

# Genome editing for the treatment of tumorigenic viral infections and virus-related carcinomas

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**Abstract** Viral infections cause at least 10%–15% of all human carcinomas. Over the last century, the elucidation of viral oncogenic roles in many cancer types has provided fundamental knowledge on carcinogenetic mechanisms and established a basis for the early intervention of virus-related cancers. Meanwhile, rapidly evolving genome-editing techniques targeting viral DNA/RNA have emerged as novel therapeutic strategies for treating virus-related carcinogenesis and have begun showing promising results. This review discusses the recent advances of genome-editing tools for treating tumorigenic viruses and their corresponding cancers, the challenges that must be overcome before clinically applying such genome-editing technologies, and more importantly, the potential solutions to these challenges.

**Keywords** genome-editing tools; tumorigenic virus; delivery method; off-target effect; virus-related carcinoma

## Introduction

Viral infections still pose a great threat to the human population. Of the many effects of these infections, cancer is the most lethal to the host. Viral infections cause at least 10%–15% of all human carcinomas [1]. The possible mechanisms for virus-induced carcinogenesis include chronic infection and inflammation [2,3], the expression of viral oncogenes that disturb normal cell cycle/functions [4–6], integration into the host genome and functional alteration of oncogenes and tumor suppressor genes [7], and host immune system deficiency against neoplasms [7].

Although no ultimate cure currently exists for most tumorigenic viruses, research in this field is growing rapidly. With the recent advancements in genome-editing technologies, artificial nucleases, for instance, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced

short palindromic repeats/CRISPR-associated (CRISPR/Cas9) system have long been used to treat tumorigenic viral infections as well as their related cancers. Furthermore, studies on these technologies have greatly progressed toward eradicating viral infections and even curing virus-induced carcinomas [8–12]. However, many obstacles remain to be overcome before patients and doctors can benefit from these technical advancements. This review provides an overview of the different genome-editing techniques used in treating viral infections and their associated tumors. It focuses on the main concerns and challenges in the clinical application of artificially engineered nucleases. Potential resolutions to concerns/challenges as regards these techniques are also discussed.

## Development of genome-editing tools and their application in treating tumorigenic viruses and associated carcinomas

In 1996, zinc finger proteins were fused to the natural-type IIS restriction enzyme-FokI to form artificial nucleases, which could cleave specific DNA sequences [13]. By replacing recognition domains, FokI-mediated cutting was

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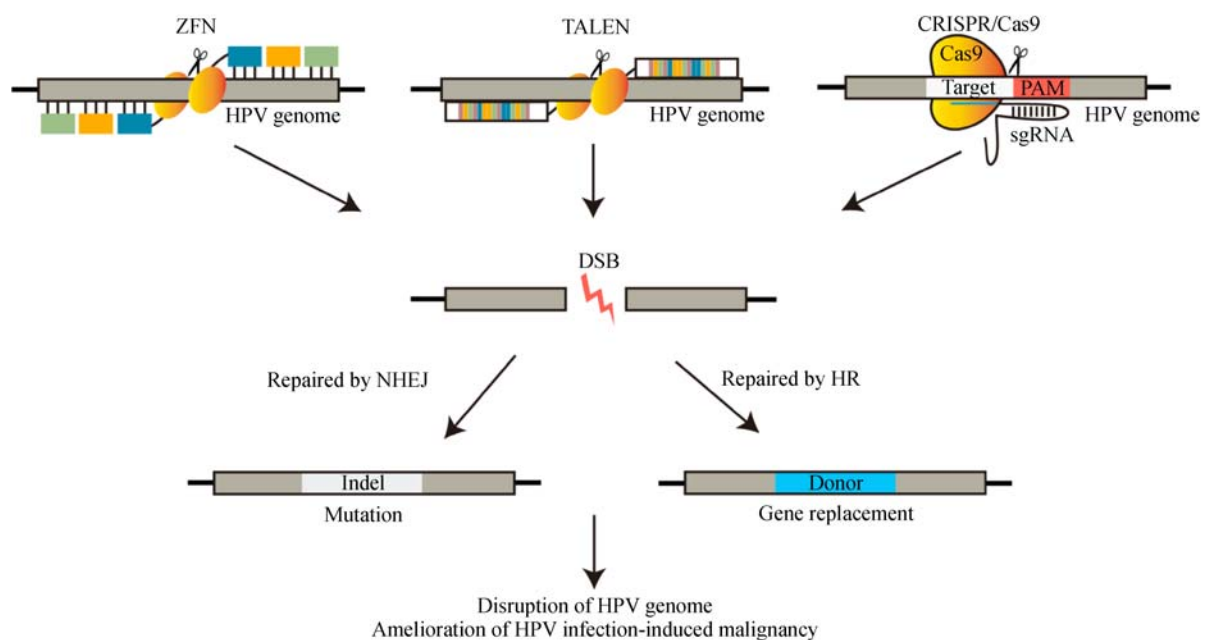
redirected to specific sites [13–15]. However, ZFN engineering requires extensive labor and sophisticated laboratory facilities. Transcription activator-like effectors (TALEs) were discovered in the plant pathogen *Xanthomonas* [16]. TALEs were fused to the catalytic domains of FokI to form TALENs [17]. Similar to ZFNs, the wild-type FokI domain fused to TALE is inactive as a monomer but can be dimerized under the guidance of repeat-variable di-residues (RVDs) from TALEs to cut specific DNA [16,17]. In many laboratories capable of routine molecular cloning, engineering effective TALENs has become easier and more accessible than engineering ZFNs. More recently, another powerful genome-editing technology known as CRISPR/Cas9 was reported [18,19]. Unlike ZFNs and TALENs, the CRISPR/Cas9 system is composed of small CRISPR RNAs (crRNAs), trans-activating crRNA (tracrRNA), and a Cas9 nuclease originally obtained from *Streptococcus pyogenes* [18]. TracrRNA and crRNA were linked together to form a single guide RNA (sgRNA), and the sgRNA guides the Cas9 protein to induce cleavage at specific genomic sites [20,21]. The system can be easily customized by simply replacing the 20 nucleotide (nt) sgRNA sequence with sequences that target different genomic sites [22]. Given its simplicity, this technique was broadly accepted by research groups. However, the off-target rates of the original version of this system were reported to be relatively high and could be the major hurdle to its clinical application. Since then, many modifications of these systems were devised to improve their efficacy and specificity.

With the rapid development of the above technologies, custom-designed artificial nucleases were applied as therapeutic strategies to specifically disrupt the DNA/RNA of tumorigenic viruses or destroy viral entry routes. Because human papillomaviruses (HPVs), hepatitis B virus (HBV), human immunodeficiency virus (HIV), and Epstein–Barr virus (EBV) have been the major targets for genome-editing tools in published studies, this review mainly focuses on these viruses and their related cancers as representative examples.

## HPV infection and HPV-related neoplasms

HPVs are double-stranded DNA viruses that mainly infect mucosa of genital tracts [23]. High-risk HPVs (HR-HPVs) were identified to be the main causes of cervical cancer [24]. Most HPV infections are transient, but a small fraction of these infections persist for decades and finally cause cancer. In the past, the prevention of HPV infections and the related cervical cancer relied heavily on prophylactic vaccines and repeated screenings, which may lead to overtreatment and potential waste of medical resources. Under such circumstances, genome-editing tools were developed as alternatives or superior solutions for current treatments of HPV-related malignancies (Fig. 1).

The HPV E2 gene regulates viral transcription and initiates HPV DNA replication [25]. In early studies, artificial nucleases were designed and engineered to target the HPV E2 gene. For example, Horner *et al.* fused the



**Fig. 1** Schematic overview of HPV genome editing mediated by ZFNs, TALENs, and the CRISPR/Cas9 system.

FokI domain to the DNA binding domain of the bovine papillomavirus type 1 (BPV1) E2 protein [26]. They observed efficient HPV18 DNA cleavage at E2 binding sites and reduced viral replication in HeLa cells.

With the advancement of genome-editing techniques, potential targets of genome-editing tools gradually expanded to include viral oncogenes *E6* and *E7*. *E6* and *E7* are involved in multiple cellular targets for maintaining HPV infection. For example, the degradation of p53 tumor suppressor protein and BAK proapoptotic protein by *E6* can increase the resistance of host cells against apoptosis [4,5]. *E7* also abolishes cell-cycle arrest and induces proliferation by inhibiting the function of the tumor suppressor retinoblastoma 1 (RB1) protein [6]. Therefore, *E6* and *E7* are dissimilar (but also complementary) target sites in HPV-related malignancies.

The HPV16/18 *E7* gene was first disrupted using ZFNs in cervical cancer cells [27]. Disrupting the *E7* gene inhibited cell growth and caused apoptosis in HPV16/18 cervical cancer cells [27]. Apoptosis and growth inhibition were also observed in HPV16-integrated SiHa and Caski cells treated with CRISPR/Cas9 against HPV16 *E7* genes [28]. Zhen *et al.* designed sgRNAs targeting the HPV16 promoter and *E6/E7* transcripts [11]. CRISPR/Cas9-treated SiHa cells showed reduced *E6* and *E7* mRNA levels, along with strong growth suppression. Similarly, Kennedy *et al.* cleaved HPV18 *E6* and *E7* genes and thus caused cell-cycle arrest of HeLa cells at the G1 phase [29].

Besides *in vitro* experiments, Zhen *et al.* confirmed the efficacy of genome-editing tools *in vivo* [11]. They reported that Balb/c nude mice inoculated subcutaneously with CRISPR/Cas9-treated SiHa cells showed a smaller tumor volume than that of control mice [11]. Similarly, Balb/c nude mice were subcutaneously injected with SiHa and HeLa cells [27], and the tumor xenografts were transfected with ZFNs plasmids. Decreased tumor sizes were observed in the ZFN-treated mice [27]. Notably, the intra-vaginal delivery of HPV16 *E7* TALENs disrupted the *E7* gene, reduced the HPV16 viral DNA load, and also reversed the malignant phenotypes in K14-HPV16 mice [12]. Therefore, HPV site-specific TALENs may be designed to eradicate persistent HPV infections in precancerous stages. Although the regional delivery of TALENs may not completely cure the advanced stages of cervical cancer, the treatment may still be beneficial.

Interestingly, low-risk HPVs (LR-HPVs), especially HPV6 and HPV11, have also become targets for genome-editing therapies. LR-HPVs are known as major risk factors for anogenital warts and laryngeal papillomatosis [30]. Anogenital warts and laryngeal papillomatosis have long been problematic because of their high recurrence rates even after surgical removal due to the persistence of LR-HPVs [31]. Liu *et al.* modified the HPV6/11 genome in cultured human foreskin keratinocytes by using CRISPR/Cas9 [32]. The scholars observed

cell growth arrest and apoptosis upon HPV6/11 *E7* gene destruction. Therefore, genome engineering tools may also become valuable therapeutic strategies to treat genital warts caused by LR-HPV infection.

## Treatments for HBV infection and hepatocellular carcinoma (HCC)

Chronic HBV infection can cause liver cirrhosis and HCC [33]. Presently, over 350 million people are chronically infected with HBV [33,34]. Patients actively infected with HBV possess a 4.6-times higher incidence of developing HCC than those of patients free of HBV infection [35]. Each year, numerous people die of complications from liver cirrhosis and HCC. HBV infection is a large burden on human society [33]. Traditional antiviral therapies against HBV infection involve using nucleoside analogs (NAs) and interferons [36]. NAs or interferons only suppress HBV replication in the cytoplasm but cannot eliminate the HBV latent-pool covalently closed circular DNA (cccDNA) in the nucleus [35,37,38]. The cccDNA in the nucleus is a pool of viral escape mutants [39] that play key roles in drug resistance and viral rebound. Thus, few patients remain free of HBV infection after withdrawing NAs and interferon treatments [40]. Besides, cccDNA is a template for viral RNA transcription; cccDNA clearance is necessary for eradicating HBV infection and curing HCC [38].

The HBV genome contains the precore/core (*C*), polymerase (*pol*), surface (*S*), and *X* open reading frames (ORFs) [41]. The *C* region encodes the core protein, the *pol* ORF is essential for producing viral DNA polymerase, the *S* region encodes the viral envelope protein, and the *X* ORF is used for viral gene transcription [41,42]. Therefore, the *C*, *pol*, *S*, and *X* ORFs are all potential targeting sites for treating HBV infection.

siRNA was used to stop HBV replication in HBV-transgenic mice [43]. Diminished HBV transcripts and reduced viral DNA were observed. siRNA also inhibited HBV cccDNA amplification [43]. However, the RNAi effect only lasted a few days and required continuous implementation for long-term treatment. In addition, unpredictable off-target effects also remain as increased concerns [44]. Highly specific genome-editing tools that last for a long period and possess few predictable off-target effects are necessary for clinical application.

ZFNs, TALENs, and CRISPR/Cas9 have gained great fame in treating HBV infection and reversing the malignant phenotypes of HCC [45–47]. Cradick *et al.* transfected Huh7 human hepatoma cells with a pTHBV2 plasmid carrying the HBV genome along with ZFN plasmids targeting HBV genomic DNA [47]. Episomal HBV viral genome disruption and lowered HBV pregenomic viral RNA were observed in Huh7 cells. Seeger and

Sohn established HepG2 cells that express the HBV receptor-sodium taurocholate co-transporting polypeptide (NTCP) [38]. These cells were permissive to HBV infection. They infected the HepG2/NTCP cells with HBV particles, which were produced from HepAD38 cells and detected cccDNA in the HepG2/NTCP cells. The group found that the cccDNA derived from infectious HBV were cleaved using CRISPR/Cas9. Bloom and colleagues were the first to disrupt the HBV genome using TALENs [46]. The team designed four TALENs targeting the *S*, *C*, and *pol* ORFs of the HBV genome. TALENs and HBV-genome-expressing plasmids were transfected into Huh7 cells. Suppressed viral replication was detected because of TALENs targeting the *S* and *C* ORFs. However, the TALENs targeting the *pol* ORF did not decrease the HBsAg levels in HepG2.2.15 cells. The team also established a mouse model by injecting HBV-carrying plasmids and corresponding TALEN plasmids by using the hydrodynamic injection method. *S* and *C* TALENs inhibited HBV viral replication in the hydrodynamic mouse model [46]. Meanwhile, Zhen *et al.* designed 12 sgRNAs targeting the *C*, *pol*, *S*, and *X* ORFs [10]. S1 sgRNA decreased HBsAg levels by 61% in HepG2.2.15 cells. Among the 12 sgRNAs, those targeting the *S* and *X* ORFs inhibited cccDNA expression prominently. HBsAg was decreased by 93% by using sgRNA X3 and S1 in the hydrodynamic mouse model. Ramanan *et al.* designed 24 sgRNAs against the *C*, *pol*, and *X* ORFs [48]. The scholars reported decreased pgRNA levels and HBsAg production in HepG2 hepatoma cells expressing HBV plasmid. *In vivo* experiments using a hydrodynamic mouse model revealed decreased HBsAg secretion and viremia. Lin *et al.* were the first to inhibit HBV replication in cultured cells and hydrodynamic mice using CRISPR/Cas9 [49]. The group designed eight sgRNAs targeting the *C*, *pol*, *S*, and *X* ORFs of the HBV genotype A. HBV plasmid, sgRNA plasmids, and the Cas9 plasmid were cotransfected into Huh7 cells. Lowered core and surface protein levels were observed. The team also detected decreased HBsAg serum levels treated with CRISPR/Cas9 using a hydrodynamic mouse model expressing HBV [49]. Chen *et al.* designed TALENs targeting conserved sequences among the A-D genotypes [36]. The team detected reduced cccDNA levels. Injecting HBV-expressing plasmid and TALEN plasmids into mice hydrodynamically resulted in reduced serum HBV DNA and liver HBV pgRNA. The results indicated viral transcription and replication inhibition *in vivo*. The combined use of TALENs and interferon- $\alpha$  *in vitro* showed synergistic effects in inhibiting cccDNA and viral transcription [36]. Kennedy *et al.* reported inhibited HBV DNA and cccDNA in HBV-infected HepAD38 and HepaRG cell lines using CRISPR/Cas9 targeting the *S* and *C* regions [45]. Meanwhile, Karimova *et al.* designed CRISPR/Cas9-nickase to inactivate HBV *in vitro* [50]. In

particular, the researchers designed sgRNAs that target the conserved sequences of the *S* and *X* ORFs. CRISPR/Cas9-nickase inactivated the HBV in chronically and *de novo* infected cells. Therefore, CRISPR/Cas9-nickase offered an alternative means to eradicate HBV infection.

Because HBV is a highly complex viral genome, disrupting only one region may not sufficiently block HBV infection. The multiplexed treatment of different HBV regions may be more effective for future clinical research [51]. Considering the heterogeneity of the HBV genome, Liu *et al.* designed eight sgRNAs that target the conserved regions of 26 different HBV genotypes and observed the HBV replication inhibition of these different genotypes. Meanwhile, Wang *et al.* designed 15 sgRNAs against the HBV A-D genotypes [52]. Eleven pairs of dual sgRNAs were produced among the 15 sgRNAs. Dual sgRNA-mediated HBsAg and HBeAg suppression was more effective than using a single sgRNA. Compared with genome editing using only one sgRNA, the collaboration of different sgRNAs could boost the inhibition efficiency, broaden the targeting range, and prevent escape caused by viral mutations [51].

## Treatments for HIV infection

HIV-1 mainly infects human CD4<sup>+</sup> T cells and causes the depletion of these cells [53]. HIV-1 is etiologically related to acquired immunodeficiency syndrome (AIDS) [53]. Until 2012, 35.3 million people were estimated to be infected with HIV-1 [53]. Highly active antiretroviral therapy (HAART) enabled the treatment of HIV infection as a chronic non-terminal disease [8]. However, HAART only temporarily inhibits viral replication. When antiviral treatment is attenuated, the latent pool of HIV infection is reactivated to produce viruses [8]. HAART causes severe side effects, including coronary artery disease [54], osteoporosis [55], and kidney failure [56]. Moreover, HAART requires lifelong treatment. High costs and lifelong treatments for HIV/AIDS have added massive burden to individuals and countries where HIV/AIDS is epidemic. siRNA has been used to combat HIV infection. However, because of its incomplete suppression and unpredicted off-target effects, siRNA is not widely applied to treat HIV infection [57,58]. Hence, an improved means of inhibiting persistent HIV infection or eliminating the virus must be developed.

Because HIV-1 involves high mutation rate [53], the virus rapidly escapes inhibition by RNAi and antiviral drugs. Therefore, disrupting highly conserved regions may be employed to treat HIV-1 infection. HIV-1 binding and entrance into cells require the CD4 receptor, either CCR5 or the CXCR4 co-receptor [53,59]. The CD4 receptor is the primary attachment receptor for HIV, but disrupting CD4 appears impractical because of the marker's essential

role in immune function [53]. A breakthrough was achieved in the field when a Berlin patient received an allogeneic bone marrow transplant to cure his myeloid leukemia [59]. After the transplant, the HIV-1 infection became undetectable, which suggested that HIV/AIDS was functionally cured in this patient [60,61]. Researchers found that the donor hematopoietic progenitor cells possess a 32-bp (base pair) deletion of the C-C chemokine receptor type 5 locus (*CCR5* $\Delta$ 32). *CCR5*-depleted cells are naturally resistant to *CCR5*-tropic HIV-1 infection [61]. This discovery highlighted that deleting the *CCR5* co-receptor could possibly cure HIV-1. In late-stage infected patients, 50% of HIV infections use *CXCR4* or *CCR5* [62–64]. Another patient who suffered from anaplastic large-cell lymphoma was transplanted with tissue from a donor bearing the *CCR5* $\Delta$ 32 mutation [65]. However, the patient eventually suffered from *CXCR4*-tropic viral infection. Therefore, *CXCR4* is also a necessary target.

Several studies have used ZFNs targeting the *CCR5* co-receptor to treat HIV/AIDS in  $CD4^+$  T cells [66–68] (Fig. 2A). Perez *et al.* established HIV-1 infection-resistant  $CD4^+$  T cells by using ZFNs [67] and disrupted 50% of *CCR5* alleles in  $CD4^+$  T cells. The mice engrafted with ZFN-modified  $CD4^+$  T cells displayed a lower HIV-1 viral load than those of the corresponding controls [67]. Holt *et al.* disrupted the *CCR5* gene in human hematopoietic stem/progenitor (HSC) cells by using ZFNs [66]. *CCR5*-edited HSC cells were proven resistant to HIV-1 infection. Tebas *et al.* described the first phase I clinical trial to treat HIV patients using ZFNs [69]. TALENs were also used to cleave the *CCR5* with high efficiency [70]. *CCR5* deletion using TALENs was reported to prevent HIV-1 infection [71]. CRISPR/Cas9 was also effective in human pluripotent stem cell (hPSC)-derived monocytes/macrophages (latent storage site for HIV-1 infection) [72]. Because the HIV-1 genome is highly mutable, viral escape mutants should be confronted using sgRNAs targeting different regions of the HIV-1 genome [73].

Because *CXCR4* is also a necessary target site for eradicating HIV-1 infection, experiments were performed to disrupt *CXCR4* by using ZFNs [68,74] (Fig. 2B). *CXCR4*-knockout  $CD4^+$  T cells were protective against *CXCR4*-tropic HIV-1 in humanized mouse models. However, anti-retroviral therapies against *CXCR4*-tropic strains resulted in the resurgence of *CCR5*-tropic strains [75]. Therefore, the target sites must be selected with care. Besides *CCR5* and *CXCR4*, the *PSIP1* gene is a potential target site for HIV-1 infection inhibition [76]. The *PSIP1* gene encodes the lens epithelium-derived growth factor (LEDGF)/p75 protein, which is a lentiviral integration cofactor. TALENs designed to knock out *PSIP1* inhibited HIV-1 integration and afforded *PSIP1*<sup>-/-</sup>  $CD4^+$  Jurkat T cells with resistance against HIV-1 infection [76].

During the HIV-1 life cycle, retroviral DNA must integrate into the host cell genome [77]. The integrated

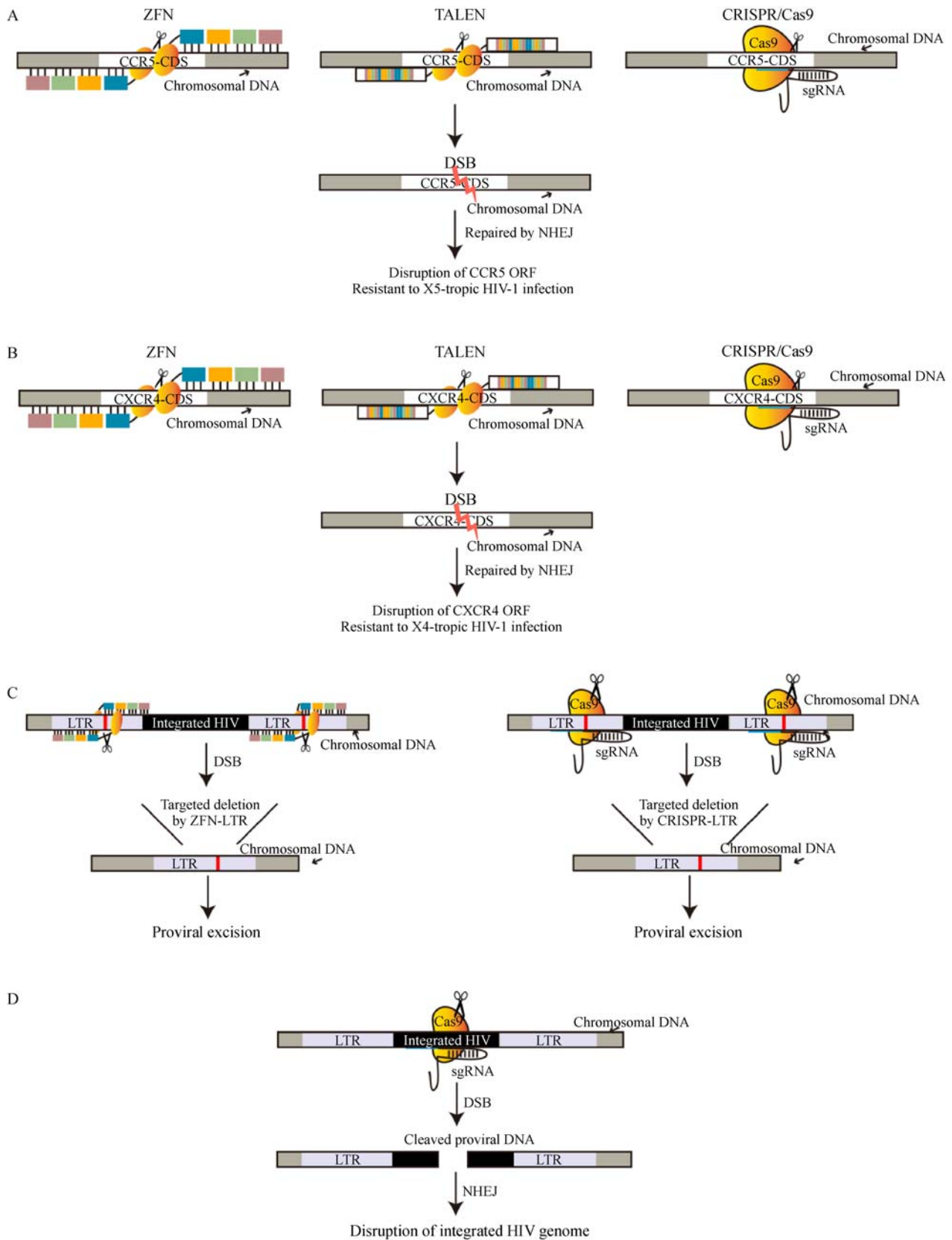
retroviral DNA is a provirus and essential to viral protein production [77]. Therefore, apart from *CCR5*, *CXCR4*, and *PSIP1*, HIV provirus DNA flanked by long-terminal repeats (LTRs) is also a potential target site for eliminating HIV-1 infection [78]. Qu *et al.* used one pair of ZFN-LTR to destroy the 5'-LTR and 3'-LTR DNA sequences [78] (Fig. 2C). The group observed 45.9% excision frequency in HIV-1 infected human cell lines. Ebina *et al.* designed sgRNAs targeting the HIV-1 LTR to eradicate the provirus by using the CRISPR/Cas9 system (Fig. 2) [77]. The study targeted the trans-activation response (TAR) region in the LTR. Because the TAR region does not comprise an excessive number of variations in different HIV-1 subtypes, it is a proper target site for eliminating the provirus [77,79]. The study successfully inhibited transcriptionally active proviruses along with provirus integrated in the latent pools [77].

## Treatments for EBV infection

EBV causes persistent infection in 95% of the adults [80]. The virus is etiologically related to Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, and 10% of gastric cancers [80]. Currently, no EBV vaccines or drugs were approved by the Food and Drug Administration [81]. However, the wide applicability of genome-editing tools may offer a potential treatment for eradicating EBV infection and curing EBV-associated malignancies.

TALENs were used to inactivate the EBV-encoded nuclear antigen-1 (*EBNA1*) gene, which is critical in EBV episome replication and persistence [81]. *EBNA1* deletion only caused cell death in EBV-infected cells and left the EBV-negative cells intact. Therefore *EBNA1* may be a potential therapeutic target site for eradicating EBV infection [81]. Meanwhile, Yuen *et al.* deleted 558 bp of the EBV promoter region of *Bam*HI A rightward transcript (*BART*) using CRISPR/Cas9 dual sgRNAs [80]. *BART* RNA encodes two clusters of microRNAs (miRNAs) that induce cell survival and promote carcinogenesis. The authors suggested that deleting the promoter region was a versatile method for ablating miRNAs and exploring the function of certain promoter regions. This work suggested that CRISPR/Cas9 can be used to treat EBV infection.

Programmed cell death protein 1 ligand (PD-L1), an immune checkpoint inhibitor, maintains high levels in some EBV-related malignancies [82]. Therefore, these malignancies may be treated using PD-1/PD-L1 associated treatments. Su *et al.* engineered CRISPR/Cas9 to disrupt PD-1 and disrupted the EBV genome in primary T cells [82]. When the genome-engineered cells are injected into tumor-bearing mice, the scholars also discovered an anti-tumor effect when combined with low-dose radiotherapy. Therefore, disrupting PD-1 by using CRISPR/Cas9 may be used to treat EBV-associated gastric cancer.



**Fig. 2** Methods to stop the HIV-1 virus from infection. (A) Disruption of CCR5 ORF mediated by genome-editing tools leads to resistance to CCR5-tropic HIV-1 infection. (B) Disruption of CXCR4 ORF mediated by genome-editing tools leads to resistance to CXCR4-tropic HIV-1 infection. (C) Provirus excision using ZFNs and CRISPR/Cas9 system targeting the 5'-LTR and 3'-LTR regions. (D) CRISPR/Cas9 cleaves the integrated HIV-1 genome.

## Comparisons among ZFNs, TALENs, and CRISPR/Cas9

ZFNs, TALENs, and CRISPR/Cas9 are highly efficient gene-editing tools. ZFNs are composed of site-specific zinc finger proteins (ZFPs) fused to FokI to mediate the cleavage of DNA strands. Meanwhile, TALENs consist of TALEs fused to FokI cleavage domains. ZFNs and TALENs must function as dimers [83–86], whereas CRISPR/Cas9 is efficient as a monomer. ZFNs recognize genomic sites by using ZFPs, and redesigning ZFPs is necessary to change the target loci. TALENs entail RVDs to mediate sequence recognition. To target other genomic sites, researchers need to synthesize another set of RVDs, which will subsequently be linked to the FokI domain. For CRISPR/Cas9, a “seed sequence” within the crRNA and a protospacer adjacent motif (PAM) sequence are required for site-specific recognition [21]. The CRISPR/Cas9 system can be reconstructed to cleave other genomic sites by redesigning the crRNA. Because tracrRNA and crRNA can be linked to form a sgRNA, redesigning the sgRNA sequence is sufficient to guide the Cas9 protein to other genomic sites [21,22].

Although genome-editing techniques have achieved great advances in treating viral infection and virus-related carcinomas, some issues remain in their clinical application. One major challenge is the delivery method. The safety, efficacy, and cost of all delivery methods must be evaluated. Off-target activity is another problem that needs to be treated with caution.

### Delivery using non-viral vectors

Transfection reagents are suitable for delivering genome-editing tools *in vitro* to many cell types. Therefore, we mainly discuss the delivery of genome-editing tools *in vivo*. For delivery *in vivo*, Hu *et al.* used the TurboFect *in vivo* Transfection Reagent (ThermoFisher Scientific) to transfect TALEN plasmids into the vagina of K14-HPV16 mice [12]. The researchers observed that the TALENs mainly accumulated in target organs (vagina and cervix) with minimized toxicity or side effects to the whole organism. Moreover, the delivery via vagina reduced hepatic first-pass metabolism and maintained a high endonuclease concentration in the target organs. Delivery via vagina is also suitable for drugs not fit for systemic use. Hu *et al.* proved the efficacy of TALENs delivery to treat cervical malignancies in K14-HPV16 mice [12]. Compared with TALENs, whether ZFNs and CRISPR/Cas9 exert the same effect, or even enhanced efficiency, remains to be investigated. In addition, some improvements should be made prior to clinical application to counteract cervical malignancies. For example, the endonucleases must be in the form of suppository, gel, or cream to maintain a long-

term exposure to the human vagina. Menstruation cycles and the vaginal pH must also be considered [12]. The duration, dosage, and cost must also be carefully examined.

Intravenous drug delivery is widely used. Because most drugs are metabolized in the liver, genome-editing tools can be delivered intravenously to cure HBV infection. However, systemic delivery may also cause cytotoxicity or side effects. Therefore, researchers should instead select a tissue-specific promoter to guarantee the endonuclease expression in specific organs [87]. Hydrodynamic injection is an alternative to systemic delivery. Yin *et al.* reported correcting a *Fah* mutation in a mouse model of human hereditary tyrosinemia by using CRISPR/Cas9 [88]. The scholars suspended CRISPR/Cas9 plasmid and a single-stranded DNA oligo donor in 2 mL saline and subsequently injected this suspension through the mouse tail vein for over 5–7 s. The group then detected corrected *Fah* mutation in 1/250 hepatocytes. However, to inject a large liquid volume over such a short time may cause high pulmonary artery pressure and acute kidney failure. Therefore, hydrodynamic injection may not be used for clinical purposes [88].

Genome-editing tools can also be delivered through other means to specific organs. In the future, respiratory viral infection, such as the Middle East respiratory syndrome coronavirus (MERS-CoV) or severe acute respiratory syndrome coronavirus (SARS-CoV) may be treated by inhaling aerosols containing genome-editing tools. Intraperitoneally, retro-orbital, intra-cranial, and rectal drug delivery are all local delivery methods that may be used to deliver genome-editing tools regionally.

### Delivery using viral vectors

Viral vectors are frequently used delivery vehicles. Adeno-associated virus (AAV) and lentivirus have been used to deliver genome-editing tools [44] but possess inadequacies. For example, AAV can only package plasmids smaller than 4.2 kb in length [44]. Given their small size, ZFNs can be delivered using AAV [89]. However, TALENs or CRISPR/Cas9 exceed the packaging capacity of AAV. Therefore, the two monomers of TALENs must be delivered separately using two AAV vectors [44]. For CRISPR/Cas9, a smaller Cas9 derived from *Staphylococcus aureus* called saCas9 can be delivered using AAV [90]. Lentivirus holds a larger delivery capacity than that of AAV; hence, TALENs or CRISPR/Cas9 are deliverable by lentiviral vectors [91,92]. High transduction efficiency is also an advantage of lentivirus delivery. Lentiviruses may integrate into target cells to maintain the stable expression of genome-editing tools to prevent the virus from resurgence. However, the viruses also tend to integrate into the host genome and cause genomic instability [92].

Integrase-deficient lentiviral vectors (IDLVs) are expressed transiently and do not tend to integrate into the genome [91]. Researchers may use IDLVs to deliver ZFNs or CRISPR/Cas9 [91,93]. However, TALENs are difficult to deliver using IDLVs [44,92]. To date, no perfect delivery method is suitable for all cases. Delivery methods should be adjusted depending on the target organs and the type of genome-editing tools being used. Researchers must also evaluate the delivery methods and choose the most suitable one.

## Off-target effects

One major concern of gene-editing tools is its off-target effect. The imprecise repair of DNA double-strand breaks (DSBs) may induce chromosomal rearrangements [94]. Unwanted chromosomal rearrangements that originate from off-target effects are the etiologies of cancer [94]. Therefore, efforts have been devoted to improving the specificity and the prevention of off-target effects in gene-editing tools.

Off-target effects vary depending on the endonucleases being tested. To reduce the off-target cleavage, scholars synthesized an obligate FokI heterodimer. DNA cleavage occurs only when the two ZFN subunits bind close to each other and form an obligate dimer [83–85]. A ZFN nickase was also developed to reduce off-target cleavage [95]. Nickase activates the homologous recombination repair mechanism instead of non-homologous end joining and thereby reduces off-targets. Similar to ZFNs, TALENs were fused to obligate FokI heterodimer to improve specificity. Because the specificity of the CRISPR/Cas9 system is only determined by the PAM sequence and the short sgRNA sequence, off-target effects can be high [21,22,96]. To reduce off-targets, researchers have applied several methods. For instance, additional two guanine nucleotides were added to the 5' end of the sgRNA sequence to reduce off-targets [97]. Short sgRNAs (17–18 nt) truncated at the 5' end of the 20 nt sgRNA sequence decreased off-target effects by 5000-fold with high on-target activity [98]. Mutation of the HNH domain or the RuvC domain of Cas9 creates a nickase [21]. Nickases only induce a nick on one DNA strand. Therefore, paired nickases with offset sgRNAs are required to induce DSBs and thereby enhancing specificity [86]. Fusing a catalytically inactive Cas9, termed dCas9, onto a FokI domain is another way to reduce off-targets [99–101]. Because dimeric endonucleases are sensitive to spacer length between left and right monomers, an optimum spacer length exists between two monomers [102,103]. Binding requires a 15–25 bp spacer between two monomers to function correctly; this spacer improves the specificity of the system without diminishing the on-target effects [101].

Additionally, a high-fidelity CRISPR/Cas9 nuclease (SpCas9-HF1) can also reduce off-target effects [104].

## Conclusions

Viral infections cause many tumor types and remain as severe threats to human society. ZFNs, TALENs, and CRISPR/Cas9 are highly efficient genome-editing tools. Given their ability to target any site in the viral genomes, genome-editing tools are potential treatments for eradicating tumorigenic viral infections and reversing the malignant phenotypes of virus-related malignancies (Fig. 2). Despite the many hurdles of clinical application, ZFNs, TALENs, and CRISPR/Cas9 technologies may substantially promote the eradication of viral infections and the cure of tumorigenic malignancies.

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## Compliance with ethics guidelines

Lan Yu, Xun Tian, Chun Gao, Ping Wu, Liming Wang, Bei Feng, Xiaomin Li, Hui Wang, Ding Ma, and Zheng Hu declare no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by a relevant institutional review board or ethics committee.

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