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Los Angeles

Improving Nuclease-Mediated Gene Editing Outcomes
in Human Hematopoietic Stem Cells

A dissertation submitted in partial satisfaction
of the requirements for the degree Doctor of Philosophy
in Molecular and Medical Pharmacology

by

Anastasia Lomova

2019

ABSTRACT OF THE DISSERTATION

Improving Nuclease-Mediated Gene Editing Outcomes in Human Hematopoietic Stem Cells

by

Anastasia Lomova

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2019

Professor Donald Barry Kohn, Chair

Autologous hematopoietic stem cell (HSC) transplantation, combined with gene editing, could provide an ideal therapeutic option for the treatment of congenital blood diseases, such as hemoglobinopathies, primary immune deficiencies, and storage disorders. Gene editing relies on site-specific induction of a double stranded break (DSB) by targeted nucleases (such as Zinc Finger Nucleases (ZFNs) or CRISPR/Cas9 system), and subsequent gene correction using endogenous cellular repair mechanisms. The two main competing pathways to repair the break are non-homologous end joining (NHEJ), an often-imprecise pathway which can result in insertions and deletions (indels), or accurate homology-directed repair (HDR) pathway which uses a homologous donor template to seamlessly repair the break and incorporate the desired changes. For certain diseases, where a knockout of a gene can result in therapeutic benefit, repair by NHEJ pathway may be favorable. However, for conditions where disruption of a gene can result in an even more severe phenotype than the original disease (such as sickle cell anemia), repair via HDR pathway is critical.

Despite advances in nuclease technologies and the ability to efficiently achieve high frequency of site-specific gene disruption, the current progress to reach clinically relevant levels of precise HDR-mediated repair still remains elusive. Therefore, our translational goal is to improve the gene editing outcomes in HSCs, specifically, increase HDR and decrease NHEJ levels, which will be beneficial for treating many diseases of the blood. This dissertation aims to identify the hindrances that limit efficient HDR-mediated editing in HSCs, and investigates several approaches to address these impediments.

Our results indicate that one major reason for low gene correction in HSCs is their heightened susceptibility to cell toxicity resulting from the electroporation of the nuclease and homologous donor template. We demonstrate that co-electroporation of mRNA encoding the anti-apoptotic protein BCL2 with gene editing reagents significantly ameliorates the cytotoxicity and increases the yield of gene-corrected HSCs.

Next, we show that cell cycle-dependent control of nuclease activity and DNA repair pathways can influence gene editing outcomes to favor the precise DNA modification (HDR) over faulty repair events (NHEJ) in human HSCs. By using a modified version of Cas9 protein with reduced nuclease activity in G1 phase of cell cycle, when HDR cannot occur, and transiently increasing the proportion of cells in HDR-preferred phases (S/G2), we achieve a 4-fold improvement in HDR/NHEJ ratio over the control condition *in vitro*, and a significant improvement in long-term gene-modified engrafted cells after xenotransplantation of edited human HSCs into immune-deficient mice.

Finally, we investigate what cellular elements govern the DNA repair pathway choice and how they can be exploited to shift the balance toward HDR from NHEJ. We test the effects of manipulating the expression levels of several DNA repair factors, that are presumed to be important for pathway choice and progression, on HDR and NHEJ levels in K562 cell line and primary human hematopoietic stem and progenitor cells (HSPCs). Interestingly, we observe

differential effects of DNA repair factor manipulation on gene editing outcomes dependent upon the delivery method employed and the types of cells used.

These strategies for improving gene editing outcomes in human HSCs have important implications for the field of gene therapy as a whole, and can be applicable to diseases where increased HDR/NHEJ ratio is critical for therapeutic success.

The dissertation of Anastasia Lomova is approved.

Thomas G Graeber

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April Dawn Pyle

Donald Barry Kohn, Committee Chair

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2019

DEDICATION

To my dearest family members:

my mom Marina, my husband Michael, and my feline companion Tima

for your eternal support and enduring encouragement.

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Lomova A, Clark DN, Romero Z, Hollis RP, Kohn DB. Improving Gene Editing in Human Hematopoietic Stem Cells by Temporal Control of DNA Repair. Poster. *American Society of Gene and Cell Therapy (ASGCT)*, Chicago, IL, May 2018.

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Chapter 1. Background and Introduction – Hematopoietic Stem Cell Gene Therapy – Progress and Lessons Learned

1.1. ABSTRACT

The use of allogeneic hematopoietic stem cells (HSCs) to treat genetic blood cell diseases has become a clinical standard but is limited by availability of suitable matched donors and potential immunologic complications. Gene therapy using autologous HSCs should avoid these limitations and thus may be safer. Progressive improvements in techniques for genetic correction of HSCs, by either vector gene addition or gene editing, are facilitating successful treatments for an increasing number of diseases. We highlight the progress, successes, and remaining challenges toward development of HSC gene therapies and discuss lessons they provide for development of future clinical stem cell therapies.

1.2. INTRODUCTION

Most inherited blood cell diseases, such as primary immune deficiencies, hemoglobinopathies, storage and metabolic disorders, congenital cytopenias and stem cell defects, can be treated by transplantation of allogeneic hematopoietic stem cells (HSCs) Table 1.2-1) (Boelens et al., 2013; Walters, 2015). The transplanted genetically normal HSCs can serve as an ongoing source of blood cells of all lineages, eliminating these disorders from a single treatment with benefits lasting life-long.

Table 1.2-1: Genetic Diseases of Blood Cells and the Transplantation Modalities that Have Been Applied Clinically as Therapies or Are in Pre-clinical Development

Category of Disease	Specific Conditions	Transplantation Modalities Applied			
		Allogeneic HSCT	γ -Retroviral Gene Therapy	Lentiviral Gene Therapy	Genome Editing
Primary immune deficiencies	ADA-deficient severe combined immune deficiency	+	+	+	n.d
	X-linked severe combined immune deficiency	+	+	+	pre-clinical
	other genetic forms of SCID (Artemis, Rag1/2).	+	n.d	pre-clinical	n.d
	Wiskott-Aldrich Syndrome	+	+	+	pre-clinical
	chronic granulomatous disease	+	+	+	pre-clinical
	leukocyte adhesion deficiency	+	+	pre-clinical	n.d
	hemophagocytic lymphohistiocytosis	+	n.d	pre-clinical	n.d
	X-linked hyper IgM syndrome	+	n.d	n.d	pre-clinical
	X-linked lymphoproliferative disease	+	n.d	n.d	n.d
	X-linked Agammaglobulinemia	Few	n.d	n.d	pre-clinical
	common variable immunodeficiency	heterogeneous genetic etiologies, often unknown; may require editing	heterogeneous genetic etiologies, often unknown; may require editing	heterogeneous genetic etiologies, often unknown; may require editing	heterogeneous genetic etiologies, often unknown; may require editing
Hemoglobinopathies	sickle cell disease	+	n.d	+	pre-clinical
	beta-thalassemia	+	n.d	+	pre-clinical
Storage and metabolic disorders	Gaucher Disease and other lipidoses	+	+	n.d	n.d
	mucopolysaccharidoses (I-VII)	+	n.d	+	n.d
	X-linked Adrenoleukodystrophy	+	n.d	+ recent phase II/III	n.d
	metachromatic leukodystrophy	+	n.d	+	n.d
	osteopetrosis	+	n.d	pre-clinical	n.d
Congenital cytopenias and stem cell defects	Fanconi anemia	+	+	+	pre-clinical
	Schwachman-Diamond Syndrome	+	n.d	pre-clinical	pre-clinical
	Kostmann's Syndrome	+	n.d	n.d	n.d

While there are generally high rates of success when an HLA-identical sibling donor is available, the outcomes of hematopoietic stem cell transplantation (HSCT) are usually not as successful with less well-matched allogeneic donors (either haplo-identical family members or unrelated donors) (Boelens et al., 2013; Walters, 2015). Reduced HLA matching between recipient and donor increases the risks of graft rejection and graft versus host disease (GVHD). Rejection of an HSC graft generally leaves the patient in a perilous position, with an urgent need to restore hematopoiesis to prevent complications from prolonged pancytopenia (anemia, infection, bleeding). The primary donor may not be available (e.g. cord blood units are not linked to their source) and a suitably matched second donor may not be identified. GVHD is a major

cause of transplant morbidity and even mortality, and can impose a chronic rheumatologic-like inflammatory/fibrotic disease, with need for persistent immune suppression and the attendant risks of infection and toxicities (Cooke et al., 2017). Immediately before and after the allogeneic transplant, high levels of immune suppression are necessary to reduce immunological risks but these treatments also add to morbidity. There has been ongoing progress with methods to reduce GVHD in allogeneic HSCT, including improved graft engineering by removal of selective T cell populations (TCR α/β depletion, naïve T cell depletion), and by use of post-transplant cyclophosphamide (Fuchs, 2015; Muccio et al., 2016). Nonetheless, immune complications and lack of suitable matched donors present significant clinical barriers to successful application of allogeneic HSCT for a wider range of disorders.

Autologous HSCT in which the patient's HSCs are gene-modified should offer complete avoidance of the major immunological complications of allogeneic HSCT, which may contribute to better outcomes for patients with genetic blood cell disorders. For specific disorders, expression of the gene introduced into HSCs is needed in cells of one or more hematopoietic lineages (e.g. red blood cells, neutrophils, lymphocytes) (**Figure 1.2-1**). The lack of immunogenicity with autologous cells allows the use of reduced intensity of the pre-transplant conditioning to make space in the marrow niche to facilitate HSC engraftment, compared to what is required for effective allogeneic HSCT (**Figure 1.2-2**).

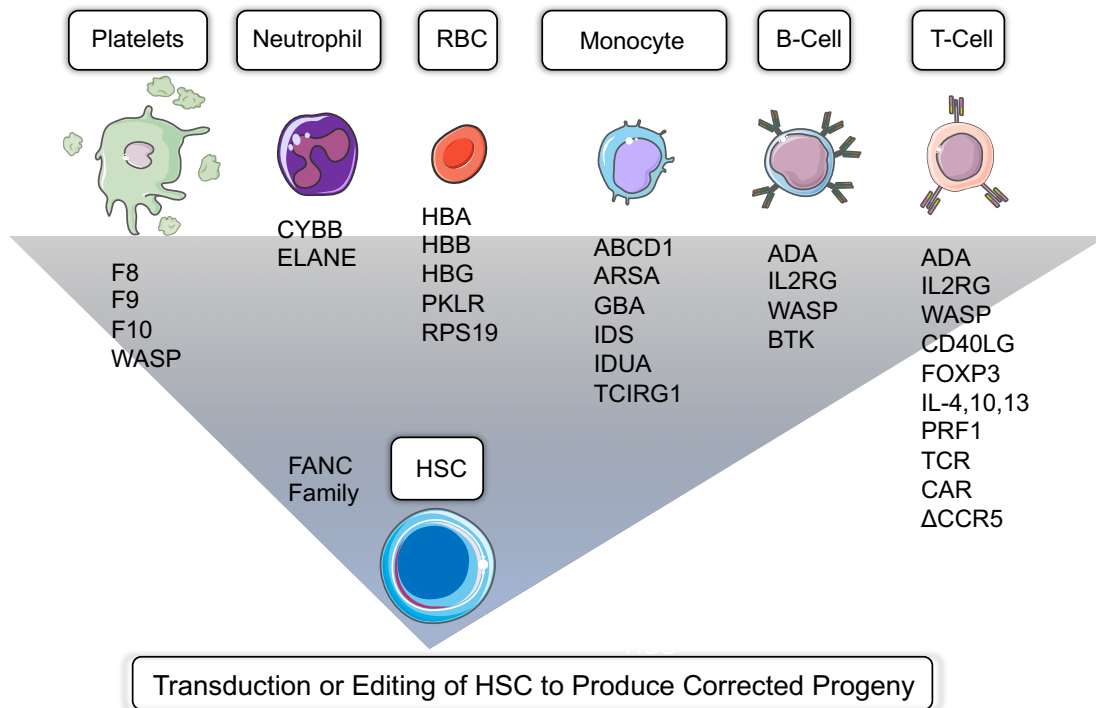


Figure 1.2-1: Overview of targets for gene therapy.

Hematopoietic stem cells (HSCs) isolated from bone marrow can be modified ex vivo and transferred back to the recipient to produce functional, terminally-differentiated cells. Specific cellular targets and the relevant diseases and genes for gene therapy include the following: HSCs: Fanconi Anemia (FANC A-F). Platelets: Hemophilia A (Factor VIII (F8)); Hemophilia B (Factor IX (F9)); Factor X deficiency (Factor X (F10)); Wiskott-Aldrich Syndrome (Wiskott Aldrich Syndrome Protein (WASP)). Neutrophils: X-linked Chronic Granulomatous Disease (Cytochrome B-245 Beta Chain (CYBB)); Kostmann's Syndrome (Elastase Neutrophil Expressed (ELANE)). Erythrocytes: Alpha-Thalassemia (Hemoglobin Subunit Alpha (HBA)); Beta-Thalassemia and Sickle Cell Disease (Hemoglobin Subunit Beta (HBB)); Pyruvate Kinase Deficiency (Pyruvate Kinase, Liver and RBC (PKLR)); Diamond-Blackfan Anemia (Ribosomal Protein S19 (RPS19)). Monocytes: X-linked Adrenoleukodystrophy (ATP Binding Cassette Subfamily D Member 1 (ABCD1)); Metachromatic Leukodystrophy (Arylsulfatase A (ARSA)); Gaucher disease (Glucosylceramidase Beta (GBA)); Hunter Syndrome (Iduronate 2-Sulfatase (IDS)); Mucopolysaccharidosis type I (Iduronidase, Alpha-L (IDUA)); Osteopetrosis (T-Cell Immune Regulator 1 (TCIRG1)). B Cells: Adenosine deaminase (ADA)-deficient Severe Combined Immunodeficiency (Adenosine Deaminase (ADA)); X-linked severe combined immunodeficiency (Interleukin 2 Receptor Subunit Gamma (IL2RG)); Wiskott-Aldrich Syndrome (Wiskott Aldrich Syndrome Protein (WASP)); X-linked agammaglobulinemia (Bruton's Tyrosine Kinase (BTK)). T Cells: Adenosine Deaminase (ADA)-deficient Severe Combined Immunodeficiency (ADA); X-linked severe combined immunodeficiency (IL2RG); Wiskott-Aldrich Syndrome Protein (WASP); X-linked Hyper IgM syndrome (CD40 Ligand (CD40LG)); IPEX Syndrome (Forkhead Box P3 (FOXP3)); Early Onset Inflammatory Disease (Interleukin 4, 10, 13 (IL-4, 10, 13)); Hemophagocytic Lymphohistiocytosis (Perforin 1 (PRF1)); Cancer (Artificial T cell receptors (TCR), Cancer; Chimeric Antigen Receptor (CAR)); Human immunodeficiency virus (C-C Motif Chemokine Receptor 5 (CCR5)).

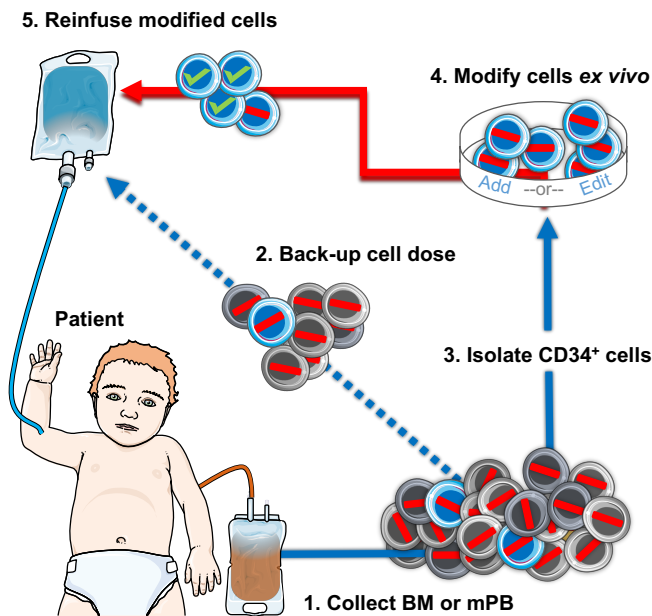


Figure 1.2-2: Autologous hematopoietic stem cell transplantation combined with gene addition or editing.

(1) Bone marrow (BM) or mobilized peripheral blood (mPB) cells are collected from the patient (red line represents a disease-causing mutation). Typically, 15-20ml of BM/Kg is an acceptable harvest target. While collecting HSCs by mobilization and apheresis is less invasive than BM aspiration, infants have small blood volumes making leukapheresis challenging. Failure to harvest adequate cell numbers can prevent therapy. (2) Modification of HSCs may reduce stem cell capacity. A back-up cell dose of non-modified cells is apportioned to restore native hematopoiesis in the event of graft failure. (3) CD34+ cells are isolated in a GMP-compliant, closed system. Purification of HSCs may reduce total cell number as CD34+ HSCs represent less than one percent of total cells. Alternatively, a CD34+/CD38- enrichment strategy may be employed to further purify HSCs and lower the amount vector required for modification. CD34+ cells may be pre-stimulated ex vivo for 1-3 days prior to modification, depending on the protocol. (4) Gene modification of HSCs must be permanent so as to be passed down to all progeny. Cells are modified by either a viral vector to add a gene (typically requires high concentration vector), or targeted nucleases with/without a donor template to disrupt, correct, or insert a gene. After ex vivo modification, the cell product undergoes release testing to assess purity, identity, safety, potency (transduction/editing efficiency), and other characteristics. If the modification strategy requires selection of corrected cells, low cell yield may prevent transplantation. (5) Prior to receiving the cell product, the patient undergoes conditioning to “make space” for engraftment of modified HSCs (green check represents successful modification of a disease-causing gene). Modified cells may be reinfused fresh or cryopreserved for delivery at a later time. While high-levels of cytoreductive agents may be toxic, inadequate conditioning may result in poor engraftment.

Current approaches to autologous transplant/gene therapy using lentiviral vectors (LVs) have produced clinical benefits similar to those from allogeneic transplant for several disorders. (Aiuti et al., 2013; Biffi et al., 2013; Cartier and Aubourg, 2010; De Ravin et al., 2016b). In multiple clinical trials (Table 1.2-1), this approach has consistently achieved quite stable frequencies of gene-corrected blood cells of all lineages, indicating engraftment, long-term persistence and ongoing generative capacity of gene-modified HSCs, with no significant diminution observed over time in human subjects (Cartier et al., 2012; Enssle et al., 2010).

Recent developments in gene editing have led to investigations toward its application for *ex vivo* gene correction in HSCs, which may have advantages compared to integrating viral vector-mediated gene addition (Carroll, 2016; Wright et al., 2016). This review will present the primary approach that is currently being used for gene modification of HSCs for clinical applications and gene addition using integrating viral vectors, as well as discuss the current status of gene editing in human HSCs for autologous transplantation. Lessons learned from advancing HSC therapies to the clinic may help inform the development of other stem cell therapies.

1.3. HSCS FOR GENE THERAPY

HSCs are long-lived and multipotent, so gene correction in HSCs should lead to persistent gene correction among the different lineages (Kondo et al., 2003). The hematopoietic system is an ideal target for gene therapy because of the ease with which HSCs can be accessed for *ex vivo* gene manipulation, effective gene-modification, and re-administration as an intravenous infusion

HSCs are traditionally harvested from bone marrow derived from the iliac crests under general anesthesia. Multiple aspirations are performed with the goal of collecting 10-20 ml of bone marrow per kilogram of recipient body weight. Alternatively, HSCs can be obtained as cytokine (e.g. G-CSF)-mobilized peripheral blood stem cells (PBSC) collected by leukopheresis. Hematopoietic growth factors, including GM-CSF and G-CSF, or CXCR4 inhibitors have been shown to increase the numbers of circulating hematopoietic stem and progenitor cells (HSPC) by 30-1000 fold (Brave et al., 2010). PBSCs are now the predominant clinical HSC source used for allogeneic and autologous transplants to routinely and successfully treat multiple blood cell disorders using current techniques.

However, the use of HSCs for gene therapy presents several challenges. HSCs are rare and delicate and are found among large numbers of more committed progenitors and mature blood cells that do not have long-term repopulating activity. While the immunophenotypic

definition of unitary human HSCs has been well-developed, (e.g. CD34+, CD38-, CD45RA-, CD90+, CD49f+ (Notta et al., 2011), purification to high levels at clinical scale may entail significant losses of cells and impair their stem cell capacity. In current clinical practice for gene therapy, the HSCs from the clinical source (bone marrow or mobilized peripheral blood stem cells) are enriched, rather than purified, usually by isolating the CD34+ fraction using immunomagnetic separation. The CD34+ population (~1% of cells in adult bone marrow) contains most long-term engrafting multipotent HSCs, but also far more numerous short-term progenitor cells. CD34 selection enables ~30-50-fold enrichment of HSCs, removing the majority of highly numerous mature blood cells and enriching the HSC targets to culture for *ex vivo* gene modification. The dosages of CD34-selected cells typically used for transplantation range from 2 to 20 million/kg, necessitating efficient processing of relatively large numbers of cells.

Because they will divide many times, any gene modification of HSCs needs to be permanent and heritable to be passed on to all successive generations of progeny cells. Currently this necessitates making changes in the genome, either by covalent gene addition with an integrating vector or direct genome editing. The critical technical challenge for successful HSC gene therapy is performing sufficient gene engineering of the autologous HSCs to provide a therapeutic level of permanent genetic correction without impairing their stem cell capacity or causing adverse effects.

Thresholds for sufficiency can be based on observations from cases where patients, allo-transplanted for these disorders, develop mixed chimerism with only a sub-fraction of the hematopoiesis coming from donor cells. Clinical improvement has been reported with donor chimerism as low as 10-30% for sickle cell disease, thalassemia, SCID, and other PIDs, making this level a reasonable target for engrafted, gene-corrected HSCs (Chaudhury et al., 2017; Hsieh et al., 2011).

1.4. VECTORS

1.4.1. Vector choice and design

An attractive property of retroviruses is their ability to convert their RNA genome into proviral DNA through reverse transcription and integration into the DNA of the host cell's genome in a quasi-random fashion. This integrating property of retroviruses allows the transmission of therapeutic information to all progeny of a transduced HSC. The initial retroviral systems used were derived from Murine Leukemia Viruses (MLV) a class of simple gammaretroviruses (gRV) that were well-known from studies of their oncogenic properties. Transduction of human HSCs with gRV vectors has remained challenging due to the quiescent nature of HSCs that typically cycle infrequently during steady state hematopoiesis (Cheshier et al., 1999; Passegué et al., 2005; Pietras et al., 2011). MLV requires the breakdown of the nuclear envelope and cellular progression through mitosis to stably integrate into host cell genome as the virus lacks active nuclear localization elements (Lewis and Emerman, 1994; D. G. Miller et al., 1990). Therefore, HSCs need to be cultured for several days with multiple cytokines to induce cycling for retroviral transduction, and this may lead to loss of stem cell capacity.

Lentiviral vectors (LVs) have subsequently become the vector platform of choice because they do not require the cells to undergo mitosis for the breakdown of the nuclear membrane to efficiently integrate their proviral DNA into host cells. Rather, LVs transit through the nuclear pores by recruitment of host cell proteins. The most widely used design of LV system used for transduction of HSCs was first developed by Naldini *et al* in 1996 and was subsequently shown to efficiently transduce HSCs by Miyoshi *et al* and Case *et al*, among others (Case et al., 1999; Miyoshi et al., 1999; Naldini et al., 1996). While clinically-effective methods have been developed for gene introduction to human HSCs using retroviral and lentiviral vectors, HSCs are relatively resistant to transduction, requiring the use of high multiplicities of infection of vector (e.g. 10-100, based on titers measured on permissive cells) to effectively modify the majority of HSCs.

1.4.2. Safety issues

The first clinical trial utilizing gene therapy to modify autologous HSCs with curative intent began in 1992. The goal of this first trial conducted by Bordignon *et al* was to correct severe combined immune deficiency (SCID) syndrome caused by deficiency in adenosine deaminase (ADA) (Bordignon *et al.*, 1995). SCID patients experience severe, recurrent and persistent infections resulting from immunodeficiency and, prior to the availability of HSCT options, the disease was lethal. This first clinical trial employing autologous gene-corrected HSCs to correct ADA-SCID utilized a vector derived from MLV to introduce an ADA cDNA into HSCs isolated from afflicted patients (Aiuti and Roncarolo, 2009; Gaspar *et al.*, 2014). These investigators and other groups in the U.K. and the U.S. have gone on to treat more than 45 ADA SCID patients with gRV vectors, with good immune recovery in most and no complications from the vectors (Candotti *et al.*, 2012; Gaspar *et al.*, 2011; Shaw *et al.*, 2017).

Additional trials using MLV-based gRV vectors, such as those conducted by groups in France and the U.K. for SCIDX1 (X-linked SCID) to correct interleukin 2 common gamma chain (IL2Rg) deficiency, demonstrated both the utility and the limitations of gRV vectors (Hacein-Bey-Abina *et al.*, 2002). Although curative in the majority of patients, five (of 20 total) patients developed T cell acute lymphoblastic leukemia (T-ALL), two to six years post treatment, as a result of the action of vectors that had integrated near proto-oncogenes (Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008). Similar leukoproliferative complications were seen in other clinical trials using gRV vector for X-linked Chronic Granulomatous Disease (X-CGD) (Ott *et al.*, 2006), and Wiskott-Aldrich Syndrome (WAS) (Boztug *et al.*, 2010). The occurrence of T-ALL or myeloid malignancies in subsets of patients from each of these clinical trials was a result of the LTR driven gRV vector landing upstream of proto-oncogenes and ectopically activating their expression (Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008). It is now known that gRVs tend to land near transcriptional start sites of genes, CpG islands, and DNase 1 hypersensitive sites (which tend to be transcriptionally active) (De Rijck *et al.*, 2013; Derse *et al.*, 2007; Emery *et al.*, 2009; Mitchell

et al., 2004; X. Wu, 2003). The LTRs of these gRV act as strong enhancers that recruit a number of transcription factors capable of overriding innate cellular transcriptional control of neighboring genes, promoting leukemogenesis (Modlich et al., 2009).

To address MLV's propensity to induce enhancer-mediated insertional mutagenesis, Gilboa *et al* developed the first self-inactivating (SIN) vector by introducing a deletion within the 3' U3 that abolishes enhancer activity. During reverse transcription, the deleted 3' U3 is copied to both ends of provirus DNA and deprives the provirus of LTR mediated enhancer and promoter activities (Yu et al., 1986). Instead, internal promoters can be introduced to drive transgene expression with higher regulated/tissue specific expression. This alteration provides SIN vectors with increased safety by reducing cellular gene activation when in proximity to neighboring promoters (Nienhuis et al., 2006). SIN gRV vectors have been used safely for subsequent studies of gene therapy for SCIDX1, indicating that this modification did achieve its goal of greatly reducing risks of insertional transformation (Hacein-Bey-Abina et al., 2014).

These studies informed the development of LV as vehicles for gene delivery. Self-inactivating deletions were introduced into the viral LTRs of LV and all sequences encoding proteins supporting HIV virulence were deleted from the provirus and all packaging constructs to create second (-Vpr, -Vif, -Vpu, -Nef) (Zufferey et al., 1997) and third (also -Tat) generation LV vector systems (Dull et al., 1998). The VSV-G glycoprotein is most commonly used to pseudotype lentiviral vectors, although other envelope proteins have shown some favorable properties (Girard-Gagnepain et al., 2014). Lentiviral vectors can transduce non-dividing cells via several mechanisms they have for nuclear import of their viral cores (Matreyek and Engelman, 2013). They also have somewhat larger carrying capacity than gRV vectors (6-9 kb) and are generally more robust for transducing human cells. They have mostly become the vector of choice for stable gene addition to human HSCs.

1.4.3. Methods to optimize vector delivery

A major limitation of gRV vectors is their inability to transduce non-dividing cells efficiently. Addition to culture dishes of a specific adhesion domain of fibronectin in a recombinant protein, CH-206, which recruits virus particles to HSCs (resulting in a higher MOI at the interface between virus and cell) was found to significantly increase transduction efficiency (Hananberg et al., 1996). *Ex-vivo* culture conditions were also found to influence HSC proliferation and transduction efficiency (Barrette et al., 2000; Sutton et al., 1999). Addition of recombinant human hematopoietic growth factors (typically ckit ligand, Flt-T ligand, thrombopoietin, and Interleukin-3) during transduction resulted in activation of CD34⁺ HSCs and therefore higher transduction rates. The combination of fibronectin and optimal ex-vivo culture conditions greatly improved transduction with proven success in the clinic, as discussed below (Millington et al., 2009).

Another method that has been used to enhance transduction is to alter the cell target specificity of a viral vector by exchanging the innate envelope protein for one derived from an alternative virus allows researchers to alter the tropism of resultant vector particle (called pseudotyping). Typically, pseudotype is chosen based on expression level and exclusivity of the envelope protein's cognate receptor (higher receptor levels equal greater gene transfer levels). HSCs can be transduced with RD114, GALV, BaEV and VSV-G pseudotype viruses, among others. Other, more specific pseudotyping strategies are being developed that include the use of diverse viral envelopes and even fusions proteins with antibodies or cytokines to target specific cell types (Gennari et al., 2009; Verhoeyen et al., 2005).

1.4.4. Advances in vector design

Surprisingly, there have been no significant improvements to the basic design of lentiviral vectors since the so-called third generation vectors were introduced almost two decades ago (Dull et al., 1998; Zufferey et al., 1998). They were designed based on a decade of experience with gammaretroviral vector design and production and have met all the safety expectations, with no

report of emergence of replication-competent lentivirus during packaging in research or clinical manufacturing.

Lentiviral vectors with relatively small and simple gene cassettes (e.g. human phosphoglycerate kinase gene or elongation alpha-1 gene minimal promoters and a cDNA) are readily produced to titers sufficiently high for effective gene modification of human HSCs at clinical-scale. However, low vector titer remains a significant problem with some LVs, especially those tasked to carry larger transgene cassettes, such as the human beta-globin gene. Several studies have shown that increasing viral RNA genome length negatively affects both titer and transduction efficiency (Cant Barrett et al., 2016; Kumar et al., 2004). Reduction of viral RNA length through removal of non-essential sequences is a viable strategy for improving LV titer for large transgene cassettes. Additionally, codon optimization may be used to improve titer by depleting secondary structures detrimental to mRNA stability (with the added benefit of improving transgene expression levels) (Moreno-Carranza et al., 2008). Other strategies for increasing LV titer include the addition of the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (wPRE), which has been shown to increase vector titer through increasing vector genomic RNA stability, export and translation (Hope, 2002; Schambach et al., 2000) or addition of elements known to improve polyadenylation of vector mRNA during packaging, such as the Bovine Growth Hormone Polyadenylation Sequence (Woychik et al., 1984). Recently, Vink et al described a reconfiguration of the basic arrangement of the key cis-regulatory elements of HIV included in the vector backbone (LTRs, primer binding site, rev-responsive element) to simplify the process of reverse transcription, which may be a limit to transduction with large vectors (Vink et al., 2017). The advantages of this design in clinical applications remain to be tested.

To date, lentiviral vectors have been produced by transient transfection of multiple plasmids (vector, packaging proteins, envelope), which is cumbersome and challenging to scale-up to levels that would be needed for commercial production. There have been extensive and long-standing efforts to develop stable lentiviral vector packaging lines, similar to those routinely

produced for gammaretroviral vectors, which could provide a master cell bank for much simpler production of vector lots. It has proven to be challenging to repress expression of the VSV-G glycoprotein (which remains the most effective pseudotype for lentiviral vectors in most cases) and some of the HIV-1 genes which may be cytotoxic to the packaging cells for cell passaging, but then rapidly and robustly inducing expression of these genes for vector production. One stable packaging cell line has been used to produce lentiviral vector for clinical trials. (De Ravin et al., 2016b; Throm et al., 2009) . It was made by successively transfecting plasmids encoding each gene cassette needed to make the virus proteins (HIV gag/pol, VSV-G) under tight, inducible expression control, and then concatamers of the vector plasmid to obtain multiple copies. This led to a cell line that was capable of scale-up to production volumes needed for production of clinical lots without loss of the packaging capacity.

1.4.5. Gene expression from lentiviral vectors

For some gene therapy applications, unregulated constitutive, ubiquitous expression of the transgene is acceptable. For example, adenosine deaminase (ADA) is expressed in all cell types and a broad range of ADA enzyme activity in all blood cell lineages is safe and sufficient to allow immune reconstitution. Thus, the vectors deployed for gene therapy of ADA SCID have used constitutive promoters, such as the MLV LTR or the Elongation Factor 1-Alpha gene promoter (Aiuti et al., 2009; Carbonaro et al., 2014). Other genes may require precise lineage, temporal or physiological-responsive expression patterns to be safe and effective. For example, molecules involved in signal transduction (receptors, intracellular signaling molecules, transcription factors) may be expressed in only specific cell types, or under specific physiologic states or in response to specific stimuli (e.g. *BTK*, CD40 ligand, *JAK3*, Stat proteins) (Brown et al., 1998). In some cases, it has been possible to build vectors using transcriptional control elements from endogenous cellular genes to apply regulated expression of transgenes (e.g. Beta-globin transcriptional control elements directing erythroid-specific expression of beta-globin

(Sadelain et al., 2000). However, it may not be possible to incorporate into a vector the necessary regulatory sequences to recapitulate endogenous gene expression patterns for vectors integrated at an array of chromosomal sites in different cells. Insulator elements have been incorporated into some vectors to attempt to mitigate potential silencing of vector expression by heterochromatinization or trans-activation of adjacent cellular genes (Browning and Trobridge, 2016; Emery, 2011). The benefits of insulators remain theoretical and, in fact, silencing and trans-activation has been a problem with current lentiviral vectors. Endogenous gene expression is often controlled by enhancer and promoter interactions that occur over long distances (up to 100kb) away (West and Fraser, 2005). Regulated gene expression can be achieved to variable extents by adding a gene's known enhancer(s) upstream of a minimal promoter within the LV. These enhancers recruit and bind specific sets of transcription factors to cause *cis* activation of the promoter (Spitz and Furlong, 2012). The best studied example is the use of multiple elements from the β -globin gene locus to achieve erythroid-specific expression for the treatment of hemoglobinopathies (Cavazzana-Calvo et al., 2010; Sadelain et al., 2000). However, it is sometimes difficult to fit all of the necessary *cis*-acting genetic elements required for precise gene expression within the size limit of vector genomes. In some cases, the function of enhancers can be antagonized by repressive chromatin structure at some LV integration sites (Hofmann et al., 2006; Yao et al., 2004). Chromatin domain insulators can be added into the LV's LTRs to overcome these positional effects and may also reduce risks from internal enhancers of vectors from affecting neighboring genes (Ramezani et al., 2008).

1.4.6. Lentiviral Vectors in Clinical Trials

Clinical trials using LVs began in the mid 2000's (Cartier et al., 2009; Cavazzana-Calvo et al., 2010) and LV have now been used safely and effectively in multiple studies for almost a dozen disorders using hematopoietic stem cells (**Table 1.2-1**) (and in many of the T cell-based immunotherapies with Chimeric Antigen Receptors and T Cell Receptor genes (Morgan and

Boyerinas, 2016). In most trials, gene delivery to HSCs was at sufficiently high levels to produce clinically beneficial levels of gene-modified HSCs and relevant mature hematopoietic cells for the treatment of disease. The absence of GVHD and the reduced amounts of conditioning chemotherapy needed for engraftment of the autologous HSCs has allowed the predicted improved safety profiles. Analyses of LV integration site in the blood cells of subjects in the different clinical trials have shown a remarkably consistent pattern, with no predilection for insertion near proto-oncogenes and no clinically significant clonal expansions (Biasco et al., 2016; Biffi et al., 2011). LV gene therapy is being developed for several other disease indications (**Table 1.2-1**), including additional primary immune deficiencies, storage and metabolic diseases, and stem cell defects such as Fanconi's Anemia. Limitations to wider applications for more clinical indications are now less biological and more logistic, as each disorder requires its specific vector and the entire pre-clinical drug development pathway. Funding by research grants to perform vector development for additional indications may become more difficult to obtain, as the scientific novelty is diminishing; while funding from pharmaceutical companies and venture capitalists relies on the expectations of financial returns, which are unknown for these relatively rare orphan disorders. Issues related to commercial marketing and reimbursement for these cell and gene therapies are complex (Brennan and Wilson, 2014; Orkin and Reilly, 2016). Additionally, the capacity to produce the large volumes of clinical-grade LVs for these studies (and also for the larger studies of CAR T cells), is limited and may slow progress. Nonetheless, gene therapy using HSCs is continuing to advance and provide effective and safe therapies for a growing list of disorders.

1.4.7. X-linked Adrenoleukodystrophy (X-ALD) HSC Gene Therapy

A recent report described efficacious HSC gene therapy for X-linked Adrenoleukodystrophy (X-ALD), a progressive neurodegenerative disorder with onset in boys during the first 1-2 decades (Eichler et al., 2017). The major premise for the approach is that the

gene-corrected engrafted HSC will produce cells that become CNS microglia and provide essential enzyme activity that can rescue very long chain fatty acid catabolism and prevent demyelination. Graft versus host disease, which seems to accelerate progression of X-ALD (Peters et al., 2004), is avoided with the use of autologous cells. The findings of beneficial stabilization of neurologic status now extend and expand upon those of the initial report of two X-ALD patients treated using a lentiviral vector into CD34+ PBSC (Cartier et al., 2009). The larger cohort studied by Eichler et al. (n=17) showed a high rate of response, with only one patient having progressive neurologic deterioration. HSC gene therapy effectively arrested disease progression in 88%, either without symptoms or with only initial progression to early neurological impairments followed by stabilization of neuro-imaging studies and clinical function.

This was the first commercially-sponsored clinical trial of HSC gene therapy (bluebird bio), with essentially all prior trials done at academic medical centers as research investigations. It was performed as a Phase II/II trial intended to obtain data to support applications for regulatory approval to market this stem cell gene therapy. Centralized manufacturing of the stem cell product was performed under full GMP conditions. Stem cell mobilization and leukopheresis was performed at several academic clinical sites enrolling subjects and shipped to a contract manufacturing organization for stem cell enrichment and transduction, yielding a cryopreserved product, which was shipped back to the clinical site following completion of product release testing. They produced consistently high-quality cell products with good cell dosages ($6-19 \times 10^6$ CD34+ cells/kg) and gene transduction levels (0.5-2.5 vector copies per cell). Patients were treated within 2-3 months from enrollment, which is an excellent time-frame to produce and certify a gene-modified stem cell product and perform all the clinical evaluations prior to an HSC transplant. The high rate of successful outcomes in terms of halting neurologic progression meet or exceed those of the clinical alternative of unrelated or haplo-identical donor transplants, strongly supporting the use of autologous gene therapy for this disorder (and by extension to many other storage diseases). Bluebird bio is pursuing regulatory drug approval for this combined

cell and gene therapy product, which would represent one of the first approved gene therapies (bluebird bio, Inc., 2017).

1.5. GENE EDITING

Viral mediated gene transfer has well established benefits and has demonstrated clinical efficiency, as described above. However, risks and drawbacks of these methods remain, such as insertional oncogenesis and modified transgene expression pattern (Cesana et al., 2014; Ng et al., 2010; Zhou et al., 2016). Targeted gene editing allows for site-specific genome modification, and thereby eliminates the risks posed by randomly inserting genes. Another advantage of targeted gene editing over viral vectors is the ability to retain endogenous control of gene expression (Barzel et al., 2014; H. Li et al., 2011). Targeted gene editing can be achieved by employing site-specific endonucleases to induce a double-stranded break (DSB) in the DNA near the mutation site. The recruitment of DNA repair proteins to the site of DNA damage stimulates DNA DSB repair via one of two main pathways: non-homologous end joining (NHEJ), or homology-directed repair (HDR) (**Figure 1.5-1**). NHEJ can be used to achieve gene disruption with site-specific induction of a DSB by a targeted nuclease and NHEJ-mediated introduction of small insertions and deletions (Indels). NHEJ is a quick but error-prone pathway (Metzger and Iliakis, 2009); HDR is precise, but is dependent on cell cycle phase (S. Kim et al., 2014). Both of these endogenous DNA repair mechanisms can be harnessed for therapeutic benefit. In mammalian cells, NHEJ is more prevalent than HDR (Chiruvella et al., 2013). For a more detailed review on these DNA damage repair pathways, please see (Chapman et al., 2012; Heyer et al., 2010; Hustedt and Durocher, 2016; Lieber et al., 2003; Shrivastav et al., 2008).

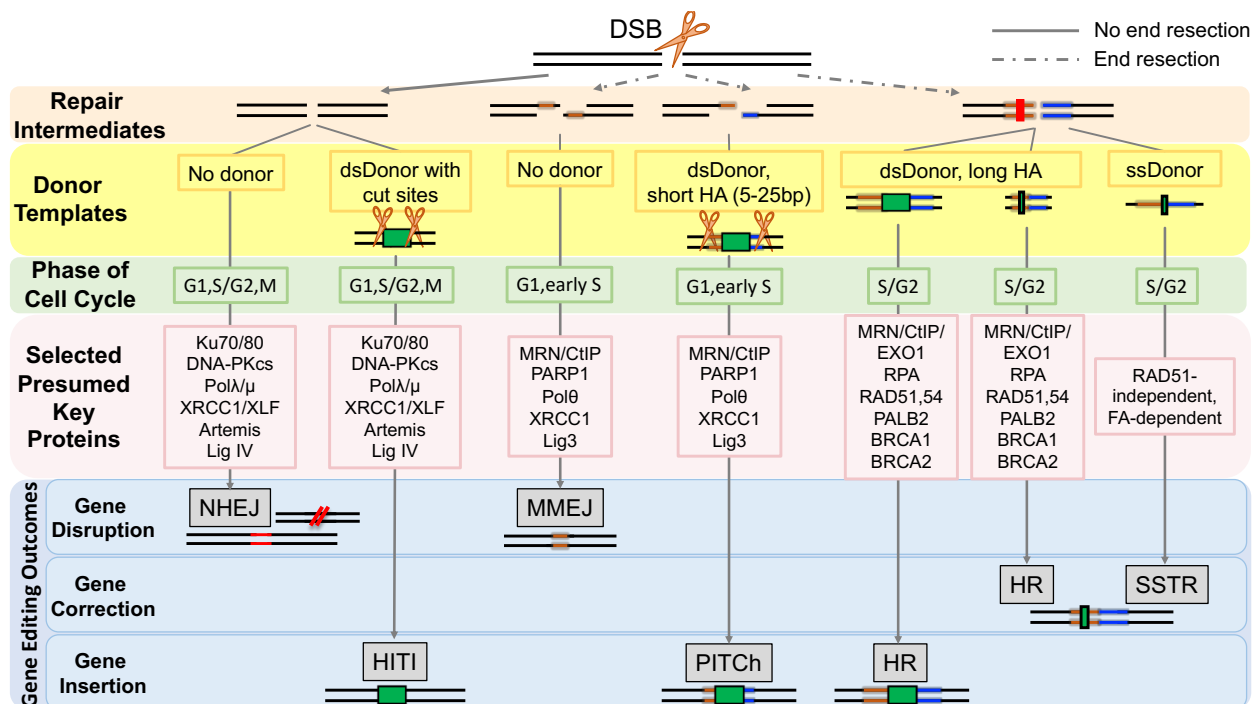


Figure 1.5-1: Summary of Gene Editing Pathways.

Double stranded break (DSB) is induced by a targeted nuclease (represented by scissors). DSB ends may or may not be resected (dashed or solid line, respectively). The ultimate gene editing outcome (light blue boxes on the bottom) depends on several factors: the type of donor template provided (yellow box), the phase of cell cycle (light green box) and the presumed DNA repair proteins available (pink box). Gray boxes indicate the names of the repair mechanisms. It should be noted that the figure illustrates the common pathways described to date, however modification of DNA repair pathways and their utilization for gene editing purposes is an area of active research.

(From left to right). A DSB with no end resection and no donor available is likely to result in insertions and deletions (indels) and lead to gene disruption via the non-homologous end joining (NHEJ) pathway. NHEJ may occur in any phase of cell cycle. Exogenously providing a double-stranded donor (dsDonor), which contains nuclease cut sites (scissors) around the gene of interest (green rectangle), may result in homology-independent targeted integration (HITI). The presence of microhomology on opposite strands of DNA around the cut site may result in gene disruption via the microhomology-mediated end joining (MMEJ) pathway. A recently reported method of gene integration, termed precise integration into target chromosome (PITCh), utilizes MMEJ machinery to integrate a gene of interest, which is provided by dsDonor with short homology arms (HA) to the DNA (HA are highlighted in orange and blue). The three pathways on the right are generally only active in S/G2 phases of cell cycle and may be used to correct a single nucleotide mutation in the DNA (represented by a red line). Exogenously providing a dsDonor with long homology arms may lead to either gene integration or gene correction via homologous recombination (HR) mechanism, depending on the length of the donor template. A new type of repair mechanism for gene correction was recently described, termed single stranded template repair (SSTR). Although resulting in the same outcome as HR-mediated gene correction, SSTR is presumed to utilize the Fanconi Anemia (FA) pathway and be RAD-51 independent.

1.5.1. Four main classes of endonucleases have been used for gene editing:

1.5.1.1. Homing Endonucleases

The first, homing endonucleases recognize DNA sequences up to 40 bp long. These proteins are naturally found in six structural families (Jasin, 1996). The LAGLIDADG endonuclease family can be engineered to modify the sequence of DNA they recognize. The

process is difficult and time consuming. An alternative being explored to facilitate retargeting HEs has been dubbed “megaTAL” (Boissel et al., 2013). These megaTALs feature a DNA binding domain composed of transcriptional activator like (TAL) effector DNA recognition motifs with the active endonuclease domain of a meganuclease.

1.5.1.2. Zinc finger nucleases (ZFNs)

ZFNs, the second generation of engineered targeted endonucleases, provide a more easily modified system than homing endonucleases (Y. G. Kim et al., 1996). The enzyme functions as a dimer, with each ZFN containing three to five Zinc Finger protein motifs which recognize 3 base pair sequences of DNA and half of the *FokI* endonuclease complex. When a pair of ZFNs with appropriate target sequence binds closely enough for their *FokI* domains to dimerize, they make a DSB. ZFNs have promising function but are limited by the complexity of engineering new pairs.

1.5.1.3. Transcription activator like effector nucleases (TALENs)

TALENs function similarly to ZFNs but use a different mechanism to recognize specific regions of DNA (Cermak et al., 2011). Instead of the zinc fingers, TALENs have 15-30 repeats of a 35 amino acid transcription activator like effector (TALE). Each TALE is composed of mostly invariable regions with only two amino acid differences known as repeat variable di-residues (RVDs). A TALE recognizes one base pair determined by which RVD a TALE contains. Adding a number of these TALEs together, fusing them to a *FokI* domain, and administering them in pairs, allows similar DSB formation to ZFNs but with an easier and more modular assembly.

1.5.1.4. CRISPR/Cas9

The most recently described targeted endonuclease, Cas9, is a monomeric protein guided by a specific type of RNA, known as a CRISPR guide (Mali et al., 2013a). The guide RNA (gRNA) contains an 18-21-nucleotide long target sequence attached to a 3' RNA scaffold loop for Cas9 protein binding. The target region must be complementary to a region in the DNA immediately upstream of a 2-5 base pair proto-spacer adjacent motif (PAM) which depends on the species of

bacteria from which the Cas protein is derived. The most obvious advantage of CRISPR/Cas9 over the other nucleases is the ease and flexibility of developing guides to target new sites.

1.5.2. Gene Editing Strategies

Depending on the disease being targeted, the type of targeted editing required may fall into one of the three categories: gene disruption, gene correction, or gene insertion (**Figure 1.5-1**).

1.5.2.1. Gene Disruption:

In certain cases, knocking out a regulatory element, viral receptor or a pathogenic gene may be sufficient to ameliorate the disease-causing phenotype. High levels of gene disruption may be achieved in hematopoietic stem and progenitor cells because this type of editing does not require a donor template and can be done via the NHEJ pathway. For example, disruption of the *BCL11A* erythroid enhancer (a repressor of fetal globin expression) can increase levels of fetal hemoglobin for the treatment of sickle cell disease and beta-thalassemia (Bauer et al., 2013; Bjurström et al., 2016; Canver et al., 2015; Chang et al., 2017). Alternatively, knockout of the *CCR5* gene in cells from HIV-infected individuals can prevent ongoing infection by the virus (Cradick et al., 2013; Hendel et al., 2015; Holt et al., 2010; L. Li et al., 2013; Mandal et al., 2014; J. C. Miller et al., 2010; Perez et al., 2008; Saydaminova et al., 2015; J. Wang et al., 2015). Trials targeting *BCL11A* are approaching the clinic (Chang et al., 2017) and several early phase clinical trials have been completed using ZFNs to modify the *CCR5* gene in HIV-infected patient peripheral blood T-cells (Tebas et al., 2014) or HSCs (DiGiusto et al., 2016).

1.5.2.2. Gene Correction:

Diseases that result from a single nucleotide substitution or other small genetic lesions may be corrected by providing a homologous donor with the corrective sequence to serve as a template for DNA repair via the HDR pathway. Applications of this approach include correction of the sickle cell mutation in the beta-globin gene and restoration of beta-globin expression in beta-thalassemia. It is challenging to engage HDR-mediated repair in the quiescent, long term HSC

population. In pre-clinical studies, gene correction levels of ~10%, ~25%, and ~35% using TALENs, ZFNs and CRISPR/Cas9, respectively, at the HBB locus were reported *in vitro* (DeWitt et al., 2016; Genovese et al., 2014; Hoban et al., 2015). However, once the gene-edited cells were transplanted into immunocompromised NOD-scid-IL2Rg^{null} (NSG) mice, the gene correction levels decreased to below 10%. These data suggest that correction was less efficient in the true stem cell population than in more differentiated progenitor cells. Further studies must be performed to improve HDR in HSCs. Recently, a new mechanism of gene correction using a single-stranded donor template was reported, which is thought to act in a RAD-51 independent, and Fanconi anemia (FA)-dependent manner (Richardson et al., 2017). Modulation of FA pathway may be beneficial for improving the efficiency of gene correction.

1.5.2.3. Gene Insertion:

In many human genetic diseases, there are a variety of different pathogenic mutations spread across the relevant gene in different patients. In general, the lengths of the gene repair tracts mediated by HDR are relatively short (<40 bp), so that it may be necessary to develop a panel of nuclease/donor template combinations capable of performing efficient editing to cover an entire gene region (Paquet et al., 2016). If this was accomplished using multiple CRISPR guides targeting different sites along a target gene or the genome, each guide might require validation for levels of activity and specificity, which could be impractical for Good Manufacturing Practice (GMP) production of the cells.

Instead, activity of the whole gene can be restored by the targeted insertion of a corrective cDNA of the relevant gene into the start of the endogenous gene locus. Examples of genes being targeted in HSCs by this method include: *IL2Rg* for X-linked Severe Combined Immune Deficiency (X-SCID), *BTK* for X-Linked Agammaglobulinemia (XLA), *CD40L* for X-Linked Hyper IgM Syndrome (XHIM), and *CYBB* for X-Linked Chronic Granulomatous Disease (XCGD) (Clough et al., 2016; De Ravin et al., 2016a; Genovese et al., 2014; Hubbard et al., 2016; Lombardo et al., 2007). Gene cassettes may be inserted into specific gene loci, such as *CCR5* for anti-HIV

strategies to knock-out the viral co-receptor gene and simultaneously insert another anti-HIV gene; or into “safe harbors” such as the AAVS1 site which safely supports sustained transgene expression (De Ravin et al., 2016a; DeKolver et al., 2010; Hockemeyer et al., 2009; Lombardo et al., 2007; Mali et al., 2013b; J. Wang et al., 2015).

The donor template or cassette is flanked by homology arms surrounding the nuclease cut site and generally consists of the gene’s full-length cDNA complete with a stop codon and a 3’ untranslated region containing the polyadenylation signal. This cDNA donor template is most often delivered via an adeno-associated virus (AAV) or other non-integrating vector (J. Wang et al., 2015). A major challenge of this approach is to achieve efficient delivery and integration of these larger donor template. In pre-clinical studies, gene insertion rates of up to 43% at the IL2RG, AAVS1 or CYBB loci were achieved *in vitro*; however, as with gene correction, the levels of gene insertion in HSCs decreased *in vivo* (De Ravin et al., 2016a; Genovese et al., 2014; Schirotti et al., 2015; J. Wang et al., 2015).

Recently, two new methods of gene integration were described: homology-independent targeted integration (HITI) and precise integration into target chromosome (PITCh), which use NHEJ and MMEJ machinery, respectively (Nakade et al., 2014; Sakuma et al., 2015; Suzuki et al., 2016). The advantage of these strategies over HDR-mediated gene integration include being able to target cells outside the S/G2 phases of cell cycle.

1.5.3. Quiescence

HSCs are quiescent and mostly reside in the G0/G1 phase of cell cycle, providing a unique challenge for gene editing, since cell cycle phase is a major factor determining which DNA repair pathway is utilized to repair DSB. NHEJ occurs throughout the cell cycle while HDR is mostly restricted to S/G2 phases, when a sister chromatid is available to serve as a homologous template for repair (Branzei and Foiani, 2008; Pietras et al., 2011). This pattern of DNA repair has caused the rates of targeted gene correction and insertion in primary human HSCs to remain relatively

low and rates of gene disruption by indels too high. Methods to increase HDR and decrease NHEJ are being developed. Current gene editing protocols utilize culture with a combination of recombinant hematopoietic growth factors (e.g. *ckit* ligand, FLT3 ligand, thrombopoietin and others) to induce cell cycling 24-72 hours prior to the delivery of nuclease and donor template. However, even with pre-stimulation the majority of HSPCs are not in S/G2 phases. Cell synchronization agents have been used successfully in 293T cells and the H9 embryonic cell line to temporarily arrest the cells in S/G2 phases of cell cycle during DNA repair to increase HDR (Lin et al., 2014). However, the downstream effects of cell synchronization on the self-renewal and differentiation potential of HSCs is not yet known.

An alternative method being explored to improve the precision of gene insertion and gene correction is by simply reducing NHEJ. One such method reported to reduce NHEJ is the inhibition of Ligase IV, which is involved in the final step in the NHEJ pathway (Maruyama et al., 2015; Srivastava et al., 2012; Van Trung Chu et al., 2015). However, other groups have not been able to achieve a significant decrease in NHEJ using this inhibitor (Gutschner et al., 2016; Pinder et al., 2015; Yang et al., 2016). A potential concern with the inhibition of Ligase IV is that decreasing NHEJ levels in the cells may not result in an increase in HDR if the cells have already committed to the end-joining pathway. The effects of this type of late repair pathway blockade are still unknown, but it may lead to lower correction efficiency or even induce apoptosis.

Rather than preventing NHEJ via its terminal step, another possibility is to control the DNA repair pathway choice more upstream at the decision-making stage. For instance, formation of the BRCA1-PALB2-BRCA2 protein complex is crucial for HDR to occur; however, it is inhibited during G1 (Orthwein et al., 2015). Modulating the interaction between BRCA1-PALB2-BRCA2 in U2OS cells allowed Orthwein and colleagues to initiate HDR in the G1 phase of cell cycle (Orthwein et al., 2015). Whether this approach can be translated to primary human HSPCs remains to be tested. Another possibility is to decrease nuclease cutting in the G1 phase of the

cell cycle by adding to Cas9 a fragment of the Geminin protein that causes Cas9 degradation during G1 when only the NHEJ repair pathway is available (Gutschner et al., 2016).

1.5.4. Methods of Delivery:

One recurring challenge of targeted editing is how best to deliver the endonuclease and homologous donor template (if necessary for the particular treatment) to HSCs. Primary human HSCs are notoriously resistant to transfection methods of gene delivery (Van Tendeloo, 2001). Electroporation methods to deliver nucleic acids have improved over time and effectively transfer nucleic acid to the majority of HSCs in a treated sample. However, there is often a mild-moderate degree of toxicity from electroporation and this is significantly worsened by delivery of plasmid DNA, in some cases resulting in up to 60% cell death 24 hours post electroporation (Hendel et al., 2015). The delivery of *in vitro* transcribed mRNA encoding the nuclease and either *in vitro* transcribed or chemically-synthesized short guide RNA is better tolerated. Co-delivery of recombinant Cas9 protein complexed to short guide RNA as ribonucleoprotein (RNP) complexes has also been shown to be effective (S. Kim et al., 2014).

Delivery of homologous donor sequences has been achieved with multiple modalities. Chemically-synthesized oligonucleotides (e.g. 50-200 bp in length) are effective donors for small sequence changes, although they may cause moderate toxicity when introduced by electroporation (Hendel et al., 2015). Integrase-defective lentiviral vectors (IDLV), and the more effective adeno-associated virus (AAV) vectors can efficiently deliver donor sequences of variable lengths (up to several kb) to HSCs with lower cytotoxicity than oligonucleotides or plasmids (Dever et al., 2016; Hoban et al., 2015; J. Wang et al., 2015).

1.6. GENE EDITING OF HSCS FOR CLINICAL APPLICATIONS

For clinical applications, multiple reagents are thus needed to perform gene editing (nuclease and donor) and each will need to be produced under standardized GMP conditions. Research scale editing is typically done with $0.2\text{-}1 \times 10^6$ CD34+ cells per experimental arm; clinical

scale will involve at least 5-10x that many CD34+ cells *per kg*, and thus 50-1,000 times more cells. Although standards for acceptable levels of off-target cutting by a nuclease for clinical editing have not been defined, it is incumbent to investigate their occurrence with the most sensitive and relevant assays that can be practically done as part of pre-clinical toxicology assessments.

1.7. CHALLENGES TO CLINICAL APPLICATION OF HSC GENE THERAPY

1.7.1. HSC harvest and expansion

While there has been much progress in applications of HSC gene therapy, many challenges remain. The numbers of HSCs that can be obtained from a patient are limited by the yields that can be isolated by bone marrow harvest or mobilization, although the combination of G-CSF and a CXCR4 inhibitor (plerixafor) generally leads to abundant cell collections (Brave et al., 2010). Some specific diseases may limit the numbers of HSCs that can be isolated, such as Fanconi anemia, which results in progressive HSC failure, or osteopetrosis, where the marrow space is progressively reduced by the accumulated bone (Daneshbod-Skibba et al., 1980; Giri et al., 2007).

The cell processing manipulations, including stem cell enrichment or gene modification--particularly when using electroporation--may lead to significant cell losses. Efforts to expand the numbers of true transplantable HSCs have been made, with several small molecules (such as SR-1, UM171, PGE₂) holding some promise, although no massive HSCs expansion has been achieved (Boitano et al., 2010; Fares et al., 2014; Goessling et al., 2011; Hoggatt et al., 2009; North et al., 2007). The goal of producing transplantable HSCs from pluripotent stem cells is advancing, with direct reprogramming to HSCs from endothelial cells also showing promise (Lis et al., 2017; Sugimura et al., 2017).

1.7.2. Gene Transfer

Gene transfer to HSCs has also advanced to a large degree, with current protocols of hematopoietic growth factor stimulation and transduction with lentiviral vectors reaching therapeutic efficacy for many disorders. Nonetheless, here too, improvements are needed. Human HSCs are relatively resistant to lentiviral vectors, evidenced by the seemingly high multiplicities of infection (M.O.I., the vector/cell ratio) needed to effectively transduce HSCs, compared to the relatively easier transduction of the cell lines typically used to gauge vector titers. The carrying capacity of lentiviral vectors has limitations, with vector titers falling off sharply as the size of the gene cassette increases. In our hands, a lentiviral vector at the small end of the size range (e.g. with a simple cDNA or transgene like GFP and a small promoter at ~4 kb proviral length) has a titer 10-30-fold higher than a vector at the large end of the size range (e.g. with a beta-globin gene cassette with exons, introns, upstream locus control region segments at ~9kb). The lower production titer necessitates a proportionately higher volume of vector preparation to produce a patient dose, increasing the costs. Additionally, the bigger vectors do not transduce HSCs as well as smaller vectors, even when adjusted to matching MOI. Improved transduction of HSCs with lentiviral vectors using small molecules (proteasome inhibitors, cyclosporine A, rapamycin) has been reported in pre-clinical studies (Petrillo et al., 2015; Santoni de Sio, 2006; C. X. Wang et al., 2014), but the effects have not been clinically validated. And, of course, the semi-random integration of the vectors throughout the genome continue to pose genotoxicity risks, although these are greatly diminished with current generation vectors that lack the strong long terminal repeat enhancers that were the major cause of insertional oncogenesis with the first generation of gRV's.

1.7.3. Gene Editing

Gene editing may avoid many of the problems specific to viral vectors, but it too remains less than ideal in several ways. The various site-specific endonucleases (HE, ZFN, TALEN, or

CRISPR) are fairly efficient and targeted gene disruption in HSCs is now in clinical trials for HIV (targeting CCR5, HIV co-receptor) and sickle cell disease (targeting BCL11a, repressor of fetal globin) (Chang et al., 2017; DiGiusto et al., 2016); clinical results have not yet been reported. However, the more elegant goals of targeted gene correction and gene insertion are more complicated to achieve, relying on the HDR pathway to perform the desired edits and thus requiring co-delivery of a homologous donor with the nuclease. Before clinical translation of gene editing, GMP methods of gene editing combining multiple GMP-grade reagents (e.g. CRISPR RNP or mRNA and short-guide RNA; AAV vector homologous donor) will need to be established. Pre-clinical work editing human HSCs using a clinically-scale approach has been reported (De Ravin et al., 2017).

1.7.4. Ex vivo processing

The ex vivo processing to enrich HSCs for clinical gene therapy has mostly been limited to CD34+ cell selection. This achieves a moderate (30-50-fold) decrease in total numbers of cells that need to be exposed to vector or gene edited (akin to a lineage-negative {lin-} murine population), but yet retains most of the HSCs. However, the CD34+ cell population is still quite heterogeneous with only a small fraction of cells being the target long-lived HSCs. Thus, a large proportion of the vector or gene editing reagents are wasted modifying the more abundant but short-term progenitor cells. Efforts to further enrich for HSCs using additional markers such as CD38(-), CD90(+), CD133(+), etc., (akin to a murine “LSK” {lin-/Sca1+/ckit+ fraction}) have been reported, but require FACS sorting which may entail long processing times and subject the cells to damaging shear forces (Baum et al., 1992). Newer sorting methodologies based on microfluidics or using serial immuno-affinity bead processes may be beneficial if they provide further enrichment without undue losses of cells (Masiuk et al., 2017; H.-W. Wu et al., 2010).

The cell culture methods used in current clinical trials are relatively standardized, using static culture in gas permeable bags or flasks in serum-free medium supplemented with multiple

hematopoietic growth factors. The use of continuous feed bioreactors and/or lower partial pressures of oxygen may provide more optimal conditions for HSC modification and preservation. Small molecules such as PGE₂ may also support HSC survival ex vivo improving the level of engraftment of gene-modified HSCs (Hoggatt et al., 2009). Additionally, the current ex vivo processing of HSCs is often done in multiple open systems, but new closed systems that continuously contain the cells are being developed that may allow processing to be done in environments less demanding than the current GMP “clean rooms”.

1.7.5. Universal donor products

Universal donor cells could largely supplant use of autologous cell products if able to achieve the ideal properties of immunogenicity absence. They can be banked as an off-the-shelf, immediately-ready source of compatible normal cells, including regenerative stem cells. Universal donor cells would have a major advantage in that they could be produced in multi-patient dose lots vs. patient-specific single lots using autologous cell products. It is possible to engineer the cells to have favorable properties, e.g. produce a therapeutic protein such as clotting factors and other serum proteins, lysosomal enzymes, anti-tumor T cell receptor or Chimeric Antigen Receptor or an immunomodulative cytokine or chemokine. Allogeneic HSCT sources may continue to have advantages for HSCT for hematologic malignancies due to their potential graft-versus-leukemia effects, although it should become possible to augment specific immune effector cell products for the positive anti-leukemia effect, but without risks for GVHD.

1.7.6. Pre-transplant cytoreductive conditioning

Finally, the pre-transplant cytoreductive conditioning used to “make space” for engraftment of the isolated and reinfused HSCs is finally advancing beyond the use of cytotoxic chemotherapy drugs or radiation. These agents are effective at ablating the marrow stem cells (myeloablation), which is necessary for engraftment of gene-modified HSCs and for suppressing the recipient’s immune system (immunoablation), which is necessary to avoid immunologic

rejection of the graft. However, they may have severe acute toxicities in multiple organ systems (heart, lungs, liver, kidney, GI) and may produce infertility or sterility, due to toxicity to germ cells. Monoclonal antibodies to HSC surface proteins (e.g. ckit, CD47, CD45) have been shown in murine models to allow improved engraftment without apparent toxicity (Chhabra et al., 2016; Czechowicz et al., 2007; Palchaudhuri et al., 2016; Xue et al., 2010). These efforts are now being translated to the clinic and may eliminate the need to use toxic preparative regimens to facilitate engraftment.

1.8. LESSONS LEARNED

1.8.1. It takes a long time

Lessons learned from the almost 30-year history of developing clinical HSC gene therapy products can inform emerging stem cell-based cellular therapies for myriad other non-hematopoietic diseases such as Duchene's Muscular Dystrophy, Huntington's Disease, Parkinson's Disease, Diabetes Mellitus and others. One clear lesson has been that development of novel therapies takes a long time. Methods for effective gene transfer to HSCs were initially developed in the 1980s and clinical trials started in the 1990's. The initial trials yielded no evidence of efficacy; the first clinical successes were not seen until the 2000's and only in the last decade are therapeutic benefits being conferred consistently for multiple disorders. Several promising HSC gene therapy cell products are advancing through early phase clinical trials (for indications including X-adrenoleukodystrophy, Metachromatic Leukodystrophy, Beta-thalassemia, Sickle Cell Disease, ADA-deficient and X-linked forms of SCID, Wiskott-Aldrich Syndrome, Chronic Granulomatous Disease) and towards licensure for commercial manufacture and sales, with the first (Strimvelis for ADA-deficient SCID) approved by the European Medicines Agency (European Medicines Agency, 2016). While it may be expected that therapies using other stem cell types will be derived in a shorter time-frame, drug development remains a slow process.

1.8.2. Support academic medical centers to develop novel cell therapies

HSC gene therapies were incubated at academic medical centers in multiple countries often at innovative HSCT programs, not via the traditional pharmaceutical company model of drug development. Universities and other research centers need to have sufficient infrastructure for early phase clinical trial performance and GMP cell processing to achieve similar academic pioneering for other stem cell therapies. Indeed, the centers that have had strong gene therapy programs with the necessary cell processing and regulatory infrastructure have been the leaders in this field (e.g. TIGET, Milan Italy; Hôpital NeckerEnfants Malade, Paris, France; University College London, London, UK; The National Institutes of Health, Bethesda MD; University of California, Los Angeles, Los Angeles CA; Boston Children's Hospital, Boston MA; St. Jude Children's Research Hospital, Memphis TN). Ongoing support of this type of research will be essential to continue the innovation of new therapies.

1.8.3. Do it right

In the early days for the field of gene therapy, the NIH RAC-provided public oversight to review clinical protocols to be performed in academic medical centers was an important forum to discuss the novel potential biohazard issues, as well as scientific and ethical concerns. This may have tempered or even slowed some advancements, but it helped provide another level of expertise, in addition to that provided by local IRB and FDA (and EMA in Europe), to ensure that trials were based on sound scientific principles, had adequate supporting pre-clinical data on potential efficacy and safety, and were well-designed and monitored. The role of the RAC in overseeing individual trials has decreased, but it still serves its federal advisory role in assessing novel biosafety issues. Other forms of stem cell therapy should also proceed with a base of strong pre-clinical data, careful consideration of the clinical setting and approach, as well as well-controlled cell manufacturing and regulatory oversight, to provide maximum safety for subjects and quality of data derived.

1.8.4. Protect subject safety by strict compliance

Any clinical trial with a novel major intervention, such as cell therapy, and especially with subjects with organ dysfunction caused by their disease, can have unexpected and potentially fatal events, either related to the cell product or not. The death of a volunteer subject in a gene therapy trial in 1999 shocked the field and the effects extended throughout much of academic clinical research (Gelsinger and Shamoo, 2008; Wilson, 2009). The response was to strengthen the quality of clinical trial performance to provide maximum protection to subjects and preserve the integrity of the data they contribute (Wilson, 2009). The field of HSCT began clinical investigations in the 1960's-70's, prior to the establishment of IRB and the other oversight bodies, as best available clinical practice for severe, generally fatal disorders. Since that time, the standards for clinical investigations have been greatly expanded for a much more complex regulatory environment. A typical clinical trial of gene therapy may undergo review by a dozen or more entities, including IRB, IBC, ISPRC, DSMB, NIH RAC, FDA, and one or more funding agencies. HSCT came to full maturity in the U.S. by the development, initiated by members of the ASBMT academic society, of the Foundation for the Accreditation of Cellular Therapy (FACT), which brought uniformity and high standards for cell processing, clinical operations, data management, regulatory management and other clinical trial activities. Commercial cell processing methods and standards have also been developed supporting several cell products that advanced to relatively late stage of investigation (e.g. neural progenitor cells derived from fetal tissue or pluripotent stem cells) and producing a licensed dendritic cell vaccine (the marketed prostate cancer dendritic cell Sipuleucel-T from Dendreon Corp.). This industry has a strong base to produce high quality cell products, but each new cell product type developed brings unique challenges, including details of the cell processing protocol, the release testing, storage, transport, and therapy administration.

1.8.5. Translational research is drug development

It is important to keep in mind that the goal of translational research is to develop a drug that is effective and safe to achieve licensure, be it a cell and/or gene therapy product such as lentiviral-transduced HSCs, iPSC derived myoblasts, dopaminergic neurons, shRNA, etc. This necessitates basic scientists learning fundamental principles of drug development, such as Good Laboratory Practices, Good Manufacturing Practice and Good Clinical practice. An important tool commonly used in drug development is the Target Product Profile (TPP), which sets goals for attributes like: clinical indication, patient population, administration route and schedule, clinical efficacy targets, potential risks, drug quality and testing methods. Early drafting of a TPP can guide subsequent studies to keep focused on the drug development goals.

1.8.6. Trials should be designed to be informative about the cell product

While the primary end-points for early phase trials mostly relate to safety, it should be possible to incorporate secondary end-points for efficacy and exploratory end-points for biomarkers that can be assessed for potential suitability as eventual primary end-points for drug approval. In the clinical trials we have done testing new vectors, there has been a primary end-point assessing safety, which is typical for a Phase I study, but also secondary end-points assessing efficacy. For ADA SCID, this has involved safety assessments by documentation of clinical adverse events, as well as ensuring absence of replication-competent viral vector emergence and absence of vector-driven clonal expansion. Efficacy assessments involved measuring expression of ADA enzyme activity in mature blood cells, quantifying engraftment of gene-modified stem cells by measuring vector copy number in cells by quantitative PCR, and performing standard clinical tests of immune function, as well as recording clinical health.

In some instances, especially with orphan diseases, non-traditional pathways of clinical trials may be accepted by regulators, with even small trials used as pivotal for registration, assuming they were done with appropriate design and rigor. The EMA approval of Strimvelis for

ADA SCID was based on a single center's Phase I/II clinical data involving 12 patients which served as a pivotal clinical trial.

1.8.7. Get the most from pre-clinical studies

The other major serious complication in the gene therapy field was the development of leukemia in subjects in several primary immune deficiency trials from insertional oncogenesis by retroviral vectors introduced into HSCs (Braun et al., 2014; Hacein-Bey-Abina et al., 2003; Stein et al., 2010). The relatively high frequency of the development of leukemia in some trials (25-75%) was not predicted by pre-clinical studies. However, pre-clinical models may not detect clinical risks that can occur in patients with much larger absolute cell dosages and longer post-treatment time periods. It may be difficult to test cell therapies by the parameters traditionally applied to drug therapies (pharmacokinetics, biodistribution, toxicity), but these aspects can be often assessed using PCR methods to quantify transgenes or cellular markers, such as human genomes in human cells against the background of murine host genomes.

Nonetheless, pre-clinical studies that are performed should be optimized to provide as much relevant information as possible. Principles of Good Laboratory Practices should be applied whenever possible, even at early stages of discovery and prior to formal IND-enabling studies. These include such key elements of GLP as following a detailed pre-defined plan for the studies, statistical plan, data capture forms, with formal data reporting. Again, even during early phases of product development, it is important to include toxicology analysis within efficacy studies to obtain initial information that can be used in the design of definitive studies for IND application. Preliminary proof-of-principles studies can also be used to begin to investigate cell dosages, potential toxicities, as well as disease-modifying activity.

1.8.8. Advancing clinical cell therapies is challenging

Clinical cell therapy requires point-to-point control of the manufacturing process and starting materials (e.g. from skin biopsy to delivery of iPSC-derived somatic cell product, whether

it is HSCs or other cell product). The GMP process requires highly trained staff and SOPs, materials specification, batch records, personnel training, in addition to the highly-controlled environment and regulated processes. To characterize the cell product for human administration, it is necessary to define release criteria – identity, purity, potency, and safety. A Certificate of Analysis is completed for each batch of cell product and the testing for each critical attribute is required with full documentation. The analytic testing for aspects of cell quality (e.g. cell counts and viability, immunohistochemistry or flow cytometry, PCR, RNA-SEQ, etc.) should be performed using well-characterized assays, which should be made more robust with advancing stages of investigation.

1.9. CONCLUSION

In conclusion, gene therapy using HSCs has progressed over three decades from ineffectiveness to being able to essentially cure several different disorders. The pathway was not linear, but required multiple iterative bench-to-bedside cycles. It is likely that therapies using other stem cells will also have progress and set-backs. But, because the underlying hypotheses for cellular therapies are so convincing, it is highly likely that multiple novel stem cell-based therapies will be developed. The lessons from the field of HSC gene therapy may provide some guidance for investigators pursuing the translational process.

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Chapter 2. Cell Cycle and Cytotoxicity May Limit Homology-Directed Repair in Human Hematopoietic Stem Cells

2.1. SUMMARY

Low efficiency of homology-directed repair (HDR) in hematopoietic stem cells (HSCs) currently limits the use of genome editing for treatment of severe genetic blood disorders. This study characterizes mechanisms that govern site-specific gene modification in human HSCs and progenitor cell populations. We used zinc finger nucleases (ZFNs) designed to target the sickle mutation in the human β -globin gene and co-delivered homologous oligodeoxynucleotide donors to identify the stages of the ex vivo gene editing process in which HSCs are less effectively modified compared to progenitor cells. HSC were more quiescent than progenitors and a smaller percentage of HSC responded to pre-stimulation with cytokines by entering S-G2/M phases when HDR may occur. The HSCs showed a higher sensitivity to cytotoxicity from the electroporation of the gene editing reagents compared to progenitors. Transient expression of the anti-apoptotic protein BCL2, co-delivered as mRNA with the gene editing reagents, attenuated this toxicity and preserved a higher number of gene-corrected HSCs. These findings may be beneficial for clinical applications.

2.2. INTRODUCTION

Most methods for genome editing currently being developed for therapeutic applications rely on induction of a DNA double stranded break (DSB) near the mutation site targeted for editing to augment the efficiency (Porteus and Baltimore, 2003). These DSBs initiate the action of cellular DNA repair pathways that serve to protect the genomic integrity of the cells after DNA damage (Lombard et al., 2005; Milyavsky et al., 2010; Niedernhofer, 2008; Rossi et al., 2008). Specific genomic edits can be made by hitchhiking on the DNA DSB repair processes to achieve gene disruption, correction or targeted addition.

DNA DSBs induce cellular DNA damage repair pathways that may proceed through non-homologous-end-joining (NHEJ) or through homology-directed-repair (HDR) using a donor template. The pathway through which the cell responds to the DSBs is not entirely understood, but largely depends on the cell cycle status and structure of DSB ends (Beerman et al., 2014; Milyavsky et al., 2010; Mohrin et al., 2010; Pietras et al., 2011; Symington and Gautier, 2011). NHEJ-mediated repair is similarly frequent in quiescent and cycling cells, whereas HDR-mediated repair appears to predominantly occur in cycling progenitor cells. Furthermore, if the DNA damage is widespread, the cells may undergo apoptotic cell death instead of DNA repair (Milyavsky et al., 2010; Nijnik et al., 2007; Vousden and Lane, 2007).

Hematopoietic stem cells (HSCs) are a clinically-relevant cell target for therapies with gene-edited autologous cells, for a large number of genetic disorders such as hemoglobinopathies, primary immune deficiencies, and storage disorders. However, to date, HDR-mediated gene editing has been constrained by relatively low efficiency in primitive long-term reconstituting HSCs (Genovese et al., 2014; Hoban et al., 2015; Wang et al., 2015). For some diseases, NHEJ-mediated error-prone DNA repair is desirable to disrupt a target gene in the HSCs by introduction of insertions and deletions (indels), e.g., therapy directed against the *CCR5* gene for the treatment of human immunodeficiency virus (HIV) (Holt et al., 2010; Tebas et al., 2014). However, for gene correction using HDR-mediated gene repair, high frequencies of undesired indels may be produced at the editing site from NHEJ, unintentionally knocking-out the target gene. DNA repair is fundamental for HSC maintenance and viability, and therefore these cells have a distinct way of responding to DNA damage (Bakker and Passegue, 2013; Beerman et al., 2014; Milyavsky et al., 2010; Mohrin et al., 2010). Milyavsky and colleagues showed that primitive HSCs are more inclined to undergo apoptosis after DSBs caused by radiation, compared to progenitor cells (Milyavsky et al., 2010). Understanding and managing the cellular DNA repair responses will be critical to achieving effective gene editing in HSCs for clinical applications.

In the present study, we used zinc finger nucleases (ZFNs) to target the point mutation in the human β -globin gene that is responsible for causing sickle cell disease (SCD). Efficient site-specific gene correction of the sickle mutation in HSCs could constitute a precise strategy to generate a life-long source of gene-corrected erythrocytes following autologous transplantation. Correction of the SCD mutation has been demonstrated in human bone marrow CD34⁺ cells; however, the assessed HDR rates after *in vivo* transplantation in most settings have been too low to be clinically therapeutic (Dever et al., 2016; DeWitt et al., 2016; Genovese et al., 2014; Hoban et al., 2015; Wang et al., 2015). The observed drop in HDR rates when analyzed *in vivo* suggests that gene correction predominantly occurs in the progenitor cell population assayed *in vitro*, and not in long-term HSCs, or that the gene-corrected HSCs are more prone susceptible to cytotoxicity and thus are lost preferentially. To achieve sustainable long-term gene correction in primitive HSCs, the rate of HDR needs to be elevated.

The objective of the present study was to identify and characterize the mechanisms which underlie the low frequency of HDR in primitive human HSCs. We treated human mobilized peripheral blood (PB) CD34⁺ cells with ZFN mRNA targeting the sickle mutation in the human β -globin gene and co-delivered homologous donors to assess the following factors in the gene editing process: (1) the efficiency of delivery by electroporation and the time-course of expression of mRNA, (2) ZFN-mediated endonuclease activity, (3) cell cycle status, and (4) cytotoxic responses in immunophenotypically defined human HSCs, multipotent progenitors (MPPs) and progenitor cell populations (Majeti et al., 2007). Our results indicate that one important reason for low gene correction in HSCs compared to progenitors is their heightened susceptibility to cell toxicity resulting from the electroporation of the nuclease and homologous donor components. We demonstrate that co-electroporation of mRNA encoding the anti-apoptotic protein BCL2 significantly ameliorates the cytotoxicity and increases the yield of gene-corrected HSCs.

2.3. RESULTS

2.3.1. Assessment of relative efficiency of nuclease delivery and activity upon electroporation of mRNA in hematopoietic stem and progenitor cells

To study whether HSCs differ regarding efficiency for delivery (electroporation) and translation of *in vitro* transcribed mRNA compared to progenitor populations, we began by analyzing these processes in the following human cell populations: HSCs (CD34⁺/CD38⁻/CD90⁺/CD45RA⁻); multi-potent progenitors (MPPs) (CD34⁺/CD38⁻/CD90⁻/CD45RA⁻); and mature stem/progenitor populations (CD34⁺/CD38⁺) (Majeti et al., 2007). Bulk CD34⁺ cells from human G-CSF-mobilized peripheral blood cells (PB CD34⁺) were cultured for two days for pre-stimulation and electroporated with *in vitro* transcribed (IVT) mRNA encoding green fluorescent protein (GFP) (0.5-7.5 µg). One to three days after electroporation, the CD34⁺ cells were analyzed by flow cytometry as HSCs, MPPs and mature stem/progenitor populations. GFP expression (%) and intensity (mean fluorescent intensity, MFI) were determined in the respective populations over the subsequent three days (**Figure 2.3-1A**). No differences were observed among the respective cell populations in regards to percentage of GFP-positive cells or the MFI (n=5) (**Figure 2.3-1B, C**). These results indicated that delivery and translation of electroporated mRNA was equivalent in HSCs, MPPs and progenitors.

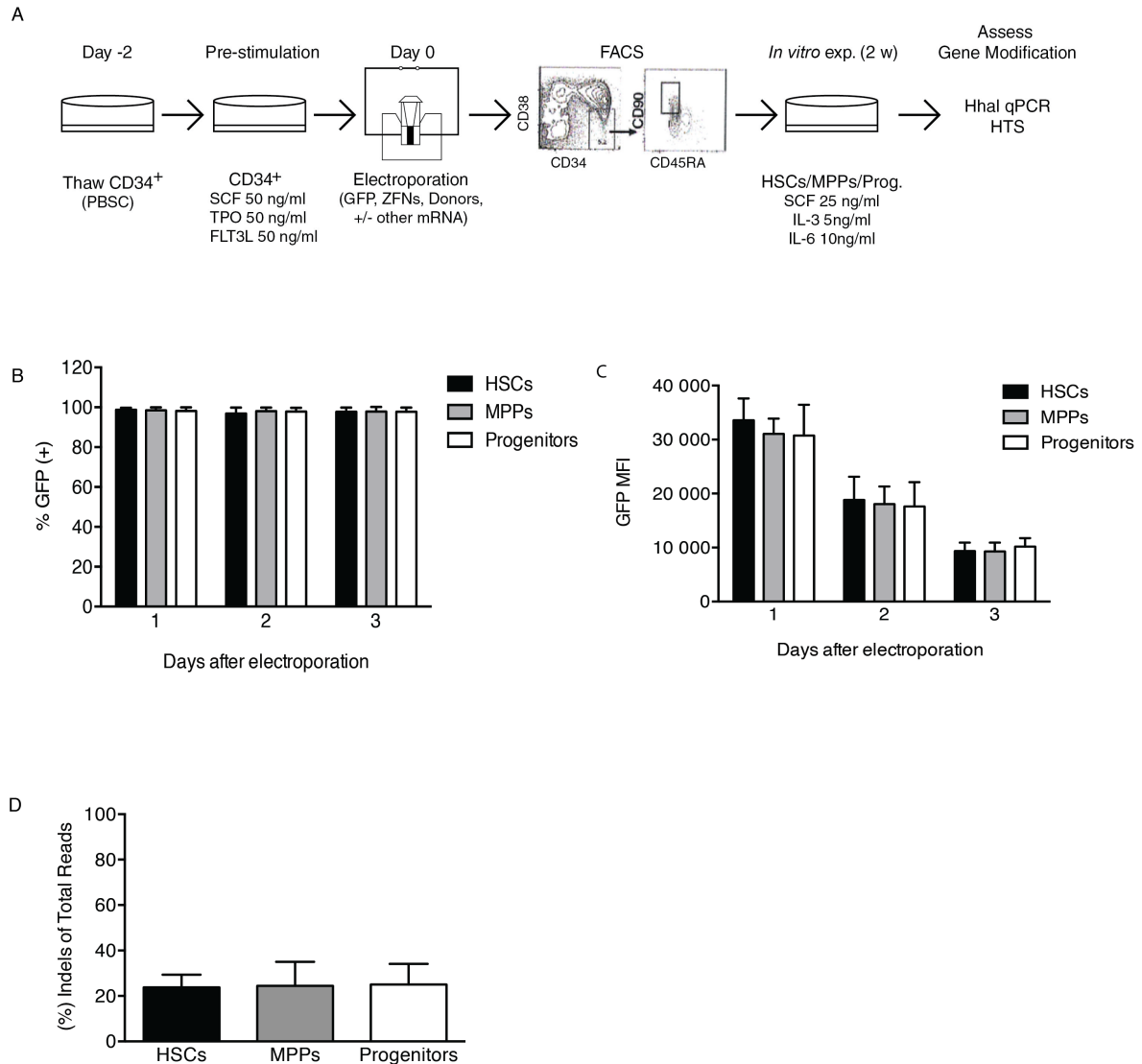


Figure 2.3-1: Expression from electroporated mRNA and nuclease activity is equivalent in HSCs and progenitors.

(A) An overview of the experimental design. (B, C) Bulk peripheral blood (PB) CD34⁺ cells were electroporated with 0.5-7.5 μ g of GFP mRNA and analyzed by flow cytometry to compare the GFP expression (%) (B) and MFI (C) in the HSCs, MPPs and progenitor cells over the subsequent three days post electroporation, (n=5). (D) Bulk PB CD34⁺ cells electroporated with 5 μ g of ZFN mRNA and analyzed by sequencing for nuclease activity by quantification of insertions and deletions (Indels) at ZFN target sequence in the HBB gene (n=5-6). Data are represented as mean $\pm$ SD.

To determine whether the HSCs were more resistant to nuclease activity from the ZFNs, compared to the progenitor cells, we delivered IVT mRNA encoding a pair of ZFNs (5 μ g/arm) targeting the sickle mutation in exon 1 of the human β -globin gene to mobilized PB CD34⁺ cells (Hoban et al., 2015). The following day, HSCs, MPPs and progenitors were fractionated by fluorescence-activated cell sorting (FACS) and each sub-population was cultured for two weeks

in myeloid expansion medium to increase the amount of genomic DNA available for high-throughput sequencing (HTS) of the human β -globin locus. The percentages of β -globin alleles containing insertions/deletions (indels) were equivalent among the three populations (n=5) (**Figure 2.3-1D**), demonstrating that equivalent endonuclease activity was achieved by electroporation of ZFN mRNA in the HSC, MPP and progenitor cell populations.

2.3.2. Effects of IDLV or DNA donor templates on gene modification

To evaluate the efficiency of site-specific HDR in HSCs, MPPs and progenitor cells, PB CD34⁺ cells were pre-stimulated for two days and electroporated with ZFN mRNA and a homologous donor template containing the corrective base to modify the single base-pair involved in SCD. In these experimental studies, the β^A -globin allele of the PB CD34⁺ cells from healthy donors was converted to a β^S -globin allele (**Figure 2.3-2A**). The homologous donor template containing the single base transversion (A/T) at the site of the sickle mutation was either a 1.1 kb fragment cloned from the 5' end of the human β -globin gene and delivered in an integrase-defective lentiviral vector (IDLV: 2×10^7 TU/ml); or a 101 bp single-stranded oligodeoxynucleotide complementary to the region of the human β -globin gene that contains the sickle cell mutation. Two versions of the oligodeoxynucleotide donor were tested in healthy donor cells: the first, "oligo", contained a sickle base pair change and a silent *HhaI* restriction site 20 bp 3' of the sickle mutation; the second, "SMS Oligo", contained two silent mutation sites (SMS) that conservatively alter base-pairs within the ZFN recognition site in the β -globin gene to prevent the ZFNs from binding and re-cleaving initially corrected alleles, in addition to the sickle and *HhaI* changes. One day after electroporation, bulk CD34⁺ cells were sorted into HSCs, MPPs and progenitor cells, which were subsequently analyzed for HDR and NHEJ events in each population by HTS of the β -globin locus.

In concordance with previous findings (Genovese et al., 2014), we observed lower levels of HDR (and NHEJ)-mediated gene modification in HSCs and MPPs compared to progenitor cells (**Figure 2.3-2B**). The ratios of HDR to NHEJ showed no significant difference among the three subpopulations (n=6) (**Figure 2.3-2C**).

Comparison of the IDLV, oligo and SMS oligo homologous donor templates with the ZFNs showed that the highest absolute levels of HDR gene modification in each cell population were achieved when using the SMS oligo donor (**Figure 2.3-2D**). Levels of NHEJ were not significantly different with any of the donor types when using the ZFNs (**Figure 2.3-2E**). The use of the SMS Oligo also led to a significant increase in the frequency of “clean” sequence reads with the only the intended HDR modification of the sickle cell base pair, with a concordant reduction in the proportion of sequence reads with “mixed” changes containing both the single base pair modification as well as an indel (**Figure 2.3-2F**). These observations indicate that the presence of the SMS, which should reduce occurrences of re-cutting and NHEJ-mediated repair in alleles where HDR-mediated gene modification has already occurred, increases the proportion of alleles containing a clean HDR modification.

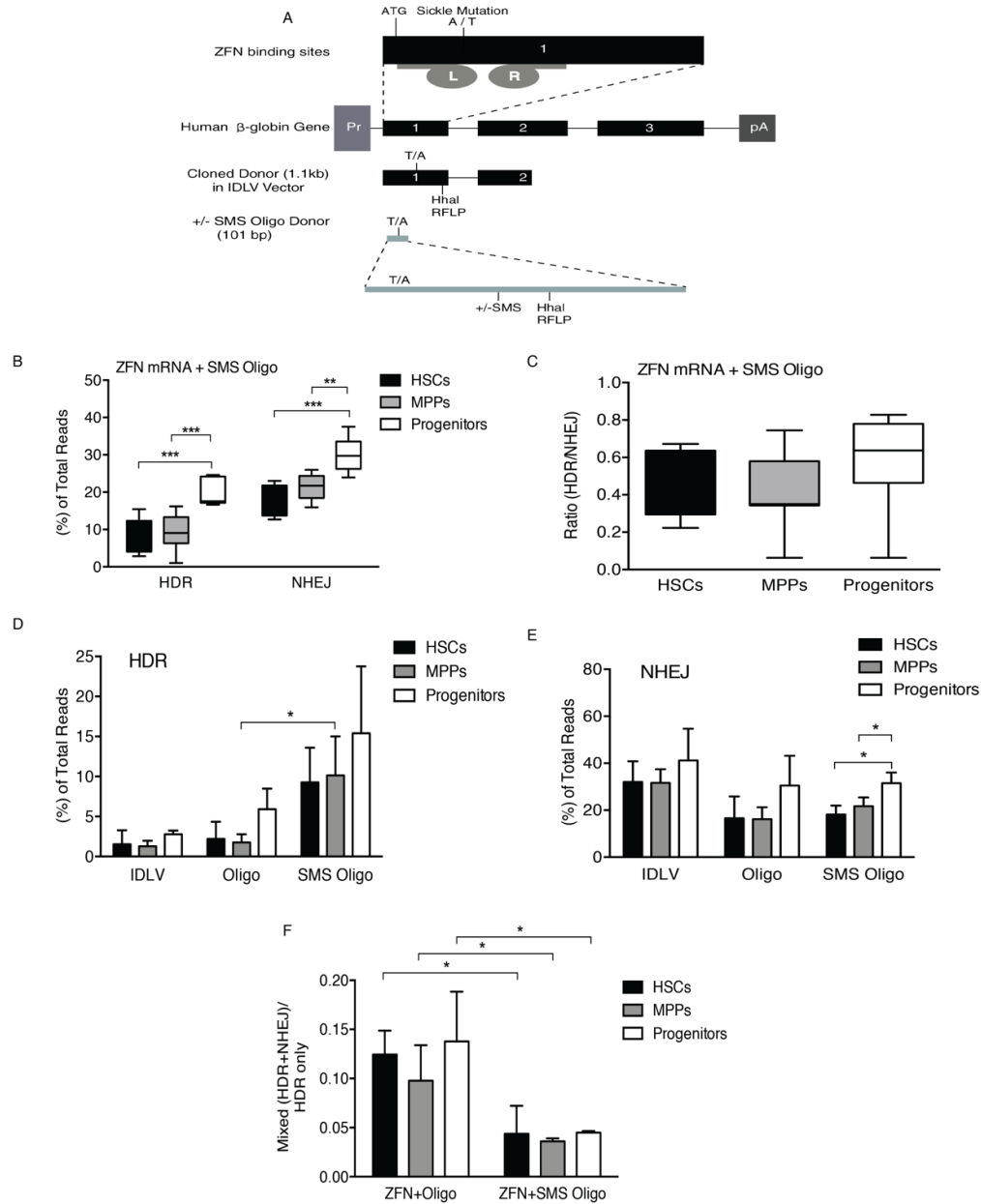


Figure 2.3-2: The gene modification rates are influenced by cell population and HDR donor template.

(A) Overview of ZFNs targeting the sickle mutation in the first exon of the human β -globin locus and the cloned 1.1 kb beta-globin gene fragment used in the integration defective lentiviral vector (IDLV) and the 101 bp oligodeoxynucleotide homologous donors. (B) PB CD34⁺ cells were pre-stimulated for two days and electroporated with 5 μ g ZFN mRNA and 3 μ M of oligodeoxynucleotide homologous donor template containing silent mutation sites (SMS) to the ZFN binding site (SMS Oligo). HDR (addition of the sickle base) and NHEJ (insertions and deletions – indels) were determined by HTS of the β -globin locus (n=6). (See also Figure 2.8-1B, using CRISPRs). (C) Ratio of HDR and NHEJ, (n=6). The ratio was calculated by dividing the percentage of sequence reads with HDR by the percentage reads with NHEJ. (D, E) Bulk PB CD34⁺ cells were pre-stimulated for two days and electroporated with 5 μ g ZFN mRNA and an IDLV donor (I) (n=3) or the SMS Oligo (n=3) or an oligonucleotide donor template without SMS (Oligo) (n=3). 20 hours after electroporation, the cells were sorted into HSCs, MPPs and progenitor populations and (D) HDR and (E) NHEJ were measured by HTS of the β -globin locus. (F) Percentages of alleles with both HDR and NHEJ (mixed) edits were significantly reduced when using the SMS oligonucleotide compared to the oligonucleotide lacking the SMS substitutions (n=3). Data are represented as mean $\pm$ SD. Statistical significance was calculated using the Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.001).

It is important to note that the differences in HDR rates among the stem and progenitor cell populations are not unique to ZFN+Oligo; similar results were observed using CRISPR/Cas9 as the editing agent with an ultramer oligonucleotide donor (**Figure 2.3-3A,B**). While there was higher HDR in progenitors than HSC or MPP with CRISPR/Cas9, rates of NHEJ were not significantly different among the populations. Thus, the ratio of HDR to NHEJ was significantly higher in progenitors among the three subpopulations when using CRISPR/Cas9+Oligo (**Figure 2.3-3C**).

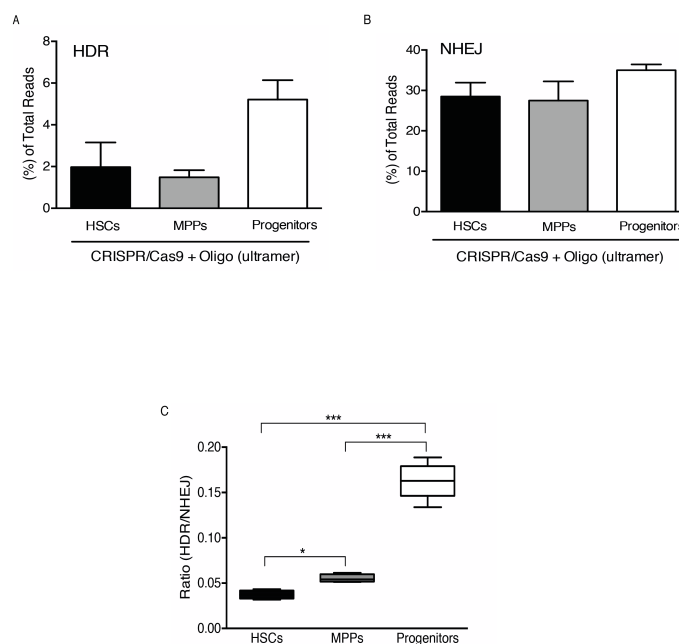


Figure 2.3-3: Quantification of gene modification outcomes using CRISPR/Cas9 in HSCs, MPPs and progenitors.

PB CD34+ cells were electroporated after two days of pre-stimulation, with CRISPR/Cas9 and an oligo donor template. The CRISPR/Cas9 was delivered as a ribonucleoprotein complex (RNP); purified Cas9 protein (200pmol) combined with gRNA (9 µg), together with an oligo ultramer 111/57 (3 µM). (A) Percentage sequence reads measuring HDR, n=2. (B) Percentage sequence reads measuring NHEJ, n=2. (C) Ratio of HDR and NHEJ, n=2.

2.3.3. Pre-stimulation of HSCs for two days increased the percentage of non-quiescent HSCs and increased frequency of HDR

Site-specific gene modification outcomes differ depending on cell cycle status, with a higher frequency of HDR in cycling progenitors, and NHEJ being favored in quiescent, primitive HSCs (Beerman et al., 2014; Mohrin et al., 2010). Because HSCs are mostly quiescent cells, pre-

stimulation with combinations of early-acting hematopoietic growth factors (e.g. *c-kit* ligand, flt-3 ligand, thrombopoietin) is often used to activate HSCs to augment gene modification. To assess the effects of 1-3 days of cytokine pre-stimulation on cell cycle and gene modification rates, we analyzed the cell cycle profile of the subpopulations by flow cytometry and measured the percentage of HDR and NHEJ (by HTS) of FACS-separated cell populations, electroporated with ZFNs+SMS oligo.

PB CD34⁺ were electroporated after one or two days of pre-stimulation (1^{PS} , 2^{PS} , respectively), and cell cycle analysis was performed immediately after electroporation, or one day post electroporation (0^{EP} , 1^{EP} , respectively), using flow cytometry to measure Ki67 antigen (proliferation) and 7-AAD (DNA content) (**Figure 2.8-1A**). Cell cycle analysis of the subpopulations, which was performed immediately after electroporation after one day of pre-stimulation ($1^{PS} 0^{EP}$), revealed that 98% of cells from all three stem and progenitor sub-populations were in G0-phase (quiescent phase) (n=1) (**Figure 2.8-1A**). After two days of pre-stimulation ($2^{PS} 0^{EP}$), approximately 70% of the HSCs and MPPs were still in G0-phase, in contrast to 20% of the progenitors. The number of cells in S-G2/M phases ranged from 11 to 22% (n=1), with an approximately 2-fold higher percentage of progenitors in S-G2/M, compared to HSCs and MPPs (**Figure 2.8-1A**).

Cell cycle analysis performed 1 day after electroporation showed that most HSCs and MPPs that had been pre-stimulated for one day ($1^{PS} 1^{EP}$) were still in the G0-phase and only a small percentage of HSCs/MPPs were in S-G2/M phases (n=2) (**Figure 2.3-4A, Figure 2.8-1B**). There was essentially no HDR in the ($1^{PS} 1^{EP}$) HSC population after 1 day of pre-stimulation (n=2) (**Figure 2.3-4B**). After two days of pre-stimulation ($2^{PS} 1^{EP}$), the percentage of HSCs in S-G2/M phases reached 20%, and there was an increase in HDR levels (5-10%) in these populations, compared to 1^{PS} (**Figure 2.3-4A,B**). The number of cells in S-G2/M phases and the HDR levels did not further increase after three days of pre-stimulation (**Figure 2.3-4B**).

The clonogenic potential of the different sub-populations cultured for 1-3 days were also assessed by plating cells in methylcellulose colony forming units assay (CFUs) from the respective sorted populations one day after electroporation (1^{PS1EP} , 2^{PS1EP} , and 3^{PS1EP}). This CFU assay showed that progenitor cells had a higher clonogenic potential compared to HSCs and MPPs, following each of the pre-stimulation durations. The clonogenic potential of HSCs increased substantially between pre-stimulation day one and two, in contrast to the MPPs and progenitors that showed similar clonogenic potential regardless of pre-stimulation length (**Figure 2.8-1C**). Overall, 2 or 3 days of pre-stimulation increased the percentages of HSCs and MPPs progressing from G0 to S-G2/M phases of cell cycle and resulted in increased frequencies of HDR-mediated gene modification.

2.3.4. High levels of cytotoxicity observed in HSCs and MPPs after delivery of ZFNs and donor template

To evaluate whether there is a difference among the different sub-populations regarding susceptibility to cytotoxic effects from gene editing, we analyzed the induced toxicity following electroporation with ZFNs and SMS oligo donor by flow cytometry, identifying HSC, MPP and progenitor cells labeled with 7-AAD and Annexin V. Six hours after electroporation, there were no significant differences in the frequency of 7-AAD⁺ cells among the HSCs compared to MPPs and progenitors (**Figure 2.3-4C**). Notably, one day after electroporation, flow cytometric analyses indicated that the HSC and MPP populations were exceptionally sensitive to cytotoxicity from the treatment, compared to the progenitor cells (n=6), with 2-3-fold more Annexin V⁺/7AAD⁺ cells (indicating late apoptosis) among HSCs and MPPs (**Figure 2.3-4D**).

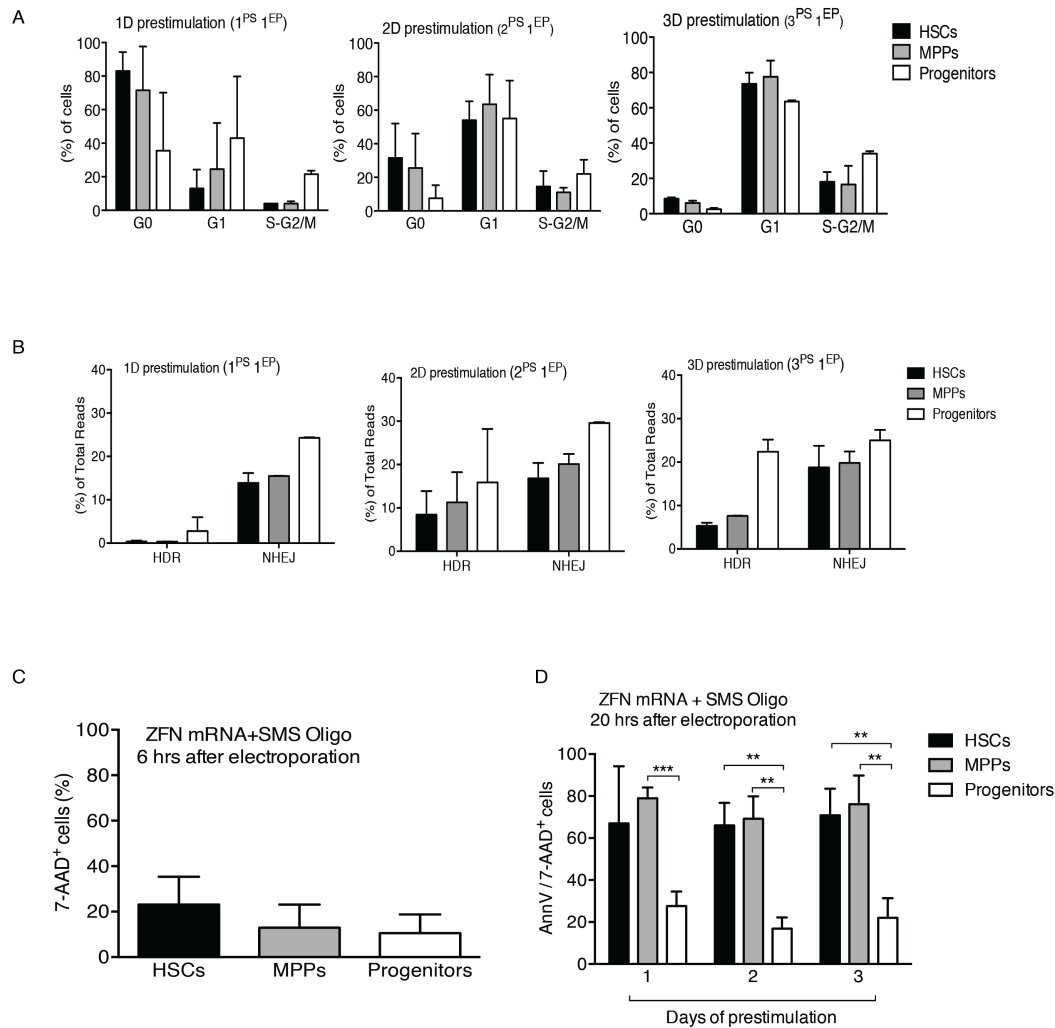


Figure 2.3-4: Assessment of different pre-stimulation times on cell cycle status, gene editing frequencies, and induced cell toxicity in HSCs, MPPs and progenitors.

Bulk CD34⁺ cells were pre-stimulated for two days and electroporated with ZFN mRNA and the SMS oligo donor and sorted the following day into HSCs, MPPs and progenitors. (A) Assessment of cell cycle status of the immunophenotypic sub-populations of cells using Ki67 and 7AAD staining of DNA to define the percentage of S-G2/M phases cells by FACS 1 day after electroporation, following 1-3 days of pre-stimulation, (n=2). (B) Gene modification (HDR and NHEJ) measured by HTS of the target locus after 1-3 days of pre-stimulation. (n=2). Acute cytotoxicity was measured by FACS after 7-AAD and AnnexinV staining in sorted HSCs, MPPs and progenitor cells 6 hours (n=3) (C) and 20 hours (n=6) (D) after electroporation with ZFN mRNA and the SMS oligonucleotide donor. Data are represented as mean $\pm$ SD. Statistical significance was calculated using the Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.001).

2.3.5. Co-delivery of *BCL2* mRNA increased numbers of gene-edited cells *in vitro*

In an attempt to compensate for toxicity induced by the gene-editing reagents, IVT human *BCL2* mRNA was co-delivered at the time of electroporation for transient expression. 2x10E5 CD34⁺ cells were electroporated with either the SMS Oligo, the ZFNs or a combination

of ZFNs and SMS Oligo, with or without *BCL2* mRNA. One day after electroporation, the amounts of cell death and apoptosis were quantified by flow cytometry in the HSPCs (CD34⁺/CD38^{low}) and progenitors (CD34⁺/CD38⁺) by DAPI and Annexin V staining. Delivery of SMS Oligo alone by electroporation resulted in 14% apoptotic cells in the HSPCs and 12% in the progenitors. Co-delivery of *in vitro* transcribed *BCL2* mRNA with the SMS oligo did not reduce the frequency of apoptotic cells (HSPCs; 18% and progenitors; 10%; n=4) (**Figure 2.3-5A**). There was no significant difference in cytotoxicity between the sub-populations when only ZFNs were delivered by electroporation. However, when the ZFNs were co-delivered with the SMS Oligo, the cytotoxicity increased to 51% in the HSPCs, while the progenitor cells remained at a frequency of 22% (n=2-3). When *BCL2* mRNA was included in the electroporation, the frequency of apoptotic cells was significantly reduced to 42% in the HSPCs and 11% in the progenitor cells.

Next, the dosage of *BCL2* mRNA yielding maximal protective effect was evaluated in cord blood bulk CD34⁺ cells. We tested a range from 1 µg to 10 µg, and as little as 1 µg of *BCL2* mRNA co-electroporated with the ZFN mRNA and the SMS Oligo reduced the frequency of apoptotic cells from 63% to 40%. Increasing the amounts of *BCL2* mRNA to 2-10 µg led to slightly better protection with 30-35% apoptotic cells (n=4) (**Figure 2.8-2A**). *BCL2* expression did not affect the HDR and NHEJ levels obtained in the bulk CD34⁺ cells, independent of the dose used (1-10 µg, n=4) (**Figure 2.8-2B**).

Because analysis of bulk CD34⁺ cells may obscure effects on the key HSC and MPP sub-populations, we analyzed the effects of *BCL2* mRNA transient expression on gene modification frequencies in cells sorted after gene modification. *In vitro* transcribed *BCL2* mRNA was co-electroporated with ZFNs and SMS oligo into CD34⁺ cells after two days pre-stimulation, followed by FACS separation of HSCs, MPPs and progenitor cells, *in vitro* expansion, and analysis of HDR and NHEJ levels through HTS.

On the day following electroporation, we quantified the cell toxicity by flow cytometry and obtained cell counts to determine cell survival. The acute cytotoxicity, observed by identifying

cells labeled with 7-AAD and Annexin V, was significantly reduced after co-delivery of 5 µg *BCL2* mRNA in each cell sub-population (n=4) (**Figure 2.3-5B**). The flow cytometry data were further supported by cell counts obtained by FACS 20 hours after electroporation, showing significantly higher numbers of cells in all three sub-populations from CD34⁺ cells treated with *BCL2* mRNA (n=4) (**Figure 2.3-5C**).

The experiment was repeated to assess the cytotoxicity induced from the respective gene-editing reagents in bulk PB CD34⁺ cells, with or without co-delivery of IVT *BCL2* mRNA. 20 hours after electroporation, the percentage of *viable cells* was determined. There was a small increase in cytotoxicity from the oligo donor alone (O) compared to the mock cells (M) and modest improved viability by inclusion *BCL2* mRNA (O+*BCL2*) (**Figure 2.8-2C**). We observed an increase of toxicity after delivery of the ZFNs alone (Z), resulting in a viability ranging between 58-77%. The viability decreased even further after combined Z+O delivery. However, when IVT *BCL2* mRNA was included in the electroporation, the viability was preserved significantly, although not completely (n=6-12) (**Figure 2.8-2C**). The experiment was repeated measuring early apoptotic cells by flow cytometry (Annexin V positive cells). Co-delivery of *BCL2* mRNA reduced the early apoptosis caused by Z+O delivery (n=4-8) (**Figure 2.8-2D**).

HSCs, MPPs and progenitors treated with ZFN + the SMS oligo, with or without *BCL2* mRNA and expanded *in vitro* were thereafter sequenced for HDR and NHEJ gene modification levels by HTS. This analysis revealed that the transient overexpression of *BCL2* resulted in significantly higher HDR gene modification in the HSC and MPP populations, with smaller effects in the progenitors (n=4). The increased NHEJ frequencies when *BCL2* mRNA was added were not significant for any of the sub-populations (**Figure 2.3-5D, E**).

Next, we investigated the differential effects of *BCL2* mRNA on toxicity using CRISPR/Cas9 for editing instead of ZFNs. Assessments were conducted of cytotoxicity induced in bulk PB CD34 after delivery of CRISPRs by electroporation, as ribonucleoprotein complex (RNP) of recombinant Cas9 (rCas9) protein and IVT gRNA, the rCas9 protein alone without gRNA

(Cas9) and oligo donors (either the SMS oligo {O} or an ultramer {U}) (**Figure 2.8-2E**). Electroporation with RNP or Cas9 protein alone caused only limited cytotoxicity; the primary cytotoxicity was caused by the oligo donors (n=4-10). Inclusion of *BCL2* mRNA partially alleviated the cytotoxicity from the oligo donors used for HDR donors with the CRISPR/Cas9 editing.

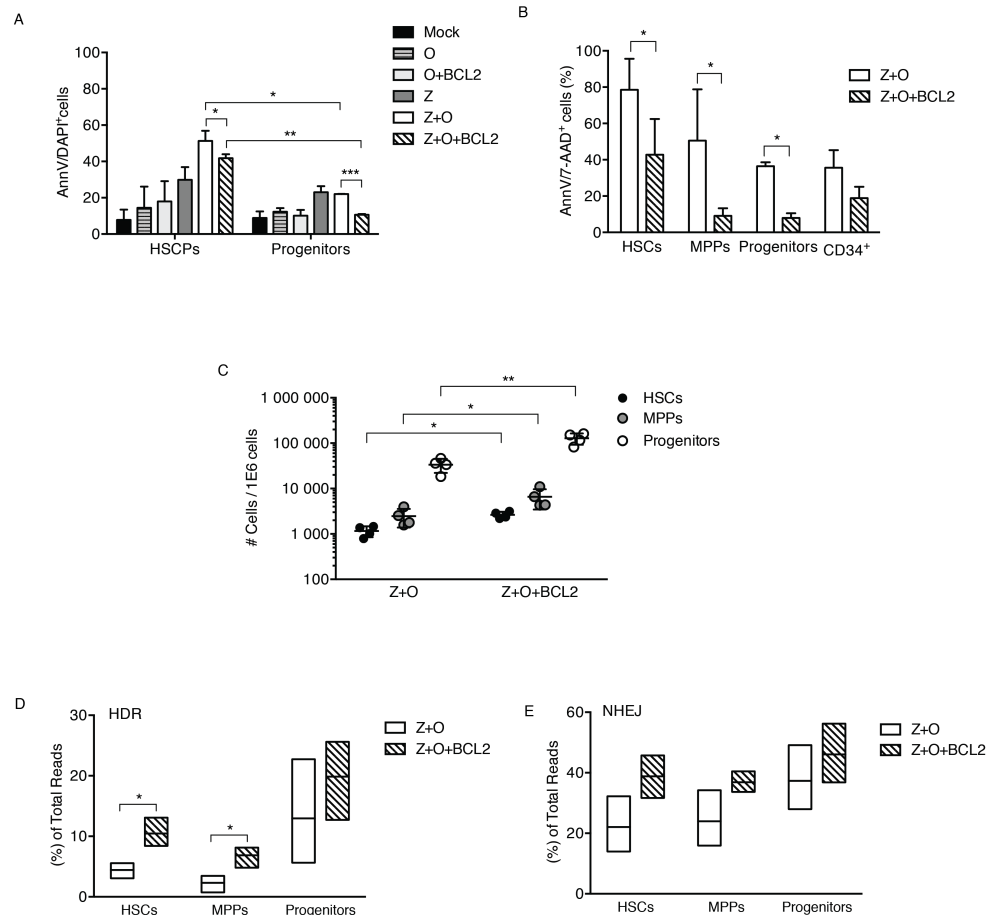


Figure 2.3-5: Transient overexpression of BCL2 mRNA decreased cell toxicity measured by flow cytometry and increased the cell numbers in vitro.

The HSPCs (CD34⁺/CD38^{low}) and progenitors were analyzed for cytotoxicity induced by SMS Oligo (O), ZFNs (Z) or a combination of ZFNs and SMS Oligo (Z+O), with or without *in vitro* transcribed *BCL2* mRNA. (A) Flow cytometry, identifying cells labeled with DAPI and AnnexinV, one day following electroporation of 2x10⁵ CD34⁺ cells, (n=2-4). The induced cytotoxicity was analyzed for HSCP and progenitor cells 20 hrs. following electroporation of 5 µg ZFN mRNA and 3 µM of SMS oligonucleotide donor, with or without 5 µg of *BCL2* mRNA. (B) Flow cytometry, identifying cells labeled with 7-AAD and AnnexinV, (n=4). (C) Cell counts measured by FACS 20 hours after electroporation (n=4). Starting cell numbers were 4x10⁶ CD34⁺ cells per treatment arm. (D, E) Determination of gene modification levels (HDR and NHEJ) in HSCs, MPPs and progenitor cells *in vitro* (n=4) with or without transient overexpression of 5 µg *BCL2* mRNA.

2.3.6. Assessment of BCL2 treated gene-edited cells *in vivo*.

To assess whether the higher HDR observed in immunophenotypically-defined HSCs *in vitro* also held true for HSCs defined functionally *in vivo*, we transplanted gene-edited bulk CD34⁺ cells, treated with or without *BCL2* mRNA, into immune-deficient recipient female NSG mice. CD34⁺ cells were pre-stimulated for two days and then electroporated with ZFN mRNA and SMS oligo donor (Z+O) or ZFN mRNA and SMS oligo donor + 5 ug *BCL2* (Z+O+*BCL2*). One day post-electroporation, the different cell doses were transplanted into female NSG mice (**Figure 2.3-6A**). We utilized a standard transplantation dose (1x10E6 viable CD34⁺/mouse) for each treatment group (mock electroporated {M}, Z+O and Z+O+*BCL2*). Also, to assess potential benefits from the improved survival of cells electroporated with *BCL2* mRNA, an additional transplant arm used the number of cells that represented the portion of starting numbers of cells that yielded 1x10E6 cells in the transplant arm without *BCL2* mRNA (approximately a 2-fold increase in number of cells across the three different transplant studies (~1.5-2.3x10⁶)).

Small aliquots of cells from each treatment arm were retained *in vitro* for determination of pre-transplant viability and gene modification levels. The day following electroporation, which was day 3 of cell culture, the cells were counted and toxicity was analyzed by flow cytometry. The groups treated with ZFNs and SMS oligo had an average of 61% viable cells (40-75% net survival and 25-60% apoptotic {7-AAD/AnnV positive} cells), compared to the groups that received ZFNs, SMS oligo and *BCL2* mRNA, that had 87.5% viable cells (80-95% net survival) and 5-20% apoptotic {7-AAD/AnnV positive} cells (**Figure 2.3-6B**). Thus, in the group that received *BCL2* mRNA with the ZFN and oligo, there was approximately 2-fold higher viability and lower percentages of the displayed markers of apoptosis.

Other aliquots of the retained gene-modified CD34⁺ cells were kept in culture for three days prior to extraction of genomic DNA and HTS sequence determination for HDR-mediated gene modification. There was a 2-fold increase of the percentage of beta-globin gene sequence

reads that showed HDR in cells from the Z+O+*BCL2* group compared to the Z+O group (**Figure 2.3-6C**).

Two months post-transplantation, the mice were analyzed by flow cytometry for human cell engraftment ($\%hCD45/(\%hCD45+\%mCD45)*100$) in PB. The engraftment levels between the mice transplanted with Z+O and Z+O+*BCL2* transplanted with the same numbers of viable cells were comparable (**Figure 2.3-6D**). As anticipated, there was a significant increase in engraftment when the relative increased numbers of surviving cells in the *BCL2* mRNA-treated group were transplanted.

At three months post-transplantation, the mice were euthanized, and engraftment and lineage analyses of human cells were done with samples from BM and spleen. The engraftment levels in the Z+O+*BCL2* group were comparable to the Z+O group when transplanted with the same numbers of viable cells, both in BM and spleens, three months after transplantation (**Figure 2.3-6E**, **Figure 2.8-3A**). Again, there was a significantly higher engraftment of human cells in the Z+O+*BCL2* group that received the relative increased numbers of surviving cells in the *BCL2* mRNA-treated group ($\sim 1.5\text{--}2.3 \times 10^6$) compared to those receiving $0.8\text{--}1 \times 10^6$ cells in the bone marrow (**Figure 2.3-6E**) and spleens (**Figure 2.8-3A**). The lineage differentiation after transplantation from the two groups did not differ in BM with a predominance of CD19⁺ B cells seen in all samples, fewer CD33⁺ myeloid cells, and very few CD3⁺ T cells (**Figure 2.8-3B**).

Three months post-transplantation, the cells from mice that had received *BCL2* mRNA-treated gene-edited cells did not show significantly higher levels of HDR-mediated gene modification in the BM (from 2.2% without *BCL2* mRNA to 2.3–3.3% with *BCL2* mRNA) (**Figure 2.3-6F**, **Figure 2.8-4**, **Figure 2.8-5**). The HDR levels in the cells in the spleen were significantly higher when *BCL2* mRNA was included in the experiment (**Figure 2.8-3C**). The NHEJ levels were similar for all groups (**Figure 2.3-6G**, **Figure 2.8-3D**). Overall, inclusion of *BCL2* mRNA led to increased absolute numbers of engrafting HDR-edited cells, with only modest increases in the percentages of edited cells *in vivo*.

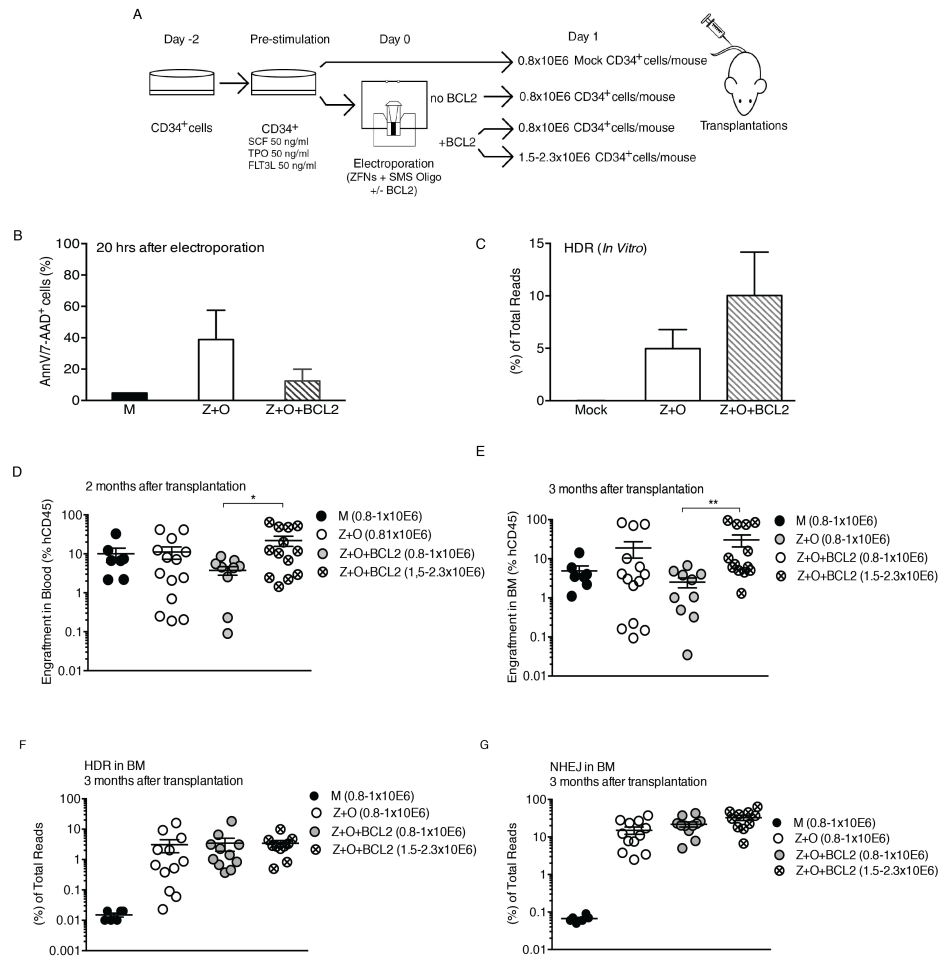


Figure 2.3-6: Transplantation of gene-modified CD34⁺ cells (+/- BCL2 mRNA) into NSG mice to assess engraftment and gene editing.

(A) An overview of the different experimental procedures and cell doses which were transplanted into NSG mice. (B) Flow cytometry, identifying CD34⁺ cells labeled with 7-AAD and AnnexinV, 20 hours after electroporation (n=3). (C) HDR-mediated gene modification of the edited CD34⁺ cells prior to transplantation was determined by HTS, after 3 days of *in vitro* culture (n=3). (D) Two months after transplantation, the engraftment (%hCD45) in PB was analyzed. (E) Three months after transplantation, the engraftment (%hCD45) levels in BM were analyzed. The levels of gene modification in BM samples from mice transplanted with CD34⁺ cells treated with or without *BCL2* mRNA was determined by HTS, measuring (F) HDR and (G) NHEJ. Data are represented as mean $\pm$ SD. Statistical significance was calculated using the Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.001). See also Figure 2.8-3A for lineage analysis of human BM cells and Figure 2.8-3B-D for human engraftment and gene modification analyses in the spleens of the transplanted NSG mice.

2.4. DISCUSSION

This study provides new insights regarding potential mechanisms responsible for low HDR gene modification efficiency in repopulating human HSCs. Previous work has successfully demonstrated that NHEJ-mediated gene modification in primitive HSCs can be efficiently

performed for diseases such as hemoglobinopathies and HIV, following disruption of the BCL11A gene or CCR5 gene respectively (Holt et al., 2010; Tebas et al., 2014; Xu et al., 2013). Considering that NHEJ is the primary DNA repair pathway of stem cells, these successful results are not surprising (Beerman et al., 2014; Milyavsky et al., 2010; Mohrin et al., 2010; Pietras et al., 2011). In contrast, studies aiming to demonstrate HDR-mediated repair in repopulating HSCs, showed that the *in vivo* HDR levels after transplantation of NSG mice were considerably lower than the initial *in vitro* HDR levels measured prior to transplantation (Genovese et al., 2014; Hoban et al., 2015; Wang et al., 2015). These findings indicate that HDR gene modification in primary HSCs is considerably less effective compared to progenitor cells. Genovese and colleagues quantified GFP⁺ cells by flow cytometry after targeted integration of a GFP-containing homology donor and found that the level of primitive cell targeting was 20-fold lower than in mature progenitors (Genovese et al., 2014). Hoban and colleagues were able to achieve 10-20% gene correction of the sickle mutation in CD34⁺ cells *in vitro* prior to transplantation by using ZFN mRNA nuclease and IDLV to deliver guide and donor template; however, the levels of gene correction in NSG mice 16 weeks post transplantation were at most 3% (Hoban et al., 2015). More recent studies have reported smaller decrements, but still significantly lower frequencies of HDR in NSG-engrafting cells compared to the progenitors analyzed in short-term culture (De Ravin et al., 2017; DeWitt et al., 2016)

The main objective of this study was to identify and characterize the molecular mechanisms that govern site-specific gene modification through HDR in the immunophenotypically defined cell populations: HSCs, MPPs and progenitor cells (Majeti et al., 2007). Our findings showed that ZFN (or GFP) mRNA delivery to and expression in hematopoietic stem and progenitor cell sub-populations were equivalent, with similar frequencies of NHEJ-mediated indels induced by the ZFN. However, we consistently observed significantly lower levels of HDR-mediated gene modification in HSCs and MPPs compared to progenitors. The different efficiencies of gene correction observed in HSC and progenitor cells highlight the importance of

analyzing the specific cell population of interest, here HSC. HDR analysis of bulk CD34⁺ cells (with a majority being progenitors) over-estimates the frequency of editing in rarer HSCs.

While NHEJ is active throughout the cell cycle, HDR is mostly limited to the S-G2 phases, which makes it challenging to achieve HDR in quiescent long-term HSCs (Beerman et al., 2014; Mohrin et al., 2010). To study the potential effect of cell cycle phase on HDR efficiency, CD34⁺ cells were pre-stimulated for 1-3 days prior to electroporation of ZFN mRNA and the oligodeoxynucleotide donor, and thereafter the cell cycle phases at the time of electroporation as well as the frequencies of HDR and NHEJ by HTS were measured. The simultaneous cell cycle and editing analyses of cells with various durations of pre-stimulation demonstrated that the percentage of HSC in S-G2/M reached a maximal value of ~15-20% after 2 days of pre-stimulation, which may set an upper limit on the percentage of cells that may undergo HDR-mediated gene modification. Interestingly, Wang and co-authors found no differences in HDR levels between HSCs and mature progenitors when fetal liver (FL) CD34⁺ cells were treated with ZFN mRNA and an AAV6 donor (Wang et al., 2015). HSCs from FL are known to cycle more frequently than adult HSCs, which could explain the differences to our findings (Babovic and Eaves, 2014; Pietras et al., 2011). Nevertheless, since FL cells have low clinical relevance in a gene therapy setting, our research was based on adult HSCs.

Another factor influencing the gene modification rates in HSC populations was the introduction of silent mutation sites (SMS) in the homologous donor template, to prevent re-cleavage and introduction of an indel in an allele that had undergone successful HDR-mediated modification. By delivering an SMS Oligo, the mixed HDR and NHEJ events were significantly reduced, which further increased the net HDR levels, compared to use of an oligo donor that retained the native ZFN-recognition sequence. Analogously, when using CRISPR/Cas9, a donor with silent base-substitutions in the proto-spacer adjacent motif (PAM) sequence may reduce production of unwanted indels by NHEJ from re-cleavage of an allele that was previously edited by HDR.

It has been suggested that human HSCs are more sensitive to DSBs and more likely to undergo p53-dependent apoptosis, compared to progenitor cells (Milyavsky et al., 2010). Therefore, we sought to determine whether the gene modification procedure used was preferentially detrimental to the survival of primitive HSCs. Our analyses of cytotoxic effects revealed a high sensitivity of HSCs and MPPs to the electroporation of ZFN mRNA and the oligonucleotide donor, resulting in ~80% cell death of immunophenotypically-defined HSCs compared to ~30% observed in progenitors, measured by flow cytometry 20 hours after treatment.

The cytotoxicity and induced apoptosis may be due to a “false alarm”, with the electroporated oligonucleotide and other gene editing components being perceived as massive chromosomal damage, triggering an apoptotic response. The *in vitro* experiment in Figure 2.3-5A suggests an increasing cytotoxicity response after each additional component included from among the gene editing reagents. The sensitivity of the HSCs appear to differ from the progenitor cells, as described by Milyavsky and colleagues who observed higher p53/ASPP1-dependent apoptosis in human HSCs in response to ionizing radiation compared to progenitor cells (Milyavsky et al., 2010).

To determine whether it was possible to block the apoptotic effects of the genome manipulations, we transiently overexpressed the anti-apoptotic protein BCL2 by introduction of *IVT BCL2* mRNA, co-delivered by electroporation with the ZFN mRNA and the SMS oligo. Cell survival analysis in the different subpopulations revealed a 2- to 3-fold decrease in apoptotic cell death with BCL2 addition, determined by cell count and flow cytometry for 7-AAD/AnnexinV negative cells. Milyavsky and colleagues demonstrated that BCL2 overexpression by stable lentiviral vector transduction alleviated cytotoxicity from gamma-irradiation to cord blood CD34⁺ cells without compromising HSC self-renewal (Milyavsky et al., 2010). Here, we show that transient expression of BCL2 by electroporation of mRNA also protected primitive PB-derived HSCs from the gene modification process and improved viability of the cells 2-3-fold. There was a non-significant increase in HDR-mediated gene correction measured in bulk CD34⁺ cells when

BCL2 mRNA was included, which is likely to be due to the more numerous progenitor cells obscuring the effect on the rarer HSCs.

This form of transient *BCL2* delivery (as mRNA) does not add the gene permanently, and should thus minimize transformation risks that prolonged *BCL2* overexpression might engender. Despite the fact that impaired or skewed HSC lineage reconstitution capacity was not observed from the transplanted mice, the risk of rescuing cells that should have undergone apoptosis due to extensive damage to the genetic integrity cannot be excluded. However, we propose that the transient expression of *BCL2* acts to prevent cells from undergoing apoptosis caused by an adverse response to the gene editing procedure and not due to mutations in the genome; this needs to be evaluated. Additional studies also will be needed to ascertain whether the transient *BCL2* expression significantly protects the survival of primitive repopulating HSCs long-term following gene editing.

In summary, these studies demonstrated the requirement to induce HSC to progress to S-G2/M phases of the cell cycle by 1-2-day pre-stimulation with early-acting hematopoietic growth factors for HDR to occur. They also revealed an elevated sensitivity to cytotoxicity from the gene editing process in HSCs compared to mature progenitor cells, which may explain the lower levels of gene modification seen in HSCs *in vivo* compared to using *in vitro* assays. A major constraint on clinical applications with HSCs is the inability to significantly expand gene-modified HSCs, and thus the numbers that can be transplanted is critically dependent on their preservation during *ex vivo* manipulations. Transient expression of *BCL2* mRNA mitigates HSC death after HDR-based gene editing, which may have useful implications for clinical development of HSC targeted gene therapy using genetically edited stem cells.

2.5. AUTHOR CONTRIBUTION

CFB designed and conducted experiments, supervised the work, analyzed data, and co-wrote the manuscript. AL, MM, SL, RPH and MK conducted experiments and AL also contributed

to the manuscript. AC, GRL, MP and SFG performed biocomputational analyses. GC and MCH provided reagents. DBK conceived the project, supervised the work and co-wrote the manuscript.

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The authors C.F. Bjurström and DB Kohn have financial relationship(s) to disclose: UCLA, Patents & Royalties: 2016-290. The authors G.J. Cost and M.C. Holmes are employees and shareholders of Sangamo Therapeutics Inc.

2.7. METHODS

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author Donald B. Kohn (dkohn1@mednet.ucla.edu).

Experimental Model and Subject Details

Mice and transplantations

Healthy donor CD34⁺ cells (previously isolated from G-CSF mobilized peripheral blood or umbilical cord blood and then cryopreserved) were thawed and cultured for two days prior to electroporation (as described above) using ZFN mRNA (5 µg), SMS Oligo (3 µM), +/- *BCL2* mRNA (5 µg). One day following the electroporation, the cells were counted and transplanted i.v. into irradiated (250cGy) 6-9-week-old female NOD/SCID/IL2R gamma^{-/-} (NSG) recipient mice (Jackson Laboratory, Bar Harbor, ME, USA).

Transplanted mice were analyzed for engraftment and lineage distribution in peripheral blood (PB), bone marrow (BM) and spleen after three months. The cells were stained with the following antibodies: V450-conjugated human CD45 (BD Pharmingen), FITC-conjugated murine CD45 (Biolegend), PeCy7-conjugated human CD19 (Biolegend), PerCP Cy5-5-conjugated human CD3 (Biolegend), PE-conjugated human CD33 (Biolegend) and APC-conjugated human CD34 (Biolegend). (Full description of antibody table in Supplementary Methods **Table 2.8-1**).

CD34⁺ cell isolation and *in vitro* culture

Adult human G-CSF-mobilized peripheral blood apheresis units were purchased from the Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center. CD34⁺ cells were isolated using either the MACS CD34⁺ enrichment kit or the CliniMACS system (Miltenyi Biotec, Inc., Bergisch Gladbach, Germany), resulting in a CD34-purity of 96-98%. CD34⁺ cells were also isolated from fresh umbilical cord blood samples obtained as anonymous medical waste from the UCLA Ronald Reagan Medical Center, deemed to not be human subjects research by the UCLA Institutional Review Board.

The CD34⁺ cells were incubated at 37°C in 5% CO₂ for 1-3 days prior to electroporation, in medium containing X-VIVO 15 (Lonza, Basel, Switzerland) supplemented with 1% Penicillin/Streptomycin/L-glutamine (Gemini Bio-products, West Sacramento, CA) and the recombinant human cytokines stem cell factor (SCF) (50ng/ml), thrombopoietin (TPO) (50ng/ml) and Fms-related tyrosine kinase 3 (Flt3)-Ligand (50ng/ml) (Peprotech, Rocky Hill, NJ) (STF). For in vitro expansion after electroporation, the cells were cultured in medium consisting of Iscove's modified Dulbecco's medium (IMDM) (Life Technologies, Grand Island, NY) supplemented with 1% Penicillin/Streptomycin/L-glutamine, 20% FBS (Gemini Bio-products), 0.5% BSA (Sigma-Aldrich, St. Louis, MO); and the cytokines: SCF (25ng/ml), interleukin (IL)-3 (5ng/ml) and IL-6 (10ng/ml) (Peprotech), for an additional two weeks prior to high-throughput sequencing.

To quantify colony forming units (CFU) from progenitor cells, PB CD34⁺ cells, pre-stimulated for 1-3 days and electroporated with ZFN and SMS oligo. 100 and 300 cells were plated in methylcellulose medium and enumerated as previously described (Romero et al, JCI, 2013).

Gene modification reagents, electroporation and gene modification analyses

mRNA encoding green fluorescent protein (GFP) or a pair of zinc finger nucleases (ZFNs) that bind and cleave sequences in the human beta-globin gene flanking the site of the canonical sickle cell mutation (Hoban et al, Blood 2015 – see also *Supplemental Methods*) were produced by *in vitro* transcription using mMESSAGE mMACHINE® T7 ULTRA Transcription Kit from Thermo Fisher Scientific). A 101 base oligodeoxynucleotide was used as HDR donor, containing the sequence from the human beta-globin gene; with the T base transversion of sickle cell as a marker of gene modification; as well as a silent restriction fragment polymorphism encoding a HhaI restriction site (Oligo) (full details described in Suppl. Methods). Another oligodeoxynucleotide used had the same features plus another pair of conservative silent mutation sites (SMS) at the site of the left ZFN binding to minimize re-cutting of corrected loci (Hoban et al., 2015). Oligos were purified by HPLC from Eurofins Genomics (Louisville, KY) or

IDT (San Diego, CA) and reconstituted in sterile TE buffer. Electroporation of CD34⁺ cells was performed using the ECM 830 Square Wave Electroporation System (Harvard Apparatus, Holliston, MA). 1x10⁶ cells/cuvette were centrifuged at 90g for 15 mins and resuspended in 100µl of BTX solution (Harvard Apparatus, Holliston, MA) together with GFP mRNA or ZFN mRNA and indicated donor template. When stated, the *BCL2* mRNA was also mixed with the ZFNs mRNA and oligo donor.

For experiments using CRISPR/Cas9 for editing, Guide RNA (gRNA) targeting the HBB locus (sequence -TTACTGCCCTGTGGGGCAAG) was produced by IVT according to previously-published protocols; In vitro transcription of guide RNAs (protocols.io dx.doi.org/10.17504/protocols.io.hdrb256) (DeWitt et al., 2016), and purified using RNeasy MinElute Cleanup Kit (Qiagen, Germantown, MD, USA). Cas9 protein was synthesized by UC Berkeley Macro Lab (Lin et al., 2014). RNP complex was formed by incubating 5 µl of Cas9 protein (200pmol) with 9 µg of guide for 15-20 minutes at room temperature prior to combining with the cells. The electroporation procedure was done as previously described (Hoban et al., 2015).

High-throughput sequencing of the human beta-globin gene at the ZFN target site

Genomic DNA was isolated using the PureLink Genomic DNA mini kit (Life Technologies). To prepare the samples for HBB targeted resequencing, the gDNA was PCR-amplified using primers to the HBB gene outside of the donor region (HBB F: 5'- atgcttagaaccgaggtagagttt- 3' and HBB R: 5'- cctgagacttcacactgatg- 3'). Next, a nested PCR was performed using inner and outer sets of primers. Inner: primers homologous to the beta globin region, and read 1 and read 2 sequences

(ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNGGCAGAGCCATCTATTGCTT and GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTCTCC TTAAACCTGTCTTG). Outer: primers homologous to read 1 and read 2 sequences, and P5 and P7 adapters for high-throughput Illumina sequencing as well as a unique index for demultiplexing (AATGATACGGCGACCACCGAGATCTACACACTCTTTCCCTACACGACGCTCTT and

CAAGCAGAAGACGGCATACGAGAT-index-

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTCTCCTTAAACCTGTCTTG). Illumina libraries were sequenced on an Illumina MiSeq at UCLA Technology Center for Genomics & Bioinformatics (TCGB).

Flow cytometry

For FACS analysis, cells were immunostained according to standard procedures with the following antibodies: Pacific blue (or APC-Cy7)-conjugated hCD34 (Biolegend, San Diego, CA), PeCy7-conjugated hCD38 (Biolegend), PerCP-Cy5.5 (or FITC)-conjugated hCD45RA (Biolegend), and PE-conjugated hCD90 (Biolegend). Dead and/or apoptotic cells were labeled with 7-AAD (or DAPI) and AnnexinV-FITC (Biolegend). The cells were analyzed or sorted on the FACS Aria, LSRII or Fortessa (BD Biosciences, San Jose, CA). Cell cycle analysis was performed by resuspending cells in 0.4% Paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for fixation for 30 min and thereafter incubating the cells in 0.2% Triton X-100 (SigmaUltra) to permeabilize for an additional hour. The cells were washed with PBS and subsequently stained with the human APC-conjugated anti-Ki67 (Biolegend) for 2 hours, washed with PBS and finally stained with 7-AAD (Biolegend) using the Annexin V Binding Buffer (Biolegend) for 1 hour prior to flow cytometry analysis. (See table with full description of antibodies in Supplementary Methods, Table).

Quantification and Statistical Analysis

Statistical variables and statistical significance are described in the Figures and the Figure Legends. The data are expressed as mean $\pm$ SEM or mean $\pm$ SD. Statistical significances for comparison between two groups were determined with 2-tailed unpaired Student's t-test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com).

2.8. SUPPLEMENTAL INFORMATION

2.8.1. Supplemental Figures

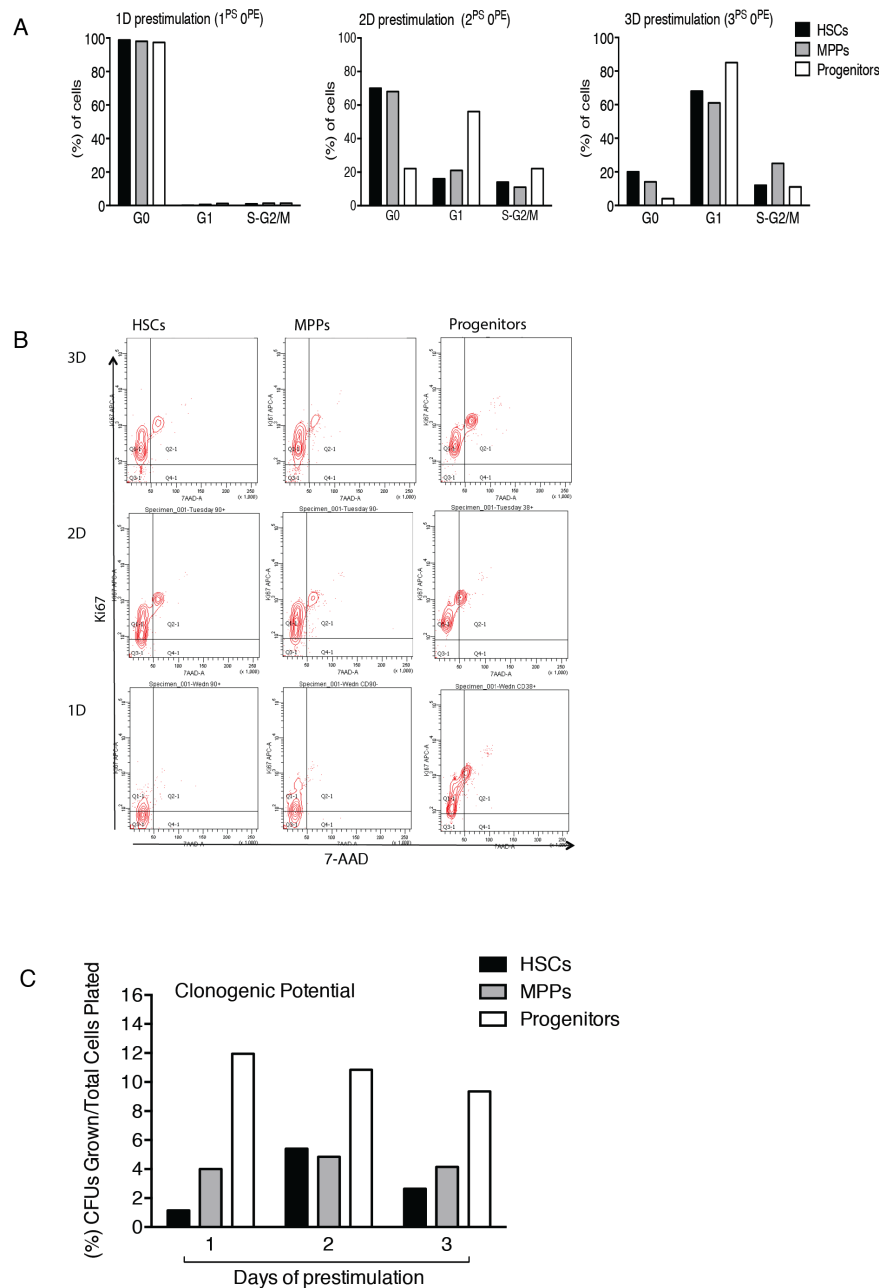


Figure 2.8-1: Cell cycle analysis and CFUs of HSCs, MPPs and progenitor cells after 1-3 days of pre-stimulation, Related to Figure 2.3-4.

(A) Cell cycle analysis on the day of electroporation, after 1-3 days of pre-stimulation. HSCs, MPPs and progenitor cells from bulk $CD34^+$ cells were treated with ZFN mRNA and SMS oligo ($n=1$). (B) Representative FACS plot of cell cycle analysis one day after electroporation, after 1-3 days of pre-stimulation in HSCs, MPPs and progenitor cells from bulk $CD34^+$ cells treated with ZFN mRNA and SMS oligo and analyzed by flow cytometry without expansion. (C) 100 or 300 edited and sorted PB HSCs, MPPs and progenitor cells were assessed for clonogenic potential after 1-3 days of pre-stimulation and electroporation with ZFN mRNA and SMS oligo.

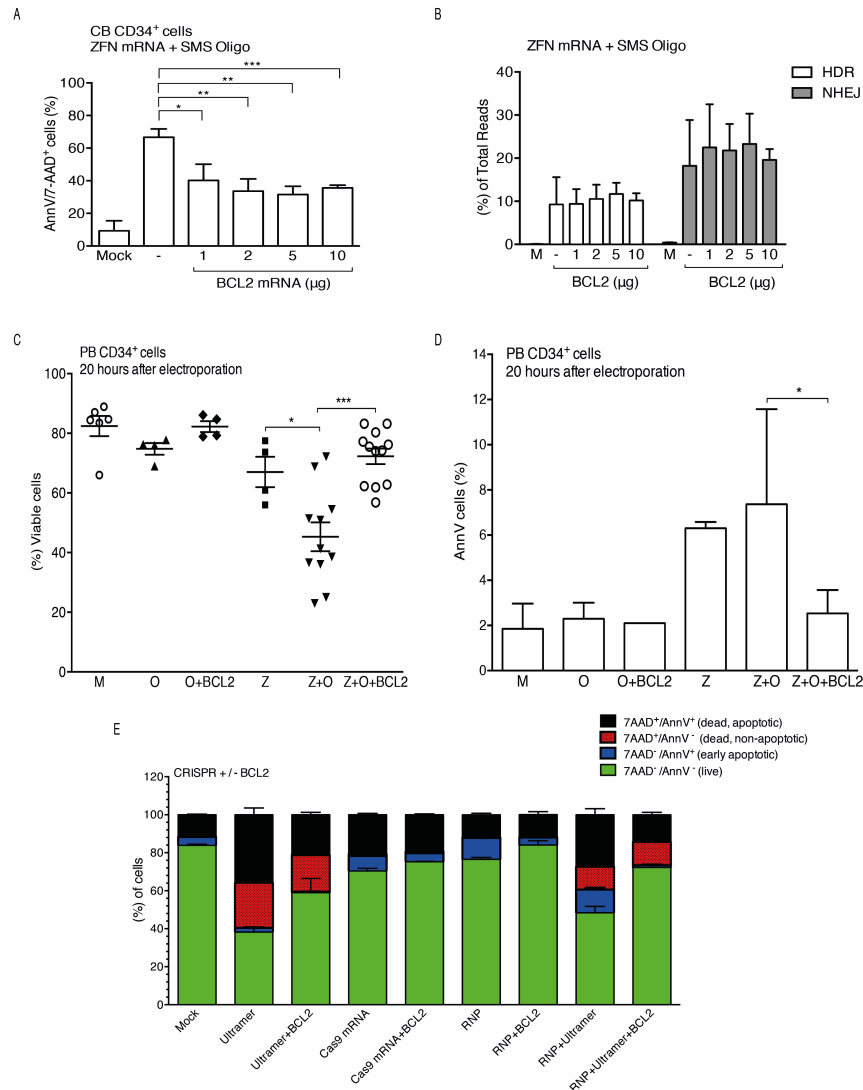


Figure 2.8-2: Assessment of the effect of BCL2 mRNA in bulk CD34⁺ cells, Related to Figure 2.3-5.

(A) Cord blood (CB) CD34⁺ cells were cultured for two days and electroporated with ZFN mRNA, SMS oligo and different concentrations of BCL2 mRNA (1, 2, 5 and 10 µg). Cell toxicity was analyzed 20 hours after electroporation by flow cytometry identifying cells labeled with 7-AAD and AnnexinV, (n=3). (B) HTS analysis was used to measure gene modification levels of HDR and NHEJ (n=3).

PB CD34⁺ cells were cultured for two days and electroporated with either ZFN mRNA only (Z), SMS oligo only (O) or ZFN mRNA and SMS Oligo (Z+O), with or without BCL2 mRNA (5 µg). Cell toxicity was analyzed 20 hours after electroporation by (C) measuring number of viable cells (n=4-12) and by flow cytometry identifying cells labeled with AnnexinV, (n=2-9). (E) Assessment of induced cell toxicity after electroporation; treatments were: mock (M), the SMS oligo (O), an ultramer oligo 111/57 (U) (3 µM), ribonucleoprotein complex of Cas9 protein and guide RNA (gRNA) (9 µg) (RNP), purified Cas9 protein alone (Cas9) (200 pmol), with or without IVT BCL2 mRNA (BCL2), alone or in different combinations. Flow cytometry identified Annexin V/7-AAD-positive cells or Annexin V/DAPI-positive cells (n=4-10). Data are represented as mean ± SD. Student's t-test was used to determine the levels of significant difference, shown as *p < 0.05, **p < 0.005 and ***p < 0.001.

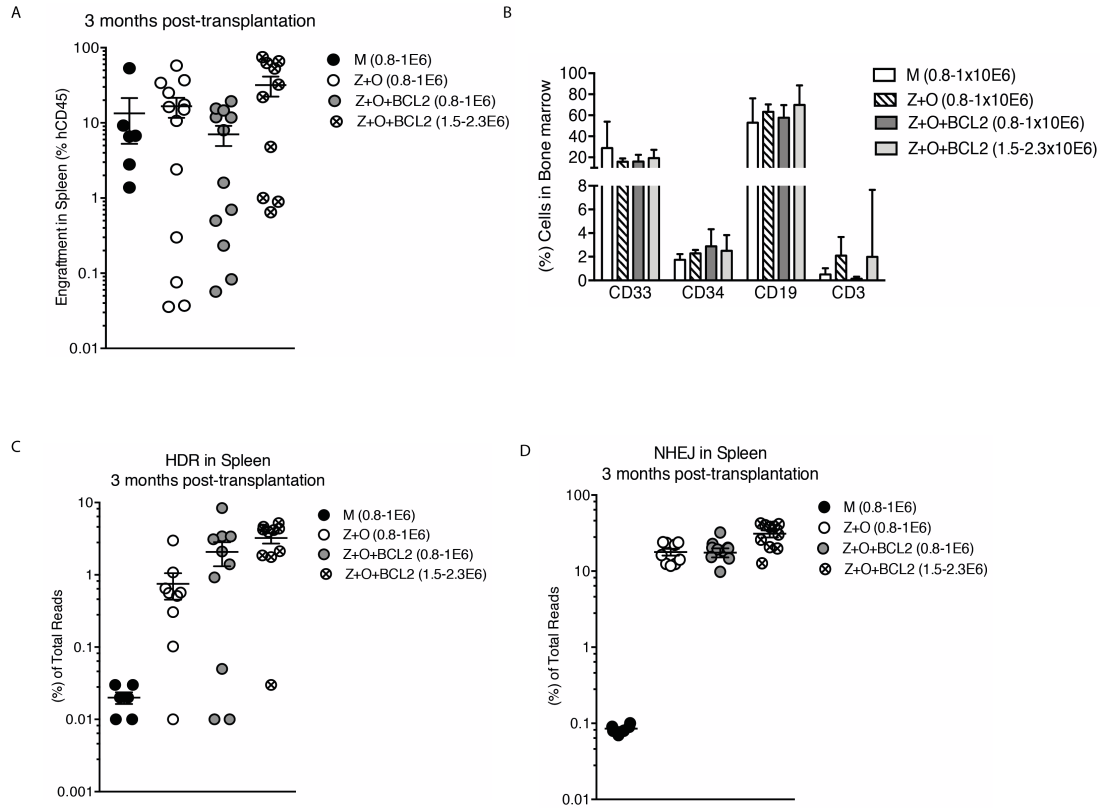


Figure 2.8-3: Lineage analysis and engraftment of human gene-edited cells in spleens of transplanted NSG mice, Related to Figure 2.3-6.

Three months after transplantation of human gene-edited CD34⁺ cells treated with or without *BCL2* mRNA, (A) the engraftment (%hCD45) of human cells in the spleen (n=5-12) was analyzed, as was (B) the lineages of human cells in the BM (n=4-11). The levels of gene modification of cells in the spleens were measured as (C) HDR and (D) NHEJ by HTS. Data are represented as mean $\pm$ SD.

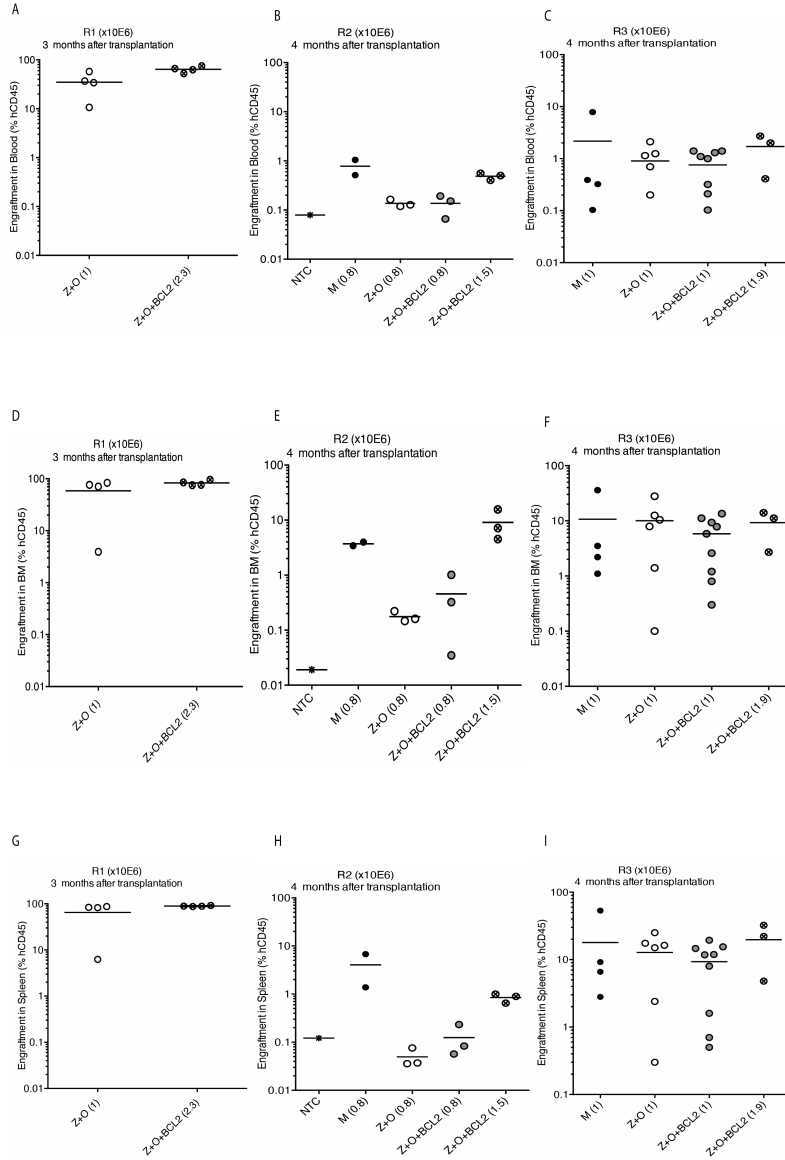


Figure 2.8-4: Engraftment in individual transplant experiments, Related to Figure 2.3-6.

Engraftment of human cells (%hCD45) for each of the three individual transplant experiments (R1, R2, R3) after 3 or 4 months is shown for (A-C) PB, (D-F) BM and (G-I) spleens. NTC = non-transplant control.

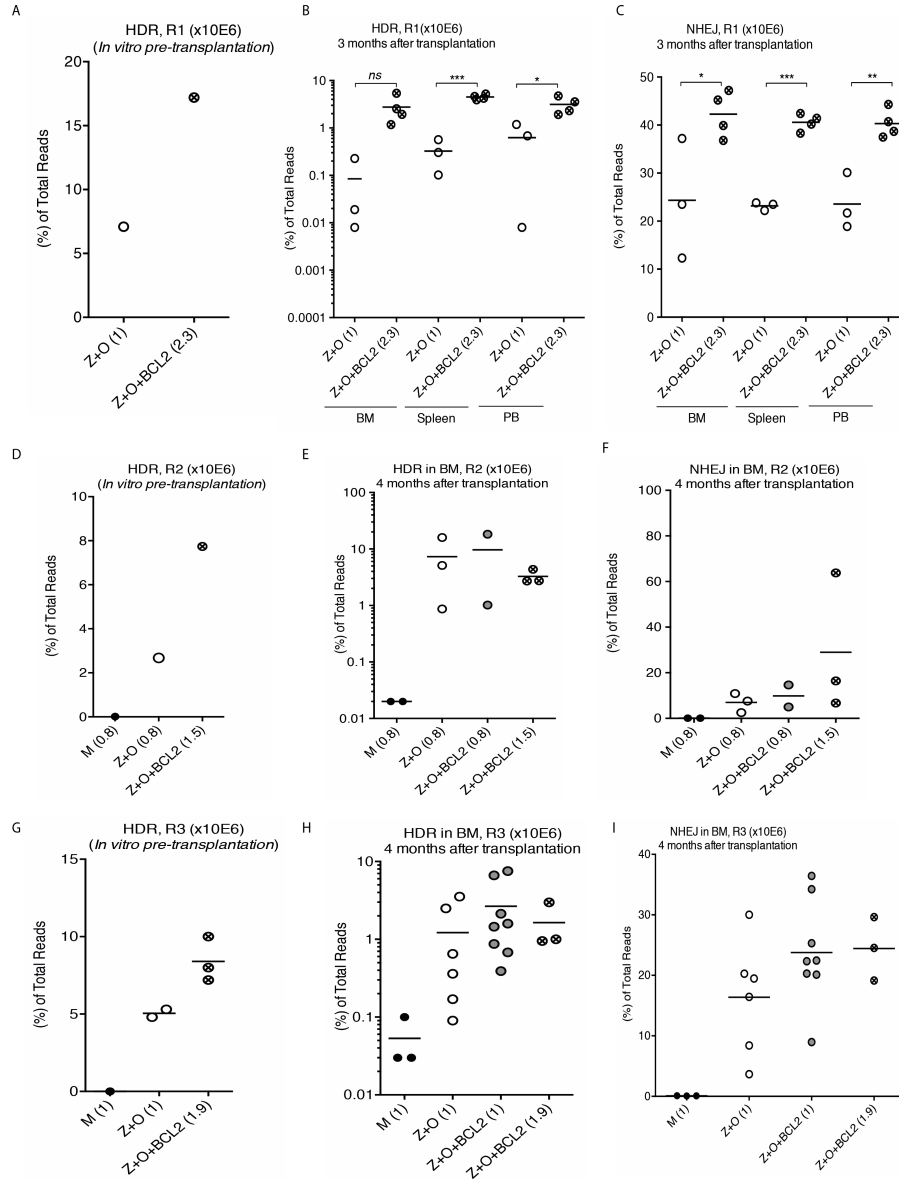


Figure 2.8-5: Gene editing in individual transplant experiments, Related to Figure 2.3-6.

HDR-mediated gene modification of the edited CD34⁺ cells prior to transplantation was determined by HTS, for each of the three individual transplant experiments (R1, R2, R3), after 3 days of *in vitro* culture (A, D, G).

Three or four months after transplantation, the levels of gene modification in BM samples from mice transplanted with CD34⁺ cells treated with or without *BCL2* mRNA was determined by HTS, measuring (B, E, H) HDR and (C, F, I) NHEJ. Statistical significance was calculated using the Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.001).

2.8.2. Supplemental Experimental Procedures

2.8.2.1. Sequences:

Globin ZFN (3 FLAG-NLS-33488-Fok) sequence

atggactacaaagaccatgacggtgattataaagatcatgacatcgattacaaggatgacgatgacaagatggcccca
agaagaagaggaaggtgggcattcacggggtagccgcccgtatggctgagaggcccttcagtgatgaatctgcatgcgtaacttca
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aaa

Globin ZFN (3 FLAG-NLS-33501-Fok) sequence

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aaa

GFP cDNA sequence

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attaaa

T7 *BCL2* cDNA sequence

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gcttgcacacccctgggtgcctatctgggccacaagtga

2.8.2.2. Nucleases and molecular cloning

The ZFNs (33501/33488) were produced at Sangamo Biosciences (Richmond, CA) and target the human β -globin gene, at the sickle mutation site. The left ZFN (recognition sequence: 5'-ACCATGGTGCATCTGAC-3') and the right ZFN (recognition sequence: 5'-GGAGAAGTCTGCCGTT-3') were each cloned into a pGEM-5Zf(+) vector. The GFP and a full-length human *BCL2* cDNA (kindly provided by John E. Dick, Ontario Institute for Cancer Research, Toronto, Canada) were cloned into a (pT7+) plasmid, between a T7 promoter and encoded poly(A) tail (~120 A's). The plasmids were linearized and used as DNA templates for *in vitro* transcription to synthesize mRNA, as previously described (Bjurstrom et al., 2016).

2.8.2.3. Donor templates

Two 101 bp single-stranded oligonucleotides were used as homologous donor templates, containing the corrective base; HBBCSNPHhaISMSSic (*cttcatccacgttcaccttgcacacagCGcagtaa*

cggcagaTttTccacaggAgtcagggtgcacatggtgtctgtttgaggttgctagtgaacacag) and

HBBCSNPHhaISic

(*cttcatccacgttcaccttgcacacagCgcagtaacggcagacttctccacaggagtcagggtgcacatggtgtctgtttgaggttgc*

tagtgaacacag) (Eurofins MWG Operon). The ultramer used for editing with CRISPR/Cas9 was as described (DeWitt et al., 2016). The donor IDLV sequence has previously been described (Hoban et al., 2015).

2.8.2.4. Human colony forming cell (CFU-S) assay

PB CD34⁺ cells were pre-stimulated for 1-3 days, electroporated with ZFN and SMS oligo and thereafter plated in methylcellulose medium as previously described (Romero et al., 2013).

Table 2.8-1: List of antibodies used in experiments

	Fluorophore	Clone	Company	Catalogue number
Mouse anti human CD3	PerCP Cy5.5	UCHT1	Biolegend	3004308
Rat anti mouse CD45	FITC	30-F11	Biolegend	103102
Mouse anti human CD33	PE	WM53	Biolegend	303404
Mouse anti human CD38	PE-Cy7	HIT2	Biolegend	303516
Mouse anti human CD90	PE	5E10	Biolegend	328110
7-AAD			Biolegend	420404
Rat anti mouse CD16/CD32	Fc block	2.4G2	BD Pharmingen	553142
Mouse anti human CD34	APC-Cy7	581	Biolegend	343514
Mouse anti human CD34	APC	561	Biolegend	343608
Mouse anti human CD45RA	PerCP Cy5.5	HI100	Biolegend	304122
Mouse anti human CD45RA	FITC	HI100	Biolegend	304106
Mouse anti human CD19	PE Cy7	HIB19	Biolegend	302216
Mouse anti human CD45	V450	HI30	BD Pharmingen	560367
Mouse anti human CD34	Pacific Blue	581	Biolegend	343512
Mouse anti human Ki67	APC	Ki67	Biolegend	350514
Annexin V	FITC		Biolegend	640906
PI			BD Pharmingen	556463

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Chapter 3. Improving Gene Editing Outcomes in Human Hematopoietic Stem and Progenitor Cells by Temporal Control of DNA Repair

3.1. ABSTRACT

CRISPR/Cas9-mediated gene editing of human hematopoietic stem cells (HSCs) is a promising strategy for the treatment of genetic blood diseases through site-specific correction of identified causal mutations. However, clinical translation is hindered by low ratio of precise gene modification using the corrective donor template (homology-directed repair, HDR) to gene disruption (non-homologous end joining, NHEJ) in human HSCs. By using a modified version of Cas9 with reduced nuclease activity in G1 phase of cell cycle when HDR cannot occur, and transiently increasing the proportion of cells in HDR-preferred phases (S/G2), we achieved a 4-fold improvement in HDR/NHEJ ratio over the control condition in vitro, and a significant improvement after xenotransplantation of edited human HSCs into immune-deficient mice. This strategy for improving gene editing outcomes in human HSCs has important implications for the field of gene therapy, and can be applied to diseases where increased HDR/NHEJ ratio is critical for therapeutic success.

3.2. SIGNIFICANCE STATEMENT

This study reports an improvement in CRISPR/Cas9-mediated gene editing outcomes in human hematopoietic stem cells. Here, we show that cell cycle-dependent control of nuclease activity and DNA repair pathways can influence gene editing outcomes to favor the precise DNA modification over faulty repair events in human hematopoietic stem cells.

This work provides important proof-of-principle findings, and can improve gene editing outcomes for the treatment of congenital diseases of the blood system.

3.3. GRAPHICAL ABSTRACT

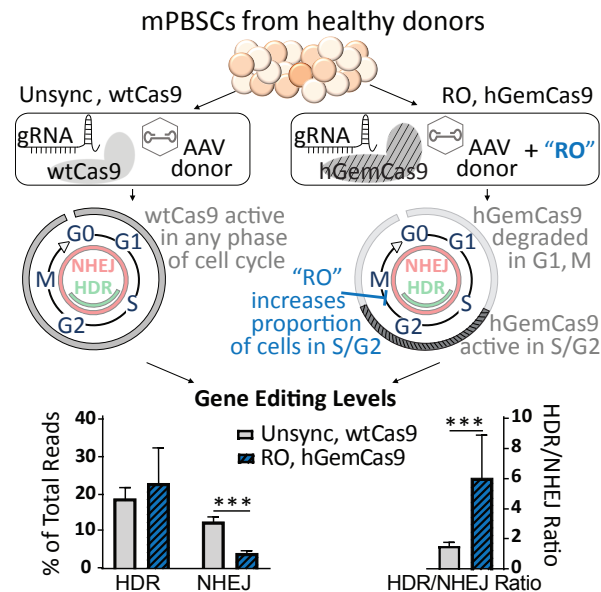


Figure 3.3-1: Graphical Abstract

A modified version of Cas9 (hGemCas9) with reduced nuclease activity in G1 phase of cell cycle, when HDR cannot occur, and transient synchronization of mobilized peripheral blood stem cells (mPBSCs) in HDR-preferred phases (S/G2) resulted in a 4-fold improvement in HDR/NHEJ ratio over the control condition *in vitro*, and a significant improvement after xenotransplantation of edited hHSCs into immune-deficient mice.

3.4. INTRODUCTION

Autologous hematopoietic stem cell (HSC) transplantation, combined with gene editing, could provide an ideal therapeutic option for the treatment of congenital blood diseases, such as hemoglobinopathies, primary immune deficiencies, and storage disorders. Site specific gene editing is achieved by inducing a double-stranded break (DSB) in the DNA near the mutation using targeted nucleases, such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas9) system, and subsequently taking advantage of the innate cellular repair pathways to fix the break. The two major DNA repair pathways are: error-prone non-homologous end joining (NHEJ), which is available throughout the cell cycle (Lieber et al., 2003), and precise homology-directed repair (HDR), which uses a repair template to accurately correct the DSB, and is restricted to S/G2 phases (Branzei and Foiani, 2008; Heyer et al., 2010).

Once modified, HSCs can be re-infused into the patient and provide a long-term self-renewing population to generate a life-long supply of healthy cells.

DSB repair via NHEJ pathway is efficient in human HSCs due to their quiescent status (Branzei and Foiani, 2008; Pietras et al., 2011; Chiruvella et al., 2013), and approaches to disrupt genes for the treatment of certain diseases have already moved to clinical trials (DiGiusto et al., 2016; Chang et al., 2017). However, for diseases where a precise correction of the disease-causing mutation is required, the HDR pathway must be employed to incorporate the provided repair template. To date, HDR-mediated gene editing has been constrained by relatively low efficiency in primitive long-term reconstituting HSCs (Genovese et al., 2014; Hoban et al., 2015; Wang et al., 2015).

In this study, we modeled the correction of the sickle cell disease (SCD)-causing mutation in human hematopoietic stem and progenitor cells (HSPCs). A single nucleotide substitution in the beta-globin gene results in the formation of sickle hemoglobin, HbS, giving rise to rigid and misshaped red blood cells under low oxygen conditions. This devastating disease has lifelong physical and psycho-social consequences: repeated painful crises and frequent costly hospitalization, anemia, kidney failure, stroke and neuro-cognitive deficiencies. There are approximately 300,000 new cases of SCD worldwide every year (Piel et al., 2017; Ribeil et al., 2017), and despite some available therapies, the remaining morbidity and mortality present a significant need for developments of improved treatment options (Piel et al., 2017). Based on results from allogeneic HSC transplants, it has been predicted that gene correction levels of approximately 10-30% in long-term primitive HSCs are required to cure SCD patients (Andreani et al., 2000; Hsieh et al., 2014; Fitzhugh et al., 2017). Although HDR levels of almost 50% are achievable in vitro currently (Hoban et al., 2015; Dever et al., 2016; DeWitt et al., 2016; Hoban et al., 2016), only 5-7% are retained in long-term cells after transplant into NOD/SCID/IL2rgnull (NSG) mice (Hoban et al., 2015; DeWitt et al., 2016). Therefore, it is critical to understand the

reason for lower HDR levels in HSCs, and to develop methods for improving gene editing outcomes in human HSCs.

Increasing the absolute amount of HDR is an obvious goal for improving gene editing outcomes. Additionally, it is equally important to consider simultaneously limiting the levels of NHEJ, as NHEJ-mediated repair may result in large deletions or genomic rearrangements (Kosicki et al., 2018). Moreover, insertions and deletions (indels) in the beta-globin gene could convert a sickle allele to a beta-thalassemia allele, potentially resulting in a worse phenotype than the initial disease. Although an acceptable background value for NHEJ in patient cells has not been established in the field yet, it is clear that the relative proportion of precise edits to indels needs to be increased in order to ensure positive therapeutic outcomes. We used a metric, designated “HDR/NHEJ ratio” to evaluate approaches of improving gene editing outcomes.

Here, we studied the temporal regulation of DNA repair pathway utilization in human HSPCs. We hypothesized that cell cycle phase-dependent manipulation of DNA repair will improve HDR/NHEJ ratio in primary human HSPCs. We showed that by using a Cas9 variant with decreased nuclease activity in G1 and M phases, and synchronizing the cells in S/G2, we can obtain a 4-fold increase in HDR/NHEJ ratio in primary human HSPCs in vitro, compared to standard conditions. More importantly, these manipulations led to a significant improvement in HDR/NHEJ ratio in vivo.

3.5. RESULTS

3.5.1. HDR Levels are Significantly Lower in Human HSCs, Compared to Progenitors

To understand the reason for the drop in gene correction levels after transplantation of edited cells into NSG mice (Hoban et al., 2015; DeWitt et al., 2016), we studied whether gene editing efficiency in long-term HSCs differs from the progenitors.

We began by comparing different phases of gene editing in the following human immunophenotypically-defined HSPC sub-populations: HSCs (CD34+/CD38-/CD90+/CD45RA-); multipotent progenitors (MPPs) (CD34+/CD38-/CD90-/CD45RA-); and mature stem/progenitor populations (CD34+/CD38+) (Majeti et al., 2007) (**Figure 3.12-1A**). We measured cell cycle profile of immunophenotypic HSPC sub-populations after two days of prestimulation with cytokines (**Figure 3.12-1B**). HSC and MPP sub-populations mainly comprised of G1 cells (75% and 87%, respectively). In contrast, in the progenitor sub-population, 55% of cells were in G1 and ~40% in S/G2 phases. To assess the efficiency of delivery and translation of Cas9 mRNA among the HSPC sub-populations, we compared the delivery and translation of electroporated green fluorescent protein (GFP) mRNA. Bulk CD34+ cells from healthy human granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood cells (“PBSCs”) were electroporated with in vitro-transcribed (IVT) mRNA encoding GFP. No statistically significant differences were observed among the HSPC sub-populations in regards to percentage of GFP-positive (GFP+) cells or the mean fluorescence intensity (MFI) on days 1-2 post electroporation, indicating that delivery and translation of electroporated mRNA is equivalent in HSCs, MPPs and progenitors (**Figure 3.5-1A-B**).

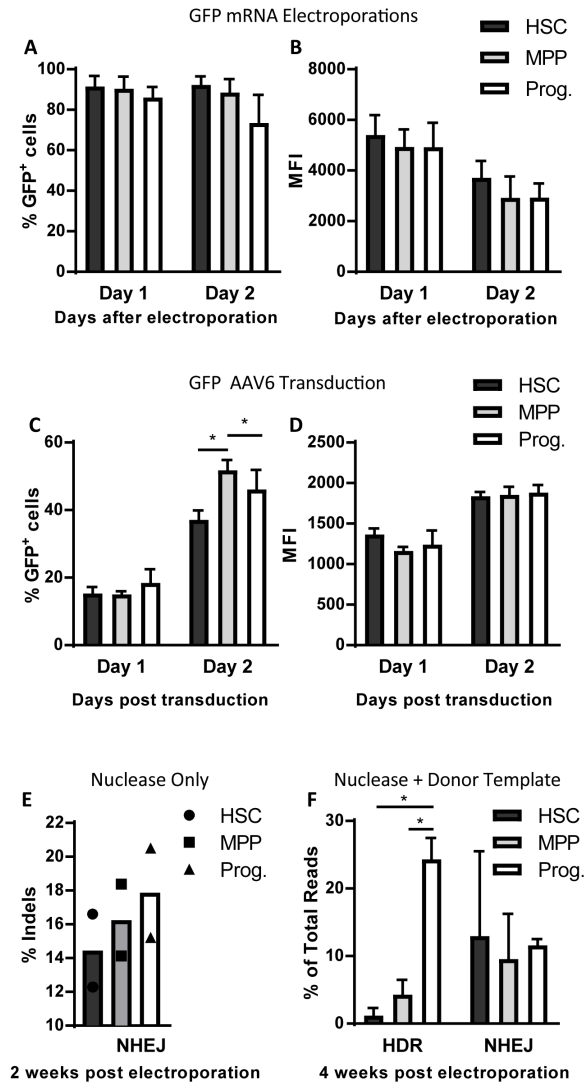


Figure 3.5-1: Homology-directed repair (HDR) levels are significantly lower in human hematopoietic stem cells (HSCs), compared with progenitors.

(A): Green fluorescent protein (GFP) expression in sub-populations (HSCs, multipotent progenitors [MPPs], progenitors) of human PBSCs, measured by flow cytometry on days 1 and 2 after electroporation of GFP mRNA; $n = 3$. Data are represented as mean $\pm$ SD. Differences between subpopulations are not significant, based on Kruskal–Wallis test. See Figure 3.12-11A for flow cytometry gating strategy. (B): Mean fluorescence intensity (MFI) of GFP expression in (A); $n = 3$. Data are represented as mean $\pm$ SD. Differences between subpopulations are not significant, based on Kruskal–Wallis test. (C): GFP expression in subpopulations of human PBSCs, measured by flow cytometry on days 1 and 2 after transducing cells with GFP-AAV6; $n = 4$. Data are represented as mean $\pm$ SD. Differences between subpopulations are not significant if not specified, based on Kruskal–Wallis test; *, $p < .05$, based on Wilcoxon rank-sum test. (D): MFI of GFP expression in (C); $n = 4$. Data are represented as mean $\pm$ SD. Differences between subpopulations are not significant, based on Kruskal–Wallis test. (E): Percentage of insertions and deletions (indels) in FACS-sorted human PBSC subpopulations (HSCs, MPPs, progenitors), measured by HTS 2 weeks after electroporating bulk PBSCs with Cas9 mRNA and sgRNA (see also Figure 3.5-2A). % indels are calculated as described in methods; $n = 2$. Data are represented as individual points, bars indicate mean. (F): Percentage of gene editing (HDR and nonhomologous end joining [NHEJ]) in FACS-sorted human PBSC subpopulations (HSCs, MPPs, progenitors), measured by HTS 4 weeks after electroporating bulk PBSCs with Cas9 mRNA and sgRNA, and transducing with AAV6 donor template (see Figure 3.5-2B for a map of AAV6). % HDR and NHEJ are calculated as described in methods; $n = 4$. Data are represented as mean $\pm$ SD. Differences are not significant if not specified, based on Kruskal–Wallis test; *, $p < .05$, based on Wilcoxon rank-sum test.

Prior to utilizing adeno-associated virus serotype 6 (AAV6) as a delivery vehicle for the donor template, we tested the transduction efficiency of GFP-AAV6 in the three sub-populations as a proxy for donor template availability in the cells. No differences in the percentage of GFP+ cells or MFI were observed among HSCs, MPPs and progenitors one day post transduction, suggesting that the delivery of the donor template would be equivalent in the three sub-populations (**Figure 3.5-1C-D**). On day 2, the percentage of GFP+ cells in the MPPs and progenitors was higher than in HSCs, however the MFI was the same.

To determine whether the three sub-populations are equally amenable to Cas9 nuclease activity, IVT mRNA encoding Cas9 and a single guide RNA (sgRNA) targeting exon 1 of the human beta-globin locus were delivered to bulk PBSCs by electroporation (refer to **Figure 3.5-2A**) (DeWitt et al., 2016; Hoban et al., 2016). The following day, HSCs, MPPs and progenitors were separated by fluorescence-activated cell sorting (FACS), and each sub-population was cultured in myeloid expansion medium for two weeks prior to performing high-throughput sequencing (HTS) of the human beta-globin locus. The percentages of beta-globin alleles containing insertions/deletions (indels) in HSPC sub-populations are shown in **Figure 3.5-1E**, demonstrating that similar endonuclease activity was achieved in the HSCs, MPPs and progenitors.

Next, to measure the levels of HDR and NHEJ in the presence of donor template, PBSCs were electroporated with Cas9 mRNA and sgRNA, and subsequently transduced with AAV6 donor template carrying a 1.1kb insert, homologous to the exon 1 of beta-globin gene, with a single-nucleotide polymorphism (SNP) to convert wild-type (wt) beta globin to sickle (along with three other SNPs described in **Figure 3.5-2B**). HSCs, MPPs and progenitors were separated by FACS the next day, and cultured in myeloid expansion medium for four weeks, prior to HTS. Unlike NHEJ levels, which were variable, but generally similar among sub-populations, gene editing via HDR pathway was significantly lower in the HSCs and MPPs, compared to the progenitors (**Figure 3.5-1F**). Of note, the levels of HDR and NHEJ in the progenitor sub-

population were repeatedly measured during the four-weeks to ensure that prolonged culture time does not affect gene editing levels (data not shown). Taken together, these data demonstrate an inherent difference between HSCs and progenitors in their ability to utilize HDR pathway for DSB repair.

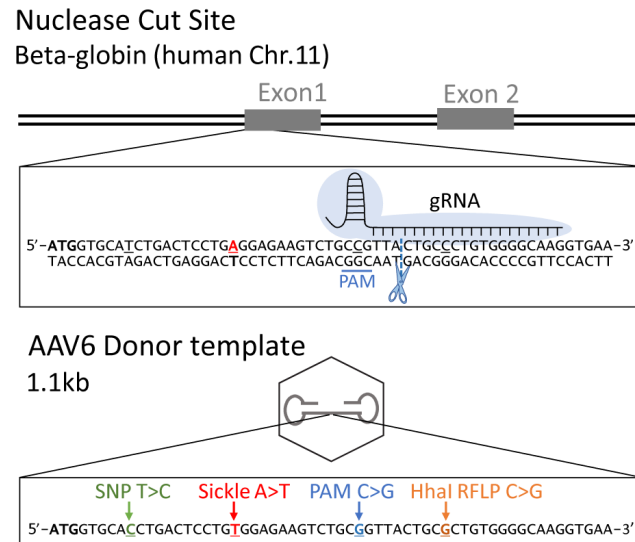


Figure 3.5-2: Overview of gene editing reagents.

(A): A map of the β -globin locus on human Chr 11 (not to scale). The inset illustrates the beginning of exon 1. The blue cloud represents Cas9 protein and the gRNA binding site is indicated. The PAM sequence is underlined in blue. The scissors and the dashed line represent the Cas9 cut site. The red "A" represents the location of the sickle mutation. (B): A schematic of the AAV donor template (not to scale). The inset shows the four mutations that are introduced by the donor template. These include: a common SNP (29 bp upstream from cut site), a sickle mutation (18 bp upstream from cut site), a PAM mutation to prevent recleavage by Cas9 after successful gene modification (5 bp upstream from cut site), and a HhaI RFLP (5 bp downstream of cut site) to be used as a surrogate marker for gene modification.

3.5.2. HDR/NHEJ Ratio is Highest in S/G2 Phases of Cell Cycle

HDR is described to be restricted to S/G2 phases of cell cycle in cell lines (Lieber et al., 2003), however it has not been previously shown whether the same temporal regulation exists in primary human CD34+ cells. To assess this, PBSCs were FACS-sorted into G1 and S/G2/M fractions (**Figure 3.5-3A**), and electroporated with gene editing reagents immediately after sort. It should be noted that the cells were not cell cycle-arrested, and continued normal cell cycle progression after FACS, therefore the populations were merely enriched for the indicated cell cycle phases at the time of electroporation. As expected based on cell line data, the levels of HDR were significantly higher in S/G2/M-sorted cells, compared to G1-sorted and bulk unsorted PBSCs

(**Figure 3.5-3B**). Additionally, NHEJ was slightly lower in S/G2/M, resulting in a ~5-fold higher HDR/NHEJ ratio (**Figure 3.5-3B, C**).

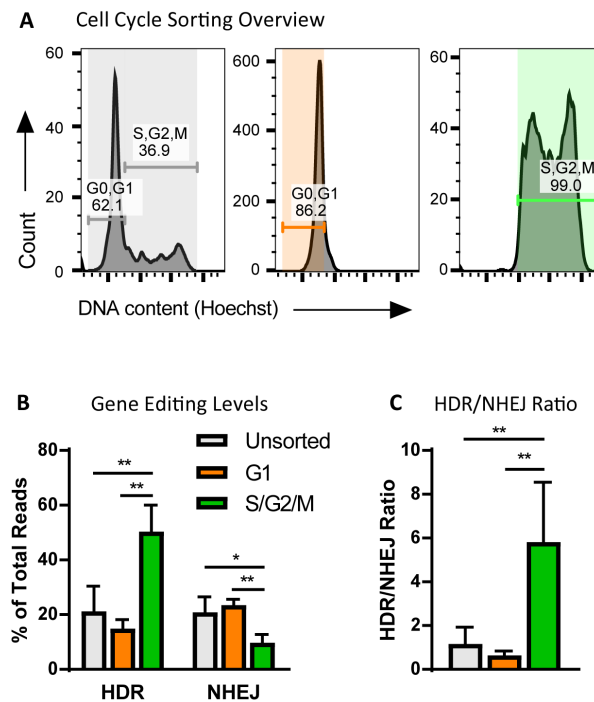


Figure 3.5-3: The ratio of homology-directed repair (HDR) to non-homologous end joining (NHEJ) is highest in S/G2 phases of cell cycle.

(A): Cell cycle analysis of human PBSCs, immediately after sorting into G1 and S/G2/M populations by fluorescence-activated cell sorting (FACS). See also Figure 3.12-2A for analysis at a later time point. (B): Percentage of gene editing (HDR and NHEJ) in unsorted, or FACS-sorted G1 or S/G2/M PBSCs, electroporated with Cas9 mRNA and sgRNA immediately after sort, and subsequently transduced with AAV6 donor template; n = 5. Data are represented as mean $\pm$ SD. Differences are not significant if not specified, based on Kruskal–Wallis test; *, p < .05; **, p $\leq$.01, based on Wilcoxon rank-sum test. (C): HDR/NHEJ ratio, calculated from (B). Data are represented as mean $\pm$ SD. Differences are not significant if not specified, based on Kruskal–Wallis test; **, p $\leq$.01, based on Wilcoxon rank-sum test.

3.5.3. hGemCas9 Allows for Lower Nuclease Activity in G1, while Retaining Specificity

Since Cas9 can generate DSBs in any phase of cell cycle, but HDR is confined to S/G2, we sought to decrease nuclease activity in G1 phase to limit unwanted NHEJ. Similar to Gutschner et al., a fragment of the human geminin domain was fused to the C-terminus of Cas9 (termed hGemCas9) (**Figure 3.5-4A**) to allow the protein to be ubiquitinated and degraded by APC/Cdh1 complex in G1 and late M phases (Gutschner et al., 2016). To confirm the reduced nuclease activity of hGemCas9 in G1, human PBSCs were FACS-sorted into G1 or S/G2/M

phases and electroporated immediately after sort with either wtCas9 or hGemCas9 IVT mRNA and sgRNA. In G1-sorted cells, nuclease activity of hGemCas9 was significantly lower than of wtCas9 (**Figure 3.5-4B**). There was also a slight decrease in activity during S/G2/M phases, possibly due to the cells progressing through cell cycle prior to analysis.

Additionally, to compare the specificity of wtCas9 vs hGemCas9, indels were quantified by HTS at the previously-identified off-target sites for this guide, termed OT1 and OT2 (DeWitt et al., 2016), in the absence and presence of donor template (**Figure 3.12-2B and D**, respectively). Nuclease specificity, calculated as the ratio of on-target to off-target indels, was similar between wtCas9 and hGemCas9 in bulk (unsorted) cells (**Figure 3.5-4C**, **Figure 3.12-2C**, **E**). However, hGemCas9 appeared to be more specific than wtCas9 in G1-sorted cells (**Figure 3.5-4C**), demonstrating a previously unreported role of hGemCas9 in preferentially decreasing off-target nuclease activity in G1 phase of cell cycle, potentially due to lower affinity for OT1.

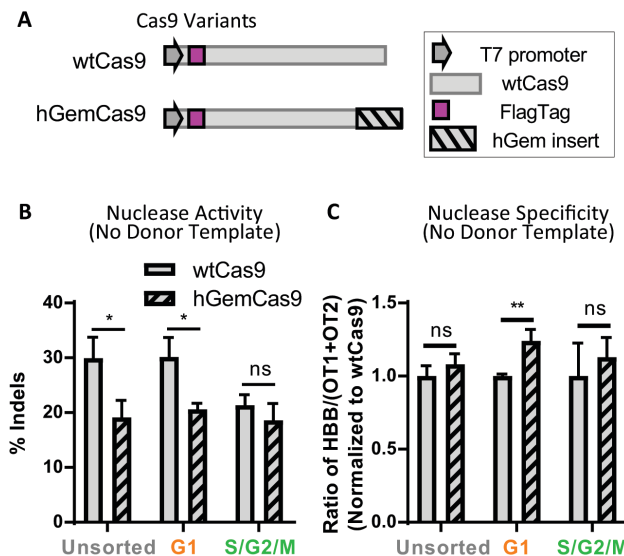


Figure 3.5-4: hGemCas9 allows for lower nuclease activity in G1, although retaining specificity.

(A): Schematic of a portion of wtCas9 and hGemCas9 plasmids used for IVT (not to scale). hGem insert at C-terminus of Cas9 consisted of the first 110 amino acids of human geminin. (B): Percentage of nuclease activity (% indels), measured by high throughput sequencing, in unsorted and G1- or S/G2/M-sorted PBSCs, electroporated immediately after sort with wtCas9 or hGemCas9 mRNA; n = 3. Data are represented as mean $\pm$ SD. Ns, not significant; *, p < .05, based on unpaired t-test (see also Figure 3.12-2B,D). (C): Nuclease specificity of wtCas9 and hGemCas9 in different phases of cell cycle, calculated from data in (B); see also Figure 3.12-2C,E).

3.5.4. Human HSPCs Can Be Transiently Synchronized in S/G2 Phases of Cell Cycle

HSCs are principally quiescent while occupying their natural niches in the body (Cheshier et al., 1999; Wilson et al., 2008; Takizawa and Manz, 2012; Walter et al., 2015); after cytokine stimulation in vitro, the majority of HSPCs are in G1 phase of cell cycle, with only about 35% present in S/G2 after two days of culture (**Figure 3.5-5A**). To increase the proportion of cells available for HDR, we used a selective inhibitor of CDK1, RO-3306 (“RO”), which transiently arrests cells at the G2-M transition, increasing the number of cells in S and G2 phases (Vassilev, 2006; Vassilev et al., 2006). RO concentration (30uM) and treatment time (20 hours) were optimized to result in a 10-12% increase in the number of cells in S/G2 phases, compared to vehicle control (DMSO), and a concomitant decrease in the number of cells in G1 phase (**Figure 3.5-5A-B**). RO treatment resulted in initial toxicity (**Figure 3.12-3A**). However, colony forming unit (CFU) assay and a competitive transplant of RO-treated and control cells indicated that the surviving cells retained their hematopoietic potential (**Figure 3.12-3B-G**).

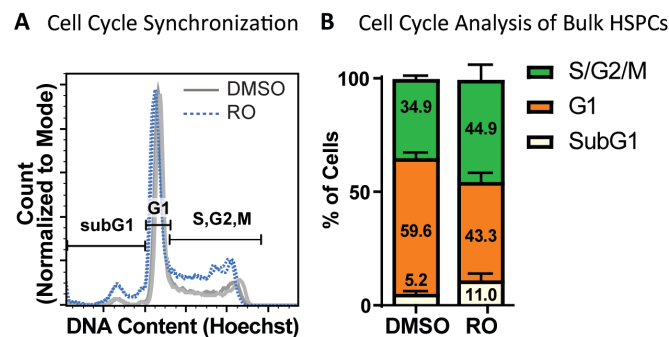


Figure 3.5-5: Human hematopoietic stem and progenitor cells (HSPCs) can be transiently synchronized in S/G2 phases of cell cycle (see also Figure 3.12-3).

(A): Representative flow cytometry plot of cell cycle analysis of PBSCs treated with 30 μ M RO or DMSO (volume equivalent) for 20 hours. SubG1 population represents apoptotic cells. (B): Quantification of flow cytometry analysis in (A); n = 6. Data are represented as mean $\pm$ SD. Numbers indicate means. Differences are not significant, based on chi-square test.

3.5.5. Reducing Nuclease Activity in G1 and Synchronizing Human HSPCs in S/G2 Improves HDR/NHEJ Ratio *in Vitro*

After confirming that hGemCas9 decreased nuclease activity in G1 and that RO treatment increased the percentage of cells in S/G2 phases, we went on to test the effects of these manipulations on gene editing levels in human PBSCs, as compared to unsynchronized (unsync) cells with wtCas9 control. As expected, hGemCas9 did not affect HDR, but significantly reduced NHEJ levels from $12.1 \pm 1.4\%$ to $6.9 \pm 0.7\%$ (**Figure 3.5-6A**). Cell synchronization with RO increased HDR slightly from $18.1 \pm 3.5\%$ to $23.5 \pm 8.3\%$, and decreased NHEJ from $12.1 \pm 1.4\%$ to $7.5 \pm 1.7\%$. Additionally, there was an additive effect between hGemCas9 and cell synchronization at reducing NHEJ to below 4%, thus improving HDR/NHEJ ratio about 4-fold over the control condition (**Figure 3.5-6B**). The use of hGemCas9 did not have any negative effects on cell viability and fold expansion (**Figure 3.5-6C-D**). Similar to earlier observations, RO treatment resulted in significant initial decrease in viability and fold expansion, measured 24 hours post electroporation.

In addition to successfully decreasing nuclease activity, hGemCas9 also did not alter nuclease specificity (**Figure 3.12-2C,E**), or the types or proportion of indels formed in bulk, unsorted PBSCs (**Figure 3.5-6E, Figure 3.12-4A**). There is a 5-base pair (bp) microhomology around the cut site, identified by an online prediction algorithm (Bae et al., 2014), which results in a 9-bp deletion via microhomology-mediated end joining (MMEJ) pathway (**Figure 3.12-4B**). Interestingly, RO pre-treatment resulted in proportionally less indels produced via MMEJ, compared to unsynchronized cells, possibly as a consequence of CDK1 inhibition (McVey and Lee, 2008; Srivastava and Raghavan, 2015) (**Figure 3.5-6E, Figure 3.12-4A**). To overcome the potential decrease of CtIP phosphorylation (which is essential for DNA end resection in homologous recombination (Huertas and Jackson, 2009; Peterson et al., 2011; 2013; Hustedt and Durocher, 2016)) by CDK1 inhibition upon RO treatment, we sought to express phosphomimic CtIP (T847E) in the cells. This constitutively active mutation has been shown to be sufficient for DSB end processing (Huertas and Jackson, 2009), which is required for HDR

(Heyer et al., 2010). CtIP T847E (CtIP*) IVT mRNA was co-electroporated with gene editing reagents. However, no differences in HDR or NHEJ levels, indel distribution or viability were observed with the addition of CtIP* mRNA (Figure 3.12-5A-E).

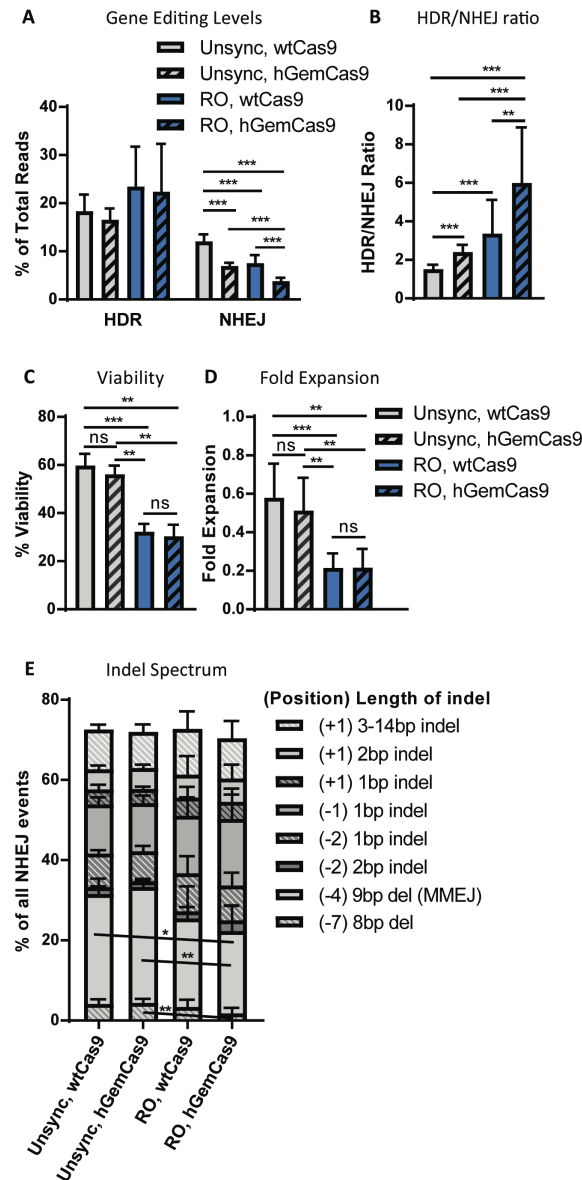


Figure 3.5-6: Reducing nuclease activity in G1 and synchronizing human hematopoietic stem and progenitor cells (HSPCs) in S/G2 improves the ratio of homology-directed repair (HDR) to nonhomologous end joining (NHEJ) in vitro.

(A): Levels of gene editing, measured by high throughput sequencing (HTS), in PBSCs, pretreated with DMSO or RO for 20 hours, electroporated with wtCas9 or hGemCas9, and subsequently transduced with AAV6 donor template. For wtCas9— $n = 10$, for hGemCas9— $n = 8$. Data are represented as mean $\pm$ SD. Differences are not significant if not specified, based on Kruskal–Wallis test; ***, $p \leq .001$, based on Wilcoxon rank-sum test (see also Figure 3.12-2D, E). (B): Ratio of HDR to NHEJ, calculated from data in (A). For wtCas9— $n = 10$, for hGemCas9— $n = 8$. Data are represented as mean $\pm$ SD. Differences are not significant if not specified; **, $p \leq .01$; ***, $p \leq .001$, based on Wilcoxon

rank-sum test. (C): Percent viability (from cells in [A]) measured by trypan blue 24 hours post-electroporation. For wtCas9—n = 9, for hGemCas9—n = 7. Data are represented as mean $\pm$ SD. Ns, not significant; **, $p \leq .01$; ***, $p \leq .001$, based on Wilcoxon rank-sum test. (D): Fold expansion (from cells in [A]), measured 24 hours post-electroporation. For wtCas9—n = 9, for hGemCas9—n = 7. Data are represented as mean $\pm$ SD. Ns, not significant; **, $p \leq .01$; ***, $p \leq .001$, based on Wilcoxon rank-sum test. (E): Indel spectrum, normalized to %NHEJ (in [A]) for each sample. Only the top indels (making up ~75% of all indels) are plotted (see also Figure 3.12-4A). Numbers in parentheses indicate position of an indel relative to cut site, numbers following parentheses indicate the length of an indel; indel—insertion or deletion (individual values for insertions or deletions events of the same length occurring at the same position were summed), del—deletion only. For wtCas9—n = 10, for hGemCas9—n = 8. Data are represented as mean $\pm$ SD. Differences are not significant if not specified; *, $p < 0.05$; **, $p \leq 0.01$, based on Wilcoxon rank-sum test.

3.5.6. Reducing Nuclease Activity in G1 and Synchronizing Human HSPCs in S/G2

Improves HDR/NHEJ Ratio *in Vivo*

Finally, to determine the effects of reducing nuclease activity in G1 (hGemCas9) and/or synchronizing cells in S/G2 (RO) on gene editing levels in long-term HSC population, bulk PBSCs were treated and electroporated as described earlier, and transplanted into 6-9-week-old sub-lethally irradiated mice 24 hours later. A small fraction of cells was kept in myeloid culture for 2 weeks to determine the levels of gene modification prior to transplantation (**Figure 3.5-7A-B**).

At 8 weeks post-transplant, the percentage of human engraftment in peripheral blood (PB) was ~3% (geometric mean) in the “RO, hGemCas9” group, and ~1% (geometric mean) in the other experimental arms (**Figure 3.5-7C**, **Figure 3.12-6A**). The mice were euthanized at 16 weeks post-transplant, and the levels of human engraftment were quantified in the mouse PB, bone marrow (BM) and spleen (**Figure 3.5-7C**). The human engraftment varied from 0.1% to 20%, typically with lower values in the male mice (**Figure 3.12-6B**), as previously observed by other groups (Notta et al., 2010). No statistically significant differences in human engraftment or lineage distribution of engrafted cells were observed among treatment groups (**Figure 3.5-7C**, **Figure 3.12-6A,C-D**).

Gene editing levels in the human engrafted cells in the mouse BM were generally lower than before transplant (**Figure 3.5-7D**). The HDR levels in the control arm (Unsync, wtCas9) decreased from over 20% in vitro to 0.03-0.4% after transplant. Similar to the in vitro data, RO pre-treatment allowed for slightly higher HDR levels, reaching 2.5%. Comparable to previously-

published data (Hoban et al., 2015; DeWitt et al., 2016), the levels of NHEJ remained high after transplant, with a geometric mean of ~5% in the “Unsync, wtCas9” group. Analogous to the in vitro data, “Unsync, hGemCas9” and “RO, hGemCas9” groups had lower NHEJ levels with a geometric mean of 0.5% and 0.7%, respectively (**Figure 3.5-7D**). Furthermore, there was an additive effect between Cas9 manipulation (hGemCas9) and cell cycle synchronization (RO), thus improving the HDR/NHEJ ratio on an average of 20-fold over the control condition (**Figure 3.5-7E**). Interestingly, the HDR levels in RO-treated cells were slightly higher than in control, despite the lower HDR pre-transplant. Because of an unexpected decrease in HDR levels in RO-treated samples before transplant, the levels of gene editing after transplant were normalized to the input values to calculate the percentage of gene editing maintained after transplant (**Figure 3.12-7**). Our data suggest that RO-treated cells have an increased ability to maintain HDR levels after transplant, and hGemCas9 is able to preserve lower NHEJ frequencies.

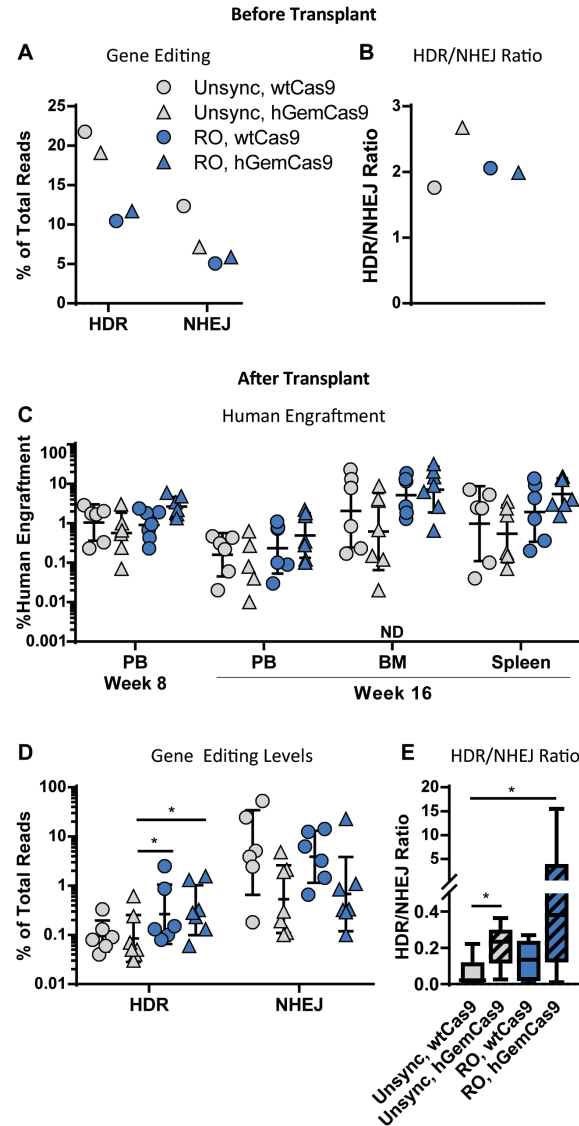


Figure 3.5-7: Reducing nuclease activity in G1 and synchronizing human hematopoietic stem and progenitor cells (HSPCs) in S/G2 improves the ratio of homology-directed repair (HDR) to non-homologous end joining (NHEJ) in vivo.

(A): Levels of gene editing (HDR and NHEJ), measured by HTS in vitro before transplant, in human PBSCs electroporated with wtCas9 mRNA and sgRNA, and subsequently transduced with AAV6; $n = 1$. (B): Ratio of HDR/NHEJ from data in (A); $n = 1$. (C): Levels of human engraftment in NSG mice at 8 and 16 weeks post-transplant in the indicated organs, measured by flow cytometry, and calculated as $(\%hCD45^+ / [\%hCD45^+ + mCD45^+]) \times 100$. PB, peripheral blood; BM, bone marrow (see also Figure 3.12-6A,B); $n = 6-7$ (3-4 female mice + 3-4 male mice). Individual points are plotted. Line—geometric (geo) mean $\pm$ geo SD. ND—zero value is not plotted on a log scale, but was used in calculating mean and SD. Differences between experimental arms are not significant, based on Kruskal-Wallis test. (D): Levels of gene editing (HDR and NHEJ) in the engrafted human cells in mouse BM at 16 weeks post-transplant; $n = 6-7$ (3-4 female mice + 3-4 male mice). Individual points are plotted. Line—geo mean $\pm$ geo SD. Data point from one of the mice in “Unsync, hGemCas9” cohort was excluded due to very low human engraftment (0.02%) resulting in unreliable gene editing values. Differences are not significant if not specified; *, $p < .05$, based on Wilcoxon rank-sum test. (E): Ratio of HDR/NHEJ from data in (D); $n = 6-7$ (3-4 female mice + 3-4 male mice). Data are represented as box-and-whisker plot, horizontal line indicates mean, boundaries of the box—the upper and lower quartiles, whiskers—the lowest and highest observations. Differences are not significant if not specified; *, $p < .05$, based on Wilcoxon rank-sum test.

3.6. DISCUSSION

In this study, we took advantage of the temporal regulation of DNA repair pathways and utilized them to improve the HDR/NHEJ ratio from gene editing in primary human HSPCs. Several methods to increase HDR and decrease NHEJ have been proposed previously. One attempt is through inhibition of the NHEJ pathway by inhibiting DNA-PK with NU7441 or KU-0060648 (Robert et al., 2015), or by blocking Ligase IV with SCR7 (Srivastava et al., 2012; Chu et al., 2015; Maruyama et al., 2015). However, we and other groups have not been able to achieve an improvement in HDR with SCR7 in our experimental systems (Pinder et al., 2015; Robert et al., 2015; Gutschner et al., 2016; Yang et al., 2016). Moreover, DNA-PK and Ligase IV act relatively late in the NHEJ, after pathway commitment. Another potential method to improve editing outcomes is to initiate HDR in the G1 phase of cell cycle by manipulating DNA repair factors to control repair pathway choice at the decision-making stage (Escribano-Díaz et al., 2013; Orthwein et al., 2015; Canny et al., 2018). Additional alternative strategies including controlling the timing of delivery and activity of nuclease reagents based on cell cycle progression, synchronizing cells in the desirable cell cycle phase, and modifying Cas9 expression based on cell cycle progression, have led to an improvement in HDR in cell lines or iPSCs (Lin et al., 2014; Gutschner et al., 2016; Yang et al., 2016). While all these manipulations were shown to be successful at increasing HDR in cell lines, their effects in primary human HSPCs have not yet been reported.

Since the goal is to provide a life-long treatment for monogenic diseases of the blood, the mutation must be corrected in the primitive stem cell sub-population of HSPCs, termed HSCs. The rare HSCs are outnumbered by progenitors, so the readout of the bulk HSPCs in vitro is only representative of the progenitor sub-populations, masking any differences in the HSCs. To determine gene editing levels in long-term HSCs, gene-modified HSPCs must be transplanted into NSG mice and assessed at 14-16 weeks post-transplant. While sufficiently high levels of editing are currently achievable in vitro (Hoban et al., 2015; Dever et al., 2016; DeWitt et al., 2016), we and others have observed that transplantation of gene-edited cells into NSG mice

results in a decrease in gene correction values (Hoban et al., 2015; DeWitt et al., 2016), unless a selectable marker was included to enrich the edited population prior to transplant (Dever et al., 2016). These observations suggest that the gene editing occurs most efficiently in the progenitor population, and less in the rare HSCs.

To determine why the HSCs are less amenable to gene editing than the progenitors, we studied the efficiency of delivery and expression of gene editing reagents in the immunophenotypically-defined HSPC sub-populations (HSCs, MPPs, progenitors). Our results suggested that the delivery and translation of nuclease mRNA are not the likely limiting factors. Also, the donor template cellular availability is unlikely restrictive, based on delivery of a GFP reporter virus of the same serotype (AAV6) to HSPCs. Additionally, the percentage of indels were similar among the sub-populations following delivery of CRISPR/Cas9 alone, indicating that the nuclease can cut DNA in HSCs as well as the progenitors. However, when combining nuclease and a donor template, the HDR levels were much lower in the HSCs and MPPs, compared to progenitors. These results indicated that the factor limiting HDR in HSCs is likely an intrinsic property of DNA repair pathway choice.

One of the characteristics that makes HSCs different from progenitors is their lack of cell cycle progression. Unlike progenitors, which are actively cycling, HSCs are normally quiescent *in vivo* (Uchida et al., 1997; Pietras et al., 2011). In culture, cytokine-stimulated HSCs enter cell cycle, but at a lower frequency than progenitors (Rossi et al., 2007). We observed that a two-day pre-stimulation with cytokines is required to increase the number of cells in S/G2 phases and to achieve detectable HDR levels (unpublished data). Interestingly, NHEJ levels were similar in HSC and progenitor sub-populations, and across different phases of cell cycle, supporting the observation that percentages of NHEJ events do not decrease in the long-term HSCs after transplant (Holt et al., 2010; Hoban et al., 2015). This observation can be explained by the fact that NHEJ can occur in any phase of cell cycle and is therefore not restricted in quiescent HSC sub-population.

The DNA repair pathways are temporally regulated, with NHEJ being active throughout the cell cycle, and HDR being confined to S/G2 phases. To take advantage of this temporal regulation, we took two approaches.

First, to decrease nuclease activity in G1 phase of cell cycle when HDR cannot occur, we produced a modified version of *Streptococcus pyogenes* (Sp) Cas9, termed hGemCas9. As expected, there were no differences in the levels of HDR, but we observed a significant decrease in NHEJ, thus resulting in a significant improvement in HDR/NHEJ ratio, both in vitro and in vivo. Additionally, the specificity of hGemCas9 was similar to that of wtCas9 in bulk unsorted cells. Interestingly, in G1 phase of cell cycle, hGemCas9 was more specific than wtCas9, suggesting that decreasing nuclease activity might result in more specificity. However, it will be necessary to compare the specificity of hGemCas9 vs wtCas9 at other loci to determine whether this observation is generally true.

In the second approach, we sought to increase the proportion of cells that are available for HDR. We used a selective inhibitor of CDK1, RO-3306, to temporarily synchronize cells in the S/G2 phases of cell cycle. Other groups have shown that synchronization of cells in the S/G2 phases increases HDR (Lin et al., 2014); however, this has not been previously tested in primary human HSPCs. We observed a >2-fold increase in the HDR/NHEJ ratio in the cells pre-treated with RO. Moreover, we observed an additive effect between hGemCas9 and cell synchronization (RO), resulting in ~4-fold improvement in the HDR/NHEJ ratio in vitro, and a significant improvement in vivo.

Although a CFU assay and a competitive mouse transplant experiment suggested that cell synchronization with RO does not affect the long-term viability and hematopoietic potential of HSCs, this drug resulted in significant initial toxicity and was only effective at increasing the S/G2 population by ~15%. Additionally, inhibition of CDK1 may be disadvantageous for improving HDR/NHEJ ratio due to its roles in DNA repair, such as phosphorylation of key proteins involved in HDR, including CtIP and MRN complex (Huertas and Jackson, 2009; Peterson et al., 2011;

2013; Hustedt and Durocher, 2016). Based on our data, replenishing phosphomimic CtIP by co-electroporation of CtIP* mRNA was not sufficient to significantly increase HDR levels. Therefore, to expand on encouraging results of improved HDR/NHEJ ratio in S/G2-synchronized cells, and in order for this strategy to be translatable to patients, it will be necessary to search for and test other synchronization reagents that do not counteract critical HDR factors and do not result in as much initial toxicity.

While the absolute levels of gene editing in NSG mice were still sub-optimal, the improvement in the HDR/NHEJ ratio relative to the control (unsync, wtCas9) provides an important proof-of-principle finding. It is essential to note that the amounts of gene editing reagents used here were chosen to provide non-plateau gene editing levels for comparative purposes, and the HDR levels may be further increased by optimizing the amounts, formulation, and delivery methods of Cas9, gRNA and donor template (Richardson et al., 2016). Several combinations for delivery of gene editing reagents are currently available, with the most popular being IVT mRNA Cas9 and guide RNA, or ribonucleoprotein (RNP) complex; and AAV6 or oligonucleotide for donor template. There is no clear ideal choice – each has its own advantages and drawbacks for the future use in patients. While in these studies we used mRNA+sgRNA+AAV6 delivery system for Cas9, guide and donor template, respectively, it may be of interest to test the effectiveness of the approaches described here utilizing other, potentially more effective, gene editing reagent delivery systems to achieve an increase in absolute HDR levels, or an even further improvement in the HDR/NHEJ ratio.

In summary, this work provides a proof-of-principle finding that HDR/NHEJ ratio can be improved in primary human HSPCs by controlling the Cas9 activity in cell cycle phase-specific manner and regulating cell cycle progression. In the future, the approaches described here can be tested with other gene editing reagent delivery systems and can be scaled up for clinical gene therapy applications.

3.7. CONCLUSION

The goal of this paper was to study the mechanistic reason for a decrease in targeted gene correction (HDR levels) in HSCs revealed after transplantation. We sought to influence the choice of DNA repair pathways to increase HDR and decrease gene disruption via NHEJ pathway, thus improving HDR/NHEJ ratio. Specifically, we focused on cell cycle phase-specific regulation of DNA repair pathways. We showed that temporal regulation of Cas9 nuclease activity and transient synchronization of primary human CD34⁺ cells in HDR-preferred phases resulted in a significant improvement in HDR/NHEJ ratio *in vitro*. Moreover, these manipulations resulted in a significant increase in the HDR/NHEJ ratio after transplant of gene-edited cells into immune-deficient mice.

3.8. ACKNOWLEDGEMENTS

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3.9. AUTHOR CONTRIBUTIONS

Conception and design: A.L., C.F.B., R.P.H., Z.R., D.B.K.; Financial support: A.L., R.P.H., Z.R., D.B.K.; Administrative support: A.L., R.P.H., Z.R., D.B.K.; Provision of study material or patients: R.P.H., Z.R., D.B.K.; Collection and/or assembly of data: A.L., D.N.C., B.C.F., M.A.D., J.E.C.; Data analysis and interpretation: A.L., D.N.C., S.F.G., M.L.K., X.W.; Manuscript writing: A.L., D.N.C., M.L.K., R.P.H., Z.R., D.B.K.; Final approval of manuscript: A.L., R.P.H., Z.R., D.B.K.

3.10. DECLARATION OF INTERESTS

The authors declare no competing financial interests.

3.11. MATERIALS AND METHODS

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Donald B. Kohn (DKohn@mednet.ucla.edu)

Experimental Model and Subject Details

Primary Human CD34⁺ Cells

Leukopaks from healthy donors were purchased from HemaCare (HemaCare BioResearch Products; Van Nuys, CA). Mobilized peripheral blood (mPB) was collected from normal, healthy donors on days 5 and 6 after 5 days of stimulation with granulocyte-colony stimulating factor (G-CSF). mPB was processed to enrich for CD34⁺ cells using magnetic beads and cryopreserved as described previously (Hoban et al., 2015).

Method Details

Cell Culture

Healthy human CD34⁺ cells from mPB (PBSCs) were thawed in pre-warmed X-VIVO 15 medium (Lonza; Basel, Switzerland) with PSQ (penicillin, streptomycin, glutamine, (Gemini Bio-Products; Sacramento, CA)), pelleted at 500g for 5 mins, and resuspended at 5x10⁵cells/mL in pre-warmed X-VIVO 15 medium with PSQ and SFT cytokines (50 ng/mL stem cell factor (SCF), 50 ng/mL fms-related tyrosine kinase 3 ligand (Elt3-L), and 50 ng/mL thrombopoietin (TPO)) (Peprotech; Rocky Hill, NJ). Cells were prestimulated at 37°C and 5% CO₂ incubator for 48 hours. Where indicated, 30uM RO (RO-3306, Sigma Aldrich; St. Louis, MO) was added to cell culture medium 28 hours after thawing. Cells were washed twice with PBS after 20 hours of RO treatment.

Electroporation

For electroporation, 2x10⁵ (or 1x10⁶ for mouse experiments) cells per condition were pelleted at 90g for 15 mins at RT, resuspended in 100ul of BTXpress Electroporation buffer (Harvard Bioscience, Inc; Holliston, MA), combined with pre-aliquoted mRNA (kept on ice), and

pulsed once at 250 V for 5 milliseconds in the BTX ECM 830 Square Wave Electroporator (Harvard Apparatus; Holliston, MA). Unless otherwise specified, 4ug of Cas9 mRNA and 4ug of sgRNA targeting the human beta-globin gene were used (See Fig 2A). GFP mRNA was electroporated at 0.3ug. After electroporation, cells were rested in cuvettes for 10 mins at RT, and then recovered with 400ul (or 2.4mL, for 1e6 cells) of X-VIVO 15 medium (with PSQ and SFT cytokines), containing AAV6 (multiplicity of infection, MOI=2e4) to introduce 4 SNPs (see Fig 2B for details) (UNC vector core; Chapel Hill, NC). The cells were cultured in a 24-well (or 6-well, for 1x10⁶ cells) plate at 37°C, 5% CO₂ incubator. 24 hours post electroporation, the cells were counted by hemocytometer using 1:2 dilution with trypan blue to determine viability (# of live cells/# of total cells x 100) and fold expansion (# of cells 24 hours after electroporation/# of cells before electroporation). Cells were replated into 1mL (or 5mL, for 1x10⁶ cells) of myeloid expansion medium (Iscove's Modified Dulbecco's Medium (IMDM, Thermo Fisher Scientific; Waltham, MA) + 20% FBS (HI FBS, Gibco/ThermoFisher; Waltham, MA) + 5ng/mL Interleukin 3 (IL3), 10ng/mL Interleukin 6 (IL6), 25ng/mL SCF (Peprotech; Rocky Hill, NJ)), and cultured for 4 days (unless otherwise specified) prior to harvesting for gDNA. Samples that were FACS-sorted into HSPC sub-populations were cultured 2-4 weeks post sort (as indicated in figure legends) to obtain enough cells for gDNA and subsequent high throughput sequencing (HTS) (electroporation of nuclease and donor template was more toxic than nuclease alone, therefore longer expansion time was required). gDNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen/ThermoFisher Scientific; Carlsbad, CA).

mRNA/sgRNA production

To make mRNA template, maxi-prepped expression plasmids were linearized with SpeI (NEB; Ipswich, MA), and purified using PCR purification kit according to manufacturer's protocol. *In vitro* transcription was carried out using mMessage T7 Ultra Kit (ThermoFisher Scientific; Waltham, MA). mRNA product was purified using the RNeasy MinElute Cleanup Kit (Qiagen; Valencia, CA) following the manufacturer's protocol.

sgRNA template was prepared as previously described (dx.doi.org/10.17504/protocols.io.hdrb256). RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen; Valencia, CA) following manufacturer's protocol.

hGemCas9

hGemCas9 was created by cloning a 110-amino acid sequence of human geminin domain to C-terminus of Cas9, based on the strategy used by Gutschner et al., 2016. Gene blocks were ordered from IDT to include homology arms for NEBuilder cloning, and cloned into SpCas9 plasmid (Bjurstrom et al., 2016; Hoban et al., 2016).

High-Throughput Sequencing (HTS)

Library prep

DNA library for HTS was prepared as described previously (Hoban et al., 2015). Briefly, an outer PCR was performed on genomic DNA to amplify a 1.1kb region of interest (using Outer PCR Fwd and Rev primers). A second PCR was performed to add a unique index to the PCR product of each sample to be sequenced (read1/read 2 and P5/P7 primers). The PCR products with the indexes were mixed at equal concentrations, which was determined by densitometry of the PCR products and analyzed by gel electrophoresis, to create a pooled library. The pooled library was purified twice using AMPure XP beads (Beckman Coulter Inc.; Brea, CA) and then quantified using ddPCR (QX 200; Bio-Rad Laboratories Inc.; Hercules, CA). High-throughput sequencing was performed at UCLA Technology Center for Genomics & Bioinformatics (TCGB) using MiSeq 2x150 paired-end reads (Illumina Inc; San Diego, CA) .

Sequencing Analysis and Calculations

Analysis of sequencing data was performed as in Hoban et al, 2015; 2016 (Hoban et al., 2015; 2016). Percentage of HDR was calculated as the (number of sequence reads containing a sickle change)/(total reads for that sample)*100. Percentage of NHEJ was calculated as the frequency of sequence reads containing an insertion or deletion -50/+36 bases around the nuclease cut site.

Flow Cytometry/FACS

All flow cytometry analysis and FACS were performed on the following instruments: BD LSRII, BD LSRFortessa, BD FACS Aria II, all with the similar 5-laser configurations: UV 355 nm, Violet 405 nm, Blue 488 nm, Yel-Grn 561 nm, Red 633 nm.

Analysis of HSC/MPP/Progenitor Populations

The cells were collected and washed with PBS+2%FBS, and stained for 30 minutes at 4°C with the following antibodies: CD34, CD38, CD45RA, CD90 (detailed antibody information in supplemental methods). Cells were washed twice with 2ml PBS+2%FBS, and resuspended in 200ul FBS+2%FBS for flow cytometry analysis, or 500ul FBS+2%FBS for FACS. Representative gating strategy is illustrated in Figure 3.12-1A. Cells were sorted into myeloid expansion medium + PSQ. After sort, the cells were spun at 300g for 15 mins, and replated into myeloid expansion medium + PSQ + cytokines (IL-3, IL-6, SCF).

Cell Cycle

For cell cycle flow cytometry analysis, 2×10^4 cells were collected after RO or DMSO treatment, fixed in 1mL ice-cold 70% EtOH and stored at -20°C for at least 2 hours (and up to 2 weeks). Fixed cells were washed twice with PBS+2%FBS and stained with 5ug/mL Hoechst 33342 (BD Biosciences; San Jose, CA) in PBS+2%FBS at a final volume of 250ul for at least 45 minutes.

For FACS-sorting of live cells, cells were cultured at 5×10^5 - 1×10^6 cells/mL and stained with 5ug/mL Hoechst 33342 for 45-60 mins at 37°C. Cells were washed with PBS + 2%FBS and resuspended at 5×10^6 cells/mL in PBS + 2%FBS + 5ug/mL Hoechst 33342.

Mouse Transplants

All research designs and procedures were reviewed by the UCLA institutional animal care and use committee (IACUC). Mouse work was performed according to the ARC protocol number 2008-167

Transplant

To evaluate the engraftment of gene-edited cells, and the levels of gene editing in long-term HSC population, the cells were transplanted into NSG mice (The Jackson Laboratory; Bar Harbor, ME). The cells were treated and electroporated as described earlier using 1×10^6 -cell electroporation protocol. For RO-treated cells, 3 μ g CtIP* was co-electroporated with gene editing reagents. 24 hours after electroporation, the cells were washed with PBS and counted. 5×10^4 cells were kept in myeloid culture for evaluating gene editing levels prior to transplant. The remaining cells were resuspended in PBS + 1% OKT3 (Biolegend; San Diego, CA) at 1.25×10^6 cells per 125 μ l. Cells were transplanted retro-orbitally into mice 3-5 hours after 250-cGy total body irradiation. The number of mice to transplant per arm was determined to give sufficient power to indicate statistically meaningful differences.

Harvest and Analysis

Retro-orbital bleeding was performed at 8 weeks post-transplant to determine the levels of human engraftment. Red blood cells were lysed using the lysing buffer (BD Biosciences, San Jose, CA). The levels of human engraftment were determined by flow cytometry (detailed flow panels in supplementary methods), and calculated as $(hCD45^+ / (hCD45^+ + mCD45^+)) \times 100$. Mice were euthanized 16 weeks post-transplant. Two femurs were collected and flushed with MACS buffer. Spleens were squeezed through a 70 μ m filter. Red blood cells were lysed using the lysing buffer (BD Biosciences, San Jose, CA). The levels of human engraftment were determined by flow cytometry. The human cells from mouse BM were isolated using hCD45+ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), gDNA was extracted, and the levels of gene editing in human cells were determined by HTS, as described earlier. Mouse harvest, flow cytometry and sequencing analyses were performed in a blinded manner.

Quantification and Statistical Analysis

For quantitative measurements related to HSPC sub-populations, gene editing levels, evaluation of hGemCas9 nuclease, engraftment, gene editing and lineage analysis in NSG mice, descriptive statistics such as number of donors/experiments/animals, mean, standard deviation

were reported and presented in figures. A two-step approach was adopted to test difference among multiple experimental groups/conditions. In the first step, a non-parametric Kruskal-Wallis test was used to evaluate if there was an overall difference. If result from the first step was significant, then unpaired Wilcoxon rank sum test was performed to identify which two groups were different. If normality assumption was met and the sample size was too small, unpaired t test was used instead. Chi-square test was used to assess if the distributions of cells are the same between experimental groups. For all statistical investigations, tests for significance were two-tailed. A p-value less than the 0.05 significance level was considered to be statistically significant. All statistical analyses were carried out using SAS version 9.4 (SAS Institute Inc. 2013).

3.12. SUPPLEMENTAL INFORMATION

3.12.1. Supplemental Figures

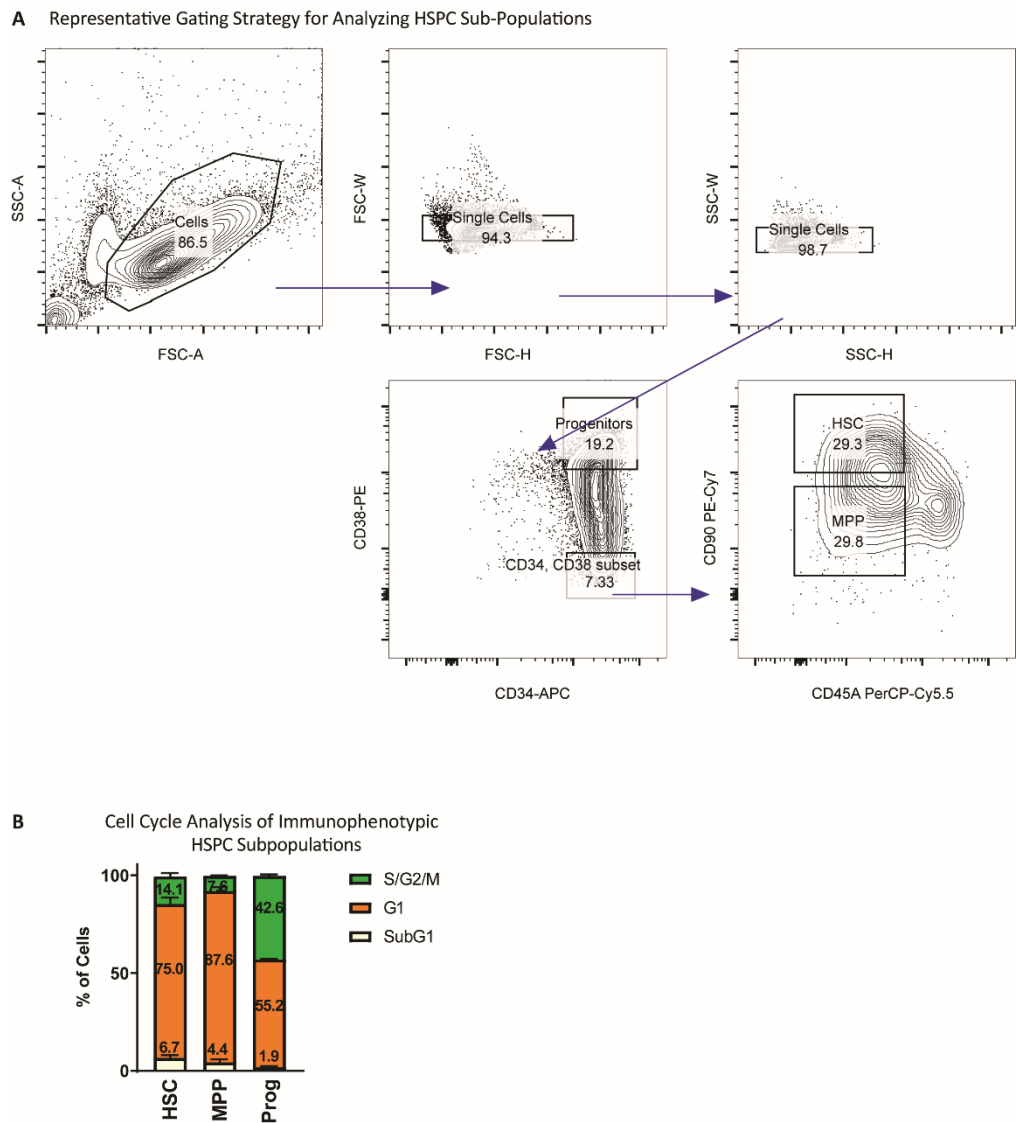


Figure 3.12-1: Analysis of HSPC subpopulations (Related to Figure 3.5-1)

A. Representative flow cytometry gating strategy for categorizing HSCs, MPPs and progenitors. Live cells were gated based on forward and side scatter, and single cells were selected based on height and width parameters of forward and side scatters. Then, progenitor population was selected as the top 20% of CD38 expression of CD34+ cells, and CD34 + 38- population was selected as the bottom 5%–10%. The CD34 + 38- population was further subgated to identify HSCs (top 30% of CD90 expression of CD45RA- cells) and MPPs (bottom 30% of CD90 expression of CD45RA- cells).

B. Cell cycle analysis of HSPC subpopulations after two days of prestimulation. N = 2. Numbers indicate means.

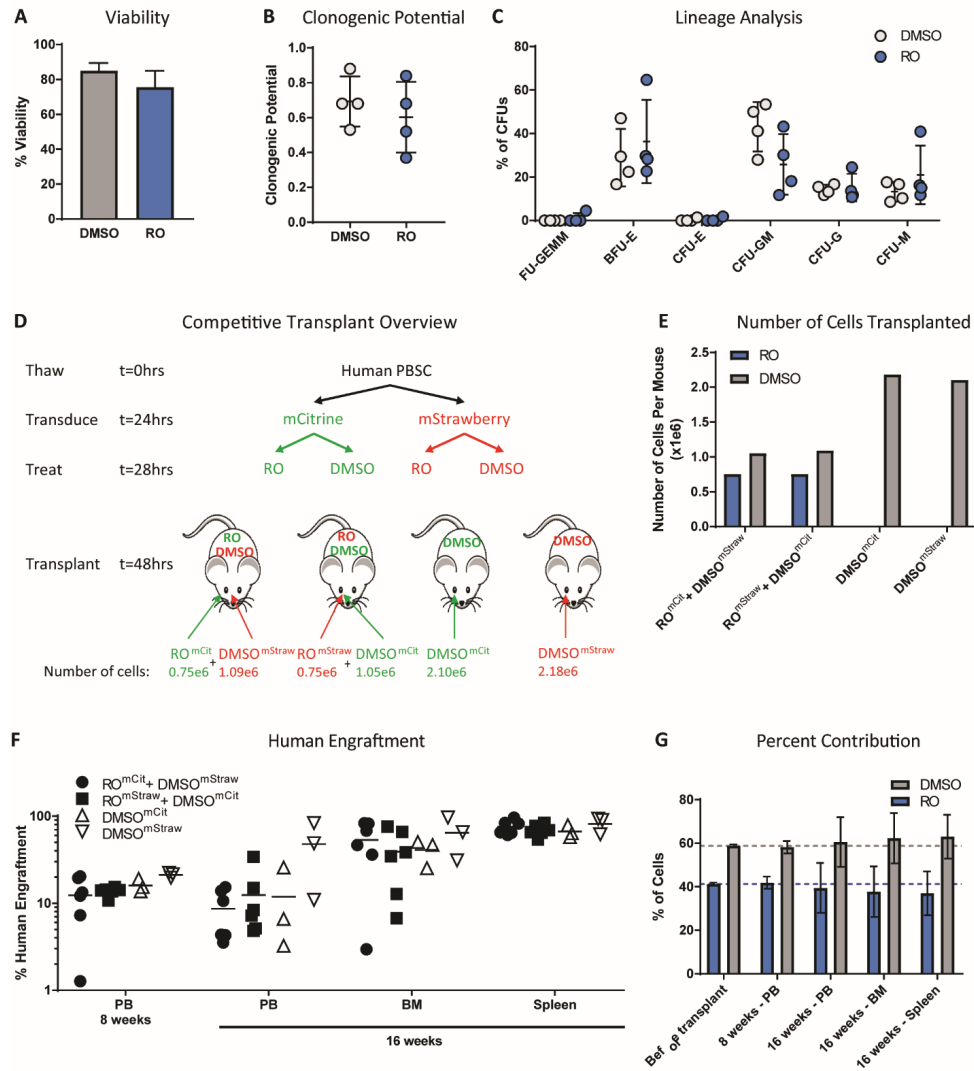


Figure 3.12-3: Synchronization of Human HSPCs with RO (Related to Figure 3.5-5)

A. Viability of cells after 20 hours of treatment with DMSO or RO. N = 6. Data are represented as bars $\pm$ SD. Differences are not significant, based on Wilcoxon Rank Sum test.

B. Clonogenic Potential of PBSCs, pre-treated with DMSO or RO, measured 14 days after plating colonies in methylcellulose medium. N = 4. Individual points plotted, line – mean $\pm$ SD. Differences are not significant, based on Wilcoxon Rank Sum test.

C. Lineage analysis of CFUs in Figure 3.12-3B. CFUs were counted and scored as the following: CFU-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM), burst forming unit-erythroid (BFU-E), CFU-erythroid (CFU-E), CFU-granulocyte/macrophage (CFU-GM), CFU-granulocyte (CFU-G), and CFU-macrophage (CFU-M). N = 4. Individual points plotted, line – mean $\pm$ SD. Differences are not significant, based on Wilcoxon Rank Sum test.

D. Schema of competitive NSG mouse transplant of RO- and DMSO-treated cells. To evaluate whether RO-pretreated cells retain their hematopoietic potential, a competitive transplant was used. Human mobilized PBSCs were pre-stimulated for 24 hours and split equally into two populations, which were transduced with fluorescent vectors (mCitrine or mStrawberry). Four hours later, RO or DMSO was added to the cells. After 20 hours of treatment, the cells were washed with PBS, counted, and RO- and DMSO-treated cells of opposite colors were mixed in equal proportions of the starting number of cells, and transplanted into mice. Numbers on the bottom indicated the number of cells transplanted (also plotted in Figure 3.12-3E). Colors were alternated to account for any potential differences in transduction efficiencies of mCitrine and mStrawberry vectors. DMSO-only mice were used as controls. Mice were harvested 16 weeks post-transplant, and the levels of human engraftment, as well as the percentage of fluorescently transduced cells were evaluated to determine the percent contribution of RO and DMSO treated cells to human engraftment.

E. Numbers of RO- or DMSO-treated cells, competitively transplanted into NSG mice.

F. Levels of human engraftment in NSG mice, 8 or 16 weeks after transplant with RO or DMSO treated cells. PB – peripheral blood, BM – bone marrow. N = 6 for RO + DMSO groups, N = 3 for DMSO-only groups. Individual points plotted. Data are represented as geo mean $\pm$ SD. Differences are not significant, based on Kruskal-Wallis test.

G. Percentage of DMSO- and RO-treated cells, contributing to all human cells in NSG mice, measured before, during (8 weeks) and after transplant (16 weeks). N = 6. Data are represented as mean $\pm$ SD. Horizontal dashed lines match the starting percentage of cells. Differences compared to the starting percentages are not significant, based on chi-square test.

A Summary of Highest Frequency Indels

-----PAM[><-----]	indel size	Unsync, wtCas9	RO, wtCas9	Unsync, hGemCas9	RO, hGemCas9
GAAGTCTGCCGTTACTGCCCTGTGGGGCAAG					
GAAGTCTGCC-----CTGTGGGGCAAG	-9	27.4 ± 3.8	22 ± 8	28.9 ± 1.6	20.5 ± 6.3
GAAGTCTGCCGTA TACTGCCCTGTGGGGCAAG	+1	8.3 ± 0.8	9.4 ± 4.3	7.5 ± 1.3	8.5 ± 3
GAAGTCTGCCGTTA--GCCCTGTGGGGCAAG	-2	5 ± 0.9	5.7 ± 4.6	5.2 ± 0.8	5.8 ± 3.4
GAAGTCT-----TGCCCTGTGGGGCAAG	-8	4.1 ± 1.1	3.4 ± 1.7	4.3 ± 1	1.7 ± 1.4
GAAGTCTGCCGTTCACTGCCCTGTGGGGCAAG	+1	3.7 ± 0.7	4 ± 1.8	4 ± 0.9	5.1 ± 4.3
GAAGTCTGCCGTTA-TGCCCTGTGGGGCAAG	-1	3.3 ± 1	3.7 ± 2.5	3 ± 0.4	4 ± 3
GAAGTCTGCCGTTTACTGCCCTGTGGGGCAAG	+1	3.2 ± 0.7	4 ± 2.9	3 ± 0.6	2.8 ± 2.1
GAAGTCTGCCGTT-CTGCCCTGTGGGGCAAG	-1	2.6 ± 0.8	3.7 ± 3.3	2.3 ± 0.9	4.1 ± 4.4
GAAGTCTGCCGTTGACTGCCCTGTGGGGCAAG	+1	2.5 ± 0.7	2.5 ± 1.5	2.5 ± 0.6	4.4 ± 2.6
GAAGTCTGCCGTTA----CTGTGGGGCAAG	-5	1.9 ± 0.3	2.2 ± 0.9	1.9 ± 0.6	1.4 ± 1.1
GAAGTCTGCCGtATTACTGCCCTGTGGGGCAAG	+2	1.8 ± 0.3	2.7 ± 2.1	1.9 ± 0.4	1.6 ± 2.9
GAAGTCTGCCGTTA-----GTGGGGCAAG	-7	1.8 ± 0.6	2.4 ± 2.2	1.3 ± 0.2	1.2 ± 1.4
GAAGTCTGCCGTTCTACTGCCCTGTGGGGCAAG	+2	1.2 ± 0.3	1.5 ± 0.7	1.3 ± 0.4	2.1 ± 1.5
GAAGTCTGCCGTTCCACTGCCCTGTGGGGCAAG	+2	1 ± 0.3	1 ± 1.1	1.2 ± 0.5	1.5 ± 1.7
GAAGTCTGCCGTTA-----GCAAG	-12	0.9 ± 0.3	1.1 ± 0.9	0.8 ± 0.2	1.1 ± 1.2

B Microhomology Around the Cut Site

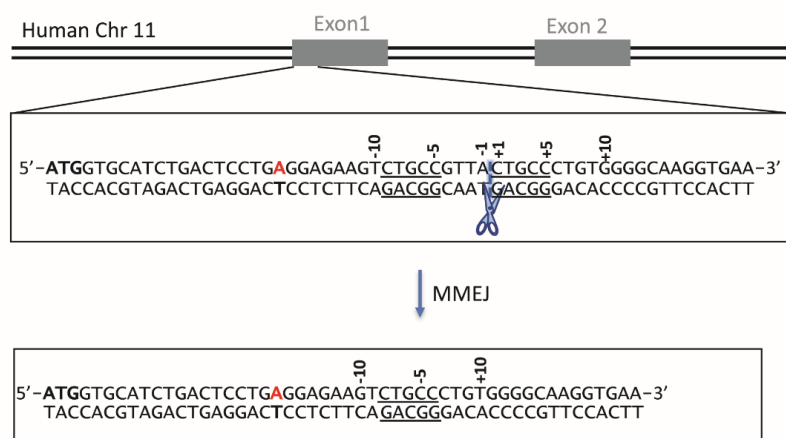


Figure 3.12-4: Indel Spectrum Analysis (Related to Figure 3.5-6)

A. Indel spectrum of samples in Figure 3.5-6E). The top 15 indels are listed. "><" and the dashed blue line indicate nuclease cut site. For wtCas9 – N = 10, for hGemCas9 – N = 8. Numbers in the table are % indels ± SD. % indel was measured by HTS and calculated as (# of sequence reads containing an indel)/(total # of sequence reads) x 100.

B. A map of beta globin locus illustrating the Cas9 cut site (dashed blue line), and the microhomology region around the cut site (underlined). Red "A" represents the site of the sickle mutation. Numbers indicate the upstream (-) and downstream (+) positions from the cut site. The bottom panel represents DNA sequence after a 9-bp deletion.

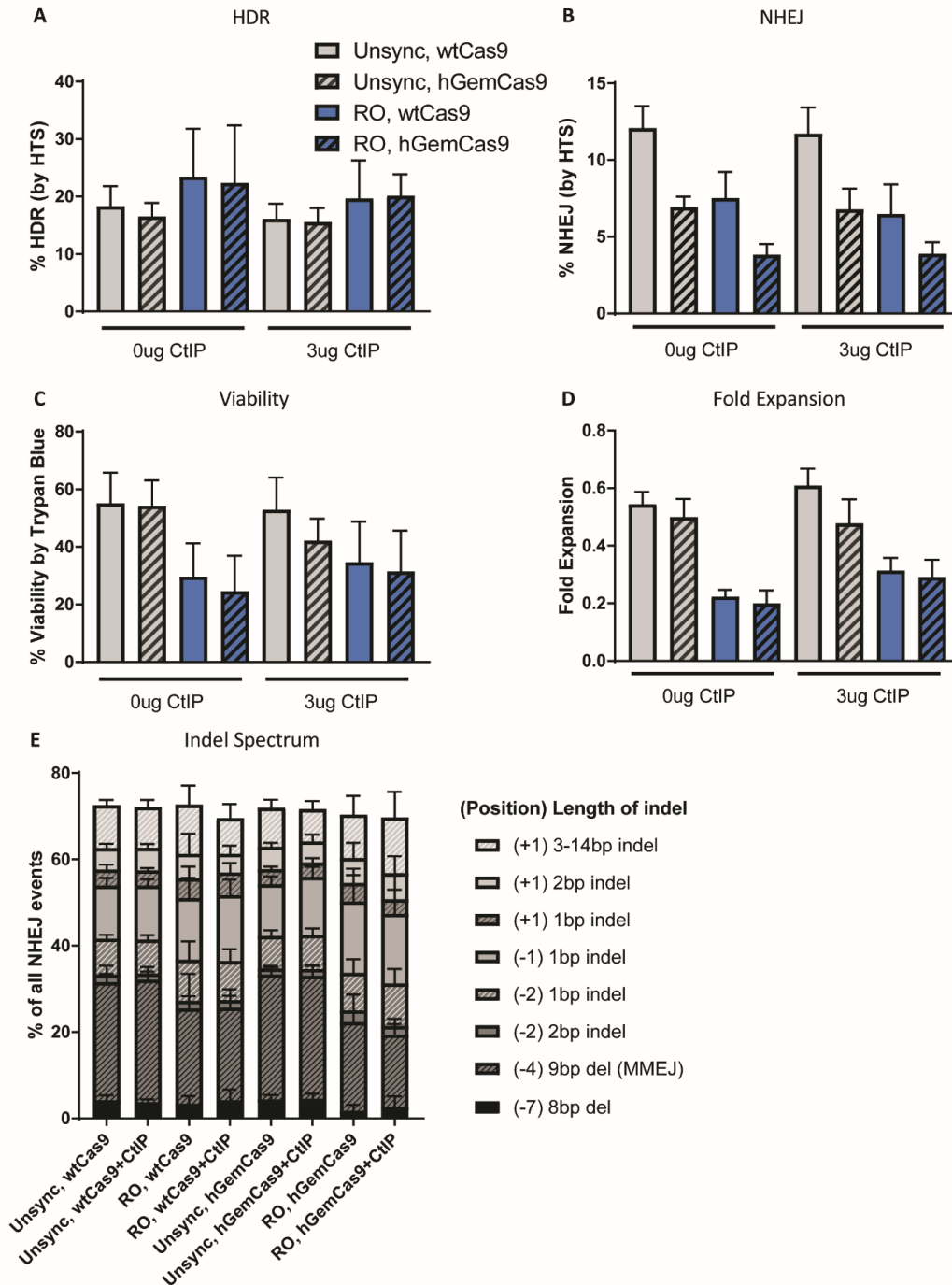


Figure 3.12-5: Evaluating the Effects of CtIP* mRNA on Gene Editing (Related to Figure 3.5-6)

A-B. Levels of gene editing (HDR and NHEJ) in PBSCs pre-treated with RO or DMSO, and electroporated with wtCas9 or hGemCas9 +/- CtIP* IVT mRNA, and subsequently transduced with AAV6 donor template. For wtCas9 – N = 10, for hGemCas9 – N = 8. Data are represented as mean $\pm$ SD. Differences between 0ug and 3ug CtIP* are not significant based on Wilcoxon Rank-Sum test.

C-D. Viability and fold expansion, measured 24 hours post electroporation in Figure 3.12-5B. Data are represented as mean $\pm$ SD. Differences between 0ug and 3ug CtIP* are not significant based on Wilcoxon Rank-Sum test.

E. Indel spectrum, normalized to %NHEJ for each sample. Numbers in parentheses indicate position of an indel relative to cut site, numbers following parentheses indicate the length of an indel, del – deletion, indel – insertion or deletion. See also Figure 3.12-4. For wtCas9 – N = 10, for hGemCas9 – N = 8. Data are represented as mean $\pm$ SD. Differences in indels between +/- CtIP* are not significant based on Kruskal-Wallis test.

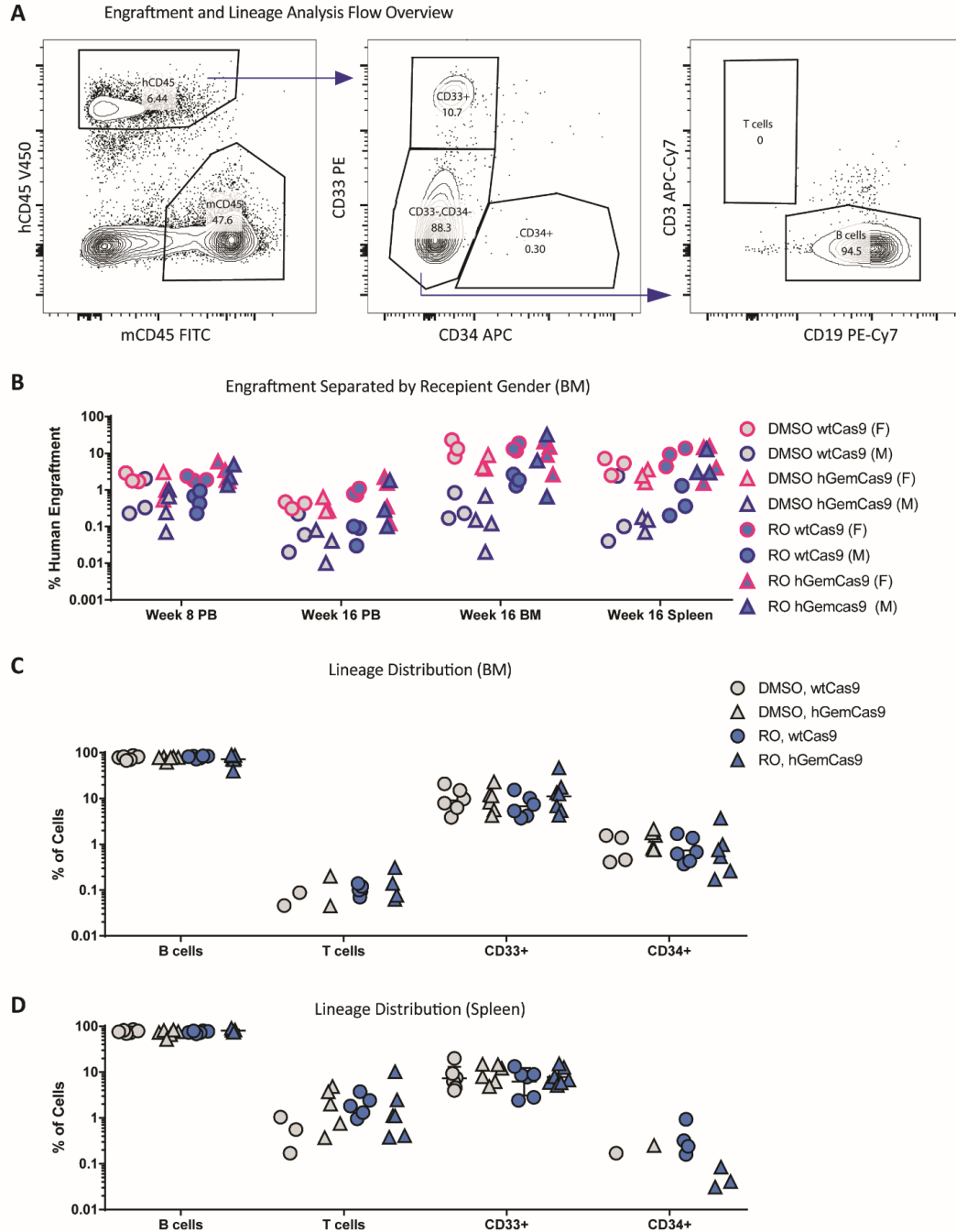


Figure 3.12-6: Engraftment and Lineage Analysis in NSG Mice (Related to Figure 3.5-7)

A. Representative flow cytometry analysis to quantify the levels of human engraftment and lineage distribution in NSG mice. hCD45⁺ cells were gated as CD33⁺ (myeloid), CD34⁺ (hematopoietic), or CD33⁻/CD34⁻, which were further subdivided into CD19⁺/CD3⁻ (B cells) and CD19⁻/CD3⁺ (T cells).

B. Levels of human engraftment in NSG mice (from Figure 3.5-6C), separated by recipient gender. N = 3–4 female mice +3–4 male mice. Individual points are plotted.

C-D. Lineage analysis of engrafted cells in BM (B) and spleen (C) of NSG mice. Analysis was performed by flow cytometry and analyzed using FlowJo software as illustrated in Figure 3.12-6A. N = 6–7 (3–4 female mice +3–4 male mice). Individual points are plotted. Line – geo mean $\pm$ geo SD. Zero values were not plotted on a log scale, but were used in calculations or SD. Differences are not significant, based on Kruskal-Wallis test.

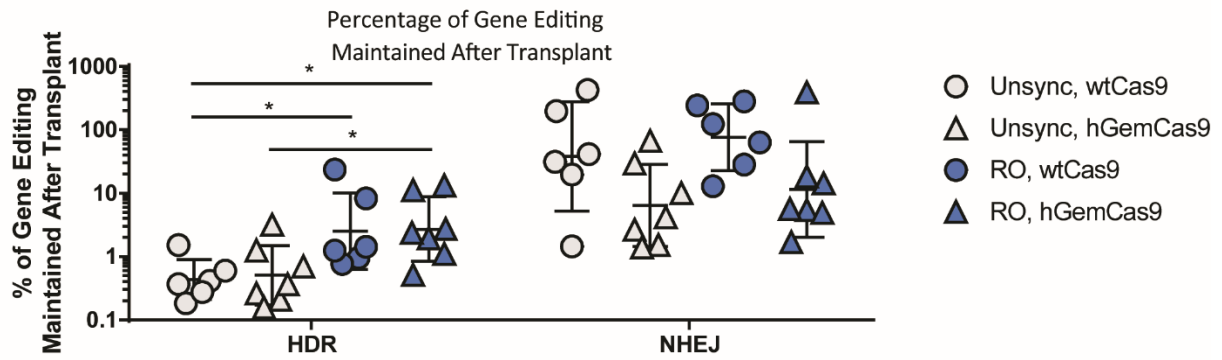


Figure 3.12-7: Gene Editing in NSG mice (Related to Figure 3.5-7D)

A. Percentage of gene editing levels maintained after transplant, calculated as (HDR or NHEJ after transplant)/(HDR or NHEJ before transplant)×100. N = 6–7 (3–4 female mice +3–4 male mice). Individual points are plotted. Line – geo mean ± geo SD. Differences are not significant if not specified, * P < .05, based on Wilcoxon Rank-Sum test.

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Chapter 4. The Effects of Manipulating DNA Repair Factors on CRISPR/Cas9-Mediated Gene Editing Outcomes in Human Hematopoietic Stem and Progenitor Cells

4.1. ABSTRACT

Gene editing of human hematopoietic stem cells (HSCs) is a promising strategy for the treatment of monogenic diseases of the blood through site-specific correction of identified causal mutations by targeted nucleases. However, the precise correction of CRISPR/Cas9-induced double stranded breaks (DSBs) via homology-directed repair (HDR) pathway remains less prevalent than the error-prone non-homologous end joining (NHEJ) pathway in HSCs. Our translational goal is to improve the frequency of HDR outcomes relative to NHEJ, which will be beneficial for treating diseases such as sickle cell disease (SCD). Therefore, it is critical to understand what governs the cellular DNA repair pathway choice, and how it can be manipulated to shift the balance toward HDR from NHEJ.

Cellular regulation of repair pathway choice depends on a complex interaction of DNA repair proteins. Previous studies have identified several factors that may influence the repair outcomes in cell lines. However, the effects of these factors have not been evaluated in disease-relevant models, such as primary human HSCs, in which it is especially challenging to achieve therapeutic levels of HDR. We tested the effects of manipulating the expression levels of several DNA repair factors that are presumed to be important for pathway choice (such as C-terminal-binding protein interacting protein (CtIP), partner and localizer of BRCA2 (PALB2), RAD52, and p53-binding protein 1 (53BP1)) on HDR and NHEJ levels in K562 cells and primary human hematopoietic stem and progenitor cells (HSPCs). We tested different methods for DNA repair factor overexpression (plasmid, lentiviral vector (LV), integrase-defective lentiviral vector (IDLV) and mRNA) at various time points, relative to delivery of CRISPR/Cas9 and donor template.

We achieved up to 3-fold increase in HDR and NHEJ levels from co-electroporating DNA RF plasmids with Cas9 plasmid in K562 cells. However, further validation experiments showed that the increase in editing levels was not due to DNA RFs but solely a response to plasmid co-electroporation, as the same increase was observed with GFP control plasmid. Moreover, no statistically significant changes on gene editing levels were observed when Cas9 was delivered as RNP, or DNA RF delivered as LV or IDLV. Interestingly, the effects of plasmid on increasing gene editing levels were not reproducible in HSPCs. Furthermore, none of the DNA RF combinations showed any statistically significant improvements on gene editing outcomes in HSPCs, regardless of their delivery method.

4.2. INTRODUCTION

Nuclease-mediated gene editing relies on precise site-specific gene disruption and gene correction. Once the double stranded break (DSB) is introduced by targeted nucleases, endogenous cell repair mechanisms are employed. The two main pathways to repair the break are non-homologous end joining (NHEJ), an imprecise pathway, which can result in insertions and deletions (indels), or accurate homology-directed repair (HDR) pathway, which uses a donor template to seamlessly repair the break. To introduce specific sequence changes to the gene, a DNA donor template with homology arms can be designed to have single nucleotide polymorphisms (SNPs) to deliver those mutations via HDR pathway.

The frequency of the two repair pathways is not equivalent, NHEJ is much more prevalent in the cells than HDR (Chiruvella et al., 2013). For certain diseases, where a knockout of a gene can result in therapeutic benefit, repair by NHEJ pathway may be favorable (Holt et al., 2010; Bauer et al., 2013; Wang et al., 2015; Bjurstrom et al., 2016; Chang et al., 2017). However, for diseases such as sickle cell disease (SCD), where disruption of a gene can result in an even more severe phenotype than the original disease, repair via HDR pathway is critical.

Here, we modeled the correction of SCD-causing mutation in primary human hematopoietic stem and progenitor cells (HSPCs), which are often referred to as CD34⁺ cells based on the expression of the marker on their surface. Human hematopoietic stem cells (HSCs) are a small subset of CD34⁺ cells, which have a unique property to engraft in a human or humanized mouse model, as well as self-renew and differentiate into all blood cell types (Doulatov et al., 2012). Therefore, correcting disease-causing mutations in HSCs is clinically-appealing, as the modified cells can engraft in a patient and provide a life-long population of corrected cells. Yet, achieving therapeutically-relevant levels of precise editing in HSCs is extremely challenging due to low abundance of these cells and their quiescent state. Furthermore, evaluating gene editing levels in the HSC population is complicated by the low HSC proportion and masking by the progenitor population. Therefore, additional assays are required, such as fluorescent-activated cell sorting (FACS) of the HSC population based on the immunophenotypic markers, or performing mouse engraftment studies, to determine gene editing levels in the long-term repopulating HSCs. While the initial experiments in this study were carried out in the bulk CD34⁺ cells, further experiments will need to be performed to evaluate gene editing levels in the HSC sub-population.

As previously discussed in Chapter 3, there is a temporal control of DNA repair, with NHEJ being active in all phases of cell cycle but HDR being confined to S/G2 phases (Branzei and Foiani, 2008; Pietras et al., 2011; Lomova et al., 2019). This temporal separation could suggest why clinically-sufficient levels of HDR are difficult to be achieved in HSCs, which are mainly in G1 phase of cell cycle. Therefore, it is critical to understand what governs the temporal regulation of the pathway choice, and how it can be manipulated to initiate HDR in G1 phase of cell cycle.

Once the DSB is introduced, the expression of and interaction among DNA repair proteins determine the outcomes of repair (**Figure 4.2-1A**). Protection of broken ends by the Ku heterodimeric complex (Ku70/Ku80) triggers a cascade of events, leading to insertions and deletions (indels) via canonical non-homologous end joining (NHEJ) pathway (Shrivastav et al.,

2008) (**Figure 4.2-1A**, left). Alternatively, the recruitment of Mre11-Rad50-Nbs1 (MRN) complex allows for translocation of the C-terminal-binding protein interacting protein (CtIP) to the DSB. When phosphorylated, CtIP acts as an endonuclease and initiates end resection, which is further continued by Exo1 nuclease (Sartori et al., 2007; Polato et al., 2014; Anand et al., 2016; Symington, 2016). Resected single stranded DNA (ssDNA) ends are protected by replication protein A (RPA). In the presence of a double-stranded donor template, Breast Cancer type 1 and type 2 susceptibility proteins (BRCA1 and BRCA2, respectively) and the Partner and Localizer of BRCA2 (PALB2) form the BRCA1/PALB2/BRCA2 complex stimulating the loading of RAD51 recombinase onto ssDNA ends and causing strand invasion (Heyer et al., 2010; Symington and Gautier, 2011), which ultimately results in gene correction by the homologous recombination (HR) pathway (Jasin and Rothstein, 2013). The same correction outcome can also be achieved by a RAD51-independent, and RAD52-dependent pathway, termed single stranded template repair pathway (SSTR), which uses a single-stranded donor template (Richardson et al., 2018). HR and SSTR are collectively referred to as homology-directed repair (HDR) (**Figure 4.2-1A**, right).

Another outcome that is possible after end resection is microhomology-mediated end joining (MMEJ) repair (not shown), which can occur if there are short regions of homology around the DSB, and the free ends can be brought together, resulting in deletion (Huertas, 2010). While targeted integration via NHEJ- or MMEJ-mediated pathways has recently been described (Nakade et al., 2014; 2015), the effectiveness of these indel-forming pathways to achieve seamless correction of disease-causing mutations remains uncertain. Therefore, currently, HDR remains the only validated pathway to achieve gene correction, and the ratio of HDR to NHEJ and MMEJ needs to be increased.

Improving gene editing outcomes can be achieved by increasing HDR levels, decreasing NHEJ levels, or both simultaneously, thus improving HDR/NHEJ ratio. In the last several years, there have been many efforts to manipulate DNA repair outcomes by either inhibiting or stimulating certain proteins responsible for specific pathways using small molecules, or mutating

DNA repair factors to skew the editing outcomes. To decrease NHEJ levels, inhibition of Ku70/Ku80 with resveratrol (Li et al., 2017), inhibition of the DNA-dependent protein kinase (DNA-PK) catalytic subunit with NU7441 or KU-0060648 (Robert et al., 2015), or inhibition of Ligase IV with SCR7 (Srivastava et al., 2012; Chu et al., 2015; Maruyama et al., 2015) have been tested. However, Ku70/Ku80 heterodimers are extremely abundant in the cells and their complete inhibition is physiologically implausible for therapeutic purposes. DNA-PK and Ligase IV act downstream of repair pathway commitment, hence, inhibition of these proteins might result in cell death as the cells are unable to switch to alternative pathways. Therefore, these proteins might not be the ideal targets for primary cells, where cell survival is critical. In addition, we and several other groups have not been able to replicate the improvements in HDR levels with SCR7 in our experimental systems (Pinder et al., 2015; Robert et al., 2015; Gutschner et al., 2016; Yang et al., 2016). Another study combined eight small molecules, termed CRISPY mix, which is comprised of inhibitors of Ku70/80, DNA-PK, DNA ligase IV, RAD51 or RAD52, and enhancers of CtIP, RAD51, or the Ataxia-telangiectasia mutated (ATM) (Riesenberg and Maricic, 2018). Although some combinations of inhibitors and enhancers improved targeted nucleotide substitutions in certain cell types, none of the factors were able to universally apply gene modification benefits to all the cell types tested, emphasizing the difference between the editing pathways in different cells. Of note, the effects of these manipulations on primary human HSPCs were not evaluated in the above-mentioned studies.

In addition to using small molecules, activating mutation of genes responsible for DNA repair pathway choice in human cell lines have also been tested. As mentioned earlier, the BRCA1-PALB2-BRCA2 interaction is critical for stimulating the loading of RAD51 to the DSB and causing strand invasion with the donor template. PALB2-BRCA2 interaction occurs in any phase of cell cycle, however BRCA1-PALB2 interaction is specific to S/G2 phases (Orthwein et al., 2015). Additionally, p53-binding protein 1 (53BP1) binds to sites on histone 2A (H2A) at DSB and serves as a recognition signal for damaged chromatin to repair by the NHEJ pathway (Fradet-

Turcotte et al., 2013; Cuella-Martin et al., 2016). 53BP1 prevents the interaction between BRCA1 and CtIP in G1, and thus inhibits end resection by CtIP (Panier and Boulton, 2014).

In human cell lines, it has been shown that a constitutively active phosphomimic form of CtIP (CtIP T847E, from here on referred to as CtIP^E), and a PALB2 with mutations in BRCA1 binding pocket (PALB2^{KR}), which allow for BRCA1-PALB2 interaction irrespective of cell cycle, along with 53BP1 inhibition (i53) can stimulate HDR in G1 phase of cell cycle (Orthwein et al., 2015). Another phosphomimic CtIP variant with an additional amino acid mutation at the BRCA1 binding site (CtIP S249D T847E, denoted as CtIP^{DE}) has been shown to increase BRCA1 recruitment to DSBs (Orthwein et al., 2015). Coupled with end-resection activation, CtIP^E or CtIP^{DE} overexpression may allow for initiation of HDR in G1 (**Figure 4.2-1B**).

Additionally, it has been shown that a truncated fragment of 53BP1 containing an identical tandem tudor domain acts as a dominant negative form of 53BP1 (dn53BP1), and competitively antagonizes 53BP1 at DSB (Paulsen et al., 2017), thus decreasing NHEJ. Coupled with ectopic expression of RAD52, dn53BP1 has been shown to improve HDR by SSTR pathway (Paulsen et al., 2017) (**Figure 4.2-1C**). However, the effects of these manipulations on primary human HSPCs have not been reported previously.

Here, we evaluated whether CtIP^E, CtIP^{DE}, PALB2^{KR}, i53, RAD52 and dn53BP1 (from here on, collectively referred to as DNA repair factors, or DNA RFs) can improve gene editing outcomes in HSPCs by increasing HDR and decreasing NHEJ levels. Additionally, we tested whether these factors can be used to stimulate HDR in G1 phase of cell cycle, which could ultimately improve gene editing outcomes in long-term repopulating HSCs.

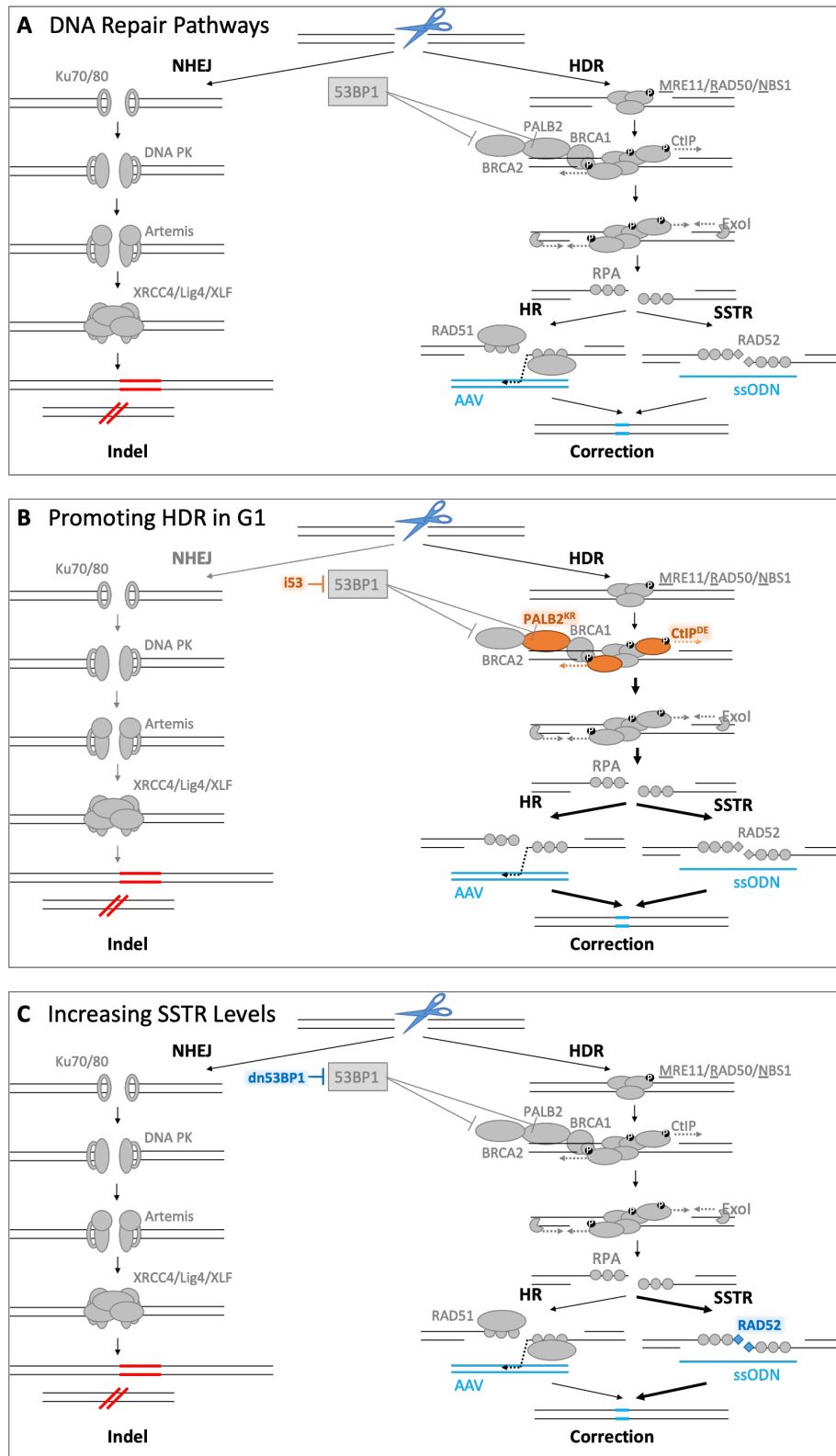


Figure 4.2-1: Diagram of DNA Repair Pathways.

(A) Overview of NHEJ (left), HDR (right) pathways and the important repair proteins. (B, C) DNA repair factors that were tested to improve HDR in G1 or to increase SSTR pathways, respectively.

4.3. RESULTS

4.3.1. Overexpression of DNA Repair Factors (RFs) in K562 Cells

To stably overexpress DNA RFs, sequences were cloned into pCCL-MNDU3 lentiviral backbone plasmids (**Figure 4.3-1A**), and packaged into lentiviral vectors (LVs). To confirm overexpression, we transduced K562 cells with LVs at multiplicity of infection (MOI) 1, and performed western blot analysis of protein expression (**Figure 4.3-1B**). The results showed basal expression of these proteins in mock (untransduced samples), and confirmed protein overexpression in LV-transduced samples. Western blots for i53 and dn53BP1 were not performed due to unavailability of antibodies for these inhibitors.

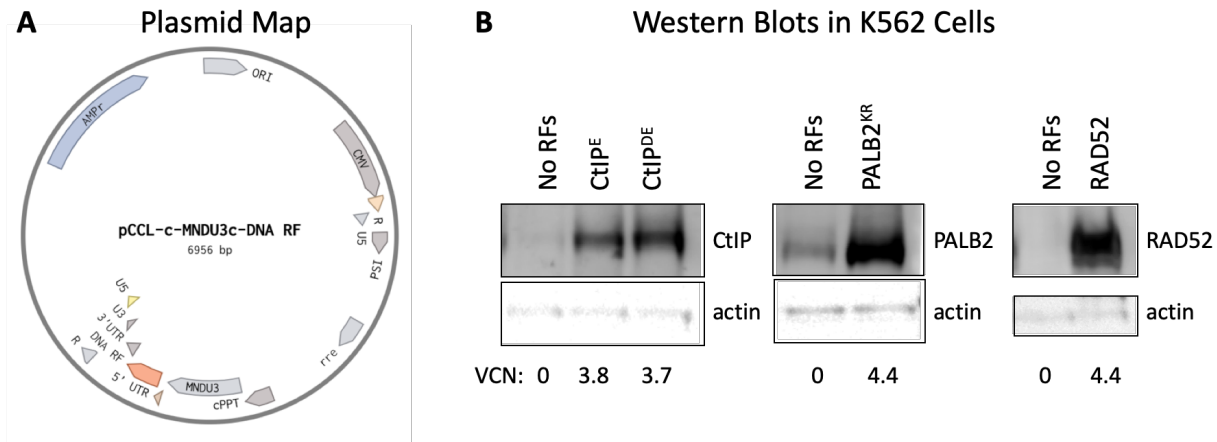


Figure 4.3-1: DNA Repair Factors Can Be Successfully Overexpressed in K562 cells.

(A) Representative plasmid map of DNA RFs that were electroporated into K562 cells and also used to package LV. (B) K562 cells were transduced with LVs expressing the indicated DNA RFs. Western Blot was performed on day 10 post transduction. Vector copy number (VCN) was determined by droplet digital PCR (ddPCR).

4.3.2. Evaluating the Effects of DNA RFs on Gene Editing Levels in K562 Cells

To evaluate the effects of DNA RFs on HDR and NHEJ levels, we began by overexpressing these factors via plasmid electroporation in K562 cells (see **Table 4.3-1** for a list of DNA RFs tested). 1ug of DNA RF plasmids were co-electroporated with gene editing reagents either individually, or in combinations (see **Figure 4.3-2A** for experimental timeline). Cas9 and guide RNA (gRNA) were delivered either as plasmid or ribonucleoprotein (RNP), and donor

template was delivered either as a single-stranded oligodeoxynucleotide (ssODN), or adeno-associated virus (AAV) (as in Lomova et al., 2019 and Romero et al., 2019). Percentage of gene correction (HDR) was measured by qPCR. When Cas9 was delivered as a plasmid, CtIP^{DE}, PALB2^{KR} and i53 did not have any effects on HDR levels when delivered individually. However, when CtIP^{DE}, PALB2^{KR} and i53 were used in combination, HDR levels increased, reaching a 3-fold improvement compared to “No RFs” condition. Similar improvement in HDR levels was observed when RAD52 and dn53BP1 plasmids were co-electroporated with Cas9 plasmid, albeit with more variability between replicates (**Figure 4.3-2B**, left).

Table 4.3-1: List of DNA Repair Factors Tested

DNA RFs:	Role:
CtIP ^E (CtIP T847E)	T847E mutant acts as a phosphomimic (Orthwein et al., 2015)
CtIP ^{DE} (CtIP S249D T847E)	S249D mutant increases BRCA2 recruitment to DSB (Orthwein et al., 2015)
PALB2 ^{KR}	K→R mutations in the BRCA1 binding pocket allow for PALB2-BRCA1 binding irrespective of cell cycle (Orthwein et al., 2015)
i53	Inhibitor of 53BP1; decreases NHEJ (Canny et al., 2018)
RAD52	Improves SSTR (Paulsen et al., 2017)
dn53BP1	Dominant negative form of 53BP1; decreases NHEJ (Paulsen et al., 2017)

Next, we tested co-delivery of DNA RFs with Cas9 RNP (**Figure 4.3-2B**, right). However, we did not see any statistically significant improvement in HDR levels with CtIP^{DE} + PALB2^{KR} + i53 with either of the donor templates. No improvements in HDR levels were detected in the context of ssODN donor when 53BP1 and RAD52 were used in combination. Although not statistically significant, a slight improvement (1.5-fold) in HDR levels in the context of AAV donor was observed with RAD52+dn53BP1. We hypothesized that the reason for not achieving improvements in HDR levels when delivering Cas9 as RNP and DNA RFs as plasmids is due to delayed kinetics of DNA RF plasmid expression and protein translation relative to Cas9 RNP, which is already in its protein form at the time of electroporation into the cells.

To get around the expression kinetics effect, we thought to express RFs prior to electroporation of nuclease and donor template. To achieve this, cells were pre-transduced with LVs expressing DNA RFs 24 hours prior to electroporation (refer to Figure 4.3-2A for timeline). However, to our surprise, even with this early compensatory expression strategy, DNA RF LVs did not have any effects on HDR levels, compared to “no RF” or GFP controls (**Figure 4.3-2C**).

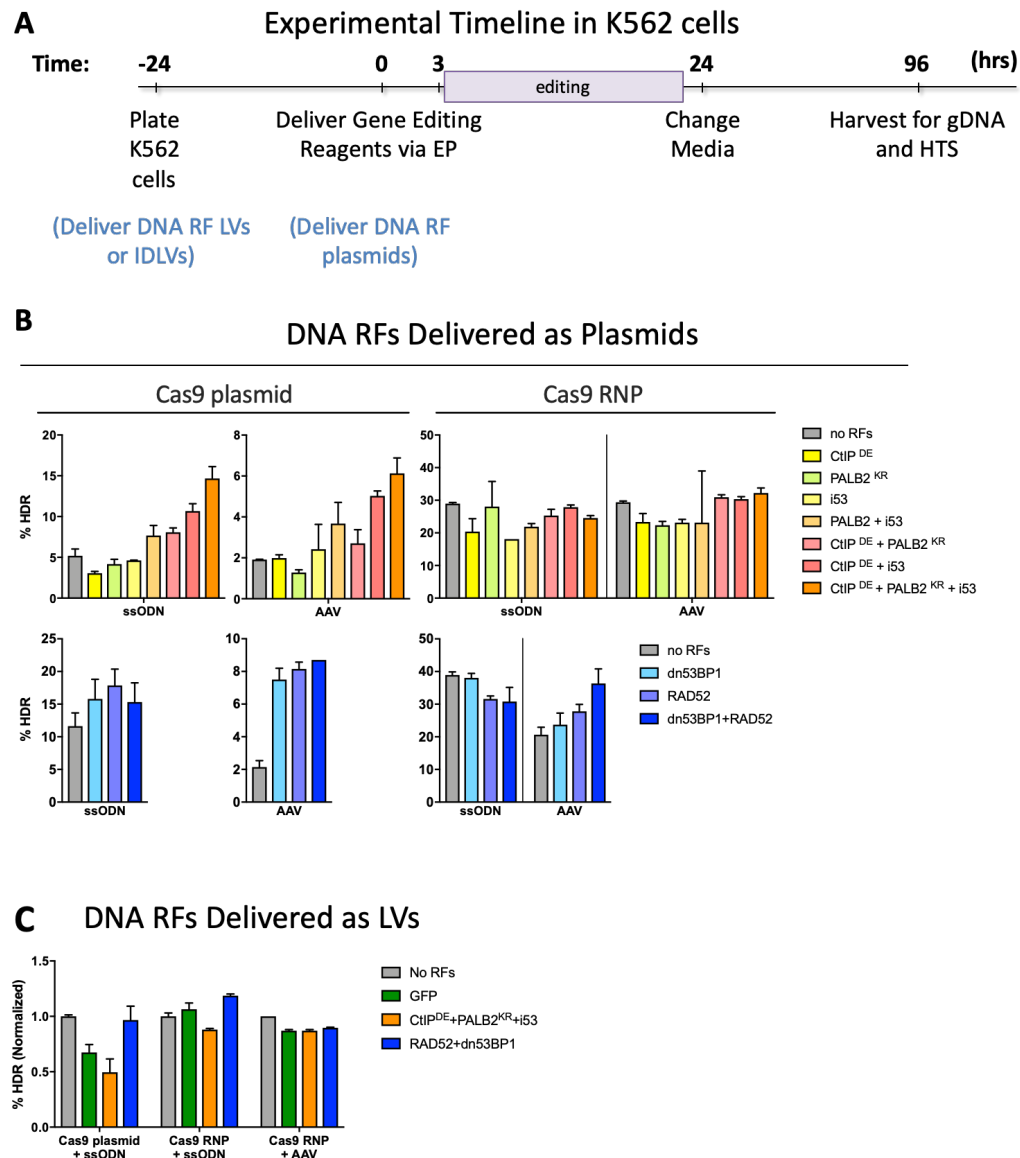


Figure 4.3-2: Evaluating the Effects of DNA RFs on Gene Editing Levels in K562 cells.

(A) Experimental timeline in K562 cells. DNA RFs were overexpressed via plasmids (in B) or LVs (in C), together with, or prior to the delivery of gene editing reagents. Cas9 nuclease was delivered either as plasmid (1ug) or RNP (100pmol Cas9 protein + 4.5ug of IVT gRNA); donor template was delivered either as ssODN (3uM) or AAV6 (MOI 2e4). HDR levels were measured by qPCR. n=2 for CtIP^{DE}/PALB2^{KR}/i53 experiments, n=6 for RAD52/dn53bp1 experiments. Bars – average ± SEM. Data in C are normalized to “no RFs” conditions for each set of experiments.

4.3.3. Comparing Different Methods of DNA RF Delivery on Gene Editing Outcomes in K562 Cells

To test whether the improvements in HDR levels reported in Figure 4.3-2B were a true effect of DNA RFs or merely a result of plasmid co-electroporation, we tested GFP control plasmid co-electroporation at varying amounts (0.3-10ug) with Cas9 plasmid delivery. The levels of gene editing were measured by high throughput sequencing (HTS). A tendency towards an increase in both HDR and NHEJ was detected with the addition of increasing amounts of GFP control plasmid (**Figure 4.3-3A**). These data suggest that the increases in HDR levels observed earlier might be an artifact of plasmid co-electroporation and not the biological effect of DNA RFs.

Next, we went on to compare delivery of DNA RFs and GFP control as LV, integrase-defective lentiviral vector (IDLV) or plasmid. First, we evaluated protein expression levels from LV, IDLV and plasmid delivery of GFP by flow cytometry. Since electroporation of 2-3ug of plasmid previously has resulted in increased HDR levels, the amounts of LV and IDLV used for subsequent DNA RF experiments were estimated based on the expression levels similar to that of 2-3ug of plasmid (**Figure 4.6-1**). Next, we transduced the cells with the chosen MOI of LV and IDLV 24 hours prior to electroporation of gene editing reagents (refer to Figure 4.3-2A for timeline). Although not statistically significant, all plasmids (CtIP^{DE}+PALB2^{KR}+i53, RAD52+dn53BP1, and GFP control) increased both HDR and NHEJ levels approximately 2-fold, while none of the LVs or IDLVs had any statistically significant effects on either HDR or NHEJ (**Figure 4.3-3B**). Of note, additional transduction timepoints and varying MOIs were tested, but still did not improve HDR levels (data not shown).

Together, these data suggest that plasmid co-electroporation induces a response in K562 cells that increases DNA repair levels via both HDR and NHEJ pathways. However, it does not appear that the DNA RFs are directly improving HDR levels.

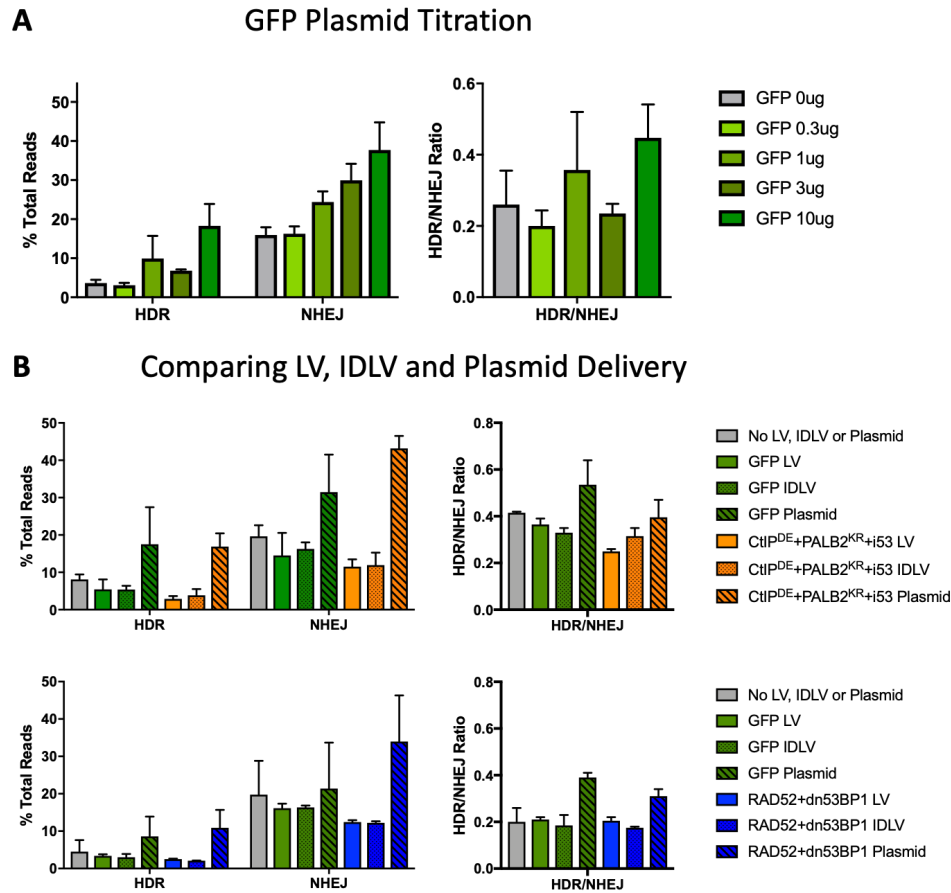


Figure 4.3-3: Comparing Different Methods of DNA RF Delivery on Gene Editing Outcomes in K562 cells.

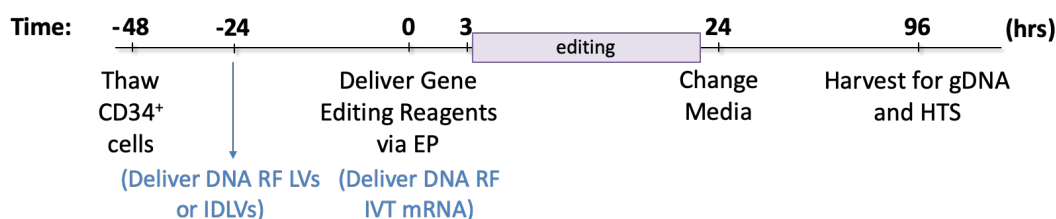
(A) Testing the Effects of Plasmid Co-Electroporation on Gene Editing Levels in K562 cells. Varying amounts of GFP control plasmid (0.3ug, 1ug, 3ug, 10ug) were co-electroporated with Cas9 plasmid (1ug). HDR and NHEJ levels were measured by HTS. $n=4$. Bars – average $\pm$ SEM. **(B)** Comparing Different Methods of DNA RF Delivery on Gene Editing Outcomes in K562 cells. K562 cells were pre-transduced with LVs or IDLVs (as indicated) for 24 hours prior to electroporation with Cas9 plasmid (1ug) and AAV donor template (MOI 2e4). HDR and NHEJ levels were determined by HTS. $n=2$. Bars – average $\pm$ SEM.

4.3.4. Evaluating the Effects of DNA RF Overexpression on Gene Editing Levels in Primary Human CD34⁺ Cells

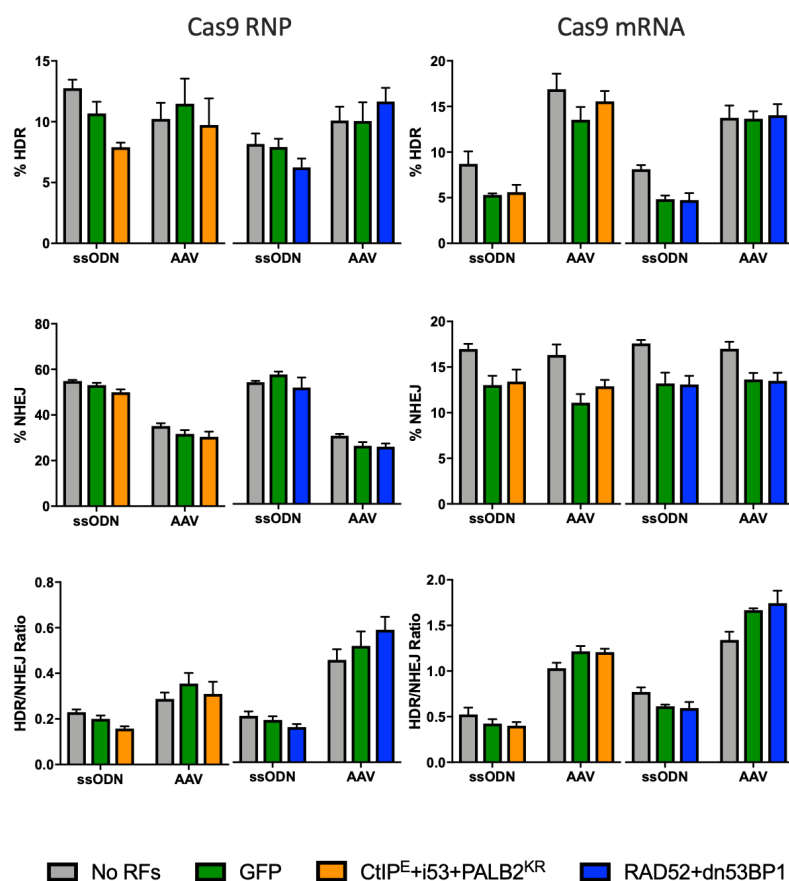
We then evaluated the effects of DNA RF overexpression on gene editing levels in primary human CD34⁺ cells. Because electroporation of plasmid is toxic to CD34⁺ cells (Hollis et al., 2006), DNA RFs were delivered as LVs or as *in vitro* transcribed (IVT) mRNAs, and GFP was used as control. Nuclease was delivered as RNP (Cas9 protein + IVT gRNA) or as IVT Cas9 mRNA + gRNA, and donor template was delivered as ssODN or AAV. As in K562 experiments, RF LVs and IDLVs were delivered to the cells 24 hours prior to electroporation, and IVT RF mRNAs were added during electroporation of gene editing reagents (**Figure 4.3-4A**). In all the conditions tested,

no statistically significant differences in HDR or NHEJ levels were observed with the addition of DNA RFs compared to controls (**Figure 4.3-4B** and **C**). Interestingly, while the levels of HDR achieved with Cas9 RNP and Cas9 mRNA were generally similar, ranging between 5 and 15 percent, the levels of NHEJ were higher with Cas9 RNP (35-60%) compared to Cas9 mRNA (12-15%), especially when ssODN was used as a donor (**Figure 4.3-4B**). These differences in Cas9 nuclease delivery and donor template utilization, although beyond the scope of this study, suggest interesting distinctions in DNA damage repair pathways.

A Experimental Timeline in CD34⁺ Cells



B DNA RFs Delivered as LV



C DNA RFs Delivered as mRNA

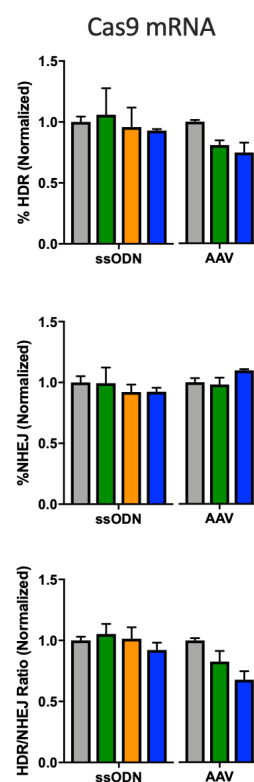


Figure 4.3-4: Evaluating the Effects of DNA RFs on Gene Editing Levels in CD34⁺ cells.

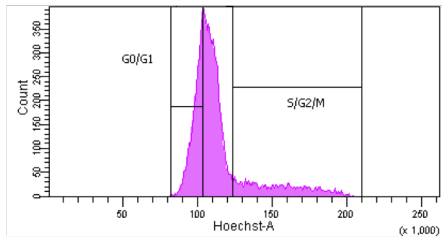
(A) Experimental Timeline in CD34⁺ cells. DNA RFs were overexpressed via LVs (B) or IVT mRNA (C) prior to, or together with, the delivery of gene editing reagents. Nuclease was delivered either as IVT HiFi Cas9 mRNA (5ug) (Vakulskas et al., 2018) + IVT gRNA (5ug), or RNP (100pmol Cas9 protein + 4.5ug of IVT gRNA); donor template was delivered either as ssODN (3uM) or AAV6 (MOI 2e4). HDR and NHEJ levels were measured by HTS four days after electroporation. In C, gene editing levels were normalized to “No RFs” control. n=4 for CtIP/PALB2/i53 experiments, n=8-10 for RAD52/dn53BP1 experiments. Bars – average ± SEM.

4.3.5. Evaluating CtIP^{DE}+i53+PALB2^{KR} Transduction in Different Phases of Cell Cycle

Because prior experiments were performed in bulk (unsorted) CD34⁺ cells, we might have been missing the effects of CtIP^{DE}+i53+PALB2^{KR}, which were being tested for their effects on initiating HDR in G1 phase of cell cycle (Orthwein et al., 2015). To evaluate whether these RFs are improving gene editing outcomes specifically in G1 phase, we transduced CD34⁺ cells with the indicated lentiviral vectors for 24 hours, performed fluorescence-activated cell sorting (FACS) to segregate the cells into G1 and S/G2/M populations, or unsorted controls (**Figure 4.3-5A**), and then electroporated each sorted cell population with gene editing reagents. As expected, the levels of gene editing were higher in the S/G2-sorted cells compared to G1-sorted and unsorted control. It is interesting to note that while the levels of HDR in unsorted and G1-sorted cells were comparable for both donor templates (5-7%), the editing in the S/G2-sorted cells with AAV donor was higher (36-50%) than that with ssODN donor (11-13%).

Disappointingly, there were no statistically significant differences in the levels of gene editing between DNA RF-, GFP-transduced cells, and untransduced controls (**Figure 4.3-5B**). These data suggest that overexpression of CtIP^{DE}+i53+PALB2^{KR} does not result in improved gene editing outcomes in G1 cell cycle phase.

A Cell Cycle Sort



B Gene Editing Levels in Sorted Sub-Populations

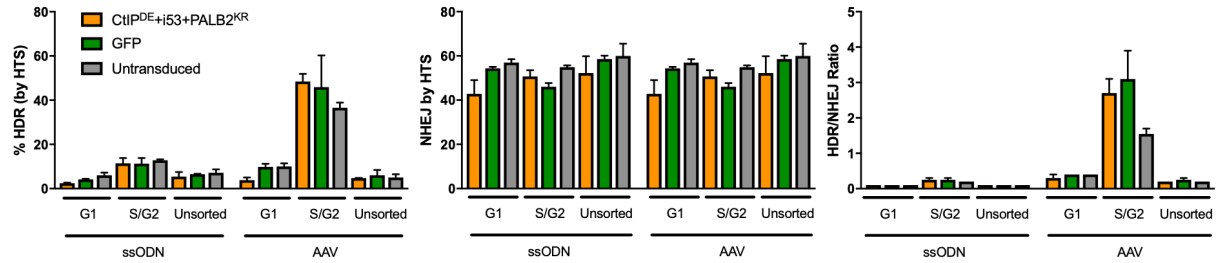


Figure 4.3-5: Evaluating CtlP^{DE}+i53+PALB2^{KR} Transduction in Different Phases of Cell Cycle.

(A) Representative FACS plot. **(B)** Gene editing levels in sorted populations. Cells were pre-transduced with the indicated lentiviral vectors, sorted into cell cycle phases and then electroporated with Cas9 RNP and ssODN or transduced with AAV donor templates. HDR and NHEJ levels were measured by HTS. n=2. Bars – average ± SEM.

4.3.6. Evaluating the Effects of Plasmid Co-Electroporation on Gene Editing Levels in CD34⁺ Cells

Because GFP control plasmid increased gene editing levels in K562 cells, we wondered whether the same phenomenon might be observed in CD34⁺ cells. Despite the expected plasmid toxicity in primary cells, we tested the effects of GFP control plasmid on gene editing levels in CD34⁺ cells. GFP plasmid was co-electroporated at varying amounts (0.3 – 5ug) with Cas9 RNP or Cas9 mRNA. Increasing levels of toxicity were observed with increasing plasmid amounts (data not shown). However, no statistically significant differences in HDR or NHEJ levels were detected with the addition of GFP control plasmid (**Figure 4.3-6A, B**). Of note, the NHEJ data for Cas9 mRNA experiments is pending.

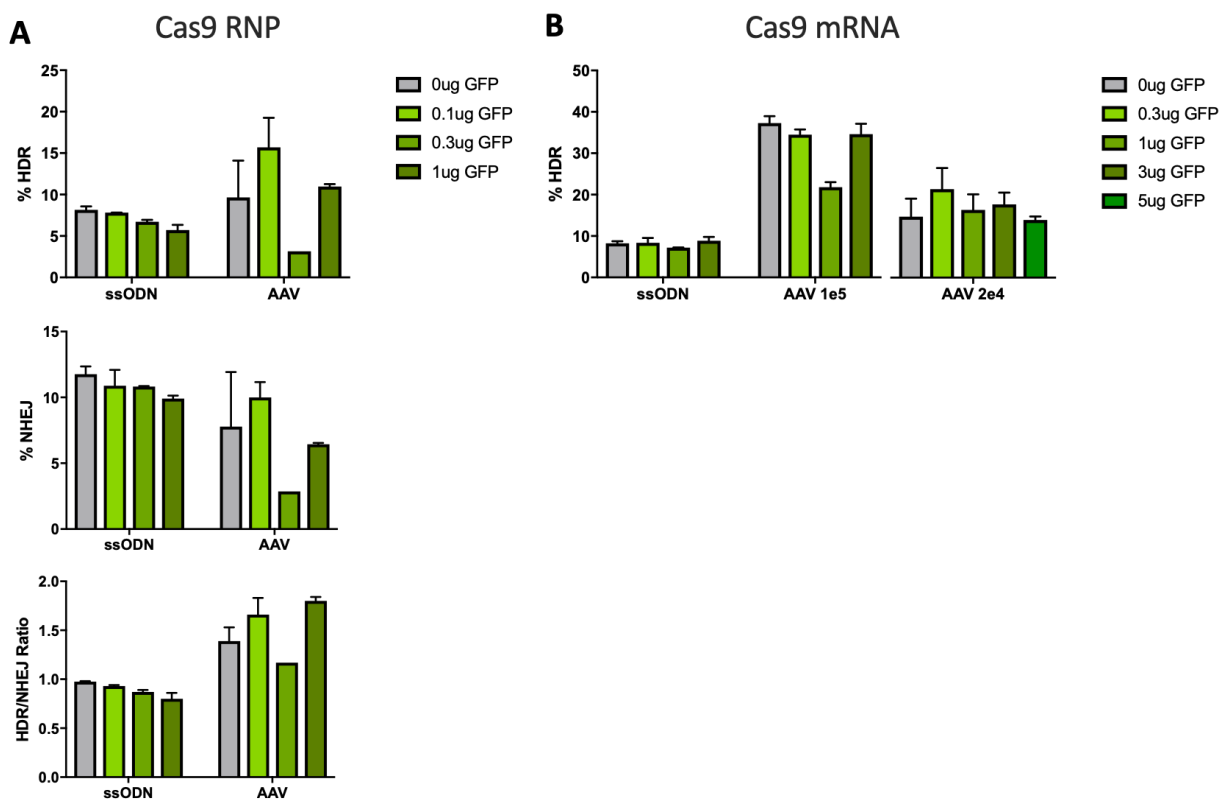


Figure 4.3-6: Evaluating the Effects of Plasmid Co-electroporation on Gene Editing Levels in CD34⁺ cells. GFP control plasmid was co-electroporated with gene editing reagents at the indicated amounts. Nuclease was delivered as RNP (100pmol Cas9 protein + 4.5ug of IVT gRNA) (**A**), or IVT HiFi Cas9 mRNA (5ug) + gRNA (5ug) (**B**); donor template was delivered as ssODN (3uM) or AAV (MOI 2e4 unless otherwise indicated). Gene editing levels were measured by HTS (in A) or qPCR (in B) four days after electroporation. n=2. Bars – average $\pm$ SEM.

4.4. DISCUSSION

In this study, we investigated whether gene editing outcomes can be improved by manipulating DNA repair pathways. Specifically, we aimed to increase HDR levels and decrease NHEJ-mediated repair.

Previous studies in human cell lines have identified certain genes responsible for regulating the temporal control of DNA repair. Although it is known that the HDR pathway is restricted to S/G2 phases of cell cycle due to the availability of sister chromatid at the site of DSB, Orthwein and collaborators identified specific DNA repair genes that impede HR in G1 phase. Additionally, the authors showed that mutating those genes in cell lines can restore the necessary DNA repair complex formation, and can allow HR to occur in G1 phase. This strategy was especially appealing to us because the majority of HSPCs are in G1 phase of cell cycle, therefore, initiating HDR in that phase would allow us to increase the proportion of precise editing in the cells of interest. Also, we tested a combination of factors that has been shown to increase the levels of precise editing via SSTR pathway.

Since it is not possible to introduce permanent mutations in HSCs as was performed in cell lines, we decided to transiently or stably overexpress the mutant versions of those DNA repair factors first in erythroleukemia cell line K562, and then in primary human CD34⁺ cells. To achieve the transient overexpression, we delivered the DNA RFs as plasmids or IVT mRNA for K562 and CD34⁺ cells, respectively, at the time of gene editing reagent delivery. Alternatively, we pre-transduced the cells with RF LVs or as IDLVs one day before delivery of gene editing reagents. We confirmed successful overexpression of certain factors, for which western blot antibodies were available – CtIP, PALB2 and RAD52. Alternative strategies for confirmation of overexpression of inhibitors of 53BP1 (i53 and dn53BP1) might be pursued in the future. However, even without direct evidence of overexpression of certain DNA RFs (such as i53 and dn53BP1), we can infer its overexpression from plasmid, mRNA, LV or IDLV delivering GFP, which was evaluated by flow cytometry.

We achieved an increase in HDR and NHEJ levels in K562 cells in the context of Cas9 plasmid and DNA RFs plasmids, but not with Cas9 RNP, or with DNA RF LVs or IDLVs (refer to **Table 4.4-1**). In fact, the increase in editing levels was not specific to the biological effect of DNA RFs as similar increase was also observed with GFP control plasmid co-electroporation. These data suggest two potential hypotheses: (1) Plasmid electroporation somehow enhances the transcription and/or translation of Cas9 plasmid and its nuclease activity, thus increasing DSBs and thereby also increasing HDR, or (2) Plasmid electroporation enhances DNA damage response in K562 cells, thus enhancing DNA repair pathways and increasing HDR and NHEJ levels in the cells.

Table 4.4-1: Summary of Data

K562 cells			<i>DNA RFs or GFP control delivered as:</i>		
			plasmid	LV	IDLV
	Cas9 plasmid	HDR	↑	—	—
		NHEJ	↑	—	—
	Cas9 RNP	HDR	—	—	—
		NHEJ	—	—	—

CD34 ⁺ cells			<i>DNA RFs or GFP control delivered as:</i>			
			mRNA	LV	IDLV	plasmid
	Cas9 mRNA	HDR	—	—	—	not tested
		NHEJ	—	—	—	tested
	Cas9 RNP	HDR	—	—	—	not tested
		NHEJ	—	—	—	tested
	Cas9 plasmid	HDR	not tested	not tested	not tested	data
		NHEJ	not tested	not tested	not tested	pending

↑ = increase in levels; — = no change in levels

To address the first hypothesis, we will compare Cas9 protein expression by western blot in the cells co-electroporated or not with GFP control plasmid. Additionally, we will test Cas9

plasmid + GFP control plasmid electroporation in CD34⁺ cells to evaluate whether the same response is observed in the primary cells.

To address the second hypothesis, we tested the effects of co-electroporating GFP control plasmid with Cas9 RNP on gene editing levels in CD34⁺ cells. However, no statistically significant changes in HDR or NHEJ levels were observed. These data suggest that even if plasmid enhances DNA damage response, it is only specific to K562 and not CD34⁺ cells. This difference between cell types could be due to increased genome preservation mechanisms in primary CD34⁺ cells.

In summary, we investigated the effects of overexpressing various DNA repair factors on gene editing levels in K562 and CD34⁺ cells. While none of the repair factors tested here resulted in statistically significant improvement of gene editing outcomes, we consider these as essential experiments with important findings. In addition, we made a curious observation that co-electroporation of additional plasmid together with Cas9 plasmid increased both HDR and NHEJ levels in K562 cells. Furthermore, we observed that Cas9 RNP produced higher NHEJ levels compared to Cas9 mRNA, while yielding similar HDR levels in CD34⁺ cells.

Currently, we are evaluating whether tethering different DNA repair factors to Cas9 will aid in more efficient delivery of these factors to the site of DNA repair, and improve HDR levels. This work underlines the complexity of DNA repair regulation, and underscores the differences in DNA damage response and DNA repair mechanisms between cell lines and primary human cells.

4.5. MATERIALS AND METHODS

Experimental Model and Subject Details

K562 cells

K562 cells were modified to contain sickle cell disease-causing mutation, as described previously (Hoban et al., 2016).

Primary Human CD34⁺ Cells

Leukopaks from healthy donors were purchased from HemaCare (HemaCare BioResearch Products; Van Nuys, CA). Mobilized peripheral blood (mPB) was collected from normal, healthy donors on days 5 and 6 after 5 days of stimulation with granulocyte-colony stimulating factor (G-CSF). Briefly, leukapheresis bags were washed three times with PBS/EDTA at room temperature (RT) and spun down at 150xg. Platelet depletion was performed from the centrifuged bags at each wash step using a plasma expessor extractor (Fenwal). The subsequent enrichment of CD34⁺ cells was done by using the CliniMACS Plus (Miltyi; Bergish Gladbach, Germany). Cells were cryopreserved in CryoStor CS5 (Stemcell Technologies; Vancouver, Canada) using a CryoMed controlled-rate freezer (Thermo Fisher Scientific; Waltham, MA).

Method Details

Cell Culture

K562 cells were cultured in RPMI medium + 10% heat-inactivated fetal bovine serum (HI FBS (Gibco/ThermoFisher; Waltham, MA)) + 1% penicillin, streptomycin, glutamine (PSQ (Gemini Bio-Products; Sacramento, CA)), and were kept at a density between 1×10^5 and 1×10^6 cells per ml.

Healthy human CD34⁺ cells from mPB (peripheral blood stem cells, PBSCs) were thawed in pre-warmed X-Vivo 15 medium (Lonza; Basel, Switzerland) with 1% PSQ, pelleted at 500xg for 5 mins, and resuspended at 5×10^5 cells/mL in pre-warmed X-Vivo 15 medium with PSQ and

SFT cytokines (50 ng/mL stem cell factor (SCF), 50 ng/mL fms-related tyrosine kinase 3 ligand (Flt3-L), and 50 ng/mL thrombopoietin (TPO)) (Peprotech; Rocky Hill, NJ). Cells were prestimulated at 37°C and 5% CO₂ incubator for 48 hours.

LV/IDLV transduction

To deliver LV/IDLV DNA RFs, cells were transduced with the MOIs indicated in figure legends for 24 hours (additional time points were tested, but data not shown). Transduction enhancers (PGE2 and Poloxamer Synperonic F108) were added during transduction, as described elsewhere (Masiuk et al., 2019b)

K562 Cell Electroporation

K562 cells were split 1:5 one day before the electroporation. Where indicated, the cells were transduced with LV or IDLV 24 hours prior to electroporation. On the day of electroporation, the cells were counted on ViCell (Beckman Coulter; Brea, CA), 2x10⁵ cells per condition were centrifuged at 90xg for 15 mins at RT, resuspended in 20ul of SF electroporation buffer (Lonza; Basel, Switzerland), combined with Cas9 plasmid or RNP, 3uM ssODN (where applicable), and DNA RF or GFP plasmids (where applicable). The cells were electroporated on Amaxa 4D Nucleofector X Unit (Lonza; Basel, Switzerland) using FF-120 setting. After electroporation, the cells were rested in electroporation strips for 10 mins at RT, and then recovered with 500ul of RPMI medium + 10% HI FBS (Gibco/ThermoFisher; Waltham, MA) + 1% PSQ (Gemini Bio-Products; Sacramento, CA). AAV6 donor template was added to recovery medium where applicable. 24 hours post electroporation, the cells were replated into fresh medium. The cells were harvested 4 days post electroporation for gDNA extraction to evaluate gene editing levels. gDNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen/ThermoFisher Scientific; Carlsbad, CA).

CD34⁺ Cell Electroporation

For electroporation, 2x10⁵ (or 1x10⁶ for FACS experiment) cells per condition were pelleted at 90xg for 15 mins at RT, resuspended in 100ul of BTXpress Electroporation buffer (Harvard

Bioscience, Inc; Holliston, MA), combined with pre-aliquoted ssODN (where applicable), RNP or mRNA (kept on ice), and pulsed once at 250 V for 5 milliseconds in the BTX ECM 830 Square Wave Electroporator (Harvard Apparatus; Holliston, MA). After electroporation, cells were rested in cuvettes for 10 mins at RT, and then recovered with 400ul (or 2.4mL, for 1×10^6 cells) of X-Vivo 15 medium (with PSQ and SFT cytokines), containing AAV6 (multiplicity of infection, MOI=2e4) to introduce 4 SNPs (Virovek; Hayward, CA). The cells were cultured in a 24-well (or 6-well, for 1×10^6 cells) plate at 37°C, 5% CO₂ incubator. 24 hours post electroporation, the cells were diluted 1:2 with trypan blue and counted manually using a hemocytometer to determine viability (number of live cells/number of total cells x 100) and fold expansion (number of cells 24 hours after electroporation/number of cells before electroporation). Cells were replated into 1mL (or 5mL, for 1×10^6 cells) of myeloid expansion medium (Iscove's Modified Dulbecco's Medium (IMDM, Thermo Fisher Scientific; Waltham, MA) + 20% FBS (HI FBS, Gibco/ThermoFisher; Waltham, MA) + 5ng/mL Interleukin 3 (IL3), 10ng/mL Interleukin 6 (IL6), 25ng/mL SCF (Peprotech; Rocky Hill, NJ)), and cultured for 4 days prior to harvesting for genomic DNA (gDNA). gDNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen/ThermoFisher Scientific; Carlsbad, CA).

Determination of Vector Copy Number (VCN)

VCN was evaluating using Psi and SDC4 primers as described previously (Masiuk et al., 2019a)

mRNA/sgRNA production

To make mRNA template, maxi-prepped expression plasmids were linearized with SpeI (NEB; Ipswich, MA), and purified using PCR purification kit according to manufacturer's protocol. In *vitro* transcription was carried out using mMessage Machine T7 Ultra Transcription Kit (ThermoFisher Scientific; Waltham, MA). mRNA product was purified using the RNeasy MinElute Cleanup Kit (Qiagen; Valencia, CA) following the manufacturer's protocol.

sgRNA template was prepared as previously described (dx.doi.org/10.17504/protocols.io.hdrb256). RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen; Valencia, CA) following manufacturer's protocol.

DNA Repair Factor (RF) production

DNA RF sequences were cloned into pCCL-MNDU3 (Logan et al., 2004) or pT7 plasmids using Gibson Assembly Cloning Kit (NEB; Ipswich, MA).

Open Reading Frame Sequences:

CtIP^E

```
ATGAACATCAGTGGGAGCTCATGCGGTAGCCCAAACAGTGCAGATACGAGTAGCGACTTTAAAGACCTTTGGAC
TAAACTGAAAGAGTGTCTATGATCGGGAAGTTCAAGGTCTTCAGGTAAAAGTGACCAAACCTTAAACAGGAGAGGAT
TTTGGATGCACAAAGGCTTGAGGAATCTTTACGAAAAACCAGCAGCTGCGAGAGCAACAAAAAGTGCTTCACG
AAACAATAAAGGTCTTGAAGATCGGCTTCGAGCGGGCCTTTGCGATCGATGCGCTGTACGGAAGAGCATATG
CGAAAAAAGCAGCAAGAGTTTGAACACATACGACAGCAGAACCTCAAGCTGATTACCGAGCTCATGAATGAAAG
AAATACGTTGCAAGAGGAAAACAAAAAGTTGAGCGAGCAATTGCAACAGAAGATAGAGAATGATCAACAACACCA
GGCAGCTGAGTTGGAGTGTGAAGAGGACGTGATTCCGACAGTCCGATTACCGCATTTAGCTTTTCAGGTGTCA
ACAGGCTGCGAAGGAAGGAAAACCCACACGTTGCTACATTGAGCAACACACACAAAACCTTGAGCATAGTGTG
TGCGCAACGAAATGCGCAAGGTTTCAAAGAGTAGTACGCACCCGCAGCATAACCCCAACGAGAACGAAATTCT
GGTAGCAGACACATATGATCAGAGCCAAAGCCCAATGGCCAAAGCACATGGCACTAGTTCTTATACTCCGGATA
AGTCTAGCTTTAATCTGGCAACGGTTGTTGCCGAGACGCTCGGCCTTGGAGTACAGGAAGAGTCCGAGACTCAA
GGACCCATGAGTCCGTTGGGCGATGAACCTTTACCATTGTCTCGAAGGAAATCACAAAAAGCAACCGTTTGAGGA
GAGCACGAGGAACACAGAGGACTCCCTGAGGTTTTGAGACTCAACCAGTAAGACACCTCCCCAGGAAGAGCTC
CCTACTCGAGTATCCGATCCAGTCTTTGGTGCAGTAGTTCTATCAAAAGTGGCCTCGACCTTAATACTTCTCTCT
CTCCCTCTCTCTCCAACCTGGGAAAAAAAACACCTCAAGACCTTGCCTTTCTCCAATACTTGACATAAGCCGAC
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RAD52

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High-Throughput Sequencing (HTS)

Library prep

DNA library for HTS was prepared as described previously (Hoban et al., 2015; Lomova et al., 2019). Briefly, an outer PCR was performed on genomic DNA to amplify a 1.1kb region of interest (using Outer PCR Forward (Fwd) and Reverse (Rev) primers). A second PCR was performed to add a unique index to the PCR product of each sample to be sequenced (read1/read2 and P5/P7 primers). The PCR products with the indexes were mixed at equal concentrations, which was determined by densitometry of the PCR products and analyzed by gel electrophoresis, to create a pooled library. The pooled library was purified twice using AMPure XP beads (Beckman Coulter Inc.; Brea, CA) and then quantified using ddPCR (QX 200; Bio-Rad Laboratories Inc.; Hercules, CA). HTS was performed at UCLA Technology Center for Genomics & Bioinformatics (TCGB) using MiSeq 2x150 paired-end reads (Illumina Inc; San Diego, CA).

Sequencing Analysis and Calculations

Analysis of sequencing data was performed as described elsewhere (Hoban et al., 2015; 2016; Lomova et al., 2019). Percentage of HDR was calculated as the (number of sequence reads containing a sickle change)/(total reads for that sample)*100. Percentage of NHEJ was calculated as the frequency of sequence reads containing an insertion or deletion -50/+36 bases around the nuclease cut site.

Flow Cytometry/ Fluorescence-activated cell sorting (FACS)

All flow cytometry analysis and FACS were performed on the following instruments: BD LSRII, BD LSRFortessa, BD FACS Aria II, all with the similar 5-laser configurations: UV 355 nm, Violet 405 nm, Blue 488 nm, Yel-Grn 561 nm, Red 633 nm.

GFP expression

To validate GFP expression from LV or IDLV transduction, or plasmid electroporation, the cells were washed twice with PBS prior to flow cytometry analysis.

Cell Cycle

Cell cycle FACS was performed as described previously (Lomova et al., 2019). Briefly, CD34⁺ cells were cultured at 5×10^5 - 1×10^6 cells/mL and stained with 5ug/mL Hoechst 33342 for 45-60 mins at 37°C. Cells were washed with PBS + 2% HI FBS and resuspended at 5×10^6 cells/mL in X-Vivo 15 + 5ug/mL Hoechst 33342. Cells were sorted into G1 or S/G2/M populations as shown in Figure 4.3-5, and recovered in X-Vivo15 medium. Immediately after sort, cells were counted, centrifuged at 90xg and electroporated.

4.6. SUPPLEMENTARY INFORMATION

4.6.1. Supplementary Figure

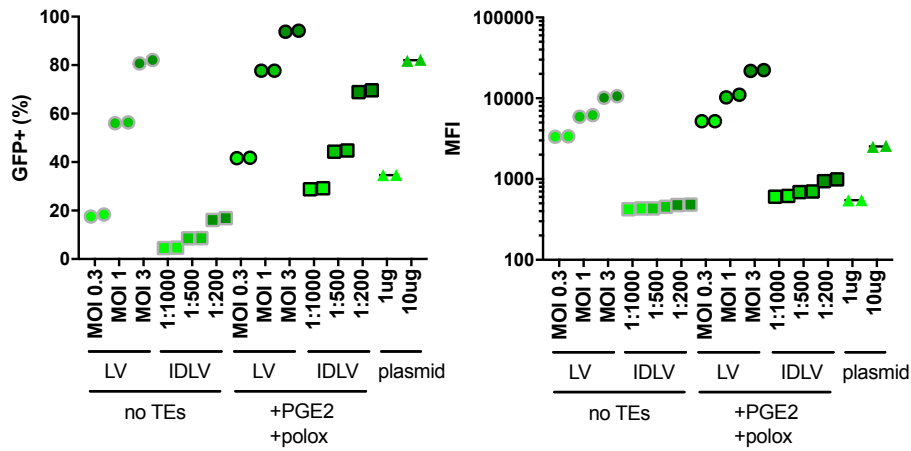


Figure 4.6-1: Comparing Protein Production from GFP LV, IDLV and Plasmid.

K562 cells were transduced in the presence or absence of the transduction enhancers (TEs): Prostaglandin E₂ (PGE2) and Poloxamer Synperonic F108 (polox) with indicated MOI and dilutions of LV and IDLV, respectively, or electroporated with plasmid. 48 hours after transduction or 24 hours after electroporation, the cells were analyzed by flow cytometry for percentage of GFP positive cells and mean fluorescent intensity (MFI). Circles: LV; squares: IDLVs; triangles: plasmid.

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