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SHORT COMMUNICATION

Extracellular vesicle-mediated delivery of CRISPR/Cas9 ribonucleoprotein complex targeting proprotein convertase subtilisin-kexin type 9 (Pcsk9) in primary mouse hepatocytes

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Abstract

The loss-of-function of the proprotein convertase subtilisin–kexin type 9 (*Pcsk9*) gene has been associated with significant reductions in plasma serum low-density lipoprotein cholesterol (LDL-C) levels. Both CRISPR/Cas9 and CRISPR-based editor-mediated *Pcsk9* inactivation have successfully lowered plasma LDL-C and PCSK9 levels in preclinical models. Despite the promising preclinical results, these studies did not report how vehicle-mediated CRISPR delivery inactivating *Pcsk9* affected low-density lipoprotein receptor recycling in vitro or ex vivo. Extracellular vesicles (EVs) have shown promise as a biocompatible delivery vehicle, and CRISPR/Cas9 ribonucleoprotein (RNP) has been demonstrated to mediate safe genome editing. Therefore, we investigated EV-mediated RNP targeting of the *Pcsk9* gene ex vivo in primary mouse hepatocytes. We engineered EVs with the rapamycin-interacting heterodimer FK506-binding protein (FKBP12) to contain its binding partner, the T82L mutant FKBP12-rapamycin binding (FRB) domain, fused to the Cas9 protein. By integrating the vesicular stomatitis virus glycoprotein on the EV membrane, the engineered Cas9 EVs were used for intracellular CRISPR/Cas9 RNP delivery, achieving genome editing with an efficacy of ±28.1% in Cas9 stoplight reporter cells. Administration of Cas9 EVs in mouse hepatocytes successfully inactivated the *Pcsk9* gene, leading to a reduction in *Pcsk9* mRNA and increased uptake of the low-density lipoprotein receptor and LDL-C. These readouts can be used in future experiments to assess the efficacy of vehicle-mediated delivery of genome editing technologies targeting *Pcsk9*. The ex vivo data could be a step towards reducing animal testing and serve as a precursor to future in vivo studies for EV-mediated CRISPR/Cas9 RNP delivery targeting *Pcsk9*.

KEYWORDS
cholesterol-lowering therapy, CRISPR/Cas9 delivery, exosomes, extracellular vesicles, gene therapy, increased LDL-C uptake, LDLR recycling, Pcsk9 inactivation

Zhiyong Lei and Joost P. G. Sluijter contributed equally to this work.

1 | INTRODUCTION

Therapeutic gene editing using the CRISPR/CRISPR-associated 9 (CRISPR/Cas9) system offers the potential for permanent modification of disease-causing genes (Jinek et al., 2013; Scott & Zhang, 2017). Among various techniques, delivering the CRISPR/Cas9 ribonucleoproteins (RNPs) directly into cells has shown fewer off-target mutations and reduced immunogenicity (Doman et al., 2020; Lattanzi et al., 2019; Liang et al., 2015; Newby et al., 2021; Suresh et al., 2017). This approach is particularly appealing because the transient presence of CRISPR/Cas9 RNPs allows for quicker and safer gene editing compared to using CRISPR/Cas9 encoded mRNA or plasmids.

However, the physicochemical properties of CRISPR/Cas9 RNPs, such as their large size and hydrophobic nature, pose challenges for intracellular delivery. Different viral delivery systems, including lentiviruses, adenoviruses, and adeno-associated viruses, have a limited packaging capacity to carry CRISPR/Cas9 RNP or are non-biocompatible affecting the RNP's integrity, stability, and function (Lee et al., 2017). As a result, developing a biocompatible delivery system for CRISPR/Cas9 RNP delivery could enable safer genome editing.

Extracellular vesicles (EVs) are natural nanocarriers in our bodies, facilitating cell-to-cell communication by transporting macromolecules. Comprising a lipid bilayer, EVs encapsulate a variety of biological materials from their parent cells, such as nucleic acids, lipids, RNAs, and proteins (Kanada et al., 2015; Zomer et al., 2015). Their biocompatibility, low immunogenicity, and innate ability to target and influence recipient cells make EVs a promising delivery system. The efficacy of using EVs for CRISPR/Cas9 RNP delivery hinges on the efficient loading of RNPs into donor cell EVs and their subsequent release into target cells. Prior research has utilized the vesicular stomatitis virus glycoprotein (VSV-G) and the rapamycin-interacting protein complex FKBP12/FRB for successful CRISPR/Cas9 RNP loading and delivery via EVs in vitro and in vivo (Campbell et al., 2019; Gee et al., 2020; Ilahibaks, Ardisasmita et al., 2023; Ilahibaks, Roefs et al., 2023; Montagna et al., 2018). However, the broader therapeutic potential of FKBP12/FRB/VSV-G engineered EVs as a delivery vehicle for CRISPR/Cas9 RNP remains underexplored.

Individuals carrying nonsense variants in the proprotein convertase subtilisin–kexin type 9 (*Pcsk9*) gene have considerably reduced plasma low-density cholesterol (LDL-C) levels with an 88% reduced risk of developing coronary heart disease (Cohen et al., 2006). *Pcsk9* is predominantly expressed in the liver and promotes LDL receptor (LDLR) degradation to the lysosome. Therapeutic intervention introducing a *Pcsk9* loss-of-function prevents LDLR degradation. It promotes LDLR recycling, enabling more LDL-C to be cleared from circulation (Figure 1). Currently, market-approved anti-PCSK9 monoclonal antibodies, alirocumab, and evolocumab, require administration every 2–4 weeks as monotherapy or add-on to statin therapy to reduce LDL-C levels over 50% and reduce the risk of developing cardiovascular events (Ray et al., 2020; Sabatine et al., 2017; Schwartz et al., 2018). Additionally, subcutaneously administration of a small-interfering RNA targeting *Pcsk9*, inclisiran, on day 1, day 90, and every 6 months after, reduced LDL-C levels up to 51% in patients with atherosclerotic cardiovascular disease (Ray et al., 2020). Although these PCSK9 interventions significantly lowered LDL-C plasma levels and the risk of developing adverse cardiovascular events, they require frequent administration. Given the genetic and pharmacological evidence that targeting *Pcsk9* is not associated with adverse health outcomes, it is an interesting target to develop a single-administration therapy by targeting *Pcsk9* with CRISPR/Cas9. Researchers have successfully delivered CRISPR/Cas9 or CRISPR-base editors via viral-, gold or lipid nanoparticles to disrupt *Pcsk9* as a potential cholesterol-lowering therapy in vivo (Chadwick et al., 2017; Ding et al., 2014; Jiang et al., 2017; Lee et al., 2023; Li et al., 2021; Musunuru et al., 2021; Rothgangl et al., 2021; Wang et al., 2016; Zhang et al., 2019). Although these reports successfully demonstrate LDL-C lowering in blood serum in vivo, these and other *Pcsk9* intervention studies did not show the functional effects of their treatment on LDLR recycling in vitro or ex vivo on different cellular levels. We therefore investigated whether FKBP12/FRB/VSV-G-engineered EVs could successfully deliver CRISPR/Cas9 RNPs targeting *Pcsk9* in primary mouse hepatocytes and analyzed the effects on different cellular levels.

In this study, different sgRNAs targeting *Mus musculus Pcsk9* were screened for their on-target activity, and the most effective combination for EV-mediated CRISPR/Cas9 RNP delivery targeting *Pcsk9* was selected. Administration of EV-mediated CRISPR/Cas9 RNP delivery led to reduced *Pcsk9* mRNA and increased LDLR levels, resulting in increased LDL-C uptake. These findings indicated that EV-mediated CRISPR/Cas9 RNP delivery successfully targets the *Pcsk9* gene, increasing LDLR recycling in vitro. Furthermore, these assays can be used as functional read-outs to evaluate further the efficacy of genome editing technologies that target *Pcsk9*.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human embryonic kidney 293FT (HEK293FT), NIH3T3, and Cas9 stoplight reporter cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, 41965-039, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin

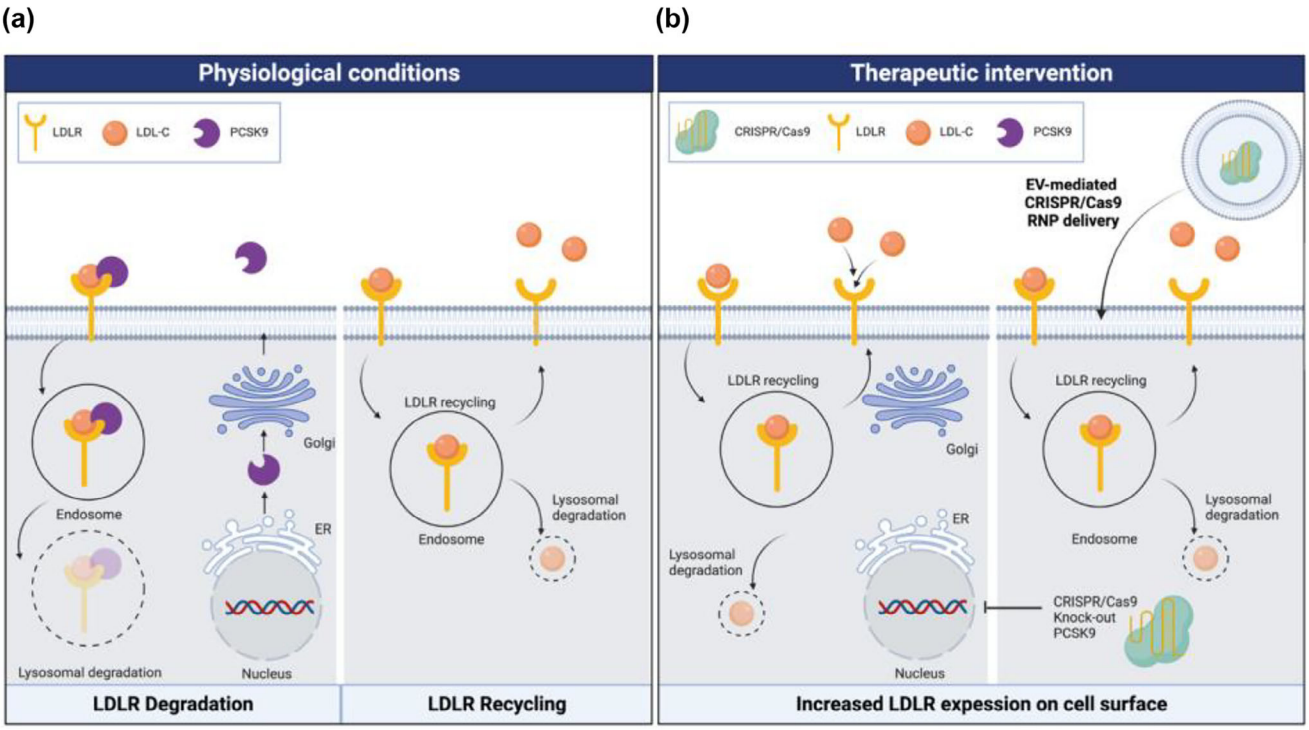

FIGURE 1 Schematic illustration of EV-mediated CRISPR/Cas9 RNP delivery knocking out *Pcsk9* leads to increased LDLR recycling and LDLR expression on the cell surface. (a) PCSK9 binds the LDLR/LDL-C complex and marks it for degradation by the lysosome. In the absence of PCSK9, the LDLR is free to bind and process plasma LDL-C, whereafter, it is recycled back to the membrane surface to clear more LDL-C from circulation. (b) Upon extracellular vesicle-mediated CRISPR/Cas9 delivery, knock-out of the *Pcsk9* gene would reduce the LDLR/LDL-C complex's degradation and increase LDLR recycling.

(P/S). Mouse hepatocytes were cultured as previously described, with modifications for 2D culture (Peng et al., 2018). Hepatocytes were either freshly isolated from mouse liver or derived from previously cultured and dissociated hepatocyte organoids. Cells were cultured on collagen-coated plastics in culture medium consisting of William's Medium E supplemented with 1% Glutamax, 1% Non-Essential Amino Acids, 1% P/S, 0.2% Normocin, 2% B27 supplement, 1% N2 supplement, 10 mM nicotinamide, 1.25 mM N-acetylcysteine, 10 μM Y27632, 1 μM A38-01, 3 μM CHIR99021, 25 ng/mL EGF, 0.1 μM dexamethasone and 2% FBS (expansion medium). 3–4 days before experiments, cells were switched to a maturation medium consisting of an expansion medium without CHIR99021 and supplemented with 3 μM dexamethasone. All cells were maintained at 37°C in a 5% CO_2 environment.

2.2 | Plasmids and gRNA

The plasmid, N-Myr-2xFKBP12/Cas9-FRB, was assembled by procuring sequences for FKBP12, N- Myristylation signal, and T82L mutant FRB from Integrated DNA Technologies. The origins of these sequences can be traced back to DrmA and DrmC in the iDimerize™ Inducible Heterodimer System (635067, Takara Bio). In the subsequent steps, these sequences were subcloned into the pcDNA5/FRT/TO® expression vector (K65001, ThermoFisher Scientific). This cloning was conducted in accordance with the guidelines provided in the manufacturer's manual for the NEBuilder® HiFi DNA assembly master mix (E2621L, New England Biolabs (NEB)). Next, Cas9 was cloned at the N-terminal of FRB with a GGSGG linker. The gRNAs utilized in this research were identified through the CHOPCHOP web tool and manual examination of the donor splicing site surrounding exon 1 or 2 in the Mus Musculus *Pcsk9* gene (refer to Table S1). Subsequently, the oligomers of the respective gRNAs underwent phosphorylation and annealing using T4 polynucleotide kinase from NEB. This process was carried out with a thermocycler protocol that included a 30-min incubation at 37°C, 5 min at 95°C, and then a gradual decrease to 25°C at a rate of 5°C per minute. Once annealed, the oligos were diluted in nuclease-free water and cloned into a BsmBI-digested pLentiCRISPR v2 backbone sourced from Addgene using T4 DNA ligase from NEB. Any residual linearized DNA was subsequently eliminated with Plasmid-Safe™ ATP-Dependent DNase from VWR. This was achieved by diluting the ligation mixture (11 μL) with 10 mM ATP (1.5 μL), 10× Plasmid-Safe™ buffer (1.5 μL), and Plasmid-Safe exonuclease (1 μL). The resulting reaction mixture was then incubated at 37°C for 30 min and at 70°C for another 30 min.

2.3 | Cas9 EV production and isolation

HEK293FT cells (1×10^7 cells/T175 flask) were seeded and cultured overnight. The HEK293FT cells were transiently transfected with the N-Myr-2xFKBP12/Cas9-FRB (15 μg), gRNA (15 μg), and VSV-G plasmid (15 μg) in a 1:2 DNA lipid ratio following the manufacturer's instructions of Lipofectamine® 3000 Transfection Reagent (L3000008, Invitrogen Corp.). After 6 h, the media was replaced with DMEM (Gibco) supplemented with exosome-depleted 10% FBS (A272080, Gibco), 1% P/S, and 500 nM of A/C Heterodimizer (635056, Takara). After 72 h, the media was collected and centrifuged at 2000 × *g* for 15 min at 4°C to remove cells and cell debris. The supernatant was recovered and centrifuged at 10,000 × *g* for 30 min at 4°C to further remove cell debris with an Open-Top Thinwall Polypropylene Tube (326823, Beckman Coulter) using an SW 32 Ti Swinging-Bucket (369694, Beckman Coulter). The supernatant was isolated and centrifuged at 100,000 × *g* for 70 min at 4°C to pellet the Cas9 EVs. The Cas9 EVs pellet was recovered by suspending the pellet in PBS, filtered through a 0.45 μm syringe SFCA membrane filter (516-1954, Corning), and stored at 4°C.

2.4 | Lentiviral production and transduction

In a 6-well plate, HEK293FT (400,000 cells/well) were seeded and incubated overnight. The next day, HEK293FT were transfected with gRNA- pLentiCRISPR v2 (1 μg), CMV (1 μg), and VSV-G (0.5 μg) plasmid in a 1:2 DNA:Lipid ratio with Lipofectamine® 3000 Transfection Reagent (L3000008, Invitrogen), following manufacturer's instructions. In a 24-well plate 48 h post-transfection, HepG2 cells (200,000 cells/well) and NIH3T3 cells (100,000 cells/well) were seeded and cultured overnight. Seventy-two hours post-transfection, the conditioned media of the transfected HEK293FT cells were collected, centrifuged at 2000 × *g* for 5 min, and the supernatant was filtered through a 0.45 μm SCFA membrane syringe filter (516-1954, Corning). Subsequently, the media containing lentivirus were added to the NIH3T3 cells. After 72 h, the media were replaced with DMEM supplemented with 10% FBS, 1% P/S, and 2 μg puromycin (A1113803, ThermoFisher Scientific). The cells were cultured with puromycin over 2 weeks.

2.5 | T7E1 assay

DNA was obtained by following the manufacturer's GeneJET Genomic DNA Purification Kit protocol (K0721, Thermo Scientific). Genomic DNA was PCR amplified using Q5® Hot Start High-Fidelity 2× Mastermix (M0494L, NEB) with respective primers listed in Table S2. The PCR product was denatured and reannealed using a thermocycler with NEBuffer 2.1 Buffer according to the manufacturer's instructions. Next, the 1 μL of T7 Endonuclease (T7E1, NEB) the reaction mixture and incubated at 37°C for 1 h. The PCR product(s) were loaded on a 2% Agarose TAE gel and analyzed using the ChemiDoc™ XRS+system (Bio-Rad) and Image Lab™ software. ImageJ software determined the mutation frequency using the following formula: (1-(1-f) ^{½}) * 100% = indel % with f representing the fraction of cleaved/uncleaved PCR product.

2.6 | Cas9 stoplight reporter assay

In a 96-well plate (655075, Greiner CELLSTAR), Cas9 stoplight reporter cells (10.000 cells/well) were seeded and cultured overnight. The next day, Cas9 EVs were administrated to the Cas9 stoplight reporter cells. Seventy-two hours post-administration, the cells were visualized with EVOS Cell Imaging System (M5000, Invitrogen) and analyzed by flow cytometry (CytoFlex, Beckman Coulter).

2.7 | Transmission electron microscopy

Cas9 EVs were produced by transiently transfecting HEK293FT donor cells cultured in the presence of the rapamycin-orthologue, isolated via differential ultracentrifugation, and suspended in PBS. Carbon-coated grids (75−200 mesh) were used to integrate Cas9 EVs for 15 min at RT. Next, the grids were washed with PBS and fixated with 2% PFA. Subsequently, the grids were treated with 0.2% glutaraldehyde in PBS for 30 min at room temperature. Uranyl-oxalate was used for staining, followed by embedding the grids in 1.8% methylcellulose and 0.4% uranyl acetate for 10 min on ice. The Cas9 EVs were visualized with a JEOL 3 microscope.

2.8 | Nanoparticle tracking analysis

Concentrated EV samples were diluted in PBS to determine the size and particle concentration with nanoparticle tracking analysis with a 405 nm laser (NS500, Malvern Nanosight). EVs were diluted in PBS for 30–100 tracks per frame. Measurements were performed in triplicate with individual measurements of 30 s at camera level 15, with a minimal track length of 10, detection threshold of 5, and screen gain of 1. Data analysis was performed with NTA software 3.3.

2.9 | Western blot

Lentivirus or EV-treated NIH3T3 were starved for 24 h before obtaining cell lysates. Similarly, EV treated mouse hepatocytes were cultured for 4 days in induction media followed by a 24 h incubation with maturation medium supplemented with 0.5% instead of 2% FBS before obtaining cell lysates. Cell lysates and EVs were lysed in RIPA lysis buffer (20-188, Sigma) supplemented with Protease/Phosphatase inhibitor cocktail (5872S, Cell Signaling Technology). Cell lysate samples were centrifuged at 14,000 x g for 10 min at 4°C. The supernatant was recovered and stored at −20°C. Protein concentrations were determined via Micro BCA Protein Assay Kit (23235, ThermoFisher). Samples were reduced with NuPAGE™ Sample Reducing Agent (NP0004, Invitrogen Corp) together with LDS sample buffer (Life Technologies), Cas9 samples included 5% 2-mercaptoethanol and heated at 95°C for 10 min. Samples were separated on Bolt™ 4–12% Bis-Tris Plus Gel (NW04125BOX, ThermoFisher Scientific), except Cas9 samples which were separated on NuPAGE™ 3–8% Tris-Acetate Protein Gel (EA0378BOX, ThermoFisherScientific), with a PageRuler Plus Prestained Protein Ladder (26619, ThermoFisher Scientific) at 130 V for 75 min. Proteins were transferred to PVDF membranes (IPVH00010, Merck). Membranes were blocked for 1 h in 5% Bovine Serum Albumin (BSA) (w/v) in Tris buffered saline (TBS).Primary antibodies included anti-rabbit DmrA (635089, Clontech), anti-mouse Cas9, (NBP2-36440, Novus Biologicals, clone 7A9-3A3), anti-rabbit TSG101 (30871, Abcam), anti-mouse CD63 (8219, Abcam), mouse anti-syntenin (TA504796, Origene), mouse anti-CD81 (SC-166029, Santa Cruz), anti-mouse PCSK9 (#MA5-32843, ThermoFisher, clone 2F1), anti-rabbit LDLR (#MA5-32075, ThermoFisher, clone SJ0197) and mouse anti-β-actin (Cell Signaling Technology, clone 8H10D10). Secondary antibodies included Alexa Fluor 680-conjugated anti-mouse antibody (LI-COR Biosciences, A-21057) and IRDye 800CW anti-rabbit antibody (1926–322, LI-COR Biosciences). Imaging was performed on an Odyssey Infrared Imager (LI-COR Biosciences) at 700 and 800 nm.

2.10 | LDL-C uptake assay

In a 96-well plate, mouse hepatocytes (10,000 cells/well) were seeded and cultured overnight. Subsequently, the media of mouse hepatocytes was changed to maturation media supplemented with 0.5% FBS. After 24 h, the medium was refreshed and supplemented with 15 μg/mL pHrodo™ Red-LDL (L34356, ThermoFisher). After 4 h, pHrodo™ Red-LDL uptake was visualized with EVOS FL Cell Imaging System (LifeTechnologies) and quantified with flow cytometry analysis (Fortessa).

2.11 | Flow cytometry analysis

For flow cytometry quantification, cells were washed with PBS and dissociated with 0.25% Trypsin-EDTA solution (Sigma-Aldrich, T4049). In a 96-well round bottom plate (650185, Greiner CELLSTAR ®), the cells were collected by centrifugation at 500 × g for 3 min and resuspended in 250 μL PBS supplemented with 2% FBS. Flow cytometry analysis for the Cas9 stoplight reporter assay was performed with CytoFlex (Beckman Coulter). Flow cytometry analysis for the LDL-C uptake assay was performed with Fortessa (BD Biosciences). The data was analyzed by Kaluza software v2.1 (Beckman Coulter Inc.).

2.12 | Statistical test

Statistical analysis was performed with GraphPad PRISM v.9.3. Comparisons between two groups were analyzed with a two-tailed unpaired t-test, and comparisons between multiple groups were analyzed by ordinary one-way ANOVA. Data is represented as mean ± SEM with p-values * <0,05 and ** <0,01 were considered statistically significant.

3 | RESULTS

3.1 | Characterization of Cas9 EVs

To produce Cas9 EVs, we transiently transfected HEK293FT cells with the N-Myr-2xFKBP12/Cas9-FRB, gRNA, and VSV-G plasmids (Figure 2a), subsequently, we cultured the cells in the presence of a rapamycin-orthologue. After 72 h, we collected the conditioned media and isolated the Cas9 EVs via differential ultracentrifugation (Figure 2b). The mean EV size, determined via nanoparticle tracking analysis (NTA), was 113,2 ± 2,3 nm (Figure 2c). Transmission electron microscopy showed that Cas9 EVs have a typical EV 'cup-shaped' morphology (Figure 2d). To verify the presence of Cas9, VSV-G, FKBP12, β-actin, and EV-marker proteins, we compared the protein composition of Cas9 EVs together with the cell lysate (CL) of their producer cells. Western blot analysis showed the presence of Cas9, FKBP12, and VSV-G in both cell lysate and isolated EV lysates (Figure 2e). In contrast, the isolated EVs were highly enriched for EV-marker proteins TSG101, Synthenin-1, CD81, and CD63 compared to the CL. These observations demonstrated that we successfully produced nano-sized EVs highly enriched with Cas9.

We next assessed the capacity of Cas9-loaded extracellular vesicles (Cas9 EVs) to deliver intracellular CRISPR/Cas9 RNPs to mediate genome editing in Cas9 stoplight reporter cells. Successful on-target non-homologous end-joining (NHEJ), resulting in a +1 nt or +2 nt frameshift, led to permanent eGFP expression in the Cas9 stoplight reporter cells (de Jong et al., 2020). We hypothesized that isolating Cas9 EVs from donor cells via differential ultracentrifugation would eliminate the rapamycin orthologue from the sample by a combination of time and procedural steps. Consequently, this would enable the FRB-Cas9 complex to disassociate from the FKBP anchor within the EV membrane upon uptake by the recipient cells. The residual rapamycin orthologue if there is any, will be further diluted facilitating its subsequent release into the cytosol of the recipient cell.

Microscopic analysis confirmed that the administration of 1×10^{10} particles of Cas9 EVs led to on-target NHEJ events, as observed by the presence of eGFP+ stoplight reporter cells (Figure 2f). Further confirmation of the intracellular delivery of CRISPR/Cas9 RNPs by Cas9 EVs was obtained through flow cytometry analysis, which revealed an on-target NHEJ efficiency of approximately 28.1% in Cas9 stoplight reporter cells (Figure 2g). These findings highlight the efficacy of Cas9 EVs as vehicles for the intracellular delivery of CRISPR/Cas9 RNPs, promoting NHEJ events.

3.2 | Selection of optimal gRNAs targeting *Mus musculus Pcsk9*

To target *Pcsk9* effectively, used gRNAs require a high on-target affinity with the genomic location to enable effective CRISPR/Cas9 genome editing. The variability of gRNAs for on-target activity remains a significant limitation. Accordingly, we tested several gRNAs targeting the exon regions or exon splice donor sites of *Mus musculus Pcsk9* (MMgRNA) (Figure 3a, Table SI). We investigated the on-target activity of the gRNAs by transducing NIH3T3 cells with a lentivirus carrying Cas9 and MMgRNA encoded plasmids and selected successfully transduced cells through puromycin selection. Subsequently, we isolated the DNA of edited cells and quantified the percentage of NHEJ through a T7 endonuclease 1 (T7E1)-based cleavage assay.

Quantification of the T7E1-cleaved PCR products showed that *Pcsk9* exon-targeting by MMgRNA 2 and 5 had the highest indel mutation frequency of 45.1 and 45.3%, respectively (Figure 3b). Regarding the MMgRNAs' targeting splice donor sites, MMgRNA 9 and 10 had an NHEJ frequency of 40.5 and 34.8%.

3.3 | EV-mediated delivery of CRISPR/Cas9 RNP targeting mouse *Pcsk9*

To investigate the Cas9 EV efficacy in mouse hepatocytes in vitro, we freshly isolated hepatocytes from mice and previously established 3D hepatocyte organoids and cultured these for several passages in 2D (Peng et al., 2018). Before adding the EVs, we switched the cells to a maturation medium to induce hepatocyte gene expression comparable to primary mouse hepatocytes. We selected MMgRNAs 5 and 9 for their on-target *Pcsk9* activity, together with MMgRNA 8, which was recently demonstrated to be effective in vivo (Ding et al., 2014). First, we matured the mouse hepatocytes for 3 days and administrated Cas9 EVs carrying either non-targeting gRNA (NTgRNA), MMgRNA 5, 8, or 9. We subsequently expanded the EV-treated mouse hepatocytes and cultured them in a maturation medium for 3–4 days before the functional read-out assays.

We also investigated the EV-mediated CRISPR/Cas9 RNP on-target NHEJ activity in mouse hepatocytes via the T7E1 assay (Figure 3c). Administration of 5×10^{11} particles of Cas9 EVs^{MMgRNA9} resulted in the highest frequency of on-target indel frequency of 21.1% compared to Cas9 EVs^{MMgRNA5} and Cas9 EVs^{MMgRNA8} which achieved 0.5% and 11.3%, respectively (Figure 3d). We subsequently investigated if the EV treatment in mouse hepatocytes led to a reduction of *Pcsk9* mRNA levels. MMgRNA 9 targets the splice donor site of exon 1, disrupting pre-mRNA processing leads to mRNA degradation. RT-qPCR of *Pcsk9*, normalized by GAPDH, confirmed that *Pcsk9* mRNA levels were reduced in mouse hepatocytes treated with Cas9 EVs^{MMgRNA9} compared

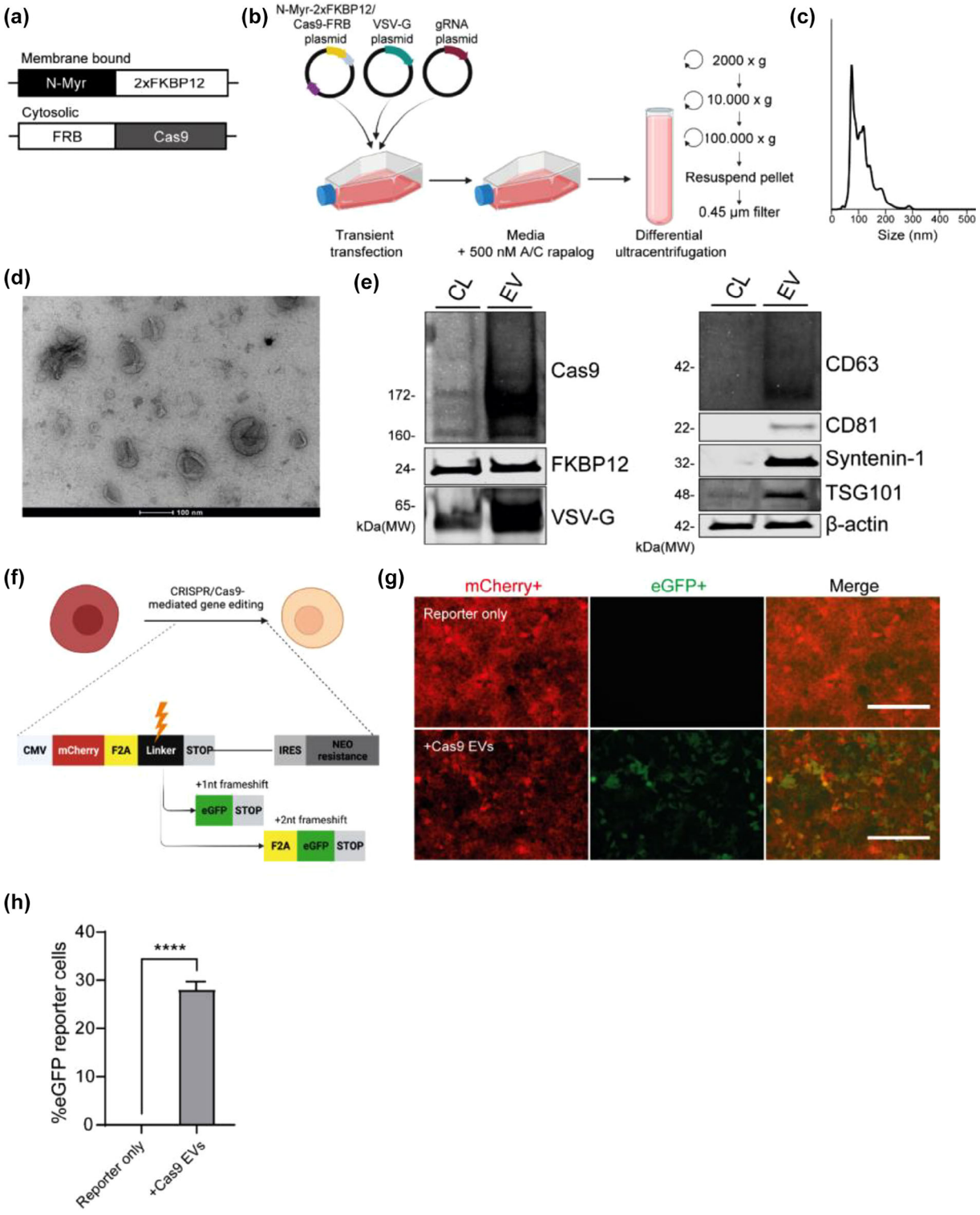

FIGURE 2 **EVs mediate CRISPR/Cas9 RNP delivery in vitro** (a) Schematic illustration of N-Myr-2xFKBP12/Cas9-FRB construct and (b) Cas9-EV production by transiently transfecting HEK293FT cells with N-Myr-FKBP12/FRB-Cas9, VSV-G and gRNA plasmids followed by differential ultracentrifugation. (c) NTA showed Cas9 EVs had a size distribution between 50-250 nm with a mean peak size of 113,2 ± 2,3 nm. (d) TEM images showed Cas9 EVs had an average size around 100 nm. Scale bar = 100 nm (E) Western blot analysis demonstrated Cas9 EVs are enriched with Cas9 protein, FKBP12, VSV-G and are positive for EV marker TSG101, CD63, CD81, synthenin-1 and β-actin. (f) Schematic illustration of CRISPR/Cas9-mediated NHEJ leading to eGFP+ expression in Cas9 stoplight reporter cells. Administration of Cas9 EVs demonstrated successful CRISPR/Cas9-mediated genome editing in Cas9 stoplight reporter cells visualized with (g) fluorescence microscopy analysis and (H) quantified by flow cytometry analysis with an efficiency of ±28,1%. Representative images of four individual experiments. Data presented as Mean ± SEM, analyzed using t-test with $^{*}p < 0{,}05$ and $^{**}p < 0{,}01$. Scale bar = 200 μm.

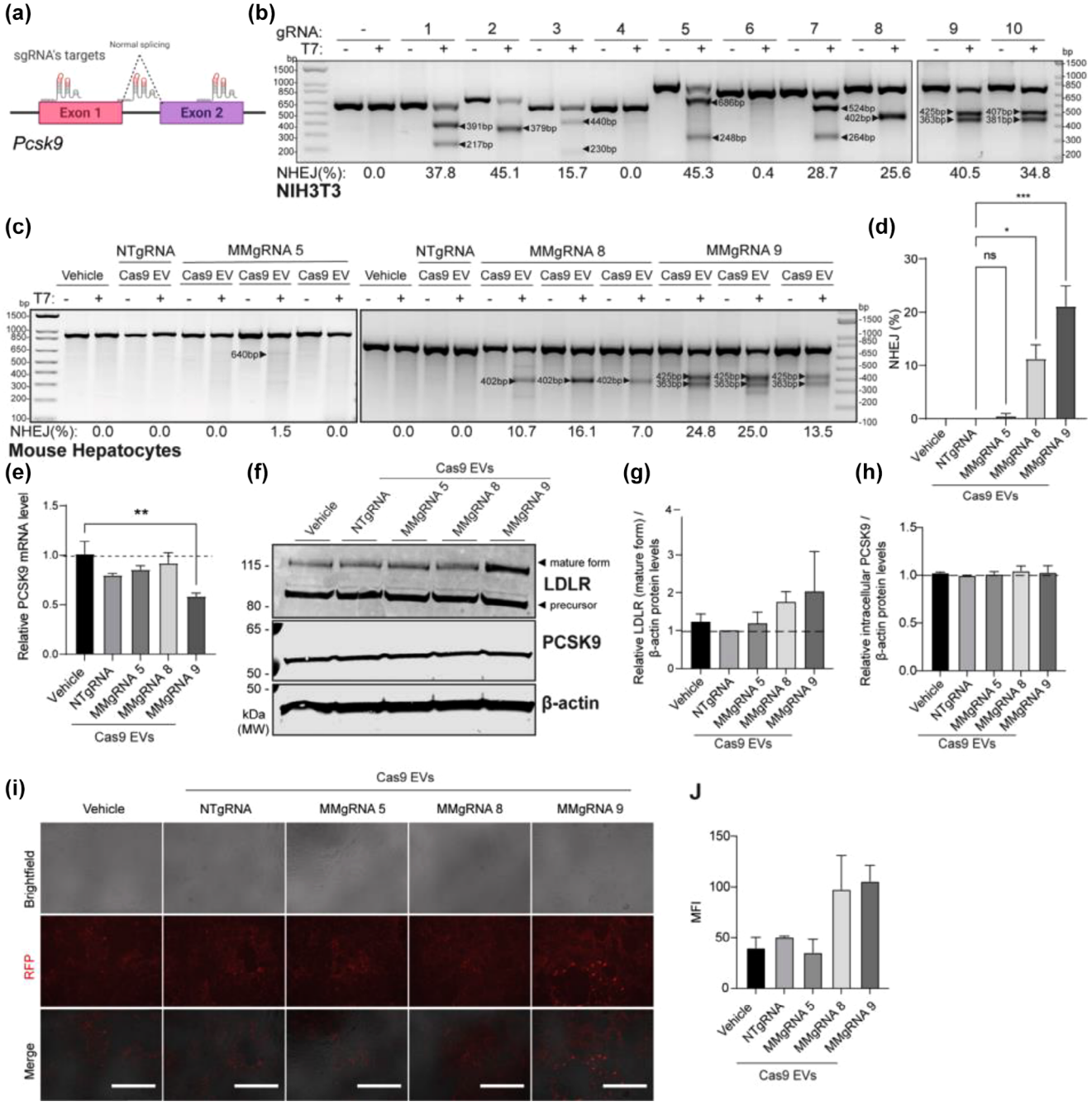

FIGURE 3 Cas9 EV mediated delivery of CRISPR/Cas9 successfully targets *Pcsk9* in mouse hepatocytes leading to increased LDLR levels and LDL-C uptake (a) Schematic illustration of MMgRNA targeting the *Pcsk9* gene (b) Agarose gel of T7E1-treated PCR products amplified from lentivirus treated NIH3T3 cells show NHEJ frequencies for the different gRNA's (c) Quantification of on-target indel mutation frequency by respective MgRNA's from lentivirus transduced cells (d) Agarose gel of T7E1-treated PCR products amplified from mouse hepatocytes treated with EVs carrying either NTgRNa, MMgRNA 5, 8 or 9 (D) Quantification of NHEJ activity of Cas9 EV^{MMgRNA} treated mouse hepatocytes (e) RT-qPCR of relative *Pcsk9* mRNA levels showed MMgRNA 9 successfully targets the exon splicing donor slice thereby interfering with *Pcsk9* pre-mRNA processing. (f) Western blot analysis was conducted on Cas9 EV-treated mouse hepatocytes using antibodies against LDLR, PCSK9, and β-actin. (g) Quantification of LDLR levels, normalized to β-actin, demonstrated that administration of Cas9 EVs loaded with gRNA promoted the expression of the mature form of LDLR. (h) Quantification of intracellular PCSK9 levels, also normalized to β-actin, suggested that PCSK9 protein levels remained relatively unchanged 3–4 days post-administration of the Cas9 EVs. (H) Microscopic analysis and (i) flow cytometry analysis of pHrodo uptake of TOP-EV treated mouse hepatocytes shows mouse hepatocytes treated with Cas9 TOP-EV carrying MMgRNA 8 or 9 results in a higher uptake of LDL-C compared to Cas9 EV^{NTgRNA} treated hepatocytes. Data expressed as mean ± SEM, analyzed using with ordinary one-way ANOVA with $^{*}p < 0.05$ and $^{**}p < 0.01$. Scale bar = 200 μm.

to Cas9 EVs^{NTgRNA} treated cells (Figure 3e). These findings demonstrated that Cas9 EVs^{MMgRNA9} treatment effectively targeted *Pck9* and resulted in aberrant *Pcsk9* mRNA levels.

Following treating mouse hepatocytes with Cas9 EVs, we evaluated the intracellular levels of PCSK9 and LDLR proteins. Western blot analysis showed that Cas9 EVs loaded with either gRNA 5, 8, or 9 increased the mature form of LDLR levels by 1.2 ± 0.4, 1.8 ± 0.4, and 2.0 ± 1.5-fold, respectively, in comparison to cells treated with Cas9 EVs carrying NTgRNA (Figure 3f, g). In contrast, PCSK9 protein levels remained relatively unchanged 3–4 days post-administration of the Cas9 EVs in the cell lysates.

Subsequently, we investigated via pHrodo LDL-C whether Cas9 EV treatment promoted LDL-C uptake. Both microscopic and flow cytometry analyses confirmed increased LDL-C uptake in the Cas9 EV-treated mouse hepatocytes (Figure 3i, j). Correspondingly, LDL-C uptake was the highest for Cas9 EV^{MMgRNA8} Cas9 EV^{MMgRNA9} and treated hepatocytes compared to the other Cas9 EVs treated cells. These findings corroborate the potential of EV-mediated delivery of CRISPR/Cas9 RNP in successfully targeting the *Pcsk9* gene in mouse hepatocytes, resulting in diminished Pcsk9 mRNA levels, increased LDLR protein expression, and subsequently, augmented LDL-C uptake, with no substantial impact on intracellular PCSK9 protein levels 3–4 days after administration.

4 | DISCUSSION

Although cholesterol-lowering therapies targeting *Pcsk9* reduce plasma LDL-C and the risk of developing adverse cardiac events, they require frequent administration to exert their therapeutic effect. Previous studies successfully inactivated *Pcsk9*, reducing PCSK9 and LDL-C serum levels in preclinical models (Ding et al., 2014; Musunuru et al., 2021; Rothgangl et al., 2021). Gene editing technologies can be developed as a single administration therapy to inactivate *Pcsk9*. Different drug delivery vehicles have been investigated to deliver plasmid- or mRNA-encoded CRISPR/Cas9 or CRISPR base editor to inactivate *Pcsk9* in vivo (Ding et al., 2014; Musunuru et al., 2021; Rothgangl et al., 2021). However, the intracellular delivery of the CRISPR/Cas9 RNPs complex is considered safer to mediate genome-editing than plasmid- or mRNA payloads, owing to RNPs transient presence in the cytosol leading to limited off-target mutagenesis effects and limited immunogenicity (Doman et al., 2020; Lattanzi et al., 2019; Liang et al., 2015; Newby et al., 2021; Suresh et al., 2017).

The investigated delivery method for intracellular CRISPR/Cas9 RNP delivery, include physical transduction (Hung et al., 2018; Kalebic et al., 2016; Kim et al., 2017), receptor-mediated (Rouet et al., 2018), induced transduction by osmocytosis (iTOP) (D'Astolfo et al., 2015; Kholosy et al., 2021), and Transmembrane Internalization Assisted by membrane filtration (TRIAMF) (Yen et al., 2018), nanoclews (Sun et al., 2015), cell-penetrating peptides (Ramakrishna et al., 2014), and nanoparticle delivery methods, such as gold-Lee et al., 2017; Mout et al., 2017; Wang et al., 2017), porous silica-Chae et al., 2022), virus-like-Banskota et al., 2022; Mangeot et al., 2019) and lipid nanoparticles (Wang et al., 2016; Wei et al., 2020; Zuris et al., 2015). While these various delivery methods have advanced gene therapy, they face limitations. For instance, physical transduction may cause cell damage, while receptor specificity requirements limit receptor-mediated transduction. Moreover, nanoparticle delivery methods, though versatile, can present challenges in terms of potential toxicity, non-specific distribution, and can possibly elicit adverse immune responses due to their synthetic nature. EVs may offer a promising alternative given their inherent biocompatibility (Gee et al., 2020; Vader et al., 2016; Zomer et al., 2016). In contrast to conventional delivery systems, EVs have all the desirable advantages, such as low toxicity, low immunogenicity, high stability in circulation, and biological barrier permeability (Elsharkasy et al., 2020; Saleh et al., 2019).

In this study, we employed rapamycin-interacting protein heterodimers FKBP12/FRB and VSV-G to load and deliver CRISPR/Cas9 RNP through EVs. Montagna et al. showed that the FKBP12/FRB system and VSV-G successfully delivered CRISPR/Cas9 RNP to inactive GFP-expression in iPSC cells, with comparable efficacy to Cas9 EVs mediating NHEJ in Cas9 stoplight reporter cells (Montagna et al., 2018). While several papers have demonstrated the effects of targeting *Pcsk9* via lipid nanoparticles (Jiang et al., 2017; Lee et al., 2023; Musunuru et al., 2021; Rothgangl et al., 2021), viral-mediated delivery (Chadwick et al., 2017; Li et al., 2021; Wang et al., 2016), or gold nanoclusters (Zhang et al., 2019-mediated CRISPR/Cas9 delivery, primarily in in vivo models, we present an efficient approach wherein CRISPR/Cas9 was actively incorporated into EVs to target *Pcsk9*, examining its impact at a multicellular level. Although light-induced dimerization has been previously utilized to deliver the Cas9 protein inside HEK293 cells to achieve *Pcsk9* targeting via EV, the outcomes were assessed through indel frequency measurements (Osteikoetxea et al., 2022). Few papers have provided a detailed cellular analysis in a relevant cell line across varied functional levels. We demonstrate that our EV-mediated CRISPR/Cas9 RNP inactivation of *Pcsk9* in primary mouse hepatocytes results in indel frequencies as determined by the T7E1 assay, reduced *Pcsk9* mRNA levels, increased LDLR protein levels, and enhanced LDL-C uptake ex vivo.

Differences in gRNA's on-target activity influence CRISPR/Cas9 genome editing efficacy. Therefore, we screened the on-target activity of gRNA's targeting *Pcsk9* exon or donor splice sites via T7E1. Moreb & Lynch found strong evidence that gRNA's on-target activity depends on CRISPR/Cas9 RNPs to find the target site (Moreb & Lynch, 2021). The different indel efficiencies of

the *Pcsk9* targeting gRNAs showed the importance of gRNA screening to identify the optimal gRNAs with on-target activity for downstream applications.

Successful targeting of the *Pcsk9* splice sites can disrupt the canonical mechanism of pre-mRNA splicing (García-Tuñón et al., 2019; Kluesner et al., 2021; Winter et al., 2019). RT-qPCR demonstrated *Pcsk9* mRNA is reduced upon Cas9 EV administration carrying splice donor site targeting MMgRNA 9. Previous studies showed that administering lipid nanoparticles (LNP) with mRNA encoded CRISPR-base editor and gRNA targeting exon 1 splice donor site effectively inactivated *Pcsk9* in vivo (Chadwick et al., 2017; Ding et al., 2014). Surprisingly, these reports did not demonstrate the efficacy of their treatment on LDLR recycling in vitro or ex vivo. Here, we report that the intracellular delivery of CRISPR/Cas9 RNP via EVs targeting the *Pcsk9* exon 1 splice donor site effectively leads to reduced *Pcsk9* mRNA levels, increased LDLR levels, and LDL-C uptake ex vivo.

In our study, we observed a reduction in *Pcsk9* mRNA levels, yet intracellular PCSK9 protein expression remained unchanged. This aligns with Rocha et al.'s findings, where *Pcsk9* inactivation using splice-switching oligonucleotides and small interfering RNA led to decreased *Pcsk9* mRNA levels and increased LDLR levels in Huh7 cells. Despite this, Rocha et al. detected consistent full-length intracellular PCSK9 protein levels in treated Huh7 cells, with only minor variations close to a magnitude of 0.005 (Rocha et al., 2015). Notably, the most pronounced effects of mutations in the *Pcsk9* gene are more evident in serum than intracellularly. Therefore, future research should consider assessing PCSK9 protein levels in serum.

Intracellular CRISPR/Cas9 RNP delivery in recipient cells was achieved with VSV-G on EVs' membrane, promoting endosomal escape upon endosome acidification (Mangeot et al., 2011; Somiya & Kuroda, 2021). Because VSV-G is a virus envelope protein, its immunogenicity should be investigated as it may pose a problem for clinical translation. Osteikoetxea et al. showed that EV-mediated delivery of CRISPR/Cas9 RNP in HEK293 cells expressing *Pcsk9* gRNA had an indel efficiency of 6%. They loaded Cas9 without a fusogenic viral envelope protein but through optically interacting proteins CRY2/CIBN with CIBN anchored to the membrane via an N-myristoylation-palmitoylation-palmitoylation lipid modification. However, the delivery of Cas9 protein without the help of VSV-G seemed only possible when supraphysiological EV doses were administered to recipient cells that already carried the gRNA (Osteikoetxea et al., 2022). Further studies are needed to explore the efficiency of EV uptake and delivery into recipient cells and the necessity for additional viral or non-viral to facilitate this process. It is also essential to examine the potential limiting factors of such proteins, such as pre-existing immunity or inflammatory reactions (Poetsch et al., 2019).

In conclusion, our study presents an efficient method for loading CRISPR/Cas9 RNP into EVs, examining its impact on *Pcsk9* targeting in primary mouse hepatocytes at different cellular levels. The use of FKBP/FRB/VSV-G engineering ensured successful EV-mediated genome editing both in vitro in stoplight reporter cells and ex vivo in primary mouse hepatocytes, achieving *Pcsk9* inactivation. Our results emphasize the significance of screening gRNAs for Pcsk9 to guarantee effective on-target activity. By targeting the splice donor site of *Pcsk9*, we noted decreased mRNA levels and enhanced LDLR expression, culminating in improved LDL-C uptake ex vivo. Given the increasing ethical focus on minimizing animal testing, ex vivo findings could guide future research in evaluating delivery methods for genome editing technologies targeting *Pcsk9*. Such ex vivo data could potentially lessen the dependence on animal testing and pave the way for future in vivo studies utilizing EV-mediated CRISPR/Cas9 RNP delivery.

AUTHOR CONTRIBUTIONS

Nazma F. Ilahibaks: Investigation; validation; formal analysis; data curation; writing—original draft. **Thomas A. Kluiver**: Investigation; validation; writing—review & editing. **Olivier G. de Jong**: Resources. **Saskia C.A. de Jager**: Funding acquisition. **Raymond Schiffelers**: Funding acquisition. **Pieter Vader**: Funding acquisition; resources; supervision; validation; writing—review & editing. **Weng Chuan Peng**: Resources; supervision; validation; writing—review & editing. **Zhiyong Lei**: Conceptualization; funding acquisition; investigation; methodology; resources; supervision; validation; writing—review & editing. **Joost P.G. Sluijter**: Conceptualization; funding acquisition; methodology; supervision; validation; writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

Pieter Vader serves on the scientific advisory committee for Evox Therapeutics. All other authors confirm they have no conflicts of interest.

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