Journal of Neuroimmune Pharmacology (2019) 14:578–594
https://doi.org/10.1007/s11481-019-09878-7

INVITED REVIEW

A Broad Application of CRISPR Cas9 in Infectious Diseases of Central Nervous System

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Received: 3 June 2019 / Accepted: 26 August 2019 / Published online: 11 September 2019

Abstract

Virus-induced diseases or neurological complications are huge socio-economic burden to human health globally. The complexity of viral-mediated CNS pathology is exacerbated by reemergence of new pathogenic neurotropic viruses of high public relevance. Although the central nervous system is considered as an immune privileged organ and is mainly protected by barrier system, there are a vast majority of neurotropic viruses capable of gaining access and cause diseases. Despite continued growth of the patient population and a number of treatment strategies, there is no successful viral specific therapy available for viral induced CNS diseases. Therefore, there is an urgent need for a clear alternative treatment strategy that can effectively target neurotropic viruses of DNA or RNA genome. To address this need, rapidly growing gene editing technology based on CRISPR/Cas9, provides unprecedented control over viral genome editing and will be an effective, highly specific and versatile tool for targeting CNS viral infection. In this review, we discuss the application of this system to control CNS viral infection and associated neurological disorders and future prospects.

Keywords CRISPR/Cas9 · Neurotropic viruses · CRISPR/Cas9 delivery system · CRISPR/Cas9-mediated viral escape

Introduction

Most cause of central nervous system infections are viral mediated and they are highly associated with significant morbidity and mortality. Furthermore, the reemergence of pathogenic neurotropic viruses exacerbates the socio-economic burden of CNS infection (Manglani and McGavern 2018). According to the new estimate, the incidence of central nervous system (CNS) viral infection is 20–30/100,000 per year, which is roughly 3 times as common as bacterial infections (Romero and Newland 2003). Given this constantly growing patient population, there is no specific therapies for CNS infections (Manglani and McGavern 2018). Antiviral drugs that are currently in use for viral CNS infections are unspecific and often ineffective in removing viral reservoirs or unable to cross CNS structural barriers to get into area of viral spread. (Nath and Tyler 2013). Furthermore, despite the CNS having complex barrier system, a diverse groups of viruses develop strategy to elude it and gain access and induce disease. Mostly this is caused by the families of Retroviridae, Herpesviridae and Polyomaviridae. Although different viruses use different approach to gain access to CNS, those viral infections that start in periphery get access to CNS either by directly infecting nerve endings or the cells of circulatory system that carry the virus passing the BBB into the CNS.

CRISPR/Cas9 System

In the past few years, the application of gene editing using nucleases such as RNA-guided nuclease system (Cas9), Zinc –finger nucleases (ZFNs) and TAL effector nuclease (TALENs) greatly revolutionized genetic engineering in vitro and in vivo (Le Rhun et al. 2019). Among available nucleases, the CRISPR/Cas9 is the nuclease of choice because it is simple and efficient and easy to use. The simplicity of CRISPR system is that a single protein, Cas9 guided by a short RNA sequence serves as a site-specific endonuclease. It was originally described by Ishino et al (1987) as DNA repeat of unknown origin and function in the genome of *Escherichia coli*. The same DNA repeats was later identified

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in other microbes and named by Mojica et al (2000) group as Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR. The CRISPR/Cas9 system is found in most *archaea* (87%) and some bacteria (45%) and serve as an RNA-based prokaryotic antiviral adaptive immune system (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005; Lino et al. 2018). The sequence homology of Cas9 proteins determine the classification of CRISPR systems into Type I, type II and type III (Wiedenheft et al. 2012). The type II CRISPR/Cas9 is well characterized and it has been extensively used as a gene editing tool of choice . CRISPR/Cas9 consists of 3 components: two RNA and a Cas9 protein. One of the RNA (CRISPR RNA or crRNA) contains a short region of homology (20 bp) that direct the CRISPR/Cas9 complex to target genetic locus. The second RNA (Trans activating crRNA or tracrRNA) base pairs with crRNA to form lops based RNA structure that leads Cas9 to the target locus where there is a PAM sequence in the target DNA to introduce double strand break (DSB) that is 3 base pair (bp) upstream of PAM (Fig. 1). This DSB can be repaired either with NHEJ which is error-prone or homology-directed repair (HDR). NHEJ creates short insertions or deletions at the cleavage site and this often results in generation of frameshift mutations to cause termination of protein translation and protein loss. On the other hand, DNA repair with HDR requires a homologous DNA template. However, DNA repair by HDR is ineffective in higher organism due to the low rate of homologus recombination and a challenge in exact site delivery of donor DNA (Bollen et al. 2018; Savic et al. 2018). Different orthologue of Cas9 use different PAM sequence to recognize the target sequence. Streptococcus pyrogenes Cas9 (SpCas9) for example identify 'NGG" PAM, on the other hand 'NNGRRT' PAM that is less common in the chromosome is used by *Staphylococcus aureus* Cas9 (SaCas9). CRISPR/Cas9 ability to edit DNA have gained enormous importance in biological research in areas such as identification of mechanism in genetic disease (Gilbert et al. 2014; Findlay et al. 2014; Lino et al. 2018; Konermann et al. 2015), creating animal model to study disease (Wang et al. 2013), understanding the mechanism of epigenetic studies (Yao et al. 2015; Vora et al. 2016; Hilton et al. 2015), controlling gene expression (Mali et al. 2013), precise base editing (Gaudelli et al. 2017), RNA dependent RNA targeting (Strutt et al. 2018), imaging of DNA loci (Barrangou and Doudna 2016) and improving genetic engineering in plans (Zhang et al. 2016). Due to the widespread presence of CRISPR/Cas in bacteria, it is currently uncertain the relative biological activity of each CRISPR/Cas system in editing mammalian genomes (Wang et al. 2018b).

The systemic evaluation of the relative performance of each CRISPR/Cas system in their ability to engineer mammalian genome provide the guidelines as to which CRISPR/Cas system to apply for the intended experimental condition (Table 1) (Wang et al. 2018b). The target specificity and cleavage efficiency of a given CRISPR system is an important determining factor in their choice for experimental application. Two independent studies have verified that both

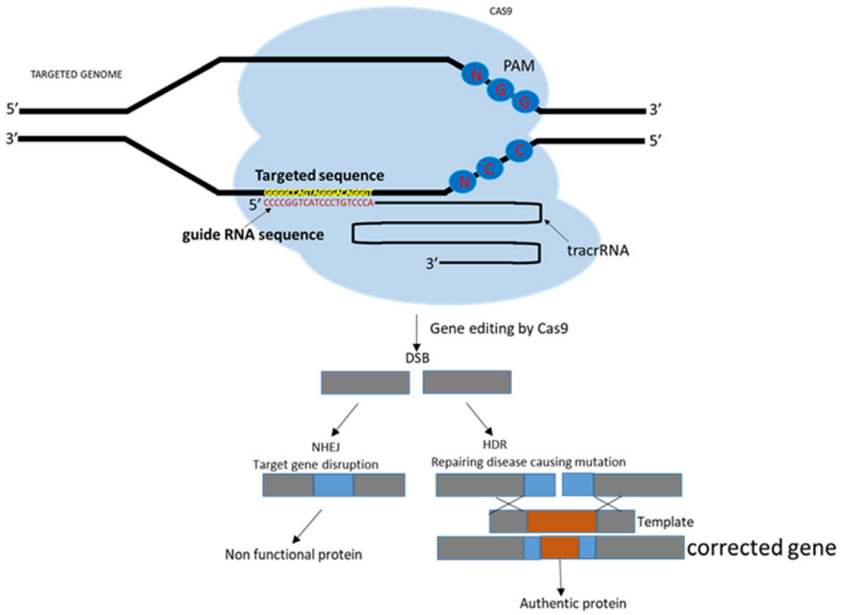

Fig. 1 CRISPR/Cas9 system. The simplicity of CRISPR/Cas9 system is that a single protein, Cas9 guided by a short RNA sequence serves as a site-specific endonuclease. CRISPR/Cas9 consists of 3 components: two RNA and a Cas9 protein. One of the RNA (CRISPR RNA or crRNA) contains a short region of homology (20 bp) that direct the CRISPR/Cas9 complex to target genetic locus. The second RNA (Trans activating crRNA or tracrRNA) base pairs with crRNA to form lops based RNA structure that leads Cas9 to the target locus where there is a PAM sequence in the target DNA to introduce double strand break (DSB) that is 3 base pair (bp) upstream of PAM. This DSB can be repaired either with NHEJ which is error-prone or homology-directed repair (HDR)

Table 1 CRISPR Cas system usage strategy for the appropriate genome editing approach in mammalian cells

CRISPR system type	NHEJ editing	HDR editing
SpCas9	+ (distinct genome locus)	+ (Asymmetric ssODN donor template)
LbCpf1	+ (distinct genome locus)	+ (Asymmetric ssODN donor template)
AsCpf1	+ (Several similar loci)	
Sacas9	+ (Several similar loci)	

AsCpf1 and LbCpf1 fulfill such requirement and they are recommended as endonuclease of choice for the future editing application (Kim et al. 2016; Kleinstiver et al. 2016; Wang et al. 2018b). It has been reported that structural analysis and random mutagenesis approach is used to create a new *S. pyogenes* Cas9 variant with enhanced specificity including eSpCas9 (Slaymaker et al. 2016), SpCas9-HF1 (Kleinstiver et al. 2016), HypaCAS9 (Chen et al. 2017) and evoCas9 (Casini et al. 2018) (Table 1). Recently, a novel function of CRISPR/Cas9 is discovered in which Cas9 in association with Tn7-like transposon mediate RNA-guided DNA insertion without homologous recombination pathway. (Strecker et al. 2019).

Crisper/Cas9 Mediated Targeting of Human Pathogenic Viruses

In addition to its enormous therapeutic potential for human genomic DNA editing, the CRISPR Cas9 system actually have been applied to target multiple human pathogens in vivo and in vitro system. We will discuss in this review various approaches used to eliminate or suppress viral infection using CRISPR/Cas9 (Fig. 1). Some of the CRISPR technology limitations that have to overcome before its practical application in clinic, will be also discussed.

Human Immunodeficiency Virus (HIV-1)

Human immunodeficiency virus (HIV-1) is a single –stranded, positive sense RNA virus that mainly infects human T cells and that leads to severe deletion of CD4+ cell and the development of AIDS. (Alimonti et al. 2003). HIV infection is a major global health problem with no vaccine insight (Bayat et al. 2018). The new report by UNAIDS indicate that more than 36.7 million people are infected with HIV worldwide and there are more than 5000 new infection each day (Dash et al. 2019). After primary infection, Viral RNA is reverse transcribed to create HIV-1 provial DNA that integrates into the host cell genome and this chromosomal integration initiate HIV latency which is defined as the integration of HIV DNA in host genome with no active viral replication or production of viral proteins. (Maartens et al. 2014). Many factors are involved in transcriptional interference in restricting HIV proviral DNA such as the absence or cytoplasmic sequestration of Transcriptional factors such as NF-kB and NFAT, the relative location of the proviral DNA promoter to the host promoter which might lead to the displacement of essential cellular transcriptional factors from the viral promoter by RNA polymerase, epigenetic mediated control of the HIV-1 promoter, the low level expression of the viral trans activator protein (Tat) and the presence of cellular transcriptional repressors (Nejat et al. 2019). The establishment of HIV latency has been shown in vivo in memory T cells and naïve T cells (Chomont et al. 2009; Wightman et al. 2010). Although monocytes-macrophages can harbor latent HIV genome, the biological significance of these cells to HIV persistence during ART therapy is uncertain (Maartens et al. 2014) Anti-retroviral therapy (ART) can successful control HIV infection but it does not affect latently infected cells and these cells serve as the source of reemerging viruses and currently there is no cellular marker to identify and therapeutically target latently infected cells (Badia et al. 2018). With a cessation of ART, the silent integrated provirus can reactivate and lead to disease progression (Panfil et al. 2018; Colby et al. 2018; Wen et al. 2018). At present, there is no effective vaccine for HIV and the development of such a vaccine faces huge challenges due to the genetic diversity of HIV, uncertainty regarding what protective immunity made of and finding and developing highly immunogenic antigens (Maartens et al. 2014). Furthermore, The characteristics of the latently infected cells that represent the HIV reservoirs, their organ distribution, their long half life time and the inability of the immune system to recognize and destroy those cells constitute major challenge for HIV eradication.(Datta et al. 2016; Finzi et al. 1999) Therefore, the main curative strategy for HIV should include an approach to eliminate the integrated provial DNA or the cells harboring the virus without causing toxic effect (Dash et al. 2019)..One such an approach is the so called "shock and kill" in which chemical agent such as histone deacetylase inhibitors applied to reactivate the virus from the latently infected cells so that these cells will be recognized and eliminated by Immune system (Shan et al. 2012; Darcis et al. 2018). This approach has considerable limitation for clinical application due to non-specificity, toxicity and inadequate induction efficiency of the chemical agents.(Archin et al. 2012) As alternative approach, Catalytically-deficient Cas9-synergistic activation mediator (dCas9-SAM) technology has been used to activate HIV from latent viral reservoirs (Zhang et al. 2015; Bialek et al. 2016).

This dCas9-SAM strategy used gRNAs targeting HIV-1 LTR as a binding site for transcriptional activator system to induce reactivation of the proviral DNA in latently infected cells that lead to death of those cells due to the toxic accumulation of the viral proteins. Several new strategies have been develop, including CRISPR/Cas9 system, to target and edit the provirus genome found in small number of resting memory T cells in patient on antiretroviral therapy without detectable HIV-1 replication (Zhu et al. 2015; Kaminski et al. 2016a, b; Wang et al. 2016c). Report from different research groups has shown that de novo infection of T cell by HIV-1 can be effectively blocked by CRISPR/Cas9 system by targeting different viral regions (Liao et al. 2015; Kaminski et al. 2016a, b; Wang et al. 2016a, b; Lebbink et al. 2017). Besides targeting viral genes which are important in viral lifecycle, the CRISPR/Cas9 system have been used to target host genes which are important for HIV infection. For example, targeting the chemokine receptor 5 (CCR5) by CRISPR/Cas9 was used to edit the CCR5 gene in human iPSCs (hiPSCs) (Kang et al. 2015) and CD4+ cells (Wang et al. 2016a). Indeed, editing CCR5 makes target cells to develop resistant to HIV infection. It has been also reported the successful application of CRISPR/Cas9 to target CXCR4 and Tat to protect human cell lines and primary human CD4+ cell from HIV infection. (Hou et al. 2015). CRISPR/Cas9 system has been also used as activator of host HIV restriction factors such as TRIM5α and APOBEC3 to inhibit HIV replication.(Bogerd et al. 2015; Dufour et al. 2018) One advantage of targeting crucial HIV host dependency factors by CRISPR/Cas9 is to prevent the generation of HIV escape mutants mediated by Cas9 editing (Lee 2019). Using adeno-associated viral vector (AAV)-mediated delivery of SaCas9 and gRNAs targeting the proviral genome in vivo mouse and rat models, Kaminski and colleagues have shown specific editing of the HIV provirus in multiple organs (Kaminski et al. 2016a) (Table 2). Although the CRISPR/Cas9 based editing system can target HIV and eliminate the virus, it is not sufficient enough by itself for the complete eradication. (Dash et al. 2019). Recently, it has been shown in collaborator effort by two groups lead by Drs. Khalili and Gendelman that combination therapy based on sequential laser ART and CRISPR can eliminate HIV-1 in a group of HIV infected Humanized Mice (Dash et al. 2019). Despite the successful application of CRISPR/Cas9 to inhibit HIV-1 replication, it has been report that the virus is capable of generating escape mutant from single gRNA based editing approach (Ueda et al. 2016). CRISPR/Cas9 mediated viral escape will be discussed in detail later section (Fig. 2).

Polyomavirus JC (JCV)

The human neurotropic Polyomavirus JC (JCV) is the etiological agent of the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). Once a rare disease seen in patient with lymphoproliferative and myeloproliferative disorders, PML has been more frequently in HIV-1 positive /AIDS patients as well as patients undergoing immunomodulatory therapy due to autoimmune disorders (Wollebo et al. 2015a; Wollebo et al. 2015b; White et al. 2016). The JCV genome has a small circular double stranded DNA that includes coding sequences for the viral early protein T-antigen, which is critical for directing viral replication and lytic infection. Seroepidemiological studies have shown that JCV infection is very common in population throughout the world and initial infection usually occurs during childhood (White and Khalili 2011). The high seroprevalence of JCV infection and the rarity of PML suggest that the immune system is able to maintain the virus in a persistent asymptomatic state, since altered immune function appears to underlie all condition that predispose to PML. Many important aspects of the JCV life cycle and the pathogenesis of PML remain unclear including whether JCV remains truly latent or persistent infection with a low level of gene expression occurs in cells. JCV DNA can be detected in oligodendrocytes and astrocytes but not neurons from normal brain (White et al. 2013). Other studies have confirmed the presence JCV DNA or viral protein Tag in the brain of individuals without PML. It is likely that JCV is latent in the brain and its presence in CNS might be accompanied by low level of viral replication. A number of treatment options have been applied to PML, including possible viral inhibitors and small molecular inhibitors of viral replication, largely without success. (Tavazzi et al. 2012) (Table 2). Therefore, alternative strategy approach are required for the treatment of PML.

The application of CRISPR/Cas9 to eradicate the genome of JCV by targeting different regions of viral genome is reported by two groups (Wollebo et al. 2015a; Chou et al. 2016). We have employed the CRISPR/Cas9 to introduce mutations in the viral genome by inactivating the gene encoding T-antigen and inhibiting viral replication. Our report showed that transient or conditional expression of Cas9 and gRNAs specifically target the DNA sequences corresponding to the N-terminal region of T-antigen introduce mutations which interfere with expression and function of the viral protein, hence suppressing viral replication in permissive cells. We have recently extended our finding using a combination approach targeting, in addition to T-antigen, NCCR and viral protein VP1 in order to show that CRISPR-associated protein 9(Cas9) is a powerful tool for editing JCV genome. Similar approach have been used by Chou et colleagues (Chou et al. 2016) targeting VP1, VP2 and NCCR to show the suppression of JCV replication in vitro. Future in vivo studies are needed in order to show whether CRISPR/Cas9 targeting JCV can be applied to treat JCV-mediated PML (Table 2) (Fig. 2).

Table 2 Applying CRISPR-Cas9 to target different sites on human viruses of clinical importance

Virus	Target gene	Cell model used	Method of delivery	Reference
HIV	LTR	HIV-1 provirus-integrated human cells such as 293 T and Hela and human T-lymphoid cells	Transfection	(Chen et al. 2017; Hu et al. 2014a)
	LTR		Transduction	(Kaminski et al. 2016b)
	LTR	Latent hmicroglia cells	Magnetic delivery	(Kaushik et al. 2019)
	Tat,Rev., EnV			(Wang et al. 2018a)
	Gag/pol			
	LTR/GagD		Transduction	(Kaminski et al. 2016a)
JCV	T-antigen	Primary human fetal glial cells	Transfection	(Wollebo et al. 2015a)
	VP-1 and NCCR	SVGA-A cells and hTERT transformed huk (1) G10 cells derived from human fetal kidney	Transduction	(Chou et al. 2016)
HSV-1	ICP0, ICP4 and ICP27	ICP0-complementing L and cell line L7 Human oligodendroglioma cell line TC620	Transfection	(Roehm et al. 2016)
	UL17	Vero cells, HEK293T cells and BALB/C mice	Transfection	(Xu et al. 2016)
	UL8, UL29, and UL52	Vero cells	Transfection	(Lin et al. 2016; van Diemen et al. 2016)
HPV	E6	Cervical carcinoma lines	Transfection	(Zhen et al. 2016)
	E7	siHa and caski cells	Transfection	(Hu et al. 2014b)
	E7	Hela cells	transfection	(Lao et al. 2018)
HTLV-1	PX region	ED T-cell	Transfection	(Ho et al. 2015)
	RNFB	Hela cells	Transduction	(Satou et al. 2016)
EBV	BVRF1	Sun719 and YCCEL1 (Gastric cancer cell lines)	Transfection	(Kanda et al. 2016)
	EBNA1, LMP1, EBNA3C	Raj cell	Transfection	(van Diemen et al. 2016)
	BART5, BART6, BART16	SNU-719	Transduction	(Wang and Quake 2014)
HCV	5'-UTR and 3'-UTR	Huh-7.5 cells	Transfection	(Price et al. 2015)
HBV	Full length	HepAD38,huh7 transfected with prcccDNA and cre-expression plasmids	Hydrodynamic injection	(Li et al. 2017)
	S, X	HepG2 cells with HBV-expression plasmid	Transfection	(Karimova et al. 2015)

Hepatitis B Virus (HBV)

Hepatitis B virus (HBV) infection is the most common chronic viral infection in the world. According to the new reports, 2 billion people have been infected, and more than 350 million are chronic carriers of the virus. (Kennedy et al. 2015; Kennedy and Cullen 2017; Soppe and Lebbink 2017). Chronic HBV infection is the major cause of liver cirrhosis and the development of hepatocellular carcinoma and mainly characterized by high viral antigen burden and in sufficient immune response (Gane 2016). After primary infection, the viral genome is transported to the nucleus to be converted to covalently closed circular cccDNA. This highly stable cccDNA serve as template for viral RNA transcription and is important for the maintenance of HBV persistence. HBV remains in the body in a latent or persistent infection in liver mainly in hepatocytes. Although many important aspect of HBV life cycle and its pathogenic potential has been well studied, the molecular mechanism that govern HBV persistence are not fully characterized. The studies by various groups indicated that different factors determine HBV persistence such as the age of infection, the viral load by the time of infection, and virus mediated suppression of both innate and adaptive immune responses (Dandri and Petersen 2016). HBV belongs to the Hepadnaviridae family. The genome is partly double stranded DNA with approximately 3.2Kbp. The present antiviral therapy against chronic hepatitis B virus failed due to their inability to remove HBV cccDNA (Arzumanyan et al. 2013). The current available HBV vaccine is effective in preventing HBV infection. However, due to the luck of availability of such vaccines around the world, chronic HBV infection is still a large global health problem (Soppe and Lebbink 2017). The formulation of alpha –interferon (INF-α) and five nucleoside analogues are standard antiviral therapeutics approved for the treatment of HBV infection (Tang et al. 2017). Although such therapeutic approach are effective on inhibiting HBV DNA polymerase activity, activation of host antiviral immune response and suppression of transcription of HBV cccDNA, they are lacking to achieve a functional cure (Block et al. 2013). Multiple strategies are being develop and are under clinical investigation to achieve a functional cure after a limited therapy time such as antiviral

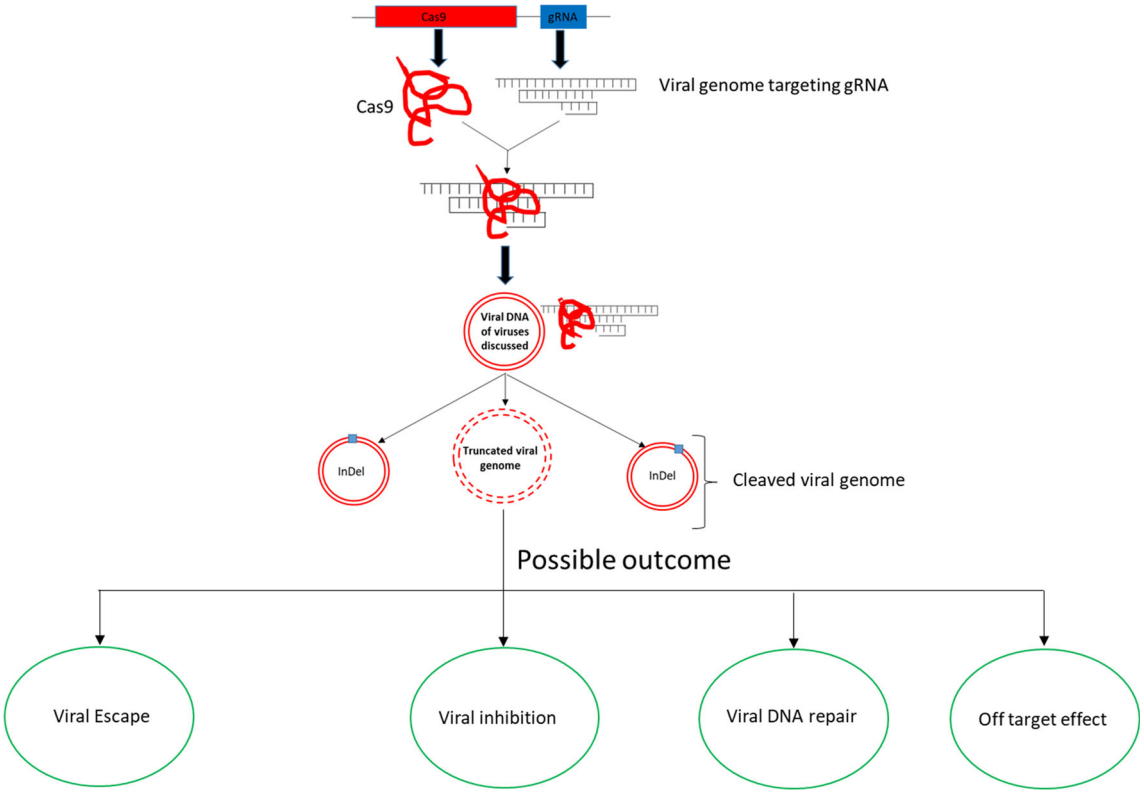

Fig. 2 CRISPR/Cas9 mediated targeting of human pathogenic neurotropic viruses. Cas9 and gRNAs, the main components of the CRISPR/Cas9 system form Cas9-gRNA complex. This complex target and cleaves the viral genome through double strand break which can be repaired by NHEJ. On the other hand, the creation of DSB result in the degradation of the virus genome. Other important outcomes like viral escape, viral DNA repair and off target effect on the host genome are shown

targeting different stage of the hepatitis B virus (HBV) replication cycle, HBV entry inhibitors, core protein inhibitors, and RNA silencers. Currently, there are immunotherapeutic approaches to activate antiviral immunity against HBV which include TLR-7 and TLR-8 agonists (Chang and Guo 2015), checkpoint inhibitors (Fisicaro et al. 2010), RIG-1 agonist, and anti-HBV immunoglobulin and therapeutic vaccines (Lopatin 2019). The successful cure for HBV would require combination of immunomodulatory, antiviral and silencing approach for the complete elimination of cccDNA in the hepatocytes (Lee et al. 2018). As novel therapy approach, CRISPR/Cas9 can be utilized to remove the cccDNA

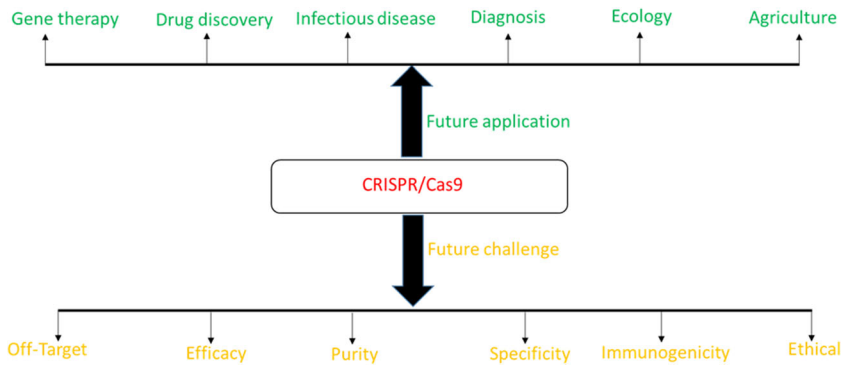

Fig. 3 Future application and challenge of CRISPR-Cas9 editing approach. Although CRISPR Cas9 system has tremendous potential for human genomic engineering application in vivo and in vitro, there are certain questions has to be addressed before its clinical use. One of the main concerns is the off-target effect of the system, in particular the CRISPR/Cas9 –mediated mosaic mutations. Moreover, the future therapeutic effect of the CRISPR/Cas9 system largely depend on the delivery of the CRISPR/Cas9 components to the target cells. The current available delivery system are not specific, efficient and have biosafety concern. Another major concern regarding future application of CRISPR Cas9 system is the recent finding of the presence of preexisting adaptive immune response in humans for a variety of Cas9 orthologs such as *S. aureus* and *S. pyogenes*. In summary, the future application of CRISPR/Cas9 for gene therapy has to improve, as CRISPR/Cas9 gene editing system has been shown powerful to target and eliminate viral infection associated with neurological complication

in vitro and vivo setting. Many research groups has reported the successful application of CRISPR/Cas9 against HBV by direct targeting HBV genome in vivo and vitro (Lin et al. 2014,Seeger and Sohn 2014, Dong et al. 2015, Karimova et al. 2015, Bloom et al. n.d.; Yang and Chen 2018). In different *vitro* cell line models, the CRISPR/Cas9 system targeting HBV core and surface antigen showed significant reduction in protein level in HepG2 (Zhen et al. 2015), HepG2.2.15 (Karimova et al. 2015; Zhen et al. 2015), HepG2-H1.3 (Karimova et al. 2015) and in Huh-7 (Lin et al. 2014; Sakuma et al. 2016) (Table 2). The study by Li and coworkers has shown the ability of CRISPR/Cas9 system to completely excised integrated HBV viral genome (Li et al. 2017). Recently, using AAV vectors as a delivery vehicle for SaCas9 and anti HBV gRNAs in a hydrodynamic in vivo HBV model, it has been reported the reduction of HBV protein without viral level. (Liu et al. 2018). As an alternative approach for AAV delivery, Jiang used lipid like nanoparticle to deliver Cas9 mRNA and gRNA targeting HBV in a hydrodynamic HBV in vivo model to show reduced HBV DNA and viral proteins expression. (Jiang et al. 2017) (Table 2). Although all these studies have shown the effectiveness of CRISPR/Cas9 system eradicating cccDNA in vivo and in vitro model, further studies are warranted if the theoretic application of this technology can effectively target and eliminate multiple copies of cccDNA in infected hepatocytes. Song et al. has reported the anti-cancer agent capability of the CRISPR/Cas9 system against an HBV-induced liver cancer by target disruption of HBsAg that lead to the significant inhibition not only proliferation but also tumorigenicity of HBV positive hepatocellular carcinoma cells (Song et al. 2018). Finally, the development of HBV infection models in vitro and vivo will greatly enhance our understanding of HBV replication, cccDNA biology and immunopathology of HBV which are important factors for basis of developing effective therapy. (Tang et al. 2017).

Herpesvirus Family

The Herpesvirus family includes large dsDNA viruses that cause widespread infection in human and causes different diseases (Xu et al. 2002). One of the common characteristics of these viruses is their ability to persist for long time in the host after primary infection. It is possible that this lifelong persistence in the host may be associated either with low level of productive infection or true latent state.(Griffin 2010). Nucleotide analogs drugs which inhibit DNA polymerase are currently used as antiviral therapy. Although these drugs control the virus replication, they are not able to eliminate the latent infection. Therefore, the most reasonable antiviral approach to achieve a functional cure for Herpes virus infection is direct disruption of herpes viral genome (Lee 2019). Several research groups have used CRISPR/Cas9 as agent to inhibit herpesvirus infections in vitro (Russell et al. 2015; Roehm et al. 2016; Van Diemen et al. 2016).

Herpes Simplex Virus Type 1 (HSV-1)

Herpes Simplex virus type 1 (HSV-1) is an alpha herpes dsDNA virus with more than 151Kbp and it infects 65% of the US population,.(Wald and Corey 2007), 52–67% in northern Europe (Pebody et al. 2004) and its global prevalence is about 90% (Nicoll et al. 2012) HSV-1 is responsible for some human diseases such as virally induced blindness, HSV-1 associated viral encephalitis and oral ulceration. The periodic reactivation of HSV-1 has been also associated with CNS diseases such as Alzheimer disease (Doll et al. 2019; Bearer 2012 and Itzhaki 2018),Multiple sclerosis (Sanders et al. 1997) and epilepsy (Sanders et al. 1996) HSV-1 is a nuclear replicating enveloped virus, usually acquired through direct contact with infected lesions or body fluid and It is a neurotropic virus with a rapid replication cycle and a wide host and cell range (Arduino and Porter 2008). HSV-1 infection occur through small lesions in epithelium and during the active phase of viral replication; the virus is then transported by retrograde flow along axons to nuclei of sensory neurons (Kennedy and Cullen 2017). There the viral genome remains latent, however viral reactivate under certain physiological conditions such as physical and emotional stress (Whitley and Roizman 2001) to facilitate dissemination. The establishment of HSV-1 latency in neurons occurs without pre-existing viral early product with the help of cellular mechanism and those the cellular factors responsible for viral reactivation mostly unknown. Surprisingly, During HSV-1 latency, there is no exact distribution of viral DNA between latently infected neurons and it has been shown that viral DNA content of infected neurons significantly differ among each other (Sawtell 1997). Although the biological significance of such variation in HSV-1 genome copy number is unclear, it is reasonable to assume that those neurons with high copy number might have advantage for faster latency exit and reactivation. This observation gives a notion that latently infected neurons not only they are not equivalent on their content of HSV-1 genome copy number but also they differ on their reactivation pattern. This observation is confirmed in vivo hyperthermia induced reactivation model in which only 1 in 2700 latently infected neurons reactivate even though the reactivation stimuli affect the whole Trigeminal ganglion (TG).(Thompson et al. 2010). Since HSV-1 is a dsDNA virus, it could be potentially target by CRISPR/Cas9. CRISPR/Cas9 have been applied by our laboratory and others to target HSV-1. Van Diemen et al. has reported that targeting viral essential genes using CRISPR/Cas9 abrogate HSV-1 infection of epithelia and fibroblast cells. However, during this study it has been shown the emerge of escape mutant after prolong incubation times as a result of CRISPR/Cas9 induced InDel mutation at

the target sites. The study by Roehm and others et al. has shown the complete inhibition of virus production and prevention of outgrowth of resistant strains using gRNAs targeting different region of HSV-1 (Roehm et al. 2016; Van Diemen et al. 2016) (Table 2).

Epstein-Barr Virus (EBV)

Epstein-Barr virus (EBV) is a gamma herpesvirus that infects and establishes an asymptomatic latent infection in more than 90% of adult population worldwide (Craddock and Heslop 2008). It does not cause any disease in health carriers due to an effective humoral and cellular immune response to EBV infection. Since its discovery in 1964 as a causative agent for Burkitt lymphoma, EBV infection is associated with 200,000 human malignancies per year (Cohen et al. 2011, Epstein (1964) such as Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and gastric cancer (Raab-Traub 2012; Craddock and Heslop 2008). Currently, there are no drugs approved for clinical use for most of EBV associated malignancies and once latency is established, it is almost impossible or difficult to eradicate EBV genome. (Lee et al. 2000; Whitehurst et al. 2013). Different approaches that suppress or eliminate EBV have shown specific therapeutic effect for some EBV associated tumors. siRNA mediated knock down of EBV essential gene such as EBNA1 has been examined for their anti-EBV effects (Ian et al. 2008; Yuen et al. 2018a, b and Hong et al. 2006). Thompson et al. 2010 group has also shown that the use of small molecular inhibitor against EBNA1 suppress EBV replication. Finally, Adoptive cellular therapy based on T cells targeting EBV derived tumor antigen has shown encouraging benefits in the treatment of EBV tumors.(Craddock and Heslop 2008). EBV latently infected cells produces less than 15 transcript from 5 to 100 copies of episomal DNA (Yuen et al. 2018a, b) while the lytic replication of EBV produces more than 100 EBV proteins. Therefore, it is highly likely that the newly develop gene editing approach based on CRISPR/Cas9 can effectively applied to eliminate latent EBV genome from infected cells.

Several studies have applied CRISPR/Cas9 as anti-viral agent to abrogate EBV infection in cell culture in vitro. Van Diemen et al. .and others reported the successful elimination of the latent EBV genome from infected cells (Van Diemen et al. 2016; Wang et al. 2016b; Yuen et al. 2017; Yuen et al. 2018a, b) (Table 2).

Human Cytomegalovirus (HCMV)

Human Cytomegalovirus is beta a double-stranded DNA virus that cause morbidity in immunocompromised individuals such as those with HIV/AIDS, newborn and organ transplant recipient (Azevedo et al. 2015; Revello et al. 2006). CMV infection during delivery cause birth defects in newborn and is the main cause of blindness, deafness and mental retardation in children (Damato and Winnen 2002).HCMV like other members of herpes viruses undergo viral latency state in which the viral genome is maintained in infected cells with no apparent production of infectious virions.. It is highly likely that HCMV reactivate from latency occur periodically in health virus carriers with no pathological complications due to effective host immune responses. (Stern et al. 2019). By contrast, HCMV reactivation from latency in an immune suppressed or compromised individual is the main causes of morbidity and mortality (Drew 1988; Rubin 1990 and Stern et al. 2019). The mechanism that govern HCMV latency and reactivation and the cellular site involved in it has been ongoing studies for many years and are not still well characterized. Different cell lines are reported to support productive HCMV infection (Sinzger et al. 2008), However, HCMV latency is limited to CD34+ progenitors, CD33+ myeloid progenitors cells and CD14+ monocytes with Surface marker B7-H4 (Reeves et al. 2005; Zhu et al. 2018 and Stern et al. 2019). Taken Together, HMCV reactivation is linked to myeloid cell differentiation and cellular differentiation is critical for reactivation as it is proven in both experimental and in vivo latency system (Reeves et al. 2005). The currently available treatment for CMV is nucleoside analogues which are associated with toxicity and development of drug strain resistance. (Biron et al. 1986). Van Diemen et al. has reported efficient application of multiplex CRISPR/Cas9/gRNA approach to limit productive infection of CMV in human cell lines.. However; in the same study, it has been reported that targeting CMV with a single gRNA led to the development of escape mutant after extended replication time (Van Diemen et al. 2016) (Table 2). Gergen et al. 2018 has demonstrated the successful inhibition of HCMV replication by using the multiplex CRISPR/Cas9 approach targeting viral essential genes such as UL122/123.

Human Papillomaviruses (HPVs)

Human papillomaviruses are non-enveloped small dsDNA viruses that infect cutaneous or mucosal epithelial cells, genital tissue and upper respiratory tract. The virus particle with icosahedral capsid symmetry has a diameter of 60 nm and contains double stranded circular DNA molecule of 8000 base pair. One of the main characteristic of HPV molecular biology is the dependence of their life cycle on differentiation status of the epithelial cells. With 14 million new cases each year and with the prevalence of 70 million cases, The anogenital tract infection by human papillomavirus (HPV) is the most common sexually transmitted diseases in the USA. (Harden and Munger 2017) At present, there are 200 genetically distinct subtypes of HPV and roughly 90 genotypes have been identified (Zhen and Li 2017). The high-risk (HR) HPVs are the main cause of cervical cancer which is the second most common cause of cancer-related death in woman worldwide

(Kennedy and Cullen 2017; Yoshiba et al. 2019; Harden and Munger 2017). HR HPV infection is also associated with 95% of anal cancers, 70% of oropharyngeal cancers, 60% of vaginal cancers (Chaturvedi et al. 2011; Gillison et al. 2008). In most of HPV mediated cancers,. there is no productive infection of HPV. Although the current available vaccines are safe and effective in preventing HPV infection, they do not give protection for those who are already infected and they do not give protective immunity against all HPV genotypes. Many progress have been achieved in studying the biology of papillomaviruses, but there is no clear evidence showing whether the human papillomaviruses infection is associated with latency (Gravitt 2012). However, according to animal model study based on rabbit oral papilloma infection, it has been shown that HPV infected basal epithelial cells with no terminal differentiation serve as HPV latent reservoirs (Maglennon et al. 2011). Non-productive infection is the hall mark of most of HR-HPV associated cancers and the integration of the viral genome in premalignant lesion is the main cause of deregulated viral genes expression viral oncoproteins E6 and E7 (Harden and Munger 2017). Despite the progress made in different treatments for HPV, there is no effective treatment approach for HPV-associated carcinogenesis (Zhen and Li 2017). Therefore targeted knock out t viral oncoproteins with CRISPR/cas9 together with the current available anti-cancer drug might be an effective treatment for HR-HPV associated cancer. Kennedy and colleagues have demonstrated intratumoral administration of CRISPR/Cas9 targeting E6 and E7 genes of HPV to induce cleavage of the HPV genome that results in the introduction of inactivating InDel mutations (Kennedy and Cullen 2015). This is associated with the induction of proteins p53 or pRb, leading to cell cycle arrest and cell death. The CRISPR/ Cas9 targeting E6 and E7 oncogenes of HPV16 have been used in combination with Cisplatin in vitro and in vivo as an effective therapy for cervical cancer. (Zhen et al. 2016). Recently study by Yoshiba et al. demonstrated that targeting E6 in high risk HPV with CRISPR/Cas9 in vitro and in vivo animal model could be an alternative approach for the treatment of patients with cervical cancer (Yoshiba et al. 2019) (Table 2).

CRISPR/Cas9 as RNA-Guided RNA Editing System to Target RNA Viruses

Most of human pathogenic RNA viruses have no dsDNA intermediate throughout their life cycle therefore they cannot be targeted by canonical CRISPR/Cas9. The report from Doudna and colleagues indicated that SpCas9 can be wired to target and cleave RNA (O'Connell et al. 2014). The delivery of a PAMmer which is a short exogenous oligonucleotide capable of serving as canonical double-stranded PAM sequence together with SpCas9 and a gRNA lead to sequence dependent cleavage of ssRNA. (O'Connell et al. 2014). The first RNA editing nucleases to discover was FnCas9 from *Francisella novicida* (Hirano et al. 2016; Green and Hu 2017) and c2c2 from *Leptotrichia wadei* (Abudayyeh et al. 2016; Green and Hu 2017). The first report of reprogramming FnCas9 to target human pathogenic RNA virus was that of HCV, a ssRNA virus with positive polarity. Price and colleagues report FnCas9 targeting 5'-UTR and 3'-UTR of HCV ssRNA genome can effectively protect hepatocellular carcinoma-derived cells from the sequent infection of HCV (Price et al. 2015). C2c2 was characterized as a single-component programmable CRISPR/Cas which utilize a CrRNA as a guide to target RNA. (Abudayyeh et al. 2016) (Table 2).

Viral Escape during CRISPR-Cas9 Application

CRISPR/Cas9 system has been extensively studied as antiviral strategy against HIV and other viral infections (Bella et al. 2018; Ebina et al. 2013; Hu et al. 2014a, b; Kaminski et al. 2016a, b; Liao et al. 2015; Yin et al. 2017). HIV is one of the potential target that replicate as integrated proviral DNA (Mefferd et al. 2018). Several studies have previously shown that CRISPR/Cas9 can target and inactivate the latent HIV-1 provirus found in resting memory T cells in patients on antiretroviral Therapy with no detectable replicating virus (Ebina et al. 2013; Hu et al. 2014a, b; Kaminski et al. 2016a, b; Wang et al. 2016a). The CRISPR/Cas9 application has been also used to protect T cells from de novo infection by HIV-1(Hu et al. 2014a, 2014b; Kaminski et al. 2016a; Lebbink et al. 2017). Despite all these promising application of the CRISPR/Cas9 system, reports from different research groups showed that HIV can develop resistance due to mutations generated during NHEJ if unique site is targeted by gRNA (Ueda et al. 2016; Wang et al. 2016a, b, c; Yoder and Bundschuh 2016). Different strategies have been proposed to prevent viral escape such as the use of multiplex gRNAs to target simultaneously multiple sites in the genome so that the chance of generating viable escape mutant would be significantly reduced. Another alternative strategy is combinatory approach using the CRISPR/Cas9 therapy with antiviral drugs and RNA interference (RNAi) or short hairpin RNA (shRNA) molecules (Herrera-Carrillo and Berkhout 2016; Wang et al. 2018a;). The inhibition of the NHEJ DNA repair pathway which is the main source of viral escape using anticancer drug such as ScR7 may reduce virus escape. (Chu et al. 2015; Maruyama et al. 2015; Wang et al. 2018a; Srivastava et al. 2012; Singh et al. 2015). one elegant strategy is to employ novel Cas-9-like nucleases such as Cpf1 which cleave the target site distal from PAM so that the targeted site is less important for gRNA binding and subsequently there will be no or significantly reduced gRNA resistance and that makes viral escape more difficult (Zetsche et al. 2015; Yamano et al.

2016; Wang et al. 2018a). Viral escape can be significantly reduced by employing RNAi or antiviral agent to inhibit viral replication so that the efficiency of CRISPR/Cas9 system can increase (White et al. 2016). Study by Ali et al. has shown that targeting noncoding, intergenic sequence is associated with viral interference activity that considerable limit creation of viral escape mutant. (Ali et al. 2016) (Figs. 2 and 3).

Methods of CRISPR/Cas9 Delivery

Though CRISPR/Cas9 has revolutionize genome editing application, there are still some challenge to overcome before their clinical usage. One of the greatest obstacles is effective delivery of CRISPR/Cas9 components both in vitro and in vivo. CRISPR/Cas9 components can be delivered in different forms such as mRNA, or as plasmid DNA encoding Cas9 or Cas9 protein. Each of these therapeutic approaches has their own advantage and disadvantage. Delivery in the forms of mRNA has advantage compare to the other forms because of small size and it can be easily packaged. The other benefit of mRNA delivery is faster gene editing activity, and control on the amount of delivery to the cells. Although plasmid-based delivery has major limitation because of the size and off target editing, they have advantage regarding stability and the flexibility in designing. There are different and well characterized delivery methods available for CRISPR/Cas9 machinery such as viral and non-viral delivery (Lino et al. 2018). Non-viral approaches such as electroporation (Matano et al. 2015; Qin et al. 2015; Zuckermann et al. 2015; Chen et al. 2016), microinjection (Yang and Chen 2018; Horii et al. 2014; Raveux et al. 2017; Crispo et al. 2015) and lipid nanoparticles (Sakuma et al. 2014; Zuris et al. 2015; Wang et al. 2016c; Yin et al. 2016) have been used for generating knock out cell lines and animal models but their therapeutic application in vivo is very limited due to their relatively poor delivery efficiency. On the other hand, viral delivery methods are by far the most successful and efficient system to deliver plasmid-based CRISPR/Cas9 in vitro and in vivo (Table 3) (Bak et al. 2018). The most used viral vectors in therapeutic approach are adeno-associated virus (AAV) (Yang and Chen 2018; Carroll et al. 2016; Hung et al. 2016; Long et al. 2016; Nelson et al. 2016; Tabebordbar et al. 2016), adenovirus (Cheng et al. 2014; Maddalo et al. 2014; Li et al. 2015; Maggio et al. 2016; Voets et al. 2017) and lentivirus (Platt et al. 2014; Shalem et al. 2014; Wang et al. 2014; Roehm et al. 2016; Zhang et al. 2016) (Table 3). Because of their wide range of serotype specificity and relatively low immunogenicity and their ability to infect dividing and non-dividing cells, AAV are most widely used viral vectors. The limitation of the packaging capacity ca 4.7Kbp is the main challenge for AAV mediated CRISPR/Cas9 delivery. One solution to this limitation is the use of the small version of Cas9 from *Staphylococcus aureus* (SaCas9), which has similar gene editing efficiency as SpCas9 but smaller size. The other alternative is the application of dual AAVs to deliver separately Cas9-encoding DNA and sgRNA. Such an approaches is used by Swiech and colleagues to disrupt a single gene (Mecp2) or multiple genes (Dnmt1, Dnmt3a and Dnmt3b) in the mouse brain via stereotatic injection (Swiech et al. 2015). Another viral delivery vector used in gene therapy is lentivirus. The lentivirus delivery has the advantage for its high infection efficiency both in dividing and non-dividing cells. This advantage is very important for gene modification in tissue such as the liver, brain and muscle (Liu et al. 2017).

Future Directions

Although CRISPR Cas9 system has tremendous potential for human genomic engineering application in vivo and in vitro, there are certain questions has to be addressed before its clinical use. One of the main concerns is the off-target effect of the system (Fu et al. 2013; Hsu et al. 2014; Wu et al. 2014; D'Agostino and D'Aniello 2017). A number of factors such as Cas9 expression level, target sequence and quantification

Table 3 Viral –based CRISPR/Cas9 delivery system used in vitro and vivo

Virus type	Adeno-associated virus (AAV)	Adenovirus	Lentivirus
Packaging capacity	4.8 kb	>37 kb	8.5-9 kb
Integration possibility	No	No	yes
Infection ability	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells
Gene expression	Transient	Transient	Stable
Transduction efficiency	high	Very high	high
Immune Response	Very low	High	low
Limitation	Small Packaging capacity and high cost of viral production process	Need of helper virus for viral replication	High possibility of Random integration
Relative transduction efficiency	70%	100%	70%

methods determine off-target cleavage rate of Cas9 (Hsu et al. 2013; Tsai et al. 2015). It has been reported that off target mutations are sometimes appear at high frequency rate than the targeted sequence (D'Agostino and D'Aniello 2017). Although Cas9 nickases and mutant versions are applied to reduce the off-target effect, our understanding the mechanism of off-target is still poor (Fig. 3). In addition to off-target, CRISPR/Cas9 –mediated mosaic mutations are major concern regarding the future gene therapy approach by CRISPR/Cas9. These mutations are likely arisen from random DNA break and repair. Moreover, the mechanism of viral DNA repair, mutation and recombination events in the host cell after cleavage by CRISPR/Cas9 remain to be examined (Zaidi et al. 2016). As a result, the future direction has to focus on developing reliable new and more sensitive method to increase the Cas9 specificity (i.e. the propensity to induce off-targets and mosaicism) (Le Rhun et al. 2019; Yan et al. 2019). For The successful application of CRISPR/Cas system in gene therapy, the new research strategy has to focus on improving the incidence and efficiency of site–specific nuclease especially on HDR-mediated genome editing which is important for the gene substitution or knock in with target specific sequence (Lino et al. 2018; Le Rhun et al. 2019). The future therapeutic effect of the CRISPR/Cas9 system largely depend on the delivery of the CRISPR/Cas9 components to the target cells. The current available delivery system are not specific, efficient and have biosafety concern. Therefore, it is essential and urgent to develop safe and effective methods of delivery (Chen et al. 2018). Another major concern regarding future application of CRISPR Cas9 system is the recent discovery of the presence of preexisting adaptive immune response in humans for a variety of Cas9 orthologs such as *S. aureus* and *S. pyogenes*. Although preexisting adaptive immune response to Cas9 may not be a major problem for the ex vivo application, this may hinder successful application of the system in vivo delivery due to the safety and toxicity. (Charlesworth et al. 2019) (Fig. 3).

In summary, the future application of CRISPR/Cas9 for gene therapy needs substantial improvement although CRISPR/Cas9 gene editing system has been proven powerful enough to target and eliminate viral infection associated with neurological complication.

Acknowledgements We thank past and present members of the Center for Neurovirology for their insightful discussion and sharing of ideas. This work was supported by seed money from Temple University awarded to HSW.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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