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Genomic Form of Rhodopsin DNA Nanoparticles Rescued Autosomal Dominant Retinitis Pigmentosa in the P23H Knock-in Mouse Model

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Abstract

Retinitis pigmentosa (RP) is a group of inherited retinal degenerative conditions and a leading cause of irreversible blindness. 25%–30% of RP cases are caused by inherited autosomal dominant (ad) mutations in the rhodopsin (Rho) protein of the retina, which impose a barrier for developing therapeutic treatments for this genetically heterogeneous disorder, as simple gene replacement is not sufficient to overcome dominant disease alleles. Previously, we have explored using the genomic short-form of Rho (*sgRho*) for gene augmentation therapy of RP in a Rho knockout mouse model. We have shown improved gene expression and fewer epigenetic modifications compared with the use of a *Rho* cDNA expression construct. In the current study, we altered our strategy by delivering a codon-optimized genomic form of Rho (*co-sgRho*) (for gene replacement) in combination with an RNAi-based inactivation of endogenous Rho alleles (gene suppression of both mutant *Rho* alleles, but mismatched with the *co-sgRho*) into a homozygous *Rho*^{P23H/P23H} knock-in (KI) RP mouse model, which has a severe phenotype of adRP. In addition, we have conjugated a cell penetrating TAT peptide sequence to our previously established CK30PEG10 diblock co-polymer. The DNAs were compacted with CK30PEG10-TAT diblock co-polymer to form DNA nanoparticles (NPs). These NPs were injected into the sub-retinal space of the KI

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Supplementary data

Supplementary information includes four figures that can be found with this article online.

Author contributions

R.N.M and M.Z. performed experiments, prepared figures and wrote the manuscript. E.R.W. helped to edit the manuscript and perform the ERG experiment. Z.H. supervised the project and helped to write and edit the manuscript.

Conflict of interest statement

The authors of this manuscript have no conflicts of interest to disclose.

mouse eyes. As a proof of concept, we demonstrated the efficiency of this strategy in the partial improvement of visual function in the *Rho*^{P23H/P23H} KI mouse model.

Graphical abstract

Keywords

Genomic DNA; Retinitis pigmentosa; Autosomal dominant; DNA nanoparticle; P23H knock-in

1. INTRODUCTION

The term Retinitis pigmentosa (RP) was conceived by a Dutch ophthalmologist Franziscus C. Donders. RP is a group of inherited neurodegenerative retinal conditions that leads to irreversible blindness and afflicts 1 in 4000 individuals worldwide [1]. Altogether, RP affects 100,000 people in the United States alone [2]. To date, mutations in over 40 RP genes (<http://www.sph.uth.tmc.edu/RetNet/>) have been identified that play a key role in the initial degeneration of rod photoreceptor cells (PRs) [1,3]. Densely packed rod cells (about 120 million) are distributed throughout the retina of human eyes and are responsible for dim-light vision. In contrast, less dense cone photoreceptor (PR) cells (about 6 million) are concentrated mostly in the macular region of human eye and are responsible for central and color vision by responding to brighter light. In the majority of RP cases, primarily rod photoreceptor cells die first. This phenomenon first leads to progressive nyctalopia (night blindness), followed by progressive peripheral visual field loss (reflecting loss of rods and cones), and may eventually end up with total blindness (loss of remaining central cones and rods). RP is genetically highly heterogeneous and is grouped into autosomal dominant (ad), autosomal recessive (ar) and X-linked inheritance [4]. To date, 25%-30% of all human RP cases are expressed as adRP due to mutations in the *rhodopsin* (*Rho*) gene, which localizes in rod PRs of the retina [5,6]. The outer segments of the rod cells are composed mostly of the abundant Rho protein, which is a member of G protein-coupled receptor (GPCR) protein family and functions as the light detector for the rod cell. The mutated *Rho* allele has a gain-of-function and/or dominant-negative effect [7] on the normal Rho function, which leads to the disassembly and destabilization of outer segments of rod cells and, ultimately, cell death. Over 100 mutations have been identified in the *Rho* gene to be responsible for this disorder

(<http://www.sph.uth.tmc.edu/RetNet/>) [1,8]. The genetic heterogeneity in the human *Rho* gene is a significant challenge in the development of an effective gene therapy for *Rho*-linked adRP. Currently, there is no clinical cure for this disease.

Previously, we and others have begun to develop gene therapy strategies using genomic DNAs, which we believe will provide appropriate levels of gene expression and restoration of protein function in a physiological way [9,10]. Our recent study, based on a nano-formulation strategy, revealed long-term transgene expression upon delivery of an intron-containing construct with exons sequences. This short-form genomic *Rho* sequence, namely *sgRho*, was able to reduce post-transcriptional epigenetic silencing caused by DNA methylation/histone modification compared to the *cRho* (cDNA) delivery alone [9,11]. These promising results caused us to consider the *sgRho* gene with further codon optimization using synonymous codons to obtain maximum *Rho* protein expression. The codon-optimized construct was designed to mismatch the shRNAs so that the transcripts of the modified sequence would remain active during the shRNA-induced silencing process. Compared to the allele-dependent method, which is designed to silence a specific mutant gene, this allele-independent approach (a suppression and replacement therapeutic strategy) has the potential to target almost every dominant mutation in the *Rho* gene. This approach solves the problem of designing specific genetic therapeutic strategies for each mutation, which is impractical due to the lack of available mouse models, the time-consuming nature of the process and financial hurdles, before translating to adRP patients for therapeutic intervention [6,12]. This approach also has advantages that can overcome drawbacks associated with several other new technologies like CRISPR-Cas9, where targeting a single mutation (e.g., P23H) can potentially cause severe off target effects [13]. The large size of our propose *sgRho* gene (>5kb) is also difficult to deliver by the widely used recombinant adeno-associated virus (AAV) vectors. Earlier studies reported potential suppression and replacement strategies using recombinant adeno-associated virus (AAV) vectors with promising functional benefits such as in the Pro347Ser (P347S) mutated adRP mouse model [14-18]. Though efficient and promising, the AAV-mediated gene delivery strategy brings several inherent drawbacks e.g. size limitation of cargo along with an immune response against the gene therapy vector and potential toxicity [19]. Therefore, development of synthetic and safe non-viral gene delivery vehicles hold great promise for treating genetic ocular disorders.

In the current study, we reengineered our previously reported [11,20-22] polyethylene glycol-substituted polylysine (CK30PEG) diblock copolymer by conjugating it with a targetable transactivator of transcription (TAT) peptide sequence. TAT is derived from the human immunodeficiency virus and is a member of the cell penetrating peptide family [23,24]. Previously, the PEGylated TAT diblock copolymer demonstrated more therapeutic efficacy compared to the PEGylated CK30 (CK30PEG) [25]. In a recent study, it was demonstrated that a TAT modified liposome protamine-based gene delivery vector could efficiently deliver a retina-specific gene into the retina and showed sustained gene expression *in vivo* [26]. Therefore, we hypothesized that covalently attaching the TAT peptide sequence to the CK30PEG diblock copolymer structure would enhance its therapeutic efficacy to deliver shRNA and *co-sgRho* cloned into two separate plasmid DNAs into the retina of the adRP mouse model. The following factors were considered and

combined in a single non-viral gene therapy strategy to make it more effective to combat adRP disorders. (i) The *co-sgRho* was designed as a replacement component with a potential for long-term gene expression in rod PR cells, (ii) rational design of shRNA to target and silence both mutated alleles to regulate gene expression at the RNA level in a mutation-independent manner, (iii) adoption of a rod cell specific promoter (MOP) upstream of *co-sgRho* to ensure rod cell specific gene expression *in vivo*, (iv) tethering the cell penetrating and safe TAT peptide to the well characterized CK30PEG diblock copolymer to ensure effective gene delivery to the retinal cells, and finally (v) use of the severe P23H KI mouse model [27] (*Rho*^{P23H/P23H}) of adRP to evaluate our gene therapy strategy *in vivo*.

In summary, we have investigated a mutation-independent strategy using interference RNA (RNAi)-mediated suppression of normal (wild type) and mutated transcripts as well as simultaneous replacement with silencing-resistant mouse *co-sgRho* delivery. To validate this challenging dual component gene delivery strategy, we separately prepared two DNA NPs (suppression and replacement segments), injected them as a mixture into the sub-retinal space of the *Rho*^{P23H/P23H} mouse model of adRP [27,28], and subsequently examined their potential efficiencies as well as their structural and functional benefits. Herein, our strategy shows expression of *co-sgRho* encoded Rho proteins in the retina resulted in partial structural and functional recovery of rod PRs as demonstrated by biochemical, histological and functional studies. Taken together, our current results provide an effective proof-of-principle non-viral therapeutic approach for adRP, which can also be applied to develop alternative therapeutic approaches for other dominant conditions.

2. Materials and methods

2.1. Materials, animals

In our experimental study, all solvents, reagents and columns were purchased from Sigma-Aldrich (USA) and Thermo Fisher Scientific (USA) unless otherwise noted. We used homozygous *Rho*^{P23H/P23H} KI (experimental) and wild type (*Rho*^{+/+}, control) mice in a C57BL/6J background (Jackson Laboratory, ME) for our studies. The homozygous P23H KI mouse [27] is one of the most severe adRP mouse models in the field. The KI animals were identified by standard polymerase chain reaction (PCR) using transgene specific forward (5'-GGTAGCACTGTTGGGCATCT-3') and reverse (5'-GACCCACAGAGACAAGCTC-3') primers and followed by manufacturer's protocol. The mutant and wild type bands appeared at ~320 bp and 150 bp respectively. The outer segment of rod PR cells is fully degenerated at postnatal day (PND) 14, and subsequently demonstrates a severe phenotype [27]. In this model, a missense mutation (CCC to CAC) in the mouse *Rho* gene (GenBank accession number NM_000539.2) encodes mutant Rho protein, where proline at position 23 (in the N-terminal domain) is substituted by a histidine (P23H, Fig. 1A). The P23H mutation is the most frequent and is responsible for 10% of all Rho-linked adRP cases in North America.[27,29] All experiments were carried out and animals were maintained in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and the guidelines of the University of North Carolina at Chapel Hill Animal Care and Use Committee. The animals were maintained in a breeding colony under cyclic light (30 lux, 12

h light-dark) conditions. The sub-retinal injections were carried out using previously described procedures. All mice were devoid of *rd8* and *rd1* genes.

2.2. Vector constructs

We redesigned and synthesized the *co-sgRho* cassette from our previously used *sgRho* (kindly provided by Dr. Muayyad Al-Ubaidi, University of Houston) [9]. In brief, the full-length mouse *sgRho* gene expression cassette, which contains four introns and five exons of the short-form of genomic *Rho* gene (Fig. 1B), was codon optimized using the GeneOptimizer technique from Life Technologies (Fig. 1B).

To evaluate their gene expression efficiencies *in vitro*, we inserted these two transgenes (*sgRho* and *co-sgRho*) in the pCAGIG vector under the control of the ubiquitous CAG promoter (pCAGIG-*sgRho*-EGFP and pCAGIG-*co-sgRho*-EGFP) as demonstrated in Fig. 1B. The *EGFP* gene (717 bp), which encodes enhanced GFP (EGFP) protein, was inserted downstream of *Rho* under the guidance of the internal ribosomal entry site (IRES) element. The CAG promoter is a hybrid element composed of the cytomegalovirus (CMV) enhancer fused to the chicken beta-actin (CBA) promoter and demonstrated to be very efficient in expressing genes in cultured cells *in vitro* [30]. The *EGFP* gene was inserted to evaluate the transfection efficiencies of the vectors in HEK293 cells (a human embryonic kidney cell line) for testing the relative expression of these two *Rho* genes (*sgRho* and *co-sgRho*).

For *in vivo* analysis, we cloned the *sgRho* (5151 bp) and *co-sgRho* (5151 bp) constructs individually under the control of mouse *Rho* promoter (MOP, rod cell specific) into the pPEI vector backbone (pPEI-MOP-*sgRho* and pPEI-MOP-*co-sgRho*, 10661 in size). The MOP promoter was inserted immediately upstream of the 5'-flanking region of the *sgRho* or *co-sgRho* expression cassette followed by the downstream potential chromosomal scaffold/matrix attachment regions (S/MAR) and the SV40 origin of replication in the pPEI vector. Earlier studies demonstrated that the MOP promoter could lead to robust gene expression in rod cells [31]. The S/MAR sequence localized downstream of the expression cassettes has been demonstrated to improve transgene expression from non-viral vectors in the retina *via* episomal replication for long-term gene expression [32,33].

Three short hairpin RNAs (shRNAs, namely shRho-1, shRho-2, and shRho-3) were designed to silence expression of the endogenous *Rho* gene (either normal or mutant but mismatched with *co-sgRho*) *via* RNA interference (RNAi). These siRNAs expressing inserts were cloned individually into the pRNA-H1.1 Shuttle vector (4881 bp, without insert, GenScript, Cat. No. 1206) at the multiple cloning site (MCS) under the control of human H1.1 promoter as demonstrated in Fig. 1C. A 19 DNA nucleotide (nt) sense siRNA sequence was linked to the complementary antisense siRNA sequence by a 10-nucleotide loop (TTGATATCCG) (Fig. 1C).

2.3. Cell culture, cell transfection and immunocytochemistry

Human embryonic kidney (HEK293) cells were used for *in vitro* transfection studies. DMEM media supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies) and 1% Antibiotic-Antimycotic (Gibco, Life Technologies). Transfections were carried out following a previously published protocol [34]. In brief, 1×10^5 cells were seeded per well in

6-well plates. The next day, the transfection was carried out using 2 µg of plasmid DNA and 5 µl of lipofectamine 2000 (Life Technologies). Untransfected cells or cells transfected with pCAGIG-sgRho-IRES-EGFP, pCAGIG-op-sgRho-IRES-EGFP, or pCBA-EGFP plasmid DNA were used as controls in serum free media. After 6 h, 2 ml of fresh DMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic were added to each well and incubated for 48 h at 37° C and 5% CO₂. Native EGFP fluorescence was imaged using a Zeiss Axio microscope (Carl Zeiss, Germany) with a GFP filter. Images were taken at 100× magnification.

The expression of the *Rho* genes in the cells and their corresponding intracellular localization were further investigated by transfection of pCAGIG-sgRho-IRES-EGFP (2 µg), pCAGIG-op-sgRho-IRES-EGFP (2 µg), pCBA-EGFP plasmid (2 g) DNAs using 5 µl lipofectamine 2000 (Life Technologies) into COS-7 (SV40 transformed fibroblast-like monkey kidney tissue) and HeLa (human epithelioid carcinoma) cells (1 × 10⁵ cells/well) cultured on poly D-lysine coated (A-003-E, Millipore Sigma) sterile glass cover slips placed in 6-well plates. Each well contained one cover slip. The same transfection procedure described above was used. After a 48-h incubation, COS-7 and HeLa cells were fixed using 1% (5 min), 2% (5 min), 3% (5 min) and finally 4% (w/v; 15 min) paraformaldehyde (PFA) in cultured media at room temperature (RT) followed by washing with Dulbecco's phosphate-buffered saline (DPBS, 3 times, 5 min each). The cover slips were then blocked with medium containing donkey serum (2.5% donkey serum, 1% gelatin from cold water fish skin, 5% BSA, 0.5% Triton X-100 in PBS, pH 7.4) for 1 h at RT. The cover slips were then incubated with a rabbit anti-rhodopsin primary antibody (ab112576, Abcam, 1:500 dilution) and a mouse anti-calreticulin antibody (FMC 75, ab22683, Abcam, 1:500 dilution) in blocking solution at 4°C overnight. On the following day, cover slips were washed with DPBS (3 times), then incubated with a mixture of the following secondary antibodies: Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L, A-21206, Molecular Probes, 1:1000 dilution) and Alexa Fluor® 647 Donkey Anti-Mouse IgG (H+L, A-31571, Molecular Probes, 1:1000 dilution) in blocking buffer at RT for 1 h. The cover slips were then washed with DPBS (3 times) and mounted with DAPI (4',6-diamidino-2-phenylindole)-containing mounting media (VECTASHIELD; Vector Laboratories, CA). Images were captured with a Zeiss LSM 710 spectral confocal laser scanning microscope (CLSM) at 40× magnification.

2.4. TAT modification of CK30PEG diblock co-polymer

The CK30PEG (10kDa of PEG contain) compounds were prepared following previously published methods [30]. We introduced the cell penetrating TAT peptide sequence (5-azido-pentanoyl-RKKRRQRRR-NH₂, 49–57, Cat. No. TAT-3734-PI, Peptides International, USA) in CK30PEG acetate using a standard bioconjugation protocol. In brief, following the referenced protocol, the CK30PEG-trifluoroacetate compound was prepared. This compound (100 mg) was then conjugated to dibenzocyclooctyne (DBCO)-PEG-NHS (10 mg, 3.4 kDa, Cat. No. PG2-DBNS-3k, Nanocs, USA) *via* standard amine reactive coupling through NHS esters in 2 ml of sodium carbonate buffer (pH 9.5) under constant stirring for 1 h at RT. Subsequently, 5 mg of TAT-azide (Peptide International, 1.463 kDa) was added to the mixture and was further stirred overnight at RT. The anchored DBCO from CK30PEG-DBCO complex was conjugated with azide terminated TAT using standard copper free

“click chemistry”. The reaction mixture was washed (10 times) with endotoxin free water and concentrated using a centrifugal concentrator (MWCO 5kDa, Vivaspin® 2, Viva Product). The unreacted small molecular weight materials were also removed by this process. The concentrated material was then lyophilized. This lyophilized material was exchanged with acetate using anion exchange resin (acetate, AG® 1-X8, Bio-Rad) and lyophilized again to obtain a white solid, the CK30PEG10k-TAT product, which was stored at –20°C until used for experiments. The product was analyzed by ¹H-nuclear magnetic resonance (NMR; Varian 400MR) spectroscopy and Fourier transform infrared spectroscopy (FTIR, Nicolet 380 FTIR spectrometer, Thermo Fisher Scientific). The NMR studies demonstrated the presence of the characteristic proton peak of the DBCO benzyl group (7.147 ppm), and PEG (CH₂, 4.661ppm) in the CK30PEG-TAT diblock copolymer.

2.5. DNA nanoparticle (NP) synthesis

Endotoxin free codon-optimized pPEI-MOP-co-sgRho, pshRho-1, pshRho-2 and psh-Rho-3 were amplified by Aldevron (Fargo, ND, USA) and separately mixed with TAT-CK30PEG-Acetate (at N: P=1:2 ratio) following the earlier protocol [11,20,22,30,33]. All of the DNA NPs were prepared and stored in saline (Sigma, Cat. No. S8776). The DNA NPs synthesis was confirmed by an agarose gel retardation assay. The intactness of DNA after compaction with diblock polymer was evaluated using a trypsin (Sigma-Aldrich, Cat. No. T4174) digestion assay. Protection of DNA from nuclease was evaluated by a DNase I (Invitrogen) digestion assay. The DNA NPs were characterized by high-resolution transmission electron microscopy (HR-TEM, Zeiss EM 910), dynamic light scattering (DLS) and zeta potential (Nano-ZS zeta sizer, Malvern, USA) experiments. The entire synthesis process was carried out using endotoxin-free water (HyClone™ Water, GE healthcare) and endotoxin-free systems.

2.6. Sub-retinal injections

In our experimental study, we performed sub-retinal injections with all mice at PND5 [9,20,35]. The mice were first anesthetized by placing them on ice for 3–5 min. The eyelids were then cut with scissors and the cornea was carefully punctured with a 27-gauge insulin syringe needle (BD, cat. No. 329412). Then a 35-gauge blunt-end needle attached to a 10 µl Nanofil syringe (World Precision Instruments, Sarasota, FL, USA) was loaded with sample (DNA NP solution or saline solution) and inserted into the eye under an operating microscope (Carl Zeiss Surgical, Incorporated, Thornwood, NY, USA). We injected 0.3 µl of either DNA NPs or saline solution into the sub-retinal space of each eye. The saline (0.3 µl), pshRho-2 NPs (2µg in 0.3 µl) and pPEI-MOP-co-sgRho NPs /pshRho-2 NPs (4µg in 0.3 µl; 2 µg of each NPs) combinations were injected into the sub-retinal space of *P23H* KI pups (PND5). During injection, the 30-gauge needle was held inside the retina for 30 seconds to confirm the delivery of samples to the sub-retinal space and then the needle was carefully withdrawn from the eye. Eyes with internal bleeding were excluded from the study. To avoid any infection after surgery, a drop of viscous triple antibiotics ointment (Equate, Walmart, Bentonville, AR, USA) was placed on each eyelid. Mice were subsequently placed on a warm bed at 37°C until they woke up.

2.7. Western blotting

Western blot analyses were carried out following standard protocols as described previously [9,30,34]. In brief, protein samples were collected from HEK293 cells and retinal tissues using RIPA buffer (Pierce, Thermo Scientific) with EDTA-free protease inhibitor cocktail (Roche) on ice. Protein concentrations were determined using a standard BCA protein assay (Bio-rad) following the referenced protocol. 10 µg of protein extract was mixed with sample buffer, fresh 2-mercaptoethanol, and then loaded onto a 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to PVDF membrane. The membrane was blocked with 5% milk in PBST (1% Tween-20 in PBS) to avoid nonspecific binding and incubated with a primary mouse monoclonal Rho antibody (1D4, 1:1000, sc57432, Santa Cruz Biotechnology, Dallas, TX) in 5% milk in PBST overnight at 4°C. The next day, after washing 3 times with PBST, the membrane was incubated with horse radish peroxidase (HRP)-conjugated anti-mouse IgG antibody (sc-2031, Santa Cruz Biotechnology, Dallas, TX) at 1:20,000 for 2 h at RT. Mouse beta-actin was used as a loading control (monoclonal anti-β-actin-peroxidase antibody, A3854, Sigma, 1:50,000). The blots were analyzed using a ChemiDoc™ MP imaging system (Bio-Rad). The densitometric analyses were calculated using Image Lab software v4.1 (Bio-Rad).

2.8. Immunohistochemistry

Immunohistochemistry (IHC) of tissue samples was performed following standard protocols [9,34,35]. In brief, the enucleated eyes were punctured and fixed in 4% paraformaldehyde (PFA) for 1 h at RT. The cornea and lens were removed during dissection of the eyes followed by further fixation (in 4% PFA for 2 h at RT) and incubation with 10%, 20% and 30% sucrose in PBS overnight. Eyes were embedded in Optimal Cutting Temperature compound (Tissue Plus, Fisher) and sectioned using a cryostat (10 µm thickness). Dried slides were washed 3 times with PBS and treated with blocking buffer (2.5% donkey serum, 1% gelatin from cold water fish skin, 5% BSA, 0.5% Triton X-100 in PBS, pH 7.4) for 1 h, followed by incubation with primary antibody (1:500 dilution) in blocking buffer at 4° C overnight. For IHC, the primary antibodies used were rabbit anti Rho (ab112576, Abcam, 1:500) and mouse anti-alpha Na⁺/K⁺ ATPase (a5, Developmental Studies Hybridoma Bank, 1:4 dilution). The slides were washed 3 times with PBST and incubated with fluorescent secondary antibodies conjugated to donkey anti-mouse Alexa Fluor 488 (A-21202, Molecular Probes) and donkey anti-rabbit Alexa Fluor 555 (A-31572, Molecular Probes) at 1:1000 dilution in the same blocking buffer for 2 h at RT. Then sections were mounted with DAPI-containing mounting media (VECTASHIELD; Vector Laboratories, CA). Images were acquired using a Zeiss LSM 710 spectral CLSM (the Microscopy Services Laboratory core facility at the University of North Carolina, Chapel Hill).

2.9. Electroretinogram

Full-field scotopic electroretinograms (ERG) was performed following a previous protocol [11,34,36]. In brief, mice were dark-adapted overnight and all ERGs were recorded under dim red light. The following day, the eyes were dilated with 1% tropicamide (Bausch & Lomb Inc., Tampa) and a drop of Genteal lubricant gel (Alcon) was placed on the eye to avoid dehydration. The mouse was placed on a heated platform (at 37 °C) throughout ERG

recordings. ERG data were collected using an Espion E² electroretinography system (Diagnosys LLC, USA). During ERG recordings, gold wire corneal electrodes were placed at the center of cornea. The reference needle electrode was placed subcutaneously at the middle of the forehead and the ground needle electrode was placed at the back but near to the tail of the mouse. A single stimulus intensity of 1.0 log cd s m⁻² (logarithm of scotopic candela seconds per meter squared) was applied to mice that were dark-adapted overnight. The scotopic a-wave amplitude (rod-mediated response) was measured from the baseline to the trough of a-wave whereas the scotopic b-wave values were taken from trough of the a-wave to the peak of b-wave. At least 7–10 mice were used per cohort. Data are presented as mean ± SEM.

2.10. Morris water maze study: visible platform test

The Morris water maze, created by Prof Richard Morris [37,38], has been used to assess visual function of rodents in a non-invasive manner[39]. We performed our water maze studies at the Mouse Behavioral Phenotyping Core Facility in the UNC Carolina Institute for Developmental Disabilities (CIDD). The maze consists of a large circular pool (diameter = 122 cm) partially filled with water (45 cm deep, 24–26 °C), located in a dimly lit room. Each mouse was given 4 trials per day for 4 days to swim to an escape platform cued by a patterned cylinder extending above the surface of the water. For each trial, the mouse was placed in the pool at 1 of 4 possible locations (randomly ordered), and then given 60 seconds to find the visible platform. If the mouse found the platform, the trial ended, and the animal was allowed to remain 10 seconds on the platform before the next trial began. If the platform was not found, the mouse was placed on the platform for 10 seconds, and then given the next trial. Measurements of latency to find the platform were taken by an observer blind to mouse genotype. Behavioral data were analyzed using a repeated measure Analysis of Variance (ANOVA). Fisher's protected least-significant difference (PLSD) tests were used for comparing group means only when a significant F value was determined. For all comparisons, significance was set at $p < 0.05$.

2.11. Statistical analysis

The current results are presented as the means ± SEM from at least 3 independent experiments. GraphPad Prism 5.0 software (La Jolla, CA, USA) was used to create the figures and perform the corresponding data analysis. These figures were further organized in Photoshop CS5 software. The Student *t*-test between two independent groups was used for the analysis of the data. Multiple group comparisons were analyzed by one-way or 2-way ANOVA as necessary. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sequence specific knock down of rho protein in HEK293 cells

The goal of our current strategy is to deliver a suppression element (shRho) that can non-specifically silence the mouse transcripts of both wild type and mutant *Rho* genes. The suppression strategy was found to be independent of the disease-causing mutation. The suppression elements were designed for co-delivery with the co-sgRho element to silence Rho protein expression. The co-sgRho was designed to be transcribed into mRNA that would

be resistant to siRNA encoded by shRho during translation. To evaluate the strategy, we designed two independent silencing studies based on *sgRho* and *co-sgRho* (Fig. 1B) expression in HEK293 cells under the control of the ubiquitous CAG promoter. We considered this highly transfectable cell line for our study to avoid any interference from endogenous mouse rhodopsin protein expression that is found in different immortalized mouse retinal cell lines (e.g. MU-PH1, R28). We included *EGFP* under the control of the IRES element in order to evaluate co-transfection efficiencies. We first transfected pCAGIG-sgRho-IRES-EGFP (Fig. 2A) and pCAGIG-co-sgRho-IRES-EGFP (Fig. 2B) with or without pshRho 1–3 (Fig. 2). We used pCBA-EGFP as a positive control (Fig. 2A). EGFP expression (~90%) confirmed the successful transfection of plasmid DNA in the cells. Co-transfections showed that only pshRho-2 plasmid DNA among the three shRho designs completely silenced wild type Rho expression encoded by the *sgRho* gene. Consistent with earlier studies, pCAGIG-sgRho-IRES-EGFP transfection expressed wild type Rho that migrated as a diffuse band on a 10% SDS-PAGE gel using the mouse monoclonal 1D4 antibody to detect the protein (Fig. 2C). pshRho-2 was able to inhibit more than 90% of Rho expression. In comparison, the other two pshRho-1 and pshRho-3 constructs did not efficiently silence wild type Rho expression. EGFP expression demonstrated no alteration in the lower panel of Fig. 2C by pshRho transfection, which indicated a specific effect of the pshRho on Rho protein expression. Next we transfected pCAGIG-co-sgRho-IRES-EGFP plasmid DNA with or without pshRho1-3 in HEK293 cells. The three silencing components were ineffective in knocking down Rho expression upon co-expression (Fig. 2D). Therefore, these results revealed that our pshRho designs were mismatched with the co-sgRho (Fig. 2B). In control studies, we found that Rho proteins migrated through the gel as a diffuse series of bands probably composed of dimers and trimers as well as heterogeneously glycosylated protein compared to rod cell opsin [11,40-42] and is consistent with the literature [11,43]. Figs. 2 and 3 also demonstrated equivalent EGFP expression from the two sets of transfection experiments. EGFP protein expression visualized by confocal imaging of co-deliveries and controls also were comparable between the 2 experiments (Fig. 3). Overall, these transfection experiments demonstrated that *co-sgRho* encoded Rho protein was completely resistant to pshRho-2 mediated silencing. Therefore, we selected pshRho-2 for further *in vitro* and *in vivo* studies to silence Rho protein expression.

3.2. Codon-optimized Rho protein was highly expressed compared to wild type rhodopsin in Cos-7 and HeLa cells

To determine the synthesis and distribution of pCAGIG-co-sgRho-IRES-EGFP compared to wild type Rho from pCAGIG-sgRho-IRES-EGFP and their localization, we transfected these two plasmids into COS-7 and HeLa cells (Fig. 3; Supplementary information Fig. S1). EGFP fluorescence was absent from untransfected COS-7 and HeLa cells as expected (Fig. 3; Supplementary information Fig. S1). We found comparable EGFP expression in both transfections. The EGFP expression exhibited equivalent levels of transfection for the two plasmids in HeLa cells (Supplementary information Fig. S2). To determine whether Rho is restricted to the endoplasmic reticulum (ER), we selected COS-7 cells because they are comparable to HeLa cells in terms of transfection efficiency but approximately 2 times larger than HeLa cells [44]. The evaluation of the cellular localization of translated Rho protein encoded by *sgRho* and *co-sgRho* plasmid DNAs are shown in Fig. 3. The results

demonstrate that neither Rho was trapped into the ER, based on calreticulin staining (calreticulin/Rho panel, Fig. 3). Calreticulin is an ER marker used to identify the ER in COS-7 cells (white, Fig. 3). We speculate that the observed high expression of Rho throughout the entire cell encoded by *co-sgRho* might be due to the codon optimization of the genomic *sgRho* gene sequence. These results demonstrate that the *co-sgRho* encoded wild type Rho is similarly expressed and distributed as *sgRho* encoded Rho. The high-level expression of Rho demonstrated that this construct is an excellent choice for the replacement of both the wild type and mutant *Rho* alleles over RNAi-mediated silencing.

3.3. Synthesis and characterizations of cell penetrating TAT-peptide tethered CK30PEG-TAT

In our prior research, we developed an acetate salt of the CK30PEG diblock copolymer and utilized it to compact DNA to generate worm-like NP formulations [20,21,30,35]. These nano-formulations resulted in notable rescue of retinal cell function by delivering therapeutic genes to the retina without any retinal toxicity. In the current research, we reengineered this diblock copolymer by chemical coupling to the TAT peptide sequence of the human immunodeficiency virus, a cell penetrating peptide, to generate a more efficient CK30PEG-TAT complex based on standard bioconjugation techniques. The synthetic scheme is presented in the Fig. 4A. The CK30 polypeptide was conjugated with polyethylene glycol-maleimide (PEG-MAL, Mol. wt. 10 kDa) following an earlier protocol [11,20,21,30,45]. The purified CK30PEG was then conjugated with dibenzocyclooctyne-containing DBCO-PEG-NHS *via* standard amine reactive coupling through NHS esters (Fig. 4A). The results of 1H-NMR (Fig. 4B) and FTIR (Supplementary information Fig. S3) analyses demonstrated that the TAT peptide was conjugated to the CK30PEG diblock copolymer.

3.4. CK30PEG-TAT compacted DNA NPs are stable, protected and intact

The compaction strategy of plasmid DNA with CK30PEG-TAT is demonstrated in Fig. 4C. To demonstrate *in vivo* gene transduction in PR cells, we utilized the rod PR cell-specific MOP promoter upstream of *co-sgRho* gene in the pPEI vector backbone as demonstrated in Fig. 5A. The pPEI-MOP-*co-sgRho* and pshRho-2 were then individually compacted with CK30PEG-TAT block copolymer at an N/P (nitrogen represents polymer and phosphate represents DNA) ratio of 2:1. Two endotoxin free plasmid DNA constructs were compacted with CK30PEG-TAT separately to maximize its *in vivo* tolerance and efficiency. The binding ability of plasmid DNA (pDNA) to CK30PEG-TAT was accomplished by strong electrostatic interactions between positively charged CK30PEG-TAT and negatively charged plasmid DNA copies. The entire compaction process was assessed using a gel retardation assay (Fig. 5B). As shown in the Fig. 5B, diblock copolymer immobilized the compacted plasmid DNA in a 1% (w/v) agarose gel. Lanes 2 and 7 demonstrate the naked pPEI-MOP-*co-sgRho* and pshRho-2 plasmid DNAs respectively. Lanes 4 and 9 show all plasmid DNAs were compacted with the CK30PEG-TAT co-polymer. We were also interested in evaluating the physical stability of NPs to nuclease exposure. Upon treatment with DNase I, we found that uncompacted or naked DNAs were completely digested (Fig. 5B, lanes 3 and 8). However, the same quantity of DNase was unable to degrade the compacted DNA NPs (with equivalent DNA content) as demonstrated in Fig. 5B (lanes 5, and 10). Therefore, the DNase digestion assay showed the stability of compacted DNA inside the diblock co-

polymer from nuclease. We further investigated the conformational integrity of encapsulated DNA in the complex using a trypsin digestion assay as demonstrated in Fig. 5B. Trypsin can digest the polypeptide backbone of the diblock polymer. Therefore, upon treatment with trypsin, DNA molecules were released (Fig. 5B, lanes 6 and 11), and demonstrated equivalent mobility with naked DNA (Fig. 5B, lanes 2 and 7). Next, we evaluated the diameter, length and morphology of these NPs using TEM. These compacted DNA NPs appeared as worm-like structures with homogeneous distribution throughout the TEM grid (Fig. 5C). Earlier studies also demonstrated the formation of worm-like structures of DNA NPs after compaction with cationic polymer. TEM measurements demonstrated the length of worm-like pPEI-MOP-co-sgRho and pshRho-2 NPs to be 740 ± 10.53 nm and 456 ± 36.61 nm, respectively (Fig. 5C and D). The insets in Fig. 5C and D represent single DNA NP formulations of pPEI-MOP-co-sgRho and pshRho-2 compacted NPs respectively. The significant difference ($***p < 0.0001$) in the length of those NPs was observed due to the large size of the pPEI-MOP-co-sgRho (10661 bp) plasmid DNA compared to the smaller pshRho-2 (4935 bp; *i.e.*, with shRho-2 insert) plasmid DNA (Fig. 5E) as demonstrated with a plausible scheme in Fig. 4C. Surprisingly, in both cases the diameter of the DNA NPs remained comparable due to the supercoil structures of both plasmid DNAs (Fig. 4C). The 4–8 nm widths of two DNA NPs revealed their critical potential to transfect cells and penetrate to the nucleus through the permeable nuclear membrane pore (diameter ~25nm) as demonstrated previously.[46,47] In the literature, worm-like DNA NPs demonstrated the highest amount of gene delivery and expression compared to the other DNA NP morphologies.[48] The zeta potential measurements of pPEI-MOP-co-sgRho NPs and pshRho-2 compacted NPs were -13.68 ± 0.09 mV and -12.04 ± 0.32 mV respectively. These negative charges were responsible for the high solubility and stability of these NPs in saline solution over long periods at 4°C. Importantly, the size measured by DLS corroborated that found with TEM. DLS size distributions for pPEI-MOP-co-sgRho NPs and pshRho-2 compacted NPs are shown in Fig. 5F and G, respectively. We also performed representative tracking analysis (NTA) of pPEI-MOP-co-sgRho NPs using Nanosight NS500 (Nanosight, USA), which showed 95.5 nm of NPs with a concentration of 1.234×10^7 particles/ml (Supplementary information Fig. S4). The NTA result demonstrated a homogeneous distribution of DNA NPs (Supplementary information Fig. S4). Together, the chemical and physical properties of our NPs clearly suggest that they are plausible therapeutic candidates for further biological evaluation.

3.5. Combinatorial co-sgRho/pshRho-2 DNA NPs treated retinas demonstrated significant levels of Rho protein expressions compared to controls by Western blot analysis

Based on the positive *in vitro* results, the suppression and replacement gene therapy was further evaluated in the *P23H* KI mouse model of adRP. The mice were genotyped before each study with specific primers using PCR according to the company protocol, as demonstrated in Fig. 5H. A 320 bp band due to the *P23H* mutation was absent in the wild type mice, which instead displayed a 150 bp band. We used these mice for our *in vivo* studies. Saline solution (0.3 µl), pshRho-2 NPs (2µg in 0.3 µl) and pPEI-MOP-co-sgRho NPs /pshRho-2 NPs (4µg in 0.3 µl) combinations were injected into the sub-retinal space of *P23H* KI pups (PND5) in three separate cohorts. Rho protein expression was evaluated at post injection (PI)-60 days as demonstrated in Fig. 6. Due to the dominant gain-of-function

mutation, there was a very little or no monomer or dimer Rho protein expression in the uninjected and saline-injected eyes, which is consistent with earlier observations [27]. Moreover, the injection of pshRho-2 NPs showed a promising knockdown of Rho protein (Fig. 6) while we observed a reduction in the amount of Rho protein expression upon treatment with pPEI-MOP-co-sgRho/pshRho-2 combinatorial NPs (Fig. 6). This pshRho-2-induced Rho suppression clearly demonstrated that combinatorial NPs silenced P23H mutated Rho expression. The saline injected control demonstrated no significant monomer or dimer Rho protein expression in the retina. Therefore, our results indicated that injections of combinatorial NPs can simultaneously deliver the therapeutic pPEI-MOP-co-sgRho and pshRho-2 NPs effectively to retinal cells and subsequently generate therapeutic amounts of Rho protein by a silencing-resistant co-sgRho gene under the control of the rod cell-specific MOP promoter.

3.6 Wild type Rho protein encoded by co-sgRho is expressed in the ONL of PRs in P23H KI mice

To evaluate the expression of wild type Rho protein after pPEI-MOP-co-sgRho/pshRho-2 combinatorial NP injections, we carried out immunohistochemistry with retinal cryosections at PI-60 days (Fig. 7). We found that injection of these combinatorial nano-formulations expressed a significant amount of Rho protein in the PRs, as determined by a rabbit polyclonal Rho antibody (Abcam, Cat. No. ab112576, USA) compared to the saline and pshRho-2 NP injected controls. The inner (labeled green) and outer (labeled red) segments are clearly distinguished in the wild type retina (Fig. 7). 3–4 ONL nuclei layers were rescued by the combinatorial NP injections (Fig. 7). In retinas from mouse eyes injected with saline, there appeared to be a single layer of nuclei in the ONL that expressed very little Rho protein at PI-60 days (Fig. 7). Injection of pshRho-2 NPs showed a knockdown of Rho protein expression compared to the saline solution injected control at PI-60 days. Surprisingly, the injection of combinatorial NPs (pPEI-MOP-co-sgRho/pshRho-2 NPs) resulted in the formation of small rod inner and outer segments as demonstrated by white arrows in Fig. 7, which were absent in the saline and pshRho-2 NP injected controls. At the same time, the codon-optimized *Rho* genomic sequence (*co-sgRho*) expressed wild type Rho protein in the rods due to escaping the pshRho-2 induced silencing pathway.

3.7. Sub-retinal injections of combinatorial co-sgRho/pshRho-2 DNA NPs resulted in delayed degeneration of rod PR cells

To evaluate retinal function in the combinatorial NP-treated eyes, we performed full field electroretinography (ERG) of dark-adapted P23H KI mice at PI-60 days (Fig. 8A-C). ERGs are determined by measuring the electrical responses from light-sensitive retinal PR cells. Rod PR activity is evaluated by measuring the scotopic a-wave, which is a negative deflection from the baseline, in dark-adapted mice (Fig. 8A). The scotopic b-wave in dark-adapted mice is a positive deflection from the bottom of the a-wave to the peak of the positive deflection from the baseline that represents a mixture of PRs and secondary neuronal cells (Fig. 8A). Saline-injected P23H KI mice were used as a negative control (Fig. 8A-D). The scotopic a-wave ($102.1 \pm 4.57 \mu\text{V}$) and b-wave ($166.9 \pm 7.17 \mu\text{V}$) values measured in wild type mice were used as a positive control. In the P23H KI mouse at PI-60 days, ERG analysis of combinatorial NP injected eyes demonstrated a significant

(*** $P<0.0001$) improvement in average scotopic a-and b-wave responses of $30.74 \pm 3.46 \mu\text{V}$ and $46.60 \pm 4.70 \mu\text{V}$, respectively, compared to the saline-injected controls, which exhibited no ERG response upon scotopic stimulation (Fig. 8A-C). The pshRho-2 NP treated eyes (Fig. 8B and C) exhibited no scotopic ERG response. Together, these results demonstrate that a single injection of combinatorial NP slows the decline of retinal function to a highly significant extent compared to saline injection in the P23H KI mouse model of adRP.

3.8. Partial recovery in the visibility of P23H KI mice on treatment with combinatorial pEPI-MOP-co-sgRho/pshRho-2 DNA NPs

Finally, we evaluated vision by performing a visually guided Morris water maze analysis. This study was performed to measure improvement in the visual acuity of the P23H KI mice injected with DNA NPs compared to controls (uninjected and pshRho-2 NPs treated). As shown in the Fig. 8D, the WT group showed proficiency in the visible platform test (* $P<0.01$), with very low latencies to escape by the fourth day of testing. In contrast, unlike the wild type and combinatorial DNA NPs injected mice, the uninjected and pshRho-2 NP injected P23H KI mice (controls) failed to demonstrate learning the task. Although, the deficits were highly significant, it is notable that the P23H KI mice with combinatorial NP injections found the platform on the majority of trials (** $P<0.05$) at day 4 compared day 1. At 4th day, the combinatorial NPs injected mice also could detect the platform more easily compared to the uninjected (* $P<0.05$) and pshRho-2 NPs injected (** $P<0.01$) controls as demonstrated in Fig. 8D. Therefore, the DNA NPs injected mice were able to find the platform, but more slowly than wild type mice. On the other hand, uninjected and pshRho-2 NPs injected controls could not find the platform and demonstrated no improvement across time. Overall, these results indicate that the combinatorial DNA NP injected P23H KI mice demonstrated learning in the water maze test at day 4 and our non-viral NP therapy approach was rescuing the function of rods in the KI mouse model of adRP.

4. Discussion

Therapeutic intervention for the treatment of irreversible blindness caused by RP is seriously challenging due to the inherited mutational heterogeneity in adRP cases. Precise suppression and replacement gene therapy is considered a conceptually achievable and ideal strategy to deal with this barrier for the development of suitable therapeutics. This strategy has demonstrated positive outcomes in several RP mouse models. In the current study, we explored the silencing potential of both the wild type and mutated *Rho* gene followed by supplementation with an RNAi resistant *co-sgRho* gene, which partially rescued the structure and function of PRs in a severe P23H KI mouse model of adRP. To our knowledge, our non-viral based suppression and replacement strategy is the first report of partial recovery in the structure and function of PRs in the retina using a *co-sgRho* gene sequence.

The design of gene delivery vectors plays an important role in *in vivo* gene therapeutic strategies [14,15,18]. In the current strategy, we use a non-viral (synthetic) gene therapy approach to overcome the hurdles associated with the viral-mediated gene delivery method. CK30PEG is a promising synthetic diblock copolymer that can efficiently bind plasmid DNA in NP formulation and has already proved to be a promising and potential non-viral

gene therapy vehicle to deliver genes into the retina. Although our previous studies demonstrated that this diblock co-polymer compacted DNA NPs were safe for the retina, even long-term, we also observed a low transduction efficiency *in vivo*. Herein, we tethered a safe TAT polypeptide, a cell penetrating peptide that is arginine rich, to enhance penetration of different entities ranging from small molecules and proteins to nucleic acids through the plasma membrane and into cells *in vitro*. The literature has shown that a TAT-modified PEI/DNA complex exhibited a significant improvement of entry into primary neurons and resulted in a 14-fold increase in gene expression without toxicity [23]. The TAT peptide conjugate also demonstrated effective translocation of the therapeutic DNA to the nucleus *via* importin receptor-mediated uptake [24]. Earlier studies demonstrated that the PEGylated TAT complex was twice as efficient at delivering genes to the retina compared to the CK30PEG complex without exhibiting any retinal toxicity [25]. Our previous study also revealed that positively charged DNA complexes could effectively deliver genes to retinal pigmented epithelial (RPE) cells possibly via phagocytosis [34]. Therefore, our current strategy was to utilize the benefits associated with the TAT sequence (49–57) by tethering it directly to the CK30PEG co-polymer in order to improve its therapeutic efficacy for retinal gene therapy. The addition of the TAT sequence did not cause any significant changes in the size and surface charge of the CK30PEG-TAT compacted DNA NPs. The size and worm-like shape of the NPs corroborated earlier gene therapy studies with CK30PEG compacted DNA NPs. The slight negative charge of the two DNA NPs not only facilitated their high solubility in water, but also might reduce the possibility of phagocytosis by RPE cells and therefore increase availability to PRs during sub-retinal administration. In addition, we also included the rod cell specific MOP promoter to ensure expression in rods. Importantly, these ideas were combined to improve delivery and expression of therapeutic cargos (both silencing and therapeutic DNA constructs) to rod cells in the P23H KI mouse model of adRP.

Gene therapy is not only dependent on the vehicle but also most importantly on the development of appropriate transgene design. In our earlier studies, we investigated whether administration of an intron-containing *sgRho* gene could reduce epigenetic silencing in *sgRho* compared to *cRho* (the cDNA) in the retina, which successfully improved long-term transgene expression of *gRho* [9]. Therefore, we decided to use the *sgRho* gene sequence with 5 exons and 4 introns in our current study. Three shRNAs, which are mismatched with the *co-sgRho*, were designed. The shRNAs were designed to target exon sequences. In our studies, we first used the ubiquitous CAG promoter to express the *Rho* genes in cultured cells. *In vitro* studies in HEK293 cells revealed a significant RNAi-mediated suppression of the corresponding gene. In these experiments, our newly designed pshRho-2 demonstrated highly specific knockdown of wild type Rho expression without negatively affecting the expression of Rho from the silencing resistant *co-sgRho* construct. Secondly, by using a codon optimization technique, we increased the protein expression efficacy of the therapeutic *Rho* gene, which improved its therapeutic potential. In this regard, higher Rho protein expression was observed in HeLa cells transfected with *co-sgRho* compared to *sgRho*. However, expression of EGFP proteins in live cell fluorescence imaging confirmed equivalent transfection efficiencies of both plasmids in HeLa cells. Both of these transfections demonstrated that Rho proteins were not retained in the ER. Therefore, these *in*

vitro experiments revealed that *co-sgRho* is likely to be a superior choice for gene replacement therapy in patients with adRP.

The final goal of the current study was to explore the effectiveness of the *Rho* gene silencing approach in combination with *co-sgRho* under the guidance of the rod cell specific MOP promoter in an adRP mouse model. To accomplish this, we mixed two separately prepared pshRho-2 and *co-sgRho* NPs and injected them into the sub-retinal space of PND5 P23H KI pups. The dose (2µg of each DNA NP) was used to maximize the expression *in vivo* based on our earlier gene therapy studies. The P23H KI mouse model was selected as it showed the characteristic retinal degeneration features found in advanced RP in patients [27]. Saline solution and pshRho-2 NP were used as controls. For *in vivo* studies, we removed the reporter *EGFP* sequence from the vector containing *co-sgRho* to eliminate any possible *EGFP*-associated toxicity. Moreover, we used the rod cell-specific MOP promoter to guide *co-sgRho* expression specifically in rod cells. At PI-60 day, we compared the treated eyes with the controls. There were no monomer or dimer Rho bands observed in retinas from the saline injected controls as found in the literature [27]. We detected only a single layer of nuclei in the ONL with very little Rho protein expression in saline-injected retinas. The pshRho-2 NPs injected mouse eyes demonstrated complete knockdown of monomer and dimer Rho proteins in the rod PR cells. Both pshRho-2 and saline injected mice exhibited a single layer of PR cell nuclei in the ONL. In contrast, co-delivery of two NPs resulted in a markedly increased amount of Rho protein expressed in the retina as demonstrated by the monomer and dimer Rho protein expression. The monomer is the most active form of Rho and the dimer form is found in the disc membrane of PR outer segments [49]. Oligomerization of Rho protein is significantly important to maintain Rho protein stability in the outer segment [50]. It has been reported that over expression of the Rho monomer might cause retinal degeneration [6,51]. However, in our current study, the monomer Rho was not over expressed compared to the WT mice. This phenomenon is likely due to the use of the short form of genomic *Rho* gene that contains both 5 exons and 4 introns to express the Rho protein. The introns were previously found to play an important role in controlling posttranscriptional gene expression [11]. Our hypothesis was to include these transcription regulatory intron sequences to improve the *Rho* gene expression efficacy *in vivo*. Thus, the strategy illustrated the improvement of Rho protein expression and further demonstrated an improvement in outer nuclear layer structure upon the treatment with the NP combination compared to the controls. The immunohistochemistry clearly demonstrated the efficacy, or success, of our constructs. Using DAPI, we illustrated the rescue of ONL. The use of anti-alpha Na⁺/K⁺ ATPase antibody demonstrated the rescue of cell bodies and the rhodopsin staining obviously validated the restoration of rhodopsin proteins. The western blot results demonstrated that the pshRho-2 treatment (control) was able to suppress endogenous Rho protein expression, which is necessary for the silencing resistant transcripts to express therapeutic Rho protein and promote structural and functional recovery in rod PR cells. The non-invasive ERG analysis of treated and control animals demonstrated a partial improvement of scotopic responses, which reflected a promising delay in degeneration of the PR cells. This conclusion was further confirmed by the behavioral water maze study. From our experiments, we observed that mice treated with the 2 NPs (pshRho-2 and pPEI-MOP-co-sgRho) learned comparatively faster on the 4th day compared to the uninjected and

pshRho-2 NP-treated controls. WT mice, which served as a positive control, learned to find the platform on all 4 days. This study demonstrated a delay in PR cell degeneration after treatment with the NP combination. Simultaneous delivery of both suppression and replacement components resulted in potential structural and functional benefits to the retina, which would not be possible without the injection of a therapeutic codon-optimized and silencing resistant short genomic form of the murine *Rho* gene. To the best of our knowledge, this is the first proof-of-principle mutation-independent suppression and replacement strategy based on non-viral gene delivery to express a *co-sgRho* precisely in the rod PR cells of an adRP mouse model. Further studies will be designed to confirm our assessments in long-term rescue using this promising combinatorial NP based gene therapy approach.

5. Conclusion

Herein, we reported a potential mutation-independent suppression and replacement strategy that can be used with multiple neurodegenerative RP disorders containing diverse genetic mutations. We demonstrated that our reengineered diblock copolymer mediated combinatorial gene therapy strategy was safe and effective in the *Rho*^{P23H/P23H} mouse model of adRP. This RNAi-mediated suppression strategy in combination with RNAi-resistant codon-optimized *Rho* replacement therapy was validated by the partial structural and functional recovery of rod PR cells. Our genomic form of *co-sgRho* is a promising therapeutic strategy with potential long-term gene expression in retina. This strategy provides therapeutic benefits to overcome the hurdles associated with mutational heterogeneity in the *Rho* gene in adRP patients and may be useful for other autosomal dominant disorders. Together with the advancement of new gene therapy strategies, the outcome of our research might offer a potential non-viral approach towards the development of therapeutics in retarding the severe progression of adRP-induced blindness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic presentations of the secondary structure of the Rho protein and the vector constructs. (A) Human rod opsin protein (NP_000530.1). The P23H point mutation is shown in red close to the N-terminus, which faces the intradiscal space. (B) Two plasmid DNA constructs are shown. The short form of the *sgRho* sequence was inserted under the control of the CAG promoter upstream of the reporter *EGFP* sequence, which is downstream of the IRES. *sgRho* short-form of rhodopsin (Rho) genomic gene; *co-sgRho*: codon-optimized *sgRho*; I: intron; E: exon; CAG: promoter; IRES: internal ribosomal entry site; *EGFP* encodes the EGFP protein; AMP: ampicillin resistance cassette. (C) Diagrams of three shRho cassettes composed of a sense loop, an antisense hairpin strand, a poly (T), and a transcription termination signal. These cassettes were cloned into the pRNA shuttle vector (4881 bp) under the control of the human H1.1 promoter. Three short-hairpin shRNA sequences were cloned into the multiple cloning site (MCS) after digestion with BamH1 and Hind III.

Fig. 2.

Transfection efficiencies and strategies for shRho screening were determined by fluorescence microscopy and Western blot after co-transfection in HEK293 at 48 h post transfection. (A) Transfection of pCAGIG-sgRho-IRES-EGFP with or without pshRho 1–3 constructs. Cells transfected with pCBA-EGFP were used as a control. The top panel represents bright field images and the lower panel represents EGFP fluorescence. (B) Transfections of pCAGIG-co-sgRho-IRES-EGFP with or without pshRho 1–3 constructs. The top panel represents bright field images and the lower panel represents EGFP

fluorescence. The fluorescence images were acquired using a Zeiss Axio microscope (Carl Zeiss, Germany) with a GFP filter at 100× magnification. Western blot analysis of rhodopsin proteins using HEK293 cell lysates after co-transfection with (C) pCAGIG-sgRho-IRES-EGFP and (D) pCAGIG-co-sgRho-IRES-EGFP plasmid DNAs. Untransfected and pCBA-EGFP-transfected cell lysates were used as negative controls. The Rho proteins were detected using the mouse monoclonal antibody 1D4. The lower panels in (C) and (D) represent the corresponding expression of the EGFP protein as control to demonstrate equivalent co-transfection efficiencies of plasmid DNAs. Beta-actin was used as a loading control.

Fig. 3.

Subcellular localization of Rho proteins in Cos-7 cells, encoded by pCAGIG-*sgRho*-IRES-EGFP and pCAGIG-*co-sgRho*-IRES-EGFP plasmid DNA, were determined by CLSM imaging. Untransfected cells (top panel) were used as a negative control. Calreticulin (white) is an ER marker. The Rho protein (red) was not retained in the ER. Rho protein was expressed throughout the cells in both gene transfections. EGFP expression (green) was used as a transfection control. The nucleus is stained with DAPI. The CLSM images were acquired at 40× magnification using a Zeiss LSM 710. Scale bar = 10 μm.

Fig. 4.

Characterization of the CK30PEG-TAT compound and schematic representation of DNA NPs compaction. (A) The synthetic scheme of CK30PEG synthesis and its conjugation with the TAT sequence. (B) ¹H-NMR analysis of CK30, CK30PEG, NHS-PEG-DBCO and CK30PEG-TAT compounds. (C) A schematic representation of DNA NP formation after compaction between the cationic diblock co-polymer and the negatively charged supercoiled plasmid DNA. The predicted structure of DNA NPs might be a worm-like morphology.

Fig. 5.

Characterization of DNA NP complexes. (A) Schematic representation of the pPEI-MOP-co-sgRho plasmid. (B) Agarose gel electrophoresis. Lane 1: 1kb plus DNA ladder; lanes 2 and 7: pPEI-MOP-co-sgRho and pshRho-2 naked plasmids, respectively; lanes 3 and 8: DNase I digestion of pPEI-MOP-co-sgRho and pshRho-2 naked plasmids, respectively; lanes 4 and 9: The CK30PEG-TAT compacted pPEI-MOP-co-sgRho and pshRho-2 NPs, respectively; lanes 5 and 10: DNase I digestion of pPEI-MOP-co-sgRho and pshRho-2 NPs, respectively; lanes 6 and 11: Trypsin digestion of pPEI-MOP-co-sgRho and pshRho-2 NPs, respectively. (C) TEM images of pPEI-MOP-co-sgRho NPs; Inset: single NP morphology, scale bar = 200 nm. (D) TEM images of pshRho-2 NPs, Inset single NP morphology, scale bar = 200 nm. Both of these NPs were uniformly distributed over the entire grid with worm-like morphologies. (E) The lengths and diameters were calculated from the TEM morphologies of both DNA NPs. Diameters were comparable but the lengths were significantly different ($***p < 0.0001$) due to the different sizes of the naked DNAs. (F) Representative DLS plot of pPEI-MOP-co-sgRho NPs. (G) Representative DLS plot of pshRho-2 NPs. (H) *Rho*^{P23H/P23H} KI mice genotyping with PCR.

Fig. 6.

The evaluation of pshRho-2 suppression and co-*sgRho* gene replacement in the retina of a $Rho^{P23H/P23H}$ KI mouse at PI-60 days. Two representative untreated WT ($Rho^{+/+}$) lysates are presented as controls. Four independent representative saline and pshRho-2 treated, and six independent representative combinatorial DNA NPs treated retinal lysates are presented. The NPs were injected into the sub-retinal space of PND5 $Rho^{P23H/P23H}$ KI pups. The arrows indicate Rho monomers and dimers.

Fig. 7.

Evaluation of Rho protein expression in the PRs of *Rho*^{P23H/P23H} retina at PI-60 days.

Rabbit anti-Rho and mouse anti-alpha Na⁺/K⁺ ATPase were used to detect Rho (Red, AF-555) and plasma membrane (green, AF-488) respectively. The images were acquired at 40× magnification using a Zeiss LSM 710 CLSM. The formation of small inner and outer segments in the retina are indicated by white arrows. OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer. Scale bar = 5 μm.

Fig. 8. Improvement of retinal function in *Rho*^{P23H/P23H} KI mice after treatment with the combinatorial NP complex at PI-60 days. Water maze experiments performed with WT (untreated), pshRho-2 NP (in saline) and pPEI-MOP-co-sgRho/pshRho-2 (in saline) combinatorial NPs treated *Rho*^{P23H/P23H} (KI) mice at PI-60 days. (A) Five representative scotopic traces (per group) were acquired from WT (untreated), saline-injected, pshRho-2 NP-injected and pPEI-MOP-co-sgRho/pshRho-2 combinatorial NPs injected KI eyes receiving 1.0 log cd.s.m⁻² light stimulus after overnight dark adaptation. (B) Average

scotopic a-wave amplitudes with $1.0 \log \text{cd.s.m}^{-2}$ light stimulus recorded from WT (untreated) along with saline, pshRho-2 NP and pPEI-MOP-co-sgRho/pshRho-2 combinatorial NPs treated KI mice eyes ($n=7-10$). (C) Average scotopic b-wave amplitudes with $1.0 \log \text{cd.s.m}^{-2}$ light stimulus recorded from WT (untreated) along with saline, pshRho-2 NP and pPEI-MOP-co-sgRho/pshRho-2 combinatorial NPs treated KI mice eyes ($n=9-10$). The scotopic ERG responses revealed a significant delay ($***p<0.0001$) in the progression of rod dysfunction upon treatment with the combinatorial NP complex. The data are presented as the mean $\pm$ SEM and were analyzed with one-way ANOVA followed by Tukey's post hoc multiple comparison test, $***p < 0.0001$. (D) The pPEI-MOP-co-sgRho/pshRho-2 combinatorial NPs-treated mice demonstrated an improved performance in a visible platform task. Data are analyzed by unpaired Student's t-tests between two independent groups. $**p < 0.01$, $*p < 0.05$, $n=4-5$.