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Targeted nonviral delivery of genome editors in vivo

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Cell-type-specific in vivo delivery of genome editing molecules is the next breakthrough that will drive biological discovery and transform the field of cell and gene therapy. Here, we discuss recent advances in the delivery of CRISPR-Cas genome editors either as preassembled ribonucleoproteins or encoded in mRNA. Both strategies avoid pitfalls of viral vector-mediated delivery and offer advantages including transient editor lifetime and potentially streamlined manufacturing capability that are already proving valuable for clinical use. We review current applications and future opportunities of these emerging delivery approaches that could make genome editing more efficacious and accessible in the future.

genome editing | CRISPR-Cas | in vivo delivery | nonviral delivery | targeted delivery

RNA-guided CRISPR-Cas enzymes are widely deployed for genome editing because of their efficacy, flexibility, and ease of use [reviewed elsewhere (1, 2)]. While CRISPR proteins like Cas9 have already shown promise in clinical trials, the reality of creating permanent changes to the human genome means that safety is of paramount importance. At the genomic level, the specificity of Cas9 has been optimized through methods to predict off-target sites (3, 4) and molecular engineering to yield high-fidelity proteins (5). However, a crucial development that will enhance both the utility and safety of genome editing is the ability to deliver CRISPR-Cas genome editing machinery exclusively to desired cell types, tissues, or organs within a patient. For many genetic diseases, only a subset of cells or a specific organ shows phenotypic signs of disease and thus would be the intended target for genome editing. Genome editing of unintended cells or organs could increase the risk of inadvertent treatment outcomes, in addition to increasing manufacturing costs due to higher dose requirements. At present, targeted delivery of CRISPR-Cas genome editors remains an important unmet need for the successful clinical translation of genome editing.

Viral vectors, devoid of their native genome and replicative capacity, are an attractive delivery strategy for gene therapies and, more recently, CRISPR-Cas genome editing [reviewed elsewhere (6)]. The most widely utilized viral vectors are retroviruses and adeno-associated virus (AAV) (7, 8). Lentiviral vectors, a subtype of retroviruses, express large transgenes (~10 kb) following genomic integration, while AAVs express a smaller transgene (~4.7 kb) from a long-lived episome; both viral vectors are capable of transducing dividing and nondividing cells. Advances in pseudotyping lentiviral vectors (9), engineering of new AAV tropisms (10), and use of tissue-specific promoters have enabled cell-specific delivery with these technologies. However, viral delivery also introduces safety concerns. Immunogenicity toward the viral vector as well as to the overexpressed transgene product may undermine therapeutic efficacy (11–13). Long-term expression of CRISPR-Cas nucleases from integrated lentiviral or episomal AAV transgenes also increases the risk of unintended off-target genomic edits. Furthermore, while primarily nonintegrating, AAVs have been found to insert into sites of DNA double-strand breaks (DSBs) including those induced by Cas9 cleavage (14, 15). Viral integration, whether at a high or low level from lentivirus or AAV, respectively, raises serious concerns over disruption of genomic integrity. Notably, a retroviral gene therapy resulted in four out of nine patients developing leukemia, which was attributed to insertional oncogenesis (16, 17).

Transient delivery of CRISPR-Cas genome editors is a promising strategy to address safety concerns related to toxicity, off-target editing, and genomic integrity. Researchers and clinicians alike have increasingly utilized both CRISPR-Cas ribonucleoproteins (RNPs), where the Cas protein is precomplexed with its single guide RNA (sgRNA), and mRNA-encoded Cas nuclease coadministered with a separate sgRNA. Both of these approaches result in transient cellular delivery and minimize the risk of insertional oncogenesis. However, unlike naturally evolved cell-specific viral tropisms, CRISPR-Cas RNPs and mRNAs lack inherent cell targeting and cell entry capabilities, necessitating significant engineering. Here, we review emerging strategies for the targeted delivery of CRISPR-Cas genome editors, with a specific focus on delivering transient

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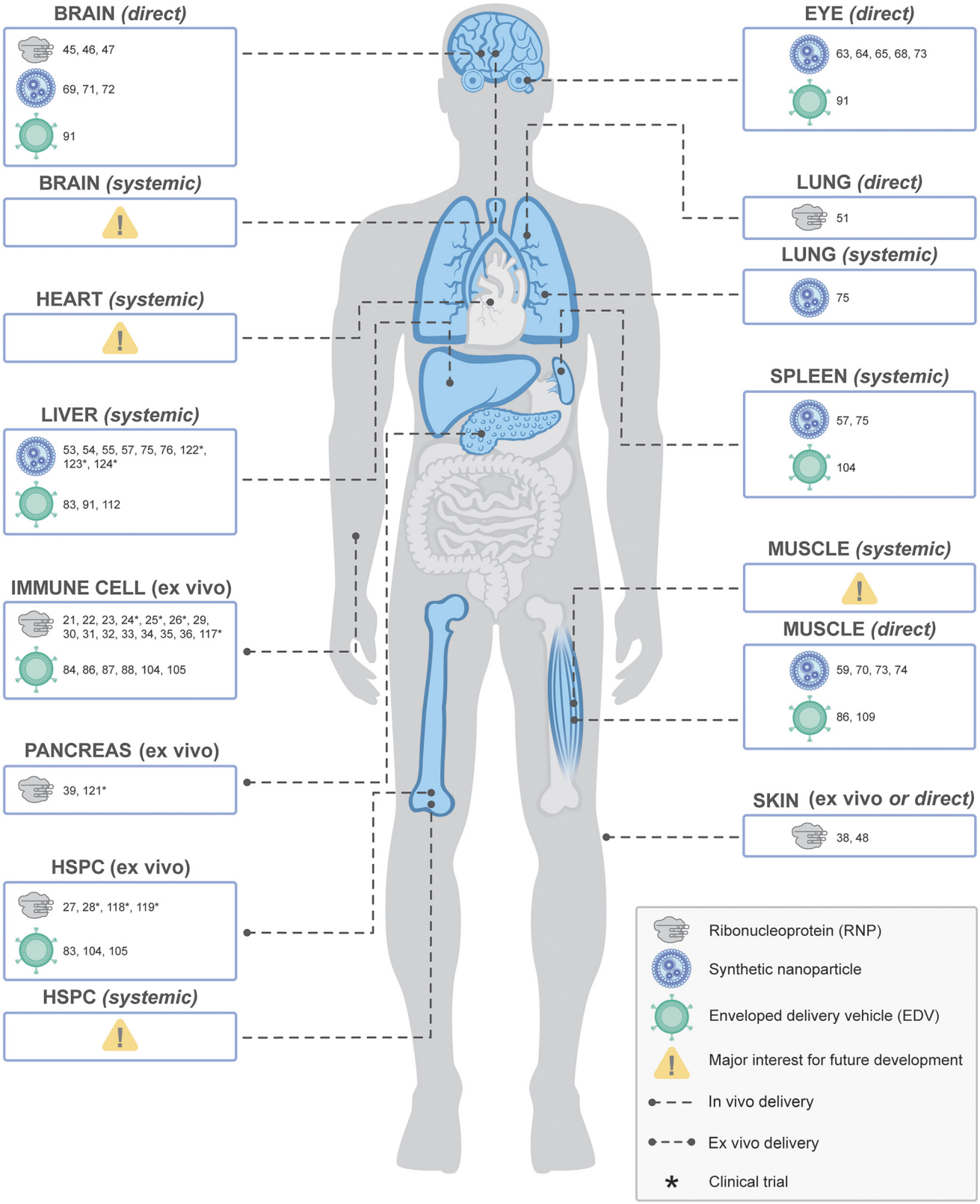

Fig. 1. Preclinical and clinical delivery methods for nonviral CRISPR-Cas genome editing. The delivery of CRISPR-Cas RNP and mRNA has been a major focus of the field. Techniques such as ex vivo delivery to T cells and HSPCs, ex vivo delivery to iPSC-derived pancreatic cells, and systemic delivery to the liver have already reached the clinic (highlighted with asterisks). While continued research efforts have developed limited delivery methods for tissues such as the lung and spleen, systemic delivery methods to the brain, heart, HSPCs, and muscle (highlighted with exclamation marks) remain a major unmet need and are poised to become the focus of future work. This image utilized assets from freepik.com.

RNP or mRNA, and discuss future developments that will be required to enable broad clinical translation (Fig. 1).

Targeted Delivery of CRISPR-Cas Ribonucleoproteins. Over the past 40 y, protein therapeutics have exploded in use, with proteins such as human growth hormone for hormone deficiency and recombinant viral protein-based vaccines becoming standards of care (18). Similarly, the use of CRISPR proteins has become increasingly popular as a therapeutic. Unlike genetically encoded proteins and sgRNAs, Cas9 RNPs exist transiently within a cell, with most degradation occurring within 24 h (19). This transient existence minimizes off-target editing and immunogenicity while retaining high genome editing efficacy. In addition, the production of recombinant Cas9 in *Escherichia coli* is significantly cheaper than the production of viral vectors encoding Cas9, which requires costly and laborious mammalian or insect cell culture (20). While Cas9 RNPs offer a number of advantages for therapeutic genome editing, a major barrier to their successful translation is targeted delivery to cell types and organs of interest.

Physical Isolation of Cells for Ex Vivo Targeted Delivery with Ribonucleoproteins. One approach for cell-type-specific delivery involves physically isolating the target cells for ex vivo, or outside of the body, genome editing. Hematopoietic cells are among the most readily isolatable cell types in the human body; both lymphoid and myeloid cells can be isolated from peripheral blood through centrifugation or surface marker-based cell sorting. T cells have been extensively engineered to reprogram their antigen specificities to combat cancer and autoimmune diseases (21). Due to the inefficiency and toxicity of plasmid DNA transfection into T cells, researchers have largely employed Cas9 RNPs for genome editing (22, 23). Electroporation of Cas9 RNPs, where high voltage temporarily increases cellular membrane permeability, has demonstrated efficient gene knockout and gene insertion, including in numerous immuno-oncology clinical trials (24–27). Hematopoietic stem and progenitor cells (HSPCs) are also a major target for genome editing in patients with various hematologic diseases. As with T cells, Cas9 RNP electroporation into HSPCs has proven to be highly efficacious; however, HSPC isolation is more involved and requires chemical mobilization from the bone marrow niche (28). In a landmark study, patients with sickle cell disease (SCD) or transfusion-dependent beta-thalassemia (TDT), which are both caused by mutations in the beta-globin gene, had their HSPCs isolated, treated ex vivo with Cas9 RNPs, and reinfused (29). Recently, additional immune cells such as myeloid cells, natural killer cells, and B cells have gained interest as targets for genome editing to enhance tumor targeting or enable therapeutic protein production. Physical isolation of each of these immune cell types and electroporation of Cas9 RNPs has proven to be a robust method for genome editing (30–35).

While electroporation of Cas9 RNPs results in high levels of genome editing, electroporation is also associated with significant cytotoxicity in primary immune cells (24). To avoid this limitation, nonelectroporation-based methods have been paired with ex vivo immune cell culture. An amphiphilic peptide fusion combining the cell penetrating ability of HIV-1 Tat with the endosomal escape capability of influenza HA2 also mediated genome editing of T cells, B cells, NK cells, and HSPCs with Cas9, Cas12a, or a Cas9-linked adenine base editor (36, 37). Notably, peptide-mediated genome editing resulted in improved cell viability, increased cell yield, and fewer changes in gene expression compared to electroporation.

Ex vivo genome editing has also become a staple of regenerative medicine, which aims to replace or regenerate specific tissues. Induced pluripotent stem cells (iPSCs) have become a pillar of regenerative medicine due to their ability to self-renew and differentiate into several different cell types. Early research demonstrating the efficacy of Cas9 RNP electroporation in iPSCs (38) laid the groundwork for novel approaches harnessing genome-edited iPSC-derived keratinocytes and fibroblasts to alleviate wounds in mice with a genetic skin disorder (39). Additionally, genome-edited iPSC-derived pancreatic islet cells have restored insulin production in models of type 1 diabetes (T1D) (40).

Physical isolation of cells prior to genome editing virtually eliminates the possibility of targeting unintended cell types, making it the most specific delivery modality. An additional advantage to ex vivo genome editing of hematopoietic cells or stem cells is that both can be expanded in culture, generating a larger therapeutic dose prior to reintroduction into the patient. Ex vivo manipulation also allows for monitoring of both phenotypic and genotypic cell quality. This may be valuable to detect and prevent off-target editing of unintended sites in the genome (41), as well as chromosomal abnormalities like translocations (25) or chromosome loss at the intended target site (42, 43). While physical isolation is a powerful method for Cas9 RNP delivery, its major limitation is its inability to translate to other tissues. Without the ability to safely isolate and replace most tissues in patients, ex vivo genome editing is limited in scope to hematologic diseases or those that can be addressed by stem cell engraftment.

Targeted In Vivo Genome Editing via Direct Delivery of Ribonucleoproteins. Unlike ex vivo genome editing, in vivo genome editing offers the advantage of not requiring the equipment or labor necessary to maintain the cell product in culture. Moreover, in vivo delivery holds the potential to target any cell type, tissue, or organ in the human body, thereby circumventing the limitations associated with physical isolation and ex vivo genome editing. However, the critical challenge for in vivo delivery is reconciling the potential to target any tissue with the necessity for cell-type specificity. When the desired genome editing target is one cell type or tissue, indiscriminate cell targeting is a major challenge to be addressed. Direct injection into the target tissue is one method for specific in vivo delivery, resulting in editing efficiency correlated to the distance from the injection site.

The brain is a major focus for therapeutic genome editing, especially for neurodegenerative diseases which currently have limited treatment options (44). Targeting the brain via systemic intravascular delivery is an incredibly difficult challenge because of the blood–brain barrier (BBB), which tightly regulates what can traffic from the bloodstream into the brain (45). To bypass this barrier, several efforts have demonstrated the feasibility of direct injections of genome editing molecules into the brain. Direct in vivo injection into the murine hippocampus, striatum, and cortex showed genome editing with Cas9 RNPs fused to six SV40 nuclear localization

sequences (NLSs), as well as with Cas12a RNPs conjugated to a neuron axonal import peptide (46, 47). Interestingly, both of these methods selectively targeted neurons over glial cells, but only within a radius of several hundred micrometers from the injection site. This biodistribution can be enhanced by conjugating polyethylene glycol (PEG) to Cas9 via a reduction-cleavable linker. PEG conjugation increased the genome-edited area by 3.7-fold after direct injection into the striatum (48).

The skin and solid tumors are also easily accessible targets for direct in vivo injection. Intradermal injection of Cas9 RNPs followed by electroporation of the injection site in postnatal mice yielded editing in the basal and suprabasal layers of the epidermis (49). Genome editing ameliorated blistering within a mouse model of dystrophic epidermolysis bullosa, which was attributed to editing of skin stem cells, since editing was observable over four months. Similarly, Cas9 RNPs fused to a cell-penetrating peptide showed genome editing activity after intratumoral injection in a mouse xenograft model (50). To enhance the tumor specificity, a matrix metalloproteinase 2 (MMP-2) sensitive linker has been employed between Cas9 and a cell-penetrating peptide, taking advantage of the up-regulated secretion of MMP-2 by cancerous cells (51).

Noninjection-based methods for direct delivery of Cas9 RNPs have also been explored. Most notably, cell-penetrating peptides have been complexed with both Cas9 and Cas12a RNPs and delivered intranasally (52). This strategy yielded significant editing of ciliated and nonciliated epithelial cells in both the small and large airways of mice.

Targeted Delivery of Synthetic Nanoparticles. Synthetic nanoparticles shield genome editing machinery from the body's inherent clearance mechanisms while also enabling cytosolic delivery of the RNA or RNP. Though multiple nanoparticle formulations have been developed, lipid nanoparticles (LNPs) are by far the most commonly used. Typically consisting of a combination of phospholipids, ionizable cationic lipids, PEG lipids, and cholesterol, LNPs rely on endocytosis for initial cell uptake [reviewed elsewhere (53, 54)], after which the ionizable lipid can disrupt the endosomal membrane, leading to cytosolic release of the cargo (55). LNPs have been utilized for the in vivo delivery of small molecule compounds for nearly three decades, but the first RNA-packaging LNP formulation to be approved by the U.S. Food and Drug Administration (FDA) was Onpattro in 2018, which was also the first approved small interfering RNA (siRNA) therapeutic.

A major advantage of LNPs with regard to genome editing is their improved capacity to package longer nucleic acid sequences compared to traditional AAV vectors. LNPs encapsulating mRNA and sgRNA for Cas9-based adenine (56, 57) and cytidine (58) base editors have already been shown to generate therapeutic levels of editing in the livers of mice and nonhuman primates. This supports dual mRNA and sgRNA-packaging LNPs as an efficient way to deliver cargos even larger than classical *Streptococcus pyogenes* Cas9, a capability that is growing in importance as the genome editing toolkit continues to expand to include Cas9 fusion proteins with broad functions including base, prime, and epigenome editing. Alternatively, LNPs may be used to deliver precomplexed RNPs to overcome size limitations while preventing overexpression of the editing machinery. Careful consideration must be made to efficiently encapsulate the protein component while preserving its structural integrity, and optimal conditions may vary depending on the cargo.

Passive In Vivo Targeting via Systemic Administration of Nanoparticles. When administered intravenously, most LNPs passively accumulate in the liver, with specific uptake by hepatocytes believed to be mediated by the accumulation of a protein corona rich in apolipoprotein E (ApoE) (59). ApoE then binds the low-density lipoprotein receptor (LDL-R), entering the cell upon LDL-R endocytosis. This inherent organ targeting has been taken advantage of by multiple groups for hepatocyte genome editing. Notably, clinical trials focused on editing the liver are ongoing for LNPs copackaging mRNA encoding Cas9 and an sgRNA. Passive targeting is not limited to LNPs, however, and other foreign particles will also accumulate in tissues like the liver and spleen. Following systemic tail vein injection in mice, untargeted gold nanoparticles decorated with Cas9 RNPs demonstrated 5 to 2,000 times higher accumulation in the liver and spleen compared to other organs such as the brain, lung, heart, intestine, and kidney (60).

Duchenne muscular dystrophy (DMD) is a degenerative neuromuscular disorder caused by mutations in the dystrophin gene that abolish functional protein expression. Multiple clinical trials have been performed using AAV as a gene-replacement vehicle, but this delivery method is believed to be limited by persistence of the transgene as well as immune responses to the viral vector that prohibit repeated administrations. In one important case, intravenous administration of AAV packaging an epigenome editor resulted in severe acute respiratory distress syndrome that unfortunately proved fatal (61). The pulmonary toxicity is believed to have resulted from an innate immune response to the AAV capsid itself, as opposed to the cargo, accentuating the need for alternative delivery methods. Genome editing offers a permanent alternative to gene replacement, though efficient delivery of the editing machinery throughout the skeletal muscles is a challenge. Cas9 mRNA-packaging LNPs were able to induce editing in mouse limb muscles after an intravenous injection in combination with a tourniquet to localize the area of effect within one limb, restoring functional gene expression throughout the leg, while direct injection was effective only for the targeted muscle (62). In addition, LNP delivery circumvented the immunogenicity of viral vectors, allowing for repeat dosing which was ineffective with AAV.

Solid tumors have also been passively targeted for genome editing by systemic delivery. Many passive targeting approaches rely on the enhanced permeability and retention (EPR) effect, which causes macromolecules to accumulate within the tumor microenvironment after systemic injection (63). PEG-coated nanoparticles and gold nanorods containing Cas9 RNP have exploited this phenomenon, resulting in preferential accumulation in the tumor, even over the liver and spleen (64, 65). In both cases, tumor-specific targeting was further engineered into the delivery vehicle by including acid-degradable or hypoxia-responsive linkers, which bias Cas9 RNP release and genome editing in the acidic and hypoxic environment of the tumor.

Targeted In Vivo Delivery via Direct Injection of Nanoparticles. Like RNPs, LNPs can also be injected directly into the target tissue to concentrate editing at a localized site. This approach is commonly utilized for tissues that are difficult to access through intravenous administration, such as immunoprivileged tissues like the eyes and the central nervous system. Though the problem of viral vector immunogenicity is believed to be less of an issue in these organs, LNPs with mRNA or RNP cargo offer a more transient expression of the genome editing machinery relative to a DNA vector.

Direct injection of mRNA or RNPs encapsulated by the commercial transfection reagent Lipofectamine has been employed in mice for editing both the retina (66–68) and the inner ear (69), but the cytotoxicity of transfection reagents will likely prevent the advancement of such a delivery vehicle beyond mouse models. Efforts to develop traditional LNPs that efficiently deliver to the neural retina are ongoing (70), but thus far, delivery of CRISPR-Cas genome editing machinery has been limited to the retinal pigment epithelium (RPE), with only limited editing of photoreceptor cells (71). Direct intrastromal injection of Cas9 RNP-packaging LNPs has also demonstrated efficient editing of the mouse cornea (72). Intratracheal administration of Cas9 mRNA-packaging LNPs has shown promise in the editing of murine lungs, and the delivery of these particles by nebulization, as has been done for other formulations, could greatly improve the ease of administration (73–75). Additionally, LNPs have even been injected directly into the brains of mice, with efficient editing observed near the injection site (76).

Non-lipid-based nanoparticles have also been demonstrated to deliver CRISPR-Cas machinery. CRISPR-Gold is composed of a gold nanoparticle with successive layers of DNA oligonucleotides, Cas9 or Cas12a RNPs, and a cationic endosomolytic polymer (77). When injected directly into the dente gyrus, hippocampus, or striatum, CRISPR-Gold facilitated genome editing of neurons, glia, and astrocytes up to 1 to 2 mm away from the injection site (78). This therapy was sufficient to alleviate multiple behavioral phenotypes in a mouse model of fragile X syndrome. Beyond indel generation, CRISPR-Gold can also mediate homology-directed repair (HDR) in order to correct mutations or insert transgenes of interest. Copackaging Cas9 RNPs and an HDR template, CRISPR-Gold has also been injected intramuscularly to produce gene correction without detectable off-target editing or immunogenicity (77). Nanoparticles encapsulating Cas9 RNPs have also shown effective editing of the brain after direct in vivo injection. Nanocomplexes, composed of an amphiphilic peptide encapsulating Cas9 RNPs, mediated genome editing of post-mitotic neurons, reduced amyloid beta plaques, and improved cognitive function in two different mouse models of Alzheimer's disease (79).

Among the more unique synthetic nanoparticles described to date are nanocapsules and DNA nanoclews. The former is a heterogenous formulation including acrylate-PEG molecules with and without a conjugated targeting ligand, an imidazole-containing monomer to enable endosomal escape, and a glutathione (GSH)-sensitive crosslinking agent to covalently bind the components until cytosolic GSH can degrade the linkages and release the cargo (80). Subretinal injection of these nanocapsules facilitated genome editing in the RPE and was further enhanced by incorporating all-trans retinoic acid (ATRA), which interacts with the interphotoreceptor retinoid-binding protein on RPE (80). These nanocapsules, along with porous silica nanoparticles loaded with Cas9 RNPs (81), efficiently generated indels after intramuscular injection into the tibialis anterior of mice. DNA nanoclews, on the other hand, consist of a repeated sequence of single-stranded DNA (ssDNA) containing partial complementarity to the sgRNA of the packaged RNP. After complexation with the RNP cargo, the nanoclew is coated with polyethylenimine (PEI) to enhance cellular uptake and endosomal escape. Intratumoral injection of these DNA nanoclews in tumor-bearing mice resulted in genome editing, although specific and efficient delivery to healthy tissues has yet to be demonstrated.

Active In Vivo Targeting via Systemic Administration of Nanoparticles. Optimization of LNP formulation is an important consideration when delivering protein cargo. However, LNP composition has consequences beyond packaging efficiency. By adding entirely new components, alteration of the stoichiometry of the classical components, or both, intravenously administered LNPs can be redirected from the liver to accumulate specifically in either the lungs or spleen, likely due to alteration of the protein corona content (59). In this selective organ targeting (SORT) system, the addition of the permanently cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) changed the target organ of one LNP formulation from the liver to the lungs in a concentration-dependent fashion in mice—the higher the percentage of DOTAP in the LNP, the more specific the localization to the lungs. Similarly, the inclusion of a negatively charged component in another LNP formulation led to LNP variants that were efficiently and specifically taken up by the spleen. Encapsulation of Cas9 mRNA with sgRNA by these LNP formulations successfully edited the targeted organs with minimal editing in nontarget tissues (82). Further research into LNP formulations may enable the delivery of genome editing machinery to an even wider array of tissues.

A clinical trial is currently ongoing using LNPs packaging an adenine base editor for the treatment of heterozygous familial hypercholesterolemia (HeFH), a primary cause of which is the decreased expression or function of LDL-R. As a result, patients with homozygous familial hypercholesterolemia (HoFH) are not amenable to passive hepatocyte targeting by traditional LNPs, which rely heavily on ApoE binding to LDL-R. To circumvent this, PEG lipids were conjugated with N-acetylgalactosamine (GalNAc) to generate LNPs that bind the asialoglycoprotein receptor, most highly expressed on hepatocytes. Using a custom HoFH nonhuman primate model, GalNAc-LNPs demonstrated efficient editing of the liver independent of LDL-R expression (83). Though inherent LDL-R targeting is not abolished in GalNAc-LNPs, this method suggests that a conjugation approach may be extended to target other tissues.

Indeed, the overexpression of surface molecules on cancerous cells has also been exploited for active targeting of genome editors. Nanoparticles encapsulating Cas9 RNPs were decorated with hyaluronic acid, which binds the membrane protein CD44 that is overexpressed in certain melanoma, colorectal, breast, and lung cancers (84). Editing following tail vein injection of these targeted Cas9 RNP nanoparticles led to reduction in tumor volume in both xenograft

and lung metastasis models. An approach combining molecular targeting and localized release has also been employed using a near-infrared (NIR)-responsive PEG nanoparticle encapsulating Cas9 RNPs and decorated with a tumor-homing peptide (85). After intravenous injection, NIR was applied to the tumor site to locally release Cas9 RNPs, resulting in decreased tumor volume and prolonged survival. This multifaceted method for targeting displayed highly selective genome editing of the tumor over any other organ.

Other retargeted LNP strategies have demonstrated efficient mRNA delivery in vivo, primarily through the incorporation of an antibody or antibody fragment into the formulation. Though these have not yet been applied to CRISPR-Cas genome editing, the concept of targeting tissue-specific antigens using biomolecules appears to be a promising route of improving nanoparticle specificity (86, 87). Notably, one synthetic nanoparticle utilized conjugated angiopep-2 to penetrate the BBB in a mouse model of glioblastoma, preferentially targeting the tumor following administration by tail vein injection (88). This result is an impressive example of minimally invasive targeted delivery to one of the most challenging organs in the body.

Targeted Delivery of Biologically Inspired Nanoparticles. The evolving field of molecular delivery is increasingly turning toward strategies inspired by biological structures, with particles derived from cellular membranes becoming a popular strategy for the targeted delivery of molecular cargo. This broad class of emerging delivery vehicles, which we term enveloped delivery vehicles (EDVs), encompasses strategies that rely on wrapping a cellular membrane fragment around a molecular cargo of interest. EDVs include enveloped virus-like particles, extracellular vesicles, and biomimetic nanoparticles (such as mechanically extruded cellular membrane fragments). Similar to an enveloped virus, a cellular membrane cloak protects packaged cargo from nucleases, proteases, and the immune system. While still at the preclinical stage of development, EDV-mediated delivery is a promising approach for the cell-type-specific delivery of genome editor complexes, both ex vivo and in vivo.

Virus-like particles (VLPs) mimic the structures of viruses but are replication-incompetent, capitalizing on the efficiency of viral delivery to link programmable target cell binding and cellular entry to the transient delivery of molecular cargo of interest (protein, RNP, and RNA). Retroviral VLPs have been engineered for the delivery of genome editing zinc finger and tale-effector nucleases (89), Cas9 RNPs (90–96), adenine base editor RNPs (97, 98), and Cas9-encoding mRNA (99). Analogous to the production process for traditional retroviral vectors, VLPs are generated through the transient plasmid transfection of producer cells to express components triggering particle budding, molecular cargo packaging, and the display of extracellular molecules that enable VLP target cell binding and entry (85, 100). These extracellular molecules are a requirement for VLP-directed genome editing, as "bald" particles are incapable of mediating delivery (95). The fact that the VLP-displayed targeting molecules define the cellular populations susceptible to genome editing is a key advantage of VLP-mediated delivery.

Extracellular vesicles (EVs) are lipid bilayer-derived particles naturally released by cells both in culture and in vivo (101, 102). Biologically, mammalian EVs have a unique dual functionality: they both trigger cell signaling events by interacting with target cells and serve as carriers for intercellular trafficking of proteins, nucleic acids, and lipids. The ability of EVs to transverse long distances in vivo (103), along with their ability to cross the BBB (104, 105), makes EVs attractive candidates for cell-targeted delivery vehicles. Loading of EVs with molecular cargo, such as genome editing machinery (either during EV biogenesis or after isolation and purification) leverages EV's inherent functionality for targeted cargo delivery (106–108). While a native mechanism for targeted EV delivery exists, EVs can be additionally functionalized for enhanced cell targeting and cell entry activity by displaying targeting and fusogen molecules, similar to VLPs (109, 110).

Physical Isolation of Cells for Ex Vivo Targeted Delivery with VLPs. VLP delivery systems have demonstrated potential in mediating genome editing in therapeutically relevant cell types when cultured in isolation ex vivo. Most engineered VLPs described to date leverage particles pseudotyped with the VSV-G glycoprotein, which uses the broadly expressed LDL-R as a receptor. Broadly transducing VLPs have demonstrated genome editing activity in primary human cells, including T cells (91, 93–95, 111, 112), B cells (112), iPSCs (90, 93), organoids (113), fibroblasts (89, 98), and CD34+ HSPCs (111, 112).

Employing viral glycoproteins with known tropisms to pseudotype VLPs is a promising strategy for directing genome editor delivery to selective cell types. Building on previous work retargeting retroviral vectors for gene therapy applications (114), multiple groups have now harnessed non-VSV-G-pseudotyped VLPs to deliver genome editors more effectively to specific cell types cultured ex vivo (90, 95, 98, 111, 112). For example, we have pseudotyped VLPs with the HIV-1 envelope glycoprotein—responsible for HIV-1's tropism for CD4+ T cells—to deliver Cas9 RNP exclusively to human CD4+ T cells within a mixed cell population ex vivo (95). Similarly, VLP "nanoblades" display the baboon retroviral envelope glycoprotein, BaEV-G (115), which circumvents the challenge of low VSV-G receptor expression on human HSPCs and B cells (90, 112, 113).

Targeted In Vivo Genome Editing via Direct Delivery of EDVs. Engineered VLPs and EVs have both demonstrated the ability to deliver CRISPR-Cas9 genome editors when locally administered in vivo. As direct delivery abrogates the need for inherent organ or cell-specific EDV targeting, these approaches have so far utilized broadly transducing pseudotypes. Direct VLP delivery of adenine base editors have been employed in subretinal injections of the eye, improving visual function, and through cerebroventricular brain injections of neonatal mice (98). Additionally, VLPs and EVs have both been used for delivering Cas9 RNPs through intramuscular injections (93, 116). Cas9-loaded EVs tested in a murine model of DMD resulted in 19% of the treated muscle fibers having the intended modification, allowing dystrophin expression in muscle fibers (116). While this is a promising approach for the treatment of DMD, achieving therapeutically meaningful levels of genome editing in muscle fibers will likely require a muscle-tropic targeting approach for delivering genome editing

reagents systemically, such as the recently functionalized muscle-specific fusogens Myomaker and Myomerger (117).

Targeted In Vivo Delivery via Systemic Administration of EDVs. A programmable delivery vehicle capable of efficiently sending genome editing machinery to specific cells or organs following systemic administration is the holy grail for in vivo genome engineering applications. So far, VLPs have demonstrated genome editing in the murine liver following intravenous injection (90, 98). Two groups utilized either broadly transducing VSV-G pseudotyped (98) or BaEV-G + VSV-G pseudotyped VLPs (90). This approach is logical, given that VSV-G's receptor is highly expressed by liver hepatocytes. Banskota et al. achieved 63% liver editing of *PCSK9* via an adenine base editor, with the highest amount of bystander genome editing being detected in the spleen (4.3%) (98).

Interestingly, the in vivo trafficking and biological characteristics of EVs are defined by their cell type of origin—even after Cas9 RNP loading, EVs produced from tumor cells preferentially accumulate in cognate tumors in mice (118), while EVs produced from hepatic cells accumulate in the liver in vivo (119). Cas9-packaged EVs can further be programmed through the display of targeting molecules in EV-producer cells. Xu et al. demonstrated that displaying an anti-CD19 chimeric antigen receptor biased EV biodistribution toward CD19-expressing Raji tumors, with ~10% increase in preferential tumor accumulation compared to nontargeted EVs (120).

One promising approach for the cell-type-specific delivery of genome editors in vivo is the VLP display of targeting molecules alongside a mutant form of VSV-G that maintains cell fusion activity but is impaired for native LDL-R binding (121–123). We recently demonstrated that codisplaying antibody-based targeting molecules alongside "VSVGmut" is a strategy for programming VLP-mediated genome editor delivery using antibody-antigen interactions (111). We leveraged this approach to develop targeting strategies for human T cells and applied this approach for the generation of genome-edited CAR T cells in humanized mice. While the codelivery of both Cas9 RNP alongside a lentiviral encoded CAR is relatively inefficient (~1.7% of all in vivo generated CAR-expressing T cells were also gene edited), this approach sets the groundwork for rational cell-specific delivery of genome editors, both ex vivo and in vivo (111).

Conclusions and Future Directions

CRISPR-Cas genome editing reached patients less than a decade after its emergence as a programmable nuclease system and continues to show remarkable success in multiple clinical trials. Initial clinical trials utilized physical isolation of the target cells and ex vivo genome editing, which avoided the challenge of targeted in vivo delivery. By electroporation of Cas9 RNP, clinicians reprogrammed antigen specificity and/or prevented premature exhaustion in isolated patient T cells. Infusion of these genome-edited T cells into patients with advanced cancers did not trigger anti-Cas9 immune responses, showed low off-target editing, and showed overall safety (25–27). Similarly, clinical trials targeting HSPCs from patients with SCD or TDT via electroporation of Cas9 RNP have exhibited exceptional clinical success. Along with no evidence of off-target editing or minimal severe adverse events, the genome editing therapy dramatically reduced vaso-occlusive crises and the need for disease-related transfusions (29, 124). This milestone therapy has benefited at least 83 patients to date (125), became the first regulatory-approved application of CRISPR-Cas9 globally when it was approved in the United Kingdom (126) and shortly after became the first FDA-approved application of CRISPR-Cas9 genome editing in the United States (127). More recently, another clinical trial employed ex vivo Cas9 RNP genome editing, dosing the first patients with immune-evasive, stem cell–derived pancreatic islet cells for the treatment of T1D (128).

While in vivo delivery of CRISPR-Cas genome editors poses a significantly greater challenge compared to ex vivo delivery, multiple clinical trials have successfully targeted the liver in vivo. LNPs encapsulating Cas9 mRNA and an sgRNA were intravenously infused into 72 patients to date with transthyretin amyloidosis. Upon passive targeting of the liver, editing of the *TTR* gene resulted in no detectable off-target editing or severe adverse events, but significant reduction of pathogenic misfolded transthyretin (129, 130). Additionally, 10 patients to date have been treated with a similar liver-targeting LNP formulation to treat the genetic swelling disease hereditary angioedema, which thus far has proven to be tolerable and clinically efficacious (130). As previously mentioned, patients with HeFH have begun to be dosed with an LNP encapsulating Cas9 base editor mRNA and sgRNA, with an aim toward editing *PCSK9* and lowering LDL cholesterol in the blood and atherosclerotic cardiovascular disease risk (131). Once again aimed at targeting the liver after systemic injection, preclinical studies in nonhuman primates demonstrated that editing was almost exclusively contained to the liver, with no detectable off-target genomic base editing (57).

Thus far, nearly all CRISPR-Cas therapies in clinical trials have utilized Cas9 RNP or mRNA, a reflection of the increased safety profile of these transient technologies. While cell-type and organ-level specificity have been achieved in these clinical trials, they have relied on physical isolation of hematopoietic cells or passive LNP targeting of the liver. In order to translate these promising genome editing therapies beyond hematologic or liver diseases, novel technologies for targeted delivery are crucial. The ideal CRISPR-Cas delivery technology is transient in nature, highly cell-type-specific in vivo, and administered via minimally invasive systemic injection. Even for hematopoietic cells, where robust ex vivo editing has proven clinically achievable, an in vivo genome editing strategy would be preferential in order to reduce the cost and changes in cellular phenotype associated with ex vivo cell culture.

While Cas9 RNP strategies have enabled efficient ex vivo and direct delivery editing, their inability to target specific cells severely limits their potential for in vivo systemic delivery. Initial engineering efforts to endow cell targeting capabilities have involved fusion of Cas9 to a liver-specific ligand (132, 133) or a HER2 specific antibody fragment (134). While both improved target cell uptake and editing in cell culture, their in vivo targeting ability remains untested. Furthermore, the presence of preexisting adaptive immune responses to the Cas9 protein raises concerns for systemic Cas9 RNP delivery, as common Cas9 orthologs are derived from human

pathogenic and commensal bacteria (12, 13). Overcoming these challenges will necessitate extensive engineering of the Cas9 protein, likely impeding the translation of unencapsulated Cas9 RNP beyond ex vivo or direct delivery genome editing applications.

Encapsulating Cas9 RNP or mRNA within a nanoparticle could overcome challenges associated with immunogenicity and intracellular delivery. While proven for efficient delivery, gold nanoparticles have shown variable toxicity after accumulation in different organs (135). Alternatively, LNPs have been extensively developed for several decades, and the COVID-19 pandemic expedited their clinical translation. The rapid development, FDA approval, and widespread deployment of multiple LNP-based mRNA vaccines have unequivocally demonstrated the safety, efficacy, and scalability of this delivery strategy (136, 137). The successful production and administration of billions of doses serve as a compelling precedent that an LNP-based mRNA genome editor can be safe, commercially viable, and broadly accessible (138). The major challenge for further translating LNPs will be targeting beyond intramuscular injection or systemic delivery to the liver. Defined chemical LNP formulations provide scaffolding for which further engineering will be required for the addition of targeting moieties. This bottom–up engineering approach may be greatly assisted by high-throughput screening, where thousands of unique formulations can be tested both in vitro and in vivo to assess cell-type or organ specificity (139, 140).

Unlike the synthetic formulation of nanoparticles, EDVs represent a biological method to deliver CRISPR-Cas genome editors, relying on naturally evolved abilities to bind, enter, and deliver cargo to targeted cells. Decorating EDVs with single-chain variable fragment has enabled further targeting of numerous tissue-specific surface molecules. Building upon a large field of antibody engineering, this platform holds exciting potential to target a vast diversity of cell types in vivo. The major challenge for translating EDVs will be production at large scale. The cost of mammalian cell culture and purification is significantly higher than that from microbial sources or synthetic production, which may limit broad accessibility of genome editing therapies with these delivery modalities. Viral gene therapies, which are similarly produced from mammalian cell culture, are some of the most expensive drugs to date (20). Improving the EDV yield from cell culture, producing biomimetic delivery vehicles from engineered cellular membranes (141–144), or constructing EDVs from synthetic building blocks would marry the targeting efficacy of these technologies with cheaper and more high-throughput production methods.

Membrane-bound ligand targeting represents the foundation for the next generation of in vivo delivery. The field of immuno-oncology has witnessed remarkable progress in refining and expanding the specificities of engineered immune cells through logic-gated activation (145–148), but current delivery vehicles do not possess the same dynamic responsiveness as an engineered cell product. To mitigate potential genotoxicity, there is a pressing need for delivery systems with enhanced precision. Recently, our lab has demonstrated that multiplexing targeting molecules on EDVs vastly improves the specificity of targeted delivery, potentially by mimicking natural intercellular signaling events. Truly logic-gated protein delivery mechanisms have yet to be developed, but the task of genome editing provides a compelling impetus for further exploration.

Early clinical successes with CRISPR-Cas genome editing has generated hope for patients with hematologic and hepatic diseases. However, in order for this technology to reach its full potential and provide cures for diseases of other organs, improved cell-type-specific delivery vehicles for in vivo therapies are critical. The development of engineered LNPs and EDVs holds tremendous promise for targeted delivery to unlock the full potential of CRISPR-Cas genome editing.

Data, Materials, and Software Availability. There are no data underlying this work.

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