CONCISE REVIEW

STEM CELLS
TRANSLATIONAL MEDICINE

Genome-edited adult stem cells: Next-generation advanced therapy medicinal products

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Abstract

Over recent decades, gene therapy, which has enabled the treatment of several incurable diseases, has undergone a veritable revolution. Cell therapy has also seen major advances in the treatment of various diseases, particularly through the use of adult stem cells (ASCs). The combination of gene and cell therapy (GCT) has opened up new opportunities to improve advanced therapy medicinal products for the treatment of several diseases. Despite the considerable potential of GCT, the use of retroviral vectors has major limitations with regard to oncogene transactivation and the lack of physiological expression. Recently, gene therapists have focused on genome editing (GE) technologies as an alternative strategy. In this review, we discuss the potential benefits of using GE technologies to improve GCT approaches based on ASCs. We will begin with a brief summary of different GE platforms and techniques and will then focus on key therapeutic approaches that have been successfully used to treat diseases in animal models. Finally, we discuss whether ASC GE could become a real alternative to retroviral vectors in a GCT setting.

KEYWORDS
adult stem cells, CRISPR, electroporation, gene delivery systems in vivo or in vitro, gene therapy, hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), pluripotent hemopoietic stem cells

Karim Benabdellah and Francisco Martin are senior authors.

1 | REASONS FOR USING ADULT STEM CELLS

Gene and cell therapy (GCT) strategies utilize multiple cell types for the treatment of different diseases.[1] The most common approaches use adult stem cells (ASCs), also known as somatic stem cells, as well as T cells. However, other differentiated cell types, such as B, NK and macrophage cells, as well as pluripotent stem cells (PSCs), have also been used.[2,3] In particular, PSCs, which are essentially embryonic stem cells (ESCs), and induced PSCs (iPSCs), have been proposed as potent therapeutic tools due to their ability to produce all types of mature cells in the human body. However, although PSCs are widely used in basic research, very few studies have been carried out on their clinical applications. PSCs are restricted to a small number of applications in clinical trials according to recent data published on the U.S. National Institute of Health's web page regarding clinical trials (https://clinicaltrials.gov). The possibility of PSC-derived products being contaminated by potentially tumorigenic undifferentiated cells, as well as the lack of clear regulatory guidelines, has delayed their clinical application. In addition, genetic alterations can be accumulated during PSC passage and/or differentiation.[4,5] Of the 25 clinical studies using PSCs, 21 use hESCs, 4 are based on iPSCs, while none use genetically modified PSCs.

Unlike PSCs, multipotent, undifferentiated ASCs, which are found in all organs of living organisms, are involved in physiological tissue regeneration.[6] ASCs, which have a self-renewal capacity, can give rise to some or all of the differentiated cells of the tissue in which they reside. They have been widely used *in clinic* due to their ability to regenerate tissues, such as blood and skin, and to dampen immune responses. Most ASCs used in clinical trials are hematopoietic progenitor stem cells (HPSCs) and mesenchymal stem cells (MSCs), with over 3000 clinical trials carried out so far (ClinicalTrials.gov 2019). A major reason for the success of ASC transplants is their safety. However, in several applications, genetic modification of ASCs is necessary in order to achieve the desired therapeutic benefits.[1] Genetically modified ASCs have been successfully employed in the treatment of several disorders through the use of integrative viral vectors.[7] These ASCs include HSPCs which are chosen due to their capacity to be grafted in bone marrow and give rise to all hematopoietic lineages. Over 120 clinical trials involving genetically modified HSPCs are ongoing or have been completed worldwide, 7 of which are now in Phase III or IV, with one medicinal treatment (Strimvelis) already approved by the Food and Drug Administration (FDA) and European Medicines agency (EMA). In addition to HSPCs, other gene-modified ASCs have also reached Phase I/II clinical trials, including MSCs, T stem cell memory (TSCM) cells, epidermal stem cells (EpSCs), endothelial stem cells (EnSCs), and neural stem cells (NSCs) (data obtained from https://clinicaltrials.gov and http://www.abedia.com/wiley/). Most of the clinical trials mentioned above rely on semi-random integration of one or more copies of the therapeutic gene introduced into the host genome using γ-retroviral or lentiviral vectors. However, this type of genetic integration has generated concerns regarding the possibility of cellular transformation and expression variability.[8] In this

Significance statement

Recent advances in adult stem cells and genome editing techniques have enabled scientists to envisage the generation of efficient and safe advanced therapy medicinal products for the treatment of untreatable diseases. Hematopoietic progenitor stem cells are now clearly regarded as the cell type of reference. Promising results have been achieved in controlling AIDS and hemoglobinopathies, resulting in several clinical trials. Promising results have also been obtained in the treatment of monogenic diseases, including X-SCID, SCID-ADA, X-CGD, and Fanconi anemia, indicating that further clinical trials will be approved in the near future.

review, we discuss the potential role of genome editing (GE) technologies in overcoming the limitations of retroviral vectors. We will focus on ex vivo strategies using ASC GE in clinical and/or preclinical settings.

2 | GE STRATEGIES

GE involves a group of technologies that enable the cellular genome to be modified. However, for its successful in-clinic application, GE needs to be used efficiently either in vitro or in vivo without affecting the normal physiology of targeted human cells. Nuclease-independent[9,10] technologies, as well as those based on the use of specific endonucleases (SENs), are used to carry out GE.[11] The nuclease-independent strategy facilitates GE without generating double strand breaks (DSBs) by using systems that improve homologous directed recombination (HDR) such as adeno-associated virus (AAV) vectors[10] or that introduce distortions in the target DNA that triggers repair mechanisms, such as triplex-forming oligonucleotides (TFOs)[9] (Figure 1).

Whether GE systems generate DNA breaks or distortions, the target cell triggers different DNA repair mechanisms, mainly through non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is a type of double-stranded break repair mechanism that does not require a DNA donor. The targeted sequences are rapidly processed by cellular machinery which generates small insertions or deletions (indels). Although a less efficient DNA repair mechanism, HDR is more precise than NHEJ. When a compatible donor DNA template is delivered to the cell, these DNA molecules are incorporated into the endogenous locus, thus enabling precise modifications to be carried out (Figure 2). The most advanced strategy in terms of preclinical and clinical applications is NHEJ-mediated GE which is highly efficient. NHEJ-based GE strategies using SENs and ASCs have already reached the clinical stage for the treatment of sickle cell disease (SCD), B-thalassemia, AIDS, and acute lymphoblastic leukemia.

There are four main types of SEN: mega nucleases (MGNs), transcription activator-like effectors nucleases (TALENs), zinc finger
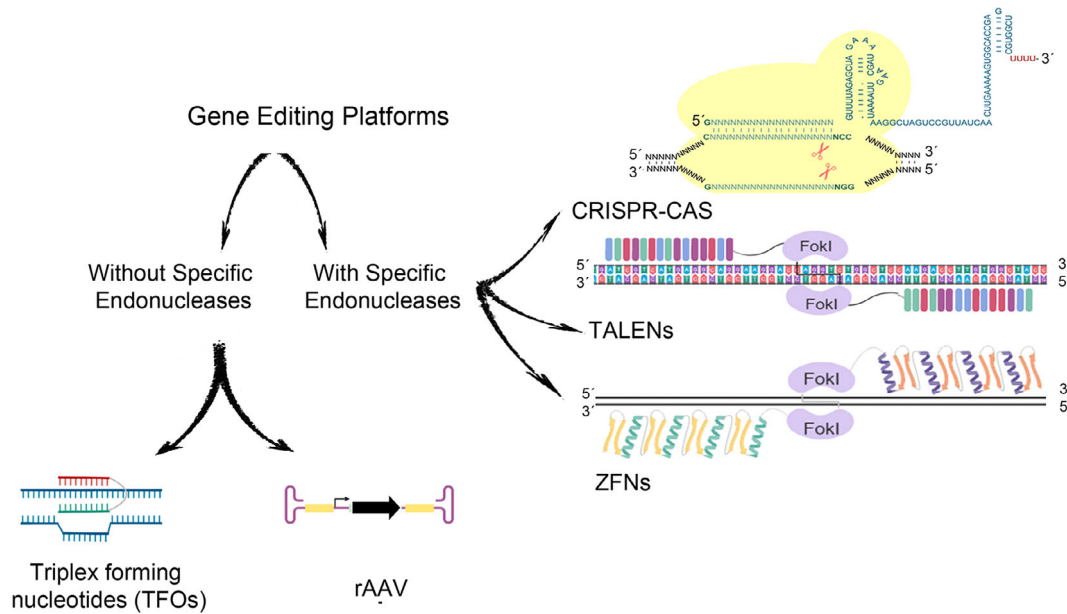
FIGURE 1 Current genome editing technology platforms can be divided into two main groups: specific endonuclease (SEN)-based (right) and nuclease-independent (left) platforms. The three main types of SEN-based genome editing platforms are the transcription activator-like effector nuclease (TALEN), zinc finger nuclease (ZFN), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) systems. The principal SEN-free gene editing platforms use recombinant adeno-associated virus (*rAAV*) vectors and triplex-forming oligonucleotides (TFOs)

nucleases (ZFNs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated system 9 (CRISPR-Cas9) systems.[11] The success of GE approaches greatly depends on the type of gene editing tools used and on how these tools are delivered to the cells and tissues. Another important feature is safety, which can be measured by the levels of unwanted off-target modifications outside the target locus. As both NHEJ and HDR strategies are capable of introducing undesirable modifications into the host genome, it is crucial to accurately determine the safest system to be used (a combination of the appropriate GE tool, delivery system, and strategy).[12–16]

In order to obtain GE-ASCs, cells must first be isolated from their original tissue and then edited ex vivo. As explained above, how GE tools (SENs and/or donor) are delivered to the ASCs is crucial for the success of the strategy. These delivery methods can be viral,[16–19] nonviral,[20–22] or a combination of both,[13] and should be transient, highly efficient, and nontoxic. Recently, a hybrid method based on murine leukemia virus particles has produced interesting results.[23,24] A more detailed review of delivery systems for GE can be found elsewhere.[25]

The most effective platforms for NHEJ-GE of ASCs are mRNA nucleofections for ZFNs and the ribonucleoprotein complex for CRISPR. Nucleofection, a type of electroporation system, is probably the most successful physical non-viral-based method for delivering macromolecules to target cell nuclei. It is important to note that nucleofection produces a spike in SEN expression, thus reducing toxicity and increasing GE specificity.[7,26] Clinical-grade electroporators, which can be used in clinical trials for the treatment of AIDS and blood disorders, have been developed. In addition to nucleofection, adenoviral (AdV), AAV, and integration-deficient lentiviral vectors (IDLVs)[16,27] are often used in HDR-GE strategies. Although capable of efficiently delivering large donor DNAs, these viral vectors can also be used to deliver specific nucleases.

A major concern with using GE technologies as a treatment option arises from the possibility of introducing off-target unwanted alterations into the modified genome.[12,28] However, none of the methods for detecting the distribution and frequency of off-targets introduced by SENs are regarded as sufficiently robust to be implemented in clinical trials.[14] Nevertheless, it is very useful to compare different off-target SEN activities in order to develop more effective and safer strategies. Some research groups have focused on CRISPR-based systems which have no endonuclease activity but maintain the capacity to bind to the site indicated by the gRNA. New CRISPR/Cas9, such as cytosine base editors and adenine base editors,[29,30] have been developed. These editors combine a catalytically dead Cas9 (dCas9) with a cytosine or adenosine deaminase domain in order to facilitate direct single-base pair substitutions (C:G to T:A and A:T to G:C) without generating DSBs. More recently, the group led by Dr. Liu has developed "prime editing" technology by combining a dCas9, a reverse transcriptase and a prime editing guide RNA (pegRNA). This technique enables DNA to be edited with unprecedented precision, with fewer errors being introduced than previous gene-editing technologies.[31]

Although technical issues still need to be addressed, 23 GE clinical trials using ZFNs (14 clinical trials), CRISPR/Cas9 (16 clinical trials), and TALENs (3 clinical trials) for the treatment of infectious
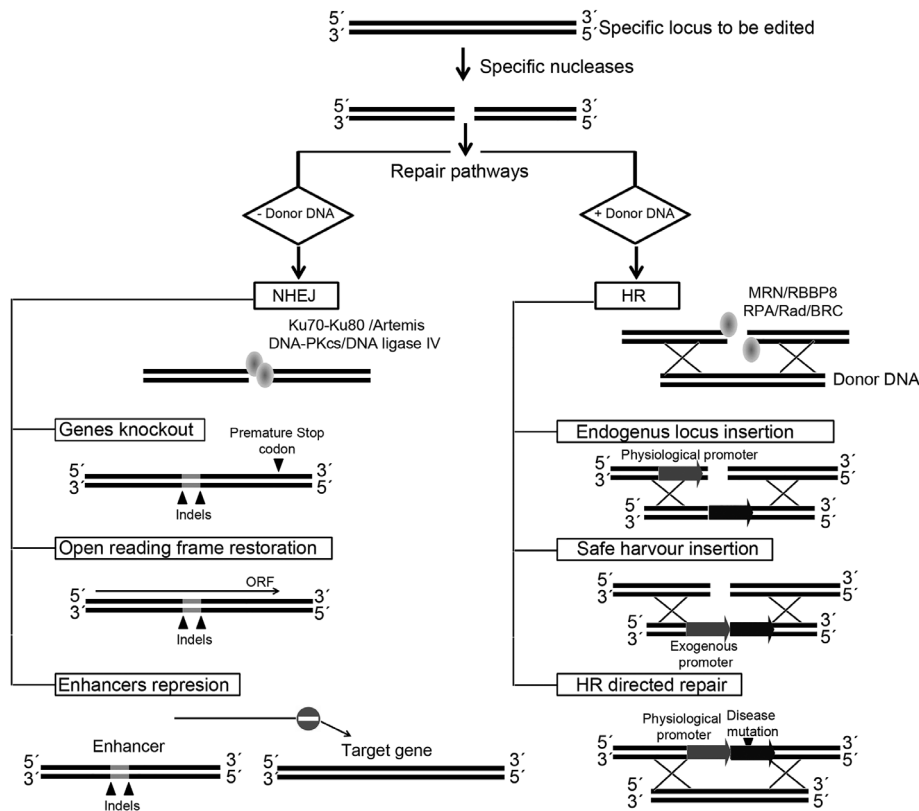
FIGURE 2   Genome editing strategies based on the activity of specific nucleases (SENs). Once SENs generate a double strand break in the target locus (top), the cell triggers two main repair pathways depending on the availability of homologous DNA and cell type. Non-homologous end joining (NHEJ, left) preferentially occurs on G1 phase and quiescence cells, whereas homologous recombination (HR, right) generally requires cell division and takes place in the S phase of the cell cycle. In the NHEJ pathway, the donor or template DNA are not available and, after SEN cleavage, the binding of the proteins Ku70-Ku80 protects the DNA ends against excessive resection and promotes DNA repair by recruiting the Artemis, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and DNA ligase IV complex. This repair pathway introduces short DNA insertions or deletions (indels) into the target site and facilitates different GE approaches: (a) generation of knockout genes by eliminating the ATG or by changing the open reading frame, thus generating premature stop codons, (b) repair of the correct open reading frame on mutated genes, or (c) elimination/alteration of enhancers/promoter regions. In contrast to NHEJ, in the HR pathway, the cell uses a donor DNA to fix DNA breaks introduced by SENs. HR repair is initiated by the combined action of the MRE11-RAD50-NBS1 (MRN) complex and RBBP8 generating single strand DNA where replication protein A (RPA), in association with Rad and BRC proteins bind and promote HR by invading the homologous template. By providing abundant homologous DNA donors, the HR pathway can also be used for different GE strategies: (a) insertion of a cDNA sequence under the regulation of a specific locus in order to provide locus-specific, physiological expression of the particular cDNA. (b) Insertion of an expression cassette (promoter and cDNA) into a safe location (harbor). (c) Alternatively, HR can be used for HR-directed repair (HRDR) of disease-causing mutations (precise gene editing) by providing DNA donor harboring the corrected DNA sequences

diseases (HIV-1 and HPV), cancer, as well as blood and metabolic disorders, are currently on-going (Clinicaltrials.gov Dec 2019). Six of these clinical trials use ex vivo ASC techniques to treat AIDS and blood disorders.

3 | GE of ASCs

As mentioned above, the vast majority of clinical trials involving gene-edited stem cells use ASCs. Although those using HPSCs are by far the most successful,[1] EpSCs, MSCs, TSCM cells, and NSCs have also produced promising results.

3.1 | Genome-edited HSPCs

The engraftment of HSPCs in a recipient's bone marrow, which gives rise to all types of hematopoietic cells, provides a wide range of intervention opportunities for a large number of disorders.[7] Despite being some of the most desirable target cells for stem cell-based therapies, HSPCs are highly resistant to gene modification and preferentially use NHEJ rather than HR pathways to repair damage to DNA.[11] This preference explains why the vast majority of HSPC GE studies and clinical trials use NHEJ-based strategies rather than HR-based approaches (Figure 3). Table 1 summarizes important preclinical studies of HSPC gene therapies (GTs).
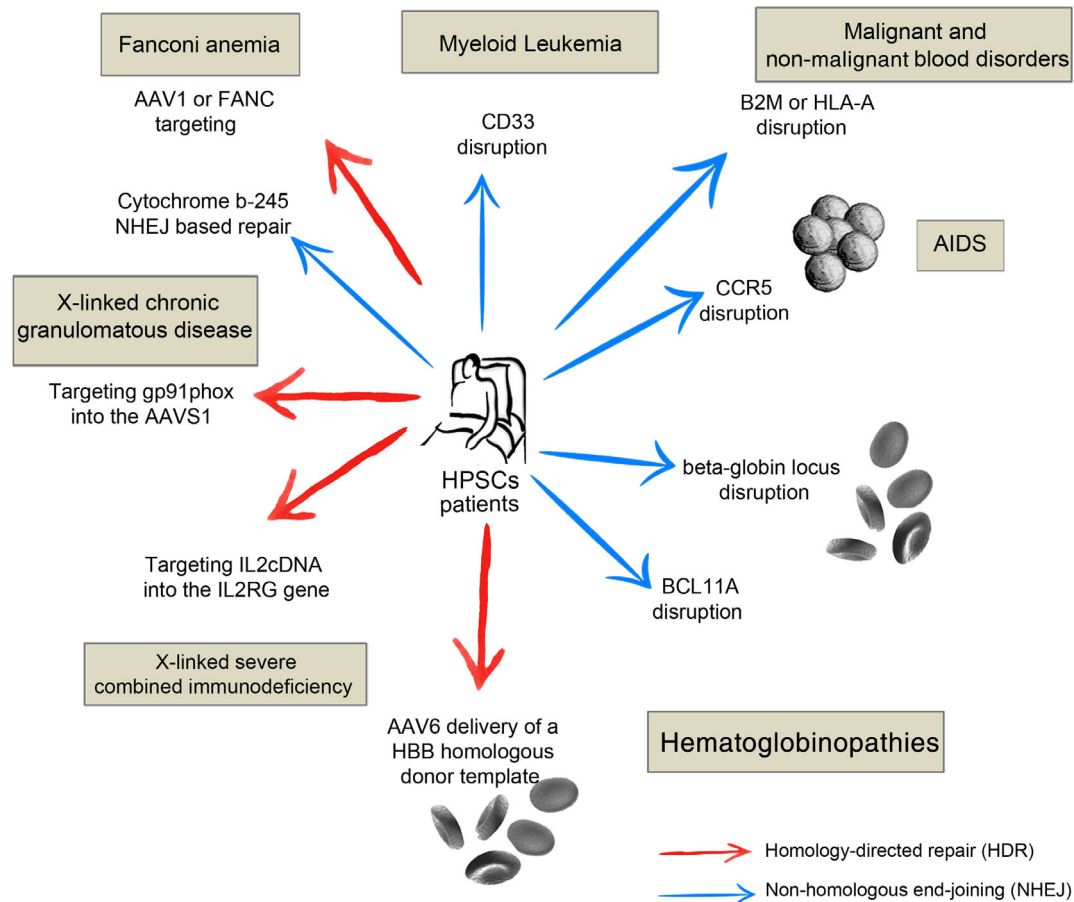
FIGURE 3 Diagram showing potential clinical HPSCs genome editing applications using either NHEJ- or HR-based approaches (blue and red arrows, respectively). The target conditions are indicated in gray boxes, and each arrow points to the locus targeted in each case

3.1.1 | HSPC GT for infectious diseases (HIV-1)

Although several strategies based on GE technology have been designed to fight different infectious agents, only human immunodeficiency virus type I (HIV-1) has been targeted using HSPC GE. Initial studies have demonstrated that long-term HSCP-CCR5-KO repopulation can be achieved using ZFN[43,44] and CRISPR/CAS9[42,54] strategies, both of which provide protection for HIV-1 in humanized NOD/SCID/IL2R gamma mice. Paterson et al. were the first to use nonhuman primates (NHPs) to demonstrate multilineage repopulation of genome-edited HSPCs.[45] These preclinical studies led to the first two clinical trials to evaluate the feasibility, safety, and engraftment of allogeneic and autologous HSCP-CCR5-KO in China (NCT03164135) and the United States (NCT02500849), respectively (Figure 4). The clinical trial being carried out in the United States using autologous HSPCs (CCR5-KO) and ZFNs, for which patients were recruited in September 2019, is sponsored by the City of Hope Medical Center in collaboration with Sangamo Therapeutics. These patients were placed on either a 2- or 3-day course of busulfan prior to product infusion in order to enhance HSPC engraftment. The clinical trial in China has already produced an initial report on HIV-1-infected patients treated with genome-edited HSPCs.[55] Paterson et al. treated HIV-infected patients with acute lymphocyte leukemia

(ALL) at the Academy of Military Medical Sciences in China using CRISPR/CAS9 to edit the CCR5 locus of allogeneic HSPCs. The patients with ALL achieved complete remission, with CD4+ cells in *CCR5KO* mice found to increase following a pause in antiretroviral therapy. However, the percentage (5%) of *CCR5* disrupted was relatively low, indicating the need for further improvement.

3.1.2 | GE-HPSCs for monogenetic diseases

Monogenetic diseases are a series of inherited pathologies associated with alterations in a single gene that can be point mutations, indels, or large deletions. These diseases include hemoglobinopathy, X-linked severe combined immunodeficiency (X-SCID), and Fanconi anaemia (FA), which were initially considered to be targets for treatment with gene-corrected HPSCs.

Hemoglobinopathies
Hemoglobinopathies, characterized by defective hemoglobin synthesis, include SCD, and β-thalassemia. Lentiviral vector (LV)-based GT has been highly successful in integrating the normal β-globin gene into *HPSCs*, a strategy which has reached clinical trial phase III using

TABLE 1 Examples of successful preclinical studies, combining different type of ASCs and genome editing tools, for the treatment of different genetic and infectious diseases

Cells type	Diseases	GE strategies	Tools	Ref
Hematopoietic progenitor stem cells	Primary immune deficiency	HR - insertion in safe harbor	CRISPR-Cas9/ZFNs	32, 33
		HR - insertion in affected locus	ZFNs	27
	Hematoglobinopathies	HR - gene repair	CRISPR-Cas9	34
			ZFNs	35
		NHEJ - therapeutic mutation	TALENs	36
			CRISPR-Cas9	37
			ZFNs	38
	Fanconi anemia	HR - insertion - safe harbor	ZFNs	39
	Cancer	HR - therapeutic mutation	CRISPR-Cas9	40, 41
		NHEJ - exon deletion		
	Infectious diseases	NHEJ - gene disruption	CRISPR-Cas9	42
			ZFNs	43–45
Other stem cells				
Mesenchymal stem cells	Liver fibrosis	HR - insertion in safe harbor	TALENs	46
	Parkinson's disease			47
Epidermal stem cells	Dystrophic epidermolysis bullosa	NHEJ - therapeutic mutation	CRISPR-Cas9	48, 49
			TALENs	50
	Junctional epidermolysis bullosa	HR - insertion in affected locus	CRISPR-Cas9	51
Neural stem cells	Krabbe disease	HR - insertion in safe harbour	CRISPR-Cas9/TALENs	52
Muscle stem cells	Duchenne muscular dystrophy	HR - gene repair	CRISPR-Cas9	53

FIGURE 4 Diagram showing the principal steps in a clinical trial using autologous as compared to allogenic HSPCs. HPSCs were harvested from patients and healthy donors and cultivated in vitro. Once an optimal number of cells with the appropriate phenotype were obtained, they were subjected to gene editing and then infused back into the patient who was treated with the appropriate conditioning regimen
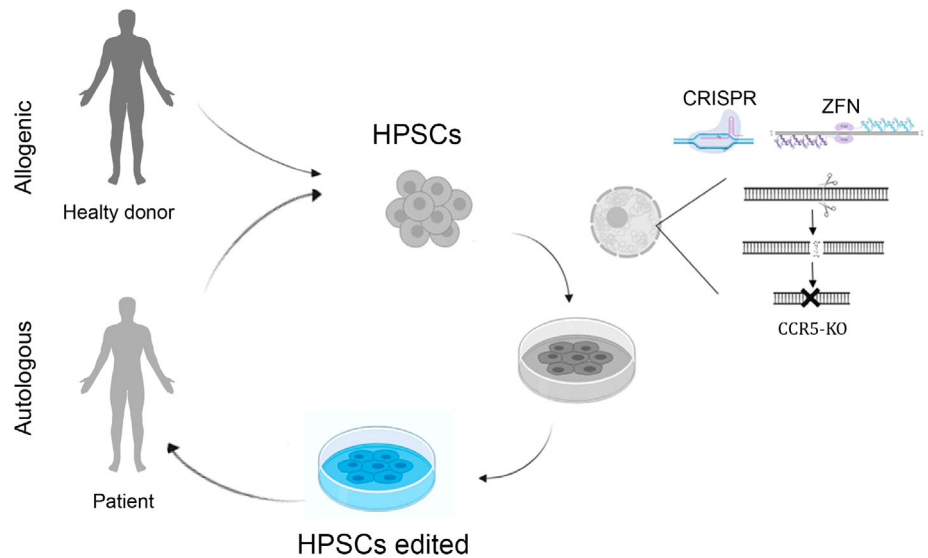
LentiGlobin (NCT03207009). Despite their considerable success, LVs, which are integrated into the transcriptionally active locus, represent a potential risk of cellular transformation.[8] GE appears to be a potentially safer alternative for restoring normal β-globin expression either through insertion of the healthy β-globin gene via the HR pathway or through reactivation of the fetal γ-globin gene[34–38,56–58] (Figure 5). The most successful strategies are aimed at reactivating fetal γ-globin gene expression by disrupting the negative regulatory region of the γ-globin gene.[59, 60] These were the first human GE strategies to be investigated in clinical trials using CRISPR/CAS9 (CTX001, NCT03745287) and later

ZFNs (ST-400; NCT03432364) for the treatment of β-thalassemia and SCD. New clinical trials have been approved for these diseases using CRISPR (iHSCs; NCT03728322) and ZFNs (PRECIZN-1; NCT03653247). Recently, CRISPR Therapeutics and Vertex published the initial results of monitoring the CTX001 trial at month 9 for a patient with β-thalassemia and at month 4 for a patient with SCD (www.crisprtx.com). The β-thalasemia patient in the transfusion-independent clinical trial had total hemoglobin levels of 11.9 g/dL, fetal hemoglobin of 10.1 g/dL, and erythrocyte-expressing fetal hemoglobin of 99.8%, with no severe side effects. The SCD patient also underwent significant
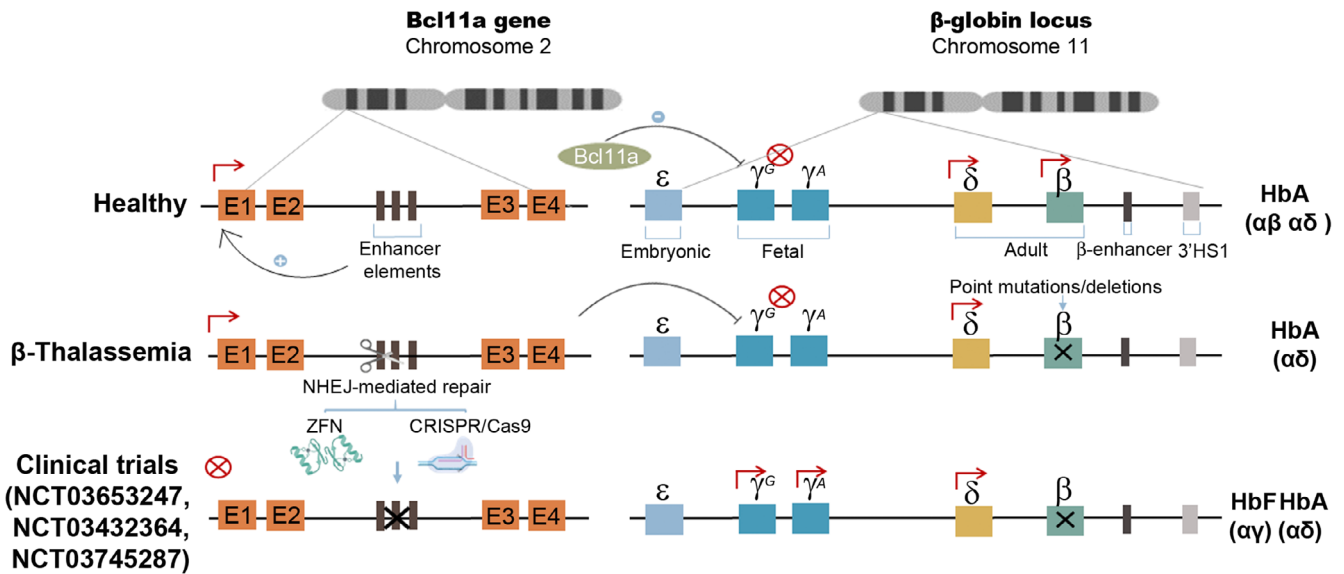
FIGURE 5 NHEJ GE strategies for treating β-thalassemic and sickle cell disease (SCD) patients. Schematic representation of the β-globin cluster in healthy individuals (top drawing). Only adult globins, δ-globin and β-globin, are expressed in healthy adult individuals. The Bcl11a gene is expressed thanks to the erythroid-specific enhancer and blocks fetal globin (γG and γA) expression. In β-thalassemia and SCD patients (middle drawing), mutations in the β-globin gene abrogate its normal expression, preventing the generation of the predominant adult hemoglobin form (αβ). Three different clinical trials are currently on-going to investigate the feasibility of eliminating the erythroid-specific enhancer of the Bcl11a gene on HSPCs (Bottom drawing) using ZFNs (NCT03432364 and NCT03745287) or CRISPR/Cas9 (NCT03653247). By disrupting Bcl11a gene expression in erythroid cells, the fetal γ-globin will be expressed in adults forming fetal hemoglobin (αγ) which should restore normal function

improvement, with the occurrence of novaso-occlusive crisis (VOC) episodes (total hemoglobin levels of 11.3 g/dL and 94.7% of erythrocytes expressing fetal hemoglobin).

Fanconi anemia (FA)

FA, characterized by congenital malformation and cancer susceptibility, with defective repair of DNA inter-strand crosslinks (ICLs), is a rare disease associated with genetic mutations in one or more of the 22 FANC genes. FA is an excellent target for genetic correction, as corrected stem cells and their progeny have a strong selective in vivo advantage.[61] Five on-going GT trials, including one at the phase II stage (NCT02931071), are investigating the efficacy and safety of HSPCs expressing the correct version of the FANCA gene through the use of LVs (ClinicalTrials.gov Sept 2019). Despite the positive outcome of the latest clinical trial using LVs,[61] GE is also being studied as a potential alternative to FA GT. However, the intrinsic problems of repairing ICL lesions in the cells of FA patients have made gene therapists cautious about using gene editing to treat these patients. Nevertheless, several studies have demonstrated the feasibility of the GE of fibroblasts from FA patients harboring mutations.[62] Diez et al., who have demonstrated the feasibility of phenotypic correction of FA HPSCs by inserting a *FANCA* donor in an *AAVS1* locus via HR, have also shown that GE can be effective in HPSCs when ZFNs are used.[39]

Primary immunodeficiencies (PIDs)

Several PIDs, including severe combined immune deficiencies (SCID-X1, SCID-ADA), Wiskott-Aldrich syndrome (WAS), chronic granulomatous disease (CGD), and X-linked hyper-IgM (X-HIM), have been successfully treated using GT-based approaches involving both γ-retroviral and LVs.[63] Given the mutagenic nature of first-generation γ-retrovirus-based vectors, some patients developed leukemia. However, the latest generation of these vectors has produced better results, with physiological promoter-driven, self-inactivating γ-retrovirus[64] and lentivirus[65,66] vectors, in particular, found to be safer and more effective in clinical settings.[1] As a result, a new advanced therapy medicinal product (ATMP), named Strimvelis, has been approved for the treatment of SCID-ADA, with several others on the way. GE has opened up the possibility of further improvements in GT strategies by expressing the therapeutic transgene in a more physiological manner through the use of endogenous regulatory sequences and/or reductions in genotoxicity caused by semi-random integration of retroviral vectors. Different cell models have been used to test the feasibility of using GE to correct genetic mutations causing PIDs.[67] However, HPSC GE is problematic due to poor permissiveness to gene transfer and limited HD DNA repair pathways in these cells. In 2014, Genovese et al. demonstrated, for the first time, successful correction of target genes in human HPSCs.[27] Using a HR-based approach based on ZFN mRNA nucleofection and IDLV-DNA donors, they repaired the mutated IL2RG gene of HPSCs from a patient with SCID-X1 and successfully engrafted genome-edited HPSCs, giving rise to functioning hematopoietic cells. In recent years, using gene-editing tools, other research groups, who have produced new studies on SCID-X1[32,33] using AAV6 for donor delivery and ZFN mRNA,[32] as well as CRISPR/Cas9 RNP[33] nucleofection, have confirmed the feasibility of correcting HPSCs from different PIDs.[32,33,68,69] Kuo et al.

have shown that AAV6 harboring the donor DNA could also be combined with TALEN mRNA or CRISPR/Cas9 RNP to restore X-linked hyper-IgM syndrome.[68] Using a different platform (Cas9 mRNA, gRNA and ssODN), De Ravin et al.[69] repaired up to 20% of HSPCs from patients with X-linked chronic granulomatous disease (X-CGD). See Table 1 for further information on GE for PID.

HLA gene editing enables the generation of HSPC universal donor cells

In recent decades, unmodified allogeneic HLA-matched *HSPCs* have been used to treat malignant and non-malignant blood disorders. However, the success of transplants depends on the existence of compatible donors, whereas the risk of graft rejection is still a major concern. A definitive approach could be developed through the genetic elimination of HLA mediated by GE. Torikai et al. have used ZFNs to fully disrupt HLA-A in T cells.[70] Recognition of GE-T cells by natural killers was circumvented by the expression of non-classical HLA molecules. This research group later demonstrated the feasibility of their approach with HSCs which maintains the engraftment of the engineered cells and reconstitutes hematopoiesis in immunocompromised mice.[71] Other strategies could benefit from using universal HLA−HSPCs as ATMP cells by, for example, enabling the manufacture of erythrocytes and/or platelets from universal HLA−HSPCs.

3.1.3 | HSPC GE for cancer GT

Genetically modified T cells expressing a chimeric antigen receptor (CAR) are a powerful new class of advanced therapy medicinal product.[72] CD19-targeting CAR-T cells were recently approved by the FDA for the treatment of refractory type B leukemia and lymphomas. This approach does not discriminate between normal and malignant B cells, although patients can live almost normal lives without B cells if treated with immunoglobulins. CD33 is an interesting target for acute myeloid leukemia (AML).[73] Unfortunately, CD33 is expressed in both malignant and in normal myeloid cells (including progenitors), which are destroyed by CD33-CAR-T cells. In order to overcome this limitation, several research groups have targeted CD33 from normal HSPCs using CRISPR/Cas9 in order to generate functional myeloid cells resistant to CAR-T αCD33.[40,41,74] Gene-edited HSPCs showed normal myeloid function and resistance to CD33 therapy mediated by T cells engineered to express CAR-targeting CD33.[40,74] It is important to note that multilineage reconstitution has been demonstrated in both mouse and NHP models.[40,41,74]

3.2 | Other stem cells

GE of other types of cells, such as EpSCs, MSCs, as well as muscle and neural stem cells, has also produced interesting results in the treatment of several diseases. However, except for EpSCs, the results have been insufficiently conclusive to test these strategies in clinical trials. The most important studies in the field are summarized below.

3.2.1 | Epidermal stem cells

The genetic modification of EpSCs has important applications in the treatment of diseases such as recessive dystrophic epidermolysis bullosa (RDEB) and junctional epidermolysis bullosa (JEB). Major advances have been made using gamma-retroviral and LV vectors to generate autologous artificial skin expressing Col7a1 for RDEB and LAMB3 for JEB; JEB can be genetically corrected by transplanting genetically modified EpSCs.[75,76] As discussed above, EPSC GE could be a good alternative to retroviral vectors, as several research groups have managed to restore Col7Aa1[48–50] and LAMB3[51] expression by genetically editing EpSCs from RDEB and JEB patients, respectively. In both these pathologies, artificial skin generated using genetically modified EpSCs has enabled long-term engraftment of phenotypically normal skin. This provides strong support for future ex vivo GE clinical trials for the treatment of RDEB and JEB patients.

3.2.2 | Mesenchymal stem cells

Due to their regenerative potential and anti-inflammatory properties, MSCs have been widely used in clinical trials for multiple diseases. However, despite the major successes of MSCs in some disorders, which have led to the approval of MSC therapies,[77–79] they have had limited therapeutic benefits in other applications. To overcome these limitations, several research groups are investigating the feasibility of genetically modifying MSCs to express different genes that enhance their therapeutic efficacy.[80] As previously described with respect to other cell types, GE has also become an alternative to GT vectors. GE-MSCs, which are being studied as a platform for the delivery of proteins into the blood stream,[81] are an interesting tool in the treatment of blood disorders caused by the absence of plasma proteins. In this setting, GE has been used to insert hFIX and hFVIII into the AAVS1 locus of MSCs through homologous recombination (HR) in order to treat hemophilia A and B.[82,83] GE-MSCS are also considered a potential alternative treatment for neurodegenerative diseases such as Parkinson's disease (PD). Using this approach, MSCs have been engineered to secrete soluble receptors of advanced glycation end products from AAVS1 loci.[47] The aim is to reduce advanced glycation end product concentrations involved in PD and Alzheimer's disease. GE-MSCs have also been used in the treatment of ischemia in an animal infarct model. Meng et al. have used TALENs to integrate the *IL-10* gene into the AAVS1 safe harbor locus of MSCs and have performed an intra-myocardial transplant in the infracted myocardium, which reduced pro-inflammatory factor expression and increased vascular density.[84,85] The same approach was used to ameliorate liver fibrosis.[46] All these data indicate that GE is becoming a real alternative to viral and nonviral vectors in generating genetically modified MSCs.

3.2.3 | Neural stem cells

Neural stem cells (NSCs), whose regenerative capacity, as with MSCs, can be improved by GE, have promising, although not immediate,

clinical potential as a cellular treatment for neurological diseases. Recently, Dever et al. demonstrated that NSCs can be modified genetically at multiple loci using Cas9 mRNA and DNA donors. They showed that, upon transplantation, GE-NSCs can migrate and differentiate into astrocytes, neurons and myelin-producing oligodendrocytes. They also highlighted the therapeutic potential of GE-NSCs by generating NSCs overexpressing the GALC enzyme which can cross-correct the GALC enzyme activity of fibroblasts obtained from Krabbe disease patients.[52] These findings highlight the therapeutic potential of GE-NSCs, not only for the regeneration of neural cells but also as a Trojan horse to deliver proteins to the central nervous system.

3.2.4 | Muscle stem cells

Muscle stem cells are undifferentiated cells capable of producing new muscle tissue and of fusing with pre-existing myofibers in order to repair damaged myofibers. Muscle satellite cells (MuSCs) are probably the most studied muscle stem cells, a population of cells that are capable of self-renewal and differentiation into muscle fibers which represent an ideal target for therapeutic GE.[86] Zhu et al. have developed a fibrin gel culture system to selectively expand MuSCs from an mdx mouse model for *Duchenne muscular dystrophy* (DMD) research. These cells were successfully corrected using CRISP/Cas9-based GE and, following transplantation to mdx mice, restored dystrophin expression in skeletal muscle.[53] This demonstrates the feasibility of using ex vivo GE-MuSCs to target and correct DMD.

3.2.5 | T stem cell memory cells

TSCM cells, which constitute a recently described 2% to 3% circulating T-cell subpopulation,[87] have a naive T-cell phenotype, express a CD62L memory marker, proliferate, self-renew, and generate effector/memory T cells. TSCM cells have emerged as a highly interesting population for adoptive T-cell therapies for cancer[88] and inherited immunodeficiency.[89] As with other ASCs, GE has also been applied to TSCM cells to enhance the potency of CAR T cells for the treatment of hematological malignancies and solid tumors. Eyquem et al.[90] inserted a α-CD19 CAR into the T-cell receptor A constant (TRAC) locus using Cas9 mRNA and gRNA electroporation followed by transduction with AAV6 harboring the donor DNA containing the CAR. This strategy generated off-the-shelf CAR-T cells (without TCR) which expressed the CAR physiologically through the endogenous TCR promoter. This approach has also been used to improve efficiency thanks to the maintenance of the TSCM phenotype following repeated exposure to the antigen. Recently Sachdeva et al. used a similar approach, involving TALENs instead of CRISPR/Cas9, to insert CAR cDNAs into the TRAC locus and to insert the proinflammatory cytokine IL12 into the CD25 or PD1 loci.[91] This resulted in the physiological expression of the CAR and a transient secretion of IL12 which depends on tumor engagement (following the expression patterns of CD25 or PD1 locus). In addition, the targeted integration of IL12 at the PD1 locus

inactivated PD1, a major T-cell immune checkpoint, and increased the cell surface of CD62L, a marker of TSCM cells. This strategy resulted in increased CAR-T cell cytotoxicity and extended survival of mice engrafted with solid tumors. A further potential improvement in generating universal off-the-shelf CAR-T cells involves simultaneously deleting TCR and beta-2 microglobulin (*B2M*) genes to reduce graft-vs-host disease (GVHD) and CAR-T-cell rejection. Recently, Choi et al.[92] generated αEGFRvIII CAR-T cells lacking the expression of the TCR, B2M, and PD1 electroporating RNP (Cas9 protein and sgRNAs targeting *TRAC*, *B2M*, and *PD1* loci) and transducing with AAV6 vectors containing donor DNAs for insertion of the EGFRvIII CAR construct at the different loci. The authors showed increased survival of mice in mouse models of glioma, which correlated with the increased presence of CAR-T cells with a TSCM phenotype.[92]

4 | CONCLUSION

In recent decades, genetic modification of ASCs using traditional GT vectors has opened up new opportunities to improve ATMPs for the treatment of several diseases. Most approaches use retroviral vectors to achieve stable transgene expression in ASCs upon expansion and differentiation. However, the use of retroviral vectors has several drawbacks associated with oncogene activation and the lack of physiological transgene expression. Recent advances in GE technologies have enabled researchers to design next-generation GM-ATMPs based on ASCs. Using cellular HR- and NHEJ-based repair pathways, GE achieves precise, and safe ASC genetic modifications such as gene disruption, addition and repair, as well as the generation of therapeutic mutations. Six ongoing phase I and II clinical trials are currently being carried out to study the safety and effectiveness of GE-ASCs in the treatment of AIDS, SCD, and β-thalassemia. The trials are based on GE-HSPCs and NHEJ disruption of CCR5 for the treatment of AIDS and on the inactivation of regulatory regions controlling the fetal γ-globin gene repression for the treatment of SCD and β-thalassemia. GE-HSPCs have also produced promising results in preclinical models for monogenetic diseases such as severe X-SCID, SCID-ADA, X-CGD, and FA, as well as for cancer and transplantation. In addition to HSPCs, several other ASCs have been studied as GE targets in therapeutic applications. GE of EpSCs, MSCs, NPCs, and MuSCs has also produced interesting results in animal models of RDEB, JEB, hemophilia, PD, and Krabbe disease.

5 | FUTURE PERSPECTIVES

It is still too early to speculate whether GE-ASC clinical trials for the treatment of AIDS, SCD, and β-thalassemia will lead to the approval of ATMPs for clinical use. For that to happen, these strategies need to demonstrate improved therapeutic effectiveness as compared to other GT approaches based on retroviral vectors already in phase III. Some of these GT techniques are about to be authorized as ATMPs.[7] For example, Lentiglobin, a HSPC-LV-based ATMP, has demonstrated

excellent, long-term therapeutic benefits in β-thalassemia patients, with no severe secondary effects. Therefore, provided GE-ASC clinical trials demonstrate a similar level of therapeutic efficiency and reduced genome alterations as compared to RV-based GT, GE-ASCs will be approved as ATMPs. Regardless of the results achieved in ongoing clinical trials, we believe that, in the near future, next-generation ATMPs will incorporate GE-ASCs in their arsenal. The field of GE is advancing at an unprecedented pace, with new, more efficient and safer tools being developed each year. Advances in the specificity and versatility of SENs,[28,93–95] in strategies to improve HR repair[96,97] and in delivery methods[13,16,98–100] have been and will remain crucial. In addition, the field of ASCs is evolving exponentially due to their safety and potential applications in regenerative medicine.[26,101–105] New developments in both GE and ASCs are bound to provide opportunities to improve the safety and efficacy of GE-ASCs in order to achieve the final objective: the approval by official medical authorities of GE-ASCs as ATMPs.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

S.S.H., A.A.G., N.M.P., A.G.G., M.T.M, M.C.G., I.R.H.: manuscript writing and illustrations; P.G.M., C.H.: manuscript review; K.B., F.M.: manuscript writing, figure artwork, final approval of manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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