A Comprehensive Review of Retinal Gene Therapy

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Blindness, although not life threatening, is a debilitating disorder for which few, if any treatments exist. Ocular gene therapies have the potential to profoundly improve the quality of life in patients with inherited retinal disease. As such, tremendous focus has been given to develop such therapies. Several factors make the eye an ideal organ for gene-replacement therapy including its accessibility, immune privilege, small size, compartmentalization, and the existence of a contralateral control. This review will provide a comprehensive summary of (i) existing gene therapy clinical trials for several genetic forms of blindness and (ii) preclinical efficacy and safety studies in a variety of animal models of retinal disease which demonstrate strong potential for clinical application. To be as comprehensive as possible, we include additional proof of concept studies using gene replacement, neurotrophic/neuroprotective, optogenetic, antiangiogenic, or antioxidative stress strategies as well as a description of the current challenges and future directions in the ocular gene therapy field to this review as a supplement.

Received 14 June 2012; accepted 8 December 2012; advance online publication 29 January 2013. doi:10.1038/mt.2012.280

INTRODUCTION

The eye is a complex sensory organ that has evolved to both promote survival and allow us an appreciation of the order and beauty of our surroundings. Rodieck wisely remarks in his prologue, "so immediate and powerful is seeing that our language gives this word additional connotations; to imagine, to comprehend, to regard, to perceive, to know from first-hand experience, to foresee."[1] Understandably, the loss of vision due to inherited or acquired retinal disease can affect one's life in significant and sometimes devastating ways. Over the past few decades, vision scientists have worked to delineate the underlying molecular events which contribute to these diseases. This knowledge, combined with thorough clinical characterization of patients has led to the development of gene-replacement strategies for numerous inherited and acquired retinal diseases. This review will summarize existing early-stage clinical trials for several forms of genetic blindness and a variety of preclinical studies in which gene transfer resulted in significant functional improvement and/or regeneration or stabilization of retinal structure in animal models of retinal disease. Focus will be given to the therapies which demonstrate the strongest potential for clinical application in the near future. Additional gene replacement, neurotrophic/neuroprotective, optogenetic, antiangiogenic, or antioxidative stress strategies as well as a description of the current challenges and future directions in the ocular gene therapy field are provided as a supplement (**Supplementary Data**).

IN CLINICAL TRIAL

RPE65-Leber congenital amaurosis

RPE65-Leber congenital amaurosis (LCA2) is associated with mutations in RPE65 (retinal pigment epithelium-specific 65 kDa protein). RPE65 is almost exclusively expressed in the RPE and functions as the retinoid isomerase responsible for converting *all-trans* retinoid to *11-cis* retinal during pigment regeneration.[2–4] Improper functioning or absence of RPE65 results in a lack of *11-cis* retinal production and an inability to efficiently form the visual pigments, rhodopsin and cone opsin. Concomitant accumulation of large amounts of all-*trans*-retinyl esters in the RPE is thought to promote photoreceptor degeneration. As in patients with LCA2, the absence of isomerase activity in the RPE65 knock-out mouse, mutant knock-in mouse and naturally occurring RPE65 mutant mouse and dog models results in retinal degeneration and severe visual impairment. The natural histories of patients with LCA2 and the aforementioned animal models as well as a summary of experiments that paved the way for clinical treatment of LCA2 has been recently reviewed by Cideciyan.[5]

Despite its rarity (less than 1 in 1,000,000 live births affected), because of its early onset and the availability of multiple animal models, a tremendous amount of attention has been focused on developing a gene-based therapy for LCA2. Results published in 2001 describing successful gene therapy using a recombinant adeno-associated virus (AAV2) vector containing RPE65 cDNA to treat three RPE65 mutant Briard dogs generated much excitement and optimism in the field.[6] In this study, subretinal delivery of a recombinant AAV2 vector carrying the canine *RPE65* gene, under the control of the hybrid cytomegalovirus/chicken β-actin (CBA) promoter, resulted in substantial improvements in visual function out to 3 months postinjection, as assessed by an improvement in the electroretinogram (ERG).[6] Subsequent follow-up studies by a number of groups would confirm and extend these results, as well as determine that subretinally delivered AAV1, AAV4, and AAV5-mediated RPE65 expression were also capable of restoring some

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level of function.[7–13] Most encouraging were the findings that gains in visual function remained stable over time.[8,9,13] Additional tests revealed that visually guided behavior was restored to treated dogs suggesting that retinal responses were being propagated to the visual processing centers of the brain.[6,7,12] Cortical responses were assessed using functional magnetic resonance imaging and, as expected, were dramatically improved as a consequence of AAV2-RPE65 treatment.[14]

In addition to the dog studies, gene-replacement studies utilizing AAV, lentiviral and adenoviral vectors carrying RPE65 were carried out in murine models of LCA2. The first LCA2 mouse model used in gene-replacement studies was the RPE65 knock-out (*Rpe65*$^{-/-}$) mouse generated and characterized by the Redmond laboratory.[15] Subretinal injection of AAV1-RPE65 resulted in measurable improvements in ERG responses in *Rpe65*$^{-/-}$ mice when treated at a variety of ages, as early as *in-utero,* with efficacy followed out to as late as 24 months of age.[16,17] Subretinal delivery of adenoviral-*RPE65* in RPE65$^{-/-}$ mice reconstituted retinoid isomerase activity as well as prevented the characteristic loss of cone photoreceptors exhibited by this model.[18] The *rd12* mouse, a natural occurring RPE65 mutant, has also been extensively utilized as a model for gene therapy.[19] AAV5-RPE65 improved ERG responses and visual guided behavior following subretinal injection in this strain.[20] Later experiments, in which *rd12* mice received subretinal injections of clinical grade AAV2-RPE65 aimed to establish an *in vivo* bioassay for characterizing AAV vector activity.[21] In this study, delivery of vector spanning 2 log units, 10e8 to 10e10 vg/μl, resulted in a clear dose-response relationship.[21] This bioassay was eventually used to evaluate stability of vector used during human clinical trials of LCA2.[22,23]

Due to their importance in daylight vision, particular attention has been focused on the fate of cone photoreceptors following AAV-RPE65 treatment. Cones utilize a noncanonical pathway for recycling of chromophore that is independent of the RPE, and enables them to function in continuous bright light.[24,25] Studies of affected patients with LCA2 suggest that, on the whole, cones are still dependent on RPE65 isomerase activity for their survival, even though some residual cone activity persists in the absence of the isomerase.[26] This is consistent with the natural histories of the *Rpe65*$^{-/-}$ and *rd12* mice; cone photoreceptors degenerate relatively quickly.[15,19] Several experiments in mouse models have specifically addressed cone survival as a consequence of gene therapy. Lentiviral and AAV-based vectors were used to treat RPE65 deficient mice that in some cases also lacked confounding rod function resulted in cone survival.[27,28] Later it would be shown that self-complementary AAV-RPE65 vectors were capable of restoring cone function and preventing cone degeneration in both a rod "functionless" RPE65 double knock-out mouse (*Rho*$^{-/-}$*Rpe65*$^{-/-}$) and the *rd12* mouse.[29]

In several of the previously mentioned studies, investigators evaluated the ability of gene therapy to improve function in older affected animals, thereby modeling treatment in mid to late stage disease. In dog studies, measurable improvements in ERG and behavioral performance were noted for dogs treated as old as 30 months of age.[30] Consistent visual improvements were possible in *rd12* mice treated at 3 months of age.[31] Even a small percentage (16%) of Rpe65$^{-/-}$ mice treated at 24 months of age showed significant improvements in ERG.[17] Taken together, these results suggested that patients with LCA2 qualifying for phase I trials (*i.e.,* as late as the 2nd decade of life or later), would have a reasonable chance of improved visual function upon treatment.

These proof of concept studies as well as Good Laboratory Practice safety studies would culminate with the multiple phase I/II gene therapy trials in human.[22,32–34] Results of four clinical trials of *RPE65* gene therapy for LCA2 have been independently initiated and interim outcomes published (NCT00481546, NCT00516477, NCT00643747; clinicaltrials.gov).[22,23,32–39] The longest follow-up to date is 3 years.[39] All three employed an AAV2 vector carrying a normal human RPE65 cDNA delivered subretinally to the worse eye. In spite of differences in the promoter employed, vector manufacturing details, dose and volumes delivered, anesthesia during vector delivery and postoperative steroid use, it was concluded by all studies that AAV-mediated subretinal gene therapy elicits no vector-related adverse events or toxic immune responses. All trials also reported clinical measures of vision improvement, but to various levels of detail and significance. A recent review of all reported clinical follow-up data up to 2010 has recently appeared.[5] Since then, one more complete 3-year follow-up study was reported by Jacobson *et al.*,[39] and the results of this study will be the primary focus here as it follows the largest cohort of treated patients for the longest time employing the most diverse measures of visual outcome.

Methods for measuring improvement in both light sensitivity and retinal function upon treatment of LCA2 had been previously developed in both mice and dog models of LCA2.[8,21,40–42] When these methods were adapted to the clinical trials, most treated subjects reported qualitative improvements in dim light sensitivity within a short time after vector administration.[22,32,34–37] To quantify these impressions, light sensitivity was quantified using a psychophysical full-field sensitivity threshold test (FST) that avoided the vision fixation instability common in these patients.[14,42,43] In the 3-year study,[39] there were statistically significant improvements from baseline in all 15 patients with posttreatment times varying among patients from 30 days to 3 years. FST improvements ranged from tenfold to 10,000-fold. The areas of dark-adapted (rod-mediated) visual field improvement, when assessed by microperimetry, corresponded well with the topological location of the subretinal bleb of vector mapped at the time of surgery, demonstrating that the retinal area responding to therapy is limited to that area receiving subretinal vector. Within this treated region, patients recovered as much as a 63,000 fold increase in their ability to detect in light. For some, this improvement was essentially all the functional gain possible given their baseline loss of photoreceptors.[22,35] In patients with the highest light sensitivity, gains in both rod and cone function could be clearly discerned. In a test aimed at assessing the usefulness of this improved retinal function, the ability of treated subjects to navigate a walking maze was quantified with improvement relative to baseline seen in five of six patients.[39]

Visual acuity, classically quantified by the smallest size of high contrast letters readable at a fixed distance,[44] was improved but with mixed statistical significance in nearly all patients in the three reported trials. However, in the context of retinal disease, particularly if foveal cones are affected, the location of highest acuity may not be within the normal human fovea.[45] When vector was delivered subfoveally, necessarily accompanied by transient

foveal detachment, patients with LCA2 often experienced no change in foveal light sensitivity but rather showed improvement extrafoveally.[22,33,35] In the 3-year study,[39] approximately half the patients experiencing a vector bleb that detached the fovea during surgery lost foveal thickness, presumably due to foveal cone loss, as assessed with follow-up optical coherence tomography (OCT) analysis. This suggests that foveal cones may be particularly sensitive to the potential damaging effects of subretinal vector-mediated foveal detachment, and that this locale for vector delivery should be cautiously approached in the future.

Related to foveal versus extrafoveal function after treatment is analysis of visual fixation, *i.e.,* the retinal location used by a patient when asked to fix their gaze on an object they could see. At baseline, most patients in the 3-year study fixated in an area encompassing their fovea.[39] Interestingly, at the 1-year follow-up visit but not before, one of the earliest patients treated reported the newly emergent ability to read the digital clock numbers in the family car.[36] In an attempt to understand this apparently new visual perception at 1 year after treatment, the patient's fixation was determined in response to a dim target that could not be perceived at baseline or at any earlier treatment follow-up visit. The treated eye now exhibited a shift in fixation away from the fovea and solidly into the superior-temporal retina that coincided precisely with the locale of the original vector bleb. This phenomenon has been described previously under quite different circumstances: as a result of macular degeneration, loss of foveal cone function can lead to a gradual fixation shift into an extrafoveal location with better function than the anatomical fovea.[46] This adaptation involves a change in central control of the ocular muscles to position images on this new, more light sensitive retinal region, the "pseudo-fovea", and likely originates as a slow visual cortex response to a new and better functioning retinal locus[47] arising through focal gene therapy. Presently, 6 of the 15 patients with LCA2 followed have developed similar pseudo-foveas upon multi-month delays after treatment (S. Jacobson and A. Cideciyan, personal communication, 2011). Such dramatic but relatively slow cortical plasticity in adults bodes well for other gene therapies for blinding retinal diseases that improve retinal function locally and suggests that the rate of generation of a useful pseudo-fovea may benefit from visual training.[48]

Most recently, readministration of AAV-RPE65 to the second, untreated contralateral eye is being investigated, with results out to 6 months indicating readministration is safe and efficacious in the previously naive eye.[49]

In summary, this first in human gene therapy for the RPE65 form of early retinal disease has been shown to be safe, free of serious complications, and effective at multiple clinical and visually useful levels. Efforts are currently underway to initiate a phase III clinical trial of AAV-RPE65 (NCT00999609; clinicaltrials.gov).

MERTK-associated autosomal recessive retinitis pigmentosa

The mer receptor tyrosine kinase (MERTK) is a member of the Axl/Mer/Tyro3 receptor tyrosine kinase family. MERTK is required for phagocytosis of photoreceptor outer segments by the RPE, and when absent leads to profound degeneration of the retina. The MERTK-associated form of arRP is very rare, with isolated patient populations identified in the Middle East and most recently the Faroe islands.[50–52] The Royal College of Surgeons rat, first described in 1938 by Bourne and colleagues, is a widely used model of recessively inherited retinal degeneration.[53,54] Retinal degeneration arises from the inability of the RPE to phagocytose shed PR outer segments, resulting in a subretinal debris field and subsequent photoreceptor loss via apoptosis.[55–57] Degeneration of the retina is accompanied by progressive attenuation of retinal function as evaluated by ERG.[58] In 2000, the retinal dystrophy locus of the Royal College of Surgeons rat was determined to be the *MERTK* gene.[59] Follow-up studies identified patients with autosomal dominant retinitis pigmentosa (ADRP) with homozygous mutations in MERTK, conclusively linking this gene to the disease.[60] Rescue experiments in the Royal College of Surgeons rat have included gene replacement as well as supplementation with neurotrophic factors aimed at arresting the degeneration and/or preserving the retina. In gene-replacement studies, adenovirus, AAV, and lentivirus have all been utilized.[61–63] The most successful of these early studies utilized a lentivirus expressing MERTK and was able to preserve measurable retinal function out to 7 months postinjection.[63] Interestingly, coadministration of lenti-*Mertk* and AAV expressing Glial-cell derived neurotrophic factor was more effective than lenti-MERTK alone.[64] Most recently, an AAV8 Y-F capsid mutant vector was shown to preserve retinal structure and function in the Royal College of Surgeons rat out to at least 1 year after treatment.[65] Presumably, the fast-acting nature of the AAV8 Y-F vector and relatively early (postnatal day 2) treatment restored MERTK expression before appreciable formation of a debris field, thus bypassing the need for supplementation with neuroprotective agents to prevent the downstream apoptosis of photoreceptors.[65] A phase I clinical trial utilizing an AAV2 vector with an RPE-specific promoter driving MERTK has been initiated in Saudi Arabia using vector manufactured at the University of Florida (NCT014822195; clinicaltrials.gov). At this point in time, three patients have been treated by subretinal injection with no adverse events recorded (F. Alkuraya and E. Abboud, personal communication, 2012).

Usher syndrome

Usher syndrome (USH) refers to a clinically and genetically heterogeneous group of autosomal recessive disorders which account for the most frequent cause of combined deafness and blindness in humans with an estimated prevalence of 3–6 per 100,000 individuals, although that is likely an underestimation given the frequent false diagnosis of retinitis pigmentosa (RP) in infants.[66,67] There are three clinical subtypes of USH (USH1, USH2, and USH3), distinguished by the severity and progression of hearing loss and the presence or absence of vestibular dysfunction, with visual loss due to RP being common to all three subtypes.[68,69] USH1 is the most severe form in terms of the onset/extent of hearing loss and RP. Currently, mutations in five genes are known to be associated with USH1.[70] The proteins encoded by these genes are expressed in cochlear hair cells of the inner ear as well as photoreceptors, both of which are highly specialized neurosensory cells with many structural and functional similarities. All five proteins are essential for the development and stability of the hair bundle in the inner ear as lack of their expression can prevent hair cell development altogether. Restoration of hearing

via gene therapy may therefore only be possible in patients with milder USH where some hair cells are retained. In contrast, photoreceptors tend to develop normally but are progressively lost due to impaired protein transport through the connecting cilium and/or synapse dysfunction. Taken together, this suggests that in terms of gene replacement, a therapeutic window may exist primarily for the retinal phenotype of various forms of USH.

The first USH1 gene identified was *myosinVIIa*[71] (*MYO7A*) which encodes an actin-based molecular motor that performs critical functions in both the inner ear and retina. Mutations in MYO7A cause Usher syndrome type 1b (Ush1b) and account for ~60% of all USH1.[68] Patients with Ush1b are born profoundly deaf, have vestibular dysfunction and develop retinal degeneration in childhood.[72] In the retina, MYO7A is expressed in RPE, the photoreceptor connecting cilia and photoreceptor synapses[73–75] and plays a role in multiple cellular processes including intracellular transport, endocytosis and cell–cell adhesion.[76] The *shaker1* mouse model of Ush1b carries a mutation in *Myo7a,* is deaf and has vestibular dysfunction. Unlike patients with Ush1b, photoreceptors in this mouse model do not degenerate. A recent study demonstrates the existence of MYO7A in the calyceal processes of human photoreceptors and suggests that defects in this structure resulting from mutations in USH1 proteins are responsible for the retinal degeneration in patients with USH1.[70] The lack of retinal degeneration in *shaker1* mice (and other mouse models of USH) could be due to the fact that mice lack calyceal processes. However, *shaker1* mice do have retinal phenotypes which distinguish them from wild type including mislocalized RPE melanosomes with defective motility and abnormal opsin transport through the connecting cilium.[77–80] The requirement of MYO7A for the apical localization of melanosomes has also been demonstrated in human RPE cells.[81]

Initial proof-of-concept studies demonstrated that RPE melanosome localization and opsin transport could be restored in the *shaker1* mouse following subretinal injection of a lentivirus carrying of *Myo7a*.[82] These results led to a phase-1 clinical trial initiated by Oxford Biomedica UK employing an equine infectious anemia virus (EIAV) lentiviral vector to evaluate safety of subretinally delivered *MYO7A* in patients with Ush1b (NCT01505062; clinicaltrials.gov). However, lentiviral transduction is restricted primarily to the RPE following subretinal injection of postnatal retina.[83,84] Because photoreceptors are the site of earliest disease expression,[85] there is an obvious need to adequately transduce this cell type in patients with Ush1b. Following the assertion that AAV vectors were capable of packaging full-length *MYO7A*[86] (~9 kb), work began to develop an AAV-based therapy for Ush1b. It is now understood that the packaging of large DNAs such as *MYO7A* initiates primarily from the 3′ end and proceeds until the AAV capsid reaches capacity (~5 kb). Resultant single-stranded vector DNAs are therefore heterogeneous in length and truncated at their 5′ ends.[87–89] It is thought that full-length large-gene reconstitution occurs from these incomplete cDNAs via either recombination between overlapping homologous internal cDNA sequences or direct annealing of vector-packaged, opposite-polarity large-transgene fragments followed by host cell DNA repair synthesis on both DNA strands primed by their free 3′ ends.[87–89] Such "heterogeneous" AAV vector technology has since been used to deliver *Myo7a* to the subretinal space of *shaker1* mice. Both serotype 2 and serotype 5 AAV vectors were capable of delivering full-length *Myo7a* to photoreceptors and RPE and correcting both melanosome localization and opsin transport in treated *shaker1* mice.[90,91] A hurdle for clinical application of heterogeneous vectors is the inability to discretely characterize the genomes within each capsid. As such, development of dual AAV vector platforms are currently underway for the treatment of Ush1b using vectors containing discretely characterized DNA payloads (**Supplementary Figure S2**). These will be described in more detail in the online supplement.

Stargardt disease, recessive *ABCA4* form

Stargardt disease is a common form of juvenile macular degeneration, the most prevalent form being recessively inherited and associated with mutations in the photoreceptor-specific ABCA4, a member of the superfamily of ATP-binding cassette (ABC) transporters.[92] ABCA4 functions as a "flippase" of *N*-retinylidene-phosphatidylethanolamine, thereby transporting it from the lumen to the cytoplasmic side of photoreceptor outer disks.[93] Mutations in ABCA4 result in decreased transport of *N*-retinylidene-phosphatidylethanolamine and phosphatidylethanolamine which is thought to lead to the formation of toxic retinoid compounds, eventually manifesting as an accumulation of lipid known as lipofuscin in the RPE, primarily composed of *N*-retinylidene-*N*-retinylethanolamine (A2E).[94] The structure/function and biology of ABCA4 have been recently reviewed by Molday *et al*. (insert same reference as above). Mice lacking abca4 (abca4$^{-/-}$ or alternatively abcr$^{-/-}$), the murine homolog to *ABCA4*, also show an increased accumulation of A2E and the formation of lipofusin granules.[95] The size of the ABCA4 cDNA is 6.8 KB and therefore beyond the conventional packaging capacity of AAV. As such, the same approaches for gene replacement discussed for MYO7A Ushers1b ("heterogeneous" AAV and EIAV lentivirus) have been employed in the abca4$^{-/-}$ mouse.[86,96] In both studies, correction of phenotype (improved recovery of photoreceptor desensitization and/or reduction of liposfuscin) in treated abca4$^{-/-}$ was observed. It is important to note that in the EIAV lentivirus study, injections were performed at P4 to P5, before photoreceptors had reached terminal differentiation, whereas AAV injections were performed at 1 month of age, in postmitotic retina.[86,96] On the basis of the positive results reported in Kong *et al*.,[96] Oxford BioMedica has initiated a phase I/II clinical trial utilizing the EIAV lentiviral platform to deliver *ABCA4* to patients with Stargardt (NCT01367444; clinicaltrials.gov). Most recently, nanoparticle delivery of *ABCA4*[97] into abca4$^{-/-}$ mice has also show to improve the retinal phenotype using the same criteria as mentioned above.

Choroideremia

Mutations in the *CHM* gene are associated with choroideremia, an X-linked retinal degeneration affecting 1:50,000 males that is readily diagnosed with fundus examination.[98–102] Initially, patients exhibit retinal thickening, followed by photoreceptor degeneration, RPE depigmentation, and retinal remodeling. Affected children and carrier females have mottled areas of pigmentation whereas affected males display loss of RPE and choriocapillaris. Affected males develop night blindness in their teens which progresses to loss of peripheral visual field and more profound blindness within three decades of onset.[103,104]

CHM encodes Rab Escort Protein 1 (REP-1), a ubiquitously expressed protein required for the efficient geranylgeranylation of *ras*-related GTPases, or Rab proteins, which are integral to the trafficking of vesicles in endocytic and exocytic pathways.[100,105,106] Geranylgeranylation, or prenylation, requires the activity of both a transferase (RabGGTase) and REP-1 which tethers the unprenylated Rab substrate to the RabGGTase and then "escorts" the newly prenylated Rab protein to a specific cellular membrane.[107] Patients with chroroideremia express little or no REP-1,[98,108,109] and it has been suggested that this lack of protein affects opsin transport to photoreceptor outer segments, apical migration of RPE melanosomes and the phagocytosis of photoreceptor outer segments by the RPE.[110] Indeed, siRNA knockdown of REP-1 in human fetal RPE cells revealed that photoreceptor outer segment internalization was unaffected but that protein clearance was delayed due to inhibition of phagosome–lysosome fusion events.[111] Generation of conditional knock-out mice revealed that disease pathogenesis involves independently triggered degeneration of photoreceptors and RPE, each associated with their own Rab prenylation defects.[112] Newer conditional mutants in which *Chm* is knocked out in either the RPE or photoreceptors, independently, reveal that despite the cell autonomous hallmarks of this disease, RPE defects accelerate photoreceptor degeneration.[113]

As a first step towards developing a clinical gene therapy trial for choroideremia, recombinant adenovirus was used to deliver full-length human REP-1 to defective lymphocytes and fibroblasts isolated from patients with choroideremia.[114] Investigators showed that vector-mediated REP-1 expression restored RabGGTase activity *in vitro*. More recently, lentiviral-mediated delivery of REP-1 to the RPE-specific *Chm* knock-out mouse revealed increases in prenylation activity in the RPE of treated mice.[115] Although this study served as proof of concept for restoring function to cells carrying *Chm* mutations, the authors acknowledged that more efficient photoreceptor transduction might be required to restore function to the neural retina. In the fall of 2011, a clinical trial for the treatment of choroideremia was initiated. This 12-subject safety study employed an AAV2 vector to deliver REP-1 to the subretinal space of affected patients.[116] Treatment safety and efficacy will be evaluated over the next 1.5 years.

Exudative (wet) age-related macular degeneration

Vascular endothelial growth factor (VEGF) is the major proangiogenic factor promoting neovascularization of the choroidal vasculature in exudative (wet) age-related macular degeneration (AMD), the leading cause of blindness in those above 65.[117,118] Inhibition of VEGF with antibodies, RNA aptamers or soluble receptors has been clinically tested for managing AMD and has shown promise.[119–122] Current practice using various versions of VEGF antibody requires long term and repeated intravitreal injections and invariably involves the cumulative risk of repeated intraocular injections and significant financial burden. Soluble FLT-1 (sFLT1) is a portion of the R1 VEGF receptor that binds extracellular VEGF and prevents it from binding to its normal cell surface vascular endothelial receptors FLT-1 and FLK. Ocular neovascularization was inhibited in animal models upon subretinal AAV2 carrying full-length sFlt1 (domains 1–7)[123,124] and treatment was also shown to be safe in nonhuman primates.[125]

Recently, an AAV2 vector with a novel soluble chimeric protein (AAV2-sFLT01) was shown to be both persistently expressed after a single injection and therapeutic at controlling neovascularization in a murine model when delivered intravitreally,[126] a much less traumatic route of administration than subretinal vector. The protein encoded by this vector is a hybrid comprised of Flt-1 domain 2 linked to a human immunoglobulin G1 heavy chain Fc fragment. When expressed, an extracellular homodimer forms that binds to VEGF with high affinity and avidity. Intravitreal AAV2-sFLT01 efficacy was confirmed in two mouse models of ocular neovascularization, the oxygen-induced retinopathy of prematurity model and the laser-induced choroidal neovascularization (CNV) model[127,128] and was recently extended to an AAV8 vector.[129] The Lukason *et al*. study[128] also included analysis of therapy in the nonhuman primate laser-induced CNV model of wet AMD. Stable sFLT01 expression was observed for 5 months in the aqueous humor. Laser treatment was initiated at 22 weeks postvector injection, and a significant reduction of CNV was documented in seven of eight treated eyes whose levels of sFLT01 in the aqueous humor exceeded 100 ng/ml, suggesting a potential minimum effective level of sFLT01 is needed to observe a reduction in CNV. Based on these data and formal safety/biodistribution studies establishing safe vector doses in the monkey, Genzyme/Sanofi has initiated a phase-1 clinical trial using intravitreal AAV2-sFLT01 for neovascular AMD[130] (NCT01024998; clinicaltrials.gov).

Endostatin, a collagen XVIII cleavage product, and angiostatin, a fibrinogen cleavage product, both inhibit tumor angiogenesis.[131,132] With adenoviral 5-endostatin vectors delivered systemically to laser CNV mice, serum endostatin levels correlated well with CNV inhibition.[133] More direct vitreal injection of either AAV-endostatin,[134] AAV-angiostatin (K1-3 domains)[135] or lentiviral vector-angiostatin[136] also significantly inhibited murine CNV. These data have led in part to a phase-1 clinical trial initiated by Oxford Biomedica (Oxford, UK) employing an EIAV lentiviral vector expressing both endostatin and angiostatin for late stage AMD (NCT01301443; clinicaltrials.gov). Results are pending.

PRECLINICAL

GUCY2D-Leber congenital amaurosis

One of the most frequently mutated *LCA* genes is guanylate cyclase-1 (*GUCY2D*).[137–139] A recent study evaluating the spectrum of causative mutations in an Asian population of 87 patients with LCA identified mutations in *GUCY2D* as the most common cause of the disease (~16%).[140] *GUCY2D* encodes the retina-specific protein guanylate cyclase-1 (GC1) which is expressed in the outer segments of rod and cone photoreceptors of human, monkey and mouse retinas.[141,142] GC1 plays a vital role in the light–dark and recovery cycle, anchoring, via cGMP, the feedback loop linking intracellular calcium levels and the polarization state of photoreceptors.[143] Like other membrane guanylatecyclases, it contains an N′-terminal signal sequence, an extracellular domain, a single transmembrane domain, a kinase-like homology domain, a dimerization domain and a C′-terminal catalytic domain, and is present likely as a homodimer.[144] LCA1-causing mutations are distributed throughout GC1,[145,146] can alter enzyme structure and stability, impact retrograde transport of other peripheral membrane associated proteins and are frequently null.[145,146] Mutations that reduce or abolish the ability of GC1 to replenish intracellular cGMP and reopen cGMP-gated cation channels, as is the case in

LCA1, are thought to create the biochemical equivalent of chronic light exposure in rod and cone photoreceptors. A hallmark feature of LCA1 is preserved retinal appearance. Patients present in infancy with a normal fundus and, despite having an unrecordable cone ERG and abnormal/unrecordable rod ERG, patients retain normal photoreceptor laminar architecture except for mainly foveal cone outer segment abnormalities.[138,146–148] Maintenance of retinal structure in LCA1 is unlike other forms of the LCA which exhibit marked retinal thinning that generally worsens with age. Taken together, the prevalence of *GUCY2D* mutations and the maintenance of retinal structure in patients with LCA1 over time suggest they may be good candidates for gene-replacement therapy.

The GC1 knock-out (GC1ko) mouse exhibits a loss of cone function that precedes cone degeneration.[149] Rod photoreceptors maintain 30–50% of their function and do not degenerate, a result likely due to the presence of guanylate cyclase-2 (GC2), a close relative of GC1, in those cells. AAV5 vectors containing *Gucy2e* (the mouse homologue of *GUCY2D*) and either the ubiquitous smCBA or photoreceptor-specific human rhodopsin kinase (hGRK1) promoter restored cone function (ERG), cone-mediated behavior and preserved cone photoreceptors in the GC1KO mouse for at least 3 months.[150] Subsequent studies have demonstrated that AAV8 and AAV5 based vectors containing the hGRK1 promoter driving either *GUCY2D* or *Gucy2e,* respectively, restore photoreceptor function, cone-mediated behavior and preserved cones over the long term.[151,152] These results suggest that therapy persists over the lifetime of the treated animal. Because patients with LCA1 have either absent or abnormal rod photoreceptor function, it was important to ask whether AAV-mediated GC1 expression could restore function to rods in a model with physiologically "silent" photoreceptors. The GC1/GC2 double knock-out (GCdko) mouse was generated[153] to suit this need. Like some patients with LCA1, these mice also exhibit complete loss of both rod and cone function. AAV5 and AAV8 (Y733F) vectors containing the hGRK1 promoter and murine GC1 proved capable of restoring both rod and cone function (ERG), rod- and cone-mediated visual behavior and preserving rod and cone structure following subretinal delivery to the GCdko mouse.[154] Related to this, a recent study showed that the hGRK1 promoter has exclusive activity in both rods and cones following subretinal injection of AAV5-hGRK1-GFP in nonhuman primate.[155] This study validated that AAV5 is capable of transducing foveal, parafoveal, and perifoveal cones as well as rods of NHP (**Supplementary Table S1**). Taken together, these results suggest that AAV5 containing the hGRK1 promoter and *GUCY2D* cDNA is a suitable choice of clinical vector intended for the treatment of LCA1.

Achromatopsia

The complete form of achromatopsia (ACHM) is an autosomal recessive disease that affects approximately 1 in 30,000 individuals and is associated with the loss or absence of cone function. ACHM is marked by poor central visual acuity (<20/200) as well as photophobia, complete color blindness and reduced cone-mediated ERG response amplitudes. It is an attractive disease for gene-replacement therapy as cone photoreceptors are present and often fairly intact in affected individuals. Although initially thought to be a stationary disease based on normal macular appearance, it has recently been shown that cone degeneration begins early in childhood, with degeneration progressing at a modest rate.[156] Mutations in genes encoding subunits of the cone-specification channel, cyclic nucleotide gated channel α and β 3, *CNGA3* and *CNGB3*, account for a combined 80% of all ACHM cases.[157,158] Mutations in two genes associated with phototransduction in cones, cone-specific α subunit of transducin, *GNAT2*, and α subunit of cone-specific phosphodiesterase, *PDE6C* are much rarer, accounting for perhaps no more than 5% of cases combined.[159–161] The first report of gene therapy for ACHM was performed in the Gnat2^{cpfl3} mouse[162] which carries a recessive mutation in *Gnat2* resulting in little to no cone-mediated ERG and poor visual acuity.[162,163] Subretinal injection of AAV5 driving *Gnat2* under the control of the human red cone opsin promoter, restored cone mediated ERG amplitudes and cone-mediated behavioral responses to levels indistinguishable from age-matched wild-type mice.[162] Gene-replacement therapy has also been tested in two large animal models of ACHM. AAV5 containing human *CNGB3* was used to treat two independent canine models of *CNGB3*-ACHM.[164] Vector-treated dogs exhibited restored cone function and day vision, however the magnitude and persistence of therapy was dependent on both the age at treatment and the promoter used.[164] Affected dogs that were treated at a relatively young age with vector utilizing the human red cone opsin promoter exhibited stable treatment effect out to at least 33 months.[164] Importantly, GNAT2 and CNGA3 which are absent or mislocalized in untreated cones, showed normalization of protein expression and localization to cone outer segments in treated eyes.[164] Gene-replacement studies in mouse models of both the *CNGA3* and *CNGB3* forms of ACHM have also shown success using AAV5 and AAV8 based vectors.[165–167] As with the *CNGB3* dog study, treated mouse eyes show a restoration of expression and normal localization of cone-specific proteins following treatment.[165–167] Given the high density of cones in the macula and the previously mentioned challenges encountered when delivering vector subretinally under the macula, the development of vectors capable of transducing photoreceptors following intravitreal delivery will be highly advantageous for moving ACHM gene-replacement therapy into the clinical trial phase.

X-linked juvenile retinoschisis

X-linked juvenile retinoschisis (XLRS) is the leading cause of monogenic macular dystrophy in males with a prevalence between 1:5,000 and 1:25,000.[168] It is characterized by localized schisis, or splitting in the retina which can occur in all layers.[169,170] Patients with XLRS exhibit an unusual electronegative ERG wherein the a-wave is relatively preserved whereas the b-wave is severely diminished[171] due presumably to a relatively preserved photoreceptor response but a loss of interaction with second order retinal neurons. XLRS is considered a stationary condition with retinal presentation in early childhood, exacerbation of symptoms in teenage years and stabilization in adulthood.[168,172] Schisis lesions become less obvious in the fourth to fifth decade but complications may still arise from macular atrophy, vitreous hemorrhage, or retinal detachment.

XLRS is caused by mutations in the *retinoschisin* (*RS1*) gene which encodes a 24 kDa protein (RS1) that is expressed in the retina and pineal gland.[173,174] RS1 is secreted from retinal neurons, most likely photoreceptors, as a homo-octamer that adheres to retinal cell surfaces.[175] Although the function of this protein is not completely

understood, its discoidin domain suggests that, like other discoidin domain-containing proteins, it may be involved in cell adhesion or cell-extracellular matrix interactions.[175,176] The RS1 knock-out (Rs1h-KO) mouse yields many hallmark features of the human condition including electronegative ERG waveforms and the presence of schisis cavities.[177–179] Several groups have reported that gene supplementation of the normal Rs1h protein in Rs1h-KO mice confers therapy to this mouse model. Initially, it was shown that intravitreal delivery at 13 weeks of age of a serotype 2 AAV vector containing the ubiquitous cytomegalovirus promoter and the murine RS1 cDNA to Rs1h-KO mice restored function (ERG) to this model out to 6 months of age.[177] Subretinal injections of serotype 5 AAV containing the photoreceptor-specific murine opsin promoter and the human RS1 cDNA into younger Rs1h-KO mice (P14) revealed more pronounced preservation of retinal structure and function.[180] Using the initial AAV2 vector, long-term (14 month) functional and structural improvements were next reported following P14, intravitreal injection.[179] Window of therapy was addressed in a subsequent report showing that AAV5-murine opsin-hRS1 vector improved retinal structure and function following subretinal injections in P15, 1 month and 2 month old Rs1h-KO mice (**Supplementary Figure S1**). Injections at 7 months of age, however, improved only retinal structure (assayed via photoreceptor survival and retention of cone outer segments) and not ERG.[181] A closer examination of protein expression and morphology of the outer plexiform layer revealed that P14 injection of AAV2-cytomegalovirus-mRS1 resulted in increases in postsynaptic density protein (PSD95) and bipolar cell metabotropic glutamate receptor subtype 5 (mGluR6), decreases in glial fibrillary acidic protein and improvements in retinal function out to 8 months of age.[182] Most recently, it was reported that a serotype 8 AAV vector containing a truncated human retinoschisin promoter (hRSp4) and the human RS1 cDNA was capable of improving structure and function out to 15 weeks after intravitreal delivery to 6–7 week old Rs1h-KO mice.[183] These studies highlight that multiple parameters including delivery method, vector serotype and promoter should be carefully considered prior to clinical application of an XLRS gene therapy vector.

Autosomal dominant RP

Mutations in at least 22 genes lead to ADRP. Diseases may be dominantly inherited for three reasons: haploinsufficiency, dominant negative gene product or toxic gain-of-function. Haploinsufficiency implies that the amount of product produced by a single wild-type allele does not support normal function. Dominant negative mutations typically interfere with the ability of the wild-type protein to reach its appropriate destination in the cell or to assemble in a multi-subunit complex. Mutations in ELOVL4 appear to be dominant for this reason.[184] Toxic gain-of-function mutations create proteins that are themselves harmful to the cell. An example in the retina would be the Q344ter mutation in rhodopsin (RHO), which causes rhodopsin to mislocalize to the cell bodies of rod photoreceptor cells.[185]

In the case of haploinsufficiency, gene therapy requires delivery of a wild-type cDNA so that the level of the normal protein becomes sufficient to support retinal health. Cai *et al*. have used compacted DNA nanoparticles to treat a dominant loss-of-function of mutation of PRPH2 in mouse model of retinal degeneration.[186] Gene therapy for dominant negative mutations or for toxic mutations is more complicated, however, since the dominant allele may require silencing. Several groups have employed small RNA technology including ribozymes and RNA interference to suppress the production of mutant. For example, in a rat model of ADRP caused by the P23H mutation of rhodopsin, AAV delivery of allele specific hairpin and hammerhead ribozymes led to long-term (6 month) rescue of photoreceptors as measured by histology and by the ERG response.[187,188] The major obstacle to mutation-specific silencing is allelic heterogeneity. There are more than 100 dominant mutations in the *RHO* gene alone, and it is impractical to generate a sequence specific inhibitor for each mutation. For this reason, a mutation-independent approach in which ribozymes, or more recently siRNAs, are being designed to target regions of mRNA that are not affected by mutation, so that both wild-type and mutant RNA are degraded.[189] As the RNA inhibitors (ribozymes, siRNAs or synthetic miRNAs) themselves lead to retinal degeneration, for therapy, they must be coupled with the delivery and expression of cDNAs bearing silent mutations that render them resistant to the codelivered ribozyme or siRNA.[190–197] Millington-Ward *et al*. recently used coinjection of 2 AAV vectors to significantly reduce the rate of retinal degeneration in a mouse line bearing a dominant P347S mutant transgene.[194] Mao *et al*. demonstrated that delivery of an siRNA designed to target mouse *Rho* and human P23H RHO mRNA as well as a resistant *RHO* gene, both contained within a single AAV vector, conferred long-term (9 month) rescue of retinal function and preservation of retinal structure following subretinal injection of P23H mice.[198]

Instead of inhibiting gene expression by degrading mutant mRNAs, Mussolino and colleagues used zinc-finger transcription factors to suppress the synthesis of P347S rhodopsin transgenic mouse model of autosomal dominant RP.[199] Silencing of the mutant human transgene led to increased viability and function of photoreceptors in the region of the retina infected by AAV. By reducing the size of the promoter of the gene targeted by the transcription factor (e.g., human RHO), a resistant wild-type gene could be delivered in conjunction with the Zn-finger protein.

However the "suppress and supplant" approach to gene therapy may not be necessary for treatment of dominant mutations in the *RHO* gene. In a mouse model of ADRP caused by a P23H human *RHO* transgene, Mao *et al*. recently demonstrated that simple AAV delivery of a wild-type mouse cDNA could significantly retard retinal degeneration as measured by ERG amplitudes and morphometry.[200] They concluded that P23H must be a dominant negative rather than a toxic gain-of-function mutation, and that treatment could be effected by altering the balance between mutant and wild-type forms of rhodopsin.

Because protein misfolding is believed to contribute to the pathogenesis associated with a large class of rhodopsin mutations, gene transfer of molecular chaperones is also an attractive approach for ADRP therapy. Gorbatyuk and colleagues used AAV to increase the expression of Grp78, an Hsp70 family member that resides in the endoplasmic reticulum[201] and reported significant improvement in ERG amplitudes and survival of photoreceptors in treated eyes. This rescue was not associated with increases in the level of properly reconstituted rhodopsin but rather with suppression of the unfolded protein response.

Leber hereditary optic neuropathy

Mitochondrial Leber hereditary optic neuropathy (LHON) is a retinal disease of ganglion cells that leads to blindness in young adults.[202] A G-to-A transition in mitochondrial DNA at position 11778 in the gene encoding subunit 4 of NADH dehydrogenase (ND4) leads to a position 340 arginine-to-histidine substitution that is responsible for approximately half of LHON cases. In contrast to other mitochondrial diseases, LHON vision loss requires essentially all copies of the mitochondrial DNA to be mutated. Therefore, a small amount of normally functioning ND4 is likely to be therapeutic and may protect against blindness. Until recently,[203] there had been no reports of successfully delivering a DNA to mammalian mitochondria, so this approach had not been an option. One alternative for ND4 LHON is to express wild-type ND4 protein in the cytoplasm by delivering an ND4 cDNA to the nucleus that is recoded in the cytoplasmic triplet code rather than the mitochondrial code and, in addition, contains an appended N-terminal mitochondrial targeting sequence to mediate efficient organelle import as has been done earlier in yeast.[204,205] Qi *et al.*[206] showed that this approach would create the LHON phenotype in a normal mouse by delivering mutant 11778 human ND4 cDNA in an AAV 2 vector to the mitochondria of retinal ganglion cells (RGCs) in a normal mouse. Using electrophoresis to transduce RGCs, Ellouze *et al.*[207] then similarly introduced the mutant 11778 human ND4 into rats and showed that mitochondrially targeted allotopic wild-type ND4 was sufficient for rescue of RGC loss and visual function induced earlier by delivery of mutant G11778A ND4. The safety of such an allotopic wild-type human ND4 gene delivery was then shown in a mouse with a FLAG-tagged, recoded, mitochondrially retargeted human ND4 delivered intravitreally by an AAV2 vector with the CBA promoter.[208] FLAG-tagged ND4 transgene product was localized to the mitochondria of RGCs. Moreover, treatment did not result in a loss of RGCs, alteration in the rates of mitochondrial ATP synthesis or loss in pattern evoked ERG signals. Together, these data support the idea that allotopic mitochondrial gene delivery can be an effective and safe mode of therapy for LHON.

SUPPLEMENTARY MATERIAL

Table S1. Transduction profiles of AAV serotypes across species.
Figure S1. Schematic of subretinal versus intravitreal injection routes.
Figure S2. Dual AAV vector platforms.
Data.

ACKNOWLEDGMENTS

We thank Jim Peterson for his editorial assistance. We acknowledge NIH grants EY021721, R24EY022012, T32 EY00713 and grants from the Macula Vision Research Foundation, Foundation Fighting Blindness, Eldon Family Foundation, Creed's Cause Foundation, Vision for Children and Research to Prevent Blindness, Inc. for partial support of this work. W.W.H. and the University of Florida have a financial interest in the use of AAV therapies, and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work. The other authors declare no conflicts of interest.

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