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Nanoparticle-mediated gene transfer specific to retinal pigment epithelial cells

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Abstract

Previously, we demonstrated that CK30PEG10k-compacted DNA nanoparticles (NPs) efficiently target photoreceptor cells and improve visual function in a retinitis pigmentosa model. Here, we test the ability of these NPs in driving transgene expression in the retinal pigment epithelium (RPE), using an RPE-specific reporter vector (VMD2-eGFP). NPs, uncompact plasmid, or saline were subretinally delivered to adult BALB/c mice. NP-based expression was specific to RPE cells and caused no deleterious effects on retinal structure and function. eGFP expression levels in NP-injected eyes peaked at post-injection day 2 (PI-2), stabilized at levels ~3-fold higher than in naked DNA-injected eyes, and remained elevated at the latest time-point examined (PI-30). Unlike naked DNA, which only transfected cells at the site of injection, NPs were able to transfect cells throughout the RPE. Subretinal injections of rhodamine labeled NPs and naked DNA showed comparable initial uptake into RPE cells. However, at PI-7 and -30 days significantly more fluorescence was detected inside the RPE of NP-injected eyes compared to naked DNA, suggesting NPs are stable inside the cell which could possibly lead to higher and sustained expression. Overall, our results demonstrate that NPs can efficiently deliver genes to the RPE and hold great potential for the treatment of RPE-associated diseases.

Keywords

Gene therapy; retinal pigment epithelium; DNA nanoparticle; subretinal injection; polyethylene glycol (PEG)

1. Introduction

Historically, non-viral gene therapy approaches have been limited by transient transgene expression, low transfection efficiency, limited uptake into some cells and low transgene expression levels [1–3]. However, recent advancements in vector engineering and delivery technologies have overcome these limitations, making non-viral gene therapy a promising approach for the treatment of human and animal diseases. Nanoparticles (NPs) containing a

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Disclosures

MJC is an employee of Copernicus Therapeutics and owns stock in the company.

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single molecule of plasmid DNA compacted with 30-mers of polylysine conjugated to 10 kDa polyethylene glycol (CK30PEG10k) is an example of a novel non-viral delivery approach [4]. This technology has been used to efficaciously deliver genes to cells of the lung, brain and retina [4–12] and yielded positive results in a phase I clinical trial for cystic fibrosis [13]. Our proof-of-principle experiments have demonstrated that DNA delivered via NPs can mediate phenotypic improvement in the structure and function of photoreceptors in the *retinal degeneration slow* mouse model of retinitis pigmentosa [5, 6]. In addition, others working with similarly compacted NPs have shown therapeutic benefits when delivered to a rat model of Parkinson's disease [12].

Many blinding inherited ocular diseases target the retinal pigment epithelium (RPE), a monolayer of cuboidal epithelial cells lining the back of the retina which functions to nourish photoreceptor cells, facilitate attachment of the retina, and metabolize retinoids [14]. Mutations in RPE-specific genes involved in the visual cycle and retinoid metabolism have been reported to cause several visual defects in patients [14]. For example, mutations in RPE65, bestrophin, lecithin retinol acyltransferase (LRAT), and retinol dehydrogenase (Rdh) genes have been shown to result in multiple ocular diseases including retinitis pigmentosa, Leber's Congenital Amaurosis (LCA), and Best's disease [15–18]. Viral vectors have shown promise in multiple RPE-associated LCA clinical trials (NCT00481546, NCT00516477, NCT00643747, www.clinicaltrials.gov) using recombinant adeno-associated viral (rAAV) vectors [19–23]. In spite of the striking positive results currently obtained with AAV-based vectors in the RPE, expanding the available repertoire of effective gene delivery agents is critical. Several of the disease-associated RPE-specific genes are too large to be packaged in AAV [24, 25], a limitation not present in CK30PEG10k NPs which can effectively deliver plasmids up to at least 20kb [26]. Although, AAV vectors are advantageous with great deal of tissue tropism, preexisting immunity due to prior exposure to wild-type (WT) AAV could limit therapeutic benefits [27, 28]. Furthermore, neutralizing antibodies to the capsid was detected in the serum of animals subretinally treated with AAV vectors [29]. In one study, some humoral response was also observed upon re-administration in a partner eye when injected intra-vitreally [30]. Importantly, NPs exhibit a positive safety profile. NPs carrying a variety of different vectors did not elicit an inflammatory response *in vivo* when delivered to the lungs of mice [31], the nares of human cystic fibrosis patients [13], the brains of rats [9], or the subretinal space of mice [5, 32]. CK30PEG10k NPs are non-immunogenic even after multiple subretinal injections (Z. Han, A. Koirala et. al., manuscript submitted). In this study, we evaluate the ability of CK30PEG10k NPs to efficiently transfect and drive gene expression in adult murine RPE cells *in vivo* as a potential therapeutic strategy for RPE-associated diseases using an eGFP reporter construct driven by a 600 bp fragment of the RPE-specific human vitelliform macular dystrophy 2 (VMD2) promoter previously shown to transduce mice RPE cells [33].

2. Materials and methods

2.1. Plasmid construction and nanoparticle formulation

pEGFP-1 plasmid (GenBank accession #U55761) was purchased from Invitrogen (Carlsbad, CA). The 600 bp human VMD2 promoter was PCR amplified from human DNA samples and cloned into the EcoRI and BamHI multiple cloning sites upstream of the eGFP sequence (Fig. 1a). pEGFP-1 encodes a red shifted variant of wild type GFP whose excitation maximum is 488nm and emission maximum is 505nm. Prior to NP formulation, plasmid DNA was processed for endotoxin removal by Aldevron (Fargo, ND) at <5 EU/mg DNA. Compaction of this vector into CK30PEG10k NPs was performed in the presence of acetate counterions and were processed with tangential flow filtration to remove excess polycations, solvent exchanged to normal saline, and concentrated to ~4.3 mg/ml DNA as described previously [10, 26]. DNA NPs were then characterized with a panel of quality control tests

[4] and had an endotoxin level of <5 EU/mg DNA. The minor diameter of the rod-like NPs was determined by transmission electron microscopy to be 8–11 nm (Fig. 1b). Electron microscopy was performed using carbon-coated grids and counterstained with uranyl acetate, as previously described [8].

Rhodamine labeling of endotoxin purified VMD2-eGFP plasmid was performed using *Label IT*® TM-Rhodamine labeling kit (Mirus Bio LLC, Madison, WI) with slight changes from the manufacturer's protocol. The number of Rhodamine molecules bound to DNA was quantified using the formula- base: dye = $(A_{\text{base}} * \epsilon_{\text{dye}}) / (A_{\text{dye}} * \epsilon_{\text{base}})$ and was determined to be 1 per 20 bp DNA (ϵ = Extinction coefficient, A= absorbance). Contribution of the dye to the absorbance was corrected by using the formula- $A_{\text{base}} = A_{260} - (A_{\text{dye}} * C.F._{260})$. $C.F._{260} = 0.27$ and $\lambda_{\text{max}} = 576$ nm for TM-Rhodamine as suggested by the manufacturer's protocol. TM-Rhodamine labeled DNA was compacted into rod-like CK30PEG10k NPs in the presence of acetate at a concentration of 3.6 mg/ml as described above.

2.2. Animals

One-month-old BALB/c female mice (25–30g) were purchased from Harlan Laboratories (Indianapolis, IN). Before use, mice were maintained in the breeding colony for at least one week under cyclic light (12L:12D) conditions; cage illumination was ~7 foot-candles during the light cycle. All experiments and animal maintenance were approved by the local Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center and were performed under the guidelines of the Association of Research in Vision and Ophthalmology (Rockville, MD).

2.3. Subretinal injections

Trans-corneal or trans-scleral (only for fundus imaging) subretinal injections were performed as previously described [8, 34]. Briefly, Balb/c mice were anesthetized by intramuscular injection of ketamine (80 mg/kg) and xylazine (14 mg/kg) in saline. Eyes were dilated with 1% Cyclogyl® (Alcon Laboratories, Ft. Worth, TX), animals were placed on a 38°C regulated heating pad under a surgical microscope (Carl Zeiss Surgical, NY) and an insulin syringe with a beveled 26 G needle was used to puncture a hole in the cornea. Next, a 33 G blunt-end needle attached to a 10 µl Nanofil® syringe controlled by a UMP3 pump controller (World Precision Instruments, Sarasota, FL) was positioned toward the superior nasal portion of the retina at which point 1 µl of either NPs, uncompacted naked DNA (both at 4.3mg/ml), or saline alone (vehicle) was injected into the subretinal space. The needle was retracted 10–15 seconds after injection when a bleb of retinal detachment was visible. Following complete removal of the injection needle, the eye was carefully observed for any indication of post-surgical complications such as iris and subretinal bleeding, pronounced retinal detachment or damage, or excessive vitreous loss. Careful evaluation of the severity of these acute post-surgical complications and subsequent long-term complications including eye infection, loss of visual function and atrophy were used to determine if animals would be excluded from the study. In the absence of any severe complications the procedure was deemed successful and the animal remained in the study. All injections were performed by a single individual. The success rate for injections was ~80%. Lastly, Gonak™ hypromellose (2.5%; Akorn Inc., Decatur, IL) was applied to the corneal surface and the animal returned to the regulated heating pad until fully recovered.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted from the enucleated whole eye using Trizol® reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. Total RNA (3 µg) was digested by RNase free DNase-I (Invitrogen, Carlsbad, CA) and purified using a phenol chloroform extraction procedure. Total purified RNA (1 µg) was used for cDNA preparation using Superscript® III

reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was further treated with RNase H (Invitrogen, Carlsbad, CA) to ensure degradation of any residual RNA. qRT-PCR using SYBR green was performed on a Bio-Rad C1000 Thermal Cycler (Hercules, CA). Relative expression was calculated using hypoxanthine-guanine phosphoribosyltransferase (HPRT) as an internal control according to the following formula: relative expression = $2^{-\Delta Ct}$, where $\Delta Ct = (Gene\ Ct - HPRT\ Ct)$. None of the samples were HPRT negative. A total of 4 to 7 independent samples were obtained for each treated and untreated cohort at each time point, with at least 3 runs each performed in triplicates. Primers used for amplification of HPRT, eGFP and RPE65 are: HPRT-F: GCAAACCTTTGCTTTCCCTGGTT; HPRT-R: CAAGGGCATATCCAACAACA; eGFP-F: CACATGAAGCAGCAGACTT; eGFP-R: AGTTCACCTTGATGCCGTTT; RPE65-F: CGGACTTGGGTTGAATCACT; RPE65-R: GGAGATATGCAGGCTTTTGC. Amplification and melting curves were both recorded to ensure proper amplifications. No-RT controls were included to ensure absence of DNA in extracted RNA and with each RNA batch converted to cDNA. Finally, PCR products were run on a 1% agarose gel to check for product size and amplification specificity.

2.5. Immunohistochemistry

Eyes were enucleated at PI-2, -7 and -30 days, corneas were punctured and placed into 4% paraformaldehyde for 15 minutes at room temperature. Subsequently, the cornea and lens were removed, and eyes were returned to 4% paraformaldehyde for an additional 30 minutes at 4°C. Samples were cryoprotected in sucrose as previously described [8] and were embedded in TissueTek® embedding media (Sakura Finetek, Torrance, CA). Cryosections (10µm) were collected on glass slides. Slides were washed with 1x PBS (3x, 5 minutes), and mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA) and finally subjected to direct fluorescence microscopy. For whole mount staining, tissues were fixed with 4% paraformaldehyde for 30 minutes at 4°C in micro-centrifuge tubes. Tissues were blocked for 1 hour at RT with blocking solution (5% BSA/PBS, 1.0% Triton X-100, 2% donkey serum) and incubated overnight with Lamin B antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a concentration of 1:300 in blocking solution. Whole mounts were briefly washed with 1X PBS (3x, 10 minutes) and incubated with secondary antibody (donkey anti-goat Alexa-Fluor 646, Invitrogen at Eugene, OR) at a concentration of 1: 500 in blocking solution, for 1 hour at room temperature. After additional PBS washes (4x, 10 minutes) whole mounts were mounted on glass slides using Prolong® Gold mounting media with DAPI (Invitrogen, Carlsbad, CA) after making radial incisions to flatten the tissue. Slides and flat mounts were imaged using a BX-62 spinning disk confocal microscope (Olympus, Japan) equipped with Slidebook® version 4.2.

2.6. Electretinography

Electretinography (ERG) was performed as described previously [8]. Briefly, mice were dark adapted overnight. A stimulus intensity of 1.89 log 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 1.46 log cd s/m² intensity and a strobe flash was presented to dilated eyes in the ganzfeld with a stimulus intensity of 1.89 log cd s/m². Amplitudes are presented as mean ± SEM.

2.7. Fundus imaging

Eyes were dilated using 1% atropine sulfate (Bausch & Lomb, Tampa, FL) prior to anesthesia and imaging. Mice were anesthetized by intramuscular injection of ketamine (80 mg/kg) and xylazine (14 mg/kg) in saline. Gonak™ hypromellose eye drops (2.5%; Akorn, Inc.) were applied on eyes for optimal refraction during imaging. Eyes were observed using both a bright light filter and a GFP filter (excitation at ~482nm and emission at ~536nm).

Images were captured using the Micron III fundus camera (Phoenix Research Laboratories, Pleasanton, CA).

2.8. Statistical analyses

All qRT-PCR, ERG analyses, eGFP distribution data are presented as mean relative values $\pm$ SEM. Two-way ANOVA was used and *P* values represent Bonferroni post-hoc tests for multiple comparisons. Values from NP injections were compared with both naked DNA and saline injected eyes.

3. Results

3.1. VMD2-eGFP nanoparticle expression following subretinal injection

To evaluate the ability of compacted DNA NPs in transducing RPE cells *in vivo*, we subretinally delivered VMD2-eGFP in NP form or as naked (uncompacted) plasmid DNA. This vector contains an eGFP reporter gene driven by a 600 bp fragment of the RPE-specific human VMD2 promoter (Fig. 1) previously well-characterized both *in vitro* and *in vivo* [33]. VMD2 expression begins at the embryonic stages and continues throughout life [5, 8, 32, 35] making it a useful choice for gene delivery. 1 μ l of NPs containing 4.3 μ g DNA, equal amount of naked DNA, or saline was delivered to the subretinal space at the superior nasal region of the eye of 5-week-old BALB/c mice. This concentration was used based on our prior dose-dependent studies of these NPs in ocular tissues that showed optimal transgene expression with no toxicity [5, 6, 8, 32, 36] (Z. Han, A. Koirala et. al., manuscript submitted). Eyes were enucleated at PI-2, -7 and -30 days and eGFP mRNA levels were assessed by qRT-PCR. At PI-2, levels of eGFP transcripts were 3 fold higher in NP-injected eyes than naked DNA-injected eyes (Fig. 2). At PI-7, levels of eGFP declined by 50% and 30% in naked DNA and NP treated eyes, respectively. Expression was further reduced for both vectors at PI-30. Importantly, levels of eGFP expression from NP-injected eyes were significantly higher than naked DNA-treated eyes at all time points (*P*<0.01 for PI-2 and *P*<0.05 for PI-7 and -30 days). No expression was detected in saline injected or uninjected eyes (data not shown) at any time point.

3.2. RPE-specific expression of VMD2-eGFP nanoparticles

To assess which cell types expressed eGFP protein, and to what extent it was expressed, cross sections throughout the treated eyes were evaluated by direct fluorescence microscopy (Fig. 3). RPE-specific eGFP expression was observed in both naked and NP-treated eyes at PI-2 and -30 days (the last time point evaluated). eGFP expression in NP-treated eyes was detected throughout the cross-section of the RPE cell layer with more eGFP positive cells observed near the injection site at both time points. In contrast, eGFP expression in naked DNA-treated eyes was confined mostly to the RPE cells at the injection site (arrows, Fig. 3a). As demonstrated by higher magnification images taken near the injection site (Fig. 3b), eGFP expression is robust and limited to the RPE cell layer in both NP and naked DNA-injected eyes at all time points. No ectopic expression was observed in any other ocular tissues, including the optic nerve, chiasm, or brain visual pathways (data not shown). No eGFP signal was detected in saline-injected eyes.

In order to confirm that the observed eGFP signal did not arise from outer segment autofluorescence, retinas were removed from the eyecups and native fluorescent signals were evaluated separately in retina and RPE whole-mounts. Consistent with Fig. 3, eGFP-positive cells were evident throughout the four quadrants (superior, inferior, nasal, and temporal) of the RPE whole-mounts prepared from NP-treated eyes at PI-2 and -30 days (Fig. 4a, middle panels). Although eGFP positive RPE cells were also detected in naked DNA-treated eyes, these cells were mostly distributed near the injection site (superior-nasal)

(Fig. 4a, asterisks in left panels). No eGFP expression was detected in saline injected RPE whole-mounts (Fig. 4a, right panels) or in retinal whole-mounts from all treatment group/time point (not shown). To further confirm the cellular origin of the observed fluorescence, higher magnification of confocal images showing binucleated RPE cells in whole mounts are presented in Fig. 4b,c.

3.3. Quantification of VMD2-eGFP nanoparticle distribution and expression

In order to quantify the percentage of the RPE cells transfected by the NPs, overlapping 10x images were captured from RPE whole mounts (Fig. 5a) to construct a composite of the entire RPE cell layer (representative 10x images shown in Fig. 5b–f). Fluorescent and non-fluorescent RPE cells were counted from images obtained from 3 mice for each cohort (7,000–10,000 cells/eye). For proper identifications of eGFP positive cells, counting was performed on 10x images. To be categorized as eGFP positive, cells had to exhibit uniform eGFP expression throughout the cell body. Cells with minor or punctuate (dotted) fluorescence patterns were considered non-expressing since similar patterns were seen in saline injected or uninjected eyes. Overall, an average of 50% of RPE-cells exhibited eGFP fluorescence in NP-injected eyes at day 2, while 10% of RPE cells exhibited fluorescence in naked DNA-injected eyes (Fig. 5g). Expression was highest in the superior (average ~62%) and nasal quadrants (average ~65%), but remarkably, NPs were able to drive expression in the regions farthest from the injection site. At PI-2, an average of 45% and 30% of the RPE cells in the temporal and inferior regions expressed eGFP, respectively (Fig. 5h, left panel). This expression pattern persisted at PI-30 (Fig. 5h, right panel). In contrast, expression in the temporal and inferior quadrants in naked DNA-injected eyes was quite low. At PI-2 days, the distribution of eGFP fluorescent cells from NP injected eyes was significantly higher than both naked DNA and saline injected eyes in all regions ($P<0.0001$). At PI-30 days, the distribution of eGFP positive RPE cells in NP-injected eyes was significantly higher than naked DNA injected eyes in the superior, nasal and temporal regions ($P<0.01$, $P<0.05$ and $P<0.01$, respectively); no significant differences were found for the inferior region ($P>0.05$). However, when compared with saline injected eyes, the eGFP distribution in NP injected eyes was significantly higher for all regions ($P<0.0001$).

In addition, live fundus imaging was performed on animals that received trans-scleral subretinal injections of NPs, naked DNA, or saline. Trans-scleral injections were used for this experiment to minimize opacification of the cornea and lens (which adversely affects image quality). Brightfield fundus images (Fig. 6a,b, upper panels) were captured using white full-spectrum light, and eGFP images were captured using a FITC filter with excitation at ~486nm and emission at ~515nm (Fig. 6a,b, bottom panels). Consistent with the data presented above, eyes injected with NPs at PI-7 and PI-14 days exhibited eGFP expression at the site of injection (Fig. 6, arrows) and in nearby regions while eyes injected with naked DNA exhibited a smaller fluorescent region and saline-injected eyes exhibited only background fluorescence. Combined, these results demonstrate that VMD2-eGFP NPs can effectively penetrate the subretinal space and efficiently transfect RPE cells both near to, and remote from the injection site.

3.4. Uptake and retention of nanoparticles by the RPE cells

The results presented above indicate that NPs mediate better gene expression than naked DNA in RPE cells. To determine whether this difference is due to differential uptake or trafficking, we subretinally delivered 1 μ l of TM-Rhodamine labeled VMD2-eGFP naked DNA or NPs (3.6mg/ml). Control eyes were subretinally injected with 1 μ l saline. Eyes were enucleated at 2 hours, 6 hours, 24 hours, 48 hours, 7 days, and 30 days PI. RPE whole mounts were immunostained with Lamin B1 antibody to label the nuclear envelope and with DAPI to counter stain the nuclei (Fig. 7). To evaluate the localization of the rhodamine-

labeled naked DNA and NPs in the RPE cells, a confocal image containing 20–25 planes (thickness of 0.5µm) was captured at 100x magnifications, deconvolved, and 3-D images were acquired. The left columns in Fig. 7 show representative confocal stacked images. Boxed regions (with ~3–4nuclei) were used to create a 3-D view as shown in the right columns of Fig. 7. By 2 hours PI, some rhodamine signals from naked DNA and NP treated eyes were detected in the RPE cells (Fig. 7a,b). The highest rhodamine signals were detected in the cytosol of both naked DNA and NP treated eyes at 6 hours PI, at which time no rhodamine signal was detected in the nucleus. Although labeled DNA was detected at 24 hours PI in the nuclei of RPE cells receiving both naked DNA and NPs, significantly more rhodamine signal was observed in the nuclei of NP treated eyes. Interestingly, at this time point higher rhodamine signal remained in the cytosol regardless of the treatment. The most striking differences were observed at PI-7 and 30 days; in naked DNA-treated eyes, only few cells exhibited rhodamine signal and at a minimal level while higher number of cells in NP-treated eyes showed higher signal consistent with the eGFP expression data shown in Fig. 2. Uptake of NPs and naked DNA were also observed in the retinal whole mounts (Fig. 7d) from the respective eyes imaged for the RPE whole mounts. Rhodamine fluorescence levels were also higher in NP injected eyes compared to that of naked DNA. Saline injected eyes exhibited no such fluorescence in the RPE (Fig. 7c). We did not detect eGFP positive cells in these studies, likely a result of transcriptional blockade by the covalently-bound rhodamine. Overall, these data demonstrate that naked DNA and NPs are rapidly taken up into RPE cells, and that compaction with CK30PEG10k protects DNA from degradation by cytosolic DNases and likely serves as a platform for gradual transport of DNA to the nucleus.

3.5. Lack of toxicity following VMD2-eGFP nanoparticle injections

In order to determine if there was gross retinal/RPE toxicities associated with NP treatment or the VMD2-eGFP vector, we conducted full-field ERG and qRT-PCR for RPE-specific genes in eyes from treated and control animals. The injection procedure induces a temporary retinal detachment which is ~60% of the total retina, with almost full reattachment occurring within 2 days PI followed by full functional recovery at PI-30 days; observations comparable to those previously reported [5, 6, 8]. Scotopic and photopic ERGs were measured at PI-30 in treated groups and uninjected controls. Consistent with our previous findings, no significant differences in scotopic or photopic a- and b-wave amplitudes were detected between any groups, suggesting that these VMD2-eGFP NPs have no deleterious effects on retinal function (Fig. 8a,b).

To determine whether NPs caused any gross toxicity to RPE cells, we evaluated the transcript levels of an RPE-specific gene, *RPE65*, as a proxy measure of RPE health. As shown in Fig. 8c, at 2, 7 and 30 days PI, there were no significant difference in *RPE65* expression levels between cohorts. Furthermore, structural evaluations of frozen sections collected from naked DNA, NPs, and saline treated eyes at PI-30 days showed no adverse effects to retinal morphology and rows of photoreceptor nuclei (10–11 rows) (Fig. 8d). The nuclei were counted on the sections taken from the site of injections where eGFP expression was distinct, such as those in Fig. 3b. Combined, these data suggest that VMD2-eGFP NPs as well as naked DNA are well tolerated after subretinal injection.

4. Discussion

Here, we systematically tested the ability of the CK30PEG10k NPs to drive gene expression in RPE cells. We show that these NPs can efficiently transfect RPE cells as early as 2 days post treatment and persisted up to 30 days, the last time-point analyzed. We have also demonstrated that the VMD2-eGFP vector drives RPE-specific expression. These

characteristics make NPs an exciting gene delivery tool with significant potential for future treatment of myriad RPE-associated blinding disorders.

A significant portion of this study was dedicated to evaluating the distribution of eGFP expression throughout the RPE cell layer because genetic therapies, both viral and non-viral, are typically restricted to a limited surface area. Effective disease improvement requires expression outside the injection site, so distribution of transgene expression throughout the eye is a critical and necessary goal. Our previous results in photoreceptors suggest that NPs are capable of driving this type of pan-retinal distribution after subretinal injection, but a systematic evaluation has not yet been performed [5, 6]. Here, we demonstrated that NPs were able to transfect cells throughout the RPE layer (~50% of the total RPE cells) although the highest levels were observed surrounding the injection site (~65% of cells near the site of injection). We attribute this capability of the NPs to their very small size and consequent ability to diffuse through the subretinal layer. The ability of these NPs to successfully drive gene expression throughout the RPE is a critical advantage in terms of clinical applicability.

In this study, both naked VMD2-eGFP DNA and NPs drove appreciable levels of expression at our initial time point (PI-2) (Fig. 2). This naked DNA-based expression was unexpected. Previously, we have extensively characterized the ability of several NPs and their corresponding naked DNAs to drive transgene expression in photoreceptors, and we had never detected expression associated with naked DNA [5, 6]. To explore this ability of RPE cells to express a transgene from naked DNA versus NPs, we evaluated, *in vivo*, the uptake and trafficking of rhodamine-labeled naked DNA and compared it to rhodamine-labeled NPs (Fig. 7). This study is essential since successful gene transfer requires effective uptake into the cell, trafficking and uptake into the nucleus, and intracellular persistence of the vector may facilitate long-term expression. Since we show a similar cellular uptake for naked DNA and NPs at the 6 hour time-point, we therefore hypothesize that the uptake is likely due to phagocytosis, a known function of RPE cells [37, 38]. This uptake pathway is likely unique to RPE cells and different from the nucleolin-mediated process by which NPs are trafficked in airway epithelial cells [41].

Though our data suggest that cellular uptake into RPE cells is similar for both naked DNA and NPs, nuclear uptake is a separate issue which often poses a barrier for non-viral vectors. Here, we demonstrate significant nuclear uptake of NPs by 24 hours (Fig. 7), which is consistent with the early robust gene expression we observed (Figs. 2–4). In contrast, the nuclear uptake for naked DNA in RPE cells seems to be inefficient as we only observed minimal rhodamine signal in the nuclei of naked DNA-treated RPE cells (Fig. 7), again consistent with the lower levels of expressions from naked DNA compared to NP injection. Studies have shown that the use of cell-type specific promoters can increase nuclear uptake of plasmid DNA *in vivo* [41] and that the inclusion of DNA sequences such as the simian virus 40 (SV40) promoter/enhancer could also increase plasmid DNA import into the nucleus [42]. The plasmid DNA used in our study contains the RPE-specific VMD2 promoter and SV40 early promoter sequences. The presence of these sequences may have facilitated the small amount of nuclear uptake of naked DNA that we observed.

We also showed that NPs persisted in the cytoplasm for up to 30 days (last time point studied) in contrast to naked DNA which only persisted few days PI. This observation suggests that NPs are stable in the cytoplasm. Resistance of NPs to cellular DNases is one of their key features. In order for the NPs to be used, each batch of NPs must pass a series of quality control tests, including resistance to DNase1 [32]. Combined, these data suggest that in RPE cells, compaction of DNA into NPs confers benefits in terms of nuclear uptake and cytoplasmic stability, but not necessarily in initial cellular uptake.

Other strategies that have shown efficacy of gene transfer into the RPE are AAV [43], equine infectious lentiviral vectors [44], polyethylene glycolated peptides [45], lipofection and electroporation [46]. These studies have shown a great deal of transgene expression in the RPE. However, a detailed analysis and distribution of transduced RPE cells comparable to those presented in Fig. 5 hasn't been demonstrated with other strategies. Thus, a direct assessment between NP-mediated gene deliveries to RPE cells to others is not applicable, however future studies from our group will be directed for such comparisons.

5. Conclusions

In this study we demonstrated that CK30PEG10k NPs efficiently transfect RPE cells *in vivo*, generate high levels of gene expression, and are distributed throughout the RPE cell layer. More importantly, with these NPs, we have shown transduction of the RPE cells away from the site of injection, an important phenomenon for successful clinical application. We show that with ~60% detachment of the retina, NP's transfected ~50% of RPE cells. As ocular gene therapy continues to develop, it has become evident that a single type of therapy will not likely be successful in all cases and the development of multiple effective delivery strategies is both prudent and necessary. Meanwhile, the current success of RPE-directed clinical trials has demonstrated that genetic targeting of the RPE is a viable treatment strategy. Our current results, therefore, have tremendous clinical potential for the treatment of myriad RPE-based diseases including: LCA, Best's disease, some forms of retinitis pigmentosa and macular dystrophy. While our study is aimed at the use of gene transfer to treat the RPE, this technology has much wider use for the treatment of a multitude of inherited or degenerative diseases.

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Fig. 1. Vector construction and Nanoparticle EM

(a) Vector map depicting the organization of the expression cassette. VMD2 promoter was cloned into EcoRI and BamHI sites in the pEGFP-1 vector purchased from Invitrogen. (b) EM image of NP formulation at 40,000x. The rod shaped particles were determined to have diameter of ~8–11 nm. Scale bar =200nm.

Fig. 2. Relative eGFP expression levels following subretinal injection

Eyes were injected at P30, enucleated at 2, 7 and 30 days PI, processed for total RNA preparation followed by cDNA synthesis and qRT-PCR. Data represent eGFP expression levels relative to HPRT. NP-driven expression of eGFP was significantly higher than naked DNA at 2, 7 and 30 days PI ($P<0.01$, $P<0.05$, and $P<0.05$, respectively). Data are presented as means $\pm$ SEM ($n=4$ for saline, $n=7$ for others).

Fig. 3. RPE specific eGFP expression

Mice were subretinally injected at P30 with 1 μ l naked DNA or NP at a concentration of 4.3mg/ml, or saline, and eyes were collected at PI-2 & 30 days. Representative confocal images of native expression of eGFP in the RPE layer are shown. Nuclei were counterstained with DAPI. Images in panel **a** and **b** were taken at 2x and 40x magnification, respectively. Images in panel **b** are taken from regions near the site of injection (indicated by arrows). Scale bar in **a** = 500 μ m, **b** = 50 μ m. (OS- outer segment, IS- inner segment, ONL- outer nuclear layer, INL- inner nuclear layer). (DAPI- Blue, eGFP- Green).

Fig. 4. Native eGFP fluorescence in RPE whole mounts

Eyes were dosed with naked DNA, NPs, or saline as presented in Fig. 3 legend. Eyes were enucleated at PI-2 and PI-30 days. The eye cup with the RPE cells layer was flattened by making radial incisions. Shown are representative planes from confocal images of RPE flat mounts (**a**) or representative regions near the site of injection (**b**, **c**) (approximate site of injections indicated by asterisks). Nuclei were counterstained with DAPI. Images in panel **a**, **b** and **c** were taken at 2x, 40x and 100x magnification, respectively. Saline injected and uninjected eyes (data not shown) were used as controls. Scale bars in **a** = 500 μ m, **b** = 20 μ m, and **c** = 10 μ m. (DAPI- Blue, eGFP- Green)

Fig. 5. Quantification of the distribution of eGFP positive RPE cells

(a) RPE whole mount image was taken at 2x (scale bar =400 μ m). (b–f) Examples of representative 10x images from a NP-treated eye used to count eGFP positive cells (b–superior, c–inferior, d–temporal, e–nasal and f–nasal) (scale bar =40 μ m). For example, the boxed region in panel a represents the 10x image in panel b. (g) The total percent of RPE cells expressing eGFP in each treatment group. (h) Quantification of the percent of total RPE cells expressing eGFP in each region. Data have been plotted as mean $\pm$ SEM ($n=3$). At PI-2 days, the eGFP distribution in NP-treated eyes was significantly higher in all regions when compared to naked DNA treatment ($P<0.0001$). Similarly, at PI-30 days, the distribution was significantly higher at the superior and temporal regions ($P<0.01$, and $P<0.01$, respectively) but no significant differences were found at the nasal and inferior region ($P>0.05$). However, compared to saline the distribution in NP treated eyes were significantly higher at both time points at all regions ($P<0.001$).

Fig. 6. *In-vivo* Fundus images showing eGFP expression in the RPE cell layer

Images were taken with two filter settings. The upper panels of **a** (PI-7 days) and **b** (PI-14 days) were taken using white light filter while the bottom panels were taken using GFP filter (excitation at ~482nm and emission at ~536nm). Injections were performed through the sclera for *in vivo* imaging purposes. NP injected eyes show a significant area of RPE transfection depicted by the bright green fluorescence over the light green background fluorescence. Approximate injection site has been indicated by arrows on bottom panels of both **a** and **b**.

Fig. 7. Cellular uptake and distribution of nanoparticles and naked DNA

For *in vivo* imaging, DNA was labeled with rhodamine. Tissues were collected from NPs (a), naked DNA (b), and saline (c) treated eyes at PI-2, 6, 24, 48 hours, 7, and 30 days for immunostaining with Lamin B (nuclear envelope marker). At 100x magnification, ~25 sections of 0.5 μm thickness were imaged. Left panels show confocal stacked images while 3D surface views are shown on the right from areas boxed in the left panels. Cytoplasmic/nuclear localization of the labeled DNA and NPs was assessed. (c) Saline injections were performed as vehicle controls and did not exhibit any rhodamine fluorescence. Scale bar in c = 10 μm . (d) Retinal whole mounts from eyes injected with rhodamine labeled naked DNA and NPs were collected at PI-48 hrs and imaged for rhodamine fluorescence to evaluate the uptake by the retina. Images were taken at 60x magnification. Scale bar in d = 20 μm . (Green- Lamin B, Red- TM-Rhodamine, Blue -DAPI).

Fig. 8. VMD2-eGFP NPs cause no gross retinal toxicity

No significant effects of naked DNA or NPs on scotopic (a) or photopic (b) retinal function are observed at PI-30 days ($P > 0.05$). Data are presented as mean $\pm$ SEM ($n = 5$). (c) qRT-PCR for *RPE65* was performed on treated eyes at the indicated time points. No significant differences in *RPE65* values (normalized to HPRT, $P > 0.05$) were detected at PI-2, -7, and -30 days in NP injected eyes when compared with naked DNA and saline treated eyes. Data are presented as mean $\pm$ SEM ($n = 5-7$). (d) Number of rows of photoreceptor nuclei counted for each cohort at PI-30 days. No significant differences in number of rows of photoreceptor nuclei were seen between cohorts ($P > 0.05$, $n = 4$). Data are presented as mean $\pm$ SEM.