Lipid Nanoparticles Efficiently Deliver the Base Editor ABE8e for *COL7A1* Correction in Dystrophic Epidermolysis Bullosa Fibroblasts In Vitro

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TO THE EDITOR

Lipid nanoparticles (LNPs) have been widely approved and used on a global scale for delivery of mRNA. LNPs can package and deliver mRNA-encoding gene editors, including adenine base editors, which convert A•T base pairs to G•C base pairs without double-stranded DNA breaks or donor DNA (Gaudelli et al, 2017). Adenine base editor is a potential treatment approach for the inherited blistering disease dystrophic epidermolysis bullosa (DEB). DEB results from pathogenic variants in *COL7A1*, leading to dysfunctional or absent type VII collagen (C7), a major component of anchoring fibrils that adhere the dermal–epidermal junction , giving stability to skin (Bardhan et al, 2020). There is currently no cure for DEB; however, ∼90% of *COL7A1* variants are single nucleotide variants, with C>T single nucleotide variants accounting for ∼60% of variants (ClinVar database; accessed August 2023). These variants are targetable by adenine base editors; our group and others have demonstrated the utility of adenine base editor in reverting pathogenic variants and restoring C7 expression (Osborn et al, 2020; Sheriff et al, 2022).

In this study, we first explore the use of novel LNPs to deliver *ABE8e* (Richter et al, 2020) in mRNA format with a single-guide RNA (sgRNA) targeting the pathogenic c.5047 C>T *COL7A1* variant in the fibroblasts of a patient with DEB in vitro. We formulated 6 different LNPs, all of which use the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), mixed at a 1:1 molar ratio with 1 of the cationic lipids 1,2-di-([Z]-octadec-9-enyloxy)-N,N,N trimethylammonium propane chloride (C18), 1,2-di-([Z]-hexadec-11-enyloxy)-N,N,N trimethylammonium propane iodide (C16), and 1,2-di-([Z]-tetradec-11-enyloxy)-N,N,N trimethylammonium propane chloride (C14) using the NanoAssemblr microfluidic system (Precision Nanosystems) to form liposomes. Liposomes were either mixed with mRNA alone at a 3:1 mass ratio or mRNA and a peptide containing an epithelial targeting motif, a cationic K_{16} motif, a hydrophobic XSX motif, and an RVRR cleavable linker (K_{16}RVRRXSXGACYGLPHKFCG) (Grant-Serroukh et al, 2022) at a 3:1:4 mass ratio to create our library of 6 LNPs (Figure 1a).

We assessed LNP toxicity and their ability to deliver 1 μg *ABE8e* mRNA and 0.5 μg sgRNA into a bulk population of patient-derived fibroblasts carrying the target variant, using Lipofectamine MessengerMAX (LFMM) as a positive control and naked (unencapsulated) mRNA as a negative control. We observed lower cytotoxicity using C14/DOPE- and C16/DOPE-based LNPs than using LFMM, measured by a lactate dehydrogenase assay (Figure 1b) and microscopy imaging (Figure 1c), demonstrating their improved safety profile and potential for in vivo use. Sanger sequencing was used to investigate editing efficiency 24 hours after transfection. The chromatograms (Figure 1d) are representative of 6 biological replicates and show a difference in the wild-type sequence (single C peak; blue) and sequence for patients with DEB (double C/T peak; blue and red) at position c.5047. As expected, delivering unencapsulated mRNA and sgRNA resulted in no editing because unencapsulated mRNA cannot penetrate cell membranes and is rapidly degraded (Yin et al, 2017). Using our LNPs, the pathogenic variant was corrected at variable degrees, with full correction displayed as a complete reversal of the double C/T peak into a single C peak, observed in both C14/DOPE LNPs, C16/DOPE + peptide LNP, and LFMM. EditR was used for in silico analysis of base editing efficiency by calculating the percentage of T>C conversions (Sheriff et al, 2022). The mean editing efficiency of the pathogenic variant (Figure 1e) was over 81% using C14/DOPE LNPs and over 61% using C16/DOPE LNPs, both with and without peptide, with individual experimental replicates reaching 100% correction. This was confirmed through next-generation sequencing (Supplementary Figure S1). Correcting the c.5047 C>T *COL7A1* variant with ABE8e can result in a silent bystander edit at position c.5052. This bystander edit was lower using our novel LNPs than using LFMM, demonstrating lower off-target editing (Figure 1e). In addition, thorough off-target analysis has been previously conducted using the same sgRNA and *ABE8e* mRNA to correct the variant targeted in this study, revealing no significant off-target effects across the genome or transcriptome (Sheriff et al, 2022). Western blot analysis (Figure 1f) revealed that correction of the target variant

Abbreviations: C14, 1,2-Di-((Z)-tetradec-11-enyloxy)-N,N,N trimethylammonium propane chloride; C16, 1,2-Di-((Z)-hexadec-11-enyloxy)-N,N,N trimethylammonium propane iodide; C18, 1,2-Di-((Z)-octadec-9-enyloxy)-N,N,N trimethylammonium propane chloride; C7, type VII collagen; DEB, dystrophic epidermolysis bullosa; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; LFMM, Lipofectamine MessengerMAX; LNP, lipid nanoparticle; sgRNA, single-guide RNA

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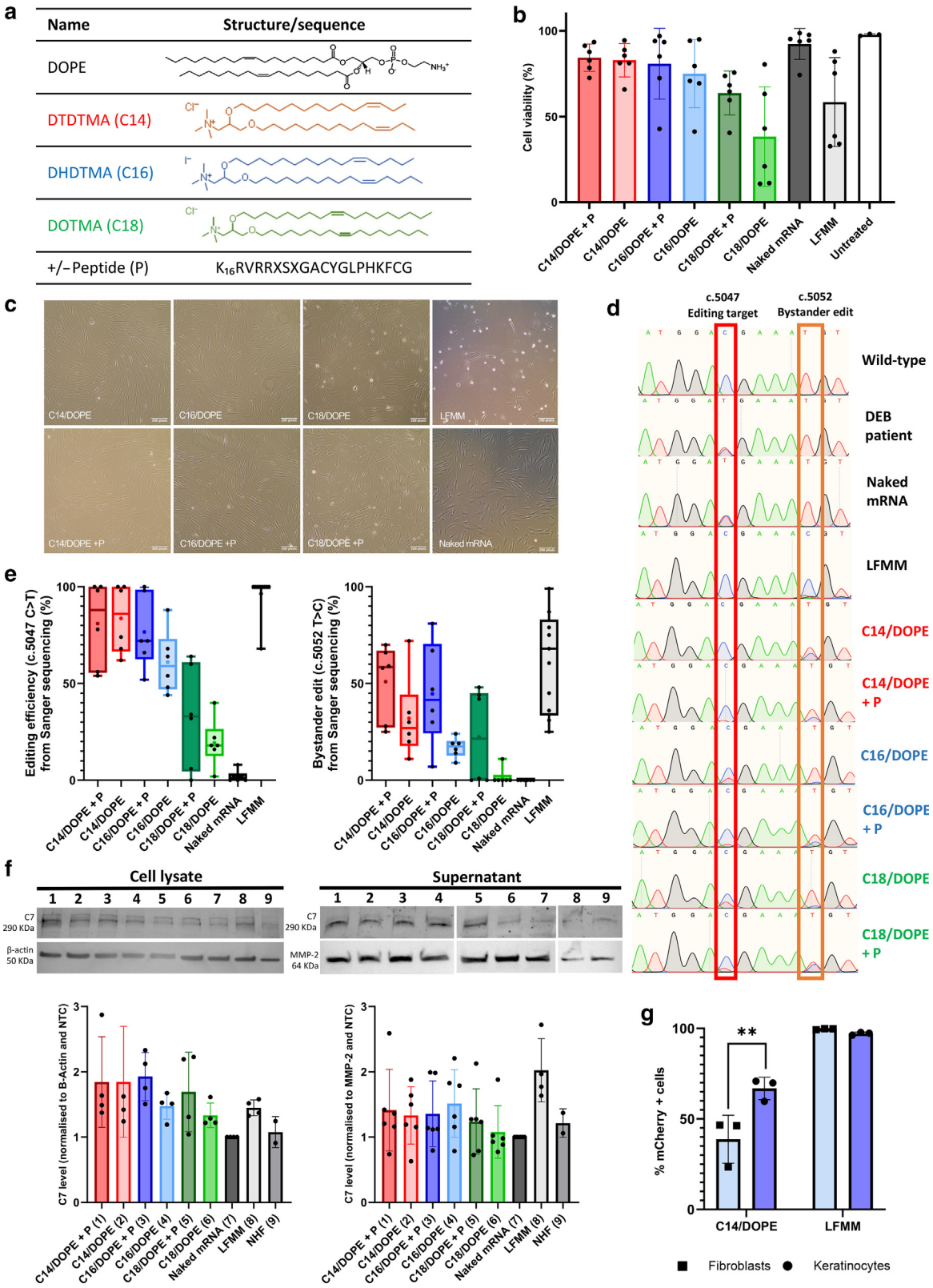

Figure 1. Correction of *COL7A1* c.5047 C>T variant using novel LNPs to deliver *ABE8e* to the fibroblasts of a patient with DEB. (**a**) Structures of lipids and sequence of peptide used in this study. P denotes K_{16}RVRRXSXGACYGLPHKFCG. (**b**) Results from a lactate dehydrogenase cytotoxicity assay reveal that C14/DOPE and C16/DOPE LNPs induce less cell damage/death in vitro than LFMM. n = 6. (**c**) Light microscopy images show that treatment with LFMM causes a change in morphology, with cells becoming shrunken, rounded, and detached. This is also accompanied by substantial cell loss. Bar = 200 μm. (**d**) Representative Sanger sequencing chromatograms showing correction of the pathogenic variant, with full correction displayed as a complete reversal of the double C/T red and blue peak into a single blue C peak as observed in both C14/DOPE LNPs, C16/DOPE + P LNP, and LFMM. (**e**) The mean editing efficiency

corresponds to an increase in C7 production and secretion, with 4 of 6 LNPs showing secretion levels comparable with that of normal human fibroblasts. Next, we compared the efficiency of transfecting nTERT keratinocytes and primary normal human fibroblasts using LFMM or C14/DOPE to deliver *mCherry* mRNA. Using C14/DOPE, nTERT keratinocytes showed a higher transfection rate than fibroblasts, with a mean percentage mCherry$^+$ cells of 66.83 and 38.80 for nTERTs and normal human fibroblasts, respectively ($P = .0013$) (Figure 1g).

Despite progress in the field of gene editing, skin remains underinvestigated for delivery of gene editing tools (Guri-Lamce et al., 2023). A recent study used ionizable LNPs to deliver *Cas9* mRNA or ribonucleoprotein to primary human keratinocytes, yielding 10−72% insertion−deletion formation depending on LNP formulation. These LNPs were then used to deliver *Cas9* mRNA or ribonucleoprotein in situ in bioengineered 3-dimensional skin models, with editing rates between 5 and 12% (Bolsoni et al, 2023). We have used novel *ABE8e* mRNA and sgRNA-loaded LNPs (C14/DOPE, C16/DOPE, C18/DOPE ± peptide K_{16}RVRRXS XGACYGLPHKFCG) to correct a recurrent DEB-causing variant and achieved high editing efficiency using C14/DOPE LNPs. We have also demonstrated that editing this variant restores functional levels of secreted C7. Previous reports indicate that much lower editing efficiencies than those achieved in this study are sufficient to restore full-length C7 at the dermal−epidermal junction (Osborn et al, 2020; Hong et al, 2022). Therefore, our C14/DOPE LNPs offer a promising foundation for clinical translation and in vivo gene editing application. In the future, we aim to topically test these LNPs in situ, with the ultimate goal of developing therapeutic topical base editing treatments (Supplementary Figure S2), bestowing a milestone transformative to the burden of DEB and potentially other skin diseases.

ETHICS STATEMENT

This study was performed in accordance with the Declaration of Helsinki. This human study was approved by Guy's and St Thomas' NHS Foundation Trust (approval: 07/H0802/104). All adult participants provided written informed consent to participate in this study.

DATA AVAILABILITY STATEMENT

Raw fastq datasets for next-generation sequencing can be requested from the corresponding author. No other datasets were generated or analyzed in this study.

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KEYWORDS

Epidermolysis bullosa; Gene editing; Gene therapy; Lipid nanoparticles

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: JJ-M, IG-L; Data Curation: IG-L, YA, JJ-M; Formal Analysis: IG-L, YA; ŁŁ; Investigation: IG-L, YA, CG, RM; Writing - Original Draft Preparation: JJ-M, IG-L; Writing - Review and Editing: JJ-M, IG-L, YA, CG, RM, SLH, JAM, ŁŁ, ER, MC

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2024.03.027

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after Sanger sequencing was over 81% using C14/DOPE LNPs and over 61% using C16/DOPE LNPs. The bystander edit was also lower using our LNPs than using LFMM. n = 6. (**f**) Western blot analysis reveals that the correction of the pathogenic variant corresponds to an increase in C7 production and secretion in cell lysates and supernatant, respectively, with 4 of 6 LNPs restoring secretion to levels close to that of NHF. Cell lysate, n = 4; NHF = 2. Supernatant, n = 6; LFMM = 4; NHF = 2. (**g**) The transfection of 1 μg of *mCherry* mRNA using C14/DOPE or LFMM was compared between fibroblasts and nTERT keratinocytes. C14/DOPE transfected nTERT keratinocytes significantly better, with a mean percentage mCherry$^+$ cells of 66.83 and 38.80 for nTERT keratinocytes and NHFs, respectively ($P = .0013$). n = 3. DTDTMA (C14), 1,2-Di-((Z)-tetradec-11-enyloxy)-N,N,N trimethylammonium propane chloride; DHDTMA (C16), 1,2-Di-((Z)-hexadec-11-enyloxy)-N,N,N trimethylammonium propane iodidet; DOTMA (C18), 1,2-Di-((Z)-octadec-9-enyloxy)-N,N,N trimethylammonium propane chloride; C7, type VII collagen; DEB, dystrophic epidermolysis bullosa; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; LFMM, Lipofectamine MessengerMAX; LNP, lipid nanoparticle; NHF, normal human fibroblast.

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SUPPLEMENTARY MATERIALS AND METHODS

Cell culture

Primary patient fibroblasts previously isolated from punch biopsies were obtained from the cell bank at St. John's Institute of Dermatology (London, United Kingdom). The fibroblasts used are from a patient with dystrophic epidermolysis bullosa, compound heterozygous for the c.5047 C>T (p.Arg1683X) mutation, targetable by ABE8e and c.7344+1 G>A (IVS95+1G>A), which we do not target. Dystrophic epidermolysis bullosa fibroblasts and normal human fibroblasts (passages 6–11) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% GlutaMAX (Gibco), and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% carbon dioxide. The culture medium was changed every 2 days, and the cells were passaged using TrypLE (Gibco) when they reached 90% confluence. nTERT keratinocytes were cultured in DMEM:Ham's F12 (1:1) supplemented with 10% fetal calf serum and RM+ (0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml epidermal GF, 5 μg/ml transferrin, 8.4 ng/ml cholera toxin, and 13 ng/ml liothyronine) and passaged using 0.25% Trypsin-EDTA (1×) (Gibco).

Lipid nanoparticle formulation

The cationic lipids 1,2-Di-((Z)-octadec-9-enyloxy)-N,N,N trimethylammonium propane chloride (C18); 1,2-di-([Z]-hexadec-11-enyloxy)-N,N,N trimethylammonium propane iodide (C16); and 1,2-Di-((Z)-tetradec-11-enyloxy)-N,N,N trimethylammonium propane chloride (C14) were purchased from Avati Polar Lipids. The lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from Cayman Chemicals. The Peptide was obtained from AMS Bio. Cationic liposomes were formulated by microfluidic mixing using the NanoAssemblr (Precision Nanosystems). Cationic lipids were mixed with (DOPE) at a 1:1 molar ratio, with 1 part ethanol phase, containing the lipids, mixed with 3 parts aqueous phase, containing nuclease-free water. After mixing, the liposomes were sonicated for 20 minutes in an ultrasonic water bath (Grant) and then dialyzed overnight against nuclease-free water using Maxi GeBAflex tubes (Generon) to remove any residual ethanol. Lipid nanoparticles were made by mixing the liposomes with mRNA at a 3:1 mass ratio or mixing liposomes with mRNA and peptide (K_{16}RVRRXSXGAC YGLPHKFCG) at a 3:1:4 mass ratio in OptiMem (Thermo Fisher Scientific) and incubated at room temperature to stabilize the lipid nanoparticles for 30 minutes before transfection.

Base editor mRNA and single-guide RNA design

ABE8e mRNA was ordered from TRILINK and included full substitution of uracil for N1- methylpseudouridine, cotranscriptional 5′ capping with the CleanCap AG analog resulting in a 5′ Cap1 structure, and a 120 nucleotide polyA tail. Single-guide RNA targeting the c.5047 C>T variant in *COL7A1* (5′- ACATTTCATC-CATCCTCTCCAGG-3′) was designed using the CRISPR RGEN guide-RNA designer tool and manufactured as synthetic single-guide RNA by Synthego, modified with 2′-O-Methyl at the first and last bases and 3′ phosphorothioate bonds between the first 3 and last 2 bases.

Transfection

Cells were seeded 24 hours before transfection in 6-well plates at 100,000 cells per well. The lipid nanoparticles were prepared as described at an mRNA mass of 1 μg per well. The DMEM was removed, and the Opti-Mem containing lipid nanoparticles was added to the cells and incubated at 37 °C in 5% carbon dioxide for 24 hours, after which the media was removed and replaced back to DMEM. Lipofectamine MessengerMAX (Thermo Fisher Scientific) was used per protocol as the positive control for transfection, and naked (unencapsulated) mRNA was used as a negative control.

DNA extraction and sanger sequencing

When the cells reached approximately 90% confluence in the 6-well plate, genomic DNA was extracted using the QIAamp DNA mini extraction kit (Qiagen) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The AmpliTaq Gold 360 master mix (Thermo Fisher Scientific) was used for polymerase chain reaction amplification along with primers to target the desired locus: (forward, CAGTGTAGCGGGTCATAGGG; revers, GCCTTCAGATGCGTGTGTGC). A GeneAmp 9700 PCR System (Applied Biosystems) was used to perform the polymerase chain reaction. The polymerase chain reaction product was visualized by gel electrophoresis using a 1.5% agarose gel containing SybrSafe and then purified using the illustra Exo-Star PCR Clean-Up Kit (GE Healthcare) and sent externally for Sanger sequencing (Source Bioscience). Sanger sequencing chromatograms were visualized with SnapGene, and editing efficiency was analyzed using EditR (http://baseeditr.com/).

Next-generation sequencing

The same DNA samples and polymerase chain reaction settings used for Sanger sequencing were used for next-generation sequencing, with sequence-specific primers with next-generation sequencing overhangs used for the polymerase chain reaction (forward, CGTCGGCAGCGTCAGATGTGTATAA GAGACAGCTGCTGCTCAGACCCTTC TC; reverse, GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGCCTTCA GATGCGTGTGTGC). Sequencing was performed on a MiSeq instrument (Illumina), using MiSeq Reagent Kit, version 2 nano (500 cycles), following the manufacturer's instructions. The sequences were obtained in fastq format, with demultiplexing performed automatically. Sequence annotation to the human genome (hg38) was done in Bowtie (https://bowtie-bio.sourceforge.net/manual.shtml), and the counts of the individual base reads against genome positions for further calculations were determined using Integrative Genomics Viewer (https://igv.org/).

Cytotoxicity assay

The lactate dehydrogenase assay (AbKine) was used to determine cytotoxicity after transfection. OptiMem media was collected from the cells 24 hours after transfection. The media was added to a clear, flat-bottomed 96-well plate at 50 μl per well and mixed with 50 μl working reagent. A media-only sample was used as a blank condition, and a 100% dead condition was prepared by adding 1% Triton-X (Sigma-

Aldrich) to cells in OptiMem media for 5 minutes before removing the media. A standard lactate dehydrogenase curve was also prepared on the same plate. The plate was incubated at 37 °C in the dark for 30 minutes, and then the absorbance was measured at 450 nm on a FLUOstar Omega Microplate Reader (BMG Labtech).

Protein extraction

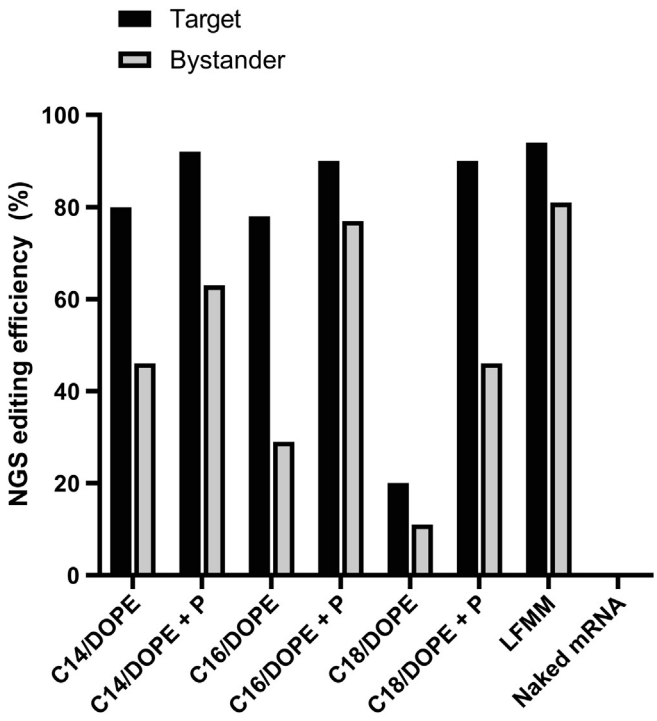
Around 70% confluence, cells were washed with PBS, and the media was replaced to OptiMem media supplemented with 50 μg/ml ascorbic acid. For the supernatant, the media was collected after 24 hours and added to 4× volume of ice-cold acetone and stored overnight at −20 °C. This was then centrifuged using a Hettich Mikro 22R Refrigerated Centrifuge at 18,000 r.p.m. at 4 °C for 10 minutes to precipitate the proteins from the supernatant. The supernatant was removed, and the protein pellet was left to dry for 5 minutes. The pellet was then resuspended in 200−300 μl of 1 M urea Tris-hydrogen chloride buffer and vortexed until dissolved. For the cell lysate, after the OptiMem media had been collected, 100 μl RIPA buffer (Thermo Fisher Scientific) supplemented with 1% protease inhibitor was added to the cells and scraped. The lysis extract was collected and stored overnight at −20 °C and then centrifuged at 8000 r.p.m. at 4 °C for 10 minutes to remove cell debris. To quantify the protein, the Thermo Fisher BCA assay was used per protocol to normalize the amount of protein loaded for the western blot.

Western blot

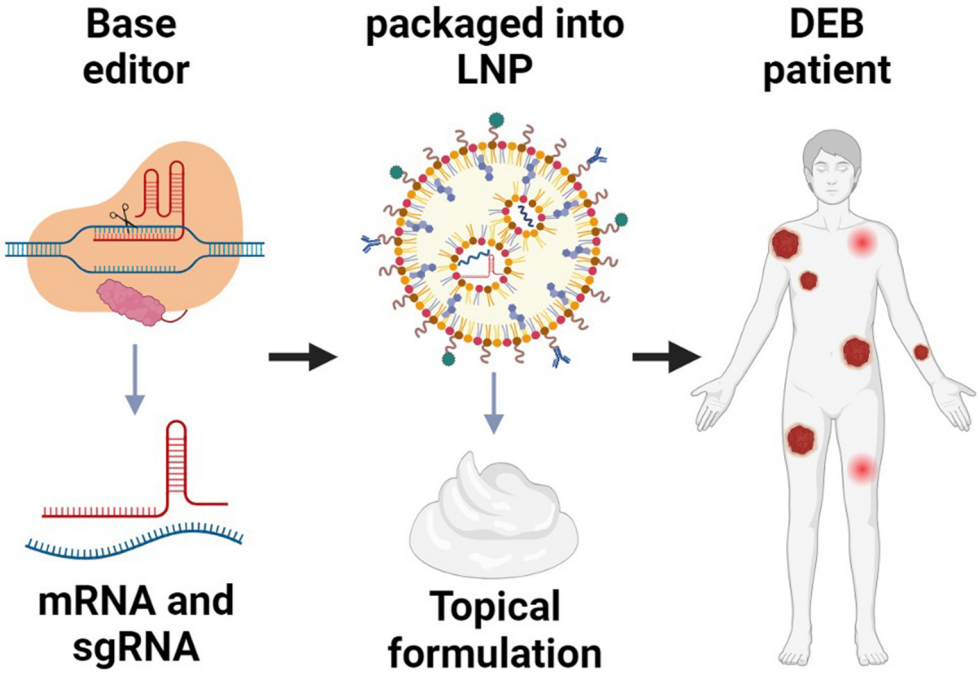
Lammeli buffer (Bio-Rad Laboratory) supplemented with β-mercaptoethanol (Sigma-Aldrich) was added to each sample and boiled for 10 minutes at 95 °C. The samples were then loaded onto Bio-Rad 4−15% acrylamide gels, at volumes determined by a BCA assay (Thermo Fisher Scientific), alongside a High Range Protein Ladder (Thermo Fisher Scientific), and ran at 80 V for an hour. The transfer was performed on a polyvinylidene difluoride membrane in the Bio-Rad Trans-Blot Turbo Transfer System. The membrane was blocked for 1 hour using a 5% skim milk powder in Tris-buffered saline-Tween20 and then incubated overnight at 4 °C with primary antibody (Bio-Rad rabbit anti collagen VII VPA00854) diluted at 1:1000. The membrane was washed with Tris-buffered saline-Tween20 and incubated for 1 hour at room temperature with secondary antibody (Dako, P044801-2, polyclonal horseradish peroxidase anti-rabbit) diluted at 1:2000. The membrane was developed with Amersham Detection Reagent (Cytiva) and imaged with the Invitrogen iBright FL1000 system. The antibody steps were repeated for the housekeeping gene, β-actin, for cell lysate blots. Primary (Santa Cruz Biotechnology, mouse monoclonal, sc-47778) and secondary (Dako, polyclonal horseradish peroxidase anti-mouse, E0354) antibodies were diluted at 1:2000. Supernatant blots were incubated with anti-matrix metalloproteinase 2 primary antibody (Cell Signaling Technology, rabbit polyclonal, 4022S) diluted at 1:1000, and the same secondary anti-rabbit was used. Cell lysates were normalized to β-actin and the negative control, whereas the supernatant samples were normalized to matrix metalloproteinase 2 and the negative control. Normalized densitometry values were calculated using ImageJ.

Flow cytometry

Transfection was performed as described earlier using EZ Cap mCherry mRNA (APExBIO, R1017). 24 hours after transfection, cells were trypsinized, washed twice in FACS buffer (2 mM EDTA and 2% fetal bovine serum in 1× PBS), and then resuspended in 300 μL of the buffer prior to being spiked with DAPI (Thermo Fisher Scientific, 62248) at 1:1000. Cells heat shocked at 65 °C for 5 minutes were mixed with normal cells at a 50:50 ratio for the live/dead control. Flow cytometry was performed on CytoFlex, and data were analyzed on FlowJo.



Supplementary Figure S1. Editing efficiency as calculated by NGS of c.5047 C>T editing target and c.5051 T>C bystander. P denotes K_{16}RVRRXSXGACYGLPHKFCG. n = 1. C14, 1,2-Di-((Z)-tetradec-11-enyloxy)-N,N,N trimethylammonium propane chloride; C16, 1,2-di-([Z]-hexadec-11-enyloxy)-N,N,N trimethylammonium propane iodide; C18, 1,2-di-([Z]-octadec-9-enyloxy)-N,N,N trimethylammonium propane chloride; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; LFMM, Lipofectamine MessengerMAX; NGS, next-generation sequencing.



Supplementary Figure S2. Envisioned strategy. Our goal is to develop therapeutic topical base editing creams/gels using LNPs for patients with DEB. DEB, dystrophic epidermolysis bullosa; LNP, lipid nanoparticle; sgRNA, single-guide RNA.