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Review Article

Review from 2018 TO 2023:

News and Perspectives on the Gene Therapy of the Eye

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Abstract

The purpose of this article is to analyze the current state of gene therapy in eye diseases.

The greatest results are on animals and in vitro and some experiments are already underway on humans especially in the Asian world.

The sector which for now has invested an applicability also in Europe and above all that of viral vectors for genetic diseases of the optic nerve such as Leber's opticopathy.

In general, the applications are more significant in the anterior segment due to ease of access but also extremely interesting for the posterior segment.

Introduction

Much can be done in the eye for genetic errors of great importance are viral vectors, in particular CRISPR-Cas9 editing for both the anterior and posterior segments.

Although some gene therapies have been approved for human use, the greatest results in this field belong to models obtained on the anterior segment carried out on animals or in vitro.

The most important studies come from China.

In Europe, positive results were seen for topical AON Aganirsen for corneal neovascularizations, the only study that completed phase 3 of the clinical trial. A trial for the use of crisp gene-editing therapy to treat viral keratitis is currently underway in China, but the study has just begun and patients are being recruited. In any case, however few, these developments give us great hopes for translational gene therapy in the anterior segment soon and in the posterior segment in the more distant future (1).

Methods

The study set out to evaluate the most significant and current prospects in the field of gene therapy substantially assessable in a reliable way from 2018 to 2023.

Whenever possible, evaluations on men are reported.

Description

Anterior Segment

The review contains information for gene therapy of the cornea, conjunctiva, trabecular meshwork, and lacrimal glands.

Corneal fibrosis and scars, dystrophies, herpetic keratitis, dry eye, glaucoma and various corneal surface diseases have shown sensitivity to gene therapy through the system of viral and non-viral vectors as well as editing techniques, especially of crsipr-cas9 and treatments epigenetics including antisens and therapeutic siRNAs. (1)

Gene therapy of corneal dystrophies is very promising, the current corneal transplant therapy often requires complex surgical procedures, appropriate donor corneas, frequent rejection so that numerous studies involving gene supplementation, gene silencing and gene editing have been developed.

Furthermore, new ex vivo genetic strategies have aided the survival of corneal transplants and the prevention of herpetic stromal keratitis, neo-vascularization, devastating scarring.

The crisp-cas9 system offers a wide range of genetic engineering that makes the surgical approach safer.

There are 22/23 mostly monogenic corneal dystrophies with high penetrance AD inheritance.

In Category 1 the genes are identified and the mutations known.

In Category 4 the clinic and genetics are unclear.

In the Cornea we have transparent avascular tissue and banks dedicated to conservation.

Negatively affect tear flow, blinking and junctions that give strong resistance against the release of external genes.

In vitro The corneal tissue to be transplanted is grown in the laboratory with injection of a viral vector or not recombinant virus carrying the gene but immune problems.

In vivo, direct intrastromal injection, intracameral or subconjunctival injection, eye drops are performed with more or less help from electroporation or ultrasound or excimer lasers to enhance gene delivery.

In vivo reporter gene delivery in herpes keratitis in vivo (10% of corneal transplants) benefits from local administration of IL10, a transgene administered by electroporation or based on adeno and lentiviral or AAV for HLAG (human leukocyte antigen G) which prevents neovascularization, T lymphocytes and

myofibroblasts.

Dominant negative mutant cyclin G1-mediated retroviral delivery prevents corneal haze after PTK.

Transferring decorin into the fibroblast genes inhibits the formation of myofibroblast and therefore corneal haze and in mucopolysaccharidosis.

The RNA-guided CRISPR/Cas is universal, the key component of which is the CAS9 protein, delivered into target cells.

The gRNA-based mechanical CRISPR deletes the genes that create knock out cell lines. CRISPR Homology

End-Joing serves as a reference model for DNA repair, useful in epidermolysis bullosa

recessive dystrophic, corneal dystrophies. It allows for genetic corrections using an RNA guideline that

is reverse transcribed at RT.

CRISPR as a molecular therapy acts like a living drug against the cancer cell.

In granular or reticular corneal dystrophies the HDR gene is repaired.

Corrections of the CRISPR gene in the cornea are possible both in ex vivo and in vivo since most genetic alterations are single-based.

However, there are several limitations associated with the management of CRISPR-cas9-based corneal treatment.

Inflammation is a very severe disease process especially in the cornea due to the relative possible neo-vascularization and current treatments have limited efficacy, side effects and short duration of action.

Gene therapy has shown great potential for these surfaces but studies have just begun and are decidedly experimental to date. (2)

Although there are encouraging results, currently the development of trials still seems very slow.

Viral and non-viral vectors used in the delivery system of major nucleic acids appear to be a good approach for the regulation of pro- and anti-inflammatory cytokines and neovascularisation.

The cornea is immune privileged (3) and can be maintained in ex vivo organ culture (4).

This is very interesting for the maintenance of endothelial cells in the corneal bank.

Corneal infections benefit from GFP, β-galactosidase like genes and gene therapy has demonstrated better control than other therapies.

Plasmid nucleic acids were used as substromal eyedrops and as a subconjunctival injection.

Furthermore, it can be aided by electroporation, iontophoresis, non-viral systems, magnetic nanoparticles, polymeric nanoparticles, non-viral vectors and mediated by inflammatory factors, such as HAV-1, IL-10 after HSV.

Gene therapy can also be driven by inflammatory lymphogenesis through modulation of anti- and pro-inflammatory cytokines.

The levels of studies are all still preclinical in animal models, scarce in human models. (5)

Characterization of the genetic basis of glaucoma could identify new targets for gene therapy.

Such therapy can deliver long-term therapeutic proteins for the treatment of glaucoma capable of lowering intraocular pressure and neo-protection in glaucomatous animal models.

Improvements in genetic engineering have developed vectors that can enable gene delivery to specific tissues such as the trabecular meshwork with minimal effects on surrounding tissue.

Future studies look for optimization of the vector to increase transductive efficiency, biocompatibility, tissue specificity and better characterization of the genetic basis of glaucoma to help identify new targets for gene therapy. (5)

Corneal Endothelium:

Conditional gene targeting is a technique that allows studying gene function in one tissue without influencing its expression in other tissues.

This is particularly useful for studying the adult function of a gene and elucidating whether deleting it from the whole organism leads to a perinatal lethal phenotype. Tissue-specific activation of Cre recombinase (Cre) coupled with flanking of a gene of interest with loxP sequences, can be used to avoid the limitations seen in some constitutive knockout models.

The Cre-loxp system is based on a recombination method typical of the P1 bacteriophage.

The two important elements of the procedure are:

- Cre: is a 38 kDa DNA recombinase, produced by the cre gene (cyclization recombinase);
- loxP (phage P1 crossover locus): a 34 bp sequence, consisting of two 13 bp palindromic sequences and an 8 bp core region.

Cre recombinase can be considered a molecular scissor that recognizes and cuts at loxP sites, which generally flank a gene of interest.

Depending on the orientation of the loxP sites, excision, inversion, or translocation of the flanked (floxed) gene can occur.

Generally the method is used for the excision - therefore, the inactivation - of the gene of interest, for which the loxp sites have a direct orientation.

Cell proliferation, migration and fibrosis, critical to restoring tissue integrity in wound healing, are features observed in the mesenchymal transition.

Mesenchymal transition is a process in which cells lose their polarity, assume a fibroblastic phenotype, and exhibit increased cell proliferation, migration, and type I collagen secretion, and corneal endothelial cells (CECs) may undergo mesenchymal transition (EnMT) in response to severe injury or inflammation.

We know that the cornea is the transparent anterior tissue of the eye that serves as the main refractive element. The maintenance of transparency is essential for the refractive function and depends on the coordinated function of its layers, i.e. epithelium, stroma, endothelium.

Type 1 collagen can lead to the formation of RCM, an opaque fibrous membrane that could lead to irreversible blindness.

- Zeb1 is a critical mediator of mesenchymal transition in many biological processes and has also been identified as a critical regulator of fibrosis in EnMT.
- Zeb1 flox/flox:UBC-CreERT2 mice were generated by crossing Zeb1 flox/flox mice with UBC-CreERT2 transgenic mice to exploit the Cre-lox system for conditional targeting of Zeb1 in the corneal endothelium.

Intracameral injection of 4-OHT into flox/flox Zeb1 in the adult UBC-CreERT2 mouse resulted in excision of exon 6 in the genomic DNA of the CECs.

Furthermore, spontaneous Cre activity is not observed in the corneal endothelium, as evidenced by the lack of 367 bp F1-R2 PCR product in Zeb1 flox/flox : UBC-CreERT2 mice that received intracameral injection of vehicle alone.

Genomic modification in the corneal endothelium is reflected at the transcriptional level in 4-OHT-injected Zeb1 flox/flox mice:UBC-CreERT2 mice.

FGF2 has been used in organ culture to stimulate Zeb1 expression. Zeb1 RT-PCR using mRNA isolated from corneal endothelium from 4-OHT-injected Zeb1 flox/flox :UBC-CreERT2 mice showed a severe decrease in the amount of intact Zeb1 mRNA.

This probably reflects the small amount of intact Zeb1 gene in the corneal endothelium and this could be due to an insufficient dosage of 4-OHT in the intracameral injection.

Intracameral injection of 4-OHT and FGF2 treatment induced Zeb1 mRNA expression in wildtype, UBC-CreERT2 and Zeb1 flox/flox mice.

Genes in the corneal endothelium can be conditionally targeted.

This opens the door to the study of the adult function of genes in the corneal endothelium that have critical developmental roles, where gene deletion leads to an embryonic or perinatal lethal phenotype.

In this report, proof of principle for spatiotemporal gene targeting in mouse corneal endothelium was demonstrated, using an inducible Cre-Lox strategy. (14)

Fuchs Syndrome:

Fuchs endothelial corneal dystrophy (FECD) causes severe vision loss and accounts for approximately 40% of all corneal transplants.

The clinical signs of FECD are: excessive production of extracellular matrix (ECM) between the corneal endothelium and Descemet's membrane (the basement membrane of the corneal endothelium) and damage to the corneal endothelial cells (CEC). The ECM forms focal growths called guttae, resulting in visual disturbances due to reduced contrast sensitivity and increased glare. Corneal endothelial decompensation due to CEC damage induces corneal edema, resulting in further severe vision loss due to corneal transparency loss.

FECD has been accepted as the most common inherited corneal disease, as it displays an autosomal dominant pattern of inheritance. However, the responsible genes remain unclear, suggesting the need for in-depth studies that make best use of current advances in genomics.

The primary goals of the investigators in this most recent study (16) were to obtain a CEC RNA-Seq dataset derived from Caucasian FECD subjects and healthy control subjects, to identify DEGs, and conduct enrichment analyzes to reveal potentially related to the pathophysiology of FECD. Multiple genetic variants have been reported, but the pathogenesis of FECD is not fully understood. In this study we used RNA-Seq to analyze differential gene expression in corneal endothelium obtained from patients with FECD.

Differential expression analysis of transcriptomic profiles revealed that the expression of 2366 genes (1092 upregulated and 1274 downregulated) was significantly altered in the corneal endothelium of FECD patients compared with healthy subjects. Gene ontology analysis demonstrated an enrichment of genes involved in extracellular matrix (ECM) organization, oxidative stress response, and apoptotic signaling.

Several analyzes have consistently indicated dysregulation of pathways associated with the ECM.

The differential gene expression findings support previously proposed mechanisms, including oxidative stress and apoptosis of endothelial cells, as well as the phenotypic clinical FECD hallmark of ECM deposits.

Further investigations focusing on the differentially expressed genes related to these pathways could be useful to elucidate the mechanisms and develop new therapies.

Posterior Segment

Retina:

All retinal disorders with visual loss are on the increase in the world showing an important therapeutic deficit in the posterior segment of the eye.

In France, led by the Italian Marco Bassetto, RNA-interference (RNAi) was found to be fundamental even if its transport into the inner retinal layer appears extremely challenging.

For this reason, the authors developed a method called MNPs - a method based on the transfer of magnetic nanoparticles capable of transporting SiRNA into all retinal layers of the retinas of adult rats. (8)

The method of these scholars called "Reverse Magnetofaction" allows the traction of the SiRNA to the MNPs.

This method is a new and non-toxic strategy both molecularly based on RNAi and as in gene therapy of the retina but hypothetically it can be transferred to all organs. (8)

The retina becomes the easiest experimental transplant with feasibility of crossbreeding between nanoparticles and transferable RNA.

An undisputed novelty is the possibility that intravitreal injections will be replaced by a microemulsified eye drop with nanostructures.

This research, patented by SIFI SpA and carried out in Catania under the guidance of Emanuela Santonocito, allows a nanostructured micro emulsion (NaMESys) to transport 0.3% (NaMESys-SOR).

It is cytocompatible in vitro on rabbit corneal cells and well tolerated up to 3 months of follow-up and was able to revascularize retinal ischemic occlusions in rats and to inhibit the expression of (TNFalpha 20.7%). Responsible for necrosis and inducing nitric oxide synthesis (INos 87.3%) mRNAs compared to controls.

Therefore capable of inhibiting neovascularization and angioretinal proliferations becoming useful also for diabetic retinopathy, in choroidal neovascularization and ultimately for all neovascular retinal diseases. (9)

In conclusion, NaMESys-SOR has been shown to be well tolerated and to deliver high sorafenib to the retina by reducing pro-inflammation and pro-angiogenesis mediators.

Speaking of genetic retinal diseases, a very difficult field, such as x-linked retinoschisis, choroideremia, Stargardt's disease, x-linked retinitis pigmentosa could be treated with intravitreal injections of AAV8 (NCT02317887) and AAV2 (NCT02416622) currently in phase 1 / 2 clinical trials. (12)

In any case, these products can be administered with a sub-retinal approach.

We do not yet know the possible immunogenicity that can cause intraocular inflammation.

Other emerging gene therapies are in the pipeline including RNAi (RNA interference) or antisense-oligonucleotide therapy.

Leber congenital amaurosis has had its greatest gene therapy success in the autosomal recessive type 2 LCA, which occurs in 1:80,000 births associated with mutations in the GUCY2D, CEP290 and RPE65 genes that are involved in 11-cis production -retinal during phototransduction.

Voretigene neparvovec-rzyl (Luxturna, Spark Therapeutics, Philadelphia) is an AAV2 that releases RPE65 via subretinal injection that demonstrates safety and benefit in Phase 3 trial, approved by the FDA in 2017.

The latest studies have shown that increases in visual function and visual field persist for 3 – 4 years after therapy with no immune response, but sadly 18 eyes of 10 patients recently showed perifoveal chorioretinal atrophy five months after treatment which it was maintained for one year after follow-up producing a central scotoma.

The most significant improvements are in children who increase by two lines of visual acuity.

However, there is the possibility of an increase in IOP and persistent inflammation, of vitreous opacities which, however, can be managed.

In March 2020, human in vivo application was performed using a CRISPR-cas system commenced to value AGN-151587(EDIT-101, allergan, NCT03872479) administered sub-retinal in 18 patients.

This study will end in 2024 by promoting in vivo genetic manipulation with the CRISPR-Cas system to eliminate genetic retinal diseases.

Despite being a small-number study, it is the first of its kind on humans. (12)

Retinal Pigmented Epithelium:

the RPE probably plays a role of cellular relay for the modulatory signals of bulb growth (ie myopia), since it is located between the retina and the two walls, namely the choroid and the sclera. Although protocols for RPE isolation have been developed for both chicks and mice, they have been shown not to be directly translatable in the guinea pig, which is a model of the mammalian myopia study.

In this study, molecular biology tools were used to examine the expression of specific genes and confirm that the samples were free from contamination from adjacent tissues. The value of this protocol has already been demonstrated in an RNA-Seq study of RPE from young pigmented guinea pigs exposed to myopia-inducing optical blur. In addition to eye growth regulation, this protocol has other potential applications in studies of retinal diseases, including myopic maculopathy, a leading cause of blindness in myopes. The main advantage of this technique is that it is relatively simple and, once perfected, produces high-quality RPE samples suitable for molecular biology studies, including RNA analysis (15)

Liquid Retina:

Several Italian groups of researchers such as the group of the Center for Synaptic Neuroscience and Technology of the Italian Institute of Technology (IIT) in Genoa directed by prof. Fabio Benfenati at the IRCCS Ospedale Policlinico San Martino in Genoa and at the Center for Nano Science and technology of the IIT in Milan, directed by prof. Guglielmo Lanzani, in collaboration with the Ophthalmology clinic of the IRCCS Sacro Cuore Don Calabria Hospital in Negrar, directed by Dr. Grazia Pertile demonstrated the effectiveness of the liquid artificial retina model presented in 2020 (Nature Nanotechnology 2020, https://opentalk.iit.it/sviluppato-il-primo-modele-sperimentale-di-protesi-liquida-di-retina/) (13) even in the more advanced stages of retinitis pigmentosa.

Photovoltaic nanoparticles called "nano sparks" are also effective in irreversible stages of retinal degeneration, completely devoid of photoreceptors and deconstructed by residual retinal circuits, so far treated with retinal prostheses (microchips).

In fact, it has been shown that nano sparks made in Italy reactivate the occipital visual cortex, regaining visual acuity and visual memory.All this would mean that this second generation, biocompatible and high resolution artificial retina could be the winning solution.

Five million people around the world are waiting for 2025/2026, when the first tests on humans will be performed.

Choroid:

Autoimmune noninfectious posterior uveitis is based on constant genetic mutations producing NIV, fibrosis, s. by Blau. (11)

Other uveitis is idiopathic and nothing emerges in the entire exome.

There are no overrepresented types of posterior segment uveitis.

The threshold of significance has been reached by 23 genes and each patient can have more variants of each gene.

Elevated levels of IL-1β have been found in vitreous uveitis.

Researchers have discovered unknown variants in chorioretinitis, especially bilateral and adolescent.

Pathogenicity predictions based on the structure of the capN5 variants are possible.

Curious that boys with dominant uveitis (Blau) can have healthy parents.

But another autoimmune form of uveitis is always present in juvenile idiopathic arthritis.

It is curious that POHS, with a controversial link to H. capsulatum infection, can give mutations in m. Crohn's disease and orofacial granulomatosis.

The structure of NLR inflammosome variants can give an example pathogenicity prediction. (multifocal choroiditis, post retinal laceration uveitis etc.); the NLR p3 variant is the most important of all.

The tyk2 gene does not cause uveitis but its mutations do (sympathetic uveitis, panuveitis, bird's eye chorioretinitis, autoimmune retinopathy, idiopathic uveitis, posterior scleritis).

We note that:

1. WES is a valuable diagnostic tool but expensive and can give false negatives.
2. NOD2, NLP!, NLRP3 and NLP4 are fundamental variants of the inflammasome capable of altering protein structure.

   If we have a high level of NOD2 it is possible to have: S. Blau, M Crohn and Psoriatic arthritis.

   If the level is lower we may have peptidoglycan uveitis.
3. NLRP1 increase promotes acute glaucoma, M. Crohn, RA, Vitiligo, its NLRP1 decrease also reduces acute glaucoma severity.
4. If NLRP3 increases, the frequency of acute glaucoma, diabetic retinopathy, age-related macular degeneration, hereditary keratoendothelitis fugax and Behçet's syndrome increases

In conclusion, therapy should be targeted to the inflammosome non-specifically.

Optic Nerve:

By removing a gene that is indirectly responsible for the pathophysiology of the disease.

As we have said, both viral (adenovirus, HSV, AAV, viral lenses) and non-viral vectors are used. Each of them has advantages and disadvantages.

Recent trabecular meshwork studies using viral lenses and AAV vectors slowed epigenetic extrinsication in glaucoma tissue.

The limit of all this is the immune response (for now in rats =).

Adenoviral vectors increase aqueous outflow in human and monkey organs.

Also important is the use of siRNAs that inhibit the myocycline gene.

in fact, the ciliary body and epithelial cells can alter the configuration of the trabecular meshwork.

MMP injected into the AC of rats has a transductive effect on the ciliary body.

Injecting slow viral vectors into AC results in expression of cyclooxygenase-2 enzyme and prostaglandin F and good reduction of IOP is obtained (in cats so far).

Recombined AAV vectors have shown a reduction in ganglion cell and Muller cell apoptosis (in chickens for now).

Inhibition of protein apoptosis inhibits the final caspase cascade inducing net neuroprotection (in rats so far).

IVT injections of adenoviral BDNF (in rats) produce selective Muller cell expression that is distinctly protective but unfortunately prone to immunosuppression.

Ultimately gene therapy can augment known mutant genes. (6)

Good news for the future of the optic nerve is caspase-3 small interfering RNA-nanoparticles therapy (neuroprotective after optic nerve damage).

In fact, it reduces the apoptosis of neuronal cells.

A new non-viral gene therapy blocks caspase-3 gene expression using a small RA (siRNA) released from polybutylcyanoacrylate nanoparticles (CaspNPs) when injected intraocularly.

CaspNPs lowered retinal immunofluorescence of capsase-3 by 57.9% in rats with pinched optic nerves.

Focal imaging showed 36.1% cell loss in these eyes versus 63.4% in control eyes.

Thus, reduction of caspase-3 protein reduces cell death of post-mitotic neurons by 50% with restoration of central visual function (non-viral therapy).

Ultimately, neuroprotection or even the restoration of damaged central vision after neurological disorders, even of a different nature, could be obtained with non-viral gene therapy with siRNA (nanoparticles).

All studies used to date have been approved by each country's animal experiment ethics committee. (7)

Perspectives also open for optic atrophies!

Conclusion

In conclusion, much can be done in the eye for genetic errors.

Great importance to CRISPR CAS9 editing where we can use viral vectors.

The least side effects are AAV vectors but they have transport limitations (5kb of DNA).

For larger genes they can be attacked by intermolecular recombination with a different vector (Usher, Stargardt).

The transgenic efficiency on the target tissue can be evaluated as a direct clinical response (Leber).

Critical Points:

1. Promoter safety.
2. Long-term side effects.
3. Costs.

Pros:

Treatability of certainly blinding diseases.

Obviously the miracle will be a new retina, a new optic nerve with in situ reconstitution and reappropriation of the visible occipital areas even when colonized by other fibers.

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