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Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy

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ABSTRACT

The clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems are efficient and versatile gene editing tools, which offer enormous potential to treat cancer by editing genome, transcriptome or epigenome of tumor cells and/or immune cells. A large body of works have been done with CRISPR/Cas systems for genetic modification, and 16 clinical trials were conducted to treat cancer by *ex vivo* or *in vivo* gene editing approaches. Now, promising preclinical works have begun using CRISPR/Cas systems *in vivo*. However, efficient and safe delivery of CRISPR/Cas systems *in vivo* is still a critical challenge for their clinical applications. This article summarizes delivery of CRISPR/Cas systems by physical methods, viral vectors and non-viral vectors for cancer gene therapy and immunotherapy. The prospects for the development of physical methods, viral vectors and non-viral vectors for delivery of CRISPR/Cas systems are reviewed, and promising advances in cancer treatment using CRISPR/Cas systems are discussed.

1. Introduction

Cancer is the main cause of disease-associated fatality despite the rapid progress in diagnostics and therapeutics [1]. A dramatic advance in our understanding of cancer biology has contributed to the licensures of many small molecular drugs, monoclonal antibodies (mAbs), gene and gene-modified cell-based products for cancer therapy [2]. These agents targeting key oncogenic signaling pathways or modulating the immune response have led to unprecedented responses or even cure in the selected cancer entities. However, many cancers are still incurable and the treatment options are limited. Therefore, new approaches are urgently needed for cancer therapy.

More recently, the clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems, stemmed from adaptive immune systems of most archaea and many bacteria, have been regarded as brilliant tools for biomedical engineering. [3–6] Owing to its simplicity, versatility and low cost, these CRISPR/Cas toolboxes offer a promising platform for targeting treatment of cancer. The CRISPR/Cas modules were first identified as adaptive antiviral immune systems and could afford protection against alien genetic materials by specific sequence-depend manner [7]. The CRISPR region contains short direct repeats (known as spacers), which derived from foreign nucleic acid fragments of invading viruses and plasmids. These spacers can offer sequence-specific memory for targeted defensing exogenous genetic materials invasion. During the immune response, the CRISPR could be transcribed into the precursor

Abbreviations: mAbs, monoclonal antibodies; CRISPR, the clustered, regularly interspaced, short palindromic repeats; Cas, CRISPR-associated protein; pre-crRNA, precursor transcript; crRNAs, CRISPR RNAs; tracrRNA, transactivating crRNA; PAM, protospacer adjacent motif; NHEJ, non-homologous end joining; HDR, homology directed repair; SaCas9, *Staphylococcus aureus* Cas9; KRAB, transcription suppressor domain; MTH1, MutT Homolog1; DNMT1, methyltransferase 1; PD-1, programmed cell death 1; TME, tumor microenvironment; VEGFA, vascular endothelial growth factor A; NGS, Next-generation sequencing; LAN, lance array nanoinjection; Ad, adenovirus; AAV, adeno-associated virus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; RHBDF1, rhomboid 5 homolog 1; GNs, gold nanoclusters; NLS, nuclear localization sequences; GO, graphene oxide; PEG, polyethylene glycol; PEI, polyethylenimine; HRECs, human primary retinal microvascular endothelial cells; HUVECs, primary human umbilical vein endothelial cells; hPRPE, human primary retinal pigment epithelial cells; VEGFR2, vascular endothelial growth factor receptor 2; iPS, human induced pluripotent stem cells; MSC, mesenchymal stem cells; MLVLPs, murine leukemia virus like particles; ORFs, open reading frames; HCC, hepatocellular carcinoma; *i.t.*, intratumoral; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; CLANs, cationic lipid-assisted polymeric nanoparticles; CML, chronic myeloid leukemia; *i.v.*, intravenous; PLNP, polyethylene glycol phospholipid-modified cationic lipid nanoparticle; Plk1, polo-like kinase 1; BPs, black phosphorus nanosheets; ARRDC1, arrestin domain containing protein 1; PARP-1, poly (ADP-ribose) polymerase-1; CPPs, cell-penetrating peptides; LMWP, low-molecular-weight protamine; PPABLG, poly(γ-4-((2-(piperidin-1-yl)ethyl)amino-methyl)benzyl-L-glutamate); TNBC, triple-negative breast cancer; NIR, near-infrared; GVHD, graft-*versus*-host-disease.

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transcripts and subsequently produced CRISPR RNA forms (called crRNA). Consequently, Cas nuclease, guided by crRNAs, can precisely recognize and cleave homologous nucleic acid of invaders [8]. Advances in the rapid development of CRISPR/Cas systems have revolutionized the field from basic research to clinical translation.

Especially as therapeutic tools against cancer, these simple and versatile systems hold great potential for manipulating the genome, transcriptome and epigenome in cancer and the immune related cells by a variety of strategies. The genome editing approach has been carried out in engineered autologous or allogeneic T cells for adoptive immunotherapy in clinical trials. More clinical translation of CRISPR/Cas systems to treat cancer mainly relies on the selection of targeting genes, the optimization of CRISPR/Cas tools with less or without off-target effects and the rational delivery strategies. In this review, we highlight the delivery strategies of CRISPR/Cas systems by physical methods, viral vectors and non-viral vectors for cancer gene therapy and immunotherapy, as well as discuss the latest advances in CRISPR/Cas tools for anti-cancer therapeutics.

2. The CRISPR/Cas systems

The new advances of prokaryote-derived manipulation systems are greatly promoting the understanding of mechanism in tumor genesis. Moreover, the CRISPR/Cas based strategies provide an innovative avenue toward clinical applications in cancer gene therapy and immunotherapy. As summarized in Table 1, the common-investigated CRISPR/Cas systems for cancer therapy are almost based on the nucleases including Cas9, Cas12a, Cas13a and their orthologues.

2.1. CRISPR/Cas9

CRISPR/Cas9 systems are commonly used as genetic engineering tools in most species (Fig. 1A). Cas9, an endonuclease guided by crRNA that consists of RuvC and HNH nuclease domains, enabled double strand DNA cleavage of the genome [11]. Meanwhile, a transactivating crRNA (tracrRNA) is also needed for the formation of RNP (Cas9: crRNA:tracrRNA ribonucleoprotein) complex. Therein, *Streptococcus pyogenes* Cas9 (SpCas9), the most common form of CRISPR/Cas9, can target to DNA through the protospacer adjacent motif (PAM) recognition [18]. After DNA cleavage by Cas9 module, the genome editing effect is produced through the non-homologous end joining (NHEJ) or homology directed repair (HDR) pathway [19]. The variants of Cas9 orthologues with distinct PAM recognition capacity were discovered to broaden the range of target locus. For example, *Staphylococcus aureus* Cas9 (SaCas9) can recognize 5′-NNGRRT (NNGAAT, NNGAGT, NNGGAT, NNGGGT) PAM sequence [20]. Additionally, the orthologues with smaller size or/and higher gene editing efficiencies have also been discovered such as CasX, the minimum size of Cas9 orthologues so far [21]. Thus far, most applications of CRISPR/Cas systems still focused on CRISPR/Cas9 technology to reach a new level of site specificity, higher editing efficiency and easier of use. The development of CRISPR/Cas9 systems has revolutionized the range from biological engineering to biomedical applications. Despite the advances, the potential of off-target effect and delivery difficulties remain challenges for CRISPR/Cas9-mediated clinical treatment.

2.2. CRISPR/Cas12a

The advances of CRISPR/Cas9 technology has encouraged sweeping exploration to discovering new systems for expanding applications. Another DNA-targeting CRISPR system for genome editing is Cas12a (known as Cpf1; Fig. 1B) [22]. Differing from the blunt ends generated by Cas9, Cas12a can product the staggered ends in its own cleavage pattern and PAM sequence, facilitating the DNA integration in a precise orientation. More importantly, Cas12a proteins are single crRNA-guided enzymes without tracrRNA assistance, and itself is responsible for pre-crRNA processing. To expand the range of targeting locus, Cas12a variants were also generated to targeting distinct PAM sequences such as 5′-TATV or 5′-VTTV, 5′-TTTT, 5′-TTCN and 5′-TATV [23,24]. In addition, CRISPR/Cas12a systems can be applied to the detection of viral DNA in patients depend on its non-specifically cleavage activity of ssDNA [25]. CRISPR/Cas12a can simplify multiplex genome editing, hence its application might be much wider in tumor therapy in the future.

2.3. CRISPR/Cas13a

Cas13a (known as C2c2), another novel RNA-guided system with the ability to target RNA, has recently been discovered (Fig. 1C) [26]. After recognizing and binding to the target RNA, Cas13a will activate the capacity of collateral cleavage to the untarget RNAs. However, the collateral cleavage activity of this system was not identified in eukaryotic species and its underlying mechanism were poorly understood [27]. So far, this kind of CRISPR-based RNA-targeting tools have already been harnessed for biomedical applications, such as detecting specific sequences of viral RNA and tumor circulating RNA in patients [28,29]. The RNA-targeting gene editing systems have great potential in the treatment of cancer by manipulating critical RNA molecules (both mRNAs and non-coding RNAs such as microRNAs, lncRNAs, *etc.*) [30]

2.4. Re-engineered CRISPR-Cas tools

Furthermore, nuclease-defective systems fused with effector domains or proteins, were repurposed into innovative CRISPR-based tools. For instance, the conversion of the most commonly used CRISPR/Cas9 tool into a trans-suppressor were achieved by fusing a transcription suppressor domain (KRAB) to dCas9 (Fig. 1D). The obtained CRISPR/dCas9 system has been utilized for transcriptional suppression of target genes within the native chromosomal context [31]. These CRISPR-derived nuclease-defective systems thus far may revolutionize the development of life sciences including anti-tumor therapy.

Table 1
Overview of diverse CRISPR/Cas systems.

Type/class	Effector	Nuclease domains	Target	Subtypes	Ref
I/1	Cas3	HD	DNA	A, B, C, D, E, F, G	[9,10]
II/2	Cas9	RuvC, HNH	DNA	A, B, C	[11–13]
III/1	Csm3, Cmr4	Autocatalytic?	RNA	A (Csm), B (Cmr), C,	[14,15]
	Csm6, Csx1	HEPN			
	Cas10	HD	DNA	D	
IV/1	Csf1	?	DNA?	A, B	[16,17]
V/2	Cas12	RuvC	DNA	A (Cpf1), B (C2c1), C (C2c3), D (Cas Y), E (Cas X)	[13]
VI/2	Cas13	HEPN × 2	RNA	A (Cas13a), B, C, D	[13]

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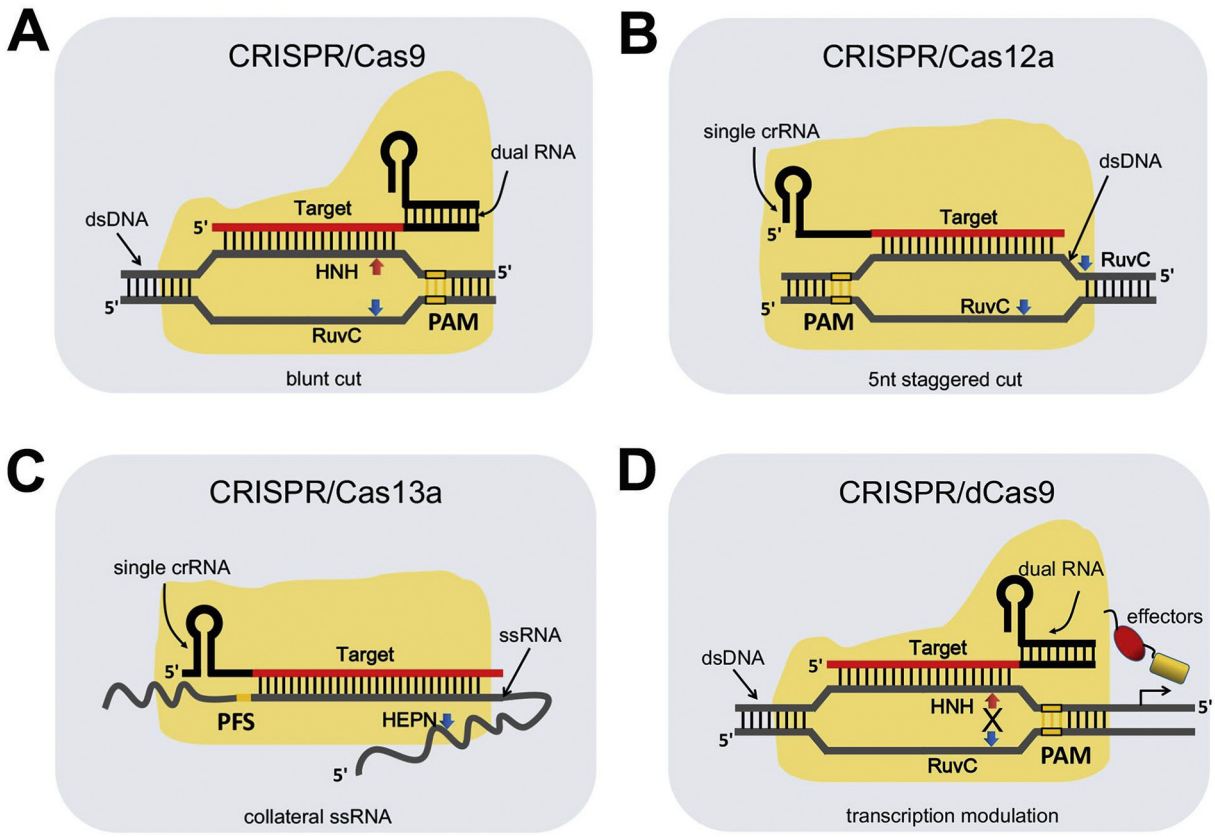

Fig. 1. Schematic diagram of frequently used CRISPR/Cas systems in cancer therapy. (A) CRISPR/Cas9 system enables a blunt DNA double-strand cleavage by complementary between guide RNA and the target locus on the presence of a PAM. (B) CRISPR/Cas12a system relies on RNA guidance (gRNA) for generating a staggered DNA double-strand break next to a PAM; (C) CRISPR/Cas13a system utilizes crRNA to cleavage the target ssRNA downstream of the PFS and also activates a nonspecific ribonuclease activity of Cas13a; (D) Inactive Cas9 (termed dCas9) with fused effectors can be repurposed as potential site-specific DNA targeting tools.

3. Anti-tumor targets for gene editing

It is critical for successful CRISPR-based antitumor therapy to select an appropriate gene target to maximize efficacy as well as minimize toxicity. The consideration of therapeutic targets for cancer therapy involves elaborate interactions among tumor, host and environment which will influence the treatment effect of CRISPR/Cas-based systems. [32–34] Here, the candidate therapeutic targets are described as follows (Table 2).

3.1. Oncogenes

In tumor cells, oncogenes are often mutated or expressed at high level. Most normal cells undergo a programmed form of apoptosis when critical functions are altered and malfunctioning. But the activated oncogenes can cause those cells designated for survival and proliferation instead. The current anti-cancer drug development has been proved formidable for directly targeting some oncogenes encode proteins (such as Kras and Myc) which were lack of specific active binding site. Benefiting from CRISPR/Cas systems, specific targeting of

Table 2
Representative therapeutic targets in current studies.

Classification	Representative targets	Comments	Ref
Oncogenes	BCR-ABL, EGFR, KRAS(G12C), HER2	Tumor maintenance often depends upon the continued activity of certain oncogenes. Efforts toward inhibition of oncogene are underway.	[35–38]
Cell death related genes	BCL2, APAF1, ATM, FAS	The loss of death control allows cancer cells to survive longer and give time to become more malignant. As more death-related targets are identified, the promising targets will be determined.	[39–42]
Epigenetic genes	DNMT1, EZH2, ARID1A, IDH1	Epigenetic enzymes have been identified as clear target for cancer therapeutics. The research for effective suppressing these targets has been a hot focus.	[43–46]
Immune-related genes	PD-1, PD-L1, PD-L2, CTLA4	Both host and tumor immune escape mechanisms can lead to a failure to amount an anti-tumor immune response and these are key targets in cancer immunotherapy.	[47,48]
Viral oncogenes	HBV(HBsAg), HPV(E5, E6, E7)	Infection of high-risk human virus is a prerequisite for cancer development. The virally oncogene could serve as targets for treatment of virus-drived cancers.	[49,50]
Tumor micro-environment associated genes	VEGF, bFGF, FGF, PDGF	Tumor microenvironment has been recognized as a significant contributor for cancer development. This concept indicated the potential of tumor microenvironment associated target therapy.	[51–54]
Others	Drug resistance gene, Long noncoding RNAs	Rapid and affordable tumor profiling has generated abundant genomic information which facilitate the discovery of candidate cancer targets.	[55–57]

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mutant Kras resulted in significantly inhibition of tumor growth *in vivo* [58]. Similarly, Evan and co-workers found that the inhibition of oncogenic Myc *via* CRISPR/Cas system triggered rapid regression of incipient and established lung tumors [59]. So far, dozens of oncogenes have been identified as the promising candidate targets for cancer treatment.

3.2. Cell death-related genes

Resistance to cell death, a hallmark of various tumor types, demonstrated potential therapeutic regimens aiming to directly kill tumor cells. For example, apoptosis-related genes expressed primarily in cancer cells are potential therapeutic targets that acts by inducing dramatic apoptosis for cancer treatment [60]. MTH1 (the MutT Homolog1), one of apoptosis-related genes, was successfully disrupted *via* CRISPR/Cas9 plasmid in ovarian tumor. The inhibition of the tumor growth and metastasis was hence achieved [61]. In addition, the other cell death types could be also good targets for CRISPR/Cas-based gene editing, involving autophagy and necroptosis.

3.3. Epigenetic genes

Epigenetics, a heritable phenotype changes without alteration in genotype, are necessary for normal development of mammals. This mechanism enables temporal and spatial control of gene activity during the formation of complex organisms. However, anomalous changes in epigenetic processes can be contributed to the malignant transformation of normal cells. [62–64] Recent advances in the study of cancer epigenetics have indicated that epigenetic-related gene might be promising target for anti-tumor therapy. It has been reported that disrupted DNA (cytosine-5) methyltransferase 1 (DNMT1) gene by CRISPR/Cas9 system can efficiently reduce DNMT1 expression. Ultimately, paclitaxel-resistant tumor growth is inhibited *in vivo* by epigenetic therapy [65].

3.4. Immune-related genes

Immune evasion is essential for cancer growth and progression. This may be also the cause of tumor resistance against conventional immunotherapy. [66–69] Thus, the immune-related genes are emerged as promising targets for therapeutic genome editing. The CRISPR/Cas9 based methods have been utilized to elevate the properties of the immune cells. The engineered CAR-T cells by disrupting the programmed cell death 1 (PD-1) enhanced its anti-tumor capacity in tumor-bearing model [70]. In addition, the disruption of CD47 displayed a potential immunotherapeutic strategy for small cell lung cancer [71]

3.5. Viral oncogenes

The oncogenic properties of high-risk human viruses account for the majority of virus-associated cancers [72,73]. The strategy targeting the key sequences of viral oncogenes have been proved effective therapeutics to the tumors induced by virus. Delivery CRISPR/Cas9 system targeting either E6 or E7 (the oncogenes of HPV) into HeLa cells successfully caused the cell death [74]

3.6. Tumor microenvironment-associated gene targets

The tumor microenvironment (TME) has been identified as a pivotal factor participating in tumor progression. With the increasing understanding of the TME, it has been shown that the components of TME could be utilized as potential targets against various types of cancer. For example, VEGF is the key angiogenic factor with multiple functions which constitutes the most important pathway in tumor angiogenesis. Bevacizumab (an antibody targeting VEGF) treatment of patients with advanced breast cancer significantly inhibited tumor growth [75]. Zhang and co-workers thus utilized CRISPR/Cas9 plasmid targeting vascular endothelial growth factor A (VEGFA) to treat osteosarcoma [76]. This new approach successfully impeded orthotopic osteosarcoma growth and reduced angiogenesis. While the promising result of anti-angiogenics, inhibition of these signaling has limited impact on overall survival of cancer patients. Prospectively, combinations of anti-angiogenics with other therapeutic modalities such as chemotherapy or checkpoint blockers may become an attractive strategy for effective cancer treatment.

3.7. Others

The appearance of next-generation sequencing (NGS) technology has facilitated rapid and affordable tumor profiling and produced a ton of genomic data. Such advances make enormous identification of candidate therapeutic gene targets in cancer research. [77–79] The versatile CRISPR/Cas systems have great potentials to take advantage of the new gene targets to achieve the highly efficient anticancer efficacy.

4. Delivery systems

The effective delivery of CRISPR/Cas gene editing system to cancer cells or immune cells is crucial to the exertion of CRISPR/Cas tools in cancer therapy. Generally, three delivery strategies (*in vitro*, *ex vivo* and *in vivo*) are throughout the investigation of CRISPR/Cas systems for cancer gene therapy and immunotherapy. Physical methods, viral vectors and non-viral vectors have been utilized to deliver CRISPR/Cas systems for gene editing in cancer cells or immune cells. The physical approaches, *via* transient membrane disruption, mainly include electroporation, membrane deformation, sonoporation, lance array nanoinjection (LAN), microinjection and hydrodynamic injection [80]. While the viral vectors, the earliest molecular tool achieving gene transfer into human cells, [81]. involve adenovirus (Ad), adeno-associated virus (AAV), retrovirus, lentivirus, Epstein-Barr virus (EBV), herpes simplex virus (HSV) and bacteriophages [82]. In addition, the non-viral vectors for the CRISPR/Cas delivery have been reviewed recently, [83]. specifically comprising lipid nanoparticles, polymer nanoparticles, polymeric hydrogel nanoparticles, gold-based hybrid nanomaterials, graphene oxide, metal-organic frameworks, black phosphorus nanosheets, *etc.*

4.1. In vitro delivery of CRISPR/Cas systems

It's efficient to obtain the optimal CRISPR/Cas tools with high gene editing activity for cancer therapy based on the *in vitro* experiment [84]. Moreover, it's quick and simple to screen the potential delivery system for the further evaluation of the *in vivo* anti-tumor efficacy. Generally, the *in vitro* delivery of CRISPR/Cas systems to cancer cells or immune cells have presently been achieved by various approaches including physical methods, viral vectors and non-viral vectors.

4.1.1. Physical methods

Electroporation is one of the most commonly used physical methods to electrotransfer CRISPR/Cas systems into tumor cells with high efficiency, which achieves enhanced permeability of the cell membrane through temporary disturbance of the lipid bilayers of the plasma membrane *via* an electrical field (Fig. 2A) [85]. It has been successfully delivered a wide range of CRISPR/Cas tools including plasmid [86–88] and protein [89]. into tumor cells *in vitro*. The CRISPR/Cas9 plasmid was efficiently transduced into the human osteosarcoma U2OS cells by electroporation and suppressed CDK11 expression (3–5-fold at 48 h *vs.* 7–15-fold at 72 h), thereby inhibiting proliferation, migration and invasion [90]. The CRISPR/Cas9 plasmid targeting ASXL1 and the repair template ssDNA were introduced into the human myeloidleukemia KBM5 cells using electroporation *in vitro* and restored ASXL1 gene expression in 0.46%–2% of KBM5 cells [91]. The human leukemia K562 cells were also reported to be effectively transfected with

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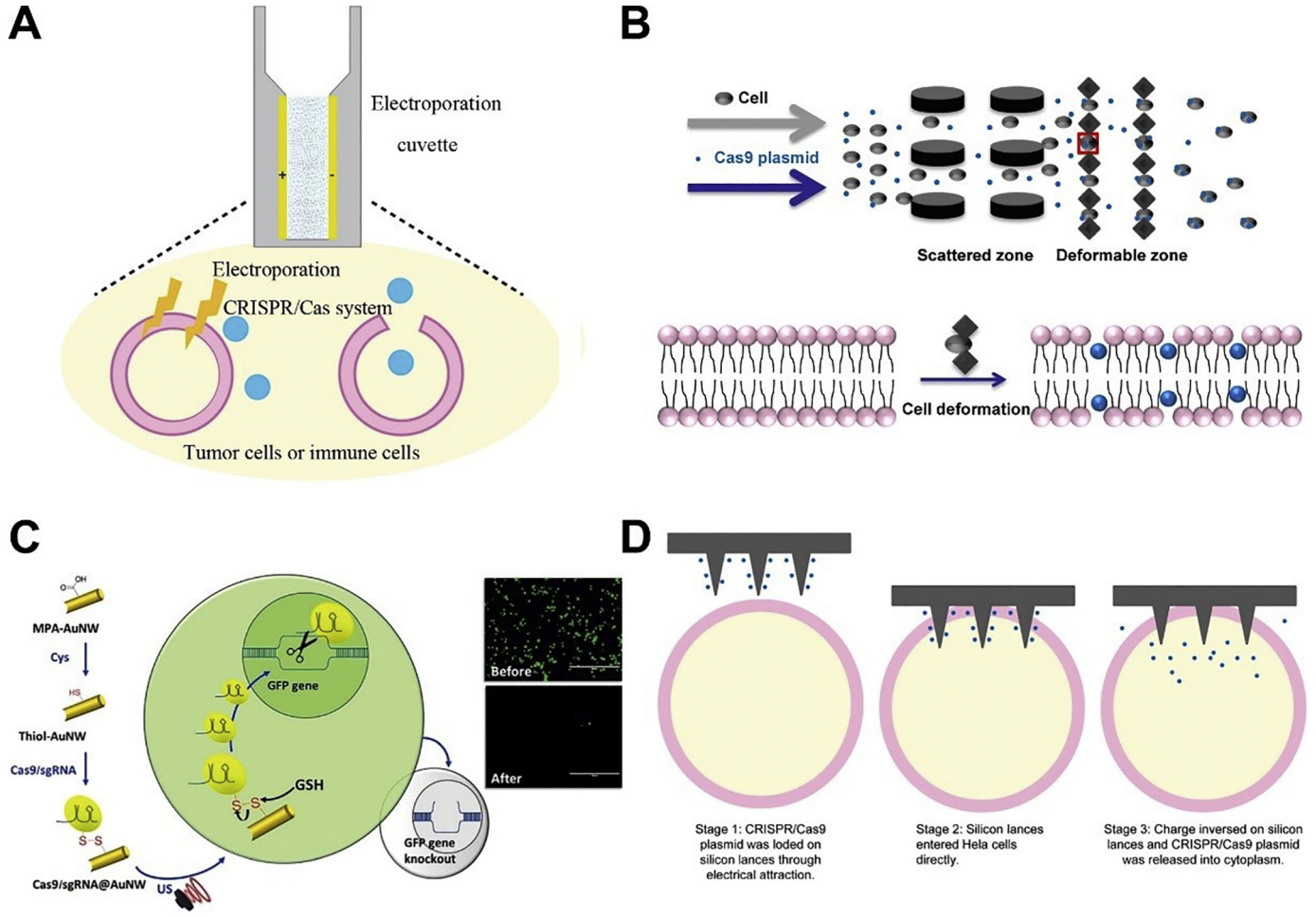

Fig. 2. Physical methods for delivery of CRISPR/Cas systems *in vitro*. (A) Electroporation delivers CRISPR/Cas systems into tumor cells or immune cells due to enhanced permeability of the cell membrane caused by transient disruption of the lipid bilayers of the plasma membrane *via* an electrical field. (B) Membrane deformation-based microfluidic device to deliver CRISPR/Cas systems into several different tumor cells *via* transient membrane disruptions or holes [85] (C) Ultrasound-propelled nanomotors mediate direct and rapid cell-membrane penetration to deliver CRISPR/Cas systems. Reproduced with permission Ref. [94]. Copyright 2018, Wiley Online Library. (D) LAN makes use of the microfabricated silicon etched array of lances to produce transient pores (1–2.5 μm in diameter) to deliver CRISPR/Cas systems [95].

the CRISPR/Cas9 plasmid, crRNA and tracrRNA by electroporation *in vitro* [92]. Electroporation is an efficient strategy for delivery CRISPR/Cas systems into tumor cells *in vitro*, however, cell death caused by electroporation might be a big issue for the *in vitro* investigation even after the improvement of the electroporation approaches, parameters and the composition of the electroporation medium [93]

The membrane distortion of cells can generate temporary membrane disturbance or holes which promotes the passive diffusion of CRISPR/Cas systems into the cytosol of tumor cells with high cell viability. Qin and co-workers designed a membrane deformation-based microfluidic device to deliver CRISPR/Cas9 system into several different tumor cells (Fig. 2B) [85]. The plasmid encoding both Cas9 and sgRNA-EGFP was successfully delivered into human breast cancer MDA-MB-231 cells expressing EGFP after 3 cycles through the same chip, achieving highly efficient genome editing (>90% EGFP knockout efficiency). The delivery of the plasmid encoding both Cas9 and sgRNA-AAVS1 *via* membrane deformation resulted in considerable cleavage at the AAVS1 locus with indels occurring at frequencies between 18% and 46% in human breast cancer MCF7 cells. The membrane deformation also successfully achieved efficient genome editing at the NUAK2 gene locus in human cervical cancer HeLa cells by introduction of the plasmid encoding both Cas9 and sgRNA-NUAK2 (with indels occurring at a frequency of about 30%). Human anaplastic large cell lymphoma SU-DHL-1 cells, one of difficult-to-transfect cell lines, exhibited >70% EGFP knockout efficiency which was much higher than the current transfection methods. Thus, the membrane deformation has the potential to deliver CRISPR/Cas systems into primary patient cells like immune cells with both higher delivery efficiency and cell viability.

Ultrasound-propelled nanomotors have shown direct and rapid cell-membrane penetration and been applied for improved intracellular delivery of miRNA, siRNA and apoptotic protease. Cas9/sgRNA complex, a temporary way of editing genes, supplies a substitute approach for the CRISPR/Cas tool. Wang and co-workers loaded the Cas9/sgRNA complex onto the nanomotor surface *via* a disulfide linkage. The obtained Cas9/sgRNA-loaded nanomotors quickly entered the cytoplasm of the murine melanoma B16F10 cells expressing GFP after ultrasound treatment for 5 min (Fig. 2C) [94]. Subsequently, 80% GFP knockout was achieved by the acoustic nanomotors loaded with only 0.6 nm of the Cas9/sgRNA complex after incubation for 2 h. This intracellular delivery method based on ultrasound-propelled nanomotors thus offers an alternative route to the transduction of Cas9/sgRNA complex into tumor cells *in vitro*.

Recently, a new physical method LAN was utilized to transfer the CRISPR/Cas9 plasmid into the GFP+/FRT HeLa cell line and achieved an average level of 93.77% EGFP gene disruption [95]. This transfection technology makes full use of the microfabricated silicon etched array of lances to produce transient pores (1–2.5 μm in diameter) in the targeting cell membranes and delivers any electrically charged molecular loads into the intracellular space *via* electrostatic attraction (Fig. 2D)

[96] LAN has much higher cell survival rates (78–91%) than conventional electroporation, [97]. providing a potential alternative strategy for the CRISPR/Cas system delivery to tumor cells *in vitro*.

4.1.2. Viral vectors

The viral vectors are one of the popular CRISPR/Cas delivery strategies [82]. Various viral delivery systems have been used for delivery of CRISPR/Cas systems into tumor cells *in vitro*. AAV has been largely applied for CRISPR genome editing due to its good safety exhibited in many clinical trials (Fig. 3). However, the carrying capacity of single AAV (4.7 kb) limits the package of large genes encoding the enzyme in the CRISPR/Cas systems (Table 3). Thus, Mizukami and co-workers combined Cas9 plasmid and AAV-sgE6 to treat HPV 18-positive human cervical cancer cell lines (HeLa, HCS-2, SKG—I) *in vitro*. E6 expression was successfully downregulated, thereby inducing improved expression of p53, enhanced apoptosis and growth inhibition in a dose-dependent manner [98]. It was reported that retrovirus coding SpCas9 (Fig. 3) can efficiently transduce human cervical carcinoma HeLa cells [99]. Lentiviral vectors (Fig. 3), a subtype of retrovirus, can package Cas9 and either E6- or E7-specific sgRNA and transduce ~90% of HeLa cells *in vitro,* which resulted in almost complete death of the transduced cells by targeted disruption of HPV E6 or E7 in HeLa cells [74]. Lentiviral libraries targeting 19,052 genes, encoding six sgRNAs per virus, can also transduce HeLa cells stably expressing Cas9 [99]. Moreover, lentiviral CRISPR/Cas9 system successfully mediated the efficient deletion of MCL-1 (80% mutation rate) and thus induced the apoptosis *in vitro* in human Burkitt lymphoma cells which required this anti-apoptotic BCL-2 protein for continuous survival and growth [100]. The lentiviral vector production is generally based on transfection of four plasmids (three packaging constructs and a transfer vector) into HEK293T cells [101]. Thus, *in vitro* optimization of CRISPR/Cas systems using the lentiviral vectors is inefficient and time-consuming.

4.1.3. Non-viral vectors

The simple easy-to-get commercial transfection reagents seem to be viable non-viral vectors for the delivery of CRISPR/Cas systems in the *in vitro* experiments (Table 4). X-tremeGENE HP DNA transfection reagent (Roche) can efficiently transduce Cas9 plasmid and sgRNA-E7 plasmid into the human cervical cancer Siha cells to enhance apoptosis and inhibit cellular viability *via* disruption of the E7 gene [102]. For the human cervical cancer Caski cells, jet-PEI polymer-based DNA transfection reagent (Polyplus) reached the similar efficacy in the delivery of Cas9 plasmid and sgRNA-E7 plasmid [102]. The common cationic lipid nucleic acid transfection reagent RNAiMAX has been shown to be capable of delivering Cas9/sgRNA complex into cultured U2OS cells in the media containing 10% serum and resulted in up to 80% genome editing efficiency in contrast to DNA transfection [103]

It's still challenging to realize high efficiency in the delivery of large genome editing tools such as CRISPR/Cas systems using the traditional cationic transfection reagents. Thus, it's urgent to explore innovative vectors. Iyer and co-workers found that Lipofectamine 2000 cannot deliver large plasmid (~10.3 kb) as well as small plasmid (~5 kb) [104]. So they designed flexible dendrimer to deliver CRISPR/dCas9-VP64 (dCas9-VP64: 9.8 kb; sgRNAs: 3.2 kb) with high transfection efficiency

Table 3
The CRISPR/Cas-loaded viral vectors potential for tumor therapy.

Virus	Insert Capacity	Features
Adenovirus	7.5 kb	Broad host range Transient expression Strong immunogenicity
Adeo-associated viral	4.7 kb	Relatively broad host range Slow expression onset Chromosomal integration Immune response
Retrovirus	8 kb	Transduction of only dividing cells Long-term expression Random integration
Lentivirus	8 kb	Broad host range Low cytotoxicity Long-term expression
Epstein-Barr virus	5 kb	Improved exogenous gene stability Stable gene expression
Sendai virus	5 kb	Broad host range Low pathogenicity Powerful capacity RNA delivery
Baculovirus	>38 kb	Low cytotoxicity Large cloning capacity Lack of pre-existing immunity

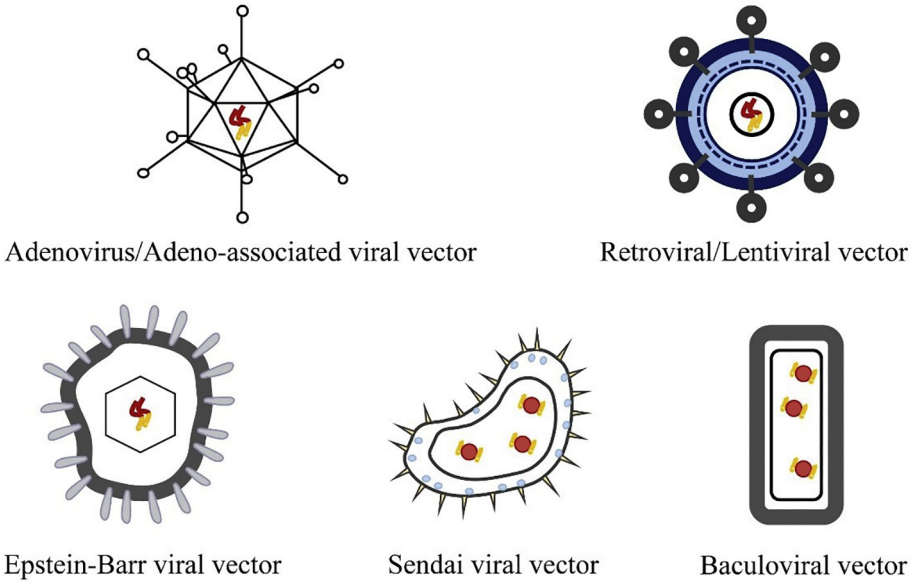

Fig. 3. The CRISPR/Cas-loaded viral vectors potential for tumor therapy involved Ad, AAV, retrovirus, lentivirus, Epstein-Barr virus (EBV), Sendai virus and Baculovirus. The former four viruses have been successfully utilized to deliver CRISPR/Cas systems to tumor cells *in vitro*, while the latter four viruses can transfer the primary cells and thus have great potential as the vectors of CRISPR/Cas systems for cancer immunotherapy. Furthermore, both Ad and lentivirus have displayed powerful delivery of CRISPR/Cas systems to tumor cells *in vivo*.

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Table 4
The CRISPR/Cas-loaded non-viral vectors for *in vitro* gene editing.

Types	Vectors	Cargos	Features	Ref.
Commercial transfection reagents	X-tremeGENE HP DNA Transfection Reagent	Cas9 plasmid and sgRNA	Commercial	[102]
	PolyJet™ *In Vitro* DNA Transfection Reagent	Cas9 plasmid and sgRNA	Commercial	[102]
	Lipofectamine RNAiMAX	Cas9/sgRNA complex	Commercial Serum resistance 80% gene editing efficiency	[103]
Innovative vectors	Flexible dendrimer	CRISPR/Cas9 plasmid	High transfection efficiency and packaging capacity for large plasmid	[104]
	Cationic polymer PEI-β-cyclodextrin	CRISPR/Cas9 plasmid	~7% gene editing efficiency	[105,106]
	Protamine and co-precipitated it with $CaCO_3$	CRISPR/Cas9 plasmid	~28.4% gene editing efficiency	[107]
	Gold nanoclusters	Cas9/sgRNA complex	~34% gene editing efficiency	[108]
	Cationic arginine-decorated gold nanoparticles	Cas9/sgRNA complex	Highly efficient cytosolic and nuclear delivery (up to 90%) ~30% gene editing efficiency	[109]
	Graphene oxide (GO)-polyethylene glycol (PEG)-polyethylenimine (PEI)	Cas9/sgRNA complex	~39% gene editing efficiency	[110]

and packaging capacity, which could significantly upregulate the tumor suppressor MASPIN in the human breast cancer MCF-7 cells at the mRNA and protein levels. Huang and *co*-workers explored cationic polymer PEI-β-cyclodextrin to deliver CRISPR/Cas9 plasmid for *in vitro* genome editing in HeLa cells, [105]. which resulted in indel rates of 7.0% at the genome locus of rhomboid 5 homolog 1 (RHBDF1) essential to epithelial cancer cell growth [106]. Cheng and co-workers combined CRISPR/Cas9 plasmid with protamine and co-precipitated it with $CaCO_3$ [107]. The co-precipitated nanoparticle was further decorated by AS1411 functionalized carboxymethyl chitosan and TAT peptide functionalized carboxymethyl chitosan to enhance the cellular uptake and nuclear transportation. This CRISPR/Cas9 plasmid loaded nanocarriers can efficiently cause the knockout of CTNNB1 gene coding β-catenin (28.4% at mRNA level) and favorable anti-cancer features involving inhibited growth, suppressed migration and invasion, and reduced cancer stemness. Gao and co-workers used gold nanoclusters (GNs) to self-assemble Cas9 protein fused with nuclear localization sequences (NLS) [108]. The obtained SpCas9 — AuNCs can induce 34% of mutation frequency in HeLa cells when combined with sgRNA specific to HPV18 E6 gene which was transfected with Lipofectamine RNAiMAX. Rotello and co-workers constructed cationic arginine-decorated gold nanoparticles capable of loading Cas9/sgRNA complex containing engineered Cas9 protein modified with a negatively charged glutamate peptide tag [109]. The formed nanoassemblies achieved highly efficient cytosolic and nuclear delivery (up to 90%) of Cas9/sgRNA complex to HeLa cells *in vitro*, leading to effective gene editing efficiency (~30%) in the target genes (AAVS1 or PTEN). Xing and co-workers developed a novel nanocarrier for cellular delivery of CRISPR/Cas9 complex based on graphene oxide (GO)-polyethylene glycol (PEG)-polyethylenimine (PEI). The CRISPR/Cas9 complex entrapped in the nanocarrier can successfully enter human gastric cancer AGS cells expressing GFP *via* endocytosis and escape from endosomes, hence reaching 39% of GFP gene editing efficiency [110]. According to the highly efficient efficacy on *in vitro* gene editing, the *in vivo* delivery efficiency is worthy of being investigated for most of the above-mentioned novel carriers.

4.2. Ex vivo delivery of CRISPR/Cas systems

In the application of *ex vivo* editing with CRISPR/Cas systems, cells derived from the body, an organism or primary cells are treated by CRISPR/Cas tools *in vitro*, and they are subsequently transplanted back into the living body. The *ex vivo* CRISPR/Cas gene editing technology allows us to adequately analyze and characterize the engineered cells, which is safer and easier to be employed to the cancer gene therapy and immunotherapy due to the minimum alteration of natural conditions [111]

Electroporation is the most commonly used physical methods in the *ex vivo* delivery of CRISPR/Cas tools. This method, independent on the cell type, can transfer CRISPR/Cas systems into most of the primary cells with high efficiency including T cells, B cells and NK cells (Table 5).

The powerful gene editing for primary human T cells holds great promise for cancer immunotherapy. The PD-1 gene can be knocked-out by electroporation of CRISPR/Cas9 plasmid on human primary T cells from cancer patients, with up-regulated IFN-γ production and

Table 5
Ex vivo delivery of CRISPR/Cas systems for cancer therapy.

Types	Approaches or vectors	Target cells	Cargos	Ref.
Physical approaches	Electroporation	Human primary T cell	CRISPR/Cas9 plasmid	[112]
	Electroporation	Human primary T cell	Cas9/sgRNA complex	[113]
	Electroporation	Human primary T cell	Cas12a mRNA + crRNA and HDR template	[115]
	Electroporation	Human primary B cell	Cas9/sgRNA complex	[116]
	Electroporation	Human primary NK cell	Cas9/sgRNA complex	[117]
Viral vectors	AAV	Human primary retinal microvascular endothelial cells (HRECs)	CRISPR/Cas9 system	[119]
	Lentiviral	Human primary nasal airway epithelial cells	CRISPR/Cas9 system	[120]
	EBV	Human primary B cell	CRISPR/Cas9 system	[122]
	Sendai virus	Human primary monocytes	CRISPR/Cas9 system	[123]
	Baculovirus	Human induced pluripotent stem cells (iPS)	CRISPR/Cas9 system	[124]
Non-viral vectors	LPEI (25 kDa)	Mesenchymal stem cells (MSC)	Plasmid DNA	[125]
	Murine leukemia virus like particles (MLVLPs)	iPS and human hematopoietic stem cells	Cas9/sgRNA complex	[126]
	NaCl-mediated hyperosmolality in combination with a transduction compound (a propanebetaine)	Various primary cells, including dendritic cells, glia cells and neurons	Cas9/sgRNA complex	[126]

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improved cytotoxicity toward cancer cells [112] A successful genome engineering by electroporated delivery of Cas9/sgRNA complex was reported in PD-1, a regulator of T-cell consumption that is a confirmed target for tumor immunotherapy [113] Cas9-mediated genetic knock-in of specific nucleotides to PD-1 with up to ~20% efficiency was achieved in primary human T cells. The electroporation of Cas9/sgRNA complex can also be employed to perform multiplex gene editing in CAR-T cells, involving TRAC (TCRα constant chain), B2M (β2-microglobulin) and PD-1 [114] 85% of double (TRAC/B2M)- and 64.7% of triple (TRAC/B2M/PD-1)-negative CAR-T cells were conducted successfully. These gene-edited CAR-T cells could efficiently induce cell death of co-cultured tumor cells in a CAR-dependent manner. In the intraperitoneal xenograft mice with FFLuc-transduced Raji lymphoma cells, the TRAC/B2M double-knockout CAR-T cells exhibited CD19-specific anti-tumor feature comparable to standard CD19 CAR-T cells after intraperitoneal administration. In addition, Chen and co-workers achieved high-efficiently targeting of both HDR-mediated dual-CAR knock-in and immune-checkpoint knockout in primary human T cells using a mixture of messenger RNA electroporation for Cas12a and AAV6 for delivery of crRNA and HDR template [115]

Except for primary T cells, CRISPR/Cas9-dediated locus-specific genome editing in primary human B cells was performed using electroporation with Cas9/sgRNA complex [116]. The engineered B cells obtained the ability to secrete therapeutic proteins potential for the application in cancer therapy. Moreover, human primary and expanded NK cells also could be edited with CRISPR/Cas systems for cancer gene therapy and immunotherapy. The TGFBR2 and HPRT1 genes in NK cells were efficiently knocked-out using Cas9/sgRNA complex *via* electroporation [117]

Theoretically, all the approaches mentioned in the above for *in vitro* experiments have potential for *ex vivo* delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy, especially those methods suitable for the transfection of human primary cells. Among these, the viral vectors have great potentials. AAV has been used to effectively infecting a wide variety of primary cells, including human primary retinal microvascular endothelial cells (HRECs), primary human umbilical vein endothelial cells (HUVECs) and human primary retinal pigment epithelial cells (hPRPE) [118]. The vascular endothelial growth factor receptor 2 (VEGFR2) was depleted by 80% with rAAV5-CRISPR/Cas9 in HRECs [119]. The lentiviral CRISPR/Cas9 system successfully knocked out MUC18 (also known as CD146 or melanoma cell adhesion molecule) in human primary nasal airway epithelial cells with high efficiency (>90%) [120]. The lentivirus also can efficiently transform Cas9/sgRNA complexes to lymphoblastoid cell lines including $CD4^+$ T cells [121]. and primary human B cells [122] EBV also have been used to efficiently transforms Cas9/sgRNA complexes to lymphoblastoid cell lines including primary human B cells (Fig. 3) [122]. Additionally, Sendai virus [123]. and Baculovirus [124] (Fig. 3), directly incorporating Cas9 protein and sgRNA, have been reported to transfer primary human monocytes and human induced pluripotent stem cells (iPS), respectively. Normally, traditional cationic non-viral vectors are difficult to transfect the primary cells. Though coordinated endosomal release and down-regulated α-tubulin deactylase, LPEI (25 kDa) successfully delivered CRISPR/Cas9 plasmid to mesenchymal stem cells (MSC) by combination with DOPE/CHEMS and HDAC6i [125] A kind of novel murine leukemia virus like particles (MLVLPs) was developed to allow the efficient transfer of Cas9/sgRNA complex to primary cells *ex vivo* including iPS cells and human hematopoietic stem cells. Furthermore, Cas9/sgRNA complex can also be highly efficiently transduced into various primary cells, including dendritic cells, glia cells as well as neurons *via* an NaCl-mediated hyperosmolality in combination with a transduction compound (a propanebetaine), independent of any vectors or setups [126]. Taken together, all these promising strategies for the transfection of the human primary cells are worthy of being investigated for the *ex vivo* delivery of CRISPR/Cas systems in cancer gene therapy and immunotherapy.

4.3. In vivo delivery of CRISPR/Cas systems

Despite lots of successful confirmation of applying CRISPR/Cas systems in cell line-based experiments, *in vivo* gene editing based on CRISPR/Cas systems for cancer treatment remains obstacle due to the difficult transport of CRISPR/Cas systems across multiple physiological barriers to cancer cells or immune cells *in vivo* [83]. It is crucial to explore riskless and efficient *in vivo* delivery systems for the translational application of CRISPR/Cas systems in tumor therapy. Both viral and non-viral vectors have been developed to deliver CRISPR/Cas systems for *in vivo* gene editing of tumor cells or immune cells.

4.3.1. Physical methods

Physical methods for delivery of CRISPR/Cas systems usually utilize vector-free DNA/mRNA/protein. The naked cargo without protection of carriers is prone to enzymatic degradation and fast clearance when delivered directly into tissues or systemic circulation [80]. Although physical methods have been widely utilized *in vitro* and *ex vivo*, only a few strategies have been used for the *in vivo* delivery of CRISPR/Cas systems, involving hydrodynamic injection [127]. and electroporation [128]. Hydrodynamic injection is one of reliable physical methods for CRISPR/Cas-based gene editing *in vivo*, which delivers plasmids into cells by a high pressure in a short time to produce temporary pores in the cell membrane [80]. With hydrodynamic injection into the tail vein of M-TgHBV mouse model, the HBV specific CRISPR/Cas9 system successfully deleted two open reading frames (ORFs) of HBV transcription template, causing significantly decrease in liver HBcAg and serum HBsAg [129]. This approach has great potential to treat HBV-induced hepatocellular carcinoma (HCC) in human. Although hydrodynamic injection has been applied in clinic, [130]. it has high risk of severe damage to the liver due to a large injection volume [80]. Electroporation has been used to deliver CRSPR/Cas systems to various organs and tissues like utero, [131]. retina [132], skeletal muscles, [133]. skin, [134]. spinal cord [135]. and neural tissues [136]. in animal experiments. Nevertheless, cell death is still problematic. Up to now, there is still no report about using electroporation for *in vivo* delivery of CRISPR/Cas systems in cancer therapy. It is clear that physical methods still need further improvement for the translational study in clinic.

4.3.2. Viral vectors

Viral vectors are regarded as effective tools for CRISPR/Cas systems to treat tumor. Liu and co-workers used a lentivirus-mediated CRISPR/Cas9 system with sgRNA-721 (LV-H721) to knock out HIF-1α for the therapy of human HCC [137]. They directly injected LV-H721 into the tumor tissues in the subcutaneous SMMC-7721 xenograft HCC model. The lentivirus-mediated CRISPR/Cas9 dramatically reduced the expression of HIF-1α in the tumor tissues 3 days post treatment. Kim and co-workers harnessed lentivirus and AAV expressing Cas9 and sgRNA to target mutant KRAS alleles in SW403 cancer cells (~50%) [138]. With intratumoral (*i.t.*) injection into the subcutaneous xenograft colon carcinoma model, the tumor growth was effectively inhibited.

Compared with lentiviral and retroviral vectors, Ad vectors (Fig. 3) can transfer CRISPR/Cas9 systems into an expansive range of dividing and nondividing cells both *in vitro* and *in vivo* [82]. Conventional Ad vectors can package ~8.1–8.2 kb of foreign DNA, much longer than AAV vectors. Thus, Ad is ideal for delivery of the entire CRISPR/Cas9 systems in one vector. Kim and co-workers used a replication-incompetent Ad vector to deliver CRISPR/Cas9 system to treat non-small cell lung cancer (NSCLC) with a mutation in the epidermal growth factor receptor (EGFR) gene [139] A mixture of Cas9-expressing Ad (Ad/Cas9) and EGFR mutation-specific sgEGFR-expressing Ad (Ad/sgEGFR) was intratumorally injected into the subcutaneous xenograft NSCLC model with H1975 cells containing the mutant alleles. The co-treatment caused indels at frequencies of 50 ± 5.3% at day 7 after the first injection, while no indels were detected in A549-derived tumors without the mutant alleles. Fast tumor regression and extended survival rate

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were observed in H1975-derived tumor models after treatment for three times (every other day). These results indicated that Ad-mediated CRISPR/Cas9 delivery can potently treat NSCLC *via* efficient gene editing of the EGFR mutant allele in H1975 cells *in vivo*. However, Ad vectors cannot achieve efficient gene transfer into cells lacking the coxsackievirus and Ad receptors (CAR) [140]. Koizumi N et al. designed a fiber-modified Ad vector (AdK7) comprising a stretch of lysine residue (K7) peptides which target heparan sulfates on the cellular surface [141]. Kazuo Takayama and Hiroyuki Mizuguchi found that AdK7 successfully transduced the high-grade cancer cell line PC-3 with lower expression of CAR [142]. They subsequently constructed a light-activated CRISPR/Cas9 system-loaded AdK7 (Opt/Cas-Ad) to upregulate the expression of Dkk-3 gene, which could inhibit the growth and metastasis of prostate cancer [143,144] Opt/Cas-Ad showed illumination-dependent spatiotemporally controllable CRISPR/Cas9-based gene editing and efficient antitumor activity after *i.t.* injection into the subcutaneous PC-3-derived tumor models.

As seen in Fig. 3, several viral vectors have been utilized to deliver CRISPR/Cas systems. Although lentivirus, AAV and Ad have shown great potential for the *in vivo* cancer therapy, other viral vectors are still worthy of further exploration for the *in vivo* delivery of CRISPR/Cas systems to treat cancer.

4.3.3. Non-viral vectors

In comparison with viral vectors, non-viral carriers are more diverse for the powerful *in vivo* delivery of CRISPR/Cas tools (Table 6). Generally, most of the non-viral carriers are in the form of nano-sized preparations which have chiefly been enlightened by traditional gene-delivery methods [145]

4.3.3.1. Common nano-sized preparations. Self-assembled micelles with a lower charge density have been designed to encapsulate CRISPR/Cas9 plasmid targeting HPV18-E7 oncogene in HeLa cells [146]. This micelle comprised quaternary ammonium-terminated poly(propylene oxide) and amphiphilic Pluronic F127, which successfully delivered the CRISPR/Cas9 system into HeLa cells in the subcutaneous HeLa xenograft model after *i.t.* injection. The Cas9-induced mutation reduced E7 expression and thus efficiently delayed the tumor growth. Wang and *co*-workers used (PEG-PLGA)-based cationic lipid-assisted polymeric nanoparticles (CLANs) to encapsulate CRISPR/Cas9 plasmid for overhanging fusion region of BCR-ABL gene in chronic myeloid leukemia (CML) mouse models by intravenous (*i.v.*) injection of K562 cells into irradiated NOD-SCID mice (Fig. 4A) [164]. After *i.v.* administration, 19.3% and 16.1% of BCR-ABL gene was knocked out in K562 cells from blood and bone marrow, respectively. The survival of CML model mice was correspondently prolonged.

A polyethylene glycol phospholipid-modified cationic lipid nanoparticle (PLNP) can also be used to encapsulate Cas9/sgRNA plasmid for the genome editing of polo-like kinase 1 (Plk1), [148]. a highly conserved serine-threonine kinase that promotes cell division and is overexpressed in various tumors [165]. An *i.t.* injection into subcutaneous A375-derived melanoma model mice resulted in significant down-regulation of Plk1 protein and inhibition of the tumor growth (>67%) *in vivo*.

The yarn-like DNA nanoclew, a novel nano-preparation, can capsulate Cas9/sgRNA complex *via* the optimal DNA sequence partially complementary to the sgRNA, [149]. following coating of the cationic polymer PEI to facilitate endosomal escape [166]. The NLS peptides fused to Cas9 can further help the Cas9/sgRNA complex be transported into the nucleus. After *i.t.* injection of Cas9/sgRNA delivery system into U2OS.EGFP xenograft tumors in nude mice, ~25% the U2OS.EGFP cells near the site of injection lost EGFP expression. Yu and co-workers subsequently loaded the NLS-engineered Cas9/sgRNA complex into a biodegradable two-dimensional black phosphorus nanosheets (BPs) [150]. This system can enter tumor cells *via* membrane penetration and endocytosis pathways, followed by a BPs biodegradation-associated endosomal escape. An *i.t.* injection significantly reduced EGFP signals in A549/EGFP tumor-bearing nude mice models.

A recent study showed that a fluorinated acid-labile branched hydroxyl-rich polycation can deliver CRISPR/Cas9 plasmid targeting survivin gene to inhibit tumor growth and enhance sensitivity to the anticancer drug temozolomide *via i.v.* injection in A549/EGFP tumor-bearing nude mice models (Fig. 4B) [151]. This carrier was reported to possess excellent acid degradability, biocompatibility and transfection performance, which showed great potential for the systemic administration of the plasmid form of CRISPR/Cas system in the cancer treatment.

Exosomes are natural nano-vesicles (30 nm to 120 nm) secreted by various cells, including immune cells, epithelial cells and tumor cells. They are capable of carrying and transferring bioactive molecules, thereby having been proposed as a new vehicle for CRISPR/Cas delivery [167,168]. Lu and co-workers prepared arrestin domain containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs) using HEK293 cells, which can package Cas9/sgRNA complex through the interaction of ARRDC1 with the WW domain in the modified Cas9 [169]. This novel carrier has great potential for the *in vivo* delivery of CRISPR/Cas system. Tumor-derived exosomes might accumulate in the tumor tissues due to their cell tropism, thus SKOV3 cell-derived exosomes (SKOV3-Exo) was used as natural carriers of CRISPR/Cas9 plasmid to treat ovarian cancer (Fig. 4C) [152]. They were harvested from the culture media of SKOV3 cells and loaded them with CRISPR/Cas9 plasmid *via* electroporation. SKOV3-Exo selectively accumulated in ovarian cancer tumors of the subcutaneous SKOV3 xenograft mice after *i.v.* injection. Both *i.v.* and *i.t.* injection of SKOV3-Exo successfully disrupted the targeting gene poly (ADP-ribose) polymerase-1 (PARP-1), a potential therapeutic target BRCA1/2 deficient breast and ovarian cancers [170,171]. Meaningfully, the inhibition of PARP-1 by CRISPR/Cas9-mediated genome editing enhanced the chemosensitivity to cisplatin.

4.3.3.2. Cell-penetrating peptides (CPPs)-mediated delivery systems. CPPs have ability to promote the delivery of CRISPR/Cas system into tumor cells through membrane destabilization to increase uptake, promote endosomal escape, and improve overall transfection efficiency [172]. Kim and co-workers used CPP to conjugate Cas9 protein and complex sgRNA targeting the CCR5 gene, respectively [173]. The obtained Cas9-m9R and sgRNA:9R led to mutation frequencies of 5.5% at the CCR5 locus in HeLa cells *in vitro*. Jang and co-workers generated a novel Cas9 fusion protein (Cas9-LMWP) expressing both NLS and a low-molecular-weight protamine (LMWP), [174]. a nature-sourced CPP powerful tool for *in vivo* macromolecular delivery [175] LMWP and NLS mediate intracellular delivery and nuclear localization, respectively. Cas9-LMWP self-assembled with the crRNA:tracrRNA hybrids targeting KRAS by electrostatical interaction. The formed ternary Cas9 RNP led to a decrease in AKT and ERK phosphorylation related to the depletion of KRAS in the subcutaneous A549 tumor xenografts by intratumoral administration. Jiang and co-workers subsequently designed core-shell nanoparticles based on TAT for the *in vivo* delivery of Cas9/sgRNA complex (Fig. 5) [154]. They firstly used TAT to modify the cationic GNs to allow enhancement in nucleus-targeting [176]. The obtained TAT-GNs with positive charge could interact electrostatically with the negatively charged Cas9/sgRNA complex to form a ternary complex (TAT-GNs/Cas9 protein/sgRNA plasmid, GCP). The negatively charged GCP, as a core, was further covered by an anionic lipid shell (DOTAP/DOPE/cholesterol), followed by post modification with DSPE-PEG on the surface of the lipid shell to form lipid-coated GCP (LGCP). After *i.t.* administration, LGCP can efficiently downregulate the expression of Plk1 protein in the subcutaneous melanoma models of A375 cells and achieved higher tumor growth inhibition (75%) than previous Cas9/sgRNA plasmid delivered by PLNP [148]. Leong and *co*-workers further designed PEGylated helical polypeptide nanoparticles (P-HNPs) to deliver Cas9/sgRNA complex to tumor cells *in vivo* [155] P-HNPs included helical polypeptide poly(γ-4-((2-(piperidin-1-yl)ethyl)amino-methyl)

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Table 6
The current *in vivo* delivery of CRISPR/Cas systems *via* non-viral vectors.

	Non-viral vectors	CRISPR/Cas systems	Targets	Cancer cells	Administration route	Mice models	*In vivo* efficacy	Ref.
Common nano-sized preparations	Micelles comprising quaternary ammonium-terminated poly(propylene oxide) and amphiphilic Pluronic F127	CRISPR/Cas9 plasmid	HPV18-E7	HeLa	*i.t.*	Subcutaneous xenograft model of cervical cancer	Reduced E7 expression; Delayed tumor growth.	[146]
	Polymeric nanoparticles containing PEG-PLGA and cationic lipid	CRISPR/Cas9 plasmid	BCR-ABL	K562	*i.v.*	Intravenous xenograft model of chronic myeloid leukemia	Knocked out 19.3% and 16.1% of BCR-ABL gene in K562 cells from blood and bone marrow; Prolonged survival.	[147]
	Polyethylene glycol phospholipid-modified cationic lipid nanoparticle (PLNP)	CRISPR/Cas9 plasmid	Plk1	A375	*i.t.*	Subcutaneous xenograft melanoma model	Downregulated Plk1 protein expression; Suppressed tumor growth.	[148]
	DNA nanoclew coating PEI	Cas9/sgRNA complex	EGFP	U2OS.EGFP	*i.t.*	Subcutaneous xenograft osteosarcoma model	Reduced ~25% EGFP expression	[149]
	Black phosphorus nanosheets	Cas9/sgRNA complex	EGFP	A549/EGFP	*i.t.*	Subcutaneous xenograft model of lung cancer	Reduced EGFP signals	[150]
	Fluorinated acid-labile branched hydroxyl-rich polycation	CRISPR/Cas9 plasmid	Survivin	A549	*i.v.*	Subcutaneous xenograft model of lung cancer	Inhibited tumor growth; Enhanced sensitivity to the anticancer drug temozolomide.	[151]
	SKOV3 cell-derived exosomes	CRISPR/Cas9 plasmid	PARP-1	SKOV3	*i.v./i.t.*	Subcutaneous xenograft model of ovarian cancer	Disrupted PARP-1; Enhanced the chemosensitivity to cisplatin.	[152]
CPP-mediated delivery systems	Novel Cas9 fusion protein expressing both NLS and a low-molecular-weight protamine	Cas9/sgRNA complex	KRAS	A549	*i.t.*	Subcutaneous xenograft model of lung cancer	Reduced KRAS expression; Inhibited tumor growth.	[153]
	TAT-modified cationic gold nanoclusters covered by an anionic lipid shell (DOTAP/DOPE/cholesterol), followed by post modification with DSPE-PEG on the outer surface (LGCP)	Cas9 protein/sgRNA plasmid	Plk1	A375	*i.t.*	Subcutaneous xenograft melanoma model	Downregulated the expression of Plk1 protein; Achieved higher tumor growth inhibition (75%) than PLNP.	[154]
	PEGylated helical polypeptide nanoparticles containing poly (γ-4-((2-(piperidin-1-yl)ethyl) amino-methyl)benzyl-L-glutamate)	Cas9 plasmid/sgRNA	Plk1	HeLa	*i.t.*	Subcutaneous xenograft model of cervical cancer	Achieved 35% of genome-editing efficiency in the Plk1 locus; Decreased 67% of the Plk1 protein expression level; Suppressed 71% of tumor growth; Prolonged survival.	[155]
Receptor-mediated delivery systems	Folate-modified liposomes	CRISPR/Cas9 plasmid	DNMT1	SKOV3	*i.p.*	Intraperitoneal xenograft model of ovarian cancer	Downregulated DNMT1 expression; Inhibited tumor growth in both paclitaxel-sensitive and -resistant ovarian cancer models.	[65]
	Osteosarcoma-specific aptamer (LC09)-functionalized PEG-PEI-Cholesterol lipopolymer	CRISPR/Cas9 plasmid	VEGFA	K7M2	*i.v.*	Orthotopic osteosarcoma model with lung metastasis	Downregulated VEGFA expression and secretion.	[76]
	Nanoparticles with a phenylboronic acid-modifed low molecular weight polyethyleneimine core and a 2,3-dimethylmaleic anhydride-modifed poly(ethylene glycol)-b-polylysine shell	CRISPR/dCas9 plasmid	miR-524	MDA-MB-231	*i.v.*	Subcutaneous xenograft model of breast cancer	Increased Pri-miR-524 levels (2.92-fold); Retardated tumor growth.	[156]
	ICAM-1 antibody modified noncationic and deformable nanolipogel containing a composition of zwitterionic and anionic lipids (DOPC and DSPE-PEG), as well as alginate hydrogel	CRISPR/Cas9 plasmid	Lipocalin 2	MDA-MB-231	*i.v.*	Orthotopical xenograft model of breast cancer	Knocked out 81% of Lipocalin 2; Supressed tumor growth.	[157]

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Receptor/CPP-mediated delivery systems	R8-dGR modified cationic liposomes	Cas9 plasmid and sgRNA plasmid	HIF-1α	BxPC-3	*i.v.*	Subcutaneous xenograft model of pancreatic cancer	Knocked out HIF-1α; Inhibited tumor growth and metastasis.	[158]
	Dual-receptor-mediated core-shell artificial virus functionalized by hyaluronan (HA) and R8-RGD tandem peptide	CRISPR/Cas9 plasmid	MTH1	SKOV3	*i.v./i.p.*	Subcutaneous xenograft model/peritoneal metastatic model of ovarian cancer	Accumulate selectively into tumor tissues; Downregulated the expression of MTH1; Possessed good antitumor efficacy.	[61]
	iRGD and cell penetration peptide (mHph3)-mediated hydrogel-core DOTAP liposomes (LHNPs)	Cas9 protein and minicircle DNA encoding sgRNA	Plk1	U87	*i.v.*	Subcutaneous xenograft glioma model	Downregulated Plk1; Achieved effective anti-tumor activity.	[159]
	LHNPs modified with Lexiscan, a small molecule known to transiently enhance BBB permeability	Cas9 protein and minicircle DNA encoding sgRNA	Plk1	U87	*i.v.*	Intracranial xenograft glioma model	Increased the accumulation of LHNPs (2.1-fold); Inhibited 60.4% expression of PLK1; Prolonged the median survival time.	[159]
Multi-modal nano-preparations	A multifunctional LACP derivative from LGCP *via* the replacement the TAT-modified cationic gold nanoclusters with TAT-modified gold nanoparticles (AuNPs)	CRISPR/Cas9 plasmid	Plk1	A375	*i.t.*	Subcutaneous xenograft melanoma model	Achieved up to 85% of tumor suppression.	[160]
	Semiconducting polymer brush	CRISPR/Cas9 plasmid	MTH1	HCT 116-GFP	*i.t.*	Subcutaneous xenograft model of colorectcal cancer	Induced indel mutations at a frequency of ≈20%.	[161]
	Upconversion nanoparticles (UCNPs)	Cas9/sgRNA complex	Plk1	A549	*i.t.*	Subcutaneous xenograft model of lung cancer	Knocked out 60.8% of Plk1; Inhibited tumor progression.	[162]
	Cationic polymer-coated Au nanorod (APC)	Cas9 plasmid driven by a heat-inducible promoter, HSP70 (HSP-Cas9)	Plk1	A549	*i.v.*	Subcutaneous xenograft model of lung cancer	Knocked out 51.4% of Plk1; Inhibited tumor progression.	[163]

benzyl-L-glutamate) (PPABLG) with high membrane-penetrating ability for enhanced cellular uptake and endosomal escape. In the subcutaneous xenograft tumor models of A549.GFP cells, P-HNPs loaded with Cas9 plasmid and sgRNA-GFP produced 29.7% of the indels at the target site by *i.t.* administration. They further investigated the antitumor efficacy of P-HNPs-based CRISPR/Cas system *via* editing of Plk1, a highly conserved serine–threonine kinase that promotes cell division and overexpressed in various tumors [165] 35% of genome-editing efficiency in the Plk1 locus was reached by P-HNP delivery of Cas9 plasmid and sgRNA-Plk1 *via i.t.* administration in the subcutaneous xenograft tumor models of HeLa cells, resulting in 67% decrease of the Plk1 protein expression level. The final tumor suppression was more than 71% with significantly enhanced survival rate of mice.

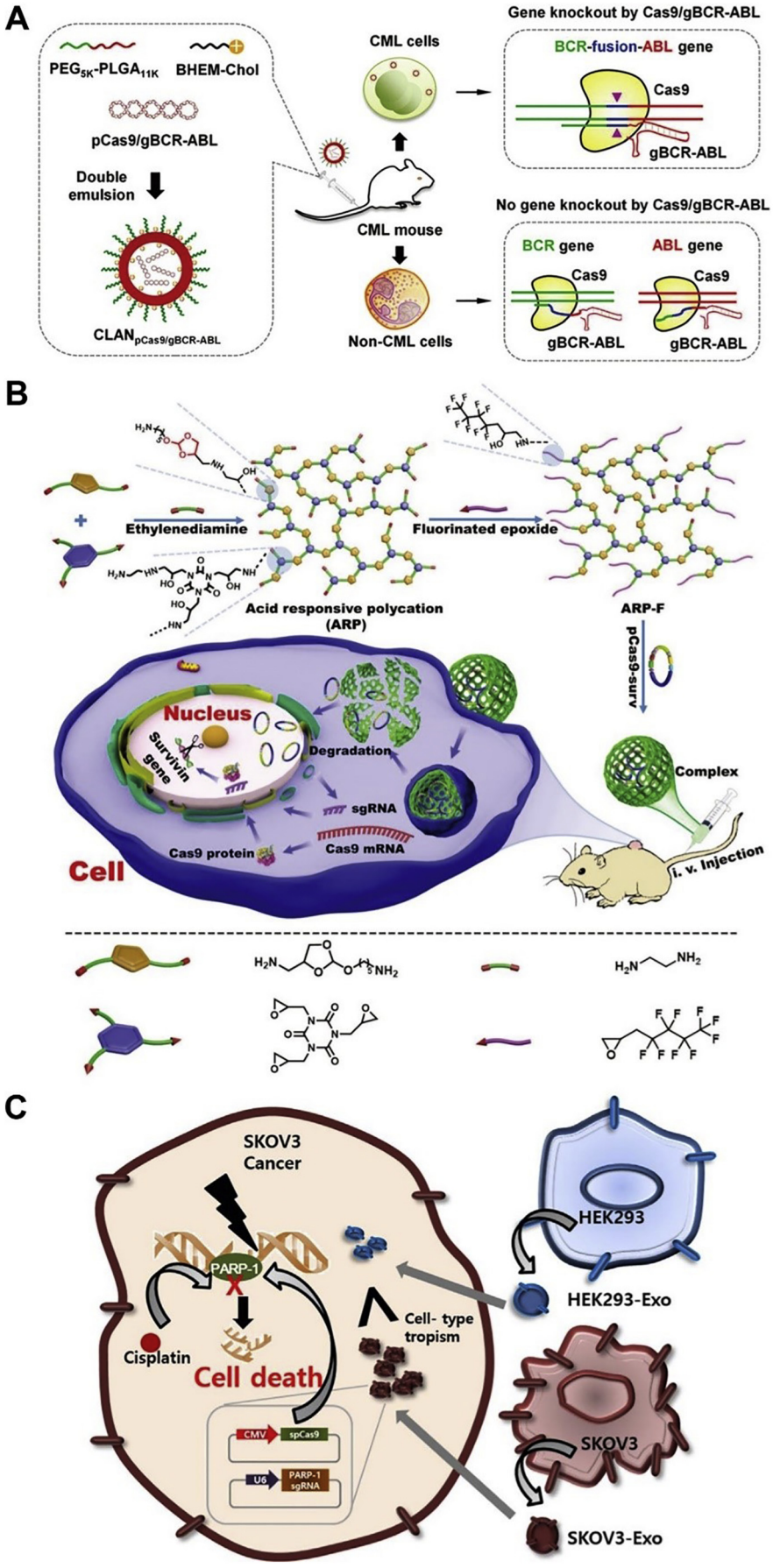
4.3.3.3. Receptor-mediated delivery systems. Based on the overexpressing folate receptor (FR) on the surface of ovarian cancer cells, our group constructed easy-to-translate folate-modified liposomes to selectively deliver the CRISPR/Cas9 plasmid targeting DNMT1 gene [65]. In the intraperitoneal SKOV3 xenograft model, CRISPR/Cas9 transcripts were detected in the tumor tissues after FR-mediated delivery of CRISPR/Cas9 plasmid by intraperitoneal administration and the Cas9 nuclease was also expressed efficiently. The expression of DNMT1 was significantly downregulated due to the precise genome editing of CRISPR/Cas9 plasmid and thus the tumor growth inhibition was achieved in both paclitaxel-sensitive and -resistant ovarian cancer models.

An osteosarcoma-specific aptamer (LC09) has been screened and used to functionalize PEG-PEI-cholesterol lipopolymer to encapsulate CRISPR/Cas9 plasmid targeting VEGFA [76]. LC09 promoted selective distribution of CRISPR/Cas9 in both orthotopic osteosarcoma and lung metastasis *via i.v.* administration in the syngeneic orthotopic osteosarcoma mouse model with spontaneous lung metastasis, causing effective VEGFA genome editing in tumor and downregulated VEGFA expression and secretion.

Phenylboronic acid (PBA) can interact specifically recognize sialylation overexpressed on the surface of cancer cells and facilitate efficient endocytosis of PBA-modified cargoes. Herein, Liu and co-workers designed a multistage delivery nanoparticle (denoted as MDNP) with a core–shell structure (Fig. 6) [156]. After *i.v.* administration into mice bearing subcutaneous MDA-MB-231 xenograft tumors, MDNP highly accumulated in the tumor tissues due to the PEGylated shell comprising 2,3-dimethylmaleic anhydride-modified poly(ethylene glycol)-*b*-polylysine. Responsive to the acidic microenvironment of tumor tissues, the shell detached, following the exposure of the cationic polyplex core containing CRISPR/dCas9 plasmid and PBA-modified low molecular weight polyethyleneimine (PBA-PEI). The enhanced uptake of CRISPR/dCas9 by cancer cells induced the transcriptional activation of the tumor suppressor gene miR-524, thus remarkable tumor growth retardation was achieved.

ICAM1 is a recently discovered nanotherapeutic target for triple-negative breast cancer (TNBC) [177]. Moses and co-workers designed a noncationic and deformable nanolipogel modified with ICAM-1 antibody (tNLG), which can effectively deliver the CRISPR/Cas9 plasmid to achieve >81% of knockout of Lipocalin 2 (a breast cancer-promoting gene) in orthotopic TNBC tumors *via i.v.* administration [157]. The tNLG contained a composition of zwitterionic and anionic lipids (DOPC and DSPE-PEG), as well as alginate hydrogel. The deformable core-shell nanostructure can efficiently encapsulate CRISPR plasmids independent of electrostatic interaction. The antibody-guided strategy improved the specific delivery of gene editing tool to the TNBC cells in which the cargo directly released into the cytosol *via* a receptor-mediated membrane fusion pathway. Thus, this innovative tumor-targeted nanolipogel system reached such highly efficient *in vivo* gene disruption efficiency.

4.3.3.4. Receptor/CPP-mediated delivery systems. R8, one of CPPs, can facilitate cell internalization and elevate the transfection efficiency [178]. It



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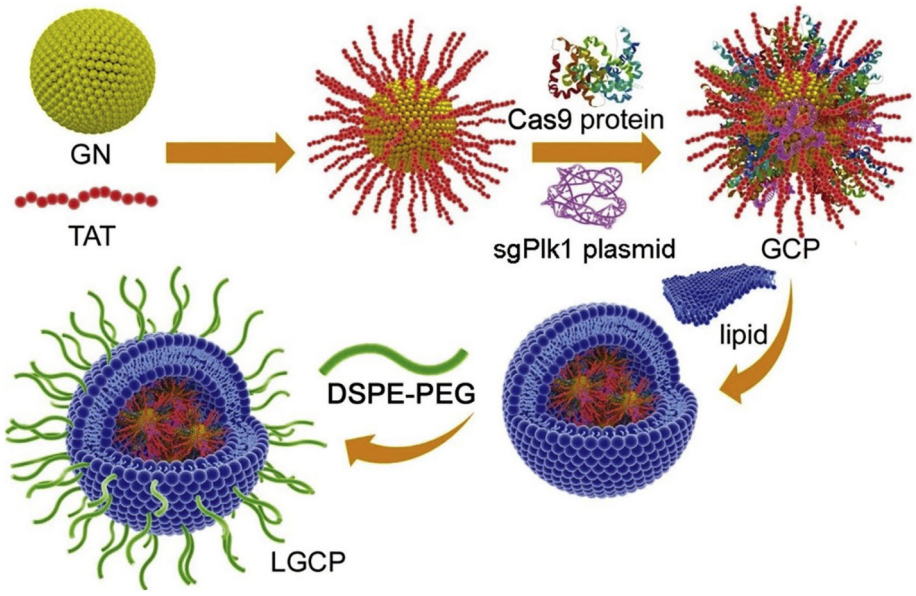

Fig. 5. CPP-mediated CRISPR/Cas9 delivery *via* intratumoral injection. Cell penetrating peptide TAT-containing LGCP. TAT-modified cationic gold nanoclusters (GN) loaded with Cas9/sgRNA complex were covered by an anionic lipid (DOTAP/DOPE/cholesterol) following the post modification with DSPE-PEG on the outer surface. Reproduced with permission Ref. [154]. Copyright 2017, Wiley Online Library. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

has been combined with targeting moiety to enhance the *in vivo* delivery of CRISPR/Cas systems for the treatment of tumor. He and co-workers co-encapsulated Cas9 plasmid and the plasmid encoding HIF-1α-targeting sgRNA in R8-dGR peptide modified cationic liposomes. R8-dGR can bind to integrin αvβ3 and neuropilin-1 overexpressing on pancreatic cancer cells (Fig. 7A) [158]. Thus, this targeting system significantly inhibited tumor growth and metastasis in BxPC-3 xenograft pancreatic tumor model after *i.v.* injection.

In order to develop a versatile delivery system with more highly efficient genome editing *in vivo* based on CRISPR/Cas9 plasmid with large size, our group designed dual-receptor-mediated core-shell artificial virus (RRPHC) potentially targeting tumor cells by CD44 receptor-recognization *via* hyaluronan (HA) [179]. and tumor vascular endothelial cells by integrin αvβ3 receptor-recognization *via* R8-RGD [180]. tandem peptide, respectively (Fig. 7B) [61]. MTH1 was chosen as the targeted gene of our CRISPR/Cas9 genome editing system (Cas9-hMTH1) to treat ovarian cancer. The RRPHC loaded with Cas9-hMTH1 (RRPHC/Cas9-hMTH1) can selectively accumulate into the tumor tissues both in the subcutaneous xenograft tumor models of SKOV3 cells after *i.v.* injection and in the peritoneal metastatic models of SKOV3 cells after *i.p.* injection. RRPHC/Cas9-hMTH1 dramatically downregulated the expression of MTH1 in tumor tissues and thus possessed better antitumor efficacy in the peritoneal metastatic models of ovarian cancer than the delivery system without R8-RGD modification.

Zhou and co-workers further designed a CPP (mHph3) [181]. mediated hydrogel-core DOTAP liposomes (LHNPs) conjugated with iRGD with high affinity for $\alpha_v\beta_3/\alpha_v\beta_5$ integrins and neuropilin-1 (Fig. 7C) [182]. LHNPs possess the ability to efficiently encapsulate both Cas9 protein and minicircle DNA encoding sgRNA-PLK1 by the PEI hydrogel-core and cationic lipid DOTAP-shell, respectively. In the subcutaneous xenograft tumor models of U87 cells, LHNPs showed enhanced accumulation in the tumor tissues mediated by iRGD ligand after *i.v.* injection and thus effective anti-tumor activity with notable downregulation of Plk1. To realize the gene editing of intracranial tumors by CRISPR/Cas9 system, LHNPs were further modified with Lexiscan, a small molecule known to temporarily improve BBB permeability [183]. The inclusion of Lexiscan improved the accumulation of LHNPs 2.1-fold in tumors by *i.v.* administration in the intracranial U87 model through an autocatalytic brain tumor-targeting mechanism [184]. The expression of Plk1 was inhibited by 60.4% and the median survival time for mice was significantly prolonged [159]

4.3.3.5. Multi-modal nano-preparations. Based on LGCP, Jiang and co-workers replaced the core GCP with TAT-modified gold nanoparticles (AuNPs) loaded with Cas9/sgRNA-Plk1 plasmid to get a multifunctional LACP (Fig. 8A) [160]. The AuNPs core can function not only as nanocarrier, but as a trigger to induce Cas9/sgRNA-Plk1 plasmid release into the cytosol of A375 cells under the photothermal conversion. Additionally, Cas9/sgRNA-Plk1 plasmid can further enter the nuclei by TAT guidance. Thus, LACP achieved up to 85% of tumor suppression in the subcutaneous melanoma models of A375 cells by *i.t.* administration.

A semiconducting polymer brush has been applied for CRISPR/Cas9 plasmid delivery with controllable endolysosomal escape (Fig. 8B), which achieved successful genome editing (~20%) of MTH1 by near-infrared (NIR) -II in HCT 116-GFP tumor models by *i.t.* injection [161]. Moreover, novel upconversion nanoparticles responsive to NIR light were also designed to deliver Cas9/sgRNA complex to edit Plk1 [162]. After *i.t.* injection, 60.8% of Plk1 was successfully knocked out by the remote-controlled NIR light in the xenograft nude mice model of A549 cells. This CRISPR/Cas delivery platform presented a huge potential for targeted cancer therapy in deep tissues.

Fig. 4. The common nano-sized preparations loaded with CRISPR/Cas9 plasmid by intravenous administration. (A) Polymeric nanoparticles containing PEG-PLGA and cationic lipid (CLANs) mediated CRISPR/Cas system delivery. The BCR-ABL fusion gene of CML cells was specifically recognized and disrupted, while non-CML cells were not affected. Reproduced with permission Ref. [164]. Copyright 2018, Royal Society of Chemistry. (B) Fluorinated acid-labile branched hydroxyl-rich polycation (ARP—F) complex with CRISPR/Cas9 plasmid (pCas9-surv) mediated the surviving gene editing in human lung cancer A549 cells. Reproduced with permission Ref. [151]. Copyright 2017, Wiley Online Library. (C) Human ovarian cancer SKOV3 cell-derived exosomes (SKOV3-Exo) mediated CRISPR/Cas9 plasmid delivery, and the subsequent knockdown of PARP-1 enhanced the chemosensitivity to cisplatin and caused SKOV3 cell death. Reproduced with permission Ref. [152]. Copyright 2017, Elsevier.

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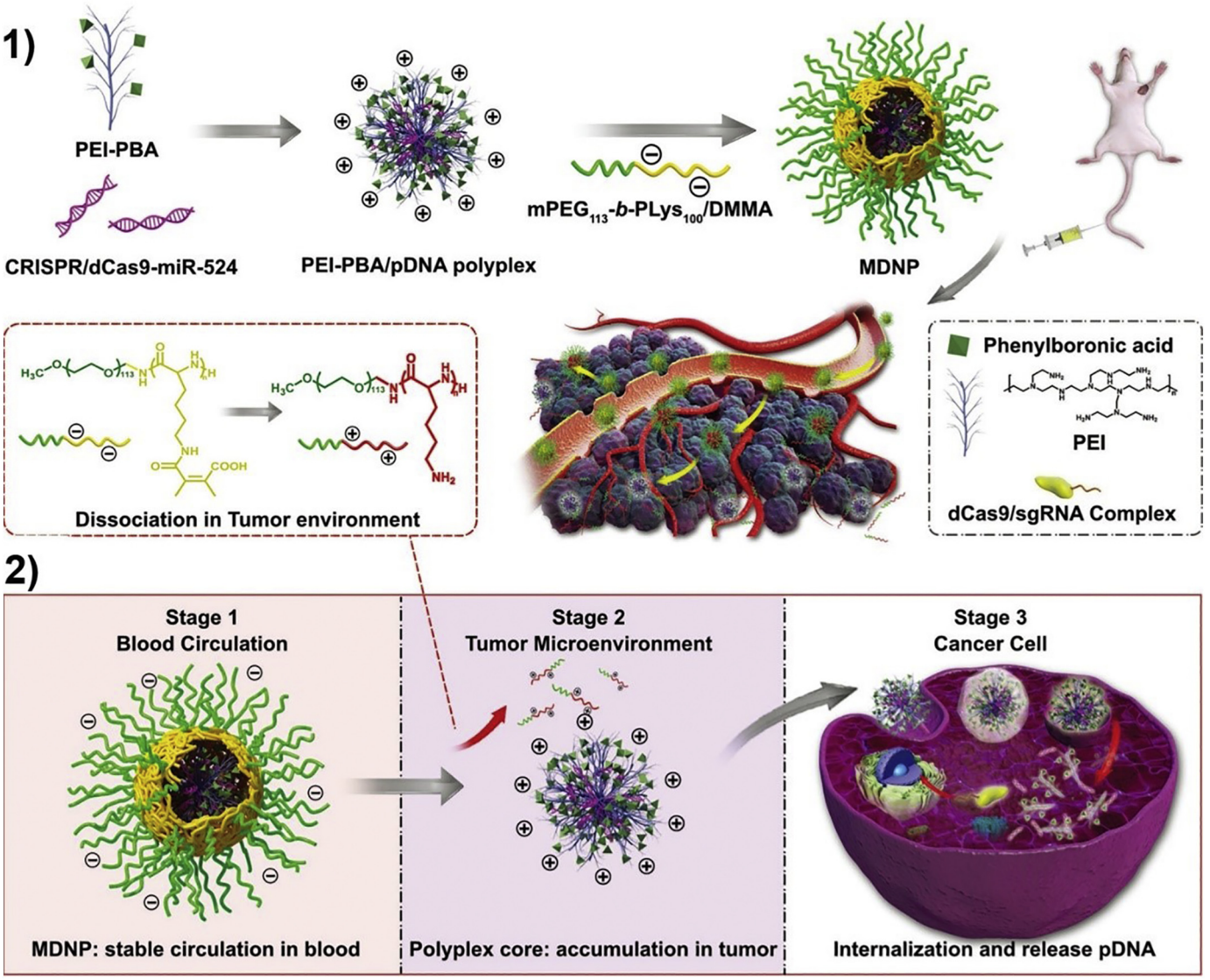

Fig. 6. Receptor-mediated delivery of CRISPR/Cas systems *in vivo*. Nanoparticles (MDNP) with a phenylboronic acid-modified low molecular weight polyethyleneimine (PEI-PBA) core and a 2,3-dimethylmaleic anhydride -modified poly(ethylene glycol)-b-polylysine (mPEG113-b-PLys100/DMMA) shell. 1) MDNP loaded with CRISPR/dCas9 plasmid treated the subcutaneous MDA-MB-231 xenograft mice *via* intravenous injection. 2) MDNP was delivered to MDA-MB-231 cells after stage 1 and stage 2, and the cationic polyplex core containing CRISPR/dCas9 plasmid was highly uptaken mediated by the specific interaction of phenylboronic acid (PBA) with sialylation overexpressing on the surface of cancer cells. Reproduced with permission Ref. [156]. Copyright 2018, Wiley Online Library.

5. Clinical translation for cancer therapy

Of all the approaches to CRISPR/Cas-mediated genome editing, the most developed is *ex vivo* delivery to human T cells in the registered clinical trials as seen in Table 7. It is now routine to get remarkably high efficiencies of gene editing on T cells *ex vivo* using electroporation or viral vectors. Normally, NHEJ-mediated gene disruption often achieves indels with efficiencies more than 80%, while HDR-mediated genome editing results in changes at a frequency of about 30% to 70% in primary human T cells [185]

The first clinical trial using CRISPR/Cas tool for lung cancer treatment was initiated at West China Hospital, Sichuan University in 2016. [186–188] In this non-randomized, open-label phase I study (NCT02793856), the immune checkpoint regulator PD-1 was firstly knocked out *ex vivo* in the recipient's blood T cells using CRISPR/Cas system. The PD-1 knockout engineered T cells were extended and subsequently infused back into the patients to assess the safety in treating metastatic NSCLC that has moved forward after all standard therapy. The similar PD-1 knockout engineered T cells were then registered to treat bladder cancer, prostate cancer, renal cell carcinoma and esophageal cancer in four clinical trials, respectively. It's promising to disrupt PD-1, the receptor inhibiting T-cell activation. However, three of the registrations have been withdrawn, probably because it's still doubtful that PD-1 knockout engineered T-cells are preferred over the antibodies neutralizing PD-1 or its ligand PD-L1 [189]

Investigators herein developed another potential approach, CAR-T cells engineered with CRISPR/Cas tool *ex vivo*. Although there are two licensed CAR-T cells products, [2]. the evidence of efficacy of conventional CAR-T cell therapy in solid tumor is still limited. Compared to engineered T cells, engineered CAR-T cells can selectively recognize a specific cancer marker mediated by the genetically introduced CAR. Theoretically, they can achieve better anti-tumor activity than CAR-T cells or engineered T cells. As first proof-of-concept studies, the patients with solid tumor are being enrolled to investigate two different kinds of engineered CAR-T cells applying *ex vivo* CRISPR/Cas gene editing (NCT03747965, NCT03545815 and NCT03399448). A first-in-human

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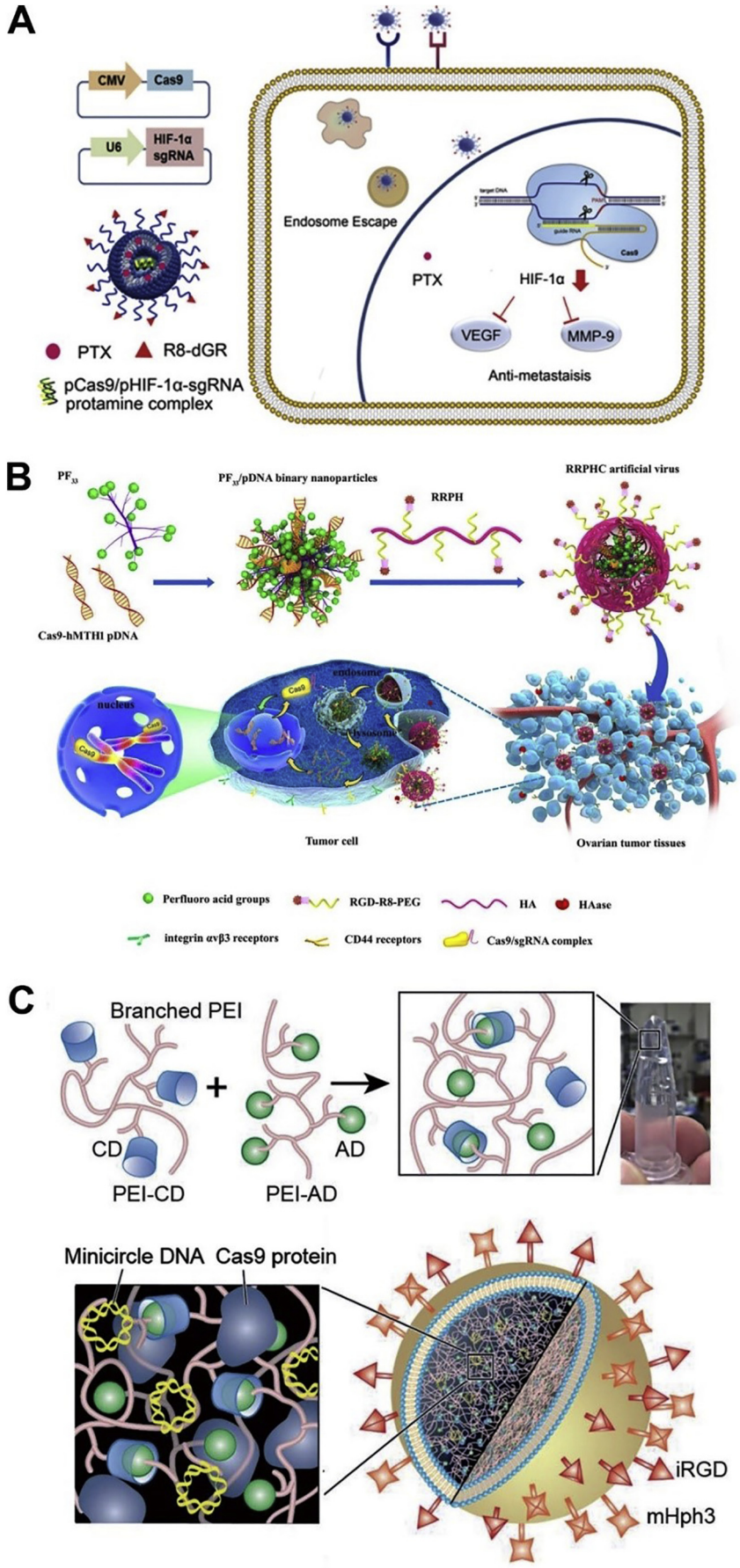

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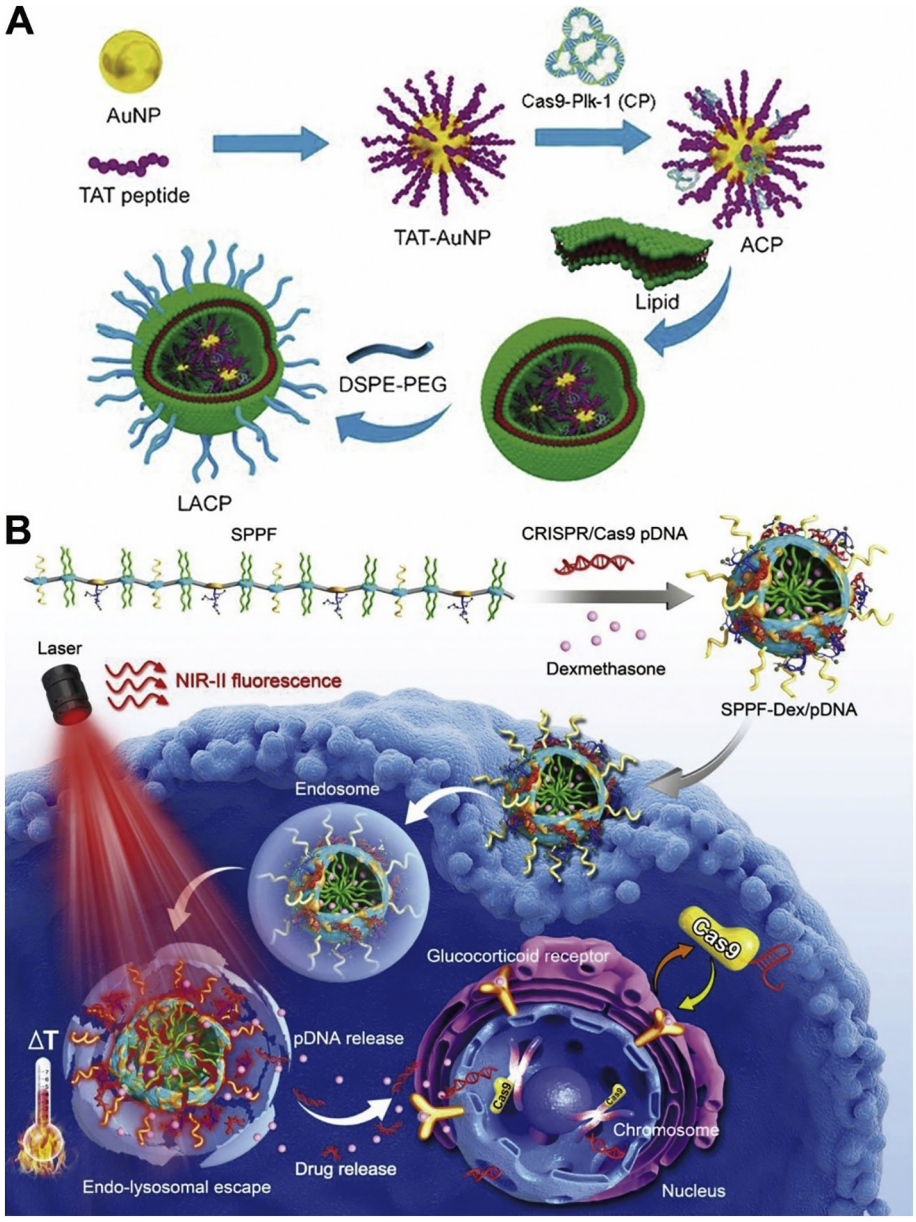

Fig. 8. Multi-modal nano-preparations for *in vivo* delivery of CRISPR/Cas systems. (A) Cas9/sgRNA-Plk1 plasmid-loaded multifunctional nanocarriers (LACP) with photothermal concersion. Reproduced with permission Ref. [159]. Copyright 2018, Wiley Online Library. (B) Semiconducting polymer brush (SPPF), co-encapsulating CRISPR/Cas9 plasmid and dexamethasone, mediated effective intracellular genome editing once NIR-II triggered endolysosomal escape upon 808 nm laser irradiation in HCT 116-GFP tumor models. Reproduced with permission Ref. [161]. Copyright 2019, Wiley Online Library.

phase I clinical trial (NCT03399448) has demonstrated the feasibility of the NY-ESO-1-directed CAR-T cells with the removal of PD-1 and TCR by Cas9/sgRNA complex editing in three patients with refractory cancer [190].

Autologous CAR-T cells have shown favorable results for the hematological cancers treatment. Nonetheless, there are still some therapeutic barriers, like limited CAR-T cell expansion in the heavily pretreated cancer patients, inadequate T-cells in infant cancer patients due to their small blood volume, and costly personalized manufacturing difficult for industrialization. Therefore, universal CAR-T cells derived from healthy donors are undoubtedly an alternative option to address above-mentioned issues. This strategy can not only avoid graft-*versus*-host-disease (GVHD), but also serve as the "off-the-shelf" therapeutic agents for large-scale clinical applications. Four allogeneic CAR-T cell therapies, becoming universal through CRISPR/Cas gene editing, are being studied in clinical trials (NCT03690011, NCT03398967,

Fig. 7. Receptor/CPP-mediated delivery systems *in vivo*. (A) R8-dGR modified liposomes mediated CRISPR/Cas9-HIF-1α delivery. The knockdown of HIF-1α enhanced the anti-tumor and anti-metastatic consequences of PTX by downregulating the downstream molecules such as VEGF and MMP-9. Reproduced with permission Ref. [158]. Copyright 2019, Elsevier. (B) Dual-receptor-mediated core-shell artificial virus (RRPHC) loaded with Cas9-hMTH1 potentially targeted tumor cells by CD44 receptor-recognization *via* hyaluronan (HA) and tumor vascular endothelial cells by integrin αvβ3 receptor-recognization *via* R8-RGD tandem peptide. Reproduced with permission Ref. [61]. Copyright 2017, American Chemical Society. (C) The iRGD and cell penetration peptide (mHph3)-mediated hydrogel-core DOTAP liposomes (LHNPs) encapsulated both Cas9 protein and minicircle DNA encoding sgRNA-PLK1 by the PEI hydrogel-core and cationic lipid DOTAP-shell. Reproduced with permission Ref. [109]. Copyright 2017, Wiley Online Library.

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Table 7
Clinical trials using genome editing for cancer therapy. From clinicaltrials.gov, accessed 2019-10-09.

Interventions	Indications	Status	Phase	Sponsor/Collaborators	Delivery strategies of CRISPR/Cas system	Study start	Trial details
PD-1 knockout engineered T cells	Metastatic non-small cell lung cancer	Active, not recruiting	1	Sichuan University	CRISPR/Cas plasmid, electroporation, *ex vivo*	2016/8/26	NCT02793856
PD-1 knockout engineered T cells	Invasive bladder cancer stage IV	Withdrawn	1	Peking University	Not disclosed	2016/9/1	NCT02863913
PD-1 knockout engineered T cells	Hormone refractory prostate cancer	Withdrawn	Not disclosed	Peking University	Not disclosed	2016/11/1	NCT02867345
PD-1 knockout engineered T cells	Metastatic renal cell carcinoma	Withdrawn	1	Peking University	Not disclosed	2016/11/1	NCT02867332
PD-1 knockout engineered T cells	Advanced esophageal cancer	Completed	Not disclosed	Hangzhou Cancer Hospital	Not disclosed	2017/5/14	NCT03081715
PD-1 knockout EBV-CTLs	Advanced stage epstein-barr virus (EBV) associated malignancies	Recruiting	1, 2	The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School	CRISPR/Cas plasmid, electroporation, *ex vivo*	2017/4/7	NCT03044743
CISH-knockout tumor-infiltrating lymphocytes (TIL)	Metastatic gastrointestinal epithelial cancer	Withdrawn	1, 2	National Cancer Institute (NCI)	Not disclosed	2018/5/17	NCT03538613
PD-1-knockout mesothelin-directed CAR-T cells	Mesothelin positive multiple solid tumors	Recruiting	1	Chinese PLA General Hospital	Cas9 protein and sgRNA, electroporation, *ex vivo*	2018/11/1	NCT03747965
PD-1 and TCR-knockout mesothelin-directed CAR-T cells	Mesothelin positive multiple solid tumors	Recruiting	1	Chinese PLA General Hospital	Cas9 protein and sgRNAs, electroporation, *ex vivo*	2018/5/1	NCT03545815
PD-1 and TCR-knockout NY-ESO-1-directed CAR-T cells	Multiple myeloma, melanoma, synovial sarcoma and myxoid/round cell liposarcoma	Active, not recruiting	1	University of Pennsylvania	Cas9 protein and sgRNA, electroporation, *ex vivo*	2018/9/5	NCT03399448
HPK1-knocknout CD19-directed CAR-T cells	Relapsed/refractory $CD19^{+}$ leukemia or lymphoma	Recruiting	1	Xijing Hospital	Electroporation with CRISPR guide RNA, *ex vivo*	2019/8/1	NCT04037566
Allogeneic CD7-directed CAR-T cells with CD7 removal on the surface	Relapsed/refractory T-cell malignancies	Not yet recruiting	1	Baylor College of Medicine	Electroporation, *ex vivo*	2019/3/1	NCT03690011
Universal TCR-knockout CAR-T cells targeting CD19 and CD20 or CD22	Relapsed/refractory CD19+ Leukemia and Lymphoma	Recruiting	1, 2	Chinese PLA General Hospital	Cas9 protein and sgRNAs, electroporation, *ex vivo*	2018/1/2	NCT03398967
Allogeneic CD19-directed BBζ CAR-T cells (UCART019) with disruption of endogenous TCR and B2M genes	Relapsed/refractory CD19+ B Cell Leukemia and Lymphoma	Recruiting	1, 2	Chinese PLA General Hospital	Cas9 mRNA+ ΔU3-sgRNA, electroporation, *ex vivo*	2017/6/1	NCT03166878
Allogeneic CD19-directed CAR-T cells with disruption of TCR and MHC I genes	Relapsed/refractory B-Cell malignancies	Recruiting	1, 2	CRISPR Therapeutics AG	Not disclosed	2019/7/22	NCT04035434
CRISPR/Cas9 (CRISPR/Cas9-HPV16 E6/E7T1 or CRISPR/Cas9-HPV18 E6/E7T2)plasmid	HPV persistency and human cervical intraepithelial neoplasia I without invasion.	Unknown	1	First Affiliated Hospital, Sun Yat-Sen University	CRISPR/Cas plasmid in gel containing C32–447, poloxamer 407 and distilled water as solvent, *in vivo*	2018/1/15	NCT03057912

NCT03166878 and NCT04035434), in which something is worth looking forward to in the future.

Compared to *ex vivo* genome editing, *in vivo* genome editing is more versatile for cancer therapy. Till now, there is only one registered clinical trial aiming at disruption of E6/E7 DNA from HPV16 and HPV18 using CRISPR/Cas9 plasmid in gel containing C32–447 and poloxamer 407 (NCT03057912). The *in vivo* genome editing will be achieved through CRISPR/Cas9 delivery to the HPV infected cervix *via* local administration [189]. One of the big challenges for *in vivo* genome editing by CRISPR/Cas system is the delivery strategy to target specific organs, tumors or even cells. The development of various *in vivo* delivery methods for CRISPR/Cas system is ongoing and some of them have shown good antitumor efficacy in tumor-bearing mice models. Of note, the rapid progress on the CRISPR/Cas delivery systems probably prompts the future clinical translational research in cancer therapy.

6. Concluding remarks

Despite the short history of CRISPR-based technologies, progression in the development of CRISPR systems has brought benefits for therapeutic applications of cancer. To date, various types of CRISPR systems have been developed, enabling genome-focused, loss-of-function (CRISPR and CRISPRi) or gain-of-function (CRISPRa), *in vitro*, *ex vivo* and/or *in vivo* studies which all the existing cancer therapeutic strategies are difficult to achieved. Importantly, cancer is a genetic disease—that is, cancer is caused by certain (*epi*) genetic changes that control the way our cells function and the (epi) genomic aberration are multifarious among patients, which renders (epi) genome manipulation in cancer a highly precision and multi-level progress. The CRISPR/Cas systems have been shown to be efficient to modulate the genome, transcriptome and epigenome of tumor cells or immune cells and thus an indispensable technology for cancer gene therapy and immunotherapy. As versatile gene editing tools, they can be utilized to intervene genetic, epigenetic and transcriptomal aberrations in tumor cells including pathogen infection-induced cancers, as well as activate or disrupt the normal endogenous genes in tumor cells or immune cells. Compared to other genome engineering systems such as meganucleases, ZFNs and TALENs, the CRISPR/Cas systems depend upon the base pairing specificity of sgRNA with the genomic DNA or RNA rather than the recognition of specific sequences by programmable protein-DNA interactions [80]. Thus, CRISPR/Cas-mediated gene editing is relatively easier, more efficient and scalable.

The targeted genomic engineering by the CRISPR/Cas systems has been applied in clinical trials to mainly improve T-cell therapy in various ways. NHEJ-based editing can remove molecules inhibiting the function of T cells like PD-1, as well as disrupt TCR mediating immunogenicity. These strategies have great potential to enhance the anti-cancer function of T cells or eliminate their alloreactivity through *ex vivo* gene editing. In contrast, HDR-based editing has been used to insert a CAR to the TCR α chain (TRAC) locus and thereby enhance T-cell potency higher than the conventionally produced CAR-T cells in the preclinical study [191]. This approach will very likely promote the initiation of new clinical trial very soon. T-cell therapies base on *ex vivo* genomic engineering have exhibited obvious benefits to cancer treatment in clinic. However, current *ex vivo* gene editing modality faces some limitations including the complex procedures and costs manufacturing genetically modified lymphocytes [192]. Indeed, most of the engineered T cells for clinical trials were transduced by electroporation which might cause cell damage. So, it's urgent to explore the clinic application of T cells experiencing *ex vivo* gene editing *via* more efficient and safe delivery strategies including novel viral vectors with low immunogenicity or non-viral vector with high transfection efficiency. Moreover, it might be an optimal alternative to program T cells *via* CRISPR/Cas editing *in situ* or *in vivo*. Of note, efficient and specific delivery of CRISPR/Cas systems is the main challenge for *in vivo* gene editing. Host T cells *in situ* have been reported to be successfully transduced by polymeric nanocarriers loaded with leukemia-specific CAR genes after *i.v.* injection in hematological cancer mouse models [193]. Hence, direct delivery of CRISPR/Cas systems *in vivo* is worth being developed to program lymphocytes in the near future.

One of *in vivo* delivery of CRISPR/Cas system to cancer cells has also been planned to investigate the clinic efficacy (NCT03057912). As shown in Table 6, there have been about 20 different delivery systems with promising efficiency for the *in vivo* gene editing, including viral and non-viral vectors for the delivery of nuclease and/or sgRNA-expressing plasmid, as well as nuclease/sgRNA complex. The key points for the clinical translation of CRISPR/Cas systems *via* direct delivery *in vivo*, probably involved not only highly efficient delivery to cancer cells or immune cells, but also the safety of the whole delivery system which might be related to the carrier itself and the off-target effect of CRISPR/Cas systems. Taken into account of these information, it's encouraging to improve the current carriers or further develop innovative *in vivo* delivery vectors with high selectivity and biocompatibility followed by the design of novel CRISPR/Cas systems for exact gene editing.

Viruses have exhibited powerful ability in delivering therapeutic agents in the recent proof-of-principle studies. Nevertheless, the clinical trial has not been initiated using viruses to deliver CRISPR/Cas systems, probably because of the limited packing capacity and safety issues [80]. Researchers have been exploring multiple approaches to overcome these shortages of viral vectors. In order to solve the packaging restriction, shorter nuclease variants can be selected, as well as the components of CRISPR/Cas systems can be packaged in several separate viral vectors. Moreover, the surface modification of viruses might facilitate the specific recognization of the targeted cells and thus reduce the security risk with the decrease of off-target effect. Indeed, the majority of the marketed products on gene therapy use viral vectors and most of the registered clinical trials likewise applied viruses to carry out the conventional gene therapy [2]. Collectively, CRISPR/Cas-based gene therapy using viral vectors will realize the clinical translation with the improvement of specificity and safety of the virus-based delivery system packaging CRISPR/Cas tools [82]

In contrast to viral approaches for CRISPR/Cas delivery, non-viral vectors have great potentials to avoid the current limitations with viral vectors [194]. Firstly, they are not restricted by the packaging restrictions due to various options suitable for the typical three modes of CRISPR/Cas systems, namely, plasmid DNA, mRNA and ribonucleoprotein (nuclease complexed with sgRNA). Secondly, the immunogenicity of non-viral vectors is normally lower than viruses. Based on organic or inorganic materials, most non-viral carriers have good biodegradability or biocompatibility. Thirdly, it's relatively easy and flexible to design the rational formulations for the delivery of CRISPR/Cas systems to the target sites. Multiple nano-sized preparations are available when the appropriate materials and processing methods are selected, including but not limited to liposomes, nanoparticles with different shape, nanosheets and micelles. Generally, investigators have developed various innovative non-viral vectors with promising functions such as cell-specific delivery with ligand targeting, membrane penetration mediated by CPP, controllable endolysosomal escape and nuclear localization. Nevertheless, only one clinical trial using non-viral strategy to deliver CRISPR/Cas plasmid has been registered to the best of our knowledge. Most routes of administration for the non-viral vectors loaded with CRISPR/Cas tools were *i.t.* injection when their anti-tumor efficacy was evaluated *in vivo* in the present studies (Table 6). This local administration is commonly limited to certain tumors accessible *via* direct injection or specific procedures such as colonoscopy, cystoscopy, bronchoscopy, thoracoscopy, coelioscopy, or even surgery [195,196]. Hence, the systemic administration should be expanded in the future research, which possibly offers patients more alternatives. The antitumor efficacy has been widely focused on in the current researches, however, the scale-up production or safety of CRISPR/Cas delivery system based on non-viral vectors will be equally important [80]. Most of the designed formulations seem complicated and the preparation process is uncontrollable. It will accelerate the clinical translation

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if the non-viral vectors for CRISPR/Cas delivery can be as simple as the lipid-based nanoparticles for siRNA delivery to liver (ONPATTRO ™, launched in 2018) [2]

Apart from the rational design of delivery vectors, it's also crucial to optimize the CRISPR/Cas systems in order to enhance the gene editing efficiency and improve safety *in vivo*. Most of the current CRISPR/Cas-mediated editing would lead to off-target effects. Although the specific delivery *via* targeting carriers can attenuate off-target effects of CRISPR/Cas systems, it's still very important to construct innovative CRISPR/Cas-based gene editing tools to increase the nuclease specificity and reduce off-target activity. Furthermore, the forms of CRISPR/Cas systems also have impact on the final gene editing efficiency. It's reported that human immune system can recognize the exogenous nuclease. The broadly investigated Cas9 nuclease in CRISPR/Cas9 system is originated in two bacterial species, *S. pyogenes* and *Staphylococcus aureus*. Many adults have been infected with these bacteria and consequently have preexisting immunity to Cas9 [197,198] A recent study found that 79% and 65% of donors had immune response to SaCas9 and SpCas9, respectively. While 46% of donors had SaCas9-specific T-cells in peripheral blood [195]. Thus, direct ribonucleoprotein delivery has risk for rapid clearance *via* systemic administration before arriving the target sites. It's very important not to expose the nuclease during circulation. This might be challenging for non-viral vectors as the cargoes are likely to leak or release. On the other hand, prolonged expression of the foreign nuclease is prone to induce an adaptive immune response, resulting in the removal of the nuclease-expressing cells [186]. It seems a good way to eliminate the cancer cells by specific introduction of CRISPR/Cas systems, but unfavorable for the immune cells. The mRNA encoding CRISPR/Cas will be an alternative option for immune-cell transduction due to its moderate duration of nuclease between protein and plasmid or viruses in the transfected cells. However, the mRNA form of CRISPR/Cas for cancer therapy has not yet been reported, probably because it's extremely unstable and its delivery is highly challenging [199]

CRISPR/Cas systems offer several advantages over conventional ones, such as simple-to-design, easy-to-use and multiplexing. Consequently, it has become a cost-effective and convenient tool for various genome editing for cancer therapy. In principle, CRISPR/Cas systems currently represent the most promising and necessary field in cancer gene therapy. However, full realization of the potential of CRISPR/Cas approaches will require addressing many challenges. Within the system itself, off-target cutting remains a major concern, especially for therapeutic and clinical applications. The off-target cutting would induce unexpected mutations at sites other than the intended on-target site. The improvement of the off-target specificity would provide solid genotype–phenotype correlations, and thus enable faithful interpretation of genome-editing data. Our understanding of off-target effects remains poor and the current clinical trials using CRISPR/Cas systems are all in the early stages and little data have been released. So CRISPR/Cas-based gene therapy and immunotherapy is still in its infancy for cancer treatment. Rational design of the delivery systems loaded with CRISPR/Cas tools has great potential to prompt the transformative therapy for patients with cancer.

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Author disclosure

The authors declare no conflict of interest.

References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (2018) 394–424.
[2] F. Wang, Z. Qin, H. Lu, S. He, J. Luo, C. Jin, X. Song, Clinical translation of gene medicine, The Journal of Gene Medicine 21 (2019), e3108.
[3] A. Bolotin, B. Quinquis, A. Sorokin, S.D. Ehrlich, Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin, Microbiology 151 (2005) 2551–2561.
[4] C. Pourcel, G. Salvignol, G. Vergnaud, CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies, Microbiology 151 (2005) 653–663.
[5] H. Deveau, J.E. Garneau, S. Moineau, CRISPR/Cas system and its role in phage-bacteria interactions, Annu. Rev. Microbiol. 64 (2010) 475–493.
[6] L.A. Marraffini, E.J. Sontheimer, CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea, Nat. Rev. Genet. 11 (2010) 181–190.
[7] R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D.A. Romero, P. Horvath, CRISPR provides acquired resistance against viruses in prokaryotes, Science 315 (2007) 1709–1712.
[8] B. Wiedenheft, S.H. Sternberg, J.A. Doudna, RNA-guided genetic silencing systems in bacteria and archaea, Nature 482 (2012) 331–338.
[9] T. Sinkunas, G. Gasiunas, C. Fremaux, R. Barrangou, P. Horvath, V. Siksnys, Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system, EMBO J. 30 (2011) 1335–1342.
[10] E.R. Westra, P.B. van Erp, T. Kunne, S.P. Wong, R.H. Staals, C.L. Seegers, S. Bollen, M.M. Jore, E. Semenova, K. Severinov, W.M. de Vos, R.T. Dame, R. de Vries, S.J. Brouns, J. van der Oost, CRISPR immunity relies on the consecutive binding and degradation of negatively supercoiled invader DNA by Cascade and Cas3, Mol. Cell 46 (2012) 595–605.
[11] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (2012) 816–821.
[12] G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) E2579–E2586.
[13] S. Shmakov, A. Smargon, D. Scott, D. Cox, N. Pyzocha, W. Yan, O.O. Abudayyeh, J.S. Gootenberg, K.S. Makarova, Y.I. Wolf, K. Severinov, F. Zhang, E.V. Koonin, Diversity and evolution of class 2 CRISPR-Cas systems, Nat. Rev. Microbiol. 15 (2017) 169–182.
[14] M. Kazlauskiene, G. Tamulaitis, G. Kostiuk, C. Venclovas, V. Siksnys, Spatiotemporal control of type III-A CRISPR-Cas immunity: coupling DNA degradation with the target RNA recognition, Mol. Cell 62 (2016) 295–306.
[15] G. Tamulaitis, C. Venclovas, V. Siksnys, Type III CRISPR-Cas immunity: major differences brushed aside, Trends Microbiol. 25 (2017) 49–61.
[16] K.S. Makarova, Y.I. Wolf, O.S. Alkhnbashi, F. Costa, S.A. Shah, S.J. Saunders, R. Barrangou, S.J. Brouns, E. Charpentier, D.H. Haft, P. Horvath, S. Moineau, F.J. Mojica, R.M. Terns, M.P. Terns, M.F. White, A.F. Yakunin, R.A. Garrett, J. van der Oost, R. Backofen, E.V. Koonin, An updated evolutionary classification of CRISPR-Cas systems, Nat. Rev. Microbiol. 13 (2015) 722–736.
[17] A. Ozcan, P. Pausch, A. Linden, A. Wulf, K. Schuhle, J. Heider, H. Urlaub, T. Heimerl, G. Bange, L. Randau, Type IV CRISPR RNA processing and effector complex formation in Aromatoleum aromaticum, Nat. Microbiol. 4 (2019) 89–96.
[18] H. Nishimasu, F.A. Ran, P.D. Hsu, S. Konermann, S.I. Shehata, N. Dohmae, R. Ishitani, F. Zhang, O. Nureki, Crystal structure of Cas9 in complex with guide RNA and target DNA, Cell 156 (2014) 935–949.
[19] M.R. O'Connell, B.L. Oakes, S.H. Sternberg, A. East-Seletsky, M. Kaplan, J.A. Doudna, Programmable RNA recognition and cleavage by CRISPR/Cas9, Nature 516 (2014) 263–266.
[20] F.A. Ran, L. Cong, W.X. Yan, D.A. Scott, J.S. Gootenberg, A.J. Kriz, B. Zetsche, O. Shalem, X. Wu, K.S. Makarova, E.V. Koonin, P.A. Sharp, F. Zhang, In vivo genome editing using Staphylococcus aureus Cas9, Nature 520 (2015) 186–191.
[21] D. Burstein, L.B. Harrington, S.C. Strutt, A.J. Probst, K. Anantharaman, B.C. Thomas, J.A. Doudna, J.F. Banfield, New CRISPR-Cas systems from uncultivated microbes, Nature 542 (2017) 237–241.
[22] B. Zetsche, J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, S.E. Volz, J. Joung, J. van der Oost, A. Regev, E.V. Koonin, F. Zhang, Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (2015) 759–771.
[23] B.P. Kleinstiver, A.A. Sousa, R.T. Walton, Y.E. Tak, J.Y. Hsu, K. Clement, M.M. Welch, J.E. Horng, J. Malagon-Lopez, I. Scarfo, M.V. Maus, L. Pinello, M.J. Aryee, J.K. Joung, Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing, Nat. Biotechnol. 37 (2019) 276–282.
[24] L. Gao, D.B.T. Cox, W.X. Yan, J.C. Manteiga, M.W. Schneider, T. Yamano, H. Nishimasu, O. Nureki, N. Crosetto, F. Zhang, Engineered Cpf1 variants with altered PAM specificities, Nat. Biotechnol. 35 (2017) 789–792.
[25] J.S. Chen, E. Ma, L.B. Harrington, M. Da Costa, X. Tian, J.M. Palefsky, J.A. Doudna, CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity, Science 360 (2018) 436–439.
[26] O.O. Abudayyeh, J.S. Gootenberg, S. Konermann, J. Joung, I.M. Slaymaker, D.B. Cox, S. Shmakov, K.S. Makarova, E. Semenova, L. Minakhin, K. Severinov, A. Regev, E.S. Lander, E.V. Koonin, F. Zhang, C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector, Science 353 (2016) aaf5573.

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[27] O.O. Abudayyeh, J.S. Gootenberg, P. Essletzbichler, S. Han, J. Joung, J.J. Belanto, V. Verdine, D.B.T. Cox, M.J. Kellner, A. Regev, E.S. Lander, D.F. Voytas, A.Y. Ting, F. Zhang, RNA targeting with CRISPR-Cas13, Nature 550 (2017) 280–284.

[28] J.S. Gootenberg, O.O. Abudayyeh, J.W. Lee, P. Essletzbichler, A.J. Dy, J. Joung, V. Verdine, N. Donghia, N.M. Daringer, C.A. Freije, C. Myhrvold, R.P. Bhattacharyya, J. Livny, A. Regev, E.V. Koonin, D.T. Hung, P.C. Sabeti, J.J. Collins, F. Zhang, Nucleic acid detection with CRISPR-Cas13a/C2c2, Science 356 (2017) 438–442.

[29] C. Dincer, R. Bruch, E. Costa-Rama, M.T. Fernandez-Abedul, A. Merkoci, A. Manz, G.A. Urban, F. Guder, Disposable sensors in diagnostics, food, and environmental monitoring, Adv. Mater. 31 (2019), e1806739.

[30] Y. Li, S. Li, J. Wang, G. Liu, CRISPR/Cas systems towards next-generation biosensing, trends in biotechnology, 37 (2019) 730–743.

[31] L.A. Gilbert, M.H. Larson, L. Morsut, Z. Liu, G.A. Brar, S.E. Torres, N. Stern-Ginossar, O. Brandman, E.H. Whitehead, J.A. Doudna, W.A. Lim, J.S. Weissman, L.S. Qi, CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes, Cell 154 (2013) 442–451.

[32] T.A. Baudino, Targeted cancer therapy: the next generation of cancer treatment, Current Drug Discovery Technologies 12 (2015) 3–20.

[33] L.T. Jia, S.Y. Chen, A.G. Yang, Cancer gene therapy targeting cellular apoptosis machinery, Cancer Treat. Rev. 38 (2012) 868–876.

[34] J. Settleman, C.L. Sawyers, T. Hunter, Challenges in validating candidate therapeutic targets in cancer, eLife 7 (2018), e32402.

[35] B.J. Druker, Inhibition of the Bcr-Abl tyrosine kinase as a therapeutic strategy for CML, Oncogene 21 (2002) 8541–8546.

[36] S.V. Sharma, D.W. Bell, J. Settleman, D.A. Haber, Epidermal growth factor receptor mutations in lung cancer, Nat. Rev. Cancer 7 (2007) 169–181.

[37] J. Canon, K. Rex, A.Y. Saiki, C. Mohr, K. Cooke, D. Bagal, K. Gaida, T. Holt, C.G. Knutson, N. Koppada, B.A. Lanman, J. Werner, A.S. Rapaport, T. San Miguel, R. Ortiz, T. Osgood, J.R. Sun, X. Zhu, J.D. McCarter, L.P. Volak, B.E. Houk, M.G. Fakih, B.H. O'Neil, T.J. Price, G.S. Falchook, J. Desai, J. Kuo, R. Govindan, D.S. Hong, W. Ouyang, H. Henary, T. Arvedson, V.J. Cee, J.R. Lipford, The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity, Nature (2019) 217–223.

[38] J. Wang, B. Xu, Targeted therapeutic options and future perspectives for HER2-positive breast cancer, Signal Transduction and Targeted Therapy 4 (2019) 34.

[39] S. Cory, J.M. Adams, The Bcl2 family: regulators of the cellular life-or-death switch, Nat. Rev. Cancer 2 (2002) 647–656.

[40] R. Shakeri, A. Kheirollahi, J. Davoodi, Apaf-1: regulation and function in cell death, Biochimie 135 (2017) 111–125.

[41] A.M. Weber, A.J. Ryan, ATM and ATR as therapeutic targets in cancer, Pharmacol. Ther. 149 (2015) 124–138.

[42] S.E. Straus, E.S. Jaffe, J.M. Puck, et al., The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis, Blood. 98 (1) (2001) 194–200.

[43] L.R. Silverman, E.P. Demakos, B.L. Peterson, A.B. Kornblith, J.C. Holland, R. Odchimar-Reissig, R.M. Stone, D. Nelson, B.L. Powell, C.M. DeCastro, J. Ellerton, R.A. Larson, C.A. Schiffer, J.F. Holland, Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B, J. Clin. Oncol. 20 (2002) 2429–2440.

[44] J. Tan, X. Yang, L. Zhuang, X. Jiang, W. Chen, P.L. Lee, R.K. Karuturi, P.B. Tan, E.T. Liu, Q. Yu, Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells, Genes Dev. 21 (2007) 1050–1063.

[45] S.V. Sharma, D.Y. Lee, B. Li, M.P. Quinlan, F. Takahashi, S. Maheswaran, U. McDermott, N. Azizian, L. Zou, M.A. Fischbach, K.K. Wong, K. Brandstetter, B. Wittner, S. Ramaswamy, M. Classon, J. Settleman, A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations, Cell 141 (2010) 69–80.

[46] J.P. Issa, CpG island methylator phenotype in cancer, Nat. Rev. Cancer 4 (2004) 988–993.

[47] D.M. Pardoll, The blockade of immune checkpoints in cancer immunotherapy, Nat. Rev. Cancer 12 (2012) 252–264.

[48] Y. Latchman, C.R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A.J. Long, J.A. Brown, R. Nunes, E.A. Greenfield, K. Bourque, V.A. Boussiotis, L.L. Carter, B.M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A.H. Sharpe, G.J. Freeman, PD-L2 is a second ligand for PD-1 and inhibits T cell activation, Nat. Immunol. 2 (2001) 261–268.

[49] H. Li, C. Sheng, H. Liu, G. Liu, X. Du, J. Du, L. Zhan, P. Li, C. Yang, L. Qi, J. Wang, X. Yang, L. Jia, J. Xie, L. Wang, R. Hao, D. Xu, Y. Tong, Y. Zhou, J. Zhou, Y. Sun, Q. Li, S. Qiu, H. Song, An effective molecular target site in hepatitis B virus S gene for Cas9 cleavage and mutational inactivation, Int. J. Biol. Sci. 12 (2016) 1104–1113.

[50] S.V. Graham, Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies, Future Microbiol. 5 (2010) 1493–1506.

[51] P. Carmeliet, VEGF as a key mediator of angiogenesis in cancer, Oncology 69 (Suppl. 3) (2005) 4–10.

[52] M. Korc, R.E. Friesel, The role of fibroblast growth factors in tumor growth, Curr. Cancer Drug Targets 9 (2009) 639–651.

[53] T. Kondo, L. Zheng, W. Liu, J. Kurebayashi, S.L. Asa, S. Ezzat, Epigenetically controlled fibroblast growth factor receptor 2 signaling imposes on the RAS/BRAF/mitogen-activated protein kinase pathway to modulate thyroid cancer progression, Cancer Res. 67 (2007) 5461–5470.

[54] J. Taeger, C. Moser, C. Hellerbrand, M.E. Mycielska, G. Glockzin, H.J. Schlitt, E.K. Geissler, O. Stoeltzing, S.A. Lang, Targeting FGFR/PDGFR/VEGFR impairs tumor growth, angiogenesis, and metastasis by effects on tumor cells, endothelial cells, and pericytes in pancreatic cancer, Mol. Cancer Ther. 10 (2011) 2157–2167.

[55] B. Mansoori, A. Mohammadi, S. Davudian, S. Shirjang, B. Baradaran, The different mechanisms of cancer drug resistance: a brief review, Advanced Pharmaceutical Bulletin 7 (2017) 339–348.

[56] E. Manchado, C.H. Huang, N. Tasdemir, D.F. Tschaharganeh, J.E. Wilkinson, S.W. Lowe, A pipeline for drug rarget identification and validation, Cold Spring Harb. Symp. Quant. Biol. 81 (2016) 257–267.

[57] A. Bhan, M. Soleimani, S.S. Mandal, Long noncoding RNA and cancer: a new paradigm, Cancer Res. 77 (2017) 3965–3981.

[58] M. Kodama, T. Kodama, J.Y. Newberg, H. Katayama, M. Kobayashi, S.M. Hanash, K. Yoshihara, Z. Wei, J.C. Tien, R. Rangel, K. Hashimoto, S. Mabuchi, K. Sawada, T. Kimura, N.G. Copeland, N.A. Jenkins, In vivo loss-of-function screens identify KPNB1 as a new druggable oncogene in epithelial ovarian cancer, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E7301–E7310.

[59] L. Soucek, J. Whitfield, C.P. Martins, A.J. Finch, D.J. Murphy, N.M. Sodir, A.N. Karnezis, L.B. Swigart, S. Nasi, G.I. Evan, Modelling Myc inhibition as a cancer therapy, Nature 455 (2008) 679–683.

[60] J.M. Brown, L.D. Attardi, The role of apoptosis in cancer development and treatment response, Nat. Rev. Cancer 5 (2005) 231–237.

[61] L. Li, L. Song, X. Liu, X. Yang, X. Li, T. He, N. Wang, S. Yang, C. Yu, T. Yin, Y. Wen, Z. He, X. Wei, W. Su, Q. Wu, S. Yao, C. Gong, Y. Wei, Artificial virus delivers CRISPR-Cas9 system for genome editing of cells in mice, ACS Nano 11 (2017) 95–111.

[62] T. Vogel, S. Lassmann, Epigenetics: development, dynamics and disease, Cell Tissue Res. 356 (2014) 451–455.

[63] R. Kanwal, K. Gupta, S. Gupta, Cancer epigenetics: an introduction, Methods Mol. Biol. 1238 (2015) 3–25.

[64] T. Ushijima, Cancer epigenetics: now harvesting fruit and seeding for common diseases, Biochem. Biophys. Res. Commun. 455 (2014) 1–2.

[65] Z.Y. He, Y.G. Zhang, Y.H. Yang, C.C. Ma, P. Wang, W. Du, L. Li, R. Xiang, X.R. Song, X. Zhao, S.H. Yao, Y.Q. Wei, In vivo ovarian cancer gene therapy using CRISPR-Cas9, Hum. Gene Ther. 29 (2018) 223–233.

[66] W. Zou, Immunosuppressive networks in the tumour environment and their therapeutic relevance, Nat. Rev. Cancer 5 (2005) 263–274.

[67] T.F. Gajewski, Y. Meng, C. Blank, I. Brown, A. Kacha, J. Kline, H. Harlin, Immune resistance orchestrated by the tumor microenvironment, Immunol. Rev. 213 (2006) 131–145.

[68] L. Zitvogel, A. Tesniere, G. Kroemer, Cancer despite immunosurveillance: immunoselection and immunosubversion, Nat. Rev. Immunol. 6 (2006) 715–727.

[69] G.P. Dunn, A.T. Bruce, H. Ikeda, L.J. Old, R.D. Schreiber, Cancer immunoediting: from immunosurveillance to tumor escape, Nat. Immunol. 3 (2002) 991–998.

[70] J. Ren, X. Liu, C. Fang, S. Jiang, C.H. June, Y. Zhao, Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition, Clin. Cancer Res. 23 (2017) 2255–2266.

[71] K. Weiskopf, N.S. Jahchan, P.J. Schnorr, S. Cristea, A.M. Ring, R.L. Maute, A.K. Volkmer, J.P. Volkmer, J. Liu, J.S. Lim, D. Yang, G. Seitz, T. Nguyen, D. Wu, K. Jude, H. Guerston, A. Barkal, F. Trapani, J. George, J.T. Poirier, E.E. Gardner, L.A. Miles, E. de Stanchina, S.M. Lofgren, H. Vogel, M.M. Winslow, C. Dive, R.K. Thomas, C.M. Rudin, M. van de Rijn, R. Majeti, K.C. Garcia, I.L. Weissman, J. Sage, CD47-blocking immunotherapies stimulate macrophage-mediated destruction of small-cell lung cancer, The Journal of Clinical Investigation 126 (2016) 2610–2620.

[72] M.K. White, J.S. Pagano, K. Khalili, Viruses and human cancers: a long road of discovery of molecular paradigms, Clin. Microbiol. Rev. 27 (2014) 463–481.

[73] K.S. Yuen, C.P. Chan, N.H. Wong, C.H. Ho, T.H. Ho, T. Lei, W. Deng, S.W. Tsao, H. Chen, K.H. Kok, D.Y. Jin, CRISPR/Cas9-mediated genome editing of Epstein-Barr virus in human cells, The Journal of General Virology 96 (2015) 626–636.

[74] E.M. Kennedy, A.V. Kornepati, M. Goldstein, H.P. Bogerd, B.C. Poling, A.W. Whisnant, M.B. Kastan, B.R. Cullen, Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease, J. Virol. 88 (2014) 11965–11972.

[75] S.B. Wedam, J.A. Low, S.X. Yang, C.K. Chow, P. Choyke, D. Danforth, S.M. Hewitt, A. Berman, S.M. Steinberg, D.J. Liewehr, J. Plehn, A. Doshi, D. Thomasson, N. McCarthy, H. Koeppen, M. Sherman, J. Zujewski, K. Camphausen, H. Chen, S.M. Swain, Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer, J. Clin. Oncol. 24 (2006) 769–777.

[76] C. Liang, F. Li, L. Wang, Z.K. Zhang, C. Wang, B. He, J. Li, Z. Chen, A.B. Shaikh, J. Liu, X. Wu, S. Peng, L. Dang, B. Guo, X. He, D.W.T. Au, C. Lu, H. Zhu, B.T. Zhang, A. Lu, G. Zhang, Tumor cell-targeted delivery of CRISPR/Cas9 by aptamer-functionalized lipopolymer for therapeutic genome editing of VEGFA in osteosarcoma, Biomaterials 147 (2017) 68–85.

[77] T.J. Pugh, O. Morozova, E.F. Attiyeh, S. Asgharzadeh, J.S. Wei, D. Auclair, S.L. Carter, K. Cibulskis, M. Hanna, A. Kiezun, J. Kim, M.S. Lawrence, L. Lichenstein, A. McKenna, C.S. Pedamallu, A.H. Ramos, E. Shefler, A. Sivachenko, C. Sougnez, C. Stewart, A. Ally, I. Birol, R. Chiu, R.D. Corbett, M. Hirst, S.D. Jackman, B. Kamoh, A.H. Khodabakshi, M. Krzywinski, A. Lo, R.A. Moore, K.L. Mungall, J. Qian, A. Tam, N. Thiessen, Y. Zhao, K.A. Cole, M. Diamond, S.J. Diskin, Y.P. Mosse, A.C. Wood, L. Ji, R. Sposto, T. Badgett, W.B. London, Y. Moyer, J.M. Gastier-Foster, M.A. Smith, J.M. Guidry Auvil, D.S. Gerhard, M.D. Hogarty, S.J. Jones, E.S. Lander, S.B. Gabriel, G. Getz, R.C. Seeger, J. Khan, M.A. Marra, M. Meyerson, J.M. Maris, The genetic landscape of high-risk neuroblastoma, Nature Genetics 45 (2013) 279–284.

[78] C. Love, Z. Sun, D. Jima, G. Li, J. Zhang, R. Miles, K.L. Richards, C.H. Dunphy, W.W. Choi, G. Srivastava, P.L. Lugar, D.A. Rizzieri, A.S. Lagoo, L. Bernal-Mizrachi, K.P. Mann, C.R. Flowers, K.N. Naresh, A.M. Evens, A. Chadburn, L.I. Gordon, M.B. Czader, J.I. Gill, E.D. Hsi, A. Greenough, A.B. Moffitt, M. McKinney, A. Banerjee, V. Grubor, S. Levy, D.B. Dunson, S.S. Dave, The genetic landscape of mutations in Burkitt lymphoma, Nat. Genet. 44 (2012) 1321–1325.

[79] Y.B. Gao, Z.L. Chen, J.G. Li, X.D. Hu, X.J. Shi, Z.M. Sun, F. Zhang, Z.R. Zhao, Z.T. Li, Z.Y. Liu, Y.D. Zhao, J. Sun, C.C. Zhou, R. Yao, S.Y. Wang, P. Wang, N. Sun, B.H. Zhang, J.S.

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Dong, Y. Yu, M. Luo, X.L. Feng, S.S. Shi, F. Zhou, F.W. Tan, B. Qiu, N. Li, K. Shao, L.J. Zhang, L.J. Zhang, Q. Xue, S.G. Gao, J. He, Genetic landscape of esophageal squamous cell carcinoma, Nat. Genet. 46 (2014) 1097–1102.

[80] H.X. Wang, M. Li, C.M. Lee, S. Chakraborty, H.W. Kim, G. Bao, K.W. Leong, CRISPR/Cas9-based genome editing for disease modeling and therapy: challenges and opportunities for nonviral delivery, Chem. Rev. 117 (2017) 9874–9906.

[81] M.A. Kotterman, T.W. Chalberg, D.V. Schaffer, Viral vectors for gene therapy: translational and clinical outlook, Annu. Rev. Biomed. Eng. 17 (2015) 63–89.

[82] C.L. Xu, M.Z.C. Ruan, V.B. Mahajan, S.H. Tsang, Viral delivery systems for CRISPR, Viruses 11 (2019) 28.

[83] T. W, D. N, C.B. Wu, F.J. Xu, G. Church, Y. P, Material solutions for delivery of CRISPR/Cas-based genome editing tools: Current status and future outlook, Materials Today 26 (2019) 40–66.

[84] H. Yin, C.Q. Song, S. Suresh, S.Y. Kwan, Q. Wu, S. Walsh, J. Ding, R.L. Bogorad, L.J. Zhu, S.A. Wolfe, V. Koteliansky, W. Xue, R. Langer, D.G. Anderson, Partial DNA-guided Cas9 enables genome editing with reduced off-target activity, Nat. Chem. Biol. 14 (2018) 311–316.

[85] X. Han, Z. Liu, M.C. Jo, K. Zhang, Y. Li, Z. Zeng, N. Li, Y. Zu, L. Qin, CRISPR-Cas9 delivery to hard-to-transfect cells *via* membrane deformation, Sci. Adv. 1 (2015), e1500454.

[86] B.P. Kleinstiver, M.S. Prew, S.Q. Tsai, N.T. Nguyen, V.V. Topkar, Z. Zheng, J.K. Joung, Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition, Nat. Biotechnol. 33 (2015) 1293–1298.

[87] F. Chen, X. Ding, Y. Feng, T. Seebeck, Y. Jiang, G.D. Davis, Targeted activation of diverse CRISPR-Cas systems for mammalian genome editing *via* proximal CRISPR targeting, Nat. Commun. 8 (2017) 14958.

[88] B.P. Kleinstiver, M.S. Prew, S.Q. Tsai, V.V. Topkar, N.T. Nguyen, Z. Zheng, A.P. Gonzales, Z. Li, R.T. Peterson, J.R. Yeh, M.J. Aryee, J.K. Joung, Engineered CRISPR-Cas9 nucleases with altered PAM specificities, Nature 523 (2015) 481–485.

[89] J.S. Chen, Y.S. Dagdas, B.P. Kleinstiver, M.M. Welch, A.A. Sousa, L.B. Harrington, S.H. Sternberg, J.K. Joung, A. Yildiz, J.A. Doudna, Enhanced proofreading governs CRISPR-Cas9 targeting accuracy, Nature 550 (2017) 407–410.

[90] Y. Feng, S. Sassi, J.K. Shen, X. Yang, Y. Gao, E. Osaka, J. Zhang, S. Yang, C. Yang, H.J. Mankin, F.J. Hornicek, Z. Duan, Targeting CDK11 in osteosarcoma cells using the CRISPR-Cas9 system, J. Orthop. Res. 33 (2015) 199–207.

[91] S. Valletta, H. Dolatshad, M. Bartenstein, B.H. Yip, E. Bello, S. Gordon, Y. Yu, J. Shaw, S. Roy, L. Scifo, A. Schuh, A. Pellagatti, T.A. Fulga, A. Verma, J. Boultwood, ASXL1 mutation correction by CRISPR/Cas9 restores gene function in leukemia cells and increases survival in mouse xenografts, Oncotarget 6 (2015) 44061–44071.

[92] S.W. Cho, S. Kim, Y. Kim, J. Kweon, H.S. Kim, S. Bae, J.S. Kim, Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases, Genome Res. 24 (2014) 132–141.

[93] Z.Y. He, K. Men, Z. Qin, Y. Yang, T. Xu, Y.Q. Wei, Non-viral and viral delivery systems for CRISPR-Cas9 technology in the biomedical field, Sci. China Life Sci. 60 (2017) 458–467.

[94] B.M. Hansen, B.E. de Ávila, G.M. Beltrán, J. Zhao, D.E. Ramírez-Herrera, P. Angsantikul, G.K. Vesterager, L. Zhang, J. Wang, Active intracellular delivery of a Cas9/sgRNA complex using ultrasound-propelled nanomotors, Angew. Chem. 57 (2018) 2657–2661.

[95] J.W. Sessions, C.S. Skousen, K.D. Price, B.W. Hanks, S. Hope, J.K. Alder, B.D. Jensen, CRISPR-Cas9 directed knock-out of a constitutively expressed gene using lance array nanoinjection, SpringerPlus 5 (2016) 1521.

[96] J.W. Sessions, T.E. Lewis, C.S. Skousen, S. Hope, B.D. Jensen, The effect of injection speed and serial injection on propidium iodide entry into cultured HeLa and primary neonatal fibroblast cells using lance array nanoinjection, SpringerPlus 5 (2016) 1093.

[97] Z.K. Lindstrom, S.J. Brewer, M.A. Ferguson, S.H. Burnett, B.D. Jensen, Injection of propidium iodide into HeLa cells using a silicon nanoinjection lance array, Journal of Nanotechnology in Engineering and Medicine 5 (2014), 021008.

[98] T. Yoshiba, Y. Saga, M. Urabe, R. Uchibori, S. Matsubara, H. Fujiwara, H. Mizukami, CRISPR/Cas9-mediated cervical cancer treatment targeting human papillomavirus E6, Oncol. Lett. 17 (2019) 2197–2206.

[99] L. Tao, J. Zhang, P. Meraner, A. Tovaglieri, X. Wu, R. Gerhard, X. Zhang, W.B. Stallcup, J. Miao, X. He, J.G. Hurdle, D.T. Breault, A.L. Brass, M. Dong, Frizzled proteins are colonic epithelial receptors for C. difficile toxin B, Nature 538 (2016) 350–355.

[100] B.J. Aubrey, G.L. Kelly, A.J. Kueh, M.S. Brennan, L. O'Connor, L. Milla, S. Wilcox, L. Tai, A. Strasser, M.J. Herold, An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo, Cell Rep. 10 (2015) 1422–1432.

[101] L.B. Ryo, E.A. Thomsen, J.G. Mikkelsen, Production and validation of lentiviral vectors for CRISPR/Cas9 delivery, Methods in Molecular Biology 1961 (2019) 93–109.

[102] Z. Hu, L. Yu, D. Zhu, W. Ding, X. Wang, C. Zhang, L. Wang, X. Jiang, H. Shen, D. He, K. Li, L. Xi, D. Ma, H. Wang, Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells, BioMed Research International 2014 (2014) 612823.

[103] J.A. Zuris, D.B. Thompson, Y. Shu, J.P. Guilinger, J.L. Bessen, J.H. Hu, M.L. Maeder, J.K. Joung, Z.Y. Chen, D.R. Liu, Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo, Nature Biotechnology 33 (2015) 73–80.

[104] J.A. Kretzmann, D. Ho, C.W. Evans, J.H.C. Plani-Lam, B. Garcia-Bloj, A.E. Mohamed, M.L. O'Mara, E. Ford, D.E.K. Tan, R. Lister, P. Blancafort, M. Norret, K.S. Iyer, Synthetically controlling dendrimer flexibility improves delivery of large plasmid DNA, Chemical Science 8 (2017) 2923–2930.

[105] Z. Zhang, T. Wan, Y. Chen, Y. Chen, H. Sun, T. Cao, Z. Songyang, G. Tang, C. Wu, Y. Ping, F.J. Xu, J. Huang, Cationic polymer-mediated CRISPR/Cas9 plasmid delivery for genome editing, Macromolecular Rapid Communications 40 (2019), e1800068.

[106] Z. Yan, H. Zou, F. Tian, J.R. Grandis, A.J. Mixson, P.Y. Lu, L.Y. Li, Human rhomboid family-1 gene silencing causes apoptosis or autophagy to epithelial cancer cells and inhibits xenograft tumor growth, Molecular Cancer Therapeutics 7 (2008) 1355–1364.

[107] X.Y. He, B.Y. Liu, Y. Peng, R.X. Zhuo, S.X. Cheng, Multifunctional vector for delivery of genome editing plasmid targeting beta-catenin to remodulate cancer cell properties, ACS Applied Materials & Interfaces 11 (2019) 226–237.

[108] E. Ju, T. Li, S. Ramos da Silva, S.J. Gao, Gold nanocluster-mediated efficient delivery of Cas9 protein through pH-induced assembly-disassembly for inactivation of virus oncogenes, ACS Applied Materials & Interfaces 11 (2019) 34717–34724.

[109] R. Mout, M. Ray, G. Yesilbag Tonga, Y.W. Lee, T. Tay, K. Sasaki, V.M. Rotello, Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing, ACS Nano 11 (2017) 2452–2458.

[110] H. Yue, X. Zhou, M. Cheng, D. Xing, Graphene oxide-mediated Cas9/sgRNA delivery for efficient genome editing, Nanoscale 10 (2018) 1063–1071.

[111] R. Barrangou, J.A. Doudna, Applications of CRISPR technologies in research and beyond, Nature Biotechnology 34 (2016) 933–941.

[112] S. Su, B. Hu, J. Shao, B. Shen, J. Du, Y. Du, J. Zhou, L. Yu, L. Zhang, F. Chen, H. Sha, L. Cheng, F. Meng, Z. Zou, X. Huang, B. Liu, CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients, Scientific Reports 6 (2016) 20070.

[113] K. Schumann, S. Lin, E. Boyer, D.R. Simeonov, M. Subramaniam, R.E. Gate, G.E. Haliburton, C.J. Ye, J.A. Bluestone, J.A. Doudna, A. Marson, Generation of knock-in primary human T cells using Cas9 ribonucleoproteins, Proceedings of the National Academy of Sciences of the United States of America 112 (2015) 10437–10442.

[114] X. Liu, Y. Zhang, C. Cheng, A.W. Cheng, X. Zhang, N. Li, C. Xia, X. Wei, X. Liu, H. Wang, CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells, Cell Research 27 (2017) 154–157.

[115] X. Dai, J.J. Park, Y. Du, H.R. Kim, G. Wang, Y. Errami, S. Chen, One-step generation of modular CAR-T cells with AAV-Cpf1, Nature Methods 16 (2019) 247–254.

[116] K.L. Hung, I. Meitlis, M. Hale, C.Y. Chen, S. Singh, S.W. Jackson, C.H. Miao, I.F. Khan, D.J. Rawlings, R.G. James, Engineering protein-secreting plasma cells by homology-directed repair in primary human B cells, Molecular Therapy 26 (2018) 456–467.

[117] M. Naeimi Kararoudi, H. Dolatshad, P. Trikha, S.A. Hussain, E. Elmas, J.A. Foltz, J.E. Moseman, A. Thakkar, R.J. Nakkula, M. Lamb, N. Chakravarti, K.J. McLaughlin, D.A. Lee, Generation of knock-out primary and expanded human NK cells using Cas9 ribonucleoproteins, Journal of Visualized Experiments : JoVE (2018) e58237.

[118] X. Huang, G. Zhou, W. Wu, Y. Duan, G. Ma, J. Song, R. Xiao, L. Vandenberghe, F. Zhang, P.A. D'Amore, H. Lei, Genome editing abrogates angiogenesis in vivo, Nature Communications 8 (2017) 112.

[119] W. Wu, Y. Duan, G. Ma, G. Zhou, C. Park-Windhol, P.A. D'Amore, H. Lei, AAV-CRISPR/Cas9-mediated depletion of VEGFR2 blocks angiogenesis in vitro, Investigative Ophthalmology & Visual Science 58 (2017) 6082–6090.

[120] H.W. Chu, C. Rios, C. Huang, A. Wesolowska-Andersen, E.G. Burchard, B.P. O'Connor, T.E. Fingerlin, D. Nichols, S.D. Reynolds, M.A. Seibold, CRISPR-Cas9-mediated gene knockout in primary human airway epithelial cells reveals a proinflammatory role for MUC18, Gene Therapy 22 (2015) 822–829.

[121] J.G. Choi, Y. Dang, S. Abraham, H. Ma, J. Zhang, H. Guo, Y. Cai, J.G. Mikkelsen, H. Wu, P. Shankar, N. Manjunath, Lentivirus pre-packed with Cas9 protein for safer gene editing, Gene Therapy 23 (2016) 627–633.

[122] S. Jiang, L.W. Wang, M.J. Walsh, S.J. Trudeau, C. Gerdt, B. Zhao, B.E. Gewurz, CRISPR/Cas9-mediated genome editing in Epstein-Barr virus-transformed lymphoblastoid B-cell lines, Current Protocols in Molecular Biology 121 (2018)31 12 31-31 12 23.

[123] A. Park, P. Hong, S.T. Won, P.A. Thibault, F. Vigant, K.Y. Oguntuyo, J.D. Taft, B. Lee, Sendai virus, an RNA virus with no risk of genomic integration, delivers CRISPR/Cas9 for efficient gene editing, Molecular Therapy. Methods & Clinical Development 3 (2016) 16057.

[124] M. Mansouri, Z. Ehsaei, V. Taylor, P. Berger, Baculovirus-based genome editing in primary cells, Plasmid 90 (2017) 5–9.

[125] Y.K. Ho, L.H. Zhou, K.C. Tam, H.P. Too, Enhanced non-viral gene delivery by coordinated endosomal release and inhibition of beta-tubulin deactylase, Nucleic acids research 45 (2017), e38.

[126] D.S. D'Astolfo, R.J. Pagliero, A. Pras, W.R. Karthaus, H. Clevers, V. Prasad, R.J. Lebbink, H. Rehmann, N. Geijsen, Efficient intracellular delivery of native proteins, Cell 161 (2015) 674–690.

[127] S. Zhen, L. Hua, Y.H. Liu, L.C. Gao, J. Fu, D.Y. Wan, L.H. Dong, H.F. Song, X. Gao, Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus, Gene Therapy 22 (2015) 404–412.

[128] M. Zuckermann, V. Hovestadt, C.B. Knobbe-Thomsen, M. Zapatka, P.A. Northcott, K. Schramm, J. Belic, D.T. Jones, B. Tschida, B. Moriarity, D. Largaespada, M.F. Roussel, A. Korshunov, G. Reifenberger, S.M. Pfister, P. Lichter, D. Kawauchi, J. Gronych, Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling, Nature Communications 6 (2015) 7391.

[129] W. Zhu, K. Xie, Y. Xu, L. Wang, K. Chen, L. Zhang, J. Fang, CRISPR/Cas9 produces anti-hepatitis B virus effect in hepatoma cells and transgenic mouse, Virus Research 217 (2016) 125–132.

[130] N. Habib, R. Havlik, L. Jiao, A. Kiri, S. Jensen, J. Nicholls, Combination of Optison™ with ultrasound and electroporation increases albumin and thrompoietin transgene expression whilst elongation factor promoter prolongs its duration, Gene Therapy and Molecular Biology 8 (2004) 1–7.

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[131] Y. Shinmyo, S. Tanaka, S. Tsunoda, K. Hosomichi, A. Tajima, H. Kawasaki, CRISPR/Cas9-mediated gene knockout in the mouse brain using in utero electroporation, Scientific Reports 6 (2016) 20611.
[132] M.C. Latella, M.T. Di Salvo, F. Cocchiarella, D. Benati, G. Grisendi, A. Comitato, V. Marigo, A. Recchia, In vivo editing of the human mutant rhodopsin gene by electroporation of plasmid-based CRISPR/Cas9 in the mouse retina, Molecular Therapy. Nucleic Acids 5 (2016), e389.
[133] L. Xu, K.H. Park, L. Zhao, J. Xu, M. El Refaey, Y. Gao, H. Zhu, J. Ma, R. Han, CRISPR-mediated genome editing restores dystrophin expression and function in mdx mice, Molecular Therapy 24 (2016) 564–569.
[134] W. Wu, Z. Lu, F. Li, W. Wang, N. Qian, J. Duan, Y. Zhang, F. Wang, T. Chen, Efficient in vivo gene editing using ribonucleoproteins in skin stem cells of recessive dystrophic epidermolysis bullosa mouse model, Proceedings of the National Academy of Sciences of the United States of America 114 (2017) 1660–1665.
[135] J.F. Fei, D. Knapp, M. Schuez, P. Murawala, Y. Zou, S. Pal Singh, D. Drechsel, E.M. Tanaka, Tissue- and time-directed electroporation of CAS9 protein-gRNA complexes in vivo yields efficient multigene knockout for studying gene function in regeneration, NPJ Regenerative Medicine 1 (2016) 16002.
[136] T. Matsuda, I. Oinuma, Optimized CRISPR/Cas9-mediated in vivo genome engineering applicable to monitoring dynamics of endogenous proteins in the mouse neural tissues, Scientific Reports 9 (2019) 11309.
[137] Q. Liu, D. Fan, D. Adah, Z. Wu, R. Liu, Q.T. Yan, Y. Zhang, Z.Y. Du, D. Wang, Y. Li, S.Y. Bao, L.P. Liu, CRISPR/Cas9mediated hypoxia inducible factor1alpha knockout enhances the antitumor effect of transarterial embolization in hepatocellular carcinoma, Oncology Reports 40 (2018) 2547–2557.
[138] W. Kim, S. Lee, H.S. Kim, M. Song, Y.H. Cha, Y.H. Kim, J. Shin, E.S. Lee, Y. Joo, J.J. Song, E.J. Choi, J.W. Choi, J. Lee, M. Kang, J.I. Yook, M.G. Lee, Y.S. Kim, S. Paik, H.H. Kim, Targeting mutant KRAS with CRISPR-Cas9 controls tumor growth, Genome Research 28 (2018)374-328.
[139] T. Koo, A.R. Yoon, H.Y. Cho, S. Bae, C.O. Yun, J.S. Kim, Selective disruption of an oncogenic mutant allele by CRISPR/Cas9 induces efficient tumor regression, Nucleic Acids Research 45 (2017) 7897–7908.
[140] T.J. Wickham, Targeting adenovirus, Gene therapy 7 (2000) 110–114.
[141] N. Koizumi, H. Mizuguchi, N. Utoguchi, Y. Watanabe, T. Hayakawa, Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob, The Journal of Gene Medicine 5 (2003) 267–276.
[142] K. Takayama, H. Mizuguchi, Generation of Optogenetically Modified Adenovirus Vector for Spatiotemporally Controllable Gene Therapy, ACS chemical biology 13 (2018) 449–454.
[143] F. Abarzua, M. Sakaguchi, M. Takaishi, Y. Nasu, K. Kurose, S. Ebara, M. Miyazaki, M. Namba, H. Kumon, N.H. Huh, Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase, Cancer Research 65 (2005) 9617–9622.
[144] H. Kumon, Y. Ariyoshi, K. Sasaki, T. Sadahira, M. Araki, S. Ebara, H. Yanai, M. Watanabe, Y. Nasu, Adenovirus vector carrying REIC/DKK-3 gene: neoadjuvant intraprostatic injection for high-risk localized prostate cancer undergoing radical prostatectomy, Cancer Gene Therapy 23 (2016) 400–409.
[145] S. Tong, B. Moyo, C.M. Lee, K. Leong, G. Bao, Engineered materials for in vivo delivery of genome-editing machinery, Nature Reviews Materials 4 (2019) 726–737.
[146] Y.H. Lao, M. Li, M.A. Gao, D. Shao, C.W. Chi, D. Huang, S. Chakraborty, T.C. Ho, W. Jiang, H.X. Wang, S. Wang, K.W. Leong, HPV oncogene manipulation using nonvirally delivered CRISPR/Cas9 or natronobacterium gregoryi argonaute, Advanced Science 5 (2018) 1700540.
[147] L. Jubair, S. Fallaha, N.A.J. McMillan, Systemic delivery of CRISPR/Cas9 targeting HPV oncogenes is effective at eliminating established tumors, Molecular Therapy 27 (2019) 2091–2099.
[148] L.M. Zhang, P. Wang, Q. Feng, N.X. Wang, Z.T. Chen, Y.Y. Huang, W.F. Zheng, X.Y. Jiang, Lipid nanoparticle-mediated efficient delivery of CRISPR/Cas9 for tumor therapy, NPG Asia Materials 9 (2017), e441.
[149] W. Sun, W. Ji, J.M. Hall, Q. Hu, C. Wang, C.L. Beisel, Z. Gu, Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing, Angewandte Chemie 54 (2015) 12029–12033.
[150] W. Zhou, H. Cui, L. Ying, X.F. Yu, Enhanced cytosolic delivery and release of CRISPR/Cas9 by black phosphorus nanosheets for genome editing, Angewandte Chemie 57 (2018) 10268–10272.
[151] Y. Qi, H. Song, H. Xiao, G. Cheng, B. Yu, F.J. Xu, Fluorinated acid-labile branched hydroxyl-rich nanosystems for flexible and robust delivery of plasmids, Small 14 (2018), e1803061.
[152] S.M. Kim, Y. Yang, S.J. Oh, Y. Hong, M. Seo, M. Jang, Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting, Journal of Controlled Release 266 (2017) 8–16.
[153] S.M. Kim, S.C. Shin, E.E. Kim, S.H. Kim, K. Park, S.J. Oh, M. Jang, Simple in vivo gene editing *via* direct self-assembly of Cas9 ribonucleoprotein complexes for cancer treatment, ACS Nano 12 (2018) 7750–7760.
[154] P. Wang, L. Zhang, Y. Xie, N. Wang, R. Tang, W. Zheng, X. Jiang, Genome Editing for Cancer Therapy: Delivery of Cas9 protein/sgRNA plasmid *via* a gold nanocluster/lipid core-shell nanocarrier, Advanced Science 4 (2017) 1700175.
[155] H.X. Wang, Z. Song, Y.H. Lao, X. Xu, J. Gong, D. Cheng, S. Chakraborty, J.S. Park, M. Li, D. Huang, L. Yin, J. Cheng, K.W. Leong, Nonviral gene editing *via* CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide, Proceedings of the National Academy of Sciences of the United States of America 115 (2018) 4903–4908.
[156] Q. Liu, K. Zhao, C. Wang, Z. Zhang, C. Zheng, Y. Zhao, Y. Zheng, C. Liu, Y. An, L. Shi, C. Kang, Y. Liu, Multistage delivery nanoparticle facilitates efficient CRISPR/dCas9 activation and tumor growth suppression in vivo, Advanced Science 6 (2019) 1801423.
[157] P. Guo, J. Yang, J. Huang, D.T. Auguste, M.A. Moses, Therapeutic genome editing of triple-negative breast tumors using a noncationic and deformable nanolipogel, Proceedings of the National Academy of Sciences of the United States of America 116 (2019) 18295–18303.
[158] M. Li, H. Xie, Y. Liu, C. Xia, X. Cun, Y. Long, X. Chen, M. Deng, R. Guo, Z. Zhang, Q. He, Knockdown of hypoxia-inducible factor-1 alpha by tumor targeted delivery of CRISPR/Cas9 system suppressed the metastasis of pancreatic cancer, Journal of Controlled Release 304 (2019) 204–215.
[159] Z. Chen, F. Liu, Y. Chen, J. Liu, X. Wang, A.T. Chen, G. Deng, H. Zhang, J. Liu, Z. Hong, J. Zhou, Targeted delivery of CRISPR/Cas9-mediated cancer gene therapy *via* liposome-templated hydrogel nanoparticles, Advanced Functional Materials (2017) 27.
[160] P. Wang, L. Zhang, W. Zheng, L. Cong, Z. Guo, Y. Xie, L. Wang, R. Tang, Q. Feng, Y. Hamada, K. Gonda, Z. Hu, X. Wu, X. Jiang, Thermo-triggered release of CRISPR-Cas9 system by lipid-encapsulated gold nanoparticles for tumor therapy, Angewandte Chemie 57 (2018) 1491–1496.
[161] L. Li, Z. Yang, S. Zhu, L. He, W. Fan, W. Tang, J. Zou, Z. Shen, M. Zhang, L. Tang, Y. Dai, G. Niu, S. Hu, X. Chen, A rationally designed semiconducting polymer brush for NIR-II imaging-guided light-triggered remote control of CRISPR/Cas9 genome editing, Advanced Materials 31 (2019), e1901187.
[162] Y. Pan, J. Yang, X. Luan, X. Liu, X. Li, J. Yang, T. Huang, L. Sun, Y. Wang, Y. Lin, Y. Song, Near-infrared upconversion-activated CRISPR-Cas9 system: A remote-controlled gene editing platform, Science Advances 5 (2019) eaav7199.
[163] X. Chen, Y. Chen, H. Xin, T. Wan, Y. Ping, Near-infrared optogenetic engineering of photothermal nanoCRISPR for programmable genome editing, Proceedings of the National Academy of Sciences of the United States of America 117 (2020) 2395–2405.
[164] Y. Liu, G. Zhao, C.F. Xu, Y.L. Luo, Z.D. Lu, J. Wang, Systemic delivery of CRISPR/Cas9 with PEG-PLGA nanoparticles for chronic myeloid leukemia targeted therapy, Biomaterials Science 6 (2018) 1592–1603.
[165] F. Eckerdt, J. Yuan, K. Strebhardt, Polo-like kinases and oncogenesis, Oncogene 24 (2005) 267–276.
[166] A.K. Varkouhi, M. Scholte, G. Storm, H.J. Haisma, Endosomal escape pathways for delivery of biologicals, Journal of Controlled Release 151 (2011) 220–228.
[167] S.L.N. Maas, X.O. Breakefield, A.M. Weaver, Extracellular vesicles: Unique intercellular delivery vehicles, Trends in Cell Biology 27 (2017) 172–188.
[168] E.L. Ai, S.I. Mager, X.O. Breakefield, M.J. Wood, Extracellular vesicles: biology and emerging therapeutic opportunities, Nature Reviews Drug Discovery 12 (2013) 347–357.
[169] Q. Wang, J. Yu, T. Kadungure, J. Beyene, H. Zhang, Q. Lu, ARMMs as a versatile platform for intracellular delivery of macromolecules, Nature Communications 9 (2018) 960.
[170] S. Li, Inhibition of poly(ADP-ribose) polymerase in BRCA mutation carriers, The New England Journal of Medicine 361 (2009) 1707.
[171] B. Pothuri, BRCA1- and BRCA2-related mutations: therapeutic implications in ovarian cancer, Annals of Oncology 24 (Suppl. 8) (2013) viii22–viii27.
[172] A. Komin, L.M. Russell, K.A. Hristova, P.C. Searson, Peptide-based strategies for enhanced cell uptake, transcellular transport, and circulation: Mechanisms and challenges, Advanced Drug Delivery Reviews 110–111 (2017) 52–64.
[173] S. Ramakrishna, A.B. Kwaku Dad, J. Beloor, R. Gopalappa, S.K. Lee, H. Kim, Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA, Genome Research 24 (2014) 1020–1027.
[174] J.L. Singulani, L. Scorzoni, N.M.S. Lourencetti, L.R. Oliveira, R.S. Concolaro, P.B. da Silva, A.C. Nazare, C.R. Polaquini, F.D. Victorelli, M. Chorilli, L.O. Regasini, A.M. Fusco Almeida, M.J.S. Mendes Giannini, Potential of the association of dodecyl gallate with nanostructured lipid system as a treatment for paracoccidioidomycosis: In vitro and in vivo efficacy and toxicity, International Journal of Pharmaceutics 547 (2018) 630–636.
[175] Y.J. Park, L.C. Chang, J.F. Liang, C. Moon, C.P. Chung, V.C. Yang, Nontoxic membrane translocation peptide from protamine, low molecular weight protamine (LMWP), for enhanced intracellular protein delivery: in vitro and in vivo study, FASEB Journal 19 (2005) 1555–1557.
[176] S.P. Borderud, Y. Li, J.E. Burkhalter, C.E. Sheffer, J.S. Ostroff, Electronic cigarette use among patients with cancer: Characteristics of electronic cigarette users and their smoking cessation outcomes, Cancer 121 (2015) 800.
[177] P. Guo, J. Huang, L. Wang, D. Jia, J. Yang, D.A. Dillon, D. Zurakowski, H. Mao, M.A. Moses, D.T. Auguste, ICAM-1 as a molecular target for triple negative breast cancer, Proceedings of the National Academy of Sciences of the United States of America 111 (2014) 14710–14715.
[178] Y. Liu, L. Mei, C. Xu, Q. Yu, K. Shi, L. Zhang, Y. Wang, Q. Zhang, H. Gao, Z. Zhang, Q. He, Dual receptor recognizing cell penetrating peptide for selective targeting, efficient intratumoral diffusion and synthesized anti-glioma therapy, Theranostics 6 (2016) 177–191.
[179] Z.R. Cohen, S. Ramishetti, N. Peshes-Yaloz, M. Goldsmith, A. Wohl, Z. Zibly, D. Peer, Localized RNAi therapeutics of chemoresistant grade IV glioma using hyaluronan-grafted lipid-based nanoparticles, ACS Nano 9 (2015) 1581–1591.
[180] J.S. Desgrosellier, D.A. Cheresh, Integrins in cancer: biological implications and therapeutic opportunities, Nature Reviews Cancer 10 (2010) 9–22.
[181] F. Salomone, F. Cardarelli, M. Di Luca, C. Boccardi, R. Nifosi, G. Bardi, L. Di Bari, M. Serresi, F. Beltram, A novel chimeric cell-penetrating peptide with membrane-disruptive properties for efficient endosomal escape, Journal of Controlled Release 163 (2012) 293–303.

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[182] K.N. Sugahara, T. Teesalu, P.P. Karmali, V.R. Kotamraju, L. Agemy, O.M. Girard, D. Hanahan, R.F. Mattrey, E. Ruoslahti, Tissue-penetrating delivery of compounds and nanoparticles into tumors, Cancer Cell 16 (2009) 510–520.

[183] A.J. Carman, J.H. Mills, A. Krenz, D.G. Kim, M.S. Bynoe, Adenosine receptor signaling modulates permeability of the blood-brain barrier, The Journal of Neuroscience 31 (2011) 13272–13280.

[184] L. Han, D.K. Kong, M.Q. Zheng, S. Murikinati, C. Ma, P. Yuan, L. Li, D. Tian, Q. Cai, C. Ye, D. Holden, J.H. Park, X. Gao, J.L. Thomas, J. Grutzendler, R.E. Carson, Y. Huang, J.M. Piepmeier, J. Zhou, Increased nanoparticle delivery to brain tumors by autocatalytic priming for improved treatment and imaging, ACS Nano 10 (2016) 4209–4218.

[185] M.H. Porteus, A new class of medicines through DNA editing, The New England Journal of Medicine 380 (2019) 947–959.

[186] D. Cyranoski, CRISPR gene-editing tested in a person for the first time, Nature 539 (2016) 479.

[187] X. Yang, Applications of CRISPR-Cas9 mediated genome engineering, Military Medical Research 2 (2015) 11.

[188] L. You, R. Tong, M. Li, Y. Liu, J. Xue, Y. Lu, Advancements and obstacles of CRISPR-Cas9 technology in translational research, Molecular Therapy. Methods & Clinical Development 13 (2019) 359–370.

[189] T. Zhan, N. Rindtorff, J. Betge, M.P. Ebert, M. Boutros, CRISPR/Cas9 for cancer research and therapy, Seminars in Cancer Biology 55 (2019) 106–119.

[190] E.A. Stadtmauer, J.A. Fraietta, M.M. Davis, A.D. Cohen, K.L. Weber, E. Lancaster, P.A. Mangan, I. Kulikovskaya, M. Gupta, F. Chen, L. Tian, V.E. Gonzalez, J. Xu, I. Jung, J.J. Melenhorst, G. Plesa, J. Shea, T. Matlawski, A. Cervini, A.L. Gaymon, S. Desjardins, A. Lamontagne, J. Salas-Mckee, A. Fesnak, D.L. Siegel, B.L. Levine, J.K. Jadlowsky, R.M. Young, A. Chew, W. Hwang, E.O. Hexner, B.M. Carreno, C.L. Nobles, F.D. Bushman, K.R. Parker, Y. Qi, A.T. Satpathy, H.Y. Chang, Y. Zhao, S.F. Lacey, C.H. June, CRISPR-engineered T cells in patients with refractory cancer, Science (2020) eaba7365.

[191] J. Eyquem, J. Mansilla-Soto, T. Giavridis, S.J. van der Stegen, M. Hamieh, K.M. Cunanan, A. Odak, M. Gonen, M. Sadelain, Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection, Nature 543 (2017) 113–117.

[192] S.A. Rosenberg, N.P. Restifo, Adoptive cell transfer as personalized immunotherapy for human cancer, Science 348 (2015) 62–68.

[193] T.T. Smith, S.B. Stephan, H.F. Moffett, L.E. McKnight, W. Ji, D. Reiman, E. Bonagofski, M.E. Wohlfahrt, S.P.S. Pillai, M.T. Stephan, In situ programming of leukaemia-specific T cells using synthetic DNA nanocarriers, Nature Nanotechnology 12 (2017) 813–820.

[194] H. Yin, R.L. Kanasty, A.A. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Non-viral vectors for gene-based therapy, Nature Reviews Genetics 15 (2014) 541–555.

[195] L. Milling, Y. Zhang, D.J. Irvine, Delivering safer immunotherapies for cancer, Advanced Drug Delivery Reviews 114 (2017) 79–101.

[196] A. Marabelle, R. Andtbacka, K. Harrington, I. Melero, R. Leidner, T. de Baere, C. Robert, P.A. Ascierto, J.F. Baurain, M. Imperiale, S. Rahimian, D. Tersago, E. Klumper, M. Hendriks, R. Kumar, M. Stern, K. Ohrling, C. Massacesi, I. Tchakov, A. Tse, J.Y. Douillard, J. Tabernero, J. Haanen, J. Brody, Starting the fight in the tumor: expert recommendations for the development of human intratumoral immunotherapy (HIT-IT), Annals of Oncology 29 (2018) 2163–2174.

[197] C.T. Charlesworth, P.S. Deshpande, D.P. Dever, J. Camarena, V.T. Lemgart, M.K. Cromer, C.A. Vakulskas, M.A. Collingwood, L. Zhang, N.M. Bode, M.A. Behlke, B. Dejene, B. Cieniewicz, R. Romano, B.J. Lesch, N. Gomez-Ospina, S. Mantri, M. Pavel-Dinu, K.I. Weinberg, M.H. Porteus, Identification of preexisting adaptive immunity to Cas9 proteins in humans, Nature Medicine 25 (2019) 249–254.

[198] D.L. Wagner, L. Amini, D.J. Wendering, L.M. Burkhardt, L. Akyuz, P. Reinke, H.D. Volk, M. Schmueck-Henneresse, High prevalence of *Streptococcus pyogenes* Cas9-reactive T cells within the adult human population, Nature Medicine 25 (2019) 242–248.

[199] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines - a new era in vaccinology, Nature Reviews Drug Discovery 17 (2018) 261–279.

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