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The Changing Landscape of Gene Editing in Hematopoietic Stem Cells: A Step Towards Cas9 Clinical Translation

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Abstract

Purpose of review—Since the discovery two decades ago that programmable endonucleases can be engineered to modify human cells at single nucleotide resolution, the concept of genome editing was born. Now these technologies are being applied to therapeutically relevant cell types, including hematopoietic stem cells (HSC), which possess the power to repopulate an entire blood and immune system. The purpose of this review is to discuss the changing landscape of genome editing in hematopoietic stem cells (GE-HSC) from the discovery stage to the preclinical stage, with the imminent goal of clinical translation for the treatment of serious genetic diseases of the blood and immune system.

Recent Findings—With the discovery that the RNA-programmable (sgRNA) clustered regularly interspace short palindromic repeats (CRISPR)-Cas9 nuclease (Cas9/sgRNA) systems can be easily used to precisely modify the human genome in 2012, a genome editing revolution of hematopoietic stem cells (HSC) has bloomed. We have observed that over the last two years, academic institutions and small biotech companies are developing HSC-based Cas9/sgRNA genome editing curative strategies to treat monogenic disorders, including β -hemoglobinopathies and primary immunodeficiencies. We will focus on recent publications (within the past 2 years) that employ different genome editing strategies to “hijack” the cell’s endogenous double strand repair pathways to confer a disease-specific therapeutic advantage.

Summary—The number of genome editing strategies in HSCs that could offer therapeutic potential for diseases of the blood and immune system have dramatically risen over the past two years. The HSC-based genome-editing field is primed to enter clinical trials in the subsequent years. We will summarize the major advancements for the development of novel autologous GE-HSC cell and gene therapy strategies for hematopoietic diseases that are candidates for curative allogeneic bone marrow transplantation.

Keywords

Cas9; genome editing; hematopoietic stem cells; CRISPR; homologous recombination

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Conflicts of interest

MHP is a consultant and has equity interest in CRISPR Tx, but CRISPR Tx had no input or opinions on the subject matter relayed herein.

Introduction

Allogeneic bone marrow transplants (now known as allo-hematopoietic stem cell (HSC) transplants (T) (allo-HSCT)) have been used since the late 1960's to offer a potential lifetime cure for a variety of monogenic hematological diseases, notably the primary immunodeficiencies¹ and β -hemoglobinopathies^{2,3}. Now, the CD34⁺ cell surface marker is used to identify a very heterogeneous population of human cells commonly referred to as hematopoietic stem and progenitor cells (HSPCs), which includes a small fraction of bona fide long-term repopulating HSCs. Once physical niche space has been created following myeloablative conditioning regimens, HSPCs have the ability to repopulate an entire hematopoietic system for >10 years of the patients life (and theoretically the entire life of the patient). The key feature is that when allo-HSCT is successful, the patient is cured for life, highlighting the concept that transplantation of HSCs that contain a non-disease causing variant of a gene can produce a functional blood and immune system. While allo-HSCT can cure multiple genetic diseases of the blood and immune systems, the challenge of finding an HLA immunologic matched donor and the subsequent immune complications of Graft vs Host Disease (GvHD) and incomplete immune reconstitution remain.

In disease specific cases where there are no suitable HLA-matched bone marrow donors, autologous HSPCs have been treated with integrating viral vectors that carry the defective gene and then reinfused back into the patient, and while this has shown to work for diseases like ADA-SCID⁴ and even more recently for SCD⁵, viral vector-based gene therapies carry the risk of insertional mutagenesis⁶. Now with state-of-the-art programmable nucleases technologies available to researchers alike, we believe that an even more promising, and collectively safer approach, would be autologous transplantation with the patients own HSCs that have been gene-edited *ex vivo* to confer an indication-specific therapeutic benefit (Figure 1). Here we review the current state of HSC-based therapeutic gene editing using engineered nucleases and discuss where we see the field heading in the future and what barriers remain for clinical translation.

State-of-the-art genome editing reagents for hematopoietic stem cells are necessary for clinical translational

The introduction of genome editing using engineered nucleases has transformed the idea that gene therapy for monogenetic diseases is attainable; but to be able to translate HSC-based genome editing technologies to the clinic, it is critical to have genome editing reagents that are: 1) efficient, 2) specific, 3) scalable and 4) non-toxic. Towards this end, there have been important improvements in classes of programmable nucleases, nuclease delivery, and homologous donor template delivery.

Genome editing is the process where a site-specific double strand break (DSB) is created using engineered nucleases that subsequently triggers the cell's endogenous repair machinery to modify (or edit) the genome in a site-specific manner. Currently there are five major classes of engineered nucleases used in genome editing (listed in chronologically order of discovery): Homing endonucleases, zinc finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), megaTALs, and more recently the RNA-

programmable (sgRNA) clustered regularly interspace short palindromic repeats (CRISPR)-Cas9 nuclease (Cas9/sgRNA) system⁷. While each nuclease platform is at different stages of clinical development (Table 1), the Cas9/sgRNA system, because of its specificity, ease of design, scalability of raw materials, and high efficiencies, is poised to be the engineered nuclease of choice for genome editing clinical therapies.

The Cas9/sgRNA system consists of the Cas9 endonuclease and a ~100-nucleotide (nt) single guide RNA (gRNA). The most commonly used Cas9/gRNA system is derived from *Streptococcus pyogenes* (SpCas9) and we briefly describe the SpCas9 system here. Target identification relies first on identification of a 3-base pair protospacer adjacent motif (PAM) and then base-pairing between a 20-nucleotide stretch of the gRNA and the DNA target site, which triggers Cas9 to cleave both DNA strands. Although the Cas9/gRNA system was shown to work in human HSPCs and a clever dual guide approach improved editing outcomes, the efficiencies were still likely too low for clinical translation⁸. Until recently when Hendel *et al.*, showed that sgRNAs modified at both termini with 2' O-Methyl 3' phosphorothioate dramatically enhanced genome editing efficiencies in primary HSPCs⁹. Another major advancement was the delivery method of the Cas9/sgRNA system into HSPCs from plasmid-based⁸ to RNA-based⁹ to more recently, as pre-complexed ribonucleoproteins (RNPs)¹⁰. We believe that the most effective method and best for clinical applications is RNP delivery as it maintains high activity (hit), while reducing duration of nuclease exposure (and-run); thus using a hit-and-run mechanism. Importantly, it also reduces the stimulation of innate immunity signaling that recognizes naked DNA and RNA, which can lead to increased toxicity and reduced editing outcomes in stem cells.

Once an engineered nuclease creates a site-specific DNA double-strand break for genome editing, the cell activates the double-strand break repair (DSBR) machinery to fix the break. There are multiple DSBR pathways but the main canonical mechanisms are non-homologous end-joining (NHEJ) and homologous recombination (HR) (Figure 1). NHEJ repair is the pathway that functions throughout the cell cycle to correct breaks through ligation of DNA ends. While usually precise in the joining of ends that created by an engineered nuclease, through the iterative cycle of break and NHEJ-mediated repair, insertions and/or deletions (INDELs), can be created at the site of the break. By creating site-specific INDELs, genetic elements, such as enhancers, TAD domains, microRNAs, and coding regions of genes can be disrupted.

If the cell uses HR to repair a DSB using an exogenous DNA donor template with arms of homology to the target site, then specific changes in the DNA can be introduced. Since the first report of engineered nuclease-stimulated HR in HSPCs, albeit at very low frequencies¹¹, there have been several homologous donor platforms that have been used to stimulate HR in human HSPCs (Table 2), including single-stranded oligodeoxynucleotides (ssODNs)^{12–16}, integrase defective lentivirus (IDLV)^{12,17}, and more recently, recombinant adeno-associated virus serotype 6 (rAAV6)^{18–21}. While all of these donors are generally classed as “Homology Directed Repair,” a more precise classification would identify rAAV6 and IDLV donors under HR-mediated editing because it is a Rad51 dependent process and ssODNs under “Single-stranded template repair (SSTR)” because it is a Rad51 independent process. That is, genome editing using IDLV or rAAV6 is mechanistically distinct from

genome editing using ssODNs and thus should be distinguished from each other when assessing different approaches to genome editing. Although current literature may suggest rAAV6 is a better donor template than IDLV²² and a more versatile donor than ssODNs because it can mediate precise nucleotide changes as well as insert gene cassettes up to 5.7kb using a dual-AAV6 platform (Bak and Porteus, manuscript in press), a more comprehensive comparison is needed to compare all donor platforms in a head-to-head fashion. Nevertheless, GE-HSC through HR could be harnessed to restore gene function in downstream hematopoietic cell lineages for therapeutic purposes. Hendel et al (2015) and Dever et al (2016) both demonstrated that an important feature of genome editing is that when high amounts of donor can be delivered to the nucleus without inducing cellular toxicity, the frequency of HR mediated editing can exceed the frequency of NHEJ mediated editing in both cell lines and primary human CD34⁺ HSPCs.

NHEJ-based therapeutic genome editing strategies to reactivate fetal hemoglobin for the treatment of β -hemoglobinopathies

The β -hemoglobinopathies are inherited monogenic blood disorders that result from mutations in the beta-globin gene (HBB) and either affect hemoglobin protein production (thalassemia) or hemoglobin tetramer function (sickle cell disease). Sickle cell disease (SCD) is an autosomal recessive disease caused by an adenine to thymidine transversion in codon 6 of the HBB gene on chromosome 11 that results in a amino acid change from a polar residue, glutamic acid (E), to a hydrophobic one, valine (V)²³. Under low-oxygen conditions, the E6V sickle hemoglobin polypeptide (HbS) promotes polymerization with another hemoglobin tetramer, ultimately causes the sickle shape (and thus its name).

Fetal hemoglobin (HbF) induction using small molecules, such as hydroxyurea, can ameliorate the severity of both SCD and β -thalassemia patients where fetal hemoglobin is normally silenced in adulthood²⁴. Building upon this knowledge, Bauer and colleagues used genome-wide association studies to identify common genetic variation in regulatory regions of a transcriptional repressor, *BCL11A*, that positively associated with HbF protein expression²⁵. Canver *et al.*, then tightly mapped the critical sequences within the *BCL11A* enhancer to identify a motif that contained a well-know erythroid specific transcription factor, GATA1²⁶, which is thought to be involved during fetal to adult hemoglobin switching in humans. Knockdown of *BCL11A* or deletion of the enhancer region represented a promising strategy to induce HbF in autologous HSPCs for the treatment of SCD.

To this end, Bjurstom, Kohn and colleagues, examined whether genome editing of *BCL11A* exon2 in HSPCs using ZFNs, TALENs, or Cas9/sgRNA could increase HbF. While they observed induction of HbF *in vitro*, the frequencies were likely too low after engraftment in NSG (4%) to be of therapeutic benefit and on top of that, plasmid delivery of the Cas9/sgRNA system resulted in very high toxicities, highlighting the importance of nuclease delivery via RNPs as discussed earlier²⁷. Because *BCL11A* is essential for lymphoid development and may be a risky therapeutic target²⁸, Tan *et al.*, compared genome editing of *BCL11A* exon 2 and the GATAA enhancer motif. Using ZFNs, researchers discovered that both strategies resulted in the increase in HbF but not surprisingly, HSPCs that were treated

with ZFNs targeting the *BCL11A* exon 2 displayed significantly reduced engraftment compared editing of the GATAA enhancer, likely because of residual *BCL11A* expression remaining when targeting the enhancer²⁹. We believe that these studies highlight the need for more preclinical studies to fine-tune *BCL11A* expression as early evidence indicates that dramatic loss of *BCL11A* expression in HSPCs negatively influences hematopoiesis.

Hereditary persistence of fetal hemoglobin (HPFH) is a benign genetic condition where individuals display an unusually high expression of HbF and interestingly, co-inheritance of HPFH in β -thalassemia or SCD patients usually results in the amelioration of clinical symptoms^{30,31}. Exciting data was recently published last year in two manuscripts by Traxler *et al.*, and Ye *et al.*, where they employed Cas9/sgRNA genome editing strategies to genetically recreate HPFH in human HSPCs. Ye, Kan and colleagues recreated the Sicilian HPFH mutation by deleting 13kb of the β -globin locus³². Using a dual guide RNA approach, they achieved 31% targeted deletion in HSPCs that resulted in significantly higher HbF expression compared to controls, albeit this was achieved via plasmid delivery of the Cas9/sgRNA components. On the other hand, Traxler, Weiss and colleagues used a single guide RNA approach to mutate a 13 nucleotide sequence that is located in the promoters of the two genes that are responsible for HbF expression, *HBG1* and *HBG2*, to recreate a naturally occurring HPFH mutation³³. Using this approach in healthy CD34⁺ HSPCs, the number of HbF expressing differentiated RBCs rose from 18% to 58%. When they employed this strategy in SCD-derived HSPCs and then differentiated edited CD34⁺ cells into RBCs, they showed robust rescue of the sickling phenotype found under hypoxic conditions. While this study is extremely encouraging, the Cas9/sgRNA system was delivered as a lentivirus, which is not therapeutically applicable. It will be interesting to see efficiencies when the Cas9/sgRNA components are delivered as an RNP. Importantly though, both studies failed to show engraftment efficiencies and multilineage reconstitution potential of edited HSPCs as well as the genome editing frequencies in the bone marrow following long-term transplantation. Nevertheless, the strategy of recreating HPFH by genome editing in HSPCs is an interesting and promising viable therapeutic option for treating SCD in the future because it does not depend on harnessing the HR machinery in HSCs and recapitulates genetic variants that human genetics has shown to be safe and effective.

HR-based therapeutic genome editing to correct *HBB* gene mutations for the treatment of β -hemoglobinopathies

Oliver smithies and colleagues first showed in 1985 that HR could be used to insert DNA sequences into the human *HBB* locus, albeit at very low frequencies (10^{-6}), but this seminal work planted the seed that HR-based therapies could be a viable therapeutic option in the future. Since engineered nucleases stimulate HR as much as 10,000 fold³⁴, it was the logical next step to see if programmable nucleases could stimulate HR at the *HBB* locus in human HSPCs.

Along those lines, Hoban *et al.*, used ZFNs to target the *HBB* locus for a DSB while supplying either an ssODN or IDLV homologous donor vector to change the SCD-causing

E6V substitution back to wildtype. Although they observed HR frequencies above 10% *in vitro* (10.5% for IDLV and 17.9% for ssODNs), there was greater than a 20-fold drop in HR efficiencies when analyzing human cells in the BM of NSG 16-weeks post transplant (.21% for IDLV and .85% for ssODNs)¹². While these frequencies are obviously too low for therapeutic applications, this important paper highlighted the problem with targeting long-term repopulating HSCs for HR at the *HBB* locus. These same researchers were the first to show that the Cas9/sgRNA system could also be used to edit the *HBB* gene by HR at the E6V position, albeit only showing *in vitro* efficiencies in HSPCs up to 18%¹⁴. Soon after, Dewitt, Corn and colleagues published very promising data showing that by combining HBB-specific Cas9 RNPs and an ssODN intended to modify the E6V region, they could achieve a mean HR frequency of 12% (and up to 30%) *in vitro* in mobilized peripheral blood-derived HSPCs. After transplantation into NSG mice, they achieved an average of 2% in long-term repopulating HSCs (the highest being 6%)¹³. At the time, this 6-fold reduction in efficiencies from HSPCs (*in vitro*) to repopulating HSCs (*in vivo*) was the gold standard.

Dever et al (2016) published a method that combined HBB-specific Cas9 RNPs (complexed with synthetic modified guide RNAs) with rAAV6 delivery of a *HBB* homologous donor template to achieve HR at the β -globin in HSPCs with a GFP/tNGFR reporter, and also to functionally correct the E6V mutation by either point mutation correction or targeted knockin of a HBB cDNA²⁰. Consistent with previous reports, there was a 4.5 fold drop from HSPCs prior to transplant (16% GFP⁺) compared to long-term engraftment in the BM (3.5% GFP⁺), but when performing large-scale genome editing studies with a tNGFR reporter (80 million HSPCs), we observed a smaller reduction (12% *in vitro* versus 7.5% *in vivo*), suggesting tNGFR may be a better reporter for optimizing HR in HSCs at the beta-globin locus.

Importantly though, they developed a robust enrichment scheme that purified >90% targeted HSPCs early in the culture period that preserved stemness and resulted in a significant increase in the number of targeted human cells in the BM. Interestingly, Agudelo *et al.*, recently published a methodology for marker-free coselection of HR at the *HBB* in cord blood-derived CD34⁺ HSPCs by selection of dominant alleles via coediting of the ubiquitous and necessary sodium/potassium pump¹⁵. It will be necessary to repeat these studies under transplants setting to address whether this enrichment scheme equally purifies HSCs as it does short-lived progenitors. Collectively, these studies hold great promise for Cas9-mediated *HBB* gene correction in autologous HSCs for the treatment of SCD.

HR-based therapeutic genome editing to restore gene expression and function to treat the primary immunodeficiencies

The monogenic primary immunodeficiencies are prime candidates for GE-HSC gene therapy because 1) they can be cured by allo-HSCT when a suitable HLA-donor is found and 2) autologous-HSC gene therapy with integrating viral vectors has provided mixed results with insertional mutagenesis being a serious concern for the safety of the patients^{35,36}. One such primary immunodeficiency, X-linked chronic granulomatous disease (X-CGD) is caused by mutations in the *CYBB* that encodes the gp91^{phox}, which is one of the five components of

the NADPH oxidase activity in phagocytes that when mutated subsequently results in deadly fungal or bacterial infections³⁷. De Ravin, Malech and colleagues used the targeted gene addition strategy to overexpress the *CYBB* gene via the AAVS1 “safe harbor” site in exon 1 of the *PPP1R12C* gene in X-CGD patient-derived HSPCs²¹. By making a DSB with AAVS1 ZFNs (delivered as mRNA) while supplying an AAVS1 homologous *CYBB* donor via rAAV6, they showed functional correction of oxidase activity in X-CGD HSPCs (10–20% of normal HSPCs). It should be noted that they needed the synthetic MND promoter, rather than the endogenous *PPP1R12C* promoter to obtain robust functional correction and highlights the difficult ability to use the AAVS1 “safe harbor” as a means to drive high-level therapeutic protein expression in HSPCs. Moreover, while the AAVS1 has been asserted to be a safe harbor, the gene encodes for a phosphatase and the long-term safety and efficacy of inserting transgenes into this locus in stem cells has not been determined. These same researchers recently used the point mutation correction strategy to repair the X-CGD C676T disease-causing SNP in X-CGD patient HSPCs using the Cas9/sgRNA system (delivered as RNA) combined with ssODN delivery of the wildtype reversion donor DNA template¹⁶. Using this strategy, they were able to achieve 12–31% HR correction (analyzed by NGS of alleles) *in vitro* and importantly, only saw at most a 2-fold reduction in efficiencies in long-term repopulating cells in the BM of NSG mice at 16 weeks post transplant. While these *in vivo* frequencies are higher than most reports, it is possible that targeting efficiencies in HSCs is locus-dependent. It should also be noted that the researchers reported that when increasing the ssODN concentration to 400ug/ml, they not only saw less targeting efficiencies *in vitro* (9%), but also reduced frequency in the BM (2%), which highlights the importance of extensive optimization of the ssODN donor molecule. Nonetheless, these results, if repeatable, would be a step in the right direction for the clinical translation of gene editing cell and gene therapies for X-CGD. While promising, the ssODN strategy is limited in that it can only be applied to specific single point mutations.

***In vivo* HSC genome editing by HR**

In vivo HSC gene editing by HR would be an exciting advancement for hematopoietic gene therapy; although it comes with greater safety risks, such as specificity, immunogenicity, bio distribution, and efficacy. Nonetheless, Bahal, Glazer and colleagues described an interesting new *in vivo* gene editing approach that is independent of engineered nucleases. Researchers injected thalassemic mice with nanoparticles encoding triplex-forming peptide nucleic acids (PNAs) along with homologous donor DNAs that resulted in sustained elevation of hemoglobin levels and up to 7% *HBB* correction in the BM *in vivo*³⁸. However, it should be noted that this experimental approach required *in vivo* delivery of stem cell factor (SCF), which needs to be investigated further to clarify its safety in humans. While not used to target HSCs *in vivo*, Suzuki, Belmonte and colleagues, described a unique methodology for targeted genome editing using Cas9/sgRNA and homology-independent targeted integration (HiTi) that was shown to target dividing and non-dividing cells in the mouse retina³⁹. It would very interesting to try this methodology to target quiescent BM-residing HSCs *in vivo* for HR.

Conclusion

With the first genome editing of autologous HSCs clinical trial being approved by the FDA for the treatment of HIV using ZFNs targeting CCR5⁴⁰, the new paradigm within cell and gene therapy has begun. Now with the variety of genome editing tools in the toolbox, we can easily typeset the genetic code with base pair precision to: 1) reactivate silenced genes through enhancer disruption, 2) replace good genes with bad genes and 3) revert disease-causing nucleotide substitutions, and with that ease of modification, comes great responsibility for all the researchers and clinicians involved. While significant effort needs to be placed on studying hematopoiesis and nuclease off-target activity following HSPC gene editing in rigorous preclinical safety and tumorigenicity models, the history of gene therapy has taught us that pre-clinical models are incomplete in their ability to fully evaluate an approach and safety and efficacy can only be tested in FDA-approved phase I/II clinical trials. Although the genome editing studies we describe herein have vastly different strategies for restoring gene function, they all have one thing in common: The development of translational genome editing research programs that will provide life-long cures for devastating genetic disease of the blood and immune system. This is why we remain optimistic and positive about the future of HSC-focused genome-editing as it will increase the breadth of knowledge of hematopoiesis and stem cell biology that will ultimately help usher in the new era of cell and gene therapies.

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Key points

- Gene editing of hematopoietic stem cells (GE-HSC) holds tremendous promise for the development of novel blood and immune system cell and gene therapies.
- Therapeutic GE-HSC by NHEJ can knockout a gene (*CCR5* or *BCL11A*) or disrupt the enhancer of a repressive transcription factor (*BCL11A*) to increase gene expression, both will result in a disease-specific therapeutic benefit.
- Therapeutic GE-HSC by HR can be used to correct disease-causing nucleotide substitutions, functional gene correction as well as targeted transgene addition into “safe harbor” sites in the genome (like AAVS1) to elicit a therapeutic benefit.
- While HR has been shown to be more refractory in HSCs compared to NHEJ, recent robust selection methods have been reported to circumvent this problem.
- Future preclinical research must focus on the long-term safety and tumorigenic potential of the GE-HSC therapeutic cell candidates.

Figure 1. Therapeutic Gene editing of autologous HSPCs

The schematic depicts the overall scheme for therapeutic gene editing of autologous HSPCs. First, HSPCs are mobilized (or harvested from the bone marrow) using either G-CSF, or Plerixafor, or the combination of both. Second, HSPCs are harvested from PBMCs using the CD34 marker. Third, patient-derived CD34⁺ HSPCs are pre-stimulated in stem cell cytokine media to promote viability, stemness, and cell cycle entry. Fourth, stimulated HSPCs are then subjected to site-specific engineered nuclease-mediated double strand breaks (DSBs), which can be repaired by 1) non-homologous end joining (NHEJ), which results in small insertions or deletions that can reactivate or silence gene expression or 2) homologous recombination (HR) if a homologous donor is added (ssODN/IDLV/AAV6), which can be used to correct point mutations or functional gene correction. Fifth, once the gene-edited hematopoietic stem cells (GE-HSC) have been released using stringent potency, efficacy, and toxicity endpoints, the patient is then conditioned using myeloablative regimens, such as busulfan, to clear non-edited resident bone-marrow HSCs. Sixth, following myeloablative conditioning to clear the bone marrow stem cell niche, GE-HSC are reinfused into the patient. Lastly, patients are monitored closely for tri-lineage engraftment, pharmacological effect, clinical adverse responses, and reduction in symptomology for 24 months, in addition to the FDA requirement of a 15-year follow-up (followed on a yearly basis).

Table 1
Summary of the available engineered nucleases for therapeutic gene editing

Table 1 summarizes the different classes of engineered nucleases that can be used for therapeutic gene editing of human HSPCs. This table breaks down the relative key parameters of each nuclease including, ease of engineering, efficiencies, on target specificity, cell toxicity, and stage of clinical development. Abbreviations: ZFN= zinc finger nucleases; TALEN= transcription activator-like effector nuclease; Cas9/sgRNA= sgRNA-guided Cas9 endonuclease adapted from the clustered regularly interspaced short palindromic repeats (CRISPR) system.

Nuclease	Engineering	Efficiencies	Specificity	Toxicity	Clinical Development
ZFN	Difficult	High	Low	Medium	Phase I/II
TALEN	Easy	Medium	Medium	Medium	Phase I
megaTAL	Difficult	Medium	ND	Low	Preclinical
Cas9/sgRNA	Very easy	High	High	Low	Preclinical

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Summary of the available homologous DNA donor templates for therapeutic gene editing by homologous recombination

Table 2

Table 2 outlines the different donor platforms to achieve homologous recombination (HR) in human HSPCs. The table summarizes: 1) when the donors were first reported to achieve HR in HSPCs, 2) the genes targeted using each donor, 3) relative *in vitro* efficiencies before transplant into NSG mice (as a readout of stem and progenitor cell targeting rates), 4) relative *in vivo* efficiencies following long-term reconstitutions in immunodeficient mice (as a readout of stem cell targeting rates), 5) the versatility of the donor template (is the donor large enough to insert large gene cassettes as well as make single nucleotide changes), and 6) the relative toxicities in HSPCs. Abbreviations: IDLV= integrase-defective lentivirus; ssODN= single stranded oligodeoxynucleotide; AAV6= recombinant adeno-associated virus serotype six

Donor Delivery	First Reported use in HSPCs	Genes Targeted	Efficiencies <i>In vitro</i>	Efficiencies <i>In vivo</i>	Versatility	Toxicity
IDLV	2007	CCR5/IL2RG/ AAVS1/HBB	Medium	Low	High	Very low
ssODN	2015	HBB/CYBB	High	Low/Medium	Low	Medium
rAAV6	2015	CCR5/AAVS1 /HBB	High	Medium/High	High	Low