JPET Fast Forward. Published on May 23, 2019 as DOI: 10.1124/jpet.119.257287
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JPET # 257287

Title: CRISPR/Cas system for genome editing: progress and prospects as a therapeutic tool

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Running title: CRISPR/Cas9 therapeutics

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Number of text pages: 21

Number of tables: 2

Number of figures: 3

Number of references: 115

Number of words

Abstract: 207

Main text: 6357

Abbreviations: CRISPR (Clustered regularly interspaced short palindromic repeats), Cas gene (CRISPR associated gene), sgRNA (Single guide RNA), tracrRNA (trans-activating CRISPR RNA), pDNA (plasmid DNA), HDR (homology-directed repair), NHEJ (non-homologous end joining), ZFNs (zinc-finger nuclease), (TALENs) transcription activator-like effector nucleases,

Special section: "Drug Delivery Technologies" (Ravi Kumar Majeti)

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) was first observed in 1987 in bacteria and archaea that was later confirmed as a part of bacterial adaptive immunity against the attacking phage. CRISPR/Cas restriction system involves the restriction endonuclease enzyme guided by a hybrid strand of RNA consisting of CRISPR RNA (crRNA) and transactivating RNA (tracrRNA) that results in a gene knock-out or knock-in followed by non-homologous end joining (NHEJ) and homology-directed repair (HDR). Owing to its efficiency, specificity and reproducibility, CRISPR/Cas restriction system was said to be the "breakthrough" in the field of biotechnology. Apart from its application in the biotechnology, CRISPR/Cas has been explored for its therapeutic potential in several diseases including cancer, alzheimer disease, sickle cell disease (SCD), duchenne muscular dystrophy (DMD), neurological disorders, etc wherein CRISPR/Cas components like Cas 9/sgRNA ribonucleoprotein (RNP), sgRNA/mRNA and plasmid were delivered. However, limitations including immunogenicity, low transfection, limited payload, instability and off-target binding pose hurdles in its therapeutic use. Non-viral vectors (including cationic polymers, lipids, etc) which are being classically used as carriers for therapeutic genes were utilized to deliver CRISPR/Cas components that showed interesting results. Herein, we have discussed the CRISPR/Cas system, its brief history and classification followed by its therapeutic applications using current non-viral delivery strategies.

Introduction

Genetic engineering is a modern tool used for direct editing of heritable or non-heritable genetic material to modulate the genotype or phenotype of the particular cell, tissue or organism. This science play with the deletion or insertion of a gene or any DNA sequence to produce revolutionary genetic changes (Collins, 2018). Gene editing have shown benefits in the management of both genetic and non-genetic conditions. Among various tools available for genome editing, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated system (Cas) (together called a CRISPR/Cas system) has shown significant advantages in terms of simplicity and specificity thereby generating interest in many research groups. CRISPR/Cas gene editing mechanism has now been well established (Doudna and Charpentier, 2014). Briefly, CRISPR locus/array contain cleaved protospacer from incoming bacteriophage. Due to protospacer addition within the CRISPR locus, it become easy for the bacteria to recognize a phage on its subsequent entry since the protospacer act as a memory for the corresponding invading phage. Now bacteria synthesize its own sgRNA and Cas9 that cleaves the phage DNA at the specific site complementary to the sgRNA and protect bacteria against phage attack (Nunez et al., 2014). The sgRNA consists of CRISPR RNA (crRNA) having a complementary sequence of phage DNA and transactivating CRISPR RNA that join to crRNA. Following its establishment as a biotechnology tool, CRISPR/Cas system has been explored for its therapeutic potential in several conditions including alzheimer's disease (Rohn et al., 2018), eye disease (Hung et al., 2016), sickle cell disease (SCD) (Park et al., 2016), duchenne muscular dystrophy (DMD) (Nelson et al., 2016), cancer (Chen et al., 2017) and neurological disorders (Rohn et al., 2018). Promising results of CRISPR/Cas9 editing were also seen in a metastatic lung cancer patient treated with Cas9 engineered T cells (Cyranoski, 2016).

For genome editing using CRISPR/Cas system, three strategies/approaches have been explored i.e. delivering CRISPR plasmid, mRNA encoding for Cas protein and sgRNA, and ribonucleoprotein complexes of sgRNA and Cas protein (Figure 1). All these approaches have their advantages as well as disadvantages in terms of efficiency, off-target effects, specificity, and cost. CRISPR plasmid delivery in one of the simple and commonly used approach wherein Cas protein and sgRNA are encoded by the same vector thus omitting the need of multiple transfections for different CRISPR/Cas components (Ran et al., 2013). However, it is more time consuming, and plasmid need to be delivered directly into the nucleus. The second approach is to deliver sgRNA and Cas9 mRNA (mRNA encoding for cas9 protein) separately. Cas9 mRNA translates to the cas9 protein that joins with the sgRNA in the cytoplasm to form ribonucleoprotein (RNP) (Niu et al., 2014). This approach decreases off-target binding and needs delivery only up to the cytoplasm. In the third approach, complex of sgRNA and Cas9 protein is delivered into the cell (Zuris et al., 2015a). This approach has gained a lot of interest owing to its reduced off-target binding, less toxicity, higher efficiency and simpler design.

CRISPR/Cas technology holds enormous potential and is very efficient, but, beyond its editing efficiency, hurdles in its *in vivo* delivery limits its use as a clinically translatable therapeutic tool. Physical methods, viral and non-viral vectors that have been earlier used for gene delivery applications are also adopted for delivering CRISPR/Cas components. These methods are have their own pros and cons in terms of off-target effects, toxicity, mutagenesis, immunogenicity and loading capacity. Non-viral carriers including cationic lipids, cationic polymer, micelleplexes, inorganic nanoparticles, have gained enormous interest because of the flexibility they offer in their design to overcome the limitations of other methods. However, these are also not devoid of delivery challenges and thus there is always a search for newer carrier materials with improved properties. Several reviews have discussed the use

of this technology in genome editing for several diseases including cancer (Martinez-Lage et al., 2018; Yin et al., 2019; Zhan et al., 2019), sickle cell disease (Demirci et al., 2019), Duchenne Muscular Dystrophy (DMD) (Lim et al., 2018) etc. Few recent reviews have also discussed the delivery aspects of CRISPR/cas components (Li et al., 2018; Lino et al., 2018). This review particularly focusses on progress and prospects of CRISPR/Cas technology followed by strategies being utilized for their delivery using non-viral carriers.

History and Origin of CRISPR

Gene editing was done since few decades through conventional homologous recombination, to produce knockout/knock-in mice (Smithies et al., 1985). Later on, two methods viz., zinc-finger nuclease (ZFNs) and transcription activator-like effector nucleases (TALENs) were used for the purpose of gene editing that works through mechanism of double-stranded break (DSB), and can fundamentally target any sequence in the human genome These techniques are one of the most widely used biotechnological tool for gene editing (Zhang et al., 2019). The ability to customize DNA is totally dependent on the DNA binding affinity as well as the specificity of the designed protein (Zinc finger and TALENs). Despite several advantages both TALENs as well as ZFNs face challenges including difficulty in engineering (Ramirez et al., 2008), limited target site selection, off-target binding (Hockemeyer et al., 2009; Hockemeyer et al., 2011) and high cost. Following the discovery of CRISPR/Cas system, interest has been diverted toward its use as a potential tool for gene editing. All these methods of genome editing have their own adaptability and application-based uses. For example, in human pluripotent stem cells, CRISPR/Cas9 brings more advantages over the other two techniques. Furthermore, CRISPR/Cas has been found to have more feasible properties like easy designing and versatility than ZFNs and TALENs (Gagat et al., 2017). CRISPR/Cas system is also cost effective and found to have more efficiency then others. Nowadays, where time is the major concern, CRISPR/Cas system

offers precise result and editing of the genome could be achieved within weeks (Ran et al., 2013).

Looking back into CRISPR/Cas history, it was first observed as a repeatedly ordered motif of less than 50 bp in the genome of bacteria and archaea (Ishino et al., 1987). Earlier it was thought to be unevenly distributed in bacteria and archaea that later on was found approximately 90% and 50% in the archaeal and bacterial genome, respectively. These motifs were found to have common features such as non-coding, different from each other (Lintner et al., 2011) and also interspaced (i.e containing foreign sequence in between) thus these were named as "Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)" (Al-Attar et al., 2011). In 2007, it was suggested that when Escherichia coli was subjected to a viral attack continuously, then a DNA was introduced in CRISPR interspacing regions, derived from phage genomic sequence thus demonstrating CRISPR/Cas as a defense system in E. coli against phage attack (Barrangou et al., 2007). It was further put forward that bacteria have an adaptive type of immunity in the form of CRISPR (Garneau et al., 2010). Mojica *et al*. sequenced 4500 CRISPR sequences from 67 strains of bacteria and archaea. On comparing these sequences in GenBank, they surprisingly found that these sequences matches with the bacteriophage sequences, invasive plasmid sequences and other genomic sequences (Mojica et al., 2005). Thereafter, Mojica *et al*. in 2009 stated that CRISPR along with spacer provide the resistance against the phage attacking the bacteria. Experiments were performed to consolidate this hypothesis wherein it was observed that resistance of bacteria against specific phage reversed when the spacer sequence was removed from the bacterial genome. The integrated spacer was then termed as CRISPR associated (Cas) gene and this whole system was named as the CRISPR/Cas system (Godde and Bickerton, 2006).

In 2010, Garneau *et al*. demonstrated that CRISPR/Cas system is one of the defence system of bacteria against virus and Cas gene (acquired from exochromosomal element i.e.

Virus) play a vital role in cleavage of plasmid and bacteriophage DNA. Many of the ongoing studies described various silent features of CRISPR/Cas system as it has all the crucial characteristics required for a biotechnology editing tool and became the subject of intensive study (Doudna and Charpentier, 2014). It has been observed that the length and sequence of the spacer may vary in same or different CRISPR with an ideal range of 26-72 bp spacer sequence and 21-48 bp repeats sequence (Haft et al., 2005; Labrie et al., 2010) . Further, the number of spacers within the CRISPR locus present in cells genome or plasmid also vary from few to hundreds (Rath *et al*., 2015). For e.g., Methanocaldococcus sp. FS406-22 and Sulfolobus tokodaii str. 7 has eighteen CRISPR with 191 spacer and five CRISPR with 458 spacers respectively (Rousseau et al., 2009). It has also been reported that Cas gene is not always present along with CRISPR loci and in this condition CRISPR depends on trans encoded factors (Rath et al., 2015). One more important postulate about CRISPR loci is that it has a Leader sequence, which is a conserved sequence located upstream to the CRISPR with respect to the direction of transcription (Pougach et al., 2010).

Gene editing mechanism of CRISPR/Cas System

Now it has been fully accepted that CRISPR/Cas system is the part of the bacterial genome and play a significant role in adaptive immunity in bacteria and archaea against attacking phage or invading plasmid wherein one genetic element destroyed the another. However, the real mechanistic role of CRISPR/Cas is still under investigation (Jiang and Doudna, 2017). It has been clearly shown that there are three distinct steps involved in CRISPR/Cas based cleavage of plasmid or dsDNA i.e. 1. Adaptation, 2. Expression and maturation, and 3. Interference (figure 2) (Amitai and Sorek, 2016). In the first stage of adaptation, a new spacer (i.e., protospacer) is incorporated in the CRISPR array by invading mobile genetic element (MGE). For this, Cas1-Cas2 complex (having two Cas 1 dimer and one Cas 2 dimer) identify the new DNA as a target that after identification and detection is incorporated into CRISPR

array as a new spacer along with same adjacent sequence (Mir et al., 2018). In the second step, a mature crRNA, composed of ribonucleoprotein (RNP) with Cas protein, is transcribed from CRISPR. While in the third stage the crRNA guide Cas protein, both present in complex form i.e. RNP, to locate Protospacer Adjacent Motif (PAM) and help Cas protein to reach target site where Cas 9 act as double scissor to cut DNA strand. Cas protein has two domains i.e. RuvC and NHN that showed distinct function by cleaving non-complementary and complementary strand respectively. Further, Cas protein makes a cut after 2-3 nucleotides from the PAM sequence (Deveau et al., 2010). This three-step mechanism was considered as the modulator in development of viral resistance in bacteria. Further, viral resistant bacteria produce different types of RNA (i.e. crRNA and tracrRNA) from two distinct regions, first is CRISPR spacer itself, and second is outside the CRISPR repeats where Cas genes are found (Jiang and Doudna, 2017). Both crRNA and tracrRNA fragments are complementary to each other and form a double-stranded DNA that acts as guide RNA (gRNA) and facilitate Cas9 along with endonuclease to blunt-ended cleavage of invading DNA (Siksnys and Gasiunas, 2016) Following this step, the repair mechanism fills the empty/cut region of the DNA with the normal sequence. This technique has been adopted in eukaryotes as a gene knock out technology with minimal cost and easy method as compared to existing techniques for many fatal diseases (Platt et al., 2014).

Classification of the CRISPR-Cas system

Literature suggested that every Cas protein is associated with unique features and diverse nature (Makarova et al., 2011). Till now more than 13 different types of CRISPR systems have been identified (Rath et al., 2015). It is very difficult to classify CRISPR system due to multiple CRISPR loci, fast evolution and horizontal transfer of the CRISPR/Cas system (Fagerlund et al., 2015). The currently adopted classification regarding CRISPR/Cas system is on the basis of CRISPR components like Cas gene similarities, Cas protein, the

organization of gene on CRISPR/Cas loci and variability within the CRISPR itself. It has been broadly classified into three distinct categories/types (i.e. Type I, II and III) on the basis of Cas gene and a rare type IV which has rudimentary CRISPR/Cas loci. These types have been further classified into various subtypes (viz. Type I A-F, Type II A-C and Type III A and B) on the basis of structural differences and the gene they encode (Koonin et al., 2017). Two major classes have been defined for CRISPR/Cas system i.e. Class I and Class II having different types as discussed below (Makarova et al., 2015).

CRISPR/Cas class I

Class I includes type I, type III (found in archaea) and type IV. The effector complexes of type I and type III CRISPR/Cas have a definite structure with a backbone containing paralogous RAMPs (Repeats-Associated Mysterious Protein), such as Cas7 and Cas5, having the RRM (RNA Recognition Motif) fold and additional 'large' and 'small' subunits. These effector complexes contain one Cas5 subunit and several Cas7 subunits. Cas 3 and Cas 10 are considered as the signature genes for Type I and Type III, respectively (Shmakov et al., 2017). Type III (B) cmr is probably rare as it has been found to cleave target RNAs (Majumdar and Terns, 2019).

CRISPR/Cas class II

In class 2, the effector system is more uniformly organized and contain simple, large and multidomain protein. Class 2 contains three types viz., Type II, Type V and Type VI. Type II has endonuclease as effector and is dominantly used as genome editing enzyme. On the other hand, Type V contain Cpf1, a RNA guide endonuclease, as an effector that cleaves the target without needing any tracrRNA. Additionally, RuvC like endonuclease is also the main feature of type II and Type V. Type VI is found to target both RNA as well as DNA and

contain two HEPN domain. Type VI is further subdivided into two subtypes viz., VI-A and VI-B containing effector protein Cas 13a and 13b respectively (Pyzocha, 2017).

Thus, distinct types of CRISPR/Cas system, with numerous effectors and Cas gene, has been identified as well as classified. Type I-III are most studied while IV-VI are newly identified CRISPR/Cas system types (Makarova et al., 2015).

CRISPR/Cas as a therapeutic Tool

Owing to the advantages of CRISPR/Cas system, research is being directed towards its use as a therapeutics tool to achieve efficient genome editing through gene knockout or gene knock in several fatal diseases of both genetic and non-genetic etiology. Table 1 summarizes the use of CRISPR/Cas9 technology for correcting mutation in genetic diseases. The use of CRISPR/Cas editing system in some of the major diseases described below.

HIV

It has been more than 3 decades but still HIV is a major health concern. Although there is anti-retroviral therapy that effectively control the viral load, however, it fails to remove the virus completely. Recently Bella *et al* showed the cleavage of HIV-1 DNA from patient immune cells by the use of lentivirus expressing CRISPR in humanized mice engrafted with patient blood (Bella et al., 2018). Result of the study showed the removal of virus DNA from the blood as well as other major organs including spleen, lung, and liver of the mice. Zhu *et al* showed that there are 10 sites in HIV-1 that could be the potential target by CRISPR/Cas system and also showed the effect of CRISPR/Cas mediated removal of mutation in HIV-1 infected JLat10.6 cells (Zhu et al., 2015)

Sickle cell disease (SCD)

SCD is a disorder caused by a point mutation in HBB gene that could be cured by allogenic hemopoietic stem cell transplantation however only a small population is compatible with this treatment. In 2016, Park *et al*. demonstrated that approximately 30% HDR could be achieved in CD4+ cell by the use of suitable CRISPR/Cas along with a donor templet strand. Cas9 RNP, when delivered along with donor templet to CD34+ hematopoietic stem/progenitor cells (HSPCs) effectively edit the genome and increase the level of wild type hemoglobin in a mouse model (Park et al., 2016). Reports have also stated that the FDA lifted the hold on CRISPR therapeutics for sickle cell disease (Baylis and McLeod, 2017).

Duchenne muscular dystrophy (DMD)

DMD is another condition wherein dystrophin gene deletion causes this x-linked genetic muscle disease. The resulting product of this dystrophin gene is responsible for the development of muscles and deletion leads to muscle weakness and muscle degeneration (Bushby et al., 2010). Mutation in exon 23 of the dystrophin gene resulted in immature protein production and responsible for the above-stated consequences. CRISPR system was delivered using AAV for DMD that enabled the DMD gene functions in mice model (Nelson et al., 2016). Results indicated that CRISPR/Cas system delete exon 23 from the dystrophin gene and leads to following events viz., modified dystrophin gene expression, recovery of functional dystrophin protein and enhancement of muscle force. Young *et al*. also reported the success in deletion of DMD exon of humans (Young et al., 2016). Further, new RNA guided endonuclease (cpf1) was found to correct the mutation in DMD in human cells as well as in animal models of DMD (Zhang et al., 2017b)

Cancer

Cancer, yet another fatal disease having multiple causes and poor treatment, have been the research agenda for genetic engineering and genome editing techniques as they provide an

alternative therapeutic tool for its cure (table 2). CRISPR/Cas system has gained a lot of interest in cancer treatment due to its efficient editing of the target gene directly along with adaptation for different delivery strategies. CRISPR/Cas9 technique has been demonstrated to knock out Ptch1 gene accountable for medulloblastoma and Trp53, Pten and Nf1 genes responsible for glioblastoma in mouse brain (Zuckermann et al., 2015). Editing efficiency of the CRISPR/Cas system was also evaluated in the genetically engineered mouse models (GEMMs) of colorectal cancer (Roper et al., 2017). Also, Pten and p53, cancer suppressor genes were also edited by CRISPR/Cas system in hepatocellular carcinoma. CRISPR/Cas9 technique was used to deplete miR-210-3p in renal carcinoma cell lines (786-O, A498 and Caki2) that significantly increased tumorigenesis along with a morphological change in A498 and Caki2 cells (Yoshino et al., 2017). Literature also provides evidence for the use of the CRISPR/Cas system in the treatment of ovarian, cervical and acute myeloid leukemia. Long non-coding RNA (lncRNA) are potential target in the bladder cancer. Although CRISPR/Cas system was not widely explored for its activity to modulate their expression, Zhen *et al*. recently showed that CRISPR/Cas gene editing tool potentially altered the expression of lncRNA expression in bladder cancer (Zhen et al., 2017a). One of the major limitation in cancer treatment is the development of resistance to the chemotherapy that could be potentially avoided by knocking out the responsible gene. For example, doxorubicin (DOX) efflux in MCF-7 cells was inhibited by knockout of the MDR1 gene (via DSB) using CRSIPR/Cas system thus providing evidence of overcoming chemo-resistance via Cas9-mediated disruption of the drug resistance-related gene (Ha et al., 2016).

Challenges in the delivery of CRISPR/Cas components

Nowadays, CRISPR/Cas9 has been under intensive research as a genetic engineering tool and is also providing satisfactory results in preclinical practices. Three major approaches differing in their properties and nature have been used for attaining CRISPR/Cas9 expression in the

target cells. These are delivering a) Plasmid DNA (pDNA) expressing Cas9 protein and sgRNA which is very simple and cost-effective method, b) mRNA (encoding Cas protein) that shows instant gene expression and reduces the risk of mutagenesis and c) RNP *i.e.* complexes of Cas9 protein and sgRNA that has the advantage of reduced off-target cleavage. The efficacy of gene cleavage not only depends on the selectivity of the CRISPR/Cas nature (pDNA, mRNA, Cas protein) but also affected by the methodology of transportation of the CRISPR gene to the target cells or tissue. Selection of a carrier for delivering the payload into the target cells have been seen as a bottleneck in achieving efficient editing. Both viral and non-viral vectors have been reported to deliver CRISPR/Cas components however following hurdles have limited their therapeutic use.

Packing

CRISPR/Cas editing could be achieved either by pDNA, mRNA or RNP, complexes, however, all of them faces packing issues in the carrier owing to their macromolecular size. For example, the size of spCas9 gene is ~4.3 kbp while negatively charged spCas9 protein has a size 160 kDa with a hydrodynamic diameter of ~7.5 nm, while sgRNA size ~31 kDa and hydrodynamic diameter 5.5 nm (Mout et al., 2017). As there is a limited capacity of various delivery vectors, the packing of CRISPR/Cas components is a major concern (Wu et al., 2010).

Targeted delivery

Although viral vectors provide targeted delivery through tissue tropism however have several disadvantages including immunogenicity, payload limitation and delivery challenges (Zincarelli et al., 2008). On the other hand delivery of CRISPR/Cas components via non-viral vectors require antibody or peptide-mediated targeting strategy to avoid off-site distribution

(Peer et al., 2007). Designing actively targeted carrier with required packaging capabilities is much more difficult.

Efficiency, off-target binding and mutagenesis

Although this technology has been demonstrated to be much more specific and efficient, however diseases like cancer require much more editing efficiency to achieve the therapeutic outcome. Off-target effects for CRISPR/Cas system is also of major concern (Choi and Meyerson, 2014). Particularly Cas9/sgRNA shows expression for a long time and could interact with other genes leading to off-target effects. Editing of a gene other than the potential site could also lead to the mutation and hence complicates the condition.

Immunogenicity

The components of the CRISPR/Cas system are derived from bacteria and potentially could induce immune responses. It has been reported that MHC class one was elicited by Cas gene and Cas protein. Literature evidence showed that in *vivo* delivery of CRISPR elicited immune responses, not against the vector but the Cas protein itself (Chew et al., 2016).

Delivery strategies for CRISPR/Cas components

Genome editing using CRISPR/Cas9 could be achieved either by gene based strategy (plasmids or viral vectors expressing Cas9 and sgRNA) or RNA based strategy (Cas9 mRNA and sgRNA) or protein based strategy (Cas9 protein and sgRNA) (Mout et al., 2017). The science of gene delivery is one of the diverse fields in biomedical science that has been under investigation for a long time wherein physical methods, viral and non-viral methods have been employed (figure 3). Conventional physical methods including microinjection and electroporation have limited *in vivo* application due to their disadvantages including off-target binding, requirement of manual operations and damage to the cells. Some newer techniques including induced transduction by osmocytosis and propanebetaine (iTOP),

hydrodynamic injection and mechanical cell deformation. CRISPR components in plasmid format along with a single stranded-DNA were delivered by tail-vein hydrodynamic injection into a mouse model of tyrosinemia that resulted in correction of *Fah* mutation in hepatocytes (Yin et al., 2014). Furthermore, this technique inhibited hepatitis B virus (HBV) replication and expression in mice (Zhen et al., 2015). However certail limitations constrained their use for gene therapy. For instance, cardiovascular dysfunction is a common consequence in case of hydrodynamic injection and iTOP.

Viral vectors mainly lentivirus (LV) and adeno-associated virus (AAV) have been reported for delivering gene editing components with high efficiency. However mutagenesis, immunogenicity and limited loading capacity poses challenges in their use as a carrier for therapeutic genes. CRISPR/Cas9 adenovirus (AVs) disrupted *Pcsk9* gene with ~50% of insertion and deletion mutation (indel) in adult mouse liver after retroorbital injection. This further resulted in a decrease of 35−40% blood cholesterol in mice (Ding et al., 2014). Although AVs does not get incorporated into the host genome; however, they can produce an immune reaction in the host (Wang et al., 2004). Adeno associated viruses (AAVs) have been used to deliver CRISPR components for rectifying the mutated dystrophin gene in Duchenne muscular dystrophy (DMD) disease (Long et al., 2016). In another study by Kim *et al*., AAVs were used to deliver CjCas9 (derived from *Campylobacter jejuni*) that induced targeted mutations with high frequencies in mouse muscle cells or retinal pigment epithelium (RPE) cells (Kim et al., 2017a).

Most trending gene delivery system in recent time rely on the non-viral methods viz., cationic polymers, lipid nanoparticles, cell-penetrating peptides (CPPs), DNA ‘nanoclews’, and gold nanoparticles (Wong et al., 2017). These carriers have shown efficient transfection with ample opportunity in the design owing to their synthetic or semi-synthetic nature. Further, hybrid systems have been proposed to confer biomimetic properties to these carriers.

Although non-viral vectors are cost-effective and with better safety profile but they also share some limitations including low transfection efficiency, irregular cellular uptake and poor delivery to target cell/tissue (Nayerossadat et al., 2012), (Ramamoorth and Narvekar, 2015). Selection of a non-viral carrier will depend on the type of payload to be delivered. As there are different approaches viz., pDNA, mRNA or RNP are used for CRISPR/Cas based editing; delivery carriers are designed accordingly. When either DNA or RNA is to be delivered, most of the nano-viral gene delivery approaches could be adopted for transfection. On the other hand, direct delivery of Cas protein has an advantage over the delivery of conventional plasmid DNA expressing Cas protein because of shorter exposure time at cellular level resulting in reduced toxicity and off-target action (Ramakrishna et al., 2014). Recently several methods were developed for delivering Cas protein to overcome existing delivery limitations such as instability in serum, poor uptake and endosomal escape, and limited in *vivo* efficiency (Zuris et al., 2015b).

Cationic polymers

A wide range of polymers, both natural and synthetic, with the desired characteristics are available for designing gene delivery vehicles. Cationic polymers, from the past decade, are one of the most explored carriers for various peptides and gene silencing oligonucleotide (such as siRNAs and miRNAs) and are available with a dispersive range of derivatives (Samal et al., 2012). Among various cationic polymers, polyethyleneimine (PEI) has been widely used for gene delivery application owing to its advantages such as efficient complexation and proton sponge effect. PEI having a branched structure with multiple amine functionality is easy to assemble, easily available and has been fully explored for its gene delivery efficacy (Ahn et al., 2008). It has been demonstrated that high molecular weight PEI with exert better transfection effect as compared to low molecular weight. The major problem associated with PEI is its toxicity that to some extent have been circumvented by the use of

branched PEI or modification of PEI with PEG (He et al., 2013). Recently, Zhang *et al* reported polyethyleneimine-β-cyclodextrin (PC) for delivering large pDNA encoding Cas9 and gRNA for in vitro genome editing. The N/P ratio of 20 or above resulted in condensation of all free pDNA molecules of different sizes ranging from 3487 to 8506 bp. Further, as the N/P ratio is increases, the size of PC/pDNA complexes decreases and all the pDNAs showed an average particle size below 200 nm at N/P ratio of 60. These complexes were also efficiently internalized by HeLa cells with negligible cytotoxicity. The genome editing efficiency was confirmed by using plasmids expressing Cas9 and sgRNA targeting the hemoglobin subunit beta (19.1%) and rhomboid 5 homolog 1 (RHBDF1) (7.0%) locus (Zhang et al., 2019). Chitosan is another natural polymer that is non-toxic and biodegradable and have been explored for delivering CRISPR components. One of the recent studies showed the polyethylene glycol monomethyl ether (mPEG) conjugated chitosan for non-viral aerosol and mucosal delivery of CRISPR/Cas system. Low and medium molecular weight chitosan was PEGylated with a high mPEG degree of substitution (DS) and complexed with pSpCas9-2A-GFP at different N/P ratios (5, 10, 20 and 30). The positively charged amines of chitosan interact with the negatively charged nucleic acid and promote the delivery significantly. It was observed that low molecular weight PEGylated chitosan showed optimal transfection at N/P ratio of 20 while PEGylated medium molecular weight chitosan showed optimal transfection at N/P ratio of 5 (Zhang et al., 2018). In another study, CRISPR/Cas9 plasmid (pCas9) was delivered intravenously using poly(ethylene glycol)-b-poly-(lactic acid-co-glycolic acid) (PEG-PLGA)-based cationic lipid-assisted polymeric nanoparticles (CLANs) that efficiently disrupted CML-related BCR-ABL fusion gene and increased the survival of a CML mouse model. (Liu et al., 2018). Kretzmann *et al*. showed the capability of poly amidoamine (PAMAM) dendrimer to efficiently load and deliver CRISPR/Cas system. A library of the dendritic copolymer was prepared by click chemistry and studied to improve

the delivery of target plasmid DNA. Literature evidence suggested that Lipofectamine 2000 could be used for delivering small pDNA (~5 kb) efficiently however for delivering large pDNA (~10.3 kb), the modified PAMAM polymer coud be better in terms of transfection effciency (Kretzmann et al., 2017).

Cationic Lipids

Lipoplexes (containing cationic lipids) is one of the most efficient non-viral vector system for the delivery of the genetic material. Cationic lipid forms a stable nanocomplex via electrostatic interaction with negatively charged Cas9/sgRNA. Zhen *et al*. delivered CRISPR/Cas 9 for the treatment of prostate cancer by using cationic liposome containing poly(ethylene glycol)-grafted 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (Zhen et al., 2017b). In cancer therapy, lipid nanoparticles showed significant delivery of the CRISPR/Cas system. Cas9/sgRNA plasmid targeting Polo-like kinase 1 (PLK-1) was encapsulated in a PEG phospholipid-modified cationic lipid nanoparticle (PLNP) to form a core-shell structure that showed an *in vitro* transfection of 47.4% in A375 cells. *In vivo* study of these PLNPs in melanoma tumor-bearing mice showed a significant downregulation of PLK-1 protein and suppression of the tumor growth (Zhang et al., 2017a).

Cas9 endonuclease proteins have a net positive charge and hence could not be complexed directly with cationic lipids. Zuris *et al*. have demonstrated that these proteins could be fused with anionic supercharged proteins or anionic nucleic acids. They efficiently delivered Cre recombinase, TALE- and Cas9-based transcriptional activators, and Cas9:sgRNA nuclease complexes into cultured human cells. Further, up to 80% genome modification was observed with Cas9:sgRNA complexes as compared to DNA transfection (Zuris et al., 2015b). In a recent study, Cho et al., have used nano-liposomes prepared using lecithin to deliver cas9-sgRNA RNPs directed against dipeptidyl peptidase-4 gene (*DPP-4*) to modulate the function

of glucagon-like peptide 1. *In vivo* delivery of these complexes in type 2 diabetes mellitus (T2DM) *db*/*db* mice disrupted DPP-4 gene expression and decline in DPP-4 enzyme activity that resulted in normalized blood glucose levels and declined in insulin resistance, and negligible impact on liver and kidney function (Cho et al., 2019). In another study, Kim et al., have complexed cas9 RNPs with lipofectamine 2000 and delivered subretinally for treatment of wet age related macular degeneration (wAMD). The authors have designed the sgRNAs targeting VEGF A gene that encodes for VEGF receptors in mouse NIH3T3 cells and human ARPE-19 cells. VEGF A sgRNA/Cas9 RNPs were delivered using lipofectamine 2000 resulted in indels at the target site with a frequency of 82% and 57% in NIH3T3 cells and ARPE-19 cells, respectively. These RNPs were further delivered sub-retinally into the adult mouse eye wherein it was observed that RNPs could induce indels in the injected area. In the mouse model of wAMD, these RNPs induced indels at a frequency of 22% and effectively reduced the concentration of the VEGF A protein in the CNV area demonstrating that subretinal injection of the VEGF A/Cas9 RNP could lead to local treatment in the eye (Kim et al., 2017b).

Bioreducible lipids are newly used as a nanocarrier for the delivery of the CRISPR/Cas system. These lipids contain disulfide linkage in the hydrophobic tail of the lipid that leads to the degradation of lipid in the reductive intracellular (Glutathione rich) environment and promote the release of loaded cargo into the cytoplasm without endosomal degradation (Wang et al., 2016) and finally, enhances the efficiency of gene delivery. Wang *et al*. showed the synthesis of cationic lipids containing a disulfide bond created by Michael addition of primary and secondary amine along with acrylate and a long chain of carbon. The head group modification leads to the synthesis of derivatives with distinct activities and act as an effective system for the delivery of Cas protein/sgRNA for editing of the allele. It was further demonstrated that RNP complex, with a super negative charge, have been more efficiently

delivered by using bio reducible lipids as compared to commercial lipids. Results of the study showed more than 70% gene knock out efficiency of Cas9/sgRNA in cultured human cells (Wang et al., 2016). In another study, cationic lipids were used delivered sgRNA/Cas (RNP) in MCF-7 cells to knockout MDR1 gene, responsible for efflux of DOX. The results showed an increase in drug uptake by four-folds relative to the untreated cells by decreasing the MDR1 gene mediated resistance (Ha et al., 2016).

Cell-penetrating peptide (CPP)

CPP has been used as a means for attaining effective Cas9 protein delivery because of their inherent ability to translocate through plasma membranes (Gagat et al., 2017). The conjugation of CPP with various cargos could be achieved through electrostatic interaction or by covalent bonding. Suresh B *et al* showed endogenous gene disruption in human cell lines mediated *via* CPP-conjugated recombinant Cas9 protein. Another report also showed the enhancement in the Cas9 delivery to nucleus by utilizing CPP. A novel CPP named as TAT-CaM was explored to deliver the cargo into nucleus effectively (Axford et al., 2017). Ramakrishna *et al*., 2014 showed that CPP mediated delivery of Cas protein as well as guide RNA with lesser off-target effects (Ramakrishna et al., 2014).

Endo-porter peptides (EPP)

Another strategy utilizing endo-porter peptides has been reported for delivering Cas protein and sgRNA. These are alpha helical and amphipathic peptides with weak basic amino acids, leucine and histidine, as their major component and could deliver non-ionic substances into the cell (Summerton, 2005). It has been reported that EPP enters the cell through endocytosis and escapes endosome, through proton sponge effect (Bartz et al., 2011). A recent study by Shen *et al*. showed efficient delivery of Cas protein and sgRNA with reduced off-target effects by complexing it with endo-porter peptide via electrostatic interaction (Shen et al.,

2018). Another study reported PEGylated nanoparticles along with a membrane disruptive and endosomolytic helical polypeptide. More than 71% suppression in the growth of cancer cells was observed in this study (Wang et al., 2018).

Gold Nanoparticle

Multiple surface functionality makes gold nanoparticle as a unique and versatile delivery system for various cargos (Yeh et al., 2012). The literature described the role of gold nanoparticle in distinct applications viz., sensing, imaging, delivery, etc. One study showed direct cytosolic delivery of ribonucleoprotein complexed with gold nanoparticle providing effective (~30%) gene-editing efficiency (Mout et al., 2017). Lee *et al*. also demonstrated the delivery of Cas9/sgRNA RNP using 15 nm gold nanoparticles, conjugated with thiolated short DNA oligos and conjugated further with donor single-stranded DNA, coated with a polymer (PAsp-DET) that disrupt endosome in mice suffering from DMD. The outcomes of the study were found to be more effective and help in correction of 5.4% of the mutated gene in DMD. Furthermore, it has been recently observed that intracranial (ICV) injection of CRISPR/gold nanoparticles, containing Cas9 CNP, edit the gene within the mice brain through metabotropic glutamate receptor 5 (mGluR5) gene (Lee et al., 2017).

Exosomes

Exosomes as an advanced delivery system have emerged as a potential area of research owing to their small size and ability to transit molecules like lipids, protein and mRNA as well as ability to cross BBB as well as a placental barrier (Shi et al., 2017). These are stable nanosized, having a diameter of 30-100 nm, vesicles that are secreted by almost every type of cell (Ibrahim and Marban, 2016). It has also been reported that exosome express surface proteins like tetraspanin thereby exhibiting cell targeting (Hoshino et al., 2015). Kim *et al*. derived natural exosome from cancer cell itself and use them as a carrier to deliver

CRISPR/Cas plasmid to treat cancer. This strategy provides a natural carrier, with less risk of immunogenicity, and effectively deliver cargo to treat ovarian cancer in SKOV3 xenograft mice (Kim et al., 2017c). The main limitation with exosome for delivering macromolecules such as proteins is its limited payload capacity. To overcome this, hybrid exosome (incorporating exosome and liposome) were prepared for delivering CRISPR/Cas component in mesenchymal stem cell (Lin et al., 2018). Another biologically inspired carrier, DNA nanoclew, have been reported to deliver CRISPR/Cas 9 in both in *vitro* and in *vivo* (Sun et al., 2015). DNA nanoclew is made up of a single strand of DNA having a yarn like structure prepared by a method named as rolling circle amplification (RCA) with palindromic sequences encoded to drive the self-assembly of nanoparticles. Cas9/sgRNA was loaded in them which were further coated with polyethyleneimine (PEI) to enable cellular internalization and endosomal escape.

Conclusion and future prospective

The CRISPR/Cas system is an adaptive (acquired) immune system in bacteria and archaea with immune memory that is stored in the form of spacer sequences derived from foreign genomes and inserted into CRISPR arrays. It has been explored as a potential biotechnology tool for genome editing required for deciphering complex components of gene expression and has been preferred over ZFNs and TALENs in terms of easiness, simplicity, and specificity. CRISPR-associated DNA endonuclease (Cas) provides a novel opportunity for therapeutic genome editing in diseased cells and tissue. CRISPR/Cas tool could be used by either delivering a Cas expression plasmid or Cas mRNA or Cas ribonucleoprotein (RNP) complex. Gene delivery using viral vectors although is the most popular choice for gene therapies however their *in vivo* application is disadvantageous for a number of reasons, including possible integration into genomic DNA, immune responses due to persistent expression of the bacterial Cas9 and off-target effects. There is still a large gap for translating

these tools to the clinic. To exploit the full therapeutic potential of this technology, it has to be merged with the advancements taking place in nanotechnology, particularly in the area of delivery strategies. Non-viral vectors, including cationic polymers and cationic lipids, used for gene delivery have been adopted for delivering CRISPR/Cas components owing to the advantages over viral constructs. However, several issues that needed to be addressed by a pharmaceutical/medical scientist are ensuring efficient packing of CRISPR components into the carrier, targeted delivery to diseased site, avoid off-target binding, improve in *vivo* efficiency, avoid mutagenesis and eliminate immunogenicity. Translating this tool for therapeutic purpose require a thorough investigation of carriers with spatiotemporal control over *in vivo* delivery to achieve the therapeutic concentrations with minimal side-effects.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Sahel, D.K., Mittal A., and Chitkara D.

Authors declare no conflict of interest.

References

Ahn HH, Lee JH, Kim KS, Lee JY, Kim MS, Khang G, Lee IW, Lee HB. (2008) Polyethyleneimine-mediated gene delivery into human adipose derived stem cells. *Biomaterials* **29**:2415-2422.

Al-Attar S, Westra ER, van der Oost J, Brouns SJ. (2011) Clustered regularly interspaced short palindromic repeats (CRISPRs): The hallmark of an ingenious antiviral defense mechanism in prokaryotes. *Biol Chem* **392**:277-289.

Amitai G and Sorek R. (2016) CRISPR-cas adaptation: Insights into the mechanism of action. *Nat Rev Microbiol* **14**:67-76.

Axford DS, Morris DP, McMurry JL. (2017) Cell penetrating peptide-mediated nuclear delivery of Cas9 to enhance the utility of CRISPR/cas genome editing. *The FASEB Journal* **31**:909.4-909.4.

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**:1709-1712.

Bartz R, Fan H, Zhang J, Innocent N, Cherrin C, Beck SC, Pei Y, Momose A, Jadhav V, Tellers DM, Meng F, Crocker LS, Sepp-Lorenzino L, Barnett SF. (2011) Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape. *Biochem J* **435**:475-487.

Baylis F and McLeod M. (2017) First-in-human phase 1 CRISPR gene editing cancer trials: Are we ready? *Curr Gene Ther* **17**:309-319.

Bella R, Kaminski R, Mancuso P, Young WB, Chen C, Sariyer R, Fischer T, Amini S, Ferrante P, Jacobson JM, Kashanchi F, Khalili K. (2018) Removal of HIV DNA by CRISPR from patient blood engrafts in humanized mice. *Mol Ther Nucleic Acids* **12**:275-282.

Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C, DMD Care Considerations Working Group. (2010) Diagnosis and management of duchenne muscular dystrophy, part 2: Implementation of multidisciplinary care. *Lancet Neurol* **9**:177-189.

Chen ZH, Yu YP, Zuo ZH, Nelson JB, Michalopoulos GK, Monga S, Liu S, Tseng G, Luo JH. (2017) Targeting genomic rearrangements in tumor cells through Cas9-mediated insertion of a suicide gene. *Nat Biotechnol* **35**:543-550.

Chew WL, Tabebordbar M, Cheng JK, Mali P, Wu EY, Ng AH, Zhu K, Wagers AJ, Church GM. (2016) A multifunctional AAV–CRISPR–Cas9 and its host response. *Nature Methods* **13**:868.

Cho EY, Ryu JY, Lee HAR, Hong SH, Park HS, Hong KS, Park SG, Kim HP, Yoon TJ. (2019) Lecithin nano-liposomal particle as a CRISPR/Cas9 complex delivery system for treating type 2 diabetes. *J Nanobiotechnology* **17**:19-019-0452-8.

Choi PS and Meyerson M. (2014) Targeted genomic rearrangements using CRISPR/cas technology. *Nat Commun* **5**:3728.

Collins JP. (2018) Gene drives in our future: Challenges of and opportunities for using a self-sustaining technology in pest and vector management. *BMC Proc* **12**:9-018-0110-4. eCollection 2018.

Cyranoski D. (2016) CRISPR gene-editing tested in a person for the first time. *Nature* **539**:479.

Demirci S, Leonard A, Haro-Mora JJ, Uchida N, Tisdale JF. (2019) CRISPR/Cas9 for sickle cell disease: Applications, future possibilities, and challenges. *Adv Exp Med Biol* .

Deveau H, Garneau JE, Moineau S. (2010) CRISPR/cas system and its role in phage-bacteria interactions. *Annu Rev Microbiol* **64**:475-493.

Ding Q, Strong A, Patel KM, Ng SL, Gosis BS, Regan SN, Cowan CA, Rader DJ, Musunuru K. (2014) Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ Res* **115**:488-492.

Doudna JA and Charpentier E. (2014) Genome editing. the new frontier of genome engineering with CRISPR-Cas9. *Science* **346**:1258096.

Fagerlund RD, Staals RH, Fineran PC. (2015) The Cpf1 CRISPR-cas protein expands genome-editing tools. *Genome Biol* **16**:251.

Gagat M, Zielinska W, Grzanka A. (2017) Cell-penetrating peptides and their utility in genome function modifications (review). *Int J Mol Med* **40**:1615-1623.

Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadan AH, Moineau S. (2010) The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**:67-71.

Godde JS and Bickerton A. (2006) The repetitive DNA elements called CRISPRs and their associated genes: Evidence of horizontal transfer among prokaryotes. *J Mol Evol* **62**:718-729.

Ha JS, Byun J, Ahn DR. (2016) Overcoming doxorubicin resistance of cancer cells by Cas9-mediated gene disruption. *Sci Rep* **6**:22847.

Haft DH, Selengut J, Mongodin EF, Nelson KE. (2005) A guild of 45 CRISPR-associated (cas) protein families and multiple CRISPR/cas subtypes exist in prokaryotic genomes. *PLoS Comput Biol* **1**:e60.

Haraguchi M, Sato M, Ozawa M. (2015) CRISPR/Cas9n-mediated deletion of the snail 1Gene (SNAI1) reveals its role in regulating cell morphology, cell-cell interactions, and gene expression in ovarian cancer (RMG-1) cells. *PLoS One* **10**:e0132260.

He Y, Cheng G, Xie L, Nie Y, He B, Gu Z. (2013) Polyethyleneimine/DNA polyplexes with reduction-sensitive hyaluronic acid derivatives shielding for targeted gene delivery. *Biomaterials* **34**:1235-1245.

Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, Miller JC, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R. (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol* **27**:851-857.

Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, Cost GJ, Zhang L, Santiago Y, Miller JC, Zeitler B, Cherone JM, Meng X, Hinkley SJ, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R. (2011) Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol* **29**:731-734.

Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I,

Peinado H, Bromberg J, Lyden D. (2015) Tumour exosome integrins determine organotropic metastasis. *Nature* **527**:329-335.

Hung SSC, McCaughey T, Swann O, Pebay A, Hewitt AW. (2016) Genome engineering in ophthalmology: Application of CRISPR/cas to the treatment of eye disease. *Prog Retin Eye Res* **53**:1-20.

Ibrahim A and Marban E. (2016) Exosomes: Fundamental biology and roles in cardiovascular physiology. *Annu Rev Physiol* **78**:67-83.

Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in escherichia coli, and identification of the gene product. *J Bacteriol* **169**:5429-5433.

Jiang F and Doudna JA. (2017) CRISPR-Cas9 structures and mechanisms. *Annu Rev Biophys* **46**:505-529.

Kim E, Koo T, Park SW, Kim D, Kim K, Cho HY, Song DW, Lee KJ, Jung MH, Kim S, Kim JH, Kim JH, Kim JS. (2017a) In vivo genome editing with a small Cas9 orthologue derived from campylobacter jejuni. *Nat Commun* **8**:14500.

Kim K, Park SW, Kim JH, Lee SH, Kim D, Koo T, Kim KE, Kim JH, Kim JS. (2017b) Genome surgery using Cas9 ribonucleoproteins for the treatment of age-related macular degeneration. *Genome Res* **27**:419-426.

Kim SM, Yang Y, Oh SJ, Hong Y, Seo M, Jang M. (2017c) Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting. *J Control Release* **266**:8-16.

Koonin EV, Makarova KS, Zhang F. (2017) Diversity, classification and evolution of CRISPR-cas systems. *Curr Opin Microbiol* **37**:67-78.

Kretzmann JA, Ho D, Evans CW, Plani-Lam JHC, Garcia-Bloj B, Mohamed AE, O'Mara ML, Ford E, Tan DEK, Lister R, Blancafort P, Norret M, Iyer KS. (2017) Synthetically controlling dendrimer flexibility improves delivery of large plasmid DNA. *Chem Sci* **8**:2923-2930.

Labrie SJ, Samson JE, Moineau S. (2010) Bacteriophage resistance mechanisms. *Nat Rev Microbiol* **8**:317-327.

Lee K, Conboy M, Park HM, Jiang F, Kim HJ, Dewitt MA, Mackley VA, Chang K, Rao A, Skinner C, Shobha T, Mehdipour M, Liu H, Huang WC, Lan F, Bray NL, Li S, Corn JE, Kataoka K, Doudna JA, Conboy I, Murthy N. (2017) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. *Nat Biomed Eng* **1**:889-901.

Li L, Hu S, Chen X. (2018) Non-viral delivery systems for CRISPR/Cas9-based genome editing: Challenges and opportunities. *Biomaterials* **171**:207-218.

Lim KRQ, Yoon C, Yokota T. (2018) Applications of CRISPR/Cas9 for the treatment of duchenne muscular dystrophy. *J Pers Med* **8**:10.3390/jpm8040038.

Lin Y, Wu J, Gu W, Huang Y, Tong Z, Huang L, Tan J. (2018) Exosome-liposome hybrid nanoparticles deliver CRISPR/Cas9 system in MSCs. *Adv Sci (Weinh)* **5**:1700611.

Lino CA, Harper JC, Carney JP, Timlin JA. (2018) Delivering CRISPR: A review of the challenges and approaches. *Drug Deliv* **25**:1234-1257.

Lintner NG, Kerou M, Brumfield SK, Graham S, Liu H, Naismith JH, Sdano M, Peng N, She Q, Copie V, Young MJ, White MF, Lawrence CM. (2011) Structural and functional characterization of an archaeal clustered regularly interspaced short palindromic repeat (CRISPR)-associated complex for antiviral defense (CASCADE). *J Biol Chem* **286**:21643-21656.

Liu Y, Zhao G, Xu CF, Luo YL, Lu ZD, Wang J. (2018) Systemic delivery of CRISPR/Cas9 with PEG-PLGA nanoparticles for chronic myeloid leukemia targeted therapy. *Biomater Sci* **6**:1592-1603.

Long C, Amoasii L, Mireault AA, McAnally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R, Olson EN. (2016) Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* **351**:400-403.

Majumdar S and Terns MP. (2019) CRISPR RNA-guided DNA cleavage by reconstituted type I-A immune effector complexes. *Extremophiles* **23**:19-33.

Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, van der Oost J, Koonin EV. (2011) Evolution and classification of the CRISPR-cas systems. *Nat Rev Microbiol* **9**:467-477.

Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJ, Charpentier E, Haft DH, Horvath P, Moineau S, Mojica FJ, Terns RM, Terns MP, White MF, Yakunin AF, Garrett RA, van der Oost J, Backofen R, Koonin EV. (2015) An updated evolutionary classification of CRISPR-cas systems. *Nat Rev Microbiol* **13**:722-736.

Martinez-Lage M, Puig-Serra P, Menendez P, Torres-Ruiz R, Rodriguez-Perales S. (2018) CRISPR/Cas9 for cancer therapy: Hopes and challenges. *Biomedicines* **6**:10.3390/biomedicines6040105.

Min YL, Li H, Rodriguez-Caycedo C, Mireault AA, Huang J, Shelton JM, McAnally JR, Amoasii L, Mammen PPA, Bassel-Duby R, Olson EN. (2019) CRISPR-Cas9 corrects duchenne muscular dystrophy exon 44 deletion mutations in mice and human cells. *Sci Adv* **5**:eaav4324.

Mir A, Edraki A, Lee J, Sontheimer EJ. (2018) Type II-C CRISPR-Cas9 biology, mechanism, and application. *ACS Chem Biol* **13**:357-365.

Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* **60**:174-182.

Mout R, Ray M, Lee YW, Scaletti F, Rotello VM. (2017) In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: Progress and challenges. *Bioconjug Chem* **28**:880-884.

Nayerossadat N, Maedeh T, Ali PA. (2012) Viral and nonviral delivery systems for gene delivery. *Adv Biomed Res* **1**:27-9175.98152. Epub 2012 Jul 6.

Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Castellanos Rivera RM, Madhavan S, Pan X, Ran FA, Yan WX, Asokan A, Zhang F, Duan D, Gersbach CA. (2016) In vivo genome editing improves muscle function in a mouse model of duchenne muscular dystrophy. *Science* **351**:403-407.

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W, Sha J. (2014) Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* **156**:836-843.

Nunez JK, Kranzusch PJ, Noeske J, Wright AV, Davies CW, Doudna JA. (2014) Cas1-Cas2 complex formation mediates spacer acquisition during CRISPR-cas adaptive immunity. *Nat Struct Mol Biol* **21**:528-534.

Park SH, Lee CM, Deshmukh H, Bao G. (2016) Therapeutic crispr/Cas9 genome editing for treating sickle cell disease. *Blood* **128**:4703.

Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. (2007) Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* **2**:751-760.

Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F. (2014) CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* **159**:440-455.

Pougach K, Semenova E, Bogdanova E, Datsenko KA, Djordjevic M, Wanner BL, Severinov K. (2010) Transcription, processing and function of CRISPR cassettes in escherichia coli. *Mol Microbiol* **77**:1367-1379.

Ramakrishna S, Kwaku Dad AB, Beloor J, Gopalappa R, Lee SK, Kim H. (2014) Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res* **24**:1020-1027.

Ramamoorth M and Narvekar A. (2015) Non viral vectors in gene therapy- an overview. *J Clin Diagn Res* **9**:GE01-6.

Ramirez CL, Foley JE, Wright DA, Muller-Lerch F, Rahman SH, Cornu TI, Winfrey RJ, Sander JD, Fu F, Townsend JA, Cathomen T, Voytas DF, Joung JK. (2008) Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat Methods* **5**:374-375.

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. (2015) In vivo genome editing using staphylococcus aureus Cas9. *Nature* **520**:186-191.

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. (2013) Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**:2281-2308.

Rath D, Amlinger L, Rath A, Lundgren M. (2015) The CRISPR-cas immune system: Biology, mechanisms and applications. *Biochimie* **117**:119-128.

Rohn TT, Kim N, Isho NF, Mack JM. (2018) The potential of CRISPR/Cas9 gene editing as a treatment strategy for alzheimer's disease. *J Alzheimers Dis Parkinsonism* **8**:10.4172/2161-0460.1000439. Epub 2018 May 31.

Roper J, Tammela T, Cetinbas NM, Akkad A, Roghanian A, Rickelt S, Almeqdadi M, Wu K, Oberli MA, Sanchez-Rivera FJ, Park YK, Liang X, Eng G, Taylor MS, Azimi R, Kedrin D, Neupane R, Beyaz S, Sicinska ET, Suarez Y, Yoo J, Chen L, Zukerberg L, Katajisto P, Deshpande V, Bass AJ, Tsichlis PN, Lees J, Langer R, Hynes RO, Chen J, Bhutkar A, Jacks T, Yilmaz OH. (2017) In vivo genome editing and organoid transplantation models of colorectal cancer and metastasis. *Nat Biotechnol* **35**:569-576.

Rousseau C, Gonnet M, Le Romancer M, Nicolas J. (2009) CRISPI: A CRISPR interactive database. *Bioinformatics* **25**:3317-3318.

Samal SK, Dash M, Van Vlierberghe S, Kaplan DL, Chiellini E, van Blitterswijk C, Moroni L, Dubruel P. (2012) Cationic polymers and their therapeutic potential. *Chem Soc Rev* **41**:7147-7194.

Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, van der Ent CK, Nieuwenhuis EE, Beekman JM, Clevers H. (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* **13**:653-658.

Shen Y, Cohen JL, Nicoloro SM, Kelly M, Yenilmez B, Henriques F, Tsagkaraki E, Edwards YJK, Hu X, Friedline RH, Kim JK, Czech MP. (2018) CRISPR-delivery particles targeting nuclear receptor-interacting protein 1 (Nrip1) in adipose cells to enhance energy expenditure. *J Biol Chem* **293**:17291-17305.

Shi Y, Jiang X, Zhang L, Pu H, Hu X, Zhang W, Cai W, Gao Y, Leak RK, Keep RF, Bennett MV, Chen J. (2017) Endothelium-targeted overexpression of heat shock protein 27 ameliorates blood-brain barrier disruption after ischemic brain injury. *Proc Natl Acad Sci U S A* **114**:E1243-E1252.

Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, Severinov K, Zhang F, Koonin EV. (2017) Diversity and evolution of class 2 CRISPR-cas systems. *Nat Rev Microbiol* **15**:169-182.

Siksnys V and Gasiunas G. (2016) Rewiring Cas9 to target new PAM sequences. *Mol Cell* **61**:793-794.

Smith C, Abalde-Atristain L, He C, Brodsky BR, Braunstein EM, Chaudhari P, Jang YY, Cheng L, Ye Z. (2015) Efficient and allele-specific genome editing of disease loci in human iPSCs. *Mol Ther* **23**:570-577.

Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. (1985) Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* **317**:230-234.

Song B, Fan Y, He W, Zhu D, Niu X, Wang D, Ou Z, Luo M, Sun X. (2015) Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. *Stem Cells Dev* **24**:1053-1065.

Summerton JE. (2005) Endo-porter: A novel reagent for safe, effective delivery of substances into cells. *Ann N Y Acad Sci* **1058**:62-75.

Sun X, Hu Z, Chen R, Jiang Q, Song G, Zhang H, Xi Y. (2015) Targeted mutagenesis in soybean using the CRISPR-Cas9 system. *Sci Rep* **5**:10342.

Wallace J, Hu R, Mosbruger TL, Dahlem TJ, Stephens WZ, Rao DS, Round JL, O'Connell RM. (2016) Genome-wide CRISPR-Cas9 screen identifies MicroRNAs that regulate myeloid leukemia cell growth. *PLoS One* **11**:e0153689.

Wang AY, Peng PD, Ehrhardt A, Storm TA, Kay MA. (2004) Comparison of adenoviral and adeno-associated viral vectors for pancreatic gene delivery in vivo. *Hum Gene Ther* **15**:405-413.

Wang HX, Song Z, Lao YH, Xu X, Gong J, Cheng D, Chakraborty S, Park JS, Li M, Huang D, Yin L, Cheng J, Leong KW. (2018) Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide. *Proc Natl Acad Sci U S A* **115**:4903-4908.

Wang M, Zuris JA, Meng F, Rees H, Sun S, Deng P, Han Y, Gao X, Pouli D, Wu Q, Georgakoudi I, Liu DR, Xu Q. (2016) Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles. *Proc Natl Acad Sci U S A* **113**:2868-2873.

Wong JKL, Mohseni R, Hamidieh AA, MacLaren RE, Habib N, Seifalian AM. (2017) Will nanotechnology bring new hope for gene delivery? *Trends Biotechnol* **35**:434-451.

Wu Z, Yang H, Colosi P. (2010) Effect of genome size on AAV vector packaging. *Molecular Therapy* **18**:80-86.

Wu Q, Madany P, Akech J, Dobson JR, Douthwright S, Browne G, Colby JL, Winter GE, Bradner JE, Pratap J, Sluder G, Bhargava R, Chiosea SI, van Wijnen AJ, Stein JL, Stein GS, Lian JB, Nickerson JA, Imbalzano AN. (2015a) The SWI/SNF ATPases are required for triple negative breast cancer cell proliferation. *J Cell Physiol* **230**:2683-2694.

Wu Y, Zhou H, Fan X, Zhang Y, Zhang M, Wang Y, Xie Z, Bai M, Yin Q, Liang D, Tang W, Liao J, Zhou C, Liu W, Zhu P, Guo H, Pan H, Wu C, Shi H, Wu L, Tang F, Li J. (2015b) Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. *Cell Res* **25**:67-79.

Xie F, Ye L, Chang JC, Beyer AI, Wang J, Muench MO, Kan YW. (2014) Seamless gene correction of beta-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. *Genome Res* **24**:1526-1533.

Xue M, Li X, Li Z, Chen W. (2014a) Urothelial carcinoma associated 1 is a hypoxia-inducible factor-1alpha-targeted long noncoding RNA that enhances hypoxic bladder cancer cell proliferation, migration, and invasion. *Tumour Biol* **35**:6901-6912.

Xue W, Chen S, Yin H, Tammela T, Papagiannakopoulos T, Joshi NS, Cai W, Yang G, Bronson R, Crowley DG, Zhang F, Anderson DG, Sharp PA, Jacks T. (2014b) CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* **514**:380-384.

Yeh YC, Creran B, Rotello VM. (2012) Gold nanoparticles: Preparation, properties, and applications in bionanotechnology. *Nanoscale* **4**:1871-1880.

Yin H, Xue W, Anderson DG. (2019) CRISPR-cas: A tool for cancer research and therapeutics. *Nat Rev Clin Oncol* **16**:281-295.

Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Koteliansky V, Sharp PA, Jacks T, Anderson DG. (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat Biotechnol* **32**:551-553.

Yoshino H, Yonemori M, Miyamoto K, Tatarano S, Kofuji S, Nohata N, Nakagawa M, Enokida H. (2017) microRNA-210-3p depletion by CRISPR/Cas9 promoted tumorigenesis through revival of TWIST1 in renal cell carcinoma. *Oncotarget* **8**:20881-20894.

Young CS, Hicks MR, Ermolova NV, Nakano H, Jan M, Younesi S, Karumbayaram S, Kumagai-Cresse C, Wang D, Zack JA, Kohn DB, Nakano A, Nelson SF, Miceli MC, Spencer MJ, Pyle AD. (2016) A single CRISPR-Cas9 deletion strategy that targets the majority of DMD patients restores dystrophin function in hiPSC-derived muscle cells. *Cell Stem Cell* **18**:533-540.

Yuen KS, Chan CP, Wong NH, Ho CH, Ho TH, Lei T, Deng W, Tsao SW, Chen H, Kok KH, Jin DY. (2015) CRISPR/Cas9-mediated genome editing of epstein-barr virus in human cells. *J Gen Virol* **96**:626-636.

Zhan T, Rindtorff N, Betge J, Ebert MP, Boutros M. (2019) CRISPR/Cas9 for cancer research and therapy. *Semin Cancer Biol* **55**:106-119.

Zhang L, Wang P, Feng Q, Wang N, Chen Z, Huang Y, Zheng W, Jiang X. (2017a) Lipid nanoparticle-mediated efficient delivery of CRISPR/Cas9 for tumor therapy. *NPG Asia Materials* **9**:e441.

Zhang H, Bahamondez-Canas TF, Zhang Y, Leal J, Smyth HDC. (2018) PEGylated chitosan for nonviral aerosol and mucosal delivery of the CRISPR/Cas9 system in vitro. *Mol Pharm* **15**:4814-4826.

Zhang Y, Long C, Li H, McAnally JR, Baskin KK, Shelton JM, Bassel-Duby R, Olson EN. (2017b) CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice. *Sci Adv* **3**:e1602814.

Zhang Z, Wan T, Chen Y, Chen Y, Sun H, Cao T, Songyang Z, Tang G, Wu C, Ping Y, Xu FJ, Huang J. (2019) Cationic polymer-mediated CRISPR/Cas9 plasmid delivery for genome editing. *Macromol Rapid Commun* **40**:e1800068.

Zhen S, Hua L, Liu YH, Gao LC, Fu J, Wan DY, Dong LH, Song HF, Gao X. (2015) Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus. *Gene Ther* **22**:404-412.

Zhen S, Hua L, Liu YH, Sun XM, Jiang MM, Chen W, Zhao L, Li X. (2017a) Inhibition of long non-coding RNA UCA1 by CRISPR/Cas9 attenuated malignant phenotypes of bladder cancer. *Oncotarget* **8**:9634-9646.

Zhen S, Takahashi Y, Narita S, Yang YC, Li X. (2017b) Targeted delivery of CRISPR/Cas9 to prostate cancer by modified gRNA using a flexible aptamer-cationic liposome. *Oncotarget* **8**:9375-9387.

Zhu W, Lei R, Le Duff Y, Li J, Guo F, Wainberg MA, Liang C. (2015) The CRISPR/Cas9 system inactivates latent HIV-1 proviral DNA. *Retrovirology* **12**:22-015-0150-z.

Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. (2008) Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther* **16**:1073-1080.

Zuckermann M, Hovestadt V, Knobbe-Thomsen CB, Zapatka M, Northcott PA, Schramm K, Belic J, Jones DT, Tschida B, Moriarity B, Largaespada D, Roussel MF, Korshunov A, Reifenberger G, Pfister SM, Lichter P, Kawauchi D, Gronych J. (2015) Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. *Nat Commun* **6**:7391.

Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR. (2015a) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat Biotechnol* **33**:73-80.

Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR. (2015b) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat Biotechnol* **33**:73-80.

Footnotes

The work was supported by the Department of Biotechnology, Government of India through research grant [BT/PR26897/NNT/28/1489/2017] under "Nano-intervention in the management of ophthalmic diseases."

Figure legends

Figure 1. Approaches for CRISPR/Cas based editing in cells with their advantages and limitations.

Figure 2. Modulation in CRISPR locus (Bacterial) in response to phage attack involving events of adaptive immunity in bacteria including requisition of protospacer into CRISPR array, maturation and expression of mRNA and interference with invading phage.

Figure 3. Strategies used for delivering CRISPR/cas components

Tables

Table 1

Genetic diseases corrected in cells using CRISPR/Cas technology

Diseases	Mutation target	Method of delivery	Target cells	Efficiency (%)	References
β-Thalassaemia	Deletion in HBB	Electroporation	Human iPSCs	17.6%	(Xie et al., 2014)
Cystic fibrosis	Deletion in *CFTR*	Lipofection	Human intestinal organoids	-	(Schwank et al., 2013)
HIV-1	CCR5Δ32	Electroporation	Human iPSCs	100%	(Zhu et al., 2015)
Hereditary tyrosinemia	Point mutation in *FAH*	Hydrodynamic injection	*in vivo* mice hepatocytes	0.40 ± 0.12 %	(Yin et al., 2014)
Duchenne muscular dystrophy	Exon deletion in dystrophin gene	Electroporation	Human iPSCs	50%	(Min et al., 2019)
α1-Antitrypsin deficiency	Point mutation in *SERPINA1*	Electroporation	Human iPSCs	18.8%	(Smith et al., 2015)
Cataracts	Deletion in *Crygc*	Electroporation	Mouse spermatogonial stem cells	29.7%	(Wu et al., 2015b)
Epstein-Barr virus	Inactivation of viral promoter	Electroporation	Human epithelial cell lines	94.2%	(Yuen et al., 2015)
LDL-C	Disruption of *Pcsk9*	Adenovirus	*in vivo* mice hepatocytes	50%	(Ran et al., 2015)
Sickle cell anemia	β-globin (HBB)	Transfection Electroporation	HEK293T, BC1, TNC1	-	(Song et al., 2015)

Table 2

CRISPR/Cas9 technology in treatment of different cancers

Cancer type	**Gene edited**	**Method of Delivery**	**Target**	**References**
Glioblastoma and medulloblastoma	Trp53, Pten, Nf1 and Ptch1	Electroporation/polyethylenimine (PEI)-mediated transfection	Patient-derived xenograft (PDX), cell-derived xenograft (CDX) and genetically engineered mouse model (GEMMs).	(Zuckermann et al., 2015)
Bladder cancer	TP53, urothelial carcinoma-associated 1 (UCA1), long non-coding RNA-related nuclear protein (ncRAN)	Hydrodynamic injection	5637 and T24 bladder cancer cell lines	(Xue et al., 2014a)
Breast cancer	Brahma (BRM) and Brahma-related Gene 1	N/A	Genetically engineered mouse model.	(Wu et al., 2015a)

	(BRG1) CDH1			
Ovarian cancer	Snail1	lipofectamine 2000	Human ovarian adenocarcinoma	(Haraguchi et al., 2015)
Acute myeloid leukemia	miRNAs	N/A	Mammalian cell phenotypes	(Wallace et al., 2016)
Renal cell carcinoma	miR-210-3p	Lipofectamine RNAiMAX transfection reagent	In *vivo* xenograft study in which Twist-related protein 1 (TWIST1) was the key target of miR210-3p.	(Yoshino et al., 2017)
Colorectal cancer	APC, TP53, KRAS, SMAD4	Lentivirus/LIPID NANOPARTICLE	Genetically engineered mouse model.	(Roper et al., 2017)
Hepatocellular carcinoma	Pten and p53 genes	Hydrodynamic injection	Embryonic stem cell targeting	(Xue et al., 2014b)

JPET Fast Forward. Published on May 23, 2019 as DOI: 10.1124/jpet.119.257287
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Figure 1

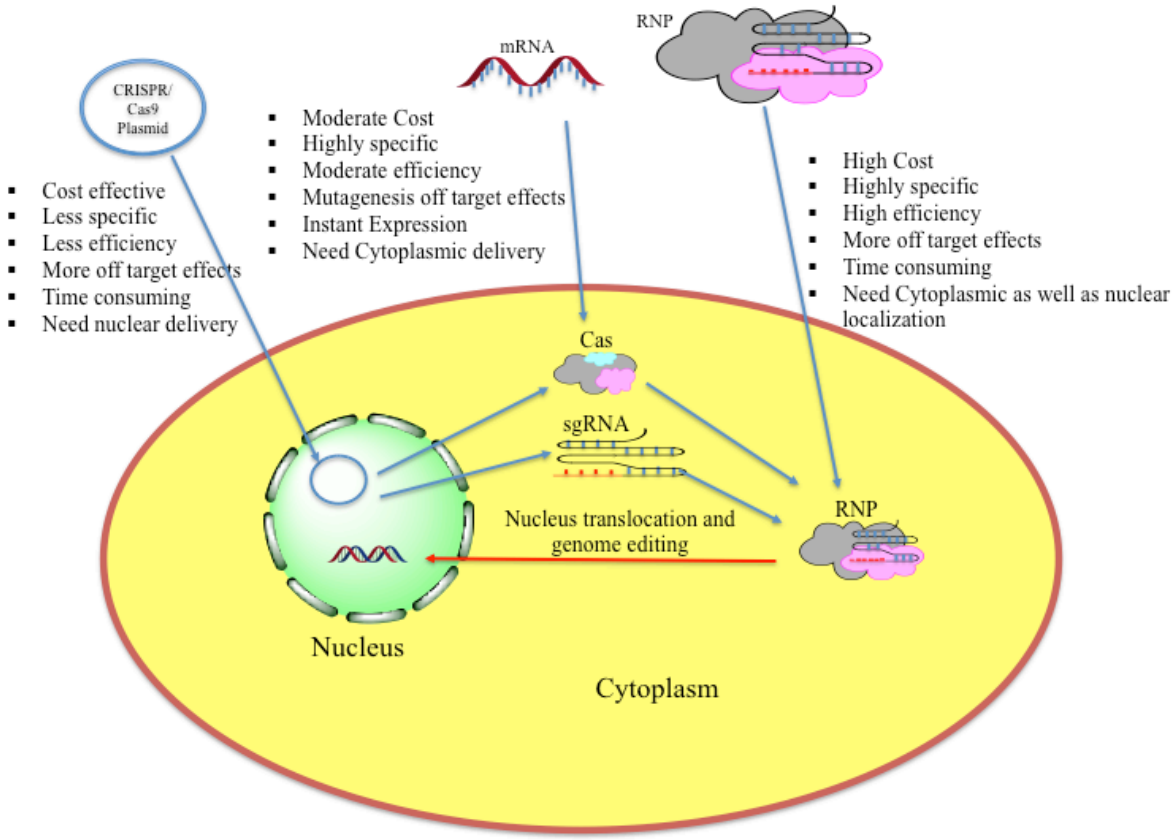

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Figure 2

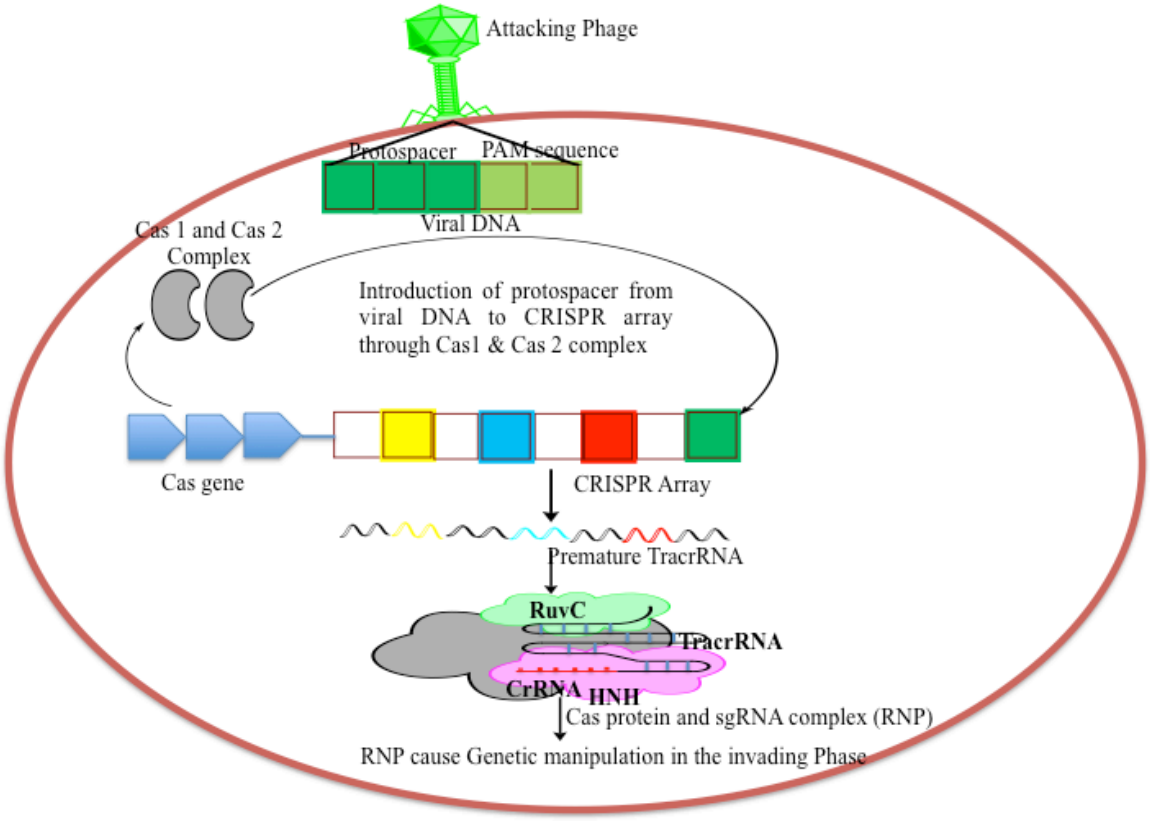

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Figure 3