Lighting a candle in the dark: advances in genetics and gene therapy of recessive retinal dystrophies

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Nonsyndromic recessive retinal dystrophies cause severe visual impairment due to the death of photoreceptor and retinal pigment epithelium cells. These diseases until recently have been considered to be incurable. Molecular genetic studies in the last two decades have revealed the underlying molecular causes in approximately two-thirds of patients. The mammalian eye has been at the forefront of therapeutic trials based on gene augmentation in humans with an early-onset nonsyndromic recessive retinal dystrophy due to mutations in the retinal pigment epithelium–specific protein 65kDa (*RPE65*) gene. Tremendous challenges still lie ahead to extrapolate these studies to other retinal disease–causing genes, as human gene augmentation studies require testing in animal models for each individual gene and sufficiently large patient cohorts for clinical trials remain to be identified through cost-effective mutation screening protocols.

Introduction

Vision is considered by many to be the most important of the five senses. Visual perception is mediated by the retina, the light-sensitive tissue that lines the inner surface of the eye (Figure 1). Light striking the retina initiates a cascade of events that ultimately triggers nerve impulses to the brain (Figure 2). The retina is a complex, layered structure consisting of neuronal and supporting cells. The photoreceptor cells are the light-sensitive cells that absorb light from the field of view and, through the phototransduction cascade, convert this information into a change in membrane potential. There are two types of photoreceptors: rods and cones. Rods mediate vision in dim light, while cones support daytime vision and the perception of color. Cones are concentrated in the central part of the retina, which is known as the macula, and the highest cone density is found in the center of the macula (an area known as the fovea), which enables high-acuity vision. The retinal pigment epithelium (RPE) supports and nourishes the photoreceptor cells and is firmly attached to the underlying vascular layer, which is known as the choroid. The RPE mediates the visual cycle, a continuous process by which the retinoids used in the phototransduction cascade are recycled.

Retinal dystrophies are characterized by degeneration of photoreceptor and RPE cells; they represent the major cause of incurable familial blindness in the Western world. The inheritance pattern of the diseases can be autosomal recessive (AR), autosomal dominant (AD), or X-linked (XL). Retinal dystrophies are generally classified based on whether the disease primarily affects the rods or the cones (and thus predominantly affects the macular or the peripheral retina) (Table 1) (1). These diseases show substantial clinical and genetic overlap (Figure 3). The distinction between some retinal dystrophies can be very subtle or even arbitrary, and mutation of a single gene can result in varied clinical diagnoses (Table 1). In addition, there is a plethora of syndromes (e.g., Bardet-Biedl syndrome, Joubert syndrome, Senior-Loken syndrome, and Usher syndrome) in which retinal abnormalities are consistently or frequently found (1, 2). These syndromes are not the topic of this Review; rather, we focus on retinal dystrophies that occur in the absence of concomitant dysfunction of other organs (i.e., nonsyndromic retinal dystrophies, in particular the AR and XL forms).

During the past few years, exciting progress has been made in developing gene augmentation therapies to correct the genetic defects causing nonsyndromic retinal dystrophies. A prerequisite for this type of therapy is that the underlying genetic defect in patients is known. In this Review, we discuss the progress that has been made toward understanding the genetic basis of AR and XL nonsyndromic retinal dystrophies. We also provide an overview of animal models of these diseases and discuss the challenges in developing successful retinal gene augmentation therapies.

Phenotypes of inherited nonsyndromic retinal diseases

Generalized and/or peripheral retinal dystrophies. Retinitis pigmentosa (RP) represents the most frequent cause of inherited visual impairment, with a worldwide prevalence of 1:4,000 (1). It encompasses a clinically heterogeneous group of progressive disorders that primarily affect the function of the photoreceptors and the RPE (2). There is a large variability in the age of onset, progression, retinal appearance, and final visual outcome. Pigment granules from the RPE migrate to perivascular sites of the neural retina secondary to photoreceptor death, forming the hallmark "bone spicules" (Figure 1C). The attenuation of the retinal arterioles and veins probably also represents a secondary effect of photoreceptor cell death. Initially, rods are predominantly affected, resulting in night blindness and tunnel vision. Later in the disease process, cones also are affected, which can result in complete blindness (1). Thirty percent of RP patients show AR inheritance, 20% show AD inheritance, and 10% show XL inheritance (2). Approximately 40% of RP patients represent isolated cases.

Conflict of interest: Jean Bennett is a co-inventor on the pending patent "A Method of Treating or Retarding the Development of Blindness" but waived any financial interest in this technology in 2002. Jean Bennett presented a seminar at Novartis in 2009.

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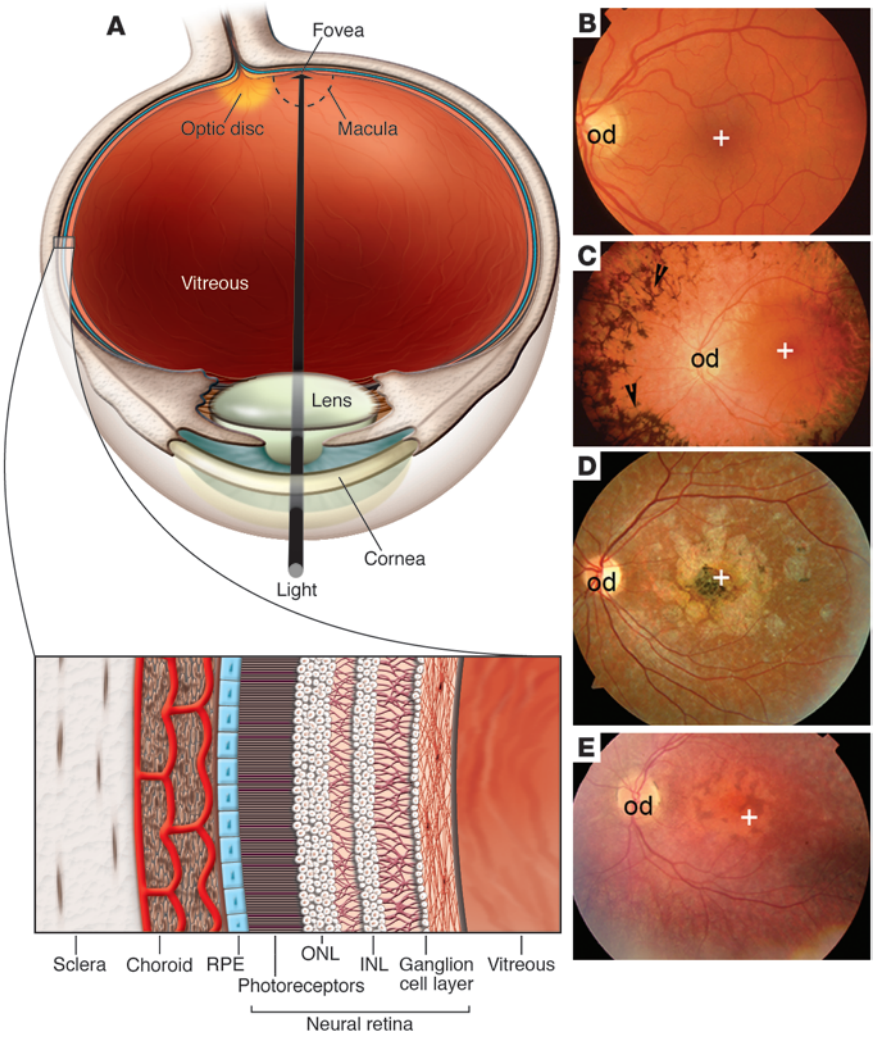

Figure 1
Anatomy of the human eye and retina. (**A**) Cross section showing the major landmarks of the human eye and retina. The borders of the macula, which is adjacent to the optic disc, are indicated with a dashed line. Fundus views of patients with (**B**) normal vision; (**C**) retinitis pigmentosa (pigmentary changes indicated by arrowheads); (**D**) STGD1 due to *ABCA4* mutations; and (**E**) LCA due to a homozygous *RPE65* mutation. +, fovea; od, optic disc; INL inner nuclear layer; ONL, outer nuclear layer.

Patients with Leber congenital amaurosis (LCA) are severely visually impaired or blind from birth. They present with nystagmus and a retinal appearance that varies from normal to mild pigment mottling with mild vascular attenuation to severe pigmentation and vascular attenuation that resemble those of the fundus in RP-like dystrophy (1) (Figure 1, C–E). A nondetectable electroretinogram (ERG) in the first year of life is pathognomonic. Some patients show a rod-cone degeneration pattern and others a cone-rod degeneration pattern. Almost all LCA patients show AR inheritance, with a worldwide incidence of approximately 1:30,000 (2).

Retinal dystrophies primarily affecting the macula. Stargardt disease type 1 (STGD1) (OMIM #248200; see Table 1 for additional listings) is the most common juvenile macular dystrophy, with a worldwide prevalence of 1:10,000 (3). STGD1 in most cases is characterized by visual acuity loss in early childhood or early adolescence, but it can also appear later in life. Macular atrophy can develop and is often characterized as having a beaten bronze appearance or a bull's-eye pattern (Figure 1D). Fundus flavimaculatus is a phenotypic variant with yellow flecks associated with secondary macular atrophy.

In the majority of patients, the accumulation of lipofuscin, which is composed of a mixture of lipids, proteins, and different fluorescent compounds, results in progressive retinal/macular changes that include alterations in fundus autofluorescence (4). Patients with AR cone-rod dystrophy (CRD) initially have a predominant loss of cone function. They show photoaversion (a preference to avoid light) and defective color vision in adolescence and early adult life, followed by decreased central vision due to progressive CRD. The macula may show a bull's-eye appearance; there are islands of impaired visual acuity and subsequently large paracentral scotomas (blind spots). Bone spicules generally are absent, and the retinal vessels show mild thinning.

Impairment or death of the cone photoreceptor cells is the clinical hallmark of cone disorders, which have an estimated prevalence of 1:35,000 (5). Achromatopsia (ACHM) is a stationary congenital AR cone disorder that is characterized by low visual acuity, photophobia, nystagmus, severe color vision defects, diminished cone ERG responses, and normal rod ERG responses. The literal meaning of ACHM (absence of color vision) does not fully capture the clinical picture, as ACHM patients generally show more severe visual acuity defects than, for example, patients with cone dystrophy (CD) in the early stage of disease (6). CD is a progressive cone disorder in which patients may initially have normal cone function but a pale optic disc predominant in the temporal side. They develop progressive loss of visual acuity, increasing photophobia, color vision disturbances, and diminished cone responses

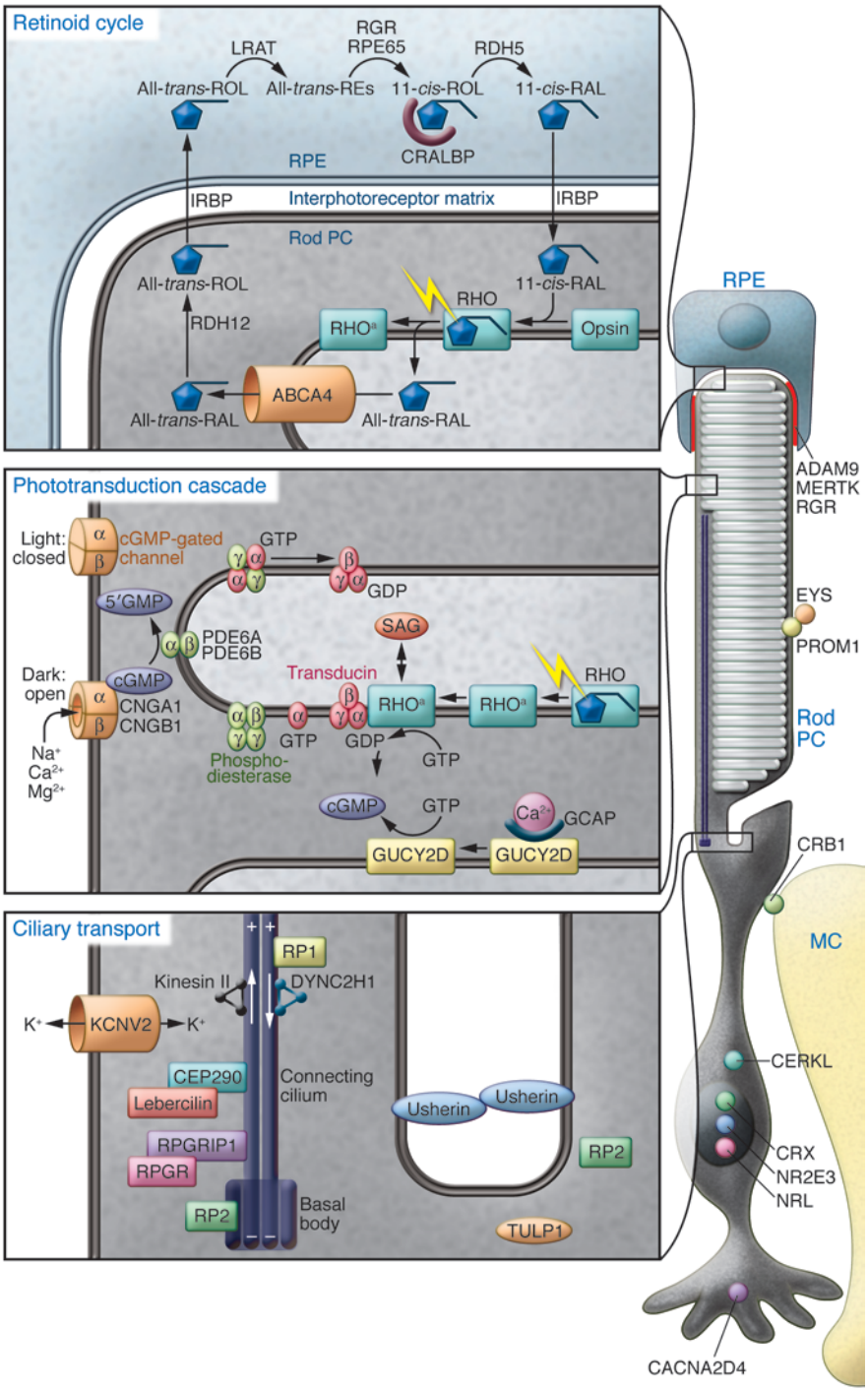

Figure 2
Schematic representation of three major processes in human rod photoreceptor cells and the RPE. Upper panel: The retinoid cycle taking place in rod photoreceptor cells (PC) and the RPE. Upon photactivation, 11-*cis*-retinal is converted into all-*trans*-retinal and dissociates from activated rhodopsin. The all-*trans*-retinal is then recycled to produce more 11-*cis*-retinal via several enzymatic steps in the RPE. ABCA4 mediates transport of all-*trans*-retinal to the outside of the photoreceptor outer segment disks. The localization and function of proteins involved in AR and XL nonsyndromic retinal dystrophies are depicted, with the exception of GCAP, a critical Ca^{2+}-binding interactor of GUCY2D, which is mutated in autosomal dominant CRD (http://www.sph.uth.tmc.edu/Retnet/home.htm). CRALBP, protein product of *RLBP1*; IRBP, protein product of *RBP3*; RAL, retinal; RE, retinyl esters; RHO^a, photoactivated rhodopsin; ROL, retinol. Middle panel: The phototransduction cascade in rod PCs. Upon photoactivation, amplification of the signal is mediated through the α-subunit of transducin and phosphodiesterase, which results in closure of the cGMP-gated channel, hyperpolarization of the cell, and reduced glutamate release at the synapse. SAG, arrestin. Lower panel: Ciliary transport along the connecting cilium. Kinesin II family motors mediate transport toward the outer segments; cytoplasmic dynein 2/1b (DYNC2H1) is involved in transport processes from the outer segments toward the inner segments. The precise roles of CEP290, Lebercilin, RPGR, RPGRIP1, and RP1 in ciliary transport processes are not yet known. AIPL1 (not indicated in this figure) is a chaperone for proteins that are farnesylated. For IDH3B and PRCD, the exact cellular functions are not known. ADAM9, MERTK, and RGR are secreted by the RPE and localize in the interphotoreceptor matrix. The *CNGA3*, *CNGB3*, *GNAT2*, and *PDE6C* genes are specifically expressed in cone PCs and therefore not indicated in this figure. At the right side, a Müller cell (MC) connects to the photoreceptor cell with the transmembrane protein CRB1. Usherin, protein product of *USH2A*.

on ERG, usually in the first or second decade of life. The visual acuity of these patients generally worsens to legal blindness before the fourth decade of life (5).

Molecular genetics of retinal dystrophies

Genetic heterogeneity. A striking characteristic of nonsyndromic AR and XL retinal dystrophies is their genetic heterogeneity (Figure 4 and Table 1). The exception is STGD1, which in nearly all cases is caused by mutations in the ATP-binding cassette, subfamily A, member 4 (*ABCA4*) gene (7). Approximately 70% of patients with recessive XL RP carry mutations in the retinitis pigmentosa GTPase regulator (*RPGR*) gene, and a small percentage carry mutations in the retinitis pigmentosa 2 (*RP2*) gene (8). The most extreme example of genetic heterogeneity is AR RP, with mutations in at least 25 genes identified to cause the condition to date, none of which is mutated in a large fraction of patients (Retnet: http://www.sph.uth.tmc.edu/RetNet/). To date, 14 genes have been linked to AR LCA, 6 genes to AR CD, 4 genes to AR CRD, and 4 genes to ACHM (Figure 4) (9). It is estimated that the identified genes account for approximately 50% of AR RP, 70% of AR LCA, 40% of AR CRD,

Table 1
Nonsyndromic recessive retinal dystrophy genes, their associated human phenotypes, animal models, and gene therapy studies

Human gene	Recessive phenotypes	Cellular expression in the retina	Mouse model (variant)	Other recessive animal models (variant[A])	Gene refs.	OMIM no. (gene)	OMIM no. (disease)	Gene therapy model	Gene therapy refs.
ABCA4	STGD1, CRD, RP	Cones and rods	KO	No	73	601691	248200, 604116, 601718	Mouse	40, 49
ADAM9	CRD	RPE	KO	No	74	602713	612775	No	
AIPL1	LCA	Rods	KO, KD	No	75–77	604392	604393	Mouse	32, 72
CACNA2D4	CD	Unknown	C57BL/10 (c.2367insC)	No	78	608171	610478	No	
CEP290	LCA, RP	Cones and rods	rd16 (Δex35–39)	Cat (c.6960+9T→G)	79, 80	610142	611755	No	
CERKL	CRD, RP	Cones and rods	KO	No	81	608381	608630, 268000	No	
CNGA1	RP	Rods	No[B]	No		123825	612095	No	
CNGA3	ACHM, CD	Cones	KO	No	82	216900	268000	No	
CNGB1	RP	Rods	KO	No	83, 84	600724	600724	No	
CNGB3	ACHM, CRD	Cones	KO	Dog	85, 86	605080	262300	Mouse	87
CRB1	LCA, RP	Müller cells	KO, KI (p.C249W), rd8	No	88–90	604210	600105	No	
CRX	LCA	Cones and rods, bipolar cells	KO	No	91	602225	120970, 268000	No	
EYS	RP, CRD	Cones and rods	No mouse ortholog	No		612424	602772	No	
GNAT2	ACHM	Cones	Cpfl3 (p.D200N)	No	92	139340	139340	Mouse	93
GUCY2D	LCA	Cones and rods	KO	rd chicken (Δex4–7)	94, 95	600179	204000	Mouse	23, 96
IDH3B	RP	Unknown	No	No	97, 98	604526	612572	No	
KCNV2	CD	Cones and rods	No	No	99–101	607604	610024	No	
LCA5	LCA	Cones and rods	No[B]	No	102	611408	604537	No	
LRAT	LCA, RP	RPE	KO	No	103	604863	613341	Mouse	104
MERTK	LCA, RP	RPE	KO	RCS rat	105	604705	604705	Rat	106, 107
NR2E3	RP, ESCS	Rods	KO, rd7	No	108	604485	268100, 604485, 611131	No	
NRL	RP	Rods	KO	No	109	162080	162080	No	
PDE6A	RP	Rods	Chemically induced	rcd3 dog	104, 110	180071	180071	No	
PDE6B	RP	Rods	rd1, rd10	Dog	111, 112	180072	180072	Mouse	41, 56, 113–116
PDE6C	ACHM, CD	Cones	cpfl1	No	117–119	600827	600827, 613093	No	
PRCD	RP	Cones and rods, RPE, GCL	No	Dog	120, 121	610598	610599	No	
PROM1	RP	Cones and rods	KO	No	122	604365	612095	No	
RBP3	RP	Cones, rods, RPE, Müller	KO	No	123, 124	180290	NA	No	
RD3	LCA	Cones and rods	rd3	No		180040	610612	No	
RDH12	LCA	Rods	KO	No	125–128	608830	612712	No	
RDH5	CD	RPE	KO	No	129, 130	601617	601617	No	
RGR	RP	RPE	KO	No	131, 132	600342	600342	No	
RHO	RP	Rods	KI, KO	No		180380	180380, 184380	Mouse	133
RLBP1	RP, RPA	RPE, Müller	KO	No		180090	1800990, 607476	No	
RP1	RP	Cones and rods	KO	No	134, 135	603937	180100	No	
RP2	XL RP	Ubiquitous	No[B]	No	136, 137	300757	312600	No	
RPE65	LCA, RP	RPE	KO, KI (p.R91W), rd12	Dog	97, 98	180069	204100	Mouse, dog	34, 36, 57, 138–140
RPGR	XL RP, XL CRD	Cones and rods	KO	XLPRA1, A2 dogs	99–101	312610	300029, 304020, 300455	No	
RPGRIP1	LCA, CRD	Cones and rods	KO	Dog	102	605446	605446, 608194	Mouse	141
SAG	RP	Rods	No[B]	No	103	181031	181031	No	
SPATA7	LCA, RP	GCL, INL, PR	No[B]	No	105	609868	609868, 604232	No	
TULP1	LCA, RP	Cones and rods	KO	No	108	602280	602280	No	
USH2A	RP, USH2	Cones and rods, OPL	KO, RBF/DnJ	No	109	608400	608400	No	

[A]Variants listed only if they are not null alleles. [B]ES cells available at EUCOMM (http://www.eucomm.org/) or KOMP (http://www.komp.org).OMIM: http://www.ncbi.nlm.nih.gov/omim; ECSC, enhanced S syndrome; KD, knockdown; KI, knock-in; NA, not available.

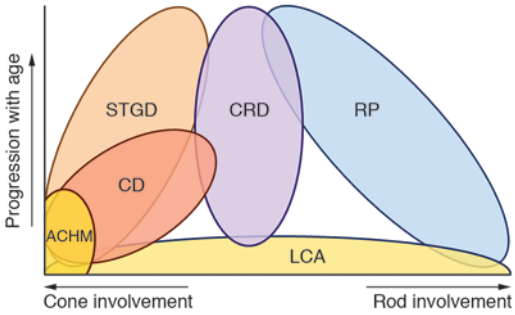

Figure 3
Phenotypic overlap among autosomal recessive retinal dystrophies. Patients with ACHM display a virtually stationary cone defect in which cones are principally defective. At end stage, CD can hardly be distinguished from CRD. Patients with STGD1 later in life show mid-peripheral defects similar to those in CRD patients. Patients with RP initially display tunnel vision due to rod defects that very often progresses to complete blindness when the cones are also affected. In patients with LCA, the defects can occur in both types of photoreceptors, or in RPE cells, and therefore clinical and molecular genetic overlap with CD, CRD, or RP can be expected.

10% of AR CD, and 80% of ACHM (9). Additional molecular genetic research is therefore warranted to identify the remaining causes of these diseases. This is important if gene augmentation therapies (such as that described below in "Gene augmentation therapy in the clinic" for LCA due to *RPE65* mutations), which are gene specific, are to be developed.

Several genes cause distinct or partially overlapping clinical phenotypes. For example, mutations in *ABCA4* cause STGD1, are a major cause of AR CRD, and are an infrequent cause of an RP-like dystrophy (10, 11). Mutations in the genes encoding crumbs homolog 1 (*CRB1*), lecithin-retinol acyltransferase (*LRAT*), MER receptor tyrosine kinase (*MERTK*), *RPE65*, spermatogenesis-associated protein 7 (*SPATA7*), and Tubby-like protein 1 (*TULP1*) can cause both LCA and juvenile-onset RP (2, 12).

The genes identified in nonsyndromic AR and XL retinal dystrophies affect a wide variety of molecular pathways and processes. In Figure 2, we depict three important processes that are affected in these conditions: the rod phototransduction cascade, the retinoid cycle, and ciliary transport (refs. 2, 9, and refs. therein).

Molecular diagnostics. Genetic testing of patients with nonsyndromic AR and XL retinal dystrophies is performed for genetic counseling purposes, that is, to estimate the recurrence risk for future offspring, and to confirm preliminary clinical diagnoses, which can be challenging in all stages of these diseases. Establishing a definite molecular diagnosis aids in the planning for clinical follow-up and allows a more accurate disease prognosis. With the advent of gene therapy and other types of treatment, the identification of a patient's gene mutation(s) is becoming increasingly important. Mutation identification not only presents technologic and economic challenges because of the enormous allelic and genetic heterogeneity displayed by the inherited retinal dystrophies, but also requires a well-balanced program to raise awareness among patients, health insurance companies, and the general public.

As discussed above (see *Genetic heterogeneity*), the genes responsible for approximately 65% of the inherited AR retinal dystrophies are currently known (2, 9). The challenge is to translate this enormous body of scientific knowledge into clinical practice, as even in the Western world, fewer than 10% of patients with inherited blindness know their genetic defect. Which techniques can be used to cost-effectively identify the underlying genetic defects?

Conventional Sanger sequencing of relatively frequently occurring variants is warranted for the centrosomal protein 290kDa (*CEP290*) c.2991+1655A→G mutation that is found in 20% of LCA patients of European descent (13), and the cyclic nucleotide gated channel β3 (*CNGB3*) p.T383fsX variant that is found in 50% of ACHM patients (14). Sanger sequencing is also preferred for diseases for which the majority of mutations can be found in one or a few genes encompassing a maximum of approximately 50 exons/amplicons, such as ACHM (caused by mutations in the 18-exon *CNGB3* gene or the 8-exon cyclic nucleotide gated channel α3 [*CNGA3*] gene), STGD1 (caused by mutations in the 50-exon *ABCA4* gene), and XL RP (caused by mutations in the 19-exon *RPGR* gene or the 5-exon *RP2* gene).

Analysis of all known LCA mutations can be performed cost-effectively using allele-specific primer extension analysis (15), which yields pathologic variants in approximately 60% of patients. This technique is available but less cost effective for AR CRD and AR RP, for which 25%–35% of alleles are known (16).

Next-generation sequencing represents a major breakthrough in cost-effective sequencing (17). The cost per base pair for this technology compared with conventional Sanger sequencing has dropped 100- to 1,000-fold. To identify novel retinal disease genes, all the exons from a sizable genomic region (e.g., established via linkage analysis [ref. 18] or by identity-by-descent mapping) can be sequenced. Alternatively, all exons of the human genome (the exome) can be sequenced for less than $10,000. Studies are in progress to tailor next-generation sequencing technology for diagnostic purposes, for example, to sequence the exons of all (approximately 180) human retinal disease genes, or a subset of these genes, for less than $1,000.

With the identification of numerous variants in many putative disease genes, it will be a challenge to discriminate pathologic from benign sequence variants. In addition, nonsyndromic retinal dystrophies in a subset of patients may be caused by the cumulative effect of mutations in more than one gene, and detailed knowledge about the interactions of proteins in networks (such as at the connecting cilium) will be required to begin to understand genetic interactions.

Animal models for recessive retinal dystrophies

Mouse models. Rodent models of retinal dystrophy have been very useful for proof of concept of gene therapy studies, in part because the degeneration process (like the lifespan of these animals) is often condensed into weeks or months. Thus, the effects of a given approach can be evaluated fairly quickly in a large number of animals. Pathologic effects of various genes/mutations can also be evaluated in these models. The primary disadvantage of mouse models of retinal dystrophies is that the mouse differs from the human in that it lacks the cone-dominated central region of the posterior fundus. Additionally, the size and anatomy of the mouse eye differ substantially from those of the human eye, thereby requiring different surgical approaches for gene delivery. Further, the lifespan of the mouse is short, which, in some cases, may limit the relevance of the model. In Table 1 we list the 44 currently known genes underlying nonsyndromic recessive retinal dystrophies in human. Mouse orthologs are known for each of these genes, except eyes shut homolog (*EYS*) (19). Naturally occurring or man-made

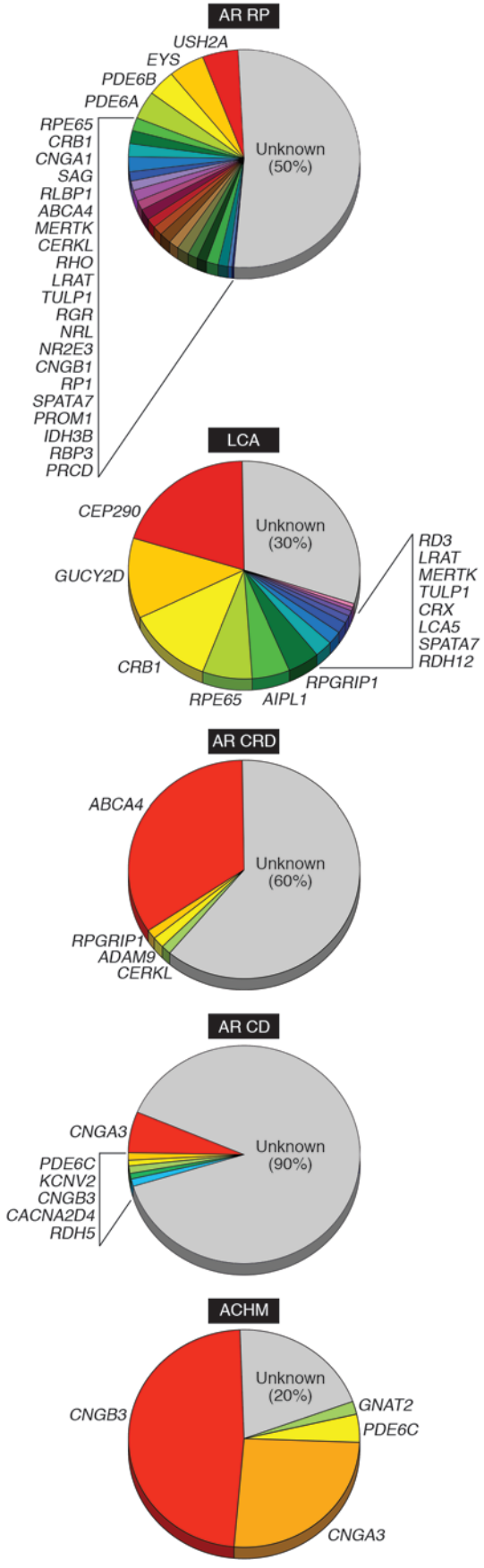

Figure 4
Prevalence of mutations in genes causing genetically heterogeneous retinal dystrophies. Estimated prevalence of mutations in genes causing AR RP, LCA, AR CRD, AR CD, and ACHM. Mutations in approximately 50% of AR RP, 30% of LCA, 60% of AR CRD, 90% of AR CD, and 20% of ACHM remain to be identified. For several genes, only one or a few families with mutations have been reported; in these cases, the gene frequency was estimated to be 1%. Estimates are based on literature searches (http://www.sph.uth.tmc.edu/Retnet/home.htm) and our own experience.

mouse models are already available for 35 of these genes, including 27 knockouts, 3 knock-ins, 1 knockdown, and 11 spontaneous mouse models (Table 1). Spontaneous models are common because blindness does not generally affect longevity or fertility (20).

In addition to the existing mouse models, large-scale research initiatives in the United States (KnockOut Mouse Project [KOMP], http://www.komp.org/; and Lexicon Pharmaceuticals, http://www.lexicongenetics.com), Canada (North American Conditional Mouse Mutagenesis Project [NorCOMM], http://www.norcomm.org/), and Europe (European Conditional Mouse Mutagenesis Program [EUCOMM], http://www.eucomm.org/) are developing and distributing mouse ES cell lines and mice carrying gene-trap or -targeted mutations across the mouse genome. As a result of these initiatives, it is now possible to obtain mouse ES cell lines lacking one of five AR nonsyndromic retinal dystrophy genes mentioned in Table 1 for which there are currently no mouse models available: mouse ES cell lines lacking *CNGA1* and *SPATA7* are available from KOMP, while mouse ES cell lines lacking LCA type 5 (*LCA5*), *RP2*, and S-arrestin (*SAG*) are available from EUCOMM. Additional mouse models are expected to become available or can be requested to enter the pipeline as these large-scale projects progress.

Large animal models. Several large animal models of AR retinal dystrophies are also available. Many of these have been identified through evaluation of visual dysfunction in family pets, and others have been identified in screens prior to training guide dogs. Some of these, such as a Briard dog with a null mutation in *RPE65* that models LCA caused by *RPE65* mutations, are enrolled in gene augmentation studies. Others may be used in future studies (e.g., a mutant Abyssinian cat with congenital blindness due to a *CEP290* splice defect and a mutant Irish setter dog with AR RP due to a *PDE6B* mutation) (Table 1). It should be noted that in all three of these models, the gene defects causing disease were not identified until the gene defect had been identified in humans. In fact, in one of these models, the Briard dog, the disease had been originally misdiagnosed as congenital stationary night blindness (21), and the diagnosis was changed to LCA after the human mutation was identified.

The retinas of dogs and cats are more anatomically similar to those of humans than are the retinas of mice. While only primates have maculas, dogs and cats have a cone photoreceptor–enriched area, the area centralis, which is functionally similar to the macula. The size of their eyes is also similar to that of humans, so that surgical approaches that would be acceptable in humans can be used. In contrast, aside from the fact that it is small, surgery in the mouse eye is difficult due to the fact that the lens occupies almost the entire vitreous cavity (Figure 5A). Thus, subretinal injections in the mouse are usually carried out through a posterior approach (Figure 5A), whereas they can be carried out under direct visualization in dogs, cats, and other large animals (Figure 5, B and D–G).

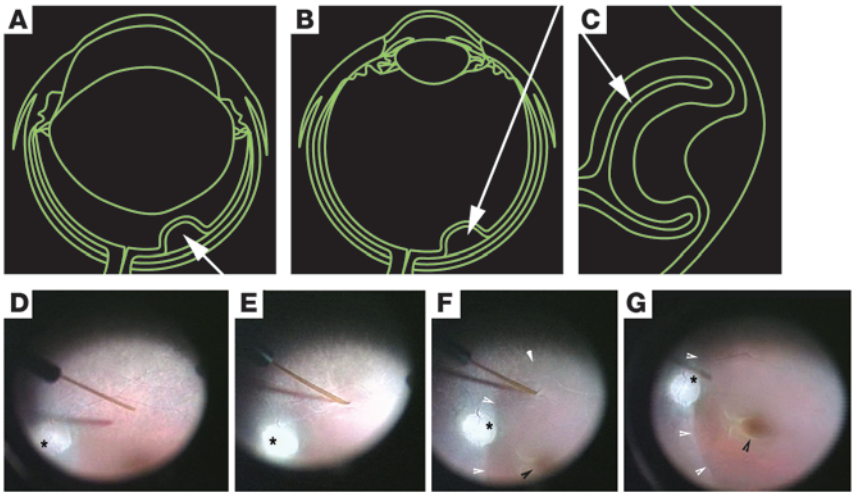

Figure 5
Approaches for surgical delivery of gene therapy vectors in retinal disease. Subretinal injection is necessary to place the gene augmentation therapy reagent in contact with the target photoreceptor and RPE cells. Arrows indicate the approaches used in the postnatal/adult mouse (**A**), large animals/humans (**B**), and fetal mice (**C**; injection into the subretinal space adjacent to retinal progenitor cells). (**D**–**G**) Frames from an intraoperative video taken during subretinal injection of the macula in a human with rAAV2.*hRPE65v2* (64). In **G**, the cannula is withdrawn, revealing the raised fovea (black arrowhead). *optic disc; white arrowheads indicate edge of the expanding detachment.

Finally, nontraditional animal models have been identified that provide additional interesting data with respect to disease pathogenesis and approaches to therapy. The rd (guanylate cyclase 2d$^{-/-}$ [*Gucy2D*$^{-/-}$]) chicken was first identified in a flock of Rhode Island Red chickens because it could not see well enough to find its food (22). This model was used successfully to demonstrate efficacy using in ovo gene transfer. The retinal and visual function improvements after gene augmentations therapy in this model provide compelling evidence that this approach could be effective in humans (23).

Gene augmentation therapy considerations

With recent progress in delineating the molecular genetic bases of nonsyndromic AR and XL retinal dystrophies in humans and animals, it is logical to ask how we can use this information to correct the diseases. Gene augmentation strategies have been tested successfully now in a dozen different animal models (Table 1). Since DNA is not able to pass through cell membranes efficiently, it is delivered through a delivery agent (vector). There are a number of details that affect the success of retinal gene augmentation, including selection of the appropriate vector and delivery method. Variables relevant to vector selection include tropism, cargo capacity, stability of expression, and/or immunogenicity.

Vector selection: viral vectors. There are a number of nonviral and viral vectors that have been used successfully in retinal gene therapy proof-of-concept studies (24, 25). Methods of nonviral delivery include the use of liposomes and DNA condensation reagents as vectors as well as electroporation, iontophoresis, and high-velocity cell bombardment ("gene gun"). Advantages of such physico-chemical approaches include the fact that there are no size limitations with respect to the transgene cassette and that the approach does not deliver additional antigens besides the DNA/DNA-binding reagents themselves. Potential disadvantages include concern about longevity of expression and efficiency of nuclear entry, although a recent report using compacted DNA nanoparticles showed evidence of marked physiologic rescue for at least 30 days after delivery (26).

Viral vectors, such as recombinant adenovirus (rAd; both early-generation and helper-dependant viruses), recombinant adeno-associated virus (rAAV), and lentivirus, have been tested extensively in vivo in the retina in safety and proof-of-concept studies. Improvements in vector design, including modification to capsids, envelopes, and surface proteins, have provided an expanded toolkit for achieving the desired transduction parameters. Although the cargo capacity of rAAVs (4.8 kb) is smaller than that of early-generation rAd or lentivirus vectors (approximately 7 kb), rAAVs generally target photoreceptors more efficiently than those vectors. Because of this and also because of the seemingly benign immune responses that it triggers, it has been used in more preclinical studies than the other vectors. Further, with the identification of scores of naturally occurring AAV variants and with the development of technology to modify the AAV capsid, a number of rAAVs are available that can deliver transgenes efficiently to a variety of different retinal cell types (27, 28). These vectors differ markedly in their cellular specificity, efficiency of transduction, and onset of transgene expression. While rAAV serotype 2 (rAAV2) vectors target RPE cells efficiently (and photoreceptors less efficiently), it takes up to 6 weeks for transgene expression mediated by this vector to plateau (29). In comparison, rAAV5 and rAAV8 vectors transduce photoreceptors with much higher efficiency than rAAV2 and result in transgene expression within 5–10 days after delivery (30, 31). It is thus not surprising that rAAV2 vectors perform well in delivering a therapeutic transgene to RPE cells in animals and humans with a relatively slowly progressing retinal degenerative disease (LCA due to *RPE65* mutations; Table 1) but an rAAV5 or -8 vector is required to target photoreceptors in an animal model with a much faster rate of retinal degeneration (e.g., RP or LCA due to aryl hydrocarbon receptor–interacting protein–like 1 [*AIPL1*] mutations; Table 1 and ref. 32). The expression time courses are affected by the time necessary for the single-stranded AAV genome to become a transcriptionally competent double-stranded form, and the time to reach peak expression levels can be reduced by selection of self-complementary (double-stranded) AAV. There are, however, limitations with the cargo capacity of self-complementary rAAV (33). Nevertheless, regardless of the capsid serotype or structure of the transgene cassette, rAAV-mediated retinal gene transfer results in stable transgene expression in target retinal cells (34), even though rAAV-delivered transgenes are maintained in an episomal fashion (35).

Similar to rAAV, lentivirus and rAd are generally trophic for the RPE and have therefore been effective in animal models of RPE disease. These include spontaneous null mutation mouse and dog models of LCA due to *RPE65* mutations and a spontaneous null rat model of AR RP due to *MERTK* mutations (Table 1)

(34, 36, 37). However, by swapping the envelope or genome (e.g., using an equine-derived versus a human-derived lentivirus vector) or pseudotyping with different fiber proteins (e.g., using a rAd containing an envelope fiber from an Ad37 type rAd instead of an Ad5-based vector; refs. 38, 39), photoreceptor transduction can be enhanced. These modified vectors have been used with some success in animal models of primary photoreceptor disease such as STGD1 (lentivirus) or AR RP caused by *PDE6B* mutations (rAd) (40, 41). Lentiviruses are integrating vectors and thus mediate stable transgene expression (assuming the appropriate promoter is used), although there is a risk of insertional mutagenesis (42). Adenoviruses are not integrating, and expression does not persist for more than a couple of months (43). This may be due, at least in part, to immune clearance, as early-generation rAd vectors carry viral open reading frames that encode immunogenic viral proteins (44). Adenovirus vector epitopes are major factors in triggering the host immune response, which can include the generation of neutralizing antibodies as well as activation of a $CD8^+$ CTL response (45). The latter is associated with release of cytokines that activate macrophages that can then phagocytose target cells. CTLs and other cytotoxic cells (such as NK cells) also directly kill virus-infected cells. Such responses can lead to systemic inflammatory response syndrome and death after systemic administration (46). The responses in animals after intraocular exposure of early-generation rAd vectors are much more benign, perhaps due to the unique immune environment of the eye, but they do limit stability of transgene expression (43) (reviewed in ref. 47). Helper-dependent adenoviruses, vectors deleted of all open reading sequences, are likely to lead to safe and stable demonstration of proof of concept of gene therapy in the eye.

Cargo capacity is generally not an issue with lentivirus vectors and first-generation rAd vectors, both of which can accommodate approximately 8 kb, but the cargo capacity of rAAV vectors (4.8 kb) can be challenging for large photoreceptor-specific cDNAs such as *ABCA4*, *CEP290*, myosin VIIA (*MYO7A*), and usher syndrome type-2A protein (*USH2A*) (48). It was thought that rAAV2/5 vectors could deliver the intact 7.8-kb *ABCA4* cDNA (49). However, subsequent analyses have shown that only a small portion of rAAVs package the intact cassette (J. Bennicelli, J.F. Wright, and J. Bennett, unpublished observations) and most of them contain partial cassettes after gene augmentation therapy in mice (J. Bennicelli, J.F. Wright, and J. Bennett, unpublished observations). It is possible that, after infection of the cell, homologous recombination between sequences in these partial cassettes allows production of the full-length ABCA4 protein. Multiple groups have proposed such a mechanism for delivery of other large cDNAs by AAV (50, 51) and suggest that for large cDNA targets, it might be possible to use two or three rAAVs that carry different portions of the large transgene cassette that recombine in the target cell to produce the full-length transgenic protein.

Alternative strategies to packaging large transgene cassettes into rAAVs include delivery of a cDNA encoding a truncated but functional protein, supply of ribonucleotides (antisense oligonucleotides) that could modulate gene expression through interference with RNA processing (as described by van Deutekom and coworkers for the severe muscular dystrophy Duchenne muscular dystrophy; ref. 52), and delivery of the cDNA in segments through a "*trans*-splicing" approach. For the latter approach, the cDNA is split into two separate rAAV vectors using an engineered intron to mediate splicing of the two cDNA segments within the cell. Feasibility of this approach using a small transgene cassette has been demonstrated in vivo in the mouse retina (53). In that study, a cytomegalovirus-promoted *lacZ* cDNA was split in half, and an intron splice donor sequence was placed on the 5′ half. A splice acceptor was placed on the 3′ half of the cDNA. The two pieces of the cassette were then packaged separately in AAV. Coinfection of cells in vitro and in vivo in the eye with the resultant AAVs resulted in transgene expression, whereas infection with either one of the AAVs alone did not (54).

Additional delivery challenges. Challenges relevant to the surgical delivery of the appropriate vectors are largely solvable. Importantly, the transgene should be delivered within the appropriate time frame — before degeneration has progressed to the point of eliminating the target cells (Figure 5G). In some AR and XL nonsyndromic retinal dystrophies, such as LCA due to *RPE65* mutations, there is a large window in which to correct the disease. Even in that disease, however, beyond a particular stage, there are no cells remaining that can be rejuvenated. In others, the disease initiates and thus must be corrected, very early in life (i.e., it has a developmental component). An example of a disease in which gene augmentation would have to occur early in life is LCA due to cone-rod homeobox (*CRX*) mutations (55). Delivery of the vector is not in itself a barrier, as approaches for performing subretinal injections have been developed in both small and large animal models and in humans and at different stages of maturation of the eye (from gestation through adulthood) (Figure 5C) (56–62). After subretinal injection, only the cells coming in contact with the recombinant viral vectors are transduced — a benefit in terms of preventing transduction to unaffected cells. The volume of injected material determines the area/number of transduced cells. The localized detachment caused by the injection (Figure 5) resolves spontaneously within several hours of the procedure, leaving little residual damage (63, 64).

Gene augmentation therapy in the clinic

To date, the first human gene augmentation studies have involved 18 subjects, all of whom had LCA due to *RPE65* mutations. All 18 of the subjects enrolled were injected once unilaterally with rAAV2 carrying the human *RPE65* cDNA in volumes ranging from 0.15–1.0 ml (60, 61, 63–65). Enrollment was completed in June 2009 in a study at the Children's Hospital of Philadelphia (CHOP) that involved 12 subjects aged 8–44 years. This study showed that delivery of up to 1.5×10^{11} vector genomes (vg) of rAAV2.hRPE65v2 to the subretinal space was safe and efficacious in all 12 individuals. The subjects showed both subjective and objective improvements in retinal and visual function, and the improvements were stable through the latest time point (2.5 years). The extent of recovery was age dependent, with children showing the greatest improvement. The five children in the study are now able to ambulate independently and to participate in normal classroom and athletic endeavors (64). Improvements in retinal/visual function have been stable for at least 2 years following vector administration (64, 65). The studies at University College London and the University of Pennsylvania/University of Florida have so far each enrolled three young adults (60–62). There were no safety concerns in any of the trials, and there was evidence of efficacy, with improvements in light sensitivity noted in some of the subjects in the University of Pennsylvania/University of Florida study and the University College London study. The various trials differ with respect to promoter sequence, the area of the retina targeted, some of the test protocols, and the exact dose

and volume administered. The data to date argue that subretinal gene transfer of rAAV2.*hRPE65* is safe and efficacious and leads to long-term improvements in retinal/visual function.

Building on the first clinical gene augmentation trials

The successes of the first human gene augmentation therapy studies involving patients with LCA caused by mutations in *RPE65* provide the foundation for gene therapy approaches for the treatment of other forms of AR and XL nonsyndromic retinal dystrophies. The primary focus once efficacy is established in animal models concerns the safety of transgene delivery. So far, the risks of toxicity/immune response due to delivery of up to 1.5×10^{11} vg rAAV2 and to exposure of RPE cells to RPE65 protein have been discharged, at least through the 2.5-year time point (64). However, many of the diseases under consideration will require gene transfer to photoreceptors instead of RPE cells, and this will necessitate use of a different vector. Further, some of the transgenes are membrane proteins and are therefore more likely to engender an immune response. Preclinical safety data in large animal models should be predictive of the risks, similar to the situation in LCA-*RPE65* studies (36, 66, 67).

A potential safety concern is posed by the report that AAV capsids can persist in retinal cells long after administration (68). The significance of this finding is unknown, particularly since no inflammation was observed in the animals that were studied. However, it does raise the possibility that delayed or chronic inflammation could evolve. In the study of LCA-*RPE65*, we have found no inflammation in animals followed as long as 10 years after rAAV injection (J. Bennett et al., unpublished observations). Finally, another safety concern is the possibility that rAAV particles could inadvertently target or spread to adjacent cells. The primary concern with respect to diseases in which photoreceptors or RPE cells are the primary cell targets is that rAAV could leak into the vitreous during the subretinal injection procedure and then transduce ganglion cells. This could result in expression in visual pathways in the brain (68–70). So far, however, there have been no reports of CNS toxicity in preclinical or clinical studies using subretinal or intravitreal delivery of rAAV.

The next questions are what diseases are next in line and what are the challenges in developing treatments for the remaining hundreds of inherited forms of retinal degeneration? The subsequent targets will be selected in consideration of the following scientific criteria: first, the targeted disease should lead to significant visual impairment; second, the transgene cassette must meet the cargo requirements for the currently available vectors; third, the selected vectors must target the primary cell population efficiently and stably; fourth, an animal model with a relevant phenotype should be available in which to demonstrate proof of concept; fifth, there should be a sufficient number of patients (more than 25) identified with the given disease so that clinical trials can be carried out; and last, there must be evidence that a sufficient number of cells exists in the patients that could be rejuvenated by gene delivery.

Given the currently available set of reagents, animal models, and knowledge base, there are more than a dozen different immediate potential targets. The design of each of the trials is likely to differ based on the disease characteristics, rate of progression of disease, and ethical issues. Likewise, the outcome measures are likely to differ from target to target, depending on the age of the subjects (and thus their ability to carry out particular test procedures), the nature of the disease, and the rate of disease progression. The final determinant in the selection of AR and XL retinal dystrophies for gene augmentation studies is the availability of funds to carry out these expensive translational studies.

While costs of proof-of-concept studies can be met by conventional funding mechanisms such as government grants and private foundations, clinical trials are usually not covered by such mechanisms, and the costs of these studies are considerable. The expenses include the generation and validation of the clinical vector, preclinical safety studies, maintaining the appropriate regulatory oversight, and the clinical trial itself. Although the diseases under consideration qualify for "orphan" status (i.e., they affect fewer than 200,000 people in the United States) and there are incentives for developing treatments for such diseases, there are very few large pharmaceutical companies willing to cover clinical trial costs given the small size of the target population. We predict that it would be difficult for a pharmaceutical company to break even let alone make a profit on a rare disease in which a drug is administered only once, unless large fees were to be billed for the drug.

An additional challenge is to determine the appropriate stage of the disease process at which to test the intervention. In many of the diseases, the degenerative component progresses more rapidly than in LCA caused by mutations in *RPE65*. Also, in diseases affecting differentiation of photoreceptors, intervention in infancy might be required to maximize the chance of restoring and preserving vision. Indeed, in most of the patients with LCA, treatment might be optimal in children younger than three years of age due to decreases in the plasticity of the retinal/central nervous system connections after that age. The Recombinant DNA Advisory Committee's approval of carrying out a gene therapy study of LCA caused by mutations in *RPE65* first in children aged eight years and older (Recombinant DNA Advisory Committee 2005, Discussion of Human Gene Transfer Protocol #0510-740; http://oba.od.nih.gov/rdna/rac_past_meeting_2005_dec_13.html) and then in children ages three years and older (http://oba.od.nih.gov/rdna/rac_past_meeting_2009_webcasts.html#dec09) will pave the way for obtaining approval for enrolling even younger subjects in retinal gene therapy trials. Another important question is whether it is safe to administer vector to the eye contralateral to that treated in the initial clinical trials. All of the subjects in the CHOP LCA-*RPE65* clinical trial have requested treatment of the contralateral eye, as now their uninjected eyes (previously their best-seeing eyes) do not function as well as their injected eyes. The concern about readministration is that the initial injection will serve to immunize the subjects against the AAV2 capsid or the transgenic protein and that the second exposure could serve as a "booster shot" and lead to a harmful immune response. This concern is a theoretical one, as, so far, the measured immune responses in these patients have been benign (63, 64). In addition, readministration of high-dose (1.5×10^{11} vg) rAAV2.hRPE65v2 to the contralateral eye in large animals that had previously been immunized with AAV proved safe and efficacious (71). The results of future readministration studies in patients with LCA due to mutations in *RPE65* will be useful in determining the appropriate trial design for new disease targets, particularly with respect to the advisability of injecting both eyes simultaneously in order to minimize the potential immunologic risks of a later readministration.

It is important not to oversell the potential of gene therapy to patients who are considering enrollment in future studies of gene therapy for retinal degeneration. The success of the LCA-*RPE65*

trial situation may be unique in that there was useful vision early in life in most of these patients. In addition, as this disease involves a deficiency of an enzyme, efficiency of transduction and RPE expression may not have to be 100% in order to achieve efficacy. In primary photoreceptor diseases, it will be important to treat the maximum possible number of photoreceptors in a given portion of the retina to maximize improvement in retinal/visual function. It will also be important to regulate the amount of protein photoreceptors produce, as too much may be toxic and too little may not result in benefit. Recent reports involving gene augmentation therapy in animal models of retinal degeneration indicate, however, that rescue of vision is possible even in some of the most severe diseases, such as LCA associated with *AIPL1* mutations (32, 72).

Conclusions

The tremendous progress in delineating the molecular bases of inherited retinal degeneration together with recent reports on the success of gene augmentation therapy for LCA caused by mutations in *RPE65* provide great promise for future applications of genetic therapies to blindness. Many obstacles lie ahead in applying gene therapy to the other potentially more challenging forms of retinal degeneration; however, the tools are available now to tackle a number of these diseases. The ultimate goal is to translate gene-based treatments to clinical practice. To make such treatments routine, there will have to be many changes in the approaches of clinicians to these diseases. Comprehensive, cost-effective screening tools still need to be developed, and clinicians need to offer genotyping tests to their patients. Progress in these ventures is underway. The work to date provides hope for patients with inherited retinal diseases, and in the next decade we believe that it is likely that novel therapeutic strategies will be developed for a number of these genetic defects.

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