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CRISPR/Cas9: The new era of gene therapy

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ABSTRACT

The Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein (CRISPR/Cas) system is present in many Bacteria and in almost all Archaea and functions in those organisms as a defense mechanism against invading viruses and plasmids. Since the function and the working mechanism of the bacterial CRISPR/Cas system were elucidated in 2007, and researchers realized its potential as a gene-editing tool in 2012, it quickly became a widely used tool to generate mutations in cells, cell lines, and various model organisms. Applications such as improvement of disease resistance of economic plants, enhancement of muscle growth, or litter size in livestock are already becoming common practice. More recently, its application to repair genetic mutations has been explored in human cells and cell lines, and currently, the first clinical trials are underway in which the CRISPR/Cas9 system is applied to cure patients from various diseases. When looking at the timeline, it is clear that the CRISPR/Cas9 system has revolutionized genome engineering in less than a decade and may well be the most versatile genome engineering tool available. Here we review the origin and function of the CRISPR/Cas9 system, its working mechanism, and its use in various model organisms. In addition, pitfalls of the method are discussed, as well as the currently running and planned clinical trials. The objectives of this review are: first, to inform readers of the working mechanism of this new technique and how it is currently used to facilitate research in model organisms and to improve the profitability of livestock and economic plants. And second, to provide insight into the application of this technique in the treatment of disease in humans.

1. Introduction

CRISPR/Cas (or Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein) systems are a typical feature of the genomes of most Bacteria and Archaea and are involved in resistance to bacteriophages and mobile genetic elements (such as plasmids). CRISPRs were first identified in the *E. coli* genome when Ishino et al. (1987) discovered the *iap* gene and loci downstream from that gene that contained repeat sequences with an unknown function. Since then, it was among others hypothesized that CRISPRs were involved in a defense system against bacteriophages. It was not until 2007, however, that the function and working mechanism were elucidated experimentally by Barrangou et al. (2007). They used the bacterium *Streptococcus thermophiles* that they infected with various bacteriophages in order to select strains for phage-resistance. They then sequenced the CRISPR loci of resistant strains. These loci had acquired spacer sequences that showed similarity to sequences present in the phages, and these were shown to provide resistance to the phages. In addition, they showed that Cas proteins were involved in the process as well (Barrangou et al., 2007). In the ensuing years, interest in the CRISPR/Cas system increased greatly, although research was focused mainly on its original defense function and its evolution in Bacteria and Archaea. At that time, applications were limited to the use of the CRISPR repeats in typing and epidemiological studies, and resistance improvement of domesticated bacteria against mobile genetic elements and viruses (Horvath and Barrangou, 2010).

It was not until 2012 that Jinek et al. (2012) suggested that the CRISPR system might have potential use as a genome editing tool, this was picked up by Carroll (2012) in an editorial summary,

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and applications soon followed. The first two reports on successfully engineered eukaryotic cells were published back-to-back in Science in 2013 (Cong et al., 2013; Mali et al., 2013). Both groups used the combination of Streptococcus pyogenes-derived CRISPR and Cas9 that has since been used widely. In the article by Cong et al. (2013), the system was used to modify two human and three mouse genes in cell lines. In the article by Mali et al. (2013), it was used to modify a human gene in a cell line as well as in induced pluripotent stem cells (iPSCs). The two articles were immediately recognized as a major breakthrough in genome engineering (Burgess, 2013; De Souza, 2013; Oost, 2013). Since then, the use of the CRISPR/Cas9 system for genome editing of eukaryotic cells has taken flight. In addition, other applications have been developed, such as epigenome-editing, which may facilitate the manipulation of stem cells and thus aid gene therapy as well (Pulecio et al., 2017). Fig. 1 illustrates the number of research articles on CRISPR that have been published since 2006.

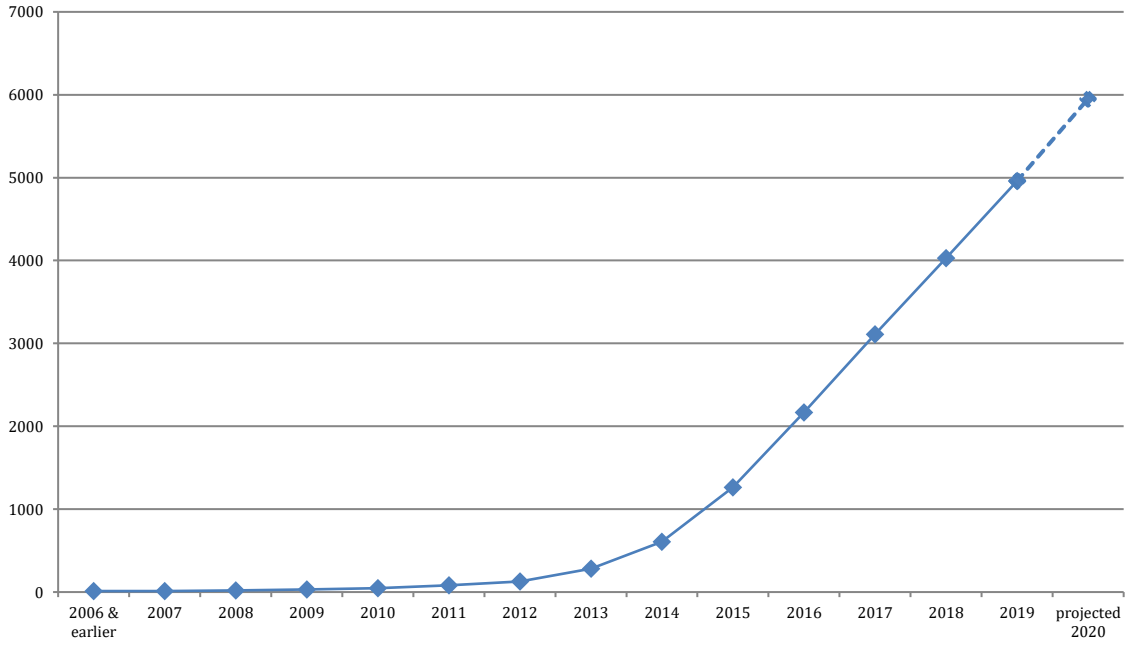

Fig. 1: Number of articles published since 2006 mentioning the CRISPR/Cas9 system

The CRISPR/Cas9 working mechanism was elucidated in 2007 by Barrangou et al. (2007). The number of articles published on CRISPR/Cas greatly increased since the suggestion was made in 2012 by Jinek et al. (2012) that the system could be used as a gene-editing tool. A search for CRISPR and date of publication was performed in PubMed to identify articles that were published mentioning the CRISPR/Cas system since 2006. Before 2006 seven articles were published mentioning CRISPR.

The objectives of this review are two-fold: first, to inform readers of the working mechanism of the CRIPR/Cas system and how it is currently used to facilitate research in model organisms and to improve the profitability of various livestock and economic plants. And second, to provide insight into the application of this technique in the treatment of disease in humans, discussing both current and potential future applications. This review contributes to the existing literature on CRISPR/Cas9 as it provides an overview of the development of the system, explains the working mechanism, and summarizes current applications in all fields. In this way, the review provides a comprehensive overview of all aspects that are important to understand the CRISPR/Cas9 system and its applications in this new era of gene therapy.

2. Method

PubMed searches and references from relevant articles were used for this review. Initial search terms used were: CRISPR/Cas, CRISPR, tracrRNAs, crRNAs, Cas9, model organism, livestock, economical plants, clinical trial, pitfall, and gene therapy and combinations thereof. For Fig. 1, we searched PubMed using the query: '(CRISPR) and ("2007"[Date-Publication]: "2007"[Date-Publication]) NOT 2008', where the years were increased at each step. Only papers published in English were reviewed.

3. Working mechanism of the CRISPR/Cas9 system

There are three CRISPR/Cas systems in bacteria: Type I, type II, and type III. The type II CRISPR/Cas system is the only one that uses only one Cas protein: The Cas9 protein. The type II CRISPR/Cas9 system has, therefore, become the most popular CRISPR/Cas system. In bacteria, the CRISPR locus in the genome contains the gene (s) for the Cas protein (s) as well as several non-coding genes that generate two guide RNAs: The trans-activating RNAs (tracrRNAs) and CRISP RNAs (crRNAs). In the

CRISPR/Cas9 system, as it is currently used, these tracrRNAs and crRNAs are combined in a single guide RNA, making it a very elegant and simple system.

The working mechanism of the CRISPR/Cas9 complex for genome editing is illustrated in Fig. 2. To use the system for genome editing a single guide RNA (depicted in red in Fig. 2) is synthetically generated to contain a sequence of about 20 bp, the spacer (the equivalent of the crRNA), that is homologous to the target DNA. The guide RNA also contains the tracrRNA sequences. The spacer binds the target DNA (depicted in blue in Fig. 2) at the homologous site, and immediately adjacent to the homologous sequence also a 2-5 nt protospacer adjacent motif (PAM) has to be present (indicated with a thin black arrow in Fig. 2). The complex of target DNA and guide RNA subsequently binds a Cas9 protein (the green oval in Fig. 2), this Cas9 protein has endonuclease activity and proceeds to cleave the DNA (the cleavage point is indicated with the thick black arrow in Fig. 2). The tracrRNA sequences are required to keep the Cas9 in an active form (Lim et al., 2016).

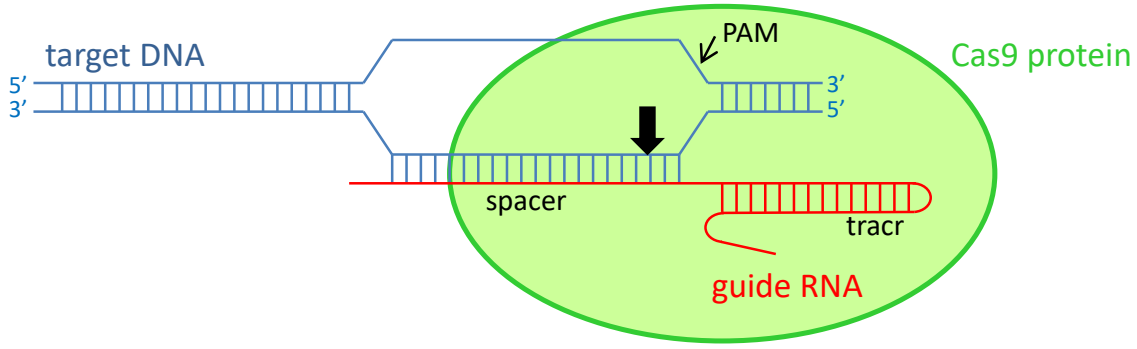

Fig. 2: CRISPR/Cas9 working mechanism

A single guide RNA (depicted in red) is synthetically generated to contain a spacer and tracr sequences. The spacer (about 20 bp long) binds the target DNA (depicted in blue) that has homology to the spacer sequence, and that also contains an adjacent PAM sequence. The complex subsequently binds a Cas9 protein (green oval). The Cas protein cleaves the DNA at the thick arrow (in black). PAM= protospacer adjacent motif; tracrRNA= transactivated CRISPR RNA.

The original Cas9 protein induces double-strand breaks and thus results in insertions and deletions when the breaks are repaired through the error-prone non-homologous end joining (NHEJ) DNA repair pathway. This is a convenient way to knock out genes. For accurate gene editing, however, the Cas9 proteins have been engineered to only cause one strand to break. To subsequently induce a specific mutation (or correction), single-stranded oligodeoxynucleotides (ssODNs, or 'donor DNA') are added that contain the desired nucleotides. These ssODNs are used as a template to repair the breakthrough homology-dependent recombination (HDR). This will allow the induction of specific mutations as well as the correction of small existing mutations as required for gene therapy.

There are currently also nuclease-deficient Cas9 proteins that only induce DNA binding. These are used to silence or activate gene expression (Qi et al., 2013). A more recent development is the use of CRISPR-based RNA targeting, which allows transcript-specific regulation (Pei and Lu, 2019). We will mainly focus here on the CRISPR/Cas9 combinations that are used to generate knock outs and specific modifications in the genome.

4. CRISPR/Cas9 gene editing in whole organisms

4.1. Model organisms

CRISPR/Cas9 gene editing has been widely used in a variety of model organisms to investigate the working mechanism, optimize the system, and determine potential side effects. It has subsequently also been used to study gene function. One of the easiest model organisms to manipulate is *Drosophila melanogaster* (the fruit fly). It was, however, not the first multicellular model organism in which gene editing with CRISPR/Cas was published. This honor was reserved for zebrafish (Chang et al., 2013; Hwang et al., 2013) and mice (Shen et al., 2013). In those first publications, mosaic animals were obtained by micro-injecting a mix of guide RNA and Cas9 encoding mRNA into either one-cell zebrafish embryos *in vivo* (Chang et al., 2013; Hwang et al., 2013) or into one-cell mouse embryos (Shen et al., 2013). The mosaicism of the offspring indicated that the CRISPR/Cas9 system was active, after the first cleavages, in multicellular embryos.

The first publication on gene editing with the CRISPR/Cas9 system in *Drosophila* was published by Gratz et al. (2013), it was incidentally also the first publication showing germline transmission of an edited gene in any model organism. They injected plasmids containing the guide RNA and Cas9 mRNA into embryos (Gratz et al., 2013). Soon after, another

group published a similar report in *Drosophila* with however a much higher (10-100 fold) efficiency due to direct injection of guide RNA and Cas9 encoding mRNA, instead of plasmids, into the embryos (Bassett et al., 2013). All modifications were insertions and deletions as the wild-type Cas9 was used that induces double-stranded breaks. Genome editing of the roundworm *Caenorhabditis elegans* soon followed, although initially not very successful (Friedland et al., 2013). The use of RNAs for injection instead of plasmids also appears to yield better results in *C. elegans* and other nematodes (Lo et al., 2013).

Single and multigene gene modifications have also been achieved in Cynomolgus monkeys by injecting embryos with mixes of guide RNAs and Cas9 mRNA. The first of such experiments resulted in mosaic animals with knock outs in the three genes targeted (Niu et al., 2014).

4.2. Livestock

Gene editing in livestock has mostly been done by knocking out genes. The first knocked out genes in livestock were reported in 2014 in pigs (Hai et al., 2014; Whitworth et al., 2014). One study knocked out the *vWF* gene thus enhancing bleeding which is an advantage during slaughter (Hai et al., 2014), and the other knocked out *CD163* which may result in improved resistance to a porcine virus (although this was not tested) and *CD1D* which removes an MHC protein (Whitworth et al., 2014). Various studies in livestock were aimed at improving muscle growth for increased meat production. In pigs, initial experiments knocking out the *FOXBO40* gene resulted in a 4% increase in muscle growth, showing that manipulation of this gene, or other genes in the same pathway, may be worth pursuing (Zou et al., 2018). Knocking out the *MSTN* gene in goats had a much greater effect on birth weight and daily weight gain, respectively ~40% and ~30% (as read from Fig. 1 in that article) (Wang et al., 2018a). In milk-producing livestock also total milk production and production of allergens in the milk, such as β-lactoglobulin (BLG), are targeted. The *BLG* gene was successfully knocked out in goats, entirely abolishing BLG production (Zhou et al., 2017). In sheep and goats, knock out of *FGF5* resulted in longer wool staple length and an increase in fleece weight (Li et al., 2017; Wang et al., 2016). Many more examples are available, showing that knocking out genes with the CRISPR/Cas system is highly efficient in livestock. Although many of the injected embryos result in mosaic animals, almost invariably, germline transmission is found in at least part of the animals as well.

More advanced gene editing experiments were performed in livestock as well. The first report of a complete gene inserted using the CRISPR/Cas9 system was by Gao et al. (2017), who introduced the *NRAMP1* gene into cattle to increase resistance to tuberculosis. Insertion of complete genes was also successfully performed by Ma et al. (2017) in sheep embryos, where *AANAT* and *ASMT* genes were introduced to produce melatonin-enriched milk. In goats, a specific amino acid change in the *GDF9* gene has been generated to affect litter size. Using guide RNA, Cas9 mRNA, and a ssODN to inject embryos, 3 out of 12 embryos had acquired the desired mutation, although also one embryo was found to have an insertion/deletion mutation. In the next experiment, after placing embryos into surrogate females, 17 kids were born, four of which had one or two copies of the desired mutation, although again, two were found to have insertion/deletions (Niu et al., 2018).

4.3. Economic plants

Not only in animals but also in plants, the CRISPR/Cas system is working well to induce genetic changes. It has already become the main technology for gene editing in plants. While in both animals and plants, mostly the *Streptococcus pyogenes* derived Cas9 is used, in plants, the *Staphylococcus aureus*-derived Cas9 was found to work just as well (Steinert et al., 2015). In addition, CRISPR can not only be used in combination with Cas9 but also with Cas12 and Cas13 (Schindele et al., 2018). The availability of multiple Cas proteins that have different sizes and different specificities may allow simultaneous induction of different types of genetic changes in the same plant cell, inducing more complex changes.

The most common model organism in plant biology is the weed *Arabidopsis thaliana*, which has been used for the study of the CRISPR/Cas system in plants, although right from the start experiments has been performed in cash crops as well (Jiang et al., 2013). So far, the CRISPR/Cas9 system has been mainly used to improve disease resistance and crop characteristics of economic plants. Improved disease resistance has, among others, been obtained to a virus in rice (Macovei et al., 2018), to powdery mildew in wheat (Wang et al., 2014), and to bacterial canker in various citrus (Jia et al., 2017). Improved crops have among others been generated with wheat, where low gluten production was achieved (Sanchez-Leon et al., 2018), and with maize, where drought-tolerance was improved (Shi et al., 2017). The system has not reached the breeding of ornamental plants, such as roses, yet. This may be because, unlike cash crops, there are many different cultivars of ornamental plants, and the economic importance of ornamental plants is not as great (Kishi-Kaboshi et al., 2018).

5. Pitfalls and points for improvement

In the few years since the first realization that the CRISPR/Cas9 system could be used for genome editing (Jinek et al., 2012), various changes have already been introduced to the system to improve efficacy and manipulate the induced effect. The first is the introduction of the single guide RNA that incorporated both the tracrRNA and the spacer RNA

that were separate molecules in the original bacterial system (Mali et al., 2013). The next change was the introduction of mutations in Cas9 that induced single-strand breaks instead of double-strand breaks (Cong et al., 2013), thus allowing, in combination with a donor DNA (ssODN), to accurately generate very specific mutations or corrections.

The main pitfall of the CRISPR/Cas system that has been observed in the past years has been off-target effects, while also other aspects, such as delivery methods, can still be improved (Peng et al., 2016). Off-target effects are mutations caused at other sites in the genome, often due to homologous sites that have only a few mismatches with the target site that is included in the spacer. An important improvement that various groups are working on is the development of new Cas9 variants that minimize the off-target effects. This has already led to the development of, among others, the high-fidelity variant SpCas9-HF1 (Kleinstiver et al., 2016), the enhanced specificity variant eSpCas9 (1.1) (Slaymaker et al., 2016), the hyper-accurate HypaCas9 variant (Chen et al., 2017), and the high fidelity, high-efficiency evoCas9 variant (Casini et al., 2018). Currently, these new Cas9 variants are in the stage of testing off-target effects in animal models. The modifications were done to create these new Cas9 variants, and their resulting reductions in off-target effects were recently reviewed (Han et al., 2020).

The design of the guide RNA is also very important to reduce off-target effects as it can affect Cas9 activity if it is too long, too short, or shows mismatches in the spacer region. The amount of guide RNA and Cas9 protein is important as well: too little is inefficient, while too much causes an increase in off-target effects. Also, the molar balance of guide RNAs versus Cas9 proteins has an effect. In addition, the Cas9 protein needs a nuclear localization signal (NLS) for it to be able to enter the nucleus where it needs to perform its job in eukaryotic cells. Since bacteria do not have a nucleus, Cas9 molecules do not have an NLS, and Cas9 needs to be engineered to include one. Finally, there are many delivery methods, many involving plasmids that encode both the Cas9 gene and the guide RNA. Plasmids, however, have the tendency to be integrated into the genome and can thus cause undesired side effects by disrupting genes or regulatory regions. The presence of plasmids can, in addition, induce immune responses that affect the gene-editing process itself. Injecting Cas9 protein directly is, unfortunately, very inefficient. Various viral vectors are also popular, although some of them induce more off-target effects.

To aid researchers in their genome engineering experiments, a range of tools is available to design the guide RNAs summarized by Brazelton et al. (2015) and Peng et al. (2016). As the efficiency of targeted mutagenesis and the adverse generation of off-target mutations will vary per locus and may also vary between different organisms, optimization of experiments and close monitoring of the results will always be warranted.

The most recent developments in the genome-editing field are the use of base editing and prime editing, which use variants of the CRISPR/Cas9 system that do not induce double-strand breaks. Base editing allows for high-efficiency editing of single nucleotides (Rees and Liu, 2018), while prime editing allows for a broader range of edits and is less dependent on the presence of specific adjacent sequences such as PAM (Anzalone et al., 2019). These are very promising developments that may make the CRISPR/Cas9 system safer and more efficient, testing in animal models will now need to verify the value of these new editing systems *in vivo*.

6. Clinical trials in patients

Currently, twenty-one clinical trials are running or starting up that involves the use of the CRISPR/Cas system in interventional studies (Table 1). These are trials designed to study safety, tolerability, biological activity, efficacy, and/or find the optimal dose. Most of these studies apply a form of adoptive immunotherapy, a therapy that -transiently- delivers cells or compounds that induce or affect the immune response to a disease. In seventeen of the studies, allogeneic or autologous T cells are re-targeted to recognize and subsequently attack malignant cells.

The most commonly administered genetically engineered T cells in these studies are the PD-1 knock out T cell. PD-1 is a cell surface receptor normally expressed on T cells and its ligand, PD-L1, is expressed on tumor cells and normal dendritic cells. PD-1 is a checkpoint that normally provides self-recognition, thus preventing autoimmunity. Due to the expression of PD-L1 on cancer cells, it, however, also prevents the immune system from killing cancer cells. Knocking out PD-1 in T cells will allow the T cells to recognize the cancer cells as foreign and kill them (Su et al., 2016; Yi and Li, 2016). Nine of the clinical trials apply this approach to treat malignancies.

Direct administration of a CRISPR/Cas plasmid or of a viral vector containing Cas to patients is performed in only two clinical trials. These are the only studies where the patients are actually treated with the CRISPR/Cas system *in vivo*. In the first study, the patients have human papillomavirus (HPV)-related cervical intraepithelial neoplasia, a malignancy that is in the majority of cases caused by infection with HPV16 or HPV18 (NCT03057912 in Table 1). The target genes of the CRISPR/Cas plasmid are encoding HPV16 and HPV18 oncoproteins. The method of administration is not specified; however, based on literature, the most likely method appears to be topical administration (Hu et al., 2015; 2014). In the second study, the patients have a retinal disease called Leber congenital amaurosis type 10 that is caused by a specific mutation in the CEP290 gene and leads to poor or no vision (NCT03872479 in Table 1). The

viral vector that is administered by subretinal injection delivers Cas9 and CEP290 gRNAs (Maeder et al., 2019).

Table 1: Currently running and planned clinical trials applying the CRISPR/Cas system

Clinical trial number	Disease or patient description	Cells or compound to administer	Target protein	Location: City, Country	Status	References
NCT03057912	HPV-related cervical intraepithelial neoplasia	CRISPR/Cas plasmid in gel	HPV16 E6/E7T1 and HPV18 E6/E7T2	Guangzhou, China	not yet recruiting	(Hu et al., 2015; 2014)
NCT03164135	HIV-infected subjects with hematological malignances	Allogeneic CD34+ hematopoietic stem cells	CCR5	Beijing, China	recruiting	(Xu et al., 2017)
NCT03399448	Multiple myeloma, melanoma, sarcoma	Autologous T cells	TCR and PD-1	Philadelphia, United States	active	(Ren et al., 2017a; 2017b)
NCT03166878	B cell leukemia, B cell lymphoma	Allogeneic T cells	TCR and B2M	Beijing, China	recruiting	n.a.
NCT03398967	B Cell Leukemia, B Cell Lymphoma	Allogeneic T cells	CD19 and CD20 or CD22	Beijing, China	recruiting	n.a.
NCT03081715	Advanced esophageal cancer	Autologous T cells	PD-1	Hangzhou, China	completed	n.a.
NCT02863913	Stage IV muscle-invasive bladder cancer	Autologous T cells	PD-1	Beijing, China	not yet recruiting	(Yi and Li, 2016)
NCT02867345	Hormone refractory prostate cancer	Autologous T cells	PD-1	Beijing, China	not yet recruiting	(Yi and Li, 2016)
NCT02867332	Metastatic renal cell carcinoma	Autologous T cells	PD-1	Not stated	not yet recruiting	(Yi and Li, 2016)
NCT02793856	Metastatic non-small cell lung cancer	Autologous T cells	PD-1	Chengdu, China	active	(Yi and Li, 2016)
NCT03044743	Advanced stage EBV-associated malignancies	Autologous EBV-CTLs	PD-1	Nanjing, China	recruiting	n.a.
NCT03545815	Mesothelin positive multiple solid tumors	CAR T Cells	PD-1 and TCR	Beijing, China	recruiting	(Hu et al., 2019)
NCT03655678	transfusion-dependent β-thalassemia	Autologous CD34+ hematopoietic stem and progenitor cells	BCL11A	Canada, Germany, United Kingdom, United States	recruiting	(Wu et al., 2019)
NCT03745287	Severe sickle cell disease	Autologous CD34+ hematopoietic stem and progenitor cells	BCL11A	Multiple locations, United States, Belgium, Canada, Germany, Italy	recruiting	(Wu et al., 2019)
NCT04244656	Relapsed or refractory multiple myeloma	Allogeneic T cells	BCMA	Melbourne, Australia; Portland and Nashville, United States	recruiting	n.a.
NCT04037566	CD19+ leukemia or lymphoma	Autologous T cells	HPK1	Xi'an, China	recruiting	n.a.
NCT04035434	Relapsed or refractory B-cell malignancies	Allogeneic T cells	CD19	Australia, United States	recruiting	n.a.
NCT03728322	β-thalassemia	Autologous induced hematopoietic stem cells	HBB	not provided	not yet recruiting	(Ou et al., 2016)
NCT03747965	Mesothelin positive multiple solid tumors	T Cells	PD-1	Beijing, China	recruiting	(Hu et al., 2019)
NCT03690011	T-cell leukemia or lymphoma	Autologous CAR T Cells	CD7	Houston, United States	not yet recruiting	(Gomes-Silva et al., 2017)
NCT03872479	Leber Congenital Amaurosis Type 10	AAV vector with Cas9 and gRNAs	CEP290	Multiple locations, United States	recruiting	(Maeder et al., 2019)

Data obtained from the clinical trial database (NIH, 2020). Note: Three other studies in the clinical trial database do not actually treat patients and have, therefore, not been included here. n.a.= not available in the database and published preliminary work was not found through literature search either; EBV= Epstein-Barr virus; CAR= chimeric antigen receptor; CTL= cytotoxic T lymphocytes

Only three of the clinical trials apply actual gene therapy in the sense that it is aimed at permanently altering the genome in (part of) the subjects' cells. The first study is aimed at HIV-infected subjects that have developed hematological malignancies. HIV uses the CCR5 receptor on hematopoietic cells to enter and infect these cells (NCT03164135 in Table 1). Hematopoietic stem cell transplantation of cells from donors that lack CCR5 has been previously successfully used to reduce HIV infection to undetectable levels (Allers et al., 2011; Hutter et al., 2009). In this clinical trial, the *CCR5* gene is knocked out in allogeneic hematopoietic stem cells before they are transfused into the patients. In a study in mice, a plasmid containing the guide RNAs and Cas9 mRNA are transfected into the hematopoietic stem cells by nucleofection (Xu et al., 2017). Whether the same transfection method will be used in the clinical trial is not clear. The second study that applies gene therapy aimed at permanently altering the genome is the one altering the *HBB* gene in β-thalassemia patients (NCT03728322 in Table 1). In that study, autologous induced hematopoietic stem cells are treated *ex vivo.* A study in mice showed that the method could successfully correct the β-thalassemia without apparent adverse effects (Ou et al., 2016). The third study that applies actual gene therapy is the one described above for Leber congenital amaurosis type 10 (NCT03872479 in Table 1). This last one is the only clinical trial applying gene therapy *in vivo* (Maeder et al., 2019).

7. Discussion

The development of the CRISPR/Cas9 system as a gene-editing tool has, in only a few years' time, revolutionized the gene-editing field and accelerated research into gene therapy. The method is much faster and easier to optimize for new targets than any of the previously available methods. While not flawless yet, optimization of the system may still improve some of the current pitfalls. Regardless of these points of improvement, clinical trials are already underway that utilize the CRISPR/Cas

system. The development of the CRISPR/Cas9 system has definitely allowed us to enter a new era in which gene therapy is a viable option for the treatment of disease.

Thus far, only one of the clinical trials applies the CRISPR/Cas system to correct mutations in patients with a genetic disease. Many genetic diseases affect multiple tissues in the body, making it difficult to reach the cells in which a gene needs to be targeted. Targeting genes *in vivo* is difficult as it requires specialized administration methods that will allow, among others, delivery of the guide RNAs and Cas9 mRNA to cell nuclei and prevent premature degradation of the RNAs. For this reason, the genetic diseases for which treatment with CRISPR/Cas9 gene therapy is developed first will be genetic diseases that affect easily accessible tissues such as retinal or hematopoietic cells, or where the transfer of induced pluripotent stem cells (iPSCs) may be an option. Indeed, several preclinical studies have been performed to investigate the possibility of using CRISPR/Cas technology for gene therapy. Some examples are hemophilia B (Lyu et al., 2018), retinitis pigmentosa (Deng et al., 2018), sickle cell disease (Park et al., 2017; Wen et al., 2017), and β thalassemia (Mettananda et al., 2017). For genetic diseases that require delivery of the CRISPR/Cas9 system into a specific tissue *in vivo*, specialized delivery methods are being developed, such as various types of lipid nanoparticles (Kulkarni et al., 2018; Wang et al., 2018b) and viral vectors. That gene therapy *in vivo* is possible was recently shown in a mouse model of the human genetic disease Duchenne muscular dystrophy. Cas9 and guide RNAs constructs were packaged into an adeno-associated virus (AAV) vector that was delivered systemically to newborn and adult *mdx/Utr* mice. Both the newborn and adult mice were found to have restored cardiac dystrophin expression, albeit much stronger in the newborn mice (El Refaey et al., 2017), which are very promising results.

A critical examination of the lack of appropriate preclinical studies and the lack of testing of in a model organism other than mice has been performed for the first clinical trial that was announced in the USA (Baylis and McLeod, 2017). It concerns the study aimed at treating patients with melanoma, sarcoma, and multiple myeloma (NCT03399448 in Table 1). This critical examination is equally valid for most of the other clinical trials currently underway. Maybe even more so since for several, no preliminary data are publicly available.

The current review focuses on explaining the working mechanism of the CRIPR/Cas system, how it is currently used to facilitate research in model organisms, and on improving the profitability of various livestock and economic plants, and on the application of this system in the treatment of disease in humans. The strength of the review is that it provides a comprehensive overview of all aspects that are important to understand the CRISPR/Cas9 system and its applications.

8. Conclusion

Regardless of these valid critical notes, the CRISPR/Cas9 system appears to be the ideal tool for genome editing, whether it involves knock outs, knock ins, or subtler changes such as inserting specific mutations or the correction of mutations as in gene therapy. Care should be taken, however, not to take shortcuts with regards to ethical obligations for preclinical evidence, as adverse events can set back the progress of this field drastically.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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