Journal of Controlled Release 244 (2016) 139–148

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

CRISPR-Cas9 gene editing: Delivery aspects and therapeutic potential

Erik Oude Blenke [a], Martijn J.W. Evers [a], Enrico Mastrobattista [a], John van der Oost [b,*]

[a] Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands
[b] Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 16 June 2016
Received in revised form 1 August 2016
Accepted 3 August 2016
Available online 4 August 2016

Keywords:
CRISPR-Cas
CRISPR-Cas9
Gene editing
Genome editing
Delivery systems
Therapeutic applications
In vivo
Ex vivo

ABSTRACT

The CRISPR-Cas9 gene editing system has taken the biomedical science field by storm, initiating rumors about future Nobel Prizes and heating up a fierce patent war, but also making significant scientific impact. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), together with CRISPR-associated proteins (Cas) are a part of the prokaryotic adaptive immune system and have successfully been repurposed for genome editing in mammalian cells. The CRISPR-Cas9 system has been used to correct genetic mutations and for replacing entire genes, opening up a world of possibilities for the treatment of genetic diseases. In addition, recently some new CRISPR-Cas systems have been discovered with interesting mechanistic variations. Despite these promising developments, many challenges have to be overcome before the system can be applied therapeutically in human patients and enabling delivery technology is one of the key challenges. Furthermore, the relatively high off-target effect of the system in its current form prevents it from being safely applied directly in the human body. In this review, the transformation of the CRISPR-Cas gene editing systems into a therapeutic modality will be discussed and the currently most realistic *in vivo* applications will be highlighted.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

The CRISPR-Cas9 gene editing system has received a tremendous amount of attention ever since the discovery of relevant mechanistic features [1–4] in 2010–2011 and the first application in eukaryotes in 2012 [1]. CRISPR is short for Clustered Regularly Interspaced Short Palindromic Repeats that direct the gene editing to a certain target and Cas9 is the associated nuclease that cuts the DNA. Applications of the system appear to be nearly endless, ranging from improving crop resistance [5] to overcoming HIV infections [6] and the controversial human embryo editing [7]. The most captivating application is the prospect of being able to correct genetic defects in diseased tissues and cells [8], although this may currently still be out of reach [9]. However, the system being named the Science Magazine's Breakthrough of the Year 2015 [10] makes it undisputed that CRISPR-Cas9 is here to stay and it is already speculated that its inventors may receive a Nobel Prize within the coming decade [7]. Similar to RNA interference, where a eukaryotic defense system against viral infections is exploited to modulate gene expression [11], this new genome editing system makes use of an adaptive immune system found in prokaryotes. There is a multitude of such systems and CRISPR-Cas9 is certainly not the first one to be described [12,13], but its simplicity and ease of use have sparked the interest of researchers in diverse fields and initiated a run to clinical applications, again very similar to the early days of RNAi [14,15]. To exploit this potential, development of carrier systems capable of delivering the CRISPR-Cas9 system to human cells is of utmost importance, taking lessons from the RNAi field where possible. In this review, the basic mechanism of CRISPR-Cas9 genome editing is explained and current and potential therapeutic applications are highlighted. A special focus will be on the delivery aspects of the system, discussing the requirements for delivery vehicles to allow safe and effective *ex vivo* and *in vivo* manipulation for therapy in human patients.

2. CRISPR-Cas9 genome editing mechanism

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), together with CRISPR-associated proteins (Cas) are a part of the adaptive immune system found in bacteria and archaea. This adaptive immune system can detect and destroy Mobile Genetic Elements (MGEs) such as unwanted viral and plasmid DNA in a highly specific manner. As mentioned before, there are other bacteria-derived targeted nucleases, like Meganucleases, TALEN (Transcription Activator-Like Effector Nucleases) or ZFN (Zinc Finger Nuclease), that are already being translated into clinical application [16–19]. The CRISPR-Cas system is a family of proteins, subdivided in Class 1 (Types I, III and IV) and Class 2 (Types II, V, VI) [12], all consisting of specific endonuclease proteins (Cas) and a guide RNA molecule [20–23]. The guide RNA molecule guides the Cas protein to a very specific MGE related DNA target (Fig. 1). This bacterial molecular machinery can be adapted for use in higher

* Corresponding author at: Laboratory of Microbiology, Wageningen University, Stippeneng 4, 6708 WE Wageningen, The Netherlands.
E-mail address: john.vanderoost@wur.nl (J. van der Oost).

http://dx.doi.org/10.1016/j.jconrel.2016.08.002
0168-3659/© 2016 Elsevier B.V. All rights reserved.

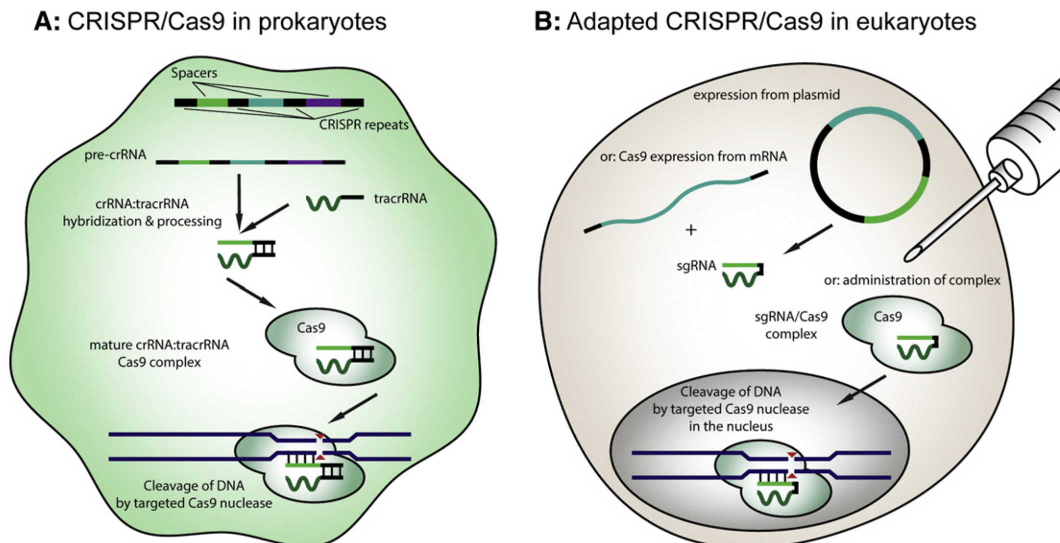

Fig. 1. Mechanism of CRISPR-Cas9 in prokaryotes and the adapted mechanism in eukaryotes. A: In prokaryotes, the protospacer sequences acquired from invading pathogens are stored as spacers in the CRISPR-loci, in the DNA flanked by CRISPR repeats. These are transcribed into a precursor (pre-crRNA) after which the repeats hybridize with anti-repeat sequences within the tracrRNA. This dsRNA is recognized and cleaved by a housekeeping ribonuclease (RNaseIII), resulting in a mature crRNA/tracrRNA hybrid that forms a stable complex with Cas9. Upon a viral invasion, it guides the nuclease to the target sequence in the DNA for cleavage. B: In eukaryotes, a sgRNA is used that combines the function of the crRNA and tracrRNA. This can be expressed from a plasmid or from mRNA, alongside the Cas9 enzyme which is not naturally present in eukaryotes. Alternatively, the sgRNA/Cas9 complex can be administered as a whole. After translocation across the nuclear membrane (due to an engineered Nuclear Localization Signal; NLS) the heterologous complex cleaves the target sequence in the chromosomal DNA.

organisms, in particular for gene-editing. To this end, the endonuclease and the guide RNA have to be heterologously expressed. For this purpose, a specific subtype of CRISPR-Cas is preferred: Class 2. The Class 2 CRISPR-Cas systems generally consist of a single multi-domain protein, such as the Type II nuclease: Cas9 [21–23]. The relatively simple architecture of Class 2 nucleases (Cas9) makes them so easy to apply, as compared to the large, multi-subunit protein Class 1 complexes.

2.1. CRISPR-Cas nucleases and guide RNAs

CRISPR-Cas based immunity in bacteria proceeds in three distinct stages. The three stages are acquisition, expression and interference [13,24].

2.1.1. Acquisition

As an adaptive defense system, bacteria and archaea collect sequences of foreign (plasmid or virus) DNA of 30–45 nucleotides long and integrate them as new spacers in the repetitive CRISPR arrays. To allow self/non-self-discrimination, foreign target sequences (protospacers) are selected on the basis of a flanking motif, the protospacer-adjacent motif (PAM).

2.1.2. Expression

During the expression stage, the CRISPR array is transcribed in one large pre-crRNA and is subsequently processed into smaller CRISPR RNAs (crRNAs). Each crRNA corresponds to one acquired foreign DNA sequence, so expression will result in a pool of crRNAs that all recognize a particular genetic element. The enzymes involved in this step vary between the different CRISPR-Cas subtypes. In the Cas9 system, the repeats of the pre-crRNA first hybridizes with a second, conserved RNA, called the transactivating CRISPR RNA (tracrRNA), after which the dsRNA is specifically cleaved by a non-Cas ribonuclease (RNaseIII). In the system adapted for gene editing (Fig. 1B), these two RNAs are fused and expressed together as a single guide RNA (sgRNA) [1].

2.1.3. Interference

In this stage, the Cas nucleases are guided by the mature crRNAs to target and (in the presence of an adjacent PAM motif) cleave the corresponding protospacer sequences in invading MGEs when present. Hybridization of the tracrRNA:crRNA/Cas9 complex - or the sgRNA/Cas9 complex to the corresponding protospacer sequence results in double stranded breaks and thereby inactivation of the invading DNA [21,22,24]. For adaptation in eukaryotes, the sgRNA and the enzyme have to be expressed, either from a plasmid or from delivered mRNA. mRNA can be used for a more transient expression. For the same reason, the Cas9 enzyme and the sgRNA complex can be directly administered, as the half-life of the enzyme is even shorter than that of exogenous mRNA. These strategies can be chosen to minimize off-target effects as will be discussed later. Expression of multiple sgRNAs from the same construct is called multiplexing, and can be used to target multiple genes or to enhance the knock-out by targeting multiple sites in the same gene [8].

2.2. PAM sequences

It should be noted that after integration of the invading DNA in the CRISPR locus, the 'foreign' sequence is also present in the bacterial genome. To avoid cleavage of the DNA in the CRISPR locus, a safety mechanism is built into the crRNA-sequence. The PAM-sequence is part of the MGE DNA, but is not copied into the CRISPR locus. Cas9-mediated cleavage of the target DNA only occurs when the PAM-sequence is present at the 3′ end. When there is base-pair complementarity but no PAM-sequence, it indicates that the crRNA is bound to the CRISPR locus itself and the sequence is then not degraded [25]. PAM sequences vary per bacterial species [26]. The most widely used Cas9 nuclease is derived from *Streptococcus pyogenes* (SpyCas9) and has GG as its PAM-sequence, meaning that every target protospacer sequence is located adjacent to two guanine bases (protospacer-NGG) [1]. In the unlikely case that this sequence is not present in the intended target DNA, another Cas9 species could be used that binds to a different PAM sequence.

2.3. Non-homologous end joining (NHEJ) and homology-directed repair (HDR)

Cleavage by the targeted nuclease results in a double stranded break (DSB) at a desired sequence-specific location in the target DNA. In eukaryotes, this DSB can be repaired by two distinct mechanisms: Non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ

generates small insertions or deletions (indels) which can inactivate the target sequence by inducing a frame-shift or introducing a pre-mature stop codon. This method is applied for the rapid generation of knock-out cell lines or animal models [27], functional genomic screens [28] and other applications of transcriptional modulation/gene silencing [29]. Alternatively, HDR repairs the DNA strands based on structural homology. In the naturally occurring situation, this could be homology to a nearby located (and structurally related) gene or in therapeutic gene editing, a co-expressed or co-delivered repair template. When two DSBs are created this can result in complete excisions of a target gene and even a provided donor DNA template sequence could be precisely inserted into a specific target site [30–32] (Fig. 2). It should be noted that the process of HDR is much less efficient than NHEJ and the knock-in or replacement of a gene happens with much lower efficiency than the knock-out of a gene using CRISPR-Cas9. This is also reflected by the number of applications of NHEJ *vs.* HDR, as will be discussed in Section 3.

To summarize, the Cas9 system is the most flexible and easiest system to adapt, because it uses only a single enzyme that mediates both the crRNA processing as well as the DNA cleavage. The specificity of the targeted nuclease can be simply altered by replacing the guide RNA, unlike ZFNs and TALENs that require protein engineering for every new target. With CRISPR-Cas9, any 22 nucleotides long DNA sequence can be targeted as long as it is flanked by the NGG motif (when SpyCas9 is used). In the simplest and most widely used application of this system, where a genetic knock-out is made, only two components need to be expressed in the host cell to cleave the target gene: the Cas9 nuclease and the sgRNA. Guide RNAs can be expressed from the same plasmid as the Cas9 protein, which is (human) codon optimized and contains a nuclear localization sequence (NLS) when applied in eukaryotes. Alternatively, the enzyme can be expressed from an mRNA for more transient expression. In the more complex situation where a gene is replaced, a DNA template has to be co-delivered to replace the excised gene. Examples of these therapeutic applications will be discussed in the following paragraphs.

3. Biomedical applications of the CRISPR-Cas9 system

There are a huge number of applications in a wide variety of research fields and various organisms. This review will be limited to applications in mammals/mammalian cells that could be of use in the biomedical field. The large majority of publications utilize a NHEJ strategy to induce knock-outs of the target gene or gain-of-function mutations in the target gene. However, the number of reports of successfully replaced genes with HDR is also growing.

Since the first report of the prokaryotic CRISPR-Cas9 system being programmable to cut isolated DNA at a desired location [1] it has literally been a race to adapt the system for use in human cells with publications from four independent groups coming out almost back-to-back [8, 33–35]. Jennifer Doudna and Emmanuelle Charpentier (University of California, Berkeley and that time at Umeå University, respectively) [1] and Feng Zhang from the Broad Institute and Massachusetts Institute of Technology, Cambridge are generally regarded as the front-runners of adapting this technology, which has led to a debate about who owns the intellectual property [36]. In the mean-time however, the Zhang lab has reported on another Type II CRISPR effector called Cpf1 [37] which could cause the patent battle to settle down, now that it has become clear that there can be alternative systems to achieve the same goal. The Cpf1 system may even have some slight advantages over the Cas9 system when it comes to gene replacement, as will be discussed briefly in the section on improvements to the system later.

3.1. Genetic knock-out animals

The first biomedical application of the CRISPR-Cas9 system was the generation of genetic knock-out mice and rats by co-injecting Cas9 mRNA and sgRNAs in one-cell stage embryos, again published by different groups very shortly after each other [27,38,39]. Conditional knock-outs could also be generated in mice and rats by integrating a donor template containing a Cre/lox recombination site as well as by a knock-in of an 11 kb template, showing that the HDR pathway could also be used for embryo engineering [40–42]. Genetic knock-outs of rabbits [43] and cynomolgus monkeys [44] were also generated by injecting one-cell stage embryos, demonstrating that this approach is feasible in the full range of preclinical animal models.

3.2. Quick genome screening and drug target identification and validation

The emergence of CRISPR-Cas9 has made the generation of knock-out animals for drug screening and target validation a routine procedure. Before, RNA interference has made an important addition to this, but gene silencing using short-hairpin RNAs (shRNAs) has several

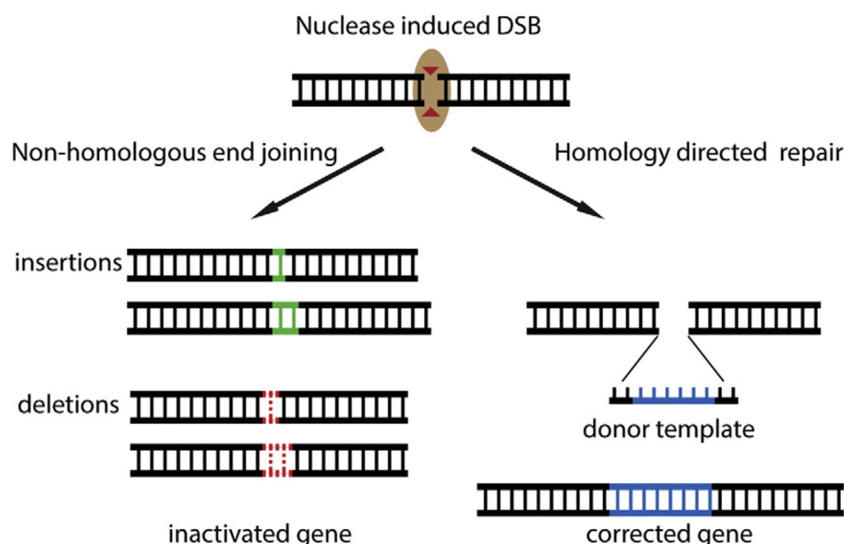

Fig. 2. Two different repair mechanisms of a double stranded break. After the targeted nuclease has created a double stranded break, there are two possible repair pathways. The first is the error-prone non-homologous end joining (NHEJ) which results in insertions or deletions in the gene, often creating a frameshift and thereby inactivating the gene. The second pathway is homology directed repair (HDR) which only takes place in the presence of a homologue part of DNA. When such a donor or repair template is co-administered, this can be used to replace or correct the gene.

drawbacks. First of all, gene silencing based on mRNA degradation is transient. In addition, it often results in only a partial knock-down of the intended target [45]. Apart from that, it turned out there is significant off-target effect due to extensive modulation of micro-RNAs [46], although admittedly, CRISPR-Cas9 in its original form is not completely free of off-target effects either. However, the ease of applicability and low costs of the CRISPR-Cas9 has removed many of the barriers to high-throughput knock-out screens for gene function [45]. Targeted endonucleases can now be expressed by lentiviruses encoding Cas9 and a genome wide array of sgRNAs [47,48]. This approach was validated by screening for resistance to lethal doses of the nucleotide analog 6-thioguanine and etoposide, and the sgRNA screen correctly identified all known genes resulting in resistance. Furthermore, through a negative selection screening, other gene sets involved in fundamental processes could be identified [47]. A similar screening against BRAF inhibitor vemurafenib led to the correct identification of all known genes involved in resistance mechanisms to that drug as well as some novel hits [48]. This Cas9-based screen was repeated with a comparable library of shRNAs and interestingly, only a fraction of the shRNAs appeared to hit the Cas9 targets, demonstrating the superiority of the CRISPR-based screening approach [48]. For a more complete overview on genome-scale knock-out screening using CRISPR-Cas9, see reviews [28,45,49].

3.3. Human embryo editing and high off-target effects

A logical follow-up to the animal embryo knock-out experiments was the editing of genes in human embryos, but ethical issues prohibit the use of normal embryos for such studies. Alternatively, tripronuclear (3PN) zygotes were used, that contain one oocyte nucleus and two sperm nuclei. These polyspermic zygotes are common byproducts of *in vitro* fertilization and are normally discarded in clinics because they are nonviable *in vivo* but do form blastocysts *in vitro*. In these zygotes, the β-subunit of the human β-globin (HBB) gene that is mutated in β-thalassemia, was cut by the CRISPR-Cas9 enzyme and a sgRNA. A replacing donor template was successfully integrated in approximately 15% of all cases, but also Homology Directed Repair with a very similar and closely located gene, HBD (delta-subunit of β-globin) was seen in 25% of the cases. Apart from that, there was a high degree of off-target cleavage and the authors concluded that the CRISPR-Cas9 system has to be improved significantly before embryo editing can be applied in a clinical setting [50]. It may have to do with the controversy around this subject [51], but it is striking how critical the authors are on these results, while such high off-target cleavage and low HDR efficiency are also seen in other studies and are a well-known limitation of the current system [52,53]. Off-target cleavage can lead to a heterogenic population of edited cells, including cells that are not cut at the intended site and more importantly, cells that are cut at the wrong site in the genome. Given the ‘permanent’ nature of the edit, the latter is of course a huge safety concern, if therapeutic gene editing is ever to be applied in human patients. The off-target effects can be partially addressed by re-designing the sgRNAs as will be discussed in the following paragraph, or by making changes to the enzyme itself (see Section 5).

Initial reports found that a single mismatch between the sgRNA and the target DNA abolished nuclease mediated cleavage [8]. This indicated that the system is highly specific and that one mismatch would lead to inactivation of the nuclease but this turned out to be highly dependent on the position in the sgRNA and on the target sequence. A more systematic investigation revealed that multiple mismatches are tolerated, at different positions depending on the sequence, the number, position and distribution [53]. Mismatches are better tolerated when they are located at the 5′ end of the sgRNA (that is, further away from the PAM sequence at the 3′, where hybridization with the target DNA is initiated). Off-target sites with as many as five mismatches were identified that were mutagenized to a comparable extent as the intended target site [52,53]. Apart from that, indel frequency at the target site is not 100% and therefore not even every cut at the intended site leads to a gene inactivating frameshift by NHEJ. This was also seen in all the experiments with the embryos. In a later stage, the embryos displayed mosaicism, indicating that cleavage in the multi-cell stages occurred with varying frequencies and efficiencies in daughter cells. Of course, in the case of the knock-out animals, several rounds of selection and cross-breeding will follow [42] but the need to select successfully engineered cells hampers the direct application of the CRISPR-Cas9 system in human patients (Fig. 3). It is impossible to say right now what an acceptable off-target cleavage rate is, because it is not known to which degree the specificity can be further optimized. Significant progress has already been made, but one could argue that it has to be 0% if it ever is to be safely applied in humans [7]. Therefore, many of the current therapeutic applications aim to engineer the target cells *ex-vivo*, thereby also avoiding the recurring delivery problem.

3.4. Ex-vivo modifications of T-cells

A cell type that is particularly suitable for *ex-vivo* engineering is the T-lymphocyte, because it can be easily harvested from the patient's blood, modified and expanded outside the body and then re-administered without any immunogenicity (Fig. 3B). This could give the immune system a boost in conditions where the body's defense mechanism is compromised such as in HIV-infection or cancer [54].

A recombinant Cas9 enzyme was pre-incubated with a sgRNA targeting CXCR4, a co-receptor for HIV entry and then electroporated into isolated human CD4+ T-cells. This resulted in knock-out of CXCR4 in ~40% of the cells, which could be sorted and enriched based on CXCR4 expression [55]. Other studies by the group of Carl June confirmed that editing of CCR5 (another HIV co-receptor) with a Zinc Finger Nuclease is safe and reduces viral DNA in HIV-infected patients [56]. In the same CRISPR-Cas study, the cell surface receptor PD-1 (PDCD1 gene) was also targeted. PD-1 is a so-called “immune check-point” that inhibits the cancer cell killing signaling in exhausted or chronically activated T-cells, and blocking the PD-1 protein has made a dramatic improvement in cancer immunotherapy. It was shown that by electroporating Cas9/PD-1 sgRNA and a HDR repair template into CD4+ cells, a gene replacement was induced in ~20% of the cells. Note, in this study a *defective* repair template was used to inactivate the PD-1 gene. This is not necessary as it was shown in the same study (and another [57]) that a knock-out could be generated by NHEJ as well, but this shows that *ex-vivo* gene replacement is also possible using the CRISPR-Cas9 system which could have potential benefit in other applications.

For example, another approach of cancer immunotherapy is endowing T-cells with a chimeric antigen receptor (CAR), usually consisting of the single-chain variable fragment (scFv) from a monoclonal antibody and a co-stimulatory signaling domain. This has the theoretical advantage that a broad repertoire of receptors with high affinity can be used, that these are applicable in every patient, and that there is minimal risk of graft-to-host immunity because the patient's own cells are used [58]. CAR T-cells are currently being evaluated in the clinic [59] and an “off-the-shelf” approach for T-cell receptor engineering was recently described using TALEN nucleases [60]. For many gene editing applications, the way has been paved by other programmable nucleases like ZFN and TALEN and it is likely that because of the low costs and tailorability, also CRISPR-Cas based applications will emerge.

In fact, while this paper was under review, the Recombinant DNA Advisory Committee (RAC) at the U.S. National Institutes of Health approved a clinical trial that proposed to use CRISPR-Cas9 for the first time to edit human T-cells in which some of the elements described above will be combined [61,62]. In the trial, led by Dr. Carl June and funded by the Parker Institute for Cancer Immunotherapy, autologous T-cells will be harvested and engineered to express an affinity enhanced T-cell receptor (TCR) recognizing the tumor antigen NY-ESO-1. This

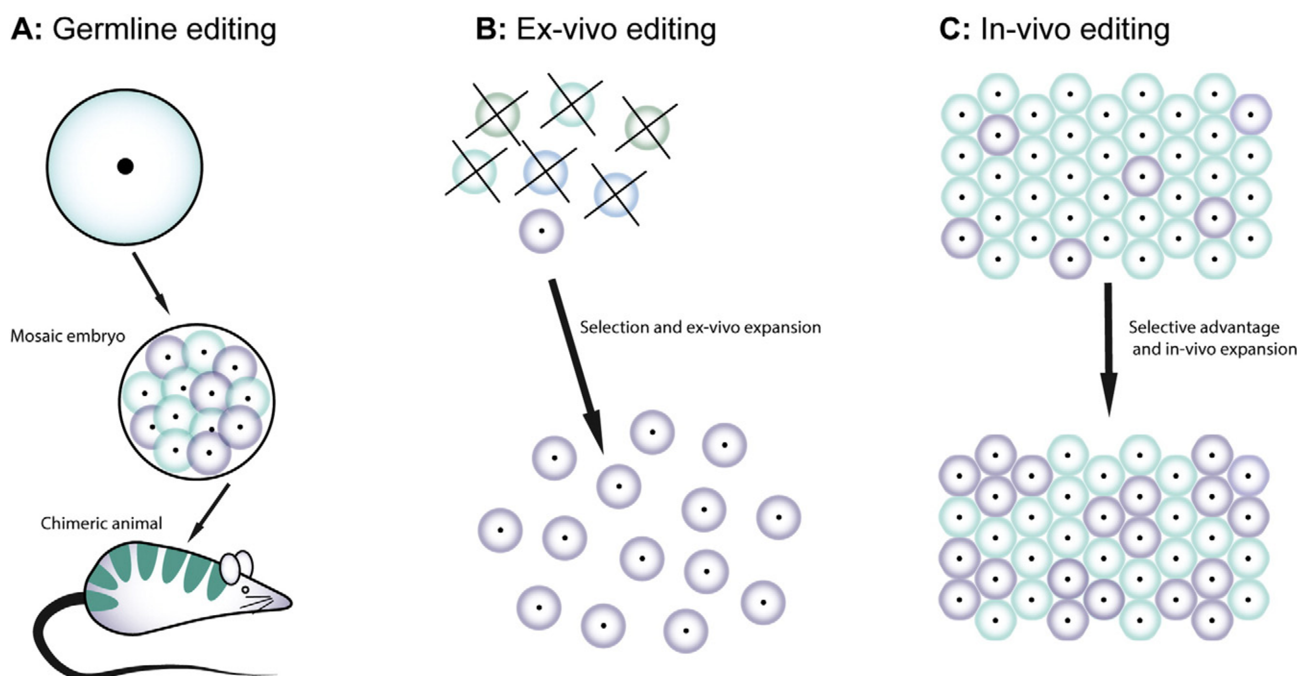

Fig. 3. Applications of CRISPR-Cas9 and effects of incomplete gene editing. A: CRISPR-Cas9 is injected into embryos in the one-cell stage, editing occurs after multiple rounds of cell division. Because not every cell is edited, this results in a mosaic embryo. The resulting pups can be selected and crossbred until a full transgenic animal is obtained. B: When cells are edited *ex-vivo*, this also results in a heterogeneous population of cells, but here the successfully edited cells can be selected and expanded before being re-administered to the patient. C: Until the editing efficiency is improved, *in-vivo* application of CRISPR-Cas9 will also result in only a partially modified population of cells. In indications where edited cells are fitter than the diseased cells, the edited cells can outgrow the diseased cells, creating 'islands' of healthy cells.

approach earlier showed promising, however very short-lived responses in clinical trials with myeloma patients, when using viral transduction of the TCR [63]. To boost the effect, the team now wants to knock-out two different parts of the primary TCR, so that the engineered receptor becomes more potent. Additionally, they want to knock-out the PD-1 gene as described above, to further potentiate the immunotherapeutic response. The simultaneous knock-down of three different gene segments demonstrates the strength of the CRISPR-Cas9 system and helps to make this already very complicated type of therapy a little less challenging [61,62].

3.5. In vivo applications

In vivo application of CRISPR-Cas9 is conceivable for indications where the current low efficacy of gene editing is sufficient to show a phenotypic - and most importantly - a clinical effect. One such example is the knock-out of Proprotein convertase subtilisin/kexin type 9 (PCSK9), involved in the Low Density Lipoprotein (LDL) clearance pathway. It was found that rare individuals that have an inactivating mutation in the PCSK9 gene not only have extremely low plasma LDL levels, but also appear to be protected against cardiovascular heart disease. Surprisingly, this knock-out did not lead to any apparent other symptoms or adverse events, making it an attractive drug target [64]. Clinical trials with PCSK9 targeting siRNA lipid nanoparticles are currently ongoing, as the protein is predominantly expressed in the liver, making it a suitable target for treatment with nanoparticles [65]. PCSK9 knock-out by adeno-associated virus-delivered CRISPR-Cas was demonstrated in mice, showing mutagenesis in ~50% of hepatocytes which resulted in decreased plasma PCSK9 levels, increased LDL receptor levels, and a 35–40% decrease in plasma cholesterol levels [66]. This study demonstrates that for certain indications, it is not essential to reach all cells, nor effectively edit all of them to show an effect, making the current incomplete targeting not necessarily an obstacle (Fig. 3C).

In fact, much lower editing efficiency was shown to still be clinically relevant. In a mouse model of hereditary tyrosinemia type I (HTI), the underlying Fah gene was corrected in the liver by hydrodynamic injection of CRISPR-Cas plasmid and a HDR repair template. This resulted in the initial expression of the wild-type Fah protein in ~1/250 cells (0.4%) but this was enough to rescue the bodyweight-loss phenotype [30]. In this particular disease, it was shown that correction of 1/10,000 hepatocytes could already reverse the disease progression, suggesting that there could be many other indications that could benefit from even such low gene editing frequencies [67]. Furthermore, it appeared that there is positive selection for the edited cells, as after 30 days, ~33% of all hepatocytes in the treated mice were expressing the corrected protein [30]. This is explained by the fact that hepatocytes that are deficient for the Fah gene are poisoned by toxic metabolites, allowing selective outgrowth of the corrected, resistant, cells.

A similar phenomenon was seen in the muscle cells of mice in a Duchenne's muscular dystrophy (DMD) model in which the defective dystrophin gene was corrected. Previously, correction of the dystrophin gene by HDR in mouse embryos was shown [68]. But as germline editing in humans is currently not feasible [51] and the homology directed repair pathway is not active in postmitotic tissues such as heart and skeletal muscle a NHEJ strategy had to be applied in adult mice. In two back-to-back papers, an AAV-CRISPR-Cas mediated excision of the defective dystrophin exon 23 was reported, which skips the premature stop-codon and restores the reading frame (indeed, this results in a shorter version of the dystrophin protein, but one that is more active than the one expressed in DMD patients). The approach described here is similar to the one currently being evaluated with "exon-skipping" anti-sense oligonucleotides. The advantage of this approach is that a similar construct could be designed for any other mutation underlying DMD, which also holds true for the sgRNA used in CRISPR-Cas studies [69]. Also in these studies, outgrow of successfully modified cells was seen, indicating that positive selection may occur on the healthier cells [70,71]. However, although the low efficiency of gene editing is not necessarily a problem, the *in vivo* delivery remains a challenge. In the following paragraph, the delivery methods of the current successes are described and suggestions for future applications are made.

4. Towards delivery systems for *in vivo* applications

In principle, utilization of the CRISPR-Cas system is as simple – or difficult – as delivering one plasmid. This plasmid should then encode the (codon optimized and NLS-tagged) Cas9 enzyme and one or more sgRNA(s). When the aim is to replace a gene, a repair template also has to be delivered or co-expressed, but this does not complicate the delivery strategy very much. What does complicate the delivery is when the gene editing is supposed to happen *in vivo*. In this regard, CRISPR-Cas is very similar to RNAi and more sophisticated delivery systems are required to translate achievements in the lab to the clinic. Granted, the development of RNAi therapeutics is in a much more mature stage, but at the same time it illustrates what a lengthy process clinical translation can be if the delivery technology is missing [72].

4.1. Ex-vivo delivery technology

Early development of the CRISPR-Cas system and most of the current applications use established methods for gene delivery, including lipofection [31], microinjection [42,73] and electroporation [74,75]. However, plasmids have to be delivered to the nucleus, which is known to be a challenge in non-dividing cells. Another potential drawback of plasmid delivery is the random integration of (part of) the plasmid in the genome causing stable expression and potentially (unwanted) mutagenesis. Furthermore, prolonged expression of a targeted nuclease (in this case a ZFN) was shown to result in more off-target effects [76]. Therefore, more recent studies aim for a fine-tuned, more transient expression by administering mRNA instead of a plasmid or by administering the Cas9/sgRNA complex directly (see Fig. 1B). When an *in vitro* transcribed sgRNA was combined with a purified Cas9 and then electroporated into the cell, the intended genome editing occurred quickly after delivery while the nuclease was already largely degraded after 12 h [77]. Apart from minimizing off-target effects, delivery of the nuclease or mRNA instead of a plasmid also avoids the nuclear barrier.

Two innovative strategies for *ex-vivo* delivery without polymers or lipids are iTOP and mechanical cell deformation. The iTOP method, short for induced transduction by osmocytosis and propanebetaine, uses a buffer composition that combines NaCl hypertonicity-induced macropinocytosis and a small-molecule transduction compound (propanebetaine) to transduce extracellular macromolecules into cells. Using this method, the Cas9 enzyme and a sgRNA were successfully delivered to a variety of cell types, with a higher indel frequency and less toxicity than cationic lipids [78]. The other method uses microfluidic devices with a diameter slightly smaller than the cells that temporarily deform the membrane and create transient membrane disruptions that facilitate passive diffusion of material into the cytosol. With this method, small single stranded DNAs, siRNAs, and plasmids encoding CRISPR-Cas9 and a sgRNA could be delivered to a wide variety of cell-types [79]. Unfortunately, as yet neither of these delivery techniques are suitable for *in vivo* delivery so more advanced delivery systems need to be developed. When designing delivery systems for CRISPR/Cas, many of the “old” challenges need to be faced again. The questions of using viral or non-viral vectors, the ideal surface charge, how to overcome the nuclear barrier and how to reach the desired target tissue are still very relevant for CRISPR-Cas delivery. In the paragraphs below, some more advanced delivery systems are discussed that could potentially also be applied *in vivo*.

4.2. In-vivo delivery systems

A study by Ramakrishna et al. demonstrated that the Cas9 nuclease and the sgRNA could also be delivered using cell penetrating peptides (CPPs) [80]. In this work, a nona-arginine based (R9) CPP was conjugated to Cas9 on a C-terminally introduced cysteine after expression and the sgRNA was complexed with a similar CPP. The sgRNA/CPP complex resulted in 300–400 nm particles with a net positive charge. When the Cas9-CPP and sgRNA-CPP were administered simultaneously, gene editing could be observed at the target site, albeit with low frequency (<15% after three rounds of treatment). Because the sgRNA is loaded into the Cas9 enzyme, it was tested whether the sgRNA/Cas9-CPP complex could facilitate cellular entry to both, but this was not the case. Possibly, the negatively charged sgRNA neutralizes the positive charge of the CPP, which also blocks the entry of the enzyme into the cell [80]. This makes the application of this system *in vivo* less likely, because the individual components need to be delivered to the same cell simultaneously, which is a significant challenge. Furthermore, the relatively large and highly cationic sgRNA/CPP complexes will probably not have very favorable circulation kinetics or biodistribution.

Another innovative approach that does deliver the enzyme and the sgRNA together, is the use of DNA nanoclews [81]. DNA nanoclews are nanoparticles based on a cage of DNA that is made by rolling circle amplification. By using structural homology to the sgRNA, the complex of the Cas enzyme and the sgRNA could in turn be complexed to the DNA cage. This was then coated with PEI to give the particle a positive charge for uptake and to enhance endosomal escape [81]. Consequently, this makes it less likely that this particle will be adaptable for delivery *in vivo* given the high toxicity of unmodified PEI.

Therefore, despite the widespread search for non-viral delivery systems, so far almost all *in vivo* success with CRISPR-Cas was achieved using viral vectors. The most widely applied vectors are Adeno Associated Vectors (AAV) because of their broad range of serotype specificity and low immunogenicity. Given the large number of targets in the liver that have already been validated for RNAi therapy, AAV8 seems to be a suitable vector for *in vivo* applications of CRISPR-Cas at this moment [67]. Looking at RNAi indications, there also seems to be a large interest in targeting the dystrophin gene in DMD. Dystrophin correction by CRISPR-Cas was already shown to be feasible and for this indication, AAV9 is a suitable vector as it provides robust expression in the major tissues affected in DMD, such as skeletal muscle, heart, and brain [68,70,71]. However, the low packaging capacity of AAV (~4.5 kb) is hardly sufficient for the packaging of the commonly used *Streptococcus pyogenes* Cas9 (SpyCas9) and a sgRNA (together ~4.2 kb) leaving no room for a DNA repair template or additional sgRNAs [82]. This can be solved by incorporating the Cas9 gene and the sgRNA in separate vectors [83] but this still requires the delivery of both viruses to the same target cell which may be challenging *in vivo*. Similarly, a split-intein Cas9 was developed that could be divided over two AAV cassettes and naturally joined after each part of the protein was expressed in the cell. This provides additional space for regulatory elements like tissue-specific promoters and multiple sgRNAs in each of the vectors but unfortunately at the cost of splicing efficiency (<35% compared to wildtype SpyCas9) [84]. A rationally designed truncated version of SpyCas9 also lost ~50% activity compared to wild-type [85]. As an alternative, the group of Feng Zhang identified different orthologs of Cas9 and found a ~1 kb shorter, but equally active Cas9 in *Staphylococcus aureus*. They packaged this SaCas9 into the hepatocyte-tropic AAV serotype 8 vector and successfully demonstrated 40% knock-down of ApoB and PCSK9 in the liver (enough to show clinically relevant effect) [82].

Promising as this improvement is, it still does not leave enough room for a DNA repair template if the therapeutic indication requires gene replacement. Furthermore, off-target mutagenesis caused by prolonged expression of the nuclease is still a risk when adenoviruses are used. To tackle both problems, a non-viral second vector was used for the delivery of Cas9 mRNA alongside a viral vector expressing the sgRNA and a HDR repair template [86]. Mice were first treated with a single dose of AAV8, which resulted in sgRNA expression after 3 days, but tenfold higher expression after 7 and 14 days. This pronounced sustained expression really demonstrates why expression of the nuclease from such a vector could cause a potential risk of mutagenesis. To maximize co-expression, a single dose of mRNA formulated in a lipid nanoparticle (LNP) containing the lipid-like material C12-200 was administered

intravenously at day 7. This delivery vehicle was previously used for siRNA delivery and just like the AAV8 vector, this type of LNP is known to predominantly target hepatocytes [87]. Initial gene correction was >6%, far more than the previously achieved 0.4% after hydrodynamic injection by the same group [30]. As a result, treatment completely rescued disease symptoms and successful homology directed repair was confirmed by deep sequencing [86].

Seeing that CRISPR-Cas therapeutics are more or less following the same path as RNAi therapeutics, it is expected that in short order, CRISPR-Cas will also completely transit to non-viral delivery systems. The demonstration that the LNPs that are in clinical evaluation for siRNA delivery are also capable of delivering CRISPR-Cas constructs is a clear indication that this transition is feasible in the near future. The fact that many metabolic diseases could be clinically reversed by restoring roughly 5% of protein expression in hepatocytes shows that the low efficiency of gene editing does not restrain therapeutic use [9]. However, the real challenge is not to adapt existing delivery systems for the delivery of CRISPR-Cas9 constructs, but rather to adapt them to reach targets beyond the liver. Here, many of the old challenges in the drug delivery field are faced again. The plasmid, or the Cas9 enzyme needs to reach the nucleus, which is still a challenge in non- or slow dividing cells. Furthermore, extrahepatic targets are still very difficult to reach with non-viral delivery systems and efforts that are made with siRNA-conjugates [88,89] are not compatible with large DNA or mRNA CRISPR-Cas9 constructs. And finally, if a gene edit is supposed to be permanent, the tissue stem cells should be targeted, rather than the highly differentiated cells that make up the bulk of the tissue and usually have a short lifespan. For many tissue types, it is not known where the stem cells are located and even when, they cannot always be targeted or reached. So delivery itself may not be the biggest bottleneck, but delivery system, target tissue and indication have to be carefully matched to reach the optimal effect.

5. Improvements on the system

Throughout this review, it has been mentioned a couple of times that not the low editing efficiency, but rather the high off-target effect of the CRISPR-Cas system could limit its application in patients. This has so far not been discussed because many improvements have already been made and it is the general opinion of the community that this will not be an issue in the long term [51]. In this section, some of these improvements will be highlighted.

The earliest attempt to minimize off-target cleavage was the use of double nickases. Already in the first applications of CRISPR-Cas9 in eukaryotes, it was described that the nuclease could be converted into a targeted nickase [1]. By making a small mutation in one of the two catalytic domains (HNH and RuvC), Cas9 would cleave only one of the two strands (Cas9n, for 'Cas9 nickase'). Two almost simultaneous reports described the use of two of such targeted nickases by expressing Cas9n and two sgRNAs each targeting the same target site, but on opposite strands. This way, only a double stranded break is created when both nickases cut on-target. Off-target single nicks are not repaired by the NHEJ-pathway but by base excision repair pathways where no insertions or deletions are formed. This paired nicking approach reduced off-target activity by 50- to 1500-fold without sacrificing on-target cleavage efficiency [90,91].

However, this approach could become technically challenging in multiplex or genome-wide library based applications. By carefully looking at the 'design rules' of sgRNAs, it was found that by shortening the sgRNAs from 20 nucleotides to 17–19 nucleotides, on-target efficiency could be significantly improved whilst minimizing off-target cleavage. This may sound counterintuitive, but it was found that the 5′ end of sgRNAs was less important for target recognition and that mismatches at that side were tolerated to a much larger extent [52]. Possibly, the nucleotides furthest away from the PAM sequence at the 3′ side compensate for mismatches elsewhere in the sequence, which would mean that shorter sgRNAs are more sensitive to mismatches and thus more specific. To test this hypothesis, a series of sgRNAs targeting the same site was designed, containing 15, 17, 19 or 20 complementary nucleotides. The 15 nucleotide sgRNA was found to be too short to induce cleavage, but the 17 and 19 nucleotide sgRNAs induced similar target site cleavage as the full length sgRNA but with >5000 times reduced off-target effects. These truncated sgRNAs (tru-gRNAs) could also be combined with the paired nickase approach to even further reduce off-target cleavage [92].

When crystal structures of the SpyCas9-sgRNA complex became available, it was hypothesized that it possesses more energy than is needed to recognize its target DNA strand. Several residues in the SpyCas9 were identified that interact aspecifically with the target DNA. These were systematically substituted to study their contribution to off-target effects. The groups of Feng Zhang and Keith Joung each produced a couple of mutants, coined eSpCas9 ("enhanced specificity") and SpCas9-HF1 ("high fidelity") respectively, that had a significant reduction in off-target effects, without abolishing on target efficacy [93,94]. Structure-based engineering of the Cas9 enzyme has led to spectacular improvements in on-target efficiency but notably, the SpCas9-HF1 and eSpCas9 each contain different mutations. So in theory, these could potentially be combined for even further improvement.

The aforementioned examples illustrate how easily the CRISPR-Cas9 system can be tailored and adapted and there is little doubt that the specificity and efficiency will be further improved. Another "improvement" however, is the transition to another enzyme than Cas9, called Cpf1, by the groups of Feng Zhang, Koonin and Van der Oost. This class 2 CRISPR-associated enzyme was recently discovered in multiple strains of *Prevotella* and *Francisella* (hence the name, CRISPR from *Prevotella* and *Francisella* 1), and out of 16 Cpf1 orthologs, two enzymes with efficient genome-editing activity in human cells were identified. Cpf1 differs from Cas9 in three ways. First, it utilizes a T-rich protospacer adjacent motif preceding the target gene, instead of a G-rich PAM following the target DNA; since such motifs are equally abundant, this is not really an advantage over the other system. Secondly, rather than using a tracrRNA and an RNaseIII-like ribonuclease to process the precursor crRNA to a mature guide, Cpf1 protein itself includes the RNA cleavage site [95,96].This has practical advantage that multiplex targeting can be done by a synthetic CRISPR array controlled by a single promoter [96]. The third difference is that Cpf1 cleaves the DNA *via* a staggered double stranded break, creating overhangs that potentially make gene replacement more efficient. The CRISPR-Cpf1 system is still in the development stage, but now that more therapeutic Cas9 gene-knockdown applications are emerging, this could accelerate the development of the elusive gene-replacement applications as well. Moreover, more class 2 CRISPR-associated nucleases are being discovered [13,97].

6. Conclusion

In a timeframe of only 3 years, CRISPR-Cas9 - originally a bacterial defense system that recognizes foreign DNA sequences and selectively cleaves them - has been repurposed to a targeted nuclease that can inactivate diseased genes or potentially even restore them. What sets CRISPR-based systems (Cas9, Cpf1, and others) apart from other targeted nucleases such as ZFN, TALEN and Meganucleases is the ease of application and tailorability, as only the Cas9/Cpf1 enzyme has to be expressed, that can be retargeted by simply changing the guide RNA sequence. There are many similarities between CRISPR-Cas and RNA interference. To start with, the ability to knock-out a gene but then on the genomic DNA level, rather than to knock it down at transcript RNA level. Furthermore, CRISPR-Cas9 has already found many applications as a biotechnological tool, and the development of therapeutic applications is already well under way. In doing so, companies should take caution not to fall into the same traps as with RNAi therapeutics development. Although the potential is huge, overcoming the delivery barrier remains crucial to achieve clinical success. Here,

many lessons can be learned from the RNAi field, as it was already shown that the same delivery technology could also deliver CRISPR-Cas9 constructs [86]. With regard to applications, it has been questioned whether RNAi and CRISPR-Cas could co-exist, as CRISPR-Cas offers a permanent solution to problems that RNAi can only address transiently. However, with regard to delivery, the RNAi field is years ahead of CRISPR-Cas and application of CRISPR-Cas in human subjects seems more challenging. Therefore, for applications like targeting the PCSK9 gene to lower plasma cholesterol levels, it is not unthinkable that RNAi therapeutics make it to the market first and are then gradually phased out when CRISPR-Cas therapeutics become a reality [72]. On the other hand, that statement is merely based on the current state of technology. The safety aspects have not been taken into account, and one could argue that permanent gene knock-outs or replacements in humans will initially only be approved for severe indications for which no alternative treatment options are available, such as Duchenne's Muscular Dystrophy, although no one can address the safety questions at this point.

Until then, work has to be done on further improving the specificity and reducing the off-target effects of CRISPR-Cas systems. At this point, gene editing in cells *ex-vivo* seems most feasible, because it overcomes both the delivery hurdle as well as the imperfect specificity (because successfully edited cells can be selected). Furthermore, complete gene replacement *via* the homology directed repair pathway has not been very efficient so far and seems further away from clinical application than gene knock-out, especially *in vivo*. So, in the current state, knock-down of genes in *ex-vivo* situations seems within reach with CRISPR-Cas9 but it is unlikely that given the state of technology, it will ever be able to compete with RNAi. The real potential lies in the correction of congenital disease and in acquired mutations like in cancer. Perhaps the comparison with plasmid gene delivery is therefore more appropriate, but with the first trial conducted in 1980 and only one approved product on the EU market as of now, progress in that field has also not been particularly rapid. If permanent gene correction is ever to be realized, a lot more knowledge should be gathered about the cell type that has to be reached and how to achieve that. Targeting the long living tissue stem cells is crucial for a permanent effect, but little is known about how to distinguish those and which carrier systems are able to reach them. But with the number of labs working on this technology, the amount of money that has been raised by companies wishing to bring this to the clinic, and the vast amount of therapeutic applications that lie on the horizon, it seems just a matter of time before these challenges are overcome. With existing knowledge and a focus on delivery technology, CRISPR-Cas based therapeutics could be developed faster than their RNAi and gene delivery counterparts, allowing safe and efficient gene editing in human patients in the near future.

References

[1] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science (80-.) 337 (2012) 816–821, http://dx.doi.org/10.1126/science.1225829.

[2] J.E. Garneau, M.-È. Dupuis, M. Villion, D.a. Romero, R. Barrangou, P. Boyaval, et al., The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA, Nature 468 (2010) 67–71, http://dx.doi.org/10.1038/nature09523.

[3] G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria, Proc. Natl. Acad. Sci. 109 (2012) E2579–E2586, http://dx.doi.org/10.1073/pnas.1208507109.

[4] E. Deltcheva, K. Chylinski, C.M. Sharma, K. Gonzales, Y. Chao, Z.A. Pirzada, et al., CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III, Nature 471 (2011) 602–607, http://dx.doi.org/10.1038/nature09886.

[5] A. Chaparro-Garcia, S. Kamoun, V. Nekrasov, Boosting plant immunity with CRISPR/Cas, Genome Biol. 16 (2015) 254, http://dx.doi.org/10.1186/s13059-015-0829-4.

[6] R. Kaminski, Y. Chen, T. Fischer, E. Tedaldi, A. Napoli, Y. Zhang, et al., Elimination of HIV-1 genomes from human T-lymphoid cells by CRISPR/Cas9 gene editing, Sci. Rep. 6 (2016) 22555, http://dx.doi.org/10.1038/srep22555.

[7] J. Travis, Germline editing dominates DNA summit, Science (80-.) 350 (2015) 1299–1300, http://dx.doi.org/10.1126/science.350.6266.1299.

[8] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, et al., Multiplex genome engineering using CRISPR/Cas systems, Science (80-.) 339 (2013) 819–823, http://dx.doi.org/10.1126/science.1231143.

[9] D.B.T. Cox, R.J. Platt, F. Zhang, Therapeutic genome editing: prospects and challenges, Nat. Med. 21 (2015) 121–131, http://dx.doi.org/10.1038/nm.3793.

[10] J. Travis, Making the cut, Science (80-.) 350 (2015) 1456–1457, http://dx.doi.org/10.1126/science.350.6267.1456.

[11] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 391 (1998) 806–811, http://dx.doi.org/10.1038/35888.

[12] K.S. Makarova, Y.I. Wolf, O.S. Alkhnbashi, F. Costa, S.A. Shah, S.J. Saunders, et al., An updated evolutionary classification of CRISPR-Cas systems, Nat. Rev. Microbiol. 13 (2015) 722–736, http://dx.doi.org/10.1038/nrmicro3569.

[13] P. Mohanraju, K.S. Makarova, B. Zetsche, F. Zhang, E.V. Koonin, J. Van der Oost, Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems, Science (80-.) (2016) (in press).

[14] D. Haussecker, The business of RNAi therapeutics in 2012, Mol. Ther. Nucleic Acids. 1 (2012), e8 http://dx.doi.org/10.1038/mtna.2011.9.

[15] D. Haussecker, Current issues of RNAi therapeutics delivery and development, J. Control. Release 195 (2014) 49–54, http://dx.doi.org/10.1016/j.jconrel.2014.07.056.

[16] G. Silva, L. Poirot, R. Galetto, J. Smith, G. Montoya, P. Duchateau, et al., Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy, Curr. Gene Ther. 11 (2011) 11–27, http://dx.doi.org/10.2174/156652311794520111.

[17] J.K. Joung, J.D. Sander, TALENs: a widely applicable technology for targeted genome editing, Nat. Rev. Mol. Cell Biol. 14 (2013) 49–55, http://dx.doi.org/10.1038/nrm3486.

[18] F.D. Urnov, E.J. Rebar, M.C. Holmes, H.S. Zhang, P.D. Gregory, Genome editing with engineered zinc finger nucleases, Nat. Rev. Genet. 11 (2010) 636–646, http://dx.doi.org/10.1038/nrg2842.

[19] T. Gaj, C.A. Gersbach, C.F. Barbas, ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering, Trends Biotechnol. 31 (2013) 397–405, http://dx.doi.org/10.1016/j.tibtech.2013.04.004.

[20] B. Wiedenheft, S.H. Sternberg, J.a. Doudna, RNA-guided genetic silencing systems in bacteria and archaea, Nature 482 (2012) 331–338, http://dx.doi.org/10.1038/nature10886.

[21] J. van der Oost, E.R. Westra, R.N. Jackson, B. Wiedenheft, Unravelling the structural and mechanistic basis of CRISPR-Cas systems, Nat. Rev. Microbiol. 12 (2014) 479–492, http://dx.doi.org/10.1038/nrmicro3279.

[22] I. Mougiakos, E.F. Bosma, W.M. de Vos, R. van Kranenburg, J. van der Oost, Next generation prokaryotic engineering: the CRISPR-Cas toolkit, Trends Biotechnol. 34 (2016) 575–587, http://dx.doi.org/10.1016/j.tibtech.2016.02.004.

[23] A.V. Wright, J.K. Nuñez, J.A. Doudna, Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering, Cell 164 (2016) 29–44, http://dx.doi.org/10.1016/j.cell.2015.12.035.

[24] J. van der Oost, M.M. Jore, E.R. Westra, M. Lundgren, S.J.J. Brouns, CRISPR-based adaptive and heritable immunity in prokaryotes, Trends Biochem. Sci. 34 (2009) 401–407, http://dx.doi.org/10.1016/j.tibs.2009.05.002.

[25] L.A. Marraffini, E.J. Sontheimer, Self versus non-self discrimination during CRISPR RNA-directed immunity, Nature 463 (2010) 568–571, http://dx.doi.org/10.1038/nature08703.

[26] F.J.M. Mojica, C. Díez-Villaseñor, J. García-Martínez, C. Almendros, Short motif sequences determine the targets of the prokaryotic CRISPR defence system, Microbiology 155 (2009) 733–740, http://dx.doi.org/10.1099/mic.0.023960-0.

[27] B. Shen, J. Zhang, H. Wu, J. Wang, K. Ma, Z. Li, et al., Generation of gene-modified mice via Cas9/RNA-mediated gene targeting, Cell Res. 23 (2013) 720–723, http://dx.doi.org/10.1038/cr.2013.46.

[28] O. Shalem, N.E. Sanjana, F. Zhang, High-throughput functional genomics using CRISPR-Cas9, Nat. Rev. Genet. 16 (2015) 299–311, http://dx.doi.org/10.1038/nrg3899.

[29] P.D. Hsu, E.S. Lander, F. Zhang, Development and applications of CRISPR-Cas9 for genome engineering, Cell 157 (2014) 1262–1278, http://dx.doi.org/10.1016/j.cell.2014.05.010.

[30] H. Yin, W. Xue, S. Chen, R.L. Bogorad, E. Benedetti, M. Grompe, et al., Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype, Nat. Biotechnol. 32 (2014) 551–553, http://dx.doi.org/10.1038/nbt.2884.

[31] J.A. Zuris, D.B. Thompson, Y. Shu, J.P. Guilinger, J.L. Bessen, J.H. Hu, et al., Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo, Nat. Biotechnol. 33 (2014) 73–80, http://dx.doi.org/10.1038/nbt.3081.

[32] H.L. Li, N. Fujimoto, N. Sasakawa, S. Shirai, T. Ohkame, T. Sakuma, et al., Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9, Stem Cell Reports 4 (2015) 143–154, http://dx.doi.org/10.1016/j.stemcr.2014.10.013.

[33] P. Mali, L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, et al., RNA-guided human genome engineering via Cas9, Science (80-.) 339 (2013) 823–826, http://dx.doi.org/10.1126/science.1232033.

[34] M. Jinek, A. East, A. Cheng, S. Lin, E. Ma, J. Doudna, RNA-programmed genome editing in human cells, Elife 2013 (2013) 1–9, http://dx.doi.org/10.7554/eLife.00471.

[35] S.W. Cho, S. Kim, J.M. Kim, J.-S. Kim, Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease, Nat. Biotechnol. 31 (2013) 230–232, http://dx.doi.org/10.1038/nbt.2507.

[36] E.S. Lander, The heroes of CRISPR, Cell 164 (2016) 18–28, http://dx.doi.org/10.1016/j.cell.2015.12.041.

[37] B. Zetsche, J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, et al., Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (2015) 759–771, http://dx.doi.org/10.1016/j.cell.2015.09.038.

[38] H. Wang, H. Yang, C.S. Shivalila, M.M. Dawlaty, A.W. Cheng, F. Zhang, et al., One-step generation of mice carrying mutations in multiple genes by CRISPR/cas-mediated genome engineering, Cell 153 (2013) 910–918, http://dx.doi.org/10.1016/j.cell.2013.04.025.

[39] W. Li, F. Teng, T. Li, Q. Zhou, Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems, Nat. Biotechnol. 31 (2013) 684–686, http://dx.doi.org/10.1038/nbt.2652.

[40] H. Yang, H. Wang, C.S. Shivalila, A.W. Cheng, L. Shi, R. Jaenisch, One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering, Cell 154 (2013) 1370–1379, http://dx.doi.org/10.1016/j.cell.2013.08.022.

[41] Y. Ma, X. Zhang, B. Shen, Y. Lu, W. Chen, J. Ma, et al., Generating rats with conditional alleles using CRISPR/Cas9, Cell Res. 24 (2014) 122–125, http://dx.doi.org/10.1038/cr.2013.157.

[42] V.T. Chu, T. Weber, R. Graf, T. Sommermann, K. Petsch, U. Sack, et al., Efficient generation of Rosa26 knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes, BMC Biotechnol. 16 (2016) 4, http://dx.doi.org/10.1186/s12896-016-0234-4.

[43] D. Yang, J. Xu, T. Zhu, J. Fan, L. Lai, J. Zhang, et al., Effective gene targeting in rabbits using RNA-guided Cas9 nucleases, J. Mol. Cell Biol. 6 (2014) 97–99, http://dx.doi.org/10.1093/jmcb/mjt047.

[44] Y. Niu, B. Shen, Y. Cui, Y. Chen, J. Wang, L. Wang, et al., Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos, Cell 156 (2014) 836–843, http://dx.doi.org/10.1016/j.cell.2014.01.027.

[45] J.D. Moore, The impact of CRISPR-Cas9 on target identification and validation, Drug Discov. Today 20 (2015) 450–457, http://dx.doi.org/10.1016/j.drudis.2014.12.016.

[46] S. Marine, A. Bahl, M. Ferrer, E. Buehler, Common seed analysis to identify off-target effects in siRNA screens, J. Biomol. Screen. 17 (2012) 370–378, http://dx.doi.org/10.1177/1087057111427348.

[47] T. Wang, J.J. Wei, D.M. Sabatini, E.S. Lander, Genetic screens in human cells using the CRISPR-Cas9 system, Science 343 (2014) 80–84, http://dx.doi.org/10.1126/science.1246981.

[48] O. Shalem, E.N. Sanjana, E. Hartenian, F. Zhang, Genome-scale CRISPR-Cas9 knockout, Science (80-.) 343 (2014) 84–88, http://dx.doi.org/10.1038/nbt.2647.

[49] J. Peng, Y. Zhou, S. Zhu, W. Wei, High-throughput screens in mammalian cells using the CRISPR-Cas9 system, FEBS J. 282 (2015) 2089–2096, http://dx.doi.org/10.1111/febs.13251.

[50] P. Liang, Y. Xu, X. Zhang, C. Ding, R. Huang, Z. Zhang, et al., CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes, Protein Cell. 6 (2015) 363–372, http://dx.doi.org/10.1007/s13238-015-0153-5.

[51] K.S. Bosley, M. Botchan, A.L. Bredenoord, D. Carroll, R.A. Charo, E. Charpentier, et al., CRISPR germline engineering—the community speaks, Nat. Publ. Gr. 33 (2015) 478–486, http://dx.doi.org/10.1038/nbt.3227.

[52] Y. Fu, J.a. Foden, C. Khayter, M.L. Maeder, D. Reyon, J.K. Joung, et al., High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells, Nat. Biotechnol. 31 (2013) 822–826, http://dx.doi.org/10.1038/nbt.2623.

[53] P.D. Hsu, D.a. Scott, J.a. Weinstein, F.A. Ran, S. Konermann, V. Agarwala, et al., DNA targeting specificity of RNA-guided Cas9 nucleases, Nat. Biotechnol. 31 (2013) 827–832, http://dx.doi.org/10.1038/nbt.2647.

[54] D.M. Barrett, S.a. Grupp, C.H. June, Chimeric antigen receptor- and TCR-modified T cells enter main street and wall street, J. Immunol. 195 (2015) 755–761, http://dx.doi.org/10.4049/jimmunol.1500751.

[55] K. Schumann, S. Lin, E. Boyer, D.R. Simeonov, M. Subramaniam, R.E. Gate, et al., Generation of knock-in primary human T cells using Cas9 ribonucleoproteins, Proc. Natl. Acad. Sci. 112 (2015) 10437–10442, http://dx.doi.org/10.1073/pnas.1512503112.

[56] P. Tebas, D. Stein, W.W. Tang, I. Frank, S.Q. Wang, G. Lee, et al., Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV, N. Engl. J. Med. 370 (2014) 901–910, http://dx.doi.org/10.1056/NEJMoa1300662.

[57] S. Su, B. Hu, J. Shao, B. Shen, J. Du, Y. Du, et al., CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients, Sci. Rep. 6 (2016) 20070, http://dx.doi.org/10.1038/srep20070.

[58] M.V. Maus, D.J. Powell, Chimeric antigen receptor T-cells - new approaches to improve their efficacy and reduce toxicity, Cancer J. 21 (2015) 475–479, http://dx.doi.org/10.1097/PPO.0000000000000155.

[59] S.L. Maude, N. Frey, P.A. Shaw, R. Aplenc, D.M. Barrett, N.J. Bunin, et al., Chimeric antigen receptor T cells for sustained remissions in leukemia, N. Engl. J. Med. 371 (2014) 1507–1517, http://dx.doi.org/10.1056/NEJMoa1407222.

[60] L.P.B. Philip, C. Schiffer-Mannioui, D. Le Clerre, I. Chion-Sotinel, S. Derniame, P. Potrel, et al., Multiplex genome-edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies, Cancer Res. 75 (2015) 3853–3864, http://dx.doi.org/10.1158/0008-5472.CAN-14-3321.

[61] J. Kaiser, First proposed human test of CRISPR passes initial safety review, Science (80-.) (2016) http://dx.doi.org/10.1126/science.aaf5796.

[62] S. Reardon, First CRISPR clinical trial gets green light from US panel, Nature (2016) http://dx.doi.org/10.1038/nature.2016.20137.

[63] A.P. Rapoport, E.a. Stadtmauer, G.K. Binder-Scholl, O. Goloubeva, D.T. Vogl, S.F. Lacey, et al., NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma, Nat. Med. 21 (2015) 1–20, http://dx.doi.org/10.1038/nm.3910.

[64] J.C. Cohen, E. Boerwinkle, T.H. Mosley, H.H. Hobbs, Sequence variations in PCSK9, low LDL, and protection against coronary heart disease, N. Engl. J. Med. 354 (2006) 1264–1272, http://dx.doi.org/10.1056/NEJMoa054013.

[65] K. Fitzgerald, M. Frank-Kamenetsky, S. Shulga-Morskaya, A. Liebow, B.R. Bettencourt, J.E. Sutherland, et al., Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial, Lancet 383 (2014) 60–68, http://dx.doi.org/10.1016/S0140-6736(13)61914-5.

[66] Q. Ding, A. Strong, K.M. Patel, S.L. Ng, B.S. Gosis, S.N. Regan, et al., Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing, Circ. Res. 115 (2014) 488–492, http://dx.doi.org/10.1161/CIRCRESAHA.115.304351.

[67] N.K. Paulk, K. Wursthorn, Z. Wang, M.J. Finegold, M.A. Kay, M. Grompe, Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo, Hepatology 51 (2010) 1200–1208, http://dx.doi.org/10.1002/hep.23481.

[68] C. Long, J.R. McAnally, J.M. Shelton, A.A. Mireault, R. Bassel-Duby, E.N. Olson, Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA, Science 345 (2014) 1184–1188, http://dx.doi.org/10.1126/science.1254445.

[69] M.C. Miceli, S.F. Nelson, The case for eteplirsen: paving the way for precision medicine, Mol. Genet. Metab. 2015–2016 (2016) http://dx.doi.org/10.1016/j.ymgme.2016.04.001.

[70] C. Long, L. Amoasii, A.A. Mireault, J.R. McAnally, H. Li, E. Sanchez-Ortiz, et al., Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy, Science (80-.) 351 (2016) 400–403, http://dx.doi.org/10.1126/science.aad5725.

[71] C.E. Nelson, C.H. Hakim, D.G. Ousterout, P.I. Thakore, E.A. Moreb, R.M.C. Rivera, et al., In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy, Science (80-.) 351 (2016) 403–407, http://dx.doi.org/10.1126/science.aad5143.

[72] D. Haussecker, Stacking up CRISPR against RNAi for therapeutic gene inhibition, FEBS J. (2016) http://dx.doi.org/10.1111/febs.13742.

[73] R.N. Cottle, C.M. Lee, D. Archer, G. Bao, Controlled delivery of β-globin-targeting TALENs and CRISPR/Cas9 into mammalian cells for genome editing using microinjection, Sci. Rep. 5 (2015) 16031, http://dx.doi.org/10.1038/srep16031.

[74] W. Qin, S.L. Dion, P.M. Kutny, Y. Zhang, A.W. Cheng, N.L. Jillette, et al., Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease, Genetics 200 (2015) 423–430, http://dx.doi.org/10.1534/genetics.115.176594.

[75] M. Hashimoto, T. Takemoto, Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing, Sci. Rep. 5 (2015) 11315, http://dx.doi.org/10.1038/srep11315.

[76] T. Gaj, J. Guo, Y. Kato, S.J. Sirk, C.F. Barbas, Targeted gene knockout by direct delivery of zinc-finger nuclease proteins, Nat. Methods 9 (2012) 805–807, http://dx.doi.org/10.1038/nmeth.2030.

[77] S. Kim, D. Kim, S.W. Cho, J. Kim, J.-S. Kim, Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins, Genome Res. 24 (2014) 1012–1019, http://dx.doi.org/10.1101/gr.171322.113.

[78] D.S. D'Astolfo, R.J. Pagliero, A. Pras, W.R. Karthaus, H. Clevers, V. Prasad, et al., Efficient intracellular delivery of native proteins, Cell 161 (2015) 674–690, http://dx.doi.org/10.1016/j.cell.2015.03.028.

[79] X. Han, Z. Liu, M.c. Jo, K. Zhang, Y. Li, Z. Zeng, et al., CRISPR-Cas9 delivery to hard-to-transfect cells via membrane deformation, Sci. Adv. 1 (2015) e1500454, http://dx.doi.org/10.1126/sciadv.1500454.

[80] S. Ramakrishna, A.-B. Kwaku Dad, J. Beloor, R. Gopalappa, S.-K. Lee, H. Kim, Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA, Genome Res. 24 (2014) 1020–1027, http://dx.doi.org/10.1101/gr.171264.113.

[81] W. Sun, W. Ji, J.M. Hall, Q. Hu, C. Wang, C.L. Beisel, et al., Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing, Angew. Chem. Int. Ed. 54 (2015) 12029–12033, http://dx.doi.org/10.1002/anie.201506030.

[82] F.A. Ran, L. Cong, W.X. Yan, D.a. Scott, J.S. Gootenberg, A.J. Kriz, et al., In vivo genome editing using *Staphylococcus aureus* Cas9, Nature 520 (2015) 186–190, http://dx.doi.org/10.1038/nature14299.

[83] N.E. Sanjana, O. Shalem, F. Zhang, Improved vectors and genome-wide libraries for CRISPR screening, Nat. Methods 11 (2014) 006726, http://dx.doi.org/10.1101/006726.

[84] E.J. Fine, C.M. Appleton, D.E. White, M.T. Brown, H. Deshmukh, M.L. Kemp, et al., Trans-spliced Cas9 allows cleavage of HBB and CCR5 genes in human cells using compact expression cassettes, Sci. Rep. 5 (2015) 10777, http://dx.doi.org/10.1038/srep10777.

[85] H. Nishimasu, F.A. Ran, P.D. Hsu, S. Konermann, S.I. Shehata, N. Dohmae, et al., Crystal structure of Cas9 in complex with guide RNA and target DNA, Cell 156 (2014) 935–949, http://dx.doi.org/10.1016/j.cell.2014.02.001.

[86] H. Yin, C.-Q. Song, J.R. Dorkin, L.J. Zhu, Y. Li, Q. Wu, et al., Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo, Nat. Biotechnol. 34 (2016) 328–333, http://dx.doi.org/10.1038/nbt.3471.

[87] K.T. Love, K.P. Mahon, C.G. Levins, K.A. Whitehead, W. Querbes, J.R. Dorkin, et al., Lipid-like materials for low-dose, in vivo gene silencing, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 1864–1869, http://dx.doi.org/10.1073/pnas.0910603106.

[88] R. Kanasty, J.R. Dorkin, A. Vegas, D. Anderson, Delivery materials for siRNA therapeutics, Nat. Mater. 12 (2013) 967–977, http://dx.doi.org/10.1038/nmat3765.

[89] H. Yin, R.L. Kanasty, A.a. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Non-viral vectors for gene-based therapy, Nat. Rev. Genet. 15 (2014) 541–555, http://dx.doi.org/10.1038/nrg3763.

[90] P. Mali, J. Aach, P.B. Stranges, K.M. Esvelt, M. Moosburner, S. Kosuri, et al., CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering, Nat. Biotechnol. 31 (2013) 833–838, http://dx.doi.org/10.1038/nbt.2675.

[91] F.A. Ran, P.D. Hsu, C.Y. Lin, J.S. Gootenberg, S. Konermann, A.E. Trevino, et al., Double nicking by RNA-guided CRISPR cas9 for enhanced genome editing specificity, Cell 154 (2013) 1380–1389, http://dx.doi.org/10.1016/j.cell.2013.08.021.
[92] Y. Fu, J.D. Sander, D. Reyon, V.M. Cascio, J.K. Joung, Improving CRISPR-Cas nuclease specificity using truncated guide RNAs, Nat. Biotechnol. 32 (2014) 279–284, http://dx.doi.org/10.1038/nbt.2808.
[93] I.M. Slaymaker, L. Gao, B. Zetsche, D.A. Scott, W.X. Yan, F. Zhang, Rationally engineered Cas9 nucleases with improved specificity, Science (80-.) 351 (2016) 84–88, http://dx.doi.org/10.1126/science.aad5227.
[94] B.P. Kleinstiver, V. Pattanayak, M.S. Prew, S.Q. Tsai, N.T. Nguyen, Z. Zheng, et al., High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects, Nature 529 (2016) 490–495, http://dx.doi.org/10.1038/nature16526.
[95] I. Fonfara, H. Richter, M. Bratovič, A. Le Rhun, E. Charpentier, The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA, Nature (2016) 1–19, http://dx.doi.org/10.1038/nature17945.
[96] B. Zetsche, P. Mohanraju, J. Van der Oost, F. Zhang, Multiplex gene editing by CRISPR-Cpf1 through autonomous processing of a single crRNA array, Nat. Biotechnol. (2016).
[97] S. Shmakov, O.O. Abudayyeh, K.S. Makarova, Y.I. Wolf, J.S. Gootenberg, E. Semenova, et al., Discovery and functional characterization of diverse class 2 CRISPR-Cas systems, Mol. Cell 60 (2015) 385–397, http://dx.doi.org/10.1016/j.molcel.2015.10.008.