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Exploring the Potential and Challenges of CRISPR Delivery and Therapeutics for Genetic Disease Treatment

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Human genetic disorders, arising from a range of genetic irregularities, can significantly affect human physiology, often with limited available treatment options. The development of the CRISPR system, facilitating precise editing of the genome, has opened new avenues for addressing a range of mutations found in various genetic disorders. However, there is currently a lack of comprehensive reviews that specifically address the application of CRISPR in genetic diseases. To bridge this gap, this review focuses on exploring the advancements in CRISPR technology and their utility in therapeutic approaches for various genetic disorders. This review introduces human genetic disorders, explains the fundamental mechanisms of CRISPR editing, and highlights the latest advancements in CRISPR technology. Additionally, it examines three CRISPR delivery techniques, including physical delivery, viral vectors, and nanocarriers. It further reviews CRISPR's applications in therapeutic approaches for genetic disorders. Finally, it identifies the primary hurdles associated with industrial development and ethics considerations that should be addressed before the application of CRISPR in a medical context.

1. Introduction

Genetic diseases arise from DNA abnormalities mainly in the nuclear or mitochondrial genome, which disrupt cellular functions and cause a range of health concerns.[1] These conditions can be inherited from parents or spontaneously arise due to individual DNA changes, affecting different body regions and organs.[1,2] Current methods of intervention for addressing genetic disorders include medications, physiotherapy, and supportive measures. In the field of medications, approaches such as genetic screening and in-utero enzyme replacement mainly focus on addressing genetic deficiencies and mutations, while the use of stem cells and small molecules provides highly specific therapeutic interventions.[3] However, obstacles persist in achieving effective therapies for genetic diseases, stemming from the absence of accurate disease models and strategies for directly rectifying mutations within patients' cells, especially in single-gene mutation cases.[1]

Addressing these challenges involves the emergence of gene editing as a crucial tool in advancing the comprehension of genetic diseases. This process enables precise DNA alterations via nucleotide additions, deletions, and alterations at the desired targeting sites, which enables disease modeling and novel gene therapies.[4,5] Prominent technologies like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR) enable precise DNA manipulation.[6] This capability empowers researchers to introduce or rectify

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specific genetic mutations, thereby paving the way for personalized medicine. This approach allows for the modification and correction of mutations within a patient's cells to yield therapeutic benefits. Furthermore, gene editing facilitates the introduction of therapeutic genes or the modification of existing genes to achieve desired outcomes.[1]

Among these genome editing techniques, ZFNs, combining a cleavage domain and DNA-binding zinc fingers, have revolutionized gene editing.[7] They have enabled efficient editing in higher eukaryotes and therapeutic applications such as engineering hematopoietic stem cells (HSCs) and T lymphocytes.[8,9] However, challenges including complex protein domain engineering and potential off-target effects have hampered their use.[10] TALENs offer a simpler approach, utilizing a DNA-binding domain and nuclease domain, though they still require extensive protein design.[10,11] CRISPR has emerged as a promising replacement for ZFNs and TALENs due to its simple preparation process, exceptional efficiency in gene editing, and its ability to edit multiple loci simultaneously.[10,12,13] The CRISPR's practical simplicity has propelled its application in disease modeling, particularly for hematological, neurological, infectious, and other genetic disorders.[14,15] Continued progress in CRISPR-associated protein (Cas) development and sgRNA design with low off-target effects has enabled CRISPR for in vivo gene editing, which paves the way for CRISPR-based therapies for human genetic diseases.[5]

This review offers an in-depth analysis of the present status of CRISPR technology in the advancement of genetic disease treatments. It begins with presenting the fundamental concepts of CRISPR gene, followed by an exploration of diverse delivery methods that aim to enhance the biological and clinical applications of CRISPR. It further discusses CRISPR's involvement in the development of therapeutic strategies for human genetic disease. Additionally, it highlights the primary hurdles that need to be overcome to fully harness CRISPR potential for clinical applications.

2. Human Genetic Diseases

Human genetic diseases arise from DNA abnormalities, either inherited or occurring spontaneously.[1] They fall into two main categories: germline diseases, stemming from mutations in germ cells and transmitted to offspring, and mitochondrial genetic diseases, resulting from alterations in mitochondrial DNA.[1] Germline genetic diseases emerge from mutations in reproductive cell DNA, passed to offspring. They include mutations in autosomal and sex chromosomes.[16] Autosomal genetic diseases come from mutations in genes on autosomal chromosomes, where dominant disorders result from a single gene mutation, while recessive conditions require mutations in both gene copies for symptoms.[16] Mitochondrial genetic diseases result from mutations in mitochondrial or nuclear DNA, impacting mitochondrial function.[1] Most mitochondrial mutations lead to nonviable oocytes and are eliminated before ovulation.[1] Accumulated somatic mitochondrial mutations in protein-coding genes are linked to various age-related conditions like Leber's Hereditary Optic Neuropathy, Mitochondrial Myopathy, Leigh Syndrome, Encephalopathy, Lactic Acidosis, and Stroke-like Episodes.[17]

In addition to the location of genetic anomalies, there are other criteria employed in the classification of human genetic diseases. Based on the affected organ or system, genetic disorders can also be categorized into neurological, cardiovascular, hematological, and connective tissue disorders.[18] Immunological disorders and cancer predisposition syndromes also contribute to the diversity of genetic diseases.[19] These categories together illustrate the complex array of human genetic diseases, and ongoing research is consistently improving our comprehension of these disorders.

3. CRISPR-Based Gene Editing Technology

3.1. CRISPR History and Development for Genome Editing

The CRISPR system's evolution history (**Figure 1**) started in 1987 with the discovery of repetitive palindromic DNA sequences in *Escherichia coli* bacteria, leading to its recognition for potential immune functions against viral infections.[20] By 2000, these sequences were found in various bacterial and archaeal species for genotyping.[21] In 2002, the term "CRISPR" was coined alongside the identification of CRISPR-associated genes.[22] Notably, Cas proteins, particularly Cas9, were acknowledged as precise genetic scissors.[22] Synthetic guide RNA (sgRNA) streamlined practical CRISPR-Cas9 system implementation for accurate DNA manipulation.[23] The discernment of associated Cas proteins and their role in the bacterial immune system laid the foundation for genome editing applications.[13,24]

The development of CRISPR-Cas9 technology reached a significant milestone in 2012 when Jennifer Doudna and Emmanuelle Charpentier demonstrated its potential for precise genome editing in a test tube.[25] Following this breakthrough, the CRISPR-Cas9 system quickly transitioned into practical application, demonstrating its effectiveness in editing DNA within mammalian cells.[26,27] A critical moment occurred in 2013 when this method was successfully applied for gene editing in human cells, particularly in independent research led by Feng Zhang and Jennifer Doudna,[15,27] representing a transformative achievement in biotechnology. In the same year, Jaenisch's lab successfully generated the first gene knock-out mouse using CRISPR/Cas9.[28] They injected Tet1 and Tet2 sgRNA along with Cas9 mRNA into zygotes, producing mice with up to 80% mutations in both genes. This was achieved through human-based homologous recombination, which facilitated the repair of DNA double breaks.[25] This technology revolutionized the capability to target and disable specific genes across a wide range of cells and organisms.[26,27] In 2016, China initiated the inaugural human clinical trial for CRISPR therapeutics, with the objective of treating non-small cell lung cancer patients.[29] This trial aimed to employ CRISPR technology in using PD-1 knockout T cells for the treatment.[29] At the same year, the first application in the CRISPR gene editing trial for cancer in the United States was initiated.[30] The autologous T cells were gene-edited using CRISPR technology to knock-out the gene loci of the TCR and PD-1, providing the potential treatment for melanoma, synovial sarcoma, and multiple myeloma.[30]

In recent years, a new advancement known as base editing technology has emerged within the realm of CRISPR gene editing systems. This innovative approach allows for precise modifications at the individual bases of RNA and DNA molecules,

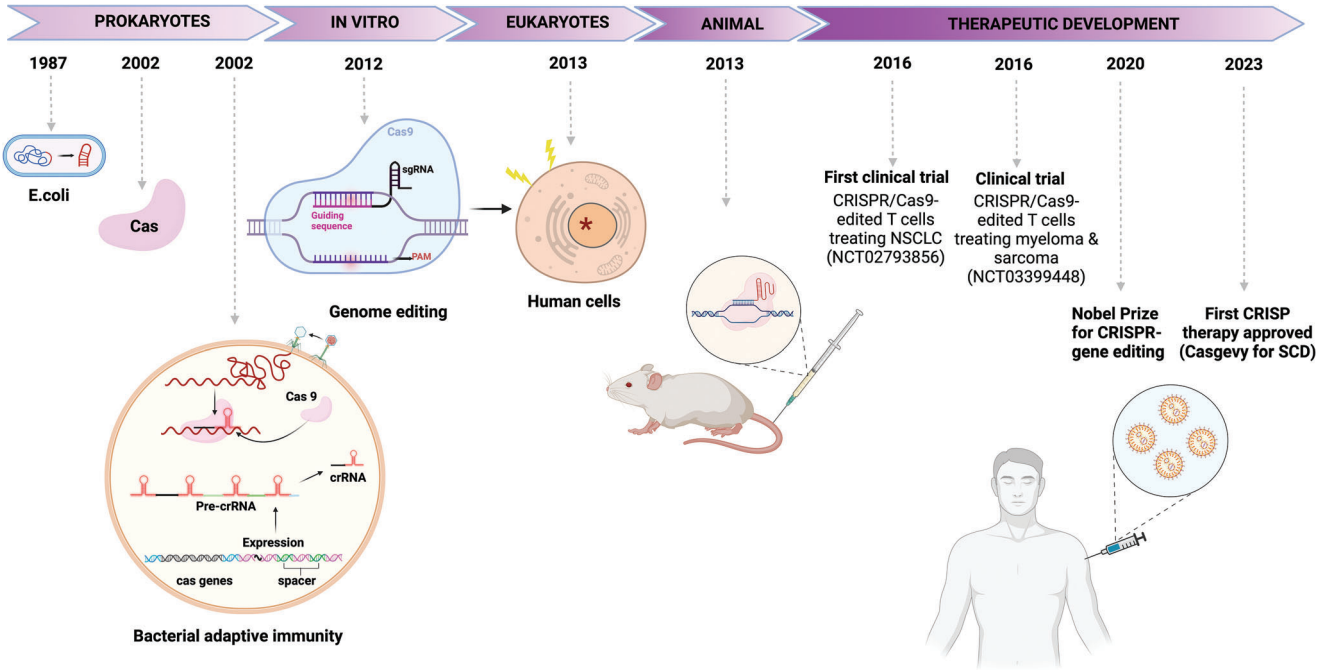

Figure 1. CRISPR Development Timeline. Highlights pivotal moments: 1987 – Ishino's discovery of palindromic DNA in E. coli, linked to viral defense. 2002 – Jansen coins "CRISPR" & identifies associated genes like Cas9. 2012 – Doudna & Charpentier demonstrate precise genome editing. 2013 – Human cell gene editing and first CRISPR mouse. 2016 – Inception of first human CRISPR therapeutics trials. 2020 – Nobel Prize in Chemistry awarded to Emmanuelle Charpentier and Jennifer Doudna for the development of CRISPR-genome editing. 2023 – Casgevy, the first CRISPR-based therapy was approved for the treatment of sickle cell disease. Created with BioRender.com.

facilitating precisely targeted alterations in both DNA and RNA sequences.[31] In 2016, David Liu and his team introduced base editing technology, marking the initial report of achieving precise modification of a single DNA base.[32] This advancement was accomplished without requiring double-stranded DNA backbone cleavage or a donor template.[32] In light of the compelling preclinical data, the first human trial of this editing approach was initiated in 2022.[33] Beam Therapeutics commenced a phase 1/2 trial in the United States, aiming to use a base editing therapy to treat severe sickle cell disease (SCD) by activating fetal hemoglobin through precise single-letter DNA modification.[33] In December 2023, Casgevy, an innovative CRISPR-based gene editing therapy developed by Vertex Pharmaceuticals and CRISPR Therapeutics, received FDA approval for the treatment of SCD.

In 2023 Feng Zhang's group discovered the latest Fanzor (Fz) protein, which is a eukaryotic programmable RNA-guided endonuclease and potential to replace the current Cas system.[34] These Fz proteins have a similar function to Cas9 but are smaller in size, with a relatively compact size of 638 amino acids compared to the standard Cas proteins of 1307 amino acids.[34] Due to their smaller size compared to the Cas9 protein, Fz systems hold the potential for easier delivery to cells and tissues as therapeutics, reducing unintended cleavage. Additionally, with its adaptability to target specific genome sites, the Fz systems could become a valuable new technology for human genome editing. However, based on the current gene editing efficiency of three wild-type Fz proteins lower than 11.8%, this strategy requires further improvement to enhance its editing efficiency.[34]

3.2. The CRISPR/Cas Components

CRISPR/Cas system generally consists of Cas effectors and guide RNA (gRNA) molecules. Based on the diversity of the Cas protein, the CRISPR system is categorized into two classes and each class also has three types.[35] Class 1 systems, encompassing types I, III, and IV, are distinguished by their utilization of multi-Cas effectors.[36] The key protein in Type I CRISPR is Cas3, which regulates precise DNA cleavage through its proficient nuclease and helicase domains.[37] Within Type III systems, the versatile Cas10, a multidomain protein, emerges as a distinctive player in DNA targeting and cleavage.[38] Conversely, Type IV systems, identifiable by Csf1, typically lack association with a CRISPR array.[39] In contrast, Class 2 systems, including Types II, V, and VI, have a singular effector such as Cas9 or Cpf1.[35,39,40] In Type II systems, the DNA endonuclease Cas9 is harnessed for CRISPR interference, marking a significant role in the process.[41] Type V systems stand out with Cas9-like nucleases, including Cpf1 (Cas12a), C2c1 (Cas12b), or C2c3, promising substantial potential for gene editing applications.[39,40] The distinctive characteristic of Type VI CRISPR lies in the presence of the C2c2 protein.[40]

CRISPR technology employs RNA molecules to guide the Cas protein to specific target sequences for gene editing. gRNA is a synthetic RNA molecule that combines two important components: CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA).[42] Within the gRNA, there is a programable region perfectly designed to match the target DNA, facilitating accurate binding and subsequent editing.[41c] Complementing the gRNA, crRNA carries the genetic information concerning the specific

DNA sequence in need of editing, with each crRNA tailored to a distinct DNA target.[43] Furthermore, tracrRNA contributes to the structural stability of the gRNA and aids in facilitating interaction between the gRNA and the Cas protein.[43] These RNA molecules collectively form an indispensable part of the CRISPR system, empowering the Cas protein to precisely identify and modify specific DNA sequences.

Within the CRISPR/Cas domain, Cas9, Cas12, and newly developed Cas14 hold significance owing to their unique interactions with crRNA, tracrRNA, and their specific nuclease functionalities crucial for achieving desired gene editing outcomes. Cas9 has a crucial structure for DNA manipulation with distinct domains, including a recognition domain for binding to target DNA guided by RNA, a flexible hinged region for conformational changes, and two nuclease domains for DNA cleavage.[42,44] The bridge helix optimally positions nuclease domains, and the protospacer adjacent motif (PAM) interaction domain enhances target specificity.[41c,44] The RNA-binding sites of Cas9 can stabilize interactions, forming a robust Cas9-gRNA complex for precise and programmable DNA editing with vast genetic and biomedical applications.[42] Similar to DNA-targeting Cas9, Cas12 solely relies on crRNAs for precise cleavage at both ssDNA and dsDNA.[45] The expanded adaptability of Cas12 empowers its efficiency across a spectrum of dynamic applications, ranging from the base editing, detection of transcriptional variations, and manipulation of epigenetic markers.[46] Cas14 can target ssDNA without the need for a PAM due to its much smaller size than other Cas proteins. Similar to Cas9, it uses tracrRNA and crRNA to locate the target DNA site.[47] Cas14 exhibits superior cleavage accuracy compared to Cas9 and Cas12, meeting the criteria for high-fidelity genome editing.[48]

3.3. Mechanism of CRISPR-Mediated Gene Editing

The CRISPR/Cas system operates by accurately targeting and altering the specific genes associated with a broad range of genetic disorders. In this section, we explain the fundamental principles that form the core of CRISPR editing for therapeutic purposes. These mechanisms include direct gene editing, base and primer editing, transcriptional regulation, and epigenetic modifications, each offering a unique avenue for potential therapeutic interventions.

3.3.1. Direct Gene Editing

CRISPR/Cas9 gene editing exploits the cell's DNA repair systems, achieving gene modifications through knock-out and knock-in methods (**Figure 2**A). In gene knock-out, sgRNA guides Cas9 protein to a specific gene site carrying PAM, thus prompting double-strand breaks (DSBs) in the DNA. In response to DNA damages, the transfected cells activate their intrinsic DNA repair processes like nonhomologous end joining (NHEJ) or homology-directed repair (HDR), often causing errors that disrupt the gene.[49] In gene knock-in, CRISPR/Cas utilizes a repair DNA template to induce DSBs in the target gene's DNA.[50] This template contains desired genetic changes to be precisely inserted into the gene. The cell's repair mechanism, guided by this template, incorporates the changes, resulting in a precise modification or "knock-in" of the gene.[51] This method allows for the introduction of specific genetic sequences or the correction of gene mutations.

3.3.2. Base Editing and Prime Editing

Traditional CRISPR gene editing has a notable downside: the potential for unintended indel (insertion or deletion) formation at the targeted site.[52] This unpredicted change in DNA can create harmful mutations, posing a challenge for precise gene edits.[53] To overcome this limitation, new techniques like base editing and prime editing have emerged (Figure 2B). Base editing involves fusing Cas effectors with enzymes capable of chemically altering DNA bases, allowing precise point mutations.[54] Cytosine base editors (CBEs) and adenine base editors (ABEs) induce specific transitions, offering highly accurate editing and reduced off-target effects.[55] Prime editing, on the other hand, enables editing in both dividing and nondividing cells, unlike HDR-based CRISPR gene editing, primarily suited for dividing cells (Figure 2C).[56] This system uses nCas9 fused with a prime editing gRNA (pegRNA) and a reverse transcriptase (RT) to edit DNA sequences. Prime editing shows promise for precise editing across various cell types and has demonstrated no detectable off-target effects in organoids and mouse models.[54,57] However, further research is needed to enhance its efficiency for broader applications.

3.3.3. Transcriptional Regulation

In addition to gene and base editing, CRISPR is employed for modifying and regulating target gene transcription and translation.[58] CRISPR activation (CRISPRa) and CRISPR-Cas13 systems control transcriptional and translational levels.[58,59] CRISPRa tools enhance gene transcription using dCas9 fused with activators like VP64 and p300 (Figure 2D,E). Advanced versions like SAM and SunTag provide improved precision and robustness in gene activation.[60,61] Meanwhile, CRISPR-Cas13 acts as a programmable translation activator by targeting RNA instead of DNA, regulating post-transcriptional translation for gene expression.[59] CRISPR achieves transcriptional and translational inhibition through CRISPRi and CRISPR-dCas13 systems.[58,59] CRISPRi blocks RNA polymerases and remodels chromatin structure, obstructing gene expression.[62] It attaches to DNA coding regions, hindering RNA polymerase binding, or employs dCas9 fused with repressors like SID4x to alter chromatin for targeted gene suppression.[60] Conversely, CRISPR-Cas13 inhibits gene expression post-translationally by targeting messenger RNA (mRNA) without cleavage, impeding mRNA translation initiation for gene suppression.[63]

3.3.4. Epigenetic Editing

CRISPR extends its utility beyond gene expression control to epigenetic editing, which manages DNA methylation and histone modifications, impacting chromatin structure and gene accessibility (Figure 2F).[64] By specifically altering epigenetic markers at precise locations, CRISPR can influence gene expression

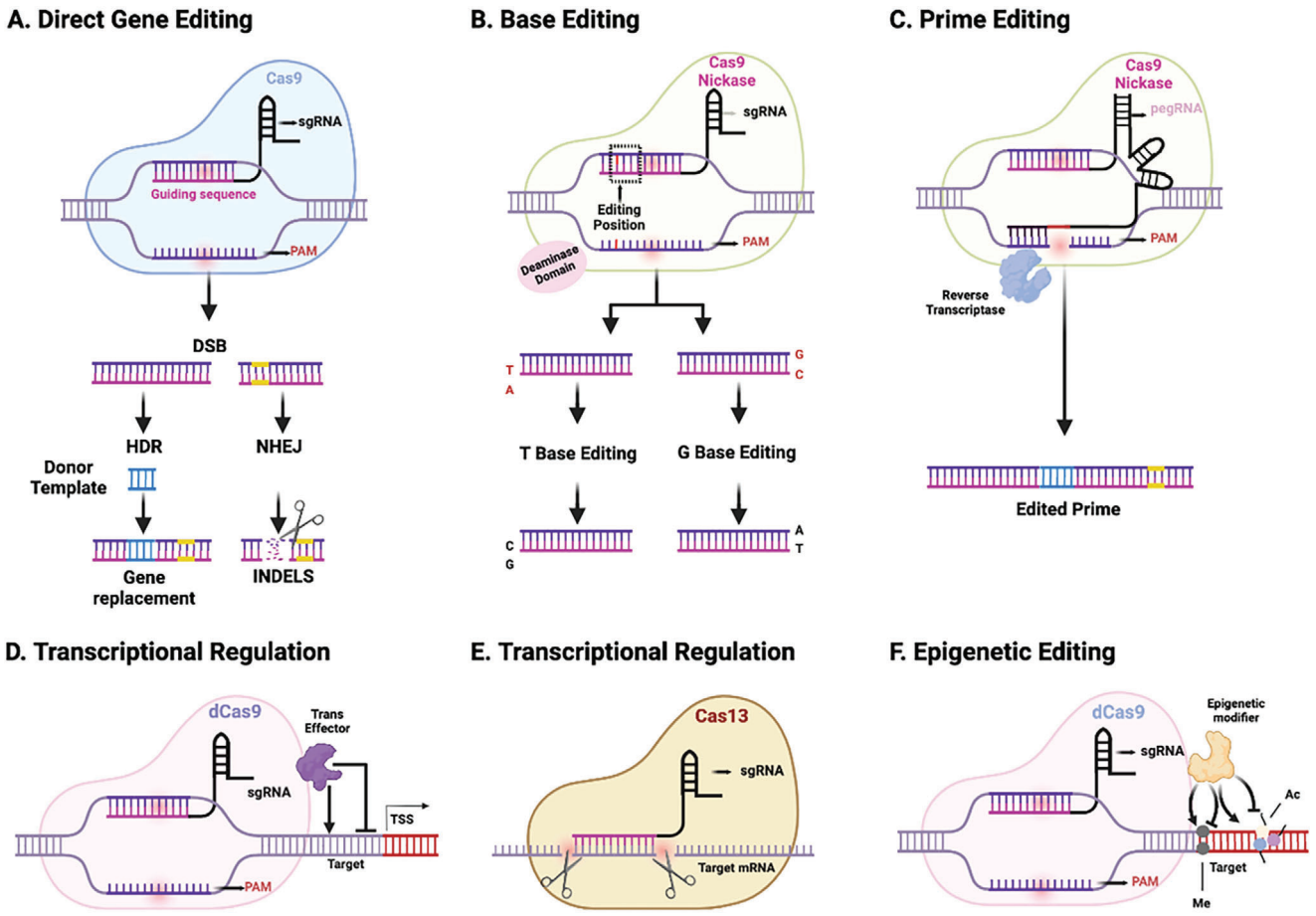

Figure 2. CRISPR Editing Mechanisms for Treating Human Genetic Diseases. A) Direct gene editing. B) Base editing. C) Prime editing. D&E). Transcriptional regulation. F) Epigenetic editing. Created with BioRender.com.

without changing the DNA sequence.[64] For instance, it modulates DNA methylation by fusing dCas9 with DNA methyltransferases or demethylation domains, repressing or activating genes, respectively.[65] CRISPR techniques precisely target histones, introducing modifications like acetylation or methylation using dCas9 fused with gene-regulatory proteins.[66] This tool allows meticulous editing of chromatin marks, impacting various histone residues and contributing significantly to understanding how histone modifications influence biological processes and genetic diseases.[67]

4. Delivery Systems for CRISPR Genome Editing

Due to its precise gene editing capabilities, CRISPR offers significant promise for understanding and treating human genetic diseases. However, for CRISPR to be effective, it must reach the specific cells or tissues affected by the disease.[68] Delivery systems are therefore essential to transport CRISPR components such as Cas proteins and gRNA to their intended sites within the cell nucleus with minimal off-target effects.[69] Moreover, efficient delivery methods can help reduce immunogenetic risks of CRISPR components, which in turn improve the tolerability of CRISPR-based therapies.[68]

CRISPR-Cas can be delivered in various forms, such as DNA plasmids encoding both Cas9 protein and gRNA; Cas9 mRNA paired with separate gRNA, or Cas9 protein combined with gRNA within a ribonucleoprotein complex.[70] Each of these cargo types possesses distinct structural, electrochemical, and stability properties. To fully exploit the therapeutic potential of CRISPR, effective delivery systems are necessary to transport CRISPR components of all forms safely and efficiently to the target tissues and cells. This section examines physical methods, viral vectors, and nanocarriers, discussing their advantages, limitations, and applications in both in vitro and in vivo settings.

4.1. Physical Method

The physical methods use the physical techniques such as microinjection and electroporation to introduce CRISPR components into target cells (**Figure 3A**). Microinjection refers to a physical technique wherein Cas9 and sgRNAs are directly injected into cells using a microscope and a fine needle.[71] This method enables the precise delivery of plasmid DNA, mRNA, or RNPs directly into the nucleus, achieving a remarkable 100% success rate.[69] Microinjection circumvents challenges linked to extracellular matrices, cellular membranes, and internal cellular

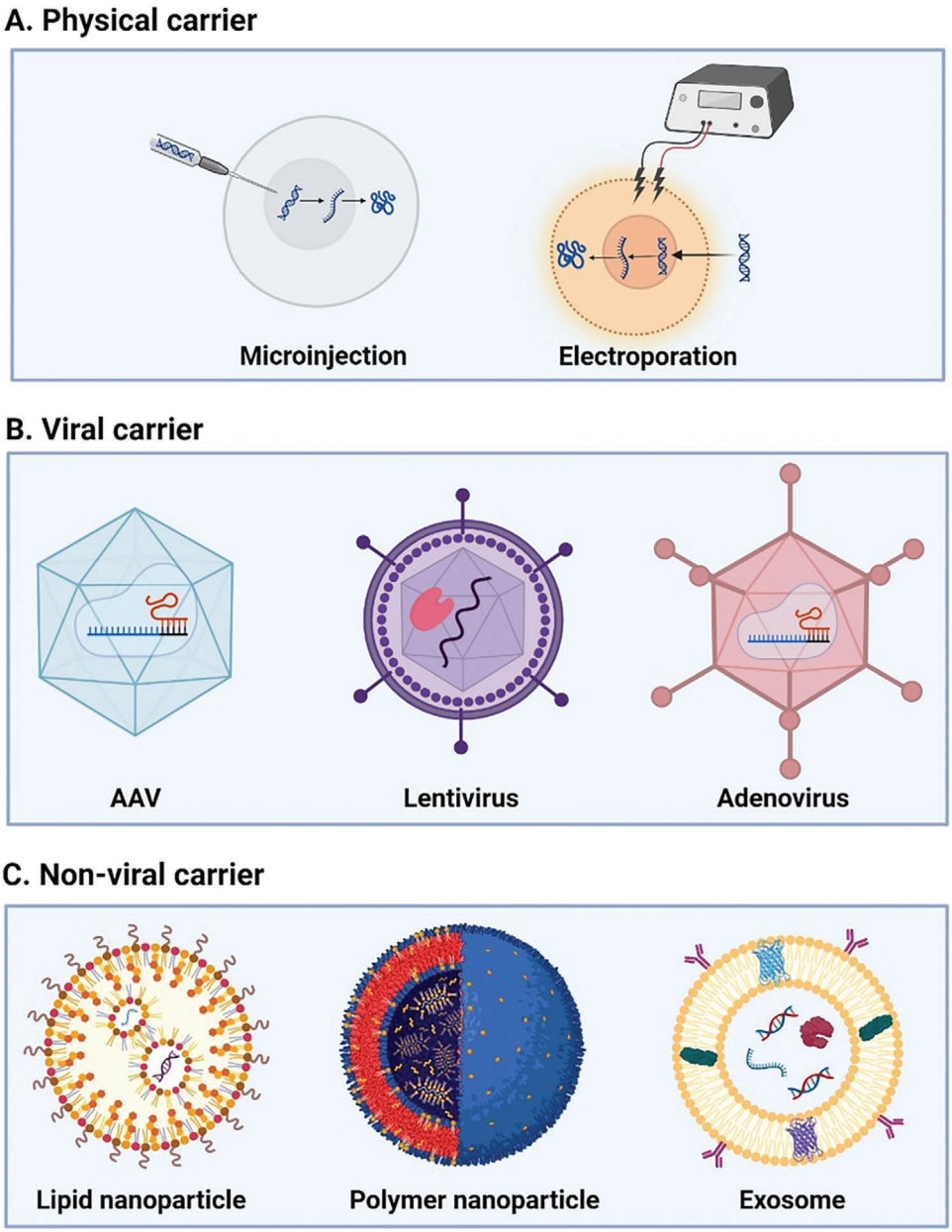

Figure 3. CRISPR delivery carriers. A) Physical carriers: microinjection, hydrodynamic injection, electroporation. B) Viral carriers: Adeno-associated virus (AAV), Lentivirus, Adenovirus. C) Non-viral carriers: lipid nanoparticles, polymer nanoparticles, exosomes.

components. It enables controlled cargo release and mitigates the risk of off-target effects.[72] However, microinjection's main challenges lie in its labor-intensive nature, where individual cells are injected manually, and scalability issues, restricting its use to in vitro or ex vivo cultured cells and precluding its application in vivo human contexts.[69]

Electroporation involves using high-voltage electrical pulses for delivery through transient pores in cell membranes.[73] Initially applied for Cas9 mRNA delivery and later optimized for CRISPR/Cas9 RNP complex delivery, this technique has demonstrated effectiveness in both rat and mouse embryos.[74] Unlike conventional transfection methods, electroporation is versatile and can be used for challenging-to-transfect cell types. Delivering the RNPs leads to more immediate activity, increasing mutagenesis efficiencies and reducing mosaicism within engineered zygotes.[75] Electroporation has played a role in advancing stem cell therapies for conditions like hemoglobinopathies through ex vivo gene editing.[76] However, its use in human in vivo experiments remains impractical due to the high voltages required to cross cell membranes.

4.2. Viral Carriers

The predominant viral vectors, such as adeno-associated viruses (AAVs), lentiviruses, and adenoviruses, are extensively utilized

for delivering CRISPR in both in vitro and in vivo settings, as shown in Figure 3B. AAVs, members of the *Parvoviridae* family, are small, non-enveloped, non-pathogenic viruses with single-stranded DNA. These characteristics render them relatively safe for infecting cells, exhibiting low initial immunogenicity in humans.[77] However, a critical limitation is their small packaging size of around 4.5–5 kb, posing a challenge for accommodating the large CRISPR/Cas9 gene-editing system.[78] Lentiviruses, with a maximum cargo size of around 10 kb, possess the capability to efficiently deliver an entire CRISPR RNP complex in a single transfection.[79] However, lentiviral vectors could potentially induce off-target mutations due to insertional mutagenesis and the prolonged expression of site-specific nucleases.[80] Adenovirus vectors have the capacity to transport approximately 8 kb of foreign DNA.[166] Unlike other viral vectors, it lacks endogenous integrase, thus having a lower risk of off-target effects and insertional mutagenesis.[81] They can efficiently transport CRISPR components into both dividing and non-dividing cells, making them suitable for specific gene-editing applications.[82] Nevertheless, significant concerns associated with adenovirus vectors include their potential to induce immune responses, tissue inflammation, and subsequent vector elimination.[82] To address these challenges, various strategies such as the addition of polymer or the use of non-human adenovirus vectors, have been shown to have a lower impact on cross-reactive immunity by CRISPR system.[83]

4.3. Nanocarriers

While physical and viral vectors have advanced, they possess limitations hindering their in vivo CRISPR delivery applications. Nanoparticles have emerged as potential carriers, ensuring precise and safe transport of CRISPR components to target cells for therapeutic outcomes.[84] Various nanocarriers such as lipid nanoparticles (LNPs), polymers, and emerging exosomes have been explored for CRISPR delivery (Figure 3C; Table S1, Supporting Information).

LNPs form stable structures with Cas9 plasmid DNA, mRNA, and gRNA, aiding cell membrane penetration and endosomal release of CRISPR content in target cells.[84] However, their permanent cationic lipid components pose issues like toxicity, instability, and low transfection efficiency.[68] To enhance the intracellular transport and transfection efficiency of CRISPR payloads, extensive efforts have been made, such as refining LNPs with ionizable cationic lipids and biodegradable lipid components. Ionizable cationic lipids enhance cargo delivery to target cells by converting LNPs to a positively charged state in the acidic late endosomal environment.[85] This modification enables LNPs to interact with the negatively charged lipids in the endosomal membrane, promoting membrane fusion and disruption. Ultimately, this process facilitates the release of CRISPR components into the cytoplasm of target cells.[84] The use of biodegradable lipid-like complexes loaded with Cas9 and sgRNA allows for highly efficient protein delivery, resulting in effective gene knockout in ≈70–90% of cultured human cells.[86,87]

Polymer nanoparticles, such as polyethylenimine (PEI), poly(L-lysine) (PLL), poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA), polyamidoamine (PAMAM), chitosan and poly(amino-co-ester)s (PAEs), offer promise for in vivo CRISPR delivery due to low immunogenicity, strong biocompatibility and ease of modification.[88] These polymers have been utilized for both in vitro and in vivo delivery of CRISPR components.[89] However, their high toxicity requires further engineering, including lipid co-complexation, to mitigate toxicity and enhance functionality.[90] Materials like PLGA, PEI, and chitosan can be modified within polymer-based nanoparticles for controlled release and targeted CRISPR delivery.[5,12]

Exosomes, small extracellular vesicles (EVs) with diameter ranging from 30 to 150 nm, are produced by nearly all cell types and are present in various body fluids.[91] Exosomes can deliver a wide range of molecules including proteins, small nucleic acids, and lipids.[92] Exosomes offer several advantages when utilized as a delivery vehicle. For example, exosomes can avoid the rapid uptake of the immune system, cross barriers such as blood-brain and placental barriers, and target specific cells or tissues due to the specific surface protein.[93] However, the small size of exosomes poses a challenge for effectively encapsulating the large components associated with the CRISPR system. To overcome this challenge, several strategies have been studied, such as exosome engineering,[94] hybrid delivery systems,[95] and direct loading techniques.[96]

5. CRISPR Application for Genetic Disease Treatment

CRISPR technology has revolutionized the field of genetic disorders by offering a wide range of applications, including the model development (Table S2, Supporting Information) and advanced therapies (Table S3, Supporting Information). This section will particularly discuss its therapeutic applications for genetic muscular diseases, cardiovascular diseases, inherited blood disorders, inherited eye diseases, neurodegenerative conditions, and genetic metabolic diseases.

5.1. Genetic Muscular Diseases

Muscular dystrophies are genetic disorders causing progressive muscle weakness due to defects in muscle structure and function, evident in biopsies.[97] Common types include Facioscapulohumeral, Duchenne, and Becker muscular dystrophies (D/BMD).[98] Among these muscular diseases, DMD, conditioned associated mutations of the X-linked DMD gene, exhibits more severe phenotypes such as muscle weakness, respiratory/cardiac problems, and reduced life expectancy.[99,100] Hence, various therapeutic approaches have been undertaken to restore dystrophin expression and functionality, aiming to alleviate symptoms associated with DMD.[99]

Currently, clinical development for DMD using pharmacological compounds faces key challenges such as inefficient delivery, lack of specificity, and inefficacy in clinical studies.[101] To address this challenge, researchers are exploring regenerative strategies using stem cell transplantation.[102] This approach utilizes various types of stem cells, including skeletal muscle-derived stem cells, mesenchymal stem cells, and induced pluripotent stem cells (iPSCs), to regenerate the damaged muscle tissue.[102] Nevertheless, despite its potential, stem cell therapy faced significant

obstacles that prevented its widespread clinical use, such as graft rejection risk and limited scalability due to the low expansion rate of undifferentiated muscle stem cells.[103] Novel therapies have emerged, including muscle-targeted approaches employing anti-inflammatory medications, myostatin inhibitors, and compounds targeting fibrotic pathways.[103] While these strategies aim at reducing inflammation and muscle fibrosis, they can only slow down DMD progression but not restore the structure and function of the degrading muscle tissue.[104]

CRISPR's potential in treating DMD is well-documented. Studies in mouse, canine, and porcine models have shown elevated dystrophin expression and improved muscle function following AAV9-CRISPR injections.[105] For instance, in a recent study utilizing a recognized porcine DMD model with a premature stop codon in exon 52 (ΔE52), scientists applied a CRISPR system (AAV9-Cas9-gE51) to excise exon 51 of the DMD gene.[106] This intervention removed the premature stop codon in the ΔE52 pigs and introduced the expression of a truncated but functional DMD gene, which lacked both exon 51 and 52 (ΔE51-52).[106] This somatic gene editing technique thus led to functional dystrophin expression in various muscles, rescuing dystrophin levels and notably increasing survival rates. It also improved skeletal function, evident in increased standing time and reduced creatine kinase levels as well as decreased muscle fibrosis. Similar improvements were observed in cardiac muscle function, specifically improved ejection function. In summary, these results imply CRISPR's potential as a promising therapeutic avenue for DMD. However, further research is required to address remaining challenges such as enhancing gene editing efficiency, exploring alternative delivery methods, and reducing reliance on viral vectors to optimize its clinical applications.

5.2. Genetic Cardiovascular Diseases

CRISPR-based therapies have developed to target various genetic cardiovascular conditions like catecholaminergic polymorphic ventricular tachycardia (CPVT), hypertrophic cardiomyopathy (HCM), and Hutchinson–Gilford progeria syndrome (HGPS).[107] CPVT is a rare genetic heart condition inherited in an autosomal dominant manner, caused by mutations in the RYR2 gene.[108] This genetic alteration leads to ventricular tachycardia, which can precipitate fainting, seizures, or sudden cardiac arrest. Currently, CPVT lacks a cure, with management primarily focused on symptom alleviation through beta-blockers and implantable cardioverter-defibrillator devices (ICDs) to regulate heart rate and rhythm.[109] However, beta-blockers pose risks of arrhythmias in non-compliant cases; ICDs have limitations like infection and inappropriate shocks and may not be suitable for all patients.[110] In addressing CPVT, a study utilized AAV9-CRISPR-SaCas9 to target the RyR2 R176Q mutation, successfully reducing RyR2 mRNA and protein levels by 30% and 25%, respectively.[111] This approach enhanced resistance to arrhythmia induction in mice and notably reduced the occurrence of inducible ventricular tachycardia, indicating CRISPR's potential in restoring cardiac signaling pathways and electromechanical coupling in CPVT.[111]

HCM is a genetic condition characterized by mutations in one of structural cardiac genes such as MYH7, MYBPC3, TNNT2, TNNI3, TPM1 or ACTC1.[112] These mutations can disrupt the normal structure and functions of sarcomere, leading to myocardium thickening, which impairs cardiac contractile function and leads to serious complications such as shortness of breath, chest pain, palpitations, and sudden death.[112] Clinical management options for HCM are limited, consisting mainly of surgical interventions or the use of medications like beta-blockers and calcium channel blockers to alleviate symptoms.[113] However, not all patients respond favorably to these treatments, and some may not be suitable for surgery, leaving them with few therapeutic options.[113] In tackling HCM, researchers have developed a small molecule known as MYK-461 designed to bind and inhibit cardiac myosin.[114] This compound has demonstrated the potential to suppress hypertrophic remodeling and myocardial fibrosis in a mouse model of HCM.[114] Furthermore, through chronic oral administration, MYK-461 has shown promise in preventing left ventricular hypertrophy.[114] Phase 3 clinical studies have revealed that MYK-461 is well-tolerated, with no significant long-term treatment-related adverse events, and has led to greater improvements in health status.[115] Along with the small molecule approach, AAV9-CRISPR base editing technology has been employed to correct the MYH6 C1211T mutation in embryos and in utero, paving the way for novel germline gene therapy to eliminate mutations in embryos.[116]

Caused by a mutation in the LMNA gene, HGPS leads to progerin accumulation which significantly impairs cardiac functions and reduces life expectancy.[117] Supportive care remains the mainstay of management, focusing on symptom relief and enhancing the quality of life of patients with HGPS. The newly developed farnesyltransferase inhibitor, Lonafarnib (ZokinvyTM), has demonstrated potential in preclinical studies for inhibiting progerin production.[118] In subsequent clinical trials, Lonafarnib extended its lifespan by approximately three months to 2.5 years during the first three years of follow-up, with effects observed up to 11 years.[119] While Lonafarnib has received FDA approval for HGPS treatment, its side effects, including changes in blood sodium and potassium levels, decreased white blood cell counts, eye toxicity, and liver function deterioration, require the long-term regular examinations.[119] Efforts to address HGPS are ongoing, with AAV9 delivery of CRISPR ABEmax-VRQR base editor showing promising results in reducing progerin expression by 87–91% in fibroblasts from HGPS patients and mice.[120] This approach corrected nuclear malformations and significantly improved aortic structure and lifespan in mouse models.[120]

In short, CRISPR holds promise for targeting genetic mutations in conditions like CPVT, HCM, and HGPS. However, concerns persist regarding the immunogenicity triggered by CRISPR/Cas9-mediated gene editing, mainly due to the persistence of Cas9 expression post-treatment.[121] Although AAV9 has shown efficient CRISPR delivery to heart tissue, it could trigger adverse immune responses, thus requiring modifications to the capsid proteins to evade immune detection.[122,123] On the other hand, LNPs have demonstrated low immunogenicity, allowing for repeated administration of CRISPR components at a much lower cost than viral vectors.[124] However, nanoparticle delivery to the heart has been challenging due to limited uptake by cardiomyocytes, which lack unique surface markers for nanoparticle uptake.[55] Therefore, there is a need to develop optimal strategies

for efficiently delivering CRISPR components to cardiac tissue with minimal immunogenicity.

5.3. Inherited Blood Disorders

Genetic blood disorders include various inherited conditions arising from genetic mutations affecting blood components like erythrocytes, platelets, and leukocytes.[125] Among these diseases, the main types of inherited blood disorders targeted by CRISPR technology are β-thalassemia, SCD, hemophilia B, and severe combined immunodeficiency (SCID).[126]

β-thalassemia and SCD are both genetic disorders caused by mutations in the HBB gene encoding hemoglobin, which affects oxygen transport by red blood cells. Gene therapy using lentiviral vectors to introduce functional copies of the β-globin gene into hematopoietic stem cells offers potential treatments.[127] Allogeneic bone marrow transplantation or drugs like hydroxyurea can also benefit patients with transfusion-dependent β-thalassemia.[128] However, access to these advanced treatments may be limited by cost and donor availability. Gene editing approach using Zinc finger nuclease targeting the BCL11A gene aims to generate patients' own hematopoietic stem and progenitor cells (HSPCs) for restoration of normal fetal hemoglobin production.[9] Clinical trials using CRISPR-edited CD34+ HSPCs showed increased fetal hemoglobin levels, relieving symptoms without requiring blood transfusions for patients with β-thalassemia and sickle cell anemia.[129] CasgevyTM, another approach using CRISPR/Cas9 to edit fetal hemoglobin genes in stem cells, has become the first-ever FDA-approved CRISPR-based therapy in the world.[130]

Hemophilia B (HB) is a rare X-linked genetic bleeding disorder characterized by deficient or defective coagulation factor IX (FIX), which is crucial for blood clotting.[131] This condition can lead to complications such as joint damage, chronic pain, and in severe cases, life-threatening bleeding episodes.[131] Treatment typically involves replacement therapy with factor IX concentrates to restore clotting. This treatment requests long-term repeated infusions to sustain sufficient clotting factor levels in the blood, due to the short half-life of factor IX drugs.[132] Emerging gene therapy using adeno-associated virus 5 (AAV5) vectors to express the Padua factor IX variant (etranacogene dezaparvovec) has been investigated for long-term or permanent correction of the clotting deficiency.[133] To avoid the risk associated with viral vectors, the new strategy employed nucleofection method to introduce SpCas9 and sgRNA to patient-derived cells, showing promise for human FIX gene correction.[126,134] In another study, electroporation of SpCas9 plasmid and sgRNA in porcine fetal fibroblasts was used to generate reconstructed pig embryos carrying knocked-out FIX gene as HB pig model.[135] The same method was employed to introduce the human FIX gene in these HB pigs, resulting in a significant reduction in bleeding incidents and ankle joint damage.[135] Further development is needed to enable in situ CRISPR gene editing as a new therapeutic approach for treating HB.

SCID is a rare group of disorders characterized by severe defects in both the adaptive and innate immune systems due to mutations in genes like IL2RG, RAG1, RAG2, DCLRE1C, and IL7RA.[136] The most effective treatment for SCID currently is hematopoietic stem cell transplantation, which can restore immune function and provide long-term immunity against infections.[137] However, this method poses high mortality rates due to the risks of graft-versus-host disease.[138] Gene therapy methods using retroviral or lentiviral gene transfer (for ADA-SCID[139] and ART-SCID),[140] introduce functional copies of the defective gene into hematopoietic stem cells, correcting disease-causing mutations and improving *T*-cell differentiation. However, these methods demand blood transfusion, which comes with labor-intensive processes, high production costs, and risks of viral infection and rejection.[137] To tackle these hurdles, CRISPR/Cas9 systems via prime editing have been explored and shown promise in correcting SCID mutations.[136,141] In proof-of-concept study, CRISPR ABEs successfully restores CD3δ in autologous hematopoietic stem and progenitor cells, achieving a correction of the pathogenic mutation by 71.2% $\pm$ 7.85%.[142] These findings suggest that suitable delivery vehicles such as LNPs, could facilitate the administration of CRISPR base editing constructs for direct in vivo gene editing.

In conclusion, CRISPR technology holds tremendous promise in revolutionizing the treatment landscape for blood disorders. The recent FDA approval of the first CRISPR-based therapy, Casgevy, highlighted the significant progress made in this field. Moving forward, continued research efforts will focus on improving delivery methods especially using nanocarriers, enhancing safety profiles, and expanding the applicability of CRISPR technology to further advance the treatment of blood disorders with the hope for improved outcomes and quality of life for patients worldwide.

5.4. Inherited Eye Diseases

CRISPR technology has been explored to treat inherited eye conditions like Leber congenital amaurosis (LCA) and autosomal dominant cone-rod dystrophy 6 (CORD6).[143] LCA, a severe retinal dystrophy, involves heterogeneous mutations in genes linked to the retinal pigment epithelium and photoreceptors such as RPE, CEP290, GUCY2D, AIPL1, CRB1, and CRX.[144] To develop effective LCA treatment, viral vectors have been employed to deliver functional copies of defective genes directly into the damaged retina.[145] This strategy led to the discovery of Luxturna, an FDA-approved gene therapy that has demonstrated efficacy in delivering a functional RPE65 gene copy into retinal cells, thereby improving patients' vision.[146] However, there are ongoing challenges associated with viral vectors in treating LCA. These challenges include the risk of viral integration, high production costs, limited accessibility, and the inability to target various mutation types effectively, hindering the advancement of personalized medicine in this context.[145] Stem cell therapy has emerged as a promising method for LCA treatment by replacing damaged retinal cells with healthy ones, but it faces a significant issue of low cell integration rate upon injection.[147] To overcome obstacles associated with the approaches mentioned above, CRISPR technology has been investigated its potential in precise and safe gene editing for LCA treatment. For instance, early studies employed CRISPR/SpCas delivery via dual rAAV5 vectors to temporarily correct the IVS26 splice mutation within the CEP290 gene through subretinal injection over a 28-day period.[148] Similarly, another investigation utilized a dual AAV vector to deliver

CRISPR ABEs to address the pathogenic RPE65 gene mutation in an LCA mouse model, achieving significant improvements in light-triggered responses in retinal degeneration.[149]

CORD6 is a condition associated with dysfunction of photoreceptor cells caused by a gain-of-function mutation in the GUCY2D gene, resulting in vision loss.[150] The dominant nature of the GUCY2D mutation poses challenges for traditional gene therapy approaches, making it difficult to suppress dominant gene expression.[151] Currently, there are no available treatments for this disease. A study employed subretinal injection of AAV5-CRISPR/Cas9, achieving a substantial knockout of GUCY2D in mouse and macaque models and reducing retGC1 expression significantly without causing ocular inflammation or inducing an immunological *T*-cell response.[152] This research indicated the potential of AAV-CRISPR/Cas9 in primate gene editing for the treatment of CORD6.

To sum up, CRISPR technology holds promise for treating inherited eye conditions. However, concerns arise with the use of viral vectors, which can induce immune responses and toxicity in the retinal pigment epithelium.[123,153] Nonviral delivery systems including nanoparticles offer an alternative method to enable transient gene editing effects, paving the way for safer and more effective CRISPR-based therapies for inherited eye disorders.[154]

5.5. Genetic Neurological Disorders

CRISPR holds promise for treating various genetic neurological disorders by targeting and correcting specific genetic mutations associated with these conditions.[155] For example, Huntington's disease (HD) is a genetic neurological disease triggered by CAG expansion in the HTT gene, which causes the build-up of mutant huntingtin protein and RNA, leading to a range of motor symptoms.[86,156] Several therapeutic strategies are being explored to slow down the progression of Huntington's disease. These include gene silencing techniques such as antisense oligonucleotides (ASOs) and RNA interference (RNAi), which aim to reduce the production of mutant huntingtin protein.[157] However, these therapies mainly focus on managing symptoms rather than addressing the underlying cause of the disease (HTT gene mutation.[158] In attempts to address HD, a research group developed a CRISPR/Cas13d system, Cas13d-CAGEX, which reduced mutant HTT RNA by over 56.2% in patient-derived neurons and improved motor function in an HD mouse model when administered via AAV vehicle.[159] In another study, AVV9 delivery of RfxCas13d (another Cas13d variant) has been found to reduce HTT protein by ≈50% in the mouse brain, indicating its potential for gene suppression in the nervous system.[160] Findings of these studies suggest that CRISPR/Cas13d systems exhibit potential in reducing mutant HTT protein levels and enhancing mouse motor coordination, while exhibiting minimal off-target effects. These characteristics are desirable for the advancement of effective therapies for clinical development in HD treatment.

Tay-Sachs disease is a rare and deadly genetic neuronal disorder caused by HEXA gene mutations affecting the essential enzyme hexosaminidase A (Hex-A).[155] Similar to HD, there is no cure for Tay-Sachs disease as current treatments focus on managing symptoms and providing supportive care to patients.[155] Current gene therapy strategies employ AAV vectors to introduce gene-encoding functional HexA enzymes in mouse and sheep models of Tay-Sachs disease via intracranial injection.[161] However, a significant limitation of AAV vectors is their restricted capacity and low transduction efficiency for co-transfecting both subunits α and β of HexA enzymes.[162] To overcome the limitations of AAV vectors, researchers have turned to the CRISPR prime editing method. This approach has been employed to edit a prevalent HEXA gene mutation, HEXA1278+TATC, in HEK293T cells.[56] This method achieved a 31% insertion efficiency with a low rate of unintended indels at 0.8%.[56] Furthermore, prime editing successfully corrected the mutation in HEXA with an efficiency rate of 33% and a low indel rate of 0.32%.[56] In conclusion, CRISPR prime editing shows promise by efficiently editing the prevalent HEXA gene mutation in HEK293T cells with high accuracy, highlighting its potential for precise genetic intervention and offering new hope for treating Tay-Sachs disorders with Hex-A deficiency.

5.6. Genetic Metabolic Diseases

CRISPR technology offers substantial potential in tackling genetic metabolic disorders like Hereditary Tyrosinemia Type 1 (HT1), Alpha-1 Antitrypsin Deficiency (AATD), and Homozygous Familial Hypercholesterolemia (HoFH).[163] HT1 stems from a fumarylacetoacetate hydrolase (FAH) enzyme deficiency, impacting tyrosine metabolism and leading to severe liver damage.[164] Current treatment methods for HT1 include nitisinone, dietary management, and liver transplantation.[165] While nitisinone can inhibit the production of toxic metabolites, patients with HT1 are life-long dependent on nitisinone as discontinuation leads to disease recurrent and liver complications.[166] Besides, nitisinone itself has side effects such as elevated blood tyrosine levels, corneal opacities, and potential effects on bone health.[166] Meanwhile, CRISPR approaches have emerged and explored for HT1 treatment. For example, studies utilized AAV8-CRISPR to correct FAH gene mutations in newborn FAH-deficient rabbits and FAH knockout mice, restoring liver function and mitigating liver and kidney damage.[167] In another study, pig embryos underwent testing through microinjection of Cas9 mRNA and sgRNA to generate FAH knock-out pigs, serving as a disease model of HT.[168] Using the same method, double knockout pigs lacking both FAH and hydroxyphenylpyruvate dioxygenase (HPD) gene, which encode the HPD enzyme downstream of the FAH metabolic pathway, were generated. In comparison with FAH knock-out pigs, these double-knockout pigs exhibited lower inflammatory responses, oxidative damage, decreased liver injury, and extended survival. Importantly, these benefits were heritable to subsequent generations. This study suggests that targeting HPD using CRISPR could represent a promising therapeutic strategy for treating HT1.

AATD impacts both the liver and lungs. It occurs due to mutations in the SERPINA1 gene, which leads to lower levels or impaired function of alpha-1 antitrypsin.[169] Current AATD treatment involves infusing purified AAT protein to boost blood AAT levels and slow lung disease progression.[170] However, it's costly and may cause adverse reactions.[170] Emerging gene therapy aims to correct the genetic defect, offering potential long-term benefits. For instance, delivering the human AAT gene with an

AAV vector has shown promising results in non-human primates and humans, but challenges like low expression and immune responses need to be addressed before widespread adoption.[171] Recently, CRISPR technology has been employed to correct SERPINA1 gene mutations in humanized mouse models and patient-derived induced pluripotent stem cells (iPSCs).[172] This correction reduced liver fibrosis, improved liver histology, and lowered inflammatory markers, suggesting potential therapeutic benefits.[172]

HoFH is a rare and severe genetic disorder marked by abnormally high LDL cholesterol levels from birth, stemming from mutations in both copies of the LDL receptor gene (LDLR) or other genes regulating LDL metabolism.[173] While liver transplantation can restore LDL receptor function in HoFH patients, this surgical procedure cannot cure HoFH and is associated with risks such as organ rejection, infection, and complications from immunosuppressive therapy.[174] The introduction of novel therapies like PCSK9 inhibitors (e.g., evolocumab, alirocumab) has expanded the treatment options for HoFHv.[175] These medications work by inhibiting PCSK9, a protein responsible for degrading LDL receptors, thus lowering LDL cholesterol levels.[175] However, these treatments may not reach the desired levels in all HoFH patients.[176] LDL apheresis removes LDL cholesterol from the bloodstream using a filtration device. However, this treatment might be expensive and thus might be unavailable for all HoFH patients.[177]

Researchers have used Cas12a to disrupt the PCSK9 gene in mouse models of HoFH and also in human primary CD34+ hematopoietic stem and progenitor cells (HSPCs).[178] In another study, GalNAc-lipid nanoparticles (GalNAc-LNP) carrying the CRISPR ABE system were used in a study to edit the ANGPTL3 gene in a non-human primate model of HoFH.[179] These nanoparticles, comprising ABE SpCas9 mRNA and ANGPTL3 gRNA, achieved up to 61% gene editing of ANGPTL3 in the liver within 40 days, resulting in an 89% reduction in ANGPTL3 protein levels (**Figure** 4A,B). Importantly, this editing was specific to the liver, without any observed off-target effects in other tissues (Figure 4C). This treatment enhanced LDL-cholesterol clearance from circulation for up to three months and maintained reduced ANGPTL3 levels for 200 days (Figure 4D,E), suggesting CRISPR therapeutics as a potential treatment for different levels of LDLR activity including in HoFH.

In conclusion, CRISPR's interventions have shown success in correcting gene mutations and improving outcomes in animal models of these genetic metabolic disorders, indicating potential therapeutic benefits for patients. These advancements highlight CRISPR's potential in revolutionizing the treatment of genetic metabolic disorders, offering hope for more effective and targeted therapies in the future.

6. Challenges of Industry Development and Ethical Consideration

6.1. Industrial Development of CRISPR Technology

The rapid development of CRISPR technology has brought industry challenges that are associated with the manufacturing CRISPR products and therapies. A primary concern is extremely high costs and maintaining rigorous quality control over CRISPR editing tools and delivery systems, a task that becomes increasingly complex with expansion and scaling up.[31] For example, manufacturing AAVs from adherent cell culture for CRISPR gene therapy delivery vector presents a challenge when it comes to managing batch-to-batch variations in plasmid yield and purity.[180] The utilization of suspension-based cell culture is considered more scalable. However, developing cell lines that efficiently produce AAVs in suspension is difficult because the cell density in suspension cultures tends to be lower than that in adherent cultures.[181] Additionally, the development and manufacturing of CRISPR-based treatments also involve the high cost of meeting regulatory requirements, ensuring product characteristics, safety, and quality control. As more CRISPR-based therapies advance into more sophisticated stages of clinical testing, industries will be required to invest to ensure that their manufacturing processes adhere to the strict standards and guidelines in current good manufacturing practices (cGMP).[31]

6.2. Clinical Development of CRISPR Technology

Off-target effects are a significant concern associated with CRISPR-based therapy since they can potentially result in unintended DNA sequence mutation carrying unpredictable and potentially hazardous consequences.[182] These off-target effects are classified into two categories: one arises from the similarity of gene sequences, and the other is due to the unpredictable binding of Cas9 to unexpected gene sites.[183] At the genomic level, off-target effects can result in significant issues, such as genomic rearrangements and substantial deletion, which potentially disrupt gene function and in extreme cases, lead to the development of cancer cells.[184] Therefore, minimizing off-target effects is essential to guarantee the safety and accuracy of CRISPR gene therapy.

To mitigate off-target effects in CRISPR gene editing, a combination of strategies can be implemented. First, the use of high-fidelity Cas9 variants, such as eSpCas9, SpCas9-HF1 or SpCas9-nCas9, is recommended, as these exhibit reduced off-target activity.[31] Additionally, the delivery of CRISPR components as ribonucleoprotein complexes (RNPs), as opposed to plasmid DNA, is an effective approach.[185] RNP delivery ensures transient expression and minimizes the risk of off-target effects.[186] Furthermore, optimization of gRNA design represents another approach to minimize off-target effects. This can be achieved by applying proper computational tools or online databases to predict potential off-target sites,[187] such as Cas-Finder, CRISPR-P, CRISPR-GE, and NCBI BLAST, etc. These tools use algorithms to predict the likelihood of binding between a gRNA and a non-target sequence based on the similarity of the sequences. Therefore, it is possible to reduce off-target effects by avoiding predicted off-target sites. Moreover, for precise modifications on the mutations, the consideration of base and prime editors is advisable due to their highly precise gene editing potential with fewer off-target effects.[31,183] In combination, these strategies contribute to the enhanced accuracy and safety of CRISPR editing while minimizing unintended genetic alterations.

Immunogenicity is another significant issue in CRISPR-based gene therapy for human genetic disorders.[188] This arises from the bacterial origin of Cas proteins, which can trigger the host's

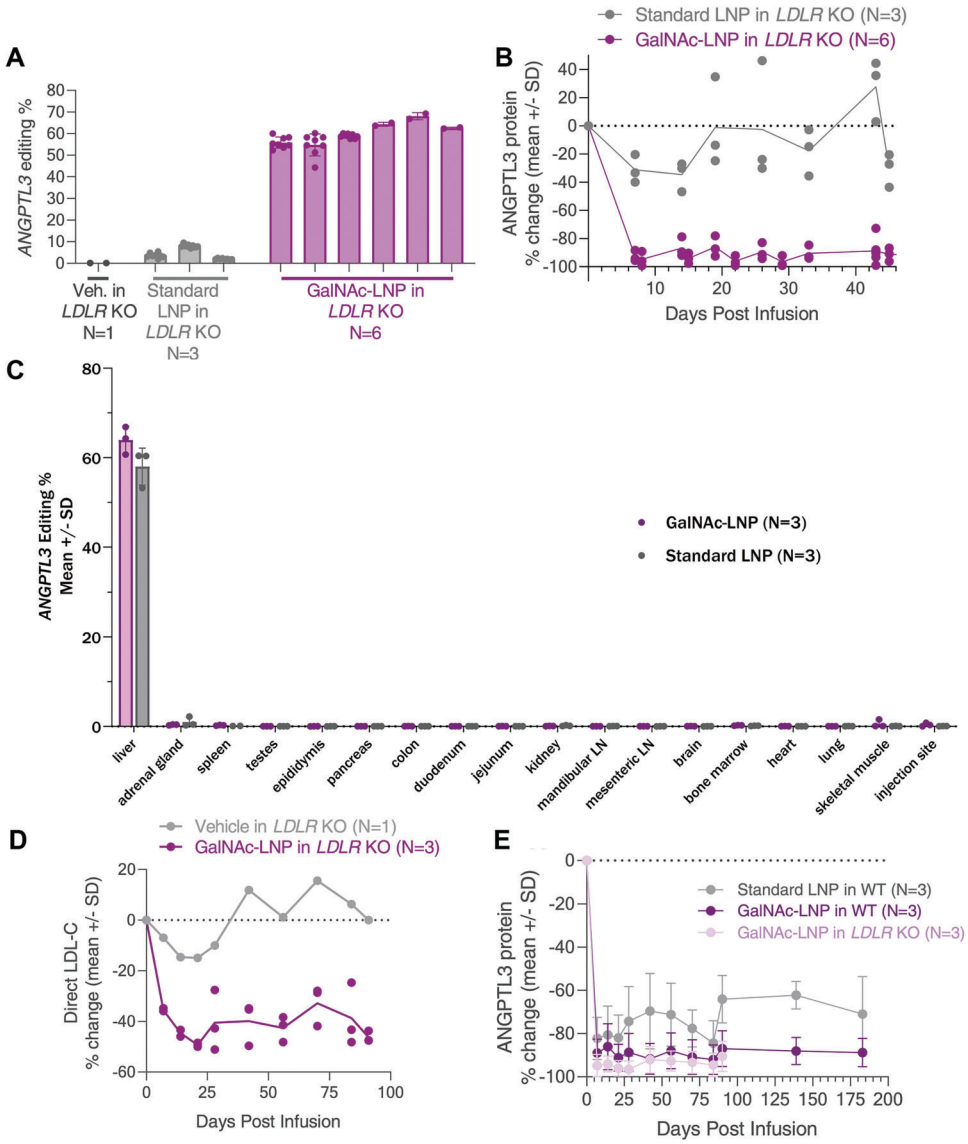

Figure 4. CRISPR adenine base editing with GalNAc-LNP targeting ANGPTL3 in the liver of a somatic LDLR deficient NHP model. A) Evaluation of ANGPTL3 gene editing efficiency in LDLR-deficient non-human primates (NHPs) in vivo with standard LNP and GalNAc-LNP using RT-PCR. B) Assessment of ANGPTL3 protein expression in LDLR KO NHPs treated with standard and GalNAc LNP through ELISA assays. C) Specific targeting of ANGPTL3 in the liver compared to non-hepatic tissues using GalNAc-LNP-CRISPR. D) Reduction of LDL-C levels by 35% due to ANGPTL3 inhibition with GalNAc-LNP. E) Average ANGPTL3 levels in three NHPs up to 200 post-LNP injection. Adapted with permission.[179] Copyright 2023, Springer Nature.

immune system, potentially leading to immune responses during in vivo genome editing.[188] Additionally, many individuals already have anti-Cas antibodies and cellular immune responses specific to Cas, possibly due to previous exposure to Cas protein through interactions with the microbiome.[189] This existing immunity could potentially impact both the editing efficiency and safety of CRISPR applications. Therefore, managing the duration of Cas9 expression is essential to minimize the risk of unwanted immune reactions in CRISPR gene editing applications.[190] To address this concern, various strategies have been employed, such as transient transfection using CRISPR mRNA or RNP,[191] utilizing less immunogenic Cas9 variants,[192] or administering immunosuppressants.[68] Overall, addressing the immunogenicity associated with the bacterial origin of CRISPR nucleases is critical for advancing in vivo CRISPR applications and ensuring their safety and effectiveness.

6.3. Ethical Considerations

CRISPR applications for treating human genetic diseases raise complex and multifaceted ethical concerns. Central among these is the concern surrounding germline modification, particularly beyond the initial 14-day embryo stage, prompting profound ethical challenges about long-term consequences.[193] The lack of understanding about genome editing further complicates these

ethical challenges, highlighting the importance of informed consent and privacy protection. Informed consent and privacy protection represent crucial facets of these ethical concerns.[194] Moreover, the current lack of comprehensive national and international regulations raises concerns about oversight of these applications and the potential for data breaches. Fear of data breaches is another ethical dimension to be addressed.[195] Ensuring that individuals contributing their genetic data for CRISPR therapies provide informed and voluntary consent is paramount. Preserving the privacy of genetic information and preventing discrimination based on genetic insights are vital ethical considerations in the genomic era.[196] To mitigate potential risks associated with gene editing, stringent regulations, and rigorous oversight are deemed essential. A global regulatory body under the United Nations could facilitate conditional worldwide use of gene editing, ensuring safety and ethical compliance while addressing potential repercussions.

In addition, ethical review boards play a pivotal role within the ethical framework. These boards are indispensable for evaluating the ethical implications of specific CRISPR applications and trials, guaranteeing the upholding of ethical standards throughout the entire research and clinical process. As CRISPR technology advances into more intricate and multifaceted clinical trials, human research ethics committees (HRECs) face the challenge of comprehensively evaluating these studies. The complexity of CRISPR trials often transcends traditional ethical review boundaries, necessitating specialized knowledge and expertise. To address this, HRECs might need to consider expanding their capabilities, either by outsourcing to members with specific expertise in genetic editing and related fields, or by inviting guest committee members for particular trials. This approach ensures that the ethical review process keeps pace with scientific advancements, maintaining rigorous ethical standards while fostering innovative medical breakthroughs. Such adaptability in ethical oversight is crucial for responsibly navigating the rapidly evolving landscape of CRISPR research and its implications for human health. Lastly, there's a strong emphasis on the necessity of international collaborations. Collaborative efforts can promote responsible development through shared research endeavors, the exchange of best practices, and the advocacy of international ethical standards. The acceptance of CRISPR technology varies across different societies and cultures, introducing further ethical complexities.[197] Recognizing the diverse perspectives on genetic editing and demonstrating respect for cultural norms and values are vital in navigating these differences while maintaining ethical integrity.[197]

7. Conclusion

Advanced CRISPR technology holds immense potential for a wide range of gene editing-based therapies for human genetic diseases. While CRISPR/Cas9 is the most used system, emerging CRISPR systems like Cas12a and Cas13d also offer promise. However, the key challenge in clinical CRISPR applications lies in achieving safe and efficient delivery to the target cells. Therefore, the selection of the Cas delivery method is crucial to achieve the right balance between the effectiveness of gene editing and the safety of therapeutic CRISPR components. This review summarises various CRISPR formats and delivery methods. CRISPR can be delivered as plasmids, RNA or RNPs, each with its own advantages and drawbacks. Viral vectors are commonly used for CRISPR delivery but carry the risk of unwanted genome integration and immunogenicity. Non-viral vectors such as nanoparticles offer better loading efficiency and safety profiles. However, they might have low transfection efficiency (such as hard-to-transfect cells) and induce unintended side effects due to their limited targeting capability. Ongoing research aims to address these challenges to achieve better therapeutic outcomes. Another critical concern is the potential for off-target effects with CRISPR-based therapies in treating human genetic disorders. Progress has been made in developing modified Cas proteins with improved specificity and engineering guide RNA to reduce off-target effects.

In summary, the application of CRISPR therapeutics for treating human genetic diseases shows great promise, as its precise editing capabilities hold the potential to cure conditions that are currently untreatable. Significant progress has been achieved in enhancing CRISPR editing precision, broadening the range of treatable conditions, developing effective delivery methods, and refining CRISPR gene editing tools. However, regulatory and ethical considerations should be addressed. Despite challenges, ongoing research and investment are critical for realizing the full potential of CRISPR in revolutionising genetic medicine and enhancing patient outcomes.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

X.Y. and T.A.B. contributed equally to this work. X.Y. and T.B. conducted the literature search, drafted the manuscript, and designed the figures. H.M., Y.A., F.D., and G.H. contributed to editing the manuscript. W.D. contributed to the conceptualization of the study and manuscript review and editing.

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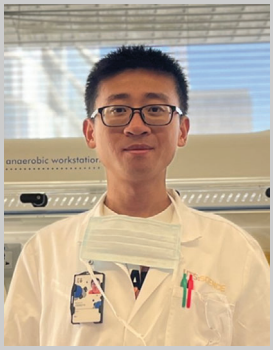

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Gyorgy Hutvagner is an expert in RNA biology, his research mainly focuses on small RNAs (miRNAs, siRNAs) and noncoding RNAs. Besides studying the molecular mechanism of small RNA biogenesis and function his laboratory is interested in developing small RNA-based cancer biomarkers and develop small RNA-based cancer therapeutics.

Wei Deng is Head of the Cancer Nanomedicine Group at the School of Biomedical Engineering at the University of Technology Sydney. Her pioneering transdisciplinary research spans nanotechnology, gene engineering, and biomedical research to create new technologies for the treatment of cancers and genetic disorders. Deng's current research was mainly focused on developing advanced therapeutic methods using nanocarriers, particularly in the areas of gene and cell therapy.