OPINION

Gene Editing Approaches against Viral Infections and Strategy to Prevent Occurrence of Viral Escape

Martyn K. White, Wenhui Hu, Kamel Khalili*

Department of Neuroscience, Center for Neurovirology and Comprehensive NeuroAIDS Center, Lewis Katz School of Medicine at Temple University, Philadelphia, Pennsylvania, United States of America

* kamel.khalili@temple.edu

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Introduction

Powerful new gene editing techniques promise groundbreaking opportunities for novel therapeutic options to important illnesses, including cancer, genetic disorders [1], and viral infections [2]. These techniques include zinc finger nucleases (ZFN) [3], transcription activator-like effector nucleases (TALEN) [4], and clustered regulatory interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9) [5, 6]. In particular, CRISPR/Cas9 provides an effective, highly specific, and versatile tool applicable to important human viruses, including HIV-1 [7]. CRISPR/Cas9 is elegant and simple compared to ZFN and TALEN because it uses one or more guide RNAs (gRNA), which are simple to produce and specifically target any sequence in an adaptable and flexible way for different targets, such as viral genes, by changing the gRNA sequence [8].

Cas9 cuts both strands of the DNA target, resulting in a double-strand break (DSB), usually repaired by nonhomologous end-joining (NHEJ), an error-prone arm of the DNA repair pathway that introduces insertions and deletions (InDels) at the break site. While InDel introduction is quite rare, the products of accurate repair are targets for recleavage by CRISPR/Cas9, whereas InDel products are not, and, hence, InDel products accumulate with repeated cleavage cycles [9, 10].

In this short review, we discuss CRISPR/Cas9 in combating human viruses by specifically targeting and disrupting essential viral genes. We will also discuss generation of viral escape mutants resistant to Cas9/gRNA and how this may be overcome.

Application to Human Viruses

Genetic editing applications, including CRISPR/Cas9, can disrupt both episomal and integrated DNA viruses and can be applied to several human viruses [2], including papillomaviruses HPV16 and HPV18 [11–13], hepatitis B virus (HBV) [14–17], Epstein-Barr virus (EBV) [18, 19], HIV-1 [7, 20–27], polyomavirus JC (JCV) [28], Herpes simplex virus-1 [29, 30], and other herpesviruses [30].

HPV16 and HPV18 are DNA viruses that cause cervical carcinoma through viral proteins E6 and E7. CRISPR/Cas9 targeting HPV E6 and E7 genes is effective with HPV-transformed cell lines and is potentially applicable to effective clinical therapy for HPV-associated tumors [12–14]. HBV is a DNA virus and causes acute and chronic liver infections and hepatocellular carcinoma (HCC). CRISPR/Cas9 can disrupt HBV in vitro and in vivo and has potential in the eradication of persistent HBV infections [11, 13, 15, 16]. EBV is a herpesvirus and causes

Burkitt's lymphoma and nasopharyngeal carcinoma. CRISPR/Cas9 can ablate expression of EBV genes and is feasible as an approach to EBV infection [18, 19].

HIV provirus permanently integrates into the host genome and becomes a persistent viral reservoir with potential to reactivate and cause disease anew, making HIV-1/AIDS a chronic, lifelong infection refractory to the immune system and antiviral drug regimens. CRISPR/Cas9 allows the excision of segments of integrated proviral DNA in different latently infected cell types by targeting sequences within the HIV-1 long terminal repeat (LTR) flanking the provirus and allowing complete provirus excision [20–22, 24–26]. These experiments were performed with T cells [20], microglia, promonocytic T cells [21], primary CD4+ T-cells cultured ex vivo [22], induced pluripotent stem cells [24], HEK293T cells [25], and Jurkat cells [26], indicating that the ability of CRISPR/Cas9 to excise HIV provirus is not restricted by cell-type–specific factors. While the fate of the eliminated DNA arising after excision of segments of integrated proviral DNA is not known for certain, we tend to think that after excision from the genome, it will be degraded. Thus, there is no published evidence that extrachromosomal double-stranded circles resulting from excision can integrate back into the chromosomal DNA. For example, we recently used CRISPR/Cas9 gene editing to eliminate HIV-1 genomes from human T-lymphoid cells and comprehensively assessed the HIV-1 eradicated cells by whole-genome sequencing to rule out any off-target effects [22]. No reintegrated viral DNA was found in this study.

The CRISPR/Cas9 approach has also allowed uninfected cells to be prophylactically protected against HIV infection [21, 22]. CRISPR/Cas9 was used in two murine models in which tail-vein or intraperitoneal injection of transgenic animals with virus vector expressing Cas9 and a multiplex of gRNAs resulted in the cleavage of integrated HIV-1 DNA provirus in many tissues, indicating proof-of-concept for in vivo eradication of integrated HIV-1 DNA by CRISPR/Cas9 [23]. Kaminski et al [27] personalized CRISPR/Cas9 activity by placing the Cas9 gene under the control of an HIV-1 promoter, which is activated by the HIV-1 Tat. Functional activation of CRISPR/Cas9 by Tat occurred during HIV-1 infection, resulting in the excision of a designated segment of integrated HIV-1 proviral DNA and consequently suppressing viral expression [27]. Thus, regulated expression of Cas9 by Tat provides a novel and safe strategy for ablation of HIV-1 at an early stage of acute infection and silent proviral reactivation in latently infected cells.

In addition to CRISPR/Cas9, the Cre recombinase gene editing approach can be used against HIV-1. This approach uses Cre recombinase from bacteriophage P1 to carry out site-specific recombination between two DNA recognition sites, known as LoxP sites, allowing precise manipulation of genomes, and has been used widely in mouse genetics [31]. Cre target specificity can be manipulated to generate novel site-specific recombinases via directed evolution [32] using substrate-linked protein evolution (SLiPE), which places the recombination target site of interest adjacent to the recombinase coding region, allowing DNA molecules with a successful recombinase coding region to be marked on the linked substrate DNA and recovered from the background of unsuccessful recombinases by PCR [32]. SLiPE has been employed to evolve recombinase-recognizing sequences within an HIV-1 LTR to efficiently excise integrated HIV-1 provirus from latently infected cells [33]. LTR-specific recombinase (Tre-recombinase) is proven to be a promising tool for excision of HIV-1 provirus from infected cells [33, 34]. Hauber et al. [34] reported conditional expression of a Tre-recombinase from self-inactivating lentivirus in HIV-infected cells, which resulted in HIV-1 provirus excision that was effective in vivo in humanized $Rag2^{-/-},\gamma^{-/-}$ mice engrafted with either Tre-transduced primary $CD4^+$ or $CD34^+$ cells [35]. Karpinski et al. [36] used SLiPE to evolve a novel recombinase (Brec1) able to recognize a sequence present in most clinically relevant HIV-1 LTRs subtypes and strains, which excised integrated HIV-1 provirus for a number of clinical isolates of HIV-1 in vitro and in vivo, including mice humanized with cells from patients [36].

The recombinase and CRISPR/Cas9 approaches are suitable for use with either double-stranded (ds) DNA viruses or retroviruses that have dsDNA as part of the life cycle (HIV-1). However, Cas9 endonuclease from *Francisella novicida* can target RNA and inhibits the RNA virus hepatitis C [37], and it may be applicable to other human RNA viruses [38].

The Problem of Viral Escape

Several reports have highlighted a caveat in CRISPR/Cas9 that is important to address when choosing gene editing strategy. CRISPR/Cas9 targeting of HIV-1 generated mutant viruses able to escape and replicate [39–42], as observed earlier with RNAi approaches [43–45]. One report described a mutant HIV-1 generated after ZFN therapy [39], and other reports indicated CRISPR/Cas9 gave profound suppression of HIV replication, but escape mutations were rapidly and consistently generated [40–42]. Escape mutants arose by insertions, deletions, and substitutions (InDels) located within the target site for Cas9 cleavage and are typical for DSBs repaired by NHEJ [40]. Cas9 cleavage inactivates the virus by introduction of mutations by NHEJ, but a subset of these retain viability and escape and are no longer susceptible to the original gRNA. Interestingly, while most InDels contributing to escape at non-coding regions were a single base pair, three base pair InDels were observed when the target was within an HIV-1 coding region; i.e., the InDel event may preserve the HIV-1 open reading frame but destroy the CRISPR gRNA sequence homology [42]. The occurrence of InDel escape mutations is a consequence of NHEJ DNA repair and so may also occur for any DNA virus or retrovirus and, indeed, was recently reported for the pseudorabies herpesvirus [46]. NHEJ is almost always the dominant mode of DNA repair [47, 48] and is the desired pathway of repair of CRISPR/Cas9-generated DSBs, because the purpose of CRISPR/Cas9 is to introduce mutations and inactivate viruses. It is also important to note that DSB and NHEJ repairs may have very different rates in some cells as compared to other cell types; e.g., T-cells relative to myeloid cells [49, 50]. However, as noted above, in studies of HIV-1 provirus excision, no cell-type-specific differences were observed.

Strategies to Combat Viral Escape

Viral escape is not insurmountable if an appropriate choice of gene editing strategy is adopted. InDels introduced by NHEJ following CRISPR/Cas9 cleavage can cause frameshifts or premature stop codons, disrupting the target gene and abrogating its function and viral viability. If a unique locus is targeted, there is a significant possibility that InDels will be generated that allow viral escape; however, if multiplex gRNAs are employed, then the probability of this is greatly reduced, because the chances of two or more viability-conferring mutations are much less, as seen for mutiplex RNA interference (RNAi) [51]. Alternatively, if two gRNAs produce DNA breaks, allowing excision of a large section of DNA, this will permanently prevent the occurrence of escape mutations, as shown in Fig 1. Several studies have demonstrated the strong suppression of HIV-1 using a multiplex approach [21–25]. Table 1 summarizes recent studies [52–75] on the use of CRISPR/Cas9 technology for editing several human viruses using single and multiplex gRNAs, resulting in the introduction of InDels and/or excision of the segment of the viral genome.

Another approach is engineering of new Cas9 variants that cleave at a site outside the target so mutations from NHEJ will not prevent binding and cleavage of Cas9 [76]; e.g., Cpf1 cleaves a region distal from target [77, 78]. Another possibility is to combine CRISPR/Cas9 with RNAi to apply a double assault. RNAi approaches exist for a number of viruses, including HIV-1 [43, 79], HBV [80], HCV [81], HPV [82, 83], JCV [84], and HSV [85]. For example, RNAi against HIV-1 is already reaching the clinic [43, 79]. Delivery and the production of escape mutants

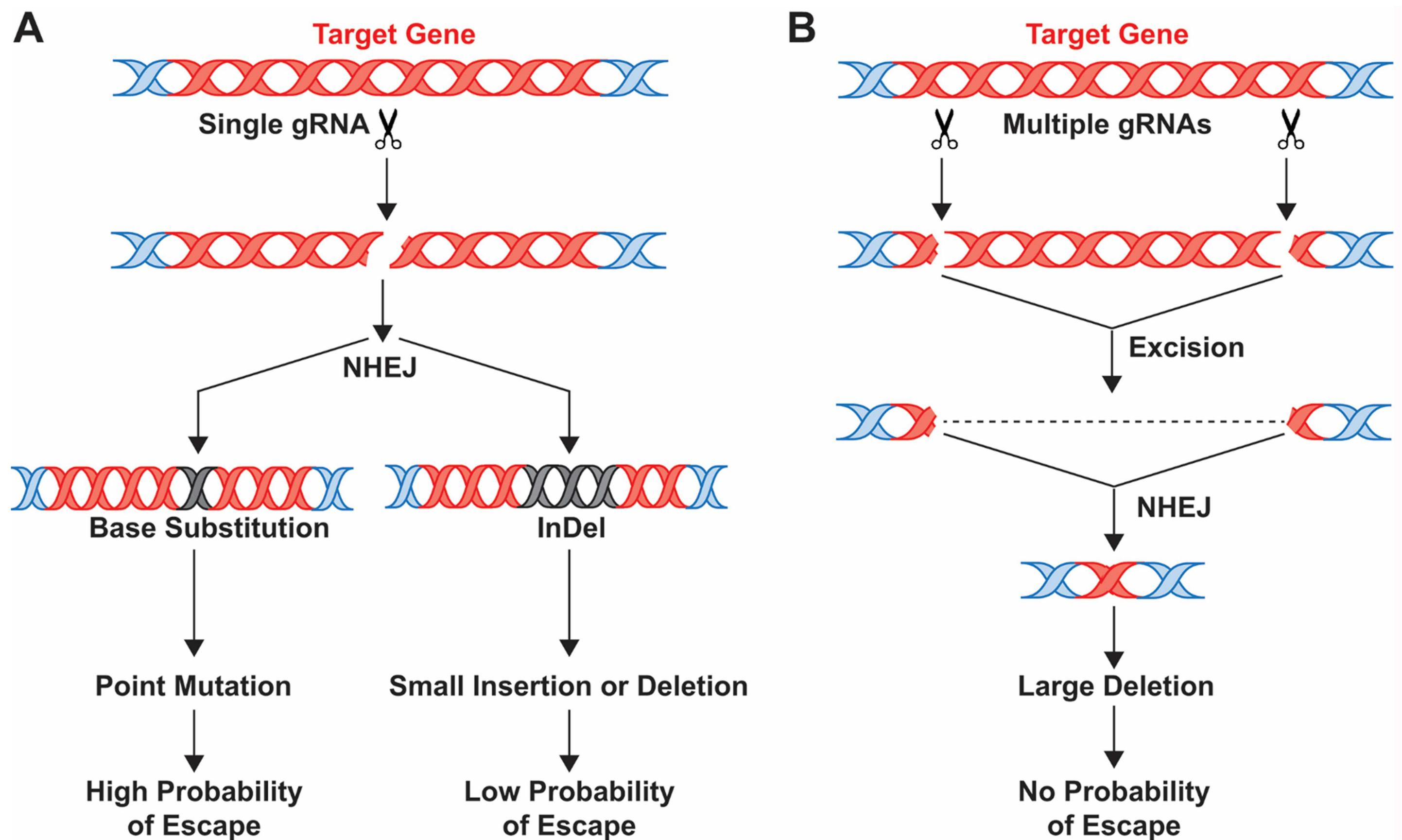

Fig 1. Schematic of the CRIPSR/Cas9 approach to viral inactivation and generation of escape mutants. CRISPR/Cas9 endonuclease can be used to cut a viral genome within an essential target gene using either a single gRNA (**A**) or multiple gRNAs (**B**). With a single gRNA, a base substitution or InDel mutation may occur on repair of the double-strand DNA break by error-prone non-homologous end-joining (NHEJ), which inactivates the virus. However, it is also possible that cleavage at a single site and error-prone NHEJ may also allow the occurrence and selection of escape mutants (**A**). However, when multiple gRNAs are used, this allows the creation of a large deletion with no protein produced and no possibility of escape mutations occurring (**B**).

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are a problem, but combination of RNAi and CRISPR/Cas9 may address these problems. Delivery of both could be performed at the same time, and, just as multiplexing gRNAs has advantages, so the combination of RNAi and CRISPR/Cas9 has advantages over either approach employed alone. Another advantage would be that reduction in viral replication rate by RNAi would be expected to reduce the viral replication rate and viral titer and, hence, render virus more susceptible to CRISPR/Cas9 cleavage [68]. Thus, the combined use of an agent such as RNAi or a small molecule antiviral that slows viral replication may enhance the efficacy of CRISPR. HIV-positive subjects eligible for treatment with CRISPR/Cas9 would also use combined antiretroviral therapy, which would significantly improve the efficacy of CRISPR/Cas9 alleviating concerns related to resistance conferred by high rates of viral replication.

Finally, two other approaches have been applied that should not be affected by CRISPR/Cas9-generated escape mutants. In the case of HIV-1, CRISPR/Cas9 can disrupt cellular HIV coreceptors, CCR5 and CXCR4 [71, 86]; cells become refractory to HIV infection, and CRISPR/Cas9 would not be expected to generate escape mutants. Another approach is the use of Cas9 mutants deficient in endonuclease activity with a transcriptional activation domain (VP64) to target the HIV promoter region in a "shock and kill" strategy [87, 88]. Because the mutant Cas9 has no nuclease activity, there is no possibility of escape mutants being generated.

Table 1. Human viral targets of CRISPR/Cas9.

Virus	Target	gRNAs		Inhibitory effects		Reference
		Monoplex (InDel)	Multiplex (InDel/excision)	In vitro	In vivo	
	-IFI16	-	+	+++	ND	[52]
HSV-1	-Torsin A and B LAP1, LULL1	+	-	+++	ND	[53]
	-ICP0, ICP4, ICP27	+	+	+++	ND	[29]
	-Promoter, E6, E7 (HPV16)	+	+	+++	ND	[13]
	-E6, E7 (HPV16, 18)	+	-	+++	ND	[11]
HPV						
	-E6, E7 (HPV16)	+	-	++	ND	[12, 54]
	E7 (HPV6, HPV11)	+	-	++	ND	[55]
	-cccDNA	-	+	+++	+++	[14–16, 56–61]
HBV	-HBsAg	+	-	++	++	[17, 62]
	-S, X	+	-	++	++	[63]
HCV	-host factor, (CLDN1, OCLN, mir122)	+	-	+++	ND	[64–66]
	-BART	-	+	+++	ND	[19]
EBV						
	-BVRF1	+	-	++	ND	[67]
JCV	-T-antigen	+	+	+++	ND	[28]
	-LTR	+	+	+++	ND	[20–22, 25,26, 68,69]
	-LTR	+	-	++	ND	[40, 41]
HIV-1	-Gag/Pol	+	+	+++	ND	[25, 26]
	-Env, Tat, Rev	+	-	+++	ND	[26]
	-host factors (CCR5, CXCR4, SAMHD1)	+	-	++	ND	[70–75]
	-LTR/Gag D	-	+	++	++	[23]

ND: Not determined

+ indicates experiment performed,—indicates no experiment

Inhibitory Effect: +++: Strong; ++: Moderate

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In conclusion, the possibility of generating escape mutants is a significant consideration when using CRISPR/Cas9 against any virus. This underlines the importance of adopting an appropriate design for gene editing strategies, but it does not detract from the promise of this approach in the treatment of human viral diseases.

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References

1. White MK, Khalili K. CRISPR/Cas9 and cancer targets: future possibilities and present challenges. Oncotarget 2016; 7: 12305–12317. doi: 10.18632/oncotarget.7104 PMID: 26840090
2. White MK, Hu W, Khalili K. The CRISPR/Cas9 genome editing methodology as a weapon against human viruses. Discov Med 2015; 19: 255–262. PMID: 25977188
3. Kim YG, Li L, Chandrasegaran S. Insertion and deletion mutants of FokI restriction endonuclease. J Biol Chem. 1994; 269: 31978–31982. PMID: 7989374
4. Wright DA, Li T, Yang B, Spalding MH. TALEN-mediated genome editing: prospects and perspectives. Biochem J. 2014; 462: 15–24. doi: 10.1042/BJ20140295 PMID: 25057889
5. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 2014; 346: 1258096. doi: 10.1126/science.1258096 PMID: 25430774
6. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell 2014; 157: 1262–1278. doi: 10.1016/j.cell.2014.05.010 PMID: 24906146
7. Khalili K, Kaminski R, Gordon J, Cosentino L, Hu W. Genome editing strategies: potential tools for eradicating HIV-1/AIDS. J Neurovirol. 2015; 21: 310–321. doi: 10.1007/s13365-014-0308-9 PMID: 25716921
8. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013; 8: 2281–2308. doi: 10.1038/nprot.2013.143 PMID: 24157548
9. Canver MC, Bauer DE, Dass A, Yien YY, Chung J, Masuda T, et al. Characterization of genomic deletion efficiency mediated by clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. J Biol Chem. 2014; 289: 21312–21324. doi: 10.1074/jbc.M114.564625 PMID: 24907273
10. Moore JK, Haber JE. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Mol Cell Biol. 1996; 16: 2164–2173. PMID: 8628283
11. Kennedy EM, Kornepati AV, Goldstein M, Bogerd HP, Poling BC, Whisnant AW, et al. Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. J Virol. 2014; 88: 11965–11972. doi: 10.1128/JVI.01879-14 PMID: 25100830
12. Hu Z, Yu L, Zhu D, Ding W, Wang X, Zhang C, et al. Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. Biomed Res Int 2014; 2014: 612823. doi: 10.1155/2014/612823 PMID: 25136604
13. Zhen S, Hua L, Takahashi Y, Narita S, Liu YH, Li Y. In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. Biochem Biophys Res Commun. 2014; 450: 1422–1426. doi: 10.1016/j.bbrc.2014.07.014 PMID: 25044113
14. Kennedy EM, Bassit LC, Mueller H, Kornepati AV, Bogerd HP, Nie T, et al. Suppression of hepatitis B virus DNA accumulation in chronically infected cells using a bacterial CRISPR/Cas RNA-guided DNA endonuclease. Virology 2015; 476: 196–205. doi: 10.1016/j.virol.2014.12.001 PMID: 25553515
15. Lin SR, Yang HC, Kuo YT, Liu CJ, Yang TY, Sung KC, et al. The CRISPR/Cas9 system facilitates clearance of the intrahepatic HBV templates in vivo. Mol Ther Nucleic Acids 2014; 3: e186. doi: 10.1038/mtna.2014.38 PMID: 25137139
16. Seeger C, Sohn JA. Targeting Hepatitis B Virus with CRISPR/Cas9. Mol Ther Nucleic Acids 2014; 3: e216. doi: 10.1038/mtna.2014.68 PMID: 25514649

17. Zhen S, Hua L, Liu YH, Gao LC, Fu J, Wan DY, et al. Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus. Gene Ther 2015; 22: 404–412. doi: 10.1038/gt.2015.2 PMID: 25652100
18. Wang J, Quake SR. RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection. Proc Natl Acad Sci USA 2014; 111: 13157–13162. doi: 10.1073/pnas.1410785111 PMID: 25157128
19. Yuen KS, Chan CP, Wong NH, Ho CH, Ho TH, Lei T, et al. CRISPR/Cas9-mediated genome editing of Epstein-Barr virus in human cells. J Gen Virol. 2015; 96: 626–636. doi: 10.1099/jgv.0.000012 PMID: 25502645
20. Ebina H, Misawa N, Kanemura Y, Koyanagi Y. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci Rep. 2013; 3: 2510. doi: 10.1038/srep02510 PMID: 23974631
21. Hu W, Kaminski R, Yang F, Zhang Y, Cosentino L, Li F, et al. RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proc Natl Acad Sci USA 2014; 111: 11461–11466. doi: 10.1073/pnas.1405186111 PMID: 25049410
22. Kaminski R, Chen Y, Fischer T, Tedaldi E, Napoli A, Zhang Y, et al. Elimination of HIV-1 genomes from human T-lymphoid cells by CRISPR/Cas9 gene editing. Sci Rep. 2016; 6: 22555. doi: 10.1038/srep22555 PMID: 26939770
23. Kaminski R, Bella R, Yin C, Otte J, Ferrante P, Gendelman HE, et al. Excision of HIV-1 DNA by gene editing: a proof-of-concept in vivo study. Gene Ther. 2016; 23: 696. doi: 10.1038/gt.2016.45 PMID: 27488023
24. Liao HK, Gu Y, Diaz A, Marlett J, Takahashi Y, Li M, et al. Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. Nat Commun. 2015; 6: 6413. doi: 10.1038/ncomms7413 PMID: 25752527
25. Yin C, Zhang T, Li F, Yang F, Putatunda R, Young WB, et al. Functional screening of guide RNAs targeting the regulatory and structural HIV-1 viral genome for a cure of AIDS. AIDS 2016; 30: 1163–1174. doi: 10.1097/QAD.0000000000001079 PMID: 26990633
26. Zhu W, Lei R, Le Duff Y, Li J, Guo F, Wainberg MA, et al. The CRISPR/Cas9 system inactivates latent HIV-1 proviral DNA. Retrovirology 2015; 12: 22. doi: 10.1186/s12977-015-0150-z PMID: 25808449
27. Kaminski R, Chen Y, Salkind J, Bella R, Young WB, Ferrante P, et al. Negative feedback regulation of HIV-1 by gene editing strategy. Sci Rep. 2016; 6: 31527. doi: 10.1038/srep31527 PMID: 27528385
28. Wollebo HS, Bellizzi A, Kaminski R, Hu W, White MK, Khalili K. CRISPR/Cas9 System as an agent for eliminating Polyomavirus JC infection. PLoS ONE 2015; 10: e0136046. doi: 10.1371/journal.pone.0136046 PMID: 26360417
29. Roehm PC, Shekarabi M, Wollebo HS, Bellizzi A, He L, Salkind J, et al. Inhibition of HSV-1 Replication by Gene Editing Strategy. Sci Rep. 2016; 6: 23146. doi: 10.1038/srep23146 PMID: 27064617
30. van Diemen FR, Kruse EM, Hooykaas MJ, Bruggeling CE, Schürch AC, van Ham PM, 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 PMID: 27362483
31. Meinke G, Bohm A, Hauber J, Pisabarro MT, Buchholz F. Cre Recombinase and other tyrosine recombinases. Chem Rev. 2016; In Press.
32. Buchholz F, Stewart AF. Alteration of Cre recombinase site specificity by substrate-linked protein evolution. Nat Biotechnol 2001; 19: 1047–1052. doi: 10.1038/nbt1101-1047 PMID: 11689850
33. Sarkar I, Hauber I, Hauber J, Buchholz F. HIV-1 proviral DNA excision using an evolved recombinase. Science 2007; 316: 1912–1915. doi: 10.1126/science.1141453 PMID: 17600219
34. Buchholz F, Hauber J. Engineered DNA modifying enzymes: components of a future strategy to cure HIV/AIDS. Antiviral Res. 2013; 97: 211–217. doi: 10.1016/j.antiviral.2012.12.017 PMID: 23267832
35. Hauber I, Hofmann-Sieber H, Chemnitz J, Dubrau D, Chusainow J, Stucka R, et al. Highly significant antiviral activity of HIV-1 LTR-specific Tre-recombinase in humanized mice. PLoS Pathog. 2013; 9: e1003587. doi: 10.1371/journal.ppat.1003587 PMID: 24086129
36. Karpinski J, Hauber I, Chemnitz J, Schäfer C, Paszkowski-Rogacz M, Chakraborty D et al. Directed evolution of a recombinase that excises the provirus of most HIV-1 primary isolates with high specificity. Nat Biotechnol. 2016; 34: 401–409. doi: 10.1038/nbt.3467 PMID: 26900663
37. Price AA, Sampson TR, Ratner HK, Grakoui A, Weiss DS. Cas9-mediated targeting of viral RNA in eukaryotic cells. Proc Natl Acad Sci USA 2015; 112: 6164–6169. doi: 10.1073/pnas.1422340112 PMID: 25918406
38. Price AA, Grakoui A, Weiss DS. Harnessing the Prokaryotic Adaptive Immune System as a Eukaryotic Antiviral Defense. Trends Microbiol. 2016; 24: 294–306. doi: 10.1016/j.tim.2016.01.005 PMID: 26852268

39. De Silva Feelixge HS, Stone D, Pietz HL, Roychoudhury P, Greninger AL, Schiffer JT, et al. Detection of treatment-resistant infectious HIV after genome-directed antiviral endonuclease therapy. Antiviral Res. 2016; 126: 90–98. doi: 10.1016/j.antiviral.2015.12.007 PMID: 26718067
40. Wang G, Zhao N, Berkhout B, Das AT. 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 PMID: 26796669
41. Wang Z, Pan Q, Gendron P, Zhu W, Guo F, Cen S, et al. CRISPR/Cas9-derived mutations both inhibit HIV-1 replication and accelerate viral escape. Cell Rep. 2016; 15: 481–489. doi: 10.1016/j.celrep.2016.03.042 PMID: 27068471
42. 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 PMID: 27404981
43. Bobbin ML, Burnett JC, Rossi JJ. RNA interference approaches for treatment of HIV-1 infection. Genome Med. 2015; 7: 50. doi: 10.1186/s13073-015-0174-y PMID: 26019725
44. Das AT, Brummelkamp TR, Westerhout EM, Vink M, Madiredjo M, Bernards R, et al. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virol. 2004; 78: 2601–2605. doi: 10.1128/JVI.78.5.2601-2605.2004 PMID: 14963165
45. ter Brake O, von Eije KJ, Berkhout B. Probing the sequence space available for HIV-1 evolution. AIDS 2008; 22: 1875–1877. doi: 10.1097/QAD.0b013e328309efe3 PMID: 18753935
46. Peng Z, Ouyang T, Pang D, Ma T, Chen X, Guo N, 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 PMID: 27507009
47. Govindan G, Ramalingam S. Programmable site-specific nucleases for targeted genome engineering in higher eukaryotes. J Cell Physiol. 2016; 231: 2380–2392. doi: 10.1002/jcp.25367 PMID: 26945523
48. Kaulich M, Dowdy SF. Combining CRISPR/Cas9 and rAAV templates for efficient gene editing. Nucleic Acid Ther. 2015; 25: 287–296. doi: 10.1089/nat.2015.0545 PMID: 26540648
49. Rooney S, Chaudhuri J, Alt FW. The role of the non-homologous end-joining pathway in lymphocyte development. Immunol Rev. 2004; 200: 115–131. doi: 10.1111/j.0105-2896.2004.00165.x PMID: 15242400
50. Felgentreff K, Du L, Weinacht KG, Dobbs K, Bartish M, Giliani S, et al. Differential role of nonhomologous end joining factors in the generation, DNA damage response, and myeloid differentiation of human induced pluripotent stem cells. Proc Natl Acad Sci USA. 2014; 111: 8889–8894. doi: 10.1073/pnas.1323649111 PMID: 24889605
51. Bos TJ, De Bruyne E, Heirman C, Vanderkerken K. In search of the most suitable lentiviral shRNA system. Curr Gene Ther. 2009; 9: 192–211. PMID: 19519364
52. Johnson KE, Bottero V, Flaherty S, Dutta S, Singh VV, Chandran B. IFI16 restricts HSV-1 replication by accumulating on the hsv-1 genome, repressing HSV-1 gene expression, and directly or indirectly modulating histone modifications. PLoS Pathog 2014; 10: e1004503. doi: 10.1371/journal.ppat.1004503 PMID: 25375629
53. Turner EM, Brown RS, Laudermilch E, Tsai PL, Schlieker C. The Torsin Activator LULL1 Is Required for Efficient Growth of Herpes Simplex Virus 1. J Virol 2015; 89, 8444–8452. doi: 10.1128/JVI.01143-15 PMID: 26041288
54. Yu L, Wang X, Zhu D, Ding W, Wang L, Zhang C, et al. Disruption of human papillomavirus 16 E6 gene by clustered regularly interspaced short palindromic repeat/Cas system in human cervical cancer cells. Onco Targets Ther 2014; 8: 37–44. doi: 10.2147/OTT.S64092 PMID: 25565864
55. Liu YC, Cai ZM, Zhang XJ. Reprogrammed CRISPR-Cas9 targeting the conserved regions of HPV6/11 E7 genes inhibits proliferation and induces apoptosis in E7-transformed keratinocytes. Asian J Androl 2016; 18: 475–479. doi: 10.4103/1008-682X.157399 PMID: 26228041
56. Dong C, Qu L, Wang H, Wei L, Dong Y, Xiong S. Targeting hepatitis B virus cccDNA by CRISPR/Cas9 nuclease efficiently inhibits viral replication. Antiviral Res 2015; 118: 110–117. doi: 10.1016/j.antiviral.2015.03.015 PMID: 25843425
57. Guo X, Chen P, Hou X, Xu W, Wang D, Wang TY, et al. The recombined cccDNA produced using minicircle technology mimicked HBV genome in structure and function closely. Sci Rep 2016; 6: 25552. doi: 10.1038/srep25552 PMID: 27174254
58. Liu X, Hao R, Chen S, Guo D, Chen Y. Inhibition of hepatitis B virus by the CRISPR/Cas9 system via targeting the conserved regions of the viral genome. J Gen Virol 2015; 96: 2252–2261. doi: 10.1099/vir.0.000159 PMID: 25904148
59. Ramanan V, Shlomai A, Cox DB, Schwartz RE, Michailidis E, Bhatta A, et al (2015). CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. Sci Rep 2015; 5: 10833. doi: 10.1038/srep10833 PMID: 26035283

60. Seeger C, Sohn JA. Complete spectrum of CRISPR/Cas9-induced mutations on HBV cccDNA. Mol Ther 2016; 24: 1258–1266. doi: 10.1038/mt.2016.94 PMID: 27203444
61. Zhu W, Xie K, Xu Y, Wang L, Chen K, Zhang L, et al. CRISPR/Cas9 produces anti-hepatitis B virus effect in hepatoma cells and transgenic mouse. Virus Res 2016; 217: 125–132. doi: 10.1016/j.virusres.2016.04.003 PMID: 27049051
62. Wang J, Xu ZW, Liu S, Zhang RY, Ding SL, Xie XM, et al. (2015). Dual gRNAs guided CRISPR/Cas9 system inhibits hepatitis B virus replication. World J Gastroenterol 2015; 21: 9554–9565. doi: 10.3748/wjg.v21.i32.9554 PMID: 26327763
63. Karimova M, Beschorner N, Dammermann W, Chemnitz J., Indenbirken D, Bockmann JH, et al. CRISPR/Cas9 nickase-mediated disruption of hepatitis B virus open reading frame S and X. Sci Rep 2015; 5: 13734. doi: 10.1038/srep13734 PMID: 26334116
64. Hopcraft SE, Azarm KD, Israelow B, Leveque N, Schwarz MC, Hsu TH, et al. Viral determinants of miR-122-independent Hepatitis C virus replication. mSphere 2015; 1: e00009–e00015. doi: 10.1128/mSphere.00009-15 PMID: 27303683
65. Hopcraft SE, Evans MJ (2015). Selection of a hepatitis C virus with altered entry factor requirements reveals a genetic interaction between the E1 glycoprotein and claudins. Hepatology 2015; 62: 1059–1069. doi: 10.1002/hep.27815 PMID: 25820616
66. Shirasago Y, Shimizu Y, Tanida I, Suzuki T, Suzuki R, Sugiyama K, et al. Occludin-knockout human hepatic Huh7.5.1-8-derived cells are completely resistant to Hepatitis C virus infection. Biol Pharm Bull 2016; 39: 839–848. doi: 10.1248/bpb.b15-01023 PMID: 26887345
67. Kanda T, Furuse Y, Oshitani H, Kiyono T. 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 PMID: 26889033
68. Ueda S, Ebina H, Kanemura Y, Misawa N, Koyanagi Y. Insufficient anti-HIV-1 potency of the CRISPR/Cas9 system for full viral replication. Microbiol Immunol. 2016; 60: 483–496. doi: 10.1111/1348-0421.12395 PMID: 27278725
69. Yan M, Wen J, Liang M, Lu Y, Kamata M, Chen IS. Modulation of gene expression by polymer nanocapsule delivery of DNA cassettes encoding small RNAs. PLoS ONE 2015; 10: e0127986. doi: 10.1371/journal.pone.0127986 PMID: 26035832
70. Bonifati S, Daly MB, St Gelais C, Kim SH, Hollenbaugh JA, Shepard C, et al. SAMHD1 controls cell cycle status, apoptosis and HIV-1 infection in monocytic THP-1 cells. Virology 2016; 495: 92–100. doi: 10.1016/j.virol.2016.05.002 PMID: 27183329
71. Hou P, Chen S, Wang S, Yu X, Chen Y, Jiang M, et al. Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection. Sci Rep. 2015; 5:15577. doi: 10.1038/srep15577 PMID: 26481100
72. Hutter G, Bodor J, Ledger S, Boyd M, Millington M, Tsie M, et al. CCR5 Targeted Cell Therapy for HIV and Prevention of Viral Escape. Viruses 2015; 7: 4186–4203. doi: 10.3390/v7082816 PMID: 26225991
73. Kang H, Minder P, Park MA, Mesquitta WT, Torbett BE, Slukvin II CCR5 disruption in induced pluripotent stem cells using CRISPR/Cas9 Provides selective resistance of immune cells to CCR5-tropic HIV-1 virus. Mol Ther Nucleic Acids 2015; 4: e268. doi: 10.1038/mtna.2015.42 PMID: 26670276
74. Li C, Guan X, Du T, Jin W, Wu B, Liu Y, et al. Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9. J Gen Virol 2016; 96: 2381–2393.
75. Wang W, Ye C, Liu J, Zhang D, Kimata JT, Zhou P. CCR5 gene disruption via lentiviral vectors expressing Cas9 and single guided RNA renders cells resistant to HIV-1 infection. PLoS ONE 2014; 9: e115987. doi: 10.1371/journal.pone.0115987 PMID: 25541967
76. Liang C, Wainberg MA, Das AT, Berkhout B. CRISPR/Cas9: a double-edged sword when used to combat HIV infection. Retrovirology 2016; 13: 37. doi: 10.1186/s12977-016-0270-0 PMID: 27230886
77. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, 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 PMID: 26422227
78. Yamano T, Nishimasu H, Zetsche B, Hirano H, Slaymaker IM, Li Y, et al. Crystal structure of Cpf1 in complex with guide RNA and target DNA. Cell 2016; 165: 949–962. doi: 10.1016/j.cell.2016.04.003 PMID: 27114038
79. Swamy MN, Wu H, Shankar P. Recent advances in RNAi-based strategies for therapy and prevention of HIV-1/AIDS. Adv Drug Deliv Rev. 2016; pii:S0169-409X(16)30091-6.
80. Gish RG, Yuen MF, Chan HL, Given BD, Lai CL, Locarnini SA, et al. Synthetic RNAi triggers and their use in chronic hepatitis B therapies with curative intent. Antiviral Res. 2015; 121: 97–108. doi: 10.1016/j.antiviral.2015.06.019 PMID: 26129970

81. Verstegen MM, Pan Q, van der Laan LJ. Gene therapies for hepatitis C virus. Adv Exp Med Biol 2015; 848: 1–29. doi: 10.1007/978-1-4939-2432-5_1 PMID: 25757613

82. Jung HS, Rajasekaran N, Ju W, Shin YK. Human papillomavirus: current and future RNAi Therapeutic strategies for cervical cancer. J Clin Med. 2015; 4: 1126–1155. doi: 10.3390/jcm4051126 PMID: 26239469

83. Jung HS, Rajasekaran N, Song SY, Kim YD, Hong S, Choi HJ, et al. Human papillomavirus E6/E7-specific siRNA potentiates the effect of radiotherapy for cervical cancer in vitro and in vivo. Int J Mol Sci. 2015; 16: 12243–12260. doi: 10.3390/ijms160612243 PMID: 26035754

84. Radhakrishnan S, Gordon J, Del Valle L, Cui J, Khalili K. Intracellular approach for blocking JC virus gene expression by using RNA interference during viral infection. J Virol. 2004; 78: 7264–7269. doi: 10.1128/JVI.78.13.7264-7269.2004 PMID: 15194802

85. Paavilainen H, Lehtinen J, Romanovskaya A, Nygårdas M, Bamford DH, Poranen MM, et al. Inhibition of clinical pathogenic herpes simplex virus 1 strains with enzymatically created siRNA pools. J Med Virol. 2016; 88(12):2196–2205. doi: 10.1002/jmv.24578 PMID: 27191509

86. Ye L, Wang J, Beyer AI, Teque F, Cradick TJ, Qi Z, et al. Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Δ32 mutation confers resistance to HIV infection. Proc Natl Acad Sci USA 2014; 111: 9591–9596. doi: 10.1073/pnas.1407473111 PMID: 24927590

87. Saayman SM, Lazar DC, Scott TA, Hart JR, Takahashi M, Burnett JC, et al. Potent and targeted activation of latent HIV-1 using the CRISPR/dCas9 activator complex. Mol Ther. 2016; 24: 488–498. doi: 10.1038/mt.2015.202 PMID: 26581162

88. Zhang Y, Yin C, Zhang T, Li F, Yang W, Kaminski R, et al. CRISPR/gRNA-directed synergistic activation mediator (SAM) induces specific, persistent and robust reactivation of the HIV-1 latent reservoirs. Sci Rep. 2015; 5: 16277. doi: 10.1038/srep16277 PMID: 26538064