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Delivery of nucleic acid based genome editing platforms via lipid nanoparticles: Clinical applications

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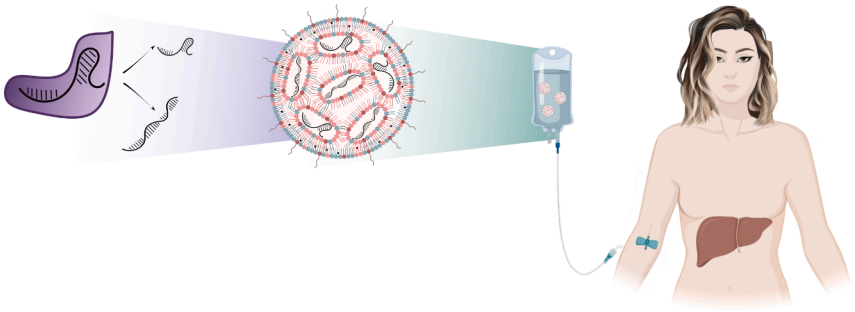
GRAPHICAL ABSTRACT



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ABSTRACT

CRISPR/Cas technology presents a promising approach for treating a wide range of diseases, including cancer and genetic disorders. Despite its potential, the translation of CRISPR/Cas into effective in-vivo gene therapy encounters challenges, primarily due to the need for safe and efficient delivery mechanisms. Lipid nanoparticles (LNPs), FDA-approved for RNA delivery, show potential for delivering also CRISPR/Cas, offering the capability to efficiently encapsulate large mRNA molecules with single guide RNAs. However, achieving precise targeting in-vivo remains a significant obstacle, necessitating further research into optimizing LNP formulations. Strategies to enhance specificity, such as modifying LNP structures and incorporating targeting ligands, are explored to improve organ and cell type targeting. Furthermore, the development of base and prime editing technology presents a potential breakthrough, offering precise modifications without generating double-strand breaks (DSBs). Prime editing, particularly when delivered via targeted LNPs, holds promise for treating diverse diseases safely and precisely. This review assesses both the progress made and the persistent challenges faced in using

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LNP-encapsulated CRISPR-based technologies for therapeutic purposes, with a particular focus on clinical translation.

1. Introduction

1.1. Nucleic acid-based genome editing platforms

1.1.1. Double-Stranded break Platforms: CRISPR-associated (CRISPR/Cas)

Clustered regularly interspaced short palindromic repeat (CRISPR) is a system utilizing Cas9 nuclease and single guide RNA (sgRNA) to target specific locations within the genome. The target is determined by a 20-nucleotide spacer sequence in the guide RNA (sgRNA), allowing the reprogramming of CRISPR by altering the sgRNA spacer sequence to target different genetic locations in the genome. [1–3] When the sgRNA matches a complementary sequence, it initiates the endonuclease activity of Cas9, inducing a double-stranded break (DSB) at a specified target. These DSBs can be resolved via non-homologous end-joining repair (NHEJ) or homology-directed repair (HDR). In NHEJ, ligation of the blunt ends of the DNA strands leads to insertion and deletion of bases (indels) and protein disruptions eventually knocking out specific genes. In HDR, CRISPR can be used to knock in the desired gene by inserting a matching sequence in specific loci. Notably, mammalian cells prefer NHEJ over HDR, leading to a predominant emphasis on NHEJ in most clinical applications involving CRISPR to date (Fig. 1). [4,5].

Other programmable nucleases (e.g., Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) utilized NHEJ for gene knockout and progressed through encouraging clinical trials using mainly viral delivery systems and addressing sickle cell and β-thalassemias diseases. However, TALENs/ZFNs approaches necessitated complex design cycles to achieve precise editing, due to unpredictable DNA binding resulting from amino acid changes. In contrast, CRISPR/Cas employs gRNA for genome recognition, streamlining the design and preparation process and enhancing its applicability across diverse research fields. [6,7].

Genome-editing methods, including CRISPR, induce DSBs at target sites. These DSBs pose risks, like large deletion, chromosomal aberrations, and cell death. [8,9] Undesired genetic alterations, known as off-targets, may occur due to partial similarity in the genome to the desired CRISPR target, leading to chromosomal rearrangements, altered oncogene expression, and cancerous transformation. [10–13] These on and off-target activities prompted the quest for DSB-free approaches to enhance CRISPR safety.

1.1.2. Dsb-free Platforms: Base and prime editors

1.1.2.1. Base editors (BEs). Base editors were first developed in 2016 by David Liu group, [14] combining either catalytically dead Cas9 (dCas9) which cannot cut the DNA, or Cas9 nickase (nCas9), which can nick one DNA strand, with a deaminase enzyme to achieve single nucleotide polymorphisms (SNPs) without inducing intentional DSBs. The initial cytosine base editors (CBEs) enable C → T substitutions, while newer adenine base editors (ABEs) perform A → G substitutions. [14,15].

Guided by a guide (g)RNA, the nCas9-deaminase complex targets DNA. After recognizing the target DNA sequence, the base-editing complex, with nCas9, induces denaturation, forming an R-loop. This exposes a short stretch of single-stranded DNA (ssDNA) in the non-

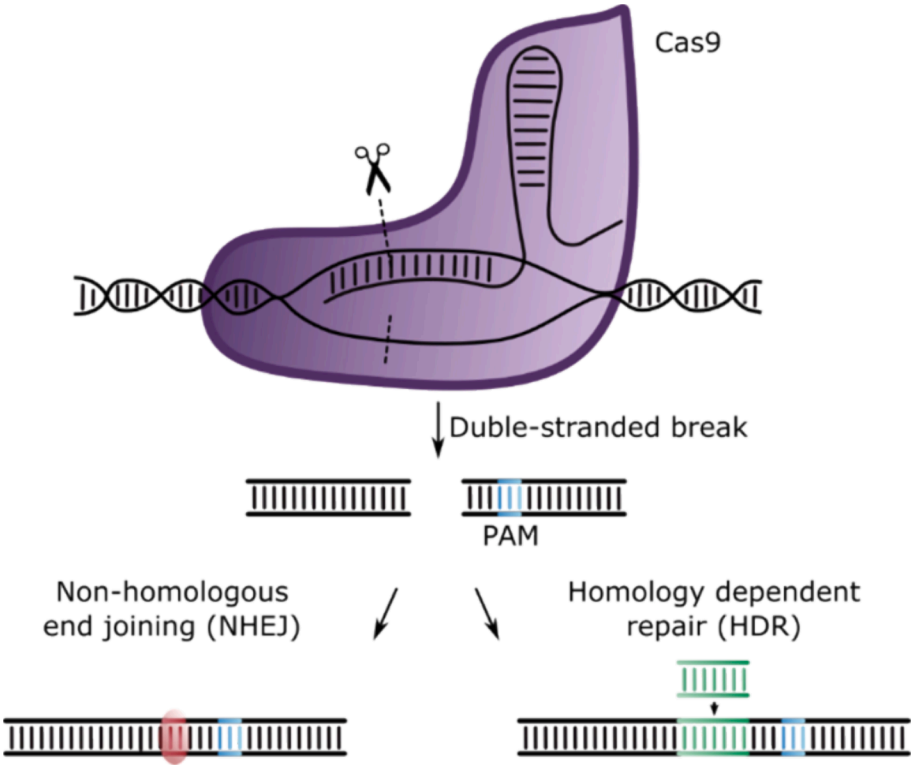

Fig. 1. Mechanism of CRISPR/Cas9 Genome Editing. The mechanism of CRISPR-Cas9 genome editing can be divided into three steps: recognition, cleavage, and repair. The gRNA directs the Cas9 protein and recognizes the target sequence within the gene of interest, leading to Cas9 nuclease activation. This forms a DSB at site 3 base pair upstream to the PAM sequence. The DSB can then be repaired by two optional mechanisms: in NHEJ the broken strands are ligated, occasionally causing insertions or deletions (indels) at the cleavage site, usually leading to gene disruption. The HDR mechanism involves a DNA template that is integrated into the cleavage site, leading to a specific gene repair.

complementary strand for deaminase activity. The deaminase chemically modifies bases within the 5–10 bp base editing window, located distal from the protospacer adjacent motif (PAM) in the target site. [14–16].

The initial version of base editors faced significant efficiency challenges. For instance, CBEs, which induce cytosine deamination, create a uracil base, prone to recognition and removal by the cell's DNA repair mechanisms. Human cells efficiently utilize the base-excision repair (BER) pathway, specifically employing uracil DNA N-glycosylase (UNG) to eliminate uracil during BER initiation. This hampers the efficiency of C-to-T edits, resulting in decreased purity and increased undesired edits like C-to-A or C-to-G. [17–19] Second-generation CBEs addressed this by introducing a uracil glycosylase inhibitor (UGI) fused with Cas9 and cytosine deaminase. UGI prevents UNG action, preserving the uracil base, and leading to a threefold increase in editing efficiency and purity (Fig. 2). [18,19].

ABEs are crucial for clinical use as G-C to A-T mutations are the most common pathogenic point mutations. [17–20] Essential for ABE creation was adapting an adenine deaminase to edit DNA, as natural ones edit only RNA. Directed mutagenesis produced a DNA-capable E.coli tRNA adenosine deaminase (TadA), with higher editing efficiency when one mutant (TadA*) and one wildtype enzyme were used. [20,21] ABEs function similarly, adenine deamination creates an inosine residue, interpreted as guanosine by DNA polymerases, achieving an A-to-G edit without the need for UGI or a similar inhibitor as inosine residues are not excised (Fig. 2). [20].

While offering advantages over DSB-gene-editing technologies, base editing does have certain limitations that are currently under investigation and being addressed. Unintended edits can occur in the editing window, where deamination affects neighboring cytosines, causing undesired effects. To mitigate this, more precise BE variants with mutated deaminase enzymes have been developed. [22,23] Furthermore not all genes are targetable by BREs because of PAM sequence preferences.[24] Unexpectedly, base editors may induce off-target RNA edits unrelated to nCas9 activity, resulting in unpredictable outcomes. Efforts are underway to modify the deaminase protein to reduce its RNA activity. [25–29] Surprisingly, indels can still occur when base editors create single-strand nicks, potentially leading to DSBs during repair by the BER pathway. [30] One strategy to prevent DSB formation involves strong inhibition of the BER pathway. [31].

1.1.2.2. Prime editors (PEs). While efficient, base editors are limited in introducing specific point mutations and cannot create deletions or insertions. This prompted the development of prime editors (PEs), which can create precise insertions and deletions with minimal unwanted modifications and virtually no off-target effects. Like BEs, PEs avoid generating DSBs, reducing the risk of chromosomal aberrations and cell toxicity, critical for safe gene editing. [32].

PEs combine nCas9 with prime editing guide RNA (pegRNA). pegRNA comprises two components; a spacer sequence which defines and locates the target locus and an extra sequence that's used as a template for the reverse transcriptase (Primer-binding site (PBS) and RT template sequence). The PE complex binds, and while pairing with a gRNA, the nCas9 nick one DNA strand forming an R-loop. This exposes a short stretch of single-stranded DNA (ssDNA) in the non-complementary strand with 3′ end where new individual bases can be added on. The PBS hybridized to the 3′ end and primes the RT which transcripts the RT template into DNA sequence. Then RT template sequence aligns 3′ flap with the complementary strand, 5′ end is removed, nick is repaired, forming a new dsDNA. Edit in non-complementary strand causes a mismatch. Mismatch repair (MMR) randomly selects a strand as a template and repairs the other, leading to rejection and reconstruction of the original sequence in half the cases. The newer PE versions come with

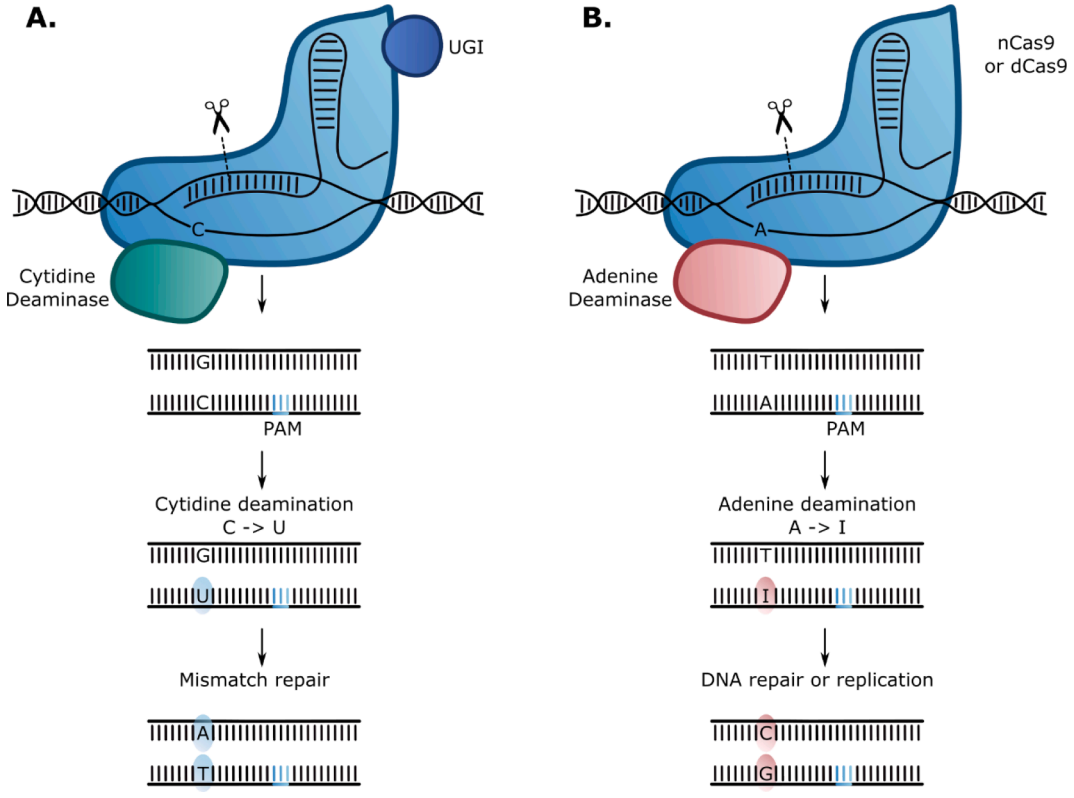

Fig. 2. Mechanism of CRISPR-Cas9 Base Editors. (**A**) Cytidine deamination. The gRNA directs the dCas9 or nCas9 to the target sequence, then exposes a short ssDNA in the free DNA strand for cytidine deaminase activity. The conversion of C to U then takes place and is protected from U excision by the addition of UGI to the complex. Then, by mismatch repair mechanisms, the U is converted to T and, the complementary A is placed. (**B**) Adenine deamination. A similar deamination mechanism leads to the conversion of A to I, followed by DNA repair or replication pathways that then convert I to G and the complementary C is being placed.

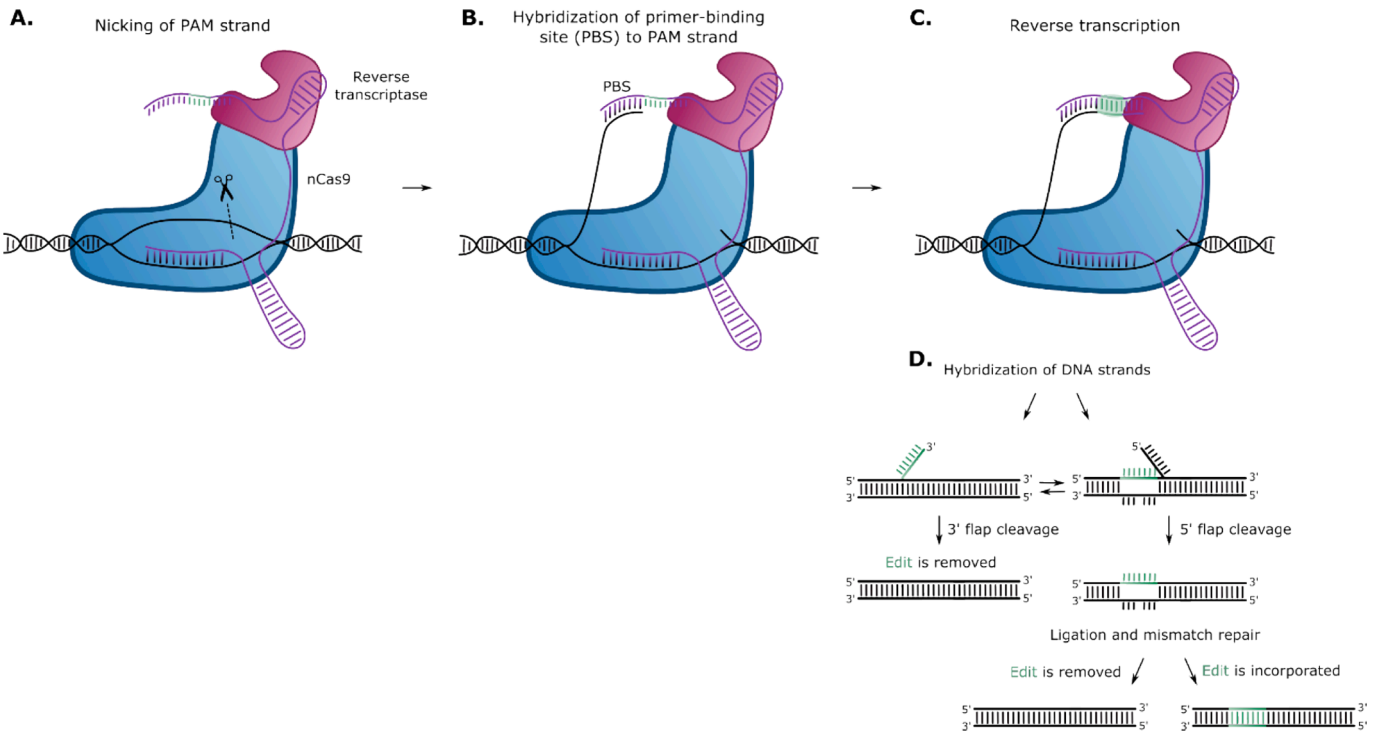

Fig. 3. Mechanism of CRISPR-Cas9 Prime Editor. The mechanism of CRISPR-Cas9 prime editing includes several steps: (**A**) Cas9 nickase is recruited to the target sequence within the gene of interest by the prime editor guide RNA (pegRNA) and nicks the target site. (**B**) The primer-binding site (PBS) of the pegRNA can then anneal to the genomic DNA flap. (**C**) This duplex is recognized by reverse transcriptase, which reverse-transcribes nucleotides extending from the PBS, copying the sequence encoded in the pegRNA. (**D**) Reverse transcription produces a 3′ flap that contains the desired prime edit as well as downstream homology to the rest of the target DNA site. The 3′ flap equilibrates with the corresponding 5′ flap, which does not contain the desired edit. Cellular degradation of the 5′ flap allows the edited 3′ flap ligation into the genome, forming a heteroduplex DNA composed of one edited strand and one original strand. These mismatches are then repaired by an intrinsic mismatch repair mechanism resulting in stable installation of the edit within the genome.

extra sgRNA, nick non-edited strand, improving MMR efficiency and achieving higher edits than older PEs without the extra sgRNA. (Fig. 3) [32,33].

Subsequently, Prime editing, unlike HDR, is performed by the molecular machine itself, directly inserting an edited DNA fragment onto the genomic DNA without the need for external repair machinery or a template. [34].

However, PEs face a key limitation in efficiency due to their multi-step nature. Scientists focus on enhancing each step's efficiency, leading to the development of new PE variants with improved features such as nuclear localization, codon optimization, additional DNA-binding domains, and altered architectures, resulting in improved prime-editing efficiency. [34,35] pegRNA design is crucial in prime editing, unlike base editing where the design of the enzyme complex determines edits more than the guide sequence, the pegRNA greatly influences prime-editing efficiency. Testing various pegRNAs with different PBS and RT template sequence lengths is crucial for efficient editing. [36,37] Structural modifications at the 3′ end protect pegRNA from degradation, significantly improving editing efficiency. [38,39] Other modifications enhancing pegRNA transcription and stability also contribute to efficiency. [37,39] Another limitation is the insert size, early PEs had limitations in generating short insertions (up to 100 bp) and deletions (up to 1000 bp). Newer PEs can achieve larger edits using pairs of pegRNAs (up to 10kB), [40] using template-jumping Prime Editing (TJ-PE), which carries two primer binding sites (PBSs),[41] or combining prime editing with recombinase-mediated integration. [42].

Nevertheless, DSB-free editing platforms, like prime editing, offer the possibility to create a wider array of possible modifications with minimal off-target events.

2. Pre-clinical applications of nucleic acid-based genome editing platforms in cancer and non-cancer diseases

Since its emergence in 2012, CRISPR has become a flexible tool for genetic manipulation, sparking intensified research efforts aimed at improving both efficacy and safety. In under a decade, human clinical trials for CRISPR gene editing have experienced a significant surge, showing encouraging initial outcomes. Achieving precise and efficient genetic modification, while avoiding interference with cell functions and unintended off-target modifications, stands as a crucial factor for the continued progress of CRISPR in clinical applications.

2.1. 2.1. Non-cancer diseases

2.1.1. CRISPR-Cas9 genome editing

CRISPR is being evaluated in clinical trials in various diseases including hematologic diseases (beta-thalassemia and sickle cell disease), hereditary metabolic disorders (Heterozygous familial hypercholesterolemia, hereditary transthyretin amyloidosis, diabetes), Immune system diseases (AIDS), and other genetic diseases (inherited eye diseases, muscular genetic diseases, and inherited lung diseases). [43].

Initially, clinical applications of CRISPR predominantly relied on DNA repair mechanisms. CRISPR operates by deactivating disease-associated genes through the induction of DSBs in coding exons. Repair through NHEJ, has the potential to introduce insertions or deletions (indels), thereby disrupting the reading frame and resulting in a truncated transcript or an unstable protein. Consequently, the early applications of CRISPR were primarily directed toward the knockout of mutant genes associated with monogenic Mendelian diseases, [44] capitalizing on the prevalence of NHEJ in mammalian cells. [45] This

approach, exemplified in its application against HIV through the knockout of the CCR5 gene, conferring resistance to HIV infection in CD4 + T cells derived from patients and imitating a natural resistance mechanism (Delta32 deletion) observed in certain populations. [46] The knock-out approach was also vastly utilized in cancer immunotherapy, CRISPR-based genetic engineering of CAR-T cells holds promise for reducing toxicity while preserving checkpoint inhibition. Research has shown the feasibility of creating PD-1-null and LAG-3 knockout T-cells using CRISPR,[47] enhancing efficacy in various mouse models of cancer. This progress has paved the way for ongoing clinical trials investigating the use of CRISPR-edited CAR-T cells in both hematological and solid cancers. [48].

In conditions like sickle cell disease (SCD) and transfusion-dependent beta-thalassemia (TDT), a knockout approach for the defected genes may be ineffective. Instead, disrupting the BCL11A erythroid specific enhancer region has been successful in recent clinical trials, reactivating fetal hemoglobin without adverse effects.[49] Patients treated no longer require transfusions in TDT or suffer painful vaso-occlusive episodes in SCD. [50] .

A pivotal clinical trial on CASGEVY (exagamglogene autotemcel) gene therapy for sickle cell disease marks the first FDA approval of a CRISPR-based treatment. CASGEVY, employing CRISPR technology, edits patients' DNA, enabling the production of a different form of hemoglobin, thereby alleviating pain episodes and complications associated with sickle cell disease. [51] CASGEVY efficacy was demonstrated in the CLIMB SCD-121 trial, a phase 3 study, that assessed exa-cell, a cell therapy utilizing ex vivo CRISPR-Cas9 gene editing of autologous CD34 + hematopoietic stem and progenitor cells (HSPCs) to reactivate fetal hemoglobin synthesis at the erythroid-specific enhancer region of BCL11A in sickle cell disease patients aged 12 to 35 years with a history of at least two severe VOCs annually. Of 44 patients receiving exa-cel, 96.7 % remained VOC-free for more than a year. Safety profile mirrored hematopoietic stem cell treatments, with no reported malignancies. [52].

Duchenne Muscular Dystrophy (DMD), a complex genetic disorder, poses challenges for traditional gene replacement due to the DMD gene's size. CRISPR can be programmed to skip mutated exon splice sites and restore reading frames to potentially alleviate DMD. This approach offers the advantage of a one-time DNA-level treatment. Promising *in-vivo* studies paved the way to demonstrate CRISPR's effectiveness in a clinical setting. [53,54].

For precise gene editing, CRISPR can utilize the HDR pathway with a donor DNA containing desired modifications. Challenges include cells preferring NHEJ over HDR and non-cycling cells not using HDR. [45] Successful efforts to enhance HDR-based approaches involve optimizing adeno-associated virus (AAV) vectors for delivering donor DNA in *ex-vivo* treatments, such as for sickle cell disease (SCD). [55] AAV is a promising gene correction method that involves Cas9-mediated genome cutting combined with AAV-mediated homologous recombination repair. This method benefits from the ability of various AAV serotypes to target specific cell types, including both dividing and quiescent cells. [55,138] In a study researchers investigated CRISPR/Cas9-mediated beta-globin (HBB) gene correction in hematopoietic stem cells (HSCs) from SCD patients. Using a humanized globin-cluster SCD mouse model, they explored Cas9-AAV6-mediated HBB correction in functional HSCs, followed by autologous transplantation. Successful gene correction in long-term multipotent HSCs *ex-vivo* led to stable hemoglobin-A production *in-vivo* after autologous transplantation. These high levels of corrected long-term HSCs, achieved with CRISPR and an AAV6 donor, paved the way for clinical trials in SCD. [55] However, the trial was recently stopped as a patient developed transfusion-dependent pancytopenia. [56].

However, HDR is generally inefficient, leading to a minority of intended edits and a majority containing undesired indels. [5,57] While exceptions exist, such as *ex-vivo* applications with blood stem cells, in many cases, especially *in-vivo* editing, it is not currently regarded as a favorable option. DSB-free editing platforms, such as BEs and PEs, do not need HDR and offer superior genetic precision compared to traditional CRISPR-Cas9 methods. BEs and PEs enable precise genetic changes with minimal undesired byproducts, ensuring high efficiency and safety. [14,16,32] Various research groups have demonstrated the efficacy of these DSB-free platforms across different contexts, justifying the excitement surrounding their application.

2.1.2. CRISPR Cas9-base editing

In-vivo utilization of ABEs was demonstrated in several genetic diseases. In a DMD-mouse model, ABEs delivered via two AAVs, corrected a DMD mutation, resulting in an improved disease phenotype.[58] Additionally, ABEs corrected the LMNA mutation in a mouse model of Hutchinson–Gilford progeria syndrome, doubling the median lifespan. [59] As for *ex-vivo*, the success of ABEs in the correction of a sickle cell disease mutation in mice led to the approval of the BEAM/ BEACON-101 phase I/II trial.[60].

Base editing has also been used to simultaneously disable CCR5 and CXCR4 receptors in CD4 + T cells, demonstrating effectiveness in making these cells resistant to HIV infection *in-vitro*. This approach holds the potential for preventing HIV infection or reinfection. [61] Additionally, another study demonstrated base editing's potential to treat spinal muscular atrophy (SMA) by introducing an A-to-G edit in the SMN2 gene, by intracerebroventricular injection of AAV9 carrying an ABE into the brain's ventricular system of SMA mice, which led to the conversion of insufficient *SMN2* genes into healthy *SMN1* genes. [62,63].

2.1.3. CRISPR Cas9-prime editing

Although prime editing is still less explored than other CRISPR systems, it shows promise in generating diverse therapeutic genome edits. Initial studies demonstrated its ability to correct mutations associated with sickle cell disease, and Tay–Sachs disease. [64,65] Prime editing allows for precise mutations beyond the capabilities of base editors, successfully correcting pathogenic mutations in a mouse model of α1-antitrypsin deficiency and Dnmt1 in mouse retinas, indicating potential in treating eye diseases. [66,67] In Duchenne Muscular Dystrophy, it effectively inserted two nucleotides (AC) into exon 52 of DMD, reframing the exon and rescuing DMD expression in cardiomyocytes. [68] Additionally, Fuchs et al, highlighted the advantages and challenges of using patient-derived organoids as disease models and prime editing for genetic disorder correction. In this study, PEs encapsulated within viral vectors, shown not to induce genome-wide off-target effects, present therapeutic potential, especially for metabolic disorders.[69] Therapeutic effects, even with low targeting efficiency, could benefit metabolic disorders like Wilson's disease, phenylketonuria, glycogen storage disease, Crigler-Najjar syndrome, and genetic diseases such as hemophilia. [69].

While prime editing's therapeutic use is in its early stages, its genomic editing flexibility holds the potential for correcting a variety of diseases. Various preclinical models are currently being examined, [70] with subsequent clinical trials planned for diverse medical conditions.

2.2. 2.2. Cancer

Adoptive cell transfer (ACT) of tumor-reactive T lymphocytes is a highly promising immunotherapeutic approach against cancer. [71] ACT involves three cell types: CAR-T cells, TCR-T cells, and TILs. [69] CRISPR-related cancer therapies mainly focus on *ex-vivo* engineering of ACT, to enhance the therapeutic potential of these cells.

2.2.1. 2.2.1. Hematological malignancies

CRISPR-engineered CAR-T cell therapies are prominent in CRISPR-based clinical trials for hematologic malignancies.

CB-010, CRISPR-edited allogeneic anti-CD19 CAR-T cell therapy for relapsed/refractory B-cell non-Hodgkin lymphoma, inserting a CD19-

specific CAR and deleting the PD-1, exhibited durable antitumor activity and favorable safety in the ANTLER trial (NCT04637763). [72] In the dose-escalation phase, with 16 patients, the overall response rate was 94 %, and 69 % achieved complete response. Among large B-cell lymphoma patients, 90 % responded, with 70 % achieving complete response. CB-010 was well-tolerated, with adverse effects comparable to other anti-CD19 CAR T-cell therapies, and no dose-limiting toxicities occurred at specified dose levels. Caribou Biosciences expanded the trial for large B-cell lymphoma (LBCL) patients needing second-line therapy, testing mid and high doses. Initial data from four patients show 100 % overall response and 50 % complete response, lasting up to a year. Preliminary expansion phase results are expected in H1 2024. [73].

2.2.2. 2.2.2. Solid cancer

CRISPR therapies for solid tumors face challenges due to easy replication of cancer cells and the obstructive tumor microenvironment.

2.2.2.1. 2.2.2.1. Ex-vivo gene editing. CRISPR ACT-based therapies while demonstrating higher editing efficiency *ex-vivo*, face further challenges that hamper its clinical application in cancer, including limited CAR-T infiltration, TME suppression, and suppressive T-cell regulatory mechanisms.

In the COBALT-RCC trial (NCT04438083),[74] the allogeneic CRISPR-Cas9 edited CAR-T therapy, CTX130, showed an 8 % objective response rate, 69 % stable disease, and a 77 % disease control rate in advanced clear cell renal cell carcinoma. Out of 13 evaluable patients, 1 had a partial response progressing to a complete response maintained for 18 months, while 4 had stable disease at 4 months. The therapy demonstrated acceptable safety, with no dose-limiting toxicities. Cytokine release syndrome (grade 1/2) occurred in 50 %, with a median onset of 1 day and a median duration of 2 days. Serious adverse events were infrequent, and one death was unrelated to treatment. [75].

CTX131, also an allogeneic CAR-T, targets CD70 using T cells from healthy donors, modified with *ex-vivo* CRISPR-Cas9 like CTX130 with additional edits to Regnase-1 and TGFBRII genes. The CTX131 CAR is precisely engineered to target CD70, inserted into the TRAC locus to disrupt native TCR, reducing graft-vs-host disease risk. Class I MHC is eliminated to prevent rejection and knock out Regnase-1 and TGFBRII genes to enhance CAR-T potency.[76,77] CTX131 is undergoing a Phase 1/2 trial (NCT05795595), [76] a multicenter, open-label study assessing safety and efficacy in adults (n = 250) with unresectable or metastatic, relapsed/refractory solid tumors, including clear cell renal cell carcinoma, cervical carcinoma, pancreatic adenocarcinoma, endometrial carcinoma, and malignant pleural mesothelioma. Recruitment is currently underway. [77].

A limitation of CAR-T cell therapy is its exclusive recognition of surface antigens, while TCRs recognize both surface and intracellular antigens. [48] Therefore, TCR-T cells can recognize epitopes at lower concentrations than CAR-T cells and are engineered to block immune checkpoints to overcome TME inhibition. [78].

NeoTCR-P1, a personalized T-cell Receptor (TCR) therapy, demonstrated safety and proof-of-concept in a phase 1a/b trial for solid tumors (NCT03970382).[79] NeoTCR-P1 redirects patients' T cells to fight cancer by targeting neo-antigens. The therapy involves sequencing patient DNA to identify these antigens, CRISPR-editing patients' T cells using electroporation with Cas9, guiding RNAs for knocking out endogenous TCR genes, and an HR template plasmid encoding the transgenic neoTCR and administering the edited cells after conditioning chemotherapy. In the trial,16 patients with cancer types including MSS-colorectal, HR + breast, ovarian, melanoma, and NSCLC were dosed with NeoTCR-P1 (four with IL-2). with 5 showing stable disease and successful cell migration to tumors post-infusion. [80].

Several clinical trials employed TCR-T therapy combined with CRISPR/Cas9-mediated PD-1 knockout to treat solid tumors; the first CRISPR/Cas9 clinical trial (NCT02793856) in China for PD-1 edited NSCLC.[81] Gene-edited T-cells infused as *ex-vivo* therapy with pembrolizumab yielded 15.5–23 % 5-year survival in 12 patients, with 5.81 % gene editing efficiency. [82] T-cells showed CD3 positivity (median 99.1 %) and CD8 positivity in some cases (73.5 %). Off-target mutations were minimal (0.05 %) in 18 potential sites via high coverage NGS. No mutations were detected in 2086 potential sites with low coverage WGS. Two years of monitoring revealed well-tolerated treatment, with no 3 + grade AEs or dose-related toxicity reported. [82].

Phase 2 trial (NCT03081715) explores PD-1 knockout T-cells in 21 esophageal cancer patients.[83] Adverse events were mild (fever, chills, skin rash). No grade 3 or 4 adverse events occurred, and no complete or partial responses were observed. Disease control rate was 35 %, with a median overall survival of 127 days. PD-1 knockout T-cells infiltrated and persisted in responsive cases. The treatment showed effectiveness and good tolerance, warranting further investigation.[84].

NCT04417764, a Phase 1 trial for PD-1 knockout engineered T cells in combination with transcatheter arterial chemoembolization (TACE) for advanced hepatocellular carcinoma (HCC). The patient-derived (autologous) T cells are CRISPR-edited to knock out the PD-1 checkpoint inhibitor. TACE, which involves blocking the hepatic artery's blood supply to the tumor aims to achieve ischemic, hypoxic, and necrotic effects by blocking tumor blood supply, while PD-1 knockout T cells, prepared using CRISPR Cas9 technology, are infused percutaneously after TACE treatment. The trial aims to enroll ten participants, evaluating safety and clinical efficacy with an estimated primary completion date in H2 2024. [48,85].

NCT03044743, assesses the safety of PD-1 knockout EBV-CTLs in treating advanced EBV-positive malignancies (gastric carcinoma, nasopharyngeal carcinoma, or lymphoma). Patients undergo 2–4 cycles based on tolerance, with autologous PD-1 knockout EBV-CTL infusion. Adverse events (CTCAE v4.0) will be evaluated after each cycle and progression-free survival (PFS) rates will be evaluated as a secondary endpoint. [86].

TILs, derived from solid tumors and expanded *ex-vivo* with IL-2, have shown promise in cancer therapy. [87].

Disrupting the CISH gene, enhances natural cytotoxicity receptor signaling and reduces NK cell exhaustion. [88] Disrupting CISH combined with TILs shows promise in preclinical studies and led to "Phase I/II Trial in Patients with Metastatic Gastrointestinal Epithelial Cancer Administering Tumor-Infiltrating Lymphocytes" (NCT04426669) which utilizes CRISPR/Cas9 to deactivate the CISH gene in tumor-infiltrating lymphocytes (TILs), boosting their ability to combat stage IV metastatic GI cancers. The estimated primary completion date for the trial is H2 2024. [88,89].

Combining TIL therapy with immune checkpoint blockade is a highly effective strategy to enhance TIL efficacy. NCT04842812, is a phase 1 trial exploring CRISPR-Cas9-edited TILs, with PD-1 knockout and expression of scFvs against PD-1 and CTLA4. Therapeutic cells, modified through CRISPR-Cas9, will be infused into 40 adult patients with refractory advanced cancers, spanning liver, colorectal, lung, breast, brain, and other solid tumor types. The trial, currently recruiting, will assess safety, and preliminary clinical efficacy, with an estimated primary completion date in 2025.[90].

2.2.2.2. 2.2.2.2. In-vivo gene editing. Most of the CRISPR/Cas9 *in-vivo* delivery experiments result in relatively low editing efficiencies which may not be sufficient for treating solid tumors and necessitate a delivery system to reach the target cancerous organ and the cancer cells. [91].

Zhang et al tried to circumvent these hurdles by developing lipid nanoparticles (LNPs) with a dual approach, incorporating a CRISPR-Cas9 system to modify the PD-L1 gene and siRNA targeting FAK expression. This strategy disrupts PD-L1 overexpression and reduces extracellular matrix density, facilitating immune cell access to the tumor microenvironment. The LNP delivery system demonstrated over a 10-fold increase in gene editing efficiency in tumor cell culture. In mouse

models, the systemic injected LNPs exhibited significant gene editing, suppressing cancer cell replication, decreasing tumor burden, and prolonging survival across various cancer types, including solid ovarian and liver tumors. The study highlights that lowering tumor tissue stiffness enhances CRISPR gene editing in solid tumors and demonstrates the capability to combine multiple therapeutic agents into a single LNP. [92].

Local injection of CRISPR/Cas9 LNP can also reach high gene editing. Cas9 mRNA and sgRNA targeting Polo-Like Kinase 1 (PLK1), a kinase essential for mitosis, were encapsulated in LNPs and injected directly into the tumor bed of mice with GBM brain cancer cells. Two days later, after euthanasia, next-generation sequencing showed that 68 % of cancer cells had undergone editing at the PLK1 locus. [93] The same CRISPR LNPs targeting PLK1 were engineered for antibody-guided delivery to disseminated ovarian tumor and led to approximately 80 % *in-vivo* gene editing, inhibition of tumor growth, and an 80 % increase in survival. [93].

Further preclinical studies are ongoing to further address both solid tumor and CRISPR-related challenges and pave the way for future clinical trials.

3. Non-viral nanoparticles for delivery of nucleic acid-based genome editing platforms

3.1. Lipid nanoparticles (LNPs)

Lipid nanoparticles (LNPs) usually have four components: amino-ionizable lipid, cholesterol, helper lipid, and PEG-lipid conjugate. When mixed they create uniform spheres that can encapsulate RNA payloads. The collective properties of LNPs stem from the interaction of these components, as they can affect LNPs' size, shape, charge, stability, and biodistribution. The component ratio crucially impacts LNP activity, toxicity, and transfection efficiency regardless of the RNA payload. [94,95].

The ionizable lipid is the key component in LNPs. It is composed of a head group (often amine groups), a stable biodegradable linker, and a tail with variable carbon lengths. These lipids carry a positive charge under acidic pH conditions and a neutral charge when exposed to physiological pH. Protonated ionizable cationic lipids bind negatively charged nucleic acid payloads. Microfluidic mixing of lipids and nucleic acid solvents forms the LNP structure. These lipids are neutral at the body's pH, ensuring electrical neutrality in the bloodstream and preventing immediate inflammatory toxicities associated with cationic lipids. Inside cells, the acidic endosomal environment protonates ionizable lipids, allowing them to interact with the endosomal membrane and facilitate the escape of nucleic acid payloads into the cytosol. [96,97] The collective properties of the ionizable lipid's head, linker, and tail influence its biodegradability, immunogenicity, potency, and control of both nucleic acid encapsulation and their escape from endosomes, ensuring successful LNP payload expression (Fig. 4). [95,98].

LNPs can deliver CRISPR components in various formats, including encapsulating (1) plasmid DNA (pDNA) encoding Cas9 protein and gRNA (2) Cas9 mRNA and gRNA, and (3) Cas9/sgRNA (protein/RNA) RNP complex. Each method has distinct advantages and limitations, requiring unique LNP-specific formulation criteria to ensure optimal compatibility without compromising function. [94].

LNP-pDNA delivery encounters challenges, primarily stemming from the plasmids' large size, which hampers encapsulation efficiency, and the negative charge of inadequately encapsulated pDNA interfering with cell membranes.[94] Strategies to enhance pDNA delivery involve condensing plasmids' volume, improving stability with DSPE-PEG, and modifying the DOTAP/DOPE ratio.[110] Zhang et al. achieved high genome editing efficiency in cancer cells, and Li et al. demonstrated PLK1 gene editing *in-vitro* and *in-vivo* without plasmid condensation reagents, emphasizing improved encapsulation efficiency and overall efficacy. [110].

Utilizing CRISPR RNPs for delivery is anticipated to yield fewer off-target effects, significantly higher editing efficiency, and up to 10-fold higher target specificity.[111] *In-vivo*, this strategy achieved significant genome editing within the inner ear hair cell population of live mice, suggesting therapeutic potential for hearing recovery. [112] RNP delivery complexities, including the large size of the Cas9 protein and the possibility of RNP denaturation during LNP formulation, have been addressed by various researchers. [94,113] Wei et al. developed an approach to systemically deliver RNPs to the muscle, liver, and lung tissues of mice using LNPs. They achieved tissue-specific gene editing in different organs by varying the DOTAP percentage in the formulations, allowing for relative tissue-specific RNP editing. [114].

mRNA-Cas9-LNPs are recognized for their efficient loading, flexible design, and biocompatibility, [115] making them extensively employed in clinical-stage gene-editing CRISPR therapies. [116] This flexibility versatility and efficiency of mRNA delivery is demonstrated in hypercholesterolemia. Besides successfully delivering Cas9-mRNA LNPs and achieving significant gene editing of proprotein convertase subtilisin/kexin type 9 (PCSK9), LNPs demonstrated efficiency in delivering ABE and CBE mRNA and gRNA for *in-vivo* targeting.[117–120] A study utilized an ionizable cationic lipid formulation to encapsulate base editor mRNA and sgPCSK9, resulting in a remarkable 63 % base editing in the cynomolgus monkey liver, accompanied by substantial reductions in

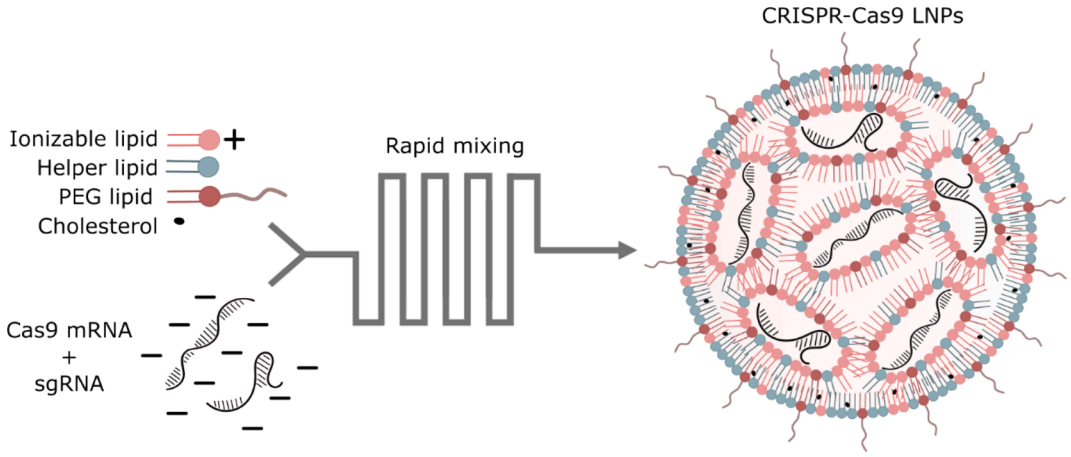

Fig. 4. CRISPR-Cas9 LNPs Generation. CRISPR-Cas9 LNPs are formulated by mixing mRNA Cas9 and a sgRNA with various lipid components: Ionizable lipid and structural lipids. The ionizable lipid interacts with the nucleic acid payloads to form inverted micelles around the CRISPR-Cas9 RNA. This leads to spontaneous self-assembly of the helper lipid and cholesterol to create LNPs. The hydrophilic PEG lipid can be found facing the exterior side of the LNPs.

serum PCSK9 and LDL cholesterol levels.[118] Moreover, LNPs encapsulating cytidine base editor mRNA and gRNA achieved a noteworthy 10.7 % editing in mice, which further increased to 18.8 % upon redosing, showcasing the adaptability of LNPs in delivering various CRISPR gene editors.[121].

Intellia Therapeutics in collaboration with Regeneron Pharmaceuticals, Inc developed a hybrid approach using LNPs and adeno-associated virus (AAV) to deliver Cas9 mRNA, sgRNA, and donor template DNA for in-vivo gene knock-in (KI) of the Human Factor 9 (hF9) gene in mice. [122] hF9 is a gene that encodes Factor IX (FIX), a blood-clotting protein that is often missing or defective in hemophilia B patients. They demonstrated robust, efficient CRISPR-mediated targeted insertion of hF9 into the liver. This strategy reduces AAV interference, minimizes off-target events, and restricts editing to matched organs. Lee et al. applied this method to alpha-1 antitrypsin deficiency (AATD), another genetic disease of the liver associated with a mutation in the *SERPINA1* gene that causes liver and lung dysfunction. By achieving both knockout of the mutated *SERPINA1* expression and KI of WT Human AAT (hAAT), they have restored the protease inhibition function [123] However, high AAV doses led to random integration in the liver, prompting caution in dosage selection. Lower doses reduced integration but compromised therapeutic effects. [124].

In essence, each component of the LNP influences its performance. For instance, modified biodegradable ionizable lipids rapidly degrade in intracellular environments, minimizing liver cytotoxicity. [98] Moreover, it is hypothesized that using these biodegradable lipids in CRISPR/Cas9 delivery ensures a higher proportion of mRNA escapes the endosomal degradation, therefore enhancing transfection efficiency, and enabling effective gene editing. [93,96,99,100] For example, BAMEA-O16B, a degradable ionizable lipid, effectively delivers CRISPR/Cas9 mRNA components *in-vivo*.[101] It accumulates in hepatocytes, leading to an 80 % reduction in serum PCSK9 levels in mice. No signs of inflammation or hepatocellular injury were observed, suggesting the biocompatibility of LNPs prepared with BAMEA-O16B for *in-vivo* gene editing in the liver.[101] Qiu et al. employed an LNP formulation with the lipid 306–012B to deliver Cas9 mRNA and gRNA targeting ANGPTL3 in familial hypercholesterolemia. This biodegradable ionizable lipid achieved a median editing rate of 38.5 %, resulting in significant reductions in serum ANGPTL3, LDL cholesterol, and triglyceride levels in a mouse model.[102] The study demonstrated a 2-fold improvement compared to MC3-based LNPs with no adverse effects on liver function or inflammatory markers.[102] Similarly, Kenjo et al. identified the ionizable lipid TCL053 for intramuscular mRNA delivery through *in-vivo* lipid screening. [101] LNP formulation TCL053 achieved ~10 % exon-skipping efficiency in a DMD model, surpassing MC3-based LNPs. [101] The formulation exhibited low immunogenicity, allowing safe re-administration for cumulative DMD editing.[103].

Other components such as PEG lipid may also affect the LNP CRISPR performance. PEG lipids enhance LNP stability and extend LNP circulation time. [100,104] PEGylated lipids coat the outer LNP surface, preventing opsonization and phagocytosis, which is crucial for maintaining concentration in circulation during *in-vivo* applications. [105,106] Adjusting concentrations and combinations of PEG lipids allows modification of LNP delivery efficiency and contributes to a higher circulation half-life. This extended circulation half-life increases the percentage of CRISPR/Cas9 molecules delivered to target cells, enhancing on-target genome editing efficiency. [94] PEGylated LNP faces a limitation known as accelerated blood clearance (ABC), involving immune activation and increased serum IgM against PEGylated lipids which often leads to clearance by the mononuclear phagocyte system and less effective gene therapy delivery, sometimes requiring multiple doses. [126].

Cholesterol enhances LNP stability by filling gaps between phospholipids and facilitates membrane fusion during cellular uptake. [107] Cholesterol stereochemistry impacts LNP delivery; for instance, 20α-hydroxycholesterol increases liver cell delivery. [108] Structural lipids like DSPC provide membrane stability. [104] Helper lipids can enhance mRNA delivery by increasing membrane fusion and enhancing endosomal escape. Yet, further research is needed to better understand the impact of different helper lipids on LNP delivery of Cas9 mRNA. [125] Moreover, helper lipid chemistry influences organ targeting; anionic lipids shift tropism to the spleen, while cationic lipids target the lungs. [109] Hence, we think that thorough testing procedures of each of the LNP components are essential to pinpoint optimal formulations for the efficient and safe delivery of CRISPR cargo. [95,98].

In general, LNPs function as efficient carriers for drugs, evading detection by the innate immune system and prolonging circulation time, [127] particularly advantageous for delivering hydrophobic drugs such as nucleic acids and proteins with short half-lives. Nevertheless, challenges like LNPs' inability to offer sustained release, rapid blood clearance, and liver accumulation can impact the efficiency of gene editing. [94] To address these challenges, further optimization of LNPs is necessary, employing combinatorial and high-throughput approaches to enhance the effectiveness of *in-vivo* CRISPR-LNP delivery.[128].

3.2. Polymers

A second type of non-viral delivery system is polymer-based nanoparticles, made from materials like PEG, PEI, or PLGA. Polymeric nanocapsules have unique morphology and architecture compared to other nanoparticles. They consist of a core containing drugs or proteins, enclosed by a polymeric membrane. [132,133] These particles encapsulate the RNP complex or encode nucleic acids for cell delivery. [129].

CLAN, a PEG-b-PLGA-based carrier, delivers Cas9 mRNA and sgRNA into macrophages, achieving 47.1 % Nlrp3 gene indels, alleviating acute inflammation in LPS-induced septic shock and monosodium urate crystal (MSU)-induced peritonitis. [130] Moreover, this treatment enhances insulin sensitivity and decreases adipose inflammation when injected systemically in high-fat diet (HFD)-induced type 2 diabetes (T2D) mouse model, presenting a promising approach for addressing NLRP3-dependent inflammatory diseases and serves as a carrier for delivering CRISPR/Cas9 into macrophages. In another study the same CLAN system delivers Cas9 mRNA and CD40-targeted sgRNA into dendritic cells, disrupting CD40 expression *in-vivo* and reducing graft damage, prolonging graft survival.[130].

Liu et al. also used PEG-PLGA-based CLANs to deliver Cas9 plasmid (pCas9) and sgRNA targeting the overhanging fusion region of the BCR-ABL gene (pCas9/gBCR-ABL), achieving up to 40 % gene editing in a CML cell line and increasing mouse survival in a xenograft leukemia mouse model. [131].

In 2022, Zou et al, designed brain-targeting CRISPR Cas9 nanocapsules against PLK1, overcoming the blood–brain barrier (BBB) by attaching angiopep-2 peptide to these nanocapsules. This ligand binds to low-density lipoprotein receptor-related protein-1 (LRP-1), highly expressed in BBB endothelial cells and glioblastoma (GBM) cells. Experiments in mice demonstrated that angiopep-2 peptide enhanced nanocapsule penetration through the BBB, exhibiting superior uptake in GBM tumor tissue compared to non-targeting nanocapsules.[134] This approach achieved significant PLK1 gene editing efficiency in brain tumors (up to 38.1 %), with minimal off-target gene editing in high-risk tissues (less than 0.5 %) when injected systemically. [134].

3.3. Inorganic nanoparticles

Another non-viral system involves inorganic nanoparticles made of gold or silica, which bind the RNP complex or nucleic acids for delivery. Both silica and gold nanoparticles have shown effectiveness in delivering Cas9 RNP complexes *in-vitro* and *in-vivo* across multiple cancer types. [135,136].

Gold nanomaterials serve as excellent carriers for the CRISPR/Cas9 system. Lee et al. utilized gold nanoparticles (CRISPR-Gold) to deliver Cas9 and Cpf1 RNPs into the brain via intracranial injection, achieving

editing in neurons, astrocytes, and microglia, alleviating behaviors in a fragile X syndrome model. [137] Brain-wide gene editing's potential benefits for neurological disorders are evident, but long-term accumulation and toxicity of gold nanoparticles in the brain require further investigation before clinical use.[138].

RNP-based CRISPR delivery offers advantages like rapid editing, low off-target effects, and no genome integration risk. [111] However, the large size and moderate negative charge of the RNP complex pose challenges for non-viral delivery. Strategies include modifying SpCas9, such as fusing it with an oligo glutamic acid tag (Cas9E), which, when combined with positively charged arginine gold nanoparticles (Arg-NPs), facilitated around 30 % gene editing *in −vitro*. [139] Targeting can be controlled by conjugating various molecules to these particles, like LNPs and polymeric particles.

4. The challenges of utilizing nanoparticle delivery systems for gene editing

4.1. Lipid nanoparticle formulation specificity

4.1.1. Passive targeting

LNPs, though holding great potential for RNA delivery, frequently accumulate in the liver, restricting their applicability to non-hepatic organs. To overcome this limitation, LNPs can be directed toward specific cells or organs using either passive or active targeting strategies.

Passive targeting involves delivering CRISPR LNPs to tissues and cells without surface modifications via targeting moieties. [140].

4.1.1.1. Organ Passive targeting. Passive targeting of CRISPR-LNPs to the liver is more feasible than to other organs, as the liver's highly perfused nature and fenestrated capillaries allow for LNP accumulation. [94] Protein corona formation also contributes to the hepatic LNPs accumulation.[146] Upon intravenous administration, LNPs' interactions with blood proteins form a "biomolecular corona," influenced by factors like opsonization. Apolipoprotein E (ApoE), a key protein coating of LNPs, interacts with LDL receptors highly expressed on hepatocytes, leading to receptor-mediated uptake into hepatoma cells and hepatocytes, contributing to hepatic accumulation and clearance. [146,147].

Other protein corona modulation is achieved via Selective ORgan Targeting (SORT) strategy. SORT LNPs, like very low-density lipoprotein (VLDL) particles, adsorb proteins from the plasma, mediating uptake in specific cells.[148] The technology enables liver-, lung-, and spleen-targeted CRISPR-Cas9 gene editing. SORT involves adding a permanently cationic lipid to the four-component LNP system with dendrimer ionizable lipids for extrahepatic targeting. Adjusting the molar percentage of the permanently cationic lipid /DOTAP in the formulation allows for specific organ targeting, with higher percentages favoring lung accumulation and spleen targeting. SORT lipids encapsulating Cas9 mRNA and gRNA against PTEN gene, reached high therapeutic potential as formulations achieved editing in both the lungs and liver. Moreover, SORT lipids demonstrated compatibility with RNP delivery of CRISPR/Cas9, enabling 13.9 % targeted editing in the liver and 15.1 % editing in the lungs.[148].

Vera et al. achieved successful BBB crossing without a targeting moiety. In the study, liposomes carried CRISPR-Cas9 to treat Mucopolysaccharidosis type I (MPS I) in mice by delivering a plasmid vector containing the functional IDUA (Alpha-L-Iduronidase) gene. Daily nasal administration for a month resulted in increased serum IDUA activity after 15 and 30 days, compensating for the mutated copy in experimental mice. [149] Despite these promising results, it should be noted that local administration methods such as nasal administration can facilitate higher doses or more frequent delivery of the CRISPR payload, however, potential toxicity and immunogenic adverse events need thorough exploration and consideration.

Nonetheless, despite the formulation strategy adjustments mentioned earlier, which facilitated selective uptake by specific organs, achieving a high level of target cell specificity remains challenging. [150].

4.1.1.2. Cell Passive targeting. Passive targeting of specific cells with CRISPR-LNPs is challenging. It involves navigating through the vascular system, avoiding clearance, extravasating to the target organ site, and finally transfecting the target cells.

Hence, in cancer, passive targeting relies on tissue-specific characteristics, such as the Enhanced Permeability and Retention (EPR) effect in solid tumors, a debated mechanism thought to significantly contribute to the increased accumulation of therapeutic agents in these tumors. [141–143] The phenomenon is closely associated with the distinctive features of blood vessels formed within cancerous tissues. These abnormal tumor vessels, characterized by fragility, leakage, and viscous blood flow, enhance the permeability of the tumor microenvironment. Dysfunctional lymphatic drainage further increases the retention of extravasated molecules. These structural and functional anomalies facilitate the passive delivery of LNP into tumors.[144] However, while the EPR effect enables passive delivery into tumors, achieving specificity to cancer cells, which is extremely vital in therapeutic CRISPR gene editing, requires additional considerations.

The administration method of injecting CRISPR LNPs is crucial for reaching target cells and ensuring therapeutic efficacy. [39,140] Upon intravenous injection, LNPs bind serum proteins, altering their trafficking and internalization pathways. Predicting serum protein binding, termed endogenous targeting, is critical for biodistribution. [140] While progress has been made in understanding how LNP formulations impact protein adsorption and biodistribution, achieving high target cell specificity without exogenous targeting remains challenging. Local administration methods for LNPs, like intramuscular, subcutaneous, or intratumoral injections, can enhance CRISPR cargo delivery efficiency, enabling more effective targeting of the intended cells. Preclinical studies of mRNA-LNP-based cancer therapies have used intratumoral injections to express CRISPR-Cas9-mediated targeting of oncogenes. For example, in Rosenblum et al., a single dose of sgPLK1-CRISPR-LNPs to the tumor bed of a murine GBM model induced apoptosis, prolonged median survival by about 50 %, and improved overall survival of GBM-bearing mice by 30 %. [93] Despite the superior therapeutic efficacy and editing demonstrated by local administration, it still lacks cellular specificity, and transfecting specific cell types with nanoparticles lacking targeting ligands remains challenging. Therefore, there is a particular need for active targeting, especially in CRISPR, to minimize off-target effects. [150].

4.1.2. Active targeting

Antibodies can be integrated into the lipid layer of nanoparticles for binding to target cells, potentially reducing liver accumulation. However, achieving effective orientation of the targeting moiety, such as an antibody, is challenging. Improper conjugation may expose the Fc region of an antibody, leading to rapid uptake by circulating leukocytes and phagocytes with Fc receptors, impacting both efficacy and toxicity. [151].

Existing chemical conjugation methods, such as EDC-Sulfo NHS or thiol-maleimide reactions, prove inefficient and can compromise the functionality of antibodies.[145] Moreover, these methods face challenges like the requirement for individual antibody optimization, potential functional damage, and random conjugation orientation. [152,153].

In response to these challenges, specialized linkers for antibody binding have been developed. A breakthrough in this field introduces a novel and flexible platform designed to enable the efficient incorporation of targeting antibodies into LNPs, demonstrating enhanced precision and efficacy in targeted drug delivery. This innovative technology

relies on a recombinant protein linker known as ASSET, an acronym for Anchored Secondary scFv Enabling Targeting. [154–156] Unlike conventional chemical coupling methods, ASSET employs a non-covalent method to anchor monoclonal antibodies to LNPs. (Fig. 5)[154].

The ASSET technology offers several advantages. Firstly, its non-covalent anchoring method minimizes the risk of damaging the antibody during the conjugation process. This ensures that the antibody retains its functionality, including its binding specificity and affinity. Additionally, the ASSET platform simplifies the conjugation process, eliminating the need for intricate optimization steps for each antibody. The flexibility of this technology enhances its adaptability, potentially making it applicable across various therapeutic scenarios. [93,140,154–159].

By utilizing ASSET, researchers and drug developers can overcome the limitations associated with conventional coupling methods, offering a more efficient and antibody-friendly strategy for precise and effective targeted drug delivery. This advancement has implications for improving the delivery of therapeutic agents to specific cells, particularly in cancer therapy, where targeted approaches are crucial for minimizing off-target effects and enhancing therapeutic efficacy. [140,157–159].

In 2020, Peer's group achieved successful *in-vivo*, cell specific editing using antibody guided LNPs to target the epidermal growth factor receptor (EGFR) expressed in ovarian cancer cells. In mouse experiments, survival after metastatic ovarian cancer increased by 80 % with double injections, Emphasizing the potential of this approach in treating cancer and underscoring the significant clinical impact achievable using ASSET-CRISPR-LNPs. [93].

4.2. Encapsulation- nucleic acid size

A major challenge in therapeutic CRISPR applications lies in packaging all components into a single delivery unit due to the large size of CRISPR nucleases. This hurdle applies to various delivery formats of CRISPR (plasmid, mRNA, and sgRNA, or RNP) and is encountered especially in viral (Adeno-associated viruses/AAV) delivery methods. On the other hand, the LNP option avoids the fundamental size restrictions commonly associated with viruses, making it an excellent choice for therapeutic applications.

Viral delivery faces size limitations for both DSB and non-DSB platforms. SpCas9 and SaCas9 differ in size (4.1 kb and 3.2 kb respectively), impacting AAV gene-based delivery where the size limit is ~ 4.7 kb. Strategies like splitting SpCas9 and sgRNA into two AAV vectors or using smaller SaCas9 help overcome this. [160] Base editors and prime editors also face size constraints, addressed through strategies like utilizing the intein system [161] or dividing editor proteins into segments for co-

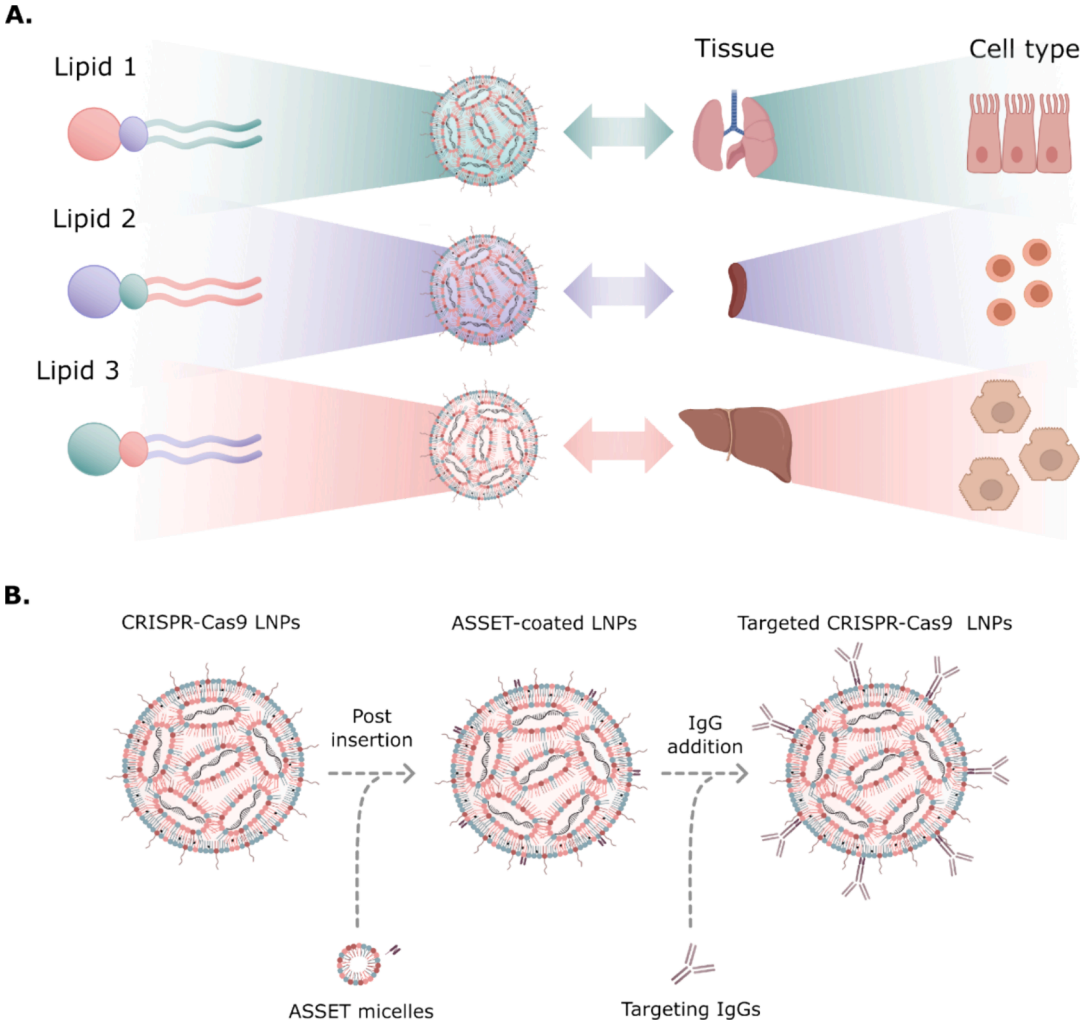

Fig. 5. Passive and Active Targeting of CRISPR-Cas9. (**A**) Passive targeting strategies rely on screening of different LNP formulations *in-vivo*, which often differ in the identity of their ionizable lipid. In this screen aspects such as specific organ and tissue biodistribution are being evaluated, for example, in the lungs, spleen, and liver. (**B**) The ASSET strategy involves forming CRISPR-Cas9 LNPs followed by post-insertion of ASSET protein purified in micelles into the LNPs. The ASSET–LNPs are then coated with the targeting IgGs through the interaction of their Fc region with the scFv domain in the ASSET to form targeted CRISPR-Cas9 LNPs.

infection and reassembly in cells. [34,67].

LNPs, unlike viral vectors, can encapsulate large payloads, overcoming encapsulation size limitation encountered in viral delivery. [100].

LNPs can co-encapsulate Cas9 mRNA and gRNA or encapsulate them separately. Co-encapsulation may enhance genome editing effectiveness by ensuring an optimal gRNA and Cas9 mRNA ratio is delivered for sufficient complexation. [93,101] Therefore, numerous biodegradable ionizable lipids have been developed for simultaneous co-encapsulation and intracellular release in mRNA delivery. [93,101,162] Nevertheless, successful outcomes have also been demonstrated using separate encapsulation methods.[101].

Modifying the composition of the LNPs formulation can overcome challenges related to the increased size and complexity of genome editors. Herrera-Barrera et al, developed Enhanced LNPs (eLNPs) with the cholesterol analog β-sitosterol to co-encapsulate PE mRNA and guide RNAs demonstrating a 54 % prime editing rate in mCherry expression in a reporter cell line which was engineered with a mammalian, codon-optimized emerald GFP-linker-TGA-mCherry construct. [163] These eLNPs, characterized by a polyhedral morphology and improved endosomal escape, enabled rapid and efficient editing within 24 h. [163] This LNP-delivery system holds promise for advancing therapies across diverse targets and applications.

4.3. Expression levels of the gene editing delivery systems

Expression levels of gene editing systems depend on several factors including administration route (systematic or local injections), delivery system, and targeting.

As mentioned earlier, optimizing the formulation of LNPs, referred to as endogenous targeting, can also influence biodistribution, potency, and safety.

Li et al. synthesized 720 new lipids with biodegradable, ionizable properties. Among these, RCB-4–8 emerged as the most potent for intratracheal delivery, exhibiting a remarkable 100-fold improvement over clinically approved MC3-LNPs.[128] Importantly, RCB-4–8 demonstrated faster clearance from lung tissues, reducing the risk of toxicity. Moreover, RCB-4–8 LNPs efficiently delivered CRISPR-Cas9 complexes in Ai9 tdTomato reporter mice. SpCas9 mRNA and sgAi9 were co-encapsulated, and administered at low or high doses, resulting in 3.0 % and 7.2 % tdTomato + cells, respectively. Additionally, they investigated the efficacy of combined viral (AAV5) for gRNA delivery and LNP delivery for Cas9 mRNA delivery, finding improved efficiency with this hybrid approach. This method for delivering RNA to the respiratory system presents a significant breakthrough by facilitating significant gene editing in specific mouse lung cell types that were previously less accessible through intravascular injection methods. [128] The high editing efficiency is attributed both to the administration route of LNPs and their optimized formula. With continued refinement and optimization, this approach has the potential to evolve into a pivotal technology for clinical applications aimed at treating lung diseases in humans.

Another way to reach high gene editing percentages in non-liver cells is to devise a strategy aimed at diminishing LNP-mediated gene editing specifically within hepatocytes. This involved pretreating with inhibitory oligonucleotides and siRNAs to suppress gene editing activity in hepatocytes.[164] Sago et al. utilized this strategy by delivering an inhibitory oligonucleotide disrupting the secondary structure of sgRNAs and siRNA targeting Cas9 mRNA before the administration of CRISPR/Cas9 mRNA loaded LNPs. This pretreatment effectively heightened the specificity of CRISPR/Cas9 mRNA-LNP-mediated gene editing in the mouse lung and spleen while reducing undesired editing in hepatocytes. This approach demonstrates a technique to refine and amplify the accuracy of gene editing, enabling more precise manipulation and increased genome editing in specific cells.[164].

In non-DSB platforms, exploring ways to enhance efficiency, including boosting the activity of the reverse transcriptase enzyme in PEs, identifying rate-limiting steps, and investigating crucial DNA repair proteins to guide the repair process for our desired edits, can significantly improve editing rates.

5. Clinical trials using LNP delivery

In 2021, gene-editing clinical trials expanded to nearly 100, addressing rare genetic diseases, other diverse diseases (diabetes, hereditary angioedema, and HIV), and an uptick in solid tumor trials. [Table 1].

The selection between viral and non-viral methods depends on factors like systemic or local administration, target tissue, genetic material size, and safety. The therapeutic gene editing field is dynamic, and the use of these methods in clinical trials may shift with advancements in technologies and delivery systems.

Viral CRISPR delivery in clinical trials has shown positive outcomes. In 2021, positive Phase 1/2 trials data for Leber congenital amaurosis 10 (LCA10), an untreatable hereditary eye disease causing blindness by the 3rd or 4th decade. EDIT 101 (NCT03872479),[165] a CRISPR-Cas9 therapy, utilized a compact Cas9 from Staphylococcus aureus and delivered by recombinant adeno-associated viruses (rAAVs). EDIT-101, aimed to correct CEP290 mutations causing LCA10 (20–30 % of cases) utilizing the HDR mechanism. No serious adverse events or dose-limiting toxicities were noted. The mid-dose cohort showed modest visual improvements. The high-dose adult cohort is ongoing, and pediatric patient dosing is set to begin.[166] Excision BioTherapeutics advances EBT-101(NCT05144386) as a potential cure for chronic HIV. [167] FDA approved its IND application for the first CRISPR-based HIV treatment. [150] In Phase 1, EBT-101, delivered via AAV, aims to cut the virus from infected cells in a one-time treatment.[150].

LNPs overcome size restrictions linked to viruses, making them an excellent option for therapeutics. Notable examples of LNP CRISPR delivery in clinical trials include Verve Therapeutics' Phase 1b trial of VERVE-101 for familial hypercholesterolemia, [168] Intellia Therapeutics' Phase 3 trial of NTLA-2001 for transthyretin amyloidosis (ATTR),[169] and its Phase 1/2 trial of NCT05120830 for hereditary angioedema (HAE). [170].

Intellia Therapeutics and Regeneron presented positive clinical data for NTLA-2001 (NCT04601051),[169] the first *in-vivo* CRISPR genome editing treatment targeting the hereditary transthyretin (TTR) gene, utilized the well-known Streptococcus pyogenes Cas9 (SpCas9) delivered via LNP. The Phase 1 trial demonstrated the safety and efficacy of this single-dose therapy for hereditary transthyretin amyloidosis with polyneuropathy (ATTRv-PN). ATTR leads to misfolded TTR protein accumulation, causing fatal complications primarily in the heart and nerves. The disease affects 500,000 people globally, with patients typically surviving 2–15 years post-onset.[171] NTLA-2001 selectively reduces mutated TTR protein levels in the blood by deactivating the TTR gene in liver cells with CRISPR-Cas9. The data was reported on the initial six patients treated in New Zealand and the UK. Administered with a single injection of two NTLA-2001 doses, all cases showed TTR reductions ranging from 52 % to 87 % compared to baseline measurements, with no significant safety issues observed by Day 28 post-treatment. [172] In 2021, Intellia received an FDA Orphan Drug Designation for NTLA-2001 in ATTR. Following this, the Phase 1 trial was extended to encompass adults with Transthyretin Amyloidosis with Cardiomyopathy (ATTR-CM). [173].

Hereditary angioedema (HAE), a painful disease causing severe inflammatory attacks, entered gene editing in 2021. Intellia Therapeutics' LNP-CRISPR-based therapy, NTLA-2002, received approval for clinical trials.[163] Administered systemically, it targets the KLKB1 gene, aiming to permanently reduce plasma kallikrein activity and halt HAE inflammation.[170].

Over the past few years, a variety of therapeutic applications for base editing have surfaced, making their way into clinical trials. Base editing

Table 1
Current clinical trials of gene editing technologies.

Disorder type	NCT	Ex-vivo/ In-vivo	Delivery	Nuclease	Target gene	Phase	Status	Conditions	Location	Completion date
Blood disorders	NCT02695160	In-vivo	AAV	ZFN	Factor IX	Phase I	Terminated	Hemophilia B	United States	2021–04
Genetic disorders	NCT03872479	In-vivo	AAV	CRISPR/ Cas9	CEP290	Phase I |Phase I I	Not Recuiting	Leber Congenital Amaurosis 10, Inherited Retinal Dystrophies, Eye Diseases, Hereditary, Retinal Disease, Retinal Degeneration, Vision Disorders, Eye Disorders Congenital	United States	2025–5
Genetic disorders	NCT05805007	In-vivo	AAV	CRISPR/ Cas9	RHO	EARLY_Phase I	Recuiting	Retinitis Pigmentosa	China	2026–04
Metabolic disorders	NCT03041324	In-vivo	AAV	ZFN	IDS	Phase I |Phase I I	Terminated	Mucopolysaccharidosis II, MPS II	United States	2021–5
Metabolic disorders	NCT02702115	In-vivo	AAV	ZFN	IDUA	Phase I |Phase I I	Terminated	MPS I	United States	2021–11
Viral diseases	NCT05144386	In-vivo	AAV	CRISPR/ Cas9	HIV-1	Phase I	Recuiting	HIV-1-infection	United States	2025–05
Viral diseases	NCT04560790	In-vivo	Lentiviral particles	CRISPR/ Cas9	BD111	NA	Completed	Viral Keratitis, Blindness Eye, Herpes Simplex Virus Infection	China	2022–7
Blood disorders	NCT05120830	In-vivo	LNPs	CRISPR/ Cas9	KLKB1	Phase I |Phase I I	Not Recuiting	Hereditary Angioedema	Australia,France, Germany, Netherlands,New Zealand,Israel	2025–12
Genetic disorders	NCT04601051	In-vivo	LNPs	CRISPR/ Cas9	TTR gene	Phase I	Not Recuiting	Transthyretin-Related (ATTR) Familial Amyloid Polyneuropathy, Transthyretin-Related (ATTR) Familial Amyloid Cardiomyopathy, Wild-Type Transthyretin Cardiac Amyloidosis	France,New Zealand,Sweden, Israel	2026–08
Metabolic disorders	NCT04601051	In-vivo	LNPs	CRISPR/ Cas9	Transthyretin Amyloidosis	Phase I	Active, not recruiting	Transthyretin Amyloidosis	France,New Zealand,Sweden, united kingdom	2026–08
Metabolic disorders	NCT05398029	In-vivo	LNPs	Base-Editor	PCSK9	Phase Ib	Recruiting	heterozygous familial hypercholesterolemia, atherosclerotic cardiovascular disease	united kingdom, new zealand	2024–12
Metabolic disorders	NCT06164730	In-vivo	LNPs	Base-Editor	PCSK9	Phase Ib	Not yet recruiting	heterozygous familial hypercholesterolemia, atherosclerotic cardiovascular disease	NA	2026–08
Viral diseases	NCT03226470	In-vivo	Plasmid	TALEN	E7, E6	Phase I	Unknown	Human Papillomavirus-Related Malignant Neoplasm	China	2022–6
Viral diseases	NCT02800369	In-vivo	Plasmid	ZFN	E7	Phase I	Unknown	Human Papillomavirus-Related Malignant Neoplasm	China	2017–7
Cancer	NCT03057912	In-vivo	Plasmid, Gel	TALEN, CRISPR/ Cas9	E7, E6	Phase I	Unknown	Human Papillomavirus-Related Malignant Neoplasm	China	2019–01
Viral diseases	NCT03057912	In-vivo	plasmid-gel	TALEN and CRISPR/ Cas9	E6/E7	Phase I	Unknown	Human Papillomavirus-Related Malignant Neoplasm	china	2019–01
Viral diseases	NCT01252641	Ex-vivo	AV	ZFN	CCR5	Phase I |Phase I I	Completed	HIV, HIV Infection	United States	2015–05
Viral diseases	NCT00842634	Ex-vivo	AV	ZFN	CCR5	Phase I	Completed	HIV, HIV Infections	United States	2013–01
Viral diseases	NCT03666871	Ex-vivo	AV	ZFN	CCR5	Phase I |Phase I I	Not Recuiting	HIV Infections	United States	2024–2
Viral diseases	NCT01044654	Ex-vivo	AV	ZFN	CCR5	Phase I	Completed	HIV Infection, HIV Infections	United States	2014–12

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Table 1 (*continued*)

Disorder type	NCT	Ex-vivo/ In-vivo	Delivery	Nuclease	Target gene	Phase	Status	Conditions	Location	Completion date
Blood disorders	NCT04443907	Ex-vivo	electroporation	CRISPR/ Cas9	HBG1 promoter, the HBG2 promoter	Phase I	Not Recuiting	Sickle Cell Disease	United States	2026–9
Blood disorders	NCT03745287	Ex-vivo	electroporation	CRISPR/ Cas9	BCL11A	Phase I I| Phase III	Not Recuiting	Sickle Cell Disease, Hematological Diseases, Hemoglobinopathies	United States, Belgium,Canada, France,Germany, Italy,Israel	2024–10
Blood disorders	NCT03432364	Ex-vivo	electroporation	ZFN	BCL11A	Phase I |Phase I I	Completed	Transfusion Dependent Beta-thalassemia	United States	2022–11
Blood disorders	NCT03653247	Ex-vivo	electroporation	ZFN	BCL11A	Phase I |Phase I I	Not Recuiting	Sickle Cell Disease	United States	2025–7
Viral diseases	NCT02388594	Ex-vivo	Electroporation	ZFN	CCR5 Delta-32	Phase I	Completed	Human Immunodeficiency Virus (HIV)	United States	2019–03
Viral diseases	NCT02225665	Ex-vivo	Electroporation	ZFN	CCR5	Phase I |Phase I I	Completed	Human Immunodeficiency Virus (HIV)	United States	2018–06
Viral diseases	NCT02500849	Ex-vivo	Electroporation	ZFN	CCR5	Phase I	Not Recuiting	HIV	United States	2024–8
Viral diseases	NCT03617198	Ex-vivo	Electroporation	ZFN	CCR5	Phase I	Not Recuiting	Hiv	United States	2027–12
Hematological malignancies	NCT03399448	Ex-vivo	Electroporation, lentiviral vector	CRISPR/ Cas9	TCRα, TCRβ, PD-1	Phase I	Terminated	Multiple Myeloma, Melanoma, Synovial Sarcoma, Myxoid/Round Cell Liposarcoma	United States	2020–10
Hematological malignancies	NCT03166878	Ex-vivo	Lentiviral (CAR), Electroporation (TCR/B2M)	CRISPR/ Cas9	βTCRα, TCRβ, β-2 microglobin	Phase I |Phase I I	Unknown	B Cell Leukemia, B Cell Lymphoma	China	2022–05
Hematological malignancies	NCT05397184	Ex-vivo	lentivirus	Base-Editor	anti CD7, CD7R, CD52R, TCR	Phase I	Recuiting	Relapsed/Refractory T-cell Acute Lymphoid Leukaemia	Israel	2025–2
Hematological malignancies	NCT04557436	Ex-vivo	lentivirus	Crispr/ Cas9	anti CD19, CD52 and TRAC loci	Phase I	Completed	B Acute Lymphoblastic Leukemia	Israel	2024–3
Hematological malignancies	NCT04142619	Ex-vivo	Lentivirus	TALEN	PD-1, CD52	Phase I	Terminated	Relapsed/Refractory Multiple Myeloma	United States	2023–6
Hematological malignancies	NCT04037566	Ex-vivo	lentivirus	CRISPR/ Cas9	HPK1	Phase I	Recuiting	Leukemia Lymphocytic Acute (ALL) in Relapse, Leukemia Lymphocytic Acute (All) Refractory	China	2024–08
Hematological malignancies	NCT04106076	Ex-vivo	lentivirus	TALEN	anti-CD123	Phase I	Withdrawn	Acute Myeloid Leukaemia	2019–12	
Hematological malignancies	NCT03190278	Ex-vivo	lentivirus	TALEN	PD-1 and CD52	Phase I	Recuiting	Relapsed/Refractory Acute Myeloid Leukemia	United States	2024–12
Hematological malignancies	NCT04150497	Ex-vivo	lentivirus	TALEN	PD-1 and CD52	Phase I	Recuiting	B-cell Acute Lymphoblastic Leukemia	United States, France	2026–1
Blood disorders	NCT05356195	Ex-vivo	NA	CRISPR/ Cas9	BCL11A	Phase III	Recuiting	Beta-Thalassemia	United States, Canada, Germany,Italy, Israel	2026–05
Blood disorders	NCT05577312	Ex-vivo	NA	CRISPR/ Cas9	BCL11A enhancer site	Phase I	Enrolling by invitaion	Beta-Thalassemia	China	2026–9
Blood disorders	NCT03728322	Ex-vivo	NA	CRISPR/ Cas9	HBB	EARLY_Phase I	Unknown	Thalassemia		2021–01
Blood disorders	NCT05444894	Ex-vivo	NA	CRISPR/ AsCas12a	γ-globin	Phase I |Phase I I	Recuiting	Transfusion Dependent Beta Thalassemia, Hemoglobinopathies, Thalassemia Major, Thalassemia Intermedia	United States, Canada	2025–12
Blood disorders	NCT06041620	Ex-vivo	NA	CRISPR/ Cas12b	HBG1/2	NA	Recuiting	Beta-Thalassemia	China	2026–6
Blood disorders	NCT06287086	Ex-vivo	NA	CRISPR/ Cas9	BCL11A	NA	Not yet recruiting	Sickle Cell Disease	2026–6	

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Disorder type	NCT	Ex-vivo/ In-vivo	Delivery	Nuclease	Target gene	Phase	Status	Conditions	Location	Completion date
Blood disorders	NCT04819841	Ex-vivo	NA	CRISPR/ Cas9	HbS to HbA	Phase I |Phase I I	Recuiting	Sickle Cell Disease	United States	2027–31
Blood disorders	NCT04925206	Ex-vivo	NA	CRISPR/ Cas9	BCL11A gene	Phase I	Not Recuiting	Transfusion Dependent Beta-Thalassaemia	China	2024–6
Blood disorders	NCT04774536	Ex-vivo	NA	CRISPR/ Cas9	HBB	Phase I |Phase I I	Not yet recruiting	Sickle Cell Disease	United States	2028–6
Blood disorders	NCT06065189	Ex-vivo	NA	Base-Editor	BCL11A	Phase I	Recruiting	β-thalassemia Major	china	2024–12
Blood disorders	NCT06107400	Ex-vivo	NA	Base-Editor	NA	Phase I	Recruiting	Alpha Thalassemia Hemoglobin H Constant Spring, Hemoglobinopathies, Hereditary Diseases	china	2026–10
Cancer	NCT05795595	Ex-vivo	NA	CRISPR/ Cas9	anti CD70	Phase I |Phase I I	Recuiting	Solid Tumors − Clear Cell Renal Cell Carcinoma, Cervical Carcinoma, Esophageal Carcinoma, Pancreatic Adenocarcinoma, Malignant Pleural Mesothelioma	United States	2030–05
Cancer	NCT05812326	Ex-vivo	NA	CRISPR/ Cas9	PD-1, anti MUC1	Phase I |Phase I I	Completed	Advanced Breast Cancer, Breast Neoplasm Malignant Female	China	2022–11
Cancer	NCT03747965	Ex-vivo	NA	CRISPR/ Cas9	PD-1, anti mesothelin	Phase I	Unknown	Solid Tumors	China	2020–05
Cancer	NCT03044743	Ex-vivo	NA	CRISPR/ Cas9	PD-1	Phase I |Phase I I	Unknown	Stage IV Gastric Carcinoma, Stage IV Nasopharyngeal Carcinoma, T-Cell Lymphoma Stage IV, Stage IV Adult Hodgkin Lymphoma, Stage IV Diffuse Large B-Cell Lymphoma	China	2022–03
Cancer	NCT04426669	Ex-vivo	NA	CRISPR/ Cas9	CISH	Phase I |Phase I I	Recuiting	Gastrointestinal Epithelial Cancer, Gastrointestinal Neoplasms, Cancer of Gastrointestinal Tract, Cancer, Gastrointestinal, Gastrointestinal Cancer, Colo-rectal Cancer, Pancreatic Cancer, Gall Bladder Cancer, Colon Cancer, Esophageal Cancer, Stomach Cancer	United States	2024–01
Cancer	NCT04976218	Ex-vivo	NA	CRISPR/ Cas9	anti EGFR, TGFβR-KO	Phase I	Recuiting	Solid Tumor, Adult, EGFR Overexpression	China	2024–12
Cancer	NCT02863913	Ex-vivo	NA	CRISPR/ Cas9	PD-1	Phase I	Withdrawn	Invasive Bladder Cancer Stage IV	China	2019–09
Cancer	NCT03545815	Ex-vivo	NA	CRISPR/ Cas9	PD-1 and TCR	Phase I	Unknown	Solid Tumor, Adult	China	2020–12
Cancer	NCT02867332	Ex-vivo	NA	CRISPR/ Cas9	PD-1	Phase I	Withdrawn	Metastatic Renal Cell Carcinoma	2020–11	
Cancer	NCT04438083	Ex-vivo	NA	CRISPR/ Cas9	anti CD70	Phase I	Not Recuiting	Renal Cell Carcinoma	United States, Australia, Canada, Netherlands	2027–04
Cancer	NCT02867345	Ex-vivo	NA	CRISPR/ Cas9	PD-1		Withdrawn	Hormone Refractory Prostate Cancer	China	2020–12
Cancer	NCT03081715	Ex-vivo	NA	CRISPR/ Cas9	PD-1	NA	Completed	Esophageal Cancer	China	2018–2
Genetic disorders	NCT06325709	Ex-vivo	NA	Base-Editor	CYBB	Phase I |Phase I I	Not yet recruiting	Chronic Granulomatous Disease (CGD), X-Linked Chronic Granulomatous Disease	United states	2032–12
Hematological malignancies	NCT05037669	Ex-vivo	NA	CRISPR/ Cas9	anti cCD19, beta-2 microglobulin, CIITA, T cell receptor alpha chain	Phase I	Withdrawn	Hematological malignancies − Acute Lymphoblastic Leukemia, Chronic Lymphocytic Leukemia, Non Hodgkin Lymphoma	2038–01	

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Disorder type	NCT	Ex-vivo/ In-vivo	Delivery	Nuclease	Target gene	Phase	Status	Conditions	Location	Completion date
Hematological malignancies	NCT05662904	Ex-vivo	NA	CRISPR/ Cas9	CD33	Phase I	Not yet recruiting	Relapsed/Refractory Acute Myeloid Leukemia (AML)	Germany	2025–02
Hematological malignancies	NCT03398967	Ex-vivo	NA	CRISPR/ Cas9	anti CD19 and anti CD20 or anti CD19 and anti CD22	I/II	Recruiting	B Cell Leukemia, B Cell Lymphoma	China	2022–5
Hematological malignancies	NCT06128044	Ex-vivo	NA	CRISPR/ Cas9	CLL-1	Phase I	Recuiting	Refractory Acute Myeloid Leukemia, Acute Myeloid Leukemia Refractory	United States	2028–04
Hematological malignancies	NCT05631912	Ex-vivo	NA	CRISPR/ Cas9	TRAC, anti CD19	Phase I |Phase I I	Recuiting	Non-hodgkin Lymphoma	China	2025–12
Hematological malignancies	NCT05066165	Ex-vivo	NA	CRISPR/ Cas9	anti-WT1	Phase I |Phase I I	Terminated	Acute Myeloid Leukemia	United States, Israel	2022–8
Hematological malignancies	NCT06014073	Ex-vivo	NA	CRISPR/ Cas9	TRAC, Power3, anti CD19	Phase I |Phase I I	Recuiting	Non Hodgkin's Lymphoma	China	2026–9
Hematological malignancies	NCT05722418	Ex-vivo	NA	CRISPR/ Cas9	anti BCMA	Phase I	Recuiting	Relapsed/Refractory Multiple Myeloma	United States	2027–02
Hematological malignancies	NCT04035434	Ex-vivo	NA	CRISPR/ Cas9	CD19	Phase I |Phase I I	Recuiting	Non-Hodgkin Lymphoma, B-cell Lymphoma	United States, Australia, Canada,France, Germany,Spain	2026–08
Hematological malignancies	NCT04767308	Ex-vivo	NA	CRISPR/ Cas9	anti CD5	EARLY_Phase I	Unknown	CD5 + Relapsed/Refractory Hematopoietic Malignancies, Chronic Lymphocytic Leukemia (CLL), Mantle Cell Lymphoma (MCL), Diffuse Large B-cell Lymphoma (DLBCL), Follicular Lymphoma (FL), Peripheral T-cell Lymphomas (PTCL)	2023–12	
Hematological malignancies	NCT04637763	Ex-vivo	NA	CRISPR/ Cas9	Anti-CD19	Phase I	Recuiting	Lymphoma, Non-Hodgkin, Relapsed Non Hodgkin Lymphoma, Refractory B-Cell Non-Hodgkin Lymphoma, Non Hodgkin Lymphoma, Lymphoma, B Cell Lymphoma, B Cell Non-Hodgkin's Lymphoma	United States, Australia,Israel	2025–09
Hematological malignancies	NCT04244656	Ex-vivo	NA	CRISPR/ Cas9	BCMA	Phase I	Not Recuiting	Multiple Myeloma	United States, Australia, Canada,Spain	2027–01
Hematological malignancies	NCT05643742	Ex-vivo	NA	CRISPR/ Cas9	CD19	Phase I |Phase I I	Recuiting	B-cell Lymphoma, Non-Hodgkin Lymphoma, B-cell Malignancy, Chronic Lymphocytic Leukemia (CLL)/Small Lymphocytic Lymphoma (SLL), Follicular Lymphoma, Mantle Cell Lymphoma, Marginal Zone Lymphoma, Large B-cell Lymphoma	United States	2030–02
Hematological malignancies	NCT05885464	Ex-vivo	NA	Base-editor	Anti-CD7	Phase I |Phase I I	Recruiting	Lymphoblastic Lymphoma, T-Cell Lymphoblastic Leukemia/Lymphoma, Lymphoblastic Leukemia	united states	2031–12
Hematological malignancies	NCT05942599	Ex-vivo	NA	Base-editor		Phase I	Recruiting	Relapsed Acute Myeloid Leukaemia	united kingdom	2026–6
Metabolic disorders	NCT05565248	Ex-vivo	NA	CRISPR/ Cas9	MHC,CD47	Phase I |Phase I I	Recuiting	Diabetes Mellitus type 1	Canada	2025–08
Metabolic disorders	NCT05210530	Ex-vivo	NA	CRISPR/ Cas9	PEC210A cells	Phase I	Completed	Diabetes Mellitus Type 1	Canada	2023–1
Viral diseases	NCT03164135	Ex-vivo	NA	CRISPR/ Cas9	CCR5	NA	Unknown	HIV-1-infection	China	2021–5
Hematological malignancies	NCT04416984	Ex-vivo	Electroporation	TALEN	anti CD19	Phase I |Phase I I	Recuiting	Relapsed or Refractory Large B Cell Lymphoma, Relapsed or Refractory Chronic	United States, Australia,	2029–05

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Table 1 (*continued*)

Disorder type	NCT	Ex-vivo/ In-vivo	Delivery	Nuclease	Target gene	Phase	Status	Conditions	Location	Completion date
Cancer	NCT02793856	Ex-vivo	Nucleofection	CRISPR/Cas9	PD-1	Phase I	Completed	Lymphocytic Leukemia, Relapsed or Refractory Small Lymphocytic Lymphoma Metastatic Non-small Cell Lung Cancer	Canada,Italy, Spain China	2020–3

has the potential to be a breakthrough for rare diseases since many mutations linked to human disease are single-point mutations. [174] Animal models support base editors' potential in correcting various rare genetic diseases like progeria,[59] Leber congenital amaurosis,[175] Sickle Cell Disease,[176] and Duchenne Muscular Dystrophy.[68].

In August 2022, Verve Therapeutics began a clinical trial using intravenously delivered LNPs carrying ABE mRNA and a gRNA to target the PCSK9 gene in patients with familial hypercholesterolemia. Verve Therapeutics' product candidate VERVE-102, expected to enter a Phase 1 clinical trial in H1 2024, utilizes targeted LNPs. VERVE-102 follows a therapeutic strategy similar to VERVE-101,[168,177] currently in Phase 1 clinical trial, using *in-vivo* base editing to permanently disrupt the PCSK9 gene in the liver to lower LDL cholesterol levels in patients with high-risk heterozygous familial hypercholesterolemia (HeFH). VERVE-101 demonstrates a sustained 50 % reduction in LDL-C using non-targeting LNPs. In contrast, VERVE-102, utilizing LNPs with a proprietary GalNAc-based ligand, shows pre-clinical success with increased liver base editing from 5 % to 61 %, minimizing editing in other tissues. Targeting LNPs enhances the company's potential to effectively lower LDL-C in patients.[177].

Another major CRISPR BEs development is Beam Therapeutics obtaining FDA approval for clinical trials of its base editor BEAM-101 to treat sickle cell disease.[60] BEAM-101, a patient-specific, autologous blood stem cell therapy, aims to provide a one-shot cure for sickle cell disease and beta-thalassemia by introducing point mutations present in some individuals with sustained fetal hemoglobin production. This strategy, while not new, is the first to incorporate a base editor.[60] Base editors, like BEAM-101, hold promise for the safe correction of single-point genetic diseases without causing DSBs marking a significant advance in CRISPR technology. Hopefully, the results will be reported soon.

While standard programmable nucleases and base editors show success in human clinical trials, limitations in cell types, required manipulations, and safety concerns persist. Prime editing emerges as a new gene-editing tool promising solutions to specific clinical needs that conventional methods may struggle to address.

Prime editing has shown promise in various therapeutic approaches *in-vivo*. For instance, in 2021, successful *in-vivo* prime editing was achieved in the retina and liver, correcting mutations in mouse models of Leber's congenital amaurosis and hereditary tyrosinemia. [178] In 2022, liver-directed prime editing was demonstrated in a mouse model of phenylketonuria, achieving a therapeutic reduction of blood phenylalanine. [179] David Liu's group also reported successful prime editing in the mouse brain, liver, and heart.[120] In 2023, Prime Medicine presented promising data on its PE approach for Glycogen storage disease type 1B (GSD1b), a rare genetic disease affecting approximately 1,500 patients. The study, conducted in non-human primates (NHPs), demonstrated efficient and precise corrections of disease-causing mutations in the glucose-6-phosphate transporter (G6PT) gene, which is implicated in GSD1b. The mutations p.L348fs and p.G339C, prevalent in 46–52 % of GSD1b patients, were targeted with Prime Editors, showing up to 50 % whole liver precise editing in NHPs at day 14 without significant unintended edits. [180] The approach restored G6PT protein expression and glucose homeostasis, with no detectable off-target edits observed. These findings provide proof of concept and support the advancement of Prime Editors for GSD1b and other liver-targeted programs. [180] These preclinical exciting developments will pave the way for future clinical trials.

Next year, Prime Medicine aims to seek FDA permission for a clinical trial of a prime-editing treatment for chronic granulomatous disease. [181] Simultaneously, researchers are advancing the technique to insert larger DNA pieces into the genome, offering potential for comprehensive gene replacement therapies, and simplifying the treatment of disorders like cystic fibrosis caused by various mutations in a single gene. [41,181] This approach could lead to universal therapies applicable to all patients with the disease.

While prime editing holds great promise, it is important to note that it is still a relatively new technology and further research is needed to optimize its efficiency, delivery methods, and safety in a clinical setting.

6. Conclusion and future outlook

Despite the significant potential of the CRISPR-Cas9 system for precise genetic editing manipulation, there remain substantial challenges in envisioning its application in *in-vivo* gene therapy. The primary challenge lies in the development of a safe and biocompatible delivery mechanism for CRISPR-Cas9. Beyond merely shielding mRNA CRISPR-Cas9 and sgRNA constructs from degradation *in-vivo*, the delivery system must possess specific attributes. Among the various options, LNPs stand out as the most advanced and FDA-approved carriers for RNA molecules. LNPs have demonstrated an ability to encapsulate relatively large mRNA molecules, making them well-suited for delivering mRNA CRISPR-Cas9 constructs, especially the CRISPR-Cas9 base and prime editors, which are even larger than the standard system. Given that nonspecific delivery of CRISPR-Cas9 can result in adverse editing effects in unintended cells, ensuring precise delivery is crucial.

Accomplishing accurate targeting within living organisms remains a significant challenge for RNA delivery systems. It is crucial to integrate varying degrees of specificity into the developed CRISPR-Cas9 delivery system to ensure precise targeting of the intended organ and specific cell type. As delineated in this review, lipid nanoparticle platforms offer exceptional versatility in delivery. By adjusting the lipid components, especially the ionizable lipid subtype, of the LNP structure, targeted delivery to specific organs and cells can be achieved passively. A more comprehensive understanding of the factors influencing the biodistribution of different LNP formulations is imperative. Specifically, a deeper exploration of the relationship between the structure of ionizable lipids, their functionality, and their influence on distribution patterns across organs and cells is required.

Additionally, decorating LNPs with target moieties such as peptides, ligands, or antibodies, facilitates active targeting and internalization of the LNPs into specific cell types. LNPs-ASSET system, designed for precise *in-vivo* cell-specific targeting, marks an early advancement in this area. We anticipate the development of innovative platforms aimed at delivering editing systems accurately to specific target cells. These dual layers for LNPs specificity, organ, and cellular specificity, are crucial requirements from the CRISPR-Cas9 delivery system, which aims to eliminate cancer cells or correct mutated genes with minimal non-specific off-target and non-specific effects. These crucial requirements from the CRISPR-Cas9 delivery system present a bottleneck in the field of CRISPR therapeutics in which we expect significant progress to be made.

To further mitigate non-specific editing effects, various molecular mechanisms can be implemented on the Cas9 mRNA construct. For example, sequences that bind to cell-specific microRNAs (miRNAs) could be integrated into the untranslated regions (UTRs) of Cas9 mRNA to modulate degradation or stabilization rates in distinct cellular populations. [140].

Besides the importance of developing stable and efficient LNP for delivering gene-editing tools to diseased cells, off-target events are a major challenge to the use of safe CRISPR LNPs in clinical applications, as they pose serious risks, requiring identification and improvement before clinical use. Scientists have explored Cas9 cleavage mechanisms and developed variants with reduced off-target risks without sacrificing efficiency. Modifying sgRNA can also mitigate off-target effects.

Despite CRISPR-Cas9's great potential as a gene editing tool, challenges also persist in its broad application, particularly in complex diseases like cancer, which led to the development of Base and prime editors, which provide a safety net, sidestepping adverse effects linked to DSBs (reducing the risk of chromosomal aberrations and cell toxicity) while impacting critical gene expression. While efficient, base editors (BEs) are limited to introducing specific point mutations, moreover, BEs have very few unwanted modifications, therefore virtually off-target effects can be possible. These limitations prompted the development of prime editors (PEs), which can create precise insertions and deletions with minimal unwanted modifications and virtually no off-target effects.

Like CRISPR/Cas9 and base editors, prime editing's initial advantages may manifest in its *ex-vivo* applications for blood disease therapies and immunotherapeutic approaches against cancer. The liver, easily accessible with LNPs, is a favorable choice for testing new gene editing tools. Intellia Therapeutics and Verve Therapeutics have conducted successful clinical trials focusing on CRISPR-Cas9 or base editor LNP delivery to the liver, suggesting a possible similar trajectory for LNPs delivery of prime editing as it exhibits significant potential in treating liver genetic diseases.

Yet the most intriguing and less explored field of prime editing investigation is *in-vivo* cancer editing which involves inducing insertions or deletions into key genes. The combination of targeted LNPs with the prime editing cargo has the potential to address challenges posed by multiple mutations within the same gene and to improve gene editing specificity while minimizing off-target effects. These attributes make it a promising avenue for future research in the field of oncology.

Finally, turning prime editing into a tool able to insert large DNA sequences, without the need for DSB, efficiently encapsulating it with precise targeted LNP, and maximizing the effort to solve any possible off-target issue, will possibility create a wider array of possible modifications, and will open up new therapeutic horizon.

Data availability

Data will be made available on request.

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Competing interests.

D.P. receives licensing fees (to patents on which he was an inventor) from, invested in, consults. (or on scientific advisory boards or boards of directors) for, lectured (and received a fee), or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNtech SE, Earli Inc., Kernal Biologics, Newphase Ltd., NeoVac Ltd., RiboX Therapeutics, Roche, SirTLabs Corporation, Teva Pharmaceuticals Inc. All other authors declare no competing financial interests.

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