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REVIEW

Advancements in CRISPR/Cas systems for disease treatment



Yangsong Xu, Hao Le, Qinjie Wu, Ning Wang^{*}, Changyang Gong^{*}

Department of Biotherapy, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China

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Abstract The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated proteins) is an adaptive immune system present in most bacteria and archaea, protecting them from infection by exogenous genetic elements. Due to its simplicity, cost-effectiveness, and precise gene editing capabilities, CRISPR/Cas technology has emerged as a promising tool for treating diseases. The continuous refinement of derivative systems has further broadened its scope in disease treatment. Nevertheless, the heterogeneous physiopathological nature of diseases and variations in disease onset sites pose significant challenges for *in vivo* applications of CRISPR systems. The efficiency of CRISPR systems in disease treatment is directly influenced by the performance of the delivery system. Additionally, concerns such as off-target effects present crucial hurdles in the clinical implementation of CRISPR systems. This review provides a comprehensive overview of the development of CRISPR systems, vector technologies, and their applications in disease treatment, while also addressing the challenges encountered in clinical settings. Furthermore, future research directions are outlined to pave the way for advancements in CRISPR-based therapies.

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^{*}Corresponding authors.

E-mail addresses: ningwang_lab@163.com (Ning Wang), chygong14@163.com (Changyang Gong).

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1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins (CRISPR/Cas) system is a naturally occurring adaptive immune mechanism found in bacteria and archaea. Its origin is rooted in the evolutionary arms race between these microorganisms and their viral predators, particularly bacteriophages¹. The CRISPR/Cas system operates by incorporating short sequences of viral DNA, derived from previous infections, into the CRISPR loci within the host genome. These sequences, known as spacers, are flanked by repeat sequences, forming a unique genetic architecture. When the host organism is re-exposed to the same virus, these spacers are transcribed into RNA molecules, which guide the associated Cas proteins to the corresponding viral DNA. The Cas proteins then cleave the viral DNA, thereby neutralizing the threat¹⁻³.

The classification of the CRISPR/Cas system is based on its structural and functional diversity. It is broadly categorized into two main classes, each with several types and subtypes (class 1: type I, III, IV; class 2: Type II, V, VI)⁴⁻⁶. Therein, type II CRISPR/Cas9 system is a revolutionary gene-editing tool derived from the *Streptococcus pyogenes*⁷. It comprises two main components: the CRISPR RNA (crRNA) and the Cas9 protein⁸. The crRNA guides the Cas9 protein to the target DNA sequence through base-pairing, while the Cas9 protein cleaves the DNA at the specified location, leading to precise modifications. This system offers several advantages over previous gene-editing technologies, including its simplicity, cost-effectiveness, and versatility^{9,10}. Unlike earlier methods such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR/Cas9 does not require custom protein engineering for each target sequence, making it more accessible to researchers¹¹⁻¹³. The applications of CRISPR/Cas9 span across various fields, including basic research, agriculture, and medicine^{5,14,15}. In disease treatment, CRISPR/Cas9 holds immense potential for correcting genetic mutations underlying various disorders, ranging from monogenic diseases to complex multifactorial conditions¹⁶. Additionally, it enables the study of disease mechanisms through precise genome editing in cellular and animal models^{16,17}.

The development of CRISPR/Cas9 has catalyzed innovation in genome engineering, leading to the development of derivative systems with enhanced functionalities and tailored applications^{4,18}. These derivative systems typically leverage the foundational principles of CRISPR/Cas9 while incorporating modifications or additional components to address specific limitations or achieve novel functionalities in physiological regulation for specific diseases^{19,20}. Incorporation of derivative systems in the genome engineering toolbox offers unprecedented precision, versatility, and control over genome editing, with profound implications for therapeutic interventions.

While various versions of CRISPR systems enable molecular-level control, their ability to exert therapeutic effects at specific disease loci necessitates efficient delivery systems^{21,22}. Different diseases manifest in distinct tissue types, each with unique physiological barriers that impede the efficient delivery of therapeutic agents. For example, solid tumors exhibit abnormal vasculature, dense extracellular matrix, and elevated interstitial fluid pressure, limiting the penetration and distribution of therapeutic payloads^{23,24}. Similarly, neurological disorders involve the blood-brain barrier (BBB), which restricts the entry of macromolecules into the brain parenchyma^{25,26}. Furthermore, the biodistribution and pharmacokinetics of delivery vehicles influence their accumulation at disease

sites, necessitating strategies to enhance specificity and retention while minimizing off-target effects²⁷⁻²⁹. Addressing the complex interplay of physiological barriers and developing innovative delivery strategies are essential steps toward realizing their therapeutic potential in specific disease contexts.

In this review, we have discussed the evolution of CRISPR technology, delineated advancements in delivery systems, and summarized the recent studies on the application of CRISPR systems in the treatment of multiple diseases. Furthermore, we have deliberated on the research frontiers and clinical challenges of CRISPR-based therapies in disease treatment. Prospectively, researchers can enhance the specificity, efficacy, and safety of CRISPR-based therapeutics by harnessing interdisciplinary approaches and leveraging emerging technologies, ultimately paving the way for precision medicine paradigms tailored to individual disease states.

2. The CRISPR/Cas9 system

CRISPR/Cas systems, an adaptive immune system, were initially discovered in prokaryotes³⁰. The CRISPR/Cas system comprises the CRISPR-encoding sequence, Cas-encoding sequence, and the CRISPR-associated sequence, which includes the leader, repeats, and spacers sequence^{1,3,31}. Over the last decade, it has transformed into a third-generation gene editing tool, profoundly influencing biomedical research^{2,32,33}. A DNA structure with numerous repetitions that reads the same forwards and backward was discovered in 1987 and dubbed CRISPR in 2002^{34,35}. In 2005, three investigations confirmed the CRISPR system as an adaptive immune system found in archaea³⁶⁻³⁸. In 2008, research demonstrated that the CRISPR/Cas system was directed by a small segment of crRNA to trigger an antiviral response in prokaryotes³⁹. Further research has shown the specificity of CRISPR for DNA targeting^{40,41}. In 2011, a *trans*-acting CRISPR RNA (tracrRNA) that is complementary to the pre-CRISPR RNA (pre-crRNA) was discovered⁴². In 2012, researchers successfully cleaved target genes using recombinant Cas9 proteins, tracrRNAs, and crRNAs *in vitro*, confirming the system's versatility in cleaving diverse double-stranded DNA (dsDNA) sites *via* distinct guide RNAs⁸. A significant milestone was reached in 2013 when scientists successfully performed gene editing in eukaryotes using the CRISPR/Cas9 system^{43,44}. Since its inception, ongoing research and development have propelled the CRISPR/Cas9 gene editing system's impact on the life sciences field. The first clinical trial utilizing CRISPR/Cas9 was disclosed in China in 2016^{45,46}, and the technique was honored with the Nobel Prize in Chemistry in 2020 (Fig. 1).

The CRISPR/Cas system serves as the adaptive immune system in prokaryotes by cleaving target DNA, resulting in double-stranded DNA breaks (DSB) for effective gene editing. Exogenous DNA entering a bacterium triggers Cas-associated proteins to identify the protospacer adjacent motif (PAM, usually composed of NGG bases) and cleave the sequence near the PAM (Fig. 2)¹. This fragment is then integrated downstream of the CRISPR leading sequence to create spacer sequences with the assistance of enzymes like Cas1 and Cas2. The pre-crRNA and tracrRNA are transcribed using the leader sequence, followed by the formation of ribonucleoprotein (RNP) complexes consisting of Cas9, crRNA, and tracrRNA. Thus, upon re-entry of the exogenous DNA into the bacterium, the RNP complexes can recognize the DNA *via* the PAM sequence, it's worth noting that different

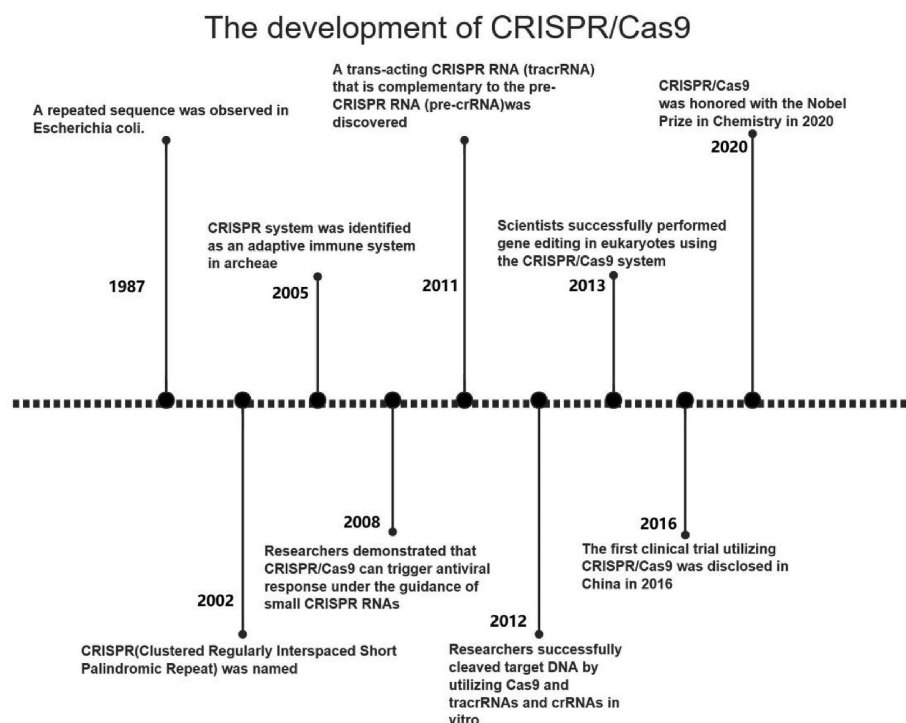


Figure 1 A milestone in the evolution of the CRISPR/Cas9 system. Created by processon.com.

CRISPR/Cas system means various PAM sequences. Subsequently, the RNP complexes can induce the DSB using the HNH and RuvC nuclease domains in the Cas protein at the third base site adjacent to the upstream of the PAM sequence. Among that, the HNH nuclease structural domain is responsible for cleaving the DNA single-strand complementary to the crRNA strand, and the RuvC nuclease domain cuts the non-complementary chains, leading to gene editing through non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms mediated gene insertion or deletion. Researchers can now achieve gene editing by creating several guide RNAs (sgRNAs) to target different sequences^{1,6,47}. Furthermore, using various CRISPR/Cas systems with PAM sequences allows researchers to achieve gene editing for more targets. The CRISPR/Cas9 gene editing method has become essential in the field of life sciences because of its efficiency, ease, and cost-effectiveness.

3. CRISPR/Cas9-based gene editing tools

Since the advent of the gene editing tools CRISPR/Cas9, significant efforts have been made to expand their application range and improve safety. Utilizing different forms of Cas-associated protein to alter the function is extensively studied^{4,6,48-50}. The CRISPR/Cas system can be categorized into two types based on the Cas-associated protein^{4,51}. Class 1 typically involves the nucleic acid endonuclease function, which necessitates a multi-protein complex comprising types I, III, and IV. On the other hand, Class 2 employs a single Cas protein for DNA cleavage, which differs from Class 1. As a result, class 2 is more commonly used due to its simplicity. Cas9, a prevalent class 2 type II protein, consisting of two endonuclease domains: HNH and RuvC. Cas12 from class 2 type V only contains the RuvC-like endonuclease domain, enabling

it to cleave two target DNA strands. In contrast, class 2 type VI Cas13 exhibits RNA targeting capabilities instead of DNA targeting (Fig. 3A–D)^{1,9,10,47}.

In 2013, Qi et al.⁵² showed that by introducing two silent mutations on the nuclease domains, RuvC1 and HNH, they created a non-functional Cas9 lacking endonuclease activity, referred to as deadCas9 or dCas9. In this context, dCas9 maintains the ability to bind specific DNA sequences guided by sgRNA. Additionally, the target gene can be suppressed or activated by incorporating a transcriptional repressor or activator into dCas9, leading to the development of CRISPRi (CRISPR interference) and CRISPRa (CRISPR activation) (Fig. 3E and F)^{9,19,53}. Various variations of transcription factors were explored for efficient gene expression regulation. The KRAB domain is the initial transcription repressor for CRISPRi systems, and dCas9-KRAB enhanced the inhibitory impact more than dCas9 alone⁵⁴. Later, additional transcription repressor domains were discovered¹⁹. The CRISPRa system encompasses two classes: dCas9 coupled with a *cis/trans*-transcription activator domain. Initial experiments employing VP64 resulted in an upregulation of target gene transcription; however, the effect was limited⁵⁵. Moreover, the tripartite activator VP64-p65-Rta was mentioned. The dCas9-VP64 two-component system can be enhanced by utilizing the three-component systems SunTag (ScFv-(GCN4)-VP64) and SAM (MS2-p65-HSF1) for more potent activation of target genes^{48,56-58}. CRISPRi/CRISPRa has transformed the role of CRISPR/Cas9 from gene editing to gene regulation. In addition to dCas9, Cas9 nickase (Cas9n), a variant of Cas9, typically with only one nucleic acid endonuclease structural domain, which induces single-stranded gaps rather than double-stranded breaks under the guidance of sgRNAs, can significantly reduce off-target effects⁵⁹.

CRISPR/Cas9 gene editing may lead to uncontrolled DSBs, particularly in certain contexts such as disorders connected to

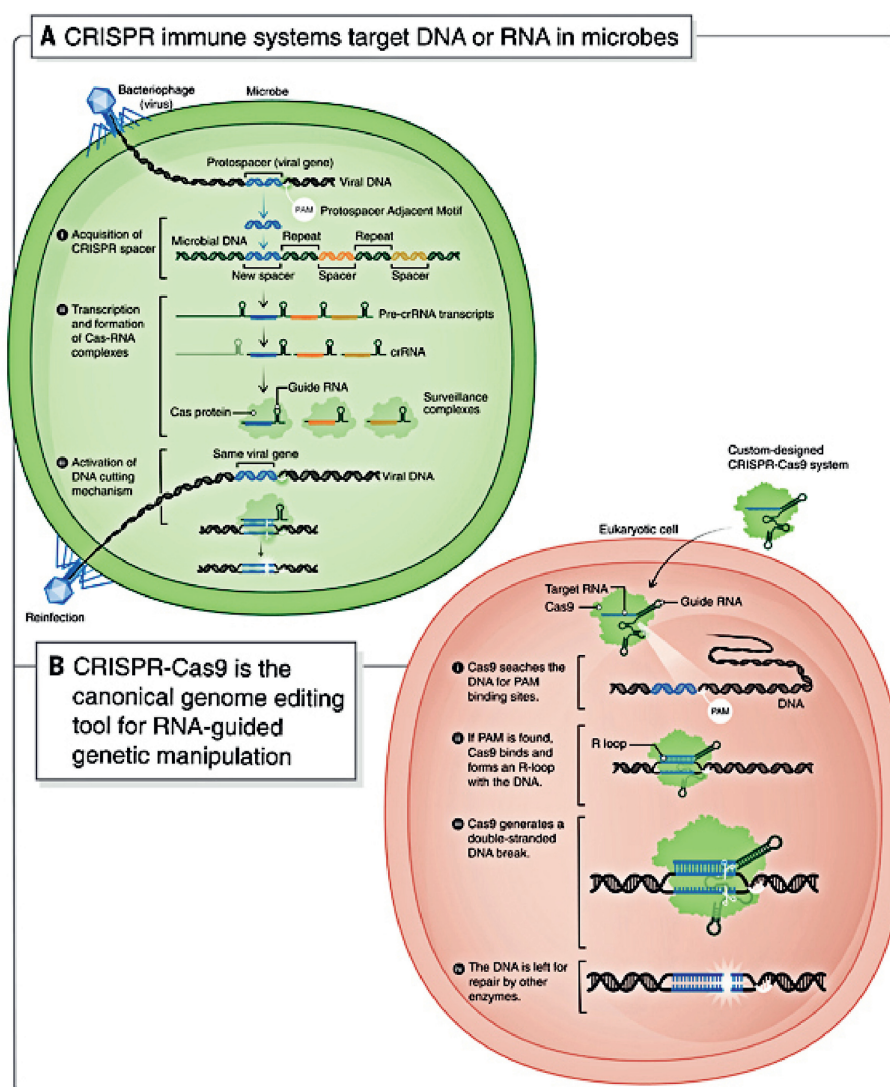


Figure 2 Mechanism of antiviral resistance in CRISPR/Cas9 system. Reprinted with the permission from Ref. 1. Copyright©2023 The American Association for the Advancement of Science.

single gene mutations where DSBs are not desirable. Komor⁶⁰ originally reported the creation of the Cytidine Base Editor (CBE) in 2016, which enables the conversion from C-G to A-T by combining cytidine deaminase with dCas9. An Adenosine Base Editor (ABE), based on Cas9n, capable of changing A-T to G-C was developed just one-year later⁶¹. The base editor has made significant advancements in accurate gene editing. However, other research has highlighted concerns over potential off-target effects and the possibility of gene mutations when using the base editor⁶²⁻⁶⁴. Therefore, extensive research has been conducted to enhance the safety of base editors, with most efforts focused on optimizing the pertinent enzymes^{65,66}. The CBE and ABE cannot facilitate the interchange of arbitrary bases. The development of Prime Editor (PE) has enabled the interchange of all bases with increased efficiency and reduced off-target effects (Fig. 3G)^{67,68}. Furthermore, high-fidelity CRISPR/Cas enzymes and multiplex genome editing have been used to broaden the application of CRISPR/Cas systems^{56,69}. The CRISPR/Cas9-based gene editing tools have been summarized in (Table 1)^{1,2,9,10,19,53,56,60,61,67-69}.

4. The application of CRISPR/Cas9 for various disease

The CRISPR/Cas9 system is a potent gene editing technology known for its accessibility, high efficiency, and specificity, revolutionizing the understanding of diseases¹⁰. The primary focus of research lies in identifying and evaluating the mechanisms of illness development, potential therapeutic targets, biomarkers, and disease susceptibility^{5,16,70}. In this section, we provide a summary of the utilization of the CRISPR/Cas9 system in various diseases, furthermore, we have summarized the CRISPR/Cas9-based clinical trials (Table 2) and clinical application (Table 3)⁷¹⁻⁷³.

4.1. Cancer

Cancer represents a significant global concern owing to its high incidence and mortality rates⁷⁴. Its formation and progression are influenced by multiple factors, notably mutations in oncogenes and tumor suppressor genes⁷⁵. These genetic abnormalities enable tumor cells to proliferate continuously, evade immune system

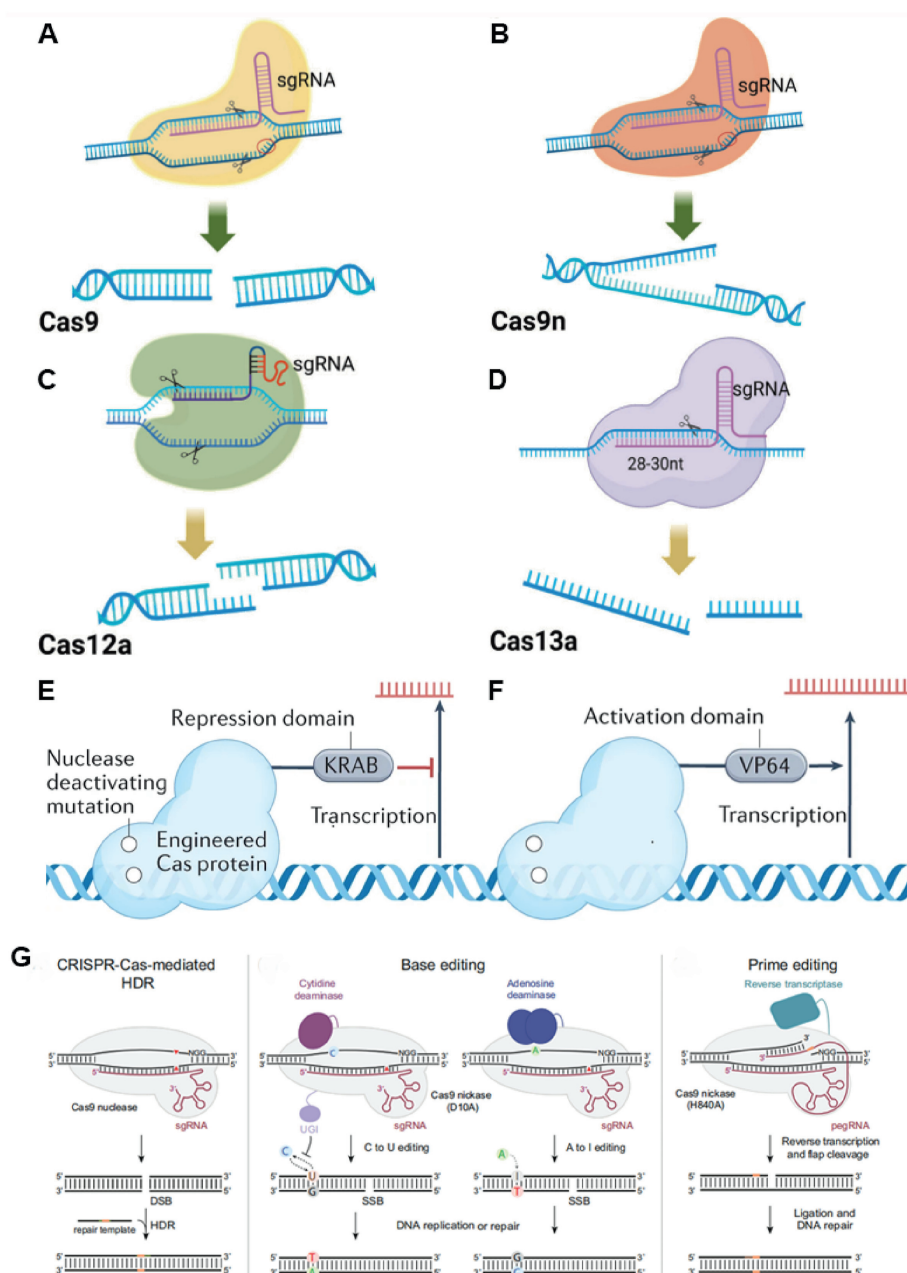


Figure 3 Schematic illustration of various Cas9-based gene editing tools. (A) The first generation of CRISPR/Cas9. Reprinted with the permission from Ref. 10. Copyright@2023, The Authors; (B) Cas9 nickase (Cas9n), a variant of Cas9, usually with only one nucleic acid endonuclease structural domain. Reprinted with the permission from Ref. 10. Copyright@2023, The Authors; (C) Cas12a and (D) Cas13a. Reprinted with the permission from Ref. 10. Copyright@2023, The Authors; (E) Cas9-based CRISPRi system by engineered Cas protein and (F) the CRISPRa system. Reprinted with the permission from Ref. 9. Copyright@2023 Springer Nature; (G) The base editing and prime editing enable the interchange of all bases. Reprinted with the permission from Ref. 68. Copyright@2023 The Authors.

surveillance, invade tissues, and metastasize to distant sites. In this context, the CRISPR/Cas9 system provides a promising avenue for cancer therapy, offering a novel approach to addressing the complexities of the disease^{33,76-78}.

Utilizing the CRISPR/Cas9 system to analyze tumor cell genomes can help identify weaknesses in tumors and potential new targets for medication development. An immunological checkpoint called protein tyrosine phosphatase (PTPN2) was discovered by genome-wide screenings utilizing the CRISPR/Cas9 system⁷⁹. The inhibitor ABBV-CLS-484 is currently undergoing Phase I

clinical trials⁸⁰. Scientists have developed a framework for conducting CRISPR/Cas9 screening in living organisms. They identified a novel immune suppressor, CD300LD, prominently present in polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), which plays a crucial function in the tumor microenvironment by influencing the STAT3-S100A8/A9 pathway⁸¹. Tumor cells often exhibit medication resistance due to the DNA repair pathway and targeting this pathway can increase the sensitivity of tumor cells to DNA damage treatments⁸². Identifying a lack of Kelchlike ECH-associated protein 1 (KEAP1) as a crucial element

Table 1 Comparison and advancements of CRISPR/Cas-based systems.

Gene editing tool	Type	Nuclease domain	Target	Description	Impact	Ref.
CRISPR/Cas9	Type II Class 2	HNH and RuvC	dsDNA	The most popular gene editing systems	Enable gene editing by causing DNA double-strand breaks	1,2
CRISPR/Cas12	Type V Class 2	RuvC	dsDNA	Cas12 targets single-stranded DNA; Cas13 targets RNA	Broadens CRISPR applications beyond DNA editing, including RNA editing and diagnostics.	9,10
CRISPR/Cas13	Type VI Class 2	HEPN	RNA			
CRISPRi/CRISPRa	Type II Class 2	—	Single-stranded DNA	dCas9 used to repress or activate gene expression without cutting DNA	Enables reversible control of gene expression, useful for functional genomics studies	19,53
Multiplex genome editing	—	—	—	Allows simultaneous editing of multiple genes	Useful for studying polygenic traits and engineering organisms with complex trait alterations	56
CBE	Type II Class 2	—	Single-stranded DNA	Converts one DNA base pair to another without double-strand breaks	Reduces off-target effects, ideal for correcting point mutations	60
ABE	Type II Class 2	—	Single-stranded DNA			61
PE	Type II Class2	—	Single-stranded DNA	Allows precise insertions, deletions, and base substitutions	Expands the range of genetic modifications with minimal errors	67,68
High-fidelity CRISPR/Cas enzymes	—	—	—	Engineered Cas9 variants (e.g., eSpCas9, SpCas9-HF1) with fewer off-target effects.	Increases safety and precision of gene editing	69

—, not applicable.

in making tumor cells more responsive to ataxia-telangiectasia mutated (ATM) inhibitors was achieved through the creation of CRISPR/Cas9 libraries in breast and lung cancer⁸³. CRISPR-Cas9 knockdown screens in *BRCA1/2*-mutated prostate cancer revealed MMS22L and CHEK2 as novel biomarkers for using PARP inhibitors in prostate cancer treatment⁸⁴. The process of tumor epithelial–mesenchymal transition (EMT) is widely recognized as a major factor contributing to the cancer spread, tissue invasion, and drug resistance. CRISPR/Cas9 screens across the entire genome identified essential genes involved in the EMT process. Elevated levels of epidermal growth factor receptor (*EGFR*) and milk fat globule-EGF factor 8 protein (*MFGE8*) reduce the sensitivity of cancer cells to IFN- γ , which hinders the killing impact of CD8 T cells⁸⁵.

Utilizing CRISPR/Cas9 to target oncogenes or immunological checkpoints is becoming a promising approach for cancer treatment⁷⁸. The *TP53* gene, a well-known tumor suppressor gene, is frequently mutated in various cancer types^{86,87}. Using CRISPR/Cas9 to restore *TP53* function has emerged as a potential approach⁸⁸. Targeting the *KRAS* gene with CRISPR/Cas9 has demonstrated efficacy in inhibiting tumor growth, both in laboratory models and living organisms^{89–91}. Evidence demonstrating the use of CRISPR/Cas9 to target *Pd-11* and enhance cytotoxic T cell infiltration into tumors has validated the efficacy of CRISPR/Cas9-based cancer therapies⁹². Combining sgRNA and Cas9 protein in a plasmid to simultaneously target *Pd-11* and *Cd47* has induced a robust innate and adaptive immune response, resulting in successful inhibition of tumor growth⁹³.

The CRISPR/Cas9 technology has revolutionized the creation of animal models, offering convenience and efficiency for researchers conducting genome-wide screens in cancer therapy studies^{32,33}. Scientists have developed a Cas9 knock-in (KI)

mouse using the Cre-dependent method to investigate the potential of CRISPR/Cas9⁹⁴. They have employed both viral and nonviral vectors to deliver sgRNA targeting tumor suppressor genes and proto-oncogenes. This approach, leveraging the high efficiency of CRISPR/Cas9, led to the successful development of a lung adenocarcinoma animal model, expanding the applications of the CRISPR/Cas9 system. Furthermore, researchers have established a prostate cancer model by transplanting genetically modified prostate epithelial cells into mice's prostate glands, a method that proves more efficient and cost-effective than conventional genetically engineered mouse models⁹⁵.

Chimeric antigen receptor (CAR)-T cell-based cancer therapy has been proven to have great potential in the field of hematological malignancy treatment since the first CAR-T was approved by the FDA for leukemia and lymphoma therapy in 2017^{96,97}. After that, researchers continuously attempt to modify and develop new generations of CAR-T therapies to overcome their deficiencies. Integrating CRISPR/Cas gene editing technology with CAR-T is emerging as a promising strategy^{98,99}. On the one hand, the CRISPR/Cas9-based genome screen has contributed to the target identification for enhancing CAR-T therapy and reducing CAR T-cell cytotoxicity; the CRISPR activation screen has uncovered the *PRODH2* function in improving CAR-T therapy, and another study also proved the critical role of death receptor signaling (TRAIL-R2) in regulating CAR-T cytotoxicity^{100,101}. On the other hand, CRISPR/Cas9-engineered CAR-T provides a new paradigm. Research points out that endogenous T-cell receptors contribute to CD19-CAR-T survival¹⁰². In addition, the elimination of adenosine A2A receptor (A2AR) by CRISPR/Cas9 augmented the CAR-T therapy in mice¹⁰³. The deletion of GM-CSF in CAR-T avoids inflammation-related adverse reactions in xenografts¹⁰⁴. A CD19/CD22 dual-

Table 2 CRISPR-based clinical trials, date provided by ClinicalTrial.gov website (accessed on 22 August).

Serial No.	NCT number	Condition	Phase	Intervention	Study start time
1	NCT06041620	Thalassemia, beta Thalassemia major	Not applicable	Biological: VGB-Ex01	2023-08-31
2	NCT03164135	HIV-1-infection	Not applicable	Genetic: CCR5 gene modification	2017-05-30
3	NCT06465537	Primary open angle glaucoma	Not applicable	Genetic: BD113vVLP	2024-06-10
4	NCT06031727	Neovascular age-related macular degeneration (nAMD)	Early phase 1	Genetic: HG202	2023-09-04
5	NCT03545815	Solid tumor, adult	Phase 1	Anti-mesothelin CAR-T cells	2018-03-19
6	NCT06128044	Acute myeloid leukemia, in relapse Acute myeloid leukemia refractory	Phase 1	Drug: CB-012	2024-02-08
7	NCT03057912	Human papillomavirus-related malignant Neoplasm	Phase 1	Biological: TALEN Biological: CRISPR/Cas9	2018-01-15
8	NCT04990557	COVID-19 respiratory infection	Phase 1 Phase 2	Drug: PD-1 and ACE2 knockout T Cells	2021-08
9	NCT03399448	Multiple myeloma Melanoma Synovial sarcoma	Phase 1	Biological: NY-ESO-1 redirected autologous T cells with CRISPR edited endogenous TCR and PD-1 Drug: Cyclophosphamide Drug: Fludarabine	2018-09-05
10	NCT04037566	Leukemia lymphocytic acute (ALL) in relapse Leukemia lymphocytic acute (all) refractory Lymphoma, B-cell	Phase 1	Genetic: XYF19 CAR-T cell Drug: Cyclophosphamide Drug: Fludarabine	2019-08
11	NCT04637763	Lymphoma, non-hodgkin Relapsed non hodgkin lymphoma Refractory B-cell non-hodgkin lymphoma	Phase 1	Genetic: CB-010 Drug: Cyclophosphamide Drug: Fludarabine	2021-05-26
12	NCT04426669	Gastrointestinal epithelial cancer Gastrointestinal neoplasms Cancer of gastrointestinal tract	Phase 1 Phase 2	Drug: Cyclophosphamide Drug: Fludarabine Biological: Tumor-infiltrating lymphocytes (TIL)	2020-05-15
13	NCT05722418	Relapsed/refractory multiple myeloma	Phase 1	Biological: CB-011	2023-02-06
14	NCT03655678	Beta-thalassemia Thalassemia Genetic diseases, inborn	Phase 2 Phase 3	Biological: CTX001	2018-09-14
15	NCT04925206	Transfusion dependent beta-thalassemia	Phase 1	Biological: ET-01	2021-08-17
16	NCT04244656	Multiple myeloma	Phase 1	Biological: CTX120	2020-01-22
17	NCT05037669	Acute lymphoblastic leukemia Chronic lymphocytic leukemia non-hodgkin lymphoma	Phase 1	Biological: PACE CART19	2022-07
18	NCT04557436	B Acute lymphoblastic leukemia	Phase 1	Drug: PBLTT52CAR19	2020-08-12
19	NCT04438083	Renal cell carcinoma	Phase 1	Biological: CTX130	2020-06-16
20	NCT05566223	Carcinoma, non-small-cell lung Metastatic non-small cell lung cancer Stage IV non-small cell lung cancer	Phase 1 Phase 2	Drug: Fludarabine Drug: Cyclophosphamide Biological: CISH inactivated TIL	2023-02
21	NCT06300723	Sickle cell disease	Not applicable	Drug: BRL-101	2024-07-29
22	NCT03747965	Solid tumor, adult	Phase 1	Biological: Mesothelin-directed CAR-T cells	2018-11
23	NCT05143307	HIV-1-infection	Phase 1	Biological: EBT-101	2023-07-11
24	NCT03872479	Leber congenital amaurosis 10 Inherited retinal dystrophies Eye diseases, hereditary	Phase 1 Phase 2	Drug: EDIT-101	2019-09-26
25	NCT05514249	Duchenne muscular dystrophy	Phase 1	Drug: CRD-TMH-001	2022-08-31
26	NCT03728322	Thalassemia	Early phase 1	Biological: iHSCs treatment group	2019-01
27	NCT06379789	Hemophilia B	Phase 1 Phase 2	Drug: REGV131 Drug: LNP1265	2024-07-25
28	NCT03745287	Sickle cell disease Hematological diseases Hemoglobinopathies	Phase 2 Phase 3	Biological: CTX001	2018-11-27

Table 2 (continued)

Serial No.	NCT number	Condition	Phase	Intervention	Study start time
29	NCT03398967	B cell leukemia B cell lymphoma	Phase 1 Phase 2	Biological: Universal dual specificity CD19 and CD20 or CD22 CAR-T Cells	2018-01-02
30	NCT06025032	Congenital hearing loss	Early phase 1	Genetic: HG205	2024-11-30
31	NCT04035434	B-cell malignancy Non-hodgkin lymphoma B-cell lymphoma	Phase 1 Phase 2	Biological: CTX110	2019-07-22
32	NCT04502446	T cell lymphoma	Phase 1	Biological: CTX130	2020-07-31
33	NCT06492304	T cell lymphoma B cell lymphoma Acute myeloid leukemia	Phase 1 Phase 2	Biological: CTX131	2024-07
34	NCT05477563	Beta-thalassemia Thalassemia Hematologic diseases	Phase 3	Biological: CTX001	2022-08-02
35	NCT02793856	Metastatic non-small cell lung cancer	Phase 1	Drug: Cyclophosphamide Other: PD-1 knockout T cells	2016-08-26
36	NCT04819841	Sickle cell disease	Phase 1 Phase 2	Genetic: Nula-cel drug product	2021-11-15
37	NCT03166878	B cell leukemia B cell lymphoma	Phase 1 Phase 2	Biological: UCART019	2017-06
38	NCT05444894	Transfusion dependent beta thalassemia Hemoglobinopathies Thalassemia major	Phase 1 Phase 2	Genetic: EDIT-301	2022-04-29
39	NCT04417764	Advanced hepatocellular carcinoma	Phase 1	Procedure: Transcatheter arterial chemoembolization Biological: PD-1 knockout engineered T cells	2019-06-20
40	NCT05643742	B-cell lymphoma Non-hodgkin lymphoma B-cell malignancy	Phase 1 Phase 2	Biological: CTX112	2023-03-10
41	NCT05565248	Diabetes mellitus Diabetes mellitus, type 1 Glucose metabolism disorders	Phase 1 Phase 2	Combination product: VCTX211	2023-01-20
42	NCT05795595	Clear cell renal cell carcinoma Cervical carcinoma Esophageal carcinoma	Phase 1 Phase 2	Biological: CTX131	2023-03-13
43	NCT03044743	Stage IV gastric carcinoma Stage IV nasopharyngeal carcinoma T-cell lymphoma stage IV	Phase 1 Phase 2	Drug: Fludarabine Drug: Cyclophosphamide Drug: Interleukin-2	2017-04-07
44	NCT04601051	Transthyretin-related (ATTR) familial amyloid polyneuropathy Transthyretin-related (ATTR) familial amyloid cardiomyopathy Wild-type transthyretin cardiac amyloidosis	Phase 1	Biological: NTLA-2001	2020-11-05

Table 3 Clinical applications of CRISPR/Cas technologies.

Name	Description/company	Time	Ref.
Casgevy	First FDA-approved CRISPR/Cas9-based gene therapy created by Vertex Pharmaceuticals and CRISPR therapeutics for the treatment of SCD	2023-12-08	71,72
Lyfgenia	Another gene therapy approved by FDA for SCD treatment	2023-12-08	73

target CAR-T demonstrated efficacy in clinical trials with no significant adverse events against relapsed/refractory acute lymphoblastic leukemia (ALL)¹⁰⁵. The various applications of CRISPR/Cas9 genetic technology suggest its potential as a groundbreaking tool in cancer research.

4.2. Inherited genetic diseases

Duchenne muscular dystrophy (DMD) is a hereditary monogenic illness caused by mutations in the X-linked dystrophin gene^{106,107}. Given its monogenic nature, CRISPR/Cas9 presents a promising method for targeting mutant exons in DMD therapy,

distinguishing it from other genetic disorders¹⁰⁸⁻¹¹¹. Initial research utilized CRISPR/Cas9 to rectify DMD by focusing on the germ line of mdx mouse models, suggesting the potential application of CRISPR/Cas9 for treating DMD in patients' muscle tissue¹¹². Applying CRISPR/Cas9 to remove the mutant exon 23 from mdx animals has resulted in improved muscle biochemistry and increased muscular force¹¹³. Most mutations of DMD are concentrated in certain regions, making it effective to target these areas during RNA splicing for DMD therapy. Researchers employ this hypothesis to select optimal guide RNAs by sgRNA screening to achieve exon skipping of DMD-specific mutations. This approach has led to the detection of restored dystrophin expression in gene-edited pluripotent stem cells (iPSCs)¹¹⁴.

Studies utilizing CRISPR/Cas9 to target DMD-related exon-44 have shown significant improvement in symptoms linked with the condition^{115,116}. Over 70% dystrophin expression was detected following intramuscular administration of CRISPR/Cas9 targeting exons-53¹¹⁷. Large-scale restoration of dystrophin expression in primary muscle-derived stem cells from DMD patients has been demonstrated through the deletion of exons^{118,119}. Exon 50 deletion was utilized in creating a mouse model¹²⁰. Integration of CRISPR/Cas9 with other technologies has enhanced our understanding of DMD pathophysiology by providing a platform for research. Furthermore, the efficacy of CRISPR/Cas9 for treating DMD in the deltaE50-MD dog model provided additional evidence of its clinical promise¹²¹. However, Cas9-specific immunogenicity was observed in dogs when CRISPR/Cas9 was used to target DMD¹²². The FDA's approval of the first gene therapy, delivering a gene encoding an anti-muscular dystrophy protein for DMD, marks a significant milestone¹²³. Additionally, CRISPR/Cas9-based gene therapy holds promise for the future of DMD treatment.

Leber congenital amaurosis (LCA) was referred to as a genetic disorder accompanied by multiple genetic mutations, such as *CEP290*, and *RPE65*, the most common gene mutation in LCA, and in 2017, the FDA approved the first gene therapy *in vivo* for treating inherited retinal diseases¹²⁴⁻¹²⁸. Studies from CRISPR/Cas9-mediated gene editing targeting *CEP290* restored the expression of *CEP290* in the LCA10 model indicating CRISPR/Cas9 can be a promising tool for LCA treatment¹²⁹. Another study using CRISPR/Cas9 in a humanized *CEP290* mouse model also made great progress¹³⁰. Additionally, CRISPR/Cas9-based gene editing toward *Rpe65* nonsense mutation in mice LCA model effectively improved the retinal functions without obvious adverse events¹³¹. Subretinal injection ABE targeting *KCNJ13* mutation corrected the pathogenic gene and restored the vision function¹³².

Phenylketonuria (PKU) was identified as an autosomal recessive disease with a lack of phenylalanine hydroxylase (PAH) and a high concentration of phenylalanine caused by mutation of *PAH* gene and the emergence of CRISPR/Cas9 provides a potential tool for PKU treatment^{133,134}. Co-delivery of saCas9 and sgRNA *in vivo* targeting *R408* mutation results in a reduction of phenylalanine¹³⁵. Similarly, a high PAH expression and low phenylalanine levels were observed in base editors treated PKU mice model¹³⁶. ABEs-based gene editing in the *P281I* variant PKU mouse model effectively corrects the *Pah* gene and rapidly normalizes the blood phenylalanine levels¹³⁷. Other studies using prime editing in the *Pah^{emu2}* mutation mice model also enabled phenylalanine to restore to a normalized concentration^{138,139}.

4.3. Allergy and immunological disorders

Research into the utilization of the CRISPR/Cas9 system to modify immune system function and investigate its role in

allergies and immunological disorders has been conducted^{16,140}. The application of CRISPR/Cas9 technology for editing allergy-related genes showcases its broad applicability and offers novel perspectives on treating allergic conditions^{14,141,142}. Genetically modified dendritic cells have been generated utilizing CRISPR/Cas9 to modify molecular expression and suppress allergen-specific Th2 immune response, thereby mitigating allergy diseases¹⁴³. *In vivo* CRISPR/Cas9 experimentation has elucidated the specific immunoregulatory function of IL-37b in Th2-related allergic inflammation in atopic dermatitis¹⁴⁴. Furthermore, a CRISPR screen revealed the homeobox protein ADNP as crucial for the Th2 cell-mediated allergy immune response¹⁴⁵.

Genome-wide CRISPR screens were employed to discover the route responsible for the differentiation of B cells into IgE⁺ plasma cells¹⁴⁶. A recent study utilizing such screens discovered that B cell factor 4 regulates Fas-induced apoptosis and T-lymphocyte maturation¹⁴⁷. Treating polyendocrinopathy enteropathy and X-linked (IPEX) syndrome by rectifying mutations in the forkhead box protein 3 (*FOXP3*) gene offers a method to address inherited autoimmune disorders¹⁴⁸. *In vivo* studies using CRISPR/Cas9 gene-edited zebrafish models alongside clinical data analysis have shed light on genetic factors contributing to inflammatory conditions¹⁴⁹. CRISPR/Cas9-mediated KI mice coupled with gene sequencing have aided in identifying causal genes in systemic lupus erythematosus¹⁵⁰. CRISPR/Cas9 libraries and models have significantly contributed to pinpointing the main causative components in this disease¹⁵¹⁻¹⁵³. Regulatory T (Treg) cells are essential for regulating the immune system to prevent an overactive immunological response¹⁵⁴. Through a CRISPR/Cas9 screening platform, various regulators of *Foxp3* have been identified¹⁵². A review has been conducted on the use of CRISPR-modified Treg cells for treating autoimmune diseases¹⁵⁴. A recent study introduced a strategy that involves using CRISPR/Cas9-edited Treg cells to regulate immune tolerance. The research focused on developing antigen-specific Treg cells with a strong and consistent *FOXP3* by genetically modifying CD4⁺ T cells. These Treg cells were found to be capable of effectively managing the immune response mediated by effector T cells¹⁵⁵. Overall, the CRISPR/Cas9 system holds great promise for treating allergy and immunological disorders.

4.4. Eye-related disorders

CRISPR/Cas9 has introduced innovative techniques and instruments for studying and treating eye-related diseases¹⁵⁶⁻¹⁵⁹. By targeting the *Nrl* gene, which encodes a protein specific to the neural retina, researchers have enhanced the survival of rod cells and improved recovery from retinal degeneration across three animal models¹⁶⁰. Furthermore, CRISPR/Cas9 has been instrumental in identifying a super-enhancer in the retina of mouse models, shedding light on the underlying mechanisms of retinal degeneration^{161,162}. Large-scale gene mutagenesis, coupled with RNA-seq analysis, has confirmed the significant involvement of the WNT pathway in the progression of retinal neural cell development¹⁶³.

Genetic editing on patient-derived iPSCs has accelerated drug discovery for age-related macular degeneration (AMD)¹⁶⁴. Removing the nonsensical mutation in *Rpe65* improved retinal function without causing any negative effects¹³¹. Additionally, research has found the role of thyroid hormones in modulating cone subtypes¹⁶⁵.

CRISPR/Cas9-mediated *in vivo* safety assessment was conducted in three patients with herpetic stromal keratitis, revealing

no off-target cleavages or adverse events during long-term treatment¹⁶⁶. Moreover, CRISPR/Cas9 has been employed to reduce intraocular pressure (IOP) as part of glaucoma treatment¹⁶⁷. These advancements highlight the potential of CRISPR/Cas9 in revolutionizing the treatments for various eye disorders.

4.5. Blood-related disorders

Transfusion-dependent β -thalassemia (TDT) and sickle cell disease (SCD) are monogenic disorders caused by a genetic mutation in the β -globin gene^{168,169}. CRISPR/Cas9 gene therapy has been used to target β -globin for homologous recombination, serving as a model for blood-related disease¹⁷⁰. Recent research has identified the heme-regulated inhibitor (HRI) as a suppressor of fetal hemoglobin (HbF), with a potential regulatory link between *HRI* and *BCL11A*¹⁷¹. Further investigations revealed the regulatory interaction between *HRI* and *BCL11A*, involving the transcription factor ATF4¹⁷². A CRISPR/Cas9 genetic screen revealed the influence of *NF1A* and *NFIX*, members of the NFI transcription factor family, in *BCL11A* expression, thus affecting HbF suppression¹⁷³.

Exploring multiplex mutagenesis to target the *HBG* promoter and *BCL11A* enhancer holds promise as a therapeutic approach to enhance HbF expression¹⁷⁴. Additionally, editing genes in the α -globin enhancer could be a viable method for treating TDT¹⁷⁵. Two clinical trials have utilized gene editing to target specific regions in hematopoietic stem cells, resulting in increased HbF levels, improved transfusion independence, and reduced vaso-occlusive events in patients with TDT and SCD¹⁷⁶. These promising outcomes suggest the potential of CRISPR/Cas9 as a treatment modality for these conditions. Both preclinical and clinical studies are currently focused on utilizing CRISPR/Cas9 for the treatment of blood-related disorders.

4.6. Cardiovascular disorders

Cardiovascular diseases remain the leading cause of death globally, and the progress in CRISPR technology has aided in the comprehension and treatment of cardiovascular disease¹⁷⁷⁻¹⁸⁰.

Genome-wide screening using CRISPR/Cas9 in mice has identified the crucial regulator in the development of cardiomyocytes¹⁸¹. Similarly, a CRISPR/Cas9-based genetic zebrafish model combined with quantitative proteomics has elucidated a regulator of heart development¹⁸². Notably, removing *Wntless* in living organisms has shown the crucial function of *Yap* in controlling Wnt signals for the process of heart regeneration¹⁸³.

Studies investigating Bicuspid aortic valve (BAV) formation underscore the potential of CRISPR/Cas9 in cardiovascular research¹⁸⁴. Furthermore, research employing CRISPR/Cas9-based knockout (KO) technology has demonstrated that early depletion of *CARMN* lncRNA accelerates atherosclerosis progression¹⁸⁵. A study conducted in 2021 utilized ABE to target the *PCSK9* gene in primates. The study examined the impact of long-term *in vivo* use of gene editors and found that *PCSK9* was completely suppressed in the liver, and 90% in the blood, leading to a 60% decrease in low-density lipoprotein cholesterol¹⁸⁶. These findings confirm the viability of clinical trials. While two clinical adverse events have raised concerns, subsequent follow-up studies have demonstrated the drug's efficacy and safety in nonhuman primate and mouse models over extended periods, providing compelling evidence for clinical investigation¹⁸⁷.

4.7. Viral infection

The CRISPR/Cas9 system, originally used by prokaryotes as a defense mechanism against viruses, shows promise as a tool to combat viral infections. This can be achieved by directly targeting the viruses or by influencing critical host variables during infection¹⁸⁸.

Numerous studies have identified protein HMGB1, RAB7A, and the *GATA6* gene as crucial host factors for SARS-CoV-2 infection, acting as regulators of the angiotensin-converting enzyme 2 (ACE2), the primary receptor for the virus¹⁸⁹⁻¹⁹¹. Genomewide CRISPR KO screens have found that the placenta-associated 8 protein (PLAC8) can prevent viral infection by decreasing the production of virus-associated RNAs¹⁹². Researchers are further investigating host factors involved in SARS-CoV-2 infection using CRISPR/Cas9 technology^{193,194}.

Utilizing the CRISPR/Cas9 system to target the reverse-transcribed products of HIV shows promise as a preventive approach against the virus¹⁹⁵. Host factors CD4 and CCR5, which are HIV co-receptors, have been studied for targeted drug development¹⁹⁶. Furthermore, *TPST2*, *SLC35B2*, and *ALCAM* have been identified as critical host factors, and notably, they were not essential for cell survival. CRISPR/Cas9 was used to assess medication combinations for their effectiveness against HIV infection.

Additionally, CRISPR/Cas9 technology has been applied to study antiviral responses against influenza virus infections^{197,198}, further expanding its utility in combating viral diseases.

4.8. Neurological disorders

Neurological disorders are diseases resulting from abnormal changes in the central and peripheral nervous systems¹⁹⁹. Despite significant efforts, understanding the mechanism and identifying therapeutic targets continues to be challenging. The use of CRISPR-based genetic technology could be a promising strategy to address these challenges^{200,201}.

Targeting mutant Huntingtin (HTT) deletion in the adult brain using CRISPR/Cas9 reduced motor impairments²⁰². This approach is considered safer and more successful compared to the embryonic model in mice, where the absence of HTT results in embryo death. Moreover, CRISPR-based genetic KI technology has been used to insert 150 CAGs into the HTT gene of pig fibroblast cells to create a large repeat associated with Huntington's disease (HD)²⁰³. CRISPR/Cas9 was then utilized to develop the HD KI model, which displayed symptoms related to HD^{204,205}. These researches highlight the potential of CRISPR/Cas9 in generating neurological animal models.

Multiple studies investigating the mechanism of Huntington's disease have highlighted the use of the CRISPR/Cas9 system in the realm of neurological illnesses, offering new avenues for research and potential therapeutic interventions²⁰⁴⁻²⁰⁶.

The APOE4 iPSC was generated through genetic modifications from APOE3. RNA-seq analysis compared the APOE4 and APOE3 iPSC-derived neurons, astrocytes, and microglia-like cells, revealing a close association between APOE4 and Alzheimer's disease²⁰⁷. Additionally, the CRISPR/Cas9 technique unveiled the molecular foundation of astrocytes, offering a framework for studying brain diseases^{208,209}. A different study utilizing CRISPR technology discovered the involvement of *TREM2* in the advancement of Alzheimer's disease²¹⁰. Furthermore, activating the *Mt1* gene with CRISPR/dCas9 significantly enhanced cognitive function in animal models with Alzheimer's disease²¹¹. Research on Parkinson's illness also supports the

potential use of CRISPR/Cas9 in treating neurological disorders, highlighting the significant promise of CRISPR/Cas9-based gene therapy for neurological diseases²¹²⁻²¹⁴.

4.9. Other disorders

Type 2 diabetes (T2D) is a metabolic disease that affects several organs. Despite limited current medications, the application of CRISPR/Cas9 in diabetes research holds promise²¹⁵. Genetically modified epidermal progenitor cells demonstrated the ability to produce glucagon-like peptide 1 (GLP-1), effectively regulating blood glucose levels impacted by a high-fat diet²¹⁶. Similarly, CRISPR-modified human brown adipocytes showed significant promise for diabetes treatment^{217,218}. CRISPR-based genetic technology revealed the genetic interactions between non-coding mutations and T2D²¹⁹. A genome-wide screening of the human pancreatic beta cell line identified the autophagy receptor CALCOCO2 as the key regulator in Type 2 Diabetes (T2D)²²⁰.

Cystic fibrosis (CF) arises from genetic abnormalities in the CFTR gene, and advancements in gene editing techniques now offer the potential to correct these mutations. The CFTR *F508* deletion mutation, which involves the deletion of phenylalanine at position 508 in exon 11, is a prevalent genetic mutation in CF patients²²¹⁻²²³. Research utilizing CRISPR/Cas9-based HDR gene insertion editing technology has advanced in rectifying CFTR²²⁴. ABE with SpCas9 and xCas9 was employed to effectively rectify the nonsense mutations in CF organoid biobank²²⁵. By employing CRISPR/Cas9-modified patient-derived iPSC macrophages and gene sequencing, insights into the impact of type I IFN signaling on CF macrophages have been elucidated²²⁶. Additionally, CRISPR/Cas9-induced gene KO helped uncover the roles of *TMEM16A* and *SLC26A4* in controlling the pH of the airway surface fluids in CF²²⁷.

The CRISPR/Cas9 gene editing technique has demonstrated significant potential in correcting genetic errors and has been applied in research linked to genetic diseases. Despite the

substantial efficacy demonstrated in preclinical animal models and clinical trials, concerns persist regarding targeted distribution and potential side effects.

5. The delivery system for CRISPR/Cas9 application

Significant advancements have been achieved in human disease research through the application of CRISPR/Cas9-based gene therapy in both pre-clinical and clinical trials. Delivering nucleic acid to the target site securely and efficiently to protect CRISPR/Cas9-based therapeutics from degradation and prevent off-target effects is a challenging task²²⁸⁻²³⁰. Various viral and non-viral vectors have been identified as potential drug delivery systems for CRISPR/Cas9-mediated gene therapy²³¹⁻²³³ (Table 4).

5.1. Viral vector

5.1.1. Lentivirus vector

Viral vector-mediated CRISPR/Cas9 delivery is being widely used for *in vivo* and *in vitro* gene therapy (Fig. 4A)²³⁴. Lentivirus (LV) is a retrovirus derived from Human Immunodeficiency Virus I (HIV-1) that contains double-stranded RNA. LV demonstrates high infection efficiency and can integrate its genome into host cells randomly to create cells expressing certain genes. However, its application in living organisms is limited due to restricted tolerance to low temperatures²³⁴⁻²³⁶. Despite this limitation, LV-mediated CAR-T cell therapy has been approved for the treatment of ALL^{237,238}.

A CRISPR/Cas9 collection provided by LV for high-throughput screening can help identify essential genes related to specific risks^{239,240}. Suh²⁴¹ utilized an LV platform to deliver ABE to restore visual function in mice. LV vectors have a greater capacity to transfer nucleic acids compared to AVVs, resulting in improved gene editing efficiency. Utilizing lentiviral vectors pseudotyped with modified hepatitis C virus (HCV) envelope

Table 4 Advantages and limitations of various delivery systems.

Delivery system	Advantage	Limitation
Lentivirus vector	High infection efficiency and it is suitable for creating cells expressing certain genes	LV randomly integrated genomes raised concerns
Adeno-associated virus vector	High safety and low immunogenicity can infect multiple cells and have a wide host range	Their efficacy is hindered by a limited packaging capacity
Adenoviral vector	AD has a safety advantage and typically has a larger genetic capacity	Their limited cell infectivity and potential to evoke immune responses hindered their application
Liposome nanoparticles	Prolonged circulation, enhanced biocompatibility, and controlled drug release	Complex processes and high costs limit their application
Polymer nanoparticles	Effectively loaded nucleic acids, high transfection efficiencies	Enhanced cationic materials are cytotoxic
Biomimetic nanoparticles	Enhanced biocompatibility and targeting ability, low immunogenicity, are cost-effective and easily accessible	Complex process, difficult to produce on a larger scale
Extracellular vesicles	Improved drug stability in biofluids for carrying cargo for long-distance transport	Limited targeting ability and transfection efficiency restrict its application
Inorganic nanoparticles	Versatile dimensions and specialized forms	Low loading capacity, limited targeting ability, and toxicity
Metal-organic framework	Displayed multifunctionality and structural flexibility	Drug release and toxicity remain to be studied extensively
Virus-like particle	Protecting cargo molecules from degradation, enhanced cell-penetrating property	High immunogenicity
Extracellular contractile injection systems	More specific binding to target cells	The delivery efficiency and <i>in vivo</i> long-term application still need to be studied extensively

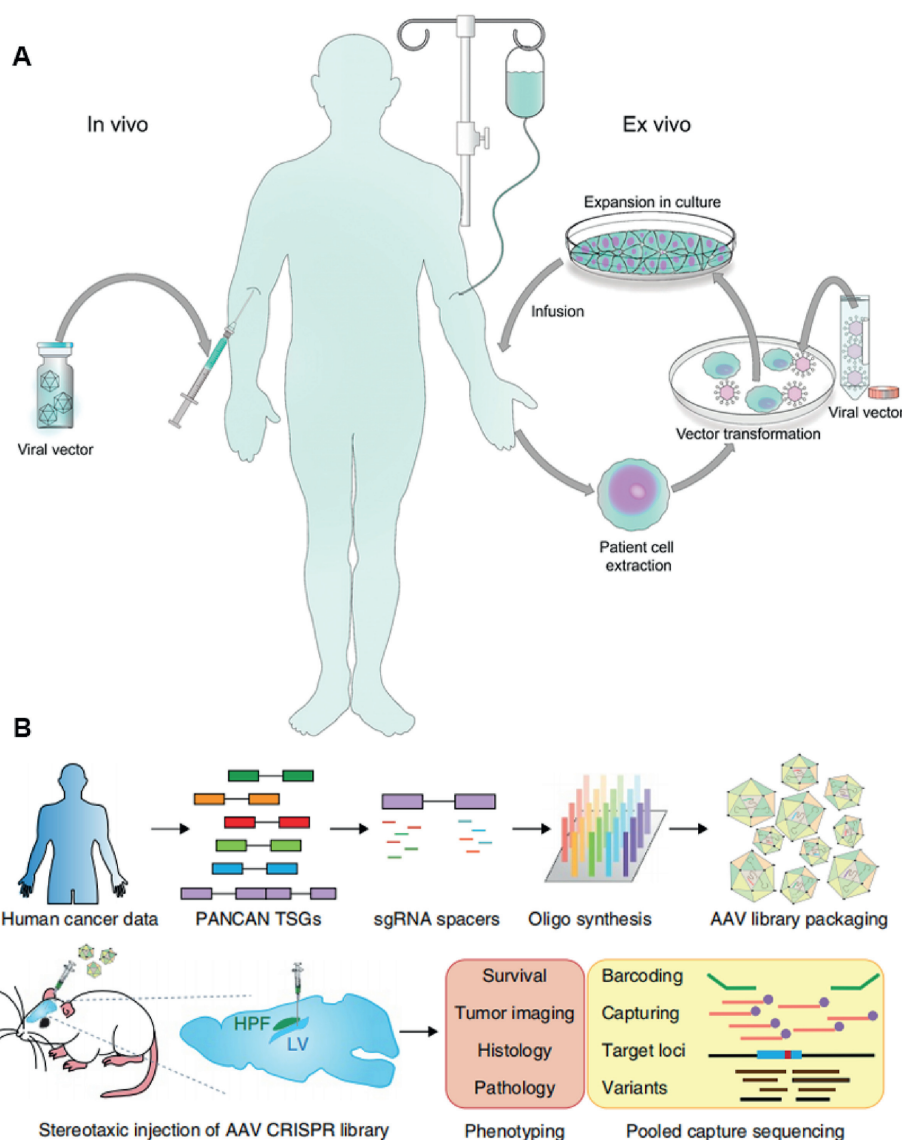


Figure 4 Schematic illustration of viral vector-mediated CRISPR/Cas9-based gene therapy. (A) Strategies of viral vector delivered CRISPR/Cas9 *in vivo* and *ex vivo*. Reprinted with the permission from Ref. 234. Copyright©2021, The Authors; (B) sgRNA library was packed by AAV to screen and identify functional suppressors in glioblastoma. Reprinted with the permission from Ref. 251. Copyright©2017 Springer Nature.

glycoproteins (E1/E2) enhanced the targeting ability and effectively transported CRISPR/Cas9 for cancer treatment²⁴². LV-mediated delivery of dCas9 addresses renal fibrosis²⁴³. Lentivirus-like bionanoparticles (LVLs) encapsulating large saCas9 and mRNA achieve robust, temporary expression, significantly reducing off-target effects²⁴⁴. mRNA containing spCas9 and sgRNA was delivered directly to retinal pigment epithelium (RPE) cells to target vascular endothelial growth factor A (*Vegfa*) using LV vectors²⁴⁵. The optimized LV efficiently transported the mRNA to the desired location and demonstrated a high level of gene editing effectiveness without causing inflammation or off-target effects. An approach utilizing integrase-deficient LV effectively achieved the insertion of large gene fragments and maintained stable expression to address the drawbacks of random insertion and the possibility of gene silencing in lentiviral vector delivery²⁴⁶.

5.1.2. Adeno-associated virus vector

Adeno-associated virus (AAV) is a non-enveloped single-stranded DNA virus known for its low immunogenicity. Unlike LV, AAV does not integrate into the genome, has a superior safety profile. However, its efficacy is hindered by a limited packaging capacity^{247,248}. Despite this limitation, numerous gene therapies based on AAV have received approval^{249,250}.

The AAV packaging of sgRNA libraries effectively overcomes the obstacles of the brain microenvironment for *in vivo* gene screening. This approach, combined with survival analysis, MRI imaging, histology, and pathology, facilitates the identification of critical glioblastoma pathogenesis factors (Fig. 4B)²⁵¹.

Mouse models are developed by inducing Cas9-specific expression in skeletal muscle fibers using Cre-mediated methods, accompanied by the delivery of sgRNA *via* AAV²⁵². Min¹¹⁵ utilized AAV as a carrier for delivering sgRNA and Cas9 to

rectify DMD exon deletion mutations both *in vitro* and *in vivo*. The work conducted in mouse models showed a positive correlation between gene editing efficiency and the dosage of AAV. However, the clinical applicability of high-dose AAV was limited.

To address this limitation, researchers developed a self-complementary AAV approach to manage high-dose AAV *in vivo*¹¹⁶. This method involves enclosing Cas9 and sgRNA in single-stranded AAV and self-complementary AAV, respectively. *In vivo* animal studies suggest that this delivery method enables effective gene editing at low doses.

Various studies have progressed in enhancing AAV-based CRISPR/Cas9 delivery methods to efficiently package sgRNA and Cas9 using minimal amounts^{253–256}. Cas9-specific immunity posed challenges for AAV-delivered gene therapy^{122,257}. Moreno²⁵⁸ confirmed the potential for enhancing the effectiveness of AAV-mediated gene editing using orthologous Cas9 proteins. A study on prolonged AAV-CRISPR genome editing reveals the possible genetic changes induced by AAV-CRISPR²⁵⁹.

5.1.3. Adenoviral vector

Adenovirus (AD) is a non-enveloped, dsDNA virus that was among the first viral vectors authorized for gene therapy. Unlike some vectors, Ad does not integrate into the genome, providing a safety advantage. Adenovirus vectors (ADV) typically have a larger genetic capacity, but their limited cell infectivity and potential to evoke immune responses are notable concerns²⁶⁰.

ADV-mediated CRISPR/Cas9 delivery holds promise in cancer therapy. Administered CRISPR/Cas9 for suicide gene insertion along with ganciclovir effectively hinders tumor progression²⁶¹. In xenograft mouse models, mutant *EGFR* alleles were precisely targeted and eliminated using CRISPR/Cas9 delivered *via* ADV²⁶². Gao⁸⁹ utilized an AD delivery system to transport sgRNA and Cas9/dCas9 targeting *KRAS G12S*, resulting in tumor regression. A gene-wide screening using CRISPR/SaCas9 delivered by ADV revealed that the *MECOM* gene could serve as a potential therapeutic target for lung squamous cell carcinoma. Deletion of *MECOM* was found to suppress cancer stem cell characteristics. To enhance gene delivery efficiency, two functional proteins were attached to the ADV: a single-chain fragment variable targeting epithelial cell adhesion molecules and a shield protein. These proteins improved the targeting ability of ADV and shielded it from degradation²⁶³. Genetically modified ADV was created by chemically attaching the RNP complex to the AD capsid surface and applying gene editing in living organisms²⁶⁴. Encapsulating AD vector encoding Cas9 and *Pd-II* sgRNA in a hydrogel resulted in prolonged and effective *Pd-II* gene KO²⁶⁵. In liver administration, CRISPR-based primer editing can be achieved alongside CRISPR/Cas9 using ADV¹³⁸. Li²⁶⁶ utilized ADV to introduce the γ -globin-encoding gene and disrupt the γ -globin repressor for SCD *in vivo*. Nonetheless, reducing immune responses and enhancing ADV targeting specificity remain challenging tasks²⁶⁷.

Viral vectors are becoming a potent tool for delivering CRISPR/Cas9. Several viral vector-based gene therapies have already been applied in clinical settings to treat various disorders. However, enhancing the safety and precision of viral vectors requires substantial effort and ongoing research.

5.2. Non-viral vector

In addition to viral vectors, non-viral drug delivery techniques are crucial for transporting therapeutic nucleic acid. Non-viral vectors

have been thoroughly studied recently for their enhanced safety and efficient packaging capacities as compared to viral vectors^{228,268}. Various cationic materials have been utilized to encapsulate gene-encoding CRISPR/Cas9 systems *via* electrostatic adsorption^{269,270}. Additionally, exosomes, which are nanoparticles derived from biological membranes, are considered efficient carriers for gene delivery^{271,272}. Inorganic nanoparticle-modulated gene therapy is frequently combined with various medications to enhance therapeutic outcomes^{273–275}. Non-viral vectors can be further improved by incorporating different molecules and peptides to enhance blood circulation and targeting capabilities compared to viral vectors^{276,277}. Furthermore, modification of non-viral vectors allows for intelligent-responsive drug release mechanisms^{229,278–281}.

5.2.1. Liposome nanoparticles

Liposomes are bilayer drug vesicles generated by lipids by self-assembly, known for their prolonged circulation, biocompatibility, and controlled drug release²⁸². These liposomes are composed of ionizable lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-lipids^{283–285}. Liposomes were initially permitted for delivering doxorubicin for cancer treatment in 1995²⁸⁶. In 2018, they were employed to transport nucleic acid drugs for treating transthyretin familial amyloid polyneuropathy, and more recently, they have played a crucial role in delivering mRNA vaccines for COVID-19^{287,288}. Liposomal vector-based nucleic acid medicines show significant promise in both preclinical and clinical trials^{289–291}.

Cholesterol, DSPC, PEG–DMG, and DSPE–PEG were selected to fabricate liposome nanoparticles (LNP) for encapsulating mRNA encoding Cas9 and sgRNA, resulting in higher gene editing efficiency compared to current LNPs²⁹². A plasmid containing Cas9 and sgRNA designed to target the *MTH1* gene was immobilized onto DSPE–PEG through electrostatic adsorption²⁹³. Kenjo²⁹⁴ developed an LNP platform using novel chemical lipids allowing multiple injections of the CRISPR/Cas9 system with reduced immune response. A study on utilizing bioorthogonal reaction for targeted tumor immunotherapy using LNP was published²⁹⁵. Lipid-polymer hybrid nanoparticles were found to encapsulate a large plasmid containing the CRISPRi system effectively²⁹⁶. Similarly, LNP can be utilized to deliver the ABE and assess its effectiveness in the luciferase reporter mouse model²⁹⁷. Liposome-coated mesoporous silica enhances nanoparticle stability and facilitates cellular uptake of the Cas9 protein/guide RNA RNP complex²⁹⁸. Furthermore, Lipid-coated gold nanoparticles enable the accumulation of nanoparticles at the tumor site and the controlled release of plasmid caused by photothermal effects²⁹⁹.

Cheng³⁰⁰ developed a method that enabled LNP to achieve targeted delivery of CRISPR/Cas9 to certain organs by strategic manipulation of lipid composition (Fig. 5). Screening a dendrimer-based library identified the optimal lipid composition for accurate HDR using CRISPR/Cas9, based on amine cores and alkyl chain lengths³⁰¹. In a previous study, an ionizable amino lipid called 5A2-SC8 was utilized to investigate an LNP capable of delivering three nucleic acids³⁰². By adjusting the mass ratio between 5A2-SC8, cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and PEG-DMG, the LNP showed not only high loading capacity but also maintained size stability for up to 72 h. Optimizing the molar ratio of 5A2-SC8/DOPE/Chol/PEG-DMG enhances the specificity of LNPs for targeting the lung and enables gene repair in CF animals²²⁴.

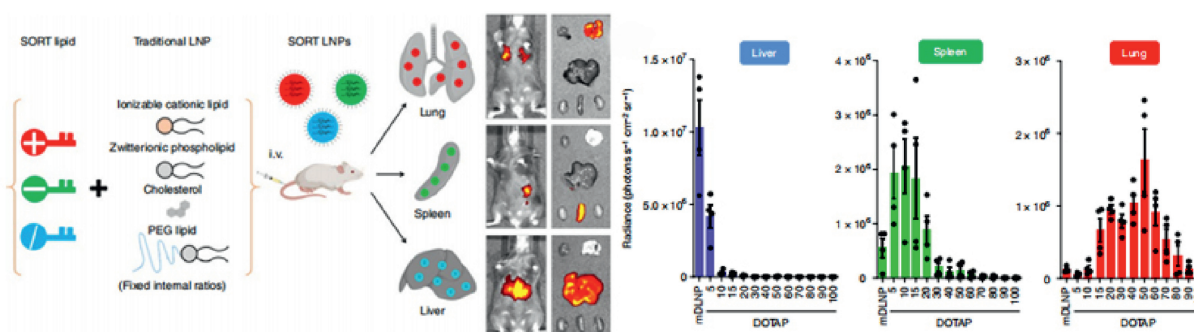


Figure 5 The development of selective organs targeting LNP via optimizing the composition of lipids, utilizing the various lipids results in the specific accumulation in the liver, spleen, and lung respectively. Reprinted with the permission from Ref. 300. Copyright©2020 Springer Nature.

Additionally, Li³⁰³ reported a three-component reaction (3-CR) LNP platform, composed of 720 lipids, for pulmonary mRNA-encoding Cas9 delivery. Intratracheal administration of mLuc-LNPs and CRISPR–Cas9 gene editors exhibited significantly higher pulmonary delivery efficiency than MC-3 LNP. Altering the size and zeta potentials of LNP has been shown to enhance gene editing effectiveness^{269,284}. Similarly, regulating the pH and structure of phospholipids allows organ targeting³⁰⁴. A study found that using cholesterol with varying charges can enable targeting specific tissues, offering a novel approach for delivering CRISPR/Cas9 through LNP technology³⁰⁵. The research demonstrated the significant potential of LNP for delivering CRISPR/Cas9 and offered a model for enhancing the effectiveness of the LNP-mediated delivery system.

5.2.2. Polymer nanoparticles

Polymer nanoparticles (PNPs) were evaluated as promising carriers for delivering CRISPR/Cas9. Polymer carriers can efficiently condense nucleic acids, enhance stability, extend blood circulation, and facilitate active targeting via material modification^{306,307}. They are formed through electrostatic interactions between cationic polymers and nucleic acids²². Importantly, cationic polymers significantly aid in attaining lysosomal escape by utilizing the proton sponge effect.

High transfection efficiency is essential for successful gene delivery. Li³⁰⁸ developed a CRISPR/Cas9 delivery system called PF33 by modifying polyethyleneimine (PEI) with fluorine (Fig. 6A). The fluorinated polymers enabled vectors to efficiently transport plasmids, resulting in approximately 90% transfection efficiency. Moreover, PF33 successfully delivered CRISPR/Cas9 to target oncogenes, leading to a high KO efficiency. In a subsequent study, researchers created a “core–shell” structure nanoparticle by coating hyaluronic acid (HA) on PF33 (Fig. 6B)⁹³. This modification endowed the nanoparticles with properties such as active targeting of tumor surface receptors and sensitivity to tumor microenvironment enzymes. Once taken up by tumor cells, HA undergoes degradation, triggering a proton sponge effect to facilitate lysosomal escape, which is crucial for gene transfection. Ultimately, PNP loaded with CRISPR/Cas9 were employed to reverse the immunosuppressive tumor microenvironment. Furthermore, plasmid-encoding CRISPR/dCas9 can be efficiently enclosed within PNP and utilized for activating endogenous therapeutic genes without inducing DSB (Fig. 6C and D)^{20,309}.

Controlling the on/off switch for gene editing may help reduce off-target effects, which is still a difficulty. A recent study detailed the use of a fluorinated dendrimer-based PNP combined with a

laser-activated specific promoter CRISPR/Cas9 system. Upon exposed to a 660 nm laser, Cas9 can be transcribed and translated, and subsequently loaded with chlorin e6 to induce an immunogenic cell death (ICD) effect³¹⁰. Semiconducting PNP offer precise control of gene editing. These PNPs were synthesized in a single step using amphiphilic PAEs and a semiconducting polymer. Cas9 enables precise gene expression through mild heating or targeted ultrasound, facilitating genetic editing of *Hsp70* and *Bag3*³¹¹. Semiconducting polymers including PF and PEG chains were used for gene editing and imaging guided by near-infrared-II (NIR-II) light³¹². Progress has also been made in delivering RNP complexes *in vitro* targeting *TP53* and following the process through pictures using PEI-mediated PNP³¹³.

Various drug release mechanisms can be achieved by utilizing different materials due to the versatility of PNP. For instance, mPEG-PC7A was selected as a pH-sensitive amphiphilic copolymer for enclosing RNP complexes³¹⁴. Additionally, a ROS-sensitive cationic polymer was employed to transport CRISPR/Cas9³¹⁵. Liu³¹⁶ developed a virus-like nanoparticle to distribute CRISPR/Cas9 and small molecule medicines simultaneously in a reducing microenvironment. This approach enhances the therapeutic efficacy by combining gene editing with traditional drug treatments. Furthermore, cationic polymers that have been functionalized and modified possess the ability to penetrate the BBB and are sensitive to glutathione³¹⁷. This feature is particularly valuable for targeting neurological disorders. A pH/GSH dual-responsive prodrug nanoparticle was modified to enhance tumor ferroptosis, a form of regulated cell death³¹⁸. Moreover, endogenous acid-responsive and exogenous light-activated CRISPR/Cas9 systems have been explored to alleviate tumor immunosuppression³¹⁹.

The PNP delivery system has revolutionized gene therapy by enhancing flexibility and diversity. PNPs can be precisely engineered, utilizing a wide range of sensitive polymer materials to release drugs in response to specific tumor microenvironments. This controllable approach to gene editing has significantly mitigated off-target effects and bolstered the safety profile of gene therapy.

5.2.3. Biomimetic nanoparticles

Researchers can utilize LNP or PNP to encapsulate CRISPR/Cas9 for passive targeting via the Enhanced Permeability and Retention (EPR) effect. By incorporating additional ligands into the nanoparticles, they can achieve active targeting and improve blood circulation. However, the complex chemistry process and expensive nature of nanoparticles have impeded their clinical application^{320,321}. Biomimetic nanoparticles have become a novel gene

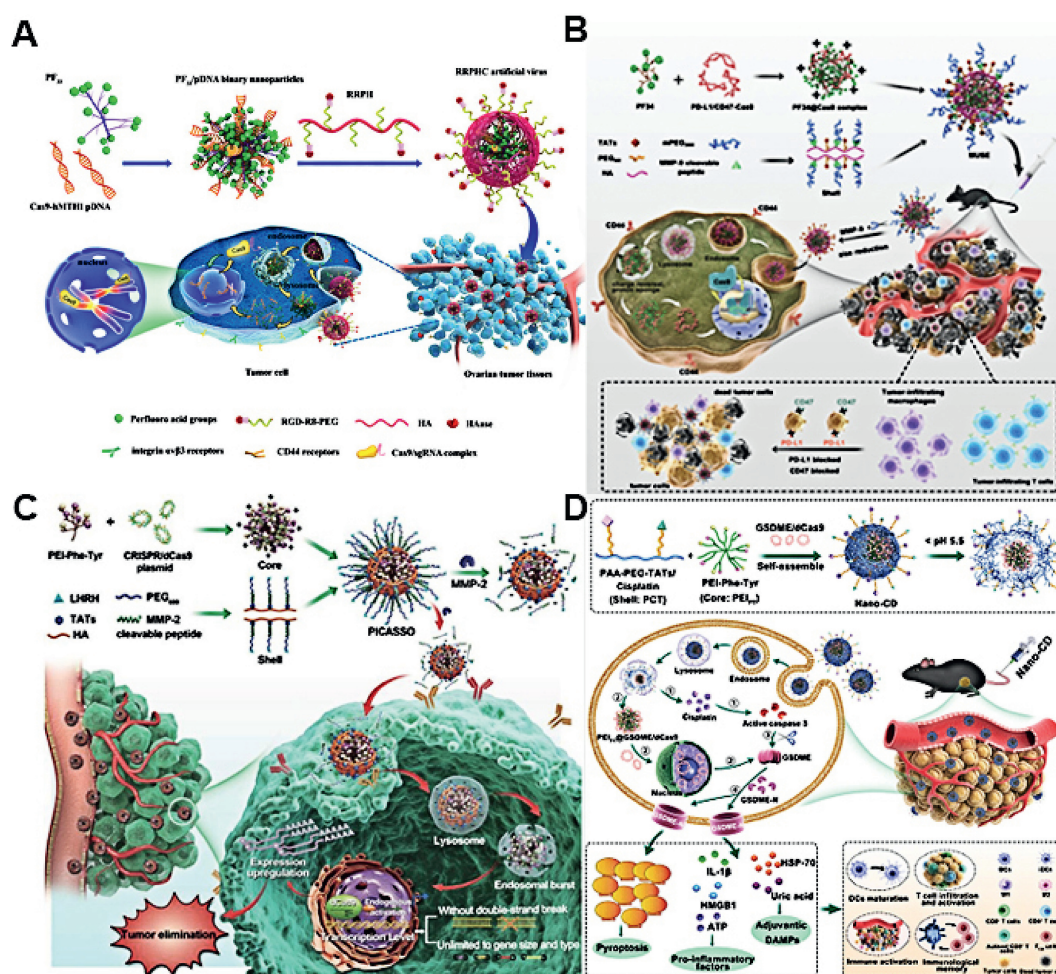


Figure 6 Representative examples of PNP delivered CRISPR/Cas9 system. (A) Synthesis of PF for highly efficient delivery CRISPR/Cas9 for gene editing *in vivo* and *in vitro*. Reprinted with the permission from Ref. 308. Copyright©2017, American Chemical Society; (B) Multistage responsive "core-shell" PNP for KO immune checkpoint PD-L1 and CD47 simultaneously. Reprinted with the permission from Ref. 93. Copyright©2020 John Wiley and Sons; (C) Reprinted with the permission from Ref. 20. Copyright©2021 The Authors and (D) PNP were used to deliver the dCas9-based CRISPRa system to activate the endogenous gene. Reprinted with the permission from Ref. 309. Copyright©2023, The Authors.

delivery system^{322,323}. Biomimetic materials enhance the biocompatibility and targeting ability of the nanoparticles, extending blood circulation. Most biomimetic nanoparticles, particularly those using cell membranes (CM) with low immunogenicity, are cost-effective and easily accessible³²⁴⁻³²⁷.

Cancer-derived CM coated on Cas9 by electrostatic interaction have facilitated the targeted delivery to tumor cells^{328,329}. A NIR-II sensitive biomimetic CRISPR/Cas9 was developed by attaching CM to the central component, enabling it to address physiological obstacles in hepatitis B virus (HBV) gene therapy³³⁰. Aghaamoo³³¹ devised a biomimetic nanoparticle platform based on CM for effective gene delivery, achieving high KO efficiency against *PTEN*. The macrophage membrane derived from RAW264.7 cells was shown to include a liver-specific promoter plasmid enabling gene editing related to liver illnesses³³². Similarly, RAW264.7-derived cell membrane can transport dCas9 to the site of inflammation³³³. Hamilton³³⁴ utilizes Cas9 RNP delivery vehicles (Cas9-EDV) as enveloped structures. Cas9-EDV can precisely target specific cells for gene editing *in vitro* and *in vivo* through

predictable antigen-antibody interactions, as opposed to viral vectors. In addition, when cancer CM are combined with zeolitic imidazolate framework (ZIF-8) and loaded with CRISPR/Cas9 for cell-type specific gene editing, the MCF-7-derived membrane nanoparticle shows a 3-fold higher gene editing efficacy in MCF-7 cells compared to nanoparticle obtained from Hela cells³³⁵. The LNP coated with mesenchymal stem cell membrane facilitated CRISPR/Cas9-mediated editing targeting *IL1RAP*³³⁶. CAR-T-cells expressing T-cell-specific antigens were used to transport an oncolytic virus and CRISPR/Cas9, named ONCOTECH, enhancing the efficacy of oncolytic virus and CAR-T therapy upon tumor site recognition, without affecting T-cell function (Fig. 7A)³³⁷.

Biomimetic nanoparticles are commonly utilized for delivering CRISPR/Cas9-based gene editing tools. Utilizing various CM with precise targeting capabilities and low immunogenicity holds great potential for delivering gene cargo. Incorporating biomimetic materials into different nanoparticles is an effective strategy for overcoming physical barriers in gene therapy.

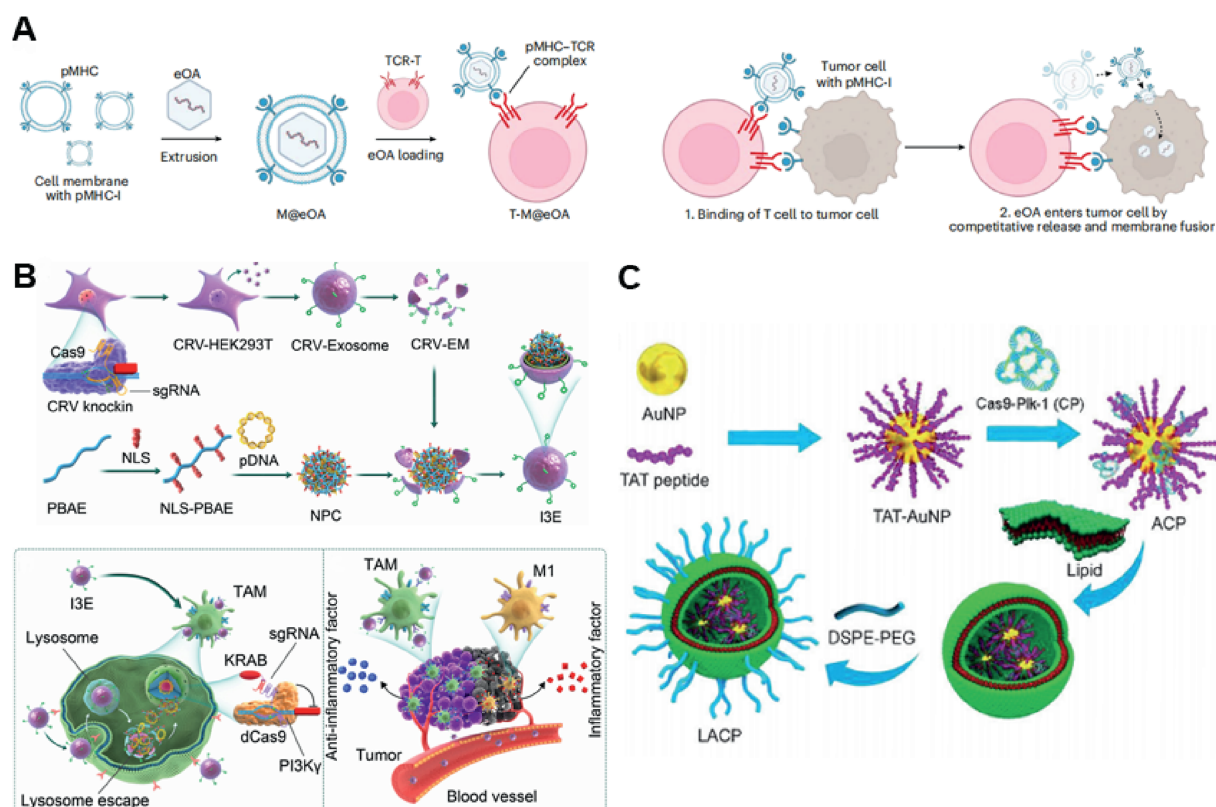


Figure 7 Representative examples of biomimetic, EVs and inorganic nanoparticles delivered CRISPR/Cas9 system. (A) The preparations of ONCOTECH, CM-expressed pMHC-I can be identified by T cells and delivered to tumor cells for gene editing. Reprinted with the permission from Ref. 337. Copyright©2024 Springer Nature; (B) Engineered exosomes for delivery of CRISPRi targeting TAM towards M1 polarization. Reprinted with the permission from Ref. 348. Copyright©2023 John Wiley and Sons; (C) AuNPs-mediated PTT was used to enhance gene editing efficiency. Reprinted with the permission from Ref. 299. Copyright©2018 John Wiley and Sons.

5.2.4. Extracellular vesicles

Extracellular vesicles (EVs) are small membrane vesicles produced by cells that can transport proteins, nucleic acids, metabolites, and enzymes within phospholipid bilayer membranes. Recent advancements in EV research have highlighted their significant role in controlling biological processes^{271,272}. EVs-based drug delivery systems are of significant interest due to the ability of EVs to remain stable in biofluids and carry cargo for long-distance transport. Exosomes are predominantly utilized as medication carriers for different types of EVs³³⁸⁻³⁴⁰.

Research has demonstrated the versatile capability of EVs in efficiently delivering various macromolecules, including the CRISPR/Cas9 system, into target cells, underscoring their significant potential for therapeutic delivery applications³⁴¹. In a study, researchers compared the efficacy of EVs and liposomes for RNA-based CRISPR/Cas9 delivery, finding that EVs are more efficient than liposomes in RNA delivery³⁴². EVs isolated from human or mouse serum using ultrafiltration and size-exclusion chromatography were utilized to bind Cas9 and sgRNA for DMD gene therapy³⁴³. Wan³⁴⁴ successfully inserted Cas9 RNP into exosomes derived from hepatic stellate cells via electroporation, facilitating gene therapy targeting liver disorders. The exosome^{RNP} achieved cytosolic delivery and particularly accumulated in the liver. Moreover, *in vivo*, ablation of *Ythdf1* through exosome mediation hindered tumor growth³⁴⁵. Integrating spCas9 into EVs enabled efficient CRISPR/Cas9 delivery to various cell types¹¹⁴.

Genetically engineered exosomes are extensively used and studied, alongside naturally occurring exosomes²⁷². Exosomes derived from epithelial cells modified with CARs exhibit selective targeting of tumor cells³⁴⁶. *In vivo*, biodistribution results indicate a higher fluorescence intensity in the CAR-EVs group compared to other groups. Moreover, EVs derived from epithelial cells, engineered with HN3 targeting, facilitate the genetic editing of cancer stem cells. Conjugating DNA aptamers to the extracellular vesicle membrane leads to the transport of RNPs into cells³⁴⁷. A peptide designed to target macrophages was attached to exosomes produced by HEK-293T cells. This was done to target tumor-associated macrophages (TAMs), and the use of CRISPRi to inhibit *Pi3kγ* resulted in M1 polarization (Fig. 7B)³⁴⁸. Liposome-exosome hybrid nanoparticles containing CRISPR/Cas9 were employed to modify the genome of chondrocytes³⁴⁹. Integrating virus-associated protein onto EVs and then combining it with Cas9 resulted in a gene editing effectiveness of around 28.1%³⁵⁰. EVs play a crucial role in intercellular communication by transporting drugs over significant distances and evading immune system detection. EVs derived from specific sources can be harnessed for targeted delivery, and genetically modified EVs further expand their targeting capabilities, rendering them a promising vehicle for gene delivery.

5.2.5. Inorganic nanoparticles

Inorganic nanoparticles, such as gold (Au) nanoparticles, mesoporous silica-based nanoparticles (MSNs), and iron oxide (Fe₃O₄),

are known for their versatile dimensions and specialized forms, often showcasing unique physical properties ideal for combined therapies^{273,351}. Their integration with CRISPR technology has significantly broadened the scope of CRISPR/Cas9 applications^{352,353}.

Positively charged gold nanorods serve as effective carriers for transporting CRISPR/Cas9 to target and disable the tumor immune checkpoint *Pd-1*⁹². Additionally, they generate ICD by gold-mediated near-infrared absorption in the second biological window, thereby enhancing the infiltration of cytotoxic T lymphocytes. Au nanoparticles-based CRISPR/Cas12a delivery was used to detect the Cell-free DNA (cfDNA) as biosensors³⁵⁴. Upconversion nanoparticles have been utilized to knockdown the *Nrf2* gene and enhance cancer photodynamic therapy by reducing cancer resistance to reactive oxygen species³⁵⁵.

Plasmid-encoding Cas9-sgPlk-1 was condensed by TAT-modified Au nanoparticle, further coated with lipids, and utilized in photothermal therapy (PTT). Under laser irradiation, AuNPs are rapidly released, leading to a lysosome burst. Moreover, a 65% reduction in PLK-1 protein expression compared to the PBS-treated group was observed (Fig. 7C)²⁹⁹.

MSNs are utilized in drug delivery due to their controllable mesopore structure, surface modification flexibility, and effective drug-loading capabilities. Engineered MSNs enable the intracellular transport of CRISPR/Cas9. MSNs-based cores efficiently co-deliver CRISPR/Cas9 and small molecule medicines because of their high biocompatibility and wide pore capacity³⁵⁶. A study demonstrated that magnetic field-controlled CRISPR/Cas9 genetic editing using magnetic nanoparticles resulted in a 35% gene editing efficiency under the magnetic field, compared to just 8% efficiency without the magnetic field³⁵⁷. Iron oxide nanoparticles responsive to light have been employed to enhance the safety of gene transfer by nanoparticles in challenging cell lines without affecting cell growth or characteristics³⁵⁸.

5.2.6. Metal–organic framework

Metal–organic frameworks (MOF) consist of metals and organic ligands, resembling inorganic nanoparticles. MOF displays multifunctionality and structural flexibility, which aids in the study of nucleic acid delivery carriers^{359,360}.

ZIF-8 was employed to simultaneously deliver Cas9 RNP and an inhibitor to control lactate metabolism³⁶¹. The nucleic acid supplied by ZIF-8 achieved a translation efficiency of 96.1% within 12 h. Laser scanning confocal microscopy (LSCM) imaging demonstrated that gene delivery mediated by ZIF-8 can rapidly evade lysosomes. ZIF-8 coated with cell-type membranes can greatly enhance targeting efficacy³³⁵. Wang³⁶² suggested a co-catalysis strategy to enhance the efficiency of delivering CRISPR using MOFs. This approach allowed for controlled activation of CRISPR/Cas9, resulting in editing efficiency against two oncogenes that were 5-fold and 7-fold higher compared to ZIF-8. Two acid-responsive MOF gene editing devices were recently developed to deliver CRISPR/Cas9 for cancer immunotherapy and osteoporosis treatment^{363,364}.

5.2.7. Others

Recently, virus-like particles (VLP), nanoparticles with hollow structures interiors, derived from viral-produced proteins as the vectors for gene therapy attracted much attention^{232,365}. VLP were formed by self-assembly with appropriate nano size, more importantly, VLP shows better safety and can not replicate in the host compared to viral vectors for their lack of viral genome^{366–368}.

Researchers reported a nanoplatfrom using leukemia-originated VLP to package RNP complexes to realize efficient gene editing in various cells³⁶⁹. Researchers also developed an engineered VLP (eVLPs) for delivering base editor and PE to carry out gene therapy *in vivo*^{370,371}. Additionally, VLP-mediated DNA nanostructure delivery exhibited efficient HDR in human primary cells³⁷². By introducing the vesicular stomatitis virus G protein, Yin et al.³⁷³ carried out a DC-targeting mRNA vaccine delivered by VLPs, which indicated the great potential of VLP for gene therapy.

Extracellular contractile injection systems (eCISs) were initially identified as a prokaryotic phage tail-like structure and other studies confirmed eCIS closely related to bacteria–cell interaction^{374–376}. Among them, bacteria can cause cell death by releasing effectors through eCISs interacting with cells^{377–379}. In 2022, Jiang et al.³⁸⁰ utilized the eCIS to deliver protein to eukaryotes, which suggests that eCIS can be a promising vector for CRISPR/Cas system. Subsequently, another research developed a programmable protein injection system for CRISPR/Cas9 cytoplasmic delivery by using eCISs, in which eCISs demonstrated specific binding to target cells with approximately 100% without obvious inflammation³⁸¹.

6. Conclusion and perspectives

Despite being in existence for just over a decade, the CRISPR/Cas9 system has profoundly transformed the landscape of molecular biology and life sciences. Currently, based on the CRISPR/Cas9 system, various variations have been discovered and applied either to improve gene editing efficiency, expand the application, or improve safety. For this concept, strategies regulating the Cas9 domain were proven to be effective. The introduction of dCas9 has enabled the transition from gene editing to gene modulation. By fusing transcription repressors or activators to dCas9, it becomes possible to manipulate the expression or repression of target genes. Importantly, CRISPRi and CRISPRa can regulate target genes without causing double-strand breaks, thereby enhancing safety. Furthermore, the development of CBE, ABE, and PE has enabled precise modification of genetic components, ushering in a new era of precise gene editing within CRISPR/Cas9-based technology.

The CRISPR/Cas9 gene editing system is extensively employed for treating human diseases caused by gene mutations due to its exceptional gene regulatory capabilities. Increasingly, research is directed towards leveraging this system to uncover disease mechanisms, establish animal models, and identify drug targets, yielding substantial success. Moreover, CRISPR/Cas9-mediated gene therapies are already holding promise in preclinical and clinical trials. Nevertheless, off-target effects, varied tissue and organ targeting, and some adverse outcomes were observed, highlighting the crucial need for safe and efficient gene delivery methods in gene therapy applications.

Although viral vectors such as AAV, LV, and ADV have shown remarkable transfection efficiency in clinical applications, concerns persist regarding the risk of random genomic insertion and integration, as well as the sustained expression of Cas9 leading to potential off-target effects. In response to these challenges, nonviral vectors are emerging as promising alternatives for delivering CRISPR/Cas9 gene editing tools. Nonviral vector-mediated delivery presents a safer option compared to viral vectors. LNP and PNP are commonly used delivery methods for CRISPR/Cas9, recognized for their effective drug loading and

rapid escape from lysosomes. Biomimetic nanoparticles and extracellular vesicle-derived delivery methods with high biocompatibility, targeting capabilities, and low immunogenicity are now experiencing significant growth. Similarly, inorganic nanoparticles and MOF-based CRISPR/Cas9 delivery strategies hold considerable promise for synergistic therapy.

Substantial progress has been achieved in preclinical and clinical investigations, leading to significant advancements in CRISPR/Cas9 technology. Despite facing numerous obstacles, this technique shows potential for treating a variety of hard-to-treat diseases. The potential for off-target effects remains a significant hurdle in its clinical application, particularly given the need for precise targeting across diverse tissues. Future research endeavors should prioritize the development of more specific sgRNAs to enable precise gene editing and the controlled expression of Cas9, offering a potential solution to mitigate off-target effects. CRISPR/Cas9 is a powerful gene-editing tool that allows for precise modifications to the genome. However, despite its precision, it can lead to unintended off-target effects, both at the gene and organ levels. A considerable effort has gone into minimizing this deficiency^{382–384}. Gene level: (1) Improved Guide RNA Design: Using bioinformatics tools to design gRNAs with minimal potential for off-target binding can reduce unintended gene editing. (2) High-Fidelity Cas9 Variants: Engineering Cas9 proteins with reduced off-target activity, such as SpCas9-HF1 or eSpCas9, can enhance specificity. Organ level: (1) Tissue-Specific Promoters: Utilizing promoters that restrict Cas9 expression to target tissues can minimize off-target effects in non-target organs. (2) Localized/Target Delivery: Direct or target delivery of CRISPR/Cas9 to specific tissues (e.g., using microinjection or localized nanoparticles) can reduce systemic distribution and off-target editing in other organs. By mitigating both gene-level and organ-level off-target effects, CRISPR/Cas9 can be more safely and effectively employed in therapeutic applications. Additionally, efforts aimed at enhancing the *in vivo* efficacy and safety of CRISPR/Cas9 gene editing technologies are imperative to facilitate their successful translation into clinical practice for gene therapy applications.

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Author contributions

Yangsong Xu: Writing — original draft, Software, Conceptualization. Hao Le: Writing — original draft, Software. Qinjie Wu: Supervision, Funding acquisition. Ning Wang: Writing — review & editing, Supervision, Funding acquisition, Conceptualization. Changyang Gong: Writing — review & editing, Supervision, Funding acquisition, Conceptualization.

Conflicts of interest

The authors have no conflicts of interest to declare.

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