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Single-cell dissection of immune system aging and acute myeloid leukemia

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Chapter 3

Utilizing Epigenetic Regulators to Improve HSC-based Lentiviral Gene Therapy

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3.1 Abstract

The curative benefits of autologous and allogeneic transplantation of hematopoietic stem cells (HSCs) have been proven in various diseases. However, the low number of true HSCs that can be collected from patients and the subsequent in vitro maintenance and expansion of true HSCs for genetic correction remain challenging. Addressing this issue, we here focused on optimizing culture conditions to improve ex vivo expansion of true HSCs for gene therapy purposes. In particular, we explored the use of epigenetic regulators to enhance the effectiveness of HSC-based lentiviral (LV) gene therapy. The histone deacetylase inhibitor quisinostat and bromodomain inhibitor CPI203 each promoted ex vivo expansion of functional HSCs, as validated by xenotransplantation assays and single-cell RNA sequencing analysis. We confirmed the stealth effect of LV transduction on the loss of HSC numbers in commonly used culture protocols, whereas the addition of quisinostat or CPI203 improved the expansion of HSCs in transduction protocols. Notably, we demonstrated that the addition of quisinostat improved the LV transduction efficiency of HSCs and early progenitors. Our suggested culture conditions highlight the potential therapeutic effects of epigenetic regulators in HSC biology and their clinical applications to advance HSC-based gene correction.

3.2 Introduction

Hematopoietic stem cells (HSCs) have been used successfully in allogeneic transplantation settings to treat hematological and immunological disorders, as they maintain homeostasis through their unique capacity of self-renewal and multipotency¹⁻³. Over the past two decades autologous HSC-based gene therapy has emerged as a compelling alternative to allogeneic transplantation as it is not dependent on finding a matched donor and avoids any risk of graft-versus-host disease. This has proven successful for several inherited diseases such as immune deficiencies, hematological disorders, but also metabolic diseases through *ex-vivo* manipulation of the patient's own HSPCs⁴⁻⁷. Lentiviral vectors (LVs) have emerged as vectors of choice in clinical settings for safer and more efficient transgene delivery to HSCs compared to γ -retroviral vectors that were used in initial gene therapy trials. Recently, gene editing has emerged as another powerful tool providing a platform for therapeutic correction of monogenic diseases. Proof of concept studies using CRISPR-Cas9 gene editing in hematopoietic stem and progenitor cells (HSPCs) have shown promise to treat hematological disorders⁸⁻¹⁰. Nevertheless, critical challenges such as the efficiency of targeting long-term HSCs, on- and off-target side effects and safety still need to be addressed.

For successful treatment outcome of hematological and immunological disorders, correction of long-term HSCs and progenitors is required to ensure long term correction of the disease¹¹. Both viral gene addition therapy as well as homology-directed recombination (HDR)-based gene editing approaches have shown preferential genetic correction of more proliferating progenitors, rather than quiescent HSCs *in vitro*, possibly due to better chromatin accessibility of relevant loci^{12,13}.

Combinations of hematopoietic cytokines are commonly used in human HSC cultures to promote HSPC expansion for gene therapy purposes¹⁴. Although these cytokines support short-term maintenance of HSCs, they fail to expand functional HSCs, particularly in genetic manipulation settings.

Great effort has been put to develop culture conditions for HSC expansion *in vitro* by incorporation of small molecules such as UM171¹⁵, StemRegening-1¹⁶ or using chemically defined cytokine free medium¹⁷. Recent approaches of using various epigenetic regulators for HSC expansion *in vitro* have been reported. Epigenetic regulation is an essential intrinsic cue for normal haematopoiesis and cell fate decisions under physiological conditions¹⁸. Small molecules targeting chromatin regulating proteins, such as histone deacetylase inhibitors (HDACi), DNA methylating agents, and Bromodomain and Extra-Terminal motif domain inhibitors (BETi) have been successfully used in culturing protocols of human HSCs¹⁹⁻²³. These findings have highlighted the importance of epigenetic regulators as a therapeutic target for HSC expansion and gene therapy purposes.

3.3 Results

CPI203 and Quisinostat support expansion of phenotypic HSCs *in vitro*

In a clinical gene therapy settings, HSPCs often are cultured in serum free medium supplemented with the essential cytokines stem cell factor (SCF), thrombopoietin (TPO), Fms-like tyrosine kinase 3 (FLT3) and Interleukin-3 (IL3)⁵. We have previously showed the adverse effect of IL3 on *ex vivo* HSC expansion and subsequently low engraftment in mice, suggesting exclusion of IL3 from culture protocols¹⁴.

CD34⁺ cells from cord blood were expanded for 4 days in a cytokine cocktail of SCF, TPO and FLT3

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(called STF), with or without addition of Quisinostat (STF+Q) or CPI203 (STF+CPI203) (Figure 1A). Flow cytometry analysis showed that addition of Quisinostat increased the expression of the HSC markers CD34, CD90, and CD201, while low expression of CD38 and CD45RA were maintained in presence of both CPI203 and Quisinostat (Figure 3.1B). This resulted in substantial increase of the frequency and count of $CD34^+CD38^{-/lo}CD90^+CD45RA^{-}CD201^+$ phenotypic HSCs (Figure 3.1C-D); in contrast, STF cytokine cocktail supported expansion of multipotent progenitors (MPPs) and different lineage progenitors after four days (Figure 3.1D-E).

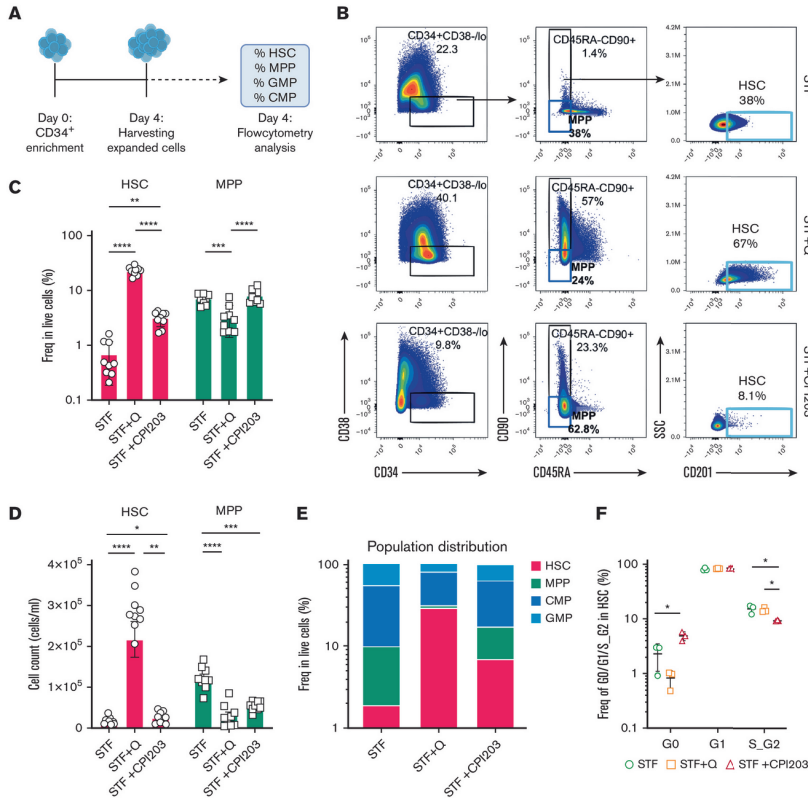


Figure 3.1: CPI203 and quisinostat support ex vivo expansion of HSCs. (A) Schematic experimental design. CD34⁺ cells were enriched from cord blood and cultured in a cytokine-supplemented medium with quisinostat (0.5 nM) or CPI203 (150 nM) for 4 days. (B) Representative gating strategy of expanded HSCs (CD34⁺CD38^{-/lo}CD90⁺CD45RA⁻CD201⁺) and MPPs (CD34⁺CD38⁻CD90⁺CD45RA⁻) in the indicated culture conditions. (C) The graph indicates the frequency of HSC and MPP after 4 days of culture in the indicated culture conditions (n = 9; mean ± standard deviation [SD]). (D) HSC and MPP count after 4 days of culture in the indicated culture conditions (n = 9; mean ± SD). (E) Stacked bar plot showing the composition of HSPCs expanded with only cytokines and in the presence of quisinostat and CPI203. (F) Frequency of cell cycle stages in the expanded HSCs (n = 3; mean ± SD). Statistical significance was determined by a 2-way analysis of variance (ANOVA) test for multiple comparisons: *P < .05; **P < .01; ***P < .001; ****P < .0001. CMP, common myeloid progenitor; GMP, granulocyte monocyte progenitor.

Both HDAC and BET inhibitors have been clinically evaluated as antitumor agents, mainly due to their antiproliferative effect on cancer cells by inducing G0/G1 cycle arrest through P13K-AKT pathway³⁰ and targeting MYC, respectively³¹. In this study, increasing concentrations of Quisinostat reduced the total nucleated count (TNC) but increased the frequency of phenotypic HSCs

(Supplementary Figure 3.1B-C). Addition of CPI203 reduced total cell counts and CD34⁺ numbers after four days of culture (Supplementary Figure 3.1D). This is consistent with the observed significant increase of G0 frequency and a decrease of proliferative states of S/G2 in HSCs, whereas no changes in cell cycle states were observed in the Quisinostat-treated HSCs (Figure 3.1F).

Quisinostat increases LV transduction efficiency *in vitro*

LV-based gene therapy in HSCs has proven to be safe and effective for correction of different blood disorders. Nevertheless, often clinical applications are limited by suboptimal transduction efficiency especially in true HSCs, due to their dormant state.¹¹ Various transduction protocols containing small molecules such as Prostaglandin E₂³², Cyclosporin H¹² and poloxamer F108 (LentiBOOST)³³ have been reported to improve transduction efficiency *in vitro* and *in vivo*.

To assess the efficiency of our suggested HSC expansion culture conditions for LV based gene therapy settings, CD34⁺ were transduced with LV carrying green fluorescent protein (GFP), in presence of either CPI203 or Quisinostat (Figure 3.2A). Both flow cytometry and vector copy number analyses showed that addition of Quisinostat in culture increased the LV transduction efficiency significantly in total cells (Figure 3.2B-C, Supplementary Figure 3.2A). Notably, Quisinostat has more than doubled the transduction efficiency in HSCs (Figure 3.2D-E) and other progenitors in comparison with other culture conditions (Supplementary Figure 3.2B).

HDACs impact gene expression status, by increasing acetylation level, leading to more transcriptionally active and accessible chromatin. Upon the introduction of Quisinostat, we have confirmed that the acetylation level of lysine 27 on Histone 3 (H3K27) in CD34⁺, HSCs, and MPPs has significantly increased (Figure 3.2F). This finding provides a potential explanation for the observed enhanced transduction efficiency.

In vivo repopulating potential of transduced HSPCs from an optimized cell culture

To evaluate the repopulation potential and *in vivo* engraftment capabilities of cultured HSPCs, we preformed xenotransplantation assays, which are the accepted gold standard for assessing multilineage potential and self-renewal of human HSCs, as reliable *in vitro* assays to address stem cell functionality are currently absent³⁴.

We transplanted 5x10⁴ and 2.5x10⁴ expanded cells from the conditions 'STF', 'STF+CPI203' and 'STF+Q' into recipient NSG mice (Figure 3.3A). All mice (5 out of 5) injected with Quisinostat- and CPI203-expanded cells showed signs of human engraftment ($\geq 0.1\%$ of huCD45⁺) at both cell doses, whereas mice that received cells expanded in cytokine cocktail only showed consistent engraftment solely at higher cell doses. Significantly higher human chimerism (huCD45⁺) in BM was detected in Quisinostat and CPI203 culture groups when mice received lower cell dose (Figure 3.3B-C). Given this difference, the subsequent sections will primarily focus on the results from mice receiving the lower cell dose of 25,000 cells. Peripheral blood analysis over time showed higher human chimerism in mice receiving CPI203-cultured cells (Figure 3.3D). Interestingly, transplantation of CPI203-expanded cells also resulted in substantial increased engraftment in the thymus (Thy), whereas no differences were observed in the spleen (Sp) (Figure 3.3E). The frequency of human CD34⁺ was significantly higher in BM in the CPI203-expanded culture group (Figure 3.3F). Distinct multilineage output in different organs demonstrated that both Quisinostat and CPI203 cultured cells yielded improved lymphoid development in BM, with significantly higher frequency of CD19⁺, common lymphoid progenitors (CLPs) and Pro-B cells (Figure 3.3F). Strikingly, cells expanded with CPI203 exhibited enhanced engraftment in thymus comparing to other organs (Supplementary Figure 3.3 A), accompanied by an increased frequency of CD3⁺, CD4⁺CD8⁺, and TCRab⁺ cells (Figure 3.3G).

Results

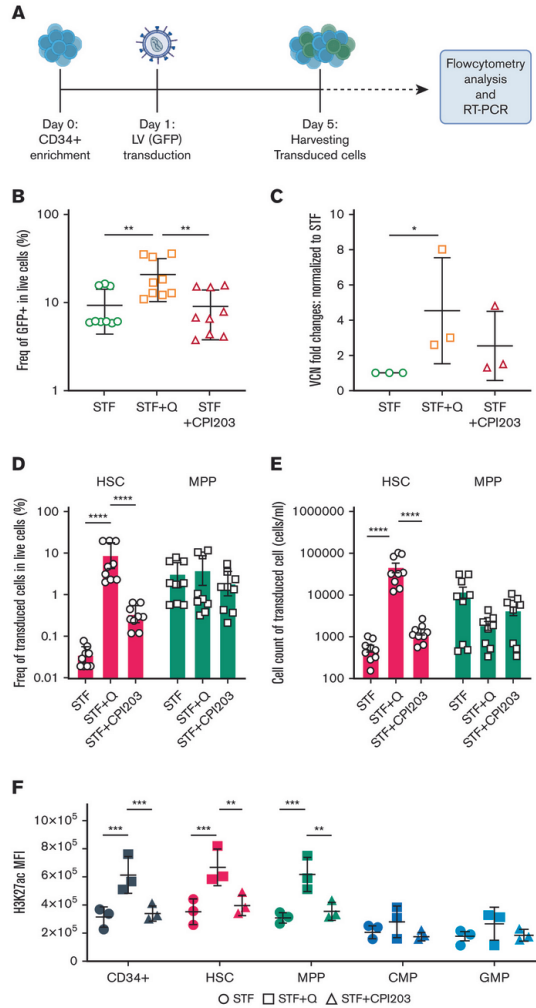


Figure 3.2: **Quisinostat increases LV transduction efficiency.** (A) Schematic experimental design. CD34+ cells were transduced with LV-GFP 24 hours after stimulation in cytokine-supplemented medium alone or the presence of quisinostat (0.5 nM) or CPI203 (150 nM). Cells were harvested for flow cytometry analysis, DNA isolation, and reverse transcription polymerase chain reaction (RT-PCR), 4 days after transduction. (B) Frequency of GFP+ 4 days after transduction in live cells (n = 9; mean $\pm$ SD). (C) VCN was determined by RT-PCR (n = 3; mean $\pm$ SD; *P < .05; unpaired t test). (D) Frequency of transduced HSCs and MPPs in the indicated culture conditions are shown (n = 9; mean $\pm$ SD). (E) Cell counts of transduced HSCs and MPPs (n = 9; mean $\pm$ SD). (F) The plot shows the mean fluorescence intensity of H3K27ac in different populations of expanded cells in the indicated culture conditions (n = 3; mean $\pm$ SD). Statistical significance was determined by 2-way ANOVA test with multiple comparisons: * P < .05; ** P < .01; *** P < .001; **** P < .0001. H3K27ac, acetylation level of lysine 27 on histone 3.

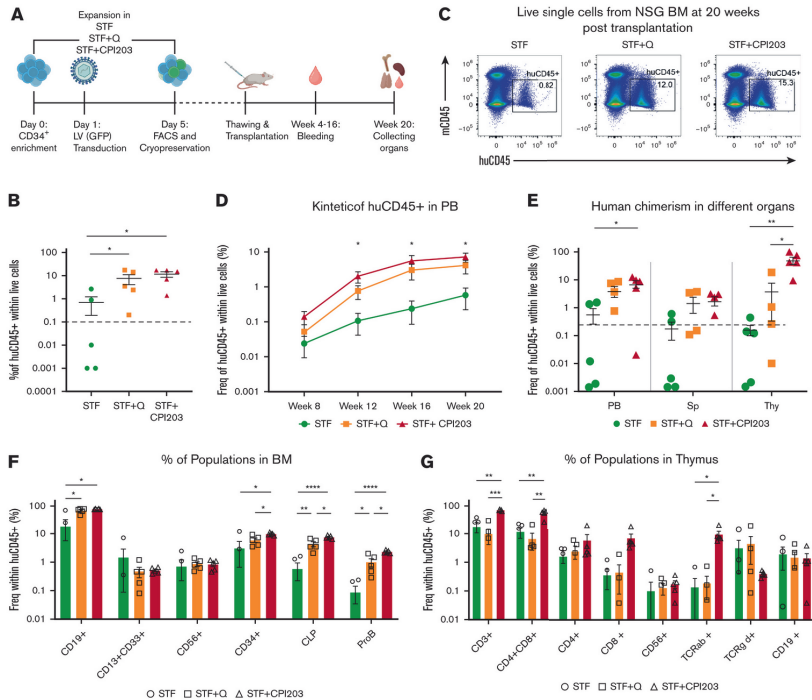


Figure 3.3: CPI203 and quisinostat support expansion of functional HSCs. (A) Schematic overview of primary transplantation. A total of 25 000 and 50 000 nucleated expanded cells in the indicated culture conditions were transplanted per mouse. (B) Human engraftment in the BM of NSG mice at week 20 after transplantation ($n = 5$ mice per group; mean $\pm$ standard error of the mean [SEM]; * $P < .05$). Statistical significance was calculated by 1-way ANOVA, with multiple comparisons. (C) Representative fluorescence-activated cell sorter (FACS) plots of huCD45 in NSG BM 20 weeks after transplantation. (D) Kinetics of chimerism in PB over time ($n = 5$ mice per group; significance of STF vs STF + CPI203 is shown in the plot). (E) Human engraftment in different organs 20 weeks after transplantation ($n = 5$ mice per group). (F) Frequency of different populations in BM ($n = 5$ mice per group). (G) Frequency of different populations in the Thy ($n = 5$ mice per group; mean $\pm$ SEM). Statistical significance was calculated by 2-way ANOVA with multiple comparisons: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

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Subsequently, we examined the frequency of transduced cells (% GFP⁺) in engrafted cells in the BM (Figure 3.4A). In alignment with our *in vitro* results, mice that received Quisinostat-expanded cells, exhibited a greater frequency of transduced huCD45⁺ in BM, PB and spleen, with a marginal increase observed in thymus when compared to other culture conditions (Figure 3.4B). Further analysis has also revealed higher transduction efficiency across various lineages in BM (Figure 3.4C). Secondary transplantation was performed to confirm long-term repopulation capacity of expanded cells in BM (Figure 3.4D-E), and engraftment of transduced cells in BM (Figure 3.4F). The data confirm that addition of CPI203 or Quisinostat maintain HSC self-renewal.

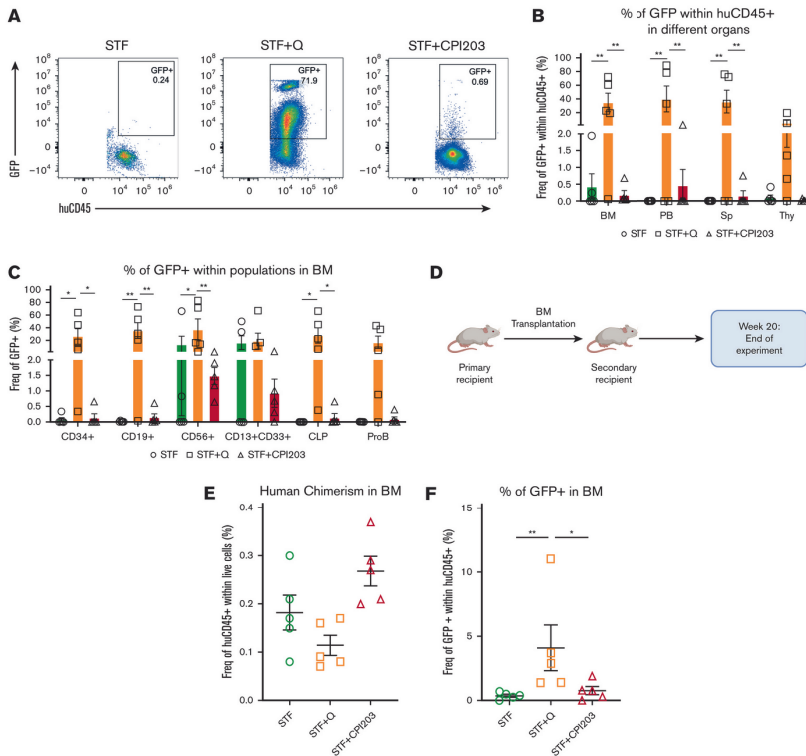


Figure 3.4: Quisinostat supports better engraftment of transduced cells in vivo. (A) Representative FACS plots of BM at 20 weeks after transplantation in primary mice. (B) Frequency of GFP⁺ cells in different organs (n = 5 mice per group; mean ± SEM; **P < .01). Statistical significance was calculated using 2-way ANOVA and multiple comparisons. (C) Frequency of transduced populations within huCD45⁺ in the BM at 20 weeks after transplantation in primary NSG mice (n = 5 mice per group; mean ± SEM; *P < .05, **P < .01). Statistical significance was calculated by 2-way ANOVA and multiple comparisons. (D) Schematic design of the secondary transplantation into NSG mice. (E) Human chimerism in the BM at 20 weeks after transplantation (n = 5 mice per group; mean ± SEM). Statistical significance was calculated using the Mann-Whitney test. (F) Frequency of transduced cells (GFP⁺) in the BM (n = 5 mice per group; mean ± SEM; *P < .05, **P < .01). Statistical significance was calculated using the Mann-Whitney test. CLP, common lymphoid progenitor.

scRNA-seq reveals epigenetic regulators support expansion of HSCs upon LV transduction

To reveal cellular heterogeneity within CD34⁺ cells among different culture conditions, we performed single cell RNA-sequencing (scRNA-seq). Specifically, we set out to compare our culture conditions as well as the effect of LV transduction on the cellular composition of the total CD34⁺

cells.

Enriched CD34⁺ cells (hereafter referred to as unstimulated CD34⁺) were used as a reference control, along with six samples that were cultured and transduced in medium supplemented with cytokines alone, Quisinostat or CPI203 (Figure 3.5A). After manual annotation of unstimulated CD34⁺ cells into multiple different progenitor cell types based on gene expression profiles, all samples were integrated and analyzed using Seurat. We identified 13 major clusters (Figure 3.5B-C) corresponding to several well recognized early differentiation stages of CD34⁺ cells. Based on the marker genes adopted from Dong *et al*²⁷, and top differential expressed genes (DEGs) (Supplementary Table 2), we identified the *bona fide* HSC population as having high expression of *AVP* and *HLF* genes, while lacking lineage specific genes such as *MPO*, *DUTT*, *GATA2*. The populations with lower expression of *HLF* and *AVP* were identified as MPP, as they have been proposed to be early progenitors¹⁷. Furthermore, progenitors of distinct lineages of myeloid, lymphoid, erythroid (Eryth) and megakaryocytic cells (Mks) were identified using their unique gene signatures (Figure 3.5C and Supplementary Figure 3.4A).

Comparing HSPC composition among different culture conditions, a higher frequency of HSCs was observed in culture conditions with CPI203 and Quisinostat compared to the STF condition (Figure 3.5 D-E, and Supplementary Figure 3.4D). In cultures supplemented with Quisinostat and CPI203, expression of HSC signature genes of *HLF*, *RORA* and *HOXA9* was higher compared to cytokine-only (STF) expanded cells, while *AVP* gene expression decreased in CPI203 expanded cells (Supplementary Figure 3.4C). Moreover, consistent with our flow cytometry analysis (Figure 3.1B), Single cell transcriptomics data confirmed higher expression of *CD34*, *THY1* and *PROCR* genes in Quisinostat-expanded cells, while expression of *CD38* gene was decreased (Figure 3.5F).

In accordance with our *in vivo* results, the scRNA-seq data also demonstrated the increase in frequencies of lymphoid multipotent progenitors (LMPPs), B cells, common myeloid progenitors (CMPs) and Mks in CPI203 expanded cells (Figure 3.5E, and Supplementary Figure 3.4D). Collectively, our *in vivo* and *in vitro* results suggest that addition of CPI203 in HSC expansion protocol not only has improved *ex vivo* expansion of HSCs, but has also supported the priming of cells towards the lymphoid lineage. On the other hand, the addition of Quisinostat reduced the frequency of granulocyte monocyte progenitors (GMPs), while increasing erythroid progenitors (MEPs) and myeloid progenitors (CMPs) (Figure 3.5E, and Supplementary Figure 3.4D).

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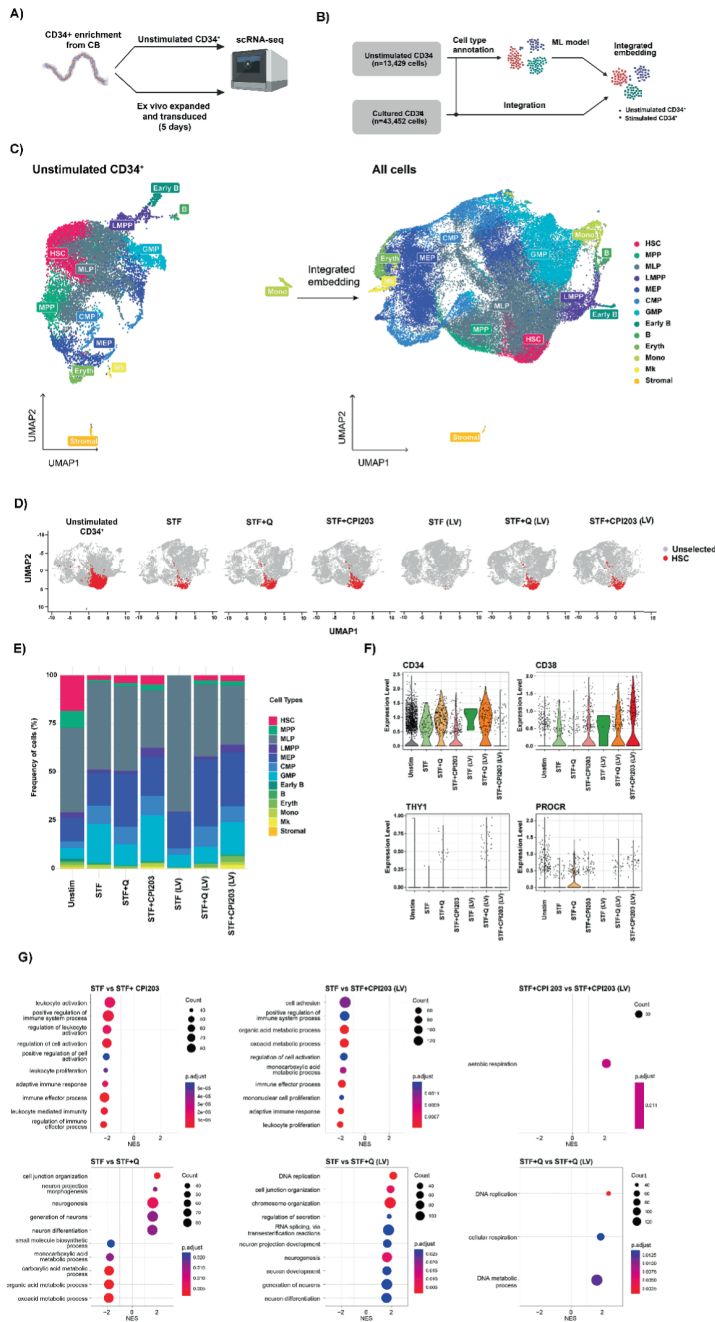


Figure 3.5: **Single-cell transcriptome analysis reveals quisinostat and CPI203 maintain HSCs upon LV transduction in CD34+.** (A) Schematic experimental design. (B) Mock-up schematic of cell-type projection. (C) Uniform manifold approximation and projection for dimension reduction (UMAP) plots of unstimulated CD34+ cells and integrated UMAP for all conditions. (D) UMAP displaying HSCs for each condition. (E) The bar plot represents population distribution. (F) Violin plots displaying the expression levels of selected HSC surface marker genes in the indicated conditions. (G) Gene set enrichment analysis and molecular processes of various conditions interrogated by scRNA-seq. LMPP, lymphoid multipotent progenitor; MEP, megakaryocyte-Eryth progenitor; ML, myeloid-lymphoid progenitor; Mono, monocyte.

Of note, our data showed higher frequency of MEPs, Eryth and Mks upon LV transduction, suggesting LVs could skew cells towards the erythroid lineage in culture conditions containing Quisinostat or CPI203. In contrast, higher frequency of myeloid-lymphoid progenitors (MLPs) have been detected in cytokine-expanded cells (Supplementary Figure 3.4D). This possible lineage skewing upon LV transduction warrants further investigation.

Finally, single cell transcriptomics results showed LV transduction in itself induces cell proliferation (Supplementary Figure 3.4B) but reduces the frequency of the HSC population compared to non-transduced conditions. This decrease is more prominent in STF condition, while inclusion of Quisinostat and CPI203 in the LV transduction protocol has helped to maintain HSCs (Figure 3.5D, and Supplementary Figure 3.4D). LV transduction induces innate immune response via indirect activation of interferon (IFN) responses through different pathways such as Toll-like receptors (TLRs)³⁵, DNA damage response³⁶ or DNA sensor responses³⁷. We have detected elevated gene expression related to the interferon response and DNA damage response, such as *TP53*, *IFITM2*, *IFITM3*, *IFI16*, *TMEM173*, *DDB2* in expanded cells. However, addition of CPI203 results in a reduction of their expression. Additionally, no changes in expression of TLRs was observed (Supplementary Figure 3.4E). Using Gene set enrichment analysis and checking the cellular processes associated with the differentially expressed genes (Figure 3.5G), we noted that the addition of Quisinostat to the LV transduction condition, resulted in differential expression of genes associated with DNA replication and chromosome organization, in line with the functional effects observed.

Collectively, these findings indicate that addition of Quisinostat and CPI203 enhances the expansion of hematopoietic stem cells (HSCs) while simultaneously priming cells for erythroid and lymphoid lineages, respectively. Furthermore, our study highlights the impact of lentiviral (LV) transduction on cellular heterogeneity, revealing a significant loss of HSCs with the commonly employed protocol in clinical settings.

3.4 Discussion

Working towards improving HSC expansion protocols for gene therapy purposes, in this study we have evaluated existing and newly tested culture conditions for HSC expansion and gene modification. We incorporated the small molecules CPI203—a BETi that has been previously reported by Hua *et al*¹⁹—and the HDACi Quisinostat for testing *ex vivo* expansion of HSCs and their genetic modification. BET proteins interact with acetylated lysins at transcriptional start sites that regulate 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 has been challenging and none have received regulatory approval³⁸. On the other hand, Quisinostat, an HDACi targeting HDAC I and II, has been evaluated in several clinical trials for different malignancies³⁹⁻⁴¹. Recent research

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suggests Quisinostat inhibits the cancer cell self-renewal by re-establishing expression of histone linker H1.0⁴². 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.

We have reported improved culture conditions for *ex vivo* expansion of true HSCs, which could be implemented in lentiviral transduction protocols for gene therapy purposes. We showed that the inclusion of epigenetic regulators of Quisinostat and CPI203 increased expansion of HSCs. In addition, the positive effect of Quisinostat on transduction efficiency provides a major benefit for the gene therapy field. This not only ensures long-term correction of true HSCs, which is the ultimate goal of gene therapy, but also reduces the costs associated with viral vectors, since less lentiviral virus would be required to achieve a desired vector copy number.

Importantly, our scRNA-seq data reveals the stealth effect of LV transduction on loss of HSCs, particularly in the conventional clinical protocol of STF-cytokine-expanded cells. LV transduction induces an innate immune response via indirect activation of interferon (IFN) responses through different pathways such as Toll-like receptors (TLRs)³⁵, DNA damage response³⁶ or DNA sensor responses³⁷. Even though LVs can escape the innate immune responses⁴³, they can be detected through TLRs³⁵ in HSPCs or trigger p53 signaling followed by robust induction of IFN responses⁴³. Human CD34⁺ cells express *TLR3*, *TLR4*, *TLR7*, *TLR8* and *TLR9* to detect infection and induce innate immune response immediately³⁵. Upon viral infection under physiological conditions, IFN- α induction leads to proliferation as a response to clear infections⁴⁴. In addition, results from mouse studies showed induction of IFN- γ impairs HSC self-renewal and restore of HSCs number upon viral infection⁴⁵. However, in our study no changes in *TLRs*' gene expressions were found. Strikingly, the benefit of CPI203 in gene editing can be investigated as it reduces interferon responses through p53 and reduced DNA damage responses.

Furthermore, our *in vivo* study suggests that CPI203 improved lymphoid development in BM and significantly in thymus without compromising other lineages, a benefit that would be valuable for patients with an impaired immune system. Moreover, based on our single cell transcriptomics data, Quisinostat primed HSCs toward the erythroid lineage, which also can hold promises for gene therapy targeting blood disorders.

In the realm of clinical trials focused on HSC-based gene therapy, substantial financial investments are dedicated to producing Good Manufacturing Practice (GMP) grade LV batches. Frequently, the expenses associated with generating these batches reach into the millions of dollars, even for a small number of patients. Compliance with regulatory standards mandates full-scale test runs in meticulously controlled clean room environments. Consequently, the limited availability of GMP-grade virus for patient treatment significantly amplifies the overall costs associated with gene therapy medicines, emerging as a critical concern within the field⁴⁶. This underscores the pressing need for advancements in HSC transduction protocols to alleviate the financial burden and streamline the production of gene therapies. Here we have reported on enhanced culture conditions that not only improve transduction efficiency but also preserve the integrity of HSCs. Such optimization is imperative for achieving a more favorable clinical outcome and emphasizes the importance of refining current protocols for expanded hematopoietic cell therapies.

3.5 Methods

Human cells and CD34⁺ enrichment

Human cord blood was obtained after informed consent from Leiden University Medical Centre. Mononuclear Cells (MNCs) were obtained from cord blood by density centrifugation using Ficoll-Amidotrizoat. CD34⁺ cells were positively selected using the human CD34 UltraPure MicroBead Kit (Miltenyi Biotec) according to the manufacturer's protocol.

CD34⁺ cell culture

100,000-250,000 enriched CD34⁺ cells/ml (>90% purity) were cultured in X-vivo15 medium (Lonza) supplemented with recombinant huSCF (300ng/ml), huTPO (100ng/ml), huFLT3 (100ng/ml) (Miltenyi Biotec), and small molecules of CPI203 (150nM) (Selleckchem) and Quisinostat (0.1, 0.5 and 1nM) (MedChemExpress) After 4-5 days of culture, expanded cells were harvested and counted using a nucleocounter 3000 (Chemometec) for subsequent immunophenotyping, transplantation in mice, and scRNA-seq.

Lentivirus production

HEK 293T cells were seeded at density of 4×10^6 cells in 10cm² plate. After 24 hours, cells were transfected using X-tremeGENE HP DNA transfection reagent (Sigma-Aldrich) at a mass ratio of 1:3 (DNA:X-tremeGene HP), with the plasmids. The amount of each viral component per million of cells was: 2.5 μ g of pSIN.SF.EGFP.WPRE; 1.25 μ g of pMDLg/pRRE; 0.6 μ g of pRSV-Rev; 0.75 μ g of pMD2.VSVG. Supernatant was harvested, clarified with 0.45 μ m filter (Whatman) at 24h and 48h post transfection. Supernatant from different time points were pooled together. Virus concentration was performed using Vivaspin20 (Sartorius) according to the manufacturer's instruction.

Lentivirus transduction

One day prior to transduction, CD34⁺ cells were cultured in cytokine supplemented X-vivo15 medium in combination with CPI203 (150nM) or Quisinostat (0.5nM). After overnight stimulation, lentiviral particles carrying GFP transgene were added to the cells at MOI of 1.5 as well as Lentiboost (Sirion) final concentration of 1ug/ml followed by spinoculation at 32 °C for 1 hour at 800 g. Four days post-transduction, transduced cells were used for *in vitro* and *in vivo* approaches.

Determination of vector copy number (VCN) by RT-qPCR

Quantitive (q)PCR was used for the quantitative analysis of proviral DNA copies in transduced cells using Hiv-psi and Albumine as housekeeping gene. Genomic DNA of transduced cells was extracted using GeneElute Mammalian Genomic DNA kit (Sigma-Aldrich). qPCR was performed using TaqMan Universal Master Mix II (Thermofisher) in combination of specific probes from universal library (Roche) on the Quantstudio system (Thermo Fisher Scientific).

Mice

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were purchased from Charles River (France). All animal experiments were approved by the Dutch Central Commission for Animal experimentation (Centrale Commissie Dierproeven, CCD).

Primary and secondary transplantations into NSG mice

For the primary transplantations, *ex vivo* expanded CD34⁺ cells (25,000 or 50,000 total nucleated cells per mouse) in Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Gibco) were transplanted by tail vein injection into pre-conditioned recipient NSG mice (n=5). For the secondary transplantations, bone marrow (BM) cells from primary recipient mice from each group were pooled and 1/7th of the pooled cells were injected into pre-conditioned secondary recipient NSG mice (n=5). 6-8-week-old recipient mice were conditioned with two consecutive doses of 25mg/kg Busulfan (Sigma-Aldrich) (48h and 24h prior transplantation). Mice used for transplan-

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tation were kept under specific pathogen-free conditions. The first four weeks after transplantation mice were fed with additional DietGel recovery food (Clear H₂O) and antibiotic water containing 0.07 mg/mL Polymixin B (Bupha), 0.0875 mg/mL Ciprofloxacin (Bayer b.v.). Peripheral blood (PB) from the mice was drawn by tail vein puncture every 4 weeks until the end of the experiment. At the end of the experiment, PB, thymus, spleen and BM were harvested. Mice were euthanized via CO₂-asphyxiation.

Flow cytometry

Ex vivo cultured cells were stained with the live/dead marker Zombie (Biolegend) according to the manufacturer's instruction. Subsequently, cells were stained with antibodies listed in Supplementary Table 1, and incubated for 30 min at 4°C in the dark in FACS Buffer (PBS pH 7.4, 0.1% azide, 0.2% BSA). Single cell suspensions from murine BM, thymus and spleen were prepared by squeezing the organs through a 70 μ M cell strainer (BD Falcon). Erythrocytes from PB and spleen were lysed in NH₄Cl (8.4 g/L)/KHCO₃ (1 g/L). Single cell suspensions were counted and stained as described above. When 7AAD and KI67 staining was needed, intracellular staining was performed using the Transcription Factor Staining Buffer Set (eBioscience), according to manufacturer's instruction. All cells were measured on an Aurora spectral flow cytometer (Cytex). The data was analysed using FlowJo software (Tree Star).

Single cell analysis quality control (QC) pipeline

QC and downstream analyses of scRNA-seq data were performed using Seurat version 4²⁴. The quality control pipeline before integration consists of three steps per sample: 1) soft filtering, 2) removal of low-quality cluster, and 3) doublet detection. In soft filtering, Seurat objects were created with cells expressing at least 200 genes and with the genes expressed at least in 3 cells. Then, standard Seurat command list was run to detect low quality clusters. We removed a cluster only if it had higher than 15% mitochondrial and less than 1000 mRNA in median. Next, we ran DoubletFinder v3 per sample with 7.5% detection rate and filtered for the singlets²⁵. To integrate the samples, CCA mode of Seurat pipeline was used with most variable 2000 features. Finally, after integration we filtered cells with higher than 15% mitochondrial mRNA.

Cell type predictions for stimulated cells

After QC, we subsetted the unstimulated CD34⁺ cells (n=13,429). Then, these cells were clustered again with a higher resolution parameter (resolution=1.2) to ensure precise annotation in downstream projections of stimulated samples. Furthermore, we checked cluster stability with *clustree* R package²⁶. To annotate each cell cluster, we used a combination of cord blood markers from Dong *et al*²⁷ and differentially expressed genes (DEGs). Top 20 DEGs per cell cluster from unstimulated cells are provided in Supplementary Table 2. To project these annotations onto stimulated samples, we trained a random forest model with 300 trees using unstimulated CD34⁺ cells via 2000 integrated features. Cross-validation on out-of-bag data showed that HSCs could be predicted with an accuracy of 81.2%.

Differential expression and gene set enrichment analysis

Differential expression analysis was conducted with MAST²⁸ with a cut-off of at least 25% difference in cells expressing a given gene. Then, clusterProfiler²⁹ was used along with biological processes subset of GO terms for gene set enrichment analysis. Pathway p-values are adjusted with Benjamini-Hochberg procedure. Pathways enriched with adjusted p<0.05 are selected. ggplot2 library was used for all visualizations unless indicated otherwise.

Statistics

Statistics were calculated and graphs were generated using GraphPad Prism9 (GraphPad Software). Statistical significance was determined by standard two-tailed Mann-Whitney U tests, unpaired t-tests and ANOVA tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

3.6 Acknowledgments

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3.7 Author Contributions

P.T designed, performed, analyzed experiments, and wrote the manuscript; E.O.K performed bioinformatic analysis; K.C.B supported in vivo experiments. B.A.E.N, S.A.V and M.C.J.A.s.E assisted with mouse experiments. M.L.H provided cord blood for this study. E.V.D.A supported with scRNA seq experimental design; K.P.O and F.J.T.S provided supervision and revision of the manuscript. All authors have read and agreed to the final version of manuscript.

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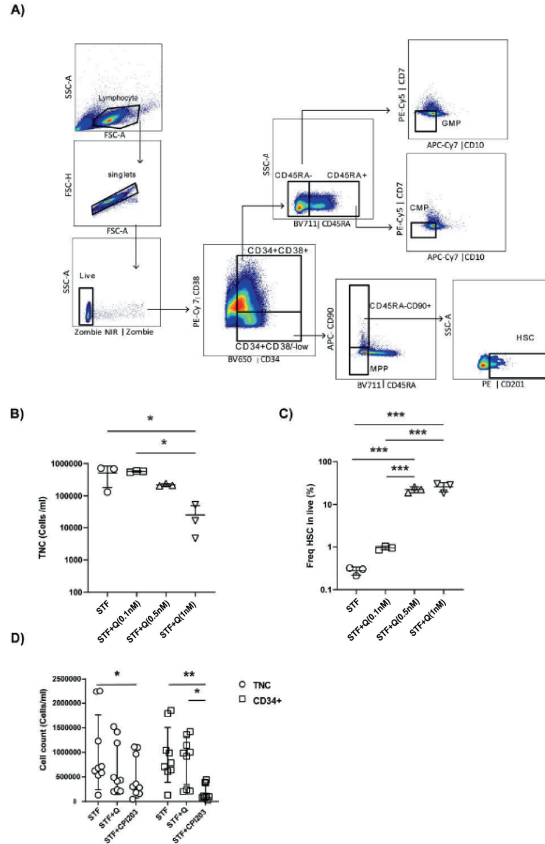
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3.9 Appendix

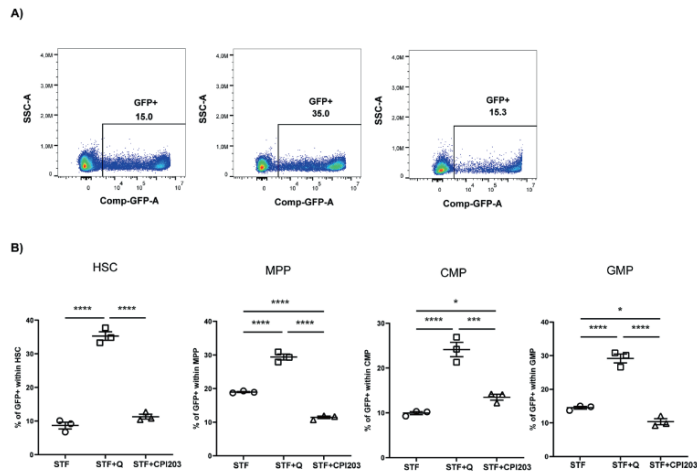
3.9.1 Supplementary Tables

The appendix was shortened due to environmental considerations. To minimize unnecessary printing and reduce material use, extensive data including 11 supplementary tables are provided online at: <https://doi.org/10.1182/bloodadvances.2024013047>.

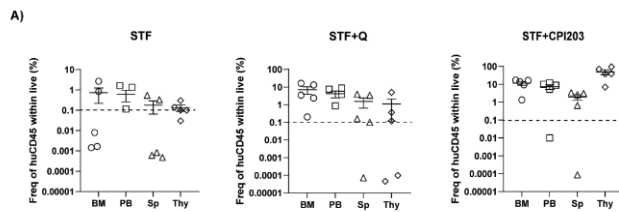
3.9.2 Supplementary Figures



Supplementary Figure 1. **Ex vivo expansion of HSPCs.** A) Representative gating strategy for phenotypic analysis of HSPCs. B) Total nucleated cell count (TNC) after 4-day culture in presence of different concentration of Quisinostat (n=3, Mean±SD). C) Frequency of HSC after 4-day culture in different concentration of Quisinostat (n=3, Mean±SD). D) TNC and CD34+ cell count after 4 days of expansion in indicated culture condition (n=9, Mean±SD). Significant statistical was determined by two-way anova with multiple comparison *p<0.05, **p<0.01, ***p<0.001)



Supplementary Figure 2. LV transduction efficiency in vitro. A) Representative FACS plots displaying GFP expression in live cells, 4 days post transduction. B) Frequency of GFP+ in different population of HSC, MPP, CMP and GMP (n=3, Mean±SD, *p<0,05, ***p<0,001, ****p<0,0001. Statistical significance was calculated by two-way anova with multiple comparison)



Supplementary Figure 3. Human engraftment in organs from primary transplanted NSG mice. A) Frequency of huCD45+ in different organs at week 20 post transplantation.

