REVIEW

A short overview of CRISPR-Cas technology and its application in viral disease control

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Abstract Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) together with CRISPR-associated (Cas) proteins have catalysed a revolution in genetic engineering. Native CRISPR-Cas systems exist in many bacteria and archaea where they provide an adaptive immune response through sequence-specific degradation of an invading pathogen's genome. This system has been reconfigured for use in genome editing, drug development, gene expression regulation, diagnostics, the prevention and treatment of cancers, and the treatment of genetic and infectious diseases. In recent years, CRISPR-Cas systems have been used in the diagnosis and control of viral diseases, for example, CRISPR-Cas12/13 coupled with new amplification techniques to improve the specificity of sequence-specific fluorescent probe detection. Importantly, CRISPR applications are both sensitive and specific and usually only require commonly available lab equipment. Unlike the canonical Cas9 which is guided to double-stranded DNA sites of interest, Cas13 systems target RNA sequences and thus can be employed in strategies directed against RNA viruses or for transcriptional silencing. Many challenges remain for these approach, including issues with specificity and the requirement for better mammalian delivery systems. In this review, we summarize the applications of CRISPR-Cas systems in controlling mammalian viral infections. Following necessary improvements, it is expected that CRISPR-Cas

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systems will be used effectively for such applications in the future.

Introduction

Numerous intracellular regulatory and defense mechanisms rely on complementary base-pairing between nucleic acid species for function. In addition to eukaryotic RNA interference (RNAi) and prokaryotic Argonaute-based systems, Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated proteins (CRISPR-Cas) systems utilize nucleic acid base-pairing to suppress or destroy genetic targets (Koonin et al. 2017). These systems are adaptable and retain the memory of sequence targets derived from previous exposure to bacteriophages (Ishino et al. 1987; Mojica et al. 2005; Pourcel et al. 2005). Targeted sequences are retained in the form of spacers of 30–40 base-pairs (bp) each, separated by a series of short palindromic repeats (25–35 bp each). The *cas* genes themselves along with these repeats and spacers form the 'CRISPR array'. In addition, the activity of CRISPR-Cas-mediated defenses requires proteins essential for Cas nuclease activity, the recognition of foreign nucleic acid and their uptake into the CRISPR array, as well as proteins critical for the recognition of the invading mobile genetic elements (MGEs).

CRISPR-Cas systems are divided into two distinct classes based on the structure of their effector complexes; Class 1 systems implement multiple Cas proteins for nucleic acid degradation while Class 2 systems use only one. These classes are further divided into six types and multiple subtypes based on differential Cas protein participation and CRISPR loci architectures: Class 1 systems include Cas types I, III, and IV while class 2 systems comprise types II, V, and VI (Makarova et al. 2018) (Table 1).

The molecular mechanisms of CRISPR-based prokaryotic adaptive immunity can be divided into two distinct processes: (i) immunization or acquistion, (ii) defense or resistance (Fig. 1). A new spacer sequence known as a protospacer is acquired from the foreign genetic elements (Yosef et al. 2012). To be acquired, the protospacer sequence for many CRISPR-Cas system types needs to be located next to a short motif called the protospacer-adjacent motif (PAM). Recognition of this motif serves as a reliable way to avoid the destruction of the host CRISPR array by its own CRISPR-Cas machinery. Cas1 and Cas2 protein activity is crucial for the adaptation stage (Yosef et al. 2012). At the crRNA biogenesis stage, precursor crRNAs (pre-crRNA) are produced by transcription of CRISPR array elements and processed into mature crRNA (Fig. 1). This maturation is performed by Cas proteins and/or cellular ribonucleases depending on the given CRISPR-Cas type (Deltcheva et al. 2011; Reeks et al. 2013). Mature crRNA is incorporated into the Cas protein effector complex. This complex uses the crRNA to interrogate foreign nucleic acids for sequence complementarity. If the requirements for both complementarity and a PAM site are satisfied, the Cas protein nuclease is activated, and the foreign genetic element is specifically targeted and degraded.

Due to its high efficiency, specificity and modular nature, the CRISPR-Cas system has emerged as a revolutionary genetic engineering technology. This technology has accelerated our capacity for genome editing, drug development, control of gene expression, as well as in the diagnostics, prevention and treatment of a broad spectrum of diseases. In this review, we focus on the application of CRISPR-Cas systems to control viral diseases and the challenges faced in that field.

CRISPR-Cas9

Cas9 is the canonical class 2 type-II system nuclease. Since the first successful use of *Streptococcus pyogenes* Cas9 (SpCas9) for eukaryotic genome engineering, the CRISPR-Cas9 system remains the most widely adopted RNA-guided DNA targeting platform for genome editing (Cong et al. 2013; Jinek et al. 2012; Mali et al. 2013b). Cas9 contains two nuclease domains termed RuvC and HNH that function in DNA cleavage, which cut the target and non-target strands of DNA, respectively. Cas9 is guided by a trans-activating-crRNA (tracrRNAs)/crRNA complex (dual RNA hybrid), or an engineered chimeric form of this complex known as a single guide RNA (sgRNA) (Jinek et al. 2012). Upon recognition of the target DNA, Cas9 generates a double-strand break (DSB) upstream of the 5′-NGG PAM site (Deltcheva et al.

Table 1 Characteristics of CRISPR-Cas systems

Class	Type/Subtype	Effector	Nuclease domains	Target	Cut structure	tracrRNA requirement	PAM/PFS	Application
1	I	Cascade	HD nuclease domain of Cas3	DNA	Single-strand cut (200-300nt)	NO	PAM	Genome editing, antimicrobials, gene regulation in bacteria and archaea
	III	Csm/Cmr complex	Cas10 PALM domain Cas7 Csm/Cmr complex	DNA RNA	Multiple sites	NO	Independent of PAM	Genome engineering and gene silencing
	IV	Complex	HD nuclease domain	DNA	Double strand	NO	PAM	Controlling plasmid propagation
2	II	Cas9	RuvC, HNH	dsDNA RNA	Blunt	Yes	3′ GC-rich PAM	Elimination of repetitive sequences specific Gene editing RNA knockdown RNA isolation (dCas 9) RNA imaging and tracking (dCas 9) Resistance against RNA viruses (Fn Cas9) Regulation of gene expression
	V-A	Cas12a (Cpf1)	RuvC, NUC	dsDNA	Staggered, 5′-overhangs(7nt)	No	5′ AT-rich PAM	Gene editing Nucleic acid detection
	V-B	Cas12b (C2c1)	RuvC	dsDNA	Staggered, 5′overhangs (5nt)	Yes	5′ AT-rich PAM	Nucleic acid detection
	VI-A	Cas13a (C2c2)	2xHEPN domain	ssRNA	Guide-dependent RNA cuts + collateral RNA cleavage	No	3′ PFS: non-G	RNA knockdown RNA imaging and tracking (dCas13a) Nucleic acid detection Resistance against RNA viruses
	VI-B	Cas13b (C2c6)	2xHEPN Domain	ssRNA	Guide-dependent RNA cuts + collateral RNA cleavage	No	5′ PFS: non-C 3′ PFS: NANA/NNA	RNA knockdown RNA editing Regulation of gene expression Nucleic acid detection

dsDNA, double-stranded DNA; ssRNA, single-stranded RNA; PAM, protospacer adjacent motif; PFS, protospacer-flanking sequence

2011; Jiang and Doudna 2017; Strich and Chertow 2019). The programmability of the CRISPR-Cas9 system to target many sites for cleavage is considered its main advantage over zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) for genome editing, technologies which
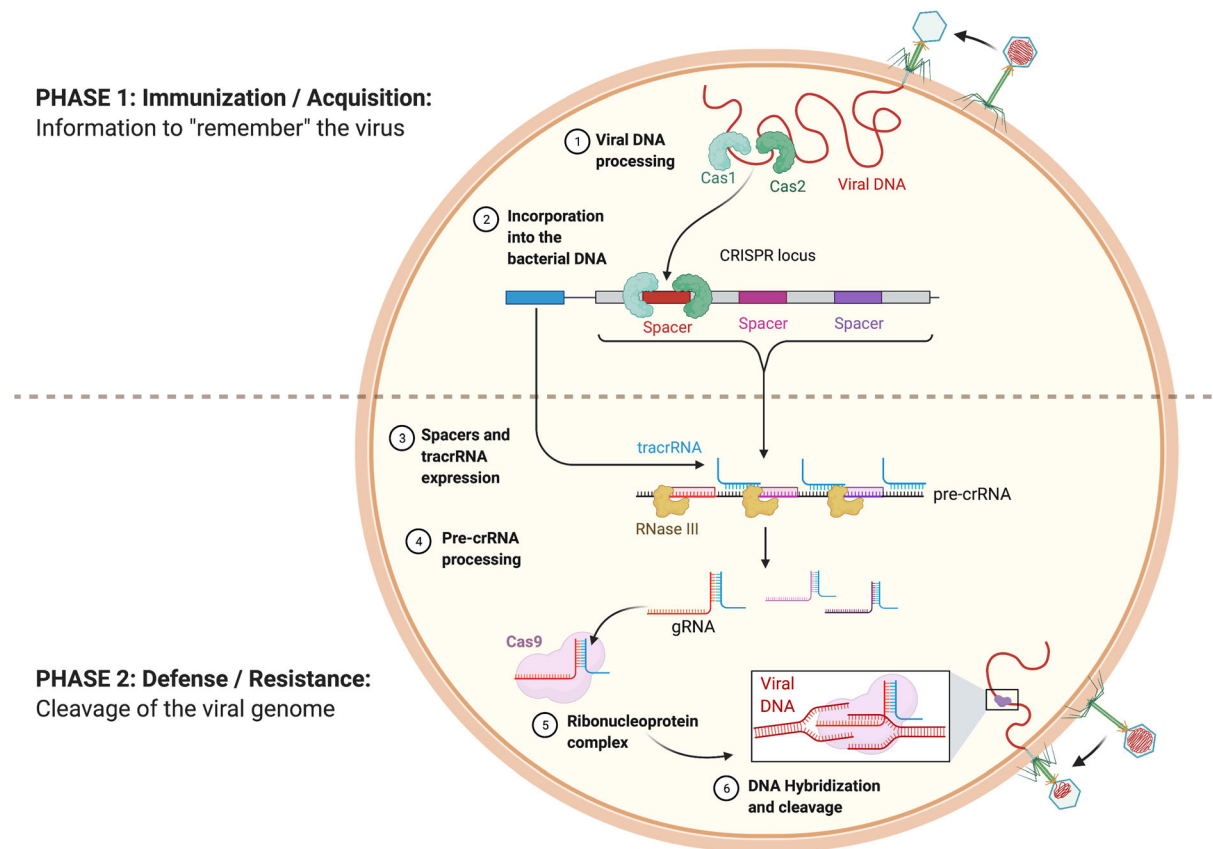
Fig. 1 Bacterial CRISPR-Cas9 systems behave as an adaptive immune response against invading bacteriophages. Following infection (Phase 1), Cas1 and Cas2 mediate incorporation of short sequences of the viral genome as spacers within the bacterial CRISPR locus. At re-exposure (Phase 2), the CRISPR locus gets expressed as pre-crRNA, along with tracrRNA. The pre-crRNA is processed to yield guide RNAs (gRNAs) which bind the ribonucleoprotein Cas9 and target this complex to complementary sequences of the infiltrating bacteriophage genome, prompting its Cas9-mediated cleavage. Reprinted from "CRISPR-Cas9 Adaptive Immune System of *Streptococcus pyogenes* Against Bacteriophages", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

were based on protein–DNA recognition (Doetschman and Georgieva 2017; Jiang and Doudna 2017). Repair of the targeted DSB and edits to the target gene are mediated by either the error-prone nonhomologous end-joining (NHEJ) or precise homology-directed repair (HDR) pathways, forming the basis of most CRISPR-Cas9 gene-editing strategies (Kim et al. 2011; Leenay et al. 2019).

Many recent studies have aimed to improve the efficiency and specificity of CRISPR-Cas9 genome editing as clinical applications of the current systems are impossible if on-target editing efficiencies are too low and non-specific off-target editing rates are too high. For one, the SpCas9 PAM requirements limit the number of targetable sites. Various strategies have been employed to expand Cas9 targeting scope, including the evolution of Cas9-derived variants with altered PAM requirements and the identification of Cas9 orthologs from other species (Chen et al. 2017; Hsu et al. 2014; Karvelis et al. 2017; Nishimasu et al. 2018).

Another strategy utilizes paired Cas9 nickases to improve site-specificity and decrease off-target DSB induction (Mali et al. 2013a; Ran et al. 2013). In this strategy, two Cas9 nickases (Cas9n) variants (D10A and H840A, respectively) are delivered together and guided to offset targets by individual sgRNAs, producing site-specific nicks on opposing strands of the DNA helix and inducing the NHEJ repair pathway. This design is proposed to reduce off-target editing activity as the repair of single-strand nicks are

predominantly regulated by the high-fidelity base excision repair pathway.

The first SpCas9 mutants reported to target altered PAM sites with improved activity were SpCas9-EQR, SpCas9-VQR and, SpCas9-VRER (Kleinstiver et al. 2015). The recognition mechanisms of the altered PAM sites in these new systems were not determined, however. High-resolution crystal structures of the three SpCas9 variants in complex with a sgRNA and their target DNA identified an unexpected change in the phosphodiester backbone of the PAM duplex (induced by multiple mutations), which was proposed to be responsible for their altered PAM specificities (Hirano et al. 2016). In addition, several enhanced fidelity variants of SpCas9 such as eSpCas9 (1.1), SpCas9-HF1, and HeFSpCas9 were produced by rational design to increase on-target editing activity and improve intrinsic SpCas9 specificity (Chen et al. 2017; Kleinstiver et al. 2016; Kulcsár et al. 2017). SpCas9-NG and xCas9 are further Cas9 variants with the potential of broadening CRISPR–Cas9 PAM compatibility (Kim et al. 2020).

CRISPR–Cas12

The defining feature of Cas12, the prototypical type V Cas protein, is its capacity to cleave double-stranded DNA (dsDNA) in a staggered fashion near a 5′ PAM (TTTV) site generating 5- to 7-nucleotide overhangs. Target DNA cleavage is mediated by the single RuvC-like nuclease domain of Cas12. Unlike Cas9, Cas12a (also referred to as Cpf1) mediates pre-crRNA processing through auto-processing of its own crRNA array (Kleinstiver et al. 2019; Li et al. 2018a; Shmakov et al. 2015; Zetsche et al. 2015). This unique feature of the CRISPR–Cas12a platform is exploited in the design of simplified systems for simultaneous editing of multiple genes in vivo (Zetsche et al. 2017). Cas12a orthologs such as Cas12f have been shown to predominantly target ssDNA molecules while others such as Cas12i mainly cleave dsDNA targets (Harrington et al. 2018; Shmakov et al. 2015).

Beyond Cas9 and Cas12a effectors, Cas12b is another system potentially useful for genome editing in mammalian cells (Strecker et al. 2019; Teng et al. 2018). The native *Alicyclobacillus acidoterrestris*-derived Cas12b (AacCas12b) is considered unsuitable for use in mammalian genome editing

applications because of the high temperatures required for nuclease activity (Shmakov et al. 2015). An engineered variant of AacCas12b that possesses nuclease activity across a wide temperature range, however, overcomes this limitation; directed by a chimeric sgRNA, this variant was demonstrated to successfully achieve genome editing in both a mammalian cell line and mice (Teng et al. 2018). The wild-type *Bacillus hisashii*-derived Cas12b (BhCas12b), though active at lower temperatures, cannot cleave target strands and in its native architecture cannot be utilized as an effective tool for genome editing. An engineered form of BhCas12b could be a promising addition to the toolkit for efficient genome editing in human cells (Strecker et al. 2019).

CRISPR–Cas13

Cas13 proteins (comprising subtypes A, B, C and D) are class 2 type VI nucleases. Type IV nucleases are programmable single-effector RNA-guided nucleases that specifically target and cleave RNA molecules. Precise RNA cleavage of single-stranded RNA (ssRNA) by Cas13 effectors is mediated by the function of dual higher eukaryotes and prokaryotes nucleotide-binding domains (HEPNs). These domains require recognition of a protospacer flanking sequence (PFS) at the 3′ end of the crRNA binding site for Cas13a and on both 5′ and 3′ ends for Cas13b.

The programmability of Cas13 effectors is promising for the development of a precise and flexible RNA editing platform (Abudayyeh et al. 2016; Cox et al. 2017; Gootenberg et al. 2017; Shmakov et al. 2015, 2017). For instance, the Cas13b ortholog from *Prevotella sp. P5-125* (PspCas13b) was identified as an efficient tool for RNA knockdown activity in mammalian cells (Cox et al. 2017). The fusion of PspCas13b to a variant of the adenosine deaminase acting on RNA (ADAR) protein family was able to facilitate editing of transcripts, providing a platform for efficient and specific RNA editing and repair of disease-associated mutations in the human cell. The RNA targetability of other Cas13 variants (e.g. Cas 13a and Cas13d) has been successfully applied as a mammalian knockdown platform with improved specificity over RNAi (Abudayyeh et al. 2017; Konermann et al. 2018). The functional diversity among Cas13 subtypes and the low potential of off-target

edits anticipates the expansion of RNA-targeting CRISPR-Cas systems into other fields, including their clinical translation to applications when DNA-targeting platforms are inappropriate.

Control of viral diseases using CRISPR-Cas systems

Cas9 systems

There are two ways in which CRISPR-Cas9 systems can be applied to the control of viral disease. Editing of mammalian genes relevant to the disease response is one approach, though many studies describe the capacity of CRISPR-Cas effectors to defend mammalian cells against DNA or RNA virus infection directly. Cas9 has demonstrated promising results in targeting both dsDNA viruses and ssRNA viruses with DNA intermediates (Liu et al. 2018; Ophinni et al. 2018; Wang et al. 2018b).

Human diseases

Human immunodeficiency virus (HIV)

HIV is an ssRNA retrovirus that infects human CD4$^+$ T-cells, macrophages and dendritic cells. Active infection results in a profound reduction of CD4$^+$ T-cells which can lead to a loss of cell-mediated immunity and increased risk of opportunistic infections (Johnson et al. 2013). Though antiretroviral treatment is successful in reducing the viral burden to undetectable levels, life-long treatment must be maintained. Following primary infection, the HIV genome is permanently integrated into the human genome as a DNA provirus. This provirus can be actively transcribed to produce progeny virus or remain dormant in a latent state. The latently infected cell can serve as a permanent viral reservoir which can reactivate upon cessation of antiretroviral treatment (Johnson et al. 2013).

CRISPR–Cas9 technology is a promising approach for inactivation of the integrated provirus or for preventing primary infection of naïve cells. The HIV long terminal repeats (LTRs), present at both 5′ and 3′ ends of the proviral DNA and serves as the binding site(s) for transcription factors, are attractive targets for CRISPR-Cas9-based therapeutic approaches.

Using such a strategy, Hu et al. induced either complete excision of the integrated viral sequence or a fragment between two target sites within the same LTR in a latently infected cell line (Hu et al. 2014). Subsequent studies have detailed the efficiency of the CRISPR-Cas9 system in targeted cleavage of various regions of the proviral DNA (Kaminski et al. 2016b; Lebbink et al. 2017; Liao et al. 2015; Wang et al. 2016). In a study by Ophinni et al., lentiviral delivery of CRISPR-Cas9 constructs targeting the HIV-1 regulatory genes *tat* and *rev* suppressed their expression and successfully inhibited HIV-1 replication in both persistently and latently infected human CD4$^+$ T-cell lines (Ophinni et al. 2018). Moreover, delivery of a recombinant adeno-associated viral (AAV) vector carrying *Staphylococcus aureus* Cas9 (SaCas9) and gRNAs targeting both LTR and *gag* sequences achieved effective cleavage of HIV-1 proviral DNA in transgenic mice and rats (Kaminski et al. 2016a). Further, the efficacy and function of CRISPR-Cas technologies have also been evaluated in humanized mouse models (Bella et al. 2018; Dash et al. 2019; Yin et al. 2017). Bella et al. demonstrated that treatment with lentiviral vectors expressing Cas9 and multiple HIV-targeted gRNAs abolished HIV proviral DNA from infected human peripheral blood mononuclear cells (PBMC) in humanized mice (Bella et al. 2018).

CRISPR-Cas9 can also target host genes critical for the HIV life-cycle. Dufour et al. used the CRISPR-Cas9 system to edit the human restriction factor TRIM5 to recognize the HIV capsid and inhibit active infection (Dufour et al. 2018). In another study, D366N mutation of the PSIP1 gene encoding host cell protein Lens Epithelial Derived Growth Factor p75 (LEDGF/p75) disrupted the integration of the HIV provirus into the host chromosome but preserved the cellular function of LEDGF/p75 (Lampi et al. 2019). In addition, the application of SpCas9 and U-modified tracrRNA in the ribonucleoprotein complex (RNP) delivery format was found to improve indel formation in LTRs and could reduce CCR5 surface receptor expression in primary CD4$^+$ T-cells (Scott et al. 2019; Xu et al. 2019)Together, these results highlight the possibilities for CRISPR-Cas9 editing systems in achieving the long-sought-after functional HIV cure.

Hepatitis B virus (HBV)

Of the *Hepadnaviridae* family, HBV causes acute and chronic liver infections and though an effective HBV vaccine exists, curing chronic infections remains a challenge. The circular HBV DNA genome is partially double-stranded, with a short sense strand and a longer anti-sense strand. The episomal covalently closed circular DNA (cccDNA) form of the HBV genome functions as an important factor in the maintenance of HBV persistence and the inability to clear the virus (Arzumanyan et al. 2013; Dandri and Petersen 2016). Multiple in vitro and in vivo studies have shown that achieving a complete HBV cure may be possible via CRISPR-Cas gene editing (Li et al. 2017; Liu et al. 2018; Scott et al. 2017; Wang et al. 2015). Application of SpCas9 with sgRNAs targeting the HBV reverse transcriptase (RT), surface antigen or core protein successfully induced the degradation of HBV DNA and depleted cccDNA levels in chronic and de novo HBV in vitro cell models (HepAD38 and HepaRG cells, respectively) (Kennedy et al. 2015). CRISPR-Cas9 HBV-targeting in liver cell lines Huh-7 (Lin et al. 2014), HepG2 (Zhen et al. 2015), HepG2.2.15 (Karimova et al. 2015; Zhen et al. 2015), HepG2-H1.3 (Karimova et al. 2015), and HepG2.A64 (Li et al. 2017) all elicited a reduction in viral replication and disruption in cccDNA.

A 2015 study found that a combination of SpCas9 and eight gRNAs targeting the conserved regions of different HBV genotypes significantly inhibited HBV replication both in vitro and in vivo (Liu et al. 2015). Liu et al. have reported efficient inhibition of HBV replication by AAV9-delivered SaCas9/gRNA in a mouse model of chronic HBV infection (Liu et al. 2018). In a similar study, recombinant AAV8-packaged CRISPR-SaCas9 was used to inhibit HBV expression in HBV transgenic mice: HBV surface antigen (HBsAg) and HBV antigen (HBeAg) serum levels significantly decreased following treatment (Li et al. 2018b). Moreover, base editing, which uses the SpCas9-BE (SpCas9-base editor) to introduce nonsense point mutations, could successfully inhibit viral gene expression in an in vitro HBV infection model (Yang et al. 2020).

Though these in vivo and in vitro studies have shown the potential of CRISPR-Cas9 in the elimination of HBV infection, further studies are needed to improve our understanding of cccDNA biology and HBV replication in order to develop effective editing systems that could be used to eradicate HBV infection.

Herpesviruses (HSV-1, EBV, HCMV)

Viruses of the Herpesviridae family (to which Herpes simplex virus type-1 (HSV-1), Epstein-Barr virus (EBV), and human cytomegalovirus (HCMV) belong) are dsDNA viruses characterised by their ability to establish life-long infections in the host. HSV-1 is the prototypical human Alphaherpesvirus and is the causative agent of herpes labialis, herpes keratitis and more severe sequelae such as viral encephalitis and neonatal herpes. Current antiviral drugs are ineffective against latent infection of HSV-1, especially in the trigeminal ganglion (TG) neurons. Many recent studies have approached this difficult virus through CRISPR-Cas9 gene editing approaches. Chen et al. attempted to inhibit HSV-1 replication in African green monkey Vero cells and cultured primary TG neurons using SpCas9 and SaCas9 systems targeting two essential HSV-1 genes (ICP0 and ICP4); demonstrating significant impairment of viral replication in both cell types (Chen et al. 2020). A study by Xu et al. reported CRISPR-Cas9-mediated knockout of the viral UL7 gene diminished genome replication, attenuated neurovirulence and decreased pathologies in mice (Xu et al. 2016). The inhibitory effect of CRISPR-Cas9 systems in HSV-1 infection by targeting different HSV-1 loci has also been reported in other studies (Li et al. 2018c; Roehm et al. 2016; Russell et al. 2015; van Diemen et al. 2016).

EBV is responsible for several human malignancies such as Burkitt's lymphoma and nasopharyngeal carcinoma. During the latent stage of viral infection, the EBV genome is maintained in an episomal form in host B cells and epithelial cells; here, only a limited number of proteins are expressed, namely those involved in the maintenance of the episomal genome (e.g. EBV nuclear antigens-1 (EBNAs-1) and proteins involved in cell transformation and induction of invasion) (Raab-Traub 2012; Yuen et al. 2018b). CRISPR-Cas9 targeting of EBV in Raji cells (a Burkitt's lymphoma cell line) derived from patients with latent EBV infection significantly reduced EBV replication and apoptotic events (Wang and Quake 2014). Similarly, complete clearance of EBV in latently infected lymphoma cells has been achieved by Cas9-targeting of viral EBNA-1 (van Diemen et al.

2016). Yuen et al. successfully deleted 558 bp in the promoter region of *Bam*HI-A rightward transcripts (BART) with CRISPR, which led to decreased levels of miR-Bart3 and reduced viral yields in latently infected EBV models (Yuen et al. 2015). Later application of multiple EBV-targeting gRNAs demonstrated down regulation of EBV DNA loads and lytic replication in latently infected nasopharyngeal carcinoma cells (Yuen et al. 2018a).

Human cytomegalovirus (HCMV) causes severe disease in neonates and immunocompromised individuals. Like all herpesviruses, HCMV can establish latent infection within the host, and reactivation is considered the main cause of HCMV-induced morbidity and mortality in immunocompromised individuals (Damato and Winnen 2002; Stern et al. 2019). Van Diemen et al. report the efficient impairment of HCMV replication by CRISPR-Cas9 gene editing in an in vitro cell model (van Diemen et al. 2016). Further, multiplexed CRISPR-Cas9 targeting of HCMV essential genes UL122/123 in human cells demonstrated successful abrogation of virus replication (Gergen et al. 2018).

While these findings highlight the potential for CRISPR-Cas9 systems in eradicating herpesviruses from the body, again additional in vitro and in vivo studies are needed to prove the capability of the system.

Human Papillomaviruses (HPVs)

HPVs are considered the etiological agents of almost all cervical carcinomas and several other cancers. E6 and E7 antigens are responsible for inducing and maintaining the oncogenic properties of HPV by disrupting the host cell tumor suppressors p53 and retinoblastoma protein (Rb), respectively (Mirabello et al. 2017). Though no effective therapy has been developed for HPV-associated carcinogenesis, CRISPR-Cas9 systems have the potential to provide effective treatment in HPV-associated cancer (Kennedy et al. 2014; Zhen and Li 2017). Cas9 targeting of the E6 or E7 oncogenes in HPV-induced cancer cell lines resulted in cleavage of the HPV genome. Successful inactivation of the oncogenes and increased p53 or pRb expression successfully induced cancer cell death (Kennedy et al. 2014). In a study by Zhen et al., in vitro and in vivo targeting of E6/E7 by Cas9 in combination with the anti-cancer drug

cisplatin provided promising results for the treatment of cervical cancer. Additionally, the potential of CRISPR-Cas9 as a highly specific alternative treatment for patients with cervical cancer was demonstrated by Yoshiba et al.: in in vitro and in vivo animal models of high-risk HPV-positive cervical cancer, transduction of an AAV carrying E6-targeting gRNA (AAV-sgE6) and Cas9 suppressed the growth of cell lines and tumor growth in a mouse model following intratumoral administration of AAV-sgE6 (Yoshiba et al. 2019). Future in vivo studies is needed to confirm the efficiency of CRISPR-Cas9 based approaches in the treatment of HPV-associated malignancy.

Human Polyomavirus 2 (or John Cunningham Virus, JC Virus (JCV))

JCV is a causative agent of progressive multifocal leukoencephalopathy (PML) and belongs to the human polyomavirus family. The small circular dsDNA genome of JCV comprises three regions: the early coding region, which encodes the regulatory T antigens; the late coding region, which encodes the viral capsid proteins (VP1-3); and the non-coding control region (NCCR), which contains the viral promoters and origin of replication (Jiang et al. 2009). Some of the currently available treatments are ineffective against the debilitating disease (Tavazzi et al. 2012). The CRISPR-Cas system has therefore been proposed as an alternative approach. Wollebo et al. introduced mutations in the JCV genome using Cas9 to specifically target the viral DNA sequences encoding the T-antigen, strongly inhibiting viral replication in primary human fetal glial cells (Wollebo et al. 2015). In another study, inhibition of JCV replication by Cas9 targeting of the late coding region and NCCR of the JCV genome was demonstrated in vitro. While in vitro studies are suggestive of the potential of CRISPR-Cas9 applications in the treatment of JCV-mediated PML, in vivo studies are still needed to confirm clinical translatability.

Though these examples demonstrate the power of CRISPR systems to tackle viral diseases of humans, there remain several hurdles on the road to widespread clinical application. Perhaps the most significant of these is reagent delivery to a large enough proportion of target cells in situ to benefit clinical outcome. While ethical considerations surrounding human germline editing can restrict delivery options, similar

constraints do not apply when considering CRISPR approaches to viral disease in other species.

Livestock diseases

Genome editing studies of host genes are possible in livestock, where ethical concerns are lesser and a precedent for cloned organisms has been established.

Classical swine fever virus (CSFV)

Classical swine fever, caused by the classical swine fever virus (CSFV), is one of the most detrimental diseases in the swine livestock industry, responsible for significant economic losses (Xie et al. 2018). A member of the *Flaviviridae* family, CSFV is an enveloped virus that possesses a single-stranded positive-sense RNA genome (Shi et al. 2016). CSFV can be spread both by horizontal and vertical transmission (De Smit 2000). Xie et al. combined CRISPR-Cas9 and RNAi technologies to generate anti-CSFV transgenic pigs with a knock-in of a defined antiviral small hairpin RNA (shRNA) at the porcine ROSA26 (pROSA26) locus. These transgenic pigs were found to effectively limit CSFV replication upon viral challenge and reduce CSFV-associated clinical signs and mortality. Importantly, disease resistance could be stably transmitted to the F1-generation (Xie et al. 2018). A more recent study using a knock-in of the antiviral porcine RSAD2 gene (pRSAD2) at the pROSA26 locus produced a pRSAD2-KI pig that constitutively overexpressed pRSAD2 and reduced CSFV infection (Xie et al. 2020).

Porcine reproductive and respiratory syndrome virus (PRRSV)

Like CSFV, PRRSV causes severe economic losses for the global swine industry. Highly pathogenic PRRSV (HP-PRRSV), originating from a genotype 2 PRRSV, is more virulent than classical PRRSV and further exacerbates the economic impact (Yang et al. 2018). PRRSV exclusively infects monocyte/macrophage cell lines and the macrophage-specific CD163 and CD169 proteins were identified as co-receptors for the virus: CD169 is expressed on the cell surface and CD163 localizes within the internalizing transport vesicles (Calvert et al. 2007; Van Gorp et al. 2008). Using CRISPR-Cas9 gene targeting and somatic cell nuclear transfer (SCNT) technologies, some studies generated CD163 knockout pigs with CRISPR-Cas9 systems that were completely resistant to HP-PRRSV infection (Whitworth et al. 2014, 2016) and Yang et al. generating CD163-knockout pig that achieved similar results (Yang et al. 2018). Other studies generating CD163 knockout pigs with CRISPR-Cas9 systems achieved similar results (Whitworth et al. 2014, 2016). Given that CD163 has a range of important biological functions (Onofre et al. 2009), CRISPR editing strategies to minimize the whole organism effects of CD163 knockout were designed to remove only domain 5 of CD163. These animals were completely resistant to PRRSV infection and retained the biological activities associated with the remaining CD163 functional domains (Burkard et al. 2017, 2018).

Transmissible gastroenteritis coronavirus (TGEV)

TGEV is an acute, rapidly spreading coronavirus disease which infects pigs of all ages. High morbidity and mortality have been reported for piglets less than 1 week old (Doyle and Hutchings 1946). Gene-edited pigs with a knockout of exon 2 of aminopeptidase N, a putative host cell receptor for TGEV, demonstrated resistance to TGEV (Whitworth et al. 2019).

In summary, though CRISPR-Cas9 systems for viral disease control in humans show significant promise, system delivery remains problematic. The production of genome-edited livestock with improved resistance and resilience to viral disease clearly demonstrates the power of CRISPR-Cas9 technology for a subset of viruses, but gaps remain.

Cas 13 systems

CRISPR-Cas13 systems complement Cas9 systems with their ability to target ssRNA viruses. Freije et al. describe the development of a CRISPR-Cas13 technology called CARVER (Cas13-assisted restriction of viral expression and readout), demonstrating an efficient antiviral response against three mammalian ssRNA viruses in cell culture (lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV)

and vesicular stomatitis virus (VSV)) (Freije et al. 2019). This programmable diagnostic and antiviral technology could be applied to a variety of ssRNA viruses.

A prophylactic antiviral CRISPR-Cas13d architecture called PAC-MAN successfully reduced SARS-CoV-2 and IAV infection in human cells (Abbott et al. 2020). This system is a promising tool for the inhibition of broad coronavirus infections, relevant in light of the current COVID-19 pandemic (Abbott et al. 2020). In a recent preprint, Xu et al. describe Cas13e.1, part of a novel family of small type VI CRISPR-Cas systems, which similarly demonstrated high cleavage activity when targeted against SARS-CoV-2 and IAV genomes (Xu et al. 2020). In another study, the PRRSV genome was completely knocked down in cell culture by the use of the CRISPR-Cas13b system (Cui et al. 2020). Cas13 has also been used to inhibit HIV-1 infection by targeting and cleaving HIV-1 RNA (Yin et al. 2020). These findings provide opportunities for more effective prophylactic and therapeutic strategies against existing and emerging viral diseases. Figure 2 descript the degradation of viral genomes using CRISPR-Cas9 and Cas13.

Pathogen detection based on CRISPR-Cas system

Cas9 has formed the basis of the majority of DNA gene editing systems and has been modified to enable the targeting of RNAs in living cells. RCas9 has been developed by incorporation of RNA-specific PAM sequences with complimentary guide RNAs. Catalytically active RCas9 has the potential to be used for diagnostic and therapeutic purposes (Batra et al. 2017; Nelles et al. 2016; O'Connell et al. 2014) (Table 2).

Several simple RNA detection strategies that do not require special instruments have been developed in recent years (Notomi et al. 2000; Piepenburg et al. 2006). Reverse transcription-recombinase polymerase amplification (RT–RPA) and reverse transcription-loop-mediated isothermal amplification (RT-LAMP) are highly sensitive RNA amplification methods that are further enhanced with sequence-specific detection modules like hybridization-based fluorescent-oligonucleotide probes (Becherer et al. 2020; Bhadra et al. 2020). RT-RPA or RT-LAMP methods have since been combined with CRISPR-Cas systems to take advantage of Cas-induced cleavage of bystander nucleic acid probes (Gootenberg et al. 2018) (Fig. 3). Non-specific cleavage of single-stranded fluorescent probes by Cas proteins can reverse reporter quenching and produce detectable signals (Fig. 3). Such assays can also be performed on lateral flow
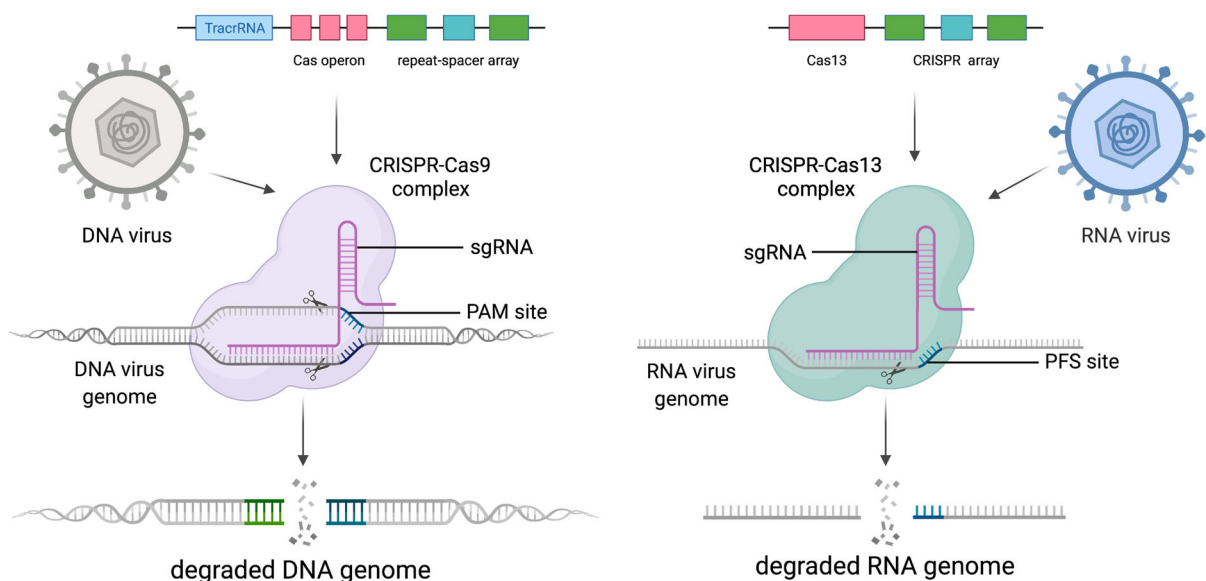
Fig. 2 Schematic of viral nucleic acid degradation using Cas9 and Cas13. Created with **BioRender.com**

Table 2 Nucleic acid detection platforms based on CRISPR-Cas systems

CRISPR-Cas system	Platform	Effector	Nucleic Acid Target	Signal Amplification Method	Fluorescent Signal	Applications	References
Cas13	SHERLOCK	LwCas13a	DNA/RNA	RPA	FAM	Virus detection, human DNA genotyping, cancer mutations	Gootenberg et al. (2017)
	SHERLOCKV2	CcaCas13b PsmCas13b LwaCas13a	DNA/RNA	RPA	FAM,TEX, Cy5,HEX	Multiple analyte detection (up to four targets)	Myhrvold et al. (2018)
	SHERLOCK + HUDSON	LwCas13a	DNA/RNA	RPA	FAM	Identify the virus directly from body fluids	Gootenberg and Abudayyeh (2018)
	CARMEN	Cas13a		PCR or RPA	Fluorescence microscopy	Multiplexed pathogen detection; more than 4,500 crRNA–target pairs on a single array	Ackerman et al. (2020)
Cas 12	DETECTOR	LbCas12a	DNA	RPA	FAM	SNP detection	Liang et al. (2019)
	HOLMES	LbCas12a	DNA/RNA	PCR; RT-PCR	HEX	Virus genotyping and human	Harrington et al. (2018)
	HOLMESv2	AacCas12b	DNA/RNA	LAMP; RT-LAMP; Asymmetric PCR	HEX,FAM	SNP detection	Li et al. (2019)
Cas14	DETECTOR	Cas14a	DNA/RNA	RPA	λex: 485 nm; λem: 535 nm	SNP identification	Aquino-Jarquin (2019)
Cas9	CAS-EXPAR	SpCas9	DNA/RNA	EXPAR	SYBR Green I	SNP genotype discrimination	Huang and Zhou (2018)
	NASBACC	SpCas9	RNA	NASBA	Luciferase	Virus detection	Pardee et al. (2016)
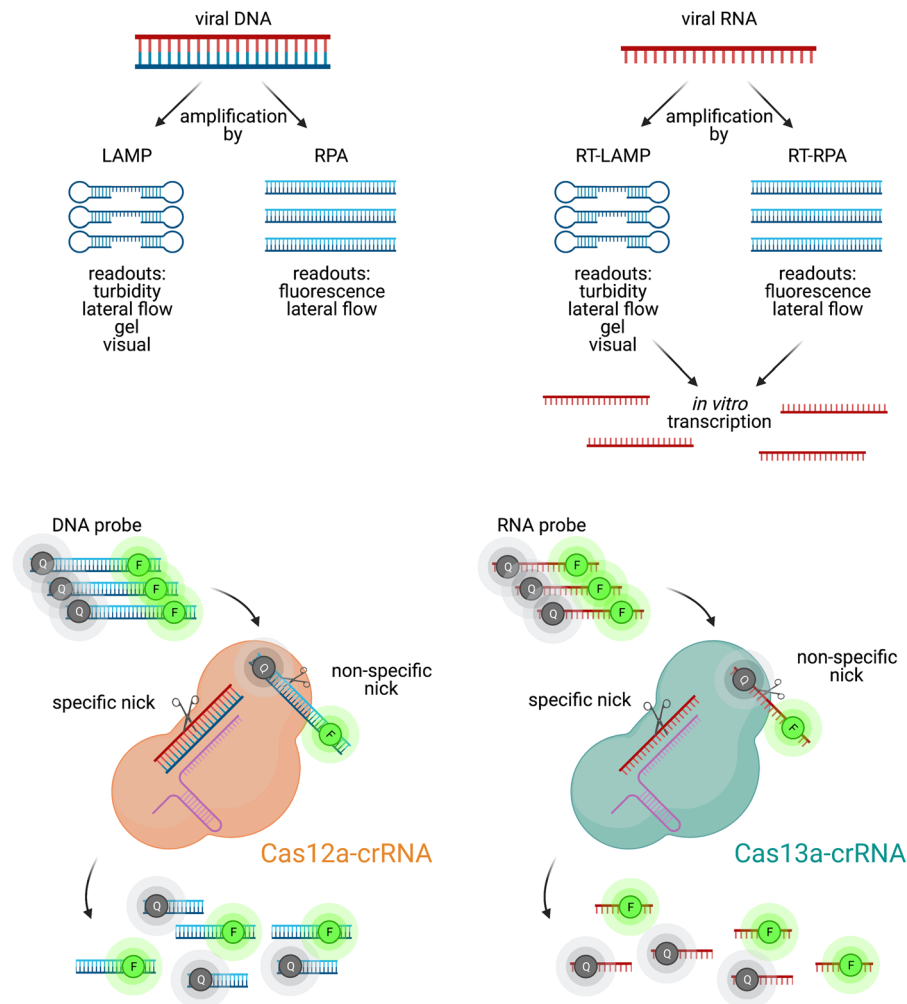
Fig. 3 Schematic of viral nucleic acid detection with CRISPR Cas12 and 13. Nucleic acid targets can be amplified by LAMP/RPA (DNA) and RT-LAMP/RPA (RNA). Cas12- and Cas13-crRNA complexes cleave introduced target-specific fluorescent probes (F) on DNA and RNA, respectively, removing quencher (Q) moieties and producing detectable fluorescent signals. Created with **BioRender.com**

strips, a method much more accessible because of strip portability and a simple visual result readout (Myhr-vold et al. 2018). These CRISPR diagnostic methods are both highly sensitive and specific (Gootenberg et al. 2018; Harrington et al. 2018). For example, a recent preprint by Zhang et al. described a protocol based on the Cas13-based Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) system for detection of SARS-CoV-2, sensitive to as few as two copies of extracted SARS-CoV-2 RNA (Zhang et al. 2020). Other CRISPR-Cas systems including Cas12, Cas13 and Cas14 have also been implemented to detect nucleic acid (Table 2).

These systems have been applied to the detection of SARS-CoV-2, Zika virus, HIV and HPV (Table 3).

Conclusion

CRISPR-Cas systems have shown themselves to be incredibly powerful tools for gene editing, viral knockout, and pathogen detection. Concerns remain, however, for clinical applications of such technologies, requiring off-target edit rates to be dramatically reduced and effective methods of delivery confirmed for in vivo use. CRISPR-Cas9 has been developed

Table 3 Viral disease detection based on CRISPR-Cas systems

Virus	Method	Cas Effector	Targeted Substrate	References
HPV	CtPCR	SpCas9	dsDNA	Wang et al. (2018a)
	CARP (ctPCR2.0)	SpCas9	dsDNA	Zhang et al. (2018a)
	ctPCR3.0	SpCas9	dsDNA	Zhang et al. (2018b)
	DETECTOR	LbCas12a	dsDNA	Chen et al. (2018)
ZIKV	NASBACC	SpCas9	RNA	Pardee et al. (2016)
	LwCas13a	SHERLOCK	dsDNA/RNA	Bhattacharya et al. (2020)
	CcaCas13b	SHERLOCK v2	dsDNA/RNA	Gootenberg and Abudayyeh (2018)
SARS COV-2	Cas13	SHINE	DNA/RNA	Arizti-Sanz et al. (2020)
	AapCas12b	STOPCovid	DNA/RNA	Joung et al. (2020)
	Cas12	iSCANSARSCOV-2	DNA/RNA	Ali et al. (2020)
HIV-1 (as integrated DNA)	AsCas12a	SCAN	DNA	Nouri et al. (2020)

predominantly for DNA editing applications. CRISPR-Cas12 is most commonly utilized in sequence detection kits. Cas13 is useful in editing RNA targets, including RNA viruses of clinical relevance. Because of their sequence programmability, much research has focused on the use of CRISPR-Cas technologies for improved accuracy in the development of disease treatment and diagnostic platforms. Despite the potential risk of non-specific RNA targeting effects, we believe that the CRISPR-Cas13 system presents a higher level of specificity over the available RNA-targeting approaches. Continued development of Cas variants with increased specificities and activities is critical for the application of these technologies to viral infection control.

Declarations

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