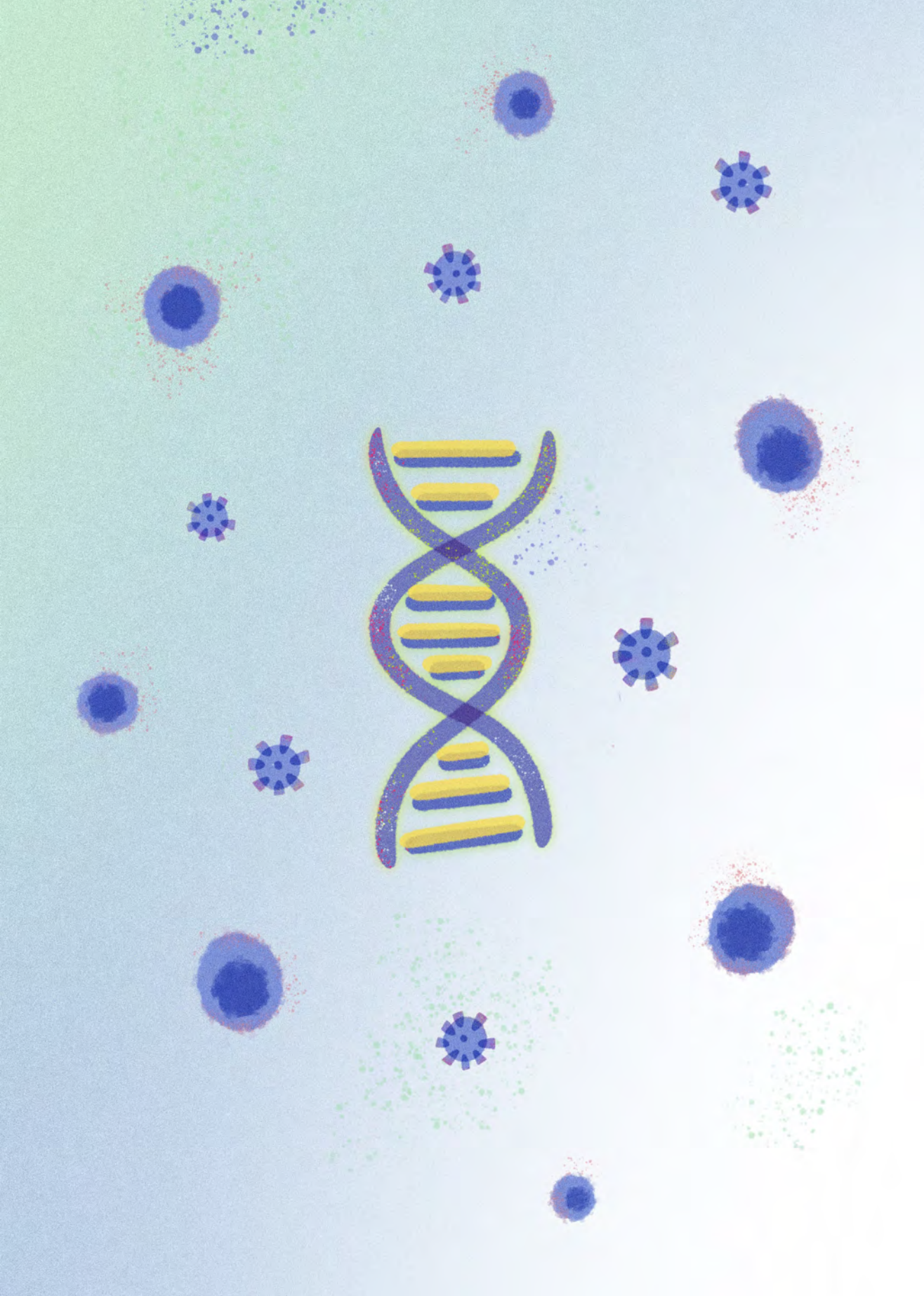
Quest for the cure: towards improving hematopoietic stem cell based lentiviral gene therapy

Tajer, S.P.



Chapter 6

General Discussion and future perspectives

General Discussion

Recent clinical successes have highlighted the transformative potential of HSC gene therapy in the regenerative medicine field. Since the early clinical trials of LV based HSC gene therapy, more than 200 patients have undergone treatment globally for various diseases including combined immune and platelet deficiency Wiskott-Aldrich syndrome (WAS)[1]; chronic granulomatous disorder (CGD)[2], primary immune deficiency (PID)[3,4], thalassemia, and sickle cell disease [5] [6,7]. Available follow up data indicate that the majority of the patients have successfully achieved stable engraftment of corrected *bona fide* HSCs leading to polyclonal hematopoietic reconstitution across all lineages. Furthermore, lack of clonal expansion and insertional mutagenesis supports the low risk for LV genotoxicity. As confidence in safety and efficacy of LV based gene therapy is growing, this platform is transitioning from confined trials in life-threatening conditions to more conditions wherever HSC transplantation is considered as a treatment option. Therefore, the rationale of patient's inclusion criteria has changed to improved safety and higher efficacy over allogeneic HSC transplantation.

The purpose of the studies described in this thesis were to improve HSC based lentiviral gene therapy for better clinical outcome. Therapeutic benefits of HSCs have transformed the gene therapy field. Autologous HSC based gene therapy where the patient's own cells are genetically modified have advantages of fewer immunological barriers of graft-vs host disease (GvHD), donor rejections and donor availability compared to allogenic transplantation. The current framework of *ex vivo* autologous HSC gene therapy is technically challenging. HSCs are a rare population in bone marrow, and they can be obtained through bone marrow aspiration or leukapheresis from peripheral blood after mobilization[8]. Enriched HSCs are genetically manipulated *in vitro*. Although, until recently *ex vivo* maintenance and expansion of true HSCs and efficient gene manipulation remains challenging. To expand our knowledge on HSC biology and their *ex vivo* expansion, we described an overview of advances in *ex vivo* expansion protocols in Chapter 2, which provides the foundation for our studies in this thesis. In Chapter 3 we challenged the currently used culture condition in clinical setting for *in vitro* manipulation, followed by Chapter 4 where we demonstrated improved culture conditions for HSC based lentiviral based gene therapy. To achieve long term correction and rapid engraftment, efficient gene correction in HSCs and early progenitors are essential. We also have showed improved lentiviral transduction efficiency in HSCs and

progenitors *in vitro* and enhanced engraftment of transduced cells *in vivo* upon incorporation of an epigenetic regulator (Chapter 4).

LV based HSC gene therapy is personalized and extremely costly. Lentiviral vectors development and their production per disease contribute to the high costs of gene therapy. Therefore, in Chapter 5 we sought to develop a novel packaging cell line for larger scale lentivirus production to reduce the high cost associated with LV production.

Improved HSCs based gene therapy

Hematopoietic stem cells are a heterogenous population

In the clinical setting, the selection of hematopoietic stem and progenitor cells (HSPCs) is achieved by the enriching stem/progenitor cells expressing CD34. $CD34^+$ cells are heterogenous pool containing different committed progenitors and only a small percentage of long-term HSCs. Hence, detailed identification of $CD34^+$ cells upon *ex vivo* manipulation would provide us essential information. In Chapter 3 and Chapter 4, we showed that changes in $CD34^+$ cells after expansion and transduction do not necessarily correspond to similar changes in true HSCs.

Moreover, enriching true HSCs for gene therapy has been proposed by different groups as a strategy to reduce the cost of gene therapy and increase safety. Refined $CD34^+CD90^+CD45RA^-$ cells for treating beta-hemoglobinopathies, resulted in substantial long-term engraftment, comparable to the entire $CD34^+$ cell population [9,10]. For clinical implementation, advanced purification systems such as CliniMACS Prodigy (Milteny Biotec) or the more complementary system of MACSQuant Tyto (Miltenyi Biotech) provide closed- systems for portable cell sorting in HSC gene therapy.

IL3 has adverse effect on HSC self-renewal

To work towards optimization of *in vitro* culture systems in clinical settings, in Chapter 3 we have challenged the inclusion of Interleukin 3 (IL3) in culture protocol. Based on early findings, combinations of hematopoietic cytokine of Stem cell factor (SCF), Flt3 ligand (FLT3), Thrombopoietin (TPO) and Interleukin 3 (IL3) have been established as crucial factors for supporting HSC and progenitor proliferation and maintenance during *in vitro* culturing, particularly in clinical applications [11,12] . However, these studies often overlooked the clinical implications of cytokine combinations on expansion and engraftment of $CD34^+$ progenitor cells, relying mainly on *in vitro* assays such as colony forming assays or long-term culture initiating cell assays to assess the functionality of LT-HSCs.

IL3 is known to support myelopoiesis and proliferation of lineage-committed progenitors in culture [13,14]. In Chapter 3 we assessed the effect of IL3 on maintenance of *in vivo* repopulating capacity of *ex vivo* expanded human $CD34^+$ cells from cord blood. Primary and secondary transplantations in NSG mice revealed that inclusion of IL3 in *in vitro* culture condition failed to preserve self-renewal and repopulation capacity of HSCs, indicating the negative effect of IL3 on the maintenance of LT-HSCs in culture. Besides, data from secondary transplantations indicated the detrimental effect of IL3 on long-term development of the lymphoid lineage (B, T, NK cells) in bone marrow, peripheral blood, spleen and thymus.

Inclusion of IL3 in a clinical protocol for expansion of $CD34^+$ for gene therapy purposes results in an increase of total $CD34^+$ cells; however, the quality of LT-HSCs is compromised in favour of the expansion of myeloid lineage-committed progenitors This observation also underscores the crucial need of a detailed identification of different subsets in clinical applications. Relying solely on $CD34^+$ frequency and counts does not necessarily align with the frequency of functional HSC which are essential cells for better gene therapy outcome.

Epigenetic regulation in HSC fate decision and their application for LV based HSC gene therapy

Hematopoiesis is a dynamic process where self-renewal and differentiation are orchestrated through a network of transcription factors, genes and signaling pathways[15,16]. Epigenetic regulation is an essential intrinsic cue for normal hematopoiesis and cell fate decisions under physiological conditions[17]. This regulation maintains the HSC pool through regulation of essential transcription factors[18,19] Changes in chromatin accessibility can alter gene expression in hematopoietic progenitors and result in disbalanced mature output [20].

Given the importance of chromatin accessibility in HSC fate decisions, and building upon previous studies[21,22,23] using various epigenetic regulators for *ex vivo* expansion of hematopoietic stem cells, we investigated the impact of incorporating epigenetic regulators in currently used protocol for HSC expansion for LV based gene therapy in Chapter 4.

We explored the potential advantages of Quisinostat- an experimental drug designed for cancer therapy- and previously described small molecule CPI203 [21]. We have shown both CPI203 and Quisinostat increase phenotypic HSCs in culture

substantially, followed by better engraftment and reconstitution of different lineages. Interestingly, we have observed, that addition of CPI203 and Quisinostat results in improved development of lymphoid and erythroid lineages without compromising other lineages, a benefit that would be valuable for patients with impaired immune system and red blood cell disorders, respectively.

Quisinostat, an HDACi targeting HDAC I and II, has been used in several clinical trials for haematological malignancies. Recent research suggests that Quisinostat inhibits cancer cell self-renewal by re-establishing expression of histone linker H1.0 [24]. H1 is highly conserved and involved in embryonic stem cell differentiation and mammalian development, although the role of H1 linker in HSCs development and fate decisions needs further investigation. Meanwhile, our studies demonstrated that Quisinostat increases H3K27 acetylation level in HSCs and progenitors, a modification believed to decrease rapidly upon *ex vivo* expansion of HSCs [25]. Higher H3K27 acetylation enhances global chromatin accessibility and hematopoietic stem cell activity[26]. To reveal the impact of Quisinostat on chromatin landscape of expanded HSCs and gain a better understanding of its role in HSC regulation, accessibility assay for transposase-accessible chromatin with sequencing (ATAC-Seq) can be employed.

The small molecule of CPI203 is a bromodomain and extra-terminal motif inhibitor (BETi). BET proteins interact with acetylated lysins at transcription start sites which regulates transcription through RNA polymerase II complex formation. BETi regulates transcription by blocking BET protein binding to histones. Therapeutic benefits of BETis have been investigated in several cancer trials, however, their clinical progression have been challenging and none have received regulatory approval [27].

Bromodomain proteins are known to regulate mouse HSC self-renewal and stemness and CPI203, a BETi, was identified through screening of small molecules where, it showed significant *ex-vivo* expansion of HSC and enhanced repopulation capacity of HSCs in bone morrow [21]. In Chapter 4 we also have corroborated these previously reported effects.

We also have further investigated the effect of Quisinostat and CPI203 in LV transduction context, which I'm going to discuss further in the coming sections.

Improved Lentiviral transduction efficiency

Often LV based clinical applications are limited by suboptimal transduction efficiency especially in true HSCs, due to their dormant state[28]. Various transduction enhancers (TEs) such as Prostaglandin E_2 (PGE2) [29], Cyclosporin H [30] and poloxamer F108 (LentiBOOST) [31] have been reported to improve transduction efficiency *in vitro* and *in vivo.* TEs can enhance the transduction efficiency by increasing cell entry, affecting intracellular processes (e.g., PGE2 , Cyclosporin H). Maximizing the transduction efficiency of LVs requires less virus per patient while ensuring sufficient transduction and transgene expression.

In **Chapter 4**, we assessed the efficiency of our suggested HSC expansion culture conditions using Quisinostat and CPI203 for LV based gene therapy settings. We demonstrated that the addition of Quisinostat to the culture significantly increased LV transduction efficiency, not only in total cells but more importantly in HSCs, compared to other culture conditions. This observed increase in transduction efficiency could be explained by an elevated acetylation level, leading to more transcriptionally active and accessible chromatin upon introduction of Quisinostat. However, no changes in LV transduction efficiency were observed in the presence of CPI203.

Furthermore, we observed that Quisinostat expanded cells showed improved engraftment of transduced cells in mouse studies, thus ensuring long term correction of HSCs. this should also reduce the costs associated with viral vectors, since less lentiviral virus would be required to achieve a desired vector copy number.

Given the encouraging results, the effect of CPI203 and Quisinostat need to be corroborated on $CD34^+$ from mobilized peripheral blood (mPB) or Bone marrow, as they are the cell sources used in clinical HSC gene therapy.

Enhancing gene edition in HSPCs

Gene editing has emerged as a potent tool for correcting monogenic diseases. The application of CRISPR-Cas9 in hematopoietic stem and progenitor cells (HSPCs) has exhibited significant promise in preclinical studies, demonstrating proof-of-principle for treating haematological disorders. Ongoing clinical trials utilizing these tools highlight their potential. Nevertheless, crucial challenges persist, including the need to enhance delivery, targeting efficiency and safety for primitive, long-term repopulating HSPCs.

The CRISPR-Cas9 platform is based on a guide RNA (gRNA) that binds to the target DNA sequence, paired with a Cas9 endonuclease. Upon binding to the DNA, Cas9 induces a double-strand break, activating two primary endogenous repair pathways: non-homologous end-joining (NHEJ) and homology-directed repair (HDR). HDR facilitates precise repair using homologous sequences to the region around the break site. This process inserts an accurate DNA sequence at the target site, making it applicable for treating diseases where correcting or introducing a genetic element could yield therapeutic benefits, resembling the gene addition approach in viral gene therapy. Repair pathways are cell cycle dependent, HDR occurs during the S and G_2 phases where cells are in proliferative state, while NHEJ happens more abundantly in G_1[32]. Increasing HDR through chemical inhibition of NHEJ has been used as a strategy to increase gene editing efficiency [33]. NHEJ is essential for cell survival, therefore, NHEJ inhibition imposes cytotoxicity and reduced cell survival. Optimized culture conditions where stemness of HSCs are maintained, while activating S/G_2 phases could improve efficiency of gene editing and reducing off target events.

Efficient gene editing also relies on the competent delivery of Cas9, gRNA, and DNA template into target cells. Nucleic acid delivery into HSCs is challenging due to their quiescent nature and low metabolic activity. Adeno-associated virus (AAV) and integrative deficient LVs (IDLV) are commonly used to deliver the DNA template to HSCs, while Cas9 and gRNA are transferred via nucleofection. However, HSCs are highly sensitive to environmental and mechanical stresses, where they can induce stress responses and interferon responses leading to the loss of HSCs and reduced viability upon nucleofection. The iTOP technology presents a promising solution by delivering recombinant Cas9 and gRNA as a Ribonucleoprotein (RNP) via macropinocytosis induction[34], potentially maintaining HSC viability and improving the efficiency of gene editing. Further investigation into the suggested culture conditions in **Chapter 4** can be beneficial for improving gene editing outcomes.

Immunity to gene therapy products

Gene manipulation in HSCs via different platforms can trigger both innate and adaptive immune responses presenting challenges for efficient gene correction in *ex vivo* gene therapies. Considering that existing and emerging gene transfer and editing technologies inevitably expose HSPCs to external nucleic acids, mainly in the form of viral vectors, the efficiency of genetic manipulation in HSPCs is significantly influenced by host antiviral factors and nucleic acid sensors.

Under physiological condition HSCs identify and respond to external pathogens or internal molecular damages through various pattern recognition receptors (PRRs) [35] including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and DNA sensors like the cGAS–STING pathway. Human $CD34^+$ cells express various TLRs to detect infections and induce innate immune response immediately [36].

Lentiviruses have become as a vector of choice in clinical settings, offer safer and more efficient transgene delivery to HSCs compared to g-retroviral vectors that were used in initial gene therapy trials. Even though lentiviruses can evade from innate immune response [37], they still can be detected through TLRs [36], activation of kinase ATM and triggering p53 signaling followed by robust induction of Interferon (IFN) responses [37]. Upon viral infection under physiological conditions, IFN-α induction leads to proliferation as a response to clear infections [38]. Moreover, HSCs are highly resistant to lentiviral transduction compared to other progenitors, mainly due to high expression of restriction factors such as interferon-induced antiviral protein (IFITM1) [39]. Therefore, a better understanding of the innate immune pathways not only upon lentiviral transduction but also in CRISPR gene editing can be beneficial for developing strategies to improve gene editing efficiency in HSCs and better clinical outcome in the patient. In Chapter 4, we have shown that lentivirus transduction has led to a substantial loss of HSCs in cytokine only expanded cells, which is the golden standard in clinical settings. The addition of epigenetic regulators such as Quisinostat and CPI203 helped to maintain HSCs upon LV transduction. The scRNA seq revealed addition of CPI203 has reduced gene expression of *TP53*, *TMEM173*, *IFITM2*, *IFTIM3*, *IF16* which are involved in induction of IFN response. Additional studies are needed to reveal the mechanism of action of Quisinostat on maintenance of HSCs upon transduction. Understanding these immune pathways can enhance gene editing efficiency in HSCs, improving clinical outcomes.

Generation of lentiviral packaging cell line

Lentiviral vectors (LVs) are essential for gene therapy due to their ability to integrate seamlessly into genomes, enabling sustained gene expression. Self-inactivating LVs (SIN-LVs) address safety concerns observed in early gamma-retroviral trials. While SIN-LVs have been successful in clinical trials and approved therapies, large-scale production remains a huge challenge. Current methods of transient transfection, face challenges like scalability, high costs, and plasmid contamination in the final viral batch. Stable producer cell lines offer a potential solution, but issues like

cytotoxicity of packaging genes of *gag/pol* and *VSV-G* and instability hinder their efficiency. Strategies involving inducible systems and alternative envelopes aim to mitigate these challenges. Therefore, in Chapter 5, we have generated a stable packaging cell line, using CRISPR-cas9 platform, integrating key viral components into human genomic safe harbours (GSHs) for enhanced stability and controlled integration. GSHs are genomic sites that accommodate the integration of new genetic material which ensure the functional predictability and genome stability without posing a risk to the host cell. This innovative approach holds promise for addressing current limitations in large-scale LV production, thereby advancing the field of gene therapy.

We generated a cell line where viral components of *REV*, *Gag pol* and *VSV-G* were introduced into three widely used GSHs of chemokine (C-C motif) receptor 5 *(CCR5)*, Adeno-Associated Virus Integration Site 1 *(AAVS*1) and Citrate Lyase Beta-Like *(CLYBL)* respectively. To control cytotoxicity of *Gagpol*, we used a point mutated *gag-pol* (T26S), known for reduced protease activity without compromising LV infectivity and maturation [40].

Regarding the envelope, non-toxic envelopes of *RD114*, *GalV*, *MLV-A4070* have been previously used to generate cell line by different groups [40-42].We have corroborated these envelopes along with *VSV.G* to produce virus, which *VSV.G* envelope resulted in higher titre (data not shown). Despite *VSV.G'*S cytotoxicity, its broad tropism and FDA approval [43] make it clinically attractive. Hence, to regulate *VSV.G* expression in the stable cell line, we implemented a "Tet-off" inducible system, offering the advantage of not requiring the addition of doxycycline /tetracycline, unlike "Tet-on" system where doxycycline /tetracycline is needed to be added to the cell culture to induce expression.

Lentiviruses were produced using our packaging cell line with physical titre of 10^6 VP/ml and functional titre of 10^5 TU/ml in small scale setting. While, higher physical titre of 10^{12} VP/ml, with substantial decrease in functional titre of 10^7 TU/ml were achieved in transient virus production. Transient virus production has led to producing higher empty viral particles, which is caused by transfection with high amount of plasmids.

Different strategies such as knocking out of antiviral responses [44] in HEK293T, or addition of sodium butyrate [40,45] have been reportedly led to increased viral titre during production. We have also investigated the effect valproic acid along with protein kinase R inhibitor (PKR-IN-C16) and PCSK9 which degrades low-density lipoprotein receptor (LDLR), a receptor known to impede lentiviral vector production [44]. Addition of PKR inhibitor and PCKS9 did not improve lentiviral titre, while marginal increase in physical titre was observed in presence of valproic acid (data not shown). Further optimization including improving transfection efficiency, medium optimization, increasing cell density are needed to increase the yield and facilitate scale up production in order to meet the demands.

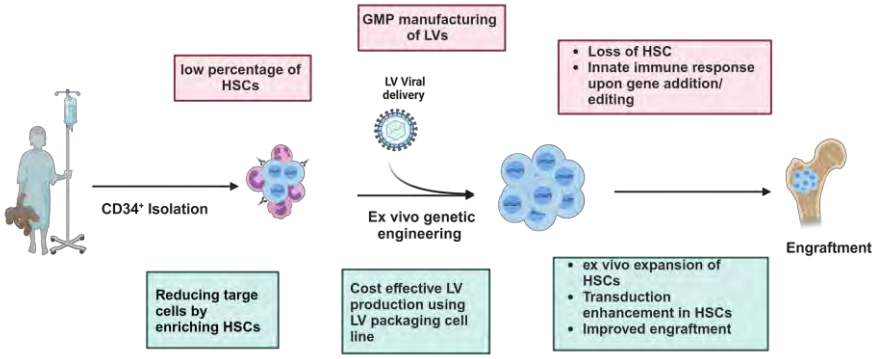


Figure 7. Challenges (in red) and improvements (in green) of LV based HSC gene therapy.

Concluding remarks and Future perspectives

Altogether, the work described in this thesis contributes towards improving and cost-effective HSC based LV gene therapy. The success of early clinical trials has paved the way for further exploration and expansion of this treatment option across a variety of diseases. This thesis focused on improving LV based HSC gene therapy through exploring the effects of specific cytokines and epigenetic regulators on HSC expansion and LV transduction efficiency. The findings highlight the importance of accurately identifying and targeting HSCs for successful gene therapy outcomes.

Additionally, the use of novel techniques such as closed-system cell sorting and portable closed-system applications can potentially increase the availability and portability of HSC gene therapy. Further research in this area will continue to

advance our understanding and application of HSC gene therapy, ultimately leading to improved clinical outcomes for patients.

The existing autologous HSC gene therapy platform is exceptionally personalized but technically challenging, contributing to high costs of gene therapy. While innovative strategies are addressing technical challenges in the field, global market access requires drug development, registration and commercialization. Withdrawal of the gene therapy product Zynteglo for β-thalassemia from European markets due to its substantial cost, approximately $2 million per patient, despite clinical approval demonstrates a case in point.

These high costs can be justified considering gene therapy products as potential one-time treatments offering improved survival and quality of life, and surpassing cumulative expense of conventional therapies.
Given that HSC gene therapies are predominantly developed and tested in high-income countries for diseases more prevalent in low- income countries such as sickle cell disease and haemophilia[46], achieving fair global access is crucial.

Innovative solutions for drug development and an expedited regulatory process are imperative for successful commercialization. Therefore, early collaboration among academia, healthcare providers, industry stakeholders, patient organizations, and global regulatory bodies is crucial to address these challenges and promote global health equity by providing cures for unmet diseases.

.

References

1. Ferrua F, Cicalese MP, Galimberti S, et al. Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. *Lancet Haematol*. May 2019;6(5):e239-e253. doi:10.1016/s2352-3026(19)30021-3
2. Kohn DB, Booth C, Kang EM, et al. Lentiviral gene therapy for X-linked chronic granulomatous disease. *Nat Med*. Feb 2020;26(2):200-206. doi:10.1038/s41591-019-0735-5
3. De Ravin SS, Wu X, Moir S, et al. Lentiviral hematopoietic stem cell gene therapy for X-linked severe combined immunodeficiency. *Sci Transl Med*. Apr 20 2016;8(335):335ra57. doi:10.1126/scitranslmed.aad8856
4. Garcia-Perez L, van Eggermond M, van Roon L, et al. Successful Preclinical Development of Gene Therapy for Recombinase-Activating Gene-1-Deficient SCID. *Mol Ther Methods Clin Dev*. Jun 12 2020;17:666-682. doi:10.1016/j.omtm.2020.03.016
5. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature*. 2010/09/01 2010;467(7313):318-322. doi:10.1038/nature09328
6. Ribeil JA, Hacein-Bey-Abina S, Payen E, et al. Gene Therapy in a Patient with Sickle Cell Disease. *N Engl J Med*. Mar 2 2017;376(9):848-855. doi:10.1056/NEJMoa1609677
7. Marktel S, Scaramuzza S, Cicalese MP, et al. Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ß-thalassemia. *Nat Med*. Feb 2019;25(2):234-241. doi:10.1038/s41591-018-0301-6
8. Ott de Bruin LM, Lankester AC, Staal FJT. Advances in gene therapy for inborn errors of immunity. *Current Opinion in Allergy and Clinical Immunology*. 2023;23(6):467-477. doi:10.1097/aci.0000000000000952
9. Radtke S, Adair JE, Giese MA, et al. A distinct hematopoietic stem cell population for rapid multilineage engraftment in nonhuman primates. *Sci Transl Med*. Nov 1 2017;9(414)doi:10.1126/scitranslmed.aan1145
10. Radtke S, Humbert O, Kiem H-P. Sorting Out the Best: Enriching Hematopoietic Stem Cells for Gene Therapy and Editing. *Molecular Therapy*. 2018/10/03/ 2018;26(10):2328-2329. doi:https://doi.org/10.1016/j.ymthe.2018.08.025
11. Metcalf D. Hematopoietic cytokines. *Blood*. 2008;111(2):485-491. doi:10.1182/blood-2007-03-079681 %J Blood
12. Hofmeister CC, Zhang J, Knight KL, Le P, Stiff PJ. Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. *Bone Marrow Transplantation*. 2007/01/01 2007;39(1):11-23. doi:10.1038/sj.bmt.1705538
13. Suda T, Suda J, Ogawa M, Ihle JN. Permissive role of interleukin 3 (IL-3) in proliferation and differentiation of multipotential hemopoietic progenitors in culture. 1985;124(2):182-190. doi:https://doi.org/10.1002/jcp.1041240203
14. Ihle JN, Keller J, Oroszlan S, et al. Biologic properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, p cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. *Journal of immunology (Baltimore, Md : 1950)*. Jul 1983;131(1):282-7.

15. Zhang CC, Lodish HF. Cytokines regulating hematopoietic stem cell function. *Current Opinion in Hematology*. 2008;15(4):307-311. doi:10.1097/MOH.0b013e3283007db5
16. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *WIREs Systems Biology and Medicine*. 2010;2(6):640-653. doi:https://doi.org/10.1002/wsbm.86
17. Rodrigues CP, Shvedunova M, Akhtar A. Epigenetic Regulators as the Gatekeepers of Hematopoiesis. *Trends Genet*. Oct 21 2020;doi:10.1016/j.tig.2020.09.015
18. Avgustinova A, Benitah SA. Epigenetic control of adult stem cell function. *Nat Rev Mol Cell Biol*. Oct 2016;17(10):643-58. doi:10.1038/nrm.2016.76
19. Goodell MA, Nguyen H, Shroyer N. Somatic stem cell heterogeneity: diversity in the blood, skin and intestinal stem cell compartments. *Nat Rev Mol Cell Biol*. May 2015;16(5):299-309. doi:10.1038/nrm3980
20. Rodrigues CP, Shvedunova M, Akhtar A. Epigenetic Regulators as the Gatekeepers of Hematopoiesis. *Trends in Genetics*. 2021/02/01/ 2021;37(2):125-142. doi:https://doi.org/10.1016/j.tig.2020.09.015
21. Hua P, Hester J, Adigbli G, et al. The BET inhibitor CPI203 promotes ex vivo expansion of cord blood long-term repopulating HSCs and megakaryocytes. *Blood*. 2020/11/19/ 2020;136(21):2410-2415. doi:https://doi.org/10.1182/blood.2020005357
22. Chaurasia P, Gajzer DC, Schaniel C, D'Souza S, Hoffman R. Epigenetic reprogramming induces the expansion of cord blood stem cells. *J Clin Invest*. Jun 2014;124(6):2378-95. doi:10.1172/jci70313
23. Chaurasia P, Gajzer D, Feldman M, Hoffman R. Histone Deacetylase Inhibitors Promote the Ex Vivo Expansion of Cord Blood CD34+ Cells in Serum Free Cultures Accompanied by the Upregulation of Pluripotency Genes. *Blood*. 2012;120(21):345-345. doi:10.1182/blood.V120.21.345.345
24. Morales Torres C, Wu MY, Hobor S, et al. Selective inhibition of cancer cell self-renewal through a Quisinostat-histone H1.0 axis. *Nat Commun*. Apr 14 2020;11(1):1792. doi:10.1038/s41467-020-15615-z
25. Chagraoui J, Girard S, Spinella J-F, et al. UM171 Preserves Epigenetic Marks that Are Reduced in Ex Vivo Culture of Human HSCs via Potentiation of the CLR3-KBTBD4 Complex. *Cell Stem Cell*. 2021/01/07/ 2021;28(1):48-62.e6. doi:https://doi.org/10.1016/j.stem.2020.12.002
26. Cabal-Hierro L, van Galen P, Prado MA, et al. Chromatin accessibility promotes hematopoietic and leukemia stem cell activity. *Nat Commun*. Mar 16 2020;11(1):1406. doi:10.1038/s41467-020-15221-z
ss27. Estoppey D, Schutzius G, Kolter C, et al. Genome-wide CRISPR-Cas9 screens identify mechanisms of BET bromodomain inhibitor sensitivity. *iScience*. Nov 19 2021;24(11):103323. doi:10.1016/j.isci.2021.103323
28. Biasco L, Pellin D, Scala S, et al. In Vivo Tracking of Human Hematopoiesis Reveals Patterns of Clonal Dynamics during Early and Steady-State Reconstitution Phases. *Cell Stem Cell*. 2016/07/07/ 2016;19(1):107-119. doi:https://doi.org/10.1016/j.stem.2016.04.016
29. Heffner GC, Bonner M, Christiansen L, et al. Prostaglandin E2 Increases Lentiviral Vector Transduction Efficiency of Adult Human Hematopoietic Stem and Progenitor Cells. *Molecular Therapy*. 2018/01/03/ 2018;26(1):320-328. doi:https://doi.org/10.1016/j.ymthe.2017.09.025

30. Petrillo C, Thorne LG, Unali G, et al. Cyclosporine H Overcomes Innate Immune Restrictions to Improve Lentiviral Transduction and Gene Editing In Human Hematopoietic Stem Cells. *Cell Stem Cell*. Dec 6 2018;23(6):820-832.e9. doi:10.1016/j.stem.2018.10.008
31. Jang Y, Kim Y-S, Wielgosz MM, et al. Optimizing lentiviral vector transduction of hematopoietic stem cells for gene therapy. *Gene Therapy*. 2020/12/01 2020;27(12):545-556. doi:10.1038/s41434-020-0150-z
32. Mao Z, Bozzella M, Seluanov A, Gorbunova V. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. *Cell Cycle*. Sep 15 2008;7(18):2902-6. doi:10.4161/cc.7.18.6679
33. Schimmel J, Muñoz-Subirana N, Kool H, et al. Modulating mutational outcomes and improving precise gene editing at CRISPR-Cas9-induced breaks by chemical inhibition of end-joining pathways. *Cell Rep*. Feb 28 2023;42(2):112019. doi:10.1016/j.celrep.2023.112019
34. D'Astolfo Diego S, Pagliero Romina J, Pras A, et al. Efficient Intracellular Delivery of Native Proteins. *Cell*. 2015/04/23/ 2015;161(3):674-690. doi:https://doi.org/10.1016/j.cell.2015.03.028
35. Piras F, Kajaste-Rudnitski A. Antiviral immunity and nucleic acid sensing in haematopoietic stem cell gene engineering. *Gene Therapy*. 2021/02/01 2021;28(1):16-28. doi:10.1038/s41434-020-0175-3
36. Nagai Y, Garrett KP, Ohta S, et al. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity*. Jun 2006;24(6):801-812. doi:10.1016/j.immuni.2006.04.008
37. Piras F, Riba M, Petrillo C, et al. Lentiviral vectors escape innate sensing but trigger p53 in human hematopoietic stem and progenitor cells. *EMBO Molecular Medicine*. 2017;9(9):1198-1211. doi:https://doi.org/10.15252/emmm.201707922
38. Passegué E, Ernst P. IFN-alpha wakes up sleeping hematopoietic stem cells. *Nat Med*. Jun 2009;15(6):612-3. doi:10.1038/nm0609-612
39. Wu X, Dao Thi VL, Huang Y, et al. Intrinsic Immunity Shapes Viral Resistance of Stem Cells. *Cell*. 2018/01/25/ 2018;172(3):423-438.e25. doi:https://doi.org/10.1016/j.cell.2017.11.018
40. Tomás HA, Rodrigues AF, Carrondo MJT, Coroadinha AS. LentiPro26: novel stable cell lines for constitutive lentiviral vector production. *Scientific Reports*. 2018/03/27 2018;8(1):5271. doi:10.1038/s41598-018-23593-y
41. Ghani K, Wang X, de Campos-Lima PO, et al. Efficient human hematopoietic cell transduction using RD114- and GALV-pseudotyped retroviral vectors produced in suspension and serum-free media. *Hum Gene Ther*. Sep 2009;20(9):966-74. doi:10.1089/hum.2009.001
42. Sanber KS, Knight SB, Stephen SL, et al. Construction of stable packaging cell lines for clinical lentiviral vector production. *Sci Rep*. Mar 12 2015;5:9021. doi:10.1038/srep09021
43. Manilla P, Rebello T, Afable C, et al. Regulatory considerations for novel gene therapy products: a review of the process leading to the first clinical lentiviral vector. *Hum Gene Ther*. Jan 2005;16(1):17-25. doi:10.1089/hum.2005.16.17
44. Han J, Tam K, Tam C, Hollis RP, Kohn DB. Improved lentiviral vector titers from a multi-gene knockout packaging line. *Molecular Therapy - Oncolytics*. 2021/12/17/ 2021;23:582-592. doi:https://doi.org/10.1016/j.omto.2021.11.012
45. Ansorge S, Lanthier S, Transfiguracion J, Durocher Y, Henry O, Kamen A. Development of a scalable process for high-yield lentiviral vector production by transient

transfection of HEK293 suspension cultures. *J Gene Med*. Oct 2009;11(10):868-76. doi:10.1002/jgm.1370
46. Cornetta K, Bonamino M, Mahlangu J, Mingozzi F, Rangarajan S, Rao J. Gene therapy access: Global challenges, opportunities, and views from Brazil, South Africa, and India. *Mol Ther*. Jun 1 2022;30(6):2122-2129. doi:10.1016/j.ymthe.2022.04.002