Application of CRISPR-Cas9 editing for virus engineering and the development of recombinant viral vaccines

Na Tang[1], Yaoyao Zhang[2], Zhiqiang Shen[1#],

Yongxiu Yao[2#], Venugopal Nair[2,3,4#]

1. Shandong Binzhou Animal Science and Veterinary Medicine Academy & UK-China Centre of Excellence for Research on Avian Diseases, Binzhou 256600, Shandong, PR China
2. The Pirbright Institute & UK-China Centre of Excellence for Research on Avian Diseases, Pirbright, Ash Road, Guildford, Surrey, United Kingdom GU24 0NF
3. The Jenner Institute Laboratories, University of Oxford, Oxford, United Kingdom
4. Department of Zoology, University of Oxford, Oxford, United Kingdom

Na Tang
E-mail: tangna0543@163.com
Tel: +86 (0)13475079769

 # Corresponding Authors

Zhiqiang Shen
E-mail: bzshenzq@163.com
Tel: +86 (0) 5433405108

Yongxiu Yao
E-mail: yongxiu.yao@pirbright.ac.uk
Tel: +44-(0)1483 231493

Venugopal Nair
E-mail: venugopal.nair@pirbright.ac.uk
Tel: +44 (0)1483 231415

Email Addresses of Co-authors:

Yaoyao Zhang yaoyao.zhang@pirbright.ac.uk

Running title: CRISPR editing for viral vaccine development
Key words: Recombinant vaccine, viral vector, CRISPR editing, herpesvirus, poxvirus, adenovirus

Abstract

CRISPR-Cas technology, discovered originally as a bacterial defense system, has been extensively repurposed as a powerful tool for genome editing for multiple applications in biology. In the field of virology, CRISPR-Cas9 technology has been widely applied on genetic recombination and engineering of genomes of various viruses to ask some fundamental questions of virus-host interactions. Its high efficiency, specificity, versatility, and low cost have also provided great inspirations and hope in the field of vaccinology to solve a series of bottleneck problems in the development of recombinant viral vaccines. This review highlights the applications of CRISPR editing in the technological advances compared to the traditional approaches used for the construction of recombinant viral vaccines and vectors, the main factors affecting their application, and the challenges that need to be overcome for further streamlining their effective usage in the prevention and control of diseases. Factors affecting efficiency, target specificity and fidelity of CRISPR-Cas editing in the context of viral genome editing and development of recombinant vaccines are also discussed.

1. Introduction

Vaccination has undeniably been one of the major successes in the fight against several major human and animal infectious diseases. Thanks to the widespread global deployment of effective vaccines, deadly diseases such as smallpox and rinderpest have been eradicated from the world, with many others in the process of eradication. Current success of several vaccines against Covid-19, developed and deployed globally in record time, further demonstrates the triumph of vaccination for protecting global health. In the last few decades, vaccines generated using recombinant DNA technologies have become revolutionary and attractive tools in human and animal disease control. Compared to traditional vaccines produced with killed or naturally attenuated pathogens, recombinant vaccines (such as subunit vaccines, attenuated recombinant vaccines and live vector vaccines) have many beneficial effects, including improved induction of immune responses, reduced side effects as a result of the precise use of the most immunogenic subunit antigen, long term persistence of immunogen, reduced safety concerns as it is not necessary to handle the causative infectious agent, and increased stability for long-term storage and shipping.[1, 2]

A number of techniques have been developed to enable gene recombination, including homologous recombination, transposition factors,[3] the use of reverse genetics approaches and cleavage by nucleases such as zinc finger nucleases (ZFN)[4] and transcription activator-like effector nucleases (TALEN).[5] Since CRISPR-Cas9 based genome editing was first reported in human and mammalian cells in 2013,[6, 7] it has become one of the most efficient, versatile and flexible tools for gene editing, particularly for the development of recombinant vaccines[8].

The CRISPR-Cas system consists of Cas nuclease and guide RNAs which guide the Cas nuclease to target and cleave the adjacent protospacer sequences. Presently, at least two classes[9] and six types of CRISPR-Cas systems have been identified and classified.[10] Among them, the type II CRISPR-Cas system with the single effector protein such as the Cas9 from *Streptococcus pyogenes* (SpCas9) is currently the most widely used CRISPR system for bioengineering, biotechnology, and translational research applications. CRISPR-Cas9 system, mainly composed of CRISPR RNA (crRNA), a Cas9 nuclease-recruiting sequence transactivation crRNA (tracrRNA) and Cas9 protein, only needs a single guide RNA (sgRNA) for the induction of a precise double-stranded break (DSB) in the target sequence. DSB can trigger DNA repair in host cells through either non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanism when homologous sequences are present.[11] Some of the other Cas9 orthologues and Cas variants (e.g., Cas9n, SaCas9, StCas9, Cas12a), recognizing different PAM sequences and performing distinct cleavage actions, have also been described.

Applications of CRISPR editing have now been extended for a variety of recombinant vaccine studies, targeting viruses,[12] yeasts,[13] bacteria,[14] animal cells,[15] parasites,[16] and plants.[17] Since most of the studies on the development of recombinant vaccines and vectors focused on viruses, this review focuses on the recent advances in the application of CRISPR-Cas9 technology on the development of recombinant viral vaccines and vectors. The review will also highlight the potential challenges of this technology, and the prospects of its future direction.

2. Classical techniques for generating recombinant vaccines

As described above, traditional methods have been widely used to generate recombination and mutations. However, in most recombination events, the sites of DNA insertion or replacement are often random, resulting in unwanted mutations. Restriction endonuclease digestion and ligation processes are unsuitable for the development of viral vaccines when genome lengths are more than 30kb. Classical genomic approaches based on spontaneous homologous recombination in mammalian cells are usually inefficient and laborious. Large fragment cloning procedures using bacterial artificial chromosome (BAC) or cosmid clones in *Escherichia coli* (*E. coli*) have been applied for recombination in viruses,[18, 19] but required multiple steps of vector cloning and selection, as well as large scale time-consuming and labor-intensive screening processes.[20] More efficient and site-specific genome editing technologies including zinc-fingers[4] and TALENs[5] have been developed in the 1980s, which can target and precisely cleave specific sites to enable more efficient gene modifications such as deletion, insertion, and replacement. However, these methods have not been widely adapted for virus recombination or vaccine development so far.

Programmable manipulation of genes facilitates the understanding of gene function and development of genetically engineered vaccines. Numerous viruses have been found to have great potential as candidate vaccines, some of which are used as recombinant vaccine vectors including poxviruses[21-25], herpesviruses[26], adenoviruses[21-25], lentiviruses[27] and alphaviruses[28-30]. These vectors can express heterologous proteins against pathogens to elicit robust immunity. However, limited by the existing methods, there are still gaps to be bridged to develop the ideal recombinant vaccines.[31]

3. CRISPR-Cas9, a new tool for viral engineering

Following the widespread application of CRISPR-Cas9 technology for the efficient editing of genomes within a broad spectrum of organisms including viruses, its potential has also been explored for vaccine development. The CRISPR-Cas9 editing system can theoretically target any dsDNA sequence in eukaryotic cells, including both the genomic DNA of cells and dsDNA of viral invaders. After several years of study, multiple human and animal viruses have been subjected to antiviral CRISPR-Cas9-based editing with the purpose of preventing viral infection. The cases in Table 1 present successful examples of CRISPR editing for studying viral gene function, generating virus mutants, and activating/deactivating viral replication.

3.1 Loss-of-function

Several studies have focused on loss-of-function through CRISPR-Cas9-based gene knock-out strategies. Different types of virus genomes, including live viruses,[32] viral genomic DNA,[33] BAC DNA,[34] and integrated viral genes in cell genomes such as human immunodeficiency virus (HIV)[35] have been successfully modified via CRISPR-Cas9 system. The knock-out efficiency (number of plaques with deletion/total number of plaques) can reach 75% mediated by NHEJ repair mechanism for MDV in chicken embryo fibroblast cells[32] or 85% by HDR pathway for vaccinia virus.[36] Multiple gene knockouts can also be achieved by simultaneous editing using several gRNAs.[37] Besides Cas9, other Cas endonucleases such as Cas12a and Cas13a also have the capability of inducing strong inhibition of virus in infected cells.[38, 39] Base editing using a Cas9 nickase or dCas9-cytidine deaminase fusion protein has also been performed to induce

premature stop codon mutagenesis in virus genomes with 100% efficiency, but the editing has been reported only in *E. coli* based on BAC system at present.[40] A majority of the past studies have obtained good (over 50%) knock-out efficiency, while a few reported low recombination rate and difficulty in purification of edited clones.[41] For some viral infections where multiple viral genomes need to be targeted within the same cell, such as Kaposi's sarcoma herpesvirus (KSHV) in endothelial cells,[42, 43] and MDV in lymphocytes,[44] there are challenges of using CRISPR to target all copies. Therefore, the research results should be cautiously tested to confirm whether the residual wildtype viral gene products influence the phenotype of interest. Cell lines stably expressing Cas9 endonuclease and sgRNAs have been constructed for successful viral gene editing. However, whether the cell lines are suitable for long-time antiviral application should be carefully verified since some reports showed that virus could escape from CRISPR-Cas9-mediated inhibition after passages.[45]

3.2 Gene insertion and replacement

The last few years have witnessed increased application of CRISPR-Cas9-based gene editing in virology for structural and functional studies of viral genes and to understand virus-host interactions.[46] These include the insertion of foreign genes as well as the replacement of viral genes in multiple viruses (Table 1). Efficiency of inserting foreign genes via HDR pathway with homologous arms,[36, 47, 48] and with non-homologous strategies have also been explored to enhance the efficiency of insertion.[49, 50] Markers and reporters have been widely used in CRISPR-Cas9 knock-in platforms to enable visualization and easy purification of viruses. For example, a stable recombinant PRV strain expressing firefly luciferase and enhanced green fluorescent protein (EGFP), developed through CRISPR-Cas9 based knock-in, was very useful for the screening of antiviral compounds and gRNAs.[51] Similarly, reciprocal swapping of gD and gI glycoprotein genes of two PRV strains to generate chimeric viruses was valuable for comparative study.[52] Assisted with single cell fluorescence-activated cell sorting (FACS), pure recombinant virus clones can be obtained in a single round of screening.[47] Gene replacement and interchange are usually done through HDR using single or dual gRNAs and donor containing homologous arms. A number of studies have used reporter gene[36] or intermediates[47] for exchange. Compared with other traditional recombination methods, CRISPR-Cas9 based gene exchange is a much easier and rapid approach for comparative study of viral gene function.[53]

3.3 Gene activation or deactivation

Infection by several viruses results in a chronic infection where the viral genome is held in a latent, epigenetically silenced state, with periodic reactivation. While latency is one of the hallmarks of herpesvirus infections, latent reservoirs of viruses, such as human immunodeficiency virus type - 1 (HIV-1), is also very significant since latency is one of the main obstacles for elimination of HIV by antiretroviral therapy. An effective therapeutic strategy would be the reactivation of HIV-1 from its latent state, making patients susceptible to antiretroviral therapy. Modified Cas9 without the endonuclease activity fused with transcriptional activators and repressors has been used for targeted CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) respectively. Cas9 variants with mutations in their two endonuclease domains deactivate the nuclease activity of Cas9 (nuclease-deficient disabled Cas9, dCas9), enabling the development of programmable RNA-dependent DNA-binding proteins.[54] Different protein or functional domains have been successfully fused to dCas9 to develop sequence-specific gene expression or cellular localization tools.[55] For example, several repurposed CRISPR-Cas9 systems have been employed to activate

latent HIV. The dCas9 fused with a tetrameric repeat of the herpes simplex virus (HSV) VP16 transactivation domain VP64[56] and/or a synergistic activation mediator (SAM) complex[56, 57] has been introduced to target the long terminal repeat (LTR) enhancer region of HIV-1, resulting in robust activation of latent HIV-1 infection in human T cells. Conversely, repurposed CRISPR-dCas9 fused to transcriptional repression domain derived from Kruppel-associated box (KRAB) has also been effective for the epigenetic silencing of the HIV proviral DNA.[58]

3.4 RNA targeting

CRISPR technology has also been recently developed for RNA targeting. Although most CRISPR-Cas9-based editing focuses on DNA viruses or their DNA replication intermediates,[59, 60] there are also some RNA targeting Cas nuclease-mediated genome editing of ssRNA viruses[61-63] and even dsRNA viruses.[64] The Cas9 endonuclease from *Francisella novicida* (FnCas9) can target diverse viruses including both +ssRNA viruses and -ssRNA viruses via an engineered RNA-targeting guide RNA (rgRNA),[61] though low RNA-cleavage efficiency and possibility of inducing off-target effect on cellular DNA have been reported.[65] The ability of class 2 type VI CRISPR effector Cas13 to target a wide range of ssRNA viruses has been demonstrated. Computational analysis of more than 350 mammalian ssRNA viral genomes has been explored for the broad utility of Cas13.[62] This study has demonstrated potent activity against three distinct ssRNA viruses including lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV), and vesicular stomatitis virus (VSV).[62] Lately, antiviral strategy to combat SARS-CoV-2 and H1N1 IAV has been developed based on Cas13d and defined crRNAs to target conserved viral gene for RNA cleavage or reduce virus replication.[63] A strategy of abrogation of Porcine reproductive and respiratory syndrome virus (PRRSV) infectivity by CRISPR-Cas13b-mediated viral RNA cleavage in MARC-145 cells has been developed to target two viral genes with an all-in-one system expressing Cas13b and duplexed crRNA cassettes.[66]

Table 1. The application of CRISPR-Cas9 based editing in virus genetics and functional study

Virus		Target gene	description
dsDNA viruses	Vaccinia virus (VACV)	TK, N1L, A46R	Dual deletions of N1L and A46R with high efficiency and no off-target effect;[36] Dual marker-free deletions[67]
	African Swine Fever Virus (ASFV)	8-DR	Using HDR mediated CRISPR-Cas9 to replace 8-DR with RFP to develop a recombinant virus[68]
		CP204L	A stable Cas9/gRNA cell line to KO ASFV essential gene[69]
		EP402R, 9GL, and A238L	CRISPR/Csn1 based KO virus editing with low recombinant rate[41]
	Herpes simplex virus type 1 (HSV-1)	gE, TK	Both gene-ablated and gene knock-in HSV were generated[70]
		UL15, UL27, UL29, UL30, UL36, UL37, UL42, UL5, UL52, UL8, UL54, UL9	Interruption of 12 essential genes can Inhibit HSV-1 replication[71]
		UL7	KO of UL7 to develop mutant HSV1 for functional study[72]
		ICP0, UL37-UL38	Efficiency study of HDR-mediated double KO by CRISPR-Cas9 in human cell line[48]
		UL7, UL41, LAT	Construction of attenuated virus strain[73]
		UL30, UL29, UL54, RS1 UL26/27, UL37/38	KO for lytic gene function study by AAV based SaCas9 system[74]
		UL23, UL26/27, UL37/38	High-efficiency nonhomologous insertion strategy[50]
		US6	Construction of all-in-one CRISPR vectors and KO US6 showed antiviral effect[75]
		ICP4	(AAV1)-mediated delivery of SaCas9 inhibits HSV-1 replication[76]

		UL39	Developing novel oncolytic Herpes simplex virus type 1 through UL39 knockout[77]
	Herpes simplex virus type 2 (HSV-2)	UL16, UL21	Generation of HSV-2 and HSV-1 UL16 mutants;[78] side-by-side comparison of HSV-2 and HSV-1 pUL21 deficient viruses[79]
		RL1 and/or LAT	construct HSV-2 mutant strains through gene deletion;[80] KI mouse IL-15 to generate a new oncolytic virus[81]
	Epstein-Barr Virus (EBV)	BART5, BART6, or BART16; EBNA1, OriP	Inhibit the targeted miRNAs and essential gene for virus infection[71]
		EBV sequence around a BssHII site	CRISPR-Cas9-mediated EBV-BAC cloning[82]
		BKRF4, BOLF1, BBRF2	KO of each gene reduced EBV infectivity[83-85]
		EBNA1, OriP, W repeats	Inhibition of EBV replication and clearance of virus from infected tumour cells[86]
	Kaposi's sarcoma herpesvirus (KSHV)	Latency-associated nuclear antigen (LANA)	Transduction of Adv5 delivered Cas9 system significantly reduced virus latency[87]
		ORF57 multiple copies (~100) in one cell	Multiple copies of gene KO or inversion by dual-gRNAs CRISPR[43]
		Viral genome wide CRISPR screen	Complementary screen for genes responsible for the cellular phonotype of interest[42]
	Human Cytomegalovirus (HCMV)	UL54, UL44, UL57, UL70, UL105, UL86, UL84, US6, US7, US11	Interruption of essential genes can Inhibit HSV-1 replication except UL84[71]
	Guinea Pig Cytomegalovirus (GPCMV)	US22, UL23, UL24, UL28, UL29, UL131, US23, US24, US26, TRS1/IRS1	CRISPR-Cas9 mutagenesis can introduce the same types of mutations as bacterial artificial chromosome recombineering with higher efficiency[88]
	Porcine pseudorabies virus (PPRV)	TK, gM, gE, gI, Us2, Us9, Us3, gG, gN, EP0	TK and gM knockout mutants displayed significantly reduced virulence;[51] gE, gI, TK deletion by CRISPR-Cas9-HDR for developing new vaccine[89]
		75 gRNAs	A stable Knock-in virus strain for the screening of antiviral compounds and sgRNAs inhibited virus replication[51]
		gB, gC, and gD	CRISPR-Cas9-HDR for gB gene exchange between two PRV strains;[53] Interchange gC and gD to generate chimeric viruses[52]
		UL41 (vhs)	Generate UL41 null virus by CRISPR-Cas9 and Gibson assembly[90]
		US7, US8, UL23, US3	Construction of a 4-genes KO vaccine virus strain for dogs[33]
	Suid herpesvirus 1 (SuHV-1)	UL24, TK	CRISPR-Cas9-HDR was adopted to generate UL24 and TK mutant viruses[91]
	Marek's disease virus (MDV)	UL6, UL19, UL27, UL30, UL49, ICP4	Multiple gRNAs targeting essential MDV genes has the ability to prevent virus replication[37]
		MicroRNA M1, M4, M9, M11, Meq, pp38	KO of Meq or pp38 to confirm the efficiency of CRISPR-Cas9 on MDV;[32] KO of multiple copies of Mir-M4 from integrated MDV genomes in transformed cell lines;[44] KO of multiple microRNAs in MDV genome[92]
	JC Polyomavirus (JCPyV)	non-coding control region (NCCR), Capsid proteins VP1 and VP2	Editing NCCR and late region inhibits virus replication[93]
	Merkel cell polyomavirus (MCPyV)	sT, LT	Frameshift mutations decreased LT protein levels and impaired cell proliferation[94]
	Human papilloma viruses (HPV-16, HPV-18, HPV-6, and HPV-11)	E6 and E7	Antiviral studies: loss of E6 and/or E7 by CRISPR-Cas9 or SaCas9 or Cas13a system reduced viral proteins or mRNA, leading to cancer cell death[95-97]
ds-DNA RT	Hepatitis B Virus (HBV)	X, S, C, P, P1, S1, PreS1, S2, PreS, PreC, PS, XCp, eE, PCE, ENII-CP/X, RT, sAg, Enhl	Reducing HBsAg, HBeAg, HBcAg, cccDNA, and viral DNA/RNA via Cas9, stCas9,[98] saCas9, Cas9n systems[95] and CRISPR-Cas-mediated "base editors"[99]
	duck hepatitis B virus (DHBV)	6 gRNAs targeted S, C, P	Two of the 6 sgRNAs efficiently inhibited on virus total DNA, cccDNA in PDHs, and total DNA in the culture medium[100]
ss-RNA RT	Human Immunodeficiency Virus (HIV)	LTR, gag, pol, tat, rev, env, vif, tet, NCR	Direct disruption of HIV genome or induction of latency reversal by Cas9 or saCas9 or dCas9 systems;[95] 1-2nt mismatches reduced the inhibition efficiency;[101] dual-gRNA/Cas9 treatment generated more site mutations than excision or inversion;[102] Epigenetically silencing of the proviral DNA with dCas9-KRAB;[58] Cas12a achieved full HIV inactivation with only a single crRNA[38]
	Human endogenous retrovirus (HERV)	env	First disruption of HERV gene and inhibition of env transcripts and proteins via SaCas9[103]
	Porcine endogenous retroviruses	pol	Genome-wide PERV inactivation in pigs[104]
	Feline immunodeficiency	U3 region of 5′ and 3′ LTR	Reduction of viral RNA in CRISPR lentivirus-treated MCH5-

	virus (FIV)		4 cells[105]
ds-RNA	Rotavirus	eGFP	dsRNA virus genome editing based on CRISPR/Csy4[64]
ssRNA(+)	Porcine epidemic diarrhea virus (PERV)	ORF3	Replacement of ORF3 gene with eGFP by CRISPR-Cas9 from BAC infectious clone[59]
	Transmissible gastroenteritis virus (TGEV)	Spike gene	224aa deletion in the N terminal domain of spike gene by CRISPR-Cas9 from BAC infectious DNA[60]
		Nsp1	Altering nsp1 gene by CRISPR-Cas9 based reverse-genetics system[106]
	Porcine reproductive and respiratory syndrome virus (PRRSV)	ORF5 and ORF7 genes	CRISPR-Cas13b-mediated viral RNA cleavage[66]
	Hepatitis C Virus (HCV)	5' and 3' LTR	Employment of CRISPR/FnCas9 to inactivate virus in mammalian cells[61]
	Dengue Virus (DENV)	NS3	Employment of CRISPR-Cas13a to inactivate virus in mammalian cells[107]
	Zika virus (ZIKV)	Inositol-requiring enzyme 1α (IRE1α)	Knockout of IRE1α dramatically reduced ZIKV replication levels[108]

3.5 Modification of cell lines for vaccine production

With most of the new generation of vaccines produced in cell culture systems, increasing the viral titres or production of antigens is a major priority for the vaccine manufacturing industry. Recent advances in genome editing techniques also open up the prospects of a relatively unexplored area of research for increasing vaccine yield through genetic engineering of host cell lines.[109-112] Viruses grown on such engineered cell lines can reach much higher titers (up to 5-7 logs higher in Vero cell line[112] compared with wild type cells). Furthermore, some of the engineered cell lines may have unexpected capability of enhancing replication of other viruses.[15] CRISPR-Cas-mediated metabolic engineering has also been extended to enhance the production of vaccine antigens.[113, 114] For example, knocking out a glutamine synthetase (GS) encoding *glul* gene from CHO cell line has been done to improve the glutamine metabolism selection system,[115] which in turn can potentially increase the production of vaccine antigen in these cells. Knockout of DNA methyltransferase Dnmt3a in CHO cells provided an enhanced long-term stable cell line of transgene expression with low methylation rate of the global DNA, providing the potential for improving the production of recombinant protein including vaccine antigens in CHO and other mammalian cells.[116] Using homology-directed DNA repair, gene expression cassettes can also be integrated into specific genomic locus in CHO cells to generate stable cell lines with manageable and predictable features for large-scale production.[117]

3.6 CRISPR-Cas9 libraries

High-throughput screening based on CRISPR-Cas9 libraries has become an attractive and powerful technique to screen target genes for functional studies. Genome-wide CRISPR-Cas9 screens are increasingly used for identifying receptors, antiviral proteins and other important genes in virus-host interaction and virus restriction factors.[89, 118-120] CRISPR-Cas9 sgRNA libraries can be designed using online tools and databases such as Cas-OFFinder (www.biootools.com),[121] Addgene (https://www.addgene.org/crispr/libraries/), Cas-Database,[122] CRISP-view,[123] DepMap,[124] BioGRID ORCS,[125] and lately iCSDB database (https://www.kobic.re.kr/icsdb/), DepMap portal and BioGRID ORCS.[126]

4. CRISPR-Cas9 applications on viral vector platforms

Since the first report of CRISPR-Cas9 mediated modification on HSV and adenovirus vectors,[127] a variety of CRISPR-Cas9 strategies have been used as basic tools for precise editing of other large

DNA viruses that can be potentially applied on further optimizing viral vectors for development and production of recombinant vaccines and therapeutics (Table 2).

4.1 Herpesviruses

Herpesvirus-based vectors have been widely used in the development of a variety of vaccines and therapeutics due to their inherent advantages, including limited host range, large genome size capable of accommodating multiple inserts, stable attenuation, ability for inducing strong cell-mediated and humoral immune responses, stable and robust expression of foreign genes, as well as good safety records. For these reasons, a number of human and animal herpesviruses have been successfully developed as recombinant vectors.

Herpes simplex virus type 1 (HSV-1) harbors a 150-kb large genome, and half of its 77 genes are dispensable for virus replication,[128] giving abundant vector capacity. Additionally, early studies have shown that the viruses lacking certain genes have replication restricted to cancer cells, demonstrating the potential for developing HSV-1 for oncolytic virus therapeutics.[129] Large fragments have been efficiently knocked into sites of HSV-1[127] and HSV-2 genomes,[81] sometimes as multiple copies,[48] suggesting the feasibility of targeted CRISPR-Cas9 mediated genome editing strategies for developing HSV-1 based attenuated vaccines and oncolytic vectors. Lately a non-homologous insertion (NHI) strategy has also been explored for rapid and high-efficiency knock-in of foreign genes.[50] The NHI strategy also paved a way for accelerating recombinant virus selection based on its inhibitory effect on wild-type and reverse-integrated viral genomes.

In animal herpesviruses, CRISPR-Cas9 has dramatically enhanced the homologous recombination efficiencies between PRV genomes and a linearized BAC transfer vector,[130] or PRV and a double gene substitution donor.[47] We have recently developed a simple and rapid CRISPR-Cas9-based strategy for constructing a recombinant herpesvirus of turkey (HVT) vector delivering the VP2 protein of infectious bursal disease virus (IBDV) through a NHEJ-dependent repair pathway and a Cre-LoxP system to eliminate the reporter protein.[49, 131] This strategy, for incorporation of genes encoding foreign antigens into the HVT genome, can lead to the rapid construction of recombinant vaccines for efficacy studies. Using this strategy, expression cassettes of infectious laryngotracheitis virus (ILTV) gD, gI and the H9N2 AIV hemagglutinin have been inserted sequentially into the distinct locations of the recombinant HVT-IBDV-VP2 viral genome, generating a triple insert recombinant vaccine candidate.[132] This offers the prospect of rapidly developing multivalent HVT-vectored vaccines capable of inducing simultaneous protection against multiple avian diseases, particularly to overcome the interference between individual recombinant HVT vaccines expressing different antigens are administered to birds. Other herpesvirus vaccine vectors such as duck enteritis virus (DEV)[133, 134] and ILTV[135] have also been developed via NHEJ-CRISPR-Cas9 and Cre-Lox Systems. Recombinant MDV vector carrying all the CRISPR-Cas9 components using an attenuated MDV vaccine strain (814 strain) for *in vivo* editing of reticuloendotheliosis virus (REV) and avian leukosis virus subgroup J (ALV-J) infection have recently been reported,[136, 137] providing efficient delivery of CRISPR components into chickens, inducing a drastic reduction of REV and ALV-J viral load, significantly diminishing clinical disease.

4.2 Poxviruses

After the successful eradication of smallpox, vaccinia virus (VACV) has been modified as a vector for vaccine and immunotherapeutic candidates. The most widely used method for modification of VACV is based on homologous recombination.[138] A highly efficient CRISPR-Cas9 method

accompanied with HDR repair, using a modified Cas9 without the nuclear localization signal (NLS) to target the cytoplasmic location of VACV replication, has been reported to generate mutations in the N1L and A46R genes of VACV.[36] Simultaneous and rapid homologous recombination of multiple target sites in both N1L and A46R genes have also been successful achieved. To obtain a marker-free VACV vector, Cre-LoxP and Flp-FRET systems have been introduced into the strategy above for marker gene excision.[67] A marker-free TK and N1L double gene deleted VACV vector was efficiently constructed by these marker-free systems, providing significant potential for developing new vaccines and vectors for clinical application. However, all studies using CRISPR-Cas9 on vaccinia virus have not given satisfactory results. For example, one recent study showed that the Cas9/gRNA complexes can cut VACV genomes efficiently, but generated very few (from <1% to no more than 10%) recombinant virus compared to the relatively high efficiency of gene editing in other viruses such as HSV (40%-60%).[139] This is thought to be due to the low efficiency of HDR and NHEJ repair pathways that leaves the cut viral genomes unable to be replicated. However in a recent study, the inefficiency of DNA repair of the VACV genome after Cas9 cleavage was used as an advantage for efficient selection method for the generation of VACV recombinants or other genetic variants.[139]

Avipox vectors such as Canarypox ALVAC have been widely studied and used as an efficacious and safe viral vector for mammals as several canarypox-vectored recombinant vaccines have been commercialized for years. Based on a bivalent ALVAC-vectored recombinant vaccine carrying the F gene and H gene of canine distemper virus (CDV), a new ALVAC recombinant virus has been constructed with the insertion of the matrix (M) gene through HDR-mediated CRISPR-Cas9 gene editing.[140] ALVAC recombinant with inserted CDV M gene has shown rapid and greater antibody responses compared to the existing vaccines, possibly because of the VLP structure assembled in the presence of the M protein.

4.3 Transgene delivery vectors

CRISPR-Cas9 complexes for targeted gene editing are currently used mainly in preclinical trials, with a few examples of therapeutics use in genetic diseases. However, CRISPR-Cas9 complexes delivered through different viral vectors are also valuable for antiviral therapies. Viral vectors used as transgene delivery vehicles of CRISPR-Cas9 complexes can be classified into two classes: integrating viruses such as murine leukemia virus (MLV),[141] foamy virus,[142] HIV, and lentivirus, and non-integrating viruses such as adeno-associated viruses (AAV),[143] adenoviruses (AdV)[144] and Sendai virus.[145] Up to now, lentivirus vectors are usually the first choice for *ex vivo* gene correction and screening, while AAVs are preferred option for *in vivo* gene transfer.[146] Lentiviruses are highly effective for genome-wide CRISPR-Cas9 screening and delivering the CRISPR-Cas9 machinery to the more difficult-to-transfect cells. Despite their advantages, lentivirus vectors carry an RNA genome which can integrate into the host genome, inducing long-term constitutive CRISPR-Cas9 expression.[147] However, lentivirus-like particles (LVLPs) have been developed to achieve transient expression of genome editing proteins,[148, 149] introducing an alternative solution for LV. AAV can efficiently infect a broad range of tissue types and yield very high viral titers, making it ideal for the delivery of antiviral CRISPR-Cas9 to the virus-infected cells.[150] However, AAV can integrate randomly into the genome at low rates [151] and the risk of such integration into the Cas9-induced DSBs during editing should be considered.[152]

Table 2. The application of CRISPR-Cas9 based editing in viral vectored vaccines

Virus/Vector	Target disease	genes	description
Canarypox virus	Canine distemper	Matrix (M) of CDV	Highly efficient system for generation recombinant canarypox virus contained CDV M, H, and F genes[140]
Vaccinia virus		Spi2, mCherry, A23R, eGFP	Knocking out of mCherry and eGFP shown inefficient NHEJ or HDR repair[139]
		N1L, A46R TK	Single or dual deletions of N1L and A46R via HDR, marker-free single or dual deletion vector[67]
herpesvirus of turkeys (HVT)	IBD, ILT, AI H9N2	IBDV VP2, ILTV gD & gI, AIV HA	Single and triple insert live avian herpesvirus vectored vaccine[49, 131, 153]
	AI H9N2	Hemagglutinin (HA) gene	Recombinant HVT harboring hemagglutinin (HA) gene from AIV H9N2 (rHVT-H9)[34]
	AI H7N9	Hemagglutinin (HA) gene	Generation of a HVT-H7N9 HA bivalent vaccine utilizing HDR and erythrocyte binding selection[154]
Infectious laryngotracheitis virus (ILTV)	Newcastle disease ILT	TK F gene	Generation of TK deleted ILTV vector and recombinant ILTV harboring F gene of NDV[135]
Duck enteritis virus (DEV)	Duck enteritis, AI	Hemagglutinin (HA) gene	Generation of recombinant DEV harboring hemagglutinin (HA) gene[133]
	AI H5N1 duck tembusu disease and duck enteritis	HA of HPAIV H5N1, E gene of DTMUV	Generation of recombinant virus harboring two foreign genes through two steps of HDR-CRISPR-Cas9 replacement[134]
Herpes simplex virus type 1 (HSV-1)	eGFP, eRFP	ICP0, UL37-38	Efficiency study of HDR-mediated double KO by CRISPR-Cas9 in cell line[48]
		UL26/UL27	Confirmation of a suitable insertion site with no effect in viral replication[155]
Foamy virus (FV)		EGFP	Foamy virus vector TraFo-CRISPR as delivery vehicle enhanced genome engineering[156]
Adenovirus		EGFP, RFP	Targeted site-specific mutations in EGFP and DsRed transgenes[127]
Sendai virus (SV)		CCR5	Construction of SeV-Cas9 system and targeting Ccr5 of monocytes[145]

5. Factors affecting the use of CRISPR-Cas9 in virus recombination

5.1 Efficiency

Several factors influencing the gene editing and transgene insertion efficiency have been identified. The cleavage and repair efficiency at the target site are dependent on the characteristics of gRNA and target DNA. To achieve the most efficient gRNA recognition and cleavage, redundant sgRNAs targeting different cleavage sites can be designed and compared via several simple methods such as T7E1 (T7 endonuclease 1 mismatch assay),[157] Surveyor mismatch cleavage assays,[158] HRM (High-Resolution Melting assay),[159] IDAA (Indel detection by amplicon analysis),[160] UDAR (Universal Donor As Reporter)[161] or targeted next-generation sequencing.[162] Dual or multiple gRNAs strategies are also helpful in improving editing efficiency.[37, 48] With the expanding repertoire of CRISPR-Cas endonucleases (SpCas9, SaCas9, Cas12a, CjCas9, and so on), the most efficacious system for target editing can be selected and optimized.[163] Compared to the expression of Cas9 from plasmid-based system or from the Cas9 mRNA, direct delivery of the synthetic Cas9 ribonucleoprotein (RNP) complex offer several advantages including its transient activity and rapid degradation with reduced off target effects,[164] in addition to the high efficiency and rapid cleavage, without the need for promoter or codon optimization.[165, 166]

CRISPR-Cas9 editing involves a two-step process of cleavage of the DNA followed by the DNA repair. In the editing of viral genomes, while the cleavage efficiency of Cas9/gRNAs can be high (often reaching 90% as detected by PCR), the efficiency of repair of cleaved fragments is always much lower (1%~7%). Although many of the multiple copies of the viral genomes are efficiently cleaved by the CRISPR-Cas9 complex, large proportions of the viral genomes cannot be efficiently repaired inhibiting viral replication. While the exact reasons for the inefficient repair of the viral genomes are not known, access of the components of the repair machinery at the sites of viral replication could be a major reason. In most cases, the efficiency of NHEJ based strategies were significantly higher than that of the HDR based strategies,[50, 167, 168] especially when insertion of large foreign genes is required.[169] However, there are also some unsuccessful cases of viral genome editing via NHEJ repair.[41, 139] For example, NHEJ repair at low efficiency following CRISPR-Cas9 targeting of vaccinia virus has been reported recently.[139] Cas9 cleavage has been shown to be efficiently achieved but recombination has been inefficiently made either by NHEJ or HDR. As VACV is a nuclear-independent virus which has the ability to replicate in cytoplasm even in enucleated cells,[170] one presumption is that VACV genome might have poor opportunity to get access to NHEJ repair mediators such as DNA ligase IV.[171] For viruses replicating in the cytoplasm, removing N- and C-terminus nuclear localization sites (NLS) from the Cas protein sequence can enhance the editing efficiency.[172] In addition, when NHEJ-directed repair is efficiently induced during genome editing, it is likely that the factors restricting the frequency of inducing HDR pathway are affected. However, chemicals such as the NHEJ inhibitor SCR7[173] and NU7026[174] have been used to increase on-target deletions and recombination efficiency.

Compared to gene editing of the host genome, the generation of recombinant progeny virus is relatively complex since the viral genome replicates very fast and constantly produce abundant target sites. Therefore, the proliferative characteristics of virus should be considered during the optimization of recombinant strategy. After transfection, Cas9 protein is detected at 4h - 8h,[139, 140]

reaching the maximum expression at approximately 20h.[140] Cas9 cleavage of the target is potentially happening very early and fast, until the limit at which genome or donor DNA could no longer be cut Is reached. The difference in the speed of CRISPR-Cas9 cleavage compared to the virus replication rates, as well as the relative inefficiency of repair pathways on the viral genome are important factors affecting viral genome editing. Adjusting of the multiplicity of infection (m.o.i.) of viruses, inoculation time and harvest time may also be helpful to enhance viral genome editing efficiency.[127] Genetic variations in viral genome may also affect the editing efficacy. Studies demonstrated that 1-nt mismatch at the cleavage site or 2 mismatches anywhere in target sequence significantly reduced the inhibitory effect on HIV-1.[101] It is also important to note that Cas9/gRNA complexes in the cells might influence the proliferation of wildtype viruses. It has been reported in different recombinant virus studies that the CRISPR-Cas9 system in eukaryotic cells significantly inhibited wild-type virus replication, leading to a higher proportion of recombinant viruses among progeny viruses.[127, 139, 175, 176]

When knock-in of foreign genes is carried out, the efficiency of recombination is also related to the features of donor construct. The length of the foreign fragment has been shown to be inversely associated with insertion efficiency of both HDR[177] and NHEJ strategies.[50] The mass ratio of Cas9 plasmid to donor plasmid can be adjusted during co-transfection to maximize the recombination efficiency.

Several delivery systems for Cas9/sgRNA complex have achieved remarkable editing efficiencies in “easy to transfect” cultured cells. However, the efficiency may drop sharply when switching to transfection of recalcitrant cell lines, exacerbated when long DNA templates were co-transfected with gRNA and Cas9 to achieve gene insertion or replacement, often resulting in no editing event, useless indels or error insertions. To achieve better delivery and gene editing efficacies, parameters such as cell confluency and culturing methods could be adjusted; varied delivery systems could be tested as well. Compared to non-viral delivery systems, viral delivery systems such as lentivirus, adenovirus, or adeno-associated viruses have been characterized by high transduction efficiency both *ex vivo* and *in vivo*. However, the application of viral vectors in developing recombinant vaccines are constrained by their exogenous viral substance, limited cargo capacity, and risk of genome integration. Baculoviral delivery vectors have a good transduction efficiency. They also have distinct characteristics that they are both replication- and integration-deficient in mammalian cells, giving good prospects for the delivery of CRISPR tools. Baculoviral vectors have large heterologous DNA cargo holding capacity, rendering them highly suitable for accommodating all components of a CRISPR machinery in a single vector.[178, 179]

5.2 Target specificity and off-target effects

Potential off-target effects remain one of the major concerns with CRISPR technology. The RNA-guided nucleases used in CRISPR systems have been shown to bind to several mismatch sequences in the binding of sgRNA and target DNA.[180] These off-target effects have the potential of generating unexpected gene mutations or chromosomal translocations. To tame off-target mutagenesis, a number of studies have identified many factors influencing off-target CRISPR-Cas9 editing. Sequence complementarity, PAM recognition specificity, target sequence homology, and Cas9 expression level are the main factors influencing off-target effects.[181, 182] In addition, other factors such as gRNA binding stability and substrate availability can also influence the likelihood of off-target editing.[183, 184] Therefore, a number of strategies have been adopted to fine-tune the

components of CRISPR-Cas9 system to enhance target specificity and eliminate off-target editing.

However, when it comes to viral genome editing, fewer off-target effects can be expected. The genome of viruses (that ranges from 1kb to 2.47 Mb[185]) are far smaller than host genome (3×10^9 nt) and no potential off-target effects were found in many viral editing studies.[37, 48, 68, 127] It is however worth noting that in viral genomes containing overlapping genes, multiple genes might be cleaved and repaired.

5.3 Fidelity of editing

CRISPR-Cas9 editing outcomes have been thought to be highly unpredictable due to its error-prone DSB repair systems like canonical NHEJ,[186] theta-mediated end joining (TMEJ), microhomology-mediated end joining (MMEJ), and even HDR repair.[167] Because of this, NHEJ based CRISPR-Cas9 system could generate mutant viruses resistant to Cas9/sgRNA.[187] Fidelity of NHEJ can is affected by the DNA sequences rather than the length of donor.[50] Some indels, especially those in coding regions, may not reduce virus replication, as these may not be recognized by the same Cas9/sgRNA complex as a result of escaping Cas9 cleavage.[188]

To reduce the occurrence of undesired mutations, certain machine learning algorithms have been developed based on the non-random nature of Cas9 induced DSB repair and abundant data available in the public domain, including inDelphi (*https://indelphi.giffordlab.mit.edu*),[189] FORECasT (https://partslab.sanger.ac.uk/FORECasT),[190] and SPROUT (https://github.com/amirmohan/SPROUT).[191] These databases available online can effectively perform predictions of the activity of gRNAs and their repair outcomes with reasonable accuracy.[186] Besides, escape variants can be prohibited using other strategies such as targeting multiple essential genes and using redundant sgRNAs,[37, 172, 192] modification of sgRNAs, and through development of new Cas enzymes such as Cas12a[193] to cleave at site outside the target. To avoid the invalid iterative cut-and-repair process of imprecise NHEJ-mediated repair, the junction sequence formed at CRISPR-Cas9 recognition site after the cleavage and integration should be carefully designed to prevent re-cuttings.

6. Conclusions and Prospects

Recent research progress shows the advantages of applying CRISPR-Cas9 technology for genetic recombination. CRISPR-Cas9 system can target genome specifically, avoiding a series of screening and analysis problems caused by early non-specific methods. It has shown high editing efficiency in the majority of cases, which makes the genomic modification and screening of recombinant virus easier and faster. The DSB and repair caused by CRISPR-Cas9 are rapid, efficient, predictable and controllable, providing effective improvement of the screening efficiency and guarantee of recombination stability. Simultaneous editing of multiple sites can be implemented by CRISPR-Cas9-based strategies, which is apt to improve the certainty and fidelity of recombination. A variety of prediction tools and analysis methods for the assessment of targeting site, repair efficiency and off-target effect have also been developed to effectively eliminate editing error and off-target effects.

These technical merits are very valuable for the development of recombinant vaccines. The

controllability and stability problems of recombinant vaccines met in other traditional methods can be well settled, as the stable and reliable gene recombination sites can be evaluated and screened on a large scale by employing CRISPR-Cas9 based strategies. The predictability of attenuation and the safety of a vaccine can also be further improved through the multiple knock-outs of different virulence genes or genetic sites. CRISPR-Cas9 systems can help to explore the mechanism and influence factors of virus attenuation and virus-host interaction, so as to reduce the possibility of host transmission and reversion of virulence and deepen and refine vaccine development. The immunogenicity of recombinant viruses can be improved via the CRISPR-Cas9 platform by implementing various measures such as optimizing promoter elements, screening recombinant sites, and making fusion of immune promoting proteins. Up to now, CRISPR-Cas9 systems have been widely applied to a broad range of cells and viruses, which will greatly expand the scope of recombinant vaccine development. In addition, the technology is simple, rapid, and does not need rounds of genetic operations and expensive reagents, which can greatly reduce the cost of vaccine research and development.

Despite the several merits of CRISPR-Cas9 system, a number of major limitations still need to be overcome to go further in the field of recombinant vaccine research and development. Better methods are needed to solve the inconsistency between the speed of Cas9/gRNA editing and the replication of virus. Further exploration should be carried out for the key factors affecting repair efficiency, thus narrowing the huge efficiency gap between Cas9/gRNA cleavage and repair in some viruses, such as cytoplasmic targeting VACV. In addition, development of strategies for RNA virus recombination also need further attention.

With its high flexibility and modification capability, CRIPSR/Cas9 can be further improved with combination of many other techniques to cover the shortcomings and promote technological progress. We believe that the highly effective, versatile, flexible, and site-specific CRISPR-Cas9 genome editing technology will promote more rapid development of recombinant vaccines and vectors against mammalian diseases.

Author Contributions: Writing—original draft, N.T.; Writing—editing, Y.Z. and Z.S.; Writing—review & editing, Y.Y. and V.N.

Conflict of interest

The authors report no conflict of interest.

Funding information

This project was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) grants BB/P016472/1, BB/L014262/1, BBS/E/I/00007032, BB/R012865/1, BB/R007896/1, BBSRC Newton Fund Joint Centre Awards on “UK-China Centre of Excellence for Research on Avian Diseases”, and the Natural Science Foundation of Shandong Province (China) grant ZR2020KC006.

References

1. Aida V, Pliasas VC, Neasham PJ et al. Novel Vaccine Technologies in Veterinary Medicine: A Herald to Human Medicine Vaccines. *Front Vet Sci.* 2021;8:654289. DOI: 10.3389/fvets.2021.654289
2. Pollet J, Chen WH, Strych U. Recombinant protein vaccines, a proven approach against coronavirus pandemics. *Adv Drug Deliv Rev.* 2021;170:71-82. DOI: 10.1016/j.addr.2021.01.001
3. Greco R, Ouwerkerk PB, Sallaud C et al. Transposon insertional mutagenesis in rice. *Plant Physiol.* 2001;125:1175-1177. DOI: 10.1104/pp.125.3.1175
4. Urnov FD, Miller JC, Lee YL et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature.* 2005;435:646-651. DOI: 10.1038/nature03556
5. Sakuma T, Hosoi S, Woltjen K et al. Efficient TALEN construction and evaluation methods for human cell and animal applications. *Genes Cells.* 2013;18:315-326. DOI: 10.1111/gtc.12037
6. Cong L, Ran FA, Cox D et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* 2013;339:819-823. DOI: 10.1126/science.1231143
7. Mali P, Yang L, Esvelt KM et al. RNA-guided human genome engineering via Cas9. *Science.* 2013;339:823-826. DOI: 10.1126/science.1232033
8. Lau CH. Applications of CRISPR-Cas in Bioengineering, Biotechnology, and Translational Research. *CRISPR J.* 2018;1:379-404. DOI: 10.1089/crispr.2018.0026
9. Makarova KS, Wolf YI, Iranzo J et al. Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. *Nat Rev Microbiol.* 2020;18:67-83. DOI: 10.1038/s41579-019-0299-x
10. Mougiakos I, Bosma EF, de Vos WM et al. Next Generation Prokaryotic Engineering: The CRISPR-Cas Toolkit. *Trends Biotechnol.* 2016;34:575-587. DOI: 10.1016/j.tibtech.2016.02.004
11. Liu M, Rehman S, Tang X et al. Methodologies for Improving HDR Efficiency. *Front Genet.* 2018;9:691. DOI: 10.3389/fgene.2018.00691
12. Soppe JA, Lebbink RJ. Antiviral Goes Viral: Harnessing CRISPR/Cas9 to Combat Viruses in Humans. *Trends Microbiol.* 2017;25:833-850. DOI: 10.1016/j.tim.2017.04.005
13. Mitsui R, Yamada R, Ogino H. CRISPR system in the yeast Saccharomyces cerevisiae and its application in the bioproduction of useful chemicals. *World J Microbiol Biotechnol.* 2019;35:111. DOI: 10.1007/s11274-019-2688-8
14. Ou B, Jiang B, Jin D et al. Engineered Recombinant Escherichia coli Probiotic Strains Integrated with F4 and F18 Fimbriae Cluster Genes in the Chromosome and Their Assessment of Immunogenic Efficacy in Vivo. *ACS Synth Biol.* 2020;9:412-426. DOI: 10.1021/acssynbio.9b00430
15. van der Sanden SM, Wu W, Dybdahl-Sissoko N et al. Engineering Enhanced Vaccine Cell Lines To Eradicate Vaccine-Preventable Diseases: the Polio End Game. *J Virol.* 2016;90:1694-1704. DOI: 10.1128/JVI.01464-15
16. Zhang WW, Karmakar S, Gannavaram S et al. A second generation leishmanization vaccine with a markerless attenuated Leishmania major strain using CRISPR gene editing. *Nat Commun.* 2020;11:3461. DOI: 10.1038/s41467-020-17154-z
17. Cao Y, Zhou H, Zhou X et al. Control of Plant Viruses by CRISPR/Cas System-Mediated Adaptive Immunity. *Front Microbiol.* 2020;11:593700. DOI: 10.3389/fmicb.2020.593700
18. Suter M, Lew AM, Grob P et al. BAC-VAC, a novel generation of (DNA) vaccines: A bacterial artificial chromosome (BAC) containing a replication-competent, packaging-defective virus genome induces protective immunity against herpes simplex virus 1. *Proc Natl Acad Sci U S A.* 1999;96:12697-

12702. DOI: 10.1073/pnas.96.22.12697

19. Reddy SM, Lupiani B, Gimeno IM et al. Rescue of a pathogenic Marek's disease virus with overlapping cosmid DNAs: use of a pp38 mutant to validate the technology for the study of gene function. *Proc Natl Acad Sci U S A.* 2002;99:7054-7059. DOI: 10.1073/pnas.092152699

20. Warden C, Tang Q, Zhu H. Herpesvirus BACs: past, present, and future. *J Biomed Biotechnol.* 2011;2011:124595. DOI: 10.1155/2011/124595

21. Parrino J, Graham BS. Smallpox vaccines: Past, present, and future. *J Allergy Clin Immunol.* 2006;118:1320-1326. DOI: 10.1016/j.jaci.2006.09.037

22. Sanchez-Sampedro L, Perdiguero B, Mejias-Perez E et al. The evolution of poxvirus vaccines. *Viruses.* 2015;7:1726-1803. DOI: 10.3390/v7041726

23. Taylor J, Trimarchi C, Weinberg R et al. Efficacy studies on a canarypox-rabies recombinant virus. *Vaccine.* 1991;9:190-193. DOI: 10.1016/0264-410x(91)90152-v

24. Bhanuprakash V, Hosamani M, Venkatesan G et al. Animal poxvirus vaccines: a comprehensive review. *Expert Rev Vaccines.* 2012;11:1355-1374. DOI: 10.1586/erv.12.116

25. Stephensen CB, Welter J, Thaker SR et al. Canine distemper virus (CDV) infection of ferrets as a model for testing Morbillivirus vaccine strategies: NYVAC- and ALVAC-based CDV recombinants protect against symptomatic infection. *J Virol.* 1997;71:1506-1513. DOI: 10.1128/JVI.71.2.1506-1513.1997

26. Kamel M, El-Sayed A. Utilization of herpesviridae as recombinant viral vectors in vaccine development against animal pathogens. *Virus Res.* 2019;270:197648. DOI: 10.1016/j.virusres.2019.197648

27. Wanisch K, Yanez-Munoz RJ. Integration-deficient lentiviral vectors: a slow coming of age. *Mol Ther.* 2009;17:1316-1332. DOI: 10.1038/mt.2009.122

28. Davis NL, West A, Reap E et al. Alphavirus replicon particles as candidate HIV vaccines. *IUBMB Life.* 2002;53:209-211. DOI: 10.1080/15216540212657

29. Thornburg NJ, Ray CA, Collier ML et al. Vaccination with Venezuelan equine encephalitis replicons encoding cowpox virus structural proteins protects mice from intranasal cowpox virus challenge. *Virology.* 2007;362:441-452. DOI: 10.1016/j.virol.2007.01.001

30. Greer CE, Zhou F, Legg HS et al. A chimeric alphavirus RNA replicon gene-based vaccine for human parainfluenza virus type 3 induces protective immunity against intranasal virus challenge. *Vaccine.* 2007;25:481-489. DOI: 10.1016/j.vaccine.2006.07.048

31. Okoli A, Okeke MI, Tryland M et al. CRISPR/Cas9-Advancing Orthopoxvirus Genome Editing for Vaccine and Vector Development. *Viruses.* 2018;10. DOI: 10.3390/v10010050

32. Zhang Y, Tang N, Sadigh Y et al. Application of CRISPR/Cas9 Gene Editing System on MDV-1 Genome for the Study of Gene Function. *Viruses.* 2018;10. DOI: 10.3390/v10060279

33. Yin H, Li Z, Zhang J et al. Construction of a US7/US8/UL23/US3-deleted recombinant pseudorabies virus and evaluation of its pathogenicity in dogs. *Vet Microbiol.* 2020;240:108543. DOI: 10.1016/j.vetmic.2019.108543

34. Liu L, Wang T, Wang M et al. Recombinant turkey herpesvirus expressing H9 hemagglutinin providing protection against H9N2 avian influenza. *Virology.* 2019;529:7-15. DOI: 10.1016/j.virol.2019.01.004

35. Vergara-Mendoza M, Gomez-Quiroz LE, Miranda-Labra RU et al. Regulation of Cas9 by viral proteins Tat and Rev for HIV-1 inactivation. *Antiviral Res.* 2020;180:104856. DOI: 10.1016/j.antiviral.2020.104856

36. Yuan M, Zhang W, Wang J et al. Efficiently editing the vaccinia virus genome by using the CRISPR-

Cas9 system. *J Virol.* 2015;89:5176-5179. DOI: 10.1128/JVI.00339-15

37. Hagag IT, Wight DJ, Bartsch D et al. Abrogation of Marek's disease virus replication using CRISPR/Cas9. *Sci Rep.* 2020;10:10919. DOI: 10.1038/s41598-020-67951-1

38. Gao Z, Fan M, Das AT et al. Extinction of all infectious HIV in cell culture by the CRISPR-Cas12a system with only a single crRNA. *Nucleic Acids Res.* 2020;48:5527-5539. DOI: 10.1093/nar/gkaa226

39. Yin L, Zhao F, Sun H et al. CRISPR-Cas13a Inhibits HIV-1 Infection. *Mol Ther Nucleic Acids.* 2020;21:147-155. DOI: 10.1016/j.omtn.2020.05.030

40. Zheng K, Jiang FF, Su L et al. Highly Efficient Base Editing in Viral Genome Based on Bacterial Artificial Chromosome Using a Cas9-Cytidine Deaminase Fused Protein. *Virol Sin.* 2020;35:191-199. DOI: 10.1007/s12250-019-00175-4

41. Wozniakowski G, Mazur-Panasiuk N, Walczak M et al. Attempts at the Development of a Recombinant African Swine Fever Virus Strain with Abrogated EP402R, 9GL, and A238L Gene Structure using the CRISPR/Cas9 System. *J Vet Res.* 2020;64:197-205. DOI: 10.2478/jvetres-2020-0039

42. Gabaev I, Williamson JC, Crozier TWM et al. Quantitative Proteomics Analysis of Lytic KSHV Infection in Human Endothelial Cells Reveals Targets of Viral Immune Modulation. *Cell Rep.* 2020;33:108249. DOI: 10.1016/j.celrep.2020.108249

43. BeltCappellino A, Majerciak V, Lobanov A et al. CRISPR/Cas9-Mediated Knockout and In Situ Inversion of the ORF57 Gene from All Copies of the Kaposi's Sarcoma-Associated Herpesvirus Genome in BCBL-1 Cells. *J Virol.* 2019;93. DOI: 10.1128/JVI.00628-19

44. Zhang Y, Tang N, Luo J et al. Marek's Disease Virus-Encoded MicroRNA 155 Ortholog Critical for the Induction of Lymphomas Is Not Essential for the Proliferation of Transformed Cell Lines. *J Virol.* 2019;93. DOI: 10.1128/JVI.00713-19

45. Peng Z, Ouyang T, Pang D et al. Pseudorabies virus can escape from CRISPR-Cas9-mediated inhibition. *Virus Res.* 2016;223:197-205. DOI: 10.1016/j.virusres.2016.08.001

46. Teng M, Yao Y, Nair V et al. Latest Advances of Virology Research Using CRISPR/Cas9-Based Gene-Editing Technology and Its Application to Vaccine Development. *Viruses.* 2021;13. DOI: 10.3390/v13050779

47. Liang X, Sun L, Yu T et al. A CRISPR/Cas9 and Cre/Lox system-based express vaccine development strategy against re-emerging Pseudorabies virus. *Sci Rep.* 2016;6:19176. DOI: 10.1038/srep19176

48. Lin C, Li H, Hao M et al. Increasing the Efficiency of CRISPR/Cas9-mediated Precise Genome Editing of HSV-1 Virus in Human Cells. *Sci Rep.* 2016;6:34531. DOI: 10.1038/srep34531

49. Tang N, Zhang Y, Pedrera M et al. A simple and rapid approach to develop recombinant avian herpesvirus vectored vaccines using CRISPR/Cas9 system. *Vaccine.* 2018;36:716-722. DOI: 10.1016/j.vaccine.2017.12.025

50. Gong Y, Bi Y, Li Z et al. High-efficiency nonhomologous insertion of a foreign gene into the herpes simplex virus genome. *J Gen Virol.* 2020;101:982-996. DOI: 10.1099/jgv.0.001451

51. Tang YD, Liu JT, Wang TY et al. Comparison of Pathogenicity-Related Genes in the Current Pseudorabies Virus Outbreak in China. *Sci Rep.* 2017;7:7783. DOI: 10.1038/s41598-017-08269-3

52. Ren J, Wang H, Zhou L et al. Glycoproteins C and D of PRV Strain HB1201 Contribute Individually to the Escape From Bartha-K61 Vaccine-Induced Immunity. *Front Microbiol.* 2020;11:323. DOI: 10.3389/fmicb.2020.00323

53. Yu ZQ, Tong W, Zheng H et al. Variations in glycoprotein B contribute to immunogenic difference between PRV variant JS-2012 and Bartha-K61. *Vet Microbiol.* 2017;208:97-105. DOI: 10.1016/j.vetmic.2017.07.019

54. Qi LS, Larson MH, Gilbert LA et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell.* 2013;152:1173-1183. DOI: 10.1016/j.cell.2013.02.022
55. Gilbert LA, Larson MH, Morsut L et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell.* 2013;154:442-451. DOI: 10.1016/j.cell.2013.06.044
56. Villamizar O, Waters SA, Scott T et al. Targeted Activation of Cystic Fibrosis Transmembrane Conductance Regulator. *Mol Ther.* 2019;27:1737-1748. DOI: 10.1016/j.ymthe.2019.07.002
57. Limsirichai P, Gaj T, Schaffer DV. CRISPR-mediated Activation of Latent HIV-1 Expression. *Mol Ther.* 2016;24:499-507. DOI: 10.1038/mt.2015.213
58. Olson A, Basukala B, Lee S et al. Targeted Chromatinization and Repression of HIV-1 Provirus Transcription with Repurposed CRISPR/Cas9. *Viruses.* 2020;12. DOI: 10.3390/v12101154
59. Peng Q, Fang L, Ding Z et al. Rapid manipulation of the porcine epidemic diarrhea virus genome by CRISPR/Cas9 technology. *J Virol Methods.* 2020;276:113772. DOI: 10.1016/j.jviromet.2019.113772
60. Wang G, Liang R, Liu Z et al. The N-Terminal Domain of Spike Protein Is Not the Enteric Tropism Determinant for Transmissible Gastroenteritis Virus in Piglets. *Viruses.* 2019;11. DOI: 10.3390/v11040313
61. Price AA, Sampson TR, Ratner HK et al. Cas9-mediated targeting of viral RNA in eukaryotic cells. *Proc Natl Acad Sci U S A.* 2015;112:6164-6169. DOI: 10.1073/pnas.1422340112
62. Freije CA, Myhrvold C, Boehm CK et al. Programmable Inhibition and Detection of RNA Viruses Using Cas13. *Mol Cell.* 2019;76:826-837 e811. DOI: 10.1016/j.molcel.2019.09.013
63. Abbott TR, Dhamdhere G, Liu Y et al. Development of CRISPR as an Antiviral Strategy to Combat SARS-CoV-2 and Influenza. *Cell.* 2020;181:865-876 e812. DOI: 10.1016/j.cell.2020.04.020
64. Papa G, Venditti L, Braga L et al. CRISPR-Csy4-Mediated Editing of Rotavirus Double-Stranded RNA Genome. *Cell Rep.* 2020;32:108205. DOI: 10.1016/j.celrep.2020.108205
65. Strutt SC, Torrez RM, Kaya E et al. RNA-dependent RNA targeting by CRISPR-Cas9. *Elife.* 2018;7. DOI: 10.7554/eLife.32724
66. Cui J, Techakriengkrai N, Nedumpun T et al. Abrogation of PRRSV infectivity by CRISPR-Cas13b-mediated viral RNA cleavage in mammalian cells. *Sci Rep.* 2020;10:9617. DOI: 10.1038/s41598-020-66775-3
67. Yuan M, Gao X, Chard LS et al. A marker-free system for highly efficient construction of vaccinia virus vectors using CRISPR Cas9. *Mol Ther Methods Clin Dev.* 2015;2:15035. DOI: 10.1038/mtm.2015.35
68. Borca MV, Holinka LG, Berggren KA et al. CRISPR-Cas9, a tool to efficiently increase the development of recombinant African swine fever viruses. *Sci Rep.* 2018;8:3154. DOI: 10.1038/s41598-018-21575-8
69. Hubner A, Petersen B, Keil GM et al. Efficient inhibition of African swine fever virus replication by CRISPR/Cas9 targeting of the viral p30 gene (CP204L). *Sci Rep.* 2018;8:1449. DOI: 10.1038/s41598-018-19626-1
70. Suenaga T, Kohyama M, Hirayasu K et al. Engineering large viral DNA genomes using the CRISPR-Cas9 system. *Microbiol Immunol.* 2014;58:513-522. DOI: 10.1111/1348-0421.12180
71. van Diemen FR, Kruse EM, Hooykaas MJ et al. CRISPR/Cas9-Mediated Genome Editing of Herpesviruses Limits Productive and Latent Infections. *PLoS Pathog.* 2016;12:e1005701. DOI: 10.1371/journal.ppat.1005701
72. Xu X, Fan S, Zhou J et al. The mutated tegument protein UL7 attenuates the virulence of herpes simplex virus 1 by reducing the modulation of alpha-4 gene transcription. *Virol J.* 2016;13:152. DOI:

10.1186/s12985-016-0600-9

73. Fan S, Xu X, Liao Y et al. Attenuated Phenotype and Immunogenic Characteristics of a Mutated Herpes Simplex Virus 1 Strain in the Rhesus Macaque. *Viruses.* 2018;10. DOI: 10.3390/v10050234

74. Oh HS, Neuhausser WM, Eggan P et al. Herpesviral lytic gene functions render the viral genome susceptible to novel editing by CRISPR/Cas9. *Elife.* 2019;8. DOI: 10.7554/eLife.51662

75. Khodadad N, Fani M, Jamehdor S et al. A knockdown of the herpes simplex virus type-1 gene in all-in-one CRISPR vectors. *Folia Histochem Cytobiol.* 2020;58:174-181. DOI: 10.5603/FHC.a2020.0020

76. Chen Y, Zhi S, Liang P et al. Single AAV-Mediated CRISPR-SaCas9 Inhibits HSV-1 Replication by Editing ICP4 in Trigeminal Ganglion Neurons. *Mol Ther Methods Clin Dev.* 2020;18:33-43. DOI: 10.1016/j.omtm.2020.05.011

77. Ebrahimi S, Makvandi M, Abbasi S et al. Developing oncolytic Herpes simplex virus type 1 through UL39 knockout by CRISPR-Cas9. *Iran J Basic Med Sci.* 2020;23:937-944. DOI: 10.22038/ijbms.2020.43864.10286

78. Gao J, Yan X, Banfield BW. Comparative Analysis of UL16 Mutants Derived from Multiple Strains of Herpes Simplex Virus 2 (HSV-2) and HSV-1 Reveals Species-Specific Requirements for the UL16 Protein. *J Virol.* 2018;92. DOI: 10.1128/JVI.00629-18

79. Finnen RL, Banfield BW. CRISPR/Cas9 Mutagenesis of UL21 in Multiple Strains of Herpes Simplex Virus Reveals Differential Requirements for pUL21 in Viral Replication. *Viruses.* 2018;10. DOI: 10.3390/v10050258

80. Liu L, Cheng J, Mou T et al. The Mutation of the Genes Related to Neurovirulence in HSV-2 Produces an Attenuated Phenotype in Mice. *Viruses.* 2020;12. DOI: 10.3390/v12070770

81. Cai L, Hu H, Duan H et al. The construction of a new oncolytic herpes simplex virus expressing murine interleukin-15 with gene-editing technology. *J Med Virol.* 2020. DOI: 10.1002/jmv.25691

82. Kanda T, Furuse Y, Oshitani H et al. Highly Efficient CRISPR/Cas9-Mediated Cloning and Functional Characterization of Gastric Cancer-Derived Epstein-Barr Virus Strains. *J Virol.* 2016;90:4383-4393. DOI: 10.1128/JVI.00060-16

83. Masud H, Yanagi Y, Watanabe T et al. Epstein-Barr Virus BBRF2 Is Required for Maximum Infectivity. *Microorganisms.* 2019;7. DOI: 10.3390/microorganisms7120705

84. Masud H, Watanabe T, Sato Y et al. The BOLF1 gene is necessary for effective Epstein-Barr viral infectivity. *Virology.* 2019;531:114-125. DOI: 10.1016/j.virol.2019.02.015

85. Masud H, Watanabe T, Yoshida M et al. Epstein-Barr Virus BKRF4 Gene Product Is Required for Efficient Progeny Production. *J Virol.* 2017;91. DOI: 10.1128/JVI.00975-17

86. Yuen KS, Wang ZM, Wong NM et al. Suppression of Epstein-Barr virus DNA load in latently infected nasopharyngeal carcinoma cells by CRISPR/Cas9. *Virus Res.* 2018;244:296-303. DOI: 10.1016/j.virusres.2017.04.019

87. Tso FY, West JT, Wood C. Reduction of Kaposi's Sarcoma-Associated Herpesvirus Latency Using CRISPR-Cas9 To Edit the Latency-Associated Nuclear Antigen Gene. *J Virol.* 2019;93. DOI: 10.1128/JVI.02183-18

88. Bierle CJ, Anderholm KM, Wang JB et al. Targeted Mutagenesis of Guinea Pig Cytomegalovirus Using CRISPR/Cas9-Mediated Gene Editing. *J Virol.* 2016;90:6989-6998. DOI: 10.1128/JVI.00139-16

89. Zhao C, Liu H, Xiao T et al. CRISPR screening of porcine sgRNA library identifies host factors associated with Japanese encephalitis virus replication. *Nat Commun.* 2020;11:5178. DOI: 10.1038/s41467-020-18936-1

90. Ye C, Chen J, Wang T et al. Generation and characterization of UL41 null pseudorabies virus

variant in vitro and in vivo. *Virol J.* 2018;15:119. DOI: 10.1186/s12985-018-1025-4

91. Ye C, Chen J, Cheng X et al. Functional analysis of the UL24 protein of suid herpesvirus 1. *Virus Genes.* 2019;55:76-86. DOI: 10.1007/s11262-018-1619-3

92. Luo J, Teng M, Zai X et al. Efficient Mutagenesis of Marek's Disease Virus-Encoded microRNAs Using a CRISPR/Cas9-Based Gene Editing System. *Viruses.* 2020;12. DOI: 10.3390/v12040466

93. Chou YY, Krupp A, Kaynor C et al. Inhibition of JCPyV infection mediated by targeted viral genome editing using CRISPR/Cas9. *Sci Rep.* 2016;6:36921. DOI: 10.1038/srep36921

94. Temblador A, Topalis D, Andrei G et al. CRISPR/Cas9 Editing of the Polyomavirus Tumor Antigens Inhibits Merkel Cell Carcinoma Growth In Vitro. *Cancers (Basel).* 2019;11. DOI: 10.3390/cancers11091260

95. Lee C. CRISPR/Cas9-Based Antiviral Strategy: Current Status and the Potential Challenge. *Molecules.* 2019;24. DOI: 10.3390/molecules24071349

96. Bortnik V, Wu M, Julcher B et al. Loss of HPV type 16 E7 restores cGAS-STING responses in human papilloma virus-positive oropharyngeal squamous cell carcinomas cells. *J Microbiol Immunol Infect.* 2020. DOI: 10.1016/j.jmii.2020.07.010

97. Li C, Guo L, Liu G et al. Reprogrammed CRISPR-Cas13a targeting the HPV16/18 E6 gene inhibits proliferation and induces apoptosis in E6-transformed keratinocytes. *Exp Ther Med.* 2020;19:3856-3860. DOI: 10.3892/etm.2020.8631

98. Kostyushev D, Brezgin S, Kostyusheva A et al. Orthologous CRISPR/Cas9 systems for specific and efficient degradation of covalently closed circular DNA of hepatitis B virus. *Cell Mol Life Sci.* 2019;76:1779-1794. DOI: 10.1007/s00018-019-03021-8

99. Yang YC, Chen YH, Kao JH et al. Permanent Inactivation of HBV Genomes by CRISPR/Cas9-Mediated Non-cleavage Base Editing. *Mol Ther Nucleic Acids.* 2020;20:480-490. DOI: 10.1016/j.omtn.2020.03.005

100. Zheng Q, Bai L, Zheng S et al. Efficient inhibition of duck hepatitis B virus DNA by the CRISPR/Cas9 system. *Mol Med Rep.* 2017;16:7199-7204. DOI: 10.3892/mmr.2017.7518

101. Darcis G, Binda CS, Klaver B et al. The Impact of HIV-1 Genetic Diversity on CRISPR-Cas9 Antiviral Activity and Viral Escape. *Viruses.* 2019;11. DOI: 10.3390/v11030255

102. Binda CS, Klaver B, Berkhout B et al. CRISPR-Cas9 Dual-gRNA Attack Causes Mutation, Excision and Inversion of the HIV-1 Proviral DNA. *Viruses.* 2020;12. DOI: 10.3390/v12030330

103. Ibba G, Piu C, Uleri E et al. Disruption by SaCas9 Endonuclease of HERV-Kenv, a Retroviral Gene with Oncogenic and Neuropathogenic Potential, Inhibits Molecules Involved in Cancer and Amyotrophic Lateral Sclerosis. *Viruses.* 2018;10. DOI: 10.3390/v10080412

104. Guell M. Genome-Wide PERV Inactivation in Pigs Using CRISPR/Cas9. *Methods Mol Biol.* 2020;2110:139-149. DOI: 10.1007/978-1-0716-0255-3_10

105. Murphy BG, Wolf T, Vogel H et al. An RNA-Directed Gene Editing Strategy for Attenuating the Infectious Potential of Feline Immunodeficiency Virus-Infected Cells: A Proof of Concept. *Viruses.* 2020;12. DOI: 10.3390/v12050511

106. Shen Z, Wang G, Yang Y et al. A conserved region of nonstructural protein 1 from alphacoronaviruses inhibits host gene expression and is critical for viral virulence. *J Biol Chem.* 2019;294:13606-13618. DOI: 10.1074/jbc.RA119.009713

107. Li H, Wang S, Dong X et al. CRISPR-Cas13a Cleavage of Dengue Virus NS3 Gene Efficiently Inhibits Viral Replication. *Mol Ther Nucleic Acids.* 2020;19:1460-1469. DOI: 10.1016/j.omtn.2020.01.028

108. Huang Y, Lin Q, Huo Z et al. Inositol-Requiring Enzyme 1alpha Promotes Zika Virus Infection

through Regulation of Stearoyl Coenzyme A Desaturase 1-Mediated Lipid Metabolism. *J Virol.* 2020;94. DOI: 10.1128/JVI.01229-20

109. Wu W, Orr-Burks N, Karpilow J et al. Development of improved vaccine cell lines against rotavirus. *Sci Data.* 2017;4:170021. DOI: 10.1038/sdata.2017.21

110. Hoeksema F, Karpilow J, Luitjens A et al. Enhancing viral vaccine production using engineered knockout vero cell lines - A second look. *Vaccine.* 2018;36:2093-2103. DOI: 10.1016/j.vaccine.2018.03.010

111. Sharon DM, Nesdoly S, Yang HJ et al. A pooled genome-wide screening strategy to identify and rank influenza host restriction factors in cell-based vaccine production platforms. *Sci Rep.* 2020;10:12166. DOI: 10.1038/s41598-020-68934-y

112. Orr-Burks N, Murray J, Wu W et al. Gene-edited vero cells as rotavirus vaccine substrates. *Vaccine X.* 2019;3:100045. DOI: 10.1016/j.jvacx.2019.100045

113. Roointan A, Morowvat MH. Road to the future of systems biotechnology: CRISPR-Cas-mediated metabolic engineering for recombinant protein production. *Biotechnol Genet Eng Rev.* 2016;32:74-91. DOI: 10.1080/02648725.2016.1270095

114. Kalemera MD, Capella-Pujol J, Chumbe A et al. Optimized cell systems for the investigation of hepatitis C virus E1E2 glycoproteins. *J Gen Virol.* 2020. DOI: 10.1099/jgv.0.001512

115. Grav LM, la Cour Karottki KJ, Lee JS et al. Application of CRISPR/Cas9 Genome Editing to Improve Recombinant Protein Production in CHO Cells. *Methods Mol Biol.* 2017;1603:101-118. DOI: 10.1007/978-1-4939-6972-2_7

116. Jia YL, Guo X, Lu JT et al. CRISPR/Cas9-mediated gene knockout for DNA methyltransferase Dnmt3a in CHO cells displays enhanced transgenic expression and long-term stability. *J Cell Mol Med.* 2018;22:4106-4116. DOI: 10.1111/jcmm.13687

117. Sergeeva D, Camacho-Zaragoza JM, Lee JS et al. CRISPR/Cas9 as a Genome Editing Tool for Targeted Gene Integration in CHO Cells. *Methods Mol Biol.* 2019;1961:213-232. DOI: 10.1007/978-1-4939-9170-9_13

118. Ma H, Kim AS, Kafai NM et al. LDLRAD3 is a receptor for Venezuelan equine encephalitis virus. *Nature.* 2020;588:308-314. DOI: 10.1038/s41586-020-2915-3

119. Petitjean O, Girardi E, Ngondo RP et al. Genome-Wide CRISPR-Cas9 Screen Reveals the Importance of the Heparan Sulfate Pathway and the Conserved Oligomeric Golgi Complex for Synthetic Double-Stranded RNA Uptake and Sindbis Virus Infection. *mSphere.* 2020;5. DOI: 10.1128/mSphere.00914-20

120. Flynn RA, Belk JA, Qi Y et al. Systematic discovery and functional interrogation of SARS-CoV-2 viral RNA-host protein interactions during infection. *bioRxiv.* 2020. DOI: 10.1101/2020.10.06.327445

121. Bae S, Park J, Kim JS. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics.* 2014;30:1473-1475. DOI: 10.1093/bioinformatics/btu048

122. Park J, Kim JS, Bae S. Cas-Database: web-based genome-wide guide RNA library design for gene knockout screens using CRISPR-Cas9. *Bioinformatics.* 2016;32:2017-2023. DOI: 10.1093/bioinformatics/btw103

123. Cui Y, Cheng X, Chen Q et al. CRISP-view: a database of functional genetic screens spanning multiple phenotypes. *Nucleic Acids Res.* 2021;49:D848-D854. DOI: 10.1093/nar/gkaa809

124. Dwane L, Behan FM, Goncalves E et al. Project Score database: a resource for investigating cancer cell dependencies and prioritizing therapeutic targets. *Nucleic Acids Res.* 2021;49:D1365-

D1372. DOI: 10.1093/nar/gkaa882
125. Oughtred R, Stark C, Breitkreutz BJ et al. The BioGRID interaction database: 2019 update. *Nucleic Acids Res.* 2019;47:D529-D541. DOI: 10.1093/nar/gky1079
126. Choi A, Jang I, Han H et al. iCSDB: an integrated database of CRISPR screens. *Nucleic Acids Res.* 2021;49:D956-D961. DOI: 10.1093/nar/gkaa989
127. Bi Y, Sun L, Gao D et al. High-efficiency targeted editing of large viral genomes by RNA-guided nucleases. *PLoS Pathog.* 2014;10:e1004090. DOI: 10.1371/journal.ppat.1004090
128. Epstein AL. HSV-1-derived recombinant and amplicon vectors for preventive or therapeutic gene transfer: an overview. *Gene Ther.* 2005;12 Suppl 1:S153. DOI: 10.1038/sj.gt.3302630
129. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. *Nat Biotechnol.* 2012;30:658-670. DOI: 10.1038/nbt.2287
130. Guo JC, Tang YD, Zhao K et al. Highly Efficient CRISPR/Cas9-Mediated Homologous Recombination Promotes the Rapid Generation of Bacterial Artificial Chromosomes of Pseudorabies Virus. *Front Microbiol.* 2016;7:2110. DOI: 10.3389/fmicb.2016.02110
131. Tang N, Zhang Y, Pedrera M et al. Generating Recombinant Avian Herpesvirus Vectors with CRISPR/Cas9 Gene Editing. *J Vis Exp.* 2019. DOI: 10.3791/58193
132. Tang N, Zhang Y, Sadigh Y et al. Generation of A Triple Insert Live Avian Herpesvirus Vectored Vaccine Using CRISPR/Cas9-Based Gene Editing. *Vaccines (Basel).* 2020;8. DOI: 10.3390/vaccines8010097
133. Chang P, Yao Y, Tang N et al. The Application of NHEJ-CRISPR/Cas9 and Cre-Lox System in the Generation of Bivalent Duck Enteritis Virus Vaccine against Avian Influenza Virus. *Viruses.* 2018;10. DOI: 10.3390/v10020081
134. Zou Z, Huang K, Wei Y et al. Construction of a highly efficient CRISPR/Cas9-mediated duck enteritis virus-based vaccine against H5N1 avian influenza virus and duck Tembusu virus infection. *Sci Rep.* 2017;7:1478. DOI: 10.1038/s41598-017-01554-1
135. Atasoy MO, Rohaim MA, Munir M. Simultaneous Deletion of Virulence Factors and Insertion of Antigens into the Infectious Laryngotracheitis Virus Using NHEJ-CRISPR/Cas9 and Cre-Lox System for Construction of a Stable Vaccine Vector. *Vaccines (Basel).* 2019;7. DOI: 10.3390/vaccines7040207
136. Liu Y, Xu Z, Zhang Y et al. Marek's disease virus as a CRISPR/Cas9 delivery system to defend against avian leukosis virus infection in chickens. *Vet Microbiol.* 2020;242:108589. DOI: 10.1016/j.vetmic.2020.108589
137. Li K, Liu Y, Xu Z et al. Prevention of Avian Retrovirus Infection in Chickens Using CRISPR-Cas9 Delivered by Marek's Disease Virus. *Mol Ther Nucleic Acids.* 2020;21:343-353. DOI: 10.1016/j.omtn.2020.06.009
138. Nakano E, Panicali D, Paoletti E. Molecular genetics of vaccinia virus: demonstration of marker rescue. *Proc Natl Acad Sci U S A.* 1982;79:1593-1596. DOI: 10.1073/pnas.79.5.1593
139. Gowripalan A, Smith S, Stefanovic T et al. Rapid poxvirus engineering using CRISPR/Cas9 as a selection tool. *Commun Biol.* 2020;3:643. DOI: 10.1038/s42003-020-01374-6
140. Gong Y, Chen T, Feng N et al. A highly efficient recombinant canarypox virus-based vaccine against canine distemper virus constructed using the CRISPR/Cas9 gene editing method. *Vet Microbiol.* 2020;251:108920. DOI: 10.1016/j.vetmic.2020.108920
141. Mann R, Mulligan RC, Baltimore D. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell.* 1983;33:153-159. DOI: 10.1016/0092-8674(83)90344-6
142. Verhoeyen E. Advances in foamy virus vector technology and disease correction could speed the

path to clinical application. *Mol Ther.* 2012;20:1105-1107. DOI: 10.1038/mt.2012.97
143. Kotterman MA, Schaffer DV. Engineering adeno-associated viruses for clinical gene therapy. *Nat Rev Genet.* 2014;15:445-451. DOI: 10.1038/nrg3742
144. Chen YH, Keiser MS, Davidson BL. Viral Vectors for Gene Transfer. *Curr Protoc Mouse Biol.* 2018;8:e58. DOI: 10.1002/cpmo.58
145. Park A, Hong P, Won ST et al. Sendai virus, an RNA virus with no risk of genomic integration, delivers CRISPR/Cas9 for efficient gene editing. *Mol Ther Methods Clin Dev.* 2016;3:16057. DOI: 10.1038/mtm.2016.57
146. Gutierrez-Guerrero A, Cosset FL, Verhoeyen E. Lentiviral Vector Pseudotypes: Precious Tools to Improve Gene Modification of Hematopoietic Cells for Research and Gene Therapy. *Viruses.* 2020;12. DOI: 10.3390/v12091016
147. Koike-Yusa H, Li Y, Tan EP et al. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol.* 2014;32:267-273. DOI: 10.1038/nbt.2800
148. Choi JG, Dang Y, Abraham S et al. Lentivirus pre-packed with Cas9 protein for safer gene editing. *Gene Ther.* 2016;23:627-633. DOI: 10.1038/gt.2016.27
149. Lu B, Javidi-Parsijani P, Makani V et al. Delivering SaCas9 mRNA by lentivirus-like bionanoparticles for transient expression and efficient genome editing. *Nucleic Acids Res.* 2019;47:e44. DOI: 10.1093/nar/gkz093
150. Senis E, Fatouros C, Grosse S et al. CRISPR/Cas9-mediated genome engineering: an adeno-associated viral (AAV) vector toolbox. *Biotechnol J.* 2014;9:1402-1412. DOI: 10.1002/biot.201400046
151. Colella P, Ronzitti G, Mingozzi F. Emerging Issues in AAV-Mediated In Vivo Gene Therapy. *Mol Ther Methods Clin Dev.* 2018;8:87-104. DOI: 10.1016/j.omtm.2017.11.007
152. Hanlon KS, Kleinstiver BP, Garcia SP et al. High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat Commun.* 2019;10:4439. DOI: 10.1038/s41467-019-12449-2
153. Tang N, Zhang YY, Sadigh Y et al. Generation of A Triple Insert Live Avian Herpesvirus Vectored Vaccine Using CRISPR/Cas9-Based Gene Editing. *Vaccines-Basel.* 2020;8. DOI: ARTN 97 10.3390/vaccines8010097
154. Chang P, Ameen F, Sealy JE et al. Application of HDR-CRISPR/Cas9 and Erythrocyte Binding for Rapid Generation of Recombinant Turkey Herpesvirus-Vectored Avian Influenza Virus Vaccines. *Vaccines (Basel).* 2019;7. DOI: 10.3390/vaccines7040192
155. Russell TA, Stefanovic T, Tscharke DC. Engineering herpes simplex viruses by infection-transfection methods including recombination site targeting by CRISPR/Cas9 nucleases. *J Virol Methods.* 2015;213:18-25. DOI: 10.1016/j.jviromet.2014.11.009
156. Lindel F, Dodt CR, Weidner N et al. TraFo-CRISPR: Enhanced Genome Engineering by Transient Foamy Virus Vector-Mediated Delivery of CRISPR/Cas9 Components. *Mol Ther Nucleic Acids.* 2019;18:708-726. DOI: 10.1016/j.omtn.2019.10.006
157. Babon JJ, McKenzie M, Cotton RG. The use of resolvases T4 endonuclease VII and T7 endonuclease I in mutation detection. *Mol Biotechnol.* 2003;23:73-81. DOI: 10.1385/MB:23:1:73
158. Vouillot L, Thelie A, Pollet N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3 (Bethesda).* 2015;5:407-415. DOI: 10.1534/g3.114.015834
159. Samarut E, Lissouba A, Drapeau P. A simplified method for identifying early CRISPR-induced indels in zebrafish embryos using High Resolution Melting analysis. *BMC Genomics.* 2016;17:547. DOI: 10.1186/s12864-016-2881-1

160. Yang Z, Steentoft C, Hauge C et al. Fast and sensitive detection of indels induced by precise gene targeting. *Nucleic Acids Res.* 2015;43:e59. DOI: 10.1093/nar/gkv126
161. Zhang H, Zhou Y, Wang Y et al. A surrogate reporter system for multiplexable evaluation of CRISPR/Cas9 in targeted mutagenesis. *Sci Rep.* 2018;8:1042. DOI: 10.1038/s41598-018-19317-x
162. Sentmanat MF, Peters ST, Florian CP et al. A Survey of Validation Strategies for CRISPR-Cas9 Editing. *Sci Rep.* 2018;8:888. DOI: 10.1038/s41598-018-19441-8
163. Li F, Wing K, Wang JH et al. Comparison of CRISPR/Cas Endonucleases for in vivo Retinal Gene Editing. *Front Cell Neurosci.* 2020;14:570917. DOI: 10.3389/fncel.2020.570917
164. Kim S, Kim D, Cho SW et al. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* 2014;24:1012-1019. DOI: 10.1101/gr.171322.113
165. Ju E, Li T, Ramos da Silva S et al. Gold Nanocluster-Mediated Efficient Delivery of Cas9 Protein through pH-Induced Assembly-Disassembly for Inactivation of Virus Oncogenes. *ACS Appl Mater Interfaces.* 2019;11:34717-34724. DOI: 10.1021/acsami.9b12335
166. Hiatt J, Cavero DA, McGregor MJ et al. Efficient generation of isogenic primary human myeloid cells using CRISPR-Cas9 ribonucleoproteins. *Cell Rep.* 2021;35:109105. DOI: 10.1016/j.celrep.2021.109105
167. Rodgers K, McVey M. Error-Prone Repair of DNA Double-Strand Breaks. *J Cell Physiol.* 2016;231:15-24. DOI: 10.1002/jcp.25053
168. Chang HHY, Pannunzio NR, Adachi N et al. Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nat Rev Mol Cell Biol.* 2017;18:495-506. DOI: 10.1038/nrm.2017.48
169. Vriend LE, Prakash R, Chen CC et al. Distinct genetic control of homologous recombination repair of Cas9-induced double-strand breaks, nicks and paired nicks. *Nucleic Acids Res.* 2016;44:5204-5217. DOI: 10.1093/nar/gkw179
170. Pennington TH, Follett EA. Vaccinia virus replication in enucleate BSC-1 cells: particle production and synthesis of viral DNA and proteins. *J Virol.* 1974;13:488-493. DOI: 10.1128/JVI.13.2.488-493.1974
171. Luteijn RD, Drexler I, Smith GL et al. Mutagenic repair of double-stranded DNA breaks in vaccinia virus genomes requires cellular DNA ligase IV activity in the cytosol. *J Gen Virol.* 2018;99:790-804. DOI: 10.1099/jgv.0.001034
172. Siegrist CM, Kinahan SM, Settecerri T et al. CRISPR/Cas9 as an antiviral against Orthopoxviruses using an AAV vector. *Sci Rep.* 2020;10:19307. DOI: 10.1038/s41598-020-76449-9
173. Vartak SV, Raghavan SC. Inhibition of nonhomologous end joining to increase the specificity of CRISPR/Cas9 genome editing. *FEBS J.* 2015;282:4289-4294. DOI: 10.1111/febs.13416
174. Riesenberg S, Maricic T. Targeting repair pathways with small molecules increases precise genome editing in pluripotent stem cells. *Nat Commun.* 2018;9:2164. DOI: 10.1038/s41467-018-04609-7
175. Tang YD, Guo JC, Wang TY et al. CRISPR/Cas9-mediated 2-sgRNA cleavage facilitates pseudorabies virus editing. *FASEB J.* 2018;32:4293-4301. DOI: 10.1096/fj.201701129R
176. Li Z, Bi Y, Xiao H et al. CRISPR-Cas9 system-driven site-specific selection pressure on Herpes simplex virus genomes. *Virus Res.* 2018;244:286-295. DOI: 10.1016/j.virusres.2017.03.010
177. Saha J, Wang SY, Davis AJ. Examining DNA Double-Strand Break Repair in a Cell Cycle-Dependent Manner. *Methods Enzymol.* 2017;591:97-118. DOI: 10.1016/bs.mie.2017.03.012
178. Mansouri M, Bellon-Echeverria I, Rizk A et al. Highly efficient baculovirus-mediated multigene

delivery in primary cells. *Nat Commun.* 2016;7:11529. DOI: 10.1038/ncomms11529
179. Mansouri M, Ehsaei Z, Taylor V et al. Baculovirus-based genome editing in primary cells. *Plasmid.* 2017;90:5-9. DOI: 10.1016/j.plasmid.2017.01.003
180. Pattanayak V, Lin S, Guilinger JP et al. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol.* 2013;31:839-843. DOI: 10.1038/nbt.2673
181. Sullivan NT, Allen AG, Atkins AJ et al. Designing Safer CRISPR/Cas9 Therapeutics for HIV: Defining Factors That Regulate and Technologies Used to Detect Off-Target Editing. *Front Microbiol.* 2020;11:1872. DOI: 10.3389/fmicb.2020.01872
182. Bayat H, Omidi M, Rajabibazl M et al. The CRISPR Growth Spurt: from Bench to Clinic on Versatile Small RNAs. *J Microbiol Biotechnol.* 2017;27:207-218. DOI: 10.4014/jmb.1607.07005
183. Fu Y, Sander JD, Reyon D et al. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol.* 2014;32:279-284. DOI: 10.1038/nbt.2808
184. Singh R, Kuscu C, Quinlan A et al. Cas9-chromatin binding information enables more accurate CRISPR off-target prediction. *Nucleic Acids Res.* 2015;43:e118. DOI: 10.1093/nar/gkv575
185. Philippe N, Legendre M, Doutre G et al. Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. *Science.* 2013;341:281-286. DOI: 10.1126/science.1239181
186. Molla KA, Yang Y. Predicting CRISPR/Cas9-Induced Mutations for Precise Genome Editing. *Trends Biotechnol.* 2020;38:136-141. DOI: 10.1016/j.tibtech.2019.08.002
187. Wang G, Zhao N, Berkhout B et al. CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape. *Mol Ther.* 2016;24:522-526. DOI: 10.1038/mt.2016.24
188. Yoder KE, Bundschuh R. Host Double Strand Break Repair Generates HIV-1 Strains Resistant to CRISPR/Cas9. *Sci Rep.* 2016;6:29530. DOI: 10.1038/srep29530
189. Shen MW, Arbab M, Hsu JY et al. Predictable and precise template-free CRISPR editing of pathogenic variants. *Nature.* 2018;563:646-651. DOI: 10.1038/s41586-018-0686-x
190. Allen F, Crepaldi L, Alsinet C et al. Predicting the mutations generated by repair of Cas9-induced double-strand breaks. *Nat Biotechnol.* 2018. DOI: 10.1038/nbt.4317
191. Leenay RT, Aghazadeh A, Hiatt J et al. Large dataset enables prediction of repair after CRISPR-Cas9 editing in primary T cells. *Nat Biotechnol.* 2019;37:1034-1037. DOI: 10.1038/s41587-019-0203-2
192. Lebbink RJ, de Jong DC, Wolters F et al. A combinational CRISPR/Cas9 gene-editing approach can halt HIV replication and prevent viral escape. *Sci Rep.* 2017;7:41968. DOI: 10.1038/srep41968
193. Zetsche B, Gootenberg JS, Abudayyeh OO et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell.* 2015;163:759-771. DOI: 10.1016/j.cell.2015.09.038