



Published in final edited form as:

Hematol Oncol Clin North Am. 2022 August ; 36(4): 647–665. doi:10.1016/j.hoc.2022.05.002.

A Curative DNA Code for Hematopoietic Defects Novel Cell Therapies for Monogenic Diseases of the Blood and Immune System

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Keywords

Hematopoietic stem cells; CRISPR/Cas9; DNA repair; Engineered viruses; Gene correction; Prime editing; Base editing; Hematological diseases

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT)^{1,2} is the most advanced treatment strategy for life-threatening conditions caused by developmental and functional defects of the mature hematopoietic lineages. The highest success rates for allogeneic (allo-) HSCT is associated with using a matched human leukocyte antigen (HLA)-identical donor graft.² However, as the demand for matched donors outweighs the supply, 75% of patients in need of a transplant rely on HLA-mismatched stem cells. Allo-HSCT confers a considerable risk of morbidity (eg, incomplete immune reconstitution, graft-versus-host disease, graft rejection) when the source of stem cells is derived from HLA-mismatched individuals.

Autologous stem cell-based gene therapy offers the greatest immunologic compatibility, delivering an ideal solution for genetic diseases of the blood and immune system while avoiding the allogeneic complications of standard treatment. This cutting-edge restorative therapy for untreatable diseases is leading to the development of regenerative medicine. The evolution from a treatment that introduces a new copy of the “healthy” gene (gene addition)³ to a treatment that corrects the “mutated gene” (genome editing),⁴ marks a critical

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DISCLOSURE

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turning point in modern medicine. Hematopoietic stem cells (HSCs) are the prototypical cells used in gene therapy due to their lifelong regenerative potential.⁵ They are defined by 2 fundamental properties—self-renewal and differentiation—with a multipotent and narrow spectrum of differentiation potential restricted to blood cells and immune lineages.⁶ These unique biological properties allow HSCs to form and replenish the hematopoietic system and act as an internal repair process during injury. These cellular attributes of HSCs give great potential for developing innovative, safer, and durable therapies.

Inherited and acquired genetic alterations originating in HSCs disrupt the output of the hematopoietic system resulting in hematological conditions whereby mature blood or immune cells fail to develop or function properly. Experiments of nature have shown that genetic somatic reversion from a pathogenic to a wild-type sequence can lead to a curative outcome,⁷ a concept that is the cornerstone of the modern era of gene therapy. In the early days of gene therapy trials, the adverse effect of a malfunctioning gene was counteracted by integrating viral vector-mediated gene transfer.⁸ This approach demonstrated clear benefits in patients with selected hematological conditions.^{9–12} However, the “gene addition” approach does not achieve the spatiotemporal gene regulation and expression that many blood and immune-related genes depend on. Clinical correction was not a consistent outcome in these gene therapy trials due to transient and low-level transgene expression unable to support robust hematopoiesis; safety was also compromised by leukemic events caused by insertional mutagenesis, reported during long-term follow-up.^{13–16}

Technological innovations in the genome engineering field through the development of genome editing tools usher in a new era of gene therapy.^{17–19} Genome engineering through editing relies on a “hit-and-run” approach to permanently modify a specific genomic sequence. Conceptually, genome editing is achieved through transient expression of an engineered nuclease that works in conjunction with the DNA repair machinery with or without a pre-designed repair template to modify a sequence of DNA permanently. When a donor/repair template is used, targeted transgene integration to achieve *in situ* reconstitution of a mutated gene while preserving robust and predictable expression can be achieved, thus abrogating the limitations previously associated with gene addition. The successful transfer of the bench to bedside gene-correction approach is contingent on overcoming cellular barriers, escaping immunologic surveillance, sustaining a physiologic gene expression, correcting abundant stem and progenitor cells to reverse the condition, and assuring that the hematopoietic system is robustly reprogrammed to maintain long-term benefits.

Genome engineering of human HSCs generates a “live biological” therapeutic drug of unparalleled complexity with potential unknown and unintended effects on patients. Combining genome editing with gene therapy, a patient’s hematopoietic system can be restored to achieve disease correction at its root cause—directly correcting the pathologic mutation at the DNA level. Preclinical data, using animal models, provided the first evidence of the efficacy, specificity, and safety of this approach in support of its advancement to first-in-human clinical trials. Early phase trials of gene editing, including direct gene correction-based approaches, have already received FDA clearance for patients with severe forms of sickle cell disease (Table 1). While this therapy is in its pioneering stage, precautionary measurements and long-term surveillance will be necessary to determine

whether the safety, efficacy, and durability of disease correction have been achieved at an acceptable risk/benefit ratio. Nonetheless, there is optimism that the curative strategy will result in the benefits outweighing the risks for patients. This article reviews the progress in advancing genome-editing technology and its application to regenerative medicine, the biological processes underpinning these innovations, and addresses the challenges and triumphs in transferring genome-editing-based gene therapies from bench to bedside. We highlight throughout examples of the key accomplishments in genome editing in blood and immune diseases, with particular emphasis on inborn errors of immunity (IEI) and severe hemoglobinopathies such as sickle cell disease (SCD) and beta-thalassemia (see also Alexis Leonard and colleagues' article, "Gene Therapy for Hemoglobinopathies: Beta-Thalassemia, Sickle Cell Disease"; Kritika Chetty and colleagues' article, "Gene Therapy for Inborn Errors of Immunity: Severe Combined Immunodeficiencies"; and Joseph D. Long and colleagues' article-Genes as Medicine: The Development of Gene Therapies for Inborn Errors of Immunity," in this issue).

MOLECULAR INTERVENTIONS FOR CORRECTING A GENE

Gene therapy relies on modifying the patient own human hematopoietic system to achieve disease correction. The approach is confined to a defined subset of cells (CD34⁺ hematopoietic stem and progenitor cells, ie, HSPCs) of patient origin, transduced by viral vectors to deliver a therapeutic payload into the cell's genome before infusion back into the patient. Until a decade ago, "gene addition" gene therapy leveraged the biology of semi-randomly integrated retro- (RV) and lenti- (LV) viruses⁸ to offset a faulty gene within a cell, which adversely affected its fitness. However, *stochastic genomic integration* of a functional gene copy did not always produce reliable clinical outcomes, with results varying among patients and trials. The rationale for redesigning the clinical vectors²⁰⁻²² to alleviate previously reported genotoxicity decreased but did not eliminate the oncogenic risk of large numbers of semi-random integrations in the clinical setting.

Advances in genome engineering technology are transforming the field of gene therapy. Rather than relying on uncontrolled vectors to deliver an exogenous therapeutic gene, *genome editing directly modifies the defective endogenous gene* using a "cut" and "paste" approach to restore the target gene's function. The ability to identify one incorrect nucleotide out of 6 billion that make up the human diploid genome and modify it precisely is an astonishing accomplishment made possible by discovering nucleases that can stimulate DNA repair responses by 1000-fold.²³⁻²⁶ As exciting as it is for translational medicine, genome editing is also a fundamental biological process crucial to developing the human adaptive immune system. For example, in the absence of our natural genome editing system, lymphocytes cannot generate a pool of receptors with a diverse repertoire required to sustain a robust immune response against infections.²⁷⁻²⁹

GENOME EDITING MEDIATED BY DNA DOUBLE-STRANDED BREAKS

The genome-editing process may be catalyzed by a nuclease designed to recognize, bind, and cut a predetermined DNA sequence.³⁰ The double-strand breaks (DSBs) generated by the nuclease mark the region for the DNA repair enzymes to insert, delete or replace a

sequence while repairing the breaks. Earlier genome editing platforms relied on homing endonucleases²³ and chimeric proteins–zinc finger nucleases (*ZFNs*)^{31–34} and transcription activator-like effectors (*TALENs*)^{35,36}–to introduce DSBs at precise genomic locations. These breaks prompt the recruitment of DNA repair enzymes that are steered by adeno-associated virus (AAV), a nonintegrating viral vector, to introduce the desired nucleotide sequence. The cellular choice of DNA repair–nonhomologous end-joining (NHEJ) or homologous recombination (HR) (see later in discussion)—determines the outcome of nuclease-based genome editing. While these earlier nuclease platforms are still being used in translational clinical trials, in the last 7 years, the *Clustered Regulatory Interspaced Short Palindromic Repeats Cas9 nuclease (CRISPR/Cas9)* has transformed the field^{17,18} because of its ease of use, remarkable activity in a variety of human cells (including HSPCs), and its surprising specificity.

While ZFNs and TALENs use proteins engineered to recognize predefined 9 to 18 or 11 to 12 nucleotides, respectively, as docking sites for DSBs generation, the CRISPR/Cas9 platform is an RNA-guided DNA endonuclease. Originally part of the bacterial adaptive immune system,¹⁷ whereby the CRISPR locus was used as an information storage mechanism for past viral infections, CRISPR/Cas9's role was to recognize and destroy viral genomes through sequence-specific DNA–RNA base pairing. The system was repurposed for genome editing applications in the biomedical field, offering significant advantages over previous platforms. Though the CRISPR locus itself is unique to the bacterial immune system¹⁸ features from the CRISPR array were used in the human genome. First, the guide RNA (gRNA), which is transcribed from the CRISPR locus to recognize incoming viral sequences in the bacterial system, was redesigned to recognize the unique 20-nucleotide human genome sequence. The gRNA complexes to the Cas9 protein and directs the Cas9 protein (which contains the nuclease activity to make a break) to the correct site in the genome. These breaks are created 3 base pairs (bp) away from the *adjacent protospacer motif (PAM)*, marking the genomic site complementary to the gRNA. Second, the dual RNA system of bacteria (crRNA and trRNA) was replaced by a single gRNA system (sgRNA), thus reducing the complexity of the system from 3 components (Cas9, trRNA, crRNA) to 2 components (Cas9 and sgRNA). The CRISPR Cas9/sgRNA system's ease of design, combined with high activity and specificity, has democratized and transformed the genome-editing field and has made it the most used nuclease platform to enable the development of preclinical “gene correction” therapies.

DOUBLE-STRAND BREAKS REPAIR PATHWAYS AND THE EFFECT OF CELL CYCLE AND CHROMATIN STRUCTURE ON GENOME EDITING

The outcome of a Cas9-mediated genome editing depends on the DNA repair pathway being used. The classical nonhomologous end joining (*cNHEJ*) repair pathway is active across all cell cycle stages. The Cas9/sgRNA ribonucleoprotein complex (RNA protein enzyme) cuts the target strand 3 bp upstream of the PAM site and 3 bp, 4 bp, or 5 bp on the nontarget strand, thus generating DSBs with blunt or staggered ends.^{37,38} *cNHEJ* ligates the blunt ends with high fidelity, but the stagger ends are processed in an error-prone way, resulting in 1 to 2 bp insertions or deletions (INDELs).³⁹ *NHEJ-based genome editing* can, therefore, be

used to (i) silence pathogenic forms of a gene, such as a dominant active gene, (ii) restore the correcting reading frame, or (iii) introduce targeted deletions of exons or enhancers. It is important to note that the nature of INDELS is highly variable between different sgRNA guides.

During the S/G₂ phase of the cell cycle, 3 repair pathways are active and in competition to resolve the DSBs: *cNHEJ*, microhomology-mediated end joining (*MMEJ*), and homologous recombination (homologous direct repair (*HDR*)). Short 3' end ssDNA overhangs can be routed into MMEJ-dependent repair by resetting and filling the gaps via DNA synthesis. Like *cNHEJ*, MMEJ does not require a DNA repair template and results in loss of sequence information. The repair outcome usually generates larger deletions than the deletions created by *cNHEJ* (>3 bp).³⁹ Inhibition of the DNA ligase (Lig I and III) active in the final step of MMEJ, using small molecule inhibitors, can bias the repair mechanism toward using a homologous repair (HR) pathway, an error-free repair approach.

HDR describes using a donor DNA molecule as a template for repairing the break to generate precise nucleotide changes in the genome (not INDELS). HDR can be harnessed using double-stranded DNA (*classic HR*) or single-stranded DNA template repair (*SSTR*).⁴⁰ The presence of either repair template will result in template-dependent, high-fidelity repair outcomes driven by different repair mechanisms. Exogenously provided double-stranded DNA donor template bearing homology arms (~400 base pairs) to the target site is incorporated into the genome by an *HR mechanism*.⁴¹ This genome-editing approach provides the flexibility of (i) changing a pathogenic single-nucleotide polymorphism (SNP) and (ii) inserting a full-length cDNA or open reading frame of a gene in-frame with the endogenous start site or at a safe-harbor locus if constitutive overexpression of the gene could provide a therapeutic benefit without inducing adverse events. Safeguarding the endogenous levels of gene expression and regulation while eliminating the risk of insertional mutagenesis is the most sought-after genome-editing outcome that would benefit larger classes of monogenic diseases (Fig. 1). HR-mediated targeted correction of point mutations has been successfully used for correcting single point mutation both for SCD^{42–46} and IEI, for example, X-linked chronic granulomatous disease.⁴⁷ The use of a full-length cDNA for *in situ* gene correction has the advantage of establishing a “*universal gene correction*” therapeutic strategy for conditions not caused by recurrent mutations but by a broader array of mutations scattered throughout the gene. The feasibility of this latter approach has been demonstrated most extensively by using adenovirus-associated viruses to deliver the cDNA, as discussed later.

SSTR-mediated genome editing can achieve small genomic changes (single to tens of base pairs), and it also occurs with high efficiency in mammalian cells, though through a mechanism that is not via the classic HR repair machinery (*SSTR* is Rad51 independent). In *SSTR*, a synthesized single-stranded oligonucleotide (ssODN) of length 70 to 150 base pairs (bps) is used, making it more accessible to investigators than classic HR donor templates. The range of changes engineered by *SSTR* is substantially more limited than what can be achieved by classic HR^{43,47} though it does offer a viable approach for introducing limited genetic edits.

Although genome editing varies considerably between different genomic sites, the nucleotides next to the Cas9 cutting site can influence the repair outcome.^{38,39,48,49} The most common outcomes observed in HSCs are small INDELS (1–2 bp), products of cNHEJ, and controlled by the nucleotide at the fourth position upstream of the PAM site. Cas9 cutting efficiency and the choice of DNA repair pathways are affected by changes in nucleosome architecture caused by chromatin remodeling. Post-translational modifications control the recruitment of DNA repair proteins such as 53BP1 and BRCA1. For example, the ubiquitylation of histone 2A (H2A) and di-methylation of histone 4 at lysine 20 (H4K20me2) recruit 53BP1 to chromatin sites next to the DSBs. These modification states (H2A and H4) were proposed to have an antagonistic effect on the recruitment of 53BP1 and BRCA1 to define cell cycle phases, affecting the choice of DSB repair pathway. A compacted chromatin (heterochromatic) structure marked by histone 3 lysine 9 trimethylation (H3K9me2/3) promotes HR and MMEJ while open chromatin (euchromatin) state stimulates the cNHEJ repair pathway.^{50,51} Although the mechanisms by which chromatin architecture promotes one repair pathway over another remain to be elucidated, fundamental insights into the genome structure can inform the development of better genome-editing strategies. Nonetheless, high frequencies of all types of editing can be achieved at loci that are transcriptionally silent in HSPCs.

NON-BREAK MEDIATED EDITING: BASE AND PRIMER EDITING

The generation of DSBs does not directly induce genome editing. It is the cellular response to the breaks that modify the DNA. DNA damage responses are intricate and tightly regulated processes. Although HR-mediated targeted correction is a highly versatile genome editing approach that corrects the DNA sequence at nucleotide resolution, it is restricted to dividing cells (the S/G₂ phase of the cell cycle). It also competes with the highly efficient NHEJ repair enzymes. This generates a heterozygous population of genome-edited alleles: some precisely corrected and others marked by INDELS. The unintended presence of INDELS as a byproduct of targeted genome editing could negatively impact the therapeutic outcome by reducing its effectiveness or generating unwanted disease-permissive genotypes. This outcome can be best exemplified by disorders of β -globin, SCD, and β -thalassemia. HR-mediated targeted genome correction of the SCD genotype (HbS/HbS) results in a large portion of the SCD patient's alleles being corrected (HbA/HbA). However, since HR-mediated genome editing cannot reach 100% efficiency, some SCD alleles will acquire INDELS that generate β -thalassemia or sickle/ β -thalassemia genotypes (INDEL/INDEL; HbS/INDEL).

To eliminate “by-product events” at the targeted locus, 2 variations to the targeted genome editing approach have been described that bypass the need for introducing DSBs: base editing and prime editing. *Base editing (BE)* uses the CRISPR-Cas9 platform to modify the chemical sequence of the DNA directly and introduce any of the 4 nucleotide transitions: C to T, T to C, A to G, and G to A.^{52–55} This is achieved by using an inactive nuclease form of Cas9 that retains nicking activity along with base-modification enzymes (*cytosine base editors, CBEs, and adenine base editors, ABEs*) active only on ssDNA. The base pairing between a DNA and an RNA molecule induces a “DNA bubble (R-loop),” allowing the deaminase enzyme to modify the DNA bases within the loop. The DNA nick created on

the nonedited strand by the catalytically disabled nuclease will be repaired using the edited strand as a template. Innovative as it is, this approach is limited to a predefined window of genomic sequences that it can act on and has a limited number of genetic changes that can be engineered.

Prime editing (PE) was developed to overcome this limitation by introducing all 12 possible base conservations (transitions and transversions) without DSBs.^{56,57} This genome editing platform is based on a prime editing guide RNA (pegRNA), containing both the primer binding site (PBS), the sequence to introduce the edit, and the Cas9 nickase (H840) carrying a reverse transcriptase (RT). RT is an RNA-dependent DNA polymerase that uses the pegRNA as a template to copy the desired genomic edit into the target DNA sequence. PE requires the expression of a foreign RT for editing, and the consequences (including genotoxicity) of expressing such an RT in cells are not fully understood.

The use of DNA base editors as a therapeutic tool has been demonstrated by efficiently correcting mutated genes (eg, *HBB* in β -thalassemia)^{58,59} and by introducing targeted deletion in gene enhancers (eg, *BCL11 A*)^{59–61} or in the promoter regions (eg, *HBG1* and *HBG2*)^{62–65} to stimulate fetal hemoglobin (HbF) upregulation. Though the latter 2 approaches are not a disease correction strategy *per se*, it alleviates the symptoms associated with β -globin-related blood conditions.

Base and prime editing technologies are exciting new tools in genome editing, but they remain less developed than nuclease-based methods in translating to patients.^{66–68} Addressing limitations concerning their restricted targetable sites, unintended off-target effects in both DNA and RNA, and bystander mutation events remain active areas of investigation as the BE, and PE tools are translated. Furthermore, in contrast to classic HR-based editing, neither BE nor PE can provide a oneshot universal approach to a genetic disease with disease-causing mutations scattered throughout the gene.

THE SCIENCE OF GENOME EDITING THE HEMATOPOIETIC SYSTEM

The unlimited self-renewal potential of HSC has always made these cells the preferred choice for gene therapy. To ensure that the genome editing modification is propagated indefinitely throughout the hematopoietic system, optimizing the *ex vivo* genome editing protocols must meet the highest specificity, efficiency, and safety standards without obstructing the cells' regenerative potential. The technological toolbox for genome editing must, therefore, be tailored to the biological properties of HSC to achieve optimal results.

Ex vivo Culturing Conditions

CD34⁺ HSPCs are purified from the bone marrow or the peripheral blood of the recipient and cultured for 2 to 3 days in the presence of growth-stimulating cytokines.^{69,70} Long-term repopulating HSCs (LT-HSCs) exit quiescence and enter the S/G₂ cell cycle phase. Under these conditions that enable genome editing, the cells are exposed to the engineered nucleases and the vector carrying the repair template. HDR-based genome editing has a limited window of action—the S/G₂ cell cycle phase—permissive to highly cycling committed progenitors but constrained in the quiescent primitive HSCs population.^{71,72} Fine-tuning

the culturing conditions,^{73,74} incorporating stemness-preserving compounds,^{74,75} and shortening the overall editing time are steps implemented to preserve the long-term multilineage repopulating capacity of the “therapeutic drug product.”⁷⁶ Achieving a balance between efficient editing and maintenance of the stemness potential of human HSPCs is vital to sustaining the long-term fitness of genome-edited HSPCs and the therapeutic benefit.

Optimizing and Delivering Engineered Nucleases and Homology-Direct Repair-Mediated DNA Repair Template

The goal of the engineered nuclease platform is to deliver the highest frequencies of genome editing while minimizing the treatment toxicity. Both the DSBs and the DNA repair template can trigger cellular responses that could adversely affect the cell fitness and the competence of the DNA repair mechanisms. Primary cells, such as HSCs, have developed heightened immune responses (eg, pathogen-associated molecular patterns, PAMPs; type I interferons, IFNs; overexpression of interferon-stimulated genes, ISG, and other cytokines) to exogenous nucleic acids and proteins by inducing exit from quiescence, promoting differentiation, reducing cellular viability, and decreasing clonogenic potential.^{77–80}

To dampen the immune responses against the genome-editing machinery, the sgRNAs are synthesized as RNA, purified using high-pressure liquid chromatography (HPLC), and cloaked with chemical modifications.⁸¹ Electroporation-based delivery of the sgRNA molecule precomplexed with Cas9 protein (ribonucleoprotein, RNP, including a high-fidelity form of Cas9)^{82,83} further shields the genome-editing molecules from inducing cellular responses against it. Studies have further shown that a pro-inflammatory transcriptional program⁷² with a subsequent decrease in the genome-edited HSPCs' clonogenic potential is generated in response to the DNA damage evoked by the nucleases. Transient p53 inhibition is one mechanism shown to enhance HR efficiency⁸⁴ and tolerability to the genome-editing process, a treatment that restores the polyclonal composition of the grafted HSPCs.^{72,74}

Nonintegrating Viral Vectors for Genome Editing

Integration deficiency lentivirus (IDLV) has been developed for different lentiviral platforms. A mutation (D116) in the catalytic domain of the integrase prevents the genomic incorporation of the viral DNA, resulting in an episomal IDLV vector that can be used as an HDR- DNA repair donor.⁸⁵ These free-ended dsDNA vectors can deliver a cargo of 10 kb⁸⁶ and are amenable to genome-editing of primary cells.^{42,87,88} Although the system avoids the risk associated with insertional mutagenesis and exhibits reduced toxicity, it does result in concatemer formation: IDLV recombines with the target site before HR occurs. While IDLV-HDR donors demonstrated reasonable HR frequencies, there seemed to be an upper limit on what could be achieved.

Adeno-associated virus type 6 (AAV6) has the best tropism for transducing the human hematopoietic system ex vivo.⁸⁹ The vector has a 4.7 to 4.9 kb transgene capacity, accommodating full-length cDNAs flanked by ~400 bp homology arms. *AAV6-HDR donor* has been reported to deliver highly efficient (20%–80%) HR-based correction in ex vivo and support long-term hematopoietic engraftment.⁶⁹ Many groups have demonstrated the

feasibility of using AAV6 to deliver a wild-type cDNA to be integrated into the endogenous locus upstream of all known pathogenic mutations, as a “universal correction” genome editing strategy for various hematological and immunologic disorders. Examples of IEI shown to be correctable in preclinical studies include X-linked SCID,^{76,90} X-linked chronic granulomatous disease,⁹¹ X-linked hyper-IgM syndrome,^{92–94} Wiskott–Aldrich syndrome,⁹⁵ X-linked agammaglobulinemia,⁹⁶ and RAG2–SCID.⁹⁷ For severe hemoglobinopathies, this approach was also successful in replacing the SCD mutation with wild-type *HBB*,⁹⁸ and replacing *HBB* in beta-thalassemia cells with *HBA*.⁹⁹

ssODN-HDR donors are short (<200 bp) oligos with even shorter homology arms (~30–60 bp) flanking the nucleotide change. The targeted correction in HSPCs delivered by this vector type is within the range of 5% to 40% and decreases by half following transplantation into immunodeficient mice.^{43,44,47} In direct comparison to AAV6-HDR donors,⁴² the HR frequencies have generally been higher (20%–80%).^{76,91,95} *ssODN* holds certain features (eg, simple design, short production time, and low cost) that make them useful for specific applications but cannot be used to insert a large transgene in HSPCs.

PERSISTENCE OF THE THERAPEUTIC STEM CELL PRODUCT

Genome-editing-based gene therapies are entering the clinical arena. The emerging Phase I/II first-in-human clinical trials (see Table 1) have and will continue to generate a wealth of information on the short- and long-term safety (eg, genomic integrity, nuclease specificity) and efficacy (eg, long-term durability of the edited HSPCs both at the intended locus and spanning the hematopoietic lineages) of this pioneering therapy. These endpoint readouts will inform the treatment’s risk/benefit ratio. Still, molecular analyses can be performed at the manufacturing stage, before its infusion into the patient, to inform the safety and efficacy of a “therapeutic drug product.” Efforts have been made to monitor the clonal composition, as a safety profile of the genome-edited HSPCs, before and after engraftment into immunodeficient mice. Studies have quantified the INDEL diversity within the genome-edited alleles^{100–103} as a surrogate readout for clonal diversity or have developed unique molecular identifiers (UMI) embedded in the HDR-corrective DNA template^{74,104} to track the HDR-modified alleles. These studies have demonstrated that human hematopoiesis, established in the immunodeficient mouse models and originated from the edited HSPCs, has an oligoclonal composition signature with multi-lineage and self-renewal potential retained in the engrafted clones. The observed loss in clonal diversity can be attributed to the suboptimal manufacturing process of edited HSPCs or the inefficiency of the murine bone marrow microenvironment to support polyclonal human hematopoiesis. These preclinical studies on edited-HSPC-derived clonal composition suggest that long-term persistence can be achieved and supports advancement in clinical testing.

VALUES AND LIMITATIONS IN EVALUATING THE THERAPEUTIC PRODUCT IN MOUSE MODELS

Immunocompetent mouse models have been used as preclinical models to assess the novel gene and cell therapies’ efficacy, toxicity, safety, and stability. The humanized murine system offers great value for the gene and cell therapy field because it bridges the proof-of-

concept of novel gene therapy and their translation into the clinic as part of IND-enabling studies. Murine models have been used to study normal and leukemic stem cells, human hematopoietic hierarchy, human immune function, autoimmune diseases, and organ and tissue transplantation. The most common immunodeficient mouse model used for human HSPC cell engraftment is the nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) $\gamma c^{-/-}$, referred to as NSG,¹⁰⁵ was engineered to lack murine T, B, and NK cells but are not fully humanized. NSG mice are less efficient in supporting human myeloid and erythroid lineage development, making it a suboptimal system for testing genetic diseases that disrupt the myeloid function and differentiation. Newer and related immunodeficient mouse models were designed—NOG, NRG, NSG-SGM3, BRGS, and MISTRG—to support better human HSPCs engraftment, and human myeloid lineage differentiation, in addition to lymphoid lineage development. These immunodeficient mice can be transplanted with human HSPCs purified from umbilical cord blood, bone marrow, fetal liver, or adult mobilized peripheral blood, allowing the cells to home to murine bone marrow, whereby they engraft, expand, differentiate, and establish a long-term human hematopoietic system.

Evaluating the potency and safety of gene-modified HSPCs, following engraftment into conditioned immunodeficient mice is a benchmark required by the FDA before approving a new medicine (eg, gene therapy) to treat monogenic blood disease. Often these xenotransplantation studies are carried out using healthy donor stem cells since obtaining patient-derived HSPCs to transplant a full human dose into an immunodeficient mouse cohort is often not feasible due to the low prevalence of the disease, as is the case for IELs (eg, ADA-SCID, SCID-X1, RAG1/2-SCID, IPEX, X-CGD).¹⁰⁶ For SCD and some forms of thalassemia, the c-Kit mutant NSG mouse model, for the first time, allowed the therapeutic efficacy to be assessed by supporting mature red blood cells development in the murine bone marrow.^{107,108} With this advancement, both toxicity and efficacy of SCD or thalassemia patient-derived and corrected HSPCs can now be evaluated using clinical-grade reagents to support initiating a clinical trial.

The data and insights generated from in vivo mouse models are essential in developing novel therapies to cure human diseases. However, humanized mouse models remain only a model, and many of them have limitations: xeno-reactive graft-versus-host disease, limited lifespan, incomplete immune function, only oligoclonal reconstitution, underdeveloped lymphoid organs, and lymphoid architecture, which require careful considerations when interpreting experimental results.

ADVANCING THIS NEW CLASS OF MEDICINE TO THE CLINIC

The Food and Drug Administration (FDA) evaluates novel therapies for safety and efficacy through clinical trials open to patients who have no available treatment or for whom current therapies are not effective. Clinical trials using CRISPR/Cas9-based gene therapies in blood disorders (see Table 1), cancer, eye disease, protein-folding disorders, and chronic infections have received FDA approval.

Recent reports of the investigational use of the first in human ex vivo CRISPR/Cas9-modified autologous HSPCs product provided therapeutic benefits that a single treatment

can offer to patients with SCD, a severely disabling condition.¹⁰⁹ In the clinical trial (CLIM SCD-121) sponsored by CRISPR Therapeutics and Vertex Pharmaceuticals, patients between the age of 18 and 35 who were diagnosed with SCD (genotypes $\beta\text{S}/\beta\text{S}$ or $\beta\text{S}/\beta^0$) and experienced more than 2 severe vaso-occlusion (VOC) episodes per year were eligible to participate for enrollment in the trial. Plerixafor mobilized patient's $\text{CD}34^+$ HSPCs expressing less than 30% of sickle hemoglobin following 8 weeks of transfusion were genome-edited ex vivo using sgRNA that directed CRISPR/Cas9 nuclease to the erythroid-specific enhancer region of *BCL11A*. Although this approach does not correct the root cause of SCD, it increases fetal hemoglobin (HbF) levels to compensate for the lack of adult hemoglobin in red blood cells. After the administration of CTX001, all 7 patients infused with the therapeutic product showed stable engraftment, which resulted in increased HbF and no VOC, 2 months postinfusion.^{109,110}

Genome editing-based gene therapy is emerging as a curative therapy that reaches beyond conventional drugs. Precision medicine enables patient-specific disease correction by delivering a stable, precise, and durable therapeutic drug. As genome-based correction therapies will progress through clinical trials and demonstrate safety, efficacy, and curative potential, pharmaceutical and regulatory sectors will have to work together to build a suitable manufacturing and product release pipeline to assure that a continued supply of these highly personalized “live biological drugs” is achieved and that all patients will benefit from these innovative therapies (Fig. 2).

ACKNOWLEDGMENTS

This work was funded in part by the Intramural Research Program, Center for Cancer Research, National Cancer Institute.

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KEY POINTS

- Precise gene correction, using programmable nucleases, enables an era of personalized medicine to treat incurable monogenic hematological and immunologic conditions.
- Direct changes to the DNA sequence of a cell are based on a growing genome editing toolset that can be applied to the hematopoietic system and extends beyond semirandom integrating viruses: nonhomologous end joining (NHEJ)-dependent gene editing, base editing, primer editing, and homology-direct repair (HDR)-mediated gene correction.
- Targeted correction of the hematopoietic system holds great promises and unique challenges on its path to a safe and effective clinical translation.
- Manufacturing advances of ex vivo gene correction therapies will be needed to reduce costs and enable cures of a broad array of ultra-rare and rare hematological disorders.

CLINICS CARE POINTS

- Genome editing of autologous hematopoietic stem cells shows great promise because of the pre-clinical data on safety and efficacy.
- Nonetheless, the clinical outcomes for patients using genome edited cells is still largely unknown, especially long term outcomes.
- Thus, clinicians should be prepared for both successes better than expected and unanticipated toxicities.

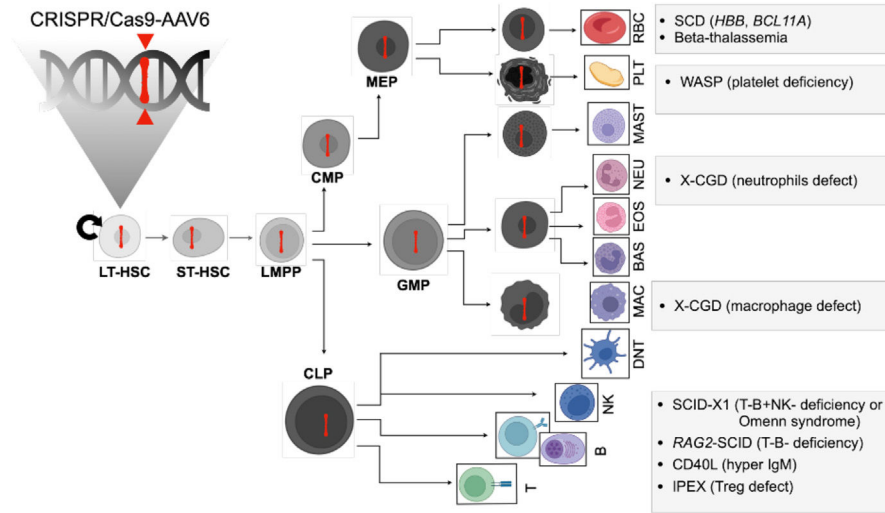


Fig. 1. Preclinical studies using a gene-editing approach for Primary Immunodeficiencies. Schematic of the human hematopoietic system and cell lineages. Gray boxes denote the hematopoietic defect causing the PID. B, B cell; BAS, basophil; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DNT, dendritic cell; EOS, eosinophil; GMP, granulocyte-monocyte progenitor; HSC, hematopoietic stem cells; LMPP, lymphoid multipotent progenitor; LT-HSCs, long-term HSC; MAC, macrophage; MAST, mast cell; MEP, megakaryocyte-erythroid progenitor; NEU, neutrophil; NK, NK cell; PLT, platelet cell; RBC, red blood cell; ST-HSC, short-term HSC; T, T cell; Treg, T regulatory cell. The image was designed using BioRender software.

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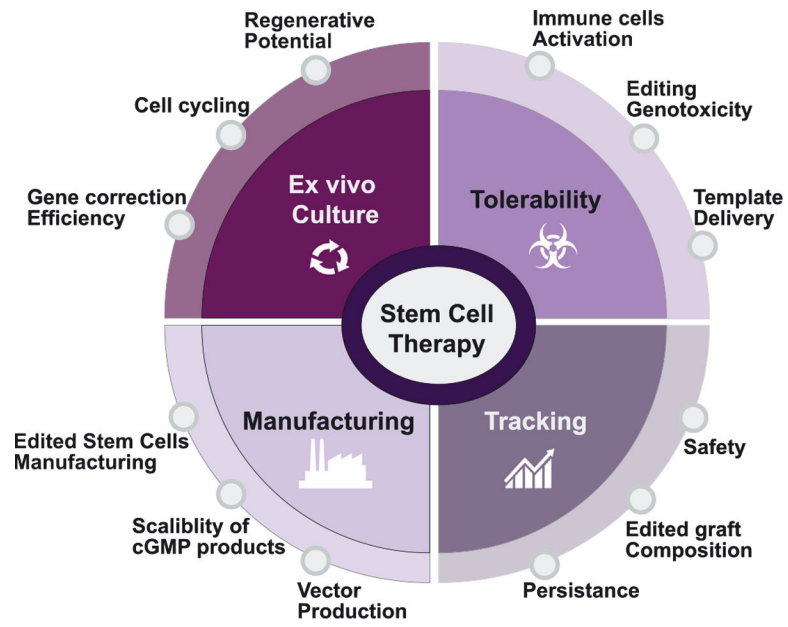


Fig. 2. Challenges in stem cell therapy. Schematic challenges toward the clinical translation of gene-editing-based stem cell therapies.

Table 1

Clinical-stage (phase I/II) gene editing candidate therapies for sickle cell disease

Product ID/Company	Clinical Trial ID	Disease	Gene Editing Approach	Description	Preclinical Data
GPH101 <i>Graphite Bio</i>	CEDAR NCT04819841	SCD	CRISPR/Cas9-AAV6 beta-globin	<ul style="list-style-type: none"> Ex vivo-edited autologous HSPC Correction of beta-globin mutation Adult and adolescents Adult and adolescents 	46
CRISPR_SCD001	NCT04774536	SCD	CRISPR-Cas9 beta-globin	<ul style="list-style-type: none"> Ex vivo-edited autologous HSPC Correction of beta-globin mutation 9 participants, 12–35 y old Sponsor: Dr. Mark Walters (UCSF) Benioff Children Hospital, UCLA and UC Berkeley 	109,110
CTX001 <i>Vertex Pharm</i> <i>CRISPR Therap</i>	CLIMB-121 NCT03745287	SCD	CRISPR-Cas9 Ablating <i>BCL11A</i> enhancer	<ul style="list-style-type: none"> Ex vivo-edited autologous HSPC Restore fetal hemoglobin (HbF) 7 adult participants Sponsor: US-based companies 	61
BIVV003 <i>Sangamo Therapeutics</i>	PRECIZN-1 NCT03653247	SCD	ZFN mRNA Ablating <i>BCL11A</i> enhancer	<ul style="list-style-type: none"> Ex vivo-edited autologous HSPC Restore fetal hemoglobin (HbF) 8 adult participants Sponsor: Sanofi, France 	59,60
OTQ923 HIX763 <i>Novartis Pharm</i>	NCT04443907	SCD	CRISPR-Cas9 Ablating <i>BCL11A</i> enhancer	<ul style="list-style-type: none"> Ex vivo-edited autologous HSPC Restore fetal hemoglobin (HbF) Section 1: OTQ923 tested in adults Section 2: HIX763 tested in adults Section 3: HIX763 or OTQ923 30 participants 2–17 y old and 18–40 y old Sponsor: Novartis and Intellia Therap. 	
EDIT-301 <i>Editas Medicine</i>	RUBY NCT04853576	SCD	CRISPR-Cas12a Enhance <i>HBG1/2</i> promoter region in beta-globin locus	<ul style="list-style-type: none"> Ex vivo-edited autologous HSPC Restore fetal hemoglobin (HbF) Tested in 0–18 y old Sponsor: US-based company 	
BEAM-101 <i>Beam Therapeutics</i>	BEACON-101 Not yet recruiting	SCD	Base-editing <i>HBG1</i> and <i>HBG2</i>	<ul style="list-style-type: none"> Ex vivo-edited autologous HSPC A to G-based editing in the <i>HBG1</i> and <i>HBG2</i> promoter region Mimics HbF natural mutation to increase HbF Sponsor: US-based company 	

Currently, no gene-editing clinical trials are opened for PIDs.

Abbreviations: BCL11A, BAF chromatin remodeling complex subunit; HbF, fetal hemoglobin; HBG1/2, hemoglobin subunit gamma 1/2; HSPC, hematopoietic stem and progenitor cell; SCD, sickle cell disease.