S., Bandeira, V. S., Alves, P. M., Hu, W. S., & Coroadinha, A. S. (2013). Metabolic pathways recruited in the production of a recombinant enveloped virus: Mining targets for process and cell engineering. *Metabolic Engineering*, *20*, 131–145.

Rodrigues, A. F., Formas-Oliveira, A. S., Guerreiro, M. R., Tomás, H. A., Alves, P. M., & Coroadinha, A. S. (2015). Single-step cloning-screening method: a new tool for developing and studying high-titer viral vector producer cells. *Gene Therapy*, *22*(9), 685–695.

Rodrigues, A., M., P., & Coroadinh, A. (2011). Production of Retroviral and Lentiviral Gene Therapy Vectors: Challenges in the Manufacturing of Lipid Enveloped Virus. *Viral Gene Therapy*.

Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., … Anderson, W. F. (1990). Gene Transfer into Humans — Immunotherapy of Patients with Advanced Melanoma, Using Tumor-Infiltrating Lymphocytes Modified by Retroviral Gene Transduction. *New England Journal of Medicine*, *323*(9), 570–578.

Sakakura, Y., Shimano, H., Sone, H., Takahashi, A., Inoue, K., Toyoshima, H., … Yamada, N. (2001). Sterol Regulatory Element-Binding Proteins Induce an Entire Pathway of Cholesterol Synthesis. *Biochemical and Biophysical Research Communications*, *286*(1), 176–183.

Schweizer, M., & Merten, O.-W. (2010). Large-scale production means for the manufacturing of lentiviral vectors. *Current Gene Therapy*, *10*(6), 474–86.

Sheridan, C. (2017). First approval in sight for Novartis' CAR-T therapy after panel vote. *Nature Biotechnology*, *35*(8), 691–693.

Stacey, G. N., & Merten, O.-W. (2011). Host Cells and Cell Banking. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 737, pp. 45–88).

Thomas, C. E., Ehrhardt, A., & Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nature Reviews Genetics*, *4*(5), 346–358.

Urlinger, S., Baron, U., Thellmann, M., Hasan, M. T., Bujard, H., & Hillen, W. (2000). Exploring the

sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(14), 7963–8.

Visiongain. (2016). *Gene therapy R&D and revenue forecasts 2016-2026 Prospects for Drugs Treating Cancer, Cardiovascular Disorders, Eye Conditions, Rare Diseases and Other Needs*.

Vodicka, M. A. (2001). Determinants for lentiviral infection of non-dividing cells. *Somatic Cell and Molecular Genetics*, *26*(1–6), 35–49.

Wirth, T., Parker, N., & Ylä-Herttuala, S. (2013). History of gene therapy. *Gene*, *525*(2), 162–169.

Wold, W. S. M., & Toth, K. (2013). Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. *Current Gene Therapy*, *13*(6), 421–33.

Zhu, Z., Ma, B., Homer, R. J., Zheng, T., & Elias, J. A. (2001). Use of the Tetracycline-controlled Transcriptional Silencer (tTS) to Eliminate Transgene Leak in Inducible Overexpression Transgenic Mice. *Journal of Biological Chemistry*, *276*(27), 25222–25229.

Annexes

Table A.1 - Primers and templates for plasmids construction

Construct	Insert			Vector	
	Fragment	Source	Primers	Parental	Primers or Restriction Enzymes
pInducible eGFP	SV40 rtTA3 Spacer	pLenti CMV rtTA3 Blast	F - aacctgcagcccaagatgtctagactggac	pLenti CMVtight eGFP Puro - Inverted PCR	F1 - atcgattaattaaacctgcagcccaagctt
					R1 - actatcgtcttgagtccaacccggta
			R - gtcatggtaagcttgctagcggtggccggctgggccgca		F2 - actcaagacgatagttaccggataa
					R2 - gtttaattaatcgattacccggtagaattcc
pInducible mCherry	mCherry	pRRLSIN CMV mCherry	F - tcgtcgactagtccaccatggtgagcaagg	pInducible eGFP	*BstXI*
			R - tcgagcggccgccacttacttgtacagctc		
pInducible CBS	CBS	pRRLSIN CBS	F - tcgtcgactagtccaccatgccttctgaga		
			R - tcgagcggccgccacttacttctggtcccgctc		
pInducible Luciferase	Luciferase	pGL4.13 luc2/SV40	F - tcgtcgactagtccaccatggaagatgcca		
			R - tcgagcggccgccacttacacggcgatcttgcc		
pInducible CTHc	CTHc	pDONR CTH	F - tcgtcgactagtccaccatgcaggaaaaag		
			R - tcgagcggccgccacttatcagctgtgacttcc		
pInducible CTHt	CTHt	pRRLSIN CTH	F - tcgtcgactagtccaccatggacctccagg		
			R - tcgagcggccgccacttagggtgtgctgccttc		
pInducible IDH1	IDH1	pRRLSIN IDH1	F - tcgtcgactagtccaccatgtccaaaaaaa		
			R - tcgagcggccgccacttactaaagtttggcctg		
pInducible IDH2	IDH2	pRRLSIN IDH2	F - tcgtcgactagtccaccatggccggctacc		
			R - tcgagcggccgccacttacaactgcctgcccag		
pInducible GSS	GSS	pRRLSIN GSS	F - tcgtcgactagtccaccatggccaccaact		
			R - tcgagcggccgccacttatacagggtatgggtt		
pInducible GSR	GSR	pRRLSIN GSR	F - tcgtcgactagtccaccatggccctgctgc		
			R - tcgagcggccgccacttaacgaagtgtgaccagctcttctgaa		
pInducible GSTM1	GSTM1	pRRLSIN GSTM1	F - tcgtcgactagtccaccatgcccatgatac		
			R - tcgagcggccgccacttacttgttgccccagac		
pInducible G6PD	G6PD	pRRLISN G6PD	F - tcgtcgactagtccaccatggcagagcagg		
			R - tcgagcggccgccacttagagcttgtgggggtt		
pInducible GPX7	GPX7	pRRLSIN GPX7	F - tcgtcgactagtccaccatggtggcggcg		
			R - tcgagcggccgccacttataagtcttctcgctt		

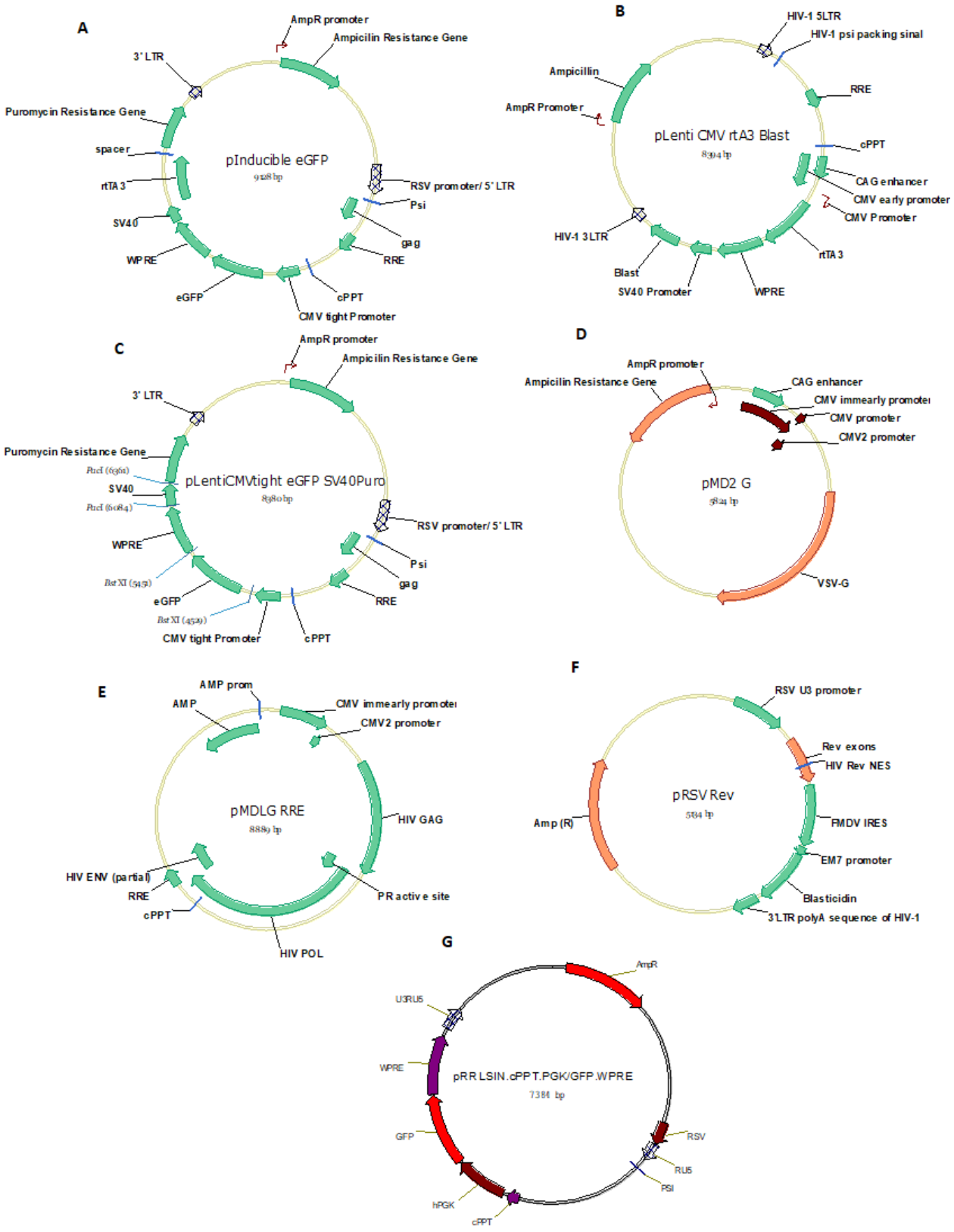


Figure A.1 - Main transcriptional units and features of plasmid constructs used in this thesis. Plasmid with inducible expression system coding for GFP and rtTA3 from which all other *pInducible* constructs were cloned **(A)**. Plasmid coding for rtTA3 **(B)**.Plasmid coding for GFP under the control of a CMVtight promoter **(C)**. Plasmid coding for VSV-G **(D)**. Plasmid coding for HIV-1 Gag-pol **(E)**. Plasmid coding for Rev **(F)**. Plasmid coding for GFP under the control of a hPGK promoter **(G)**.

Table A.2 - Primers for RT-qPCR.

Primer application	Target / Gene	Orientation	5' to 3' sequence
Universal LV Titration method	HIV-1 long terminar repeat (LTR)	Forward	GCTAACTAGGGAACCCAC
		Reverse	GCTAGAGATTTTCCACACTGA
Gene expression - Inducible system components	eGFP	Forward	CAGAAGAACGGCATCAAGGT
		Reverse	CTGGGTGCTCAGGTAGTGG
	rtTA3	Forward	GGAAACTCGCTCAAAAGCTG
		Reverse	CGATGTGAGAGGAGAGCACA
Gene expression and copy number - Retroviral vector components	Transgene (LacZ)	Forward	ACTATCCCGACCGCCTTACT
		Reverse	TAGCGGCTGATGTTGAACTG
	Gag-pol	Forward	GTCCACTATCGCCAGTTGCT
		Reverse	CTGGGTCCTCAGGGTCATAA
	Envelope (GaLV)	Forward	GGACCAAAATAGCGAATGGA
		Reverse	GGTGAACTGTACGCCTGGAT
Gene expression - Lipid Metabolis	ATP citrate synthase (ACLY)	Forward	TGTAACAGAGCCAGGAACCC
		Reverse	CTGTACCCCAGTGGCTGTTT
	Acetyl-CoA carboxylase (ACACA)	Forward	CTCAGTCATGGTGCTGCTGT
		Reverse	CCTGCCACTCTTGCTTTAGG
	Fatty acid synthase (FASN)	Forward	GCACCAACTCCATGTTTGG
		Reverse	TGGAGATCACATGCGGTTTA
	HMG-CoA reductase (HMGCR)	Forward	ACAATAAGATCTGTGGTTGGAATTATGA
		Reverse	GCTATGCATCGTGTTATTGTCAGAA
	3-hydroxy-3-methylglutaryl-CoA synthase 1, cytosolic (HMGCS1)	Forward	CCATTGAAGAGGCTTCTGGT
		Reverse	CTGCCCCTATTCTTCCCTTC
	3-hydroxy-3-methylglutaryl-CoA synthase 2, mitochondrial (HMGCS2)	Forward	GCTTCTCCCCGTGAATCATA
		Reverse	ACCATAAGCCCAGGACAGTG
	Acetyl-CoA acetyltransferase 2 (ACAT2)	Forward	AAAAGCAGGTTGGTCACTGG
		Reverse	CGACTTCTGCCCATTCTCTC
	Sterol regulatory element binding transcription factor 2 (SREBF2)	Forward	TGGCTTCTCTCCCTTACTCCA
		Reverse	GAGAGGCACAGGAAGGTGAG
	Sterol regulatory element binding transcription factor 1 (SREBF1)	Forward	TGCATTTTCTGACACGCTTC
		Reverse	CCAAGCTGTACAGGCTCTCC
	Lanosterol synthase (LSS)	Forward	TCCCGGACTATCTCTGGATG
		Reverse	ACCTGTGAGACCTCAGGA
	Mevalonate kinase (MVK)	Forward	AGGTGGACCAAGGAGGATTT
		Reverse	GGTCAGCAGGATCTGGAGA
Gene expression - Glutathione metabolism	Cystathionine-beta-synthase (CBS)	Forward	TCATCGTGATGCCAGAGAAG
		Reverse	TTGGGGATTTCGTTCTTCAG
	Cystathionine gamma-lyase (CTH)	Forward	ATCCACAGCATGAGTTGGTG
		Reverse	CTCAGCAAGGCTTTCGAATC
	Glutathione S-transferase mu 1 (GSTM1)	Forward	TCGTGTGGACATTTTGGAGA
		Reverse	GGGCTCAAATATACGGTGGA
	Glutathione synthetase (GSS)	Forward	CAGCGTGCCATAGAGAATGA
		Reverse	GACGTGCTTCCCAATTCTGT
	Glutathione peroxidase 7 (GPX7)	Forward	TTCACAGACCAGCACTACCG
		Reverse	GTCTGGGCCAGGTACTTGAA
	Glutathione-disulfide reductase (GSR)	Forward	CAGTGGGACTCACGGAAGAT
		Reverse	AAACCCTGCAGCATTTCATC
	Glucose-6-phosphate dehydrogenase (G6PD)	Forward	GAGGCCGTGTACACCAAGAT
		Reverse	AGCAGTGGGGTGAAAATACG
	Isocitrate dehydrogenase (NADP(+)) 1, cytosolic (IDH1)	Forward	GCTTCATCTGGGCCTGTAAA
		Reverse	GCTTTGCTCTGTGGGCTAAC
	Isocitrate dehydrogenase (NADP(+)) 2, mitochondrial (IDH2)	Forward	TGGCTCAGGTCCTCAAGTCT
		Reverse	CTCAGCCTCAATCGTCTTCC
Reference gene	Ribosomal protein L22 (RPL22)	Forward	CTGCCAATTTTGAGCAGTTT
		Reverse	CTTTGCTGTTAGCAACTACGC