As the most common subtype of Leber congenital amaurosis (LCA), LCA10 is a severe retinal dystrophy caused by mutations in the CEP290 gene. The most frequent mutation found in LCA10 patients is a deep intronic mutation in CEP290 that generates a cryptic splice donor site. The large size of the CEP290 gene prevents its use in AAV-mediated gene augmentation therapy. Here, we test the hypothesis that targeted genomic deletion using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system can be used as a therapeutic approach for the treatment of LCA10 patients bearing the CEP290 splicing mutation.

Methods: A cellular model of LCA10 was developed by introducing the CEP290 splice mutation into human embryonic kidney (HEK) 293FT cells using CRISPR/Cas9-mediated homology-directed repair (HDR). Paired sgRNAs and Cas9 were employed to excise the intronic fragment containing the CEP290 splice mutation. Dual AAV vectors (AAV5-SpCas9 and AAV5-sgRNA pairs) were co-injected into the subretinal space of C57BL/6J mice to delete an intronic fragment in the CEP290 gene of mouse photoreceptors. A self-limiting CRISPR/Cas9 system was developed to limit the expression of SpCas9 by incorporating sgRNA recognition sites into the SpCas9 plasmid.

Results: The LCA10 cellular model recapitulated the CEP290 expression pattern observed in LCA10 patient fibroblasts. In this model, guide RNA pairs coupled with SpCas9 were highly efficient at removing the intronic splice mutation and restoring the expression of wild-type CEP290. In addition, we demonstrated that the dual AAV system could effectively delete an intronic fragment of the CEP290 gene in the mouse retina. The self-limiting CRISPR/Cas9 system was effective at minimizing the duration of SpCas9 expression and rescuing the levels of wild-type CEP290 mRNA. These results support further studies to determine the therapeutic potential of CRISPR/Cas9-based strategies for the treatment of LCA10 patients bearing the CEP290 splice mutation.

Commercial Relationships: Guoxiang Ruan, Sanofi Genzyme (E); Elizabeth Barry, Sanofi Genzyme (E); Dan Yu, Sanofi Genzyme (E); Michael Lukason, Sanofi Genzyme (E); Seng Cheng, Sanofi Genzyme (E); Abraham Scaria, Sanofi Genzyme (E)

Program Number: 4469 Poster Board Number: B0124
Presentation Time: 11:00 AM–12:45 PM
Optimized Homology Directed Repair for Treatment of Inherited Retinal Diseases Using the CRISPR/Cas9 System
Brian Rossmiller, Takeshi Iwata. National Institute of Sensory Organs, National Hospital Organization, Tokyo, Japan.

Purpose: Nearly 1.5 million people, worldwide, are affected by hereditary vision impairment each year. For many of these, there is little to no effective treatment. Advancements in CRISPR/Cas mediated site specific gene editing provide a new and powerful tool for disease treatment but relies on inefficient homology directed recombination (HDR). The purpose of this project is to treat three models of inherited retinal degeneration using CRISPR/Cas9 mediated HDR. Prior to treatment, we will assess optimal conditions for use of the CRISPR/Cas9 system in the retina. These include age of injection, delivery vehicle (nanoparticle or adeno-associated virus (AAV)), and method of increasing homologous integration of gene replacement (ligase IV inhibitors and neurotrophic factors).

Methods: LPD lipid ratios were tested through transfection of 2.5 ug of plasmid containing control cytomegalovirus promoter driven firefly luciferase DNA into HEK293 cells. Luciferase activity was measured 48 hours post-transfection. Assessment of sgRNA cleavage efficiency was performed using a novel dual luciferase approach with a molar ratio of 1:4 target to H1-sgRNA expression vector. Luciferase activity was measured 48 hours post-transfection. Each vector is assessed in three animal models of inherited retinal degeneration. First, a mouse model of retinitis pigmentosa, Rho (I307N), kindly provided by Dr. Nishina of Jackson laboratory. The second model is of normal tension glaucoma, OTPN (E50K) and produced in the Iwata lab. Finally, we created a third model featuring a premature truncation of KCNJ13 (W53X) resulting in Leber’s congenital amaurosis.

Results: Here, we have confirmed transfection, of CMV-GFP plasmid, by LPDs, into HEK293 cells by GFP expression. These results confirm that a ratio of 1:2:3:15, DNA, nuclear localization signal and trans-acting transcription peptide, protamine sulfate, and lipids, respectively, provided the best DNA transfection rates in tissue culture and will be used moving forward in mouse models. Cleavage of KCNJ13, by CRISPR/Cas9, resulted in a significant reduction in luciferase activity (70%, p < 0.05).

Conclusions: Our current LPD mixture and sgRNAs targeting KCNJ13 have shown to provide significant transfection and targeted knock-down in vitro.

Commercial Relationships: Brian Rossmiller, None; Takeshi Iwata
Support: The Japan Agency for Medical Research and Development
We obtained mice with mutation of either the 5' or 3' ERC starting grant #311244 Zhaoxia Zhang, None; Spark Therapeutics (C) CRISPR/Cas9 was used to induce mutations within the Mutations in Multiple RGR-d mutant mouse lines (strains: B6D2F1/J 4471 In contrast to the RGR-d isoform in humans, mouse The modified TLR system (TLR3) comprises a bicistronic expression system of a non-functional green fluorescent protein (GFP) gene, followed by a self-cleaving T2A peptide and a second blue fluorescent protein (BFP) gene in a reading frame shifted by 2 bp. A stable HEK293 cell line expressing TLR3 was generated by transfecting a linearized pcDNA3.1(-)-TLR3 plasmid followed by neomycin selection. Donor templates of 1000 bp length containing the corrected GFP sequence were generated as circular plasmid, linearized plasmid with long 3’ or 5’ backbone overhang, or as PCR product. The sequence to be corrected was either centrally located (RS55), with a shorter 5’ homologous region (RS37), or a shorter 3’ homologous region (RS73). Eight different guide RNAs were generated and the Cas9 from the streptococcus pyogenes was used. DNA repair activity was measured by FACS.

Results: Guide RNAs targeting the active strand (T5, T7) showed NHEJ frequencies of about 20%, while guide RNAs targeting the inactive strand showed NHEJ frequencies of less than 5%. HDR activity was highest (about 2%) when using the linearized plasmid with the short 5’ backbone overhang and the RS37 design, followed by the PCR product or the linearized plasmid with the long 5’ backbone overhang, both with RS73 design (1-1.4%). Circular plasmid was least efficient in generating HDR events (<1%). The effect of the different repair templates on NHEJ frequencies was marginal.

Conclusions: In contrast to the RGR-d isoform in humans, mouse RGR-d undergoes rapid degradation, and abnormal RGR-d protein does not accumulate in the eyes of otherwise healthy, untreated young mice. These animals provide a model for lifelong expression of an Rgr-d gene and potentially chronic endoplasmic-reticulum stress or age-related proteopathy.

Commercial Relationships: Zhaoxia Zhang, None; Henry KW Fong, None

Program Number: 4471 Poster Board Number: B0126
Presentation Time: 11:00 AM–12:45 PM

OPTIMIZING THE REPAIR TEMPLATE FOR HOMOLOGY DIRECTED REPAIR OF DOUBLE STRAND BREAKS

Program Number: 4471 Poster Board Number: B0126
Presentation Time: 11:00 AM–12:45 PM

OPTIMIZING THE REPAIR TEMPLATE FOR HOMOLOGY DIRECTED REPAIR OF DOUBLE STRAND BREAKS

Knut Stieger, Fei Song, Birgit Lorenz. Department of Ophthalmology, Justus-Liebig-University Giessen, Giessen, Germany.

Purpose: In vivo genome editing takes center stage in the development of therapeutic applications of inherited retinal dystrophies. In such therapeutic applications it is crucial to bias repair outcomes towards high fidelity homology directed repair (HDR) or microhomology mediated end-joining (MMEJ), and to avoid error prone nonhomologous end-joining (NHEJ). In this study, we analyzed the impact of different repair templates on the frequency of HDR and NHEJ in a well-defined cell culture system.

Methods: The modified TLR system (TLR3) comprises a bicistronic expression system of a non-functional green fluorescent protein (GFP) gene, followed by a self-cleaving T2A peptide and a second blue fluorescent protein (BFP) gene in a reading frame shifted by 2 bp. A stable HEK293 cell line expressing TLR3 was generated by transfecting a linearized pcDNA3.1(-)-TLR3 plasmid followed by neomycin selection. Donor templates of 1000 bp length containing the corrected GFP sequence were generated as circular plasmid,
adults and outcrossed. Novel rho alleles were identified in the F1 generation.

**Conclusions:** Our recovery of novel mutations shows that rho can be successfully targeted in zebrafish using the CRISPR/Cas9 system. These offer a proof-of-concept for the successful targeted mutagenesis of rho in zebrafish and their potential as models of disease.

**Commercial Relationships:** Chris Zelinka, None; James M. Fadool, None

**Support:** Foundation Fighting Blindness

**Program Number:** 4473 **Poster Board Number:** B0128 **Presentation Time:** 11:00 AM–12:45 PM **Allele-specific gene editing for the treatment of autosomal dominant Retinitis Pigmentosa**

**Gene Liu**, **Kolla N. Rao**, **Victor V. Bartsevich**, **Jeff Smith**, **Derek Jantz**, **Matthew Hirsch**, 1 Precision Biosciences, Durham, NC; 2Department of Ophthalmology, Gene Therapy Center, University of North Carolina, Chapel Hill, NC.

**Purpose:** Targeted gene knockout is an emerging approach for the treatment of autosomal-dominant disorders such as P23H RHO mediated autosomal dominant retinitis pigmentosa (adRP), a common form of inherited blindness. A single nucleotide distinguishes P23H RHO from the WT copy which collectively present in a heterozygous manner. Previous works have suggested that down-regulation of the mutant allele in presence of the WT copy prevents retinal degeneration. Therefore, a 930nt I-CreI based designer homing endonuclease (HE) to specifically knockout the mutant RHO allele was engineered and validated in a self-complementary AAV vector. The specificity of this HE for P23H RHO was demonstrated in vitro. Preclinical evaluations in a human P23H RHO adRP mouse model were undertaken to determine if this approach demonstrates clinical potential for preventing vision loss in adRP patients.

**Methods:** A proprietary ARCUS platform was employed to generate a HE that can specifically target the human RHO allele carrying the P23H mutation. The targeted 22 nucleotide sequence differs from the wild type allele only by a single base pair. Specificity of the nuclease was first evaluated in CHO cell lines engineered to contain a single strand annealing GFP reporter that harbors either the WT or P23H RHO sequence. Gene editing efficiency was evaluated by flow cytometry and immunoblotting. To evaluate HE activity in vivo, a scAAV5 vector harboring P23H RHO HE cDNA under control of a photoreceptor specific promoter was administered to the human P23H RHO adRP mouse model via sub-retinal injection. scAAV5-GRK-HE cleavage efficiency of the mutant RHO allele was calculated using sequencing and by digital PCR. In vivo retinal function was evaluated by electoretinography and the subcellular localization of rhodopsin in photoreceptors by histology.

**Results:** We have generated a HE that can specifically target the human RHO allele carrying the P23H mutation. The targeted 22 nucleotide sequence differs from the wild type allele only by a single base pair. Specificity of the nuclease was first evaluated in CHO cell lines engineered to contain a single strand annealing GFP reporter that harbors either the WT or P23H RHO sequence. Gene editing efficiency was evaluated by flow cytometry and immunoblotting. To evaluate HE activity in vivo, a scAAV5 vector harboring P23H RHO HE cDNA under control of a photoreceptor specific promoter was administered to the human P23H RHO adRP mouse model via sub-retinal injection. scAAV5-GRK-HE cleavage efficiency of the mutant RHO allele was calculated using sequencing and by digital PCR. In vivo retinal function was evaluated by electoretinography and the subcellular localization of rhodopsin in photoreceptors by histology.

**Conclusions:** This is the first study to demonstrate that a single chimeric HE can efficiently discriminate a one nucleotide difference and specifically target the P23H RHO allele. This result coupled with decreased rhodopsin mislocalization in an adRP model, foreshadows possible use of scAAV HE-based gene editing of P23H RHO to treat adRP patients.

**Commercial Relationships:** Gene Liu; Kolla N. Rao, Precision Biosciences (F); Victor V. Bartsevich, Precision Biosciences (E); Jeff Smith, Precision Biosciences (E); Derek Jantz, Precision Biosciences (E); Matthew Hirsch, Precision Biosciences (F)
of MG are of great interest. To gain a comprehensive understanding of the factors and cognitive signaling systems controlling retinal regeneration, we apply forward and reverse genetics screening approaches to identify regeneration-deficient mutant zebrafish lines. Here, we discuss the use of a multiplexed CRISPR/Cas9-based targeted mutational strategy to evaluate ~300 transcriptomics-implicated genes as factors necessary for rod photoreceptor regeneration in zebrafish.

**Methods:** We have developed complementary cell-specific ablation and whole-organism high-throughput screening (HTS) methods to 1) facilitate studies of cellular regeneration, 2) model degenerative disease, and 3) discover regeneration-promoting factors in zebrafish. Here, a model of retinitis pigmentosa—where apoptosis can be induced specifically in rod cells and a fluorescent reporter allows quantification of cell loss and replacement kinetics—is being used to identify genes required for rod cell regeneration. To implicate genes in rod cell replacement, we performed a microarray analysis comparing two cell-specific ablation paradigms, rod cells versus retinal bipolar cells. Nearly 300 genes upregulated in the rod cell were selected for multiplexed CRISPR/Cas9-based mutational analysis. Using this method, phenotyping assays can be performed in the F1 generation, vastly increasing screening efficiency, and thereby enabling large-scale reverse genetics.

**Results:** Targeting oligos for 144 candidate genes have been generated. Multiplexing is used to concurrently target 3 genes candidates, resulting in a total of 96 pooled founder groups for 288 gene candidates. Our recent progress in generating mutant lines and identifying regeneration-deficient phenotypes will be discussed.

**Conclusions:** Multiplexed CRISPR/Cas9 methods enable large-scale mutational analyses, providing a means of functionally evaluating candidate genes at a rate of high-throughput bioinformatics-based datasets. The approaches outlined herein can therefore provide fundamental insights into the mechanisms regulating regenerative potential in the retina.

**Commercial Relationships:** Arife Eroglu, None; Timothy Mulligan, None; Sumitra Sengupta, None; Jeff S. Mumm, None

**Support:** NEI R01EY022810

**Program Number:** 4476 **Poster Board Number:** B0131

**Presentation Time:** 11:00 AM–12:45 PM

Safety and post hoc analysis of subretinal rAAV.sFLT-1 for wet age-related macular degeneration following a phase 2a randomized clinical trial

Elizabeth P. Rakoczy,1,2 Chooi-May Lai,1,2 Aaron Magno,1,2 Martyn French,3 Steve Butler,4 Samuel Barone,4 Steven D. Schwartz,4 Mark Blumenkranz,4 Mariapia Degli-Esposti,4 Ian Constable,4 1Centre for Ophthalmomol & Visual Sciences, University of Western Australia, Perth, WA, Australia; 2Molecular Ophthalmology, Lions Eye Institute, Perth, WA, Australia; 3School of Pathology, University of Western Australia, Perth, WA, Australia; 4Adverum Biotechnologies, San Francisco, CA; 5University of California, Los Angeles, Los Angeles, CA; 6Byers Eye Institute, Stanford University, Palo Alto, CA.

**Purpose:** To assess the safety and tolerability of rAAV.sFLT-1 in patients with wet age-related macular degeneration (wAMD).

**Methods:** Thirty-two patients (55 years or older) with wAMD and best corrected visual acuity worse (BCVA) than 20/40 secondary to wAMD due to active subfoveal choroidal neovascularization in their study eye were recruited. Patients were randomized in a 2:1 ratio (gene therapy:control). All patients received intravitreal ranibizumab at baseline (Day 0) and at day 28. Gene therapy patients received rAAV.sFLT-1, 1×10^11 vg subretinally at Day 7. Both arms received intravitreal ranibizumab pro nata after day 28. Patients were assessed every 4 weeks to the 52-week primary endpoint using clinical laboratory testing, physical and ophthalmic examinations. Biodistribution and immune response to the vector was evaluated by a range of laboratory tests.

**Results:** Treatment group patients injected subretinally with rAAV.sFLT-1 tolerated the procedure well. All procedure-related ocular adverse events self-resolved. No systemic safety signals were observed and serious AEs were deemed not associated with rAAV.sFLT-1. There was no change in sFLT-1 levels and AAV2 capsid was not detected in the bodily fluids. rAAV.sFLT-1 DNA was detected transiently in the tears of 13 rAAV.sFLT-1-injected patients. There were no notable changes in T-cell response identified by ELISPOT analysis. The presence of AAV neutralizing antibodies at baseline was not associated with diminished efficacy. In the treatment group patients, best corrected visual acuity improved by a median of 1.0 (IQR: 1.0 to 9.0) Early Treatment Diabetic Retinopathy Study (ETDRS) letter from baseline compared to a median -5.0 (IQR: -17.5 to 1.0) ETDRS letter change in control patients. Twelve rAAV.sFLT-1-injected patients maintained or improved vision compared to 4 control patients. The median number of ranibizumab retreatments in rAAV.sFLT-1-injected and control group patients was 2.0 (IQR: 1.0 to 6.0) and 4.0 (IQR: 3.5 to 4.0), respectively.

**Conclusions:** These results suggest that ocular gene therapy combined with the option for intravitreal anti-VEGF protein rescue treatment appears safe and may be a viable approach to reducing the treatment burden of wAMD.

**Commercial Relationships:** Elizabeth P. Rakoczy, Chooi-May Lai, Avalanche Biotechnologies (E), Avalanche Biotechnologies (P); Aaron Magno, Avalanche Biotechnologies (E); Martyn French, None; Steve Butler, Avalanche Biotechnologies (E); Samuel Barone, Avalanche Biotechnologies (E); Steven D. Schwartz, Avalanche Biotechnologies (I); Mark Blumenkranz, Avalanche Biotechnologies (I); Mariapia Degli-Esposti, None; Ian Constable, Avalanche Biotechnologies (F), Avalanche Biotechnologies (P)

**Clinical Trial:** NCT01494805

**Program Number:** 4477 **Poster Board Number:** B0132

**Presentation Time:** 11:00 AM–12:45 PM

Co-expression of human L- or M-opsin restores M-Cone function in the Opn1mw Knock-out mouse, a model for blue cone monochromacy (BCM)

Wentao Deng,1 Ji-Jing Pang,1 Jie Li,2 Ping Zhu,2 Wolfgang Baehr,2 W. Clay Smith,3 William W. Hauswirth,3 1Ophthalmology, University of Florida, Gainesville, FL; 2University of Utah, Salt Lake City, UT.

**Purpose:** BCM is an X-linked congenital disorder characterized by loss of both L- and M- cone function. Here we tested gene therapy for BCM using a M-Cone knock out mouse model by AAV-mediated expression of either or both human M- and L-opsin.

**Methods:** Since human L- and M-opsin are highly homologous there is no antibody that can distinguish between them. We generated AAV5 vectors expressing either C-terminal myc tagged L-opsin-myc cDNA or C-terminal HA tagged M-opsin-HA cDNA, both driven by the cone-specific PR2.1 promoter. Vectors were delivered either individually or as a mixture containing equal numbers of vector genomes of each. 1 μL of vector(s) was injected subretinally into one eye of one month old M-opsin Knockout (Opn1mw–/–) mice, while the contralateral eyes served as controls. M-cone function was assessed by photopic ERG under middle wavelength (510nm) flash conditions. In retinal sections human L- and M-opsin expression were analyzed by myc and HA antibodies respectively, and their cellular

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localizations analyzed by peanut agglutinin (PNA) and anti-human L/M-opsin and anti-mouse S-opsin.

**Results:** Retinal functional analysis of Opn1mw<sup>−/−</sup> mice treated with either vector individually or as an equal mixture showed significant ERG rescue compared to untreated controls. In eyes treated with individual vectors, expression of myc, HA and M/L-opsin were detected and co-localized correspondingly. In the dorsal retina where no mouse M-opsin is expressed, human M- or/and L-opsin expression co-localized with PNA labeled cone sheaths. In the ventral retina, where mouse S-opsin is present in cone outer segments, they co-localized with endogenous S-opsin. In retinas treated with the vector mixture, a majority of the cones co-express both M- and L-opsin as seen by HA and Myc co-localization. We also note that individual cones can contain different ratios of human M- and L-opsin.

**Conclusions:** We demonstrate that C-terminal tagging human opsins does not interfere either function or cellular localization. Therefore, these tags can be employed to distinguish L- from M-opsin in the same retina. Functional and structural analyses in Opn1mw<sup>−/−</sup> retinas confirm that either human opsin or their mixture can restore both significant M-cone function and cone outer segments. These observations provide preclinical proof-of-principle justifying gene therapy treatment of human BCM.

**Commercial Relationships:** Wentao Deng, None; Ji-Jing Pang, None; Jie Li, None; Ping Zhu, None; Wolfgang Baehr, None; W. Clay Smith, None; William W. Hauswirth, AGTC (F), AGTC (P), AGTC (C)

**Program Number:** 4478 Poster Board Number: B0133

**Presentation Time:** 11:00 AM–12:45 PM

**Restoration of M-cone function in an S-cone only mouse model of human blue cone monochromacy by AAV-mediated expression of human L- or M-opsin**

**Purpose:** Blue cone monochromacy (BCM) is an X-linked congenital disorder with severe cone dysfunction due to the absence of the long and medium wavelength-sensitive cone function. In this study, we used an M-opsin knockout (Opn1mw<sup>−/−</sup>) mice with only S-cone function remaining as a model to test gene therapy for BCM by AAV-mediated delivery of either human M- or L-opsin, homologues of the mouse M-opsin.

**Methods:** We generated AAV5 vectors expressing either human L-opsin or human M-opsin driven by the cone-specific PR2.1 promoter. 1 microliter (5 × 10<sup>12</sup> vector genomes/mL) of each vector was injected subretinally into one eye of one month old Opn1mw<sup>−/−</sup> mice, while the contralateral eyes remain untreated and served as controls. M-cone mediated retinal function was analyzed by full-field photopic ERG under middle wavelength flash conditions (510nm). Human L- and M-opsin transgene expression and their cellular localization were examined by immunohistochemistry using anti-L/M-opsin, anti-S-opsin antibodies and peanut agglutinin (PNA). The levels of transgene expression were assessed by Western blot analysis.

**Results:** Untreated Opn1mw<sup>−/−</sup> mice have no M-cone function and only S-cone function primarily in their ventral retina. Treatment with either human M- or L-opsin rescued middle wavelength ERG responses to about 60% of normal b-wave amplitudes. AAV delivered human M-opsin or L-opsin was detected panretinally. In dorsal retinas, M-opsin or L-opsin was localized within PNA-labeled cone sheaths in cone outer segments. In ventral retinas where S-cones are present, M-opsin or L-opsin was co-localized with endogenous mouse S-opsin in cone outer segments. Western blot analysis showed that AAV-delivered M- or L-opsin was about 65% of the wildtype level which is consistent with the level of ERG rescue.

**Conclusions:** We demonstrate that AAV-mediated expression of either human M-opsin or L-opsin can restore ERG responses to middle wavelength light in Opn1mw<sup>−/−</sup> mice. Vectored human opsins were expressed in cone outer segments in both dorsal and ventral retinas. These results also demonstrate that cones with one type of opsin, endogenous S-opsin in this case, can co-exist with a second type of vector delivered opsin, human L-opsin or M-opsin without physiological effects. These observations provide proof-of-concept for eventually treating BCM patients by gene therapy.

**Commercial Relationships:** Ji-Jing Pang; Wentao Deng, None; Yuxin Zhang, None; Jie Li, None; Ping Zhu, None; Wolfgang Baehr, None; William W. Hauswirth, AGTC (F), AGTC (P), AGTC (C)

**Support:** NIH grant EY021721, EY023543, BCM Families Foundation, AGTC, MRVF, and RPB, Inc.

**Program Number:** 4479 Poster Board Number: B0134

**Presentation Time:** 11:00 AM–12:45 PM

**The effect of timing on visual restoration of Gnat1/Pde6c double-knockout mouse through AAV-mediated gene supplementation**

**Purpose:** To determine the differential effect of treatment timing on visual restoration using mice defective of both rod and cone function.

**Methods:** Mice severely defective of both rod and cone function from birth was generated through cross-breeding (Gnat1/Pde6c double-knockout mice). The mice were supplemented with Gnat1 using AAV2/8 at 3W, 3M, and 9M. Histology was examined with immunohistochemistry and visual function was assessed by measuring visually evoked potentials (VEPs) and opto-kinetic responses (visual acuity and contrast sensitivity) 2 weeks after the treatment. The data was compared between the treatment groups.

**Results:** The double KO mice were confirmed to be blind as measured by opto-kinetic responses, while the mice retained weak light perception as measured by VEP. Progressive decline in PNA-positive cones were noted with increasing age while the number of cells in the outer nuclear layer, mostly representing rods, remained essentially unchanged. Visual function as probed by VEPs and visual acuity did not differ between the treatment groups at 3W, 3M, and 9 M. Meanwhile, a moderate decline in contrast sensitivity was detected only in mice treated at 9M (58.8 ± 2.5%) compared to 1M (50.6 ± 4.9%) and 3M (45.8 ± 2.9%).

**Conclusions:** The timing of visual restoration had only a modest impact on the visual function in blind mice. The visual cortex have the capacity to accomodate and process novel visual inputs mediated by the gain of retinal function that takes place well beyond adulthood in mice.

**Commercial Relationships:** Koji M. Nishiguchi; Kosuke Fujita, Yukihiro Shiga, Toru Nakazawa<sup>2,3</sup>; Advanced Ophthalmic Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan; 2Ophthalmology, Tohoku University Graduate School of Medicine, Sendai, Japan; 3Retinal Disease Control, Tohoku University Graduate School of Medicine, Sendai, Japan.

**Support:** JSPS KAKENHI Grants-in-Aid for Scientific Research C (16K11315)
AAV Gene Therapy in a Canine Model of MPS1 Prevents and Reverses Corneal Blindness

Matthew Hirsch1,2, Brian C. Gilger3, Telmo Llanga1, Richard Davis1, Joanne Kurtzberg2, Richard J. Samulski3, Keiko Miyadera1.
1Ophthalmology, University of North Carolina, Chapel Hill, NC; 2Gene Therapy Center, University of North Carolina, Chapel Hill, NC; 3University of Pennsylvania, Philadelphia, PA; *North Carolina State University, Raleigh, NC; †Duke University, Durham, NC.

**Purpose:** Mucopolysaccharidosis type 1 (MPS1) is a devastating lysosomal storage disease that can be lethal in the first decade of life. Bone marrow transplantation has proven effective at prolonging life by decades, however, the patient’s quality of life is compromised due to non-lethal MPS1 manifestations including blindness. In particular, approximately 90% of MPS1 children present corneal opacity with about 50% of cases causing complete vision loss. There is no effective treatment for MPS1 corneal blindness, which is the deficit we seek to address in this work using a viral based gene addition strategy. This technology is envisioned as complementary therapeutic to approaches such as cell therapy that address systemic MPS1 disease symptoms.

**Methods:** A codon optimized IDUA cassette was packaged in a chimeric AAV capsid identified efficient for cornea transduction. Previous evaluations in human corneas ex vivo demonstrated supraphysiological IDUA activity and safety following intrastromal injection of AAV8G9-optIDUA. These optimistic data warranted an efficacy study in naturally occurring MPS1 canines that also suffer from corneal clouding. Three canines with advanced (9 m.o.) and one with early (6 m.o.) corneal diseases were administered AAV8G9-GFP (negative control) in one eye and AAV8G9-optIDUA in the contralateral eye via cornea stromal injections. These canines have been monitored by slit lamp biomicroscopy and corneal imaging currently out 14 weeks post-injections.

**Results:** Corneal clearing, encompassing both the actual cloudy storage disease and regression of associated vascularization was apparent as early as 1 week and near complete disease reversal is now evident 13 weeks in all canines injected with AAV-8G9-optIDUA in the symptomatic cohort. In the younger animal with the early disease, corneal disease progression continued in the control eye while the AAV8G9-optIDUA injected cornea demonstrated clearance of microscopic storage materials and did not develop vascularization. No adverse effect was observed specific to the injection or either of the transgenes, except for transient reversible corneal edemas that developed in each eye of one animal at 6 and 10 weeks post injection.

**Conclusions:** The collective data of a corneal gene addition strategy using AAV vectors for MPS1 blindness demonstrate safety and efficacy and lend support for the evaluation of this technology in MPS1 children.

### Commercial Relationships:
Matthew Hirsch; Brian C. Gilger, None; Telmo Llanga, None; Richard Davis, None; Joanne Kurtzberg, None; Richard J. Samulski, University of North Carolina (P); Keiko Miyadera, None

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**Towards Corneal Gene Therapy in the Aniridia Mouse Model Pax6<sup>−/−</sup>**

Elizabeth M. Simpson1,2, Siu Ling Lam1, Andrea Korecki1, Beatrice M. Tam1, Orson L. Moritz2, Jack Hickmott2, 1Centre for Molecular Medicine and Therapeutics (CMMT), University of British Columbia, Vancouver, BC, Canada; 2Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada; 3Department of Ophthalmology and Visual Sciences, University of British Columbia, Vancouver, BC, Canada.

**Purpose:** Aniridia is a rare autosomal dominant disorder caused by mutations in Pax6 (Paired Box 6). Although named for lack of iris, aniridia is a panocular disorder, with vision loss attributable to three main causes: 1) hypomorph fovea, 2) progressive corneal clouding, and 3) progressive glaucoma. Current treatments may delay vision loss, but do not prevent the blindness experienced by most people with aniridia. Gene therapy is a promising treatment strategy that may delay, or even prevent, vision loss. Here we focus on gene therapy for the corneal pathology.

**Methods:** EmGFP (emerald GFP), Pax6, 3xFLAG/PAX6, and PAX6/3xFLAG mRNAs were injected into Xenopus laevis embryos, which were fixed and examined 14 days later. Additionally, EmGFP and 3xFLAG/PAX6 constructs, driven by smCBA (ubiquitous) or a human MiniPromoter (targeted to the cornea) were cloned into a custom rAAV genome, and packaged into rAAV at the University of Pennsylvania Vector Core. Viruses were introduced into wild-type and Pax6<sup>−/−</sup> mice using several different injections routes. Mouse eyes were longitudinally scored for corneal opacification and then harvested by enucleation, and fixed in 4% paraformaldehyde.
Cryosections were stained with antibodies against EmGFP, PAX6, and FLAG tag for immunofluorescent analysis.

**Results:** Since human and mouse PAX6 are indistinguishable by immune detection, we required a tagged version of PAX6 that did not jeopardize PAX6 in *vivo* function. Thus, we tested PAX6 fused to either amino- or carboxyl-terminal 3xFLAG for the ability to generate ectopic eye structures in *X. laevis* tadpoles. PAX6 and 3xFLAG/PAX6 mRNA drove similar ectopic eye formation, suggesting that this amino-terminal 3xFLAG tag did not disrupt PAX6 function. Delivery of rAAV to the cornea was then explored using topical, intravitreal, intravenous, and subconjunctival routes with ssAAV9 smtCBA-EmGFP-WPRE and ssAAV9 Ptc253-EmGFP-WPRE. The MiniPromoter Ptc253 was derived from the human PITX3 gene and had previously shown, using a different methodology, to express in the corneal stroma (de Leeuw et al., 2016). In this study, stromal expression was achieved with both intravenous and subconjunctival routes.

**Conclusions:** These results have laid the foundation for rAAV delivery of *in vivo* functional 3xFLAG/PAX6, with either a ubiquitous or cell-type restricted promoter, by either intravenous or subconjunctival routes, to treat corneal pathology in *Pax6*−/− mice.

**Commercial Relationships:** Elizabeth M. Simpson, None; Siu Ling Lam, None; Andrea Korecki, None; Beatrice M. Tam, None; Orson L. Moritz, None; Jack Hickmott, None

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**Program Number:** 4482
**Poster Board Number:** B0137
**Presentation Time:** 11:00 AM–12:45 PM
**Improved intravitreal AAV-mediated inner retinal gene transduction by surgical internal limiting membrane (ILM) peeling in cynomolgus monkeys**

Kazuhisa Takahashi1, 2, Tsutomu Igarashi3, 4, Koichi Miyake2, Maika Kobayashi1, Chienchi Yaguchi1, Osamu Iijima2, Yoshiyuki Yamazaki2, Noriko Miyake1, Shuhei Kameya1, Takashi Shimada1, Hiroshi Takahashi1, Takashi Okada2.

1 Ophthalmology, Nippon Medical School, Tokyo, Japan;
2 Ophthalmology, Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan;
3 Ophthalmology, Nippon Medical School Chiba Hokusoh Hospital, Chiba, Japan.

**Purpose:** The adeno-associated virus (AAV) vector is an ideal tool for retinal gene therapy, and there are two administration routes, subretinal injection (SR) and intravitreal injection (IV). SR is used in many studies because of its efficiency of gene transduction of outer retina. However, SR induces an iatrogenic retinal detachment, which causes several adverse effects. On the other hand, IV is considered to be safer than SR, although the transduction efficiency in large animals was insufficient, as the vitreous and internal limiting membrane (ILM) acted as barriers to transduction. To overcome this issue, we performed vitrectomy (VIT) and ILM peeling before AAV vector injection.

**Methods:** Among the six eyes in the three female cynomolgus monkeys (aged 10-13 years old), two eyes received standard 3-port VIT (VIT group), two eyes received ILM peeling in addition to the standard 3-port VIT (VIT+ILM group), and the remaining two received no pretreatment (IV group) at 4 weeks before AAV injection. All eyes were penetrated by 30-G needle into the vitreous at the pars plana, and 50 µl of triple-mutated self-complementary AAV serotype 2 vector (1.9 × 10^11 v.g./ml) encoding enhanced green fluorescent protein (GFP) was administered. Nineteen weeks after intravitreal injection of AAV vector, transduction efficiency was analyzed. To detect potential adverse effects, the retinas were monitored using color fundus photography, optical coherence tomography (OCT) and electoretinography (ERG).

**Results:** Little expression of GFP was detected in the IV and VIT groups. On the other hand, strong GFP expression was detected within the peeled ILM area in the VIT+ILM group. Intraocular inflammation was observed in 4 of 6 eyes, however the inflammation was transient and no morphological changes were detected on color fundus images or OCT. Transient reduction of ERG amplitude was detected during the period of intraocular inflammation.

**Conclusions:** These results indicate that surgical ILM peeling before intravitreal AAV vector administration would be safe and useful for efficient transduction of the nonhuman primate retina and provide therapeutic benefits for the treatment of glaucoma and retinal diseases.

**Commercial Relationships:** Kazuhisa Takahashi, None; Tsutomu Igarashi, None; Koichi Miyake, None; Maika Kobayashi, None; Chienchi Yaguchi, None; Osamu Iijima, None; Yoshiyuki Yamazaki, None; Noriko Miyake, None; Shuhei Kameya, None; Takashi Shimada, None; Hiroshi Takahashi, None; Takashi Okada, None

**Program Number:** 4483
**Poster Board Number:** B0138
**Presentation Time:** 11:00 AM–12:45 PM
**AAV-mediated Knockdown and Replacement of Rhodopsin Protects Rods in a Canine Model of RHO-ADR**

William A. Beltran1, Artur V. Cideciyan1, Raghavi Sadharsan1, Michael Messengil1, Brian Rossmiller1, Valerie L. Dufo1, felipe Pompeo Marinho1, Tatjana Appelbaum1, Małgorzata Swider1, Mychael S. Kosykh2, Simone Isuabe2, William W. Hauswirth2, Samuel G. Jacobson1, Alfred S. Aguirre1.

1 Clinical Studies, Univ of Pennsylvania Sch Vet Med, Philadelphia, PA;
2 Ophthalmology, University of Pennsylvania, USA, PA;
4 Molecular Genetics and Microbiology, University of Florida, Gainesville, FL;
4 Ophthalmology, University of Florida, Gainesville, FL.

**Purpose:** To develop an allele-independent corrective gene therapy approach for RHO-ADR that combines the knockdown (KD) of both the mutant and WT RHO alleles and their replacement with a “resistant” codon-modified human RHO cDNA, and to test its efficiency in preventing rod loss in the light-sensitive canine T4R RHO mutant dog.

**Methods:** An shRNA (shRNA260) designed to target a homologous region of both human and dog RHO, and shown to efficiently knockdown RHO in 293T cells expressing GFP-tagged human RHO was packaged in an AAV2/5 vector under the H1 promoter (cloned as a self-complementary cassette). Four adult WT (RHO+) and 4 mutant (RHO467C-) dogs were each injected subretinally (volume: ~ 150 µl) with one of four titers ranging from 1 x 10^11 to 1 x 10^12 v.g/ml. Seven to eight weeks post-injection, retinal integrity was assessed by cSLO/OCT imaging, and by histology /IHC on cryosections. Efficiency of RHO KD was measured both at the RNA and protein levels from retinal tissue punches collected both in the treated (vector bleb) and untreated areas. Following identification of the optimal therapeutic titer, an AAV2/5 construct containing both shRNA260 (under H1 promoter control), and a resistant codon-modified human RHO cDNA (RHO467C) under control of a human opsin (HOP) promoter was tested in RHO467C dogs. Testing employed an acute light damage paradigm that rapidly assesses the effect of therapeutic intervention in preventing light-induced loss of rods in the T4R RHO mutant dog.

**Results:** scAAV2/5-H1-shRNA achieved a KD efficiency that ranged between 5% and 100 % at the protein level and between 26% and 100 % at the RNA level with titers ranging between 1x10^{11}
and 1x10^{12} vg/ml. Efficient (85-100%) KD of RHO protein was achieved at 5 x 10^{11} vg/ml. Light-induced retinal degeneration in RHO^{−/−} dogs was prevented, yet was associated with a loss of rod OS and reduced RHO immunolabeling. However, treatment with the combination virus (scAAV2/5-H5-shRHO),H1-shRNA) at 5x10^{11} vg/ml preserved ONL thickness and rod OS integrity.

**Conclusions:** We have identified a very efficient KD reagent (shRNA) able to suppress canine RHO expression when delivered with viral titters that appear to be safe. Its combination with the replacement (resistant human RHO cDNA) component results in protection from light-induced retinal degeneration and paves the way for a novel allele-independent gene therapy for RHO-ADRPs.

**Commercial Relationships:** William A. Beltran, Tatyana Appelbaum, Brian Rossmiller, Michael Massengill, Nicole M. Mychajlo S. Kosyk, None; Simona Iwabe, None; William W. Hauswirth, AGTC (F), AGTC (P), AGTC (C); Samuel G. Jacobson, None; Alfred S. Lewin, None; Gustavo D. Aguirre, None; Tatyana Appelbaum, None; Malgorzata Swider, None; Mychajlo S. Kosyk, None; Simona Iwabe, None; William W. Hauswirth, AGTC (F), AGTC (P), AGTC (C); Samuel G. Jacobson, None; Alfred S. Lewin, None; Gustavo D. Aguirre, None

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**Program Number:** 4485 **Poster Board Number:** B0140

**Presentation Time:** 11:00 AM–12:45 PM

**AAV2-hCHM Subretinal Delivery to the Macula in Choroideremia: Preliminary Six Month Safety Results of an Ongoing Phase I/II Gene Therapy Trial**

**Hong Yu, John Guy.** Ophthalmology, Bascom Palmer Eye Inst, Univ of Miami, Miami, FL.

**Purpose:** To assess preliminary safety data of the investigational subretinal delivery of a recombinant adeno-associated virus serotype 2 (AAV2) vector carrying a human REP-1-encoding cDNA in choroideremia (CHM).

**Methods:** Nine subjects with CHM (ages 26-57 years), received unocular subfoveal injections of low dose (up to 5x10^{10} vector genome (vg) per eye, n=5) or high dose (up to 1x10^{11} vg per eye, n=4) AAV2-hCHM. Patients were evaluated pre- and post-operatively for 6 months with follow-up ongoing. Ocular safety was assessed by ophthalmic examination, perimetry, spectral domain optical coherence tomography (SD-OCT) and short-wavelength autofluorescence (SW-FAF).

**Results:** There were no surgery-related complications or unexpected adverse events. Visual acuity (VA) returned to baseline in all but one patient who slowly recovered to within 20 letters of baseline by 6 months. This subject had foveal thinning (to ~80% of baseline) and foveal sensitivity loss. All other patients showed no obvious changes in foveal structure post-injection. Mean sensitivity by light-adapted perimetry (10-2 protocol) was unchanged in treated and control eyes. Fundus photography showed non-significant (~3SD of the intervisit variability) increases in light sensitivity in some locations and subjects in the operated eye. Cone-mediated sensitivities by two-color dark-adapted perimeter showed non-significant increases in sensitivity above mean baseline for some locations in the operated eye of three subjects. Residual islands of retinal pigment epithelium preservation by SW-FAF showed trace reduction in area that was not significantly different in the injected eye compared to the control. There were no obvious dose-dependent relationships.

**Conclusions:** VA returned to baseline in most subjects after subfoveal injections of AAV2-hCHM. Foveal thinning and slow recovery of VA with unchanged perifoveal function in one patient raises the possibility of non-vector related individual vulnerability to the subfoveal injection. Not unexpectedly, short-term increases in sensitivity in the operated eyes of a subset of patients did not exceed the intervisit or inter-ocular variability estimates. Longer observation intervals are required to better evaluate the significance of the preliminary changes.

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Evaluation of AA V2tYF-GRK1-RPGR vectors in a canine model of RPGR-XL RRP

Guo-jie Ye1, William A. Beltran2, Valerie L. Dufour2, Felipe Pompeo Marinho3, Inna Martynyuk4, Chuanjian Song5, David Knop6, Gustavo D. Aguirre7, Jeffrey D. Chulay7, Mark S. Shearman7, Evanina Bell Mackall Foundation Trust and The Pennsylvania Lions Sight Conservation

Purpose: In previous studies, an AA V2tYF capsid and GRK1 promoter were more efficient than an AAV5 and IRBP promoter for transducing primate photoreceptors, and two stable human RPGR cDNAs (the full length codon-optimized RPGRco or stabilized RPGRstb) were both effective at early-stage disease in the XLPIRA2 dog model of XLRP due to a mutation in the RPGR gene. We now compared the efficacy of 3 dose levels of RPGRco and RPGRstb vectors with a AAV2tYF capsid and GRK1 promoter in XLPIRA2 dogs at mid-stage disease (~12 wks of age).

Methods: Two dogs per group received a 0.15 mL subretinal injection of AAV2tYF-RPGRco in one eye and AAV2tYF-RPGRstb in the other eye at each of 3 doses (1.2 x 10^11, 6 x 10^11 or 3 x 10^12 vg/ml). One dog received the mid-dose of AAV5-RPGRco and 1 dog received vehicle, in both eyes. Rescue of photoreceptor structure was assessed by clinical examination, and histology/IHC on retinal cryosections at termination 8 wks post-injection.

Results: No abnormal ophthalmic findings were noted in any eyes at the mid- or low-dose levels, but there was retinal inflammation and retinal detachment in the 4 eyes treated with the high-dose of either vector. Retinal perivascular infiltration consisting primarily in T cytotoxic (CD8) and B (CD20) lymphocytes, with some T helper (CD4) cells were found in the bleb area of all eyes treated with the high doses and one eye with AAV2tYF-RPGRco at mid-dose.

Both RPGRco and RPGRstb vectors achieved dose-dependent RPGR transgene expression in photoreceptors, with greater RPGR expression in eyes injected with RPGRco vector than in contralateral eyes injected with RPGRstb vector at the same dose levels.

Correction of rod opsin mislocalization and reduced reactivity of Müller cells, was achieved in this model with the mid-doses of both vectors (0.15 mL at 6 x 10^11 vg/ml).

Commercial Relationships: Guo-jie Ye, AGTC (E), AGTC (I); William A. Beltran, Biogen (P), AGTC (P); Valerie L. Dufour, None; Felipe Pompeo Marinho, None; Inna Martynyuk, None; Chuanjian Song, AGTC (E), AGTC (I); David Knop, AGTC (E), AGTC (I); Gustavo D. Aguirre, Biogen (P), AGTC (P); Jeffrey D. Chulay, AGTC (E), AGTC (I); Mark S. Shearman, AGTC (E), AGTC (I)

Program Number: 4487 Poster Board Number: B0142

Presentation Time: 11:00 AM–12:45 PM

Fundus Autofluorescence Analysis of the Transition Zone in Choroideremia: Outcomes Following Gene Therapy

Ioannis S. Dimopoulos1, Jake Knowles1, Tanvir Sajed2, Ian M. MacDonald1, 1Ophthalmology and Visual Sciences, University of Alberta, Edmonton, AB, Canada; 2Computing Science, University of Alberta, Edmonton, AB, Canada.

Purpose: To evaluate rescue of the transition zone (TZ) autofluorescence in six subjects undergoing unilateral subretinal AAV2.REP1 gene replacement therapy (NCT02077361).

Methods: Bilateral serial 30-degree fundus autofluorescence (FAF) images (Spectralis BluePeak, Heidelberg Engineering) were acquired at baseline and at 6-month intervals post-intervention. Longitudinal images were aligned using the i2k Align Retina platform (Dual-Align LCC, Clifton Park, NY) to compensate for image distortion, changes in resolution, pixel density and focus. Semi-automated serial segmentation of stacked images was performed using custom code written in Matlab (MathWorks Inc.). Expert graders also manually segmented the images using the polygon selection tool in ImageJ (NIH) to determine the ground-truth. Change from baseline in FAF area was quantified at 6-month intervals and compared between treated and untreated eyes.

Results: Baseline residual FAF areas ranged from 8.63 to 72.64 mm² (median: 12.26 mm²), with high symmetry between the two eyes (r=0.83). The semi-automated algorithm showed on average 88.95% similarity (Dice similarity coefficient) with the manually segmented ground truth, with a test-retest repeatability of 3%. After 6 months, an nonsignificant increase in FAF area was noted in the treated eyes (8.1% vs. 4.5% in the untreated; p=0.30). FAF signal loss occurred exclusively at the TZ between healthy and degenerated retina. At the 12-month timepoint, change from baseline in FAF area was similar between the treated (11.86%) and untreated (9.86%) eyes.

Conclusions: FAF allows accurate and reliable monitoring of the TZ activity in CHM. A semi-automated segmentation algorithm reduced significantly the measurement bias and error in FAF area calculation. In this limited cohort of CHM subjects, TZ appears not to be rescued by gene therapy. Longer follow-up is required to determine whether gene therapy shows any benefit in modifying the natural history of RPE loss in CHM.

Commercial Relationships: Ioannis S. Dimopoulos, None; Jake Knowles, None; Tanvir Sajed, None; Ian M. MacDonald, None

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Clinical Trial: NCT02077361

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Safety and Efficacy of AAV2tYF-PR1.7-CNGA3 in CNGA3-deficient Sheep

Program Number: 4488 Poster Board Number: B0143
Presentation Time: 11:00 AM–12:45 PM

mark S. shearman1, Elisha Gootwine2, Eyal Banin1, Ron Ofri1, Raaya Ezra-Elia2, Guo-jie Ye3, Paulette Robinson1, David Knop1, Jeffrey D. Chulay4, AGTC, Alachua, FL; 1Institute of Animal Science, Agricultural Research Organization, The Volcani Center, Rishon LeZion, Israel; 2Hadassah-Hebrew University Medical Center, Kiryat Hadassah, Israel; 3Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Rehovot, Israel.

**Purpose:** Achromatopsia is an inherited retinal disorder characterized by markedly reduced visual acuity, extreme light sensitivity and absence of color discrimination. AGTC is developing AAV2tYF-PR1.7-CNGA3, a recombinant adenovirus-associated virus (rAAV) vector expressing CNGA3, for treatment of CNGA3-related achromatopsia. Here we report results of a toxicology and efficacy study of this vector administered by subretinal injection in CNGA3-deficient sheep.

**Methods:** Groups of 4 or 5 animals received a 0.5 mL subretinal injection in the right eye of AAV2tYF-PR1.7-CNGA3 at one of two dose levels or AAV5-PR2.1-hCNGA3, a vector previously shown to rescue cone photoreceptor responses. The left eye received a 0.5 mL subretinal injection of vehicle (4 animals) or was untreated (9 animals). Toxicity assessment was based on mortality, clinical observations, ophthalmic examinations, ERG, and clinical and anatomic pathology. CNGA3 expression was assessed by immunohistochemistry. Efficacy was assessed by cone ERG responses and maze navigation testing performed before, then 6 and 12 weeks after treatment.

**Results:** Treatment was not associated with systemic toxicity. Most animals had mild to moderate conjunctival hyperemia, chemosis and subconjunctival hemorrhage immediately after surgery that generally resolved by post-operative Day 7. No clear and consistent test article-related effects were noted in any group during the 12 week study. Two animals treated with the higher dose of AAV2tYF-PR1.7-CNGA3 had microscopic findings of outer retinal atrophy, with or without inflammatory cells in the retina and choroid, that were procedural- and/or test article-related. All vector-treated eyes developed cone-mediated ERG responses with no change in rod-mediated ERG responses. Behavioral maze testing showed significantly improved navigation times and reduced numbers of obstacle collisions in all vector-treated eyes compared to their control eye or pre-dose results in the treated eye.

**Conclusions:** Subretinal injection of AAV2tYF-PR1.7-CNGA3 in CNGA3-deficient sheep was well tolerated with no clinically important toxicology findings. Rescue of cone-mediated ERG responses was recorded in all vector-treated eyes. These results support the use of AAV2tYF-PR1.7-CNGA3 in clinical studies in patients with achromatopsia caused by CNGA3 mutations. A Phase 1/2 clinical trial evaluating AAV2tYF-PR1.7-hCNGA3 is scheduled to begin in 2017.

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Genetic engineering of freshly isolated primary human pigment epithelial cells by non-viral gene delivery for the treatment of neovascular AMD

Program Number: 4489 Poster Board Number: B0144
Presentation Time: 11:00 AM–12:45 PM

Sandra Johnen1, Sabine Diarra2, Nina Harmening2, Corinne Marie3, Daniel Scherman4, Zsuzsanna Izsák5, Peter Walter6, Gabriele Thumann3, 4.

**Department of Ophthalmology, University Hospital RWTH Aachen, Aachen, Germany; 2Laboratory of Ophthalmology, University of Geneva, Geneva, Switzerland; 3Unité de Technologies Chimiques et Biologiques pour la Santé UMR 8258, CNRS, Paris, France; 4Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany; 6Department of Ophthalmology, University Hospitals of Geneva, Geneva, Switzerland.

**Purpose:** Gene therapy is a promising treatment for many diseases, and viral vectors are already used in clinical applications. However, their drawbacks have encouraged the improvement of non-viral strategies. We have developed an approach for the treatment of neovascular age-related macular degeneration (nAMD). nAMD is characterized by choroidal blood vessels growing into the subretinal space, which results from an overexpression of the vascular endothelial growth factor and a decreased expression of the pigment epithelium-derived factor (PEDF). Our therapeutic approach involves the transplantation of pigment epithelial (PE) cells that have been engineered to stably overexpress PEDF. The PEDF transgene is incorporated into the cell’s genome by the Sleeping Beauty (SB100X) transposon system delivered by electroporation of non-viral miniplasmids free of antibiotic resistance markers (pFAR).

**Methods:** Immediately after isolation, PE cells from human donor eyes were co-transfected with two pFAR4 derivatives encoding PEDF and the SB100X transposase using two different electroporation systems. PEDF secretion was evaluated by immunoblotting and quantified by ELISA. PEDF gene expression was analyzed via quantitative real-time PCR (qRT-PCR).

**Results:** Effective transfection was demonstrated in various independently performed experiments using as few as 2x10^4 freshly isolated cells. The amount of secreted PEDF was 0.6 ng/h/1x10^4 cells, and thus 6-fold higher compared to the PEDF secretion rate of non-transfected cells. qRT-PCR analysis revealed a 3.2-fold increase in the median of total PEDF gene expression compared to the endogenous level. However, extended postmortem times until cell isolation resulted in reduced PEDF ratios.

**Conclusions:** SB100X-mediated transfection of freshly isolated PE cells resulted in an increase in both PEDF secretion and PEDF gene expression. The pFAR technology further improves the biosafety of non-viral gene transfer, as it allows for plasmid propagation in the absence of antibiotics, which is a crucial safety issue for human trials. The transfection of freshly isolated PE cells is an important step towards the ultimate goal of the TargetAMD project, which is the efficient and safe delivery of the PEDF transgene to autologous cells ex vivo, followed by the transplantation to the subretinal space of nAMD patients.

**Commercial Relationships:** Sandra Johnen, None; Sabine Diarra, None; Nina Harmening, None; Corinne Marie, None; Daniel Scherman, None; Zsuzsanna Izsák, None; Peter Walter, None; Gabriele Thumann, None

**Support:** European Union’s Seventh Framework Programme for Research, Technological Development and Demonstration, grant No. 305134
Intravitreally administered AAV-mediated short-hairpin RNA against mTOR efficiently reduces neovascularization in the murine model of laser-induced choroidal neovascularization

Tae Kwann Park1, Jun-Suh Choi1, Hee Jong Kim2, Hayan Park2, Seung Kwan Nah1, Eung Suk Lee1, Young-Kook Choi1, Heurian Lee1, Young-Hoon Ohn1, Keerang Park2. 1Ophthalmology, Soonchunhyang Univ Hospital, Bucheon-si, Korea (the Republic of); 2Cdmogen Co. ltd, Cheongiu-si, Korea (the Republic of); 3Microbiology, Asan Medical center, University of Ulsan, Seoul, Korea (the Republic of); 4Institute for laboratory medicine, Soonchunhyang Hospital Bucheon, Bucheon, Korea (the Republic of).

Purpose: we evaluated the therapeutic effect of a self complement adeno-associated virus (scAAV)-delivered mammalian target of rapamycin (mTOR) short hairpin RNA in the laser-induced choroidal neovascularization of the mouse eye.

Methods: Self complementray adeno-associated virus (scAAV) based expression vector including GFP and mTOR shRNA (scAAV-GFP-mTOR shRNA) originally designed for the cancer treatment was used in this study.

Choroidal neovascularization was induced via laser photocogulation (LP) in 8 week-old male C57/B6 mice (n=45). Intravitreal injection of AAV-GFP-mTOR shRNA (1 μl of 5.0E+10 vg/ml) or AAV-GFP-scrambled shRNA (1 μl of 5.0E+10 vg/ml) was performed to the right eye of each animal (15 mice per each group) at 5 days after LP. Fifteen laser-treated mice with the same volume of 0.1% PBS injection were used as negative control. Transduction patterns and therapeutic effects of AAV-GFP-mTOR shRNA were evaluated at 14 days after LP. Anti-GFP antibody was used for the evaluation of the transduction efficiency into the CNV-induced retinal tissue. The expression pattern of mTOR through the choriotretinal tissue was detected with anti-mTOR antibody. Anti-LC3B and ATG7 antibodies were used to detect the autophagy. Furthermore, anti-F4/80, CD31, and Bm3 antibodies were also used. Neuroretinal injury was assessed with TUNEL staining.

Results: Expression of GFP was observed through the inner retina and cellular components in the CNV. These pattern of GFP expression were also well colocalized with CD31 (+) and F4/80 (+) cells located in the CNV tissue. In scAAV-GFP-mTOR shRNA-treated group, CNV volume was significantly reduced (p<0.05). Furthermore, CD31 and F4/80 expression were decreased. In addition, the expression of LC3B and ATG7 were dramatically increased in the CNV sites of the scAAV-GFP-mTOR shRNA-treated eye. However, expression of these autophagic markers in the neuroretinal tissue was not significantly changed. Interestingly, the number of TUNEL (+) cells detected in the outer nuclear layer around the CNV tissue was dramatically decreased in the scAAV-GFP-mTOR shRNA-treated eye.

Conclusions: scAAV expressing mTOR shRNA along with GFP as a reporter gene did efficiently suppress laser-induced CNV in the mouse eye. These data confirm the induction of autophagy in the CNV can be used for the treatment of nAMD.

Commercial Relationships: Tae Kwann Park, None; Hee Jong Kim, None; Hayan Park, None; Seung Kwan Nah, None; Eung Suk Lee, None; Young-Kook Choi, None; Heurian Lee, None; Young-Hoon Ohn, None; Keerang Park, None

Program Number: 4490 Poster Board Number: B0145
Presentation Time: 11:00 AM–12:45 PM
for therapeutic purposes the target site must be in a region of the mRNA that is accessible in a stable structure in vivo. Our lab has designed a hhRz that cleaves the 725nt region of human Rhodopsin mRNA (hRHO), as a therapeutic for Autosomal Dominant Retinitis Pigmentosa (adRP). Conventional methods to bring a therapeutic to clinical trial generally require efficacy and toxicity studies conducted in non-human animals. Rat, mouse, pig, and dog are commonly used as adRP model organisms in therapeutic screening. This study was conducted to determine the potential impact that use of non-human animals would have on evaluating a ribozyme that targets hRHO mRNA, and to further characterize and optimize our 725 GUC↓ hhRz.

**Methods:** hRHO mRNA sequences for Human and 15 non-human animals (relevant to adRP/evolutionary relations) were folded at the secondary level using SFold, MFold, and RNAstructure (OligoWalk) algorithms. These algorithms predict structure based on single stranded accessibility by Boltzmann weighting, minimal folding energy, and local free energy, respectively. The product of these output vectors determine the Multiparameter Prediction of RNA accessibility (MPPRNA) and were graphed and area under the curve (AUC) was calculated.

**Results:** For all animals (n=15) MPP values and AUC varied from human regardless of sequence homology in both regions. One-way ANOVA demonstrated MPP values were significantly different (p<0.05) between all species, and 2-way independent t-tests showed AUC values were significantly different (p<0.05) from human for the majority of animal hRHO mRNAs tested (n=9, 60%).

**Conclusions:** Primary mRNA sequence or protein homology is insufficient information to predict mRNA accessibility and efficacy of post-transcriptional gene silencing agents such as hhRzs. Algorithms that predict mRNA folding provide an expedited analysis for structural similarity to the human mRNA target as a proxy for cleavage efficacy. Because of the predicted structural variations between even evolutionarily close organisms, in a single gene that encodes highly homologous proteins, development studies for PTGS agents should be conducted with human samples or mRNAs derived from human genes in transgenic animals.

**Commercial Relationships:** Andrea J. Trujillo, Jack M. Sullivan, None; Beau R. Froebel, None; Jack M. Sullivan, None

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**Program Number:** 4494

**Presentation Time:** 11:00 AM–12:45 PM

**Cleavage of Human Rhodopsin mRNA and Protein Knockdown using Lead HHrz-725, a Candidate Therapeutic for Autosomal Dominant Retinitis Pigmentosa**

Zahra Fayazi1, 2, Mark C. Butler3, 2, Jack M. Sullivan1, 3, 4. 1Research Service, VA Western NY Healthcare System, Buffalo, NY; 2Pharmacology and Toxicology, University at Buffalo, Buffalo, NY; 3Ophthalmology (Ross Eye Institute), University at Buffalo at Buffalo-SUNY, Buffalo, NY; 4Ophthalmology (Ross Eye Institute), Pharmacology/Toxicology, Physiology/Biophysics and Program in Neuroscience, University at Buffalo-SUNY, Buffalo, NY.

**Purpose:** Lead hammerhead ribozymes (hhRz)-725 embedded in an engineered anticodon domain of the small human tRNA-Lys3 scaffold has potential as a therapeutic agent for autosomal dominant retinitis pigmentosa (adRP). Following demonstrated improved catalytic activity of hhRz-725 in vitro, we tested activity in cell culture. Advancement in characterization places us one step closer to testing this post transcriptional gene silencing (PTGS) agent in an adRP animal model.

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Methods: tRNA-Ribozymes were cloned into a plasmid with T7 promoter to drive in vitro transcription. Full length human RHO (hRHO) including 5 UTR and part of 3 UTR was cloned into plasmids driven by T7 promoter for in vitro and CMV for expression into HEK-293S cells. In vitro cleavage reactions used tRNA polymerase for co-transcription and cleavage of hhRz and hRHO RNAs with analysis by PAGE. Real time PCR for hRHO was performed using primers spanning exon1 and 2 and an internal probe containing the fluorescent dye (FAM) at the 5' and a quenching dye (BHQ1) at the 3' end and b-actin as endogenous control and analyzed by comparative CT method (ΔACT method).

Results: We studied cleavage activity of hhRz-725 in vitro and cell culture on short and full length versions of hRHO mRNA. Since we found hhRz-725 has superior cleavage activity in the context of a smaller tRNA scaffold than prior adenoviral VAI scaffold, we tested it with no scaffold and four different constructs containing a smaller stem-II in hhRz-725 (Mini-725) but showed no enzymatic improvement. Looking at the combination of hhRz-725 and hhRz-266 and hhRz-1364 with hRHO the catalytic function showed independent cleavage activity for each hhRz. Quantification of hRHO mRNA by real time RT/PCR and protein using 1D4 antibody to hRHO in cells transfected with VAI-hhRz-725 showed ~40% knockdown both in mRNA (p=1.58856E-4) and protein level (p=0.1048E-6). Also tRNA-hhRz-725 showed ~40 % hRHO mRNA (p=0.01688) knockdown. While less specific PTG agent shRNA at the (725 GUC↓) showed more than 90% (p=2.92915E-7) RHO mRNA knockdown by real time RT/PCR.

Conclusions: We are optimizing our lead hhRz-725 in the human tRNA scaffold and showed hRHO mRNA cleavage in vitro and also knockdown of hRHO mRNA and protein in cell culture. We are now in the process of advancing the ribozyme for testing in a humanized mouse model of adRP.

Commercial Relationships: Zahra Fayazi, Mark C. Butler, None; Jack M. Sullivan, Research Foundation of SUNY (P)

Support: NIH/NEI R01EY013433 (JMS); Veterans Administration Merit Award 1101 BX0000669 (JMS); SUNY Health Now Award (JMS); Research to Prevent Blindness Unrestricted Award (Univ. Buffalo)

Program Number: 4496 Poster Board Number: B0151

Presentation Time: 11:00 AM–12:45 PM

RNA Mango - Finding a Needle in a Haystack: Tracking Rhodopsin mRNA using RNA Aptamers

Jennifer B. Breen, Jack M. Sullivan, Sriganesh Ramachandra Rao, Zahra Fayazi, Mark C. Butler, Mark C. Butler, None; Jennifer B. Breen, None; Jack M. Sullivan

Support: NIH/NEI R01EY013433 (JMS); Veterans Administration Merit Award 1101 BX0000669 (JMS) SUNY Health Now Award (JMS); Research to Prevent Blindness Unrestricted Award (Univ. Buffalo)

Purpose: Specific 3'UTR mRNA sequences (e.g. zip codes) traffic RNA out of the nucleus and into the cytoplasm for protein production, and this knowledge is critical for success of post-transcriptional gene silencing agents (e.g. ribozymes) as colocalization is essential to enzymatic efficacy. We hypothesize a newly developed RNA Aptamer “MANGO”, conjugated with a small molecule fluorescent dye “TO1-Biotin”, can label a target mRNA, track the path/distribution, and aid in biotechnological applications (e.g. SELEX) to develop more efficient hammerhead ribozymes.

Methods: Two MANGO aptamers (M and M-C4) were inserted in the 1410 nt stem/loop of a 1532 nt human rhodopsin (hRHO) cDNA and in vitro transcribed. The 1410 nt region was chosen based on RNA structure algorithm predictions of accessibility and maintenance of hRho overall structure. Equal molar concentrations of TO1-Biotin were added, and fluorescence observed on a gel-imaging box. Controls include WT hRho, buffer, and dye alone. Cells transfected with hRho M and M-C4 cDNA constructs were incubated with TO1-Biotin, Alexa647-1D4 antibody and DAPI and visualized on a confocal microscope (EX/EM 488/535nm). Fluorescence intensity was quantified using ImageJ, and analyzed by two-tailed Student’s t-test. HEK293S cells alone, cells with WT hRho serve as controls.

Results: With an intrinsic 260nm excitation, visualization of TO1-Biotin binding to in vitro transcribed hRho M, hRho M-C4 and WT hRho RNA at 100nM is seen with RFUs at 66.2, 50.2, 24.5 respectively. Using ImageJ, WT hRho mRNA (n=12) Corrected Total Cell Fluorescence (CTCF) for TO1-Biotin binding is 4605.96 (p=0.94); while hRho M-C4 (n=11) had a CTCF of 43158.36 (p=1.91E-3), which indicates specificity to the MANGO aptamer in mammalian cells. Images show DAPI (blue) stained cells containing Alexa647 (red) bound to hRho proteins, overlaid with clusters of TO1-Biotin (green), which indicates that hRho MANGO mRNA is present during and does not disrupt protein production.

Conclusions: Preliminary data suggests that MANGO aptamer may be used to track hRho mRNA produced and shuttled within the cell and cytoplasm. High-resolution information for mRNA localization requires further optimization. Spatial assessment of mRNA hRho trafficking may allow optimal hammerhead ribozyme delivery to co-localize within photoreceptors of the eye.

Commercial Relationships: Jennifer B. Breen, None; Jack M. Sullivan, None; Sriganesh Ramachandra Rao, None; Jack M. Sullivan

Support: NIH/NEI R01EY013433 (JMS)

Program Number: 4496 Poster Board Number: B0151

Presentation Time: 11:00 AM–12:45 PM

Hammerhead ribozyme in a tRNA scaffold: Effects of RNA processing and anti-sense flank length on cleavage in-vivo

Jason Myers, Zahra Fayazi, Mark C. Butler, Jack M. Sullivan

Methods: tRNA hhRz processing and mRNA were assayed by endpoint RT/PCR and real time RT/PCR. hhRho knockdown by various 725 hhRzs and varying anti-sense (AS) flank lengths was assayed by RT/PCR, Alexa-647-1D4 IHC, and a reporter from a bicistronic construct (pRHO-ires-SuperNova). hRhRz kinetic model of Stage-Zimmermann and Uhlenbeck (1998) and RNAstructure were used for modelling.

Results: tRNAs are transcribed by RNA Pol III, but addition of an upstream H1 promoter/enhancer increases tRNA levels substantially. Fractionation and RNA isolation from H1 tRNA hhRz transfected cells with a 3' CCA lead to loss of hRhR during processing, while lack of 3' CCA allows nuclear export where the tRNA hhRz reaches a cytoplasmic steady-state (SS) concentration which modulates collision frequency with hRHO mRNA. When SS is reached cleavage efficiency depends on the hhRz enzyme kinetics which are influenced.
by the length of the hhRz AS flanks. Kinetic modelling of our initial hhRz with 11 nt 5' and 7 nt 3' AS flanks predicted reaction kinetics with a lack of binding specificity (K_m in μM = 1.74E-17 M) and product inhibition (product leaving rates of 6.67E-4 min⁻¹ and 2.1 min⁻¹). In transfected cells this tRNA hhRz led to an increase in hRHO levels (mRNA, protein) compared to tRNA scaffold alone (p=0.01099). Decreasing 725 AS flanks to 7/7, 6/6 and 5/5 dropped RHO levels significantly (>50%) compared to Scaffold (7/7 p=0.02072, 6/6 p=1.42E-5, 5/5 p=1.16E-4). Our new lead candidate H1 725 6/6 -CCA had a binding affinity of 0.49 nM and leaving rates of 5 min⁻¹ and 17.8 min⁻¹ which provides specificity without product inhibition. Using Supernova as a reporter for hhRz cleavage, H1 tRNA 725 6/6 –CCA suppressed fluorescence levels by 72% compared to scaffold (p=3.25E-52).

**Conclusions:** A therapeutic 725 hhRz driven by the H1 promoter/enhancer with 6nt/6nt AS flanks suppresses levels of membrane bound hRHO protein by 50% in transient transfection experiments. This tRNA hhRz delivered by AAV to photoreceptors has potential to rescue light-induced damage of the ABCA4/RDH8 double knockout mouse (Stargardt, dAMD model) and intrinsic retinal degeneration in our humanized adRP hRHO mouse.

**Commercial Relationships:** Jason Myers; Zahra Fayazi; None; Mark C. Butler; None; Jack M. Sullivan, Research Foundation of SUNY (P)

**Support:** NIH/NEI R01EY013433 (JMS); Veterans Administration Merit Award 1101 BX000669 (JMS); SUNY Health Now Award (JMS); Research to Prevent Blindness Unrestricted Award (Univ. Buffalo)

**Program Number:** 4497 Poster Board Number: B0152

**Presentation Time:** 11:00 AM–12:45 PM

Adeno-associated virus dual vector-mediated gene therapy for ABCA4 Stargardt Disease

**Frank M. Dyka, Vince A. Chiodo, William W. Hauswirth.** Ophthalmology, University of Florida, Gainesville, FL.

**Purpose:** ATP binding cassette transporter A4 (ABCA4) is a transmembrane protein present in the RPE, photoreceptors, and retinal ganglion cells. Mutations in the ABCA4 gene cause the autosomal recessive disease Stargardt Disease. The aim of this study was to develop a gene therapy strategy to deliver the ABCA4 complementing gene to slow or halt the progression of Stargardt disease.

**Methods:** We used the Adeno-associated virus (AAV) vectors, the first generation of which cannot infect dividing or postmitotic cells. To overcome this limitation, we developed a second generation dual vector system for gene delivery. The first vector, containing the ABCA4 gene, was produced using the HDHt packaging system while the second vector was produced using the HDHt packaging system. The first vector, containing the ABCA4 gene, was produced using the HDHt packaging system while the second vector contained the H1 promoter/enhancer and 11 nt 5' and 7 nt 3' AS flanks. The first and second vectors were then co-infected into the humanized Stargardt mouse (our humanized Stargardt mouse model). The vector was injected into the vitreous cavity of the mouse eye and the expression of ABCA4 was assessed using immunohistochemistry and in vivo electroretinography.

**Results:** We observed a significant increase in ABCA4 expression in the retina of the mouse model following gene therapy. In vivo electroretinography followed by lipofuscin/A2E autofluorescence employing a confocal scanning laser ophthalmoscope.

**Conclusions:** Use of the human ABCA4 coding sequence was cloned into AAV dual vector pairs, where one vector contained the promoter and the 5' portion of the cDNA sequence and the second vector contained the 3' portion and a polyA signal. ABCA4 knock out mice were injected with AAV dual vector pairs and retinal function was measured by electrophysiology and lipofuscin/A2E autofluorescence employing a confocal scanning laser ophthalmoscope.

**Supported by:** This research was supported by the National Eye Institute, National Institutes of Health (R01EY013433, JMS) and the Macular Vision Research Foundation, Research to Prevent Blindness, Inc., and the Vision Research Foundation.
Preclinical evaluation of rAAV8.CNGA3 in the Cnga3 knockout mouse model of ACHM2

Purpose: Mutations in the cyclic nucleotide-gated channel alpha 3 subunit (CNGA3) gene are known to cause achromatopsia type 2 (ACHM2) characterized by poor visual acuity, photophobia and lack of color discrimination. Here, a novel recombinant adeno-associated virus (AAV) vector for gene supplementation therapy of CNGA3-linked ACHM2 was developed and tested for efficacy in the preclinical Cnga3 knockout (KO) mouse model of ACHM2.
Methods: An AAV-based gene supplementation vector coding for full length human CNGA3 under control of the human, cone-specific cone arrestin promoter was generated and used for packaging of AAVV-pseudotyped vectors. The resulting vector (rAAV8.CNGA3) was tested for efficacy by subretinal injections in two-week-old Cnga3 KO mice with short-term and long-term analysis at 8 weeks and 12 months post-injection, respectively. AAV vector-mediated expression of human CNGA3 protein was assessed by immunohistochemistry using a CNGA3-specific antibody combined with confocal microscopy. Efficacy was assessed in vivo by electroretinography (ERG) with protocols designed to isolate cone-specific light responses.
Results: We designed and generated rAAV8.CNGA3, a novel AAV vector optimized for efficient CNGA3 gene expression in human cone photoreceptors. Transgene expression assay of rAAV8.CNGA3 in Cnga3 KO mice confirmed efficient and specific human CNGA3 protein expression that localized to cone photoreceptor outer segments. A biological activity assay for rAAV8.CNGA3 based on ERG analysis confirmed a beneficial effect of the treatment at both observation time points. Additionally, we found prolonged survival of cone photoreceptors up to 12 months after treatment.
Conclusions: The novel rAAV8.CNGA3 vector supports efficient and specific transgene expression and biological activity in the preclinical Cnga3 KO mouse model of ACHM2.
Commercial Relationships: Stylianos Michalakis, Christian Schön, None; Regine Mühlfriedel, None; Vithiyanjali Sothilingam, None; Bernd Wissinger, None; Mathias W. Seeliger, YEESERV (P); Martin Biel, YEESERV (P)
Support: Tistou and Charlotte Kerstan Foundation
Program Number: 4499 Poster Board Number: B0154
Presentation Time: 11:00 AM–12:45 PM
Preclinical evaluation of rAAV8.PDE6A in the Pde6aKO mutant mouse model of RP43

Purpose: Mutations in the phosphodiesterase 6 alpha (PDE6A) gene are known to cause retinitis pigmentosa type 43 (RP43) – a currently cureless blinding disease. Here, a novel recombinant adeno-associated virus (AAV) vector for gene supplementation therapy of PDE6A-linked RP was developed and tested for efficacy in the preclinical Pde6aKO mouse model of RP43.
Methods: An AAV-based gene supplementation vector coding for full length human PDE6A under control of the human, rod-specific rhodopsin promoter was generated and used for packaging of AAVV-pseudotyped vectors. The resulting vector (rAAV8.PDE6A) was tested for efficacy by subretinal injections in two-week-old Pde6aKO mutant mice with short-term and long-term analysis at 4 weeks and 6 months post-injection, respectively. AAV vector-mediated expression of human PDE6A protein was assessed by immunohistochemistry using a PDE6A-specific antibody combined with confocal microscopy. Efficacy was assessed by measuring morphological preservation in vivo using optical coherence tomography (OCT) and ex vivo by analyzing histological cross-section of treated and untreated retinas.
Results: We designed and generated rAAV8.PDE6A, a novel AAV vector optimized for efficient PDE6A gene expression in human rod photoreceptors. Transgene expression assay of rAAV8.PDE6A confirmed efficient and specific human PDE6A protein expression that localized to rod photoreceptor outer segments. A biological activity assay for rAAV8.PDE6A based on analysis of morphological preservation confirmed a beneficial effect of the treatment at both observation time points. Additionally, we found a preservation of rod outer segment structure and prolonged survival of cone photoreceptors up to 6 months after treatment.
Conclusions: The novel rAAV8.PDE6A vector supports efficient and specific transgene expression and biological activity in the preclinical Pde6aKO mouse model of RP43.
Commercial Relationships: Christian Schön, EyeServ (P); Regine Mühlfriedel, None; Vithiyanjali Sothilingam, None; Francois Paquet-Durand, None; Bernd Wissinger, None; Mathias W. Seeliger, EyeServ (P); Martin Biel, EyeServ (P); Stylianos Michalakis, EyeServ (P)
Support: Tistou and Charlotte Kerstan Foundation
Program Number: 4500 Poster Board Number: B0155
Presentation Time: 11:00 AM–12:45 PM
Preclinical evaluation of rAAV8.PDE6A in the Pde6aKO mouse model of RP43

Purpose: Mutations in the cyclic nucleotide-gated channel alpha 3 subunit (CNGA3) gene are known to cause achromatopsia type 2 (ACHM2) characterized by poor visual acuity, photophobia and lack of color discrimination. Here, a novel recombinant adeno-associated virus (AAV) vector for gene supplementation therapy of CNGA3-linked ACHM2 was developed and tested for efficacy in the preclinical Cnga3 knockout (KO) mouse model of ACHM2.
Methods: An AAV-based gene supplementation vector coding for full length human CNGA3 under control of the human, cone-specific cone arrestin promoter was generated and used for packaging of AAVV-pseudotyped vectors. The resulting vector (rAAV8.CNGA3) was tested for efficacy by subretinal injections in two-week-old Cnga3 KO mice with short-term and long-term analysis at 8 weeks and 12 months post-injection, respectively. AAV vector-mediated expression of human CNGA3 protein was assessed by immunohistochemistry using a CNGA3-specific antibody combined with confocal microscopy. Efficacy was assessed in vivo by electroretinography (ERG) with protocols designed to isolate cone-specific light responses.
Results: We designed and generated rAAV8.CNGA3, a novel AAV vector optimized for efficient CNGA3 gene expression in human cone photoreceptors. Transgene expression assay of rAAV8.CNGA3 in Cnga3 KO mice confirmed efficient and specific human CNGA3 protein expression that localized to cone photoreceptor outer segments. A biological activity assay for rAAV8.CNGA3 based on ERG analysis confirmed a beneficial effect of the treatment at both observation time points. Additionally, we found prolonged survival of cone photoreceptors up to 12 months after treatment.
Conclusions: The novel rAAV8.CNGA3 vector supports efficient and specific transgene expression and biological activity in the preclinical Cnga3 knockout mouse model of ACHM2.
Commercial Relationships: Stylianos Michalakis, Christian Schön, None; Regine Mühlfriedel, None; Vithiyanjali Sothilingam, None; Bernd Wissinger, None; Mathias W. Seeliger, YEESERV (P); Martin Biel, YEESERV (P)
Support: Tistou and Charlotte Kerstan Foundation
Program Number: 4501 Poster Board Number: B0156
Presentation Time: 11:00 AM–12:45 PM
In-frame exon skipping fails to rescue retinal or hair cell defects in zebrafish models of PCDH15 (USH1F)
Jennifer B. Phillips, Jeremy Wegner, Monte Westerfield. Inst of Neuroscience, University of Oregon, Eugene, OR.

Purpose: The R245X mutation in PCDH15 R245X mutation causes a severe form of Usher Syndrome. The affected codon lies in an in-frame exon that encodes part of two consecutive E-cadherin domains in the extracellular region of PCDH15. This region is proximal to a protein-protein interaction domain required in stereocilia, but of unknown function in retinal tissue. To evaluate in-frame exon-skiing as a therapy for PCHD15(R245X) patients, we deleted
Exon 8 from zebrafish *pcdh15* genes and assayed visual and auditory function.

**Methods:** CRISPRs with zebrafish-optimized Cas9 were injected into zygotes to generate germline mutations. Sequence analysis of gDNA and cDNA confirmed the deletions and revealed production of transcripts lacking exon 8. Behavioral and histological analyses were performed to assay hearing, balance, cellular morphology and cell death. Images were captured with an iOS camera or Zeiss LSM5 Confocal microscope.

**Results:** We analyzed behavior, cell morphology and photoreceptor cell death in zebrafish *pcdh15aΔE8* and *pcdh15bΔE8* lines, each containing in-frame deletions of Exon 8. We also examined truncating mutations in exon 8 of both zebrafish *PCDH15* orthologs (*pcdh15aΔEX* and *pcdh15bΔEX*, respectively) showing partially overlapping functions with a greater requirement for *pcdh15a* in inner ear and for *pcdh15b* in retina. We used these lines as positive controls for experiments with the in-frame exon deletions, *pcdh15aΔE8* and *pcdh15bΔE8* animals recapitulated the swimming and balance phenotypes seen in the E8 truncating mutants for each gene, with no improvements or differences noted in mechanosensory hair cell structure or function. Mild photoreceptor cell morphology defects and low levels of photoreceptor death that was not exacerbated by exposure to increased illumination observed in *pcdh15aΔE8* larvae were recapitulated in *pcdh15bΔE8* larvae. Severe PRC morphology defects and marked PRC death that increased with elevated light intensity was the same in *pcdh15bΔE8* and *pcdh15bΔE8* larvae.

**Conclusions:** We conclude that disruption of EC1 and EC2 domains is incompatible with normal PCDH15 protein function in auditory and visual sensory cells, even when more C-terminal portions of the protein remain intact. Our rapid and inexpensive evaluation of this therapeutic approach in zebrafish disease models informs future approaches for alternative allele-specific treatments of the R245X mutation.

**Commercial Relationships:** Jennifer B. Phillips; Jeremy Wegner, None; Monte Westerfield, None

**Support:** NIH P01HD22486, Vision for a Cure and the Usher 1F Collaborative

**Program Number:** 4502 Poster Board Number: B0157

**Presentation Time:** 11:00 AM–12:45 PM

**Intracellular expression of mini-chaperone confers resistance to apoptosis from oxidative and thermal stress in cos7 cells**

*Pottur Santhoshkumar, Krishna Sharma.* Ophthalmology, University of Missouri-Columbia, Columbia, MO.

**Purpose:** Therapeutic potentials of peptide chaperone derived from α-crystallin is well established. The objective of this study is to prepare a mini-gene construct capable of expressing functional peptide chaperone(s) in mammalian cells.

**Methods:** A stable cell line expressing a mini-chaperone (MGKFFIFLDVKHFSPEDLTVK) was prepared by transfecting Cos7 cells with linearized pCDNA3.1(+) vector containing the engineered mini-gene inserted between EcoRI and XbaI sites and grown on Geneticin selective medium including 5% serum and maintained at 37°C and 5% CO₂. Cos7 cells transfected with linearized empty vector served as control. The anti-apoptotic activity of the intracellularly expressed mini-chaperone was tested by treating the cells grown on a 96-well plate with 75, 150 and 300 μM H₂O₂ for 16 h followed by TUNEL assay. The anti-apoptotic activity of the mini-chaperone against hyperthermia was investigated by incubating the culture plates at 55°C for 1 h and the live/dead cell in the two groups were visualized by staining the cells with HCS NuclearMask™ Red stain and Image-IT® DEAD Green™ Viability Stain at 37°C for 30 min. The plates were imaged using the SpectraMax Minimax 300 Imaging Cytometer (Molecular Devices) and the live/dead cells were quantified using Softmax Pro software.

**Results:** Cell cultures transfected with mini-gene showed 55 – 80% less TUNEL positive cells when compared to control groups. Cells transfected with empty vector (positive control) failed to provide any protection against H₂O₂ and was comparable to untransfected cells (negative control). In the Live/Dead assay, cells transfected with empty vector showed increased number of dead cells (8.6 + 2.3%) compared to cells transfected with mini-gene (2.2 + 1.2%). Our data suggests that the intracellular expression of mini-chaperone protects Cos7 cells from oxidative and thermal stress.

**Conclusions:** Functional mini-chaperones can be expressed in cells. Mini-gene therapy can be a possible strategy for treating diseases involving oxidative stress, protein aggregation, and apoptosis.

**Commercial Relationships:** Pottur Santhoshkumar, None; Krishna Sharma, None

**Support:** NIH Grant EY 023219

**Program Number:** 4503 Poster Board Number: B0158

**Presentation Time:** 11:00 AM–12:45 PM

**Overexpression of human Nrf2 rescues cone photoreceptors and retinal pigment epithelial cells from degeneration in the rd1 mouse**

*David M. Wu, Xike J. Ji, Brian P. Hafler, Yantu Xue, Sophia Zhao, Parimal Rana, Wenjun Xiong, Connie Cepko.* 1Retina Service, Massachusetts Eye and Ear Infirmary, Boston, MA; 2Genetics, Harvard Medical School, Boston, MA; 3Biomedical Sciences, City University of Hong Kong, Kowloon, Hong Kong.

**Purpose:** Overexpression of mouse Nrf2 in the rd1 mouse, a model of retinitis pigmentosa, results in rescue of cones and retinal pigment epithelial cells. The next step towards evaluating the potential for Nrf2 for human disease therapy is to assess its ability to rescue retinal degeneration in larger animals. In planning such studies, it would be useful to know whether the product of the human Nrf2 gene is able to rescue across species, since this is the gene product of most interest in the development of human therapies.

**Methods:** An AAV 2/8 was constructed using a CMV promoter and human-beta globin intron to drive expression of the human Nrf2 gene (AAV 2/8 CMV human Nrf2). A construct with the same promoter complex encoding GFP strongly drives GFP expression in cone photoreceptors and retinal pigment epithelial cells in mouse retina (AAV 2/8 CMV GFP). The AAV 2/8 driving the human Nrf2 gene was delivered to the subretinal space of rd1 mouse eyes at p0. For controls, some eyes were left uninjected and other eyes were injected with AAV 2/8 with the same promoter complex driving GFP or mouse Nrf2 gene (AAV 2/8 CMV mouse Nrf2). The mice were sacrificed at P40 or P70. Cones were identified by immunostaining for cone arrestin and retinal pigment epithelial cells were identified by phallolidin.

**Results:** All rd1 eyes at P40 and P70 showed evidence of retinal degeneration, with cone and retinal pigment epithelial cell loss higher in the central eye compared to the peripheral eye. However, there was significantly less loss in eyes injected with AAV 2/8 CMV human Nrf2 or AAV 2/8 mouse Nrf2 than in those that had been uninjected, or injected with AAV 2/8 CMV GFP. There was not a marked difference in eyes receiving AAV 2/8 CMV human Nrf2 compared to AAV 2/8 CMV mouse Nrf2.

**Conclusions:** Overexpression of human Nrf2 in the degenerating eyes of rd1 mice rescues cone photoreceptors and retinal pigment epithelial cells. There is significant homology between the coding sequence and protein sequence for human and mouse Nrf2 (83 and 82% respectively). Given that sequence homology is similar or greater in Nrf2 genes from large animals (89% in dogs and 99% in...
Characterization of hypoxia–regulated, glial cell specific AAV vector for targeting retinal neovascularization


**Purpose:** The Muller cell is known to secrete angiogenic factors under conditions of hypoxia exposure similar to the mouse oxygen induced retinopathy (OIR) model or in diabetic retinopathy. Previously, we constructed adeno-associated virus vector (AAV) gene therapy vectors whose transgene expression is driven by a hypoxia responsive domain combined with the glial fibrillary associated protein (GFAP) promoter. Here we developed and tested a hypoxia regulated and glial cell specific vector encoding human decorin which functions as an antiangiogenic protein.

**Methods:** Plasmids for AAV generation were constructed with the human decorin cDNA together with the CMV promoter enabling generation of the virus AAV-CMV-Decorin or with the GFAP promoter giving virus AAV-GFAP-Decorin or with the GFAP promoter PLUS a hypoxia responsive domain (HRE) giving AAV-HRE-GFAP-Decorin. AAVs were then packaged using these plasmids for subsequent infection of primary rat brain astrocytes, PC12 neuronal cells or retinal epithelial ARPE-19 cells. After infection cells were subjected to 72 h of hypoxia. Levels of mRNA for human decorin were measured by quantitative real time RT-PCR.

**Results:** Following infection of primary astrocytes with AAV-HRE-GFAP and hypoxia treatment there was a significant induction in decorin mRNA of greater than 5 fold relative to normoxic cells. Infection with AAV-GFAP-Decorin resulted in high level expression of decorin mRNA either in the absence or presence of hypoxia. Neither AAV-GFAP-Decorin nor regulated AAV-HRE-GFAP-Decorin infected into ARPE-19 cells or PC12 cells resulted in significant decorin mRNA expression with or without hypoxia. By contrast AAV-CMV-Decorin infection resulted in high level expression of decorin mRNA in ARPE19 cells and in PC12 cells with or without hypoxia.

**Conclusions:** Our data indicates that AAV-HRE-GFAP-Decorin drives hypoxia inducible, as well as glial cell specific expression, of decorin mRNA. AAV-GFAP-Decorin showed glial cell specific expression that was not further modified by hypoxia exposure. However, AAV-CMV-Decorin was ubiquitously expressing decorin mRNA in all cell types tested in both conditions of hypoxia and normoxia. These studies contribute to comparing the relative efficacy of hypoxia responsive and glial cell specific AAV-HRE-GFAP-decorin to glial cell specific vector AAV-GFAP-decorin for antiangiogenic treatment in diabetic retinopathy.

**Commercial Relationships:** Janet C. Blanks, None; Howard Prentice, None; James Sullivan, None

**Support:** NIH NEI EY023993-03, Massachusetts Lion Eye Research Fund, Inc., HHMI

Program Number: 4504 Poster Board Number: B0159
Presentation Time: 11:00 AM–12:45 PM

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AAV-mediated delivery of ABCA4 to the photoreceptors of Abca4-/- mice using an overlapping dual vector strategy

Michelle E. McClements1, Alun R. Barnard2, Mandeep S. Singh3, Zhichun Jiang4, Peter Charbel Issa5, Roxana A. Radu6, Robert E. MacLaren7, 8. Nuffield Department of Clinical Neurosciences (Ophthalmology), University of Oxford, Oxford, United Kingdom; 2(John Hopkins University, Baltimore, MD; 3Ophthalmology, Stein Eye Institute, David Geffen School of Medicine, California, CA; 4Oxford Eye Hospital, Oxford, United Kingdom.

**Purpose:** Gene therapy development for disorders caused by mutations in large genes is an advancing field. A dual vector approach for delivering the large gene split in two parts and carried in two separate adeno-associated viral (AAV) vectors is feasible, but given the likely reduction in transduction efficiency associated with this strategy, achieving therapeutic levels of protein in target cells has to date remained challenging. Here we reveal that a customized overlapping dual vector strategy can successfully deliver robust levels of ABCA4 protein to the photoreceptor outer segments of the Abca4-/- mouse as assessed by immunohistochemistry.

**Methods:** Overlapping transgenes were packaged in AAV8 Y733F capsids. The upstream transgene contained the human rhodopsin kinase (GRK1) promoter and an upstream portion of the ABC4 CDS. The downstream transgene contained a downstream portion of the ABCA4 CDS, Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and polA signal (pA). Both the upstream and downstream transgenes carried a region of ABCA4 CDS overlap. Abca4-/- mice received a 2 μl sub retinal injection at 4-5 weeks of age containing a 1:1 mix of upstream and downstream vectors (1 x 10e13 gc/ml). Eyes were harvested 6 weeks post-injection for immunohistochemical (IHC) assessments. Eyes were fixed, frozen and sectioned prior to IHC staining. Staining for Hcn1 was used as a photoreceptor inner segment marker.

**Results:** Uninjected Abca4-/- and injected controls did not stain for Abca4/ABCA4 in the photoreceptor outer segments. However, dual vector treated eyes did show robust ABCA4 staining in the photoreceptor outer segments. No off-target ABCA4 expression was observed in other cell types.

**Conclusions:** An overlapping dual vector approach using the AAV8 Y733F serotype generates readily detectable levels of therapeutic protein in the photoreceptor outer segment structures.

**Commercial Relationships:** Michelle E. McClements, University of Oxford (P); Alun R. Barnard, None; Mandeep S. Singh, None; Zhichun Jiang, None; Peter Charbel Issa, None; Roxana A. Radu, None; Robert E. MacLaren, University of Oxford (P)

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Novel AAV variants isolated by directed evolution in primate display enhanced retinal transduction following intravitreal injection

Shreyasi Choudhury1, Damien Marseic, James Peterson1, Diego Fajardo1, Antonette Bennett1, Paul D. Gamlin1, Mavis Aghbandie-Mckenna1, Sergei Zolotukhin, Sanford L. Boye1, Shannon E. Boye1. 1Department of Ophthalmology, University of Florida, Gainesville, FL; 2Department of Pediatrics, University of Florida, Gainesville, FL; 3Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL; 4Department of Ophthalmology, University of Alabama at Birmingham, Birmingham, AL.

Purpose: Directed evolution of AAV capsid libraries in mice has identified variants with enhanced transduction of mouse retina following intravitreal (Ivt) injection. In primate retina, improvements were less substantial. We developed a method to generate sortatable retinal cells in primate which enabled screening of a highly complex AAV2-based capsid library to identify AAV variants capable of efficient retinal transduction following Ivt injection. In parallel, the same library was screened in mice. After 3 rounds of selection in mice and 2 in macaque, a subset of the most prevalent variants was characterized. The purpose of this study was to evaluate their transduction profiles following Ivt injection in mice and to measure physicochemical parameters previously shown to correlate with increased transduction efficiency by this injection route.

Methods: Capsid variants contained a self-complementary AAV genome carrying the truncated CBA promoter driving mCherry (sc-smCBA-mCherry). Transduction was quantified in vitro using ocular cell lines. Vectors were Ivt injected into Nrl-GFP mice and transduction was evaluated 4 weeks later by funduscopy and FACS. AAV2wt and AAV2quadY-F+F-T-V were included for comparison. Measurements of capsid stability, as determined by differential scanning fluorimetry (DSF), and affinity for heparan sulfate by heparin column chromatography are underway.

Results: After 3 rounds of screening in mice, two heavily enriched variants, M3-A (32% relative frequency) and M3-B (21%) were chosen for analysis. After two rounds of selection in macaque, the 4 most prevalent variants P2-V1, P2-V2, P2-V3 and P2-V4 were analyzed. Interestingly, P2-V1 is identical to M3-B and P2-V4 is identical to M3-A. All variants displayed substantially improved transduction in vitro compared to AAV2wt. When compared to our most efficient rationally designed capsid, AAV2quadY-F+F-T-V, P2-V1, P2-V2 and P2-V3 are all improved, with P2-V3 displaying a >2-fold increase in the number of retinal cells transduced following Ivt injection.

Conclusions: AAV capsids identified by screening in primate and mouse retina show enhanced transduction efficiencies both in vitro and following Ivt injection. An additional round of screening in both primate and mouse has been completed and results are pending.

Ivt-mediated transduction of enhanced AAVs.

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Gene augmentation therapy improves inner and outer segment morphology and preserves retinal structure in a large animal model of CNGB1-retinitis pigmentosa

Laurence M. Occelli1, Paige A. Winkler1, Vince A. Chiodo2, Sanford L. Boye3, William W. Hauswirth4, Simon M. Petersen-Jones5. 1Small Animal Clinical Sciences, Michigan State University, East Lansing, MI; 2University of Florida, Gainesville, FL.

Purpose: Mutations in cyclic nucleotide gated channel beta 1 (CNGB1) cause autosomal recessive RP type 45 (RP45). Cngb1-/- dogs have marked loss of rod function and a slowly progressive photoreceptor degeneration recapitulating RP45. We showed at ARVO 2016 that gene augmentation therapy in Cngb1-/- dogs rescues rod function. Treated dogs had a dramatic restoration of rod electroretinogram (ERG) and improved vision in dim light. The purpose of this study was to investigate long-term effects of gene therapy in Cngb1-/- dogs.

Methods: Seven eyes of 5 Cngb1-/- dogs received a subretinal injection of 1x10^5 vg of AAV2/5-hGRK1-c-Cngb1. Dark- and light-adapted electroretinograms (ERGs), vision testing, fundus imaging and structural preservation assessment by spectral-domain optical coherence tomography (SD-OCT) imaging was performed regularly post-injection. Immunohistochemistry (IHC) was performed on eyes collected 3, 6, 9 and 23 months post-injection.

Results: Gene augmentation rescued rod function (as measured by ERG and vision testing) up to 18 months post-injection (latest time point assessed). SD-OCT showed that there was improved definition of the zones representing the external limiting membrane to the interdigitation zone which was maintained over time. Following an initial decline in Receptor plus (REC+) thickness after subretinal injection further decline halted and thickness was preserved in the longer term (up to 23 months in two eyes). IHC confirmed photoreceptor preservation but only in the treated regions.

Conclusions: Rescue of rod function by gene augmentation therapy in Cngb1-/- dogs was maintained long term. There was also structural preservation with improved morphology of photoreceptor inner/outer segments. The initial continued decline in REC+ thickness following treatment may represent loss of rods prior to full expression of the transgene coupled with loss of any rods that had inadequate transgene expression or were already irreversibly committed to cell death. This study shows successful long-term gene therapy in a large animal model of RP45 making this an attractive disease for translational therapy.

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Subretinal delivery of RGX-314 AA V8-anti-VEGF Fab gene therapy in NHP
Anna Tretiakova1, Tomas S. Aleman3, Arkady Lyubarsky3, Elaine J. Zhou4, Erik Wielechowski3, Gui-Shuang Ying2, Erin Bote1, Leah Makaron1, Stephen Yoo1, Jean Bennett4, Albert M. Maguire3, James Wilson1.
1Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA; 2Center for Preventative Ophthalmology and Biostatistics, University of Pennsylvania, Philadelphia, PA; 3Center for Advanced Retinal and Ocular Therapeutics, Scheie Eye Institute, University of Pennsylvania, Philadelphia, PA; 4Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; 5REGENXBIO, Rockville, MD; 6Center for Cellular and Molecular Therapeutics, The Children’s Hospital of Philadelphia, Philadelphia, PA.

**Purpose:** To evaluate safety and define an upper dose limit of an anti-VEGF Fab-containing AA V8 gene therapy vector for wet age-related macular degeneration in non-human primates (NHP) following subretinal delivery.

**Methods:** 20 NHPs were used in the study. Animals received a single subretinal injection of 1e10 (low), 1e12 (high) AA V8 GC/eye or a control. Production of anti-VEGF Fab was assessed by VEGF ELISA of anterior chamber (AC) fluid. A separate NHP study assessed a 1e11 (middle) GC/eye dose. Retinal structure was evaluated with spectral domain optical coherence tomography (SD-OCT) imaging at 3 months. Retinal function was evaluated using full-field electroretinography (ERG). Retinal distribution of anti-VEGF Fab mRNA and protein was evaluated.

**Results:** At 30 days post injection, expression in control NHPs (n=4) was not detectable. Median expression in low dose NHPs (n=6) was 889 ng/ml (ranging 679 - 2116 ng/ml) and in high dose NHPs (n=6) 2452 ng/ml (ranging 966 - 3500 ng/ml). The middle 1e11 dose (n=8, separate study) showed median expression of 1505 ng/ml (ranging 431 - 2346 ng/ml). Anti-VEGF Fab mRNA and protein were distributed widely throughout the retina well beyond the injection site. Retinal demelanization was present in all injection sites, with no signs of intraocular inflammation. Low dose NHPs showed no significant difference in retinal thickness relative to vehicle controls. There was 10% decrease in total retinal thickness at injection site in high dose NHPs. 1e11 dosed NHP’s SD-OCTs are under evaluation. At 10 months, 4 eyes from 2 NHPs in 1e11 group were normal histologically; minimal perivascular infiltrates were present in one optic nerve. Histopathologic changes at 3 months in the low dose eyes included minimal inflammation in the choroid and in the high dose eyes included inflammation in the retina, choroid, and sclera. No significant changes in thicknesses were observed in the control animals. Control or low dose NHPs did not show measurable changes in retinal function. High dose resulted in a statistically significant (p<0.05) decline in the amplitude of ERG as compared to baseline, formulation control, and low dose.

**Conclusions:** The study demonstrated presence of anti-VEGF Fab in AC at 30 days in all RGX-314 doses and the feasibility of using OCT and ERG as structural and functional measures and to define an upper dose limit in NHP dose escalation studies.

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