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Mechanism of Human Hematopoietic Stem Cell Loss During Ex Vivo Manipulation and Gene Transfer

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**Mechanism of Human Hematopoietic Stem Cell Loss During *Ex Vivo* Manipulation
And Gene Transfer**

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fulfillment of the requirements for the degree of

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ABSTRACT

Gene therapy (GT) which is a gene transfer into human hematopoietic stem cells (HSCs), offers an attractive treatment strategy for curing several hematopoietic disorders. However GT requires *ex-vivo* manipulation and culture of HSCs, which largely results in their differentiation to progenitors. Currently, the numbers of genetically modified human HSCs that repopulate after autologous transplant are a major limitation to effective gene transfer. High intensity myelo-ablative conditioning is therefore often required to destroy resident HSCs, and to allow an engraftment advantage to genetically modified HSCs. Success achieved in many immune deficiency disorders is due to the selective advantage of gene modified progeny.

Apart from non-human primate models, which have limitations in availability and high costs, there is a lack of a good experimental model of adult human HSCs. Immune deficient NOD/SCID/IL-2R gamma null (NSG) mice can robustly engraft human hematopoietic stem/progenitor cells (HSPCs), giving rise to myeloid, B- & T-lymphoid cell progeny, and have been used to study human hematopoiesis *in vivo*. However, most studies in NSG mice use umbilical cord blood derived HSPCs that engraft readily and do not mimic bone marrow or mobilized peripheral blood derived adult HSPC kinetics or engraftment. The latter are primarily used for gene therapy. We optimized and developed a robust model of adult human HSC engraftment to test long term repopulating potential (LTRP) of HSCs during *ex vivo* manipulation.

Our study show that extended time of *ex vivo* culture of HSPCs results in substantial loss of LTRP. Furthermore, genetically manipulated HSPCs transduced with

retroviral vectors showed a relative loss of lymphoid potential at the expense of increased myeloid lineages, suggestive of HSC aging. Mechanistically, we found increased reactive oxygen species and increased p38 MAPK signaling with increased time in culture. Using a highly potent p38 MAPK inhibitor, we retain secondary engraftment to a level similar to that of un-manipulated cells, and partially revert the myeloid skewing.

We further investigated the mechanism of loss of HSC LTRP in our *ex vivo* culture conditions. We observed increased DNA damage response (DDR) in HSCs that was significantly reduced by p38 MAPK inhibition. We also showed that p38 inhibition increased the time HSCs spent in the G0 (quiescent) phase of the cell cycle, suggesting a protective mechanism for preventing HSC loss. Interestingly, we found significant accumulation of HSCs in the late (S-G2-M) phase of the cell cycle only if we introduce double strand breaks (DSBs) to actively cycling HSCs either with viral vectors or gene editing nucleases. Addition of eicosanoids such as prostaglandin E2 (PGE2) along with p38 MAPK inhibition completely restored a balanced myelo-lymphoid progeny through the mechanism of HIF-1 α stabilization and diminished DDR.

Overall, our study has discovered substantial loss of HSC function during *ex vivo* culture and gene manipulation. The mechanistic insights gained from our research can be utilized to optimize the conditions for the effective clinical application of both gene editing and viral mediated gene transfer protocols using the combination of small molecule inhibitors.

PREFACE

The work in this dissertation is being submitted:

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TABLE OF CONTENTS

Page

Abstract.....	i
Preface.....	iv
Acknowledgments	v
Table of Contents	vi

Chapter I: Introduction.....	1
I. Gene Therapy	2
II. Hematopoietic stem cells and its therapeutic application.....	3
III. Maintaining and/or Expanding HSCs during <i>ex vivo</i> culture	6
IV. p38 MAPK in regulating HSC function	12
V. Prostaglandin E2 effects in HSCs.....	14
VI. Cell cycle, ROS, and DDR in <i>ex vivo</i> cultured HSCs	15

Chapter II: Mechanism of Human HSC Loss during Ex vivo Manipulation and Gene Transfer.....	17
I. Abstract.....	18
II. Introduction	19
III. Results.....	21
Ex vivo HSC division and gene transfer results in loss of LTRP and a myeloid skewed gene-modified progeny.....	21
Mechanism of action of p38 inhibition in human HSC fate.....	25
Crosstalk between S-G ₂ M checkpoint and HIF-1 α links RV-induced DSB, cell cycle and HSC fate.....	27
p38i combined with PGE2 restore LTRP and lineage fate of transduced HSCs.....	30
IV. Discussion	31
V. Acknowledgements.....	33
VI. Methods	33

VII.	References	39
VIII.	Figure Legends	45
IX.	Figures	56
X.	Supplementary Figures	62
Chapter III: Discussion		76
I.	Overview	77
II.	Conclusion and Future Directions	80
References		83

Chapter I

Introduction

Gene Therapy

Gene therapy involves the transfer of therapeutic genes into cells, using viral or non-viral methods either *in vitro* or *in vivo*¹. Gene editing is a new form of gene therapy that uses site specific cleavage by a variety of nucleases. If a donor template is provided, it can result in homology directed repair (HDR) by the cellular DNA repair process^{2,3}. If no donor template is provided, the cell repairs the DNA double strand break via non homologous end joining (NHEJ), resulting in an error prone repair and gene disruption³. Viral gene delivery using lentiviral vectors (LV) or γ -retroviral vectors (RV) are promising system due to their high gene transfer efficiency^{1,4,5}. Retroviruses were developed in the laboratory for the gene delivery purposes more than three decades ago^{4,6}. The fact that RV are equipped with various structural and functional proteins enables them to reverse transcribe their RNA into complementary DNA (cDNA) which integrates into the genome of the host cell which they infect⁷.

Based on the history of gene therapy, the initial RV used for gene transfer were derived from the murine Moloney leukemia virus (MLV), which belongs to the gamma-retrovirus family^{4,8}. Gamma retroviral vectors have been successfully utilized in gene therapy clinical trials for the treatment of various immunodeficiency disorders (IDDs). However, they can only infect dividing cells, as they need the nuclear membrane to be ruptured for their entry⁷. This possesses a challenge for transduction of hematopoietic stem cells (HSCs) which are mainly in their quiescent G₀ phase of the cell cycle. RV with their long terminal repeats (LTR) region containing viral promoters and enhancers have tendency to integrate near cellular proto-oncogenes, resulting in hematological malignancies⁹. Substantial improvements have been accomplished in generating self-

inactivating (SIN) RV¹⁰ with less risk of generating replication competent virus and vector mobilization, and also reduced chances of cellular oncogene activation¹¹. Gene editing via HDR, like RV is dependent on cycling HSCs. LVs are a subset of retroviruses that have been increasingly used for gene delivery purposes because of their ability to transduce quiescent cells¹¹. Although LV are better than RV in preventing the deterioration of stem cell function during ex vivo manipulation, they still can be integrated next to oncogenes¹². LV can also accommodate larger sizes of inserts than RV^{11,13}. As studies in ex vivo expanded cells show loss of HSC's long term reconstituting potential¹⁴ and LV can transduce non-dividing cells, prolonged ex-vivo pre-stimulation time of the cells (necessary to bring quiescent cells into cycle) could be reduced before transduction to preserve HSC function. Gene editing via NHEJ, like LV transduction, is cell cycle independent.

Hematopoietic Stem Cells and its Therapeutic Application

HSCs are known for their unique properties of self-renewal and differentiation into a series of lineage restricted hematopoietic progenitor cells (HPCs) that eventually give rise to various mature blood cell types¹⁵⁻¹⁸ (Figure 1). There are three major types of division that decide HSC fate: a) symmetric self-renewal (giving rise to two HSCs), b) asymmetric maintenance (giving rise to one HSC and one HPC), c) symmetric commitment (giving rise to two daughter HPCs) (Figure 2). A fine balance between the self-renewal and differentiation of rare stem cells is critical for HSC function in life-long replenishment of mature blood lineages¹⁹.

Figure 1. Hematopoietic Stem and Progenitor Compartment

Figure 2. Hematopoietic Stem Cell Division

Because of the unique properties of HSCs, they are an excellent target for treating several hematopoietic disorders including cancers via either normal or gene modified HSC transplantation (HSCT) therapy^{20,21}. Several clinical trials have been carried out in which autologous HSCs were isolated from patients, followed by gene transfer performed *in vitro*, and finally transfusion of the gene corrected HSCs back into the patient. The gene therapy field began with this promising approach to treat several immunological and genetic disorders such as X-linked severe combined immunodeficiency (SCID-X1), adenosine deaminase deficiency (ADA-SCID), adrenoleukodystrophy (ALD), thalassemia, and chronic granulomatous disease (CGD)^{22,23}. However, early trials largely failed due to insufficient transgene expression, insertion of target genes near oncogenes and/or insufficient engraftment of HSCs long-term^{22,24}.

Cord blood (CB), mobilized peripheral blood (MPB) and bone marrow (BM) are used as sources of HSCs in therapeutic applications, including allogeneic hematopoietic stem cell transplant (HSCT). A major challenge in use of MPB or BM derived HSCs in clinical stem cell therapy is identification of proper human leukocyte antigen (HLA)-matched donors for bone marrow transplantation. CB has been candidate for unrelated allogeneic HSCT as CB banked samples are increasingly available; however one cord unit has a limited number of HSCs available for transplantation into adults, and it also may result in delayed myeloid engraftment carrying the risk of early-transplant related mortality^{15,25,26}. Therefore, it is essential to explore new ways to expand HSCs during *ex vivo* culture before transplantation²⁷⁻³⁰. Consequently, developing a protocol that can

maintain/expand HSCs without predisposing them for leukemic transformation has been one of the major goals in the field of HSC biology ³¹.

Maintaining and/or expanding HSCs during *ex vivo* culture

HSCs have been extensively studied to identify extrinsic and intrinsic factors affecting their fate decisions ^{32,33}. By applying the knowledge gained from studying the effects of these factors, numerous experimental approaches have been followed to expand and/or maintain HSPCs derived from BM, CB, or MPB. These approaches have led to progressive increase in knowledge in HSC biology, its fate decisions and success ^{34,35}.

The majority of HSCs *in vitro* expansion protocols are designed for expanding human CB ³⁶. However, adult HSCs derived from MPB have been increasingly used in clinical stem cell based therapies for the treatment of hematopoietic disorders ³⁷. MPB derived HSCs differ from CB HSCs in many aspects, including gene expression profiles, cloning efficiency, and response to cytokines ³⁸. Thus, an efficient protocol for expansion of MPB derived adult HSCs is crucial for success of HSC transplantation and improved graft size ^{37,39}.

Expansion of human HSCs was initially attempted with various combination of cytokines such as thrombopoietin (TPO), Interleukin 3 (IL-3), IL-6, IL-11, stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt-3L), and granulocyte-macrophage colony-stimulating factor (GM-CSF) ^{16,40-44}. Results of HSC expansion were mostly based on *in vitro* assays which only reflect HPC progeny rather than human HSC potential. Bhatia *et al.*, introduced the importance of some of the more primitive cells

that are capable of repopulating non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice as SCID-repopulating cells (SRC) for the quantitation of human HSCs⁴⁵.

Since HSCs reside in BM niches that supply regulatory signals for the proper maintenance of HSC properties⁴⁶, co-culture of HSCs with stromal cells *in vitro* tends to mimic the natural environmental niches⁴⁷, thus allowing the interaction of stroma-derived factors for supporting the growth of HSCs. Stroma-derived factors such as granulocyte colony-stimulating factor (G-CSF), GM-CSF, stem cell factor (SCF), stromal cell-derived factor-1 α (SDF1 α), and leukaemia inhibiting factor (LIF) have been shown to promote *ex vivo* expansion and maintenance of human HSCs⁴⁸⁻⁵⁵. The heparin-binding growth factor pleiotrophin is expressed by stromal cells and has been shown to improve expansion of both mouse and human HSCs *ex vivo* and their long-term repopulating ability *in vivo*^{56,57}. However, the expansion effects were less in human HSCs than in mouse HSCs^{41,56}.

More than a decade ago, a serum-free culture medium with added cytokines was demonstrated to be superior in expanding human HSC-enriched populations with higher engraftment potential in NOD-SCID mice and non-human primates^{45,58}. However, the use of these cytokine cocktail-based expansion protocols was not sufficient to enable the long-term re-population of the *ex-vivo* cultured cells, demonstrating the need of additional supporting factors or molecules for maintaining/increasing human HSC population *in vitro*⁴¹.

Investigation of key regulatory elements that govern self-renewal and differentiation of HSCs have identified several relevant transcription factors significant

for primitive hematopoietic cell proliferation and differentiation. Retroviral overexpression of the transcription factor HOXB4 in murine HSCs resulted in a significant increase of HSC self-renewal, increased *ex vivo* expansion of HSCs, and increased *in vivo* regenerative potential without causing functional damage^{59,60}. Furthermore, to obviate the need for retroviral gene transfer, ectopic expression of HOXB4 was used. Stromal cells were cultured to secrete HOXB4 protein for HSCs, or recombinant human HIV trans-activating protein (TAT)-HOXB4 was directly delivered. After short exposure to this protein, both *in vitro* expansion of HSCs and a long-term repopulating potential were achieved^{61,62}. However, the *ex vivo* expansion effect of HOXB4 was variable among several species including human and non-human primates⁶³. Later, recombinant degradation-resistant HOXB4 protein was also directly added to human adult HSCs, leading to increased maintenance of HSCs both *in vitro* and *in vivo*⁶⁴. Recently a novel approach was implemented for the expansion of HSC population *ex vivo*, by culturing them with fusion proteins containing protein transduction domain of the HIV1 transactivation protein (Tat)-MYC or Tat-Bcl-2³⁰. However questions remain regarding safety and efficacy of use of these proteins in a clinical setting. Based on the murine data, HOXB4 gene transfer was performed in dog CD34⁺ HSPCs. All Dogs developed leukemia due to overexpression of HOXB4 and not because of retroviral mediated gene transfer⁶⁵. There was also expansion of murine HSCs using engineered fusions N-terminal domain of nucleoporin 98 (NUP98) and the homeodomain of the HOX transcription factor, HOXA10 (NUP98-HOXA10hd fusion)⁶⁶, but after leukemia with HOXB4 in large animal models, success in murine HSCs was not considered to be

equivalent to that in HSCs from large animal models or human, and this approach was discarded.

While exploring pathways responsible for HSC self-renewal, factors affecting embryonic development were found to play role in human HSC regulation. These include wingless-type (Wnt), Notch, and Sonic hedgehog (Shh) signaling^{29,41,67-70}. For instance, work on notch signaling by addition of notch-ligands, resulted in expansion of both mouse and human HSCs²⁹. However, canonical Notch signaling is not required for *in vivo* expansion of human HSCs⁷¹. Studies on Wnt proteins show that they promote HSC expansion *in vitro*⁷²⁻⁷⁴. However, the effect of the canonical Wnt signaling in regulating HSC function is not well understood and the results are controversial⁷³. Surprisingly, exogenous Wnt3a protein has recently been shown to suppress the expansion of CB-derived highly enriched HSC population⁷⁵.

Numerous epigenetic approaches are currently being explored to expand human HSCs *ex vivo*³⁶. For instance, the stable introduction of polycomb (PcG) group gene Bmi1 into the CB derived HSPCs (constitutive expression) resulted in long-term maintenance of the stem-cell pool and enhanced self-renewal of HSPCs^{76,77}. Enhancer of zeste homolog 2 (Ezh2) prevented HSC exhaustion after replicative stress⁷⁷. Chromatin-modifying agents (CMAs): 5-aza-2'-deoxycytidine (5azaD) and trichostatin A (TSA) were also reported to *ex vivo* expand HSCs derived from CB, BM, and MPB^{37,78,79}. Recently, Chaurasia et al. utilized the histone deacetylase inhibitor (HDACI) and valproic acid (VPA) for the robust *ex vivo* expansion of CB HSCs, resulting in increased self-renewal activity as displayed in terms of SCID-repopulating cells (SRCs) via induction of pluripotency genes³⁶. Studies by Chen et al., showed that adult human

HSPCs treated with UNC0638, a G9a/GLP (methyl transferases) small molecular inhibitor, preserves stem cell phenotype and function during *in vitro* expansion⁸⁰. Their study suggests G9a/GLP activity promoting progressive histone H3K9me2 patterning during HSPC lineage specification and inhibiting that modification, delays HSPC lineage commitment⁸⁰.

Various small molecules have also been used for *ex vivo* expansion of HSCs, including the retinoic acid receptor agonist all-trans retinoic acid (ATRA), the copper chelator tetraethylenepentamine (TEPA), the Wnt signaling modulator 6-bromoindirubin-3'-oxime (BIO), an aryl hydrocarbon receptor (AhR) antagonist: stemregenin1 (SR1), the c-MPL agonist NR-101, and the lipid mediator prostaglandin E2 (PGE2)^{29,34,41,81-83}.

Very recently, work of Gao *et al.* identified a novel small molecule inhibitor of INK4 protein p18 (INK4C) that can enhance *ex vivo* expansion of murine HSCs⁸⁴. Whether this inhibitor will have same effect on human HSCs, is questionable. The use of small molecule inhibitors will help in identification of signaling pathways implicated in human HSC self-renewal for their use in *ex vivo* expansion.

Since cytokines are known to stimulate quiescent HSCs leading to their expansion and commitment to HPCs at the cost of HSC self-renewal¹⁶, an alternative strategy has been reported in which mTOR inhibition and Wnt/ β -catenin activation (via rapamycin and GSK-3 inhibitors, respectively) maintain both human and mouse HSCs *ex vivo*⁸⁵. The *ex vivo* culture conditions in this case, were free of cytokines and the cultured cells engrafted long term⁸⁵. Similarly, a study by Heinz et al showed that

resveratrol, a SIRT1 activator was able to efficiently preserve the CB derived HSC during *in vitro* culture, enabling robust multi-lineage human engraftment in NSG mice⁸⁶.

Another approach for *ex vivo* expansion that holds promise for the use of expanded HSCs was reported by Csaszar et al. They introduced an automated "fed-batch" culture system. Extra medium was added at frequent intervals to dilute inhibitory factors, maintaining stem cell density⁸⁷. However the cost effectiveness of this approach and its scalability is yet to be defined⁸⁸.

Most recently, *ex vivo* maintenance of mouse HSCs has been shown after reprogramming the cellular metabolism⁸⁹. HSCs use glycolysis for energy production rather than mitochondrial oxidative phosphorylation⁹⁰. Liu *et al.* used a selective inhibitor of the mitochondrial phosphatase Ptpmt1 to switch metabolism toward glycolysis, resulting in *ex vivo* maintenance of HSCs⁸⁹. Intriguingly, one of the four Yamanaka reprogramming factors (i.e. factors that are able to convert somatic cells into induced pluripotent stem cells), OCT4, has been shown to significantly increase the rigorously defined phenotypic HSC population as well as to increase SRCs *in vivo*⁹¹. This study was conducted using a small molecule OAC1 (Oct4-activating compound 1) and the mechanism was shown to be mediated via upregulation of HOXB4,⁹¹ which has been previously shown as a powerful intrinsic regulator of HSC expansion⁵⁹⁻⁶², although constitutive prolonged expression has led to leukemia in large animal models.

p38 MAPK in regulating HSC function

p38MAPK (p38), a serine-threonine kinase, is a member of mitogen-activated protein kinase (MAPK) superfamily. p38 is activated by stress. The p38 family comprises of four isozymes: p38 α , p38 β , p38 γ , and p38 δ ^{92,93}. Among these isozymes, p38 α is highly expressed in HSPCs⁹². The role of p38 MAPK in various cellular processes such as proliferation, apoptosis, senescence and differentiation is documented⁹³⁻⁹⁶. Knockout of p38 α is embryonically lethal as it is required for definitive erythropoiesis⁹⁷ but p38 α is dispensable in normal steady-state adult hematopoiesis⁹⁸. However, in stress hematopoiesis, a major role of p38 MAPK has been documented⁹². Multiple factors can induce the activation of p38 MAPK pathway in hematopoietic cells, including environmental stress and inflammatory cytokines⁹⁹. Progeny of HSCs are able to produce various suppressive cytokines. For example, tumor growth factor- β (TGF β) can act upstream of p38 and stimulate p38 activation¹⁰⁰⁻¹⁰².

Studies performed by Ito *et al.* have shown increased accumulation of reactive oxygen species (ROS) in ATM $-/-$ HSCs from old mice. This leads to loss of HSC self-renewing potential and eventually to bone marrow failure¹⁰³. Later, the same group showed that in ATM $(-/-)$ HSCs, p38 MAPK was activated downstream of increased ROS and loss of HSCs long term repopulating potential (LTRP)¹⁰⁴. Treatment with either N-acetylcysteine (NAC) or with a p38 inhibitor restored long term reconstitution capacity and maintained HSC quiescence *in vivo*¹⁰⁴. Another elegant study by Miyamoto *et al.* showed impaired long-term reconstitution potential in Foxo3a $(-/-)$ mice which increased phosphorylation of p38 MAPK, increased ROS levels, and loss of

quiescence¹⁰⁵. These studies implicate relationships among ROS, p38 MAPK, and quiescence in regulation of HSC self-renewal function.

Regenerative stress is believed to lead to exhaustion of HSC pool^{106,107}. Serial bone marrow transplantation adds a strong regenerative stress to HSCs, leading to limited repopulating ability and eventually leading to HSC loss¹⁰⁸. Interestingly, elevated ROS production occurs with serial transplantation and induces p38 activation, thereby resulting in premature HSC exhaustion^{104,105}. Hence, p38 inhibition has been used as a strategy to expand both murine and human cord blood derived HSPCs *ex vivo*^{109,110}.

Besides self-renewal, HSCs are responsible for replenishing with all the hematopoietic cell types in response to loss of hematopoietic stress. Recently, Karigane et al. demonstrated that p38 activation is a novel modulator of HSC repopulation /recovery after various hematological stresses, including bone marrow transplantation, cytokines or chemotherapeutic agents. Their study shows that hematological stress can activate p38 α , which activates purine metabolism and initiates cycling of HSCs, leading to bone marrow repopulation⁹². Furthermore, very recently, the role of a tumor suppressor with a deubiquitinase activity, cylindromatosis (CYLD), has been shown to regulate HSC repopulation and self-renewal potential by preventing p38 MAPK activation thereby preserving quiescence¹¹¹. Mechanistically, they showed that CYLD intreracts with tumor necrosis factor–associated factor 2 (TRAF2) which is known to control the activity of MAPK family¹¹¹.

Prostaglandin E2 effects in HSCs

Prostaglandin E2 (PGE2) belongs to eicosanoid family which mediates tissue responses including inflammation and infection¹¹². Roles of PGE2 in HSCs have been explored by several studies performed in zebrafish, mice, and in human cells^{82,113}. A short *ex vivo* treatment of PGE2 was shown to increase kidney marrow recovery after irradiation in zebrafish, and increased LTRP of HSCs in murine bone marrow after limiting dilution transplant⁸². However, a discrepancy was found in the *ex vivo* versus the *in vivo* effect of HSC treatment with PGE2. *In vivo* treatment with PGE2 only increased the short-term HSCs and MPPs¹¹⁴. In human CB HSPCs exposed to PGE2 *ex vivo* followed by xenotransplants, as well as similar treatment of HSPCs followed by transplants of nonhuman primates, PGE2 boosted engraftment level¹¹⁵. The mechanism of action of PGE2 was dissected by Pelus and colleagues, who showed increased engraftment after PGE2 *ex vivo* treatment, resulted from higher homing efficiency (via CXCR4 upregulation), and survival of HSCs (via upregulation of surviving gene expression)¹¹³. Interestingly, the same group performed further studies to explore the role of endogenous PGE2. They surprisingly detected HSPCs egress from the bone marrow after non-steroidal anti-inflammatory drug (NSAID) treatment, which inhibits endogenous PGE2⁸³. These studies led to utilization of PGE2 in a phase I clinical trial in double cord HSPC transplantation¹¹⁶. As shown earlier, PGE2 mediates homing in HSCs by increasing CXCR4 receptor expression via stabilization of hypoxia-inducible factor-1 α (HIF-1 α)¹¹⁷.

Cell cycle, ROS and DDR in *ex vivo* cultured HSCs

Our studies highlight several stress signaling pathways induced during *ex vivo* culture. The homeostasis of HSCs is well-maintained in their hypoxic bone marrow niche^{118 119}. HSCs reside in their niches in a quiescent phase (G_0) of cell cycle. Upon stress, HSCs may undergo either self-renewing or differentiation divisions upon entering the cell cycle. Studies have shown that HSCs entering cell cycle allows repair of DNA damage albe the cycling per se leads to a functional decline of the HSC pool¹²⁰. It seems that it is preferable for HSCs to undergo differentiation upon genotoxic stress, so that the mutated DNA will not accumulate, which could result in a carry forward of leukemia causing muttions, and conceivably convert a normal HSCs into cancer stem cells (CSCs)¹²⁰⁻¹²².

Upon oxidative stress (or any other genotoxic stress), DNA develop single strand breaks (SSBs) and double strand breaks (DSBs)¹²³. The check point kinases ATM and ATR are then recruited to SSBs or DSBs site and recruit a cascade of proteins to repair DNA. After the DNA repair, the cells progress through the cell cycle¹²⁴. A downstream consequence of DNA damage response (DDR) is γ H2AX phosphorylation (Histone H2AX phosphorylated at serine 139). After γ H2ax foci form, other repair factors are recruited and the complex works together to repair the damage.

HSCs are susceptible to DNA damage from replication stress, ROS, and various extrinsic DNA damaging factors¹²⁵. Recently, studies have shown that DNA replication associated with aging and chronic inflammation results in progressive accumulation of DNA damage leading to loss of HSC function^{122,126}. Furthermore, as HSCs age, their

capacity to proliferate and regenerate slows down¹²⁷. Interestingly, aged HSCs, are increased in numbers, but have diminished long term repopulating potential, and produce myeloid biased progeny at the cost of reduced lymphoid progeny¹²⁸⁻¹³⁰. Regarding the molecular mechanism of HSC aging, the involvement of WNT signaling pathway has been reported in many studies¹³¹. WNT signaling is differentially expressed in young (human CB derived) and aged (human MPB derived) HSCs¹³². Of many WNTs, WNT5A and WNT11 are involved in non-canonical signaling¹³³, whereas WNT1, WNT 3A and WNT8 are involved in canonical WNT signaling. During aging of HSCs, the non-canonical WNT predominates over canonical WNT signaling¹³⁴. This finding is supported by the study of Zanette et al., who showed an induction of non-canonical WNT signaling in *ex vivo* expanded HSCs based on their microarray studies¹³⁵. It has been suggested that due to the competition for binding with the WNT receptor, canonical WNT signaling pathway is at least partially inhibited by the activation of non-canonical WNT^{134,136}.

In summary, various mechanisms that are involved in regulating murine HSC function may not correlate well with human HSC function. Moreover, many studies performed in human CB derived HSCs are not relevant for gene therapy. Hence, studies in human HSCs derived from adult sources are essential for determining the success of gene therapy.

Mechanism of Human Hematopoietic Stem Cell Loss during *Ex Vivo* Manipulation and Gene Transfer

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Conflict of interest: None

Abstract

Hematopoietic stem cells (HSCs) are excellent targets for gene therapy (GT) to cure inherited disorders. However, genetic manipulation of HSCs causes tremendous loss of long-term repopulating potential (LTRP), which has resulted in failure of numerous GT trials, or necessitated very high doses of HSCs and pre-transplant conditioning for success. We show that non-cycling HSCs were more tolerant of genetic manipulation. Cycling HSCs, when genetically manipulated however, developed an exhausted/aged HSC phenotype with a myeloid-biased progeny. Mechanistically, we show that exit of HSCs from dormancy *in vitro* increased p38MAPK stress signaling, which activated the DNA damage response pathway (DDR), resulting in loss of HSC LTRP. Furthermore, induction of DNA double-strand break (DSB) in cycling HSCs (by γ -retrovirus/lentivirus integrase or Cas9-nuclease) triggered the G₂M cell-cycle checkpoint resulting in accumulation of transduced/edited HSCs in the G₂M phase, depletion of HIF1 α , and exhausted/aged HSCs. The combination of p38 inhibition with prostaglandin-E2 was necessary to reduce the DDR and stabilize HIF1 α to reverse the G₂M accumulation to restore the lineage-fate of transduced-HSCs with LTRP. Herein, we have identified fundamental mechanisms of loss of LTRP and HSC fate change with genetic manipulation that can be targeted, and have broad, important implications for the success of GT.

Introduction

Gene therapy (GT) is a promising alternative to allogeneic hematopoietic stem cell transplantation (HSCT) to cure patients with monogenic disorders who lack a suitable transplant donor^{1,2,3-15}. Its success depends on the ability of the gene-modified hematopoietic stem cells (HSCs) to differentiate into hematopoietic progenitor cells (HPCs) to regenerate the hematopoietic system, while simultaneously self-renew and regenerate themselves to sustain life-long blood cell production¹⁶⁻¹⁸. For GT, CD34⁺ hematopoietic stem and progenitor cells (HSPCs) containing the rare HSCs (CD34⁺38⁻90⁺45RA⁻49f⁺)¹⁹ are cultured for 2-4 days in cytokine-rich medium that enforces HSC division, making them more amenable to genetic manipulation²⁰, and then transplanted following pre-transplant chemotherapy conditioning.

GT trials using γ -retroviral vectors (RV), which only transduce dividing cells, were largely unsuccessful, or modestly successful only with 5-30 times higher transduced CD34⁺ HSPCs than that used for HSCT²¹, except for in treating immune deficiency disorders (IDDs)^{1,2}. Success in IDDs occurred because a few engrafting gene-modified HSCs are capable of producing the long-lived lymphoid progeny with tremendous amplification potential^{1,2}. Gene editing (GE) approaches require conditions similar to RV gene transfer – i.e. cycling HSCs, especially for homology directed repair (HDR)²²⁻²⁷. Like RV, with GE, high editing efficiencies *in vitro* do not translate to high LTRP *in vivo*^{23,28,29}. Lentivirus vectors (LV) that transduce non-dividing cells have had more success, but, other than in IDD³⁰, success has been achieved with very high HSPC dose, and myeloablative chemotherapy conditioning. LV-transduced HSCs are otherwise unable to compete with the residual HSCs, despite residual HSCs being compromised with the high-dose chemotherapy³¹⁻³³. A testament to the tremendous HSC loss, even with LV, is presence of a few hundred to thousand common integrants in multiple blood cell lineages in patients, which is 2-3 orders of magnitude less than the number of transduced HSCs transplanted.

These underscore the tremendous loss of long-term repopulating potential (LTRP) of HSCs with gene transfer/editing. Hence, failure of GT results from the poor understanding of the fundamental mechanisms of HSC loss and pathways which alter human HSC fate and their LTRP with genetic manipulation ; If these are identified and targeted, HSCs will be maintained

during genetic manipulation (with RV, LV or GE), exploiting the full therapeutic potential of GT.

Attempts to expand cord blood (CB) HSCs *in vitro*, a readily available source for HSCT, have largely failed^{34,35}. Recently, few promising compounds - p38/MAPK inhibitors^{36,37}, angiopoietin-like proteins³⁸ prostaglandin E2 (PGE2)³⁹⁻⁴², Stemregenin-1 (SR1)^{43,44}, UM171⁴⁵ and UNC0638⁴⁶ have shown a potential to increase the LTRP of CB HSPCs, but their mechanisms of action are either diverse or unknown; and most do not expand adult HSPCs. Indeed, CB HSCs, besides being a largely irrelevant source for GT, are distinct from adult bone marrow (BM)- or mobilized peripheral blood (MPB)-derived HSCs. CB HSCs are either cycling or more rapidly enter cell-cycle⁴⁷. Adult HSCs, on the other hand, are largely quiescent, and protect their genome through quiescence⁴⁸. Removing adult HSCs from their hypoxic BM-niche and manipulation of their genome after enforced cycling could induce HSC genotoxic stress signaling. p38 MAPK, a stress-activated kinase with diverse roles in various cellular processes⁴⁹, has been shown to impede CB CD34⁺ cell expansion and is induced by ROS^{36,37,50}. However, the mechanism by which it promotes loss of HSCs is unknown. Prostaglandin E2 (PGE2) is another recently studied factor that is known to increase HSC homing and survival^{39,41,42,51,52}. However, pathways that result in HSC survival are distinct from those promoting HSC self-renewal⁵³. It is conceivable that survival and self-renewal pathways intersect in HSCs undergoing gene manipulation to alter HSC fate.

Herein, using an adult model of human HSCs, we identified the fundamental mechanisms that affect the LTRP of quiescent and cycling HSCs with genetic manipulation. We show that HSC stress signaling and an excess DDR results from viral vector/gene editing nuclease-induced DNA double strand break (DSB), together, alter HSC cell cycle kinetics to change HSC fate, resulting in loss of LTRP and HSCs with a predominantly myeloid-biased progeny. We show that targeting these pathways restores genetically manipulated HSC fate and allows long-term maintenance of gene marking in both myeloid and lymphoid lineages. The findings have broad implications for the future success of GT using either viral vectors or gene edited human HSCs.

Results

Ex vivo HSC division and gene transfer results in loss of LTRP and a myeloid skewed gene-modified progeny. We first adapted the NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mouse xenograft model to an adult (BM/MPB-derived) HSC model. We transplanted 1 million MPB CD34⁺ cells in lethally irradiated NSG mice, and assessed serial engraftment in bone marrow (BM) at 6, 12 and 24-weeks (circulating human cells do not correlate with engraftment in BM; **Supplementary Fig. S1a-b**). A multi-potential graft, composed of B-, T- and myeloid cells was only evident by 24-weeks (**Supplementary Fig. S1c-d**), when 1:1 secondary transplants (2T) yielded successful human secondary engraftment (>0.01% human CD45⁺ cells). Multi-potential engraftment was assessed at 24-week in primary (1T) mice, and LTRP at 1.5-3 months in 2T mice (**Supplementary Fig. S2**).

We utilized two commonly clinically utilized protocols for LV and RV transductions. These protocols were specifically chosen to encompass viral vector-mediated gene transfer or gene editing (GE): The LV gene transfer is similar to that used for inducing non-homologous end joining (NHEJ)-mediated gene disruption by gene editing (GE) nucleases (zinc finger nucleases, homing endonucleases, TALENs or Cas9 nucleases), since both are HSC cell-cycle independent. RV gene transfer is similar to homology directed repair (HDR) to correct gene mutations by GE nucleases, as both require cycling HSCs. MPB CD34⁺ HSPCs were transduced with (a) GFP-LV within 18-24h and either immediately transplanted, or kept in culture and transplanted at 36-42h into irradiated NSG mice; (b) Alternatively, CD34⁺ cells were pre-stimulated for 2 days *ex vivo*, transduced with a GFP-RV at days 2 and 3 (44h and 68h), and transplanted into NSG mice between 72-96h (**Supplementary Fig. S2 a-b**). As a control, NSG mice were transplanted with unmanipulated CD34⁺ HSPCs immediately following their isolation (0h) (**Supplementary Fig. S2**).

Robust human hematopoietic engraftment (human CD45⁺ cells) was observed in BM under all conditions with no major variations at 24 weeks after 1T (**Fig. 1a**). However, there was a significant loss of LTRP in 2T animals in conditions where HSPCs were cultured for greater than 24h: the loss in LTRP was the highest in the RV 72-96h group (**Fig. 1b**). Gene transfer efficiency in CD34⁺ cells *in vitro* at 2 weeks (using the conventional colony forming assay) with

RV and LV was comparable. While LV-transduced (GFP⁺) human cells declined from an average of 80% *in vitro*, to ~50% by 24-weeks *in vivo*, there was a higher progressive reduction in RV-transduced (GFP⁺) cells from 80% *in vitro* to 5-8% *in vivo* (**Supplementary Fig. S3**). Hence, RV-transduced human xenografts were lost with time in the NSG mice, while those transduced with LV vectors were better sustained.

Multi-lineage reconstitution at 6, 12 and 24 weeks in BM in 1T mice was examined in untransduced (GFP⁻) and transduced (GFP⁺) progeny of HSPC. At 6-weeks, lineage output was similar in both the GFP⁺ and GFP⁻ populations and comparable to that derived from unmanipulated 0h controls. However, by 12-weeks, the 72-96h RV-gene modified population showed a significant increase in myeloid cell progeny, which occurred at the expense of reduced B lymphoid cell progeny (**Supplementary Fig. S4**); and by 24 weeks, the myeloid bias was extreme in this group, but also apparent in the 36-42h LV graft, which occurred at the expense of significantly reduced B and T lymphoid population; there were also significantly less GFP⁺ CD34⁺ cells in both the 36-42h LV and 72-96h RV grafts (**Fig. 1c-j**). It was noteworthy that there was no such lineage skewing seen in the untransduced progeny in any of the conditions, demonstrating that this lineage skewing was not an effect of the cytokine/culture conditions; furthermore, the untransduced lineage pattern was similar to the graft derived from 0h HSPCs. Hence, the myeloid lineage bias was secondary to gene transfer and increased time in culture. It is noteworthy that the self-inactivating RV used herein is devoid of viral enhancers and has not shown the insertional adverse events or lineage-skewing in experimental models^{54,55} and human trials^{56,57}. Similarly, the LV has also been tested *in vitro* and in human trials with no adverse events from vector insertion^{31,58}.

Collectively, gene transfer and ex-vivo culture beyond the first 24h resulted in a significant loss of LTRP in 2T mice, and the transduced CD34⁺ progeny was myeloid-biased, at the expense of the lymphoid progeny. The loss of LTRP mimic results of numerous RV GT trials and gene-edited xenografts^{28,29}, wherein there was either short-term engraftment (most unpublished,⁵⁹) or modest gene-marked long-term engraftment, despite a high HSPC dose and robust gene transfer *in vitro*²¹; In addition, the model also simulates successful LV GT trials that have shown stable gene marking with a short culture protocol³⁰⁻³³, but modest success/failure in

other LV trials where LV transductions were performed similar to the RV protocol⁶⁰, collectively validating this model as a preclinical model of gene-manipulated HSC engraftment and LTRP.

We investigated mechanisms for the loss of LTRP and myeloid-lineage biased progeny. RV gene transfer and GE-mediated HDR occurs in dividing cells. LV gene transfer and GE-mediated NHEJ can occur independent of the cycling status of cells, although LV also have a preference for dividing cells⁶¹. It is possible that the failure of RV trials was from preferential integration of transgenes into HPCs or multipotent progenitors (MPPs), that readily cycle and comprise 99% of the CD34⁺ HSPCs; and poor transduction of the quiescent HSCs, comprising ~1% of CD34⁺ cells. Using the phenotype of human HSCs identified by Dick and colleagues¹⁹, we find that the clinical transduction conditions utilized herein are indeed optimized to transduce human HSCs (CD34⁺CD38⁻CD90⁺CD45RA⁻CD49f⁺), MPP (CD34⁺CD38⁻CD90⁻CD45RA⁻^{19,62}) and total CD34⁺ cells comparably both with RV and LV (**Supplementary Fig. S5**).

Hence HSC transduced while cycling likely change fate to HPC or are lost. A careful assessment of the HSPC compartment showed no cell death of CD34⁺ cells or apoptosis of the HSCs; in fact there was an increase in the phenotypic HSCs with increased time in culture (**Supplementary Fig. S6 a-c**). EdU labeling showed that the highly HSC-enriched CD34⁺38⁻90⁺ cells entered cell-cycle only after 24h, and majority had undergone at least one cell division by 72h (**Supplementary Fig. S6 c-d**).

We hypothesized that HSCs are physiologically maintained in their hypoxic niches⁶³, and inducing cell division in cytokine-enriched culture in ambient oxygen conditions likely induces high oxidative stress⁶⁴. Indeed, reactive oxygen species (ROS) were significantly increased in HSCs with 72-96h of culture, and these ROS were generated from mitochondria (**Supplementary Fig. S6 e-f**). N-acetylcysteineamide was able to decrease ROS in the HSCs, but unexpectedly, resulted in a reciprocal decline in gene transfer efficiency (**Supplementary Fig. S6 g-h**). Therefore, we investigated the downstream pathways activated by increased oxidative stress.

High ROS has been shown to induce stress signaling, especially mitogen-activated protein kinases (MAPKs)³⁷. We analyzed the phosphorylation status of ERK, JNK and p38 MAPKs in the different culture protocols and found significant activation only of p38 MAPK (p38) with *ex vivo* culture beyond 24h (**Supplementary Fig. S7a-f**). Interestingly, there was significantly higher p38 activation in cycling HSCs, although not in transduced HSCs (**Supplementary Fig. S7g-i**). All p38 inhibitors (p38i)^{65 66} decreased p38 phosphorylation in HSCs, showing specificity of activation of this pathway (**Supplementary Fig. S7 j-k**). A previous study has shown that CB CD34⁺ cells cultured for a week with p38 inhibition have increased human engraftment at 4 months in xenografts³⁶. This time-point may still reflect HPC output. Hence we explored the effect of p38 inhibition on LTRP of adult gene-modified HSCs.

We chose Birb-796, a selective p38 α inhibitor⁶⁷, at concentrations far below those causing non-specific inhibition; and observed significantly lower phospho-p38 (p-p38), to levels in unmanipulated HSCs in all conditions (**Fig. 2 a-b**). To distinguish the effect of gene transfer into non-cycling HSCs versus cycling HSCs from the effect of culture duration, we performed serial transplants of human CD34⁺ HSPCs transduced with LV within 18-24h (non-cycling HSCs) and transplanted after 24, 36-42 or 72-96h, or transduced with RV at 44h and 68h (cycling HSCs) and transplanted at 72-96h (**Supplementary Fig. S2**), with or without the p38i. At 24 weeks, human CD45⁺ cell engraftment in the NSG BM was similar among all the groups with/without p38i, and indeed improved with p38i for the 72-96h LV and RV groups (**Fig. 2c**), similar to results reported in CB CD34⁺ cells³⁶.

Interestingly, p38i restored the LTRP in 2T mice transplanted with 36-42h and 72-96h groups to the levels observed in 0h and 24h groups (**Fig. 2d and Supplementary Fig. S8**): nearly one-third 2T mice did not engraft when CD34⁺ cells were cultured for longer than 24h without p38i; but addition of a p38i restored the LTRP in 2T mice to levels seen with un-manipulated HSPCs, regardless of vector type or when they were transduced.

P38i also restored the multi-potentiality of the graft at 24 week (1T) to levels similar to 0h controls, if gene transfer into HSCs occurred in the first 24h (when not in cycle) of culture, even if they were kept in prolonged cultures thereafter and then transplanted. However, when

cycling HSCs were transduced (the RV group), the pronounced myeloid skewing of the transduced progeny was somewhat reduced, but still significant, (**Fig. 2 e-f**), with a corresponding reduction in B cells (**Fig. 2 g-h**) and CD34⁺ HSPCs (**Fig. 2i-j**). This effect was specific only to genetically manipulated HSC progeny (**Fig. 2 e-j**). Taken together, p38i during gene transfer maintained transduced HSC LTRP during prolonged *ex vivo* culture conditions, but the extreme myeloid biased progeny and reduced lymphoid potential with transduction of cycling HSCs was only partly rescued.

Mechanism of action of p38 inhibition on human HSC fate

We next investigated the mechanism by which p38 inhibition can retain the LTRP of cultured and transduced HSCs. p38i had no effect on the total number of CD34⁺ cells, or viability *in vitro* and also no effect on gene transfer efficiency *in vitro* or *in vivo* (**Supplementary Fig. S9 a-c**). The HSC compartment also showed no difference in apoptosis, transduction efficiency, or ROS levels with p38 inhibition; and increased phenotypic HSC population was similar except in prolonged cultures, when phenotypic HSCs were higher with p38i (**Supplementary Fig. 10**).

RV/LV integrase^{8 68} or GE nuclease mediated DNA double-strand break (DSB) could evoke DNA damage response and repair (DDR) pathways. We therefore examined whether p38 inhibition reduces the DDR with HSC division and gene transfer, in FACS-sorted HSC by immunofluorescence. Indeed, increased γ H2AX foci/cell were seen in transduced HSCs in either the 42h or 72h group; which were highly significantly reduced with p38 inhibition (**Fig. 3 a-c**); 53bp1 staining, concurrently performed, ensured that increased γ H2AX was associated with DDR foci (**Fig. 3 a-c**).

We also compared the DDR pathway activation in transduced and untransduced HSCs by flow cytometry for γ H2AX. As compared to unmanipulated HSCs (0h), increased γ H2AX MFI and increased number of γ H2AX positive HSC were seen even at 24h (a period where HSC are not in cycle), which returned to levels seen in unmanipulated HSCs with p38i treatment (**Fig. 3d-f**). Notably, this population is transduced, although GFP protein expression is not present at this early 24h time point. Hence, increased DDR at 24h is likely caused by the gene transfer induced DSB. After 36 hours, the untransduced (GFP⁻) versus transduced (GFP⁺) HSC

population could be separately analyzed. The transduced (GFP⁺) population had higher γ H2AX levels, and percentage of γ H2AX positive HSCs than the untransduced (GFP⁻) population, both in the 36-42h and 72-96h group; p38i significantly reduced the percentage of γ H2AX⁺ cells, although at these later time points, the levels of γ H2AX did not return to levels seen in 0h HSCs [both the percentage of HSCs that stain for γ H2AX (**Fig 3d-f**) and the γ H2AX MFI (**Fig. 3d-e**)]. Inducing dormant HSCs to cycle also triggers DDR⁶⁹. Our data show that p38 inhibition significantly reduces the DDR in HSCs both with increased cycling and with transduction, although not to baseline levels seen in unmanipulated HSC.

P38 activation was highest in cycling HSCs (**Supplementary Figs. S6 d and S7 g-h**). Therefore, we examined the role of p38i on HSC cell-cycle kinetics. Nearly all HSCs were in G₀ phase when freshly isolated from MPB (**Fig. 4a**) or BM (**Supplementary Fig. S12**); by 24 hours, a third of them transitioned to the G₁ phase, but most HSCs were not cycling (not in S-G₂M phase). p38i significantly increased the proportion of HSCs in the G₀ quiescent phase, and decreased the proportion of HSCs in the S-G₂M phase, before the first HSC division *in vitro* (24h). However, after HSCs progressed through cell cycle, the effect of p38i on increasing the G₀ population was lost (**Fig. 4a-b**). Hence when the transduction/DSB occurs when HSC are not in cycle, the p38i-mediated delay in transition from G₀ to G₁ and G₁ to S phase (**Fig. 4 a-b, Supplementary Fig. S11**), conceivably allowing DDR to subside; hence HSC fate is maintained, as shown by rescue of LTRP and a balanced lineage production. The same phenomenon was also seen in HSCs derived from adult human bone marrow (**Supplementary Fig. S12**). We also noted significantly lower GFP⁺ HSCs in G₀ phase at 42h, even though they were transduced within 24h, suggesting that LV preferably transduced G₀ cells that were not deeply dormant, and by 42h, these had progressed to G₁ and S-G₂M phase. However, when HSCs were transduced when they were actively cycling, as with RV, there was no difference in the percentage of cells in the different phases of cell cycle, with or without p38i (**Fig. 4 a-b; supplementary Fig. S11**).

Remarkably, only the transduced (GFP⁺) 72h RV population showed a highly significant reduction in HSC in G₁ phase and increase in those in S-G₂M phase, regardless of p38

inhibition. Since in this condition, the transplanted HSCs produced a myeloid-biased progeny that p38i did not significantly rescue, we examined the S- G₂M phase in depth (**Fig. 2**).

Crosstalk between S-G₂M checkpoint and HIF-1 α links RV-induced DSB, cell cycle and HSC fate

The S-G₂M kinetics of transduced and untransduced non-cycling and cycling HSCs showed that when DSB occurred in cycling HSCs (RV transduction after 2 days of culture) there was accumulation of HSCs in the late S and G₂M phases (**Fig. 4c**). However, when vector integration occurred within the first 24h (with LV), this phenomenon was not seen (**Fig. 4d**). Furthermore, this effect was not specific to RV, but specific to cell cycle phase when HSCs were transduced. CD34⁺ cells from the same donor were cultured for ~2 days (44h) and transduced either with LV (LV late or LV^L), or with RV at 44h and 68h, or with LV within 24h but kept in culture for 72h (LV^E) (**Supplementary Fig. S13 a**). We observed the late S and G₂M accumulation only in transduced (GFP⁺) cycling HSCs in the LV^L or RV groups (**Fig. 4 e-f**). The G₂M accumulation was lesser in the LV^L than RV, since LV transduces both cycling and non-cycling HSC, and therefore the GFP⁺ population comprised of a mixed population. Notably, this G₂M accumulation did not result in apoptosis (rather, HSC numbers were increased, **Supplementary Figs S6c and S10c**), and HSCs transitioned out of the G₂M phase when followed serially (**Fig. 4e**). This G₂M accumulation was not seen when non-cycling HSCs were transduced (24h LV or LV^E; **Fig. 4e**), even when cell cycle analysis was performed at the same time points after transduction with LV^E versus RV (**Fig. 4 c-d**).

The increase in phenotypic HSCs, which fail at maintaining LTRP and produce a myeloid biased progeny, is reminiscent of aged/exhausted HSCs. We sorted the HSC population after LV and RV at 24h and 72h, respectively, from one experiment for RNAseq analysis, to get an overview of the transcriptional profile of aging-related genes. Wnt5a and Wnt4 (non-canonical Wnt genes associated with aging of mouse HSC^{70,71}) mRNA levels were undetectable in the human CD34⁺38⁻90⁺RA⁻49f⁺ HSC, and only Wnt5b, Wnt11, and Fzd3 (a downstream target of Wnt signaling⁷²) expression was at detectable levels. A qPCR for these genes in 24h LV^E and 72h LV^L sorted HSCs from different MPB donors, using P34 (Cdk2) mRNA expression to

validate the non-cycling and cycling phases of HSCs at these time points, showed increased Fzd3 and Wnt family genes in the LV^L group (**Supplementary Fig. S13b**).

To further validate that this phenomenon is not vector induced, but occurs from any induced DSB in cycling HSCs, we determined if this G₂M accumulation would also occur with a gene editing nuclease. To prove the concept, we performed an experiment where CD34⁺ cells were cultured for the same 44h period as LV^L and RV groups, and then nucleoporated with a Cas9/hCD45gRNA ribonucleoprotein complex, that would induce a rapid DNA DSB in the human CD45 alleles; controls cells were nucleoporated with vehicle, without the Cas9/gRNA ribonucleoprotein complex. We observed the G₂M accumulation in the HSC population with Cas9 nuclease-induced DSB (shown by loss of CD45 expression by FACS in HSCs; **Supplementary Fig. 13e-f**).

The delay of HSC in the G₂M phase changes HSC lineage fate, resulting in a myeloid biased progeny: the 24h LV and 72h LV^E groups at 6 months post-1T showed no myeloid biased, but 72h LV^L or RV groups had a myeloid-biased transduced HSC progeny at the expense of loss of B-lymphoid potential (**Supplementary Fig. S13 c-d**). Collectively, our data shows that when a DSB is induced in cycling HSCs via vector integration or a nuclease, the DDR stalls the HSC in the G₂M phase, and this delay results in HSC that primarily produce a myeloid progeny. A concomitant increased number of HSC with increased expression of Fzd3 and Wnt5b suggests a HSC phenotype associated with exhaustion or aging⁷¹; alternatively, it is conceivable that the lymphoid biased HSC population may be more vulnerable⁷³ to fate change with accumulation of HSCs in G₂M phases.

We sought for mechanisms of G₂M accumulation, since their identification and targeting could restore normal cell-cycle progression in the HSCs transduced when cycling; is essential for HDR-mediated gene editing since few human diseases can be corrected with NHEJ-mediated gene disruption. DNA DSB classically triggers the cell cycle check point, ATM/Chk2 kinase, but if DSB occurs in S and G₂ phase, it can trigger ATR/Chk1 kinase⁷⁴⁻⁷⁷. More recently, activation of MK2/p38 MAPK by DNA DSBs during V(D)J recombination in thymocytes has been shown to induce a G₂M cell-cycle checkpoint⁷⁸; and besides the canonical ATR/Chk1 and ATM/Chk2

kinases, MK2/ p38 MAPK has recently been shown to be the non-canonical check-point kinase that controls the cell-cycle response to DNA damage in parallel to Chk1⁷⁹. We used a specific Chk1 inhibitor (MK-8776; Chk1i), a Chk2 inhibitor (PV1019; chk2i) and p38i alone or in combination with Chk1i or Chk2i to determine their role in HSCs accumulated in G₂M phase. We found that fewer transduced-HSCs accumulated in the G₂M phase with Chk1i (albeit not statistically significant), but not with Chk2i (**Supplementary Fig. S14a, Fig. 5a**). However, remarkably fewer transduced-HSCs accumulated in G₂M phase with the combination of Chk1i and p38i, but not Chk2i and p38i, suggesting that Chk1 kinase and p38 MAPK induce the G₂M checkpoint, when DSB is induced in cycling HSCs (**Fig. 5b**). In fact, Chk1 alone did not reduce γ H2AX levels in the transduced HSCs, which required p38i; and the combination of Chk1i and p38i did not have any additive effect in reducing γ H2AX (**Fig. 5b**).

Studies in HIF-1 α deficient mouse embryonic fibroblasts show constitutive Chk1 expression⁸⁰, suggesting that HIF-1 α is upstream of Chk1 activation and HSCs in HIF-1 α deficient mice develop an aged HSC phenotype⁸¹. We hypothesized that delay in cell-cycle progression in HSCs could result in destabilization of HIF-1 α , mediating the transduced HSC phenotype. We observed a significant decrease in HIF-1 α levels by immunofluorescence, when we cultured HSCs for 72 hours, as compared to 0h HSCs; moreover, HIF-1 α levels were further reduced in HSCs that were transduced when cycling (**Fig. 5d**). Recently, prostaglandin E2 (PGE2), which has been shown to enhance HSC survival and homing in murine and CB HSCs^{42 52 82}, has been shown to stabilize HIF-1 α in HSCs⁸². Therefore, we investigated if PGE2 would stabilize HIF-1 α in cycling, transduced, adult HSCs, and whether this stabilization would prevent G₂M accumulation and restore the HSC lineage fate. Indeed, immunofluorescence analysis for HIF-1 α in sorted highly HSC-enriched CD34⁺38⁻90⁺ cells demonstrated that HIF-1 α was significantly reduced by RV induced DNA DSB in cycling HSC, and it could be stabilized with PGE2, albeit, the highest HIF-1 α levels were seen when PGE2 was combined with p38i (**Fig. 5c-e**). We confirmed the immunofluorescence data with flow cytometry (**Supplementary Fig. S14b**). Furthermore, increased HIF-1 α in transduced cycling HSCs was associated with their reduced accumulation in the G₂M phase of cell cycle. Importantly, PGE2 alone tended to reduce the number of cycling transduced CD34⁺38⁻90⁺ cells in G₂M phase, but a significant reduction only occurred with the combination of PGE2 and p38i. Notably, PGE2 alone had no

effect on reducing the DDR (reducing γ H2AX levels) in these HSC-enriched populations. However, p38i significantly reduced the γ H2AX, and the combination of p38i and PGE2 resulted in a highly significant reduction of γ H2AX (**Fig 5g and supplementary Fig. S14c**).

p38i combined with PGE2 restore LTRP and lineage fate of transduced HSCs.

We performed a limiting dilution transplant with the combination treatment of p38i and PGE2 in NSG mice. Here, we cultured CD34⁺ cells overnight and then transduced them with LV 12h apart (at 18 and 30h), for a total culture period of 42 hours, and transplanted them into NSG mice with limiting doses of CD34⁺ cells, starting with half the dose (500K CD34⁺ /mouse) used in prior experiments. We observed myeloid skewing and loss of B-lymphoid potential at 6mo in the 500K and 250K cell groups, similar to RV transduced cells (**Fig. 6 a-d**). Remarkably, at further limiting HSC doses, which impose severe regenerative stress on engrafted HSC, we observed a myeloid skewing and loss of B lymphoid potential even in the untransduced CD34⁺ cells, suggesting that excessive HSC proliferation induces the myeloid biased progeny. Treatment with p38i alone was sufficient to restore lineage balance in untransduced progeny at higher HSPC dosage, but had less effect on transduced HSCs progeny. PGE2 alone was sufficient to restore the lineage skewing, although at the lowest cell doses, where there was a combination of excess cycling and transduction stress, the combination of PGE2 and p38i was most effective at reversing the myeloid skewing and restoring the B lymphoid potential, rather than PGE2 alone (**Fig. 6 a-d**). Moreover, the total human engraftment in the bone marrow at 24 weeks after 1T revealed higher engraftment levels with the p38i and PGE2 combination in the higher CD34⁺ dose groups, where engraftment could be meaningfully analyzed (**Fig. 6e**). The competitive repopulating units (CRU) in BM at 6 months were enhanced nearly five-fold with the combination treatment, as compared to the single treatment with p38i or PGE2 (**Fig. 6f**). Furthermore, the assessment of LTRP in 2T mice at 3 months confirmed the effect of the p38i and PGE2 combination treatment, at increasing both the LTRP and reversal of the myeloid skewed phenotype (**Fig. 6 g, i-l**). It is to be noted that the use of p38i or PGE2 or the combination did not have any effect in the GFP marking, even in the long-termed secondary human grafts (**Fig. 6 h**).

Discussion

Herein, we show fundamental and distinct mechanisms by which *in vitro* genetic manipulation of HSCs results in HSC loss and fate change. p38 stress signaling has been shown to be induced by ROS³⁷, and by HSC cycling in CYLD (Cylindromatosis) mutant mice *in vivo*⁸³; and its inhibition improves engraftment in CB-derived CD34+ cells and mouse HSCs^{36,50,84}.

However, the mechanism by which p38 activation mediates these effects is unknown. We show that p38 activation increases the DDR, which, that reduces the LTRP of HSCs. Our study also explains the mechanism of increase DDR with exit from dormancy and HSC attrition seen *in vivo* in mouse HSCs⁶⁹, and shows that the DDR response is further exaggerated by gene transfer-mediated DSB, which is also reduced with p38 inhibition.

In addition we show that the DNA-DSBs, induced by vector integrase or gene editing nucleases in cycling HSCs destabilize HIF-1 α and evoke Chk1/p38-mediated G₂M checkpoint, altering the cell cycle progression of HSC, which appears to age HSC (increased numbers of HSCs that have poor LTRP, a myeloid biased progeny and upregulation of Wnt signaling). While our data supports p38 as a non-canonical checkpoint kinase in cycling HSC, where it acts in concert with chk1 to evoke the G₂M checkpoint, increased p38 signaling may also hasten HSCs into cycle, since p38 inhibition increases HSCs in G₀ phase, and this rapid transition of HSCs from G₀ phase alters their fate to HPCs, that have reduced LTRP. Cell cycle and cell fate have been intimately linked through chromatin remodeling in many stem cell types⁸⁵. Our data reveals that alteration in specific cell-cycle phase duration during the first HSC division *in vitro* is sufficient to alter HSC fate.

While PGE2 can also mediate improved LTRP via HSC survival and homing, we observe only slightly increased CRU with PGE2 alone; while PGE2 alone however, significantly reverses the myeloid biased HSC progeny. However, both PGE2 or Chk1i require concomitant p38 inhibition to significantly abrogate the G₂M accumulation, and more importantly to reduce DDR in HSCs, and these combined effects improve the CRU potential of cycling adult HSCs by nearly five-fold. It is also conceivable that PGE2 mediates survival of the lymphoid-biased HSCs, which are more vulnerable to genotoxic stress⁷³. Regardless, this study provides critical

mechanistic insight based solution for both vector-mediated gene transfer or nuclease mediated gene correction of cycling HSCs without alteration of their lineage fate and LTRP. Recently, Stemregenin-1 (an aryl hydrocarbon receptor inhibitor) shown to expand CB HSPC, and PGE2 together were shown to increase gene targeted CB CD34+CD133+CD90+ HSPC that produced lymphoid progeny; while this increase may be secondary to their overall expansion with Stemregenin-1 prior to targeting, PGE2 may have helped HSC-lineage fate retention.²⁷

Additionally, we observe that DDR is activated even in non-cycling transduced-HSCs, but HSCs appear much more tolerant of this genotoxic stress when quiescent. Hence GE methodologies geared towards gene disruption via NHEJ could be targeted to quiescent HSCs without sustaining the genotoxic stress associated with GE of cycling HSC; and here, p38i can dampen the DDR and likely reduce the regenerative stress. From a translational perspective, our studies explain the basis of the outcomes of RV and LV GT trials, and explain the poor *in vivo* engraftment of gene targeted HSPCs (where HDR occurs in cycling HSC), despite high *in vitro* gene editing efficiencies.

In conclusion, by identifying the mechanism of loss of LTRP and HSC fate change with gene transfer, we provide important insights into gene transfer/repair at different cell-cycle phase of HSCs; and provide the means of genetic manipulation of cycling HSCs by inhibition of p38 stress signaling and stabilization of HIF-1 α , that is applicable to vector-mediated and GE-mediated HDR.

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Methods

Human CD34⁺ cell Isolation, culture and transduction

Fresh G-CSF mobilized peripheral blood cells were collected via apheresis from normal healthy volunteers using an IRB approved protocol from the Cell Processing Laboratory, CCHMC, and were subjected to a positive selection using anti-CD34 antibody on the CliniMacs (Miltenyi Biotech, Germany) or Indirect CD34 Micro Bead Kit, human (Miltenyi Biotech, Inc. Bisley, Germany) as described previously²¹ to a greater than 95% purity. Fresh or cryopreserved CD34⁺ was used freshly for ex vivo culture and transduction. For controls, freshly isolated CD34⁺ cells were immediately transplanted into NSG mice. In some experiments, normal bone marrow donor derived CD34⁺ cells were purchased from Cell Processing Core at Cincinnati Children's Hospital Medical Center.

Freshly isolated CD34⁺ cells were used, wherever possible, and especially for 0h controls. In some experiments cryopreserved CD34⁺ cells were thawed for 4hrs in IMDM containing cytokines, then washed and used for experiments. CD34⁺ cells were transduced in X-VIVO 10 (Lonza) medium supplemented with 2% human serum albumin (Baxter), recombinant human Flt-3 ligand (Flt3-L; 200 ng/mL), stem cell factor (SCF; 300 ng/mL), thrombopoietin (TPO; 100 ng/mL) (all cytokines obtained from PeproTech), and penicillin-streptomycin (ThermoFisher Scientific) on non-tissue culture treated plates coated with retronectin (4 μ g/cm², CH-296, Takara Bio Inc.). For LV transduction, CD34⁺ cells (2-5 x 10⁶/mL) were pre-stimulated for 4-8 hours and transduced twice with lentivirus vector (LV) maintaining the vector concentration

between 5×10^7 to 1×10^8 infectious unit (IU) per mL of media volume, 12-14 hours apart. Cells were kept in culture for a total of either 18-24 hours, 36-42 hours or 72-96 hours. For RV transduction, human CD34⁺ cells were pre-stimulated for ~2 days (42-48 hours) at a cell density of 3×10^5 cells/mL. Retroviral coated tissue culture flasks or plates were preloaded at room temperature with GALV pseudotyped RV twice for one hour each. The vector supernatant was removed and the cells were loaded into the RV loaded retroviral coated flasks. RV transduction was also performed twice 24 hours apart at an MOI ranging from 5 to 10. After total of 72-96 hours in culture, cells were harvested. LV and RV transductions were performed at 37°C under 5% CO₂ in ambient oxygen concentrations (20% O₂). For some experiments, LV was added just like RV after 2 days of pre-stimulation. p38 MAPK inhibitors Birb 796 (600nM), Vx-745 (1µM), or LY 2228820 (500nM) (all from Selleckchem) were supplemented to the culture media. The Chk1 inhibitor: MK-8776 (1µM) (Selleckchem S2735) or Chk2 Inhibitor: PV1019 (1 µM) (Calbiochem 220488) were added only with transductions; 16,16 dimethyl Prostaglandin E2 (PGE2) (10µM) (Cayman) was added at the beginning of culture, at first transduction and one hour before harvest. N-acetyl cysteine amide (NACA) (Sigma A0737) was used in the concentrations as described.

Following transduction, cells were harvested and washed and resuspended in PBS, and injected intravenously (1×10^6 cells per mouse) into NSG mice that had received 280 cGy radiation, using a 135Cs source (Mark I Model 68A Irradiator, J.L. Shepherd and Associates, San Fernando, CA). In some experiments, a portion of the cells were plated on MethoCult GF 4434 (Stem Cell Technologies, Vancouver, Canada) to determine the gene transfer efficiency at day 14 in colony forming unit-cells (CFC), and in liquid cultures to estimate the percentage of eGFP⁺ cells at days 7.

Xeno-transplantation

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were bred and maintained in a specific pathogen-free environment and all experiments were approved by the Institutional Animal Care and Use (IACUC) Committee of Cincinnati Children's Research Foundation. Eight to 14 week old NSG mice were fed doxycycline chow (TestDiet; Modified Prolab RMH-1500 with 0.0625%

doxycycline) a week prior to, during and for 2 weeks post irradiation. Human engraftment was analyzed in bone marrow (BM), via BM aspirates performed at 6 and 12 wks from left and right femurs. After sacrifice at 24 wks, marrow from all of the primary mice (1T) was harvested. Peripheral blood analysis was also performed in some experiments. Secondary transplants (2T) were performed after mouse CD45⁺ cells were depleted from BM harvested from each 1T mouse separately, using Biotin Rat Anti-Mouse CD45 (BD Biosciences 553078) and Streptavidin Particles Plus-DM (BD Biosciences 557812) and injected into one irradiated (280 cGy) secondary NSG mouse. For calculating competitive repopulating units (CRU) from limiting dilution transplantation, the L-Calcul software (Stem Cell Technologies) was used.

Lentivirus and γ retrovirus vector constructs

Lentiviral vector (LV) pRRL.SIN.cPPT.MNDU3.eGFP.WPRE⁸⁶ encodes the enhanced green fluorescent protein (GFP) under the control of MNDU3 promoter. The vector was packaged using the VSV-G envelope. The retroviral vector (RV) pSRS11.EFS.GFP.PRE (GALV)⁸⁷ encodes GFP under the control of a short EF1 α (EFS) promoter, and was packaged using the GALV envelope.

Flow cytometry analyses

PE-Cyanine 7 or APC-eFluor 780, APC- or PE- conjugated antibodies directed to human CD34 (BD Biosciences 560710 or 555824), CD38 (ebiosciences, San Diego, CA 47-0389-42), CD90 (BioLegend 328120), CD45RA (eBiosciences 17-0458-42), CD49f (BD Biosciences 555736), CD45 (BD 55485 or Biolegend 304026), CD33 (eBiosciences 25-0338-42), CD19 (BD Biosciences 557791), or CD3 (BD Biosciences 555340). Cells ($\sim 5 \times 10^5$ - 1×10^6) were used to label cells as per manufacturer instructions with appropriate isotype controls. For phospho-flow, primary antibodies phospho-p38 (Thr180/Tyr182; 4092S), phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204; 4094S), and phospho-JNK (Thr183-Tyr185 PE conjugate; 5755S) (all from Cell Signaling) were used for staining overnight followed by wash and secondary antibody stain including Goat anti-Rabbit IgG (H+L) or Goat anti-Rabbit IgG (H+L) or Pacific Blue (P-10994) antibody. Apoptosis assay was performed using PE Annexin V Apoptosis Detection Kit (BD Biosciences 559763) according to the manufacturer's instructions. Total ROS and mitochondrial specific ROS were detected using 10 μ M CM-H2DCFDA (ThermoFisher

Scientific C6827) and 5 μ M MitoSOX Red (ThermoFisher Scientific M36008) respectively, following manufacturer's instructions.

For cell cycle analysis, cells were first labeled with surface markers, fixed and permeabilized using BD Fixation/Permeabilization solution (BD Biosciences 554714) and then stained with PerCP-CyTM5.5 Mouse anti-Ki-67 (BD Biosciences 561284), Alexa Fluor 647 Mouse anti-Ki-67 (BD Biosciences 558615) and Hoechst 33342 (Sigma-Aldrich B2261) at 10 μ g/mL, as described previously⁸⁸. For EdU incorporation assay, cells were incubated with 10 μ M EdU throughout the culture period and the Click-it Plus EdU Pacific Blue Flow Cytometry Assay Kit (ThermoFisher Scientific C10636) were used for staining. For DDR staining, fixed and permeabilized cells were stained with PE mouse anti-H2AX (pS139) (BD Biosciences 562377). Samples were run through a FACSCanto, Fortessa, or LSR 2 flow cytometers (BD Biosciences), and data was analyzed by FACSDiva software (BD). In some experiments, cells were stained with the appropriate antibodies as stated above and then sorted using BD FACS Aria II cell sorter.

Immunofluorescence

FACS sorted CD34⁺ 38⁻ 90⁺ cells were fixed in 2% paraformaldehyde and permeabilized using 0.1% Triton X-100 (Sigma-Aldrich). Cells were then stained with primary antibodies Anti-phospho-H2AX (Ser 139) (Millipore, 05-636), anti-53BP1 antibody (Novus Biologicals, NB100-904), or anti-HIF-1 α (ab103063). For secondary antibodies, Alexa Fluor 594 conjugated Goat anti-Rabbit IgG (H+L) (A-11037) or, Alexa Fluor 647 conjugated Goat anti-Rabbit IgG (H+L) (A-21245) (both from ThermoFisher Scientific) were used for 1 hour at 37 $^{\circ}$ C. Cells were counterstained and mounted with vectashield antifade mounting medium with DAPI (H-1200 Vector Laboratories). Images were obtained using a Nikon 90i upright microscope.

Nucleoporation of Cas9/gRNA RNP in human CD34⁺ cells

Cas9 protein was obtained from UC Berkeley Macro Lab. CD45 sgRNA was synthesized by assembly PCR and *in vitro*-transcription using the GeneArtTM Precision gRNA Synthesis Kit. The quality of the sgRNA sample was determined by running it on a 10% NovexTM TBE-Urea Gel and a discreet band at 100 bases indicated intact sgRNA. Cas9 buffer was prepared with

20 mM HEPES pH 7.5, 150 mM KCl, 1 mM MgCl₂, 10% glycerol and 1 mM TCEP. Thawed and 44 hours pre-stimulated human MPB derived CD34⁺ cells were subjected to nucleofection with Cas9 RNPs. Cas9 RNPs were assembled immediately prior to nucleofection of CD34⁺ cells as described previously²⁹. To nucleofect a 20µL cell suspension (150,000- 200,000 cells) with Cas9 RNP, a 5 µL solution containing 120pmol of sgRNA in Cas9 buffer was prepared. A 5 µL solution containing 100pmol Cas9 protein in Cas9 buffer was prepared and added to the sgRNA solution slowly over ~30 seconds, and incubated at room temperature for 20 minutes. For each nucleofection, 150,000 to 200,000 CD34⁺ cells were re-suspended in 20 µL P3 solution (Lonza), and mixed with 10 µL Cas9 RNP. This mixture was then nucleofected using the Lonza 4D nucleofector using the E0100 program. Nucleofected cells were recovered in the fresh media (supplemented with human cytokines as described above) and cultured at 37 °C for further time periods. Cells were then harvested at 6 and 24 hours post-nucleofection for cell cycle staining. The remaining cells were cultured for 8 additional days prior to genotyping and CD45 staining for flow cytometry.

Quantitative PCR analyses

RNAseq analysis was performed on RNA from FACS sorted cultured and transduced CD34⁺CD38⁺CD90⁺CD45RA⁺CD49f⁺ HSC at 24h and 72h with and without treatment with p38i in one experiment. For qRT PCR, cultured and transduced cells were harvested and sorted for CD34⁺CD38⁺CD90⁺ markers using FACS Ariel cell sorter (BD) directly into TRI Reagent (Molecular Research Center, Inc). Total RNA was purified with phase separation by chloroform and precipitation by isopropanol. cDNA was generated using the SuperScript IV VILO Master Mix (ThermoFisher Scientific 11756050). The cDNA was amplified by qPCR using the primer/probe sets as follows: FZD3 Hs00184043_m1, WNT5B Hs01086864_m1, p34 CDC2 Hs00938777_m1 (ABI/ThermoFisher, 4331182) and analyzed on ABI 7900 Fast Real-time PCR system. The master mix used for qPCR set up was iTaq Universal Probes Supermix (BioRad 1725134). Gene expression level was calculated using $\Delta\Delta C_t$ method, normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABI/ThermoFisher).

Statistical Analyses

Data are expressed as Mean $\pm$ standard error of the mean (SEM). Depending upon the groups, data was analyzed by performing Mann-Whitney U test, paired Student's t- test or Wilcoxon test, log rank test as indicated in the figure legends, using the GraphPad Prism (V.7) software. P values ≤ 0.05 were considered significant.

Author Contributions

AS designed and performed the experiments, plotted, analyzed the data and wrote the manuscript, TH, SF, AL, AH, RJ, DMP assisted with critical experiments and analyzed data, MDF helped with developing critical techniques, experimental design and interpretation of the data and writing of the manuscript, PM conceptualized the project, designed the experiments, analyzed and interpreted the data and wrote the manuscript, all authors reviewed/edited the manuscript.

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Figure Legends

Figure 1. *Ex vivo* manipulation and gene transfer in human CD34⁺ HSPC for longer than 24 hours results in significant loss of LTRP and a myeloid-skewed gene-modified progeny.

(a) Freshly isolated or thawed human mobilized peripheral blood derived CD34⁺ cells were cultured and transduced with lentiviral vector (LV) or γ -retroviral vector (RV) for the indicated time period. An equivalent input of 1 million CD34⁺ cells were injected per irradiated NSG mouse. The percentage of human CD45⁺ cells in bone marrow (BM) of primary NSG mice at the indicate time points is shown. The x-axis denotes the number of weeks after primary transplant (1T). Data are representative of mean $\pm$ SEM; for 0h mock n=10, for 18-24h n= 20, for 36-42h n= 9, for 72-96h n= 19 mice for the total of 58 mice. **(b)** Total human cells in BM of primary NSG mice were transplanted one to one into irradiated secondary mice. Human engraftment in bone marrow (Y-axis) 6 weeks after secondary transplantation (2T) is shown (for 0h mock n=7, for 18-24h n= 5, for 36-42h n= 14, for 72-96h n= 14 for the total of 40 mice. Multi-lineage reconstitution was analyzed 24 weeks post 1T. Both untransduced (GFP⁻) and transduced (GFP⁺) human CD33⁺ myeloid population **(c, d)**, human CD19⁺ B-Lymphoid **(e, f)**, human CD3⁺ T-Lymphoid **(g, h)**, & human CD34⁺ HSPC population **(i, j)** are shown. Data expressed as mean $\pm$ SEM; n = same as **Fig. 1 a**. Statistics: Mann Whitney U test, **** P<0.0001, *** P<0.001, **P<0.01,*P<0.05.

Figure 2. Inhibition of p38 MAPK during *ex vivo* culture rescues the long term repopulating potential (LTRP) of HSCs and partially reverts the myeloid skewing phenotype. Human CD34⁺ cells were cultured and transduced with lentiviral vector (LV) or γ -retroviral vector (RV) expressing green fluorescent protein (GFP) for the indicated time points.**(a)** Representative flow cytometric histogram plot of p-p38 MAPK (number represents

mean fluorescence intensity (MFI). **(b)** Quantitative fold change of p-p38 MAPK MFI ($\pm$ SEM) in human CD34⁺ 38⁻ 90⁺ 45RA⁻ 49f⁺ HSCs with or without p38 inhibitor (p38i). **(c)** Human CD45⁺ engraftment in NSG mice with (striped bars) or without p38 inhibitor (solid bars) after 24 weeks post 1T **(d)** Human engraftment in NSG mice with or without p38i after 6 weeks post 2T. Non-engrafted mice (<0.01% CD45⁺ cells in the whole BM) over total mice transplanted is shown as percentage. Statistics: Fisher's exact test. Human CD33⁺ myeloid **(e & f)**, CD19⁺ B-Lymphoid **(g & h)** and CD34⁺ HSPCs **(i & j)** re-constitution with or without p38i (left panel, **e, g, & i**: GFP⁻ untransduced; right panel **f, h & j**: GFP⁺ transduced) in 1T mice 24 weeks post transplantation. Data expressed as mean $\pm$ SEM from 5 independent experiments. For 0h: n=15 mice, 18-24h no treatment (Φ) n=19 mice, 18-24h p38i n=21 mice, 36-42h Φ n=17 mice, 36-42h p38i n=14 mice, 72-96 LV Φ n=12 mice 72-96 LV p38i n=7 mice, 72-96h RV Φ n=17 mice, 72-96h RV p38i n=17 mice for the total of 139 mice. Statistics: Mann Whitney U test, *P<0.05, **P<0.01, ***P<0.001.

Figure 3. p38MAPK inhibition reduces DNA Damage Response (DDR) during ex vivo culture. Human CD34⁺ cells were cultured and transduced with lentiviral vector (LV) or γ -retroviral vector (RV) expressing green fluorescent protein (GFP) for the indicated time points. Representative Immunofluorescence images of sorted human CD34⁺ 38⁻ 90⁺ population treated with or without p38i for 72 hours stained with anti- γ H2AX and 53BP1 **(a)** and quantification of γ -H2AX and 53BP1 as MFI/cell in HSCs transduced with LV for 36 hours **(b)** and with RV for 72 hours **(d)**. Representative histogram plot from flow cytometric analysis of HSCs stained for γ -H2AX with or without p38 inhibitor (p38i) and quantification of fold change in mean fluorescence intensity (MFI) of γ -H2AX in HSCs **(e)** and percent γ H2AX⁺ HSCs **(f)** at indicated time period. Percentage shown in histograms are cells positive for γ H2AX and the

numbers below are mean fluorescent intensity (MFI) (n= 5 independent experiments).

Statistics: Mann Whitney U test and paired t test, *P<0.05, **P<0.01, ***P<0.001.

Figure 4. p38 MAPK inhibition retains HSCs in G₀ quiescent phase during early time period of *ex vivo* culture and transduction of human HSC enriched cells after longer *ex vivo* pre-stimulation time shows increased G₂M phase accumulation. Human MPB derived CD34⁺ cells were cultured and transduced as described in **Fig. 3 a**. **(a)** FACS plot representing HSCs in G₀ (quiescent), G₁, and S-G₂M phase of cell cycle with or without p38i and at indicated time in culture and the quantification **(b)** is shown. (Φ=no treatment) Percentage of (± SEM) HSCs in G₀, G₁ and S-G₂M phase of cell cycle is depicted on the y-axis. (n=4 independent experiments for 24h LV and n=7 for 42h LV and n=5 for 72h RV)). **(c-d)** Histogram plot representing the analysis of G₂M phase accumulation in HSCs either with LV^E (LV Early) **(c)** or RV **(d)** and analyzed 18 hours after the last transduction (n=1). **(e)** Histogram plot representing time course analysis of G₂-M phase accumulation in HSCs transduced with LV either at early (LV^E) or late time period (LV^L) or with γ-retrovirus vector (RV). Quantification of fold change in GFP⁻ (untransduced) versus GFP⁺ (transduced) cells at G₂M phase is shown **(f)** (n=3 independent experiments). Statistics: paired Student's t test, *P<0.05, **P<0.01, ***P<0.001.

Figure 5. Increased G₂M phase accumulation and decreased HIF-1α in late transduced HSCs is reversed by p38 inhibitor (p38i) in combination with either Chk1 kinase inhibitor (Chk1i) or prostaglandin E2 (PGE2). **(a)** Quantification of fold change in GFP⁻ (untransduced) versus GFP⁺ (transduced) HSCs at G₂M phase by flow cytometry after 72 hours of RV transduction (n=6) (paired student's t test, *P<0.05. **P<0.01), and **(b)** quantification of fold change in γ-H2AX mean fluorescent intensity (MFI). n=9 independent

experiments; statistics: Paired wilcoxon test, * $P < 0.05$. ** $P < 0.01$. **(c)** Quantitative fold change in HIF-1 α MFI in HSCs (n=3 independent experiments using 3 different MPB donors; paired student's t test). **(d)** Representative immunofluorescence image of HSCs stained for HIF-1 α and **(e)** quantitative MFI of HIF-1 α per cell are shown (Paired wilcoxon test, * $0.05 > P$). Quantitative fold Change in HSC enriched cells at G2-M phase **(f)** (n=8) and fold change in γ -H2AX MFI of HSCs **(g)** with indicated treatments are shown (n=9 independent experiments). Statistics: paired t test, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$.

Figure 6. Combined treatment of p38i and PGE2 increased human engraftment and a complete reversal of myeloid skewing phenotype in CD34⁺ cells transplanted mice at limiting doses. Limiting Dilution transplant was performed as described in methods. Mice were injected with a fraction of the final culture equivalent to the indicated number of starting CD34⁺ cells after 42 hours of lentiviral (LV) transduction and culture with no treatment (ϕ : open green circles), p38 inhibitor (p38i: filled blue circles) or PGE2 (P: filled purple circles) or p38i +PGE2 (p38i+P: filled red circles). The percentage of human CD33⁺ myeloid cells (untransduced; GFP⁻ : **a** & transduced GFP⁺ : **b**) and CD19⁺ B-lymphoid cells (untransduced; GFP⁻ : **c** & transduced GFP⁺ : **d**) in the bone marrow (BM) at 6 months post 1T are shown (n=5mice/treatment each CD34 input doses except for P:500K dose n=4, p38i+P:50K dose n=3, and ϕ : 250K dose n=4, for a total of 76 NSG mice). **(e)** % Human CD45⁺ cells in the bone marrow at 6 months (6mo) post primary transplant (1T) (y axis) and equivalent number of starting CD34⁺ cells (X-axis) are shown. **(f)** Plotted are the percentages of negative (un-engrafted) mice containing less than 0.17% human CD45⁺ cells at 6 months post-primary transplant. Frequency of functional HSCs (Competitive repopulation units: CRU) is shown on the side. As described in methods, secondary transplant was performed after 24 weeks post

primary limiting dilution transplant from the highest input dose used in 1T. % Human CD45⁺ cells (**g**), GFP⁺ hCD45⁺ cells (**h**), and % Human CD33⁺ myeloid cells (untransduced; GFP⁻ : **i** left & transduced GFP⁺ : **j** right), CD19⁺ B-lymphoid cells (untransduced; GFP⁻ : **k** & transduced GFP⁺ : **l**) in the BM at 12 weeks post-secondary transplant (2T) are shown. For ϕ n=9, for p38i n=7, for P n=8, and for p38i+P: n= 8 for a total of 32 NSG mice. Data expressed as mean $\pm$ SEM. Statistics: Mann Whitney U test, exact P values shown.

Supplementary Figures

Figure S1. Human xenograft model of adult hematopoietic stem cells. Freshly isolated human mobilized peripheral blood (MPB) derived CD34⁺ cells (1 million cells/mouse) were transduced with a lentiviral vector (LV) within 18 hours and injected into irradiated NSG mice. Primary human engraftment was analyzed in mice at 6 weeks, in bone marrow (BM) (**a**) and in peripheral blood (PB) concurrently (**b**). Each symbol represents an individual mouse, lines show mean $\pm$ S.E.M. (BM for 0h n=13, 18-24h n=14, 36-42h n=15; PB: for 0h n=6, 18-24h n=7, 36-42h n=8 mice). (**c**) At 6, 12 and 24 weeks post primary transplant (1T), BM was analyzed for the different human cell populations by flow cytometry . Representative FACS plots showing human CD45⁺ cells gated for GFP⁺ (transduced) versus GFP⁻ (untransduced) cells at 24 weeks post primary transplant (1T). From the GFP⁻ and GFP⁺ human CD45⁺ populations, human CD33⁺ myeloid cells, human CD19⁺ B-Lymphoid and CD3⁺ T-Lymphoid cells and human CD34⁺ HSPCs, that were negative for CD19 are shown. (**d**) The percentage multi-lineage engraftment analyzed at 6, 12, and 24 weeks after 1T of uncultured MPB derived CD34⁺ cells is shown. Data plotted as mean $\pm$ S.E.M. For 6 weeks n=20, 12 weeks n=12, 24weeks n=10 mice).

Figure S2. A preclinical model for studying adult human HSC *ex vivo* manipulation and gene transfer and its effect on LTRP. (a) Freshly isolated or thawed mobilized peripheral blood (MPB) derived CD34⁺ cells were utilized for the study. Two different protocols were utilized for the study where lentiviral vector (LV) or γ -Retroviral vector (RV) mediated gene transfer was performed at indicated time points. 0h, 18-24h, 36-42h, and 72-96h indicates the total amount of time where cells were exposed in the *ex vivo* culture after CD34⁺ cell isolation before injection into NSG mice. (b) After indicated time in culture and transduction, 1 million CD34⁺ starting equivalent cells were transplanted per NSG mouse after total body irradiation intravenously (primary transplant = 1T). Primary human engraftment and multi-lineage reconstitution was analyzed in mice at indicated time periods after bone marrow harvest at 6 and 12 weeks from left and right femurs, and after sacrifice at 24 weeks, from all bones. A portion of cells were analyzed for FACS and the rest were depleted of mouse CD45⁺ cells and transplanted one to one, into secondary mice (secondary transplant = 2T).

Figure S3. GFP Marking *in vitro* and *in vivo*. Human MPB CD34⁺ cells were cultured and transduced with the LV or RV for the indicated hours (hrs) as described in Fig. S2 a, and colony forming unit cells (CFUc) plated on a small proportion of CD34⁺ cells, while the rest (1 million starting equivalent) were transplanted intravenously into NSG mice. BM was aspirated at 6 and 12 weeks from right and left femurs, and mice sacrificed at 24 weeks; BM was analyzed for GFP⁺ hCD45⁺ cells at 6, 12 and 24 weeks (for 18-24 hrs n=20, for 36-42 hrs n=9, and for 72-96 hrs n= 7 for the total of 36 mice; data presented as mean $\pm$ S.E.M).

Figure S4. Multi-lineage engraftment of *ex vivo* manipulated and cultured MPB derived hCD34⁺ cells in NSG mice. Human mobilized peripheral blood (MPB) CD34⁺ cells were cultured for the indicated hours (X-axes), and transduced with lentiviral vector (LV) or γ -

retroviral vector (RV) encoding enhanced green fluorescent protein (GFP) as a marker of transduced cells; an equivalent input of 1 million CD34⁺ cells were injected per irradiated (280 cGy) NSG mouse thereafter. Bone marrow (BM) was analyzed for multi-lineage reconstitution at 6 weeks (**a, c, e, g**) and 12 weeks (**b, d, f, h**) after primary transplant. Both untransduced (GFP⁻) and transduced (GFP⁺) human CD33⁺ myeloid population (**a, b**), human CD19⁺ B-Lymphoid (**c, d**), human CD3⁺ T-Lymphoid (**e, f**), and human CD34⁺ HSPCs population (**g, h**) are shown. Data expressed as mean $\pm$ SEM. For 0h n= 12, 18-24h n= 7, 36-42h n= 11, 72-96h n= 20 for the total of 50 NSG mice. Statistics: Mann Whitney test, ** P<0.01.

Figure S5. Both LV and RV transduce human HPCs and HSCs comparably.

Representative fluorescence-activated cell sorting (FACS) plot showing green fluorescent protein (GFP) marker expression in various hematopoietic stem/progenitor (HSPC) subsets after LV transduction for 42 hours (**a** left) and the quantification (**a** right) (n=4 independent experiments), in human CD34⁺ HSPCs versus CD34⁺ 38⁻ 90⁺ 45RA⁻ 49f⁺ HSCs post LV or RV transduction for 72 hours (left) and the quantification (right) (n=3 independent experiments) (**b**) and in MPP versus HSCs after LV (**c**) or RV (**e**) transduction for 72 hours along with the quantification of the respective groups (**d** and **f**) are shown as a bar graph with mean $\pm$ SEM (n=3 independent experiments).

Figure S6. *Ex vivo* manipulation is not associated with reduced viability or apoptosis of HSCs, but with increased phenotypic HSCs with higher ROS; reducing ROS decreases gene transfer. Human CD34⁺ cells were cultured and transduced with LV or RV for the indicated time points. (**a**) Total cell viability after harvest was determined by trypan blue exclusion method. (**b**) Annexin V⁺ (apoptotic) CD34⁺ 38⁻ 90⁺ cells after *ex vivo* culture was detected by flow cytometry. (**c**) Fold increase in phenotypic human HSCs (CD34⁺ 38⁻ 90⁺

45RA⁻ 49f⁺ cells) was calculated after flow cytometric analysis (n=5). Statistics: paired t test. (d) Proliferation status of human CD34⁺ HSPC versus CD34⁺ 38⁻ 90⁺ HSC enriched population during *ex vivo* culture determined by the proportion of EdU⁺ HSPCs and HSCs in the EdU incorporation assay (n=3). (e) Total intracellular ROS levels were determined using CM-H2DCFDA fluorescence; fold change in DCFDA MFI in LV and RV transduced human CD34⁺ 38⁻ 90⁺ cells is shown as mean of $\pm$ SEM (n=3). Statistics: Student's t test, *p<0.05. (f) Representative histogram plots showing MitoSOX for the measurement of mitochondrial-specific ROS in human CD34⁺ 38⁻ 90⁺ cells cultured for 24 hours versus 72 hours. The numbers represents MFI. Histogram plots showing MitoSOX levels (g) and transduction efficiency in terms of % positive cerulean fluorescent protein (CFP) (h) with increasing doses of the anti-oxidant N-acetylcysteine amide (NACA) in CD34⁺ 38⁻ 90⁺ cells.

Figure S7. Increase in time in culture activates p38 MAPK in HSC. Human MPB derived CD34⁺ cells were cultured and transduced as described in Fig. S3. Representative histograms plots showing phospho-ERK (p-ERK) (a), phospho JNK (p-JNK) (b), and phospho-p38MAPK (p-p38) (c) levels in human CD34⁺ 38⁻ 90⁺ HSC-enriched population by flow cytometry. Quantitative fold-change in mean fluorescent intensity (MFI) of p-ERK (d), p-JNK (e) and p-p38 (f) at indicated times are shown. Data expressed as mean $\pm$ SEM from 3 independent experiments using three different MPB donors. Statistics: Student's t test, *p<0.05, **p<0.01, ***p<0.001. (g) Representative histogram plot of p-p38 level in non-cycling (in G0-G1 phase of cell cycle) HSC-enriched populations versus cycling HSC-enriched cells (in S-G₂-M phase of cell cycle, determined by Hoechst 33342 staining). Numbers indicate MFI. Quantitative fold-change in p-p38 MFI in cycling vs non-cycling (h) and untransduced (GFP⁻) versus transduced (GFP⁺) HSC enriched cells (i) from 3 independent experiments is shown as mean $\pm$ SEM.

Statistics: Student's t test, * $p < 0.05$. (j-k) Human MPB derived CD34⁺ cells were cultured with or without various p38 inhibitors (B= Birb 796, Vx= VX 745, and Ly= Ly2228820) and transduced with retrovirus (RV) for 72 hours. Representative histogram plots of p-p38 levels in human CD34⁺ 38⁻ 90⁺ cells are shown (j). Quantitative fold change in p-p38 mean fluorescence intensity (MFI) in human CD34⁺ 38⁻ 90⁺ cells compared to unmanipulated (0h) is shown (k). Data is expressed as mean $\pm$ SEM from 7 independent experiments using 7 different MPB donors. Statistics: Student's t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure S8. Inhibition of p38 MAPK during *ex vivo* culture retains the human engraftment in the secondary transplanted NSG mice. Human engraftment in NSG mice with or without Birb 796 after 6 weeks of secondary transplant. Engrafted mice ($>0.01\%$ CD45⁺ cells in the whole bone marrow) are shown above the middle bar, engrafted/Total mice number is shown on the side. Empty triangles are control & filled triangles are Birb 796 treated. Each symbol represents an individual mouse. Data expressed as mean $\pm$ SEM from 5 independent experiments (n=7-29 mice per group). Statistics: Mann Whitney U test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FigureS9. GFP Marking in bone marrow of NSG mice. Human CD34⁺ cells were cultured as described in Fig. 2 and transplanted into NSG mice. Total GFP⁺ cells were analyzed *in vitro* before transplant (a) and in vivo at 6 weeks (b) and 24 weeks (b) post primary transplant (1T). In (a) 18-24 hours was not sufficient time for measuring GFP expression and thus not shown. Data expressed as mean $\pm$ SEM from 5 independent experiments (n=12-21 mice per group). Statistics: Paired t test, ** $p < 0.01$.

Supplementary Figure S10. p38 inhibition does not change total CD34⁺ cell number/viability, apoptosis, transduction efficiency, and ROS level but may retain the

percentage of phenotypic HSCs. Human CD34⁺ cells were cultured and transduced as described in Fig. 2a. Harvested cells were stained with trypan blue for viability, total CD34⁺ cell number **(a)**, percent annexin V⁺ (apoptotic) CD34⁺ 38⁻ 90⁺ cells **(b)** (n=3), fold change in percent human CD34⁺ 38⁻ 90⁺ 45RA⁻ 49f⁺ (HSCs) **(c)** (n=6), fold change in transduction efficiency (based on GFP marker percentage) over non-treated CD34⁺ 38⁻ 90⁺ cells **(d)** (n=5), and fold change in MitoSOX MFI in CD34⁺ 38⁻ 90⁺ cells **(e)** (n=3) are shown. Data represents mean ± SEM. Statistics: Paired t test, *p<0.05.

Figure S11. p38MAPK inhibition retains HSCs in G₀ quiescent phase during early time period of ex vivo culture and gene transfer. Human MPB derived CD34⁺ cells were cultured and transduced as described in **Fig. 3**. Quantitative plot representing HSCs in G₀ and G₁-S-G₂-M phase of cell cycle with or without p38i at 24 hours (LV) **(a)**, 42 hours (LV) **(b)**, and 72 hours (RV) **(c)** of ex vivo culture. (n=4 independent experiments for 24h LV, n=7 for 42h LV and n=5 for 72h RV). Statistics: paired t test, *p<0.05, **p<0.01, ***p<0.001.

Figure S12. p38MAPK inhibition also reduces DNA Damage Response (DDR) in human CD34⁺38⁻90⁺ cells derived from bone marrow (BM). Human BM derived CD34⁺ cells were cultured and transduced with lentivirus (LV) for 48 hours. **(a)** Representative FACS plot representing bone marrow derived HSC enriched CD34⁺ 38⁻ 90⁺ population in G₀ (Quiescent), G₁, and S-G₂-M phase of cell cycle with or without p38i **(left)** and the corresponding representative histogram plot of γH2AX at indicated time and treatment **(right)**. Percentage shown in histogram are cells positive for γH2AX and the numbers below is mean fluorescent intensity (MFI). Quantitative plots of HSCs in various cell cycle phase **(b)** and its corresponding γH2AX MFI **(c)** are shown. n=1 experiment.

Figure S13. Transduction of non cycling HSCs (6 and 18h with LV; LV^E) retains HSC lineage fate, but transduction of cycling HSCs (at 44h and 68h with RV or LV [LV^L]) results in a myeloid biased HSC fate. (a) *Ex vivo* culture and transduction schema. **(b)** mRNA expression of genes associated with aging in 24h versus 72h culture analyzed by qPCR. GAPDH was used as a reference gene. n=4 for Fzd3 and n=3 for all other genes. Log2 Scale. Data mean $\pm$ SEM. Unpaired t test. *p<0.05, **p<0.01, ***p<0.001. Myeloid **(c)** and B lymphoid **(d)** lineage proportions within transduced (GFP⁺) CD45⁺ cells in BM of NSG mice 24 weeks after 1T. Data expressed as mean $\pm$ SEM (n=5 mice for 24LV and 72 LV^E, n=4 mice for 72 LV^L or 72 RV). Statistics: Mann Whitney U test, *p<0.05. **(e)** Transfection Schema **(top)** G2M accumulation in ϕ (no transfection control) versus gene edited (GE) (transfected) HSCs **(below: left)**. GE was performed by transfection of RNPs containing Cas9 and gRNA after 44 hours of pre-stimulation and cell cycle analyzed either at 6 hours or 24 hours post transfection. Quantitative plot showing time course of G2-M accumulation 6 hours versus 24 hours after transfection **(below: right)**. **(f)** % hCD45 expression with or without GE on day 7.

Figure S14. Increased HSC enriched cells at G2-M phase is specific to CHK1, not CHK2 and transduction during ex vivo culture decreased HIF1 α and increased γ H2AX which are reversed by the combination of p38 inhibitor (p38i) and prostaglandin E2 (PGE2). (a) Representative histogram plot of G2-M phase accumulation (% shown) in HSCs transduced with RV for 72 hours with indicated compounds. **(b)** Representative histogram plot HSCs stained for HIF-1 α ; DEF (Diethyl Fumarate) and α -KG (α -Keto Glutarate) used as a positive and negative control for HIF-1 α staining. n=1 **(c)** Representative histogram plot of γ H2AX in HSCs after 72 hours of culture and RV transduction with indicated compounds. Numbers represents mean fluorescent intensity (MFI). ϕ = No transduction, Control= no treatment.

Figure 1

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Chapter 3:

DISCUSSION

Overview

We define two major HSC stress arising during *ex vivo* culture that can affect long term HSC function *in vivo*. HSC stress #1 is the enforcement of HSC division *ex vivo*. HSC stress #2 is the genetic manipulation of actively cycling HSCs. In fact, our studies also show a third stress, the regenerative stress following transplant. Previous studies showed that in a genetic knockdown of p190-B Rho GTPase-activating protein (RhoGAP), murine HSC self-renewal¹³⁷ is mediated via decreased TGF β 1 activation followed by decreased p38 MAPK activity (Hinge *et al.* ASH abstract 2013). p38 MAPK inhibitors (p38i) have previously been reported to expand HSPCs in both murine and human (CB derived HSPCs) mostly in 5 to 7 days long expansion culture^{109,110,138}. In these studies, human HSC assays were almost exclusively performed using CB derived HSPCs, and not adult HSPCs, that differ remarkably in cell cycle kinetics engraftment and expansion potential and additionally, lacked serial secondary transplant data^{109,110}. Hence these studies did not represent a true adult human hematopoietic stem cell functional readout. Here we present, for the first time, progressive increase in activation of p38 MAPK in adult MPB-derived, rigorously defined, phenotypic HSCs during 2-4 days of short *ex vivo* gene therapy. We identified a novel mechanism in which increasing time of *ex vivo* culture leads to tremendous loss of adult human HSC LTRP via the activation of p38 MAPK pathway. Inhibiting p38MAPK pathway with small molecule inhibitors *ex vivo* resulted in significant retention of the secondary adult human grafts in NSG mice.

Next, we for the first time show that viral transduction performed when HSCs are actively cycling in culture leads to a tremendous skewing of human grafts into myeloid

lineages, (which is only observed in gene modified progeny), suggestive of HSC aging. p38 inhibition was able to partially yet significantly reverse the myeloid skewing phenotype. We observed a large loss of GFP gene marking in RV transduced HSC progeny, but not with LV transduced ones in our xenografts (Supplementary Fig. S3). We speculated that there is preferential gene transfer into the cycling HSPCs; however, careful analysis of gene transfer into the entire HSPC compartment showed no preferential gene transfer to HSPCs versus HSCs (Supplementary Fig. S5). This suggests that the loss of gene marking in RV groups is in fact due to the loss of HSC LTRP. While we did not find any obvious loss of HSCs in culture (at least with our apoptosis assay for a limited time period), it is possible that a portion of HSCs (transduced when they were actively cycling) could have died later on, after transplant or the ones that survived changed fate to HPCs after undergoing transplantation.

Our study shows that more than 90% of human CD34⁺38⁻90⁺ highly HSC-enriched cells are in the quiescent G0 phase of the cell cycle before *ex vivo* culture, and start entering G1 phase by 24 hours. A previous report found that increased HSC cycling results from increase in p38 activation in CYLD (Cylindromatosis) mutant mice *in vivo*¹¹¹. Here we show that even a short *ex vivo* treatment of p38i results in a significant increase in cells in the quiescent G0 phase of the cell cycle (Fig 4a-b). Additionally, we present evidence of increased DDR within 24 hours of *ex vivo* culture and transduction (Fig.3) and show that the addition of p38i can significantly reduce DDR at all tested conditions. Diverse conditions have been shown to activate p38 MAPK pathway in murine HSCs including ROS¹⁰⁴, DNA damage¹⁰⁵ and increased cycling¹²⁶. We have identified the presence of all these stress inducers during *ex vivo* culture, and found that

ultimately all of these converge onto activation of the p38 MAPK pathway, leading to loss of HSC LTRP. In addition to these stress, viral transduction may have selectively led to loss of lymphoid-biased HSCs, leading into myeloid skewing progeny.

Although RV trials are largely being replaced by LV trials and gene targeting, culturing of HSCs *ex vivo* remains necessary to allow cells to enter the cell cycle (S-G2M) for homology directed repair (HDR) for gene editing^{2,3}. As shown by Walter et al., increasing rounds of replicative stress (activation of HSCs out of quiescence) results in accumulation of DNA damage, leading to profound loss of LTRP and myeloid biased HSC progeny similar to accelerating aging¹²⁶. We show that the replicative stress associated with DDR is from the p38 activation. Furthermore, in our limiting dilution assay, we show that the extreme regenerative stress ages the untransduced HSCs as well, suggesting a role of p38 inhibition *in vivo* soon after transplant. Our data also shows that the imbalance of lineages in our HSC grafts obtained from late-transduced HSC progeny could be the consequence of transduction/double strand breaks (DSBs) induced either by LV, RV, or nucleases used in gene editing which adds further stress to already-stressed cycling HSCs.

We have shown that the treatment of HSCs with PGE2 (previously reported to increase murine and human CB HSPC engraftment^{82,83}) was able to reverse the myeloid lineage skewing phenotype in our limiting dilution analysis (LDA) xenografts under extreme stress conditions (i.e. culture plus transduction and regenerative stress) (Fig. 5). Although PGE2 alone was sufficient to revert the myeloid skewed phenotype, its combination with p38i was necessary to restore the LTRP. Previous studies showed a profound loss of HSC LTRP with regenerative stress such as serial bone marrow

transplantation^{106,139,140}. Here, we did not perform LDA on un-manipulated HSPCs, which would have shown whether the combination completely restored the LTRP.

We found that addition of p38i to PGE2 was essential to reduce culture and transduction stress. This combination decreased DNA damage and G2-M accumulation (Fig. 5). Our data showing decreased HIF-1 α with increased time in culture and transduction may be partly explained by our normoxic culture conditions and suggests interplay between the cell cycle, ROS, and DNA damage (induced by DSBs).

Our purpose in using Chk1inhibitor (Chk1i) in culture was to decrease the G2-M accumulation of HSCs, as Chk1i has been reported to allow cells to progress through the cell cycle¹⁴¹. However, Chk1i may allow increase accumulation of un-repaired DNA damage as shown by increased γ H2AX (Fig 5 a-b). Hence, we turned to use of a safer compound, PGE2 (currently undergoing clinical trial¹¹⁶), which appears to provide adequate HIF-1 α to the stressed HSCs to maintain stemness during *ex vivo* culture and gene transfer. Here, it would be interesting to study the effect of Chk1 (or Wee1) inhibition with or without p38i at restoring LTRP and lineage fate of HSCs.

Conclusions and Future Directions

In spite of enormous progress in maintaining HSCs in culture, several challenges still exist in therapeutic use of *ex vivo* expanded HSCs. Cytokines are known to bring quiescent HSCs into an active cell cycle phase and thus induce differentiation of HSCs into HPCs at the cost of their self-renewal^{142,143}. A current barrier to success in HSC *ex vivo* expansion and its therapeutic application includes insufficient HSC output which

may be mainly due to the cytokines induced differentiation of these cells in the *ex vivo* culture conditions ⁴¹.

Increasing effort toward developing optimal laboratory conditions to produce HSCs on a clinical scale is essential ²⁷. A majority of the *ex vivo* expansion methods investigated so far are designed for CB derived HSCs, and whether they are compatible with BM or MPB derived adult HSCs is still questionable ⁸⁸. Development of approaches designed for HSCs derived from all sources, and supported by long term xenograft data, will greatly assist the field of regenerative medicine.

Using a new, robust, adult HSC xenograft model, our study provides novel mechanistic insights into the tremendous loss of functional capacity of HSCs during *ex vivo* culture and gene transfer. We find that using stress inhibitors during *ex vivo* culture greatly increases HSC fitness. This insight should benefit the fields of both gene therapy and targeted gene editing for the treatment of monogenic disorders.

Small molecules offer an additional advantage over cytokines and cell intrinsic factors, as they can be used transiently *in vitro* ¹⁴⁴. However, utilization of small molecules in clinical therapy demands a thorough and careful study. Confirmation of our results in the non-human primate model may provide a better pre-clinical data than murine models, as there are difference between mice and human in terms of cell cycle kinetics and response to cytokines ^{145,146}.

We have shown that the combination of PGE2 and p38i significantly lowers the G2M accumulation of HSCs, as well as the DDR response as shown by our γH2AX status. Moreover, only when we use the combination of PGE2 and p38i, we noticed an increase in quiescence of HSCs, at least up to 42 hours of *ex vivo* culture. It is possible

that HSCs remaining longer in G0 and G1 during the transduction period provide time for HSCs to repair from the *in vitro* insults such as DSBs and ROS induced with *ex vivo* genetic manipulation. Further studies should be performed to determine the exact time or delay in cell cycle phases with or without p38i in our culture conditions such as by utilizing time lapse microscopy.

The massive loss of HSC LTRP and a myeloid skewed phenotype in RV transduced HSC progeny could have resulted from increased ROS in culture conditions. The use of p38i was not able to reduce ROS (although it was not surprising, since ROS is upstream of p38 activation), and, whether PGE2 or its combination with p38i reduces ROS remains to be seen. As various inflammatory cytokines can be generated by HSC progeny, and culturing HSCs in cytokine rich medium could have stimulated inflammatory pathway activation¹⁴⁷. It will be interesting to dissect the activation of specific inflammatory pathways in each of our culture conditions to explore ways by which specific factors regulate the human HSC function. Another possible avenue for future studies would be to explore the epigenetic status of HSCs during *ex vivo* culture and after transduction. Several epigenetic factors have been shown to affect gene expression in HSCs undergoing stress and thus affecting stem cell function¹⁴⁸⁻¹⁵⁰.

A recent study has highlighted the role of mitochondrial clearance for maintaining stemness in highly purified Tie2⁺ HSC population¹⁵¹. It would be interesting to determine if the changes in the metabolic landscape occur in stressed HSCs, because this could possibly explain both the loss of LTRP and HSC aging.

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