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Chapter 14

Protocol for Delivery of CRISPR/dCas9 Systems for Epigenetic Editing into Solid Tumors Using Lipid Nanoparticles Encapsulating RNA

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Abstract

Genome editing tools, particularly the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems (e.g., CRISPR/Cas9), and their repurposing into epigenetic editing platforms, offer enormous potential as safe and customizable therapies for cancer. Specifically, various transcriptional abnormalities in human malignancies, such as silencing of tumor suppressors and ectopic re-expression of oncogenes, have been successfully targeted with virtually no off-target effects using CRISPR activation and repression systems. In these systems, the nuclease-deactivated Cas9 protein (dCas9) is fused to one or more domains inducing selective activation or repression of the targeted genes. Despite these advances, the efficient in vivo delivery of these molecules into the target cancer cells represents a critical barrier to accomplishing translation into a clinical therapy setting for cancer. Major obstacles include the large size of dCas9 fusion proteins, the necessity of multimodal delivery of protein and gRNAs, and the potential of these formulations to elicit detrimental immune responses.

In this context, viral methods for delivering CRISPR face several limitations, such as the packaging capacity of the viral genome, the potential for integration of the nucleic acids into the host cells genome, and immunogenicity of viral proteins, posing serious safety concerns. The rapid development of mRNA vaccines in response to the COVID-19 pandemic has rekindled interest in mRNA-based approaches for CRISPR/dCas9 delivery. Simultaneously, due to their high loading capacity, scalability, customizable surface modification for cell targeting, and low immunogenicity, lipid nanoparticles (LNPs) have been widely explored as nonviral vectors. In this chapter, we first describe the design of optimized dCas9-effector mRNAs and gRNAs for epigenetic editing. We outline formulations of LNPs suitable for dCas9 mRNA delivery. Additionally, we provide a protocol for the co-encapsulation of the dCas9-effector mRNAs and gRNA into these LNPs, along with detailed methods for delivering these formulations to both cell lines (in vitro) and mouse models of breast cancer (in vivo).

Key words Lipid nanoparticle, RNA delivery, CRISPR/dCas9

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1 Introduction

Conventional genome editing via the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system relies on the catalysis of the Cas9 endonuclease. Cas9 is recruited in the genome by a chimeric single-guide RNA (sgRNA) complementary to the target DNA sequence flanking a protospacer adjacent motif (PAM), inducing DNA cleavage. This system has been adapted for epigenome engineering to facilitate highly specific transcriptional activation or repression. In epigenetic editing platforms, nuclease-deactivated Cas9 (dCas9) is fused to epigenetic effectors such as the transcriptional repressor, Krüppel associated box (KRAB) domain, for gene repression (CRISPRr) or the tripartite transactivator VP64, p65, and Rta (VPR) for gene activation (CRISPRa). Epigenetic editing approaches represent promising targeted therapies for diseases with epigenetic and transcriptional alterations, such as cancers with poor outcomes. In particular, CRISPRr has demonstrated the ability to silence overexpressed genes, including "hard to drug" oncogenic transcription factors [1], while CRISPRa reactivates epigenetically silenced tumor suppressor genes [2]. However, a significant challenge in developing CRISPR systems as cancer therapies lies in efficiently delivering their biomolecular components into cancer cells. DNA delivery methods, such as those using adeno-associated viruses or lentiviral vectors, face limited therapeutic potential due to concerns about immunogenicity, toxicity, and the possibility of integration into the genome [3, 4].

One alternative is to deliver CRISPR as molecular complexes combining Cas9 protein and gRNA (ribonucleoprotein (RNP) complexes). While RNP delivery offers advantages such as high on-target specificity and a short half-life suitable for "hit and run" epigenetic approaches, it also has limitations, including the antigenicity of Cas9 protein [3] and the large molecular weight of Cas9, making it challenging to deliver into target cells and organs [5].

Recently, the field of mRNA therapeutics has rapidly advanced due to the acceleration of siRNA technology and the development of mRNA vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This makes mRNA an attractive choice for CRISPR delivery [6]. In this approach, sgRNAs are delivered in synthetically modified forms, and Cas9 (or dCas9-effector fusions) is encoded in a mRNA format, which is translated in the cytosol of the host cell into a functional protein. Cas9 proteins form complexes with the sgRNA and translocate into the nucleus of the target cell via nuclear localization sequences. RNA delivery offers advantages such as the relatively smaller molecular weight of Cas9 mRNAs compared to corresponding Cas9 proteins, facilitating effective uptake and expression of CRISPR in the cell. However,

mRNA delivery for cancer therapy faces limitations, including the instability and potential immunogenicity of mRNA, though these can be addressed through RNA modifications [3] and nano-delivery vehicles [5]. Chemical modification of gRNA is crucial for protecting it from premature degradation [3]. Cas9 mRNAs can also be modified, for example, by incorporating pseudouridine and 5-methylcytosine to reduce innate immune responses and improve stability [7]; these modifications are discussed in more detail in Subheading 3.3.

Until recently, Cas9 mRNA has only been used to edit the genomes of embryos, zygotes, and cultured cells [8, 9] due to the difficulty in developing an effective in vivo delivery strategy. Several studies have described mRNA delivery of conventional Cas9 with sgRNA [10–13]. Recently, lipid nanoparticles encapsulating Cas9 mRNA and a single guide RNA targeting transthyretin (TTR) have entered clinical trials for the treatment of transthyretin amyloidosis [14]. In the field of epigenetic editing, the first mRNA delivery protocols were developed and optimized as a tool for CRISPR screens in vitro [15]; however, only a few studies have successfully delivered mRNA for dCas9 activators and repressors in vivo. CRISPRa and CRISPRr RNA platforms induced transient and programmable gene regulation in human primary cells, including hematopoietic stem and progenitor cells (HSPCs) [16]. Furthermore, CRISPRa RNA delivery in vivo has been achieved to activate the erythropoietin gene in the mouse liver [17].

The strategies for delivery of Cas9/dCas9 mRNA and gRNAs include electroporation, polymeric nanoparticles and hydrogels, and lipid nanoparticles (LNPs) [18]. Recently, virus-like particles (VLPs) and extracellular vesicles (EVs) have been adapted for the delivery of CRISPR systems, enabling the encapsulation of both RNA and protein. VLPs have improved safety features relative to lentiviral and retroviral systems; however, they can still potentially elicit immune responses in vivo [19, 20]. Alternatively, LNPs have been increasingly explored for RNA delivery in vivo due to low immunogenicity, high circulation time, and its versatility as a therapy modality. While lipid-based carriers may have some cytotoxic and inflammatory effects, the development of novel synthetic ionizable cationic lipids and LNP formulations has reduced toxicity, making LNP-mediated delivery of RNA-based CRISPR therapies a realistic prospect. Current research actively focuses on optimizing LNP formulations by varying the lipid formulations and coating of LNPs to improve cellular uptake rates and avoid endosomal escape [4].

This chapter describes a protocol for the assembly of LNPs encapsulating RNAs for the transfection of CRISPR/dCas9 systems in vitro, with a focus on cancer cell epigenetic editing and reprogramming. Furthermore, we also provide methods for the administration of LNPs in vivo focusing on mouse models of breast

cancer. To illustrate the method, we present a specific example describing the design of optimized dCas9-KRAB mRNA and gRNAs for CRISPRr, aiming to downregulate oncogenic transcription factors in triple-negative breast cancer. The LNP delivery methods described herein have successfully been employed to deliver conventional Cas9 in vivo to brain tissue [10]. Moreover, this approach can also be applied to various experimental models (e.g., gene activation, repression, several cancer/cell types) and to edit one or multiple genes. It is suitable for delivering a wide variety of therapeutic RNAs, including chemically modified siRNAs [4].

2 Materials

2.1 sgRNA Design

1. UCSC Genome Browser (https://genome.ucsc.edu): used as a source of genomic data [21].
2. Benchling (https://benchling.com): allows the import of genomic data to rank and select appropriate gRNAs.

2.2 Lipid Nanoparticle (LNP) Formulation

1. Lipids: The encapsulation of nucleic acids within LNPs is crucial for both in vitro and in vivo studies for various therapeutic applications. LNPs are usually prepared using four lipid components: ionizable cationic lipids, helper phospholipids, PEG-lipids, and cholesterol.
   (i) Ionizable cationic lipids: These lipids often consist of a tertiary amine that becomes deprotonated under neutral pH and holds a positive charge at pH levels lower than the acid-dissociation constant (pKa) of the lipid. Its primary roles involve aiding in the encapsulation of nucleic acids in LNPs and mediating the disruption of the endosomal membrane to facilitate the release of nucleic acids into the cytosol. Examples include: (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl4(dimethylamino) butanoate (D-Lin-MC3-DMA) or MC3 (MedKoo, cat. # 555308).
   (ii) PEG-lipids: These lipids aid in preventing aggregation and improving stability. An example is: 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2000) (Avanti Polar Lipids, cat. # 880151).
   (iii) Helper lipids: Helper phospholipids in LNPs play a crucial role in stability, delivery, and functionality. These lipids aid in maintaining the structural integrity of LNPs, enhancing their stability during circulation, and promoting cellular uptake, for example: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Avanti Polar Lipids, cat. # 850725).

(iv) Cholesterol (Sigma-Aldrich, cat. # C8667).

2. For purification of LNPs: Pur-A-Lyzer Midi Dialysis Kit (Sigma Aldrich, cat. # PURD35100).

2.3 In Vitro and In Vivo Delivery of mRNA for dCas9 Effectors and gRNA LNP Formulations

1. Cell line pertaining to the model of interest: For in vitro proof of principle internalization studies, we have used the human embryonic kidney 293 T (HEK293T) obtained from the American Type Culture Collection (ATCC). For in vivo studies, we transfected optimized formulations in breast cancer triple negative SUM159 cells (Asterand Biosciences). Moreover, internalization studies can be performed with any cell line of interest.
2. Cell culture media: HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose-pyruvate supplemented with heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Anti-Anti, Gibco). SUM159 were grown in Ham's F12 medium containing 1% antibiotic-antimycotic supplemented with 5% heat-inactivated FBS, 1 μg/mL hydrocortisone, 5 μg/mL insulin, and 10 mM HEPES buffer.
3. Opti-MEM Reduced Serum Medium (Gibco): Optional for in vitro delivery (*see* **Note 1**).
4. Matrigel Basement Membrane Matrix: Required for inoculation of cells to form tumors for in vivo delivery; Corning Matrigel Matrix High Concentration, cat. # 354248.
5. LNP formulations (as described in Subheadings 2.2 and 3.4).
6. 25 mM citrate buffer, pH 4.0 (ThermoFisher, cat. # 258585000).
7. dCas9-effector mRNA (1 μg/μL): see Subheading 3.2 for details of the design of dCas9-effector mRNA.
8. Alt-R CRISPR-Cas9 sgRNA (IDT, described further in Subheading 3.1, *see* **Note 2**): Resuspend sgRNA to 100 μM in duplex buffer (IDT), aliquot and store at −80 °C. Prepare working aliquots of 15 μM sgRNA in TE buffer. If more than one gRNA is encapsulated (multiplexing), 15 μM must be the total concentration of all gRNAs. Working aliquots can be stored at −80 °C but avoid multiple freeze-thaw cycles.
9. Control mRNA for in vitro studies: CleanCap EGFP mRNA (1 μg/μL, Trilink Bio Technologies Inc., cat. # L-7601).
10. Control mRNA for in vivo studies: Firefly Luciferase mRNA (1 μg/μL) (Trilink Bio Technologies Inc., cat. # L-7602), requires the substrate D-Luciferin (potassium salt), Cayman Chemicals.

2.4 Visualization of dCas9-Effector Transfection Efficiency In Vitro and In Vivo by Immunofluorescence

1. UV-sterilized 13 mm circular glass coverslips (No 1.5H thickness is recommended for confocal microscopy) coated in Poly-L-Lysine (0.1 mg/mL in H_2O): required for seeding of cells in vitro.
2. Pierce 4% formaldehyde (ThermoFisher) in 1× phosphate-buffered saline (PBS).
3. Washing solution: for cell lines use 1× PBS, for tissue sections use Tris-buffered saline (TBS): 150 mM NaCl, 20 mM Tris, pH 7.4, containing 0.1% Tween-20 (TBST).
4. Antigen retrieval buffer: 10 mM Sodium Citrate, pH 6. Add 2.94 g sodium citrate trisodium salt dehydrate to 1000 mL ultrapure water, adjust pH to 6 with concentrated HCl.
5. Permeabilization buffer (for tissue sections): 0.2% Triton X-100 in TBST.
6. Blocking buffer: For cell lines, 5% normal goat serum, 1% bovine serum albumin (BSA) 0.3% Triton X-100 in 1× PBS. For tissue sections, 10% normal goat serum, 1% BSA, 0.3% Triton X-100 in 1× PBS.
7. Antibody diluent: 1% bovine serum albumin (BSA), 0.3% Triton X-100 in 1× PBS.
8. Primary antibodies: Anti-Cas9 antibody produced in mouse (Abcam, cat. # ab191468), and Anti-ZEB1 antibody produced in rabbit (Sigma-Aldrich, cat. # HPA027524).
9. Secondary antibodies: Alexa Fluor 488 goat anti-mouse (ThermoFisher), Alexa Fluor 594 goat anti-rabbit (ThermoFisher).
10. Nuclear staining: Hoechst 33258 (10 mg/mL Sigma-Aldrich).
11. Mounting media: SlowFade Diamond Antifade mounting (ThermoFisher).

3 Methods

3.1 gRNA Design

Correct design and optimization of gRNA is crucial for effective and specific CRISPR gene regulation activity. Targeting the dCas9 protein to specific genomic loci, while minimizing off-target activities first requires an accurate annotation of the relevant genomic data [22]. This includes the location of the relevant protospacer adjacent motif (PAM) sequences, any known target RNA isoforms, the position of the transcriptional start site (TSS), and the location of any relevant promoter/genomic features such as CpG islands, chromatin accessibility, and the position of nearby genes [23] (Fig. 1a). The UCSC genome browser (https://genome.ucsc.edu) is a widely used source of genomic data. In addition to promoter sequences, enhancer sequences can also be harnessed to target genes with CRISPRa/r, and these enhancers can be

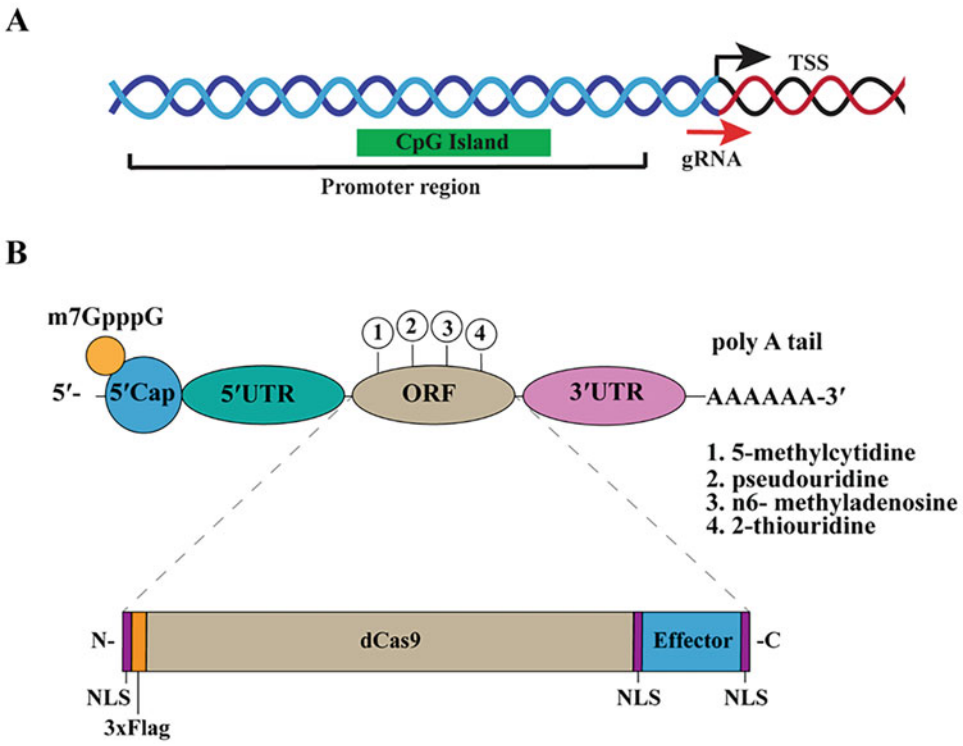

Fig. 1 Schematic representation of a targeted promoter and a typical mRNA template. (**a**) Schematic representation of a targeted promoter. Annotation of the TSS (transcription start site) and other features such as CpG islands are important considerations when designing gRNAs. (**b**) Upper part: A schematic of a typical mRNA template with the five components, the 5′ cap, 5′ UTR, ORF, 3′ UTR, and the poly (A) tail. Numbers 1–4 represent possible chemical modifications that should be considered when designing a therapeutic mRNA. Lower part: A schematic of the ORF translated by the cell, indicating the molecular components of dCas9-effector fusions (bottom). Multiple NLS ensure nuclear localization of the dCas9 fused to an effector protein, for example, for repression, KRAB, or activation, VPR domains. *CpG* 5′-C-phosphate-G-3′, *TSS* transcription start site, *gRNA* guide RNA, *UTR* untranslated region, *ORF* open reading frame, *NLS* nuclear localization sequence

identified and analyzed by tools such as FANTOM5 enhancer atlas, http://fantom.gsc.riken.jp/5.

Available online tools for designing gRNA sequences enable the selection of high-fidelity gRNAs. Platforms such as Benchling (https://benchling.com/) allow the import of genomic data for ranking and selection of appropriate gRNAs. Important considerations to make when designing gRNAs include few or no off-target sites (which are scored in the program), high binding specificity, the distance from the TSS (for CRISPRa within 500–50 bp of the TSS, for CRISPRr in proximity to the TSS [24, 25]), and the directionality of the gRNA, which determine the positioning of epigenetic effectors and affect activity [26]. Finally, it is recommended that several gRNAs, typically ~four per targeted promoter, that are well spread across the target region are selected; these should be tested

in vitro, for example, using lentiviral vectors. Specific details of the design of the ZEB1 gRNA (*see* Subheading 3.2.1) is further described by Waryah et al. [1].

3.2 dCas9-Effector mRNA Design

There are five basic functional components in mature eukaryotic mRNAs: the 5′Cap, 5′ untranslated region (5′UTR), the open reading frame (ORF), the 3′ untranslated region (3′UTR), and the poly (A) tail (Fig. 1b). All these components should be considered when designing mRNA therapeutics. Modification of these components has been shown to increase stability, increase translation, and lower the immunogenicity of mRNA [27–29].

Firstly, the 5′ Cap, an N7-methylated guanosine tethered to the first RNA base can be adapted with a 3′-O-Me-m7G(5′)ppp(5′)G anti-reverse cap analog (ARCA). ARCAs contain a modified cap structure containing a 5′-5′ triphosphate bridge [30], which protects the synthetic mRNA from degradation and improves translation efficiency and stability. Furthermore, additional chemical modification of the ARCA cap analogs at the elongated 5′-5′ phosphate bridge or 3′- (or 2′-) position further improve both translation efficiency and stability [31, 32]. Other possible modifications to the 5′Cap include a co-transcriptional capping method (called CleanCap [7]) that forms a natural Cap 1 structure. The CleanCap further enhances RNA stability by inhibiting the de-capping metalloenzymes (DCP2).

The 5′ UTR and 3′ UTR regions have been also modified to improve the therapeutic effect of mRNAs. For example, the incorporation of an artificial 5′ UTR containing a strong Kozak signal and the 3′ UTR from the human α-globin mRNA improved protein expression in fibroblasts that were reprogrammed into pluripotent stem cells [33]. Another report screened various combinations of 5′ UTR and 3′ UTR regions and demonstrated that 5′ and 3′ UTR regions from complement factor 3 (C3) and cytochrome p4502E1 (CYP2E1), respectively, consistently displayed a larger increase in protein expression, but not in mRNA stability compared to a reference UTR [30].

Finally, the nucleoside composition of a therapeutic mRNA can be modified to improve stability, increase translational efficiency, and reduce immunogenicity [28]. One key modification, pioneered by Kariko, Weissman, and colleagues (who won the 2023 Nobel Prize for Physiology or Medicine), is replacement of cytidine and uridine nucleosides by 5-methylcytidine and pseudouridine, respectively [27, 34, 35]. Incorporation of pseudouridine, found naturally in tRNAs, rRNA, and small nuclear RNAs, into mRNAs, enhances the translational efficiency while suppressing RNA-mediated immune activation by reduced activation of RNA-dependent protein kinase (PKR) [27]. Furthermore, other modifications such as N6- methyladenosine, N6- methyladenosine-5′-triphosphate, 5-methylcytidine-5′-triphosphate, and

2-thiouridine-5′-triphosphate reduce immunostimulation and increase protein expression (reviewed in [36]).

3.3 In Vitro Transcription of dCas9-Effector mRNA

dCas9-effector mRNA fusions are produced by in vitro transcription utilizing a template encoding the optimized dCas9-effector as DNA downstream of a phage promoter sequence for mRNA transcription, commonly T7. dCas9-effector DNA is then transcribed into mRNA using RNA polymerases specific to the promoter, for example, T7 RNA polymerase [3]. In vitro transcription of the dCas9-fusion constructs is challenging due to the large size of the mRNA (~4 to 5 kb). It is recommended that a commercial kit suitable for the generation of 5 kb RNA transcripts is used, such as mMESSAGE mMACHINE™ T7 Transcription Kit (Thermofisher) or HiScribe T7 Kit with CleanCap® Reagent (NEB) [37]. Alternatively, custom dCas9-effector mRNA synthesis is commercially available from several sources (e.g., GenScript Biotech, Singapore, or Trilink Bio Technologies Inc). Below we outline a brief protocol, outlining the specific considerations for the in vitro transcription of dCas9-effector mRNA.

1. Clone the synthetic DNA fragments encoding the dCas9-effector mRNA into a plasmid vector, containing sequences corresponding to a T7 promotor, a 5′UTR and 3′UTR, and a 100 bp polyA tail.
2. Linearize plasmid DNA with a restriction enzyme downstream of the insert to be transcribed to generate a template with no additional nucleotides beyond polyA. Purify linearized plasmids by ethanol precipitation.
3. Assemble a reaction at room temperature of 1 μg plasmid DNA per 20 μL reaction, 10 mM uridine 5′-triphosphate, adenosine 5′-triphosphate, cytidine 5′-triphosphate and guanosine 5′--triphosphate and T7 RNA polymerase mix in reaction buffer. If modifications are required, replace the natural ribonucleoside triphosphate (NTP) with the modified NTP. RNA can be capped in the same reaction by including a T7 CleanCap Reagent and trinucleotide cap analog such as CleanCap Reagent AG (NEB). Incubate reaction at 37 °C for at least 3 h, though the reaction can be incubated for up to 16 h, which may increase yield of long transcripts such as the dCas9-effector mRNA.
4. Purify the RNA using a commercial kit, such as Monarch RNA Cleanup Kit column purification kit purification (NEB), or lithium chloride precipitation to remove the DNA template.
5. Before use assess the quality of the RNA using spectrophotometry and gel electrophoresis.

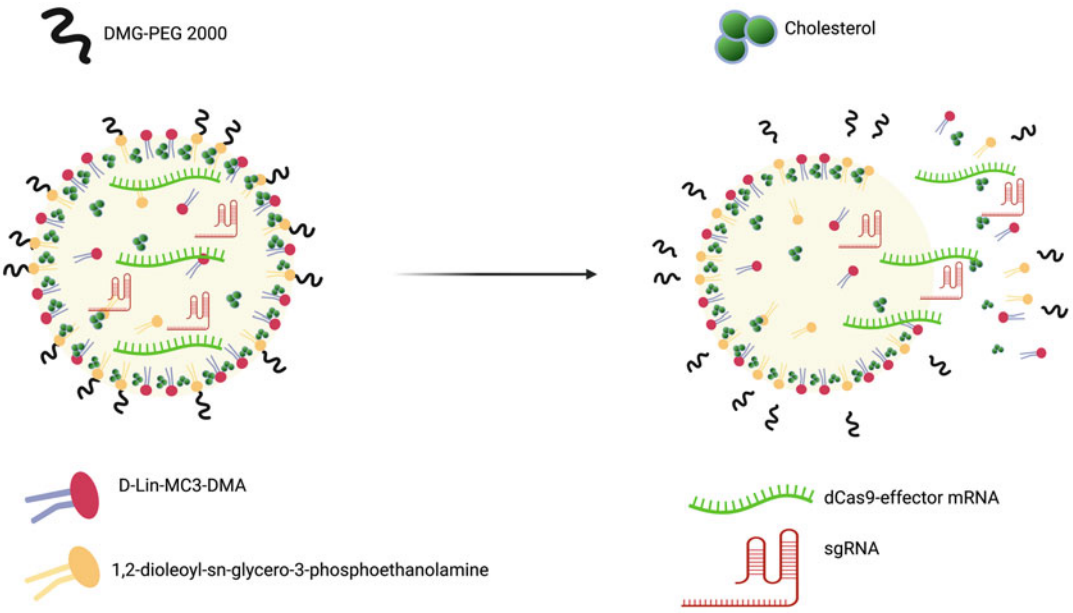

Fig. 2 Schematic representation of the proposed structure of LNPs containing dCas9-effector mRNA and sgRNA and the mechanism of delivery of RNAs into the cytosol. DMG-PEG = 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (PEG-lipid); D-Lin-MC3-DMA = (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl4(dimethylamino) butanoate (Ionizable cationic lipid); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine = DOPE (Helper lipid)

3.4 LNP Formulation

1. Prior to LNP assembly, take out lipid solutions (MC3, DOPE, cholesterol, and DMG-PEG), keep on ice, and vortex whenever necessary, to avoid precipitates. The cholesterol solution should be warmed up to >37 °C to dissolve the crystals that form during cold storage.
2. Combine MC3, DOPE, cholesterol, and DMG-PEG in optimized molar ratios. The molar ratios influence the morphology of LNPs and should be optimized depending on the delivery system and cargo being delivered. As an example, we have used 36.8:23.8:38.2:1.2 molar ratios, respectively, for successful in vitro delivery of Cas9 mRNA with the sgRNA [10].
3. Proceed to Subheading 3.4.1 or 3.5 to encapsulate nucleic acid (mRNA/sgRNA) into LNPs. LNP formulations can also be stored at −80 °C and thawed prior to this step. The proposed structure of LNPs and encapsulation and release of dCas9-effector mRNA with sgRNA into the cytosol is outlined in Fig. 2.
4. Purification of LNPs: To remove the excess ethanol from the LNPs, dialyze the LNP suspension against the 100-fold volume of phosphate-buffered saline (PBS) at pH 7.4 for 12 h at 4 °C. For dialysis, utilize a molecular weight cutoff (MWCO)

of 3500 Da tubing (Pur-A-Lyzer Midi Dialysis Kit). After dialysis, LNP size distribution and polydispersity should be determined by a particle size analyzer (Malvern Zetasizer, Malvern).

3.4.1 LNP-Mediated In Vitro Delivery of dCas9-Effector mRNA and gRNAs

The following protocol is for adherent cells grown in 24 well plates in 450 μL media; for each well prepare 6 μL mRNA/LNP. This protocol can be scaled up or down to suit other plate formats; the gRNA/mRNA LNP mix described below is sufficient for 4.5 wells of a 96 well plate. For 6 well plates we recommend scaling the mixture up by a factor of 4.5. The 293 T cells are grown at 37 °C and 5% CO_2. Prepare formulations in sterile conditions under a Class II Biological Safety Cabinet.

1. Seed cells onto plates 16–24 h prior to transfection of LNP to provide a confluency of ~80% at time of delivery. For 24 well plates 80,000 293 T cells were recommended per well.
2. Dilute nucleic acid (mRNA/sgRNA) in a 25 mM citric acid buffer of pH 4.0 in a 1:2 volumetric ratio. To prepare a 6 μL complex of liposome/RNA first add 2.25 μL of 25 mM citrate buffer, pH 4.0, 1 μL dCas9 mRNA (1 μg/μL), and 0.5 μl of 15 μM gRNA (~0.25 μg) and 0.75 μL nuclease-free water to a 1.5 mL sterile tube (*see* **Notes 3** and **4**). Mix gently by pipetting a few times (avoid vortexing). A no-gRNA control and a no-RNA control (LNP only) should also be included. To quantify the transfection efficiency of the LNP formulations, add 1 μL of 1 μg/μl EGFP mRNA instead of the dCas9 mRNA/gRNA. For these controls use nuclease-free water as a substitute for the RNA to bring up the final volume of the tubes to 4.5 μL.
3. To encapsulate nucleic acid, the two solutions, lipids in the organic phase and nucleic acid in the aqueous phase, should be mixed by a pipette at a 1:3 volumetric ratio. To make a 6 μL complex of LNP/RNA add 1.5 μL LNP to the 4.5 μL buffer/RNA mixture and mix gently by pipetting a few times (avoid vortexing).
4. Incubate the mixture at room temperature for 10 min.
5. After the incubation period add 450 μL prewarmed cell media.
6. Remove the old media from the wells and replace with the transfection mix.
7. Immunofluorescent imaging (*see* Subheading 3.4.2, Fig. 3) or flow cytometry analysis can be performed 24 h after transfection to assess/quantify protein expression of dCas9 effectors and the target gene/s. If using GFP mRNA as a control to assess transfection efficiency of the LNPs, the expression of

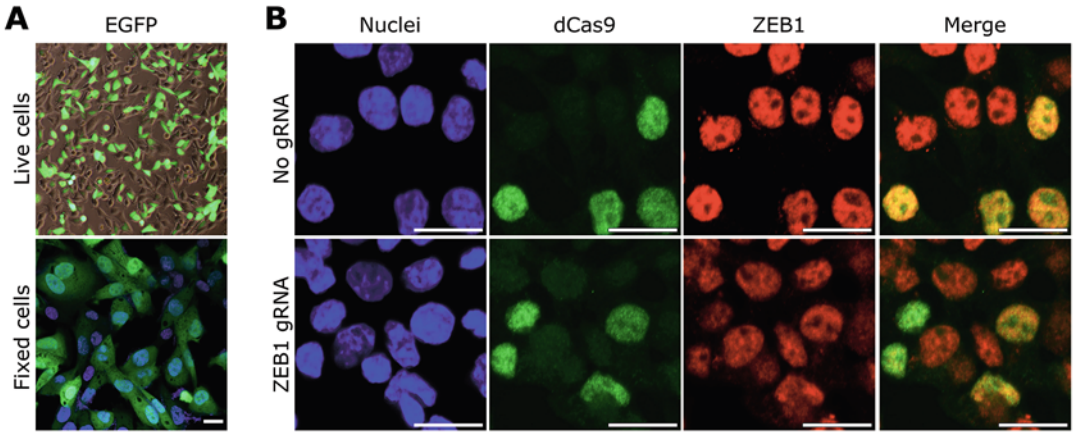

Fig. 3 Assessment of EGFP mRNA and dCas9 mRNA transfection in vitro. (**a**) Representative images of HEK293T cells 24 h after transfection with LNP + EGFP mRNA to assess the efficiency of LNPs in delivering mRNA in vitro. Top panel shows live cells imaged with light microscopy and GFP fluorescence. Bottom panel shows fixed cells imaged with confocal microscopy to visualize GFP fluorescence (*green*), with nuclear staining (Hoechst) shown in blue. (**b**) Representative images of HEK293T transfected with LNP + dCas9-KRAB mRNA in the absence of gRNA (No gRNA) or with a gRNA targeting ZEB1 (ZEB1 gRNA). dCas9 (green) entered the nuclei (blue) and in the presence of ZEB1 gRNA reduced intensity of ZEB1 expression (red); this is particularly visible in cells expressing high dCas9. Scale bars indicate 20 μm

GFP can also be visualized by fluorescent microscopy or flow cytometry (*see* **Note 5**).

3.4.2 Determination of LNP Transfection Efficiency by Immunofluorescence

The dCas9-effector mRNA and transfection efficiencies are visualized 24 h after transfection using immunofluorescence methods. Co-staining of LNP-transfected cells with antibodies against Cas9 and the target gene of interest enables the assessment of target gene repression/activation. For the purpose of an example, we have provided representative results (Fig. 3) of an LNP transfection experiment encapsulating dCas9-KRAB mRNA and sgRNA specific for ZEB1 repression. As mentioned previously, LNP transfection efficacy can directly be monitored with encapsulation of an mRNA encoding EGFP, followed by quantification of the green fluorescence in living cells.

1. Seed cells onto UV sterilized glass circular coated with Poly-L-Lysine and perform transfection protocol described above in Subheading 3.4.1.
2. Twenty-four hours after transfection, aspirate media and wash cells twice with 1× PBS then fix cells by incubating 20 min in 4% paraformaldehyde.
3. Wash three times with 1× PBS and block with 200 μL blocking buffer for 1–2 h.

4. Aspirate block buffer and incubate overnight at 4 °C with the Cas9 primary antibody diluted in antibody diluent, the target of interest can also be co-stained to assess repression/activation. We have used Cas9 Mouse antibody (Abcam, 1/100), with ZEB1 Rabbit antibody (Sigma, 1/300).
5. Aspirate primary antibody and wash three times in 1× PBS. Incubate for 1 h at room temperature with the following secondary antibodies diluted in antibody diluent: goat α-mouse 488-conjugated antibody, goat α-mouse, or α-rabbit 594-conjugated antibody (1:500) with Hoechst 33258 (1: 5000) for nuclei staining.
6. Mount coverslips onto slides with antifade mountant (ThermoFisher). Leave slides overnight at room temperature prior to imaging to allow mountant to set.
7. Image using a fluorescent or confocal microscope, for the images used in Fig. 3, a Nikon A1Si inverted confocal microscope was used.

3.5 In Vivo Delivery of LNPs Encapsulating RNA for Tumor Targeting

The following protocol is designed to prepare tumor cells for subcutaneous injection into immunocompromised mice, followed by administration of LNPs (previous sections) either as localized (orthotopic or intratumoral) or systemic (via tail vein) treatment modalities (Fig. 4). It is intended to be a general protocol for tumor cell lines (i.e., SUM159) capable of forming tumors in mice. Therefore, it is important to adjust cell culture and inoculation conditions when necessary. All animal experiments must comply with local regulations and protocols.

3.5.1 Inoculation of Tumor Cells

Mouse strain, specific cell line, and number of cells inoculated should be tailored to the needs of the specific experiment. In this protocol, we implant TNBC SUM159 cell line in Foxn1^{nu} (nude) mice.

1. Approximately, 2×10^6 cells resuspended in serum-free media are mixed with Matrigel in a 1:1 volume of media for a total volume of 100 μL/subcutaneous injection/mouse (*see* **Note 6**).
2. Using a 29G 0.5 mL insulin syringe, nude mice are inoculated with SUM159 cells (2×10^6) by subcutaneous injection in the hind flank. For noninvasive assessment of tumor growth by bioluminescence imaging (BLI), SUM159 cells engineered with a luciferase reporter gene can be implanted, although growth kinetics of cells with and without the luciferase transgene might vary.
3. Observe the mice two to three times a week for tumor development and growth. Once tumors become palpable

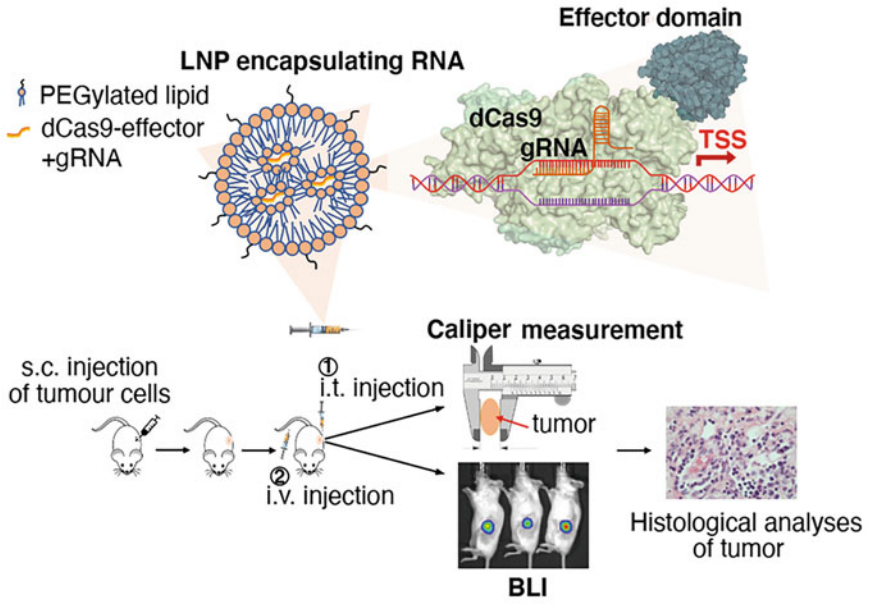

Fig. 4 Schematic representation of LNP-mediated delivery of CRISPR/dcas9 systems in a mouse model of triple-negative breast cancer. The dCas9-effector mRNA and gRNAs are encapsulated in the LNP and delivered to a luciferase-expressing tumor cell line (SUM159) engrafted in the flank of the mice and monitored by caliper to assess tumor growth or by BLI. LNP injection modalities include (1) intratumoral injection and (2) systemic (intravenous) administration. PEG-lipid conjugates improve pharmacokinetics and efficiency for long systemic circulation of LNPs. *s.c.* subcutaneous, *i.t.* intratumoral injection, *i.v.* intravenous injection, *BLI* bioluminescence imaging, *LNP* lipid nanoparticle, *PEG* polyethylene glycol, *TSS* transcriptional start site, *eGFP* enhanced green fluorescent protein

(~50 mm^3; within 1–2 weeks), mice are randomized into different mRNA-LNP treatment group.

3.5.2 LNP Encapsulating RNA Preparation for In Vivo Delivery

1. To prepare a 40 μL LNP RNA complex, transfer 15 μL 25 mM citrate buffer, pH 4.0 into a 1.7 mL tube.
2. Add 10 μL 1 μg/mL dCas9 mRNA and 5 μL 15 μM gRNA into the tube and mix gently by pipetting a few times (avoid vortexing). A no-gRNA control and a no-RNA control (LNP only) should also be included. To visualize the uptake of LNP RNA complex by tumors, 10 μL 1 μg/mL firefly luciferase (Luc)-encoding LNP RNA and 5 μL nuclease-free water can be encapsulated instead of the dCas9-effector mRNA. However, in this case, tumor cells inoculated in mice should not express the luciferase reporter construct.
3. Add 10 μL LNP to the PBS/mRNA mixture and mix gently by pipetting about five times (avoid vortexing). The ratio of citrate buffer: mRNA: LNP should be 3:3:2 and can be scaled up accordingly.
4. Incubate the mixture at room temperature for 10 min.

5. Use an air blow device to remove the 10 μL of ethanol (this comes from lipids as they are soluble in ethanol). Be cautious of the airflow and start with a low setting to avoid liquid spillage. This step typically takes less than 5 min.
6. After the air blows, incubate the mixture at room temperature for another 10 min.
7. Add more PBS to make up the injection volume (50 μL or 100 μL).
8. These LNPs are time sensitive and therefore must be administered within 1 h.

3.5.3 Intratumoral/ Orthotopic Delivery of LNP Formulations

Intratumoral (i.t.) delivery of LNPs allows higher doses to reach the tumor without an increase in systemic toxicity. Such localized treatments are often utilized as proof of principle for the efficacy of LNP formulations in delivering CRISPR, and to verify target gene regulation.

1. Place a 26G 1 mL syringe in the center of the tumor and inject 100 μL of the LNP formulations directly into the tumor. One syringe/needle is recommended per mouse.
2. Follow tumor growth and assess expression of dCas9-effector and target gene regulation as described in Subheadings 3.5.6 and 3.5.7, respectively.

3.5.4 Intravenous/ Systemic Delivery of LNP Formulations

Intravenous (i.v.) injection administers the formulations directly into the bloodstream of the mice, which typically reach the tumor by the retention effect, particularly in tumors that are highly vascularized. Alternatively, LNPs can be decorated with several ligands, peptides, or antibodies to confer specific tumor targeting [38, 39].

1. Prior to injection, warm the animal for 5–10 min to dilate the veins. Mice may be warmed by placing them in a small animal warming box with operating temperature adjustable to 37 °C. If an overhead heat lamp is used, extra care must be taken to prevent overheating the mice.
2. Restrain mice using a commercially available restraint device designed for small animals.
3. Draw up the 100 μL of LNP formulation to be administered into the syringe. Ensure that no air bubbles are present in the syringe or substance to be injected.
4. Insert the needle (27G—30G) into the tail vein, bevel facing up, toward the direction of the head. Keep the needle/syringe parallel to the tail. No resistance should be felt when depressing the plunger.

5. Follow tumor growth and assess expression of dCas9-effector and target gene regulation as described in Subheadings 3.5.6 and 3.5.7, respectively.

3.5.5 Bioluminescence Imaging (BLI) for Control LNPs Incorporating a Luciferase-Encoding mRNA

As mentioned earlier, luciferase mRNA encapsulated into the LNPs can be used as a control to assess the efficacity of LNPs in delivering RNA into the target tissues in vivo. Mice are injected with the chemiluminescent substrate of firefly luciferase, D-luciferin, and BLI is conducted in a 4–48-h interval post-treatment, with in vivo imaging equipment, such as the IVIS Lumina II In Vivo Imaging System [40, 41].

1. Inject the mice intraperitoneally (i.p.) with D-luciferin (150 mg/kg in 200 μL) with a 26G 1 mL syringe before anesthesia.
2. Approximately 4 h after D-luciferin injection, anesthetize the mice with inhaled isoflurane in an induction chamber with the isoflurane level at 4% until unconsciousness is achieved (within 3–5 min) and then lower it to 2% for the remainder of the procedure. Repeat imaging at 24, 48, and 72 h to determine the half-life of the luciferase expression.
3. Acquire sequential images of luminescence at 1 min interval for 30 min. 60 s exposure, medium binning, blocked excitation filter, and open emission filter.
4. Quantify the region of interest (ROI) and the total signal intensity in the ROI (photon/sec/m^2) of the tumor luminescent area using Living Image software 3D (Xenogen) [40, 41].
5. Perform ex vivo imaging on isolated tumor and organs to obtain stronger luminescent intensity.
6. In vivo studies need to be complemented by ex vivo biodistribution analyses by quantification of the luciferase signal in tumor and other organs such as the liver, spleen, and kidneys, normalized to a no RNA control (LNP only).

3.5.6 Tumor Measuring and Collection

1. Monitor tumor volume (mm^3) every 2–3 days using a digital calliper and calculate the volume using the modified ellipsoid formula $V = ((\text{width})^2 \times ½ \times \text{length})$ [42, 43] as the product of the largest perpendicular diameters.
2. Humanely euthanize the mice once tumors reach a volume of ~1000 mm^3. Resected tumors can be stored and used for downstream analysis. If histological analysis is required, tumors can be fixed for 24 h in 4% paraformaldehyde. For analysis of RNA expression, tissue can be snap frozen and stored at −80 °C.

3.5.7 Immunofluorescence Staining of Tissue Sections for Detection of dCas9-Effectors in the Target Tissues

Resected tumors are investigated by immunofluorescence for the detection of dCas9-effectors and the expression of target gene to evaluate efficiency of the Cas-effector editing (e.g., activation or repression) and the efficacity of the in vivo LNP delivery. The following protocol is tailored for formalin-fixed paraffin-embedded samples.

1. Cut tumor sagittally into 4 μm sections and mount onto slides.
2. Deparaffinize sections in 3 × 5 min washes of xylene and rehydrate in 2 × 10 min washes of 100% ethanol followed by 2 × 10 min 95% ethanol.
3. Subject sections to antigen retrieval by heating slides in antigen retrieval buffer (10 mM Sodium Citrate, pH 6) for 10 min.
4. Permeabilize sections by immersing slides in permeabilization buffer for 10 min.
5. Wash slides 2 × 5 min with TBST.
6. Use a liquid-repellent slide marker (Pap pen) to draw a hydrophobic barrier around the section and add enough blocking buffer to cover the section. Incubate for 1.5 h at RT.
7. Remove blocking buffer and add α-Cas9 primary antibody generated in mouse (1:100, Abcam) diluted in antibody buffer. Incubate overnight at 4 °C. Repression or activation of the target gene can also be assessed by co-staining with antibody of a different species, for example, the ZEB1 Rabbit antibody (1:300, Sigma-Aldrich) to assess repression of ZEB1.
8. The next day, rinse slides twice for 5 min in TBST and add secondary antibodies diluted in antibody diluent, for example, Alexa Fluor 488 goat anti-mouse (1:250, ThermoFisher), Alexa Fluor 594 goat anti-rabbit secondary antibody (1:250, ThermoFisher), and Hoechst (1:5000, Sigma-Aldrich). Incubate 2 h at room temperature.
9. Mount slides with antifade mountant (ThermoFisher) and seal with nail polish. Leave slides overnight at room temperature prior to imaging to allow mountant to set.
10. Acquire images with a fluorescent tissue microscope or confocal microscope.

4 Notes

1. LNP transfections can be carried out in reduced serum media such as Opti-MEM, provided it is replaced with complete media 4 h after transfection. However, we have found in our cell lines that Opti-MEM does not improve mRNA LNP transfection efficiency and reduces cell viability of some cell lines, including SUM159.

2. The amount of dCas9 mRNA can be increased, but the ratio of buffer/mRNA: LNP should be 1:3, therefore the mix should be scaled up accordingly.
3. Chemical modifications are critical in order to improve the stability of gRNAs [3]. Such gRNAs are commercially available from several suppliers such as IDT (Alt-R CRISPR-Cas sgRNA), which incorporate proprietary chemical modifications to stabilize the RNA, increasing resistance to nuclease activity, while reducing toxicity and innate immune responses.
4. The ratio of gRNA to dCas9 mRNA can be adjusted; however, it is not recommended to increase gRNA concentration much beyond this ratio as gRNA is smaller and may compete with the larger dCas9 mRNA for liposomal packaging [17].
5. We have found that the GFP signal fades within days after mounting slides and it is recommended to also image live cells using a standard fluorescent microscope for GFP prior to fixing, then image fixed cells within 2 days.
6. To thaw Matrigel, place them on ice overnight at 4 °C (refrigerator or cold room).

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