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Crispr/Cas9 HR

CRISPR/Cas9 genome engineering in hematopoietic cells

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The development of genome editing tools capable of modifying specific genomic sequences with unprecedented accuracy has opened up a wide range of new possibilities in targeted gene manipulation. In particular, the CRISPR/Cas9 system, a repurposed prokaryotic adaptive immune system, has been widely adopted because of its unmatched simplicity and flexibility.
In this review we discuss achievements and current limitations of CRISPR/Cas9 genome editing in hematopoietic cells with special emphasis on its potential use in *ex vivo* gene therapy of monogenic blood disorders, HIV and cancer.

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Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) are defining components of the genomes of most bacteria and archaea and are part of their adaptive immune system defending against phage and plasmid DNA infection [1]. The most widely used CRISPR/Cas9 system of *Streptococcus pyogenes* (SpCas9/sgRNA) consists of three components: the CRISPR-associated DNA cleaving endonuclease Cas9 protein (~160 kDa, ~4.2 kb), a target DNA sequence-recognizing RNA transcribed from short DNA sequences known as protospacers (crRNA), and a trans-activating crRNA (tracrRNA) required for crRNA transcription [2,3].

For genome editing, fully functional fusions between crRNAs and tracrRNAs are used as single guide RNAs (sgRNA) for Cas9 targeting to pre-specified genomic sites [4]. For target identification, SpCas9 requires a trinucleotide sequence (*i.e.* 5′-NGG) located downstream of the sgRNA called protospacer adjacent motif (PAM), which determines the exact position of DNA binding [3,5]. Unlike other designer endonucleases such as Zinc Finger Nucleases and TALENs involving DNA recognizing protein domains, CRISPR/Cas9 targeting is based on RNA/DNA base-pairing, thus circumventing the laborious design and engineering of target specific proteins consisting of concatenated proteins whose specificity is dependent on domain interactions that are difficult to predict. Overall, CRISPR/Cas9 RNA-guided nucleases (RGNs) are easy to make even by laboratories not specialized in genome engineering.

Like all the other genome editing systems, RGNs generate DNA double strand breaks (DSB) at their target sites which are repaired either by homologous recombination or by nonhomologous end-joining (NHEJ). While homology directed repair (HDR) requires a template and is precise, NHEJ re-ligates the DNA ends without requiring a template in an error prone process that is associated with random nucleotide **in**sertions and/or **del**etions (indels) [6,7]. Both mechanisms have been

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exploited for targeted gene knock-outs, gene replacements and *in situ* gene repair in hematopoietic cells [8–12].

Here we discuss CRISPR/Cas9 strategies employed for the treatment of inherited blood disorders, HIV and cancer by the *ex vivo* manipulation of hematopoietic stem and progenitor cells (HSPCs) or T-lymphocytes.

Ex vivo *genome editing*

For *ex vivo* genome editing HSPCs or T-lymphocytes are isolated from patients by apheresis and kept in tissue culture in the presence of dedicated cytokine cocktails stimulating proliferation and preventing differentiation. Following genetic manipulation, the engineered cells are reintroduced into the patients (Fig. 1). Overall, *ex vivo* gene therapy is a well-established procedure that has been implemented in numerous clinical trials [13–17].

One of the most challenging issues of the *ex vivo* approach is gene delivery to cultured hematopoietic cells. As most current gene delivery protocols do not employ drug selection, gene transduction methods need to be good enough to yield the minimum number of functionally reconstituted cells required for a therapeutic response, which varies by disease. In instances where positive selection for gene corrected cells occurs *in vivo* (*e.g.*, X-linked severe combined immunodeficiency (X-SCID), ADA-SCID, Wiscott–Aldrich syndrome (WAS)) [18–20] significantly fewer cells are required than for diseases without selection (chronic granulomatous disease (CGD), ß-thalassemia, *etc.*).

For successful genome editing all RGN components have to be present simultaneously in the target cell. Thus, sgRNA, Cas9 and the homologous recombination template (donor template) need to be delivered either as separate molecules or in combination using dedicated expression vectors. Typically, Cas9 and sgRNA are combined on either plasmid or viral expression vectors and delivered together with oligonucleotide or plasmid donor templates by electroporation and/or infection (Fig. 2) [21,22]. The most frequently employed viral expression vectors are non-integrating integrase-deficient lentiviral vectors (IDLVs) [23,24] and adeno-associated virus vectors (AAVs) [9], both expressing RGNs only transiently in target cells. This substantially reduces genotoxic and off-target effects caused by integrating vectors and sustained RGN expression. More recently, highly efficient cell transduction protocols have been developed enabling virus free delivery of Cas9 as mRNA [10,11] or protein or as pre-assembled Cas9/sgRNA ribonucleoprotein particles (RNPs) along with oligonucleotide donor templates by electroporation [9,11,27,28]. In particular, the RNP/oligonucleotide strategy appears quite promising as it shortens the cell's exposure to Cas9 and thus reduces the frequency of off-target effects without affecting on-target mutation rates.

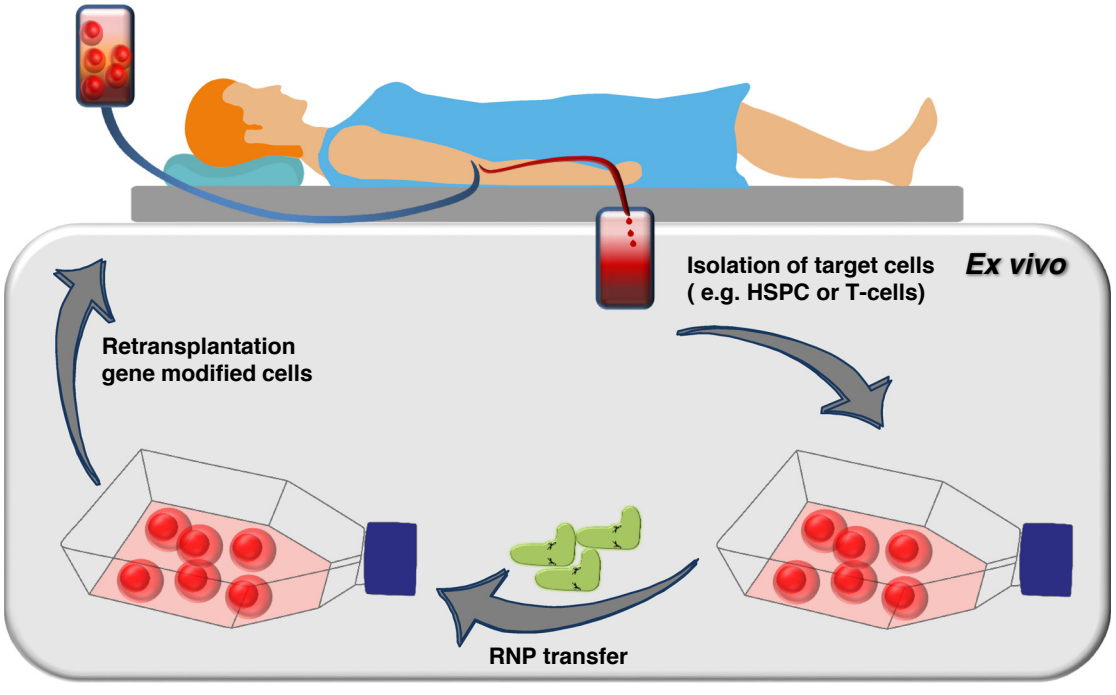

Fig. 1. *Ex-vivo* gene therapy. Hematopoietic cells isolated from patients are transduced in tissue culture with the desired RGNs and reintroduced into the patient usually without any selection. HSPC, hematopoietic stem and progenitor cells; RNP, ribonucleoprotein.

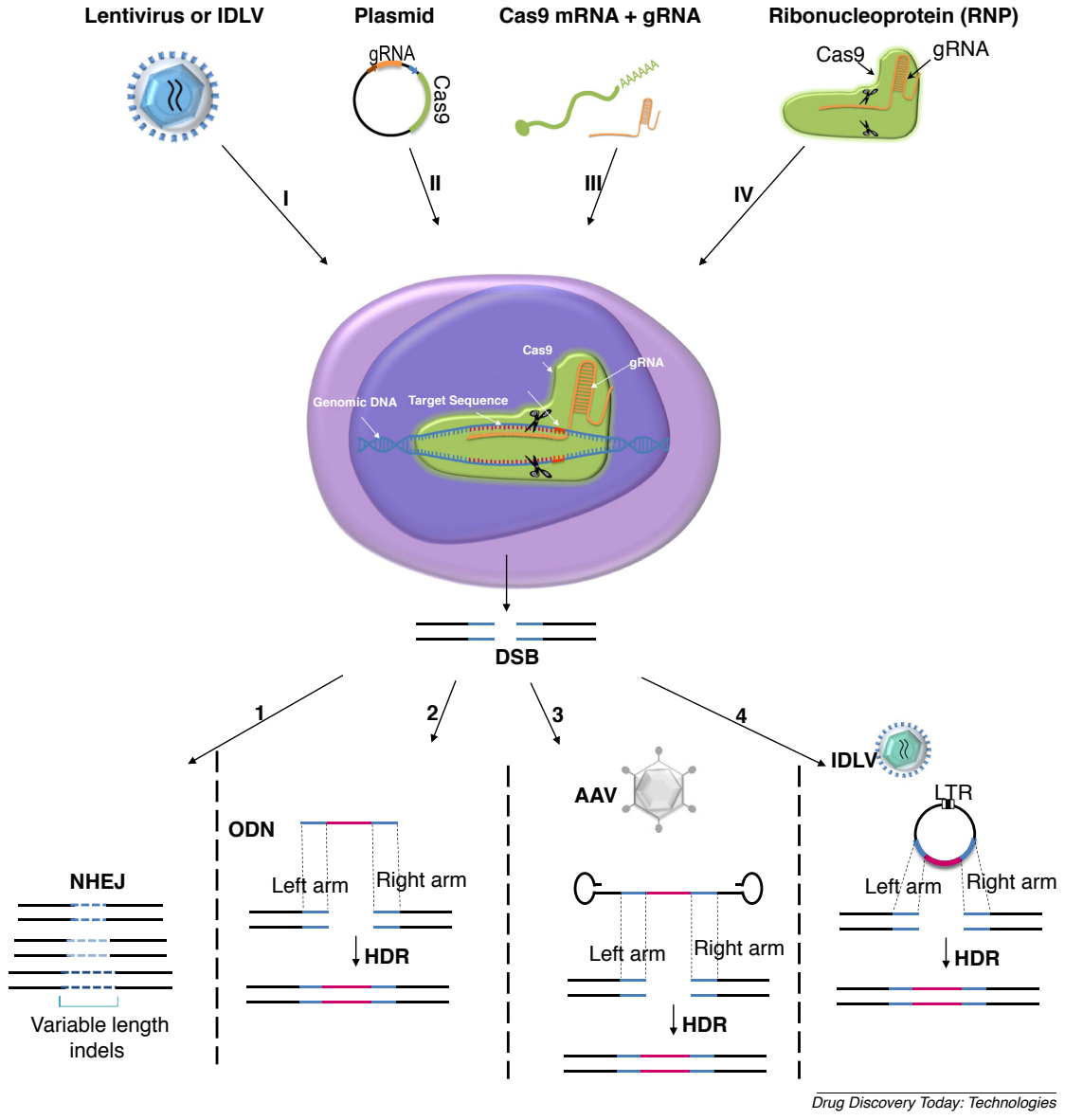

Fig. 2. CRISPR/Cas9 for genome editing and homology-directed repair in primary HSPCs. Cas9 and sgRNAs can be delivered into cells *in vitro via* lentiviral transduction or plasmid-, mRNA + sgRNA- or RNP-based electroporation. After Cas9/sgRNA delivery, Cas9 is directed to the target site by the sgRNA *via* its specific base-pairing with the targeted DNA sequence they can bind to the target site and cleaved into DNA and generates DSBs. DSBs at the target site are subsequently repaired by HDR or NHEJ. gRNA, guide RNA; IDLV, integration defective lentivirus vector; DSB, double strand break; AAV, andenovirus associated virus; HDR, homology directed repair.

Gene repair in HSPC

Over 250 hereditary monogenic disorders affecting the hematopoietic system [33] are caused by unique inactivating mutations dispersed over the entire locus of the affected genes. Most of these mutations affect the patient's immune system, resulting in increased susceptibility to infections, allergens, autoimmune diseases or cancer. Although *ex vivo* gene therapy based on gene replacement proved successful in several clinical trials, insertional mutagenesis and unregulated transgene expression remain a major concern. These problems can be largely circumvented by genome editing using appropriate RGNs [17,34]. For example, patient specific mutations causing SCID, CGD, sickle cell anemia (SCD) or ß-thalassemia could be corrected *ex vivo* by exploiting the HDR pathway [10,27–29,32]. Alternatively, delivery of donor constructs containing a healthy copy of the affected gene along with dedicated RGNs enable precise insertion of the healthy gene into the endogenous locus [9,10]. In both instances and unlike random gene addition, gene expression remains under endogenous control. While RGN delivery to HPSCs is still not optimal and requires improvement, for most monogenic blood disorders wild type protein restoration in about 10% of the physiologically relevant cells is sufficient for a therapeutic effect, suggesting that even suboptimal *ex vivo* HSPC

reconstitution can be successful [35]. Moreover, for immunodeficiency diseases where positive selection of gene repaired HSPCs occurs (*e.g.*, X-SCID, adenosine deamidase (ADA)-SCID, WAS) [18–20], less than 10% of corrected cells sufficed for immune system reconstitution [36]. Table 1 lists the diseases for which RGN targeted homologous recombination was successful in preclinical experiments.

However, gene correction in HSPCs by RGN-induced HDR requires HSPCs to be in active cell cycle. As most primitive hematopoietic stem cells are quiescent, cell cycling needs to be induced in order to exploit this pathway. Although the cytokine cocktails used in *ex vivo* cultures promote cell cycling, they also induce differentiation and thus reduce the number of self-renewing stem cells required for hematopoietic regeneration. Therefore, for some applications it might be useful to exploit the NHEJ pathway because it is several times more efficient than HDR [37] and, unlike HDR, operates in all phases of the cell cycle [38,39] thereby rendering even quiescent cells amenable to gene repair. Moreover, because NHEJ does not require donor templates it simplifies RGN delivery and circumvents insertional mutations induced by randomly integrating donor templates. We recently found that transient delivery of RGNs by IDLVs can effectively correct frameshift mutations by frameshift restoring indels [40]. Considering that about 25% of all mutations causing immunodeficiency diseases are frameshift mutations, a substantial number of patients could benefit from this approach [41–43].

The first monogenic blood disease to which CRISPR/Cas9 has been applied in patients is SCD (ClinicalTrials.gov Identifier: NCT03167450) [44]. SCD is an attractive CRISPR/Cas9 target because it is caused by a single missense mutation in the ß-globin gene (D6V) which is identical in each patient, and can therefore be treated by generic RGNs. A recent application for a phase I/II clinical trial using CRISPR/Cas9 SCD treatment (CTX001) has been submitted by CRISPR Therapeutics to the European Medicines Agency (EMA).

Gene knock-outs in HSPCs and T-cells

CRISPR/Cas technology has been widely used for gene knockouts in a large variety of cells and organisms [45]. Unlike gene repair, RGN-mediated knockouts rely entirely on DSB repair by NHEJ, whose associated indels effectively disrupt their targets. Major therapeutic applications include the human immunodeficiency virus (HIV) and cancer. Both involve specific *ex vivo* gene inactivation in T-lymphocytes.

HIV/Aids

Although effective combined antiretroviral (cART) therapies are available and proved largely successful in controlling the HIV infection, they do not eliminate the virus and therefore require lifelong application. Moreover, cART resistant variants frequently arise, requiring cART modifications that are often associated with severe adverse effects [46]. Therefore, curative approaches are urgently required; CRISPR/Cas9 mediated inactivation of the CCR5 receptor in $CD4^+$ T-cells currently appears to be the most promising. CCR5 is required for HIV entry into their natural CD4 cell hosts [47]. Accordingly, individuals with CCR5 mutations are protected from HIV infection [48], as was first demonstrated in the so called ''Berlin'' patient whose HIV infection was eradicated by a bone marrow transplant from an HLA-matched donor with a 32 bp deletion in *CCR5* [47,49]. Subsequent studies showed that CCR5 can be effectively knocked out in HSPCs by CRISPR/Cas9 technology and that the *ex vivo* manipulated HSPCs can readily engraft and protect immunodeficient mice from HIV infection [50,51]. The first clinical trial evaluating this strategy in HIV patients is currently underway (ClinicalTrials.gov Identifier: NCT03164135).

Additional studies targeting the CCR5 by RGNs in T cells have also been successful [52–55]. Although the strategy is more straightforward because T cells are much easier to grow and manipulate in tissue culture than HSPCs, it is unlikely to eradicate the disease because latent HIV reservoirs persisting in HSPCs [56] continue to be a source of infection for their own CD4 cell progeny as well as for re-infused CD4 cells that initially escaped RGN transduction. Similar experiments showed that inactivation of the CCR5 co-receptor – CXCR4 – can also block virus entry into CD4 cells [53]. Finally, RGN inactivation of HIV proviruses was reported as an alternative approach, although the emergence of escape mutants in the long run could lessen its curative potential [57].

Cancer

In addition to HIV treatment, CRISPR/Cas9 gene knockouts in T-cells combined with the expression of chimeric antigen receptors (CAR) are revolutionizing cancer immunotherapy. Most approaches involve the inactivation of the programmed

Table 1. Examples of applications of genome editing to therapeutic model disease.

Disease	sgRNA and Cas9 delivery	Donor delivery	References
X-linked chronic granulomatous disease (X-CGD)	Cas9 mRNA and sgRNA electroporation	ssODN electroporation	[29]
X-linked severe combined immunodeficiency (X-SCID)	Cas9 protein/mRNA and sgRNA (RNA/RNP) electroporation	Plasmid electroporation	[10]
	Cas9 protein and sgRNA (RNP) electroporation	AAV and IDLV transduction	[32]
Sickle cell disease (SCD)	Cas9 protein and sgRNA (RNP) electroporation	ssODN electroporation	[28]
Sickle cell disease (SCD) and β-thalassaemia	Cas9 protein and sgRNA (RNP) electroporation	AAV transduction	[27]

death-1 (PD-1) receptor in T-cells [52,58–62]. PD-1 regulates the anti-cancer cell immune response and is inhibited by receptor blocking ligands produced by cancer cells to evade an immune response. Several recent studies showed that targeting PD-1 with dedicated RGNs significantly increases the abundance of anti-tumor cytotoxic T cells (CTLs) in both solid and hematologic cancers [58,59,61]. A phase 1 clinical trial based on this strategy was initiated in 2016 in China with non-small cell lung cancer patients (ClinicalTrials.gov Identifier: NCT02793856) [63]. Further ongoing trials include patients with bladder, prostate and renal cancer (ClinicalTrials.gov Identifier: NCT02863913, NCT02867345 and NCT02867332), and Epstein–Barr-Virus (EBV) associated cancers (NCT03044743).

Presently, T-lymphocyte activity enhancing CRISPR/Cas9 knock outs of PD-1, MHC-I associated beta-2 microglobulin (B2M), lymphocyte activation gene-3 (LAG-3) and of the endogenous T-cell receptor are used in combination with lentivirally transduced CARs in the treatment of leukemias and lymphomas (NCT03166878, NCT03398967 and NCT03399448) [52,62,66,67]. Finally, several more recent improvements of the CAR-T cell approach includes RGN knock out of CD33 in HSPCs, which decreases overall toxicity [64], and the RGN knock-out of CD7 in anti-CD7-CAR-T cells, which enhances anti-tumor activity [65].

CRISPR/Cas9 adverse effects

In the most widely used SpCas9 system, sgRNAs targeting a gene of interest typically consist of a 20 nt protospacer element complementary to the target DNA upstream of an NGG PAM [3,5]. While effectively recognizing the pre-specified targets, most sgRNAs also recognize off-targets displaying one or more nucleotide mismatches, resulting in off-target mutations. These can cause disease by disrupting the function of essential genes. Despite numerous online tools assisting the design of sgRNAs devoid of off-target effects, off-target mutations with the SpCas9 system cannot be entirely avoided and pose potential safety problems for clinical applications. Therefore, much work has been invested in CRSPR/Cas9 systems to minimize or even eliminate off-target effects.

For example, by introducing a point mutation (D10A) into the catalytic domain of Cas9, its DSB activity was converted into inducing single strand nicks [68,69]. To generate DSBs, this modified Cas9 nuclease (Cas9 nickase) requires two sgRNAs, each of which targets the nickase to prespecified sequences on opposite strands. While still effectively generating on-target DSBs, the strategy reduces off-target activity by 50–1000 fold because single nicks are repaired by high-fidelity base excision (BER) and not by NHEJ [68,69].

Other strategies such as fusing mutationally inactivated Cas9 (dead Cas, dCas) to Fok1 nuclease [21,70], small molecule or light inducible Cas9 [37,71,72], or the development of mutagenized high-fidelity Cas9 versions [73,74] were also quite effective in reducing off-target mutations [28].

However, the frequency of RGN induced off target mutations is not only influenced by the target sequence but also by the cell type subjected to gene editing. In HSPCs the off target mutation rate was found to be extremely low suggesting that the probability of adverse effects caused by *ex vivo* genome editing is rather low [50].

Conclusions and future perspectives

The application of the CRISPR/Cas9 system to hematopoietic cells has enormous therapeutic potential. Unlike classic gene replacement therapies performed with randomly integrating vectors, CRISPR/Cas9 enables *in situ* gene repair largely circumventing side effects induced by insertional mutations and/or inadequate exogenous control of gene expression. Moreover, the highly efficient gene targeting surpasses classical gene targeting by homologous recombination by several orders of magnitude, thus providing an unprecedented tool for knocking out genes involved in the pathogenesis of various diseases. Although off-target effects are still a matter of concern, the benefit-to-harm ratio of the CRISPR/Cas9 system is significantly higher than that of classical gene therapy relying on randomly integrating retroviral or transposon vectors.

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