

CURRENT RESEARCH

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Gene Therapy for Genetic and Acquired Retinal Diseases

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Abstract. We present an overview of the current status of basic science and translational research being applied to gene therapy for eye disease, focusing on diseases of the retina. We discuss the viral and nonviral methods being used to transfer genes to the retina and retinal pigment epithelium, and the advantages and disadvantages of each approach. We review the various genetic and somatic treatment strategies that are being used for genetically determined and acquired diseases of the retina, including gene replacement, gene silencing by ribozymes and antisense oligonucleotides, suicide gene therapy, antiapoptosis, and growth factor therapies. The rationales for the specific therapeutic approaches to each disease are discussed. Schematics of gene transfer methods and therapeutic approaches are presented together with a glossary of gene transfer terminology. (*Surv Ophthalmol* 47:449–469, 2002. © 2002 by Elsevier Science Inc. All rights reserved.)

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Over the past decade there has been an explosion of research that uses gene transfer techniques to probe the molecular mechanisms directing retinal differentiation and development and applies gene therapy to the treatment of ocular diseases. The delivery of foreign genes to ocular tissues to modify the genotype and phenotype of cells is enabling novel approaches to the understanding and treatment of many eye diseases.

We review current gene transfer methods and the strategies with which these are being applied to gene therapy of eye disease, with particular emphasis on diseases of the retina. Because of the availability of genetically well-defined and -characterized animal models and the ease of gene delivery to the retina

and vitreous, most research in ocular gene therapy has been directed at the treatment of retinal degenerative diseases. The literature demonstrates well the varied approaches to gene delivery in the eye and the strategies being employed to correct the genetically disparate molecular defects that lead to retinal degeneration and to modify abnormal cellular phenotypes to ameliorate disease progression.

The ideal gene transfer method would *transfect* genes to a specific cell type with high efficiency; deliver a replacement gene with a regulatory sequence to the nucleus, where it would become integrated into the host genome in a non-mutagenic fashion and be expressed or regulated; *transduce* cells efficiently, independent of

the mitotic potential of the recipient; be non-infectious, non-toxic, and non-immunogenic; and be easy to manufacture and apply clinically. Each of the gene transfer methods presently in use shares some, but not all, of these ideal properties. Each method has been used successfully to transduce foreign genes into retinal cells, and each has specific advantages and disadvantages for certain recipient cell types.

Mechanisms of Gene Transfer

ADENOVIRUS VECTORS

Adenoviruses are non-enveloped, double-stranded DNA viruses that infect a broad range of human and non-human cell types by binding to specific cell-surface receptors. Receptor-mediated endocytosis internalizes the viral DNA, which then escapes the endosome and is trafficked to the nucleus, where it binds to the nuclear envelope and enters the nucleus. The virus is not incorporated into the host genome, but remains as a transcriptionally active episome within

the nucleus (Fig. 1). Because it is not incorporated into the host cell genome, the episome is eventually degraded or lost, which accounts for the transient nature of adenoviral vector gene expression.

Concerns about the infectious nature of adenoviral vectors has led to the development of replication-defective, also called helper-dependent, adenoviruses for gene transfer. Deletion of replication specific genes and non-essential DNA sequences from these viral vectors prevents viral replication. The crippled virus acts as a vector to shuttle up to 8 kilobases (kb) of the foreign DNA of interest to the nucleus of the recipient cell where it is transiently expressed. These viral vectors are easily grown to high-infectivity titers (using helper viruses) and have a high transfection efficiency and broad range of transducible target cells in the eye.^{25,44,102} Adenoviral vectors can infect both dividing and non-dividing cells, which makes them particularly useful to transfer genes to post-mitotic cells. Adenoviruses have been effective in transducing retinal photorecep-

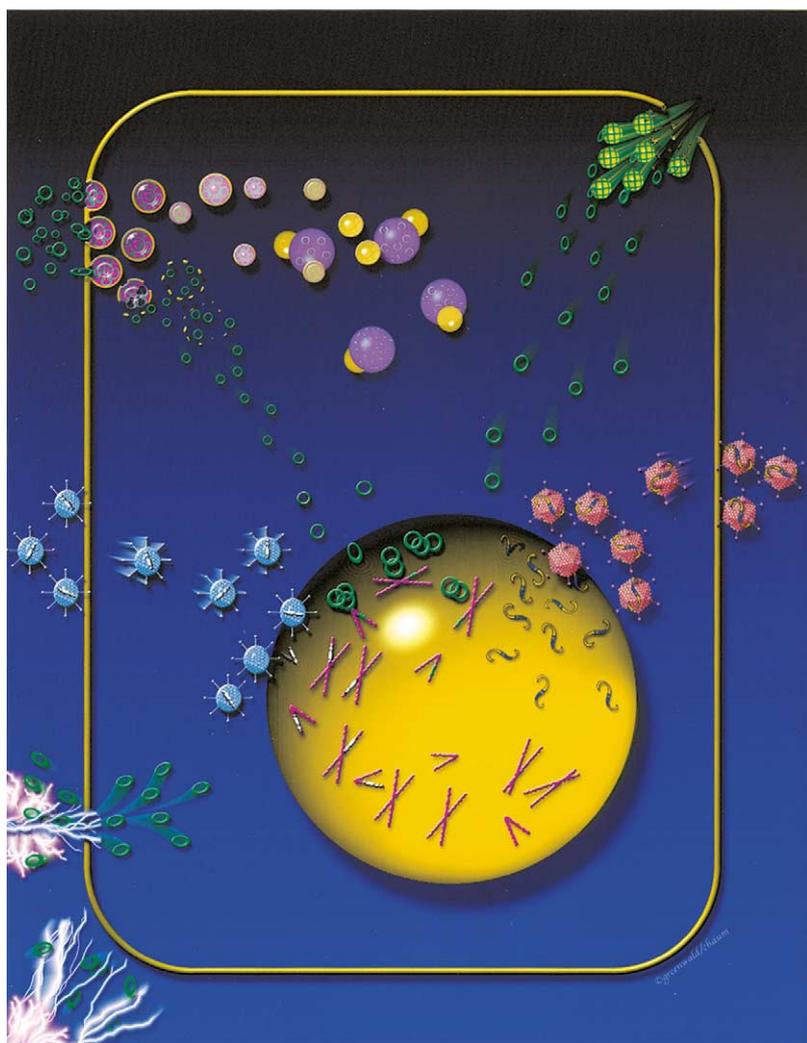


Fig. 1. Gene transfer methods. Clockwise from upper left. Lipid-mediated gene transfer: Plasmid vectors are taken up by endocytosis and are released into the cytoplasm by breakdown of the endosome. Plasmids migrate to the nucleus by passive diffusion through the cell. Many endosomes fuse with lysosomes where plasmid DNA is broken down. Plasmids that reach the nucleus may remain episomal or integrate into the host genome. *Mechanical delivery:* "Gene guns" deliver vector-coated particles directly through the cell wall into the cytoplasm. *Adenoviral gene transfer:* Adenoviruses are taken up by a receptor-mediated mechanism and deliver viral DNA to the nucleus. The adenoviral DNA and any transgene contained in the viral vector remain as episomes within the nucleus. *Electroporation:* Vectors enter the cell through membrane pores opened in response to an electrical charge delivered to the cell. *Retroviral gene transfer:* Retroviruses are taken up by a receptor-mediated mechanism. The virus is delivered to the nucleus, where the viral DNA, and any transgene contained within it integrates into the host genome.

tors,^{13,24,75,133,145} ganglion cells,^{25,43,133} Müller cells,^{75,102} and retinal pigment epithelial cells.^{13,25,55,56,102,133}

Adenoviral vectors have some inherent limitations with respect to potential clinical application. Peak marker transgene expression occurs 2 days to 2 weeks after gene transfer^{25,102} and is typically limited to a few weeks because there is no integration into the host DNA; however, expression for as long as 13 weeks has been reported.²⁵ The broad range of target cells is a potential disadvantage if only one cell layer or cell type is targeted for transduction. Adeno-viral transduction can also cause tissue inflammation and a significant immune response to viral proteins in the host, limiting the duration of gene expression and exposing transduced cells to cytotoxic responses.^{32,174,210}

Deletion of many of the native viral genes has reduced but not eliminated the immunogenicity of adenoviral vectors. In addition, immune memory from repeated applications of the vector may produce neutralizing antibodies to both viral proteins and the transgene protein thus decreasing the effectiveness of repeated gene transfers. The T-cell-mediated immune response of the host has been shown to play a role in limiting the duration of adenovirus-mediated transgene expression in the eye,^{94,174,210} although studies suggest that the subretinal space has some immune privilege.^{2,23,94,200,230} Modified adenoviral vectors may improve the duration of gene expression by reducing the number of viral proteins presented to the host immune system. The longer duration of transgene expression in immune-deficient mice suggests that host immune suppression may be a useful adjunct to gene therapy to prolong the expression of adenoviral vectors, although this has not yet been demonstrated in retinal transfections.⁹⁴

Replication-deficient adenoviral vectors can theoretically regain the ability to replicate by recombining with wild-type adenoviruses within the host cell, although large deletion replication-deficient viruses have decreased this risk. Finally, the vector itself may have potentially toxic effects on the retina. Intravitreal or subretinal injection of an adenoviral vector containing a marker gene can affect retinal function, measured by a reduction in the electroretinogram (ERG).¹⁸³ The risk of damaging retinal function by gene transfer must be addressed in the treatment of diseases of the retina in which there may be little tolerance for additional injury.

Another form of adenoviral gene transfer uses encapsidated adenoviral minichromosomes (EAMs),¹¹⁵ which are deletion mutants of the adenovirus in which all of the viral genes have been removed. EAMs have large DNA shuttling capacity (up to 30 kb), are less immunogenic than replication-deficient adenoviruses and they also demonstrate transgene expression in transfected cells for up to 18 weeks.¹¹⁵

Because EAMs lack all viral genes, they must be packaged in the virion coat using a replication-competent helper adenovirus. The difficulty in completely removing the wild-type helper adenovirus from EAM preparations may, however, limit the clinical application of this vector.

Despite some limitations, the adenoviral DNA vectors have been shown to be effective. They are easy to apply experimentally *in vitro* and *in vivo*, and they may be particularly useful for gene therapy in which transgene expression of limited duration is sufficient to achieve the desired clinical effect.

ADENO-ASSOCIATED VIRUS VECTORS

Adeno-associated viruses (AAVs) are non-enveloped, single-stranded DNA parvoviruses that, like adenoviruses, can efficiently infect dividing and non-dividing cells. In contrast to adenoviruses, AAVs insert into the host genome at a specific locus, thus increasing the likelihood of stable transgene expression.¹⁸⁴ Site-specific AAV integration decreases the theoretical risk of random insertional mutagenesis. Recombinant adeno-associated viruses (rAAVs) have been developed that integrate randomly in the host genome and have been shown to effect stable transduction of the retina for more than 1 year.^{22,84,89} These rAAV vectors do introduce the additional risk of insertional mutagenesis from random integration. AAVs are not associated with any known human infectious disease; thus, there is minimal theoretical risk of human disease from recombination with a pathogenic wild-type virus in the host cell.

AAVs, like adenoviruses, have been shown to transduce rod photoreceptors,^{7,22,71,81,103} ganglion cells,^{81,84} and RPE.^{7,81,121,167} However, AAV vectors have a more limited cloning capacity (5 kb vs. 8 kb maximum insert size) and so, may not be able to transduce larger genes of interest.⁴⁰ There is a strong correlation between the viral titer and the efficiency of gene transfer using viral vectors; thus, the lower titers seen with rAAVs compared to adenoviruses may reduce transfer frequencies. While transgene expression is rapid following transfection with adenoviruses, gene expression in the retina may be delayed several weeks following *in vivo* rAAV gene transfer.^{19,22,71} Delayed transgene expression may complicate the analysis of the efficacy of AAV gene transfer in clinical trials.

AAVs cause less tissue inflammation than do adenoviruses. While the host does generate an immune response against AAVs, repeated injection of an rAAV vector into the subretinal space does not appear to interfere with sustained gene transfer.¹¹

HERPES SIMPLEX VIRUS VECTORS

The herpes simplex virus (HSV) is a DNA virus that has a natural tropism for neurons; thus, it may

be potentially useful in applying gene therapy to diseases of the nervous system, including the retina. HSV can infect non-neuronal tissue as well and does not require cell division to integrate into the host genome.

Gene transfer using HSV utilizes either replication-defective mutants or multiple deletion mutants, termed amplicons, which have had most of the HSV genes removed. Amplicons are produced similarly to EAMs, using helper viruses, and they have similar problems with contamination by the replication-competent helper virus. Replication-competent, but attenuated, HSV vectors have been proposed for use in ocular gene transfer.^{138,197} These mutants have a limited capacity for replication, but they may be able to deliver genes locally within tissue from a limited infection. The ability of HSV to exist in a latent state likely makes this vector efficacious in producing stable transgene expression within host neurons. HSV vectors with a ribonuclease reductase gene deletion cannot reactivate from a latent state and may have applications in transgene delivery to the retina with a reduced risk of infection.³⁴

The HSV viral genome is large and has the capacity to carry more than 30 kb of foreign DNA, significantly more than other viral vectors.³⁵ This packaging capacity is advantageous for the delivery of large genes and genes with upstream regulatory elements and with promoters to enhance transgene expression. HSV infection is frequently, although not invariably, associated with significant tissue inflammation¹⁹⁷ and cytotoxicity, which may severely limit its clinical application. This problem with viral-induced inflammation may be mitigated with new deletion mutant vectors that reduce immunogenicity or by adjunct suppression of the host immune response following gene transfer.

The HSV thymidine kinase gene (*HSV-tk*) has been used extensively in suicide gene therapy studies. The viral thymidine kinase has a markedly higher affinity for the prodrug ganciclovir than does the cellular thymidine kinase.⁶⁵ Ganciclovir is converted to a cytotoxic metabolite with significantly higher efficiency by *HSV-tk*-transduced cells and thus, preferentially kills the transduced cell. The *HSV-tk* gene can be transfected using other viruses, for example, in an adenoviral vector for transient expression, in retinoblastoma tumors,^{97,98} or a retroviral vector that selectively targets dividing cells for gene delivery.^{111,181}

RNA VIRUS VECTORS

Retroviruses are a family of RNA viruses that can infect cells and integrate into the genome of the host cell (Fig. 1). Transgenes delivered by retroviruses demonstrate long-term, stable gene expres-

sion and may be vertically transferred to daughter cells. Most retroviral vectors cannot enter the nucleus directly; they require cell division to infect the host cell following breakdown of the nuclear envelope. This requirement precludes the use of standard retroviral vectors to transduce terminally differentiated cells, such as photoreceptors or tissues in which cell division occurs at a minimal rate. Retroviral vectors can be packaged with up to 8 kb of foreign DNA and, so, have the same DNA-shuttling capacity as adenoviruses. Retroviruses insert into host chromosomes at random locations; thus, there is a risk of insertional mutagenesis from these vectors. Finally, despite the potential for sustained transgene expression, there is often host modification of the transgene, such as promoter inactivation, which may affect gene expression depending on where integration into the host genome occurs.

Lentiviruses are a subclass of retroviruses that includes the human immunodeficiency virus (HIV) family. Like all retroviruses, lentiviruses integrate a DNA copy into the host genome. However, unlike murine retroviruses, lentiviruses encode proteins that allow them to form a complex with the nuclear envelope and to transit the pores in an intact nuclear membrane. Thus, lentiviruses can infect dividing and non-dividing cells.^{160,161} Modifications of this virus have resulted in the development of efficient gene transfer vectors that permit stable transgene expression.¹⁶¹ Transgenes integrate into the host genome but viral genes do not, thus, there is less risk of generating recombinant retroviruses.

Lentivirus vectors are constructed with the protein coat of the vesicular stomatitis virus (pseudotyped) to circumvent the T-cell lymphocyte specificity of the native virus. These pseudotyped constructs have been used to infect a broad range of retinal cells including retinal photoreceptors^{153,206} and RPE.^{76,117,153} Transgene expression has been demonstrated for up to 6 months following *in vivo* lentiviral gene transfer in animal models.^{110,117,161,167}

Despite modifications to render the virus replication-incompetent, there is appropriate concern about its clinical use, because of the theoretical possibility of generating wild-type HIV virus during production or from viral recombination in the host. New viral constructs are being developed to try to address this issue and minimize or eliminate the risk.²³¹

PHYSICOCHEMICAL GENE TRANSFER

Lipofection

Lipofection reagents are families of molecules composed of phospholipids that contain both hydropho-

bic and hydrophilic domains. These reagents form complexes with DNA in a purely physicochemical manner by electrostatic interactions between the positively charged (cationic) lipid and the negatively charged DNA.⁷⁰ The DNA condenses with the lipofection reagent in a non-enzymatic fashion to form lipid/DNA complexes⁷⁸ that can be used to deliver foreign DNA to cells *in vitro* and *in vivo*.^{15,39,46,48,49,69,114,146} Lipophilic polyamines have also been used to transfect DNA and to enhance the efficacy of cationic phospholipid gene transfer.^{1,16} Virus-coated liposomes have also been used to transfer genes to the anterior segment, retina, and choroidal neovascular tissue.^{86,87,168}

The lipid/DNA complexes fuse with the plasma membrane and are internalized by endocytosis (Fig. 1).²²² After endocytosis, much of the DNA is degraded by fusion of the endosome with lysosomes.⁷⁴ However, some of the DNA is released into the cytoplasm from the endosomes and makes its way to the nucleus in what is probably a concentration-dependent manner.^{74,227} Active transport of DNA to the nucleus following lipofection has not been demonstrated.

There are several theoretical advantages to lipofection-mediated gene therapy that uses plasmid vectors. Plasmids can carry larger DNA sequences than viral vectors, and so can be constructed containing promoters and regulatory elements controlling the gene of interest and thereby permit regulation of transgene expression in the host. Plasmids can also be manufactured in high purity by large-scale fermentation without the risk of generating infectious or recombinant viruses. Plasmid vectors are easy to use and construct, and they cause minimal toxicity *in vitro*.^{39,48,159,198}

Disadvantages of lipofection include poor target selectivity, reduced efficiency compared with viral vectors, and short duration of expression. Transient transgene expression results from the episomal status of the plasmid vector after it reaches the nucleus, with low frequency of integration into the host genome. However, a small percentage of transfected retinal cells do show transduction with sustained transgene expression *in vitro*. Thus, unselected integration into the host cell genome does occur, albeit at a low frequency.⁴⁸ The efficiency of gene transfer by lipofection may ultimately be improved by targeting specific cell-surface receptors, inhibiting lysosome digestion, and directing transport to the nucleus via nuclear-localizing sequences.

Other Physicochemical Gene Transfer Methods

Other methods of delivering plasmid vectors to the eye include microinjection, and microprojectiles ("gene gun") (Fig. 1).²⁰⁷ Calcium/phosphate precip-

itates of plasmid DNA have been used to deliver genes *in vitro* for almost 3 decades.⁸⁰ This technique is effective but is unlikely to have clinical applications due to cytotoxicity and the low efficiency of gene transfer relative to that of newer commercially available reagents and viral vectors.

DNA can also be transfected into cells by electroporation, generating an electric potential across the target cell membrane (Fig. 1). Such approaches are effective *in vitro* but are limited, in part, by the ability to generate the electrical field *in vivo*. However, this approach may be effective for gene delivery to surface tissues such as cornea and conjunctiva.^{166,182} Iontophoretic delivery of genes or antisense oligonucleotides across the sclera may have practical applications in treating diseases of the retina and choroid.⁵⁹ Bioimplants containing genetically modified cells have been shown to deliver growth factors in the eye and to have a therapeutic effect on retinal degeneration.^{17,208,214} *Ex vivo* gene therapy with bioimplantation may have broad applications, not only for the delivery of growth factors, but also viral vectors, antisense DNA, and suicide gene therapy. This approach may help to circumvent the transient effects of some gene therapies by providing a reservoir of vectors within the eye that are delivered over an extended period.

Therapeutic Approaches to Retinal Diseases

Most research in ocular gene therapy is being applied to the retinitis pigmentosa family of diseases because the phenotypes of these conditions are well characterized in animal models and many of the genes have been cloned. However, significant research effort is also being directed toward other genetic and acquired retinal diseases, including retinal pigment epithelial (RPE) and metabolic dystrophies, retinoblastoma, proliferative vitreoretinopathy, age-related macular degeneration, and choroidal neovascularization. In addition, gene therapy is being applied to cells *ex vivo* for pharmacotherapeutic application in ocular disease. In developing strategies to treat retinal diseases using gene therapy, it is instructive to consider the genetic and somatic events leading to these conditions. At the beginning of each section, the rationale for each strategy will be presented in a brief review of the pathophysiology and molecular events leading to retinal degeneration.

Retinitis pigmentosa (RP) comprises a genetically and clinically heterogeneous group of retinal degenerations caused by mutations in genes of the phototransduction cascade,^{169,171} genes encoding structural proteins integral to photoreceptor outer segment integrity,¹⁷¹ and genes involved in systemic

metabolism.¹⁹⁴ RP occurs in autosomal dominant (adRP), autosomal recessive (arRP) and X-linked recessive (XLRP) forms. There are more than 150 known rhodopsin and peripherin mutations that give rise to adRP.¹⁷¹ There are several animal models for RP and RP-like diseases; the best characterized of these are mutations in the mouse. The retinal degeneration (*rd*) autosomal recessive mutation in the cyclic GMP phosphodiesterase (*BPDE*) gene leads to rapid retinal degeneration by postnatal week 8 as a result of the accumulation of toxic levels of intracellular cyclic GMP.¹³⁹ The retinal degeneration slow (*rdS*) autosomal recessive mutation in the peripherin gene causes a growth arrest of rod outer segments and progressive retinal degeneration over 1 year.¹⁸⁶ Mutant knockout mice have been generated to create new models of hemizygous and homozygous recessive rhodopsin mutations.⁹⁶

In the Royal College of Surgeons (RCS) rat model, the RPE is unable to phagocytize photoreceptor outer segment membranes. A mutation in the receptor tyrosine kinase gene (*rdy/Mertk*) results in accumulation of toxic debris in the subretinal space and induces apoptotic retinal degeneration.^{4,124} No specific human analog of RCS-like retinal degeneration is known. Biological models of retinal degeneration are also found in larger animals, including cats (*Rdy*),¹²⁹ and dogs (*rd I*),⁵² and have been developed in transgenic pigs.^{135,192} An animal model of a Leber's congenital amaurosis caused by a mutation in the *RPE65* gene is found in the Briard dog.⁵

The identification of mutations in the phototransduction genes that cause retinal degeneration in animal models and the cloning of the homologous human genes have contributed greatly to the development of the genetic vectors used to treat these diseases. These animal models have permitted testing of the efficacy of gene transfer for the treatment of genetically determined and acquired retinal degenerations and they have established the proof of principle for gene therapy in these diseases.

DOMINANT RETINAL DEGENERATIONS

Autosomal dominant retinal degenerations in animal models and the known autosomal dominant human forms of these diseases (adRP) are characterized by the production of normal and abnormal outer segment proteins in photoreceptor cells. The abnormal, mutant form of the protein is in some way toxic to the retina and causes progressive retinal degeneration with the final common pathway being apoptotic cell death.^{140,212} Gene therapy for adRP has focused on the use of ribozymes to inhibit the translation of the mutant protein by directed enzymatic cleavage of the mutant mRNA transcript.^{89,90,91,127,131,192}

Antisense oligonucleotides can also inhibit protein translation from the transcript.¹³¹ Gene-replacement therapy has also been shown to rescue photoreceptors in a mouse transgenic model of adRP induced by a rhodopsin mutation.¹⁵¹ Growth factor and anti-apoptotic therapies have also been shown to be somewhat effective in slowing the disease process in dominant retinal degenerations.

Ribozyme Therapy

Ribozymes are RNA enzyme molecules that can cleave specific messenger RNA (mRNA) sequences.^{88,201} Ribozymes contain variable sequences that determine the specificity of the ribozyme for its target molecule and highly conserved sequences that direct catalytic hydrolysis of the mRNA at specific trinucleotide motifs.²⁹ Mutation-specific cleavage of the transcript functionally "silences" the mutant allele by preventing synthesis of the abnormal protein from the transcript (Fig. 2). The strategy used is to construct ribozymes that identify unique mutations or that permit binding to targeted, accessible sites in the mRNA transcript. Ribozymes are synthesized *in situ* from vectors, and each molecule can catalyze the hydrolysis of many transcripts within the cell. Recently, a mutation-independent approach to ribozyme therapy has been used in which both mutant and wild type transcripts are cleaved but a modified wild-type transcript encoding the normal protein is introduced. These modified transcripts are not cleaved by the ribozyme and, thus, permit translation of the wild-type protein in the cell.¹⁶³ Mutation-independent ribozymes have been synthesized for rhodopsin and peripherin transcripts and will likely have a broad application in gene therapy for the large number of heterogeneous mutations seen in adRP.

The catalytic activity of ribozymes engineered to target and cleave specific opsin mRNA mutations seen in adRP has been demonstrated in animal models *in vitro*.^{63,192} Ribozyme therapy is effective in delaying retinal degeneration in animal models of dominant retinal degeneration (Fig. 3). Adeno-associated virus-mediated (AAV-mediated) transfer of a ribozyme targeting a rat rhodopsin transcript with a proline to histidine mutation (P23H) was shown to delay the onset of photoreceptor degeneration for 3 months.¹³¹ Additional studies to assess the longevity of ribozyme therapy in the model demonstrated preservation of photoreceptors and ERG signal even after 8 months.¹²⁷ Ribozyme therapy was also able to delay retinal degeneration even after the degenerative process had begun.

Ribozymes can also be directed against wild-type alleles.^{92,163} Ribozymes directed against the *BPDE*

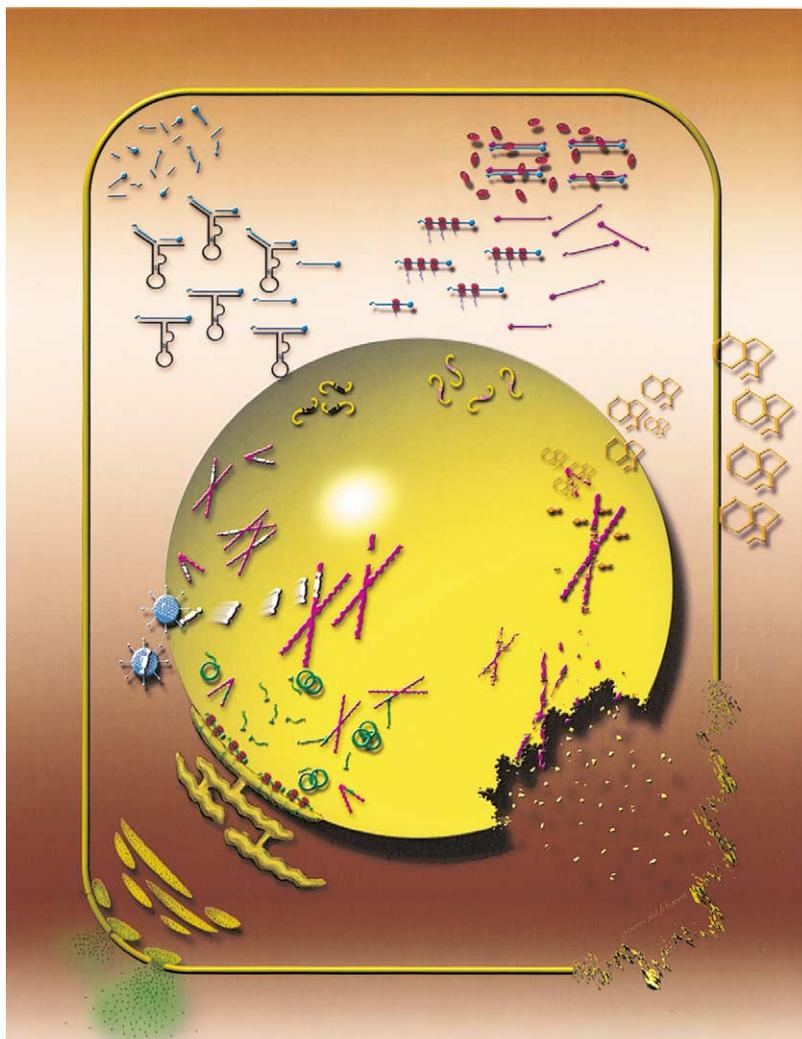


Fig. 2. Therapeutic approaches to gene therapy. Clockwise from upper left: *Ribozyme therapy*: Ribozymes hybridize with mRNA from a mutated gene. The ribozyme enzymatically cleaves the mRNA and functionally silences the gene by preventing synthesis of the abnormal protein from the transcript. Upper right: *Antisense therapy*: Complementary DNA molecules hybridize with the mRNA from the mutated gene. The ribosomes cannot bind to the double stranded heteroduplex, preventing synthesis of the abnormal protein. Lower right: *Suicide gene therapy*: Gancyclovir is converted to a cytotoxic nucleotide by a transfected viral thymidine kinase. The drug inhibits DNA synthesis, leading to cell death. Dying cells are also cytotoxic to nearby untransduced cells, causing the "bystander effect". Lower left: *Growth factor therapy*: Growth factors are synthesized and secreted from cells expressing growth factor transgenes. The growth factors have positive neurotrophic effects on surrounding retinal cells and also on the secreting cells via specific receptors. Middle left: *Gene replacement therapy*: Mutated genes causing autosomal recessive retinal degenerations can be replaced using viral or non-viral methods.

transcript in rd^-/rd^+ heterozygous mice induce significant retinal degeneration and functional visual impairment.⁹² Thus, ribozymes can also be used to silence wild-type genes to generate *in vivo* models of retinal degeneration.

Growth Factor Gene Therapy

Gene therapy using trophic growth factors is another approach to enhancing the survival of retinal photoreceptors in dominant retinal degeneration. Upregulation of basic fibroblast growth factor (bFGF) expression in response to laser photocoagulation of the retina has been demonstrated *in vivo*,²²³ and intravitreal injection of bFGF delays retinal degeneration in the RCS rat,^{66,170} as well as in models of light- and ischemia-induced retinal injury.^{67,125,126,213} Adenoviral-mediated transfer of the bFGF gene to the retina via subretinal injection delayed retinal degeneration in the RCS rat.⁶ Similar results have been observed in the transgenic rat model (Fig. 4).¹²³ Growth factor gene therapy has resulted in improved photoreceptor

survival both histologically and by functional ERG testing.⁸

Successful treatment with growth factors may also be achieved by genetically modifying cells *ex vivo* and then implanting them within the eye to act as a reservoir of trophic factors that bathe the retina by slow release into the vitreous. Intravitreal transfer of fibroblasts, expressing the bFGF gene, encapsulated in a biocompatible polymer, delayed retinal degeneration up to 3 months after transfer in the RCS rat.²¹⁴

Antiapoptosis Gene Therapy

Apoptosis appears to be the final common pathway of cell death in retinal degeneration.^{140,212} A somatic, mutation-independent approach to prolong retinal survival by modulating the genes that commit the photoreceptor cell to apoptotic cell death may show efficacy in adRP and arRP models (Fig. 2). Several pro- and antiapoptotic genes have been identified.^{10,142} Transfer of the antiapoptotic gene *bcl-2* to the retina delays retinal degeneration in rd^-/rd^- ,^{26,50,104} rds^-/rds^- ,¹⁶² and $Pdeg^{tm1}/Pdeg^{tm1}$ mice.²¹¹

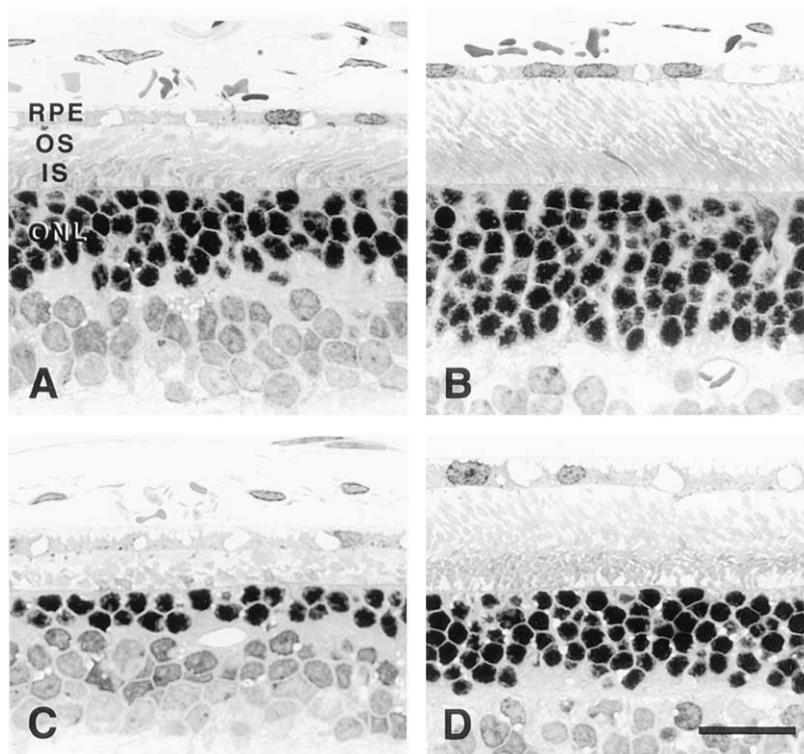


Fig. 3. Light micrographs of P23H transgenic rat retinas taken at post-natal day P130 (A and B) or 240 (C and D). A: Uninjected eye from a rat at P130, which shows 3–4 rows of photoreceptor nuclei in the outer nuclear layer, reduced from the normal 8–9 at this age. Photoreceptor inner segments and outer segments are shorter than normal. B: Retina from the opposite eye from the same rat as in A, which was injected subretinally with Hh13 ribozyme at day P15. About 6–7 rows of photoreceptor nuclei are present, and the photoreceptor inner and outer segments are about 70–80% of normal length. C: Uninjected eye from a rat at P240, which shows only 1–2 rows of photoreceptor nuclei surviving, and only remnants of inner and outer segments. D: Retina from the opposite eye of the same rat in C, which was injected with Hh13 ribozyme at P15. About 4–5 rows of photoreceptor nuclei are still present and the inner and outer segments are almost as long as at P130 (B). (Bar = 20 μm .) (Reproduced with permission of the Proceedings of the National Academy of Sciences, USA from LaVail et al.¹²⁷ Copyright 2000, National Academy of Sciences, USA.)

Interestingly, *bcl-2* transfer to retinal ganglion cells increased their susceptibility to glutamate-induced apoptosis rather than preventing it.¹⁹⁵ The protective effects of *bcl-2* transfer to the retina may be tissue specific or may be mediated by secondary pathways that differ between retinal cell types.

The proapoptotic protein caspase-3 is upregulated in retinal degeneration in the transgenic rat¹³⁷ and may be specifically inhibited by transgene expression of an X-linked inhibitor of apoptosis.²²⁴ More research is needed in this important area of investigation to elucidate the efficacy of antiapoptosis therapy for retinal degeneration.

Antisense Gene Therapy

Antisense gene therapy is based upon the use of synthetic, short DNA sequences (oligodeoxynucleotides, ODN) that are designed to be complementary to a targeted mRNA molecule. The ODN is capable of forming a stable DNA-RNA heteroduplex with the mRNA and, thus, prevent translation of the

protein from the transcript (Fig. 2). The mRNA of the heteroduplex is enzymatically destroyed by RNase H, but the ODN is not, so that the ODN is free to bind to other identical mRNA transcripts, and function catalytically, in a manner similar to ribozymes, to suppress translation of a specific gene transcript.³⁶ The above results from the P23H ribozyme studies by Lewin et al. showed that catalytically inactive ribozymes also slowed the progression of retinal degeneration, and they demonstrated a protective effect of gene silencing by an antisense-type molecule.¹³¹

Despite the efficacy of ribozymes to eliminate mutant transcripts and growth factors to provide trophic support to photoreceptors or to modulate apoptosis, their use delays retinal degeneration in animal models of adRP, but it does not prevent degeneration. Effective therapy in human disease will likely require repeat treatments, sustained delivery devices, or transduction by integrating vectors to achieve sustained transgene expression.

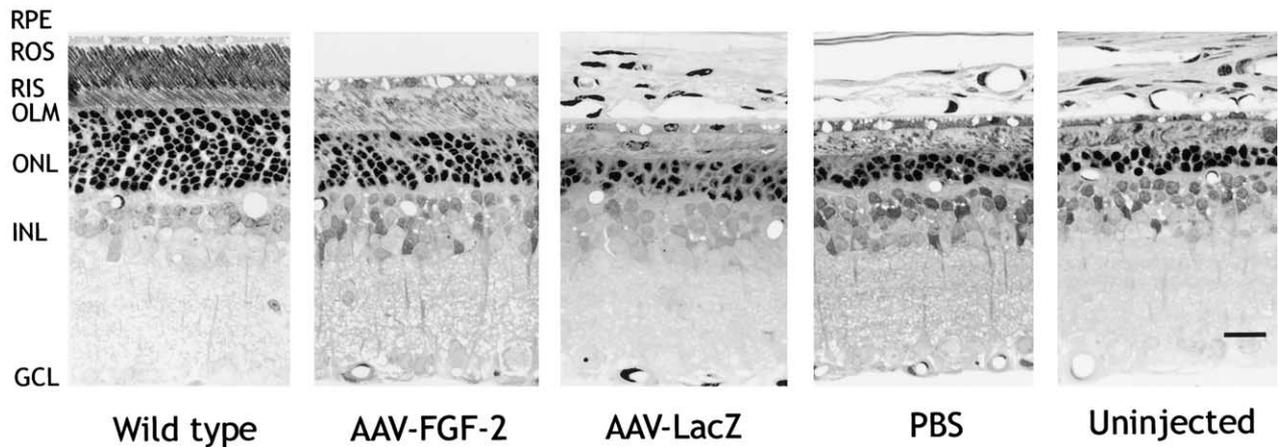


Fig. 4. Morphologic rescue of degenerated S334ter-4 rat retinas at postnatal day P60 using an adenovirus vector encoding bFGF (AAV-FGF-2). In the region of uninjected S334ter-4 retinas, the photoreceptors degenerated from 9–10 cells thick, as seen in wild-type retinas, to 2–3 cells thick. Retinas injected with AAV-FGF-2 had an outer nuclear layer that was significantly thicker than uninjected, β -galactosidase-injected (AAV-LacZ), or saline-injected (PBS) retinas. ROS = rod outer segments; RIS = rod inner segments; OLM = outer limiting membrane; GCL = ganglion cell layer. Scale bar = 20 μ m. (Reproduced with permission of *Investigative Ophthalmology and Visual Science* from Lau et al.¹²³ Copyright 2000, Association for Research in Vision and Ophthalmology.)

RECESSIVE RETINAL DEGENERATIONS

Recessive degenerations (arRP and XLRP) are characterized by the inability to produce a functional gene product. The absence of the normal gene product, such as a structural protein of the outer segment (*rom1*) or an enzyme of the phototransduction cascade (β PDE), to cite two examples, results in the expression of the mutant phenotype. In arRP, there are germline mutations at both alleles as is seen in *rd⁻/rd⁻* and *rds⁻/rds⁻* mice. In arRP, all of the photoreceptor cells harbor the mutation and undergo degeneration. The principle of gene therapy in recessive degenerations is to cure the disease by replacing the missing gene with a wild-type copy in each photoreceptor cell (Fig. 2). This principle was first demonstrated in the seminal studies by Lem et al.¹²⁸ and Travis et al.²⁰⁹ in which retinal degeneration in *rd⁻/rd⁻* and *rds⁻/rds⁻* mice were rescued by transgenic expression of a bovine cGMP phosphodiesterase β -subunit, and a wild-type (peripherin)*rds* gene. The goal of photoreceptor rescue requires efficient transduction of most of the photoreceptor cells because in theory, any cell expressing a wild-type gene will survive. However, the genetic and somatic processes causing retinal degeneration may require more complex intervention than just simple gene replacement.¹⁰⁹ Somatic therapies aimed at enhancing survival using growth factors and antiapoptosis genes have also been shown to improve photoreceptor cell survival in recessive degenerations, but are not curative.

Gene-Replacement Therapy

The *rd⁻/rd⁻* mouse develops progressive retinal degeneration shortly after birth from a mutation in the

gene encoding the β -subunit of cyclic GMP phosphodiesterase, β -PDE.¹²⁸ A mutation in the human homologue of this gene has also been identified in a subset of patients with retinitis pigmentosa.¹⁵⁰ Several gene delivery techniques have been employed to modify the phenotype of the *rd⁻/rd⁻* mouse by transfecting a wild-type copy of the β -PDE gene to the retina.^{24,103,115,206} Adenovirus-mediated transfer of β -PDE to the retina of the *rd⁻/rd⁻* mouse resulted in increased PDE activity within the retina and delayed retinal degeneration up to 12 weeks after gene transfer.^{24,115} The use of second-generation adenoviral vectors improved the duration of expression of the wild-type gene, perhaps by diminishing the host immune response through minimizing the number of adenoviral genes expressed.¹¹⁵

Successful transfer of β -PDE to the retina of the *rd⁻/rd⁻* mouse and photoreceptor rescue has also been accomplished with AAV¹⁰³ and retroviral vectors.^{110,206} In the study by Takahashi et al., photoreceptors were present at 24 weeks in animals receiving the β -subunit of PDE, but they were absent by 6 weeks in control *rd⁻/rd⁻* mice.²⁰⁶ AAV-mediated transfer in *rd⁻/rd⁻* and *rds⁻/rds⁻* mice resulted in photoreceptor rescue and also improved retinal function, demonstrated by ERG testing.^{8,103}

Growth Factor Therapy

Gene transfer and expression of ciliary neurotrophic factor (CNTF) delays photoreceptor degeneration in *rd⁻/rd⁻* and *rds⁻/rds⁻* mice,^{42,43,147} as well as in rhodopsin knockout mice, P23H rhodopsin transgenic rats, *red1/red1* dogs¹⁸ and in ischemic injury.²¹³ CNTF transgene expression was correlated

with photoreceptor rescue in these models, but did not preserve ERG function. Conversely, CNTF did not demonstrate photoreceptor rescue a transgenic pig trial.⁸⁹ In addition, CNTF gene therapy was associated with intraocular inflammation, including uveitis and cataract, particularly in the large animal studies.^{18,89}

The protective effects of growth factors on photoreceptors have also been demonstrated using bFGF^{6,123} (Fig. 4), brain-derived neurotrophic factor (BDNF),^{132,147} neurotrophin-3,¹²⁵ and a glial-derived neurotrophic factor.⁷² However, not all neurotrophic factors demonstrate protective effects,¹⁴ and neuroprotection does not always preserve functional responses. Transgene expression of neurturin (a glial-derived neurotrophic factor) and a neurturin receptor in the *rd⁻/rd⁻* mouse retina failed to demonstrate histological or functional rescue of degenerating photoreceptors.¹⁰¹ This suggests that the neuroprotective effects of growth factors may be cell specific or perhaps mediated through cell-specific secondary messenger pathways.

Antiapoptosis Gene Therapy

Antiapoptosis strategies applicable to photoreceptor rescue have been developed in *Drosophila* models of apoptosis-induced cell death. The *Drosophila* reaper (*rpr*) and head involution defective (*hid*) genes are important proapoptosis regulators of programmed cell death in the fly. When over-expressed in the developing retina, these genes result in ablation of the eye. The apoptosis-inducing effects of the *rpr*²¹⁹ and *hid*⁸² genes are suppressed by the ectopic expression of an antiapoptotic baculovirus protein p35 and this suggests a possible role for antiapoptotic therapy in recessive retinal degenerations.

GENETIC DISEASES OF THE RPE AND SYSTEMIC METABOLISM

Gene-Replacement Therapy

There are animal models of genetic diseases of the RPE and systemic metabolic diseases, in which the retinal manifestations are seen primarily in the RPE. In Sly syndrome (mucopolysaccharidosis, MPS type VII), a mutation in the β -glucuronidase gene causes skeletal deformities and ocular findings of corneal clouding, optic atrophy, and retinal degeneration. Accumulation of glycosaminoglycans in the RPE results in an abnormal distribution of proteoglycans in the interphotoreceptor matrix and leads to photoreceptor degeneration.¹⁹⁹

Correction of the genetic defect in MPS type VII by gene therapy has been demonstrated in the dog *in vitro*¹⁹⁹ and in the mouse *in vivo*.¹³⁴ Photoreceptor rescue in the mouse was achieved by intraocular de-

livery of the β -glucuronidase gene, but not by systemic therapy to the hematopoietic system,^{185,220} probably from lack of transport of β -glucuronidase across the blood-retinal barrier. However, AAV transduction of neonatal mice following intravenous injection of a vector encoding β -glucuronidase did result in photoreceptor rescue, perhaps due to an immature blood–retinal barrier in the postnatal mouse.⁵⁸

Gyrate atrophy (GA) is an autosomal recessive degeneration of the choroid and retina caused by a deficiency in the mitochondrial matrix enzyme ornithine- δ -aminotransferase (*OAT*).¹⁹⁴ The pathophysiology of the degeneration is unknown. If elevated levels of ornithine or the byproducts of ornithine metabolism are responsible for the retinal degeneration, then a mechanism for clearing ornithine from the circulation may treat the ocular disease. Toward this goal, the *OAT* gene has been successfully transfected into many cell types *in vitro* including, Chinese hamster ovary cells,¹¹⁶ mouse embryonal fibroblasts,¹⁷⁷ keratinocytes from a patient with GA,²⁰³ and human RPE cells.²⁰² *OAT* transgene expression and synthesis of an enzymatically active protein was demonstrated in these studies. Ornithine clearance was less than predicted based on enzyme activity in keratinocytes and may be limited by clearance of ornithine metabolites from the transduced cells.²⁰³ Cytotoxicity was seen in RPE cells expressing high levels of *OAT* activity, but may have been caused by the use of a first-generation adenoviral vector.²⁰²

The *RPE65* gene encodes an RPE-specific protein that participates in the recycling of photobleached retinaldehyde. Mutations in the *RPE65* gene have been identified in patients with Leber's congenital amaurosis (LCA),¹⁴⁴ and in the Briard dog.⁵ Retinas of patients with LCA appear normal, thus, the *RPE65* mutation may cause photoreceptor dysfunction without significant degeneration and transduction of the *RPE65* gene may restore functional vision in these patients. This concept was recently demonstrated in the Briard dog model of LCA.³ Correction of the genetic defects, photoreceptor rescue, and restoration of functional vision in animal models of retinal dystrophy and degeneration provide the "proof of principle" for gene replacement therapy in diseases of the retina and RPE.

RETINOBLASTOMA

Malignant transformation in retinoblastoma (RB) is a multi-step process that requires the development of germline homozygosity at the RB locus or functional inactivation of the pRB protein.^{105,112} The germline mutation that causes retinoblastoma is a

dominantly transmitted, but genetically recessive, mutation of a prototypical “tumor suppressor” gene. The loss of *RB* gene function confers a predisposition to malignant transformation in primitive retinal neuroblasts.^{41,64} The pRB protein is a nuclear, regulatory protein that functions as a transcriptional repressor and controls progression of the cell through the S-phase of the cell cycle by inhibiting E2F transcription factors.^{105,217} The loss of pRB function removes an important transcriptional control checkpoint and can lead to proliferation and malignant transformation.

Gene-Replacement Therapy

Suppression of the malignant phenotype in tumors may be achieved theoretically by reintroduction of a functional *RB* gene or pRB protein in tumor cells;^{95,106} however, there have been conflicting results from *in vitro* and *in vivo* studies using this approach.^{141,155,225,226} Retroviral transduction of a functional *RB* gene into retinoblastoma cell lines does not inhibit *RB* tumor growth within the eye,^{204,225} nevertheless it does repress subcutaneous *RB* tumor formation in immune-deficient mice^{95,204,225} by what may be local tissue factors. It is likely that lack of *RB* tumor inhibition in these models also results from the presence of additional acquired somatic mutations, which confer proliferative potential in these cell lines independent of the *RB* mutation.

Suicide Gene Therapy

A more effective treatment for *RB* will likely be suicide gene therapy (Fig. 2). HSV ribonuclease reductase mutants are cytotoxic only to dividing cells and have been used to inhibit the growth of an established *RB* tumor cell line, Y79, *in vitro* and in the nude mouse.¹¹³ HSV-*tk*-transduced cells are killed by ganciclovir due to the conversion of this prodrug to a toxic metabolite by the activity of the viral-*tk* gene.⁶⁵ Dying cells also demonstrate a local cytotoxic effect on non-transduced cells known as the “bystander effect”.^{28,54,73} This local effect may reduce the need for high efficiency gene transfer in suicide gene therapy. Retroviral transduction of the HSV-*tk* gene into Y79 cell line retinoblastoma cells has demonstrated both sensitivity to the cytotoxic effects of ganciclovir, and a significant bystander effect.⁹³ Ganciclovir also inhibited subcutaneous tumor growth in nude mice by HSV-*tk*-transduced Y79 cells.

The cytotoxic effect of ganciclovir on HSV-*tk*-transduced Y79 cells has also been demonstrated *in vivo* in a murine model of intraocular *RB*.⁹⁷ Intraocular HSV-*tk* suicide gene therapy was shown to be effective in reducing tumor burden with 70% of animals showing tumor ablation following intravitreal injection of ganciclovir. Based on the results of

these preliminary studies, a gene therapy trial based on HSV-*tk* suicide therapy for *RB* is currently underway at the Baylor College of Medicine.⁹⁸ The clinical trial is a phase I, maximum-tolerated dose study to determine dose-limiting toxicity and minimal effective dose of adenovirus-mediated transfer of the HSV-*tk* suicide gene to intraocular *RB* tumors, followed by ganciclovir treatment. Three patients have been enrolled and completed treatment and two additional patients are currently being evaluated for inclusion in the trial. Clinical evidence of tumor cytotoxicity was seen in all treated patients, and all patients are currently alive and well, however, one patient did require enucleation due to progressive vitreous seeding.⁹⁸ The trial has been expanded to include unilateral and bilateral *RB* to attempt to salvage the eye in those patients who would otherwise require enucleation.

The prospects for suicide gene therapy of *RB* are encouraging. The therapy requires only short-term gene expression to be effective and has demonstrated a significant bystander effect, which reduces the requirement for highly efficient transduction within the tumor. There are obvious medical and ethical concerns about the risk of iatrogenic metastases and inflammatory eye disease from suicide gene therapy that need to be addressed in phase I and phase II clinical trials, but to date, there have been no complications from treatment. The debilitating effects of our current therapies for *RB*—severe retinal scarring with visual impairment, and radiation-induced complications including, orbital malformations, non-ocular tumors in patients with heritable disease, and blinding radiation retinopathy—warrant serious investigation into gene therapy for *RB*.

PROLIFERATIVE VITREORETINOPATHY

Proliferative vitreoretinopathy (PVR) is an acquired, intraocular scarring process in the posterior segment that commonly occurs after retinal detachment and penetrating trauma.⁴⁷ Although the pathogenesis of PVR is not completely understood, it is characterized by the growth of hypocoelular membranes on the retina and within the vitreous gel. Proliferation of several cell types; including RPE cells, fibroblasts, and glia within the posterior segment is accompanied by deposition of extracellular matrix proteins, including fibronectin, laminin, and collagen, types I, II, and IV.⁷⁹ Cell-mediated contraction of collagen fibrils leads to tractional retinal folds and detachment. Somatic therapies directed at inhibiting cellular proliferation and the deposition of extracellular matrix have been efficacious in reducing the severity of disease in experimental animal models of PVR.

Suicide Gene Therapy

Cultured fibroblasts and RPE cells produce PVR when injected into the vitreous cavity of the rabbit.^{181,188} Experimental models of PVR have been developed in which proliferating cells are selectively targeted for suicide with the HSV-*tk* in a retroviral vector.^{111,181} HSV-*tk* is selectively transduced in dividing cells by this method and renders them susceptible to the cytotoxic effects of ganciclovir.

Selective killing of dividing cells has been demonstrated in several studies. In mixed cultures of non-dividing rat neurons and proliferating RPE cells, the RPE cells were preferentially killed by ganciclovir following transduction with the HSV-*tk* gene.²²¹ Fibroblasts transduced with the HSV-*tk* gene demonstrate less severe PVR and a decreased incidence of retinal detachment following intravitreal injections of ganciclovir in vivo.¹⁸¹ Reduced severity was also seen using mixtures of fibroblasts in which only 10% of cells expressed the HSV-*tk* gene, which demonstrated a bystander effect in PVR. This bystander effect has also been demonstrated in vivo and in vitro in studies examining the effect of ganciclovir on human RPE cells transduced with the HSV-*tk* gene.^{111,188}

Intravitreal injection of a retroviral vector into eyes with experimental PVR results in expression of a marker gene within the PVR membranes; however, transduction frequency is low.¹¹¹ Nevertheless, ganciclovir therapy following HSV-*tk* gene transfer significantly reduces PVR, even at a transduction frequency of 1%. The presence of vitreous during gene transfer in vitro reduces the transduction efficiency in a dose-dependent manner.¹¹¹ Mechanical effects of the vitreous may also limit the efficiency of transduction following intravitreal injection. The role of vitrectomy in reducing the mechanical limitations imposed by the vitreous to improve the efficiency of transduction to the retina in PVR and perhaps in genetic degenerations is worth exploring in future studies.

Antisense Gene Therapy

Other strategies for treating PVR may emerge from a better understanding of the molecular signals regulating cell proliferation and cell-mediated contractility. Modulation of growth factor, receptor, or structural gene expression by antisense gene therapy may have a role in gene therapy for PVR. Platelet-derived growth factor (PDGF), appears to contribute to the development of PVR in animal models and in human disease.^{37,178} Fibroblasts from α - and β -PDGF-receptor knockout mice induced significantly less PVR than cells that expressed these receptors.¹² In particular, α -PDGF-receptor mutants and knockouts have a reduced ability to induce PVR,

which suggests a role for antisense-inhibition of PDGF-mediated cellular proliferation in the disease.⁹⁹ Antisense inhibition of cell proliferation has also been demonstrated in human RPE cells by blocking *c-myc* expression³⁸ and in the deposition of basement membrane (a component of epiretinal membrane formation in PVR) by blocking fibronectin gene expression.¹⁷⁹

Growth Factor Gene Therapy

Intravitreal injection of BDNF in a cat model of retinal detachment significantly reduced Müller cell glial proliferation and indicates a possible role for growth factor gene therapy in retinal detachment and PVR.¹³² BDNF also maintains better organization of the outer segments in the setting of retinal detachment, which suggests a role for this growth factor, and perhaps others, in promoting photoreceptor survival.

While the results of suicide therapy for experimental PVR are encouraging, PVR membranes in the rabbit model are cellular and develop very rapidly, whereas membranes in human PVR are hypocellular and progress more slowly. The success of transduction by viral vectors and the bystander effects seen in the rabbit models may not be equivalent in human PVR, and remains to be proven. These studies do show that the transcripts of the α -PDGF receptor, *c-myc*, fibronectin, and perhaps other genes may be targets for antisense or ribozyme gene therapy for PVR.

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is an acquired disease of the RPE, which results in a secondary degeneration of the overlying neurosensory retina.²²⁸ Degenerative changes of the RPE and Bruch's membrane are the primary factors responsible for the disease. The pathophysiology of the disease is still incompletely understood, but AMD results from a combination of environmental, aging, and genetic factors in the RPE and retina.²²⁸ Unlike the degenerative process in RP in which specific mutations give rise to the disease phenotype, the putative role of specific genes in the degenerative process in AMD is less clear. Although certain genes may predispose some patients to develop AMD, the genetic linkage is controversial, and to date, the genetics of AMD remains largely unknown.^{9,229} Genetic susceptibility to AMD is probably multifactorial and thus will not be amenable to gene therapy directed at the germline. In the absence of a well-defined genetic defect which gives rise to AMD, gene therapy will likely focus on somatic therapy using growth factors and antiapoptosis therapy to prolong the survival of the RPE and retinal photoreceptors.

Growth Factor Gene Therapy

There is experimental evidence that growth factors play an important role in maintaining the health of RPE cells and in enabling them to respond to injury. Basic-FGF, insulin-like growth factor (IGF-1), PDGF, and CNTF have all been shown to stimulate DNA synthesis and RPE cell proliferation in vitro.^{37,83,189,190,205,216} RPE cells in vivo co-express growth factors and their receptors demonstrating the autocrine and paracrine functions of these hormones. Theoretically, it may be possible to enhance RPE cell survival by somatic modulation of growth factor gene expression in patients with AMD.

Defective phagocytic function by the RPE results in the retinal degeneration seen in the RCS rat model.¹²⁴ While the RCS rat is not an animal model for human AMD, age-related phagocytic dysfunction and incomplete lysosomal digestion of photoreceptor membranes by the RPE results in the accumulation of drusen, the hallmark of AMD in humans.²²⁸ This accumulation ultimately results in loss of RPE cells and in geographic atrophy, perhaps due to the cytotoxicity of these deposits on the surrounding cells. Enhancing phagocytic activity in aging RPE cells using gene therapy is a potential approach to the treatment of AMD. Basic-FGF has been shown to stimulate phagocytic activity and prolong retinal survival in the RCS rat model.¹⁴⁹ Basic-FGF,^{6,223} CNTF,^{42,43,126} IGF-1,¹⁹⁰ BDNF,^{62,131} and other growth factors and secondary messengers of the intracellular signaling pathways have demonstrated neuroprotective effects on the retinal neurons in animal models of retinal degeneration and detachment.⁷⁷ Gene transfer and expression of these growth factor proteins may similarly inhibit retinal degeneration by a neuroprotective effect in AMD.

The high intrinsic phagocytic activity of the RPE cells has been used to direct labeled plasmids and reporter genes to the RPE using liposome-mediated DNA transfer.^{46,48,49} RPE cells are also competent to express foreign genes delivered by viral vectors with high transfection frequencies.^{55,134,202} Preliminary studies using growth factors have suggested that growth factor transgene expression can effect changes in the growth characteristics and morphology of human RPE cells in vitro.⁴⁹ However, it remains to be proven whether the RPE phenotype can be selectively modulated by growth factors and whether induced changes in the RPE will have a beneficial effect on the RPE degeneration seen in AMD.

Antisense Gene Therapy

Antiapoptosis gene therapy, discussed in the Retinal Degeneration section, has similar potential applicability for the treatment of AMD. Inhibiting the

degeneration of RPE and photoreceptor cells by apoptotic mechanisms may slow disease progression and visual loss. Transfer of the gene for the oxidative stress protein heme oxygenase-1 has been demonstrated in the rabbit retina² and may be a mechanism by which the retina and RPE can be protected from oxidative injury in patients with AMD. Development of somatic therapy for atrophic AMD is hampered by the lack of a good in vivo animal model for the disease. Aged monkeys do develop drusen, but true macular degeneration is rare in the non-human primate. An in vitro model to mimic the decline in membrane digestion in the RPE has been developed. Antisense transcripts directed to cathepsin S reduce aspartic protease activity in RPE cells and may provide an in vitro model for the phagocytic dysfunction in AMD,¹⁷² as well as an alternative model for photoreceptor degeneration.¹¹⁹ For the foreseeable future, progress will likely depend upon in vitro work using eye bank tissue donated from patients with AMD.

CHOROIDAL NEOVASCULARIZATION

Degeneration of the RPE and defects in Bruch's membrane, which occur in AMD and other diseases characterized by RPE atrophy and/or chorioretinal scarring, predispose some patients to the formation of choroidal neovascular membranes (CNV).²²⁸ The RPE plays an active role in inhibiting angiogenesis by hormones such as vascular endothelial growth factor (VEGF).¹⁵⁴ The RPE synthesizes proteins that are antiangiogenic, such as tissue-inhibitors of metalloproteinases¹⁸⁰ and pigment epithelium-derived factor.¹⁵⁴ Growth factors secreted by the RPE that have angiogenic activity, such as bFGF and IGF-1, are tightly regulated by the RPE to control this deleterious activity.²¹⁶

Potential gene therapy applications to CNV include (antiangiogenic) growth factor gene therapy,¹¹⁸ antisense or ribozyme therapy directed at angiogenic factors, and suicide gene therapy directed at neovascular tissue. In a laser-induced model of CNV, marker gene transfer to neovascular membranes has been demonstrated using retroviral,¹⁵⁶⁻¹⁵⁸ adenoviral,^{120,157} and AAV vectors¹¹⁸ via intravitreal¹⁵⁷ and subretinal injection.^{118,120,156-158} Recently, it was demonstrated that expression of angiostatin in experimental CNV significantly reduces the size of CNV lesions.¹¹⁸

Antisense oligonucleotides can be introduced into laser-induced CNV via intravitreal injection; however, the duration of expression is brief, and introduction of antisense oligonucleotides to VEGF failed to decrease the amount of neovascularization.¹⁶⁴ The antifibronectin antisense therapy demonstrated in

PVR¹⁷⁹ may have some efficacy in CNV therapy but has not been experimentally tested.

EX VIVO GENE THERAPY

One way to overcome the problems of intraocular cell targeting is to remove target cells, genetically modify them *ex vivo*, and return them to the host.^{33,68,165} This approach has several potential advantages. It facilitates formerly difficult tasks, such as improving the efficiency of transduction and selection and cloning of transduced cells, which are more easily accomplished *in vitro*. Another advantage is that *in vivo* targeting may be avoided as happens when implants containing genetically modified cells are used to deliver growth factors to the retina. Finally, this approach permits assessment of safety profiles of modified cells and pharmacotherapeutic efficacy prior to clinical application.

The main disadvantage of *ex vivo* transduction is that certain cells, most importantly photoreceptors, cannot be removed and surgically reintroduced. Surgical reimplantation of a modified target tissue, such as the RPE, may also be difficult, and, depending on the transduction method used, transgene expression may still be of limited duration.¹⁵⁶ *Ex vivo* genetic modification of cells followed by intraocular implantation has been employed to rescue photoreceptors in the RCS rat²¹⁴ and in the *rd1* dog model²⁰⁸ of retinal degeneration. This type of pharmacotherapeutic approach to gene therapy using growth factors and antiapoptotic factors will likely have broad applications in many ocular diseases.

Summary

There is considerable excitement about the prospects for gene therapy for the treatment of ocular diseases. Progress is being made not only in the treatment of retinal disease, but also in the areas of glaucoma,^{31,87,108} neuroprotection,^{77,130} uveitis,¹⁷⁶ corneal disease,^{68,122} and lens epithelial proliferation.^{53,107,143} Gene therapy for genetic and acquired retinal disease presents many unique and difficult challenges to researchers and clinicians committed to the translational application of these novel therapies. While some success may be achieved treating specific germline mutations, it is likely that broad clinical application, especially in non-retinal diseases, will require further development of mutation-independent somatic therapies such as the ones discussed above.

The chronic, progressive nature of many of these retinal diseases will require long-term, stable expression of transgenes over years or even decades. Although the preliminary results of long-term gene expression are encouraging, the duration of expression that will be required to confer a therapeutic ef-

fect over the lifetime of the patient has yet to be achieved. Numerous technical objectives also need to be accomplished before gene therapy can be safely and effectively applied to the treatment of retinal disease. Given the pace of research and the success obtained to date, it is likely that many, if not all of these technical objectives will be met in the coming decade. These include the following:

1. Inducible transcription⁴⁵ and/or translational control of transgene expression;
2. Optimizing efficient vector transduction for specific cell types within the eye
3. Modulation of the host immune response to transgene expression and vectors
4. Minimizing the risk of viral vector infectivity
5. Development of better animal models for human ocular disease

Although the risk of systemic toxicity in ocular gene therapy is likely small, there are potential risks from clinical application. The risk of generating wild-type infectious virus or new genetic recombinants, in particular those derived from HIV-based vectors must be critically assessed before and during clinical trials. The early onset and progressive nature of many of these diseases may require early intervention, perhaps even prior to the clinical manifestation of disease. The risk of vision loss from gene therapy in eyes that are not yet visually impaired presents serious ethical issues that need to be addressed within the medical and lay communities.

In the coming decades gene therapy will, no doubt, become an increasingly important therapy for the treatment of what are presently blinding retinal diseases. Those of us in the ophthalmic community should view the body of basic science and translational research in ocular gene therapy over the past decade with great satisfaction and look forward to the future of gene therapy with great optimism, tempered with a dose of realism.

Method of Literature Search

We performed Medline searches covering the years 1996 through 2001, without language restrictions, using the keywords: *gene therapy, gene transfer, retinoblastoma, retinitis pigmentosa, retinal degeneration, retinal dystrophy, proliferative retinopathy, pigment epithelium of the eye, growth factors, retina, retinal degeneration, animal models, choroid, neovascularization, liposomes, viral vectors, adenovirus, adeno-associated virus, herpes simplex virus, retroviruses, transduction, antisense oligonucleotide, apoptosis, transfection, thymidine kinase gene, suicide gene therapy, green fluorescent protein, β galactosidase gene, ribozyme, knockout mice, transgenic animals, neuroprotection*. Additional references were obtained from the bibliographies of articles obtained from the Medline

searches and from web-based articles from the Fourth Great Basin Visual Science Symposium, August 2000.

The authors would like to point out a large body of unpublished work on gene therapy encompassing *in vitro* and *in vivo* animal studies, knockout and transgenic retinal degeneration models, elucidation of new genetic mutations in human ocular disease, somatic gene therapies and new vector constructs for gene therapy, to name a few, which have been presented in paper and poster format at the Association for Research in Vision and Ophthalmology yearly meetings. Due to space constraints, only selected examples of this work were referenced for this article. Readers interested in the most current areas of investigation in gene therapy are encouraged to search the annual IOVS Supplement referencing the ARVO meetings. Other reviews of gene therapy have also been published in the ophthalmic literature.^{20,21,27,30,89,152}

Numerous papers pertaining to retinal gene therapy have been published in the literature since this manuscript was accepted for publication. These include studies in gene transfer methods,^{61,85,218} regulated transcription,^{60,148} growth factors,^{136,196} gene therapy in animal models of genetic disease,^{57,187,191,215} neovascularization,⁵¹ immunological responses,^{100,175,193} and a human clinical trial in neovascular AMD.¹⁷³

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Glossary

Antisense: A therapeutic approach to inhibit translation of mRNA molecules into proteins by using small, complementary DNA sequences to bind to the transcripts. These DNA/RNA heteroduplexes do not allow the ribosomes to attach and initiate protein synthesis.

Apoptosis: A process by which the initiation of specific molecular pathways results in programmed cell death. Apoptosis is the final common pathway of cell death in genetic retinal degenerations.

Episome: A DNA sequence (plasmid or adenoviral) which resides in the nucleus but does not integrate into the host cell genome. Episomes contain autonomous promoters that permit gene expression, but because they do not integrate, gene expression is transient.

Ex vivo: Performed outside the host with subsequent delivery in vivo. For example, gene transfer to RPE cells in vitro followed by transplantation of those cells beneath the retina.

Genome: The cell's genetic information, in the form of DNA in the nucleus.

In vitro: Performed in tissue culture. For example, gene transfer to RPE cells in a petri dish.

In vivo: Performed within the host. For example, gene transfer by injecting a viral vector directly into the vitreous cavity.

Knockout: Genetically engineered animal models (usually mice) in which specific genes have been mutated.

Lipofection: Non-viral gene transfer performed with phospholipid-based molecules.

Marker gene: A gene which encodes a protein which can be used to identify the cells that express it. Also called a reporter gene. Marker genes are used to determine the success and duration of foreign gene transfer into cells.

Murine: Mouse.

Plasmid: Extrachromosomal DNA molecules, which can be used as vectors to transfer genes to target cells using physicochemical methods.

Ribozyme: An RNA enzyme that has the ability to cleave mRNA molecules at specific trinucleotide sequences. They are used to deplete the cell of specific mRNA transcripts.

Transcription: The transfer of the genetic information from a DNA template (gene) to a

messenger RNA (mRNA) molecule. The mRNA is also called a transcript.

Transduction: Stable gene expression following gene transfer. The term indicates sustained transgene expression. Transduction is usually achieved using integrating vectors such as AAV or retroviruses, but also occurs at low frequency using plasmid vectors.

Transfection: Gene transfer using viral or plasmid DNA. The term is generally used to indicate transient transgene expression.

Transgene: A foreign gene transferred to and expressed by the target cell.

Translation: The process of forming a protein molecule at a ribosomal site of protein synthesis from a messenger RNA.

Vector: A vehicle, viral or non-viral, used to transfer a gene to a target cell.