**REVIEW****Applications of CRISPR-Cas System in Tumor Biology**Mengdan Ma<sup>1,2</sup>, Yuchen Liu<sup>1,\*</sup> and Weiren Huang<sup>1,\*</sup><sup>1</sup>Shenzhen Institute of Translational Medicine, Health Science Center, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, 518035, China<sup>2</sup>Shantou University Medical College, Shantou, 515041, China

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**ABSTRACT**

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system, which is an RNA-guided nuclease system, plays an important role in the adaptive immune response of bacteria, and it is a rapidly developing gene editing technology. It has been widely used in a variety of cells, microorganisms, plants, and animals. This technique has helped to overcome the limitations of previous gene editing methods, and it has promoted the development of synthetic biology, genetics, and proteomics. The ability of the CRISPR-Cas system to modify the genetic components of a system has led to various practical applications, such as base editing, transcription regulation, and epigenetic modification. Moreover, the CRISPR-Cas system can accelerate the development of animal tumor models, thereby promoting *in vivo* tumor research. In this review, first, we briefly introduce the CRISPR-Cas gene editing technology, followed by a discussion on how this technology plays a vital role in tumor modeling, researching the mechanism of cancer development, drug delivery system, therapy, and genome-wide screening for novel drug targets. Finally, we analyze the current technical problems of this system and highlight its future prospects in clinical applications. Our aim is to explore the applications of CRISPR in cancer research and treatment by discussing these research advances.

**KEYWORDS**

CRISPR-Cas; tumor modeling construction; mechanism study; delivery system; immunotherapy; screening drug targets

**Nomenclature**

CRISPR-Cas Clustered regularly interspaced short palindromic repeats-associated

ZFN	Zinc-finger nucleases
TALEN	Transcription activator-like effector nucleases
gRNA	Guide RNA
crRNA	CRISPR RNA
RNP	Ribonucleoprotein
tracrRNA	Trans-activating CRISPR RNA
sgRNA	Single guide RNA
PAM	Protospacer adjacent motif
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference



neoCOR	Neoplastic cerebral organoid
P53	Tumor protein 53 gene
PTEN	Phosphatase and tensin homologue gene
RB1	Retinoblastoma 1
NF1	Neurofibromin-1 gene
SCLC	Small cell lung cancer
PC	Prostate cancer
FRMD6	FERM domain-containing protein 6
ATT	Antiangiogenic therapy
GeCKO	Genome-wide CRISPR knockout
dCas9	Dead Cas9
CBE	Cytosine base editor
SHERLOCK	Specific High-Sensitivity Enzymatic Reporter Unlocking
RPA	Recombinant polymerase amplification
TME	Tumor microenvironment
CAR	Chimeric antigen receptor
TCR	T cell receptor
CRS	Cytokine release syndrome
CRES	CAR-T-cell-related encephalopathy syndrome
TanCARs	Tandem CARs
CSC	Cancer stem cell
GBM	Glioblastoma
TCR $\alpha$	T cell receptor $\alpha$
TCR $\beta$	T cell receptor $\beta$
PD-1	Death protein 1
NYCE	NY-ESO-1 transduced CRISPR 3X edited cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
HPK1	Hematopoietic progenitor kinase1
hPSC	Human pluripotent stem cell
ESC	Embryonic stem cell
iPSC	Induced pluripotent stem cell
ITRs/IRs	Inverted end repeat sequences
AAV	Adeno-associated virus
DSB	DNA double-strand break
ABE	Adenine base editor
m6A	RNA N6-methyladenosine
miRNA	MicroRNA
METTL3	Methyltransferase 3
CRC	Colorectal cancer
lncRNA	Long non-coding RNA
GACAT3	Gastric cancer associated transcript 3
CTLs	Cytotoxic T lymphocytes
ATM	Ataxia telangiectasia mutated
PARP	Polyadenosine diphosphate-ribose polymerase
ATR	ATM and Rad3-related
pDNA	Plasmid DNA
LNP	Lipid nanoparticles
HTVI	Hydrodynamic tail-vein injection

NC	Nanocapsule
GSH	Glutathione
RPE	Retinal pigment epithelium
EV	Exosomes
ABP	Aptamer-binding protein
LBP	Lactose derived branch cationic biopolymer
HCC	Hepatocellular carcinoma
iPhos	Ionizable phospholipids
iPLNP	Multi-component lipid nanoparticles
SEND	Selective endogenous encapsidation for cellular delivery
PEG10	Paternally expressed 10
nCas9	Cas9 nickase
NHEJ	Non-homologous end joining

## 1 Introduction

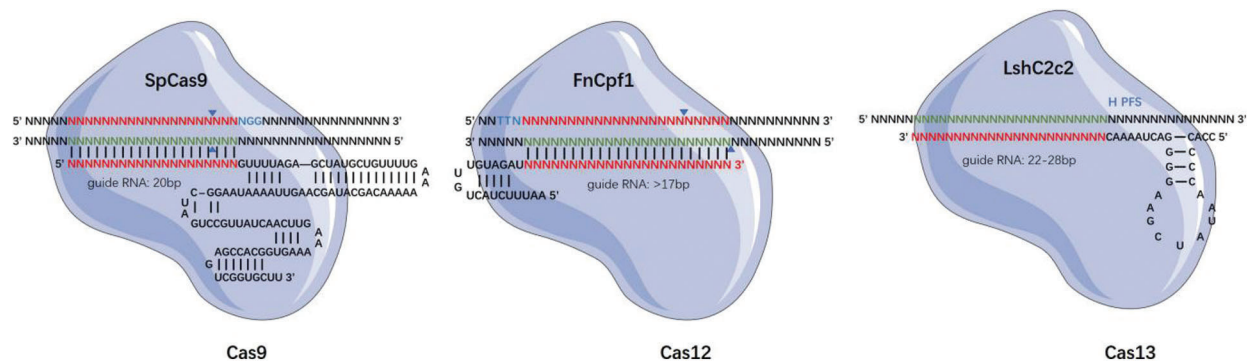
The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system is a precise, RNA-guided gene-editing technique. It has a great potential in the field of basic tumor study and a wide range of clinical applications. In an attempt to solve the existing clinical problems, many studies have used the CRISPR technology to establish cell, organoid, and animal cancer models [1,2] to study the underlying molecular mechanisms of occurrence and development of cancer, to screen drug-resistant strains [3,4], and to conduct genome-wide screening of drug-resistant genes in patients. This technology can screen targeted drugs with a high specificity, thereby improving the effects of gene therapy and immune cell therapy on cancer patients [5–11]. Therefore, the CRISPR technology has undoubtedly revolutionized biotechnology as well as medicine.

Previously, the gene-editing technologies that were widely used included zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The ZFNs are formed by the fusion of the zinc finger DNA-binding domain to the DNA-cutting domain of a nuclease [12]. Similarly, TALENs are obtained by the fusion of the DNA-binding domain of a TAL effector with the DNA cleavage domain of a nuclease. The DNA-binding element of the TALENs consists of a series of TALE subunits [13], and altering this DNA-binding domain alters the specificity of the DNA cutting site. The ZFNs and the TALENs have played an important role in the field of gene editing, but they are associated with problems, such as cytotoxicity, time-consuming design, or low editing efficiency [14]. In contrast to these programmable nuclease enzymes, the CRISPR-Cas system uses an RNA molecule (guide RNA, gRNA) to locate the target DNA sequence, and hence, it functions as an RNA-guided nuclease. The CRISPR-Cas gene array is mainly composed of a leader sequence, followed by short repeating sequences of similar length that are separated by unique interval sequences, and small clusters of cas genes [15]. This CRISPR-Cas system, which is a part of the natural immune system of bacteria, functions in three stages. During the adaptation stage, when exogenous plasmids or phages invade bacteria/archaea, some Cas proteins insert specific gene fragments from exogenous DNA into the original CRISPR sequence, known as the proto-spacer. Next, at the expression stage, CRISPR RNA (crRNA) expression and maturation occur, including transcription of repeat and spacer sequences, and pre-crRNA is formed. The sequence is stored with exogenous genetic information and further cleaved by the CRISPR-Cas system. Each repeating sequence-spacer sequence unit is separately cleaved and processed into crRNA. The crRNA interacts with Cas protein to form an RNA-protein complex. At the interference stage, each crRNA only contains a spacer sequence. When the foreign substance invades again, ribonucleoproteins (RNPs) scan the genetic material. Once the spacer sequence on the crRNA is matched with the original spacer sequence on the foreign invading DNA, Cas

is activated and interferes with the foreign aid DNA, causing it to cleave [15]. The difference in the expression stage is that at the Cas9 loci, there is another sequence preceding Cas that can be transcribed into trans-activating CRISPR RNA (tracrRNA). crRNA is complementary to the tracrRNA sequences, and the two form a crRNA:tracrRNA double-stranded complex, which mediates site-specific DNA recognition and cleavage by Cas9. The crRNA:tracrRNA double-stranded complex was artificially redesigned into a single transcript, such as a single-guide RNA (sgRNA) or guide RNA chimera, and the important characteristics of the crRNA:tracrRNA double-stranded complex were incorporated into it. This could bind to Cas9 and recognize DNA targets [16].

The earliest discovered and the most widely used among the Cas proteins is the Cas9; thereafter, several Cas proteins of different sizes and with different targets and protospacer adjacent motif (PAM) preference sequences have been discovered [17]. The CRISPR-Cas system can be divided into two broad categories depending on whether it is a single- or multi-effector protein complex. The Class 1 system consists of multi-subunit protein-crRNA effector complexes, and it includes three Cas types, namely I, III, and IV [18,19]. On the contrary, the Class 2 system consists of single effector protein complexes, and it includes three Cas types, namely II, V, and VI [20,21]. The Class 2 systems are widely used in gene editing technology, especially type II Cas9, type V Cas12, and type VI Cas13. In addition to targeting DNA, Classes III, VI systems, as well as the modified RCas9 can specifically bind RNA, and Class V systems that can target both DNA and RNA [22].

The targeting specificity of the CRISPR-Cas gene editing system is determined by two conditions, namely the specific binding between the sgRNA and the genomic DNA sequences and the ability of the Cas proteins to specifically recognize specific short DNA sequences on the genomic DNA as PAM sequences (Fig. 1). Hence, by changing the sequence of the gRNA, we can target and edit almost all gene sequences, including DNA and RNA [23]. Concurrently, the development of various Cas derivatives has helped diversify the functions of the CRISPR/Cas system. Some examples are mentioned as follows. Gene editing: gene editing using the base editor, or prime editing for the insertion or deletion of short or long fragments, has been implemented [24–26]. Epigenetic modification: abnormal DNA methylation and its effect on gene expression can cause many complex human diseases, such as cancer [27]. The Cas protein is able to change its genetic expression without altering the DNA sequence by methylation with DNA [28]. At the transcriptional level, CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) can be used to activate or inhibit RNA expression, respectively, from genes [29]. Therefore, CRISPR, with its high editing efficiency, high specificity, and simple design, is of groundbreaking and innovative significance in the field of gene editing.



**Figure 1:** Structure of CRISPR Cas system

Cancer remains the leading cause of disease-related deaths despite rapid advances in diagnosis and treatment. In 2020, there were approximately 19.3 million new cancer cases worldwide, which is more than the 18.1 million cases detected in 2018 [30]. Therefore, it is particularly important to explore the underlying mechanisms of the occurrence, development, and spread of cancer and consequently develop appropriate treatments. The CRISPR-Cas system may be termed as a revolutionary “gene scissors”-based technology, which has brought a new dawn for cancer research and treatment [31]. In this review, we discuss the role of the CRISPR system in basic tumor research and its subsequent applications. The CRISPR-Cas9 system is highly effective in treating invasive cancers, and it is an important step in the search for a cure for cancer. The CRISPR-Cas9 system has been utilized by many researchers to study the characteristics of different cancers, to identify the mechanisms of drug resistance, to carry out research work related to cell death, functional genomics, signaling pathways, drug discovery, drug responses, cell therapy, and even in cancer treatment. The applications of the CRISPR-Cas9 gene editing technology in tumor research are summarized in Table 1.

**Table 1:** Contribution of CRISPR-Cas9 gene editing tool in cancer

Cancer type	Oncogenes	Tumor suppressor genes	Drug resistance genes
Breast cancer	FLI1 [32], miR-23b and miR-27b [33], and ASPH [34]	MEG3 [35]	HER2 [36] and PIK3CA [37]
Lung cancer	CTNND2 [38], SLC2A1 [39], and CDC20 [40]	DHODH [41], GOT1 [42], p107 [43], and miR-1304 [43]	ABCG2 [44] and NRF2 [45]
Colorectal cancer	MUC5AC [46]	STK11 [47], Acvr1b, Acvr2a, and Arid2 [48]	WRN [49] and TRAF5 [50]
Prostatic cancer	HNRNPL [51] and TRAF6 [52]	PGC1 $\alpha$ [53], Lcn2 [54], and FRMD6 [55]	PARP, ATR [56], and AR-V7 [57]
Gastric cancer	ARID1A [58], GMAN [59], and PDEF [60]	SST [61] and XPO1 [62]	ILK [63], CSK, and PTEN [64]
Liver cancer	Ctnnb1 [65]	BAP1 [66]	PHGDH [67]
Cervical cancer	E6 [68], E7 [69], and Trio [70]	WRN [71]	ST6GAL2 [72], Cdc25A, HAUSP [73], and miR-214 [74]
Thyroid cancer	LIMD2 [75], miR-17-92 [76], HDAC1, and HDAC2 [77]	SNHG3 [78]	PPP1R15A and CDK7 [79]
Esophagus cancer	PLCE1 [80] and MAP2K6 [81]	PARK2 [82], TNXB [83], and Notch1 [84]	p53 [85] and miR-432-3p [86]
Bladder cancer	ARID1A, GPRC5A, MLL2 [87], and ARRB1 [88]	STAG2 [89]	MSH2 [90]

## 2 Model Construction

With gene sequencing and whole-genome screening, we can identify the commonly mutated genes in cancer patients. Thereafter, based upon the knowledge of the mutated genes, the CRISPR-Cas technology can be used to simulate the tumor gene mutations via gene knockout, knockin, activation, or inhibition of

the identified genes. This will help us to build relevant cellular or animal models rapidly and accurately for studying the genetic determinants as well as the underlying mechanisms of tumor development.

### **2.1 *In Vitro* Cellular Model Construction**

Cellular models have a short cycle and a clean background, and they provide results easily. Neuroblastoma is a neuroextracranial tumor that most commonly affects children, but it has a poor prognosis. Certain studies indicate that the poor prognosis of neuroblastoma may be associated with mutations in *ATRX*, but it is difficult to confirm due to the lack of available models and preclinical studies. To assess the effect of the *ATRX* loss of function in neuroblastoma, George et al. [91] used the CRISPR-Cas9 technology to generate neuroblastoma cell lines with an *ATRX* mutation via homologous gene-targeting, and they used these cellular models to design innovative approaches that may be used for neuroblastoma treatment.

Organoids are essentially *in vitro* tissue constructs that mimic the patient's tumor; therefore, organoid culture is a powerful tool for studying the characteristics of a cancerous tumor in an *in vitro* culture medium. Organoids reproduce many basic properties of primitive human tissues, including three-dimensional structure, multilineage differentiation, signal nodes, histology, and high-fidelity pathology. Hence, they can be used to construct disease models, personalize tumor therapy, explore cell growth and development mechanisms, and detect efficacy and toxicity [92]. They can grow to about 1 mm in diameter, resemble the patient's tumor in shape, and share several molecular and genetic characteristics. Additionally, they can undergo genetic changes over time; this phenomenon is known as clonal evolution, and it is a major contributor to tumor progression and drug resistance [93]. Therefore, it represents a new method for investigating cancer biology [94]. Brain tumors are one of the deadliest and most devastating cancers, but their studies have been hampered due to genetic heterogeneity and a lack of models. A new model of neoplastic cerebral organoid (neoCOR) was established *in vitro*. In this model, oncogenic mutations were introduced in the organoids by transposon and CRISPR-Cas9 mediated mutations, thus, reproducing the development of brain tumors. The establishment of this model provided a valuable supplement to the basic and preclinical models that are currently used in brain tumor biology research [95]. Furthermore, to explore the causes of breast cancer, Cas9 was used to knockout four breast cancer-related genes, namely the tumor protein 53 gene (*P53*), phosphatase and tensin homologue gene (*PTEN*), Retinoblastoma 1 (*RB1*), and neurofibromin-1 gene (*NFI*), in breast progenitor cells. These mutant breast organoids gained the ability of long-term culture, formed estrogen-receptor positive luminal tumors, and were sensitive to chemotherapeutic drugs [96]. Therefore, the development of these organoid models with tumor-like characteristics provides a novel approach for further research in tumor biology.

### **2.2 *In Vivo* Model Construction**

Animal models can show tumor growth, thereby facilitating the convenient and rapid detection of potential tumor- and metastasis-related genes and providing assistance in the investigation of tumor immune escape, drug resistance, and other mechanisms. Based on these functions, animal models are now considered an important tool for preclinical drug testing. Since mutations and deletions in the tumor suppressor genes *Pten* and *p53* have been found in several human cancers, a Cas9 sgRNA system specifically targeting *PTEN* and *P53* was administered into the liver of mice, thereby leading to the development of mice with tumor-like characteristics [97].

Small cell lung cancer (SCLC) is characterized by rapid disease progression, high recurrence after treatment, poor prognosis, poor treatment effects, and many other problems. Several SCLC-related gene mutations have been identified by sequencing, but their functions remain unclear. Some researchers have used the CRISPR-Cas9 technology to selectively mutate the identified genes in mice and construct SCLC

mouse models; these models helped to explore the gene functions, thereby leading to the discovery of the tumor suppressor gene *P107* [43].

Through RNA sequencing of prostate tissues in prostate cancer (PC) patients and normal individuals, Haldrup et al. [55] found that *FRMD6* (FERM domain-containing protein 6) was abnormally hypermethylated and significantly downregulated in PC; moreover, it was associated with postoperative recurrence of PC. By knocking out or overexpressing *FRMD6* in cells, it was observed that *FRMD6* could inhibit cell proliferation, and the same phenomenon was seen in 3D spheres. Upon subcutaneous injection of the constructed *FRMD6* knockout cell line into mice, they exhibited a faster rate of tumor growth than that seen in the control groups. Therefore, by using these animal models, the signaling pathway of *FRMD6* was determined. Moreover, to study the role of *FRMD6* in a non-tumorigenic environment, an *FRMD6/PTEN* double knockout *in situ* tumor mouse model was constructed. The results showed that the deletion of *FRMD6* might accelerate the formation of tumor and play a driving role in the early stage of PC development. Therefore, *FRMD6* has been identified as a novel tumor suppressor gene and a biomarker candidate in PC prognosis.

The establishment of animal models that can stably maintain the characteristics of tumor cells is conducive to further investigations on the tumor microenvironment and the mechanism underlying gene mutation as well as the development or screening of targeted drugs on this basis.

### 3 Studying the Mechanism of Tumor Development

The CRISPR-Cas9 technology can be used to study tumor genesis mechanisms, particularly the role of single nucleotide mutations, chromosomal ectopic events, and other factors involved in tumor development; additionally, it can be used to screen the functional genes present in tumor cells. These genetic associations correspond to the changes in gene function that occur in tumorigenesis, thereby helping to study the disease mechanism [98]. Numerous studies have shown that in the case of benign tumors, angiogenesis is rare and vascular growth is slow; on the contrary, most malignant tumors exhibit dense angiogenesis and rapid vascular growth [99]. Therefore, angiogenesis plays an important role in tumor development and metastasis, and inhibiting this process will significantly prevent the development, diffusion, and metastasis of tumor tissues. Incidentally, antiangiogenic therapy (ATT) has been successfully used to inhibit abnormal angiogenesis in the clinical treatment of tumors. However, the cancerous tumors often portray resistance to ATT. The CRISPR-Cas9 technology can be used to study the mechanisms of tumor angiogenesis as well as the probable causes of drug resistance [100,101]. The *ARID1A* mutation is one of the most common molecular aberrations in human cancers, but its carcinogenic mechanism is not clear. The *ARID1A* knockout model was established in *TP53*<sup>-/-</sup> human gastric organs. These engineered *ARID1A* deficient organs reflected several clinicopathological features of *ARID1A* mutant gastric cancer. Together with regulatory network-based analysis and high-throughput drug screening, researchers used this human organoid model to discover the potential mechanism of *ARID1A* in gastric epithelial carcinogenesis [58].

Another application of the CRISPR-Cas9 technology is the development of genome-scale CRISPR knockout (GeCKO) libraries, which are capable of rapidly producing loss-of-function mutants in human or mouse cells and subsequently screening for the desired phenotypes. These libraries can be used to identify the genes necessary for cell viability in cancer and pluripotent stem cells as well as to screen for missing genes related to drug resistance of the cancer cells [102].

Many CRISPR derivatives have also played an important role in cancer research. Upon fusion of the dead Cas9 (dCas9) protein with a transcriptional activator or suppressor protein domain and its subsequent association with gRNA sequences, it is possible to target the upstream regulatory regions of genes. This allows the transcription regulatory complex to effectively control the expression level of genes, and then explore their functions. The construction of a genome-wide targeted gRNA library and

the upregulated expression of the whole genome by the cooperative activity of dCas9 and transcriptional activator proteins can be applied to high-throughput and rapid functional acquisition screening. The single-base editor, namely the cytosine base editor (CBE) is used to generate nucleotide mutations of multiple DNA damage response genes, which leads to adaptive changes in the cells when DNA damage occurs to screen and identify the DNA damage responses [103].

#### 4 CRISPR in Cancer Diagnostics

Early diagnosis of cancer can facilitate early detection and treatment of patients, thereby reducing the physical and economic burden of patients. The existing methods of cancer diagnosis have certain limitations with respect to sensitivity, specificity, cost, and time. The ability to rapidly detect nucleic acids in a highly sensitive and single-base-specific manner using a portable platform may help improve disease diagnosis and surveillance, epidemiology, and general laboratory tasks. The Cas13a protein can remain active after cleaving the target RNA and continue to cleave other non-target RNAs. Gootenberg et al. developed a CRISPR-based diagnostic system named SHERLOCK (Specific High-Sensitivity Enzymatic Reporter Unlocking). The system combines Cas13 with recombinant polymerase amplification (RPA), which transcribes amplified DNA into RNA using T7 polymerase, which is then detected by Cas13a. Two human tumor gene hotspot mutations (EGFR L858R and BRAF V600E) were selected. SHERLOCK could distinguish between the normal genotype and the hotspot mutation, and the two human tumor gene mutations could be detected with 0.1% sensitivity [104]. The assay involved the addition of all reagents, including CRISPR-Cas12a and its guide RNA, fluorescent reporter molecules, and the RPA system, in a single reaction. When heated to body temperature, the copy number of the target DNA was rapidly increased via RPA, which made it easier for Cas12a to detect and cleave the target DNA, followed by the arbitrary cleavage of a nearby single-stranded DNA, which induced fluorescence in the reporter molecule. The technique can be used for the instantaneous and easy detection of small quantities of DNA in clinical samples. DETECTR is a technology based on DNA testing that can potentially be used for any type of rapid diagnosis, including the diagnosis of cancer and infectious diseases [105].

#### 5 CRISPR in Immuno-Oncology

Cancer is a genomic disease characterized by an overall genomic instability, a large accumulation of point mutations, and structural changes during tumor progression. These genomic mutations may produce tumor antigens that may be recognized by the immune system as non-autoantigens, thereby triggering cellular immune responses. Therefore, the immune system plays a crucial role in immune surveillance, as immune cells from the adaptive and innate immune systems infiltrate the tumor microenvironment (TME) and help to regulate the tumor progression [106]. Immune cell therapy has shown excellent efficacy in the treatment of cancer, and a variety of immune cell therapies have been successfully tested in clinical trials.

As an emerging gene editing technology, the CRISPR-Cas9 system has been widely used in the field of tumor therapy for targeted knockout of tumor immune checkpoint molecules or for rapid and simple gene editing, which significantly reduce the operational difficulty of tumor immunotherapy and greatly promote the development of research in this field.

##### 5.1 Adoptive Cell Transfer Therapy (ACT)

The ACT is used to isolate immunoactive cells from tumor patients, amplify them, identify their functions *in vitro*, and deliver them back to the patients to directly kill the tumors or to stimulate immune responses that will ultimately kill the tumor cells [107]. The most widely used ACT techniques include the chimeric antigen receptor (CAR) T cell therapy and T cell receptor (TCR) T cell therapy that have been engineered using the CRISPR-Cas9 technology [108,109]. This technique is the most mature and



widely used immune cell therapy that has shown good targeting, tumor destruction, and persistence in both *in vivo* and *in vitro* situations [110].

The CAR-T cell therapy works by recognizing membrane surface antigens, and it is highly effective against hematological tumors [111]. The combination of radiotherapy and CAR-T cell therapy may enhance the therapeutic effects [108]. A number of clinical trials have been conducted or are underway to address the safety, feasibility, and efficacy of CAR-T cell therapy in patients with hematological malignancies or solid tumors. CAR-T cell therapy has been shown to be effective in hematoma owing to the fact that hematoma tumor cells have an ancestral target, CD19 (found only in tumor cells but not in normal cells), which can be used to guide CAR-T cells to detect and destroy cancer cells in the treatment of hematological malignancies. CAR-T cell therapy is an important breakthrough in the treatment of leukemia and lymphoma, but unlike other cancer therapies, its success has been accompanied by drastic side effects, including cytokine release syndrome (CRS) and CAR-T-cell-related encephalopathy syndrome (CRES) [112]. A Phase II trial performed with 74 participants with recurrent or refractory Mantle cell Lymphoma showed that KTE-X19 CAR-T cell therapy induced lasting remission, but the intervention also induced serious and life-threatening toxic effects, which was consistent with findings from previous reports [113]. Anti-CD19 CAR-T cell therapy can lead to severe CRS and neurotoxicity. Ying et al. generated a new type of CD19 BBz (86) CAR-T cells with costimulation, which showed lower cytokine levels, higher anti-apoptotic molecule production, and slower proliferation than other cell types previously studied. In clinical trials, CD19 BBz (86) CAR-T cell therapy produced an effective and lasting antilymphoma response without causing neurotoxicity or severe CRS [114]. To reduce the failure rate and recurrence rate after CAR-T cell treatment, Tong et al. [115] designed a series of tandem CARs (TanCARs). It was found that TanCAR-T cells possess double antigens targeting CD19 and CD20. In clinical trials, the safety and tolerance of TanCAR-T cells were evaluated. The results showed that TanCAR-T cells mediated a long-lasting and effective antitumor response without causing CRES of Grade 3 or a higher grade.

A clinical study evaluated the safety and efficacy of CD30 CAR-T cell therapy in patients with recurrent/refractory Hodgkin's lymphoma. The results showed that the effects of autologous CD30 CAR-T cell therapy were long-lasting, and the therapy achieved complete remission and showed good safety. They suggested that CD30 CAR-T cells may be a safe and effective choice for patients with Hodgkin's lymphoma and other lymphomas expressing CD30. At present, autologous CAR-T therapy has achieved clinical and commercial success. The development of more targets is an important strategy to benefit more patients and expand the applicability of the therapy. In addition, general CAR-T therapy is an important direction of CAR-T in the future. We expect these leading technologies to be transformed into products of significant utility and provide patients with a wider range of treatment options [116].

Certain results have also been reported in the treatment of solid tumors, such as gastrointestinal tumors [117]. CD133 is a marker of cancer stem cells (CSCs). CSCs are cancer cells with stem cell-like properties, such as self-replication and multicellular differentiation. These cells are considered to have the potential to form tumors and develop cancer. They are important cellular sources in the process of cancer metastasis. Singh et al. designed three immunotherapy methods based on human anti-CD133 antibody fragments and studied their targeting effect on CD133+ cells in a glioblastoma (GBM) humanized mouse model. CD133 CAR-T cells showed highest effectiveness in the GBM mouse model, without exerting any adverse effects on normal CD133+ hematopoietic stem cells, and did not induce acute systemic toxicity in a GBM humanized mouse model *in situ* [118]. CD133 CAR-T cell therapy also showed antitumor activity and safety in the clinical treatment of liver cancer [119].

Likewise, TCR-T therapy has made significant breakthroughs in clinical trials of melanoma and synovial sarcoma, and it has achieved preliminary efficacy in treating other advanced solid tumors [120].

Compared to the 2D-cultured cells, the 3D-cultured cells are more similar to the *in vivo* physiological conditions, especially the cell-cell interactions, cell proliferation, anaerobic conditions, and gene expression. Studies have shown that the 3D cell culture technology is a potential model for the pre-drug testing or drug screening of TCR-T and CAR-T based immunotherapy [121]. The researchers used CRISPR to knock out three genes in T cells, namely the T cell receptor  $\alpha$  (TCR $\alpha$ ) and T cell receptor  $\beta$  (TCR $\beta$ ), two genes that encode the endogenous TCR chain, and *PDCDI*, a gene that encodes the cell death protein 1 (PD-1). Moreover, one cancer-specific TCR transgene, NY-ESO-1, was inserted. Therefore, the modified T cells were named NYCE (NY-ESO-1 transduced CRISPR 3X edited cells). Eventually, after four rounds of editing, the T cells were injected back into the patient. The results showed that the engineered T cells had the ability to kill tumor cells *in vivo*, and there were no obvious immune rejections or related toxicity [122].

### 5.2 Antibody Targeting Therapy

Immune checkpoint molecules are the key factors that regulate the balance between the activation and suppression of immune responses. Cancer cells typically evade immune surveillance by upregulating the immune checkpoint molecules; hence, blocking this process can significantly enhance anti-tumor immune responses. The PD-1 and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are well-known immune checkpoints that can significantly inhibit the activity of T cells, thus making it easy for the tumor cells to escape from the body's immune mechanism; this leads to the poor results of T cell therapy [123]. At present, to improve the therapeutic effects of T cells, it is common to use immune checkpoint inhibitors, which are antibodies generated against the common immune check-points, and which form an effective method to overcome the inhibitions of the T cell immune response. Anti-tumor immune responses can also be achieved by using the CRISPR-Cas9 gene editing technology to knock out the genes that are involved in negative immune regulation. For instance, knockout of immune checkpoints using targeted gene editing techniques has been a promising strategy for improving the outcome of the CAR-T therapy in solid tumors [123]. Moreover, in the first human phase I clinical trial of CRISPR-Cas9 gene-edited T cell therapy in patients with advanced non-small cell lung cancer, Lu et al. [124] demonstrated that such a targeted immunotherapy is clinically feasible. Likewise, Si et al. [125] conducted a comprehensive study on the role of hematopoietic progenitor kinase1 (HPK1) and its related mechanisms in the regulation of T cell dysfunction. They found that gene depletion, pharmacological inhibition, or proteolysis targeting chimeric mediated degradation of HPK1 enhanced the efficacy of CAR-T cell-based immunotherapy in a variety of hematological and solid tumor mouse models. HPK1 is an attractive drug target for improving immunotherapy. The CD-19 HPK1<sup>KO</sup> CAR-T cells prepared by the CRISPR-Cas9 technology have been used in clinical trials, which are a part of the second clinical exploration in the world to improve T cell function through gene editing [125].

### 5.3 Tumor Vaccine

Unlike tumor immunotherapy, which targets immune checkpoints, tumor vaccines utilize antigen-antibody responses [126]. Cancer vaccines are of two main types: prophylactic and therapeutic. Prophylactic vaccines prevent cancer by preventing certain viral or bacterial infections or killing the corresponding pathogens, whereas therapeutic vaccines restrict tumor cell proliferation or kill tumor cells by activating specific immune functions in the body through cancer cell antigens. Tumor antigens that are generated after cell canceration can be used as the target for the adaptive immune response; this is the basis for preparing tumor vaccines. Additionally, sequencing mutated genes and obtaining new antigens help to prepare personalized tumor vaccines. These personalized cancer vaccines are an emerging treatment option that uses a patient's own cancer cells to develop vaccines that are designed to train the immune system to recognize and destroy the cancer cells [127,128]. NeoVax, a personalized cancer vaccine, has shown excellent results in clinical trials with patients with melanoma and produced

long-lasting immune responses [129]. Along with immune checkpoint inhibitors or other cancer therapies, personalized cancer vaccines may show a high clinical efficacy [130]. Ravindranathan et al. [131] used the CRISPR-Cas9 technology to screen cytokines affecting the immunogenicity of breast cancer cells and enhance the effectiveness of autologous tumor vaccines. Guo et al. [132] found that STEAP1 was expressed at high levels in both human and mouse prostate cancer cells, and constructed a fusion protein vaccine that could inhibit tumor growth and improve the tumor microenvironment *in vivo*. When the antigenic target was knocked out using the CRISPR-Cas9 system, the immune response against prostate cancer was considerably suppressed. There is a broad scope for further explorations in tumor vaccine development.

#### **5.4 Cell Therapy Based on Pluripotent Stem Cells**

Human pluripotent stem cells (hPSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have the potential for self-renewal and differentiation, thereby making them the most advantageous sources of cells for cell therapy [133]. One of the most significant obstacles in the clinical application of gene-edited hPSCs is the risk of cancer development [134,135]. Two drugs have been developed to inhibit the normal functions of hPSCs. The first drug depletes undifferentiated hPSCs, and the second drug kills cells derived from hPSCs [136]. The two main categories of hPSCs that are currently being used in clinical research are ESC and iPSC, and they have also been used in several clinical trials. It is expected that these hPSCs will provide scientists with an unprecedented opportunity to develop cell therapies for refractory diseases and injuries [137]. Methods for iPSC-based immunotherapy, which involves iPSC-derived MSCs and iPSC-derived NK cells, have been developed by organizations in the private sector for the treatment of hematological malignancies [138]. In patients with acute myeloid leukemia, the CD33 protein is expressed at high levels on the surface of tumor cells; however, treatments targeting CD33 may damage healthy cells. CD33 knockout in hPSCs, which induces hematopoietic resistance to CD33-targeted therapy and specific targeting of AML using CAR-T cells, can effectively eliminate leukemia without causing myelotoxicity [139].

#### **5.5 Identification of Potential Specific Targets**

The CRISPR technology can screen for targets that can improve the immune cell functioning, thereby improving immunotherapy [9,140]. Ye et al. [141] embedded an sgRNA and the “*Sleeping Beauty*” transposon in between the inverted end repeat sequences (ITRs/IRs) in the genome of adeno-associated virus (AAV). Transfection into mouse T cells and an effective gene editing screening can improve immunotherapy targets in glioblastoma mouse models. Therefore, by using the CRISPR screening *in vivo*, an epigenetic transcription network was established to screen new targets for enhanced immunotherapy [142].

### **6 CRISPR in Gene Therapy**

At present, most of the anticancer drugs require repeated administration; this not only increases the treatment cost and the drug toxicity, but also reduces the patient’s quality of life to a great extent. However, with the progress of technology, it has been demonstrated that the CRISPR-Cas9 gene editing system has the potential to destroy the tumor survival genes permanently; this can overcome the difficulties of traditional cancer treatment procedures, improve the efficacy of the treatment, and unlock new approaches for cancer treatment [143].

Fusion genes are present in all major types of cancer cells. It can inhibit tumor formation by inducing death of cancer cells containing fusion genes. In fact, the CRISPR-Cas9 gene editing system has successfully eliminated tumor cells in cell lines and mouse models of Ewing sarcoma and chronic myeloid leukemia by inducing cell death in the cancer cells containing fusion genes [144].

The use of the CRISPR technology to edit genes for the treatment of diseases caused by gene dysfunction is quite efficient. Theoretically, the CRISPR-Cas9 gene editing technology can permanently disrupt the tumor survival genes, thereby overcoming the repeated dose limits of traditional cancer therapies and improving the effectiveness of treatment. This is a promising option for treating the root cause of several human diseases. Undoubtedly, the CRISPR technology plays an important role in the treatment of numerous diseases, including genetic diseases [145].

At present, the CRISPR technology for gene therapy is mostly based on generating a DNA double-strand break (DSB) and inducing the cell DNA repair pathway, thereby resulting in a change in the target site sequence. Most diseases, including cancer, are caused by point mutations. Therefore, it is necessary to develop tools that can target specific point mutation sites. At present, the existing single base editing technologies include the CBE, which can convert C:G to T:A and the adenine base editor (ABE) that can convert A:T to G:C [146–149]. The newly discovered prime editor can carry out accurate gene editing, including long fragment replacement, insertion, and deletion, by editing the sgRNA and introducing a template at a DSB [150,151]. It is very important to explore the potential of guided editing to realize this application, including the research and treatment of genetic diseases [152].

In general, gene therapy is still at the pre-clinical exploration stage, and the criteria for estimating its safety, efficacy, and ability to exert long-lasting effects should be further refined. Cell and gene therapy may be truly life-changing and/or yield life-saving outcomes. Current regulatory models that require tests to be conducted on a large number of patients for determining the safety and efficacy of treatment methods are inappropriate for therapeutic techniques that target mutations found in a single patient or in a very small patient population. The development of a single composition that can be used to treat a larger patient population is a promising strategy. In the near future, the field of innovations in cell and gene therapy will face unique challenges, with the shift of traditional therapies to personalized therapies. This will be accompanied by an unprecedented level of control over nucleic acid delivery, immune system regulation, and precise manipulation of the human genome. Concurrently, insights on a new world of technological potential have inspired entirely new areas of research, such as synthetic biology, cell reprogramming, and high-throughput functional genomics, that will undoubtedly continue to reshape the face of biomedical research.

## 7 CRISPR Screening for Novel Drug Targets

The CRISPR technology can also accelerate the discovery of genes that may be targeted for new cancer treatments. Genome-wide screening in cancer models allows a large-scale screening of cancer-specific genes as well as the genes necessary for growth and development. By using the CRISPR-Cas9 screening in the cancer models, we can systematically study the carcinogenesis, development, and metabolic dependence of cancer cells to identify the potential therapeutic pathways [153,154]. It is possible to integrate the drug sensitivity of specific anticancer drugs with genome-wide CRISPR loss-of-function screening in cell lines to investigate the cellular pathways that support drug sensitivity; identification of these protein-protein networks facilitate drug development [155,156]. Multiple drug combinations have been discovered based upon the mechanism of drug resistance in tumor cells [3,56,157].

Over the years, there has been an increase in the number of mutated genes observed in cancer, but the functional genes, i.e., the genes necessary for the survival and proliferation of cancer cells, are largely unknown. To identify these cancer driver genes, lung cancer cells were cultured in both 2D monolayers and 3D lung cancer spheroids, followed by a genome-wide screening that revealed that some genes were necessary for cell growth in the 3D spheroids but not in the 2D levels [158]. Behan et al. [159] used the CRISPR-Cas9 technology to disrupt every gene in more than 300 cancer models developed from 30 types of cancer to identify the genes necessary for cancer cell survival. Additionally, a new system was developed to prioritize the 600 most promising drug targets for developing novel cancer treatments.

These results can accelerate the development of targeted therapies and help scientists to complete the development of a Cancer Dependency Map, which is a detailed rulebook for enlisting the hundreds of cancer gene dependencies, and which can help to develop targeted cancer treatment for patients [159].

The RNA N6-methyladenosine (m6A) modification can regulate gene expression and play an important role in many life processes. Previously, a study has shown that m6A modification plays a vital role in the occurrence and development of human tumors by regulating RNA stability, microRNA (miRNA) processing, mRNA cutting, and translation [160]. Using CRISPR-Cas9 knockout screening, methyltransferase 3 (METTL3) was identified as an important m6A regulatory enzyme in colorectal cancer (CRC). Silencing METTL3 inhibited the occurrence of CRC tumors at the cellular, organoid and mouse levels. The signal pathway of mettl3 promoting CRC was determined by m6A sequencing, RNA sequencing, ribosome sequencing and functional verification. The efficacy of targeting the *METTL3* in the treatment of CRC was established in CRC cell lines, patient-derived CRC-like organs, and *METTL3* knockout mouse models [161]. Ever since it was discovered that RNA-guided RNA targeting the CRISPR/Cas effector Cas13 (previously known as C2c2) can be used for RNA knockout in mammalian cells, the use of this technique in cancer research has increased considerably [162]. Long non-coding RNAs (lncRNAs) are closely associated with the occurrence of cancer. They can regulate DNA methylation, histone modification, chromatin remodeling, and act as precursors of miRNAs through a variety of pathways [163]. Incidentally, the lncRNA gastric cancer associated transcript 3 (*GACAT3*) was significantly expressed in bladder cancer tissues. After knockout of *GACAT3* by CRISPR-Cas13 technology, it was found that cell proliferation ability was inhibited and enhanced the apoptosis of the cancer cells. Therefore, *GACAT3* is a potential target for bladder cancer diagnosis and therapy [164].

Metastasis is one of the major factors that makes cancer treatment difficult, and it is associated with the poor prognosis of cancer. In a recent study, the genes encoding cell surface proteins were screened by Cas9-based CRISPRa to identify the cell surface molecules that enhance metastatic abilities of cancer cells. The scientists found that overexpression of the gene *LRRN4CL* increased the ability of mouse as well as human melanoma cells to metastasize to the lungs. They also tested the cellular models of colon cancer, breast cancer, and bladder cancer, and they confirmed that *LRRN4CL* was transferred to all lungs in all these cells. This is the first time that *LRRN4CL* has been associated with cancer. Moreover, the results show that reducing the *LRRN4CL* expression can help to prevent metastasis to the lungs, thereby making it a potential drug target [165].

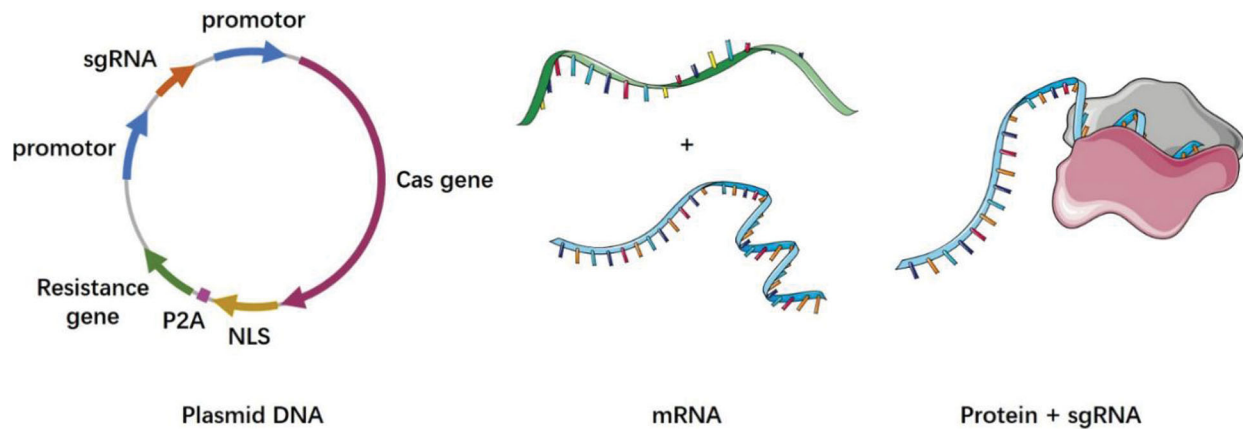
To screen the genes and signaling pathways that enable cancer cells to escape destruction by the host's immune system, a group of genetically diverse mouse cancer cell lines that were cultured in the presence of cytotoxic T lymphocytes (CTLs) were screened using genome-wide CRISPR technology. A total of 182 genes were identified in this experiment, the individual interference of which increased either the sensitivity or the resistance of the cancer cells to CTL-mediated toxicity. Further exploration revealed the coordinated manner in which the identified genes and signaling pathways function in cancer cells to evade the action of CTLs [166].

To study the characteristics of ataxia telangiectasia mutated (ATM)-deficient fatal PC and discuss its comprehensive lethal treatment strategy, the ATM function was impaired in modified *in vitro* cell line models by using CRISPR-Cas9 technology. These models were used to test drug sensitivity and function analysis, followed by validation in patient-derived models. The results showed that the combined inhibition of polyadenosine diphosphate-ribose polymerase (PARP) and ATR (ATM and Rad3-related) had good antitumor activity in these models [56].

## 8 Delivery of CRISPR-Cas *in vivo*

Although the CRISPR gene-editing technology is already being used in clinical trials, the efficiency of its safe delivery remains a major challenge. Commonly used delivery methods include physical methods,

viral vectors, and non-viral vectors. Currently, therapeutic technologies based on the CRISPR-Cas9 system are mainly realized through targeted delivery [7]. The various ways in which the CRISPR system can be delivered is discussed in the following section (Fig. 2).



**Figure 2:** CRISPR cas system delivery forms

Plasmid DNA (pDNA): a plasmid containing the Cas9 and sgRNA sequences; mRNA: Cas9 is transcribed into mRNA *in vitro*, and then delivered into cells via a vector with the sgRNA; protein +sgRNA: Cas9 is translated into the protein *in vitro*, and then delivered into cells via a vector with the sgRNA

## 8.1 Delivery Forms

### 8.1.1 Plasmid DNA

The CRISPR-Cas9 system encoding the Cas9 protein and the specific sgRNA that is required by the CRISPR-Cas9 system are integrated into the same plasmid DNA vector. This technique has the advantages of a simple operation and low cost, and is widely used [167]. In one experiment, a  $\beta$ -catenin-targeting CRISPR-Cas9 plasmid was constructed, which was modified by aptamer and enriched significantly in tumor nuclei. This system can effectively eliminate  $\beta$ -catenin and inhibit the Wnt/ $\beta$ -catenin pathway, reversing the immunosuppressive effect of tumors [168]. However, the large amount of encoded pDNA increases the delivery difficulty, and the long-term expression of Cas9 in the target cells may lead to an increase in the off-target effects and immune responses [169,170].

### 8.1.2 mRNA and Guide RNA

The off-target effect of the CRISPR-Cas9 system can be significantly reduced by transcribing the Cas9 mRNA *in vitro* and directly delivering the Cas9 mRNA to cells. This ensures a controlled dosage of the mRNA introduction as well as a rapid initiation of the gene editing process [171]. Owing to the poor stability of mRNA, a special mechanism is required to protect it from extracellular nuclease degradation during delivery [172–174]. In a clinical trial, Gillmore et al. [175] delivered sgRNA targeting the TTR gene and optimized the mRNA sequences of the spCas9 protein to the liver via a lipid nanoparticle (LNP) delivery vector for the treatment of transthyroxine protein amyloidosis. Phase I clinical trials showed good efficacy and safety of the treatment method.

### 8.1.3 Cas Protein and Guide RNA

To minimize the off-target effects of the CRISPR-Cas9 system, the most efficient approach for its delivery is to directly introduce the Cas9 protein and its specific sgRNA instead of the intracellular expression of pDNA or mRNA for Cas9 protein and sgRNA [176,177]. RNP-based delivery methods can strike a good balance between reducing cytotoxicity and maintaining genome rearrangement efficiency

[178]. Two essential conditions for the direct delivery of the Cas9 protein and efficient genome editing are vector assistance for successful intracellular delivery of Cas9 protein and efficient lysosome escape. A nanodelivery system loaded with Cas9 can assist the Cas9 protein to enter the target cell, where it can be rapidly degraded by endogenous proteases immediately after cutting the target to achieve higher gene editing efficiency and lower off-target effects. There are three main methods of protein delivery, namely direct conjugation of proteins and carriers, physical adsorption of proteins and carriers based on charge or hydrophilic interactions, and protein nanocapsules based on emulsion dispersion [179]. Currently, majority of the Cas9 protein delivery systems are in the form of RNP [180].

## 8.2 Delivery Systems

Direct *in vivo* application of CRISPR-Cas9 system requires delivery systems that can target the CRISPR-Cas components to specific tissues or cells in the human body without triggering immune activation or causing high frequencies of off-target effects. Currently, physical methods, lentivirus, adenovirus, AAV, and LNP are being used as delivery vectors for human gene therapy; however, some of these delivery vectors are associated with problems like random integration into the genome, poor efficiency, and triggering unnecessary immune responses. At present, the most commonly used physical methods for the CRISPR delivery include microinjection [181], electroporation [182], and hydrodynamic tail-vein injection (HTVI) [183]. These methods are not applicable in case of the *in vivo* clinical trials because of their high experimental conditions, operation techniques, and subjects. Retroviruses, adenoviruses, and AAV are three major groups of viruses that have been used extensively to provide genetic material for treatment purposes. However, building viral vectors is a laborious and costly process with certain problems, such as insertion size limitations as well as the risk of developing cancer or other immune responses in humans. For example, retroviruses can cause insertional mutations that lead to cancer [184], and high intravenous doses of AAV for gene therapy can also cause toxicity [185]. Compared to these viral vectors, the non-viral vectors have advantages of low immunogenicity and no endogenous viral recombination, thereby minimizing the chances of short- and long-term adverse reactions; moreover, they have lower payload constraints and are generally easier to synthesize and produce on a large scale [186]. Therefore, many studies have focused on non-viral vectors, including polymer-liposome nanomaterials and emerging exosomes. Nevertheless, the safe and effective delivery of the CRISPR-Cas9 system into organisms, followed by a successful gene editing function still faces several challenges.

### 8.2.1 Nonviral Delivery

The CRISPR-lipid nanoparticle, which is a new delivery system based on lipid nanoparticles, has been developed to specifically target cancer cells and destroy them through genetic manipulation [187]. The system carries a genetic messenger (mRNA) that encodes the Cas9 protein, which acts as the “molecular scissors” that cuts into the DNA of cancer cells, thereby disabling it and permanently preventing replication. This method does not involve any chemotherapy drugs, and hence, does not have any side effects; moreover, the cancer cells treated in this way will never be active again. To test the feasibility of using this technology to treat cancer, two of the deadliest cancers, glioblastoma and metastatic ovarian cancer, were studied. The overall survival was improved in mice treated with CRISPR-LNPs in both the cancer models. Therefore, this technology reveals many new possibilities for treating various types of cancer as well as rare genetic diseases and chronic viral diseases such as AIDS [187].

Chen et al. developed a nanocapsule (NC) that was synthesized from a shearable, covalently cross-linked polymer coating of glutathione (GSH) that can encapsulate a preassembled RNP complex of Cas9 and sgRNA. These NCs effectively performed a targeted gene editing *in vitro*, without any significant cytotoxicity. Additionally, after local administration, the NCs exhibited powerful gene-editing functions in the mouse retinal pigment epithelium (RPE) tissue and skeletal muscles. Hence, these

customizable NCs provide a nanoplatform that effectively delivers CRISPR-RNP complexes for somatic cell gene editing *in vitro* as well as *in vivo* [188].

Recently, exosomes/extracellular vesicles (EVs) are being increasingly used as delivery vectors for small-molecule therapies, proteins, RNA, DNA, and potentially new CRISPR technologies because they form a natural means of intercellular communication that is biocompatible, immunoinert, and effective in reaching their targets [189]. The RNPs were integrated into the EVs by the specific interaction between an RNA adaptor and an aptamer-binding protein (ABP). The RNA adaptor was inserted into an sgRNA, and the ABP COM was fused at both ends of the exosome-rich tetra protein CD63. A three-component complex consisting of CD63-COM fusion protein, modified sgRNA, and Cas9 or ABE was formed as a result of the abovementioned interaction, and the Cas9 and ABE RNPs were enriched into the EVs. This system can deliver gene modifications to multiple sites with high efficiency and safety [190].

Qi et al. [191] developed a lactose-derived branched cationic biopolymer (LBP) with a large number of reducible disulfide bonds and hydroxyl groups as a potential delivery vector of the CRISPR-Cas9 system for efficient *in vivo* genome editing in the treatment of hepatocellular carcinoma (HCC) *in situ*. The LBP-mediated delivery of pCas9-survivin demonstrated effective gene editing in the *in vitro* conditions by inducing apoptosis and inhibiting the proliferation of HCC cells. Furthermore, the LBP/pCas9 complex significantly enhanced the antitumor effect of the drug by inducing survivin knockout. Hence, a safe method was developed to efficiently edit the genome of the CRISPR-Cas9 system *in vitro* and *in vivo*.

In a recent study, the researchers have explored the combinatorial synthesis of multi-tailed ionizable phospholipids (iPhos) that can deliver messenger RNA or mRNA/sgRNA *in vivo* for gene editing. The structure-activity relationship shows that the chemical structure of iPhos can control the efficacy and organ selectivity in the *in vivo* conditions. Furthermore, the iPhos lipids cooperate with various auxiliary lipids to form multi-component lipid nanoparticles (iPLNPs) for selective organ targeting. Zwitterions, ionizable cations, and permanent cationic auxiliary lipids can carry out tissue-specific mRNA transmission and CRISPR-Cas9 gene editing in the spleen, liver, and lungs after intravenous administration [192]. This discovery proves that the regulation of the chemical structure of the lipids as well as the nanoparticle components can achieve organ selectivity. This, in turn, provides an idea for the development of next-generation gene vectors, and it can promote the research for organ-specific disease treatment based on mRNA delivery and the CRISPR-Cas gene editing.

Recently, Feng Zhang's team developed a new RNA delivery platform named selective endogenous encapsidation for cellular delivery (SEND). The paternally expressed 10 (PEG10) protein, which is a retrovirus-like protein engineered to bind to its mRNA and form a protective globule around it, is used to package and deliver RNA. Using SEND, the CRISPR-Cas9 system was successfully delivered to human and mouse cells in mRNA form, and specific genes were edited. Compared with other delivery vectors, the immune response was lower and the safety was greater [145].

## 9 Technical Limitations

Since the birth of the CRISPR technology, there have been two main obstacles hindering its clinical application, namely the off-target effects and the low delivery efficiency. Several variants of Cas proteins have been developed until now, among which the most widely used ones are dCas9 [193] and Cas9 nickase (nCas9) [194], which are inactivated at the restriction site, and xCas9 [195], spCas9-NG [196], SpG, and SpRY [197], which have a wide PAM sequence and high specificity. The initial concerns about the off-target activities have been overcome with the improvements in the CRISPR-Cas system and its delivery strategies [195,198–200]. At present, the most serious problem is the genome damage caused by DSBs and the toxicity to organisms.



### 9.1 DSBs Causing DNA Damage

The CRISPR system can activate the *p53*, thereby leading to DNA damage [134,135]. In a comprehensive systematic study of mouse and human cells, the researchers found that the CRISPR-Cas9 system caused a wide range of mutations and quite a few genetic rearrangements, such as DNA deletions and insertions in a number of cells; these changes can cause important genes to be turned on or off, thereby leading to pathogenic consequences [201]. Xu et al. found that the Cas9 protein as well as its various mutations could directly bind to KU86, which is a key factor in the DNA repair pathway, and interfere with the formation of the DNA-PK complex; this can inhibit DNA damage repair mediated by the non-homologous end joining (NHEJ) repair pathway and lead to DNA damage and genomic instability [202]. Hence, further research and specificity tests are needed before the CRISPR-Cas9 system can be used clinically.

### 9.2 Toxicity of CRISPR-Cas System

Previous studies have shown that the CRISPR-Cas9 gene editing can lead to the activation of *p53*, which, in turn, causes DNA damage; therefore, it is possible that the *p53* in the successfully gene-edited cells is defective. This increases the risk of cancer, and it can potentially cause cancer in individuals who have been treated by these gene-edited cells [134,135]. It was subsequently discovered that CRISPR-Cas9 edited mice exhibited frequent and large base deletions in their fertilized eggs [203,204]. Wagner et al. found that almost all healthy human subjects possessed T cells that reacted against Cas proteins; this is possibly because of the pre-existing immune memory against Cas9 protein since *Streptococcus* infection is quite common in humans. Incidentally, the Cas molecules from other bacteria also produce a similar immune response, which may be due to the high degree of similarity between these enzymes. Hence, these immune cells may produce adverse immune responses during gene therapy, thus compromising the safety and effectiveness of the CRISPR technology [205]. These studies suggest that the body's immune response can disrupt gene therapy and pose a health threat to the patients who are receiving it. If antibodies against Cas9 bind to the Cas9 in blood, then the Cas9 can be inactivated even before it performs the gene editing. Additionally, the Cas9-specific T cells may actively eliminate the Cas9-modified cells, resulting in treatment failure [206]. Recently, Leibowitz reported that DSBs introduced during CRISPR-Cas9 gene editing may lead to chromosome fragmentation, which is a highly destructive form of genome rearrangement that may lead to the emergence of oncogenic fusion proteins or dysregulation of specific gene expression [207]. However, we can avoid immune rejection by searching for a new Cas9 enzyme from a bacterium that has never infected humans or by artificially redesigning the Cas9 enzyme.

The discovery of a series of problems caused by the CRISPR-Cas system indicates that our understanding of the mechanisms of CRISPR-Cas9 gene editing technology is still insufficient, and we need to be cautious regarding the safety evaluation of this technology.

## 10 Conclusions

In the past ten years, the CRISPR technology has developed by leaps and bounds from the initial gene knockout to modular tools, and today, it plays a significant role in disease treatment and research. For example, it has applications in transcriptional regulation [29,208], epigenetic modification [28,209], chromatin imaging [210,211] and base editing tools [212]. The key challenge is to apply new technologies for clinical treatment. It is believed that the CRISPR technology will be more widely used in disease treatment in the future with the development of safe and effective gene editing tools as well as improved delivery systems. The development of DNA sequencing and its large-scale applications have led to the recognition of genetic variation between the patient population and the general population; moreover, it has expanded our understanding of how genetic variation relates to disease predisposition,

disease development, and treatment response [156]. These advances have spurred the development of personalized or precision medicine. The CRISPR-Cas system is also suitable for large-scale screening, and it has been used in a variety of settings. It has shown good results in the construction of disease models, drug development, screening of drug targets, and preclinical research. The CRISPR-Cas9 gene editing system also functions as a self-regulating switch for the CRISPR-Cas9 gene array. Its role in turning on or turning down CRISPR-Cas9 activity could help scientists to develop new ways of genetically engineering cells for research purposes [213]. The development and application of smaller Cas proteins, which ease the delivery process, can be developed as a varied genetic engineering tool for gene regulation and therapy [214]. With the progress in research, the CRISPR-Cas system has been used in clinical applications, and it is believed that with the improvement of the safety measures and the development of personalized therapy, the large-scale use of this technology in disease treatment will soon be possible.

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