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Technical Considerations for the Use of CRISPR/Cas9 in Hematology Research

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Abstract

The hematopoietic system is responsible for transporting oxygen and nutrients, fighting infections, and repairing tissue damage. Hematopoietic system dysfunction therefore causes a range of serious health consequences. Lifelong hematopoiesis is maintained by repopulating multipotent hematopoietic stem cells (HSCs) that replenish shorter-lived, mature blood cell types. A prokaryotic mechanism of immunity, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 nuclease system, has recently been “repurposed” to efficiently mutate mammalian genomes in a sequence-specific manner. The application of this genome-editing technology to hematology has afforded new approaches for functional genomics and even the prospect of “correcting” dysfunctional HSCs in the treatment of serious genetic hematological diseases. In this *Perspective*, we provide an overview of three recent CRISPR/Cas9 methods in hematology: gene disruption, gene targeting, and saturating mutagenesis. We also summarize the technical considerations and advice provided during the May 2017 International Society of Experimental Hematology New Investigator Committee webinar on the same topic.

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Introduction

The mammalian hematopoietic system plays an essential role in health and disease, carrying oxygen and nutrients around the body, fighting infection, and helping to repair tissue damage. A small number of multipotent hematopoietic stem cells (HSCs) are responsible for the lifelong balanced production of mature blood cells (Eaves, 2015). Under homeostasis, HSCs are located in the bone marrow and maintained in a long-term quiescent state (Wilson et al., 2008). However, following stress or bone marrow transplantation, hematopoietic stem and progenitor cells (HSPCs) can be activated to expand and re-establish homeostasis (Osawa et al., 1996, Haas et al., 2015, Eaves, 2015). The ability of HSCs to efficiently repopulate and drive long-term blood formation following transplantation makes them an attractive and widely used tool in many clinical settings (Felfly and Haddad, 2014). The ability to precisely modify the genomes of HSCs has therefore been a long-standing desire in clinical hematology and would also represent a powerful tool for studying basic hematological science.

The field of genome editing has been recently revolutionized by the introduction of engineered nucleases, promising a controlled genomic engineering approach (Gersbach, 2014). Zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALENs) and, in particular, the clustered regularly interspaced short palindromic repeats (CRISPR) technology have been developed to introduce precise genomic alterations (Cho et al., 2013, Cong et al., 2013, Mali et al., 2013, Hsu et al., 2014, Sander and Joung, 2014, Porteus et al., 2003). The CRISPR/Cas9 technology was adapted from a prokaryotic immune system and consists (among others) of the endonuclease Cas9 and a single guide RNA (sgRNA), which targets the Cas9 to a desired region of genome through Watson-Crick base-pairing (Figure 1). CRISPR/Cas9 induced DNA double-strand breaks can be repaired by the error-prone non-homologous end-joining (NHEJ) pathway, which frequently results in the introduction of insertions or deletions (indels). Alternatively, homologous recombination (HR) can be exploited to introduce precise genomic modifications using homologous DNA donor templates. Great progress has been made in the hematology field regarding CRISPR/Cas9 applications and while manuscripts describing efficient protocols for gene disruption and HR-mediated gene targeting approaches in various hematopoietic cell types have been published (Gundry et al., 2016, Dever et al., 2016, Canver et al., 2015, Mandal et al., 2014, Tzelepis et al., 2016, Heckl et al., 2014), there are no reviews that describe the technical considerations for the use of CRISPR/Cas9 in hematology research.

In May 2017, Daniel Bauer, Michael Gundry, and Daniel Dever presented in an International Society of Experimental Hematology (ISEH) webinar organized by the New Investigator Committee and moderated by David Yudovich. This webinar focused on the technical considerations for the use of CRISPR/Cas9 genome editing in hematology research. During the webinar, three methods were covered: CRISPR/Cas9 for gene disruption; CRISPR/Cas9

for gene targeting; and CRISPR/Cas9 for saturating mutagenesis. This *Perspective* provides a synthesis of the topics covered during the webinar, including protocols summarizing the technical advice for designing, troubleshooting, and optimizing these CRISPR/Cas9 applications. It aims to provide the hematology research community with a useful reference for reproducing experimental CRISPR/Cas9 methodologies.

Experimental Considerations

Before starting CRISPR/Cas9 experiments, there are several important technical aspects to consider. In particular, the design of sgRNAs, the choice of Cas9/sgRNA reagents, and the delivery mechanism should be considered, as outlined below.

Design of sgRNAs

Bioinformatics tools for sgRNA design—There are a wide variety of web-based bioinformatics tools available for sgRNA design and each has a slightly different algorithm for scoring on-target efficiency and specificity. For example, CRISPRscan (Moreno-Mateos et al., 2015) has UCSC browser track functionality, which can be useful in visualizing target exons/regions. Additionally, the scoring system for CRISPRscan takes into account that sgRNAs transcribed from a T7 promoter (necessary for efficient *in vitro* transcription) must begin with two 5′ guanine nucleotides prior to the start of the target transcript. Mismatches between the target locus and these two 5′ guanine nucleotides can lead to variable cleavage efficiencies. There are a range of other similar bioinformatics tools available, such as CHOPCHOP (Labun et al., 2016) and CRISPOR (Haeussler et al., 2016) among others.

Exon targeting—When planning an experiment to test for the consequence of loss-of-function of a gene, it is important to target the appropriate exons. Conserved exons can be identified using RNA-seq data from your cell type of interest (or closest available cell type). Exons that are included in all transcripts should be used for sgRNA targeting. It is also best to target early exons because mutation of later exons may not result in nonsense-mediated decay (Popp and Maquat, 2016). In addition to these guidelines, it is also recommended to confirm protein changes by western blotting and/or flow cytometry following gene targeting.

Accurate reference genome—It is important to have accurate genomic sequences for the cell lines you are working with. Unknown SNPs, indels, and other variants can alter existing protospacer adjacent motifs (PAMs), change the sgRNA spacer sequence, and/or generate new PAMs in a target sample. If targeting repetitive elements, there may be many off-target sites within the genome, which can complicate any downstream analysis and can also lead to cellular toxicity (Munoz et al., 2016, Aguirre et al., 2016, Canver et al., 2017).

Numbers of sgRNAs per target—When planning a gene disruption experiment, it is recommended to test 2–3 exons per gene with 3–5 sgRNAs per exon. For gene disruption experiments, sgRNAs can be combined for the same exon into a single electroporation. This often results in co-deletions between sgRNAs (Gundry et al., 2016). Notably, the hands-on time required to make 10–20 sgRNAs is only marginally greater than that required to make a single sgRNA.

Pre-screening sgRNAs—For knocking out a gene in primary samples (where material is limited), it is recommended to first screen sgRNAs in representative cell lines (K562 cells or HL-60 cells are commonly used) in order to select sgRNAs with the highest gene disruption efficiency for use with primary samples.

Cas9 and sgRNA reagents and delivery

Cas9/sgRNA reagents—The early protocols available for CRISPR/Cas9 involved either plasmid-based delivery through transfection or lentiviral-based delivery of Cas9 and sgRNA (Figure 1) (Gersbach, 2014). These remain common for CRISPR/Cas9 screening approaches including saturating mutagenesis. However, very low efficiency rates were seen when using primary human HSPCs (Gundry et al., 2016, Mandal et al., 2014). Ribonucleoprotein (RNP)-based delivery provides an alternative delivery approach for CRISPR/Cas9 (Figure 1). In this strategy the biologically active components, Cas9 protein and sgRNA, are complexed and then directly delivered into target cells. Allelic disruption rates exceeding 85% have been observed in both hematopoietic cell lines and primary HSPCs in the absence of selection. Importantly, the RNP method may minimize off-target effects since it delivers only a brief pulse of Cas9 and also eliminates use of exogenous DNA, which could randomly integrate into the target cell genome. Recombinant Cas9 protein can be purchased from various vendors (e.g. Thermo Scientific, IDT). sgRNAs can be either *in vitro* transcribed (IVT) in house (Moreno-Mateos et al., 2015) or synthesized by various vendors (TriLink, Synthego). For the latter, it is recommended to use chemically-modified synthetic RNAs (Hendel et al., 2015a).

Cas9/sgRNA delivery—While lentiviral constructs can be used to deliver Cas9/sgRNA, electroporation is commonly used for RNP (and plasmid) Cas9/sgRNA delivery. The most frequently used devices for delivery of RNPs into HSPCs are the Invitrogen Neon instrument (program: 1600V, 10ms, 3 pulses), Lonza Nucleofector 4D (program: DZ100), and the Lonza Nucleofector IIb (program: U-014). All devices are capable of allelic disruption rates exceeding 85% when optimized (although maximal efficiency is target specific).

Protocol Summaries

Having considered how to design sgRNAs, the types of Cas9 and sgRNA available to use, and specialized equipment necessary for these experiments, we next outline the basic protocols for use of CRISPR/Cas9 in: (A) gene disruption; (B) gene targeting; and (C) saturating mutagenesis. Rather than specific point-by-point guides, these protocols focus on general advice for each step. Specific experimental details are described elsewhere (Gundry et al., 2016, Dever et al., 2016, Canver et al., 2015).

(A) CRISPR/Cas9 for gene disruption

CRISPR/Cas9 editing can be used to establish relationships between the absence of specific gene products and the resulting changes in cellular function and structure. The following RNP-based protocol affords efficient gene disruption in primary HSPCs (or cell lines) and can be completed in just three days (Gundry et al., 2016, DeWitt et al., 2016, DeWitt et al., 2017).

1. HSPC culture—When working with primary human HSPCs, short-term pre-culture in the presence of cytokines is recommended, to increase the gene disruption efficiency. A 36–48 hour pre-culture is optimal prior to electroporation. Starting cell densities of 2.5×10^5 cells/ml is recommended. StemSpan II media (STEMCELL Technologies) supplemented with 100 ng/ml hSCF, 100 ng/ml hTPO and 100 ng/ml hFLT3L is recommended.

2. RNP generation and delivery—After choosing the target sequences, custom sgRNA primers are ordered containing a T7 promoter, the target sequence and the first 15bp of the sgRNA scaffold sequence. These primers used in a six-cycle overlap-PCR performed using a high fidelity polymerase and a universal scaffold reverse primer (Gundry et al., 2016). The resulting dsDNA templates are then purified and used for IVT for 4–12 hours. The IVT sgRNAs are then purified and incubated with Cas9 protein at a molar ratio of 2.5:1 for 15 minutes at room temperature to form the gene-specific RNPs.

3. RNP delivery—HSPCs or cell lines are electroporated in the presence of RNPs using Invitrogen Neon or Lonza Nucleofactors. Following electroporation, a 24-hour recovery is used prior to assessing knockout efficiency.

4. Genetic validation assays—A number of assays can be used for estimating indel frequencies, although PCR of the target locus followed by amplicon sequencing of the PCR products to obtain accurate estimates of indel rates is recommended (Hendel et al., 2015b, Brinkman et al., 2014). Amplicon sequencing libraries can be spiked into larger high-throughput sequencing runs at nominal cost (Gundry et al., 2016).

5. Functional assays—Following gene disruption, the next step is often *in vivo* or *in vitro* assays to study the consequences of gene loss-of-function. Following electroporation, the cells can be cultured further or used for *in vivo* experiments (e.g. transplantation into NSG mice). It is of vital importance to use the appropriate controls. IVT sgRNAs can be toxic, particularly to primary cells. Using several control sgRNAs is recommended, including a cell surface marker expressed on the cell, as well as a second target gene not expressed by the cells.

Summary—Using the CRISPR/Cas9 RNP-based gene disruption approach described above, a targeted editing frequency of over 85% in human CD34⁺ HSPCs can be achieved (Gundry et al., 2016). Electroporation enables robust delivery of the RNP to virtually all CD34⁺ HSPCs. Furthermore, the short half-life of RNP minimizes off-target cleavage and limits HSPC toxicity: RNP-treated HSPCs did not show reduction in methylcellulose clonogenic capacity or cell viability (Gundry et al., 2016). This method opens the door for cost effective generation of genetic models, with quick turnaround times from biologic question to efficient CRISPR/Cas9 perturbation.

(B) CRISPR/Cas9 for gene targeting

Unlike the method described above that relies on random indels via NHEJ, CRISPR/Cas9 can also be used for targeted genome editing via HR. Here, a homologous DNA repair construct must be used as a template to introduce changes in the DNA. While plasmid or

linear DNA can be used, the adeno-associated virus serotype six (AAV6) system provides an efficient template, particularly for primary human HSPCs. Its use in CRISPR/Cas9-mediated homologous recombination in human HSPCs is outlined below (Dever et al., 2016). Please note that this protocol does not cover AAV donor design or production, which have been described in detail elsewhere (Khan et al., 2011, Grieger et al., 2006, Rago et al., 2007). Alternatively single-stranded DNA oligonucleotides (ssODN) donor templates may be utilized, which although potentially of lower efficiency may be easier to synthesize (DeWitt et al., 2017).

1. Human HSPC pre-culture—As above, it is recommended to culture the HSPCs for 36–48 hours before gene targeting to promote growth and cell viability, which enables high levels of HR. Following pre-culture, HSPCs should be >80% viable and expanded up to three-fold (depending on HSPC source).

2. RNP formation—Use of chemically-modified synthetic sgRNAs is recommended for this protocol. RNP formation can be achieved by mixing sgRNA and Cas9 in a 2.5:1 molar ratio followed by a 10-minute incubation at room temperature. However, it is recommended to empirically determine the optimal Cas9/sgRNA ratio for each target. Additionally, use high enough concentrations of Cas9 and sgRNA to avoid taking up more than 20% of final electroporation volume.

3. RNP electroporation and AAV transduction—Nucleofection is recommended for electroporating human HSPCs in this protocol. When using nucleofection cuvettes, electroporation of $0.5\text{--}1 \times 10^6$ HSPCs is optimal. Inclusion of the appropriate controls is important, and should include: a mock electroporated sample (receiving no AAV or RNP); a mock-electroporated sample receiving AAV; a RNP-electroporated cells receiving no AAV (RNP-only); and a RNP-electroporated sample receiving AAV (RNP + AAV). Resuspend the HSPCs in nucleofection solution, gently mix with the RNP, and then electroporate. Following electroporation, dilute HSPCs to $2.5\text{--}5 \times 10^5$ cells/ml with media. Remember to save 10–20% of the sample for the RNP-only control. Immediately add rAAV6 to the media with RNP electroporated HSPCs and incubate for 12–24 hours.

4. Genetic assays—Time course for indel and HR frequencies may be performed from day 1–4 post-targeting. Clonal gene targeting in hematopoietic progenitors can be tested following methylcellulose colony forming assays. This assay can also be used to evaluate the influence of gene targeting on progenitor function.

5. Cellular assays—Where GFP (or another cellular marker) is inserted by HR, efficiencies within phenotypically-defined HSPC populations can be assessed by flow cytometry. However, the gold standard for assessing gene-targeting frequencies in HSCs is transplantation into immunocompromised NSG mice. Bulk targeted and/or HR-enriched HSPCs should be transplanted into the femur or tail-vein of sub-lethally irradiated 6–8 week-old NSG mice and human chimerism in the bone marrow analyzed at 16 weeks post-transplant. Secondary transplants are recommended to truly assess self-renewal capacity and long-term multi-lineage repopulation of gene-targeted human HSCs. For further details see (Park et al., 2008).

Summary—The HR-based repair in HSPCs is a potentially transformative approach for both basic and clinical applications. Remarkably, this CRISPR/Cas9-mediated HR protocol can achieve efficiencies of over 20% of primary human CD34⁺ HSPCs. Significantly, within AAV6-transduced colony-forming HSPCs, over 90% of the cells underwent a single HR repair, of which over 40% achieved homozygous modification (Dever et al., 2016). While gene correction is likely the most clinically relevant application of this method, various types of HR donor designs are possible, including: a gene insertion knock-in; a knock-in reporter cassette for precise gene disruption; or an SNP-type modification for amino acid substitution. Combined, this approach has far-reaching implications, both for studying precise genetic alterations in disease modeling and the clinical setting of “gene correcting” hereditary diseases.

(C) CRISPR/Cas9 for saturating mutagenesis

While the methods described above use CRISPR/Cas9 to disrupt or repair gene function, the role of non-coding regions of the genome can also be interrogated by similar methods. By tiling sgRNAs throughout a region of interest, it is possible to achieve saturating mutagenesis screening (Canver et al., 2015, Canver et al., 2017). This approach has recently been applied to study non-coding regions that determine globin gene expression (Canver et al., 2015). Given the large numbers of cells needed for such screening, this approach is most easily undertaken using cell lines, preferably one that is already engineered to constitutively express Cas9.

1. sgRNA library generation—All possible 20bp sgRNA target sequences upstream of PAM (or other recognition) sites should be identified within the genomic region of interest. Importantly, the PAM-restriction of the nuclease used will determine the density of mutagenesis possible within the region of interest. DNA oligos for these sgRNA sequences can be ordered as a pool and cloned via Gibson Assembly into a lentivirus backbone. Previous studies have used lentiGuide-Puro (Addgene 52963) for this approach (Canver et al., 2015). Following transformation into competent *E. coli* and maxiprep plasmid purification of the pooled sgRNA plasmids, library coverage should be verified by NGS sequencing. Lentivirus libraries can be generated by standard 293T transfection methods, described elsewhere (Canver et al., 2015).

2. Screening approach—Following lentiviral sgRNA library generation, the Cas9-expressing cell line of interest can be transduced. Lentiviral transduction should be titrated to achieve low multiplicity of transduction (i.e. so that one vector has transduced each cell). It is important that each cell only receives a single sgRNA. Following transduction (and commonly selection), cells can be cultured and/or differentiated as required. Typically at least 1000 transduced cells per sgRNA are maintained throughout the experiment to fully represent the library. Transduced cells can be collected before or after additional culture and/or differentiation, or FACS-purified based on marker gene expression (e.g. fetal hemoglobin level).

3. Analysis of sgRNA enrichment—Following cell collection, gDNA can be isolated from the different cell populations and lentiviral sgRNA sequences amplified for NGS

sequencing. From this analysis, enrichment of a particular sgRNA can be calculated, typically as a relative ratio, e.g. enrichment = $\log_2(A/B)$ where A and B are different cell populations or time points (Canver et al., 2015). By mapping sgRNA sequences back to the genome, target regions can be identified.

4. Analysis of genomic mutations—While mapping sgRNAs back to the genome allows target regions enriched in a particular cell population to be identified, it does not identify the type of genetic mutation that has occurred. Amplification and deep sequencing of the mutated region from the gDNA is therefore important to validate genomic mutation. Several bioinformatics platforms such as CRISPResso (Pinello et al., 2016), can be used to quantify and visualize the genome editing outcomes.

Summary—This method exemplifies the application of the CRISPR/Cas9 system for functional characterization of native enhancers. This type of saturating mutagenesis approach was first used to identify critical positions in the *BCL11A* enhancer (Canver et al., 2015) and more recently to interrogate the *HBS1L-MYB* locus (Canver et al., 2017). Interestingly, a high-resolution positional comparison between human and mouse screens highlighted that there are structural differences between the *BCL11A* enhancers despite extensive sequence homologies and conserved hemoglobin switching function (Canver et al., 2015). It is worth noting that although these saturating mutagenesis screens were carried out in the context of a non-coding enhancer, similar tiling approaches can be extended and applied to coding gene sequences too. The use of the CRISPR/Cas9 system for screening purposes has also been utilized for genome-wide gene disruption (Shalem et al., 2014).

Optimization Advice

Although the CRISPR toolbox offers tremendous flexibility in genomic perturbation, it can present several challenges. In particular, researchers need to address issues concerning reagent delivery and genome-editing efficiency, and particular care is required for HSPC assays. The following recommendations identify good practices for CRISPR/Cas9 experiments. While these recommendations are focused towards hematopoietic cell types, many should be broadly applicable to other mammalian systems.

Improving reagent delivery

Cas9/sgRNA reagent choice—The first step to productive genome editing is delivery. The size of the Cas9 protein creates a strain on the coding capacity and packaging efficiency of commonly used delivery vectors. This limitation can be addressed in part by switching to smaller Cas9 variants or optimized lentiviral backbones (Ran et al., 2015). However, the protocols described above address this issue by delivering pre-complexed Cas9 protein and IVT (or synthetic) sgRNA RNPs via electroporation. Of course, this may be less feasible for screening approaches where many sgRNAs are required.

Improving Cas9 genomic coverage—Use of different Cas9 variants can help to increase genomic coverage, for example in saturating mutagenesis. These include SaCas9, AsCpf1, LbCpf1, or other CRISPR modalities such as CRISPR-mediated repression or activation (Friedland et al., 2015, Kleinstiver et al., 2016, Gilbert et al., 2014). Additionally,

use of such complementary tools can validate positive hits further, and help to resolve mechanistic details through increasing genomic targeting space and direct regulatory control.

AAV optimization—Recombinant AAV serotype six (rAAV6) should be used for HSPC gene targeting (Dever et al., 2016) and should be purified via iodixanol or cesium chloride gradients. It is recommended to generate rAAV6 that have homology arms centered on the cut site and also introduce an SFFV-GFP-pA (or UbC-GFP-pA) cassette to allow assessment of targeting frequencies. It is also important to use high-titer, purified rAAV6 so that the rAAV6 volume is less than 20% of HSPC media (preferably less than 10%).

Reagent quality—Targeting inefficiencies can also occur due to poor quality reagents, including plasmids and virus preparations, Cas9 protein, and sgRNAs. As such, different reagent batches should be compared for consistent targeting efficiencies and similar cell viabilities.

Validating genome editing efficiency

sgRNA design and validation—Good sgRNA design is critical for a successful experiment and the design process starts from a good reference sequence and transcript isoform expression data. As mentioned above, targeting an early exon may be advantageous because a late truncated gene product is not guaranteed to produce nonsense-mediated decay (Popp and Maquat, 2016), and a non-targeted isoform can also result in residual expression and/or confounding effects. Avoiding off-target effects is also important. Additionally, good sgRNA target is also conditional on available compatible validation tools. Good validation primer design is therefore essential for each sgRNA sequence in testing.

Testing sgRNAs—When testing designed sgRNAs it is important to realize that some sgRNAs are inherently poorly performing in editing or toxicity, either due to repeats in the genome, particular off-target effects, or poor locus accessibility. Different sgRNAs may also have a different propensity for producing frame-shift alleles. As such, empirical testing of several sgRNAs should be undertaken to select the optimal targeting sequences. It is also worth considering use of multiple sgRNAs in combination. Multiple sgRNAs that target nearby regions (same exon) have been shown to increase the total editing frequency at the locus, up to near complete efficacy (Hendel et al., 2015a, Mandal et al., 2014).

Control sgRNAs—DNA damage response in HSCs has been previously linked to increased apoptosis and reduced clonogenic capacity (Milyavsky et al., 2010, Walter et al., 2015). Edited cells must be therefore compared to control sgRNA-edited cells, and not only compared to mock treatment. To account for this, sgRNAs can be designed from non-coding regions of the genome or genes that are not expressed in the target cell type. As further controls, multiple targeting and non-targeting sgRNAs should be tested to validate an on-target functional effect in the cells of interest.

Validation assays—Cas9/sgRNA can generate mixtures of editing products and can lead to an incomplete targeting efficiency, complex heterozygous products, and codon-frame

preserving edits. Generally, editing validation assays should be carried out at early time points to avoid the risk of positive and negative selection. It is recommended that validation assays are be conducted at both DNA and protein levels. Assays such as exon co-deletion PCR, Surveyor, Sanger sequencing, and NGS provide a relative estimate of editing efficiency (Hendel et al., 2015b). Notably, amplicon NGS provides robust results at relatively small coverage costs (Gundry et al., 2016). However, flow cytometry and especially western blots should be used to confirm functional gene deletion. As validation assays often pose demands on cell number and culture logistics, surrogate cell lines can be used for early stage validation.

Improving HSPC assays

HSPC culture—HSPCs are sensitive cells that cannot endure significant stress without altering functional capacity. Cell densities need to be closely monitored and should be kept below 5×10^5 cells/ml. As noted above, pre-culture of HSPCs is recommended. In this context, the previously described HSC supporting compounds such as SR1 and UM171 may be helpful in promoting HSPC maintenance (Boitano et al., 2010, Fares et al., 2014), as well as regular medium changes.

Xenograft assays—To retain maximal HSC activity, primary HSPCs should be cultured for as short a time as possible prior to use in functional assays. Transplantation into NSG mice is recommended no later than 24 hours post-electroporation. Assessing gene-targeting frequencies in HSCs via xenograft transplantation in NSG mice is essential for determining repopulating capacity of edited HSCs (Park et al., 2008). It is important to keep in mind that HSCs are targeted less frequently than short-lived progenitors. It is therefore recommended to transplant higher numbers of HR-enriched targeted cells for each mouse ($>5 \times 10^5$ cells).

HSPC heterogeneity—Different CD34⁺ HSPC sources have different baseline ability to undergo editing, presumably due to the cell cycle status and absolute frequencies of HSCs within the CD34⁺ fraction. Cord blood (CB) CD34⁺ HSPCs appear to be highly amenable for genome-editing purposes. By contrast, mobilized peripheral blood CD34⁺ HSPCs, the most common source of HSPCs for clinical applications, may perform less well (Dever et al., 2016). It is therefore recommended to use CB HSPCs to establish the methodology and then use the HSPC source that is most relevant to your end goals thereafter. It is also worth noting that gene targeting efficiencies in HSPCs can be gene-dependent as well as donor-dependent (Gundry et al., 2016). Given this heterogeneity, it is recommended to perform at least three independent experiments using three biological replicates (i.e. three unique donors) to distinguish experimental variability from biological variability.

Conclusions

Although CRISPR/Cas9 technology is promising to revolutionize basic and translational biomedicine, its technical application is complicated, particularly its delivery and efficiency in primary cell types. The application of the complex CRISPR/Cas9 methodology to the study of hematopoiesis represents a significant challenge where optimal experimental configurations and treatment modalities must be crafted. We hope that this *Perspective*

provides a useful community resource for those wanting to develop robust experimental procedures and helps users to meet the challenges of the CRISPR/Cas9 genome editing toolbox.

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Highlights

- CRISPR/Cas9 is a powerful tool for genome editing in hematopoietic cells, including primary hematopoietic stem and progenitor cells
- CRISPR/Cas9 can be used for gene disruption, gene targeting, and/or genomic screening
- Several technical aspects of the CRISPR/Cas9 toolkit, particularly single guide RNA design and Cas9 delivery, should be considered during assay optimization

Figure 1. Cas9/sgRNA for genome editing

Cas9 and sgRNAs can be delivered to cells via lentiviral transduction, or DNA plasmid-based or ribonuclearprotein (RNP)-based electroporation. Following Cas9/sgRNA delivery, there are a range of downstream applications of this technology in hematology research including: (1) gene disruption (via generating frameshift mutations) for studying of gene function; (2) gene targeting (via AAV6-based homologous recombination) for repairing deleterious alleles, introducing precise mutations, or tagging endogenous genes; and (3)

saturating mutagenesis for identifying functional genomic components within a locus of interest.

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