Molecular Therapy
Nucleic Acids
Review

Advanced delivery systems for gene editing: A comprehensive review from the GenE-HumDi COST Action Working Group

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In the past decade, precise targeting through genome editing has emerged as a promising alternative to traditional therapeutic approaches. Genome editing can be performed using various platforms, where programmable DNA nucleases create permanent genetic changes at specific genomic locations due to their ability to recognize precise DNA sequences. Clinical application of this technology requires the delivery of the editing reagents to transplantable cells *ex vivo* or to tissues and organs for *in vivo* approaches, often representing a barrier to achieving the desired editing efficiency and safety. In this review, authored by members of the GenE-HumDi European Cooperation in Science and Technology (COST) Action, we described the plethora of delivery systems available for genome-editing components, including viral and non-viral systems, highlighting their advantages, limitations, and potential application in a clinical setting.

INTRODUCTION

Genome editing represents a promising approach for curing a broad range of genetic diseases either by permanently inactivating disease-causing genes or restoring the functions of mutated genes.[1] The fast development of genome editing largely relies on advances in sequence-specific nuclease technologies and versatile delivery systems. Since the first discovery on the harnessing of the bacterial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system from *Streptococcus pyogenes* for gene-editing purposes,[2] the CRISPR-derived gene-editing tools have been largely broadened from classical double-stranded DNA breaks (DSBs) to more specific and precise gene-editing reagents, such as base editing and prime editing by fusing Cas9 nickases to deaminase and reverse transcriptase domains, respectively.[3–5] In mammalian cells, DSBs are mostly repaired by non-homologous end joying (NHEJ), resulting in the frequent installation of small insertions or deletions (known as indels) at the break site. Homology-directed repair (HDR) is another pathway that cells have evolved to precisely repair DSBs, which is, however, less efficient when compared to NHEJ and requires the presentation of a donor DNA template.[6,7] Thus, the gene-editing outcomes are largely defined by the efficiency of delivering gene-editing tools with or without a donor DNA template into target cells.

Delivery technologies, including those based on both viral and non-viral vectors, have been revolutionized in the past decades, and a wide spectrum of delivery systems is now being explored. Striking examples include lentiviral vectors, virus-like particles, adenoviral vectors (AdVs), and lipid nanoparticles for efficient *in vitro* and *in vivo* delivery of DNA and RNA molecules into cells and tissues.[8,9] Natural or engineered extracellular vesicles offer another option for encapsulating therapeutic agents, benefiting from immune tolerance, stability, and specificity. Additionally, polymeric nanoparticles, such as poly (lactic-co-glycolic acid (PLGA) and cationic polymers, provide customizable solutions for genome editing, enhancing both efficacy and safety. However, the therapeutic application of genome-editing technologies has been restricted by method-specific shortcomings in delivery efficiency, capacity, and specificity to targeted cells and tissues.[10] This is particularly true considering the plethora of different gene editors and DNA repair pathways that can be now exploited to achieve the desired therapeutic outcome, which dramatically increases the complexity of selecting the right delivery tool. Indeed, while base editing, prime editing, and CRISPR-Cas NHEJ-based approaches require delivery of only the main gene-editing tools, such as Cas enzyme and single guide RNA (gRNA or sgRNA), with CRISPR-Cas HDR-based approaches, suitable delivery methods for HDR templates are also needed. The paucity of efficient, non-toxic, and/or cost-effective delivery methods for DNA/RNA donor templates has significantly hindered the progression of HDR-based platforms into the clinics compared to NHEJ-based ones, highlighting how delivery is indeed a crucial bottleneck that must be addressed to advance CRISPR-based therapeutics. Moreover, the efficacy of each delivery tool can drastically vary when employed for an *ex vivo* versus an *in vivo* gene-editing approach and when targeting different cell types and tissues. To advance the translation of gene-editing technologies in treating human diseases, the European Cooperation in Science and Technology (COST) is supporting a gene-editing research network (GenE-HumDi) of more than 300 researchers and innovators from 39 countries.[11] This comprehensive review, authored

Table 1. Limitations of gene-editing delivery methods

Delivery tool	Delivery type	Limitations
IDLV	viral	limited cargo capacity, lower transduction efficacy, lower gene expression, chances of vector integration
Virus-like particles	pseudoviral	limited cargo capacity and stability, challenging manufacturing
Adenovirus	viral	immune response, transient transgene expression, prominent liver targeting, challenging manufacturing
Adeno-associated virus	viral	limited cargo capacity, transient transgene expression, potential immune response, challenging manufacturing
Lipid nanoparticles	non-viral	low bioavailability, lack of target specificity, possible instability
Extracellular vesicles	non-viral	complex composition, challenging isolation and characterization, limited reproducibility
Synthetic nanoparticles	non-viral	challenging manufacturing, unclear safety, heterogeneity
Inorganic nanoparticles	non-viral	low intracellular delivery, limited targeting specificity, scalability, design complexity
Microinjection	physical	labor intensive, time consuming, potential physical cell damage
Electroporation	physical	potential physical cell damage and alteration in cellular homeostasis

by members of the GenE-HumDi COST Action Working Group 3 "Delivery Strategies," discusses various delivery methods and comments on the challenges associated with editing difficult cell types. Here, we conduct a comprehensive survey of currently available genome-editing delivery methods with emphasis on delivery into specific cell types and organs and on the complexities of reaching and effectively editing challenging organs *in vivo*. This aligns with the primary objectives of the Working Group, which focuses on evaluating *ex vivo* delivery systems and identifying ideal delivery methods for each cell type and gene-editing technology, as well as assessing *in vivo* delivery systems to determine the most fitting set of tools for each individual animal model, route of administration, and target disease.

Viral and viral-like delivery methods

Viral vectors have gained prominence as gene-editing delivery tools owing to their efficiency in introducing genetic material into target cells both *in vitro* and *in vivo*. They take advantage of the natural mechanisms of viral infection to deliver nucleic acids into host cell nuclei. Indeed, viral vectors can be adapted for transferring gene-editing tools into therapeutically relevant cell types and organs, and their ability to target specific cell types further facilitates efficient intracellular delivery, making them one of the most widely used delivery methods. Among the viral-based delivery tools, integrase-defective lentiviral vectors (IDLVs) together with AdVs and adeno-associated viral vectors (AAVs), have gained popularity due to their potential use for *in vivo* gene editing. Other delivery strategies, such as those based on the use of virus-like particles (VLPs), have also been developed to match the efficacy of viruses in delivering therapeutic payloads with the safety of non-infectious platforms. In the following sections, we discuss the utility of each of these viral vector methods as well as their limitations (Table 1).

Integrase-defective Lentiviral Vectors (IDLVs)

Retroviruses, including gamma retroviruses and lentiviruses, are RNA viruses with an envelope that can semi-randomly insert their reverse transcribed DNA genomes into the chromosomal DNA of target cells upon infection. Their large genomes enable the efficient transfer of up to 9 kb of genetic material into host cells, a feature that, once these viruses are engineered into their viral vector counterparts, has been harnessed to deliver a range of therapeutic genes to transplantable cells, aiding in the development of ground-breaking *ex vivo* gene therapies. However, their conventional design, which facilitates stable integration into the host cell genome, is not ideal for gene-editing applications where transient expression of endonucleases is preferable to minimize off-target DNA cleavage. In this regard, the development of IDLVs has significantly benefited the field, providing safer and transient delivery tools (Figure 1B). In 2007, Cathomen et al. published a seminal paper demonstrating the use of IDLVs for gene correction through HDR. In their proof-of-concept study, an IDLV containing a repair donor template was co-delivered with an I-SceI homing endonuclease expression vector to rescue a defective enhanced green fluorescent protein (EGFP) gene.[12] Seven years later, the team of Marina Cavazzana applied a similar approach to correct Artemis deficiency in murine hematopoietic stem cells (mHSCs) by delivering both the I-SceI enzyme and the Artemis correction template.[13] Programmable zinc-finger nucleases (ZFNs) have also been successfully delivered by using IDLVs. Naldini's team demonstrated that IDLV-mediated ZFN delivery yields high editing rates (13%–39%) at the IL-2 receptor common gamma-chain gene (*IL2RG*) across different cell types and facilitates site-specific gene addition through ZFN cleavage and HDR/homologous template DNA, achieving up to 50% of gene knockin (KI) in human cell lines and human embryonic stem cells.[14] Building on these developments, Coluccio et al. focused on targeted gene addition in human epithelial stem cells through ZFN-mediated HDR by targeting the "safe-harbor" locus, adeno-associated virus integration site 1 (*AAVS1*). This approach resulted in >20% targeted gene addition in a human keratinocyte cell line, >10% in immortalized keratinocytes, and <1% in primary keratinocytes.[15] Further advancing the field, in 2013, Kohn et al. investigated the use of IDLVs for the transient delivery of ZFNs and donor templates for site-specific modification of the human adenosine deaminase (*ADA*) gene. This work highlighted the

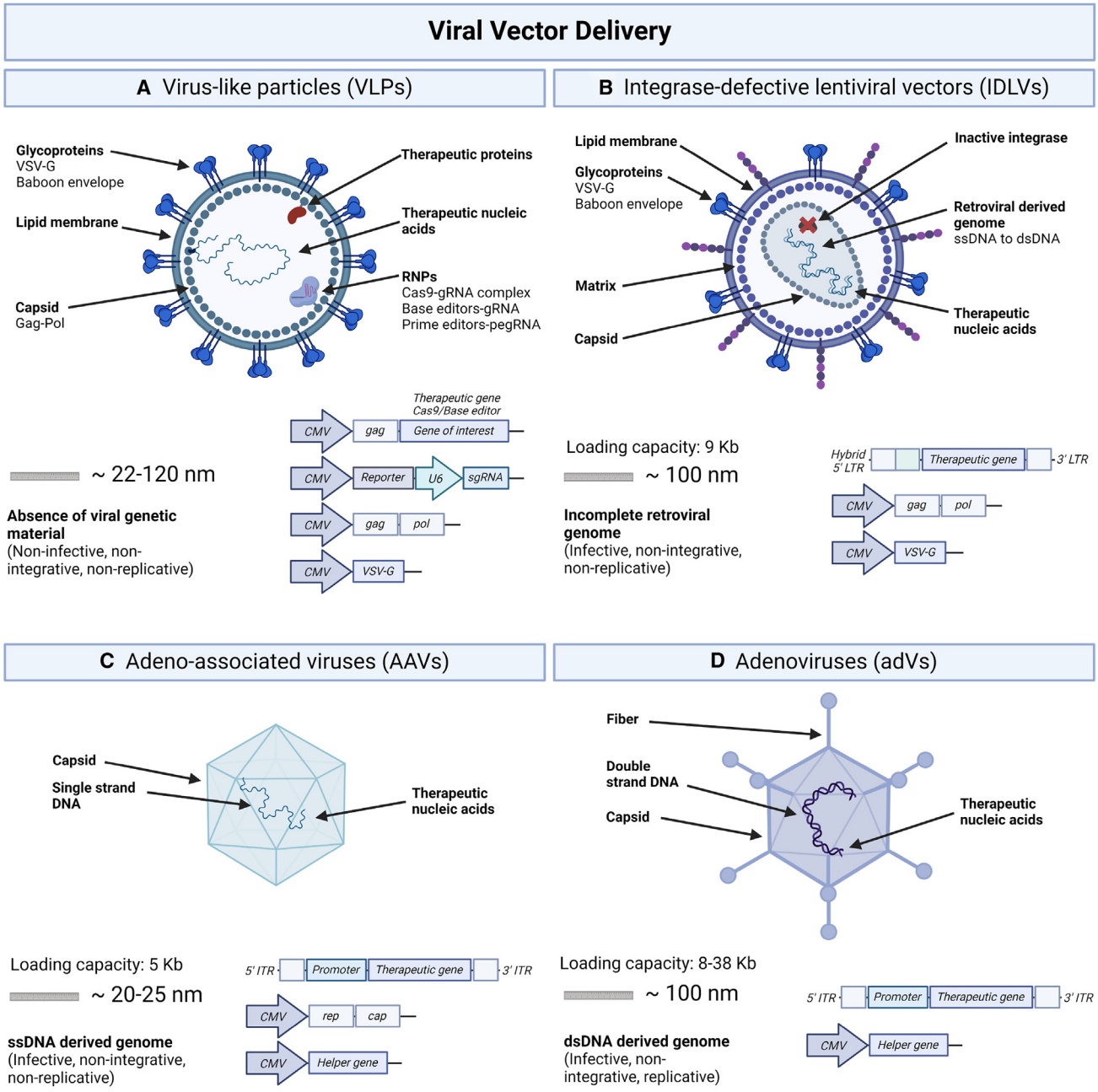

Figure 1. Structure of viral and viral-like methods used as gene-editing delivery agents

(A) Virus-like particles (VLPs) can be used to deliver therapeutic proteins and nucleic acids and are devoid of viral genetic material, rendering them non-infectious, non-integrative, and non-replicative. (B) Integrase-defective lentiviral vectors (IDLVs) contain therapeutic nucleic acids with an incomplete retroviral genome, making them able to transduce cells while being non-integrative and non-replicative. (C) Adeno-associated viral vectors (AAVs) can transfer therapeutic nucleic acids in a single-stranded DNA-derived genome; they are non-integrative and non-replicative. (D) Adenoviral vectors (AdVs) deliver therapeutic nucleic acids in a double-stranded DNA-derived genome; they transduce cells, are non-integrative, and are replication defective. Created with Biorender.com.

importance of vector design modification for the co-delivery of highly similar sequences in genome-editing nucleases, showing significant improvements in the use of IDLVs for delivering ZFNs.[16] Although IDLV delivery of specific nucleases was demonstrated to be efficient and relatively safe,[17] it can lead to weak transgene expression. In this regard, a recent study by the Benabdellah team investigated enhanced configurations of IDLVs aimed at boosting transgene expression while maintaining targeted cell specificity. They observed

IS2-containing episomes relocating to DAPI-low nuclear regions, suggesting improved transcriptional activity.[18] Alternatively, Gonçalves et al. used HDAC inhibitors to improve IDLV-mediated ZNF expression and thus site-directed mutagenesis to levels comparable to integrating vectors.[19] IDLVs have also been shown to be efficient vehicles for delivering CRISPR gene-editing components *in vitro* and *in vivo*. By introducing the transcription factor Sp1-binding site(s) into the CRISPR lentiviral vector, Ortinski and colleagues reported the generation of functionally enhanced IDLVs for efficient delivery and expression of an all-in-one CRISPR system (i.e., Cas9 and gRNA) and ensuing gene editing *in vitro* and *in vivo*.[20] More recently, Uchida and colleagues showed delivery of Cas9 protein, gRNA, and donor DNA via IDLVs into hematopoietic stem/progenitor cells (HSPCs) to correct the sickle cell disease (SCD) mutation.[21]

Recent work by Naldini's team highlighted the superiority of the IDLV platform when serving as a carrier of donor template for HDR-mediated gene KI in HSPCs. Indeed, they found that IDLVs display reduced viral DNA load upon entering the cell, leading to weaker DNA damage responses when compared to those triggered by AAV genomes bearing palindromic inverted terminal repeats (ITRs), thereby improving clonogenic capacity and editing efficiency in long-term repopulating hematopoietic stem cells (HSCs). Importantly, IDLVs showed lower frequencies of viral DNA fragment insertions, mitigating the genotoxic risks associated with AAV-based HDR editing.[22] The same team demonstrated successful correction of the CD40 ligand gene (*CD40LG*) for the treatment of hyper-immunoglobulin (Ig)M1 syndrome and developed a good manufacturing practice (GMP)-compliant and scalable process for *in situ* CD4+ T cell gene editing using an IDLV as the corrective donor template.[23]

In conclusion, IDLVs offer a safer, transient delivery system for gene-editing tools, but they face several challenges and limitations. Their transduction efficiency can be lower than that of integrating vectors, particularly in certain primary and non-dividing cells, and they generally exhibit lower expression levels compared to their integrative counterparts, limiting their effectiveness in applications requiring robust gene expression. Additionally, their limited cargo capacity (~8–10 kb) restricts the delivery of larger gene-editing systems. To address the first limitation, it has been shown that incorporating the chimeric sequence element IS2 into the long terminal repeat (LTR) of IDLVs significantly enhances gene expression levels.[18] To overcome the cargo capacity issue, a double-IDLV construct was developed, where the gene-editing tools are split into two parts and delivered using two separate IDLVs instead of a single vector, effectively solving this challenge.[16]

Virus-Like Particles (VLPs)

VLPs have emerged as versatile and powerful vehicles for delivering gene-editing agents. These non-infectious assemblies of viral proteins (usually derived from the Moloney leukemia virus and the human immunodeficiency virus) can package mRNAs, proteins, or ribonucleoproteins (RNPs) instead of genetic material encoding these molecular entities.[24] VLPs harness the natural advantages of viruses for efficient intracellular delivery, such as their ability to encapsulate cargoes and escape endosomes, and can be assembled with diverse envelope proteins to target various cell types. However, unlike traditional viruses, VLPs deliver gene-editing agents transiently as mRNAs, RNPs, or proteins, instead of DNA constructs, which significantly lowers the risks of off-target effects and vector genome integration.[25–27] This combination of viral and non-viral delivery benefits makes VLPs attractive for gene-editing applications.

Taking advantage of fusing cargo proteins to retroviral Gag proteins, desired proteins can be packaged into virions during the capsid self-assembly process in producer cells. For instance, Cas9 nuclease has been successfully incorporated into VLPs by fusing it to the HIV-1 Gag protein, achieving significant gene-editing efficiencies in various cell types and allowing for targeted delivery to specific cell subpopulations by using different envelope glycoproteins.[28,29] Using a similar approach, Liu and co-workers developed engineered VLPs (eVLPs) containing base editors and prime editors by incorporating a protease-cleavable linker between the MMLV (Moloney Murine Leukemia Virus) Gag and the cargo moieties, thereby reducing premature cleavage. Moreover, they included nuclear export sequences (NESs) to control Gag-cargo localization in producer cells, boosting cargo loading into eVLPs. This strategy enabled therapeutic levels of editing when tested *ex vivo* and *in vivo*.[25,26] With several advantages as discussed above over viral vector delivery approaches, VLPs also hold tremendous potential for personalized medicine tailored to individual mutations owing to their potentially safe, stable, and cost-effective nature. As *in vivo* gene-editing technology advances toward clinical application, VLPs might prove to be promising vehicles potentially enhancing the efficacy of gene therapies.

Due to their innate ability to encapsulate nucleic acids, VLPs are an attractive choice as DNA/RNA delivery vehicles. However, despite being advantageous for safe and transient Cas9 (with or without gRNA) delivery, VLPs face challenges such as limited cargo size, stability, and challenging large-scale production. The ability to regulate VLP particle size would be advantageous, as, for example, it would provide more surface area for enhanced antigen presentation and a larger internal volume for greater cargo capacity. However, altering the number of capsid protein subunits or the bonds between them, which is required to expand the overall size, is difficult without compromising the structural integrity of VLPs. To address this, Beila et al. suggested the production of "bespoke VLPs" where the introduction of modified capsid monomers is able to increase the VLP diameter significantly, resulting in larger sizes with adjusted symmetries.[30] Stability is also paramount for the success of VLPs in therapeutic gene editing; we envisage that robust design and appropriate formulation, such as surface modifications (e.g., PEGylation), could improve VLP stability by reducing protein aggregation and protecting against immune clearance, thus enhancing their longevity and effectiveness. Finally, optimization of large-scale production methods will allow us to streamline and scale up the manufacture of gene-editing agents carrying VLPs, facilitating comprehensive preclinical testing before its clinical translation. This scaling effort will also allow for the efficient production of high-quality base and prime editing agents, ensuring their availability for rigorous testing in complex biological models.[31]

Adenoo-Associated Viruses (AAVs)

Their robust *in vivo* transduction efficiencies, benign nature, extensive tissue tropism, infrequent genomic integration, and capability to endure within non-dividing cells collectively establish AAVs as exceptionally proficient carriers for genetic modification.[32] AAVs are small, non-enveloped viruses from the *Parvoviridae* family, with a 4.9-kb genome and a protein capsid composed of VP1, VP2, and VP3. The genome includes two open reading frames, *Rep* and *Cap*, and is flanked by ITRs, essential for AAV DNA replication and packaging. AAVs enter a latent state without a helper virus, enhancing their suitability as gene delivery vectors.[33] Recombinant AAV vectors (rAAVs) are made without AAV Rep/Cap genes and, hence, are replication deficient even in the presence of a helper virus (e.g., AdV or herpes simplex virus [HSV]). Their capsids can be sourced from natural AAV serotypes or engineered variants.[34] rAAVs possess a low immunogenic profile and demonstrate limited efficiency in transducing antigen-presenting cells,[35] making them of great interest as vectors both *in vitro* and *in vivo* for facilitating genome-editing-mediated therapeutic gene correction.[36,37]

Recombinant AAV vectors are increasingly employed to deliver DNA repair templates alongside endonucleases for therapeutic purposes.[32] The initial applications focused on introducing DSBs in the genome to promote homologous recombination with a provided template. Both ZFNs and CRISPR-Cas9 nucleases have been combined with AAV to induce targeted donor DNA integration in various settings. As discussed later in this review, AAV vectors carrying transgenes for clinical use have been employed to target several organs and tissues, including the brain and liver.[38–42]

Among the latest advancements is the use of rAAVs for the *in vivo* delivery of base editors; however, these vectors face the challenge of limited packaging capacity. As a result, ongoing research is focused on developing innovative strategies to overcome this limitation. Within this framework, Levy and collaborators have successfully developed dual rAAVs to deliver split cytosine base editors (CBEs) and split adenine base editors (ABEs), which are reconstituted by *trans*-splicing inteins. This approach enabled efficient base editing in various tissues (e.g., liver, skeletal muscle, heart, retina, and brain) and corrected mutations causing Niemann-Pick disease type C, delaying neurodegeneration and increasing lifespan.[43] Furthermore, Yeh et al., developed a base-editing strategy using dual rAAVs to address a recessive *Tmc1* mutation (c.A545G) causing deafness in Baringo mice. By packaging optimized CBEs and gRNAs into rAAVs and injecting them into the inner ears, they achieved up to 51% mutation reversion, restoring sensory transduction and partial hearing recovery.[44] Zhang et al. engineered and characterized a compact ABE, Nme2-ABE8e, derived from Nme2Cas9 of *Neisseria meningitidis*. This editor features a distinct protospacer adjacent motif (N4CC) and reduced off-target effects when compared to traditional Cas9-based ABEs. Delivered via a single rAAV vector, Nme2-ABE8e achieved efficient editing in mammalian cell cultures and mouse models. Its compact size and single-vector compatibility suggest significant potential for safer and more effective therapeutic applications.[45] In the same way, Davis et al. recently demonstrated that size-optimized rAAVs with ABEs provide efficient editing in mice, surpassing dual-rAAV systems. Single-AAV-encoded ABEs achieve up to 66% editing efficiency in liver, 33% in heart, and 22% in muscle, outperforming dual-rAAV systems. Three minimized ABE8e variants cover about 82% of the human genome, enhancing AAV-based research and therapeutic uses by streamlining production and minimizing required doses.[46]

As discussed above, prime editing enables precise genome modifications by facilitating base pair substitutions, insertions, or deletions without generating DSBs or requiring exogenous donor DNA. However, the ~6.3-kb coding sequence of prime editors exceeds the ~4.7-kb packaging limit of AAVs, which has prompted several studies to aim at overcoming this barrier. Liu et al. developed an NLS-optimized SpCas9-based prime editor that significantly enhances the effectiveness of genome editing at both reporter and native genetic loci. This optimized system enabled tumor formation through somatic cell editing involving the installation of oncogenic mutations in mice and successfully corrected a pathogenic mutation in the liver using dual-rAAV delivery, underscoring the technology's potential for *in vivo* disease modeling and correction, respectively.[47] Further advancements were made by Gao et al., who described smaller prime editors with enhanced expression. This optimization led to improved rAAV titers and editing efficiency, achieving up to 6% precise editing of the *Pcsk9* gene in mouse liver with dual rAAV8 vectors. This work highlights the strong potential of optimized rAAV-prime editing systems for *in vivo* applications.[48] Moreover, aiming at developing more precise editing tools, Zheng et al., identified a compact prime editor with consistent editing efficiencies *in vitro* and *in vivo*, which were comparable to those achieved by its full-length counterpart. Moreover, its delivery via dual-rAAV8 enabled efficient editing of the *Pcsk9* gene.[49] In a related development, Lan et al. introduced a miniature prime editor (mini-PE) maintaining editing efficiency while reducing size. The optimized mini-PE achieved up to 10% precise editing in human and mouse cells. Nevertheless, in mouse retinas, combining the mini-PE with rAAVs demonstrated less than 1% efficiency in editing the *Hsf1* gene, indicating a need for further improvements to enhance editing efficiency and explore therapeutic applications for human genetic disorders.[50] In the same vein, Davis et al. developed enhanced rAAV-prime editor vectors with improved editor expression and gRNA stability. When delivered as a dual rAAV, it achieved up to 46% editing efficiency in mouse liver and 42% in the cortex. These systems enable precise editing for conditions such as Alzheimer's and coronary artery disease without off-target effects, representing a significant advancement in therapeutic gene editing.[51] In summary, the relatively low immunogenicity of rAAV and their inability to integrate frequently into the genome, combined with a large bulk of clinical data regarding their safety in gene therapy clinical trials, make rAAVs a promising delivery vehicle for gene editing, particularly for *in vivo* applications in post-mitotic tissues where AAV episomes can persist for a long time. Future research should focus on further refining these delivery systems to enhance their efficacy and safety, on defining affordable and

optimized manufacturing processes, as well as on strategies to reduce immune response to AAV in patients with pre-existing immunity, in order to boost accessibility to AAV-based therapies and to ultimately pave the way for effective treatments of genetic disorders.

Adenoviruses (AdVs)

Adenoviruses (*Adenoviridae* family; *Mastadenovirus* genus) comprise numerous viral serotypes that are regularly being identified in a wide range of vertebrates, including humans (currently over 100). This natural diversity, and associated broad cell tropisms, is offering the possibility to construct vectors that either bypass pre-existing humoral immunity to prototypic human type-5 vectors or that engage specific cell-surface receptors on target cell populations.[52] Structurally, adenoviruses and their vector derivatives consist of a protein-capped linear double-stranded DNA genome packaged in an icosahedral protein capsid (∼100 nm) with protruding cell receptor-interacting fiber proteins.[52,53] First-generation recombinant AdV vectors (rAdVs) lack the viral early region 1 (E1) alone or together with E3; while second-generation rAdVs combine deletions in E1 or E1 and E3 with deletions in other regions, namely E4 or E2.[52,53]

The high production yields, non-integrating character, and robust transduction of dividing and post-mitotic cells have set the stage for initial studies exploring these AdV systems for the delivery of programmable nucleases such as ZFNs,[54] transcription activator-like effector nucleases (TALENs),[55] CRISPR-Cas9 nucleases[56] and, more recently, prime editors (PEs).[57] However, to blunt cytotoxicity *in vitro* and immunogenicity *in vivo*, associated with "leaky" viral gene expression from early region-deleted AdVs, helper-dependent, or high-capacity rAdV systems (HC-rAdVs) have also started to be investigated for genome-editing purposes (Figure 1D).[53,58] Importantly, the lack of viral genes combined with their vast payload capacity (up to 36 kb) make HC-rAdVs particularly suitable sources of exogenous (donor) DNA substrates for targeted gene KIs via HDR[59,60] and advanced genome-editing tools, such as base editors and PEs.[61,62] Indeed, HC-rAdVs are capable of transducing complete base editing and prime editing complexes consisting of Cas nickases fused to deaminase and reverse transcriptase effector domains, respectively, together with their cognate gRNAs.[63,64]

Compared with nucleases, nickase-based genome-editing technologies present lower cytotoxicity and genotoxicity, as chromosomal nicks are poor stimuli for DNA damage response and error-prone DNA end joining, respectively. Hence, integrating HC-rAdV and advanced genome-editing technologies is starting to offer exciting prospects for the treatment of monogenetic disorders. Examples include cystic fibrosis,[63] Duchenne muscular dystrophy (DMD),[65] and hemoglobinopathies.[61,63,66] In fact, regarding the latter conditions, recent studies using capsid-modified HC-rAdV particles provide proof of concepts for *in vivo* base editing and prime editing in HSPCs with evidence for the rescue of pathogenic phenotypes.[61,63,66]

Notwithstanding the herein-described developments, *ex vivo* and *in vivo* HC-rAdVP-based gene therapies will profit from further research aiming at overcoming or mitigating well-defined limitations. Notably, research on dissecting particle-cell interactions causing pro-inflammatory cytokine release (e.g., interleukin [IL]-6 and tumor necrosis factor [TNF]) and activation of intracellular innate immunity sensors (e.g., Toll-like receptors and cGAS-STING), is warranted.[67] The resulting insights are expected to yield approaches or regimens capable of transiently modulating said extracellular and intracellular pathways to bring about improved vector performance and ensuing genome-editing outcomes. Moreover, in the context of *in vivo* gene therapies, vector particle engineering achieving liver de-targeting and strict cell- or tissue-specific transduction should become ever-more critical.[68] Equally of translational relevance, improved upstream and downstream pipelines for HC-rAdV production are in demand in that the assembly of these vectors is substantially more complex than that of their earlier-generation counterparts due to their requirement for a complementing E1-deleted helper rAdV.[58]

Non-viral delivery methods

Although viral vectors have shown high efficiency in delivering genetic material for gene editing, their safety concern constitutes a critical issue. Therefore, non-viral delivery methods have been explored for gene editing in addition to viral delivery. On the non-viral side, lipid nanoparticles (LNPs), synthetic or polymeric nanoparticles, extracellular vesicles (EVs), and inorganic nanoparticles provide customizable solutions for genome editing, enhancing efficacy and safety. In parallel, physical methods, including electroporation, and microinjection, offer direct and efficient delivery of gene-editing tools directly into target cells (Figure 2). In the following sections, we discuss the advantages and disadvantages of each of these methods.

Lipid nanoparticles (LNPs)

LNPs have emerged as a potent non-viral delivery system for CRISPR-based genome-editing tools that can be exploited *in vitro* and *in vivo*.[69,70] From the first US Food and Drug Administration (FDA)-approved LNP-based drug, Patisiran, in 2018 for the treatment of hereditary transthyretin amyloidosis, which encapsulates a small interfering RNA,[71] to the widespread use of LNPs in the form of mRNA vaccines against COVID-19,[72] several advances have been made in order to drive the use of LNPs for CRISPR delivery, opening the path to new drug discoveries and clinical translation. A vast repertoire of research reports delivery of the CRISPR system via LNPs, with cargo in the form of plasmid DNA,[50,73] mRNA,[50,73–76] and RNPs,[50,77,78] encompassing a variety of CRISPR-derived gene editors. LNPs usually consist of four main components, which are part of the ethanol phase: cholesterol, neutral helper lipid (e.g., 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine, Distearoylphosphatidylcholine), poly (ethylene glycol) (PEG)-lipid conjugate (e.g., PEG_{2000}-C-DMG, ALC0159), and, most important, amino-ionizable lipids (e.g., DLin-MC3-DMA, SM-102, ALC3015). The LNP cargo is added in the aqueous phase (nucleic acids in low-pH buffer, proteins such as RNPs in neutral buffer). When mixing these components, LNP self-assemble thanks to noncovalent interactions between all components. However, LNP production can be

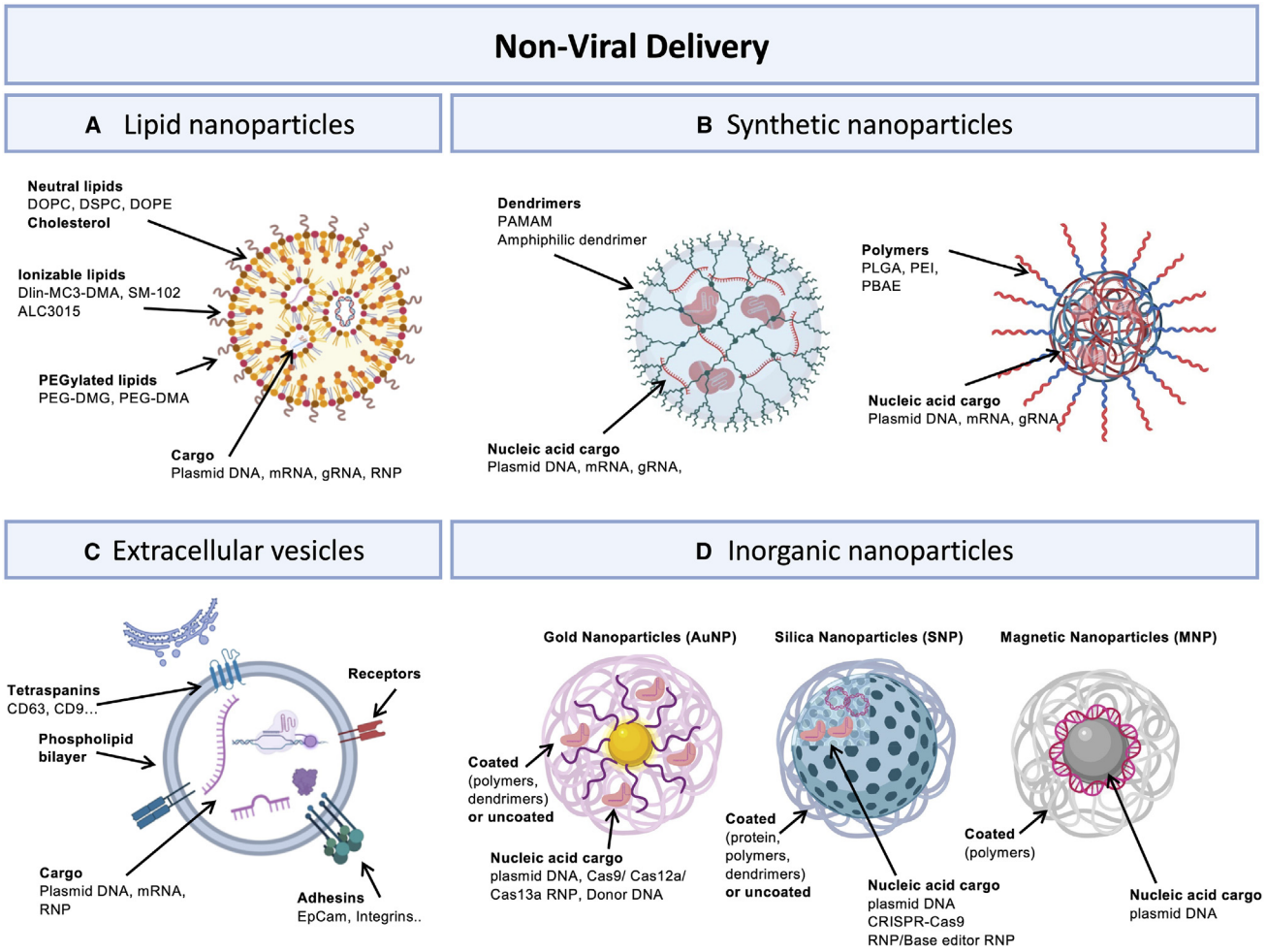

Figure 2. Schematic of non-viral methods for delivering gene-editing tools

The CRISPR-Cas system can be delivered in various forms: DNA, mRNA/sgRNA, or ribonucleoprotein (RNP). Non-viral delivery methods include (A) Lipid nanoparticles (LNPs): efficient carriers that encapsulate and protect gene-editing tools, facilitating their cellular uptake. (B) Extracellular vesicles (EVs): naturally occurring particles that transport genetic material between cells, offering a biocompatible delivery system. (C) Synthetic carriers (such as dendrimers and polymers): engineered molecules designed to improve the stability and delivery efficiency of gene-editing tools. (D) Inorganic nanoparticles (including gold, silica, and magnetic particles): robust delivery platforms that can be functionalized for targeted delivery and controlled release of gene-editing agents. Created with Biorender.com.

enhanced and regulated via thin-film hydration, ethanol injection, T-junction mixing, or microfluidic mixing.[70,79] The component ratios as well as the conditions of the mixture influence the physical properties of the LNPs, such as shape, size, surface charge, and compactness, which in turn influence their stability and biodistribution. Recent advancements in LNP composition, particularly in the development of biodegradable ionizable lipids, can improve particle stability, increase circulation time, reduce toxicity, and lower immunogenicity, thus enabling higher cargo transfer.[69,70,80,81]

In late 1980s, Felgner et al.[82] reported that electrostatic interactions of cationic lipids with the negatively charged phosphate backbone of the DNA lead to lipid-nucleic acid complex formation, which enables cellular uptake by interacting with the negative charges on the cell membrane. This is now exploited by the most important component of the lipid LNP formulation, the ionizable lipids, that undergo protonation in low-pH environments during LNP production, whereas, when exposed to neutral pH, they carry a neutral charge, which ensures electrical neutrality and prevents inflammatory risks, associated with cationic lipids.[70] Inside the cells, the acidic endosomal environment re-protonates the ionizable lipids, which causes endosomal membrane disruption, followed by endosomal escape of the cargo and subsequent cytosolic translocation. Endocytosis therefore represents a key process of LNP uptake,[78] and is usually mediated by the binding of blood-circulating apolipoprotein E (ApoE) to LNPs, which can in turn bind to low-density lipoprotein receptor (LDL-R), resulting mainly in uptake by the liver.[70,75,79,83] This remains a limiting factor for the use of LNPs as a specific organ-targeted

drug-delivery system. To avoid LDL-R mediated uptake, a multivalent N-acetylgalactosamine (GalNAc)-targeting ligand can be introduced into the LNPs to allow for uptake via the asialoglycoprotein receptor. The approach was used to drive base editing for the modification of the *ANGPTL3* gene, which resulted in approximately 60% of editing in the liver with minimal nontargeted organ genome modification.[78] Base-editing mRNA, delivered using LNPs, was also used to modify *Pcsk9* mutations that drive the occurrence of familial hypercholesterolemia, and therapeutic relevance was demonstrated in primates.[84] The *Pcsk9* gene was also modified in mice by Cas9 RNP delivered via LNPs.[78] In the study, led by Wei et al.,[14] LNPs modified with DOTAP (permanent cationic lipid) were used to achieve RNP packaging in nanoparticles, therefore overcoming the limitation of packing different species inside LNPs and allowing specific organ targeting (liver, lung, muscles, and brain).[78] Another important LNP modification to facilitate RNP packaging is the addition of guanidinium-rich lipopeptides to the LNP mixture. Guanidinium-rich agents aid in RNP loading as they present high affinity toward the anionic side chains of proteins and the phosphate backbone of gRNAs, which is why guanidinium-based LNPs exhibit higher cell membrane penetration and thus facilitate RNP delivery into cells and tissues.[85] In this study, Zhu et al. used single-stranded DNA (ssDNA)/Cas9 RNP, transferred by LNPs via intramuscular injection, to restore dystrophin expression in muscles and significantly improved muscle strength in a DMD mouse model. Mohanna et al. reported for the first time successful delivery and genome modification via ssDNA/RNPs LNP transfer into the cornea via intrastromal LNP injection,[86] again demonstrating encapsulation and delivery of pre-complexed RNPs and ssDNA HDR templates within a single lipid nanoparticle. LNP-based co-delivery of ssDNA as a template to drive HDR in lungs was also reported by Wei et al.[87] Here, an HDR template in the form of single-stranded oligodeoxynucleotide (ssODN), co-delivered with Cas9 mRNA and gRNA, was used to tackle cystic fibrosis.[87] Another approach using degradable, ionizable, dendrimer-based lipids achieved delivery of an HDR DNA template.[88] A recent report states that specific design strategies for ssODN should be considered, with the highest activity achieved at a melting temperature near room temperature.[77] In addition to base editors, other types of editors can be delivered via LNP. A recent publication by Herrera-Barrera et al. shows that LNPs are also capable of delivering prime editor mRNA to a human HAP1 reporter cell line and to HEK293 cells.[89] Approximately 60% of prime editing was observed with LNPs containing the cholesterol analog β-sitosterol.

LNPs have been used to modify the genome in a wide array of different cell types (e.g., T cells, B cells, hepatocytes, HSPCs, muscle cells).[50,78,79,89–92] For efficient action of the CRISPR cargo *in vivo*, the LNPs, injected intravenously, must overcome the obstacles of enzymatic blood degradation, phagocytosis, plasma protein sequestration, reticuloendothelial system entrapment, and high kidney clearance.[79] To achieve cell- and/or organ-specific targeting, several improvements can be made to avoid abundant liver accumulation of the injected LNPs.[81] While passive targeting of the liver by exploiting ApoE/LDL-R interactions can be utilized, the selective organ targeting (SORT) system, where a lepidic SORT molecule is incorporated, shows extrahepatic delivery.[87,90] A more straightforward approach is active targeting, where chemical coupling interactions between the PEG lipid, modified with a reactive moiety such as maleimide, and antibodies are used.[70,75,93–95] A safer approach to achieve active targeting and avoid the possibility of damaging the antibodies is the anchored secondary single chain variable fragment (scFv) enabling targeting (ASSET) method, which allows for noncovalent coupling of the antibody to CRISPR loaded LNPs.[69,75] With more research still needed to optimize LNP specific delivery, LNPs have been shown to be very efficient in genome alteration *in vivo*, which has already led to several clinical trials.[96] VERVE-101, a phase I clinical trial, aims to cure familiar hypercholesterolemia by ABE mRNA LNP delivery to inactivate *PCSK9* gene.[84,91] Similarly, CTX310 uses Cas9 mRNA LNP-based delivery to modify *ANGPTL3* gene to treat patients with atherosclerotic cardiovascular disease in a phase I clinical trial.[97] NTLA-2001, where Cas9 mRNA is delivered to inactivate the *TTR* gene to treat transthyretin amyloidosis, is being tested in a phase III clinical trial.[98] Inactivation of kalikrein K1 (*KLKB1* gene), part of the NTLA-2002 phase I/II clinical trial, is seeking therapeutic solution for patients suffering from hereditary angioedema. Taken together, LNPs are recognized as one of the most promising and widely used non-viral delivery methods due to their ability to safely alter the genome *in vivo*. Compared to some viral delivery mechanisms, they offer transient cargo expression,[99] minimizing unwanted side effects; they are relatively non-immunogenic, allowing for repeated administrations[100]; and they provide protection for the packaged cargo. Although they have been shown to be very efficient, there are still several limitations, such as rapid body clearance and low bioavailability. The main drawback is the lack of cell or tissue specificity, as well as the inability to cross the blood-brain barrier (BBB), which remains a strong limiting factor. Although various advances are being made (for instance, the use of sorting lipids, targeting moieties, or Trojan-horse molecules), there are no reports of specific cell targeting.[101–103] Also, to our knowledge, there are no reports yet on efficient LNP-mediated delivery of long coding templates for HDR.

Extracellular Vescicles (EVs)

EVs are lipid bilayer vesicles encapsulating endogenous biomolecules and are actively secreted by all cells from prokaryotes to eukaryotes.[104,105] EVs contain biologically active molecules, including various RNAs, proteins, and bioactive lipids derived from their producer cell. The horizontal transfer of these macromolecules renders EVs essential mediators of intercellular communication. As nature's very own nanoparticles, EVs inherently benefit from immune tolerance, stability in circulation, as well as the ability to cross biological barriers to reach distant organs such as the brain.[106] These unique properties of EVs have inspired many scientists to use them as a next-generation drug-delivery tool. Therapeutic EV research has shown an exponential transition in the past decade with the development of engineering tools to harness its potential for biotherapeutics delivery.[104] When applied as vehicles for drug delivery, EVs could be engineered exogenously or endogenously for cargo loading. Exogenous loading usually involves disruption of the membrane using

electroporation or some surfactants after the isolation of EV, while endogenous loading happens during the EV production where the producer cells are engineered to overexpress the cargo fused to an EV scaffold, which is then incorporated into the secreted vesicles during EV biogenesis. Recent studies have demonstrated the effectiveness of EVs in delivering CRISPR-Cas9 for various therapeutic applications. For instance, engineered EVs have been successfully used to deliver CRISPR-Cas9 RNPs for treating dominant progressive hearing loss in the Shaker-1 mouse model.[107] This was achieved using a novel high-throughput microfluidic electroporation system, which enhanced loading efficiency and preserved integrity of the EVs, leading to significant recovery of hearing function. In cancer treatment, EVs have been employed to deliver CRISPR-Cas9 systems to target and modify cancer cells. A study by Kim et al. demonstrated that cancer-derived exosomes could serve as effective delivery vehicles for CRISPR-Cas9-mediated genome editing with less immunogenicity. They successfully disrupted poly (ADP-ribose) polymerase-1 (PARP-1) expression in SKOV3 cells with up to 27% indels, which induced apoptosis in ovarian tumor cells and enhanced chemosensitivity to cisplatin.[108] Another significant study involved using EVs to deliver CRISPR-Cas9 for the treatment of DMD, leading to improved muscle function and increased expression of dystrophin in treated animal models,[109] in which 19% of the extracted cDNA contains the intended editing outcome. In another study, EVs engineered with CRISPR-Cas9 RNP achieved 90% editing efficiency in primary skeletal muscle cells derived from DMD patient induced pluripotent stem cells (iPSCs).[110] A recent study developed novel systems termed VSV-G plus EV-sorting domain-intein-cargo (VEDIC) and VSV-G-foldon-intein-cargo (VFIC), which use an engineered mini-intein protein with self-cleavage activity to link cargo to an EV-sorting domain and release it from the EV membrane inside the EV lumen. These systems utilize the fusogenic protein VSV-G to facilitate endosomal escape and cargo release into the cytosol of recipient cells. This approach has demonstrated nearly 100% recombination efficiency and close to 80% genome-editing efficiency in CRISPR-Cas9 GFP reporter cells like Hela-TL and B16F10 cells by EV-transferred Cre recombinase and Cas9/sgRNA RNPs. Moreover, the developed system showcased efficient delivery of Cre recombinase enzyme, where a single intracerebroventricular injection in Cre *LoxP* fluorescent reporter mouse Ai9 leads to nearly 60% recombination of cells in the hippocampus and the cortex. Despite these promising developments, challenges remain related to the complex and in large part unknown composition of EVs, the difficulty in the standardization of protocols for EV isolation, purification and characterization, and the associated challenges in reproducibility and comparability of results across studies.[111] Furthermore, improving the stability and efficiency of EV-mediated CRISPR-Cas9 delivery *in vivo* and enhancing the targeting ability and optimizing the cargo-loading capacity of EVs are critical areas for future research to ensure the safe and effective application of this technology in clinical settings.

Other synthetic nanoparticles

Many different synthetic nanoparticles (e.g., polymer nanoparticle, dendrimer nanoparticle, synthetic peptide-based nanoparticles) are emerging as an alternative approach to address the challenges in advancing gene editing to widespread therapeutic use. Due to the large size of genome-editing enzymes and increasingly complex microenvironment of targeted tissues, improved delivery systems are required to achieve effective delivery to desired tissues and interaction with specific cell types, facilitate endosome escape, avoid clearance by off-target organs, and minimize innate immune toxicity. Here, we present some exemplary studies on how non-viral delivery vectors have been evolved to enable rapid progress and overcome current challenges of genome editing beyond LNPs.

Among synthetic nanoparticles, polymers or polymeric materials have been extensively studied for genome-editing applications. PLGA is a commonly adopted polymer that has been approved by the FDA for small-molecule delivery.[112] Alternatively, scientists have successfully used PLGA polymers to deliver triplex-forming peptide nucleic acids (PNAs) to catalyze genome editing.[113,114] Polymers containing cationic components, such as polyethylenimine (PEI) and poly(l-lysine) (PLL), can form nanocomplexes with genome-editing cargoes via electrostatic interactions. For example, polymers composed of hyperbranched poly(β-amino ester) (PBAE) have been used to deliver CRISPR-Cas9 RNPs intracranially. PBAE nanoparticle represents an ideal carrier for encapsulation and delivery of CRISPR-Cas9 RNPs, enabling robust gene knockin (4%) and knockout (75%) in murine glioma cell line and human mesenchymal stem cells (MSCs).[115] However, a concern in the use of cationic polymers is their tolerability. To diminish their toxicity, researchers introduced degradable crosslinker to polymers to facilitate the degradation of polymer nanoparticles when exposed to cytosolic microenvironment, representing a safe and effective delivery vector for *in vivo* genome editing.[116] An exceptional class of polymers is represented by dendrimers, which are structurally well-defined and highly symmetric branched molecules built up from repetitive branching units emanating from a central core. As a specific category of dendrimer, amphiphilic dendrimer, featuring a hydrophobic alkyl chain and a hydrophilic dendron head, leverages the advantages of both lipids and cationic polymers and offers a promising alternative for macromolecule delivery and genome editing.[117] An amphiphilic dendrimer-based LNP (dLNP) was designed to deliver a Cas9 mRNA/gRNA/donor DNA complex to achieve non-viral HDR-mediated correction of mutations *in vivo* at single-nucleotide scale. This "all-in-one" strategy provided a convenient and effective approach for combined nucleic acid delivery with various lengths and origins.[88] Similarly, and as mentioned above, a SORT system where LNPs are composed of cationic dendrimers and a fifth lipid component to ensure effective mRNA delivery and release has also been successfully used for RNP delivery for the treatment of multiple genetic diseases, including hypercholesterolemia, DMD, and cystic fibrosis.[78] Fusing cargoes with synthetic cell-penetrating peptide (CPP) is another approach to enable high genome-editing efficiency. By conjugating Cas9 protein and complexing gRNA with CPP, condensed and positively charged nanoparticles can be formulated to enhance effective genome editing. CPP-Cas9/gRNA nanocomplexes enabled direct Cas9 and gRNA delivery and reduced RNA-guided endonuclease

(RGEN) working time, leading to drastically lower off-target cleavage effect as compared with plasmid transfection.[118] A follow-up study screened CPPs to enable RNP delivery for primary human lymphocyte-targeted CRISPR editing (PERC).[119] Screening from 37 amphiphilic peptides, A5K derived from chimeric HA2-TAT fusion scaffold was identified as the most potent peptide for RNP transfection. A5K peptide outperformed electroporation in sequential RNP delivery with improved cell viability and minimal perturbation of cell transcriptome and phenotype, thus enabling sequential administration and avoiding potential genotoxicity caused by simultaneous edits.

Overall, synthetic nanoparticles such as polymer or dendrimer-based nanoparticles offer promising prospects as nucleic acid carrier thanks to their tunable traits including charge, molecular weight, and degradability, which influence nucleic acid delivery.[120] However, clinical translation of synthetic nanoparticles still faces multiple challenges, such as complicated manufacturing process and unclear safety profile.[121] In addition, the chemical and pharmaceutical activity of synthetic nanoparticles should be consistent across many batches to maintain biological effect in the body.

Inorganic nanoparticles

Inorganic nanoparticles are also attractive and practical for delivering genetic materials into cells for genome editing due to their unique properties, including thermal, optical, electric, and magnetic properties alongside high stability and ease of synthesis.[122] They can be engineered to vary in size, shape, and porosity to protect encapsulated molecules from degradation. Commonly studied materials in this category include gold, silica, magnetic compounds, quantum dots, and carbon nanotubes.

Gold nanoparticles (AuNPs) have unique optical, electronic, and thermal properties that make them highly versatile for gene editing, diagnosis, and biosensing applications.[123,124] The first use of AuNPs for gene editing was described in a study carried out by Mout and colleagues in 2017, where the direct delivery of CRISPR-Cas9-ribonucleoprotein (Cas9-RNP) into the cytosol and nucleus achieve high delivery (~90%) and effective gene editing (~30%) *in vitro* in HeLa cells, validated also in other cell lines, including human embryonic kidney cells (HEK293T) and mouse macrophage (Raw 264.7) cells.[125] Other studies used AuNPs conjugated with DNA serving as HDR template and cationic polymers to deliver Cas9 RNP for correcting DMD,[125] as well as fragile X syndrome in mouse models, with undetectable levels of toxicity at the doses used with Cas9 and Cas12 (Cpf1) RNP.[126] AuNPs have also been used to deliver Cas9 and Cpf1 RNPs to HSPCs with no detected toxicity *in vitro* and a gene-editing efficiency within the 17%–65% range, which was sustained for 12 weeks post *ex vivo* HSPC transplantation into immunodeficient mice.[127,128] AuNPs can form self-assembled nanoclusters with CRISPR-Cas9 at physiological pH to facilitate its delivery into the cell nucleus to effectively knock out target genes, such as oncogenes in cancer cells, restoring tumor-suppressive functions.[129] For instance, AuNPs were loaded with dendrimers to develop core-shell tectos responsive to both reactive oxygen species and pH for efficient delivery of the CRISPR-Cas9 system to permanent disrupt the *PD-L1* gene in cancer cells, achieving almost 60% efficiency, thereby boosting antitumor immunity.[130] Moreover, the combination of the *cis*-cleavage activity of Cas9, Cas12a, and Cas13a proteins, as well as the *trans*-cleavage activity of Cas12a and Cas13a proteins with gold nanomaterials, has been used to develop CRISPR-Cas-based biosensors.[131–133] These biosensors enable signal readout modes such as fluorescence, colorimetry, and electrochemistry to facilitate diagnostic applications for infectious diseases, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[134]

Silica nanoparticles (SNPs) offer a number of advantageous properties, including a large pore volume, efficient encapsulation, ease of surface modification, and biodegradability.[135] The surface of SNPs can be modified with a variety of ligands or targeting moieties, including cationic polymers or peptides, in order to enhance cellular uptake and transfection efficiency.[136] The porous structure of SNPs enables the encapsulation or adsorption of nucleic acids, including plasmid DNA and small interfering RNA (siRNA). Moreover, they have been identified as a promising nanoplatform for the systemic administration of various anticancer drugs. For this reason, SNPs have been employed to deliver CRISPR-Cas9 plasmid with small drugs for the purpose of efficiently targeting cancer therapies, demonstrating customizable combination therapies,[135,137] as well as for inflammatory diseases.[138] Furthermore, Cas9 and base-editor RNPs have been delivered by a biocompatible sponge-like silica nanoconstruct in human and mouse cells (HeLa, HEK293T, and NIH3T3) as well as for an *in vivo* solid-tumor model, achieving greater efficiency in editing (5× with Cas9 RNP and 2× with base-editor RNP) with minimal off-target effects in comparison to commercialized materials such as lipid-based methods.[139]

Magnetic nanoparticles (MNPs) have emerged as a highly effective tool in the field of gene editing, markedly enhancing the delivery and precision of CRISPR-Cas9 systems. MNPs offer distinctive benefits in terms of their rapid transfection process, magnetic targeting, isolation and positioning of transfected cells, and molecular imaging.[140] A number of studies have highlighted the potential of MNP-mediated CRISPR-Cas9 delivery. Rohiwal et al. developed PEI-modified MNPs for the delivery of CRISPR-Cas9 plasmids, demonstrating that their gene-editing efficiency was very similar to that of lipofectamine transfection (6% for NHEJ and 0.5% for HDR) in a stable HEK293 cell line expressing the traffic light reporter (TLR-3).[141] Magneto-electric nanoparticles have been employed to traverse the BBB, exhibiting potential for the prevention of latent HIV-1 infection.[142] MNPs have been demonstrated to be effective in controlling the spatial aspects of gene editing *in vivo*.[142] Recently, the Magnetic Nanoparticle-Assisted Genome Editing (MAGE) platform has been described as a potential method for correcting the mutated *MeCP2* gene in iPSC-derived neural progenitor cells from a patient with Rett syndrome.[143] Based on previous results, inorganic nanoparticles hold great promise for gene-editing applications, particularly for the delivery of CRISPR-Cas9 systems. At present, the focus when using MNPs is on plasmid DNA, but there is potential

for efficiency improvements through the co-delivery of gRNA and Cas9 mRNA or protein, which, however, necessitates the creation of new carrier designs. Despite promising results, MNP-based delivery is a developing field that faces a number of challenges, such as low intracellular delivery efficiency, targeting specificity, endosomal escape, biocompatibility, scalability, and design complexity. Addressing these limitations is crucial for advancing the use of these nanoparticles for gene-editing therapies.

Physical delivery

Two commonly used physical methods for genome editor delivery are microinjection and, notably, electroporation (EP).[144,145] Microinjection is a precise and efficient method for delivering various substances, including CRISPR-Cas9 RNPs, into cells.[146,147] This technique offers advantages such as accurate dosage control, high efficiency, and low cytotoxicity. However, manual microinjection is labor intensive and time consuming, and it can also cause physical damage to the cell, limiting its application to single-cell systems as zygotes.[147] EP is an alternative, highly efficient, non-viral method for delivering nucleic acids and proteins to cells. This technique involves applying short, high-voltage pulses to create temporary pores in cell membranes, allowing the entry of various molecules. EP offers numerous advantages, including increased efficiency, reduced variability, and virtually unlimited cargo size delivery.[148,149] It is known for its easy operation, controllable parameters, and cost-effectiveness, making it a preferred method for delivering biomolecules into cells. Recent advancements in micro/nanofluidic technologies have enabled single-cell electroporation with high throughput and cell viability.[150–152] The nanostraw-electroporation system allows for precise dosage control and effective delivery into various cell types, including primary cells.[153–155] Notably, EP has been successfully used to genetically edit human primary cells, overcoming their resistance to viral gene delivery methods and achieving high levels of gene editing with good cell viability. Nevertheless, especially in more sensitive cell populations such as stem cells, careful optimization of electroporation parameters is crucial, as membrane disruption may cause significant alterations in cellular homeostasis, DNA damage, and mitochondrial stress, potentially triggering cell death.[156]

Delivery of gene-editing tools for preclinical and clinical applications

Initial genome-editing efforts have mainly focused on primary cells, including T cells, HSPCs, and iPSCs, because of their therapeutic potential and relatively easy manipulation outside of the patient's body. Indeed, many current gene-editing clinical trials focus on *ex vivo* editing, where cells are taken from a patient, modified in the lab, and then reinfused back to the patient. While this method is suitable for certain cell types, it is not applicable to organs like the liver, brain, or muscle, for which *in vivo* delivery is essential. Moreover, modification using CRISPR-Cas9-mediated editing of some primary cells, such as neurons, still represents a significant challenge, limiting the effectiveness of gene therapy for neurological disorders. In the subsequent sections, we explore the various scenarios of therapeutic *ex vivo* and *in vivo* gene-editing delivery attempts to different organs, their limitations, and requirements for efficient gene-editor delivery (Figure 3).

Targeting HSPCs to tackle blood disorders

Despite the relatively recent adaptation of genome-editing tools for therapeutic purposes, there has been a remarkable interest on implemented genome editing for the modification of HSPCs. Gene editing utilizing hematopoietic cells is mostly performed *ex vivo*, following a process in which bone marrow- or peripheral blood-derived HSPCs are isolated from the patient, genetically, and then reinfused back to the patient where they engraft, undergo self-renewal, and differentiate to establish a population of modified cells that pass the transgene to daughter blood cells (Figure 4). For *ex vivo* delivery of gene-editing reagents to HSPCs, electroporation is currently the gold standard in the field, which introduces preassembled ribonucleoprotein (RNP) complexes consisting of recombinant Cas9 protein and synthetic sgRNA or a fully mRNA-based platform. This approach has been successfully used to perform deletions of regulatory regions or genes via NHEJ repair, as exemplified by the recent approval of Casgevy for the treatment of β-hemoglobinopathies, where induced insertions and deletions (indels) in the erythroid enhancer of the *BCL11a* gene activate γ-globin, compensating for the absence of functional β-globin. On the other hand, gene KI via HDR has been demonstrated in numerous preclinical studies. In this setting, following electroporation and delivery of the gene-editing reagents targeting a specific locus, a repair template with homology to the regions flanking the nuclease cut site must be provided to the cells to allow site-specific integration through the HDR pathway. Initial proof-of-concept studies of efficient correction of point mutations or of short stretches of DNA were carried out by using single-strand oligo DNAs, which are well tolerated by HSPCs.[157,158] However, given the narrow applicability of approaches targeting single mutations, targeted gene insertion is considered the preferred methodology for addressing recessive monogenic disorders, providing a universal solution that could be applied to all the patients affected by a specific disease. While first attempts favored the use of IDLVs for this scope, recently the field has transitioned to non-integrating rAAVs, especially to the HSPC tropic rAAV6, as more efficient delivery vehicles of homology templates into HSPCs, easily reaching up to 80% of KI frequency *in vitro*.[159] However, HDR-based approaches have been shown to be relatively inefficient in long-term repopulating HSCs, and a reduction in the repopulation capacity of *ex vivo* manipulated HSPCs has been observed in numerous preclinical studies.[160,161] These issues are mostly due to inefficient HDR in quiescent primitive stem cells and to exposure of HSPCs to stress stimuli, such as reactive oxygen species and DNA damage, which can result in a loss of self-renewal capacity or apoptosis.[156] Indeed, one clinical trial (NCT04819841) employing an HDR donor delivered via rAAV6 to HSPCs for the treatment of SCD demonstrated strong preclinical efficacy[162] but unsuccessful therapeutic outcome, as the first patient treated experienced pancytopenia, likely due to cell toxicity caused by the editing procedure. Overall, there are more

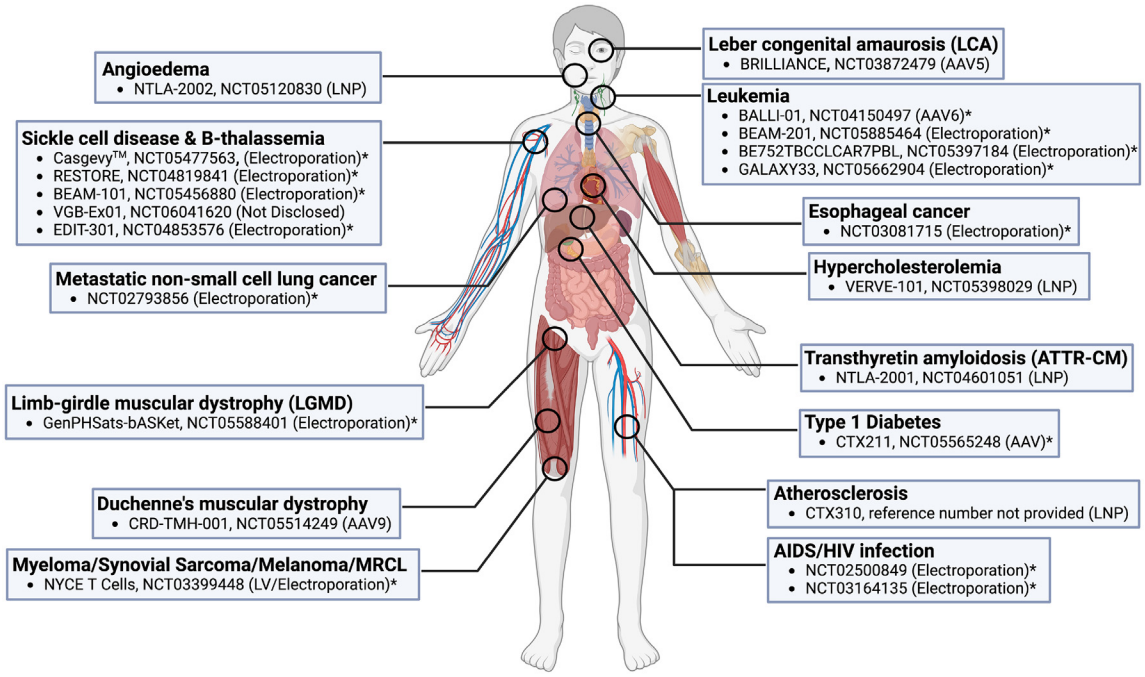

Figure 3. Overview of selected ongoing gene-editing clinical trials for various diseases

For each example, the official name of the clinical trial, ClinicalTrials.gov ID, and gene-editing delivery method are provided when available. An asterisk (*) indicates *ex vivo* cell treatment performed before infusion or transplantation.

than 30 clinical trials registered to date, providing transplantation of gene-modified HSPCs obtained by various genome-editing technologies such as CRISPR-Cas9, CRISPR-Cas12, ZFNs, TALENs, and base and PEs. β-thalassemia and SCD are diseases caused by mutations affecting the production of the adult hemoglobin and currently dominate the application of genome-edited HSPCs. These trials often aim to reactivate the expression of the fetal hemoglobin (HbF) by inactivating *BCL11A*, a gene encoding a major HbF transcriptional repressor. Other strategies and genome-editing tools have also been explored, including the adult β-globin gene correction via HDR (NCT04819841) and HbF reactivation using CRISPR/AsCas12 or base editors (NCT04853576, NCT05456880). Two gene-editing-based treatments for two forms of another inherited bloodborne disease, chronic granulomatous disease (CGD), have also recently been tested in clinical trials using base editing to amend a major X-CGD mutation (*CYBB*, NCT06325709)[163] or prime-editing tools targeting the p47-CGD-causing mutation (*NCF1*, alias PM359, NCT06559176). Other therapies that are being investigated using gene-edited HSPCs include prevention from lethal HIV infection by disrupting the CCR5 co-receptor, enhancing cancer immunotherapies by targeting genes responsible for T cell exhaustion (UCART22, NCT04150497; BEAM-201, NCT05885464; BE CAR7 T, NCT05397184), or eliminating surface markers targeted by chemo/immunotherapies in HSPCs to reduce toxicity of the treatment (Galaxy33, NCT05662904). Of particular note is the rapid integration of newer genome-editing technologies into clinical trials. Six and two trials feature base and prime editing, respectively, despite these technologies being relatively recent discoveries. The most resourced clinical delivery system for genome editing of HSPCs is electroporation (www.Clinicaltrials.gov). However, there are several options including proprietary protocols, and the details are hard to compare as not all trials disclosed detailed manufacturing procedures. Interestingly, all trials using either base or prime editing, ZFNs or TALENs are based on mRNA electroporation, while 90% of CRISPR-Cas9 trials, and two trials using CRISPR-Cas12, employ RNP electroporation. There is only once exception in the TDT trial NCT04925206 targeting *BCL11A*, where Cas9 is delivered via mRNA electroporation. Also, the pioneer NCT04819841 trial to correct Sickle *HBB* mutation in SCD HSPCs combining Cas9 RNPs and an AAV6 as HDR donor template also resources on electroporation for the RNP delivery.

Irrespective of which approach is taken and despite being a promising therapeutic option, *ex vivo* gene editing comes with certain limitations, including (1) complex *ex vivo* HSPC manipulations that reduce their fitness (e.g., electroporation and *in vitro* culture), (2) the need of a conditioning regimen prior to infusion of corrected cells to make room in the bone marrow for transplanted HSPCs, (3) requirement of specialized transplantation units, and (4) high costs. As such, it would be hugely beneficial for safety, simplicity, and treatment cost if genetic correction of HSPCs could be achieved *in situ*, without affecting the bone marrow niche (Figure 4). Attempts to deliver editing components *in vivo* in murine HSPCs have been recently made. Li

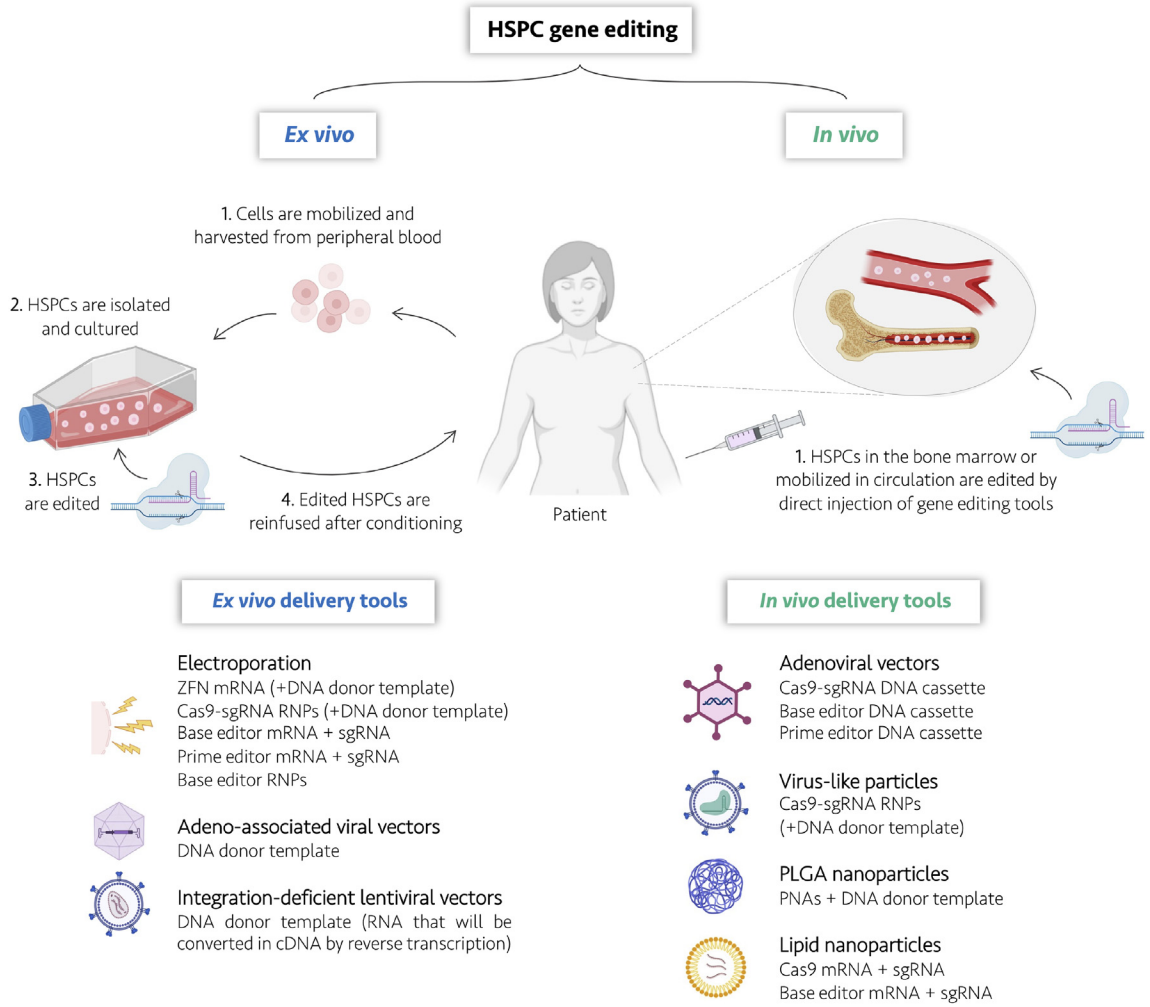

Figure 4. *Ex vivo* and *in vivo* approaches for HSPC gene editing

Ex vivo protocols involve multiple steps: HSPC mobilization, collection via peripheral blood stem cell apheresis, isolation and culture, gene-editing tool delivery, and reinfusion of edited HSPCs into the patient after myeloablative conditioning. In *ex vivo* approaches, gene-editing tools are typically delivered by electroporation as mRNA or RNP. For HDR-based strategies, a DNA donor template is delivered via electroporation (e.g., as ssODN) or viral delivery (using AAVs or Integration-deficient lentiviral vectors). In contrast, *in vivo* strategies simplify the treatment process by directly injecting gene-editing tools intravenously or into the bone, resulting in the editing of HSPCs either mobilized in circulation or *in situ* in the bone marrow. Various delivery tools for *in vivo* approaches are currently under preclinical development, including AdVs, VLPs, and non-viral polymeric (e.g., PLGA) or lipid nanoparticles.

et al. used an AdV with high affinity for CD46 (expressed in HSPCs but also on other cell types) in CD46/Townes mice, achieving over 40% prime editing efficiency in peripheral blood mononuclear cells only after the use of a complex system for selection of edited cells.[164] However, there are concerns related to the immune response to these vectors[165,166] and the use of a chemotherapeutic drug to select edited cells, which might induce secondary malignancies.

Several groups have developed retrovirus-derived VLPs to transiently deliver CRISPR-based RNP complexes to several cell types, including HSPCs.[167] Preliminary data showed up to 30% of editing efficiency in human hematopoietic cells following *in vivo* injection of VLPs in immunodeficient mice pre-engrafted with human HSPCs. However, these VLPs were pseudotyped with the envelope protein of baboon endogenous retrovirus that binds to ASCT receptors expressed on HSPCs but also on other cell types. Polymeric nanoparticles and LNPs represent an appealing alternative delivery system to viral vectors for RNA therapeutics. Attempts to use polymeric nanoparticles to deliver editing reagents (e.g., to correct a β-thalassemic mutation)[113] did not specifically target HSPCs in mice and reached up to 7% gene editing in hematopoietic cells. Conversely, two groups successfully used CD117/c-kit antibody-targeted LNPs encapsulating Cre recombinase mRNA to access HSCs in the adult mouse bone marrow niche, achieving, respectively, over 50% and 90% editing in

long-term HSCs in mouse models for gene editing.[168,169] However, most of the vector was non-specifically targeted to hepatocytes (which uptake LNP with high efficiency), causing liver toxicity. Interestingly, Lian and colleagues recently tested numerous lipid compositions to develop LNP that specifically home to the murine bone marrow. These LNPs targeted different hematopoietic cell types and, when loaded with mRNAs encoding the CRISPR-Cas9 system, achieved 2%–5% gene-editing efficiency in a mouse model of SCD.[170] Finally, different LNP formulations were recently tested in human HSPCs achieving about 40% of editing efficiency in *ex vivo* approaches.[92] Of note, treatment of human HSPCs could elicit a transcriptional response (e.g., in genes involved in cholesterol metabolism that could alter their properties).[92] Thus, these approaches require additional improvements to increase specificity reduce toxicity and improve the editing frequency to attain the desired efficacy and safety standards essential for therapeutics.

Targeting T cells for immunotherapy approaches

Gene editing of T lymphocytes (T cells) plays a crucial role in gene therapy and immunotherapy. By redesigning not only the antigen to which the T cells respond but also the type of response, this field has become highly attractive for combating cancer, autoimmune diseases, and infectious diseases.[171] Researchers mainly focus on two primary sources of T cells for genome editing: peripheral blood T cells (to improve Chimeric Antigen Receptor [CAR] and T cell receptor [TCR]-T cell therapies)[171–173] and tumor-infiltrating T lymphocytes.[174,175] In both cases, *ex vivo* genetic modification is the preferred approach due to the ease of cell collection and their relatively straightforward genetic manipulation, expansion and subsequent reinfusion into patients, making it compatible with clinical practice. *In vivo* approaches are also being pursued due to the advantages explained above; however, their efficiency in T cells remains insufficient, and safety data are still lacking for clinical application.

Ex vivo gene editing of T cells been pursued using almost all editors available, including ZFNs,[176] TALENs,[177] and CRISPR-Cas systems.[178–180] The aim in all cases has been to eliminate certain genes (KO strategy) such as PD1, LAG3, TRAC, CD52, and CD7, or to specifically integrate a DNA cargo (KI strategy) such as CAR or IL15 into a desired locus like TRAC or PD1. The delivery requirements of the two strategies are quite different since knocking out genes requires a relatively straightforward delivery of the specific nucleases in the form of mRNA or RNP, while KI of DNA sequences also requires the delivery of large DNA donors, a more challenging approach frequently resulting in higher cell toxicity and lower editing efficiencies.

KO strategies are the method of choice in the clinic due to their simplicity and efficiency, with over 100 clinical trials ongoing (www.clinicaltrial.org). For therapeutic applications, the delivery of gene-editing tools into the target cells must be short term to avoid undesired side effects. Therefore, traditional integrative systems such as retroviral vectors are of no use, and electroporation of mRNA or RNP represents the method of choice due to its high efficiency and transient expression of the editing components, with editing efficiencies over 80%. RNP electroporation is used more frequently due to the shorter lifespan of nuclease activity and lower toxicity. Also, in some approaches, non-integrative viral vectors (rAdVs and rAAVs) have been used and reached clinical trials.[181–183] However, despite the great success, there is still room for improvement to reduce toxicity and increase efficacy. New non-viral systems are being investigated, such as exosomes,[184,185] nanomaterials,[186–189] and, recently, peptide-assisted genome editing (PAGE)[190] offering new alternatives to improve the results achieved with electroporation.

Similar to HSPCs, for KI strategies, rAAV6 vectors are most frequently used to deliver the donor DNA coupled to electroporation of RNP or RNAs to deliver the specific nucleases, with efficiencies over 70%.[191–193] However, several approaches have been developed using viral vectors[183,194] and non-viral systems[186,188,195] to deliver both elements. In general, the viral-free systems have fewer safety concerns and lower costs, being more suitable for clinical translation.[196]

Targeting hepatocytes for the treatment of liver diseases

The advent of gene-editing technologies has heralded a new era of potentially curative therapies for human inherited diseases in which hepatocytes are the target cell type by correcting the genetic errors *ex vivo* and subsequent re-introduction of the edited hepatocytes into the organism to regenerate the diseased liver. This attractive approach for liver diseases was investigated for a urea-cycle defect, ornithine transcarbamylase (OTC) deficiency, a severe disease affecting the metabolism of ammonia to urea for elimination from the body, which is frequently treated by orthotopic liver transplantation. Primary hepatocytes isolated from an OTC-deficient patient were genetically corrected *ex vivo* by electroporation of RNPs consisting of the Cas9 nuclease and combinations of pairs of gRNAs flanking a newly created aberrant intronic splice site, resulting in its deletion in >60% of cells. Immunocompromised and conditionally hepatocompromised FRGN (Fah(-/-)), Rag2(-/-) and Il2rg(-/-)) mice transplanted with genetically edited patient hepatocytes displayed a repopulated liver, restored urea cycle, normal liver OTC activity, and a significant phenotypic correction of the disease symptoms, as compared with mice transplanted with unedited hepatocytes.[197] However, these results are based on using a mouse strain that facilitates liver repopulation to high levels. In other disease contexts, this approach may not be applicable unless the edited, corrected hepatocytes gain a liver-repopulating advantage, as is the case in hereditary tyrosinemia type I (HTI).[198] HTI is caused by a deficiency in fumarylacetoacetate hydrolase (FAH), the final enzyme in the catabolism of tyrosine. The buildup of toxic metabolites results in high risk of cirrhosis, hepatocellular carcinoma, and liver failure. Concerning *ex vivo* hepatocyte gene-editing approaches for HTI, different studies have reported the application of CRISPR-Cas9, base, and prime editor systems, using various delivery methods: electroporation and lentiviral and AAV vectors, with either single or dual strategies (preferentially using rAAV8, with strong hepatotropism).[198–201] Transplanted corrected hepatocytes successfully repopulated the liver of *Fah*$^{-/-}$ mice, restored metabolic functions, and significantly improved

survival and health markers in the recipient mice. These promising research efforts offer hope for less invasive, more sustainable treatments for this disease and potentially for other liver disorders.

Effective *in vivo* viral and non-viral delivery systems targeting the liver have also been developed; however, challenges remain, as most current versions of CRISPR tools are too large to be packaged into viral vectors such as rAAV. Therefore, innovative approaches such as intein-split gene editors are being used, necessitating dual-rAAV strategies.[202,203] To date, encapsulation of gene editors in LNPs is the predominant delivery tool, owing to their titratability, ease of manufacturing, and capacity to carry Cas, editor- or prime editor-encoding mRNA, and the gRNA. In addition, the liver is composed of different cell types that interact with each other, and thus editors need to be delivered to the appropriate cell type, while modification of non-target tissues must be avoided. Current delivery strategies using rAAV, rrAdV, and LNPs tend to reach hepatocytes, but other cell types, such as cholangiocytes or endothelial cells, with potential pathological roles in liver diseases may not be easily targeted. Another critical issue is that edited cells may be lost over time, as the injured liver is constantly regenerating, unless corrected cells are positively selected, as in the case of HTI.[204] For pediatric diseases, hepatocyte proliferation during development may also dilute out the corrective effect. Therefore, liver genome-editing technologies have focused on targeting diseases where corrected cells have a selective growth advantage or where the correction of only a small percentage of cells can confer therapeutic benefit. Within liver diseases, one area of CRISPR research with great potential is the correction of pathogenic mutations in genes specifically expressed by the liver, as is the case for several inherited metabolic diseases; e.g., phenylketonuria (PKU), urea cycle disorders, or hereditary tyrosinemia. Recent studies have described the use of base editors to correct specific mutations in mouse models of PKU,[202,203,205,206] while, in other cases, prime editing has been the method of choice.[207] Generally, when comparing similar delivery strategies, prime editing correction rates were often lower than those for base editing. In general, intravenous injection of editors (tail vein or retro-orbital) results in efficient delivery to liver hepatocytes, with technically challenging hydrodynamic tail-vein injection (HTVI) being the most effective. Editing efficiencies ranged from 10% to 35% of the hepatocytes in the PKU mouse models used, with a good safety profile and limited off targets. The gene-editing treatment fully and stably normalized blood phenylalanine levels, biomarker of the disease, and reverted the pathological phenotype.[202,203,205–208] Notably, LNP administration of the base or prime editor mRNAs allowed for a more rapid normalization of blood phenylalanine levels, usually within a week, compared to rAAV delivery of intein-split editors, which required several months to reach the therapeutic threshold. Furthermore, the only study that attempted an HDR strategy via dual-rAAV vectors required co-administration of vanillin as an NHEJ inhibitor to achieve a significant reduction in phenylalanine levels.[40]

For OTC deficiency, intravenous infusion of one rAAV expressing Cas9 and another expressing a guide RNA and a wild-type (WT) donor DNA into newborn *Spf*^{ash} mice, a model of the disease, resulted in reversion of the *OTC* mutation in 10% of hepatocytes and increased survival.[209] However, in adult *Spf*^{ash} mice, correction was lower and a high number of more complex and extensive indels that affected *OTC* gene expression were detected, leading to lethal hyperammonemia. This could reflect different NHEJ DNA repair mechanisms in non-dividing adult hepatocytes compared to dividing newborn hepatocytes.[209] In HTI, delivery of plasmid CRISPR-Cas9 and a donor ssDNA corrected the homozygous mutation in the Fah5981SB mouse model through HDR[204] when delivered via HTVI. Transient expression of Cas9, sgRNA, and ssDNA were sufficient to correct the disease phenotype, possibly favored by the strong positive selection and expansion of *Fah*+ hepatocytes in the *Fah* mutant liver, as the editing rate was initially estimated to be ∼1/250 cells.[204] In a subsequent study, the authors used intravenous LNP-mediated delivery of Cas9 mRNA combined with rAAV encoding an sgRNA and a repair template to induce the mutation correction in the same mouse model. The editing efficiency was >6% of hepatocytes at 7 days after injection.[210] Correction of this gene through HDR has also been attempted through delivery of a dual rAdV system to a rat FahΔ10/Δ10 model, resulting in up to 95% *Fah*+ cells at 9 months after treatment owing to their selective enrichment.[211] Prime editors and ABEs have also been shown to efficiently correct the disease mutation and phenotype in *Fah* mutant mice.[212,213] The CRISPR components were delivered via HTVI or tail-vein injection in the form of plasmids or mRNAs encapsulated in LNPs, demonstrating the versatility of CRISPR delivery to the liver. Other well-known examples of liver diseases under study for CRISPR-Cas9 and HDR therapy with viral delivery include alpha-1 antitrypsin deficiency, to correct the most common mutation[212] or hemophilia B.[214] Gene silencing by CRISPR tools has also been used for hepatitis B infection[215] and is being investigated to target oncogenes in hepatocellular carcinoma.[216] A complete update of preclinical liver gene-editing studies is shown in Table 2. Currently, four liver-directed gene-editing therapies are in clinical trial, for familial hypercholesterolemia, OTC deficiency, transthyretin amyloidosis, and hereditary angioedema,[98,217,218] highlighting the rapid development of the field and its potential to treat a variety of liver diseases. Several recent studies described the successful *in vivo* use (in mice and non-human primate models) of CRISPR base editors to effectively reduce low-density lipoprotein (LDL) levels, via targeting of *PCKS9* (Proprotein Convertase Subtilisin/Kexin type 9), a negative regulator of LDL receptor, as a proof of concept of a potential therapeutic strategy for familial hypercholesterolemia. The strategy involved LNP-mediated delivery of adenine base editor mRNA targeting and disrupting a splice site, achieving up to 30% of edited hepatocytes in macaques, concomitant with a reduction of plasma PCKS9 and LDL levels. A GalNAc-based asialoglycoprotein receptor ligand on the LNP surface effectively increased liver editing with minimal editing in nontargeted tissues,[83] representing an optimal strategy for patients lacking sufficient LDL receptor activity, specifically those with familial hypercholesterolemia, as LNPs typically deliver their cargo via LDL-receptor-mediated endocytosis. An ongoing phase I clinical trial sponsored by Verve therapeutics (NCT05398029) will evaluate the base-editing approach designed to disrupt the expression

Table 2. Description of liver-directed gene-editing approaches attempted so far

Disease (model)	Strain	Target genes	Editing system	Delivery system	Reference
PKU (mice)	*PAH* R408W	*PAH* c.1222C>T	v3em PE3-AAV	dual-AAV intein split	Brooks et al.[207]
PKU (mice)	*PAH* R408W	*PAH* c.1222C>T	ABE8.8 SpRY	LNP-mRNA + sgRNA	Brooks et al.[205]
PKU (mice)	*PAH* P281L	*PAH* c.842C>T	ABE8.8 SpCas9	LNP-mRNA + sgRNA	Brooks et al.[205]
PKU (mice)	*Pah*^{enu2}	*PAH* c.835T>C	PE$^{\Delta RnH}$	AdV dual AAV8 intein split + sgRNA	Bock et al.[57]
PKU (mice)	B6. BTBR-*Pah*^{enu2}	*PAH* c.835T>C	miniBE-PLUS SaCasKKH	dual AAV8 (GCN-ScFv)	Zhou et al.[206]
PKU (mice)	*Pah*^{enu2}	*PAH* c.835T>C	SaKKH-CBE3	LNP-mRNA + sgRNA	Villiger et al.[203]
PKU (mice)	*Pah*^{enu2}	*PAH* c.835T>C	SpCas9 +repair template (HDR) + vanillin	dual AAV2/8	Richards et al.[40]
PKU (mice)	*Pah*^{enu2}	*PAH* c.835T>C	nSaKKH-BE3	dual AAV	Villiger et al.[202]
HoFH (mice)	C57BL/6 (WT)	*PCSK9* Q152H	v3em PE3-AAV	dual-AAV intein split	Davis et al.[51]
HoFH (NHP)	*Macaca fascicularis LDLR* KO	*ANGPTL3*	ABE8.8 SpCas9	GalNAc LNP-mRNA + gRNA	Kasiewicz et al.[83]
HoFH (NHP)	*M. fascicularis* (WT)	*PCSK9*	ABE8.8 SpCas9	LNP-mRNA + sgRNA	Musunuru et al.[84]
HoFH (NHP)	*M. fascicularis* (WT)	*PCSK9*	ABEmax	LNP-mRNA + sgRNA	Rothgangl et al.[221]
HoFH (NHP)	C57BL/6 (WT)	*PCSK9*	SpCas9	LNP (5A2-DOT5)-RNP (Cas9+sgRNA)	Wei et al.[78]
HTI (mice)	*Fah*$^{mut/mut}$	*FAH*	PE3	plasmids (HTVI)	Jang et al.[212]
HTI (mice)	*Fah*$^{mut/mut}$	*FAH*	ABE6.3/RA6.3	plasmids (HTVI) LNP-mRNA + sgRNA	Song et al.[213]
HTI (rat)	*Fah*$^{\Delta 10/\Delta 10}$	*FAH*	SpCas9n+ repair template (HDR)	dual AdV	Shao et al.[211]
HTI (mice)	*Fah*$^{mut/mut}$	*FAH*	SpCas9n+ repair template (HDR)	LNP-mRNA (Cas9) AAV2/8 (sgRNA+HDR donor)	Yin et al.[210]
HTI (mice)	*Fah*$^{mut/mut}$	*FAH*	SpCas9n+ repair template (HDR)	plasmids (HTVI)	Yin et al.[210]
Hemophilia B (mice)	*FIX* R333Q	*ROSA26*	SpCas9 + repair template (HDR)	dual AdV	Stephens et al.[214]
Hemophilia B (mice)	*FIX* KO	*FIX*	SaCas9 + repair template (HDR)	dual AAV8	Ohmori et al.[222]
AATD (mice)	PiZ (AAT-Glu348Lys)	*SERPINA1* Glu348Lys	Cas9 + repair template (HDR)	dual AAV8+AAV9	Song et al.[223]
AATD (mice)	PiZ (AAT-Glu348Lys)	*SERPINA1* Glu348Lys	SpCas9	AdV	Bjursell et al.[224]
OTC deficiency (mice)	*Spf*^{ash}	*OTC*	SaCas9 + repair template (HDR)	dual AAV8	Yang et al.[209]
HCC (mice)	BEL7402Luc tumor	*Survivin*	SpCas9	LBP	Qi et al.[216]

PKU, phenylketonuria; HoFH, homozygous familiar hypercholesterolemia; HTI, hereditary tyrosinemia type I; AATD, α-1-antitrypsin deficiency; OTC, ornithine transcarbamylase deficiency; HCC, hepatocellular carcinoma; NHP, non-human primates; LBP, lactose-derived branched cationic biopolymer; HDR, homologous directed repair; RNP, ribonucleoprotein; AdV, adenovirus; AAV, adeno-associated virus; LNP, lipid nanoparticles.

of the *PCSK9* gene in the liver and to lower circulating PCSK9 and LDL in patients with familial hypercholesterolemia, atherosclerotic cardiovascular disease, and uncontrolled hypercholesterolemia. A gene-editing trial for treatment of OTC deficiency, sponsored by iECURE, relies on the intravenous infusion of two rAAVs carrying an ARCUS nuclease targeting the well-characterized *PCSK9* gene locus, and a functional *OTC* gene, respectively (NCT06255782). The cut in the *PCSK9* site serves as the insertion site for the functional *OTC* gene, providing a potential path to its permanent expression. Other similar editing approaches using rAAV vectors to deliver ZNFs and a functional gene for insertion into the albumin locus have also entered the clinical trial phase.[219] Transthyretin amyloidosis is a progressive fatal disease characterized by accumulation in tissues, predominantly the nerves and heart, of amyloid fibrils composed of misfolded transthyretin (TTR) protein. Circulating TTR is produced almost exclusively by the liver, and preclinical *in vitro* and *in vivo* studies have shown durable KO of TTR protein after a single dose of LNP-mediated delivery of an mRNA coding for Cas9 and a gRNA targeting the

TTR gene.[74] Intellia Therapeutics is funding a clinical trial to evaluate this approach as treatment for transthyretin amyloidosis with cardiomyopathy (NCT04601051).[98,220] A similar knockdown approach is being tested for hereditary angioedema, a rare genetic disease that leads to severe, unpredictable, and potentially fatal swelling attacks, primarily in the gastrointestinal tract and cutaneous and submucosal tissues of the body, resulting from the dysregulation of the contact activation pathway. Plasma kallikrein is directly responsible for the production of bradykinin, a peptide that leads to increased vascular permeability and subsequent tissue swelling. Gene editing based on LNP-mediated delivery of mRNA encoding the Cas9 endonuclease and a gRNA targeting the *KLKB1* gene responsible for the production of plasma prekallikrein in the liver has resulted in a one-time treatment to reduce the total plasma kallikrein protein level. A recently launched clinical trial funded by Intellia Therapeutics is validating this approach for the treatment and prevention of angioedema attacks (NCT05120830).[218] In summary, advancements in genome-editing technologies and liver-targeted gene delivery have made gene editing for human liver diseases both feasible and realistically achievable as a therapeutic option in the near future.

Targeting iPSCs and hMSCs for disease modeling and regenerative medicine

After Yamanaka's pioneering work in 2006,[225] demonstrating the ability of four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, to reprogram somatic cells into pluripotent cells (iPSCs), this technology rapidly expanded into the fields of disease modeling and tissue engineering. For human studies, the human iPSCs (hiPSCs) present the advantages of self-renewal and differentiation capacity without the ethical concerns of embryonic-derived material. Additionally, the derivation of hiPSCs from patients has become a powerful tool to overcome the limitations of cancer cell lines and animal models to recapitulate essential features of human disease. Recent biotechnological advances have made available a complete toolkit for genome editing, but some limitations exist for efficient hiPSC modification. When editing involves the introduction of DNA DSBs, gene KOs are easier to generate than gene insertions or point mutations. Important bottlenecks in this process include low transfection and transduction efficiencies; low survival under stress conditions such as drug selection, flow sorting, and single-cell culture; and less HDR activity compared to the competing NHEJ pathway. Some successful strategies to overcome these problems include the use of Rho-kinase inhibitor to increase single-cell survival,[226] RNA interference to downregulate the NHEJ pathway, limiting the apoptosis mechanism via antiapoptotic miRNA-21,[227] and the combination of pro-survival molecules with inhibition of the p53 pathway.[228] Besides DSBs, the development of epigenome editing in hiPSCs has prompted the generation of platforms for functional genomic studies in combination with libraries of gRNA.[229] Regarding the more advanced tools, base editors can modify hiPSCs more efficiently than HDR-based methods,[230] and prime editing has been shown to generate heterozygous point mutations in hiPSCs more efficiently than other GE methodologies.[147] The delivery of the gene-editing machinery in the form of plasmid DNA, mRNA, or RNP into hiPSCs has been achieved via electroporation,[231] nucleofection,[232] viral vectors,[233,234] and non-viral systems.[110] Plasmids are useful for delivering large gene-editing tools, as is the case with prime-editing components.[147] Viruses have a limited cargo capacity but smaller-sized nucleases have been successfully used in iPSCs to overcome this limitation.[235] There are also new developments regarding non-viral delivery systems; some examples are EVs engineered with viral envelopes, used to assay a therapeutic exon-skipping technology,[110] or a hexane dithiol-conjugated polyethyleneimine system capable of co-delivering the gene-editing platform in a plasmidic form for gene correction in a Fabry disease model.[236] Disease modeling, regenerative medicine, and functional genomics are the main fields of application of gene editing in iPSCs. The creation of isogenic cell lines with gene editing have provided the needed controls for the intrinsic variability of human samples, making the information gathered from patient-derived models much more powerful and uncovering the molecular mechanisms of many genetic diseases, including neurodegenerative, cardiac, and neuromuscular diseases.[237–239] To apply this strategy to complex polygenic traits, multiple gene targets have been modified in parallel to get insight into diseases such as colorectal cancer[240] or Parkinson's.[241] For colorectal cancer, CRISPR-Cas9 was delivered by electroporation into dissociated human intestinal organoids to introduce mutations in key tumor suppressors and oncogenes through both NHEJ and HDR, achieving high efficiency via selective growth of edited clones. In Parkinson's disease, CRISPR-Cas9 targeted non-coding regulatory elements within the *SNCA* gene in pluripotent stem cells, using electroporation and generating isogenic lines with either risk or protective enhancer variants. Edited cells were differentiated into neural precursors and neurons to assess allele-specific effects on *SNCA* expression, providing a controlled model to study genetic risk in neurodegeneration. Another application of gene editing in hiPSCs is the preclinical proof of concept of the efficacy of a CRISPR therapy, such as for the correction of DMD[242] and of retinal degeneration.[243] For DMD, CRISPR-Cas9 delivered via nucleofection targeted a large deletion in the *DMD* gene through NHEJ, achieving functional dystrophin restoration in patient-derived myotubes and engrafted muscle in NSG-mdx mice. In retinal degeneration, plasmid-based CRISPR-Cas9 with HDR corrected a *MERTK* frameshift mutation in hiPSCs from a retinitis pigmentosa patient, leading to full functional restoration in retinal pigment epithelium (RPE) cells. As a tool for regenerative medicine, antigen-presenting molecules have been modified in hiPSCs to obtain a cellular product with a reduced risk of immune rejection.[244] In this study, CRISPR-Cas9 was used to knock out human leukocyte antigen (HLA) class I and II components (B2M and CIITA) in hiPSCs via lentiviral delivery, efficiently generating clones lacking these immunogenic markers. Edited hiPSCs, differentiated into cardiomyocytes, formed spheroids that maintained normal electrophysiological properties while significantly reducing T cell activation in coculture assays, thus presenting a viable model for universal donor cells in allogeneic transplantation.

Human MSCs (hMSCs) have proved to be effective in autologous and allogenic cell therapy.[245] The clinical safety of approved hMSCs-based

therapies, their diverse therapeutic mechanisms of action, and the establishment of efficient protocols for their genetic modification *in vitro* and *in vivo* hold promises for the treatment of a variety of genetic or acquired diseases such as cancer and cardiovascular, lung, liver, kidney, gastrointestinal, and neurodegenerative diseases.[246] hMSCs have been successfully genetically modified using ZFNs, TALENs, and CRISPR with their different variants.[247] The ZFN technology was the first one to be used for gene editing in hMSCs.[248] Park et al. demonstrated that engineered hMSCs integrating anti-inflammatory or angiogenic factors significantly alleviated kidney dysfunction.[249] Additionally, hMSCs were genetically modified using TALENs to express the stromal cell-derived factor-1 (SDF-1) to investigate angiogenic potency in a hindlimb ischemia model,[250] or IL10 and CXCR4 to promote tube formation and anti-inflammatory potential,[251] as well as granulocyte chemotactic protein-2 (GCP-2) and stromal-derived factor-1alpha (SDF-1alpha) to assess their therapeutic potential in the context of experimental ischemia.[252] However, most research outcomes on hMSCs are reported using the CRISPR-Cas9 system, with delivery often via non-viral methods such as electroporation and nucleofection. Electroporation has been shown to be effective in hMSCs; KO of the *PPARG* gene by introducing the CRISPR-Cas9 system through electroporation in adipose hMSCs showed over 90% efficiency. Schary et al. recently developed and optimized an improved electroporation protocol for delivering hMSCs from the heart and epicardial fat of patients with ischemic heart disease, achieving a 68% success rate.[253] An alternative approach involves using exosome-liposome hybrid nanoparticles to deliver the CRISPR-Cas9 system to hMSCs through endocytosis.[254] rAAV-based platforms have also been used in hMSCs; for instance, Srifa et al. developed a Cas9-rAAV6-based gene-editing platform capable of integrating up to 3.2 kb of exogenous DNA into the genome of human umbilical cord blood MSCs while preserving their phenotypic characteristics.[255] The *SIRT1* gene was successfully inserted into the *AAVS1* "safe-harbor" locus in amniotic MSCs via HDR using TALENs. The transfection efficiency was less than 10% of GFP-positive cells; however, it reached 99% with puromycin and fluorescence-activated cell sorting (FACS) isolation of knocked-in cells. Edited cells were confirmed to significantly express the *SIRT1* mRNA through qPCR.[256]

Targeting muscle cells to treat neuromuscular disorders

Neuromuscular diseases (NMDs) are a heterogeneous group of rare inherited conditions characterized by muscle weakness. There are more than 600 disease-causative genes associated with NMDs. DMD and spinal muscular atrophy (SMA) are two of the most prevalent and most widely investigated NMDs, and pioneer treatments have been developed and approved for these two degenerative conditions. Splice-switching antisense oligonucleotides were approved and commercialized for the treatment of DMD and SMA between 2017 and 2018,[257,258] and a gene therapy to deliver the *SMN* gene to SMA patients was approved in 2019 in the US and 2020 in Europe.[259] In addition, several gene therapy clinical trials are underway for DMD, X-linked myotubular myopathy (XMTM), and other muscular dystrophies.[260,261] Despite these advancements, there are still many challenges to overcome in this field. On one hand, RNA-based therapies require continuous administration. On the other hand, AAV-mediated gene therapy (referring to gene addition) does not correct the underlying genetic defect and the exact duration of the effect of transgene expression is not known. If required, repeated administration would be hampered by immune response against the viral vectors commonly used for delivery. Gene-editing technologies represent a more versatile and permanent correction for a wide range of genetic defects associated with NMDs, including large deletions, dominant mutations, and triplet repeat expansions. This is why there is a wealth of studies on cell models (mainly human iPSC) and animal models (murine but also dogs and other large animals) of NMDs.[262] Different gene-editing approaches (standard Cas9 nucleases, base editors, and PEs) have been investigated in the context of different NMDs, including DMD and SMA,[262–264] but also others such as myotonic dystrophy (DM1),[265] limb-girdle muscular dystrophy,[266] and congenital muscular dystrophy.[267] However, translating the results of those *in vitro* studies to the clinic remains challenging. In most cases, muscle diseases affect several muscle groups in the body, and thus the first challenge is to efficiently and safely distribute the gene-editing components to all the affected tissues. Furthermore, there is a variety of cell types that require genetic correction, depending on the precise etiology of the NMD of interest. This would include cardiomyocytes, as well as muscle fibers when the heart is affected, the muscle stem cell within its specialized niche, or interstitial cells within skeletal muscle.

Viral vectors (mostly rAAVs) and synthetic nanoparticles (e.g., lipid, polymeric, and inorganic) are the two main classes of delivery systems being investigated for NMDs, although other options, such as EVs and VLPs, are also being explored.[8,268] While viral vectors and nanoparticles each offer distinct advantages and drawbacks, both share the primary limitation of achieving site-specific delivery to target organs and tissues beyond the liver and kidneys. For skeletal muscle delivery in particular, AAV vectors are currently the most promising option due to their low integration rate, high transgene expression, muscle tropism, promising results in large animal models, and the approval of existing rAAV-based therapies by regulatory agencies. However, as discussed above, the cargo capacity of AAVs is below the required size to pack most editors and thus only systems such as *trans*-splicing intein double-vector systems or smaller proteins can be delivered. Moreover, immunity to common AAV serotypes requires immunosuppression and limits repeated administration. Conversely, nanoparticles have a much larger cargo capacity than AAVs and generally show a better safety profile with reduced immunogenicity.[78] The safety concerns and lack of specificity for the target organ of natural AAV capsids have driven research to evolve novel capsids that specifically target skeletal muscle while de-targeting the liver.[269,270] To harness the full potential of LNPs as delivery vectors, there is a need for a better understanding of the mechanisms that control their homing and internalization in skeletal muscle and its component cell types. For example, homing of nanoparticles to skeletal muscle is likely enhanced by certain biochemical cues such as those found in the regenerating muscle.[271] Similar to other non-liver target organs,

the application of *in vivo* gene editing to treat muscle diseases in humans is still in its early infancy. A search in the clinical trials NCBI database (clinicaltrials.gov) using the terms "neuromuscular disease" and "gene editing" retrieves two results for DMD. One of them is a single-patient study carried out in the US with fatal consequences. In this case, a rAAV9 containing dSaCas was used to activate the expression of cortical dystrophin via epigenome editing in a 27-year-old DMD patient. Acute respiratory distress syndrome 6 days after transgene treatment occurred and the patient died 2 days later.[272] The second one is a first-in-human study (NCT06392724) of a drug (GEN6050X) consisting of base editors delivered with a dual-rAAV9 vector system to modulate exon 50 skipping in the *DMD* gene in three ambulatory patients with DMD. There is also an ongoing phase I/II clinical trial (NCT05588401) using autologous cell transplantation of muscle stem cells for limb-girdle muscular dystrophy (GenPHSats) edited using ABEs to correct a splicing exonic point mutation in exon 2 of the *SCGA* gene (https://crisprmedicinenews.com/). Overall, bridging the gap between preclinical research and clinical research of potential gene-editing therapeutics for muscle diseases requires a better understanding and validation of the safety of gene editing at a global level. This includes analyzing genome-wide off-target effects at non-predicted sites and understanding how these may impact gene expression and cell function. Additionally, addressing pre-existing immunity to Cas proteins and delivery vectors, as well as developing optimized delivery tools for skeletal and cardiac muscle that can selectively target specific cell types, is crucial for achieving more precise and efficient gene editing.

Targeting the brain to tackle neurological disorders

Gene editing holds immense promise for treating neurological disorders by disrupting targeted genes, introducing functional ones, or editing specific alleles responsible for neurological disorders. However, as with traditional gene replacement strategies, efficient delivery methods are crucial for the successful application of gene-editing technologies in the context of neurological disorders. The central nervous system (CNS) presents unique challenges to treatment development due to the BBB, which acts as a highly selective barrier between the bloodstream and the CNS. This protective mechanism prevents harmful substances from reaching the brain but also interferes with the systemic delivery of therapeutic agents.

Traditional gene therapy delivery is based on vectors, such as viral vectors, that cannot cross the BBB after systemic administration. As a consequence, invasive procedures such as intrathecal or intracerebral injection are needed for direct delivery into the CNS.[273] rAAVs are the most widely used viral vectors *in vivo*, with AAV9 being the most used serotype for delivery due to its CNS tropism.[274] However, most natural AAV serotypes have limitations to cross the BBB. Researchers are actively developing strategies to overcome these limitations by introducing modifications to AAV capsids to increase their BBB permeability and target specific CNS areas and cell types. Most of the efforts are directed toward the development of engineering rAAV libraries specific for the CNS, such as the Cre recombination-based rAAV targeted evolution library (CREATE),[275] which identified the highly efficient PHP capsid variants. However, the evolved capsids are usually selected in mice and show strain and species specificity, which limits their translation into humans. With this in mind, Sabeti's group applied the directed evolution of AAV capsids leveraging *in vivo* expression of transgene RNA (DELIVER[269]) strategy to non-human primates and identified a family of capsids with increased CNS tropism in macaques following systemic administration.[276] A promising brand-new study based on this approach has recently described an engineered AAV capsid that binds the human transferrin receptor (TfR1) to efficiently cross the human BBB, resulting in 40–50 times greater reporter expression in the CNS of human *TFRC* KI mice with high CNS-specific tropism.[277] These directed-evolution approaches can be complemented using structure-guided rational design strategies to enhance CNS delivery, transduction efficiency, and the ability to evade pre-existing humoral immunity.[278]

There are several examples of approaches targeting genes involved in neurological diseases *in vivo* using CRISPR-Cas9 technology. Most studies use dual-rAAV systems to fit the Cas9 nuclease and the sgRNA expressing genes. In Alzheimer's disease, CRISPR-Cas9 was used to disrupt the APPswe (Swedish) mutation in the amyloid precursor protein (*APP*) gene, which causes dominantly inherited Alzheimer's disease (AD). Fibroblasts from human APPswe carriers were transfected with *S. pyogenes* Cas9-2A-GFP and gRNAs targeting APP mutant allele. As measured by ELISA, conditioned media of targeted patient-derived fibroblasts displayed around 60% reduction in secreted β-amyloid (Aβ). The decrease on pathogenic Aβ was also observed in Tg2576 transgenic mice that were co-transduced unilaterally in hippocampus with rAAV9-Cas9 and rAAV9-gRNA 2 months post surgery.[279] In Huntington's disease (HD), both allele- and non-allele-specific approaches have been evaluated to knock out the *HTT* gene. In one study, a non-allele-specific CRISPR-Cas9-mediated gene-editing approach was used to permanently suppress endogenous HTT expression in the striatum of HD140Q-KI mice using two separate AAV vectors encoding Cas9 nuclease and two gRNAs targeting mHTT upstream and downstream of the CAG repeat in exon 1. The treatment effectively depleted HTT aggregates, attenuated early neuropathology, and alleviated HD-associated motor deficits and neurological symptoms for 3 months. In the same year, another group reported a reduction in the expression of mutant HTT expression to 40% using CRISPR-selective editing of the mutant allele *in vitro* and *in vivo* via intracerebral rAAV delivery of CRISPR-Cas9 components.[280,281] Other diseases in which CRISPR-Cas9-mediated gene targeting has been evaluated as a therapeutic approach *in vivo* include amyotrophic lateral sclerosis (ALS),[282,283] Parkinson's disease (PD),[284] DMD,[285] and fragile X syndrome.[126]

More recently, other genome-editing strategies such as base editing or transcriptome editing with CRISPR-Cas13 system have been evaluated *in vivo*.[46,264,286–289] In 2020, Lim et al. reported base editing in the G93A-SOD1 mouse model of ALS after intrathecal injection of dual-rrAAV particles encoding a split-intein cytidine base editor. Despite deep-sequencing analysis of the bulk tissue only revealing

that ~1.2% of *SOD1* reads have been edited, the authors consider an "effective editing rate" of ~20% in the dual-transduced cells. The strategy lowered mutant SOD1 *in vivo* and reduced the rate of muscle atrophy, improved neuromuscular function, and reduced SOD1 immunoreactive inclusions by up to 40%, providing a therapeutic benefit in the adult mouse model.[286] More recently, the group of David Liu reported an 87% base-editing efficiency of the *SMN2* gene after intracerebroventricular (ICV) injection of a dual-rAAV9 vector encoding a split-intein base editor and a gRNA in a neonate mouse model of SMA, rescuing the disease pathology phenotype and extending lifespan.[264] In the near future, the use of single-rAAV base-editing systems, as described by the same group, may simplify the development of base-editor therapies in clinical settings.[46] Regarding transcriptome editing, the group of Thomas Gaj has achieved *in vivo* silencing of HTT, SOD1,[287] and more recently of ATXN2 mRNAs using both intravenous and CNS-directed injections of single rAAV1 or rAAV9 particles expressing the RfxCas13d nuclease and gRNAs.[288] However, the use of CRISPR-Cas13 *in vivo* remains controversial due to the reported collateral activity of Cas13 nuclease, which can induce lethality in mice.[274] To address this, recent studies have been employing high-fidelity CRISPR-Cas13 nucleases. In a study performed in a mouse model of Angelman syndrome (AS), paternal expression of *Ube3a* was restored by targeting an antisense transcript that selectively silences the *Ube3a* paternal allele. Delivery of CRISPR-Cas13 system directly in the brain using AAV-PHP.eb particles alleviated AS-related symptoms, including obesity and motor dysfunction.[289]

In addition to AAV, the field is actively exploring alternative methods for brain-targeted gene therapy. Non-viral vectors, such as nanoparticles, liposomes, and EVs, offer low immunogenicity, good biocompatibility, and low toxicity but often suffer from lower transfection efficiency and the need for invasive methods to deliver them to the brain. Researchers developed the CRISPR-Gold non-viral delivery vehicle to deliver CRISPR-Cas9 ribonucleoprotein to efficiently edit all major cell types in the brain, including neurons, astrocytes, and microglia. Interestingly, exaggerated repetitive behaviors shown in *Fmr1* KO mice were specifically rescued by disrupting the metabotropic glutamate receptor 5 (*mGluR5*) gene, which efficiently reduces local mGluR5 mRNA levels by 40%–50% in the striatum after intracranial injection.[126] Another study has evaluated CRISPR-Cas9 nanocapsules to deliver single Cas9/sgRNA complexes within a glutathione-sensitive polymer shell to treat glioblastoma. The authors achieved a dual-action ligand that facilitates BBB penetration, tumor cell targeting, and Cas9/sgRNA selective release that led to high (up to 38%) *PLK1* gene-editing efficiency in a brain tumor with negligible (less than 0.5%) off-target gene editing in high-risk tissue.[290]

As one of the emerging frontiers in the field of gene-therapy delivery, researchers are also using physical methods to temporarily disrupt the BBB. Ultrasound and focused ultrasound disruption (FUS) are emerging non-invasive techniques that use focused sound waves to create temporary openings in the BBB,[291] potentially allowing the passage of gene therapy vectors delivered peripherally without the risks associated with surgery. A recent study in non-human primates tested low-intensity FUS for delivery of rAAV9 to regions of the brain involved in PD. The results showed that the procedure was well tolerated, generally without abnormal magnetic resonance imaging signals, and resulted in successful gene delivery to the desired brain regions. Similar results were safely reproduced in three patients with PD, where the BBB opening was followed by ^{18}F-choline uptake in the putamen and midbrain regions, demonstrating that the less invasive nature of this methodology could facilitate focal viral vector delivery for gene therapy and allows early and repeated interventions to treat neurodegenerative disease.[292] Despite significant progress, several challenges remain. Precise targeting of specific brain regions and control of gene expression levels are critical aspects that require further refinement. In addition, the long-term safety and cross-species translatability of the results from mouse models to non-human primates and humans must be carefully assessed. Recent advances in rAAV engineering, the exploration of novel lipid-derived vehicles and nanoparticles, and FUS offer a promising future for gene therapy in brain disorders. As these technologies mature and clinical trials progress, we may be on the cusp of a new era in neurological treatment, offering life-changing therapies to patients with currently untreatable conditions.

Gene editing strategies for retinal and cardiac diseases

AAVs are the preferred vectors for delivering gene-editing tools to various organs, including the retina, pancreas, and heart. However, efficiently targeting cells within these organs poses unique challenges due to their complex structures. Different AAV serotypes are selected based on their suitability for specific organs: rAAV9 and rAAV2 vectors are commonly used for the retina due to their effectiveness in transducing retinal cells; rAAV8 mediates efficient delivery to pancreatic tissue; while for cardiac applications, rAAV1, rAAV6, rAAV8, and rAAV9 are preferred due to their strong tropism for heart cells, facilitating effective gene transfer.

Retina. AAV9 and 2 serotypes have been widely used in the treatment of several inherited retinal disorders (IRDs) through gene-editing approaches. These disorders can result from mutations in over 200 different genes, with the most prevalent types often caused by mutations in genes crucial for the visual cycle or retinal maintenance. Given that autosomal recessive inheritance is the most common pattern observed in IRDs, gene editing has long been considered a promising therapeutic approach for these patients, as correcting just one of the two affected loci could provide significant benefits. In preclinical studies, CRISPR-Cas9 has been used to correct mutations in various IRD genes, such as *Rho* and *Rpgr*, in animal models.[293] For instance, correcting the P23H mutation in the *Rho* gene, a common cause of autosomal dominant retinitis pigmentosa, significantly preserved photoreceptor cells and improved visual function in rats. This was achieved using CRISPR-Cas9 delivered via rAAV2/8, targeting rod photoreceptors through subretinal injection. The editing efficiency ranged from 5.97% in homozygous P23H rats to 14.8% in heterozygous models, with no off-target effects detected. Treated eyes demonstrated sustained photoreceptor preservation,

higher electroretinography (ERG) amplitudes, and improved visual acuity, maintained for up to 15 months.[294] Similarly, CRISPR-Cas9 targeting the *Rs1* gene in X-linked retinoschisis has shown functional improvement in retinal cells. Using 3D retinal organoids derived from patient-specific hiPSCs, CRISPR-Cas9 was delivered via plasmid transfection for HDR and base editing, achieving correction efficiencies of over 50% with minimal off-target effects. Gene correction restored RS1 protein secretion, resolved the retinal splitting phenotype, normalized ciliary marker expression, and improved the structural integrity of the photoreceptor outer segments.[295] Current research in gene-editing therapeutics for the retina focuses on improving efficacy, safety, and delivery of treatments. Early studies with conventional CRISPR-Cas9 highlighted its potential but also revealed limitations such as off-target activity, low efficacy in correcting point mutations, and DSB-related cytotoxicity.[296,297] Advanced technologies such as prime and base editing offer significant advantages, including the ability to correct point mutations without causing DSBs. For example, base editing has been successfully employed to correct a point mutation in the *Rpe65* gene, restoring retinal function in a mouse model of Leber congenital amaurosis (LCA). Using subretinal delivery via lentivirus and a dual-rAAV strategy, ABEs achieved up to 82% correction efficiency at the target site, with 40% of transcripts precisely edited. Treated rd12 mice demonstrated significant restoration of cone survival and function, with corrected opsin localization and upregulation of cone-specific phototransduction genes.[298] The first-in-human application of CRISPR-Cas9 for IRDs has shown promising results, particularly in LCA caused by *CEP290* mutations. The BRILLIANCE clinical trial (using EDIT-101) demonstrated that subretinal injections of a rAAV5 vector delivering CRISPR-Cas9 components effectively targeted photoreceptor cells, restoring normal splicing and improving retinal function. Participants showed biological activity with up to a 1.3 logMAR improvement in best-corrected visual acuity (BCVA), enhanced full-field stimulus testing sensitivity, and improved visual navigation scores (https://classic.clinicaltrials.gov/ct2/show/NCT03872479).[299]

Heart. Cardiovascular diseases are the leading cause of death worldwide.[300,301] Among these diseases are cardiomyopathies, or genetic heart conditions, which affect up to 1 in 250 individuals.[302] Over the past 40 years, the underlying genetic causes of various cardiomyopathies have been revealed, opening opportunities for treatments based on gene therapy and/or gene-editing approaches. Despite initial safety issues associated with the delivery of gene therapies in cardiac diseases, the development of AAV vectors has helped advance their clinical potential. The development of AAV vectors has overcome some of the initial safety issues associated with the delivery of gene therapies in general and gene-editing approaches in particular in cardiac diseases. The natural tropism of certain AAV serotypes such as AAV1, 6, 8, and 9 to the heart can be harnessed for cardiac therapies.[303–306] For instance, the H530R mutation in the *Prkag2* gene, which causes an autosomal dominant form of the Wolff-Parkinson-White syndrome, was targeted using CRISPR-Cas in a mouse model of the disease. A single systemic injection of rAAV9-Cas9/sgRNA at postnatal day 4 or day 42 substantially restored the morphology and function of the heart in H530R Prkag2 transgenic and KI mice. Treatment with this rAAV9-Cas9/sgRNA led to great cardiac improvement with significant decreases in left ventricular wall thickness and a ∼70% reduction in myocardial glycogen, despite only a 20% reduction in mRNA.[307] Another example is catecholaminergic polymorphic ventricular tachycardia caused by *Ryr2* mutations, corrected in mice via a single rAAV9-SaCas9 injection. The disease-causing R176Q allele was disrupted through frameshift deletions caused by the SaCas9 with no detected off-target mutations. This editing resulted in phenotype rescue, with none of the R176Q/+ treated mice developing arrhythmias when electrically stimulated, and a ∼30% decrease in total *Ryr2* mRNA.[308] Dual base editors (SpRY-ABE8e) were used in another study, and were delivered as split inteins via rAAV9 to correct *Mybpc3* nonsense mutations in *Mybpc*$^{R946X/R946X}$ mice. Six months after administration, the editing efficiency of a 1×10^{14} vg/kg per AAV dose was ∼30% in cardiomyocytes, and 78%–110% of MYBPC3 protein expression was restored. This was sufficient to prevent cardiac hypertrophy and restored normal heart function with minimal off-target effects. Systolic function, chamber dilation, and wall thickness were improved and retained over the course of 6 months in *Mybpc*$^{R946X/R946X}$ mice.[309] Furthermore, another significant study identified an adenine base editor and single-guide RNA system that efficiently corrected the dominant-negative c.1208G>A (p.R403Q) pathogenic variant in β-myosin (*MYH7*), a common variant leading to hypertrophic cardiomyopathy (HCM), with minimal bystander and off-target editing.[310] This study showed that delivering base-editing components rescued pathological manifestations of HCM in iPSC-derived cardiomyocytes and in a humanized mouse model, demonstrating the potential of base editing to treat inherited cardiac diseases. In the iPSC-derived cardiomyocytes, 98% of on target editing was achieved, whereas, in the *Myh6*$^{h403/+}$ humanized mouse model, ∼33% editing of the pathogenic adenine was reached. Significantly, in this mouse model, treatment with base editors reduced HCM symptoms, resulting in levels of cardiac wall thickness, heart weight, collagen area, and echocardiograph readings that were comparable to WT mice. The author used rAAV9 for delivery into mice; however, as the full-length base editor (approximately 5.4 kb) exceeds the packaging limit of a single rAAV9, the authors used *trans*-splicing inteins to reconstitute the full-length base editor in cells upon protein expression. Similarly, another study assessed two different genetic therapies—an adenine base editor (ABE8e) and a potent Cas9 nuclease delivered by rAAV9—to prevent disease in mice carrying a heterozygous HCM pathogenic variant. One dose of dual-rAAV9 vectors, each carrying one-half of RNA-guided ABE8e, corrected the pathogenic variant in over 70% of ventricular cardiomyocytes and maintained durable, normal cardiac structure and function.[311] Despite these advances, targeting genetic therapies to the heart still faces significant challenges. Previously, intracoronary and intramyocardial injections have been used to administer therapies; however, these methods are highly invasive. The intravenous route is less invasive but often leads to a wide biodistribution throughout the body and thus unwanted off-target effects, as even highly cardiotropic AAVs are largely taken up by the liver. Consequently, it is important to consider optimizing affinity to the heart through strategies such as identification of specific receptors

present in the organ[269] or reducing liver targeting. Moreover, the limited packaging capacity of rAAVs, their immunogenicity, and potential off-target effects have pushed the field to explore non-viral delivery systems to deliver gene editors in the form of mRNA and protein.[312–314]

Conclusions

To conclude, this review addresses a primary goal of the Delivery Strategies Working Group 3 within the COST GenE-HumDi network: evaluating *ex vivo* and *in vivo* delivery systems to determine the most effective method for each cell type and gene-editing technology. While viral delivery systems are well established, they have drawbacks such as immunogenicity and limited payload capacity. These challenges have spurred the development of innovative non-viral delivery technologies, including LNPs and EVs, which show great promise. Initial gene-editing efforts have predominantly targeted primary cells, such as T cells, HSPCs, and iPSCs, due to their therapeutic potential and ease of manipulation *ex vivo*. Although organs such as the liver have demonstrated significant progress in gene-editing applications, more complex organs such as the eye, the brain, or the pancreas present greater challenges for *in vivo* targeting and still remain under development. Further research is essential to refine these gene-editing techniques, particularly *in vivo*. This includes exploring and optimizing vector particle-cell interactions, addressing the immunogenicity of gene-editing tools, and assessing their potential effects on genome integrity, all of which require ongoing and iterative research and development efforts.

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