On Retinal Gene Therapy

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Key Words

Gene therapy · Retinal dystrophy · Vitreo-retinal surgery

Abstract

Mutations in a large number of genes cause retinal degeneration and blindness with no cure currently available. Retinal gene therapy has evolved over the last decades to become a promising new treatment paradigm for these rare disorders. This article reflects on the ideas and concepts arising from basic science towards the translation of retinal gene therapy into the clinical realm. It describes the advances and present thinking on the efficacy of current clinical trials and discusses potential roadblocks and solutions for the future of retinal gene therapy.

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Introduction

Gene therapy can be defined as bringing a therapeutic nucleotide sequence such as DNA into affected cells of a patient. The molecule then helps cells to help themselves by giving them new information they can use to counteract a relevant disease mechanism. One example is the introduction of a healthy copy of the *RPGR* coding sequence in patients with X-linked retinitis pigmentosa type 3,

which is caused by mutations in the *RPGR* gene. After providing them with a healthy copy of the gene, the transduced photoreceptors can produce the normal gene product and restore a physiologically steady state.

This simple but elegant idea was born after it became evident from the studies by Avery et al. [1] in the 1940s (1) that nucleotides – not proteins – hold our genetic code and (2) that transfer of nucleotides into a cell can enable the receiving cell to utilise this novel information and to perform new functions. After the discovery that gene transfer was already an established part of nature's repertoire and routinely performed by viral particles [2], Rogers and Pfunderer [3] were among the first to promote the idea of a virus-mediated gene transfer for therapeutic purposes in the late 1960s. After the principal idea had been stated, several obstacles were readily defined. Key areas of research were regarded to be recombinant DNA technology (how to make the therapeutic nucleotide sequence) and efficiency of gene transfer (how to get it into the target cell). These areas of basic research were increasingly successful in the late 20th century and were matched by the rapid advances in the clinical and molecular characterisation of hereditary retinal diseases (HRDs). *RPGR* for example was the first HRD gene to be cloned and characterised in the early 1990s. Since then, more than 250 genes and loci spread over all 22 autosomal chromosomes, the X-chromosome and the mitochondrial ge-

nome have been associated with one or several HRDs. In consequence, there is a large and clearly defined yet unmet need to develop therapies for these blinding disorders.

Advances in the Field of Diagnostics

Ophthalmology has played a pioneering role in terms of understanding hereditary conditions ever since the early days of medical genetics for two important reasons. Genetic defects leading to visual dysfunction cause considerable disability, which is readily detected and reported by patients and easily linked to the affected organ by the physician even at times of relatively limited diagnostic resources and before detailed knowledge of genetic mechanisms was available. Secondly, the eye with all its affected structures could be investigated in great detail early on. Since the introduction of the ophthalmoscope by Hermann von Helmholtz in 1850, Frans Cornelis Donders (1818–1889) in Utrecht, Albrecht von Graefe (1828–1870) in Berlin, Robert Walter Dooyne (1857–1916) in Oxford and other early champions of ophthalmology have used the unique advantage of directly observing neuroretinal tissue in vivo in microscopic detail to describe pathologic changes in relation to inheritance patterns. As such, the first description of bone spicule pigmentation – a clinical hallmark of HRDs – was given by Donders [4] in *Graefe's Archives* as early as 1857. Today, a vast array of diagnostic tools aids the detailed description of morphological and functional changes in retinal diseases [5–7]. However, the low incidence of HRDs often makes the performance of useful prospective observational trials a challenge to single centres when attempting to define the most suitable outcome measures and study designs for an interventional trial. This calls for old-fashioned collaboration between clinical centres and new models to include non-academic stakeholders such as patient interest groups and funding bodies with the common goal to coordinate efforts and pool data from patients with rare disorders. International databases with standardised clinical data would greatly aid the identification of relevant endpoints and intelligent trial designs for interventional studies.

Next to clinical workup, molecular genetic testing is a *sine qua non* for gene therapy. Sanger sequencing remains the gold standard for diagnosing mutations in target regions. However, it is difficult and costly to cover vast genomic areas by this direct sequencing approach, and all analyses are hypothesis driven: certain genes are investi-

gated according to type based on categorical clinical patterns (e.g. cone dystrophy vs. primary rod dystrophy or macular dystrophy). As a result, the 'usual suspects' are efficiently picked up, but mutations beyond the known catalogue remain undetected in routine diagnostic analyses. This changed with the advance of next-generation sequencing, which accelerated the identification of novel disease-causing mutations. However, the non-hypothesis-driven quality of this analysis often leads to a vast amount of data which is challenging to interpret. For example, there is increasing evidence of multiple mutations across different loci in HRD patients, challenging the concept of HRDs being single-gene defects [8]. This may in part explain the heterogeneity of their phenotypic presentation: patients with the same diagnosed mutation might have additional genetic defects or polymorphic modifiers explaining differences, for example, in disease severity or rate of progression. Besides impacting on the genotype-phenotype correlation, this also has implications for gene therapy trials, where patient selection has to be carefully tailored so as not to include patients with additional disease-causing mutations. For example, achromatopsia patients with two homozygous mutations in *CNGA3* and *CNGB3* would not likely benefit from *CNGA3* augmentation gene therapy as the β subunit of the CNG channel would still be missing even after successful gene therapy. The concept of multi-genetic causes of HRDs is also of interest for the development of future therapies for diseases such as age-related macular degeneration, where a multitude of genetic loci (*ABCA4*, *ARMS2*, *C2*, *C3*, *CFB*, *CFH*, *ERCC6*, *FBLN5*, *HMCN1*, *HTRA1*, *RAX2*, *TLR3* and *TLR4*) determine the genetic risk of developing the disease.

Tools for Retinal Gene Therapy

Early viral gene therapy was performed with integrating virus classes, which integrate into the genome of the target cell. In dividing cells, daughter cells will inherit the 'treated' genome, thereby leading to a sustained therapeutic effect, e.g. in the bone marrow or other mitotic target tissues. This prevents a washout of the gene therapy in following generations of, for example, bone marrow-derived lymphocytes. The potential risk of integration lies in the chances of disrupting important genetic information, such as a tumour suppressor gene. As retinal tissue is post-mitotic, the benefit of integration is less relevant, tilting the benefit/risk ratio in favour of non-integrating virus vectors such as the adeno-associated virus (AAV).

AAVs belong to the genus *Dependovirus* of the family of Parvoviridae [9]. ‘Parvus’ is Latin for ‘small’, thus indicating the small size of Parvoviridae. AAVs are approximately 20 nm in diameter with a packaging capacity of approximately 4.8 kb of single-stranded DNA. They are free of envelope structures and are less immunogenic than, for example, adenovirus [10]. The recombinant AAV (rAAV) used in all clinical studies so far features elements of the serotype 2 AAV of human origin. The trials targeting Leber’s congenital amaurosis (LCA) and choroideraemia used AAV2 capsid proteins to package the therapeutic transgene. Capsid proteins are the first to interact with target and off-target cells and determine the tissue tropism and infection efficiency. rAAV2 virions gain entry into target cells by using the cellular receptor heparan sulphate proteoglycan. Internalisation is enhanced by interactions with one or more of at least six known co-receptors including $\alpha_v\beta_5$ integrins, fibroblast growth factor receptor 1, hepatocyte growth factor receptor, $\alpha_v\beta_1$ integrin and laminin receptor [11–13]. Binding of rAAV2 to a cell surface receptor initiates internalisation (receptor-mediated endocytosis) through clathrin-coated pits. Once internalised, the virus rapidly moves to the cell nucleus and accumulates perinuclearly within 30 min after the onset of endocytosis. Virus particles then slowly penetrate into the nucleus, possibly through the nuclear pore complex [14]. Within 2 h, viral particles can be detected within the cell nucleus, suggesting that rAAV enters the nucleus prior to uncoating, though full-level expression of the transgene takes days to weeks depending on tissue, cell type and AAV serotype. As such, the release of the therapeutic single-stranded nucleotide, its subsequent second-strand synthesis and configuration as monomeric and/or concatemeric chromatin structures all help to dictate expression dynamics [15, 16]. Integration events have been made exceedingly unlikely due to the removal of wild-type virus genes for Rep78 and Rep68, crucial elements for vector genome integration, thereby rendering the risk of insertional mutagenesis negligible [17, 18]. This is also supported by the observation that rAAVs have been used for more than 20 years in ocular gene transfer studies in animal models and more than 10 years in human clinical trials with no sign of malignant transformation [19].

One interesting topic for scientists and clinicians alike is the development of different ‘flavours’ of AAV vectors, which could be optimised to the application route and/or target cell population. The ideal vector would be highly specific to the target cell type (e.g. cone photoreceptors) and get there in the least invasive manner possible. This

can be achieved by targeted design or directed evolution among other strategies [20, 21]. Additionally, regulatory elements in the therapeutic sequence can be designed to increase specificity of expression [22] and even allow regulation of expression levels over the course of the disease [23].

Does Gene Therapy Work?

In those years, the research community attained extraordinary success in the (pre-)clinical development of AAV vectors for HRDs [24–32]. This culminated in landmark clinical trials with first-in-man ocular gene therapy applications [28, 29, 33] to treat Leber congenital amaurosis type 2 (OMIM 204100) with AAV2.RPE65. RPE65 is an isomerase found in the retinal pigment epithelium (RPE) and is responsible for recycling vitamin A derivatives, which in turn are necessary for light detection in the photoreceptors. Mutations in RPE65 lead to drastically decreased sensitivity of the retina to light stimuli, degeneration of RPE and photoreceptor cells and ultimately blindness. These phase I/II trials were successful in showing the safety of gene addition in patients with missense as well as predicted null mutations [34, 35]. This was important, since it had previously not been clear whether patients with null mutations would stage an immune response against the therapeutic transgene product as it would be new to the immune system. Bennett et al. [36] were the first to demonstrate the lack of a clinically significant immune response after second administration of AAV2.RPE65 to the contralateral eye years after the first injection. Based on this pioneering work, several conclusions could be drawn:

- High numbers of AAV particles can be administered in the subretinal space without clinically evident toxic effects
- Subretinal AAV does not lead to a clinically significant immune response
- Gene addition to RPE cells can restore metabolic activity to clinically relevant levels

However, some of the more optimistic expectations of restoring vision in LCA2 patients were not met in these first trials. These had been fuelled by the exceptional results from pre-clinical studies on animal models of LCA2, with Lancelot being the most prominent recipient having visited the US Senate [37]. Lancelot was one of the Briard dogs with a naturally occurring mutation in RPE65 that were treated using AAV2.RPE65. The therapeutic effect was remarkable with robust electro-physiological evi-

dence of efficacy and very apparent behavioural change. While most patients from the first trials showed some form of improvement in psychophysical tests measuring retinal sensitivity under mesopic or scotopic conditions, no patient was demonstrated to have gained an objective electrophysiological response. This can be seen as difficult given the unmasked nature of the trial design.

The long-term follow-up of the patients from the first trials additionally points towards a possible decline of the initial functional improvement [34, 35]. Several potential explanations were put forward on why the therapeutic benefit was not more obvious and why there was an indication of continued degeneration. One maintains that the intervention had come too late to effectively stop the degeneration of the remaining retinal tissue. Support for this comes from observations in animal models, where treatment success was highly dependent on the timing of the intervention [38, 39]. Treatment at very early disease stages showed functional and structural rescue, while a later intervention showed only transient functional improvements with continuous structural degeneration eventually leading to complete blindness. Some authors argue that the disease stage found in the enrolled patients equated to the second group and, thus, only allowed for a transient functional benefit. Data published by Maguire et al. [30] support this line of argument with a greater treatment effect seen in younger patients. However, Bainbridge et al. [35] and Jacobson et al. [34] could not find a clear correlation between age of participant and response to treatment. In fact, Bainbridge et al. [35] reported the greatest improvements in older participants.

Indirectly, this leads to the second line of argument: the applied dose might not have reached the required therapeutic threshold. The greater success in older participants could be related to less tissue being left at a later disease stage, thus increasing the ratio of AAV particles applied to cells available for transduction (i.e. increasing the multiplicity of infection). Further support for this hypothesis comes from canine studies, which demonstrate that sub-threshold doses of AAV2.REP65 can lead to limited functional rescue without electroretinography improvements (as observed in patients), while higher doses lead to robust measurable responses on electroretinography, which were also associated with protection against continuous degeneration [35, 39].

A third hypothesis focused on secondary structural changes in retinal tissue of LCA2 patients: Cideciyan et al. [40] suggested that the therapeutic effect of restored recycling of all-*trans*-retinyl esters into 11-*cis*-retinal by the RPE65 isomerase transgene product could be limited

by a pathological alteration of the degenerating retinal tissue, such as disorganised photoreceptor outer segments and accumulating lipid droplets forming a 'resistive barrier'.

A fourth possible explanation of limited functional rescue is the prolonged and extensive deprivation of the visual cortex in LCA2 patients, who typically experience severe vision loss from birth (hence the name 'congenital'). One could, therefore, argue that even with the best possible restoration of the recycling activity of RPE65 and phototransduction in the remaining photoreceptors, the perception of light on a cognitive level might be dictated by a limited cortical plasticity. An often cited example is the development of amblyopia in patients in whom strabismus is not treated until at least their early teens. Correction of such a strabismus in adults usually does not achieve significant improvement in the vision of the amblyopic eye. However, Ashtari et al. [41] demonstrated significantly enhanced activity in the visual cortex in LCA2 patients following gene therapy. The activity was specifically found in the cortical area corresponding to the treatment area in the retina and correlated with improvement in other clinical outcome measures. Maybe more importantly, the improvement was clearly evident even 3 years after gene therapy, suggesting a sustained beneficial effect.

Another clinical trial also shows a more optimistic picture. Using AAV2.REP1, MacLaren et al. [42] successfully treated 14 patients with choroideraemia to date. Preliminary data showed good safety and an unexpected increase in visual function in some of the patients. This is remarkable as most previous reports on choroideraemia emphasised the preservation of visual acuity until the very last stages [43], and most trial participants went into the operation with (near-)normal visual acuity. Hence, the initial goal was to show safety and efficacy in terms of preventing further deterioration of vision. For ethical reasons, the worse eye was treated. The latest follow-up data from trial participants now suggest that the treated (initially worse) eye has now become the functionally better eye in the majority of patients. With a follow-up of more than 3 years, this suggests that gene therapy can lead to a sustained treatment effect in human patients just as we have seen in animal models.

Taken together, there are a number of reasons which might explain why some of the first trials were 'only' successful in showing the safety of subretinal AAV gene therapy but limited in demonstrating its efficacy. It is important to acknowledge the enormous contributions made by these groups to the field of retinal gene therapy.

In their wake, a large number of gene therapy trials have started or are planned, and an unprecedented investment from the financial sector in gene therapy companies, such as Oxford BioMedica plc., Spark Therapeutics, NightstaRx Ltd., Avalanche Biotechnologies, etc., has made the translation of current (pre-)clinical gene therapy programmes into an approved treatment more likely.

Potential Roadblocks and Solutions for Future Retinal Gene Therapy

Some potential roadblocks can be identified which could possibly delay the development of retinal gene therapy. These can be divided into practical aspects and more abstract issues.

One practical bottleneck for retinal gene therapy is the surgical application of vector suspension in order to transduce the target cell population efficiently. Most retinal gene therapies are geared towards photoreceptor and/or RPE cells. Both cell populations are immediately adjacent to the potential subretinal space, which can be filled with balanced salt solutions using fine needles with minimal trauma. This route of delivery has several advantages over intravitreal or intracameral injections, including greater transduction efficiency and greater control of biodistribution. Additionally, the subretinal space has been shown to feature a deviant immune response similar to the anterior chamber-associated immune deviation [10, 44]. By actively suppressing potentially harmful immune responses, anterior chamber-associated immune deviation may have played a role in the success of subretinal gene therapy in the second eye 1.7–3.3 years after treatment of the contralateral eye [36].

Subretinal delivery of therapeutic agents is an established procedure in vitreoretinal surgery, one example being solutions containing recombinant tissue plasminogen activator for the treatment of submacular haemorrhages in neovascular age-related macular degeneration [45, 46]. The microsurgical procedures and instruments for subretinal delivery are therefore available, but a number of unique factors, such as small injection volumes (~100–500 µl) and the minimal surplus solution for priming and flushing, need to be considered as well as biomechanical alterations of ocular tissue due to the underlying disease.

The different retinal gene therapy trials so far have all applied the vector suspension to the subretinal space, each using protocols with small but important differences:

Bainbridge et al. [28] used 1,000 µl with gas-fluid exchanges to push the bleb into the target area. Maguire et al. [29] injected 150 µl vector suspension with 0.001% PF-68 directly into the subretinal space of the target area, and a similar approach was used in the trial by Hauswirth et al. [33] with a volume of 150–300 µl applied directly using a 39-gauge cannula. Bennicelli et al. [47] have pointed out that without addition of 0.001% of PF-68, 90% of the AAV2 particles are retained in the injection system. Therefore, the actual dose given in the trial by Bainbridge et al. [28] (which did not use PF-68) was likely one log unit below what is nominally reported.

Surprisingly, another bottleneck can be the lack of robust clinical data on the natural history of disease progression, degree of symmetry, test/re-test variability and/or comparison of relevant outcome measure analyses. Most existing papers report retrospective case series-type clinical studies, where the cohort might be defined based on clinical diagnosis alone. However, data from prospective trials are much more appropriate to design a sound interventional trial with suitably defined in-/exclusion criteria, follow-up schedules and primary/secondary outcome measures. These data are also more likely to help identify potential treatment windows for HRDs; however, more relevant data might come from current and future interventional trials.

A more abstract hazard in the way of gene therapeutic development is the effort needed to actually reach trial (let alone market) approval by various regulatory bodies in relation to the financial and human resources available for the academic investigator. The Orphan Drug Designation programmes by the FDA and EMA are only one part of the solution. More lobbying by patient interest groups seems necessary to streamline regulatory procedures and make investigational new drug applications more economical for academic investigators. For example, it might be possible to provide biodistribution data on, for example, AAV8 after subretinal application in non-human primates and use these data in trials with different transgene constructs as long as the capsid, vector solution and delivery methods are identical. Consolidating regulatory requirements across national borders (e.g. within the EU) would make much sense, especially when dealing with orphan diseases, where large trials would benefit from multiple centres participating due to the low prevalence within each country. The regulatory bodies and patient interest organisations are key stakeholders, but it is the responsibility of the academic investigator to point out ways to improve current practice.

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