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Persistence of non-viral vector mediated RPE65 expression: case for viability as a gene transfer therapy for RPE-based diseases

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Abstract

Mutations in the retinal pigment epithelium (RPE) gene RPE65 are associated with multiple blinding diseases including Leber's Congenital Amaurosis (LCA). Our goal has been to develop persistent, effective non-viral genetic therapies to treat this condition. Using precisely engineered DNA vectors and high capacity compacted DNA nanoparticles (NP), we previously demonstrated that both plasmid and NP forms of VMD2-hRPE65-S/MAR improved the disease phenotypes in an *rpe65*^{-/-} model of LCA up to 6 months post-injection (PI), however the duration of this treatment efficacy was not established. Here, we test the ability of these vectors to sustain gene expression and phenotypic improvement for the life of the animal. NPs or naked DNA were subretinally injected in *rpe65*^{-/-} mice at postnatal day (P) 16 and evaluated at 15 months PI. Quantitative real-time PCR (qRT-PCR) and immunofluorescence were performed at PI-15 months and demonstrated appreciable expression of transferred RPE65 (levels were 32% of wild-type [WT] for NPs and 44% of WT for naked DNA). No reduction in expression at the message level was observed from PI-6 month data. Spectral electroretinography (ERG) demonstrated significant improvement in cone ERG amplitudes in treated versus uninjected animals. Most importantly, we also observed reduced fundus autofluorescence in the eyes injected with NP and naked DNA compared to uninjected counterparts. Consistent with these observations, biochemical studies showed a reduction in the accumulation of toxic retinyl esters in treated mice, suggesting that the transferred hRPE65 was functional. These critical results indicate that both NP and uncompacted plasmid VMD2-hRPE65-S/MAR can mediate persistent, long-term improvement in an RPE-associated disease phenotype, and suggest that DNA NPs, which are non-toxic and have a large payload capacity, expand the treatment repertoire available for ocular gene therapy.

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CONFLICTS OF INTEREST

Mark Cooper is an employee of Copernicus Therapeutics, Inc. and owns stock in the company.

Keywords

RPE65; non-viral gene therapy; retinal pigment epithelium; DNA nanoparticle; S/MAR

INTRODUCTION

Leber's Congenital Amaurosis (LCA) is an autosomal recessive blinding disease that affects 1 in 81,000 newborns every year [1] and currently has no curative therapies. Mutations in the retinal pigment epithelium (RPE)-specific isomerohydrolase RPE65 account for 16% of all LCA patients [2]. RPE65 activity is critical for the visual cycle and regeneration of the visual chromophore 11-cis-retinal, and in its absence, retinyl esters accumulate in the RPE. This accumulation of toxic intermediates eventually leads to RPE atrophy and subsequent death of cone and rod photoreceptors [3]. In addition, since the visual chromophore is not regenerated, rhodopsin does not function and dim light vision is completely absent in LCA patients [3].

Since many causative LCA genes are known, development of genetic therapies has been a research priority. The most common vectors, adeno-associated viruses (AAV), are currently being tested in Phase I/II clinical trials for RPE65-associated LCA [4-6]. Though these trials have exhibited positive results, improved efficacy is needed and some concerns remain [7, 8]. We have been developing non-viral gene therapies; specifically compacted DNA nanoparticles (NPs) combined with engineered plasmid vectors, for the treatment of genetic ocular diseases. These NPs/vectors have several advantages [9]; in particular they transfect mitotic and post-mitotic cells and have a large capacity (tested up to 20 kb in the lung [10] and 14 kb in the eye [11]) enabling compaction of vectors carrying large disease genes or vectors containing beneficial regulatory DNA elements. Importantly, they do not elicit any toxic responses after subretinal delivery in the eye [12-14], even after repeat injections, an issue that may be a concern with some AAVs [15].

In contrast to AAV, which has been shown to last for 3 or more years in animals as well as patients, one of the key limitations of some non-viral gene therapies has been transient gene expression. To overcome this, we have previously tested beneficial DNA elements, specifically a scaffold/matrix attachment region (S/MAR) in our vector and demonstrated correction of the prolonged photoreceptor expression and correction of the *Abca4*^{-/-} Stargardt's dystrophy disease phenotype for up to 8 months, respectively [11]. Subsequently, we extended this work to the RPE. We showed that an eGFP reporter gene in an S/MAR vector drove RPE-specific gene expression for up to 2 years (longest timepoint tested) when delivered as either nanocompacted or uncompacted (naked) DNA. When this vector contained hRPE65 instead of eGFP, we showed it could mediate improvements in cone function in the *rpe65*^{-/-} model of LCA at post injection (PI) 6 months [16]. While these data are encouraging, truly long-term studies are needed due to the chronic nature of disease targets and our previous work showing that the persistence of gene expression can vary with the gene and promoter of interest [17-19]. Here we fully characterize the long-term efficacy of compacted DNA NPs in the RPE. We observe persistent RPE65 expression in NP or naked DNA treated *rpe65*^{-/-} mice (a well-studied model of RPE65-associated LCA) for up to PI-15 months (longest timepoint tested). More importantly, our biochemical analyses suggest that the transferred RPE65 is enzymatically active (something we have not assessed previously), and we show long-term, appreciable improvement in the LCA disease phenotype.

MATERIALS AND METHODS

Animal Care

All animal experiments and maintenance were approved by the Institutional Animal Care and Use Committee (University of Oklahoma, Oklahoma City, USA), and were performed under the guidelines of the Association of Research in Vision and Ophthalmology (Rockville, MD, USA). *Rpe65*^{-/-} mice were obtained from Dr. Michael Redmond (National Eye Institute) and were maintained in the breeding colony under cyclic light (12L:12D, 30 lux) conditions. All mice used for these studies except for fundus imaging were albino.

Plasmid DNA construction, NP compaction, and subretinal injection

The human VMD2 promoter (-585/+38) and hRPE65 cDNA sequence were cloned upstream of the S/MAR sequence in the pEPI vector [20] generously shared by Dr. Isa Stehle/Dr. Hans Lipps (University of Witten/Herdecke). Plasmids were amplified and processed for endotoxin removal (Aldevron L.L.C., Fargo, ND) prior to NP formulation and use in animal studies. Plasmid DNA was compacted with CK30PEG10K using acetate as the lysine counterion as previously described [10, 21, 22]. NP minor diameter was determined to be 8-11 nanometers by transmission electron microscopy and final concentration was 4.3 mg/ml in saline. TEMs of the NPs used in this study are found in [16]. As described previously, all NPs underwent a series of quality control tests to confirm stability, homogeneity, charge, lack of aggregation, and DNase resistance [16, 22, 23]. Trans-corneal or trans-scleral (to minimize corneal and lens opacification in fundus imaging experiments only) subretinal injections in the superior central region were performed in *rpe65*^{-/-} mice at post-natal day (P) 16 as described previously [24].

Quantitative Real-Time PCR

qRT-PCR was performed for RPE65 (F: 5'-GAGATATGTACTTCCTTG-3', R: 5'-CTTCTGGTAATTGATTGAG-3') and data were analyzed as previously described [22]. RPE65 levels were normalized to the housekeeping gene HPRT (F: 5'-GCAAACCTTTGCTTCCCTGGTT-3', R: 5'-CAAGGGCATATCCAACAACA-3'). The RPE65 primer sequences are common to human and mouse and were used to enable direct comparison of expression levels. N=5-6 eyes/group.

Immunohistochemistry

Immunohistochemistry was performed as previously described [18, 25]. Retinal cryosections or whole mounts were blocked in 5% BSA and incubated overnight with the following antibodies: mouse monoclonal anti-RPE65 (ab13826, Abcam, Cambridge, MA), rabbit polyclonal anti-RPE65 (shared by Dr. J. X. Ma, University of Oklahoma HSC, OK), goat polyclonal anti-S-opsin (sc-14363, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-M-opsin (generously shared by Dr. Cheryl Craft, University of Southern California), followed by washing and incubation with appropriate fluorescent secondary antibodies (Life Technologies, Grand Island, NY). Slides were observed and images acquired with a BX-62 spinning disk confocal microscope (Olympus, Japan) and Slidebook 4.2 software. Exposure times and display settings for all images were normalized to a no primary control section. N=3-4 eyes/group.

Ocular Fundus Photography

Fundus imaging was performed as previously described [25]. Briefly, anesthetized mice were placed on a pedestal and the eye was placed directly under the lens of the MICRON III camera (Phoenix Research Laboratories, Pleasanton, CA) and images were collected. N=3-5 mice/group.

Electroretinography (ERG)

Mice (N=5-9 mice/group) were dark adapted overnight, anesthetized and ERG was performed as described previously [18, 25]. Briefly, a stimulus intensity of 157 cd s/m² was presented to dark-adapted, dilated eyes in a Ganzfeld (Model GS-2000; LKC Technologies, Gaithersburg, MD) to record scotopic a- and b-wave responses. Subsequently, photopic responses were evaluated after light adapting the animals for 5 minutes under a light source of 29.03 cd/m² and strobe flashes of photopic green light (510 nm peak), and ultra violet light (360 nm peak) light were presented to the dilated eyes in the same Ganzfeld with stimulus intensities of 12.5 cd s/m², and 0.79 cd s/m², respectively.

Retinoid measurements

Mouse eyes (1-4 eyes pooled/sample and 3-7 independent samples for all groups) from each individual experiment were homogenized in 1 ml DPBS (1×) containing 4 M hydroxylamine and incubated at ambient temperature for 30 min. All-trans-retinyl acetate (atRAc) was added to the homogenate as an internal standard after which the mixture was extracted with chloroform (3 × 2 ml). After filtration, the organic layers were combined and dried under argon. The sample was re-dissolved in 10 µl hexane and 10 µl was injected into Waters Acquity UPLC-ESI/MS (Waters, Milford, MA). Normal phase chromatography was employed using an Acquity UPLC BEH Amide Column (130Å, 1.7 µm, 2.1 × 100 mm; Waters, Milford, MA), and solvent elution with hexane (A) and hexane-dioxane-isopropanol (60:36:4, v/v/v) (B) with the following gradient of solvent A: 0-3 min, 99.8%; 3-13 min, 99.8 to 95%; 13-28 min, 95 to 80%. The flow rate was 0.5 ml/min. UV absorbance peaks were identified by comparison with external standards of all-trans-retinyl palmitate (Sigma-Aldrich, St. Louis, MO), 11-cis-retinol (reduced from 11-cis-retinal [26]), all-trans-retinol (Sigma-Aldrich), and retinal oximes of all-trans-retinal (Sigma-Aldrich) and 11-cis-retinal (a gift to JRS from Dr. Rosalie Crouch, Medical University of South Carolina). Identification was confirmed by mass to charge ratio (m/z). The recovery of atRAc was used to calibrate for loss during extraction. Molar quantities per eye were calculated by comparison to standard concentrations determined spectrophotometrically using published extinction coefficients [27], and values were normalized to total sample volumes. Peak areas were calculated using Waters Empower Software.

Statistics

Graphpad Prism was used to conduct statistical analyses. To determine whether there were statistically significant differences between mean gene expression levels, mean ERG amplitudes, and mean retinoid levels, results were analyzed using 1-way ANOVA with post-hoc analysis by Bonferroni's multiple comparison test. Results are plotted as means ± SEM. * P<0.05, ** P<0.01, *** P<0.001.

RESULTS

Long-term hRPE65 expression analysis in treated *rpe65*^{-/-} mice

Previously, we demonstrated that S/MAR-containing nanocompacted and uncompacted DNA both drove persistent eGFP expression in the RPE which did not significantly decrease over time (for up to two and a half years PI [24]). To confirm that similar persistence of expression was observed with therapeutic S/MAR vectors, we used NPs in which we replaced eGFP with the human RPE65 cDNA [16]. NPs or uncompacted VMD2-hRPE65-S/MAR was subretinally delivered to *rpe65*^{-/-} mice at P16. At PI-15 months, qRT-PCR and immunofluorescence were conducted to examine long-term hRPE65 expression. We show that there is no significant reduction in expression of hRPE65 message in NP or naked DNA treated animals at PI-15 months compared to PI-6 months (PI-6 month data replotted from

[24], Fig. 1A). At PI-15 months, mean hRPE65 message levels in NP and naked DNA treated eyes are 30-40% of WT. There was no significant difference in expression levels at either PI-6 months or PI-15 months between naked DNA and NP treated animals. Immunofluorescence labeling of retinal sections (Fig. 1B, top) and RPE flat mounts (Fig. 1B, bottom) with antibodies against RPE65 (red) shows that RPE65 protein expression is robust at PI-15 months, and as expected, is specific to the RPE cell layer.

Long-term functional improvement is seen in treated *rpe65*^{-/-} mice

To determine whether this long-term gene expression led to preservation of retinal function, scotopic and spectral photopic electroretinography (ERG) was conducted on treated *rpe65*^{-/-} and age-matched control animals at PI-15 months. We previously observed no significant improvement in scotopic a-wave amplitudes at PI-6 months, and only small improvements in scotopic b-wave [24]. This result was recapitulated at PI-15 months; although means were higher in NP and naked DNA treated than untreated animals, the improvement was not statistically significant (Fig. 2A-B). In contrast, treated animals exhibited significant improvement in both UV photopic b-wave (short wavelength [SWL]-cone response, Fig. 2C) and green photopic b-wave (medium wavelength [MWL]-cone response, Fig. 2D) compared to uninjected animals. Green cone photopic b-wave amplitudes in treated animals were not significantly different from age-matched WT animals and mean values were 75.9% (NP) and 78.5% (naked) of WT. There were no significant differences in ERG responses between naked DNA-treated and NP-treated eyes, consistent with our data in Figure 1. Importantly, there were no significant reduction in ERG amplitudes in treated eyes from 6 months [24] to 15 months, suggesting that the vectors may prevent further loss of photoreceptor cells over the long term.

Maintenance of cone photoreceptor cells in treated *rpe65*^{-/-} mice

Cone ERG results showed that treated animals exhibited improved cone function and survival compared to uninjected *rpe65*^{-/-} animals. To assess this improvement on the structural level, retinal sections from treated and control eyes at PI-15 months were co-labeled with antibodies against RPE65 (magenta), SWL-opsin (red), and MWL-opsin (green). Consistent with what has been previously shown for the *rpe65*^{-/-} model [28], uninjected *rpe65*^{-/-} eyes show significantly fewer MWL-labeled cone outer segments (OSs), virtually no SWL-opsin labeled OSs, and no expression of RPE65 (Fig. 3A-B, second row). In addition, the MWL-opsin labeling that is present is detected throughout the photoreceptor, rather than being specifically localized to the OS as it is in the WT (Fig. 3A, first and second rows). The site of injection is in the superior central region, and consistent with our previously observed pattern of distribution of expression (superior > nasal > temporal > inferior) [24], we observe that treatment with either naked DNA or NPs led to preservation of MWL-cones in the central superior and central nasal regions (Fig. 3A), but much less or none in the central inferior or temporal regions (inferior shown in Fig. 3B). While cones are preserved (in contrast to uninjected animals), MWL-opsin remained mislocalized throughout the cone cell body. We also observe preservation of some SWL-cones, but the improvement compared to uninjected is less pronounced.

Long-term structural rescue in *rpe65*^{-/-} mice

Increased fundus autofluorescence, detected on fundus images as bright white speckles (black arrows Fig. 4), is a distinguishing feature of *rpe65*^{-/-} and *rpe65*^{rd12} mice [24, 29]. This phenotype is thought to arise from accumulation of retinyl esters as a result of interruption of the visual cycle in the absence of RPE65 [3]. To determine if treatment with naked DNA or NPs has any effect on this bright white speckle like phenotype or accumulation of retinyl esters, we assessed both of these parameters at PI-15 months in WT

eyes (Fig. 4A) and in *rpe65*^{-/-} mice in which one eye was treated with NPs or naked DNA (Fig. 4B-C, right column) and the contralateral eye was untreated (Fig. 4B-C, left column). Shown in Fig. 4 are three representative WT and treated animals for each group. These bright white speckles in the fundus of the *rpe65*^{-/-} eye start to appear at ~2 months of age and are very pronounced at 15 months (Fig. 4B-C, left column). In contrast, the treated cohorts show tremendous reduction in this phenotype suggesting improvement in photoreceptor/RPE health by reduction in retinyl ester accumulation.

To biochemically quantify the amount of various retinoids, including retinyl esters, we performed UPLC-ESI/MS (ultra-performance liquid chromatography-electrospray tandem mass spectrometry) analysis in treated and untreated eyes as well as in age-matched WT samples. As expected, we observed a steep increase in the retinal ester all-trans retinyl palmitate in uninjected eyes (Fig. 5A). This accumulation was significantly reduced by an average of 41% in NP injected and 36% in naked DNA treated eyes (Fig. 5A). 11-cis retinol and 11-cis retinal levels were below the limit of detection in all knockout groups (treated and untreated), although normal levels were observed in WT eyes (not shown). Similarly, all-trans retinal levels were extremely low in all knockout groups while WT levels were normal (Fig. 5B). All-trans retinol levels were not significantly different in any cohort (Fig. 5C).

DISCUSSION

Here we show that delivery of S/MAR-containing NP or uncompact plasmid DNA carrying the VMD2-hRPE65-S/MAR expression cassette can mediate long-term (PI-15 months) structural and functional improvement in the LCA phenotype of the *rpe65*^{-/-} mouse. We observe long-term exogenous gene expression at levels ~40% of WT. This gene expression is sufficient to mediate significant improvements in survival of cone photoreceptors with consequent improvement in cone ERGs. Furthermore, we observe a striking reduction in fundus autofluorescence and accumulation of retinyl esters. Together these data strongly suggest that RPE health in aged *rpe65*^{-/-} mice is improved in the presence of transferred hRPE65 cDNA and that the expressed protein is functional. Combined with our previous work showing reporter gene expression for up to two and a half years with S/MAR containing vectors [24], these results suggest that S/MAR containing NP or uncompact DNA may be an effective long-term non-viral strategy for the treatment of RPE-based diseases.

Our results are generally consistent with what has been observed in previous studies using AAVs, lentivirus, and adenovirus to deliver RPE65 to mouse models of RPE65-associated LCA. Several studies have shown cone preservation (with early treatment) [30-32], improvement in cone function [30, 31, 33], some reduction in retinyl ester accumulation [33], and improvements in the fundus phenotype [33]. One of the interesting outcomes of this study is that, in common with our previous results [24], we did not observe a substantial increase in scotopic ERG amplitudes (although means in treated mice were higher than those in controls). This phenotype has also proven to be highly refractory to treatment in studies utilizing AAVs [15, 34], and even in studies reporting improvements in rod function, ERG amplitudes did not meet WT levels [30, 31, 33]. The mechanism underlying limited rod rescue is unclear. It could relate to variations in efficacy of the human vs. mouse RPE65 protein due to, for example, differential post-translational modifications. But both human and mouse RPE65 proteins have 6 putative conserved palmitoylation sites (CSS Palm 3.0 software) suggesting that this may not play a significant role. A second issue is that many visual transduction genes (in rods and cones) are downregulated in *rpe65*^{-/-} mice [35, 36], and it is not clear whether increasing hRPE65 alone is enough to compensate for (or reverse) those changes. However, our retinoid analyses may provide a better understanding of the

mechanism underlying this low scotopic function. Visual transduction mediated by rhodopsin activation results in photoisomerization of 11-cis to all-trans retinal. During the recycling of 11-cis from all-trans retinal, RPE65 plays the key role of converting all-trans retinyl palmitate and other esters to 11-cis retinol [37]. In the case of *rpe65* mutations (or in the absence of RPE65) this cascade is interrupted; as a result the recycling of 11-cis retinal is absent and 11-cis retinal levels are extremely low (resulting in very low scotopic function). Consistent with this, our data here show that visual cycle retinoids (11-cis retinal, all-trans retinal, and 11-cis retinol) are all highly reduced in the absence of RPE65, and that levels of these retinoids are not significantly improved with treatment. The lack of significant improvements in scotopic, rod-based vision is thus clearly understandable in light of the absence of recycled chromophore. We do observe significant, persistent improvement in cone function, likely a function of the fact that cones require significantly less retinoid overall (as cones make up only 3% of photoreceptors in the rodent) and may have the ability to regenerate their own chromophore [38, 39]. Importantly, although we do not see significant improvement in 11-cis retinal or 11-cis retinol levels, the significant reduction in all-trans retinyl palmitate (the ester which accumulates in the absence of RPE65) we observed suggests that the expressed protein is functional and is important for preserving RPE integrity.

The therapeutic question that arises from these data then is how to improve levels of 11-cis retinal (and thus further improve visual outcomes). One answer may be concurrent supplementation with retinoids. Studies have shown positive effects of 9-cis retinal (a stable chromophore precursor) and 9-cis-retinyl acetate supplementation in LCA models [40, 41], and clinical trials are underway to assess the utility of this approach in patients (clinicaltrials.gov, NCT01521793, NCT01014052). An additional approach would be to increase transduction efficiency and distribution of expression of genetic therapies. By incorporating S/MARs into our treatment vectors we have overcome one of the most vexing problems facing non-viral gene therapy for RPE-associated diseases (transient gene expression [25]), and future objectives will be on these other two parameters. Our data here suggests that RPE65 gene expression levels are ~40% of WT, and previous studies using eGFP reporter genes have shown that at two and a half years post-injection ~21% of all RPE cells express the transferred vector [24], with the highest concentration of expressing cells near the site of injection. However, our data suggest that increasing distribution of transferred vectors throughout the eye will be critical. As a result of the phagocytic activity of the RPE, both NP and uncompact DNA are efficiently taken up into the cell. While this efficient transfection is a beneficial feature of the RPE, future work will likely include testing novel NP compaction formulations to allow broader distribution of vector throughout the subretinal space prior to uptake and an increase in the total number of transfected cells.

We hypothesize that the longevity of the plasmid, which is a critical feature of this work and a required characteristic for efficacious non-viral gene therapy for chronic ocular diseases, results primarily from the inclusion and location of the S/MAR region in the vector. *In vivo*, S/MARs are thought to organize the nucleosomes into chromatin loops and play a critical role in nuclear architecture and function [42]. S/MARs have distinct affinity for the active centers of the nuclear matrix and play a role in stress induced DNA duplex destabilization [43], functions that are thought to be mediated by interactions between S/MARs and several nuclear factors [20, 44, 45]. Importantly, the S/MAR sequence and vector we use have been shown to augment gene transcription and promote the stability of virally and non-virally delivered DNA in other systems [46]. In our case, positive results likely arise from three features of S/MARs, namely their ability to augment transcription (by targeting to an active transcriptional hub via the aforementioned interactions with other nuclear factors [20, 44]), their ability to shield from repression effects, and their ability to promote episomal maintenance and DNA stability. Interestingly, augmentation of transcription was only seen

when the S/MAR was directly downstream of the expression cassette and upstream of the polyadenylation site [47] as it is in our vector, highlighting that location of the S/MAR within the vector may be a critical feature. We have not conducted extensive studies regarding silencing of our non-viral constructs, however, we do observe a precipitous drop in expression from PI-2 days to PI-120 days with non-S/MAR vectors [16], suggesting that shielding against repression/silencing effects [48], may be a beneficial effect of S/MARs in our system. Finally, it has been shown that S/MARs help plasmid DNA replicate episomally over several generations in cell culture and *in vivo* in liver cells [47, 49]. While RPE cells are non-dividing, and we have not assessed whether vector replication occurs in our system, episomal maintenance of gene therapy vectors is highly desirable over random integration for toxicity and safety-related reasons, but loss of episomal vectors over time leads to reduced expression. Thus, the ability of S/MARs to promote episomal maintenance is of substantial benefit. Our future studies may assess the mechanisms underlying plasmid maintenance in our system, and we have added a discussion of these issues to the current manuscript.

An important finding of this study is that both naked and compacted DNA generate similar levels and duration of hRPE65 transgene expression in the RPE cells, likely due to their phagocytic activity. In contrast, in most tissues naked DNA generates low levels and transient expression [50]. For example, in the lung [23], brain [51], and retinal photoreceptors [12, 18], DNA NPs generate several fold higher levels of transgene expression than naked DNA, and in each tissue long-term transgene expression has been documented for NPs [11, 51-53] but not for naked DNA. Furthermore, naked DNA can generate significant toxicities due to CpG island activation of toll-like receptors, whereas the same plasmid compacted as a NP does not [54, 55]. Importantly, DNA NPs have been evaluated in a human clinical trial in cystic fibrosis patients and no adverse events were attributed to the NPs [56]. In total, while naked DNA and NPs may be effective in the RPE, detailed toxicology studies are warranted for naked DNA as a potential therapeutic vector in the eye prior to further development.

In conclusion, we here show data demonstrating appreciable correction of the cone defects (structural and functional) and improved RPE health (assessed by fundus speckle phenotype and reduction in accumulation of toxic retinyl esters) in *rpe65*^{-/-} eyes treated with either NP or uncompact VMD2-hRPE65-S/MAR DNA. Most importantly, these vectors mediate this rescue for the long-term (here shown at 15 months PI, the longest timepoint evaluated), indicating that gene expression and rescue for the life of the animal are possible with a single non-viral treatment. The safety and large size capacity of DNA NPs is well described. Combined, these data suggest that S/MAR-containing NP vectors may be a suitable addition to the available repertoire of genetic treatments for many types of RPE-associated diseases, not just RPE65-associated LCA.

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Figure 1. Naked and NP VMD2-hRPE65-S/MAR drives long-term gene expression

NP or naked VMD2-hRPE65-S/MAR were subretinally injected at P16 in *rpe65*^{-/-} mice. **A.** qRT-PCR was conducted at PI-15 months on cDNA prepared from injected eyes as well as age-matched WT and uninjected *rpe65*^{-/-} controls. PI-6 month data are replotted from [24]; experiments here were conducted and analyzed by the same person and under the same conditions as those in [24] and HPRT values were consistent. N=5-6 eyes per group. **B.** Immunofluorescence was performed at PI-15 months on retinal cryosections (top) and RPE flat mounts (bottom) using rabbit polyclonal antibodies against RPE65 (red). Blue shows DAPI counterstain. Scale bar 20 μ m. RPE: retinal pigment epithelium, OS: outer segments, IS: inner segments, ONL: outer nuclear layer, INL: inner nuclear layer. N=3-4 eyes per group.

Figure 2. VMD2-hRPE65-S/MAR treatment results in improved photopic ERG function at PI-15 months

Full field scotopic and photopic ERGs were performed at PI-15 months on animals from all cohorts. Shown are mean scotopic a-wave (**A**), scotopic b-wave (**B**), photopic UV b-wave (**C**), and photopic green b-wave (**D**) amplitudes ($\pm$ SEM). No significant improvement is observed in scotopic amplitudes, but photopic amplitudes are increased significantly (* $P < 0.05$ in 1-way ANOVA with Bonferroni's post-hoc comparison) compared to uninjected. $N = 5-9$ mice/group.

Figure 3. Cones are preserved at PI-15 months after treatment with VMD2-hRPE65-S/MAR Retinal cryosections collected at PI-15 months from WT uninjected *rpe65*^{-/-}, naked VMD2-hRPE65-S/MAR, and NP-VMD2-hRPE65-S/MAR were labeled with mouse monoclonal anti-RPE65 (magenta), goat polyclonal SWL-opsin (red), rabbit polyclonal MWL-opsin (green), and DAPI (blue). Cone preservation is observed in the superior and nasal regions (A) but not the inferior (B) Scale bar, 50 μ M. RPE: retinal pigment epithelium, OS: outer segments, ONL: outer nuclear layer, INL: inner nuclear layer. N=3-5 eyes/group.

Figure 4. Fundus autofluorescence is reduced in VMD2-hRPE65-S/MAR treated eyes
Fundus photographs were taken at PI-15 months from pigmented WT (A) or pigmented *rpe65*^{-/-} mice (B-C). **A.** WT mice do not show any signs of fundus autofluorescence. **B-C.** Shown are three representative animals (one animal in each row) in which one eye was injected (right column) while the contralateral eye remained uninjected (left column). Observe significant fundus autofluorescence (black arrows) in the uninjected eye. Autofluorescence is significantly reduced, although still present to a minor degree (white arrows), in treated eyes. N=3-5 eyes/group.

Figure 5. Retinyl ester accumulation is attenuated in eyes treated with VMD2-hRPE65-S/MAR
Retinoid analysis was conducted on eyes collected from each group at PI-15 months. Shown are the retinyl ester all-trans retinyl palmitate (A), all-trans retinal (B), and the precursor all-trans retinol (C). N=3-7 independent samples per group, each sample contained between 1-4 eyecups. *** P<0.0001 by 1-way ANOVA with Bonferroni's post-hoc comparisons.