

ADENO-ASSOCIATED VIRUS 8-MEDIATED GENE THERAPY FOR CHOROIDEREMIA: PRECLINICAL STUDIES IN *IN VITRO* AND *IN VIVO* MODELS

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ABSTRACT

ADENO-ASSOCIATED VIRUS 8-MEDIATED GENE THERAPY FOR CHOROIDEREMIA: PRECLINICAL STUDIES IN *IN VITRO* AND *IN* *VIVO* MODELS

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Choroideremia (CHM) is a slowly progressive X-linked retinal degeneration that results ultimately in total blindness due to loss of photoreceptors, retinal pigment epithelium, and choroid. *CHM*, the gene implicated in choroideremia, encodes Rab escort protein-1 (REP-1), which is involved in the post-translational activation via prenylation of Rab proteins. We evaluated AAV8.CBA.hCHM, a human *CHM* encoding recombinant adeno-associated virus serotype 8 (rAAV8) vector, which targets retinal cells efficiently, for therapeutic effect and safety *in vitro* and *in vivo* in a murine model of CHM. *In vitro* studies assayed the ability of the vector to produce functional REP-1 protein in established cell lines and in CHM patient derived primary fibroblasts. Assays included Western blots, immunofluorescent labeling, and a REP-1 functional assay which measured the ability of exogenous REP-1 to prenylate Rab proteins. The *in vivo* work used unilateral subretinal delivery of AAV8.hCHM to treat a murine model of CHM, with the effects of treatment evaluated with pupillometry,

ophthalmoscopy, histology, and immunofluorescence analysis. The contralateral eye was treated with an AAV8.EGFP control. The results of the *in vitro* analysis demonstrated that the AAV8.CBA.hCHM vector was capable of inducing robust expression of REP-1 protein in a dose-responsive fashion in cultured cells, with the prenylation assay indicating that the exogenous REP-1 protein produced was capable of assisting in the activation of Rab proteins. This functional assay was also applied to CHM patient fibroblasts transduced with an AAV serotype 2 version of the vector and demonstrated that exogenous REP-1 produced in these cells was functional. The *in vivo* subretinal treatment of CHM mice with AAV8.CBA.hCHM resulted in the improvement of pupillary response in the treated eyes of some animals, as well as slowing of progression as judged by ophthalmoscopy. Histology of treated animals showed properly localized expression of human REP-1 and a significant improvement in outer retina health as determined by an increase in the thickness of the outer nuclear layer in treated eyes compared to controls. Combined, these results indicate that transduction with AAV8.CBA.hCHM reduces the biochemical and pathogenic defects in CHM both *in vitro* and *in vivo* and supports the application of AAV8 vectors in CHM gene therapy.

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GENERAL INTRODUCTION

CHOROIDEREMIA

Disease Pathology

Choroideremia (CHM) is a form of X-linked retinal disease which is characterized by the degeneration of the retinal pigment epithelium (RPE), photoreceptors, and choriocapillaris. In affected males, impaired night vision (nyctalopia) is apparent early in childhood and progressive loss of peripheral vision is also noted by the second decade of life. As the disease progresses, the visual field steadily constricts as retinal cells gradually die, and this eventually impinges on the central macula, the region of fine visual discrimination and color sensation. The disease culminates in complete blindness as early as the fourth decade of life. Initial diagnosis of new cases is remarkably simple, with fundoscopic imaging displaying a unique pattern of degeneration of peripheral RPE and retina, allowing the direct observation of the normally hidden choroid (1-3). Confirmation is made by molecular diagnosis. Female carriers of the disease are typically asymptomatic, however, the fundus examination often reveals a patchy degeneration of the retina and RPE, likely due to lyonization (4).

The *CHM* Gene

Choroideremia is caused by mutations in the *CHM* gene and results in the lack of expression of its protein product, Rab escort protein-1 (REP-1). The *CHM* gene is located at the Xq21.2 locus on the X chromosome and is composed of 15 exons spanning an approximately 186Kb genomic region (5). The *CHM* mRNA is ~5.5Kb and encodes a 653 amino acid protein, REP-1.

REP-1 Protein

A ubiquitously expressed protein, REP-1 plays an important role in cellular endocytosis and vesicular transport due to its involvement in the post translational modification of the Rab small GTPases (1, 5-8). Rab GTPases are members of the Ras superfamily and, when integrated with membranes, serve as controllers of tethering, docking, and fusion (9-10). In humans, more than 60 Rabs have been so far identified (11). Before newly produced Rabs can integrate with membranes, they must be post-translationally modified through the addition of prenyl groups to 1-2 cysteines located near the C-terminus of the Rab protein. In order for this modification to occur, REP-1 must associate with the Rab GTPase and present it to Rab Geranylgeranyltransferase (Rab GGTase) for prenylation. Once prenylated, the Rab protein is escorted by REP-1 to its target membrane, REP-1 then disassociates and returns to the cytosol (1, 7-11). In

cells lacking REP-1, Rab GTPases are hypoprenylated, it is this shortage of functional Rabs which is thought to cause the CHM phenotype (9, 12-13).

Molecular Basis of Disease

Historically, there has been an ongoing debate as to the molecular causes of choroideremia pathology as well as the tissue types that are directly affected by the lack of REP-1 protein. Because Rab proteins are vital for proper cellular function throughout the body, it is immediately remarkable that individuals with *CHM* mutations do not display systemic disease, instead only showing a disease phenotype in their ocular tissues. It is thought that normal cellular function in the non-ocular tissues of CHM affected individuals is maintained thanks to the presence of Rab escort protein-2 (REP-2), a protein with many sequence similarities to REP-1. REP-2 is encoded by the autosomal *CHM*-like (*CHML*) gene, which bears a 71% homology to *CHM*. Like REP-1, REP-2 is expressed ubiquitously (14-15) and functions very similarly to REP-1, assisting in the prenylation and transport of Rab proteins. It is thought to substitute for REP-1 in most of the tissues of CHM patients (13-14). However, REP-2 does not assist in the prenylation of all Rabs to the same extent as REP-1, with certain Rabs, such as Rab27a, being prenylated much less efficiently when associated with REP-2 (12-14). It is believed that some of the Rab proteins which are under-prenylated in ocular tissues are more vital for proper cellular function than in the rest of the body, resulting in cellular dysfunction and eventual death.

Precisely how a shortage of certain prenylated Rab proteins causes choroideremia has been hard for researchers to pin down. While tissue degeneration is observed in the choriocapilaris, RPE, and photoreceptors, these tissues are highly interdependent, and the loss of function of one can quickly lead to the collateral degeneration of the neighboring tissues (16). Therefore, it has been difficult to ascertain which cell types are degenerating in an autonomous manner, and which are degenerating due to the loss of neighboring tissues. Examining the retinas of female carriers of choroideremia has proven instructive in elucidating this problem, due to the lyonization of REP-1. In the female carrier, the wildtype version of REP-1 is expressed in some cells and the mutant version in others, resulting in a patchy appearance in the retina (17). This expression does not necessarily overlap for the RPE and photoreceptors, allowing for regions where the photoreceptors express REP-1, but, the underlying RPE does not, or vice versa (4). This situation allows us to examine the effects of REP-1 loss in one tissue type, without the confounding effects of it also being lacking in the neighboring tissues. Post-mortem examinations of female carrier retinas have shown that the photoreceptor and RPE layers appear to degenerate independently, even when the neighboring layers still appear healthy (4, 18). Work in a conditional knockout animal model of the disease, wherein REP-1 expression is eliminated in either the RPE or photoreceptors, supports these findings, demonstrating degeneration in both tissues regardless of the REP-1 expression in the neighboring tissue (16, 19).

The molecular causes of degeneration in the RPE and photoreceptors of choroideremia patients have yet to be fully understood, and likely are not due to the same dysfunction in each cell type. Experiments in RPE cells with REP-1 siRNA silencing have demonstrated slowed processing of phagocytosed photoreceptor outer segments due to defects in phagosome-lysosome fusion (20). It has also been observed that RPE melanosomes are not properly trafficked to the apical processes in REP-1 lacking cells in both a murine model of the disease and female carriers (4, 18, 21). In the photoreceptors, lack of REP-1 has been shown to result in inefficient opsin transport to the outer segments, a function essential to proper photoreceptor function (18). While these observed cellular dysfunctions may not all be culpable in cell death and vision loss, they do demonstrate that the delicate balance of intracellular transport is perturbed in both cell types, and help support the concept of cell autonomous degeneration in choroideremia.

Choroideremia Murine Model

No naturally occurring animal models of choroideremia have so far been identified, and the engineering of disease models has been hampered by differences in REP-1 necessity between humans and some species widely used for producing animal models (22-24). Attempts to produce murine *CHM* KOs proved embryonic lethal for both KO males and heterozygous females born from carrier mothers (22). The fact that heterozygous females who received the mutant gene from their mothers also were nonviable suggested that the lethality

may be due to impairment of extraembryonic tissue, such as the placenta, which is known to undergo paternal X chromosome silencing (22). Further research supported this hypothesis, with defective vascularization of the extra-embryonic tissue observed for affected embryos (24). *CHM* KO also proved embryonic lethal in zebrafish (23).

As of this time, the only vertebrate model for choroideremia is a conditional mouse knockout of *Rep1*, where female mice are heterozygous for *REP-1* KO, having inherited the mutant gene paternally (16, 22). Produced by the lab of Dr. Miguel C. Seabra, I will from here on refer to this strain as *Chm*^{WT/null}. The *Chm*^{WT/null} mouse model was generated by first creating a mouse strain with loxP sites flanking exon 4 of the *Chm* gene. This strain was then mated to mice who express Cre as a fusion protein with the tamoxifen-responsive estrogen receptor (MerCreMer). Resulting male mice who received both the floxed *Chm* gene and the MerCreMer gene undergo recombination of the loxP sites when treated with tamoxifen, this results in the excision of exon 4 of the *Chm* gene and the introduction of an early stop codon. By first treating these males with tamoxifen, and then mating them to wild-type females, female pups who are heterozygous for the *Chm* KO can be produced. Due to the embryonic lethal nature of *Chm* KO in mice, no male pups carrying the KO gene can be produced from this crossing. The heterozygous females produced in this manner exhibit a degeneration of the retina similar to but much more severe than the degeneration observed in human female CHM carriers, with patchy hypopigmentation,

progressive loss of RPE and photoreceptors, and reduced retinal/visual function in affected eyes (16).

GENE THERAPY

Gene Therapy Methods Targeting the Genetic Basis of a Specific Disease

Gene therapy directed at the molecular cause of a particular disease is approached by the editing of the mutations or the addition of a non-mutated copy of the disease-associated gene, methods respectively referred to as gene correction and gene augmentation. In gene correction, the general strategy is to induce a double stranded break (DSB) in the genome near the site of the deleterious mutation. This DSB initiates the homologous repair machinery for the genome and, if homologous sequences are available, the homologous repair machinery uses these as a template to replace the section where the double stranded break occurred. In normal cellular function, the most common template for homologous repair is the matching sequence in the sister chromatid, which results in an error free repair of the damaged sequence. In gene correction, this repair mechanism is co-opted by supplying an additional potential template in the form of a WT transgene cassette with flanking sequences homologous to the DSB site. In this case, it is also possible for the homologous repair machinery to use the transgene cassette as a template, thereby integrating it into the genomic sequence. Gene augmentation is substantially less complicated than gene correction. Rather than attempt to correct the genome itself, gene augmentation

seeks to simply supply the affected cells with a new WT copy of the mutated gene, which will be expressed in parallel with the cell's endogenous copies of the gene. This method can work well for recessive mutations, but, often falters in the face of autosomal dominant disorders, wherein the disease phenotype is not caused by a lack of protein product, but, instead the presence of an innately harmful mutant protein product. Due to its simplicity and the prevalence of recessive genetic diseases, gene augmentation is currently the form of gene therapy for which we have the most experience.

Non-viral

Non-viral gene therapy methods focus on techniques to efficiently introduce DNA into cells without the use of viral vectors. The advantages of non-viral gene therapy include avoiding potential immune responses to viral vector capsids and the ability to introduce transgene cassettes which are larger than the packaging capacity of commonly used viral vectors. A major area of research in non-viral gene transfer involves the complexing of DNA with cationic lipids or polymers. In this situation, the negatively charged phosphate groups on the DNA interact electrostatically with the positively charged amine groups present in the cationic compounds causing the DNA to form tight complexes with the cationic molecules (25-26). These cationic complexes then interact electrostatically with anionic cell membranes, facilitating endocytosis of the complexes (27-28). As the pH of the endosomes decrease, some DNA will manage to escape into the cytosol, where it then must make its way to the nucleus before the transgene can be expressed.

There are a number of challenges which still need to be overcome with regards to cationic lipid and polymer based systems. Both systems suffer from significant cytotoxicity, the reduction of which has been a major area of research (29-30). Efficient entry of transfected DNA into the nucleus is another point of difficulty and is currently thought to occur through a combination of diffusion during cell division, and directed transport through nuclear pores (31-34). In the case of cationic polymers, endosomal escape is another challenge, as the polymers do not possess the membrane destabilizing hydrophobic domains that lipids do (35). Despite their potential promise, these combined complications have led to cationic gene transfer not yet reaching the point where they are viable for clinical treatments.

Retroviral

Retroviral vectors are single stranded RNA viruses capable of transducing and integrating into the genomes of dividing cells (36-37). This genomic integration allows transgenes carried by retroviral vectors to be expressed long term in tissues with a high cell turnover rate. However, serious adverse events associated with this integration ability have led to retroviruses being viewed as undesirable vectors for gene therapy.

Retroviral vectors were the first viral vectors to be used in a successful clinical gene therapy, wherein a vector derived from the murine leukemia virus (MLV) gamma retrovirus was used to deliver a transgene expressing the γ c cytokine receptor subunit ex vivo to hematopoietic stem cells of patients suffering

from X-linked severe combined immunodeficiency (38). This resulted in improved immune function in most of the treatment cohort (38-39). Unfortunately, later patient follow-ups discovered the clonal expansion T cells in 4 individuals, resulting in acute lymphoblastic leukemia (40-41). Further investigation demonstrated that the retroviral vectors were integrating near proto-oncogenes, resulting in their activation and oncogenic cell behavior (39, 41). Later studies show that MLV vectors have an integration target site preference for the transcriptional start regions of genes (42-43). This, combined with the gene activating properties of retroviral LTRs, resulted in the overexpression of genes immediately downstream of the integration site (44-46).

Both due to their inability to transduce non-dividing cells, low viral production titers, and the occurrence of multiple cases of oncogenic insertional mutagenesis (39-41, 47), retroviral vectors are currently poor candidates for translational gene therapy applications.

Lentiviral

Lentiviral vectors are, in fact, themselves a subgroup of retroviral vectors, however, due to possessing significantly different transduction and integration profiles than MLV-based gamma retroviral vectors, they are typically referred to as a separate viral vector type. Unlike retroviral vectors, recombinant lentiviral vectors are capable of transduction and integration in non-dividing cells (48-49); they also can be produced at greater titers than recombinant retroviruses, allowing for easier production (50). Perhaps the greatest strength of lentiviral

vectors is their currently observed lack of harmful insertional mutagenesis. Unlike retroviral vectors, lentiviral vectors do not favor transcriptional start sites for genomic integration, instead, they prefer to integrate near genes which are already highly expressed within the cell (42, 51). Because most previous examples of insertional mutagenesis involve the activation of quiescent proto-oncogenes by nearby enhancing LTRs, lentiviral transgenes favoring integration near already active genes should significantly reduce the chances of proto-oncogene activation.

Recent examples of successful lentiviral gene therapy include the treatment of Wiskott-Aldrick Syndrome (52-53), β -thalassaemia (54), X-linked adrenoleukodystrophy (55), and metachromatic leukodystrophy (56). Harmful insertional mutagenesis has yet to be observed in any of these trials, some of which have had upwards of 5 years of follow-up. This fact helps to bolster recombinant lentiviral vectors' reputation as a safer method for integrating gene augmentation when compared to recombinant gamma retroviral vectors. However, there are additional features that would be desirable in lentiviral vectors, including the ability to target a more diverse set of cells and an expansion of its cargo capacity, so further improvements in vector design are warranted (46).

Adenoviral

Recombinant adenoviral vectors were at one time one of the most widely used clinical gene therapy vectors. This was due to a large transgene capacity of

approximately 36kb, the ability to transduce a wide range of dividing and non-dividing cells, and lack of genomic integration, thereby avoiding potential insertional mutagenesis. Instead of genomic integration, the adenoviral genome persists in the cell nucleus as a replicating episome (57). The downside of this gene transfer method is that the episomes are rarely passed on to daughter cells, resulting in the loss of transgene production in cell populations with high turnover rates. However, in terminally differentiated cells and the appropriate immunologic milieu, these episomes can persist for many years, allowing for long term correction of genetic diseases (58).

Adenoviral vectors dramatically fell out of favor at the end of the 20th century in what would be the first known death due to gene therapy (59). In September of 1999 a patient named Jesse Gelsinger, with partial ornithine transcarbamylase (OTC) deficiency received a high dose recombinant adenoviral vector expressing the OTC protein as part of a gene therapy pilot study. 18 hours following vector infusion in the portal vein Jesse Gelsinger began to show signs of confusion and jaundice and by 35 hours he was intubated and responsive to deep pain only. At 98 hours he was removed from life support and expired. Cause of death appeared to be due to a massive systemic innate immune response to the recombinant vector. The fallout from this event was immense, with a collapse in gene therapy's public opinion and a substantial increase in regulations pertaining to gene therapy. It took years for gene therapy research in the US to recover, and adenoviral vectors are now considered better candidates

for immunologic application (such as vaccination or anti-cancer approaches) than for gene augmentation therapy.

Adeno-associated virus

Recombinant adeno-associated viruses (rAAVs) are currently a favored viral vectors for gene therapy due to their low immunogenicity, ability to transduce dividing and quiescent cells, natural replication incompetence, and lack of genomic integration. There is also an excellent safety profile for these vectors. AAVs were first isolated as contaminants of adenovirus preparations in the 1960s (60), and were later demonstrated to neither cause disease nor to be capable of replication without concurrent adenoviral infection (61-62). Wild-type (WT) AAV has a single stranded DNA (ssDNA) genome of ~4.7Kb and is non-enveloped. It is a member of the parovirus family in the dependovirus genus. While WT AAV has been shown to undergo site specific genomic integration (63), rAAV vectors are prominently non-integrating, with their genomes instead existing in the nucleus as episomes (64). These episomes do not replicate upon cell division, thus in cells with constant turnover, rAAV transgene expression will soon be lost due to dilutional effects. This makes rAAV vectors best suited either for cases requiring transient expression, or the transduction of terminally differentiated cells with little or no turnover. Due to their terminally differentiated state and low turnover, this makes many of the cell types in the retina, including the RPE and photoreceptors, well suited for transduction with rAAV vectors.

The tissue tropism and transduction efficiency of rAAV can be greatly affected by the serotype of AAV used (65-66). For the retinal gene therapy trials targeting the form of Leber's congenital amaurosis (LCA) due to *RPE65* mutations, the AAV2/2 serotype was utilized. In this nomenclature the first number indicates the serotype of the viral genomic sequence, while the second number indicates the serotype of the capsid used. AAV2/2 shows preferential transduction of the RPE, with lower transduction of rod photoreceptors and little to no transduction of cone photoreceptors (67-68). In the case of LCA-RPE65, this was ideal, as the retinal degeneration exhibited in LCA-RPE65 originates from RPE dysfunction. However, in situations where degeneration occurs in more than one cell type autonomously, such as may be the case in CHM, serotypes which show broader and more equal tissue transduction in the retina are desirable. Of the current tested serotypes, AAV5 and AAV8 both show improved transduction of the RPE and photoreceptors when compared to AAV2 (69-70), and, along with AAV9, demonstrate transgene expression in cone photoreceptors, which are not transduced by AAV2 (70).

Retinal Gene Therapy

The retina is an ideal target for gene therapy for a number of reasons. The retina forms as an outpouching of the brain and thus possesses the blood-brain barrier, which restricts the penetration of many substances into or out of this tissue. It is immunoprivileged, with the aqueous humor containing factors which suppress the innate immune response and promote regulatory activity which

desensitizes the adaptive immune system to foreign antigens within the eye (71-73). Finally, the retina is a relatively small organ to treat and possesses contralateral redundancy, providing both a control for the treated eye and a reserve if the treatment harms visual function. Also, the doses used to treat the retina are much smaller than those that would be used to treat a systemic disease.

The retina was the target of one of several successful human gene therapy trials, for the treatment of Leber's congenital amaurosis 2 (LCA2), a congenital retinal dystrophy caused by a lack of the RPE65 protein product (74-75). The disease is congenital and is thus one of the most severe forms of retinal degeneration. There is significant impairment of vision and random eye movements (nystagmus) observed in infancy and complete blindness occurs in mid to late adulthood. *RPE65* is expressed almost exclusively within the RPE of the eye, where it is vital for the production of *11-cis* retinal, a molecule necessary for the proper function of the photoreceptors. Lack of *11-cis* retinal quickly leads to the dysfunction and death of photoreceptors as well as the loss of the RPE in affected individuals. Multiple concurrent gene therapy trials were undertaken to supplement mutant endogenous copies of RPE65 in LCA2 patients with transgenes expressing the wild-type copy of RPE65 (75-78). rAAV vectors were used in all trials due to the terminally differentiated nature of the RPE, the low immunogenicity exhibited by rAAV, and the relatively small size of the RPE65 transgene being within the limits of rAAV vector capacity. In the years following the studies most patients showed improved visual function, especially among

younger members of the treated cohorts who had greater levels of persisting RPE and photoreceptors at the time of treatment (75, 79-80). No negative outcomes due to immune response to either the rAAV capsid or transgene were observed in the trials, and in one trial it was shown that there was a benign immune response even after treatment of the contralateral (previously uninjected) eye as well (81). Further, improvements in retinal and visual function were observed in the majority of the newly treated eyes.

The success in the LCA-RPE65 trials, as well as the lack of severe adverse events associated with vector administration, has spurred researchers on to test viral gene therapy in other congenital and progressive retinal dystrophies. Diseases for which recombinant vectors are currently in clinical trial include exudative age related macular degeneration, Usher syndrome, Stargardt disease due to ABCA4 dysfunction, and MERTK-associated autosomal recessive retinitis pigmentosa. There is also a clinical trial in progress targeting choroideremia (82).

MATERIALS AND METHODS

Generation of a recombinant adeno-associated virus (AAV) carrying the full-length human REP-1-encoding cDNA

A recombinant AAV proviral plasmid carrying the wild type human *CHM* cDNA (hCHM), pAAV.CBA.hCHM, was generated by cloning the human *CHM* cDNA into the transgene cassette harbored in an AAV proviral plasmid such that it was placed under control of the cytomegalovirus immediate early enhancer, the proximal chicken β actin (CBA) promoter, and CBA intron 1 sequences flanking the CBA exon 1. The proviral plasmid also contains AAV2 inverted terminal repeats and a bovine growth hormone-derived PolyA sequence (Figure 1). The proviral plasmid backbone contains a 4.7 Kb lambda phage fragment stuffer followed by the kanamycin bacterial selection gene and the bacterial origin (Figure 1). The proviral plasmid was used to generate recombinant AAV2 and AAV8 vectors by triple transfection of HEK293T cells using a helper plasmid encoding the AAV2 or AAV8 capsid, respectively. The AAV2.CBA.hCHM and AAV8.CBA.hCHM vectors were generated by the research vector core facility at the Center for Cellular and Molecular Therapeutics at the Children's Hospital of Philadelphia (CHOP). DNA sequences were confirmed by sequence analysis. The control vector, AAV8.EGFP, was provided by the Penn Vector Core facility.

Cell lines and tissue culture conditions

CHO cells were cultured in Ham's F-12K (Kaighn's) (Invitrogen, Carlsbad, CA) with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA).

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/L glucose and 10% FBS (Invitrogen, Carlsbad, CA). All cells were grown

at 37°C and 5% CO₂. Transfections in the cells were carried out using

Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or Fugene-6 transfection reagent (Roche applied Sciences, Indiana Police, IN) according to the manufacturer's

protocol.

Immunofluorescence analysis

Immunofluorescence of cell cultures was performed using the monoclonal REP-1 antibody 2F1 (1:500, sc-23905, Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence of retinal cryosections was performed using a polyclonal REP-1 antibody (1:500 dilution, HPA003231, Sigma, St. Louis, MO).

Fluorescence was visualized with a Zeiss Axio Imager .M2 microscope and

captured using an AxioCam MR camera with Axiovision software (Carl Zeiss

Microscopy, LLC, Thornwood, NY).

Assessment of transgene production and activity

In order to verify the ability of AAV8.CBA.hCHM to produce exogenous human REP-1 *in vitro* we infected CHO cells at multiple MOIs with the vector.

CHO cells were infected with AAV8.CBA.hCHM at 1E4, 1E5, or 1E6 vector

genomes (vg) per cell. Cells were harvested 48 h post-infection. Western blot analysis was performed using the anti-REP-1 2F1 antibody (1:1000, see above) as the primary antibody and a secondary HRP conjugated anti-mouse IgG antibody (1:5000, NA931-1ml, Amersham Biosciences, Piscataway, NJ). The blots were developed by the chemiluminescent method using ECL reagents according to the manufacturer's instructions.

An *in vitro* prenylation assay was performed using [3H]-geranylgeranyl pyrophosphate (GGPP) (Perkin Elmer, Boston, MA, USA) as a prenyl group donor, in the presence of recombinant Rab geranylgeranyl transferase and Rab27 (Blue Sky Biotech, Worcester, MA) as previously described (83). Incorporation of radiolabeled prenyl groups into Rab27 was measured by scintillation counting using a Beckman Coulter LS 6500 (Beckman Coulter, Brea CA). All experiments were performed in triplicate, and statistical comparison of prenylation between experimental and control groups was evaluated using Student's *t*-test. Two different prenylation assays were performed. In one, COS-7 cells were transduced at 1E6 MOI with AAV8.CBA.hCHM, the functional assay was then performed on cell lysates. Endogenous REP-1 is present in COS-7 cells, and the assay was performed with the idea that overexpression of exogenous REP-1 would result in a significant increase in prenylation in the treated group. The other cells transduced were primary fibroblasts from choroideremia-affected individuals. These cells had been collected under an approved Institutional Review Board protocol, #808828. We found that AAV8 does not efficiently transduce primary fibroblasts. Therefore, we generated an

AAV2.CBA.hCHM vector using the same proviral plasmid. Primary fibroblasts were transduced with AAV2.CBA.hCHM at an MOI of 1E6 and cell lysates were subsequently assayed for prenylation activity. Data are presented as the mean \pm Standard deviation (SD). *P* values were calculated using the 2-tailed Student's *t* test. A *P*-value less than <0.05 is considered as a statistically significant value.

Animal Studies

Animal studies were carried out under an Institutional Animal Care and Use Committee-approved protocol, 200902. A limited number ($n=18$) of heterozygous female *Chm*^{WT/null} mice were available for long-term follow-up. Mice were maintained on a 12 h light- dark cycle. Mice were anesthetized with ketamine/xylazine and 1E9 AAV8.CBA.hCHM in $\sim 1 \mu\text{l}$ DPBS was delivered subretinally when they were 6 weeks of age (84). The contralateral eye was injected with the same concentration of AAV8.EGFP as control.

Pupillometry: Measures of the amplitude of the pupillary light reflex were performed serially following injection. Mice were dark adapted for 24 hours before analysis. A NeurOptics A-1000 Pupillometer (NeurOptics Inc., Irvine, CA) was used to measure changes in pupillary diameter of eyes of the AAV8-injected mice over time following exposure to five 0.1 second flashes of $4.5 \mu\text{W}/\text{cm}^2$ intensity at 10 second intervals. Spurious lines due to eye blinks were manually removed.

Retina fixation, cryosectioning, and histology: At 50 weeks of age

Chm^{WT/null} mice were sacrificed, eyes harvested, and fixed in 4% PFA. Eyes were then cryoprotected, embedded in Optimal Cutting Temperature (OCT) media (Fisher Scientific), and frozen. Cryosections were made using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). Retinal sections were then stained with hematoxylin and eosin (H&E) or evaluated for REP-1 protein by immunofluorescence. The number of rows of nuclei in the ONL was measured at 6 prescribed points throughout the retina, and then these were compared for control and AAV8.CBA.hCHM treated retinas. Thicknesses of designated regions of the retina were graphically compared as the mean \pm standard deviation (SD), while total nuclear thickness was presented as the median. *P* values were calculated using signed rank tests and paired *t*-tests for medians and means respectively. A *P*-value less than <0.05 is considered as a statistically significant value.

PRODUCTION AND *IN VITRO* FUNCTIONAL VERIFICATION OF A REP-1 PRODUCING AAV VECTOR

SUMMARY

Choroideremia is an X-linked congenital retinal dystrophy caused by mutations in the *CHM* gene which result in the lack of expression of its protein product Rab escort protein-1 (REP-1). In choroideremia patients, there is a progressive loss of vision and the disease is symptomatic in the first decade of life with nyctalopia. By the end of the second decade loss of peripheral vision is usually observed, causing a progressive constriction of the visual field which culminates in complete loss of vision as early as the 4th decade. In the ocular tissues, degeneration of the choriocapilaris, RPE, and photoreceptors are observed, with the loss of retinal layers beginning in the peripheral retina and slowly constricting upon the fundus.

In this study we produced pAAV.CBA.hCHM, a rAAV proviral plasmid with the human *CHM* cDNA under the control of a constitutive chicken beta-actin (CBA) promoter. Initial transfection experiments in Chinese hamster ovary (CHO) cells with the proviral construct revealed robust expression of REP-1 in transfected cells via western blot. An AAV8 version of the vector was then produced from the proviral plasmid and also showed strong REP-1 expression when transducing CHO cells.

A REP-1 functional assay was then developed and used to test lysates from AAV8.CBA.hCHM transduced cells, with a nearly 2-fold increase in REP-1 activity observed. Attempts to test AAV8.CBA.hCHM in CHM patient primary fibroblasts initially failed because fibroblasts are not efficiently transduced by the AAV8 serotype. In order to cope with this, we used the same pAAV.CBA.hCHM proviral plasmid to produce an AAV2 serotype version of the vector, AAV2.CBA.hCHM. The AAV2 serotype is capable of transducing primary fibroblasts, and functional analysis of lysates from AAV2.CBA.hCHM transfection patient fibroblasts indicated a 3-fold increase in REP-1 activity as compared to untransduced cells.

INTRODUCTION

Choroideremia (CHM) is an X-linked inherited retinal disease which typically presents in childhood with nightblindness (nyctalopia) and progresses rapidly during adolescence and young adulthood to destroy peripheral vision. Central vision (visual acuity) often remains good until mid-life, but then is also ultimately affected. Choroideremia is often diagnosed by the unique appearance of the retinal fundus, which shows scalloped areas of confluent loss of retinal pigment epithelium (RPE) and choriocapillaris. Underlying photoreceptors in these areas degenerate as well. These changes are readily apparent with ophthalmoscopy and can be confirmed by fluorescein angiography and optical coherence tomography (OCT). Female carriers of the disease often show a patchy degeneration in the fundus, and can also be symptomatic, although usually their findings are subclinical. The extent of disease in carriers is affected by the extent of retinal cell lyonization (random X chromosome inactivation).

Choroideremia is caused by loss-of-function mutations in the *CHM* gene, which encodes the protein Rab escort protein-1 (REP-1). REP-1 consists of 653 amino acids with a molecular weight of ~83 kDa. There are more than 110 known mutations in *CHM*, including nonsense, splicing, deletions, and insertions (85). Most of these mutations result in a lack of production of the REP-1 protein and the remainder, presumably, result in its loss-of-function. The lack of production of the REP-1 protein forms the basis of a commonly used test to diagnose this

condition (86). REP-1 plays a key role in the post-translational lipid modification of Rab small GTPases (Rabs) (87). Rabs are members of the Ras superfamily and, when integrated with membranes, serve as controllers of tethering, docking, and fusion (88). Before newly produced Rabs can integrate with membranes, they must be post-translationally modified through the addition of prenyl groups to 1-2 cysteines located near the C-terminus of the Rab proteins. In order for these modifications to occur, REP-1 must associate with Rab GTPases and present them to Rab Geranylgeranyltransferase (Rab GGTase) for prenylation. Once prenylated, the Rabs are escorted by REP-1 to their target membrane, REP-1 then disassociates and returns to the cytosol (87). Lack of REP-1, as observed in CHM, leads to the accumulation of unprenylated, and hence nonfunctional, Rab proteins, causing the CHM phenotype (12-13).

Choroideremia is an ideal target for gene therapy for a number of reasons. It is a monogenetic disease, whose cDNA has already been successfully cloned. Initial diagnosis is quicker and simpler than with most forms of inherited blindness, due to the readily identifiable unique pattern of retinal degeneration. The degeneration is slow and significant impairment in vision is generally not recognized until the second decade of life, allowing for a large window of time for potential intervention and allowing use in a clinical trial of adult subjects. Finally, the effects of choroideremia are isolated to the eye, an ideal target for gene therapy due to its immunoprivileged state, small size, and relative physical isolation from the rest of the body (89-90). Proof of concept of gene augmentation therapy has been successfully performed in dozens of animal

models for different retinal diseases (91-92) and the retina was one of the first organs in which gene therapy was found to be successful in humans (74, 76, 80, 92-94).

Proof-of-concept of gene augmentation therapy for CHM was first described using a first generation recombinant adenoviral vector. This vector successfully rescued REP-1 function in lymphoblasts and fibroblasts isolated from affected individuals, showing both robust protein expression and function in cell culture (95). However, the adenoviral vector is not ideal for human application due to the transient nature of adenovirus-mediated gene transfer and the potential for a toxic inflammatory response (59, 96). Tolmachova *et al* have used both a recombinant lentivirus and a recombinant adeno-associated virus (AAV) serotype 2 (AAV2) to test for rescue and these have proven effective in restoring REP-1-mediated prenylation activity *in vitro* and *in vivo* (97-98). Lentivirus preferentially transduces RPE (99) and carries a risk of insertional mutagenesis (41). Recombinant adeno-associated virus vectors, however, have a strong safety and efficacy record in both animals and humans, and, by modifying the AAV capsid, one can target a diverse set of retinal cells and control onset of expression (89, 100). More than half a dozen human clinical trials targeting retinal disease are now in progress using AAV2 (<http://clinicaltrials.gov>), including one targeting choroideremia. Because AAV serotype 8 (AAV8) targets photoreceptors more efficiently than AAV2 and thus could potentially be used at a lower dose to treat choroideremia (101), we carried out proof-of-concept studies using that vector. AAV8, like AAV2, also targets RPE cells efficiently.

We carried out preliminary tests of safety and efficacy in a mouse model of choroideremia. Since knockout of the murine *Chm* gene is embryonic lethal, we used a conditional knock-out mouse generated using the Cre/loxP system of site-specific recombination (102). In this model, heterozygous null female carriers exhibit hallmarks of CHM: slowly progressive degeneration of the photoreceptors, patchy depigmentation of the RPE, and similar biochemical deficits as are found in CHM patients.

Our results show that administration of AAV8.CBA.hCHM reverses the prenylation defect and sustains retinal morphology and function *in vivo*. There was no inflammation or other sign of toxicity in retinas injected subretinally with AAV8.CBA.hCHM. These results establish the potential therapeutic use of AAV8.CBA.hCHM constructs in treating retinal degenerations caused by lack of functional REP-1 protein in choroideremia.

RESULTS

Expression of AAV8.CBA.hCHM in cultured cells

To verify the appropriate expression of the wildtype human *CHM* protein encoded in the proviral construct (Figure 1), CHO cells were transfected with pAAV.CBA.hCHM and western blot analysis was performed using the human REP-1 specific 2F1 antibody. A band of the expected size (83 kDa) for human REP-1 was observed in transfected cells with no band observed in control, untransfected cells (Figure 2). The pAAV.CBA.hCHM proviral plasmid was then used to produce a recombinant AAV8 serotype vector using the triple transfection method. In order to verify that transfection with AAV8.CBA.hCHM would result in the production of exogenous REP-1, we transduced aliquots of CHO cells with 1E4, 1E5, or 1E6 vector genomes (vg) per cell. 48 h later, cell lysates were collected and analyzed via western blot using 2F1 antibody. A clear dose dependent production of human REP-1 was observed in the transduced cell lysates (Figure 3), while lysates from untransduced cells showed only very faint expression, likely due to 2F1 cross-reactivity to endogenous REP-1.

Verification of exogenous REP-1 function in cultured cells

In order to verify function of the human REP-1 produced by AAV8.CBA.hCHM, we compared REP-1 activity in vector-treated COS-7 cells to that of untreated cells. COS-7 cells were infected with AAV8.CBA.hCHM at an

MOI of 1E6 vg/cell and over-expression of human REP-1 in the treated cells was confirmed via western blot (Figure 4A) and immunofluorescence (Figure 4B) using the 2F1 antibody. Results of both these assays demonstrated elevated levels of REP-1 protein in the cells when compared to endogenous levels.

Cell lysates from AAV8.CBA.hCHM treated and control cells were then assayed for REP-1 activity by measuring the ability of REP-1 in the cell lysates to prenylate exogenous Rab27. Lysates from transduced cells showed a nearly 2 fold increase in Rab27 prenylation over control cells (Figure 5, $P=0.0034$), clearly illustrating that the exogenous human REP-1 produced by the AAV8.CBA.hCHM vector is capable of normal REP-1 function with regards to the prenylation of Rab proteins.

We attempted to demonstrate functional REP-1 expression in AAV8.CBA.hCHM-infected cells derived from individuals with choroideremia. Unfortunately, the AAV8 serotype poorly transduces primary fibroblasts rendering it unfit for use in such an experiment. Instead, we produced an AAV2 serotype derived from the same proviral plasmid. We successfully transduced CHM patient primary fibroblasts at an MOI of 1E6 AAV2.CBA.hCHM. Cell lysates were then assayed for REP-1 activity and the results indicated an almost 3-fold increase in Rab27 prenylation in transduced cells as compared to control (Figure 6, $P=0.017$).

DISCUSSION

It has previously been concluded that choroideremia is caused by the lack of REP-1 protein expression or function in retinal cells, and, more specifically, the reduction in functional Rab proteins due to lack of REP-1 to facilitate prenylation. Therefore, in order to treat choroideremia via gene therapy, it is necessary to design a platform for initiating the production of WT functional REP-1 in retinal cells.

Viral vector gene therapy is currently the most effective means with which to deliver transgenes to cells *in vivo*, and rAAV vectors have previously been demonstrated to be capable of delivering transgenes for prolonged expression in terminally differentiated retinal tissues. In this study we have produced a rAAV vector expressing the human REP-1 gene under the control of a constitutive CBA promoter. This construct, as a proviral plasmid, was capable of strong expression of human REP-1 in CHO cells following transfection with a cationic liposome reagent. A rAAV2/8 vector, AAV8.CBA.hCHM, was produced using this proviral plasmid and further experiments in CHO cells demonstrated a robust REP-1 expression dose response following transduction at varying MOIs.

While the verification of REP-1 expression via Western blot was heartening, it was desirous to verify that the exogenous REP-1 produced from AAV8.CBA.hCHM was capable of normal REP-1 function, namely the prenylation and activation of Rab proteins. In order to determine this, we devised a functional

assay for REP-1, wherein lysates from transduced cells were used in an *in vitro* prenylation assay which would radiolabel Rab proteins in the presence of functional REP-1. This functional assay scales with the amount of functional REP-1 available, allowing the assay to be used in cases of REP-1 over-expression in cells already containing endogenous REP-1 as well as for expression in cells lacking endogenous REP-1. Via this assay, we demonstrated that the AAV8.CBA.hCHM vector was capable of producing functional REP-1 in COS-7 cells, with rates of Rab prenylation in transduced cells being nearly 2 times that observed in cell lysates containing only endogenous REP-1. Initial attempts to measure REP-1 activity in transduced CHM patient fibroblasts proved unsuccessful, as it soon became clear that the AAV8 serotype, while excellent for transducing retina tissue, was not capable of transducing primary fibroblasts efficiently. Previous work in our lab has indicated that primary fibroblasts are more permissive to transduction by AAV2, so we used the proviral pAAV.CBA.hCHM plasmid to produce an AAV 2 serotype vector. As predicted, this new vector efficiently transduced CHM patient fibroblasts, and produced functional human REP-1 protein as observed via Western blot and Rab prenylation assay. While this AAV2.CBA.hCHM vector possess a different serotype than the AAV8.CBA.hCHM used previously, it is produced using the same proviral plasmid and carries an identical transgene, thus, results gained from it pertaining to the transgene product should apply equally to AAV8.CBA.hCHM.

In conclusion, we have demonstrated that an AAV8 vector encoding the human *CHM* gene is capable of expression robust levels of functional REP-1 protein *in vitro* in both established cell lines and primary patient fibroblasts. This is an important step towards the potential use of an AAV8 gene therapy vector containing a *CHM* transgene to treat CHM in humans.

FIGURES

pAAV.CBA.hCHM

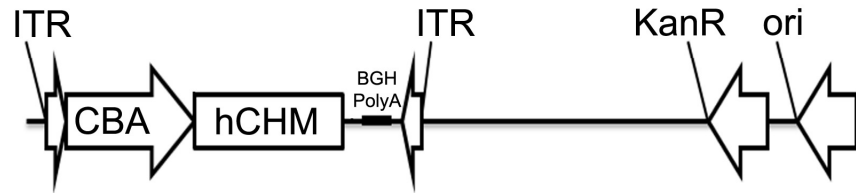


Figure 1. Generation of a CHM expression vector, pAAV.CBA.hCHM.

Schematic of the AAV proviral plasmid, carrying human CHM under the control of the cytomegalovirus enhancer chicken β actin (CBA) promoter. Flanking inverted terminal repeats (ITR) guarantee proper packaging of the transgene cassette into AAV capsids while the replication origin (ori) and kanamycin resistance gene (KanR) allow for efficient production of the plasmid transformed bacteria.

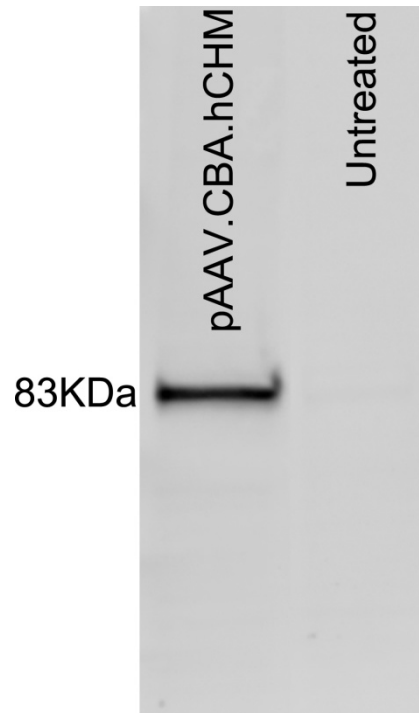


Figure 2. Western blot verification of expression from pAAV.CBA.hCHM.

Immunoblot reveals no REP-1 protein in control, untransfected, CHO cells, while there is a band of the expected size of REP-1 (83 kDa) in CHO cells transfected with pAAV.CBA.hCHM.

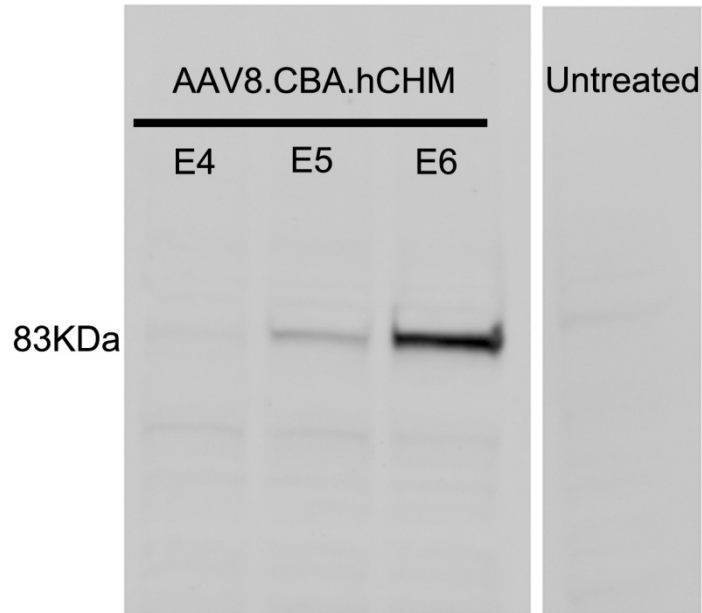


Figure 3. Dose response analysis of AAV8.hCHM AAV8 vector in vitro.

AAV8.CBA.hCHM vector was used to infect CHO cells at 1E4, 1E5 or 1E6 vg/cell. Immunoblot shows a clear dose dependent production of human REP-1 in the transduced cell lysates, whereas lysates from untreated cells showed only very faint expression, likely as a result of 2 F1 cross-reactivity to endogenous REP-1.

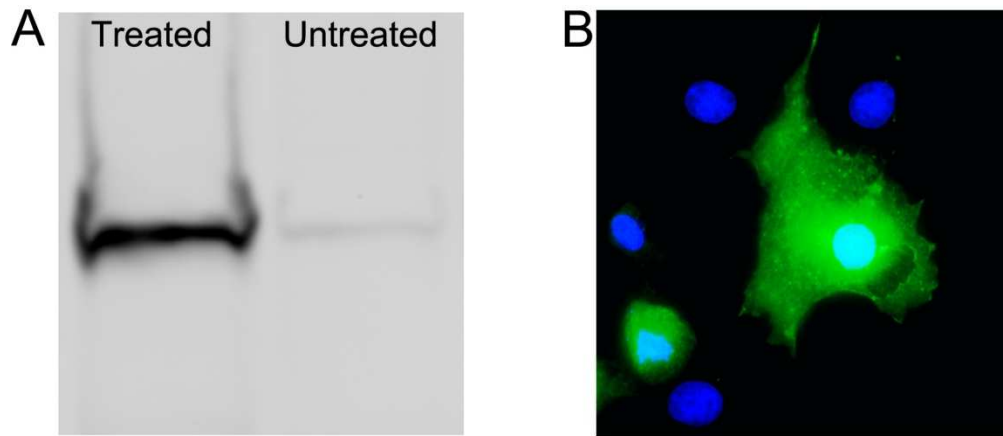


Figure 4. Immunoblot and immunofluorescence of COS-7 cells transduced with AAV8.CBA.hCHM.

AAV8.CBA.hCHM was used to transduce COS-7 cells with $1E6$ vg/cell and expression of human REP-1 was verified by western blotting (A) and (B) immunofluorescence.

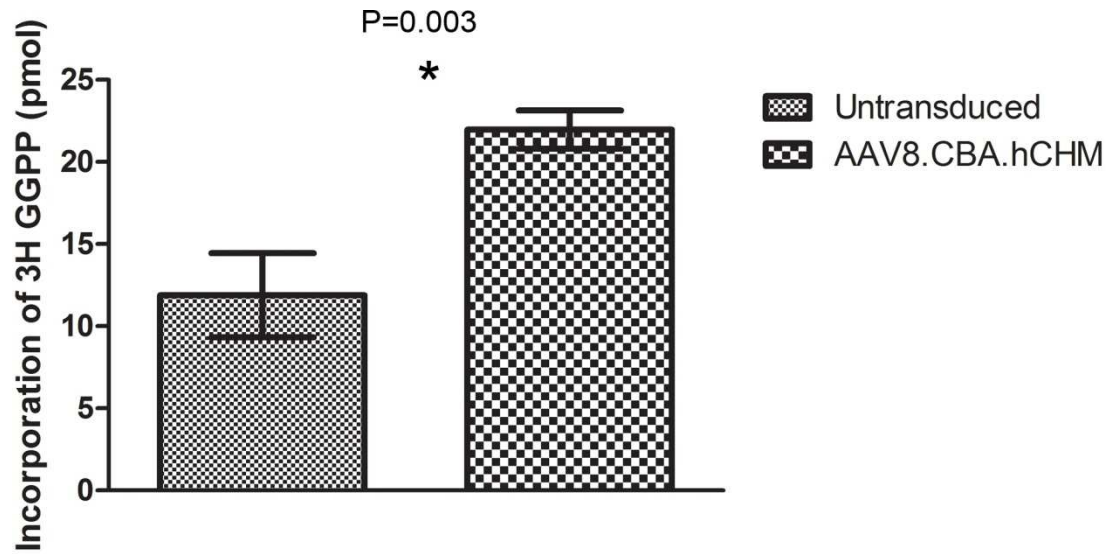


Figure 5. Verification of exogenous REP-1 function from AAV8.CBA.hCHM transduced COS-7 cells.

Cell lysates from AAV8.CBA.hCHM and control COS-7 cells were assayed for REP-1 activity using a prenylation assay. There is an almost two-fold increase in Rab27 prenylation in transduced compared to control cells ($p=0.0034$). These assays demonstrate greatly elevated levels of functional REP-1 protein in COS-7 cells after transduction with AAV8.CBA.hCHM compared to endogenous levels.

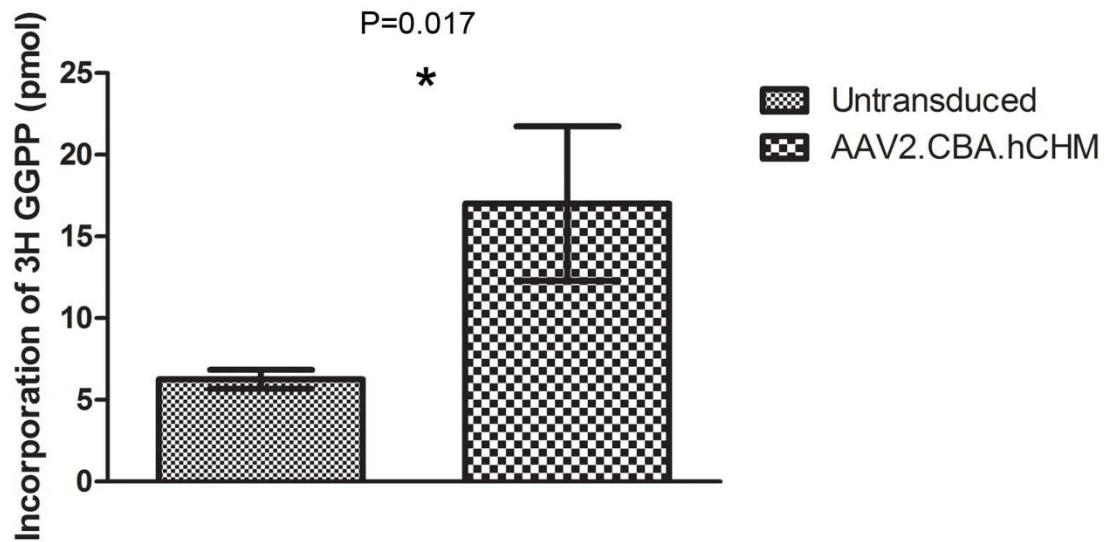


Figure 6. Verification of exogenous REP-1 function from AAV8.CBA.hCHM transduced choroideremia patient fibroblasts.

Unfortunately AAV8.CBA.hCHM (1E6 vg/cell) does not efficiently transduce CHM patient fibroblasts. Therefore, we produced an AAV2 serotype of or vector, AAV2.CBA.hCHM, as the AAV2 serotype has previously been observed to transduce human fibroblasts successfully. Patient fibroblasts treated with AAV2.CBA.hCHM at 1E6 vg/cell showed an almost three-fold increase in REP-1 activity compared to control (p=0.017).

TREATMENT OF A CHOROIDEREMIA MURINE MODEL WITH A REP-1 PRODUCING AAV VECTOR

SUMMARY

In this study we subretinally injected 6 week old *CHM^{WT/null}* mice in one eye with our experimental vector AAV8.CBA.CHM, while contralateral eyes were injected with an AAV8.EGFP control. The pupillary responses of treated mice were assayed regularly via pupillometry until the mice were 50 weeks of age, with clear divergences in response between treated and control eyes becoming apparent at approximately 32-39 weeks of age in some animals. At 50 weeks of age the mice were sacrificed and retinal tissue harvested and cryosectioned. Immunostaining analysis of treated and control retinal sections demonstrated expression of human REP-1 protein in the photoreceptors and RPE of treated retinas, with no corresponding expression in control eyes. Analysis of H&E stained retinas showed a significant difference in retinal health between treated and untreated eyes, with ONL layer thickness being significantly higher in treated retinas as compared to untreated retinas.

INTRODUCTION

The road to the production of an animal model for CHM has not been a smooth one. Initial attempts to produce a murine animal model, the *Chm*^{null/Y} mouse, revealed that lack of *Chm* in mice is embryonic lethal, with the malformation of placental extra-embryonic tissue appearing to be the cause (24). It was also discovered that *Chm*^{WT/null} mothers were incapable of producing carrier female offspring when crossed with WT males, likely due to the inactivation of the paternal X-chromosome in murine extra-embryonic tissue (103-104). This theory was supported by the discovery that *Chm*^{null/WT} and *Chm*^{null/Y} embryos produced by *Chm*^{WT/null} females crossed with WT males could be rescued by aggregation at the 8-cell stage with tetraploid WT embryos. The aggregation, referred to as a tetraploid complementation assay, exploits the fact that tetraploid embryos are capable of forming extra-embryonic tissue, such as the placenta, but not a functional fetus. By aggregating these tetraploid cells with mutant diploid embryos it is possible to rescue extra-embryonic defects in these mutant embryos, verifying that defects in extra-embryonic development are the cause of the embryonic lethality (105). Unfortunately, large scale production of a disease model by the use of a tetraploid complementation assay is not viable, and a different method needed to be found. The solution occurred when it was observed that *Chm*^{WT/null} females exhibited a slow patchy degeneration of their retinas similar to that observed in human female carriers of CHM (22). This patchy degeneration is ascribed to the lyonization of cells within female retinas,

wherein the X-chromosome of one chromatid is inactivated throughout the cells of the retina. This results in a diffuse degeneration of the retina where some cells are dysfunctional and others are not. Lyonization need not be uniform, and can differ between animals, potentially resulting in some animals displaying greater degeneration than others. Unlike in human female carriers, the degeneration observed in murine carriers is not asymptomatic, and by 6 months of age the mice display aberration in electroretinograms and significant thinning of the ONL (16).

Our lab has previously produced an AAV serotype 8 vector which expresses the human REP-1 gene under the control of the CBA promoter. This vector has been shown to produce functional human REP-1 protein in COS-7 cells and an AAV2 serotype version of the vector did the same in CHM patient fibroblasts. While the AAV2 serotype already has a successful history as a gene therapy vector, it demonstrates a marked transduction preference in the retina for the RPE, with much less targeting of the photoreceptors. Choroideremia is a disease which causes dysfunction and autonomous degeneration in both the RPE and photoreceptors, which makes it highly desirable to use a vector which will target both cell types well at reasonable vector doses. The AAV8 serotype has been shown to target both the RPE and photoreceptors in murine and non-human primate models and results in expression of transgenes in both tissue types at higher levels than observed with AAV2 at equivalent vector doses (106). Because of this, we chose to use an AAV2/8 serotype for our animal studies.

In this study, we subretinally injected *Chm*^{WT/null} mice with AAV8.CBA.hCHM in one eye, and contralaterally with AAV8.EGFP as a control. Animals were then monitored via ophthalmoscopy and pupillometry until sacrifice, after which retinal tissue was harvested and analyzed.

RESULTS

Safety of subretinal injection of AAV8.CBA.hCHM

Chm^{WT/null} mice were injected unilaterally and subretinally with 1E9 vg of AAV8.CBA.hCHM. The mice were evaluated by ophthalmoscopy at baseline, 1 week after injection, and at monthly intervals thereafter. There was no evidence at any timepoint of inflammation resulting from exposure to the experimental or control vector. Media remained clear and the retina had reattached by the first post-operative examination. The *Chm*^{WT/null} retinas before and after treatment with AAV8.CBA.hCHM showed diffuse small hypo-pigmented lesions (Figure 7). Lesions remained similar in size in the case of AAV8.CBA.hCHM-injected eyes but expanded in size in AAV8.EGFP-injected eyes (Figure 7). Retinas that had been injected subretinally with the AAV8.EGFP control vector possessed high levels of EGFP protein by the first post-operative examination and continuing through the latest timepoint (Figure 7).

Pupillometry and histology results indicate efficacy in *Chm*^{null/WT} mice

Analysis of retinal function in experimental and control eyes was performed regularly and non-invasively using evaluation of the amplitude of the pupillary light reflex (PLR) following injection. While the PLR was similar between treated and control eyes at baseline, differences in response appeared between 32 and 39 weeks of age with diminished responses in the control eyes. (Figure

8). The amplitude of the PLR was similar in *Chm*^{WT/null} AAV8.CBA.hCHM-injected eyes as in untreated eyes of age-matched wildtype mice (Average amplitude in AAV8.CBA.hCHM-injected *Chm*^{WT/null} eyes was 0.39 mm; Average amplitude in wildtype mice was 0.34 mm, *P*= 0.08).

At 50 weeks of age, the mice were sacrificed and their eyes harvested and cryosectioned. Histology displayed severe loss of the photoreceptors (decrease in outer nuclear layer thickness) in control retinas (Figure 9B). In the AAV8.CBA.hCHM-treated retinas, degeneration was significantly stalled, as evidenced by an average of 7 rows of photoreceptor nuclei in the outer nuclear layer (ONL) versus <1 row of nuclei in the control retinas (Figure 9E). Measures of retinal thickness across the retina (Figure 10) showed an increased ONL thickness for all retinal points in the treated eye, with two neighboring points reaching significance (*P*=0.018, 0.038). When all the points across the treated and untreated eyes are combined, we again observe significant improvement in the ONL of the treated eyes as compared to the untreated (Figure 11). There were no inflammatory cells observed in either the AAV8.CBA.hCHM or the AAV8.EGFP-injected eyes.

Transgenic human REP-1 is successfully expressed in *Chm*^{WT/null} mouse retinas

To confirm that AAV8.CBA.hCHM subretinal injection results in the localized expression of human REP-1 in treated *Chm*^{WT/null} mice, we performed immunofluorescent labeling of retinal cryosections using a polyclonal human

specific REP-1 antibody produced in rabbits. In treated mouse eyes, labeling of human REP-1 was observed in the RPE and photoreceptor inner segments (Figure 9D), this recapitulates the REP-1 localization observed in immunofluorescent labeled human retina sections (107). No human REP-1 labeling was observed in the control retinas (Figure 9A), with robust EGFP expression in the remaining RPE and neural retina observed instead (Figure 9C).

DISCUSSION

In this study we hypothesized that the introduction of functional human REP-1 to the RPE and photoreceptor cells of the retina would help ameliorate the retinal degeneration observed in a murine model of choroideremia. In order to test this, we produced an AAV8.CBA.hCHM vector which will express human REP-1 constitutively in transduced cells and injected CHM mice subretinally in one eye, while injecting an AAV8.EGFP expressing a reporter transgene as control in the contralateral eye.

Treated mice were followed up at regular intervals via pupillometry and ophthalmoscopy, with a divergence in pupillary response observed between treated and control eyes in some mice at 32-39 weeks of age. In these mice the pupillary response in treated eye was more robust than that observed in control eyes, indicating improved retinal function in those eyes. While specific animals showed significant improvements in pupillary response, this was not observed across the entire treated population, with average pupillary response for all animals combined not showing a significant improvement in treated eyes ($P=0.08$). However, the remarkable improvement observed in some animals does suggest that treatment had a beneficial effect on pupillary response in those animals.

Previous research involving the choroideremia mouse model used in this study indicated that there was significant outer nuclear layer loss at

approximately 8-16 months (16), therefore, we targeted this age range for a more invasive analysis of retinal histology. At 50 weeks of age the treated mice were sacrificed, and treated and control eyes were harvested and cryosectioned. Immunostaining of treated and control eyes for human REP-1 confirmed that we had induced the robust expression of REP-1 protein in treated retinas, with localization to the photoreceptor outer segments and RPE. This expression closely mirrored that observed in endogenous REP-1 localization in the human retina, and supports the conclusion that human exogenous REP-1 is functional and will localize correctly when expressed in retinal cells.

The ONL of the retina is composed of the nuclei of photoreceptors cells. This means that the number of nuclei in the ONL directly corresponds to the number of rod and cone photoreceptors in the retina, with the thickness, in nuclei, of the ONL being considered a clear indicator of photoreceptor number and health. In order to quantitatively determine the health of treated retinas, as compared to controls, we measured the ONL thickness of treated and control retinas at set points along the retinas, and compared nuclei numbers between treated and controls. Our results indicated that at two adjoining points in the retinas there was a significant improvement in health in the treated retina. When these points were then combined to look at retinal thickness across all points, it was again demonstrated that the treated retinas were significantly more healthy than the untreated retinas with regards to ONL thickness. While it could not be proven with this study, it is possible that the region of confluent improvement seen across the treated retinas may correspond to the subretinal injection site

where the AAV8.CBA.hCHM vector was delivered. However, this can't be verified, as the injection sites could potentially vary among treated animals.

In conclusion, we have demonstrated that an AAV8 vector expressing human REP-1 protein is capable of transducing the RPE and photoreceptor cells in a murine model of CHM, and reducing the observed degeneration in the treated retina as compared to control eyes. These results strongly support future attempts to move this potential therapeutic treatment to clinical trial.

FIGURES

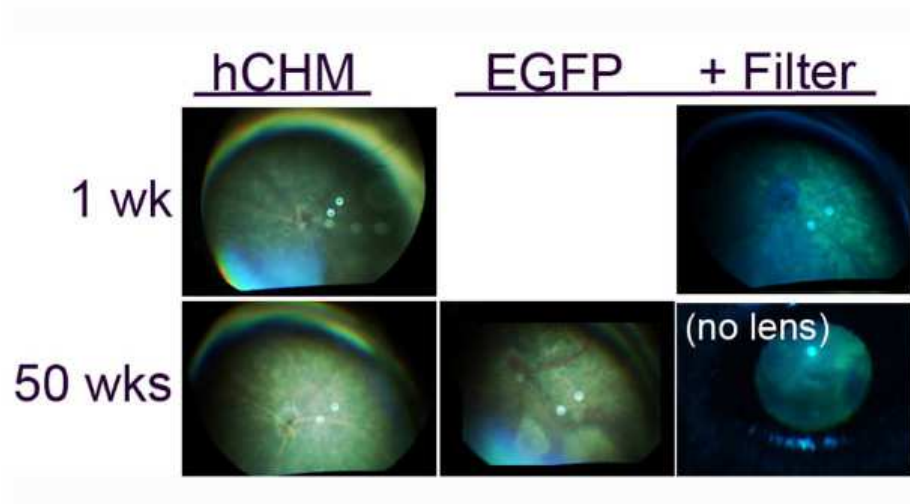


Figure 7: Ophthalmoscopy findings in AAV8.CBA.hCHM- and control (AAV8.EGFP-injected) retinas.

Representative retinas from AAV8.CBA.hCHM-injected and AAV8.EGFP-injected *Chm*^{WT/null} mice 1 and 50 weeks post-injection show diffuse pigmentary changes, similar to those observed prior to injection. EGFP is visible in the AAV8.EGFP-injected retina (only) using a fluorescein filter (shown at the 1-week timepoint but continuing through termination of the study and visible even without using a lens to focus on the retina). There were greater areas of depigmentation in AAV8.EGFP-injected retinas 50 weeks after injection, although the appearance of AAV8.CBA.hCHM-injected retinas was similar to that observed soon after injection.

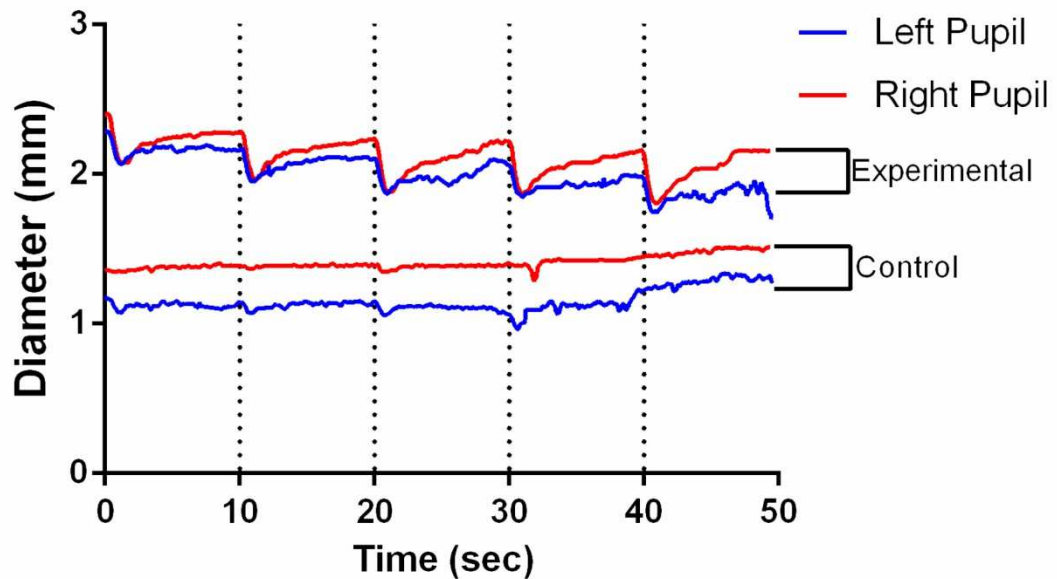


Figure 8. Pupillometry analysis of AAV8.CBA.hCHM treat CHM mice.

Representative pupillary light reflex traces 49 weeks after injection of the right eye with AAV8.CBA.hCHM and the left eye (control) with AAV8.EGFP. Brisk pupillary constriction is observed after the right eye (but not the left eye) is stimulated. Left and right pupils are indicated by blue and red traces, respectively, as a function of time after each flash of light (vertical dotted lines).

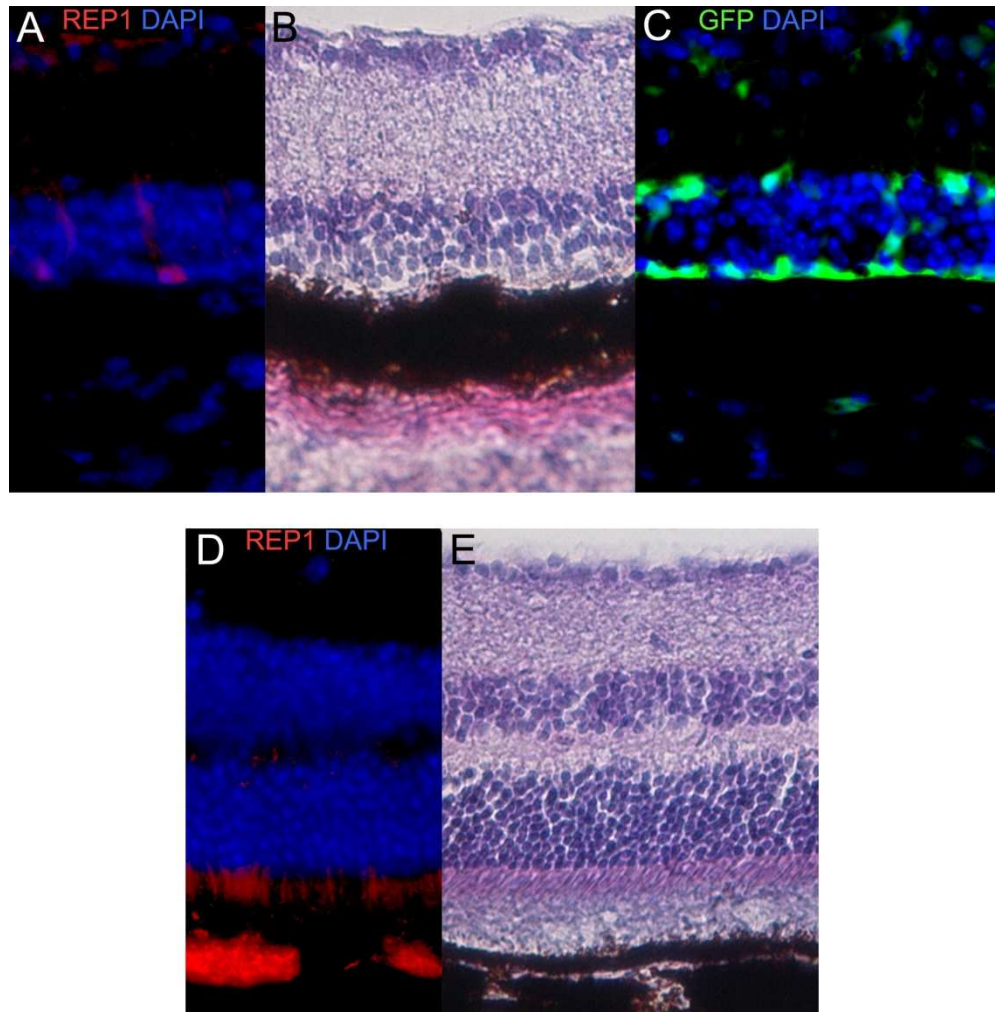


Figure 9. Histological and immunofluorescent analysis of retinas from AAV8.CBA.hCHM treated CHM^{WT/null} mice.

REP-1 immunofluorescent and H&E staining collage of control (A–C) and treated (D–E) CHM mouse retinas. (A) There is no detectable immunofluorescence specific to human REP-1 in the AAV8.EGFP-injected retina. (B) H&E staining reveal loss of ONL. (C) GFP is present in the treated portion of the retina at high levels in the RPE and remaining neural retina. By contrast, REP-1 protein is immunofluorescently detectable in RPE and photoreceptor inner segments in AAVCHM-treated retinas (D). (E) H&E staining shows preservation of the ONL.

Representative AAV8.CBA.hCHM-treated retina is shown at approximately 50 weeks of age.

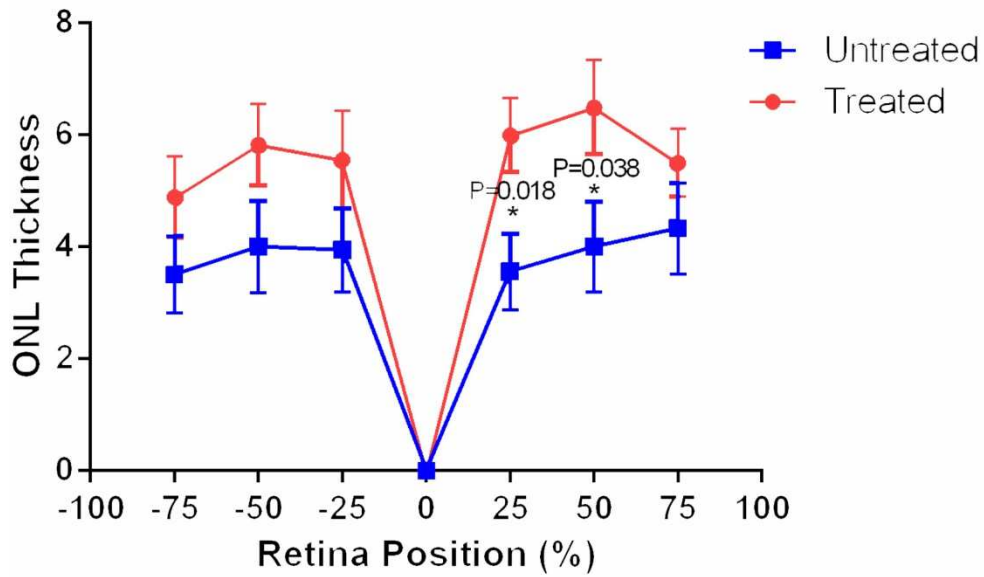


Figure 10. Spidergram of retinal outer nuclear layer (ONL) thickness.

Graph depicting average ONL layer thickness for untreated and treated eyes across six points in the retina. Two neighboring points show significant improvement in the treated eye ($p=0.018$ and 0.038). Analyzed retinas harvested from animals at approximately 50 weeks of age.

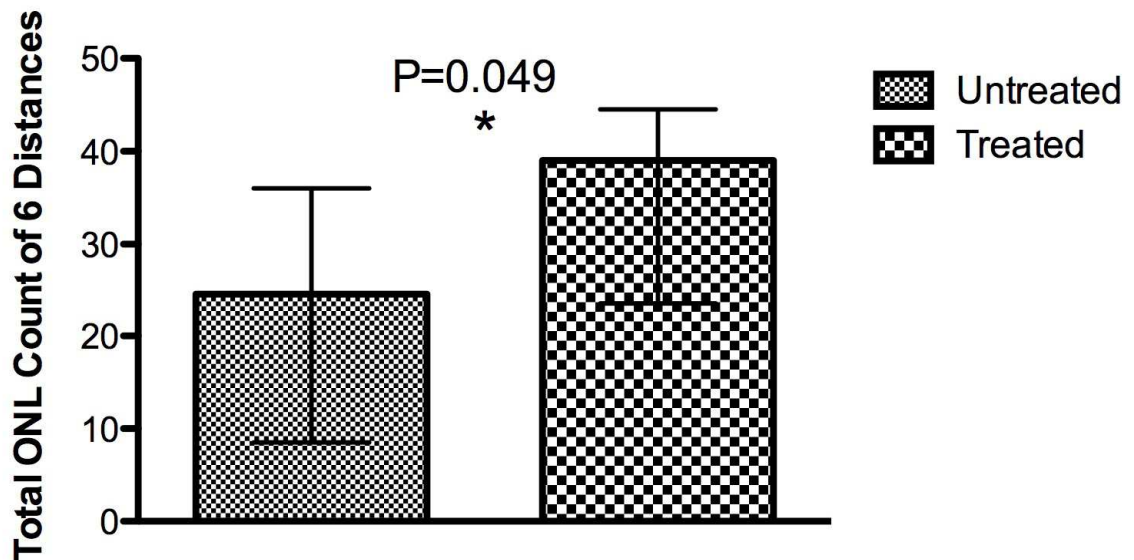


Figure 11. Combined ONL across all measured points.

Comparison of untreated and treated ONL thickness using an average of all measured points in the retinas. The treated retina shows a significant improvement in ONL thickness compared to the untreated retina ($p=0.049$). Error bars represent IQR. Analyzed retinas harvested from animals at approximately 50 weeks of age.

CONCLUSION AND FUTURE DIRECTIONS

CONCLUSION

Although the molecular basis of choroideremia was first characterized and the gene cloned more than 23 years ago (108-109), progress in demonstrating proof-of-concept of gene augmentation therapy has only recently emerged for this disease. This is for two main reasons: 1) it has been difficult to generate an accurate animal model of the disease phenotype since lack of REP-1 is lethal in the mouse (110). This has made it difficult to unequivocally identify the primary cell type initiating the disease and also to test therapies; and 2) it was not until recently that vectors capable of delivering genes safely and efficiently to the appropriate retinal target cells were identified. There is now a large body of data relating to the safety and efficacy after subretinal injection of rAAV serotype 2 (AAV2) generated in human clinical trials for Leber congenital amaurosis due to *RPE65* mutations (74-76, 80, 92-94, 111-113). Soon there will be additional data available on the safety profile of AAV2 used to treat choroidal neovascularization in wet age-related macular degeneration and choroideremia (<http://clinicaltrials.gov>).

It was sensible to move forward with gene therapy for choroideremia using AAV2, as has been done by MacLaren, During, Seabra and colleagues, as this vector serotype can target RPE and photoreceptors safely and efficiently and in a

stable fashion (68). Preliminary results from their Phase 1 clinical trial show a high degree of safety and evidence of efficacy in two of six subjects. In an effort to develop a vector which could be used to target RPE and photoreceptor cells even more efficiently (and thus potentially at a lower dose) than AAV2, we explored applications of rAAV8 to choroideremia. AAV8 is known to target a diverse set of retinal cells, including RPE cells and photoreceptors, safely in mice, dogs and non-human primates (70, 101). AAV8-mediated transgene expression reaches maximal levels much sooner than does AAV2-mediated expression. Furthermore, AAV8 can be used at ten times less than the dose of AAV2 to achieve equivalent expression in the same set of cells (70, 89, 101). AAV8 has been used successfully in a gene augmentation therapy approach in animal models of achromatopsia, LCA, autosomal recessive retinitis pigmentosa, and retinoschisis (114-117). It has also been applied to humans with hemophilia, without any adverse safety issues (118). Similar to the way that use of AAV2 in LCA-RPE65 has expedited development of AAV2 for other blinding human conditions, use of AAV8 in a human clinical trial for choroideremia could not only result in robust safety and efficacy data but could also serve as a stepping stone for using AAV8 in other, less common, human retinal degenerative conditions.

Recently, Tolmachova et al described safety and efficacy of subretinal delivery of an AAV2.CBA.CHM vector after subretinal injection in *Chm*^{WT/null} mice (98). Their data demonstrated transduction of the RPE and improvement in electroretinogram (ERG) a- and b-waves in comparison to sham-injected eyes. The ERG response reflects improvement of function in the neural retina. In the

present study, we have evaluated the effects of a vector, AAV8, which transduces photoreceptors more efficiently than AAV2. This vector, like AAV2, also transduces RPE cells. We demonstrated, through use of pupillometry, that signals originating in the AAV8.CBA.hCHM-injected *Chm*^{WT/null} retinas are relayed to the brain. We also demonstrated that AAV8-mediated delivery of the wildtype human *CHM* cDNA resulted in efficient production of REP-1 in CHO cells and COS7 cells. Although CHO and COS7 cells have endogenous (hamster and monkey, respectively) REP-1, we were able to take advantage of a human-REP-1-specific antibody to demonstrate expression. We then proved that this exogenous REP-1 functions properly, with regards to the prenylation of Rab proteins, both in COS7 cells transduced with AAV8.CBA.hCHM and CHM patient derived fibroblasts transduced with AAV2.CBA.hCHM produced using the same proviral plasmid. The next step was to evaluate safety and efficacy in an animal model of choroideremia, the *Chm*^{WT/null} mouse. For this, we carried out subretinal injections of AAV8.CBA.hCHM in one eye and injected the contralateral eye with AAV8.EGFP as control. The animals were followed serially over the next half year for safety and efficacy. There was no evidence at any timepoint of inflammation due to exposure to AAV8 or to the human *CHM* transgene. Results showed robust preservation of pupillary response and retinal morphology in the eyes treated with AAV8.CBA.hCHM but not in the control eyes. Rescue was confirmed histologically, with the photoreceptor and RPE layers of experimental eyes overall remaining more intact than those of the control eyes. Furthermore, the REP-1 protein could be detected in the experimental but not control eyes.

One challenge with interpretation of the data is that we cannot exclude the possibility that exposure to EGFP protein resulted in toxicity in the control eyes. Due to the limited number of animals, we were not able to assess this possibility. We have carried out numerous studies using *EGFP* as a control transgene and have only seen evidence of a cell-mediated immune response directed at EGFP in one experiment (101). That experiment involved non-human primates, not mice. Since we did not have enough animals to add an additional cohort of uninjected (or sham-injected) control eyes, we cannot rule out the potential of EGFP-mediated toxicity. Nevertheless, we can still conclude that exposure to AAV8 and the *CHM* transgene was not toxic and delivery of AAV8.CBA.hCHM resulted in longterm rescue of retinal-cortical function in *Chm*^{WT/null} mice without any apparent local or systemic toxicity. The data reported here is the first step in developing proof-of-concept of AAV8-mediated retinal gene therapy for choroideremia.

FUTURE DIRECTIONS

Improved *In Vitro* Models

The cells of the neural retina are highly specialized, with little resemblance to commonly used immortalized cell lines. With the exception of RPE cells, attempts to isolate or produce immortalized cell lines from retinal tissues have met with very limited success, with the generated line often retaining very few unique characteristics of the cell types they were originally derived from. Working with primary tissue cultures harvested from animals and humans can correct some of these problems, but, result in new difficulties including cells only being viable for a short time and the need to obtain fresh tissue regularly, a real challenge if you are working with human tissue. These limitations are complicated further when the subject of research is a genetic disease, as ideally you would wish to conduct investigations of potential treatments in cells derived from an affected individual. In the case of fibroblasts and lymphoblasts this is a possibility, but, to work with retinal tissue, for example, would require harvest within hours of death of ocular tissue from a molecular characterized choroideremia patient. So far, such tissue has not been available. If it were, the harvest could potentially yield a primary RPE cell model of choroideremia, but not a line of neuronal cells. Therefore, means by which to produce retinal cells, particularly RPE and photoreceptors, with known CHM mutations would provide

researchers with a powerful platform from which to study the molecular mechanisms of the disease and test potential therapeutics. Recent advances in the field of induced pluripotent stem (iPS) cell derived tissues may offer the answer to this need. It has become possible to reliably produce iPS cells from patient peripheral blood mononuclear cells (PBMCs) (119) and fibroblasts (120), and derivation protocols now exist to produce iPS-RPE cells whose function and appearance very closely match that of primary RPE cells (121-122). In theory, iPS cells derived from a CHM affected individual can be differentiated into these iPS-RPE cells, providing a reliable supply of affected RPE cells for studies of disease mechanisms and the effects of exogenous REP-1 introduction on cell function. In particular, photoreceptor outer segment phagocytosis assays are a commonly used method to analyze RPE function, and phagocytosis has been shown to be perturbed in immortalized RPE cells with REP-1 siRNA KD (20). REP-1 deficient iPS-RPE cells would allow us to verify this abnormality in patient cells as well as ascertain whether potential treatments are capable of ameliorating the dysfunction.

The production of functional photoreceptors from iPS cells is a challenging prospect due to the increased complexity of photoreceptors and their high dependence upon neighboring tissues for proper function and development. However, advances are currently being made in this area. We and others are now able to generate neural retina precursor cells reliably from iPS cells and these cells can have features of differentiating photoreceptors. In fact, the cells congregate into pseudo-eye cups containing multiple retinal layers (123). It is

likely that steps will be identified in the near future allowing these eye cups to fully recapitulate the development/function of neural retinal cells *in vivo*. This will allow studies of disease mechanism and will expedite the development of therapeutic strategies, including gene therapy.

Improved Animal Models

One of the greatest challenges faced by those attempting translational research of choroideremia is the lack of animal models which faithfully reproduce the human disease phenotype. This is compounded by the fact that the (one) murine model of the disease exhibits a slow degeneration (detectable after 50 weeks). This poses practical problems for a laboratory as the timeline of experiments is long.

Recently generated models have attempted to improve upon this original system by instead producing mouse lines with tissue specific Cre expression in either the RPE, photoreceptors, or both tissues concurrently. By crossing this tissue specific Cre expressing strain with the already existing floxed-*Chm* strain (*Chm^{flox}*), it is possible to produce a mouse strain which will spontaneously KO the *Chm* gene in only the RPE and photoreceptors (19). Some of the immediate benefits of this method include a much easier breeding strategy for the disease model and a disease phenotype which should theoretically be more true to that observed in affected human males. This is due to the new murine model disrupting REP-1 expression in all RPE and photoreceptor cells, rather than

depending upon lyonization in the retinas of *Chm*^{WT/null} heterozygous females to achieve a mosaic KO. Instead, this method should result in a complete KO of *Chm* in both the RPE and the photoreceptors. Unfortunately, while these conditional KO models did allow for a better understanding of CHM tissue pathology, they did not show retinal degeneration which was significantly more severe than that observed in the *Chm*^{null/WT} model (16, 19). Therefore, the generation of animal models of choroideremia which both exhibit a disease phenotype more similar to that seen in affected humans and experience a faster disease onset to allow for shorter studies is highly desirable. Ideally these models would include both a small animal model for easy studies with high numbers of treated animals, and a large animal model for therapy treatment in a system which more closely resembles humans.

Murine disease models are a gold standard for potential therapy testing due to their quick maturation, large litter numbers, relatively low resource requirements, ease of handling, and vast pre-existing research community resources. However, generation of a murine choroideremia model has been complicated due to KO of *Chm* being embryonic lethal in mice. As previously mentioned, methods have been found to circumvent this, but, degeneration in the mice is still dissimilar to that observed in humans, and, most importantly, occurs over a timespan that is substantially longer than ideal. Therefore, the generation of a murine model with a quicker disease onset and progression would be a powerful tool in the research of choroideremia therapies.

Seabra's work with the newest conditional tissue-specific Cre expressing *Chm^{flox}* model suggests that even mostly complete loss of Rep-1 expression in the RPE and photoreceptors is not sufficient to produce a disease model with efficient disease pathology. Therefore, approaches by which this degeneration can be accelerated, either by further disrupting the Rab prenylation pathway, or by otherwise enhancing the degeneration in a *Chm* mutation dependent fashion, are a promising area of research.

As previously described, Rep-1 is involved in the activation-by-prenylation of Rab proteins. These proteins associate with the cell membrane and are vital for intracellular trafficking. In humans, it is thought that the lack of functional REP-1 is mitigated by the presence of REP-2, a protein which shares a 71% homology with REP-1. REP-2 has been shown to also be capable of presenting Rab proteins for prenylation, although at a lesser efficiency than REP-1 for some Rab proteins (12-14). Little work has so far been done examining the murine homologue of REP-2, Rep-2, but, it has been demonstrated *via in situ* hybridization that Rep-2 mRNA is present in the same area of the murine retina as is the mRNA of Rep-1 (15). This strongly suggests that Rep-2 protein is also present in those tissues and is likely assisting in the prenylation of Rab proteins. This makes the Rep-2 gene an excellent candidate for KD or KO to further exacerbate the decrease in the Rab prenylation caused by Rep-1 KO. It has already been observed in a zebrafish model of CHM, which lacks the Rep-2 gene, that the complete loss of Rab prenylation is disastrous for proper cellular function, quickly resulting in the death of fertilized embryos (23). Presumably,

eliminating both Rep-1 and Rep-2 in the murine eye could have a similar effect, resulting in an abrupt degeneration of the RPE and photoreceptors which should still be mostly treatable with the reinstatement of Rep-1 expression via gene therapy. Potential complications of this model include that, without any Rab prenylation, degeneration will be so fast that it will be difficult to treat mice before the complete loss of the target cell layers, and the possibility that the reinstatement of Rep-1 expression will not be enough to compensate for the lack of Rep-2. The former complication would be a difficult one to ameliorate, and could either require *in utero* treatment of the model to transduce still healthy cells or the use of a less stringent means of Rep-2 KD, such as siRNA, so as not to entirely eliminate its expression.

Another potential method to further reduce the prenylation of Rab proteins in a retinal tissue-specific *Chm^{flox}* model would be to cross it with mice with the *gunmetal (gm)* mutation. *Gm* mice possess a G to A substitution mutation in a splice acceptor site at the start of exon 1 in the Rab geranylgeranyl transferase gene (124). This mutation results in exon 1 being spliced out in most mature mRNAs, causing the loss of the gene's normal start codon. The resulting mRNA is unlikely to be translated. To a much lesser degree, a cryptic splice acceptor and donor site allows for an mRNA retaining exon 1 to be produced in *gm* mice, which enables the production of geranylgeranyl transferase at approximately a fourth the level seen in wt mice. Geranylgeranyl transferase interacts with REP-1 and Rab proteins to effect the addition of geranylgeranyl prenyl groups to the Rab proteins. In the *gm* mouse the reduction in geranylgeranyl transferase

corresponds with a significant drop in the levels of prenylated Rab proteins, similar to that observed in CHM patients (124). These mice exhibit partial cutaneous albinism, prolonged bleeding, platelet abnormalities, and thrombocytopenia, but, no obvious visual defects. By crossing tissue-specific Cre expressing *Chm^{fllox}* mice with the *gm* model, it may be possible to generate mice with a greater reduction in Rab prenylation in the RPE and photoreceptors than is observed in either model alone. This could result in a model which will exhibit more severe degeneration in the retina, but, still be amenable to rescue with the addition of a REP-1 transgene.

While small animal models are excellent for fast, inexpensive proof of concept experiments, they often falter at being direct translations of the disease phenotype observed in humans due to large differences in genetics and physiology. Attempts to produce a *Chm^{null/Y}* mouse model is an excellent example of this, as it was discovered that the KO of *Chm* was embryonic lethal in mice (24), whereas the only observed disease phenotype in humans is retinal dystrophy. Physiological differences also play a significant role, as mice and many other small animal models do not possess organs that closely mimic those seen in humans. In the case of the eye, mice do not possess a macula, a cone dense central region seen only in primates, and overall have very low levels of cones, approximately 1%, compared to humans, ~5% (125-126). Mice are dichromats, with two types of cones that express two different light sensitive opsins, for medium and ultraviolet wavelengths (127), humans, on the other hand, are trichromats, with 3 different types of cone opsins that are sensitive at

short, medium, and long wavelengths (128-129). The morphology of murine and primate eyes is also vastly different, with the lens in murine eyes taking up much of the interior of the eye, greatly restricting the size of the vitreous chamber. In comparison, the primate lens composes much less of the interior of the eye, resulting in a far larger vitreous chamber relative to the size of the eye. This results in treatments that might target the vitreous chamber, such as intra-vitreal injections, having drastically different mechanics depending upon whether you are testing them in murine and other small rodent models, or in large animal models, as dilution factors in the vitreous are vastly different in rodents versus large animal models. These differences mean that while murine models might be useful and efficient for translational work, it is important to correlate results between small and large animal models.

Swine are considered an ideal large animal model when pursuing translational research for human diseases due to their similar genetics, physiology, and anatomy (130). In fact, swine organs so closely resemble those of humans that swine are now being engineered for xenotransplantation (131-132). In the eye, swine lack a macula, as do all non-primates, but, they do possess a cone-rich horizontal temporal to nasal retinal streak across the retina, as well as a rod-rich retinal periphery (133), a configuration analogous to a macula. Remarkably, swine retinas are richer in cone photoreceptors than human retinas, with levels of approximately 20-30% (134). Like mice, swine are dichromats, however, they possess short- and medium-wavelength cones, both

of which are shared with trichromat humans, while mice possess medium- and ultraviolet-wavelength cones, only one of which is shared with humans (133).

Engineering swine disease models is a process that has undergone rapid progress over the last two decades. While initially only crude random transgene addition was possible via pronuclear injection of zygotes (135), methods of targeted transgene introduction or gene modification have become possible through the modification of swine somatic cell genomes via homologous recombination (136), zinc finger nucleases (137), transposases (138), CRISPR/Cas9 (139), and other genome editing methods, followed by somatic cell nuclear transfer (SCNT) of desired genetic modifications into porcine zygotes. At this time a number of swine models for human disease have been produced, including for retinitis pigmentosa (140), autosomal dominant Stargardt-like macular dystrophy type 3 (141), hypertriglyceridemia (142), and cystic fibrosis (143-144). The cystic fibrosis model is of particular interest because it highlights the complications which can occur when you attempt to model human diseases in small animal models. Murine models of cystic fibrosis do not exhibit all the classic symptoms of cystic fibrosis (145) and have been of only moderate use in modeling disease progression. In comparison, the swine models of cystic fibrosis so far produced have displayed disease phenotypes which very closely resemble those observed in humans and have allowed the discovery of hereto unknown disease mechanisms (146). While it is not assured, the much greater similarities in development between swine and humans as compared to mice and humans supports the idea that generation of a CHM model in swine may result in

a viable large animal model which presents a disease phenotype more in step with what it observed in affected humans.

There a number of potential complications which could arise when attempt to produce a swine model of choroideremia. The most obvious of these is that the KO of *CHM* will prove to be embryonic lethal in swine as well as mice. This outcome would not be recoverable, and research would need to progress in other directions. However, chances of this occurring are hopefully low, due to swine having much more in common with humans than mice. The next danger is that while the *CHM^{null/Y}* model production will be successful and display retinal degeneration, it will be a very slow degeneration, as seen in the mice. Given that miniature pigs, which are typically used as the background for swine models, can live 12-28 years (147), it is possible that a swine model of CHM could take upwards of 5 years to display a disease phenotype, a time span that would not be ideal for translational studies but which would more accurately model the human condition, This problem could be approached using methods similar to those suggested above to address the slow degeneration in murine models, either by targeting expression of REP-2 for KD, or by attempting to hinder another part of the Rab prenylation pathway so as to exacerbate the degeneration.

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