Retinal dystrophies, genomic applications in diagnosis and prospects for therapy

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Abstract: Retinal dystrophies (RDs) are degenerative diseases of the retina which have marked clinical and genetic heterogeneity. Common presentations among these disorders include night or colour blindness, tunnel vision and subsequent progression to complete blindness. The known causative disease genes have a variety of developmental and functional roles with mutations in more than 120 genes shown to be responsible for the phenotypes. In addition, mutations within the same gene have been shown to cause different disease phenotypes, even amongst affected individuals within the same family highlighting further levels of complexity. The known disease genes encode proteins involved in retinal cellular structures, phototransduction, the visual cycle, and photoreceptor structure or gene regulation. This review aims to demonstrate the high degree of genetic complexity in both the causative disease genes and their associated phenotypes, highlighting the more common clinical manifestation of retinitis pigmentosa (RP). The review also provides insight to recent advances in genomic molecular diagnosis and gene and cell-based therapies for the RDs.

Keywords: Retinal dystrophy (RD); retinitis pigmentosa (RP); massively parallel sequencing (MPS); CRISPR/Cas9

Introduction

Retinal dystrophies (RDs) are a group of conditions that have a range of clinical manifestations which are estimated to affect as many as 1 in 4,000 individuals (1). Cases may be syndromic or non-syndromic. Vision impairment may vary from poor peripheral or night vision to complete blindness, and severity usually increases with age. Cases may be familial with autosomal recessive, autosomal dominant or X-linked modes of inheritance described, with sporadic cases also observed (2-4). Due to the high genetic heterogeneity underlying these disorders, prioritisation in examining the >120 genes known to be associated with the inherited RDs is challenging (5). This has led to a lack of readily available testing in many countries for examination of all associated genes in a cost-effective and timely manner. Recent advances in genomic analysis technologies, including next generation sequencing (NGS) and chromosome microarrays, allow the prospect of genome-wide approaches to be feasible for the first time in a diagnostic setting. Studies examining copy number variation in RD are limited and have used multiplex ligation-dependant probe amplification (MLPA) techniques to target regions of interest (6). Until now, the usual diagnostic approach has been to use array-based primer extension (APEX) technology or Sanger sequencing to examine specific mutations, exons or gene targets. These techniques are reported to have a diagnostic yield of approximately 10-20% in RD patients (7). There are currently no cure or treatment options for patients with RD, with only an inevitable progression to blindness. New NGS genomic strategies and genome engineering technologies provide revolutionary opportunities in improving both diagnostic
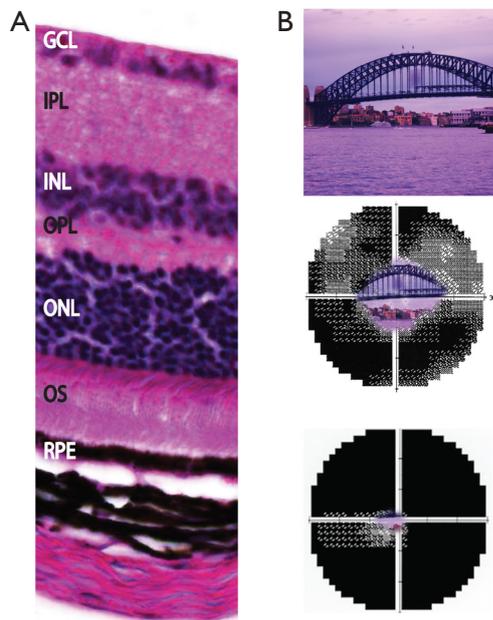
Figure 1 Retinal layers and clinical impact of retinal dystrophy. (A) Expansion of retinal region with histology section showing normal retinal layers of the mouse, which has close homology with histology of the human eye. The ONL contains the nuclei of the photoreceptors, and the OS layer contains the outer segments of the photoreceptors; (B) progressive visual field loss as experienced by retinitis pigmentosa patients. Upper image shows normal full visual field, middle image shows constricted field and lower image shows almost no central vision remaining. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments of photoreceptors; RPE, retinal pigment epithelium.

and therapeutic approaches in the RDs, emphasising the need for understanding of these conditions and applications of these new technologies.

RD can be categorized into broad groups depending on the type of photoreceptor affected and the manifestations, or degree, of atrophy within the retina. Rod and cone photoreceptors are the primary cellular units that facilitate the conversion of light energy to a neural action potential in the retina and facilitate an image to be perceived in the brain (*Figure 1*). RD groups can include rod-dominated diseases, cone-dominated diseases and generalised retinal degenerations involving both rod and cone photoreceptors. Syndromic forms whereby the phenotype extends to more organ systems than just retinal degeneration also exist, however are beyond the scope of this review. RD occurs due

to abnormalities of retinal cellular structures including the photoreceptors as well as defects in the phototransduction and visual cycle pathways which are required to facilitate the conversion of light energy into a neuronal signal that is perceived by the brain [reviewed in (8)]. Phototransduction describes the process whereby a neural action potential is generated and propagated along the photoreceptor allowing an amplified response. In the visual cycle light sensitive pigments are generated and recycled and this involves the movement of intermediates through different cell layers of the retina. This review aims to highlight our understanding of non-syndromic RD, primarily regarding the rod and cone dominated dystrophies while also making reference to generalised RDs, specifically leber congenital amaurosis (LCA) and choroideremia. Molecular pathogenesis, application of new genomic technologies for molecular diagnoses and provision of gene-based therapeutic strategies will also be discussed.

Rod and rod-cone dystrophies

The rod and rod-cone dystrophies particularly affect the rod photoreceptors or the rod photoreceptors are the first affected. This group of disorders can be further delineated into progressive degenerative forms including retinitis pigmentosa (RP) and stationary forms called congenital stationary night blindness (CSNB). Syndromic forms of the disease also exist and have clinical presentations which extend to more than the affected retina. All Mendelian inheritance patterns, including autosomal dominant, autosomal recessive and X-linked have been observed.

Retinitis pigmentosa (RP)

The most common clinical manifestation of RD is RP. RP is a progressive non-syndromic rod-cone disease and has high levels of clinical and genetic heterogeneity. Variation exists at multiple levels with locus and allelic heterogeneity, incomplete penetrance and variable expression and penetrance all observed (9). The onset of the disease varies with early onset or juvenile RP sufferers affected from as early as the first years of life whereas adult or late onset RP symptoms develop significantly later. Clinical presentations manifest with progressive deterioration of the ability to see in dim light causing night blindness, followed by loss of peripheral vision that slowly encroaches toward the centre of the visual field resulting in tunnel vision (*Figure 1*). Later stages of the disease can result in
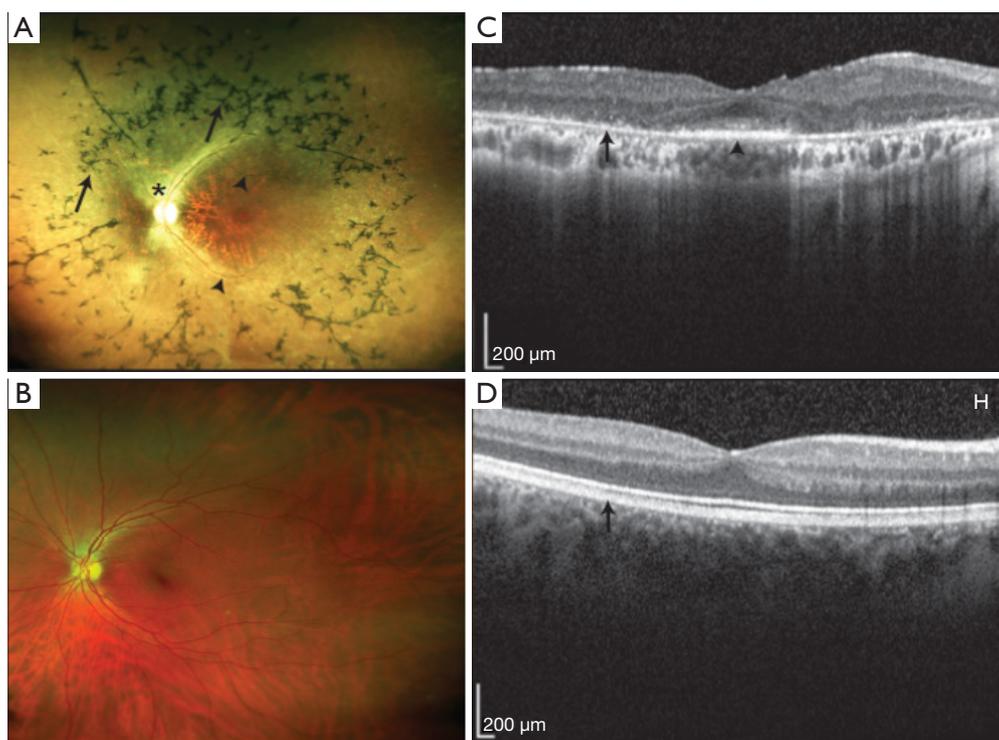
Figure 2 Retinitis pigmentosa: fundal images. (A,B) Wide field fundus photography illustrating retinal features of retinitis pigmentosa, including pigmentary changes (arrows), waxy pallor of the optic disc (asterisk) and retinal arteriolar attenuation (arrowheads) in (A), compared with the normal retinal image in (B); (C,D) OCT imaging showing thinning of the rod photoreceptor outer segments in retinitis pigmentosa in (C), compared with normal in (D) (arrows). There is relative preservation of the cone photoreceptor outer segments present in the foveal region in retinitis pigmentosa (arrowhead). OCT, optical coherence tomography.

complete blindness where the cone photoreceptors are also implicated. Affected photoreceptors undergo apoptosis, which is evident with the thinning of the outer nuclear layer and pigmented deposits or lesions present in the diseased retina (*Figure 2*). The loss in visual acuity has been shown to be proportionate to the level of deterioration of the fundus. Other clinical manifestations associated with RP include posterior subcapsular cataracts, dust like particles in the vitreous, white dots deep within the retina and Hyaline bodies affecting the optic nerve. The affected region of the retina may be restricted to a specific site, adding further complexity to disease identification (10).

Over 60 disease genes are reported to associate with RP (*Table 1*) (5,122). Known functions of the encoded proteins can be grouped into five broad categories including: phototransduction; retinal metabolism; RNA splicing; tissue development and maintenance; and cellular structure. Modes of inheritance vary with 15-20% autosomal dominant, 5-20% autosomal recessive, 5-15% X-linked, and simplex or unknown inheritance observed in 40-50%

of cases (9). Digenic inheritance has also been observed where heterozygous mutations in two genes *ROM1* and *PRPH2* have been shown to cause the RP phenotype (77). Due to such complex clinical presentations and genetic factors, even with the latest genetic diagnostic techniques, including NGS strategies, molecular diagnosis is only achieved in approximately 50% of tested RP patients (123). There is also further genetic heterogeneity with some genes implicated in other forms of RD.

Mutations in the gene *RHO*, encoding rhodopsin which is critical in phototransduction, are a leading cause of RP. Rhodopsin is a 7-transmembrane spanning protein making up approximately 80% of the protein found in the disc membrane of rod outer segments. *RHO* contains five exons that encode 348 amino acids. The primary function of the protein is to initiate the phototransduction cascade by facilitating the conformational change of 11-cis retinal into 11-trans retinal. Mutations in this gene are seen in ~20-30% of autosomal dominant RP while autosomal recessive inheritance is also observed with certain mutations, but much more rarely.

Table 1 Disease genes: human retinitis pigmentosa

Gene¯	Inheritance*	Potential function	Estimated frequency	Studies
RHO	Dominant & recessive	Phototransduction	20-30% & <1%	(11,12)
GUCA1B	Dominant	Phototransduction	Rare (4-5% in Japan)	(13)
RDH12	Dominant	Phototransduction	Unknown	(14,15)
PDE6A	Recessive	Phototransduction	2-5%	(16,17)
PDE6B	Recessive	Phototransduction	2-8%	(18,19)
SAG	Recessive	Phototransduction	2-3% in Japan	(20)
CNGA1	Recessive	Phototransduction	1-2%	(21,22)
CNGB1	Recessive	Phototransduction	<1%	(23,24)
PDE6G	Recessive	Phototransduction	<1%	(25)
RPE65	Dominant & recessive	Retinal metabolism	Rare & 2-5%	(26,27)
LRAT	Recessive	Retinal metabolism	<1%	(28,29)
RBP3	Recessive	Retinal metabolism	<1%	(30,31)
RGR	Recessive	Retinal metabolism	<1%	(32)
RLBP1	Recessive	Retinal metabolism	<1%	(33,34)
ABCA4	Recessive	Retinal metabolism	2-5%	(3,35)
MVK	Recessive	Retinal metabolism/unknown	Unknown	(36)
IDH3B	Recessive	Citric acid cycle	<1%	(37)
PRPF31	Dominant	Splicing	5-10%	(38-40)
PRPF8	Dominant	Splicing	2-3%	(39,41)
PRPF3	Dominant	Splicing	1%	(39,42)
PRPF4	Dominant	Splicing	Unknown	(43,44)
PRPF6	Dominant	Splicing	Rare	(45)
RP9/PAP1	Dominant	Splicing	Rare	(46,47)
SNRNP200	Dominant	Splicing	1-2%	(48-50)
DHX38	Recessive	Splicing	Unknown	(51)
NR2E3	Dominant & recessive	Transcription factor	1-2% & Rare	(52,53)
CRX	Dominant	Transcription factor	1%	(54,55)
ZNF513	Recessive	Transcription factor	<1%	(56,57)
RP1	Dominant & recessive	Tissue development & maintenance	3-4% & <1%	(58,59)
NRL	Dominant & recessive	Tissue development & maintenance	Rare & <1%	(60,61)
SEMA4A	Dominant	Tissue development & maintenance	Rare (3-4% in Pakistan)	(62)
FAM161A	Recessive	Tissue development & maintenance	<1%	(63,64)
TULP1	Recessive	Tissue development & maintenance	<1%	(65,66)
CRB1	Recessive	Tissue development & maintenance	6-7% in Spain	(67,68)
RP2	X-linked	Tissue development & maintenance	10-20% (X-linked)	(69,70)
ARL2BP	Recessive	Photoreceptor maintenance & function	Unknown	(71)
IMPDH1	Dominant	Regulates cell growth	2-3%	(2,72)
USH2A	Recessive	Cellular structure	10-15%	(73,74)
FSCN2	Dominant	Cellular structure	Rare (3% in Japan)	(75,76)
ROM1	Dominant	Cellular structure	Rare	(77,78)
IMPG2	Recessive	Cellular structure	<1%	(79,80)

Table 1 (*continued*)

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Gene˜	Inheritance*	Potential function	Estimated frequency	Studies
MAK	Recessive	Cellular structure	<1%	(81,82)
PROM1	Recessive	Cellular structure	<1%	(83,84)
PRPH2	Dominant	Photoreceptor OS structure	5-10%	(85-87)
CLRN1	Recessive	Photoreceptor structure	<1%	(88)
DHDDS	Recessive	Photoreceptor structure	<1%	(89,90)
MERTK	Recessive	Transmembrane protein	<1%	(91,92)
TTC8	Recessive	Transmembrane protein	<1%	(93)
ARL6	Recessive	Transmembrane protein	<1%	(94,95)
BEST1	Dominant & recessive	Anion channel	Rare & <1%	(96,97)
RPGR	X-linked	Intraflagellar transport	70-90% (X-linked)	(4,98,99)
KLHL7	Dominant	Ubiquitin—proteasome protein degradation	1-2%	(100,101)
TOPORS	Dominant	Ubiquitin-protein ligase	1%	(102,103)
EYS	Recessive	Cell signalling	Common in China (10-30% in Spain)	(104,105)
CERKL	Recessive	Cell signalling	3-4% in Spain	(106,107)
KIZ	Recessive	Cell division	<1% in North African Sephardic Jews	(108)
NEK2	Recessive	Cell division	Unknown	(109)
CA4	Dominant	Unknown	Rare	(110,111)
C2orf71	Recessive	Unknown	<1%	(112,113)
C8orf37	Recessive	Unknown	<1%	(114,115)
PRCD	Recessive	Unknown	<1%	(116,117)
SPATA7	Recessive	Unknown	<1%	(118,119)
EMC1	Recessive	Unknown	Unknown	(120)
GPR125	Recessive	Unknown	Unknown	(120)
KIAA1549	Recessive	Unknown	Unknown	(120)
SLC7A14	Recessive	Unknown	~2%	(121)

˜, adapted from sources http://www.ncbi.nlm.nih.gov/books/NBK1417/ & https://sph.uth.edu/Retnet/; *, dominant, autosomal dominant; recessive, autosomal recessive.

Mutations that lead to recessive forms of inheritance are suggested to still confer a phenotype in the heterozygous state, however it is milder or with onset later in life (124). The severity of the phenotype appears to depend on the location of the mutation in the protein. Patients with mutations leading to abnormal amino acids in the parts of rhodopsin located in the intradiscal space show a less severe phenotype with better visual acuity and improved dark light adaptation, compared with those where mutations affect amino acids in the cytoplasmic space. Patients with mutations occurring within the transmembrane regions of rhodopsin showed an intermediate outcome (125). Intrafamilial variation is also noted amongst patients indicating the likely presence of genetic modifiers and/or environmental factors contributing to the phenotypic effects that are seen (124).

Other RP disease genes implicated in the phototransduction process have an expected frequency of less than 2-5% amongst affected individuals. Autosomal dominant inheritance is observed due to mutations in *RDH12*, encoding retinol dehydrogenase-12, which is responsible for metabolising all-trans and -cis retinols (14). *GUCA1B* encodes guanylate cyclase-activating protein 2, which is responsible for activating photoreceptor guanylate cyclases for the conversion of cGMP to cGTP involved with the

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hyperpolarisation response to light (13). Mutations in this gene lead to autosomal dominant RP. Autosomal recessive inheritance is seen associated with mutations in *PDE6A* and *PDE6B*, which encode phosphodiesterase 6A & 6B responsible for the α and β subunits respectively of a key enzyme that maintains cytoplasmic cGMP concentration crucial for rod cell phototransduction (16,18). Mutations in *CNGA1* and *CNGB1* also follow an autosomal recessive inheritance pattern and these genes encode the α and β subunits of cyclic nucleotide gated ion channels responsible for opening of calcium channels after binding of the cGMP/cGTP ligand (21,23). The *SAG* gene encodes the arrestin protein responsible for the inactivation of the phototransduction cascade, specifically acting on the activated rhodopsin molecule. While mutations in *SAG* are implicated in a form of CSNB, discussed below, a homozygous 1-bp deletion (c.1147delA) has been observed in three unrelated individuals with RP (20).

Mutations in RP disease-causing genes that encode proteins associated with retinal metabolism generally follow an autosomal recessive inheritance pattern. These include *ABCA4*, which encodes the ATP-binding cassette subfamily A member-4, a transmembrane protein that facilitates the removal of all-trans retinaldehyde from the photoreceptor (3); the *LRAT* gene which encodes lecithin retinol acyltransferase, an enzyme located in the RPE that initiates the reactions where all-cis retinal is derived from all-trans retinol (vitamin A) (28); the *RBP3* gene which encodes retinal binding protein 3 that is secreted from rod photoreceptors and responsible for the transportation of retinoids from the photoreceptor to the RPE and for the binding of fatty acids in the interphotoreceptor matrix (30); the *RGR* gene which encodes the G protein-coupled receptor retinal that is located in the RPE and preferentially binds all-trans retinal facilitating its conversion into 11-cis retinal (32); and the *RLBP1* gene which encodes retinaldehyde-binding protein 1 found in the RPE (33). *RPE65* that encodes retinal specific protein 65 kD is responsible for the conversion of all-trans retinyl ester to 11-cis retinol in the RPE. Mutations in this gene are most usually associated with a severe autosomal recessive form of RD called LCA (26). Interestingly, mutations in *RPE65* have also been observed segregating in an autosomal dominant pattern in RP, highlighting further the variation of outcomes from mutations within the same gene (27).

Genes encoding splicing factors have also been implicated in the expression of an RP phenotype and all follow an autosomal dominant inheritance pattern. These

genes include *PRPF31*, *PRPF8*, *PRPF3*, *PAP1*, *SNRNP200* and *PRPF6* (38,41,42,45,46,48). It is interesting that despite deficiencies in splicing having an effect on processes of the entire cell, mutations in the above-mentioned genes only confer an RP phenotype, and there are various reasons considered for this. These include that the splicing factor affected acts specifically on genes which are expressed in the retina, such as *RHO* (126), or that the splicing of genes outside the retina may be affected, but the phenotype is only evident in the retina due to its specific rapid turnover requirements (127). Some genes may be important in spliceosome assembly and maturation, and studies are ongoing to understand the mechanisms causing the splicing abnormalities (128,129).

Retinal tissue development, differentiation and maintenance is critical for proper photoreceptor function, and mutations in genes encoding factors critical in these processes can cause RP including: *RP1*, *NR2E3*, *CRX*, *NRL*, *SEMA4A*, *FAM161A*, *TULP1*, *RP2*, *CRB1* and *IMPDH1* (2,52,58,60,62,63,65,67, 69,130). A subgroup of these genes, namely *CRX*, *NRL* and *NR2E3* are known to interact during retinal neurogenesis. *CRX* is a transcription factor that regulates retinal cellular differentiation and also plays a role in the maintenance of neural bipolar cells in the adult retina (54). Interestingly a p.Arg41Gln missense mutation has been shown to cause RP in one study while being associated with cone-rod dystrophy (CORD) in another (55,131). *NRL* encodes a neural retina leucine zipper transcription factor and *NR2E3* encodes a ligand-activated transcription factor. These are exclusively expressed in rod photoreceptors and are required for their differentiation during retinal development (52,60).

The functions of the photoreceptors can only take place through specialised cellular structures and development and maintenance of these are critical for normal retinal function. Mutations in genes encoding proteins in this group that have been associated with autosomal recessive RP include: *PROM1*, *MAK*, *IMPG2*, *DHDDS*, *CLRN1*, and *USH2A* (73,79,81,83,88,89) while genes associated with autosomal dominant RP include: *FSCN2*, *ROM1* and *PRPH2* (75,77,85,86). *PROM1* encodes Prominin-1, which is a highly conserved protein across the animal kingdom that plays a crucial role in disc membrane morphogenesis in rod photoreceptors (83). *MAK* encodes the male germ cell-associated kinase involved in regulation of cilium length that connects the outer segments to the cell bodies within the photoreceptor (81). The interphotoreceptor matrix proteoglycan is encoded by the gene *IMPG2*, and is a part of the extracellular matrix which connects the photoreceptor

Table 2 Disease genes: human congenital stationary night blindness

Genes⁻	Inheritance*	Phenotype	Studies
GNAT1, PDE6B, RHO	Dominant	Night blindness	(138-140)
CABP4, CACNA2D4	Recessive	Incomplete night blindness	(141,142)
GRM6, TRPM1	Recessive	Night blindness	(137,143-145)
SAG, GRK1	Recessive	Oguchi disease	(146,147)
CACNA1F	X-Linked	Incomplete night blindness (CSNB2)	(148,149)
NYX	X-Linked	Night blindness (CSNB1)	(150)

⁻, adapted from source https://sph.uth.edu/Retnet/; *, dominant, autosomal dominant; recessive, autosomal recessive.

outer segments to the RPE (79). *DHDDS* encodes a dehydrodolichyl diphosphate synthase, which localises to the inner segments of the photoreceptor and plays an important role in the glycosylation of rhodopsin (89). *CLRN1* and *USH2A* encode clarin-1 and usherin, respectively, and are both implicated in autosomal recessive forms of syndromic and non-syndromic RP. The clarin-1 protein contains five transmembrane domains that secure the protein into the plasma membrane and is thought to include roles in both hair cell and photoreceptor synapses (88). Usherin is a plasma membrane bound protein with a large extracellular domain, which performs structural and signalling functions through interactions with the cilium of photoreceptors and hair cells (73). Usherin knockout mouse models reveal progressive degeneration of the retina indicating usherin is involved in photoreceptor maintenance (132). Mutations in *CLRN1* and *USH2A* are predominantly associated with Usher syndrome, a disorder which presents with both retinal degeneration and sensorineural hearing loss (132,133).

BEST1 mutations are found in a group of RDs termed 'Bestrophinopathies' inherited in both autosomal dominant and recessive patterns (96,134). The BEST1 protein is predominately expressed in the basolateral membrane of the RPE and has been proposed to be an ion channel regulator interacting with calcium activated chloride channels (CaCC). The majority of missense mutations are found in the conserved N-terminal of the protein which contains the membrane spanning and calcium interaction domains (135).

Congenital stationary night blindness (CSNB)

CSNB is a non-progressive form of night blindness, also known as nyctalopia, where patients find it difficult to see in relatively low light intensities. Presentation onset is from an early age and can also include decreased visual acuity, myopia, nystagmus and strabismus (136). Photoreceptor function as measured by electroretinogram (ERG) may indicate absence of rod pathway function, or there may be incomplete rod and cone dysfunction. Varying abnormalities may be observed on fundus examination. Complete CSNB results from defects in bipolar cell signalling pathways, resulting in only one intact alternate pathway (137). There are currently 11 genes where mutations have been identified in patients with CSNB (*Table 2*). Known functions of these genes include roles in calcium channel function with respect to *CACNA1F*, *CACNA2D4* and *TRPM1* (137,141,148), calcium-binding in *CABP4* (142), glutamate receptor functions in *GRM6* (143) and involvement in the phototransduction cascade in *GNAT1*, *GRK1*, *PDE6B*, *SAG* and *RHO* (138-140,146,147).

The incomplete form of X-linked CSNB (CSNB2) is caused by disruption of the *CACNA1F* gene, which encodes the α1F subunit of calcium channels located at the synaptic connection between the rod photoreceptor and the bipolar cell (148,149). The calcium channel regulates the release of glutamate into the synaptic cleft depending on the membrane potential of the bipolar cell or photoreceptor. It has been shown that loss of function mutations of the gene disrupt the calcium ion flow at this synapse resulting in a non-functional channel and therefore loss of function of the photoreceptor (151). The CSNB2 phenotype that is observed includes a diminished scotopic b-wave on ERG, indicating a diminution in the signalling between rod photoreceptors and bipolar cells (149). There have been more than 50 reported mutations in this gene that result in various forms of protein truncation and calcium channel loss of function (136,152).

The complete form of X-linked CSNB (CSNB1) is caused by mutations in the gene *NYX*, which is predominantly expressed in the retina. Although the exact mechanism is yet to be known, the encoded protein nyctalopin is a small leucine-rich proteoglycan that when mutated is believed to disrupt bipolar amacrine and ganglion cell signalling (150). Truncated proteins are typically non-functional and would be expected to cause the phenotype observed. The CSNB1 phenotype is distinguished from CSNB2 with ERG as both rod and cone photoreceptor

function is affected (150). Deletions within the gene have also been observed in mice which have resulted in the no b-wave ("nob") phenotype (153).

Autosomal recessive forms of CSNB can be broken up into 3 clinical sub-types including incomplete forms, complete forms and Oguchi disease. Oguchi disease is clinically distinct from the other autosomal recessive CSNBs due to characteristic ERG abnormalities including an absent rod response with normal cone responses. Fundal changes known as the Mizuo-Nakamura phenomenon are also observed whereby during light exposure and adaption the retina has a metallic, golden brown appearance. This appearance disappears however after the retina is returned to a complete dark adaptation (154). There are now two forms of Oguchi disease known, type 1 and type 2, which are caused by mutations in *SAG* and the G-protein dependent receptor kinase 1 gene *GRK1* respectively. Investigations into a cohort of Japanese patients indicated that arrestin and rhodopsin kinase genes are possible candidates for the phenotype due to their association with the inactivation of rhodopsin in the recovery phase of phototransduction (146). A *SAG* c.1147delA mutation was observed more frequently in affected unrelated Japanese individuals, which reflects a founder effect (147). Mutations in *SAG* have also been found in patients with an RP phenotype, suggesting variable expression associated with mutations within the gene (20). *GRK1* mutations are mainly reported to cause an Oguchi phenotype, although there have also been reports of an RP phenotype (155).

Autosomal dominant forms of CSNB are caused by mutations in *GNAT1*, *PDE6B*, and *RHO*, all of which are involved in aspects of the phototransduction cascade (138,139,146). *GNAT1* encodes Guanine nucleotide-binding protein α, α-transducing activity polypeptide 1 which assembles the α subunit in the rod transducing protein that stimulates the coupling of rhodopsin and GMP during the photoreceptor visual response. *PDE6B* codes for the β subunit of a membrane bound enzyme, phosphodiesterase 6B, that is responsible for the hyperpolarization of the rod photoreceptor. Mutations in *RHO* are more usually seen in patients with autosomal dominant RP but have also been identified in CSNB depending on the amino acids that are affected (156). One study identified the *RHO* c.884C>T:p.Ala295Val missense mutation in a family with autosomal dominant CSNB, where patients had a diminished sensitivity and response to light at lower intensities without any additional retinal degeneration (157).

Cone and cone-rod dystrophies

Cone and cone-rod dystrophies present as more severe compared with the rod or rod-cone dystrophies, as it is high acuity vision and the perception of colour that is lost. Nystagmus and photophobia also occur, and in the cone-rod dystrophies, complete blindness occurs in the later stages because the rod photoreceptors also undergo degeneration (158). As in the rod dominated dystrophies, progressive and stationary forms of cone and cone-rod dystrophies may occur.

Progressive cone and cone-rod dystrophies

The onset of progressive cone (COD) and CORD is usually during early childhood or adolescence. Affected individuals usually present with only cone photoreceptor involvement (COD) or cone followed by rod degeneration (CORD). Differences between the two dystrophies are apparent with additional rod involvement leading to an increase in severity with most sufferers reaching legal blindness by the age of 40 (158). On fundus examination, appearance of the macula varies with some cases presenting with an atrophic appearance (*Figure 3*), or retinal pigment deposits. Currently there are over 30 genes described with reported disease-causing mutations (*Table 3*), with various roles in similar functional groupings as those described for the rod dystrophies, but with specificity of function to the cone photoreceptors. As shown in previous review of the literature, molecular causes are able to be identified in approximately 20% and 74% of autosomal dominant and X-linked COD/CORD respectively, while 23-25% of autosomal recessive pedigrees can be resolved (200). This indicates that many underlying disease genes remain to be identified especially in the autosomal dominant and recessive forms of these conditions.

Subgroups of genes where mutations lead to COD and CORD are also associated with other forms of RD, presumably based on their relatively ubiquitous functions across the photoreceptors and/or retina. As an example, *ABCA4* mutations are further implicated in RP, and a form of juvenile macular degeneration also known as Stargardts disease (3,201). *ABCA4* is the most frequently identified disease gene in COD and CORD of autosomal inheritance with studies reporting its frequency in 9% and 26% of cases respectively (158). Furthermore, truncating mutations involving *ABCA4* are more evident in the CORD cohort
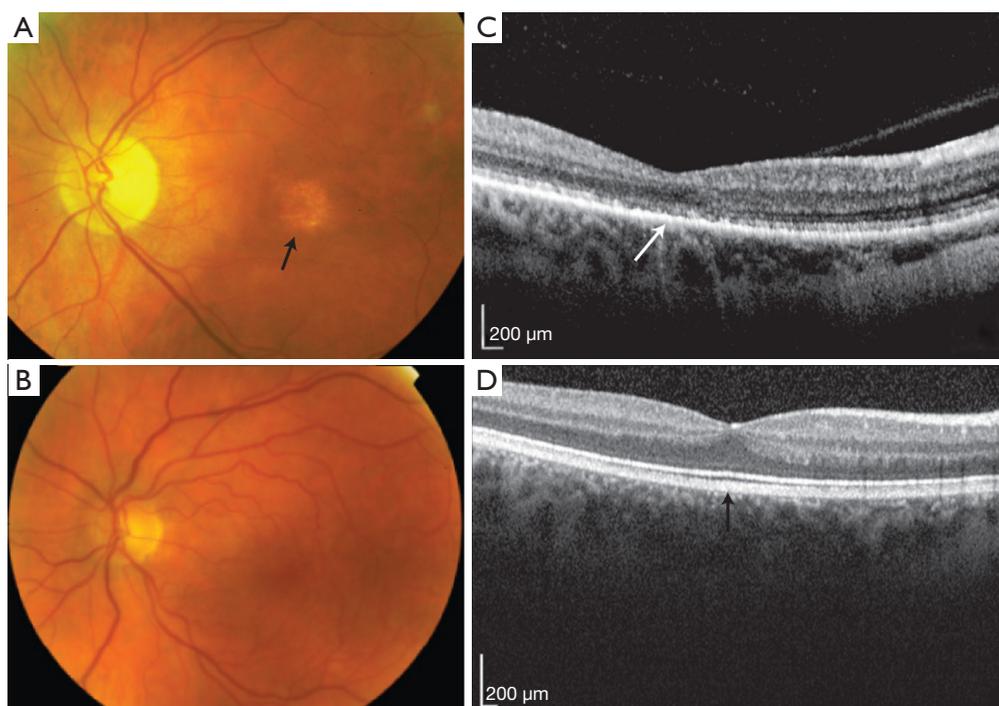
Figure 3 Cone dystrophy: fundal images. (A,B) Wide field fundus photography illustrating retinal features of cone dystrophy, showing macular atrophy in (A) (arrow), compared with the normal macular appearance present in (B); (C,D) OCT in cone dystrophy illustrates the loss of the foveal photoreceptor outer segments in (C), compared with normal in (D) (arrows). OCT, optical coherence tomography.

compared to the COD cohort, reportedly seen in 76% and 63% respectively (158,200).

Achromatopsia

The stationary forms of cone dystrophy can exist in two forms whereby complete or incomplete achromatopsia results in the loss of all colour perception, or the perception of only a specific colour respectively. Tritanopia, or defective blue vision is an autosomal dominant phenotype that is caused by mutations in the gene *OPN1SW*, which encodes the short wave sensitive opsin that detects blue light (202). Genes currently known to be associated with autosomal recessive complete achromatopsia include *CNGA3*, *CNGB3*, *GNAT2*, *PDE6C* and *PDE6H*. *CNGB3* alone is responsible for up to 50% of complete achromatopsia in affected individuals (203). *CNGA3* and *CNGB3* encode the α and β subunits of cGMP-gated channels located in the cone photoreceptor, which are involved in key steps of phototransduction (204,205). *GNAT2* encodes the cone-specific α subunit of transducin, which is the cone visual pigment that induces one of the first steps of the phototransduction cascade (206). *PDE6C*

and *PDE6H* encode the cone specific α and gamma subunits of a cGMP phosphodiesterase respectively, which is an enzyme responsible for the conversion of cGMP to 5'-GMP during light exposure (163,164).

The genetic overlap across the non-syndromic RDs including RP, COD/CORD and the stationary forms is quite complex and is illustrated in *Figure 4*. Genetic overlap is expected to a certain degree due to fundamental similarities in photoreceptor structures and cellular processes despite independence of their scotopic and photopic roles.

Generalised non-syndromic RDs

RDs involving the simultaneous degradation of both rod and cone photoreceptor functions are termed generalized RDs. The majority of cases present with progressive, often severe, deterioration of vision. Both syndromic and non-syndromic forms exist.

Leber congenital amaurosis (LCA)

The most common non-syndromic generalised RD is

Table 3 Disease genes: human cone and cone-rod dystrophy

Gene[~]	Inheritance[*]	Potential function	Studies
GUCA1A	Dominant	Phototransduction	(159,160)
GUCY2D	Dominant	Phototransduction	(161,162)
PDE6C	Recessive	Phototransduction	(163)
PDE6H	Recessive	Phototransduction	(164,165)
CNGB3	Recessive	Phototransduction	(166,167)
PRPH2	Dominant	Phototransduction	(168,169)
ABCA4	Recessive	Retinal Metabolism	(170,171)
RDH5	Recessive	Retinal metabolism	(172,173)
CRX	Dominant	Transcription factor	(130,131)
RAX2	Recessive	Transcription	(174,175)
RPGRIP1	Recessive	Interacts with RPGR	(176)
ADAM9	Recessive	Cell/matrix interaction	(177)
AIPL1	Dominant	Transport, protein trafficking	(178,179)
TTLL5	Recessive	Cilia function	(180)
CACNA1F	X-Linked	Calcium channel	(181)
CACNA2D4	Recessive	Ion channel	(142)
HRG4	Dominant	Neurotransmitter release	(182)
KCNV2	Recessive	Ion channel subunit	(183,184)
RIMS1	Dominant	Neurotransmitter release	(185,186)
PITPNM3	Dominant	Transport	(187)
SEMA4A	Dominant & recessive	Axon guidance	(62,188)
CERKL	Recessive	Cell signalling	(189)
RPGR	Dominant & recessive	Intraflagellar transport	(190,191)
PROM1	Dominant	Cellular structure	(192,193)
CDHR1	Recessive	Cellular structure	(194,195)
C21orf2	Recessive	Unknown	(120)
C8orf37	Recessive	Unknown	(114,115)
CNNM4	Recessive	Unknown	(196,197)
RAB28	Recessive	Unknown	(198,199)

[~], adapted from source https://sph.uth.edu/Retnet/; [*], dominant, autosomal dominant; recessive, autosomal recessive.

LCA. The onset of LCA is early with affected individuals developing symptoms within the first year of life. Clinical features include poor vision, nystagmus, and no measurable light response on ophthalmic examination with ERG (207).
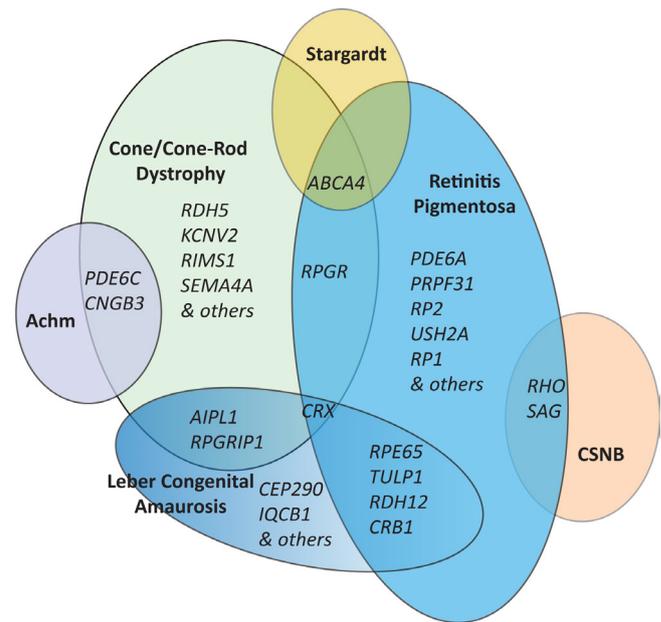

Figure 4 Genetic heterogeneity in retinal dystrophies. Diagrammatic representation of overlap between genetic causes of various forms of retinal dystrophy with some example genes shown. Achm, achromatopsia; CSNB, congenital stationary night blindness.

A characteristic finding is Franschetti's oculo-digital sign where patients repeatedly rub and poke their eyes. The physical appearance of the retina varies in early stages, however retinal pigmentary changes can be observed with progression of disease (207). Interestingly, there have been suggestions of genotype-phenotype patterns of retinal appearance including a translucent RPE appearance with white dots with *RPE65* gene mutations and progressive macular atrophy prominent in *NMNAT1*, but which is also seen in patients with *AIPL1* and *CRB1* mutations (208). To date there are over 20 genes associated with LCA with nearly all following an autosomal recessive inheritance pattern (*Table 4*). Despite this, the underlying genetic causes of LCA are not fully known (231), with genetic overlap with other forms of RD present (*Figure 4*).

Choroideremia

The only X-linked form of non-syndromic generalised RD is choroideremia, which is caused by mutations in the gene *CHM* (232). Patients present during the second decade of life with night blindness and progressive degeneration of

Table 4 Disease genes: Leber congenital amaurosis

Gene~	Inheritance*	Potential function	Estimated frequency	Studies
GUCY2D	Recessive	Phototransduction	6-21%	(209,210)
RDH12	Recessive	Phototransduction	~4%	(14,15)
LRAT	Recessive	Retinal metabolism	<1%	(28,211)
RPE65	Recessive	Visual cycle	3-16%	(209,212)
RD3	Recessive	Splicing	Rare	(213,214)
CRX	Dominant & Recessive	Transcription factor	~3%	(215,216)
OTX2	Dominant	Transcription factor	Rare	(217)
CRB1	Recessive	Tissue development and maintenance	10%	(209,218)
TULP1	Recessive	Tissue development & maintenance	1-2%	(209,219)
IMPDH1	Dominant	Regulates cell growth	Rare	(72,220)
GDF6	Recessive	Growth factor	Unknown	(221)
CABP4	Recessive	Cell signalling	Unknown	(222)
AIPL1	Recessive	Transport, protein trafficking	4-8%	(178,209)
CEP290	Recessive	Centrosomal & ciliary protein	<30%	(223)
IQCB1	Recessive	Interacts with RPGR & connecting cilia	Unknown	(224)
LCA5	Recessive	Centrosome protein with ciliary function	1-7%	(225,226)
NMNAT1	Recessive	Photoreceptor maintenance	5%	(208,227)
RPGRIP1	Recessive	Interacts with RPGR	~5%	(209,228)
KCNJ13	Recessive	Potassium channel	Unknown	(229)
DTHD1	Recessive	Unknown	Unknown	(120)
SPATA7	Recessive	Unknown	2%	(119,230)

~, adapted from sources http://www.ncbi.nlm.nih.gov/books/NBK1298/ & https://sph.uth.edu/Retnet/); *, dominant, autosomal dominant; recessive, autosomal recessive.

photoreceptors, the RPE and the choroid. Characteristically, affected males have an appearance of chorioretinal scalloped atrophy in the midperipheral fundus. *CHM* encodes REP-1, a subunit of the intracellular trafficking protein rab protein 1 which is responsible for the intracellular transport of proteins and organelles (233). Multiple non-synonymous mutations along with insertions and deletions have been reported to be associated with the disease (234). Heterogeneity is evident among choroideremia patients with some atypical presentations being first identified as RP on clinical examination. Studies using genomic tools to provide molecular diagnosis are proving useful in refining clinical diagnosis (235).

This review of clinical features and underlying genetic causes in the non-syndromic degenerative and stationary RDs including RP, COD/CORD, LCA, CSNB and achromatopsia indicates that while there are specific groupings, there is also a degree of clinical overlap and genetic causes in these conditions (*Figure 4*). Knowledge of these overlapping clinical features and genetic causes is important in design of molecular diagnostic approaches, molecular genetic result interpretation and work towards therapies in these conditions.

Molecular diagnosis and NGS

Providing a molecular diagnosis in RD is challenging due to the large numbers of genes responsible, variable expression, incomplete penetrance, oligogenic inheritance and frequent clinical and genetic overlap, as discussed above. The value of traditional technologies such as Sanger sequencing for detecting mutations in diseases with high genetic heterogeneity is limited, due to the large amounts of time and labour required to individually sequence many genes and the consequent high costs. Other technologies, such as APEX genotyping microarray chips, can examine multiple variants in multiple genes simultaneously and have provided some advantages in detecting genetic

aberrations (236). This technology has been applied to several forms of RD including non-syndromic CORD and syndromic forms of RD such as Usher syndrome (237,238). However, the APEX technology has limited resolving power as the genotyping array only detects a fixed number of mutations from a fixed number of genes. In RD, many of the causative mutations are novel, so the value of a chip with a limited number of known mutations is limited. The contribution of copy number variation (CNV) in RD is yet to be fully explored, with only limited studies so far reported using low resolution comparative genomic hybridisation (CGH) arrays or limited targeted approaches such as MLPA in specific genes (6,239). These technologies are limited in RD diagnosis due to the low effective mean resolution or relatively small number of targets that can be realistically examined.

The advent of NGS or massively parallel sequencing (MPS) has seen a rapid increase in the amount of genetic information that can be examined in a single sequencing assay. NGS is a relatively new technology that allows sequencing of targeted exonic regions, whole exomes and whole genomes of patients in a fast and relatively cheaper manner than ever before (240). The ability to simultaneously sequence regions in parallel allows for hundreds or thousands of sequencing fragments or 'reads' to cover a single region. This approach achieves accurate large scale sequencing, resulting in possible applications in a diagnostic setting (241). The use of NGS technologies for CNV detection is yet to be fully explored and appears to be a promising method of examining the whole genome at a much higher resolution than previously possible. NGS approaches can be used to detect CNVs via the quantification of the number of reads; however this relies heavily on the depth of coverage and the quality of data (242). Various bioinformatics tools have been designed to aid in the analysis of the large amounts of data generated from NGS (243,244). Some of these including those for: alignment (245), variant annotation (246) and conservation (247) are publicly available or in commercially distributed software packages (245,246). Powerful web tools such as 1,000 genomes (248) allow for minor allele frequencies (MAF) to be obtained while others such as SIFT (249), MutationTaster (250) and PolyPhen2 (251) generate pathogenicity prediction scores, which are essential in prioritising the often extensive list of variants detected.

In the last 5 years, efforts have been focused on applying NGS technology to mutation detection in RD (241,252). Some groups have opted to develop their own purpose-built panels which sequence a predetermined list of known disease genes. This approach provides a significant advance over the previous laborious gene by gene Sanger sequencing approach. It is also an advance on APEX technologies which only examined specific variants in specific genes. It has provided capacity for variant detection in approximately 50% of patients with RP where previously most patients were unable to receive a molecular diagnosis because of the cost and inefficiencies of the previous detection methods (123). By only examining known disease genes in a predetermined list, this approach reduces the number of variants detected, therefore only providing information where a clinical interpretation can be made with relative assurance (252,253). However, novel disease genes in the RDs are being discovered at a rapid rate, so this approach is limited in its flexibility to include more disease genes as they are discovered. In addition, these approaches are limited in their ability to detect variants in regulatory regions that are not included in the targeted strategy or may be less efficient in detection of copy number and structural variants (242).

A more broad-based NGS strategy such as whole exome sequencing (WES) or whole genome sequencing (WGS) can provide an enhanced method of investigation of disease gene regions over a predetermined targeted sequencing and capture approach (254-256). Variants in non-relevant genes can be filtered using bioinformatics strategies to reduce incidental findings. WES and WGS provide the flexibility to examine newly identified disease genes since all genes are captured and the bioinformatic filter can be modified to include examination of novel disease genes. WGS provides additional capacity in identification of copy number and structural variants (257). With multiple commercial companies investing in various types of NGS technology, competition is encouraging regular product improvements, enhancements and lowering of costs, making this a viable diagnostic technology.

Therapies and future directions

Advances in stem cell and genome editing technologies are the catalysing factors in the development of gene-based therapies and treatment options in RD. Various treatment strategies investigating the applications of gene based technologies, cell based therapies and retinal implant or transplantation are actively being sought in the RD's. The type of RD, the extent of retinal deterioration and the cellular structures or processes affected ultimately determine the appropriateness of which treatment strategy is applied.

Table 5 Examples of therapies applied in the retinal dystrophies

Approach	Context	Strategy summary	Studies
Gene therapy	Human clinical trial	*RPE65* delivered by Adeno-associated Virus (AAV)	(263)
	Mouse model	*CHM* delivered by Adeno-associated Virus (AAV)	(264)
	Human clinical trial	*CHM* delivered by Adeno-associated Virus (AAV)	(270)
	In vitro model	*CEP290* delivered by Lentivirus vector	(271)
	Rat model	*MERTK* delivered by Adeno-associated Virus (AAV)	(272)
	Mouse model	CRB1 and *CRB2* delivered by Adeno-associated Virus (AAV)	(273)
Cell replacement	Rat model	Human RPC transplantation restores mature rat retina cells	(274)
	Mouse model	Adult mouse iPSC differentiate into photoreceptors	(267)
	Mouse model	RPCs differentiate and integrate into functional photoreceptors	(269)
	Mouse model	RPCs differentiate into photoreceptors	(268)
Retinal implants	Clinical trials	Argus II Retinal Prosthesis System	(260,261)

The rate and amount of disease progression is the limiting factor determining the therapeutic approach which is likely to be successful. Improvements in diagnostic technologies and the understanding of molecular pathogenesis are expected to decrease the time needed to reach a molecular diagnosis, and therefore initiate a treatment before subsequent disease progression. As an example, clinical trials of *RPE65* gene replacement therapy delivered via viral vectors have shown promise, with children affected by LCA followed for three years post treatment all showing improvements in rod and cone photoreceptor function (258). In patients with more advanced disease progression, it is likely that a gene therapy approach will not be as effective due to the extent of deterioration. In these cases, a cell replacement or retinal implant approach is likely to be more appropriate, whereby stem cells or a visual prosthesis are delivered to the diseased retina.

Retinal implants or prosthetics are an area actively being explored with the potential of treating the RDs. Also sometimes referred to as 'bionic eyes', patients have electronic devices inserted into the retina and utilise the remaining intact neural network to transmit the signals to the visual centres of the brain. The detection of light is performed via a light sensing microchip inserted into the central or peripheral visual field. Implantation of an electrode into the neural layers of the retina enables a connection bridge to the existing neural network (259). Due to the rather intrusive nature of surgery required, applications are often restricted to preserve any remaining limited vision the patient may have (260). This technology has been successfully applied in RP patients with varying stages of disease progression and has resulted in improvements to light detection and some restoration of visual perception (260,261).

More than a decade ago there was success in gene replacement therapies applied to RD using animal models (262). More recently there has been progress in human trials, specifically in the delivery of functional cDNA to the retina via adeno-associated virus (AAV) vectors (263-265). There is also hope in the prospect of use of pluripotent stem cells in replacing disease-affected retinal cells. Stem cells are capable of differentiating into specific retinal cell types and an unlimited number of identical daughter cells can be produced. Work is progressing in delivery of these cells via injection to the accessible eye. Embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and retinal progenitor cells (RPCs) are examples of stem cells that have been applied, and have demonstrated regeneration in diseased retinal mouse models (266-268). The use of ESCs is practical and documented in mouse models with success shown by transplantation and restoration of retinal function (269). However the expansion of both ESC and RPC applications using human-derived cells is limited due to ethical considerations. In contrast, iPSCs are derived from child or adult fibroblasts and therefore have the advantage of increased availability, and since they can be derived from an affected patient, there is a reduced risk of host immune system rejection. IPSCs can be differentiated to retinal cells and can deliver healthy retinal cells into the diseased retina in the mouse, facilitating the repair and restoration of function (267). Examples of successful gene therapy, cell replacement and retinal implant strategies are illustrated in *Table 5*.

Further development of iPSC applications for

treatment of RD in human patients, requires use of efficient genome engineering to reverse or alter the mutation. Advances in genome editing tools have allowed *in vitro* DNA modification to be as precise as to the single base pair level (275). This has important uses in illustrating the mechanisms and pathophysiology of genetic disease with the induction of targeted mutations, while also aiding in the development of treatments and therapies with the modification of pathogenic mutations back to normal. The most promising of these technologies is the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, whereby precise genomic regions can be targeted through easily synthesised guide RNA (276). The system then edits the genome through inducing double stranded breaks (DSBs) and subsequent homology-directed repair (HDR) at a precise location, resulting in the newly modified target still being in its same position in the genome so still under the influence of its endogenous control elements such as promoters, enhances and repressors. This is particularly important as it prevents incorrect or inappropriate levels of expression of the newly modified gene. Recently, the specificity of the CRISPR/Cas9 system approach has been improved with the modification of the Cas9 nuclease to induce only a single strand break, or nick, as opposed to DSBs where significant misalignment and pairing has been reported. The single strand break approach allows for the endogenous base excision repair pathway to facilitate repair and results in more specific and efficient modification (277).

Application of the CRISPR/Cas9 system in conjunction with stem cell technologies is likely to pave the way for 'precision medicine' and catalyse the future understanding and treatment of genetic disease. It is anticipated that future work will be more individualised, whereby a patient has a blood and skin sample collected for the identification of the pathogenic mutation and the generation of a fibroblast cell line. Once the mutation is identified, the effect of therapy and treatment options can be observed in the fibroblast cell line to examine their efficiency. This could be expanded to include the development of iPSCs from the patient fibroblast line, genome editing to correct the DNA mutation, differential to retinal cells and transplant into the affected retina (278). In highly heterogeneous genetic diseases such as RD where the underlying genetic cause is likely to be unique, this approach is one of the most promising avenues of future research and exploration.

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