Gene-independent therapeutic interventions to maintain and restore light sensitivity in degenerating photoreceptors

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**ABSTRACT**

Neurodegenerative retinal diseases are a prime cause of blindness in industrialized countries. In many cases, there are no therapeutic treatments, although they are essential to improve patients’ quality of life. A set of disease-causing genes, which primarily affect photoreceptors, has already been identified and is of major interest for developing gene therapies. Nevertheless, depending on the nature and the state of the disease, gene-independent strategies are needed. Various strategies to halt disease progression or maintain function of the retina are under research. These therapeutic interventions include neuroprotection, direct reprogramming of affected photoreceptors, the application of non-coding RNAs, the generation of artificial photoreceptors by optogenetics and cell replacement strategies. During recent years, major breakthroughs have been made such as the first optogenetic application to a blind patient whose visual function partially recovered by targeting retinal ganglion cells. Also, RPE cell transplantation therapies are under clinical investigation and show great promise to improve visual function in blind patients. These cells are generated from human stem cells. Similar therapies for replacing photoreceptors are extensively tested in pre-clinical models. This marks just the start of promising new cures taking advantage of developments in the areas of genetic engineering, optogenetics, and stem-cell research. In this review, we present the recent advances of gene-independent approaches that are currently under clinical evaluation. Our main focus is on photoreceptors as these sensory cells are highly vulnerable to degenerative diseases, and are crucial for light detection.

1. Introduction

1.1. Structure and function of the retina

The mammalian retina contains five neuronal cell classes (Masland 2001) as well as Müller glia (MG) (Reichenbach and Bringmann 2013), which are organized in distinct retinal layers (Fig. 1). There are two major photoreceptor types, rods for dim-light and cones for high-acuity, color, and daylight vision. Photoreceptor nuclei are clustered in the outer nuclear layer (ONL). Their light-sensitive outer segments (OSs) are surrounded by microvilli of retinal pigment epithelium (RPE) cells (George et al., 2021; Lakkaraju et al., 2020) and their terminals form synaptic connections with bipolar and horizontal cells in the outer plexiform layer (OPL). The cell bodies of bipolar, horizontal, and amacrine cells, as well as MG, are located in the inner nuclear layer (INL). The processes of bipolar and amacrine cells stratify into the inner plexiform layer (IPL) where they form synaptic connections with retinal ganglion cells. The ganglion cells’ axons form the optic nerve relaying visual information to higher brain areas. MG span the entire retina, and their tips and endfeet form the outer and inner limiting membranes. The retina is inversely layered: the photoreceptors face inwards. Therefore, all light information first passes through the entire retinal tissue before photons are detected and converted into electrical signals by photoreceptor OS. Some species, including humans, have a specialized retinal area for high-acuity vision, the macula, with the fovea in its center that consists only of cone photoreceptors (Provis et al. 1998).

The function of the retina is relatively complex, and to-date still not entirely understood. Lots of signal processing occurs within the retinal network, mediated by inhibitory horizontal cells in the OPL and inhibitory amacrine cells in the IPL. There are two major vertical signal pathways that diverge downstream of photoreceptors, the ON and OFF pathways: these each have specialized bipolar and ganglion cells. The ON pathway is activated by increments and the OFF by decrements of light. Some ganglion cells, called ON-OFF cells, receive input from both pathways. In addition, there are also intrinsically light-sensitive ganglion cells, called melanopsin cells, which contribute to non-image forming vision such as the pupillary reflex and circadian rhythm (Hattar et al., 2002; Foster and Hankins 2002). Ganglion cell types are organized as mosaics across the retina (Hoon et al., 2014). Each ganglion cell type can be considered as a specialized channel extracting one feature of the visual scene (Roska and Werblin 2001; Werblin et al. 2001; Baden et al., 2016; Borst and Euler 2011; Wasle 2004). These parallel channels then send their information to higher brain areas.

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where the image is further processed and assembled. During daylight, when they were once thought to be inhibited and silent, it has now been shown that rods contribute to cone-driven surround inhibition via horizontal cells (Szikra et al., 2014). As well as being a light-detecting tissue, the retina also does sophisticated biological computing.

### 1.2. Photoreceptors

Here, we specifically focus on photoreceptor cells: in many degenerative retinal diseases these sensory cells are progressively lost, eventually causing blindness (Bramall et al., 2010). The characteristic morphology of rod and cone photoreceptors determine their function. Their tips are called OSs and are the subcellular compartments needed to convert photons into electrochemical signals. They are specialized cilia structures with membranous assemblies, discs in rods and invaginations in cones (Richardson 1969), which are covered in photopigments, rhodopsin (RHO) for rods and three classes of opsins in cones. These membrane-spanning molecules are G-protein-coupled receptors, which are activated by the chromophore retinal. When a photon of the proper wavelength hits the chromophore, the 11-cis-retinal is transformed to all-trans-retinal, triggering the phototransduction cascade resulting in the closure of cyclic guanosine 3’-5’ monophosphate (cGMP)-gated ion channels. This results in a hyperpolarization upon light activation (Arshavsky et al. 2002). Of note, the large surface area of the membranous structures including their high density of opsin increases the likelihood that a photon is detected (Sommer et al. 2011). In addition, the phototransduction cascade itself amplifies the light signal, resulting in a high sensitivity. Rodopsin and cone opsins lack catalytic activity to isomerize the chromophore: RPE cells perform this task for rods and MG predominantly for cones. RPE cells also contribute to the maintenance of OS by phagocytosing parts of the disks and invaginations, which are renewed from the base. Outer and inner segments (ISs) are linked by a connecting cilium. Within IS, mitochondria provide large amounts of energy to maintain the resting potential: photoreceptors are depolarized in the dark by open cGMP-gated ion channels leading to sodium influx, which is constantly reversed by adenosine triphosphate (ATP)-driven sodium-potassium pumps. The IS are adjacent to the nuclei-containing cell bodies linked to short axons, which project to the OPL where the photoreceptor terminals are synaptically connected to bipolar and horizontal cells. Of note, photoreceptors are also interconnected via electrical synapses called gap junctions (Cangiano and Asteriti 2021). The light-induced hyperpolarization is relayed to the terminals, and blocks the secretion of the neurotransmitter glutamate to the synaptic cleft. Reduced glutamate levels are then detected by the downstream neurons. Due to this steady glutamate release and ongoing depolarization in the dark by open cGMP-gated ion channels leading to a sodium influx, which is constantly reversed by adenosine triphosphate (ATP)-driven sodium-potassium pumps. The IS are adjacent to the nuclei-containing cell bodies linked to short axons, which project to the OPL where the photoreceptor terminals are synaptically connected to bipolar and horizontal cells. Of note, photoreceptors are also interconnected via electrical synapses called gap junctions (Cangiano and Asteriti 2021). The light-induced hyperpolarization is relayed to the terminals, and blocks the secretion of the neurotransmitter glutamate to the synaptic cleft. Reduced glutamate levels are then detected by the downstream neurons. Due to this steady glutamate release and ongoing depolarization in the dark, photoreceptors have an enormous energy consumption and metabolic activity. The phototransduction cascade members of rod and cone photoreceptors differ in their isoform combinations but are functionally quite similar. However, this high specialization and functional interdependence of proteins and enzymes means that each element, when malfunctioning, can cause the whole process to stop functioning: dysfunction and progressive degeneration of the cell.
compartments can result in entire photoreceptor degeneration. Disease-causing mutations have been described at every structural and functional element within photoreceptors. Dysfunctional RPE, MG, and immune responses also trigger photoreceptor degeneration.

Progressive dysfunction and death of photoreceptors is the major cause of adult visual impairment and blindness in industrialized countries (Wright et al., 2010). Photoreceptor degenerative diseases can be divided into inherited retinal diseases (IRDs) caused by mutations and age-related macular degeneration (AMD) caused by a combination of genetic and environmental risk factors. In-depth knowledge of pathophysiology and underlying disease-causing mutations is key to developing tailored therapeutic interventions to counteract photoreceptor dysfunction and degeneration, and the resulting blindness. As for every degenerative disease, a cure is always a race against time: intervention is needed before the damage manifests, i.e., the cells are lost (Fig. 2). In-depth knowledge of photoreceptor structure and function also helps in understanding the impact of mutations that lead to blindness: an excellent starting point for counteracting the cellular consequences. For diseases not affecting the macula, rodent models with naturally-occurring mutations or transgenic lines in which the mutations have been introduced can be a powerful research tool, mimicking the human retinal disease phenotype. For some retinal diseases, there are excellent model systems that have been crucial for testing novel therapies (Slijkerman et al., 2015). The nature of the disease-causing mutations is also of high importance: autosomal recessively inherited mutations can be treated with a wildtype gene copy, whereas dominant mutations must be inactivated. To date, more than 300 genes are known to be associated with retinal inherited diseases: in theory, specific gene therapies could be tailored to each gene or mutation. However, not all disease-causing mutations have yet been identified, so mutation-independent approaches are also required. While a perfect cure, which reverses the disease phenotype, is hard to achieve, stopping or delaying the progressive vision loss is important for maintaining and improving patients' quality of life. Therefore, neuroprotective therapies are also being developed. These mutation-independent therapies do not reverse the genetic insult but rather stabilize and protect, and prevent further loss of light sensitivity. A neuroprotective approach is particularly appropriate when cone function is not primarily affected by the mutation and thus cone-mediated daylight vision is preserved, such as in retinitis pigmentosa (RP). Preventing photoreceptor degeneration by reprogramming the affected cell type into one unaffected by the disease-causing mutation is another intriguing approach; as well as inducing intrinsic regenerative potentials. Neuroprotection by non-coding RNAs that regulate photoreceptor gene expression represents another intervention. Neuroprotection is powerful when the underlying disease-causing mutations are unknown, or gene supplementation is not compatible with established and approved gene-therapy vectors. At later stages, optogenetics is an option to convert remaining retinal cell types into artificial photoreceptors. As a last resort, cell-transplantation therapies for RPE and photoreceptor cells are emerging to substitute for the degenerated cell types. Here, we specifically focus on gene-independent and CRISPR-based approaches to maintain photoreceptor function, to generate artificial photoreceptors by optogenetics and to replace photoreceptors in degenerative diseases. Of note, but not featured within this article, are gene supplementation (Garafalo et al., 2020), antisense nucleotide approaches (Gupta et al., 2021; Xue and MacLaren 2020), read-through compounds to overcome nonsense mutations (Nagel-Wolfrum et al., 2014; Samanta et al., 2019), biosynthetic retinal implants (Yue et al., 2016) and light-sensitive polymers (Maya-Vetencourt et al., 2020); these also have the potential to counteract blindness.

2. Neuroprotection

Aggressive loss of photoreceptors across various retinopathies causes loss of vision. Although apoptosis is considered the final cell death pathway in RP and in AMD, other pathways such as autophagy and proteasome activity may as well add to the complex reaction of photoreceptors to the environmental stress (Wenzel et al., 2005). As several mechanisms, such as lack of energy, oxidation, and inflammation, contribute to photoreceptor death in retinal dystrophies, research is focusing on specifically targeting these pathways. Such alternative gene-independent therapies target a larger population of diseases by prolonging the survival of photoreceptors using neurotrophic factors, anti-oxidants, or anti-inflammatory proteins, or by targeting metabolic deficits that can occur during the degeneration process in the retina.
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miRNAs as therapeutic gene regulators are also an option. At advanced stages, the main goal is to prevent further progression and vision loss. At late stages (all photoreceptors are degenerated, or only dormant cones remain) transplantation approaches and optogenetic interventions are promising.

2.1. Neurotrophic factors

Neurotrophic factors are small molecules, mostly peptides, that enhance the growth and survival of mature neurons. They assist developmental processes and provide guidance cues for maturing neurons. Rescue of damaged photoreceptors has been shown using different trophic factors such as FGF, CNTF, GDNF, BDNF, and many others (Wenzel et al., 2005). Receptors for these factors are present in the ONL which, after activation, instigates a protective stimulus to the injured cells and promotes photoreceptor survival. It has been shown that microglia activated by light damage, and increased GDNF then FGF-2 levels, enhanced cell survival (Joly et al., 2007; Cao et al., 2001). Pigment epithelium-derived factor (PEDF) has been shown to switch stress-induced cell-death signals into cell-survival mechanisms (Barros-stable and Tombran-Tink, 2006). bFGF has also been shown to have a similar impact on cell survival. However, these factors can also have negative effects; growth factors have angiogenic and mitogenic properties (Faktorovich et al., 1990). Delivery of therapeutic genes using adeno-associated viruses (AAVs) that target specific retinal cell types has been shown to ameliorate cell death and accelerate photoreceptor survival in a more targeted and efficient form. Specifically, targeted delivery of GDNF into glial cells by intravitreal injections of an AAV variant ShH10 has shown to significantly slow down retinal degeneration (Dalkara et al., 2011). In the future, use of such cell-specific targeted AAVs can be used to simultaneously infect multiple cell types such as the glial cells for trophins and photoreceptors for gene replacement without compromising efficacy, and to significantly enhance the therapeutic benefits.

2.2. Glycolysis and oxidative stress

Factors released from rods, e.g., rod-derived cone viability factor (RdCVF), are known to preserve the integrity of cones during retinal degeneration. RdCVF is an inactive thioredoxin that prevents the binding partner, microtubule-associated protein TAU, from oxidation and aggregation. This is performed by binding to a transmembrane protein called basigin-1 (BSG1) expressed specifically in photoreceptors. BSG1 then binds to the glucose transporter GLUT1 that then initiates aerobic glycolysis, thereby increasing glucose production. Renewal of OS of photoreceptors and maintenance of their length requires a high intake of glucose. Therefore, RdCVF could significantly help vision restoration, especially in RP patients, where the cones seem to survive at advanced stages of degeneration though with shorter and impaired OS (Ali et al., 2015). The Naxn1 gene is responsible for producing RdCVF which, via alternative splicing, creates a second product called RdCVFL. RdCVFL is a putative thioredoxin enzyme that now exclusively demonstrates a protective role towards cone survival. Oxidative stress via hyperphosphorylation of TAU is known to cause cell death. RdCVFL has now been shown to inhibit this phosphorylation process, thus protecting the photoreceptors from phototoxic stress followed by secondary cell death (Fridich et al., 2009). Most RP mutations primarily affect rod function; this subsequently leads to cone dysfunction and, ultimately, complete loss of vision. RdCVF as described before has been shown to significantly promote cone survival and to prevent further retinal damage. Its clinical relevance was demonstrated in a P23H rat model where an injection of RdCVF led to a significant increase in fully-preserved, physiologically viable, and functional cone cells (Yang et al., 2009). In RP, 90% of rods degenerate, irrespective of kinetics, while cones are killed by extreme phototoxic stress. Due to high energy consumption and continuous OS maintenance, cones are an easy target for cellular stress. Several therapies to preserve cones focus on energy maintenance, e.g., the activation of the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 activation results in improved glucose uptake via an increase in the metabolite NADPH thereby generating more energy. Low NADPH levels trigger caspase 2 apoptosis, causing cone death. This study therefore also suggests NADPH plays a crucial role in protecting cones. This is a promising therapeutic advantage for preserving cones and restraining further vision loss (Venkatesh et al., 2015).

Another study investigated alleviating the shortfall of nutrients in RP progression. They screened 20 genes related to energy generation and identified Txnip, a member of the alpha-arrestin family responsible for binding to thioredoxin, facilitating removal of GLUT1 and thereby forcing cells to use non-glucose metabolites. Txnip was delivered using...
AAV gene therapy. Another Txnip allele, called C247S, was best at prolonging cone survival by forcing cones to use lactate instead of glucose to generate ATP. Moreover, mitochondria looked healthier in treated cones. This, in addition to anti-inflammatory agents, also resulted in higher cone rescue and protection from structural degeneration (Xue et al., 2021). Glucose synthesis, maintenance, and transfer from RPE cells plays a crucial role in cone survival and is highly impacted during RP. To test this, an autosomal dominant RP pig model was developed. It was shown that glucose becomes sequestered in the RPE cells during disease progression and rod degeneration, and is therefore not transported to the cones. Transplantation of rod precursors restored the glucose transfer and even improved cone function. A similar effect was seen with a glucose injection in the sub-retinal space. This proved that cones have their own metabolic pathway; trying to preserve this mechanism independent of rods could be essential for delaying vision loss (Wang et al., 2016). The oxidative species in the blood such as reactive oxygen species and nitrogen species (ROS/NOS) are byproducts of oxidation. Contrary to the belief that they have an obvious deleterious impact on cell death, under homeostatic conditions ROS/NOS play a crucial role in maintaining the cell’s physiological processes. Therefore, to directly introduce anti-oxidative agents seems to be a rather blunt therapeutic approach with minimal benefits. Nuclear-factor erythroid-factor 2 (NRF2) is a transcription factor that, on activation, binds to the antioxidant response element and improves cytoprotection, as well as promoting anti-inflammatory genes and mechanisms. Using AAVs, NRF2 has been shown to prolong cell survival for cones as well as retinal ganglion cells, and slow down cone degeneration, when delivered into three different RP models. Hence, this study is a perfect illustration of an alternative therapeutic avenue that is effective across a spectrum of cell types, promoting more sophisticated treatments for several diseases triggered via oxidation (Xiong et al., 2015).

2.3. Cell death pathways

The overactivation of microglia has also been implicated in nearly all neurodegenerative disorders causing phagocytosis of live neurons, termed as “phagoptosis” (Brown and Neher 2014). Microglia are the resident immune cells of the central nervous system and the retina. Additionally, the activation and secretion of IL-1β from microglia activates pro-inflammatory mechanisms directly or indirectly through MG, accelerating stress in rods thus flaring degeneration in a positive feedback manner (Zhao et al., 2015). Phagoptosis in turn causes death of cone photoreceptors, although the mechanism is rather unclear. To protect cones from microglial phagoptosis, transforming growth factor beta (TGF-β), an anti-inflammatory cytokine, was delivered to RP mouse models. It was concluded that TGF-β does indeed play a crucial role in enhancing cone survival; however, overexpression of the TGF-β system was shown to also affect the viability of cones due to nonresponsiveness of the cells. Hence, TGF-β supports a more immunomodulatory strategy. In the future, this strategy could be used to provide years of meaningful vision by enhancing cone photoreceptor survival (Wang et al. 2020). Apoptotic cascade signaling in cells eventually leads to their death. There are several factors and proteins that contribute to this process. Premature cone death could be prevented or delayed by obstructing these cell death signals using external blockers. One such component of the apoptotic pathway is the complement scheme leading to phagocytosis, of which C1q is a key protein. In degeneration mouse models, C1q has been shown to have neuroprotective properties: increasing C1q during pathology clears diseased cellular components. However, C1q is not the obvious choice for neuroprotective attributes: its increase can also trigger phagocytosis even though an absence of C1q is most definitely linked to cone cell death. Therefore, as a future therapy, maintaining or enhancing C1q levels might help ameliorate retinal degeneration (Humphries et al., 2012). Complement dysregulation leading to irregular phagocytosis has been shown in geographic atrophy, secondary to AMD. Therefore, several complement inhibitors were tested at human level to improve AMD pathology. A lack of genetic association between AMD and complement inhibitors indicates that several inhibitors fail to reduce atrophy. Results from inhibitors with positive effects were inconclusive due to discontinuation of trial or dose-related risks (Halawa et al., 2021); complement pathways and their manipulation to ameliorate disease pathology and improve cone survival require further investigation.

Cones cell death has also been shown to occur through a non-apoptotic pathway characterized by accumulation of cGMP, increased production of which triggers a subsequent increase in histone deacetylase (HDAC) and other protein kinases. A common non-apoptotic pathway for highly-variable IRDs can thus be harnessed for targeted therapeutic ventures, increasing the chance of rescuing cone cells or preventing their death. A promising option in current medicine is the use of a HDAC inhibitor called trichostatin (TSA). Injection of TSA in both in vivo and in vitro samples has provided significant protection to cone photoreceptors. A single injection protected cones for over 16 days post injection in vivo. Moreover, it also significantly improved impaired cone migration in degenerating retina (Trifunovic et al., 2016). More specifically, HDAC4 is known to regulate cell survival by repressing apoptotic signals during bone and muscle development. A murine model of retinal degeneration was used to test its role in the retina: loss of HDAC4 resulted in massive photoreceptor loss, whereas overexpression in a diseased pathology improved photoreceptor survival. The protective effects of HDAC4 were seen when combined with hypoxia-induced factor (HIF-1α) in the cytoplasm (Chen and Cepko 2009). Therefore, HDACs prove to be a promising therapeutic avenue for ameliorating degeneration.

3. CRISPR-Cas9 for gene silencing and correcting

Adaptive immunity in prokaryotes known as the clustered regularly interspaced short palindromic repeats (CRISPR), along with their CRISPR-associated (Cas) nucleases have been used extensively as a genome-engineering technique for the last decade (Jinek et al., 2012; Gasanias et al., 2012; Cong et al., 2013; Mali et al., 2013; Barrangou and Marraffini 2014). Cas9 endonucleases are targeted by a 20 bp long complementary single-guide RNA (sgRNA) to a specific genomic region, where they introduce a double-strand break (DSB). In addition to the sgRNA, Cas9 requires the presence of a specific protospacer-adjacent motif (PAM) sequence, located proximal to the sgRNA-complementary target sequence in the genome (Jinek et al., 2012). The DSB induced by the Cas9 endonuclease can be repaired by different DNA repair mechanisms present in the cells, mainly by non-homologous end-joining (NHEJ) repair and the homology directed repair (HDR). The HDR takes place in presence of an exogenous donor template and leads to a precise correction at the DSB locus. In the absence of the exogenous template, the Cas9-induced DSBS are repaired by the NHEJ, leading to the so-called “indels” - insertions and deletions - at the target site in the genome (Yeh et al. 2019). Therefore, the disease-causing allele in autosomal dominant variants can be inactivated specifically, but only if the gene variant generates a unique PAM site that makes it possible to design a gRNA that contains the variant in the seed sequence. The most popular and widely-used CRISPR-Cas9 system is derived from Streptococcus pyogenes (SpCas9), which recognizes the NGG PAM site (Ran et al., 2013). Although this PAM site is abundant in the genome, it’s likely that some mutations cannot be targeted, limiting its use for allele-specific targeting. Of note, in addition to NGG PAM, SpCas9 can recognize non-canonical PAMs including NAG and NGA, but with lower specificity (Zhang et al., 2014). Therefore, different Cas9 orthologs and engineered variants with different PAM sequences have been developed to expand the targeting range of the CRISPR-Cas9 system. For instance, the Cas9 orthologue from Staphylococcus aureus (SaCas9), the triple SpCas9 mutant (Cas9-VQR), and the evolved xCas9 recognize NNGRRT, NGA, and a broad range of novel PAMs, respectively (Ran et al., 2015; Kleinstiver et al., 2015; Hu et al., 2018).
3.1. CRISPR-Cas9 therapy for autosomal dominant RP

CRISPR-based therapeutic approaches to treat autosomal dominant RP are based on specific targeting of the mutated allele by sgRNAs, followed by the introduction of DSBs by the Cas9 protein and subsequent formation of indels by the post-mitotic predominant repair pathway NHEJ. Such an approach with a sgRNA specific for the P23H RHO mutation and Cas9 VQR variant which disrupts only the mutated RHO allele has been developed and tested in RHO-P23H mice (Giannelli et al., 2018; Li et al., 2018). Knockout of the mutated RHO allele in sgRNA- and Cas9-electroporated eyes slowed photoreceptor degeneration: OS/IS tracts were longer four weeks after the delivery, and ERG recordings showed a significant functional rescue compared to control eyes (Giannelli et al., 2018). Furthermore, two different AAV9-PHP.B vectors were intravitreally injected into a P23H mouse model. One vector carried an inducible Cas9-VQR and the other one carried the sgRNA together with the transactivator under the hRHO promoter, enabling Cas9 expression only in rod photoreceptors. The AAV9-PHP.B had a high transduction efficiency in neural tissues (Deverman et al., 2016) and showed high gene delivery in the retina when administered intravitreally. Again, RHO was only disrupted in the mutated allele and not in the wild type. The same CRISPR-based strategy was applied for the specific disruption of the P347S mutated allele, a mutation which occurs frequently in Europe (Fernandez-San Jose et al., 2015). In a study of RHO.P347S mice (Patrizi et al., 2021), AA-V-mediated SpCas9 or the high-fidelity SpCas9-VQR variant (Cas9-VQRHF1) and the allele-specific sgRNA were delivered subretinally and the mice examined one month after injection. The ERG analysis showed significant improvement, and eyes showed significantly greater pupillary constriction compared to sgRNA scramble-injected eyes. However, the ONL structure was not significantly improved by the treatment, indicating that reduction of the P347S mutated protein improved the function of surviving rods but did not significantly prevent their death.

Another therapeutic option is the suppression and replacement approach based on the Cas9-induced knockout of both wild-type and disease-causing RHO alleles, together with replacing the lost gene with a copy that is resistant to silencing. Here, two AAV2/8 vectors were developed to deliver the CRISPR system into two mouse models of RHO-dependent autosomal dominant RP (RhoP23H and RhoD190N) (Tsai et al., 2018). One vector encoded the SpCas9 protein and the second vector both sgRNAs targeting mouse Rho and a copy of human RHO cDNA as a replacement. sgRNAs were designed to target the Cas9 protein to exon 1 of the Rho gene, leading to a deletion that permanently destroyed both alleles. Delivering the sgRNAs and a replacement RHO on a different vector from Cas9 facilitated gene disruption only in cells receiving gene supplementation. Three months after treatment, the ONL was 17–36% thicker than with gene replacement therapy alone. In addition, the ERG with mixed rod-cone components showed significant functional improvements (Tsai et al., 2018). Another dual vector CRISPR-based strategy is currently in preclinical studies (Meng et al., 2020). Here, an AAV5 vector encodes a SaCas9 and the second vector sgRNAs, as well as codon-optimized RHO cDNA, both under the minimal RHO promoter. Tested in human retinal explants, predominantly frameshift deletions in RHO alleles were induced, while the expression level of the exogenous RHO was comparable to normal endogenous RHO expression. This intervention has a potential to ameliorate disease progression and treat RP caused by different RHO mutations. Also, it is continuously being improved and has great potential to soon enter clinical trials for treating autosomal dominant RP.

3.2. CRISPR-Cas9 therapy for ciliopathies

CRISPR-based gene editing also has the potential to treat autosomal recessive diseases, such as Leber’s congenital amaurosis (LCA) ciliopathy caused by mutations in the CEPI290 gene, the most common one being an intronic mutation in intron 26 generating a novel splice donor site (Maeder et al., 2019). One strategy for correcting such a mutation is to use two sgRNAs to target Cas9 and remove the aberrant splice donor created by the mutation, resulting in a shorter, but functional, CEPI290 protein. Subretinal delivery of an AA-V with double sgRNAs and a SaCas9 in humanized CEPI290 mice and in non-human primates (NHP) showed productive editing rates in >10% of photoreceptors, meeting the target therapeutic threshold (Maeder et al., 2019). There was no detectable adaptive immune response to SaCas9 and only mild inflammation due to AAV5 in the immunsuppressed primates. Therefore, the CRISPR-based therapy for the LCA10 proved safe, and could become the first in vivo gene editing be authorized for clinical trials (NCT03872479).

Another recessive disease targeted for treatment using genetic engineering is the RP associated with the RPGR gene. The majority of the disease-causing mutations in this gene cause framen shifts in a highly repetitive, purine-rich region known as the ORF15 exon. This intervention was tested in an RPGR mouse model (Gumerson et al., 2021). Subretinal dual AA-V injections delivering corresponding sgRNA and SpCas9 restored the reading frame of RPGR in a subpopulation of cells (3.3–10.7%) across the entire retina. These preliminary results are promising, but require improvements for targeting more cells, and demonstrating functional readouts and safety.

3.3. Limitations and future directions of CRISPR-Cas9 approaches

The CRISPR-Cas9 system has great potential as a therapeutic option for IRDs as gRNA designs facilitate to cover several mutations at once, to manipulate entire strings of genomic DNA. However, there are still safety and efficiency concerns that are currently being addressed. One of the main limitations of the CRISPR-Cas9 system is that Cas9 protein can tolerate some mismatches in its pairing with DNA, leading to so-called off-target cuts. These can potentially be deleterious for the cell, posing risks in clinical applications. Different methods to in silico predict off-target cuts have been developed. Sequencing of the target and in silico predicted off-target sites should be performed to rule out any unwanted effects before the approach is tested in patients. As off-target cuts can also happen randomly in a cell type-specific manner (Zischewski et al., 2017), whole genome sequencing to verify potential off targets is preferred. In order to increase their specificity to avoid off-targets and to improve their overall efficiency, Cas9 proteins and sgRNAs are constantly being improved (Cong et al., 2013; Ran et al., 2015; Slaymaker et al., 2016). Smaller Cas orthologues such as SaCas9 can be delivered together with sgRNAs using one AA-V vector for a long-term expression in cells (Ran et al., 2015). However, long-term Cas9 expression raises the possibility of a disruption of the endogenous genetic material by off-target effects. Transient, instead of long-term, expression of Cas9 might be beneficial. A transient presence of a Cas9 protein can be achieved by directly transferring a Cas9 protein/sgRNA complex which is degraded within three days in a mouse retina (Kim et al., 2017). Alternatively, self-inactivating Cas9 proteins could be used (Merienne et al., 2017; Li et al., 2019). There is also evidence of pre-existing humoral and cell-mediated adaptive immune responses to Cas9 in humans (Charlesworth et al., 2019). However, recent preclinical testing of CRISPR-Cas9 therapy for LCA showed no adaptive immune responses in NHP (Maeder et al., 2019), making it safe for translation and the start of clinical trials. Finally, the HDR repair pathway is limited to the S and G2 phases of the cell cycle, and therefore cannot achieve efficient activity in postmitotic retinal cells (Ye et al. 2019). This is a major drawback for mutation-correction strategies where the Cas9-induced DSBs should be corrected by the HDR in the presence of an exogenous DNA template. The efficiency of the HDR pathway could be increased by inhibiting certain components of the NHEJ pathway in vitro (Maruyama et al., 2015) or by adding small molecules such as beta-adrenergic receptor agonist or an inhibitor of intracellular protein transport (Yu et al., 2015). However, methods that alter DNA repair pathways are hardly translatable to the clinic. Therefore, a new CRISPR-Cas9 strategy called homology-independent targeted
integration (HITI) has been developed: the NHEJ pathway in post-mitotic cells is used to repair mutations accurately (Suzuki et al., 2016). In a proof-of-concept study, a homozygous mutation in the Mertk gene in a rat model was successfully corrected.

It has also been shown that another DSB repair pathway, namely microhomology-mediated end joining (MMEJ), might be active in postmitotic photoreceptors (Pasquini et al., 2020; Gumerson et al., 2021; Shen et al., 2018) (Fig. 3A). By introducing a DSB in a highly repetitive genomic region in ORF15 of the RPGR gene, the 32-bp long repetitive fragments are thought to represent the microhomologies needed for the alternative MMEJ repair (Gumerson et al., 2021). In the future, other DNA repair pathway mechanisms might be exploited for a precise in vivo gene correction. Alternatively, different approaches that do not need HDR for a precise mutation correction are currently being developed (Fig. 3B and C). Base editors comprise a catalytically disabled nuclease fused to a nucleobase deaminase enzyme. Targeted by a sgRNA to a specific genomic locus, base editors directly convert one base or base pair into another, enabling mutations in non-dividing cells to be corrected efficiently (Rees and Liu 2018). There are two classes of base editors: cytosine base editors (CBEs) with cytidine deaminase (rAPOBEC1) (Komor et al., 2016) which convert G-C base pairs to A-T; and adenine base editors (ABEs) with adenosine deaminase (TadA) which convert A-T base pairs to G-C (Gaudelli et al., 2017) with high precision and efficiency. A recent study showed the applicability of base editors for treating IRDs: a lentiviral vector carrying ABE and a sgRNA targeting the mutation site was subretinally injected into a rd12 mouse model carrying a nonsense mutation in the Rpe65 gene on exon 3 that leads to a premature stop codon and shorter, nonfunctional protein (Suh et al., 2021). This mutation has recently been identified as causing LCA within the Chinese population (Zhong et al., 2019). Four weeks after the injection, target base editing was observed at a rate of 15.95 ± 2.87% with a maximum correction of up to 29% in the RPE tissue. Of note, gene correction in rd12 mice using a recently-reported HDR approach resulted in correction efficiency of around 1% (Jo et al., 2019). ABE-treated mice produced 11-cis retinal, indicating a restored visual cycle. Furthermore, functional ERG responses indicated a substantial level of vision restoration showing evidence of the clinical potential of base editors. Another CRISPR-based gene-editing strategy that does not rely...
on DSBs and HDR is called prime editors. Prime editors comprise a catalytically impaired Cas9 endonuclease fused to an engineered reverse transcriptase, programmed with a prime editing guide RNA (pegRNA) that both specifies the target site and encodes the desired edit (Anzalone et al., 2019). In this way, prime editors directly create new genetic information at a specified DNA site. Prime editors allow point mutations, as well as bigger deletions or insertions, to be corrected: they are a powerful genome-editing tool.

Genomic-engineering approaches represent a new class of therapy; their translation to clinical situations will require further intensive testing. Especially, using retinal organoids from patient-derived cell lines carrying the disease-causing mutations might prove useful. The editing would in this way be studied in a more appropriate genomic and cellular context than mouse models. Also, as it applies to all gene-therapy approaches, developing novel viral or non-viral delivery systems which allow more efficient and specific targeting of photoreceptor cells in the retina will be crucial for their translation into clinical situations.

4. **In-situ reprogramming**

4.1. Direct reprogramming to prevent retinal degeneration

Many mutations causing retinal degeneration primarily affect specific cell types, as these genes or isoforms are not expressed in other retinal cell types. Therefore, overwriting the cellular identity of the

**Fig. 4. In-situ reprogramming of retinal cells.** Genetic engineering is used to transform remaining cell types into photoreceptors. (A) CRISPRa is used to activate Opn1, a cone-opsin, to make rhodopsin-deficient rods functional. (B) Knocking out Nrl, deterministic for rod development, is used to reprogram rods to cone-like cells. (C) Expressing Ascl1 in MG is used to reprogram them into rod photoreceptors.
affected cell types represents an intriguing strategy to prevent the ignition of the pathological cascade, from cellular dysfunction to cell death and progressing retinal degeneration (Fig. 4A and B). To this end, it has been shown that rod photoreceptors can be programmed into cone-like cells by knocking out the transcription factor Nrl, which is deterministic for rods. This was first shown in transgenic animals lacking Nrl during retinogenesis (Roger et al., 2012), but is also efficient upon inducible Nrl knockout in adult rods (Montana et al., 2013). Programming rods to cones was efficient at preventing rod-driven retinal degeneration. The disruption of the Nrl locus in postmitotic rods was also efficient when using CRISPR-Cas9 genomic engineering in combination with AAV-mediated gene delivery (Yu et al., 2017). Overall, targeting Nrl to directly program rods into cone-like cells improves their survival and also preserves the function and survival of native cones that would normally undergo further degeneration in RP. When the exact disease-causing mutation is known, this approach can be further refined to substitute the dysfunctional rod homolog with the cone one. CRISPR-Cas9 transcriptional activation (CRISPRa) was used in combination with guide RNAs for Omp1mw to activate this cone opsin in a rhodopsin-deficient RP mouse, which led to improved function and delayed degeneration without showing any adverse immune reaction (Bohm et al., 2020).

Converting rods into cone-like cells, or repairing the rod phototransduction cascade with cone homologs, is a clever therapeutic intervention to prevent rod degeneration and thereby maintain cone vision. CRISPR-Cas9-mediated gene disruption still requires further improvements to avoid off-target activity. Overall, the CRISPR-Cas9 components for gene disruption or transcriptional activation exceed the cargo limits of AAVs. Although this can be overcome by using split AAV approaches, improvements in efficiency are still required for a robust gene therapy (Liu et al., 2021).

4.2. Unlocking the regenerative potential of Müller glia

MG are the major glial cells in the vertebrate retina, taking over many important functions (Bringmann et al., 2018; Reichenbach and Bringuier, 2020). In zebrafish, these cells can enter a stem-cell-like state after retinal damage (Hitchcock et al., 2004). MG have the potential to divide and provide progenitor cells for various cell types which can then integrate and repair the retina (Fig. 4C). Because of their ability to re-enter the cell cycle and their distribution throughout the whole retina, they are a valuable target for therapeutic approaches to restore vision: this is an active field of investigation in the mammalian retina (Wohl 2022). These cells have the potential to proliferate and differentiate into neurons in vivo in mice after stimulation with specific growth factors (Karl et al., 2008). Another study looked at the potential for direct reprogramming of MG into neurons by overexpressing the Ascl1 transcription factor (Jorstad et al., 2017). The combination of β-catenin gene transfer, reactivating the cell cycle, and treating with rod-specific transcription factors resulted in targeted differentiation of MG into rod photoreceptors (Yao et al., 2018). Mice showed visual responses after treatment. MicroRNAs (miRNAs) have also been investigated for their ability to reprogram MG into neurons (Wohl et al. 2019). Other studies have shown that retinal ganglion cells can be created from MG (Zhou et al., 2020; Guimaraes et al., 2018). For a more detailed overview concerning the potential of MG for regenerative therapies in the retina, there are some specialized review articles focusing on this topic (Jadhav et al. 2009; Eastlake et al., 2021; Martin and Poche 2019). This line of research still requires extensive experimental work to find an efficient and safe therapy; the potential side effects of expended MG within the in-situ reprogrammed retinas over time should be studied, but it is exciting to use reprogramming strategies to unlock the regenerative potential within mammalian retinas.

5. Small non-coding RNAs

In recent years, non-coding RNAs have come into the spotlight as more molecules and their diverse roles in retinal function have been discovered. In this section, we focus on miRNAs, which are small, non-coding RNAs of about 22 nucleotides length (Ghiladali and Zamoer, 2009). MiRNAs act as master regulators by targeting hundreds of genes at once (Kutsche et al., 2018) and pathways are frequently accompanied by altered miRNA profiles and thus represent biomarkers for diseases (Velerti et al., 2015; Zuzic et al., 2019). Therefore, dissecting the diverse functions of miRNAs at the molecular level in healthy and diseased photoreceptors is key to harnessing their regulatory potential to protect and repair these sensory cells.

MiRNAs regulate gene expression, thereby maintaining cellular functions and homeostasis (Sundermeier and Palczewski, 2016). They originate either from direct transcription or splicing events when residing in intronic regions of protein coding genes (Lee et al., 2004; Borchart et al. 2006; Westholm and Lai 2011) and their biogenesis is tightly regulated and requires several steps (O’Brien et al., 2015) (Fig. 5A). These non-coding RNAs form characteristic secondary RNA structures, so-called stem loops, which are further processed and exported to the cytoplasm. Finally, the mature single-stranded miRNAs are bound to the Argonaute (AGO) proteins within the RNA-induced silencing complex (RISC), where they are active. Unlike shRNAs and mirtons, miRNAs are the natural RNA substrate for RISC becoming active. RISC complex mediates miRNA binding to target mRNA molecules, mostly in the 3 untranslated region (3’ UTR) of the transcript (Wang et al., 2008; Lytle et al. 2007). Partial complementary binding blocks translation, and initiates deadenylation and mRNA decay (Wightman et al. 1993; Wang et al., 2008; Bartel 2004). The length and sequence of mature miRNA sequences is highly variable (Morin et al., 2008), with changes of nucleotides within the miRNA sequence or additions, deletions and substitutions at the 3’ and/or 5’ end (Landgraf et al., 2007; Marti et al., 2010) resulting in miRNA variants, so-called isomiRs. Therefore, single nucleotide substitutions in the seed regions can change the target specificity and thereby impact miRNA functionality (Cammaerts et al., 2015). In a non-canonical mode, miRNAs also activate translation by changing the function of Argonaute protein 2 (AGO2) from a repressor to an activator (Vasudevan et al. 2007). Of note, one miRNA has up to thousands of potential mRNA target molecules, and each mRNA molecule has annotated miRNA binding sites for multiple miRNAs, highlighting how important miRNAs are in gene regulation. The fold-change reduction of target mRNA levels is quite moderate, which implies that miRNAs have buffer-like stabilizing gene regulatory features. MiRNAs provide stability to biological systems that are continually exposed to internal and external stress factors (Ebert and Sharp, 2012). One of the major internal stress factors of photoreceptor cells is their high metabolic activity (Sung and Chiang, 2010). Studies have also revealed that the turnover of miRNAs accelerates in connection to light exposure in photoreceptors (Krol et al., 2010), which directly links miRNAs to neuronal activity.

5.1. The importance of miRNAs for retinal development and function

MiRNA expression is known to be tissue specific and important for development (Lagos-Quintana et al., 2002). There is a catalog of all retinal miRNAs which shows that a small subset of miRNAs accounts for almost 90% of the retinal miRNome (Karali et al., 2016). When looking specifically at photoreceptors, transcriptomic studies have revealed that a subset of this miRNome is highly expressed in photoreceptor cells, controlling their development and correct function (Sundermeier et al., 2014; Lumayag et al., 2013). This includes a miRNA cluster consisting of mir-182, mir-183, and mir-96, as well as mir-124 and mir-9. Of note, temporally-controlled expression of mature miR-183/182/96 molecules during photoreceptor development is required for the photoreceptor layer to be organized correctly (Krol et al., 2015). Dysregulation of
miRNA regulatory networks by the deletion of Dicer during development leads to failures in retinal architecture as well as neurodegeneration (Georgi and Reh 2010; Damiani et al., 2008). The complete lack of the miR-183/182/96 cluster during photoreceptor development alters morphogenesis, phototransduction, synaptogenesis, and synaptic transmission (Lumayag et al., 2013). The miR-183/182/96 cluster impacts on terminal maturation of photoreceptors as shown by a miRNA knockout model (Fan et al., 2017). The transcription factor Pax6, whose spatio-temporal expression pattern is crucial for eye development, has also been shown to be miRNA-regulated (van Heyningen and Williamson 2002; Kaspi et al., 2013). MiR-204 has been shown to support retinal and lens development by targeting Meis2.
transcription factor (Conte et al., 2010). Different miRNAs have been identified as regulators of developmental states and transitions of retinal progenitor cells. When overexpressed, these miRNAs accelerate retinal development (La Torre, Georgi, and Reh 2013). MiR-124 is directly linked to cone photoreceptor development, reduced opsin expression and progressive cell death (Sanuki et al., 2011). A miR-211 knockout model resulted in a progressing cone dystrophy with reduction of visual function, highlighting the importance of this miRNA to cone photoreceptor function and survival (Barbato et al., 2017).

5.2. miRNA dysregulation and the impact on retinal degeneration

Altered miRNA profiles, especially miR-183/182/96, have been found in RP mouse models, although, causes of or consequences due to retinal degeneration were not determined (Loscher et al. 2007, 2008). As mentioned earlier, this cluster is highly expressed in photoreceptors and upregulated upon light exposure (Krol et al., 2010). When knocking out the miRNA processing machinery postnatally by specifically targeting DGC8 in cone photoreceptors, their cellular development and function was initially unaltered (Busskamp et al., 2014a). DGC8 was found to be a stable protein; the miRNA knockout effect first became visible when the remaining DGC8 protein degraded over time. Upon DGC8 loss, the cones lost their OS. Consequently, photopic cone function was lost, but no further degeneration of cone cell bodies was detected. Transcriptomic analyses further linked morphological and functional findings to the specific downregulation of cone phototransduction cascade genes, suggesting that miRNAs must be regulated to maintain the structure and function of OS. Of note, the pathogenic phenotype was prevented by administering only miR-183 and miR-182 before the onset of outer-segment loss. An in-depth systems-level analysis over the course of outer-segment loss revealed that several essential biological pathways, including membrane trafficking and cilium-associated pathways, were altered (Busskamp et al., 2014a). The genesis of OS during photoreceptor development also required shifts in gene expression (Daum et al., 2017), highlighting the overall importance of transcriptional control within this cellular compartment. When depleting Dicer in mature rod photoreceptors, a similar degenerative phenotype was detected, and rod-specific miRNAs were discovered (Sundermeier et al., 2014). Additional work has demonstrated that a specific miR-182 knockout in mouse photoreceptors did not show a structural phenotype (Jin et al., 2009), likely because the function of miR-182 was performed by miR-183 and miR-96. A miR-183 and miR-96 knockout induced a progressive retinal degeneration, suggesting that these two miRNA species are crucial for proper photoreceptor development and maintenance. Slc6a6, coding for a taurine transporter, was identified as a key target gene that is indispensable for photoreceptor function (Xiang et al., 2017). Another study deleted miR-183 genomically, which led to functional alterations, and identified Rnf217 and cilium-related genes to be regulated by miR-183 (Zhang et al., 2020).

miRNA dysregulation has also been linked to AMD (Wang et al., 2014; Chu-Tan et al., 2018). The identification of retinal miRNA/miRNA interactions in healthy and diseased retinas showed a shift towards miRNAs targeting genes in inflammatory pathways in the diseased retina (Chu-Tan et al., 2021). Chronic inflammation is one of the main factors associated with retinal neurodegeneration (Petrillo et al., 2021). Neuroinflammation is thereby connected to the activation of the innate immune system (Shastri et al. 2013; Edwards et al., 2005; Hageman et al., 2005). Transcriptomic studies have indicated that miRNAs targeting mRNAs involved in immune responses, were upregulated after light-induced retinal damage (Szazena et al., 2015). Of note, miR-124 was shown to have immunomodulatory effects. It is highly expressed in the central nervous system and cells of the retina (Karali et al., 2007). MiR-124 has been directly linked to neuroinflammation (Chu-Tan et al., 2018). It has been shown that miR-124 targets the CC-chemokine ligand 2 (Ccl2), which is a pro-inflammatory chemokine secreted by MG upon retinal damage, attracting macrophages. High levels of CCL2 can be detected in tear fluids and vitreous fluids of AMD and diabetic retinopathy (DR) patients (Liu et al., 2010; Wakabayashi et al., 2011; Chernykh et al., 2015). CCL2 is also upregulated in wet and dry AMD (Newman et al., 2012) and RP (Newton and Megaw 2020). In summary, altered miRNA expression patterns are directly linked to degenerative processes that hit photoreceptor cells. Therefore, miRNA pathways may serve as excellent therapeutic interventions.

5.3. miRNAs as therapeutic agents to counteract retinal degeneration

The anti-inflammatory and anti-apoptotic functions of miRNAs can be directly harnessed by overexpressing these non-coding RNAs in photoreceptors. The anti-inflammatory effects of miR-124 mentioned before have also been demonstrated in several studies. Intravitreal administration of miR-124 mimics triggered downregulation of Ccl2, therefore diminishing the inflammatory response and supporting photoreceptor survival and improvement of retinal function (Chu-Tan et al., 2018). The anti-inflammatory effect of miR-124 via down-regulating CCL2 expression has also been shown in ganglion cells (Dong et al., 2015). The inhibition or knockout of miR-155 attenuates the immune response upon retinal degeneration (Aggio-Bruce et al., 2021). Hence, miR-155 inhibitors may be used for neuroprotection to prevent a deleterious immune response. The neuroprotective and therapeutic potential of miR-204 has been shown in a mouse model of LCA (Karali et al., 2020). Here, AAV-mediated delivery of miR-204 slowed retinal degeneration by attenuating microglia and thereby photoreceptor cell death. The delivery of chemically engineered anti-miR-132 oligonucleotides that are complementary to miR-132 also prevented angiogenic sprouting in the developing mouse retina. Choroidal neovascularization is the major cause of vision loss in patients suffering from neovascular forms of AMD (Chappelow and Kaiser 2008; Veleri et al., 2015). Another miRNA approach worth testing for its clinical impact is applying miR-183 and miR-182 to cone photoreceptors in order to prevent the loss of their OS (Fig. 5B and C) (Busskamp et al., 2014a). Here, the timing of the miRNA substitution is critical: it needs to start before the OS are completely degraded. While these two miRNAs induce OS in photoreceptors within mouse stem-cell-derived retinal organoids, which subsequently become light-sensitive, these miRNAs failed to regenerate these subcellular compartments in vivo after the degeneration had started.

Therapeutic applications of non-coding RNA also have some limitations, particularly in their immunogenicity (Winkle et al., 2021). Pathogen-associated molecular pattern (PAMP) receptors such as toll-like receptors (TLRs) detect single- or double-stranded RNA molecules extra- and intracellularly as part of the normal virus defense mechanism (Kumar et al. 2011). The immune reaction caused by RNA-interference (RNAi) was first described in an in vitro system where different small interfering (si) RNAs caused an interferon-γ-mediated (IFNγ) immune response (Slezd et al., 2003). Later intraocular injections of anti-vascular endothelial growth factor receptor 1 (VEGFR1) siRNAs bevasiranib (NCT00499590) and AGN 211745 (NCT00395057) triggered an immune response by activating TLR3 in vivo (Kleiman et al., 2008). The recognition of RNA therapeutics by TLR signaling may lead to a variety of downstream immune responses (Barton and Medzhitov 2003). miRNAs are recognized by TLR7 and TLR8 depending on their GU content, leading to an INFγ inflammatory response, activation of nuclear factor ‘kappa-light-chain-enhancer’ in activated B-cells (NF-κB), and production of inflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor (TNF) in macrophages and dendritic cells (Heil et al., 2004; Sioud 2005; Judge et al., 2005; Forsbach et al., 2008). Also, single-stranded RNAs elicit a stronger immune response than double-stranded RNAs (Sioud 2006). Modifications of non-coding RNA therapeutics that reduce their immunogenicity are currently under investigation (Winkle et al., 2021; Chen et al., 2013). However, not all miRNA therapeutics elicit the same immune response (Winkle et al., 2021).
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6. Optogenetics for vision restoration

Optogenetics refers to light-sensitive proteins (rhodopsins) that, upon light activation, induce biological processes such as ion channel opening or closure, ion pumping across the cell membrane, or trigger intracellular signaling cascades. Channelrhodopsins (channels) and halorhodopsins (pumps) induce electrical signals via membrane currents. Depending on the translocated ions, the cells ectopically expressing these optogenes either depolarize or hyperpolarize. Optogenes are found in many microbial organisms and are linked to several biological processes. For example, channelrhodopsins (ChR) are found in the eye spot of green algae, controlling their phototaxis (Nagel et al., 2002). These microbial rhodopsins are membrane proteins with seven transmembrane helix motifs. Of note, the chromophore retinal, which undergoes a light-dependent conformational change, is covalently bound via a Schiff base to helix 7. The photocycle kinetics, including the re-isomerization of the retinal, determine the channel or pump activity. Although mammalian rhodopsins are GPCRs, which require an entire protein cascade to function, microbial rhodopsins translocate ions directly. While the phototransduction cascade of GPCRs leads to a signal amplification and thereby high light-sensitivity, microbial optogenes require high light levels to function. Of note, the light-sensing GPCRs in mammalian photoreceptors require other retinal cell types for the re-isomerization of the chromophore. Some light-sensitive retinal ganglion cells, which are important for non-image-forming vision, express the GPCR melanopsin, which has intrinsic retinal isomerase activity (Panda et al., 2005). Optogenes also have broader action spectra, impeding the possibility of using multiple tools at once without cross-activation. As the chromophore retinal is abundant in mammalian cells, microbial optogenes are also functional in these cells upon ectopic expression. Within neurons, depolarization leads to an activation resulting in the secretion of neurotransmitters at their synaptic terminals to relay the electrical signal to downstream neurons within neuronal circuits. In contrast, hyperpolarization induces functional inhibition by blocking synaptic communication. Overall, optogenetically-modified neurons are rendered to artificial photoreceptor cells.

Optogenetics has been adapted from basic biophysical research to broader applications by ectopically expressing these tools in neurons (Boyden et al., 2005); it has revolutionized the functional neuroscience field for dissecting neuronal functions and connections within neuronal circuits. First, halorhodopsins were used to inhibit neuronal functions (Han and Boyden 2007; Zhang et al., 2007) and since then many additional optogenes have been discovered or engineered to provide additional or improved features such as spectral tuning, light-sensitivity, and cellular expression levels. For example, light-gated anion channels were first engineered (Berndt et al., 2014; Wietek et al., 2014), before being discovered in algae (Govorunova et al., 2015; Wietek et al., 2016; Oppermann et al., 2019). Fused optogenes have also been developed for spectral control over de- and hyperpolarization (Kleinlogel et al., 2011; Vierock et al., 2021). The field of optogenetic tool development is very active, and more tools with advanced features are expected to become available soon. Especially for in vivo applications, a spectral tuning towards higher wavelengths is an advantage: red light penetrates deeper into tissues and the corresponding photons carry less energy, reducing potential photochemical damage in the host tissue. Blue light triggers pupil contraction via photoreceptors and melanopsin-expressing ganglion cells, which is an important factor for in vivo applications in the eye (Foster and Hankins 2002; Bonmati-Carrion et al., 2018). As the light levels for activating blue-shifted optogenetic tools need to be very high, the pupillary-light reflex based on remaining melanopsin-expressing ganglion cells will be triggered and the overall light levels reaching the retina will therefore be further reduced.

6.1. Optogenetic vision restoration of retinal ganglion and bipolar cells

One of the first in vivo applications of optogenetics was the functional reactivation of retinal ganglion cells in a mouse model of retinal degeneration (Bi et al., 2006) (Fig. 6A and B). Recombinant AAV particles carrying ChR2 fused to a green fluorescent protein driven by a ubiquitous promoter element were injected into the intravitreal space of blind mice. This treatment generated light-sensitive retinal ganglion cells that relayed light information to the visual cortex. Melanopsin has also been shown to improve vision in formerly blind mice by rendering retinal ganglion cells light sensitive (Lin et al., 2008). While melanopsin is more sensitive to light, its kinetics are suboptimal for image-forming vision. Recently, cone opsin was also shown to render retinal ganglion cells light sensitive (Berry et al., 2019). The ease of targeting retinal ganglion cells with AAVs, and the availability of strong and ubiquitous promoter elements to drive robust optogen expression, makes this intervention a priority for clinical translation. Safety and efficacy of optogenetic vision restoration in ganglion cells have also been demonstrated in non-human primates (Chaffiol et al., 2017; McGregor et al., 2020; Gauvain et al., 2021; Sengupta et al., 2016). Injecting a red-shifted channelrhodopsin, ChRimsonR, into the eye of a blind patient resulted in partial recovery of visual function (Sahel et al., 2021) (NCT03326336). Image processing towards the optimal wavelength and intensities has been achieved by engineered goggles (Medical Device - GS030-MD; (Sahel et al., 2021). This first in-human application of optogenetics for vision restoration is an exciting step forward and it clearly demonstrates the overall feasibility, safety, and efficacy of this mutation-independent approach to overcome blindness. This pioneering work paves the way for future, more sophisticated therapeutic optogenetic interventions.

Resensitizing retinal ganglion cells bypasses several retinal signaling pathways that normally each extract, process, and relay information about different visual features to higher brain areas: using depolarizing optogenetic tools in retinal ganglion cells phases all pathways to ON cells. It is hoped that the brain of blind patients will be plastic and learn how to interpret these kinds of signals. With more patients receiving optogenetic activation of ganglion cells, we will soon be able to determine the quality and efficiency of this treatment. One workaround would be to use cell-type-specific promoter elements to direct different kinds of spectrally-shifted optogenes to subsets of retinal ON ganglion cells, or to also apply hyperpolarizing tools specifically to OFF ganglion cells. However, the field currently lacks specific promoter elements to drive optogenetic tools in a ganglion cell subtype-specific manner.

Another way of maintaining the major retinal signaling pathways, is to sensitize retinal bipolar cells upstream of ganglion cells (Fig. 6A and C). Based on specific promoter elements for ON bipolar cells, ChR2 was expressed in these cells in a mouse model of retinal degeneration: this...
results in intraretinal signal processing specific for ON signals (Lagali et al., 2008). Specifically, downstream ON ganglion cells were activated, retinal processing features such as lateral inhibition and center-surround organization of the ganglion cells were detected, and the light information was sent to the visual cortex. These formerly blind animals performed better in behavioral tests than their blind littermates. Many groups have improved specific promoter elements (Hulliger et al. 2020) and AAV capsids (Mace et al., 2015) to advance this approach. Of note, different non-microbial optogenetic tools have been successfully tested, including engineered ones (van Wyk et al., 2015) and rhodopsin (Gaub et al., 2015; Cehajic-Kapetanovic et al., 2015), which further improved light sensitivity. Many groups around the world are contributing to optogenetic vision restoration approaches which target retinal ganglion

6.2. Optogenetic reactivation of persisting cone photoreceptors

Nowadays, human vision depends mostly on cone photoreceptors enabling color, daylight, and high-acuity vision. Due to artificial light

![Fig. 6. Optogenetic approaches for vision restoration. (A) Different strategic cell types have been targeted by AAVs delivering optogenetic tools. The main targets are (remaining) photoreceptors, and bipolar and ganglion cells. Optogenetic tools include the hyperpolarizing chloride pump eNpHR, or depolarizing TRP channels conjugated with gold nanorods. ChR2 and ChRimmonR are depolarizing ion channels that have been used to render bipolar and ganglion cells light sensitive. (B) Delivering optogenetic tools to ganglion cells leads to activation of all cells upon light exposure, thus phasing all targeted cells to ON cells, totally removing OFF pathways. (C) Targeting subpopulations of bipolar cells like the ON type leads to preservation of ON-signal-specific intraretinal signal processing. (D) Resensitizing remaining cone photoreceptors most closely resembles natural retinal function, resensitizing all cone pathways. (E) Resensitized cones at postnatal day 495 still express eNpHR-EYFP (green) upon AAV-mediated transfer at postnatal day 21 (left) and remain functional (right) as shown by light-evoked action potentials recorded by micro-electrode arrays. Cones are also labeled with a mCAR antibody (magenta) and cell bodies with DAPI (white). Scale bar, 20 μm. f-RD corresponds to the rd1 mouse model that undergoes a fast retinal degeneration. This data is a follow-up study to (Busskamp et al., 2010) and modified from Journal of Bioenergetics and Biomembranes volume 50, page 502.](image-url)
during the dark, rod photoreceptor-mediated night vision has become less important for visual tasks. Therefore, preventing cone photoreceptors from dysfunction and subsequent degeneration is of utmost importance to maintain the quality of life. In RP, it was thought that cones degenerate fast after becoming insensitive to light. However, clinical reports have shown that there are cone photoreceptor cell bodies, especially in the fovea, in blind RP patients. In mouse models of aggressive RP, cone cell bodies merged into the inner nuclear layer and were therefore considered as degenerated. These so-called “dormant” cone photoreceptors were then targeted with optogenetic tools for resensitizing (Fig. 6A and D). As photoreceptors hyperpolarize upon light exposure, an enhanced light-sensitive chloride pump from the halobacterium *Natronomonas pharaonis*, called eNpHR, was used (Buskamp et al., 2010). As unspecific halorhodopsin expression in other retinal cell types would alter and reduce downstream signal processing, photoreceptor-specific expression was essential and achieved via defined promoter elements. These eNpHR-expression cassettes were delivered via AAV-mediated gene transfer into the subretinal space of blind mouse models. It was shown that eNpHR-expressing “dormant” cones, which persist for long time periods (Fig. 6E), were still synaptically connected to downstream bipolar cells and were efficiently hyperpolarized by light. All cone pathways, including the ON and OFF channels, as well as all tested retinal signal processing features were working in these formerly blind animals. These light responses were relayed to the visual cortex, and robust behavioral responses to light were measured. Furthermore, it was shown that eNpHR also hyperpolarized human cones in *post-mortem* retinal tissue (Buskamp et al., 2010). The red-shifted tuning of this halorhodopsin was also an advantage, avoiding pupillary contraction upon light stimulation during the behavioral tests. The light levels required were also considered as safe. However, there are still some limitations: the overall light sensitivity of microbial optogenetic tools is lower and the activity range, based on phototransduction cascades, is very narrow compared to normal rod and cone vision. This impedes adaptation to different light levels. More sensitive hyperpolarizing optogenetic tools have been discovered and characterized, of which Jaws, a more red-shifted light-sensitive chloride pump, resensitized dormant mouse cone photoreceptors well (Chuong et al., 2014). Another study engineered a near-infrared optogenetic tool based on temperature-sensitive TRP channels, which require gold nanorods for activation (Nelidova et al., 2020). The gold nanorods were conjugated to antibodies binding specifically to the ectopic TRP channel of the targeted neuron. Photoreceptors of blind mice were resensitized despite the polarity reversal, meaning that the dormant photoreceptors reacted to light with depolarization. The light signals were sent to higher brain areas, resulting in robust behavioral responses. This method also succeeded in sensitizing human photoreceptors in *post-mortem* retinal explants. The authors have also demonstrated that intrinsic photoreceptors were not activated by near-infrared light, which is important to specifically activate dormant cones in partially-degenerated retinas. In this way, remaining and optogenetically-targeted photoreceptors can contribute to vision simultaneously. Still, halorhodopsin or TRP-mediated vision restoration targeting remaining cone photoreceptors also requires the usage of specific signal-amplifying goggles to set the image to the optimal wavelengths and intensity.

The preclinical studies and the successful applications of optogenetic photoreceptor reactivation in *post-mortem* human retinas have been very promising. This approach has several advantages over targeting other retinal cell types such as reactivating all retinal cone signaling pathways. Of note, the natural resolution limit is set to the diameter of a photo-receptor OS (Domdei et al., 2021; Tuten and Harmening 2021). In dormant optogenetically-reactivated cones, the resolution corresponds to the diameter of the cell body. For other retinal cell types, their processes such as dendritic arbor and axons have to be taken into account to estimate the best possible regained resolution. Here, protein localization tags that cluster optogen gene expression to cell bodies have been explored and are likely beneficial (Greenberg et al. 2011).

Overall, reactivating dormant cone photoreceptors in RP - as long as these cells persist - represents a very promising strategy to restore visual function with optogenetics. However, when all photoreceptor cell bodies are degenerated, downstream cell types must be targeted, first bipolar cells and then ganglion cells.

### 7. Retinal cell transplantation approaches

As there are no sophisticated regenerative properties to replace degenerated cell types in order to maintain or restore visual properties in the human retina, cell replacement therapies represent one option (Fig. 7 and Table 1). Potential therapeutic strategies include providing RPE cells to maintain the functionality of host photoreceptors, or transplanting photoreceptors to either replace the degenerated or protect the remaining sensory cells by secreting neurotrophic factors. Depending on the disease state, replacing both cell types might be necessary. Efforts in this line of research over the last few decades have been specifically summarized in recent review articles (Gasparini et al., 2019; Jin et al., 2019; Singh et al., 2020). As photoreceptor function depends on healthy RPE, this cell class was first tested for cell therapy approaches. RPE cells form a monolayer, and separate the retina from the choroid. As mentioned previously, RPE cells take over essential functions to maintain vision (Strauss 2005) and their dysfunction negatively impacts on photoreceptor function and survival. Hence, replacing RPE cells to restore vision was pioneered almost forty years ago (Gouras et al. 1984) and the first transplantations of fetal RPE cells into AMD patients were performed three decades ago (Algvere et al., 1994). Since the early days of RPE grafts, the clinical efficacy of these approaches has been significantly improved: non-fetal human RPE cell sources have been identified by many contributing groups. Specifically, human stem cell technology boosted this field, as many RPE differentiation protocols have been developed to use this almost unlimited cellular source as starting material (Vitillo et al. 2020). To avoid graft rejection, immunological aspects for establishing RPE cell transplantation must be considered as a routine treatment (Sugita et al., 2021). Several clinical trials based on human stem cell-derived RPE cells have been performed for treating AMD and Stargardt disease (da Cruz et al., 2018; Mandai et al., 2017; Schwartz et al., 2015; Mehat et al., 2018), of which some have led to partial visual improvement (Schwartz et al., 2015; da Cruz et al., 2018).

Only limited functional improvements could be expected from phase I studies as they are usually performed in late stage of the disease, when photoreceptors are already degenerated. Two different approaches were used in these clinical studies to deliver RPE cells: cell suspensions and RPE sheets. While grafted cells perform better in terms of their morphology, physiology, and survival when transplanted as sheets, the use of quickly-formed RPE strips has recently been explored (Nishida et al., 2021). The clinical data obtained so far, based on a large and sophisticated body of experimental preclinical work, has given rise to optimism that an RPE transplantation therapy to counteract progressive vision loss in larger patient groups will be achieved soon.

#### 7.1. Photoreceptor transplantation

Similar to the RPE transplantation endeavors, the replacement of degenerated photoreceptors in the outer retina represents a promising strategy to restore visual function (Chacko et al., 2009). Several comprehensive review articles specifically focus on this topic (Jayakody et al., 2015; Stern et al., 2018; Santos-Ferreira et al. 2016; Seiler and Aramant 2012; Gagliardi, Ben M’Barek, and Goureau 2019). Replacing these sensory cells is quite challenging: graft survival, function, and...
Fig. 7. Transplantation of cells and cell sheets into the subretinal space. Cells (photoreceptors or RPE) or sheets derived from 2D cultures or 3D retinal organoids are generated in vitro and transplanted to replace degenerated cells.

| Disease Type of a transplant | Number of patients | Country | Start | Study identifiers |
|------------------------------|--------------------|---------|-------|-------------------|
| AMD ESC-RPE on membrane      | 16                 | USA     | 10/2015 | NCT02590692      |
| AMD ESC-RPE suspension       | 12                 | South Korea | 09/2012 | NCT01674829      |
| AMD ESC-RPE suspension       | 11                 | USA     | 02/2013 | NCT02463344      |
| AMD ESC-RPE suspension       | 3                  | South Korea | 05/2016 | NCT03305029      |
| AMD ESC-RPE suspension       | 13                 | USA     | 04/2011 | NCT01344993      |
| Neovascular AMD ESC-RPE suspension | 5  | Japan | 02/2017 | UMIN000026003    |
| Stargardt ESC-RPE suspension | 12                 | USA     | 01/2013 | NCT02941991      |
| Stargardt ESC-RPE suspension | 13                 | USA     | 07/2012 | NCT02445612      |
| Stargardt ESC-RPE suspension | 12                 | USA     | 11/2011 | NCT01469832      |
| Stargardt ESC-RPE suspension | 13                 | USA     | 04/2011 | NCT01345006      |
| Stargardt ESC-RPE suspension | 3                  | South Korea | 09/2012 | NCT01625559      |
| Retinitis Pigmentosa ESC-RPE suspension | 10 | China | 05/2020 | NCT03944239      |
| Retinitis Pigmentosa ESC-RPE suspension | 10 | China | 09/2015 | ChiCTR-OCB-15007055 |
| Retinitis Pigmentosa iPSC-Retina-sheets | 2 | Japan | 10/2020 | jRCTs0002000027    |
| Retinitis Pigmentosa ESC-RPE suspension | 12 | France | 08/2019 | NCT03963154      |
| Retinitis Pigmentosa Allogeneic RPC suspension | 84 | USA | 03/2017 | NCT0373733      |

Several leading groups in the field of photoreceptor transplantation have independently and simultaneously discovered that transplanted mouse photoreceptors exhibit a material transfer to remaining host photoreceptors (Pearson et al., 2016; Santos-Ferreira et al., 2016; Singh et al., 2016). The exchanged material, likely via nanotube-like processes (Kalargyrou et al., 2021; Ortin-Martinez et al., 2021), itself beneficially impacted on the remaining host photoreceptors, potentially leading to restoring visual functions. As long as there are some non-functional intrinsic photoreceptors, grafted cells might regenerate these host cells, which also represents a strategy to restore visual function. This type of material transfer was frequently detected when transplanting mouse photoreceptors into mouse host retinas, but has not yet been detected when transplanting human photoreceptors into mouse retinas (Gasparini et al., 2022). Material transfer upon photoreceptor transplantation requires further investigation, also in larger animal models including non-human primates (Shirai et al., 2016; Abooualizadeh et al., 2020; Tu et al., 2019). Another interesting approach to study the function of transplanted photoreceptors and synaptic connections to bipolar cells are stem-cell-derived photoreceptors tagged with optogenetics (Garita-Hernandez et al., 2019, 2021); functional integration of grafted cells is crucial for this intervention. Overall, at the preclinical stage, photoreceptor transplantation therapies represent a promising strategy to substitute degenerated photoreceptors in order to restore high-performance vision efficiently.

7.2. Limitations of RPE and photoreceptor transplantation approaches

The cell quantities and quality required for a broad clinical application of photoreceptor transplantation therapies represents a major bottleneck. The use of primary photoreceptor cells or precursors derived from fetal tissues is accompanied by severe ethical, legal, and technical constraints. Alternative strategies to differentiate photoreceptors from other human cell lines, such as fibroblasts or pluripotent stem cells, have
been widely investigated. For example, fibroblasts have been demonstrated to undergo photoreceptor differentiation using transcription factors (Seko et al., 2014) or small molecules (Mahato et al., 2020). Also, human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have been used to produce photoreceptors in 2D cultures (Mellough et al., 2012; Lamba et al., 2006; Osakada et al., 2008; Hirami et al., 2009; Zhou et al., 2015). However, the photoreceptor yields, subsequent downstream processing, developmental stages, and differentiation duration of these direct 2D reprogramming protocols are not yet sufficient for a viable photoreceptor cell source.

The ability to generate entire retinal tissues, so-called retinal organoids, from pluripotent stem cells (Eiraku et al., 2011; Nakano et al., 2012) has boosted the photoreceptor transplantation field by providing a source of human photoreceptor cells (Gasparini et al., 2019) for further investigating the efficacy and safety of cell therapy. Still, adapting and upscaling photoreceptor production using retinal organoid technology is not trivial: several technological as well as economic challenges must be overcome. A clinical-grade medical cell product requires good manufacturing practices (GMP) such as the removal of all animal products from cell culture and standardized, preferably automated, production including efficient quality control. Significant progress has been made towards GMP for photoreceptors (Zhu et al., 2018; Wiley et al., 2016) and this highlights the overall feasibility of GMP retinal organoids. There have also been attempts towards a more standardized organoid production using mouse ESCs in bioreactors (DiStefano et al., 2018), which still need to be adapted for human stem cells. Still, all existing protocols require extended culture times including multiple manual steps and extensive media feeding. The protocols started with visually-guided sections of retinal tissue requiring fluorescent reporter cell lines from other cell masses (Eiraku et al., 2011), and have progressed to unbiased tri-section (Volkner et al., 2016) then checkerboard scrapping (Cowan et al., 2020), which has simplified time-consuming manual steps and led to higher organoid yields. Supplementation with small molecules has further increased the number of photoreceptors per organoid, or has shifted rod versus cone fate (Volkner et al., 2016; Zerti et al., 2020; Kelley et al., 2020). However, more than 100 days in culture are required to harvest and purify photoreceptors cells for transplantation (Gasparini et al., 2019). These long cultivation times include high and complex media consumption, and require highly-trained manpower for production, maintenance, harvesting, and down-stream processing, which significantly increase the price of the medical product. Another important point is that established retinal organoid protocols only work with very few human stem-cell lines (Cowan et al., 2020), which has to be taken into account when attempting to use patient-derived and mutation-corrected hiPSCs. Autologous cells avoid immune rejection caused by the human leukocyte antigen (HLA) (Nakatsuji et al. 2008). The de novo reprogramming of patient iPSCs and the subsequent gene editing to correct the disease-causing mutation renders personalized medicine also unaffordable for a general therapeutic intervention. Here, HLA superdonor lines, which are homozygous for one HLA allele, have been established to provide immunological compatibility to larger patient groups. These individual lines still require further testing of their competence to generate photoreceptor-rich retinal organoids.

Retinal organoids are an amazing model system for advancing the photoreceptor transplantation field: however, they come with the aforementioned technical and economic disadvantages. Therefore, developing accelerated photoreceptor differentiation protocols starting from hiPSCs to photoreceptors, without the step of 3D cellular structures, will be a step towards affordable cell therapies. For example, the therapeutic replacement of dopaminergic neurons in Parkinson disease (Studer 2017) greatly benefits from the availability of a robust and direct midbrain dopamine neuronal differentiation protocol (Krikis et al., 2011; Kim et al., 2021). Here, defined differentiation media supplemented with small molecules, WNT and SSH signaling activators, and dual-SMAD inhibition generates transplantable dopaminergic neurons within a few weeks, overcoming extensive culturing times and steps (Kim et al., 2021). As mentioned before, similar attempts to induce photoreceptor genesis from fibroblasts or stem cells by manipulating signaling cascades have so far not been very efficient at providing the required quantity of clinical-grade photoreceptors. However, this does not mean that it is impossible to trigger photoreceptor differentiation in 2D in an efficient way, just that the ideal recipe has not yet been discovered. The present line of study represents a good starting point for further improvements and discoveries. So far, the tested signaling cascade modifiers, morphogens and transcription factors have been in vivo inspired by retinal development. As signaling cascade modifiers finally impact on transcription factors which alter transcription in order to induce differentiation, forced expression of transcription factors in human stem cells represents an alternative differentiation route. For example, transcription factors of the neurogenin family have resulted in robust one-step protocols to transform human stem cells efficiently into neurons (Fig. 8) (Zhang et al., 2013; Busskamp et al., 2014b). Implementing this methodology to generate photoreceptors requires the knowledge of which transcription factors are effective. There is a long list of transcription factors which have been found to be essential for in vivo development. However, these factors have not yet proven to robustly drive photoreceptor genesis in vitro, likely due to the different cellular ground states of retinal progenitors in vivo, and fibroblasts or stem cells in vitro. Hence, it is likely that additional transcription factors that act in concert with retina-specific ones are needed to drive human stem cells to photoreceptors. To this end, we have assembled a comprehensive human transcription factor library and robust screening platforms to screen for factors that initiate stem-cell differentiation (Fig. 9) (Ng et al., 2021). In combination with modified hiPSC lines carrying photoreceptor-specific reporter constructs (Gagliardi et al., 2018; Gasparini et al., 2022), unbiased transcription factor screenings to identify the genetic recipe for driving human stem cells to photoreceptors are ongoing. Combining forced transcription-factor overexpression with small-molecule protocols might further improve the 2D generation of clinical-grade human photoreceptors for replacing degenerated photoreceptors in retinal disease to overcome blindness. Overall, there is a lot of work in progress in this field and cell therapies becoming a viable treatment option in the future looks promising. This therapeutic intervention represents the last resort to substitute degenerated photoreceptors.

8. Future directions and conclusions

Understanding the pathophysiology of retinal degenerative diseases is key to identifying therapeutic entry points. A lot of research on the genetic basis and the processes involved has resulted in a toolbox of different strategies that are currently under investigation. Such a wide range of options is needed to adapt the interventions to the specific subtype of disease and the stage of progression. Moreover, a combination of different therapies could in the end prove to be more efficient than a single approach.

Many different approaches have been pioneered and have inspired complementary interventions for other retinal diseases. The presence of rodent models, which efficiently mimic some monogenic diseases, has boosted the gene therapy field for IRDs. However, the field so far lacks sophisticated AMD animal models. Here, it is likely that human stem cell-derived retinal organoids will lead to new discoveries and treatment options (Sharma et al., 2021). It is of utmost importance to reveal the molecular pathological events that lead to AMD. So far, the available clinical imaging and genomic data only describe the phenotypes but have not yet identified the molecular causes triggering the onset of AMD. Maintaining or restoring intrinsic photoreceptor functions using mutation-independent therapies will provide the best functional outcome. After the onset of photoreceptor degeneration in both IRDs and AMD, several mechanisms such as hyperoxia and lack of energy have been identified as contributing to secondary photoreceptor loss.
Therefore, gene therapies targeting those biological pathways are now recognized as an attractive strategy for promoting photoreceptor survival. CRISPR-Cas9 has been tested for gene correction, as well as for in situ reprogramming and has shown great potential to prevent retinal degeneration. Cell types at risk can be reprogrammed into another type to avoid disease progression. Promising genetic targets are under investigation. Müller glia cells have been shown to be a well-suited target for in situ reprogramming because of their ability to reenter the cell cycle and differentiate to other cells such as neurons. Although there are still some safety and efficiency concerns, the latest studies have not shown any immune responses to the Cas9 protein (Maeder et al., 2019). Novel CRISPR-Cas9-based gene editing approaches such as base and prime editors are being developed to overcome the problems of low gene-editing efficiency in post-mitotic cells. MiRNAs are a promising therapeutic tool to counteract retinal degeneration, due to their anti-inflammatory and anti-apoptotic effects as well as their importance for proper retinal development and photoreceptor maintenance. However, the immunogenicity of individual miRNAs is still a limiting factor and requires further investigation before it enters the transition of therapeutic application. At final disease stages with severe photoreceptor degeneration, optogenetics and cell therapies look promising for restoring some level of vision, enhancing the patients’ quality of life. Efforts have been made to provide RPE cells as therapeutic strategies to maintain functionality of host photoreceptors, transplant photoreceptors to replace degenerated sensory cells, or to replace both photoreceptor and RPE. Being able to generate retinal organoids from pluripotent stem cells has advanced the photoreceptor transplantation field by providing a source of photoreceptor cells for further investigating the efficacy and safety of cell therapy. However, accelerating the photoreceptor differentiation protocols is necessary for overcoming the economic challenges associated with using 3D cellular structures as a cell-therapy source.

When a recessive disease-causing mutation is known, photoreceptor degeneration can be prevented by supplementing the cell with a wild-type copy. Dominant mutations can be targeted by knocking down or knocking out of the corresponding mutated allele. However, these approaches require and optimal time window, before the onset of degeneration, and combability with approved gene therapy techniques such as fitting cargo sizes for AAV transfer. Otherwise, mutation-independent approaches such as the ones featured in this article are therapeutic options. Over the years, the entire vision restoration field has produced invaluable data, increased pathophysiological knowledge, and pioneered innovative therapies. There are already examples of successful clinical translations and there are more to come.

Fig. 8. Overexpression of transcription factors (TFs) to induce neuronal differentiation. (A) The induced Neurogenin (iNGN) cell line has the TFs neurogenin 1 and neurogenin 2 under the doxycycline-inducible Tet-On promoter system. Overexpression of the TFs leads to rapid and robust differentiation into neurons. (B) After plating of the iNGN cells, they are kept for 1 day in mTeSR medium under ROCK pathway inhibitor to increase survival. The next day induction is started by adding doxycycline to the medium. (C) Cells differentiate to neurons in 4 days when induced with doxycycline while uninduced cells remain stem cells. Figures modified from Busskamp et al. (2014b).
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Author statement

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Fig. 9. The human TFome expression library and its application for cell fate engineering (A) All-in-one lentiviral expression vector containing a doxycycline-inducible promoter (Tet-On) driving a TF open reading frame (ORF) followed by an in-fusion V5 C-terminal epitope tag and a constitutive promoter driving the puromycin resistance gene (PuroV5). (B) V5 epitope antibody staining assessing ectopic TF expression after lentiviral transduction in hiPSCs and doxycycline treatment. Scale bar, 300 μm. (C) Schematic of human TFome screening for stem cell differentiation. hiPSCs were transduced with the TFome using the multiplicity of infection (MOI) of 0.1 to ensure single TF integration per cell, treated with doxycycline for 4 days, stained for the pluripotency marker Tra1-60, and sorted based on its low and high expression. Single-sorted cells underwent NGS-based TF identification and scoring. (D) Validation of top TF hits identified from the screening in different hiPSC lines at 4 dpi. Percentage of differentiation was calculated by loss of pluripotency markers using flow cytometry. Mean ± SEM, n = 3, two-sided Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001. Figures modified from (Ng et al., 2021).

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