

Expert Opinion

1. Introduction
2. The nature of inherited blindness and its treatment via gene therapy
3. The structure and function of *cis*-regulatory elements and their application in the design of gene therapy vectors
4. Expert opinion

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Gene therapy

The role of *cis*-regulatory elements in the design of gene therapy vectors for inherited blindness

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Background: Hereditary retinal disease is currently known to involve nearly 200 different genetic loci. There has been remarkable recent progress in the treatment of retinal disease via gene therapy in animal models using virus-based vectors. The majority of retinal diseases affect one of several cell types. In order to target expression of a rescue transgene specifically to the cells in need of therapy, it is necessary to employ a *cis*-regulatory element (CRE) to drive expression of the transgene specifically in those cells. **Objective/Methods:** This review discusses the repertoire of CREs currently available for use in gene therapy vectors for treatment of retinal disease and outlines the issues that must be taken into consideration in the development of novel CREs for the purpose of gene therapy in the retina. **Conclusion:** There have been a number of important recent advances in the identification and characterization of retinal CREs and their utilization in gene therapy vectors. Nevertheless, future efforts to rationally manipulate existing CREs and design novel synthetic CREs for therapeutic purposes will require a better understanding of the *cis*-regulatory rules that govern CRE activity *in vivo*.

Keywords: adeno-associated virus, blindness, *cis*-regulatory elements, gene therapy, photoreceptors, retina

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1. Introduction

Hereditary diseases of the retina comprise one of the most genetically heterogeneous groups of disorders that affect humans. At the last count, there were nearly 200 genetic loci implicated in inherited blindness [1]. For nearly 75% of these loci, the causative gene has been identified [1]. Knowledge of these genes has led to marked improvements in our ability to pinpoint the causes of blindness in individual patients. Furthermore, advances in the design of virus-based vectors, has set the stage for the treatment of many of these disorders using gene therapy [2-4]. A key requirement for successful gene therapy is accurate targeting of the rescue construct or transgene to the cells in need of therapy. Such targeting is affected by a number of different factors including delivery of the gene therapy vector to the correct anatomical location, the cellular tropism of the viral vector and the *cis*-regulatory elements (CREs; enhancers/promoters) used to drive expression of the rescue transgene [2]. The purpose of this review is to discuss the factors affecting cellular targeting of gene therapy with a focus on the role of CREs in this process.

2. The nature of inherited blindness and its treatment via gene therapy

The retina is a laminated neural structure which lines the back of the eye and mediates the first steps in processing of visual information [5]. It consists of three cellular layers which contain the primary sensory neurons or photoreceptors (rods and cones), interneurons (horizontal cells, bipolar cells and amacrine cells) and output neurons (ganglion cells), which transmit visual information to the brain. Immediately external to the photoreceptors lies an epithelial layer known as the retinal pigment epithelium (RPE) which performs a number of functions required for normal photoreceptor activity [6]. The majority of hereditary retinal diseases affect either photoreceptors or the RPE [1]. A subset of hereditary retinal diseases affect other retinal cell types specifically. For example, the optic neuropathies, a class of diseases which involve mitochondrial dysfunction, affect retinal ganglion cells and their axons in the optic nerve [7,8]. In addition, a few diseases target other retinal cell types such as bipolar cells [9].

Hereditary retinal diseases can be classified according to inheritance pattern, clinical features and a variety of other parameters [10,11]. An important parameter from the standpoint of gene therapy targeting is the cell type(s) in which the disease gene is normally expressed. The clinical features of the various forms of hereditary retinal disease typically correlate with the spatial and temporal expression pattern of the affected gene [11]. For example, many forms of retinitis pigmentosa, a progressive degeneration of rods with late, secondary involvement of cones, are caused by mutations in genes that are expressed specifically (and often exclusively) in rod photoreceptors [12]. In contrast, the three genes that have been implicated in achromatopsia [1,13], a disease that specifically affects cones and causes total colorblindness and loss of acuity, are all preferentially expressed in cone photoreceptors within the retina [14-16]. In addition to expression specifically in rods or cones, retinal disease genes can show varying degrees of rod/cone co-expression [15]. Lastly, the expression pattern of retinal disease genes can vary along the dorsal-ventral and nasal-temporal axes of the retina [15].

On top of variation in spatial expression pattern, retinal disease genes can also vary in their temporal patterns and quantitative levels of expression. Whereas photoreceptor degeneration can be caused by mutations in developmental regulators such as the transcription factors cone-rod homeobox (*Crx*) and neural retina leucine zipper (*Nrl*), which are turned on early during photoreceptor development, it can also be caused by mutations in genes that are turned on during the latest stages of photoreceptor differentiation [17]. The transcript levels of many photoreceptor genes (and presumably a number of retinal disease genes) can also vary according to the circadian cycle [18-20]. Lastly, retinal disease genes can vary markedly in their levels of expression. semiquantitative analysis of retinal transcript levels via serial

analysis of gene expression in mice suggests that the transcript levels of photoreceptor genes can vary over nearly three orders of magnitude [17,21]. Clearly, efforts to restore cellular function via gene therapy must take into consideration the precise spatiotemporal patterns and levels of expression of the gene whose activity is to be rescued.

For successful gene replacement therapy in an individual patient with hereditary blindness, one must first identify the causative gene in order to engineer a rescue transgene consisting of a normal copy of the gene. Secondly, one must target the rescue transgene to the cells in need of therapy. In treatment of patients with recessive (loss-of-function) alleles, the simplest gene therapy scenario, expression of a rescue transgene at endogenous levels in the right cells should, in principle, be sufficient to restore normal cellular function. In patients with disease caused by dominant alleles, especially those with a gain-of-function or dominant-negative mechanism of action [22], a treatment strategy must be used which first removes the abnormally functioning gene product [23]. After this is accomplished, it may be desirable in some cases to then resupply the targeted cells with a normal copy of the gene in question. One approach to this problem is to use RNA interference (RNAi) to knock down the levels of the abnormal transcript while simultaneously expressing a codon-modified version of the wild type gene that is immune to degradation by RNAi [24].

The three most critical factors affecting transgene targeting in intraocular gene therapy are: i) delivery of the gene therapy vector to the correct anatomical location; ii) the tropism of the viral delivery vector (i.e., which cell types the virus preferentially infects); and iii) The CRE used to drive expression of the rescue transgene once it is delivered to the target tissue. When gene therapy is to be directed to a widely dispersed target such as bone marrow, it is usually necessary to introduce the delivery virus via the bloodstream. Anatomical delivery becomes a significant problem in such cases as the virus must not only evade the immune system while in the bloodstream but it must also exit the bloodstream, usually by passing through the vascular endothelium, in order to enter the target tissue [25]. In gene therapy for most intraocular diseases, these problems can largely be circumvented by direct inoculation of the virus into specific anatomic compartments within the eye (Figure 1) [3]. Effective targeting of transgenes to photoreceptors or the RPE can be achieved by introducing the transducing virus into the subretinal space between the outer segments of photoreceptors and the RPE (Figure 1). Targeting of transgenes to retinal ganglion cells, in contrast, requires introduction of the virus into the intravitreal space (Figure 1). Another advantage of intraocular gene therapy over systemic therapy is that the immune-privileged status of the retina may reduce the likelihood of unwanted immune responses to the therapeutic virus [26].

Lentiviral, adenoviral and adeno-associated viral vectors have all been used to treat animal models of blindness [16,27-29].

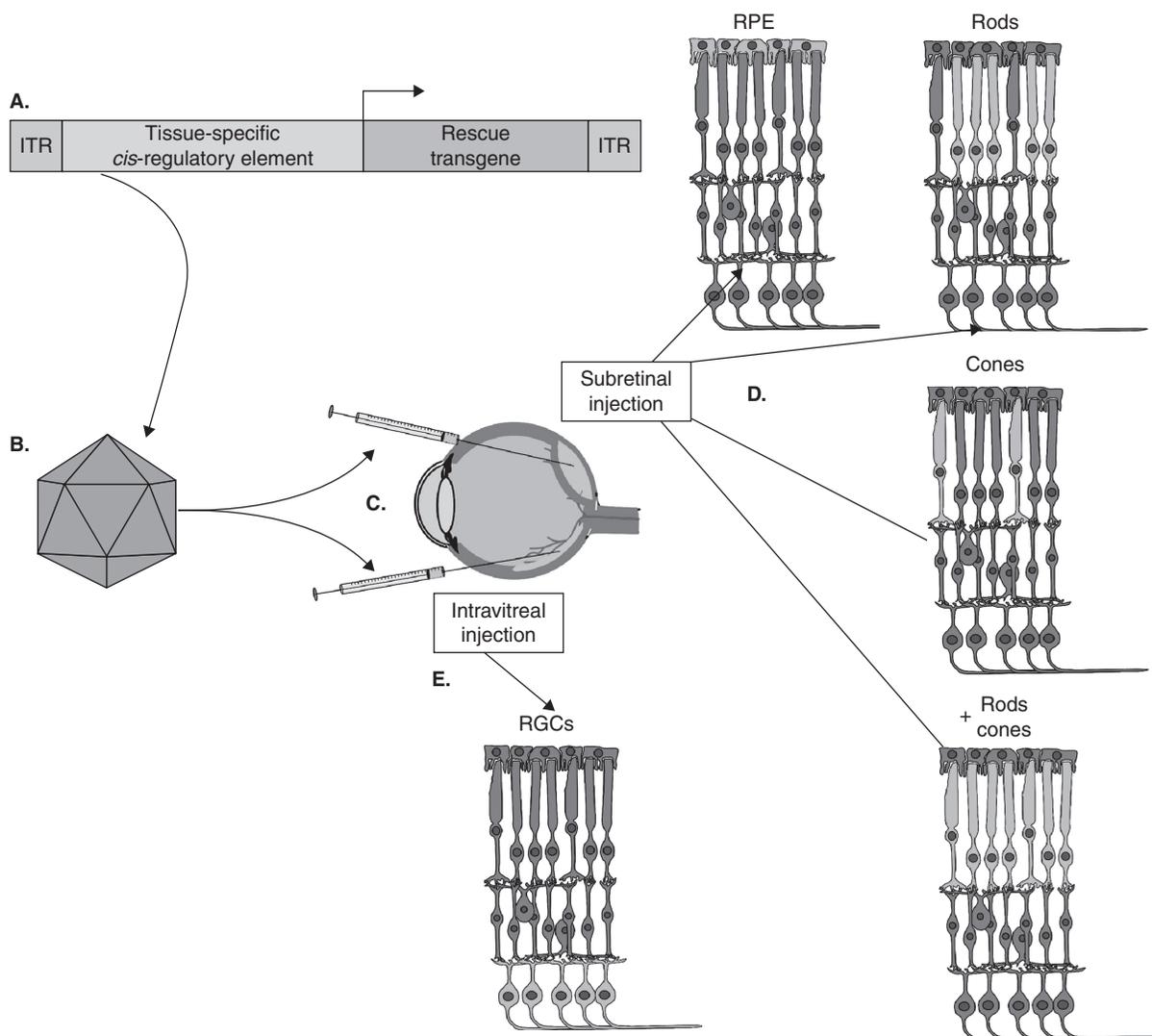


Figure 1. Targeting retinal gene therapy to specific cell types. **A.** Structure of a generic gene therapy vector containing a rescue transgene and a tissue-specific cis-regulatory element (CRE) flanked by inverted terminal repeats (ITR) derived from the recombinant adeno-associated virus (rAAV). **B.** The viral capsid in which the genome depicted in **A** is packaged. The particular set of proteins present in the capsid determine the infectivity of the virus for particular cell types (viral tropism). **C.** Injection into the subretinal space (between the retina and the retinal pigment epithelium (RPE)) is used to target virus to photoreceptors or RPE. Injection into the intravitreal space is used to target virus to retinal ganglion cells (RGCs). **D** and **E.** Once the virus infects cells, cell type specific CREs drive expression of the rescue transgene (in green) in specific cell populations within the retina or RPE.

Each of these viral vectors has advantages and disadvantages with regard to intraocular gene therapy and specifically the issue of cellular targeting. Three important aspects of these vectors that impinge on their ability to target specific cell populations are their DNA carrying capacity (which determines the size of the rescue transgene as well as the associated CRE that can be accommodated in the vector), their cellular tropism and whether or not the vectors integrate into the host chromosome. Lentiviral vectors have a moderate DNA carrying capacity (8 – 9 kb) [30] but appear to have a somewhat restricted cellular tropism in the eye and normally integrate into host chromosomes.

These vectors have been used to effectively target gene therapy to RPE [31,32]. However, although lentiviral vectors have been used to target photoreceptors in the early post-natal period in the mouse [28], they are not particularly effective at targeting adult photoreceptors [33-35]. Integration into host chromosomes can be problematic from the perspective of transgene regulation since individual targeted cells can experience insertion of a variable number of copies of the viral genome as well as varying sites of integration [32]. Variation in integration site can result in non-uniform transgene expression within the targeted cell population [32] due to variation in the chromatin context of the insertion

site as well as differences in the proximity of endogenous CREs to the transgene cassette. Potential approaches to ameliorate these drawbacks with regard to cellular targeting of expression include the use of chromatin insulators to protect the transgene from the effects of chromosomal context [36,37] or the use of integrase-deficient lentiviruses, which do not integrate into the chromosome [38].

Unlike lentiviruses, adenoviral vectors do not integrate into chromosomes and, in addition, have a large DNA carrying capacity (up to 36 kb) [30]. The large carrying capacity of these vectors is useful for the purposes of cellular targeting since it makes it possible to utilize relatively large CREs, which are more likely to contain the genomic regulatory elements necessary for robust, cell-type-specific expression of a transgene. Unfortunately, early generation adenoviral vectors were only able to effectively transduce photoreceptors when delivered during the early stages of retinal development [29,39,40]. This limitation motivated Cashman and colleagues [41] to engineer improvements in the adenoviral vector including the use of a photoreceptor-specific CREs to drive transgene expression. These changes resulted in dramatically improved transduction of- and expression in adult photoreceptors in the mouse [41]. This study has revealed new promise for adenoviral vectors as therapeutic vectors for common retinal diseases.

The most commonly used vector for intraocular gene therapy is recombinant adeno-associated virus (AAV) [2,3]. AAV is a naturally replication-incompetent virus which consists of a small viral genome containing two inverted terminal repeats (ITRs) and two open reading frames, which encode several different viral proteins [2]. AAV is generally maintained episomally but can integrate into chromosomes [42,43]. It is possible to replace the entire viral genome between the two ITRs with cargo DNA consisting of a rescue transgene and its associated tissue-specific CRE. One limitation of recombinant AAV (rAAV)-based gene therapy is that the size of the DNA cargo is limited to ~ 4.7 kb due to packaging constraints within the viral capsid [44]. Viral tropism is largely determined by the viral attachment proteins expressed on the viral capsid [25,45]. It is possible to alter the tropism of the rAAV virus by packaging one viral genome with capsid proteins from another strain of virus, a procedure known as pseudotyping [25]. For example, serotypes rAAV2/1 and rAAV2/4 (i.e., the rAAV2 genome packaged with the capsid proteins from serotypes 1 or 4, respectively) preferentially target RPE when introduced into the subretinal space [45]. In contrast, rAAV2/5 targets photoreceptors as well as RPE when injected into the same location [46]. It was recently reported that rAAV2/7 and rAAV2/8 are even more efficient than rAAV2/5 at targeting photoreceptors [45,46]. Clearly, rapid progress is being made in developing viruses with enhanced tropism for the cell types most commonly affected by heritable blinding diseases.

Many non-viral approaches to gene therapy could benefit from the use of appropriately chosen CREs to drive cell

type-specific transgene expression [47,48]. Given recent improvements in delivery of non-viral vehicles to target cell populations within the retina and the development of viral strains with improved cellular tropism within the eye [45], the role of CREs in driving cell-type-specific expression in gene therapy experiments has taken on increased importance. It is therefore important to review the structure of CREs and their role in driving gene expression, prior to discussing recent advances in the use of cell-type-specific CREs in intraocular gene therapy.

3. The structure and function of *cis*-regulatory elements and their application in the design of gene therapy vectors

Transcriptional regulatory networks (TRNs) underlie the basic developmental and physiologic processes of all multicellular organisms [49-54]. A TRN consists of a hierarchy of transcription factors (TFs), which alter the expression of individual target genes by binding to the tissue-specific CREs of those genes. Since individual TFs can activate the expression of other TFs, the early expression of a small set of master regulatory TFs can set in motion a hierarchical cascade of gene expression that serves to spatially subdivide an embryo or organ anlage and thereby foster the formation of distinct cell types. TFs within endogenous gene networks control the spatiotemporal pattern and levels of expression of their target genes by binding to CREs, short (~ 300 – 600 bp) stretches of genomic DNA that can lie upstream, downstream, or within the introns of the genes they control [53,55]. CREs typically consist of multiple clustered binding sites for both transcriptional activators and repressors. They serve as logical integrators of transcriptional input, giving a unitary output in the form of spatiotemporally precise and quantitatively exact transcriptional activity. Given the critical importance of CREs in controlling gene expression, the non-coding DNA containing tissue-specific CREs often shows high degrees of phylogenetic conservation [56].

As discussed above, the genes that have been implicated in hereditary diseases of the retina show wide variation in spatiotemporal pattern and levels of expression. Photoreceptor disease genes, for example, can show variation in expression with respect to a number of parameters, including extent of expression in rods vs cones, extent of expression in non-photoreceptor cell types, regional distribution of expression within the retina, timing of the onset of gene expression, expression according to daily (circadian) rhythms and variation in quantitative levels of expression [11,15]. Given this type of variation and the imprecise nature of viral tropism, it is not possible to engineer a single, universal CRE suitable for all gene therapy purposes in the retina. Instead, it is necessary to identify and evaluate CREs that drive the spatiotemporal patterns and levels of expression needed for a given rescue transgene. In the following paragraphs the use of CREs in

targeting expression to photoreceptors is discussed but similar considerations apply to targeting other retinal cell types and RPE [2,57,58].

Early efforts to achieve *in vivo* gene transfer and expression in mammalian retina relied exclusively on the ubiquitously expressing cytomegalovirus (CMV) promoter to drive reporter expression [59,60]. This promoter drove expression predominantly in RPE with only sporadic, low-level expression in photoreceptors [61]. Reliable photoreceptor-specific expression was first achieved by using the mouse Rhodopsin (*Rho*) proximal promoter to drive reporter expression [61]. This promoter remains to this day the most widely used in rAAV-mediated gene therapy experiments that target photoreceptors [27,62]. Although very useful, the *Rho* proximal promoter has a variety of deficiencies that make it less than ideal. First, whereas the *Rho* gene is expressed exclusively in rods, the *Rho* proximal promoter drives leaky expression in cones in addition to rods, especially when delivered via rAAV [62-64]. Furthermore, in transgenic mice this promoter drives relatively weak, patchy expression throughout the retina [65]. Although constructs containing longer pieces of DNA upstream of the *Rho* locus tend to drive higher, more consistent expression in transgenic mice [65], they display an unusual gradient of expression in transgenic mice such that there is high level expression in the superior-temporal retina and almost no expression at all in the inferior-nasal half [66]. It is not known to what extent these *Rho* CREs display a gradient of expression when introduced via rAAV but failure of transgene expression in half of a patient's treated eye could represent a significant clinical problem.

Recently two groups reported successful targeting of transgene expression specifically to cone photoreceptors using the promoter regions of the human blue opsin and X-linked opsin loci [16,67,68]. Furthermore, it was shown that the human X-linked opsin CRE can be used to effectively target transgene expression to cone photoreceptors and rescue function in a mouse model of achromatopsia [16]. The authors did caution, however, that the use of such a cone subtype-specific promoter might not be ideal for use in human patients where rescue of all cone subtypes is desired [16]. Another recent report showed reliable transgene expression in both rods and cones using an rAAV vector carrying a promoter from the upstream region of G protein-coupled receptor kinase 1 (*Grk1*) [69]. This compact pan-photoreceptor promoter is a welcome addition to the armamentarium of CREs available for retinal gene therapy because rod/cone co-expression is seen with many photoreceptor disease genes [15] including several that have been implicated in Leber's congenital amaurosis [27], a severe, early-onset form of blindness.

An additional issue that is rarely addressed in studies of CREs for use in retinal gene therapy is the question of quantitative levels of expression driven by virus-delivered CREs. Of course, one can argue that some expression, even

at an inappropriate level, is better than none when trying to rescue cellular function within a target population. This is undoubtedly true but studies of the *Rho* gene suggest that transgene overexpression, even of wild type *Rho*, can lead to photoreceptor degeneration [70]. Furthermore, since *Rho* is the most highly expressed gene in rod photoreceptors [17], use of the *Rho* promoter to drive expression of rescue transgenes for disease genes that are normally expressed at much lower levels, could be detrimental to photoreceptors. These considerations strongly suggest that CREs that drive gene-appropriate patterns and levels of expression are needed for optimal results in human gene therapy.

4. Expert opinion

Despite the importance of CREs in targeting expression of therapeutic transgenes to specific cell types, the number of CREs available for gene therapy in the retina is quite limited. This dearth of CREs is attributable to two main causes. First, the identification of native cell-type-specific CREs has been hampered by the lack of effective computational algorithms to predict their location. Second, there are currently no high-throughput *in vivo* assays for testing fragments of genomic DNA for promoter activity in mammals. These difficulties stem from the fact that nearly 98% of the mammalian genome consists of non-coding DNA, and CREs can be found nearly anywhere within these vast regions: upstream, downstream or within the introns of the gene whose expression they control [56]. Unlike our detailed knowledge of the rules that govern the structure of coding sequences, we know very little about the grammar rules that govern the structure of CREs in any mammalian cell type [56]. This latter fact is what makes the design of effective computational algorithms to find CREs within non-coding DNA so difficult.

To complicate matters, most computational studies which have produced sets of CRE predictions for specific cell types have been unable to validate their computational 'hits' on account of the lack of rapid *in vivo* assays for testing such predictions. For most mammalian tissues, the costly and time-consuming creation of transgenic mice carrying CRE-reporter constructs remains the 'gold standard' for testing genomic DNA for CRE activity [71]. Given the paramount importance of transcriptional networks in all cells and the fact that CREs mediate the interactions between transcription factors and their target genes within these networks, it is frustrating that so little progress has been made toward their systematic characterization. The pressing need for cell-type-specific CREs to direct transgene expression in gene therapy vectors further underscores the importance of current efforts to develop effective computational algorithms to identify endogenous CREs within genomic DNA and to analyze these elements in a high-throughput fashion.

In the ideal scenario, individual gene replacement therapy vectors should be engineered to contain the native

cis-regulatory regions of the gene to be rescued so as to faithfully recapitulate the endogenous spatiotemporal pattern and levels of expression of that gene. Two recent studies have begun to make progress toward the systematic elucidation of photoreceptor-specific CREs [72,73]. This knowledge may eventually be useful in engineering gene-specific CREs for therapeutic purposes in the retina [72,73]. In the first study, the authors developed a computational algorithm to identify CREs around photoreceptor genes based on the presence of Crx, Nrl and/or nuclear receptor subfamily 2, group E, member 3 (Nr2e3) binding sites in a 2.2 kb region around the transcription start sites (TSSs) of retina-enriched transcripts [72]. This approach is reasonable given the fact that CREs occur with increased frequency in the region immediately upstream of the TSS and within the first intron of the gene they control [74] and that Crx, Nrl and Nr2e3 are three of the most important transcription factors known to regulate photoreceptor gene expression in mammals [14,75-79]. The authors predicted a number of CREs computationally and proceeded to validate five of them in a variety of biochemical and cell culture assays [72].

In a series of parallel studies [15,73,79], the author of the present review and colleagues carried out a combined computational and experimental analysis of the photoreceptor transcriptional networks regulated by Crx, Nrl and Nr2e3 [73]. This permitted the definition of a typology of photoreceptor gene expression patterns in the mouse [15] and the creation of a comprehensive model of the mammalian photoreceptor transcriptional network containing over 600 genes including most retinal disease genes expressed in photoreceptors [73]. In one of these studies, a computational algorithm was developed to identify the CREs that control the expression of genes within the photoreceptor gene network [73]. This algorithm identified putative CREs around hundreds of genes in the network based on the presence and clustering of phylogenetically conserved binding sites for Crx, Nrl and Nr2e3 [73]. Electroporation of CRE-reporter fusion constructs into living mouse retinae permitted validation of 19 novel CREs around mouse orthologs of retinal disease genes. By coupling an effective computational algorithm to identify photoreceptor CREs with a rapid, *in vivo* validation system, it has been possible to more than double the number of photoreceptor CREs available for gene therapy [73].

Of course, translating these results into useful gene therapies for human patients will require that a number of additional hurdles be overcome. First, it is not clear whether the CREs identified around retinal disease genes contain the full regulatory activity for the gene in question. Prior studies of the Rho locus have shown that strong CRE activity is mediated by at least two spatially discrete CREs: a proximal promoter and an upstream enhancer element [80]. In addition, computational algorithms often detect the presence of more than one regulatory peak around a given locus [73]. It is therefore likely that, at least in some cases,

the full CRE activity for a gene may depend on the combined activity of multiple spatially discrete CREs. If this is the case, then it may be necessary to combine all such CREs into a single element in order to faithfully recapitulate the endogenous expression pattern and levels of the gene in question. Given the limited cloning capacity of the rAAV vector, it will be necessary to engineer such combined CREs to be as short as possible while still retaining the full functionality of the endogenous regulatory regions. Alternatively, other viral vectors with larger DNA carrying capacities such as adenovirus may be needed to deliver relatively long CREs.

Another issue that needs to be addressed is the extent to which CRE-driven expression matches the quantitative levels of the endogenous transcript. In the mouse, this problem can be studied by fluorescence-activated cell sorting of rAAV-transduced photoreceptors expressing GFP under the control of the CRE in question followed by comparison of GFP transcript levels with those of the endogenous gene. However, determining the extent to which the levels of expression of a given gene in the mouse match those in human photoreceptors remains a problem. Similar considerations apply to differences between orthologous human and mouse photoreceptor genes as regards spatiotemporal patterns of expression. One way to address these issues is to evaluate CRE-driven expression in non-human primate models which are likely to have patterns and levels of expression of most photoreceptor genes similar to those seen in humans [68].

Although CREs derived from endogenous photoreceptor gene loci will have a number of useful applications in gene replacement therapy, numerous circumstances can be envisioned in which it would be desirable to modulate the activity of such endogenous CREs either up or down. For example, it is possible that a specific gene defect or stage of retinal degeneration may require that a therapeutic transgene be expressed at either sub or supranormal levels relative to wild-type expression in order to achieve effective rescue. A patient with a blinding disease caused by a hypomorphic allele that results in a reduction but not a complete absence of activity of a given gene product, may require delivery of a therapeutic transgene expressed at levels below those driven by that gene's endogenous CRE. In such a case, it would be very useful to be able to fine-tune an endogenous CRE to drive a level of expression appropriate for a given patient.

It is possible to pharmacologically regulate expression of a transgene within a viral gene therapy vector by the use of a variety of drug-inducible CREs [81,82]. However, most inducible CREs that have so far been tested drive expression in all infected cell types upon induction. A number of therapeutic applications can be envisaged in which it would be desirable to engineer a CRE that drives expression only in a specific cell type upon induction. Such a CRE would permit spatially precise delivery of a therapeutic transgene with dosage controlled pharmacologically. This type of system

would be useful in delivering therapeutic transgenes that might be toxic when expressed continuously or in excessive quantity or might have adverse consequences when expressed in an undesired location.

One potential application of a drug-inducible, cell-type-specific CRE would be in the delivery of neurotrophic factors or antiapoptotic agents to the degenerating retina. Since apoptosis is the proximate cause of degeneration in many forms of blindness [83,84], its prevention should, in principle, slow disease progression. Likewise, expression of neurotrophic factors may help prolong the life of vulnerable populations of cells in the retina. Such therapies do not require that the therapeutic transgene be expressed in the affected cell population. Nevertheless, there is likely to be an optimal pattern of expression for any given therapeutic transgene, which must be determined on a case by case basis. Ciliary neurotrophic factor (CNTF) has been shown to slow retinal degeneration in a variety of animal models [85-87], however, there are some concerns about toxic effects of CNTF at high doses [88,89]. In addition, the consequences of long-term CNTF expression in 'off target' tissues is currently unknown. The use of a drug-inducible, tissue-specific CRE to drive CNTF expression would not only permit clinicians to fine-tune the levels of expression within a relatively narrow therapeutic window but would also allow them to alter the dosage of the therapeutic agent over time as the degeneration progressed. Furthermore, the additional level of specificity conferred by tissue-specific expression would reduce the likelihood of side effects due to expression of the transgene outside of the target tissue.

Another type of 'hybrid' CRE that could have a range of useful clinical applications would be one that drives cell type-specific expression but only in response to local environmental conditions. A variety of diseases that affect photoreceptors, including diabetic retinopathy and age-related macular degeneration, are associated with neovascularization in the context of hypoxia [90,91]. For the treatment of such diseases, it would be desirable to engineer a photoreceptor-specific, hypoxia-inducible CRE that could be used to target antiangiogenic agents directly to the cells subjected to the hypoxic event. In this manner, it would be possible to restrict expression of the transgene to the cells that need it and to avoid promiscuous expression under conditions when it might be undesirable. Early efforts have demonstrated the possibility of achieving hypoxia-inducible expression in the retina via viral gene therapy [92]. Furthermore, the feasibility of engineering a hypoxia-inducible and tissue-specific CRE has recently been demonstrated by Su and colleagues [93], who were able to achieve cardiac-specific, hypoxia-inducible

transgene expression by engineering a chimeric CRE containing both a cardiac-specific CRE and binding sites for hypoxia-inducible factor.

Clearly, there are a number of therapeutic approaches that will necessitate fine-tuning of endogenous CREs and that will require the *de novo* engineering of synthetic cell type-specific CREs with special properties such as drug- or hypoxia-inducibility. How far are we from being able to 're-engineer' endogenous CREs or create entirely synthetic CREs for therapeutic purposes? One prerequisite for precise fine-tuning of endogenous CREs is a better understanding of the 'grammar' rules that define the cell type-specificity and quantitative levels driven by a given CRE. In the case of photoreceptor-specific CREs, the presence of binding sites for Crx, Nrl and Nr2e3 are important but there is currently little understanding of the roles of binding site affinity, intersite spacing and orientation of individual sites on the overall levels of transcriptional activity driven by a CRE. In addition, a variety of other TFs have been implicated in the control of photoreceptor gene expression (e.g., neurogenic differentiation 1 (*Neurod1*), retinoid X receptor gamma (*Rxrg*) and thyroid hormone receptor, beta 2 (*Thrb2*)) [94-96] but their role (if any) in the regulation of most photoreceptor CREs is currently unknown. A more complete understanding of the *cis*-regulatory rules governing the activity of CREs is clearly needed in order to carry out rational engineering of CREs. Specifically, we need a quantitative understanding of the effects on CRE activity of altering the affinity, spacing and orientation of key TF binding sites. In addition, we need to elucidate the manner in which the addition of novel binding sites (such as sites for hypoxia-inducible factor) to a cell-type-specific CRE will affect the overall activity of the *cis*-element. The development of high-throughput *in vivo* approaches to CRE analysis in mammals will greatly facilitate the engineering of novel CREs. As knowledge of the functional architecture of cell type-specific CREs improves, it may someday be possible to rationally design synthetic CREs that drive any desired pattern or level of expression within photoreceptors or other retinal cell types [73]. Such rationally designed CREs will permit clinicians to customize gene therapy vectors for individual patients depending on their particular mutation and stage of disease.

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Bibliography

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

1. Available from: <http://www.sph.uth.tmc.edu/RetNet/> [Last accessed 7 March 2008]
2. Dinculescu A, Glushakova L, Min SH, Hauswirth WW. Adeno-associated virus-vectored gene therapy for retinal disease. *Hum Gene Ther* 2005;16(6):649-63
- **An excellent overview of the use of adeno-associated virus in retinal gene therapy.**
3. Bainbridge JW, Tan MH, Ali RR. Gene therapy progress and prospects: the eye. *Gene Ther* 2006;13(16):1191-7
4. Bainbridge JW. Gene therapy clinical trials for inherited eye disease. *Expert Rev Ophthalmol* 2007;2(4):517-9
5. Rodieck RW. *The First Steps in Seeing*. Sunderland, MA: Sinauer; 1998
6. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev* 2005;85(3):845-81
7. Newman NJ. Hereditary optic neuropathies: from the mitochondria to the optic nerve. *Am J Ophthalmol* 2005;140(3):517-23
8. Carelli V, La Morgia C, Iommarini L, et al. Mitochondrial optic neuropathies: how two genomes may kill the same cell type? *Biosci Rep* 2007;27(1-3):173-84
9. Gregg RG, Kamermans M, Klooster J, et al. Nyctalopin expression in retinal bipolar cells restores visual function in a mouse model of complete X-linked congenital stationary night blindness. *J Neurophysiol* 2007;98(5):3023-33
10. Rattner A, Sun H, Nathans J. Molecular genetics of human retinal disease. *Ann Rev Genet* 1999;33:89-131
11. Montana CL, Corbo JC. Inherited diseases of photoreceptors and prospects for gene therapy. *Pharmacogenomics* 2008;9(3):335-47
12. Hartong DT, Berson EL, Dryja TP. Retinitis pigmentosa. *Lancet* 2006;368(9549):1795-809
13. Kohl S, Varsanyi B, Antunes GA, et al. CNGB3 mutations account for 50% of all cases with autosomal recessive achromatopsia. *Eur J Hum Genet* 2005;13(3):302-8
14. Chen J, Rattner A, Nathans J. The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. *J Neurosci* 2005;25(1):118-29
15. Corbo JC, Myers CA, Lawrence KA, et al. A typology of photoreceptor gene expression patterns in the mouse. *Proc Natl Acad Sci USA* 2007;104(29):12069-74
16. Alexander JJ, Umino Y, Everhart D, et al. Restoration of cone vision in a mouse model of achromatopsia. *Nat Med* 2007;13(6):685-7
- **The first successful treatment of a cone-based disease using a gene replacement construct driven by a cone-specific CRE.**
17. Blackshaw S, Fraioli RE, Furukawa T, Cepko CL. Comprehensive analysis of photoreceptor gene expression and the identification of candidate retinal disease genes. *Cell* 2001;107(5):579-89
18. Pierce ME, Sheshberadaran H, Zhang Z, et al. Circadian regulation of iodopsin gene expression in embryonic photoreceptors in retinal cell culture. *Neuron* 1993;10(4):579-84
19. Yu CJ, Gao Y, Li P, Li L. Synchronizing multiphasic circadian rhythms of rhodopsin promoter expression in rod photoreceptor cells. *J Exp Biol* 2007;210(Pt 4):676-84
20. Storch KF, Paz C, Signorovitch J, et al. Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. *Cell* 2007;130(4):730-41
21. Blackshaw S, Harpavat S, Trimarchi J, et al. Genomic analysis of mouse retinal development. *PLoS Biol* 2004;2(9):E247. Published online 29 June 2004, doi:10.1371/journal.pbio.0020247
22. Wilson JH, Wensel TG. The nature of dominant mutations of rhodopsin and implications for gene therapy. *Mol Neurobiol* 2003;28(2):149-58
23. Farrar GJ, Kenna PF, Humphries P. On the genetics of retinitis pigmentosa and on mutation-independent approaches to therapeutic intervention. *EMBO J* 2002;21(5):857-64
24. O'Reilly M, Palfi A, Chadderton N, et al. RNA interference-mediated suppression and replacement of human rhodopsin in vivo. *Am J Hum Genet* 2007;81(1):127-35
25. Waehler R, Russell SJ, Curiel DT. Engineering targeted viral vectors for gene therapy. *Nat Rev Genet* 2007;8(8):573-87
- **An excellent discussion of the issues involved in engineering viral gene therapy vectors with altered cellular tropism.**
26. Allocca M, Tessitore A, Cotugno G, Auricchio A. AAV-mediated gene transfer for retinal diseases. *Expert Opin Biol Ther* 2006;6(12):1279-94
27. Pawlyk BS, Smith AJ, Buch PK, et al. Gene replacement therapy rescues photoreceptor degeneration in a murine model of Leber congenital amaurosis lacking RPGRIP. *Invest Ophthalmol Vis Sci* 2005;46(9):3039-45
28. Takahashi M, Miyoshi H, Verma IM, Gage FH. Rescue from photoreceptor degeneration in the rd mouse by human immunodeficiency virus vector-mediated gene transfer. *J Virol* 1999;73(9):7812-6
29. Bennett J, Tanabe T, Sun D, et al. Photoreceptor cell rescue in retinal degeneration (rd) mice by in vivo gene therapy. *Nat Med* 1996;2(6):649-54
30. Breyer B, Jiang W, Cheng H, et al. Adenoviral vector-mediated gene transfer for human gene therapy. *Curr Gene Ther* 2001;1(2):149-62
31. Bemelmans AP, Kostic C, Crippa SV, et al. Lentiviral gene transfer of RPE65 rescues survival and function of cones in a mouse model of Leber congenital amaurosis. *PLoS Med* 2006;3(10):e347. Published online 10 October 2006, doi:10.1371/journal.pmed.0030347
32. Hashimoto T, Gibbs D, Lillo C, et al. Lentiviral gene replacement therapy of retinas in a mouse model for Usher syndrome type 1B. *Gene Ther* 2007;14(7):584-94
- **A good example of the need to evaluate a number of different CREs in order to identify one that functions effectively in a gene therapy vector.**
33. Bainbridge JW, Stephens C, Parsley K, et al. In vivo gene transfer to the mouse eye using an HIV-based lentiviral vector; efficient long-term transduction of corneal endothelium and retinal pigment epithelium. *Gene Ther* 2001;8(21):1665-8
34. Gruter O, Kostic C, Crippa SV, et al. Lentiviral vector-mediated gene transfer in adult mouse photoreceptors is impaired by the presence of a physical barrier. *Gene Ther* 2005;12(11):942-7
35. Pang J, Cheng M, Haire SE, et al. Efficiency of lentiviral transduction

- during development in normal and rd mice. *Mol Vis* 2006;12:756-67
36. Recillas-Targa F. Multiple strategies for gene transfer, expression, knockdown, and chromatin influence in mammalian cell lines and transgenic animals. *Mol Biotechnol* 2006;34(3):337-54
 37. Wallace JA, Felsenfeld G. We gather together: insulators and genome organization. *Curr Opin Genet Dev* 2007;17(5):400-7
 38. Yanez-Munoz RJ, Balaggan KS, MacNeil A, et al. Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med* 2006;12(3):348-53
 39. Kumar-Singh R, Farber DB. Encapsidated adenovirus mini-chromosome-mediated delivery of genes to the retina: application to the rescue of photoreceptor degeneration. *Hum Mol Genet* 1998;7(12):1893-900
 40. Pang J, Cheng M, Stevenson D, et al. Adenoviral-mediated gene transfer to retinal explants during development and degeneration. *Exp Eye Res* 2004;79(2):189-201
 41. Cashman SM, McCullough L, Kumar-Singh R. Improved retinal transduction in vivo and photoreceptor-specific transgene expression using adenovirus vectors with modified penton base. *Mol Ther* 2007;15(9):1640-6
 - **An exciting study that demonstrates the possibility of engineering adenoviruses to target adult photoreceptors effectively.**
 42. Donsante A, Vogler C, Muzyczka N, et al. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Ther* 2001;8(17):1343-6
 43. Donsante A, Miller DG, Li Y, et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 2007;317(5837):477
 44. Goverdhana S, Puntel M, Xiong W, et al. Regulatable gene expression systems for gene therapy applications: progress and future challenges. *Mol Ther* 2005;12(2):189-211
 45. Allocca M, Mussolino C, Garcia-Hoyos M, et al. Novel adeno-associated virus serotypes efficiently transduce murine photoreceptors. *J Virol* 2007;81(20):11372-80
 - **An important study, which presents novel adeno-associated virus serotypes with an improved ability to infect adult photoreceptors in mice.**
 46. Natkunarajah M, Trittbach P, McIntosh J, et al. Assessment of ocular transduction using single-stranded and self-complementary recombinant adeno-associated virus serotype 2/8. *Gene Ther* 2008;15(6):463-7
 47. Peeters L, Sanders NN, Demeester J, De Smedt SC. Challenges in non-viral ocular gene transfer. *Biochem Soc Trans* 2007;35(Pt 1):47-9
 48. Andrieu-Soler C, Bejjani RA, de Bizemont T, et al. Ocular gene therapy: a review of nonviral strategies. *Mol Vis* 2006;12:1334-47
 49. Alberts B, Johnson A, Lewis J, et al. *Molecular Biology of the Cell*. New York: Garland Science; 2002
 50. Arnone MI, Davidson EH. The hardwiring of development: organization and function of genomic regulatory systems. *Development* 1997;124(10):1851-64
 51. Bolouri H, Davidson EH. Modeling DNA sequence-based cis-regulatory gene networks. *Dev Biol* 2002;246(1):2-13
 52. Carroll SB, Grenier JK, Weatherbee SD. From DNA to diversity: molecular genetics and the evolution of animal design. 1st edition. Malden, Mass: Blackwell Science; 2001
 53. Davidson EH. *Genomic regulatory systems: development and evolution*. 1st edition. San Diego: Academic Press; 2001
 54. Ptashne M, Gann A. *Genes & signals*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2002
 55. Levine M, Davidson EH. *Gene regulatory networks for development*. *Proc Natl Acad Sci USA* 2005;102(14):4936-42
 56. Elnitski L, Jin VX, Farnham PJ, Jones SJ. Locating mammalian transcription factor binding sites: a survey of computational and experimental techniques. *Genome Res* 2006;16(12):1455-64
 57. Esumi N, Oshima Y, Li Y, et al. Analysis of the VMD2 promoter and implication of E-box binding factors in its regulation. *J Biol Chem* 2004;279(18):19064-73
 58. Boulanger A, Liu S, Henningsgaard AA, et al. The upstream region of the Rpe65 gene confers retinal pigment epithelium-specific expression in vivo and in vitro and contains critical octamer and E-box binding sites. *J Biol Chem* 2000;275(40):31274-82
 59. Bennett J, Wilson J, Sun D, et al. Adenovirus vector-mediated in vivo gene transfer into adult murine retina. *Invest Ophthalmol Vis Sci* 1994;35(5):2535-42
 60. Li T, Adamian M, Roof DJ, et al. In vivo transfer of a reporter gene to the retina mediated by an adenoviral vector. *Invest Ophthalmol Vis Sci* 1994;35(5):2543-9
 61. Flannery JG, Zolotukhin S, Vaquero MI, et al. Efficient photoreceptor-targeted gene expression in vivo by recombinant adeno-associated virus. *Proc Natl Acad Sci USA* 1997;94(13):6916-21
 62. Glushakova LG, Timmers AM, Issa TM, et al. Does recombinant adeno-associated virus-vectored proximal region of mouse rhodopsin promoter support only rod-type specific expression in vivo? *Mol Vis* 2006;12:298-309
 63. Gouras P, Kjeldbye H, Zack DJ. Reporter gene expression in cones in transgenic mice carrying bovine rhodopsin promoter/lacZ transgenes. *Vis Neurosci* 1994;11(6):1227-31
 64. Woodford BJ, Chen J, Simon MI. Expression of rhodopsin promoter transgene product in both rods and cones. *Exp Eye Res* 1994;58(5):631-5
 65. Nie Z, Chen S, Kumar R, Zack DJ. RER, an evolutionarily conserved sequence upstream of the rhodopsin gene, has enhancer activity. *J Biol Chem* 1996;271(5):2667-75
 66. Zack DJ, Bennett J, Wang Y, et al. Unusual topography of bovine rhodopsin promoter-lacZ fusion gene expression in transgenic mouse retinas. *Neuron* 1991;6(2):187-99
 67. Glushakova LG, Timmers AM, Pang J, et al. Human blue-opsin promoter preferentially targets reporter gene expression to rat s-cone photoreceptors. *Invest Ophthalmol Vis Sci* 2006;47(8):3505-13
 68. Mancuso K, Hendrickson AE, Connor TB Jr, et al. Recombinant adeno-associated virus targets passenger gene expression to cones in primate retina. *J Opt Soc Am A Opt Image Sci Vis* 2007;24(5):1411-6
 - **This study demonstrates the feasibility of targeting an adeno-associated viral transgene specifically to primate cones using a CRE that combines the proximal promoter and locus control region (LCR) enhancer from the human X-linked opsin locus.**

69. Khani SC, Pawlyk BS, Bulgakov OV, et al. AAV-mediated expression targeting of rod and cone photoreceptors with a human rhodopsin kinase promoter. *Invest Ophthalmol Vis Sci* 2007;48(9):3954-61
- **This study presents a compact CRE that drives expression in both rods and cones when transduced by adeno-associated virus.**
70. Olsson JE, Gordon JW, Pawlyk BS, et al. Transgenic mice with a rhodopsin mutation (Pro23His): a mouse model of autosomal dominant retinitis pigmentosa. *Neuron* 1992;9(5):815-30
71. Pennacchio LA, Ahituv N, Moses AM, et al. In vivo enhancer analysis of human conserved non-coding sequences. *Nature* 2006;444(7118):499-502
- **A large-scale analysis of human CREs via creation of transgenic mice carrying CRE-reporter fusion constructs.**
72. Qian J, Esumi N, Chen Y, et al. Identification of regulatory targets of tissue-specific transcription factors: application to retina-specific gene regulation. *Nucleic Acids Res* 2005;33(11):3479-91
- **An important study, which demonstrates the feasibility of identifying photoreceptor CREs around photoreceptor genes by searching for clusters of Crx, Nrl and Nr2e3 binding sites.**
73. Hsiao TH, Diaconu C, Myers CA, et al. The cis-regulatory logic of the mammalian photoreceptor transcriptional network. *PLoS One* 2007;2:e643. Published online 25 July 2007, doi:10.1371/journal.pone.0000643
- **Another study, which uses the presence of phylogenetically conserved Crx, Nrl and Nr2e3 binding sites to predict the location of photoreceptor-specific CREs.**
74. Schroeder MD, Pearce M, Fak J, et al. Transcriptional control in the segmentation gene network of *Drosophila*. *PLoS Biol* 2004;2(9):E271. Published online 31 August 2004, doi:10.1371/journal.pbio.0020271
75. Chen S, Wang QL, Nie Z, et al. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* 1997;19(5):1017-30
76. Furukawa T, Morrow EM, Li T, et al. Retinopathy and attenuated circadian entrainment in Crx-deficient mice. *Nat Genet* 1999;23(4):466-70
77. Mears AJ, Kondo M, Swain PK, et al. Nrl is required for rod photoreceptor development. *Nat Genet* 2001;29(4):447-52
78. Haider NB, Jacobson SG, Cideciyan AV, et al. Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. *Nat Genet* 2000;24(2):127-31
79. Corbo JC, Cepko CL. A hybrid photoreceptor expressing both rod and cone genes in a mouse model of enhanced S-cone syndrome. *PLoS Genet* 2005;1(2):e11. Published online 5 August 2005, doi:10.1371/journal.pgen.0010011
80. Nie Z, Chen, S, Kumar R, Zack D. RER, an evolutionarily conserved sequence upstream of the rhodopsin gene, has enhancer activity. *J Biol Chem* 1996;271(5):2667-75
81. Folliot S, Briot D, Conrath H, et al. Sustained tetracycline-regulated transgene expression in vivo in rat retinal ganglion cells using a single type 2 adeno-associated viral vector. *J Gene Med* 2003;5(6):493-501
82. Stieger K, Le Meur G, Lasne F, et al. Long-term doxycycline-regulated transgene expression in the retina of nonhuman primates following subretinal injection of recombinant AAV vectors. *Mol Ther* 2006;13(5):967-75
83. Portera-Cailliau C, Sung CH, Nathans J, Adler R. Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. *Proc Natl Acad Sci USA* 1994;91(3):974-8
84. Reme CE, Grimm C, Hafezi F, et al. Apoptotic cell death in retinal degenerations. *Prog Retin Eye Res* 1998;17(4):443-64
85. LaVail MM, Yasumura D, Matthes MT, et al. Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest Ophthalmol Vis Sci* 1998;39(3):592-602
86. Tao W, Wen R, Goddard MB, et al. Encapsulated cell-based delivery of CNTF reduces photoreceptor degeneration in animal models of retinitis pigmentosa. *Invest Ophthalmol Vis Sci* 2002;43(10):3292-8
87. Liang FQ, Dejneka NS, Cohen DR, et al. AAV-mediated delivery of ciliary neurotrophic factor prolongs photoreceptor survival in the rhodopsin knockout mouse. *Mol Ther* 2001;3(2):241-8
88. McGill TJ, Prusky GT, Douglas RM, et al. Intraocular CNTF reduces vision in normal rats in a dose-dependent manner. *Invest Ophthalmol Vis Sci* 2007;48(12):5756-66
89. Bok D. Ciliary neurotrophic factor therapy for inherited retinal diseases: pros and cons. *Retina* 2005;25(8 Suppl):S27-8
90. Arden GB, Sidman RL, Arap W, Schlingemann RO. Spare the rod and spoil the eye. *Br J Ophthalmol* 2005;89(6):764-9
91. Zhang SX, Ma JX. Ocular neovascularization: Implication of endogenous angiogenic inhibitors and potential therapy. *Prog Retin Eye Res* 2007;26(1):1-37
92. Bainbridge JW, Mistry A, Binley K, et al. Hypoxia-regulated transgene expression in experimental retinal and choroidal neovascularization. *Gene Ther* 2003;10(12):1049-54
93. Su H, Joho S, Huang Y, et al. Adeno-associated viral vector delivers cardiac-specific and hypoxia-inducible VEGF expression in ischemic mouse hearts. *Proc Natl Acad Sci USA* 2004;101(46):16280-5
- **An interesting study, which demonstrates that it is possible to engineer a 'hybrid' CRE which is both cardiac-specific and hypoxia-inducible.**
94. Pennesi ME, Cho JH, Yang Z, et al. BETA2/NeuroD1 null mice: a new model for transcription factor-dependent photoreceptor degeneration. *J Neurosci* 2003;23(2):453-61
95. Roberts MR, Hendrickson A, McGuire CR, Reh TA. Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. *Invest Ophthalmol Vis Sci* 2005;46(8):2897-904
96. Ng L, Hurley JB, Dierks B, et al. A thyroid hormone receptor that is required for the development of green cone photoreceptors. *Nat Genet* 2001;27(1):94-8

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