REVIEW ARTICLE

Viral vectors and extracellular vesicles: innate delivery systems utilized in CRISPR/Cas-mediated cancer therapy

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Gene editing-based therapeutic strategies grant the power to override cell machinery and alter faulty genes contributing to disease development like cancer. Nowadays, the principal tool for gene editing is the clustered regularly interspaced short palindromic repeats-associated nuclease 9 (CRISPR/Cas9) system. In order to bring this gene-editing system from the bench to the bedside, a significant hurdle remains, and that is the delivery of CRISPR/Cas to various target cells in vivo and in vitro. The CRISPR-Cas system can be delivered into mammalian cells using various strategies; among all, we have reviewed recent research around two natural gene delivery systems that have been proven to be compatible with human cells. Herein, we have discussed the advantages and limitations of viral vectors, and extracellular vesicles (EVs) in delivering the CRISPR/Cas system for cancer therapy purposes.

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INTRODUCTION

Cancer is one of the top fatal diseases in the world, which causes numerous complications in many aspects. Despite all the advances in diagnostics and therapeutics, it remains too challenging to be effectively treated [1, 2]. Various therapeutic methods have been developed, including small molecules [3], and gene therapies [4], to fight cancer by targeting oncogenic signaling pathways or modulating the immune system, which in some cases has led to complete remission. Nevertheless, novel therapeutic approaches are still needed to cure different kinds of cancer.

The advances achieved by prokaryote-derived gene alteration systems have greatly aided the understanding of diseases mechanisms and finding new treatment strategies. In this regard, the Clustered regularly interspaced short palindromic repeats-associated nuclease 9 (CRISPR/Cas9)-based approaches provide a novel strategy toward gene editing for clinical utilization [5]. Hence, knowing the history of the CRISPR/Cas system and its features can help comprehend this revolutionary technology.

Initially, In 1987, when Ishino and colleagues attempted to discover an alkaline-phosphatase-isozyme convertase in Escherichia coli, they encountered repetitive sequences interspersed with spacers belonging to the CRISPR/Cas system [6]. The next encounter with such sequences was in Haloferax mediterranei, where the repeats contained 35 bp spacer sequences [7]. During the identification phase of the CRISPR/Cas, these spacer regions caught the attention of scientists. Afterward, finding similar enigmatic orders of repeats and spacer in archaea and bacteria emphasized the value of their biological importance [8]. In 2002, the repetitive sequences found in the DNA of Haloferax mediterranei, near the region related to the DNA repair system were defined as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs). These genes accompanied by CRISPR-associated (Cas) genes were initially thought to be involved in the DNA repair system [9]. Investigations in 2005 could connect the link between the origin of the spacers and bacteriophages [9, 10]. The Koonin et al. study followed it in 2006, where they found the connection related to the activity of CRISPR and Cas genes as an entirety, which showcased a degree of resemblance between CRISPR-Cas system and prokaryotic RNA interference (RNAi) participating in the immune system [11]. CRISPR has been used for many purposes and in 2020 Emmanuelle Charpentier and Jennifer Doudna received the Nobel prize for introducing the CRISPR/Cas9 system as a gene editing tool for precise manipulation human genome.

CRISPR–Cas9-targeted fragmentation of DNA can be used as a means to pinpoint the changes in cancer-specific sequences. Moreover, in recent years, the development of chimeric antigen receptors (CAR) has made a milestone in cancer therapy. Although CAR T-cell therapy has shown some encouraging results, there are still some limitations that must be addressed, and the CRISPR/Cas system has shown promise for improving CAR T-cell-based cancer immunotherapy [12].

Moreover, in gene therapy, oncogenic viruses have been a significant concern for many years, and CRISPR appears to offer a solution to preventing them from causing cancer [13]. CRISPR-Cas system can be delivered into mammalian cells using various strategies such as viral vectors, extracellular vesicles (EVs), physical methods, and nanocomplexes [14]. In this review, we discussed the advantages and limitations of viral vectors and EVs for delivering the CRISPR/Cas system for cancer diagnosis and treatment.

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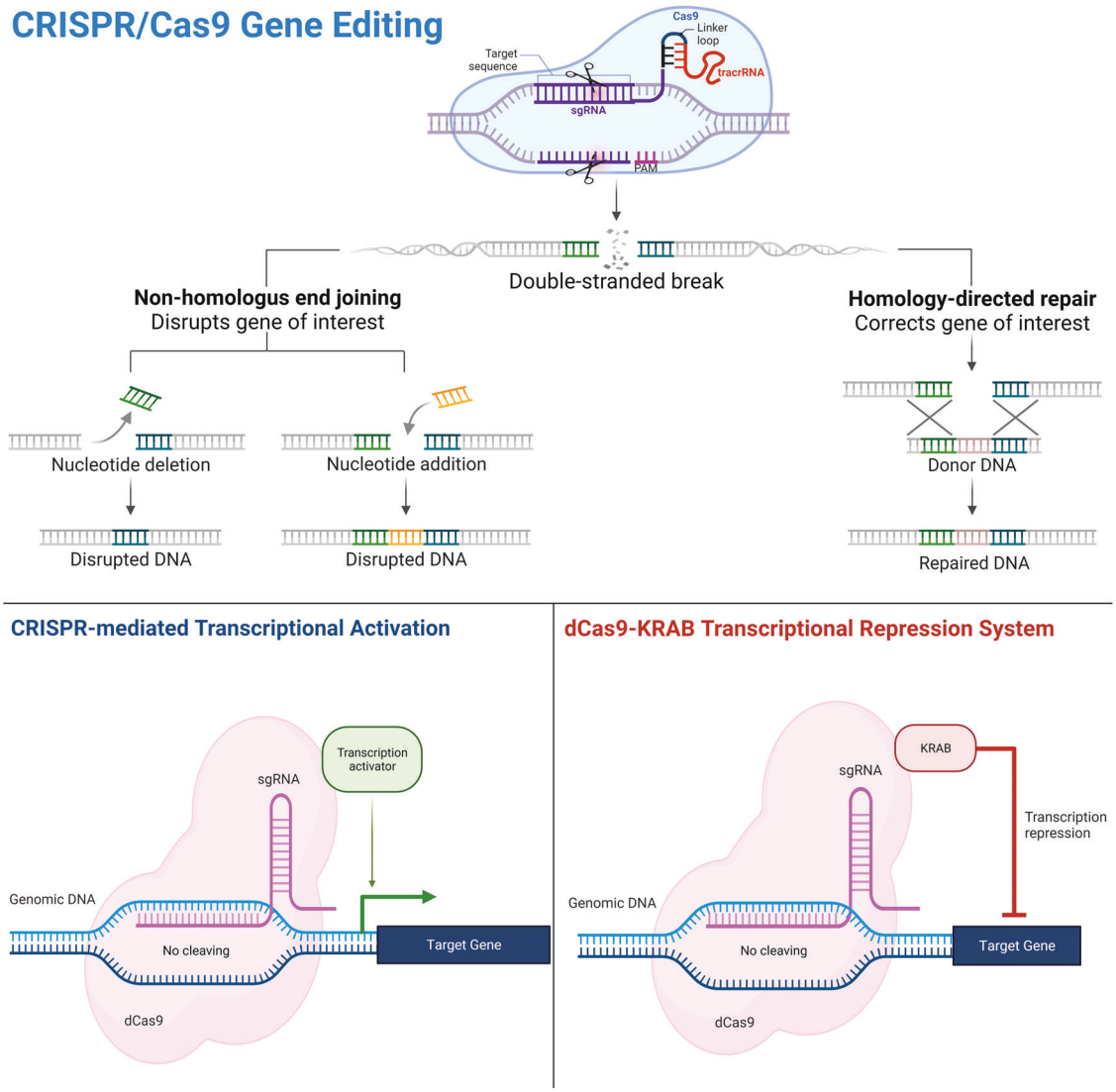

Fig. 1 Overview on different mechanisms of action carried out by CRISPR/Cas9 systems. One of the significant advantages of the CRISPR/Cas system is that it can be modified readily to carry out various functions. Cas9 can induce DSB and edit genes with high accuracy or with the help of modified Cas9 enzymes like dCas9; this system can be used as a transcription activator (CRISPRa) or transcription repressor (CRISPRi).

THE CRISPR/CAS SYSTEMS

Recent advancements in prokaryote-isolated editing systems have paved the way for a better understanding of tumorigenesis mechanisms. CRISPR/Cas-based methods offer an ingenious path toward utilizing gene therapy and immunotherapy for cancer treatment. The CRISPR/Cas systems used in cancer treatment approaches are mostly based on the Cas nucleases (Cas9, Cas12a, and Cas13a) and their orthologs [15].

CRISPR/Cas9 systems are generally utilized as genetic engineering tools in most species. Cas9 is a crRNA-guided endonuclease that contains two domains called RuvC and HNH, participating in cleaving double-strand DNA (dsDNA) [16]. Furthermore, CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) are required to form the RNP complex (Cas9–crRNA–tracrRNA ribonucleoprotein). The most frequent kind of Cas9 is Streptococcus pyogenes Cas9 (SpCas9) which is able to target DNA via protospacer adjacent motif (PAM) recognition (Fig. 1) [17]. Following Cas9-mediated DNA cleavage, the gene editing effect occurs via either the non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathway [18]. Staphylococcus aureus Cas9 (SaCas9), a variant of Cas9, has a distinctive PAM recognition capability, which can target the 5′-NNGRRT PAMs [19]. Recently, CasX, a variant of Cas9 that is both smaller and more efficient at gene editing, was discovered and is regarded as the smallest of them all [20]. Moreover, by modifying CRISPR/Cas9 into a nuclease-deficient system (called dCas9) and fusing an additional effector domain, this system can be repurposed for a variety of purposes. Specifically, CRISPR/dCas9 systems fused with the KRAB domain (CRISPR/dCas9-KRAB), a transcriptional repressor domain, are used to interfere with target gene transcription [21]. The CRISPR interference (CRISPRi) system created in this way can be used for numerous purposes, such as cancer diagnosis and treatment.

The breakthroughs achieved by the CRISPR/Cas9 system have prompted sweeping exploration to uncover novel methods for extending applications. Another endonuclease with unique features for the CRISPR system is Cas12a (so-called Cpf1) [22]. In contrast to Cas9, which creates blunt ends in DNA, Cas12a is able to make the staggered ends in a distinctive cleavage pattern, promoting the DNA integration in a stringent position. Of note, the Cas12a enzymes do not require tracrRNA and process the pre-crRNA on their own. In order to cover a wide range of targeting locus, variants of Cas12a were developed to target different PAMs (5′-TATV, 5′-VTTV, 5′-TTTT, 5′-TATV, 5′-TTCN) [23, 24]. Moreover, it has been shown that CRISPR/Cas12a system potentially detects

viral DNA in samples, though it hinges on the non-specific cutting of single-stranded DNA (ssDNA) [25]. This particular CRISPR system seems to have the potential to provide an opportunity for multiplex genome editing; therefore, its utilization might be much broader in cancer therapy.

Cas13a also termed C2c2, is a novel form of RNA-guided endonuclease with the ability to target RNA [26]. Upon recognizing and attaching to RNA, Cas13a can collaterally cut untargeted RNAs. As of yet, the Cas13a function in eukaryotes has not been discovered, and its mechanism of action is not fully understood [27]. CRISPR systems able to target RNAs have been used in clinical research for the detection of RNA viruses and tumor-derived circulating RNA [28, 29]. Altering different types of RNAs such as messenger RNA (mRNA), microRNA (miR), long non-coding RNA (lncRNA), and circular RNA (circRNA) by gene-editing methods, especially CRISPR, offers splendid potential in cancer therapy [30]. CRISPR/Cas is originally a system for defending bacteria against invaders, which is now being used for gene editing in mammalian cells and is able to provide immunity that transcends bacterial boundaries [31, 32].

CRISPR/Cas systems are categorized into two classes based on how many Cas endonucleases participate in their machinery. Class I operates using several Cas proteins, whereas class II requires a single Cas enzyme [16, 33]. As compared to the class I CRISPR-Cas system, elements (Cas protein, crRNA, and tracrRNA) involved in class II can be generated more readily; therefore, it would be a more appealing option for gene editing [16, 34–36]. Cas9 is a crRNA-guided endonuclease that contains two domains, HNH and RuvC, which cut complementary and non-complementary DNA strands, respectively [16, 31]. To facilitate mammalian gene manipulation, the crRNA and tracrRNA elements of CRISPR/Cas9 have been replaced with single-guide RNA (sgRNA) [37]. SgRNA's 20 nucleotides at the 5′-terminus bind the target gene, while its 3′-duplex enables interaction with Cas9, ensuring accurate and guided gene editing [16]. The Cas9 should recognize the PAM right next to the target DNA prior to Cas9-induced double-strand break (DSB). Afterward, Cas9 undergoes a conformational change that enables it to execute a DSB three to four nucleotides upstream of the PAM [16, 35, 38]. Following that, a conformational transition occurs to the Cas9 letting it execute a DSB three to four nucleotides upstream of the PAM [16, 35, 38]. Upon Cas9-induced DSB, DNA repair pathways, such as NHEJ and HDR, become activated.

Since NEHJ, as the leading DSB repair pathway, ligates DNA break ends regardless of homologous templates, there is a risk of error in the ligation process due to arbitrary nucleotide insertions and deletions (indels), which could lead to frameshifts and nonsense mutations in genes [39]. In addition to gene alteration, NHEJ can result in deletions when it executes DNA repair with two distinct sgRNAs. These effects of NHEJ can be beneficial in the treatment of diseases indicated by an overproduction of a particular protein.

The HDR pathway repairs DNA with greater precision because a piece of DNA template homologous to the targeted part of chromosomal DNA is required for gene insertion. This allows transgenes to be integrated with more specificity after DSBs [40] HDR-based genome editing can be beneficial for diseases resulting from gene deletion, such as X-linked retinitis pigmentosa [41], hemophilia A/B [42, 43], and phenylketonuria [44]. However, NHEJ is preferred over HDR in mammalian cells for several reasons: NHEJ is active during the entire cell cycle, whereas HDR is only active in the S/G2 phase and NHEJ is faster than HDR. The use of NHEJ exceeds that of HDR, particularly in terminally differentiated cells, such as neurons, cardiac myocytes, and mature muscle cells [45, 46].

Since these repair pathways provide a simple approach to edit genes, they have become the most commonly used strategies in cancer research, even though there are alternatives such as homology-independent targeted integration (HITI), homology-mediated end joining (HMEJ), and microhomology-mediated end joining (MMEJ) [47–50].

VIRAL VECTORS AND EXTRACELLULAR VESICLES USED IN CRISPR-CAS9 DELIVERY

CRISPR-Cas system can be delivered into mammalian cells using various strategies such as viral vectors and extracellular vesicles (Table 1). In this section, we will discuss these vectors in more detail. However, other approaches, such as physical methods and nanocomplexes, have not been addressed in this review. For further information on these methods, readers can refer to the cited papers discussing them [51–54].

Viral vectors

Viruses are genuine vehicles for gene delivery. Mammalian gene therapy has been made possible with recombinant and pseudotyped viral vectors. The most frequently used viral vectors for CRISPR/Cas delivery are adeno-associated viruses (AAVs) [55], adenoviral vectors (AdVs) [56], and lentiviral vectors (LVs) [57] (Fig. 2), which are being used in clinical trials.

Adeno-associated viruses (AAVs). AAVs are small, non-enveloped single-stranded DNA viruses that are not pathogenic to humans. These members of the Parvoviridae family have attracted the attention of researchers as gene delivery systems (Fig. 3) [58]. Despite the fact that around 80% of the general population is sero-positive for these viruses, no connection between AAVs and human diseases has been reported. Various characteristics of AAVs, including relatively low immunogenicity, cytotoxicity, and chromosomal integration probability, make them quintessential delivery systems for CRISPR/Cas, particularly in vivo [59, 60]. Furthermore, different types of AAVs are available for gene delivery into a variety of cells, such as lung, heart, neuron, and muscle cells, thus making them excellent tropism vectors for tissue-specific applications [61].

To some degree, AAVs still have the ability to integrate their genes into the host genome, to overcome this limitation recombinant AAV has been developed [62]. AAVs' gene called Rep is responsible for the production of Rep proteins (Rep78, Rep68, Rep52, Rep40), involved in the packaging of the viral genome, replication, gene expression, and integration of the genetic material [63]. For accurate site-specific integration, AAVs require Rep proteins, particularly Rep78 and 68, although in recombinant forms of AAVs the Rep gene has been removed [64]. Overall, these recombinant AAVs have proven to be effective gene delivery systems; for instance, delivering CRISPR/Cas9 to mice bearing a mutation in the low-density lipoprotein receptor (LDLR) gene exhibited therapeutic effects [65]. Thus far, many AAV-based gene therapies have achieved FDA approval, for example, in Pompe disease and inherited retinal disease (IRD) retinal pigment epithelium (RPE)65-LCA (LCA2), which indicates the effectiveness of AAVs vectors [66]. Furthermore, efficient delivery of the CRISPR/Cas9 system by AAVs has paved the way for disease modelings, such as neurodegenerative abnormalities, muscular dystrophy, liver diseases, and sickle cell disease [67–71].

Although AAVs have impressive achievement records, their limited packaging capacity and genome length limit their ability to harbor genetic payloads that exceed 5 kb. In this case, carrying large cargoes like SpCas9 protein remains a considerable limitation [72]. A number of methods have been designed by scientists to circumvent the barricade of the AAVs' limited packaging capacity. One of which is to transduce cells with AAVs only carrying sgRNA, knowing that these cells have already been induced to express the Cas9 protein. Another strategy is to co-transduce the cells using two AAVs tagged distinctively, one carrying sgRNA and another the Cas9 protein. Nevertheless, there

Table 1. Overview of recent studies that used exosomes and viral vectors for CRISPR/Cas delivery in cancer cells.

Study	Vector	Type of cancer	Approach	Outcome
A New Tool for CRISPR-Cas13a-Based Cancer Gene Therapy [55]	AAVs	HCC	Decoy minimal promoter (DMP)-controlled CRISPR-Cas13a system used to knock down several oncogenes (TERT, EZH2, and RelA)	Significant inhibition of HCC cell growth
CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma [56]	AAV6	Glioblastoma (GBM)	Allogeneic EGFRvIII CAR T-cell deficient in PD-1, TCR and B2M generated by CRISPR-Cas9	Enhanced antitumor efficacy in preclinical models of GBM.
Targeting HPV16 DNA using CRISPR/Cas inhibits anal cancer growth in vivo [57]	AAVs	Anal cancer	Cleaving the HPV16 E6 or E7 genes in primary human anal cancer cells utilizing CRISPR/Cas9	Significant and selective tumor suppression
CRISPR/Cas9-mediated cervical cancer treatment targeting human papillomavirus E6 [58]	AAVs	Cervical cancer	Causing multiple mutations in targeted HPV E6 gene in cervical cancer cells by CRISPR/Cas9	Increased expression of tumor suppressors, tumor growth inhibition, and increased apoptosis in the cancer cells
Disruption of PD-1 Enhanced the Anti-tumor Activity of Chimeric Antigen Receptor T Cells Against Hepatocellular Carcinoma [59]	Lentivirus	Hepatocellular Carcinoma (HCC)	The PD-1 disruption in the second-generation GPC3-targeted CAR T cells by CRISPR/Cas9.	Promoted Anti-tumor activity of the CAR T cells against HCC
CRISPR knock out of programmed cell death protein 1 enhances anti-tumor activity of cytotoxic T lymphocytes [60]	Lentivirus	Multiple Myeloma (MM)	Impairing PD-1/PD-L1 pathway in CTLs with CRISPR-Cas9 system.	CTLs repressed MM tumor growth and prolonged survival.
Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer [61]	Lentivirus	Refractory non-small-cell lung cancer (NSCLC)	CRISPR-Cas9-mediated knock down of PD-1 of T cells in patients with NSCLC	Results showcased the clinical safety and feasibility of CRISPR-Cas9 gene-edited T cells in NSCLC patients.
CRISPR-engineered T cells in patients with refractory cancer [62]	Lentivirus	Refractory cancer	NY-ESO-1 TCR-expressing engineered cells deprived of PD-1 (PDCD1), TCRα (TRAC), and TCRβ (TRBC)	Enhanced anti-tumor immunity and feasibility multiplex CRISPR-Cas9 editing at clinical scale
Activation of concurrent apoptosis and necroptosis by SMAC mimetics for the treatment of refractory and relapsed ALL [63]	Lentivirus	Refractory acute lymphoblastic leukemia (ALL)	Disruption of receptor-interacting protein kinase 1 (RIP1) by using CRISPR and pharmacologic interference (SMAC mimetics).	Hampering cancer cells evading apoptosis, and activating concurrent apoptosis and necroptosis
Genome-wide CRISPR screen identifies HNRNPL as a prostate cancer dependency regulating RNA splicing [64]	Lentivirus	Prostate cancer	CRISPR/Cas9-based gene knockout of essential spliceosome and RNA binding protein (RBP) genes.	A RBP gene aclled HNRNPL is capable of increasing prostate cancer growth.
Genome-wide CRISPR/Cas9 library screen identifies PCMT1 as a critical driver of ovarian cancer metastasis [65]	Lentivirus	Ovarian cancer	PCMT1 (protein-L-isoaspartate (D-aspartate) O-methyltransferase) knockdown by CRISPR/Cas9	PCMT1 increases in vivo metastasis formation and its serum levels may serve as a potential metastatic marker.
Genome-wide CRISPR screen reveals SGOL1 as a druggable target of sorafenib-treated hepatocellular carcinoma [66]	Lentivirus	HCC	In combination with NGS, the genome-wide CRISPR screen was used to determine loss-of-function mutations bestowing sorafenib resistance upon HCC cells.	SGOL1 found to be a druggable target that its inhibition may reduce drug resistance against sorafenib treatment.
Genome-Wide CRISPR-Cas9 Screen Identifies MicroRNAs That Regulate Myeloid Leukemia Cell Growth [67]	Lentivirus	Acute Myeloid Leukemia (AML)	miRNA loss-of-function screening was exerted by CRISPR-Cas9 technology.	Disruption of miR-150 (targeting p53) and miR-155 are therapeutic targets in AML.
Genome-wide CRISPR screen identifies *LGALS2* as an oxidative stress-responsive gene with an inhibitory function on colon tumor growth [68]	Lentivirus	Colon cancer	The CRISPR-based screening alongside NGS evaluated the genetic factors involved in the regulation of oxidative stress.	It is reported that Glycan-binding protein Galectin 2 (Gal2) overexpression reduces the human colon tumor growth.
Genome-wide CRISPR-Cas9 screen identified KLF11 as a druggable suppressor for sarcoma cancer stem cells [69]	Lentivirus	Osteosarcoma	The genome-wide CRISPR screening of cancer stem cells (CSCs) of Osteosarcoma identified the regulator of osteosarcoma.	Results showed that Low KLF11 correlates with osteosarcoma's poor prognosis and inadequate chemotherapy response.
Genome-Scale CRISPR-Cas9 Transcriptional Activation Screening in Metformin Resistance Related Gene of Prostate Cancer [70]	Lentivirus	Prostate cancer	CRISPR-based screening of metformin resistance in prostate cancer to find genes involved in metformin insensitivity.	Activation of ECE1, ABCA12, BPY2, EEF1A1, RAD9A, and NIPSNAP1 associated with in vitro resistance to metformin.
Genome-wide CRISPR-Cas9 knockout library screening identified PTPMT1 in cardiolipin synthesis is crucial to survival in hypoxia in liver cancer [71]	Lentivirus	HCC	Genome-wide CRISPR-Cas9 screening showcased therapeutic factors responsible for hypoxic survival in HCC.	Knockout of PTPMT1 provokes ROS and apoptosis in hypoxic HCC cells.

Table 1. continued

Study	Vector	Type of cancer	Approach	Outcome
Identifying novel therapeutic targets in gastric cancer using genome-wide CRISPR-Cas9 screening [72]	Lentivirus	Gastric cancer	The genome-scale CRISPR-Cas9 knock-out library of gastric cancer cells	Among 184 novel genes involved in gastric cancer, methyltransferase 1 (METTL1) inhibition was the most validated approach for cancer-targeted therapy.
In vivo CRISPR/Cas9 targeting of fusion oncogenes for selective elimination of cancer cells [73]	Adenovirus	PDX (patient-derived xenograft) cancer models	CRISPR/Cas9-mediated targeting of two introns of the translocated genes, results in	Disruption of the fusion oncogene in cancer cells, followed by a selective and efficient activity for cancer cell elimination.
Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing [74]	Lentivirus and Adenovirus	Pancreatic cancer	CRISPR/Cas9-mediated genomic manipulation of pancreatic cancer cells to develop transgenic mouse lines, allowing titratable initiation of pancreatic tumors	This method paves the way for the investigation of molecular alterations, driving each step of pancreatic cancer development.
Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting [75]	Exosome	Ovarian cancer	Tumor-derived exosomes loaded with cas9 and PARP-1 sgRNA expression plasmids via electroporation	CRISPR/Cas9-induced inhibition of PARP-1 resulted in ovarian cancer cell apoptosis and increased sensitivity to chemotherapeutic agent (cisplatin).
Exosome-mediated delivery of CRISPR/Cas9 for targeting of oncogenic KrasG12D in pancreatic cancer [76]	Exosome	Pancreatic cancer	Exosomes loaded with CRISPR/Cas9 capable of targeting the mutant KrasG12D oncogenic	Suppressed proliferation and hampered tumor growth in syngeneic subcutaneous and orthotopic models of pancreatic cancer.
Tropism-facilitated delivery of CRISPR/Cas9 system with chimeric antigen receptor-extracellular vesicles against B-cell malignancies [77]	EVs	B cell malignancies	The CRISPR/Cas9 system aiming at the MYC oncogene, was loaded into selective EVs having anti-CD19-CAR on their surface.	The induced CRISPR/Cas9-mediated loss-of-function mutations of the MYC gene in CD19 + cells exhibited the significant potential of this approach.
Efficient RNA drug delivery using red blood cell extracellular vesicles [78]	RBC extracellular vesicles (RBCEVs)	AML M5	Electroporation of HA-tagged Cas9 mRNA and gRNA of human *mir-125b-2* into RBCEVs, and used them to treat MOLM13 cells.	Exosomes successfully transfected both human cells and xenograft mice, with no notable cytotoxicity.
Exosome–Liposome Hybrid Nanoparticles Deliver CRISPR/Cas9 System in MSCs [79]	Exosome	Mesenchymal stem cells (MSCs)	Hybrid exosomes-liposomes capable of carrying large cargoes such as CRISPR/Cas9 System	Hybrid exosomes efficiently delivered CRISPR/dCas9 to inhibit the expression of mRunx2 and hCTNNB1 in MSCs
In vitro and in vivo RNA inhibition by CD9-HuR functionalized exosomes encapsulated with miRNA or CRISPR/dCas9 [80]	Exosome	Recipient cells	Since Hur is an RNA binding protein, CD9-HuR exosomes could efficiently encapsulate the miR-155 or CRISPR/dCas9	Increased RNA cargo loading into engineered exosomes
Activation of Necroptosis by Engineered Self Tumor-Derived Exosomes Loaded with CRISPR/Cas9 [55]	Exosome	GBM, Thyroid cancer, lung adenocarcinoma	Engineered exosomes for TNFR activation and impairment of IAP 1/2 and Caspase 8 expression loaded with CRISPR/CAS9	Activation of TNFR and subsequently inactivation of IAP 1/2 and Caspase results in blocked cell survival and Necroptosis activation.

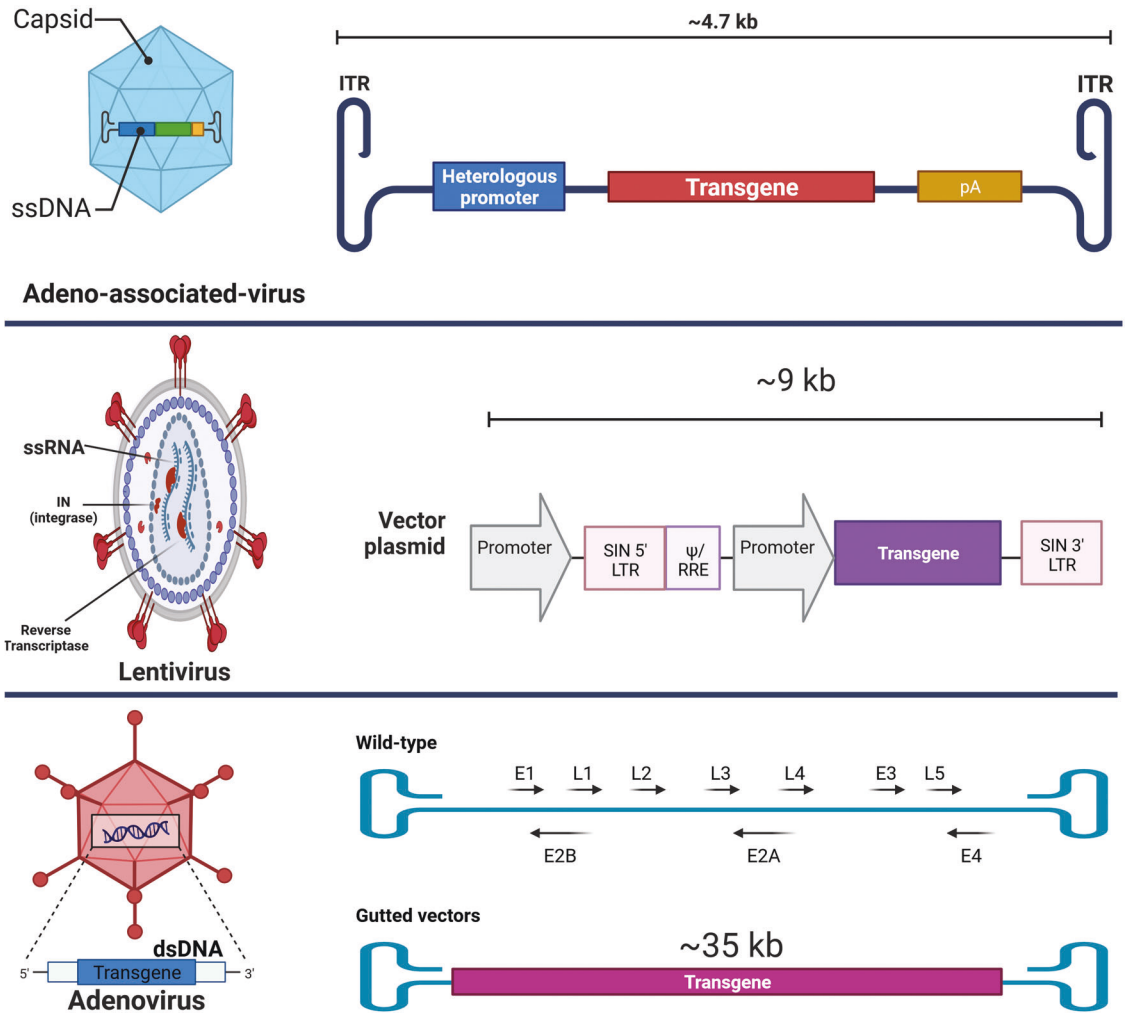

Fig. 2 Commonly used viral vectors for gene delivery. AAVs are single-stranded DNA viruses with no envelope and small size that are not pathogenic to humans. The gutted Adenoviral vectors are double-strand DNA viruses devoid of most genes from their wild-type, although still capable of transducing a broad spectrum of both dividing and non-dividing cells with a capacity of carrying genetic cargo up to 35 kb. Lentiviral **vectors**, primarily derived from HIV-1, are capable of integrating their transgene (~9 kb) into the human genome and are suitable for long-term gene expression.

are concerns about this approach since it demands a high viral dose and accurate tracking of the vectors in order to confirm co-transduction [73, 74].

There are some genes that are even larger than the capacity of dual AVV vectors (9 kb); for example, the CDH23 (Usher syndrome) and DMD (muscular dystrophy) genes are about 10 kb and 11.1 kb, respectively. In order to deliver these kinds of genes into host cells, scientists have designed a triple AAV system [75, 76].

Since a novel approach called CRISPR/Cas-mediated base and prime editing uses dead or Cas9 nickase (nCas9) enzymes, it can modify the CRISPR-Cas system so that it is possible to use single-vector delivery and overcome challenges pertaining to limited viral-vector packaging capacity [77]. Further characteristics of this system include not inducing DSB, does not need a DNA donor template, and has great capability for editing non-dividing cells [78]. AAVs have proven to be potential systems for delivering CRISPR DNA base-editing tools [58]; for instance, a study reported that in vivo delivery of the CRISPR/Cas-based cytidine-base editor by dual AAVs could treat amyotrophic lateral sclerosis (ALS) in an animal model. It has been shown that the AAV-delivered prime editor is an effective tool for correcting pathogenic alleles and cancer modeling in adult mice, because it has a significant lower off-target effect than the CRISPR/Cas-based base editor [78, 79]. Although AAV-base and prime editing systems improve some drawbacks related to AAV-mediated CRISPR/Cas9 delivery, the limitations, including viral-induced immune response, vector persistency, and off-target activity, are still present to some extent [77].

The packaging capacity limitation of AAVs can be managed by utilizing other classes of Cas protein like Cas12a or different forms of Cas9 having smaller sizes, such as SaCas9 and St1-Cas9 [55, 80–82]. Moreover, breaking large transgenes into two separate cuts and packaging each half into two sets of AAVs can extend AAVs' delivery capacity. This approach is carried out by a process called intein-mediated trans-splicing, which to some degree is similar to mRNA splicing [83, 84]. This method has been used to deliver base and prime editing systems, which were found to be quite promising [85, 86]. Another challenging issue for in vivo delivery is the pre-existing immunogenicity against the bacterial Cas9 protein and AAV capsid. However, the AAV capsid can be improved by altering its antigens, creating a chimeric AAV capsid to decrease antibody response and evade the immune system [87, 88].

The characteristics of AAVs, which include low immunogenicity and cytotoxicity, as well as a slim chance of chromosomal integration, make them the ideal delivery vessels for CRISPR/Cas

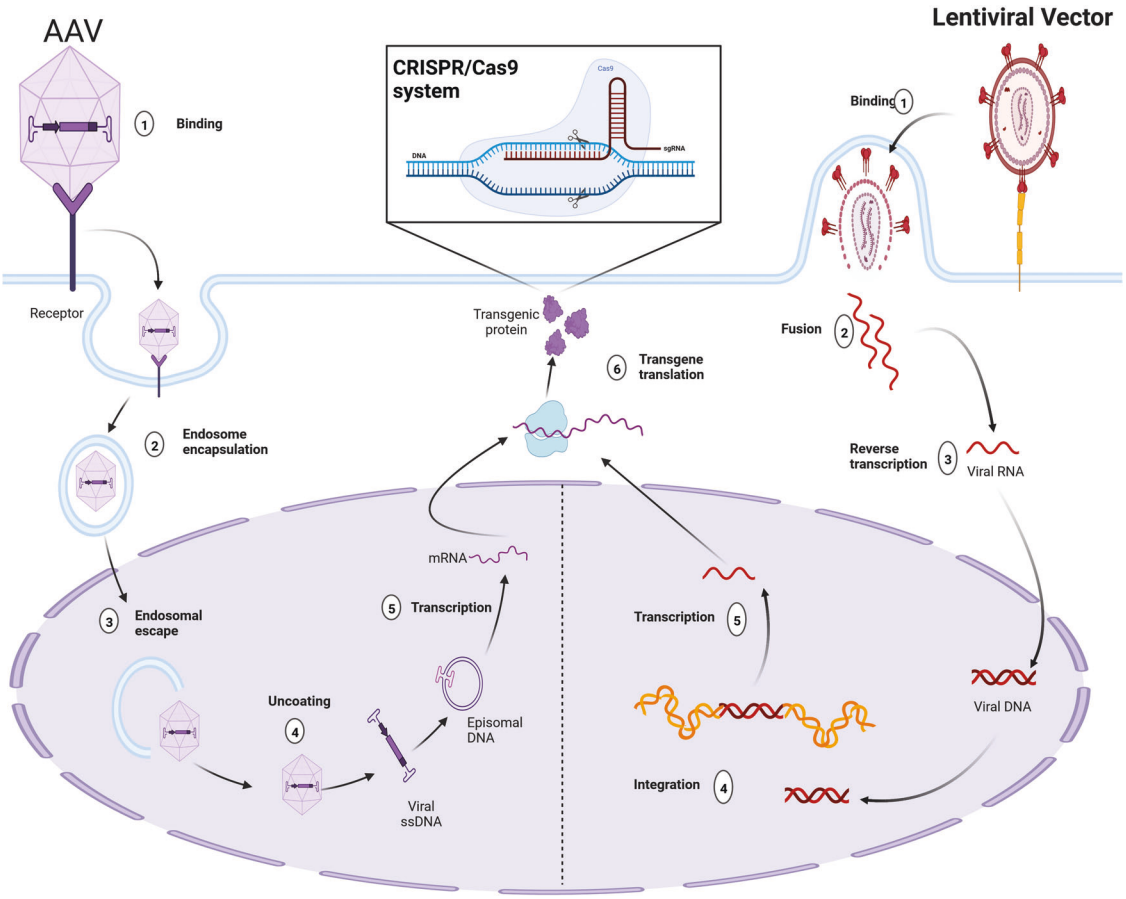

Fig. 3 Viral Vectors encoding CRISPR/Cas. Mechanism of action of two types of viral vectors for delivery of CRISPR/Cas systems: integrative (lentiviral vectors) and non-integrative (Adenoviral and Adeno-associated viral (AAV) vectors).

system. Alongside these, having various types and diverse delivery approaches, like dual and triple AAV vectors, covers their limitations compared to other viral vectors and brings them to the forefront of gene delivery.

Adenoviral vectors. The AdV is a double-strand DNA virus that can transduce a broad spectrum of both dividing and non-dividing cells. These vectors can carry a genetic cargo up to 37 kb, and following transduction, they create an episomal DNA adjacent to the host DNA instead of integrating into the genome. Noteworthy that the episomal gene expression of AdVs removes the off-target effects, a limitation in CRISPR/Cas-based gene editing [89, 90].

There are different generations of AdVs; in the first one, the E1 gene was deleted, although using this generation could provoke acute and chronic immune responses [88]. Furthermore, in the second generation, the E2 and E4 genes of AdV were removed to diminish the immune response. Of note, the capacity of second-generation AdVs (~14 kb) for incorporating transgene is substantially superior to the first-generation (~8 kb) [91, 92]. The helper-dependent vectors (gutted vectors), which represent the third generation of AdV vectors, do not contain viral genes that allow cloning up to 35 kb; therefore, they move beyond the size limitation barrier for delivering the CRISPR/Cas9 system. It is worth mentioning that, like second-generation AdVs, this generation does not trigger chronic immune responses [93, 94].

The recombinant AdV5 has shown potential for in vivo CRISPR/Cas9-mediated knock-in of the human alpha-1-antitrypsin gene in mice, and it has also been demonstrated that gene expression continued for more than 200 days [95]. In a recent study, AdV-delivered CRISPR/Cas12a to human hepatocytes indicated its potential toward gene editing of human cells, although the provoked immune system against the vector and Cas protein was reported [96]. Furthermore, AdV structural proteins (Hexon, penton, and fiber) are quite manipulatable, which is advantageous for creating AdVs with tissue-specific tropism. Moreover, these properties, along with the fact that AdVs are safe for clinical trials, can be manufactured at large scales, and are cost-effectively manufactured, make them indispensable for CRISPR/Cas delivery [56, 97]. These beneficial features could be verified by the fact that AdVs became the top choice for creating mRNA vaccines for Coronavirus disease 2019 (COVID-19). This betokens the idea that AdVs can be produced as a delivery system on a global scale [98].

All in all, in CRISPR/Cas-based gene editing, AdVs' episomal gene expression eliminates off-target effects. Also, its huge packaging capacity allows for loading large genetic payloads, which is a concern in other viral vectors.

Lentiviral vectors. The HIV-1-derived LVs are single-stranded RNA (ssRNA) viruses primarily used for integrating the desired transgene into dividing and non-dividing cells (Fig. 3). Their delivery capacity for genetic cargo is around 9 kb, encompassed by a lipid-enriched capsid. There are four generations of LVs, where the third and fourth generations are mostly safe for clinical applications [99].

The effectiveness of CRISPR/Cas-based gene editing greatly depends on the delivery of its components into the host cell. As LVs are a great armamentarium for gene delivery, and the CRISPR/Cas system was proven to be functional in human cells, LVs expressing the whole system were chosen [100]. Like shRNAs, LVs

carrying CRISPR/Cas9 systems have also been designed initially in a library model with numerous sgRNAs [101]. A study showed that an LV-based genome-scale CRISPR-Cas9 knockout (GeCKO) library that targets more than 18,000 human genes is quite valuable for negative and positive selection screening, which are generally utilized for in vitro assays to determine disturbances in cells, especially cancer cells affected by various stimuli [100]. In particular, this library has been used to specify genes critical for the viability of cancer cells and pluripotent stem cells. It also allowed for screening of loss of function in genes that cause resistance to vemurafenib in melanoma cells [100]. Further studies have also developed LV-based CRISPR/Cas libraries which have resulted in the identification of novel tumor-suppressor genes involved in myeloid leukemia (Nf1, Ezh2, Dnmt3a, Tet2, and Runx1), and fetal hemoglobin reinduction (BCL11A) [102, 103].

With time, the capabilities of the LV-delivered CRISPR/Cas9 system are expanding into the fields of targeted therapy for HIV-1 and HBV infections as well as the treatment of genetically defective diseases, such as cystic fibrosis and neurodegenerative diseases [104]. Additionally, other methods, including base and prime editing CRISPR systems and epigenetic modifiers, have also been paired with LVs [105]. Since LVs integrate the encoding CRISPR/Cas transgene into the host genome, and due to their long-lasting gene expression, the possibility of off-target effects could be raised as a concern. To address this risk, integration-deficient lentiviral vectors (IDLV) were developed to provide an impermanent CRISPR/Cas expression [106].

In unresectable hepatocellular carcinoma, HIF-1α expression is capable of worsening the prognosis of the disease and hampering the overall improvement of patients. A recent study knocked out the HIF-1α in mice using an LV-delivered CRISPR/Cas9 system where it drastically diminished the HIF-1α expression in the tumor tissues three days post-injection, showcasing valuable antitumor effects [107]. Chromosomal translocations creating fusion oncogenes are frequent in certain cancers and are substantial tumorigenesis factors [108, 109]. For example, a tyrosine kinase produced by BCR-ABL rearrangement can develop chronic myeloid leukemia (CML) [110]. Tyrosine kinase inhibitors (TKIs) such as Imatinib can significantly inhibit the product of BCR-ABL, though there are reports of drug resistance against these TKIs [111]. Accordingly, Martinez-Lage and colleagues used an LV-based CRISPR/Cas9 system to induce deletion in introns of the BCR-ABL rearranged gene, which resulted in a significant deletion of the BCR transactivation domain as well as frameshift mutation in the DNA-binding domain of ABL [112].

LV has always been an intriguing viral vector for delivering genes, particularly for clinical purposes. This is because the transgene can be integrated into the host genome and expressed for a long time. Furthermore, the desired gene can be passed along to new cells following cell division, thus ensuring that CRISPR/Cas functions are expressed for a long time to come.

Extracellular vesicles (EVs)

Extracellular vesicles are nano-scaled non-viral delivery vessels that can be used for various purposes, one of which is to be used as a targeted delivery system. EVs are lipid-coated particles produced by different cells with the innate objective of cell–cell transportation of cargoes such as genetic material, and proteins [113]. EVs can be grouped into three main categories: microvesicles (MVs), exosomes, and apoptotic bodies. They are different in packaging capacity, biogenesis, function, and releasing mechanism. Among them, exosomes having 30–150 nm in diameter are genuine options for selective delivery of proteins, and genetic components, including CRISPR/Cas system (Fig. 4) [113–115].

Exosomes have drawn much attention for being used in cancer diagnosis and therapy. Exosomes secreted from tumor cells could be an excellent option for tumor-targeted therapy since they are similar to their source and, therefore, more likely to be taken up by the cells. Cancer therapy with a cell-specific tropism approach can be facilitated by controlling this feature and loading desired cargoes into tumor-derived exosomes. Accordingly, a study indicated that loading tumor-derived exosomes with a cancer therapeutic agent called Doxil and injecting it systemically into the tissue of origin can result in promoted tumor suppression compared to the application of the drug alone [116]. In an intriguing strategy, biocompatible porous silicon nanoparticles (PSiNPs) harboring doxorubicin called DOX@E-PSiNPs were introduced to isolated tumor cells (Fig. 5). Afterward, tumor cell-released exosomes sheltering DOX@E-PSiNPs were injected systematically into mice, which were uptaken by both bulk cancer cells and cancer stem cells (CSCs), resulting in considerable tumor suppression [117]. The promising potential of exosomes for CRISPR/Cas delivery in tumor cells can be extrapolated from their ability to deliver selective and efficient delivery systems.

According to the potency of exosomes, a study used tumor-derived exosomes for in vivo targeted delivery of CRISPR/Cas9 into SKOV3 xenograft mice cells with ovarian cancer. They compared epithelial cell-derived exosomes and cancer-derived exosomes to deliver a CRISPR/Cas9 system capable of suppressing the expression of poly (ADP-ribose) polymerase-1 (PARP-1). The results showed significant apoptosis in ovarian cancer cells and a synergic enhancement of chemosensitivity to cisplatin. The combination of both therapeutic approaches resulted in 57% inhibition of cancer proliferation, almost twice the effect of exosomes- or cisplatin-sole treatment [118]. One serious concern about using exosome-mediated gene delivery, especially in the case of delivering the CRISPR/Cas system, is the potential for off-target negative impacts on peripheral and distant tissues.

In addition to the packaging capabilities of exosomes, engineering them to be targeted delivery vehicles is an essential goal. Alvarez-Erviti and colleagues produced brain-targeting exosomes carrying short interfering RNA (siRNA) from engineered dendritic cells capable of expressing Lamp2b, an exosomal membrane protein, fused with nervous system-specific rabies viral glycoprotein (RVG). The results of in vivo delivery of these exosomes in mice showcased strong therapeutic potential and no non-specific uptake by other tissues [119]. Another study used a similar approach to deliver miRNA in the cartilage as a treatment for osteoarthritis, which exhibited a significant potential in targeting hard-penetrating tissues [120, 121].

Necroptosis is a form of necrosis in response to a pathogen or inflammation, in which cells undergo non-programmed cell death. Of note, in this pathway caspase 8 and IAP1/2, which are involved in cell survival and apoptosis, should be suppressed. In a recent study, a particular kind of tumor-derived exosomes was designed to have TNF receptor (TNFR) ligands on their surface. These exosomes were carrying CRISPR/Cas9 systems which are capable of inhibiting caspase 8 and IAP1/2; therefore, following exosome-mediated activation of TNFR signaling and CRISPR/Cas9-mediated inactivation of caspase 8 and IAP1/2, tumor cells underwent necroptosis (Fig. 6). The advantage of necroptosis over apoptosis is that the former also provokes T-cells to eliminate the remaining cancer cells [122].

Unlike viruses, exosomes can be modified in a matter of size. Hybrid exosomes, a combination of cell-derived exosomes and synthetic liposomes, have been developed to carry large cargoes such as CRISPR/Cas system [123]. Not only do hybrid exosomes have greater packaging capacity but also, due to the positive charge of the liposomes, they interact more efficiently with negatively charged RNA and DNA to uptake them by membrane fusion. This approach can remove the need for loading genetic material into the exosomes by mechanical methods such as electroporation [123]. Noteworthy that this alteration to exosomes had no effects on the efficiency of their cell-type tropism and uptake [124]. This method has been used by Lin and colleagues to

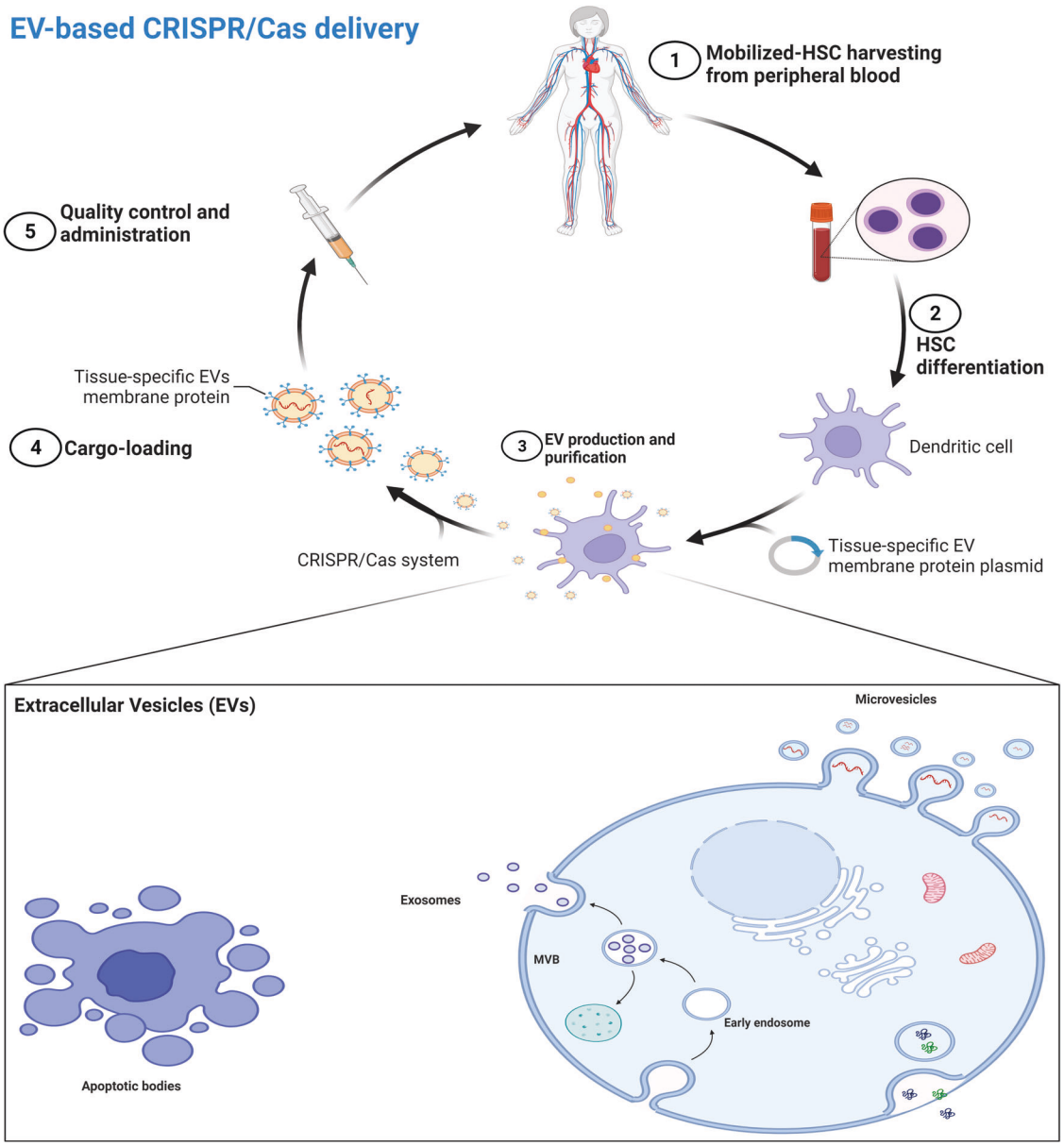

Fig. 4 EV-based gene delivery. There are different types of EVs, including microvesicles, exosomes, and apoptotic bodies, that can be collected from human cells. These EVs can carry proteins, DNA, and different types of RNA from one cell to another. These exosomes can be engineered to target a specific tissue while carrying our desired cargo like CRISPR/Cas system. This approach allows for autologous tissue-specific gene editing.

efficiently deliver CRISPR/Cas9 expression vectors to mesenchymal stem cells (MSCs), which are hard to transfect [125].

Exosomes can also be optimized with DNA aptamer, a short synthetic oligonucleotide, in order to use exosomes as targeted delivery systems. DNA aptamers are cost-effective, readily available, and non-provocative for the immune system; thus making them a suitable alternative to antibodies or other probes [126, 127]. A recent study used cholesterol-anchored valency-controlled tetrahedral DNA nanostructures (TDNs) conjugated with DNA aptamer on the surface of exosomes to selectively deliver the CRISPR/Cas system inhibiting the WNT10B gene into hepatocellular carcinoma cells in vitro, ex vivo, and in vivo. The promising results of targeted gene suppression in hepatocellular carcinoma cells by this method highlight the outstanding potential of EVs for being used as labeled and targeted delivery systems for CRISPR/Cas system [128].

The risk of nucleated cell-derived EVs-mediated horizontal gene transfer could be viewed as a limitation for EVs, though RBC-derived EVs bypass this safety-related issue. Since RBCs are non-nucleated, O blood group RBCs can be used as a universal source for the harvesting of EVs without DNA. The RBC-EVs have been used in vivo and in vitro for delivering CRISPR/Cas9, demonstrating high transfection efficiency and without detectable cytotoxicity [129]. Further, Pham and colleagues reported that RBC-EVs could be targeted for selective delivery of cargoes. Accordingly, they conjugated the epidermal growth factor receptor (EGFR)-targeting peptide to paclitaxel-carrying EVs by Sortase A and OaAEP1 ligase. The results indicated that EVs could efficiently deliver the chemotherapeutic agent to EGFR-positive lung cancer cells, causing significant apoptosis and shrinking the tumor [129]. It is plausible to extrapolate the considerable potential of targeted RBC-EVs for the delivery of CRISPR/Cas9 systems to specific cancer cells.

EVs, especially tumor-derived EVs have been shown to be extremely effective in bypassing tumor defense mechanisms in CRISPR/Cas-based cancer therapy. They come in different sizes

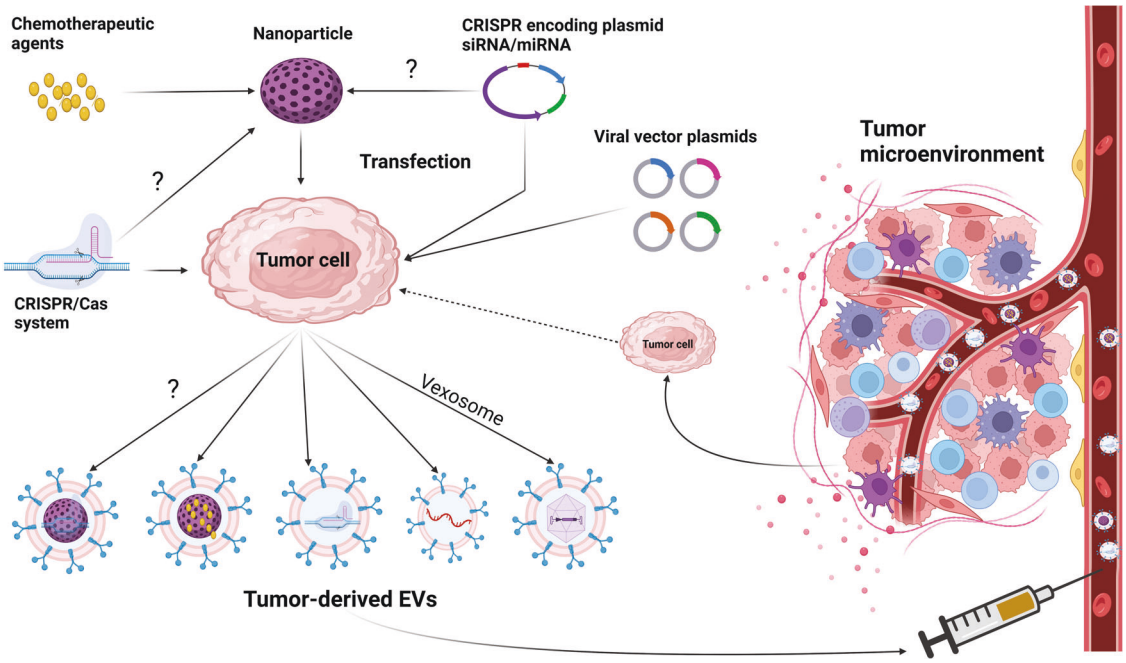

Fig. 5 Tumor-derived exosomes. Transfecting tumor cells with DOX@E-PSiNPs, CRISPR/Cas system, and viral vector plasmids while inducing the tumor cells to produce exosomes can provide tumor-derived exosomes encapsulating our desired cargo. Since these exosomes originated from the tumor, they can be harvested and injected systematically back into tumor cells for therapeutic purposes.

and can contain different genetic materials. These qualities, as well as many others, including easy manipulation, reliable gene delivery, and high safety, make EVs formidable cancer therapy weapons.

Vexosomes: exosome-enveloped viral vectors

Exosomes-enveloped viral vector or vexosome is a novel gene delivery approach that confers exosome features such as a broad spectrum of cell tropism, almost non-immunogenic, and scalability, to viral vectors for enhancing gene therapy [130]. The production of vexosomes requires infecting the packaging cells with plasmids encoding the viral genome. The cells' cytoplasm becomes enriched with the produced viral genome and proteins, which are recognized by the cell's receptors in the plasma membrane, endosomes, and phagosomes. Therefore, EVs encapsulate the viral components upon their secretion from the cells. Saari and co-workers used this method to produce capsid-free EV-based vectors carrying oncolytic AAV components (AAV/EVs) into cancer cells [131]. Vexosomes have not been used for delivery of CRISPR/Cas system, though their potential in combining the characteristics of viral vectors and EVs is promising for efficient gene editing.

AAV/EVs can be modified to deliver genes more selectively by including targeting peptides on their surfaces. Multiple studies have reported benefits from using targeted AAV/EVs in vivo and in vitro; for example, Wood et al. reported that these vectors can pass the blood-brain barrier and effectively transduce neural cells [132]. Different serotypes of AAV, including AAV1, AAV6, and AAV9, have been used for targeted delivery of transgenes into cochlear and vestibular hair cells, neurons, and oligodendrocytes, respectively [133–136]. It indicates the compatibility of this approach for gene delivery into various cell types.

The EVs encapsulating the AAVs can be used as a trojan horse to circumvent the pre-existing immunity against the viral vectors. In this context, a recent study systematically injected AAV9/EVs into mice with pre-existing immunity to AAV9, and the results demonstrated successful evading from the immune system [137]. Furthermore, this approach has shown that it can reduce the number of injections required for efficient delivery of vectors, reducing the risk of AAV-induced cytotoxicity [119, 138–140]. Despite the benefits of this delivery method, it has not yet been explored as a delivery method for the CRISPR/Cas system.

Combining the power and potential of EVs and viral vectors promises a great future in the field of gene delivery, especially for CRISPR/Cas delivery. Due to the fact that this approach is new, it will be more interesting for researchers to explore its full potential as a cancer therapy.

CLINICAL TRANSLATION FOR CANCER DIAGNOSTICS AND THERAPIES

The CRISPR/Cas technology provides a quintessential gene-editing application [80, 141], though in the case of clinical cancer therapy it has not been a major player. That said, the potential of CRISPR-based genome editing is promising for cancer diagnostics and therapies in the near future (Table 2).

CRISPR–Cas9-targeted fragmentation of DNA can be used as a means to pinpoint the changes in cancer-specific sequence. For example, CRISPR/Cas9 can detect microsatellite sequences, called short tandem repeats (STRs), which can serve as cancer markers. Most STR assays depend on PCR amplicons, which are limited to a few. In comparison, CRISPR/Cas9-mediated STR-sequencing is capable of accurately and sensitively analyzing more than 2000 STRs in parallel [142, 143]. There are regions in the human genome housing complex megabase-sized fragments, containing biologically essential genes. Owing to their complexity and variations, these regions are not thoroughly uncovered, although Baker and colleagues used a potential approach called CISMR (CRISPR-mediated isolation of specific megabase-sized regions of the genome), for targeted sequencing of these fragments [144]. Moreover, if CRISPR-mediated fragmentation of genomic DNA is combined with duplex sequencing, correcting sequencing errors, it can result in ultra-accurate sequencing with low DNA input, termed CRISPR-DS [145]. As compared to duplex sequencing, CRISPR-DS had a superior capability in detecting TP53 mutations in the peritoneal fluid of ovarian cancer patients using 10 to 100-

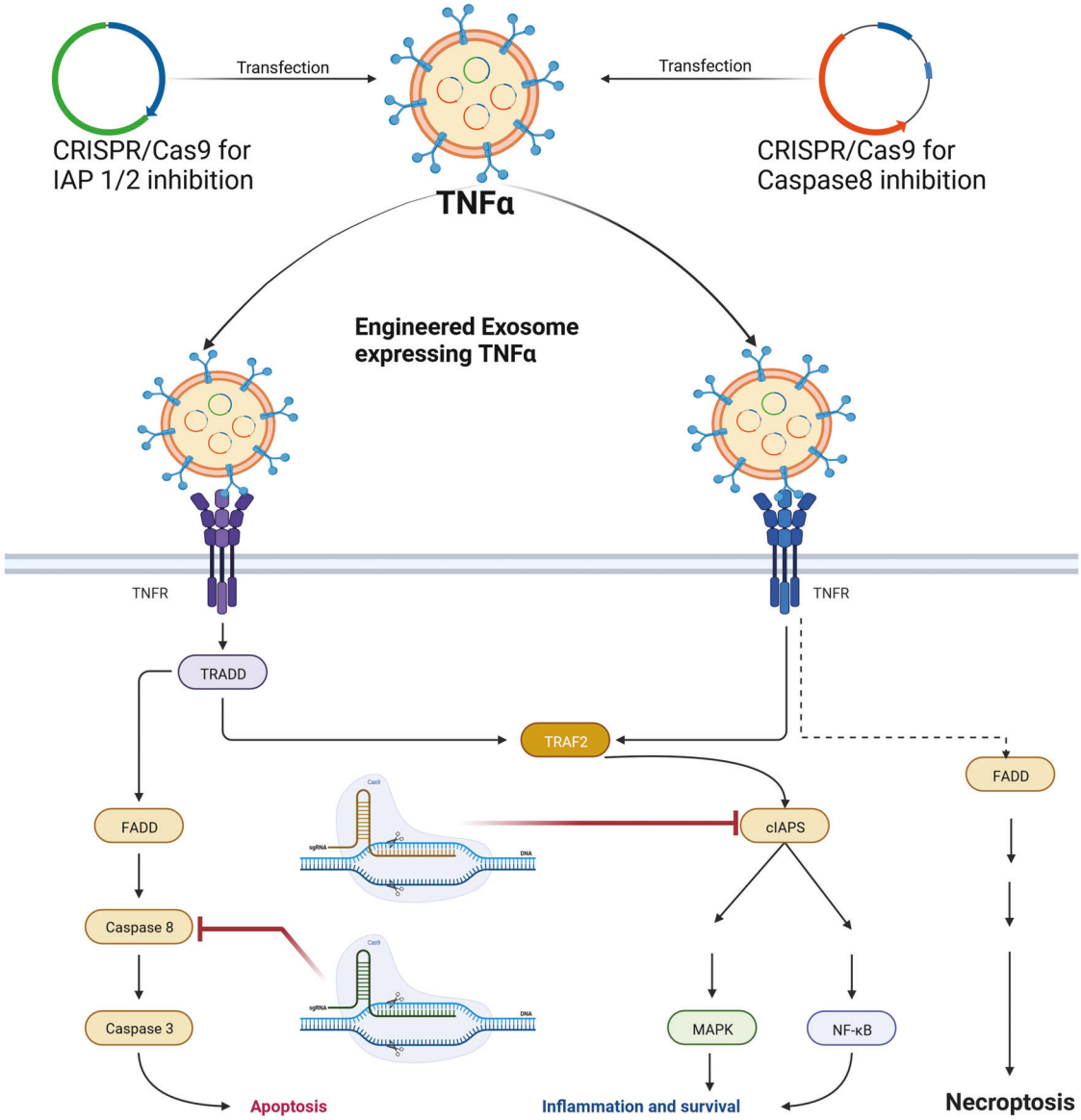

Fig. 6 Necroptosis induction using exosomes. Engineered exosomes with TFNα on their surface, carrying two vectors of the CRISPR/Cas system capable of inhibiting cIAPs and caspase 8. In this approach, the exosome-TNFα ligand provokes TNFR signaling pathways, which can activate the cIAPs, propelling the cell toward survival. CRISPR/Cas9-mediated inhibition of cIAPs alongside active caspase 8 results in apoptosis of cancer cells. Moreover, dual inhibiting of cIAPs and caspase 8 alongside TNFR signaling lead to necroptosis. The advantage of necroptosis over apoptosis is that the former also provokes T-cells to eliminate the remaining cancer cells.

fold less DNA than the latter [145]. In light of CRISPR-DS' diagnostic potential, it has now reached the stage of clinical trials [NCT03606486]. Other highly accurate enzymatic nucleic acid detection systems have also been developed for cancer-related mutations, called DETECTR and SHERLOCK, which use Cas12a and Cas13, respectively [25, 28, 146, 147].

Additional to cancer diagnosis, CRISPR has made its way to be applied for clinical cancer treatment, which demonstrates the astonishing potential of a recently developed gene-editing tool. Initially, the CRISPR/Cas9 system was used for treating non-small-cell lung carcinoma (NSCLC) at West China Hospital, Sichuan University, in 2016 [148–150]. In that study, patients' T-cells were genetically engineered not to express the T-cell activation inhibitor, PD-1. Accordingly, this approach has also been used for cancer of other tissues, including bladder, prostate, renal, and esophageal [150]. It is noteworthy that some of these clinical trials have withdrawn their studies [151].

The development of CAR T cell receptors has made significant strides in cancer treatment, with Kymriah and Yescarta (CAR T-cell against CD19) being approved by the FDA for the treatment of B-cell leukemia and lymphoma [152]. Despite all the encouraging results from autologous CAR T-cell therapy, there are still some limitations that need to be tackled. For example, in some cases, such as infants, there may not be enough T-cells to generate CAR-T cells and perform autologous transplantation. The CRISPR/Cas system can be integrated into CAR T-cell engineering to produce universal CAR T-cells from healthy donors [153].

Viral vectors integrate CAR genes into T-cells in a random manner, not in a site-specific manner, which might adversely affect the genome. Eyquem et al. used a CRISPR/Cas9-based site-specific integration of the CD19-specific CAR gene to the T-cell receptor α constant (TRAC) locus. These engineered CAR T-cells outperformed the previous generations regarding safety, precision, and effectiveness in targeting acute lymphoblastic leukemia cells [154].

Table 2. Clinical trials on CRISPR/Cas-mediated cancer therapy (https://www.clinicaltrials.gov).

Study title	Status	Conditions	Interventions	Gender	Age	Phases	Clinical trial identifier
Study of CRISPR-Cas9 Mediated PD-1 and TCR Gene-knocked Out Mesothelin-directed CAR-T Cells in Patients with Mesothelin Positive Multiple Solid Tumors.	Recruiting	Solid Tumor	Biological: anti-mesothelin CAR-T cells	All	18–70	Phase 1	NCT03545815
A Study of Metastatic Gastrointestinal Cancers Treated with Tumor Infiltrating Lymphocytes in Which the Gene Encoding the Intracellular Immune Checkpoint CISH Is Inhibited Using CRISPR Genetic Engineering	Recruiting	Gastrointestinal Cancer	Drug: Cyclophosphamide|Drug: Fludarabine| Biological: Tumor-Infiltrating Lymphocytes (TIL)| Drug: Aldesleukin	All	18–70	Phase1 and Phase 2	NCT04426669
A Safety and Efficacy Study of TALEN and CRISPR/Cas9 in the Treatment of HPV-related Cervical Intraepithelial Neoplasia	Unknown status	Human Papillomavirus-Related Malignant Neoplasm	Biological: TALEN|Biological: CRISPR/Cas9	Female	18–50	Phase 1	NCT03057912
Study of PD-1 Gene-knocked Out Mesothelin-directed CAR-T Cells with the Conditioning of PC in Mesothelin Positive Multiple Solid Tumors	Unknown status	Solid Tumor	Biological: Mesothelin-directed CAR-T cells	All	18–70	Phase 1	NCT03747965
A Safety and Efficacy Study Evaluating CTX110 in Subjects With Relapsed or Refractory B-Cell Malignancies (CARBON)	Recruiting	B-cell Malignancy|Non-Hodgkin Lymphoma|B-cell Lymphoma| Adult B Cell ALL	Biological: CTX110	All	18<	Phase 1	NCT04035434
PD-1 Knockout Engineered T Cells for Advanced Esophageal Cancer	Completed	Esophageal Cancer	Other: PD-1 Knockout T Cells	All	18–80	Not Applicable	NCT03081715
PD-1 Knockout Engineered T Cells for Metastatic Non-small Cell Lung Cancer	Completed	Metastatic Non-small Cell Lung Cancer	Drug: Cyclophosphamide| Other: PD-1 Knockout T Cells	All	18–70	Phase 1	NCT02793856
Stem Cells in NF1 Patients With Tumors of the Central Nervous System	Suspended	Neurofibromatosis Type 1| Tumors of the Central Nervous System	Diagnostic Test: Collection of Stem Cells	All	Child, Adult, Older Adult		NCT03332030
A Safety and Efficacy Study Evaluating CTX130 in Subjects With Relapsed or Refractory T or B Cell Malignancies (COBALT-LYM)	Recruiting	T Cell Lymphoma	Biological: CTX130	All	18<	Phase 1	NCT04502446
First-time-in-human (FTIH) Study of GSK3145095 Alone and in Combination With Other Anticancer Agents in Adults With Advanced Solid Tumors	Terminated	Neoplasms, Pancreatic	Drug: GSK3145095|Drug: Pembrolizumab	All	18<	Phase 2	NCT03681951

Table 2. continued

Study title	Status	Conditions	Interventions	Gender	Age	Phases	Clinical trial identifier
Lavage of the Uterine Cavity for Diagnosis of Ovarian Cancer	Recruiting	Ovarian Cancer	Other: Biospecimen Collection|Other: Laboratory Biomarker Analysis|Device: Lavage|Other: Pap Smear	Female	18<	Not Applicable	NCT03606486
TGF²R-KO CAR-EGFR T Cells in Previously Treated Advanced EGFR-positive Solid Tumors	Recruiting	Solid Tumor| EGFR Overexpression	Biological: TGF²R-KO CAR-EGFR T Cells	All	18–75	Phase 1	NCT04976218
PD-1 Knockout Engineered T Cells for Castration Resistant Prostate Cancer	Withdrawn	Hormone Refractory Prostate Cancer	Biological: PD-1 Knockout T Cells|Drug: Cyclophosphamide|Drug: IL-2	Male	45–85		NCT02867345
PD-1 Knockout Engineered T Cells for Muscle-invasive Bladder Cancer	Withdrawn	Invasive Bladder Cancer Stage IV	Biological: PD-1 Knockout T Cells|Drug: Cyclophosphamide|Drug: IL-2	All	18–75	Phase 1	NCT02863913
CRISPR (HPK1) Edited CD19-specific CAR-T Cells (XYF19 CAR-T Cells) for CD19 + Leukemia or Lymphoma.	Recruiting	Leukemia Lymphocytic Acute (ALL) in Relapse|Leukemia Lymphocytic Acute (All) Refractory|Lymphoma, B-Cell|CD19 Positive	Genetic: XYF19 CAR-T cell|Drug: Cyclophosphamide|Drug: Fludarabine	All	18–55	Phase 1	NCT04037566
Safety and Efficacy of CT125A Cells for Treatment of Relapsed/Refractory CD5 + Hematopoietic Malignancies	Not yet recruiting	CD5+ Relapsed/Refractory Hematopoietic Malignancies|Chronic Lymphocytic Leukemia (CLL)|Mantle Cell Lymphoma (MCL)|Diffuse Large B-cell Lymphoma (DLBCL)|Follicular Lymphoma (FL)|Peripheral T-cell Lymphomas (PTCL)	Biological: CT125A cells|Drug: Cyclophosphamide, fludarabine	All	18–70	Early Phase 1	NCT04767308
A Safety and Efficacy Study Evaluating CTX120 in Subjects With Relapsed or Refractory Multiple Myeloma	Recruiting	Multiple Myeloma	Biological: CTX120	All	18<	Phase 1	NCT04244656
Study of Base Edited CAR7 T Cells to Treat T Cell Malignancies (TvT CAR7)	Recruiting	Relapsed/Refractory T-cell Acute Lymphoid Leukemia	Biological: Cryopreserved BE CAR7 T cells (BE752TBCCLCAR7PBL)	All	6–16	Phase 1	NCT05397184
PD-1 Knockout EBV-CTLs for Advanced Stage Epstein-Barr Virus (EBV) Associated Malignancies	Unknown status	Stage IV Gastric Carcinoma|Stage IV Nasopharyngeal Carcinoma|T-Cell Lymphoma Stage IV|Stage IV Adult Hodgkin Lymphoma|Stage IV Diffuse Large B-Cell Lymphoma	Drug: Fludarabine|Drug: Cyclophosphamide|Drug: Interleukin-2	All	18–75	Phase 1|Phase 2	NCT03044743
Study Investigating NTLA-5001 in Subjects With Acute Myeloid Leukemia	Recruiting	Acute Myeloid Leukemia	Genetic: Arm 1: NTLA-5001|Genetic: Arm 2: NTLA-5001	All	18<	Phase 1|Phase 2	NCT05066165
A Study Evaluating UCART019 in Patients With Relapsed or Refractory CD19+ Leukemia and Lymphoma	Unknown status	B Cell Leukemia|B Cell Lymphoma	Biological: UCART019	All	12–75	Phase 1|Phase 2	NCT03166878
A Long-term Follow-up Study of Patients Who Received VOR33	Recruiting	Leukemia, Myeloid, Acute	Genetic: VOR33	All	Child, Adult, Older Adult		NCT05309733

Table 2. continued

Study title	Status	Conditions	Interventions	Gender	Age	Phases	Clinical trial identifier
NY-ESO-1-redirected CRISPR (TCRendo and PD1) Edited T Cells (NYCE T Cells)	Terminated	Multiple Myeloma |Melanoma| Synovial Sarcoma |Myxoid/ Round Cell Liposarcoma	Biological: NY-ESO-1 redirected autologous T cells with CRISPR edited endogenous TCR and PD-1| Drug: Cyclophosphamide| Drug: Fludarabine|Device: NY-ESO-1 expression testing	All	18<	Phase 1	NCT03399448
A Safety and Efficacy Study Evaluating CTX130 in Subjects With Relapsed or Refractory Renal Cell Carcinoma (COBALT-RCC)	Recruiting	Renal Cell Carcinoma	Biological: CTX130	All	18<	Phase 1	NCT04438083
CRISPR-Edited Allogeneic Anti-CD19 CAR-T Cell Therapy for Relapsed/Refractory B Cell Non-Hodgkin Lymphoma	Recruiting	Lymphoma	Genetic: CB-010|Drug: Cyclophosphamide|Drug: Fludarabine	All	18<	Phase 1	NCT04637763
Programmed Allogeneic CRISPR-edited T Cells Engineered to Express Anti-CD19 Chimeric Antigen Receptor (PACE CART19) in Patients With Relapsed Or Refractory CD19 + Leukemia and Lymphoma	Withdrawn	Acute Lymphoblastic Leukemia | Chronic Lymphocytic Leukemia| Non-Hodgkin Lymphoma	Biological: PACE CART19	All	18<	Phase 1	NCT05037669
TT52CAR19 Therapy for B-cell Acute Lymphoblastic Leukemia (B-ALL)	Recruiting	B Acute Lymphoblastic Leukemia	Drug: PBLTT52CAR19	All	6 Months to 18 Years	Phase 1	NCT04557436
A Feasibility and Safety Study of Universal Dual Specificity CD19 and CD20 or CD22 CAR-T Cell Immunotherapy for Relapsed or Refractory Leukemia and Lymphoma	Unknown status	B Cell Leukemia Cell Lymphoma	Biological: Universal Dual Specificity CD19 and CD20 or CD22 CAR-T Cells	All	12–70	Phase 1| Phase 2	NCT03398967
TACE Combined With PD-1 Knockout Engineered T Cell in Advanced Hepatocellular Carcinoma.	Unknown status	Advanced Hepatocellular Carcinoma	Procedure: Transcatheter arterial chemoembolization Biological: PD-1 knockout engineered T cells	All	18–70	Phase 1	NCT04417764
PD-1 Knockout Engineered T Cells for Metastatic Renal Cell Carcinoma.	Withdrawn	Metastatic Renal Cell Carcinoma	Biological: PD-1 Knockout T Cells Drug: Cyclophosphamide Drug: IL-2	All	18–75	Phase 1	NCT02867332

CAR T-cell-induced cytokine storms and neuroinflammation are not favorable outcomes and are thought to be the effects of GM-CSF. Thus, to address these limitations, lenzilumab has been used to suppress the GM-CSF, which not only reduces the side effects of CAR-T cell therapy but also increases its proliferation, and improves the control of leukemia. According to that, Sterner and colleagues manufactured GM-CSF knocked-out CAR-T cells with CRISPR/Cas9 system, which showcased significant in vivo anti-tumor activity while keeping the side effects at their lowest [155].

Targeting CD33, a myeloid marker in acute myeloid leukemia (AML) by CAR T-cells leads to induced toxicity due to the destruction of normal myeloid cells. An intricate approach, with CRISPR/Cas9-mediated knock-out of the CD33 gene from normal stem cells, followed by autologous transplantation in the rhesus monkey, paved the way for CD33-specific CAR T-cell-mediated elimination of leukemic cells, without affecting normal hematopoietic stem cells [156]. Additionally, Multiplex CRISPR-Cas9 system has been used for simultaneous knock-out of multiple genes in T-cells, including T cell receptor (TCR) chains, and PD-1. This could create cancer-specific T-cells (NY-ESO-1) having features such as minor mismatch pairing to the target, and enhanced durability, as well as offering risk-free graft-versus-host disease (GVHD) [157]. All these efforts have made it clear that CRISPR/Cas9 system is a great armamentarium in improving immunotherapy against cancer.

Given that CRISPR/Cas system can be introduced to human cells, it might be a potent tool to protect us against carcinogenic viruses [158]. Some viruses are capable of initiating cancer in humans, such as hepatitis B and C viruses (HBV and HCV), human papillomavirus (HPV), and Epstein-Barr virus (EBV), which can cause hepatocellular carcinoma (HCC) [159], cervical cancer [160] and Burkitt lymphoma [161], respectively. Given that CRISPR/Cas system can be introduced to human cells, it might be a potent tool to protect us against carcinogenic viruses [162].

The role of HBV in HCC is tightly pertained to the activity of covalent closed-loop DNA (cccDNA) of the HBV in hepatocytes [163]. A number of studies have shown that CRISPR/Cas9-induced mutations in HBV cccDNA can reduce its levels, impairing virus replication [164–166]. Moreover, with the help of CRISRP/Cas system it was demonstrated that a lncRNA PCNAP1 contributes in HBV replication and promotes hepatocarcinogenesis by modulating miR-154/PCNA/HBV cccDNA signaling [167]. In addition, HCV also contributes to HCC development. In a recent study, Francisella novicida (FnCas9), a type of Cas9 that can target both DNA and RNA viruses, was used to inhibit the ssRNA of HCV inside HCC cells [168]. The potential of FnCas9 seems to be promising for targeting different types of oncogenic viruses.

The persistence of HPV infection can lead to the development of cervical cancer, and it is responsible for the death of nearly 200,000 patients each year [168]. Among HPV functional proteins, E6 and E7 have been associated with the carcinogenic properties of HPV [169]. These two proteins are capable of sabotaging the major tumor suppressors in cells, including p53 and Rb. Based on that, researchers suppressed the E6 and E7 genes of HPV-16 and HPV-18 using the CRISPR/Cas9 system, which then restored the expression of p53 and Rb, leading to apoptosis in the cancer cells [170, 171].

EBV, another oncogenic virus mostly related to Burkitt lymphoma, has been targeted by CRISPR/Cas9. Wang et al. stated that targeting EBV genes, such as EBNA-1, LMP-1, or EBNA-3C by CRISPR/Cas9 in Burkitt's lymphoma cells derived from a patient with latent EBV infection resulted in a drastic reduction in cell proliferation and a decrease in viral load [172]. This approach has also been supported by other studies [172], and since EBV causes several types of malignancies, CRISPR/Cas9-based targeted therapies can be effective in preventing these diseases.

CONCLUSION

Though the CRISPR system was only recently developed, it has already made significant advances in cancer diagnosis and treatment. Researchers can inhibit or induce gene expression in vitro, in vivo, or ex vivo using CRISPR/Cas. Although CRISPR has some challenges ahead, it has attracted much attention in recent years due to its potential for precise cancer therapy and immunotherapy.

Against the wide spectrum utility of CRISPR in cancer diagnosis and therapy, there are still limitations and concerns that should be addressed in the future. One of these is the probability of CRISPR/Cas-induced DSBs, causing unwanted large deletions in the genome, and in the worst case scenario, it can lead to chromothripsis, which can disrupt tumor suppressors and cell regulatory systems [172, 173]. Another issue is the off-target effects of the CRISPR/Cas system, although in this review some solutions have been mentioned to avoid them. There has been concern about off-target effects, which might lead to CRISPR-induced cancer growth, but the good news is there is no evidence that this is the case. Noteworthy is that these effects can be minimized by using precise protocols and standards [173–175]. As widely used Cas9 comes from bacteria, pre-existing immunity to it can be considered a limitation of the CRISPR/Cas system [176–178]. Modification of Cas enzymes, and creating variants with different antigenic properties may help in this case [179].

Delivering the CRISPR/Cas system to target cells either in vivo or in vitro has its own challenges. To address them different delivery systems have been used, including physical methods, viral vectors, extracellular vesicles, nanocomplexes, etc. Among all, viral vectors and EVs are non-synthetic and natural systems that are destined to deliver their genetic cargoes. In recent years these two genuine systems have been combined together creating a novel vector called vexosomes. Overall, advances in delivery systems promise a bright future for efficient CRISPR/Cas-based cancer therapy.

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MS conceived, edited, and revised the manuscript; SEA, MS, MAZ, MDF, FS, and MO wrote the manuscript. SEA, MS, and MDF designed the figures. FS, MAZ, and SEA prepared tables. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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

ADDITIONAL INFORMATION

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