Chapter

Perspective on Gene Therapy for Glaucoma

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Abstract

Glaucoma is a chronic and multifactorial neurodegenerative disease marked by structural damage to the optic nerve with axonal loss, progressive retinal ganglion cell degeneration, and optic disc excavation. Both high intraocular pressure and aging are important risk factors, but not essential to the progression of glaucomatous neurodegeneration. Current treatments are based on controlling intraocular pressure, which is not always effective in avoiding the progression of visual loss. In this sense, novel therapeutic strategies to glaucoma should aim to promote the neuroprotection of both the cell soma of retinal ganglion cells and the axons of the optic nerve. Gene therapy is a new therapeutical approach to glaucoma with a great capacity to overcome neurodegeneration. It consists of the transfer of exogenous genetic material to target cells with a therapeutic purpose. Gene therapy strategies for glaucoma include both the neuroprotection aiming to prevent cell soma and axonal loss and the regeneration of optic nerve axons. In this chapter, we review the most promising current gene therapies for glaucoma that address the various aspects of glaucoma pathology. We also discuss the potential of combining neuroprotective and regenerative strategies to reach a synergic effect for the treatment of glaucoma.

Keywords: glaucoma, retinal ganglion cell, optic nerve, gene therapy, neuroprotection, neuroregeneration

1. Introduction

Glaucoma is a heterogeneous group of highly prevalent ocular disorders that can progress to blindness, impacting functional capacity, social relations, and quality of life. It is now the leading cause of irreversible blindness in the world [1]. Furthermore, it affects mainly the elderly, and its prevalence is expected to increase in the next decades, in parallel with the progressive aging of the world population [2]. The high incidence of glaucoma with continuous growth, combined with its outcome of progressive and irreversible blindness, makes this disease a major public health problem. The pathophysiology of glaucoma is still not completely understood, and the disease has no cure. Glaucoma is a multifactorial, chronic disease characterized by structural damage to the optic nerve, thinning of the nerve fiber layer, and the

degeneration of retinal ganglion cells (RGCs). These changes result in corresponding visual field impairment that progresses to complete vision loss. RGCs transmit visual information to the brain through the axons of the optic nerve. RGC axons converge to the optic disc and exit the globe through the lamina cribrosa to form the optic nerve. In glaucoma, the progressive cupping of the optic disc occurs due to damage to the lamina cribrosa and loss of RGC axons [3]. Long-standing evidence describes elevated intraocular pressure (IOP) and aging as the most prevalent stressors for RGCs in glaucoma. However, glaucomatous optic neuropathy may also develop in normal IOP conditions, in which damage occurs to the optic nerve without eye pressure exceeding the normal range [4].

Current treatments for glaucoma are related to IOP reduction, since high IOP is a manageable known risk factor. The procedure uses hypotensive eye drops or surgical interventions [5]. However, such approaches are often not sufficient to impair the death of RGCs and the progression of blindness, which may affect about half of the treated individuals [6, 7]. Recently, novel therapeutic approaches have searched for an efficient way to overcome neurodegeneration, focusing directly on preventing cell death and ensuring axonal integrity, including promising strategies based on gene therapy. This method consists of the transfer and expression of exogenous genetic material to cells and was originally developed to correct genetic diseases by supplying the cells with a normal copy of a defective gene [8]. Advances in the safety and efficacy of viral vectors capable to deliver therapeutic genetic material, as well as the recent approval of gene-based medicines by regulatory agencies of various countries, put gene therapy on center stage. A widespread panel of possible applications includes studies aimed at the treatment of complex, multifactorial diseases, such as glaucoma. Gene therapy strategies for glaucoma include the manipulation of a variety of intra- and extracellular factors involved in different cellular processes, such as apoptosis, metabolism, and axonal regeneration pathways. Such approaches may prevent neurodegeneration, and promising preclinical results strongly suggest translational potential.

2. Glaucoma: a neurodegenerative disease with early axonal damage

RGC cell death is the common outcome in glaucomatous neuropathies. It is believed to be a consequence of chronic stress, such as caused by IOP, which is expected to affect mainly the unmyelinated, initial portion of the RGC axons located in the optic nerve head (ONH). Such stress is associated with axon dysfunction, such as the biomechanical interruption of axonal transport [9]. Clinical and experimental evidence identified factors that may contribute to optic nerve head damage, such as mitochondrial dysfunction, oxidative stress, excitotoxicity, deprivation of neurotrophic factors, genetic susceptibility, reduced blood flow, vascular dysregulation, and neuroinflammation [9–11]. These alterations form an interconnected network of pathogenic processes that culminate in the degeneration of RGCs. However, each part of the RGC structure—soma, axon, and synapses—shows both the temporally and mechanically distinct degenerative patterns [12].

The degeneration of RGCs can be influenced by damage that affects their synapses and dendrites, as well as by signs from an axonal insult [13]. Early-onset modifications in dying RGCs include the silencing of RGC-specific gene expression, which precedes loss of neurons in certain animal models of glaucoma [14]. The pruning of RGC dendritic trees, cell body atrophy, nuclear shrinkage, and loss of RGC synapses with amacrine and bipolar cells are also among the initial changes detected in the

glaucomatous retina [15]. These events activate several signaling pathways, such as those involving the mitogen-activated protein kinase p38 and Jun N-terminal kinases, which transmit the degeneration message to RGC soma [16]. As a key mechanism of RGC death in glaucoma, programmed cell death by apoptosis has been demonstrated in different species, such as rodents [17], nonhuman primates [18], and humans [19]. The cell death pathway is mediated by protein interactions of the BCL2 gene family, such as BAX or BAK, stimulators of apoptosis, while others, such as BCL-X and BCL2, have antiapoptotic functions. Activated BAX protein aggregates in the outer mitochondrial membrane and induces membrane instability and permeabilization, leading to the release in the cell cytoplasm of cytochrome c, which activates a cascade of caspases to induce cell death. On the other hand, BCL-X inhibits the mitochondrial activation of BAX, keeping the latter in the cytosol. RGC apoptosis depend on the activation of BAX, with the participation of mitochondrial components. BAX knockout animals ($BAX^{-/}$) submitted to acute optic nerve injuries are resistant to cell death by apoptosis, although BAX deficiency is not sufficient to prevent the axonal dysfunction of RGCs [20], suggesting that the mechanisms of cell death and axon degeneration are independent. RGC body loss, however, follows a spatially defined pattern. In rodents subjected to IOP by either a genetic or experimental approach, an asynchronous degeneration of individual RGCs leads to a sectorial pattern of neuron loss [21]. These experimental observations are akin to the pathological and clinical studies of glaucomatous humans, who show localized abnormalities and remodeling of the inner plexiform layer of the retina, correlated with a reduction in visual field function usually seen in early disease stages [9].

The ONH is considered the primary site of damage to RGCs in glaucoma. Despite the difference in lamina composition between humans and rodents, either IOP-dependent or IOP-independent insults to ONH can give rise to distal and proximal signs for the axonal degeneration of RGCs [12]. Among molecular changes triggered in this region, axonal transport failure due to mitochondrial dysfunction and an unbalanced axonal supply of neurotrophins such as brain-derived neurotrophic factor (BDNF) by oligodendrocytes stand out [13, 22]. Decreased blood flow, oxidative stress, reactive gliosis, and extracellular matrix remodeling are also molecular actors that regulate axonal degeneration in glaucomatous retina [9]. However, the exact contribution of each factor to RGC degeneration in glaucoma is not well established. Damaged axons in the optic nerve undergo degeneration, alter functional connectivity of neural circuits, and, consequently, cause a progressive loss of visual function. Axonal degeneration can be classified according to distinct parameters, such as the spatial relationship with the site of damage (proximal vs. distal) and time course (acute vs. chronic). Traumatic damage, as mimicked by optic nerve crush (ONC), results in complete axon degeneration through a series of well-defined events. First, there is acute axonal degeneration (AAD) close to the injury site, where rapid axon disintegration occurs at up to about 500 μm distal and proximal to injury site. This initial process of AAD is followed by a latency period of several hours, in which the rest of the injured axon remains unchanged. Then, two distinct degeneration processes begin: (i) abrupt granular disintegration of axon distal portion, a process known as Wallerian degeneration (WD), where there is cytoskeleton breakdown and organelle destruction; (ii) retrograde degeneration of the axon proximal portion (dying back). In addition, there may be secondary degeneration of cells not affected by the initial injury [23, 24]. In contrast with acute injuries, in chronic conditions, axons gradually degenerate toward a death process that progresses in a distal-to-proximal pattern from the synaptic region to the cell body. In the experimental

models of glaucoma, both dying back and WD have been proposed as the mechanisms of axonal loss, while the role of AAD in glaucomatous degeneration is not understood. The heterogeneity of lesion sites highlights the need for further studies to better understand the time course and the complex processes of anterograde and retrograde degeneration of different subcellular regions of RGCs in experimental glaucoma [12].

An aggravating factor of neuronal degeneration in the adult central nervous system (CNS) of mammals is its low regenerative capacity. Once an injury occurs, damaged axons cannot regenerate and recover their integrity to prevent neuron death, therefore resulting in irreversible deficits. For this reason, numerous studies investigate the inhibition mechanisms of axonal regeneration in the CNS. The manipulation of these events can mediate the regrowth of axons and potentially benefit individuals affected either by acute injuries in the CNS or by neurodegenerative diseases associated with axonal dysfunctions, such as glaucoma.

3. Gene therapy for glaucoma

3.1 Strategies for neuroprotection

Over the past few decades, several strategies for neuroprotection of RGCs have been explored. Among those, gene therapy techniques have been developed and refined to allow an efficient targeting of this cell type. Considering RGC death, the critical cellular event of glaucomatous degeneration, the main targets of gene therapy strategies rely on antiapoptotic approaches, as well as on neurotrophic factors, Rho/Rho-associated protein kinase (ROCK) pathway, and mitochondrial disbalance, as summarized in **Table 1**.

Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), are essential for neuronal survival in the CNS, including RGCs. Acting through Tropomyosin receptor kinase B (TrkB) receptor, present on RGC dendrites and cell bodies, the BDNF can activate metabolic pathways for cell survival. Unbalanced physiological BDNF levels or its receptor have been shown in the experimental animal models of glaucoma as well as in patients [25], providing the rationale for new therapies based on BDNF supplementation. The viral vector-mediated overexpression of BDNF promoted robust neuroprotection in a variety of experimental glaucoma models, including acute injuries by NMDA injection [26], ischemia/reperfusion induced by an abrupt elevation of IOP [27], partial optic nerve transection [28], and surgically induced chronic OHT [29]. However, a sustained expression of exogenous BDNF has proved neurotoxic and led to downregulation of its high-affinity TrkB receptor, thus reducing BDNF/TrkB downstream signaling and therapeutical efficacy [30]. To overcome this transient effect, a simultaneous gene therapy with BDNF and TrkB receptor transgenes was tested. After a single intravitreal (IVT) injection, axonal transport was enhanced, and visual functional recovery was achieved in a laser-induced ocular hypertension rat model [31]. Ciliary neurotrophic factor (CNTF) is another well-characterized neurotrophic factor with neuroprotective effects demonstrated when overexpressed by different viral vector platforms in multiple RGC degeneration models, such as ONC [32], vascular occlusion [33], and OHT-induced models [34].

Rho/ROCK signaling pathway plays an important role in the pathogenesis of glaucoma and has been studied as a possible target to promote the neuroprotection of RGCs [35]. This pathway regulates several cellular processes, including cytoskeletal

Target	Mechanism of action	Animal models
1. Growth and neurotrophic factors		
BDNF	Overexpression of neurotrophic factor BDNF	ON transection; photocoagulation of TM; NMDA ivt.; cannulation of AC; partial ON transection
BDNF + TrkB	Overexpression of BDNF + receptor	ONC; photocoagulation of TM
BMP4	Overexpression of growth factor BMP4	Microbeads
FGF2	Overexpression of neurotrophic factor FGF	ON transection; NMDA ivt.
CNTF	Overexpression of the cytokine CNTF	ON transection; ONC; focal crush + retinal vessels occlusion; photocoagulation of TM
GDNF	Overexpression of neurotrophic factor GDNF	ON transection
GDNF + BIRC4	Overexpression of GDNF + caspase inhibitor BIRC4	ON transection
PEDF	Overexpression of PEDF	Cannulation of AC; NMDA ivt.
VEGFD	Overexpression of growth factor VEGFD	NMDA ivt.
2. Antiapoptotic factors		
BAG1	Overexpression of co-chaperone BAG1	ON transection; ONC
Bcl-X_L	Overexpression of antiapoptotic factor Bcl-X_L	Hypertonic saline injection in episcleral vein; DBA2J mouse
BIRC4/XIAP	Overexpression of caspase inhibitor BIRC4	Hypertonic saline injection in episcleral vein; ON transection; microbeads
sFasL	Overexpression of antiapoptotic factor FasL	DBA2J mouse; microbeads
3. Transcription factors		
ATF3	Overexpression of ATF3	ONC
Brn3b	Overexpression ofBrn3b	Hypertonic saline injection in episcleral vein
CREB	Overexpression of a constitutively active variant of CREB	NMDA ivt.
KLF7	Overexpression of KLF7	Cannulation of AC
4. Oxidative stress components		
Catalase	Overexpression of antioxidant enzyme, scavenger of hydrogen peroxid	Cannulation of AC
NRF2	Overexpression of transcription factor NRF2, which mediates transcription of several antioxidant elements	ONC
SOD2	Overexpression of antioxidant enzyme SOD2	Cannulation of AC
SOD2 + Catalase	Overexpression of SOD2 + catalase	ONC
5. Rho/ROCK pathway		
Exoenzyme C3	Overexpression of an inhibitor of Rho proteins	Cannulation of AC

Target	Mechanism of action	Animal models
RhoA	Silencing of RhoA	ONC
ROCK2	Silencing of ROCK2	ONC
6. Mitochondria-related targets		
NMNAT1	Overexpression of NAD production related enzyme	DBA2J mouse
OPA1	Overexpression of mitochondrial fusion protein OPA1	DBA2J mouse
Neuroglobin	Overexpression of the hemoprotein neuroglobin	DBA2J mouse
8. Other targets		
ABCA1	Overexpression of ABCA1 phospholipid transporter	Cannulation of AC
MCT2	Overexpression of monocarboxylate transporter MCT2	DBA2J mouse; microbeads
CaMKII	Overexpression of constitutively active CaMKII, enzyme in the Ca^{+2} signaling pathway	NMDA ivt.; ONC; microbeads; Glast-deficient mice
S100A4	Overexpression of S100A4, a Ca^{+2} binding protein	Cannulation of AC
CR2-Crry	Overexpression of complement inhibitor CR2-Crry	DBA2J mouse
CRMP2	Overexpression of CRMP2, a cytoskeleton regulator	Partial ON transection
Hsp70	Overexpression of chaperone Hsp70	ONC
MEK1	Overexpression of MEK1, an ERK1/2 activator	ON transection; hypertonic saline injection in episcleral vein
Shp2	Silencing of protein-tyrosine phosphatase shp2	Microbeads
ULK1	Overexpression of a dominant-negative form of autophagy activating kinase 1	ONC
miRs-17-5p + 30c-2 + 92a	Delivery of multiple miRNAs with a variety of targets	ONC
miRs-92a + 292 + 182		

Note: OHT: Ocular hypertension; I/R: Ischemia/reperfusion; ON: Optic nerve; TM: Trabecular meshwork; AC: Anterior chamber; ONC: Optic nerve crush; ivt: Intravitreal.

Table 1.
Gene therapy strategies for neuroprotection.

remodeling and synthesis of extracellular matrix components. Intravitreal injections of rAAV2 vectors carrying shRNA to knockdown RhoA expression can protect RGC from death caused by optic nerve injury [36]. In a similar study, the rAAV2-mediated knockdown of another member of this pathway, such as ROCK2, confers structural neuroprotection to RGC soma and axons after ONC [37]. Moreover, the inhibition of ROCK by the overexpression of BAG1 [38], an inhibitor of Rho/Rock signaling, can rescue RGC from apoptosis induced by axon injuries.

The modulation of apoptotic pathways has also been explored with gene therapy platforms. The overexpression of Bcl-XL, an antiapoptotic member of the Bcl-2 protein family, using an rAAV2 vector with phosphoglycerate kinase gene promoter (Pgk), robustly ameliorated RGC soma pathology and axonal degeneration in the chronic OHT mouse model, DBA/2 J, and provided a long-term somal neuroprotection after acute ONC [39]. Mechanisms involved in this therapy rely on blocking apoptosis induced by the activation of BAX, limiting its fusion to the mitochondria compartment. Alternatively, the overexpression of caspase inhibitor BIRC4 using rAAVs led to neuroprotection in a glaucoma model of OHT induced by the injection of magnetic microbeads in the anterior chamber, showing the preservation of RGC function as evaluated by pattern electroretinogram (PERG), and axonal integrity in the optic nerve [40]. Additionally, apoptosis in neuronal cells has been associated with the subcellular localization of Annexin A1 (ANXA1), since the nuclear localization of this molecule can modulate transcriptional factors such as p53 and p65 and trigger this type of cell death. As related to this pathway, Luo et al. described a strong neuroprotective action mediated by the overexpression of ATP-binding cassette (ABC) transporter A1 (ABCA1), which reduced the nuclear localization of ANXA1, and was associated with robust RGC survival in an I/R model induced by the cannulation of the anterior chamber [41].

A known outcome of RGC injury is the disruption of intracellular Ca^{+2} homeostasis, an ion that acts as an important intracellular signaling molecule [42]. Ca^{+2}/calmodulin-dependent protein kinase II (CaMKII) is a key responder in this pathway and has transcription factor CREB as an important downstream effector [43]. Guo et al. reported a decrease in phosphorylated CaMKII after RGC lesion by NMDA-induced excitotoxicity and ONC, indicating lower protein activity. The reactivation of CaMKII, mediated by the rAAV overexpression of a constitutively active mutant, robustly enhanced RGC survival after NMDA lesion, ONC, glaucoma models of microbead injection and in Glast-deficient mice. CREB activation was necessary and sufficient for the protective action of CaMKII. Furthermore, the neuroprotective effect of CaMKII had a long-lasting effect, was present even if overexpression was induced after the lesion, and led to the preservation of visual function [44].

In addition to those pathways, mitochondria dysfunction is another target explored to slow down glaucoma progression. ONH damage leads to an unbalance of mitochondrial homeostatic activity, compromising oxidative phosphorylation due to the dysregulation of intracellular calcium concentrations, thus contributing to reduced energy availability, increased production of reactive oxygen species (ROS), and activation of RGC apoptosis [45]. Selectively targeting specific ROS-mediated signaling pathways using rAAV2 constructs encoding the transcription factors NRF2 and/or PGC1a promoted the scavenger of ROS and protected RGCs from oxidative stress triggered by ONC [46]. However, the overproduction of stress response transcription factors Nrf2 and PGC1a can be toxic to neurons; therefore, adequate levels of expression are required. Moreover, reduced nicotinamide adenine dinucleotide (NAD) levels have been closely correlated with mitochondrial dysfunction and were implicated in glaucomatous degeneration [47]. NAD is a key component for healthy mitochondrial metabolism and an important redox cofactor essential for RGC function. Intravitreal viral gene therapy overexpressing Nmnat1, the terminal enzyme for NAD production, robustly protected DBA/2 J RGC against neurodegeneration, and prevented several early changes such as axoplasmic transport impairment and decline in RGC functional activity [48].

3.2 Strategies for axonal regeneration

Axonal damage is an early event during RGC degeneration in glaucoma. In this sense, besides preventing cell degeneration, gene therapy strategies to glaucoma should also aim at axonal regrowth after axon loss. However, axonal regeneration in mammalian CNS is not easy, since after development is completed, axons lose their ability to regrow. This is opposed to the peripheral nervous system, in which after axon damage, the distal portion of the lesion, not connected to cell body, degenerates, but a growth cone may develop in the axon's proximal part, which will regrow again. In this case, successful axonal regeneration leads to target reconnection, and usually, the neuron does not die. In the CNS, a scar develops in the lesion site, axons do not regenerate, and the neuros eventually die [49, 50]. This inability to regenerate has been associated with a few different factors, divided into two major groups known as cell intrinsic and cell extrinsic. Cell intrinsic factors include mostly genes related to axonal growth, which have their expression modulated after development, comprising several transcriptional factors as well as components of signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt (PI3K/Akt) and Janus kinase/signal transducer and activator of transcription protein (Jak/STAT) [51]. Cell extrinsic factors are mostly molecules associated with astrocytes and oligodendrocytes, such as chondroitin sulfate proteoglycans (CSPGs), NOGO myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMGp), which are present in the glial scar and act as inhibitors of axonal regeneration. Yet, such molecules activate the Rho/Rho-associated protein kinase (Rho/ROCK) intracellular pathway, which mediates the intracellular responses to the extrinsic inhibitor molecules [52].

Numerous strategies have been tested for the regeneration of RGC axons. All used the ONC model to induce rapid axonal degeneration followed by RGC death, where axons completely degenerate distal to the injury site, thus facilitating the identification of regrown axons [53]. A handful of those approaches include gene transfer by viral vectors promoting the overexpression of proregenerative genes or, alternatively, silencing of antiregenerative ones. Gene manipulations that are capable of inducing axon regrowth are, in general, related to either intrinsic or extrinsic mechanisms that impair axonal regeneration, with a great diversity of targets. An overview of the mechanisms identified to date to enhance axonal regeneration based on viral vector delivery to the optic nerve is presented in **Table 2**.

PI3K/Akt is a well-known pathway related to axonal growth, and modifying different steps of it can lead to axonal regeneration. The activation of PI3-K converts phosphatidylinositol (4,5) bisphosphate (PIP2) into phosphatidylinositol (3,4,5) trisphosphate (PIP3), which activates the protein kinase Akt. One of the main consequences of Akt activation is phosphorylation and activation of mechanistic target of rapamycin (mTOR), a protein involved in a high diversity of cellular processes, including cell growth, motility, survival, and protein synthesis [52]. One of the first identified strategies to promote axonal regeneration is the inhibition of phosphatase and tensin homolog (PTEN). PTEN is a protein phosphatase that converts PIP3 into PIP2 and, therefore, inhibits Akt/mTOR, opposing the action of PI3K. The silencing of PTEN gene mediated by an intravitreal injection of rAAV-shRNA.PTEN vectors promotes axonal regeneration in the optic nerve [54]. This strategy was especially effective when used with a mutant capsid designed to enhance transduction. The intravitreal injection of rAAV2(Y444F)-shRNA.PTEN led to robust axonal regeneration, with some axons found all the way through the optic nerve, past the chiasma and into the optic tract [55]. The manipulation of several other targets in PI3K/Akt/

Target	Mechanism of action	Extent
1. PI3K/Akt pathway		
PTEN	**Silencing of an inhibitor of PI3K/Akt pathway**	**OT**
PI3K	Overexpression of a catalytic subunit of PI3K	ON
Akt	Overexpression of a constitutively active form of Akt	ON
cRHEB	Overexpression of a positive regulator of mTOR signaling	ON
S6K1	Overexpression of a downstream effector of mTOR	ON
GSK3	Overexpression of dominant negative form of GSK3β	ON
eIF2B	Overexpression of a constitutively active mutant of eIF2Bε	ON
FGF2	Overexpression of growth factor FGF2	ON
IGF1	Overexpression of growth factor IGF1	ON
Neuritin	Overexpression of neurotrophic factor neuritin	ON
2. Jak/STAT pathway		
CNTF	**Overexpression of a mutant peptide with higher affinity for CNTFRα**	**OT**
IL-6	**Overexpression of a hyperactive form of IL-6**	**CH**
IL-22	Silencing of IL22, a cytokine	ON
STAT3	Overexpression of constitutively active variants of STAT3	ON
SOCS4	Silencing of a suppressor of cytokine signaling	ON
Pim1	Overexpression of a downstream effector molecule of Jak/STAT	ON
3. Rho/ROCK pathway		
RhoA	Silencing of RhoA	ON
ROCK2	Silencing of ROCK2	ON
LIMK-1	Silencing of a downstream target of ROCK2	ON
LOTUS	Overexpression of a Nogo receptor antagonist	ON
PirB	Silencing of a receptor of myelin-associated inhibitors (MAIs)	ON
4. Transcription Factors		
KLF9	**Silencing of KLF9**	**CH**
c-myc	Overexpression of c-myc	ON
KLF4	Delivery of miRNA-135 s, which targets KLF4	ON
p53	Overexpression of p53	ON
SOX 11	Overexpression of SOX 11	ON
5. Other targets		
Lin 28	**Overexpression of Lin 28, an RNA-binding protein**	**CH**
Cpeb1	Overexpression of Cpeb1, an RNA-binding protein	ON
Armcx1	Overexpression of Armcx1, a mitochondrial protein	ON
BAG 1	Overexpression of co-chaperone BAG1	ON
DCLK2	Overexpression of DCLK2, a cytoskeleton regulator	ON
HDAC5	Overexpression of histone deacetylase HDAC5	ON
Set-β	Overexpression of Set-β, a transcriptional regulator	ON
Tceal3	Overexpression of Tceal3, a transcriptional regulator	ON

Target	Mechanism of action	Extent
Melanopsin	Overexpression of photopigment melanopsin, a G-protein coupled receptor	ON
Lipin1	Silencing of Lipin1 (biosynthesis of triglycerides)	ON
Pcyt1a	Overexpression of constitutively active Pcyt1 (biosynthesis of phospholipids)	ON
Pcyt2	Overexpression of Pcyt2 (biosynthesis of phospholipids)	ON
ULK1	Overexpression of a dominant-negative form of autophagy activating kinase 1	ON
MLP*	Overexpression of MLP, a cysteine-rich protein	ON
NDNF*	Overexpression of NDNF, a neurotrophic factor	ON
PRPH*	Overexpression of PRPH, a neuronal intermediate filament protein	ON
TIMP2*	Overexpression of TIMP2, tissue inhibitor of metalloproteinases 2	ON
UCN*	Overexpression of UCN, corticotropin-releasing factor	ON
THBS1*	Overexpression of THBS1, a secreted glycoprotein	ON
RASSF3*	Silencing of Rassf3, associated with the Ras family	ON
TBC1D22B*	Silencing of Tbc1d22b, a GTPase-activating protein for Rab family	ON

**identified by large-scale screening; OT: Optic tract; ON: Optic nerve; CH: Optic chiasma.*

Table 2.
Gene therapy strategies for axonal regeneration. Targets and most efficient strategy for each one after ONC.

mTOR pathway with the use of gene therapy vectors also led to axonal regeneration, even though restricted to the optic nerve. Strategies included the use of rAAVs to overexpress a constitutively active form of Akt [56], the catalytic subunit of protein kinase PI3K [57], and ras-homolog-enriched-in-brain 1 (Rheb1), a positive regulator of mTOR signaling [58]. The activation of Akt also leads to phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3). GSK3, on the other hand, leads to the inhibition of translation initiation factor 2B epsilon (eIF2Bε). Using rAAVs to overexpress either a dominant negative form of GSK3β or a constitutively active eIF2Bε mutant also led to axonal regeneration [59].

Another common signaling pathway related to axonal regeneration is Jak/STAT. This pathway is usually activated after cytokine biding to extracellular receptors associated with protein kinases JAKs, leading to its activation and phosphorylation of STATs. An important negative feedback mechanism of this pathway is mediated by the proteins of the suppressor of cytokine signaling (SOCS) family, which inhibits Jak/STAT signaling [52]. Two highly efficient rAAV-mediated regenerative strategies involve the overexpression of two of the major cytokines that can activate the Jak/STAT pathway, interleukin 6 (IL-6) and ciliary neurotrophic factor (CNTF). When the overexpression of mutant CNTF peptide exhibiting a higher affinity for CNTF receptor alfa (CNTFRα) was driven by a ShH10 vector, an rAAV variant that preferentially infects Müller glia in mice, axonal regeneration was identified all the way into the optic tract [60]. The overexpression of a designer, hyperactive, form of IL-6 led to axonal regeneration until the chiasma [61]. Other successful strategies related to Jak/STAT and regeneration of the optic nerve involved the overexpression of a constitutively active variants of STAT3 [62] and the inhibition of SOCS4 with shRNA [63].

Furthermore, several transcriptional factors are associated with regenerative pathways and have been so far studied with gene therapy platforms. Among strategies for high-distance regeneration, silencing of KLF9 using rAAV-KLF9.shRNA mediated axonal regeneration up to the chiasm after intravitreal injection in rats [64]. The

manipulation of other transcriptional factors led to regeneration in the optic nerve, including rAAV-mediated overexpression of SRY-box transcription factor 11 (SOX 11) [65, 66] and c-myc [67].

Rho/ROCK pathway is also important in the control of axonal regeneration. It is a convergence pathway activated in response of receptor binding of extrinsic inhibitory factors, that activates RhoA and its downstream target ROCK, the activation of which led to the collapse of the growth cone and impaired axonal growth [52]. The intravitreal injection of rAAVs associated with either RhoA-shRNA, ROCK2-shRNA, or LIMK-1-shRNA, targeting LIM domain kinase (LIMK), a downstream target of ROCK2, led to enhanced axonal regeneration in the optic nerve [36, 37]. Similarly, the overexpression of BAG 1, which inhibits ROCK2 activity, increased regeneration [38].

Some other proregenerative manipulations have also been described, which are not directly linked to the above-mentioned pathways. An especially robust strategy was the overexpression of Lin 28, an RNA-binding protein that is expressed mainly during early embryogenesis in mammals and the reactivation of which is associated with tissue repair mechanisms. Axonal regeneration after the intravitreal injection of rAAV-Lin28a in mice was identified until the chiasma [68].

Recently, many novel targets for axonal regeneration have been described based on large-scale screenings, capable of identifying a myriad of potential genes associated with this mechanism. Those studies were based on the transcriptional profiling of RGC subtypes with a higher regenerative ability, or under conditions in which a regenerative response was favored, or alternatively, in a genome-wide loss of function *in vitro* screen using an shRNA library [69–72].

The most efficient proregenerative strategies identified so far are related to the manipulation of more than one factor. In fact, several combinatorial strategies using rAAVs have been reported to lead to long-distance axonal regeneration. The overexpression of four transcriptional factors, Oct4/Pou5f1, Sox2, and Klf4 genes combined within a same rAAV particle, led to efficient axonal regeneration up to the chiasma [73]. Another successful example is combining KLF9 knockdown by rAAV-KLF-9shRNA and injection of PTEN, a chelator of mobile zinc, which mediated high-distance axonal regeneration until the optic tract [74]. Similarly, using the combination of PTEN silencing by rAAV- shPTEN4, CNTF overexpression using rAAV-CNTF, and injection of a cAMP analog, some axons reached the chiasm and followed along the contralateral nerve, reaching central nervous system targets [54]. A combination of cRheb1 overexpression and induction of neuronal activity by visual stimulation even partially recovered visual function of injured animals, leading to robust axonal regeneration and enabling reinnervation of central targets with a partial recovery of optokinetic reflex after ONC [58].

3.3 Combinatorial gene therapy

Pathways to promote RGC survival and axonal regeneration are not usually overlapping. As discussed above, different signaling pathways and regulatory molecules seem to be critical for either promoting neuroprotection or inducing axonal regeneration. In this sense, a combination of both strategies in a single-gene therapy approach would likely be highly beneficial for glaucoma. With an efficient neuroprotective approach, more RGCs will survive the injury and, thus, be available to successfully regenerate their axons in response to a proregenerative stimulus. On the other hand, an effective regenerative approach will guarantee the integrity of the axons of RGCs that have been already partially or completely lost, with the potential to recover

neuronal function and favor cell survival at a long term, inclusive of retrograde neurotrophic support from the axonal targets. There is evidence that neuroprotective and regenerative pathways do not always overlap, and gene manipulation strategies can even have opposite consequences in each one. Clear examples are the genetic manipulation of apoptosis-related genes BAX and Bcl-2. The gene knockout of the proapoptotic protein BAX and the constitutive overexpression of the antiapoptotic protein Bcl-2 are very efficient strategies to prevent the neurodegeneration of RGCs, with survival of almost all cells in the ganglion cell layer of the retina but cannot efficiently regenerate their axons [75, 76]. Yet, dual leucine zipper kinase (DLK/MAP3K12), sphingosine 1-phosphate receptor 1 (S1PR1), and BDNF have neuroprotective properties, although they act as the inhibitors of axonal regeneration [77–79]. The transcriptional factor Sox 11, on the other hand, has been associated with both the proregenerative and prodeath responses [80]. Examples mentioned above depict well the complexity of the neurodegenerative and regenerative responses of RGCs, which needs to be considered when designing a gene therapy strategy to glaucoma. Still, some studies highlight the potential of combining neuroprotective and proregenerative strategies. For example, the intravitreal injection of rAAV-CNTF or rAAV-THBS is more efficient in promoting axonal regeneration when BAX protein is depleted [70, 76]. Similarly, the overexpression of CNTF in mice engineered to overexpress Bcl-2 had a stronger effect over axonal regeneration than in wild-type mice [32]. These examples of combined genetic manipulations show the potential of such strategies. However, they remain to be further explored in a gene therapy approach.

3.4 Challenges

Gene therapy involves the transfer and expression of exogenous genetic material in target cells for therapeutic purposes. Currently, gene therapy trials are on the rise, with more than six products reaching commercial approval by regulatory agencies such as U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA), and more than 40 products, targeting a variety of pathological conditions, are expected to be approved for clinical use in next decade [81]. Besides the latest growth in the field, gene therapy products are still very expensive, especially because of high manufacturing costs combined to the fact that most current gene therapy products treat rare diseases and benefit a restricted number of patients [81]. Expansion in gene therapy research, including other targets and high prevalent diseases, such as glaucoma, might contribute to decrease costs in the long run.

Recent successes in ocular gene therapy with LUXTURNA—a gene therapy product to improve and maintain vision in patients with Leber's congenital amaurosis—have paved the path for more studies in the field [82]. Ideally, for a therapy to be successful, the transduction of target cells involved in the pathology must occur. Thus, gene therapy studies for glaucoma need to efficiently transduce RGCs and reach the therapeutic level of gene expression. The transfer of genetic material to cells depends on the use of carriers that facilitate the entry of nucleic acid into target cells. In the retina, recombinant viral vectors derived from AAV have been the most efficient tool for gene transfer *in vivo* [83]. Despite recent evidence of genotoxicity mediated by rAAV vectors due to insertional mutagenesis into genomic DNA that culminated in tumor generation and alteration in liver function [84], no adverse effects of this magnitude have been described to date, after several safety ophthalmological clinical trials [85].

The delivery of gene therapy vectors to the retina may follow two major intraocular injection routes, namely subretinal (SR) for retinal epithelial cells and photoreceptors

transduction and intravitreal (IVT), reaching preferentially the ganglion cell layer [86]. In higher species, both the SR and IVT injections induce mild and transient inflammatory responses [87], which are stronger when the doses of injected vector are increased. Inflammation can result in the clearance of transduced cells by cytotoxic T-cells, thus reducing therapy efficacy and worsening patient condition. Cellular immune responses prevent vector readministration due to the generation of neutralizing antibodies against rAAV capsid [88]. Other factors can influence ocular immunogenicity, such as rAAV cassette elements. rAAV incorporating ubiquitous promoters derived from viral sequences, such as CMV or CAG, led to microglia activation and inflammatory cytokine expression, triggering RPE and photoreceptor death after subretinal injections, while photoreceptor-specific promoters were not toxic to these cells even when higher doses were administered [89]. Further studies conducted in large animals, using other cell-type-specific promoters and a wider range of doses, will provide more insight into the correlation between toxicity and genetic material.

In small animals, the IVT injection of rAAV vectors efficiently transduces RGCs, but in nonhuman primates, the transduction is very inefficient [90]. This may be related to physical and biological barriers, such as the large size of primate eye when compared with rodents, which causes a significant dilution of the injected vector, as well as the thickness of the internal limiting membrane that hinders the passage of vectors to the retina [91]. These barriers make it difficult to translate preclinical studies to humans. Several recent studies have tried to enhance rAAV transduction efficiency after IVT injections, especially the use of mutant rAAV capsids [92]. However, the translation of these strategies to larger animals is still a challenge. Tyrosine-mutant rAAV vectors were not as efficient in dogs as they were in mice [93]. Digestion of ILM [94] and subILM injections [95] are also proposed strategies to increase transduction in primates through vitreous. However, until now, efficient and widespread transduction of nonhuman primates' RGCs after IVT injection has not been achieved.

Although the route of vector administration is important for directing gene expression in the region of interest, retinal tissue is complex, with a wide variety of cell types and rAAV vectors have been shown to transduce all of those. The use of an RGC-specific promoter can restrict gene expression to target cells, thus reducing unwanted off-target effects. For example, a Thy1 promoter confers high expression levels with some selectivity for RGCs; however, owing to its size of more than 6 kB, it is not suitable for rAAV [96]. A promoter less than 200 bp of NEFH gene, on the other hand, showed a more restricted expression to this cell type, and owing to its small size, it may serve as a tool for the insertion of genes or larger regulatory sequences in space-constrained vectors [97]. Moreover, hSYN promoter, despite being very efficient in mice, were shown to be inefficient by IVT in primates, making it difficult to translate its use [90]. Recently, PLE345 (NEFL) showed robust expression in RGC bodies and nerve fibers localized on the site of injection, with also a small number of cells of the inner nuclear layer [98]. Still, a promoter based on the regulatory region of the gamma-synuclein gene (SNCG) drove strong expression in RGCs in both mice and primates, allowing gene editing on this cell type and optogenetic restoration of vision [99, 100]. Those promoters may benefit future gene therapy applications in the path to clinical translation.

4. Conclusions

Despite the different subtypes of glaucoma, such as open-angle, angle-closure, pseudoexfoliative, and normal-tension, among others, the common outcome converges

to RGC death. In the past two decades, promising gene therapy strategies to glaucoma have been developed, focusing on both the neuroprotective and proregenerative mechanisms to overcome RGC degeneration, and, in theory, will be able to cover all the glaucoma subtypes. However, the translation to clinic is far much complex. For example, animal models do not cover the pathophysiology aspects of the different subtypes of glaucoma, and a lot of animal studies do not predict with sufficient certainty what will happen in humans. Finding a successful strategy is still a big challenge. An ideal gene therapy approach still needs to surpass issues related to vector delivery platforms, such as safety and efficacy, besides efficient promotion of long-term cell survival and axonal regrowth. For this, the manipulation of a single gene will most likely not be enough and will probably require the combinatorial use of distinct strategies.

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Conflict of interest

Authors declare no conflict of interest.

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