Let there be light: gene and cell therapy for blindness.

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Abstract

Retinal degenerative diseases are a leading cause of irreversible blindness. Retinal cell death is the main cause of vision loss in genetic disorders such as retinitis pigmentosa, Stargardt disease and Leber congenital amaurosis, as well as in complex age-related diseases such as age-related macular degeneration (AMD). For these blinding conditions, gene and cell therapy approaches offer therapeutic intervention at various disease stages. The present review outlines recent advances in therapies for retinal degenerative disease, focusing on the progress and challenges in the development and clinical translation of gene and cell therapies. A significant body of preclinical evidence and initial clinical results pave the way for further development of these cutting edge treatments for patients with retinal degenerative disorders.

Introduction

Blinding diseases of the retina

Retinal diseases are a major cause of irreversible blindness. These conditions can be caused genetically or acquired later in life. Complex diseases have both genetic and acquired counterparts. Most common forms of multi-factorial retinal diseases include macular degeneration, glaucoma, diabetic retinopathy and retinoblastoma. Inherited retinal degenerations on the other hand are entirely linked to mutations in retinal neurons and their underlying epithelium. Retinal cell death is the main cause of vision loss in many blinding conditions for which gene and cell therapy approaches offer intervention at various stages (see Figure 1 for an example). Basic research on how and why retinal cells die in different diseases is crucial for the development of treatment strategies to prevent or reverse vision loss using gene and cell therapy. This review will focus on the latest developments in laboratory and clinical aspects of gene and cell therapy for retinal diseases.

Why the eye?

The eye represents an ideal target for gene and cell therapies: it is easily accessible and small (requiring a low volume of virus/active dose), highly compartmentalized (permitting different ocular tissues -anterior chamber, vitreous cavity or subretinal space- to be specifically targeted), and separated from the rest of the body by the blood-retinal barrier (ensuring ocular immune privilege and minimal systemic dissemination). As the retinal cells normally do not divide, the cell population remains stable making it possible to use non-integrating vectors for sustained transgene expression (for review: [1-3]). Other important reasons why the eye has been on the forefront of gene and cell therapies is the fact that the contralateral eye can serve as an internal control which is extremely helpful in evaluation of outcomes. Lastly, the progress in

imaging technologies (such as optical coherence tomography, adaptive optics) for visualizing this accessible part of the body has been of great value in both diagnostics and follow-up after treatments.

Gene therapy

Gene therapy is an emerging therapeutic approach to treat, cure or prevent a disease by providing a gene with therapeutic action. Diseases associated with loss-of-function mutations can be treated by gene replacement therapy (also referred to as gene supplementation), while those associated with gain-of-function mutations require eradication of mutant alleles in addition to supplementing the gene. In all instances, the genetically modifying factors (DNA or RNA and/or their interacting proteins) need to be delivered into the relevant target cells. The advancements in the design and production of gene delivery vectors have been an important part of gene therapy progress and are discussed below. Additionally, recent advances in molecular genetics and rapidly evolving knowledge of retinal biology allowed significant progress to be made in gene therapy for retinal disorders, with promising results not only in animal models, but also in humans.

Gene delivery: viruses, nanoparticles, physical methods

Most gene therapy studies use viral vectors, such as adenovirus (Ad)-, adeno-associated virus (AAV)- or lentivirus (LV)- to enable gene delivery to the retina. There are two local administration routes that allow viral vectors to access retinal cells. Viral vectors can either be injected into the vitreous cavity through an intravitreal injection or they can be injected into the subretinal space created through a transient retinal detachment. Intravitreal injections deliver the vector in proximity to the retinal ganglion cells and are the preferred delivery route

for targeting the inner retina. Subretinal injections deliver the vectors between the photoreceptors and their underlying retinal pigment epithelium (RPE). As most inherited retinal degenerations are caused by mutations found in the photoreceptor and RPE cells, subretinal injections have been used in most gene therapy studies.

Depending on the cell target Ad, LV and AAV have been studied. Following subretinal delivery both Ad and LV transduce the RPE efficiently. Ad however has been associated with cytotoxic T lymphocyte–mediated removal of the transduced cells that express the encoded Ad proteins [4] leading to transient gene expression. More recently, helper-dependent Ad vectors devoid of sequences encoding viral proteins have been developed and shown to target the RPE stably [5, 6]. Thus far photoreceptor transduction remains elusive with Ad and Lv vectors despite the great diversity of new serotypes and pseudotypes tested [7]. Further development of Ads and discovery of new Ad serotypes might enable photoreceptor transduction using Ad [8-10] in the future. If Ad can be modified to enable photoreceptor transduction, its large carrying capacity will be of interest for treating inherited retinal degenerations like Usher syndrome (USH) where the genes involved have very long open reading frames. LVs are an alternative to Ad. LV based vectors are deleted of all viral genes and, thus, do not activate the immune system [11, 12]. However, LVs are integrating vectors and this implies the possibility of insertional mutagenesis, potential mobilization in human cells and vector replication [12]. Most studies showed that subretinal delivery of LVs lead to efficient RPE transduction [13-15], but post-mitotic photoreceptors seem refractory to transduction by LV [16, 17]. The recent development of the non-primate equine infectious anemia virus has raised hopes for overcoming this limitation [18]. This has been the basis for the ongoing clinical trials to treat exudative age-related macular degeneration (AMD) (ClinicalTrials.gov Identifier: NCT01301443), Stargardt disease (STGD) (ClinicalTrials.gov Identifier: NCT01367444), and

USH type IB (USH1B) (ClinicalTrials.gov Identifier: NCT01505062) using this vector. These clinical trials are discussed below. However, the transduction of mature neural retina remains a fundamental limitation of LVs and Ads as vectors for retinal gene therapy. This is one of the reasons why AAV has been the preferred vector of choice for gene delivery to the retina in the recent years. AAVs have an excellent safety profile (lack of pathogenicity and low immunogenicity) and provide long-lasting transgene expression (for review: [19]). AAV has the additional advantage of being a small virus, which can diffuse easily across biological barriers and within neural tissue. It is the only vector that can provide gene delivery to the inner retina after intravitreal delivery [20-22]. Although the small size of the AAV particle is an advantage, it is also its weakness: the 25-nm AAV particles can only package 4.7 kB of genetic material limiting its application in some diseases. Nevertheless, AAV has yet another advantage that has contributed to its development as a gene delivery vehicle: it is a non-enveloped virus and its capsid can be easily modified using genetic engineering techniques. As such, it has been extensively explored for its ability to target different groups of cells in the retina [23-28]. As an example, it has been engineered to provide gene delivery into deeper layers of the retina after intra-vitreal administration removing the need for subretinal detachment [23-25, 29]. One such AAV variant called 7m8 was able to ensure efficient pan-retinal delivery of the therapeutic gene from the vitreous, with a long-term histological and functional rescue of X-linked retinoschisis and Leber congenital amaurosis (LCA) phenotypes in mice and provided superior retinal gene delivery in nonhuman primates [25].

Studies in the past pointed towards the higher efficiency of viral versus nonviral vehicles [30] for retinal gene delivery. However, recent reports on nanoparticle-mediated retinal gene therapy showed an improvement compared with previous studies with non-viral agents [31]. Non-viral (lipid or nanoparticle) carriers provide a complementary approach (for review: [32]).

These include naked DNA, DNA encapsulating liposomes, compacted-DNA nanoparticles (cationic liposome/DNA complexes). In general, they allow transfection of cells with larger pieces of DNA and carry lower risk of immune responses associated with viral gene delivery [33, 34]. However, lack of long-term gene expression is a major limitation of such vectors. For example, clinical trials using polyethylene glycol-substituted 30-mer lysine peptide based nanoparticles and lipid-mediated vectors to deliver therapeutic genes to the nasal mucosa of patients with cystic fibrosis reported no detectable gene expression, although vector DNA was detectable for at least 2 weeks [35]. Although their use has been limited thus far, their additional development for increased efficacy could make them versatile tools for gene delivery in the years to come.

The first clinical success of retinal gene therapy

Encouraging results from animal studies (mouse, rat, dog) showed that AAV-mediated gene therapy has the potential to slow down or reverse vision loss, and paved the way towards first application in humans. The first success of gene therapy today has been documented with the clinical trials for LCA, a severe retinal dystrophy characterized by visual impairment from birth [36]. It is caused by mutations in at least 19 different genes (22 mapped and identified genes, after http://www.retnet.org, accessed July 8th 2015). Mutations in the gene encoding the RPE-specific protein RPE65 appear to account for ≈5-10% of LCA cases [37, 38]. For this specific form of human LCA (LCA2), the first clinical trial of gene replacement therapy started in 2007 at i) the UCL Institute of Ophthalmology and Moorfields Eye Hospital (UK), ii) the Scheie's Center for Hereditary Retinal Degenerations, University of Pennsylvania and the University of Florida College of Medicine in Gainesville (USA), and iii) the Children's Hospital of Philadelphia and the University of Pennsylvania (USA); Naples Second University and TIGEM (Italy). Patients received a single subretinal injection of AAV2 vector

carrying the *RPE65* gene in the most affected eye. In 2008, the independently working groups reported the first safety and efficacy results of the AAV-mediated *RPE65* transfer [39-41]. In addition to excellent safety, improvements in some measures of vision (including best-corrected visual acuity, kinetic visual field, nystagmus testing, pupillary light reflex, microperimetry, dark-adapted perimetry, dark-adapted full-field sensitivity testing) have been demonstrated in these Phase I clinical trials. Since then, results of follow-up and dose-escalation studies have been published [40, 42-46] and confirmed the feasibility and benefits of gene therapy in retinal degenerative diseases. In view of these encouraging results, re-administration of *RPE65* gene-based treatment was performed for the first time in the contralateral eye of adult patients with LCA, three years after the initial gene therapy administration [47]. This intervention leads to positive improvements in the second eye. In addition to improved visual outcomes, functional magnetic resonance imaging (fMRI) studies provided evidence that the human visual cortex responds to gene therapy-mediated recovery of retinal function [45]. fMRI evaluation found correlation between preserved light sensitivity and cortical projection zone of pseudo-foveas developed in treated retinal regions (observed 9-12 months after therapy and persisted for up to 6 years [48]. Multimodal non-invasive neuroimaging has recently revealed long-term structural plasticity in the visual pathways of LCA patients that received single eye gene augmentation therapy [49]. It has been suggested that the visual experience gained by gene therapy may promote reorganization and maturation of synaptic connectivity in the visual pathways of the treated eye in LCA patients. Today, retinal gene therapy has entered into Phase 3 (ClinicalTrials.gov Identifier: NCT00999609). At least 24 LCA patients (age 3-years or older), will be recruited at either the Children's Hospital of Philadelphia or University of Iowa. They will receive a subretinal administration of AAV2-*RPE65* to both eyes. Prospective open label gene therapy (AAV4-*RPE*65) study for *RPE65*-

associated retinal dystrophy was also run and completed in the Nantes University Hospital (ClinicalTrials.gov Identifier: NCT01496040) (results still to be published).

The initial positive results from LCA gene therapy studies were recently challenged by new findings. In 2013, two groups [50, 51] reported that early visual improvements in *RPE65*-treated LCA patients persist up to 3 years, with no detectable decline in visual improvements. However, retinal degeneration continued to progress. This observation was also seen in *RPE65*-mutant dogs. Two years later, The New England Journal of Medicine published the long-term follow-up results of two independent studies on *RPE65*-gene therapy. Jacobson and colleagues (US) described follow-up data from three *RPE65*-treated patients [52]. The patients all had improvement in visual sensitivity in the treated region that was sustained between 1-3 years after gene therapy. Unexpectedly, 4.5-6 years after treatment, the areas of improved vision were found progressively diminished in all three patients. The authors concluded that the degeneration continued at the same rate as in untreated retina, despite the initial improvement. The study from the UK (Bainbridge and colleagues) [53] involved 12 patients (in this study the fovea was also targeted in order to improve both central and extrafoveal vision). Six of these patients had improvements in visual sensitivity that peaked at 6 to 12 months after treatment. Similarly to the US study, the effect declined or was lost by 3 years post-injection. These new findings prove that at least for several years gene therapy can improve vision but also indicate that the photoreceptors continue to die after the peak improvement, regardless of treatment.

As of today, it is not clear what caused the effects of gene therapy to be transient in these two clinical studies. The study by Bainbridge and colleagues concluded that there is a species difference in the amount of RPE65 required to drive the visual cycle and that the demand for

RPE65 in affected persons in their study was not met to the extent required for a durable, robust effect ("too little" therapeutic protein). Indeed, the demand for RPE65 is likely higher in humans than in dogs [54]. Another potential culprit for the transient effects is related to the stage of the retina at the time of intervention and progressive loss of trophic support (especially regarding the cones). The study by Jacobson and colleagues [52] speculated that healthier photoreceptors survived in the treated retina, whereas other more stressed rods were already in a pre-apoptotic ("at the point of no return") state and continued to die. The loss of visual function at later times after treatment is in line with this natural progression of the degeneration. Furthermore, the reduction in the number of rod photoreceptors in spite of the therapy may eventually lead to a loss of trophic support for the cone photoreceptors that initially had a response to the therapy. In both studies, there were no improvements in foveal function despite vector having been delivered to the fovea in some of the patients. The question of why *RPE65* gene supplementation improves the function of extra-foveal cones but not that of foveal cones remains unresolved but might well be a long-term complication of surgery. Indeed, the connections between the RPE and the cones are different in the fovea and at the periphery. Though successful results have been obtained in the macula in gene supplementation therapy for choroideremia [55, 56], it has been proposed that detaching the foveal cones has detrimental consequences in LCA2 [46].

Whatever might be responsible for the reported transient effects, there is a clear need to improve the initial approaches. A recent study [57] suggests, at least in retinitis pigmentosa (RP) due to mutant rod-specific cyclic GMP (cGMP) phosphodiesterase 6b (PDE6b), the photoreceptor cell death can be halted, no matter at what stage of the disease gene therapeutic intervention is provided. It is unclear if the findings of this study also apply to LCA but it is noteworthy that with appropriate amount of therapeutic protein delivered to all mutant cells,

one can stop the course of cell loss challenging the 'point of no return' hypothesis for photoreceptor cell death. One need for refinement is to better understand the visual cycle of human foveal cones and their reaction to detachment in order to make the treatments efficacious for visual acuity. Another need, identified through structural studies in dogs and patients, would be to seek a more complete therapeutic outcome that involves both visual improvement and structural rescue. A combination gene therapy where trophic support is provided at the same time as gene supplementation might prove most effective in the long run. Further developments in vector systems that can deliver genes to the foveal region without need for subretinal detachment will likely be beneficial for therapeutic outcome [24, 25].

Implementation of gene therapy for other inherited retinal diseases

Today, gene therapy is being implemented for other retinal degenerative diseases. Positive outcomes have been published for the treatment of choroideremia using AAV as a vector [55, 56]. These and other ongoing studies are discussed below. Further in the pipeline are gene therapies for other forms of LCA caused by mutations in different genes. *GUCY2D* is one of the most frequently mutated genes (12%) and responsible for LCA1 disease form [36]. Recent studies provided evidence that AAV-mediated subretinal delivery of *Gucy2e* preserves the photoreceptor morphology and restores the retinal function of mouse models over lifetime [58] [59], suggesting that gene-replacement therapy for people with LCA1 gene could be feasible. Orphan designation (EMA/COMP/97253/2014) for development of AAV vector serotype 8 containing the human *GUCY2D* gene was recently granted for treatment of LCA1 [60]. As mutations in *GUCY2D* are also associated with autosomal recessive forms of cone-rod dystrophy (reviewed in [59]), this gene-replacement therapy may offer vision restoration to a larger group of patients. Although very preliminary, strategies for development of gene therapy for *CEP290*-associated LCA (LCA10) are also under consideration. Burnight, and

colleagues [61] provided evidence that LV vector expressing full-length human *CEP290* can correct *CEP290* disease-specific cellular phenotype in patient-derived fibroblasts but it is not clear if the LV mediated approach will be able to deliver to photoreceptors in patients. Another approach might be the use of Crispr-Cas9 mediated gene editing; this is currently being developed by Editas. Potential treatment of LCA4 due to *AIPL1* mutations is also under consideration [62]. High level of *AIPL1* photoreceptor expression and no toxicity were documented in *Aipl1* null mice and porcine eyes that received subretinal administration of AAV2/8-*AIPL1* [54]. As some patients with *AIPL1*-associated disease have a late-onset and slow progression rate, it may be a good candidate for gene augmentation therapy [63].

Current clinical trials

Stargardt disease (STGD) is the most common hereditary macular dystrophy and the most common cause of central visual loss in young people. In majority of cases (90-95%), the disease is inherited as autosomal recessive trait and associated with mutations in the photoreceptor-specific *ABCA4* gene (that codes the ATP-binding cassette transporter involved in the clearance of retinoid byproducts) [64]. Proof of concept studies in *Abca4*$^{-/-}$ mouse [65] demonstrated that the subretinal administration of LV- vector containing the human *ABCA4* gene was associated with reduced A2E accumulation, corrected lipofuscin levels, and improved RPE morphology and retinal function. Based on these findings, the first gene-based therapy clinical trial for treatment of STGD moved into human studies. SAR422459 (LV-*ABCA4*) is currently underway (ClinicalTrials.gov Identifier: NCT01367444) at the Casey Eye Institute, Oregon Health & Science University, US and the National Eye Hospital of Quinze-Vingts, Paris, France. No serious adverse events related to dose level 1 or the method of administration were reported so far (Data Safety Monitoring Board, 2012, http://www.oxfordbiomedica.co.uk/press-releases/oxford-biomedica-announces-positive-

dsmb-review-of-ongoing-retinostat-r-and-stargen-clinical-studies/). More recently dual AAV systems have also successfully been implemented in bringing a gene therapy solution to Stargardt disease [66]. Subretinal delivery of *ABCA4* via optimized DNA-nanoparticles also resulted in persistent transgene expression and significant structural and functional correction in the *Abca4*$^{-/-}$ mice [31] suggesting a relevant alternative approach for *ABCA4* gene delivery.

Choroideremia (CHM) is an X-linked recessive disease that leads to progressive retinal degeneration and blindness caused by mutations in the *CHM* gene. The first clinical trial for this monogenic retinal disorder without extraocular manifestations has been undertaken at the Oxford University to assess the safety and tolerability of the AAV2.*REP1* vector administered at two different doses to the retina in 12 CHM patients (ClinicalTrials.gov identifier: NCT01461213, PI: Robert MacLaren). So far, no major safety issues have been reported [55] and some improvements above baseline were reported.

X-linked retinoschisis (XLRS) is characterized by a splitting of the neurosensory retina and progressive macular atrophy. Proof-of-principal for gene replacement therapy (AAV8-*RS1*) in mouse models has been achieved for both structural and functional recovery [67], and the first gene therapy trial is now undertaken by the group of Prof. Paul Sieving (ClinicalTrials.gov Identifier: NCT02416622) and by AGTC (ClinicalTrials.gov Identifier: NCT02416622).

Leber hereditary optic neuropathy (LHON) is a maternally inherited disease caused by mitochondrial DNA point mutations in complex I and characterized by acute (or subacute) painless loss of central vision resulting from degeneration of the retinal ganglion cell layer and optic nerve. Replacement of normal *ND4* and *ND1* gene transcript in fibroblasts of patients harboring mutations in these genes restored electron transport chain activity and

intravitreal viral delivery of normal gene rescued vision in an animal model of LHON [68-71]. First-in-man dose-escalation safety studies are completed and ongoing in several centers (ClinicalTrials.gov Identifiers: NCT01267422, NCT02161380, NCT02064569). No serious adverse reactions related to the treatment or the study procedures have been documented ([72] and Sahel JA, Uretsky S. Gene therapy for Leber Hereditary Optic Neuropathy. ISOPT Clinical, Berlin, Germany, July 2015). Preparation for the upcoming pivotal Phase III of the drug development will be undertaken.

There are other examples of ongoing and completed gene therapies for retinal diseases, including Usher syndrome 1b (MYO7A) (UshStat®, ClinicalTrials.gov Identifier: NCT01505062; the Institut de la Vision/Clinical investigation center at the National Eye Hospital of Quinze-Vingts and the Casey Eye Institute, Portland, Oregon) and autosomal recessive retinitis pigmentosa caused by *MERTK* mutations (AAV2-VMD2-*hMERTK*). In 2013, an AAV vector containing the human *CNGB3* gene received orphan drug designation (EU/3/13/1099) for treatment of achromatopsia. AGTC Inc. plans to treat both CNGB3 and CNGA3 forms of achromatopsia.

Beyond gene supplementation

Secretion of anti-angiogenic factors

Age related macular degeneration (AMD) is the most frequent cause of vision impairment among the elderly. Wet AMD accounts for 90% of AMD-related blindness in these patients. The majority of current treatments for wet AMD aim to prevent choroidal neovascularization through the delivery of anti-angiogenic factors (i.e. bevacizumab (Avastin) or Ranibizumab (Lucentis)). These compounds inhibit vascular endothelial growth factor A (VEGF-A), which is thought to be responsible for the growth and increased permeability of new blood vessels [73].

As AMD is a complex disease, it was not considered a likely candidate for gene therapy. However, the success of VEGF-antagonists requiring frequent readministration and the possibility of long-term expression of anti-angiogenic molecules through its AAV mediated expression sparked interest. In two ongoing phase I clinical trials, pigment epithelium-derived factor (PEDF) and soluble fms-like tyrosine kinase 1 (sFLT) are being tested as potential candidates to for the treatment of wet AMD (ClinicalTrials.gov Identifiers: NCT01024998 and NCT01494805). All of the above-mentioned clinical trials are summarized in Table 1.

Neuroprotection

Neuroprotective agents can prevent and reverse the oxidative stress and its damaging effects, and restore the normal cell function. One retina-specific trophic factor, called Rod-derived cone viability factor (RdCVF) has been shown to induce cone survival and functional rescue in animal models of retinitis pigmentosa (RP) [74, 75], in a gene-independent manner. AAV-RdCVF prolonged cone survival and function in RP mice [76]. It has been suggested as a particularly well-suited therapy for preventing secondary cone degeneration in rod-cone dystrophies and treating RP at a stage of night blindness associated with moderate central visual impairment (STAGE I and II in Figure 1) [77]. A recent study showed that retinal cone survival promoted by RdCVF is associated with accelerated glucose entry of into photoreceptors and enhanced aerobic glycolysis, uncovering an entirely novel mechanism of neuroprotection [78]. Structural and functional rescue in retinal diseases has also been reported for intraocular gene transfer of other vector-delivered neuroprotrophic factors in pre-clinical animal models, e.g. ciliary neurotrophic factor (CNTF) [79-81], pigmented-epithelial derived factor (PEDF) [82] and glial-derived neurotrophic factor (GDNF) [83]. Although promising, no clinical trials have thus far been conducted where trophic factors are provided in form of gene therapy. Encapsulated cell technology has been used in ongoing and completed clinical trials

to provide neuroptorection through CNTF secretion in atrophic macular degeneration (NCT00447954), retinitis pigmentosa (NCT00447980, NCT00447993), achromatopsia (NCT01648452), macular telengectesia (NCT01949324) and glaucoma (NCT01408472).

Once photoreceptors stop capturing light: What next?

When the photoreceptor degeneration is too advanced (STAGE III and IV in Figure 1), patients will have a little chance to benefit from gene replacement therapy or neuroprotection. In these cases, new strategies for vision restoration should be explored. These include retinal prosthesis -designed to stimulate responses from surviving inner retinal neurons- [84-86] or optogenetics, a technique allowing control of neural activity via genetic introduction of light-sensitive proteins such as channelrhodopsin and halorhodopsin [87-90]. Vertebrate opsins such as melanopsin [91] and rhodopsin [92, 93], as well as a chimera between melanopsin and mGluR6 receptor [94] have also been used for vision restoration in late stage RP. The common feature between these approaches is the use of a gene encoding a light sensitive protein that transforms light-insensitive cells of the retina into artificial photoreceptors. This strategy has enjoyed success in preclinical studies, in a number of rodent models of IRD. Currently, optogenetics is being moved towards the clinic by several companies (Gensight Biologics and Restrosense Therapeutics) that have shown interest in the use of microbial opsins for vision restoration. Approaches to evaluate candidate patients for optogenetic therapy is also on its way [95].

Cell Therapy

Cell therapy represents an alternative to repair the degenerated retina. Transplantation of retinal cells has been historically viewed as a potential vision restoration strategy for retinal

degenerative diseases, particularly in disease or disease stages associated with significant cell damage (STAGE II and IV in Figure 1). This therapeutic approach aims at replacing the lost retinal cells using stem cells, progenitor cells and mature neural retinal cells. The main advantage of cell therapies as a source for regenerative therapy is that they are mutation-independent and can be used in a wide range of retinal degenerative conditions. Patients with retinal degeneration typically lose RPE cells, photoreceptors, or both. Therefore, two main cell sources can be considered: first, RPE cells to replace dysfunctional or degenerated RPE and prevent photoreceptor cell loss and, second, photoreceptor precursors to repair the degenerating neural retina.

Several novel stem cell-based therapies addressing inherited and age-related retinal degenerative diseases are currently under development and/or clinical evaluation. They are based on significant body of evidence showing that human pluripotent stem cells (PSCs) can be expanded indefinitely in culture and can be used as an unlimited source of retinal cells (RPE cells, photoreceptors and retinal ganglion cells) for treatment of retinal degeneration. Since their first establishment in 1981 [96], human embryonic stem cells (ESCs) have been intensively studied by many groups worldwide. Furthermore, the recent discovery that somatic cells can be reprogrammed into an ES cell-like pluripotent state, known as induced pluripotent stem cells (iPSCs) offers the same applications in regenerative medicine, bypassing human ESCs which have major ethical restrictions. After reprogramming mouse somatic cells into iPSCs [97], the group of S. Yamanaka was able to reprogram human fibroblasts into iPSCs by over-expressing the four transcription factors OCT4, KLF4, SOX2 and C-MYC [98]. Today, these two types PSCs represent major cell sources in regenerative medicine.

In the last decades, different groups reported encouraging morphological and functional results in animal models of retinal degeneration after transplantation of RPE cells, retinal progenitor cells, photoreceptors precursors or full thickness retinal sheets (see recent reviews: [99-102]). Integration into the host retina and reconstruction of functional neural circuitry, have been seen as major hurdles for successful cell transplantation. For these reasons, the most advanced studies today concern the transplantation of RPE.

Cell transplantation using human PSCs

Human PSCs for RPE cell replacement

Currently, the most plausible approach for development of cell therapy for macular degeneration consists of replacement of the lost or dysfunctional RPE with healthy RPE cells, which are essential for photoreceptor shedding, maintenance and survival. Indeed, different groups have already demonstrated that human PSCs can be differentiated into RPE cells with morphological and functional characteristics similar to those of human RPE cells (for review: [103]).

The first-in-man safety and tolerability prospective clinical trial to evaluate subretinal injection of human ES-derived RPE cells (specifically line MA09-hRPE) in patients with dry AMD and STGD is currently underway. It has been sponsored by *Ocata Therapeutics, Inc. MA, USA* (formally *Advanced Cell Technology*), and conducted at four centers in the USA: Jules Stein Eye Institute (University of California Los Angeles); Wills Eye Hospital (Philadelphia, PA); Bascom Palmer Eye Institute (Miami, FL); and Massachusetts Eye and Ear Infirmary (Boston, MA). Doses of 50,000, 100,000, and 150,000 cells (cell suspensions) have been administered to one eye of 9 patients with dry AMD and 9 STGD patients (3 patients in each cohort). Patches of increasing subretinal pigmentation consistent with the

transplanted RPE cells were documented in 13 out of the 18 patients (72%), but were not correlated with visual acuity improvement. Follow-up testing showed that 10 out of 18 treated eyes had substantial improvements in the first year after transplantation. Stable improvement of visual acuity over 22 months was reported in 7 patients, but decreased by more than 10 letters in one patient. Untreated eyes did not show similar visual improvements, but no correlations between visual acuity improvement and the number of transplanted cells was reported[104, 105]. Median follow-up at 22 months suggest no major safety concerns (no signs of hyperproliferation, tumorigenicity, ectopic tissue formation or apparent rejection). Adverse events were associated with surgery and immunosuppressive treatment but were not considered related to the human ESC-derived cells [104, 105]. These results provide evidence of the medium- to long-term safety, graft survival and biological activity of injection of human ES-derived RPE cell suspensions in patients with macular degeneration.

To date, at least 15 ongoing clinical trials are registered at the International Clinical Trials Registry Platform (ICTRP) of the World Health Organization to test stem cell-based replacement therapies for treatment of retinal dystrophies (Table 1). Some examples include the phase I/II clinical trials with human ES-derived RPE cells sponsored by *Chabiotech Co. Ltd.* (S. Korea), *Cell Cure Neurosciences Ltd.* (Israel) and *Pfizer* (UK) [106]. The London Project to Cure Blindness (sponsored by *Pfizer*) will insert a monolayer sheet of human ES-derived RPE cells cultured on polyester membrane in 10 patients with wet AMD and rapid recent vision decline. This polyester matrix has ben reported to maintain polarized human RPE cells after grafting into the rabbit subretinal space [107]. Similarly, the California Project to Cure Blindness will use differentiated polarized monolayer of RPE cells attached to a non-degradable parylene membrane possessing permeability properties of a healthy Bruch's membrane. A human phase I/II clinical trial will evaluate the safety and the tolerability of this

tissue-engineering product (TEP) in patients with gyrate atrophy [100, 108]. The proof of concept with this TEP has been reported in RCS rats and in Yucatan pigs [100, 109].

As human iPSCs can be obtained directly from the patient, they have the advantage of being autologous and therefore less immunogenic than ESCs for future cell transplantation studies. In this context, the group of Masayo Takahashi at RIKEN Center for Developmental Biology (Kobe, Japan), is currently setting up human clinical trials with human iPS-derived RPE for treatment of AMD. Based on safety studies in rodents and monkeys, this group started to implant a sheet of RPE differentiated from iPS cells previously derived from fibroblasts of one patient suffering from exudative form of AMD (http://www.riken.jp/en/pr/press/2013/20130730_1; http://www.nature.com/news/japanese-woman-is-first-recipient-of-nextgeneration-stem-cells-1.15915). This pilot clinical study is assessing the safety (inducing immune reactions, cancerous growth) and feasibility of the transplantation of autologous iPSCs. No further details have yet been reported.

Human PSCs for photoreceptor cell replacement

While RPE replacement alone may be used for specific disease indications, transplantation of photoreceptors -as retinal sheet or as suspension of dissociated cells- is required after extensive photoreceptor degeneration. Retinal neurons, including photoreceptors have been generated from human iPSCs by different laboratories worldwide (for review: [110, 111]), but so far cell transplantation to restore neural retina is restricted to animal models. Prior groundbreaking studies in mice revealed that the ontogenetic stage of transplanted cells is crucial for successful integration into the adult host retina and recovery of vision [112-114]. Indeed, the group of Robin Ali demonstrated in mice that only stage-specific photoreceptor precursors, corresponding to post mitotic committed photoreceptors, are able to efficiently

integrate into the degenerating retina, differentiate into mature photoreceptors, form synaptic connections and possibly lead to recovery of visual function [112, 114]. Furthermore, a direct relationship between cellular integration and functional recovery has been clearly established [114]. From these pioneering studies emerged the importance of identifying the in vitro equivalent of post-mitotic postnatal photoreceptor precursors derived from PSCs. These photoreceptor precursor cells (PPCs) derived from mouse ES cells in which GFP allowed to trace the photoreceptor lineage, have been successfully differentiated and transplanted into the mouse retina [115, 116]. To date only one study has reported transplantation of photoreceptors derived from human PSCs in mouse models of photoreceptor degeneration [117]. Following subretinal transplantation of virally labelled GFP-positive photoreceptors, Lamba and colleagues [117] demonstrated cell integration into the remaining neural retina and partial restoration of visual function. The use of fluorescent reporter cell lines to isolate the photoreceptor precursors is not compatible with future clinical applications. The surface antigen CD73, previously used to isolate precursors of photoreceptors from mouse postnatal retina for transplantation [118, 119], could be a promising candidate. Our group recently demonstrated that photoreceptor precursors differentiated from human iPSCs specifically expressed CD73 [120]. Based on data with mouse ES cells, the use of a five cell surface biomarker panel (CD73-CD133-CD47-CD24 positive and CD15 negative) could be used to improve the isolation of photoreceptor precursors [121].

In the case of very severe degenerations and loss of outer nuclear layer (ONL), transplantation of retinal sheets rather than dissociated cells could be required. Recently, Takahahsi and colleagues transplanted mouse PSC-derived retinal sheets containing a defined ONL into *rd1* mice (a model of advanced retinal degeneration associated with lost ONL) and observed host-graft synaptic connections [122]. The development of recent innovative protocols

allowing the generation of neuro-retinal structures from human PSCs [120, 123, 124] will be very helpful to assess the capacity of human retinal sheet to make contact with the recipient retina after subretinal transplantation.

Alternative source of human cells for retinal cell replacement

It has been reported that many types of stem cells, such as neural stem cells (NSCs) and mesenchymal stem cells (MSCs), possess inherent neuroprotective properties when transplanted in animal models of retinal disease (for review: [125]). Even though the generation of new retinal cells directly derived from MSCs and NSCs remains unlikely, paracrine effects (such as anti-apoptotic and anti-inflammatory signalling) could explain how these cells contribute to prolonged retinal cell survival. *StemCells, Inc*. is sponsoring a study to determine the safety and potential benefits of subretinal injection of human NSCs in patients with geographic atrophy due to AMD. The trial is based on findings that subretinal transplantation of human NSCs (grown as neurospheres) derived from the foetal brain can partially protect photoreceptor degeneration and preserve the visual function in RCS rats [126]. Very early results at 6 months follow-up showed maintenance or improvement in best corrected visual acuity and contrast sensitivity with no safety concerns (http://investor.stemcellsinc.com/phoenix.zhtml?c=86230&p=irol-newsArticle_print&ID=1941107). Autologous bone marrow derived stem cells (BMSC) are under evaluation in phase I/II clinical trials in AMD patients in South Florida and California (Davis) and in various locations worldwide (Table 2). Safety studies using of human umbilical tissue-derived cells (UTCs) -subretinal administration of CNTO 2476- are currently performed in patients with advanced RP and in subjects with visual acuity impairment associated with geographic atrophy secondary to AMD. No clinical data has yet been reported. Similar concept is implemented in the RP project of the California Institute for

Regenerative Medicine, with *jCyte* company. They will explore the safety of intravitreal injection in RP patients of human retinal progenitor cells obtained from foetal retina and expanded in culture.. It is expected that these cells will not only exert neurotrophic support but also will also differentiate and integrate into the retina.

Another type of cell-based therapy for retinal degeneration approaching clinical translation is the use of human cells to provide neurotrophic factors in order to improve the survival of photoreceptors and their function. An example in this respect is the implantable cell-encapsulation device NT-501 developed by *Neurotech Pharmaceuticals* [127] that consists of human RPE cell line transfected with a plasmid encoding ciliary neurotrophic factor (CNTF) encapsulated within a semi-permeable polymer membrane and supportive matrices. Phase I clinical trial indicated that CNTF is safe for the human retina even with severely compromised photoreceptors and may have application beyond disease caused by genetic mutations [128]. Currently, the device is under evaluation in Phase II studies for treatment of dry AMD and RP.

Challenges

Although stem cell therapy carries great potential for treatment of retinal degeneration its advancement to clinical translation faces multiple challenges. Among the most important are health and ethical issues associated with the nature of most stem cell types, such as risks of tumorigenesis associated with reprogramming and immunogenic responses. Risks associated with the surgery and microbiological safety can also be limiting factors for the use of stem cells. The type and number of cells needed for effective treatment, transplanted cell survival and the functional outcomes remain questions of major importance for successful stem cell-

based therapies in the clinic. Finally, production and delivery of clinical grade stem cells involve unique regulatory and quality control requirements that need to be clearly established.

Concluding remarks

Gene and cell therapies opened new doors in the treatment of currently incurable retinal degenerative disorders and promise to be the therapeutics of the future. To accelerate the advancement of this innovative field, expert groups recently proposed key steps and recommendations. These recommendations address the most pressing needs for the development and delivery of effective treatments for retinal dystrophies in the next decade. The meeting of the National Eye Institute in collaboration with the National Institutes of Health Center for Regenerative Medicine [129] and the Monaciano Symposium [130] strongly recommended collaborative translational efforts, including creation of international databases of correlative phenotype-genotype information, standardized protocols and outcome measures, common regulatory protocols and technology transfer mechanisms [131]. There is a strong conviction that efficient partnerships between academia, industry, funding agencies and policy makers are needed to translate laboratory discovery into development of innovative gene and cell therapeutic strategies in retinal degenerations.

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