Bioactive Materials 21 (2023) 394–402

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: www.keaipublishing.com/en/journals/bioactive-materials

Genome editing in cancer: Challenges and potential opportunities

Dor Breier [a,b,c,d], Dan Peer [a,b,c,d,*]

[a] Laboratory of Precision Nanomedicine, Shmunis School of Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel
[b] Department of Materials Sciences and Engineering, Iby and Aladar Fleischman Faculty of Engineering, Tel Aviv University, Tel Aviv, Israel
[c] Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv, Israel
[d] Cancer Biology Research Center, Tel Aviv University, Tel Aviv, Israel

ARTICLE INFO

Keywords:
Genome editing
CRISPR/Cas systems
Cancer therapy
Off-target activity

ABSTRACT

Ever since its mechanism was discovered back in 2012, the CRISPR/Cas9 system have revolutionized the field of genome editing. While at first it was seen as a therapeutic tool mostly relevant for curing genetic diseases, it has been recently shown to also hold the potential to become a clinically relevant therapy for cancer. However, there are multiple challenges that must be addressed prior to clinical testing. Predominantly, the safety of the system when used for *in-vivo* therapies, including off-target activity and the effects of the double strand break induction on genomic stability. Here, we will focus on the inherent challenges in the CRISPR/Cas9 system and discuss various opportunities to overcoming these challenges.

1. Introduction

Genome editing is a method in which engineered nucleases are utilized to induce double-stranded breaks (DSB) at specific genomic loci in order to harness the cellular endogenous DNA repair mechanisms to introduce genomic modifications [1,2]. Following the formation of a DSB, the cell will utilize one of two repair mechanisms – non-homologous end-joining (NHEJ) and homology dependent repair (HDR) – and both can be used to induce changes in the DNA [3,4]. During NHEJ, the cell ligates the broken ends of the DNA back together – a process that is fast but often inaccurate, with the repaired strands often containing small mutations in the form of small deletions and insertions [5,6]. In Genome editing, NHEJ is utilized to inactivate gene function through loss of function mutations. HDR is a more complex process, requiring a donor DNA with homology to both sides of the break. In HDR, the cell processes the ends of the DSB to leave 3′ overhangs that invade the donor DNA at the homologous sites, using it as a template for DNA synthesis, thereby correcting the break and making it identical to the donor DNA [7]. While in nature the donor DNA is the sister chromatid, in genome editing an exogenous DNA is introduced into the cells to serve as a template to introduce desired changes into the genome [8] (Fig. 1). Over the years, several types of engineered nucleases have been used to induce the DSB required for genome editing, including the zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeat/CRISPR-associated (CRISPR/Cas) systems [9,10].

Ever since its mechanism was discovered back in 2012, the CRISPR/Cas family have revolutionized the field of genome editing. The CRISPR/Cas system originates in bacteria and archaea, where the various Cas proteins serve as an acquired immune system against viral infections [11]. The CRISPR/Cas systems are generally classified into 2 classes based upon the organization of the effector protein complex – while class 1 systems require a multi-protein complex, the class 2 systems utilize a single-protein effector module. Unlike the older nucleases which utilized complex protein-DNA interactions, the CRISPR/Cas systems are based upon a simple Watson-Crick base pairing between a guide RNA molecule and targeted nucleic acids (which could be either DNA or RNA, depending on the Cas used) [9]. The gRNA is comprised of 2 distinct RNA molecules – the CRISPR RNA (crRNA) and the trans-activating CRISPR RNA (tracrRNA), which hybridize to form the tracrRNA: crRNA hybrid that complexes with the Cas nuclease to guide it to the target site [12]. In Bacteria, CRISPR loci respond to invasion of foreign DNA by integrating short fragments of the foreign sequence into the proximal end of the CRISPR array [11]. The repeat-spacer element is transcribed and processed into the mature crRNA which allows the Cas nuclease to target the invading nucleic acid. The target site is

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author. Department of Cell Research and Immunology, Tel-Aviv University, Ramat Aviv, Tel-Aviv, 69978, Israel.
E-mail address: peer@tauex.tau.ac.il (D. Peer).

https://doi.org/10.1016/j.bioactmat.2022.08.013
Received 1 June 2022; Received in revised form 21 August 2022; Accepted 24 August 2022
Available online 14 September 2022

determined both by the base pairing complementarity between the gRNA and the target sequence and by the protospacer adjacent motif (PAM) – a sequence of 3 nucleic acids that is specific for each type of Cas – in the case of the Cas9 system, the motif is NGG (N being any nucleotide) [13]. As of today, the most studied and commonly used CRISPR/Cas system in genome editing is the CRISPR/Cas9 system. The Cas9 protein recognizes and hybridizes with sites containing the PAM motif, and when the gRNA matches the DNA sequence, the protein cleaves the target strand 3 bps upstream to the PAM [14]. In genome editing, one can use either a two-part system formulation, using two separate synthetic molecules annealed to form a single complex, or a single guide RNA formulation (sgRNA) – a single synthetic molecule containing both the tracrRNA and crRNA [11]. To increase editing efficiency, it is common to use chemical modifications on the gRNA to stabilize it and protect it from RNAses [15]. As of today, genome editing is tested for various applications, including therapies for genetic diseases, both *ex-vivo* and *in-vivo*, cancer immunotherapies by checkpoint inhibition, and cancer therapies by gene silencing [16,17].

2. Genome editing in cancer – or, why CRISPR and not siRNA?

As of today, cancer is 2nd leading cause of death worldwide, with a 2022 estimated projection of 609 thousand deaths in the US alone [18]. As it was becoming clearer that new therapies are required, the idea of RNA-based gene therapy in cancer rose to prominence. Many of these therapies are based upon small interfering RNA (siRNA) – a class of 20–27 bps long, double-stranded RNA molecules that are encoded in the genome in the form of long double-stranded RNAs that are cleaved by the Dicer enzyme into short segments. The antisense strand of the siRNA is integrated into a complex consisting of the RNA-induced silencing complex (RISC) and the Argonaute 2 (AGO2) endoribonuclease. When the siRNA binds an mRNA via perfect base-pairing, the AGO2 endoribonuclease cleaves it [19]. In the case of cancer, siRNA can be used to downregulate oncogenes as well as other genes which are critical for the cancer's survival [20]. As of today, there are multiple clinical trials using siRNAs to eradicate tumors [21,22]. Similar to siRNA, genome editing can be used to silence genes critical for cancer. Thus, a natural question arises – what are the advantages of genome editing over siRNA? The first advantage of genome editing over siRNA is that since genome editing works on the DNA, on a cell-by-cell comparison, its effect is stronger compared to siRNA – when targeting a gene that is transcribed many times, siRNA is unable to silence all copies of the mRNA, meaning that some protein level will be maintained in the cells. Genome editing however, does not face that problem – since it targets the DNA rather than the mRNA, it matters not how much a gene is transcribed as all future copies of the mRNA will carry the silencing mutations. The second advantage of genome editing is the permanence of the effects – while siRNA is only effective for as long as it is in the cell, the effects of genome editing are permanent [23]. Therefore, while siRNA is limited only to genes whose silencing will have immediate effects, genome editing

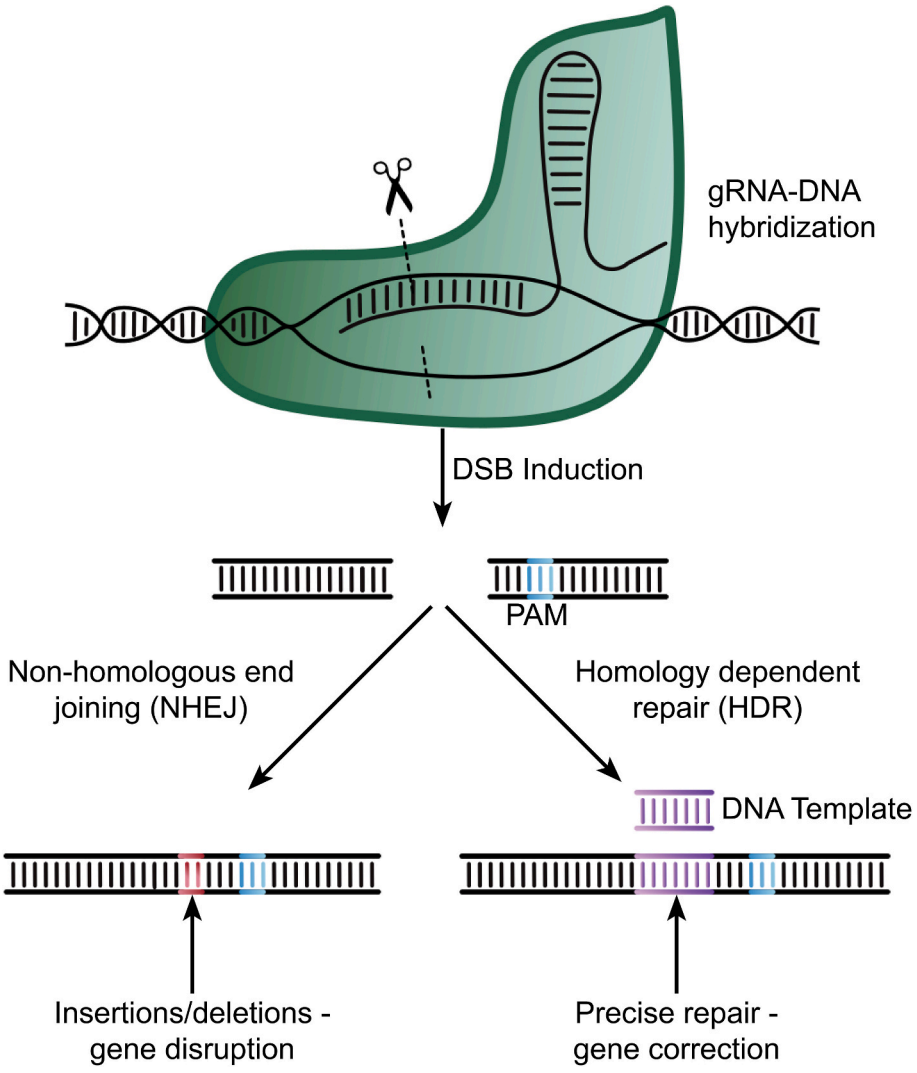

Fig. 1. CRISPR/Cas9 induction of double-stranded breaks and the cellular repair mechanisms. The gRNA found in the Cas9 ribonucleoprotein (RNP) complex hybridizes with the DNA at the cut site, leading to nuclease activation which induces the formation of a genomic DSB. The DSB is then repaired with one of two pathways: in NHEJ, the broken strands are ligated back together, often leading to mutations in the form of insertions and deletions leading to gene disruption. In HDR, a matching DNA template is copied unto the break, repairing the gene in a precise manner.

allows for a wider repertoire of targets such as stable proteins with a long half-life. So as can be seen, genome editing offers a valid approach to cancer therapies, and the effects of CRISPR/Cas9 based editing in cancer has been shown both *in-vitro* and *in-vivo* [24].

3. The inherent challenges of *in-vivo* CRISPR/Cas9 editing

While its effectiveness against cancer has been proven, there are still various challenges remaining before CRISPR/Cas9 based therapies will be ready for clinical translation. The first type of challenges is "on-target – off-tumor" activity. While the CRISPR/Cas9 system shows high levels of precision (even if not perfect, as will be discussed later), it would work in any cell it reaches. While choosing a target gene that is transcribed in cancerous cells or that is only critical for them could somewhat alleviate this matter, it should be noted that several recent studies have shown that the DSB induced by the Cas9 nuclease could lead to a large spectrum of chromosomal abnormalities, such abnormalities include chromosomal deletions that can include thousands of base pairs which are likely to lead to cellular damage, and chromothripsis – mutational rearrangements of large segments of the chromosome that might lead to oncogenic transformation, meaning that the therapy meant to cure one type of cancer would instead lead to the future onset of another type [25–28]. The second type of challenges is "off-target – off-tumor" activity – while the gRNA:Cas9 complex activation usually requires perfect base-pairing between the gRNA and the DNA, DSBs can also be formed at lower efficiency when there are a few mismatches [29,30]. Even though the occurrence of such events in the cancerous cells are likely to be meaningless and have no consequences on the disease's progression or prognosis, especially under the assumption that the loss of the target gene will lead to apoptosis, they may have dire ramifications when occurring in healthy cells. While the exact consequences of off-target activity would differ greatly according to where the DSB was formed, it is logical to assume it will lead to either cellular damage or cancerous transformation. For example, it has been shown in both primates and rodents that DSBs formed at oncogenes such as p53 could serve as a trigger for oncogenic transformation of the cells once enough time has passed [31,32]. A second possible effect would be translocations between loci where DSBs were formed, such translocations could occur either between the on-target and an off-target, or between two off-target sites, and while the exact consequences are unpredictable, it has been proven that translocations arising between cut sites can lead to cancerous transformations [33]. Therefore, it is clear that these challenges need to be addressed, and the danger minimized before genome editing can be utilized against cancer in the clinic.

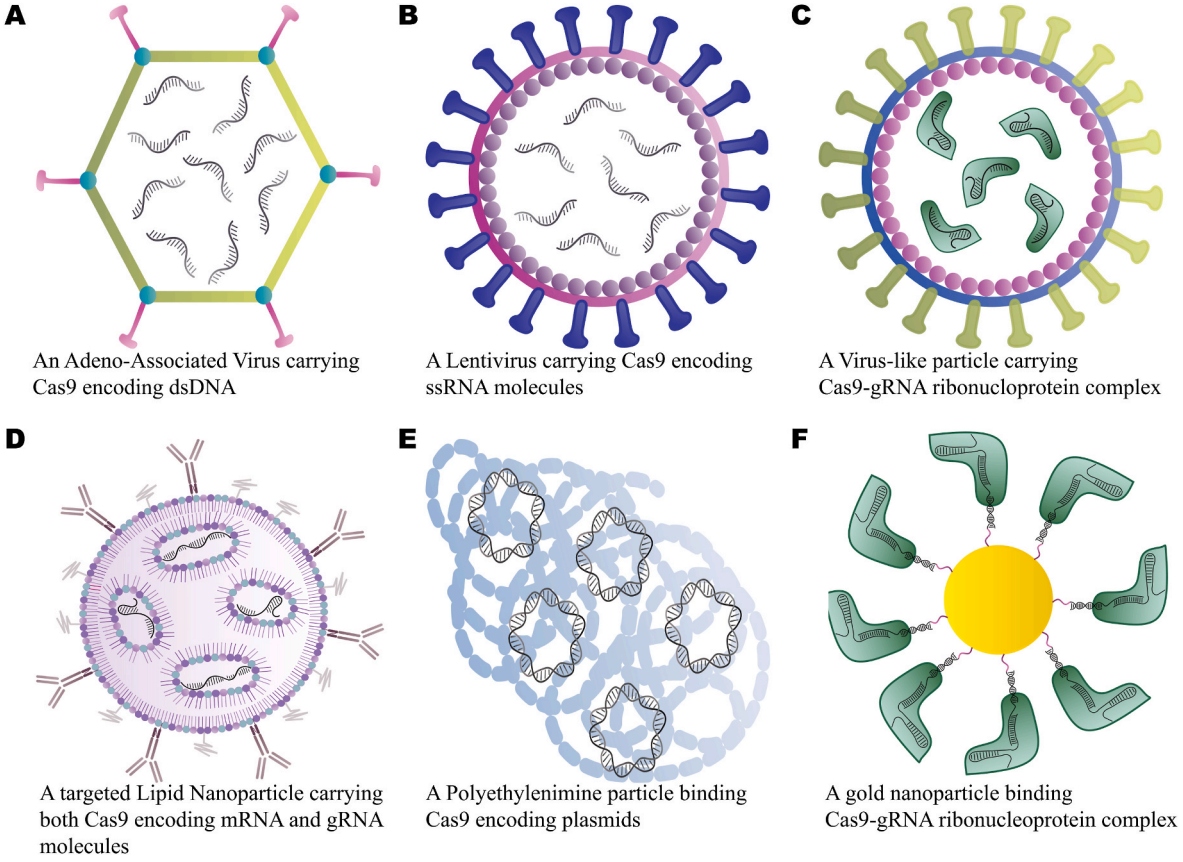

Fig. 2. CRISPR/Cas9 delivery systems. A – an adeno-associated virus (AAV) carrying a single-stranded DNA encoding the Cas9 nuclease. The DNA will undergo transcription in the nucleus, leading to Cas9 expression. B – a lentivirus carrying a single-stranded RNA molecule encoding both the Cas9 nuclease and the gRNA molecule. The RNA would undergo reverse transcription, leading to either genomic integration and subsequent constitutive expression (in a normal lentivirus), or transient expression as extra-genomic DNA (in an integration deficient lentivirus). The glycoproteins enveloping the capsid allow for selective targeting of specific cells by the virus. C – a virus-like particle (VLP) carrying a complete sgRNA-Cas9 RNP complex. Similar to lentiviruses, the glycoproteins enveloping the capsid allow for selective targeting of the particle. D – a lipid nanoparticle (LNP) carrying both Cas9 encoding mRNA and sgRNA molecules. Upon entering the cytoplasm, the mRNA will undergo transcription and the sgRNA will hybridize with the expressed Cas9. The LNP is coated by antibodies which enable specific targeting. The conjugated antibodies represent the way various targeting moieties can be conjugated to particles at large. E – Polyethylenimine binding a plasmid encoding for both the Cas9 nuclease and the gRNA. Upon entering the nucleus, the plasmid will undergo transcription, giving rise to both the Cas9 mRNA and the gRNA. F – a gold nanoparticle binding a complete sgRNA-Cas9 RNP complex via DNA-oligomers.

4. Towards solving the challenges present in *in-vivo* CRISPR/Cas9 therapies

4.1. Targeted delivery systems

While the challenges mentioned present a hurdle on the way to the clinic, current research is giving rise to various possible solutions. First, as both groups of challenges mentioned occur in healthy cells that aren't the target of the therapy, limiting its effects only to cancerous cells should prevent them. As of today, there are multiple delivery systems capable of delivering either a complete, hybridized Ribonucleoprotein (RNP) complex or the nucleic acids encoding it [34,35]. Such systems include both viral and non-viral vectors. First, let us focus on the viral vectors – as of today, Cas9 encoding nucleic acids have been delivered using recombinant adeno-associated viruses r(AAVs) or lentiviruses. rAAVs deliver a single-stranded DNA of up to 4.7 kb that reaches the nucleus and utilizes natural polymerases to synthesize the complementing strand and then transcribe the encoded gene (Fig. 2A) [36]. As there are many serotypes of AAV, it is possible to target different types of cells depending on which serotype is used. One of the major drawbacks regarding rAAVs is that the carrying capacity, since Cas9 is a large protein, and since AAVs also need to contain other elements such as a promoter and polyA signal, using a single AAV to encode both the Cas9 and the gRNA is problematic. Hence, rAAV based Cas9 systems usually use dual vectors, the first encoding the Cas9 protein, and the other encoding the gRNA [37]. The problem when using the dual vectors is that it increases both the complexity of the therapy and the viral load in the patient. In addition, it has been found that past infections with natural AAVs of the same serotype lead to the presence of neutralizing antibodies, meaning that the same therapy might not work or even illicit a potentially severe immune response in some patients, especially if there is need for multiple transfusions [38]. A second method of viral delivery is using lentiviruses. Unlike the non-integrating AAVs, lentiviruses pack RNA that undergoes reverse transcription, followed by integration in the genome of the targeted cell (Fig. 2B). One advantage of the lentivirus over AAVs is that it has a much higher packaging ability, removing the need for dual vectors. While its integration might interfere with regular gene expression, recent advances in the field have lowered this risk. A risk that is common to both AAVs and lentiviruses is that long-term Cas9 expression is known to lead to increased off-target activity and hence, increase the inherent risk in the therapy whenever the viruses transfect healthy cells [39]. In addition, the long-term expression of the protein might be immunogenic as studies have shown that as Cas9 is a bacterial protein, some humans have both T cells and antibodies targeting it [40–42]. One solution was using integration deficient Lentiviruses instead of the regular variant, a modified, non-integrating variant of lentiviruses, which has successfully delivered Cas9 encoding RNA alongside the gRNA, leading to transient expression of the Cas9 protein [43,44]. A second solution to lower this risk can be found in synthetic virus-like particles (VLPs) – an engineered form of lentiviruses that has been shown to efficiently carry either a completed Cas9-gRNA RNP complex or the mRNA encoding it to cells, leading to transient activity rather than the long-term effects seen in AAVs or Lentiviruses (Fig. 2C). In VLPs the capsid contains variants of the natural lentiviral capsid proteins, such as a Cas9 fused gag protein that leads to its packaging in the VLP or a PEG10 protein that packs any RNA that contains the PEG10 UTRs. Furthermore, it has been shown that by modifying the envelope glycoproteins, it is possible to target specific cell types, thus limiting the effects to cancerous cells [45–48].

The second family of delivery systems are non-viral. The most advanced and clinically approved are lipid nanoparticles (LNP). LNPs are made from a mixture of ionizable amino lipids, structural lipids, cholesterol, and polyethylene glycol (PEG)-based lipids [49–51]. It has been shown that targeted LNPs can deliver Cas9 mRNA alongside the gRNA to cancer cells in a precise manner and high efficiency, leading to therapeutic genome editing (Fig. 2D) [24]. The biodistribution of the LNP is based upon various factors such as the formulation of the LNP and which ionizable lipid is used, enabling the initial targeting [52]. Furthermore, several targeting moieties have been developed and shown to be effective for targeting specific cells – such targeting moieties include conjugating or integrating antibodies, peptides, sugars, or other molecules, which enable precise targeting of the LNP [53–55]. A second type of non-viral delivery system is polymer-based nanoparticles. These particles are made of polymeric materials such as PEG, polyethylenimine (PEI) or poly(lactic-*co*-glycolic-acid) (PLGA) and more. The polymeric particle can encapsulate either the RNP complex or a nucleic acid encoding it and deliver it to target cells (Fig. 2E) [56,57]. Similar to LNPs, various targeting moieties can be conjugated to the polymeric particles in order to target specific cells [58]. Finally, a third non-viral delivery system is inorganic nanoparticles made of gold or silica that bind the RNP complex or nucleic acid for delivery. Silica nanoparticles have been shown to be effective in delivering Cas9 RNP complexes both *in-vitro* and *in-vivo* in multiple types of cancer [59,60]. In addition, while not shown specifically in cancer, efficient Cas9 RNP complexes delivery was achieved both *in-vitro* and *in-vivo* by using gold nanoparticles (Fig. 2F) [61,62]. Similar to LNPs and polymeric particles, it is possible to control both targeting and efficacy by conjugating various molecules to the particles [63].

As mentioned above, it is possible to enhance the targeting of non-viral particles by adding various targeting moieties. While there are multiple types of targeting moieties, they all share the same general strategy – the targeting moiety binds a surface marker located on the membrane of the targeted cell population, allowing for precision targeting [64]. Targeting moieties include peptides, sugars, and antibodies as well as other molecules. One example of a targeting moiety is the addition of sugars such as mannose and glucan to facilitate delivery to macrophages through expressed mannose receptors [65,66]. A second example is chemically conjugating antibodies targeting surface markers on cancer cells to deliver siRNA to cancer cells [67]. A suitable replacement for chemical conjugation is directly integrating the targeting moiety in the particle. One such system is the Anchored Secondary single-chain FV Enabling Targeting (ASSET). The ASSET system consists of a self-assembling lipoprotein which integrates into the LNP. The single-chain FV interacts with the Fc domain of antibodies, creating a modular targeting system without the need for chemically conjugating the antibodies to the particle [54,68,69]. By utilizing the various delivery systems and targeting moieties it is possible to minimize the delivery of the Cas9 (whether encoded in nucleic acids or in RNP form) into healthy cells without interfering with its delivery to the cancerous cells.

4.2. Conditioned Cas9 expression and activity

A second approach to reducing the off-tumor activity of the Cas9 therapy can be found in synthetic biology. In recent years, much has been written about integrating logic gating into biological systems in order to control protein expression and activity. One system that could be relevant for Cas9 based therapies is conditioned gRNA (also known as cgRNA). cgRNAs are gRNAs which include a secondary RNA structure that enables their inactivation or activation in the presence of specific trigger RNAs (Fig. 3A) [70,71]. Using a cgRNA that will only unlock in the presence of an mRNA that is exclusive to cancerous cells will prevent off-tumor activity. A second system is the toehold – a secondary structure integrated into the mRNA which blocks translation by making the Kozak sequence and start codon unavailable to the Ribosome. A trigger RNA – that can be either an mRNA or a miRNA – hybridizes with the mRNA and unlocks the secondary structure to enable translation (Fig. 3B) [72,73]. Finally, a third type of systems is based upon conditioned degradation of the mRNA, which can be done either by using miRNA binding sites located in the 3′ UTR of the mRNA to lead to mRNA degradation in cells expressing a specific miRNA, or by integrating an aptazyme riboswitch that cleaves the polyA segment upon interaction with the trigger RNA (Fig. 3C) [74,75]. Due to the known differences in

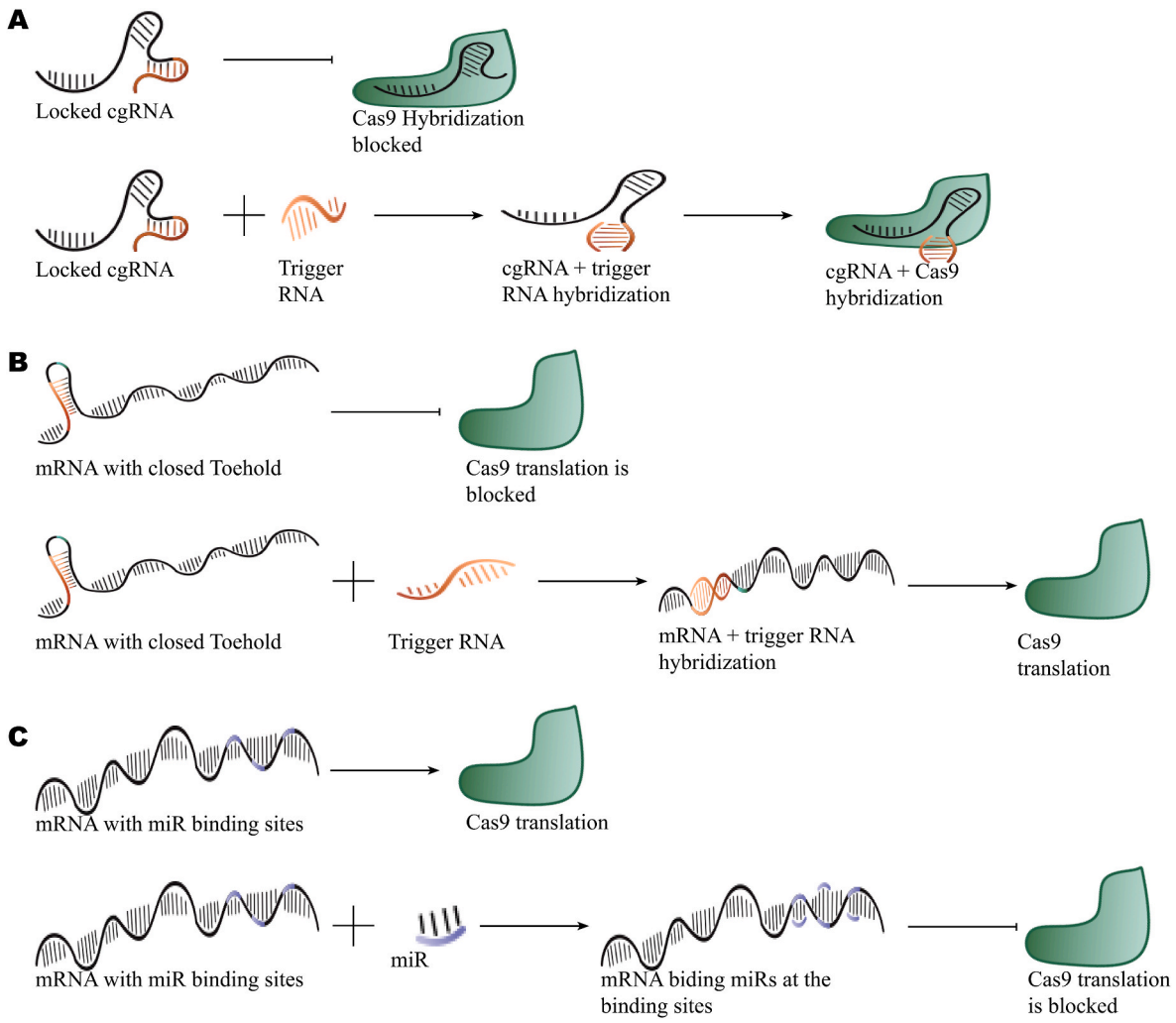

Fig. 3. Conditioned Cas9 expression and activity. A – Upper: a conditioned gRNA (cgRNA) without the trigger RNA, with the target site (shown in orange) folding upon itself and creating a secondary structure which prohibits the cgRNA from hybridizing with the Cas9 to form the active RNP complex. Lower: a cgRNA in the presence of a trigger RNA. The trigger RNA binds the target site (shown in orange) and unfolds the secondary structure, allowing the cgRNA to hybridize with the Cas9 and form the active RNP complex. B – Upper: an mRNA containing a closed toehold without the trigger RNA. The trigger RNA binding site (shown in orange) folds into the toehold stem, while the ATG start codon (shown in green) is found at the loop. The toehold's secondary structure prevents the Ribosome from accessing the start codon, thus blocking Cas9 translation. Lower – an mRNA containing a closed toehold with the trigger RNA. The trigger RNA binds the binding site (shown in orange) and unfolds the toehold's secondary structure. Due to the unfolding of the toehold, the ATG start codon (shown in green) is accessible to the ribosome, allowing Cas9 translation. C – Upper – an mRNA containing multiple miR binding sites without the target miR. The mRNA undergoes normal translation leading to Cas9 expression in the cells. Lower – an mRNA containing multiple miR binding sites in the presence of the target miR. The miRs bind to the binding sites found at the mRNA, leading to translation blockage and mRNA degradation, thus preventing Cas9 expression in the cells.

both gene and miRNA expression in cancer, utilizing such systems could limit Cas9 expression and activity only to the cancerous cells.

4.3. Off-target analysis

Since no system is perfect, even the integration of both targeting and logic gating into the system might not be enough to completely prevent off-tumor activity. Therefore, additional safety measures will be needed to prevent unwanted activity. First, let us focus on the off-target activity as that is more likely to cause cancerous transformation compared to on-target activity. The first approach regarding off-target activity is mapping the potential off-target sites. As the saying goes – "knowing is half the battle" – while it wouldn't prevent off-target activity, correct mapping of the off-target sites will allow for precise assessment of the danger involved. As of today, there are many different methods allowing for the detection of off-target sites such as Circularization *In-Vitro* Reporting of Cleavage Effects by Sequencing (CIRCLE-seq), Selective Enrichment and Identification of Adapter-Tagged DNA Ends by Sequencing (SITE-seq), Discovery of *In-Situ* Cas Off-Targets and Verification by Sequencing (DISCOVER-seq) and Genome-wide, Unbiased Identification of DSBs enabled by Sequencing (GUIDE-seq) [76–79]. The editing in the off-target sites can then be further validated and quantified using methods such as rhAmpSeq, with some enabling to quantify translocations which arise as a result of the editing [80,81]. Since each gene has multiple possible guide targets, and since different guides would have differing levels of off-target activity, screening multiple gRNAs to find the safest and best gRNA would allow to minimize the dangers of off-target activity.

4.4. High-fidelity Cas9 variants

Seeing that even the best of gRNAs might still have off-target activity, other options are needed to further minimize the risk. In the years since its mechanism was first discovered, researchers have developed multiple variations of the Cas9 nuclease, attempting to increase its precision and efficiency, as well as changing its PAM sequence in order to expand the

potential cut sites. Cas9 variants such as the SpCas9-HF1, eSpCas9, HypaCas9 and SuperFi-Cas9 were shown to have increased accuracy without greatly lowering the on-target activity. These variants were made by introducing various modifications to the Cas9 structure – these changes include weakening the DNA binding capability of the nuclease (eSpCas9) and affecting the Cas9 conformational changes to make it stricter (HypaCas9) [82–85]. Another notable variant of Cas9 is the *Fok*I-fused Cas9 – unlike other variants aiming for higher fidelity, this variant of Cas9 uses a catalytically inactive Cas9 variant (commonly known as dead Cas9 or dCas9 for short) that is fused to a *Fok*I nuclease, requiring 2 different target sites that are located near each other in order to work – which greatly reduces the odds of off-target activity [86]. These Cas9 variants and future ones could further minimize the risk of off-target activity while maintaining high levels of on-target activity (Table 1).

4.5. Non-nuclease Cas9 variants

While the variants mentioned up to this point would minimize the off-target activity of the Cas9, the issue of on-target – off-tumor activity remains. While using a gene that has no effect on healthy cells would alleviate some of the danger, the potential for the other adverse effects of the DSB mentioned before is still a challenge. One solution to this problem could be to prevent gene expression without the induction of the DSB. First, it was shown that dCas9 can physically block transcription – while effective, the effects might be too short-term for cancer as the effect will pass once the protein is degraded [87]. However, over the years researchers have fused various effector proteins to the dCas9 platform, using the dCas9 as a targeting system guiding the effector proteins to the desired genomic location. One such system is the dCas9-KRAB protein, a hybrid of dCas9 with the Kruppel-associated box (KRAB) domain of the Kox1 protein. The dCas9-KRAB system recruits chromatin-modifying complexes which silence transcription [88]. A second form of dCas9 based genome editing is Base Editors – a dCas9 or Nickase-Cas9 (nCas9 – a Cas9 mutant only capable of nicking the DNA without inducing the full DSB) fused to either a cytidine aminase or an adenosine aminase enabling the change of single base pairs from A-T to G-C or the other way around [89–92]. This system could be used to induce critical mutations that would deactivate the gene without the need for a DSB, increasing the safety of the system as the various chromosomal abnormalities reported wouldn't occur. A completely different approach to cancer therapy could be to activate silenced genes in the cancerous cells. As of today, several dCas9 systems have been developed to activate gene expression – many of these systems are the fusion of the dCas9 with a chain of VP16 domains or other similar proteins that lead to transcription activation [88]. These systems could potentially reactivate genes silenced by the cancerous cell to reactivate pathways leading to cellular death or in order to lower resistance to current therapies [93]. A final Cas9-based system worthy of a mention is prime editing. Prime editing utilizes an nCas9 fused to an engineered reverse transcriptase to introduce small genomic modifications including both insertions, deletions, and base replacements without the need for a DSB. While most Cas9 technologies utilize similarly sized gRNAs, prime editing gRNAs (pegRNA) are longer, as they contain both the targeting gRNA and the template encoding the desired edit. Following the formation of a nick in the genome, the pegRNA hybridizes with the PAM containing strand, serving as a primer for reverse transcription of the rest of the RNA template. The newly formed DNA strand replaces the original, leading to the insertion of the desired mutation into the genome [94,95]. A slightly more complicated system, known as twin prime editing, utilizes a pair of pegRNA as well as a recombinase to further increase the size of the possible edits [96]. While this technology holds an incredible potential in the field of genetic diseases, it could also be used against cancer by inducing critical mutations into key genes by inducing deletions or insertions. The various catalytically inactive Cas9 variants mentioned here could potentially circumvent the potential adverse effects inherent in the formation of DSBs by interfering with critical gene expression without inducing them (Table 2).

4.6. Other Cas nucleases as a replacement for Cas9

Finally, a completely different approach would be to use other types of Cas effectors – as the years pass, the scientific community keeps

Table 1
CRISPR/Cas9 variants designed for enhanced nuclease accuracy.

Name of Cas9 variant	Source of Nuclease activity	Novelty leading to off-target reduction
SpCas9-HF1	Cas9	Switching strategic amino acids with alanine interfered with Hydrogen-bond forming with the DNA, leading to the need for stricter base-pairing [82]
eSpCas9	Cas9	Neutralizing positive charges in the nontarget strand groove weakened charge-based DNA binding, leading to dependency on gRNA-DNA base-pairing [83]
HypaCas9	Cas9	Mutated REC3 domain to be more stringent and lock the HNH nuclease domain more tightly in the presence of mismatches [84]
SuperFi-Cas9	Cas9	Mutated the loop found in the RuvC domain to reduce off-target tolerance without interfering with on-target activity [85]
*Fok*I-fused Cas9	*Fok*I	Based upon the activity of the *Fok*I nuclease using a dCas9 as a guidance system. Due to the need of hybridization between two *Fok*I enzymes, 2 close gRNAs are needed for activity, making the system more stringent [86]

Table 2
CRISPR/Cas9 variants designed for gene therapy and gene editing without DSB induction.

Name of Cas9 variant	Fused protein/domain	Activity
Dead Cas9 (dCas9)	None	The dCas9 sits on the target site and physically blocks transcription [87]
dCas9-KRAB	Kruppel-Associated box domain	The dCas9 reaches the target site and the KRAB domain recruits chromatin modifying complexes to silence transcription [88]
Adenine Base Editor (ABE)	Mutated tRNA adenine deaminase (TadA)	There are multiple variants of the ABE system, but most include a variant of TadA – after the dCas9 reaches the target site, the TadA deaminates an Adenine into an Inosine [89]
Cytosine Base Editor (CBE)	Cytdine deaminase, Uracil DNA glycosylase inhibitor (UGI)	There are several generations of the CBE system using different cytidine deaminase variants. Once the nCas9 reaches the target site a cytidine is deaminated into an uracil. The UGI prevents uracil excision, and the nickase activity on the other strand ensures the mismatch will be corrected according to the edited strand [90]
dCas9-VP64	4 copies of the herpes simplex viral protein 16 (VP16) oligomer	The VP16 domains promote transcriptional machinery assembly at the target site to induce gene expression [88]
Prime editing	Reverse Transcriptase	The nCas9 forms a nick, followed by reverse transcription of the relevant parts of the pegRNA beginning at the edge of the nick next to the PAM sequence. The transcribed DNA sequence replaces the original strand, inserting the desired mutations [94]

Table 3
summary of works utilizing Cas9 editing against cancer.

Target Gene	Delivery System	Administration Route	Cas9 Cargo type	Biological effect
HPV16 [104]	AAV	Intratumoral injection	dsDNA encoding both Cas9 and the gRNA	Tumor size reduced by 50% *in-vivo*
KSP [105]	Lentivirus	Intraperitoneally	RNA encoding both Cas9 and 2 gRNAs	Tumor size reduced by 90% *in-vivo*
PLK1 [24]	LNP	Intracerebral and intraperitoneally	mRNA encoding Cas9 alongside gRNA	Tumor growth inhibited, mice survival improved by 80%
PD-L1 [106]	LNP	Local or intravenous	mRNA encoding Cas9 alongside gRNA	Tumor size reduced by 50% and 90% (with and without the addition of FAK targeting siRNA), mice survival improved by over 40 days
BCR-ABL [56]	PEG-PEI	Intravenous	Plasmid encoding both Cas9 and the gRNA	hCD45+ cells level reduced by 50%, survival increased by 60%
EGFR [59]	Silica Nanoparticle	Intravenous	Plasmid encoding both Cas9 and the gRNA	The combined effect of EGFR KO and Sorafenib delivery led to tumor growth inhibition
PD-L1 [60]	Silica nanoparticle	Intravenous	Plasmid encoding both Cas9 and the gRNA	The combined effect of PD-L1 KO and Axitinib delivery led to tumor growth inhibition

looking for other forms of nucleases in the Cas family. One DNA targeting Cas nuclease that has been identified is the Cas12a (also known as Cpf1), which just like the Cas9 nuclease, is capable of inducing DSBs [97]. While currently less characterised in comparison to the Cas9, the Cas12a nuclease uses a PAM in the form of TTTN, meaning the cut sites would greatly differ compared to Cas9, with a preference for A-T rich areas in the genome. It should be noted that it has been found that Cas12a has a relatively high off-target activity levels which includes both DNA degradation and DNA nicking, which might make it less safe in comparison to Cas9 [98]. However, there has also been some work regarding lowering said off-target activity, for example by using a DNA-RNA hybrid as a guiding molecule as it lowers the binding capability of the guide with DNA that contains mismatches [99]. Therefore, while it may hold the same potential to be used as a cancer therapy as Cas9, the system would require more characterization and engineering before it will be ready for clinical translation.

A second type of Cas nuclease that has been identified and whose use is growing more common as time passes is Cas13. Unlike Cas9 and Cas12a which are DNA targeting nucleases, Cas13 is an RNA targeting nuclease. Cas13 scans RNA transcripts, and upon finding a match with its gRNA, it cleaves the transcript. A second difference between Cas9 and Cas12 and Cas13, is that Cas13 doesn't require a PAM sequence which increases its flexibility [100]. Cas13 has been shown to be an effective tool for knocking down gene expression without risking the long-term effects of DNA editing [101]. Furthermore, several works have shown that just like was done with dCas9, it is possible to harness a catalytically inactive Cas13 as an RNA targeting system for various RNA modifying enzymes, allowing RNA editing instead of simply knocking down gene expression [102]. While this technology holds a great potential for therapeutic applications, especially when it comes to curing disease causing mutations, it is our belief that as of today it is less relevant for cancer therapy as siRNA achieves similar effects within roughly the same timeframe without requiring the complex delivery of the complex as was detailed regarding Cas9. Furthermore, as the effects are temporary there is good reason to expect the need for several treatments to eradicate the disease, and recent works have shown that just like with Cas9, the human immune system is primed against the bacterial protein [103]. Despite that, further developments in the field may one day lead to the clinical translation of Cas13-based cancer therapies.

5. Conclusions

In summary, as of today, multiple works have shown the effectiveness of Cas9 as a potential cancer therapy *in vivo* (Table 3). This review has shown that while there are still many remaining challenges before genome editing can be used in the clinic as a therapy for cancer, there are currently many possible ways to overcome those challenges that are under development. By combining various aspects of biological research such as targeted therapy and conditioned Cas9 activity it should be possible to minimize off-tumor activity down to clinically relevant levels. Hence, it is likely to assume that the inherent challenges will be solved, and that Cas9 based therapies will become a reality in the near future, leading to a revolution in the field of cancer therapeutics. And to summarize with a quote that perfectly concludes this review paper: "There is a way out of every box, a solution to every puzzle; it's just a matter of finding it" (Capt. Jean-Luc Picard, Star Trek: The Next Generation).

Ethics approval and consent to participate

We declare our consent to participate in this special issue.
D.B. and D.P.

Credit authorship contribution statement

Dor Breier: conceived the research, wrote the original draft and revised the manuscript. **Dan Peer:** conceived the research, wrote the original draft and revised the manuscript, Supervised and received funding.

Funding

This work was funded by the Shmunis Family Foundation awarded to D.P.

Declaration of competing interest

D.P. declares the following competing financial interest(s): D.P. receives licensing fees (to patents on which he was an inventor) from, invested in, consults (or on scientific advisory boards or boards of directors) for, lectured (and received a fee) or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNtech SE., Kernal Biologics, Merck, Newphase Ltd., NLC Pharma Ltd., NeoVac Ltd., Roche, SirTLabs Corporation, Teva Pharmaceuticals Inc.

D.B. declares no competing financial interests.

References

[1] D. Carroll, Genome engineering with targetable nucleases, Annu. Rev. Biochem. 83 (2014) 409–439, https://doi.org/10.1146/annurev-biochem-060713-035418.

[2] M. Porteus, Genome editing: a new approach to human therapeutics, Annu. Rev. Pharmacol. Toxicol. 56 (2016) 163–190, https://doi.org/10.1146/annurev-pharmtox-010814-124454.

[3] M. Jasin, J.E. Haber, The democratization of gene editing: insights from site-specific cleavage and double-strand break repair, DNA Repair 44 (2016) 6–16, https://doi.org/10.1016/j.dnarep.2016.05.001.

[4] C. Wyman, R. Kanaar, DNA double-strand break repair: all's well that ends well, Annu. Rev. Genet. 40 (2006) 363–383, https://doi.org/10.1146/annurev.genet.40.110405.090451.

[5] M. Bibikova, et al., Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases, Genetics 161 (3) (2002) 1169–1175.

[6] M.H. Porteus, A new class of medicines through DNA editing, N. Engl. J. Med. 380 (10) (2019) 947–959, https://doi.org/10.1056/NEJMra1800729.

[7] W. Sun, et al., Strategies for enhancing the homology-directed repair efficiency of CRISPR-cas systems, Crispr J. 5 (1) (2022) 7–18, https://doi.org/10.1089/crispr.2021.0039.

[8] N. Bharathkumar, et al., CRISPR/cas-based modifications for therapeutic applications: a review, Mol. Biotechnol. 64 (4) (2022) 355–372, https://doi.org/10.1007/s12033-021-00422-8.

[9] T. Gaj, C.A. Gersbach, C.F. Barbas 3rd, ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering, Trends Biotechnol. 31 (7) (2013) 397–405, https://doi.org/10.1016/j.tibtech.2013.04.004.

[10] C. Mussolino, T. Mlambo, T. Cathomen, Proven and novel strategies for efficient editing of the human genome, Curr. Opin. Pharmacol. 24 (2015) 105–112, https://doi.org/10.1016/j.coph.2015.08.008.

[11] M. Jinek, et al., A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (6096) (2012) 816–821, https://doi.org/10.1126/science.1225829.

[12] P.D. Hsu, E.S. Lander, F. Zhang, Development and applications of CRISPR-Cas9 for genome engineering, Cell 157 (6) (2014) 1262–1278, https://doi.org/10.1016/j.cell.2014.05.010.

[13] R.M. Gupta, K. Musunuru, Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9, J. Clin. Invest. 124 (10) (2014) 4154–4161, https://doi.org/10.1172/jci72992.

[14] F.A. Ran, et al., In vivo genome editing using Staphylococcus aureus Cas9, Nature 520 (7546) (2015) 186–191, https://doi.org/10.1038/nature14299.

[15] A. Hendel, et al., Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells, Nat. Biotechnol. 33 (9) (2015) 985–989, https://doi.org/10.1038/nbt.3290.

[16] A. Lattanzi, et al., Development of β-globin gene correction in human hematopoietic stem cells as a potential durable treatment for sickle cell disease, Sci. Transl. Med. 13 (598) (2021), https://doi.org/10.1126/scitranslmed.abf2444.

[17] S. Rafii, et al., Current status of CRISPR/Cas9 application in clinical cancer research: opportunities and challenges, Cancers 14 (4) (2022), https://doi.org/10.3390/cancers14040947.

[18] R.L. Siegel, et al., Cancer statistics, 2022, CA A Cancer J. Clin. 72 (1) (2022) 7–33, https://doi.org/10.3322/caac.21708.

[19] G. Kara, G.A. Calin, B. Ozpolat, RNAi-based therapeutics and tumor targeted delivery in cancer, Adv. Drug Deliv. Rev. 182 (2022), 114113, https://doi.org/10.1016/j.addr.2022.114113.

[20] E.S. Smith, et al., Clinical applications of short non-coding RNA-based therapies in the era of precision medicine, Cancers 14 (6) (2022), https://doi.org/10.3390/cancers14061588.

[21] S. Mirzaei, et al., Pre-clinical and clinical applications of small interfering RNAs (siRNA) and Co-delivery systems for pancreatic cancer therapy, Cells 10 (12) (2021), https://doi.org/10.3390/cells10123348.

[22] H. Zogg, R. Singh, S. Ro, Current advances in RNA therapeutics for human diseases, Int. J. Mol. Sci. 23 (5) (2022), https://doi.org/10.3390/ijms23052736.

[23] M. Boettcher, M.T. McManus, Choosing the right tool for the job: RNAi, TALEN, or CRISPR, Mol. Cell 58 (4) (2015) 575–585, https://doi.org/10.1016/j.molcel.2015.04.028.

[24] D. Rosenblum, et al., CRISPR-Cas9 genome editing using targeted lipid nanoparticles for cancer therapy, Sci. Adv. 6 (47) (2020), https://doi.org/10.1126/sciadv.abc9450.

[25] M.L. Leibowitz, et al., Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing, Nat. Genet. 53 (6) (2021) 895–905, https://doi.org/10.1038/s41588-021-00838-7.

[26] G. Cullot, et al., CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations, Nat. Commun. 10 (1) (2019) 1136, https://doi.org/10.1038/s41467-019-09006-2.

[27] M.V. Zuccaro, et al., Allele-specific chromosome removal after Cas9 cleavage in human embryos, Cell 183 (6) (2020) 1650–1664, https://doi.org/10.1016/j.cell.2020.10.025, e15.

[28] A.D. Nahmad, et al., Frequent aneuploidy in primary human T cells after CRISPR-Cas9 cleavage, Nat. Biotechnol. (2022), https://doi.org/10.1038/s41587-022-01377-0.

[29] Y. Fu, et al., High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells, Nat. Biotechnol. 31 (9) (2013) 822–826, https://doi.org/10.1038/nbt.2623.

[30] P.D. Hsu, et al., DNA targeting specificity of RNA-guided Cas9 nucleases, Nat. Biotechnol. 31 (9) (2013) 827–832, https://doi.org/10.1038/nbt.2647.

[31] L. Zhong, et al., Generation of in situ CRISPR-mediated primary and metastatic cancer from monkey liver, Signal Transduct. Targeted Ther. 6 (1) (2021) 411, https://doi.org/10.1038/s41392-021-00799-7.

[32] W. Xue, et al., CRISPR-mediated direct mutation of cancer genes in the mouse liver, Nature 514 (7522) (2014) 380–384, https://doi.org/10.1038/nature13589.

[33] R.B. Blasco, et al., Simple and rapid in vivo generation of chromosomal rearrangements using CRISPR/Cas9 technology, Cell Rep. 9 (4) (2014) 1219–1227, https://doi.org/10.1016/j.celrep.2014.10.051.

[34] M. Behr, et al., In vivo delivery of CRISPR-Cas9 therapeutics: progress and challenges, Acta Pharm. Sin. B 11 (8) (2021) 2150–2171, https://doi.org/10.1016/j.apsb.2021.05.020.

[35] D. Rosenblum, et al., Progress and challenges towards CRISPR/Cas clinical translation, Adv. Drug Deliv. Rev. 154–155 (2020) 176–186, https://doi.org/10.1016/j.addr.2020.07.004.

[36] C. Li, R.J. Samulski, Engineering adeno-associated virus vectors for gene therapy, Nat. Rev. Genet. 21 (4) (2020) 255–272, https://doi.org/10.1038/s41576-019-0205-4.

[37] E. Senís, et al., CRISPR/Cas9-mediated genome engineering: an adeno-associated viral (AAV) vector toolbox, Biotechnol. J. 9 (11) (2014) 1402–1412, https://doi.org/10.1002/biot.201400046.

[38] E. Hastie, R.J. Samulski, Adeno-associated virus at 50: a golden anniversary of Discovery, research, and gene therapy success—a personal perspective, Hum. Gene Ther. 26 (5) (2015) 257–265, https://doi.org/10.1089/hum.2015.025.

[39] X. Wu, A.J. Kriz, P.A. Sharp, Target specificity of the CRISPR-Cas9 system, Quant. Biol. 2 (2) (2014) 59–70, https://doi.org/10.1007/s40484-014-0030-x.

[40] A. Mehta, O.M. Merkel, Immunogenicity of Cas9 protein, J. Pharmaceut. Sci. 109 (1) (2020) 62–67, https://doi.org/10.1016/j.xphs.2019.10.003.

[41] C.T. Charlesworth, et al., Identification of preexisting adaptive immunity to Cas9 proteins in humans, Nat. Med. 25 (2) (2019) 249–254, https://doi.org/10.1038/s41591-018-0326-x.

[42] V.L. Simhadri, et al., Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population, Mol. Ther. Methods Clin. Dev. 10 (2018) 105–112, https://doi.org/10.1016/j.omtm.2018.06.006.

[43] S. Ling, et al., Lentiviral delivery of co-packaged Cas9 mRNA and a Vegfa-targeting guide RNA prevents wet age-related macular degeneration in mice, Nat. Biomed. Eng. 5 (2) (2021) 144–156, https://doi.org/10.1038/s41551-020-00656-y.

[44] P.I. Ortinski, et al., Integrase-deficient lentiviral vector as an all-in-one platform for highly efficient CRISPR/Cas9-Mediated gene editing, Mol. Ther. Methods Clin. Dev. 5 (2017) 153–164, https://doi.org/10.1016/j.omtm.2017.04.002.

[45] J.R. Hamilton, et al., Targeted delivery of CRISPR-Cas9 and transgenes enables complex immune cell engineering, Cell Rep. 35 (9) (2021), 109207, https://doi.org/10.1016/j.celrep.2021.109207.

[46] S. Banskota, et al., Engineered virus-like particles for efficient in vivo delivery of therapeutic proteins, Cell 185 (2) (2022) 250–265, https://doi.org/10.1016/j.cell.2021.12.021, e16.

[47] A. Gutkin, D. Rosenblum, D. Peer, RNA delivery with a human virus-like particle, Nat. Biotechnol. 39 (12) (2021) 1514–1515, https://doi.org/10.1038/s41587-021-01124-x.

[48] M. Segel, et al., Mammalian retrovirus-like protein PEG10 packages its own mRNA and can be pseudotyped for mRNA delivery, Science 373 (6557) (2021) 882–889, https://doi.org/10.1126/science.abg6155.

[49] M. Schlich, et al., Cytosolic delivery of nucleic acids: the case of ionizable lipid nanoparticles, Bioeng. Transl. Med. 6 (2) (2021), e10213, https://doi.org/10.1002/btm2.10213.

[50] X. Han, et al., An ionizable lipid toolbox for RNA delivery, Nat. Commun. 12 (1) (2021) 7233, https://doi.org/10.1038/s41467-021-27493-0.

[51] X. Han, M.J. Mitchell, G. Nie, Nanomaterials for therapeutic RNA delivery, Matter 3 (6) (2020) 1948–1975, https://doi.org/10.1016/j.matt.2020.09.020.

[52] M.M. Żak, L. Zangi, Lipid nanoparticles for organ-specific mRNA therapeutic delivery, Pharmaceutics 13 (10) (2021), https://doi.org/10.3390/pharmaceutics13101675.

[53] E.A. Taha, J. Lee, A. Hotta, Delivery of CRISPR-Cas tools for in vivo genome editing therapy: trends and challenges, J. Contr. Release 342 (2022) 345–361, https://doi.org/10.1016/j.jconrel.2022.01.013.

[54] R. Kedmi, et al., A modular platform for targeted RNAi therapeutics, Nat. Nanotechnol. 13 (3) (2018) 214–219, https://doi.org/10.1038/s41565-017-0043-5.

[55] R.M. Ray, et al., Enhanced target cell specificity and uptake of lipid nanoparticles using RNA aptamers and peptides, Beilstein J. Org. Chem. 17 (2021) 891–907, https://doi.org/10.3762/bjoc.17.75.

[56] Y. Liu, et al., Systemic delivery of CRISPR/Cas9 with PEG-PLGA nanoparticles for chronic myeloid leukemia targeted therapy, Biomater. Sci. 6 (6) (2018) 1592–1603, https://doi.org/10.1039/c8bm00263k.

[57] G. Chen, et al., A biodegradable nanocapsule delivers a Cas9 ribonucleoprotein complex for in vivo genome editing, Nat. Nanotechnol. 14 (10) (2019) 974–980, https://doi.org/10.1038/s41565-019-0539-2.

[58] A. Gagliardi, et al., Biodegradable Polymeric Nanoparticles for Drug Delivery to Solid Tumors, vol. 12, 2021, https://doi.org/10.3389/fphar.2021.601626.

[59] B.-C. Zhang, et al., Co-Delivery of sorafenib and CRISPR/Cas9 based on targeted core–shell hollow mesoporous organosilica nanoparticles for synergistic HCC therapy, ACS Appl. Mater. Interfaces 12 (51) (2020) 57362–57372, https://doi.org/10.1021/acsami.0c17660.

[60] Q. Liu, et al., Virus-like nanoparticle as a co-delivery system to enhance efficacy of CRISPR/Cas9-based cancer immunotherapy, Biomaterials 258 (2020), 120275, https://doi.org/10.1016/j.biomaterials.2020.120275.

[61] K. Lee, et al., Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair, Nat. Biomed. Eng. 1 (2017) 889–901, https://doi.org/10.1038/s41551-017-0137-2.

[62] B. Lee, et al., Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours, Nat. Biomed. Eng. 2 (7) (2018) 497–507, https://doi.org/10.1038/s41551-018-0252-8.
[63] Y. Zhou, et al., Mesoporous silica nanoparticles for drug and gene delivery, Acta Pharm. Sin. B 8 (2) (2018) 165–177, https://doi.org/10.1016/j.apsb.2018.01.007.
[64] D. Tarab-Ravski, L. Stotsky-Oterin, D. Peer, Delivery strategies of RNA therapeutics to leukocytes, J. Contr. Release 342 (2022) 362–371, https://doi.org/10.1016/j.jconrel.2022.01.016.
[65] N. Leber, et al., α-Mannosyl-Functionalized cationic nanohydrogel particles for targeted gene knockdown in immunosuppressive macrophages, Macromol. Biosci. 19 (7) (2019), e1900162, https://doi.org/10.1002/mabi.201900162.
[66] M. Zhang, et al., Non-viral nanoparticle delivers small interfering RNA to macrophages in vitro and in vivo, PLoS One 10 (3) (2015), e0118472, https://doi.org/10.1371/journal.pone.0118472.
[67] S. Weinstein, et al., Harnessing RNAi-based nanomedicines for therapeutic gene silencing in B-cell malignancies, Proc. Natl. Acad. Sci. U. S. A. 113 (1) (2016) E16–E22, https://doi.org/10.1073/pnas.1519273113.
[68] N. Veiga, et al., Leukocyte-specific siRNA delivery revealing IRF8 as a potential anti-inflammatory target, J. Contr. Release 313 (2019) 33–41, https://doi.org/10.1016/j.jconrel.2019.10.001.
[69] N. Veiga, et al., Cell specific delivery of modified mRNA expressing therapeutic proteins to leukocytes, Nat. Commun. 9 (1) (2018) 4493, https://doi.org/10.1038/s41467-018-06936-1.
[70] M. Jin, et al., Programmable CRISPR-cas repression, activation, and computation with sequence-independent targets and triggers, ACS Synth. Biol. 8 (7) (2019) 1583–1589, https://doi.org/10.1021/acssynbio.9b00141.
[71] L.M. Hochrein, H. Li, N.A. Pierce, High-performance allosteric conditional guide RNAs for mammalian cell-selective regulation of CRISPR/Cas, ACS Synth. Biol. 10 (5) (2021) 964–971, https://doi.org/10.1021/acssynbio.1c00037.
[72] S. Wang, N.J. Emery, A.P. Liu, A novel synthetic toehold switch for MicroRNA detection in mammalian cells, ACS Synth. Biol. 8 (5) (2019) 1079–1088, https://doi.org/10.1021/acssynbio.8b00530.
[73] E.M. Zhao, et al., RNA-responsive elements for eukaryotic translational control, Nat. Biotechnol. (2021), https://doi.org/10.1038/s41587-021-01068-2.
[74] G. Zhong, et al., Rational design of aptazyme riboswitches for efficient control of gene expression in mammalian cells, Elife 5 (2016), https://doi.org/10.7554/eLife.18858.
[75] R. Jain, et al., MicroRNAs enable mRNA therapeutics to selectively program cancer cells to self-destruct, Nucleic Acid Therapeut. 28 (5) (2018) 285–296, https://doi.org/10.1089/nat.2018.0734.
[76] S.Q. Tsai, et al., CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets, Nat. Methods 14 (6) (2017) 607–614, https://doi.org/10.1038/nmeth.4278.
[77] P. Cameron, et al., Mapping the genomic landscape of CRISPR-Cas9 cleavage, Nat. Methods 14 (6) (2017) 600–606, https://doi.org/10.1038/nmeth.4284.
[78] B. Wienert, et al., Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq, Science 364 (6437) (2019) 286–289, https://doi.org/10.1126/science.aav9023.
[79] S.Q. Tsai, et al., GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases, Nat. Biotechnol. 33 (2) (2015) 187–197, https://doi.org/10.1038/nbt.3117.
[80] J. Shapiro, et al., Increasing CRISPR efficiency and measuring its specificity in HSPCs using a clinically relevant system, Mol. Ther. Methods Clin. Dev. 17 (2020) 1097–1107, https://doi.org/10.1016/j.omtm.2020.04.027.
[81] I. Amit, et al., CRISPECTOR provides accurate estimation of genome editing translocation and off-target activity from comparative NGS data, Nat. Commun. 12 (1) (2021) 3042, https://doi.org/10.1038/s41467-021-22417-4.
[82] B.P. Kleinstiver, et al., High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects, Nature 529 (7587) (2016) 490–495, https://doi.org/10.1038/nature16526.
[83] I.M. Slaymaker, et al., Rationally engineered Cas9 nucleases with improved specificity, Science 351 (6268) (2016) 84–88, https://doi.org/10.1126/science.aad5227.
[84] J.S. Chen, et al., Enhanced proofreading governs CRISPR–Cas9 targeting accuracy, Nature 550 (7676) (2017) 407–410, https://doi.org/10.1038/nature24268.
[85] J.P.K. Bravo, et al., Structural basis for mismatch surveillance by CRISPR-Cas9, Nature 603 (7900) (2022) 343–347, https://doi.org/10.1038/s41586-022-04470-1.
[86] J.P. Guilinger, D.B. Thompson, D.R. Liu, Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification, Nat. Biotechnol. 32 (6) (2014) 577–582, https://doi.org/10.1038/nbt.2909.
[87] L.S. Qi, et al., Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression, Cell 152 (5) (2013) 1173–1183, https://doi.org/10.1016/j.cell.2013.02.022.
[88] L.A. Gilbert, et al., CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes, Cell 154 (2) (2013) 442–451, https://doi.org/10.1016/j.cell.2013.06.044.
[89] N.M. Gaudelli, et al., Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage, Nature 551 (7681) (2017) 464–471, https://doi.org/10.1038/nature24644.
[90] A.C. Komor, et al., Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage, Nature 533 (7603) (2016) 420–424, https://doi.org/10.1038/nature17946.
[91] M.F. Richter, et al., Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity, Nat. Biotechnol. 38 (7) (2020) 883–891, https://doi.org/10.1038/s41587-020-0453-z.
[92] K. Nishida, et al., Targeted Nucleotide Editing Using Hybrid Prokaryotic and Vertebrate Adaptive Immune Systems, vol. 353, 2016, p. aaf8729, https://doi.org/10.1126/science.aaf8729, 6305.
[93] M.M. Rahman, T.O. Tollefsbol, Targeting cancer epigenetics with CRISPR-dCAS9: principles and prospects, Methods 187 (2021) 77–91, https://doi.org/10.1016/j.ymeth.2020.04.006.
[94] A.V. Anzalone, et al., Search-and-replace genome editing without double-strand breaks or donor DNA, Nature 576 (7785) (2019) 149–157, https://doi.org/10.1038/s41586-019-1711-4.
[95] A.V. Anzalone, L.W. Koblan, D.R. Liu, Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors, Nat. Biotechnol. 38 (7) (2020) 824–844, https://doi.org/10.1038/s41587-020-0561-9.
[96] A.V. Anzalone, et al., Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing, Nat. Biotechnol. 40 (5) (2022) 731–740, https://doi.org/10.1038/s41587-021-01133-w.
[97] B. Zetsche, et al., Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (3) (2015) 759–771, https://doi.org/10.1016/j.cell.2015.09.038.
[98] K. Murugan, et al., CRISPR-Cas12a has widespread off-target and dsDNA-nicking effects, J. Biol. Chem. 295 (17) (2020) 5538–5553, https://doi.org/10.1074/jbc.RA120.012933.
[99] H. Kim, et al., Enhancement of target specificity of CRISPR-Cas12a by using a chimeric DNA-RNA guide, Nucleic Acids Res. 48 (15) (2020) 8601–8616, https://doi.org/10.1093/nar/gkaa605.
[100] N. Huynh, et al., A versatile toolkit for CRISPR-Cas13-based RNA manipulation in Drosophila, Genome Biol. 21 (1) (2020) 279, https://doi.org/10.1186/s13059-020-02193-y.
[101] O.O. Abudayyeh, et al., RNA targeting with CRISPR-Cas13, Nature 550 (7675) (2017) 280–284, https://doi.org/10.1038/nature24049.
[102] T. Tang, et al., Programmable System of Cas13-Mediated RNA Modification and its Biological and Biomedical Applications, vol. 9, 2021, https://doi.org/10.3389/fcell.2021.677587.
[103] X.-Z.E. Tang, et al., Pre-existing adaptive immunity to the RNA-editing enzyme Cas13d in humans, Nat. Med. (2022), https://doi.org/10.1038/s41591-022-01848-6.
[104] D.S. Hsu, et al., Targeting HPV16 DNA using CRISPR/Cas inhibits anal cancer growth in vivo, Future Virol. 13 (7) (2018) 475–482, https://doi.org/10.2217/fvl-2018-0010.
[105] S. Lee, Y.-Y. Kim, H.J. Ahn, Systemic delivery of CRISPR/Cas9 to hepatic tumors for cancer treatment using altered tropism of lentiviral vector, Biomaterials 272 (2021), 120793, https://doi.org/10.1016/j.biomaterials.2021.120793.
[106] D. Zhang, et al., Enhancing CRISPR/Cas gene editing through modulating cellular mechanical properties for cancer therapy, Nat. Nanotechnol. 17 (7) (2022) 777–787, https://doi.org/10.1038/s41565-022-01122-3.