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Quest for the cure: towards improving hematopoietic stem cell based lentiviral gene therapy

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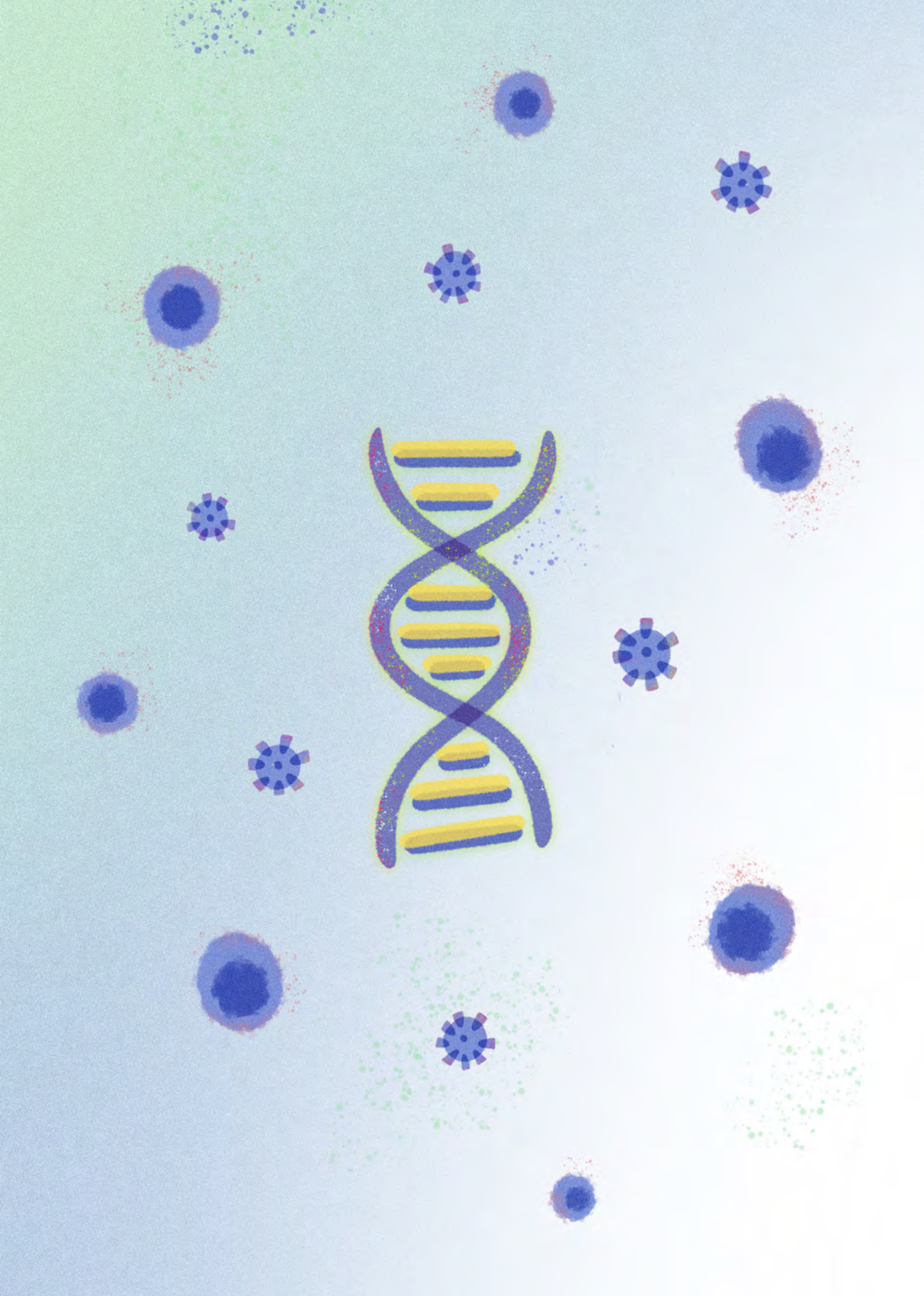

Chapter 1

General Introduction

Gene Therapy

Genes make up who we are biologically, and carry hereditary information codes that determine functions within our body. Genes can mutate and faulty ones can be passed to offspring and cause genetic diseases like hemophilia or cancer. Gene therapy offers hope to cure inherited genetic disorders as well as acquired ones. Gene therapy refers to introduction of genetic material either by adding a new copy of a gene or correcting the defective genes in target cells for therapeutic purposes. The early concept of introducing an exogenous DNA into the cell, goes back to 1970s when recombinant DNA technology offered the possibility of gene modification[1]. Although it was not only till the 1980s that the first attempts to deliver genes into mouse cells using viral vectors were made[2-4]. These steps helped the field in finding its momentum and paved the way for further development of gene therapy as we know it today.

The first authorized gene therapy was conducted in the 1990s, to treat a form of severe combined immunodeficiency (SCID), with a defect gene encoding adenosine deaminase (ADA)[5]. SCID is a rare genetic disorder that impairs immune system, making the patient highly susceptible to infection, due to lack of T cell development in thymus. In this trial, peripheral T lymphocytes of the patient were used for gene therapy. Although, the survival of corrected T cells were demonstrated, it is difficult to evaluate the clinical benefit of this trial, as the patient has continued to receive enzymatic replacement therapy [6]. One of the reason that SCID was selected as a target for gene therapy in initial trials is due to intriguing evidences of successful allogeneic hematopoietic stem cell transplantation to restore immune system in SCID patients [7,8].

Subsequently, European scientists achieved success in curing two patients with X1-linked SCID, with defect in *IL2RG* gene, using corrected hematopoietic stem cells (HSCs) from bone marrow, reporting the first successful gene therapy[9]. X1-linked SCID is recognized by an early block in T and natural killer (NK) lymphocyte differentiation, due to signaling impairment for development, growth and activation of lymphoid progenitors [10-12].

Despite the initial promising result of the first trial, later trials experienced some setbacks as some patients developed Leukemia due to the integration of retro viral vectors used for gene delivery in proto-oncogenes [13,14], which raised safety and efficacy concerns about gene therapy, leading to increased regulatory monitoring and developing safe and more efficient vectors for gene delivery [15,16].

Nevertheless, despite these setbacks, gene therapy continued to progress and over the years has evolved into different approaches, mainly *in vivo* and *ex vivo* based gene therapy depending on the target disease and delivery method. *In vivo* gene therapy is based on direct delivery of therapeutic genes using for instance adeno-associated virus (AAV) to the patient's body through injection. While *ex vivo* based gene therapy (autologous gene therapy) involves manipulation of patient's cells using approaches such as lentiviral vectors (LVs), or CRISPR cas9 gene editing *in vitro* and infusing cells back to the patient.

Over the years, gene therapy has shown remarkable success in treating different disorders. In 2017, approval of some gene therapy products such as Luxturna -an Adeno-associated virus (AAV) based gene therapy product for inherited retinal dystrophy [17,18], and also Kymriah- a CAR-T cell modification for acute lymphoblastic leukemia (ALL)[19,20]- by FDA, marked a significant milestone in the field and established new paths forward for the treatment of other diseases.

Gene therapy is an evolving field, and the safety and efficacy of gene therapy products need to be carefully evaluated in pre-clinical studies and clinical trials according to the established guidelines by FDA and EMA, as the long term safety and efficacy of gene therapy is not clear.

Moreover, on the technical front, one of the main bottleneck in the development of gene and cell therapy is the high cost of the products, mainly due to complexity involved in manufacturing of viral vectors in substantial scale. As a result, the large-scale production becomes challenging at a reasonable cost, which can in the end limit accessibility of patients to gene therapy products.

Yet, gene therapy faces several challenges. This field is a highly regulated, with a lengthy and costly approval process of a gene therapy product. The development of a new product requires thorough testing and clinical trials that can take years to complete. Not to mention, different regulatory frameworks in different countries, making it challenging to develop and market gene therapy globally [21].

In the remainder of this thesis I will focus on hematopoietic stem cell based gene therapy and challenges around *ex vivo* expansion of hematopoietic stem cells, gene delivery efficiency and large scale production of lentiviral vectors.

Hematopoietic stem cell-based gene therapy

Hematopoietic stem cells (HSCs) are a rare population of cells residing in bone marrow, supporting the lifelong haematopoiesis of blood system, through the unique ability of both self-renewal and differentiation. A genetic mutation in HSCs could result in a wide range of diseases, either by directly affecting the HSC

functionality or its differentiated progeny. Correction of mutations in HSCs can be curative for many haematological diseases.

Allogenic HSC transplantation (HSCT) has been widely applied using HSCs from healthy donor for transplantation into a patient, to reconstitute healthy haematopoiesis. However, HSCT is limited by the availability of a matching donor. Moreover, there are immunological barriers with allogeneic transplantation such as graft versus host disease (GvHD), and the need of immunosuppression after transplantation [22]. In the last two decades, the autologous HSC based gene therapy has become an attractive alternative for allogeneic transplantation for many disorders, including primary immunodeficiencies, haematological disorders and even metabolic diseases. Autologous HSC gene therapy, whereas the patient's modified HSCs are the source for transplantation, can overcome the above mentioned limitations of allogenic transplantation.

The correct gene is transferred into the patient's hematopoietic stem and progenitor cells (HSPCs) *in vitro* using integrating viral vector or CRISPR editing systems, and modified cells reinfuse to the patient (Figure 1) [23]. HSC-based gene therapy for various disorders is growing rapidly as novel gene editing approaches are evolving, however this field faces some challenges.

HSCs are a rare population that can be isolated from different sources such as cord blood, bone marrow or mobilized peripheral blood. *Ex vivo* expansion of functional HSCs for genetic modification has been challenging, which limits the ability to maintain and modify of true HSCs. Furthermore, efficient delivery of therapeutic genes into HSCs is often a barrier in the field. Viral vectors have been used commonly to deliver the genes into HSCs, even though large-scale production of viral vectors can be cumbersome and costly.

As novel gene editing technologies matures, there are substantial interests in HSC based gene therapies. Thus, improvement in *ex vivo* expansion of HSCs in order to maintain the HSC functionality, increasing efficiency of therapeutic gene delivery into HSCs, as well as cost efficient viral vectors are needed in order to extend accessibility of HSC based gene therapy to the patients.

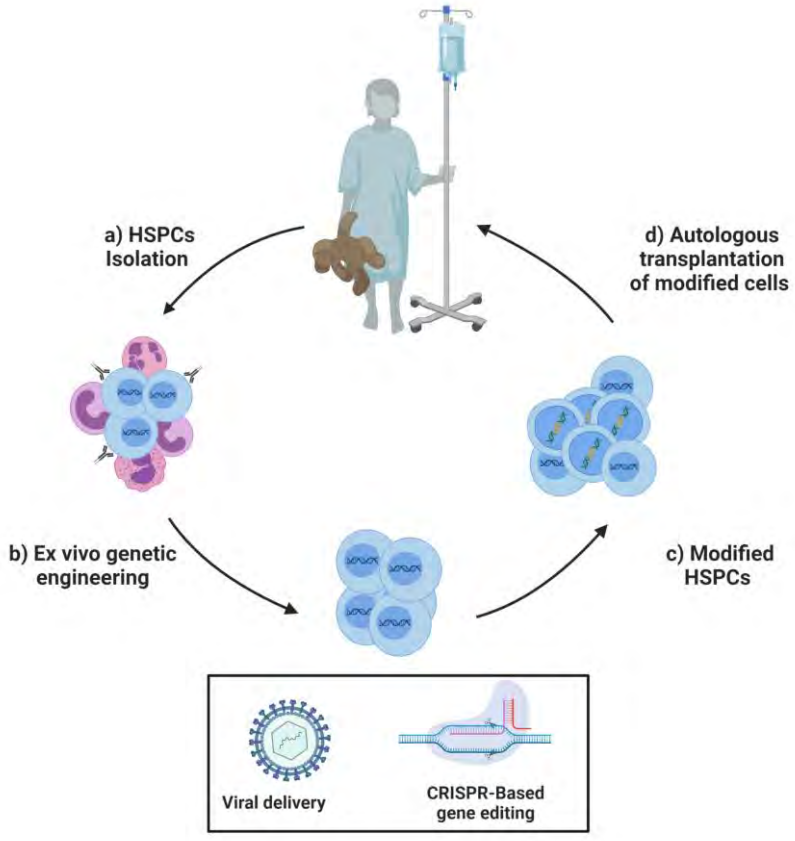

Figure 1. Overview of Hematopoietic Stem cell-based gene therapy. a) Isolation of hematopoietic stem and progenitor cells (HSPCs) from the patient, which contains long term HSCs. B) *Ex vivo* gene modification of HSPCs using viral vector delivery or CRISRP based tools. c) culturing and harvesting modified cells *in vitro*. d) Transplantation of genetically modified cells back into the patient, which eventually results in reconstitution of healthy hematopoietic system. Created by Biorender.com

Ex vivo expansion of hematopoietic stem cells

Hematopoietic stem cells are the target cells for HSC based gene therapy as their curative potential has been proved by allogeneic HSC transplantation. Utilizing HSPCs for gene therapy and gene modification requires *ex vivo* culturing and manipulation of true HSCs, however, *ex vivo* expansion of HSCs has been the holy grail in the field for many years. For decades, combinations of different cytokines have been used in clinical setting to support expansion of lineage committed progenitors but also of HSCs. Although, most attempts for *ex vivo* expansion using

commonly used cytokines have been unsuccessful due to loss of true HSCs and expansion of the lineage committed cells [24].

HSPCs can be enriched using $CD34^+$ surface marker, which consist of a very heterogenous population containing of different lineages progenitors and small percentage of true HSCs. Combinations of different surface markers are used to identify each population *in vitro* and *in vivo,* although some of the markers are not stable during *ex vivo* culture such as CD38. therefore, identification of reliable markers, such as CD201, is essential for proper characterization of true HSCs *in vitro* [25,26].

Furthermore, recent advances in single cell technologies helped with a better understanding of hematopoietic stem cell biology and their development stages [27]. Single cell RNA sequencing findings have reshaped the notion of HSC hierarchy and heterogeneity, proposing novel definition of "continuum of low-primed undifferentiated hematopoietic stem and progenitor cells-" defined as "CLOUD-HSCs" challenging the classical view of HSC hierarchy and gradual differentiation [28-30].

High-throughput analysis have helped the field progressing in identifying small molecules for expansion of hematopoietic stem cells while preserving their stemness *in vitro* such as UM171 [31], StemRegenin1 [32] and BET inhibitors like CPI203 [33]. In Chapter 2 the importance of *ex vivo* expansion of HSCs in gene therapy has been extensively discussed. We, also assessed the influence of interleukin 3 (IL3) on expansion of HSCs in murine transplantation setting in Chapter 3. Furthermore In Chapter 4 we demonstrated the novel culture conditions for expansion of hematopoietic stem cells and improvement of LV based gene modification and gene editing.

Gene therapy using viral vectors

In nature, many viruses have evolved to infect cells and integrate their genetic material into host's genomes to replicate. As a result, viral vectors have been used widely for delivering therapeutic genes to cells. Retroviruses have been an attractive vehicle for gene delivery, as they integrate the viral genes into the host's genome by leveraging host's cells replication machinery [34]. Retroviral' s long terminal repeats (LTRs) possess a strong promoter enhancer activity to drive transgene expression. Initially, gamma retroviruses (gRVs)-derived vectors were deployed for SCID gene therapy trials. gRV integration into the genome is dependent of breakdown of nuclear envelope, which requires cell divisions. Therefore, gRVs show limited efficiency of gene transferring into HSCs, due to quiescent nature and infrequent cell

division of these cells. Consequently, the transduction of HSCs using gRV requires culture of cell to induce cell cycling, which leads to loss of stemness. Furthermore, use of gRVs for gene delivery possess the risk of adverse events associated with insertional mutagenesis in or near proto-oncogenes. In early clinical trials for X-linked SCID four patients from French center and one patient from English trial developed leukemia couple of years after treatment [35-37]. Also, similar adverse effects were reported for other immunodeficiencies like X-linked chronic granulomatous disease (X-CGD)[38] and Wiskott-Aldrich syndrome (WAS) [39]. These occurrences accelerated the development a new generation of vectors for more efficient and safer gene delivery into HSCs [40,41].

Self-Inactivating (SIN)Lentiviral based system

HIV-derived lentiviral vectors were developed and became the vector of choice due to their ability to integrate efficiently into both non-dividing and dividing cells, and sustain long term expression[42],with favorable insertion site pattern compared to γ-RVs [43].

Different lentiviral systems have been developed for safety consideration due to HIV-1 pathogenicity. Crucial virulence factors such as *Vpr, Vpu, tat* and *Net* have been removed, left only essential elements encoding the structural proteins and enzymes for generating viral particles. For safety concerns, this system use the split genome design instead of packaging all the elements in one vector to reduce the risk of replication competent lentiviruses (RCLs) generation during viral production (Figure 2) [44]. Moreover, in order to reduce the risk of proto-oncogene activation due to insertional mutagenesis caused by 3' LTRs promoter/enhancer in gRVs as well as non-SIN LVs, SIN-LVs were developed. LTRs are DNA sequences at end of viral genes, which are important for driving gene expression and facilitating integration into the host's genome. In SIN vectors, the potential transactivation activity on neighboring genes is diminished by modifying 3' LTRs region. As a result of this diminished promoter/enhancer activity, an internal promoter is needed to drive the expression of the transgene [45]. Collectively, these developments in the third generation of SIN-LVs significantly improved their safety and efficacy in gene therapy. They have been successfully used in a large number of preclinical and clinical studies for treatment of different genetic disorders[46,47].

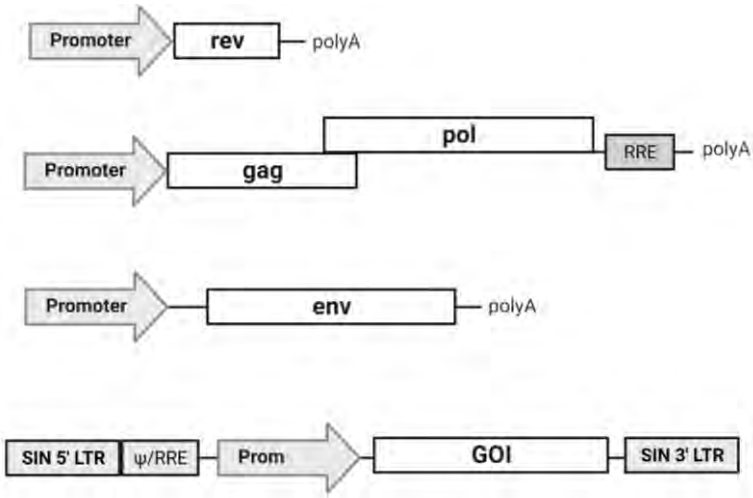

Figure 2. Schematic representation of third generation lentiviral vector system. Crucial elements for lentiviral packaging have been divided over four plasmids for safety reasons. SIN vectors which carry the gene of interest (GOI) with modified 3' and 5' long terminal repeats (LTRs) were developed to reduce transactivation of neighbouring genes. Created by Biorender.com

Lentiviral production system

The predominant method employed to date for lentivirus vector production involves co-transfection of packaging genes (*gag/pol*, *rev*, envelope protein) and transfer vector transiently in a packaging cell line (Figure 3a). Transient transfection saves time, allows expression of cytotoxic viral genes and with most method provides titer of 10^7 to 10^9 TU/ml [48]. Various factors including the quality of DNA, the transfection method, the packaging cell line and the size of transfer vector, can determine transfection efficiency.

However, the transient production of LVs has some challenges including, inadequate scalability for large-scale production, the high cost of production associated with good manufacturing practice (GMP) of plasmids, the potential plasmid contamination in harvested. virus, and optimizing transfection condition. As a result, LV production is primarily restricted to transient small-scale production using cell factories with titer yield ranging from 10^9 to 10^{11} TU/ml, sufficient enough to treat only one or few patients, which limits the reproducibility across patients in large clinical trials. Extensive optimization of large-scale production of LVs for clinical trials such as using suspension cell culture, different packaging cell lines have been done. However, the main bottleneck in large-scale production of LVs is large amount plasmid DNA, which makes the process extremely costly.

Stable producer cell lines can provide affordable system compared to transient system for LV production in industrial setting.

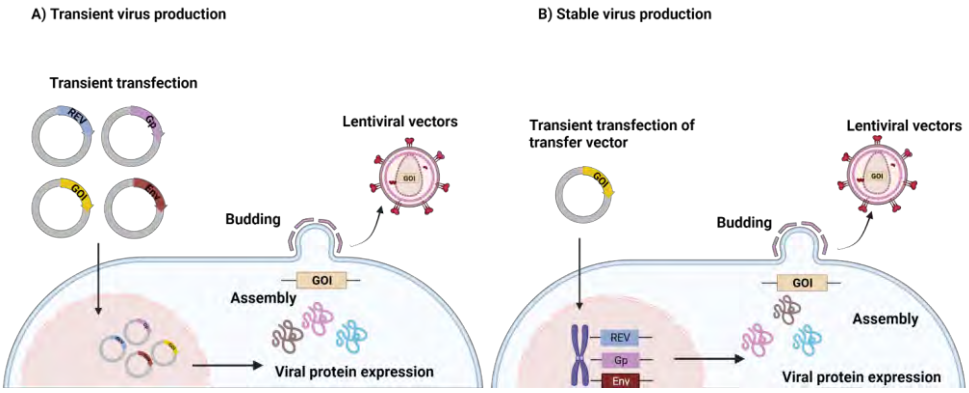

Figure 3. Transient vs stable lentiviral production. In transient system (A), viral vectors are produced by co-transfection of four plasmids using packaging cell line, while in stable production system (B), packaging elements of Rev, gag/pol(Gp) and envelop are expressed stably in packaging cell line, and only transfer vector is introduced transiently. Created by Biorender.com

Generation of stable producer cell lines

To overcome the limitations associated with transient production, stable LV producer cell lines have become a preferred choice, as they offer lower production cost and improved reproducibility among different batches, which are currently the main limitations of transient system for large-scale production of LVs. Since the 90s, extensive efforts have been employed to generate stable cell lines that express viral proteins constitutively, applying different strategies to introduce genes and utilizing different pseudo-typed envelopes to overcome cytotoxicity associated with vesicular stomatitis virus G glycoprotein (VSV-G) a commonly used envelope for LV production (Table 1). A lentiviral packaging cell line (PCL) that stably expresses the packaging elements of *gag/pol*, *rev* and/or envelope protein, can be used to produce viral particles upon transient introduction of the transfer vector. The STAR packaging cell line was generated using murine leukemia virus (MLV) to introduced viral elements, and nontoxic envelope of RD114 was employed. Nevertheless, this cell line constrained from clinical setting due to use of non-SIN MLVs for cell line generation. RD2.Molpack was generated using baculo-AAV transduction and employing RD114 envelope. Although, safety concerns were arouse for this cell line due to co-expression of *rev* and *gagpol* from the same plasmid and use of non- SIN

virus to introduced viral genes for cell line generation. However, these concerns were corrected in RD3.Molpack cell line [49,50].

Although, nontoxic envelopes have been used for generation of cell lines, VSV-G has more advantage for clinical settings due to its broad tropism and FDA approval for LV based gene therapy [51].

The ideal packaging cell line should be able to produce a high titer of functional viral particles and stably express the packaging genes over different generations (Figure 3b). However, low viral titer and reduced stability due to cytotoxicity associated with *gag/pol* and the commonly used envelope protein of *VSV-G* result in limited utilization of such cell lines.

Inducible systems, such as Tet-on and Tet-off systems have been mainly used to control the expression of *gag/pol* and *VSV-G* through addition or removal of the tetracycline/doxycycline in the culture medium. Alternatively, non-toxic envelopes have also been used for generation of constitutive packaging system of LV production.

Nonetheless, stable cell line generation using a transfection system poses some challenges such as low integration efficiency, low expression of the transgene,silencing of transgene expression and limitation in cell metabo-lism. In Chapter 5, we described our method for generation of stable cell line using safe harbor loci targeting via CRISPR/cas9 to ensure stable pro-duction of LVs during large scale production.

CRISPR based gene editing

CRISPR-cas9 system provides a powerful platform for gene correction specially in monogenic diseases. Ongoing clinical trials for treating genetic blood disorders of sickle cell disease and β-thalassemia using CRISPR-cas9 in patients HSPCs showing promising results [59]. CRISPR-cas9 system, is based on guide RNA (gRNA) a complementary sequence to the target DNA and Cas9 an endonuclease that creates a double strand break on the target site of DNA, which inducing the activation of DNA repair pathways of non-homologous end joining (NHEJ) and homology- directed repair (HDR). HDR pathway is essential for the accurate DNA repair and insertion of a correct DNA sequence at the target site, therefore high frequency of HDR- mediated gene insertion is required. Recent successes of HDR mediated gene insertion in HSPCs has been reported [60,61]. Although, some challenges ranging from culturing of the cells, cas9 and DNA template delivery and efficiency of gene correction specially in the true HSC fraction need to be addressed.

Table 1. Overview of published stable producer cell lines for lentivirus production.

PCL name	Year	Envelope	Method of expression	Titer (TU/ml)	Reference
STAR	2003	RD114	MLV based transduction Constitutive codon optimize *gag-pol*, *RD114pro*	10^7	[52]
293SF.pacLV	2008	VSVG	Inducible cumate and Tet-on to control *Rev* and *VSVG*	10^7	[53]
GPRG	2009	VSVG	Inducible Tet-off to control *Rev* and *VSVG*	10^7	[54]
RD2.Mol-pack	2013	RD114-TR	Constitutive expression of *Rev* and *gagpol, and RD114*	10^6	[50]
WinPack-RD	2015	RD114-PR	Constitutive expression of *Rev* and codon optimized *gagpol*	10^7	[55]
RD3.Mol-pack	2016	RD114-TR	LV transduction of envelope	10^6	[56]
LentiPro	2018	4070A	Constitutive expression of *Rev* and mutated *gagpol* (T26S)	10^6 (per day)	[57]
EuLV	2021	VSVG	Inducible	10^7	[58]

To achieve successful treatment of blood disorders, long term correction of true HSCs and progenitors are necessary. Hence, tailoring culture condition to facilitate gene editing in HSCs while preserving long term HSCs is essential for *ex vivo* gene editing field. HDR-mediated gene editing is cell cycle dependent, therefore in quiescent HSCs this pathway is restricted [62,63]. Several strategies such as using cell cycle modulator to increase HDR- mediated editing by inducing cell cycling in HSCs have been explored [64]. Despite the high level of gene editing *in vitro*, relatively modest outcomes in animal transplantation receiving *ex vivo* edited HSCs have been reported [65].

Thesis outline

In this thesis, we aim to tackle several key challenges in HSC-based gene therapy. Our primary objectives include the development of culture protocols for the *ex vivo* expansion of HSCs and the improvement of LV transduction efficiency in HSCs to facilitate gene therapy applications. Additionally, we seek to create a novel packaging cell line for LV production to reduce the high costs associated with gene therapy product manufacturing.

Chapter 2 of this thesis provides an overview of the challenges associated with the *ex vivo* expansion of HSPCs. This chapter highlights the significance of optimizing HSPCs' culture conditions for clinical applications, setting the foundation for our research in Chapter 3 and Chapter 4.

Chapter 3 explores our findings regarding the detrimental effects of IL3 on HSC expansion in a murine transplantation setting. Our results suggest that IL3 should be excluded from clinical protocols. as inclusion of IL3 in culture conditions resulted in reduced engraftment and repopulating capacity of HSCs, even though IL3 supported the proliferation and expansion of $CD34^+$ cells, but not true HSCs. Furthermore, in Chapter 4, we report on novel culture conditions for the *ex vivo* expansion of HSPCs for gene therapy applications. Our research reveals that the addition of a small molecule, Quisinostat, an epigenetic regulator, has significantly improved LV transduction efficiency in HSCs and progenitors both *in vitro* and *in vivo.* Moreover, we also have reported the LV transduction leads to prominent loss of HSCs in culture. Chapter 5 addresses the costly and cumbersome nature of LV production. We have successfully generated a packaging cell line for LV production, which serves to reduce production costs, enhance scalability, and ensure reproducibility in LV titer.

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