Letters in Animal Biology

Journal homepage: www.liabjournal.com

Advancements of CRISPR/Cas9 technology and its value in antiviral therapeutics

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Article info

Received: 21 August 2021
Received in revised form: 24 September 2021
Accepted: 25 September 2021
Published online: 26 September 2021

Keyword:
CRISPR/Cas9
Antiviral therapy
Delivery
Challenges
Immunogenicity
Off-target effects

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Abstract

CISPR/Cas9 system is a natural immune mechanism adopted by bacteria and archaea on exposure to invading phages and plasmids. The field of genome editing has been revolutionized with the advent of CRISPR/Cas9 technology. The CRISPR/Cas9 based gene editing has offered a promising therapeutic platform for many animal and human diseases, particularly viral diseases because viruses evolve constantly and hence escape vaccine-induced immunity. The targeted genome editing by RNA-guided nucleases is rapid, easy, economical, and efficient compared to previous editing technologies. It not only helps in the direct destruction of viruses, but also helps us understand the host-virus interactions, gene functions, and develop recombinant vaccines. It has been widely experimented in the field of antiviral therapy, starting with HIV in 2013 to SARS CoV-2 recently, with a series of modifications in structure and composition of CRISPR/Cas9 and delivery mechanisms to achieve the ever-increasing promising results. Herein, this review focused on the origin of CRISPR/Cas9 system, mechanism of action, advantages over existing gene-editing tools, its progress in antiviral therapy, vaccine development, delivery approaches, and challenges faced in the application of CRISPR/Cas9.

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1. Introduction

CRISPR (clustered regularly interspaced short palindromic repeats) – Cas system is a natural adaptive immune mechanism of bacteria and archaea against the invading foreign genetic plasmids and phages (Tang et al. 2018; Teng et al. 2021). The CRISPR was first time observed in 1987 by Ishino et al. (1987) in Escherichia coli and later on, similar structures were observed in other bacteria and archaea species (Teng et al. 2021; Khadempur et al. 2019). They are specific coded areas owing their origin to phage or plasmid genomes, called spacers, and integrated in-between the repeated sequences in the host DNA. During the exposure of bacteria to foreign genetic elements, the CRISPR associated (Cas) nucleases break down the invading genetic material, part of which is placed between two repeated sequences as a spacer. The sensitivity of bacteria to foreign invading genetic elements depends upon the contents of spacer sequences. This CRISPR has been shown to work in coordination with Cas endonucleases and the genes coding them are located in the vicinity of the CRISPR locus (Khadempur et al. 2019). In prokaryotes, the CRISPR/Cas system precisely recognises and binds the DNA or RNA of invading phage or plasmids through CRISPR RNA (crRNA), and uses the trans-activating

crRNA to direct Cas enzymes to recognise and cleave the invading DNA or RNA (tracrRNA) (Barrangou et al. 2007). In 2012, a ground-breaking research demonstrated the ability of a DNA endonuclease Cas9, guided by two RNAs, to introduce a double-stranded break in target DNA in vitro (Gasiunas et al. 2012; Jinek et al. 2012) making it the first gene-editing technology under in vitro conditions. Subsequently, crRNA and tracrRNA were combined into a single guided RNA (sgRNA) which made gene editing more efficient (Jinek et al. 2012) and the introduction of sgRNA has greatly simplified the CRISPR/Cas9 system.

The CRISPR/Cas9 system is composed of two major elements – sgRNA and a Cas9 endonuclease. The sgRNA, complexed with Cas9, forms a complementary base pairing with a target sequence and makes the Cas9 introduce a specific cleavage in double-stranded DNA (Doudna and Charpentier 2014; Jiang and Doudna 2017). The CRISPR/Cas9 system has been effectively used in the correction of genetic defects and the effective generation of genetically modified cells and animal models since the introduction of sgRNA instead of two RNAs (Doudna and Charpentier 2014; Jinek et al. 2012; Cong et al. 2013; Strecker et al. 2019; Mali et al. 2013; Ran et al. 2013; Zhang et al. 2017). Owing to its protective immunity property in bacteria against invading phages CRISPR/Cas system has been successfully employed in modifying the genomes of several DNA and RNA viruses which have opened up a new advanced horizon of antiviral therapy. In CRISPR/Cas system the most commonly employed DNA and RNA targeting endonucleases in the antiviral therapy are Cas9 and Cas13, respectively.

2. Principal/Mechanism of action of CRISPR/Cas9 system

The CRISPR/Cas9 system induces a heritable adaptive immunity in bacteria and archaea against invading foreign genetic elements (Barrangou and Marraffini 2014) and has revolutionised the field of genome editing which was not that feasible with the previous technologies such as gene editing mediated by zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Doudna and Charpentier 2014; Hsu et al. 2014; Sander and Joung 2014). It was only in 2012 that the mode of action of Cas9 guided by RNA was demonstrated under in vitro conditions, whereby it cleaved the target DNA through a small synthetic RNA (Gasiunas et al. 2012). And, in the following year the CRISPR/Cas9 genome editing was demonstrated in mammalian cells (Cong et al., 2013; Mali et al., 2013). A Cas9 helicase and a sgRNA made of crRNA and tracrRNA constitute the CRISPR/Cas9 system's functional components. The Cas9 binds the tracrRNA, transcribed from the palindromic repeats of host DNA, and cleaves the invasive DNA paired with the RNA spacers (crRNA) which are transcripts of small DNA stretches acquired from extra-chromosomal elements (Xiao et al. 2019). The sgRNA directed Cas9 induces double stranded break in the invasive DNA in the Protospacer adjacent motif (PAM) region. The Cas9 has two domains of nuclease activity – histidine-asparagine-histidine (HNH) domain mediating the cleavage of target strand and RuvC domain mediating the cleavage of a non-target strand (Jinek et al. 2012; Nishimasu et al. 2014). Thereafter, the endogenous cellular repair machinery, consisting of a series of polymerases and ligases, carries out the repair process by adding or removing nucleotides at the breakpoints before re-joining the broken strands (Sander and Joung 2014).

The repair mechanism is mediated by either non-homologous end joining (NHEJ) DNA replication or by the homology-directed repair (HDR) pathway depending on the availability of homologous DNA template (Teng et al. 2021; Luther et al. 2018). In the NHEJ pathway the random nucleotides are incorporated at the cleavage site till a small overlapping strand is built which allows the DNA polymerases and ligases to ligate the broken ends. This mechanism results in frameshift mutation affecting the transcription and translation of the target gene which in turn results in a knock-out of gene expression or loss of protein function (Ran et al. 2013; Teng et al. 2021). On the other hand, availability of the foreign homologous 'repair template' DNA strand induces the HDR pathway whereby this homologous scaffold, complimentary to either end of the cleavage, gets incorporated between the broken ends (Zhang et al. 2017; Teng et al. 2021; Luther et al. 2018). The NHEJ pathway is more effective than the HDR pathways because former takes place through the cell cycle and latter takes place only take place during the S and G2 phases of the cell cycle (Wang et al. 2013; Yang et al. 2013).

3. Advantages of CRIPSR/Cas9 over previous recombinant techniques

Before the advent of CRISPR/Cas9 technology as a versatile tool for genomic editing, the researchers used DNA-binding nucleases such as mega-nucleases (MN), zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALEN) (Xu et al. 2015; Jo et al. 2015; Scharenberg et al. 2013). The genome editing mediated by CRISPR/Cas9 is having more flexibility and efficiency because it is simple and base pairing between sgRNA and target DNA is more accurate compared to its predecessor techniques (Gaj et al. 2013; Hsu et al. 2014). All these previous techniques as well as CRISPR/Cas9 specifically introduced the double-stranded breaks in target DNA, but previous techniques required the design and engineering of the protein detecting DNA-binding domain which is laborious, time-consuming, and less economical. These practical issues were overcome by the advent of CRISPR/Cas9 technology where the detection of DNA-binding domain is RNA dependent (Lander 2016; Khadempar et al.

2019).

Another advantageous feature of CRISPR/Cas9 over its predecessors is that it allows for sophisticated gene editing by its ability to retarget new DNA sequences to introduce multiple double-strand breaks easily because only the redesigning of complementary sgRNA is required and Cas9 nuclease remains the same in all cases. In comparison to the de novo synthesis of large guide proteins required by earlier gene editing technologies, the organisation in the CRISPR/Cas9 system is more simpler (Cong et al. 2013; Chena et al. 2019; Xiao-Jie et al. 2015). The property of multiplexing of CRISPR/Cas9 system, whereby multiple sgRNAs target different loci of target genome resulting in editing of these loci simultaneously, is not shown by predecessor gene-editing tools (Cong et al. 2013; Doudna and Charpentier 2014). The CRISPR/Cas9 system also enables the desired activation or suppression of the target gene's expression through the fusion of an inactive Cas9 mutant with an effector domain, such as CRISPRa or CRISPRi, respectively (Gilbert et al. 2014; Konermann et al. 2015).

However, certain limitations of the CRISPR/Cas9 system has been observed over other gene-editing tools. The mitochondrial DNA of patient-derived cells can be targeted and cleaved by modified TALEN technology, whereas, targeting of mitochondrial genome by CRISPR/Cas9 is yet to be established (Bacman et al. 2013). Additionally, PAM region and guanine at the 5' end of the target DNA sequence are the restrictions for CRISPR/Cas9, but the only prerequisite for TALEN targets is the presence of thymine at the 5' end (Kim and Kim 2014; Gaj et al. 2013). The extent of off-target DNA cleavage and the consequent effects in different gene-editing tools are not yet established well. There are reports where CRISPR/Cas9 mediated gene editing is shown to have limited off-target cleavage compared to other nucleases including ZFNs, TALENs, and homing endonucleases by ChIP-seq (Duan et al. 2014). On the other hand, TALEN has reportedly been shown to have fewer off-target effects than the CRISPR/Cas9 does (Kim et al. 2013; Wei et al. 2013) because FokI nuclease has heterodimeric nature in TALEN (Xiao-Jie et al. 2015).

4. CRISPR/Cas technology in antiviral therapy

Since the inception of CRISPR/Cas as a tool for gene editing, it has been applied to organisms with cellular structure as well as to non-cellular organisms, such as DNA and RNA viruses. Because of its precise targeting of specific viruses in the infected cells it has emerged as a novel approach in the antiviral treatment (Vilela et al. 2020). The mechanism of action of the CRISPR/Cas9 system was demonstrated in 2012 and in the following year, it was applied against human immunodeficiency virus (HIV) with promising results.

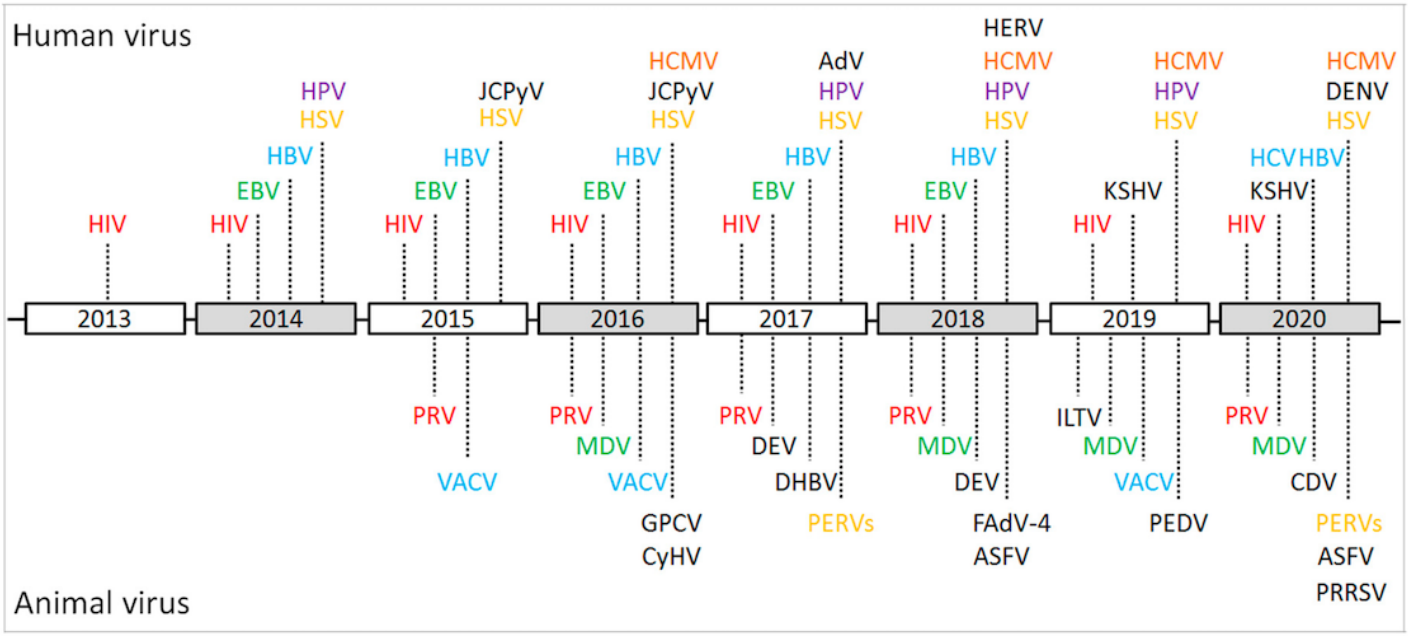

Fig. 1. Important events and achievements made in the field of viral genome editing through CRISPR/Cas9 during 2013–2020. HIV: human immunodeficiency virus; EBV: Epstein–Barr virus; HSV: herpes simplex virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HPV: human papillomavirus; HCMV: human cytomegalovirus; JCPyV: JC polyomavirus; KSHV: Kaposi's sarcoma-associated herpesvirus; AdV: Adenovirus; HERV: human endogenous retrovirus; DENV: dengue virus; PRV: pseudorabies virus; VACV: vaccinia virus; MDV: Marek's disease virus; GPCV: guinea pig cytomegalovirus; CyHV: cyprinid herpesvirus; DEV: duck enteritis virus; DHBV: duck hepatitis B virus; PERVs: porcine endogenous retroviruses; ASFV: African swine fever virus; FAdV-4: Fowl Adenovirus 4; ILTV: infectious laryngotracheitis virus; PEDV: porcine epidemic diarrhea virus; CDV: canine distemper virus; PRRSV: porcine reproductive and respiratory syndrome virus.

(Source: Teng et al. 2021)

Thereafter, its use against different species of viruses increased year after year, however, its use against animal viruses started only in 2015 when it was targeted against pseudorabies virus (PRV) (Fig. 1). Most of the research on the antiviral therapeutic side of CRISPR/Cas9 targeted the viral genome responsible for gene expression and replication (Roehm et al. 2016), along with the viral structure, transformation, and latency (van Diemen et al. 2016; Yuen et al. 2018). In addition to the development of antiviral therapy, it has been used for functional study of virulence factors, virus-host interactions, identification of host factors required for viral replication, and development of genetically engineered viral vectors for vaccine development (Teng et al. 2021).

PRV was the first animal virus to be co-transfected with CRISPR/Cas9 complex into PK15 cell line and almost 100% efficiency was obtained in gene editing of virus (Xu et al. 2015). In another study, 75 sgRNAs targeting different genes across the PRV genome were designed and transfected into the Vero cell line, a significant decline of PRV infection and replication was observed with most of the designed sgRNAs (Tang et al. 2017). A significant decrease in virus replication and yield in each passage was observed in PRV infected cell line (PX459) which stably expressed Cas9 nuclease and specific sgRNA targeting UL30 gene which is conserved in PRV (Ren et al. 2018). This indicates that it is the efficient editing of CRISPR/Cas9 which has led to a significant decline of viral replication. However, the use of multiple sgRNA simultaneously significantly increases the gene-editing efficiency. The use of two sgRNAs demonstrated a much better knock-out or knock-in efficiency in the PRV genome compared to one sgRNA based transfection-infection approach (Tang et al. 2018).

Herpesvirus of turkey (HVT) based Marek's disease (MD) vaccine is a first-generation vaccine that has been successfully used in poultry for the past five decades. With time it has been used as a vector for the expression of heterologous antigens against several avian diseases such as Newcastle disease, avian influenza, infectious bursal disease, infectious laryngo-tracheitis, avian leukosis, and Eimeria infections (Bublot 2004; Croneberg et al. 1999; Li et al. 2011; Tsukamoto et al. 2002). These recombinant vaccines conferred excellent long-lasting immunity against MD as well as other intended diseases in poultry. The generation of recombinant HTV vaccines by conventional homologous recombination in virus-infected cells or by bacterial artificial chromosome mutagenesis (Tsukamoto et al. 2002; Li et al. 2011) is laborious and time-consuming because construction of transfer vectors followed by several rounds of plaque purifications are required. CRISPR/Cas9 has gained popularity as a versatile tool for identifying different viral genes as targets to prevent MD virus replication (Hagag et al. 2020). However, it was Tang et al. (2018) who demonstrated the feasibility of the CRISPR/Cas9 approach in inducing mutations for the generation of HVT vectors. The use of two sgRNAs targeting meq and pp38 genes of MD virus-1 successfully knocked out these genes for the genome of Rispens vaccine stain without affecting its replication (Zhang et al. 2018). In another study, optimal sgRNA and Cas9 expression cassettes were inserted into the genome of Marek's disease vaccine strain to establish the antiviral property of CRISPR/Cas9 against Avian leukosis virus (ALV), a lymphoproliferative disease of poultry (Liu et al. 2020). By targeting the long terminal repeats (LTR) of ALV under in vitro and in vivo conditions CRISPR/Cas9 optimised different target sites and generated excised indel mutations within the ALV genome resulting in its disruption and the consequent protection against ALV infection.

Soon after the animal/avian herpesviruses, the application of the CRISPR/Cas9 system as an antiviral strategy started in human herpesvirus infections under in vitro conditions (Van Diemen and Lebbink 2017). The complete inhibition of herpes simplex virus (HSV) replication in host cells was achieved through CRISPR/Cas9 gene editing by targeting gE, TK, ICP0, ICP4, and ICP27 genes of HSV (Suenaga et al. 2014; Roehm et al. 2016). Another herpesvirus infection is Epstein-Barr virus (EBV) infection which is correlated with malignant conditions such as nasopharyngeal carcinoma, Hodgkin's lymphoma, and Burkitt's lymphoma (Taylor et al. 2015; Ok et al. 2015). CRISPR/cas9 significantly reduced virus load and cell proliferation and enhanced cell apoptosis by employing seven anti-EBV gRNAs in a Burkitt's lymphoma patient-derived B cell line (Raji cells) with latent EBV infection (Ang and Quake 2014). The designing of different gRNAs targeting different regions of the EBV genome followed by transfection into C666-1 reduced EBV DNA by 50% and established the feasibility of EBV gene editing by CRISPR/Cas9 technology. However, the survival of C666-1 cells and their sensitivity to anti-cancerous drugs were not affected which suggests that CRISPR/Cas9 system can potentially make EBV- transformed cancer cells more sensitive to anti-cancerous drugs (Yuen et al. 2017; Yuen et al. 2018). Human cytomegalovirus (HCMV) is another herpesvirus infection in humans commonly associated with breast cancers. In a study, three specific g RNAs were designed against the UL122/123 gene of HCMV followed by transfection into primary fibroblasts and U-251 MG cell line (Gergen et al. 2018). The targeting of the UL122/123 gene, crucial for lytic replication and reactivation from latency, reduced the virus replication by 90% and also prevented replication of new HCMV virus particles significantly. Furthermore, targeting the IE region of the HCMV genome, the delivery of lentivirus based CRISPR/Cas9/sgRNA into HFF primary fibroblasts and THP-1 monocytic cell line significantly reduced viral gene expression and production of virus particles; and inhibited DNA production and virus reactivation, respectively (Xiao et al. 2020).

Human papillomavirus (HPV) is another oncogenic virus causing cervical cancers in humans by encoding oncogenic genes E6 and E7 which are responsible for malignant transformation cells and maintenance of malignancy. It has been reported that the transfection of SiHa and CaSki cell lines by specific CRISPR/Cas9-gRNA complexes targeting the HPV-16 E7 gene resulted in E7 inhibition, upregulation of tumour suppressor protein – pRb, tumour cell apoptosis, and finally inhibition of tumour growth (Hu et al. 2014). On similar lines targeting of HPV-16 E6/E7 resulted in inhibition of E6/E7 protein expressions, upregulation of tumour suppressor proteins – p53 and p21, and inhibition of SiHa cell proliferation as well. In the mice model, it was also possible to see the in vivo regression of the subcutaneously transplanted tumour (Zhen et al. 2014). Additionally, studies have demonstrated that CRISPR/Cas9, which specifically targets the malignant genes E6 and E7 as well as their promoters, inhibits the formation of cervical tumours and also reverses malignancy (Kennedy et al. 2014; Zhen et al. 2015). Hepatitis B virus is also strongly linked to hepatocellular cancer in humans (HBV). In an attempt to employ CRISPR/Cas9 against HBV, eight gRNAs was designed against HBV (genotype A) and transfected in Huh-7 cells. Out of these eight gRNAs, two were successful in reducing the expression of HBV core and surface proteins (HBcAg and HBsAg) (Lin et al. 2014). Further, a specific cleavage of covalently closed circular DNA (cccDNA) of HBV in infected cells was tried by CRISPR/Cas9 complex (Kennedy et al. 2015) and researchers presented it as a potential tool for clinical therapy against HBV. Subsequently, the use of the CRISPR/Cas9 system against HBV (genotype A-D) was evaluated by using single as well as combined gRNAs targeting the regulatory region of HBV. Significant reduction in HBsAg or HBV e antigen (HBeAg) expression was observed with superiority of dual gRNAs over single gRNAs and dual gRNAs efficiently inhibited HBV expression and destroyed the cccDNA reservoirs in HepAD38 cell line (Wang et al. 2015). However, only partial scavenging of the virus occurs (Chena et al. 2019) which demands further modifications in the CRISPR/Cas9 system and identifications of new targets to completely inhibit HBV cccDNA replication. Therefore, these studies establish the potential of the CRISPR/Cas9 system in the treatment of cancers caused by viruses. However, variability in viral targets is a challenge in the therapeutic approach of the CRISPR/Cas9 system which requires simultaneous targeting of different target loci (Chena et al. 2019).

HIV is the first virus in which gene editing was achieved efficiently by CRISPR/Cas9 system. A specific gRNA complexed with Cas9 nuclease, transfected into Jurkat cells, efficiently cleaved and induced mutations in LTR of HIV-1 (Ebina et al. 2013) and can potentially remove the integrated viral genes from the host chromosome which indicates the potential of CRISPR/Cas9 in curing HIV infection. In another study, a new gRNA was designed, targeting the HIV-1 LTR U3 promoter region, where Cas9 nuclease could completely excise the proviral DNA integrated into microglia, promonocytic, and T cells (Hu et al. 2014). However, the use of more than one gRNAs targeting different regions of the HIV genome prevents viral replication more efficiently (Lebbink et al. 2017). The higher efficiency of gene editing by simultaneous use of multiple gRNAs was further established by Wang et al. (2018) who demonstrated effective removal of latent HIV-1 virus, inhibition of virus reactivation, and destruction of HIV-1 genome by using Staphylococcus aureus Cas9 and multiple gRNAs. And, the latest addition to the applications of the CRISPR/Cas system is the cleavage of SARS-CoV-2 RNA by employing Cas13 (RNA-guided RNA endonuclease) and crRNA (Abbott et al. 2020). The authors testified that the use of a cocktail of different crRNAs has a pan-coronavirus application which has significantly inhibit viral replication.

5. CRISPR/Cas system and vaccine development

Traditional methods for the development of viral vectored vaccines are time-consuming, laborious, and inefficient which require many rounds of plaque purification, and thus have not the ability to meet the urgent requirements in pandemics (Liang et al. 2016; Zou et al. 2017). Further, to optimise precise targets in viruses while vaccine development it is important to precisely trace the mechanism of viral disease – infection, replication, and spread (Puschnik et al. 2017). In this regard, the application of CRISPR/Cas9 is a novel strategy to identify the genes involved in viral pathogenesis and design the specific gRNAs against them which not only leads to the rapid and efficient development of recombinant vaccine but also can generate multivalent recombinant vaccines conferring protection against multiple diseases simultaneously (Vilela et al. 2020). According to reports, the CRISPR/Cas9 system considerably expedites the development of a PRV vaccine by a straightforward, one-step multiple-gene recombination procedure (Liang et al. 2016). Herpes simplex virus type I, herpesvirus of turkey and duck enteritis virus, guinea pig cytomegalovirus, Epstein-Barr virus, adenovirus, pseudorabies virus, vaccinia virus, SARS Cov-2, HIV-1, HBV, and HPV are a few examples of the viruses against which the CRISPR/Cas9 system has been successfully used to edit genes in their vaccine development process (Suenaga et al. 2014; Xu et al. 2015; Yuan et al. 2015; Bierle et al. 2016; Zou et al. 2017; Chang et al. 2018; Abbott et al. 2020; Ebina et al. 2013; Lebbink et al. 2017; Kennedy et al. 2015; Hu et al. 2014). The methods of western blotting, immunofluorescence, and molecular detection were used to successfully establish the stability and expression of the inserted genetic elements in the recombinant viruses in cell cultures for at least 15 passages (Tang et al. 2018; Atasoy et al. 2019; Chang et al. 2019).

6. CRISPR/Cas9 delivery approaches under *in vivo* conditions

The delivery of crRNA, tracrRNA (or sgRNA), and associated Cas9 endonuclease into the cell and further the nucleus is a major bottleneck in the CRISPR/Cas9 application in biological systems (Luther et al. 2018). Several delivery formats have been tried over the years which can be broadly classified as viral and non-viral formats because of the significant contribution of viruses in the delivery of CRISPR/Cas9 gene-editing machinery. The viral formats of delivery have achieved considerable success from cell culture to animal models (Chiou et al. 2015; Long et al. 2014). This success is attributed to the natural design of viruses to carry genetic elements to be introduced and expressed in the cells. However, there are certain issues with the use of viral formats of delivery which has led to the emergence of non-viral formats of CRISPR/Cas9 delivery, such as plasmid-based, mRNA based, protein-based, etc. Among these approaches or formats used for delivery of CRISPR/Cas9 system in cell lines, for *in vivo* application only a few are feasible due to lack of stability or compatibility issues (Luther et al. 2018).

Most commonly used approach for delivery of the CRISPR/Cas9 system into cells is the viral approach; and lentiviral, adenoviral, and adeno-associated viral (AAV) delivery methods are its common constituents. Among the viral methods adenoviral delivery has been used most frequently which commonly features extrachromosomal expression rather than integrating into the host genome (Schmidt and Grimn 2015; Jager and Ehrhardt 2009). However, adenoviral vectors show considerable immunogenicity and massive hepatomegaly was observed in mice one week after delivery of CRISPR/Cas9 components (Wang et al. 2015). Thereafter, a high-capacity adenoviral vector was designed to carry CRISPR/ Cas9 components, targeting E6 oncogene of HPV, dystrophin gene associated with Duchenne muscular dystrophy, and HIV co-receptor C-C chemokine receptor-5, into primary cell lines and achieving an editing efficiency of about 93% (Ehrke-Schulz et al. 2017). However, this high-capacity vector has not been yet tested under *in vivo* conditions. On the other hand, lentiviral vectors are very efficient in the delivery of CRISPR/Cas9 components into the cell and further integration of desired genes into the host genome. However, this integration is random which may occasionally lead to undesired mutations, such as insertion near protooncogenes may activate them and lead to tumorigenesis (Luther et al. 2018). Attempts to develop non-integrating lentiviral vectors have resulted in decreased efficiency (Sarkis et al. 2008) which has led to the emergence of other vectors for delivery of CRISPR/Cas9 components. In comparison to adenoviral and lentiviral vectors, AAV vectors have been developed for delivery of CRISPR components which specifically integrates the desired genes at the AAV – integration site 1 locus in mammalian cells (Kotin et al. 1990). This site-specific integration of genes is considered safe for *in vivo* application because of less off-target effects. The use of AAV vectors for delivery of *Streptococcus pyogenes* Cas9 (spCas9) and sgRNA targeting mutated dystrophin gene in muscle tissue successfully restored normal production of dystrophin and skeletal muscle function in mice (Tabebordbar et al. 2016). But the inherent problem with AAV is their ability to carry small size CRISPR elements only. The use of smaller variants of Cas9, such as *Streptococcus aureus* Cas9 (saCas9), overcomes this size limitation to a considerable extent (Ran et al. 2015). However, the highly immunogenic nature of saCas9 compared to spCas9 again limits the therapeutic application of AAV based CRISPR/Cas9 delivery (Li et al. 2015).

The plasmid-based delivery formats, conceptually similar to viral vectors, have been introduced for the delivery of CRISPR/Cas9 elements to avoid the issues faced while using viral vectors. The delivery of CRISPR/Cas9 components in plasmid format has been tried in a mouse model for correcting hereditary tyrosinemia (Platt et al. 2014). However, the success in the correction of the hereditary defect was insignificant. Therefore, to achieve the high transfection efficiency observed with viral vectors a nucleus targeting artificial virus was synthesized to deliver plasmid encoding Cas9 and sgRNA targeting ovarian cancer (Li et al. 2016). A significant disruption of the involved oncogene with a corresponding decrease of tumour size was observed. Furthermore, in the pursuit of developing the organ- or tissue-specific delivery vehicles a cell-specific aptamer (LC09) was functionalised onto a lipopolymer moiety and coupled with CRISPR/Cas9 plasmid system and a selective tumour specificity was observed in osteosarcoma and lung metastasis (Liang et al. 2017). This study has demonstrated the cell- or tissue-specific targeting of plasmid-based CRISPR/Cas9 delivery with potentially lesser off-target effects.

The delivery of mRNA coding for Cas9 into the cell along with CRISPR machinery is another commonly used delivery format. This approach of delivery is rapid, but transient, in action with lesser chances of being integrated into the host genome (Nelles et al. 2016; Zetsche et al. 2015). But, the problem with this delivery format is that the individual carriers are required for CRISPR/Cas9 components. To correct hereditary tyrosinemia in murine model a lipid-mediated delivery of Cas9 mRNA coupled with AAV delivery of sgRNA and HDR template and a correction of >6% hepatocytes was reported (Yin et al. 2016). However, in addition to the instability of the mRNA stability of sgRNA also gets compromised during mRNA translation to Cas9 which is to be complexed with sgRNA. Therefore, to improve the sgRNA stability for better editing efficiency synthetic modifications in sgRNA have been carried out by changing the RNA 2'OH to 2'OMe or 2'F, or by forming

phosphorothioate bonds (Yin et al. 2017). Along the same lines, modified sgRNA and Cas9-coding mRNA were combined into a single lipid nanoparticle vehicle, and when administered to mice, it effectively shut down the transthyretin gene, resulting in a more than 97% decrease in the relevant blood protein (Finn et al. 2018). Another study has recently demonstrated an effective non-viral co-delivery of Cas9 mRNA and a sgRNA in a single zwitterionic amino lipid vector with 95% editing efficiency (Miller et al. 2017).

The use of a suitable synthetic delivery vector for direct delivery of Cas9 protein associated with sgRNA (Cas9-RNP) constitutes another strategy. It is a transient delivery technique with practically no off-target effects, limited immunogenicity (Paix et al. 2015), and high editing efficiency in a short period time. Though the problem of sgRNA degradation is overcome by this method of delivery the endosomal entrapment of protein complex, the laborious expression of Cas9 protein, gradual loss of nuclease activity after synthesis are the problems associated with this method (Kelley et al. 2016; Hendel et al. 2015; Ran et al. 2013). In addition to these methods of delivery, several other methods such as electroporation/nucleofection, lipid-based transfection, gene-gun delivery, along with other mechanical and non-mechanical approaches have been attempted under *in vitro* conditions with variable successes (Chen et al. 2016; Horii et al. 2014).

7. Challenges in CRISPR/Cas9 antiviral therapy and potential solutions

Since the CRISPR/Cas9 components are macromolecules, the effective delivery CRISPR/Cas9 complex into the cell cytoplasm and then the nucleus for gene modification is the first major obstacle in CRISPR/Cas9 antiviral therapy. Different delivery vectors discussed above are laced with advantages as well as disadvantages. Adenoviral vectors have the ability to incorporate large DNA fragments (Wold and Toth 2013) but the development of a recombinant adenoviral vector is still a major obstacle (Afkhami et al. 2016). Lentiviral vectors deliver the CRISPR/Cas9 components efficiently and integrate them into the host genome which results in their stable expression, but they are marred with increased risk of off-target effects (Wang et al. 2014; Khalili et al. 2017). AAV vectors are considered safe and efficient delivery formats but have the disadvantage of small packaging size. Furthermore, targeting the viral infections in the central nervous system, such as HIV-1, by CRISPR/Cas9 blood-brain barrier (BBB) is a major obstacle. Only small size lipophilic molecules can cross BBB. This hurdle has been overcome to some extent by the introduction of nanoparticle-based CRISPR/Cas9 delivery to the brain, such as polymer nanoparticles (Fornaguera et al. 2015), magnetic nanoparticles (Nair et al. 2013), and gold nanoparticles (Mout et al. 2017). Even the organ/tissue-specific nanoparticle delivery vehicles have been developed where CRISPR/Cas9 complex show a predilection for specific organs such as the liver and lungs (Givens et al. 2018). Though considerable success has been achieved in the delivery of CRISPR/Cas9 system into the cells for gene editing, significant challenges still exist in transforming this progress into in vivo application.

Undesirable immunogenicity of Cas9 protein, potential binding epitopes of MHC, can potentially lead to life-threatening immune reactions. However, compared to spCas9 the immunogenicity of saCas9 is more intense (Mehta and Merkel 2020). It has been reported that the administration of AAV-CRISPR/Cas9 complex intramuscularly in mice targeting different genes elicited the immune response against the complex (Chew et al. 2016). However, the immune reaction was not so severe to negate the significance of CRISPR/Cas9 and the reaction was ascribed to Cas9 protein only, not the whole AAV-CRISPR/Cas9 complex. Even the pre-existing humoral and cell-mediated immunity against Cas9 protein in humans has been reported which need to be considered while conducting trials (Charlesworth et al. 2019). A moderate level of innate immunity leading to cell toxicity has been observed against the RNP/spCas9 complex which was prevented by chemical synthesis and phosphatase treatment of sgRNA for 5'-ppp removal (Kim et al. 2018).

The potential off-target gene editing by CRISPR/Cas9 system is a major concern, particularly with lentiviral vectors, with dangerous clinical implications. Since permanent genetic alterations are induced by CRISPR/Cas9 system, its off-target editing needs careful monitoring before to in vivo application. With six or more mismatches in sgRNAs significant off-target gene editing or effects were detected (Wang et al. 2015). The study on off-target editing effects of CRISPR/Cas9 targeting human β-haemoglobin and CCR5 genes revealed more than 50% off-target editing (Cradick et al. 2013). However, several strategies have been adopted to reduce the off-target effects of CRISPR/Cas9 therapy. The most practical method for reducing the off-target effects is the choice of an appropriate target site that has no homology throughout the genome, as the composition and structure of the sgRNA have a direct impact on the off-target effects (Cho et al. 2014). The use of paired Cas9 nickases to generate paired nicks (one by each nickase) in target DNA sequence increases target specificity because off-target single-nicks are faithfully repaired (Ran et al., 2013; Doudna and Charpentier 2014; Xiao-Jie et al. 2015). Furthermore, truncation of sgRNAs helps reduce the off-target effects significantly because the shorter the sgRNAs lesser is the mismatch tolerance (Fu et al. 2014). Reduced off-target effects have also been proposed by dimerization of CRISPR/Cas9 with other nucleases, such as dimerization of FokI nuclease with dead Cas9, because of increased specificity (Tsai et al. 2014). A strategy of placing Cas9 under the control of HIV-1 promoter, activated by transcriptional activator – Tat, resulted

in controlled expression of Cas9 in cells which have the potential of reducing the off-target gene editing (Kaminski et al. 2016). Furthermore, the minimum or undetectable off-target effects have been observed with the direct delivery of Cas9-RNP (Schumann et al. 2015; Vakulskas et al., 2018) because Cas9 RNPs will be degraded following target DNA editing.

8. Conclusions

On exposure to invasive phages and plasmids, bacteria and archaea develop the CISPR/Cas9 system as a natural defence mechanism. This natural mechanism of defence has been co-opted to introduce gene editing in cells and viruses under in vitro conditions followed by its in vivo translation. It has revolutionised the field of gene editing by surpassing the previous techniques – MN, ZFN, and TALEN, in terms of flexibility, economy, and efficiency. With a series of changes in structure and composition of CRISPR/Cas9, as well as the use of various delivery systems, it has been extensively tried in the field of antiviral medicine, from HIV in 2013 to SARS CoV-2 lately, with increasingly hopeful outcomes. CRISPR/Cas9 technique has enriched the knowledge on viral disease mechanisms and has opened up new avenues of developing multivalent recombinant vaccines against different diseases. The refinement of delivery approaches of CRISPR/Cas9 into the cells is evolving day by day to remove the disadvantages of the existing delivery formats. And, theoretically in the near future CRISPR/Cas9 technique is expected to be a panacea against all diseases in humans and animals.

Declarations

Funding: No funding from any organisation was received for this manuscript

Conflicts of interest: The authors declare no conflicts of interest

Ethical approval: Not applicable

Acknowledgements: None to acknowledge

References

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Citation

Dev K, Begum J, Mir NA, Kant R. (2021). Advancements of CRISPR/Cas9 technology and its value in antiviral therapeutics. Letters in Animal Biology 01(01): 46 – 57.