

Genome editing: A perspective on the application of CRISPR/Cas9 to study human diseases (Review)

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Received November 25, 2017; Accepted August 1, 2018

DOI: 10.3892/ijmm.2019.4112

Abstract. Genome editing reemerged in 2012 with the development of CRISPR/Cas9 technology, which is a genetic manipulation tool derived from the defense system of certain bacteria against viruses and plasmids. This method is easy to apply and has been used in a wide variety of experimental models, including cell lines, laboratory animals, plants, and even in human clinical trials. The CRISPR/Cas9 system consists of directing the Cas9 nuclease to create a site-directed double-strand DNA break using a small RNA molecule as a guide. A process that allows a permanent modification of the genomic target sequence can repair the damage caused to DNA. In the present study, the basic principles of the CRISPR/Cas9 system are reviewed, as well as the strategies and modifications of the enzyme Cas9 to eliminate the off-target cuts, and the different applications of CRISPR/Cas9 as a system for visualization and gene expression activation or suppression. In addition, the review emphasizes on the potential application of this system in the treatment of different diseases, such as pulmonary, gastrointestinal, hematologic, immune system, viral, autoimmune and inflammatory diseases, and cancer.

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1. Introduction

In order to determine the function of a gene, the gene can be inactivated by homologous recombination or by blocking its messenger RNA through RNA interference (1). This approach can be applied in cultured cells by transfection or in living organisms by transgenesis (1). Recent advances in genome editing allow the manipulation of any gene at its own locus in a broad variety of species and tissues, including cultured cells and animal organs. Genome editing is a powerful tool for biomedical research and provides hope for correcting some inherited diseases.

Genome editing is based on the use of highly specific and programmable nucleases, which produce specific changes in regions of interest in the genome by introducing double-strand breaks (DSBs) that are later repaired by cellular mechanisms. These repair mechanisms include the non-homologous end-joining (NHEJ) that is prone to error, and the homology-directed repair (HDR) that is error-free (Fig. 1). Repair permits the generation of insertions, deletions or substitutions in the target area (2-4). These mutations may interrupt, eliminate or correct the defects in genes. The latter possibility affords the ability to correct inherent errors in DNA that cause diseases. The 'programmable' nucleases are mainly meganucleases (5), zinc-finger nucleases (6), transcription activator-like effector nucleases (7), and the CRISPR/Cas9 system [involving the Clustered Regularly Interspaced Short Palindromic Repeats and nuclease(s) associated to the CRISPR locus] (8). Usually, the CRISPR/Cas9 system is comprised of a

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Key words: CRISPR/Cas9, genome editing, disease models, pulmonary disease, gastrointestinal disease, hematologic disease, viral disease, cancer, autoimmune disease, inflammatory disease

guide RNA (gRNA) that directs the Cas9 nuclease to create a DSB in a specific place of the genome. In the last decade, this system has gained wide acceptance over other systems due to its simplicity, speed and efficiency for modifying endogenous genes in any cell or target tissue, even in the most traditionally difficult-to-treat organisms.

2. CRISPR/Cas9 system

Discovery of the CRISPR/Cas9 system. The eubacteria and archaea possess a defense system that adapts, through RNA, to recognize and destroy external DNA and RNA. This provides acquired immunity against invading plasmids and viruses (9). This system is found in approximately 50% of the bacterial genomes that are sequenced and in 87% of the genomes of archaea (9-12). It is also important in a range of additional functions, including replicon divisions (13), high-temperature adaptation (14), chromosomal rearrangements (15) and DNA repair (16).

CRISPR was discovered in the *Escherichia coli* genome in 1987 as a series of repeated fragments of 29 nucleotides (nt) in length interspaced with variable sequence fragments of 32 nt (17). Interest in the CRISPR system and its associated Cas genes led to the discovery of similar short-repeat palindromic sequences of 24-40 nt in several groups of bacteria and archaea. The repeat sequences are separated by unique variable sequences of 20-58 nt (13,18). The associated genes (Cas) were identified invariably adjacent to a CRISPR locus, suggesting a functional association (19). The initial hypothesis regarding the function of the CRISPR locus proposed roles in cellular DNA repair and replicon partitioning processes; however, in 2005, the first evidence that the CRISPR/Cas system is part of an adaptive prokaryotic immune system was reported through the observation that the majority of the sequences intercalated between the identical repeats were derived from invading phage and plasmid genomes (20-22). In 2007, the incorporation of new spacers was demonstrated in a CRISPR/Cas locus of *Streptococcus thermophilus* (23), while the CRISPR transcription processing to small mature CRISPR-RNAs (crRNAs) that guide the Cas complex of *Escherichia coli* was validated experimentally in 2008 (24). In 2010, Cas of *Streptococcus thermophilus* was demonstrated to create a single DSB at a precise position in target DNA (25), and the following year it was reported that the maturation of crRNA requires trans-encoded small crRNA (tracrRNA), Cas9 and an RNase III in *Streptococcus pyogenes* (26). Evidence of function in a heterologous system was obtained in 2011 when it was shown that the CRISPR/Cas system of *Streptococcus thermophilus* on transfer to *Escherichia coli* provided heterologous immunity against plasmids and phage infection (27). In 2012, the simplification of the CRISPR/Cas9 of *Streptococcus pyogenes* system was achieved by replacing a tracrRNA and a crRNA with a synthetic single gRNA to direct Cas9 to its target and to perform the cleavage (8). Finally, in 2013, the use of the CRISPR/Cas9 system (type II, *Streptococcus pyogenes*) was described as a genome editing tool for the induction of site-specific DSBs and subsequent mutagenesis in plant, mouse and human cells, and clinical trials (28-32).

Operation of the CRISPR/Cas9 system as a genome editing tool. The type II CRISPR/Cas system is the most commonly

used system for genome editing, using the well-characterized Cas9 endonuclease of *Streptococcus pyogenes*. In the endogenous system, the mature crRNA joins with a tracrRNA (small RNA that is complementary to the CRISPR sequences) to form a tracrRNA:crRNA complex, which guides the Cas9 to a target site. Thus, CRISPR/Cas9 performs sequence-specific cleavage by simple interaction of crRNA by base pairing at the target site. After joining the target site, the two DNA strands are cleaved by the nuclease domain of Cas9, a HNH domain that cleaves the complementary target strand to the gRNA and a RuvC-like nuclease domain, which cleaves the non-target strand (33-35). The gRNA designed by Jinek *et al.* (8) in 2012 was a chimerical RNA, which contains all the essential components of crRNA and tracrRNA to guide Cas9. Since then, multiple variants of CRISPR/Cas9 have been developed, which recognize sequences of 18-24 nt of the gRNA, and 2-4 nt of protospacer adjacent motif (PAM) in target sites (3,36). Therefore, CRISPR/Cas9 can theoretically be directed to a specific sequence of DNA of 22-29 nt, which is unique in most of the genomes, although it has been noted that CRISPR/Cas9 has a high-tolerance for non-specific mating of base pairs between gRNA and its complementary target sequence. This specificity is sensitive to numbers, position and distribution of wrong interactions (3,8,28,29). For instance, the CRISPR/Cas9 of *Streptococcus pyogenes* (SpCas9) tolerates up to six imbalances of base pairs at target sites (8). The genome editing mediated by CRISPR/Cas9 depends on the generation of the DSB and the subsequent process of DNA repair. The DSB generated by the CRISPR/Cas9 triggers the process of cell repair in DNA, as a NHEJ, which is prone to error and thus can produce mutations involving small insertions and deletions (indels) in target sites, which can interrupt or eliminate the function of the genes or the genomic target elements (such as regulatory regions). Another repair process that can also be triggered is the HDR error-free, which can potentially correct innate disease-causing errors of DNA (genes or regulatory elements) (37).

PAM sequence and off-target cuts. The specificity of CRISPR/Cas9, besides the complementarity of the gRNA/target sequences, requires a PAM sequence that is located immediately after the target sequence. The reliance on the PAM sequence for the cleavage of the DNA restricts the frequency of the cleavage sites in the genomes, thus target sites are found more frequently for small PAM sequences than for longer ones; consequently off-target cut sites are less likely to exist for long PAM sequences than for short ones. The identified PAM sequences vary between different microorganisms, and the following sequences have been reported: 5'-NGG-3' in *Streptococcus pyogenes* (SpCas9) (8), 5'-NGG-3' or 5'-NNAGAAW-3' in *Streptococcus thermophilus* (St1Cas9) (25,38,39), 5'-NNGRRT-3' or 5'-NNGRR(N)-3' in *Staphylococcus aureus* (SaCas9) (40,41), 5'-NNRRRT-3' or 5'-NNNNGMTT-3' in *Neisseria meningitidis* (NmCas9) (42), and 5'-NGG-3' in *Francisella novivida* (FnCpf1) (43,44), where N refers to every nucleotide, R to purines A or G, M to nucleotide A or C, and W to weak bonds A or T (3,11,36,45).

Although the DSB activity of CRISPR/Cas9 is based on the complementarity of target sequences with the gRNAs of ~20 nt in length, the system allows cleavage at genomic

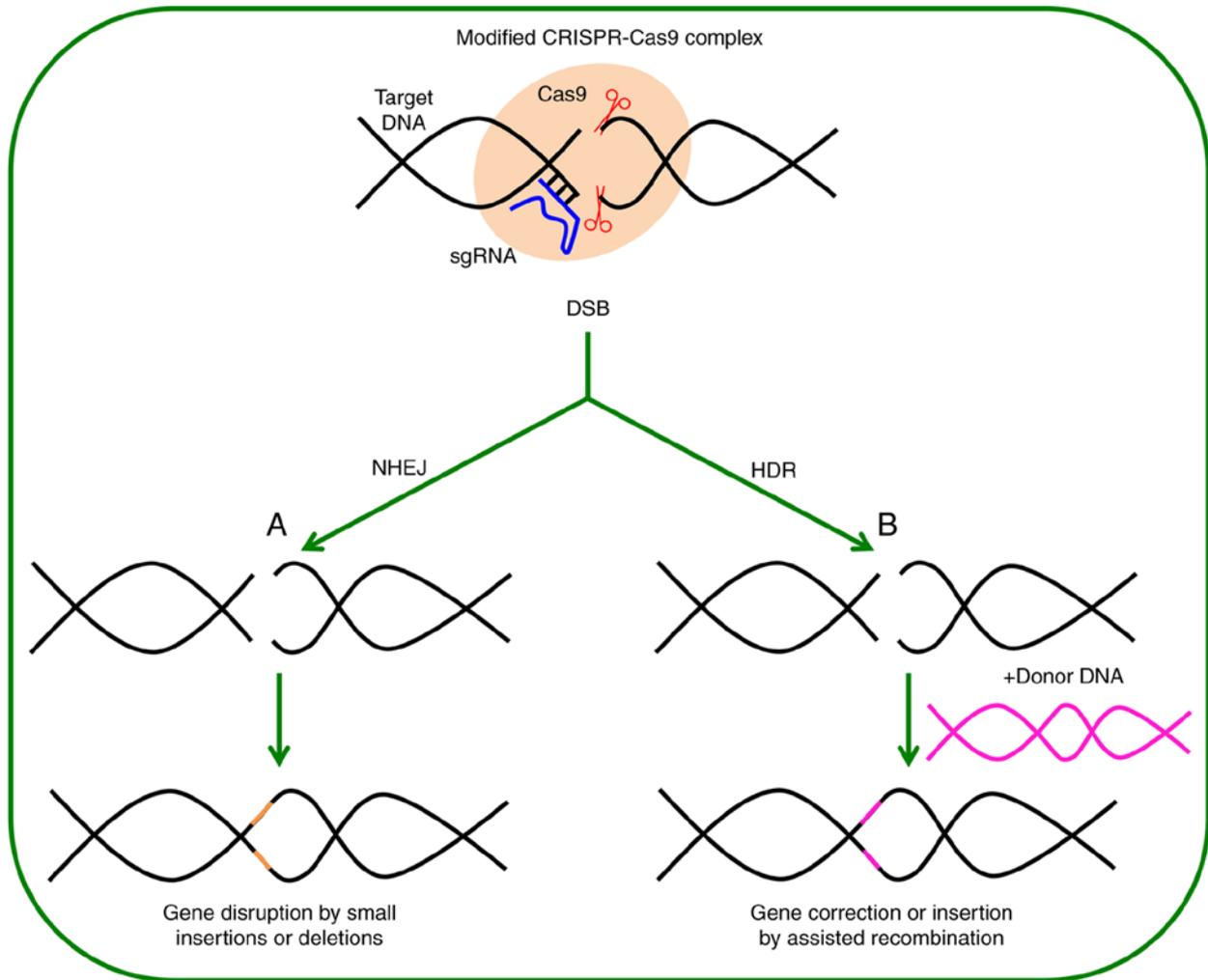


Figure 1. Genome editing through the CRISPR/Cas9 system. Cas9 and the gRNA create a complex that binds with the DNA close to the PAM site. A DSB is generated in the target site that could be repaired via NHEJ or HDR. (A) The repair by NHEJ usually results in insertions or deletions, or in frameshift that causes the gene knockout by interruption. (B) If a DNA donor with homology in the ends is provided, this DNA can be inserted to the target site to modify the gene, introducing the nucleotides and leading to frameshifts or insertion of cDNA. gRNA, guide RNA; PAM, protospacer adjacent motif; DSB, double-strand break; NHEJ, non-homologous end joining; HDR, homology-directed repair.

locations partially complementary to the gRNA, because the gRNA allows mismatch pairings between the DNA and gRNA (3). These off-target cuts of CRISPR/Cas9 are one of the biggest issues that currently remain unresolved (causing certain undesirable consequences) and differ between different target sites due to the diversity of nucleotide sequence and the genomic context. The CRISPR/Cas9 system has been implemented successfully for gene editing, and for the control of different types of biological systems; however, there is little evidence of the consequences of off-target genome editing. Indeed, there are only a few assays of genotoxicity based on cells in culture that allow to quantify, stratify and help prevent biological side effects of gene editing in a given target cell population. Although a number of gene editing studies have reached the phase of clinical trial, clinical evidence showing that gene editing is truly capable of treating disorders is still scarce (30-32).

Several methods have been proposed to optimize gRNA design and minimize off-target cuts to reach the reliability and specificity necessary for safety in therapeutics applications (46). Identification of the optimal gRNA

among various candidates for a given target site, and the localization of potential off-target sites can be supported by different bioinformatics tools (47); for example: CRISPRdirect (48), E-CRISPR (49), WU-CRISPR (50), CRISPR gRNA design tool (<https://www.atum.bio/eCommerce/cas9/input>), sgRNA Designer (51), sgRNA Scorer 2.0 (52,53), CRISPRscan (54), CRISPR-ERA (55), CCTop (56), CRISPOR (57), Breaking-Cas (46), CHOPCHOP (58), CRISP MultiTarget (59), GT-Scan (60), ge-CRISPR (47), CRISPR Design (61), Cas-Designer (62), Cas-OFFinder (63), COSMID (64), DESKGEN Guide Picker (65), and CRISPR Genome Analyzer (66) (Table I). These software programs have different characteristics and applications. The optimal choice will depend on the type of organism (eukaryotic or prokaryotic) for which the gRNA is designed, the variety of PAM to be used, the size of the DNA target, and the variety of recently-reported Cas-like nucleases (46).

Strategies and modifications of the enzyme Cas9 to minimize off-target cuts. The design of variations in the traditional SpCas9 system has been examined for controlling its

performance, such as using an inducible system for temporary expression of the nuclease (67). Another strategy to decrease activity off-target is to replace the SpCas9 enzyme for mutant nSpCas9 (nCas9), which cuts a single strand through the inactivation of a nuclease domain RuvC or HNH. In this case, in order to perform the DSB, two nCas9 are needed to target opposite strands of DNA in close proximity (separated by no more than 100 bp), with each nCas9 guided by its own sgRNA that is able to cleave only the strand complementary to the sgRNA (45,68). When two nCas9 are used to perform a DSB, off-target activity is reduced by 50 to 1,500 times (40,45,69,70). Furthermore, another strategy consists in increasing the specificity by mitigating the helicase activity (eSpCas9) to disrupt the off-target sites without interfering with specificity and activity (71). Fusing the nuclease FokI with dead SpCas9 (dSpCas9) also improves specificity; in this case, dSpCas9 lacks its cleavage activity by inactivation of its two-nuclease HNH and RuvC domains. Consequently, the fusion forms RNA-guided FokI nucleases, thus, the excision activity for the DSB will depend only on the bounds of the two gRNAs to the DNA with a well-defined spacing and orientation, allowing the dimerization of monomers FokI-dSpCas9 to form a catalytically active FokI dimer, which reduces the possibility that a suitable target sequence appears again in the genome, and therefore improves specificity (72-74). Another modification is the binary system Split-SpCas9 that uses the expression the nuclease lobe and the α -helical domain independently. These two are naturally in the enzyme Cas9 alone or attached to the DNA through gRNA. The domains do not interact on their own, but the gRNA recruits all of them in a ternary complex that recaps the activity of Cas9 and catalyzes site-specific DNA cleavage. The uses of modified gRNA annul the Cas9 activity dividing the dimer, which allows the development of an inducible and adjustable dimerization system for genome editing applications. This system has been tested *in vitro* and *in vivo* in a mouse model (75,76). Among other modifications to date, the SpCas9-cytidine deaminase can be found, which is a fusion of dSpCas9 with the cytidine deaminase enzyme. This action by cytidine deaminases, converts cytosine (C) to uracil (U), working on single-stranded DNA accessible in the ternary complex between Cas9, gRNA and the target DNA for the introduction of point mutations (77).

New variants of system CRISPR. Studies have identified other enzymes of the CRISPR family, including enzymes encoded by *Cas* genes smaller than *SpCas9* (4.2 kb), such as *SaCas9*, *StlCas9*, and *NmCas9* (3.2, 3.4, and 3.2 kb, respectively) (41,45). These enzymes would facilitate its packaging into viral vectors (41,45). Another recently identified enzyme called Cpf1 with shorter crRNA sequences can be used instead of SpCas9 (43,78). Also, the most recently discovered C2c2 (Cas13a) and C2c6 (Cas13b), which can cleave RNA (78-82). Cas13b has already been developed as an RNA base editing technology, having been used as a tool for RNA editing using catalytically-inactive Cas13b fused to the adenosine deaminase domain of ADAR2 for programmable adenosine to inosine replacement in transcripts (82).

Other applications of CRISPR/Cas9. Besides the use of CRISPR/Cas9 to generate indels (Fig. 2), modified versions

have been developed for different applications, such as activation or repression through regulation of gene expressions by fusing heterologous domains for modifying epigenetic signatures on histones (i.e. changing methylation patterns) or transcriptional activators/repressors (83-85). Furthermore, CRISPR/Cas9 has been used for the selective labeling of the genome, such as for the monitoring of dynamic chromatin processes (86,87).

3. CRISPR/Cas9 in disease models

The CRISPR/Cas9 system has an extraordinary therapeutic potential for treating different diseases in which the genetic cause of dysfunction is known, or for the study of these diseases through the creation of cell or animal models. Therapy based on genome editing can lead to the restoration of gene function or compensation of the mutation. Single nucleotide polymorphism (SNP) editing has been approached with different strategies, such as: knocking out the gene that causes the disease (88), introducing a protective mutation (89) or adding a therapeutic transgene (90). When the disease is caused by a virus, cleavage of viral DNA can be performed (70,91-94). Fig. 3 shows certain of the strategies already mentioned and others that could be used to approach different diseases. The purpose of this figure is to expand the panorama of the therapeutic possibilities of CRISPR/Cas9. In the case that a mutation in a gene is difficult to repair due to its genomic context, there may be a pseudogene that could be activated to replace the mutated gene (95). However, if the cause of the disease is a protein that causes damage to the organism by its anomalous characteristics (such as by misfolding and accumulation in a tissue) (e.g. amyloidosis), its expression could be down regulated at several points in its pathway of expression (96).

CRISPR/Cas9 in pulmonary and gastrointestinal diseases. The homozygous $\Delta 508$ mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene was corrected using CRISPR/Cas9 *in vitro* in intestinal stem cells of cystic fibrosis patients (97). Edited stem cells were differentiated into intestinal organoids and exhibited a functional CFTR product (97). CRISPR/Cas9 also shows potential in cases requiring a liver transplant, such as drug therapy-refractory metabolic liver disorders (88,98). CRISPR has been used to suppress genes, thus reprogramming the metabolic pathway, achieving a benign phenotype with treatment of hereditary type I tyrosinemia in mice (88). By eliminating the 4-hydroxyphenylpyruvate dioxygenase (*HPD*) gene in order to inhibit the second step in tyrosine catabolism, the modified hepatocytes (*FAH^{-/-}/HPD^{-/-}*) exhibited a growth advantage over the unedited hepatocytes (*FAH^{-/-}/HPD^{+/+}*). In a number mice, the replacement was almost complete (92-99%) in 8 weeks. The *HPD* mutation increased tyrosine catabolism avoiding the pathologic effect of FAH deficiency and the accumulation of tyrosine and toxic metabolites, such as fumarylacetoacetate and succinylacetone in hepatocytes, which in turn results in severe liver damage with increased risk of hepatocarcinoma (88).

The CRISPR/Cas9 system can also be used as therapy in Hirschsprung disease and the megacystis-microcolon-intestinal hypoperistalsis syndrome (MMIHS), in which certain

Table I. Bioinformatics programs for the design of gRNA and search of off-target cuts.

| Name | Website | Available species | Use | |
|-------------------------|---|---|---|---|
| CRISPR direct | http://crispr.dbcls.jp/ | >200 | Design of gRNAs | Naito <i>et al</i> , 2015 |
| E-CRISPR | http://www.e-crisp.org/E-CRISP/ | 55 | Design of gRNAs | Heigwer <i>et al</i> , 2014 |
| WU-CRISPR | http://crispr.wustl.edu | 2 | Design of gRNAs | Wong <i>et al</i> , 2015 |
| CRISPR gRNA design tool | https://www.atum.bio/eCommerce/cas9/input | 5 | Design of gRNAs for genome editing for Cas9 | https://www.atum.bio/eCommerce/cas9/input |
| sgRNA Designer | https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design | 2 | Design of gRNAs for <i>S. aureus</i> and <i>S. pyogenes</i> Cas9 | Doench <i>et al</i> , 2014 |
| sgRNA Scorer 2.0 | http://crispr.med.harvard.edu/sgRNAScorerV2 | 2 | Design of gRNAs for Cas9s from <i>S. aureus</i> and <i>S. thermophilus</i> 3 and Cpf1 | Chari <i>et al</i> , 2015 and 2017 |
| CRISPRscan | http://www.crisprscan.org | 19 | Design of gRNAs for Cas9s and Cpf1 of <i>Acidaminococcus</i> and <i>Lachnospiraceae</i> | Moreno-Mateos <i>et al</i> , 2015 |
| CRISPR-ERA | http://CRISPR-ERA.stanford.edu | 9 | Design of gRNAs for genome editing, repression and activation | Liu <i>et al</i> , 2015 |
| CCtop | http://crispr.cos.uni-heidelberg.de | 50 | Design of gRNAs for Cas9s from <i>S. pyogenes</i> and its variants, <i>S. thermophilus</i> , <i>S. aureus</i> , <i>N. Meningitidis</i> , <i>Treponema denticula</i> , <i>Campylobacter jejuni</i> , and Cpf1 of <i>Acidaminococcus</i> and <i>Lachnospiraceae</i> | Stemmer <i>et al</i> , 2015 |
| CRISPOR | http://crispor.tefor.net | 191 | Design of gRNAs for Cas9s from <i>S. pyogenes</i> and its variants, <i>S. thermophilus</i> , <i>S. aureus</i> , <i>N. Meningitidis</i> , <i>Campylobacter jejuni</i> , and Cpf1 of <i>Acidaminococcus</i> and <i>Lachnospiraceae</i> | Haeussler <i>et al</i> , 2016 |
| Breaking-Cas | http://bioinfogp.cnb.csic.es/tools/breakingcas | Any eukaryotic genome available at Ensembl/Ensembl Genomes (>1,000 genomes) | To design gRNAs for Cas9s from <i>S. pyogenes</i> , <i>S. aureus</i> , Cpf1 of <i>Acidaminococcus sp.</i> and <i>Natronobacterium gregoryi</i> (No PAM needed). To evaluate putative undesired off-targets for CRISPR/Cas applications | Oliveros <i>et al</i> , 2016 |
| CHOPCHOP | http://chopchop.cbu.uib.no | 91 | Identifying gRNA targets Cas9 and its variants, and Cpf1 | Montague <i>et al</i> , 2014 |
| CRISP Multi Target | www.multicrispr.net | 12 | Identifying gRNA targets common to several similar sequences or unique to each of these sequences | Prykhozhij <i>et al</i> , 2015 |
| GT-Scan | http://gt-scan.csiro.au | >25 | Identifying unique genomic targets | O'Brien <i>et al</i> , 2014 |
| ge-CRISPR | http://bioinfo.imtech.res.in/manojk/gecrispr/ | 4 | Prediction and analysis of gRNAs genome editing efficiency | Kaur <i>et al</i> , 2016 |
| CRISPR Design | http://crispr.mit.edu | 15 | Selection and validation of gRNAs, as well as prediction of off-target loci for specificity analyses | Hsu <i>et al</i> , 2013 |
| Cas-Designer | http://www.rgenome.net/cas-designer/ | 33 | Design of gRNAs for user-defined PAM sequence with 10 different enzymes in a given DNA sequence. Identifying potential sites off-target using Cas-OF finder | Park <i>et al</i> , 2015 |

Table I. Continued.

| Name | Website | Available species | Use | |
|-------------------------|---|---|---|-----------------------------|
| Cas-OFFinder | http://www.rgenome.net/cas-offinder/ | Any given genome or user-provided sequence | Identifying potential off-target sites for given gRNAs | Bae <i>et al</i> , 2014 |
| COSMID | http://crispr.bme.gatech.edu | 7 | Identifying and validating CRISPR/Cas Off-target sites | Cradick <i>et al</i> , 2014 |
| DESKGEN Guide Picker | https://www.deskgen.com/guide-picker/ | 2 | Meta tool for designing CRISPR experiments | Hough <i>et al</i> , 2017 |
| CRISPR Genome Analyzer | http://crispr-ga.net | Any genomic locus of any organism for which the sequence is available | Assess the quality of gene editing using next gen sequencing data | Güell <i>et al</i> , 2014 |

gRNA, guide RNA; PAM, protospacer adjacent motif.

mutations were identified through complete exome sequencing in patients diagnosed with those diseases (99,100). In Hirschsprung disease, four genes (*DENND3*, *NCLN*, *NUP98* and *TBATA*) have been linked to the neuronal processes shared by the central nervous system and the enteric nervous system; this function was verified *in vivo* through a gene knockout by CRISPR/Cas9 (99,100). A zebrafish model was used to observe that the knockout of the afore mentioned genes resulted in the loss of function and interruption of the development of the enteric nervous system (loss of enteric neurons) causing a similar Hirschsprung phenotype *in vivo* (99). The *LMOD1* gene is involved in establishing normal smooth muscle cytoskeletal-contraction coupling, and a mutation causes MMIHS. Mice with knockout of *LMOD1* gene exhibited a similar phenotype to this syndrome (protein levels and pathology consistent with MMIHS), displaying beginning of bladder distention at 18.5 days of embryonic development. In certain cases, the developing bladder expanded until it encroached the abdominal cavity, while histological analysis detected early onset thinning of the detrusor muscle of the bladder of *Lmod1*^{-/-} mice. These results suggest a role for the *LMOD1* gene in establishing normal smooth muscle cytoskeletal-contraction coupling (100).

CRISPR/Cas9 in hematologic diseases. β -hemoglobinopathies, including sickle-cell disease (SCD) and β -thalassemia, are caused by mutations in the β -globin (*HBB*) gene, and affect millions of people worldwide. Several studies have used CRISPR/Cas9 and donor sequences to achieve homologous recombination in the *HBB* gene in induced pluripotent stem cells (iPSCs) and hematopoietic stem cells (HSCs) (101-104). For instance, efficient correction of the Glu6Val mutation responsible of the SCD was achieved using progenitor cells that were derived from patients and differentiated to erythrocytes, resulting in the expression of the mRNA of adult β -globin (HbA) and confirming the intact transcriptional regulation of modified *HBB* alleles (101,102). Another genome editing strategy to treat hemoglobinopathies is inserting a mutation to generate a benign genetic condition. In the hereditary persistence of fetal hemoglobin (HPFH), a benign

genetic condition, the mutations attenuate the shift of γ -globin to β -globin, causing a high level of fetal hemoglobin expression (HbF) throughout life, which can relieve the clinical signs of β -thalassemia or SCD. In a previous study, CRISPR/Cas9 was used to mimic the HPFH mutation in promoters of the *HBG1* and *HBG2* genes in human blood progenitor cells. The edited progenitor cells produced red blood cells (RBCs) with increased HbF levels that were sufficient to inhibit the pathological hypoxia of RBCs found in SCD (105). Another strategy applied to increase the HbF levels was CRISPR/Cas9 mutation of *BCL11A* erythroid enhancer, a validated repressor of HbF and therapeutic target for β -hemoglobin disorders (106). Furthermore, in β -thalassemia caused by the expression-suppression point mutations or deletions in the β -globin gene, effective correction of the *HBB* mutations by CRISPR/Cas9 was achieved in iPSCs cells derived from a patient, which differentiated in erythroblasts having restored the expression of β -hemoglobin (103,104).

Finally, CRISPR/Cas9 has been applied in the treatment of multiple other hematological diseases, such as alloimmune bleeding disorders, including fetal and neonatal alloimmune thrombocytopenia and post-transfusion purpura, to transform Leu33⁺ megakaryocyte-like DAMI cells and iPSCs to the Pro33 allotype, which is responsible for generating the human platelet alloantigen 1a and 1b epitopes (107). Furthermore, this technology has been used for treating Fanconi anemia by correcting point mutation in patient-derived fibroblasts (108), as well as in hemophilia for the restoration of factor VIII deficiency in mice (109-112). Notably, CRISPR/Cas9 has been used for the generation of mutant pigs in the *vWTF* gene to serve as an animal model for von Willebrand disease, or to facilitate bleeding prior to the meat processing in the meat industry (113).

CRISPR/Cas9 in viral diseases. Viruses are obligate intracellular pathogens that infect cells through specific receptors and depend on cellular components of the host for their replication. Upon entering the cell, the viral genome is reproduced, transcribed and translated to complete its life cycle (114). A number of genomes of DNA viruses and retroviruses are integrated

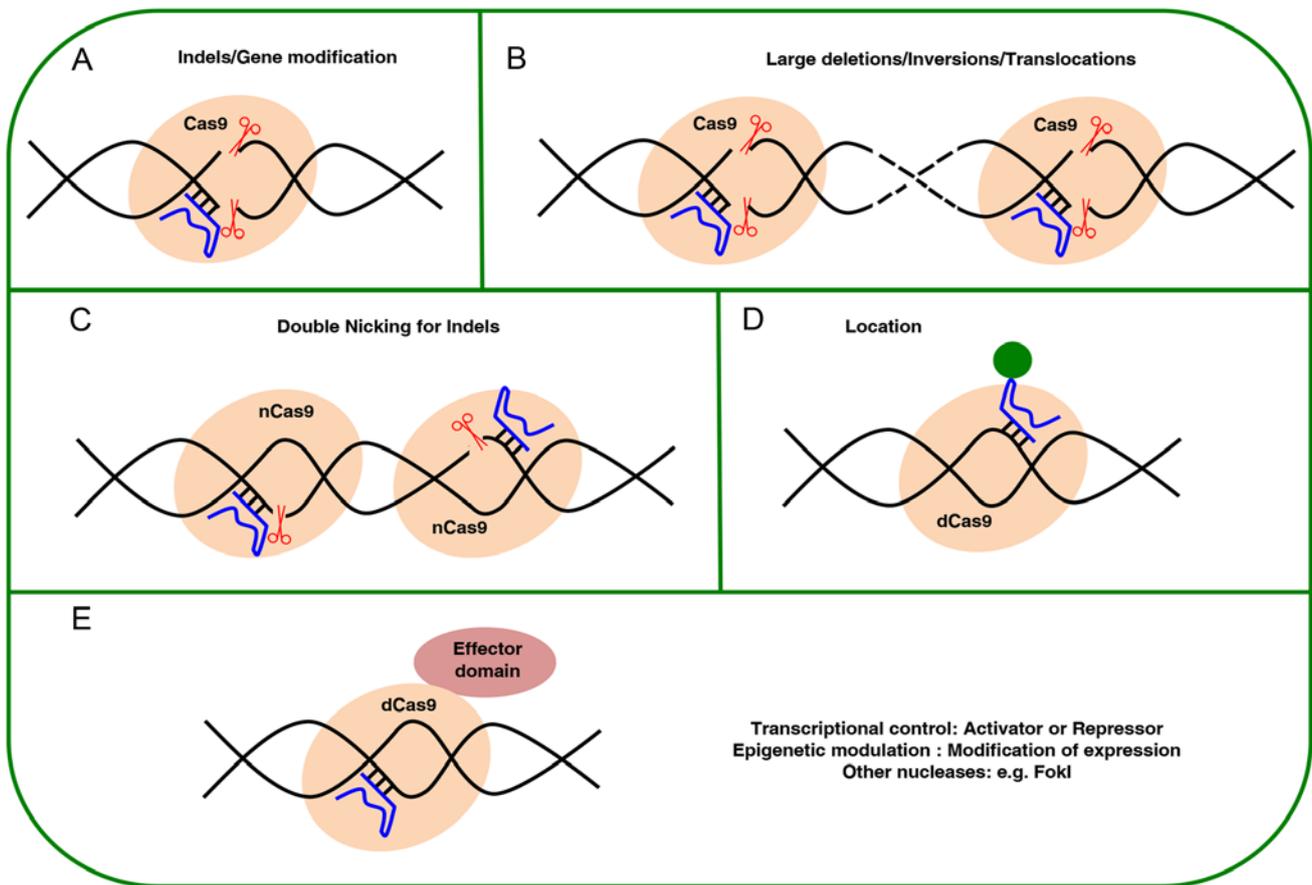


Figure 2. Overview of applications based on Cas9. (A) Nuclease Cas9 directed by a gRNA can induce insertion or deletion mutations; (B) a pair of Cas9 nucleases directed by a gRNA may induce sequence-specific replacement or insertion, large deletions or genomic rearrangements (such as inversions or translocations); (C) double cutting by nCas9 to improve the specificity of editing; (D) visualization of specific sites in the genome by dCas9; (E) dCas9 can mediate the regulation of specific endogenous genes by heterologous effector domains or performing histone modifications or DNA methylation. gRNA, guide RNA.

into the cellular genome. When a virus infects a human, it can cause severe disease with high mortality, morbidity and/or subsequent transmission to other people. Certain viral infections can be reduced by vaccination immunity, while this is not possible for others. Viral infections that require more attention due to their nature and social impact are those caused by the human papilloma virus (HPV), the human immunodeficiency virus (HIV) and the hepatitis B virus (HBV).

HPV16 expresses variants of the viral oncoproteins E6 and E7, which are tightly linked to the development and maintenance of malignant phenotypes that can result in cervical cancer (93), the second most frequent cause of cancer in women worldwide (92). CRISPR/Cas9 has been used alone or in combination with other treatments in *in vitro* and *in vivo* studies to combat HPV16 and HPV18 etiologic factors of cervical cancer (92,93,115). CRISPR-Cas9 targeting HPV16 and HPV18 oncogenes E6 and E7 in cervical carcinoma cell lines, including HeLa and SiHa cells, led to the arrest of the cell cycle and eventual death of the malignant cells (115). In another study, mutation of the HPV16 E6 and E7 viral oncogenes inhibited tumor growth *in vivo*, demonstrating that treatment with CRISPR/Cas9 works as a therapy with cisplatin, one of the first-line treatments most commonly used as a chemotherapeutic agent (93).

In HIV, the chemokine receptor 5 (CCR5) works as an essential co-receptor to HIV-1; thus, loss of CCR5 receptor

function protects against viral infection. CRISPR/Cas9 was used to edit the *CCR5* gene in CD4⁺ cells (116) and in human iPSCs (hiPSCs) cells (89). The mutant hiPSCs differentiated into macrophages that became resistant to trophic CCR5 HIV-1 virus (89).

The persistence of covalently closed circular DNA (cccDNA) of HBV is a major barrier to antiviral therapy for chronic hepatitis B eradication. A cure would require the elimination of persistent cccDNA or removal of the hepatocytic viral load (91). With specific gRNAs against the HBV in multiple studies, the CRISPR/Cas9 system significantly reduced the production of HBV core and surface proteins in Huh-7 (70,91), HepG2 (117), HepG2.2.15 (117,119) and HepG2-H1.3 cell lines (119), which were transfected with a HBV expression vector. Furthermore, in a mouse model, this system cleaved the intrahepatic plasmid containing the HBV genome and facilitated its clearance *in vivo*, resulting in a reduction of the serum antigen surface levels. This suggests that the CRISPR/Cas9 system was able to disrupt HBV both *in vitro* and *in vivo*, indicating its potential in the eradication of persistent infection by this virus (91,118,119). CRISPR/Cas9 has not only been used in the treatment of this virus, but also in the study of cellular mechanisms that lead to carcinogenesis (120).

In the case of hepatitis C virus (HCV) that causes chronic hepatitis, liver cirrhosis and hepatocellular carcinomas, CRISPR/Cas9 has been used in the identification of critical

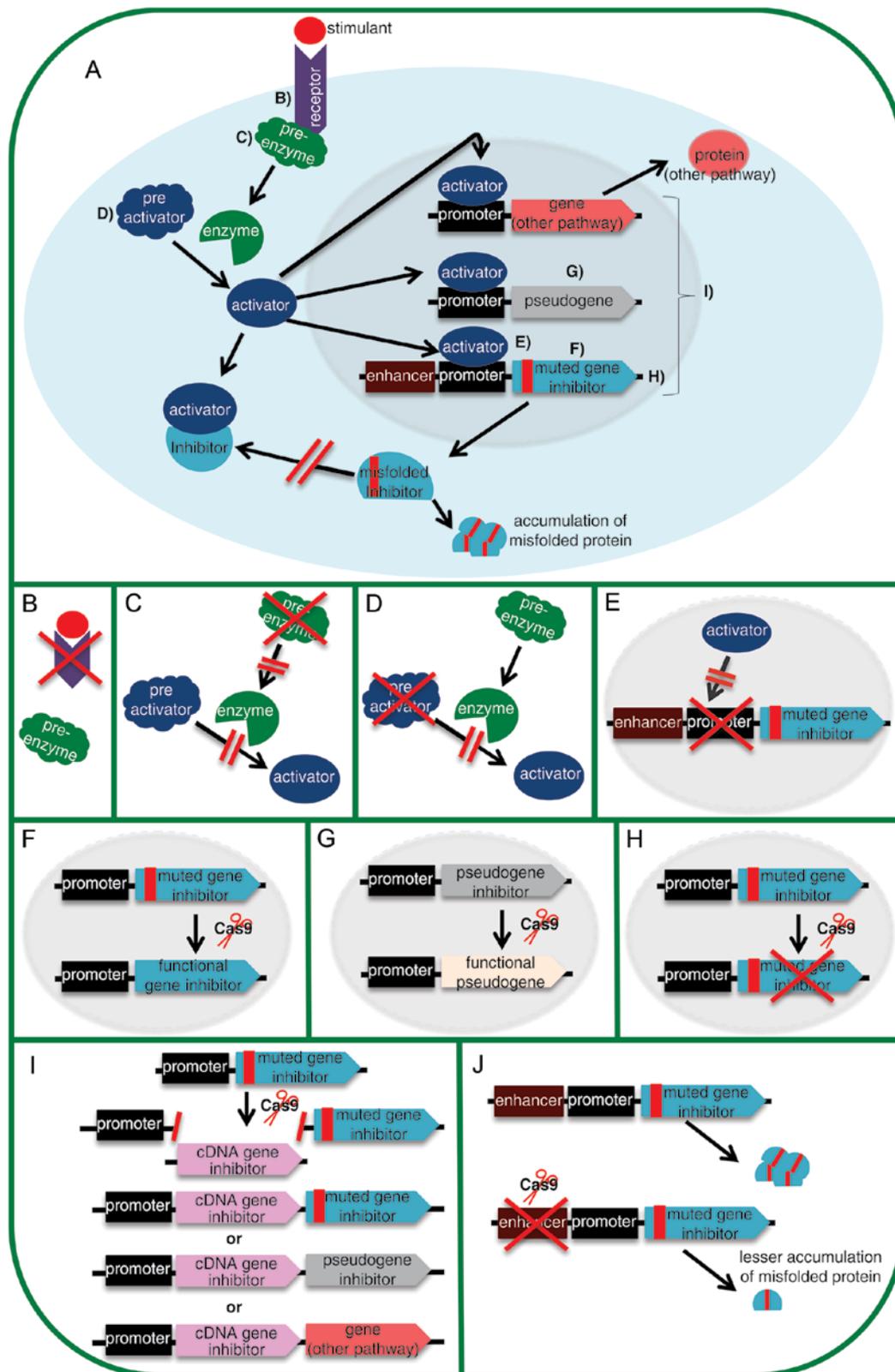


Figure 3. Strategies for editing a signaling pathway. (A) Signaling pathway where there is a mutation in the inhibitory gene that prevents the correct folding of encoded protein and therefore its function as an inhibitor. Indents B-E display different strategies that can be approached for preventing that inhibitory protein from being produced. (B) Knockout of the gene that codes for receptor, preventing it from acting on the pre-enzyme and from producing activator protein. (C) Knockout of the gene that codes for pre-enzyme, preventing it from acting on the pre-activator protein and from producing activator protein. (D) Knockout of the gene coding for pre-activator protein, preventing the enzyme acting on it from producing activator protein. (E) Mutation of the binding site of promoter so that the activator protein cannot bind. Indents F-I display different strategies that can be applied for the production of inhibitory protein. (F) Edition of a defective gene to restore production of an inhibitory protein to produce a functional inhibitory protein. (G) In the case that mutations in the inhibitor gene are difficult to repair, the pseudogene inhibitor is repaired to produce a functional inhibitory protein. (H) If a deleterious mutation is difficult to repair and causes the accumulation of a misfolded protein, the gene could be totally inactivated and the pseudogene can be reactivated to produce a functional protein. (I) Another strategy is the addition of the functional cDNA of the inhibitor gene in any of the genes or pseudogene stimulated by the activator protein. (J) Finally, mutation of the enhancer results in reduced production of inhibitory protein.

host components for HCV infections (121). In addition, the Epstein-Barr virus (EBV) establishes a persistent infection throughout life in 90-95% of the adults. Although it does not cause disease in healthy carriers, the infection is etiologically associated with lymphoid and epithelial neoplasias, such as Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma (122). The CRISPR/Cas9 system has been used *in vitro* against EBV in the Raji cell line, demonstrating a marked reduction in proliferation and viral load, as well as restoring the apoptotic pathway in cells subsequent to treatment (123). Another strategy applied to this virus was the removal of the BART promoter region (558 nt), which is one of two clusters that codes for 22 differentially expressed pre-microRNAs during latent EBV infection and is believed to be involved in epithelial cell transformation (122). This region was deleted in specific human epithelial cell lines with latent EBV infection, including nasopharyngeal carcinoma C666-1 cells. This was tested in order to determine if it is required for infection and transformation of epithelial cells, resulting in the loss of BART miRNA expression and activity. It was identified that EBV performance with pBART deletion was lower in comparison with that of WT virus measured in Raji cells, indicating the importance of miR-BARTs in the viral infection of epithelial cells, and that it will be of great interest to investigate whether they are particularly required for the infection and transformation of epithelial cells (122).

CRISPR/Cas9 in vector diseases. In the case of diseases transmitted by a vector, CRISPR/Cas9 has been used for the study of gene function and for gene drive to eradicate important vector-transmitted diseases, such as dengue, chikungunya, yellow fever and malaria (124,125).

In order to investigate the function of specific genes, studies have been conducted on the *Plasmodium sp.* genome, the parasite that causes malaria (125-127). Since the parasite resides in the RBCs, the transfection efficiency is lower as it has to cross four membranes (the RBC membrane, parasitophorous membrane, parasite cytoplasm membrane and parasite nuclear membrane). However, using CRISPR/Cas9 technology, 100% efficiency of gene deletion, 22-45% efficiency in tagging and 25% efficiency in nucleotide replacement were achieved, which is a significant advance in new studies of this parasite (125). Furthermore, CRISPR/Cas9 can be directed to the primary vector that transmits the disease, such as *Aedes aegypti*, which is the primary vector of several viruses. In this study, CRISPR/Cas9 was used to research the genetic and neurological basis of innate chemosensory behavior to achieve stable and precise loss-of-function mutations in five genes (124).

CRISPR/Cas9-mediated gene drive involves stimulating biased inheritance of particular alleles, such as gene knockouts, gene replacements and genetic transformations, in order to alter entire populations of organisms. This methodology is important since several species harm human interests, including human health or agriculture (128). CRISPR/Cas9 as part of a gene drive tool can be used to provide a deleterious trait (such as distorted sex ratio, reduced fertility and chemical sensitivity) (129). For instance, in *Anopheles gambiae*, the main vector for malaria, the CRISPR/Cas9 system was used to confer a recessive female-sterility phenotype upon disruption of three target genes: *AGAP005958*, *AGAP011377* and

AGAP007280 (130). This study demonstrated that disrupting *AGAP007280* gene alone was sufficient for a successful gene drive targeting female reproduction in an insect population (130).

CRISPR/Cas9 in cancer. Cancer is a group of diseases characterized by multiple genetic and epigenetic alterations in oncogenes and tumor suppressor genes. Experimentation to manipulate normal and cancerous cell genomes is vital for modeling the disease, as well as for the systematic study of genes involved in the process of initiation, progression and therapeutic response of cancer.

The fast modeling of genetic events has taken on a major relevance due to the need to elucidate the importance of genetic alterations present in human tumors, detected by large-scale sequencing of the cancer genome. This is necessary to discern between passenger mutations that are assumed to not directly affect the tumorigenic process, and those that directly or indirectly induce mutations that promote the transformation of normal cells into cancer cells by mutating oncogenes (promoting gain of function) and/or inactivation of tumor suppressor genes (promoting loss of function) (131).

Through the CRISPR/Cas9 technology, it was identified that *NANOG* and *NANOGP8* genes contribute to the high malignant potential of prostate cancer. Knockouts of *NANOG* and *NANOGP8* in human prostate DU145 cells significantly attenuated the malignant potential, including sphere formation, anchorage independent growth, migration ability and drug resistance, as compared with that of parental DU145 cells. Cell proliferation was not inhibited *in vitro*, but in immune deficient mice the tumorigenic potential decreased significantly *in vivo* (132). To assess the impact of *BCR-ABL* fusion on the leukemic processes in the Boff-p210 cell line, a hematopoietic cell line that is independent of interleukin (IL)-3 and expresses *BCR-ABL* p210, CRISPR/Cas9 was used to eliminate expression of the p210 oncoprotein. This resulted in the loss of ability of Boff-p210 cell line to grow in the absence of IL-3 and showed a significant increase in apoptosis levels (133). In immunosuppressed mice, the edited *BCR/ABL* cells developed smaller tumors compared with those originating from the parental Boff-p210 cells (133). Furthermore, a single-cell clone of edited *BCR/ABL* cells with a unique frameshift mutation (averting expression of the p210 oncoprotein) was unable to develop tumors, similar to the results in the Baf/3 parental line (133).

Although important, prior to CRISPR, the generation of animal models to test anti-cancer agents and undetected drug resistance mechanisms was an expensive and slow process (131). To provide a flexible and effective method to investigate the somatic alterations of loss of function, and their influence in tumorigenesis, CRISPR/Cas9 was used for the disruption of somatic genes, by the individual deletion of *PTCH1* or the triple deletion of *TRP53*, *PTEN* and *NF1* genes in the mouse brain, resulting in the development of medulloblastoma and glioblastoma, respectively (134). In some instances, chromosomal rearrangements serve a central role in the pathogenesis of human cancer, such as the oncogenic fusion between the echinoderm microtubule-associated protein like 4 (*EML4*) gene and anaplastic lymphoma kinase

Table II. Clinical gene editing trials with CRISPR/Cas9.

| Disease | Target | Strategy | Edited cells | Clinical Trials. gov identifier | Status |
|---|---------------------------------|----------|-------------------|------------------------------------|--------------------|
| HPV-related malignant neoplasm | HPV <i>E6/E7</i> , 16 and 18 | NHEJ | HPV16 and 18 | NCT03057912 | Not yet recruiting |
| HIV-infected subjects with hematological malignances | <i>CCR5</i> | NHEJ | CD34 ⁺ | NCT0316435 | Recruiting |
| Relapsed or refractory CD19 ⁺ on B-cell leukemia and lymphoma | <i>TCR</i> and <i>B2M</i> | NHEJ | UCART19 | NTC03166878 | Recruiting |
| Advanced esophageal cancer | <i>PD-1</i> | NHEJ | T cell | NCT03081715 | Recruiting |
| Muscle-invasive bladder cancer stage IV | <i>PD-1</i> | NHEJ | T cell | NCT02863913 | Not yet recruiting |
| Hormone refractory prostate cancer | <i>PD-1</i> | NHEJ | T cell | NCT02867345 | Not yet recruiting |
| Metastatic renal cell carcinoma | <i>PD-1</i> | NHEJ | T cell | NCT02867332 | Not yet recruiting |
| Metastatic non-small cell lung cancer | <i>PD-1</i> | NHEJ | T cell | NCT02793856 | Recruiting |
| Advanced stage EBV associated malignancies (stage IV gastric carcinoma; stage IV nasopharyngeal carcinoma; T-Cell lymphoma stage IV; stage IV adult Hodgkin lymphoma; stage IV diffuse large B-cell lymphoma) | <i>PD-1</i> | NHEJ | T cell | NCT03044743 | Recruiting |

HPV, human papilloma virus; HIV, human immunodeficiency virus; EBV, Epstein-Barr virus; CCR5, chemokine receptor type 5; TCR, T-cell receptor; B2M, β -2-microglobulin; PD-1, programmed cell death protein 1; NHEJ, non-homologous end joining.

(*ALK*) gene. The resulting *EML4-ALK* oncogene is detected in a subset of human non-small cell lung cancer (NSCLC), and this is clinically relevant since it imparts sensitivity to inhibitors of ALK. CRISPR/Cas9 was used to generate a mouse model of lung cancer driven by the *EML4-ALK* gene rearrangement. The resulting tumors harbored the *EML4-ALK* inversion, expressed the *EML4-ALK* fusion gene, presented typical histopathological and molecular characteristics of human ALK⁺ NSCLC, and responded to treatment with ALK inhibitors (135).

Currently clinical protocols in cancer are underway to assess the application of the CRISPR/Cas9 technology in lung, prostate, renal, esophageal and bladder cancer, as well as in neoplasias associated with HPV and EBV (Table II; <https://clinicaltrials.gov/ct2/results?cond=&term=crispr&entry=&state=&city=&dist=>).

Autoimmune and inflammatory diseases. The wide range of rheumatic diseases extends from rare monogenic auto-inflammatory diseases to complex polygenic autoimmune diseases (136). High levels of IL-1 characterize monogenic auto-inflammatory diseases. Genetically, certain of these syndromes result from mutations in the *NLRP3* gene, with autosomal dominant inheritance and variable penetrance (136,137). In the complex polygenic autoimmune diseases, such as rheumatoid arthritis (RA), multiple genetic factors and environmental triggers are involved; for instance, the heritability of RA is considered to be ~65% (138). A number

of these diseases are potential clinical targets for CRISPR/Cas9, where this technology could be used for *in vitro* and *in vivo* functional genomics studies to elucidate the single and combined role of single nucleotide polymorphisms identified by association studies at the genomic level through the rapid creation of cellular and animal models. Additionally, this technology can be applied in individualized therapy as a tool to correct mutations, in strategies adapted for each patient.

In this sense, CRISPR/Cas9 has been used for the creation of a rat chondrosarcoma cell line that stably expresses Cas9 for the study of complex interactions that regulate function, differentiation and chondrocyte homeostasis, and to examine the role of genes associated with cartilage degenerating diseases (139). CRISPR/Cas9 can accelerate the *in vitro* and *in vivo* functional elucidation of the role played by genes in the inflammatory and bone components of these diseases. In this regard, CRISPR/Cas9 technology was applied to a murine macrophage cell line to demonstrate that the *RAS-GRP3* gene limits the inflammatory response by activating Rap1 (140). In another study, microRNA (miR)-155 was shown to exert pro-inflammatory and pro-osteoclastogenic effects through the CRISPR/Cas9-induced mutation of the miR-155 binding site to the *SHIP1* gene, a negative inflammation regulator (141). In addition, CRISPR/Cas9 technology may also incorporate a cell therapy strategy, for instance in RA, a disease characterized by deregulated responses to pro-inflammatory cytokines such as IL-1 and tumor necrosis factor- α (TNF- α). CRISPR/Cas9 was used to program murine induced iPSCs

with the ability to respond to an inflammatory stimulus with potent and autonomously regulated production of anti-cytokines. TNF- α and IL-1 are two of the most potent stimulators of *CCL2* gene expression. If an antagonistic gene to TNF- α or IL-1 is placed under the control of the *CCL2* promoter, it will respond to cytokine levels and cause a self-regulated inflammatory process. This allows the control of the cell expression of biological therapies (90).

CRISPR/Cas9 in primary immunodeficiency. Primary immunodeficiency (PID) is a group of heterogeneous and rare chronic diseases, in which part of the immune system functions inappropriately or does not function at all. They are caused by numerous genetic defects, of which >230 have been identified with very variable clinical manifestations. Among the PID diseases, severe combined immunodeficiency (SCID) results in a blockage of T cell development with an additional primary or secondary defect in B cells, and natural killer (NK) cells may or may not be affected. The most common form of SCID is the X chromosome-linked syndrome X-SCID, which is caused by mutations in the gene that encode the γ receptor of IL-2 (namely the *IL2RG* gene) (142).

PID, including SCID, can be treated by allogeneic transplantation of healthy HSCs; however, histocompatibility problems may be faced, together with the risk of acquisition of transmitted diseases (143). These problems can be overcome by the correction of the patient's own HSCs through inserting a copy of the functional gene by a viral vector. Although there are success cases in clinical trials, serious complications may also occur due to viral integration close to oncogenes and hence, their activation (144,145). A promising alternative is the use of CRISPR/Cas9 to induce a targeted homologous repair, having a clear advantage over traditional gene therapy. Alternatively, in certain immune deficiencies, there is loss-of-function or gain-of-function in genes subjected to a strict control of expression. Examples included the X-linked agammaglobulinemia and the signal transducer and activator of transcription 3 (STAT3) loss-of-function, as well as the STAT3 and STAT1 gain-of-function, or activated PI3K- δ syndrome. These diseases ideally require the correction of the existing gene to restore normal function or regulation, which may be achieved by CRISPR/Cas9 (146).

It is important to mention that the therapeutic efficacy of gene editing depends on several factors, including editing efficiency, which varies significantly depending on the cell type, senescence status, and cell cycle status of the target. Other factors that also influence therapeutic efficiency include cell aptitude, which refers to the feasibility of reaching a therapeutic modification threshold, and the efficient delivery of programmable nuclease system to the target tissue, which is only considered to be effective if the programmable nuclease system arrives safely and efficiently to the nucleus of the target cell. Finally, the specificity of the editing is another important factor, which refers to only editing the target DNA without affecting any other genes (4,147). Since hematopoietic cells are the most common target in immunological diseases, autologous hematopoietic stem cell transplantation is a viable grafting method of cells that are already corrected *ex vivo* by CRISPR/Cas9, replacing all or part of the hematopoietic stem cell compartment (146).

CRISPR/Cas9 technology was recently applied in iPSCs to develop an *in vitro* model of Janus kinase 3 deficiency. A blockage in T cell development was eliminated after performing a CRISPR/Cas9-mediated editing (148). Chronic granulomatous disease (CGD) is characterized by severe and persistent infections due to the lack of an anti-pathogenic oxidative burst normally performed by phagocytic cells to contain and eliminate bacterial and fungal growth. CRISPR/Cas9 was used in iPSCs derived from a patient with CGD to correct a single mutation in the *CYBB* gene intron and to restore oxidative function in phagocytes (149). Additionally, CRISPR/Cas9 was used to delete GT dinucleotide in the *NCF1* gene, which encodes human p47phox protein, in an acute myeloid leukemia cell line (PLB-985) to generate a CGD cell model that reacts to the genetic background of the disease in order to be used for preclinical vector tests (150).

CRISPR/Cas9 has been used to generate 'knockout' models that had not been susceptible to efficient genetic modification for the study of immune system diseases, such as rabbits with knockout of 1-5 genes at the same time (*IL2RG*, *RAG1*, *RAG2*, *TIK11*, and *ALB*) with efficiencies ranging from 100% (for 1 gene) to 33.3% (for 5 genes) by microinjection into pronuclear stage embryos cytoplasm (151). Rabbits with *IL2RG* and *RAG1* gene knockout are an important animal model of immune deficiencies characterized by the absence of mature T, B and NK cells (151). Other model examples involve hamsters carrying knockouts of the *STAT2* gene for the study of viral infections, of the *KCNQ1* gene for cardiovascular function investigation and of the *PPP1R12C* gene for transgenic integration (152). In addition, pigs carrying knockout of the gene coding for the JH region of the IgM heavy chain, which is crucial for the development and differentiation of B cells, resulted in piglets lacking antibody-producing B cells. The generation of a B cell-deficient mutant is the first step in producing human antibody repertoires in large animal models (153).

CRISPR/Cas9 in other immune diseases. Regarding the use of CRISPR/Cas9 to investigate allergic diseases, this technology has been used to examine the role of certain genes, such as the *MUC18* gene, which is also known as CD146 or melanoma cell adhesion molecule. Through CRISPR/Cas9, the knockout of *MUC18* gene was conducted in human nasal airway epithelial cells, which is the first line of defense against environmental factors, such as pathogens and pollution. This led to a reduced response of IL-8 following stimulation of the Toll-like receptor agonist, suggesting a pro-inflammatory role of *MUC18* gene in response to bacterial or viral stimulation (154). Another example is the use of this technology in X-linked hyper IgM syndrome, where CRISPR/Cas9 was used to correct mutations in the CD40 ligand (155). Furthermore, CRISPR/Cas9 was used to induce recombination of IgH chain class changes in desired subclasses in murine and human B cells. It was also used to produce Fab fragments instead of the whole IgH molecule in mouse hybridoma cells, with the aim of a more careful scrutiny of Ig subclasses and novel methods for accessible production of Fab fragments for research or therapeutic uses (156).

Monkeys serve as one of the most valuable animal models for the development of therapeutic strategies due to their close similarities with humans (157). CRISPR/Cas9 was successfully used in the generation of monkeys with knockout in the *NROB1*, *PPAR- γ* and *RAG1* genes (158), being able to serve as models

of X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism (159), lipodystrophy metabolism (160), insulin sensitivity, obesity and inflammatory disease (161). Furthermore, monkey knockout models of RAG gene could be used in regenerative medicine, allograft and xenograft transplantation, and reconstitution experiments associated with the immune system (162).

CRISPR/Cas9 in other diseases. The CRISPR/Cas9 system has been applied in cellular and animal models to study and search treatments for different neurological disorders, such as Parkinson's disease (163). Mutation in *PARK2* or *PINK1* genes leads to early onset Parkinson's disease, as an autosomal recessive disease in humans (164). The *PARK2* gene encodes a protein called parkin, which is a component of the multiprotein complex E3 ubiquitin ligase, while the *PINK1* gene encodes for the PTEN-induced putative kinase 1, a mitochondrial serine/threonine kinase protein. The CRISPR/Cas9 system has also been applied in amyotrophic lateral sclerosis (165,166), Huntington's disease (167), schizophrenia (168) and autism (168). It has also been applied in movement diseases, such as Duchenne muscular dystrophy (169-172), in metabolic diseases, such as type I diabetes (173) and hypercholesterolemia (174), and in inherited diseases that affect vision, such as retinitis pigmentosa (175), among numerous others.

4. Conclusion

It is expected that CRISPR/Cas9 technology will soon be validated through *ex vivo* clinical protocols already in course in humans to test against various types of cancer. In the meantime, it is fully applicable to cell therapy experiments and deserves to be vigorously developed as a research tool in an unusually diverse range of biological systems. This will allow the necessary improvements for future biomedical technologies and applications.

Acknowledgements

The authors would like to thank Alec Christopher Escalante Gomez (School of Nutrition of the Universidad Autónoma de Nuevo León, Monterrey) for aid in the translation of this review and Jessica García for valuable suggestions.

Funding

Authors wish to acknowledge Tecnológico de Monterrey and Mission XXI for their financial support regarding the publication charges.

Availability of data and materials

Not applicable.

Authors' contributions

DRRR reviewed the basic science literature and wrote the manuscript; MAGE reviewed the clinical issues; RRS and MDLGR supported DRRR in revisions of the manuscript;

HABS conceived the project and supervised the writing of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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