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► **To cite this version:**

Mario Amendola, Aurélie Bedel, Ana Buj-Bello, Mathieu Carrara, Jean-Paul Concordet, et al.. Recent Progress in Genome Editing for Gene Therapy Applications: The French Perspective. *Human Gene Therapy*, 2021, 32 (19-20), pp.1059-1075. 10.1089/hum.2021.191 . hal-03420183

**HAL Id: hal-03420183**

**<https://hal.science/hal-03420183>**

Submitted on 27 Nov 2022

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**Review article****Recent progress in genome editing for gene therapy applications: the French perspective**

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**Running title** (50 characters): Genome editing for gene therapy in France

**Keywords** (6): Genome editing, CRISPR/Cas9, gene therapy, HSCs, gene editing toxicity, T cell

**Abstracts (300 words)**

Recent advances in genome editing tools, especially the novel developments in the clustered regularly interspaced short palindrome repeats associated protein (CRISPR/Cas)-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 in order to disrupt or correct genes or insert expression cassettes in safe harbors in the genome underlines 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 a muscular dystrophy and the potency of T cell gene editing to increase anti-cancer activity of chimeric antigen receptor (CAR) 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 towards 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.

## Introduction

### Gene or genome editing: the basics

Gene editing is a type of genetic engineering where nucleotides or bigger DNA sequences are either inserted, deleted or replaced in the genome using nucleases, which create site-specific double-strand breaks (DSB) in genomic loci. There are different types of nucleases: Meganucleases (MGNs), transcription activator-like effector nucleases (TALENs), zinc fingers nucleases (ZFNs) and clustered regularly interspaced short palindromic repeats associated to Cas9 nucleases (CRISPR/Cas9). The cellular machinery repairs the DSB induced by these nucleases either by non-homologous end joining pathways (Non-Homologous or Microhomology-Mediated End Joining, NHEJ or MMEJ) 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.<sup>1</sup> MMEJ is also frequently involved in repair of DSBs induced by Cas9 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. In contrast, HDR results in gene correction/insertion by homologous recombination 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 either by plasmids, integration-deficient lentiviral vectors (IDLVs), recombinant adeno-associated viruses serotype 6 (rAAV6) or by electroporation of double stranded DNA or oligonucleotides (ODN).<sup>2,3</sup> 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 knock-out and knock-in genome editing due to its high specificity, activity, easy design and highly efficient gene editing in cell lines and primary cells.<sup>4</sup> The CRISPR/Cas9 component can be introduced in the cell of interest using different methods, e.g. by using CRISPR/Cas9 encoding retroviral vectors<sup>5</sup> or plasmids<sup>6</sup> and RNAs<sup>3</sup> encoding these components introduced by electroporation. Currently though, 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

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2  
3 guide RNA(s) (gRNA) and Cas9 proteins.<sup>7</sup> In contrast to retroviral delivery, RNP delivery  
4 offers a major advantage since the Cas9/gRNAs are only transiently present in the cell, thereby  
5 avoiding insertional mutagenesis and cellular toxicity related to persistent Cas9 activity<sup>8</sup>,  
6 implying a safety benefit essential for clinical applications.  
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### 10 11 12 13 **Therapeutic gene editing** 14

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17 The multiple advantages of gene editing over gene addition in the gene therapy field are: 1) the  
18 capacity of modifying/correcting specific endogenous DNA sequences; 2) the normal  
19 transcriptional regulation of the gene is maintained since correction can be introduced at the  
20 targeted genomic locus thus allowing a spatio-temporal and thus physiological regulation of  
21 transgene expression<sup>2</sup> and 3) the insertional mutagenesis risks and the activation of oncogenes  
22 are strongly reduced. Thus, precise genetic manipulation of cells by gene editing provides  
23 unprecedented opportunities for correction of immune-deficiencies such as X-linked severe  
24 combined immunodeficiency (SCID-X1)<sup>9</sup> or Wiskott-Aldrich syndrome (WAS)<sup>10</sup> or bone  
25 marrow failures, such as Fanconi Anemia (FA) and many other disease indications.<sup>11</sup> HSPC-  
26 based gene therapy is very attractive treatment for FA because corrected stem cells have a  
27 selective advantage.<sup>12</sup> Interestingly, NHEJ was utilized to create an insertion/ deletion (indel)  
28 next to an FA mutation leading to correction of FA phenotype at high efficiency.<sup>13</sup>  
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39 Additionally,  $\beta$ -hemoglobinopathies ( $\beta$ -thalassemia and sickle cell disease) are attractive  
40 targets for therapeutic gene-editing using HDR.<sup>14</sup> However, NHEJ might offer alternative  
41 correction strategies for gene therapy of  $\beta$ -thalassemia and Sickle cell disease. Gene editing  
42 strategies for  $\beta$ - hemoglobinopathies have rather focused on disruption of silencing factors/  
43 regulators such as BCL11A in hematopoietic stem cells (HSCs) to induce *de novo* expression  
44 of fetal hemoglobin.<sup>15,16</sup> In this review, two collaborating French research teams will extend  
45 further on the more recent gene editing approaches for correction of hemoglobinopathies but  
46 also other monogenetic diseases such as blood clotting diseases and lysosomal storage  
47 disorders.  
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54 In addition to HSCs, T cells are very powerful gene therapy target cells and are highly amenable  
55 to gene editing. Gene editing in T cells is being used currently to generate potent chimeric  
56 antigen receptor (CAR) T cells for combating cancer.<sup>17</sup> CARs are laboratory designed T cell  
57 signaling receptors which upon encounter with a cancer specific antigen will get activated and  
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3 eliminate the cancer cells. One example for improving CAR T cell design is gene editing  
4 mediated knock-out of the endogenous T cell receptor (TCR) to avoid the graft-versus-host  
5 disease. This might permit to produce a universal CAR T cell.<sup>18</sup> This allows to move away from  
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7 the up to now obligatory costly autologous CAR T cell therapies for treatment of cancer  
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9 patients. We will focus in this review on some of the newest gene editing strategies employed  
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11 by a French research team to improve CAR T cell efficiency.  
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15 Interestingly, not only hematopoietic cells such as T, B and HSCs are gene therapy targets for  
16 gene editing but recently a French team has developed a gene editing approach to correct a type  
17 of muscle disease<sup>19</sup>. They will report in this review on Myotonic Dystrophy type 1 (DM1)  
18 correction via gene editing of a gene that is mainly expressed in smooth, skeletal, and cardiac  
19 muscles. For the French gene therapy landscape and beyond it is also important to underline  
20 that quite recently the Technological Research Accelerator in Genomic Therapy (ART-TG)  
21 was put in place to help the gene therapy labs in France to conduct preclinical studies and to  
22 manufacture products and materials in GMP for clinical trials which will include gene editing  
23 protocols (<https://www.art-tg.com/about-us>).  
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### 33 **Cellular and genomic toxicity induced by genome editing tools**

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36 Genomic toxicity caused by gene editing nucleases remains a major safety concern in  
37 therapeutic applications since they can introduce unwanted DSBs in non-targeted DNA  
38 sequences of the genome called 'off-targets'. The off-target activity is linked to areas of the  
39 genome which share high homology with the specific site targeted for gene editing.<sup>20</sup> When an  
40 off-target cutting event occurs, it can be repaired via the NHEJ and MMEJ pathways,  
41 potentially resulting in an indel mutation; or, if it occurs simultaneously with an on-target or a  
42 second off-target cutting event, the off-target cutting activity can generate a chromosomal  
43 rearrangement. In this review, we are not focusing on detection or prediction of off-target  
44 effects for gene editing but refer to an excellent recent review.<sup>21</sup>  
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51 To increase the precision of gene editing and reducing off-target cuts, other CRISPR genome  
52 editing tools are continuously developed, with enhanced targeting scope, and improved editing  
53 specificity. Three main classes of CRISPR-based genome editing agents are available today,  
54 namely, nucleases as mentioned above, base editors and prime editors. Base editors generate  
55 single-nucleotide changes in DNA, while prime editors use Cas9 fused to an engineered reverse  
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3 transcriptase, programmed with a prime editing guide RNA (pegRNA) that both specifies the  
4 target site and encodes the desired sequence edit template.<sup>20</sup> In this review French researcher  
5 discuss strategies designed to increase the efficiency of precise gene editing by HDR and finally  
6 base editing and prime editing, which represent improved alternative tools for precise gene  
7 editing applications in gene therapy.  
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11 As mentioned above off-target modifications by the CRISPR/Cas9 first-generation nuclease  
12 have been widely reported. However, the on-target NHEJ genotoxicity in response to DNA  
13 DSBs has often been underestimated and is not well studied yet. This can lead to huge  
14 chromosomal deletions or rearrangements and is not well understood up to now.<sup>22,23</sup> Therefore,  
15 a French research team will dedicate here a section to CRISPR/Cas9 induced on-target genomic  
16 toxicity by presenting their recent work from a French team elaborating on how to resolve these  
17 unwanted side-effects.  
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21 Finally, the different delivery systems such as stable retroviral gene transfer and electroporation  
22 of plasmids encoding the gene editing tools, induce still cellular toxicity.<sup>10,24</sup> This is  
23 problematic when we want to gene modify primary human T, B and HSCs since it is clear now  
24 that a minimum of living modified cells needs to be reinfused in the patient for therapeutic  
25 efficiency. Improved protocols for delivery of CRISPR/Cas9 with the associated gRNAs by  
26 electroporation of RNPs have reduced cellular toxicity to some extent. In this review, a French  
27 team will elaborate on new delivery tools for the gene editing machinery, which especially  
28 avoids cell death in primary gene therapy target cells.  
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## 41 **Recent developments in genomic editing for gene therapy**

### 42 **Novel genome editing approaches for $\beta$ -hemoglobinopathies**

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45  $\beta$ -hemoglobinopathies are caused by mutations affecting the production of the  $\beta$ -globin chain  
46 of the adult hemoglobin tetramer (Hb) (**Figure 1A and B**). In particular, sickle cell disease  
47 (SCD) is caused by a single nucleotide mutation in the sixth codon of the  $\beta$ -globin gene, which  
48 leads to the E6V amino acid substitution. Hemoglobin tetramers containing the defective sickle  
49  $\beta^S$ -globin (HbS) polymerize under hypoxia, and red blood cells (RBCs) assume a sickle shape  
50 and become inflexible (**Figure 1A**). Sickle RBCs have a short half-life and obstruct micro  
51 vessels causing a chronic multi-organ disease associated with poor quality of life and short life  
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3 expectancy.  $\beta$ -thalassemia is caused by mutations that reduce ( $\beta^+$ ) or abrogate ( $\beta^0$ )  $\beta$ -globin  
4 production. The uncoupled  $\alpha$ -globin chains precipitate, causing apoptosis of erythroid  
5 precursors and hemolytic anemia (**Figure 1B**). Transplantation of autologous, genetically  
6 modified hematopoietic stem cells (HSCs) is an attractive therapeutic option for patients  
7 lacking a suitable allogeneic HSC donor.  
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12 The clinical severity of  $\beta$ -hemoglobinopathies is alleviated by the co-inheritance of  
13 mutations causing fetal  $\beta$ -like  $\gamma$ -globin expression in adult life - a benign condition termed  
14 hereditary persistence of fetal Hb (HPFH).<sup>25</sup>  $\gamma$ -globin exerts a potent anti-sickling effect in  
15 SCD and compensates for  $\beta$ -globin deficiency in  $\beta$ -thalassemia.  
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19 Two classes of HPFH mutations have been described, large deletions in the  $\beta$ -globin  
20 locus or mutations in the  $\gamma$ -globin promoters. HPFH deletion mutations are large deletions  
21 usually encompassing the adult  $\beta$ - and  $\delta$ -globin genes. They are thought to either remove  $\gamma$ -  
22 globin inhibitory sequences or to juxtapose the  $\gamma$ -globin genes ( $G\gamma$  and  $A\gamma$ ) to distal  
23 transcriptional enhancers (**Figure 1C**). HPFH mutations in the two  $\gamma$ -globin promoters cluster  
24 in the -200, -175 and -115 regions upstream of the transcriptional start sites. They are mainly  
25 point mutations or small deletions or insertions that either disrupt the binding sites (BS) of fetal  
26 Hb (HbF) repressors (e.g., LRF and BCL11A in position -200 and -115, respectively) or  
27 generate BS for transcriptional activators (e.g., KLF1, TAL1 and GATA1 in position -200, -  
28 175 and -115, respectively).  
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32 We have recently generated a 13.6-kb HPFH-like large deletion in the  $\beta$ -globin locus  
33 using the CRISPR/Cas9 nuclease system in primary HSPCs from SCD patients (**Figure 1C**).<sup>15</sup>  
34 NHEJ-mediated deletion of the 13.6-kb region led to a robust reactivation of HbF synthesis in  
35 the erythroid progeny of edited HSPCs and to a substantial amelioration of the sickling cell  
36 phenotype. Interestingly, HbF reactivation upon generation of HPFH-like deletions in adult  
37 erythroid cells was associated with increased interaction of the  $\gamma$ -globin promoters with potent  
38 enhancers located within the  $\beta$ -globin locus. Furthermore, we compared the efficiency of  
39 different methods to deliver the CRISPR/Cas9 system into HSPCs and generate HPFH-like  
40 deletions. RNP delivery exhibited a good balance between cytotoxicity and efficiency, while  
41 minimizing the off-target activity.<sup>26</sup>  
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45 Furthermore, we have used the CRISPR/Cas9 RNP complexes to disrupt the LRF or  
46 BCL11A repressor BS in the  $\gamma$ -globin promoters by NHEJ and microhomology-mediated end  
47 joining (MMEJ) inducing Indel generation, thus mimicking the effect of HPFH mutations  
48 (**Figure 1C**).<sup>27</sup> In primary SCD patient-derived HSPCs, the efficient targeting of LRF or  
49 BCL11A BS resulted in a high proportion of  $\gamma$ -globin expressing HSPC-derived RBCs and  
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3 correction of the sickling cell phenotype. Editing efficiency was minimal at the vast majority  
4 of the predicted off-target sites. Importantly, xenotransplantation of HSPCs treated with gRNAs  
5 disrupting the LRF or BCL11A BS in immunodeficient NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice showed a high  
6 editing efficiency in long-term repopulating HSCs.  
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10 Overall, these studies identified several genomic sites in the  $\beta$ -globin locus as potent  
11 targets for genome-editing treatment of SCD. Interestingly, this strategy can be potentially  
12 applied also to  $\beta$ -thalassemia.  
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15 To further improve the efficacy of HSC editing-based therapy for  $\beta$ -thalassemia, we  
16 have devised a novel gene replacement strategy. Clinical data have shown that the severity of  
17  $\beta$ -thalassemia directly correlates with the number of  $\alpha$ -globin (*HBA*) genes, with deletions of  
18 *HBA* genes having a beneficial effect for patients.<sup>28</sup> In addition, CRISPR/Cas9-mediated  
19 downregulation of  $\alpha$ -globin genes expression ameliorated the globin balance in  $\beta^+$ -thalassemia  
20 patients.<sup>29</sup> To treat both  $\beta^0$ - and  $\beta^+$ -thalassemia, we successfully combined the expression of a  
21 therapeutic  $\beta$ -like globin chain and the down-regulation of  $\alpha$ -globin expression (**Figure 1D**).<sup>8</sup>  
22 In particular, we used CRISPR/Cas9 RNP containing a gRNA targeting the 2 identical 5'UTRs  
23 of the  $\alpha$ -globin genes ( $\alpha 1$  and  $\alpha 2$ ) to delete the  $\alpha 2$ -globin allele that was replaced via HDR by  
24 a therapeutic  $\beta$ -like globin gene (delivered using AAV6). Editing of HSPCs from  $\beta$ -thalassemia  
25 patients led to correction of the  $\alpha/\beta$  globin imbalance. Xenotransplantation experiments in  
26 immunodeficient NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice showed long-term repopulating capacity of edited  
27 HSCs *in vivo*. These results were already confirmed by an independent group, validating the  
28 robustness of this editing approach.<sup>30</sup>  
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### 42 **Advances in genome editing approaches for systemic diseases**

43 Enzyme replacement therapy (ERT) consists in the periodic intravenous administration  
44 of specific enzymes produced to supplement a protein that is deficient because of a genetic  
45 defect. ERT is approved or under investigation to treat more than 40 inherited disorders, mostly  
46 involving blood factors and lysosomal enzymes. Although life saving for some patients this  
47 requires frequent costly injections with a peak-and-trough serum kinetics, which reduce  
48 patients' compliance to the therapy and efficacy of treatment. Sometimes these therapies are  
49 affected by development of antibodies against the administered drugs, which negatively  
50 influence drug bioavailability and activity. Instead, gene therapy can provide constant serum  
51 levels of therapeutic proteins with a single treatment and can induce immune tolerance to the  
52 expressed transgene.<sup>31,32</sup> Autologous HSCs can be successfully engineered *ex vivo* by LV to  
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3 express a transgene of interest; however, the semi-random integration pattern is intrinsically  
4 associated with the risk of inactivating an oncosuppressor and transactivating an oncogene.  
5 Targeting a selected genomic harbour can reduce insertional mutagenesis risk, as also enables  
6 the exploitation of endogenous promoters, or selected chromatin contexts, to achieve specific  
7 transgene expression levels/patterns.<sup>33</sup>  
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11 We recently identified the  $\alpha$ -globin as a suitable locus for CRISPR/Cas9-mediated  
12 targeted gene addition. The idea is to combine the strong transcriptional output of the  $\alpha$ -globin  
13 promoter with the abundance of transgene-expressing erythroblasts to maximize protein  
14 production, reducing the number of integration events required to reach therapeutic levels. In  
15 addition, the  $\alpha$ -globin locus is a safe harbour, since there are 4  $\alpha$ -globin genes per cell (**Figure**  
16 **1 D**) and the loss of up to 3  $\alpha$ -globin alleles is mostly asymptomatic.<sup>14</sup> We edited human HSPCs  
17 with an RNP complex combining Cas9 with a gRNA targeting the 5' UTR of the  $\alpha$ -globin genes  
18 (**Figure 1D**). The therapeutic transgenes were delivered with an AAV6 vector and integrated  
19 via the HDR pathway. We first demonstrated that the selected gRNA provided efficient and  
20 precise editing without affecting viability and differentiation potential of HSPCs and  
21 hemoglobin expression in HSPC-derived erythroid cells or inducing off-target genome  
22 modifications. Then, we demonstrated that the expression of the integrated transgene was  
23 specific to the erythroid lineage and was induced during erythroid differentiation, mimicking  
24 the endogenous  $\alpha$ -globin physiological expression pattern. In addition, we tested this strategy  
25 with therapeutic transgenes for different monogenic diseases, such as hemophilia B and  
26 lysosomal storage disorders (LSD).<sup>14</sup>  
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39 Hemophilia B is a coagulation disorder caused by the absence of functional Factor IX  
40 in the blood. We demonstrated that targeted integration in HSPCs of a F9 gene under the control  
41 of the HBA2 promoter resulted in FIX mRNA expression and protein secretion in their  
42 erythroid progeny and that secreted FIX was functional in reducing blood clotting time *in vitro*.  
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46 LSD are inherited metabolic conditions characterized by an abnormal build-up of toxic  
47 metabolites in lysosomes as a result of enzyme deficiencies. Wolman disease (WD) in particular  
48 is a life-threatening genetic condition due to the accumulation of cholesterol and triglycerides  
49 caused by mutations in the gene encoding lysosomal acid lipase (LAL). We demonstrate that  
50 LAL secreted from erythroid cells derived from edited HSPCs was functional and capable of  
51 cross-correcting patient's cells *ex vivo*, reducing both toxic cholesterol and lipid accumulations.  
52 Finally, we confirmed that edited HSCs conserve their *in vivo* homing, engraftment and multi-  
53 lineage potential by performing xenotransplantation experiments in immunodeficient  
54 NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice.<sup>14</sup> Overall, we established a safe and versatile CRISPR/Cas9-based HSC  
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platform for different therapeutic applications, including haemophilia 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 non-coding region of the causative genes. Most of these diseases affect the central nervous system or the neuromuscular system, such as fragile X syndrome (FXS), Huntington disease (HD), Friedreich ataxia (FRDA), spinocerebellar ataxias (SCAs), spinal and bulbar muscular atrophy (SBMA), amyotrophic lateral sclerosis/frontotemporal dementia (C9-ALS/FTD) and myotonic dystrophies (DM1 and DM2).<sup>34</sup> 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.<sup>35</sup> Here we provide an overview on the approaches that have been explored for myotonic dystrophy type 1 (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 8000 individuals. It is a multisystemic autosomal dominant disorder caused by a CTG repeat expansion in the 3' untranslated region (3' UTR) of the *DMPK* gene.<sup>36</sup> 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.<sup>37</sup> 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 ribonucleoprotein aggregates named foci, leading to defects in the alternative splicing of many pre-mRNAs.<sup>38</sup>

Early attempts to correct CAG/CTG repeat expansions at the genomic level were performed using meganucleases, 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

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3 pluripotent stem cells (iPSCs) resulting in transcripts without repeats.<sup>39,40</sup> With the emergence  
4 of the CRISPR/Cas system as a powerful gene-editing tool, the potential of this technology to  
5 treat MRE disorders was revealed using a CRISPR/Cas9 D10A nickase in a reporter cellular  
6 model demonstrating its ability to induce CAG/CTG repeat contractions.<sup>41</sup> Later, several  
7 CRISPR/Cas-based therapeutic strategies targeting various regions of the *DMPK* gene or  
8 transcripts were evaluated in cellular and mouse models of DM1, opening new perspectives for  
9 the treatment of this disorder.<sup>19,42-46</sup>

10 Our team developed a dual viral vector strategy to excise the pathogenic *DMPK* CTG repeat  
11 region by co-expression of the small sized *Staphylococcus aureus* Cas9 (SaCas9) and selected  
12 pairs of guide RNAs targeting genomic sequences surrounding the trinucleotide repeats.<sup>19</sup> This  
13 approach was initially tested in cultured DM1 patient-derived myoblasts carrying a large CTG  
14 repeat expansion (2600 CTG repeats) to evaluate the ability of the CRISPR/Cas9 system to  
15 delete large expansions, which resulted in the disappearance of nuclear foci and correction of  
16 splicing abnormalities in edited cells. Based on these promising results, we further expanded  
17 our work in a DMSXL disease mouse model, which contains a human *DMPK* transgene with  
18 ~1200 CTG repeats, by intramuscular administration of serotype 9 adeno-associated viral  
19 (AAV9) vectors expressing CRISPR/SaCas9 components and established the proof-of-concept  
20 that genome excision of a large CTG expansion is also feasible *in vivo* in skeletal muscle.  
21 Injection of AAV9 vectors in tibialis anterior muscle of homozygous DMSXL mice was able  
22 to reduce the overall amount of myonuclei with toxic *DMPK* RNA foci but also showed some  
23 limitations. Therefore, additional preclinical studies will be required to further optimize and  
24 evaluate the therapeutic benefit and safety of this approach at the level of the whole body by  
25 targeting the entire musculature, including heart, and eventually other tissues affected in the  
26 disease.

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

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29 The adoptive transfer of CAR T cells represents a highly promising strategy to fight  
30 against multiple cancer indications. This strategy relies on the engineering of T cells to redirect  
31 their cytolytic activity toward malignant cells via transgenic expression of a tumor antigen-  
32 specific receptor at their surface. Today, the current protocols of treatment consist in autologous  
33 adoptive T cell transfer. In this approach, T lymphocytes recovered from patients, are  
34 genetically modified, and expanded *ex vivo* before infusion back into patients. Clinical results  
35 gathered over more than 10 years show impressive rates of complete remission in different

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3 indications.<sup>47</sup> These highly positive clinical outcomes led to the FDA-approval of 4 different  
4 products named tisagenlecleucel, axicabtagene ciloleucel, brexucabtagene autoleucel and  
5 lisocabtagene maraleucel to treat acute lymphoblastic leukemia, large B cell lymphoma, mantle  
6 cell lymphoma and multiple myeloma, respectively.<sup>48-50</sup>  
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10 While highly promising, autologous CAR T-cell therapies have been hampered by many  
11 practical and clinical challenges. Practical challenges include the production time and high cost,  
12 the dependence on patients T-cells' fitness that is reduced by the disease or previous lines of  
13 therapies and the logistical conundrum associated to the coordination of CAR T-cell production  
14 and injection. Clinical challenges include the life threatening cytokine storm observed in most  
15 patients<sup>51</sup>, the transient persistence of CAR T-cell fitness and their difficult access to tumor. It  
16 also includes the tumor-dependent inhibitory signals and tumor-associated suppressor cells that  
17 are usually found in the microenvironment of tumors and contribute, directly or indirectly, to  
18 the impairment of CAR T-cell antitumor activity.  
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25 These multiple challenges cannot be easily addressed with standard cell engineering  
26 methods. However, implementation of advanced gene editing technologies in cell culture  
27 processes brought a wealth of solutions that are now revolutionizing the field of CAR T-cell  
28 therapies and more broadly, immunotherapies. The following section describes how gene  
29 editing technologies, especially those based on TALEN, could be leveraged to overcome some  
30 of the practical and clinical challenges faced by CAR T-cell therapies.  
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36 One of the first important challenge tackled by gene editing technologies was to enable  
37 the production of universal CAR T-cell compatible with adoptive transfer in allogeneic settings.  
38 In contrast to the autologous approach, universal CAR T-cell could be mass produced from  
39 healthy donor T-cells and can then be theoretically transferred as an off-the-shelf medicine to  
40 any HLA-mismatched patients (**Figure 2A and 2B**, respectively). However, for this approach  
41 to be successful, the graft versus host (GvH) and the host versus graft (HvG) reactions must be  
42 avoided to safely allow CAR T-cells to engraft and express their antitumor function in HLA-  
43 mismatched patients (**Figure 2C** top panel). Indeed, in this scenario, GvH reaction would  
44 consists in the recognition of the Major Histocompatibility Complex (MHC) marker exposed  
45 at the surface of host cells by an alloreactive  $\alpha\beta$  T-cell receptor ( $\alpha\beta$ TCR) exposed on the CAR  
46 T-cell surface. This recognition would promote an acute, non specific and globale depletion of  
47 host tissues and lead to an eventual fatal outcome (**Figure 2C**). HvG reaction would involve  
48 the same recognition mechanism but results in the depletion of CAR T-cell by alloreactive host  
49 T-cells and thus lead to a poor antitumor activity and therapeutic outcome. Thus, without  
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3 additional genome engineering, adoptive transfert of CAR T-cell in allogeneic settings would  
4 be simply unsafe and inefficient.  
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6 In the past 10 years, several different genome engineering strategies were developed to  
7 prevent GvH and HvG. The first strategy developed to prevent GvH and HvG was to engineer  
8 a  $\alpha\beta$ TCR and CD52 double knocked out (KO) CAR T-cell through TALEN-mediated gene  
9 inactivation.  $\alpha\beta$ TCR inactivation was shown to be highly efficient and to robustly prevent GvH.  
10 In addition, CD52 inactivation enabled CAR T-cell to resist to alemtuzumab, an FDA-approved  
11 antibody designed to bind and promote depletion of CD52 expressing immune cells.<sup>52</sup> Such  
12 resistance enabled Alemtuzumab-dependent host lymphodepletion led to an efficient  
13 prevention of HvG and created a receptive environment for  $\alpha\beta$ TCR/CD52-deficient CAR T-  
14 cell engraftment in the clinic. <sup>53</sup> A second strategy consisted in the TALEN-mediated  
15 inactivation of  $\alpha\beta$ TCR and dCK, which enabled to produce  $\alpha\beta$ TCR deficient CAR T-cells  
16 resistant to purine nucleotide analogues (PNAs, including fludarabine clofarabine and  
17 decitabine) that are commonly used to lymphodeplete patients prior CAR T-cell infusion  
18 (**Figure 2C**, middle panel).<sup>54</sup> A third strategy aimed at inactivating  $\alpha\beta$ TCR along with B2M to  
19 create  $\alpha\beta$ TCR/MHC deficient CAR T-cells (**Figure 2C**, right panel). This approach enabled  
20 the generation of non-alloreactive CAR T-cell capable of evading alloreactive T-cell attack and  
21 is currently evaluated in clinic to assess its safety and efficacy profile.  
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34 Genome engineering technics were also used to mitigate the cytokine release syndrome  
35 (CRS) associated to CAR T-cell infusion. CRS is characterized by fever, hypotension and  
36 respiratory insufficiency and is correlated to elevated pro-inflammatory cytokines in patients'  
37 following CAR T-cell infusion. Its severity is mainly mediated by IL-6, IL-1 and other factors  
38 produced by patient macrophages, that are commonly activated in the vicinity of CAR T-cell.<sup>55</sup>  
39 Two independent studies<sup>56,57</sup> identified Granulocyte-macrophage colony-stimulating factor  
40 (GMCSF) as one of the main mediators of macrophage activation by CAR T-cell (**Figure 2D**,  
41 left panel), opening opportunities to mitigate CRS through genome engineering of CAR T-cell.  
42 In that context, TALEN- and CRISPR/Cas9-mediated inactivation of GMCSF in CAR T-cells  
43 was shown to drastically decrease CAR T-cell dependent-secretion of GMCSF and to abolish  
44 subsequent macrophage-dependent secretion of multiple CRS biomarkers, including IL-6  
45 (**Figure 2D**, right panel). While this approach may not fully eliminate CRS symptoms, it could  
46 be combined with other engineering approaches and improve the overall safety of CAR T-cell  
47 therapies for cancer patients.<sup>58</sup>  
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58 Finally genome engineering was also used to improve CAR T-cell antitumor function  
59 in the hostile tumor microenviroment. For instance, among the multiple strategies published  
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3 in the past years, one consisted in rewiring PD1 expression into the secretion of a powerful  
4 immunostimulatory agent named IL-12 (**Figure 2E**).<sup>58</sup> This was achieved by inserting an IL-  
5 12 expression cassette in frame with the PD1 gene using a PD1-specific TALEN and an AAV6  
6 matrix bearing the IL-12 expression cassette. This strategy resulted in the inactivation of PD1,  
7 one of the main actor of tumor-dependent inhibition of CAR T-cell and in the localized and  
8 tumor-dependent secretion of IL-12. This approach improved antitumor activity and  
9 proliferation capacity of CAR T-cell while mitigated the risks of adverse events commonly  
10 observed when IL-12 is systemically infused in patients.<sup>59</sup>  
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## 20 **Toxicity and recent improvements in gene editing for gene therapy**

### 21 **ON-target genotoxicity in gene therapy target cells upon CRISPR/Cas9 nuclease** 22 **challenge**

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29 CRISPR/Cas9 is a powerful technology for genome editing. However, high efficiency of the  
30 editing nuclease is not void of downsides due to unwanted and uncontrolled activity. The most  
31 studied and almost resolved side effect is OFF-target genotoxicity. By contrast, the ON-target  
32 genotoxicity of CRISPR/Cas9 nuclease due to DNA DSB at the targeted locus has received  
33 little attention and is still underestimated. Using Cas9 nuclease-induced DSB at the  
34 Uroporphyrinogen III Synthase (*UROS*) locus (chromosome 10q) to model and correct  
35 congenital erythropoietic porphyria (CEP), we demonstrated that HDR is rare compared with  
36 the competitive unwanted NHEJ pathway. Indeed, the edition is often bi-allelic, and  
37 uncontrolled small indels are concomitant to precise correction. They lead to disrupted targeted  
38 sequences and cause unwanted dysfunctional protein in cell lines and iPSC.<sup>60,61</sup> We  
39 demonstrated that uncontrolled indels induced by the NHEJ are very frequent in corrected  
40 HEK293T, with a precise genome editing ratio (HDR/NHEJ) of 0.5. Several approaches have  
41 been proposed to improve this ratio e.g. by NHEJ inhibition or activating the HDR pathways  
42 (for review see Sledinski et al. <sup>62</sup>). It is also possible to exploit the design of the gRNA. We  
43 tested an original approach to correct compound heterozygous recessive mutations. We  
44 compared editing efficiency and genotoxicity using a biallelic gRNA *versus* a mutant allele-  
45 specific gRNA in iPSCs derived from a CEP patient carrying compound heterozygous *UROS*  
46 mutations. We reported that, unlike the biallelic one, the mutant allele-specific gRNA was free  
47 of ON-target collateral damage and allows a precise gene correction without concomitant indels  
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3 in the same iPSC clone. This design that avoids genotoxicity with ON-target scarless gene  
4 correction should be recommended for recessive diseases with frequent cases of compound  
5 heterozygous mutations.<sup>61</sup>  
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8 In addition to small indels, a single ON-target DSB (without a second DSB at OFF-  
9 target genomic loci) can also lead to interstitial large deletions of several kilobases, symmetrical  
10 or not at the targeted site, in mouse hematopoietic progenitors, in human immortalized  
11 differentiated cells or in mouse embryos.<sup>63</sup> Recently, larger deletions (up to three hundred  
12 kilobases) in mouse zygote were reported.<sup>64</sup>  
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17 Unexpectedly, even larger genomic rearrangements may occur. We reported that  
18 CRISPR/Cas9 can cause megabase-scale chromosomal terminal truncations targeting *UROS*  
19 (Chromosome 10q) in two human cell lines (HEK-293T and K562) and in human primary  
20 fibroblasts deficient for the tumor suppressor p53. This megabase-scale deletion was mapped  
21 by array-CGH (comparative genomic hybridization). This by-product starts at DSB cut site and  
22 deletes the chromosomal end (7.5 Mb). It removes 43 genes including five proto-oncogenes  
23 and seven tumor suppressors.<sup>60</sup> This risk of inducing Megabase-scale deletions was recently  
24 confirmed in other human primary cell-types (iPSC and human embryos)<sup>65,66</sup>, suggesting that  
25 CRISPR-mediated large rearrangements are not locus or cell-type dependent.  
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32 We then evaluated if unexpected large rearrangements can occur targeting globin genes  
33 for gene editing (important gene therapy targets for hemoglobinopathies as described above) in  
34 chromosome 11p. Again, we observed that genome editing targeting globin genes induces  
35 megabase-scale losses of heterozygosity (LOH) from the globin CRISPR/Cas9 cut site to the  
36 telomere (5.2Mb). In established lines, CRISPR/Cas9 nuclease induced frequent (up to 20%)  
37 terminal chromosome 11p truncations and rare copy-neutral LOH (without loss of genomic  
38 material). Targeting the  $\beta$ -globin cluster in primary HSPCs, we detected 1.1% of clones (7/648)  
39 with acquired megabase LOH induced by CRISPR/Cas9. In-depth analysis by CGH/SNP-array  
40 revealed the presence of copy-neutral LOH from the cut site to the telomere without deletion.  
41 So, the cell-type seems to be crucial to determine the type of DNA rearrangement that might  
42 occur after CRISPR/Cas9 DSB. In HSPCs, copy-neutral (CN) LOH led to 11p15.5 partial  
43 uniparental disomy, comprising two Chr11p15.5 imprinting centers (*H19/IGF2:IG-DMR/IC1*  
44 and *KCNQ1OT1:TSS-DMR/IC2*) and impacting *H19* and *IGF2* expression (Boutin et al.,  
45 Nature Com; 2021, in press). It is possible that CN-LOH is a survival repair response to the  
46 loss of an allele in p53-proficient cells. The mechanism of extra-large terminal CN-LOH post  
47 CRISPR/Cas9-mediated DSB is probably consistent with Break-induced replication (BIR)  
48 recently described in eukaryotic cells. It involves extensive DNA synthesis from the DSB to  
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3 the telomere. It can even provoke chromothripsis, an extensive and complex chromosome  
4 rearrangement restricted to the chromosome targeted by CRISPR-Cas9.<sup>67</sup> In parallel, interstitial  
5 smaller kilo-base CN-LOH by gene conversion in the close surroundings of the DSB was also  
6 described in the globin cluster.<sup>68</sup>  
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10 This ON-target genotoxicity, at base/kilobase and megabase-scale could be a safety  
11 concern for CRISPR clinical trials and has to be confirmed in other gene *loci* and *in vivo*  
12 settings. These new data highlight the necessity to develop preclinical tests to evaluate carefully  
13 not only the well-known OFF-target but also the ON-target genotoxicity risks. Further studies  
14 to understand the mechanisms of the appearance of these extra-large chromosomal  
15 rearrangements will be necessary to find solutions/alternatives to prevent them. Interestingly,  
16 nickases, inducing a DNA single strand break, allow HDR without indels or truncations in cell  
17 lines.<sup>60</sup> Unfortunately, its use is still challenging for clinical studies due to low efficiency in  
18 human primary cells. Modulating DSB DNA repair pathways or developing clinical trials with  
19 DSB-free genome editing are currently under study. Novel and safer gene editing strategies and  
20 tools are also mentioned in the next section.  
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### 30 **Improving gene editing precision: the safer DNA repair perspective**

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34 The goal of genome editing experiments is to change a targeted DNA sequence into a desired  
35 DNA sequence in the genome of cultured cells or organisms. In most cases, especially in gene  
36 therapy approaches aimed at precise gene correction or gene insertion as exemplified in the first  
37 section, a single sequence product is pursued at high efficiency. However, in some applications,  
38 heterogeneous edited sequences may be acceptable; for example it is the case in gene therapy  
39 approaches developed in by some French teams to treat SCD and  $\beta$ -thalassemia by disruption  
40 of binding sites for transcriptional repressors in HBG promoters mimicking HPFH mutations  
41 (<sup>27</sup> and see above) and we can also cite diseases such as Huntington disease or DM1, caused by  
42 trinucleotide expansions that can be reduced or deleted by targeting the repeats with CRISPR  
43 nucleases as shown by us and the GF Richard lab at the Pasteur Institute in Paris.<sup>69</sup> Improved  
44 CRISPR genome editing tools are continuously evolving, with enhanced targeting scope,  
45 improved editing specificity and importantly, enhanced precision of genome editing activity,  
46 which are all important issues when clinical applications are envisioned. Three main classes  
47 of CRISPR-based genome editing agents are available today, namely, nucleases, base editors  
48 and prime editors, expanding the solutions that can be chosen and optimized for a given genome  
49 editing application. We discuss in this section strategies designed to increase the efficiency of  
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3 precise gene editing by HDR and finally base editing and prime editing, which are promising  
4 alternative tools for precise gene editing in gene therapy.

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6 The basic principle of precise gene editing in living cells was first pioneered with the I-SceI  
7 nuclease isolated by the Dujon lab (Pasteur Institute, Paris). Together with several other groups,  
8 they used I-SceI to introduce a targeted double strand break into a model gene carrying the 16  
9 pb I-SceI target sequence and strongly stimulated precise gene modification by homology-  
10 dependent repair (HDR)<sup>70</sup>, triggering the new era of gene editing with programmable nucleases.  
11 ZNF, TALEN and CRISPR/Cas9 were next used to target genomics sites of interest and more  
12 recently, successive generations of programmable nucleases were engineered providing even  
13 greater flexibility and easy use for precise gene editing based on HDR. End-joining pathways  
14 of DNA DSB repair, however, were found to generally prevail over HDR in mammalian cells.  
15 Hence, precise modification by HDR, copying from template DNA with the desired sequence  
16 change, is not the most common outcome and imprecise modifications are generally much more  
17 frequent (<sup>71</sup> and see the above section). Therefore, approaches to improve genome editing  
18 precision are actively explored.

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20 Different types of donors can be used as homology templates, single-stranded or double-  
21 stranded DNA. HDR pathways differ depending on the nature of donor DNA, and are broadly  
22 classified into homologous recombination (HR) for double-stranded donors and single-stranded  
23 templated repair (SSTR) for single-stranded donors, that likely share early HDR steps but  
24 require different factors necessary for downstream incorporation of donor information.<sup>72</sup>  
25 Importantly, this expands the possibilities for precise genome editing. Single-stranded donors  
26 are indeed a promising option for gene editing. We have shown that short oligonucleotides  
27 (ssODN) require only short homology regions (around 35 nt), and when modified with  
28 phosphorothioate linkages they can exhibit very high precise editing activity to introduce short  
29 DNA sequences (<100 bp); in addition, they have the advantage to be easy to design and are  
30 synthesized commercially.<sup>73</sup> To integrate longer sequences, protocols for efficient synthesis of  
31 long single-stranded DNA were successfully developed and used to generate precise  
32 modifications in different systems.<sup>74</sup> The mechanism of HDR with single-stranded donors is  
33 not entirely characterized but was shown to involve factors of the Fanconi repair pathway, that  
34 are shared with the HR pathway. Donor and nuclease delivery can also have a strong impact.  
35 For example, AAV vectors promote efficient precise gene editing, as shown by the Porteus  
36 lab<sup>75</sup> and further exemplified by work<sup>14</sup> from the Amendola lab at Généthon (Evry, France).  
37 This is possibly related to AAV vector sequences binding to nuclear factors favorable to HDR  
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3 or to differences in final nuclear concentration of donor sequences. We have also shown that  
4 increased HDR genome editing can be obtained using Cas9 protein compared to mRNA.<sup>76</sup>  
5 Efficiency of precise gene modification by HDR nevertheless remains too often very limited.  
6 Pioneer studies from D Carroll found that genetic inactivation of Ligase 4, an essential NHEJ  
7 protein, increased the frequency of HDR in *Drosophila melanogaster* and therefore showed  
8 that manipulating DNA repair pathways can help to increase the efficiency of precise gene  
9 editing.<sup>71</sup> Pharmacological modulators, such as DNAP-PKc inhibitor nedisertib<sup>77</sup>, can thus  
10 improve gene editing but unfortunately are associated with non-specific toxicity. Several  
11 research teams, including ours, have next shown that direct fusion of DNA repair proteins to  
12 Cas9 can also bias the outcome of DNA repair and be used to favor precise gene editing by  
13 HDR (**Figure 3 A, B**). The potential advantage of such targeting of the repair protein of interest  
14 at the DSB site is to avoid global effects on cellular DNA repair induced by pharmacological  
15 inhibitors. Since processing of DSB ends via 5' to 3' resection is the major determinant of repair  
16 pathway choice, CtIP, a protein that promotes Mre11 exonuclease activity at this early step of  
17 HDR, was fused to Cas9. The fusion of Cas9 with a minimal N-terminal fragment of CtIP was  
18 sufficient to stimulate HDR<sup>78</sup>. This fragment contains the cell-cycle-dependent phosphorylation  
19 sites of CtIP that represent one of the main control points of HDR (**Figure 3B**). Another option  
20 is to perform co-targeting of a gene with a phenotype that can be conveniently screened to  
21 enrich for cells carrying the modifications of interest.<sup>79</sup>  
22 Finally, novel tools developed by the Liu lab at MIT bypass the limited activity of HDR by  
23 avoiding DSB repair and engaging other repair pathways; these tools raise exciting possibilities  
24 currently being investigated. For example, base editing is achieved by fusing C or A deaminase  
25 moieties to D10A Cas9 nickase (**Figure 3C**).<sup>80</sup> Co-targeting of a selectable gene with a base  
26 editor can also be used to increase efficiency<sup>81</sup> and such tools have been used in animals to  
27 generate models of human disease, for instance cancer modelling in zebrafish<sup>82</sup> by the Del Bene  
28 team at the Institute for Vision. A major limitation, however, is that all target nucleotides in a  
29 specific window will be modified (**Figure 3C**), limiting precision of the approach. In addition,  
30 base editors can currently install only 6 (C→T, A→G, C→G, G→A, T→C, G→C) of the 12  
31 possible types of point mutations. An alternative, potentially universal approach for small  
32 sequence changes, point mutations, small insertions and small deletions, is prime editing. It  
33 implies a fusion of the H840A Cas9 nickase to the Reverse transcriptase of the Moloney Murine  
34 Leukemia Virus and modified guide RNAs carrying sequence templates to be copied into the  
35 target genomic site (**Figure 3D**).<sup>83</sup> Prime editing is actively investigated to address its highly  
36 variable efficiency and overcome delivery challenges. Both base editing and prime editing are  
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3 based on targeting an enzyme that will directly modify DNA (respectively C or A deaminase  
4 or reverse transcriptase) to the target sequence of interest with the CRISPR/Cas9 system.  
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6 However, only one DNA strand is modified and stable introduction of the mutation depends on  
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8 copying the modification onto the second DNA strand. DNA repair pathways involved need to  
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10 be better understood and may also be manipulated to effectively increase gene editing  
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12 efficiency.

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

### 26 27 28 29 **Nanoblades for efficient non-toxic cellular delivery of the gene editing machinery into** 30 **gene therapy targets**

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34 To obtain efficient gene editing in primary blood target cells, the delivery systems to introduce  
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36 the endonuclease, to produce DSB and the donor DNA for Knock-in (KI) into the nucleus of  
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38 the target cell, have to be very efficient. There are different methods to deliver the gene editing  
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40 machinery into the cells: transduction by integrating lentiviral vectors (LV), or adeno-  
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42 associated viral (AAV) vectors, transfection with DNA or RNA or electroporation with  
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44 ribonucleoprotein CAS9/gRNA complexes (RNPs) (**Figure 4A**). For HDR strategies the donor  
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46 template can be supplied by infection with an AAV vector, integrative deficient LV (IDLV) or  
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48 electroporation of single-stranded DNA or oligonucleotides encoding the template DNA. All  
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50 these methods have delivered the gene editing tools with different degrees of efficiency, toxicity  
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52 and off-target effects.

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54 To introduce the gene editing machinery in primary cells, the method of choice at the moment  
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56 is electroporation of RNPs, which allows efficient editing of human T and B cells and HSPCs.<sup>7</sup>  
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58 For B cells, other methods were employed like electroporation of Cas9 mRNA or the  
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60 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.<sup>3</sup> In the case of



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3 HSPCs, Cas9 has been efficiently introduced by electroporation of CAS9/gRNA complexes  
4 (RNPs).

5  
6 For gene therapy strategies based on NHEJ in HSPCs, diseases such as  $\beta$ -hemoglobinopathies  
7 (see first section), HIV or Fanconi anemia have been successfully corrected by gene editing.  
8 For example in Fanconi anemia the introduction of Cas9/gRNA complexes by electroporation  
9 of RNPs introduced DSBs in the mutated FANCA gene, which when repaired by NHEJ,  
10 restored the FA gene function.<sup>13</sup> For HDR strategies in HSPCs, the donor template is being  
11 introduced preferentially using AAV6 which allowed genetic repair of multiple diseases such  
12 as chronic granulomatous disease (CGD), SCID-X or WAS.<sup>84</sup> Although these approaches were  
13 successful, some drawbacks were reported such as cell death and off-target effects.

14  
15 The ideal gene editing tool should be precise, fast, non-toxic and induce as less as possible off-  
16 target effects. Recently, two collaborative French teams (CIRI, Lyon, France) developed a new  
17 gene editing tool delivery system, the “nanoblades”. This is a vehicle in which the Cas9/gRNA  
18 RNP is packaged into a modified virus like particles (VLP) derived from a murine leukemia  
19 virus (MLV) or HIV (**Figure 4B**).<sup>85,86</sup> The endonuclease, Cas9, is fused to a viral structural  
20 protein gag, associated with its gRNA and is by this means actively incorporated into the VLPs.  
21 These nanoblades allow rapid and transient RNP delivery and importantly are devoid of viral  
22 genomic sequences. They produce DSBs rapidly and efficiency in immortalized cells, iPSCs  
23 and primary cells.<sup>86</sup> More interestingly, since nanoblades are viral-vector-derived particles  
24 (VLP), they carry a viral envelope at their surface to allow VLP cell entry. They can thus easily  
25 be pseudotyped as their counterpart viral vectors with different envelope glycoproteins (gps).  
26 We have previously shown that the baboon endogenous virus (BaEV) envelope gp incorporated  
27 into a LV, allowed efficient cell entry into human T, B and HSPCs (**Figure 4C**).<sup>84,87-89</sup> Indeed,  
28 nanoblades displaying at their surface a combination of BaEV and the vesicular stomatitis virus  
29 G (VSV-G) envelope glycoproteins deliver the Cas9/gRNA transiently and rapidly into slightly  
30 activated T, B cells and HSPCs without any change in their phenotypes nor cellular toxicity.<sup>85</sup>

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32 We demonstrated that nanoblades confer efficient delivery of the gene editing machinery in  
33 HSPCs reaching up to 80% editing, without obvious detection of gene editing at off-target sites,  
34 in contrast to other methods. Finally, treatment of HSPCs with nanoblades in combination with  
35 a donor DNA template encoding rAAV6 vector resulted in up to 40 % stable expression cassette  
36 knock-in into a specific gene locus (**Figure 4D**). Importantly, no toxicity was detected upon  
37 nanoblade-mediated KI in HSPCs since no significant effect was seen on cell survival and  
38 proliferation in nanoblade treated versus untreated cells. Different other methods to deliver the  
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3 gene editing tools such as electroporation, adenoviruses, AAVs and LVs have been used,  
4 conferring different degrees of efficiency, toxicity, and off-target effects. The nanoblades  
5 combine actually the low to undetected toxicity of retroviral delivery (VLP) and the transient  
6 expression of Cas9/gRNA RNP-mediated gene editing. Indeed, nanoblades confer efficient  
7 NHEJ-mediated gene editing in HSPCs and in T and B cells, but not at expense of significant  
8 induced cellular toxicity.  
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15 Nanoblades represent an easy to use, flexible and efficient platform for gene editing in gene  
16 therapy targets. Easy to use since only the plasmid coding for the gRNAs needs to be redesigned  
17 to target another genomic locus. Flexible since they can harbor multiple gRNAs to permit  
18 knock-out of multiple genes at once.<sup>86</sup> Continuously Cas9 proteins are improved to reduce off-  
19 target activity or increase efficiency. For example other targetable nucleases were identified  
20 e.g. Cpf1 nucleases, high fidelity Cas9, nickases, hyper-accurate Cas9<sup>90,91</sup> or to increase the  
21 precision of editing, base editors were engineered for therapeutic applications.<sup>92</sup> All these new  
22 components might readily be incorporated into nanoblades by fusing them to MLV or HIV gag  
23 proteins.  
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33 In the same line, other groups have developed CRISPR/Cas9 vehicles that resemble our  
34 nanoblades.<sup>93-95</sup> The three systems were highly efficient for gene editing in cell lines and some  
35 in primary cells such as iPSCs. However, these transient CRISPR/Cas9 delivery systems were  
36 not evaluated for gene editing in human T, B and HSPCs gene therapy targets yet.  
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39 Summarizing, these nanoblades are simple to implement, show high flexibility for different  
40 targets including primary immune cells of human and murine origin, is relatively inexpensive  
41 and therefore provides important perspectives for basic and clinical translation in the area of  
42 gene therapy.  
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## 48 **Conclusion**

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

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12 We thank J. Boutin, G. Cullot, F. Prat, J. Rosier and D. Cappellen, the biotherapy team  
13 (Inserm U1035), Vect'Ub platform (TBM core), Bordeaux hospital genetic laboratory,  
14 Bordeaux hospital tumor Biology and Tumor Bank Laboratory for their main contributions.  
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17 We thank M. Jimena A. Recalde, C. Costa, O. Bernadin, S. Périan, F. Fusil, G. Froment, F-L.  
18 Cosset, E. Ricci and P. E. Mangeot (CIRI, Lyon, France) for their contributions to the  
19 nanoblade work. M.A. and A.B-B. thank past and present members of their laboratory at  
20 Genethon (Evry, France). We thank current and past members of the GE2R team at MNHN  
21 lab (Inserm U1154/CNRS UMR7196).  
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## 27 **Authorship confirmation statement**

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30 A.B., F.M.G. wrote the manuscript. A.M., M.A., M.L. and G.F. wrote the manuscript and  
31 prepared Figure 1. J.V. wrote the manuscript and prepared figure 2. M.C., J-P.C., C.G., D.G.  
32 wrote the manuscript and prepared Figure 3. EV and A.G-G. wrote the manuscript and prepared  
33 Figure 4. C.S. and A.B-B. wrote the manuscript.  
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## 39 **Author(s') disclosure statement(s)**

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42 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., J.F. have no  
43 conflict of interest to disclose. J.V. is currently a Collectis. SA employee, TALEN® is a  
44 Collectis' patented technology. A.B-B. has a patent application related to gene editing of DM1.  
45 J-P. C. and C.G. have a patent application related to gene editing with Cas9 fusion to a CtIP  
46 Nter domain.  
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## 52 **Funding statement**

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55 We thank the Agence de Biomédecine, INSERM, Bordeaux University and French ministry of  
56 higher education and research for financial support. We thank the EU (Euronanomed, AAV-  
57 Chem), Cancéropole (CRISPR/Cas9 Screen Action) and AFM for financial support. This work  
58 was supported by State funding from the Agence Nationale de la Recherche (ANR) (ANR-10-  
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IAHU-01, ANR-16-CE18-0012-01, ANR-II-INSB-0014, ANR-16-CE18 STaHR) and the European Research Council. This work was supported by the AFM-Telethon, Genopole (Chaire Fondagen). We thank Canceropôle Grand-Ouest et AVISAN Plan Cancer for financial support.

## Figure legends

**Figure 1** Editing human HSPCs as therapeutic strategy for genetic disorders. Genetics and pathophysiology of SCD **(A)** and  $\beta$ -thalassemia **(B)**.  $\beta^S$ , sickle  $\beta$ -globin.  $\beta^0$  and  $\beta^+$  indicate  $\beta$ -thalassemia mutations that cause absent or reduced  $\beta$ -globin expression, respectively.  $\delta$ ,  $\delta$ -globin  $\beta$ -like chain that accounts for  $\leq 3\%$  of the total  $\beta$ -like chains in adult RBCs. **(C)** Editing the  $\beta$ -globin locus to reactivate HbF expression. Dark red scissors indicate CRISPR/Cas9 nuclease editing the  $G\gamma$ - and  $A\gamma$ -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).  $\epsilon$ , embryonic  $\beta$ -like globin gene;  $G\gamma$  and  $A\gamma$ , fetal  $\beta$ -like globin gene;  $\psi\beta$ ,  $\beta$ -globin pseudogene;  $\beta_{mut}$ ,  $\beta$ -globin gene harboring SCD or  $\beta$ -thalassemia mutations. **(D)** Editing the  $\alpha$ -globin locus to correct  $\beta$ -thalassemia or lysosomal storage disorders. Grey scissors indicate CRISPR/Cas9 nuclease deleting the  $\alpha_2$  gene, which is replaced by a therapeutic  $\beta$ -like globin gene or by a transgene expressing a secreted protein.  $\zeta$ , embryonic  $\alpha$ -like globin gene;  $\psi\zeta$ ,  $\zeta$ -globin pseudogene;  $\alpha_1$  and  $\alpha_2$  fetal/adult  $\alpha$ -globin genes;  $\beta_{like}$ , therapeutic  $\beta$ -like globin gene; GoI, gene of interest; F9, Factor IX; LAL, lysosomal acid lipase; GLA,  $\alpha$ -galactosidase; IDUA,  $\alpha$ -L-iduronidase.

**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 Graft versus Host (GvH) and Host versus graft (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.

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7 **Figure 3** Genome editing strategies.

8 Different classes of CRISPR-based genome editing agents are shown: nucleases, wt (**A**) or  
9 fused to HDR enhancer (**B**), base editors (**C**), and prime editors (**D**). (PAM) protospacer  
10 adjacent motif; (HDR) homology-directed repair; (RT): transcriptase.  
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19 **Figure 4.** Delivery methods for delivery of CRISPR/Cas9 into primary gene target cells

20 (**A**) Electroporation of RNP complexes, AAV6 encoding CRISPR and lentiviral vectors  
21 encoding the CRISPR/Cas9 and the gRNA. (**B**) Improvement of delivery into primary cells;  
22 HSC, T cells and B cells by pseudotyping with heterologous viral envelopes. Primary cells  
23 express receptors such as ASCT1/2, SLAM and CD46, which allow LVs displaying envelopes  
24 from the measles virus (HF) or the Baboon endogenous virus (BaEV) to deliver their cargo into  
25 primary cells efficiently. However, unstimulated T, B cells and HSCs do not express the  
26 receptor for the Vesicular stomatitis virus G (VSV-G) envelope, prohibiting efficient  
27 transduction in non-stimulated cells. (**C**) Schematic representation of nanoblades and its  
28 components. (**D**) HDR repair using CRISPR/Cas9 introduced by nanoblades and a donor DNA  
29 template. The donor template is provided into the cell by an AAV6 vector, CRISPR/Cas9 RNP  
30 delivered by nanoblades make a DSB and the homology arms in the donor DNA allow  
31 homology recombination introducing a transgene into the cell genome in a specific gene locus.  
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### Publication in press

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56 CRISPR-Cas9 globin editing can induce megabase-scale copy-neutral losses of heterozygosity  
57 in hematopoietic cells Boutin J, Rosier J et al Accepted July 2021, Nature communication  
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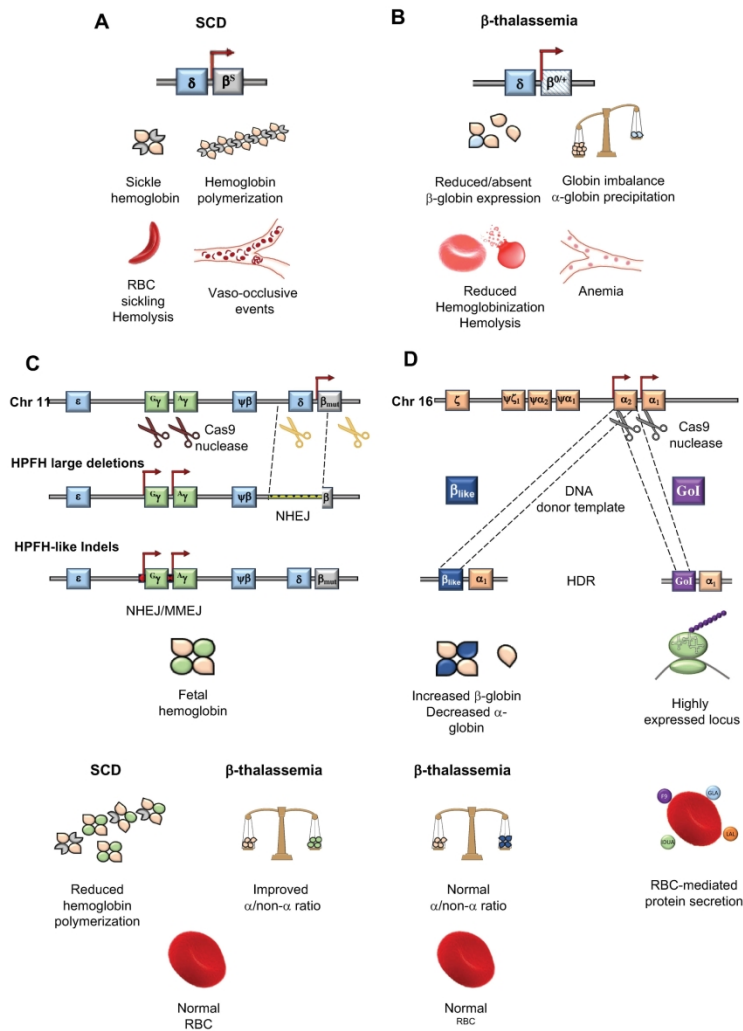


Figure 1

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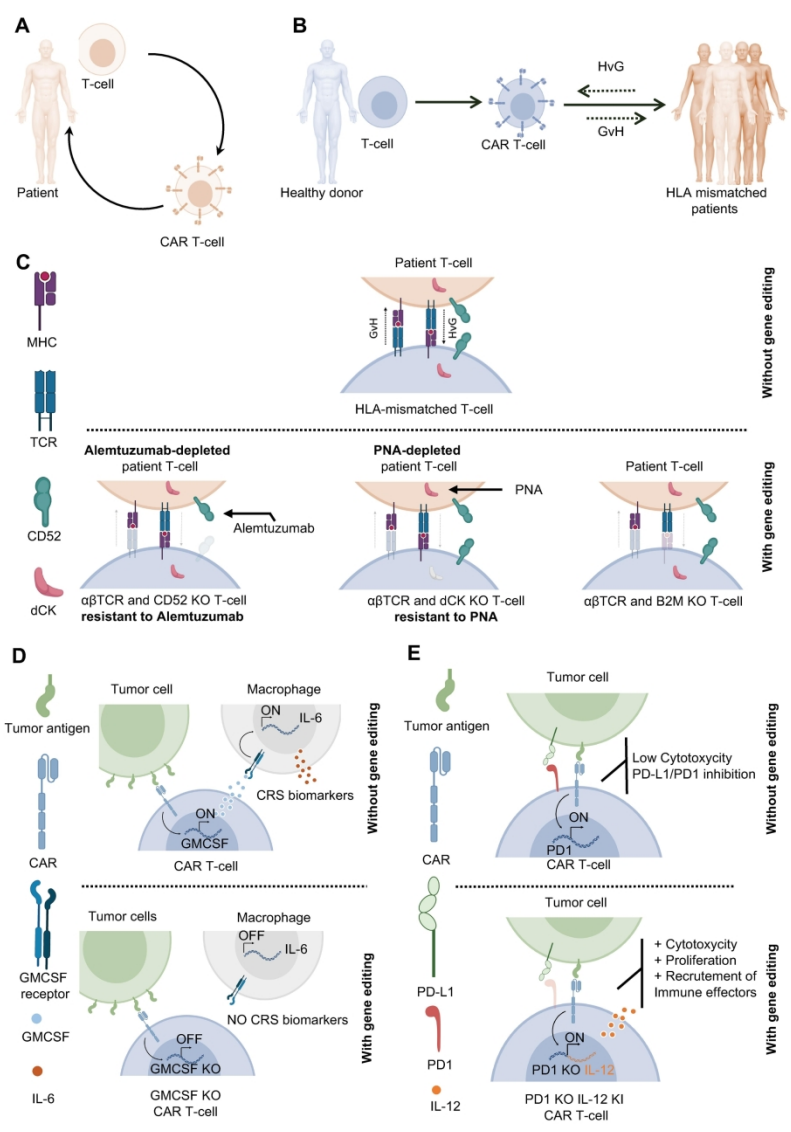


Figure 2

209x297mm (300 x 300 DPI)



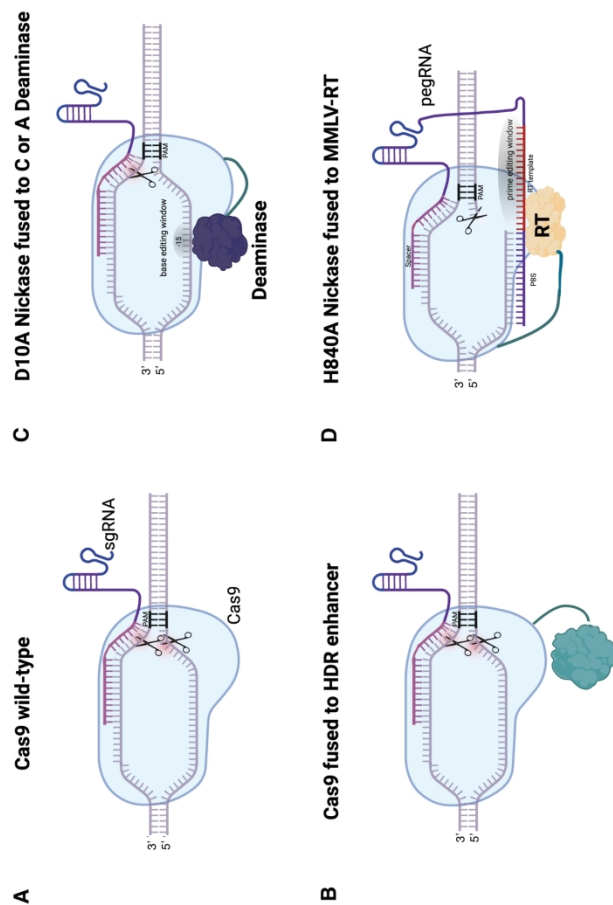


Figure 3

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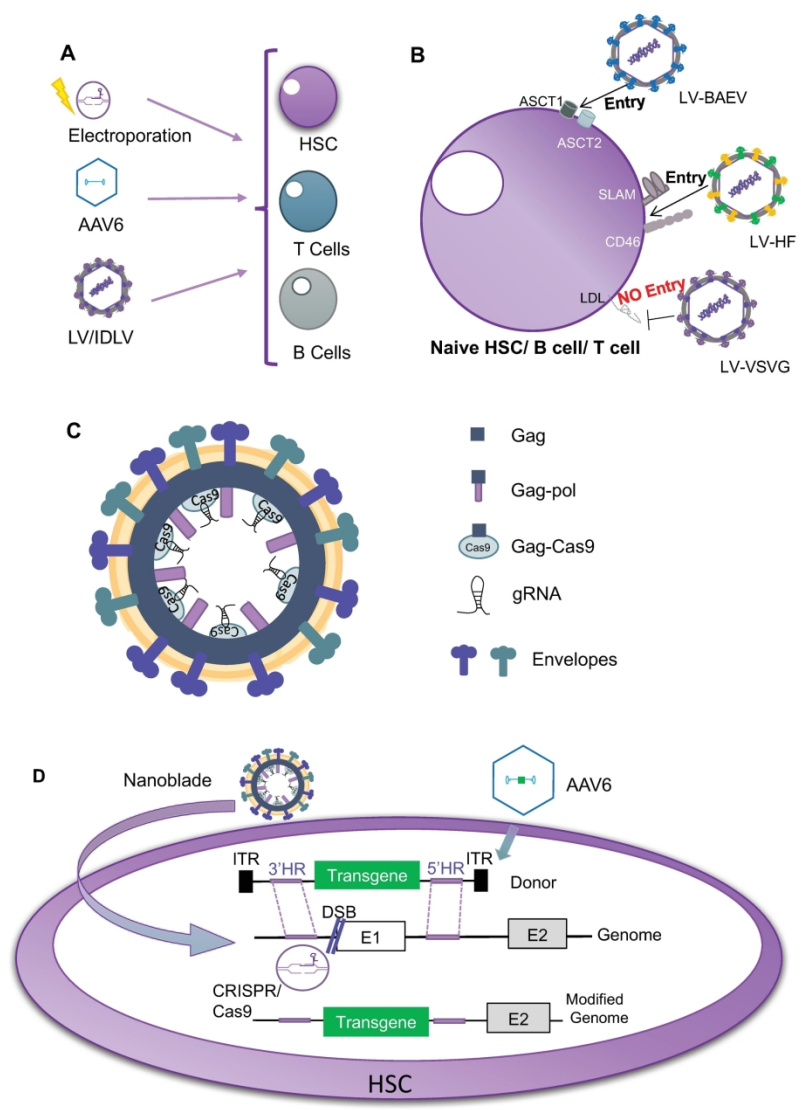


Figure 4

190x275mm (300 x 300 DPI)