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Exploring the potential of cell-derived vesicles for transient delivery of gene editing payloads

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Abstract

Gene editing technologies have the potential to correct genetic disorders by modifying, inserting, or deleting specific DNA sequences or genes, paving the way for a new class of genetic therapies. While gene editing tools continue to be improved to increase their precision and efficiency, the

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Declarations of interest:

B.P.K. is an inventor on patents or patent applications filed by MGB that describe genome engineering technologies. B.P.K. is a consultant for EcoR1 capital and Novartis Venture Fund, and is on the scientific advisory boards of Acrigen Biosciences, Life Edit Therapeutics, and Prime Medicine. B.P.K. has a financial interest in Prime Medicine, Inc., a company developing therapeutic CRISPR-Cas technologies for gene editing. B.P.K.'s interests were reviewed and are managed by MGH and MGB in accordance with their conflict-of-interest policies. L.P.A. laboratory has received funding from Bioblast, Wave Life Sciences, Roche, PTC Therapeutics. L.P.A. laboratory members are inventors on patents or patent applications filed by UC that describe gene therapy applications.

limited efficacy of *in vivo* delivery remains a major hurdle for clinical use. An ideal delivery vehicle should be able to target a sufficient number of diseased cells in a transient time window to maximize on-target editing and mitigate off-target events and immunogenicity.

Here, we review major advances in novel delivery platforms based on cell-derived vesicles - extracellular vesicles and virus-like particles - for transient delivery of gene editing payloads. We discuss major findings regarding packaging, *in vivo* biodistribution, therapeutic efficacy, and safety concerns of cell-derived vesicles delivery of gene-editing cargos and their potential for clinical translation.

Keywords

biovesicles; extracellular vesicles; virus-like particles; CRISPR-Cas9; Cre recombinase; delivery vehicles

1. Introduction

The development of gene editing tools has enabled targeted editing of DNA sequences in human cells, increasing the repertoire of DNA targeting therapeutics to address genetic disorders [1]. Gene editing expands the current ability of gene augmentation and gene silencing therapies by allowing to address the root cause of genetic disorders, correct point mutations, target gene insertion or gene knock out. Several leading technologies including zinc-finger nucleases (ZFNs), endonucleases, transcription activator-like effector nucleases (TALENs), and particularly the clustered regularly interspaced short palindromic repeat (CRISPR)–Cas-associated nucleases have promoted tremendous progress in gene editing from basic research to applied biomedical and biotechnological research [2]. Although the potential of therapeutic gene editing has prompted widespread efforts to pursue clinical applications, one major issue has been *in vivo* delivery [3]. The concerns regarding safety related to off-target mutagenesis and the potential for gene editors to trigger immune responses have led to the development of progressively more precise gene editing tools [4], [5] and delivery methods that restrict long-term exposure to editing agents [6], [7], [8]. Tackling the challenge of *in vivo* delivery and tissue targeting will require the transportation of highly efficient gene editing agents to edit a significant population of cells to allow optimal therapeutic efficacy [9].

So far, these efforts have mainly focused on the use of viral vectors as delivery agents, using adeno-associated viral vectors (AAV) and lentiviral vectors (LVs), which allow long-term expression of gene editing tools in target tissue and have shown therapeutic efficacy in multiple disease models [10]. Nonetheless, the persistent expression of genome editing technologies encoded from these vectors increases the likelihood of off-target effects and may induce immune responses [11], [12], [13]. Transient delivery modalities offer an advantage through short-term expression of gene editing tools. Lipid based delivery platforms, such as lipid nanoparticles (LNPs) underwent tremendous recent development, even though they are better suited for nucleic acid encapsulation rather than protein and ribonucleoprotein (RNP) delivery [9]. In the last years, cell-derived vesicles emerged as an

alternative platform to transiently deliver gene editing agents in the form of RNA and/or protein.

In this review, we shed light on a unique approach to deliver gene editing applications, focusing on cell-derived vesicles as promising delivery vehicles. While the spotlight remains in viral vectors and LNPs as primary delivery strategies, our focus diverges towards the promising realm of extracellular vesicles (EVs) and virus-like particles (VLPs). Notably, there has been a surge in publications throughout the last years harnessing the potential of cell-derived vesicles as a delivery strategy for genome editing tools. Herein we address the critical aspects for transient and efficient delivery of CRISPR RNAs and proteins *in vivo*, highlighting the intrinsic advantages of cell-derived vesicles compared to viral vectors and synthetic lipid-based systems. We focus on the loading methods of RNA and protein therapeutic gene editing tools, particularly CRISPR-Cas technologies in EVs and VLPs.

2. Genome editing technologies

Genome editing technologies enable the precise modifications of specific DNA sequences. Over the past three decades, various platforms of programmable endonucleases have been meticulously developed to facilitate targeted genome editing, including meganucleases [14], zinc-finger nucleases (ZFNs) [15], transcription activator-like effector nucleases (TALENs) [16], [17] and CRISPR endonucleases [18], [19], [20].

The initial stage of genome editing started with the use of meganucleases as restriction enzymes for editing the genome at specific sites, followed by ZFNs and TALENs. ZFNs and TALENs are chimeric proteins that combine characteristics from a specific DNA-binding domain with customizable specificity and a nuclease domain derived from the bacterial class *Flavobacterium okeanokoites* (FokI) [15], [21], [22], [23], [24]. Both ZFNs and TALENs required the tailored design of DNA-binding modules, with a zinc finger module (~ 30 amino acids) recognizing a 3 bp DNA sequence and a TALEN module (34 amino acids) recognizing a single nucleotide in the DNA [15], [17], [25]. FokI operates as a dimer making the design of these nucleases a complex and laborious task since it requires a pair of these nucleases to bind opposite DNA strands in close proximity for efficient cleavage of the target DNA site [26]. However, since both DNA-binding and cleavage domains work in an independent manner [27], the generation of nucleases with new DNA-binding specificities was simplified in comparison with meganucleases. Nevertheless, TALENs were advantageous in terms of specificity but their requirement of reengineering for each target locus, large protein size and repetitive regions make them challenging and less efficient for delivery purposes [16], [17].

Then emerged the revolutionary CRISPR-Cas nucleases. For more than a decade, CRISPR-Cas enzymes have been transforming genome editing research by facilitating genetic alterations at the DNA level [2], [28]. CRISPR's ease of use, adaptability, and potential for clinical translation reshaped the landscape of genome engineering, eliminating the need for laborious protein engineering efforts associated with the previous methods. CRISPR and their associated proteins (Cas) were found to be key components of a bacterial adaptive immune system [29], [30]. The most used CRISPR system for targeted genome editing in

human cells employs the class II Cas9 endonuclease from *Streptococcus pyogenes* (SpCas9) that is directed to a specific genomic locus via a programmable guide RNA (gRNA) molecule, mediated by complementary DNA–RNA base pairing. Once bound to its target sequence, the HNH and RuvC SpCas9 catalytic domains mediate nicking of each DNA strand to induce double-strand breaks (DSBs) [18], [20], [31], [32]. For SpCas9, the DSBs typically occur three base pairs upstream of an 'NGG' protospacer adjacent motif (PAM), where the PAM is located directly adjacent to the target site protospacer that pairs with the gRNA spacer. The requirement for Cas enzymes to recognize a PAM defines the targeting scope of that enzyme, since it is a critical requirement for initial target DNA recognition prior to subsequent cleavage. Over 200 new CRISPR associated functional systems have been identified [33], [34], offering a variety of targeting options to modify a wide range of genetic sequences within the human genome. To further broaden the targeting scope of Cas enzymes, various Cas9 endonucleases have been engineered to alter their PAM compatibility to alternative [35], [36] or broader and more flexible [37], [38] targeting sequences.

CRISPR-Cas9 nucleases are typically deployed to introduce DNA DSBs, which are generally resolved via DNA repair pathways including non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homology-directed repair (HDR) (from the sister chromosome) [39]. The repair of DNA DSBs via NHEJ or MMEJ typically results in nucleotide insertions or deletions (indels) that can disrupt noncoding sequences or lead to gene silencing if a particular coding sequence is targeted and its frameshift generates a premature stop codon [40], [41], [42], [43], [44]. Alternatively, HDR can be exploited in the presence of an exogenous DNA template to incorporate user-defined sequences at the cleaved DNA site. While HDR has been widely used to achieve precise genetic modifications, its application is generally restricted to dividing cells given the restriction of HDR factors to certain phases of the cell cycle. Additionally, NHEJ-related proteins are typically more abundant than HDR factors in cells, which often leads to indels being introduced more efficiently than precise HDR-mediated edits [45], [46].

To overcome challenges related to genome editing precision when using nucleases, modified CRISPR tools have been developed by altering the DNA cleavage activity of SpCas9. Inactivation of the HNH or RuvC catalytic domains switches the enzymatic activity to a nickase Cas9 (nCas9), which avoids DSBs by cleaving only one of the DNA strands. Mutation of both catalytic domains produces a dead or deactivated Cas9 (dCas9) that retains DNA targeting ability but does not cleave either DNA strand [19], [31]. Next-generation CRISPR enzymes that leverage nCas9 or dCas9 have been developed by combining their DNA targeting precision with fusions to enzymatic effector domains, resulting in a versatile CRISPR toolbox capable of executing a broad spectrum of (epi)genetic modifications that are largely independent of DSBs. Development of dCas9-based artificial transcription factors allow precise control over gene expression without requiring genetic modifications, having utility to decipher the function of genes, to understand noncoding regulatory sequences, and offering potential therapeutic avenues for genetic disorders [47]. For example, dCas9 fusions to transcriptional activation domains promote gene expression, artificially elevating expression of genes targeted by gRNAs [48], [49]. Alternatively, dCas9 enzymes can be fused to transcriptional repression domains that silence gene expression

[50], [51], [52]. Beyond up-or-down regulation of gene expression via transcriptional perturbation, several other epigenetic modifying enzymes have been developed [53].

Another variation, base editors (BEs), were created to facilitate the installation of targeted point mutations without requiring DSB or exogenous DNA templates [54]. BEs are comprised of a nCas9 fused to nucleotide deaminases to achieve transition base substitutions [55], [56]. Recently, more efficient versions of BEs have been engineered that overcome certain limitations to enable higher levels of target base editing [57], [58], [59], [60], [61], [62]. These revolutionary tools have been at the cutting edge of efforts to treat a wide range of genetic disorders by offering the potential to modify and correct single DNA bases, addressing the root cause of many diseases [63], [64], [65], [66]. Current BEs, however, are largely limited to transition mutations and some transversions, and remain prone to editing unwanted bases near the target bases (leading to so-called “bystander editing”).

Prime editors (PEs) were developed to enable a wider diversity of small genetic edits, marking another milestone in the advancement of genetic modification technologies. PEs are comprised of nCas9 fused to a reverse transcriptase (RT) domain. They are co-delivered along with a prime editing guide RNA (pegRNA) that serves the typical function of a gRNA to guide the enzyme to specific genomic sites, while also acting as a template to encode genetic alterations of interest. The pegRNA can anneal to the nicked non-target DNA strand, creating an RNA:DNA substrate for the RT domain to extend via reverse-transcription [67]. PE technologies overcomes certain limitations of BEs by employing a unique molecular strategy that enables the full spectrum of nucleotide transitions, transversions, and controlled insertions or deletions of short or large-sized DNA sequences [68], [69], [70], [71]. Recently, DNA-dependent DNA polymerases (DDPs)-based editing has emerged as a complementary system to prime editing expanding the ability of writing into the genome [72], [73]. The DDPs Phi29 and EcKlenow were shown to be able of writing at a Cas9-nicked site from a single strand DNA template. For instance, click editors (CEs) associate EcKlenow polymerase with nCas9 tethered to an HUH endonuclease domain that covalently binds a single stranded DNA template (clkDNA) [72] to install the edit when directed to the target site by a gRNA [72].

Together, the extensive development of the CRISPR toolbox offers new technologies to treat genetic disorders whose genome-targeting precision can be maximized by using CRISPR-Cas9 variants capable of targeting nearly any DNA base in the genome [37], [74]. For clinical translation, there is a need for delivering genome editing tools *in vivo* in an efficient and safe manner [75]. Unfortunately, there are limited options to transiently deliver CRISPR technologies due to size of the requisite genetic payload (especially for viral vectors such as those derived from AAVs where the genome size is limited) and also delivery vector targeting limitations. Exploration of novel Cas9 orthologs [76] and CRISPR ancestral nucleases [77] with smaller sizes can help to overcome the limitation of larger enzyme coding sequences. Beyond viral vectors, recent engineering possibilities to deliver CRISPR enzymes through cell-derived vesicles in a transient manner became a promising approach for therapeutics less susceptible to strict size constraints of the delivered enzymes.

3. Challenges for *in vivo* delivery of genome editing technologies

Gene editing tools can be delivered in the form of DNA or messenger RNA (mRNA) molecules that encode the necessary enzymes or gRNAs, or they can be delivered directly as active RNP enzymes. Delivery of genetic material (resulting in DNA expression) guarantees long-term or even permanent expression in post-mitotic cells and is mostly achieved through viral vectors, namely LVs, AAVs [78] or adenoviral vectors [79]. However, long-term expression of gene editing tools through DNA delivery is undesirable as it increases the chances of off-target gene editing [6], [80] and immunogenicity [81], [82]. In contrast, short-term expression of gene editing tools has been found to reduce the occurrence of non-specific cleavage at undesirable sites [6], [80]. Development of more active nucleases with increased activity, often comes with the cost of more off-target editing when permanently expressed in the cell. For this reason, transient expression of a highly active DNA endonuclease is desirable to minimize potential genotoxicity caused by off-target events [83]. While viral-vector mediated long-term expression of gene editing tools has generally been more effective in living organisms [3], advances in the use of cell-derived vesicles have increased the effectiveness of temporary delivery of these systems [6], [80]. Transient expression of CRISPR editing enzymes is generally attained through mRNA or RNP delivery, which are degraded over time. Transient delivery of mRNA ensures the production of multiple CRISPR enzymes for each delivered molecule, increasing the likelihood of gene editing. Lastly, delivery of gene editing proteins or RNPs in their active form allows immediate and potent on-target edition followed by rapid cell clearance, without the requirement of the cellular machinery to express the active genome editing effectors [7]. This approach, particularly the delivery of CRISPR RNPs, has emerged as a promising therapeutic strategy for genetic disorders in animal models, offering precise and efficient corrections to the underlying genetic mutations [7], [84]. Underneath we review transient delivery approaches of gene editing tools for gene therapy.

To achieve *in vivo* therapeutic efficacy, delivery vehicles must incorporate their cargo in a highly efficient manner and overcome several biological barriers to deliver these agents to the intracellular compartment of target cells. Delivery vehicles must 1) protect their cargo from degradation or immune recognition, 2) bind to target cells, 3) readily internalize cell membranes, 4) escape intracellular degradation and release their cargo, and 5) reach the suitable intracellular compartment (Figure 1).

Protection of gene editing cargo can be achieved by encapsulation in biological or synthetic nanoparticles, which prevents their direct degradation and immune recognition, enabling circulation within body compartments until internalized by the target cell [9]. For that, it needs to avoid recognition by the immune system which can identify delivery vehicles as foreign entities and promote their degradation, either by the mononuclear phagocytic system or by antibody-mediated recognition [9]. Some EV-subtypes (e.g. exosomes and microvesicles) [85] and some VLPs that do not rely on the outer surface expression of viral scaffolds may remain undetected since their surface composition resembles the one found in cell membranes and naturally occurring in the body. Cell-derived vesicles evasion of the immune system is highly dependent on the progenitor cell type (autologous or heterologous) and the expression of surface modifications that might be recognized as

exogenous entities. Additionally, apoptotic bodies or vesicles expressing exogenous epitopes can be more readily cleared [86]. To overcome body clearance, EVs and VLPs can be modified to express CD47 on their surface to elude macrophage phagocytosis [87]. The short intracellular lifespan of CRISPR-based systems is crucial, as a prolonged expression of these elements in cells can elicit their cell-targeted destruction by the immune system [81], [88], [89]. A possible strategy to circumvent this issue is protein engineering of nucleases to reduce their epitope immunoreactivity [90].

The ability of cell-derived vesicles to achieve efficient internalization strongly depends on their half-life within the target tissue and their interaction with the intended cells, representing one of the major challenges in therapeutic delivery. In the case of LNPs, this can be achieved by fine-tuning the lipid composition, molar ratios, and particle charge [91], or by modifying the surface composition with small molecules, aptamers, peptides, and antibodies that interact with target cell receptors [92]. Other factors influencing efficient target engagement and internalization include the presence of biological barriers.

For example, cell-derived vesicles delivery to the central nervous system (CNS) is highly challenging due to biological barriers such as the blood brain barrier (BBB) [93] and the short half-life of the vesicles [94]. Direct CNS administrations could be achieved with injections into the parenchyma or cerebrospinal fluid (CSF). However, these can pose a risk of safety at the injection site due to their invasiveness, potentially causing damage to the brain tissue and making them unsuitable for multiple administrations. Alternatively, intranasal and intravenous routes are less invasive approaches which have been explored for brain delivery [95], [96], [97].

Delivery to CNS requires active permeation through the BBB and targeting of specific neural cells [98]. One solution is expressing rabies virus glycoprotein (RVG) peptide on the surface of EVs to facilitate the crossing of the BBB by binding to acetylcholine receptors in neural and endothelial cells [99]. Alternatively, targeting the transferrin receptor (TfR) in brain capillary endothelial cells is highly explored due to its high expression in these cell types and can result in 20-30 times increase in brain concentrations [100], [101], [102]. Recently, CD98hc has also been shown to be an alternative target for brain delivery with slower and prolonged kinetics [103].

Additionally, cell engagement typically depends on the interaction of surface moieties with receptors on the surface of target cells to promote their internalization [104]. Native EVs' targeting ability depends on their origin since they carry specific signatures from their progenitor cells [105], such as integrins and proteoglycans [106], [107], although the efficiency and the mechanisms by which EVs are internalized are still poorly understood [108]. VLPs' targeting is typically dependent on the viral scaffolds expressed on their surface and their progenitor origin for enveloped VLPs. In either case, these carriers can be engineered to express surface targeting moieties to promote their interaction with cells in a specific tissue [99], [109], such as RVG for neuron-specific targeting [97], [110].

Following cell engagement, cellular internalization of cell-derived vesicles is usually mediated through endocytosis of the delivery vehicles into endosomes [108]. To release

their content in cytoplasm and reach the target compartment, delivery vehicles must escape endosomal degradation and the acidic environment of endosome compartments [111]. Viruses and VLPs are equipped with viral moieties that take advantage of endosomal acidification to trigger structural modifications that promote endosomal escape and cargo release [112]. On the other hand, EVs' mechanism for cytosolic delivery still requires further understanding [108]. A study suggests that around 30% of internalized EVs release their cargo to the cytosol [113]. For gene editing tools to be effective after escaping the endosomal compartment, they must be directed to the nucleus to access and modify the DNA which is generally attained by fusing these with nuclear localization signal (NLS) peptides.

In conclusion, the development of effective delivery vehicles that can efficiently transport and safeguard their cargo while evading the numerous biological barriers represents a crucial frontier in advancing targeted therapies and holds promise for transformative advancements in the field of genome editing.

4. Cell-derived vesicles

Cell-derived vesicles are an heterogenous population of naturally occurring membrane vesicles originated and secreted by cells [114], [115]. Here, we define cell-derived vesicles as an umbrella term for two groups of particles: EVs and VLPs, both cell-derived vesicles which can be harnessed as promising delivery vehicles.

4.1 Extracellular Vesicles

EVs are lipid-bilayer membrane vesicles secreted by all cells which are responsible for intercellular communication and mediate the exchange of molecular information in the form of nucleic acids, proteins and lipids [108], [116]. EVs are further divided into subtypes, some of the most well-reported include exosomes (30-150nm), microvesicles (100-1000nm) and apoptotic bodies (100-5000nm) [116]. Each subtype of EVs is defined based on characteristics including size, density, composition, and biogenesis process, although the overlap of these features among EV-subtypes hampers their characterization [117]. Given their complexity and heterogeneity, the International Society for Extracellular Vesicles released the Minimal Information for Studies of Extracellular vesicles (MISEV) guidelines to develop and implement best practices and scientific considerations for the study of EVs [117], [118]. For example, EVs' size is commonly reported within the nanometer range, although such a large umbrella terminology can comprehend particles ranging from 30 to 1000nm, up to 5000nm in some cases. The size of EVs, among other properties, are influenced by isolation methods, biogenesis process, cell type and cellular state, buffer storing conditions and ultimately by the equipment of analysis. These aspects should be carefully considered and streamlined to obtain homogenous and reproducible EVs' preparations.

Vesicle properties such as size and surface composition affect EVs' biodistribution, altering their ability to cross tissues, biological barriers, and cell membranes [104]. While small EVs (<100nm) were shown to be mostly retained in the liver and kidney within the first hour of intravenous administration, peaking in the lungs and spleen between 2-12h, large

EVs (>200nm) were shown to be most abundant in lungs within the first hour, shifting towards the liver as the levels in the lungs decreased [119], suggesting that EVs' size alters their biodistribution. Moreover, understanding the biogenesis' processes that promote and ultimately lead to secretion of a specific subtype of EVs allows the precise loading of therapeutic agents into their lumen and modification and functionalization of their surface [104]. The versatility to modify EVs' properties has motivated intense efforts to engineer them as delivery vehicles, making EVs promising candidates for transient *in vivo* delivery of gene editing agents with reduced immunogenicity due to their membrane biocompatibility. Engineering EVs' surface and composition allows a better understanding of their *in vivo* behavior, harnessing their potential to deliver therapeutic payloads to human cells.

4.2 Virus-like particles

Beyond the native release of EVs from parental cells, several viral pathogens can hijack the cell machinery and take advantage of EVs' biogenesis mechanisms to promote their survival and further spread virions to the extracellular space [112]. The study of viral biogenesis and assembly allowed the engineering of viral scaffold architectures to generate VLPs, virus-derived particles composed of one or more viral scaffolds, possessing the ability to self-assemble, but lacking viral genetic material [120], [121]. Budding of enveloped viruses is primarily driven by either a viral envelope protein, a viral capsid protein or a combination of both. Expression of these components alone in eukaryotic cells is sufficient to promote the assembly of VLPs. Given the large diversity of viruses, VLPs comprise a large group of particles with virus-like characteristics, from cell membrane derived vesicles coated with virus-derived proteins to more complex architectures largely resembling viruses but lacking a viral genome.

VLPs exploit several native features of viruses to generate effective delivery vehicles, such as the ability to target specific cell types, efficiently internalize cells and escape endosome degradation, making them ideal to transiently deliver gene editing agents [111], [112]. In this review, we categorized VLPs' based on the subunits required for their self-assembly: viral envelope proteins or viral capsid proteins for VLP generation. Other approaches engineer self-assembling structures resembling viruses, such as enveloped protein nanocages (EPNs) [122]. Cells expressing viral scaffolds secrete a heterogenous population of cell-derived vesicles, from native EVs to VLPs, both groups sharing overlapping features (Figure 2) leading to their co-isolation through different isolation methods [123].

Overall, EVs and VLPs can transiently deliver gene editing tools in the form of mRNA or protein, allowing potent on-target efficiency in recipient cells, while being rapidly degraded to improve their safety profile [7], [124]. The loading of therapeutic gene editing agents into cell-derived vesicles can be achieved exogenously through sonication and freeze-thaw cycles [125], electroporation [126], and others, or endogenously by genetically modifying parental cells to load and secrete therapeutic particles [127], [128], [129], [130], [131].

5. Cre-lox system as a gene editing model to study cell-derived vesicles delivery

To investigate the intercellular signaling of EVs, the Cre-lox recombinase system has been employed to study communication and delivery across different tissues [132], [133]. Cre is a bacteriophage tyrosine-type site-specific recombinase that mediates DNA recombination of sequences flanked by 34 bp sites named Lox [134], [135]. Depending on the location and orientation of these Lox sites, Cre can promote the insertion, deletion, inversion or translocation of target DNA sequences, making this system a primary tool to modify DNA [134], [135]. For these reasons, Cre recombinase is often used as a primary proof of concept for DNA targeting, either to study EVs' communication *in vivo* or the functional delivery of particles [132], [133], [136].

The ability of Cre recombinase to induce permanent changes in DNA is often used to activate reporter genes, facilitating the differentiation of cells that uptake EVs, as demonstrated in Cre reporter mouse models. When applied in an *in vivo* reporter model, this strategy can be used to study the role of EVs in: a) the transport of cancer associated RNAs, such as microRNAs (miRNAs) and mRNAs, that trigger immunosuppression and lead to tumor progression [132], [133], b) cell senescence due to the transport of interferon-induced transmembrane protein 3 (IFITM3) molecules [137]; c) brain inflammation by studying the distinct miRNA profile transferred by hematopoietic-derived EVs [138]; d) brain communication between specific type of cells [139] and different brain regions [136]. Using Cre tools in a *in vivo* setting is an advantage to study transfer of low amounts of EVs, however their used is limited to reporter models.

5.1 Cell-derived vesicles delivery *in vivo* using Cre-lox systems

The capacity of EVs to load and transport genome editing molecules to specific tissues can be explored using Cre recombinase as a primary proof of concept for DNA targeting (Figure 3). Many strategies for incorporation of biomolecules in EVs rely mainly on packaging of plasmid DNA and small nucleic acids, such as miRNAs and small mRNAs, which were found to be naturally contained in EVs, with a peak size of 200 nucleotides but stretching up to 5kb or beyond [140], [141], [142]. There are still only a few packaging strategies for proteins [143].

5.1.1 Extracellular Vesicles—One approach took advantage of the interaction between the WW tag domains from the neuronally expressed developmentally downregulated 4 (Nedd4) ubiquitin ligases family with the late-domain (L-domain) present in Ndfip1 protein (Nedd4 Family Interacting Protein 1), a ubiquitin ligase adaptor protein which participates in the budding of EVs. The fusion of the WW domains with Cre protein leads to interaction with Ndfip1, driving Cre protein inside of vesicles. After showing functional deliver to reporter cells *in vitro*, the engineered EVs containing Cre protein were administered through intranasal route to Ai14 reporter mice, which activates tdTomato expression upon Cre activity. Interestingly, tdTomato expression was found within the brain mainly associated with neurons and microglia in the olfactory bulb, cortex, striatum, hippocampus,

and cerebellum, indicating functional delivery on WW-Cre containing EVs *in vivo* upon intranasal administration [96].

Light-dependent optogenetic dimerization has also been demonstrated to improve the loading of proteins of interest inside EVs. One particular tool named EXPLORs (exosomes for protein loading via optically reversible protein–protein interactions) was developed [144] to attain this purpose by assembling three major components: 1) the photoreceptor cryptochrome 2 (CRY2) which binds the cargo protein; 2) a truncated version of CRY-interacting basic-helix-loop- helix 1 (CIBN) fused to the EV-specific tetraspanin CD9, and 3) the 488nm blue light that triggers protein-protein interaction. Exposure to blue light during EVs biogenesis promotes the dimerization between CRY and CIBN, allowing the protein cargo to be efficiently packaged into CD9-positive EVs. One advantage of this system is that dimerization is transient as protein-protein interaction is interrupted following removal of blue light emission, resulting in the release of the cargo to the intraluminal space of EVs. Moreover, EVs containing Cre molecules (derived from HEK293T cells) were functionally internalized to neurosphere-derived reporter cells allowing 95% of Cre mediated recombination measured by GFP expression. The same system was then delivered to the brain of a transgenic reporter mouse by intracranial injection into the striatum, being mainly internalized by neurons [144]. EXPLOR-derived EVs carrying Cre protein isolated from HEK293T cells were also found to cross the placental barrier in mice upon intraperitoneal injection in the maternal side. These EVs switched tdTomato to GFP expression in fetal reporter cells, suggesting a role for EVs signaling during pregnancy [145].

5.1.2 Virus-like particles—A robust cytosolic delivery *in vitro* and *in vivo* was demonstrated upon expression of VSV-G on the surface of two delivery systems: VSV-G plus EV-sorting Domain-Intein-Cargo (VEDIC) and VSV-G-Foldon-Intein-Cargo (VFIC) [146]. These systems combined a fusogenic protein (VSV-G) and a small intein protein with self-cleavage activity to link cargo to an EV-sorting domain (CD63) and release it from the membrane. Remarkably, these cell-derived vesicles containing Cre protein were able to mediate nearly 100% of Cre recombination when compared to non-VSV-G vesicles *in vitro*. Alongside the robust Cre protein delivery *in vitro*, this strategy achieved successful *in vivo* Cre delivery through intratumoral, intracerebroventricular and intraperitoneal delivery. Remarkably, this platform was then used to deliver Cas9:gRNA RNPs in cell-derived vesicles achieving nearly 80% genome editing efficiency compared to non VSV-G particles *in vitro* [146], paving the way to deliver more advanced genome editing tools such as BEs and PEs.

5.2 Limitations in studying Cre transfer and functional delivery through cell-derived vesicles

The use of Cre-lox reporter systems to study EVs transfer is growing in the field, although some considerations about the use of this tool should be highlighted. The use of Cre recombinase allows to study the *in vivo* role of EVs produced in physiological amounts [132], [133], [136] due to the capacity to distinguish recombined cells in an entire population. Also, Cre mRNA and protein can be used as a tool to study loading properties of

EVs, *in vivo* biodistribution and targetability. However, the use of Cre recombinase to study EVs is limited to reporter models and is lacking translation in disease or clinical settings. For that reason, Cre should be seen as a tool to study EVs and their ability to deliver functional cargo to the intended cells. It is relevant to characterize the EVs population and evaluate which type of Cre molecules are associated with each subpopulation. For instance, Cre plasmid, mRNA or protein can be found associated with EVs depending on the isolation methodology.

When small and large EVs were differentially isolated to evaluate which type of Cre molecules are present in each EV subpopulation, it was found that Cre plasmid is preferentially packaged in large EVs and it is the main active form in target cells. Large EVs showed functional delivery of cargo to luciferase reporter *in vitro* and *in vivo* models that start expressing bioluminescence upon functional Cre delivery [147]. This study warns for the presence of plasmid DNA in a specific type of EVs that may impact the understanding of the functional effects of the loaded Cre mRNA in EVs. A different study warned to the difficulty of detecting mRNAs inside of endothelial-derived EVs, including Cre mRNA [148]. Caution should be made in terms of amount of starting material to isolate EVs, methods of isolation and characterization of Cre-derived EVs to particularly understand which type of Cre molecules are driving the recombination.

Despite these concerns, the use of Cre derived EVs is becoming a more widespread tool to study communication and functional delivery of EVs. Overall, the simplicity and precision of Cre-lox systems to target DNA at loxP sites makes it a useful tool to advance our comprehension of intercellular communication mediated by EVs and its impact in health and disease (Figure 3).

The study of EVs and VLPs functional delivery with Cre-lox reporter systems can support the development of CRISPR editing tools by informing where these are delivered, and therefore guide the engineering of cell-targeted EVs and VLPs that minimize the likelihood of off-target delivery with consequent toxicity, a major bottleneck in the translation of CRISPR therapeutics. While the limitation of Cre-lox recombinase system to specific Lox sequences of the DNA restricts its versatility and broad applicability, it has undoubtedly demonstrated its worth in investigating paracrine communication of EVs *in vivo*.

6. Extracellular Vesicles delivery of genome editing technologies

EVs are an attractive option to deliver genome editing technologies given their suitable characteristics for *in vivo* therapeutic delivery such as nanoscale size, natural low immunogenic composition, and ability to assemble proteins for enhancing packaging or targeting efficacy. EVs offer the possibility to deliver genome editing tools in a transient manner in the form of RNA or protein, facilitating the temporary introduction of gene editing enzymes that undergo rapid turnover. This helps mitigate potential geno- and immunotoxicity associated with long-term exposure, as observed with viral vector-based delivery methods [6], [81][124]. Transient delivery of CRISPR tools in EVs can be achieved by loading Cas RNPs (Cas protein and gRNAs) or Cas9 mRNA and synthetic gRNAs. The encapsulation of molecules of interest in EVs falls into two major categories: endogenous or

exogenous loading. Table 1 and Table 3 summarize the main findings of studies using EVs as a delivery vehicle for CRISPR-Cas systems *in vivo* and *in vitro*, respectively.

6.1 Endogenous loading

Endogenous loading occurs in the parental cell through cellular packaging mechanisms during EVs' biogenesis or release, resulting in incorporation of the molecules of interest in EVs. This can be achieved by passive loading of molecules of interest in EV-donor cells (relying on stochastic loading) or by overexpressing the payload in producer cells. Direct incubation of molecules of interest in EV-donor cells is generally performed when these were produced synthetically and cannot otherwise be expressed in cells, for example for loading drugs [149]. In the case of mRNA and protein, endogenous loading usually relies on overexpression in EV-donor cells of plasmid DNA or viral vector transgenes that encode the mRNA and/or protein to be incorporated in EVs.

To encapsulate CRISPR-based machinery in EVs, most studies rely on plasmid DNA transfection in EV-donor cells. Overexpression of CRISPR-encoding systems results in the detection of CRISPR machinery within secreted EVs [129], [143]. EVs passively loaded with Cas9 nuclease or dCas9-VPR were shown to mediate gene editing and gene activation, respectively, both *in vitro* and *in vivo* [144], [145]. Although overexpression of plasmid DNA in EV-donor cells results in the detection of CRISPR elements in EVs, this process relies on the stochastic packaging of CRISPR machinery in vesicles being formed within the cell, resulting in limited loading efficiencies [115], [131]. Endogenous cargo loading is more efficient by fusing molecules of interest to EV-sorting motifs that are selectively enriched into EVs (Figure 4). For example, SpCas9 was more efficiently loaded in EVs when fused with a packaging system based on CD63 tetraspanin [131].

Another important aspect to take into consideration when developing methods to incorporate cargo in EVs is the engagement of these molecules to the correct subcellular compartment target. Fusion of EV-sorting motifs to gene editing enzymes can compromise their nuclear localization in target cells potentially leading to a re-secretion in a newly formed EV. Regarding the association of gene editing tools with EVs, they can be: 1) covalently tethered to EV-sorting motifs, being difficult to dissociate both proteins in the target cell; 2) tethered to EV-sorting motifs through reversible linkers, to optimize loading in EV-donor cells and functional delivery in target cells.

6.1.1 Fusion to EV-sorting motifs—Endogenous loading of genome editing proteins or RNAs can be achieved by tethering EV-sorting motifs. For proteins, this happens when genome editing enzymes are connected to EV-enriched proteins or motifs that undergo post-translational modifications, recruiting them into EVs or tethering them to the cell membrane. Examples include tetraspanins CD63, CD9, and CD81 [146], [147], or motifs that undergo palmitoylation and/or myristoylation that attach proteins to the cell membrane (Figure 4). Fusion of GFP to CD63 (C-terminus) and anti-GFP nanobody to SpCas9 (C-terminus) loaded more efficiently SpCas9 in EVs than without the use of CD63 as EV-packaging system [131]. gRNAs were also co-enriched in EVs, through their interaction with SpCas9, resulting in Cas9:gRNA RNP loading in EVs. SpCas9 RNP loaded EVs were shown to

switch on DsRed expression by deleting a sequence encoding an in-frame stop codon *in vitro* [131]. Their biodistribution in TdTomato reporter mice remained limited after five intravenous injections with dim tdTomato signal observed only in the liver. No signal was detected in other organs such as heart, lung, kidney and spleen [155].

In another study, Cas9 was attached to arrestin domain containing protein 1 (ARRDC1) [124]. ARRDC1 was used to package Cas9 RNPs in EVs [124] through its interaction with the cytoplasmic side of the plasma membrane. ARRDC1 recruits the endosomal sorting complexes required for transport (ESCRT)-I machinery to initiate membrane budding of ARRDC1-mediated microvesicles (ARMMs). Since ARRDC1 specifically interacts with WW-domain containing proteins, WW-domains were fused to Cas9 to promote its enrichment into ARMMs. Incubating ARMMs loaded with Cas9 RNPs targeting GFP in GFP-expressing cells significantly increased the number of GFP-negative cells, rising from 4.8% in the control condition to as high as 13.4% in Cas9 RNP ARMMs [124]. The authors hypothesized that ARMMs might avoid the lysosomal degradation machinery by directly fusing to the plasma membrane.

Other EV-enriched proteins have been engineered to load CRISPR-Cas9 in EVs, namely members of immunoglobulin superfamily EWI and MARCKS protein families [149]. It was demonstrated that MARCKSL1, BASP1, MARCKS, and PTGFRN could load higher levels of GFP molecules in EVs compared to commonly used tetraspanins CD9, CD63 and CD81, suggesting that the same might work for other macromolecules such as Cas9 [149]. Truncated forms of Basp1 loaded on average 4 to 5 molecules of Cas9 protein per EV, measured through densitometry quantification of western blot experiments with various amounts of recombinant Cas9 [150].

6.1.2 Fusion to EV-sorting motifs through reversible linkers—Fusing proteins that drive CRISPR systems from the cytosol into EVs in producer cells might negatively affect their routing to the nucleus in target cells [128], [151]. For this reason, several studies engineered dimerization systems that allow transient interactions between proteins of interest, such as Cas proteins, and EV-packaging proteins [122], [127], [128], [129], [152]. Using CD9 and Myristoylation-Palmitoylation-Palmitoylation (MysPalm) signals, four dimerization systems were compared: CRY2 to CIBN; truncated Phytochrome B (PHYB) to phytochrome-interacting factor 6 (PIF6); vivid-based Magnet positively charged (pMags) to vivid-based Magnet negatively charged (nMags) and FK506-binding protein (FKBP) to FKBP-rapamycin-binding domain (FRB) [130]. From the four systems tested, CRY2-CIBN proved to be superior in Cas9 enrichment, achieving over 20 molecules of Cas9 loaded per EV [130]. In a reporter system where CRISPR-Cas9 editing leads to RFP expression, the authors demonstrated that EVs loaded with CRISPR-Cas9 via the MysPalm-CRY2-CIBN system resulted in approximately 42% RFP-positive cells *in vitro* [130] Additionally, CRISPR-Cas9 EVs targeting the *PCSK9* gene mediated up to 4.4% gene editing *in vitro* [130].

Overall, engineering reversible linkers and EV-sorting motifs to promote the endogenous loading of genome editing tools in EVs seems to improve editing efficiency in target cells, possibly by favouring routing of genome editing enzymes towards the nucleus.

6.2 Exogenous loading

Exogenous loading, which involves loading cargos of interest into EVs after their isolation, predominantly relies on physical or chemical-based methods such as sonication, electroporation, freeze and thaw cycles, transfection reagents, saponin treatment, and incubation [160], [161] [162]. These methods are particularly suitable for molecules with low endogenous loading capacity or those that are synthetically produced, like drugs [126], [163], [164]. Exogenous loading methods also improve the scalability of production and eliminate the risk of horizontal gene transfer. Red blood cells (RBC)-derived EVs [126] were used to electroporate Cas9 mRNA and gRNAs. RBC-derived EVs loaded with Cas9 mRNA and gRNA targeting the human mir-125b-2 locus resulted in approximately 98% reduction of mir-125b expression and 90% reduction of mir-125a expression after 2 days of treatment in MOLM13 cells [126]. However, the degree of luminal loading of RNAs in EVs through electroporation might be difficult to assess and result in RNA aggregation and membrane disruption [165]. For example, electroporation of small interfering RNAs (siRNA) in EVs was shown to result in high siRNA aggregation and retention in EVs' pellet, leading to the overestimation of loading efficiency and urging the necessity for alternative loading methods [165].

In addition to electroporation, sonication and freeze-thaw cycles were also employed to load Cas9 RNPs into EVs. Tumor-targeting EVs loaded with Cas9 RNPs via sonication or freeze-thaw cycles were able to downregulate GFP expression *in vitro* by 43% [125]. Furthermore, these EVs, when loaded with Cas9 RNPs, successfully knocked out *WNT10B* expression, leading to a significant decrease in WNT10B protein expression and subsequent reduction in tumor growth both *in vitro* and in xenograft tumor models [125]. Another study loaded serum EVs with SpCas9 RNPs through protein transfection using CRISPRMAX [166]. Serum EVs loaded with SpCas9 RNPs targeting introns 22 and 24 of the *Dmd* gene were administered into the tibialis anterior muscle of Rag/mdx mice, resulting in 18.6% restoration of dystrophin expression in fibers and up to a 19% deletion of exons 23 and 24 in cDNA transcripts [166].

Another promising exogenous loading technique utilizes positively supercharged proteins to load cargoes of interest in EVs. These supercharged proteins possess the ability to cross through negatively charged membranes, facilitating the internalization in EVs [167]. Nucleic acids can also be loaded through conjugation with supercharged proteins while proteins of interest can potentially be directly reprogrammed to express positively charged amino acids, without losing their function [167].

Taken together, exogenous loading methods are promising strategies to load genome editing tools in EVs, potentially improving the scalability of production and pharmaceutical formulation compared to endogenous loading which relies on genetic engineering of EV-donor cells. However, several aspects still require further development, including variability in loading efficiencies and the risk of membrane disruption, which could compromise EVs' integrity [165].

Overall, there is compelling *in vitro* and *in vivo* data demonstrating EVs can be harnessed as vehicles to deliver functional therapeutic cargo. Nevertheless, most studies using EVs as

delivery agents for CRISPR-based systems [124], [125], [126], [130], [131], [151], [152], [155], [166], zinc finger epigenetic repressors [168] and activators [169], and TALE-based transcription regulators [170] were performed *in vitro*, limiting our understanding of their biodistribution and delivery effectiveness *in vivo*. Moreover, there is still considerable room for improvement in editing efficiencies. Numerous limitations significantly impact CRISPR editing efficiency, including lack of tissue and cell-specific tropism and sub-optimal cytoplasmic delivery, probably due to endosomal degradation.

7. Virus-like particles delivery of genome editing technologies

VLPs are a promising method for delivering protein or genetic material such as CRISPR components. They combine the advantages of cell-derived lipid membrane nanovesicles with the efficient cargo-packaging capabilities of viral scaffolds. In the following discussion, we summarize key findings from studies using VLPs to deliver CRISPR-Cas systems both in *in vitro* (Table 3) and *in vivo* (Table 1). So far, VLPs for therapeutic delivery of gene editing technologies have been created by engineering particles in two main ways: 1) incorporating viral envelope glycoproteins, primarily utilizing VSV-G; 2) engineering viral capsid proteins, including gag proteins (Figure 4).

7.1 Viral envelope proteins for VLP generation

Viral envelope proteins play a crucial role in localizing, attaching and entering host cells, as well as improving intracellular delivery of macromolecules [120]. The main viral envelope protein used to generate VLPs for macromolecules' delivery is VSV-G, also known from its use in lentiviral vector packaging systems. VSV-G is a coating protein with glycosylated moieties derived from the vesicular stomatitis virus with high fusogenic characteristics that plays an important role in cell engagement, internalization and endosomal escape. VSV-G mediates cell internalization through recognition of ubiquitously expressed receptors from the LDL family [171], [172]. In acidic environments, VSV-G mediates fusion between viral and endosomal membranes inducing the release of viral cargo in the cytosol [173]. VSV-G-engineered vesicles were reported to deliver functional exogenous proteins to recipient cells [121]. Delivery of gene editing enzymes by VSV-G fusogenic vesicles was first reported by passive loading of Cas9 RNPs in VSV-G VLPs by overexpressing these components in producer cells [174]. VSV-G VLPs were shown to deliver *Sp*Cas9:gRNA to HEK 293T cells, resulting in over 50% reduction in eGFP fluorescence. Additionally, they successfully delivered nCas9:gRNAs targeting the GFP coding sequence, leading to gene ablation and 50% decrease in GFP fluorescence [174]. In vivo data showed intra-cardiac injection of VLPs in 5-day-old newborn GFP transgenic mice resulted in 30% eGFP-negative cardiomyocytes [174].

To increase packaging of therapeutic agents in VLPs, molecules of interest can be actively attached to elements enriched in these particles instead of loaded in a stochastic fashion (Figure 4). To that end, protein myristoylation was used to anchor Cas9 protein to the cell membrane [175]. An octapeptide derived from Src kinase was tethered to Cas9 to promote subsequent myristoylation (mCas9) and enrichment [175]. VSV-G was co-expressed to enhance internalization efficiency [175]. VSV-G particles loaded with

mCas9:gRNA resulted in 42% eGFP loss with high rates of indels in HEK 293T eGFP cells [175].

As described in the previous section, release of genome editing tools from packaging motifs can be achieved by engineering linkers, such as through non-covalent binding [159] or inducible/cleavable tethering approaches [7], [129], [176], [177] (Figure 4). To this end, a heterodimer system containing two different binding motifs, DmrC and DmrA, was fused to CRISPR-Cas9 and a membrane associated protein [178]. Cas9 was fused with the DmrC domain which physically associates with the DmrA domain present on the membrane-associated protein CherryPicker Red, when promoted by the A/C heterodimerizer [176]. VSV-G VLPs loaded with Cas9 RNPs were able to edit the HIV LTR region in a microglial cell line, achieving of 8% indels by Tracking of Indels by Decomposition (TIDE) [176].

VSV-G has also been used to directly load Cas9 RNPs in VLPs [159]. Engineered VSV-G and Cas9 were tethered to one another via a split GFP system [159], [179]. In this system, GFP is split into 2 fragments between the tenth and eleventh β-strand, resulting in a 16 amino acid fragment (GFP11) and the remaining protein fragment (GFP10), which bind to one another to reestablish a full-length fluorescent protein [179]. VSV-G VLPs loaded with *Sa*Cas9 RNP targeting the *PINK1* gene, a kinase that recruits Parkin ubiquitin ligase to mitochondria, presented a 40% reduction in Venus-Parkin mitochondrial recruitment [159]. Intravenous injection of VSV-G VLPs loaded with *Sa*Cas9 RNP targeting proprotein convertase subtilisin/kexin type 9 (PCSK9), predominantly expressed in the liver, led to a reduction in total cholesterol levels for at least 14 days after the initial injection [159].

While the packaging of genome editing enzymes allows the co-enrichment of different gRNAs, specific packaging of gRNA through aptamer binding proteins allows the co-enrichment of different genome editors. For example, RNA aptamer (termed *com*) fusion to gRNAs and aptamer binding protein (ABP) fusion to both termini of CD63 was developed to enrich Cas9 RNPs in EVs [180]. VSV-G was co-expressed, generating VLPs. This strategy allowed vesicles' enrichment with SaCas9 or SpCas9 nucleases, and Cas9 adenine base editors (ABE) RNPs, relying on the interaction of CD63-ABP to *com*-gRNA and gRNA to Cas9. Additionally, multiplex gene targeting was attained by co-packaging different gRNAs or both SpCas9 and SaCas9 RNPs with different targeting sites. A single preparation of particles loaded with RNPs targeting two different genes showed higher editing efficiency than particles individually packaging RNPs with a single targeting gRNA [180]. To test *in vivo* efficacy, particles loaded with Cas9 RNPs targeting Duchenne muscular dystrophy (DMD) exon-53 were injected in mouse tibialis anterior muscle of del52hDMD/mdx mice, achieving up to 0.2% indels rates and dystrophin expression as observed by immunostaining [180].

Overall, VSV-G engineered VLPs improve the intracellular delivery of genome editing tools, achieving considerable levels of editing *in vitro* and *in vivo.* Nevertheless, expanding the current library of viral envelopes and engineering scaffolds with increasing cell-type specific tropism is still an unmet need.

7.2 Viral capsid proteins for VLP generation

Viral capsid proteins are also able to promote the self-assembly of VLPs. Retroviral gag proteins are one of the most utilized viral capsid proteins to generate VLPs for the delivery of macromolecules. Gag is a structural protein from human immunodeficiency virus (HIV) and other retroviruses which is essential for the assembly, budding and maturation of viral particles [181]. Gag protein multimerizes at the cell membrane and induces budding and release of VLPs in the extracellular environment [181]. So far, most studies developing VLPs for the delivery of genome editing tools used a combination of capsid gag proteins and envelope proteins, mainly VSV-G, resembling the architecture of lentiviral vector packaging systems.

Gag-engineered VLPs, were first described to load CRISPR-Cas9 RNPs upon fusion of *Sp*Cas9 to Gag from Murine Leukemia Virus (MLV) [177]. Expression of Gag-*Sp*Cas9 with MLV structural and accessory proteins Gag-Pol, along with a gene-targeting gRNA, VSV-G and Baboon Endogenous retrovirus Rless glycoprotein (BaEVRless) resulted in *Sp*Cas9 RNP-enriched nanovesicles [177]. The generated VLPs achieved high levels of editing, exhibiting a dose dependent effect ranging from 35% to 77% editing of *EMX1* gene (by TIDE analysis) [177]. VLPs were also optimized for insertion of genetic material through HDR, achieving over 50% knock in efficiency in HEK293T cells. Their effectiveness was also demonstrated *in vivo*, in which retro-orbitally injected VLPs resulted in 7% to 13% editing of xyphenylpyruvate dioxygenase (*Hpd*) gene, measured by T7 endonuclease 1 mismatch detection assay (T7E1). A similar packaging system was developed by engineering a chemically induced dimerization system to incorporate Cas9 protein and gRNAs into VLPs [129]. Gag fused to FKBP12 and Cas9 to FRB were tethered together by chemical induction with rapamycin analog AP21967, working as an inducible packaging system by promoting the FKBP12:FRB dimerization [129]. GRNA was actively enriched into VLPs by fusion with the ψ packaging signal, a domain that specifically binds to the nucleocapsid of Gag. Self-cleaving ribozymes flanking gRNA allowed its release inside particles. VLPs loaded with Cas9 RNPs achieved up to 48% of indels at *CCR5* gene in Jurkat T-lymphocyte cells (by T7E1). These VLPs were also able to induce exon skipping in human induced pluripotent stem cells (iPSCs), achieving 22% and 29% exon 45 deletion by targeting the splice acceptor (SA) and splice donor (SD) sites of the *DMD* gene, respectively [129]. Multiplexed VLPs targeting the SA and SD sites of *DMD* gene, achieved up to 92% of exon skipping in iPSC-derived skeletal muscle cells, restoring dystrophin protein expression [129]. To validate this system in vivo, an animal model was employed in which disruption of SA and SD sites restores luciferase expression. VLPs injected into the gastrocnemius muscle led to sustained luciferase expression up to 160 days after injection, indicating stable genomic editing over this period [129].

Beyond the delivery of CRISPR-Cas RNPs, VLPs were shown to delivery CRISPR-Cas encoding RNAs by engineering a system in which the MS2 protein binds to the MS2 target site RNA hairpin structure [128], [182]. To generate retroviral-based particles pseudotyped with VSV-G and loaded with CRISPR-Cas9 RNA transcripts, the MS2 coat protein was incorporated into the MLV-Gag precursor and the MS2 target site RNA hairpin structures were introduced within the sequence of SpCas9 and gRNAs expression plasmids [128],

[182]. The resulting VLPs achieved up to 44.1-55.6% *RFP647* knockout in reporter cells and an average of 43% *CXCR4* knockout in human Jurkat cells [182]. The Gag.MS2.CRISPR-Cas9 particles were further improved by transferring the system from gammaretroviral to an alpharetroviral vector platform [128], achieving a 4- to 26-fold increase in *RFP647.Tet2* knockouts, with an average knockout rate over 70% [128].

Apart from delivering CRISPR nucleases, the utility of VLPs was broadly expanded to package and deliver larger genome editing technologies, such as CRISPR BEs and PEs [7], [84]. For this purpose, the adenine base editor ABE8e was fused to the Gag polyprotein from Friend murine leukemia virus (FMLV) via a cleavable linker, which is cleaved by FMLV protease upon particle maturation [7]. Additionally, three nuclear export signals (NES) were fused to MLV-Gag immediately before the cleavable linker to promote cytoplasmic localization in producer cells, increasing packaging efficiency into VLPs [7]. ABE8e was flanked by nuclear localization signals to promote nuclear importation upon delivery [7]. These VLPs achieved very high base editing efficiency *in vitro*, close to 100% for several targets in HEK293T cells. BE VLPs were able to reduce the serum levels of Pcsk9 by 78%, after 63% of liver editing in mice injected by systemic retro-orbital injection [7]. In a mouse model of genetic blindness, BE VLPs improved visual function following subretinal injection, leading to up to 12% correction of the R44X mutation with no detectable bystander editing, thus far being one of the most effective applications of VLPs for the delivery of gene editing tools *in vivo*. The optimal VLP architecture seems to be dependent of the type of editor loaded, since a simple replacement of BEs with PEs in the previously described VLP architecture yielded very low functional delivery [84]. A systematic engineering of the system was taken to maximize prime editing efficiencies through VLPs delivery. The development of PE-VLPs system benefits from the engineering of PEmax architecture, epegRNAs and DNA mismatch repair evading mutations and additional gRNA recruitment through the MCP-MS2 recruitment mechanism or the COM–Com protein–RNA aptamer pair [84]. Moreover, a thorough optimization of the protease cleaving sites was performed to determine the optimal ratio and positioning between nuclear localization signals (NLS) and nuclear export signals (NLS). Alternatively, a system was engineered that eliminates the need for covalent binding of the PE to the GAG protein. This is achieved by utilizing a coiled-coil peptide-dependent recruitment of the PE. The editing levels were 79-fold higher in Neuro-2A cells and 170-fold higher in HEK293T cells when compared to the first architecture [84]. Remarkably, efficient *in vivo* delivery of PE with VLPs upon a single subretinal injection was shown for the first time in 2 different mouse models. It achieved 15% efficiency in correcting a 4-bp deletion in *Mfrp* in the rd6 model of retinal degeneration and 7.2% efficiency correcting an Rpe65 substitution that partially rescue visual function in the rd12 model [84].

Another strategy incorporated a cell type-specific antibody in the delivery vector architecture to allow more directed targeting, separating the fusion and targeting functions of fusogens, named Delivery to Intended Recipient Cells Through Envelope Design (DIRECTED) [183]. A chimeric antibody binding protein or a SNAP-tag was introduced between the secretion signal and the transmembrane domain of VSV-G, creating a modular system for antibody recruitment [183]. This strategy was used to target Cas9-RNP VLPs to Jurkat E6 cells through a αCD5 antibody, with gRNA targeting B2M, which is highly

expressed in this cell type. After 4 days of delivery, flow cytometry analysis showed that αCD5 DIRECTED particles led to approximately 55% loss of B2M protein, whereas the absence of antibody resulted in less than 10% B2M loss [183]. The DIRECTED platform allows the integration of targeting ligands to improve cell-type specific delivery.

Other genome editing tools have delivered in VLPs, such as [184], [185]. ZFNs and TALENs were fused to the N-terminus of Gag to be recruited into the VLPs, using a lentiviral packaging system harboring a mutation that renders the integrase incapable of mediating vector insertion [185]. VLPs facilitated the delivery of ZFNs proteins to cells, promoting gene disruption and homologous recombination *in vitro*. Notably, ZFNs-VLPs exhibited a more favorable on-target/off-target cleavage ratio compared to transfection with plasmid-encoded ZFNs [185]. TALEN-VLPs were also able to mediate gene disruption in cellular models [185]. VLPs were also able to deliver TALENs as mRNA *in vitro*, abrogating the expression of CCR5 and TCR in multiple cell lines [184], suggesting that VLPs can deliver a wide range of genome editing platforms.

Overall, VLPs have been shown to deliver genome editing technologies with high editing efficiency *in vitro*, and increasing evidence shows their therapeutic potential *in vivo.* So far, transient delivery of CRISPR systems through cell-derived vesicles has been shown to reduce off-target editing relative to viral vector-based delivery.

8. Synthetic lipid-based nanoparticles and cell-derived vesicles for the delivery of genome editing tools

Besides EVs and VLPs, other non-viral methods including synthetic LNPs have emerged for the delivery of CRISPR machinery [187], [188]. In Table 2, we compare the main nanoparticle properties between EVs, VLPs and LNPs. LNPs have facilitated the delivery of RNA *in vivo* [187], thus increasing interest in expanding their applicability domain with delivery of CRISPR components, such as mRNA encoding Cas9 and synthetic gRNAs to target cells [187]. LNPs contain controllable lipid moieties in their formulations, mainly constituted of four primary lipid components: ionizable cationic lipids, polyethylene glycol (PEG) lipids, zwitterionic phospholipids, and cholesterol [9], [189]. These components work synergistically to enable payload encapsulation, transport, and cargo delivery following LNPs cellular uptake by endocytosis [190].

LNPs have been demonstrated to deliver various CRISPR-Cas9 components to cells: plasmid DNA (pDNA) that encodes both Cas9 protein and gRNA, pDNA encoding Cas9 protein in combination with synthetic gRNAs, Cas9 mRNA and synthetic gRNAs, and Cas9:gRNA (protein/RNA) RNP complex [187], [188], [191], [192], [193]. Ionizable cationic lipids have been demonstrated to be particularly useful for Cas9 RNP delivery, due to the negative charge of Cas9 RNP in association with the gRNA which leads to a spontaneous assembly due to electrostatic interactions with cationic lipids [13], [187]. There are advantages and limitations to each CRISPR-Cas9 modality, so each delivery approach might require an LNP-specific formulation to ensure optimal cargo/delivery platform compatibility.

LNPs provide a protective environment for CRISPR cargo, shielding it from degradation by nucleases and other cellular components, thereby increasing its stability and bioavailability. However, LNPs have limited cargo capacity [194], limiting the absolute amounts of genome editing components that can be loaded, which may hinder the packaging of larger genome editing technologies. LNPs can be engineered to have low immunogenicity, reducing the risk of triggering an immune response upon delivery into the body. LNP formulations have been optimized to improve particle stability, increase circulation time, reduce toxicity, and lower immunogenicity [195], [196], [197], thanks to the PEG lipids added to the LNPs formulation [9]. A persistent bottleneck of LNPs application is the intrinsic ability to be un-specifically uptaken by different cell types and accumulate mostly in liver and spleen. To overcome this, tissue- and cell-specific targeting has been achieved by modifying LNP surface charge [198] and composition, the last with lipids associated with moieties for targeting specific organs and de-targeting the liver and spleen [198], [199], [200], [201]. Unspecific uptake of LNPs can lead to CRISPR off-targets and raise potential safety concerns that should be carefully evaluated in preclinical studies. While LNPs have been explored to deliver gene therapy and gene editing cargos to various organs, a big challenge yet to overcome is their limited ability to cross biological barriers such as BBB. Studies have demonstrated that LNPs properties can be fine-tuned to enable targeting LNPs to cross the BBB [202]. In conclusion, the homogenous formulation and the easy scalability of LNPs make them a promising delivery platform for CRISPR-Cas9 modalities. However there remain challenges that need to be overcome to untap their full potential and translate their application to the bedside.

In contrast to LNPs, EVs and VLPs have intrinsic tissue-specific tropism depending on the cellular source and are natural carriers of DNA, RNA and proteins, properties that make them suitable for delivering multiplex payloads necessary for genome editing therapeutics. In addition, as natural mediators of cell-cell communications, EVs and VLPs have innate abilities to cross barriers such as BBB. Depending on the cell source (and whether they are from autologous or heterologous origin), EVs can be immunologically silent. This property is being leveraged to mask genome editing cargos from recognition of immune cells. Furthermore, EVs can carry large protein payloads, whereas LNPs are more suitable to deliver nucleic acids. However, a major drawback of EVs is that these are less homogenous than LNPs (e.g. package cytosolic cellular components) and their production has limited scalability due to the necessity of keeping large batches of cells in culture, scalable isolation protocols and need for quality control in each step which make the production streamline longer [203], [204], [205], [206]. EVs require large cultures of producing cells, posing challenges in terms of production costs and regulatory aspects regarding their biological origin [206]. While there is a gap in data directly comparing EVs, VLPs or synthetic LNPs as delivery agents for CRISPR modalities, we believe there is need for case-specific evaluations of the most appropriate vehicle platform depending on target tissue, biological barriers and cargo payloads.

9. Concluding Remarks

Gene editing tools are revolutionizing the way we pursue treatments for genetic disorders by enabling DNA targeting and precise corrections of genetic mutations. In recent years, there has been continuous improvement of CRISPR-Cas9 technologies, with research focusing on

multiple expanding targeting capabilities through the engineering of Cas9 variants [35], [36], [37], [38] increasing specificity and reducing off-target activity [4], [226], [227], [228] and improving their characteristics for optimal delivery [7], [127], [229].

One of the remaining challenges for more widely applicable *in vivo* gene editing is to overcome the barriers for efficient delivery to specific target tissues. Thus far, most applications to deliver gene editing agents *in vivo* outside of the liver have relied on viral vectors, particularly AAVs, which have serotype-dependent tissue tropism and can lead to long-term expression of transgenes [230], [231], [232]. However, they have a limited packaging capacity of approximately 4.7 kilobases, which greatly restricts the ability to encode and deliver large CRISPR-based enzymes along with gRNAs and regulatory elements [233]. While viral vector-based delivery of genome editing technologies can result in overall higher editing efficiencies, it also increases the length of time genomes are exposed to editing enzymes, which can result in increased levels of off-target mutagenesis [6], [80], immunogenicity [81], [82] and integration of the AAV into CRISPR-induced or stochastic DNA DSBs [234]. Addressing these drawbacks would ensure the continued development of safe CRISPR-based therapies. The initial identification of off-target events has led to concerns about adverse consequences, ranging from point mutations, insertions, deletions, inversions, that can lead to loss or gain of function of genes [235], [236]. This has prompted researchers to develop AAV vectors with controllable expression [237], [238], [239] or to directly limit their expression through cell specific promotors or tissue specific capsids [230], [240], [241], [242]. Even though controllable transgene expression can result in a safer profile, these systems can be prone to a certain degree of expression leakiness [243]. Because DNA edits are durable, most genome editing tools can elicit a permanent genomic change from transient expression. Persistent expression from viral vectors *in vivo* is therefore not necessary for most genetic perturbations and often carries the potential for undesirable consequences. To overcome these obstacles, vehicles that allow the delivery of gene-free, short-lived mRNA, protein or RNP coding gene editing agents may be preferable.

Novel delivery vehicles that transiently deliver editing agents without long-term expression in target tissues offer several advantages. Ultimately, transient delivery of mRNA and especially protein and RNPs offers a more favorable safety profile. Transient expression of genome editing tools would allow potent on-target editing and low off-target activity by being rapidly degraded within the cell. Synthetic nanoparticles such as LNPs and cell-derived vesicles such as EVs and VLPs, can protect therapeutic cargo and facilitate entry into cells without resulting in long-term gene expression as observed with viral vectors [244]. However, cell-derived vesicles' cellular origin carries challenges in manufacturing and large-scale production when compared to LNPs that allow a precise control of their composition, easy scalability, and manufacturing [245], [246]. Despite having an edge in these aspects, LNPs still present dose-limiting toxicity, and their efficient delivery is mainly confined to the liver, with their efficiency relying on prolonged circulation and passive cellular uptake [219], [247]. In this regard, cell-derived vesicles present specific surface and luminal signatures derived from producer cells, such as proteins, glycoproteins and other surface interactors that ultimately improve cellular uptake and biocompatibility. When comparing the drug delivery efficiency of EVs and LNPs, EVs were shown to deliver RNAs several orders of magnitude more efficiently than LNPs [224], which can be decisive for

safe delivery of gene editing agents. Additionally, EVs have a low immunogenic profile [85], with several clinical studies in human subjects administered with autologous EVs showing good safety outcomes [206]. However, as delivery vehicles, EVs should be derived from clinical safe grade human cells and each formulation evaluated for its immunogenicity and biocompatibility [248].

VLPs, on the other side, have the potential to induce antibody and cell-mediated adaptive immune responses *in vivo* resulting from the viral scaffolds used in their production [249]. For this reason, VLPs might rely on a single administration or in developing strategies to either reduce their immunogenicity or eliminate neutralizing antibodies form circulation. So far, the use of VLPs for the delivery of gene editing agents relies mostly on retroviral Gag and VSV-G, with some studies using RVG and FuG-B2 [7]. One concern regarding the use of cell-derived nanoparticles, such as EVs and VLPs, is the carry-over of cell-derived cargo such as RNAs and proteins that is dependent on the producer cell state and may alter homeostasis of the receptor cell [250], [251]. The carry-over of unwanted cargos into EVs and VLPs largely depends on the producer cell and may be overcome using a clinical grade cell source compliant with clinical safety [206].

Regarding gene editing efficacy, native EVs were shown to mediate the functional delivery of the CRISPR-Cas9 systems *in vitro*, but available data *in vivo* is limited. EV-based delivery for genome editing is still far from viral-vector based efficacy, with EV-delivered gene editing reaching up to 10% editing at the DNA level *in vitro* [124], [130], [151], with one study achieving 30% indels with surface modified EVs [125]. On the other hand, VLPs have shown much higher gene editing efficacy, reaching up to 97% gene editing at the DNA level *in vitro* [7], with several independent studies showing more than 50% efficacy [128], [129], [174], [177], [180], [182]. Moreover, the use of VLPs *in vivo* also shows promising results, with phenotypical improvements in several disease animal models, such as rescue of visual function in a mouse model of Leber congenital amaurosis [7], [84]. Besides the advantages of high editing efficiency observed with VLPs, they have also been shown to significantly reduce off-target activity when compared to plasmid or viral-vector delivery due to expression and rapid clearance of protein/RNPs [7]. While EVs still require further improvement to increase their delivery efficiency, VLPs have shown potent on-target editing while avoiding off-target mutagenesis. The higher delivery efficacy of VLPs might be related to their increased uptake in recipient cells and higher cytoplasmic delivery, possibly due to the viral scaffolds' properties to escape endosomal degradation. Nevertheless, the fusogenic nature of these proteins might compromise their biodistribution and targetability. Further biodistribution and immunogenicity studies need to be carried out to prove the safety of VLPs administered through non-invasive routes for therapeutic delivery.

10. Future Perspectives

In recent years, both EVs and VLPs have motivated great efforts as delivery vehicles for gene editing tools. Most strategies have relied on endogenous loading in EVs with transmembrane (such CD63 and CD9) and membrane-bound (such as Basp1) proteins while VLPs have mostly relied on retroviral gag or VSV-G proteins. Despite the use of several packaging strategies, the field would still profit from screening additional packaging

proteins that would further increase the number of packaged cargos per particle and the refinement of the optimal affinity to allow cargo release in target cells. To this end, introducing cleavable linkers that allows cargo release from packaging motifs in recipient cells seems to be a critical step towards increasing efficiency of delivery. Another loading strategy which so far has been less investigated is exogenous loading of gene editing agents. Exogenous loading would significantly improve the scalability and manufacturability of cell-derived vesicles loaded with gene editors without relying on complex to establish packaging cell lines and endogenous loading mechanisms that show limitations in cargo release in receptor cells. So far, the critical steps which need to be addressed to efficiently load cargos exogenously in cell-derived vesicles are finding a mechanism to cross the membrane without disrupting nanoparticle integrity and cargo functional stability and avoiding the precipitation of cargos at particles' surface.

While most studies focus on delivery of CRISPR-Cas9 nucleases, EVs and VLPs can be engineered to deliver more precise CRISPR-based strategies, such as BEs, PEs and CEs for which there are additional packaging limitations due to their larger coding sequences. Despite their large size, BEs, PEs and CRISPR activators have been shown to be packaged in VLPs, suggesting that VLPs do not have strict packaging limitations as the most used viral vectors.

So far, the most significant challenges in the field are targetability and endosomal escape. Tissue-tropism of both EVs and VLPs largely depend on passive cellular uptake and specific internalization mechanisms through surface interactors which are ubiquitously expressed. Continued efforts are still required to improve tissue targetability while mitigating unintended targeting of typical filtering organs (from instance the liver, if not the intended target) and optimize minimally invasive administration routes for single and multiple administrations. Upon reaching the target tissue, EVs and VLPs must escape endosomal degradation to allow efficient cargo release within cells. On this matter, VLPs have the upper hand since viral scaffolds such as VSV-G mediates endosomal escape which in the end improves editing efficiency.

Overall, the main challenge of efficient delivery with EVs and VLPs is related to targeting to specific tissues and cell types, evade degradation, and reach their intracellular destination. With the fast pace of development and enhancement of gene editing tools, novel delivery strategies that allow precise targetability while avoiding collateral editing of unwanted tissues will help the translation of gene editing therapies to the clinic.

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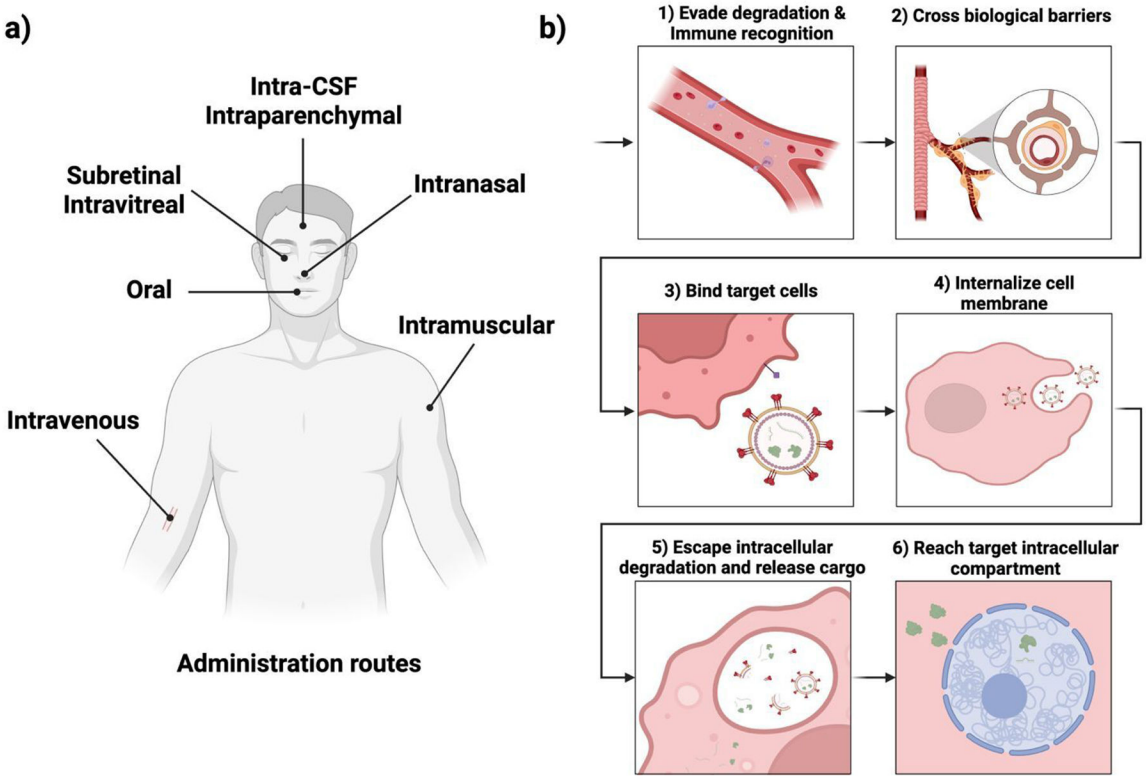

Figure 1. Challenges for in vivo delivery of genome editing technologies.

a) Several administration routes have been used for in vivo delivery of cell-derived vesicles loaded with genome editing agents, such as: intra-CSF, intraparenchymal, subretinal, intravitreal, oral, intravenous, intranasal and intramuscular injections. b) Upon administration, cell-derived vesicles must: 1) evade degradation and immune recognition and 2) cross biological barriers depending on the target tissue. They must then 3) recognize target cells in specific tissues and 4) be readily internalized. Upon internalization, the functional cargo in cell-derived vesicles must 5) escape intracellular degradation and achieve cytoplasmic delivery. Lastly, free therapeutic cargos must 6) reach the target intracellular compartment, specifically the nucleus for CRISPR-Cas9 systems.

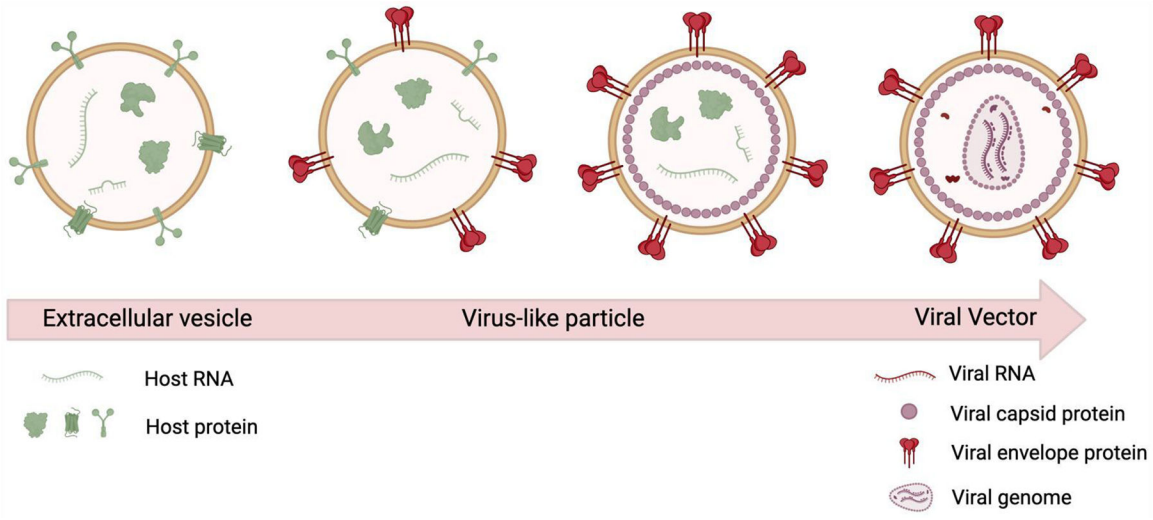

Figure 2. Cell-derived vesicles: from native EVs to VLPs.

Native EVs are related with their producer cell since the lipidic membrane resembles the cell membrane and carries host proteins and nucleic acids. Upon viral infection, or expression of viral components in cells, there is formation of VLPs, which still resemble the host cell while carrying viral scaffolds on their surface and/or lumen, being devoid of viral genome and infectious properties. On the other extreme, there are viral vectors, which carry a viral genome and share structural similarities with virions, without their infectivity properties. Studying cell-derived vesicles typically requires different levels of modulation with viral components to facilitate the loading, internalization, and release of content in the target cell.

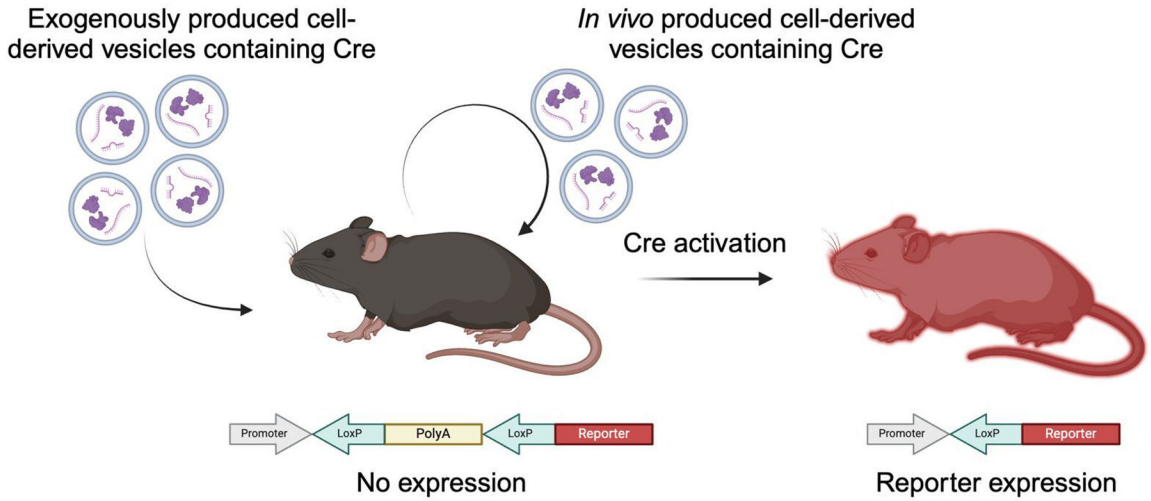

Figure 3. Cre-lox systems to study cell-derived vesicles delivery in vivo.

The Cre-lox system represents a valuable tool for gaining insights into the in vivo fate of cell-derived vesicles. Cell-derived vesicles containing Cre molecules can be produced in a living animal (in vivo produced cell-derived vesicles) or isolated from cell culture media (exogenously produced cell-derived vesicles containing Cre molecules). Both types will benefit from Cre reporter models for studying the endogenous release, organotropism, and uptake of vesicles in different tissues of a living animal. Typically, Cre reporter mouse models are engineered to express reporter proteins following Cre recombination of the reporter DNA. This enables the sensitive detection of functional delivery events that permanent modified the reporter DNA.

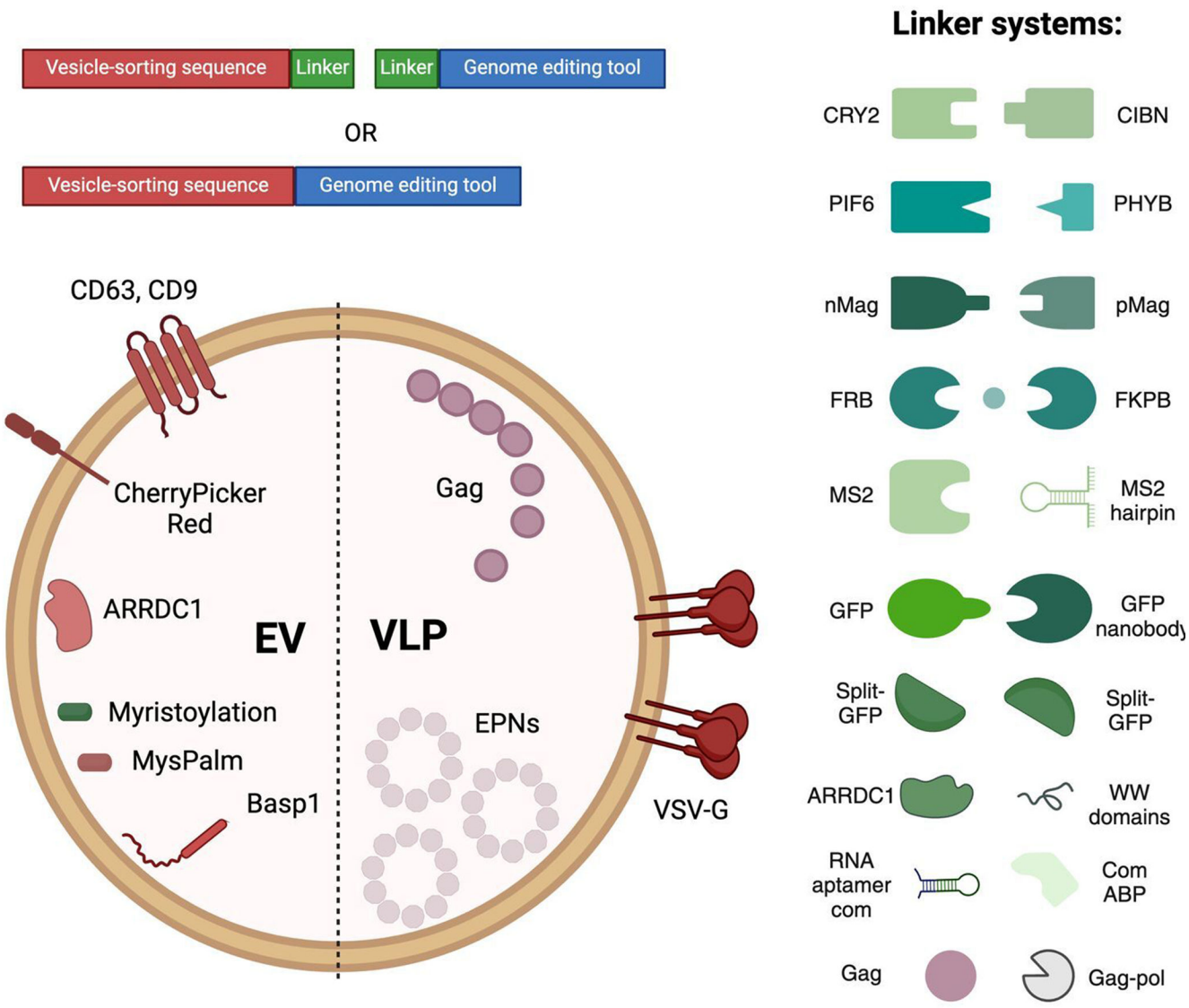

Figure 4. Strategies for the endogenous loading of genome editing tools in cell-derived vesicles.

Endogenous packaging of CRISPR-Cas payloads in cell-derived vesicles has been achieved by fusing an EV-sorting sequences with genome editing RNPs or to a linker system which is then tethered to CRISPR components. EV-sorting proteins and motifs used so far include CD63, CD9, CherryPiker Red, ARRDC1, Myristoylation motif, MysPalm motif, Basp1, Gag, EPNs and VSV-G. Linker systems used include: CRY2 – CIBN; PIF6 – PHYB; nMag – pMag; FRB – FKBP; MS2 – MS2 hairpin; GFP – GFP nanobody; Split GFP; ARRDC1 – WW domains; RNA aptamer com – Com ABP; Gag – Gag pol.

Table 1.

***In vivo* studies using EVs and VLPs for the delivery of CRISPR-Cas agents.**

Abbreviations: ABE – Adenine base editor; ARRDC1 – arrestin domain containing protein 1; BE – Base editor; PE – Prime Editor; dCas9 - dead Cas9; *DMD* – Duchenne muscular dystrophy; eGFP; Enhanced green fluorescent protein; EVs – Extracellular Vesicles; fLUC – Firefly luciferase; GFP - green fluorescent protein; Indels – Insertions and deletions; LPS – Lipopolysaccharide; MMLV – Moloney Murine Leukemia Virus; N.A. – Not applicable; NGS – Next generation sequencing; PCR – Polymerase Chain Reaction; *Mfrp - membrane-type frizzled-related protein; PCSK9 – Proprotein convertase subtilisin/kexin type 9*; qPCR – Quantitative PCR; qRT-PCR Quantitative Reverse Transcription PCR; RNP – Ribonucleoprotein; SaCas9 – Staphylococcus aureus Cas9; gRNA – guide RNA; SpCas9 – Streptococcus pyogenes Cas9; T7E1 – T7 endonuclease 1; tPA – Tissue plasminogen activator; VPR - VP64-p65-Rta transcriptional activator; VSV-G – Vesicular stomatitis virus G protein.

Delivery Vehicle	CRISPR System	Packaging system	Disease	Mouse Model	Target	Administration route	Efficiency	Detection Method	Phenotypic analysis	Off-targets	Reference
EVs	dCas9-VPR RNP	Passive loading	N.A.	BALB/c implanted with HEK293T fLUC cells	fLUC reporter	Intravenous (tail vein injection)	Increase in luciferase activity	Bioluminescence	N.A.	N.A.	[151]
				BALB/c with chemical induced hepatotoxicity	*Hgf*	Intravenous (Hydrodynamic tail vein injection)	Increased HGF expression Reduction in liver damage markers	ELISA	N.A.	N.A.	
EVs	dCas9-VPR RNP	Passive loading	N.A.	C57BL/6 mice	*HNF4α*	Intravenous (tail vein injection)	Increased HNF4α mRNA and protein expression (liver tissue)	qPCR & western blot	N.A.	N.A.	[152]
EVs	SpCas9 RNPs	CD63-GFP SpCas9-GFP nanobody	N.A.	tdTomato reporter mouse	*STOP-tdTomato*	Intravenous (tail vein injection)	TdTomato expression in liver	Microscopy	N.A.	N.A.	[155]
EVs (with surface targeting ligand)	SpCas9 RNPs	Sonication and repeated freeze-thaw cycles	Xenograft tumor	BALB/c nude mice bearing HepG2 xenograft	*WNT10B*	Intravenous	Suppression of tumor development (tumor volume and weight)	Xenograft analysis	N.A.	N.A.	[125]
							Up to 23% indels in tumors	High-throughput sequencing			
Serum EVs	SpCas9 RNPs	Protein transfection CRISPRMAX	N.A.	Ai9 mice	*TdTomato*	Tibialis anterior muscle injection	20% of fibers expression TdTomato	IHC	N.A.	N.A.	[166]
							Up to ~8.7%Ai9 gene mutation	qPCR			

Delivery Vehicle	CRISPR System	Packaging system	Disease	Mouse Model	Target	Administration route	Efficiency	Detection Method	Phenotypic analysis	Off-targets	Reference
							Indels	Sanger sequencing			
							Deletion of exons 23 and 24	PCR			
							18.6% restored dystrophin in fibers	IHC			
			DMD	Rag/mdx mice	*Intron 22 and 24 of Dmd gene*	Tibialis anterior muscle injection	Up to 19% deletion of exons 23 and 24 in cDNA transcripts	qRT-PCR	N.A.	N.A.	
							Detection of edited band	PCR			
			DMD	hDMD/mdx mice	*Exon 50 and 54 of human DMD gene*	Tibialis anterior muscle injection	Edited gene	Sanger sequencing	N.A.	N.A.	
			Acute lung injury/ acute respiratory distress syndrome model	C57BL/6 mice with intratracheal instillation of LPS	*IL-6, TNF and IL-1 (3 gRNAs)*	Intrabronchial administration	Reduced IL-6, IL-1β and TNF mRNA expression in lung tissue and bronchoalveolar lavage fluid	qPCR	Reduced infiltration of monocytic cells and neutrophils, septal thickening, interstitial edema, and collagen accumulation in the bronchioles and alveoli Ameliorated lung injury score	N.A.	
EVs	Cas9Rx RNPs	tPA-Cas9Rx							Prolonged survival Reduced septal thickening, interstitial edema, and collagen accumulation in the lung tissue Reduced aggregation of inflammatory cells in the		[186]
			Septicemia model	C57BL/6 and BALB/c	*IL-6, TNF and IL-1 (3 gRNAs)*	Intraperitoneal injection	N.A.	N.A.		N.A.	

Delivery Vehicle	CRISPR System	Packaging system	Disease	Mouse Model	Target	Administration route	Efficiency	Detection Method	Phenotypic analysis	Off-targets	Reference
									liver Alleviated damage in renal tubular cells		
VLPs (VSV-G)	SpCas9 RNP	com (aptamer)-gRNA Com-CD63-Com (aptamer binding protein)	DMD	del52hDMD/ mdx mice	DMD exon 53	Local injection in tibialis anterior muscle	0.2% indels rates Dystrophin expression	NGS Immunostaining	N.A.	N.A. N.A.	[180]
	ABE8e RNPs		N.A.	C57BL/6J mice	*Dnmt1*	ICV injection (neonatal mice)	6.1% editing in cortex (53% in GFP$^+$ sorted cells) 4.4% editing in mid-brain (55% in GFP$^+$ sorted cells)		N.A.	N.A. N.A.	
v4 VLPs (MMLV-Gag, MMLV protease, VSV-G)	ABE8e-NG RNPs	MMLV-Gag-BE with linker peptide (cleaved by MMLV protease)			*Pcsk9*	Intravenous (retro-orbital injection)	63% editing in bulk liver 21% correction of the R44X mutation	Illumina MiSeq sequencing	78% reduction of Pcsk9 in serum Minimal rescue of visual function	No detectable off-target editing in 14 sites (CIRCLE-seq) Substantial levels of bystander editing	[7]
	ABE7.10-NG RNPs		Leber congenital amaurosis	rd12mouse	*Rpe65*	Subretinal injection	12% correction of the R44X mutation		Rescued visual function	No bystander editing No RNA off-target editing at Perp and Mcm3ap	
v3 PE-eVLPs	PE3 RNPs	PE recruitment via P3-P4 interaction; gRNA recruitment via MCP:MS2 interaction	N.A.	C57BL/6J mice	*Dnmt1*	ICV injection (neonatal mice)	2.3 % editing in cortex (36% in GFP$^+$ sorted cells)	Illumina MiSeq sequencing	N.A.	N.A.	[84]
v3b PE-eVLPs	PE3 RNPs	PE recruitment via P3-P4	N.A.	C57BL/6J mice	*Dnmt1*	ICV injection (neonatal mice)	3.2 % editing in cortex (47% in	Illumina MiSeq sequencing	N.A.	N.A.	

Delivery Vehicle	CRISPR System	Packaging system	Disease	Mouse Model	Target	Administration route	Efficiency	Detection Method	Phenotypic analysis	Off-targets	Reference
							GFP$^+$ sorted cells)				
v3 PE3b-eVLPs	PE3b RNPs	interaction; gRNA recruitment via COM:COM interaction	autosomal recessive retinal degeneration	*rd6* mouse	*Mfrp*	Subretinal injection	15% correction of the 4-bp deletion	Illumina MiSeq sequencing	Restoration of MFRP protein expression	No detectable off-target editing in 10 sites (CIRCLE-seq)	
	PE3b RNPs		Leber congenital amaurosis	*rd12* mouse	*Rpe65*	Subretinal injection	7.2% correction of the Rpe65 R44X mutation	Illumina MiSeq sequencing	Partial rescue of visual function	No detectable off-target editing in 10 sites (CIRCLE-seq)	
VLPs (MLV-Gag, VSV-G)	SpCas9 RNPs	Chemical dimerization of Gag-FKBP12 to SpCas9-FRB	DMD	Luc reporter mice (CAG-Luc2-hDMD Ex45 KI)	*SA and SD sites of exon 45 in the human dystrophin gene (2 gRNAs)*	Gastrocnemius muscle injection	Luciferase activity after 3 days and sustained up to 160 days	IVIS imaging	N.A.	N.A.	[129]
							Up to 7% exon skipping	RT-PCR			
							~ 7% genomic deletion	MiSeq deep sequencing			
					mdx point mutation near SA and SD sites in dystrophin gene (2 gRNAs)		1.1% deletion	PCR			
			DMD	Mouse model of DMD		Tibialis anterior muscle injection	1.6% exon 23 skipping	RT-PCR	N.A.	N.A.	
VLPs (MLV-Gag, MLV protease, VSV-G, BaEVRless)	SpCas9 RNPs	MMLV-Gag-SpCas9 (cleaved by MLV protease)	Hereditary tyrosinemia type I	(NODFah−/−/ Rag2−/−/ Il2rg−/−) mice	*Hpd*	Intravenous (retro-orbital injection)	Between 7% and 13% editing in liver	T7E1 assay	N.A.	N.A.	[177]
VLPs (VSV-G)	SaCas9 RNPs	VSV-G-GFP11 and Sa-Cas9-GFP1-10 ± CD47-GFP11	N.A.	BALB/c mice	*Pcsk9*	Intravenous (tail vein injection)	Significant reduction of serum Pcsk9 and LDL levels	ELISA, LDL-Cholesterol kit	N.A.	N.A.	[159]
VLPs (VSV-G)	SpCas9 RNPs	Passive loading	N.A.	eGFP transgenic mice	*eGFP*	Intracardiac injection	~30% of GFP negative cardiomyocytes	Microscopy	N.A.	N.A.	[174]

Table 2.

Comparison of nanoparticles' properties of extracellular vesicles (EVs), virus-like particles (VLPs) and lipid nanoparticles (LNPs).

Several properties impact the effectiveness of delivery vehicles: immunogenicity, loading capacity and efficiency, protein and nucleic acid delivery, natural ability to cross biological barriers, half-life in circulation, potential for engineering targeting moieties, stability, scalability, cost of production, overall engineering capacity, clinical safety, heterogeneity, cell uptake, endosomal escape, complexity of regulatory landscape, among others. For simplicity, "++" indicates very favorable feature, "+" a favorable feature, and "-" an unfavorable feature.

NANOPARTICLE PROPERTIES	EXTRACELLULAR VESICLES (EVS)	VIRUS-LIKE PARTICLES (VLPS)	LIPID NANOPARTICLES (LNPS)	REFERENCES
LOW IMMUNOGENICITY	++	−	+	[85], [207], [208], [209]
LOADING CAPACITY	+	+	+	[84], [125], [210]
LOADING EFFICIENCY	+	+	++	[211], [212]
PROTEIN PACKAGING	++	++	−	[7], [129], [186]
LARGE NUCLEIC ACIDS PACKAGING	−	−	++	[210], [213]
SMALL NUCLEIC ACIDS PACKAGING	+	+	+	[97], [159], [214]
NATURAL ABILITY TO CROSS BIOLOGICAL BARRIERS	+	+	−	[96], [215], [216]
HALF-LIFE	−	−	+	[119], [217]
ENGINEERING TARGETING MOIETIES	+	+	−	[7], [198], [218]
STABILITY	−	−	+	[206], [219], [220]
SCALABILITY	−	−	++	[206], [219]
LOW COST OF PRODUCTION	−	−	++	[206], [219]
MULTIPLE ENGINEERING CAPACITY	++	++	−	[7], [218], [221]
CLINICAL SAFETY	+	−	++	[206], [222]
HOMOGENEITY	−	−	++	[118], [223]
NATURAL CELL UPTAKE	+	++	−	[159], [224]
NATURAL ENDOSOMAL ESCAPE	−	++	+	[113], [146], [225]
SIMPLICITY OF REGULATORY LANDSCAPE	−	−	++	[206], [219], [222]

Table 3.

In vitro *studies using EVs and VLPs for the delivery of CRISPR-Cas systems.*

Abbreviations: ABE – Adenine base editor; ARRDC1 – arrestin domain containing protein 1; BE – Base editor; BMDMs – Bone marrow-derived macrophages; CCK8 – Cell Counting Kit-8; CIBN - Cryptochrome-interacting basic helix-loop-helix 1 truncated version; CRY2 - Cryptochrome 2; dCas9 - dead Cas9; DMD – Duchenne muscular dystrophy; eBFP – Enhanced blue fluorescent protein; eGFP ; Enhanced green fluorescent protein; EVs – Extracellular Vesicles; fLUC – Firefly luciferase; FMLV – Friend; Murine Leukemia Virus; GFP - green fluorescent protein; HBB - human beta haemoglobin; HBE – human bronchial epithelial cells; HIV - human immunodeficiency virus; HSCs - Hepatic stellate cell; HSPCs – hematopoietic stemand progenitor cells; Indels – Insertions and deletions; iPSCs – Induced pluripotent stem cell; LPS – Lipopolysaccharide; MEFs – Mouse embryonic fibroblasts; MMLV – Moloney Murine Leukemia Virus; MysPalm - Myristoylation-Palmitoylation-Palmitoylation; N.A. – Not applicable; N2a – Neuro2a cells; NanoLuc – Nano lucifesase; NF-κB – Nuclear factor kappa B; NGS – Next generation sequencing; NOS2- nitric oxide synthase 2; NuFF – Human Newborn Foreskin Fibroblasts; PCR – Polymerase Chain Reaction ; *PCSK9 – Proprotein convertase subtilisin/kexin type 9*; PHH – primary human hepatocytes; PINK1 – PTEN induced kinase 1; qPCR – Quantitative PCR; qRT-PCR Quantitative Reverse Transcription PCR; RFP – Red fluorescent protein; RNP – Ribonucleoprotein; RSV – Respiratory syncytial virus; SaCas9 – Staphylococcus aureus Cas9; gRNA – guide RNA; SpCas9 – Streptococcus pyogenes Cas9; T7E1 – T7 endonuclease 1; tPA – Tissue plasminogen activator; VPR - VP64-p65-Rta transcriptional activator; VSV-G – Vesicular stomatitis virus G protein.

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
EVs VSV-G	SaCas9 RNP	com (aptamer)-gRNA Com-CD63-Com (aptamer binding protein)	HBB-IL2RG-eGFP^{-}	HEK293T	±25% GFP positive cells	Flow Cytometry	N.A.	[180]
					48.3% indels rate	NGS		
	SpCas9 RNP				±11% GFP positive cells	Flow Cytometry		
			DMD-eGFP^{-}	HEK293T	51.4% indels rate	NGS		
	ABE		Site 5	HEK293T	39.7% indels rate	NGS		
			Site 5	MDA-MB-231	24.2% indels rate			
	SaCas9 RNP		DMD intron 50 DMD intron 51	N.A.	Target sequence removal	PCR		
			DMD intron 50	N.A.	Target sequence removal			
	SpCas9 RNP		DMD intron 53	N.A.				
EVs	SpCas9 RNPs	CD63-GFP SpCas9-GFP nanobody	Stop DsRed	A549	Target sequence removal	Sanger Sequencing	N.A.	[131]
					DsRed expression	Microscopy		

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
EVs	SpCas9 RNPs	CD63-mCherry SpCas9-mCherry nanobody	Stop GFP	HBE	GFP expression	Microscopy	N.A.	[155]
				A549				
[ARRDC1]-mediated microvesicles	Cas9 RNPs	WW-Cas9 interaction with ARRDC1	GFP$^+$	U2OS	13.4% GFP negative cells (4.8% controls)	Flow cytometry	N.A.	[124]
					indels	T7E1 Assay Sequencing		
EVs VSV-G	Cas9 RNP	Protein myristoylation (octapeptide from Src kinase)	eGFP	HEK293T	42% eGFP loss	Flow cytometry	RPS27 (RNA sequencing)	[175]
					indels	Sanger Sequencing		
EVs	Cas9 RNP	Cas9-CRY2 & MysPalm-CIBN (Dimerization system)	LoxP sites	HEK293T	42% RFP expression	Fluorescence microscopy	N.A.	[130]
			PCSK9	HEK293T	4.4% gene editing	NGS	N.A.	
EVs	Cas9 RNP	Passive loading	*MYD88*	HEK293T	10.1% indels	T7E1 Assay	N.A.	[151]
	dCas9-VPR RNP	Passive loading	fLUC reporter	HEK293T	Increase in luciferase activity	Bioluminescence	N.A.	
			ACTC1	HEK293T N2a	ACTC1 mRNA fold-change	qPCR	N.A.	
EVs	dCas9-VPR RNP	Passive loading	*HNF4α*	HSCs	Increased HNF4α mRNA and protein expression	qPCR & western blot	N.A.	[152]
v1 VLPs (FMLV-Gag, FMLV protease, VSV-G)	ABE8e RNPs	FMLV-Gag-BE with linker peptide (cleaved by FMLV protease)	*Site 2*	HEK293T	>97% editing	Illumina MiSeq sequencing	N.A.	[7]
			Site 3					
			BCL11A	HEK293T	73% editing			
v4 VLPs (MMLV-Gag, MMLV protease, VSV-G)	ABE8e RNPs	MMLV-Gag-BE with linker peptide (cleaved by MMLV protease)	*BCL11A*	HEK293T	95% editing	Illumina MiSeq sequencing	N.A.	
			Dnmt1	3T3 mouse fibro-blasts	>90% editing			
			BCL11A + Site 2	HEK293T	>95% Site 2 editing >75% BCL11A editing			
v4 VLPs (MMLV-Gag, MMLV protease VSV-G)	SpCas9 RNPs	MMLV-Gag-SpCas9 with linker peptide (cleaved by MMLV protease)	*EMX1*	HEK293T	>95% editing	Illumina MiSeq sequencing	N.A.	
v4 VLPs (MMLV-Gag, MMLV protease, FuG-B2)	ABE8e RNPs	MMLV-Gag-BE with linker peptide (cleaved by MMLV protease)	*Dnmt1*	Neuro-2a	>80%	Illumina MiSeq sequencing	N.A.	

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
v4 VLPs (MMLV-Gag, MMLV protease, VSV-G)	ABE8e RNPs	MMLV-Gag-BE with linker peptide (cleaved by MMLV protease)	*BCL11A*	HEK293T	>70%	Illumina MiSeq sequencing	5% OT1 0-2% OT2	
			Site 2		>95%		0-2% OT1	
			Site 3		>95%		0-2% OT1 0-2% OT2 0-2% OT3	
v4 VLPs (MMLV-Gag, MMLV protease, VSV-G)	ABE8e RNPs	MMLV-Gag-BE with linker peptide (cleaved by MMLV protease)	N.A.	HEK293T	N.A.	Illumina MiSeq sequencing	0-1% Cas-independent off-target	
v4 VLPs (MMLV-Gag, MMLV protease, VSV-G)	ABE8e RNPs	MMLV-Gag-BE with linker peptide (cleaved by MMLV protease)	*COL7A1*	Primary human fibroblasts	>95%	Illumina MiSeq sequencing	Minimal Cas-dependent off-targets in 10 sites	
			Idua	Primary mouse fibro-blasts	>95%		N.A.	
			B2M	primary human T cells	>45%		N.A.	
			CIITA		>55%			
VLPs (MLV-Gag-MS2, MLV protease, VSV-G)	SpCas9 mRNA and gRNAs	MLV-Gag-MS2 binding to stem loop sequence on RNAs (SpCas9 and gRNAs)	*RFP657.Tet2*	NIH3T3 RFP657.Tet2 reporter cells	~45% RFP negative cells	Flow cytometry	N.A.	[182]
				HT1080 RFP657.Tet2 reporter cells	~45% RFP negative cells			
			CXCR4	Jurkat cells	20-45% CXCR4 negative cells	Flow cytometry	N.A.	
					~43% indels	T7E1 assay	N.A.	
			TP53	DsRed^{+} NuFF cells	Growth advantage	Growth advantage in co-cultures by DsRed^{+} cells	N.A.	
					Up to 85% indels	Sanger Sequencing		
VLPs (avian RSV-based a.Gag-MS2, VSV-G)	SpCas9 mRNA and gRNAs	Avian RSV-based a.Gag-MS2 binding to stem loop sequence on RNAs (SpCas9 and gRNAs)	*RFP657.Tet2*	HT1080 RFP657.Tet2	Up to 75% knockout	Flow cytometry	N.A.	[128]
				iPSC-based RFP657.Tet2	Up to 55% knockout	Flow cytometry	N.A.	
			RFP657.Tet2 + CXCR4	Jurkat RFP657.Tet2	37-53% double knockout	Flow cytometry	N.A.	

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
			RFP657.Tet2 + CXCR4 + eGFP	Jurkat RFP657.Tet2 and eGFP	Up to 31% triple knockout	Flow cytometry	N.A.	
			TP53	NuFF	Up to 93% indels	Sanger Sequencing	N.A.	
			TP53	PHH	Up to 55% indels	Sanger Sequencing	N.A.	
			TP53	hCD34+HSPCs	Up to 36% indels	Sanger Sequencing	N.A.	
			TP53	CF1 MEFs	31% indels	Sanger Sequencing	N.A.	
			TP53	C3H MEFs	25% indels	Sanger Sequencing	N.A.	
	SpCas9 mRNA and gRNAs Donor template		*Knock-in of eBFP in eGFP sequence*	HT1080	2% eBFP$^+$ cells	Flow cytometry	N.A.	
				NuFF	2% eBFP$^+$ cells	Flow cytometry	N.A.	
VLPs (MLV-Gag, VSV-G)	SpCas9 RNPs	Chemical dimerization of GAG-FKBP12 to SpCas9-FRB	*Dystrophin in EGxxFP reporter gene*	HEK293T	>15% GFP$^+$ cells	Flow cytometry	N.A.	[129]
			Dystrophin in EGxxFP reporter gene	C2C12 mouse myoblast cells	>25% GFP$^+$ cells	Flow cytometry	N.A.	
			Dystrophin in EGxxFP reporter gene	Hu5 human myoblast cells	>50% GFP$^+$ cells	Flow cytometry	N.A.	
			Dystrophin	iPSCs	>50% indels	T7E1 assay	N.A.	
			Dystrophin (2 gRNAs)	Healthy iPSCs	Up to 38% deletion	PCR amplification	N.A.	
					>40% indels	T7E1 assay		
				DMD patient iPSCs	Up to 36% deletion	PCR amplification	N.A.	
					>60% indels	T7E1 assay		
				DMD iPSCs derived skeletal muscle cells	Up to 92% exon 45 skipping	PCR amplification	N.A.	
					Restoration of dystrophin expression	ProteinSimple Wes	N.A.	
			VEGFA	HEK293T	32.5% indels	T7E1 assay	Nearly eliminated	

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
			EMX1	HEK293T	Up to 20% indels	T7E1 assay	Nearly eliminated	
VLPs (VSV-G)	SpCas9 RNPs	Heterodimerization of CherryPicker Red (with DmrA domain) to SpCas9 (with DmrC domain)	LTR of HIV	HIV-NanoLuc CHME-5	8% indels	Sanger Sequencing	0.6%	[176]
					Decreased luminescence	Luminescence assay		
					Decreased in viral protein Nef	WES and immunofluorescence		
VLPs (MMLV-Gag, MMLV protease, engineered VSV-G, targeting antibody)	SpCas9 RNPs	MMLV-Gag-SpCas9 with linker peptide (cleaved by MMLV protease)	*B2M*	Jurkat E6 cells	Up to ~55% loss of B2M protein	Flow cytometry	N.A.	[183]
VLPs (MLV-Gag, MLV protease, VSV-G, BaEVRless)	SpCas9 RNPs	MMLV-Gag-SpCas9 (cleaved by MLV protease)	*EMX1*	HEK293T	Up to 76.6% editing	Sanger Sequencing	Not detected at MFAP1 off-target site	[177]
				hiPSCs	67% editing	Illumina MiSeq sequencing	N.A.	
			GFP	Mouse GFP BMDMs	75% reduction GFP	Flow cytometry	N.A.	
					60-65% editing	T7E1 assay	N.A.	
			EMX1	Mouse primary bone marrow cells	71% editing	T7E1 assay	N.A.	
			Fto locus		76% editing	Sanger Sequencing	N.A.	
			Myd88 (2 gRNAs)	human hematopoietic stem cells	Deleted amplicon	PCR	N.A.	
					50% editing	T7E1 assay		
	SpCas9 RNPs, DNA template		*DDX3 (flag knock-in)*	HEK293T	Flag tag insertion	PCR	N.A.	
						Immunoprecipitation & Western blot		
			AAVS1 (puromycin resistance gene knock-in)	HEK293T	Puromycin cassette insertion	PCR	N.A.	
						Puromycin selection		
	SP-dCas9-VPR RNPs	MMLV-Gag-SP-dCas9-VPR (cleaved by MLV protease)	*promoter region of human Titin*	MCF-7	Up to 200-fold increase expression (2 gRNAs)	RT-PCR	N.A.	
					Up to 400-fold increase			

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
					expression (4 gRNAs)			
	SpCas9 RNPs	MMLV-Gag-SpCas9 (cleaved by MLV protease)	*Tyr*	Mouse zygote	Gene editing in 16 of 40 blastocysts	T7E1 assay	N.A.	
						HinfI restriction		
			Tyr (2 gRNAs)	single-cell embryos	5 of 8 F0 mice with gene editing (up to 78%)	PCR	N.A.	
						Sanger Sequencing		
VLPs (VSV-G)	SaCas9 RNPs	VSV-G-GFP11 and Sa-Cas9-GFP1-10	*PINK1*	Venus-Parkin HeLa cells	40% reduction of cells positive for Parkin recruitment	Confocal microscopy	N.A.	[159]
				PINK1-eGFP HeLa Cells	Partial loss of GFP signal	Flow cytometry	N.A.	
						Western blot		
EVs	SpCas9 mRNA and gRNAs	Electroporation	*human mir-125b-2 locus*	MOLM13 cells	~98% reduction of miR-125b and 90% reduction of miR-125a	qRT-PCR	N.A.	[126]
					indels	HiSeq		
VLPs (VSV-G)	SpCas9 RNPs	Passive loading	*eGFP*	HEK293T eGFP cells	~50% of eGFP negative cells	Cytometry	N.A.	[174]
			CXCR4	HEK293T cells	~60% editing	Sanger Sequencing	N.A.	
			VEGFA		~30% editing		Background levels of indels at 2 off-target sites	
							1 off-target (GUIDE-seq)	
			eGFP (2 gRNAs)	HEK293T eGFP cells	17% deletion of the eGFP locus	PCR	N.A.	
			eGFP	iPSCs eGFP	~30% of eGFP negative cells	Flow Cytometry	N.A.	
				J-Lat-A1 eGFP	~14% of eGFP negative cells			
				HeLa eGFP	~12% of eGFP negative cells			
	SpCas9 nickase RNPs		*eGFP (2 gRNAs)*	HEK293T eGFP cells	~50% of eGFP negative cells	Flow Cytometry	N.A.	

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
VLPs (VSV-G, Gag or MinimalGag)	SpCas9 RNPs	Gag-SpCas9 or MinimalGag-SpCas9	*eGFP*	HEK293T eGFP cells	~40% of eGFP negative cells	Flow Cytometry	N.A.	
			CXCR4	HEK293T cells	~30-40% of editing	Sanger Sequencing	N.A.	
			VEGFA		~35-40% of editing			
EVs (with surface targeting ligand)	SpCas9 RNPs	Sonication and repeated freeze-thawcycles	*GFP*	GFP HepG2 cells	43% reduction of GFP fluorescence	Flow Cytometry	N.A.	[125]
			WNT10B	HepG2 cells	Reduction of WNT10B protein expression	Western Blot	N.A.	
					41% reduction of cell viability	CCK8 viability assay		
					30% indels	Sequencing		
			WNT10B	Human liver organoids from patients with primary liver cancer	~60% apoptosis of tumor organoids	Tumor inhibition assay (caspase3/7 probe)	N.A.	
EVs	SpCas9 RNPs	Protein transfection CRISPRMAX into EVs	*TdTomato*	HeLa/Ai9 cells	17% TdTomato^{+} cells	Flow cytometry	N.A.	[166]
					Deletion of poly(A) sequence	PCR		
				N2a/Ai9 cells	6.4% TdTomato^{+} cells	Flow cytometry		
EVs	Cas9Rx RNPs	tPA-Cas9Rx	*mCherry*	HEK293T mCherry cells	~60% supression of mCherry fluorescence	Fluorescence microscopy	N.A.	[186]
				N2a mCherry cells				
			VEGFA	HEK293T	~80% reduction mRNA	qPCR	N.A.	
				N2a	~50% reduction mRNA			
				GL261	~50% reduction mRNA			
				Bend.3	~60% reduction mRNA			

Delivery Vehicle	Gene Editing Agent	Packaging system	Target	Cell line	Efficiency	Detection method	Off-targets	Publication
			IL-6, *TNF* and *IL-1* (3 gRNAs)	Raw264.7 cells	Reduced mRNA levels of IL-6, IL-1β and TNF	qRT-PCR	N.A.	
					Downregulated; NF-κB , JAK-STAT and TNF signaling pathways, and cytokine–cytokine receptor interaction pathway	RNA sequencing		
					Downregulated IL-6, IL-1β, TNF, NOS2, CD86 and NF-κB levels			
				BMDM (LPS stimulation)	~70% reduction of NOS2 (marker of activated macrophages) expression	Western Blot		
					Lower ratio of $CD86^+$ cells (marker of M1 phenotype macrophages)	Flow cytometry		