CRISPR to fix bad blood: a new tool in basic and clinical hematology

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ABSTRACT

Advances in genome engineering in the last decade, particularly in the development of programmable nucleases, have made it possible to edit the genomes of most cell types precisely and efficiently. Chief among these advances, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is a novel, versatile and easy-to-use tool to edit genomes irrespective of their complexity, with multiple and broad applications in biomedicine. In this review, we focus on the use of CRISPR/Cas9 genome editing in the context of hematologic diseases and appraise the major achievements and challenges in this rapidly moving field to gain a clearer perspective on the potential of this technology to move from the laboratory to the clinic. Accordingly, we discuss data from studies editing hematopoietic cells to understand and model blood diseases, and to develop novel therapies for hematologic malignancies. We provide an overview of the applications of gene editing in experimental, preclinical and clinical hematology including interrogation of gene function, target identification and drug discovery and chimeric antigen receptor T-cell engineering. We also highlight current limitations of CRISPR/Cas9 and the possible strategies to overcome them. Finally, we consider what advances in CRISPR/Cas9 are needed to move the hematology field forward.

Introduction

Genome engineering is defined as the deliberate modification of an organism’s genetic material. It has been used since the early 1980s to study the impact of DNA mutations in human disease precisely and has helped to unravel the genetic basis of many malignancies and to advance their diagnosis, prevention, and treatment. Genome engineering to introduce defined alterations has traditionally employed homologous recombination strategies to modify a gene of interest (gain- or loss-of-function) using segments of exogenous DNA.1 To achieve homologous recombination in the “pre-nuclease” era, large DNA sequences homologous to the target sequence, containing sequence changes designed to produce the desired modification, were introduced into the nucleus of the receiving cell. This technology depends heavily on chance since the DNA construct is expected to interact with the target and induce gene conversion upon recombination of DNA homology arms. The success rate of this technology was historically extremely low, which together with the complexity in designing targeting vectors, and the time and resources required, put it out of reach of some researchers. However, with the advent of highly-specific chimeric nucleases (which are able to recognize 18 or more base pairs) to induce locus-specific DNA double-strand breaks (DSB), the efficiency of homologous recombination rose substantially (e.g., becoming more than 40,000 times more efficient), depending on the experimental system. The use of such nucleases has increased in recent years with the development of meganucleases, zinc-finger nucleases, and transcription activator-like effector nucleases.
(TALEN), opening new horizons for genome manipulation (Figure 1A). Nevertheless, designing the aforementioned nucleases to induce DSB in specific loci relies on predicting protein-DNA interactions, which remains technically challenging, and so these nucleases are not practicable in every laboratory. By contrast, the recent breakthrough of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) technology, which is based on nucleic acid interactions, has enabled specific genome editing in a versatile and uncomplicated manner over previous nucleases, and has revolutionized the field of genome engineering (Figure 1B, Table 1).

CRISPR sequence repeats were first reported in *Escherichia coli* and were later characterized in *Haloferax mediterranei*, an archaeon isolated from a hypersaline environment in Alicante (Spain). Soon after, these sequence repeats were identified as a part of a primitive adaptive immune system in prokaryotes. In 2012, Doudna and Charpentier demonstrated the first use of CRISPR/Cas9 to introduce site-specific DSB in target DNA based on the ability of a single guide RNA (gRNA) to direct sequence-specific targeting.

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**Figure 1.** Nucleases used in genome engineering. (A) Pre-CRISPR nucleases such as meganucleases, zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) are proteins that bind directly to DNA. Meganucleases are naturally occurring restriction enzymes that recognize between 12 to 40 base pair sequences, although they allow for some restricted level of engineering to make them specific to certain loci. Engineered ZFN induce specific double-strand breaks (DSB) acting as dimers. Each monomer is composed of a non-specific cleavage domain from the FokI endonuclease and a zinc-finger protein array where each domain bind three base pairs. ZFN dimers are able to recognize 18–24 base pairs in the target sequence, allowing for highly specific targeting. TALEN are designed combining the same non-specific endonuclease FokI domain and transcription activator-like effector (TALE) proteins. TALE proteins present a central domain responsible for DNA binding, which interacts specifically with just one nucleotide. One of these domains consists of monomers of 34 amino acid residues, two of which are responsible for nucleotide recognition. This makes the design of TALEN very straightforward in principle. (B) In contrast to the nucleases described in (A), the Cas9 endonuclease of the CRISPR/Cas9 system binds to the target DNA through the guide RNA (gRNA) by Watson-Crick base pairing. The gRNA is composed of two molecules of RNA: (i) the CRISPR RNA (crRNA) (green nucleotides) of which 20 nucleotides (white bold in top panel in (A), black bold in middle and bottom panels in (A)) show strict homology to the target and (ii) the trans-activating crRNA (tracrRNA), which binds to the crRNA and to the Cas9 nuclease (yellow structure). The gRNA brings Cas9 the target sequence, which is always adjacent to a protospacer adjacent motif (PAM) sequence. The PAM sequence for the most used Cas9, isolated from the bacteria *Streptococcus pyogenes*, is NGG (TGG in the white box). Notes: white arrows in (A) represent hydrogen bonds between amino acids from proteins and DNA base pairs; thick black arrows point to the site of cleavage of the nucleases.
specific Cas9 double-stranded DNA cleavage, illustrating the wide-ranging application of CRISPR as a genome-editing technology. Indeed, the CRISPR/Cas9 system was first successfully used in human cells in 2013. The essential components of this technology include a gRNA that binds specifically to a 20-base pair sequence of interest, and the Cas9 enzyme – an endonuclease that introduces a DSB. Additionally, a conserved dinucleotide-containing protospacer adjacent motif sequence upstream of the gRNA-binding site is required by the endonuclease to recognize and cleave the sequence. In the case of Cas9 isolated from Streptococcus pyogenes, the most widely used nuclease, the protospacer adjacent motif sequence is NGG (Figure 2A). If these conditions are fulfilled, CRISPR/Cas9 can be directed to cleave any genomic sequence. Subsequently, the DSB (in eukaryotic cells) triggers endogenous cellular DNA-repair pathways that can be exploited either to generate gene knock-outs based on the introduction of insertions or deletions (indels) at the DSB by non-homologous end joining (NHEJ), or for genome editing by introducing an engineered template DNA via homology-directed repair (HDR) (Figure 2A). In contrast to the protein-DNA interactions of other nuclease editing systems, CRISPR relies on Watson-Crick pairing between RNA and DNA. Thus, researchers keen to perform gene editing require only a basic knowledge of molecular biology to design a targeting system against a locus of choice. Here, we will focus mainly on work done with the Cas9 nuclease, although it is worth mentioning that the CRISPR/Cas9 system can include many other enzyme variants with numerous functions that are suitable for applications beyond gene editing (Table 2).

In comparison with engineered nucleases, CRISPR/Cas9 is an easy-to-use genome-editing tool, and several different CRISPR/Cas9-component delivery methods are available for in vitro, ex vivo and in vivo applications (Table 3). Generally, Cas9 and gRNA can be introduced into cells in several formats, such as plasmid DNA, lentiviral vectors, mRNA, or more recently by using pre-assembled ribonucleoprotein complexes (Table 4). Indeed, ribonucleoprotein complexes are perhaps the best choice for clinical applications given their high efficiency and short window of action, which reduces the duration of nuclease exposure and, consequently, the possibility of undesired off-target effects. In the hematopoietic setting, CRISPR/Cas9 gene editing has been applied both in research and in clinical translation studies (Figure 2B). In disease modeling, CRISPR/Cas9 technology coupled to next-generation genomics allows researchers to faithfully recapitulate the genetic mutations seen in patients with clonal hematopoiesis or leukemia. In the clinical setting, the main goal is to employ CRISPR/Cas9 to treat diseases of the blood and immune system. With this view, several biotechnology companies have pipelines to develop and...
evaluate therapeutic CRISPR/Cas9 gene editing to correct mutations in pathologies such as autoimmune diseases and multiple myeloma.

Given the exponential output of scientific publications using CRISPR in the last years, there is a clear need to synthesize the latest data on gene editing. With this in mind, here we describe recent advances in the use of CRISPR/Cas9 in hematologic research and clinical translation. We also consider the limitations of CRISPR/Cas9 technology for therapeutic applications, their possible solutions, and how the field of hematology may move forward. While CRISPR/Cas9 gene editing is hoped to be a treatment for many hematologic diseases, large clinical trials are needed to evaluate the efficacy and safety of CRISPR/Cas9 for patients, a promising area that will undoubtedly expand in the near future.

CRISPR/Cas9 in hematology research

**In vitro gene editing of blood cells**

Hematopoietic cell lines are a robust model for validating gRNA specificity and CRISPR/Cas9 experimental design because of their easy manipulation and expansion, and enrichment of edited cells. In this respect, cell lines have been employed: (i) to analyze gene function upon NHEJ-mediated gene disruption; (ii) to insert a point mutation or a DNA fragment; (iii) to correct a point mutation, and (iv) to create chromosomal translocations. Although valuable as a proof-of-concept approach, editing success cannot always be extrapolated to difficult-to-edit primary cells.

Many studies have reported successful CRISPR/Cas9-mediated gene editing in hematopoietic cell lines. As a consequence of indels introduced by NHEJ-mediated DSB

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**Figure 2. CRISPR/Cas9-mediated genome editing in hematology.** (A) Illustration of the CRISPR/Cas9 system. Site-specific DSB are produced by CRISPR/Cas9 and are either repaired by NHEJ, introducing indels that provoke gene disruption, or by HDR that, in the presence of a DNA template, creates insertions, translocations, or point mutations. gRNA: guide RNA; DSB: double-strand break; NHEJ: non-homologous end joining; HDR: homology-directed repair; indel: insertions and deletions. (B) Applications of CRISPR/Cas9 technology in hematologic research and human therapy. HIV: human immunodeficiency virus; CAR: chimeric antigen receptor; CHIP: clonal hematopoiesis of indeterminate potential.
repair, the resulting frameshift or nonsense mutations can give rise to truncated proteins that have a gain- or loss-of-function. This is a considerably faster approach than conventional homologous recombination-based gene targeting to create gene knock-outs and, given its simplicity, CRISPR/Cas9 is poised to become the method of choice for knock-out studies in most cases. Moreover, CRISPR/Cas9 is superior to RNA interference approaches for deciphering gene function, since the latter produce hypomorphic phenotypes that do not always mirror the complete loss-of-function that often occurs with genetic mutations.

Table 2. Comparison of the most widely used Cas nucleases.

| Cas nuclease | Identified from* | Targeted molecule | Key features | Most frequent application |
|--------------|------------------|-------------------|--------------|--------------------------|
| Cas9 | Streptococcus pyogenes | DNA | • DSB proximal to PAM (blunt ends) | Knock-out/Knock-in |
| dCas9 | Mutant form of Cas9 | DNA | • Lacks endonuclease activity | Regulation of gene expression (CRISPRi/Cas9) |
| Cas9 nickase | Mutant form of Cas9 | DNA | • Single-strand break | Knock-in |
| Cas12a (Cpf1) | Acidaminococcus sp. Lachnospiraceae bacterium | DNA | • DSB distal to PAM (staggered ends) | Knock-out/Knock-in |
| Cas13a (C2c2) | Leptotrichia wadei Leptotrichia buccalis Leptotrichia shahii | RNA | • Lacks a DNase domain | Regulation of gene expression (CRISPRa) |

Table 3. Summary of delivery approaches for CRISPR/Cas9 components.

| Delivery vehicle | Advantages | Disadvantages |
|------------------|------------|--------------|
| Physical approaches | | |
| Microinjection | • Delivered directly into cell of interest | • Time-consuming |
| Electroportation | • Standardized protocols available | • Requires expertise |
| • High efficiency | • Limited to *in vitro* and *ex vivo* cells | |
| • High efficiency with plasmids | • Cell cytotoxicity | |
| | • Some cells are not susceptible | |
| Viral-based approaches | | |
| Lentivirus | • Robust, stable expression | • Immune response, but low |
| • Allows delivery in complex and primary cells | • Limited packaging capacity (18 kb) | |
| • Efficiency variable with construct length | • Random genome integration | |
| | • Off-targets from Cas9-constitutive expression | |
| | • Expertise and safety issues | |
| Adenovirus | • No genome integration | • High immune response |
| • Transient expression | • Limited packaging capacity (35 kb) | |
| • Reduced off-targets | • Expertise and safety issues | |
| • High efficiency | | |
| Adeno-associated virus | • No genome integration | • Immune response, but very low |
| • Reduced immunogenicity and cytotoxicity | • Limited packaging capacity (4.5 kb) | |
| • Reduced off-targets | • Costly | |
| • High efficiency | • Expertise and safety issues | |
| Non-viral approaches | | |
| Lipid nanoparticles | • Simple manipulation | • Dependent on cell type |
| | • Low cost | • Endosomal degradation | |
| | • Reduced off-targets | | |
| Cell-penetrating peptides | • Deliver intact ribonucleoproteins | • Variable penetrating efficiency |
CRISPR/Cas9-mediated cleavage followed by HDR has been employed to introduce point mutations or gene fragments into specific loci using donor template DNA flanked by 3’ and 5’ sequences homologous to the target region. However, creating a knock-in allele by homologous recombination of a targeting construct using embryonic stem cells (which could be used to produce a mouse model) or by CRISPR/Cas9 is not so different in terms of cost and time.23 CRISPR/Cas9 gene editing can help to elucidate the role of patients’ mutations by generating cellular models carrying these lesions. Along this line, patients with myelodysplastic syndromes frequently have mutations in splicing genes such as the P95H mutation in Additional sex combs like 1 (ASXL1) gene, frequently mutated in myelodysplastic syndrome, chronic myelomonocytic leukemia, and AML.24,25

In acute myeloid leukemia, driver mutations can also cause and/or maintain leukemia24 and precise AML models are needed to develop novel, target-focused therapeutic potential.23 In acute myeloid leukemia, driver mutations were successfully used in human cell lines and human HSC to generate chromosomal translocations resembling those described in acute leukemia, such as t(8;21)/RUNX1-ETO, t(11;19)/MLL-AF4, t(4;11)/KMT2A-AFF1/AFF1-KMT2A and t(11;19)/MLL-ENL.26-28 This achievement is relevant because the model-generated K562 cell line and the K562/Ph+ model system in the long-term remains a challenge for clinical translation.29

In addition to modeling disease, CRISPR/Cas9 has been employed to correct mutations in disease-associated genes using single-stranded donor oligonucleotides as DNA donor templates for HDR. For example, a loss-of-function mutation in the Additional sex combs like 1 (ASXL1) gene, which regulates a suitable model for drug testing. For example, the R140Q mutation in the Krebs cycle enzyme isocitrate dehydrogenase 2 (IDH2) endows cells with neomorphic enzyme activity, generating an oncometabolite that interferes with epigenetic cell regulation and contributes to malignant transformation. To study the molecular and functional characteristics of this driver mutation, genome editing was used in K562 cells to introduce the IDH2/R140Q mutation.25 Cells carrying this mutation recapitulated the genetic, epigenetic and functional changes seen in IDH2-mutated patients, offering a suitable model for drug testing.

In modeling disease, CRISPR/Cas9 has been used to correct mutations in disease-associated genes using single-stranded donor oligonucleotides as DNA donor templates for HDR. For example, a loss-of-function mutation in the Additional sex combs like 1 (ASXL1) gene, frequently mutated in myelodysplastic syndrome, chronic myelomonocytic leukemia, and AML was corrected in a chronic myeloid leukemia cell line.26 Similarly, AML blasts (precursor cells) containing the IDH2P95H mutation were corrected to restore cell function to wild-type status.25 These results constitute a proof-of-concept that CRISPR/Cas9 gene correction of primary hematopoietic cells is feasible. Beyond hematopoietic cells, CRISPR/Cas9 genome editing of human induced pluripotent stem cells (iPSC) has been used to correct disease-relevant mutations. For example, correction of the HCLS1 associated protein X-1 (HAX1) gene by CRISPR/Cas9-mediated HDR reversed the severe congenital neutropenia phenotype in patient-specific iPSC.27 This is important given that iPSC are excellent platforms to model disease and also hold promise for use in patient-specific, cell-based regenerative therapy. Accordingly, hematopoietic cells carrying a mutation could be isolated from the patient, reprogrammed to iPSC, edited, differentiated to hematopoietic stem cells (HSC) and re-introduced by autologous HSC transplantation. However, the capability of iPSC-derived HSC to reconstitute the blood system in the long-term remains a challenge for clinical translation.27

Table 4. Comparison of the different formats available for CRISPR/Cas9 components.

| CRISPR format option | Advantages | Disadvantages |
|----------------------|------------|---------------|
| Cas9 and/or gRNA-encoding plasmids | Simple-to-use approach, Multiple gRNA can be integrated into the same plasmid, Repositories available, Economical | Off-targets from Cas9-constitutive expression, Activation of innate immune system against plasmids |
| Cas9 mRNA and gRNA | Lack of genome integration, Less off-targets than integrative plasmids | Issues with mRNA stability, Immunogenicity, Expensive |
| Ribonucleoprotein complex | Few off-target effects due to transient expression, Fast, avoiding cell transcription and/or translation, Highly efficient | Transient expression not sufficient in some contexts, More expensive than previous options |

The main CRISPR component formats are: (i) DNA plasmids encoding the Cas9 protein and a guide RNA (gRNA), either individually or together; (ii) mRNA for Cas9 translation applied to the cell, together with a separate gRNA. (iii) Ribonucleoprotein complexes, formed by pre-assembled Cas9 protein and gRNA. We highlight the most relevant pros and cons for each option. gRNA: guide RNA.
CRISPR/Cas9 gene editing in hematology

CRISPR/Cas9 and gRNA were microinjected into NSG mouse zygotes to generate mice with hemophilia A or hemophilia B. These models should allow the evaluation of the efficacy and safety of novel gene therapy vectors in hemophilia.

Given the importance of reporter mouse lines in biomedical research, it is not surprising that CRISPR/Cas9 technology has been applied in the study of early developmental processes. Recently, a knock-in mouse strain was created for dynamic tracking and enrichment of the MEIS1 transcription factor during hematopoiesis. This GFF-HA epitope tag reporter strategy and CRISPR/Cas9 gene editing could be employed to develop new reporter mouse lines to study other transcription factors important for hematopoiesis.

Mice carrying mutations in multiple genes have traditionally been generated by sequential recombination in embryonic stem cells and/or intercrossing of mice with single mutations. CRISPR/Cas9 technology allows the generation of mice bearing different gene mutations in a more affordable, less labor-intensive and time-consuming manner than traditional approaches. Similar to the hemophilia models described above, mice with bi-allelic mutations in TET1 and TET2 were created by co-injection of targeting gRNA into mouse zygotes, which is a much faster approach compared with traditional techniques and allows one-step generation of animals with precise mutations. Accordingly, targeting multiple genes using CRISPR/Cas9 should facilitate, for example, the in vivo study of a family of genes with redundant functions. Indeed, Cas9 mRNA and multiple gRNA targeting B2M, IL2RG, PRF1, PRKDC, and RAG1 genes were microinjected together into mouse embryos to produce different immunodeficient mouse strains, thus generating new valuable tools to advance research in human HSPC xenotransplantation.

There is increasing evidence that the acquisition of somatic mutations in HSC, leading to clonal hematopoiesis, is a cardiovascular risk factor. Indeed, DNMT3A and TET2 somatic mutations are drivers of clonal hematopoiesis of indeterminate potential, a state that predisposes to subsequent development of a hematologic malignancy or cardiovascular death. This recent study used CRISPR/Cas9 to inactivate DNMT3A and TET2 genes in HSPC and showed that atherosclerotic plaque size was markedly increased in reconstituted mice.

Somatic CRISPR/Cas9 mouse models

Mouse models with somatic genome editing can be built by CRISPR/Cas9 modification of ex vivo cells followed by transplantation (murine cells) or xenotransplantation (human cells). For instance, the ability to modulate CRISPR/Cas9 activity has been exploited to perform doxycycline CRISPR/Cas9-inducible Trp53-knockout/mutation. When HSPC isolated from a lymphoma transgenic model (Em-Myc) were transplanted, this resulted in accelerated lymphoma development in vivo. Thus, a highly efficient inducible CRISPR/Cas9 vector system can be used to identify novel gene mutations that drive tumorigenesis or to knock-out essential genes that are required for cell survival in vitro.

As mentioned in the previous section, one of the unique features of the CRISPR/Cas9 system is its simplicity in enabling simultaneous disruption of several sites in
the genome. Multiplex CRISPR/Cas9 editing of genes mutated in human leukemias has been demonstrated in mouse and human cells using either lentiviral or ribonuclease-protein approaches. Edited cells were then transplanted into conditioned animals and the identity of the disrupted genes was revealed by next-generation sequencing from clones expanded in sick mice. Moreover, ex vivo CRISPR/Cas9 gene editing of HSPC is also useful for studying clonal hematopoiesis of indeterminate potential. Multiplex ribonuclease-protein-editing and tracking clonal dynamics by high-throughput sequencing revealed the expansion of mutant clones resembling human clonal hematopoiesis of indeterminate potential, some of which continued to expand and cause death, by hematopoietic failure or AML, in transplanted mice. Accordingly, multiplex CRISPR/Cas9 gene editing is an advantageous tool for functional genomics and for modeling the mutational complexity and co-occurrence patterns observed in hematologic patients at diagnosis, who in the case of AML, carry an average of 2.3 genomic mutations. A number of publications on the use of CRISPR/Cas9 gene editing in hematologic research are listed in Table 5.

### Gene editing as a therapeutic application in hematologic disorders

Allogeneic HSC transplantation is the frontline treatment for many hematologic disorders; however, this option is only available when a suitable donor exists. Nevertheless, transplanted patients can develop graft-versus-host disease and die of transplant-associated causes. In this scenario, ex vivo gene therapy using viral vectors and ex vivo gene editing by TALEN or zinc-finger nucleases in hematopoietic cells followed by autologous HSC transplantation represent therapeutic alternatives that are currently being investigated in clinical trials. However, permanent viral integration into the host genome and/or insertional activation of proto-oncogenes that could lead to secondary leukemia are potential pitfalls related to integrative vector-based gene therapy. Site-specific endonucleases, especially CRISPR/Cas9, offer the possibility of delivering non-integrative editing components into target cells, such as mRNA and ribonucleaseproteins, constituting a promising approach for HSC gene editing.

### Inherited diseases

Clinically, CRISPR/Cas9 gene editing holds promise for monogenic hematologic disorders and, thus far, it has been mainly employed in hemoglobinopathies. β-thalassemia is caused by mutations in the human hemoglobin beta (HBB) gene and is characterized by reduced β-hemoglobin production, resulting in hemoglobin clumping, hemolytic anemia, and ineffective erythropoiesis. One strategy to remedy this defect using CRISPR/Cas9 is to repair the HBB mutation as has been achieved in iPSC from patients with β-thalassemia. Another strategy is to reactivate the fetal hemoglobin gene via disruption of the BCL11A gene, an erythroid enhancer regulator of the

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**Table 5. List of studies on CRISPR/Cas9 gene editing in hematologic diseases.**

| Disease | Gene/s | Aim/Repair pathway | Target cells | Format/Delivery | Reference |
|---------|--------|---------------------|--------------|-----------------|-----------|
| Myeloid malignancies | TET2, RUNXI, DNMT3A, NF1, EZH2 and SMC3 | Knock out/NHEJ | LSK | Two-vector system/Lentivirus | 35 |
| Myeloid malignancies | 192 chromatin regulatory domains | Knock out/NHEJ | RN2 with constitutive Cas9 expression | One-vector system/Lentivirus | 36 |
| MDS | SRSF2 | Point mutation/HDR | K562 | CRISPR vector and ssODN/Electroporation | 23 |
| MDS, CMML, AML | ASXL1 | Mutation correction/HDR | KBM5 | CRISPR vector and ssODN/Electroporation | 26 |
| MLL | MLL and AF4 | Chromosomal rearrangements/ HDR | HEK293 | CRISPR vector and template plasmid/Lipofection | 31 |
| AML | IDH2 | Knock in/HDR | K562 | CRISPR vector and template plasmid/Nucleofection | 25 |
| AML | IDH2 | Mutation correction/HDR | Primary AML blasts | Two-vector system/Lentivirus | 25 |
| SCN | HAX1 | Mutation correction/HDR | iPSC | CRISPR vector and ssODN/Lipofection | 27 |
| Pediatric AML | MLL and ENL | Chromosomal rearrangements/NHEJ | Human HSPC | One-vector system/Lentivirus | 32 |
| AML and MDS | TET2, ASXL1, DNMT3A, RUNXI, TET3, NF1, EZH2, STAT2, SMC3, SRSF2 and U2AF1 | Knock out/NHEJ | Human HSPC | One-vector system/Lentivirus | 48 |
| AML | RUNXI and ETO | Chromosomal rearrangements/NHEJ | Human HSPC | One-vector system/Electroporation | 30 |
| MDS | ASXL1 | Knock out/NHEJ | U937 | Two vector system/Electroporation | 20 |
| XCGD | CYBB | Mutation correction/NHEJ | PLB | One vector system/Lentivirus | 29 |
| CHIP | DNMT3A and TET2 | Knock out/NHEJ | Human HSPC | One vector system/Lentivirus | 44 |
| CHIP | FLT3, DNMT3A, SMC3, EZH2, RUNXI and NF1 | Knock out/NHEJ | LSK | RNP/Electroporation | 19 |

MDS: myelodysplastic syndromes; CMML: chronic myelomonocytic leukemia; AML: acute myeloid leukemia; MLL: mixed lineage leukemia; SCN: severe congenital neutropenia; XCGD: X-linked chronic granulomatous disease; CHIP: clonal hematopoiesis of indeterminate potential; NHEJ: non-homologous end joining; HDR: homology-directed repair; LSK: Lin-Sca-1+c-Kit+; iPSC: induced pluripotent stem cells; HSPC: hematopoietic stem and progenitor cells; ssODN: single-stranded donor oligonucleotides; RPN: ribonucleaseprotein.
CRISPR/Cas9 gene editing in hematology

Table 6. Current clinical trials in hematology using the CRISPR/Cas9 system.

| Disease                      | Product | Aim/title                                                                 | Phase          | CT identifier | Industry/Academy |
|-----------------------------|---------|---------------------------------------------------------------------------|----------------|---------------|------------------|
| β-thalassemia               | CTX001  | A safety and efficacy study evaluating CTX001 in subjects with transduction-dependent β-thalassemia | Enrolling      | NCT03655678   | CRISPR Therapeutics |
| iHSC treatment group        | CTX001  | iHSC with the gene correction of HBB                                   | Not yet recruiting | NCT03723222   | Allile Medical Science and Technology |
| SCD                         | CTX001  | A safety and efficacy study evaluating CTX001 in subjects with severe sickle cell disease | IND & CTA approved | NCT03745281 | CRISPR Therapeutics |
| HIV-1 infection             | CCR5 gene modification | Safety of transplantation of CRISPR CCR5 modified CD34+ HSPC in HIV-infected subjects with hematologic malignancies | Enrolling | NCT03161435 | Beijing, China |
| B-cell leukemia             | UCART019 | A study evaluating UCART019 in patients with relapsed or refractory CD19+ leukemia and lymphoma | Phase I/II     | NCT03166578 | Beijing, China |
| CD19+ leukemia              | CTX101  | A feasibility and safety study of universal dual specificity CD19 and CD20 CAR-T-cell immunotherapy | Phase I/II     | NCT03398967 | Beijing, China |
| CD19+ lymphoma              | CTX110  | Anti-CD19 allogeneic CAR-T cells with TCR and B2M knocked-out          | Initiates in first-half of 2019 | NA          | CRISPR Therapeutics |
| Multiple myeloma            | NYCT T Cells | NY-ESO-1 Redirected CRISPR (TCR endogenous and PD1) edited T cells | Preclinical | NCT03399448 | Pennsylvania, USA |
| T-cell ALL                  | CTX120  | Anti-BCMA allogeneic CAR-T cells with TCR and B2M knocked-out           | Research       | NA           | CRISPR Therapeutics |
| T-cell lymphoblastic lymphoma | CD7.CAR28zeta | Cell therapy for high risk T-cell malignancies using CD7-specific CAR expressed on autologous T cells (CRIMSON) | Phase I       | NCT03500011 | Houston, USA |

SCD: sickle cell disease; HIV-1: human immunodeficiency virus type 1; ALL: acute lymphoblastic leukemia; iHSC: induced hematopoietic stem cell; CAR: chimeric antigen receptor; HBB: β hemoglobin; HSPC: hematopoietic stem and progenitor cell; UCART: universal CAR-T cells; TCR: T-cell receptor; B2M: β2-microglobulin; BCMA: B-cell maturation antigen; IND & CTA: investigational new drug and clinical trial authorization; CT: clinical trial; NA: not applicable.
promise for the clinical translation of CRISPR/Cas9 gene editing therapies for primary immunodeficiencies.

Acquired immunodeficiencies

Human immunodeficiency virus type-1 (HIV-1) infection gives rise to acquired immune deficiency syndrome (AIDS). Several different CRISPR/Cas9 gene-editing strategies have been used to target HIV-1 along its replication cycle. For example, disruption of the CCR5 receptor gene, which is present in the host cell and is a co-factor for entry of HIV into the cell, represents a promising strategy to combat the disease, and several studies have reported NHEJ-mediated CRISPR/Cas9 inactivation of CCR5 and other co-factors in lymphocytes. In one study, generation of iPSC homozygous for the naturally occurring CCR5Δ32 mutation through CRISPR/Cas9 genome editing conferred resistance to HIV infection. Antiretroviral therapy fails to cure HIV-1 infection because of the persistence of HIV reservoirs harboring integrated HIV DNA. CRISPR/Cas9-mediated deletion or inactivation of proviral DNA could eliminate this source of HIV persistence, thereby being a potentially curative treatment. In preclinical studies, CRISPR/Cas9 efficiently mutated and deactivated HIV proviral DNA in latently infected Jurkat cells. However, complete eradication of HIV latent infection is challenging because of the development of mutations resistant to attack by DNA-shearing enzymes. Clinically, the safety of transplantation of CRISPR CCR5-modified CD34+ cells in HIV-infected patients with hematologic malignancies is under evaluation in clinical trials (Table 6).

Cancer immunotherapy using chimeric antigen receptor T cells

Cancer immunotherapy can be defined as the induction or enhancement of cancer-specific immune responses against malignant tumors. One approach to this is the ex vivo manipulation of patients’ T cells to express a chimeric antigen receptor (CAR) including an intracellular chimeric signaling domain capable of activating T cells and an extracellular binding domain that recognizes an antigen specific for and strongly expressed on tumor cells. CAR-T cells are re-infused into patients to attack cancer cells in vivo. Currently, CAR-T cells expressing CD19, CD20, CD22, or dual B targeting CAR-T cells are available to treat acute lymphoblastic leukemia, non-Hodgkin lymphoma, and chronic lymphocytic leukemia. Unfortunately, CAR-T-cell administration can have adverse effects, such as neurotoxicity, cytopenia and cytokine release syndrome, which can be life-threatening. CRISPR/Cas9 can be utilized to complement CAR-T-cell therapy, for example, via disruption of the endogenous T-cell receptor (TCR). Upon its interaction with engineered, transgenic TCR in patients’ cells, endogenous TCR can alter the antigen specificity of CAR-T cells. In a study using CRISPR/Cas9 to knock out endogenous TCR-β, with simultaneous introduction of CAR-T cells, the authors found that this replacement strategy resulted in more robust transgenic anticancer T cells. The CRISPR/Cas9 system has also been applied to eliminate other genes that encode inhibitory T-cell surface receptors, such as programmed cell death protein 1 (PD1), to improve the efficiency of T-cell-based immunotherapy. To exploit CAR-T-cell therapy beyond the autologous setting, allogeneic universal T cells derived from healthy donors could be engineered by CRISPR/Cas9 upon disruption of TCR to prevent graft-versus-host disease, or beta-2-microglobulin, to eliminate major histocompatibility complex I (MHC I) expression, or by integrating a CAR precisely at the disrupted T-cell receptor α constant (TRAC) locus to improve safety and potency. Thus, CRISPR/Cas9 technology holds enormous promise to advance the field of cancer immunotherapy and several clinical trials are running to assess the efficacy of CRISPR/Cas9-edited CAR-T cells (Table 6).

Challenges and opportunities for CRISPR/Cas9 therapeutic applications

Delivery of editing tools

Delivery platforms that ensure the access of editing components into a large number of target cells are critical for the clinical development of this technique. Ribonucleoprotein is the cargo format preferred over other transient delivery methods such as mRNA and non-integrating viral vectors because of its hit-and-run mechanism, which reduces the risk of undesired off-target effects, and also because of its ability to efficiently modify cells with low translation rates. Nevertheless, the benefit-to-harm ratio of the CRISPR/Cas9 system must be maximized. Possible solutions include the development of novel approaches to integrate ribonucleoprotein and donor template DNA for gene correction in a unique system.

Safety

Off-target DSB can result from non-specific Cas9 cleavage at unwanted genome sites, which is perhaps the major concern regarding therapeutic CRISPR/Cas9 editing. Accordingly, genome-wide sequencing approaches should be employed to thoroughly examine for modifications at unanticipated genome locations, or at anticipated off-target sites indicated by in silico prediction tools. The issue of off-target activity is, nevertheless, controversial since studies have yielded contrasting results. Along this line, several methods have been developed in the last years to detect CRISPR off-target mutations; however, there is a lack of consensus on how to predict which putative off-target sites should be examined via deep targeted sequencing. Additionally, the possibility of Cas9-induced on-target mutagenesis, including large deletions and rearrangements that may have pathogenic consequences, has been highlighted as another safety concern. Accordingly, more research is needed for a definitive understanding of the in vivo genomic effects of CRISPR/Cas9. Indeed, the possibility of producing undesired gene modifications raises concerns about the use of the CRISPR/Cas9 system for therapy in humans. For instance, infused gene-edited HSC could have the potential to expand clonally and induce leukemia, and so clinical gene editing might cause panic. Possible solutions include the substitution of Cas9 with a different nuclease, for example, Cas12a (also known as Cpf1), which prohibits mismatches between the 18 nucleotides next to the protospacer adjacent motif. Other alternatives include the use of paired nickases, guided by two different gRNA targeting the same locus but on opposite DNA strands, or “base editors” editing nucleotides without inducing a DNA break.

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Efficiency

Suboptimal DNA repair outcomes or insufficient target conversion might prevent an intervention from reaching a critical gene-editing threshold necessary to rescue the genetic defect. Strategies to enhance the frequency of HDR in CRISPR/Cas9-mediated transgenesis have been reported and need to be tested in the clinical context. Moreover, it should be considered that efficacy could be reduced if the CRISPR/Cas9-induced mutation is detrimental to the cells, having a negative, non-reversible effect.

Immunogenicity

The immune system reaction to in vivo administration of gene editing reagents or ex vivo genetically modified cells is also a cause for concern. The presence of antibodies against Cas9, mainly isolated from Staphylococcus aureus or Streptococcus pyogenes, is common in neonates and adults. Similarly, T lymphocytes against Staphylococcus aureus Cas9 constitute an obstacle to CRISPR/Cas9 therapeutic gene editing. Accordingly, the possible immune response must be examined in depth to ascertain whether it could compromise the efficacy of CRISPR-based treatments. Strategies to minimize/eliminate immunogenicity include the use of nucleases other than Cas9 that have not been exposed to the human immune system, or novel nucleases that do not activate an immune response. Other strategies could be: (i) to design an in silico prediction tool for immunogenic predisposition; (ii) to understand the innate immune mechanism against CRISPR/Cas9 in order to help in vector choice and engineering; (iii) to identify antigenic regions on CRISPR/Cas9 to enable deimmunization and epitope masking; and (iv) to employ immunosuppression by using drugs and/or regulatory T cells to reduce undesired immune reactivity.

p53-mediated DNA damage response

CRISPR/Cas9 genome editing has recently been shown to induce a p53-mediated DNA damage response in some human cell types, which is in part responsible for the low targeting efficiencies observed in these cells. Consequently, p53 inhibition may improve the efficiency of genome editing in wild type cells; however, a caveat to this approach would be the increased likelihood of cancerous transformation of cells in which the “guardian” activity of p53 is inhibited. p53 gene sequence and function should, therefore, be monitored closely in cells destined for therapy when developing CRISPR/Cas9 cell-based therapies.

Bioethical regulation

CRISPR/Cas9 gene editing is associated with several ethical issues; for example, its application to humans, embryos or germline cells. While the clinical application in human somatic cells to treat hematologic diseases is generally accepted, there is consensus among geneticists that its application in human embryos and germline cells (except for research purposes), in which genetic changes would be inherited by future generations, should be impermissible. That being said, some alarming news was recently reported about the use of CRISPR/Cas9 in human embryos to inactivate the CCR5 receptor and provoke resistance to HIV infection. The biophysicist He Jiankui presented limited (and non-peer-reviewed) data on the birth of twin girls genetically edited with CRISPR/Cas9. This claim, whether true or not, urgently imposes the establishment of strict regulations on human CRISPR/Cas9 genome editing such that it should only be considered for therapeutic uses, but not for human enhancement or eugenics, although it could be used as a research tool to understand early human development or disease pathogenesis. Thus far, no patients have been clinically treated with in vivo CRISPR-based therapy, whereas patients have been given infusions of ex vivo modified T cells (Table 6).

The ethical and regulatory aspects of therapeutic CRISPR/Cas9 genome editing are very complex. Given the proven potential of CRISPR/Cas9 to modify the human genome, there are naturally great expectations for future applications. To discuss all these concerns, a multi-disciplinary regulatory committee, composed of geneticists, lawyers, society representatives and clinicians, should be created to define a legislative framework to regulate permission or prohibition of CRISPR applications and any genome engineering technique in the future. Global scientific and biological ethics communities must take the lead and establish standards and procedures that reduce the dangers of these powerful new technologies without forgetting the benefits.

What is necessary to move the hematology field forward

As with any new treatment, safety and efficacy are very important. Efforts should be made to develop novel vector systems to maximize delivery to target cells with minimal side effects. In this sense, platforms for systemic and local administration of CRISPR/Cas9 would be highly desirable, including non-integrative vectors for gene therapy or systems based on nanoparticle technology, ribonucleoproteins or other novel approaches.

In the last decade, next-generation sequencing has led to enormous advances in the molecular diagnostics of hematologic malignancies. Somatic mutations have been revealed in many diseases and some may have important prognostic value. The functional impact of these mutations on tumor initiation and/or maintenance needs to be addressed in the next years, and CRISPR/Cas9-based screens in patient-derived cells will be powerful tools to undertake this endeavor. In addition, some hematologic malignancies are characterized by mutations in epigenetic modifiers, proteins that modify DNA at cytosine residues or cause post-translational histone modifications. Some therapies already exploit epigenetic targets, such as DNA methyltransferase 3A (DNMT3A) or histone deacetylase (HDAC), and hypomethylating agents, including the DNA methyltransferase inhibitors azacytidine and decitabine, are used to treat myelodysplastic syndrome and AML. Accordingly, targeted epigenome editing, which is the modification of the epigenome at specific sites as opposed to whole-genome modification, could be an area for research development in hematology – for example, for fine-tuning gene expression by locally modulating DNA methylation or determining the function of specific methylations sites. Because epigenome editing does not involve genetic changes, it may also be less hazardous with respect to off-target effects. The challenges will be how to administer epigenome-editing tools in vivo, to achieve reversible epigenetic modifications at precise sites
and to ensure that epigenetic modification is heritable upon cell division.

Last but not least, the contribution of CRISPR/Cas9 to multiplex editing in CAR-T cells to create safer and more effective treatments cannot be underestimated, and it is highly likely that this technique will move forward the field of cancer immunotherapy for personalized T-cell-based therapies. The engineering of novel CAR-T cells by pharmaceutical industries, resulting in costly and unaffordable treatments for the general population, should be accompanied by their production by academia institutions, which could make it easier to tailor CAR-T cells for each patient. Thus, drug regulatory authorities should facilitate their academic production and provide resources for CAR-T cell manufacturing processes, so that these can be simplified and automated to enable scaling up of these cell products.

Conclusions

CRISPR/Cas9 technology is a revolutionary approach for genome editing with wide applications in molecular biology, genetics, and medicine. It has great potential for dissecting gene function, modeling diseases and editing human genes for curative treatment. The number of publications in this field has doubled every year since its introduction, and the CRISPR/Cas9 system is now more widely used in biotechnology and research laboratories than other, more time-consuming and expensive approaches such as zinc-finger nucleases or TALENs. In hematology, CRISPR/Cas9 can be used to model diseases using cultured cells or model organisms, but perhaps more importantly, it can be a valuable approach to correct ex vivo mutations and chromosomal aberrations in cells from patients with blood disorders for autologous HSC transplantation. However, many pitfalls and challenges need to be overcome for the translation of CRISPR/Cas9 gene editing to the clinic. For example, we do not know the minimum number of edited cells needed to functionally correct a genetic defect or if gene editing can be applied to treat multigenic diseases. Further research is necessary to implement CRISPR/Cas9 in the clinical context, so that genome editing-based treatments are available to patients. In conclusion, the CRISPR/Cas9 revolution brings us a specific, efficient and versatile tool for editing genes. Nowadays, technology is no longer a limitation and scientists can probably do any genetic manipulation they can dream of.

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