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An analysis of retinal gene therapy clinical trials: successful translation will depend on successful translation

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Abstract

In 2008 the initial results from the first three retinal gene therapy trials using adeno-associated viral vectors to treat an inherited retinal degeneration were published. There were no significant vector-related side effects and there was evidence of successful gene transfer with improved vision in several patients. The success of these trials heralds the beginning of a new era in treatments for retinal diseases. Much can be learnt by comparing the results of the studies as each has subtle differences, both in surgical technique and in vector design. In contrast to laboratory models, humans generally harbour missense rather than null mutations and are treated much later, when recipient cells are compromised by the disease process. Intracellular stress responses, such as those regulated by endoplasmic reticulum protein kinase (PERK) and the mammalian target of rapamycin kinase (mTOR) pathways, are likely to inhibit translation of transgenic mRNA by mechanisms that will not be evident in null laboratory models treated early in the disease process. Understanding ways to overcome stress responses is likely to be a critical step in translating applications of gene therapy from animal models to other human retinal diseases.

Keywords

RPE65; LCA; AAV; clinical trial; retinitis pigmentosa; RP; genetics

Introduction

The photoreceptor can capture a single photon of light and convert this energy into a neuronal response; a process that is dependent on an intricate protein structure and high energy demand, both of which require a large number of genes for normal function. This dependency may explain why genetic diseases seem disproportionately to affect the photoreceptor compared to other cell types. Certain photoreceptor genes, such as those regulating transcription and translation of rhodopsin, need such a high level of expression that malfunction can result from haploinsufficiency of general splicing factors which may not be manifest elsewhere in the body. Furthermore, the high energy environment of the subretinal space, coupled with sustained exposure of these cells to light, may explain why malfunctioning photoreceptor cells frequently undergo a progressive degeneration by apoptosis. The mechanism of sight loss in the estimated 1 in 3,000 people with a diagnosis of retinitis pigmentosa is therefore a combination of both loss of function and loss of cells. The relative contribution from each for any given retinitis pigmentosa sufferer has until recently been academic, but successful reports emerging from three independent retinal gene therapy trials this year have shown that the loss of function element may be treatable [1-3], thereby raising new questions. It is not yet clear if the progressive retinal degeneration will stop if the defective gene is replaced at a late stage of the disease when many cells have already been lost. It is also not yet clear if missense mutations have latent dominant negative

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effects that become manifest following viral vectored replacement of the wildtype gene. The answers to these questions will determine the future success and widespread applicability of retinal gene therapy in clinical practice. Humans differ from mouse knockout models of retinal degeneration in generally having missense rather than null mutations that cause recessive disease and treatments are likely to be applied at much later stages when mRNA translation may be suppressed by stress responses. The clinical trials have at last provided us with the opportunity to find out if our laboratory predictions over the last decade will also be valid for patients in practice. This brief review will therefore discuss the clinical applications of retinal gene therapy in light of the recent clinical trials.

Disease

Gene therapy in its least complicated application might be considered a means of replacing or augmenting the expression of a wild-type gene, the deficiency of which would otherwise lead to a chronic degenerative disease process. This concept of gene replacement has been the aim of all three current clinical trials, using adeno-associated viral (AAV) vectored replacement of the gene encoding a retinal pigment epithelium-specific 65 kilodalton protein (RPE65) vital for the visual cycle. Preclinical work leading up to the trials has shown efficacy of the concept of gene therapy in the naturally occurring Briard dog [4] and mouse [5] models of inherited RPE65 deficiency. Prior to the current RPE65 clinical trials, two clinical studies explored more complex applications for ocular gene therapy using recombinant adenoviral vectors to elicit transient therapeutic effects. One trial used adenovirus to deliver herpes simplex thymidine kinase followed by ganciclovir to eliminate retinoblastoma cells seeded into the vitreous in 8 patients and showed some efficacy without significant side effects [6]. In the other study, adenovirus was used to deliver human pigment epithelium-derived factor (PEDF) also by intravitreal injection of vector to inhibit choroidal blood vessel growth in 28 patients who had advanced neovascular age-related macular degeneration. This adenoviral vectored gene therapy was also well tolerated, with no significant side effects and some evidence of efficacy during the study period [7]. The earlier studies with adenovirus, however, were designed to give temporary therapeutic effects and must be distinguished from the current clinical trials using AAV, which employ a vector that is designed to give indefinite expression of a naturally occurring gene that is deficient in the diseased state.

The choice of RPE65 for gene replacement in all three of the clinical trials reflects a general agreement that this disease is ideal for testing the concept of retinal gene therapy. This is because RPE65 is an essential enzyme in the visual pathway and any deficiency will lead to a loss of vision that is functional as well as anatomical, particularly in earlier stages of the degeneration. RPE65 has been identified as the isomerase in the pathway that recycles all-trans retinal to the visually sensitive 11-cis form [8]. This is also a key process in dark adaptation, whereby vision becomes clearer during the first few minutes of entering a darkened room. Hence successful RPE65 gene replacement should be manifest as improved vision, particularly in darker (scotopic) conditions, in addition to halting or slowing the photoreceptor cell loss. This provides almost instant feedback on the success of retinal gene transfer, which is ideal for testing the concept of retinal gene therapy for the first time. Indeed all three RPE65 clinical trials showed evidence of improved visual function. The Bainbridge study showed improved perimetry and navigation through a dimly lit maze in one patient [1]. The Maguire study showed improvements in visual acuity, visual field testing, navigation and pupil responses in all three patients [2]. The Hauswirth study also showed improvements in rod and cone function in transduced areas in a detailed follow-up analysis of visual function in all three patients [9].

It is rare that three independent but very similar clinical trials report findings together and so positively, but this accidental consequence has provided a unique opportunity to compare results directly to gain further information on the best approach for future clinical treatments. Unanswered questions still remain relating to the optimal serotype and dose of AAV vector, together with the optimal promoter and regulatory sequences. But the key question is whether or not the underlying degeneration will stop following full correction of the functional deficit. In this regard starting the trials in patients with RPE65 deficiency is ideal, because assessments of improvement in visual function provide a proxy for successful transgene delivery. Most retinal degenerations occur due to deficiencies in genes that are not directly involved in visual transduction and affected patients initially have a near normal level of vision, which declines as a result of loss of photoreceptor cells. In these cases an improvement in vision after gene therapy would not be expected and it would be difficult to determine if progression of the degeneration was not simply because transgene delivery and/or its expression had been unsuccessful. For this reason future monitoring of the retinal thickness in treated and untreated regions in treated patients enrolled in the RPE65 clinical trials will be essential. It will provide key information on whether successful gene replacement in a degenerate retina can also prevent further cell loss.

Targeting of vector

The first report of successful retinal gene transfer was performed using AAV serotype 2 over ten years ago [10] and despite the development of new retina specific AAV vectors in recent times, probably more is known about AAV2 than any other serotype. AAV2 was also the vector used to treat the RPE65 deficient Briard dog in the early large animal studies [4, 11]. More recent studies have confirmed the long term efficacy of AAV2 in dogs [12] and safety at higher doses in non-human primates [13]. It is therefore entirely reasonable that the first human clinical trials would start with AAV2, however, other serotypes have since been characterised. AAV4 for instance has been shown to be as effective as AAV2 in restoring visual function to the RPE65 deficient Briard dog [14]. Similarly in the non-human primate, AAV4 transgene expression is limited to the retinal pigment epithelium after subretinal injection with little if any expression in photoreceptor cells [15].

Limiting ectopic expression of a transgene to the defective tissue would theoretically confer additional safety benefits, but for the RPE65 clinical trials the concept is more complex, because it is still not entirely clear if human cones express RPE65; cone transduction would not be expected using an AAV4 vector [15]. A previous study identified RPE65 protein in cone photoreceptors of the mouse, rabbit and cow. This was further confirmed by immunostaining showing RPE65 co-localised with peanut agglutinin in mouse cones and with evidence of absence in RPE65 deficient mice [16]. Conversely, a more recent study in the cynomolgus monkey failed to show RPE65 expression in foveal cones by immunohistochemistry, which may reflect differences between mammalian species [17]. Unless it is proven conclusively that human vision is not dependent on the expression of RPE65 within foveal cones, it seems reasonable to continue with a vector such as AAV2, which also targets photoreceptors in addition to the retinal pigment epithelium.

The question of targeting however is further complicated because 11-cis retinal, the putative visual cycle product of the RPE65 isomerase enzyme, can diffuse across cell membranes. Indeed in RPE65 deficient mice, visual responses can be restored by intraperitoneal administration of 11-cis retinal which leads to improvement in the electroretinogram (ERG) [18]. Hence even if foveal cones in humans do express RPE65, ectopic expression of the enzyme in other cells may still improve vision in patients, as long as the transduced cells can isomerise sufficient amounts of 11-cis retinal to diffuse back into cone photoreceptor outer segments and interact with cone opsins. Cone opsin mislocalization and cone-specific ERG

function is restored in RPE65 deficient mice following systemic 11-cis retinal administration, providing further evidence that exogenously applied vitamin A derivatives can reach the retina [19]. Delivering RPE65 by HIV-1-based lentiviral vector restores cone mediated function in RPE65 deficient mice and rescues cones overlying non-transduced regions of retinal pigment epithelium [20]. Since this vector does not readily transduce adult mouse rods or cones [21], it also provides good evidence of rescue by a diffusible molecule. Hence in mice at least, where cones probably express RPE65, rescue can be achieved by limiting transgene expression to the retinal pigment epithelium and this most likely occurs because 11-cis retinal can diffuse back into cone outer segments across the subretinal space [20].

The diet of humans is far more varied than that of laboratory mice and orally administered vitamin A derivatives taken to treat acne (13-cis retinoic acid), reach the retina as evidenced by their ability to interfere with the visual cycle through inhibition of the retinol dehydrogenases [22]. It is therefore conceivable that gross fluctuations in dietary 11-cis retinal or 9-cis retinal intake might also influence vision in patients with severe RPE65 deficiency [23]. It is interesting to note that the first RPE65 mutation was identified as a cause of early onset recessive retinal dystrophy only ten years before the first gene therapy clinical trials [24] and the isomerase function of RPE65 was resolved as late as 2005 [4, 25], by which time preparation for the clinical trials was already underway. This speed and momentum towards gene therapy for RPE65 appears to have bypassed clinical trials to assess the potential of oral retinoids, despite compelling evidence of their efficacy in RPE65 deficient mice [18, 19, 23]. Nevertheless a Phase 1 clinical trial to assess the role of oral retinoid QLT091001 in the treatment of LCA has just been completed (NCT00765427). It may be that a combination of oral retinoid and gene therapy injection will give the most effective improvement in vision in these patients – a ‘carrot and stick’ approach. This has been shown in mice deficient of lecithin retinol acyl-transferase (LRAT), which has a function closely related to RPE65 in the visual cycle [26].

Treating missense mutations

Another factor relevant to the local diffusibility of 11-cis retinal relates to the potential role of ectopic expression of RPE65 in cones. Rodent cones are believed to express RPE65 [16] and even if this protein is no longer expressed in the cones of primates [17], it is possible that other visual cycle enzymes remain, enabling cones to isomerise 11-cis retinal when RPE65 is present. This may be relevant in patients whose retinal pigment epithelium function is so severely compromised that transgene expression levels are sub-therapeutic.

Mice and dogs with null RPE65 mutations readily express the transgene in the retinal pigment epithelium when vector is administered early in the disease process [14], but transduction of the diseased human retinal pigment epithelium cannot be assumed to be as efficient. This is partly because the diseased human retinal pigment epithelium will become severely compromised by the chronic accumulation of all-trans retinyl esters within large intracellular lipid inclusion droplets, which are likely to impact on normal cell function progressively over time and confound the expression of vector mediated transgenes [4, 27]. This may explain why AAV mediated gene therapy has been noted to be considerably less effective in RPE65 deficient dogs beyond their first year, despite the relative preservation of the photoreceptor layer [14]. In some specific missense mutations, misfolding of mutant RPE65 protein is likely to inhibit mRNA translation through activation of the endoplasmic reticulum stress response [28]. Even in the absence of misfolding, RPE65 protein levels are tightly regulated by post-transcriptional mechanisms that can silence wildtype mRNA transcripts in vitro [29, 30]. The presence of mutant and/or misfolded protein in patients with missense mutations may also interfere with expression of RPE65 at the translational

level (which would also include cell specific transcription factors). Thus although mRNA may be effectively transcribed from an AAV vectored transgene, in the presence of a missense mutation, misfolded RPE65 protein has the potential to inhibit translation of the therapeutic mRNA in ways that would not operate in a null mutation.

The translational silencing of RPE65 mRNA occurs through a sequence within the first 170 nucleotides immediately 3′ to the TGA stop codon, possibly through a microRNA binding site at some point within this region [29]. This appears to be the main regulatory region of the whole 3′untranslated region (UTR), which spans almost 1000 nucleotides. Details of the cloning of each RPE65 transgene from the three studies are not readily available, but it may be critical to ensure that the spacer region between the stop codon and the polyadenylation signal of the transgene does not contain even a few nucleotides of residual 3′UTR from the human RPE65 gene. This region can suppress RPE65 translation completely in dedifferentiating RPE cells in tissue culture [29, 30]. Furthermore this inhibition may be species specific and only apparent in retinal pigment epithelium that becomes stressed [30]. Hence it is possible that a human RPE65 transgene with a short residual 3′UTR would be effective at treating animal models with null mutations early in the disease but subject to translational inhibition in the diseased RPE of patients with degenerate retinas.

So the situation in adult patients with RPE65 missense mutations is inherently different to the rodent and dog models. With most reported experiments in the animal models, the retinal pigment epithelium is treated early whilst still relatively healthy and the resulting mRNA is likely to be translated highly effectively. In adult human patients, however, it seems likely that substantially higher levels of AAV transgene expression would be required to elicit similar rescue effects in the diseased cells that contain mutant RPE65 protein. In some patients, because 11-cis retinal may diffuse short distances across the retina, it is however theoretically possible that ectopic expression of RPE65 in cones could be sufficient to elicit detectable visual improvements, even without successful transduction of the retinal pigment epithelium. Ectopic RPE65 expression and slow diffusion of 11-cis retinal is a possible explanation for the very slow rod kinetics seen in some patients after AAV gene therapy with a ubiquitous promoter, which would be predicted also to target cone photoreceptors [9].

Inverted terminal repeats and capsid

All three transgenes used in the clinical trials were similar in having AAV2 inverted terminal repeats and human DNA coding sequences for RPE65, but key differences lay with the promoters, the use or not of surfactant in the injection system and the final vector preparation protocols. These subtle differences are likely to have implications for the effectiveness of transgene expression in the diseased human retina and it is therefore crucial to compare results between these three trials as closely as possible. The three studies were otherwise very similar in terms of patient demographics, although each study incorporated patients from a different country. A direct comparison can be made between Patient 1 of the Bainbridge study and patient P3 in the Hauswirth study – both were males between 18-21 years with an identical homozygous Y368H / Y368H missense mutation and virtually identical visual acuities (6/85 vs. 6/86) prior to surgery [1, 3]. Neither patient had a significant improvement in visual acuity after surgery, but vector was not injected under the fovea in the Hauswirth study. In perimetry, however, the patient in the Hauswirth study had a significant improvement in visual sensitivity over the transduced area, which was not seen in the patient in the Bainbridge study [1, 9]. Hence this comparison between only two (but virtually identical) patients may give clues to the effectiveness or not of the different transgenes and specifically promoters used for this particular missense mutation. As explained above, the complexities of RPE65 mRNA translation regulation and the unfolded protein response mean that the results with human missense mutations cannot easily be

predicted from the laboratory models, which are almost exclusively null. The Maguire study differed from the other two in using surfactant which may result in a greater effective dose of viral particles entering the eye [31]. That study contained twins with identical E102Y/E102K mutations and both showed similar improvements in vision from a low baseline and similar improvements in visual field [2]. As patient numbers increase across the three trials it will be of particular interest to compare the results between patients with the same mutations, both within and between trials. This should provide further information on the efficacy of each gene therapy approach in treating missense mutations, which has until now been lacking in most of the preclinical data.

There are few published details on the coding sequence of the AAV-2 inverted terminal repeats used in the three trials, but this may be relevant in terms of efficiency of transduction following internalization of vector particles. The wild-type AAV2 inverted terminal repeat is 145 nucleotides in length, with a 125 nucleotide palindromic repeat sequence, but there is heterogeneity in the arrangement of these repeats and for the non-palindromic segments [32, 33]. In future it would be interesting to know precisely which sequences were used and some agreement and standardization for clinical trials may be helpful. Similarly there is little detail on the AAV2 capsid protein coding sequences on the helper plasmids. This is likely to be far more relevant for clinical trials because it is generally the capsid protein that generates the immune responses to AAV2. Around 30% of adult subjects have a significant neutralising antibody titre to AAV2 and even more harbour antigen-specific memory CD8+ T cells - most likely generated from previous exposure to wild-type AAV2, particularly in childhood [34]. Since memory T cells are more readily triggered than immature lymphocytes, patients being re-exposed to AAV2 capsid protein through gene therapy will have a different outcome to experimental animals with no previous AAV exposure. In the clinical trials, neutralizing antibody titres to the AAV2 capsid temporarily increased in one patient in the Maguire study [2] and showed a marginal increase in another in the Hauswirth study [3], although both results were of borderline significance. There were no detectable antibody responses to the capsid protein in the Bainbridge study, despite the use of a higher overall dose [1]. Collectively these results suggest that there are unlikely to be significant immunological problems when using these titres of AAV2 in the immune privileged subretinal space of humans, which is in contrast to observations in the liver using much higher systemic doses [35]. This is probably the most significant and wide ranging conclusion from the current studies, because it provides the necessary safety data to continue with AAV2 for other retinal gene therapy applications in future.

There is still a question, however, about the best approach to treat the fellow eye at a later date because this would by definition represent a second dose of AAV2 vector. Some variability within epitopes for the AAV2 capsid proteins may facilitate designing the second vector with a slightly different sequence to the first, or even using another serotype altogether. This may reduce the chance of activation of memory T-cells primed during the first gene therapy treatment and AAV2 exposure. Small changes in AAV capsid protein amino acid residues can also increase transduction efficiency significantly [36]. It may therefore be beneficial in future to develop mutant capsid sequences as they may also be less prone to generate an immune response in patients who have had previous exposure e to wild type AAV2.

Transgene

The most significant difference in the vector designs between the three clinical trials relates to differences in the DNA promoter sequences used. The Bainbridge study employed a 1400 nucleotide human RPE65 promoter which had been shown to be effective in RPE65 deficient dogs in a previous study, with about one tenth the strength of the CMV promoter

[14]. The Maguire and Hauswirth studies used a cytomegalovirus (CMV) enhancer - chicken beta-actin (CBA) promoter sequence containing a hybrid intron in the 5′ untranslated region (UTR). A previous study directly comparing the efficiency of this to a pure CMV promoter, driving a 1.3-kb human alpha-1-antitrypsin transgene in AAV, showed that the CBA promoter yielded over 130 times the level of protein compared to CMV [37]. Comparing these two studies as a rough guide suggests that the CBA promoter would give in the region of three log units higher expression compared to the human RPE65 promoter, at least in dogs with a null RPE65 mutation. A direct comparison of the relative strengths of all three promoters used in the study is, however, further compounded by the use of a modified Kozac consensus upstream of the start codon in the Maguire study [2]. This may also influence efficiency of translation which, as explained above, is likely to be a critical issue in cells undergoing a stress response. It does however seem reasonable to assume that substantially more vector genomes would be required to elicit comparable levels of expression from the cell-specific RPE65 promoter compared to CBA, which might explain the less impressive vision changes in the Bainbridge compared to Maguire studies, despite a vector dose almost one log unit higher.

These rough estimates of relative gene expression levels are based on previous observations on null backgrounds and with transduced cells that are still relatively healthy [14, 37]. For patients in the trials, however, it is likely that levels of protein expressed from the transgenic DNA would be considerably lower, due to inhibitory effects at the level of translation for two principal reasons. First the retinal pigment epithelial cells treated much later in the human disease are likely to be stressed by chronic dysregulation of the retinoid cycle and possibly lipid inclusions as the disease progresses [27]. Activation of the mTOR (mammalian target of rapamycin) kinase as a result of this stress response would block translation by inhibition of the mRNA 5′-cap recognition process via the phosphorylation of eukaryotic initiation factor 4 (eIF4) binding proteins [38]. Second, any accumulation of misfolded proteins in the endoplasmic reticulum would further inhibit assembly of the translation complex through PERK-mediated phosphorylation of eIF2-alpha [28]. A transgene sequence that is dependent on a cell-specific promoter would be doubly affected, because the stress response is global and is likely to block translation of cell specific proteins, which would include transcription factors. Hence in a stress response, cell-specific transgene expression would be inhibited at transcriptional and translational levels. Viruses have evolved to use translation initiation sequences that avoid the 5′-cap initiation complex, such as the internal ribosome entry site (IRES), which presumably facilitates translation of viral RNA sequences in stressed cells [39].

It is therefore likely that expression from a cell-specific promoter would be considerably less than with an intron-containing ubiquitous CBA promoter - and even more so in diseased cells harbouring missense mutations. It should be remembered however that the first retinal gene therapy studies are Phase 1 clinical trials and safety is the primary concern. The great advantage of a cell-specific promoter is that it is much less likely to be active in distant sites inadvertently transduced by vector spread. This theoretically reduces the risk of insertional mutagenesis, which has been a concern in mice receiving large AAV2 doses to the liver [40]. This needs however to be balanced with the risk of immune reactions to AAV2 capsid proteins if far greater numbers of vector particles are required to achieve therapeutic levels of gene expression.

Conclusion

Most patients with inherited retinal degenerations have missense mutations, but virtually all our translational research is developed in animal models with null mutations. True null mutations in RPE65 do exist in humans but are rare [41]. Some RPE65 missense mutations

may even have mild latent dominant negative effects, as evidenced by a mild phenotype in heterozygotes [42]. A key to translating our laboratory research into successful human gene therapy will be to understand the effects of missense mutations on the translational machinery. In some cases eliciting the unfolded protein response may lead to cell death, whilst in others it may simply block translation. In diseases such as RPE65, the chronic accumulation of intracellular waste products resulting from defective retinoid recycling will further compromise the cell by mTOR related stress responses, which are largely irrelevant in animal models treated very early in the disease process. For these reasons it seems likely that higher levels of transgene expression will be required to treat patients with missense mutations. The best results of retinal gene therapy will almost certainly be seen in patients with true null mutations that do not result in significant stress responses in affected cells.

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