Current advances regarding CRISPR/Cas and its potential as a gene therapy for monogenic diseases

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Keywords: CRISPR, Cas, gene therapy, monogenic disease, kidney disease

Abstract

Current advances regarding the application of CRISPR/Cas in both *ex vivo* and *in vivo* systems have paved a promising way for its application as a gene therapy. Amongst many others, monogenic (kidney) diseases present themselves as potential targets for treatment with CRISPR/Cas gene therapies, as they are caused by mutations in a single gene, often leading to a loss of function, which allows for the implementation of CRISPR/Cas knock-in strategies as a therapeutic agent for these diseases. Therefore, in this review, a short overview of the current variety of possible CRISPR/Cas techniques is presented, including Homology-directed repair (HDR) and Non-homologous end-joining (NHEJ) repair pathways, in addition to their application in more advanced and novel CRISPR/Cas techniques, such as Homology-independent targeted integration (HITI), Microhomology-dependent targeted integration (MITI), base- and prime editing. Hereafter, recently developed *ex vivo* and *in vivo* CRISPR/Cas techniques together with CRISPR/Cas clinical trials for the treatment of several monogenic diseases are described and discussed. Moreover, the potential limitations that come along with the use of CRISPR/Cas as a potential tool for gene therapy are also presented and critically discussed, including delivery methods, off-target effects, possible immune responses and the costs of the CRISPR/Cas. Lastly, we also present and theorize about the advances and adaptions needed for CRISPR/Cas to be a realistic gene therapy for monogenic kidney diseases.

Layman's summary

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated system (Cas) have been discovered around a decade ago, however since then it has slowly been developed into a gene editing tool. The improvements that have been made to the CRISPR/Cas system, allow for it to be used in both cells in a culture or directly in the body. One type of diseases that can be especially helped by further development of the CRISPR/Cas system, are monogenic diseases. These types of diseases are caused by a mutation in only one gene. Often, due to this mutation the function of the gene is lost as it is partly, or even completely missing. The fact that only one gene is affected, makes it a lot easier for the CRISPR/Cas system to fix. Recently, the number of CRISPR/Cas techniques that are available have increased and thus a growing number of mutations can be fixed. Besides the repair of a (partly) missing gene or the removal of a gene, now the editing of specific bases is, amongst others, also possible. Moreover, in this review the different techniques that have recently been developed for use in cultured cells and directly into animal models of monogenic diseases are discussed. From these studies, it

can be seen that repair of these mutated genes is often possible via CRISPR/Cas techniques. Moreover, even some studies have made it onto the clinical trial phases and are now being tested in humans. Preliminary results from these trials show great promise. Furthermore, we discuss the potential ways in which the discovered CRISPR/Cas techniques can be used for the treatment of monogenic kidney diseases, as currently no curative treatments are available and often a kidney transplant is needed. However, since the fact that repair of only one copy of the gene could already lead to disappearance of disease symptoms and thus present a clinical benefit for these patients, in theory, the success rate of gene repair in these monogenic kidney diseases could be high. Especially with the information from the current studies, a lot of possibilities are available, which are briefly discussed in two examples. However, with use of the CRISPR/Cas system, some limitations also arise, such as how to get the system into the body and possible side-effects that can come along with this. Nevertheless, there are also options available which can overcome these limitations and improve the technique. When all this information is taken together, it is obvious that CRISPR/Cas is a powerful tool for gene editing. Yet, it is also obvious that the technique still needs to be improved and adapted before it can be, safely, used in humans. However, overall, CRISPR/Cas proves to be a realistic therapy for the treatment of monogenic diseases and could therefore benefit this group of patients.

1. Introduction

Since the discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) around a decade ago, great improvements have been made with regards to its applications in both *in vitro* and *in vivo* systems, offering a powerful tool towards gene therapies[1]. Originally, the genomic structure of the CRISPR/Cas system was discovered in prokaryotes, when researchers observed non-repeating sequences separated by unusual repeated segments in a palindromic pattern[2–4]. In 2002, Jansen et al., discovered that such sequences, now called CRISPR loci, were associated with a set of adjacent genes; the CRISPR-associated system (Cas)[5]. Following studies reported, that this system was used as an adaptive immune response in prokaryote organisms, which use the CRISPR/Cas system to protect them from exogenous DNA, such as viruses[6]. Moreover, it was discovered that there are different types of CRISPR systems among different organisms, which can be grouped into two classes based on the different Cas proteins. The best-known and most widely used type, is type II CRISPR, which makes use of Cas9[7]. This Cas protein, together with its guiding RNA (gRNA), searches for foreign DNA in the cell by recognizing protospacer adjacent motifs (PAMs), which are short motifs that lie adjacent to the target sequence. When such motifs are recognized, the DNA is unwound by the Cas9:RNA complex and, if a base-complementary is detected, Cas9 will cleave the target strand, resulting in double-strand breaks (DSBs)[1]. This technique was later translated to be used as a gene disrupting system, which nowadays is known as a CRISPR/Cas knock-out[1].

In 2012, the CRISPR/Cas9 technology was polished and adapted into a genomic editing tool[8,9]. Since then, CRISPR/Cas9 genome editing has become a topic of great interest in biological and biomedical fields. The technique has been intensively studied and modified since its first discovery, and several different forms of application have since then been developed[6]. With the current advances of this technique and the great interest within the scientific community, the therapeutic potentials of editing, regulating and monitoring individual genes and thereby, potentially curing genetic diseases becomes more realistic[6]. Therefore, in this review we present

the current state of the art of the CRISPR/Cas system. In addition, we critically discuss the main challenges faced nowadays regarding its application as a cure for monogenic diseases.

2. The state of the art of the CRISPR/Cas system

To date, several different techniques have been optimized from the original prokaryotic CRISPR/Cas system and adapted to eukaryotic cells (Figure 1). CRISPR/Cas applications are mostly based on the different mechanisms of DNA double-strand break (DSB) repair and can be categorized into two groups: knock-out and knock-in, being the latter of more interest in the gene repair field. There are several CRISPR/Cas adaptations that allow the insertion of exogenous DNA into a specific position in the endogenous nuclear genomic DNA, each of them with its own pro's and con's (Table 1).

Table 1. Different CRISPR/Cas techniques. *This table displays an overview of the main CRISPR/Cas techniques, including their application, and general pro's and con's. The recently developed techniques HITI and MITI are derived from the original NHEJ technique. HDR = Homology Directed Repair, NHEJ = Non-Homologous End-Joining, HITI = Homology-Independent Targeted Integration, MITI = Microhomology-Dependent Targeted Integration.*

Technique	Function	Based on	Pro's	Con's
HDR	Knock-in	Cas9	Less error prone Highly efficient in fast dividing cells	Only small insertions Limited to cell cycle phase
NHEJ	Knock-out Silencing Knock-in	Cas9	Active throughout the cell cycle	Error prone
HITI	Knock-in	Cas9	Highly efficient in non/slowly dividing cells Active throughout the cell cycle Large insertions possible Dominantly error free Higher chances of forward integration	Off-target effects, introduction of INDELS and 5' and 3' ends of the insertion
MITI	Knock-in	Cas12a	Generates sticky ends Seamless junctions	Homology of the 5th base pair need to be known

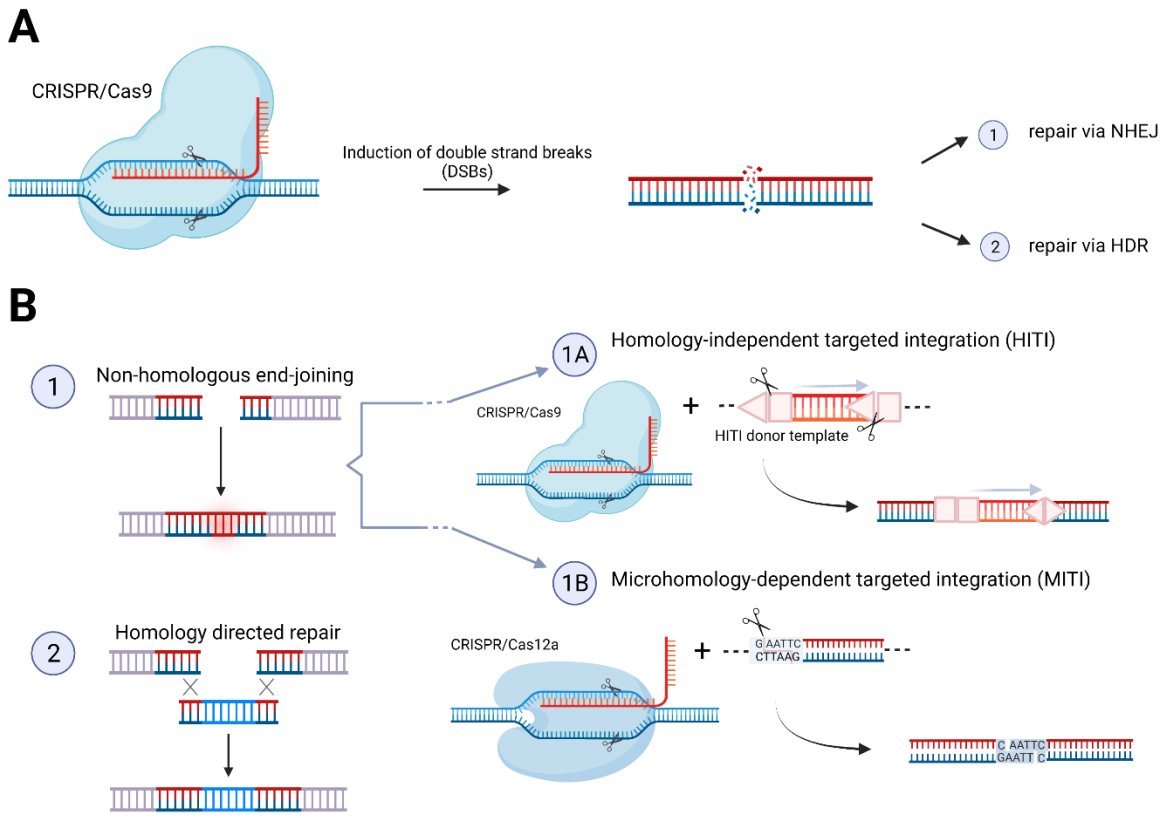

Figure 1. The CRISPR/Cas system and its different gene editing techniques *A) Here the original CRISPR/Cas9 system is depicted. The system introduces double strand breaks (DSBs) into the DNA, which can be subsequently repaired via two repair mechanisms; non-homologous end-joining (NHEJ) or homology-directed repair (HDR). B) On the left, the two primary mechanisms of repair are explained; NHEJ and HDR. At the right is depicted how two new techniques have been developed from NHEJ; HITI and MITI.*

2.1 Homology-directed recombination repair

Homology-directed recombination repair (HDR) makes use of a repair template via homology "arms" at the 5' and 3' sites of the DSB. Via the resection at the DSB sites, a terminal 3'-OH single-strand DNA is generated and the single-strand DNA subsequently initiates recombinant-dependent DSB repair. Since the DNA ends are non-ligable, non-homologous end-joining (NHEJ) repair pathways cannot be activated[10]. Moreover, HDR involves formation of a D-loop, in which, the non-base-paired strand of the invaded DNA is displaced. Therefore, the 3'-OH end can extend past the DSB to use the homologous DNA template for the reparation of the DSB. Moreover, due to its use of a template, the technique is more accurate in its restoration of genetic information when repairing DSBs[10]. The process then involves resolution, annealing and ligation to eventually complete repair of the DSB. As this technique of repair does not make use of indels, it is an error-free pathway, as opposed to the NHEJ pathway[10].

However, this repair mechanism only occurs during the G2 and S phase of the cell cycle. Moreover, due to its use of a template, the technique is more accurate in its restoration of genetic information when repairing DSBs[10]. Nevertheless, this template also requires cloning of the homology arm for each specific gene that is targeted. Although very specific, HDR is therefore also more time-consuming[11]. Additionally, although HDR is a less error-prone and therefore a more convenient technique for gene therapy, in practice, the use of this technique to

repair DSBs remains to be much lower than that of others. The reported efficiency of HDR to repair DSBs, is only 0.5-20% in mammalian cells, since most of the repairs of DSB still occur through the NHEJ mechanism. NHEJ accounts for 20-60% of the repairs caused by CRISPR/Cas9 editing[1]. To overcome this challenge, several techniques have been developed. These techniques aim to increase HDR efficiency, while subsequently also suppressing NHEJ. Small molecular NHEJ inhibitors, cell cycle synchronization and use of cell lines which are lacking NHEJ components have been developed amongst others for this purpose[1]. .

2.2 Non-homologous end-joining

NHEJ is a quick repair technique and is most often used by cells to repair DSBs, as it is active during all cell cycle phases[6]. Despite this, during the G1 phase NHEJ is most active[10]. The DSBs in the DNA can be recognized by a heterodimer protein Ku, which binds to the ends of the DNA that are broken. Via this binding, a scaffold is created that can recruit various other proteins and factors that are needed for NHEJ, which together ensure the proper positioning and sealing of the broken ends[10]. Eventually, with this method, ligable ends are formed, and subsequently, nucleotides can be added or deleted from the ends when needed. However, since this process mostly occurs without the need for an homologous DNA duplex, errors and mutations are more prone to occur during the NHEJ pathway[10]. Additionally, when CRISPR/Cas is used, the cut that is made in the DNA often results in a loss of several nucleotides and frameshift changes can therefore often occur. This can promote the silencing of edited genes. Therefore, the NHEJ pathway is most beneficial if silencing or knockout of a gene is required in regards to gene therapy[6].

2.2.1 Homology-independent targeted integration

As stated before, although HDR is less error-prone when repairing DSBs overall, NHEJ is more efficient when repairing DSBs in mammalian cells, since NHEJ is active in both proliferating- and non-proliferating cells. However, as NHEJ is more error-prone, improvements of this gene therapy would be highly beneficial[12]. In 2016, Suzuki et al., developed a NHEJ-based homology-independent strategy for gene therapy, which makes use of the CRISPR/Cas9 system to integrate double-stranded (ds)DNA[13]. This technique was named homology-independent targeted integration (HITI)[13] (Figure 2). With HITI, donor DNA which does not contain homology arms is used and, therefore, DNA repair via the HDR pathway is not possible. Moreover, the donor DNA sequence for the HITI method is flanked by Cas9 cleaves sites and the gRNA sequences in the reverse orientation. This leads to cleavage of both the genomic target and the donor DNA and prevents the sequence to get inserted in the reversed direction. Thereby, blunt ends are generated and subsequently, the donor DNA is used by the cell for the repair of the DSBs through NHEJ[12].

With HITI, in both dividing and non-dividing cells, DNA knock-in is possible and it can be used both *in vivo* and *in vitro*[13]. Moreover, the repair via this mechanism is dominantly error-free. In addition, with the use of specific gRNAs specification of the direction in which the repaired gene is inserted is possible. Hereby, the efficiency of the technique is heightened even further[12].

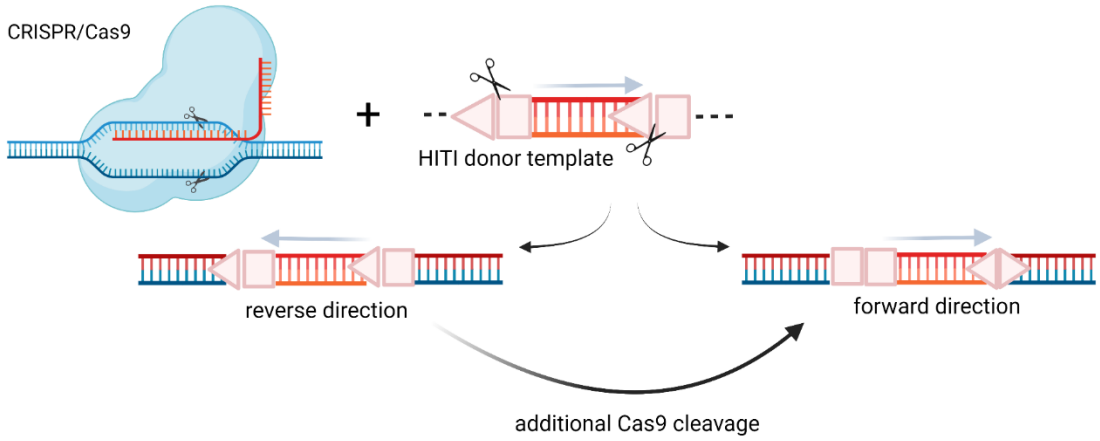

Figure 2. Homology-independent targeted integration (HITI). *The HITI technique makes use of the CRISPR/Cas9 system combined with a specific donor template that gets inserted into the target DNA. Via the inclusion of specific gRNAs and PAM sequences, inserted in the reversed orientation at the 5' and 3' ends of the template, the orientation of the inserted donor template can be specified. The orange/red base pairs represent the donor template. The triangle represents the gRNA sequence while the rectangle represents the PAM sequence (Cas9 cut site). After DSBs are induced by Cas9, if the two PAM sequences and the two gRNA-binding sequences land next to each other, Cas9 will not recognize the cut site and the sequence will get inserted in the forward direction. In contrast, if the PAM sequence lands next to the gRNA-binding sequence, Cas9 will recognize the cut site and will cut again, preventing the sequence to get inserted In the reverse direction.*

2.2.2 Microhomology-dependent targeted integration

Besides the widely used CRISPR/Cas9 combination, there are also several other types of Cas nucleases. Amongst them Cas12a, which can, unlike Cas9, process CRISPR RNA arrays into mature CRISPR RNAs. Hereby, Cas12a can target multiple genes at once. Moreover, whereas Cas9 generates blunt ends when cutting DSB, Cas12a generates sticky ends. Therefore, it was thought that Cas12a potentially has a better accuracy regarding gene knock-in when compared to Cas9. The method of gene therapy that was subsequently developed with this knowledge, depends on microhomology between five base pairs and is called microhomology-dependent targeted integration (MITI) (figure 3)[11]. This Cas12a-based method has a recognition site engineered into its donor vector, which is introduced via either PCR or T4 ligation techniques. The orientation of this target sequence in the donor vector lies in the opposite direction of the genome target, however, the last five base pairs of the genome target site sequence are identical to the five base pairs of the donor vector that lie distal to its PAM sequence. These identical base pairs enable the repair to generate a seamless junction[11]. When the previously mentioned HITI approach was compared with MITI, results showed that integrations mediated by Cas12a MITI were performed with an accuracy of 70% in comparison with HITI-mediated integration[11].

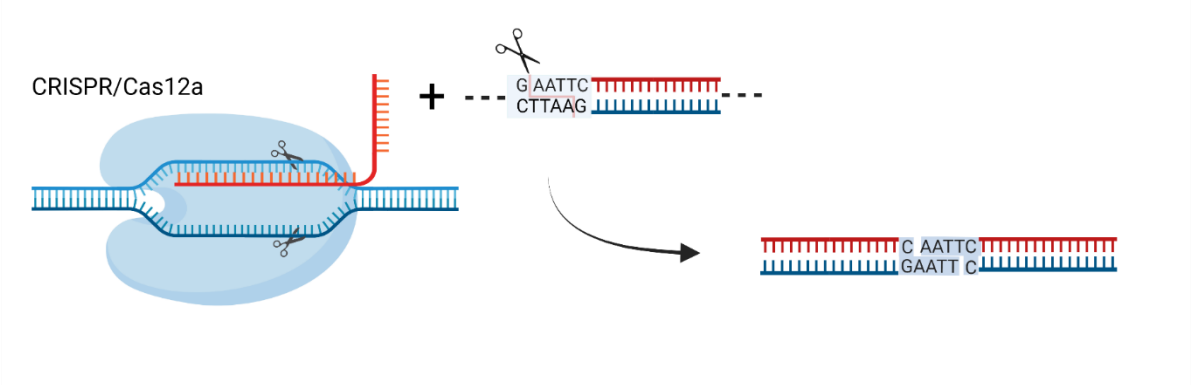

Figure 3. Microhomology-dependent targeted integration (MITI). *The MITI technique makes use of Cas12a instead of Cas9 and depends on microhomology between 5 base pairs as can be seen in the figure. These 5 base pairs induce sticky ends when the Cas9 nickase cuts here, depicted by the scissor. The Cas9 nickase cuts at a complementary place in the target DNA, inducing a complementary sticky end to that of the donor vector, making it so that they fit perfectly together.*

2.3 Base-editing

Besides editing of complete genes or parts of genes, the CRISPR-Cas system can also be used to edit specific bases. Cas9 has two functional domains that can be modified in order to deactivate them without affecting the overall binding activity of Cas9. In this context, deactivated Cas (dCas) and Cas nickase (nCas) can be generated and used for screening or monitoring. Moreover, when fused with other proteins or molecules, these modified Cas proteins can be used for editing of a specific base[6]. Via the fusion of nCas9 endonuclease with a base-modification enzyme, such as deaminases, a CRISPR/Cas base editor (BE) technology emerges[14]. Currently, there are adenine base editors (ABEs) and cytosine base editors (CBEs) (Figure 4). These two classes of base editors can together convert all four possible nucleotide changes. More recently, a dual-deaminase CRISPR/Cas base editor was developed which allows the combination of ABE and CBE simultaneously[15–17]. Since most genetic disorders in humans are often caused by either a point mutation or single-nucleotide polymorphism (SNP), DNA base editors hold great potential as therapeutic agents[6]. For instance, In 2016, Komor et al., showed that the CBE

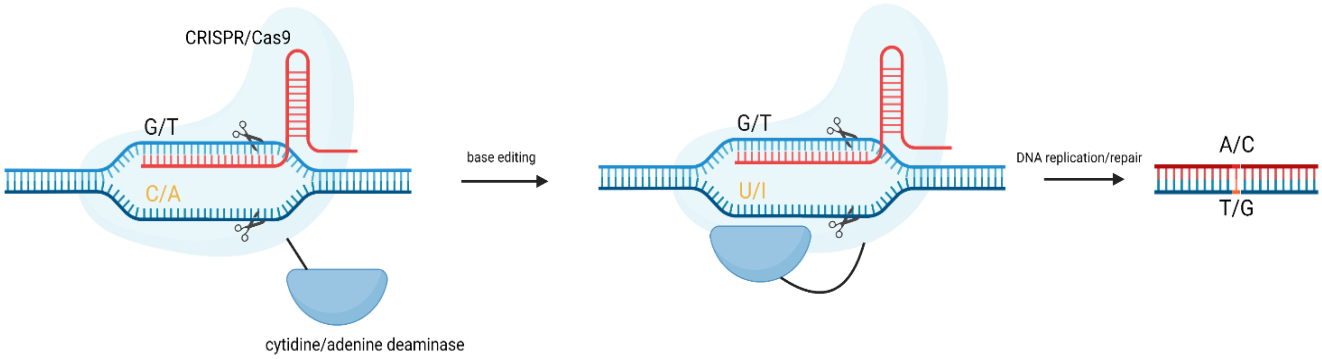

Figure 4. Cytidine and adenine base editing. *In this figure both adenine and cytidine base editing is showcased. On the left can be seen how a cytidine or adenine deaminase is added to the CRISPR/Cas9 system. In the next step, on the right, can be seen how this deaminase can change either an C to U or an A to I. Subsequently, after replication or repair of the DNA, a base is changed from A to T or C to G and CRISPR/Cas9 base editing has taken place.*

technique, which had an on target editing efficiency of 35-75%, can be used to repair point mutations associated with cancer and Alzheimer's disease in both mouse and human cell lines[14].

2.4 Prime-editing

In 2019, Anzalone et al., created a new application of the CRISPR/Cas technique[18]. To develop this technique, so-called prime editing (Figure 5), the CRISPR/Cas9 system was changed in two major ways. The first change involves the fusion of a reverse transcriptase to the Cas9 nickase. The second change constitutes of the traditional gRNA being changed by a prime editing guide RNA (pegRNA), which is linked to a gene-specific RNA sequence. This specific sequence contains a primer binding site (PBS), which is complementary to the target region on the edited DNA[18].

With prime editing, the CRISPR/Cas9 is guided to its target sequence as naturally occurs. However, when it binds and nCas9 cuts, a single strand break (SSB) is formed and the broken DNA sequence then serves as a primer at which the PBS from the sgRNA can bind. Consequently, a new DNA fragment is synthesized via CRISPR/Cas9-fused reverse transcriptase. This new DNA fragment is then inserted, repairing the break. Via this mechanism, specific DNA sequences can be replaced with CRISPR/Cas9 prime editing without the necessity of a DNA template[18].

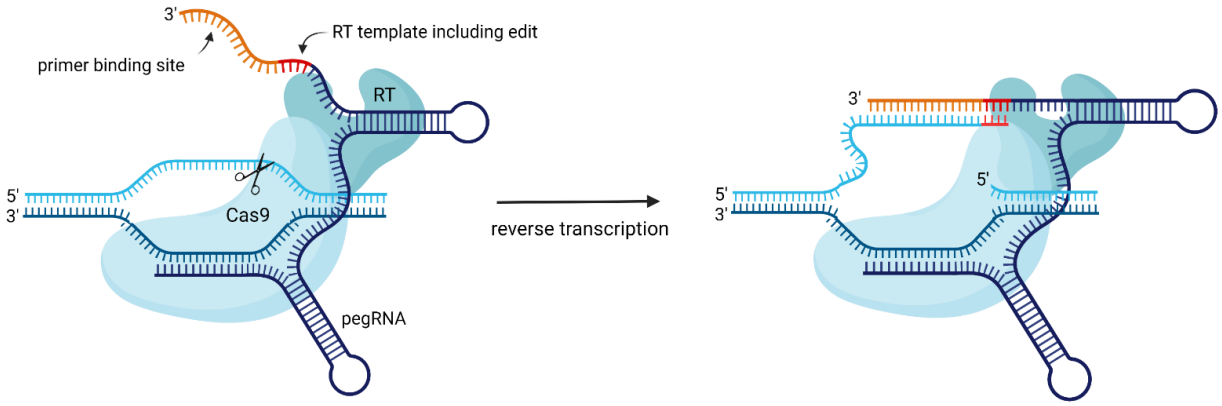

Figure 5. Prime editing. *Here, the process of prime editing is shown. At the left can be seen that a reverse transcriptase (RT) is added to the Cas9 protein. Moreover, the system now also contains a pegRNA together with a primer binding site (PBS) which can be seen in orange. When reverse transcription takes place, the target DNA binds to the PBS after Cas9 has created a single strand break. After binding, reverse transcription can take place and the DNA is repaired.*

3. Current progress and challenges

Since the discovery of the CRISPR/Cas system and its potential as a therapeutic agent, a lot of advances have been made regarding research into its therapeutical implementations. Monogenic diseases are of special interest for treatment with the CRISPR/Cas system as they are caused by a single mutated gene. In this section, relevant studies that implemented the CRISPR/Cas9 system *ex vivo* and *in vivo* to date will be discussed. Additionally, the CRISPR/Cas-based therapies that are currently in clinical trials stages are also summarized.

3.1 *Ex vivo* techniques

Many studies have been done regarding *ex vivo* applications of the CRISPR/Cas technique. Below these will be discussed, these applications of CRISPR/Cas mostly focus on both the knock-in and knock-out of genes to cure monogenic diseases *ex vivo*.

3.1.1 Targeting the R803X mutation in a ADPKD cell line

In 2020, Liu et al,. aimed to develop a gene corrected human isogenic cell line from polycystic kidney disease with the use of CRISPR/Cas9[19]. Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent monogenic kidney disease and often results in severe kidney failure, therefore, the creation of a gene therapy is of great importance. ADPKD is caused by a mutation in the gene R803X, however the exact mechanisms by which R803X is involved in the ADPKD pathogenesis is unknown. To elucidate this mechanism, an isogenic cell line was created with the use of a CRISPR/Cas9 system. This system, together with sgRNAs targeting the *PKD2* mutation (R803X, Chr4: 88989098C > T), was transfected via a plasmid containing single-stranded oligodeoxynucleotides (ssODN). These ssODNs of 35 base pairs were designed to have the same sequence as the wild type allele of the gene. Subsequently, the plasmids were nucleofected into IBMS-iPSC-014-05, after which a selection was made of successfully transduced cells. Subsequently, IBMS-iPSC-014-C cells were taken in which the mutation had been reverted back to the wildtype X803R from this selection. With this technique, an isogenic control (IBMS-iPSC-014-C) was successfully created from the original IBMS-iPSC-014-05 cell line with the use of a CRISPR/Cas9 system. The results showed that the mutation was successfully corrected, while normal pluripotency, morphology and differentiation capacity of the cells was maintained, hereby a first step towards a new gene therapy for treatment of ADPKD was made.

3.1.2 Targeted integration of an AGT transgene for PH1 treatment

In another study, Estève et al., (2019), aimed to create an *ex vivo* technique for the treatment of primary hyperoxaluria type 1 (PH1)[20]. PH1 is also a metabolic monogenic disease that is caused by endogenous overproduction of hepatic oxalate, due to a mutation in the gene that encodes for alanine-glyoxylate aminotransferase (AGT). This enzyme is mainly responsible for the catalyzation of glyoxylate into glycine, when mutated glyoxylate is overproduced and is then converted to oxalate through catalyzation by lactate dehydrogenase (LDH), leading to build up of oxalate. This can subsequently cause end-stage renal disease to arise[21]. In this study, the aim was to create autologous gene-corrected hepatocytes, which after correction can be transplanted back into the patient[20]. To do so, they aimed to integrate the *AAVS1* site of patient derived PH1-iPSCs with a PKG promotor-driven codon-optimized AGT transgene via targeted integration. When the iPSCS subsequently differentiate into hepatocyte-like cells (HLCs), rescue of AGT expression can be achieved. This was done via the transfection of plasmids containing sgRNAs targeting the *AAVS1* locus and the CRISPR/Cas9 system into PH1-iPSCs, which successfully altered the mutation and restored AGT expression after *in vitro* differentiation of hepatic cells. Importantly, no off-target effects were observed.

3.1.3 CRISPR/Cas mediated knock-out of IVS26 in a cellular LCA10 model

Leber congenital amaurosis type 10 (LCA10) is a monogenic disease caused by bi-allelic loss-of-function mutations in the CEP290 gene, which leads to poor to no vision presenting in early infancy[22]. Ruan et al., (2017)

aimed to develop an *ex vivo* technique to treat LCA10 patients with a specific IVS26 mutation with CRISPR/Cas9 based gene therapy[23]. They targeted the IVS26 mutation, a splice mutation in the *CEP290* gene, with a plasmid containing specific pairs of sgRNAs and Cas9 to excise the intronic fragment that contains the mutation. Moreover, the plasmid contained ssODNs with 75 nucleotide homology arms flanking both sides of the mutation. This technique was used in a cellular model of LCA10, which was created by introduction of the IVS26 mutation via CRISPR/Cas9 and HDR repair in a HEK293FT cell line. Subsequent transfection of this cell line with the CRISPR/Cas9 system plasmid, lead to efficient correction of the aberrant splicing of the *CEP290* variant and expression of the wild-type *CEP290* was restored.

3.1.4 Adenine base editing as a treatment for SCDs

Lastly, Newby et al., (2021) aimed to create a CRISPR/Cas9 based gene therapy as a treatment for sickle cell disease[24], which is caused by a mutation in the *HBB* gene encoding for β-globin. This mutation results in hemoglobin polymerization and a subsequent sickled shape of red blood cells, leading to various symptoms and microvascular occlusions. Here, they tried to create an adenine base editor (ABE8e-NRCH) to convert the sickle cell disease allele into a Makassar β-globin, HBB^{G}, which is a nonpathogenic variant. To target the mutant allele, a phage-assisted continuous evolution (PACE)-generated Cas9-NRCH was used, combined with TadA-8e, a deoxyadenosine deaminase which is highly effective for base editing. Via this mechanism, ABE8e-NRCH was generated, which was then co-delivered into HBB^{S} HEK293T cells with sgRNAs targeting HBB^{S}. Hereby, a 58% conversion of HBB^{s} to HBB^{G} was achieved. Moreover, this system was also used to edit human HSPCs from patients with sickle cell disease, in which editing of HBB^{s} to HBB^{G} was achieved with an efficiency of up to 80%, while an efficiency of 68% was observed in bone marrow repopulating HSPCs. This led to a reduction of 47.7% to 16.4% of sickling frequency in these cells. Moreover, no significant off-target effects were observed and when these corrected cells were transplanted into adult humanized SCD mice, efficient bone marrow editing of the mutation was observed and the gene correction threshold was exceeded.

3.2 *In vivo* techniques

Besides the achievement in *ex vivo* research and despite the fact that most of the CRISPR/Cas-based research is still at an early pre-clinical stage, especially focused on 2D *in vitro* disease models, several potential therapies have made it to the *in vivo* trials. Most of them are based on either gene knock-in or knock-out and target monogenic diseases, especially metabolic diseases are often focused on.

3.2.1 Epigenetic remodeling of the mdx gene with CRISPR/Cas packaged AAVs

In 2017, Liao et al., aimed to *in vivo* activate endogenous target genes by using trans epigenetic remodeling via a CRISPR/Cas system[25]. Here, they sought to develop a system in which the fused nuclease dCas9 was separated from the transcriptional activators so the whole complex could be fitted into an AAV delivery system. To do so, amongst others, they designed short sgRNAs from 14 to 15 base pairs that guide the wild-type Cas9 to its target sequence. With this technique, no DSBs are made while gain-of-function can be achieved due to the use of dead sgRNAs. This CRISPR/Cas technique was optimized to treat several different genetic diseases as a proof-of-concept such as acute kidney injury, type 1 diabetes, and Duchenne muscular dystrophy. When this optimized system was injected into the fore- and hindlimb muscles of *mdx* mice, a model for Duchenne muscular dystrophy,

disease symptoms were ameliorated. Altogether, with this technique they achieved the modulation of histone marks and, hereby, transcriptional activation of target genes *in vivo*. This proves that physiological relevant phenotypes can be generated without the need to create DSBs, avoiding unwanted genetic mutations. Despite the great benefit that this technique offers, it does still need to be determined if it possibly activates the host immune response[25].

3.2.2 Knock-in of the FAH gene in HT1 rabbits

In a study by Li et al., 2021, they aimed to treat hereditary tyrosinemia type 1 (HT1) with CRISPR/Cas9-mediated gene correction[26]. HT1 is a monogenic metabolic disease resulting from a mutation in the enzyme fumarylacetoacetate hydrolase (FAH), which leads to the accumulation of toxic metabolites and subsequently severe liver failure, and renal tubular dysfunction in the first weeks of life. In this study, CRISPR/Cas9 donor templates, targeting the *FAH* gene were delivered *in vivo* using an AAV and 15 days old HT1 rabbits. This *FAH* mutation, that carried a 10 base pair deletion, was targeted for a knock-in of the correct FAH gene via the CRISPR/Cas9 system. The AVVs were 8-packaged Cas9, FAH sgRNA and contained donor templates. The chosen sgRNA showed a 35% efficiency in creating a 3N+1 base pair insertion or 3N+2 base pairs deletion, both sufficient to transform the out-of-frame mutation into in-frame mutations. The DSBs that were created were repaired via both NHEJ- and HDR, with an efficiency of 0.90%-3.71% and 2.39%-6.35%, respectively. The results showed that with this CRISPR/Cas9 system, the gene could be successfully corrected, resulting in recovery of liver function, decrease in damage to both the liver and kidneys and, ultimately, the lethal phenotype of HT1 rabbits could be rescued. Importantly, no significant off-target effects were found when 10 potential off-target sites were screened for. None of these potential off-target sites showed more alterations in their sequences than were observed in wild-type animals. Lastly, when the rabbits reached adulthood, they could even produce healthy offspring.

3.2.3 Targeting knock-out of the LDHA gene to cure PH1

In a study done by Zheng et al., (2020), they aimed to disrupt the efficiency of LDH by creating insertions and deletions with CRISPR/Cas9, as a potential cure for PH1[21]. To achieve this, they created two separate AAV vectors; one containing Cas9 and the liver-specific promotor-1 (LP1) and another which contained sgRNAs specifically targeting the *LDHA* gene[21]. The experiments were carried out in seven days old *Agxt*^{D205N} rats, in which they observed that in 18.6% of the *LDHA* alleles, indels could be detected, which correlated with the decrease in LDH expression. Furthermore, treated rats showed significantly lower levels of urinary oxalate then PH1 controls groups. These results showed that the CRISPR/Cas9 treatment was efficacious in treating the symptoms and phenotypes of the PH1 rat model. In addition, no off-target effects nor liver injury were detected.

3.2.4 Disruption of the Hao1 gene in mice to cure PH1

A second group also aimed to use a CRISPR/Cas9 based therapeutic approach for PH1. Zabaleta et al., (2018), aimed to create an *in vivo* CRISPR/Cas9 mediated substrate reduction therapy targeting non-essential enzymes of the glyoxylate pathway[27]. More specifically, they established an AAV-based CRISPR/Cas9 system that targets glyoxylate oxidase via the disruption of the murine *Hao1* gene. To do so, they designed a single AAV system, containing Cas9 and two specific *Hao1* sgRNAs. This vector was subsequently transfected in *Agxt1*$^{-/-}$ mice via an intravenous route, after which the presence of indels in the livers of the treated mice was reported in 42.12%

and 56.32% of the cases. Furthermore, western blot analysis also showed a significant reduction in protein expression of glyoxylate oxidase. When analyzing the symptoms of treated mice, they presented with significantly less symptoms and lower levels of urine oxalate were observed, which were similar to those of wild type animals. In addition, the treatment also showed protection against the formation of kidney stones, a common symptom of PH1. When looking at potential off-target effects, none were observed. These results indicate that the here used CRISPR/Cas9 therapeutic approach is tissue-specific.

3.2.5 Targeting IVS26 with EDIT-101 in a LCA10 mouse model

In a study, done by Maeder et al., (2019), they aimed to develop a gene therapy to target the most common LCA10-causing mutation; IVS26 (A>G)[22]. The method by Maeder et al., used an AAV5 vector, containing both Cas9 and a sgRNA specific for CEP290 and named this method EDIT101. To test the technique they first used a human CEP290 IVS26 knock-in mouse model; HuCEP290 knock-in mice. These mice were treated with 1 microliter of 1 x 10^{12}vg ml $^{-1}$ of EDIT-101 via subretinal injection, here the editing rate of the therapy was 21.4%. However, to achieve a clinical benefit 10% of the focal cones need to be functionally rescued, which can be achieved by administration of a minimum dose of 3 x 10^{11}. In this dosing scheme 62% of the treated eyes achieved this functional rescue. Moreover, at a dosage of 1 x 10^{12} to 1 x 10^{13} vg ml^{-1} a functional rescue was observed in >94%. However, to establish the level of productive editing in a retina more similar to humans, the experiments were also carried out in non-human primates by using a surrogate pair of gRNAs. In cynomolgus monkeys, it was shown that productive editing rates of 3.5%, 16.1% and 27.9% were achieved at dosages of 1 x 10^{11}, 7 x 10^{11} and 1 x 10 vg ml^{-1} dose groups, respectively. When looking at possible side effects, such as an immune response against Cas9, none were detected. Nevertheless, in non-human primates an immune response against AAV5 was observed, however gene-editing efficiency remained high. Moreover, no significant off-target effects were observed[22].

3.3 Clinical trials

Besides all the current efforts for *in vivo* and *ex vivo* CRISPR/Cas9 techniques, three potential therapeutic techniques for monogenic disorders have advanced towards clinical trials recently. Additionally, clinical trials for CRISPR/Cas9-based therapies for other diseases, such as cancers and infectious diseases, have also started clinical trial stages.

In 2020, the first attempt at treating a monogenic disease with CRISPR/Cas9 was made. Researchers tried to treat sickle cell disorders, more specifically, transfusion dependent Beta-thalassemia (TDT) and sickle cell disease (SCD) via an *ex vivo* mechanism[28]. Both diseases are caused by mutations in the hemoglobin β-subunit gene (*HBB*). While in TDT this mutation leads to little to no β-synthesis, in SCD the mutation causes deoxygenated hemoglobin molecules to polymerize, which subsequently leads to erythrocytes with a sickled form. Previous therapies and studies have shown that when fetal hemoglobin levels are increased, both in TDT and SCD, it improves morbidity and mortality parameters. Therefore, in this clinical trial they aimed to heighten the production of fetal hemoglobin via repression of the *BCL11A* gene. Single-nucleotide polymorphisms (SNPs) in the *BCL11A* locus, which downregulate its expression, have been proven to be associated with increased expression of fetal hemoglobin in adults. Here, they used CRISPR/Cas9 to reduce BCL11A expression at the erythroid-specific

enhancer region of *BCL11A* in hematopoietic stem- and progenitor cells (HSPCs). When these cells were treated with the CRISPR/Cas9 system, namely CTX001, approximately 80% of the BCL11A alleles were modified. Moreover, no evidence of off-target effects were found. Consequently, synthesis of fetal hemoglobin was reactivated and restored. When these autologous CRISPR/Cas9-edited CD34+ HSPCs were subsequently injected into patients with TDT and SCD, both patient groups showed high levels of allelic editing in both their blood and bone marrow during a one year later follow-up, together with increases in fetal hemoglobin and an independency of transfusions.

Also in 2020, a clinical trial for the treatment of ocular disease with CRISPR/Cas9 was set up[29], which was the first *in vivo* CRISPR/Cas9 clinical trial to be started. The trial, called BRILLIANCE, aims to treat LCA10 with a CRISPR/Cas9 system; EDIT-101. Preliminary results, released by Editas medicine at the end of 2021 showed positive data, including a positive safety profile, during the 15 months of observation. Only mild adverse events were observed, which were mostly related to the injection of the drug into the retina. Moreover, immune responses against Cas9 were not observed. Furthermore, in the mid-dose cohort ($1.1 \times 10^{12-}$ vg/ml), clinical evidence of gene editing has been observed. Here, patients showed improvements in full-field light sensitivity threshold (FST) testing, best corrected visual acuity (BVCA) and/or improvement in the ability of patients to navigate standardized navigation courses with varying difficulty levels. As these preliminary results are promising, the trail goes onwards.

In 2021, Gillmore et al., aimed to find a therapeutic CRISPR/Cas9 system for the treatment of transthyretin amyloidosis (ATTR)[30]. This disease, caused by a mutation in the TTR gene, results in accumulation of misfolded transthyretin (TTR) protein, which mainly takes place in the nerves and heart, but also in other tissues. Here, they developed an *in vivo* gene-editing agent called NTLA-2001, which is designed to reduce the concentration of TTR in the serum and hereby alleviating ATTR symptoms. In contrast to the other studies, were they often used an AAV to deliver the therapy, here they chose to use a lipid nanoparticle. This particle encapsulates the messenger RNA for the Cas9 protein and contains a sgRNA which specifically targets the *TTR* gene. Lipid nanoparticles can be used in this instance, as TTR is mostly produced in the liver. The lipid nanoparticle is specifically designed so that, once in the liver, plasma apolipoprotein E can bind to the surface of the lipid nanoparticle leading to its opsonization. Hereafter, the particle is actively endocytosed by these hepatocytes, leading to the release of the CRISPR/Cas9 system. By making use of lipid nanoparticles, the efficacy is maximized while simultaneously minimizing potential systemic toxic effects. Moreover, NTLA-2001 will be administered via intravenous infusion, when the liver is reached, TTR in hepatocytes will be edited, which will ultimately lead to a decrease in the production of mutant, but also wild type TTR. In earlier preclinical studies, a single dose of NTLA-2001 showed promising results with a >95% reduction in serum TTR protein. When infused in patients, during the first 28 days, little to no adverse events were observed, while adverse events that did occur were only mild. Moreover, when the mean reduction in serum TTR protein levels were measured at day 28 compared to baseline, a reduction of 52% was observed in the 0.1 mg/kg dose. In the group that received a 0.3 mg/kg dose this reduction was measured to be 87%. This shows that the targeted knockout of *TTR* by use of NTLA-2001 seems to be effective in patients with hereditary ATTR amyloidosis.

4. Future applications of CRISPR/Cas-based gene therapies for monogenic (kidney) diseases

As became evident from the studies discussed above, one of the major challenges when using CRISPR/Cas is the efficacy of the system, which is often <50%. Although this is something that still can be improved, studies have shown that, in most cases, a clinically significant effect could already be observed. In most studies, amelioration of symptoms and/or a significant lower level of the targeted protein was shown. This points towards the possibility that only a small percentage of all the genes or protein needs to be altered to reach a clinical benefit. The study from Maeder et al., (2019) where a CRISPR/Cas9 therapy for LCA10 was developed further proves this point[22]. As in this study, only 10% of the focal cones needed to be rescued for a clinical benefit to be observed amongst patients.

Moreover, although, as discussed below, off-target effects are often a great worry when CRISPR/Cas as a therapeutic agent is discussed, in the (pre)clinical studies mentioned above, off-target effects often proved to be very limited if not even present at all. This lack of observed off-target effects, even when predicted off-target effects were extensively searched for, seems to be very promising in regards to the safety of the techniques. Therefore, it can be hypothesized that the CRISPR/Cas techniques developed above, are more safe than originally expected.

A further observation, and potential limitation, that can be made from the few studies that have made it onto the clinical trial phases, is that the therapeutic agents are all targeted towards a specific tissue, such as EDIT-101 which is directly injected into the eye[22] or the *in vivo* technique for ATTR amyloidosis which makes use of lipid nanoparticles which specifically target hepatocytes in the liver where the disease is most prominent[30]. From this we can learn that to be most successful, at least in this stage of CRISPR/Cas technology, it is most effective to make a system that can be directly targeted towards the target tissue. On one side, this is a great benefit as targeted delivery of the CRISPR/Cas system heightens efficacy and limits off target effects. However, on the other side this can also be a limitation as targeted delivery to a specific tissue is not always possible. In the case of monogenic kidney disease, it would therefore be ideal to design either an AAV or lipid nanoparticle that can be directly targeted towards the kidneys, or in the case of other diseases, other tissues. However, currently, such specific delivery methods are not on the market yet for the kidneys, therefore it would prove beneficial to do more research in this field. Currently, there is some research being done regarding low molecular weight protein (LMWP) lysozymes. These lysozymes are especially suitable as drug carriers for targeted delivery to the kidneys[31]. However, as these carriers are quite small, the CRISPR/Cas system is too big to be packaged in there. Nevertheless, it would be of great interest to develop this technique further and to find a solution for the weight limitations. Moreover, one possibility would be to inject the therapy directly into the kidneys via the back. However, as this is a very invasive procedure, research into other delivery methods is needed.

When looking at chronic kidney diseases (CKD) alone, approximately 450 monogenic causes have been identified to date[32]. As these disorders are caused by a genetic mutation, the current treatments remain to be only symptom relieving, rather than curative, although they are especially suited for gene therapy as only one gene needs to be targeted. Therefore, the need for gene therapies is pressing. It is estimated that there are around 5,000-8,000 monogenic diseases[33].

Of the total cases of CKD, about 30% of the pediatric cases can be attributed to monogenic causes, while in the adult population, around 5-30% of total CKD cases has a monogenic origin. However, these numbers are possibly still underestimated, as not all monogenic CKD disorders have been identified yet[32]. Moreover, within monogenic diseases a division can be made between autosomal recessive (AR) diseases and autosomal dominant (AD) diseases. In AR monogenic diseases, both parental alleles of the gene need to be mutated in order for the disease to be present, as opposed to AD monogenic diseases, in which the diseased phenotype can already arise when only one parental allele is affected[32]. Since most of the monogenic kidney disease are due to a loss of function of the gene, these types of diseases are especially viable for treatment with CRISPR/Cas knock-in techniques. By implementing knock-in strategies to either introduce the part of the gene that is missing or to re-introduce the whole gene that is deleted, these diseases could potentially be cured. One such monogenic kidney disease that could benefit from CRISPR/Cas treatment, is Nephronophthisis. This renal disorder comprises different types, of which type 1 is the most prevalent and could benefit the most of a CRISPR/Cas based therapeutic agent. Nephronophthisis type 1 is tubulo-interstitial kidney disease, in which the kidneys become progressively smaller over time[34]. Type 1 Nephronophthisis already arises in juvenile years and end-stage renal disease is already observed around 13 years of age[35]. This specific type is caused by a homozygous deletion mutation in the *Nephrocystin-1* (*NPHP1*) gene on chromosome 2q13[36]. With the use of a CRISPR/Cas knock-in the deleted gene can be put back in via use of a template. Another example that could benefit from a CRISPR/Cas based therapy is Nephropathic Cystinosis. This disease leads to a lysosomal storage disorder and presents with renal failure at the age of 10 years. The symptoms are caused by mutations in the *CTNS* gene, being the most prevalent one a deletion of 57 kilobases. Since the most prevalent mutations are deletions in the *CTNS* gene, all mutant variants could be potentially repaired via a CRISPR/Cas knock-in method by which the healthy gene sequence can be reintroduced[37].

Overall, most genetic kidney disorders arise in an AR manner. Therefore, since both copies of the gene need to be affected, repair of only one allele could already ameliorate disease symptoms and present a clinical benefit. Therefore, in theory, the success rate of gene repair in these monogenic kidney diseases could be high.

5. Potential hurdles

Although CRISPR/Cas and its various applications have shown potential as a gene editing technique, it also holds some limitations that need to be addressed, such as the delivery methods used, off-target effects and potential immune responses.

5.1 Delivery methods

Throughout the years, several delivery methods have been developed, which on their own all come with separate benefits and limitations. However, an effective delivery method is as important as an effective CRISPR/Cas system itself, since without it, the therapy cannot reach its target. Although a vast majority of delivery methods has been developed, many can still not be used directly in human gene therapy since they show damage to cells, toxicity, cause an immune response or have significant off-target impact.

5.1.1 Viral delivery systems

AAV is wildly used as a delivery method for CRISPR/Cas therapies, as it holds many benefits. First, when used in humans, it does not cause nor relate to any diseases. Moreover, there are many different AAV serotypes, which opts for choosing the most suiting serotype for targeting of specific cell types or a specific function[38]. Second, they are applicable *in vitro*, *ex* vivo and in *vivo*[1].

Although little to no immune responses are observed against AAVs and they show little toxicity, when administered more often, immune responses and toxicity can potentially develop[39]. In addition, AAVs generally can only be packaged with ~4.5-5 kilobases of genomic material[40] and CRISPR/Cas9 systems often exceed this number, especially when other elements such as DNA templates or multiple sgRNAs are used[1]. To overcome these drawbacks, several altered AAV methods have been developed. For instance, the delivery of the CRISPR/Cas system via packaging into two separate AAV particles that can then be used for co-infection. Moreover, recently Xiaoshu et al., (2021) created an engineered miniature CRISPR/Cas system named CasMINI[41] where they applied RNA- and protein engineering to create a functional type V-F Cas12f system which is a much smaller Cas compared to Cas9 or Cas12a, making it easier to package into AAVs, or other delivery systems. Although smaller, this CasMINI showed the same activity as Cas12a and had no detectable off-target effects[41].

Besides the commonly used AAVs, there are also other viral delivery systems such as lentiviruses and adenoviruses. However, these are much less used as they often elicit strong immune responses and integration into the host cells cannot be ruled out. Furthermore, more off-target effects are observed with these techniques making them even less suitable[1].

5.1.2 Lipid nanoparticle delivery methods

Another delivery method that has been briefly discussed and is on the rise is the use of lipid nanoparticles. This non-viral method of delivery is more attractive as it does not contain any viral components, leading to less safety and immunogenicity concerns. Moreover, these particles can also be used for *in vitro*, *ex vivo* and *in vivo* delivery, making them very useful. However, when using lipid nanoparticles as a CRISPR/Cas delivery method, these need to be transported through both external and internal barriers which can form a problem for delivery to various organs. Furthermore, lipid nanoparticles, once in the cell, become part of the endosome. This endosomal pathway can rapidly change into a lysosomal pathway, leading to the degradation of the lipid nanoparticle and its content, which is a major limitation to the efficacy of the gene editing. Moreover, even when the particle does not go into the lysosomal route, it needs to escape the endosome and translocate to the nucleus. These are all points at which the efficacy of the technique potentially lessens[1]. In addition to the lipid nanoparticle designed to treat ATTR amyloidosis[30], other techniques have also been developed recently. In 2017, Miller et al., developed a zwitterionic amino lipid (ZAL) for the co-delivery of long RNAs such as Cas9 and sgRNAs[42]. This method led to permanent DNA editing observed through a >90% reduction in protein expression. Moreover, in 2020, Cheng et al., developed a selective organ targeting (SORT) nanoparticle which can deliver CRISPR/Cas systems and mRNA in a tissue-specific manner[43]. Here, they systematically engineered multiple classes of lipid nanoparticles, by which the lung, spleen and liver could be targeted after intravenous administration.

5.2 Off-target effects

It has been shown that the CRISPR/Cas system, besides editing the targeted gene, often leads to off-target effects. Although many efforts have already been put into limiting off-target impact, this effect is still commonly observed in preclinical studies, which leads to a suboptimal safety of the technique and also limits further in human application of this gene therapy technique[6]. The off-target effects are most often caused by the sgRNAs, when the 18-20 long nucleotide protospacer sequence in the sgRNA does not recognize the target sequences, this leads to off-target cleavage[44]. However, these off-target effects, amongst others, can be contained by improvements of the Cas proteins. For instance, when Cas9 is edited so that it only induces SSBs, unless the forward and reverse DNA sequences are both bound by a Cas9, off-target effects are limited since these are then only SSBs, which can be repaired through a simple DNA ligase mechanism[44,45]. Moreover, designing and choosing the right delivery method for the CRISPR/Cas system can also aid in the limitation of off-target effects[6]. As described above, besides advances in the CRISPR/Cas system, many advances are also being made in the development of delivery systems, leading to a decreasing number of observed off-target effects.

5.3 Immune responses

Besides the delivery and efficacy of the CRISPR/Cas system itself, a burden also lies with the reaction of the human body to the CRISPR/Cas system. As the CRISPR/Cas system is adapted from bacteria and archaea, the human body has an immune response against these infectious agents and therefore also against the CRISPR/Cas system. The chance of an immune response is especially significant, as the Cas enzymes are often obtained from *Streptococcus pyogenes* and *Staphylococcus aureus*, both known for infection of humans[6]. In a study by Charlesworth et al., (2019), it was shown that patients had IgG antibodies against *Streptococcus pyogenes* and *Staphylococcus aureus* at a rate of 78% and 58%, respectively[46], which suggests that humans already have a pre-existing immune response against these wildly used Cas9 proteins. This immune response can influence the efficacy of the therapies and therefore is something to keep in mind. Moreover, immune responses can not only be caused by the Cas enzymes themselves, but also by the delivery methods such as AAVs. However, to overcome these challenges, both the AAVs and Cas enzymes can be adapted. For instance, in a previous study, they modified a specific domain of Cas9, leading to a change in the T-cell bound epitope and therefore a decrease in immune response was observed[47]. Via these mechanisms and modifications, the natural immune response can be evaded.

5.4 Costs

Besides the more biological hurdles, the CRISPR/Cas system and use of it as a therapy also come with great costs. Although the technique is already less expensive than previous gene editing techniques such as Zinc fingers, there is still a large price attached to it. When this is considered, together with the fact that gene therapies are often developed for smaller, often very specific, groups of patients, the question is if these patients can even afford such therapies. Therefore, when developing new CRISPR/Cas gene therapies, the affordability and possibly the insurance of said therapies should be considered as it is of great importance that these highly beneficial therapies can actually treat patients.

6. Conclusion

From all the information currently available, it is obvious that CRISPR/Cas is a powerful gene editing tool. Nevertheless, it is also obvious that the technique still needs to be improved and adapted for in-human use and safety. However, when taken together, CRISPR/Cas proves to be a realistic therapy that could benefit monogenic disease patients. Since most monogenic diseases currently can only be treated for their symptoms, rather than a curative option, it is still of great importance that better therapies are developed. In the long run, these patients often still rely on kidney transplants and experience a severe burden on their life. Moreover, as monogenic kidney diseases are caused by a single mutated gene, they are the perfect candidate for treatment with the emerging CRISPR/Cas techniques. From all the information presented here, it can be concluded that great advances are being made with regards to CRISPR/Cas as a realistic therapeutic candidate for monogenic (kidney) diseases. Besides the vast amount of *ex-* and *in vivo* studies, there are also several clinical studies currently ongoing, which points to a promising future of CRISPR/Cas as an approved therapeutic agent for the treatment of monogenic diseases.

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