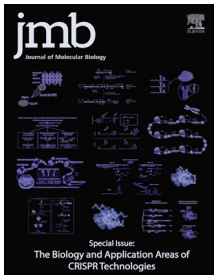

A Versatile Tool for the Quantification of CRISPR/Cas9-Induced Genome Editing Events in Human Hematopoietic Cell Lines and Hematopoietic Stem/Progenitor Cells

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Abstract

The efficient site-specific DNA double-strand breaks (DSB) created by CRISPR/Cas9 has revolutionized genome engineering and has great potential for editing hematopoietic stem/progenitor cells (HSPCs). However, detailed understanding of the variables that influence choice of DNA–DSB repair (DDR) pathways by HSPC is required for therapeutic levels of editing in these clinically relevant cells. We developed a hematopoietic-reporter system that rapidly quantifies the three major DDR pathways utilized at the individual DSB created by CRISPR/Cas9—NHEJ, MMEJ, and HDR—and show its applicability in evaluating the different DDR outcomes utilized by human hematopoietic cell lines and primary human HSPC.

Introduction

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system has enabled us to engineer the genome by creating a site-specific DNA double-strand break (DSB) and exploiting the endogenous DNA–DSB repair (DDR) pathway. The CRISPR/Cas9 induced DNA–DSB may engage in any one of the three prevalent DDR pathways: non-homologous end-joining (NHEJ), microhomology-mediated end-joining (MMEJ), or homology-directed repair (HDR), each resulting in distinct gene-editing outcomes [1]. NHEJ is reported to occur highly efficiently and is applied therapeutically to target dominant gain-of-function mutations or to remove the normal function of a gene [2]. In the presence of an exogenous homologous DNA template (donor template), error-free HDR can occur, which allows precise homology-directed correction of a disease mutation or insertion of the desired sequence at the targeted site [3]. However, the efficiency of HDR and MMEJ is much lower. MMEJ can be useful, in removing small regulatory regions of genes that can be therapeutic [4]. In addition, the PITCh system [5] (Precise Integration into Target Chromosomes), which allows MMEJ-mediated knock-in of exogenous DNA might be supportive in cells where HDR occurs rarely.

Gene correction in a small number of hematopoietic stem cells (HSC) results in expanded life-long gene correct progeny, and therefore HSC remains the most promising targets for gene-editing [6-8]. Human HSC, which have been phenotypically defined as CD34+CD38-CD90+CD45RA-CD49f+ cells [9], are about 1-2% of the clinically used CD34+ hematopoietic stem and progenitor cells (HSPC) [10]. Molecular analysis of edited HSPC, to precisely quantify different DSB-repair outcomes following gene-editing, is laborious and exorbitantly expensive [11]. Therefore, there is an important need

to accurately and rapidly quantify the gene-editing efficiency in HSPC/HSC to optimize and translate gene editing therapy to the clinic. Following gene editing of CD34+ HSPC by site-specific nucleases, DSB are repaired less efficiently by HDR, while NHEJ is the predominant DSB repair pathway [12]. This background mutagenic NHEJ limits the usefulness of translating gene-editing for gene correction [13-17]. Several factors are known to influence DSB repair pathways, including the cell cycle status [18-21], expression levels of DNA repair proteins in a cell type [22-24], and the availability of donor template [25-27]. In the past year, few studies reported HDR rates >50%, demonstrating that therapeutic levels of gene correction can be achieved [17,28]. While strategies have been developed to bias gene editing outcome toward HDR over NHEJ [29], the approaches to improve MMEJ have not been explored. An important limitation in developing such applications has been the lack of a method to rapidly assess all three DDR outcomes—NHEJ, MMEJ, and HDR occurring at an individual DNA–DSB. Previously reported quantification systems have all focused on either one or two DDR pathways [30-33], and to obtain information on competition between repair processes at a single DNA–DSB from these systems would require high-throughput DNA sequencing [34]. Therefore, we developed the CD45 hematopoietic gene editing reporter system that possesses two key advantages over previously described systems: (1) It does not require generation of reporter cell lines and therefore can be applied easily in primary cells or animal models, and (2) it provides a rapid positive readout of all three DDR outcomes: NHEJ, MMEJ, and HDR, allowing direct assessment of the competition between DDR mechanisms.

Results

The gRNA-induced DSB repair pattern is unique to each gRNA, creating specific indel patterns that are consistent across species and cell types [35]. We therefore screened multiple gRNA and chose a single gRNA that targets exon 2 of the human *PTPRC* gene (encoding the CD45 surface receptor), just downstream of the start codon (ATG) such that (i) all subsequent indels caused by NHEJ result in a frame shift mutation (1-bp insertion in >90% of indels and rarely a 2- and 4-bp deletion), resulting in CD45 knockout (KO) detected by flow cytometry (FACS) and TIDE assay [36] [Fig. 1 (i)]. (ii) Knock-in of a promoter-less GFP reporter via HDR was detectable by FACS [Fig. 2 (i)]. (iii) In addition, a 3-bp micro-homology around the Cas9 cut site would result in a 6-bp in-frame

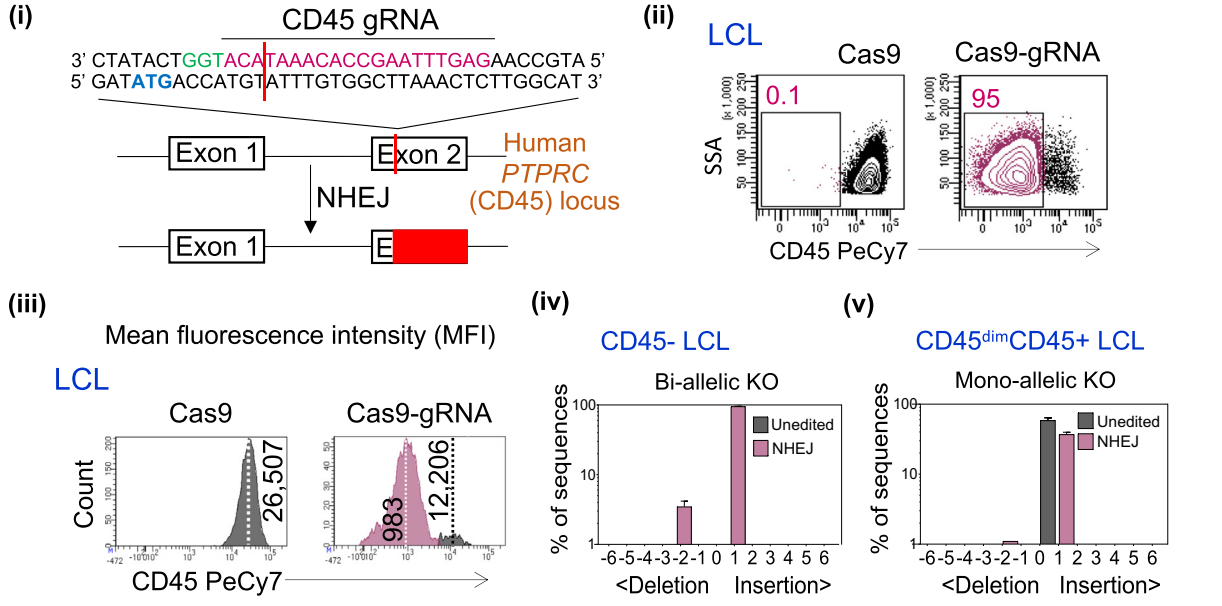

Fig. 1. The CD45-gRNA design allows highly efficient CD45 allele KO with all bi-allelic NHEJ-mediated repair detectable by flow-cytometry. (i) A gRNA was designed to target the CD45 gene sequence immediately downstream of its start codon; the PAM sequence is shown in green,gRNA in pink and start codon in blue. The double strand break [DSB] (denoted as red vertical line) when repaired by NHEJ mediates frame-shiftindel leading to knockout [KO] of the CD45 allele. (ii) Representative flow cytometry analyses at day 7 in LCL treated with Cas9 alone/no gRNAor with Cas9/CD45-gRNA ribonucleoprotein complex (RNP) showing the efficiency of bi-allelic NHEJ causing loss of CD45 expression. (iii) Histogram plots showing mean florescence intensity [MFI] of unedited (Cas9 alone/no gRNA) LCL or edited (Cas9/CD45-gRNA RNP) LCL labelled with the CD45-PeCy7 Ab. (iv-v) TIDE assay results in LCL showing the type of indels (X-axis) and the percentage of alleles (Y-axis) with the respective indels giving the indel patterns and the efficiency of NHEJ in CD45- LCL and in CD45^{dim}CD45+ LCL.

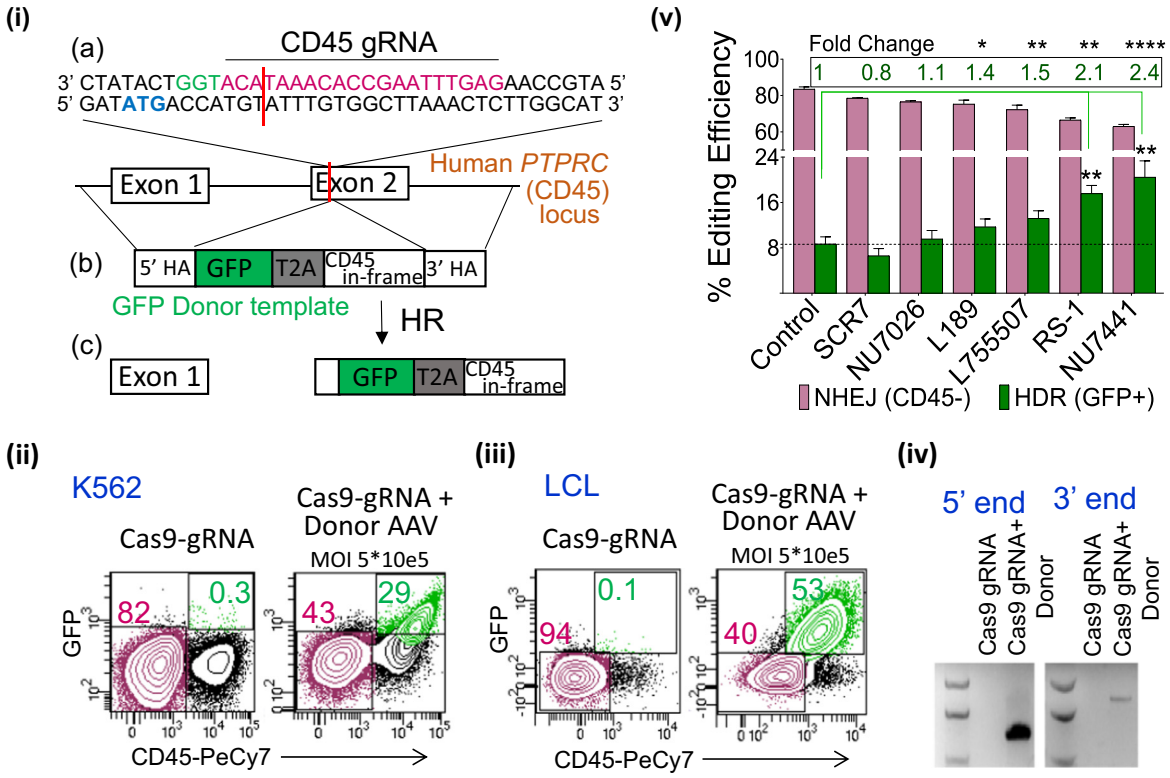

Fig. 2. The Gene Editing Reporter System allows detection and optimization of HDR-mediated repair by flow-cytometry at a single hematopoietic cell level. (i) (a) Schematic around the target site in the CD45 gene. (b) The GFP donor template, which has homology sequences/arm [HA] on the 5' and 3' side of the CD45-gRNA cut site. Within the homology arms are the sequences that encode a promoter-less GFP cDNA, followed by a self cleaving peptide (T2A) and few additional bases of theCD45 gene that place the CD45 gene in frame. (c) The expected configuration of the CD45 gene after editing via HDR in the presence of donor template. Hence, whenthe DSB is repaired by HDR in the presence of the GFP donor template, the cells would express both GFP and CD45. (ii) Flow cytometry analyses at day 7, after electroporation of Cas9-gRNA RNP with or without the GFP donor template that was delivered via an AAV2/6 vector, showing the efficiency of HDR in LCL and (iii) in K562 cells. (iv) Agarose gel image showing the expected size amplicons of the PCR products run with primers external to the 5' HA and 3' HA, and internal primers in GFP, following HDR. (v) The percentages of all-alleleic NHEJ (pink bars) and HDR (green bars) quantified by flow-cytometry as shown in (Sup. Fig. S2) are plotted as bargraph. The blank horizontal dotted line represent the baseline HDR percentage in untreated condition when delivering both Cas9/CD45 gRNA and donor template using the plasmid system. The numbers on the top represent the fold change in HDR with various treatments. All data are shown as mean ± s.e.m. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ by Unpaired t test with Welch's correction).

deletion from MMEJ, which would not disrupt CD45 expression and could be quantified by the TIDE assay [Fig. 3 (i)].

We tested the system in EBV-transformed B lymphocytes (LCL), K562 cells, and human CD34+ hematopoietic stem and progenitor cells (HSPC).

NHEJ mediated gene editing

LCL that repaired the Cas9/CD45-gRNA-induced DSB via NHEJ on both alleles resulted in CD45 – cells, detectable by FACS [Fig. 1 (i-ii)]. The expression of CD45 (reflected in the mean fluorescence intensity, MFI) in the CD45+ edited cell population was nearly half that of the unedited control cells [Fig. 1 (iii)], suggesting that this $CD45^{dim}$CD45+ fraction contains mono-allelic CD45 KO. By sorting these populations, we confirmed that in the CD45 – cells, there were no unedited alleles, and the NHEJ editing was predominantly due to a 1-bp insertion (and a 2-bp deletion in a small number of remaining alleles). Nearly half the $CD45^{dim}$CD45+ fraction showed mono-allelic CD45 KO by TIDE assay [Fig. 1 (v)]. Importantly, at this locus, all indels repaired by NHEJ resulted in an out of frame CD45 allele, detecting NHEJ 100% of the time [Fig. 1 (iv)]. Overall, we could precisely quantify the bi-allelic editingevents, and estimate the mono-allelic editing events in separate cell populations by flow-cytometry, as opposed to the molecular assays that quantify total editing events.

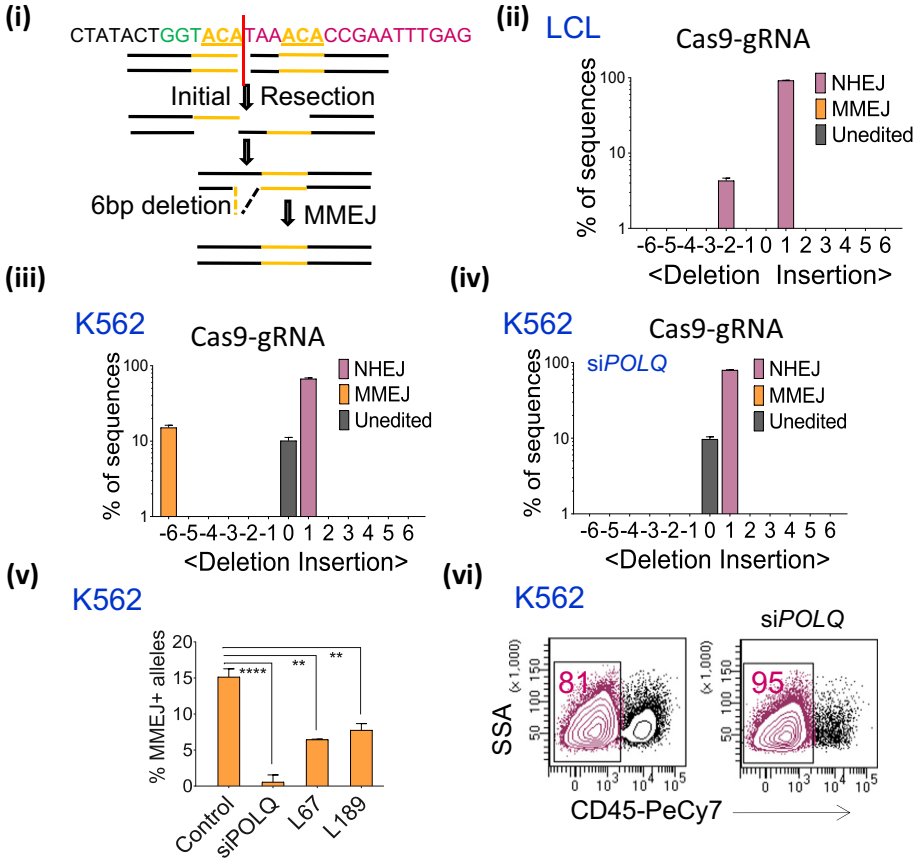

Fig. 3. Detection of MMEJ Using the Gene Editing Reporter System. (i) The CD45 gRNA (pink) was designed to have three base pair micro-homologies (gold) adjacent to the Cas9 cut site. After the introduction of the DSB by Cas9/CD45-gRNA, initial resection of the DNA overhangs can occur. The 3bp micro-homologies from either side of the DSB in the resected strands complement each other, deleting the 6bp via MMEJ-mediated repair process. (ii) TIDE assays results showing the efficiency of 6bp deletion caused by MMEJ as gold bars, while all others (NHEJ-mediated) are in pink bars; unedited allele percentage is shown in gray bars, in LCL, (iii) in K562 cells and (iv) in K562 cells treated with si*POLQ*. (v) TIDE assay based quantification of % alleles repaired via MMEJ in edited K562 cells that were either untreated (controls), or treated with si*POLQ*, orwith small molecules antagonists of the MMEJ pathway. (vi) Flow-cytometry analyses on K562 cells 7 days after electroporation with Cas9/CD45-gRNA RNP + scramble siRNA (left panel) or Cas9/CD45-gRNA RNP + si*POLQ* (right panel). The numbers in pink quantify the percentage of cells repaired via NHEJ.

HDR mediated gene editing

To accurately detect the efficiency of HDR by flow-cytometry at a single cell level, we designed a GFP HDR donor, which would facilitate the insertion of promoter-less GFP just after the CD45 start codon by HDR, while retaining CD45 in-frame [Fig. 2 (i)]. The provision of the GFP HDR homology donor via an AAV2/6 vector with Cas9/CD45-gRNA detected the efficiency of HDR at a single-cell level, (CD45+ GFP+ cells). This reporter allowed optimization of HDR to 30% in K562 cells [Fig. 2 (ii)and 50% in LCL [Fig. 2 (iii)], with a corresponding reduction in NHEJ events [Fig. 2 (ii-iii)]. HDR was confirmed by PCR, using primers external to the 5' and 3'homology sequences and internal for GFP [Fig. 2 (iv)]. It is to be noted that while unedited LCL are all CD45+, some K562 cells in culture spontaneously differentiate into erythroid cells which do not express CD45. Hence, 10%–15% CD45− cells were present in unedited control K562 cells (Sup. Fig. S1a, b).

Next, to demonstrate manipulation of editing outcomes using this platform, we targeted different proteins in the DNA repair pathway using small-molecule inhibitors of NHEJ and agonists of HDR in K562 cells. Cas9-CD45 gRNA and the CD45-GFP donor were delivered by nucleofection of plasmid constructs. We found that RS-1 (an agonist of Rad51, a key molecule in HDR) and NU7441 (an inhibitor of DNA-PK, a key effector in NHEJ mediated repair) significantly increased the percentage of HDR (measured as CD45+ GFP+ cells) in K562 cells from an average of 8.6% to 17.6% and 21.5%, respectively [Fig. 2 (v) and Sup. Fig. S2]. However, unlike previous reports in 293T cells [30], we did not find SCR7 to improve HDR or reduce NHEJ in K562 cells.

MMEJ mediated gene editing

In addition to measuring the NHEJ and HDR events, the CD45-gRNA was targeted to have a 3bp micro-homology around the DSB, such that repair by MMEJ causes an 'in-frame' 6bp deletion [Fig. 3 (i)] that can be analyzed by TIDE assay. Surprisingly, however, no MMEJ was detectable in LCL in multiple experiments [Fig. 3 (ii)]. We found that 12-15% K562 cells repaired Cas9/CD45-gRNA induced DSB via MMEJ through the in-frame 6bp deletion [Fig. 3 (iii)]. To confirm that the 6bp deletion events occurred via MMEJ, transfection of siRNA against Polymerase θ (*POLQ*), the key enzyme mediating MMEJ before Cas9/CD45-gRNA RNP electroporation completely prevented the 6bp MMEJ-mediated deletion at the expense of increasing NHEJ-mediated 1bp insertion [Fig. 3 (iv)]. Furthermore, small molecule inhibitors of other MMEJ pathway proteins

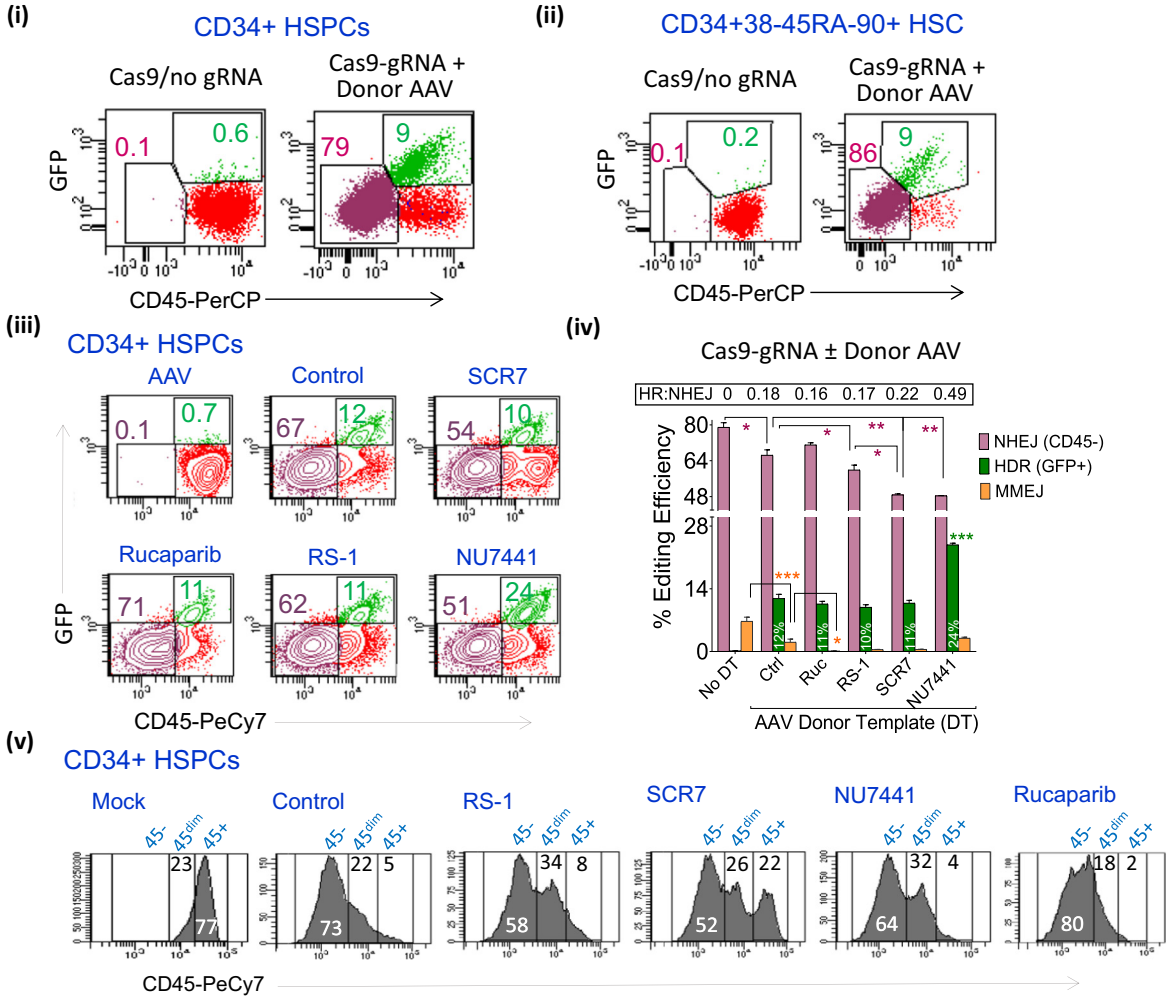

Fig. 4. High Efficiency Gene Editing in Hematopoietic Stem and Progenitor Cells (HSPC) and Hematopoietic Stem Cells (HSC). Representative flow cytometry analyses for CD45 and GFP at 7 days following electroporation of human mobilized peripheral blood (MPB)-derived CD34+ HSPC with either Cas9 alone/no gRNA, or Cas9/CD45-gRNA RNP and donor AAV2/6 (MOI – 10,000), showing bi-allelic NHEJ and HDR (i) in the total CD34+ HSPC or (ii) in the CD34+38-45RA-90+ HSC. (iii) Flow-cytometry analyses for CD45 and GFP at 7 days following electroporation of human mobilized peripheral blood (MPB)-derived CD34+ HSPC with donor AAV2/6 only or Cas9/CD45-gRNA RNP and donor AAV2/6 with/without the presence of different small molecule antagonists of NHEJ-mediated repair (SCR7 and NU7441), MMEJ-mediated repair (Rucaparib) or agonist of HDR-mediated repair (RS-1). The CD45+GFP+ cells (green population) are cells edited with HDR-mediated repair, CD45-GFP- cells (pink population) are those with bi-allelic NHEJ-mediated repair, and the red population show unedited/mono-allelic CD45 KO. The numbers in green and pink quantify the percentage of CD45+GFP+ cells and CD45- cells, respectively. (v) Bar graph showing the quantification of NHEJ, HDR and MMEJ in MPB-derived CD34+ HSPCs with various treatments. All data are shown as mean ± s.e.m. (∗∗ $p < 0.01$ and ∗∗∗∗ $p < 0.0001$ by Unpaired t test with Welch's correction). (iv) Histogram plots showing the expression of CD45 (including bi-allelic/mono-allelic editing/unedited) in CD34+ HSPC under various treatments labelled with the CD45-PeCy7 Ab.

also reduced the 6bp deletion [Fig. 3 (v)]. Reduction in MMEJ was accompanied by an increase in the NHEJ events [Fig. 3 (vi)].

Primary Human HSPC and HSC editing

We then tested this rapid hematopoietic gene editing reporter system in primary human CD34+ HSPC. Here, we achieved around 80% CD45 KO and 9% HDR events in both CD34+ HSPC and CD34+38–90+RA-49f+ HSC [Fig. 4 i, ii)]. Using the TIDE assay, we found around 10% MMEJ events in CD34+ HSPC in the absence of donor AAV2/6 [Fig. 4 (iv)]. However, upon provision of an AAV2/6 donor template, MMEJ frequency was reduced, likely due to competition with the HDR repair pathway [Fig. 4 (iv)]. Indeed, a small-molecule inhibitor of MMEJ, Rucaparib, further reduced MMEJ, and here, we observed higher NHEJ instead of improved HDR [Fig. 4 (iii, iv)]. We also tested the effect of an agonist of HDR (RS1), but found that unlike in K562 cells, RS-1 did not increase HDR in HSPC; however, it did significantly reduce NHEJ- and MMEJ-mediated repair [Fig. 4 (iii–iv)]. We then tested two small-molecule inhibitors of NHEJ (SCR7 and NU7441). While both SCR7 and NU7441 significantly reduced NHEJ-mediated repair, only NU7441 improved HDR in HSPC by two fold [Fig. 4 (iii–iv)]. Interestingly, SCR7 did not increase HDR, but reduced the proportion of CD45− HSPC (bi-allelic NHEJ) and resulted in significantly higher mono-allelic NHEJ ($CD45^{dim+}$) or unedited (CD45+) HSPC [Fig. 4 (iii-iv)]. It is to be noted that most small-molecule inhibitors of DNA repair pathways exhibited significant toxicity to HSPC, with the greatest loss of viability seen with MMEJ inhibitors Talazoparib and L189, followed by NU7441 and RS1 [Sup. Fig. S3a].

Discussion

In this study, we developed a rapid method to precisely assess three different types of DSB repair outcomes in hematopoietic cells including HSPC and HSC following editing by CRISPR/Cas9. This platform allows rapid enhancement of gene-editing efficiencies and manipulation of the DNA repair pathways utilized in HSPC. While developed for CRISPR/Cas9 system, this platform can be adapted to other site-specific nucleases. This platform led to several unique observations which can now be used to analyze mechanisms and improve genome editing: (1) RS-1 and NU7441 significantly increased the efficiency of HDR in hematopoietic cell lines, but only NU7441 had the same effect in HSPC. Both reduced HSPC viability. Global inhibition of NHEJ, even temporarily, and deficiencies of NHEJ pathway proteins, including Ku70, XRCC4, DNA-PKcs, and Ligase-4, have been associated with bone marrow failure, stem cell aging, oncogenic mutations, and lack of Tand B cell development [37-42]. Therefore, clinical use of these inhibitors would need to be carefully evaluated for safety. While agonists of HDR may be considered clinically safer, RS-1, a Rad51 agonist, only improves HDR in hematopoietic cell lines, not HSPC. Therefore, better or more potent HDR agonists may be necessary to improve HDR in human HSPC. (2) MMEJ occurred in CD34+ HSPC and myelogenous leukemia cell lines but not in immortalized primary B cells. Perhaps lymphocytes that depend on recombinase 1 and 2, do not express MMEJ pathway genes. Therefore, DNA repair pathways of lymphocytes could be further explored for future gene-editing based immunotherapies. Some gene-editing strategies for the treatment of β-globinopathies, such as generation of MMEJ-mediated 13bp deletion to mimic the hereditary persistence of fetal hemoglobin mutation [43] or knockout of core region the *BCL11A* core enhancer [44] could optimize MMEJ-based gene-editing efficiency using this platform.

In summary, we have carefully designed a rapid and precise reporter that allows determination of, and optimization of, the different DDR pathways utilized after site-specific nuclease-mediated DSB in hematopoietic cells, and specifically in the clinically relevant human CD34+ HSPC and the rare HSC population within them. This system allows possible studies on factors that affect the different DDR outcomes and ways to manipulate these pathways to achieve the desired editing outcome. These factors can then be applied to any cell type.

Methods

Human cell culture

Human hematopoietic cell lines: (i) The K562 cell line was obtained from American Tissue Culture Consortium; and (ii) EBV immortalized B-lymphocytes (LCL) were generated by immortalizing primary B lymphocytes with EBV by the Diagnostic Immunology Laboratory, CCHMC. LCL were cultured in RPMI-1640 (Sigma) supplemented with 20% fetal bovine serum (FBS,VWR), 1% L-glutamine (MP Biomedicals) and 1% Penicillin-Streptomycin (Lonza). K562 were cultured in DMEM (Corning) supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin-Streptomycin.

Human CD34+ HSPC isolation and culture

Mobilized Peripheral Blood (MPB) product from healthy donors was obtained from the Cell Manipulation Laboratory core at CCHMC. CD34+ cells were enriched using the Miltenyi Biotec CD34+ isolation kit (130-046-702), following manufacturer's instructions, to a >90% purity. CD34+ cells were cultured in X-VIVO10 (Lonza) supplemented with 300 ng/mL

FLT3-ligand, 100 ng/mL thrombopoietin and 300 ng/mL stem cell factor (all purchased from Peprotech) for 48 h prior to electroporation with Cas9/CD45-gRNA RNP.

Synthesis of Cas9 RNPs

SpCas9 protein was purchased from the QB3 Macro Lab, UC Berkeley (Berkeley, California). Protospacer sequences for CD45 (*PTPRC* gene) were identified using the Benchling software. The sgRNA including the CD45 20bp (5' GAGTTTAAGC-CACAAATACA 3') target sequence was synthesized by assembly PCR and in vitro-transcription using the GeneArt Precision gRNA synthesis kit (ThermoFisher Scientific: A29377) or purchased modified sgRNA from Synthego. The concentration of the synthesized sgRNA was determined using a Nanodrop spectrophotometer (Life Technologies, Inc). Cas9 RNP complex was assembled immediately prior to electroporation/nucleofection of the target cells. For K562 cells and LCL, 3 μg Cas9 and 1 μg sgRNA were incubated at room temperature for 30 min in 2 μL Buffer R.

Delivery of gene editing components

For electroporation, 1–2×10^5 LCL cells were re-suspended in 10μL Buffer R with 3μg Cas9 protein complexed with 1μg sgRNA and electroporated using the Neon™ electroporation system 10μl kit (ThermoFisher: MPK1025). Briefly, the cell suspension was transferred to a 10 μL Neon™ tip and electroporated. The optimized electroporation parameters used for LCL was 1350V/30ms/1pulse. For nucleofection, 1–2×10^5 K562 cells were re-suspended in 20 μL nucleofector solution (Lonza SF cell line kit: V4XC-2032) with 3 μg Cas9 protein complexed with 1 μg sgRNA. The cell suspension was transferred to 16-well nucleofector strips and nucleofected (Program FF-120) using Lonza 4D-Nucleofector™ system (AAF-1002B, AAF-1002X). MPB derived CD34+ cells were electroporated using Neon electroporation system. Briefly, a solution containing 9 μg Cas9 protein and 2 μg sgRNA in 2 μl Buffer T were incubated at RT for 30 minutes. The solution was transferred to the 10 μL CD34+ HSPC suspension (1-1.5×10^5 cells in Buffer T) and electroporated using the electroporation parameter:1650V/10ms/3pulse. For the HDR experiments, AAV2/6 vector (detailed methodology of AAV2/6 production is in Supplement Materials) expressing the donor template was also added along with the RNP. For the experiment with different small molecule inhibitors or agonists, 1–2×10^5 K562 cells were re-suspended in 20ul nucleofector solution (Lonza SF cell line kit: V4XC-2032) with 1ug U6-CD45sgRNA-CBH-Cas9-T2A-mCherry plasmid and/or 1ug GFP donor plasmid. The cell suspension was transferred to 16 well nucleofector strips and nucleofected (ProgramFF-120) using Lonza4D-Nucleofector™ system (AAF-1002B, AAF-1002X). After transfection, cells were transferred into pre-warmed complete medium and maintained until analysis.

Small molecules

SCR7(Xcessbio M60082-2), L67 (Sigma SML1797), L189 (Tocris 3561), RS1 (Sigma R9782-5MG) and L755507 (Xcessbio M60237-2s) were purchased. NU7026, NU7441 was a gift from Dr. Susanne Wells' lab at CCHMC. For the MMEJ assay, 4h before the Cas9 RNP electroporation, K562 cells were treated with 3 μM small molecules and washed off during electroporation. For the HDR assay with different small molecule inhibitors or agonists in K562 cells, 8h after nucleofection with 1ug U6-CD45-sgRNA-CBH-Cas9-T2A-mCherry CRISPR-Cas9-P2A-mCherry-U6-sgRNA plasmid and/or 1ug GFP donor plasmid. Electroporated K562 cells were maintained in complete medium supplied with 3 μM small molecules and washed after 24 hours. For the HDR assay in CD34+ HSPCs with different small molecule inhibitors or agonists, the cells were re-suspended in the small molecules for 5h before RNP electroporation and AAV transduction and washed 16h later.

Small inhibitory (si) RNA

SMARTpool: Accell POLQ siRNA, (Dharmacon, CO:E-015180-00-0005) was added at 1 μM concentration 24 h prior to nucleofection with Cas9/CD45-gRNA RNP.

Flow cytometry

Transfected cells labeled with anti-human PeCy7-CD45 (BD-557748) prior to analysis on a FACS Fortessa-1 (BD). HSPC were labelled with anti-human PerCP-CD45, and HSC markers: PeCy7-CD34, APC-Cy7-CD38, Alexa Fluor 700-CD90 and APC-CD45RA. Labelled cells were analyzed on FACS Canto (BD).

Genomic DNA isolation and TIDE assay

Transfected (edited) cells were cultured and harvested at day 7. Pools of edited cells were lysed using Cell Lysis solution (Qiagen, 158908). Other contaminants, such as proteins, were removed by Protein Precipitation solution (Qiagen, 158912). Finally, the genomic DNA was recovered by precipitation with isopropanol, washed twice with 70% ethanol, and dissolved in TE buffer. A 728bp region around the Cas9 cut site was amplified by PCR using EconoTaq PLUSGREEN 2X Master Mix

(Lucigen, 30033) with *PTPRC* gene (CD45) specific primers, (forward primer [FP] 5' GTCATCTTGCCAACACCCATT 3' and reverse primer [RP] 5'AGCAGGCTTCTCACTTCCAGTT 3') using the following conditions: 94°C for 2 min; 35 cycles (94°C for 30 sec, 56°C for 30 sec, 72°C for 45 sec) and 72°C for 5 min. PCR products were run on 1% agarose gels, purified and subjected to Sanger sequencing using the FP2 primer (5' TCATCACCTAGCAGTTCATGCAG 3') at the CCHMC DNA Sequencing and Genotyping Core. The sequencing results were analyzedin the TIDE software (https://tide-calculator.nki.nl). Percentages of sequences with indels were plotted. For HDR, genomic DNA was amplified using primers listed in the Supplementary Table 1.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2018.05.005.

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Keywords:
Hematopoietic stem cells;
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NHEJ;
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Abbreviations used:
CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; DSB, double-strand break; DDR, DNA–DSB repair; NHEJ, non-homologous end-joining; MMEJ, microhomology-mediated end-joining; HDR, homology-directed repair; KO, knockout.

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