Recent Progress in Genome Editing for Gene Therapy Applications: The French Perspective

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Recent advances in genome editing tools, especially novel developments in the clustered regularly interspaced short palindromic repeats associated to Cas9 nucleases (CRISPR/Cas9)-derived editing machinery, have revolutionized not only basic science but, importantly, also the gene therapy field. Their flexibility and ability to introduce precise modifications in the genome to disrupt or correct genes or insert expression cassettes in safe harbors in the genome underline their potential applications as a medicine of the future to cure many genetic diseases. In this review, we give an overview of the recent progress made by French researchers in the field of therapeutic genome editing, while putting their work in the general context of advances made in the field. We focus on recent hematopoietic stem cell gene editing strategies for blood diseases affecting the red blood cells or blood coagulation as well as lysosomal storage diseases. We report on a genome editing-based therapy for muscular dystrophy and the potency of T cell gene editing to increase anticancer activity of chimeric antigen receptor T cells to combat cancer. We will also discuss technical obstacles and side effects such as unwanted editing activity that need to be surmounted on the way toward a clinical implementation of genome editing. We propose here improvements developed today, including by French researchers to overcome the editing-related genotoxicity and improve editing precision by the use of novel recombinant nuclease-based systems such as nickases, base editors, and prime editors. Finally, a solution is proposed to resolve the cellular toxicity induced by the systems employed for gene editing machinery delivery.

Keywords: genome editing, CRISPR/Cas9, gene therapy, HSCs, gene editing toxicity, T cell

INTRODUCTION

Gene or genome editing: the basics

Gene editing is a type of genetic engineering where nucleotides or bigger DNA sequences are inserted, deleted, or replaced in the genome using nucleases, which create site-specific double-strand breaks (DSBs) in genomic loci. There are different types of nucleases: meganucleases (MGNs), transcription activator-like effector nucleases (TALENs), zinc fingers nucleases, and clustered regularly interspaced short palindromic repeats associated to Cas9 nuclease (CRISPR/Cas9).

The cellular machinery repairs the DSB induced by these nucleases either by nonhomologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) pathways or by homology-directed repair (HDR) when a DNA template encoding sequences that are homologous to the targeted genomic locus is available. The most frequent DNA repair pathway that takes place after DSB is NHEJ. In this case, DNA ends are fused without a repair template and this leads to insertion or deletion of nucleotides, often introducing frameshift mutations, totally or partially blocking gene transcription and translation.[1] MMEJ is also frequently involved in repair of DSBs induced by nucleases and typically results in deletions flanked by short stretches of microhomology that may be predicted for highly efficient disruption of the target open reading frame. MMEJ also allows using short terminal homologies to integrate transgenes.[2–4] In contrast, HDR results in gene correction/insertion by

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homologous recombination (HR) with the sister chromatid or delivery of a donor DNA repair template. The DSB induced by endonucleases at a specific locus can be sealed by HDR when an exogenous DNA template is provided carrying homology arms to the targeted genomic locus. This template is provided by plasmids, integration-deficient lentiviral vectors (IDLVs), recombinant adeno-associated viruses serotype 6 (rAAV6), or electroporation of double-stranded DNA, long ssDNA, or oligonucleotides (ODN).[5,6] However, since HDR is restricted to the S/G2 phase of the cell cycle, gene modification remains a challenge for the scientific community, in particular, in primary gene therapy target cells.

One type of these nucleases, the bacteria-originated CRISPR/Cas9 system, has revolutionized the methodology to produce knockout and knock-in (KI) genome editing due to its high specificity, activity, easy design, and highly efficient gene editing in cell lines and primary cells.[7] The CRISPR/Cas9 component can be introduced in the cell of interest using different methods, for example, by using CRISPR/Cas9 encoding retroviral vectors[8] or plasmids[9] and RNAs[6] encoding these components introduced by electroporation. Delivery of mRNA coding for the nucleases combined with guide RNA (gRNA) as RNA is also employed. Currently, although, the method of choice to obtain efficient gene editing in primary human T and B cells and hematopoietic stem and progenitor cells (HSPCs) is electroporation of ribonucleoproteins (RNPs), incorporating gRNA and Cas9 proteins.[10] In contrast to retroviral delivery, RNP delivery offers a major advantage since the Cas9/gRNAs are only transiently present in the cell, thereby avoiding insertional mutagenesis and cellular toxicity related to persistent Cas9 activity,[11] implying a safety benefit essential for clinical applications.

Therapeutic gene editing

The multiple advantages of gene editing over gene addition in the gene therapy field are as follows: (i) the capacity of modifying/correcting specific endogenous DNA sequences; (ii) the normal transcriptional regulation of the gene is maintained since correction can be introduced at the targeted genomic locus, thus allowing a spatiotemporal and thus physiological regulation of transgene expression;[5] and (iii) the insertional mutagenesis risks and the activation of oncogenes are strongly reduced. Thus, precise genetic manipulation of cells by gene editing provides unprecedented opportunities for correction of immune deficiencies such as X-linked severe combined immunodeficiency (SCID-X1)[12] or Wiskott-Aldrich syndrome (WAS)[13] or bone marrow failures, such as Fanconi anemia (FA), and many other disease indications.[14] HSPC-based gene therapy is very attractive treatment for FA because corrected stem cells have a selective advantage.[15] Interestingly, NHEJ was utilized to create an insertion/deletion (indel) next to an FA mutation leading to correction of FA phenotype at high efficiency.[16]

In addition, β-hemoglobinopathies (β-thalassemia and sickle cell disease [SCD]) are attractive targets for therapeutic gene editing using HDR.[17] However, NHEJ might offer alternative correction strategies for gene therapy of β-thalassemia and SCD. Gene editing strategies for β-hemoglobinopathies have rather focused on disruption of silencing factors/regulators such as BCL11A in hematopoietic stem cells (HSCs) to induce *de novo* expression of fetal hemoglobin.[18,19] In this review, two collaborating French research teams will extend further not only on the more recent gene editing approaches for correction of hemoglobinopathies but also other monogenetic diseases such as blood clotting diseases and lysosomal storage disorders (LSD).

In addition to HSCs, T cells are very powerful gene therapy target cells and are highly amenable to gene editing. Gene editing in T cells is being used currently to generate potent chimeric antigen receptor (CAR) T cells for combating cancer.[20] CARs are laboratory designed T cell signaling receptors, which upon encounter with a cancer-specific antigen will get activated and eliminate the cancer cells. One example for improving CAR T cell design is gene editing-mediated knockout of the endogenous T cell receptor (TCR) to avoid the graft versus host (GvH) disease. This might permit to produce a universal CAR T cell.[21] This allows to move away from the up to now obligatory costly autologous CAR T cell therapies for treatment of cancer patients. We will focus in this review on some of the newest gene editing strategies employed by a French research team to improve CAR T cell efficiency.

Interestingly, not only hematopoietic cells such as T and B cells and HSCs are gene therapy targets for gene editing but also, recently, a French team has developed a gene editing approach to correct a type of muscle disease.[22] They will report in this review on myotonic dystrophy type 1 (DM1) correction through gene editing of a gene that is mainly expressed in smooth, skeletal, and cardiac muscles. For the French gene therapy landscape and beyond, it is also important to underline that quite recently, the Technological Research Accelerator in Genomic Therapy (ART-TG) was put in place to help the gene therapy laboratories in France to conduct preclinical studies and to manufacture products and materials in good manufacturing practice for clinical trials, which will include gene editing protocols (https://www.art-tg.com/about-us).

Cellular and genomic toxicity induced by genome editing tools

Genomic toxicity caused by gene editing nucleases remains a major safety concern in therapeutic applications since they can introduce unwanted DSBs in nontargeted DNA sequences of the genome called ''off-targets.'' The off-target activity is linked to areas of the genome that share high homology with the specific site targeted for gene editing.[23] When an off-target cutting event occurs, it can be repaired through the NHEJ and MMEJ pathways,

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potentially resulting in an indel mutation, or, if it occurs simultaneously with an on-target or a second off-target cutting event, the off-target cutting activity can generate a chromosomal rearrangement. In this review, we are not focusing on detection or prediction of off-target effects for gene editing, but refer to an excellent recent review.[24]

To increase the precision of gene editing and reduce off-target cuts, other CRISPR genome editing tools are continuously developed, with enhanced targeting scope and improved editing specificity. Three main classes of CRISPR-based genome editing agents are available today, namely, nucleases as mentioned above, base editors and prime editors. Base editors generate single-nucleotide changes in DNA, while prime editors use Cas9 fused to an engineered reverse transcriptase, programmed with a prime editing gRNA that both specifies the target site and encodes the desired sequence edit template.[23] In this review, French researchers discuss strategies designed to increase the efficiency of precise gene editing by HDR and finally base editing and prime editing, which represent improved alternative tools for precise gene editing applications in gene therapy.

As mentioned above, off-target modifications by the CRISPR/Cas9 first-generation nuclease have been widely reported. However, the on-target NHEJ genotoxicity in response to DNA DSBs has often been underestimated and is not well studied yet. This can lead to huge chromosomal deletions or rearrangements and is not well understood up to now.[25,26] Therefore, a French research team will dedicate here a section to CRISPR/Cas9 induced on-target genomic toxicity by presenting their recent work from a French team elaborating on how to resolve these unwanted side effects.

Finally, the different delivery systems, such as stable retroviral gene transfer and electroporation of plasmids encoding the gene editing tools, induce still cellular toxicity.[13,27] This is problematic when we want to gene modify primary human T and B cells and HSCs since it is clear now that a minimum of living modified cells need to be reinfused in the patient for therapeutic efficiency. Improved protocols for delivery of CRISPR/Cas9 with the associated gRNAs by electroporation of RNPs have reduced cellular toxicity to some extent. In this review, a French team will elaborate on new delivery tools for the gene editing machinery, which especially avoids cell death in primary gene therapy target cells.

RECENT DEVELOPMENTS IN GENOMIC EDITING FOR GENE THERAPY

Novel genome editing approaches for β-hemoglobinopathies

β-Hemoglobinopathies are caused by mutations affecting the production of the β-globin chain of the adult hemoglobin tetramer (Hb) (Fig. 1A, B). In particular, SCD is caused by a single-nucleotide mutation in the sixth codon of the β-globin gene, which leads to the E6V amino acid substitution. Hemoglobin tetramers containing the defective sickle β^S-globin (HbS) polymerize under hypoxia, and red blood cells (RBCs) assume a sickle shape and become inflexible (Fig. 1A). Sickle RBCs have a short half-life and obstruct microvessels causing a chronic multiorgan disease associated with poor quality of life and short life expectancy. β-Thalassemia is caused by mutations that reduce (β^+) or abrogate (β^0) β-globin production. The uncoupled α-globin chains precipitate, causing apoptosis of erythroid precursors and hemolytic anemia (Fig. 1B). Transplantation of autologous, genetically modified HSCs is an attractive therapeutic option for patients lacking a suitable allogeneic HSC donor.

The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of mutations causing fetal β-like γ-globin expression in adult life—a benign condition termed hereditary persistence of fetal Hb (HPFH).[28] γ-globin exerts a potent antisickling effect in SCD and compensates for β-globin deficiency in β-thalassemia.

Two classes of HPFH mutations have been described, large deletions in the β-globin locus or mutations in the γ-globin promoters. HPFH deletion mutations are large deletions, usually encompassing the adult β- and δ-globin genes. They are thought to either remove γ-globin inhibitory sequences or juxtapose the γ-globin genes (Gγ and Aγ) to distal transcriptional enhancers (Fig. 1C). HPFH mutations in the two γ-globin promoters cluster in the –200, –175, and –115 regions upstream of the transcriptional start sites. They are mainly point mutations or small deletions or insertions that either disrupt the binding sites (BS) of fetal Hb (HbF) repressors (*e.g.*, LRF and BCL11A in position –200 and –115, respectively) or generate BS for transcriptional activators (*e.g.*, KLF1, TAL1, and GATA1 in position –200, –175, and –115, respectively).

We have recently generated a 13.6-kb HPFH-like large deletion in the β-globin locus using the CRISPR/Cas9 nuclease system in primary HSPCs from SCD patients (Fig. 1C).[18] NHEJ-mediated deletion of the 13.6-kb region led to a robust reactivation of HbF synthesis in the erythroid progeny of edited HSPCs and to a substantial amelioration of the sickling cell phenotype. Interestingly, HbF reactivation upon generation of HPFH-like deletions in adult erythroid cells was associated with increased interaction of the γ-globin promoters with potent enhancers located within the β-globin locus. Furthermore, we compared the efficiency of different methods to deliver the CRISPR/Cas9 system into HSPCs and generate HPFH-like deletions. RNP delivery exhibited a good balance between cytotoxicity and efficiency, while reducing the off-target activity.[29] Of note, this strategy involves the use of two gRNAs to simultaneously cleave two genomic loci flanking the target region to reproduce the 13.6-kb deletion, thus potentially increasing the off-target effects. In addition, failed deletion of this region results in the generation of NHEJ-mediated indels at the gRNA target sites, whose consequences have not yet been investigated.

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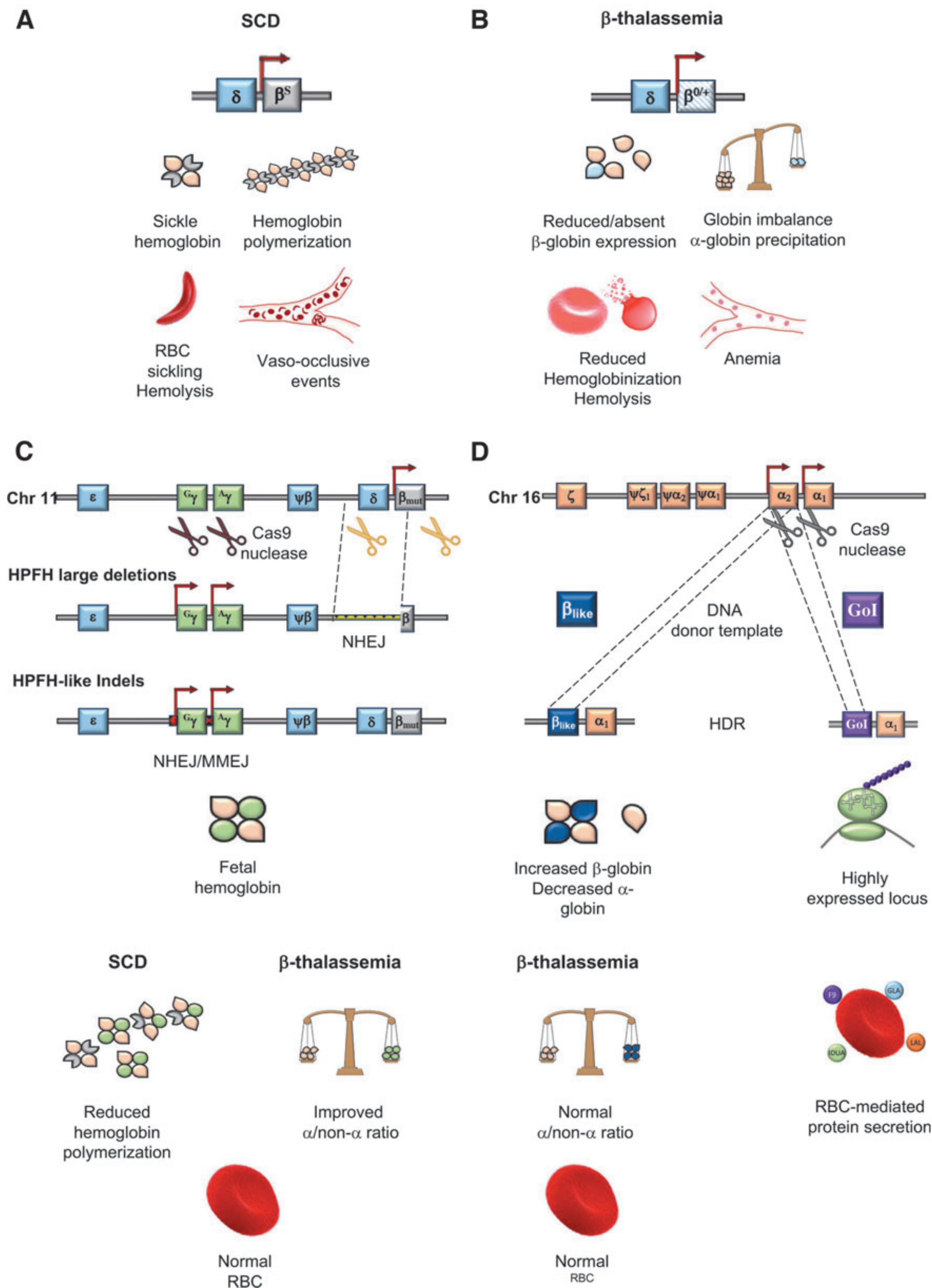

Figure 1. Editing human HSPCs as therapeutic strategy for genetic disorders. Genetics and pathophysiology of SCD **(A)** and β-thalassemia **(B)**. β^S, sickle β-globin. β^0 and β^+ indicate β-thalassemia mutations that cause absent or reduced β-globin expression, respectively. δ, δ-globin β-like chain that accounts for ≤3% of the total β-like chains in adult RBCs. **(C)** Editing the β-globin locus to reactivate HbF expression. *Dark red* scissors indicate CRISPR/Cas9 nuclease editing the Gγ- and Aγ-globin promoters (edited promoters are indicated with *red boxes*). *Yellow* scissors indicate CRISPR/Cas9 nuclease generating an HPFH-like large deletion (the deleted region is indicated with a *yellow box*). ε, embryonic β-like globin gene; Gγ and Aγ, fetal β-like globin gene; $\psi\beta$, β-globin pseudogene; β_{mut}, β-globin gene harboring SCD or β-thalassemia mutations. Hb tetramers contain only γ-globin or both γ-globin and β^S-globin inhibit Hb polymerization.[119,120] **(D)** Editing the α-globin locus to correct β-thalassemia or lysosomal storage disorders. *Gray* scissors indicate CRISPR/Cas9 nuclease deleting the α_2 gene, which is replaced by a therapeutic β-like globin gene or by a transgene expressing a secreted protein. ζ, embryonic α-like globin gene; $\psi\zeta$, ζ-globin pseudogene; α_1 and α_2 fetal/adult α-globin genes; β_{like}, therapeutic β-like globin gene; F9, Factor IX; GLA, α-galactosidase; GoI, gene of interest; IDUA, α-L-iduronidase; LAL, lysosomal acid lipase. CRISPR/Cas9, clustered regularly interspaced short palindromic repeats associated to Cas9 nuclease; HbF, fetal Hb; HPFH, hereditary persistence of fetal Hb; HSPC, hematopoietic stem and progenitor cell; RBC, red blood cell; SCD, sickle cell disease. Color images are available online.

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Furthermore, we have used the CRISPR/Cas9 RNP complexes to disrupt the LRF or BCL11A repressor BS in the γ-globin promoters by NHEJ and MMEJ inducing indel generation, thus mimicking the effect of HPFH mutations (Fig. 1C).[30] In primary SCD patient-derived HSPCs, the efficient targeting of LRF or BCL11A BS resulted in a high proportion of γ-globin expressing HSPC-derived RBCs and correction of the sickling cell phenotype. Editing efficiency was minimal at the vast majority of the predicted off-target sites. Importantly, xenotransplantation of HSPCs treated with gRNAs disrupting the LRF or BCL11A BS in immunodeficient NOD/SCID/$\gamma c^{-/-}$ mice showed a high editing efficiency in long-term repopulating HSCs.

Overall, these studies identified several genomic sites in the β-globin locus as potent targets for genome-editing treatment of SCD. Interestingly, this strategy can be potentially applied also to β-thalassemia. Notably, genome editing strategies for β-hemoglobinopathies aimed at reactivating HbF are currently being tested in clinical trials. In particular, Frangoul *et al.*[31] recently reported promising clinical results of gene therapy trials aimed at downregulating the expression of the HbF repressor BCL11A by targeting its erythroid-specific enhancer. Clinical trials aimed at reactivating HbF through the targeting of the β-globin locus will uncover the therapeutic potential of these alternative approaches.

To further improve the efficacy of HSC editing-based therapy for β-thalassemia, we have devised a novel gene replacement strategy. Clinical data have shown that the severity of β-thalassemia directly correlates with the number of α-globin (*HBA*) genes, with deletions of *HBA* genes having a beneficial effect for patients.[32] In addition, CRISPR/Cas9-mediated downregulation of α-globin gene expression ameliorated the globin balance in β^+-thalassemia patients.[33] To treat both β^0- and β^+-thalassemia, we successfully combined the expression of a therapeutic β-like globin chain and the downregulation of α-globin expression (Fig. 1D).[11] In particular, we used CRISPR/Cas9 RNP containing a gRNA targeting the two identical 5′ untranslated regions (UTRs) of the α-globin genes ($\alpha 1$ and $\alpha 2$) to delete the $\alpha 2$-globin allele that was replaced through HDR by a therapeutic β-like globin gene (delivered using AAV6). Editing of HSPCs from β-thalassemia patients led to correction of the α/β globin imbalance. Xenotransplantation experiments in immunodeficient NOD/SCID/$\gamma c^{-/-}$ mice showed long-term repopulating capacity of edited HSCs *in vivo*. These results were already confirmed by an independent group, validating the robustness of this editing approach.[34]

Advances in genome editing approaches for systemic diseases

Enzyme replacement therapy (ERT) consists of periodic intravenous administration of specific enzymes produced to supplement a protein that is deficient because of a genetic defect. ERT is approved or under investigation to treat more than 40 inherited disorders, mostly involving blood factors and lysosomal enzymes. Although life saving for some patients, this requires frequent costly injections with a peak-and-trough serum kinetics, which reduce patients' compliance to the therapy and efficacy of treatment. Sometimes these therapies are affected by development of antibodies against the administered drugs, which negatively influence drug bioavailability and activity. Instead, gene therapy can provide constant serum levels of therapeutic proteins with a single treatment and can induce immune tolerance to the expressed transgene.[35,36]

Autologous HSCs can be successfully engineered *ex vivo* by lentiviral vector (LV) to express a transgene of interest; however, the semirandom integration pattern is intrinsically associated with the risk of inactivating an oncosuppressor and transactivating an oncogene. Targeting a selected genomic harbor can reduce insertional mutagenesis risk, as also enables the exploitation of endogenous promoters, or selected chromatin contexts, to achieve specific transgene expression levels/patterns.[37] An elegant example of this approach is the targeted integration of AAV-delivered transgenes under the control of the endogenous albumin promoter in the liver.[38,39] The strong transcriptional activity of this promoter allows efficient protein expression with limited transgene integration. Until today, this strategy has been applied to hemophilia A and B[38,40,41] and metabolic disorders.[42–44] Although promising, this approach still presents some concerns: (i) long-term AAV-mediated expression of endonucleases can result in off-target editing[45,46] and unwanted AAV insertions[47]; (ii) immune responses against AAV vectors[48] or nucleases[49,50] severely limit the number of eligible patients; and (iii) albumin mutations have been observed in human hepatocellular carcinoma (Cancer Genome Atlas Research Network, 2017[51]).

To avoid these issues, we recently identified the α-globin as a suitable locus for CRISPR/Cas9-mediated targeted gene addition. The idea is to combine the strong transcriptional output of the α-globin promoter with the abundance of transgene-expressing erythroblasts to maximize protein production, reducing the number of integration events required to reach therapeutic levels. In addition, the α-globin locus is a safe harbor, since there are four α-globin genes per cell (Fig. 1D) and the loss of up to three α-globin alleles is mostly asymptomatic.[17] We edited human HSPCs with an RNP complex combining Cas9 with a gRNA targeting the 5′ UTR of the α-globin genes (Fig. 1D). The therapeutic transgenes were delivered with an AAV6 vector and integrated through the HDR pathway. We first demonstrated that the selected gRNA provided efficient and precise editing without affecting viability and differentiation potential of HSPCs and hemoglobin expression in HSPC-derived erythroid cells or inducing off-target genome modifications. Then we demonstrated that

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the expression of the integrated transgene was specific to the erythroid lineage and was induced during erythroid differentiation, mimicking the endogenous α-globin physiological expression pattern. In addition, we tested this strategy with therapeutic transgenes for different monogenic diseases, such as hemophilia B and LSD.[17]

Hemophilia B is a coagulation disorder caused by the absence of functional Factor IX in the blood. We demonstrated that targeted integration in HSPCs of an F9 gene under the control of the *HBA2* promoter resulted in factor 9 (FIX) mRNA expression and protein secretion in their erythroid progeny, and that secreted FIX was functional in reducing blood clotting time *in vitro*.

LSD are inherited metabolic conditions characterized by an abnormal build-up of toxic metabolites in lysosomes as a result of enzyme deficiencies. Wolman disease in particular is a life-threatening genetic condition due to the accumulation of cholesterol and triglycerides caused by mutations in the gene encoding lysosomal acid lipase (LAL). We demonstrate that LAL secreted from erythroid cells derived from edited HSPCs was functional and capable of cross-correcting patient's cells *ex vivo*, reducing both toxic cholesterol and lipid accumulations. Finally, we confirmed that edited HSCs conserve their *in vivo* homing, engraftment, and multilineage potential by performing xenotransplantation experiments in immunodeficient NOD/SCID/$\gamma c^{-/-}$ mice.[17] Overall, we established a safe and versatile CRISPR/Cas9-based HSC platform for different therapeutic applications, including hemophilia and inherited metabolic disorders.

Genome editing as a valid option to treat neurological disorders

Microsatellite repeat expansion (MRE) diseases are a group of at least 50 inherited disorders with unmet medical need due to a pathological increase in the number of short tandem nucleotide repeats within the coding or noncoding region of the causative genes. Most of these diseases affect the central nervous system or the neuromuscular system, such as fragile X syndrome, Huntington disease (HD), Friedreich ataxia, spinocerebellar ataxias, spinal and bulbar muscular atrophy, C9-amyotrophic lateral sclerosis/frontotemporal dementia, and myotonic dystrophies (DM1 and DM2).[52] Gene editing strategies to correct pathological nucleotide repeat expansions at the DNA or RNA level have been investigated for some of these disorders during the past years.[53] In this study, we provide an overview on the approaches that have been explored for DM1, with a particular focus on the work performed by our laboratory.

DM1, also known as Steiner's disease, is the most prevalent form of muscular dystrophy in adults with a global incidence of about 1 in 8,000 individuals. It is a multisystemic autosomal dominant disorder caused by a CTG repeat expansion in the 3′ UTR of the *DMPK* gene.[54] The number of *DMPK* CTG repeats ranges from 51 to several thousands in affected individuals, correlates with disease severity and inversely with age of onset, and increases during successive generations. Clinical manifestations include myotonia, muscle weakness, respiratory insufficiency, cardiac arrhythmias, gastrointestinal symptoms, somnolence, diabetes, and cataracts. Premature death occurs generally due to cardiovascular disease, sudden death, and respiratory insufficiency.[55] The disease mechanism has been extensively studied in animal models and results from a toxic gain of function of *DMPK* RNA transcripts containing expanded CUG repeats, which accumulate in the nucleus as stable RNP aggregates named foci, leading to defects in the alternative splicing of many pre-mRNAs.[56]

Early attempts to correct CAG/CTG repeat expansions at the genomic level were performed using MGNs, ZNFs, and TALENs in various cell types, the latter appearing more efficacious and specific in inducing repeat contractions, at least in yeast models. TALENS were also used to insert a premature polyA signal upstream of *DMPK* CTG repeats in DM1-induced pluripotent stem cells (iPSCs) resulting in transcripts without repeats.[57,58] With the emergence of the CRISPR/Cas system as a powerful gene-editing tool, the potential of this technology to treat MRE disorders was revealed using a CRISPR/Cas9 D10A nickase in a reporter cellular model demonstrating its ability to induce CAG/CTG repeat contractions.[59] Later, several CRISPR/Cas-based therapeutic strategies targeting various regions of the *DMPK* gene or transcripts were evaluated in cellular and mouse models of DM1, opening new perspectives for the treatment of this disorder.[22,60–64]

Our team developed a dual viral vector strategy to excise the pathogenic *DMPK* CTG repeat region by co-expression of the small sized *Staphylococcus aureus* Cas9 (SaCas9) and selected pairs of gRNAs targeting genomic sequences surrounding the trinucleotide repeats.[22] This approach was initially tested in cultured DM1 patient-derived myoblasts carrying a large CTG repeat expansion (2,600 CTG repeats) to evaluate the ability of the CRISPR/Cas9 system to delete large expansions, which resulted in the disappearance of nuclear foci and correction of splicing abnormalities in edited cells. Based on these promising results, we further expanded our work in a DMSXL disease mouse model, which contains a human *DMPK* transgene with ~1,200 CTG repeats, by intramuscular administration of serotype 9 adeno-associated viral (AAV9) vectors expressing CRISPR/SaCas9 components, and established the proof-of-concept that genome excision of a large CTG expansion is also feasible *in vivo* in skeletal muscle. Injection of AAV9 vectors in tibialis anterior muscle of homozygous DMSXL mice was not only able to reduce the overall amount of myonuclei with toxic *DMPK* RNA foci but also showed some limitations. As mentioned earlier, DM1 is manifested in many organs, not just in muscle, and the difficulty of targeting multiple affected tissues *in vivo* will present a significant challenge.

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Therefore, additional preclinical studies will be required to further optimize and evaluate the therapeutic benefit and safety of this approach at the level of the whole body by targeting the entire musculature, including heart, and eventually other tissues affected in the disease.

Advanced genome editing strategies to unleash the full potential of CAR T cells

The adoptive transfer of CAR T cells represents a highly promising strategy to fight against multiple cancer indications. This strategy relies on the engineering of T cells to redirect their cytolytic activity toward malignant cells through transgenic expression of a tumor antigen-specific receptor at their surface. Today, the current protocols of treatment consist in autologous adoptive T cell transfer. In this approach, T lymphocytes recovered from patients are genetically modified and expanded *ex vivo* before infusion back into patients. Clinical results gathered over more than 10 years show impressive rates of complete remission in different indications.[65] These highly positive clinical outcomes led to the FDA approval of four different products named tisagenlecleucel, axicabtagene ciloleucel, brexucabtagene autoleucel, and lisocabtagene maraleucel to treat acute lymphoblastic leukemia, large B cell lymphoma, mantle cell lymphoma, and multiple myeloma, respectively.[66–68]

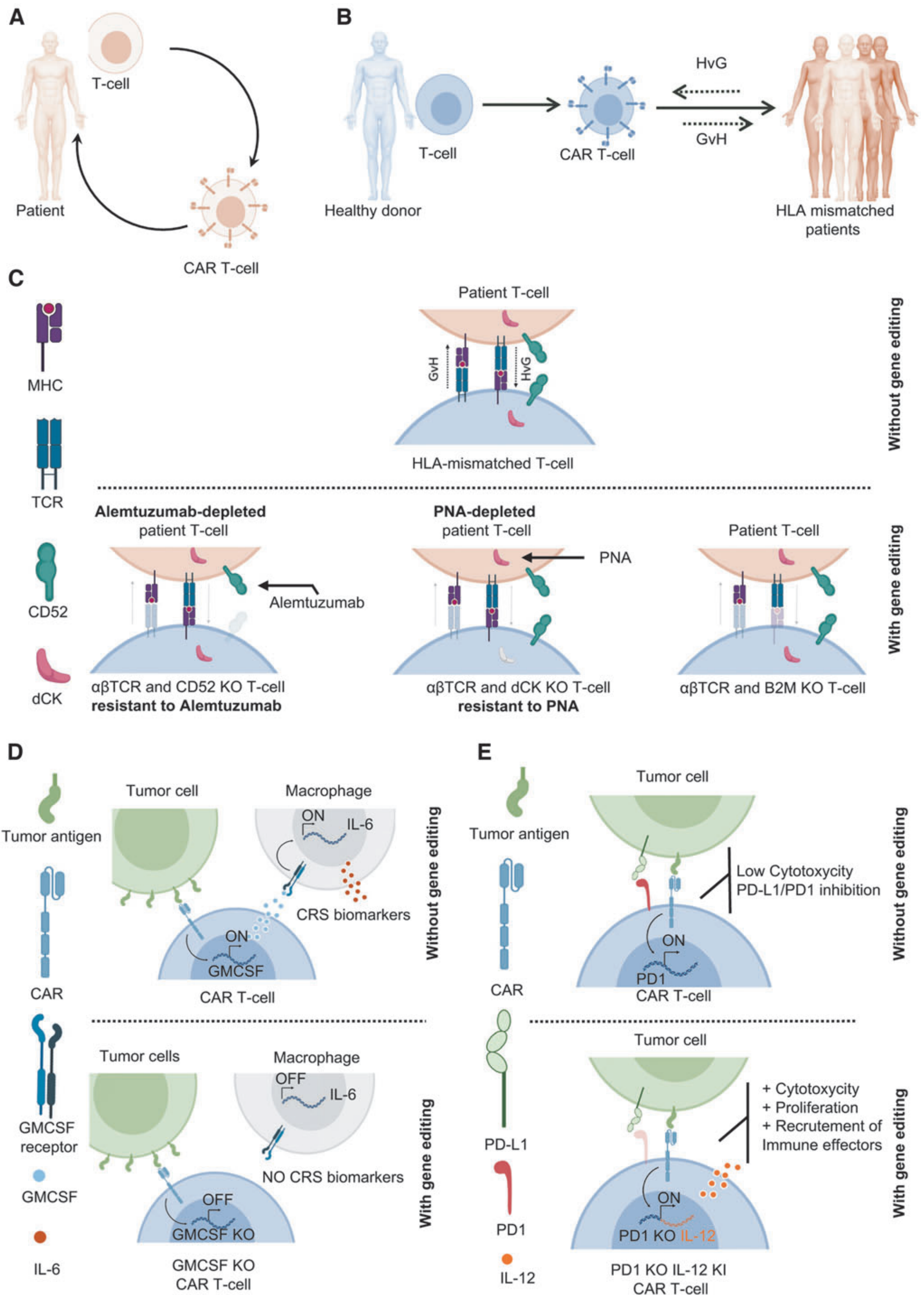
While highly promising, autologous CAR T cell therapies have been hampered by many practical and clinical challenges. Practical challenges include the production time and high cost, the dependence on patients T cells' fitness that is reduced by the disease or previous lines of therapies, and the logistical conundrum associated to the coordination of CAR T cell production and injection. Clinical challenges include the life-threatening cytokine storm observed in most patients,[69] the transient persistence of CAR T cell fitness, and their difficult access to tumor. It also includes the tumor-dependent inhibitory signals and tumor-associated suppressor cells that are usually found in the microenvironment of tumors and contribute, directly or indirectly, to the impairment of CAR T cell antitumor activity.

These multiple challenges cannot be easily addressed with standard cell engineering methods. However, implementation of advanced gene editing technologies in cell culture processes brought a wealth of solutions that are now revolutionizing the field of CAR T cell therapies and more broadly, immunotherapies. The following section describes how gene editing technologies, especially those based on TALEN, could be leveraged to overcome some of the practical and clinical challenges faced by CAR T cell therapies.

One of the first important challenge tackled by gene editing technologies was to enable the production of universal CAR T cell compatible with adoptive transfer in allogeneic settings. In contrast to the autologous approach, universal CAR T cell could be mass produced from healthy donor T cells and then be theoretically transferred as an off-the-shelf medicine to any HLA-mismatched patients (Fig. 2A, B, respectively). However, for this approach to be successful, the GvH and the host versus graft (HvG) reactions must be avoided to safely allow CAR T cells to engraft and express their antitumor function in HLA-mismatched patients (Fig. 2C, top panel). Indeed, in this scenario, GvH reaction would consist in the recognition of the major histocompatibility complex (MHC) marker exposed at the surface of host cells by an alloreactive $\alpha\beta$ T cell receptor ($\alpha\beta$TCR) exposed on the CAR T cell surface. This recognition would promote an acute, nonspecific, and global depletion of host tissues and lead to an eventual fatal outcome (Fig. 2C). HvG reaction would involve the same recognition mechanism, but results in the depletion of CAR T cell by alloreactive host T cells, and thus lead to a poor antitumor activity and therapeutic outcome. Thus, without additional genome engineering, adoptive transfer of CAR T cell in allogeneic settings would be simply unsafe and inefficient.

In the past 10 years, several different genome engineering strategies were developed to prevent GvH and HvG. The first strategy developed to prevent GvH and HvG was to engineer an $\alpha\beta$TCR and CD52 double knocked out CAR T cell through TALEN-mediated gene inactivation. $\alpha\beta$TCR inactivation was shown to be highly efficient and to robustly prevent GvH. In addition, CD52 inactivation enabled CAR T cell to resist to alemtuzumab, an FDA-approved antibody designed to bind and promote depletion of CD52-expressing immune cells.[70] Such resistance-enabled alemtuzumab-dependent host lymphodepletion led to an efficient prevention of HvG and created a receptive environment for $\alpha\beta$TCR/CD52-deficient CAR T cell engraftment in the clinic.[71] A second strategy consisted in the TALEN-mediated inactivation of $\alpha\beta$TCR and dCK, which enabled to produce $\alpha\beta$TCR-deficient CAR T cells resistant to purine nucleotide analogs (including fludarabine clofarabine and decitabine) that are commonly used to lymphodeplete patients before CAR T cell infusion (Fig. 2C, middle panel).[72] A third strategy aimed at

Figure 2. Genome engineering strategies used to unleash the full potential of CAR T cell therapies. **(A)** Schema describing the concept of autologous CAR T cell therapy. **(B)** Schema describing the concept of universal CAR T cell therapy compatible with adoptive transplant in allogeneic settings. **(C)** *Top panel*, schema describing the concept of GvH and HvG reactions occurring during adoptive transplant of T cells in allogeneic settings. **(C)** *Bottom panel*, genome engineering approaches developed to prevent GvH and HvG. **(D)** Genome engineering approach to prevent cytokine release syndrome. **(E)** Genome engineering approach to improve CAR T cell potency by enabling a tumor-dependent secretion of the IL-12 immunostimulatory agent. CAR, chimeric antigen receptor; GvH, graft versus host; HvG, host versus graft. Color images are available online.



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inactivating $\alpha\beta$TCR along with B2M to create $\alpha\beta$TCR/MHC-deficient CAR T cells (Fig. 2C, right panel). This approach enabled the generation of nonalloreactive CAR T cell capable of evading alloreactive T cell attack and is currently evaluated in clinic to assess its safety and efficacy profile.

Genome engineering technics were also used to mitigate the cytokine release syndrome (CRS) associated to CAR T cell infusion. CRS is characterized by fever, hypotension, and respiratory insufficiency and is correlated to elevated pro-,inflammatory cytokines in patients' following CAR T cell infusion. Its severity is mainly mediated by IL-6, IL-1, and other factors produced by patient macrophages, which are commonly activated in the vicinity of CAR T cell.[73] Two independent studies[74,75] identified granulocyte-macrophage colony-stimulating factor (GMCSF) as one of the main mediators of macrophage activation by CAR T cell (Fig. 2D, left panel), opening opportunities to mitigate CRS through genome engineering of CAR T cell. In that context, TALEN- and CRISPR/Cas9-mediated inactivation of GMCSF in CAR T cells were shown to drastically decrease CAR T cell dependent-secretion of GMCSF and to abolish subsequent macrophage-dependent secretion of multiple CRS biomarkers, including IL-6 (Fig. 2D, right panel). While this approach may not fully eliminate CRS symptoms, it could be combined with other engineering approaches and improve the overall safety of CAR T cell therapies for cancer patients.[76]

Finally, genome engineering was also used to improve CAR T cell antitumor function in the hostile tumor microenvironment. For instance, among the multiple strategies published in the past years, one consisted in rewiring PD1 expression into the secretion of a powerful immunostimulatory agent named IL-12 (Fig. 2E).[76] This was achieved by inserting an IL-12 expression cassette in frame with the PD1 gene using a PD1-specific TALEN and an AAV6 matrix bearing the IL-12 expression cassette. This strategy resulted in the inactivation of PD1, one of the main factors of tumor-dependent inhibition of CAR T cell and in the localized and tumor-dependent secretion of IL-12. This approach improved antitumor activity and proliferation capacity of CAR T cell, while mitigating the risks of adverse events commonly observed when IL-12 is systemically infused in patients.[77]

TOXICITY AND RECENT IMPROVEMENTS IN GENE EDITING FOR GENE THERAPY

ON-target genotoxicity in gene therapy target cells upon CRISPR/Cas9 nuclease challenge

CRISPR/Cas9 is a powerful technology for genome editing. However, high efficiency of the editing nuclease is not void of downsides due to unwanted and uncontrolled activity. The most studied and almost resolved side effect is OFF-target genotoxicity. By contrast, the ON-target genotoxicity of CRISPR/Cas9 nuclease due to single DNA DSB at the targeted locus was initially underestimated, but has received recently much more attention. Using Cas9 nuclease-induced DSB at the uroporphyrinogen III synthase (*UROS*) locus (chromosome 10q) to model and correct congenital erythropoietic porphyria (CEP), we demonstrated that HDR is rare compared with the competitive unwanted NHEJ pathway. Indeed, the edition is often biallelic, and uncontrolled small indels are concomitant to precise correction. They lead to disrupted targeted sequences and cause unwanted dysfunctional protein in cell lines and iPSCs.[78,79] We demonstrated that uncontrolled indels induced by the NHEJ are very frequent in corrected HEK293T, with a precise genome editing ratio (HDR/NHEJ) of 0.5. Several approaches have been proposed to improve this ratio, for example, by NHEJ inhibition or activating the HDR pathways (for review see Sledzinski *et al.*[80]).

It is also possible to exploit the design of the gRNA. We tested an original approach to correct compound heterozygous recessive mutations. We compared editing efficiency and genotoxicity using a biallelic gRNA versus a mutant allele-specific gRNA in iPSCs derived from a CEP patient carrying compound heterozygous *UROS* mutations. We reported that, unlike the biallelic one, the mutant allele-specific gRNA was free of ON-target collateral damage and allows a precise gene correction without concomitant indels in the same iPSC clone. This design that avoids genotoxicity with ON-target scarless gene correction should be recommended for recessive diseases with frequent cases of compound heterozygous mutations.[79]

In addition to small indels, a single ON-target DSB (without a second DSB at OFF-target genomic loci) can also lead to interstitial large deletions of several kilobases, symmetrical or not at the targeted site, in mouse hematopoietic progenitors, in human immortalized differentiated cells[81] or in mouse embryos.[25,82] Recently, larger deletions (up to 300 kb) in mouse zygote were reported.[83]

Unexpectedly, even larger genomic rearrangements may occur. We reported that CRISPR/Cas9 can cause megabase-scale chromosomal terminal truncations targeting *UROS* (chromosome 10q) in two human cell lines (HEK-293T and K562) and in human primary fibroblasts deficient for the tumor suppressor p53. This megabase-scale deletion was mapped by array-CGH (comparative genomic hybridization). This by-product starts at DSB cut site and deletes the chromosomal end (7.5 Mb). It removes 43 genes, including 5 proto-oncogenes and 7 tumor suppressors.[78] This risk of inducing megabase-scale deletions was recently confirmed in other human primary cell types (iPSCs and human embryos),[84,85] suggesting that CRISPR-mediated large rearrangements are not locus or cell-type dependent.

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We then evaluated if unexpected large rearrangements can occur targeting globin genes for gene editing (important gene therapy targets for hemoglobinopathies as described above) in chromosome 11p. Again, we observed that genome editing targeting globin genes induces megabase-scale losses of heterozygosity (LOH) from the globin CRISPR/Cas9 cut site to the telomere (5.2 Mb). In established lines, CRISPR/Cas9 nuclease induced frequent (up to 20%) terminal chromosome 11p truncations and rare copy-neutral (CN)-LOH (without loss of genomic material). Targeting the β-globin cluster in primary HSPCs, we detected 1.1% of clones (7/648) with acquired megabase LOH induced by CRISPR/Cas9. In-depth analysis by CGH/SNP array revealed the presence of CN-LOH from the cut site to the telomere without deletion. So, the cell type seems to be crucial to determine the type of DNA rearrangement that might occur after CRISPR/Cas9 DSB.

In HSPCs, CN-LOH led to 11p15.5 partial uniparental disomy, comprising two Chr11p15.5 imprinting centers (*H19/IGF2:IG-DMR/IC1* and *KCNQ1OT1:TSS-DMR/IC2*) and impacting *H19* and *IGF2* expression.[86] It is possible that CN-LOH is a survival repair response to the loss of an allele in p53-proficient cells. Indeed, CRISPR-induced single DSB can be lethal due to a transient p53-mediated DNA damage response in primary cells,[87–89] and therefore, the selection for clones with low p53 activity or p53 mutations should be evaluated in preclinical and clinical studies. The mechanism of extra-large terminal CN-LOH post CRISPR/Cas9-mediated DSB is probably consistent with break-induced replication recently described in eukaryotic cells. It involves extensive DNA synthesis from the DSB to the telomere. It can even provoke chromothripsis, an extensive and complex chromosome rearrangement restricted to the chromosome targeted by CRISPR-Cas9.[90] In parallel, interstitial smaller kilobase CN-LOH by gene conversion in the close surroundings of the DSB was also described in the globin cluster.[91]

This ON-target genotoxicity, at base/kilobase and megabase scale, could be a safety concern for CRISPR clinical trials and has to be confirmed in other gene *loci* and *in vivo* settings. These new data highlight the necessity to develop preclinical tests to evaluate carefully not only the well-known OFF-target but also the ON-target genotoxicity risks. Further studies to understand the mechanisms of the appearance of these extra-large chromosomal rearrangements will be necessary to find solutions/alternatives to prevent them. Interestingly, nickases, inducing a DNA single-strand break, allow HDR without indels or truncations in cell lines.[78] Unfortunately, its use is still challenging for clinical studies due to low efficiency in human primary cells. Modulating DSB DNA repair pathways or developing clinical trials with DSB-free genome editing is currently under study. Novel and safer gene editing strategies and tools are also mentioned in the next section.

Improving gene editing precision: the safer DNA repair perspective

The goal of genome editing experiments is to change a targeted DNA sequence into a desired DNA sequence in the genome of cultured cells or organisms. In most cases, especially in gene therapy approaches aimed at precise gene correction or gene insertion as exemplified in the first section, a single sequence product is pursued at high efficiency. However, in some applications, heterogeneous edited sequences may be acceptable; for example, it is the case in gene therapy approaches developed in by some French teams to treat SCD and β-thalassemia by disruption of BS for transcriptional repressors in HBG promoters mimicking HPFH mutations (Weber *et al.*[30] and see Novel genome editing approaches for β-hemoglobinopathies section) and we can also cite diseases such as HD or DM1, caused by trinucleotide expansions that can be reduced or deleted by targeting the repeats with CRISPR nucleases as shown by us and the GF Richard laboratory at the Pasteur Institute in Paris.[92] Improved CRISPR genome editing tools are continuously evolving, with enhanced targeting scope, improved editing specificity, and importantly, enhanced precision of genome editing activity, which are all important issues when clinical applications are envisioned. Three main classes of CRISPR-based genome editing agents are available today, namely, nucleases, base editors, and prime editors, expanding the solutions that can be chosen and optimized for a given genome editing application. We discuss in this section strategies designed to increase the efficiency of precise gene editing by HDR and finally base editing and prime editing, which are promising alternative tools for precise gene editing in gene therapy.

The basic principle of precise gene editing in living cells was first pioneered with the I-*Sce*1 nuclease isolated by the Dujon laboratory (Pasteur Institute, Paris). Together with several other groups, they used I-*Sce*1 to introduce a targeted DSB into a model gene carrying the 16 pb I-*Sce*1 target sequence and strongly stimulated precise gene modification by HDR,[93] triggering the new era of gene editing with programmable nucleases. ZNF, TALEN, and CRISPR/Cas9 were next used to target genomics sites of interest, and more recently, successive generations of programmable nucleases were engineered providing even greater flexibility and easy use for precise gene editing based on HDR. End-joining pathways of DNA DSB repair, however, were found to generally prevail over HDR in mammalian cells. Hence, precise modification by HDR, copying from template DNA with the desired sequence change, is not the most common outcome, and imprecise modifications are generally much more frequent (Yeh *et al.*[94] and see the above section). Therefore, approaches to improve genome editing precision are actively explored.

Different types of donors can be used as homology templates, single-stranded or double-stranded DNA. HDR pathways differ depending on the nature of donor DNA,

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and are broadly classified into HR for double-stranded donors and single-stranded templated repair for single-stranded donors, which likely share early HDR steps, but require different factors necessary for downstream incorporation of donor information.[95] Importantly, this expands the possibilities for precise genome editing. Single-stranded donors are indeed a promising option for gene editing. We have shown that short ODN require only short homology regions (around 35 nt), and when modified with phosphorothioate linkages, they can exhibit very high precise editing activity to introduce short DNA sequences (<100 bp); in addition, they have the advantage to be easy to design and are synthesized commercially.[96] To integrate longer sequences, protocols for efficient synthesis of long single-stranded DNA were successfully developed and used to generate precise modifications in different systems.[97] The mechanism of HDR with single-stranded donors is not entirely characterized, but was shown to involve factors of the Fanconi repair pathway, which are shared with the HR pathway. Donor and nuclease delivery can also have a strong impact. For example, AAV vectors promote efficient precise gene editing, as shown by the Porteus laboratory[98] and further exemplified by work[17] from the Amendola laboratory at Généthon (Evry, France). This is possibly related to AAV vector sequences binding to nuclear factors favorable to HDR or to differences in final nuclear concentration of donor sequences. We have also shown that increased HDR genome editing can be obtained using Cas9 protein compared to mRNA.[99]

Efficiency of precise gene modification by HDR nevertheless remains too often very limited. Pioneer studies from D Carroll found that genetic inactivation of ligase 4, an essential NHEJ protein, increased the frequency of HDR in *Drosophila melanogaster* and therefore showed that manipulating DNA repair pathways can help increase the efficiency of precise gene editing.[94] Pharmacological modulators, such as DNAP-PKc inhibitor nedisertib,[100] can thus improve gene editing, but unfortunately are associated with nonspecific toxicity. Several research teams, including ours, have next shown that direct fusion of DNA repair proteins to Cas9 can also bias the outcome of DNA repair and be used to favor precise gene editing by HDR (Fig. 3A, B). The potential advantage of such targeting of the repair protein of interest at the DSB site is to avoid global effects on cellular DNA repair induced by pharmacological inhibitors. Since processing of DSB ends through 5′ to 3′ resection is the major determinant of repair pathway choice, CtIP, a protein that promotes Mre11 exonuclease activity at this early step of HDR, was fused to Cas9. The fusion of Cas9 with a minimal N-terminal fragment of CtIP was sufficient to stimulate HDR.[101] This fragment contains the cell cycle-dependent phosphorylation sites of CtIP that represent one of the main control

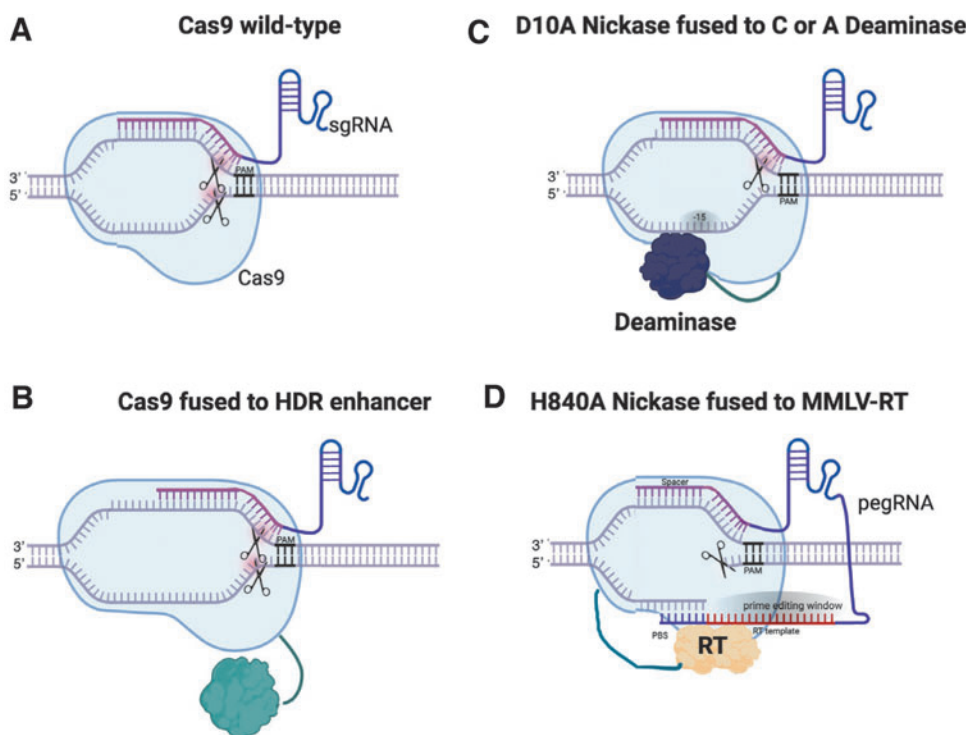

Figure 3. Genome editing strategies. Different classes of CRISPR-based genome editing agents are shown: nucleases, wt **(A)** or fused to HDR enhancer **(B)**, base editors **(C)**, and prime editors **(D)**. HDR, homology-directed repair; PAM, protospacer adjacent motif; RT, transcriptase. Color images are available online.

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points of HDR (Fig. 3B). Another option is to perform co-targeting of a gene with a phenotype that can be conveniently screened to enrich for cells carrying the modifications of interest.[102]

Finally, novel tools developed by the Liu laboratory at MIT bypass the limited activity of HDR by avoiding DSB repair and engaging other repair pathways. They designed their base editors and prime editor specifically to take advantage of knowledge about DNA repair processes, particularly by the introduction of a nick in the unmodified strand to direct mismatch repair to favor the desired change. These tools raise exciting possibilities currently being investigated. For example, base editing is achieved by fusing C or A deaminase moieties to D10A Cas9 nickase (Fig. 3C).[103] Co-targeting of a selectable gene with a base editor can also be used to increase efficiency[104] and such tools have been used in animals to generate models of human disease, for instance, cancer modeling in zebrafish[105] by the Del Bene team at the Institute for Vision. A major limitation, however, is that all target nucleotides in a specific window will be modified (Fig. 3C), limiting precision of the approach. In addition, base editors can currently install only 6 (C→T, A→G, C→G, G→A, T→C, G→C) of the 12 possible types of point mutations. An alternative, potentially universal approach for small sequence changes, point mutations, small insertions, and small deletions, is prime editing. It implies a fusion of the H840A Cas9 nickase to the reverse transcriptase of the Moloney murine leukemia virus (MLV) and modified gRNAs carrying sequence templates to be copied into the target genomic site (Fig. 3D).[106] Prime editing is actively investigated to address its highly variable efficiency and overcome delivery challenges. Both base editing and prime editing are based on targeting an enzyme that will directly modify DNA (respectively, C or A deaminase or reverse transcriptase) to the target sequence of interest with the CRISPR/Cas9 system. However, only one DNA strand is modified and stable introduction of the mutation depends on copying the modification onto the second DNA strand. In base and prime editing strategies, Cas nickases are used to nick the nonedited DNA strand to direct DNA repair to that strand and to use the edited strand as a template for resynthesizing the nonedited nicked strand. DNA repair pathways involved may also be manipulated to effectively increase gene editing efficiency.

The improvement of the first described CRISPR/Cas genome editing system resulted in the development of sophisticated novel gene editing tools with more precision and flexibility genome editing reagents. From a gene therapy point of view, efforts are still needed to improve editing capacities, and also to characterize and understand all the consequences of CRISPR/Cas treatments, as well as to propose innovative approaches to deliver editing agents into cells, as discussed below.

Nanoblades for efficient nontoxic cellular delivery of the gene editing machinery into gene therapy targets

To obtain efficient gene editing in primary blood target cells, the delivery systems to introduce the endonuclease, to produce DSB and the donor DNA for KI into the nucleus of the target cell, have to be very efficient. There are different methods to deliver the gene editing machinery into the cells: transduction by integrating LVs, or adeno-associated viral (AAV) vectors, transfection with DNA or RNA, or electroporation with RNP CAS9/gRNA complexes (RNPs) (Fig. 4A). For HDR strategies, the donor template can be supplied by infection with an AAV vector, IDLV or electroporation of single-stranded DNA, or ODN encoding the template DNA. All these methods have delivered the gene-editing tools with different degrees of efficiency, toxicity, and off-target effects.

To introduce the gene-editing machinery in primary cells, the method of choice at the moment is electroporation of RNPs, which allows efficient editing of human T and B cells and HSPCs.[10] For B cells, other methods were employed like electroporation of Cas9 mRNA or the combination of Cas9 protein with chemically modified gRNA, which in combination with an AAV6 vector encoding the donor template resulted in efficient KI in B cells.[6] In the case of HSPCs, Cas9 has been efficiently introduced by electroporation of CAS9/gRNA complexes (RNPs).

For gene therapy strategies based on NHEJ in HSPCs, diseases such as β-hemoglobinopathies (see Novel gene editing approaches for gene therapy section), HIV, or FA have been successfully corrected by gene editing. For example, in FA, the introduction of Cas9/gRNA complexes by electroporation of RNPs introduced DSBs in the mutated FANCA gene, which, when repaired by NHEJ, restored the FA gene function.[16] For HDR strategies in HSPCs, the donor template is being introduced preferentially using AAV6, which allowed genetic repair of multiple diseases such as chronic granulomatous disease, SCID-X, or WAS.[107] Although these approaches were successful, some drawbacks were reported such as cell death and off-target effects.

The ideal gene-editing tool should be precise, be fast, b nontoxic, and induce as less as possible off-target effects. Recently, two collaborative French teams (CIRI, Lyon, France) developed a new gene-editing tool delivery system, the ''nanoblades.'' This is a vehicle in which the Cas9/gRNA RNP is packaged into a modified virus-like particles (VLPs) derived from an MLV or HIV (Fig. 4B).[108,109] The endonuclease, Cas9, is fused to a viral structural protein gag, associated with its gRNA, and is by this means actively incorporated into the VLPs. These nanoblades allow rapid and transient RNP delivery and importantly are devoid of viral genomic sequences. They produce DSBs rapidly and efficiency in immortalized cells,

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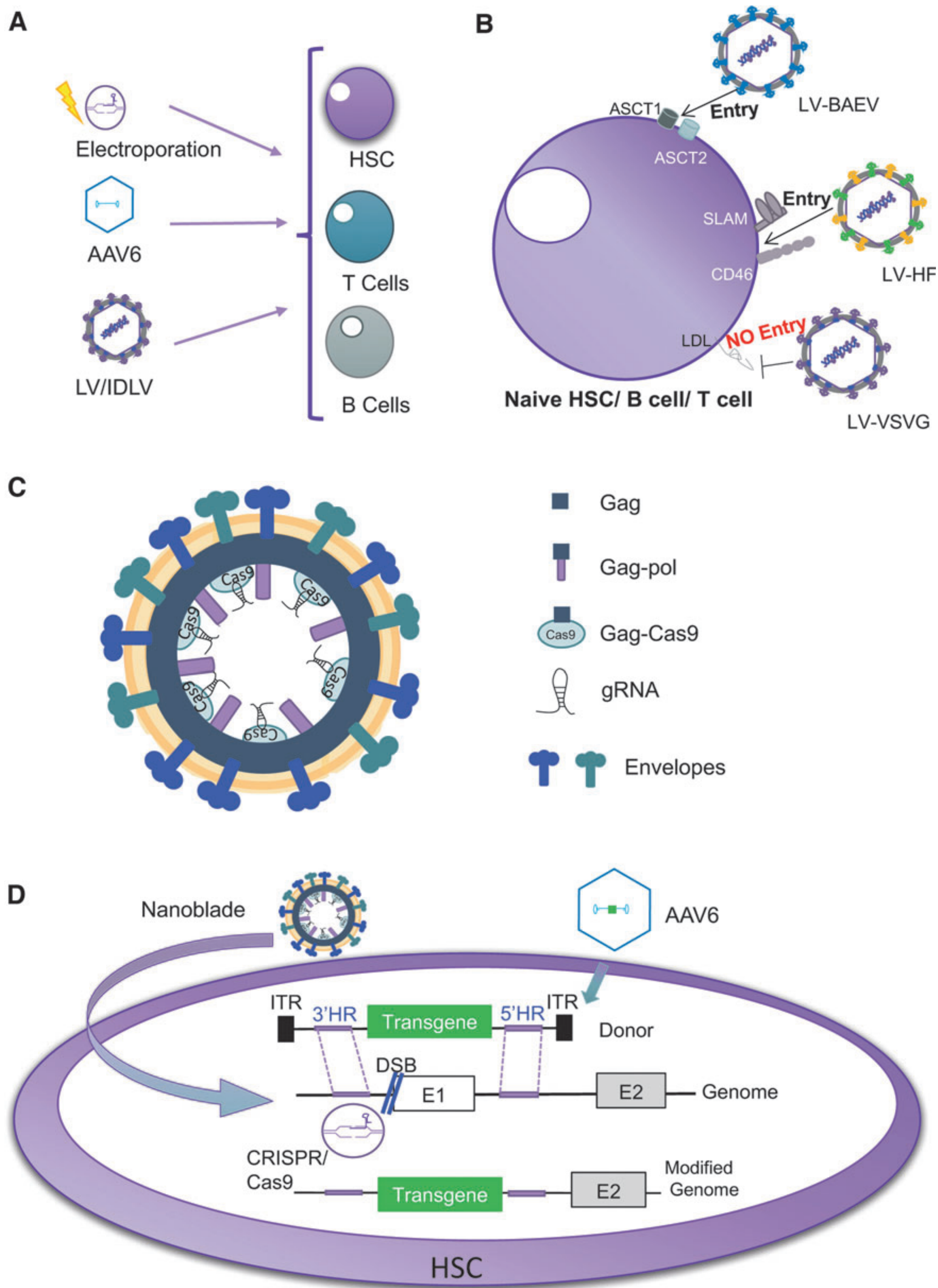

Figure 4. Delivery methods for delivery of CRISPR/Cas9 into primary gene target cells. **(A)** Electroporation of RNP complexes, AAV6 encoding CRISPR and lentiviral vectors encoding the CRISPR/Cas9 and the gRNA. **(B)** Improvement of delivery into primary cells; HSC, T cells and B cells by pseudotyping with heterologous viral envelopes. Primary cells express receptors such as ASCT1/2, SLAM, and CD46, which allow LVs displaying envelopes from the measles virus (HF) or the BaEV to deliver their cargo into primary cells efficiently. However, unstimulated T and B cells and HSCs do not express the receptor for the VSV-G envelope, prohibiting efficient transduction in nonstimulated cells. **(C)** Schematic representation of nanoblades and its components. **(D)** HDR repair using CRISPR/Cas9 introduced by nanoblades and a donor DNA template. The donor template is provided into the cell by an AAV6 vector, CRISPR/Cas9 RNP delivered by nanoblades make a DSB and the homology arms in the donor DNA allow homology recombination introducing a transgene into the cell genome in a specific gene locus. BaEV, baboon endogenous virus; DSB, double-strand break; gRNA, guide RNA; HSC, hematopoietic stem cell; LV, lentiviral vector; RNP, ribonucleoprotein; VSV-G, vesicular stomatitis virus G. Color images are available online.

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iPSCs, and primary cells.[109] More interestingly, since nanoblades are viral vector-derived particles (VLP), they carry a viral envelope at their surface to allow VLP cell entry. They can thus easily be pseudotyped as their counterpart viral vectors with different envelope glycoproteins (gps). We have previously shown that the baboon endogenous virus (BaEV) envelope gp incorporated into an LV allowed efficient cell entry into human T and B cells and HSPCs (Fig. 4C).[107,110–112] Indeed, nanoblades displaying at their surface a combination of BaEV and the vesicular stomatitis virus G (VSV-G) envelope gps deliver the Cas9/gRNA transiently and rapidly into slightly activated T and B cells and HSPCs without any change in their phenotype or cellular toxicity.[108]

We demonstrated that nanoblades confer efficient delivery of the gene-editing machinery in HSPCs reaching up to 80% editing, without obvious detection of gene editing at off-target sites, in contrast to other methods. Finally, treatment of HSPCs with nanoblades in combination with a donor DNA template encoding rAAV6 vector resulted in up to 40% stable expression cassette KI into a specific gene locus (Fig. 4D). Importantly, no toxicity was detected upon nanoblade-mediated KI in HSPCs since no significant effect was seen on cell survival and proliferation in nanoblade-treated versus untreated cells. Various other methods to deliver the gene-editing tools such as electroporation, adenoviruses, AAVs, and LVs have been used, conferring different degrees of efficiency, toxicity, and off-target effects. The nanoblades combine actually the low to undetected toxicity of retroviral delivery (VLP) and the transient expression of Cas9/gRNA RNP-mediated gene editing. Indeed, nanoblades confer efficient NHEJ-mediated gene editing in HSPCs and in T and B cells, but not at the expense of significantly induced cellular toxicity.

Nanoblades represent an easy to use, flexible, and efficient platform for gene editing in gene therapy targets. Easy to use since only the plasmid coding for the gRNAs needs to be redesigned to target another genomic locus. Flexible since they can harbor multiple gRNAs to permit knockout of multiple genes at once.[109] Continuously, Cas9 proteins are improved to reduce off-target activity or increase efficiency. For example, other targetable nucleases were identified, for example, Cpf1 nucleases, high-fidelity Cas9, nickases, and hyperaccurate Cas9,[113,114] or to increase the precision of editing, base editors were engineered for therapeutic applications.[115] All these new components might readily be incorporated into nanoblades by fusing them to MLV or HIV gag proteins.

In the same line, other groups have developed CRISPR/Cas9 vehicles that resemble our nanoblades.[116–118] The three systems were highly efficient for gene editing in cell lines and some in primary cells such as iPSCs. However, these transient CRISPR/Cas9 delivery systems were not evaluated for gene editing in human T and B cells and HSPC gene therapy targets yet.

Summarizing, these nanoblades are simple to implement, show high flexibility for different targets, including primary immune cells of human and murine origin, is relatively inexpensive, and therefore provides important perspectives for basic and clinical translation in the area of gene therapy.

CONCLUSION

Gene editing has now emerged as a realistic approach for treatment of both inherited and acquired diseases. After giving an overview of some recent particular preclinical gene editing applications for a variety of different diseases (*e.g.*, hematopoietic, muscle, and cancer indications) developed by French researchers, we wanted to underline here that gene editing has entered a new era of research focusing on improving precision, delivery, and safety of these tools in primary gene therapy target cells. Nevertheless, first results from clinical trials indicate that therapeutic gene editing can be safe and might provide a therapeutic option for treatment of many human diseases.

AUTHORS' CONTRIBUTIONS

A.B. and F.M.G. wrote the article. A.M., M.A., M.L., and G.F. wrote the article and prepared Fig. 1. J.V. wrote the article and prepared Fig. 2. M.C., J-P.C., C.G., and D.G. wrote the article and prepared Fig. 3. E.V. and A.G.-G. wrote the article and prepared Fig. 4. C.S. and A.B.-B. wrote the article.

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AUTHOR DISCLOSURE

A.B., F.M.G., E.V., A.G.-G., M.A. A.M., G.F., M.L., M.C., J.-P.C., C.G., D.G., and J.F. have no conflict of interest to disclose. J.V. is currently a Cellectis. SA employee, TALEN® is a Cellectis' patented technology. A.B.-B. has a patent application related to gene editing of DM1. J-P.C. and C.G. have a patent application related to gene editing with Cas9 fusion to a CtIP Nter domain.

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