

Gene editing and its application for hematological diseases

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Abstract The use of precise, rationally designed gene-editing nucleases allows for targeted genome and transcriptome modification, and at present, four major classes of nucleases are being employed: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases (MNs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. Each reagent shares the ability to recognize and bind a target sequence of DNA. Depending on the properties of the reagent, the DNA can be cleaved on one or both strands, or epigenetic changes can be mediated. These novel properties can impact hematological disease by allowing for: (1) direct modification of hematopoietic stem/progenitor cells (HSPCs), (2) gene alteration of hematopoietic lineage committed terminal effectors, (3) genome engineering in non-hematopoietic cells with reprogramming to a hematopoietic phenotype, and (4) transcriptome modulation for gene regulation, modeling, and discovery.

Keywords ZFN · TALEN · CRISPR/Cas9 · Hematopoiesis · Blood disorders · Hematopoietic stem cells

Introduction

The accessibility of progenitor and terminal effector cells of the hematopoietic system for isolation and the ability to culture and manipulate them *ex vivo* has placed hematological disorders at the forefront of gene-based therapeutic medicine.

Classically, this has involved isolation of the cells from the marrow or peripheral blood and the delivery of a functional copy of the defective, disease-causing gene via integrating viral or non-viral gene transfer vectors and delivery back to the patient. In this strategy, gene regulation is typically governed by exogenous regulatory elements that mediate sustained and supraphysiological levels of the cargo. The integrating properties of the vector result in transmission to progeny cells that allows for life-long therapy. Moreover, the ability to correct a patient's own cells mitigates the incidence of graft-versus-host disease (GVHD) that can result in significant morbidity and mortality when allogeneic hematopoietic cell transplantation (HCT), the standard of care for multiple disorders, is employed. However, the integrating and sustained gene expression properties of gene therapy vectors can result in significant genotoxicity and/or dysregulation of endogenous gene(s) due to integration of the therapeutic cassette [1, 2]. Furthermore, genes not subject to the regulatory cues that govern normal gene expression may be undesirable [3]. Gene editing is a powerful approach that maintains the advantage of hematopoietic compartment-based therapies (cell availability, isolation, and *ex vivo* modification), while drastically improving safety. This approach refers to the precision alteration of a sequence of DNA at the genome level making it a permanent event that maintains gene control under endogenous elements subject to the regulatory mechanisms of the cell. Presented here are the options, considerations, and applications to date for gene editing in cells of the hematopoietic system.

At present, four major classes of nucleases are available for human genome engineering: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases (MNs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (Fig. 1). Each reagent shares the ability to recognize and bind a target sequence of DNA. Depending on the properties of the

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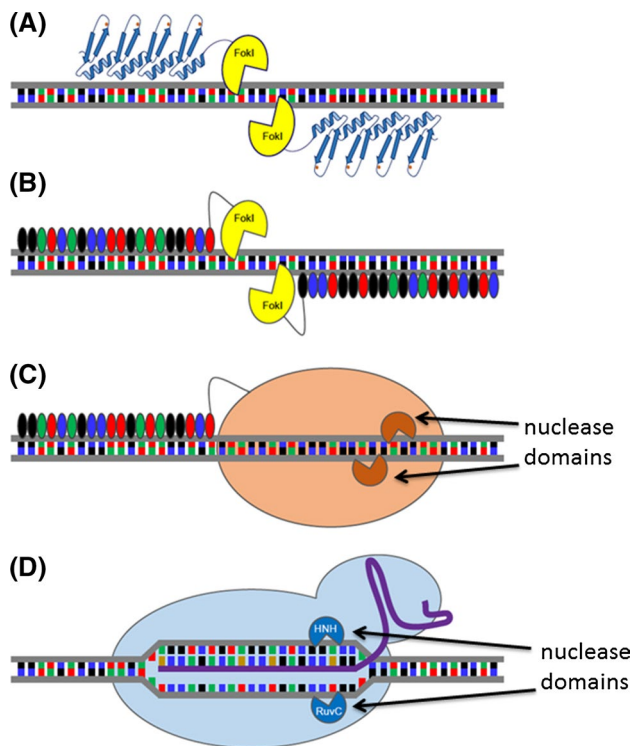


Fig. 1 Engineered nuclease architecture. **a** Zinc finger nucleases. ZFNs are dimeric arrays with DNA binding fingers (blue) that are tethered to the nuclease domains of *FokI* (yellow) that mediate the DNA cleavage event. The coordinating zinc ion required for DNA binding is shown in orange. **b** Transcription activator-like effector nucleases. TALENs act as heterodimers that are linked to a *FokI* nuclease domain. The TAL binding domains (colored ovals) interact with a single base of DNA by virtue of the repeat variable diresidues that recognize the four bases. The RVD HD (blue) binds DNA base C, NN (black) recognizes G, NI (green) interacts with A, and NG (red) binds T. **c** MegaTAL nuclease. TAL RVD units are fused to a custom engineered homing endonuclease (orange) that generates the DNA break. **d** Clustered regularly interspaced short palindromic repeats/Cas9. Watson-Crick base pairing of the gRNA (purple) complexed with the Cas9 protein (light blue) results in unwinding and cleavage of each strand of the DNA helix by the HNH and RuvC nuclease domains. In other configurations, the nuclease domains of Cas9 can be inactivated, and alternative functional domains included. Bases are all color-coded: A (green), C (blue), G (black), T (red), U (gold)

reagent, the DNA can be cleaved on one or both strands, or epigenetic changes can be mediated. These novel properties can impact hematological disease by allowing for: (1) direct modification of hematopoietic stem/progenitor cells (HSPC), (2) gene alteration of hematopoietic lineage committed terminal effectors, (3) genome engineering in non-hematopoietic cells with reprogramming to a hematopoietic phenotype, and (4) transcriptome modulation for gene regulation, modeling, and discovery (Fig. 2).

The critical considerations for gene-editing strategies are summarized in Table 1 and include: reagent choice, cost, availability of starting material reagents, engineering

process complexity/length of time, ability to be packaged in efficient gene delivery vehicles, and potential for broad applicability (e.g., ability to be multiplexed, activate, or repress genes). Moreover, the ability to isolate gene edited cells is an important consideration that will be impacted by the end user's cell type of choice, disease model, and purity required for infusion to achieve therapeutic effect based on the pathobiology of the individual and unique disorders broadly characterized as hematological diseases.

ZFNs

The Zif268 transcription factor has served as the template for generating custom Cys₂-His₂ ZFNs, with each zinc finger (ZF) comprised of approximately 30 amino acids in a $\beta\beta\alpha$ configuration that typically binds three nucleotides [4]. ZFNs are structured as dimeric left and right arrays, each containing between 3 and 6 ZF modules capable of targeting 18–36 bases of genomic sequence (Fig. 1a). In their basal state, ZFs lack intrinsic nuclease activity, necessitating their linkage to a non-specific catalytic domain of the type IIS restriction enzyme *FokI* [5]. The *FokI* nuclease functions only as a dimer, and thus a pair of ZFNs can be engineered, whereby they flank a target genomic sequence, allowing dimerization and cleavage of the intervening DNA, resulting in a double-strand break (DSB). To maximize specificity and prevent activity of the left–left or right–right arrays, the *FokI* domain has been engineered to function as an obligate dimer, such that only the left–right arrays create a DSB [6]. The DNA binding property of ZFs is such that adjacent finger modules appear to influence neighboring ZF recognition and binding, and this context specificity has frustrated approaches to construct ZFNs [7, 8]. Therefore, screening procedures were developed to identify three ZF arrays that accurately target their intended sequence, thus surmounting the problem of context-specificity [9–14]. Oligomerized pool engineering [9] and context-dependent assembly [13] allowed designer ZFNs to be obtained with greater affinity and higher specificity for the desired target sequence. Using these approaches, ZFNs are predicted to have a targeting capacity of approximately one site per 500 bp of human genomic DNA. This restricted targeting capacity, coupled with complicated generation procedures, makes ZFNs limiting [13, 15]. However, a valuable attribute of ZFNs is their small size that allows for simplified delivery as DNA, RNA, or in any viral expression/delivery vehicle.

TALENs

The pathogenic bacterial genus *Xanthomonas* uses specific DNA recognition proteins to regulate the transcription of target genes in the genomes of the plants they infect [16, 17].

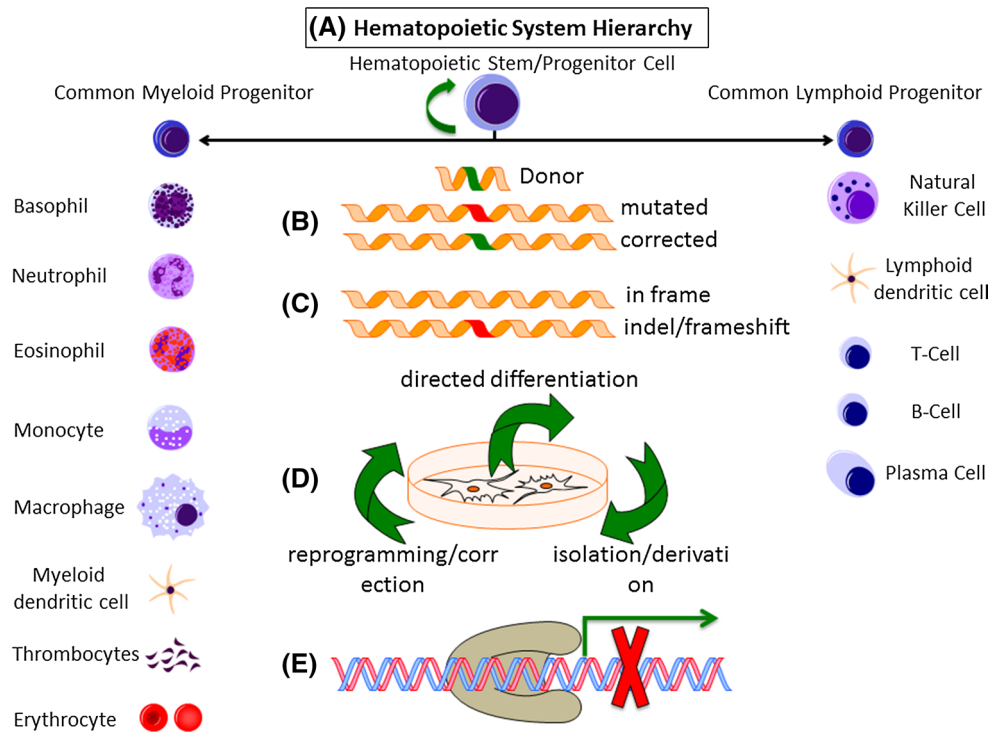


Fig. 2 Hematopoietic system and gene editing. **a** The hematopoietic system is derived from a hematopoietic stem/progenitor cell that is self-renewing and differentiates into the common lymphoid (CLP) and myeloid progenitors (CMP). The CL and CMPs give rise to the effector cells of the lymphoid and myeloid compartments, respectively. These cells include T- and B- lymphocytes, NK cells, plasma cells, and mononuclear, red blood and dendritic cells and thrombocytes. Gene editing. **b** Homology-directed repair. A single- or double-stranded DNA donor can be utilized as a repair template for the correction of disease-causing mutations (shown as a reversion of the red base to green). **c** Non-homologous end joining. DNA lesions can

be repaired by direct ligation of free ends by the error prone NHEJ pathway that results in insertions and deletions. These can cause frameshift mutations that disrupt the open reading frame of genes and cause loss of protein activity. **d** Autologous cellular engineering. Terminally differentiated cells can be obtained and modified by gene editing nucleases before or after reprogramming to pluripotency. The multipotent progenitors can then be employed for therapeutic lineage commitment. **e** Epigenome modification. The DNA binding properties of engineered proteins can be employed for transcriptome modulation for increased or decreased gene expression

Table 1 Nuclease hematological applications summary

Protein class	Cost (\$)	Engineering components	Generation Process	Function	Multiple	Vector packaging
MN	>5000	Restricted	Complicated	Nuclease	Yes (low)	Multiple
ZFN	5–10,000	Available	Complicated	Nuclease, nickase	Yes (low)	Multiple
TALEN	<1000	Freely available	Simplified	Nuclease, nickase, activator, repressor	Yes (low)	Few
CRISPR	<100	Freely available	Simplest	Nuclease, nickase, activator, repressor	Yes (expansive)	Multiple

Nuclease protein classes are shown at left followed by the approximate cost required to generate a single, gene specific candidate reagent. The engineering components refer to the core architecture that confers functionality and whether the building blocks are restricted to industry, available through and academic collaboration/purchase, or readily and freely available from not for profit agencies or commercial DNA synthesis. Function indicates the properties of the classes as nickases, activators, or repressors. Multiplex and vector packaging refer to simultaneous multigene targeting and the candidate's ability to be packaged and delivered in multiple delivery vehicles. MN, ZFN, and TALEN can target a small number of genes, while CRISPR/Cas9 possess a genome level multiplex ability. The size of TALENs makes them the most restrictive in regard to vectorability profile. To date, only one version (derived from *S. aureus*) of CRISPR/Cas9 can be packaged in an adeno-associated viral vector

These transcription activator-like (TAL) effectors are DNA binding proteins containing between 13 and 28 repeats, each of which is composed of 33–35 amino acids. The 12th and 13th amino acids of each repeat are known as the repeat variable diresidues (RVDs) and are responsible for DNA binding. Each RVD interfaces with a single nucleotide: RVD NI recognizes adenosine, HD interacts with cytosine, and NG binds thymine, while NN, NH, and NK all have varying specificities for guanine [18–20]. TAL effector nucleases (TALENs) utilize the modularity of these TAL effector RVDs to create novel DNA binding domains that are then linked to the nuclease domain of *FokI*. Akin to ZFNs, TALENs are designed in pairs that flank the target sequence, allowing the two *FokI* monomers to dimerize and create a DSB (Fig. 1b).

Because of the highly repetitive nature of the TAL effector, construction of each TALEN pair via PCR is challenging. To remedy this, a Golden Gate assembly procedure utilizes plasmid templates with the desired RVD building block flanked by type IIS restriction enzyme sites. This allows for ordered assembly of repeats to create the desired TAL effector DNA binding sequence [21]. These specifically designed sequences are then attached to the *FokI* nuclease domain to create the final TALEN construct [22–24]. Like ZFNs, specificity is increased due to the necessity of the TALEN pair to dimerize to cleave the desired genomic DNA sequence. The DNA binding capability of TAL arrays has been exploited further by fusing them to transcriptional activators to allow for gene expression modulation [25]. The ability to expand functionality beyond DNA cleavage, the greatly simplified engineering process using context-independent modules, and the higher frequency of target sites in the human genome (approximately one TAL site per 35 bp of DNA [15, 22]) has made the use of TALENs widespread. Furthermore, TALENs are readily deliverable as mRNA; however, their large, repetitive nature limits their use in some DNA and viral vectors [26].

MNs

Multiple MN families exist, and the class containing the LAGLIDADG protein motif has been primarily used for reengineering to recognize unique target sites [27]. These monomeric enzymes are derived from bacterial homing endonucleases and are constructed around a ‘central 4’ base pair recognition sequence [28, 29]. Recently, a hybrid MN-TAL (megaTAL) protein has been employed for mammalian genome modification (Fig. 1c) [30–32]. The small size and robust activity of MNs and megaTALs are a decided advantage; however, the complicated engineering procedure, requirement for the presence of the ‘central 4’ base composition in the target gene of interest, and potential for promiscuous activity at other genomic loci represent hurdles for expansive application [33, 34].

CRISPR/Cas9

The most recent addition to the genome engineering toolbox is derived from a so-called bacterial adaptive immune system that utilizes an RNA/protein complex [35]. This endogenous system operates in response to phage infection, resulting in the integration of short stretches (approximately 20 nucleotides) of invading phage DNA into the host genome between palindromic repeats [36–38]. These palindromic repeats provide for the ability of the bacterium to differentiate self from non-self to direct DNA cleavage of phage DNA while preserving the integrity of the bacterial genome [39]. The endogenous CRISPR/Cas9 system consists of two RNAs, one that targets the Cas9 protein to the desired DNA sequence (crRNA), and the other that hybridizes to the crRNA and helps to stabilize the Cas9 protein (tracrRNA) [40, 41]. Upon RNA/DNA hybridization, the HNH nuclease domain of Cas9 cleaves the complementary target DNA strand, whereas the RuvC nuclease domain cleaves the opposite strand (Fig. 1d). This cleavage requires the presence of a three nucleotide sequence termed the protospacer adjacent motif (PAM), the sequence of which is 5'-NGG-3' in the CRISPR/Cas9 system from *Streptococcus pyogenes* (Sp.), which to date has been utilized the most frequently for mammalian gene editing [42–45]. Shortly after delineation of how the CRISPR/Cas9 system functioned, it was shown that the crRNA and the tracrRNA could be shortened and combined into a single hybrid RNA, called a guide RNA (gRNA) [40]. This minimal RNA can be expressed as a PolIII transcript from a DNA vector or delivered as an in vitro-transcribed RNA species [43, 46]. A major power of the CRISPR/Cas9 system is that the Cas9 component is constant and can recognize multiple, independent gRNAs. Such a multiplexed targeting approach that is not easily achievable with ZFNs, MNs or TALENs, which require protein engineering for each genomic target [47–49]. Limitations of the CRISPR/Cas9 system include the PAM requirement and the potential for off target (OT) effects. Toward expanding the targeting capacity of Cas9, new variants with alternate PAMs have been described [50, 51], including a Cas9 system from *Staphylococcus aureus*, whose functional components are able to be packaged into the size-restricted adeno-associated virus (AAV) platform [52]. Rationale engineering of the existing Cas9 proteins and the discovery of new candidates, such as the recently described Cpf [53], have further expanded the PAM recognition repertoire and improved specificity, allowing for access to larger portions of the genome of interest [54–58].

Due to initial concerns related to high-level OT activity of CRISPR/Cas9 reagents [56], further modifications to the Cas9 or gRNAs have been undertaken. Truncated gRNAs

Table 2 Candidate nuclease properties and application matrix

Nuclease class	Cell type	Gene	Repair pathway	Disease	References
Meganuclease	HSPC, T-cell	<i>CCR5, TRAC</i>	NHEJ, HDR	HIV, leukemia	[31, 76]
ZFN	HSPC, T-cell	<i>CCR5, IL2RG, AAVS1, CXCR4, TRAC, TRBC</i>	NHEJ, HDR	HIV, leukemia, SCID-X1	[69, 70, 75, 78, 79, 81, 82]
TALEN	T-cell	<i>CD52, TRAC</i>	NHEJ	Leukemia	[32, 83, 90]
CRISPR/Cas9	T-cell	<i>TRAC</i>	NHEJ	Leukemia	[34]

The candidate nuclease and blood lineage cell type that they have been utilized in are shown with corresponding manuscript reference at far right. The DNA repair pathway employed for cell engineering is either NHEJ (non-homologous end joining) or HDR (homology-directed repair). The disease model and targeted gene are also shown (*CCR5* C–C chemokine receptor type 5, *TRAC* alpha chain of the T-cell receptor, *AAVS1* adeno-associated virus integration site 1, *IL2RG* interleukin 2 receptor, gamma, *CXCR4* chemokine (C–X–C motif) receptor 4, *TRBC* beta chain of the T-cell receptor)

appear to result in a more targeted activity profile [59, 60]. Direct alteration of Cas9 [57, 58, 61] or the generation of paired Cas9 nickase mutants capable of cleaving a single strand of DNA have been developed, such that only one of the nuclease domains is catalytically active [62]. Then, by designing two gRNAs complementary to opposite DNA strands, two single-strand breaks are generated to make a structure analogous to a staggered DSB [63, 64]. In addition, complete nuclease mutants lacking all catalytic activity can be fused to the nuclease domain of *FokI*, such that they function much like ZFNs and TALENs [65]. In addition, engineering studies have shown that Cas9 is amenable to modifications that retain its DNA binding ability allowing different domains to be fused to the complex to mediate effects, such as transcriptional activation, repression, and histone acetylation [66–68].

Hematopoietic cell genome engineering

Each of the major classes of gene editing proteins has been employed in hematopoietic lineage cells, primarily for HSPC and/or T-cells, and work to date is summarized in Table 2. HSPC gene modification is highly desirable, as these cells are the source of all lineages of the immune system over the lifespan of an individual (Fig. 2a). Furthermore, allogeneic HCT is a major therapeutic option for a multitude of disorders that are due to defects in the hematopoietic cell compartment (e.g., leukemia, Fanconi anemia, immunodeficiencies, Wiskott–Aldrich syndrome, and hemoglobinopathies) as well as those without an overt blood cell developmental defect (e.g., lysosomal storage disease, Wilms' tumor, and hemophilia). As such, the ability to precisely modify HSPC in an autologous manner would result in life-long therapy that greatly mitigates the side effects related to GVHD, and transcriptional- and integrating vector-related genomic perturbation.

Following nuclease-mediated DNA cleavage, two competing DNA repair pathways are employed for resolution of the break that left untended is highly toxic: homology-directed repair (HDR) or non-homologous end joining (NHEJ). HDR is an exquisitely precise repair pathway utilizing a homologous sequence of endogenous or exogenous DNA as the repair template—the latter of which allows for the acquisition of a user-defined sequence at the targeted locus (Fig. 2b). NHEJ is a mutagenic DNA repair pathway where broken DNA ends are processed and ligated back to one another in a manner that commonly results in insertions/deletions (indels) that can disrupt the targeted open reading frame, resulting in a loss of function mutation (Fig. 2c).

Initial efforts to mediate HDR in HSPC showed low overall rates of <1 % using ZFNs specific for the *CCR5* locus and a donor, each delivered as integrase-deficient lentiviral vector (IDLV) particles [69]. In 2014, members of this same group improved HDR rates in HPSCs from both healthy donors and a patient with X-linked severe combined immunodeficiency caused by an *IL2RG* gene mutation [70]. To accomplish this, nuclease/donor delivery and HSC culture conditions were optimized to allow for the HDR donor derived insertion of exons 5–8 into the *IL2RG* locus [70]. This resulted in restoration of the proper reading frame, with transcription mediated by the endogenous promoter, and is a broadly applicable strategy for the correction of SCID-X1 mutations that occur downstream of exon 4 [70]. The IDLV donor was delivered first followed by ZFN mRNA to cells cultured in the presence of 16,16-dimethyl-prostaglandin E2 (PGE2) [71, 72] with or without StemRegenin 1 (SR1) [73]. PGE2 promotes HSC survival, homing, and expansion [74], and SR1 is an aryl hydrocarbon receptor antagonist that facilitates ex vivo expansion and retention of engraftment potential [73]. The use of both agents was additive in the resultant ability to recover the highest levels of gene-corrected cells that were

used for primary and secondary engraftment of immunodeficient mice [70].

A second study by Wang assessed HDR in CD34+ HSPCs derived from fetal liver or cord blood using ZFNs delivered as mRNA and a donor template borne on an AAV serotype 6 vector [75]. HDR-driven insertion of a GFP expression cassette was observed at rates of 17 % at *CCR5* and 26 % at the *AAVS1* loci in G-CSF mobilized peripheral HSPCs and 19 % (*CCR5*) and 43 % (*AAVS1*) in HSPCs from fetal liver [75]. Importantly, this group showed that the CD34+ cells treated in bulk resulted in modification of the subfraction of cells with a CD34⁺CD133⁺CD90⁺ phenotype that represents a primitive population of highly engraftable precursors [75]. In addition, using a donor template packaged in AAV-6, Sather et al. further employed electroporation of megaTAL nuclease mRNA that targeted the *CCR5* locus in mobilized CD34s cultured with SR1 and achieved maximal HDR rates of 35 % [76]. In this same study, AAV-6 was also highly capable of mediating HDR in primary T-cells, utilizing an HDR targeting construct containing homology arms to the *CCR5* locus flanking cargo comprised of: the C46 gene that encodes an HIV restriction protein that confers resistance to HIV, the PGT145 anti-HIV chimeric antigen receptor (CAR), or the CD-19 CAR, respectively [76]. As such, HDR-mediated insertion of the exogenous expression cassettes is paired with loss of *CCR5* to confer greater resistance to HIV infection by *CXCR4* HIV strains, HIV target lysis mediated by PGT145, or dual HIV resistance and ability to abolish secondary CD19+ B cell tumors [76]. These studies complement another study reported by Wang and colleagues that showed robust HDR in CD4 and CD8 cells, further supporting nuclease and AAV-based cellular engineering for infections, autoimmunity or malignancies [77].

The ability to selectively delete receptors/genes that contribute to the primary or secondary disease process holds tremendous potential for engineering cells for therapy. At present, two disorders with a hematological component are at the leading edge for translational application of engineered nucleases for mutagenic NHEJ-based cellular therapy. The first in human use of gene-editing nucleases employed a *CCR5*-targeted ZFN and is being utilized as part of an autologous therapy in HIV patients [78]. The next-generation strategies utilizing tandem *CCR5* and *CXCR4* ZFNs, as well as the above HDR-based strategies, have been used preclinically to establish proof of principle for engineering pan HIV resistant T-cells [76, 79].

B-cell leukemia is a common adult and childhood malignancy with a poor prognosis with relapse. The treatment option of choice at present is allogeneic HCT with the rationale that HLA-mismatched donor cells will recognize tumor cells as foreign as part of a graft-versus-leukemia

(GVL) effect. However, due to off-tumor GVHD-associated pathology, autologous-based therapies employing CARs for the CD19 epitope on malignant (and normal) B-cells have been used as part of an individualized treatment strategy that has resulted in some extraordinary successes [80]. However, this personalized approach can result in variability in transduction efficiencies and cell numbers [80] that can be delivered to patients and thus is impractical for wider spread use. As a solution to this, engineered nucleases specific for components of the T-cell receptor (TCR), a multiunit complex that is responsible for antigen recognition has been developed. This strategy has allowed for the generation of cells that are greatly reduced in their GVHD-based reactivity toward healthy tissue and represents an off the shelf, universal donor therapy option.

Provasi et al. in 2012 employed ZFNs to selectively disrupt the genes that encode the alpha and beta chains of the TCR to render the cells TCR null [81]. The TCR negative cells were then paired with a CAR for Wilms' tumor to mediate anti-tumor effects [81]. In the same year, Torikai et al. employed ZFNs for the alpha chain of the TCR to generate non-alloreactive cells that express the CD19 CAR [82]. Robust TCR disruption rates have also been reported employing megaTALs and TALENs, respectively [31, 83]. Extending this approach to confer greater therapeutic benefits, Poirot et al. employed a multiplex strategy utilizing TALENs for the TCR-alpha chain and CD52 genes, respectively [32]. CD52 encodes a receptor that can be targeted by the monoclonal antibody alemtuzumab that is used therapeutically for leukemia [84]. However, because CD52 is present on both T- and B-cells, T-cells can be depleted and TALEN-mediated disruption of the gene confers a survival advantage to the engineered cells, following administration of alemtuzumab, which may potentiate their therapeutic value [32]. An important finding from this study was that the simultaneous generation of DNA DSBs at the TCR and CD52 loci resulted in translocation events [32]. An extension of this consideration is the potential for overlapping areas of sequence homology to the intended target site, giving rise to OT DNA DSBs. Genome-level screens have shown that these OT events can result in translocations and/or indels [34, 85, 86]. Previous work has shown that the *CCR5* ZFN, TALEN, and CRISPR/Cas9 reagents can have OT activity at the *CCR2* locus that shares close sequence homology to *CCR5* [87–89]. In an effort to define the potential for OT effects at the TCR-alpha locus, members of this group performed a genome-level comparative analysis for a TCR-alpha megaTAL, TALEN, and CRISPR/Cas9 reagent, and observed OT effects for the megaTAL but not the other classes of reagents [34]. Comprehensive OT mapping is of great importance, as it will aid in safer cellular engineering and in the development/re-engineering of more target-restricted reagents for translational application.

Despite the presence of OT sites, both ZFNs and TALENs have been utilized in humans. The use of TALENs maximized safety considerations due to the engineering process by including a suicide gene to allow for targeted deletion of infused cells. Using the co-delivery/targeting approach that was validated in a murine model [32], Qasim and colleagues engineered allogeneic T-cells to be TCR-alpha and CD52 null that expressed a CD19 CAR. The cells were infused into a pediatric patient with relapsed B-cell leukemia, who has subsequently shown cytogenetic and molecular remission [90]. As such, the ability to deliver genome-editing reagents to hematopoietic progenitors and effectors represents a translational pipeline for both individualized and universal approaches for hematological disorders.

Future directions

While a major advantage for hematopoietic cell modification is their relative accessibility for explant, modification, and reinfusion, the extended manipulation and culture ex vivo have the possibility of negatively impacting cellular phenotype and engraftability rates [91]. Advances in culture techniques with small molecule regulators that maintain and sustain primitive phenotypes (e.g., SR1 [73]) aid in circumventing this obstacle. However, for disorders such as the bone marrow failure syndromes, where progenitor cells are in short supply, future therapies may include in vivo gene editing or the development of the next-generation cellular engineering-based differentiation methodologies.

Blood development occurs within a complex milieu of progenitor cells and a microenvironment with instructive and supportive signals that are a crucial part of the multimodal maturation process. Hematopoiesis occurs in two waves: embryonic and definitive. The embryonic phase is characterized by the generation of transient cell populations and occurs in the yolk sac giving rise to myelo-erythroid effectors [92]. The definitive wave results in the self-renewing hematopoietic stem/progenitor cell that develops in the aorta/gonad/mesonephros region of the embryo and gives rise to all of the mature blood lineages (erythroid, myeloid, and lymphoid) [92, 93]. These cells then migrate and expand in the fetal liver before transiting to and taking up residence in the bone marrow cavity where they remain through life [94]. This latter developmental process involves endothelial and hematopoietic precursors ultimately giving rise to the definitive HSPC [95].

A decided advantage of in vivo gene editing would be that HSPCs could be targeted in situ, with the native bone marrow stroma providing the proper developmental programming instructions. Indeed, murine models of non-hematopoietic in vivo genome modification have been reported, whereby a gene-specific nuclease and a donor

template are delivered to a target organ(s), and the modification is done at the organ and DNA levels [96]. However, modification of HSPCs at sufficient frequency may be limiting in the context of gene delivery, HDR rates, and therapeutic effect related to whether the targeted/corrected cell possesses a preferential selective outgrowth profile. Therefore, the generation of primitive or terminally differentiated hematopoietic effector cells from an induced pluripotent stem cell (iPSC) intermediary holds tremendous promise for future regenerative medicine therapies.

The introduction of the iPSC reprogramming cocktail of *c-myc*, *Oct3/4*, *Klf4*, and *Sox2* into terminally differentiated cell types results in a population of renewable cells with multilineage potential [97]. Megakaryocytes are able to be derived from iPSCs under GMP conditions [98] and such a strategy would allow for a renewable population of cells for a bank of off-the-shelf platelet products. Employing an allogeneic approach for T-cell immunotherapy, Themeli et al. [99] generated iPSCs from T-cells, redifferentiated them into T-cells that resulted in a $\gamma\delta$ T-cell phenotype, and transduced them with a CAR for tumor therapy in a murine model. Such an approach would result in the standardization of cell doses and transduction levels that have been variable in trials to date [80, 100, 101]. Furthermore, this procedure could be optimized to produce T-cells with a stem memory phenotype, a cell that is normally at low levels in the peripheral blood and is highly desirable due to its ability to self-renew, and give rise to memory and effector cells [102, 103]. Toward developing red blood cells (RBCs) in vitro, Giani and colleagues used a population-based genetics analysis and discovered a naturally occurring loss of function variant of the *SH2B3* gene that results in increased RBC counts [104]. Using a CRISPR/Cas9 gene deletion strategy, they were subsequently able to inhibit *SH2B3* expression and recapitulate the variant phenotype resulting in enhanced RBC precursor formation in vitro [104].

The ability to correct disease-causing mutations in terminally differentiated cells with the subsequent capacity to differentiate them into repopulating blood progenitors would be a paradigm shift for treating disorders of the hematopoietic system. Gene correction of fibroblast cells from patients with Fanconi anemia has been reported using ZFNs and CRISPR/Cas9 reagents [105, 106], as has their ability to be induced to pluripotency, albeit with no in vivo blood development potential [106, 107]. Using iPSCs as a starting point for HSPC generation, however, has largely resulted in progenitors of the primitive type that do not allow for long-term repopulation and engraftment [95, 108]. Toward recapitulating the critical supportive bone marrow microenvironment, groups led by Amabile [109] and Suzuki [110] injected human iPSCs into immune-deficient animals resulting in teratoma-derived CD34+CD45+ that arose from a complex, multilineage formative environment with the cells being capable of

serial engraftment. Due to the clinical hurdles of a teratoma-based approach, a highly promising avenue of investigation is the use of in vitro vascular cell co-culture to provide the instructive niche required for HSPC development [95]. Sandler and colleagues utilized stable adenovirus E4ORF1 gene expression to generate VeraVec™ cells that serve as a supportive matrix of cells. This group further defined a transcriptional cocktail of four genes *FOSB*, *GFII*, *RUNX1*, and *SP11* (FGRS) that when upregulated in primary endothelial cells that were cultured with VeraVec™ resulted in progenitors capable of multilineage engraftment with the ability to be serially transferred [111]. As such, gene correction/modification of endothelial cells, either primary or derived from iPSCs, could be potentially used as a foundational template for autologous HSPC generation.

The modulation of the FGRS gene set observed to drive HSPC development represents an ideal platform for the application of genome-level screens for genes that can be expressed/repressed to enhance the further/refined development of therapeutically viable HSPCs in vitro (Fig. 2d). The most powerful system for such an approach is the CRISPR/Cas9 system by virtue of its multiplex ability, whereby multiple different gRNAs can simultaneously target genes and the Cas9 can act as a nuclease, transcriptional activator, or repressor to modulate the intended genes [41, 47, 112]. In 2014, Shalem et al. generated over 64,000 gRNAs capable of targeting more than 18,000 genes and performed genome-level knockout studies to discover genes previously unknown in melanoma drug resistance [113]. In 2015, a study to achieve saturating mutagenesis of the *BCL11A* erythroid enhancer that is a direct regulator of hemoglobin F (HbF) levels [114] was undertaken to define and disrupt critical sequences to mediate HbF induction [115]. As such, the ability to precisely target this non-coding sequence may facilitate threshold levels of functional HbF for treating β -hemoglobinopathies [115].

Less permanent, transcriptome/epigenome modifications may also be employed to determine gene candidates that drive HSPC development, and this platform may hold direct therapeutic value for primary patient-derived cells. Konermann et al. designed more than 70,000 gRNAs that target upstream of every human coding isoform and performed a genome-level screen with a Cas9 transcriptional activator system to discover genes that were upregulated resulting in resistance to a *BRAF* inhibitor involved in melanoma drug resistance [49]. Specific to hematopoietic therapeutic intent, Hilton and colleagues used the epigenome modifying properties of a Cas9-acetyltransferase fusion complex to an enhancer region of the β -globin locus control region and observed significant upregulation of the downstream *HBE*, *HBG*, and *HBD* genes [116].

Permanent (HDR and NHEJ) and less permanent (gene/chromatin regulation) modifications represent

powerful tools for improving existing, developing, and discovering new treatment approaches in cells of the hematopoietic lineage. These methodologies will intersect with novel stem cell development and differentiation approaches and will synergize for the development of the next-generation cell- and genome-based therapies. Guiding principles for the design and use of gene editing reagents for hematopoietic cell modification and therapy will encompass multiple points of emphasis: the cell and disease model will inform the choice of reagent whose activity, specificity, and deliverability properties will dictate their application in the cell and model of choice. Each reagent has attributes and limitations that must be considered in the context of the cell target and/or disease model. For instance, ZFNs and MNs can be readily delivered to most cell types using a multitude of delivery vehicles; however, their complex engineering process can make their application limiting. The more easily generated TALEN and CRISPR/Cas9 reagents level the playing field by placing the technology in the hands of the entire scientific community; however, their size and the target sequence requirements can make delivery and access to certain portions of the genome challenging. Importantly, the rapidly evolving area of study with discovery of new gene-editing reagents is additive to the field and increases the flexibility of the applications for scientists but does not relegate previous classes of nucleases to obscurity.

The transition from bench to bedside for the application of gene editing reagents has been described for T-cells in the context of HIV therapy and leukemia treatment [78, 90]. As such, the developmental pipeline has been established, and the continued application of gene editing in novel ways in cells of the hematopoietic system will aid in more accurate disease modeling that will facilitate more effective autologous, gene-based, individualized therapies for more uniform therapeutic responses. This process will be further augmented by the largely freely available starting materials and enhanced culture techniques that allow the hematological community at large to consider, plan, and execute novel gene editing strategies.

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