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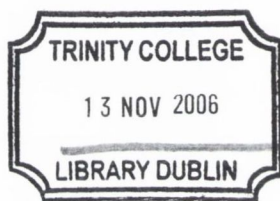
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Exploration of novel therapeutics for Autosomal Dominant Retinitis Pigmentosa

A Thesis Submitted to the University of Dublin for the Degree of Doctor
of Philosophy

by

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THESIS
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DECLARATION

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Thérèse Cronin

January 2006

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Abstract

This thesis is concerned with exploration of a suppression and replacement therapeutic approach for the dominant form of Retinitis Pigmentosa (ADRP). Retinitis Pigmentosa is the most heterogeneous of all hereditary disorders and as such presents special challenges as a disease model for intervention by molecular therapies. The main focus of this thesis has centred on the gene encoding the opsin pigment, rhodopsin - the source of many ADRP-causing mutations. Essentially siRNA-expressing vectors have been employed to downregulate transcript levels from this gene, and the effect has been assessed in transformed cell lines as well as in a retinal cell culture model.

At the same time, attempts have been made to restore the functional gene product of the rhodopsin gene by delivering an artificial replacement rhodopsin gene. Translated protein derived from this replacement gene should be identical to the endogenous wild-type rhodopsin protein, with the added property of immunity to the suppressing agent. Due to its mutation-independent nature, this strategy is of particular value for ADRP - rather than targeting the source of the primary mutation, the suppression agent targets any degenerate region of gene-sequence that can be altered in a replacement gene sequence. Thus, in principle, a photoreceptor cell of the retina may be relieved of the pathogenic mutation and at the same time have normal function restored. Furthermore, this 'suppression and replacement' approach is applicable to many human autosomal dominant mendelian disorders where the number of potential mutations causing a single disease may make mutation-specific therapies less suitable for intervention.

Chapter 3 is focused on the potency and specificity of a series of siRNAs targeting the first exon of the human rhodopsin gene. This RNAi assay was carried out against a rhodopsin target cotransfected in mammalian cell culture with presynthesised siRNA molecules. Following on from this, hairpin vectors were engineered to express the most effective siRNA sequences and stable gene silencing of rhodopsin was assessed using shRNAs in three transformed cell lines. RNA and protein down-regulation was measured in these cell lines. Dose curves of RNAi activity and toxicity levels within the cells following siRNA delivery were also examined *in vitro*. The

suppression achieved by one particular siRNA, termed siC, was further examined against an endogenous human rhodopsin target in transgenic animals. The NHR+/- (MouRho-/-) mouse model carries the normal human rhodopsin gene on an endogenous mouse rhodopsin knockout background. Primary retinal cell culture, derived from the retinas of neonatal mice of the NHR+/- (MouRho-/-) genotype, was used as an experimental system in which to test siC-based silencing of human rhodopsin transcripts in the retina. FACS analysis combined with real-time rtPCR, as well as immunostaining of dissociated explant cells and explant sections were the techniques employed to determine suppression of RNA by siC and to determine the resulting human rhodopsin protein levels in this animal model.

Chapter 4 is focused on the resistance of an artificial rhodopsin gene to the RNAi-mediating agents. This replacement gene carries five nucleotide changes from the normal human rhodopsin target. Two of these changes are covered by one of the selected siRNAs, siB, and five changes are covered by a second selected siRNA, siC. The replacement rhodopsin gene expression and protein levels were assessed *in vitro* following cotransfection of the replacement rhodopsin cDNA with the siRNAs (where the siRNAs are in the form of both presynthesized molecules and as shRNA molecules). A transgenic mouse expressing this replacement rhodopsin gene was available for an assessment of the resistance of the replacement rhodopsin to the siRNAs in the retina. Similar assays to those carried out in chapter 3 were used for this evaluation in explant cell culture derived from neonatal retinas of the transgenic mice (i.e. FACS analysis, real-time rtPCR and immunostaining of dissociated cells and retinal sections). As such, the resistance of a replacement human rhodopsin transgene to shRNA-mediated silencing could be determined in the retinal environment.

Chapter 5 focuses on further examination of the siRNA-mediated silencing of rhodopsin gene expression in an animal model of ADRP, the pro23his mouse. However the rapid degeneration in this model made it somewhat less suitable for demonstrating therapeutic intervention. The consequences of this were considered for future suppression studies using the pro23his mouse model. An alternative vector for stable expression of siRNA molecules in the retina was also considered in this chapter.

A hairpin loop derived from an endogenous microRNA was incorporated into the original shRNA vector to determine whether this hairpin modification might enhance the level of silencing specific to normal human rhodopsin (in the region altered in the replacement gene of chapter 4). The rationale behind this is that the microRNA loop may be more efficiently processed by the cell and result in more potent gene silencing. In addition, this chapter presents data on attempts to suppress a second gene, the human peripherin/RDS gene. This gene encodes a structural protein of the photoreceptor cell, and, as for the rhodopsin gene, is a common source of mutations leading to ADRP. The suppression tools used in this case were hammerhead ribozymes. The ribozymes were cotransfected with a human peripherin/RDS cDNA into mammalian cell culture to determine their effect on peripherin expression levels *in vitro*. The efficacy of the hammerhead ribozyme as a suppression tool *in vitro* is considered in light of the potent silencing that can now potentially be achieved with siRNA.

Chapter 6 is concerned with the use of delivery agents for stable siRNA expression. At the preliminary stage of this study, non-viral delivery methods were investigated, however subsequently a viral approach was adopted. The Φ c31 integrase enzyme was used in conjunction with shRNA-expressing plasmids, which were engineered to carry the attachment sequence required for a homologous integration event to occur in mammalian cells. An initial evaluation of this vehicle was carried out *in vitro*. At this time, AAV viral-delivery technology became established within the lab. The reported successes of AAV viral vectors for retinal-specific transgene-delivery, led to the use of this vector for assessment of AAV-mediated gene expression *in vivo*. The photoreceptor-specific expression of the AAV2/5 serotype (expressing a reporter EGFP transgene) was assessed over a 42-day period. In addition, the shRNA cassette used in previous chapters for suppression and replacement studies on the rhodopsin gene was cloned into the transgene cassette of the recombinant-AAV vector system. The down-regulation of rhodopsin expression from this viral-derived shRNA was determined *in vitro*. This leads up to future studies of AAV-mediated suppression and replacement gene therapy in animal models of ADRP, where heterogeneity of the disease at the molecular level has previously been a barrier to success.

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Abbreviations

ADRP	Autosomal Dominant Retinitis pigmentosa
RDS	Retinal Degeneration Slow
cGMP	Cyclic Guanine Monophosphate Dehydrogenase
CRALBP	Cellular Retinaldehyde Binding Protein
RPE	Retinal Pigment Epithelium
B-PDE	Beta-Phosphodiesterase
ARRP	Autosomal Recessive Retinitis Pigmentosa
ROS	Rod Outer Segment
CRX	Cone-Rod Homeobox
NRL	Neural Retina Leucine Zipper
IMPDH	Inosine Monophosphate Dehydrogenase
CSNB	Congenital Stationary Night Blindness
ERG	Electroretinogram
CNCG	cGMP-gated cation channel
EGFP	Enhanced Green Fluorescent Protein
GAPDh	Glyceraldehyde phosphate dehydrogenase
18SrRNA	18S ribosomal RNA
CMV	Cytomegalovirus
UTR	Untranslated Region
QrtPCR	Quantitative Reverse-transcriptase PCR
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescent Activated Cell Sorter
IRES	Internal Ribosomal Entry Site
RNAi	RNA interference
SiRNA	Small interfering RNA
ShRNA	Small hairpin RNA
ODN	Oligodinucleotide
AS	Antisense
RISC	RNA-induced silencing complex
ISG	Immune-stimulated gene
PKR	Protein Kinase Response
IFN	Interferon
dsRNA	Double-stranded RNA
RdRP	RNA-dependent RNA Polymerase
SMN	Survival of Motor Neuron
rAAV	Recombinant Adeno-associated Virus
ITRs	Inverted Terminal Repeats
OAS-1	OligoAdenylate Synthetase-1
Ifi- α	Interferon-inducible protein α

Chapter 1

Introduction

CHAPTER 1

1.1 Vision

The aim of this study is to investigate the application of a suppression and replacement strategy as a means of therapeutic intervention in the inherited retinal dystrophies. The retina, where the process of visual transduction is carried out and where the hereditary dystrophy Retinitis Pigmentosa (RP) occurs shall be discussed in this introduction. The unique features of the retina that challenge treatment strategies shall be considered throughout the thesis.

1.1.1 The Retina

The retina occupies the internal wall of the posterior segment of the eye, (fig. 1.1). This membrane is of neuro-ectodermal origin and can be divided into two sections, the neural retina, comprising the crucial photoreceptor cells and the retinal pigment epithelium (RPE). Subretinal space separates the neural retina from the RPE. Signal transduction is carried out within the seven-layered neural retina. Light enters via the ganglion cell layer (GCL) and penetrates various tissue layers before reaching the photoreceptor cells that amplify and process the signal, the rods and cones. The signal is then transported by the neurotransmitter glutamate from the photoreceptor cells to bipolar cells to ganglion cells and leaves the retina via the optic nerve. From here it is conducted to the brain where an image is formed. Despite the fact that photoreceptor cells are at the front-line in the processing of light signals, these cells lie at the back of the retina, therefore light rays must pass through many retinal layers before reaching their target sensory cells. The proposed reason for this awkward design feature of the retina is that the pigment-bearing membranes of photoreceptor cells must be in contact with the pigment epithelial layer of the eye, which provides these cells with the vital molecule retinal or vitamin A and discussed further in section 1.1.2. Retinal tissue is estimated to produce 30% of the sensory input to the brain (Ogden, 1989) hence degeneration of this tissue can cause profound change in the signals being carried by higher order neurons for processing.

1.1.2 Photoreceptors

Photoreceptor cells of the retina mediate vision. In humans, approximately 97% of these cells are rod photoreceptors and allow for vision in dim light, termed scotopic vision (Nathans, 1992). The remaining 3%, cone photoreceptors, are mainly clustered in the macula at the centre of the retina. These cells mediate detailed and colour vision operating in conditions of bright light, termed photopic vision. The variations in cone cell number and distribution among animal eyes reveal adaptations to the different environments in which they live. While fish, frog, turtle and bird retinas have three to five types of cones allowing for very good colour vision, most mammals have retinas in which rods dominate. Moreover the cones are often concentrated in specialised regions. In humans, the cone photoreceptor cells are concentrated within the cone dense macular region at the centre of the retina, devoid of rods, called the fovea maximising detailed vision. Eagles and hawks also possess a fovea, enabling the fine-focus and discrimination of images. In mammals such as squirrels and rabbits the corresponding cone-rich region of the retina is present as a long horizontal strip called the visual streak allowing them to detect the fast movement of predators.

The highly specialised photoreceptor cells absorb photons of light by means of rod- and cone-specific photopigments termed opsins. The change in opsin conformation that results from this absorption of light energy is the activating signal for phototransduction to occur. The phototransduction cascade amplifies the signal until it reaches the second order neurons and ultimately the brain. It is in the rod-dominated peripheral retina that the primary defect in the hereditary degenerative condition Retinitis Pigmentosa, (RP) becomes manifest. As a result, an impact on scotopic vision, a restricted field of vision and compromised motion detection are the common early symptoms in RP.

The photoreceptor cell is divided into sections according to its position in the retina: the outer segment (OS), of which the apical surface is embedded in the retinal pigment epithelium, (RPE) and the inner segment connecting the outer segment to the cell body, (fig. 1.2). Finally the synaptic terminus links up with cells of the neural pathway to the brain. Multiple invaginations of the outer segment, the photoreceptor discs, characterise this segment of the rod cell. These discs are continually shed, phagocytosed by the RPE, and renewed from the top, adjacent to the inner segment. Opsin pigments used by these specialised cells to capture photons of light are

localised to the multiple disc foldings. The folds allow for an extensive surface area to maximise the energy that can be captured by the opsins.

1.1.2.1 Opsins

Rhodopsin, (RHO): As shown fig. 1.2, this photosensitive protein is present in both the plasma membrane and the lamellar side of photoreceptor discs. Rhodopsin is a vital photopigment, as well as the major structural protein of the rod outer segment (ROS). Rod photoreceptors are concentrated in the peripheral retina, facilitating an adequate field of vision and motion detection and making optimal use of the rhodopsin pigments contained within. The rhodopsin gene serves as a useful model for understanding the largest receptor class in the human genome, the G protein-coupled receptors, (GPCR) (Bockaert and Pin, 1999). The rhodopsin family is the largest in the GPCR group of proteins; hence, this gene has been the subject of intense study in the past. The common structural feature of all GPCRs is a seven-helical transmembrane region that anchors the receptor to the plasma membrane of the cell, with the N-termini exposed to the extracellular space (Unger *et al.*, 1997). It is estimated that 40 to 50% of all current drug targets are GPCRs (Fredriksson *et al.*, 2005). As the most common source of RP-causing mutations the rhodopsin gene is a target for the suppression and replacement strategy in this thesis (Farrar, 2002).

Cone opsins: In addition to rhodopsin there are three cone pigments known, which are absent in rod photoreceptor cells. Cones operate under conditions of bright light and in humans these cells provide trichromatic colour vision. Unlike most mammals, which have only two cone opsins, primates have three types of cones — red-sensitive as well as blue- and green-sensitive cells (Kolb, 2003). Cone dystrophies show a loss of central and colour vision. Such dystrophies are said to be stationary in contrast to the typically progressive degeneration caused by rod dystrophies (reviewed by Simunovic and Moore, 1998).

1.1.2.2 Structural proteins of the photoreceptor outer segment

Peripherin/rds: Present in the curved rim of photoreceptor discs, the peripherin protein is expressed in both rods and cones (Connell and Molday, 1990). The protein is responsible for the structural curvature in the discs (fig 1.2) and facilitates the connection between the disk rims and the plasma membrane (Goldberg *et al.*, 1995, 1996). This gene is a target for suppression by ribozymes in chapter 5 of this study.

Rod Outer Segment Protein1, (ROM-1): Highly homologous to peripherin in structure and function, this protein differs mainly in expression pattern as, in humans, it is found to be solely expressed in rod photoreceptor cells (Bascom *et al.*, 1992). However the presence of ROM-1 in cone cells of the bovine retina has been reported (Moritz and Molday, 1996). ROM-1 shows 55% sequence homology to peripherin/RDS (Bascom *et al.*, 1992). The protein forms a dimer, which can interact with peripherin dimers to form a functional tetrameric structure. In the absence of ROM-1 it appears that tetramers may form from four peripherin/RDS molecules (Goldberg *et al.*, 1995).

1.1.3 Retinal Pigment Epithelium

This single layer of insulating cells beneath Bruch's membrane and the neurosensory retina provides a protective barrier between the blood and the neural retina. A vital function of the pigment epithelial layer is the provision of retinal to the photoreceptor cells, thus enabling phototransduction to take place. Once retinal molecules have been exposed to light and undergone a conformational change, they are recycled back into the pigment epithelium. The tips of photoreceptor cells are regularly shed, displaced along the length of the cell body and phagocytosed by the RPE (Young, 1976). The RPE tissue is usually very dark because its cells are full of melanin granules. The pigment granules absorb stray photons, preventing their reflection back into the photoreceptor cells, which may result in blurred images being processed by higher order neurons (Ogden, 1989). Among the major proteins localised to the photoreceptor and RPE layers are cellular retinaldehyde binding protein, (CRALBP); 11-cis-retinol dehydrogenase (c-RDH); all-trans-retinyl ester isomerohydrolase (RPE65); and rim protein (ABCR). The RPE and retinal endothelial blood vessels collectively form the blood retinal barrier (BRB) and act in

conjunction to prevent the exchange of molecules between the systemic circulation and the posterior chamber of the eye (Stuer *et al.*, 2004).

The architecture of the eye is arranged such that the retina is thoroughly insulated by the RPE. This is necessary to protect the delicate tissue from external insult, but may also hamper efforts to deliver therapies for degenerative diseases of the retina. The complications associated with retinal delivery have to be taken into account when selecting agents for potential therapeutic value. Therefore, in addition to the assessment of therapeutic elements for the retina in this study, the practical aspects of retinal delivery are also addressed.

1.1.4 The phototransduction cascade

Phototransduction is a well-characterised biochemical process that converts light into an electrochemical signal within the photoreceptors ready for processing by higher-order neurons (reviewed by Molday 1998).

The phototransduction cascade appears to be similar in both rods and cones, however most research to date has focused specifically on rod cells. Photons of light are capable of converting the 11-cis-retinal form of rhodopsin to its all-trans isomer. This activated form of the rhodopsin protein, termed meta-II rhodopsin, binds transducin catalysing the exchange of GDP for GTP on its α -subunit thus converting transducin to its activated form. Transducin in turn activates cGMP-phosphodiesterase (cGMP-PDE), an enzyme which causes the hydrolysis of channel-bound cGMP to 5'-GMP. The cGMP-gated channels of the rod outer segment (ROS), which are normally open under dark conditions, close in response to the rapid decrease in intracellular cGMP levels. The rod cell becomes hyperpolarised, as Na^+ and Ca^+ ions can no longer flow freely into the ROS. Closure of the channels also causes inhibition of glutamate release at the synaptic junctions and, as a result, an electrical signal can be transmitted from the activated cell via the synapses through the layers of the retina and on to the brain via the optic nerve.

After photoexcitation the cell returns to its dark state: rhodopsin kinase phosphorylates rhodopsin causing arrestin to bind and preventing any further interactions with transducin; the intrinsic GTPase activity of transducin results in the hydrolysis of GTP to GDP on the transducin α -subunit, thus inactivating both transducin and PDE; cGMP levels are regenerated via a calcium feedback mechanism involving the enzyme responsible for the synthesis of cGMP from GTP, and as the

cGMP levels once again rise the cGMP-gated channels can re-open and the photoreceptor cell is returned to its depolarised state. Amplification of the light-induced signal is based on the fact that each molecule of rhodopsin can activate several hundred transducin molecules, and similar amplification occurs throughout the phototransduction cascade (Fig 1.3).

Inevitably, with its crucial position at the apex of the cascade, anything that will disrupt the proper function of rhodopsin will have severe and far-reaching implications.

1.2 RETINITIS PIGMENTOSA

Approximately one third of all human inherited diseases include defects of the eye (www.ncbi.nlm.nih.gov/OMIM). The non-lethality of such defects goes some way in explaining this high proportion. However the eye is composed of non-dividing tissues with intense oxygen consumption and is host to complex inter-relationships between many specialised retinal cells including rods, cones and RPE cells. As such the eye may be uniquely sensitive to genetic defects that fail to show pathological effects elsewhere in the body. For example, mutations that arise in ubiquitously expressed housekeeping genes, such as the splicing factor genes may only become evident as dominant retina-specific disease (Chakarova *et al.*, 2002). Retinitis pigmentosa (RP) refers to a group of degenerative retinopathies, which show a unique level of genetic complexity.

1.2.1 Clinical Findings

RP represents the largest group of visual handicap in the developed world with an estimated prevalence of 1 in 3500 (Inglehearn *et al.*, 1998). However, it is difficult to obtain accurate incidence figures, since many patients remain undiagnosed or are not identified in clinical studies. The term “retinitis pigmentosa” was originally used in 1857 by the German physician, Donders to describe the pigmentation of the retina he observed in certain forms of blindness. The name, despite being technically misleading as no real inflammation occurs in the primary defect of RP, applies to hereditary retinal dystrophies, which mainly affect the rod system. Primary RP involves progressive night blindness, electroretinograph (ERG) decline, and constriction of the visual field and a loss of visual acuity. Despite some phenotypic variability among RP patients, with detailed examination certain common features of the disease emerge. Pigmentation of the retina is clearly evident and is thought to result from migration of pigment-containing RPE cells into the degenerating retina. In 1945, Karpe showed certain distinctive features upon examination of an eye affected by RP. In addition to pigment deposits scattered over the retina, the RP-eye may show attenuated retinal vessels, and a pale appearance of the optic disk. The most common findings from ophthalmoscope images of the affected eye include a “bone spicule” pattern on the retina (Li *et al.*, 1995). This describes pigment

deposited on retinal vessels in a bone corpuscular arrangement (fig. 1.4). Although it may chart progress of the disease, such pigment is a secondary effect and not a direct cause of the visual deterioration. However, pigment deposition may further compromise retinal function. Some investigators suggest that pigment deposits may be derived from atrophy, or depigmentation of the RPE (Bird, 1995). Narrowing of the retinal arterioles may also occur and almost half of RP patients will develop cataracts, usually in advanced stages of the disease (Fagerholm *et al.*, 1985). Fortunately, in most patients the macular region can show some resilience to the disease and central vision is preserved until the end-stages of RP (Kaplan *et al.*, 1990).

1.2.2 Classification

Whilst there are some shared symptoms and clinical findings among RP patients, huge variability at the genetic and biochemical level makes it difficult for researchers to classify the disease. This may affect the patient's diagnosis and the management of the degeneration. Accurate phenotype–genotype correlations may be necessary for therapies to be developed and appropriately applied in clinical settings. Classification starts with division into primary RP, where degeneration is confined to the eyes, and secondary RP where there may be multiple organs affected, as seen in Usher's or Bardet-Biedl syndromes. In addition to the clinical history and supplementary tests such as colour vision testing, four main tests may be used to narrow down diagnosis: these are ERG, visual field range, visual acuity, and pedigree analysis.

1.2.2.1 ERG

An electroretinogram (ERG) is a graphic recording of a response evoked from the retina by an electrical stimulus on the corneal surface. Karpe first identified the ERG as a useful tool in diagnosis of RP in 1945. The ERG response is proportional to the severity of disease and in more advanced cases it may not be possible to record any ERG response (Cideciyan *et al.*, 1993). The normal ERG output shows a negative “a-wave” representing hyperpolarisation of the photoreceptors. A positive “b-wave” measures response from the mid-retinal bipolar cells. Cone-rod and rod-cone degenerations can be distinguished with an ERG (Parrish *et al.*, 1984). In the

light-adapted state a so-called photopic ERG can be performed to assess function of the cone system. The rods are bleached out in these lit conditions and will not contribute to the ERG response. To assess exclusive rod-response (the scotopic ERG), the patient is dark-adapted for 30 minutes and then shown a blue or white-flash below cone threshold. It is essential that controlled test conditions be established before waveforms can be analysed. In typical RP, rods are primarily affected and show preferential loss over cones. On ERG-testing rod-cone degeneration can be identified with the photopic b-wave amplitude larger than the scotopic b-wave amplitude. As the b-wave is likely to be significantly reduced in an RP patient, computer averaging is often used to enhance RP ERGs (Armington *et al.*, 1961). The time for the b-wave to reach its peak has also been reported as delayed in those affected by retinal degeneration (Berson *et al.*, 1969).

1.2.2.2 Visual Field Range and Visual Acuity

The clinical findings on visual field range depend on the stage of the disease. Over time the visual field will usually become progressively restricted although some patches of peripheral vision may be preserved (Heckenlively *et al.*, 1988). A core of macular sight will often be retained, although the precise reason for this preservation is not understood. An indicator that hereditary disease may be the cause of a retinal degeneration is found in the symmetry of visual field disruption between left and right eyes (Massof *et al.*, 1979).

Visual acuity, in the typical form of RP, may start to decline at a certain point even without complications such as cataract, glaucoma, or macular edema (Hirakawa *et al.*, 1999). The loss of visual acuity has been linked to the annual loss of visual field range, and as such measurements of the visual field perimeter have been assessed for their potential to predict the point at which central visual acuity may start to decline in RP patients (Seiple *et al.*, 2004). For the purposes of advising patients on their visual prognosis, this data may be more informative than information based solely on family history of the disease, as the rate at which visual function is lost varies greatly even among affected members of the same family.

1.2.2.3 Pedigree analysis

In addition to being a reliable way of classifying this diverse group of diseases, tracing the inheritance pattern of RP mutations has proven invaluable for genetic counselling. Genetic subdivisions are decided by the mode of segregation in a family, i.e., autosomal dominant (ADRP), autosomal recessive (ARRP), and sex-linked RP (XLRP). In almost half of all cases the absence of family history means that segregation pattern cannot be deduced and the disease is denoted as simplex RP (SRP) (Jay, 1982). Multiple gene loci for each mode of segregation have been identified and are compiled in the database:

<http://utsph.sph.uth.tmc.edu/www/utsph/retnet/home.htm>.

Autosomal dominant inheritance, (ADRP): ADRP pedigree maps may show full penetrance of the disease or trait with only one copy of the dominant gene present. However autosomal dominant genes may also show occasional nonpenetrance of the mutation in certain individuals, further compounding difficulties in diagnosing ADRP (Heckenlively *et al.*, 1988).

Autosomal recessive inheritance, (ARRP): In this case two copies of the gene must be present for the disease to manifest itself. While most autosomal recessive carriers with only one RP gene will be asymptomatic, they may show a “subclinical” RP disease state, with minor fundus or ERG changes. It may be difficult to distinguish this patient from an ADRP patient who shows nonpenetrance of the ADRP gene (Dryja *et al.*, 1995b).

X-linked recessive inheritance (XLRP): This mode of inheritance has been demonstrated with a recessive gene for RP carried on the X chromosome (Farrar *et al.*, 1998). X-linked dominant disease has yet to be demonstrated in the retinal dystrophies (Dahl, 1991, Maubaret and Hamel, 2005).

Sporadic inheritance: Population surveys in Europe and the US have found a wide range of “sporadic” inheritance, from 18% to as much as 58% in some reports (Boughman and Caldwell, 1982). Some of these estimates may be from simplex autosomal recessive pedigrees where by chance only one individual is affected. However, there is clearly an excess of simplex families than is predicted by probability (Jay and Jay, 1982). Hence a mutational or environmental influence must be taken into account.

1.2.2.4 Genotype-Phenotype and Molecular Data

Molecular data arising from newly discovered genes allows for continued modification of the genetic and clinical subtypes of RP. Subsequent to grouping by inheritance patterns, subdivision of these groups has been made based on the course and symptoms of the disease. For example, ADRP cases have been grouped according to the presence of macular involvement, ARRP by age of onset and severity and XLRP cases by the first symptom experienced (Kaplan *et al.*, 1990). ADRP, the focus of this study, has also been further divided into subtypes based on rod photoreceptor ERGs and associated fundus changes (Fishman *et al.*, 1985) and will be further discussed in section 1.3. Increased understanding of the molecular aspects of RP should enable a more comprehensive classification of the disease, as subdivisions based on clinical observations and on genetic data may be reconciled. There are currently no obvious clinical markers to characterise the specific genetic types of RP, with variability in age of onset and progression being the most distinct features of each type. With so many incomplete pedigrees the precise frequencies of each subgroup of RP are unknown. Hence, emphasis has been placed on biochemical markers of the disease.

A more detailed classification system that would take advantage of the growing molecular data being gathered by RP laboratories has more recently been proposed (Van Soest *et al.*, 1999). Three major groupings are proposed and are based on mutations affecting: 1) disc shedding and renewal, 2) visual transduction, 3) retinol metabolism. A fourth grouping distinguishes those mechanisms disrupted by mutations in unidentified genes or genes of unknown function. This category illustrates the complexity of the task. However, this study has led the way in linking the molecular and clinical data currently available. An updated and somewhat simplified classification groups the mutations directly according to the proteins encoded and is used in the following section to help describe the molecular genetics of this disease (Bessant *et al.*, 2001).

1.2.3 Overview of the molecular genetics of RP

Complex biochemical and genetic processes are responsible for the proper functioning of the retina. As such many mutations, distinct in biochemical nature, might affect the photoreceptors and RPE yet result in a common degenerative pathway. To help understand the range of defects that can result in RP, it is useful to group the mutations according to class of gene product affected (Bessant *et al.*, 2001). A generalised classification of such affected proteins include those involved in phototransduction, in photoreceptor structure, in photoreceptor and RPE metabolism, and proteins that regulate gene expression, as well as genes of other or unknown function.

1.2.3.1 Phototransduction proteins

The process that converts a photon of light into an electrochemical signal within the photoreceptors is termed phototransduction (reviewed by Molday, 1998). Mutations causing retinal disease have been discovered in every known protein that participates in photoactivation or the recovery process.

Rhodopsin mutations affecting phototransduction: Such mutations have been found to cause RP or congenital stationary night blindness (CSNB). In some cases the mutation may double up, having an effect on the biochemistry as well as the structure of the cell. This is especially evident with the rod photopigment rhodopsin, which triggers amplification of a light-induced signal and constitutes up to 85% of the protein of the ROS (Humphries *et al.*, 1997). Most alleles of rhodopsin display dominant inheritance (Wilson *et al.*, 2003). Mice heterozygous for null alleles of the rhodopsin gene do not suffer serious degeneration despite expressing only half of the normal amount of protein (Humphries *et al.*, 1997). Hence, it is the presence of rhodopsin encoded by a dominant mutated allele that causes degeneration; whether this is due to toxic gain-of-function or an interfering dominant-negative has in some cases of RP yet to be established.

Over 100 rhodopsin RP-causing mutations have been identified to date and a database containing the complete reference set for each reported mutation maintained by Daiger *et al.* (<http://utsph.sph.uth.tmc.edu/www/utsph/RETNET/home.htm>).

In 1990 researchers were able to build on the work of Hogness and Nathans, (1986) in cloning the rhodopsin gene to screen this candidate gene and identify the first mutations in rhodopsin that cause ADRP (Dryja *et al.*, 1990; Farrar *et al.*, 1990;

Hogness *et al.*, 1994). Rhodopsin consists of an opsin, (a typical seven transmembrane G-protein-coupled receptor) covalently bound to a chromophore, 11-cis-retinal. This opsin is found in rod photoreceptor cells, (fig. 1.2). In many cases, the consequences of the mutations, whether they interfere with phototransduction or compromise overall photoreceptor structure and stability have yet to be elucidated. However, in cultured cells it was found that the characteristics of proteins expressed from rhodopsin transcripts carrying a variety of mutations have revealed two distinct classes of biochemical defects (Sung *et al.*, 1993). Class I mutants (15%) show normal subcellular localisation, while class II mutations (85%) interfere with folding and/or stability of the protein often causing accumulation in the rough endoplasmic reticulum. The class I mutations most likely affect the correct functioning of rhodopsin at the apex of the phototransduction cascade. The possible implications include aberrant activation of transducin or an increased incidence of spontaneous isomerisation by the rhodopsin molecule itself (Dryja *et al.*, 1993). However, most rhodopsin mutations that affect the biochemical pathways tend to cause congenital stationary night blindness (CSNB) rather than RP (Al-Jandal *et al.*, 1999).

β -Phosphodiesterase mutations causing RP: Autosomal recessive forms of RP have been associated with mutations in the gene encoding β -phosphodiesterase (Reiss *et al.*, 1992, Danciger *et al.*, 1995, Huang *et al.*, 1995). A defect in this protein causes a decrease in phosphodiesterase activity, resulting in an increase in cyclic GMP levels within the rod photoreceptors. Many nonsense and missense mutations of the α - and β -subunits have been found to cause ARRP (Bayes *et al.*, 1995), however no mutations affecting the twin γ subunits have been as yet identified in RP (Hahn *et al.*, 1994). A homozygous loss-of-function mutation in the β subunit of PDE causes the murine retinal dystrophy rd (Bowes *et al.*, 1990). This commonly used model displays a phenotype similar to classic retinal degeneration seen in humans with complete loss of photoreceptor cells caused by apoptotic degeneration.

CNCG mutations causing RP: Mutations of the rod cGMP-gated cation channel (CNCG) have also been found to cause recessive RP. Five different mutations of the α -subunit of the cGMP-gated cation channel have been associated with ARRP (Dryja *et al.*, 1995). The cGMP-gated channels may be held open permanently resulting in toxicity from the constant influx of Na^+ and Ca^{2+} ions.

1.2.3.2 Photoreceptor structure

Rhodopsin mutations affecting structure: Defective folding and transport of rhodopsin to the membrane is the primary cause of defects in autosomal dominant RP (Naash *et al.*, 1993). Hence rhodopsin and associated mutations is discussed in more detail in section 1.3. In contrast, rhodopsin mutations causing ARRP are rare. The fact that most mutations result in a dominantly segregating disease, illustrates the importance of the protein to photoreceptor structure. Accumulation of rhodopsin protein affected by class II mutations in photoreceptor cells can disrupt the renewal and shedding of outer segments and thus may perhaps trigger cell death (Huang *et al.*, 1995) (fig. 1.2 of the figures section at the back of this chapter shows the location of this protein in a photoreceptor cell). Rhodopsin is the most common source of mutations in ADRP, accounting for 20% of dominant RP cases (<http://www.retina-international.org/sci-news/rhomut.htm>) and is the target of suppression and replacement studies in this thesis.

Peripherin/rds: The transmembrane protein peripherin/rds, (retinal degeneration slow) is abundant in the outer segment disc rims of both rods and cones. In addition to the naturally occurring mutation of the rds mouse, over 40 different mutations of this gene have been identified in humans (Travis *et al.*, 1989). Peripherin/RDS mutations display wide phenotypic variation causing many types of retinal dystrophy. These mutations cause a dominant form of RP (discussed further in section 1.3.2) as well as a digenic form that requires heterozygous mutations in both RDS and its homologous structural partner ROM-1 (Kajiwara *et al.*, 1994).

1.2.3.3 Photoreceptor and RPE metabolism

RPE cells and photoreceptor outer segments collaborate in two major retinal functions: (a) Shedding and Renewal of outer segment discs, and (b) retinol metabolism.

(a) Shedding and Renewal of outer segment discs: As discussed in section 1.1.2 the RPE plays an essential role in the shedding and degradation of rod outer segment (ROS) discs. Disruption of the delicate balance between disc loss and renewal inevitably leads to retinal degeneration. The mutations that interfere with this process are found in the crucial photoreceptor genes, rhodopsin, peripherin/RDS and ROM1.

This disruption of RPE metabolism is a secondary defect caused when the RPE is overwhelmed by an increased rate of photoreceptor death due to an unrelated RP mutation. The extent of the downstream consequences of mutations for RPE cells suggests an additional reason for the phenotypic variability seen in RP. The retina, with its unusually large number of mitochondria inevitably suffers toxic effects from metabolic stress. In addition, with so many unique and specialised cell types interacting with each other to support retinal metabolism, the source and variety of error is immense.

Many rhodopsin mutations, especially dominant mutations, result in defective folding and transport so that the protein never gets incorporated into the outer segment membrane of the rod photoreceptor (Naash *et al.*, 1993). The ectopic accumulation of these mutant gene products may result in a shortening of the rod outer segment as is observed in several animal models of retinal degeneration (Roof *et al.*, 1994). This condition is associated with reduced oxygen consumption by the cell and may cause cellular degeneration due to the toxic level of oxygen. It has also been suggested that this effect is compounded by the shortened cell body being in closer physical proximity to the oxygen-rich choroid layer (Hims *et al.*, 2003).

(b) Retinol Metabolism: A critical phase of the visual cycle involves regeneration of 11-cis-retinal in preparation for further photoisomerisation reactions. Many enzymes and retinoid-binding proteins of the RPE and rod photoreceptors are involved in the cycle and have been identified as sources of mutations in many forms of RP.

Cellular Retinaldehyde Binding Protein (CRALBP) acts as a substrate carrier for 11-cis-retinol and promotes oxidation of 11-cis-retinol to 11-cis-retinal, following the bleaching of photoreceptor cells (Crabb *et al.*, 1991). Mutations in this gene cause atypical ARRP termed retinitis punctata albescens in which numerous white dots can be observed scattered over the fundus (Maw *et al.*, 1997). These mutations inhibit or completely block the regeneration of 11-cis-retinal and rhodopsin.

The 61-kda RPE65 protein encompasses 10% of total protein found in the RPE. Through association with other proteins RPE65 is believed to play a role in retinoid metabolism (Bavik *et al.*, 1992). The phenotype resulting from ARRP-causing RPE65 mutations often involve partial or complete inability to regenerate 11-

cis-retinal. This causes a severe early or even congenital onset of the disease (Gu *et al.*, 1997).

ARRP mutations have also been found in the gene encoding the RPE-retinal G-protein coupled receptor (RGR), a rhodopsin homolog that facilitates the reverse reaction of rhodopsin photoisomerisation, i.e. isomerisation of all-trans-retinal to 11-cis-retinal (Bernal *et al.*, 2003).

1.2.3.4 Proteins involved in regulation of gene expression

Many transcription factors are involved in retinal development and in the tissues subsequent maintenance. Two such factors that dominate are neural retina leucine zipper (NRL) and cone-rod homeobox (CRX), which act synergistically in controlling expression of photoreceptor-specific genes such as rhodopsin (Kumar *et al.*, 1996; Furukawa *et al.*, 1997). A mutation in NRL (Ser50Thr) has been identified in a number of families affected by ADRP where expression from the rhodopsin promoter is upregulated (Bessant *et al.*, 1999).

A relatively recent addition to the list of genes affected by ADRP-causing mutations includes three pre-mRNA splicing factors (McKie *et al.*, 2001; Vithana *et al.*, 2001; Chakarova *et al.*, 2002). The feature making these mutations unique and distinct from other RP mutations is that they affect ubiquitously expressed genes. It has been suggested that the high metabolic rate of retinal cells make them particularly vulnerable to defects in housekeeping genes that may not show any pathological effects in other tissues.

1.2.3.5 Proteins involved in trafficking and intracellular interactions

Genes involved in intracellular interactions and transport have also been associated with RP phenotypes. For example usherin, a protein with cell adhesion properties is mutated in specific cases of recessive RP (Rivolta *et al.*, 2000).

The product of the gene TULP1, a retina-specific member of the tubby-like gene family is believed to be involved in transport of rhodopsin from the inner to the outer segment (Hagstrom *et al.*, 2001). This gene has been mapped to the short arm of chromosome 6 close to a locus for ARRP. Linkage analysis of this gene has

suggested that TULP1 mutations may be involved in certain rare forms of recessive RP (Hagstrom *et al.*, 1998).

1.2.3.6 Genes of other or unknown function

Inosine Monophosphate dehydrogenase 1 (IMPDH1), encodes a protein involved in nucleotide biosynthesis (Kennan *et al.*, 2002). Research is currently active to determine by what mechanism a defect in this gene may result in the RP phenotype. RP9 is a ubiquitously expressed protein of as yet unknown function, which has been implicated in RP (Keen *et al.*, 2002). The PAP-1 gene encoding a splicing factor protein that binds pim-1 kinase has recently been identified, as the defective gene in the RP9 form of adRP (Maita *et al.*, 2004).

1.3 ADRP

ADRP is estimated to account for up to 15–20% of all cases of RP according to the averaged data from five independent studies (Heckenlively *et al.*, 1988; Dryja *et al.*, 1995b; Phelan *et al.*, 2000; <http://www.retina-international.org/sci-news>). Autosomal dominant classification is primarily based on parent-to-child transmission. Traditionally it has been thought that the progression of visual dysfunction is slowest in those with ADRP. This inheritance pattern is associated with mutations in genes important to the architecture of the retinal layers. Defects within the phototransduction cascade almost always lead to recessive RP. The one exception to this is in rhodopsin, which may in fact have an additional structural role. Mutations in two retinal genes, rhodopsin and peripherin/RDS, account for around 35% of all dominant cases of RP (Sohocki *et al.*, 2001). Of the remaining twelve known dominant RP genes, four have mutation ‘hotspots’ that account for most if not all the mutations associated with the genes (<http://www.sph.uth.tmc.edu/RetNet/>).

1.3.1 Rhodopsin

The first dominant RP gene to be found was localised to chromosome 3q and tightly linked to that encoding the photoreceptor pigment rhodopsin (McWilliam *et al.*, 1989; Farrar *et al.*, 1990). The first mutations in the gene were quickly documented (Dryja *et al.*, 1990, 1991), since then over 150 different mutations in rhodopsin have been identified conferring the ADRP phenotype (Millington-Ward *et al.*, 1997; Phelan and Bok, 2000; O’Neill *et al.*, 2000; Cashman *et al.*, 2005; Kiang *et al.*, 2005; <http://www.retina-international.org/sci-news/rhomut.htm>). The consequences of the mutations, whether it interferes with phototransduction or compromises overall photoreceptor structure and stability have, in many cases, yet to be elucidated. Cell culture studies to characterise the defects have led to their division into class I and class II dominant RHO mutations (Sung *et al.*, 1993). Class I mutants, (15%) resemble the wild-type protein in yield, regeneration with 11-cis-retinal, and subcellular localisation. These mutations are to be found in the first transmembrane domain and near the carboxy terminus of the protein and usually affect the visual transduction cascade. The class II mutants (85%) are misfolded or unstable proteins that tend to accumulate in the rough endoplasmic reticulum and interfere with the visual cycle (Sung *et al.*, 1993). Additional studies in transgenic mice suggest that the

primary rhodopsin defect in ADRP involves defective folding and transport of the protein to the membrane (Roof *et al.*, 1994).

The first mutation in rhodopsin identified as causing ADRP was the Pro23His mutation, encountered in 12% of patients in the US, but less so in other populations (Van Soest *et al.*, 1999; Chan *et al.*, 2001). The relatively mild phenotype associated with the P23H mutation may be caused by gradual photoreceptor degeneration due to degradation of P23H rhodopsin by the ubiquitin proteasome system (Illing *et al.*, 2002). Mutations in rhodopsin may cause cell death of photoreceptors when modified by only one amino acid (Pannarale *et al.*, 1996). Such a dramatic effect may not be surprising given the importance of this protein to phototransduction of the rod outer segments. However it may be the secondary effect of such death that causes the phenotype seen in ADRP. The retinal pigment epithelium, (RPE), is involved in phagocytosis of dying photoreceptors and the pathological consequences of the rhodopsin mutation may in some cases be traced to its effects on the RPE. With the increased rate of photoreceptor cell death, the RPE can become overwhelmed, thus a failure in RPE metabolism can cause the symptoms of some cases of RP. This illustrates how genotype–phenotype correlations can occasionally be a poor indicator of the molecular genetics of RP as downstream consequences of the primary mutation may be more difficult to trace. It also goes some way in explaining how the disease may show variance in severity and onset among patients of the one affected family, where a single common rhodopsin mutation has been found. Additional environmental influences and/or other genes have to be factored in before the true molecular nature of the disease can be uncovered.

1.3.2 Peripherin/rds

The second locus for ADRP was identified on chromosome 6p (Farrar *et al.*, 1991). The mutations were subsequently found within the gene encoding RDS-peripherin, a vital structural protein of the outer segment disc membranes (Farrar *et al.*, 1991; Kajiwarra *et al.*, 1991). The RDS mouse carries a natural mutation in the gene encoding the peripherin/rds protein (Sanyal *et al.*, 1995; Travis *et al.*, 1989). No protein is translated in this model as a result of a large insertion in the gene. Mice homozygous for this mutation develop no outer segments (Ma *et al.*, 1995), while heterozygous mice have shortened, disorganised outer segments (Hawkins *et al.*, 1985). This phenotype illustrates the critical importance of this gene to the

architecture and maintenance of photoreceptor segments. With the exception of a role in digenic RP, all mutations in the *peripherin/rds* gene have been found to cause dominant disease (reviewed by Molday *et al.*, 2004). A lack of the protein leads to instability of the ROS and affects the continuous recycling of outer segment fragments by the RPE. General mutations of ubiquitous genes which result in aberrant protein translation may also cause ADRP through the affect on *peripherin/rds* yield (Fishman *et al.*, 1997).

1.4 Animal Models of Retinal Degeneration

1.4.1 Use of animals in retinal gene therapy research

Animal models of retinal degeneration have been studied for many years in the hope of discovering the cause of photoreceptor cell death. Since the emergence of research in retinal gene therapy, animal models have been invaluable in the contributing to the field in three vital areas. Firstly, these models have provided candidate genes for testing in the human; second, the pathology of a mutant gene and the gene product can be analysed in animals to understand the mechanism of degeneration, this information can identify potential drug targets; and finally treatment strategies and delivery methods can be assessed for efficacy and safety in preclinical trials in the animal model.

While the best-characterised mutations occur in the mouse, biological models of retinal degeneration are also found in larger animals, including the cat (Rdy) (Leon and Curtis, 1990), and dog (rcl 1) (Clements *et al.*, 1993), and have been developed in transgenic pigs (Li *et al.*, 1998; Shaw *et al.*, 2000). The pig model expressing a mutated opsin gene (Pro347Leu) shows a similar phenotype to the human, with early rod loss and slower cone cell degeneration, making this model very suitable for preclinical efficacy and safety trials.

A mutation in the RPE65 gene found in the briard dog has led to the current use of this model for the development of therapies for Leber's congenital amaurosis (Aguirre *et al.*, 1998). The Royal College of Surgeons (RCS) rat model has helped elucidate an important pathological mechanism underlying many of the occurrences of apoptosis in retinal degenerations. The RCS rat carries a mutation in the receptor tyrosine kinase gene (rdy/Mertk), which makes the RPE incapable of phagocytosing photoreceptor outer segment membranes. The resulting accumulation of toxic debris in the sub-retinal space triggers apoptosis in the retina. Similarly the effects that light has on the typical degenerative progress have been investigated (Zhang *et al.*, 2004). Animal models have also been useful in assessing combination therapies such as the anti-apoptotic approach combined with Vitamin A supplementation (Eversole-Cire *et al.*, 2000). The use of these models has been further extended with the need to establish effective means of drug delivery to the retina. Thus, the aim of administering sufficient doses of therapeutic agents to the posterior segment of the

eye has necessitated further animal experimentation in this newly emerging area of research.

1.4.2 Mouse models

A mutation of major importance, and found in many inbred laboratory mice is *rd1* (Keeler *et al.*, 1966). This mutation affects exon 7 of the *Pde6b* gene encoding the beta subunit of cGMP-PDE, and homozygous mice have an early and severe onset retinal degeneration (Pittler and Baehr, 1991). The *rd1* mutation is common in mice. Therefore when embarking upon the construction or use of mouse retinal models it is important to avoid strains or stocks carrying the *rd1* allele (Chang *et al.*, 2002). As well as phenotypic assays, a simple PCR and RFLP *DdeI* digest may be performed to exclude the possibility of contamination from the *rd1* allele (Pittler and Baehr, 1991). This model has been instrumental in our understanding of the rod-cone progression of photoreceptor cell death, as well as the morphological, physiological and biochemical characteristics of retinal degenerations (Farber *et al.*, 1994). It has also been used to show how the pathological effects of retinal mutations may extend even to second order neurons, showing that cellular interactions are vital for the formation of functional synapses in normal mice (Strettoi *et al.*, 2000). Among the important findings yielded, at least in part, by animal models is the characterisation of the apoptotic pathway leading to photoreceptor cell death (Chang *et al.*, 1993). It is now known from studies on transgenic mice that apoptotic cell death induced by light damage is dependent on the presence of functional rhodopsin (Naash *et al.*, 1996). This has important therapeutic implications that may allow intervention at this final common pathway. Mouse models have been especially crucial in assessing the benefits of somatic gene therapy in treating hereditary retinal conditions (Bennett *et al.*, 1996; Kiang *et al.*, 2005).

Mouse models carrying three forms of a human rhodopsin transgene (a normal, a mutant, and a modified replacement form), all on an endogenous mouse rhodopsin knockout background, have been used for generating *ex vivo* data for this thesis. Each model is discussed in detail in the relevant chapters.

1.4.2.1 Producing transgenic animals

Pronuclear microinjection of DNA into a fertilised mouse egg is one of the current methods used for production of transgenic animals (Olsson *et al.*, 1992; Okabe

et al., 1997). The DNA is typically a transgene constructed as a chimeric fusion gene containing a 5' upstream regulatory region to confer tissue-specific expression. Heterozygote offspring are bred to homozygosity and the functional effect determined.

A number of techniques are employed to achieve tissue-specific expression of a transgene. The 5' regulatory sequences from murine (Lem *et al.*, 1991), bovine (Zack *et al.*, 1991), and porcine (Petters *et al.*, 1997) rod opsin genes, as well as the human interphotoreceptor retinoid binding protein gene (May *et al.*, 2003) may be used to target the transgene expression to photoreceptor cells. Unfortunately it is difficult to control the level of transgene expression. The transgenes integrate randomly in the chromosome, and a number of copies may insert at different loci. This has implications, for example, in a comparative assessment of expression in normal human rhodopsin and replacement human rhodopsin models in this thesis (results presented within chapters 3 and 4). Moreover, the sequences flanking the insertion site may have even greater effect than transgene copy number, as local regulatory epigenetic effects may be imposed on the site. Hence, it is necessary to characterise more than one line of transgenic mice for expression level by immunohistochemical techniques. Gene targeting can overcome some of these problems, as a specific and stable modification may be made to an endogenous gene. This technique has been successfully employed in production of the rho-/- knockout animal (Humphries *et al.*, 1997).

1.4.3 Genotype-phenotype correlations in transgenic animals

While animal models that mimic the human disease genetically will generally faithfully produce phenotypic results similar to those seen in the human, exceptions have been noted and should be considered when validating a model for use in research for retinal degeneration. In humans a mutation at codon 296, (the 11-cis-retinal binding site) of the rhodopsin gene, was believed to cause degeneration through continuous activation of the cascade as shown in cell culture (Robinson *et al.*, 1992). However the opposite effect of the corresponding mutation was observed in transgenic mice. In these models the mutant protein was permanently phosphorylated and inactivated by arrestin, resulting in shutoff of the cascade (Tiansen *et al.*, 1995). It may be argued that natural models are the 'true' models as many genes mutated in animals that result in retinal degeneration are similarly abnormal in the human

(Chader, 2002). However most of the natural animal models are for the recessive form of RP and a good natural model for ADRP has yet to be found. Therefore transgenic animals have been used in this project, however the limitations associated with the ‘artificial’ transgene expression in these models is taken into account. The most common currently available transgenic animal models for retinal degenerations are listed in table 1 of the Figures and Tables section at the end of this chapter. The animal models used in this thesis shall be discussed in greater detail in the relevant chapters (these animal models are the wild-type rhodopsin ‘NHR’ mouse in section 3.1.11; a replacement rhodopsin mouse, ‘HR5m’ in section 4.1.6; and the pro23his mouse in section 5.1.2).

1.5 Suppression & Replacement

Successful gene therapy for dominant disorders, where pathology is due to a gain of function mutation may require a strategy that suppresses the disease allele in concert with the provision of a functional gene copy (O'Neill *et al.*, 2000). There are over 500 mutations identified across 12 genes in the dominant form of RP. Designing a therapy for each mutation would be costly and time-consuming. For example, approximately 150 ADRP-causing mutations are associated with the rhodopsin gene (<http://www.retina-international.org/sci-news>). As such, many individual therapies would be necessary to tackle the level of heterogeneity associated with the rhodopsin gene alone. The possibility of developing mutation-independent therapeutics is therefore being explored for ADRP. Certain characteristics of the genome enable this approach; namely a) untranslated regions, b) intragenic polymorphisms, c) degeneracy of the genetic code. Targeting these features will enable the mutation to be silenced at sites (a, b, or c above) that can be altered in the replacement. Thus, without affecting the replacement protein's function at an amino acid level, suppression of the replacement gene can be avoided. Inevitably, the level of flexibility at these sites is not without limits. For example, care must be taken not to interfere with regulatory sequences in the UTR. In addition, given possible constraints due to codon usage, sequence alterations at all degenerate sites may not always be possible for a given gene.

The suppression and replacement strategy is to be assessed for intervention where pathology is due to mutations affecting two critical aspects of vision: visual transduction (rhodopsin-based mutations) and photoreceptor cell structure (i.e. mutations in both rhodopsin and peripherin/rds). Proof-of-concept experiments have been designed and carried out to achieve this aim. While the silencing effect of dsRNA (as used in this thesis) on Cytomegalovirus(CMV)-driven rhodopsin transcripts is helpful in that it identifies suitable targets based on sequence alone, it by no means reflects what may happen to the rhodopsin transcript in its normal physiological environment of the photoreceptor cell. Assessing the effect of stable rhodopsin knockdown in the retina is therefore necessary and steps toward this goal are made in this thesis. This includes the validation of a vector system for stable expression of dsRNA in vitro and in retinal explants. Some preliminary results for the effect of a replacement protein in these systems will also be presented. Toxicity and

immune responses in the systems used were monitored throughout the study. Means of delivery of the therapeutic to the retina are considered in chapter 6. Positive results have encouraged further working up of suitable suppressor reagents that are compatible with a replacement component of a therapeutic. This thesis represents further work towards the long-term goal of an in vivo suppression and replacement treatment strategy for ADRP and allied retinal dystrophies.

1.6 FIGURES and TABLES

Transgenic models for retinal degenerations

Strain	Gene	Mutation type	Cell layer	Degeneration time-course
B6D2F1	rhodopsin	substitution (Pro23His)	ONL, RPE	starts at p20
C57L/6J	rhodopsin	substitution (Pro347Ser)	ONL	p21-1 year
C57BL/6 × SJL	rhodopsin	substitution (Val20Gly, Pro23His, Pro27Leu)	ONL	p20-sp250
C57L/6J	rhodopsin	nonsense (Gln344ter = Q344ter)	ONL	starts at p14
FVBN × C57BL/6	peripherin	substitution (Pro216Leu)	ONL	1 month-7 months
C57BL/6J	IRBP	knockout (IRBP ^{-/-})	ONL	starts at p11
C57BL/6 × MF1	γPDE	knockout (Pdeg ^{-/-})	ONL	p 0-8 weeks
C57BL/6	Rpe65	knockout (Rpe65 ^{-/-})	RPE, ONL	starts at 7 weeks
C57BL/6	—	insertion of diphtheria toxin A gene	ONL	p7-3 months
C57BL/6 × 129Sv	cyclic nucleotide-gated channel (CNG3)	knockout (CNG3 ^{-/-})	ONL (rods)	2 months-8 months
C57BL/6	ABCR	knockout (RmP ^{-/-})	ONL	—
C57BL/6	rhodopsin	knockout (Rho ^{-/-})	ONL	p24-3 months
C57BL/6	rhodopsin kinase (RK)	knockout (RK ^{-/-})	ONL (rods)	depends on illumination state
C57BL/6 × 129Sv	arrestin	knockout (arrestin ^{-/-})	ONL (only rods)	3 months-12 months
rat	rhodopsin	substitution (S344ter)	ONL	p 8-p20
rat	rhodopsin	substitution (P23H)	ONL	p 15-1 year
pig	rhodopsin	substitution (Pro347Leu)	ONL	p 14-20 months

Table 1: Commonly used animal models of retinal degeneration (Chader *et al.*,2002).

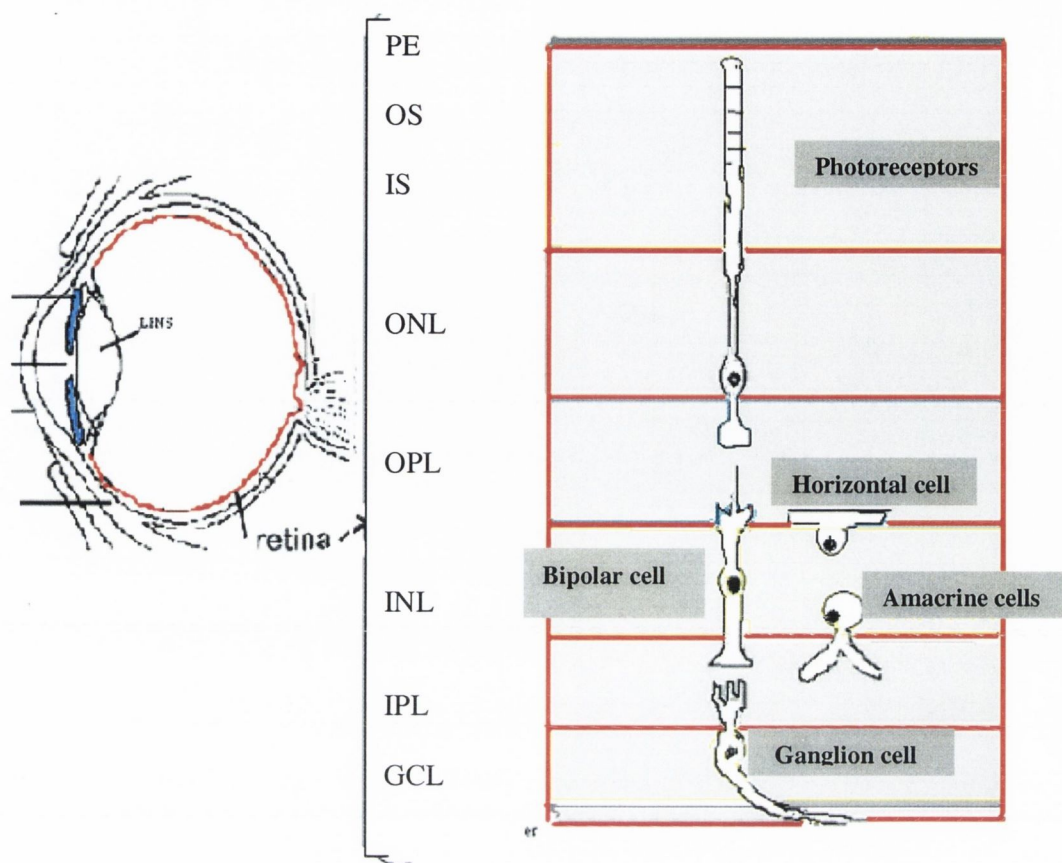


Fig. 1.1

Diagram showing the cellular layers of the retina. The retina is a thin layer of tissue found at the back of the eye and is comprised of the neural retina and the retinal pigment epithelium (RPE). Subretinal space separates the neural retina from the RPE. Signal transduction is carried out within the seven-layered neural retina.

PE: Pigment Epithelium; OS: Outer Segment; IS: Inner Segment; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

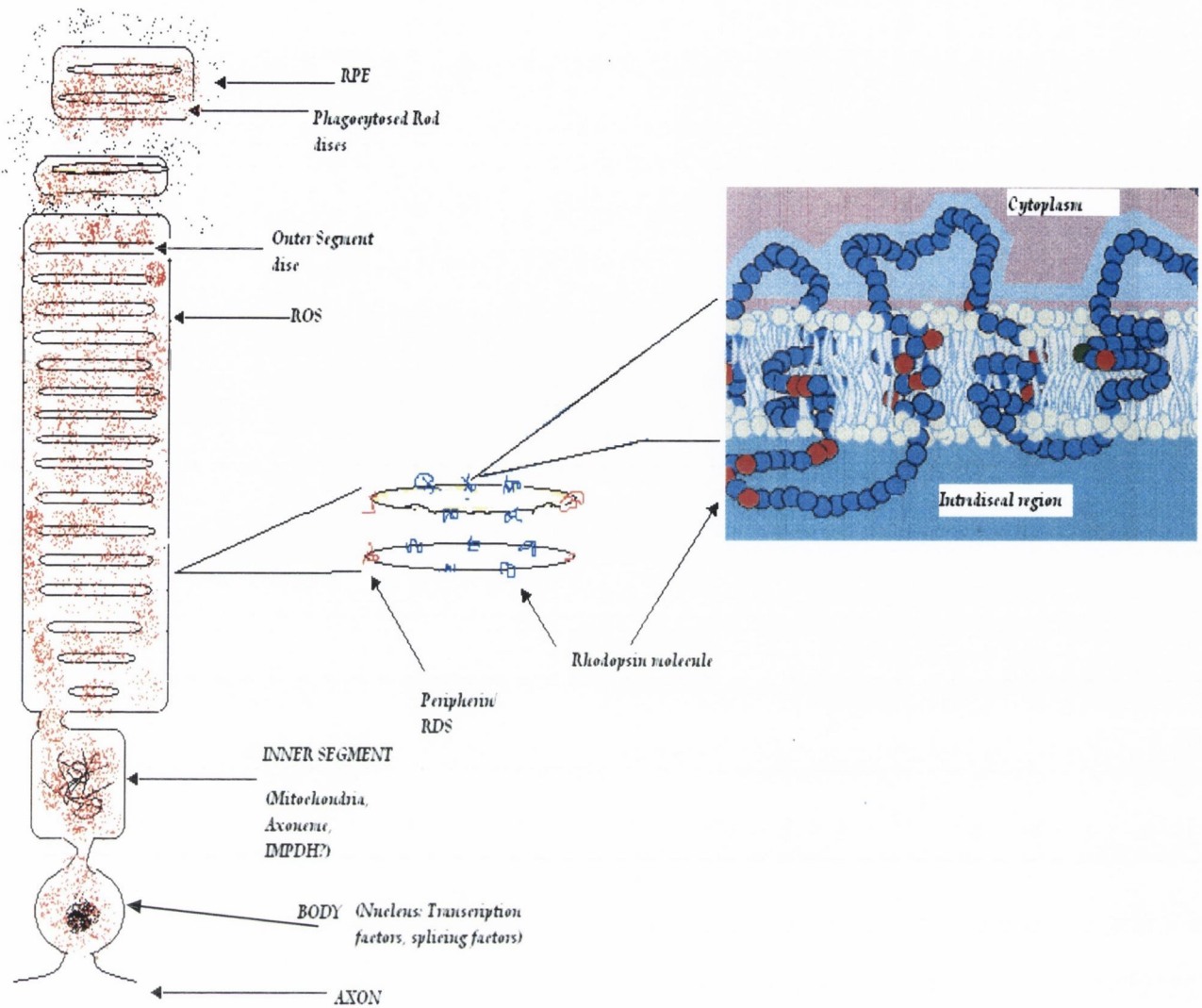


Fig. 1.2:

Diagram of the rod photoreceptor cell, with the location of rhodopsin in the photoreceptor discs. The highly specialised rod photoreceptor cell absorbs the photons of light by means of the photopigment rhodopsin. The change in opsin conformation that results from this absorption of light energy is the activating signal for phototransduction to occur. The phototransduction cascade amplifies the signal until it reaches the second order neurons and ultimately the brain where the image is formed. RPE: Retinal pigment epithelium; ROS: Rod Outer Segment; RDS: Retinal Degeneration Slow; IMPDH: Inosine Monophosphate Dehydrogenase.

Rod photoreceptor discs

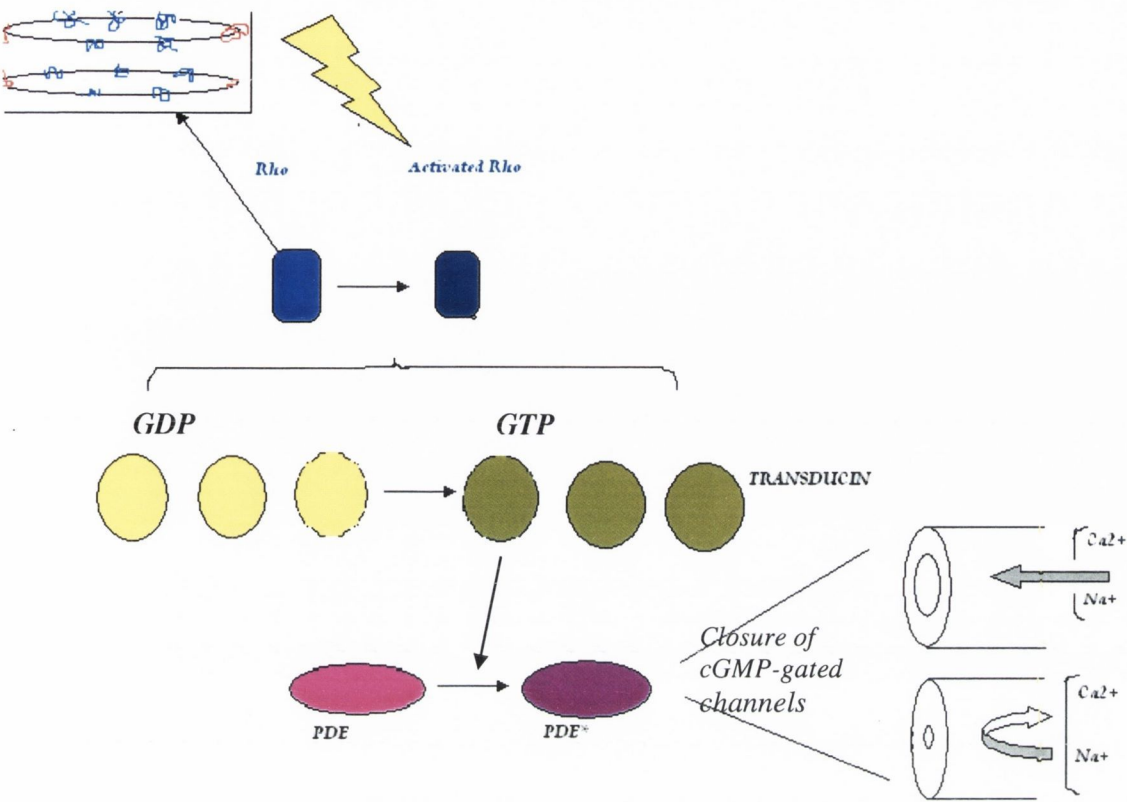
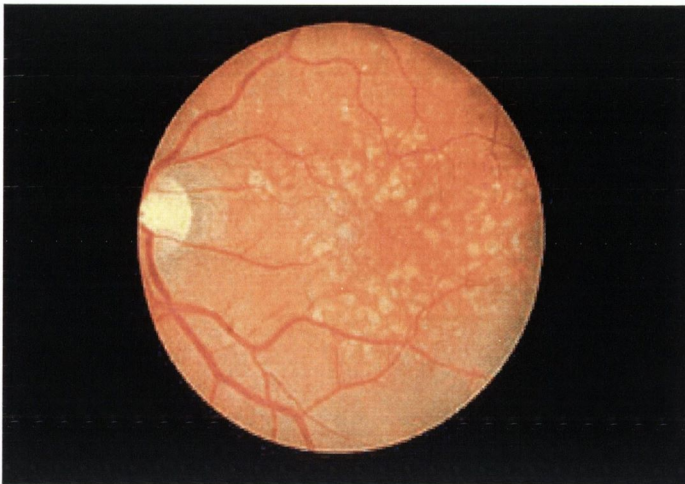


Fig. 1.3 :

Diagram of the phototransduction amplification cascade. Photons of light convert the 11-cis-retinal chromophore to all-trans isomer. This activated form of the rhodopsin protein catalyses a series of events that results in hydrolysis of channel-bound cGMP to 5'-GMP. The cGMP-gated channels of the ROS, normally open under dark conditions, close in response to the rapid decrease in intracellular cGMP levels. The rod cell becomes hyperpolarized, as Na^{+} and Ca^{+} ions can no longer flow freely into the ROS. Closure of the channels also causes inhibition of glutamate release at the synaptic junctions, and an electrical signal can be transmitted from the activated cell via the synapses through the layers of the retina and on to the brain via the optic nerve. Rho: Rhodopsin; GDP: Guanine diphosphate; GTP: Guanine triphosphate; PDE: Phosphodiesterase.

Normal Retina



RP Retina

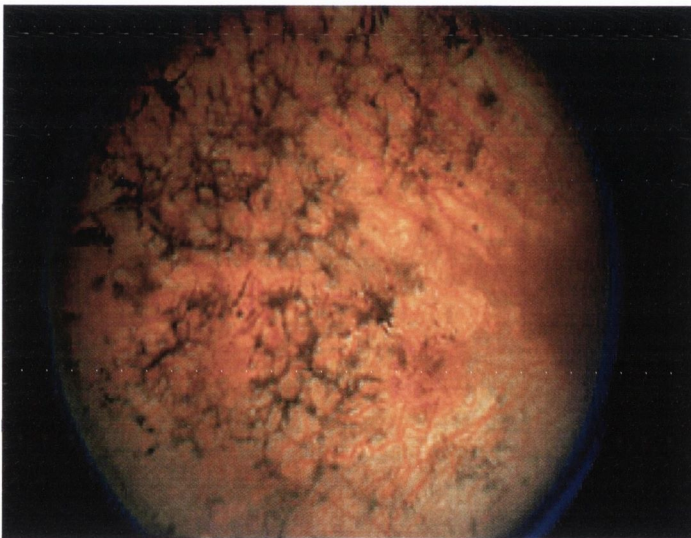


Fig. 1.4:

Contrasting images of a normal retina and retina affected with Retinitis Pigmentosa. The pigment deposits and palour of the optic disk can be seen clearly in the degenerating retina compared to the healthy retina. Attenuated retinal blood vessels are also distinguishable in the diseased tissue. Pictures taken by Donnell Creel and available on <http://webvision.med.utah.edu/>).

Chapter 2

Materials and Methods

CHAPTER 2

2.1 MATERIALS used throughout the thesis

2.1.1 Buffers and stock solutions for cloning:

1M Tris

60.55 g of Tris base was dissolved in 400 ml of ddH₂O. The pH was adjusted to a pH of 7.4, 7.6 or 8.0 using concentrated HCl. With each addition of HCl the solution was allowed to cool before re-examining the pH. Once the desired pH was achieved the solution was made up to 500 ml with ddH₂O.

0.5M EDTA (pH8.0)

93.6 g of disodium ethylenediaminetetra-acetate·2H₂O was added to 400 ml of ddH₂O. Following mixing on a magnetic stirrer, the pH was adjusted to 8.0 with NaOH and the corrected solution made up to 500 ml with ddH₂O.

Tris-acetate/EDTA electrophoresis buffer (50×TAE)

The following 50X stock solution was made and stored at room temperature:

242	g	Tris base
57.1	ml	Glacial acetic acid
100	ml	0.5 M EDTA (pH 8.0)

Made up to 1L with ddH₂O.

Diluted down to 1× TAE with ddH₂O prior to use.

(The above solutions were dispensed into aliquots and sterilised by autoclaving).

10% Sodium dodecyl sulfate (SDS)

100 g of electrophoresis-grade was dissolved in 900 ml of ddH₂O and heated to 68°C to aid dissolution. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl and the volume adjusted to 1L with ddH₂O.

Gel-Loading Dye

Made up to 6×:

0.25%	bromophenol blue
0.25%	xylene cyanol
30%	glycerol in water

LB

10 g	Tryptone
5 g	Yeast
10 g	NaCl

Made up in 1L of ddH₂O before autoclaving.

Antibiotics	stock soln.	working conc.
Ampicillin:	50 mg/ml in sterile ddH ₂ O	100µg/ml
Kanamycin:	50 mg/ml in sterile ddH ₂ O	50 µg/ml
Tetracycline:	50 mg/ml in ethanol	50 µg/ml

(Ampicillin and kanamycin sterilised through a 0.22-micron filter prior to use).

Lysis solution for XL1B

50 mM	Tris pH 8.0
25%	Sucrose
2 mM	EDTA pH 8.0
950 ml	sterile ddH ₂ O

M-Stet

5%	Triton X-100
50 mM	EDTA pH 8.0
50 mM	Tris pH 8.0
5%	Sucrose

Made up to 200ml with sterile ddH₂O

2.1.2 Buffers and stock solutions for protein work:

Tris-buffered saline, (TBS, 25 mM)

25 ml	1M Tris
8 g	NaCl
0.2 g	KCl
975 ml	ddH ₂ O

SDS Loading Buffer, (5×)

250 mM	Tris Cl
500 mM	dTT
10%	SDS

0.5% Bromophenol blue

50% Glycerol

This buffer was mixed in a 1:5 ratio with the protein dilution for loading on a gel.

Resolving gel for Tris-glycine SDS-PAGE, 10%

3.3 ml 30% Acrylamide

2.5 ml 1.5 M Tris (pH 8.8)

0.1 ml 10% SDS

0.1 ml 10% Ammonium persulfate

0.004 ml TEMED (N,N,N',N'-tetramethylethylenediamine)

4.0 ml sterile ddH₂O

Stacking gel for Tris-glycine SDS-PAGE, 5%

0.83ml 30% Acrylamide mix

0.63 ml 1.0 M Tris (pH 6.8)

0.05 ml 10% SDS

0.05 ml 10% Ammonium persulfate

0.005 ml TEMED

3.4 ml sterile ddH₂O

SDS-PAGE running buffer

3.02 g 25 mM Tris

18.8 g 250 mM Glycine

1 g 0.1% SDS

Made up to 1L with ddH₂O

SDS-PAGE transfer buffer

39 mM 2.9 g Glycine

48 mM 5.8 g Tris

0.037% 0.37 g SDS

25% 200 ml Methanol

Made up to 1L with ddH₂O

2.1.3 Buffers and stock solutions for mammalian cell culture work

Dulbecco's Modified Eagle's Medium+

450 ml Dulbecco's Modified Eagle's Medium (Sigma, D6046)

50 ml Fetal Calf Serum (Invitrogen)

5 ml	Sodium Pyruvate
5 ml	L-Glutamine (for all cells used with the exception of HeLa cells which received 10 ml L-Glutamine for 500 ml of media).

Lysis Solution for mammalian cells

3 ml	Inhibitor (Protease Complete Mini tablet, Roche) in TBS
2 ml	10 % Triton in TBS
5 ml	TBS (as listed in section 2.1.2 above)

Freezing Solution

20%	dimethyl sulphoxide
60%	fetal calf serum
20%	complete DMEM

X-Gal Stock Solution

40 mg/ml X-gal dissolved in DMSO

β-Gal Stain Base Solution (prepared in PBS, pH7.2)

5 mM potassium ferricyanide
5 mM potassium ferrocyanide
2 mM MgCl₂
0.02% NP-40
0.01% SDS

β-Gal Fixative (prepared in PBS, pH7.2)

2% (v/v) paraformaldehyde
0.2% (v/v) glutaraldehyde
prepared in PBS

Tail Lysis Buffer:

50 mM Tris pH 8.0
100 mM EDTA
100mM NaCl
1% SDS

2.2 General Methods

(THE CLONING OF CONSTRUCTS AND ANY METHODS SPECIFIC TO INDIVIDUAL EXPERIMENTS ARE AVAILABLE WITH THE RELEVANT RESULTS CHAPTER).

2.2.1 Preparation of oligonucleotides and DNA templates

2.2.1.1 Oligonucleotide synthesis:

All oligos were ordered presynthesised from Sigma-Genosys (UK). Primers were ordered purified by desalting, with the exception of primers for quantitative rtPCR work, which were HPLC purified. Primers for PCR were designed 18 to 24 bases in length with approximately 50% GC content. Only primers with little secondary structure and a low chance of forming primer-dimers were selected for purchase. They were chosen exhibiting similar melting temperatures in the range of 55–65°C and with complete complementarity to the target. The melting temperature, T_m , of the primers was considered a crucial aspect of the design, as it may affect the specificity of a PCR reaction. The following formula was used in estimating the melting temperature:

$$T_m (^\circ\text{C}) \approx 2(N_A + N_T) + 4(N_G + N_C),$$
 where N equals the number of bases and the A, T, G and C subscripts refer to each of the four nucleotides.
(Thein SL, 1986).

Stock concentration of primer was kept at 100 pmol. Annealing temperatures in PCR reactions were kept at 5–10°C below the lowest primer melting temperature.

2.2.1.2 Extraction and precipitation of nucleic acids:

Nucleic acid samples were mixed with equal volumes of phenol, vortexed for 30 seconds and centrifuged at 13,200 X G for 5 minutes. The upper aqueous phase carrying nucleic acid was transferred to an equal volume of phenol/chloroform, vortexed and centrifuged for a further 5 mins at 13,200 X G. This step was repeated with the aqueous phase and then added to an equal volume of chloroform for a final separation of phases by centrifugation. A 1/10 volume of 3 M sodium acetate was added to each sample. The sodium acetate was at neutral pH for precipitations, unless the DNA solution was in alkaline buffer, in which case acetate of lower pH (generally 5.5) was used. A 2X volume of absolute ethanol was added for plasmid DNA precipitation and 2.5X volume for RNA precipitation. Samples were left at –20°C for 30 minutes before being centrifuged at 13,200 X G for 20 minutes. The nucleic acid pellets were twice washed with 70% ethanol, left on the bench to dry and then resuspended in ddH₂O.

2.2.1.3 Polymerase Chain Reaction (PCR):

PCR amplifications were performed using a DNA engine PTC-200 (MJ Research) using a heated lid to prevent evaporation. PCR reactions contained 200 ng template, 25 pmol of forward and reverse primers, 0.2 M of each dATP, dTTP, dCTP, and dGTP, and 1 unit of Taq polymerase. The MgCl₂ buffer concentration was optimised for each reaction within the range 10 mM, 15 mM, 20 mM, and 25 mM. The volume was then brought up to 50 µl with dH₂O. The standard PCR run was; 94°C for five minutes denaturation, then 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for elongation at 1 minute/kb of product expected. The 55°C annealing temperature may be altered depending on GC-content of the primers and was usually set at 5°C below the T_m of the primers. The number of PCR amplification cycles may also be altered depending on the efficiency of the PCR reaction. Each run was ended with a final extension step of 72°C for 10 minutes.

2.2.1.4 Purification of PCR products:

In addition to the normal wash and precipitation step as in 2.2.1.2, for sequencing reactions PCR products were purified using Qiaquick PCR purification columns, (Qiagen, GmbH, Hilden) according to the manufacturers instructions. For DNA products isolated from agarose gel bands, the Qiagen Gel Extraction kit (Qiagen, GmbH, Hilden) was used according to the manufacturers instructions prior to sequencing.

2.2.1.5 Site-directed mutagenesis PCR:

The PCR cycle was modified from the standard PCR reaction by optimizing initially in control reactions. Base substitutions were incorporated into primer design and used to insert mutations in the DNA template. Approximately 1 pmole of DNA template was added to a PCR cocktail of a 25 µl total volume, containing 1X *Pfu Turbo* buffer (Stratagene), 20 pmole of each primer, 250 µM of each dNTP, and 2.5 U of *Pfu Turbo* enzyme (Stratagene). The *Pfu Turbo* enzyme was used as polymerase in the reaction for its proofreading ability, as it exhibits the lowest error rate of any thermostable DNA polymerase studied (Lundberg *et al*, 1991). The PCR cycling parameters were altered from the standard PCR reaction, as the *Pfu Turbo* enzyme is sensitive to the incorporation of unwanted second-site mutations. The PCR involved, initial denaturation at 94°C for 1 minute, 25 cycles of 94°C for 45 seconds, 54°C for 1 minute, and 1 minute at 72°C before a final 10-minute extension step at 72°C. The P1 and

P2 products were generated initially from the parental template with template primers ($T_{forward}$ and $T_{reverse}$) and mutagenesis primers ($M_{forward}$ and $M_{reverse}$).

P1: $T_{forward}$ & $M_{reverse}$ and P2: $M_{forward}$ & $T_{reverse}$.

The flanking template primers, $T_{forward}$ and $T_{reverse}$ were used to generate P3 from the combined P1 and P2 products.

P3: ($T_{forward}$ & $T_{reverse}$) primers on (P1 + P2) template

The mutated PCR product, P3, was cloned back into the original digested backbone to create the altered clone.

2.2.1.6 SNP genotyping:

DNA was extracted from tail tips of adult NHR+/-, P23H+/-, NHR5m+/-, L129 and C57 wild-type mice using the method outlined in section 2.2.6. A PCR reaction was performed using primers designed from the construct with which the animal model was made, depending on whether this was derived from the cDNA of rhodopsin, (NHR, Replacement models), or from genomic DNA (P23H model). (All primer sequences are given in appendix 1A). Sequencing reactions were carried out on the PCR products, as outlined in section 2.2.2.7, and the presence of double peaks in the location of the SNP as predicted in dbSNP (www.ncbi.nlm.nih.gov/dbSNP) used as evidence that the polymorphism occurs in the animal.

2.2.2 Cloning Techniques

2.2.2.1 Restriction Enzyme Digests:

Enzyme digests were typically carried out in a final reaction volume of 50 μ l depending on the quantity of DNA being cleaved. Buffer was added to a 1X concentration. Enzyme concentration for the digest reactions was kept between 2–5 U/ μ g of DNA. The final volume was made up with ddH₂O and the reaction incubated at 37°C for 2–3 hours. Restriction enzymes were purchased from New England Biolabs.

2.2.2.2 Gel Isolation and Extraction:

DNA was visualised on 1 to 2% agarose gels. 1 μ l of ethidium bromide was added per 30 ml agarose solution and the gel poured. Loading dye (2.1.1) was added to the DNA at a dilution of 1 in 5. Following electrophoresis of 1–20 μ l of the products in 1X TAE buffer at 80 volts

the DNA could be visualised by placing the gel over a UV light source. The DNA could be extracted from the gel section using the QIAquick gel extraction kit from Qiagen.

2.2.2.3 Ligations:

In 15 µl volumes, varying concentrations of insert DNA were added to 100 ng of dephosphorylated vector using Calf Intestinal phosphatase, (CIP), Boehringer Mannheim, (GmbH). A 1/10 volume of 10X ligase buffer and rATP was added with 10 U of ligase enzyme, (Roche). The final volume was made up to 15 µl with ddH₂O and the mix left at 16–18°C overnight.

2.2.2.4 Transformations:

5 µl of the ligation mix was diluted in 10 µl of dH₂O. 200 µl of competent cells was added and samples were left on ice for forty minutes. Cells were heat-shocked at 42°C for 5 minutes and returned to the ice. 1 ml of Luria-Bertani broth, (2.1.1, reagents from Oxoid, Hampshire) was added and samples incubated for 1 hour at 37°C. 200 µl of each transformation was plated on LB agar plates containing appropriate antibiotic concentrations: tetracycline at a concentration of 50 µg/ml; Ampicillin at 60 µg/ml; Kanamycin at 50 µg/ml. All antibiotics were ordered from Sigma-Aldrich.

2.2.2.5 Plasmid Mini-preparations:

Bacterial colonies from the transformed plates were used to grow 10 ml cultures of LB broth containing antibiotic. The cultures were left shaking overnight at 220 X G at 37°C to reach a log phase of growth and maximize plasmid retrieval. The supernatant from each culture was removed and the pellet resuspended in 60 µl of lysis solution (2.1.1) and transferred immediately to an eppendorf on ice for further resuspension by pipetting. 20 µl of 40 mg/ml of lysozyme (Fluka) made up in 250 mM Tris was added to the lysis solution and left on ice for approximately 10 minutes. 550 µl of MStet (2.1.1) was added and left at room temperature for 10 minutes. The lids were opened and the eppendorfs boiled for 1 – 1.5 minute(s). The tubes were centrifuged at 32,000 X G for 30 minutes. The pellet was removed with a sterilized tooth-pick and the supernatant heat treated with 5 – 10µl of RNAase (Stratagene) at 10 mg/ml and left at 37°C for 15 minutes. The DNA was washed once with 1X phenol (Invitrogen) and the supernatant moved to a fresh eppendorf. At this stage, 0.6X volume of cold isopropanol was added to precipitate the DNA out of the supernatant. After mixing and leaving for 15 minutes at room temperature the solution was centrifuged at 32,000

X G for 10 minutes. The pellet was washed with 1.5 ml of 80% ethanol before resuspension in 50 – 100 µl of ddH₂O.

2.2.2.6 Large-scale plasmid purification:

The Qiagen High Speed Plasmid Maxi kit was used for all large-scale maxiprep plasmid purification. Colonies, identified as positive for a clone, were used to seed 2 mls of LB containing appropriate antibiotics. These cultures were left in an orbital shaker at 37°C for 8 hours. 1 ml of the seeded broth was added to 200 ml LB broth containing appropriate antibiotics and incubated with shaking at 220 X G at 37°C overnight. The culture was centrifuged at 5000 X G for 15 minutes (Sorvall RC-5B refrigerated superspeed centrifuge – rotor GSA). The pellet was then used with the Maxi kit to produce 1 ml of DNA from the bacterial cells. Harvested DNA was quantified using the Cecil Ce3021 Spectrophotometer by measuring a 1/100 dilution of the DNA at a wavelength of 260 nm. The readout was multiplied by 50 and by the dilution factor to obtain the concentration (units: µg/ml). The ratio between the readings at 260 nm and 280 nm was used to determine the purity of the DNA sample. A ratio of 1.8 is achieved for pure samples and lower ratios suggest protein contamination in the DNA sample.

2.2.2.7 Capillary Sequencing:

500 ng of DNA template for sequencing, 3 pmol of a single primer, and 8 µl of Taq-mix from the Dye terminator cycle sequencing ready reaction kit, (Perkin Elmer), were used in a PCR reaction made up to 20 µl with ddH₂O. The PCR sequencing program run involved 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1.2 minutes, with a final 72°C primer extension step for 10 minutes. After the PCR reaction the mix was precipitated with 50 µl of 100% ethanol and 2 µl 3M NaAc pH 5.2 at –20°C for 30 minutes. This was followed by a 70% ethanol wash. The pellets were allowed to air-dry. A mix of 100 µl formamide with 20 µl of 50 mM EDTA, pH 8.0 was used as resuspension buffer. Pellets were resuspended in 25 µl of the resuspension buffer, heated to 95°C and then placed on ice to maintain the template products in a denatured state. The samples were loaded and sequenced on an ABI 310 DNA sequencer.

2.2.3 RNA Techniques

2.2.3.1 *Creating ribonuclease-free conditions:*

It is necessary that all glass-ware, tips, eppendorfs, solutions, and buffers used for RNA work be RNase free. Prior to use all equipment and benchtops were treated with RNase-Away (Gibco/BRL). Promega and Amgen provided nuclease-free tips and eppendorfs respectively. Finally aseptic techniques were used and gloves worn at all times.

2.2.3.2 *RNA extraction from tissue culture cells:*

For most assays total RNA was extracted from 3×10^5 mammalian cells using Trizol reagent (Molecular Research Centre, Inc). All volumes of reagent used were adjusted to account for different cell density requirements and according to plate surface area. For the 24-well plate, 400 μ l of trizol was added to each well. This was transferred, after 30 seconds of agitation, to a pre-cooled 1.5 ml eppendorf. 80 μ l of chloroform was added and vortexed for 30 seconds. After centrifugation at 13,000 X G for 15 minutes at 2–8°C, 160 μ l of the supernatant was transferred to a fresh cooled eppendorf. The nucleic acid was precipitated with 133 μ l of isopropanol at room temperature for 10 minutes. The pellet from a 10 minute centrifugation at 2–8°C was washed with 1 ml of 75% Ethanol and resuspended in 40 μ l of nuclease-free H₂O (Promega). To remove any contaminating DNA the RNA samples were then treated with 2.5 μ l DNase and 5 μ l DNase buffer, (Promega) and incubated for an hour at 37°C. To stop the DNase reaction, a stop solution supplied with the enzyme was used with 10-minute 65°C incubation.

2.2.3.3 *Quantitative real-time PCR Analysis:*

Quantitative RT-PCR was performed using the Quantitect Sybr Green RT-PCR kit, (Qiagen GmbH, Hilden). Primers for human rhodopsin and human peripherin/*rds* cDNA yield, approximately, a 100 bp product. Gapdh, 18S rRNA, beta-actin and neomycin-resistance genes were used as house-keeping genes with primers also giving a 100 bp product. All primers were HPLC purified and flanked an intron to control for DNA contamination. These primers are listed in the appendix section. The Roche lightcycler machine was used in all analysis. Initial PCR conditions were established in optimization experiments using cDNA and taq polymerase on a PCR machine. Reactions involved a reverse transcription step for 20 minutes starting at 50°C with increases of 20°C/second(s). An initial PCR step of 95°C for

15 minutes to facilitate activation of the HotStar Taq polymerase (Qiagen), followed by 35–45 cycles (depending on abundance of target) of the PCR reaction: 94°C for 15 s, annealing at 55°C for 30 s, and extension step at 72°C for 30 s. While optimizing the technique, products were checked in PCR reactions to ensure that larger, intron-containing DNA contaminants were not present in the RT reaction. The PCR products were analysed by electrophoresis on a 2% agarose gel. In typical assays, standard curves were generated for each the gene interest and the reference gene by preparing reactions containing serial dilutions of a given sample. Dilution series were adjusted according to estimated RNA yield from the cells. The relative concentration of transcript in samples could be derived from the standard curve. The level of expression of the gene of interest could then be determined from the ratio (relative transcript concentration of gene of interest)/(the relative transcript concentration of the reference gene).

2.2.4 Protein Analysis techniques

2.2.4.1 Protein extraction from transfected mammalian cells for Elisa:

Transfected cells were washed with ice-cold TBS, (2.1.3) and collected in 200- μ l TBS volume using plastic cell scrapers. The cells were solubilised by adding in a 1:1 ratio to 2% Triton/ TBS/inhibitors, (Complete Mini inhibitor tablet, Roche). This lysis solution and cells were pipetted up and down, and then placed on ice on a stirrer plate with a small magnetic stirbar for 1 hour. The solution was centrifuged for 20,000–40,000 X G, at 4°C for 2 minutes. The supernatant was retained and centrifuged again under the same conditions to pellet unsolubilised material. The supernatant was collected and stored in aliquots at -20°C.

2.2.4.2 Quantifying cellular protein, Bradford Assay:

The Bradford spectroscopic protocol is routinely used to measure the concentration of protein in a solution (Stoscheck, 1990). This dye assay is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acid conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form. To ensure equal loading of protein samples a standard curve was generated using Bovine Serum Albumin (BSA, Sigma-aldrich) protein. Dilutions of 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml of BSA was prepared and 5 μ l of each was assayed in 250 μ l of BioRad protein assay (Biorad GmbH). The BSA standard dilutions were read in duplicate on a benchtop spectrophotometer (BioRad *SmartSpec* 3000) at 595 nm and generated absorbance values between 0.1 and 0.65. The equation of the line generated

from these standards was used to calculate concentrations (x-values) by inputting absorbance values (y-values) of protein samples derived from cells. Protein yield ranged between absorbance values of 0.25 and 0.4. Sample volumes were adjusted accordingly to ensure that an equal quantity of total protein was loaded for each sample in an ELISA plate and on an SDS Page gel for western blot.

2.2.4.3 Elisa from transformed cells:

The protein samples were diluted in TBS and 50 µl aliquots of each sample pipetted into the wells of an ELISA plate. The ELISA plates were left to dry at 37°C overnight. The wells were then treated with four 5-minute washes in 200 µl PBST (Phosphate Buffered Saline + 0.05% Tween-20). The samples were then incubated with 100 µl of 1:2000 dilution of anti-rhodopsin mouse primary antibody (kind gift from Prof RS Molday) + 0.1 mg of BSA. The incubation was carried out in a humidified chamber (prepared by lining a sealed plastic dish with wet tissue) for 1–2 h at room temperature. After four more 5-minute PBST washes, the samples were incubated with the secondary antibody. This involved addition of 100 µl of 1:4000 anti-mouse IgG-peroxidase, (Sigma Aldrich) + 0.1 mg BSA to the wells for 1 hour at room temperature in a humidified chamber. Four final 5-minute washes in PBST were carried out. A substrate for the peroxidase-mediated colour development in the ELISA reaction is necessary. The substrate used was 3,3'-5,5'-tetramethylbenzidine (TMB, Roche). 100 µl of TMB solution was added to the cells for 30 minutes. To stop the ELISA reaction 50 µl of 0.5 M H₂SO₄ was added to the wells. A multi-channel pipette was used to ensure that samples being compared received antibody and reagent at the same time.

2.2.4.4 Western Blot analysis:

Proteins were electrophoresed on SDS-PAGE gels (2.1.3) at 50 V until samples reached the separating gel and thereafter at 80 V for 3 hours. Samples were transferred to a polyvinylidene difluoride (PVDF) membrane, (Funakoshi Co., Tokyo) by electroblotting at 120 mA for 2 hours. Blots were blocked against non-specific protein binding by soaking for 1 hour in 5% milk TBST solution on a rotator and then probed overnight at 4°C with an appropriate dilution, (1/250 for rhodopsin 4D2 antibody and 1/4000 for actin) of primary antibody. The blots were washed for 1 hour in 3 changes of TBST and probed for a one hour at 37°C with a peroxidase-conjugated goat anti-mouse secondary antibody, (dilution, 1/1000; Amersham, UK). Immune complexes were visualized with an enhanced chemiluminescence kit, (Supersignal west pico, Pierce). The actin antibody (abcam, cambridge) was used as a loading control in the western blots.

2.2.5 Mammalian Tissue Culture techniques

2.2.5.1 Sub-culturing mammalian cells:

Adherent cells were grown to confluency in 100 mm tissue culture dishes (Nunc) with complete DMEM in a incubator (Forma Scientific) at 37°C and 5% CO₂. Medium was removed from the cells and 3 ml of phosphate buffered saline (PBS, Sigma) was added to remove traces of medium. This wash step was repeated once. With PBS removed 1 ml of 2X EDTA-Trypsin (Sigma) was added to dissociate the cell monolayer. After 10 minutes at 37°C the cell layer could be removed by pipetting and the cells were centrifuged at 1,000 X G for 5 minutes. Pellets were resuspended in 5 mls of DMEM and 1 ml was removed for counting. Upon reaching 70–80% confluency, cells were split 1:4 (5×10^5 cells) in new dishes containing 15 mls medium. Medium was renewed every 2–3 days. All tissue culture procedures were carried out in a laminar air-flow cabinet (ESI securigarde 1200), with the exception of those involving microscope work and centrifugation.

2.2.5.2 Haemocytometer cell counting:

The edges of a coverslip were dampened and placed over the counting area on a haemocytometer slide (Hawksley) to achieve a secure seal. The cell sample was diluted 1/10, mixed and gently pipetted along the edge of the coverslip, capillary action allowing the fluid to be drawn underneath. The slide was viewed at 10X magnification under the microscope. The number of cells occupying 16 squares was counted for four quadrants of the slide and the average for a quadrant calculated. The number of cells = average cell count x dilution x $10^4/\text{ml}$.

2.2.5.3 Freezing down stocks of cells:

Cells grown to 70–80% confluency in 100 mm dishes were harvested and pellets resuspended in 2 mls DMEM (Sigma) supplemented with 10% fetal calf serum (Gibco, Brl). 500 µl volumes were added to labeled, pre-cooled cryotubes, (Nunc) containing an equal volume of 2X freezing solution (2.1.4). Cryotubes were stored at –70°C for up to 6 months and thereafter transferred to liquid nitrogen for storage up to 8 months.

2.2.5.4 Transfection of cells with LipofectAMINE 2000:

LipofectAMINE 2000 (GibcoBRL) is a polycationic lipid reagent that can be used to deliver nucleic acid molecules to cells (Ciccarone *et al*, 1999). One day prior to transfection cells were harvested, counted and plated on tissue culture dishes with antibiotic-free medium, so that they were 50–80% confluent on the day of transfection. For transfection of 1 µg of DNA, the DNA was diluted in 100 µl of medium. 4 µl of the LipoFectAMINE 2000 reagent was diluted in 100 µl of serum-free medium and complexed with the DNA for 30 minutes at room temperature. The lipid-DNA complex at a total volume of 200 µl was then pipetted directly onto the cells and the medium replaced the following day. The protocol for transfection of dsRNA was as above with Oligofectamine, (GibcoBRL) used as a lipid carrier. In addition, it was necessary to add the lipid-dsRNA complex to serum-free medium initially and 30% FCS added 4 hours after transfection. The medium is replaced after 24 hours with DMEM containing 10% serum.

2.2.5.5 In Situ staining of transfected cells (6-well plate):

24 hours post-transfection with a β-galactosidase reporter plasmid, medium was removed and the cells washed twice with PBS. 2 mls of fixing solution was pipetted onto the cells and the cells left at room temperature for 10 minutes. The cells were then rinsed with rinse solution. Cells were stained with 420 µl of stain solution at 37°C overnight. Following colour development, the cells were again rinsed before counting. Counting involved totaling the number of stained cell bodies observed under the microscope compared to clear cells in a minimum of four microscope fields of view.

2.2.5.6 Generating stable, clonal transformed mammalian cells:

48 hours post-transfection, cell medium was supplemented with 600 µg/ml G418 or 400-µg/ml zeocin (Invitrogen) depending on the antibiotic-resistance gene on the plasmid. Cells were selected over 3–4 weeks. The concentration of antibiotic was dropped to a maintenance level (200 µg/ml) after the control untransfected antibiotic-treated plate had died. Sterile, greased silicone cloning cylinders (Sigma) were used for single colony selection. The cells selected within the cylinder were trypsinised, seeded in 96-well plates and cultured through approximately 6 to 8 passages to reach confluency in a 100 mm dish.

2.2.5.7 MTT Cytotoxicity test:

Cells were seeded at 2×10^4 in a 24-well tissue culture dish with 400 μ l of DMEM and transfected with DNA (using liposfectAMINE 2000 reagent) or dsRNA (using Oligofectamine reagent, GibcoBRL) at 24 hours. After a further 24 hours, the cells were split across a 96-well tissue culture dish, with one well from the 24-well plate occupying 3 wells of the 96-well plate (150 μ l of media/well). After a further 24 hours the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) assay was performed. Testing involved removing media and staining cells over a 3-hour period with MTT. The MTT was made up to a concentration of 5 mg/ml in complete medium. 200 μ l of a 1 in 10 dilution of the MTT media was added to each well. After 3 hours at 37°C, 200 μ l of DMSO was added to each well and the colour development read at 595 nm.

2.2.5.8 DNA extraction from mammalian cell culture:

Approximately 1×10^6 cells were trypsinised, harvested and resuspended in 400 μ l of 10 mM tris-HCl (pH 8.0), 10 mM EDTA. Sodium dodecyl sulfate (Scharlau, Barcelona) and Proteinase K (Invitrogen) were added to a final concentration of 0.5% and 200 μ g/ml, respectively. The cell solution was mixed and incubated at 55°C for 2 hours. NaCl was added to a final concentration of 0.2 M. The nucleic acid was extracted twice with 1:1 phenol: chloroform and once with chloroform. Ribonuclease A (Stratagene, La Jolla) was added to a final concentration of 25 μ g/ml and incubated for 1 hour at 37°C. The DNA was extracted once with 1:1 phenol:chloroform, once with chloroform and precipitated with 1.5 volumes of ethanol. The DNA was pelleted by centrifugation at 1000 X G for 5 minutes. The DNA was resuspended in 100 μ l of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and used in subsequent PCR reactions.

2.2.6 Explant techniques:

2.2.6.1 Tail DNA extraction for genotyping

Tail tips from transgenic mice were digested overnight at 55°C in 400 µl of tail lysis buffer (2.1.5) and 15 µl Proteinase K (Invitrogen). The nucleic acid was extracted twice with 1:1 phenol: chloroform and once with chloroform. The DNA was precipitated with 1.5 volumes of ethanol, spooled on a plastic pipette tip and resuspended in approximately 80 µl of water.

2.2.6.2 In vitro electroporation and Retinal explant culture (Dr. Marius Ader carried out the following technique in the unit)

Dissected retinas from P0 or P1 newborn mice were transferred to a micro electroporation chamber (3 mm X 10 mm X 5 mm) filled with Hanks balanced salt solution (HBSS) carrying DNA for electroporation at a concentration of 1 µg/µl. Five 30 V pulses of 50-ms duration were applied to the tissue in the chamber using a pulse generator. The electroporated retinas were then cultured at 37°C on Nucleopore polycarbonate filters (Whatman, 0.2 µm pore) with neurobasal medium (Invitrogen) containing 2 mM taurine and B-27 supplement (Invitrogen).

2.2.6.3 Fluorescent Activated Cell Sorter (FACS) analysis of explanted tissue

Explanted retinas on the wells were delivered to the cytometer facility in the Conway Institute, University College Dublin, where they were dissociated on site and filtered through 50-µm Filcon filters (Dakocytomation; CN192). The samples were eluted in PBS and injected into the BD FACSaria sorter. The sorter incorporates three lasers at 488 nm (blue), 633 nm (red) and 407 nm (violet) which enable detection of two scatter signals, from side and forward deflection of the laser beam. In addition a FITC optical filter was used for detection of fluorescence. The sorting flow rate of 4 was used to maximise the purity of the final sample. Following sorting the cells were concentrated on Ultrafree-MC filter units (Amicon, PTFE membrane) at a concentration of <100,000 cells/unit, by spinning through the filters for 1 minute at 1300 X G. In addition, this allowed for separation of cell debris from the final population to be used for sorting. The filter units were stored in dry ice for transportation from the facility.

2.2.6.4 Dissociation of retinal explants from the membrane

P14 retinal tissues from cultured explants were washed twice on the polycarbonate filter membranes with PBS. A volume of 2 mls of trypsin (Sigma T-1005) at a concentration of 10 mg/ml was added to the explants. Following incubation at 37°C for 10–20 minutes, the retinas were removed from the membrane by pipetting up and down and transferred to a 15 ml tube. 200 µl of trypsin inhibitor (Roche 109878) was added to the cell solution to make a final concentration of 2 mg/ml. A 20-µl volume of DNase 1 (Sigma D5025) was also added to the dissociated cells giving a final DNase concentration of 100 µg/ml. The cells were dissociated by pipetting gently up and down 10 times with a P1000 pipette tip. The cells were pelleted by centrifugation for 5 minutes at 1200 X G. The pellets were resuspended in HBSS and plated onto PLL-coverslips at 500 µl/well.

2.2.6.5 RNA extraction and analysis of explant tissue following FACS sorting

To remove cell debris sorted cells were concentrated on amicon filters (as described in 2.2.6.3) and eluted with RLT buffer (Qiagen RNeasy Kit) to which 1 % v/v of 10-βmercaptoethanol (BDH chemicals, England) had been added. The buffer was passed through the filters by centrifuging at 6600 X G for two minutes. The filtrate was reapplied to the filter and eluted a second time through the filter by centrifuging at 6600 X G for two minutes. This step was repeated once more and the final filtrate used for RNA extraction. Qiagen RNeasy Mini Kit was used to extract RNA from the filtrate of sorted retinal cells. The RNA was extracted immediately using Qiagen RNeasy kit according to manufacturers instructions.

2.2.6.6 PLL-coating of coverslips

Approximately 1000 x 13 mm coverslips (SLS, Nottingham) were placed in a beaker and filled with 100% ethanol. The contents were left shaking overnight at room temperature. The coverslips were now washed in sterile water two to three times for 10–15 minutes. Water was removed and coverslips removed to 100 mm tissue culture dishes where they were arranged in separate rows and washed twice more with sterile water. 2 mls of Poly-L-Lysine solution, (Sigma-Aldrich, MO), was added to 100 ml of sterile water and mixed well. The coverslips, after a further wash, were added to a dish carrying a 1 in 50 dilution of the 2% PLL solution. The dish was agitated at regular intervals and coverslips turned. The PLL was left coating the coverslips overnight in the tissue culture hood. The liquid was removed from the plates and the coverslips washed three times with sterile water. They were allowed to air-dry in the

tissue-culture plate with the lid removed overnight in the tissue culture cabinet. To minimise degradation of the PLL protein, the coverslips were not exposed to any UV light.

2.2.6.7 Immunostaining of explanted cells

Following two PBS-washes of the explants on the polycarbonate filter membranes used for explant culturing, the explant cells were dissociated as described in section 2.2.6.4). A 500 µl volume of cells in HBSS was added to wells of a 24-well tissue-culture dish each well containing a single PLL-coated coverslip. The cells were left to settle on the PLL-coverslips for 30 minutes at 37°C. The supernatant was removed and the cells that remained clinging to the PLL-coverslips were fixed onto the coverslips by pipeting 500 µl of 4% paraformaldehyde onto the coverslips, which were then left to incubate for 30 mins at 37°C. To avoid non-specific immunostaining, the cells were blocked with a 500-µl volume of HBSS containing 10% goat serum (SigmaAldrich) and 0.3% triton (Sigma) for 1 hour in the dark at 37°C. The cells were then incubated with a 100-µl volume of a 1/150 dilution of 4D2 anti-rhodopsin antibody at 4°C overnight. Following three 5-minute washes with 500-µl volumes of PBS, the cells were incubated for 2 hours at 37°C with 100 µl of 1/700 dilution of anti-mouse alexafluor label (568nm, Sigma Aldrich). After a further PBS-wash, nuclear staining was performed by pipetting a 100-µl volume of 4'6-diamidine-2-phenylindole-dihydrochloride (DAPI, SigmaAldrich), at a dilution of 1/5000 onto the cell-coated coverslips for five minutes. This stain was removed and the cells washed once more with PBS as before. To fix the cells a drop of aqua polymount (Polyscience Inc.) was pipetted directly onto the cells, and the slides were covered with glass covers and stored at 4°C in the dark for future microscopy work.

2.2.6.8 Cryosectioning of retinal tissue and immuno-staining:

Before sectioning, the explant tissue was fixed on the membrane by replacing the culture media with 500 µl of 4% paraformaldehyde and incubation at 37°C for 30 minutes. Following fixation with 4% paraformaldehyde the explant membranes were washed by incubation with 500 µl PBS for 5 minutes. This wash step was repeated. The outlines of the retinas on the polycarbonate membranes were cut out and the retinas were suspended in OCT (optimal cutting temperature) compound (Tissue-tek) contained within plastic moulds. The OCT sections were snap frozen by liquid nitrogen and stored at -20°C prior to sectioning. The sections were made on a Leica CM 1900 cryosectioner in 7–10 µm widths.

2.2.7 Bioinformatic tools

2.2.7.1 www.ncbi.nlm.nih.gov

The NCBI database was used for all sequence retrieval and for sequence homology searches (Blast program). Pubmed was the most common literature database used for this thesis work.

2.2.7.2 *Secondary plotfold analysis*

Input sequence from the sequence retrieval database at NCBI was used in a widely available RNAdraw program, (Ole Matzura and Anders Wennborg, 1996). Default enthalpy and entropy settings for energy calculations at 37°C were used in all analyses.

2.2.7.3 <http://www.sph.uth.tmc.edu/Retnet/>

This retinal information network was frequently used to monitor newly identified retinal mutations.

Chapter 3

Suppression of human rhodopsin gene expression using siRNA

CHAPTER 3

3.1 Introduction

3.1.1 Targeted suppression of genes involved in Retinitis Pigmentosa

Gene therapy for dominant disorders where pathology is due to a gain of function mutation will often require suppression of the disease allele. A key aim of the study described in this chapter was to assess the effect of suppressing agents targeting genes implicated in the autosomal dominant forms of the degenerative retinal condition, Retinitis Pigmentosa, (ADRP). As discussed in chapter 1, (1.1.2), rhodopsin occupies an apical position in the visual transduction cascade and is the source of the most common mutations causing ADRP. In terms of potency of gene silencing, RNAi technology has surpassed ribozyme and antisense technologies over the last three years. However alternative suppression agents may be necessary to achieve complete suppression of the target gene (for example, ribozymes are discussed in chapter 5 for suppression of the peripherin/*rds* gene). In order to realise a long-term therapeutic goal, the specific activities of suppression agents (targeting the gene of interest) must be determined accurately. This thesis is primarily concerned with the application of RNAi to suppress expression of the rhodopsin gene, one of the most commonly mutated genes in ADRP. The most effective approach for suppression of mutant genes involved in the pathogenesis of ADRP is considered.

3.1.2 Suppression of the target gene Rhodopsin by RNAi

Various lines of transgenic mice have been generated carrying human mutant rhodopsin transgenes (Bernstein *et al.*, 2001). For example, a mouse expressing the pro23his human rhodopsin transgene has been created for research into this relatively common ADRP-causing genotype (Dryja *et al.*, 1992). In this model, Dryja and colleagues observed a close correlation between severity of disease and level of expression of the mutant transgene. In the context of suppression-based gene therapies, this finding suggests that reduction in levels of mutant rhodopsin protein should in principle provide some therapeutic benefit. Hence there is a clear rationale for developing molecules such as siRNA, ribozymes, and antisense, which suppress rhodopsin effectively. Although complete suppression of a disease allele may be

difficult to achieve, in some cases low levels of mutant protein may be tolerated (Hutvágner *et al.*, 2001). Gene silencing has been achieved with varying success in the past using antisense DNA and RNA, ribozymes, triple helix, peptide nucleic acids, and oligo decoys amongst other agents (Bass *et al.* 2001; Bernstein *et al.*, 2001; Sijen *et al.*, 2001; Hutvágner *et al.*, 2002; Zhang *et al.*, 2002; Bernstein *et al.*, 2003). Of these approaches, antisense, ribozyme and siRNA technologies, in particular, have been developed as potential nucleotide-based therapies. During the course of the studies described in this thesis, RNAi has been used to achieve knockdown of the rhodopsin gene, with the long-term goal of utilising the suppression of rhodopsin expression as a potential gene therapy strategy for ADRP. A short description of the mechanism of RNAi is provided below, together with commentary on how this relatively new technology is being used for experimental research and therapeutic development.

3.1.3 Milestones in the discovery and application of RNAi:

The phenomenon termed cosuppression was originally observed in petunia flowers, whereby overexpression of the gene responsible for purple pigmentation caused the flower to lose its purple colour (Jorgensen *et al.*, 1996). However the mechanism of cosuppression was unexplained until a link was made with a gene silencing study in *Caenorhabditis elegans*. Fire *et al.* reported the potency of combined antisense and sense nucleotide strands for silencing of endogenous homologous targets in a seemingly independent pathway from the pathways involved in classical antisense inhibition (1998). This seminal 1998 paper was the first in which such a phenomenon was described and the authors coined the term RNA interference (RNAi) to describe this effect.

In January 2001, sequence-specific silencing was demonstrated for the first time, without inducing activity from the dsRNA-dependent protein kinase (PKR) or triggering synthesis of 2'-5' polyadenylic acid, both of which cause global, non-specific shutdown of translation (Elbashir *et al.*, 2001b). Inspired by the observation of small RNase-III like digestion products of dsRNA in *Drosophila* lysate, Elbashir *et al.* were able to induce specific gene silencing by utilising so called siRNAs of 21–25 nucleotides in length. The potential therapeutic benefit associated with RNAi-

based suppression was shown in early cell-based assays, when poly-glutamine cytotoxicity was reduced in *Drosophila* embryonic S2 cells by the siRNA-mediated targeting of CAG repeats for down-regulation, (Caplen *et al.*, 2002).

In February 2002, Paddison *et al.* became the first to demonstrate that long-term gene silencing can be stably achieved with the expression of long hairpin dsRNAs from an RNA Pol-III promoter in mammalian cells (Paddison *et al.*, 2002). Yu *et al.* further refined this method (2002), while Brummelkamp and colleagues designed the first vector system based on hairpin dsRNA to achieve stable and specific gene silencing, (Brummelkamp *et al.*, 2002a). Such progress widened the application of the technology to a therapeutic setting. During this same time Donze and Picard devised an alternative method of synthesizing dsRNA by using recombinant T7 RNA polymerase to produce dsRNA *in vitro* (Donze and Picard, 2002). Using array-based expression profiling and other methods, it was established that the major requirements for expression of dsRNA included short oligos with a 2-nucleotide 3' overhang and no 5'UTR leader sequence (Kumar *et al.*, 2003). Subsequent to these initial findings, RNAi has been widely adopted in academia and industry alike as a means to achieve potent suppression of expression of a target gene.

3.1.4 The mechanism of RNAi mediated suppression in cells

The proposed model for dsRNA-directed mRNA cleavage, as it occurs endogenously, involves processing of dsRNA into 21–23nt fragments by the enzyme dicer or a dicer homologue (Jorgensen *et al.*, 1996; Fire *et al.*, 1998). The resulting fragments with 3' blunt ends are incorporated into a distinct nuclease complex, the RNA-induced Silencing Complex, (RISC) which targets mRNA for degradation (refer to fig. 3.1 in the figures and tables section at the end of this chapter). Cleavage is performed relative to the center of the targeting dsRNA, usually around positions 10 and 11. Additionally, an RdRp such as that encoded by the *ego-1* gene in *C.elegans* might amplify the dsRNA signal producing long-lasting post-transcriptional gene silencing in a process called transitive interference (Dykxhoorn *et al.*, 2003). The absence of identified RdRp homologs in *Drosophila* and mammals suggests that this amplification of gene silencing is unlikely to be present in mammalian systems.

Dicer does not function in mammalian cell culture when an exogenous long dsRNA targeting a gene is delivered (Elbashir *et al.*, 2001a). Instead a global shut down of gene expression results. The phosphorylation of translation factors by PKR precipitated by the longer molecule may be avoided by delivering instead, short dsRNAs of approximately 21 nucleotides in length. Since these discoveries dsRNA has been utilised to elicit gene silencing in primary mammalian cells, stem cells, and transgenic mice and it seems that the range of applications for this powerful suppression technology continues to expand (Rubinson *et al.*, 2003).

3.1.5 Components of the RNAi pathway

Post-transcriptional gene silencing mechanisms have been shown to require the formation of a ribonucleic protein complex termed the RNA-induced silencing complex (RISC). Martinez *et al.*, (2002), have analysed the reconstitution of human RISC in extract prepared from HeLa cells. In this study the RISC complex was partially purified and its size estimated between 90 and 160 kDa. A number of important conclusions have been reached resulting from observations in this cell-free system. The relatively small size of RISC suggested that the dicer enzyme is not present as part of the RISC complex. Streptavidin selection of biotinylated siRNA substrate strands in the cell-free system revealed that only the antisense siRNA strand gets incorporated into RISC. In addition, RISC activity was observed in cytoplasmic HeLa extract but not in nuclear extract, suggesting that the RNAi machinery exists predominantly in the cytoplasm. This finding supported the theory that RNAi may act primarily on mature rather than nuclear precursor mRNA (Montgomery *et al.*, 1998). Mass spectrometry on affinity-purified RISC identified two proteins of the Argonaute family, eIF2C1 and eIF2C2 (human GERp95), in the complex. Although there is limited biochemical information currently available on argonaute proteins the PAZ and Piwi domains they contain have previously been linked to processes involving posttranscriptional gene silencing (Cerutti *et al.*, 2000). Human eIF2C1 is more than 80% homologous to eIF2C2 and therefore it is possible that one of the two proteins may be functionally redundant (Martinez *et al.*, 2002). Alternatively eIF2C1 and eIF2C2 may form heterodimers necessary for RISC formation. Human eIF2C2 has been shown in HeLa cells to be associated with a species of dsRNA in the cell termed microRNAs (Dostie *et al.*, 2003). EIF2C2 has been identified as a component of the

SMN (Survival of Motor Neuron) complex, a regulator of ribonucleoprotein assembly (Mourelatos *et al.*, 2002). The role of microRNAs as translational regulators is further discussed in chapter 5.

3.1.5.1 Dicer's role in RNAi:

The generation of small RNAs from larger precursor transcripts is an essential feature of the RNAi pathway. Exogenous dsRNA when introduced into invertebrate cells becomes cleaved by an RNase-III enzyme, Dicer, into siRNA (Bernstein *et al.* 2001; Elbashir *et al.* 2001c). Human dicer was identified by its unique domain structure, (which includes an ATP-dependent RNA helicase domain, RNase-III motifs and COOH-terminal dsRNA-binding domain), and by its sequence homology to *Drosophila* dicer (Bass *et al.* 2001; Bernstein *et al.* 2001).

The length of siRNA processed by dicer is species-specific and may reflect differences in the spacing or structure of the RNase-III domains of dicer homologs (fig. 3.2). Studies on recombinant dicer *in vitro* suggest that the enzyme's cleavage activity is ATP-independent. The ATP requirement in the reaction may be to ensure sufficient product release to sustain the multiple turnover kinetics of the enzyme (Zhang *et al.*, 2002; Haley *et al.*, 2004). It has been suggested that the helicase domain of the dicer enzyme may have an additional role as a translocase dragging the enzyme down the length of the mRNA rather than simply unwinding the transcript for localized cleavage (Hutvagner *et al.*, 2002).

Dicer is also required for the maturation of microRNAs involved in the regulation of developmentally essential genes (further discussed in chapter 5). Hutvagner *et al.* have shown how the naturally occurring 21 nucleotide small temporal RNA, (stRNA) *let-7*, which regulates developmental timing in *c. elegans*, is cleaved by a dicer homologue in *Drosophila* (Hutvagner *et al.*, 2001). This essential role may explain why disruption of the mouse dicer gene results in an embryonic lethal phenotype (Bernstein *et al.*, 2003).

3.1.5 (ii) RNA-dependent RNA Polymerase in RNAi:

There is evidence of amplification and spreading of the silencing effect in plants, *Neurospora*, and *C. elegans* (Tang *et al.*, 2004; Sijen *et al.*, 2001, Chicas *et al.*, 2004). RNA-directed RNA polymerase, (RdRP) was among the genes identified as

being involved in this process using genetic screens for mutants of siRNA amplification (Cogoni *et al.* 1999). The involvement of RdRP in the self-sustaining nature of RNAi has been suggested and the effect has been characterised most extensively in *C.elegans* (Fire *et al.* 1998; Sijen *et al.* 2001). In plants this spread of gene silencing may have evolved as a systemic resistance to viral infections, however the RdRp gene, termed SDE-1 in plants, is required only for transgene-induced PTGS and not for virus-induced PTGS (Dalmay *et al.* 2000). Amplification is not believed to occur in vertebrates or fruitflies, as genes for the necessary RNA polymerase have not been found in these organisms. Lipardi *et al.* provided convincing evidence that characteristics of the siRNA may be critical to enable the molecule to serve as a primer for RdRp (Lipardi *et al.* 2001). This study involved analysis of the dsRNA-dependent degradation of GFP mRNA in a cell-free extract of *Drosophila* embryos using recombinant RdRp. The work revealed how the RdRp chain reaction is initiated by siRNA-primed conversion of target mRNA and amplifies dsRNA suppression. The resulting products can then add to the substrate pool for dicer. Although this work was carried out in cell-free extract of *Drosophila*, an RdRp homologue has yet to be identified in the fruitfly. The RdRP gene in *C. elegans* encodes EGO-1, (the SDE-1 homologue in nematodes) which is essential for RNAi but only in germline cells (Smardon *et al.*, 2000).

Winston *et al.* identified a second protein in *C. elegans* required for systemic silencing termed SID-1 (2002). The *sid1* gene encodes a transmembrane protein that may act as a channel for import of the silencing signal. Curiously, SID-1 homologues are present in mammals raising the possibility that some aspects of systemic silencing may exist in mammals, although current evidence suggests otherwise. Transitive RNAi has not been observed in HeLa cell lysate and is likely to be rare in higher vertebrates (Martinez and Tuschl. 2002). Further evidence against this primed propagation of gene silencing using guide siRNAs in mammalian systems include the fact that the silenced mammalian genes return to normal levels at 5 to 9 days post transfection (Elbashir *et al.*, 2001a). A mechanism for the systemic spread of silencing may have been selected against in higher vertebrates as secondary siRNA generation can also cause amplification of any non-specific effect, potentially putting a significant fraction of the genome at risk.

3.1.6 Differential requirements of the sense and antisense strands in siRNA

In September 2002, Martinez *et al.* manipulated extract prepared from HeLa cells to create a human biochemical cell-free system that could recapitulate RNAi, thus unraveling some mechanistic details. By focusing on the mRNA targeting step and formation of RISC, they were able to demonstrate that the 5'-phosphorylated antisense siRNA strand resides in the complex with the crucial accessory proteins (Martinez and Tuschl., 2002). This incorporation of the antisense strand is necessary for effective inhibition to occur. In the design of siRNAs it is therefore important to ensure that the free energy of the 5'antisense strand will be in excess of the 3' end and thus ensure its preferential early incorporation into RISC.

3.1.7 *In vitro* synthesis of Small Interfering RNA

Elbashir *et al.* carried out the first reported experiment involving siRNA-induced silencing of an endogenous transcript by targeting lamin A/C following transfection of duplex siRNA into HeLa cells (Elbashir *et al.*, 2001a). Currently there are four methods used to produce siRNA *in vitro*; these are chemical synthesis using RNA phosphoramidites, Pol-III or pol-II promoter driven expression of siRNA, *in vitro* T7 RNA polymerase transcription and annealing of siRNA, and finally restriction enzyme-generated siRNA.

3.1.7.1 Chemical synthesis

Chemical synthesis of oligonucleotides is a readily used procedure in molecular biology. However production of siRNA requires additional, more specialized steps, including the annealing *in vitro* of two homologous strands, addition of chemical entities to increase stability, and synthesis of two nucleotide overhangs to ensure activation of RISC. A 3' hydroxyl group and 5'phosphate group are also required by the siRNA for functionality (Dykxhoorn *et al.* 2003). Over the last four years a number of commercial ventures have emerged specializing in production of ultra-pure small dsRNA species. Many of these companies also offer the application of patented algorithm programs to provide predictions of the most active siRNAs against any given target. Phosphoramidates are the unit building blocks for synthesizing oligonucleotides and for the chemical synthesis of twenty-one nucleotide RNAs. These oligos are usually HPLC purified and sense and antisense strands can be reconstituted and annealed immediately prior to use (Genset oligos,

SA). The efficacy of chemically synthesized siRNA for inducing endogenous RNAi was demonstrated in the early RNAi experiment involving silencing of cytoskeletal protein lamin A/C in human cell lines. Importantly cell viability was not compromised by the use of artificial siRNA (Elbashir *et al.* 2001a).

3.1.7.2 T7 siRNA Synthesis

The high cost of chemical siRNA synthesis combined with the uncertainty of effective silencing had originally been an obstacle to the development of RNAi-mediated gene silencing as a standard laboratory procedure. In 2002 Donze and Picard devised a simple alternative to chemical synthesis (Donze and Picard, 2002). The process involves transcription in a test-tube of siRNA using T7 RNA polymerase and has been shown to produce a relatively large amount of dsRNA. Owing to its short termination signal, the T7 promoter has been widely used for *in vitro* transcription of RNAs over the past two decades. The method requires a 38-mer oligonucleotide with the target sequence preceded by the complementary sequence of the T7 promoter annealed to an 18-mer oligonucleotide encompassing the T7 promoter, which is used as a primer for the test-tube reaction. The transcribed sequence is 19-nt plus two nucleotides. Since the antisense strand has been shown to guide target recognition, the two nucleotides on the antisense strand should be specific for the target but may be random on the sense strand (Lipardi *et al.*, 2001). Over the last two years as an understanding of the sequence-specific requirements for effective siRNA has emerged, a major shortcoming of this procedure has also been highlighted. During transcription, the last guanosine of the T7 promoter becomes the first ribonucleotide to be incorporated into the RNA. Therefore all siRNAs designed by this method will start with a G. This clearly may not be optimal, as potency rankings of siRNA have shown that the most active siRNAs, with few exceptions, start with a U (Hall *et al.*, 2004). However, the simplicity and low cost of this protocol make it an attractive alternative to conventional chemical methods of siRNA synthesis.

3.1.7.3 Restriction enzyme-generated siRNA

A major limitation of most forms of presynthesized siRNA is the varied inhibitory ability of different siRNAs targeting the same gene (Harborth *et al.*, 2001; Holen *et al.*, 2002). Each mRNA must be screened for efficient silencing in a

relatively laborious and costly process. Specific post-transcriptional gene silencing with a single long dsRNA in excess of 26-nucleotides is generally not possible, without activating interferon response genes and precipitating a global shutdown of gene expression. However, a transcript spanning the entire length of the target gene's cDNA may be generated *in vitro* using T7 RNA polymerase. The nascent transcript is then chopped up to produce 21-nt siRNAs using an appropriate recombinant enzyme (Yang *et al.*, 2002). An example of such an enzyme is RNase-III from *E. coli*. In effect a library of siRNA may be produced for a given gene by random RNase-III-mediated cleavage along the length of the transcript. Although the most active RNAi species within this mix may be difficult to determine, suppression is generally guaranteed. However, one obvious disadvantage of this approach is the likelihood of significant 'off-target' effects; these effects are discussed further in chapters 4 and 5.

3.1.7.4 Expression vectors for production of siRNA

Mammalian cells have the cellular machinery to convert short hairpin RNA into siRNA to mediate specific gene silencing (Paddison *et al.* 2002; Brummelkamp *et al.*, 2002a). Upon transfection of plasmid expressing a short hairpin RNA, (shRNA) a double-stranded transcript would be produced and recognized by dicer for cleavage into siRNA in the cell. Constitutive expression of siRNA may allow for long-term silencing of a target gene. Moreover, cell lines stably expressing shRNAs could be selected by antibiotic resistance and maintained for *in vitro* work. The crucial factor required to achieve such a system was the use of suitable promoters. The RNA Pol-III promoters U6 and H1 cause termination after the second uridine in a short termination signal (of five uridines) and hence result in formation of transcripts similar to those siRNAs, which are produced by dicer cleavage, i.e., containing two symmetrical 3' overhanging U nucleotides. A temporal or tissue-specific control on suppression may be added to the construct. For example, a tetracycline-inducible U6-driven siRNA vector has been delivered to an *in vivo* model of prostate cancer (Czauderna, *et al.* 2003).

3.1.8 Stable expression of RNAi for therapeutic purposes

The transient nature of translational inhibition by siRNA in cell culture, which typically peaks at around 48 hours post-transfection, can be overcome by a vector-driven expression system. The vehicle chosen will depend in part on the target tissue

and on the efficacy of the siRNA molecule for targeting a gene of interest. Subsequently the appropriate transfection reagent, delivery method or route of administration needs to be optimised for the vector. Assessing and developing the most suitable siRNA suppression within a vector system is the principle focus of this chapter. However, it is also important to be aware of the effects that these vector-systems may have on gene expression independent of the expressed siRNA sequence. Hence possible vector competition effects from the cotransfection of target and suppressor plasmids in cells shall be considered. In addition the potential toxicity and immune responses provoked shall be addressed in this chapter and in chapter 5.

3.1.8.1 RNA Pol III-dependent siRNA construct:

Transcriptional termination by RNA polymerase-III occurs at runs of four consecutive T residues in the DNA template (Booth and Pugh, 1997; Tazi *et al.*, 2003). RNA Pol-III-based vectors have been shown to elicit efficient synthesis of short RNA molecules in mammalian cells (Noonberg *et al.*, 1994, Good *et al.*, 1997). Specifically, the U6 and H1 promoters in the RNA Pol-III class are useful for transcription of small RNA species in mammalian cells. This is due to the fact that the promoters carry their regulatory gene expression elements in a discrete promoter located 5' to the transcript, unlike other Pol-III-dependent genes where the cis-acting regulatory elements occur within the transcript itself (Baer *et al.*, 1990). Studies using cellular extracts have revealed that U6 recruits trans-acting factors other than those normally used for RNA pol-III-based *in vitro* transcription. Yu *et al.* were among the first to inhibit gene expression using an expression vector carrying the mouse U6 promoter (Yu *et al.*, 2002). In addition to four terminal U residues and a 3-nucleotide hairpin loop, Yu *et al.* chose target sequences that began with a G. This is the first nucleotide of the mouse U6 gene and may make the construct more resilient to addition of extra nucleotides at 5'UTR of the siRNA sense strand. In this work the pol-III-dependent H1 promoter is adapted for the development of a stable siRNA expression system, H1-shRNA (small hairpin RNA). The H1 promoter drives expression of the RNA component of the human nuclear Rnase P and the unusually compact organisation of this promoter has been characterised (Myslinski *et al.*, 2001). Unlike most known cases of Pol-III transcription, intragenic sequences (with the possible exception of the initiating G nucleotide in the case of the U6 promoter) are

not required for efficient and accurate transcription of the U6 or H1 gene *in vitro* (Das *et al.*, 1988).

3.1.8.2 RNA Pol II-dependent siRNA construct:

Recently Lee *et al.* have shown the effect of a microRNA-based molecule, (miRNA) in the post-transcriptional regulation of gene expression in the HeLa cell line with the miRNAs transcribed by RNA pol II-based promoters (Lee *et al.*, 2005). The rationale behind the use of pol II-based promoters for driving siRNA expression is that the cell itself can produce siRNA optimally with this promoter. Moreover the regulatory elements of the promoters driving miRNA expression are capable of restricting expression to specific tissues or developmental stages (Dickins *et al.*, 2005). The possibility of using such a construct to achieve Pol II-based tissue-specific stable siRNA expression is therefore relevant for therapeutic RNAi strategies. Indeed most recently, miRNA expression cassettes have been used in essence as factories for siRNAs. The use of microRNA-based loops in shRNA design is further explored in chapter 5.

3.1.8.3 Hairpin siRNA vectors (shRNA):

A number of designs utilizing Pol III-based promoters to drive expression of functional siRNAs have been explored (fig. 3.3). The use of a single promoter to drive expression of a hairpin construct has been shown to be the most powerful method of achieving inhibition (Brummelkamp *et al.*, 2002a). Other groups have since optimised the hairpin construct used in the initial studies, primarily with variations in the length of the hairpin loop (Das *et al.*, 1998). The success of shRNAs in achieving gene silencing may be attributable to the innate processing of microRNA molecules that occurs in mammalian cells from a hairpin precursor element (Boden *et al.*, 2004). The cell readily recognises and processes such hairpin constructs following their transport by exportin-5 from the nucleus to the cytoplasm. It has been suggested that transfected siRNA-expressing hairpin vectors can recruit this microRNA-processing pathway (further investigated in chapter 5). It has also been demonstrated that the length and nucleotide sequence of the loop can affect the efficiency of gene silencing (Kawasaki *et al.*, 2003b). This aspect of shRNA design is explored in chapter 5 with the use of a microRNA loop on a shRNA vector. Early successes with the shRNA vector system included the generation of stable knockdown

cell lines, transgenic animals and the inducible regulation of RNAi (Leung and Whitaker, 2005). While some of these applications have yet to be fully validated, the success of this system for stable delivery of siRNA *in vitro* and *in vivo* has been repeatedly confirmed and shall be further discussed in section 3.4.

In addition to Pol-III promoters and polymerase-II vectors, viral vector-based expression systems have been used effectively to express siRNA (Tiscornia *et al.*, 2003). In every case the effect caused on gene expression by the vector system independent of the siRNA has to be assessed (Brummelkamp *et al.*, 2002; Robinson *et al.*, 2003). A Pol III-expression vector-based method of siRNA production was explored during the course of this study. This approach, as described in this thesis, may enable long-term production of a potential therapeutic for ADRP.

3.1.9 Choosing target sites

The appropriate choice of target site from the open reading frame of a cDNA sequence is crucial for effective down-regulation by siRNA. Holen *et al.* originally observed wide variation in siRNA-based suppression in mammalian cells depending on target sequences (Holen *et al.*, 2002). A basic set of criteria was established early on in the development of RNAi technology. These included selection of a target sequence no less than 50 nucleotides from the start codon, as complexes involved in translation initiation may block access of RISC to the mRNA. A 50% GC content and a target mRNA sequence 5'-AA(N19)UU were also recommended (Tuschl, 2004). Most recently, as prediction algorithms for siRNA designs have been updated and compared, additional considerations have been applied to selection. Ui-Tei *et al.* extensively analysed 62 target sequences of four exogenous and two endogenous genes in mammalian and *Drosophila* cells (Ui-Tei *et al.*, 2004). Highly effective RNAi was found to occur in mammalian cells if the following four sequence conditions were met: 1) A/U at the 5' end of the antisense strand; 2) G/C at the 5' end of the sense strand; 3) AU richness at the 5' terminal one-third of the antisense strand; 4) The absence of GC stretches of more than 9 bp in length. Typically, those siRNAs with sequence opposite in aspect to the first three conditions above failed to induce any silencing of the target genes. Even a 1-nt variation in the target sequence, i.e. one nucleotide up- or down-stream had a considerable effect on RNAi activity in mammalian cells. *Drosophila* cells were far less sensitive to this slight sequence variation.

It has been demonstrated using positive control siRNAs of known activity (targeting the EGFP gene), that the presence of A/U at the 5' end of the antisense strand is even more essential than the requirement of absolute sequence homology between dsRNA and the target (Ui-Tei *et al.*, 2004). This prompted the authors to suggest that the 5' antisense end of siRNA molecules plays a crucial role in the formation of the RISC complex. It is believed that the presence of the lesser hydrogen bonded A/U pairing at this terminus allows for greater flexibility in unwinding of the duplex ensuring helicase recruitment and RISC formation. Khvorova *et al.* found a strong link between siRNA function and low internal energy across the duplex and especially in the region of nt 9-14 (2003). Other investigators however have not observed as strong a correlation (Ui-Tei *et al.*, 2004).

The secondary structure of the target transcript, which is a key consideration for antisense and ribozyme designs, seems to be less relevant for siRNA. The target sequence rather than overall structure appears to be essential for siRNA activity. Thus far, the suggested siRNA design rules allow sufficient scope for selection of optimal molecules to achieve RNAi in mammals. Rational design strategies should in future minimize the time and cost involved in down-regulation of gene expression using this technology. An example of an online tool for selection of siRNA target sites is the freely available siDirect software system at <http://design.RNAi.jp/>, (Naito , 2004) and MWG's siMAX program (Schramm and Ramey, 2005). Companies involved in chemical manufacture of presynthesized siRNA molecules similarly offer support in target site selection (<http://www.oligoengine.com/>, <http://www.dharmcon.com>, and <http://www.qiagen.com/siRNA>).

3.1.10 Replacement rhodopsin protein: achieving functional rescue

A replacement gene for human rhodopsin was generated by Dr. Mary O'Reilly in our laboratory that encompasses five changes at degenerate base pairs in codons of the first exon of the human rhodopsin gene. Notably this replacement gene (termed HR5m in this study) has been chosen to express functional rhodopsin protein *in vivo* by restoring rod photoreceptor activity in a transgenic mouse on a rhodopsin knockout background, (rho-/-). More specifically, transgenic mice expressing the replacement human rhodopsin gene were generated and subsequently bred to a mouse line in which the mouse rhodopsin gene has been disrupted. Offspring show a normal physiological response to light (as evaluated by electroretinogram) and normal

histology when dissected retinas were sectioned and examined by microscopy (discussed in chapter 4, figs. 4.2a-b). The rhodopsin replacement gene was originally designed for use in conjunction with a multimeric ribozyme and is now being used for siRNA targeting the same region of human rhodopsin gene (O'Neill *et al.*, 2000). This illustrates how, in principle, the suppression and replacement strategy can allow for a generic replacement gene to be used with many potential suppression agents.

In the current study, a range of small interfering RNAs has been designed covering the region of the sequence that has been altered in the human rhodopsin replacement gene. These dsRNA molecules target wild-type human rhodopsin sequence (without the incorporated replacement base pair changes). Hence, in principle, transcripts from the replacement rhodopsin gene should be spared from suppression by dsRNAs targeting the wild-type rhodopsin sequence. Results from a study exploring if transcripts from a modified human rhodopsin gene can indeed be protected from siRNA-based suppression shall be presented in chapter 4, together with a focus on the specificity of RNAi.

The resulting data provides the impetus for the design of an *in vivo* experiment evaluating siRNA-mediated suppression and replacement of the human rhodopsin gene (discussed in chapter 7).

3.1.11 Animal Models used in the experiments described in chapter 3

3.1.11.1 Rho^{-/-} knockout mouse:

The rho^{-/-} transgenic mouse model with an inherited retinopathy was engineered to carry a targeted disruption of the rhodopsin gene (Humphries *et al.*, 1997). In this mouse model, rod outer segment fail to develop, although the animal does possess the expected number of photoreceptor nuclei at birth. At two months the ERG is no longer recordable and by 3 months nearly all photoreceptors are lost, with the exception of some residual cones (Humphries *et al.*, 1997). The degeneration is found to be more rapid when bred onto a L129 rather than a C57 murine background (Humphries *et al.*, 2001). Rho^{-/-} mice have been used in the past to assess the therapeutic benefit of transplantation of schwann cells to affected retinas (Keegan *et al.*, 2003).

3.1.11.2 Human Rhodopsin transgenic mouse model, NHR mouse:

Murine and human rhodopsins differ in their amino acid sequences at 18 residues. Despite variability around the chromophore-binding lysine residue at codon 296, an area expected to show conservation between species, the human and mouse rhodopsin proteins appear to be functionally equivalent. It has been shown that human rhodopsin can be used to rescue the photoreceptors of *rho*^{-/-} mice by crossing these animals with mice expressing a wild-type NHR transgene (Normal Human Rhodopsin mouse) at levels, which approximate to those normally existing in the mouse retina (Olsson *et al.*, 1992; McNally *et al.*, 1999). A cross between the NHR mouse model (on an endogenous mouse rhodopsin knockout background) and the *Rho*^{-/-} model results in a substantial long-term rescue of structure and function of the rod photoreceptors in pups, which were of the genotype NHR^{+/-}(MouRho^{-/-}). The offspring showed 75% normal maximum ERG amplitude and normal wave thresholds at 20 weeks (McNally *et al.*, 1999). Notably, in the context of this thesis, the NHR mouse is used for *ex vivo* testing of siRNAs targeting human rhodopsin (section 3.3.8).

3.1.12 Retinal Explants

Primary cultures, though useful for their similarities to living systems, present special challenges to the researcher as it requires time and skill to produce a suitably organotypic culture. Explant cultures of whole or partial retina preserve the overall tissue architecture of the retina *in situ*. Transgenic animals provide a valuable source of genetically altered cells for such organotypic cultures.

In a series of explant experiments undertaken as part of the suppression study described in this chapter, retinal explants were used as a model for assessing the effect of siRNA-based rhodopsin transcript downregulation in retinal cells. Retinas from NHR^{+/-}(MouRho^{-/-}) mice were taken at post-natal day 0 (carried out by Dr Marius Ader) and cultured for two weeks. The suppressor on an EGFP-tagged plasmid (fig. 3.5) was electroporated into retinal cultures on the first day of explant seeding.

The model used in this case was an animal carrying one copy of normal human rhodopsin on a mouse rhodopsin knockout background, NHR^{+/-}(MouRho^{-/-}). Animals were genotyped using DNA extracted from tail-tips of mice. To optimise cell survival in explants, it was necessary to seed the day old retinas as soon as the animals were sacrificed, therefore all retinas from a single litter were harvested and

used in generation of explant material. Explants subsequently shown to be positive for the correct genotype were used for further experimentation. (It is not practically or ethically possible to tail-tip neonates while they are still alive, therefore all pups had to be sacrificed, retinas extracted, seeded on a membrane and electroporated with the siRNA constructs before the genotyping results were obtained). RNA levels as well as the immunostaining and histology of retinal cultures were assessed at 14 days post-electroporation. The findings from this *ex vivo* study in retinal explants are presented in this chapter of the thesis. While *in vitro* findings usually require *in vivo* validation, the gap between conventional tissue culture to animal studies has been bridged using the explant system. Parameters for full-scale animal experiments can be established in the explant model thereby possibly reducing the overall time required deriving *in vivo* data.

3.1.13 Plasmid Delivery in retinal explants

Electroporation is a promising technique for gene delivery to neurons. This approach utilizes an electric field to create transient pores in the cell membrane thus allowing entry of cDNA from the extracellular medium (reviewed by Inoue and Krumlauf, 2001). An efficient non-viral, electroporation-based transfection technique that has been optimised for neural progenitor cells has been described by Richard I and Ader M (Richard and Ader, 2005), and has been further adapted by Dr. Ader for the retinal explants used in this thesis.

3.1.14 Summary of topics explored in this chapter

1) The most effective siRNA molecules targeting the region of the human rhodopsin cDNA from nucleotides 177 to 189, (mRNA loop-14, as illustrated in fig. 3.4) are determined and developed for further use in a suppression and replacement therapeutic strategy for ADRP. The siRNAs targeting human rhodopsin shown in fig. 3.7 have been cotransfected into mammalian cells with a plasmid expressing human rhodopsin cDNA and subsequently rhodopsin expression levels evaluated. Each siRNA-based suppression agent was assessed by a combination of i) microscope analysis of cells transfected with constructs containing the target rhodopsin gene together with an EGFP marker, ii) quantitative rt-PCR on RNA from stable and transiently transfected cells using two different rhodopsin-expressing constructs and a

variety of house-keeping controls, and iii) the most promising siRNAs were further evaluated in ELISA on protein purified from the cotransfected cells.

2) This study also initially involved the stable expression of the most active suppression agents in an *in vitro* system. In April 2002, Brummelkamp *et al* detailed their design for pSuper, an optimised expression vector for siRNA (2002). The promoter chosen to drive expression in this case was the polymerase III-dependent H1-RNA promoter. The group demonstrated that they achieved 2 months of stable suppression of an endogenous gene in MCF-7 cells. Given the success in the literature for shRNA-based suppression, this vector has been adapted in the current study to explore stable knockdown of the human rhodopsin gene. The generic construct, as used in mammalian cell culture is illustrated in fig. 3.6.

3) The second half of this chapter of the thesis is concerned with the validation of siRNA-mediated suppression of rhodopsin in the region of interest (mRNA loop-14, as illustrated in fig. 3.4), in an *ex-vivo* organotypic model using retinal explants. As mentioned above, the mouse model chosen for assessment of the suppression carries the NHR transgene on an endogenous mouse rhodopsin knockout background. Results from this section of the work will provide the impetus to proceed towards *in vivo* analysis of siRNAs/shRNAs targeting the rhodopsin gene.

3.2 Methods

(General methods are presented in section 2.2)

3.2.1 *Predicted secondary structure of human rhodopsin mRNA*

A plotfold based on the thermodynamic profile of human rhodopsin mRNA was produced using the web-based application “RNAdraw”. The full-length rhodopsin cDNA including 5’UTR was used as input sequence (obtained from the sequence retrieval database at NCBI). Default enthalpy and entropy settings for energy calculations at 37°C were used in all analyses. The predicted open loops of the transcript are numbered from the start of transcription; ‘loop-14’ identifies the site targeted in previous suppression studies (O’Neill *et al.*, 2000) and altered in the replacement rhodopsin gene used in chapter four.

3.2.2 *Cloning pIRES-EGFP-HR (used in the in vitro analyses 3.3.1–3.3.7)*

The human rhodopsin cDNA clone, (HR) was originally obtained from Dr. W. Baehr. The cDNA and full 5’-UTR was subsequently cloned into pcDNA3 by Dr. J. Farrar and Dr. S. Kiang. This clone was then used to produce the pIRES-EGFP-HR construct. To link expression of rhodopsin with a fluorescent marker, the cDNA, (HR, 1.7kb) was digested out of the plasmid pcDNA3-HR with Hind III (2hrs at 37°C, as described in section 2.2.2.1). The 1.7 kb HR product was extracted from an agarose gel using the Qiagen Gel Extraction kit (Qiagen, GmbH, Hilden). The HR cDNA was end-filled using Klenow enzyme by incubation at 37°C for 30 min (Roche), and Eco R1 digested (2hrs at 37°C), before purifying and precipitating the DNA as described in section 2.2.1.2. The pIRES2-EGFP vector backbone was digested using EcoR1 and purified (as described in section 2.2.1.2). The HR cDNA was ligated with the cut pIRES2-EGFP backbone overnight as described in section 2.2.2.3. The ligated product was used to transform XL1blue E.coli (2.2.2.4), and the broths plated on kanamycin resistant LB-agar. After overnight incubation at 37°C, colonies were screened for the presence of the recombinant plasmid by PCR: Each colony was restreaked on kanamycin resistant LB-agar plates and also used to inoculate 5 mls of 10X Tris-Triton solution (2.1.1). The bacteria in the 5 mls of Tris-Triton were lysed at 95°C for 5 minutes and the solution then used as template in a PCR reaction (2.2.1.3) with primers Rho124F and Rho258R (Appendix 1A). 8 mls of colony PCR product was run on a 1% agarose gel and products of the correct size used to identify those colonies positive for the HR transgene. Positive colonies were further expanded to produce large quantities of the plasmid shown in fig. 3.5. The plasmids were maxiprepmed using the protocol described in section 2.2.2.6 and sequenced as described in section 2.2.2.7.

3.2.3 Cloning shRNA (*pSuper*, Brummelkamp *et al.*, 2002) constructs

The vector backbone was kindly provided by Dr. Kiang (Kiang *et al.*, 2005, originally based on a construct designed by Brummelkamp *et al.*, 2002), and was digested using Hind III and Eco R1. A pair of 64-nt oligonucleotides (forward and reverse) was used to generate the shRNA and ordered as desalted oligonucleotides from Sigma_genosys. The generic sequences are listed in appendix 1A (H1-siRNAF/R). The design of the oligos included (i) the incorporation of the sense and antisense 19-nt sequence derived from the target site (for silencing), (ii) a Bgl II restriction site at the 5' end of one strand and (iii) a Hind III overhang at the 5' end of the homologous strand. The oligonucleotides were resuspended to a concentration of 200 mM in nuclease-free ddH₂O. 1 µl of the forward and reverse oligos were mixed with 48 µl of annealing buffer (100 mM NaCl and 50 mM HEPES pH 7.4). The mixture was incubated for 4 mins at 90°C, followed by 10 mins at 70°C. Consecutive drops of 10°C in temperature allowed for slow cooling of the annealed nucleotide strands over a period of 30 mins until reaching a final temperature of 10°C. The annealed oligos could then be used for cloning as described in section 2.2.2.

3.2.4 Preparing presynthesized siRNA

Before use of presynthesized siRNA, efforts were made to create Rnase-free working conditions (2.2.3.1). The siRNA oligonucleotide (Qiagen and Dharmacon) was reconstituted in the tube provided with sterile buffer (100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) to obtain a 20-µM solution. The tube was then heated to 90°C for 1 min and incubated at 37°C for 60 mins before use in RNAi experiments.

3.2.5 Cloning pEGFP-siC (used to generate results in sections 3.3.8–13)

The pEGFP vector (Clontech), with the addition of a 300-bp spacer sequence was kindly donated by Dr. Arpad Palfi (Kiang *et al.*, 2005). The backbone of this vector was digested with Hind III and Eco R1. These sites were also used to excise the H1-shRNA cassette from the pH1-shRNA construct (3.2.3) and this cassette cloned directly into the pEGFP backbone (as outlined in methods section 2.2.2).

3.2.6 Assessing suppression agents targeting human rhodopsin *in vitro*

The pIRES-EGFP-HR construct (3.2.2) was cotransfected with each of the siRNAs or the shRNAs using lipofectamine 2000 (Invitrogen) as described in Methods chapter 2 (section 2.2.5.4). The culturing of COS7, HeLa, and HEK293 cells is described in section 2.2.5.

RNA was extracted using Trizol reagent (Molecular Research Centre, Inc) as described in section 2.2.3.2. The extraction and analysis of protein samples from the transfected cells is outlined in chapter 2, section 2.2.4.

3.2.7 *Real-time rtPCR analysis of the human rhodopsin gene*

The Roche Lightcycler machine was used for all real-time rtPCR analyses in the experiments of this thesis. This machine can be used to determine the relative concentration of a given transcript in a sample by using a house-keeping gene as a reference for steady-state transcript levels. Two rtPCR reactions are carried out on the same sample for each the gene of interest and the house-keeping gene, however the two reactions are set up in separate tubes and efforts must therefore be made to ensure that the pipetting remains consistent while setting up the parallel reactions. Figure 3.8a shows an example of a standard curve report from one quantitative rtPCR run analysing rhodopsin levels in RNA extracted from HeLa cell culture. This curve plots the crossing cycle number (i.e. the time-point where amplification of the PCR product begins) versus the log of the concentration. Each dilution in the series was made in duplicate or triplicate, depending on whether the concentration range involved the use of three or four dilutions to generate the curve. The analysis algorithm 'fit-points' was used, whereby the region of minimum error was selected by manually fitting points to the slope of the standard curve. Data from the calculated regression line, such as the slope of the line and the intercept, is listed below the graph, and can be used to infer the relative efficiency of the run. The slope represents overall efficiency of the rtPCR reaction, the error indicates the extent of pipetting variation between individual samples and the coefficient 'r' offers an estimate of the accumulated error in the standard series.

In the graph of fig. 3.8b (from the same rtPCR run as shown in fig. 3.8a) a plot of fluorescence versus cycle number, with each curve representing a given sample, is presented. The chemistry of this quantitative reaction is based on fluorescence from SYBR green reagent (Qiagen). Each SYBR green molecule is capable of binding to the major groove of double-stranded DNA. Once bound to the DNA, the SYBR green molecule can emit a fluorescent signal upon excitation at 470 nm. Therefore the level of fluorescence is dependent on the quantity of bound SYBR green and hence on product concentration. The data from real time rtPCR runs in every case within this thesis was generated as expression level relative to a reference house-keeping gene.

Note: In figs. 3.8a and 3.8b of the figures section, only the typical readout for the rhodopsin gene is shown. It must be compared to the results from a parallel reaction on a house-keeping reference gene (e.g. GAPDH, 18s rRNA or β -actin) for the relative level of

rhodopsin to be assessed. Detection of house-keeping mRNA was routinely used to normalise quantitative rt-PCR data, i.e. to control for several variables that may exist in the protocol. Being essential for cell viability, such genes are ubiquitously expressed, showing similar levels and patterns of expression in different types of cells. However it was taken into account during real-time PCR analysis that no internal control is perfect, with reports suggesting that levels of these genes may vary across tissues and cell types during cell proliferation and stages of development. For the initial cell experiments, mRNAs from GAPDH, neomycin-resistance (on the transfected plasmids) and 18S-ribosomal RNA were considered suitable controls.

3.2.8 Statistical Analysis

Unless otherwise stated, the data shows the averaged results of three repeated experiments. Error values were estimated as $\pm$ standard deviations from the mean (Excel), and the Student's T-test (Excel) used to determine statistical significances (where $t < 0.05$ was deemed statistically significant). Pooled t tests were calculated using Data Desk 6.0 PPC (Data Description, Inc. New York, NY, USA). Differences were deemed statistically significant where $P < 0.05$ with 95% confidence interval.

3.2.9 Primary cell culture

The culturing and electroporation of retinal explants, as performed by Dr. Ader is described in section 2.2.6. Flow cytometry and RNA extraction from the sorted cells is outlined in section 2.2.6.3–5. Immunostaining of the explanted cells is described in 2.2.6.6–9. Vibratome sectioning of the explant tissue is described in section 2.2.6.8.

3.2.10 Animal crosses

In the following results section and throughout this thesis, “HR” denotes the ‘Human Rhodopsin’ cDNA used in cell culture, while “NHR” is used to denote ‘Normal Human Rhodopsin’ genomic DNA found in the mouse model used for retinal explant studies. The NHR $\pm$ mouse (Dryja *et al.*, 1992) was mated to a mouse knock-out background. The NHR $\pm$ -(MouRho $\pm$) genotypes were selected by PCR analysis of tail-tip DNA from the offspring (as outlined in materials and methods chapter, 2.2.6.1). In this way the transgene target level was kept constant between all animals tested for siRNA-based suppression.

3.3 Results

3.3.1 The effect of a series of siRNA molecules on the expression of human rhodopsin (pIRES-EGFP-HR) in COS7 cell culture.

A range of siRNAs, targeting human rhodopsin, termed A, B, C, D, and E has been evaluated for potency in cell culture. The siRNAs cover the region of the human rhodopsin transcript termed loop 14 (illustrated in a plotfold of fig. 3.4). Silencer A covers just one of the nucleotide changes present in the replacement gene and as such could be used, in principle, to test whether siRNA sequence-discrimination could extend to just a single nucleotide. Silencers B, C, D and E on the other hand cover a greater number of nucleotide changes in the replacement rhodopsin gene and in principle would be expected to have little or no effect on the expression of the replacement gene.

The human rhodopsin gene was cloned into the pIRES2-EGFP vector (BD Biosciences, Clontech) to generate the construct pIRES-EGFP-HR (fig. 3.5). The pIRES-EGFP-HR vector included a gene expressing EGFP, (a fluorescent protein derived from the jellyfish *Aequorea Victoria*), linked to the human rhodopsin cDNA by a sequence for an internal ribosomal entry site (IRES) on the message (Jackson *et al.*, 1990). The presynthesized siRNAs listed in figure 3.10 were cotransfected with this construct into COS7 cells and the levels of rhodopsin transcript measured by real-time rtPCR. This data is presented in figure 3.11.

PCR primers flanking introns in human rhodopsin, GAPDH and the EGFP gene were used for rt-PCR amplification (primer sequences listed in appendix 1A). The percentage of siRNA-based suppression achieved was determined relative to a non-targeting siRNA control (siNT), commercially available from Qiagen. Studies suggest that the RNAi machinery, which plays a crucial role in chromosome architecture, may become saturated in standard siRNA assays (Kamath *et al.*, 2000). In principle, it should be possible to monitor and differentiate any pathogenic effects of cellular function that may arise from the method of delivery or the carrier vector used, compared to that due to RNAi. A completely foreign siRNA sequence with no homology to any gene in the organism being studied should be used in tandem with the targeting siRNA.

For this initial study, cotransfections using siNT and the target plasmid, (pIRES-EGFP-HR), were set as showing 100% expression of the rhodopsin target. All other siRNA cotransfections were thus expressed as a percentage of the expression from the HR/siNT cotransfected cell population. The GAPDH gene was used as a measure of steady-state gene expression level for all samples analysed in deriving fig. 3.11.

In addition an siRNA targeting EGFP, siEGFP, served as a positive control for suppression. This siRNA has previously been shown to specifically and effectively target EGFP transcripts *in vitro* (Caplen *et al.*, 2000). As the marker EGFP gene is expressed on the same transcript as the human rhodopsin gene, (in the pIRES-EGFP-HR plasmid), siEGFP may be expected to show equivalent suppression of human rhodopsin and of EGFP expression levels. Although the siRNAs did not carry any stabilising chemical modifications, the positive control, siEGFP, was found to be active in the 46–50 hour period during which the experiments were carried out and therefore this was considered an appropriate time-frame in which to evaluate other siRNAs (data not shown).

Using this approach, the most potent siRNAs were found to be ‘siB’ and ‘siC’ achieving over 56% and 70% suppression of the target gene respectively, as evaluated by real-time rtPCR (table 3.1), and hence these siRNAs were therefore chosen for further analysis. To further explore the potency of these siRNAs, the siB and siC sequences were cloned in the form of short hairpin dsRNA (shRNA) constructs driven by a H1 promoter for continued suppression studies.

3.3.2 Suppression of human rhodopsin transcript from the pIRES-EGFP-HR construct, following cotransfection with RNAi constructs: H1-siB, H1-siC, H1-siEGFP.

The siRNAs that were chosen to be optimal for targeted rhodopsin suppression, that is siB and siC, (section 3.3.1) were cloned as small-hairpin-RNAs (shRNAs) into the pH1-siRNA construct (fig. 3.6) to examine the effect of stable shRNA expression on target rhodopsin levels. The vectors pIRES-EGFP-HR and pH1-siB or pH1-siC were cotransfected into mammalian cell culture using lipofectamine 2000 (as outlined in section 2.2.5.4). After 48 hours in culture, RNA from these cells was extracted and analysed by real-time rtPCR. The siRNAs chosen showed enhanced suppression activities when stably expressed from a H1-driven

expression plasmid, with approximately 73% suppression obtained using H1-siB and 72% suppression using H1-siC, (fig. 3.12).

The scrambled non-targeting dsRNA sequence used in section 3.3.1 (siNT, Qiagen) was similarly cloned into the H1-cassette as a non-targeting vector control. When analysing data-sets from multiple siRNAs, expression levels for non-targeting controls are often set at 100% (as for fig. 3.11). However, in this shRNA-based study, it was of interest to evaluate what level of variance is present in the data generated from transfections with vector driving expression of control non-targeting scrambled siRNA from the H1 promoter. Therefore, rhodopsin expression level following cotransfection with H1-siNT was treated initially as a variable.

Quantitative rtPCR on RNA extracted following cotransfections in mammalian cells would suggest that variation in the levels of message may be effected by the reference gene chosen. It became clear from these expression studies using COS7 cells that some conventional house-keeping genes are less capable than others of holding steady state expression levels. This variation is possibly dependent on the health of the cell. Experiments were carried out on cells between passage number 3 and 10, in an effort to limit this variation as much as possible. In addition to consistency of expression, the level of transcription is also an important parameter in choice of reference gene. Error values may be increased if the gene chosen as a reference is expressed at a significantly higher level than the gene of interest, or indeed vice versa. In some cases, the use of the 18S-rRNA gene as a reference point may exacerbate any error as the 18S-rRNA primers start to amplify the message by cycle number 5 of a standard rtPCR reaction, compared to amplification starting at cycle 15 for the rhodopsin message. As such the RNA data generated using the highly expressed 18S-rRNA gene as a reference in transfected cells is limited in the current study, with no more than two runs performed per sample (therefore no standard deviations were recorded for some data-sets using this gene, fig. 3.12).

The ideal situation to allow accurate quantification of the target transcript between samples is to normalise expression to a transfected reference gene in addition to a cellular house-keeping reference. The data presented in table 3.2a suggests that the neomycin resistance gene (neoR) on the target plasmid serves as a good reference for assessing rhodopsin-specific suppression in COS7 cells. The level of expression from the neoR gene is intermediate between that of GAPDH and 18S-rRNA. As the neoR gene is also expressed from pIRES-EGFP-HR, it may be considered to offer an

accurate reflection of the level of expression from the episomal plasmid carrying the target gene. However a cellular house-keeping reference gene remains essential, as it reveals the difference in levels of target transcript between samples relative to the overall level of transcription being carried out by the cell. The combined results for rhodopsin expression values relative to the three genes GAPDH, 18s-rRNA, and neomycin-resistance have been used to estimate shRNA-based suppression of rhodopsin in mammalian cells.

Finally, as part of this investigation of stable expression of siB and siC *in vitro*, the consistency of the level of H1-siB and H1-siC rhodopsin-suppression between different mammalian cell lines was determined. The highest level of suppression was observed in HEK293 cells (fig. 3.13). It has been suggested that complexes involved in the regulation of transcription/translation may be compromised by the presence of the foreign dsRNA (Zamore *et al.*, 2003). This may have consequences on steady-state expression levels and, such effects may vary between different cell types. However, the difference in suppression observed in siC-transfected samples between HEK293 cells and COS7 cells was not found to be statistically significant, (p -value: 0.153). Notably, the level of rhodopsin suppression achieved was relatively consistent between different cell lines for a given siRNA.

In summary, overall results derived with H1-driven shRNA constructs generally correlate or show greater suppression than results obtained using presynthesized siRNAs (table 3.1, and 3.2b). Results using either synthesised siB and siC or shRNA vectors expressing these siRNAs would suggest that these sequences warrant further analysis.

3.3.3 Dose Curve showing the effective range of siRNA concentration to achieve suppression of human rhodopsin in the pIRES-EGFP-HR construct.

The aim of this experiment was to determine the optimal range of siRNA activity in cell culture. It has been suggested that at high concentrations of siRNA, the RISC machinery may become saturated causing a drop in silencing. This so-called 'derepression' of target has been observed in other cell systems and may be a factor for consideration before further development of specific siRNAs (Zamore *et al.*, 2003). Based on the greater number of nucleotide changes covered by siC in the replacement rhodopsin transgenic mouse, it was decided to further the development of

this siRNA and maximise the specific targeting of the wild-type rather than the replacement rhodopsin transcript (results from the analysis of siB and siC against replacement rhodopsin cDNA are presented in chapter four). The dose curve experiment, the results of which are shown in fig. 3.14, was therefore focused on siC. In addition, the presynthesized dsRNA molecule, siC, and not the plasmid-driven H1-siC was used. Therefore plasmid and promoter based effects on the dose curve were not considered and a dose curve specific to silencing effects alone was determined.

The dose curve should reveal the limiting concentration of siRNA needed to achieve suppression. Furthermore, in order to examine the extent to which saturation of RNAi machinery in the cell may affect our results, we tested the level of target suppression at different concentrations of siRNA. There is considerable literature suggesting that 'off-target' effects can be minimised by using the lowest concentration of siRNA as possible whilst still achieving optimal gene silencing (Li *et al.*, 2004). Hence, it is valuable for any siRNA to explore the dose range over which the molecule can act to promote potent suppression of its target.

It was concluded from this experiment that the range of 10–20 pmols appears optimal for siC siRNA activities (fig. 3.14). Furthermore, suppression is sustained by siC even at very low concentrations, with down-regulation of rhodopsin target evident down to 1 pmol siC concentration. The dose curve experiment was repeated and variation in suppression was only evident at very low concentration between these two experiments.

3.3.4 MTT assay of cytotoxicity of siRNA in mammalian cells

It was of value to explore whether the presence of siRNA in cells had any cytotoxic effect, possibly induced by the metabolic implications associated with active siRNA molecules in cell culture. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, was first described by Mosmann in 1983, and is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT reagent. As a result, dark blue formazan crystals are formed which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. The formazan crystals are insoluble in aqueous solutions, but can dissolve in DMSO, producing a purple colour, which can be measured at 595nm. The number of surviving cells is directly proportional to the level of the formazan product created, as intact mitochondria are

required for the colour development. The MTT assay was carried out twice on HeLa cells plated in triplicate in a 96-well plate. The six values obtained for each sample were pooled yielding average absorbance readings (fig. 3.15).

The number of cells seeded for the MTT assay was chosen based on a standard curve of absorbance readings from untransfected cells treated with the MTT reagent at a range of cell densities. The cell density of 5×10^4 lying in the linear range of the curve (corresponding to absorbance values of 1–1.25) was chosen for the assay. Values outside of the linear range of this curve (with end-points 0.75 and 1.75) may not yield an accurate quantification of changes in the rate of cell proliferation. The assay (as shown in fig. 3.15) suggests that overall cell viability is not affected by the siRNA cotransfection procedure used in experiments in this chapter.

While a minor increase was observed between the levels of cell death caused by transfection procedure alone and by transfection of siRNA, this difference was only found to show minor significance for data-sets of rhodopsin alone compared to rhodopsin cotransfections with 60 nm XerSiC or 120 ng H1-siC, (DataDesc., NY). In these cases increased cell survival was recorded in the cotransfected samples. It appears likely that the siRNAs do not cause a significant level of cell death, as cellular metabolism in the treated wells clearly remains active.

3.3.5 ELISA showing siRNA-based suppression of human rhodopsin protein in HEK293 cells.

As mentioned in section 3.3.3, the siRNA termed siC was chosen for further evaluation in cell culture, as crucially this molecule covers all five changes of the intended replacement rhodopsin gene (further discussed in chapter 4), and therefore is less likely to target transcripts from the replacement gene. On the other hand siB covers only two of the replacement changes (fig. 3.10), and as the suppression results obtained using siB were less consistent between cell lines it was not prioritised initially. However this suppressor does elicit potent suppression of human rhodopsin expression and will likely be developed further, as it is advantageous to have more than one siRNA targeting a gene of interest.

The Human-Anti-Mouse-Antibody (HAMA)-ELISA allows for quantitative *in vitro* determination of protein in culture medium. The assay uses an antibody-bridging technique that will discriminate the very low amount of rhodopsin antibody in the large pool of IgG. The peroxidase-conjugated anti-rhodopsin 4D2 antibody

(Molday *et al.*, 1984) binds to its C-terminal epitope on the rhodopsin protein. Following washing, the horseradish peroxidase (HRP) in the 4D2-IgG-HRP complex is developed by the substrate tetramethylbenzidine (TMB, Roche) and resulting luminescence reaction determined photometrically.

Three batches of HEK293 cells, at densities of $4-5 \times 10^5$, were transfected with approximately 350 ng of rhodopsin plasmid, (pcDNA3-HR). A 20-nM concentration of siC dsRNA was used in cotransfections with pcDNA3-HR as consistent effective suppression of the target rhodopsin gene had already been obtained at this concentration (fig. 3.11). Protein was extracted as described in the materials and methods chapter, section 2.2.4 and quantified by Bradford assay before being used in this experiment. At the lowest dilution of protein it was found that approximately 65% suppression of rhodopsin protein was achieved by siC (fig. 3.16). This level of suppression drops as the sample becomes more dilute, as competing background effects of the highly sensitive peroxidase-mediated POD substrate reaction are likely to take precedence over the primary and secondary antibody reaction. The results from this protein assay mirror and support those obtained for RNA assays using siC in cell culture.

3.3.6 Fluorescent microscopy on HeLa cell culture transfected with pIRES-EGFP-HR and pH1-siC.

In section 3.3.5 the suppression of rhodopsin by a synthesised siRNA termed siC (Qiagen), was quantified at the protein level by an antibody-substrate reaction. The target rhodopsin gene (HR, Human Rhodopsin) was originally cloned into the pIRES2 vector (Clontech). As described in section 3.3.1, this construct expresses the marker EGFP gene on the same transcript as the cloned gene of interest, thus generating pIRES-EGFP-HR. An IRES site links the target message to the EGFP protein thereby making it possible to demonstrate post-transcriptional down-regulation at the protein level by fluorescent microscopy as well as by ELISA. Hence, the suppression achieved using the H1-driven shRNA vector, H1-siC, was analysed in HeLa cell culture using EGFP protein as readout for suppression. Notably, reduced green fluorescence is evident in the cells cotransfected with pIRES-EGFP-HR and H1-siC (fig. 3.17b), compared to the cells cotransfected with pIRES-EGFP-HR and H1-siNT, (fig. 3.17a) and is similar to the level of downregulation observed in the H1-siEGFP positive control cell transfections (fig. 3.17c). The above

analysis is purely qualitative and is used to support the quantitative data obtained in section 3.3.5 and furthermore, is used to confirm that suppression at the protein level is maintained when the siC suppressor is expressed as a hairpin construct from the H1 promoter. In summary, the protein results described in sections 3.3.5 and 3.3.6 together with the RNA suppression data presented in sections 3.3.1–3.3.4, suggest that siC is indeed an effective suppressor of human rhodopsin.

3.3.7 FACS analysis of explanted tissue from NHR+/- (MouRho-/-) transgenic mice, following electroporation with pEGFP-siC:

Given that results from both RNA and protein assays suggest that siC can elicit potent suppression of the human rhodopsin gene, a study of siC-based suppression was undertaken using a system that more clearly resembles the target tissue than do the cell cultures used up to this point of the study. Hence, the following results are concerned with data derived from electroporation of the siC siRNA into retinal explants from the NHR+/- (mouRho-/-) mouse (this animal model is described in section 3.1.11). The construct used for silencing, pEGFP-siC (illustrated in figure 3.7) carries an EGFP marker gene expressed in reverse orientation to siC, thus allowing for isolation of a fluorescent, transfected retinal cell population by FACS sorting.

Only those retinas identified by genotyping from tail-tip DNA to be positive for the NHR transgene were FACS analysed (BD FACSAria). Scatter and granularity parameters achieved by two scatter signals (from side and forward deflection of the FACS laser beam) were used to identify intact photoreceptor cells. From this population those intact cells transfected with shRNA constructs were identified by detection of a fluorescent signal through a FITC optical filter (fig. 3.18a-c).

3.3.8 Level of expression of human rhodopsin from explanted tissue of NHR+/-, MouRho-/- transgenic mice, following electroporation with pEGFP-siC.

A combination of shRNA construct design, retinal explants, FACS cell sorting, transgene genotyping and quantitative rtPCR have been used to explore suppression of human rhodopsin expression in retinal explants from mice expressing the genomic sequence of the wild-type human rhodopsin gene (figs. 3.18 and 3.19). The results indicate that significant suppression of the target gene was achieved and mirror those findings obtained previously in cell culture.

69% suppression of the wild-type human rhodopsin transgene message was estimated in the NHR+/- (MouRho-/-) rhodopsin knockout murine retina (fig. 3.19). The primers used in the quantitative rtPCR flanked an intron within the target gene and control PCR reactions were performed on the products of the Lightcycler reaction. None of the samples yielded PCR products larger than that expected from the cDNA, suggesting that DNA contamination was not a factor for concern with these results.

3.3.9 Dissociation, immunostaining and cell counts of cells from explanted tissue of NHR+/-, MouRho-/- transgenic mice, following electroporation with pEGFP-siRNA.

While RNA analysis provided evidence suggesting that siC-based suppression of rhodopsin is observed when constructs are electroporated into retinal explants, it was of value to explore if expression was also evident using protein assays. Therefore electroporated p0 explants (NHR+/-/MouRho-/-) were dissociated after two weeks in cell culture and immunostained for rhodopsin protein expression (4D2 anti-Rho antibody). Counting was carried out in divided sections of the coverslips ensuring that no section was counted twice. Each green cell was numbered as 1 under the green filter (i.e a cell which was successfully electroporated with the siRNA expression cassette) before switching to the red filter and numbering the same cell as 1 or 0 for positive or negative rhodopsin expression, respectively.

The results indicate that the percentage of pEGFPsiC-transfected photoreceptor cells still expressing rhodopsin is lower than the percentage of pEGFPsiNT-transfected cells expressing rhodopsin (Table 3.7). It was estimated that 72% of NHR cells receiving pEGFPsiNT still showed rhodopsin staining, while 34% of NHR cells receiving pEGFPsiC still showed rhodopsin expression. The apparent suppression by siNT likely reflects rhodopsin-negative müller cells and may be used to normalise the suppression level observed with siC. Therefore actual siC-induced full suppression is likely to be more than the 40% estimated from the counts of cells used to compile table 3.7. However, this value reflects full suppression and does not take into account that the highly sensitive anti-rhodopsin 4D2 antibody is still capable of picking up the residual rhodopsin in cells where, for example, a proportion (possibly a large proportion) of rhodopsin protein may be ablated. Therefore achieving total rhodopsin knockdown in 40% of cells, to the extent that these cells are

as negative as müller cell types is encouraging and suggests that siC warrants further development as a potential therapeutic.

In addition to dissociated cell stainings, cryosections of a NHR+/--(MouRho-/-) retinal explant transfected with pEGFP-siC were immunostained for the human rhodopsin transgene. The immunostained cryosection pictures of fig. 3.22 are however purely qualitative and are used to support the more quantitative protein data from this section of the work.

3.4 Discussion

3.4.1 Selection of siRNAs targeting rhodopsin for analysis

Most siRNAs selected for testing had targets within a relatively confined stretch of the rhodopsin transcript (nt 170-188). However, the ability of these oligos to achieve targeted suppression varied widely. This variation ranged from suppression levels of 65–75% (siB and siC), to 0% (siD) (fig. 3.11). Understanding the reasons for this variation would be valuable if high-through-put assessment of effective siRNA targets in the human genome is to be achieved. The application of siRNA would thus become an extremely cost-effective approach for reverse-genetics and functional analyses. In addition, discrimination of siRNAs with differing potencies would be facilitated and hence the appropriate suppressor applied to different clinical settings whereby anything from moderate to full suppression may be required.

Early experiences with antisense and ribozyme agents suggested that secondary structure considerations of the target transcript were crucial for the identification of potent suppression agents. Open loop structures at least 100 nucleotides in from the transcription start site were optimal targets, being easily accessible to bulky hammerhead ribozyme oligos and at a sufficient distance from the region closer to the start of transcription, which is often blocked by complexes involved with transcription (O'Neill *et al.*, 2000). The large open loop structure, termed loop 14, (loops numbered relative to transcription start site) occurs in the region of nucleotides 174 – 206 of the human rhodopsin cDNA and is considered a suitable target for suppression (fig. 3.4). The secondary structure is altered dramatically when five consecutive changes are introduced to the sequence and transformed again when 7 changes are introduced. These altered secondary structures may possibly have implications for securing protection of a replacement rhodopsin gene from suppression and are discussed in chapter four of this thesis. However, studies to date suggest that the 3D structure of the transcript is not influential in determining RNAi potency.

3.4.1.1 Microarray-based selection of siRNA target sites:

The explosive growth of RNAi literature over the last four years has provided many examples of targeted suppression in the mammalian genome (for a comprehensive recent review see Dillon *et al.*, 2005). However, until recently, selection of siRNA target regions has essentially been a random process of sequence selection. During the course of selecting and testing siA–E, microarray-based assays for the identification of optimal siRNA targets in the human genome, have become available (Parrish *et al.*, 2000; Reynolds *et al.*, 2004). The siRNAs used in these studies were designed prior to such array-based analyses and a post-hoc analysis of their features compared to those identified using new design rules is useful.

While features of the whole secondary structure of the rhodopsin gene may be important for successful transcript downregulation using ribozyme and traditional antisense approaches (O'Neill *et al.*, 2003), such requirements on structure are less rigorous for an RNAi approach. These siRNAs siA, siB, siC, siD and siE assessed in section 3.3.1 are clustered very close together and yet show highly divergent activities in cell culture (figs. 3.10 and 3.11). Therefore each individual 21–22mer and not the overall landscape of the target mRNA, may be responsible for the suppression achieved. Libraries of siRNAs have been developed and their suppression ability against control targets such as luciferase and lamin A/C assessed (Reynolds *et al.*, 2004). As a result, the importance of the position and energy profile of nucleotides in dsRNA has been highlighted.

3.4.1.2 Thermodynamic criteria for selection:

Thermodynamic attributes of a siRNA are believed to be important only in the initial siRNA-RISC recognition step. A trend emerging from the research analysed to date is that siRNAs with a low G/C content show greater activity. In a systematic analysis of 180 siRNAs targeting the mRNA of two genes, Reynolds *et al.*, (2004) were able to confirm this observation as well as identifying other target sequence features that were important in influencing siRNA functionality. A GC content of 30–52%, conferring lower overall thermodynamic stability on the siRNA, was chosen as the first criterion for selection. All siRNAs tested in the open loop, number 14 of rhodopsin (targeted in this study) had approximately 50% GC content. More significant than this requirement is the need for A/U bps at positions 15–19. The thermodynamic flexibility of this end of the siRNA correlated with an increase in

silencing (Parrish *et al.*, 2000). SiB and siC carried 3 and 2 A/U's in this region, respectively. This is in contrast with the less effective suppressors A and E, which carried only one A or U in bases at positions 15–19.

3.4.1.3 Sequence-specific criteria for selection:

Sequence-related determinants of functional siRNAs were found in array-based analyses to have a higher impact on the probability of achieving RNAi than purely thermodynamic features. Sequence-specific characteristics may affect siRNA-protein interactions. An A at position 19 of the sense strand has significant influence on potency. None of the siRNAs evaluated in the current study obey this rule. Even more vital however was the presence of an A at the third base in the sense strand. Both B and C meet this requirement, in contrast to the less effective siRNAs A, D, and E. None of the siRNAs evaluated carried a U at position 10, the next factor shown to increase probability of silencing. Negative criterion included the absence of a G or C at position 19 of the sense strand and absence of a G at position 13 of the sense strand (only siD meets both these criterion. This siRNA was the oligo favourably selected by an early Dharmacon algorithm; however, it failed to induce rhodopsin silencing – fig. 3.11). The choice of siRNAs was somewhat restricted by the desire to ultimately use a transgenic animal model carrying a human rhodopsin replacement gene which was sequence-modified in the narrow region surrounding nucleotides 174–186 of the gene. However, despite falling short on some of the hypothesised requirements for siRNA functionality, siB and siC were active in cell culture and thus chosen for further investigation of human rhodopsin suppression and replacement. The study highlights that many of the parameters governing siRNA potency remain elusive.

3.4.2 Controls for siRNA experiments

Early successes with siRNA for gene knockdown experiments led to a flurry of papers showing RNAi in action and the field has rapidly begun to acquire a large volume of data (reviewed by Dillon *et al.*, 2005). To establish consistency in these studies, a consensus set of essential and useful controls has been proposed for RNAi researchers. The following controls were applied in this study on RNAi-based suppression of human rhodopsin expression:

(i) A scrambled siRNA in the form of a sequence totally unrelated to the target or any other region of the mammalian genome is necessary. In the current study, a commercially available non-targeting siRNA was utilised, (Qiagen, Gmbh; Appendix 4A). In this regard it is notable that components of the RNAi machinery may play a role in chromosomal architecture (reviewed in Bernstein *et al.*, 2005; Bailis and Forsburg, 2002; Dernburg and Karpen, 2002). Altered global gene expression patterns may reflect effects on chromatin structure arising from the presence of siRNA in the cell. This will ultimately require a full-scale microarray analysis. For initial *in vitro* siRNA validation experiments, non-targeting siRNAs are essential to monitor the potential for such non-specific effects in the experimental system of choice. Perhaps the most appropriate control in this regard, is a non-targeting siRNA that is functional in the cells and will be incorporated into RISC for processing (further discussed in chapter four).

(ii) Inclusion of a positive control siRNA in the study was necessary to ensure that the appropriate concentrations of dsRNA were used. Research has shown how the RNAi machinery may be saturated in cell culture potentially leading to derepression of the target (Zamore *et al.*, 2003). As the target gene of interest was expressed from a bicistronic plasmid carrying the EGFP gene, a commercially available siRNA shown to down-regulate EGFP by up to 90% could be used to ensure that the experimental protocol was working and that RNAi could be achieved (fig. 3.11).

(iii) Mismatch siRNA containing one or two changes in the antisense strand is also recommended. Notably, mismatched siRNAs are investigated in the replacement stage of this study and shall be further discussed in chapter five.

(iv) The possibility that translational attenuation rather than post-transcriptional inhibition may be occurring makes it vital that down-regulation of mRNA and protein is demonstrated. Therefore suppression levels were also determined by ELISA (fig. 3.16), and supported by fluorescence microscopy pictures (fig. 3.17) and immunostaining of retinal tissue treated with siRNA (fig. 3.21). A functional read-out is also useful and will take the form of electroretinograms when the siRNAs come to be tested *in vivo*.

(v) A rescue control is often recommended for RNAi used in loss-of-function studies whereby the functional phenotype of the silenced gene is restored (Bennett 2004). Assessing the potential of the replacement rhodopsin gene to overcome the

phenotypic effect of rhodopsin-specific dsRNA may serve this purpose and is discussed in Chapter four.

(vi) As a quantitative control, titration of siRNA is vital and titration down as far as 2-pmol of siRNA still resulted in potent suppression of the target gene in this study. Suppressors siB and siC were optimal at 10-pmol and less consistently effective at lower concentrations (fig. 3.14).

(vii) In addition, it is preferable to show the same gene silencing effect with a number of siRNAs targeting different regions of the same mRNA (as shown using si-A, -B, -C, and -E in fig. 3.11).

These controls help guarantee the authenticity of any positive results and make the data comparable with data generated in other laboratories.

3.4.3 Plasmid-expression of dsRNA: H1-siB & H1-siC

Having chosen a suitable siRNA by testing various synthetic siRNAs in transformed cells (fig.3.11), the most effective suppressors, siB and siC were further developed for stable suppression of the target gene by cloning shRNA sequences into the H1-based pSuper vector. Fig. 3.12 shows the level of rhodopsin silencing achieved from vector-derived siRNA. Over 70% suppression was demonstrated with H1-siC. Using H1-siB, a greater level of suppression was achieved (<80%), when dsRNA targeting this region is plasmid-derived rather than as a synthetic molecule (>65% suppression).

A factor to be considered when driving stable RNAi with Pol-III-based vectors is the variation in H1 and U6 expression patterns across different organs and the possibility that different cell types may experience varying degrees of suppression. This is of concern for the generation of knockout mice using siRNA technology. Seibler *et al.* have generated mice using a well-defined single-copy shRNA plasmid by targeted transgenesis in ES cells (Seibler *et al.*, 2005). The embryos derived from an insertion into the *rosa26* locus showed a broad level of suppression across all tissue types using cassettes with both the U6 and the H1 pol III promoters. A lower degree of RNAi was observed for lymphocytes, testes and in some cases the liver. However, the authors suggest, that this effect is not due to limited RNA pol III activity in these organs but rather to other factors such as availability of RISC, which may vary between tissue types. Notably, the siRNA-based suppression results

obtained in the current study are similar across three mammalian cell lines, (COS7, HeLa, HEK293), used for cotransfections of vector expressing the target gene and shRNA (fig. 3.12).

3.4.4 Optimising siC for further development

The siRNA molecule termed siC was chosen for further analysis as it may offer the best chance of recovery of rhodopsin function when teamed with the replacement rhodopsin gene (discussed in chapter 4). Nevertheless, siB was also found to be functional in synthesised siRNA and shRNA forms and is currently being developed for further study.

3.4.4.1 Dose Curve:

Saturation of RISC and other components of the cell's RNAi machinery may be a concern with such studies as performed in this chapter; therefore a dose curve of optimal effect of specific suppression (siC) relative to a non-specific effect (siNT) was determined in HeLa cells (fig 3.14). The siRNA, siC was found to function optimally in the 10- to 20-pmol range, when compared to siNT-treated cells. In fact, siC was found to be active even down to subnanomolar levels. The results highlight the need for an assessment of RNAi activity across a range of concentrations for each target and suppressor used.

3.4.4.2 Effect of siC on protein levels

An assessment of rhodopsin protein downregulation using siC was undertaken by an ELISA. At the most concentrated dilution of protein it was found that approximately 65% targeted suppression of rhodopsin protein was achieved (fig. 3.16). As such the specific silencing by this siRNA is further supported in the reduced level of translated rhodopsin protein.

The cell culture experiments have served as preparation for the further development of RNAi-based suppression of rhodopsin *in vivo*. However, primary cell culture studies were used in this study to bridge the leap from *in vitro* to *in vivo* analysis. It is possible to assess human rhodopsin expression driven by a human rhodopsin-specific promoter in a retinal explant, and thus to model the *in vivo* environment in which therapeutic agents may ultimately be tested. The necessary conditions and assays may be optimised using *ex vivo* experimental techniques. We

have electroporated such a retinal model system with siC and examined the effect of this siRNA on human rhodopsin expression when compared to retinal models electroporated with a control non-targeting siRNA.

3.4.5 Treatment of primary retinal culture with rhodopsin-targeting siRNA

Neonatal mice carrying one copy of a wild-type human rhodopsin, (NHR) transgene on a mouse rhodopsin null background were sacrificed and enucleated at post-natal day P0 or P1. The retinas were grown on membranes *in vitro* and cultured with neurobasal media supplemented with growth factors (culturing of all explants was performed in conjunction with Dr. Marius Ader). These retinal explants were harvested for analysis of human rhodopsin RNA and protein levels at 14 days post-electroporation with either a control non-targeting construct, (pEGFP-NT) or a construct targeting NHR (pEGFP-siC). The results from a minimum of 6 retinas were pooled for each RNA and protein analysis. The retinas within each analysis were derived from NHR+/- (MouRho/-) littermates.

3.4.5.1 RNA Analysis:

For each retinal explant, approximately 15% of the retinal explant cells were successfully electroporated with the shRNA constructs (fig. 3.18). Hence, RNAi-based suppression may not be obvious in RNA extracted from the total cell pool. Therefore before RNA quantification could be undertaken, it was necessary to dissociate the explants into individual cells and to isolate the relevant cell population by FACS analysis (i.e. identifying those cells positive for fluorescence and therefore carrying the shRNA cassette). Cells with the appropriate fluorescent and scatter profile were collected and RNA extractions performed at the flow cytometry site to ensure minimal loss of sample. This procedure yielded a well-defined cell population and allowed us to achieve definitive quantitative real-time PCR results (fig. 3.19). Approximately 69% suppression of the wild-type human rhodopsin transgene message was achieved in the NHR+/- (MouRho/-) transgenic retina. Results from RNAi-based suppression of human rhodopsin in retinal explants are encouraging and now require further validation *in vivo*. However, the endogenous mouse rhodopsin knockout background of this NHR+/- (MouRho/-) animal model has some further consequences for the interpretation of this RNA data. These will be discussed

subsequent to the exploration of a replacement rhodopsin model on the same mouse rhodopsin knockout background in chapter 4 (section 4.4.3, pg 142). It is possible that following siC-treatment, a mutant rhodopsin gene may be capable of producing sufficient mutant protein from surviving transcripts such that retinal damage will still occur. The dominant pathology may be evident even in the presence of a functional therapeutic protein, as would be envisaged in the suppression and replacement approach. *In vitro* results offer clues to this vital question. Analyses of the effect of siC-based suppression on transcripts from the rhodopsin replacement gene and a mutant rhodopsin gene (pro23his) are undertaken in chapters 4 and 5.

3.4.5.2 Protein analysis:

The problems listed in 3.4.5.1 above regarding the limited transfected retinal population available to us from the explant culture, also applied in the protein analysis phase of this experiment. However, it was hoped that use of an immunostaining assay would lessen the requirement for an isolated population of transfected rod photoreceptor cells. Visualising individual dissociated cells (from NHR+/- (rho-/-) retinal explants) stained for rhodopsin protein and also expressing the EGFP tag should offer some view as to the efficacy of the RNAi-based suppression strategy. The antibody used was obtained from Dr. Robert Molday and recognises an epitope at the carboxyl terminal region of the rhodopsin protein. This antibody cross-reacts with the alexa-fluor label, (568nm) and can be used to visualise the rhodopsin protein under a fluorescent red light.

Using this approach, it was estimated that 72% of NHR cells receiving si-NT still show rhodopsin staining, while 34% of NHR cells receiving si-C show rhodopsin expression (Table 3.7). The apparent suppression by siNT is likely to be due to transfection of rhodopsin-negative müller cells and may be used to normalise the suppression level observed with siC. Therefore actual siC-induced full suppression is estimated at 40%. It is also notable that the incomplete suppression in the cells that stain positive for rhodopsin has not been determined in this particular assay. Residual rhodopsin will react with the 4D2 antibody and as the alexafluor labelling of this reaction is non-quantitative a partial suppression of rhodopsin expression will not be evident. In essence, the assay counts only those cells in which significant suppression has been achieved (such that antibody is no longer capable of binding and producing a fluorescent signal). Clearly this assay must underestimate the suppression obtained.

In an attempt to identify localised regions of suppression in the retinal explant, cryosections from NHR+/- (MouRho-/-) retina electroporated with the non-targeting construct, pEGFP-NT and with the pEGFP-siC construct, were immunostained and examined by fluorescence microscopy. Upon evaluation of sections from four retinal explants there was a suggestion that photoreceptor cells electroporated with non-targeting siRNA showed more intense rhodopsin antibody-staining than those electroporated with siC construct, (site A compared to site B in figures 3.19a–c). Clearly a method enabling higher levels of transduction of retinal cells in these explants than that obtained using electroporation (approximately 15–20%) would be highly preferable. Hence, a method to achieve this has been explored utilising a viral vector for delivery, Adeno-associated virus (AAV) and is discussed in detail in chapter 6.

3.4.6 Summary of chapter 3

The choice of RNAi target sequences within a confined region of the human rhodopsin gene with the aim of pursuing a suppression and replacement strategy, as outlined in chapter 1, has been carried out thus far and an siRNA termed ‘siC’ chosen for further analysis. The dose of siRNA for optimal suppression of human rhodopsin in transformed cells has also been assessed. Further optimisation of the vector pH1siC may be necessary for efficient down-regulation of the target gene, and shall be investigated in the context of microRNA loops in chapter 5.

Furthermore, the research carried out and presented in this chapter suggests that siC targeting the loop-14 region of human rhodopsin transcript should in principle be capable of down-regulating rhodopsin expression in a retinal environment, as evaluated in retinal explants. This result encouraged further investigation of the use of siC as an agent to suppress expression of human rhodopsin *in vivo* and for incorporation into a suppression and replacement therapeutic strategy for rhodopsin-linked ADRP.

3.5 FIGURES and TABLES

The mechanism of RNA interference

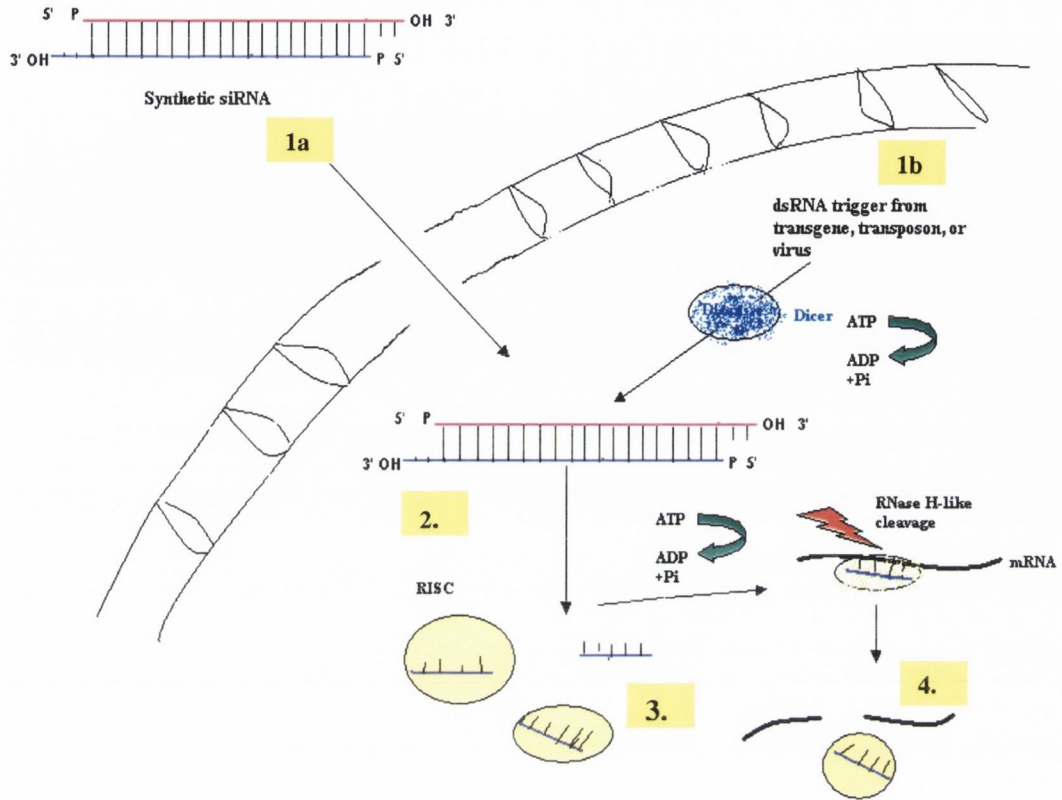


Fig. 3.1:

The mechanism of RNAi

1. RNAi may be triggered in the cell by introduction of an *in vitro* synthesised dsRNA molecule, (1a) or as a cellular, dicer-dependent response to dsRNA, (1b) from invading viruses, or by jumping transposons and transgenes.
2. These siRNAs are incorporated into an inactive ribonucleoprotein complex.
3. ATP-dependent unwinding of the siRNA duplex generates an active RNA-induced silencing complex (RISC). The ATP is necessary to maintain the 5' phosphates on the siRNAs.
4. In an ATP-independent reaction, the RNA target is cleaved.

Human Dicer

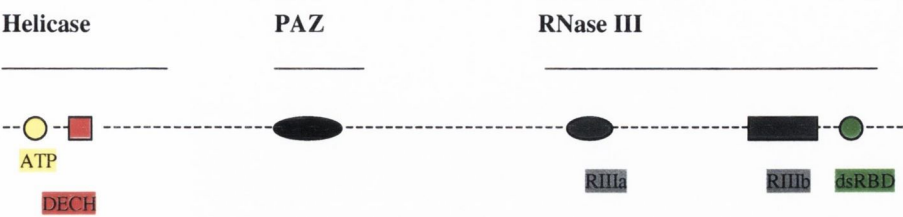


Fig 3.2: Domain Structure and expression of Human Dicer. Three major domains have been identified in the protein: an N-terminal helicase domain, a Piwi/Argonaute/Zwille (PAZ) domain, and a C-terminal RNase-III domain. The PAZ domain is involved in RNA binding, (Kelley *et al*, 2003) however, the biochemical function of this domain is not, as yet, fully elucidated. The RNase-III domain comprises a dsRNA binding domain (dsRBD) as well as tandem ribonuclease III motifs. Adapted from Provost *et al.*, 2002.

DNA-based siRNA constructs

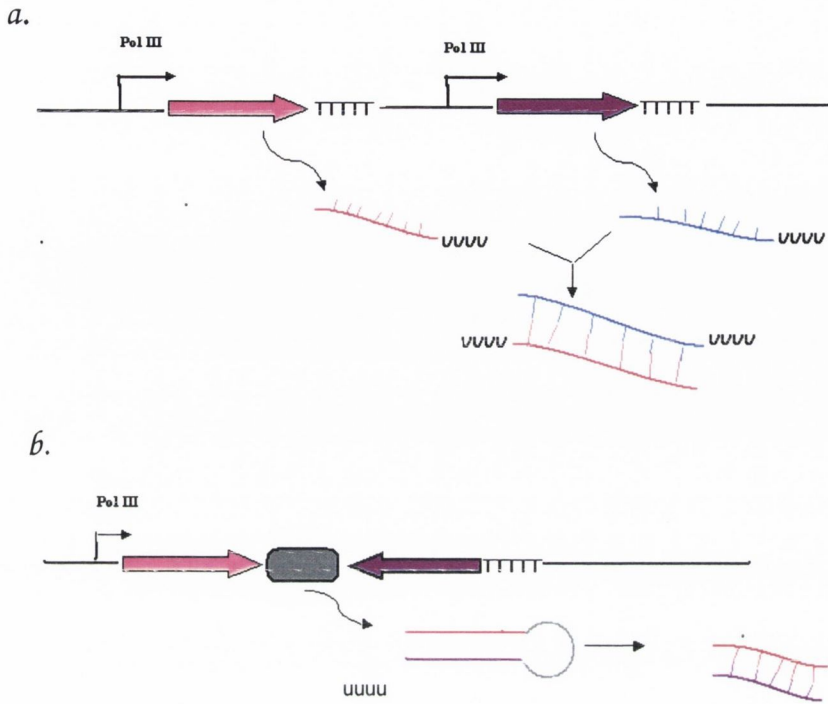


Fig. 3.3:

- a.* Tandem Pol-III promoters that express individual sense and antisense strands of the siRNA that associate in *trans*.
- b.* A single Pol-III promoter that expresses short hairpin (sh)RNA with the sense antisense strands of the siRNA that associate in *cis*.

Human rhodopsin cDNA plotfold

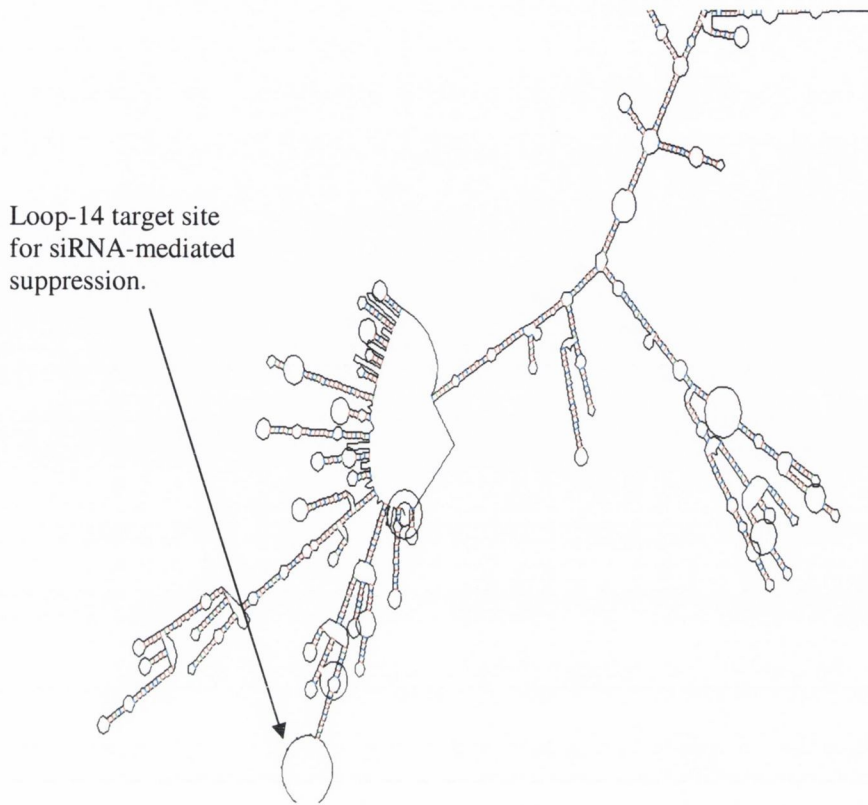


Fig. 3.4:

A plotfold of the most favourable secondary structure for human rhodopsin cDNA based on energy profiles. The HR cDNA was analysed in a widely available RNAdraw program, (Matzura and Wennborg, 1996). The full-length rhodopsin cDNA including 5'UTR was used as input sequence (obtained from the sequence retrieval database at NCBI). Default enthalpy and entropy settings for energy calculations at 37°C were used in all analyses. The open loop number 14 (loops numbered relative to the start of transcription) identifies the region containing the RNAi target site used in this thesis.

pIRES-EGFP-HR construct

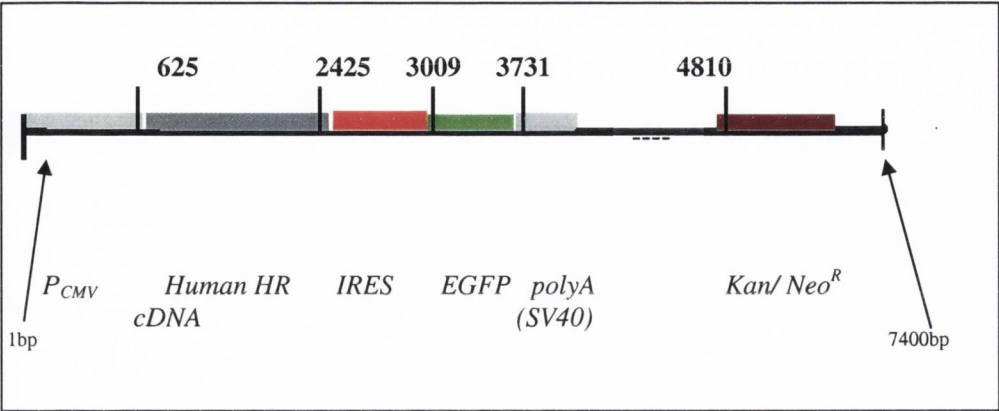


Fig. 3.5:

Diagram of the pIRES-EGFP-HR construct, carrying the human rhodopsin cDNA target (for experiments involving siRNA-mediated silencing). *P_{CMV}*:Cytomegalovirus promoter; *IRES*: Internal Ribosome Entry Site; *EGFP*: Enhanced Green Fluorescent Protein; *Kan/ Neo^R*: Kanamycin and Neomycin resistance gene.

pH1-siRNA construct

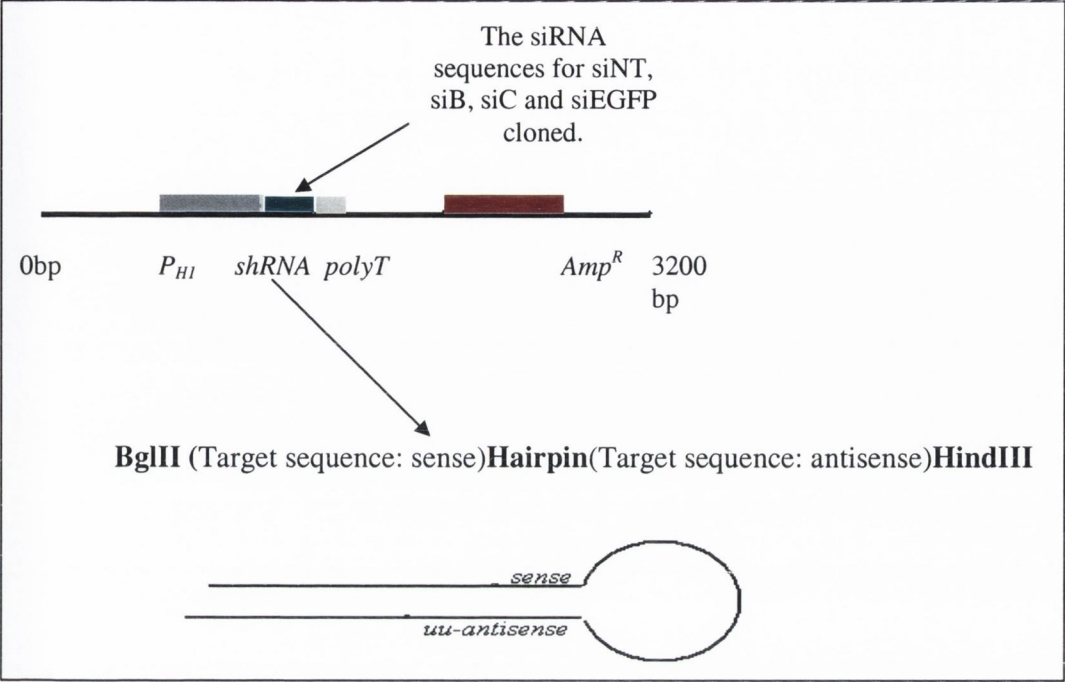


Fig. 3.6:

Diagram of the pH1-siRNA construct: The oligonucleotide sequences each contain the 19-nt target, in both sense and antisense orientation, separated by a 9-nt spacer sequence. The resulting transcript can thus fold back on itself to form the 19-base pair stem-loop structure. P_{H1} : H1 promoter; Amp^R : Ampicillin resistance gene.

pEGFP-shRNA

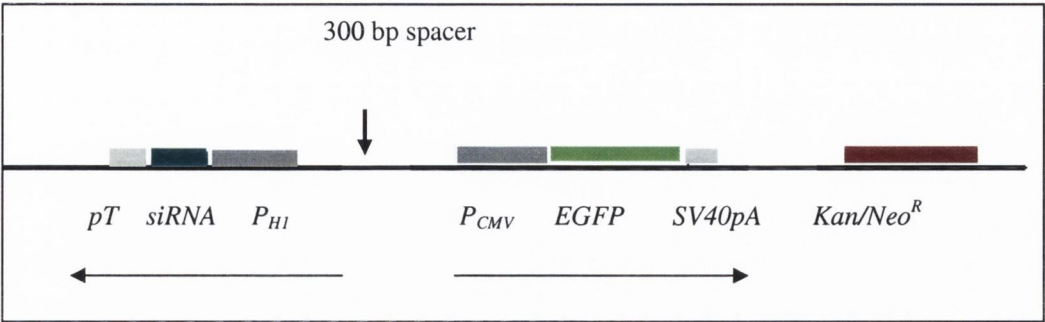


Fig. 3.7:

pEGFP-shRNA construct. The Pol-III-based H1 promoter is used to drive expression of an siRNA (The siRNA, siC, was selected based on rhodopsin gene silencing as shown in the results section 3.3.1, with siNT as a control). The CMV promoter is used to achieve a high level of marker EGFP expression. A 300-bp spacer comprising the ubiquitin C intron sequence separates the two cistrons. EGFP: Enhanced Green Fluorescent; *P_{H1}*: H1 promoter; *Kan^R*: Kanamycin-resistance gene; SV40pA: Simian virus-40 poly-A.

Real-time rtPCR data from rhodopsin (HR cDNA) analysis.

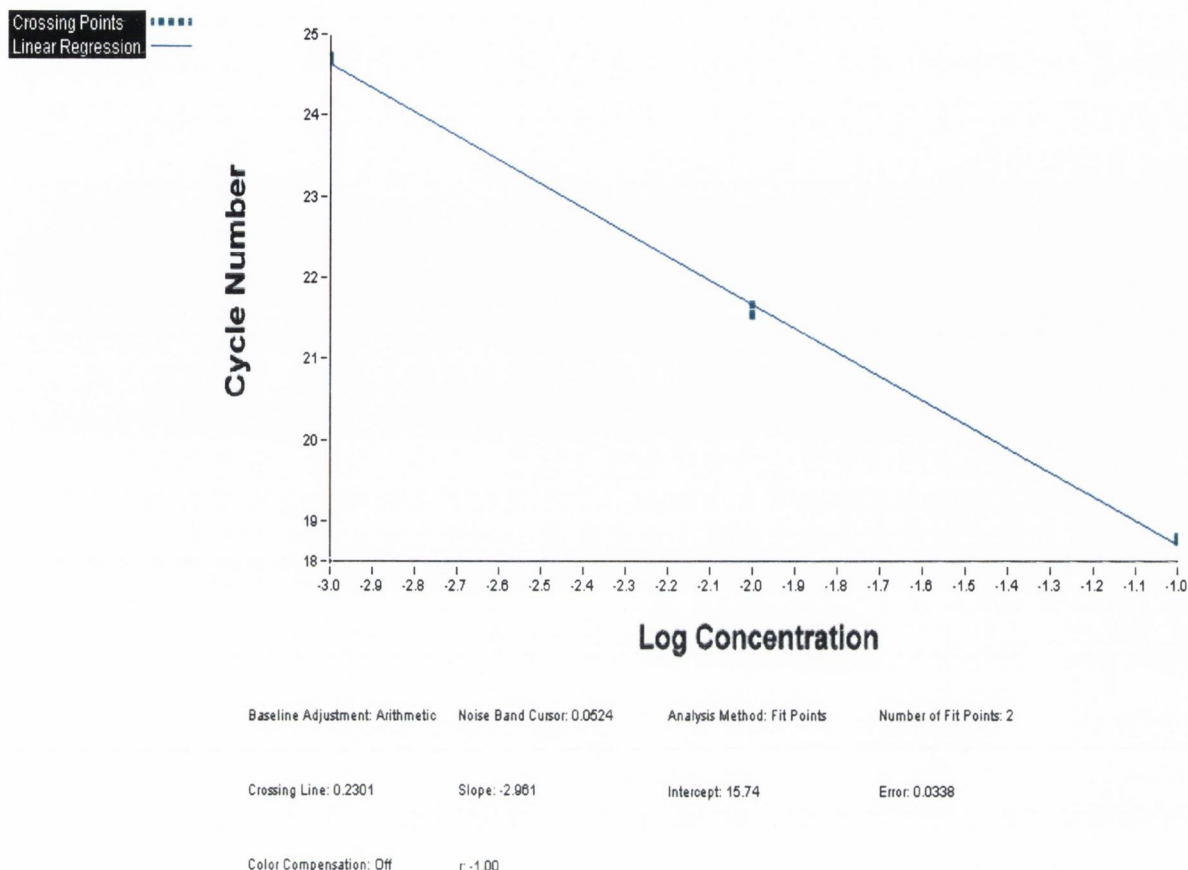


Fig. 3.8a:

Diagram showing a standard curve report from one quantitative rtPCR run analysing rhodopsin levels in RNA extracted from HeLa cell culture. The curve plots the crossing cycle number versus the log of the concentration. Data listed below the graph can be used to infer the relative efficiency of the run. The slope represents overall efficiency of the rtPCR reaction, the error indicates the extent of pipetting variation between individual samples and the coefficient 'r' offers an estimate of the accumulated error in the standard series.

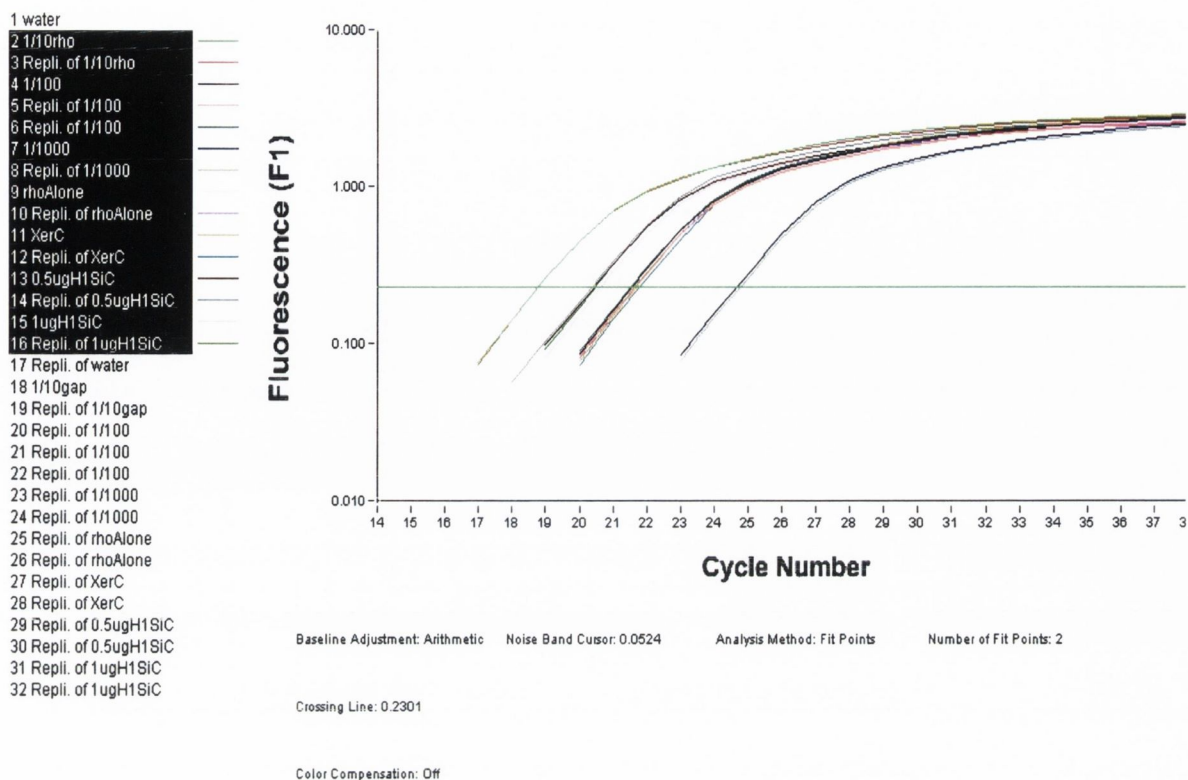


Fig. 3.8b:

Analysis report for quantifying rhodopsin levels (HR cDNA) in RNA samples extracted from HeLa cell culture. Each graph represents a plot of the level of fluorescence from a given sample in a tube at a given PCR cycle number. The sample list highlighted in black on the left of the plot shows the label given to each rtPCR reaction. The labels numbered 2 to 8 refer to tubes carrying specific dilutions of the RNA. The graphs generated by plotting the fluorescence reactions in these tubes against PCR cycle number can be used to set up a rhodopsin standard curve for the RNA. This is compared to the parallel standard curve generated for a reference gene at equivalent dilutions of the RNA and set up in tandem with the rhodopsin-amplified samples. The graphs for this standard curve are not shown in this figure and are represented by the unhighlighted lower half of the list on the left). It becomes possible to determine the relative rhodopsin expression level in many unknown samples by comparing the rhodopsin/reference ratios for the samples.

Animal matings for *ex vivo* analysis of NHR suppression.

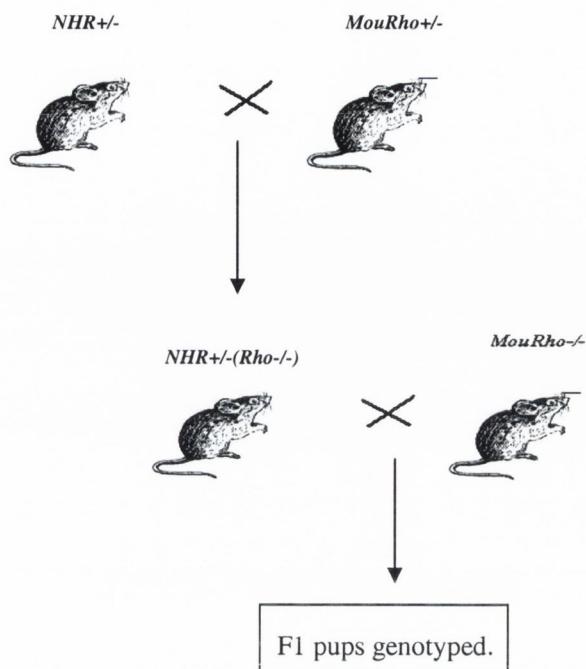


Fig. 3.9:

The matings set up for the explant experiment of section 3.3.7-9. Animals positive for the NHR transgene and negative for endogenous mouse rhodopsin were identified by PCR genotyping of tail-tip DNA (as described in section 2.2.6.1) and were used in the *ex vivo* experiments outlined in this chapter.

SiRNA target sequences compared in the wild-type and replacement rhodopsin cDNAs

	nt 177					nt 189				
Amino acids		L	Y	V	T	V				
Wild type:	CTC	ACG	CTC	TAC	GTC	ACC	GTC	CAG	CAC	
Replacement	CTC	ACG	CTG	TAT	GTG	ACG	GTG	CAG	CAC	
Amino acids		L	Y	V	T	V				
siRNA Silencer A -----										
<i>(1 nucleotide difference between wt and replacement)</i>										
siRNA Silencer B -----										
<i>(2 nucleotide differences between wt and replacement)</i>										
siRNA Silencer C -----										
<i>(5 nucleotide differences between wt and replacement)</i>										
----- siRNA Silencer D										
<i>(2 nucleotide differences between wt and replacement)</i>										
siRNA Silencer E -----										
<i>(5 nucleotide difference between wt and replacement)</i>										

Fig. 3.10:

SiRNA Targets: regions in the wild-type and replacement rhodopsin protein. While Si-A, -B, -C and -E were chosen by eye, si-D was picked from a Dharmacon prediction algorithm for the most effective silencer molecule in the region (www.dharmacon.com).

Rhodopsin RNA expression levels following cotransfection with presynthesised siRNA in mammalian cells

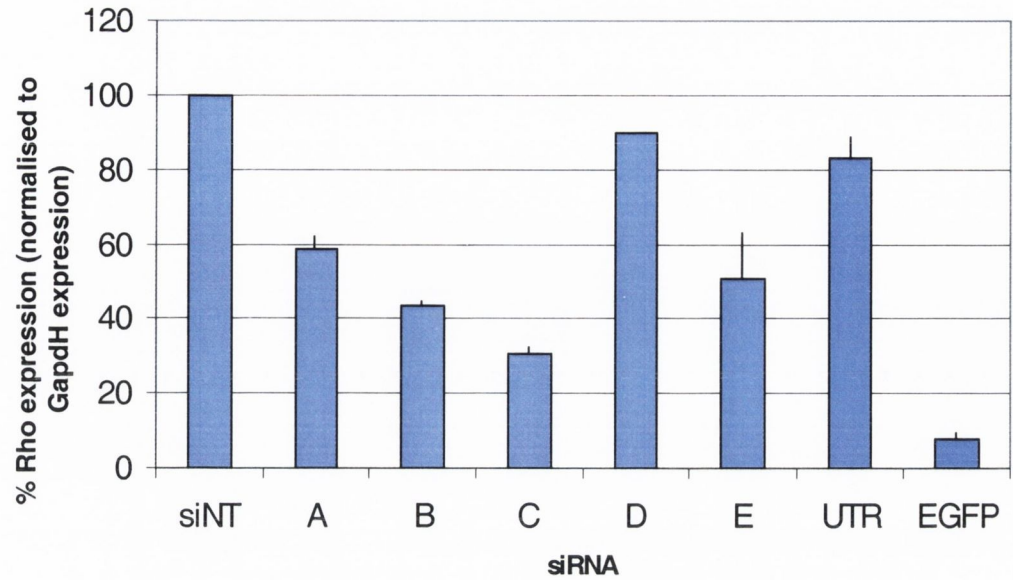


Fig. 3.11: Rhodopsin RNA expression levels normalised to GAPDH expression levels following targeted RNAi suppression in mammalian cells. Cotransfections of synthetic siRNAs and pIRES-EGFP-HR were carried out in COS7 cells and analysed by real-time rtPCR. The rhodopsin expression level for the siNT cotransfection was set at 100% (siNT from Qiagen, nucleotide sequence shown in Appendix, 4A).

	SiNT	SiA	SiB	SiC	SiD	SiE	siUTR	siEGFP
Average HR expression (%)	100	58.67	43.7	26.6	90	51.05	83	8
Std deviation	neg	3.06	1.06	6.58	n/a	11.9	5.66	1.41

Table 3.1: Rhodopsin expression following cotransfection with presynthesised siRNA *in vitro*. The results from three sets of cotransfections were averaged to produce the data. Errors are based on standard deviations calculated using Microsoft excel spreadsheet; no error was recorded for the siD analysis as this transfection was carried out only twice. All cotransfection data-sets, with the exception of siD and siUTR, were found to differ significantly from the siNT cotransfection data-set ($t < 0.05$). The siRNA siUTR was used to assess the possibility of silencing in the UTR region over an SNP of human rhodopsin and is discussed further in chapter 4, section 4.3.1.

Rhodopsin RNA expression levels normalised to cellular and plasmid gene expression levels following plasmid-driven RNAi suppression.

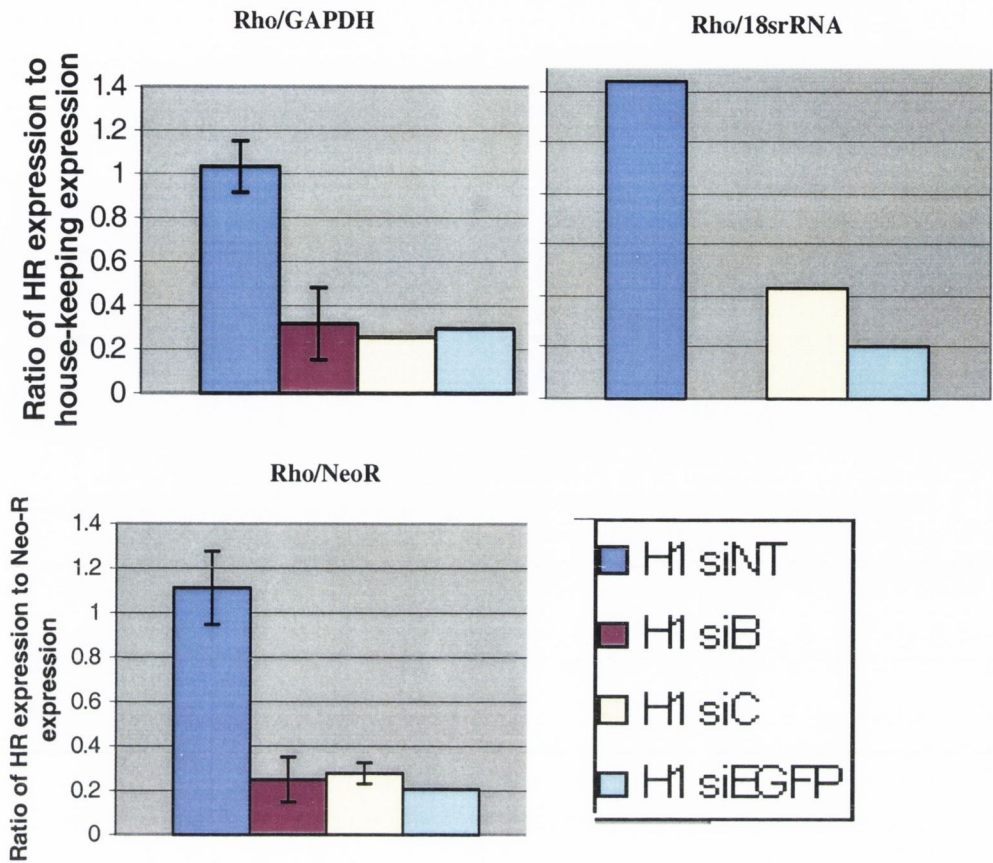


Fig. 3.12:

Rhodopsin RNA expression levels following stable RNAi suppression of rhodopsin by H1-SiB and H1-siC. The raw data for this figure is presented in table 3.2. In total the transfections were carried out three times. Analysis was carried out by real-time rtPCR. To normalize the data to a house-keeping gene it was necessary to set up each rtPCR reaction for real-time analysis in duplicate: once with rhodopsin primers and using house-keeping gene primers for the second reaction (this set up of real-time rtPCR reactions is described in section 2.2.3.3). RNA samples were analysed by normalizing the data to the relevant house-keeping gene (three data-sets for GAPDH and NeoR and two data-sets for 18S-rRNA). The plasmids H1-siNT and H1-siEGFP serve as negative and positive controls for suppression, respectively. The results were most consistent with the siC cotransfections data-set. The analysis of H1-siB cotransfections was not performed

relative to an 18s-rRNA reference reaction as further development of this siRNA was delayed in favour of siC.

H1-SiRNA	Rho/GAPDH		Rho/NeoR		Rho/18srRNA
NT	1.0344		1.1135		1.237
B	0.31655		0.2491		n/a
C	0.255		0.2775		0.431
GFP	0.2945		0.2052		0.21
H 1-SiRNA	S.D.	p-value	S.D.	p-value	S.D.
NT	0.2318		0.214	0.021	0.0037
B	0.3287	0.043	0.101	0.028	
C	0.0867	0.0137	0.048	0.015	Neg

Table 3.2a:

Data used to generate fig. 3.12: average rhodopsin expression ratios relative to reference gene. Overall percentage suppression values are listed in table 3.2b below. Data from three sets of cotransfections were used for both the GAPDH and neoR genes. The 18S-rRNA housekeeping gene was only used as a reference gene in two sets of transfections and was not used to normalise data for H1-siB cotransfections. S.D. refers to standard deviation from the mean. Student’s T-test was used to generate values of significant differences relative to H1siNT data, ($p<0.05$ denotes significance).

H1-SiRNA	Rho/Gap	Rho/Neo	Rho/rRNA	Ave. expression of NHR
H1-SiNT	100%	100%	100%	100%
H1-SiB	30.6%	22.37%	n/a	26.5%
H1-SiC	24.65%	24.92%	34.84%	28.14%
H1-SiGFP	28.47%	18.43%	16.98%	21.29%

Table 3.2b:

Level of rhodopsin expression from pIRES-EGFP-HR, relative to a 100% expression level in cells transfected with H1-construct with non-targeting siRNA (Data from fig. 3.12).

Rhodopsin expression levels following plasmid-driven RNAi suppression in three different mammalian cell lines

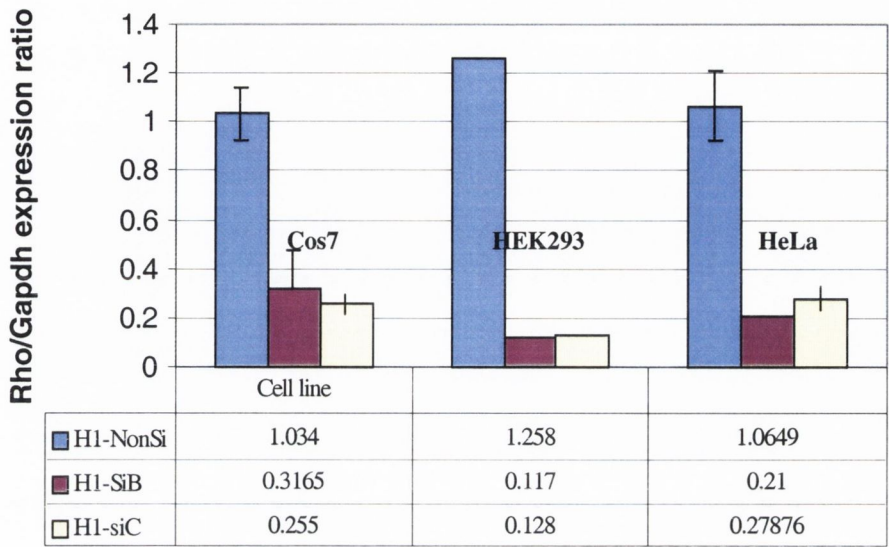


Fig. 3.13:

Rhodopsin expression levels normalised to cellular GAPDH expression levels following plasmid-driven RNAi suppression. Analysis of each RNA extraction was performed twice following 3 sets of cotransfections in COS7 and HeLa cells. Data was limited for cotransfections in HEK293 cells. All data-sets from each cell line generated a strong overall statistical significance of the difference in expression level between H1-siNT and the targeting constructs, ($p = 7e-7$ and $p = 5e-9$, for H1-siB and H1-siC, respectively, where $p < 0.05$ denotes statistically significant difference between data-sets).

H1-SiRNA	COS7	HEK293	HeLa
H1-SiNT	100%	100%	100%
H1-SiB	30.6%	9.3%	19.8%
H1-SiC	24.66%	10.17%	26.18%

Table 3.3:

Level of rhodopsin expression from pIRES-EGFP-HR in three different cell lines, treated with siRNA (relative to rhodopsin expression in H1-siNT-treated sample fixed at 100%).

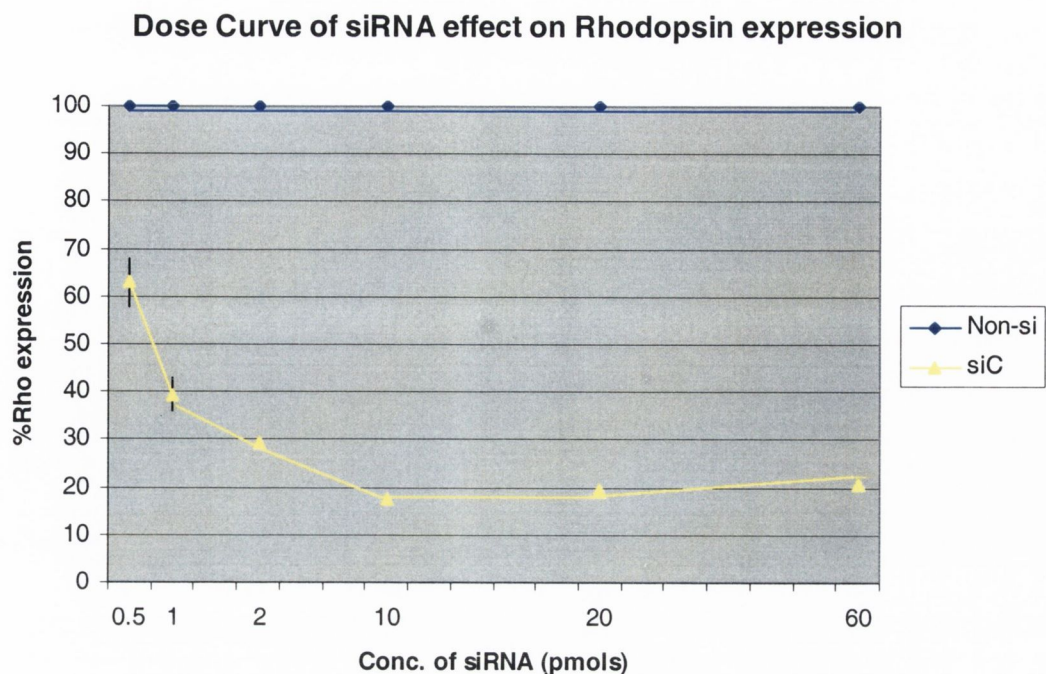


Fig. 3.14: Rhodopsin expression levels following cotransfections of 400 ng of pIRES-EGFP-HR with different concentrations of siC in COS7 cells (yellow line). The expression data generated for cotransfections with the non-targeting control siRNA was set at 100% (blue line). Transfections of siRNA and target (specifically for the dose curve) were carried out twice, and variation was only apparent for the cotransfections with 0.5 pmol and 1 pmol of siRNA.

Conc. siRNA (nM)	No. of pmoles	SiRNA,(20nm) volume (µl)	SiC
60	60	3	20.6
20	20	1	19.4
10	10	1/2	17.6
2	2	1/10	29.5
0.5	1/2	1/40	63

Table 3.4: Dose curve for presynthesised siRNA activity *in vitro*. The experiment was carried out twice. Variation in the expression levels recorded from cells transfected with lower concentrations of siRNA led to significant error (at 0.5 pmol, S.D.= 4.9; at 2 pmol S.D.=3.4).
(Rhodopsin expression in cells transfected with the control non-targeting siRNA set at 100%).

MTT analysis following siRNA-mediated silencing of rhodopsin expression in mammalian cells

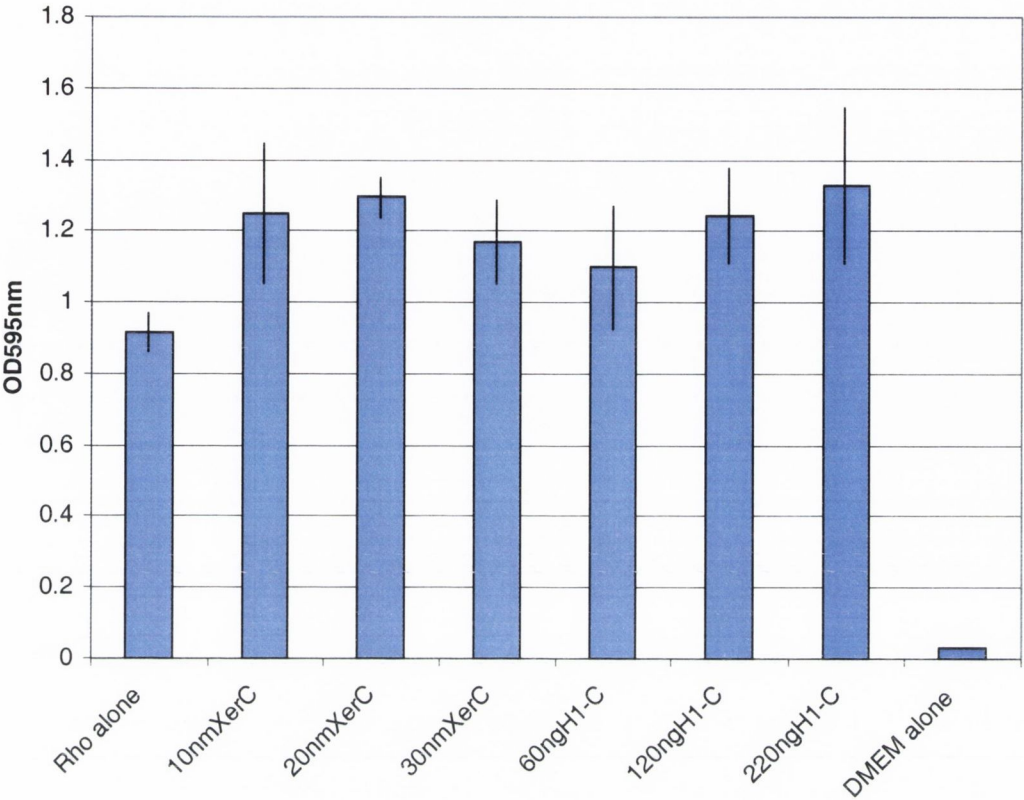


Fig 3.15:

Optical density readings showing the level of formazan metabolite formed in cells treated with silencing agents. “Rho alone” refers to the transfection of pIRES-EGFP-HR plasmid alone; “XerC” denotes cotransfection of the presynthesized siRNA, siC (ordered from Xeragon.com, now Qiagen) with pIRES-EGFP-HR, and “H1-C” refers to the hairpin plasmid, and used in cotransfection with pIRES-EGFP-HR. The data was normalised to the absorbance reading from a control well of mock-transfected cells treated with lipofectamine 2000 (invitrogen) alone. “DMEM alone” refers to wells that have medium but no cells. The samples were pipetted in triplicate wells and the experiment repeated. The results from these six readings were pooled to generate the graph.

ELISA on protein purified from cells following siRNA-mediated silencing of rhodopsin gene expression.

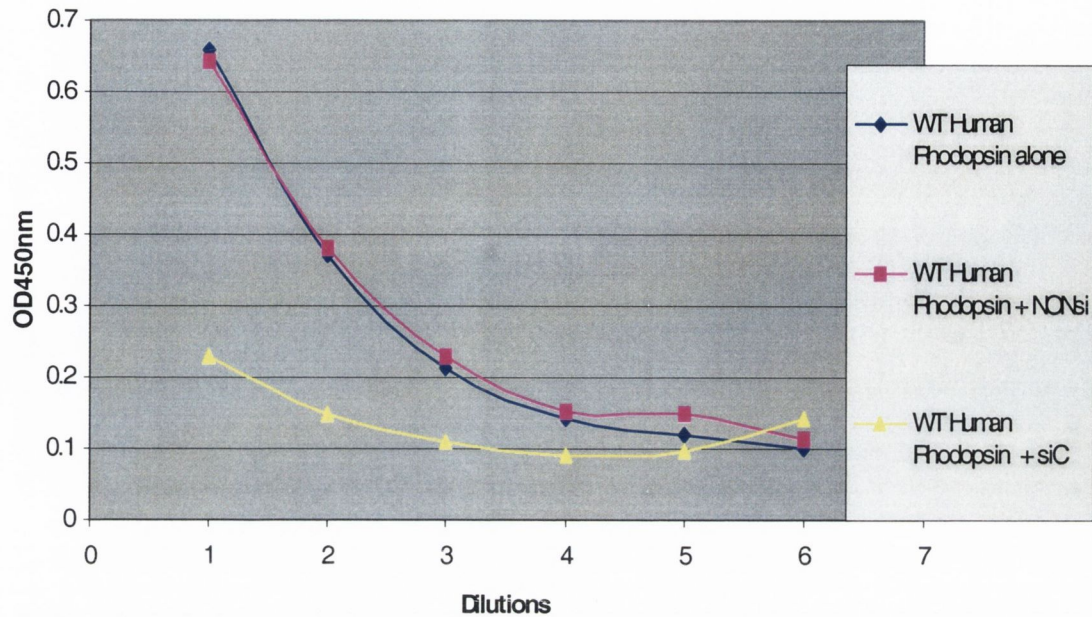


Fig. 3.16: Protein expression as determined by ELISA from cotransfected HEK293 cells. The protein samples from 3 individual sets of transfections (of 4×10^5 cells) were pooled for this analysis. The blue and pink lines represent controls of *target alone* and *target* treated with a non-targeting siRNA, respectively. The yellow line shows the level of rhodopsin expression following cotransfection of the rhodopsin-expressing plasmid with siC. Specific rhodopsin protein expression was detected using the peroxidase-conjugated anti-rhodopsin 4D2 antibody (Molday *et al.*, 1984), with the level of antibody binding determined by colorimetric assay.

Protein Dilution	Dilution factor	HR alone	HR+NonSi	HR+siC	HR alone expression%	HR+NonSi expression%	HR+siC expression%
1	1/40	0.659	0.642	0.2275	100	97.4	34.5
2	1/80	0.3725	0.3795	0.149	100	101.9	40
3	1/160	0.2145	0.23	0.111	100	107.2	51.7
4	1/320	0.1435	0.151	0.0895	100	105.2	62.4
5	1/640	0.119	0.147	0.0955			
6	1/1280	0.1	0.112	0.141			

Table 3.5: OD450nm readings for protein dilutions taken from HEK293 cells treated with wild-type rhodopsin and siRNA following 4D2-antibody staining and POD substrate ELISA reaction. The percentage values for the final two dilutions are not included as background levels of the antibody-substrate reaction quench any suppression effects.

Cell Microscopy pictures following siRNA and pIRES-EGFP-HR cotransfections

Dapi stain

EGFP fluorescence

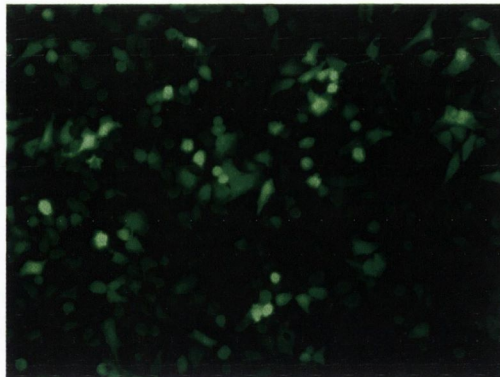
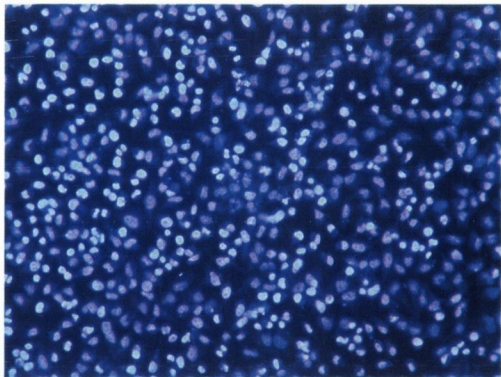


Fig 3.17a:

pIRES-EGFP-HR & pH1-NT cotransfection in 1×10^5 HeLa cells. Two poly-L-Lysine-treated coverslips coated with the cells from two separate cotransfection experiments were analysed under white light.

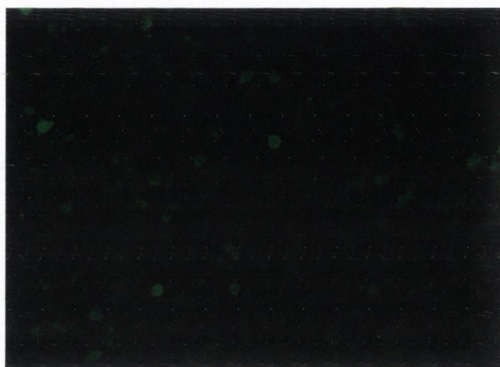
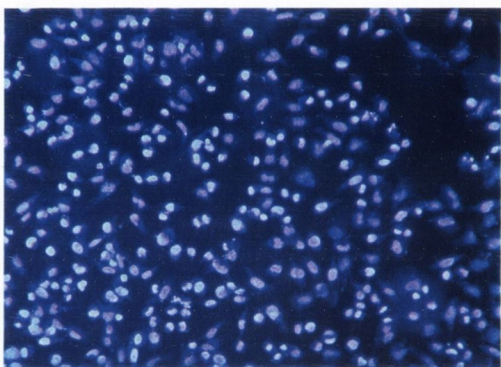


Fig 3.17b:

pIRES-EGFP-HR & pH1-C cotransfection in HeLa cells, (1×10^5 HeLa cells; two coverslips).

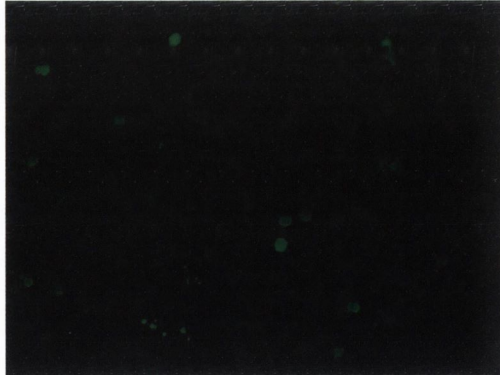
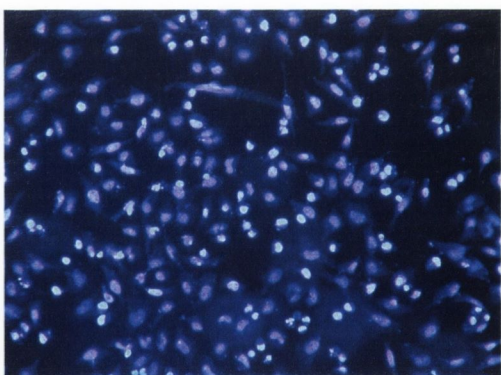


Fig 3.17c:

pIRES-EGFP-HR & pH1-siEGFP cotransfection in HeLa cells, (1×10^5 HeLa cells; two coverslips).

Fluorescent Activated Cell Sorter (FACS) analysis of NHR+/--(MouRho-/-) mouse explanted retinal tissue (outlined in materials and methods section 2.2.6.3)

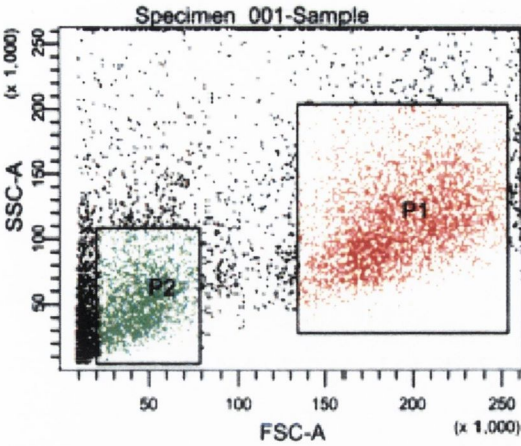


Fig 3.18a:

The P1 population, composed of photoreceptor cells, was selected according to size by side scatter, (SSC) and granularity parameters (FSC: Forward Scatter). Smaller particles, from the P2 population, were also analysed by real time rt-PCR and shown to be composed of mainly debris from segmented RPE cells.

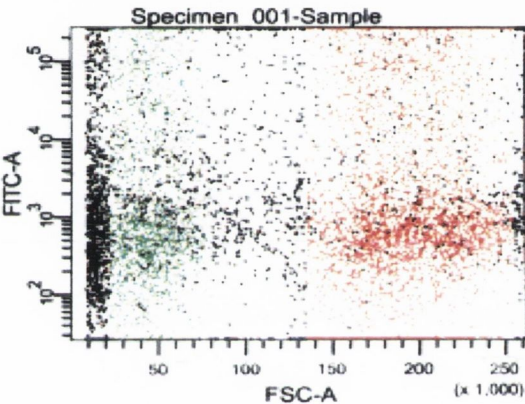


Fig 3.18b:

The P1 pool of cells, from the sort selecting for granularity was expected to be composed largely of Müller glia cells and photoreceptor cells. The final P3 cell population was selected according to size and fluorescence (FITC) and was shown by quantitative rtPCR to contain transfected photoreceptor cells.

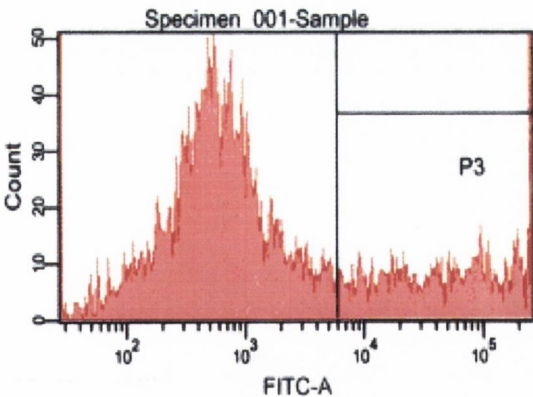


Fig 3.18c:

The P3 photoreceptor population carried the electroporated pEGFP-siC or control pEGFP-siNT constructs was counted and isolated for RNA extraction (using the Qiagen RNeasy Mini Kit) and further analysis.

Suppression of endogenous human rhodopsin transgene by electroporation of siRNA in mouse retinal explants and assessed by real time rt-PCR

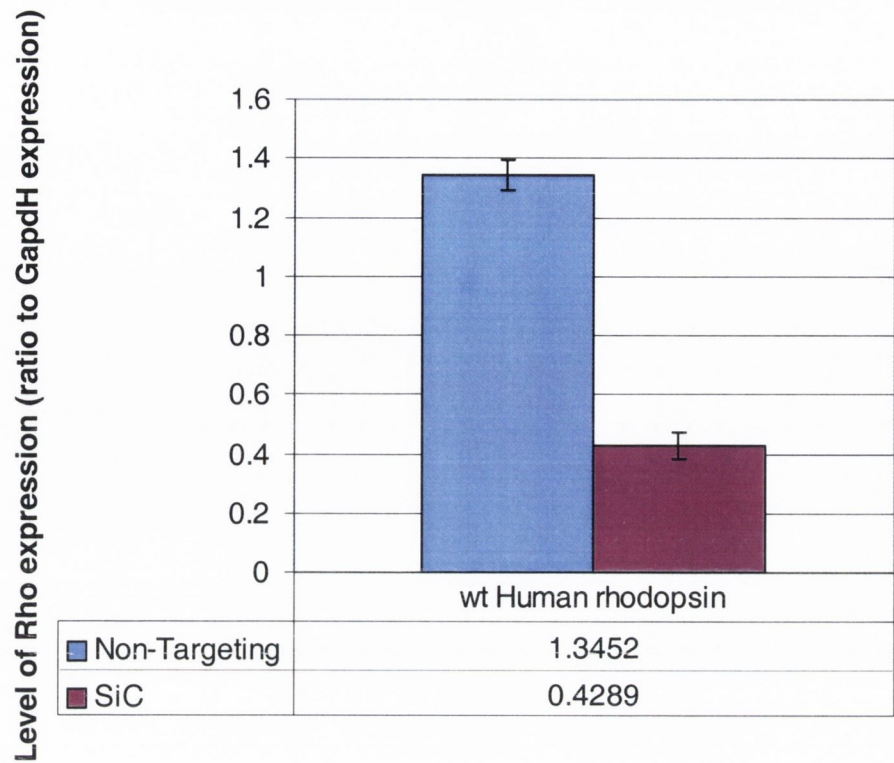


Fig 3.19: Suppression of endogenous human rhodopsin transgene in mouse retinal explants assessed by quantitative rt-PCR. The FACS and real-time rtPCR analysis was performed three times using RNA from a total of 6 retinas.

Rhodopsin expression relative to GAPDH in transfected explanted retina		
Suppressor	pEGFP-H1siNT	p EGFP-H1SiC
NHR expression (%)	100	31.88
Standard Deviation	0.053	0.0459
p-value	2.117e-7	

Table 3.6: Percentage suppression of NHR mRNA from retinal explanted cells. The average ratio of rhodopsin to GAPDH gene expression was fixed at 100% for the siNT electroporation, yielding approximately 68% total siC-mediated suppression of the human rhodopsin transgene in the NHR+/--(MouRho-/-) explant. Standard deviations (+/-) were calculated on the array of gene expression ratios for both siNT and siC data-sets; and Student’s paired T-test (excel) was used to assess the significance of the difference between the siNT and siC expression ratio data-sets.

Dissociation and immunostaining of cells from explanted tissue of NHR+/- (MouRho/-) transgenic mice, following electroporation with pEGFP-siNT.

Representative pictures of the cell-coated coverslips examined are presented below. A single retina was used to coat each coverslip.

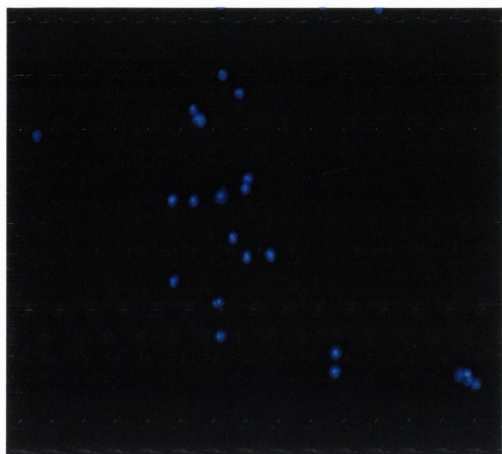


Fig 3.20a: Dapi stain of cells from 4 NHR+/- retinas electroporated with pEGFP-siNT.

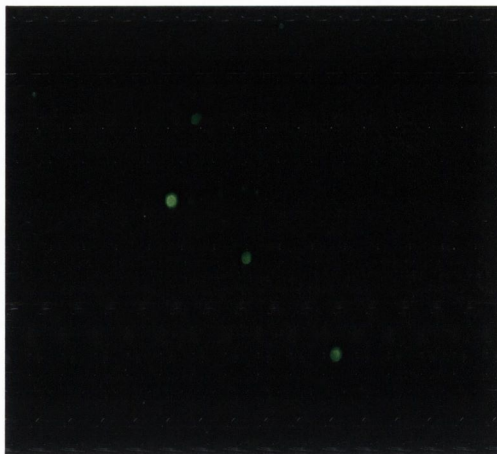


Fig 3.20b: EGFP expression of cells from 4 NHR+/- retinas electroporated with pEGFP-siNT.

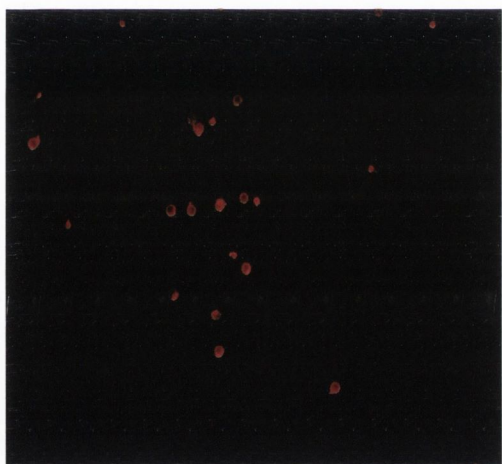


Fig 3.20c: 4D2 anti-rho immunostain and secondary alexa fluor (458nm) labelling of cells from 4 NHR+/- retinas electroporated with pEGFP-siNT.

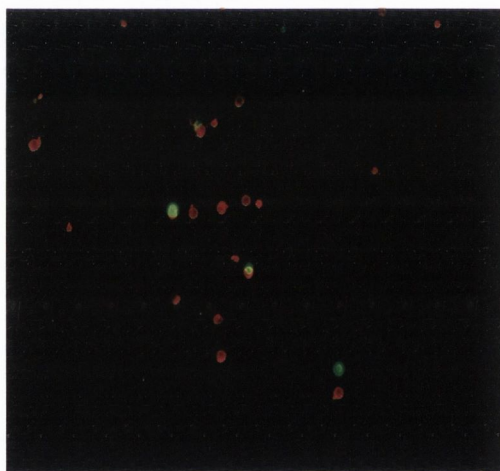


Fig 3.20d: Overlay of Figs. 3.20b and c, EGFP transfection superimposed on rhodopsin-positive cells.

Dissociation and immunostaining of cells from explanted tissue of NHR+/-, MouRho-/- transgenic mice, following electroporation with pEGFP-siC.

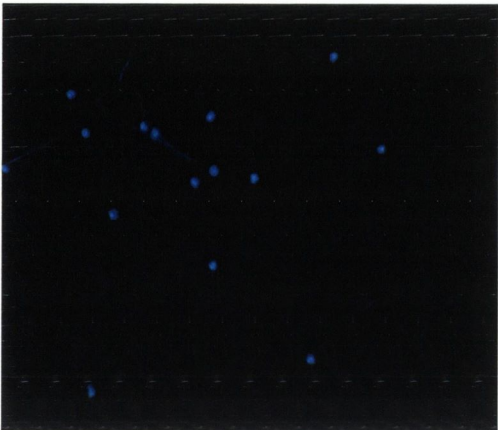


Fig 3.21a: Dapi stain of cells from 4 NHR+/- (MouRho-/-) retinas electroporated with pEGFP-siC.

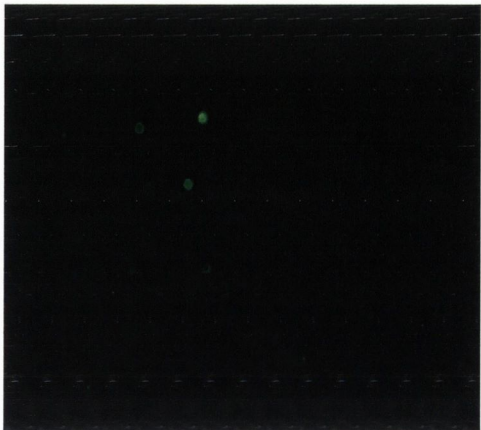


Fig 3.21b: GFP expression of cells from 4 NHR+/- (MouRho-/-) retinas electroporated with pEGFP-siC.

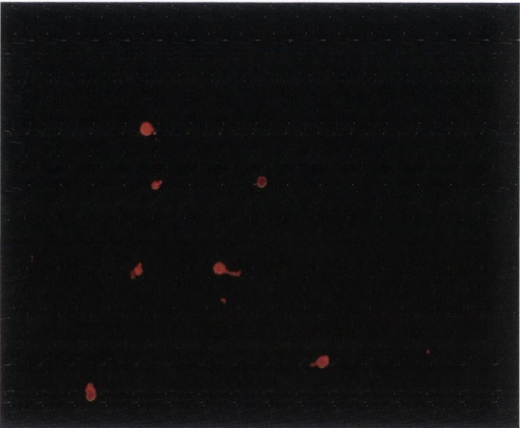


Fig 3.21c: 4D2-antiRho immunostain and secondary alexa fluor (458nm) labelling of cells from 4 NHR+/- (MouRho-/-) retinas electroporated with pEGFP-siC.

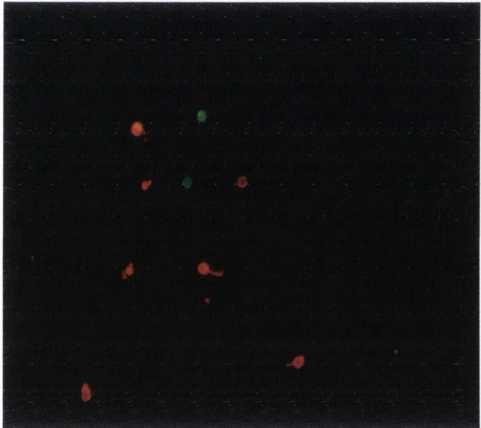


Fig 3.21d: Overlay of pictures Figs 25 and 26 showing transfected cells superimposed on 4D2 stained pictures.

	No. of green cells	No. of red cells
pEGFP-siNT	291	210
pEGFP-si-C	112	39

Table 3.7: NHR cell count from four NHR+/- (MouRho-/-) retinas electroporated with pEGFP-siRNA (Figs. 3.20 and 3.21). The retinas were dissociated and immunostained for counting under a fluorescent microscope (as described in section 2.2.6.7).

Immunostaining of cryosections from explanted tissue of three NHR+/- (MouRho-/-) transgenic mice, following electroporation with pEGFP-siC.

Fig. 3.22a :

Dapi stained cross-section of NHR+/- retina electroporated with pEGFP-siC. Grown for two weeks as an explant before being harvested, cryosectioned, and stained. The rosette formation typical of outer segments grown in culture can be seen in A and B. The cryosections presented here are 7µm in thickness.

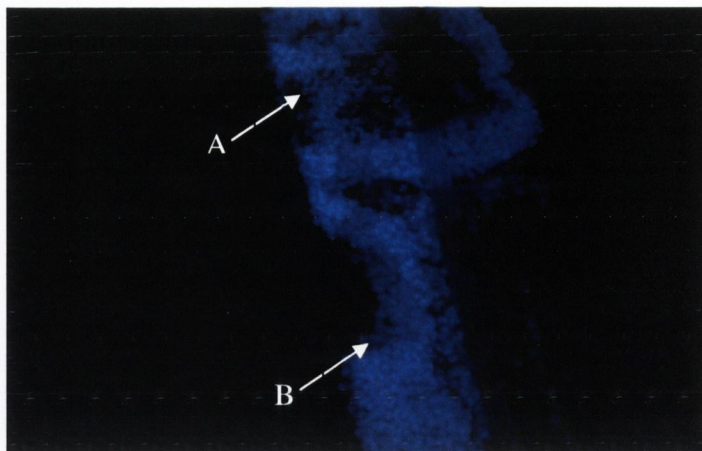


Fig. 3.22b:

Cross-section of NHR+/- retina electroporated with pEGFP-siC as shown in fig. 3.22a. Sections were visualised under a fluorescent green light. The transfected cells are highlighted by A, and an untransfected cluster of cells highlighted by B.

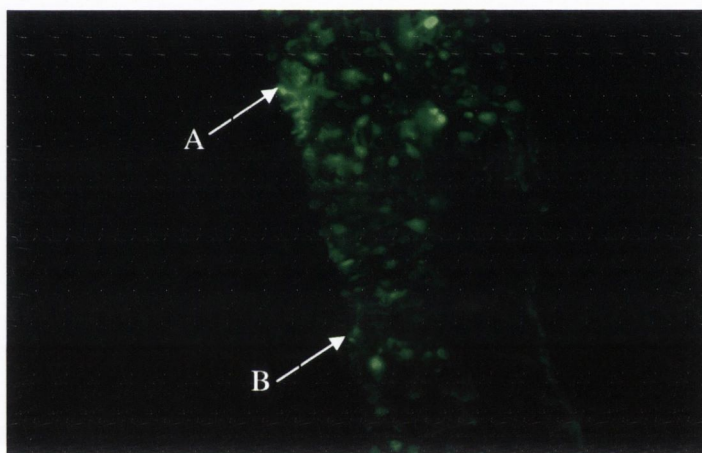
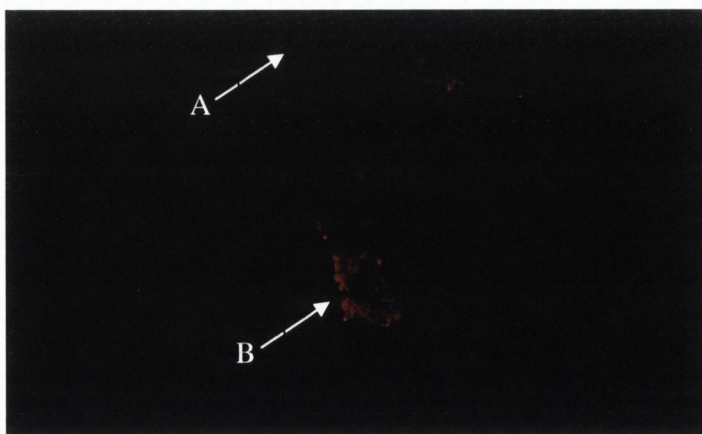


Fig. 3.22c:

The section as shown in 3.22a and 3.22b has been immunostained with rhodopsin antibody and secondary stained with a cross-reacting alexa fluor label. Hence rhodopsin protein should be visible under red light. Reduced signal for rhodopsin is evident in the transfected cluster A compared to stronger staining in the lesser transfected cluster B.



Chapter 4

Effect of siRNA on replacement human rhodopsin gene expression

CHAPTER 4

4.1 INTRODUCTION

4.1.1 Aims of chapter 4

Results from recent studies have suggested that RNAi technology is amenable to a mutation-independent suppression and replacement therapeutic approach (Millington-Ward *et al.*, 2004; Kiang *et al.*, 2005, Palfi *et al.*, 2005). This chapter is primarily concerned with the replacement aspect of this strategy for the design of treatments targeting rhodopsin-linked ADRP and allied dominantly inherited degenerative disorders. The rhodopsin gene exhibits a high degree of intragenic mutational heterogeneity and is a major cause of dominant RP; it therefore serves as a good model in which to develop mutation-independent therapeutic strategies. In this chapter, a codon-modified version of the wild-type human rhodopsin gene is assessed for resistance to the suppressing agent (i.e. the siRNA sequences selected in chapter 3). This replacement gene shall be discussed in the context of the design of potential therapies targeting a dominant gain of function disorder that exhibits extensive mutational heterogeneity.

4.1.2 RNAi specificity

The specificity of RNAi technology remains an issue under controversy and requiring further exploration. Jackson *et al.* reported suppression of ‘off-target genes’ containing as few as 11 contiguous bases of complementarity to the siRNA (Jackson *et al.*, 2003). It has been suggested that during RNAi, the overall stability of the RNA duplex formed between the antisense siRNA strand and mRNA target site may determine the success of silencing (the higher the stability the greater the silencing) rather than the absolute number of mismatches (Saxena *et al.*, 2003). Therefore factors additional to sequence that may confer specificity need to be considered when attempting to optimise the use of RNAi in any suppression and replacement therapeutic strategy. A level of control can be achieved by rational selection of targets with favourable thermodynamic profiles. RNAi suppression, if applied appropriately, (i.e. with rational selection criteria), should, in principle, offer the level of specificity necessary for suppression and replacement therapeutic designs.

4.1.3 Allele-specific preference

As an alternative to a suppression and replacement approach, an allele-specific approach has also been considered for treatment of rhodopsin-based ADRP. The essence of the approach is the exploitation of intragenic polymorphisms. Such polymorphisms have been utilised extensively over the past two decades to enable localisation of disease genes and more generally have been used in various genome-mapping projects (Myers *et al.*, 2005). It is proposed that such polymorphisms may be used as a means of discriminating between mutant and wild-type alleles of a gene, but in a mutation-independent manner. A suppression agent may be used to target one allele of a polymorphic variant while in principle leaving the alternative allele intact. If the polymorphic variant is frequent in a given population, a suppression agent targeting this allele may be used to treat patients independent of which mutation they have in a given gene, as long as the polymorphic variant and mutation reside on the same allele of the gene.

RNAi-based targeted suppression of an allele carrying a dominant disease mutation may be achieved by designing a siRNA molecule covering an SNP site and targeting the variant polymorphism carried on the disease allele. Thus the wild-type allele would, in principle, avoid suppression and remain capable of expressing the wild-type protein in the absence of the dominant, mutant form. It is likely that such a therapy would be of considerable interest in the long-term as it may require less intervention than the two-pronged suppression and replacement approach (described in section 4.1.4) and may allow some endogenous wild-type protein to remain *in vivo*. However suitable SNPs need to show a sufficient level of heterozygosity in order to be feasible candidates for suppression. Furthermore, in many cases, it is unlikely that allele-specific suppression with a single nucleotide resolution will be achievable. It may be that RNAi will show 'allele-preference' rather than full allele-specificity when applied as a suppressing agent in dominant disease (Millington-Ward *et al.*, 2004).

The success of such a strategy is therefore dependent on two factors: as mentioned, the fine discrimination of RNAi at a single nucleotide level, and the availability of single nucleotide polymorphisms in the coding sequence of the target gene. Unfortunately there are few SNPs in the coding sequence of the rhodopsin gene, and the heterozygosities of each of the known SNPs are low. In the current study, only the published SNPs for which allele frequencies were available were

investigated (section 4.3.1) and of these, only those SNPs that occurred in the transgene animal models that were available to us were assessed as target sites for suppression. An SNP at nucleotide 70 of rhodopsin cDNA, (dbSNP: rs7984), shows a moderate level of heterozygosity and hence an siRNA covering this region has been designed for testing (Primer sequences used for sequencing the rhodopsin gene over SNP sites listed in Appendix 1A; siSNP covering rs7984 listed in 4A).

However, in the context of dominant disease it is worth noting that in many cases it is unclear whether the dominant phenotype is conferred by a gain-of-function mutation or haplo-insufficiency or a combination of both mechanisms, and hence use of a replacement gene in conjunction with suppression may ultimately be necessary for some disorders. A suppression and replacement strategy has been proposed for dominant disorders, which are mutationally heterogeneous (such as ADRP). Validation of the replacement element of this strategy involving the use of a 5 and 7-codon-modified versions of the rhodopsin cDNA has been undertaken and shall be discussed in more detail in the following section.

4.1.4 Suppression & Replacement therapeutic approach

To date there are very few published studies involving RNAi-based suppression of genes associated with ocular disease. The original study showing photoreceptor preservation by the use of siRNA techniques was carried out in *Drosophila*, where degeneration in a constitutively active TRP heterozygote was rescued following mutation-independent suppression of TRP by siRNA (Geng *et al.*, 2004). An autosomal dominant mutation *Trp*^{P365} causes the TRP channels to be permanently open. The opening of these channels is normally only achieved by photostimulation of rhodopsin protein. In this mutant fly, the continuously open channel causes an excessive influx of calcium ions leading to photoreceptor degeneration. The results described in this paper demonstrated, using protein and ERG data, how reduction of the mutant TRP mRNA by dsRNA does not necessarily need to be complete to achieve a substantial rescue of the degeneration (Geng *et al.*, 2004).

A key aim of the study described in this chapter was to validate a therapeutic approach flexible enough to remain mutation-independent whilst being directly applicable to rhodopsin mutations, caused by either toxic gain-of-function effect(s) or

interfering dominant-negative effects. A combination approach involving regulated suppression of rhodopsin twinned with a tight control on replacement rhodopsin expression is desirable. Notably, the use of a modified replacement gene whose sequence is altered such that transcripts from this gene are resistant to siRNA-based silencing could, in principle, provide a means of overcoming the mutational heterogeneity in disorders such as rhodopsin-linked RP. In this regard there has been some progress recently on the use of suppressor-resistant replacement genes in a suppression and replacement therapeutic approach for dominant disease (Kiang *et al.*, 2005; Roth *et al.*, 2004). Similarly, Kim and Rossi, (2004), have demonstrated RNAi-mediated down-regulation of EGFP in tandem with the expression of a codon-modified replacement gene in cell culture.

The replacement gene used in the study has already been validated as functioning *in vivo* using transgenic technologies (O'Reilly *et al.*, personal communication) and we hope to target suppression of wild-type rhodopsin to the region altered in this replacement rhodopsin sequence. The replacement gene is arguably the more tricky aspect of the design, as it may often require substantial engineering to achieve an appropriate expression level. Many replacement constructs containing a variety of rod-specific promoters, a range of promoter lengths, 5' and 3' UTRs, heterologous introns and so on may need to be tested in order to achieve optimal expression in the retina. A transgenic mouse carrying the human rhodopsin replacement gene used in the current study has been generated and when mated to mice with a targeted disruption of the rhodopsin gene, completely rescued the phenotype in these rho-/- mice. Given the availability of the replacement gene and associated transgenic mouse, siRNA-based suppressing agents were chosen such that they were targeting the same position modified in the rhodopsin replacement gene (as shown in fig. 4.1 in the figures section at the end of this chapter). The desired feature of an optimal suppression agent would be that it silences expression of the wild-type rhodopsin gene whilst leaving transcripts from the modified replacement gene intact.

The research described in chapter 3 was focused on the identification of optimal siRNA molecules targeting the wild-type human rhodopsin sequence but over the region modified in the replacement (approximately 70% suppression of rhodopsin expression was achieved in cell culture, section 3.3.8). The aim in the current study is to progress the research forward towards the generation of potentially therapeutic vectors - hence design of

constructs carrying two elements – a suppression agent and a replacement gene. The research described in this chapter explores the second element of these constructs, the replacement gene.

4.1.5 RNAi & secondary structure

The variable potencies of si-A, -B, -C, -D, and -E as suppression agents (section 3.3.1), suggested early on that it is likely that each individual 21–22-mer and not the overall landscape of the target mRNA, may be responsible for the efficiency of target downregulation that can be achieved. This is in contrast to other antisense and ribozyme knockdown strategies, in which the accessibility of the mRNA appears to have a significant influence on suppression (Xing *et al.*, 1992). Despite this, it was of interest to evaluate how the predicted secondary structure of the replacement transcript may possibly have altered as a result of the introduction of 5 or 7 base changes. It is possible that secondary structure may influence RNA stability and translation. While these two features were not ultimately explored specifically, it was nevertheless worthwhile to take note of any changes in secondary structure that could potentially influence them. A computer program based on thermodynamic energy calculations of bonding nucleotide pairs, devised by Ole Matzura and Anders Wennborg, was used for this secondary structure calculation (Figures and Tables section, table 4.3).

4.1.6 Replacement rhodopsin transgenic mouse model

A transgenic mouse model was generated by Dr. Mary O'Reilly, TCD, in collaboration with Dr. Therese Tuohy in the University of Cincinnati. This animal model expresses a replacement rhodopsin gene and can therefore be used to evaluate *in vivo* if transcripts from the replacement gene are resistant to suppression. The replacement rhodopsin protein is derived from a transgene carrying five sequence modifications at degenerate sites between nucleotides 177 to 189 of the human rhodopsin cDNA (fig. 4.1).

The rhodopsin transgene in this transgenic mouse is driven by 3.8 kb of the mouse rhodopsin promoter. In addition to carrying all five exons of rhodopsin, the transgene has intron 9 of the HPRT gene with the necessary exon/intron junctions available for splicing. The use of heterologous introns interrupting the coding sequences of a gene has been reported to enhance transgene expression (Lacy-

Hulbert, 2001). The replacement transgenic mouse was generated on a mixed C57/L129 mouse genetic background and subsequently bred solely on a L129 background. Initially, transgenic mouse lines expressing the human rhodopsin replacement gene were bred onto Rho^{-/-} mice. Subsequently, human rhodopsin replacement transgenic mice on a mouse rhodopsin knockout background (Rho^{-/-}) were analysed for rhodopsin expression and retinal function. Functional rhodopsin expression in the replacement animal was shown by retinal histology and electroretinography at 4 months of age (figures 4.2a and 4.2b).

Notably, the replacement rhodopsin mouse on a Rho^{-/-} background (termed HR5m(MouRho^{-/-}) in this thesis) represents a powerful tool with which to investigate if transcripts from the replacement gene are indeed resistant to siC-based suppression of human rhodopsin as would be predicted due to the incorporation of 5 sequence changes between the siC target site and the replacement rhodopsin transcript. Hence retinal explants from the HR5m(MouRho^{-/-}) model have been used in an *ex vivo* investigation of whether siC, (as a wild-type human rhodopsin-specific suppressor), will spare transcripts from the replacement gene. The study described in this chapter utilises similar technologies to those described at the end of chapter three to explore if the replacement rhodopsin gene is resistant to siRNA-mediated down-regulation in an organotypic culture.

4.2 METHODS

4.2.1 *Plotfold analysis*

The wild-type human rhodopsin cDNA sequence including the 5'UTR, (NCBI nucleotide database: NM_000539), as used in all clones tested in mammalian cell culture was the input sequence in the web-based 'RNAdraw' program, (Matzura and Anders Wennborg, 1996). The alternative sequences for replacement rhodopsin, HR5m and HR7m with changes between nucleotides 174–192 of the cDNA, (corresponding to nucleotides 270–288 of the mRNA), were used for structure comparisons. Default enthalpy and entropy settings for energy calculations at 37°C were used in all analyses.

4.2.2 *Generating pIRES-EGFP-HR5m*

The original replacement rhodopsin construct, termed pRz40M-2, was generated to evaluate protection from ribozyme-mediated suppression. This construct contains the human rhodopsin cDNA altered at 5 consecutive codons between bases 177 and 189 (the cDNA is denoted HR5m throughout this thesis) cloned in the pcDNA3.1 vector (Invitrogen). This plasmid was kindly donated by Dr. Mary O'Reilly. The 1.8kb HR5m sequence was excised from this vector using restriction digest sites XbaI and BamHI. This was cloned into the XbaI/BamHI digested pIRES2-EGFP vector (Clontech) to generate the pIRES-EGFP-HR5m construct illustrated in figure 4.3. Cloning was carried out as outlined in the general methods section 2.2.2.

4.2.3 *Generating pc3-HR7m (used to generate results section 4.3.6)*

The site-directed PCR mutagenesis strategy as outlined in section 2.2.1.6 was used to generate the human rhodopsin cDNA with 7 mutations. Base substitutions were incorporated into primers MP2f and MP1r (G to an A and G to a U; primer sequence in Appendix 1A) and were used to insert two mutations at nucleotides 192 and 195 of the HR5m sequence in the DNA template, pIRES-EGFP-HR5m. A forward primer, HRho+1f(NHE 1) at the beginning of the human rhodopsin cDNA and a reverse primer downstream at position 852, HRho852r(BST EII) flank the target site where the mutations are introduced by primers MP2f and MP1r. The amplification products P1, (primers MP2f with HRho852r) and P2, (primers HRho+1f with MP1R) were produced by PCR reactions catalysed by high fidelity Taq polymerase (Gibco Brl). P1 and P2 are subsequently combined as the template DNA for a third PCR reaction between flanking primers HRho+1f and HRho852r to generate P3. The

primers are designed across the restriction sites BST EII and NHE 1 in the NHR gene. Thus, cloning of the altered product back into the NHR backbone to generate the complete HR7m cDNA can be readily achieved. A BST EII enzyme digest, (50°C for two hours) and a NHE 1 enzyme digest, (37°C for two hours) was performed on the original human rhodopsin construct in the pcDNA3 vector. The digested vector backbone was then ligated with the mutated 852 bp HR7m fragment (also cut with the same enzymes) before cloning in an XL1B host as described in section 2.2.2.4. The final pcDNA3-HR7m product (fig. 4.4) was purified as described in 2.2.2.6.

All retinal explant techniques including the dissociation of cells, FACS, immunostaining and sectioning of explant tissue is described in chapter 2, section 2.2.6.

4.3 RESULTS

(In the following sections wild-type human rhodopsin cDNA is denoted HR, and replacement human rhodopsin with 5 and 7 changes are denoted HR5m and HR7m respectively).

4.3.1 Investigating the application of an allele-specific approach to suppression of human rhodopsin transcripts

Targeting the human rhodopsin SNP, rs7984, for suppression in cell culture:

Data from dbSNP (www.ncbi.nlm.nih.gov/dbSNP), was used to select possible sites in the human rhodopsin mRNA suitable for targeted allele-specific suppression. Only three SNPs with significant heterozygosity values were available in the mRNA, and of these only one occurred in available animal models (NHR, HR5m, wild-type C57 and wild-type L129) making it a suitable candidate for an *in vivo* assessment of allele-specific rhodopsin suppression (Table 4.1). The siRNA, siUTR, originally tested in chapter 3 for the possibility of silencing the untranslated regions of genes, spans the SNP, Rs7984. This siRNA failed to achieve any silencing of human rhodopsin cDNA in mammalian cell culture (section 3.3.1). An allele-specific approach may not be feasible at this time, without the identification of other polymorphisms in the coding sequence of human rhodopsin. Furthermore, the specificity of RNAi may not be sufficient in many cases to enable discrimination at the level of a single nucleotide. Nevertheless, the principle of using intragenic polymorphisms to discriminate between mutant and wild-type alleles of a gene remains an interesting one and hence may be pursued further in the future.

4.3.2 Structural analysis of replacement human rhodopsin cDNAs:

The HR, HR5m, and HR7m cDNA sequences were used as input in the web-based RNAdraw program (Matzura and Wennborg, 1996). This program calculates the most probable secondary transcript structure based on the lowest possible energy requirements for folding. The large loop 14 of the HR cDNA is split into three smaller loops, 14, 15, and 16 when the five sequence changes are introduced in HR5m. The predicted structure is again changed in the HR7m construct, when the target site becomes spread across small hairpin loops formed on the periphery of a much larger global loop in the cDNA (fig. 4.6).

4.3.3 The effect of synthesised dsRNAs, siB and siC, on expression of human rhodopsin from the pIRES-EGFP-HR5m construct.

The two dsRNA molecules identified in chapter 3 as potent suppressors of human rhodopsin (siB and siC) were evaluated to establish if codon-modified replacement genes evade suppression by siB and siC. The level of modified rhodopsin mRNA, HR5m, was determined by real-time rtPCR, following cotransfection of 20-pmol concentrations of the siB and siC siRNAs with pIRES-EGFP-HR5m (figs.4.7a and 4.7b) into COS7 cells. Transcript levels were compared to the level of transcript when pIRES-EGFP-HR5m was cotransfected with siNT siRNA (concentration, 20 pMol). Notably, in this experiment presynthesized siRNAs, (Qiagen), rather than plasmid driven siRNAs were used to avoid any possible artefact caused by different plasmids competing in cotransfections. The concentration of the HR5m plasmid was fixed at 400 ng for all cotransfections.

At 20 pmoles of siRNA, no suppression of the HR5m cDNA occurs over the averaged data from six quantitative rtPCR analyses for each siB and siC (two rtPCR runs on each RNA pool from three separate cotransfections of siRNA and pIRES-EGFP-HR5m) (fig. 4.7 a-b). However, significant error was recorded during the course of the siB analyses, this took the form of both up and down-regulation of the HR5m transcript compared to the siNT control (the Qiagen non-targeting siRNA as used in chapter 3 and listed in appendix 4A). The high error was not observed in previous real-time assays using the same controls (chapter 3). It was also not evident in the siC and HR5m cotransfections (where there are five mismatches preventing HR5m silencing). Overall, the results suggest that the modified HR5m gene used to generate the HR5m replacement rhodopsin mouse model (section 4.3.8) is resistant to silencing by siRNAs specific for the normal rhodopsin gene. The *p*-values for these experiments show that there was no statistical difference in RNA analysed from the siNT and siB or siC transfected cells (table 4.4a–4.4b). The possibility that the inconsistency may be specific to the siB sequence (which contains two nucleotide changes compared to the HR5m target) required further investigation. In chapter 3, it was demonstrated that both siRNAs, siB and siC, showed silencing activity in the form of short hairpin RNA (H1-shRNA) constructs when tested against the wild-type rhodopsin gene (section 3.3.2). Therefore, in the experiments undertaken in section

4.3.4, transcripts from the HR5m gene are examined for potential protection from suppression by shRNA incorporating the siB and siC sequences, i.e. H1-siB and H1-siC (as described in methods section 3.2.3).

4.3.4 Expression of human replacement rhodopsin cDNA in the pIRES-EGFP-HR5m construct, following treatment with the H1-siB or H1-siC dsRNA construct.

The averaged real time rtPCR results of expression values following six separate cotransfections of shRNA and HR5m in mammalian cell culture are presented in fig. 4.8. Specifically, the results from three transfections in COS 7 cells, two sets of transfections in HeLa cells and one transfection of HEK293 cells were averaged. Both target (pIRES-EGFP-HR5m) and shRNA plasmids were transfected at concentrations of 400 ng per 5×10^4 cells. (See chapter 2, section 2.2.5 for details of the transfection protocol).

When the replacement rhodopsin cDNA was cotransfected with H1-siB, (fig. 4.8) over 30% down-regulation of HR5m expression was estimated to occur. The difference in expression between H1-siNT and H1-siB cotransfected cells was found to be statistically significant when both GAPDH and NeoR primers were used in normalisation rtPCR reactions ($p=0.037$; $p=0.046$). Downregulation of gene expression is not entirely unexpected from a siRNA that covers only two nucleotide changes present in the replacement gene. When expressed as an H1-driven hairpin molecule the risk of off-target inhibition may possibly be increased. This may be because the shRNA may mimic the precursor microRNA hairpin molecule and may elicit silencing through non-specific binding with a mismatched target. Hence a dsRNA molecule that can suppress expression of the HR cDNA using a siRNA-based mechanism may still be capable of having a microRNA effect on the HR5m cDNA.

The dsRNA siC, however, covers all five sequence changes in the HR5m cDNA and, notably, also in the transgenic mouse replacement model (introduced in section 4.1.6). When cotransfected with the HR5m mismatched human rhodopsin target into mammalian cells, H1-siC, preserved almost equivalent levels of the target cDNA expression when compared to the H1-siNT control. The 10% loss of transcript estimated when data was normalised to GAPDH is within the error of the experiment ($p=0.24$), hence the difference in this case is not significant and transcript considered to be unaffected by the rhodopsin-targeting siRNA. The difference in rhodopsin

expression for H1-siC compared to H1-siNT is deemed to have reached statistical significance ($p=0.05$) when the RNA for the siC experiments is normalised for rhodopsin expression using the plasmid-derived NeoR gene. However, in this case the H1-siC cotransfected cells show slight increase in rhodopsin expression compared to H1-siNT. This upregulation is, however relatively slight (116%) and may possibly be attributable to variation in transfection efficiencies between experiments and the inherent error associated with real-time rtPCR experiments.

It is worth noting that the error associated with the experiments using the HR5m/H1-siNT sample was high and the significant variation between transfections must be taken into account in the interpretation of these results. This error value is unrelated to pipetting error as replicate samples showed similar values. Each sample did show variation in the level of expression from transfected plasmids between the three different cell lines transfected, HeLa, HEK293, and COS7 (similarly shown for the wild-type pIRES-EGFP-HR plasmid cotransfection with siRNA in section 3.3.2, fig. 3.13). However, in general, the real-time rtPCR data from experiments using either synthesized siC (fig. 4.7b) or H1-driven siC (fig. 4.8) suggest that this siRNA results in minimal suppression of expression of the modified replacement gene HR5m, as evaluated *in vitro* in cell culture.

The variation in the rhodopsin levels when treated with non-targeting shRNA has implications for this replacement study where an accurate measure of any potential targeting of the mismatched replacement gene is necessary. To further investigate the error associated with the HR5m/H1-siNT cotransfection, the expression level from an alternative non-targeting shRNA construct (H1-Col1A1) was analysed. H1-siNT is a scrambled sequence with little or no extended homology to sequences in the NCBI database (Qiagen, GmbH). As such it may not be capable of activating the RISC machinery, and may not be the ideal control for assessing the therapeutic potential of siRNA for gene inhibition. H1-siCol1A1, on the other hand, is specific to the collagen-1 gene and is a functional dsRNA molecule, (McMahon HP, personal communication), which can be used as an alternative non-targeting shRNA control. There was little overall difference calculated between the H1-siNT and H1-siCol1A1 cotransfections for the level of HR5m gene expression (normalised to GAPDH gene expression) (fig. 4.9). H1-siNT rhodopsin gene expression level and the associated error in treated cells are similar to that of H1-siCol1A1-treated cells.

As such it was decided to continue for the present time with Qiagen's siNT sequence expressed from the H1-promoter as the shRNA negative control.

Given that much of siC-based suppression of human rhodopsin gene expression is eliminated using the HR5m human rhodopsin gene with the 5-nucleotide modifications, siC was deemed a more suitable dsRNA molecule to carry forward for further development. A dose curve in fig. 4.10 shows the resistance of transcripts from HR5m cDNA to siC-based suppression. The results with the HR5m construct are compared to a dose-curve showing the effect of siC on the expression of wild-type HR cDNA. The error detected in normalised rhodopsin gene expression for the replacement rhodopsin gene is far greater than for the equivalent wild-type rhodopsin gene. However, the dose curve suggests that minimising the quantity of siRNA cotransfected with the target gene may maximise the potential of a suppression and replacement strategy using siRNA (for example <10-pmols appears to be optimal for maximum silencing of HR while preserving HR5m transcript levels). The study described in this chapter to date with the HR5m replacement rhodopsin gene has been focused on the evaluation of the resistance of this gene to RNAi-mediated suppression using RNA assays. Hence, the study was expanded to evaluate this feature of the HR5m construct using protein assays. Therefore in section 4.3.5, an assessment and comparison of wild type and replacement protein expression following cotransfection with siC was undertaken using ELISA.

4.3.5 Protein expression from HEK293 cells cotransfected with siRNA, (siNT and siC) and rhodopsin-expressing constructs, (HR and HR5m).

The chart of fig. 4.11 and the accompanying data, (table 4.6a), show the comparison of protein expression levels for wild-type (HR) and replacement rhodopsin (HR5m) from transfection of HEK293 cells. A Biorad assay was carried out on initial protein extractions to ensure equal amounts of total protein were used for each sample. (Further details of this assay can be found in chapter 2 methods, section 2.2.4). In chapter 3 for the wild-type human rhodopsin protein, approximately 65% suppression of HR rhodopsin protein was achieved by siC when compared to treatment with siNT (fig. 3.16). In a similar experiment to that outlined in chapter 3 (section 3.3.5), the Human-Anti-Mouse-Antibody (HAMA)-ELISA was used for quantitative determination of protein in culture medium. The peroxidase-conjugated anti-rhodopsin 4D2 antibody (Molday *et al.*, 1984) was employed in a bridging

technique, whereby the peroxidase reacts photometrically with the substrate tetramethylbenzidine (TMB, Roche).

1×10^6 HEK293 cells were cotransfected with 1.6 μg of the pIRES-EGFP-HR5m rhodopsin plasmid and a 20-nM concentration of siC or siNT dsRNA. Protein was extracted as described in the materials and methods chapter, section 2.2.4. The protein analysis was carried out twice on pooled protein extracted from two transfections of HEK cells. Approximately 35% suppression of HR5m is detected by the substrate reaction in this assay when protein from the HR5m/siC transfected cell pool is compared to protein from the HR5m/siNT cells. The level of suppression observed tends to drop as the protein sample becomes more dilute. This may be due to competing background effects as the highly sensitive peroxidase-mediated POD substrate reaction starts to take precedence over the primary and secondary antibody reaction.

It is possible that despite the presence of 5-nucleotide changes, the HR5m transcript is targeted by the siC siRNA albeit at a far reduced level to that of the wild-type transcript. The down-regulation of a message carrying as many as 5 sequence differences to a siRNA has similarly been reported (Raoul and Aebischer, 2004). In this case the five nucleotide changes engineered into wild-type SOD1 gene failed to confer complete resistance to silencing by a SOD1-specific shRNA for treatment of ALS. The risk of suppression of the HR5m gene warranted the construction and testing of a rhodopsin cDNA construct with an increased number of nucleotide changes, to examine whether this would enhance protection of a codon-modified rhodopsin cDNA that would remain capable of coding for wild-type rhodopsin protein.

4.3.6 Expression of human replacement rhodopsin cDNA from the pcHR7m construct, following treatment with the H1-si-C dsRNA construct.

To examine whether siC-based suppression of replacement transcripts could be completely abolished, a replacement rhodopsin cDNA termed HR7m was generated. The two further nucleotide modifications (as shown in table 4.2) were incorporated into the HR7m cDNA over the sequence targeted by siC. Subsequently, levels of expression of HR7m following cotransfection with siC and siNT were assessed by real-time rt-PCR.

A PCR site-directed mutagenesis strategy was used to incorporate two further base-pair changes into human rhodopsin cDNA. This technique is outlined in Methods section 2.2.1, and section 4.2.3, (for primer sequences see appendix 1A). To eliminate any possibility, however remote, that the EGFP portion of the original pIRES-EGFP-HR5m transcript was in some way affecting siRNA and target interaction, the mutated HR7m transcript was expressed from pcDNA3 vector (Invitrogen). (The suppression of the unmodified wild-type HR cDNA was also assessed when expressed from the pcDNA3 vector – data not shown. In this case no difference in expression levels was detected between the pIRES vector and the pcDNA3 vector systems following cotransfection with siRNA. It was therefore predicted that the presence of EGFP as part of the transcript from the expression vector, pIRES, should not alter suppression results. However, it remained of interest to evaluate expression of a replacement rhodopsin transcript, in this case HR7m, when this modified rhodopsin cDNA is transcribed without an EGFP tag following cotransfection with siRNA).

A relatively minor percentage suppression of the HR7m target was observed - approximately a 12% drop in relative levels of HR7m transcript was estimated by real-time rtPCR analysis in the siC-treated sample. However, this analysis was performed only twice and therefore the results serve as a preliminary data-set suggesting the use of a cDNA with 7 nucleotide changes as the replacement transcript (fig. 4.12). Additionally, the human replacement rhodopsin transgenic mouse currently available, as stated previously, carries 5 nucleotide changes around the target site for siC (the same nucleotide changes as for the HR5m cDNA tested in section 4.3.3–5). A key aim of the work described in this thesis is the validation of a replacement strategy combined with RNAi targeting a human rhodopsin transgene. It was decided that for the purposes of completion of this chapter, it would be valuable to utilise the HR5m replacement mouse model to explore sequence-specific protection from siC-suppression in the target tissue, the retina. In addition, the potential value of the HR7m rhodopsin replacement gene as a means to avoid siC-based suppression of rhodopsin gene expression was acknowledged and further work-up of the HR7m gene study may be undertaken in the future.

4.3.7 Retinal explants in the replacement rhodopsin study:

The drop in HR5m protein levels obtained when HR5m is cotransfected with siC in HEK293 cells, is estimated at 37% (by ELISA, table 4.6b); the latter number is however based on protein samples pooled from 3 separate cotransfections of cells. Even given that the 37% suppression of the replacement gene in cell culture system is indeed accurate (which, given the inherent error of the cell transfections, it may not be), this suppression may not translate through to equivalent levels of suppression *in vivo*. Hence, to further explore siRNA-based suppression of expression of the HR5m gene, a retinal explant approach was adopted. This system more closely resembles the target retinal tissue; results obtained from this work are detailed below.

4.3.8 Level of expression of human replacement rhodopsin from explanted tissue of HR5m+/- (MouRho-/-) transgenic mice, following electroporation with pEGFP-si-C:

Methods of gene delivery to the photoreceptor cells of an adult live animal are not yet fully developed for plasmid DNA. However, electroporation of plasmid vectors into the neonatal mouse retina may enable retinal expression of shRNA, and has been optimised by Matsuda and Cepko (2003). To evaluate the potential of siC to suppress the target gene rhodopsin, this technique has been combined with the culturing of retinal explants, (optimised by my colleague Dr. Marius Ader), to achieve delivery of pEGFP-siRNA plasmids (fig. 4.13 and fig. 4.15) to the photoreceptor cells. The aim of this work is to explore the siRNA-based suppression of expression of the rhodopsin gene in a system, which more closely resembles the target tissue (hence the use of retinal explants). While the work described in chapter 3 was concerned with suppression of the wild-type rhodopsin gene by siC, the explant study described in this chapter is focused on exploration of the resistance of transcripts from a replacement rhodopsin transgene to siRNA-based suppression subsequent to administration of shRNA constructs via electroporation. The animal model HR5m, described in section 4.1.6, was used to test the resistance of this transgene to siC-induced suppression *in vivo*.

4.3.8.1 Animal Matings:

The pups in this experiment were derived from a cross between two transgenic mouse lines, one carrying a disruption of the mouse rhodopsin gene and the other engineered to express the human rhodopsin replacement gene, HR5m. The following mating was set up:

$$\text{MouRho-/-} \times \text{HR5m+/-}(\text{MouRho-/-})$$

This breeding ensured that the number of copies of the HR5m transgene is kept constant in all tested animals, with only one available copy in each breeding pair. Following electroporation of constructs into P0 or P1 newborn animals, and culturing for two weeks in collaboration with Dr. Ader, (as described in chapter 3 and methods section 2.2.6), the harvested retinas were analysed in the three following ways: 1) for RNA analysis, the dissociated cells were FACS-sorted and RNA from cells positive for fluorescence (and therefore successfully transfected) analysed by real-time rt-PCR; 2) for protein analysis, the dissociated cells were immunostained using alexa-fluor-labelling of the rhodopsin antibody 4D2; 3) for analysis of overall morphology the intact explant was fixed, cryosectioned and immunostained. In summary, the experimental approach requires the use of many novel technologies, including retinal explant culture, electroporation, FACS analysis, RNAi and real-time rtPCR.

4.3.8.2 FACS Analysis:

The explanted retina is typically composed of 60–70% photoreceptor cells with the remainder of cells being Müller glia cells and other peripheral cell types. The rhodopsin transgene present in the retinal explant is the mutagenised HR5m form coding for wild-type human rhodopsin protein and, as all matings were carried out on an endogenous rhodopsin knockout background, no mouse rhodopsin protein should be present (Humphries *et al.*, 1997). On average 15–20% of cells are electroporated, therefore to ensure that any rhodopsin suppression present can in fact be observed, it is necessary to select for transfected photoreceptors by fluorescence (facilitated by the EGFP gene on the plasmid) as well as by size fractionation (distinguishing photoreceptor cells from RPE segments or glial cells) (fig. 4.16). FACS analysis revealed that in this case approximately 10–15% of the photoreceptor cells were electroporated with the shRNA plasmid (pEGFP-siNT and pEGFP-siC). This FACS sorted cell population was subsequently utilised to evaluate the effect of siRNA on replacement rhodopsin transcript levels.

4.3.8.3 Real-time rtPCR analysis on RNA from sorted cells:

The RNA data for fig. 4.17 was gathered from a pooled sample of 6 retinas for each of the control and targeted electroporations. The RNA was extracted from FACS sorted EGFP fluorescing cells (using the protocol described in section 2.2.6) and analysed in two separate quantitative rt-PCR runs. The chart shows the ratios of rhodopsin expression to mouse 18S rRNA expression in the retinal RNA taken from explants electroporated with either pEGFP-siNT (non-targeting control), or pEGFP-siC. There was approximately 30% loss of rhodopsin message in the pEGFP-siC treated retinas compared to those treated with pEGFP-siNT. Notably, a few housekeeping genes were assessed for suitable reference expression levels from mouse retinal RNA of the replacement model. The genes assessed were mouse GAPDH, mouse β -actin, mouse peripherin, and mouse ribosomal RNA, (18s rRNA). In this case it was found that the 18s rRNA gene offered the highest level of expression and as the RNA yield was relatively low, it was decided to use this high-expressing gene to normalise the data from the HR5m mouse.

The suppression obtained in the study described above is in excess of what was expected for the replacement rhodopsin transgene in the retinal explant, furthermore, it contradicts the RNA cell culture data, which suggests complete preservation of the HR5m transgene is possible (fig. 4.8). It is worth highlighting that the final sample yield following genotyping, sorting, and optimisation of rtPCR steps was relatively low. Therefore, the analysis of the RNA was repeated no more than twice before evidence of RNA degradation became apparent in the sample (the 18s rRNA signal started appearing later; at cycle 14 in subsequent runs as opposed to cycle 5 as expected). Moreover, the result is not found to be statistically significant with a high error recorded between real-time rtPCR runs (generating a *p*-value of 0.6). However we can compare this initial result to the data gathered in chapter 3 (section 3.3.9) for siRNA-based suppression rhodopsin in retinal explants from the NHR (Normal Human Rhodopsin) mouse. In summary, the level of 69% suppression of the NHR gene was repeated three times in retinal cells pooled from three animals. Further analysis of retinal explants from the HR5m mouse is necessary to achieve the same level of statistical significance in the HR5m mouse as that obtained with retinal explants from the NHR mouse and thus determine if some residual siC-based suppression continues to be evident at the RNA level.

However, this initial result led to a further analysis of the knockout rhodopsin background used for this retinal explant experiment and for the explant experiments of chapter 3. The matings were set up with the Rho^{-/-} animal as illustrated in fig. 3.9 and 4.14. This mouse model carries a targeted insertion of the neomycin resistance gene in the second exon of the rhodopsin gene (Humphries *et al.*, 1997). Real time rtPCR analysis using rhodopsin primers on RNA extracted from the Rho^{-/-} mouse revealed that the endogenous rhodopsin gene is capable of producing transcript. As the Rho^{-/-} mice fail to elaborate rod outer segments, and as anti-Rho immunostaining of dissociated cells from this mouse show that the transcripts do not get translated (results not shown), the knockout animal may be more suited for deriving protein data. However the presence of possibly unstable and truncated endogenous rhodopsin transcripts in the MouRho^{-/-} mouse has implications for the RNA data derived from explant studies carried out to date, as illustrated in fig. 4.19.

4.3.9 Cell count of dissociated cells (20X magnification), from explanted tissue of HR5m+/- (MouRho^{-/-}) transgenic mice, following electroporation with pEGFP-si-NT/C:

As an alternative to evaluation of H1-siC-based suppression in retinal explants using RNA assays, dissociated cells from electroporated retinal explants may be immunostained to assay for protein (cells positive for rhodopsin) and EGFP (positive for electroporation of siRNA plasmid) expression. In this assay, cells from four retinal explants were dissociated, immunostained and counted for each pEGFP-H1siNT and pEGFP-H1siC electroporations. As with the previous experiment, retinas were removed for culturing as explants from HR5m+/- (rho^{-/-}) mice at p0 and electroporated with a 1 µg/µl of pEGFP-H1siRNA DNA solution. The explants were dissociated into single cells by trypsin-mediated digestion and immunostained for rhodopsin protein levels. A 1:150 dilution of rho4D2 (Molday RS, 1984) was used for immunocytochemistry and the primary 4D2 mouse antibody visualised using Alexa fluor 568 nm goat anti-mouse secondary antibody (Molecular Probes) as described in methods section 2.2.6.8. Overall fewer pEGFP-siC cells were available for counting than pEGFP-siNT cells, as the electroporation efficiency for the pEGFP-siC plasmid in this case was lower than for pEGFP-siNT. While 86% of replacement cells receiving pEGFP-H1siNT still showed rhodopsin staining, 52.2% of replacement cells receiving pEGFP-H1siC still showed rhodopsin expression. This suggests that

there was approximately a 30% decrease in HR5m replacement rhodopsin protein levels in photoreceptor cells transfected with the siC rhodopsin-specific siRNA. However, if you account for the cells of possible müller glial origin, that do not stain in the siNT-treated HR5m explant, (thus setting the rhodopsin expression value in this control explant at 100%) the result for suppression at the protein level may be closer to 40%.

While it is somewhat surprising that some residual suppression of transcripts from a replacement gene with five nucleotide changes to the target site for siC may occur, the result may possibly be interpreted in the context of overall rhodopsin expression between the HR5m retinas evaluated in the current study and NHR retinas evaluated in chapter 3, section 3.3.7. For example, it may be that the HR5m retina has significantly lower levels of expression of rhodopsin protein, when compared to the NHR retina. While ERG and histological analysis of the HR5m model showed photoreceptor function and outer segment morphology to be similar to wild-type mice (figs. 4.2a and 4.2b), the overall transcript level between NHR and HR5m animals may be significantly different and may account, at least in part, for the resulting suppression observed in the study (the results of which are shown in table 4.8). Therefore rhodopsin levels were compared in retinal RNA extracted from three NHR and three HR5m animals at 3 months of age (both sets of animals were bred on an endogenous mouse rhodopsin knock-out background, as was also the case for the retinal explants). The transcript levels of rhodopsin were found to be significantly reduced in the HR5m mouse compared to the NHR mouse model (fig. 4.18). The HR5m model was estimated to express approximately half the level of rhodopsin transcript expressed in the NHR mouse model. This result is further discussed in section 4.4. However it highlights the need for a quantitative assessment at the protein level in these models.

As an alternative to quantitative protein assessment, immunostaining of retinal vibratome sections from the HR5m animal model was carried out (fig 4.20). Localised regions of co-expression of rhodopsin and the pEGFP-siC construct can be observed in the site identified as 'A' in figure 4.20. However, this type of analysis provides only limited qualitative data that may be suggestive that protection from silencing can be achieved. A quantitative ELISA to determine the relative rhodopsin protein expression levels between the NHR and HR5m animal models treated with pEGFP-siC may resolve the question of suppression and replacement of protein by

siRNA targeted to loop-14 region of rhodopsin. However, electroporated explant cells may not yield sufficient protein to undertake such ELISAs. One alternative would be to achieve efficient delivery *in vivo* to quantify the rhodopsin protein, and to this end, developments in *in vivo* delivery techniques are considered in chapter 6.

4.4 DISCUSSION

4.4.1 Allele-specific silencing

Web-based searches for single nucleotide polymorphisms of human rhodopsin revealed only one SNP, (rs7984 from dbSNP), with sufficiently high heterozygosity to warrant investigation for an allele-specific RNAi-based silencing approach. However this SNP is located in the 5'UTR of the rhodopsin gene, a region typically found to be resistant to suppression, at least using an RNAi approach. No suppression was achieved using an siRNA, siUTR (as shown in chapter 3, fig. 3.11; siRNA sequences listed in 4A) in the region of this SNP. Investigation of RNAi-based tools up- or down-stream of siUTR, (incorporating the SNP target site) may be carried out at a future date to examine whether the site can be used to selectively silence mutant alleles of rhodopsin. However, silencing of this region may be difficult to achieve. A ribozyme-based suppression strategy, (as outlined for human *rds*/peripherin gene in chapter 5), may possibly be more appropriate when targeting this SNP of the human rhodopsin gene.

As stated above, it is notable that few polymorphisms have been identified in the rhodopsin cDNA sequence to date (table 4.1). Hence, the main body of research described in this chapter is concerned with the further development of a siRNA-based suppression and replacement strategy, rather than with the use of intragenic polymorphisms in the development of an allele-specific therapeutic approach for rhodopsin-linked RP.

4.4.2 Analysis of siRNA targeting the replacement rhodopsin gene in mammalian cells

In this study the neomycin-resistance, (neoR) gene on the target plasmid and the endogenous housekeeping gene, GAPDH, were used to normalise levels of rhodopsin gene expression. By quantifying expression relative to the neoR gene on the target plasmid, differences in transfection efficiencies may be accounted for, as well as accounting for the ability of the cellular machinery to transcribe from an episomal plasmid.

4.4.2.1 The effect of the siB/C siRNAs on expression of the replacement rhodopsin gene, HR5m:

Cotransfection of siRNA with the HR5m replacement mismatched rhodopsin target cDNA was undertaken in mammalian cell culture. The results obtained from transfections of three mammalian cell lines were averaged to provide an estimate of levels of rhodopsin expression (fig. 4.7 and 4.8). As presynthesized siRNA molecules, both siB and siC do not cause suppression of the HR5m gene (fig. 4.7a and 4.7b). However, the data suggests that when stably expressed as a hairpin construct, siB (covering two changes in the replacement gene) does not provide sequence specificity and results in approximately 30% suppression of transcripts from the HR5m cDNA (averaged data from the normalisation of rhodopsin to GAPDH and NeoR house-keeping genes – fig. 4.8). In contrast, cotransfection of H1-siC, covering all 5 changes in the rhodopsin replacement) with the HR5m replacement gene does not cause suppression of the replacement (fig. 4.8, table 4.5). The degree of complementarity between a dsRNA and its target probably determines the specific mode of post-transcriptional repression to be carried out by the cell. Full complementarity, (HR/siB and HR/siC figures 3.11 and 3.12) results in cleavage of the mRNA at a single phosphodiester bond located near the centre of the sequence relative to the complementary siRNA. The resulting cleavage products will be rapidly destroyed by endonucleases. Where complementarity is not complete, in particular where a mismatch is located near the centre of the siRNA:mRNA helix (as for HR5m/siB), microRNA-induced translational repression may result in off-target effects (further investigated in chapter five).

In summary, the HR5m mRNA appears to be resistant to cleavage by both the plasmid-derived and the presynthesised forms of the siRNA siC. Therefore, siC was carried forward for further analysis in this suppression and replacement study.

4.4.2.2 ELISA on protein from cotransfection of siC and HR5m in mammalian cell culture:

The siC siRNA made use of all five nucleotide changes in the HR5m cDNA (moreover the mouse replacement transgene model, HR5m, was generated using the same HR5m cDNA). Therefore, it was decided to further assess the activity of this suppressor by evaluating rhodopsin protein levels subsequent to cotransfection of HR5m and siC in HEK 293 cells.

Over 30% down-regulation of the HR5m protein was observed using an ELISA to analyse protein extracted from cells transfected with siC and compared to cells transfected with siNT (fig. 4.11). Possible interpretations of this result include:

i) The siC molecule is not highly specific and can target HR5m as well as HR cDNA thus resulting in the downstream reduction in HR5m protein. Studies to date from other groups, have suggested otherwise with the high level of siRNA specificity making it an attractive tool for studies of gene function as well as therapeutic suppression. However, it remains possible that mRNAs may show different levels of accessibility to RNAi, with the number of changes that can be accommodated before suppression is eliminated varying from one to five and possibly beyond (Lin *et al*, 2005); Jackson *et al*. have reported knockdown of a message with as few as 11 bases matching the siRNA (, 2003). Similarly, Lin *et al*. recently reported off-target gene silencing by a siRNA with no more than 7 nucleotide complementation to the downregulated messages (Lin X, 2005). However, it is notable that in these studies the regions of complementarity were contiguous. In contrast, in the HR5m modified gene the mismatches are spaced at three nucleotide intervals from each other making it somewhat unlikely, in principle, that stable base-pairing will be achieved between the siC and HR5m message.

ii) Although 30% suppression of the replacement gene (HR5m) represents a considerable reduction in expression, it may yet be an artefact of transfection in a cell culture system and variation in transfection efficiencies in addition to inherent experimental error associated with real time rtPCR, may account, in part, for the expression differences.

iii) The non-targeting control siRNA, H1-siNT may downregulate house-keepin gene expression levels to a greater degree than H1-siC, such that rhodopsin levels appear artificially reduced in the siC cotransfection. In this case a functional non-targeting siRNA that does target another gene in the system and thus is guaranteed to effectively activate the RISC complex may be more suitable. The construct H1-siCol1A1, (a functional silencing construct which does not, however have any targets in the COS7 cells) was employed to test the siNT control used in this study thus far. There was no difference in expression ratios of rhodopsin/GAPDH for two non-targeting siRNA constructs, H1-siNT and H1-siCol1A1, when the RNA from cotransfection of either construct with HR5m was analysed in COS7 cells (fig. 4.9).

Two approaches were used to investigate whether the factors (i) or (ii) above may be resulting in the *in vitro* suppression observed. To assess the significance of sequence-specificity to the silencing detected thus far at the protein level in transformed cells, two additional nucleotide changes were added to the HR5m construct to generate HR7m. In order to examine whether knockdown of HR5m protein levels would persist when the HR5m target is endogenously expressed in the cell, retinal explants were adopted as an experimental system to assess siC activity.

4.4.2.3 siC and HR7m:

In general, RNAi molecules are thought to better tolerate a mismatch at the 5'-end of the RNAi molecule, while the central positions, between bases 9 and 11, and the 3'-end exhibit lower tolerance (Haley B, 2004). In principle, it is therefore unexpected that siC-induced suppression of the replacement rhodopsin gene should occur, although preliminary results from studies using the HR5m cDNA suggested that some residual suppression might be occurring (fig. 4.11). However, to investigate whether suppression can be completely eliminated the rhodopsin gene was further modified. The addition of two extra changes to the HR5m cDNA in the region targeted by siC, thus generating HR7m should, in principle, add to sequence-specific resistance of transcripts from the replacement gene to siC-based suppression. Initial results suggest that the protection of the HR gene from silencing by siC may be achieved when 7-nucleotide changes are incorporated into the targeted region compared to 5 changes (fig. 4.12). It is notable that an HR7m construct carrying both the suppressor, siC, with the replacement rhodopsin gene containing seven base-pair changes is being developed for its potential value in future siRNA-mediated suppression and replacement studies. This construct is discussed in chapter 7.

However, as the replacement rhodopsin transgenic mouse model currently available in the lab, carries the 5-nucleotide changes present in HR5m, it was decided to continue with an analysis of the protection of the modified HR5m replacement gene from siC-based suppression using this transgenic mouse model. In essence, using the retinal tissues from the HR5m mouse, resistance of HR5m transcripts to siC-based suppression could be investigated in the target tissue, the retina, as opposed to a culture system, which does not accurately represent the tissue of interest.

4.4.3 Explant data from the replacement rhodopsin animal model

In order to confirm initial results, that the HR5m replacement gene is resistant to siC-mediated cleavage at the RNA level (see fig. 4.7 and 4.8), further investigation was undertaken in a more biologically relevant system, that is, using retinal explants. We chose explanted tissues from one-day-old transgenic mouse lines with appropriate genotypes (NHR and HR5m) as suitable models in which to test the potential therapeutic value of a suppression and replacement strategy – i.e. utilising siRNA for suppression and the degeneracy of the genetic code to achieve protection of the replacement gene from suppression. Transgenic animals provide a valuable source of genetically altered cells for such organotypic cultures. (Explant retinal cultures are further discussed as suitable model systems for these investigations in the context of a degenerating retina in chapter 5, section 5.4.2).

The time-frame chosen for electroporation of retinal explants was day 1 post-natally as, at this early stage, progenitor photoreceptor cells have been shown to be more accessible by electroporation (Cepko *et al.*, 2004). FACS analysis was undertaken from 2-week-old retinal explants and revealed that, subsequent to selection for cell size, granularity and fluorescence, approximately 15% of the cells were transfected (fig. 4.16). In the subsequent RNA analysis, approximately 30% down-regulation of endogenous HR5m mRNA was estimated in the siC-electroporated retinas compared to siNT-electroporated retinas from HR5m+/- (MouRho-/-) mice (figure 4.17). However further assessment of the mating set up in fig. 4.14 has led to a re-analysis of the knockout mouse rhodopsin background of the mice used to produce the litters for this explant study. The disruption of the endogenous rhodopsin gene in the Rho-/- mice was achieved by insertion of the neomycin resistance gene into exon 2 of the mouse rhodopsin gene (Humphries *et al.*, 1997). While a complete knockdown of rhodopsin protein is evident in the Rho-/- animals, the rhodopsin transcript appears to be produced in spite of the targeted disruption of this gene (as assessed by real time rtPCR analysis on RNA from the Rho-/- mouse). Even in an unstable truncated form, this transcript (prior endonuclease-mediated destruction) may be detected by the real-time rtPCR assay used in section 4.3.8. As such, in the HR5m transgenic mouse on a rho-/- background, both HR5m and the mouse rhodopsin transcripts are present and represent potential targets for siC-mediated silencing in the explants tested in this section. The residual suppression observed for the HR5m gene in retinal explants

may therefore be as a result of down-regulation of transcripts expressed from the disrupted mouse rhodopsin gene.

The normal human rhodopsin (NHR) in the NHR mouse used in matings of chapter 3, (3.13), was determined to be expressed at equivalent levels to mouse rhodopsin in wild-type animal (Dryja *et al.*, 1992). Notably, for chapter 3, there is one base-pair difference between the human rhodopsin and mouse rhodopsin genes in the region targeted by siC. The transcripts from the disrupted mouse gene for the explant study of chapter 3 may therefore be less optimal targets for siC-mediated cleavage than transcripts from the NHR gene (fully complementary to siC sequence) but nevertheless may be suppressed in part by siC. Indeed given that transcripts from the modified replacement gene (HR5m) have five mismatches with siC, mouse rhodopsin transcripts are expected to be far more susceptible to siC-mediated cleavage than transcripts from the modified form of the rhodopsin gene. In this case siC is more capable of targeting a transcript with one base-pair difference (MouRho) than of targeting transcripts with 5 base-pair differences to siC-sequence (HR5m). Notably, suppression of mouse rhodopsin transcripts may in part account for the residual suppression of the replacement rhodopsin transcripts observed in cells from retinal explants from HR5m mice. The potential effects of such suppression dynamics on the RNA data derived from the explant experiments are considered in fig. 4.19. The sensitivity of the sybr-green-based assays being used may complicate the possibility of fully validating a useful set of human-rhodopsin specific primers to obtain accurate real time rtPCR data on the HR5m+/--(mouRho-/-) model. Therefore, future retinal explant experiments using the knockout mouse rhodopsin background may preferably be undertaken using protein analysis to simplify the subsequent analysis of suppression and replacement of transgenes in the retina.

For the protein analysis, each retina was analysed separately. With no more than approximately 10-15% electroporation efficiency possible in these explant systems, the FACS would likely not have yielded enough surviving cells for protein immunostaining. Therefore retinal explants used for analysis of protein expression were not FACS sorted. Instead rhodopsin levels were assessed by immunostaining for rhodopsin protein from the replacement rhodopsin gene in transfected as well as untransfected retinal explant cells. The cryosection of the HR5m explant treated with pEGFP-siC did show preservation of a rhodopsin-specific signal picked up with alexa

fluor label at 495nm (fig. 4.20). However, overall cell counts (table 4.8) suggested that suppression may be occurring in the retinal explants from the HR5m animal model electroporated with siC compared to electroporation with siNT. Approximately 30–40% decrease was determined from these cell counts (table 4.8). However the number of cells in this immunostained sample is low and a larger sample is required to obtain a sufficiently robust result.

One possible explanation for the residual suppression observed at the protein level in this initial study involving siC-treatment of HR5m retinal explants, might be the relative level of rhodopsin transgene expression in the HR5m replacement animal model compared to that in the NHR animal model. For example, if the level of rhodopsin expressed in retinas of HR5m mice was significantly lower than that expressed in NHR mice, discrepancies may result when comparing changes in relative protein levels. A minor amount of suppression of the replacement rhodopsin transgene may be sufficient to prevent the alexafluor-labelled-antibody from detecting the protein. By contrast, a far greater level of suppression may be necessary to prevent alexafluor-staining of rhodopsin in retinal cells from NHR mice (analysed in chapter 3, section 3.3.9), if these mice express greater levels of rhodopsin in the first place. If the rhodopsin protein levels expressed in these two transgenic animal models are significantly different, a comparable assessment of the rhodopsin protein levels subsequent to treatment with siC between the two models may not be possible by immunostaining. In this regard it is notable that approximately a 43% difference was estimated in rhodopsin transcript levels of retinal RNA taken from age-matched NHR and HR5m mice (fig. 4.18) and normalised to the 18srRNA gene. Three mice of each NHR and HR5m genotypes were sacrificed at 3-months of age and the RNA analysed by real-time rtPCR. It would seem from this initial result that the HR5m mice express lower levels of rhodopsin than NHR mice. Therefore differences in the relative rhodopsin transgene levels in the respective animal models may account at least in part for the apparent residual suppression estimated using protein assays and presented in section 4.3.9.

4.4.4 Suppression and replacement analysis of the human rhodopsin gene to date

For RNAi to be harnessed as a useful therapeutic tool, it is critical that siRNA-mediated transcriptional silencing be specific. The *ex vivo* data we have accumulated

suggests that the modified rhodopsin gene is at least partially protected from siC-targeted suppression in the cells of the replacement animal model. It is as yet not clear whether minimal suppression of the rhodopsin expression persists and whether this would have any detrimental effects. Down-regulation of mutant rhodopsin transcripts must be sufficient such that the ADRP phenotype is rescued or at least the progression of the disease is slowed down. In parallel, a replacement rhodopsin transcript should be protected from RNAi suppression to a degree that will result in the supply of sufficient functional rhodopsin protein and thereby avoid degeneration. Detailed investigation of the suppression and replacement elements of the therapeutic this issue will likely from here on require further *in vivo* analysis.

Gene delivery is the common bottleneck in retinal gene therapy and indeed more generally in the development of many gene-based medicines. In this regard, viral and non-viral methods of transfecting the photoreceptor cells for delivery of siRNA have been investigated and associated results are described in chapter 6. Prior to a description of a study on methods of delivery (chapter 6), siC-based suppression of rhodopsin was investigated in a disease model of ADRP, the pro23his mouse (chapter 5). In addition, the possibility of using microRNA-based tools to enhance the downregulation of human rhodopsin was examined.

4. FIGURES and TABLES

Single nucleotide polymorphisms as targets for achieving suppression of gene expression from the rhodopsin gene

SNP	Nucleotide of NHR mRNA (NM_00539)	Nucleotide of NHR genomic DNA (HSU49742)	Heterozygosity (2pq)
Rs7984	70	269	A: 0.412 G: 0.518
Rs2855558	2054	6190	A: 0.425 G: 0.575
Rs2269736	45	244	G: 0.332 A: 0.668

Table 4.1:

Table showing single nucleotide polymorphisms (SNPs) in the mRNA of human rhodopsin selected from dbSNP of the NCBI database. Only those SNPs with high heterozygosity level (i.e. with significant chance of being prevalent in the population) were investigated. Primers (1A) flanking the SNPs were used to sequence DNA taken from the NHR and HR5m transgenic mouse models, as well as DNA from wild-type mice of C57 and L129 genetic backgrounds. SNPs identified to date as having high heterozygosity levels in the human rhodopsin gene are outside of coding regions, and clustered in introns and UTRs. The siRNA, siUTR, originally tested in chapter 3 for the possibility of silencing the untranslated regions of genes, spans the SNP, Rs7984. This siRNA failed to achieve any silencing of human rhodopsin cDNA in mammalian cell culture (3.3.1). Therefore the targeting of known rhodopsin-SNPs for siRNA-mediated suppression was not at this time considered feasible. Altering the degenerate base-pairs in the coding sequence of human rhodopsin was therefore considered the most suitable method of applying a siRNA-based suppression and replacement therapeutic approach (outlined in section 1.1.5) to rhodopsin-linked ADRP.

Replacement Rhodopsin mouse model: replacement changes designed based on the degeneracy of the genetic code

		nt 177						nt 189				
Amino acids			L	Y	V	T	V					
(a) Wild type (NHR)	CTC	ACG	CTC	TAC	GTC	ACC	GTC	CAG				
(b) Replacement (HR5m)	CTC	ACG	<u>CTG</u>	<u>TAT</u>	<u>GTG</u>	<u>ACG</u>	<u>GTG</u>	CAG				
Amino acids			L	Y	V	T	V					

Fig. 4.1:

Figure showing the preservation of amino acid sequence in the replacement human rhodopsin gene, following alteration of third base pairs in five consecutive codons. (a) The wild-type human rhodopsin sequence (NHR); (b) The replacement rhodopsin sequence (HR5m). A replacement rhodopsin transgene incorporating the altered sequence shown in (b) was used to generate the HR5m mouse model on a Rho^{-/-} background (described in section 4.1.6). A retinal section from this animal is shown in fig. 4.2a and an electroretinogram of retinal function from the same animal is shown in fig. 4.2b.

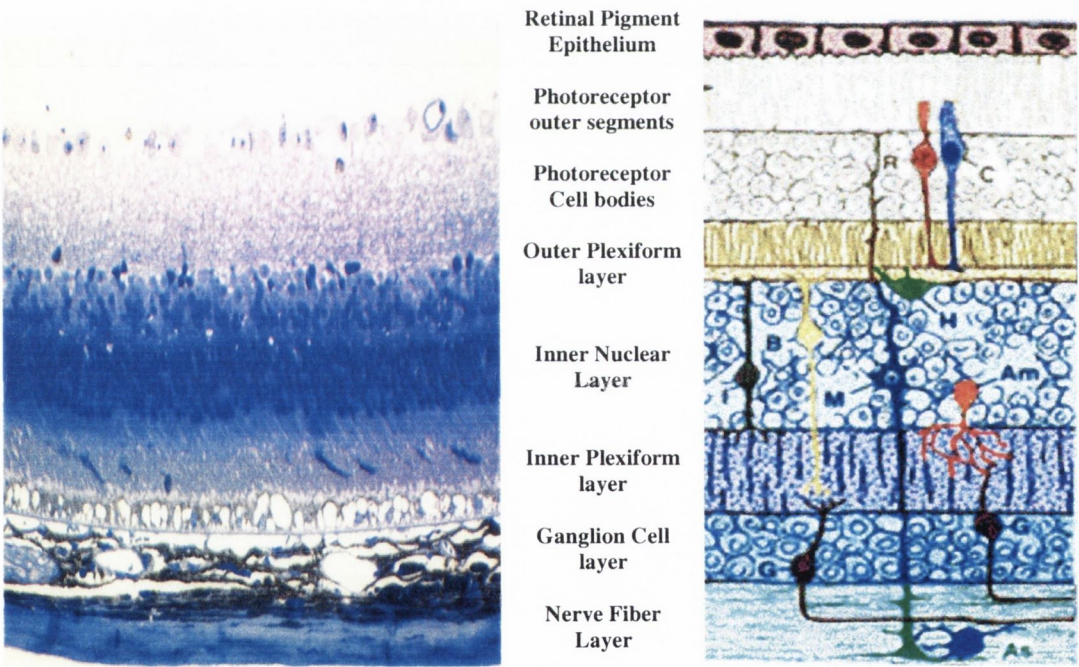
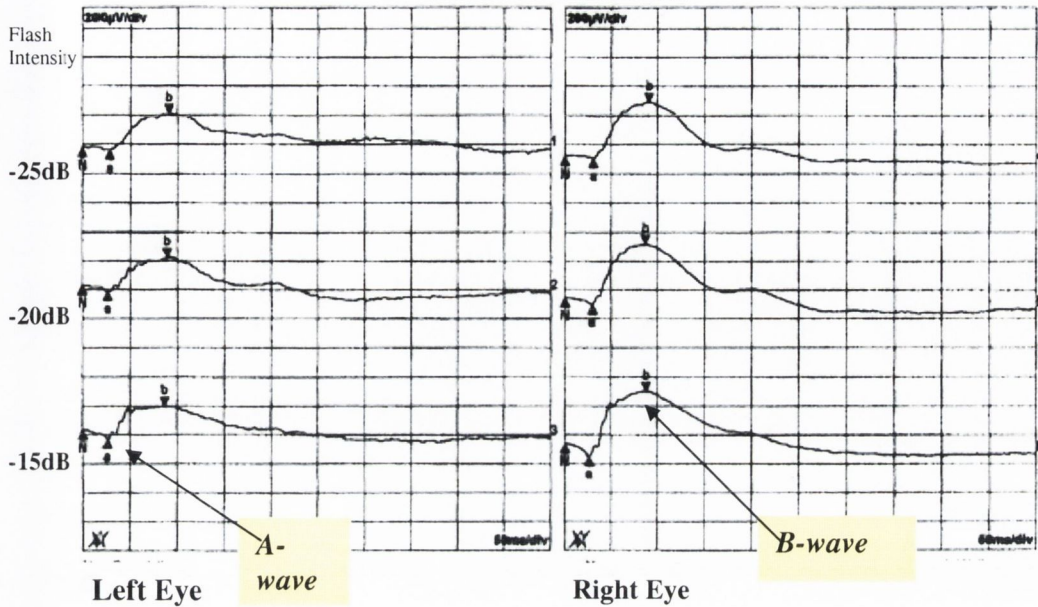


Fig. 4.2a: The left panel of this figure shows the resin-embedded section of retina from a 4-month-old mouse. This mouse carries a modified human rhodopsin transgene on an endogenous mouse rhodopsin knockout background (HR5m^{+/-(MouRho^{-/-})}). The retinal layers are juxtaposed with the illustration on the right panel of the figure. H: horizontal cell, Am: amacrine cell, M: Muller glial cell, B: Bipolar cell, R and C: Rod photoreceptor cells (Sections courtesy of Dr. M. O'Reilly).

**Dark-Adapted ERG corresponding to the
above retinal section**

ERG -25db-15dB erste Gruppe



(ERG data courtesy of Dr. PF Kenna)

Fig. 4.2b:

Electroretinogram (ERG) read-out from a HR5m+/--(MouRho-/-) (human rhodopsin replacement transgene on a mouse rhodopsin knockout background) mouse at 4 months of age. The corresponding retinal section for this animal is shown in fig. 4.2a. Functional rhodopsin expression in the replacement animal is shown by measuring photoreceptor responses to flashes of bright light. The normal B-wave amplitude and timing in fig. 4.2b suggests this HR5m(MouRho-/-) animal has functional retinal tissue at 4-months as is also suggested by the retinal section of fig. 4.2a.

Constructs generated for chapter 4

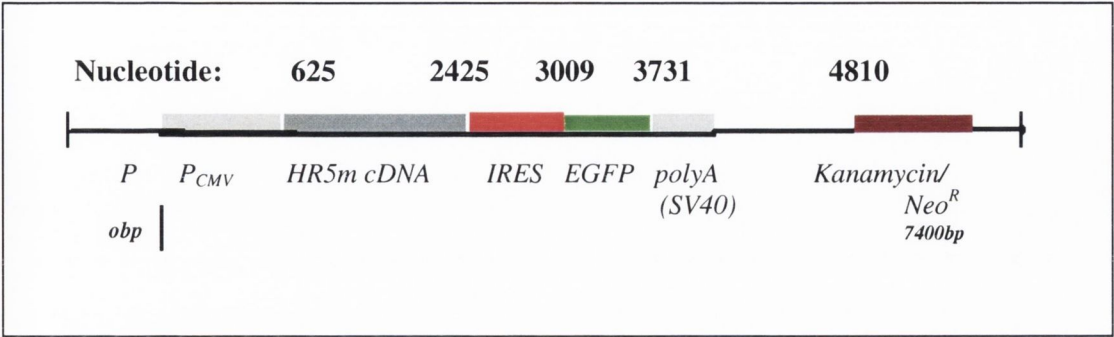


Fig. 4.3:

Diagrammatic representation of the pIRES-EGFP-HR5m construct. This construct was used for *in vitro* experiments investigating the resistance to siRNA-mediated silencing achieved by 5 nucleotide changes over the siRNA-target site. *P_{CMV}*: Cytomegalovirus promoter; *IRES*: Internal Ribosome Entry Site; *EGFP*: Enhanced Green Fluorescent Protein; *Kan/ Neo^R*: Kanamycin and Neomycin resistance gene; *HR5m*: Human rhodopsin cDNA with 5 nucleotide changes.

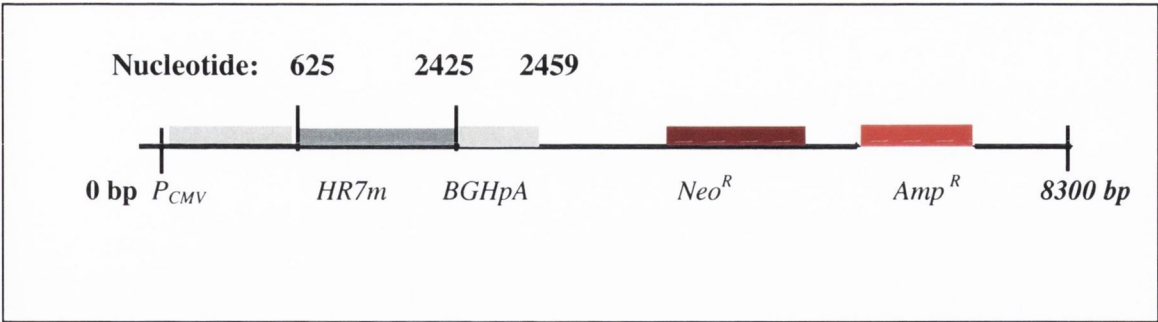


Fig. 4.4:

Diagrammatic representation of pcDNA3-HR7m construct. The human rhodopsin replacement gene was further modified and expressed from the pcDNA3 vector (Invitrogen). This construct was used for *in vitro* experiments investigating the resistance to siRNA-mediated silencing achieved by 7-nucleotide changes over the siRNA-target site. P_{CMV}: Cytomegalovirus promoter; BGHpA: Bovine Growth Hormone polyA; Amp^R : Ampicillin resistance gene and Neo^R : Neomycin resistance gene; HR7m: Human rhodopsin cDNA with 7 nucleotide changes.

Electropherogram sequences for the wild-type rhodopsin cDNA, (HR), and two replacement human rhodopsins, HR5m and HR7m are presented.

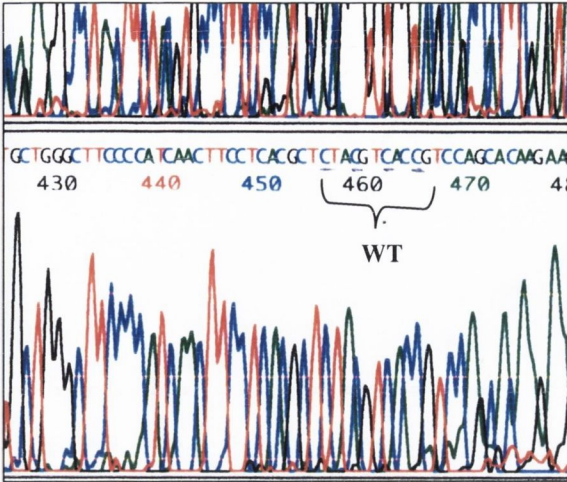


Fig. 4.5a: Wild-type HR cDNA

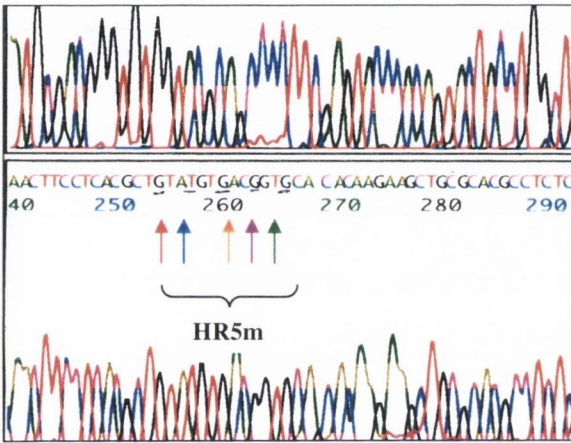


Fig. 4.5b: HR5m replacement rhodopsin cDNA

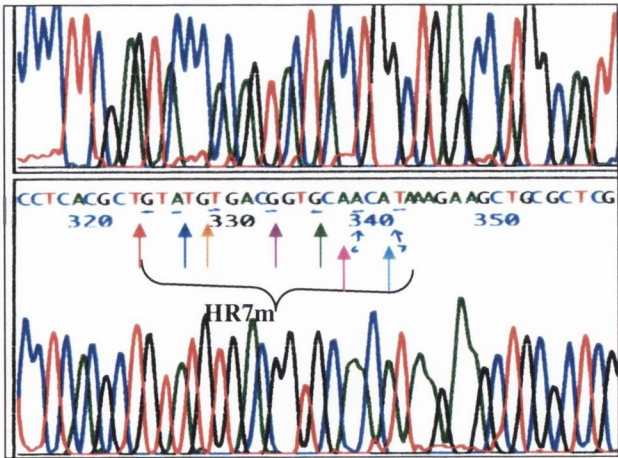
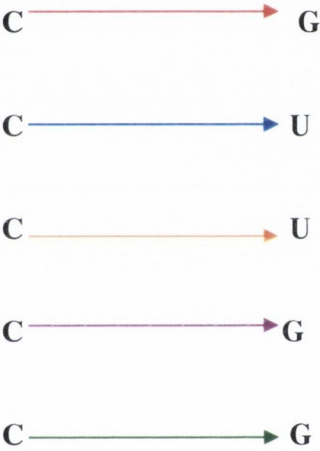


Fig. 4.5c: HR7m replacement rhodopsin cDNA

A PCR site-directed mutagenesis strategy was used to incorporate changes into human rhodopsin cDNA. This strategy is outlined in chapter 2, methods 2.2.1, and 4.2.4.

HR5m (nt 177-189) replacement sequence of the rhodopsin transgene in the mouse model, HR5m.



HR7m (nt 192-195) cDNA used in section 4.3.6



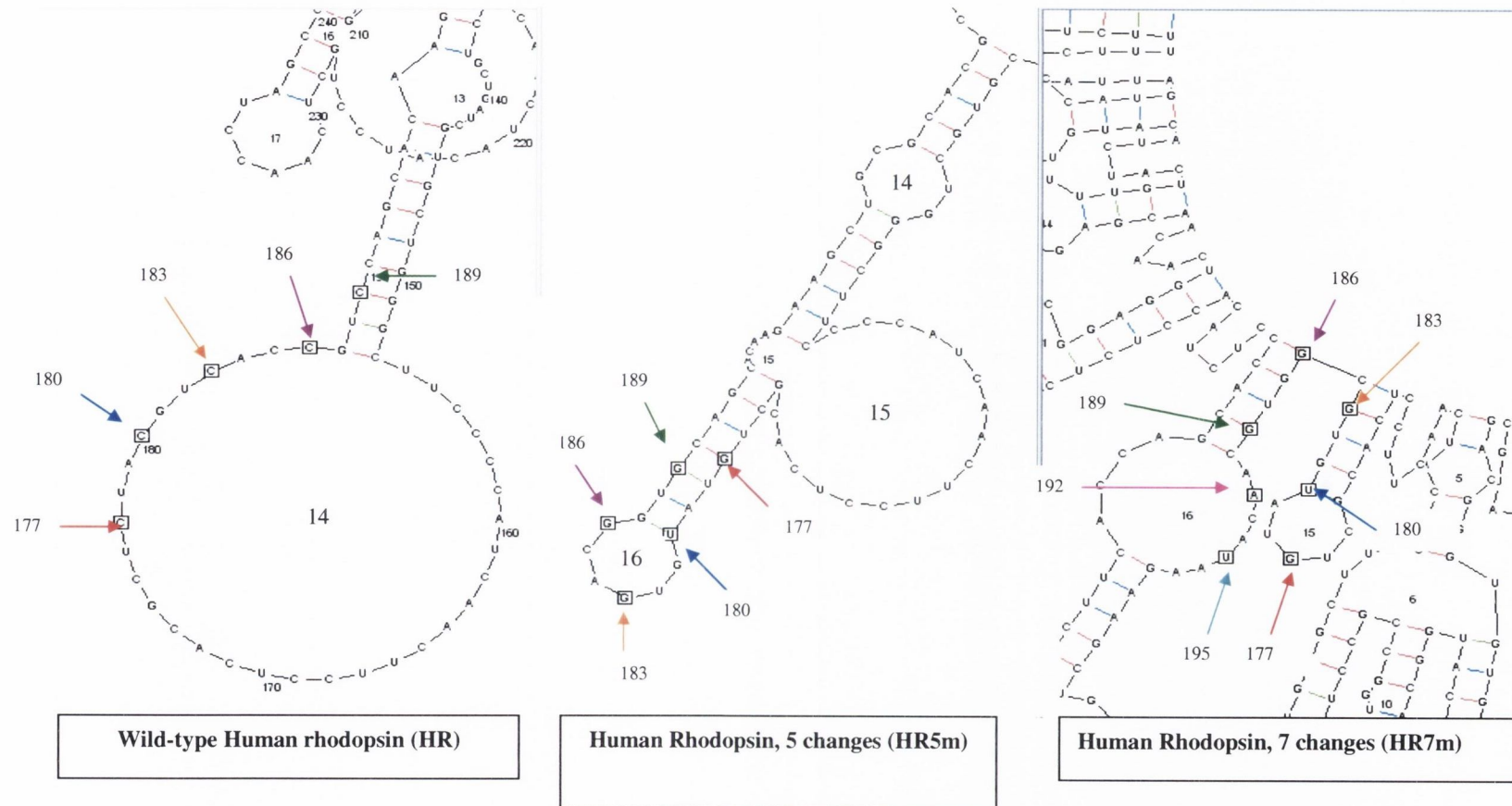


Fig. 4.6:

Predicted Secondary structure of wild-type human rhodopsin transcript compared to that for human rhodopsin replacement transcripts, HR5m and HR7m (RNAdraw). The presented view is of the same scale for all three structures. See table 4.2 overleaf for sequence changes.

Changes shown in rhodopsin mRNA plotfolds of fig. 4.6 and sequence electropherogram of fig. 4.5:

7m

5m

MRNA	cDNA	Changes
273	177	C → G
276	180	C → U
279	183	C → G
282	186	C → G
285	189	C → G
288.	192	C → A
291	195	G → U

Table 4.2:
Nucleotide changes incorporated into the replacement human rhodopsin cDNAs, HR5m and HR7m.

	No. of base-pairs	A-U	G-C	G-U	No. of stem-loops	Energy
WtHR	828	288	451	89	179	-721.35
HR5m	831	290	450	91	180	-722.38
HR7m	831	287	448	96	181	-719.72

Table 4.3:
Structural information derived from the RNAdraw secondary structure calculation. The RNAdraw prediction is based on the minimum energy requirement of the mRNA structure. Default temperature settings of 37°C were used for the calculation. The highest hit in the output from the RNAdraw program is presented in fig. 4.6 and shows the structure requiring minimum energy for its formation, for each HR, HR5m and HR7m sequences.

Effect of presynthesised siRNA on rhodopsin replacement gene, (HR5m) expression:

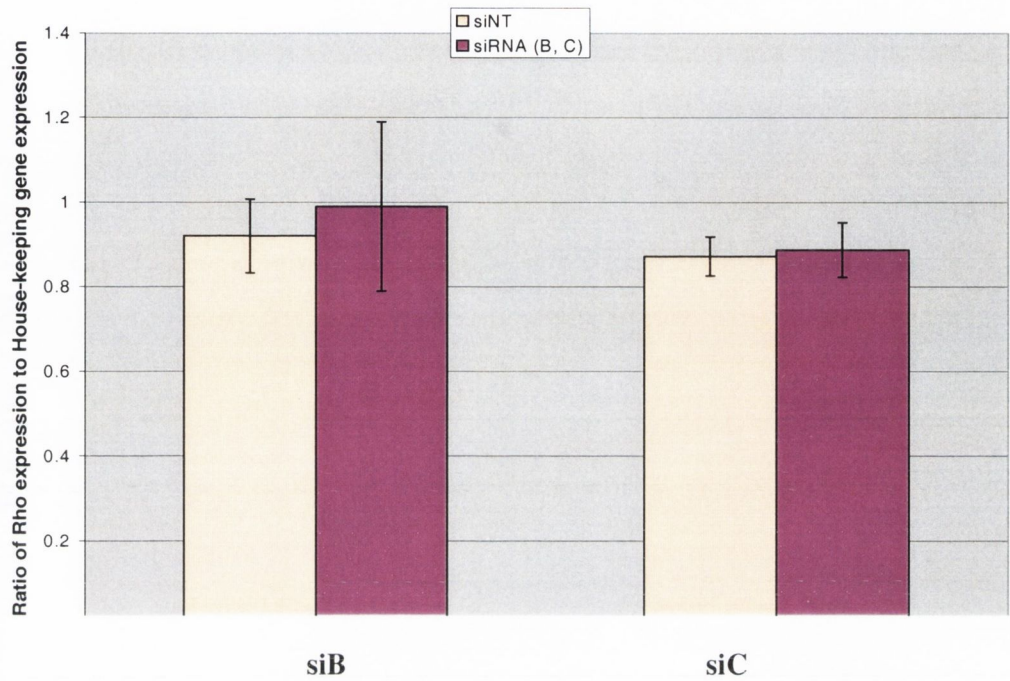


Fig. 4.7: Graph showing rhodopsin expression level normalised to GAPDH level, following cotransfection of pIRES-EGFP-HR5m with dsRNAs (Qiagen). HR5m carries two nucleotide differences to the target site for siB and five nucleotide differences to siC.

	SiNT/HR5m	SiB/HR5m	SiNT/HR5m	SiC/HR5m
Ave. ratio of expression: Rho/GAPDH	0.92	0.99	0.872	0.887
Normalised %age HR5m	100	107.6	100	101.7
Std. Dev.	0.174	0.402	0.09	0.129

Table 4.4

Table showing the data for fig.4.7. Transfections were repeated three times and RT-rtPCR runs performed twice for each RNA pool. No statistically significant difference was detected between the siNT and the siB data-sets (*p*-value: 0.557) between the siNT and the siC data-sets (*p*-value: 0.603).

Suppression of rhodopsin replacement gene, (HR5m) by plasmid-generated siRNA

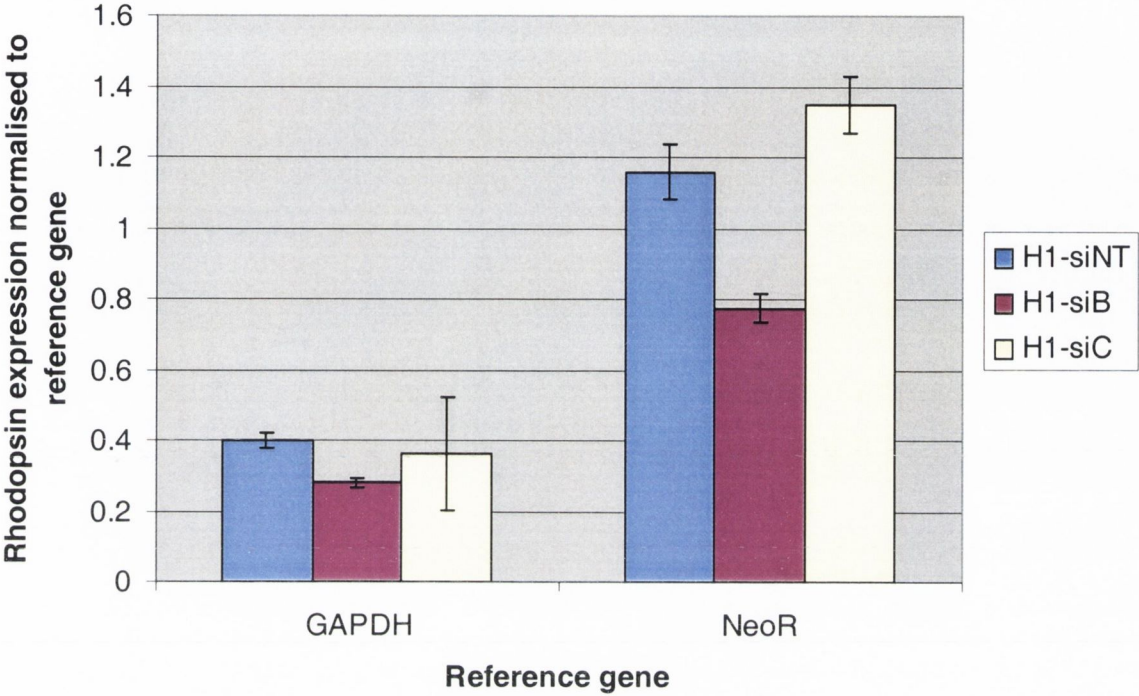


Fig. 4.8:

Graph showing the expression levels of the replacement rhodopsin gene normalised to cellular GAPDH and target-vector neomycin resistance expression levels following plasmid-driven RNAi. Cotransfection of pIRES-EGFP-HR5m and pH1-siRNA, (sequences NT, B and C, appendix 2A) carried out in COS7 (three transfections), HeLa (two transfections) & HEK293 cells (one transfection). The data represents a pooling of the values derived from each cell line. The statistical significance of this data is presented in table 4.5 overleaf.

ShRNA	Rho/Gap	Std.		Rho/Neo	Std.	
		Deviation	Average (%)		Deviation	Average (%)
H1-siNT	0.4	0.029	100	1.16	0.156	100
H1-siB	0.28	0.03	70	0.776	0.056	66.9
H1-siC	0.363	0.188	90.8	1.35	0.162	116.4
Student's t-test: GAPDH; NeoR						
H1-siB vs H1-siNT		p-value: 0.037; 0.046				
H1-siC vs H1-siNT		p-value: 0.24; 0.05				

Table 4.5:

Table showing the data for fig.4.8, the average HR5m expression ratios following cotransfections with H1-siRNA in mammalian cells. Error values for expression levels relative to both GAPDH and neoR genes were pooled to obtain +/- standard deviations. A paired Student's T-test was used to compare the ratios obtained between H1-NT treatments to ratios obtained for both the H1-siB and H1-siC treatments. The data was analysed individually when normalised to the cellular GAPDH reference gene and the plasmid-reference NeoR gene. While the H1-siC data-set shows no statistically significant difference to the H1-siNT data-set ($p= 0.24$ (GAPDH) and 0.05 (NeoR)), statistical significance in the data is evident for H1-siB treatments compared to H1-siNT treatments ($p= 0.037$ (GAPDH) and 0.046 (NeoR)).

Comparing two non-targeting controls (sense and non-sense) for effect on expression from the HR5m replacement rhodopsin gene.

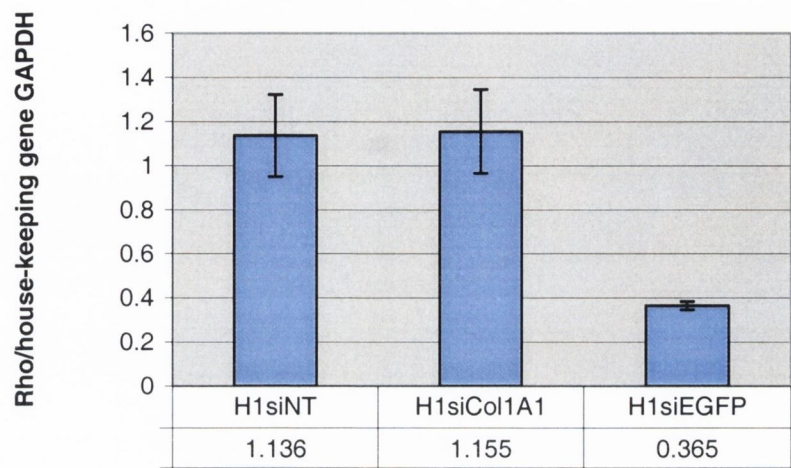


Fig. 4.9: Comparison of rhodopsin expression from cells cotransfected with control shRNAs and pIRES-EGFP-HR5m into HeLa cells. The shRNAs include a ‘nonsense’ non-targeting siRNA (H1-siNT), and a functional non-targeting siRNA (H1-siCol1A1), thus comparing the effects on gene expression of the negative control siRNA when it is incorporated into RISC (H1-siCol1A1), to when it is not incorporated into RISC, (H1-siNT). H1-siEGFP serves as a positive control for targeted silencing of the pIRES-EGFP-HR5m plasmid.

Dose Curve of siRNA on replacement rhodopsin gene expression

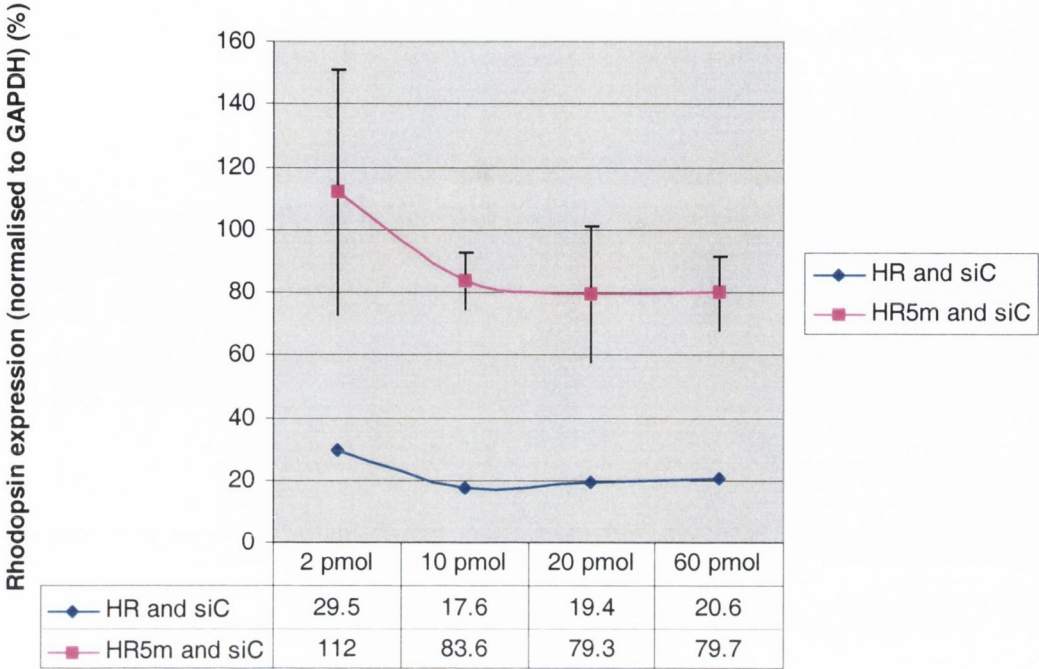


Fig. 4.10:

Dose curve showing rhodopsin expression (normalised to GAPDH expression) for a range of siC concentrations and a fixed concentration of target plasmid (400 ng) following cotransfection into mammalian cells (COS7 and HeLa cells). The pink line shows the level of rhodopsin expression from the pIRES-EGFP-HR5m replacement rhodopsin construct cotransfected with a range of concentrations of presynthesised siC siRNA. This level is expressed relative to a fixed value of 100% rhodopsin expression for a control transfection of non-targeting siRNA (20 pmol, from Qiagen) and replacement rhodopsin expressed from pIRES-EGFP-HR5m (400 ng) (not shown on the graph). For each siC concentration, the data from three transfections is averaged.

For comparison the blue line shows the percentage rhodopsin expression from a dose curve of wild-type target, pIRES-EGFP-HR, and a range of cotransfections with siC (as presented in chapter 3, fig. 3.14). This percentage is relative to 100% for the expression ratio of 20 pmol of siNT (Qiagen) and pIRES-EGFP-HR (400 ng).

Analysis of protein expression from cells cotransfected with rhodopsin and siRNA

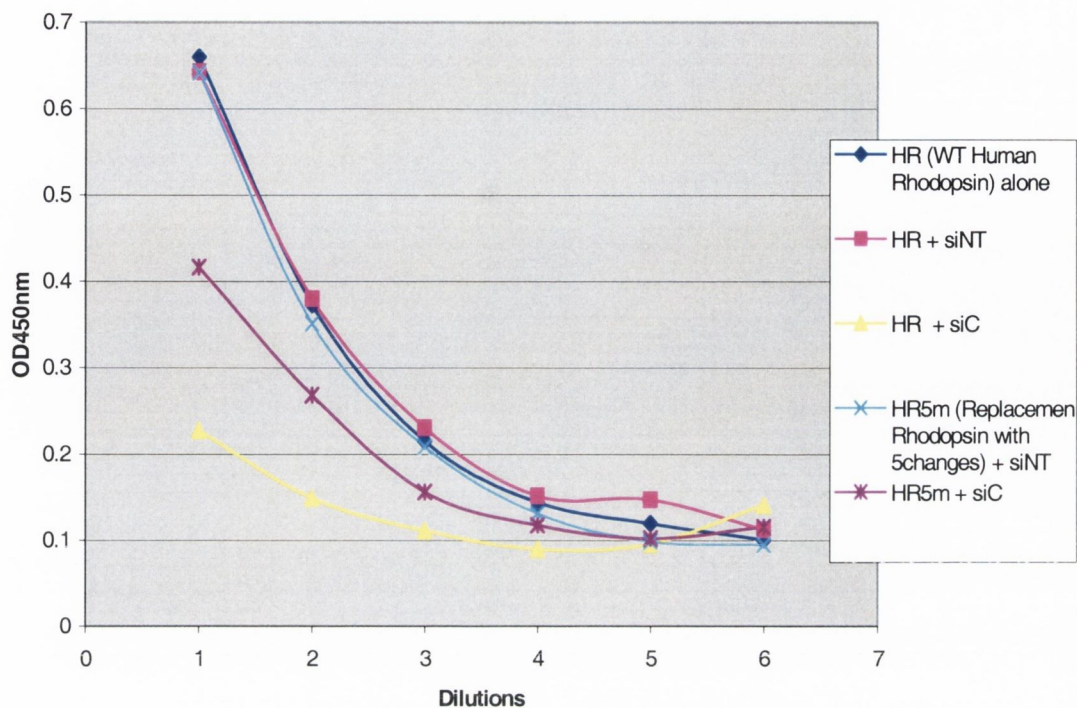


Fig. 4.11: Chart showing expression of human rhodopsin replacement protein, (translated from the HR5m cDNA) vs wild-type rhodopsin protein (translated from the HR cDNA) following treatment with siRNA in HEK293 cells. A targeting siRNA (siC) specific to the loop 14 region of human rhodopsin (and modified in the replacement cDNA) was assessed for silencing activity when compared to a control non-targeting siRNA (siNT).

Protein Dilution	Dilution factor	HR alone	HR+SiNT	HR+siC	HR5m+SiNT	HR5m+siC
1	1/40	0.659	0.642	0.2275	0.641	0.416
2	1/80	0.3725	0.3795	0.149	0.3505	0.268
3	1/160	0.2145	0.23	0.111	0.208	0.1555
4	1/320	0.1435	0.151	0.0895	0.1305	0.117
5	1/640	0.119	0.147	0.0955	0.098	0.1015
6	1/1280	0.1	0.112	0.141	0.094	0.115

Table 4.6a: Table showing data for the ELISA of fig.4.11. Results are averaged from protein samples taken from two sets of transfections in HEK293 cells. The figures represent the read-out at 450nm from the ELISA colorimetric reaction. The OD450 nm read-out of table 4.6a represents a linear relationship with the concentration of the protein in the cells.

NHR alone (% expression)	NHR+siNT (% expression)	NHR+siC (% expression)	HR5m+siNT (% expression)	HR5m+siC (% expression)
100	97.4	34.5	97.3	63.1

Table 4.6b: Table showing the overall percentage values of rhodopsin expression from the lowest protein dilution (1/40) in the ELISA experiment shown in fig. 4.11.

Analysis of replacement rhodopsin gene expression following addition of two further changes to the HR5m sequence to generate the HR7m cDNA

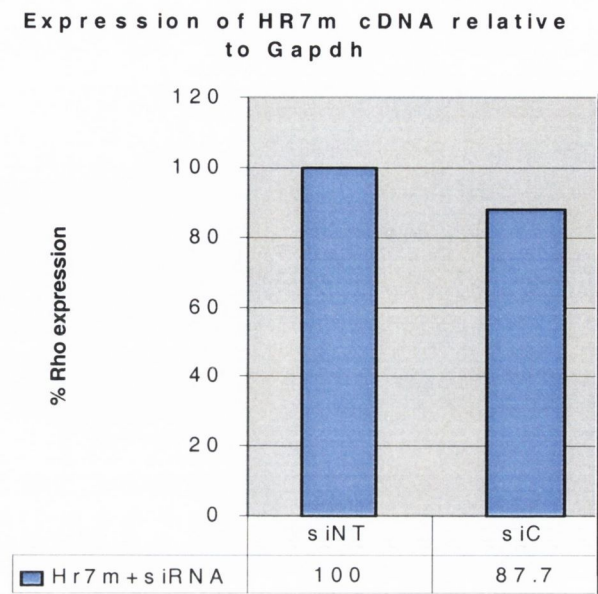
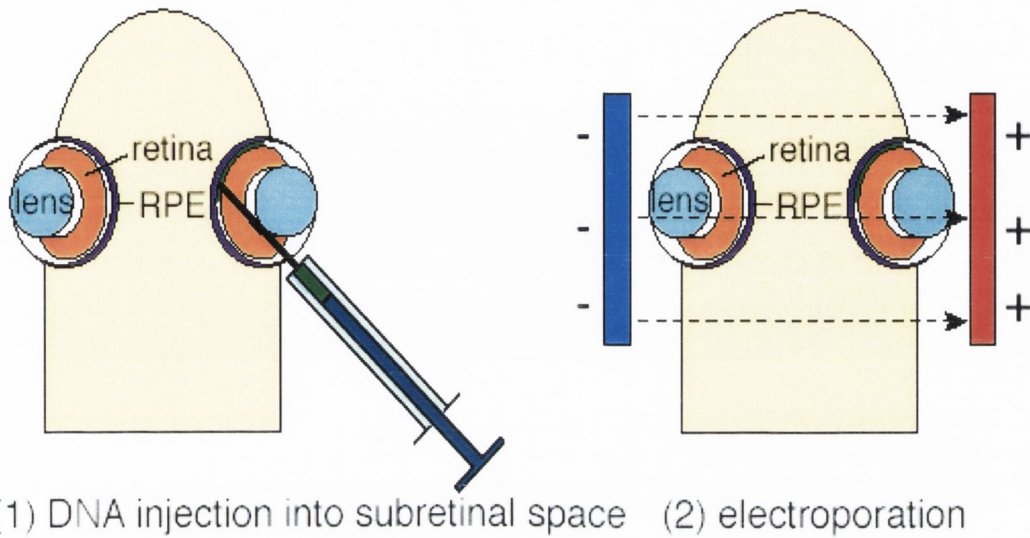


Fig. 4.12:

Expression of HR7m cDNA in HeLa cells following cotransfection with the shRNA construct H1-siNT or H1-siC. Each of the H1-plasmids was cotransfected alongside 400 ng of pcDNA3-HR7m into 5×10^4 cells. RNA was extracted and analysed by real-time rtPCR after 48 hrs. Real-time expression values for treatment of HR7m cDNA with H1-siC were compared to non-targeting siRNA following normalisation of the data to GAPDH expression. The experiment was performed three times, and analysed once with each RNA sample. The 12% suppression suggested by the limited data is not statistically significant, (*p*-value: 0.636).

Two methods of drug delivery to the mammalian retina.



(reproduced from Matsuda and Cepko, 2004).

Fig. 4.13:

Current approaches to achieving delivery of therapeutic agents to the mammalian retina. Both techniques have been applied successfully to mice at post-natal day 0–2. DNA constructs may be injected into the subretinal space of newborn mouse pups. Tweezer type electrodes, placed on the heads of the pups may be used to deliver approximately five electric pulses (80V of 50-ms duration) in the direction shown. The aim is to deliver the DNA to the scleral side of the retina, where undifferentiated mitotic and newly postmitotic cells exist. Unfortunately the region that can be transfected with simple plasmid-based and non-viral vectors is limited *in vivo*. Figure 4.15 overleaf, however, shows how the above delivery technique may be adapted to an *in vitro* system for deriving primary retinal culture carrying the DNA construct of interest.

Mouse matings for *ex vivo* experiments

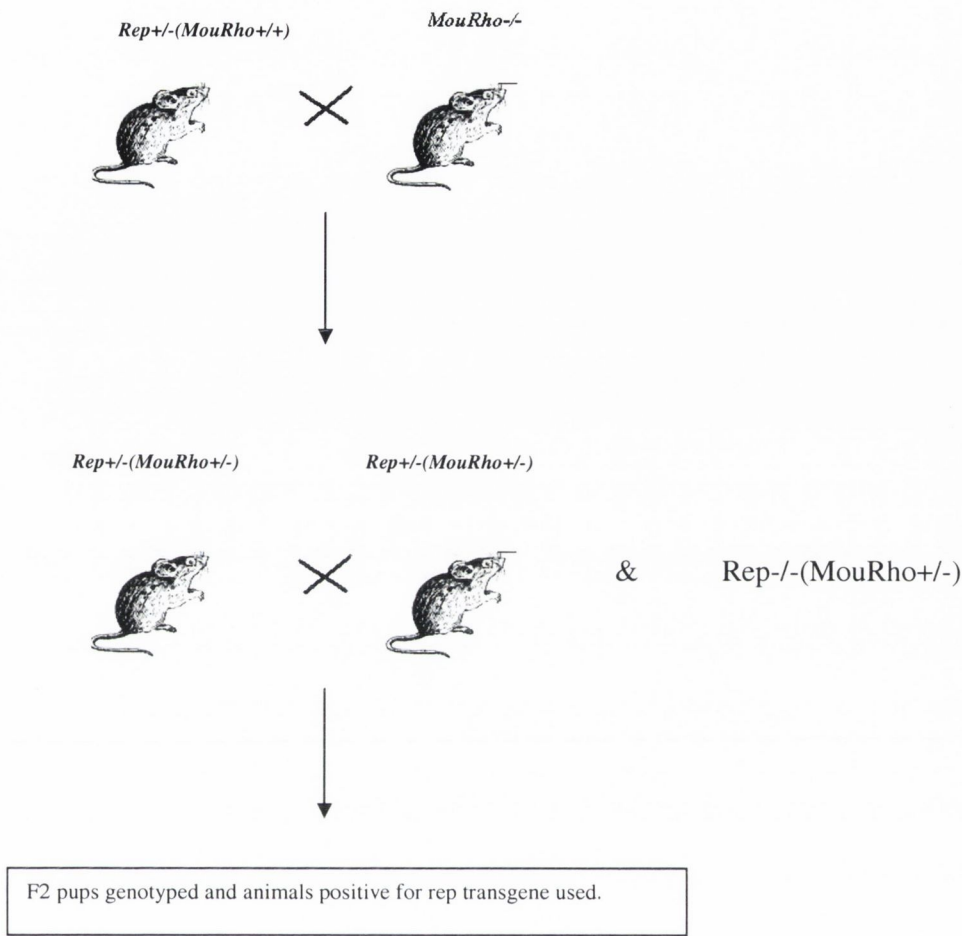


Fig. 4.14:

The mouse matings set up for the *ex vivo* experiment of section 4.3.8-9 are shown above. $Rep^{+/-}(MouRho^{-/-})$ refers to the replacement rhodopsin mice, which carry the HR5m $^{+/-}$ transgene. Siblings carrying the HR5m transgene from the F1 generation were mated to produce the F2 litter. Animals positive for the HR5m transgene and negative for endogenous mouse rhodopsin were identified by PCR-genotyping of tail-tip DNA (as described in section 2.2.6.1) and were used in the explant set-up shown in figure 4.19.

Electroporation of retinal explants with plasmid DNA solution.

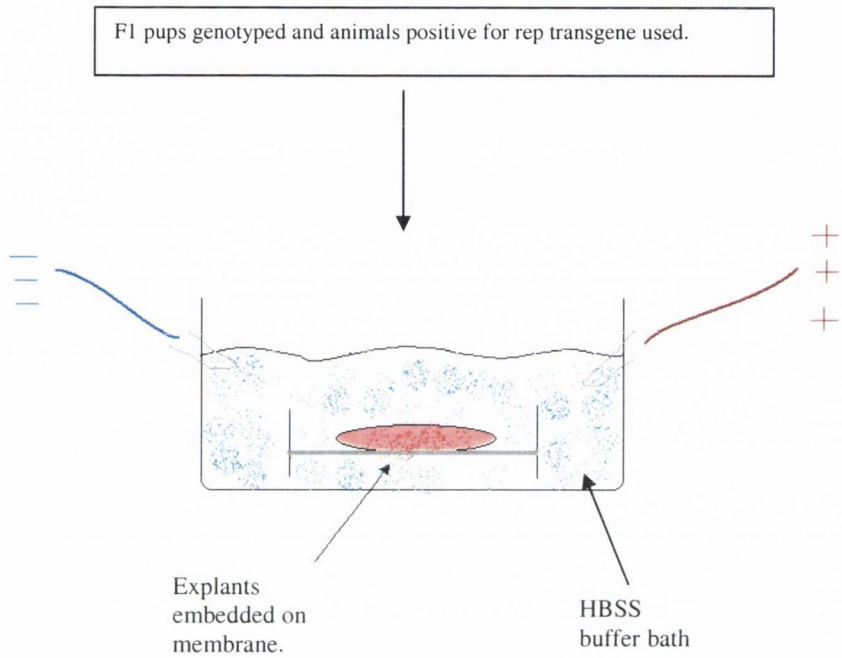


Fig. 4.15:

The HR5m+/- (MouRho-/-) retinas, embedded on polycarbonate membrane and placed in Hank's Balanced Salt solutions (HBSS), were electroporated with plasmid DNA by driving a current through the HBSS bath containing the DNA solution at 1µg/µl. The retinas were cultured over a two-week period before analysis for HR5m expression.

HR5m+/--(MouRho-/-) retinal explant study

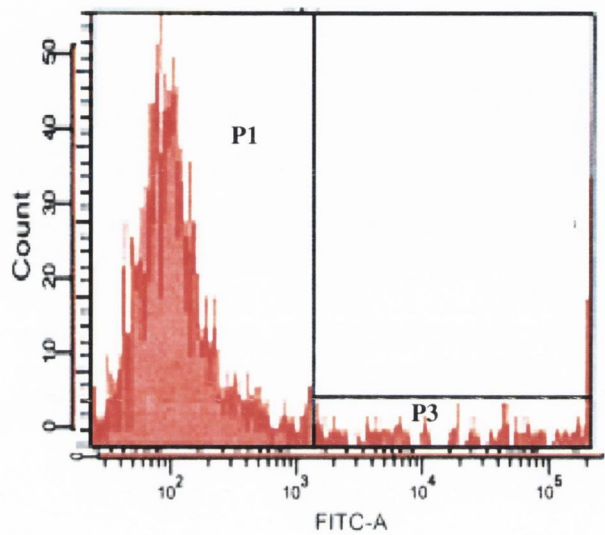


Fig 4.16:
FACS analysis of HR5m+/--(MouRho-/-) retinal explants. The intact cells were selected initially by size fractionation, and the photoreceptor cells identified by side scatter profile (P1). The EGFP-tag on the electroporated shRNA plasmid allows for selection of the transfected photoreceptor cell population (P3) from the P1 population pool.

RNA data from the HR5m+/--(MouRho/-) retinal explant study

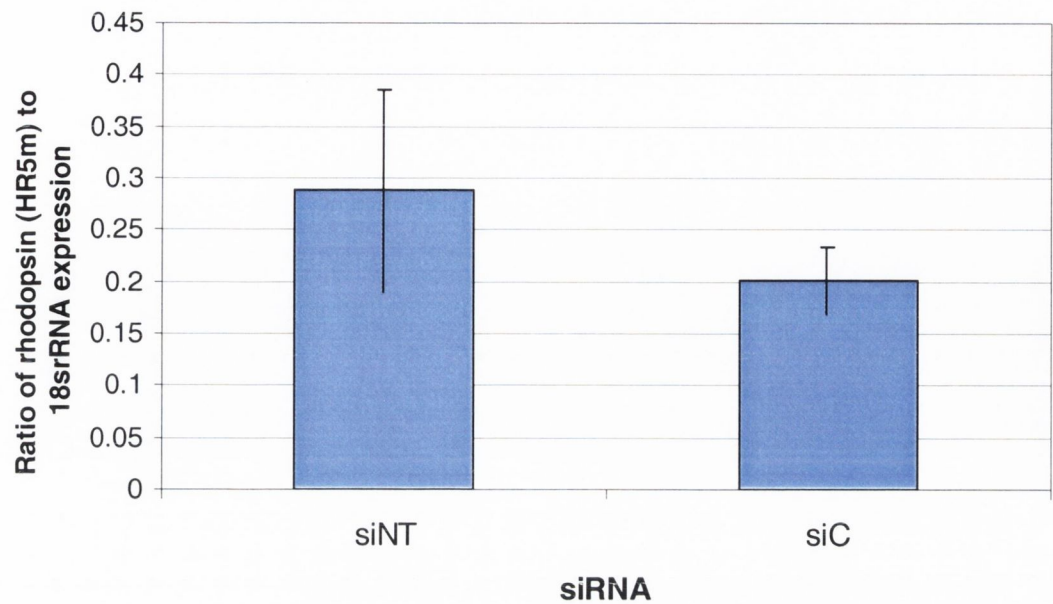


Fig. 4.17:

Rhodopsin transcript levels normalised to 18S rRNA transcript levels in RNA samples taken from the FACS-analysed explants shown in fig 4.13. The retinal explants used are derived from the HR5m+/--(MouRho/-) transgenic mouse model electroporated with the shRNA constructs, pEGFP-siNT or pEGFP-siC. Three mice were electroporated for each construct.

	<i>rho/18srRNA</i>	<i>%HR5m expression</i>	<i>Std Deviation</i>
pEGFP-siNT	0.2877	100	0.1961
pEGFP-siC	0.20085	69.86	0.06459

Table 4.7:

Table showing the relative rhodopsin expression in RNA extracted from retinal HR5m+/- explants electroporated with shRNA (as illustrated in the chart of fig. 4.14 on the previous page). The data was taken from two real-time rtPCR runs using 6 explanted retinas for each pEGFP-siNT and pEGFP-siC electroporations. Student’s Paired t-test yielded a p-value of 0.6 between the pEGFP-siNT and pEGFP-siC data-sets (t<0.05 deemed statistically significant with a 95% confidence interval). Hence, a significant suppression occurring on

rhodopsin levels in the pEGFP-siC-treated retina compared to the pEGFP-siNT-treated retina cannot be inferred based on this data.

Protein data from the HR5m+/- (MouRho-/-) retinal explant study

	<i>No. of green cells (+ve for shRNA)</i>	<i>No. of green and red cells) (+ve for shRNA and rhodopsin)</i>	<i>% Rhodopsin expression</i>
pEGFP-siNT	157	136	86.6
pEGFP-si-C	115	60	52.

Table 4.8:

Table showing data from a protein study on HR5m+/- (MouRho-/-) retinal explants. The numbers represent counts of dissociated cells from four retinas for each pEGFP-H1siNT and pEGFP-H1siC electroporations. Immunocytochemistry (AntiRHO4D2 1:150) was performed following dissociation of cells from retinal explants by trypsin digestion and attachment of single cells to poly-L-Lysine- coated coverslips. Green cells are positive for the pEGFP-shRNA plasmids (siC and siNT) while red cells are positive for rhodopsin expression. This is a non-quantitative assay, which may not reveal the true level of comparative expression in the two retinas. The endogenous levels of transgene expression in the HR and HR5m models may affect antibody-labelling.

Factors effecting the results of the retinal explant experiments of chapters 3 and 4

(i) Analysis of rhodopsin mRNA levels in the NHR mouse model (expressing the normal human rhodopsin transgene) compared to the HR5m mouse model (expressing the replacement human rhodopsin transgene).

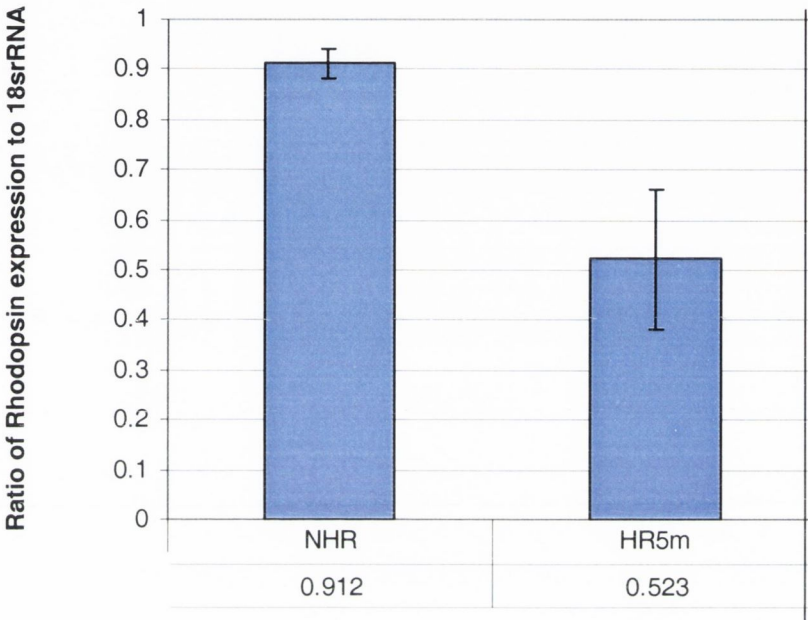


Fig. 4.18:

Chart showing the relative expression levels of the human rhodopsin transgene in NHR and HR5m retinas from 3-month-old mice. Three mice of each NHR+/- (MouRho/-) and HR5m+/- (MouRho/-) genotypes were analysed. Two real-time rtPCR reactions were carried out on each animal and the data from the six reactions pooled. The data shows 43% reduction in the level of transgene in the HR5m model compared to the NHR model.

(ii) Effect of the genetic background of the NHR and HR5m mice on the explant RNA analysis experiments of chapters 3 and 4.

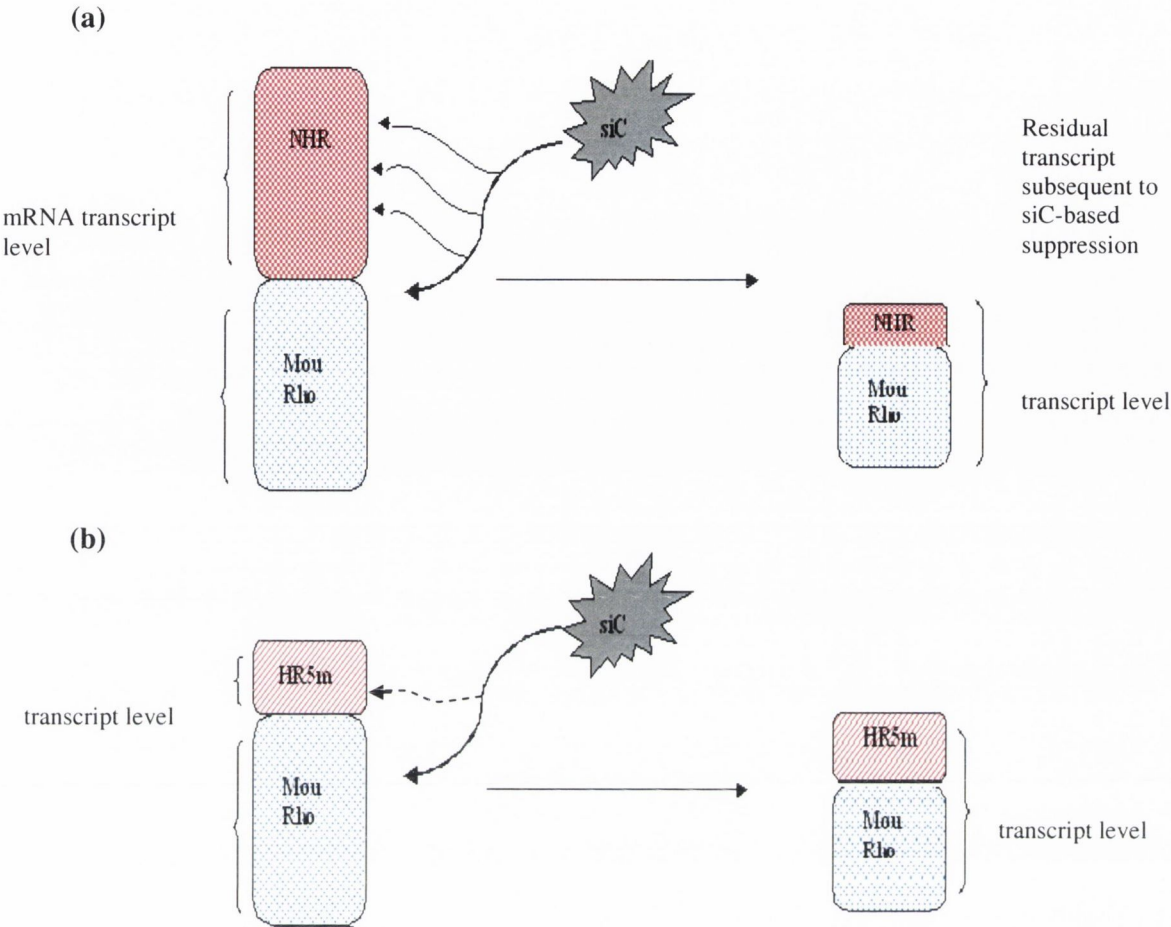


Fig. 4.19:

Endogenous mouse rhodopsin transcript has since these experiments been shown to continue to be produced in the rho-/- knockout animal model. The mouse rhodopsin transcript may therefore be a target for siC-mediated down-regulation in the retinal explant experiments carried out for the purposes of generating suppression and replacement data for the human rhodopsin gene. This figure shows the effect such a background may have on the retinal explant experiments described and may clarify some of the results obtained using explant material to date. The potential consequence of endogenous rhodopsin transcripts in the experimental system for the NHR explant used in chapter 3 is illustrated in (a). There is one base-pair change between the siC sequence and the corresponding region in the mouse rhodopsin gene. As such, the mouse transcript may not be suppressed with the same efficiency as transcript from the human transgene may be suppressed. At the same time, the expression

from the disrupted mouse rhodopsin gene will be detected in the real time rtPCR run, thus potentially leading to an underestimate of the percentage of suppression of the human rhodopsin transgen when estimated using this assay. The residual transcript subsequent to siC-based suppression of rhodopsin may be predominantly mouse rhodopsin. The real-time rtPCR assay utilised does not discriminate between mouse and human rhodopsin transcripts. For the purposes of the clarity of figure 4.19, the proportions of the mouse and human transcripts have been set at 50%. However it is notable that the relative proportion of the human rhodopsin and the mouse rhodopsin transcripts in the NHR(MouRho^{-/-}) mouse have not been determined. (For example, the transcripts from the disrupted rhodopsin gene may be less stable than from a wild-type gene).

For the rhodopsin replacement gene study described in chapter 4, endogenous mouse rhodopsin transcripts may also affect explant data as illustrated in (b) of this figure. However in this case the level of modified human rhodopsin transcript as evaluated by real-time rtPCR may be considerably less than the level of normal human rhodopsin transcript in the NHR model (fig. 4.18). The relative level of HR5m transcript compared to mouse rhodopsin transcript (expressed from the disrupted gene) has not been determined for HR5m(MouRho^{-/-}) mice and hence the proportions provided in the diagram of fig. 4.19b are hypothetical. However, as for A, the mouse rhodopsin transcript carries the base-pair difference to the siC sequence, however, the competing human rhodopsin transcript has been modified to carry 5 differences to the siC siRNA and therefore siC may elicit a far greater level of suppression on the mouse rhodopsin mRNA. The presence of residual mouse rhodopsin transcript which may be more efficiently suppressed by siC from the modified HR5m replacement transcript would result in residual siC-based suppression being observed in retinal explants from HR5m(MouRho^{-/-}) mice and hence may account at least in part for the 30% of suppression observed in cells from retinal explants (fig. 4.17).

Immunostaining of cryosections from explanted tissue of HR5m+/- (MouRho-/-) transgenic mice, following electroporation with pEGFP-siC

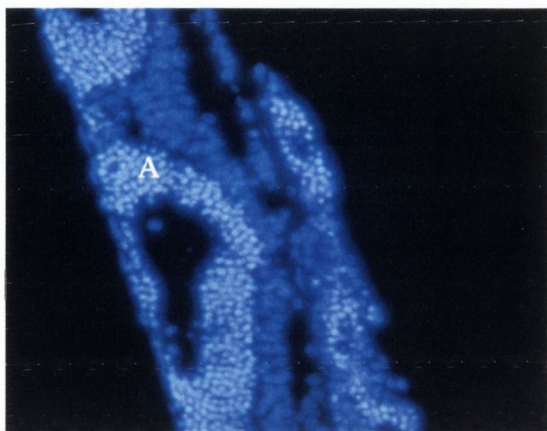


Fig. 4.20a: Dapi stained cross-section of HR5m+/- retina Electroporated with pEGFP-siC.

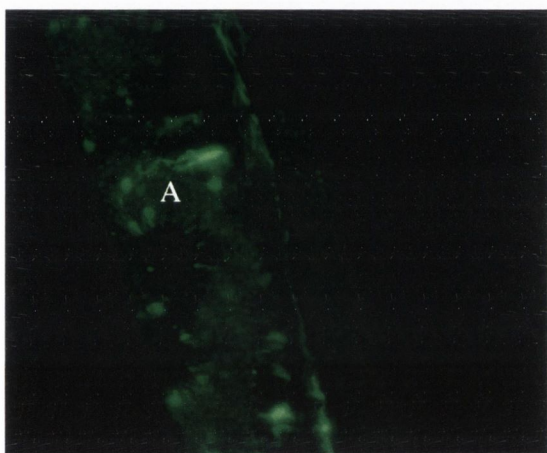


Fig. 4.20b: Cross-section of HR5m+/- retina electroporated with pEGFP-siC. Visualised under a fluorescence microscope.

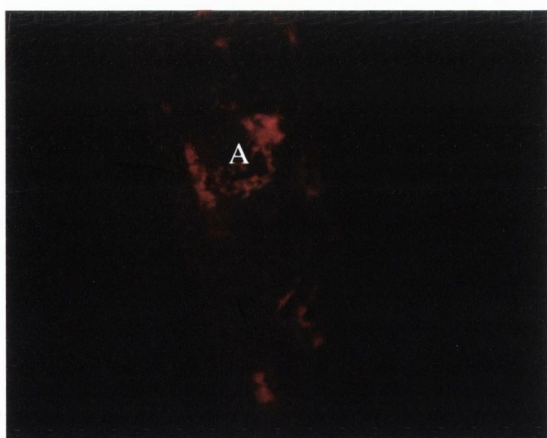


Fig. 4.20c: Cross-section of 4D2-immunostained HR5m+/- retina electroporated with pEGFP-siC.

Fig. 4.20:

The retinal explant derived from a p0 NHR5m+/- (rho-/-) mouse was electroporated with pEGFP-siC as described in sections 4.3.9 and 4.3.10 (Methods section 2.2.6.2 and 2.2.6.10).

After two weeks in culture the retinal explant was fixed with paraformaldehyde, snap frozen and cryosectioned (Methods section 2.2.6.10). A region of the rod outer segment is labelled A in the dapi-stained section of figure 4.20a.

The same region is observed under fluorescent green light in fig. 4.20b. The strong green fluorescence suggests successful transfection with the pEGFP-siC construct.

Fig. 4.20c shows a fluorescent microscope picture of the section visualised under red light. The explant had been immunostained with rhodopsin antibody and secondary stained with a cross-reacting alexa fluor label. Hence rhodopsin protein should be visible. Signal for rhodopsin is evident in region A. Although a purely qualitative result, this image suggests that the rhodopsin protein in the HR5m+/- (rho-/-) explant is protected from siC-induced gene silencing.

Chapter 5

Further exploration of suppression of ADRP-linked gene expression

CHAPTER 5

5.1 INTRODUCTION

5.1.1 Objectives of chapter 5

The research described in this chapter is concerned with attempts to optimise rhodopsin-specific suppression prior to engineering the suppression element of the therapeutic into a delivery for *in vivo* studies, discussed in chapter 6. There are three main focuses in this chapter and are outlined below;

i) One aim of the research described in this thesis is to demonstrate application of the tools selected for silencing in chapter 3 to a disease context. Thus, one of the questions explored in this chapter is whether these suppression agents can be used to suppress expression of a mutant rhodopsin allele previously implicated in ADRP. The pro23his model was chosen to explore this therapeutic approach and the results of *in vitro* and *ex vivo* analyses are presented. The siRNA molecule, siC, appears to offer the best combination of targeted rhodopsin-suppression in the region of interest, (nt177–189) with a marginal amount of down-regulation of the replacement gene (discussed in detail in chapters 3 and 4) and therefore was chosen to evaluate rhodopsin suppression in the pro23his mouse model.

ii) The replacement rhodopsin transgenic mouse described in this thesis, HR5m, offers potential as an *in vivo* model for testing the resistance of endogenous replacement rhodopsin transcripts to a suppressing agent specific for ‘normal’ human rhodopsin, (NHR). However, use of the HR5m model as a research tool restricts the design of the NHR-suppressing agent, to targeting the loop14 region of the NHR cDNA (fig. 3.10). It is in this region that the nucleotide changes have been engineered in the HR5m gene of the replacement model. Approaches to optimise the silencing thus far achieved targeting this region of the rhodopsin transcript are therefore of interest. One possibility in this regard, is based on that of Zeng *et al.* (2002), who used a microRNA-based dsRNA as a way of avoiding potential immune activation caused by dsRNA-mediated gene silencing. Hence, it was investigated in the current study as to whether silencing targeted to the loop14 region of the NHR cDNA might be safely enhanced by designing a microRNA-based molecule that covers the siC site found to be a suitable target for suppression in chapter 3.

iii) Finally, in order to demonstrate the flexibility of the suppression and replacement approach, a second gene implicated in ADRP has been targeted using an alternative suppression tool and applying an alternative angle on the gene replacement strategy. Hence, ribozymes targeting the human peripherin/*rds* gene in the 5' untranslated region were assessed in the current study. In principle, as the 5' and 3'UTRs do not contribute to the sequence of the transcript translated into protein, the UTRs may therefore provide an opportunity to introduce sequence alterations in replacement genes which do not alter the encoded protein, but enable transcripts from replacement genes to be protected from suppression.

5.1.2 Pro23his mouse model

The pro23his mutation is the most common mutation causing ADRP, accounting for 15% of all cases in the US (Olsson *et al.*, 1992). The pro23his mouse model of ADRP derived by Dryja and Olsson *et al.*, (Olsson *et al.*, 1992) carries an insertion into the germline of mice of the mutant allele from a 40-year-old patient with ADRP. The retinopathy that occurs in the pro23his line of transgenic animals used for the purposes of this study is more severe than the human equivalent. Photoreceptor degeneration is evident from postnatal day 5 and is triggered as soon as rhodopsin starts to be expressed in the retinas. In these pro23his mice, as used in the lab in TCD, the majority of photoreceptor cells have degenerated by two weeks postnatally.

The substitution of a proline for histidine results in a change in the molecular structure of the N-terminus of rhodopsin that ultimately leads to photoreceptor cell death via a largely unknown mechanism. Wu *et al.* have shown that the absorbance spectrum and photic sensitivity of opsin from the pro23his animal is similar to that of wild-type opsin, as shown by its ability to regenerate normally upon addition of 11-*cis*-retinal *in vitro*. In HEK293 cells expression of pro23his cDNA has been shown by immunostaining to result in accumulation of the protein in the endoplasmic reticulum/Golgi apparatus of the cell. This, it has been suggested, implies that the protein may be abnormally trafficked within the context of a photoreceptor cell and perhaps diverted from the disc membranes of the outer segment (Sung *et al.*, 1991). There is some discrepancy in the literature regarding the extent of the degeneration in the pro23his mouse and there may be variation in severity depending on the genetic background onto which the colony is bred or the transgene copy number in the

pro23his line used (Dryja *et al.*, 1993, Jin *et al.*, 1995). It may be that while the expression of the transgene may be uniform between different colonies, the extent of disc shedding may vary, with some lines having a more rapid disc loss for unrelated biochemical reasons.

The research described in this chapter, is focused on the suppression of the expression of mutant rhodopsin in the pro23his animal using the siRNA molecule, siC, as defined in chapter 3. Thus results from the study may represent in essence a case study, which suggests proof of principle for the application of RNAi technology for the treatment of ADRP in humans.

5.1.3 Micro-RNA

Various classes of naturally occurring short RNAs have been identified in normal mammalian cells. These elements include microRNAs, (miRNAs), antisense siRNAs, tiny noncoding RNAs, (tncRNAs) (Ambros *et al.*, 2003), heterochromatic siRNAs (Reinhart, 2002), and repeat associated small RNAs (Aravin *et al.*, 2003.). These classes of RNAs are distinguished based on their precursor molecules and the nature of the suppression effect they have on the genome (fig. 5.1). Recently discovered miRNA functions include determination of transitions between larval stages in post-embryonic development in the worm (Novina *et al.*, 2004); neuronal patterning in nematodes (Johnston and Hobert, 2003); control of cell proliferation, cell death and fat metabolism in flies; control of crucial developmental transitions in plants (Bartel *et al.*, 2004.); and modulation of haematopoietic lineage differentiation in mammals (Chen *et al.*, 2004). However computational approaches have suggested that these examples are a small fraction of the multiple roles that a microRNA species may have in a cell. These bioinformatic analyses have led to experimental efforts to determine the individual functions of highly expressed miRNAs.

5.1.3.1 Defining characteristics of miRNA and siRNA:

Mature miRNAs are single-stranded RNA molecules of 21–22 nts that function in repression of gene translation usually by binding to the 3' UTR of the mRNA. It is clear that miRNAs and siRNAs have similarities: both are approximately 21–25 nt and have the 5'-phosphate and 3'-hydroxyl characteristic of a Dicer product; both require members of the Argonaute family for their accumulation in cells; both use sequence complementarity to act on mRNA target; both require

RISC formation to orchestrate suppression of the target gene. However their differences are significant and may be crucial in understanding the dynamic of their respective roles. siRNAs can be generated from exogenous long dsRNA, are processed such that numerous siRNAs accumulate from both strands of these dsRNAs, and yield two complementary RNAs in equal abundance; miRNA on the other hand derive from endogenous precursors that form hairpin structures with a miRNA generated from one arm of the stem-loop. This arm usually contains mismatches and is generated in vast excess over its complement (Krol *et al.*, 2004). It seems probable that endogenous siRNAs generally target the genes they originated from, whereas miRNAs regulate separate genes, with possibly hundreds of targets per miRNA. These miRNAs exhibit a dose effect with the level of translational inhibition achieved determined by the number of miRNAs bound to the target. (Doench *et al.*, 2003).

In attempts to maximise a silencing effect, some researchers have used hairpin siRNA vectors designed with a miRNA-based loop (Zeng *et al.*, 2002; Boden, 2004). By recruiting an endogenous microRNA-processing pathway it was hoped that the efficiency of siRNA production and turnover in the cell might be improved. Despite some overlap in their processing and also their activities, it appears that the miRNA and siRNA processing pathways have genetically and biochemically distinct elements. For example, the *C. elegans* mutant RDE-1 is defective in RNAi, whereas RDE-1 paralogs ALG-1 and ALG-2 (argonaute-like genes) are essential for generation of the *lin4* and *let7* miRNAs needed for development (Grishok *et al.*, 2001). Identifying the point of divergence in these two silencing pathways may aid in ensuring that a miRNA response is not provoked. This information alongside bioinformatics tools can be used to identify potential miRNA targets and may allow for safer design of siRNA for therapeutic application in the future.

5.1.3.2 Functions of known miRNA:

The first microRNA to be discovered, *lin-4*, found in *C. elegans*, is complementary to 7 sites in the 3'UTR of *lin14*, and inhibits translation of this target during development (Lee *et al.*, 1993). *Let7*, a microRNA discovered in *C. elegans* in 2000 was subsequently also shown to exist in flies, humans and apparently all bilaterally symmetrical animals (Reinhard *et al.*, 2000). An RNase-III endonuclease identified in *Drosophila* termed Drosha is believed to be involved in the generation of

60–70 nt stem loop miRNA intermediates (Lee *et al.*, 2003). Typically excised from these 60- to 70- nucleotide hairpin precursor RNA structures, only one of the strands from the microRNA hairpin molecule accumulates following Dicer digestion. This may be due to protection by accessory proteins from RNA degradation (Hammond *et al.*, 2001). Studies on the miRNAs thus far discovered suggest that translational inhibition is achieved through binding of the miRNAs to mismatched target sites in 3'UTR regions. The bulges formed by the mismatches are believed to block the progress of the ribosome along the mRNA, thus blocking the target mRNA from making its protein.

A H1 shRNA vector with a microRNA-based hairpin loop has been designed with the intent of optimising the early processing of the siRNA. It was examined whether a shRNA hairpin molecule with miRNA sequence could be used to mimic a typical precursor miRNA thus enabling the processing pathway in mammalian cells to be harnessed more efficiently. It may enhance the siRNA-based suppression that has already been achieved (chapter 3) which is targeted to sequences in the first exon of the rhodopsin gene (over the region where the replacement sequences in genes have been altered in the HR5m mouse model). It is notable that Miyagishi *et al.* have shown previously that inclusion of a natural microRNA-derived loop is advantageous for achieving gene silencing (Miyagishi *et al.*, 2005).

5.1.3.3 Human miRNAs:

Zeng *et al.* have demonstrated that posttranslation regulation by miRNAs is most probably conserved in mammalian cells, (Zeng *et al.*, 2002). Overexpression of miR-30 in mammalian cells resulted in repression of a reporter gene with partial complementarity to miR-30 binding sites in its 3'UTR without affecting mRNA stability of the reporter gene. Computational methods have led to an early estimation of 200–255 miRNA genes in human (Lim *et al.*, 2003). Burge and Bartel have since presented evidence that up to one third of all human genes may be regulated by miRNA (Burge and Bartel, 2005), and the number of microRNAs has been estimated to be closer to 800 (Bentwich *et al.*, 2005). Many miRNAs may only be evident under certain environmental conditions. Poy *et al.* have shown how a microRNA specific to pancreatic cells, miR-375, modulates the secretion of insulin in the cell in a pathway independent of changes in glucose metabolism or intracellular Ca^{2+} (Poy *et al.*, 2004). MiRNAs may also be temporally regulated in neurons during mammalian

brain development (Krichevsky *et al.*, 2003). Tissue-specific expression of miRNAs has been demonstrated in humans. For example, Lagos-Quintana *et al.* found miR124 to be enriched in the human eye (2003). Recently Ruvkun *et al.* have reported the discovery of a group of miRNAs expressed predominately in the brain, and which they have termed neuromiRNAs (Ruvkun *et al.*, 2004). However these molecules have been termed miRNAs purely on the basis of size and localisation to polysomes, which constitute the protein translation machinery, and their targets in the brain are currently unknown.

5.1.3.4 MiR-23C:

Zeng *et al.* have shown that by substituting the stem sequences of the mir-30 precursor with an unrelated base-paired sequence, novel miRNAs can be made to achieve targeted gene silencing (Zeng *et al.*, 2002). Using assays performed in cultured human cells, they were able to demonstrate that endogenous human miR-21 miRNA, or overexpressed forms of the human miR-30 could induce the cleavage of mRNAs bearing fully complementary target sites, a phenotype previously viewed as characteristic of siRNAs. Conversely, they also showed how a synthetic siRNA is capable of down-regulating the expression of an mRNA bearing partially mismatched target sites, without inducing detectable mRNA cleavage or reducing mRNA expression levels. Similarly Doench *et al.* investigated whether a siRNA can function like a miRNA, by repressing gene expression without a concordant decrease in mRNA stability (Doench *et al.*, 2003). In the research described in this thesis, a microRNA that has been identified in human cells – miR23 is used to modify the shRNA vector used for RNAi-based silencing. It is well established that many miRNAs are limited in their expression to certain stages in development or to certain tissue or cell types (Bartel *et al.*, 2004). There was, at the time of design of this construct, limited information on microRNAs specific to ocular tissue and none as yet identified to be exclusively active in retinal tissue. However some information regarding microRNAs specific to neural lineages was becoming available through extensive array-based searches of the human genome. The miR-23 loop was chosen based on results of Kawasaki *et al.* where it was suggested that miR-23 may have a regulatory role in the retinoic acid (RA)-induced neural differentiation of NT2 cells (Kawasaki *et al.*, 2003b), however Kawasaki *et al.* were unable to clarify the

physiological role of miR-23 and have since retracted their earlier finding (Kawasaki *et al.*, 2003c).

In addition to the use of a loop derived from the mi23 microRNA, the design used in the current study (illustrated in fig. 5.2) incorporates a bulge in the centre of the sense strand of the dsRNA (for reference compare fig. 5.2 to the siC sequence shown in appendix 1A). It is possible that recruiting a microRNA mechanism may enhance silencing using siRNA-based sequences and hence this possibility has been explored in the context of the human rhodopsin target gene. Miyagishi *et al.* have presented results from an optimised siRNA-expression system achieving enhance suppression of the firefly luciferase gene. Features in this design included (i) a (Pol III)-promoter driven hairpin with a 21-nt stem sequence, (ii) mutations introduced into the sense strand to create a mismatched bulge in the stem, and (iii) a microRNA miR30-derived hairpin loop. The inclusion of a natural microRNA-derived loop was found to be advantageous, similarly the mismatch-induced ‘bulge’ was deemed to make the construct more mutationally stable and efficient in production than others to date. Maximising efficiency of the suppression agent and maintaining mutational stability is of relevance when shRNA molecules come to be adapted for viral delivery (for example, with AAV in chapter 6 of this thesis).

As ADRP is a dominant disease and the siRNA-based suppression study described in this thesis is confined to targeting the loop 14 region of the rhodopsin transcript (based on the HR5m replacement animal model available for testing the strategy), enhancing the potency of the suppressor is desirable. However, retaining sequence specificity of the suppressing agent is vital for achieving the aims of this thesis, i.e. *in vivo* validation of a suppression and replacement therapeutic strategy.

5.1.4 Immune response to siRNA

In keeping with a theme of this chapter of attempting to optimise the activity of suppression tools before applying them *in vivo*, immune responses to the siRNA agents used thus far were examined. A dsRNA-induced immune response may be detected when long dsRNA, (in excess of 25 nt) is introduced to the cell and results in genome-wide inhibition of translation (Elbashir *et al.*, 2001a). This immune reaction is believed to have evolved in mammalian cells as an antiviral defence mechanism with the dsRNA viral genome as the trigger signal for the response. An exception applies to mammalian oocytes, embryonic stem cells and pre-implantation embryos

where specific homologous target suppression has been observed with longer dsRNA species, without the induction of an immune response. Model systems such as *Drosophila* and *c.elegans* are similarly equipped to suppress specific target without inducing an immune response. However, as discussed in chapter 3, section 3.1.3, sequence-specific suppression is not achieved in adult mammalian systems challenged with longer dsRNA molecules. It is believed that the dsRNA, which may appear to the cell to be a common viral replicative intermediate, will provoke activation of the interferon (IF) system in mammals (Elbashir *et al.*, 2001b). Interferons are cytokines at the frontline of the defense against a viral assault. The interferons initiate a signalling cascade that includes the Janus family of tyrosine kinases, signal transducers, and transcription factors (Jak-Stat signalling pathway), culminating in transcription of Interferon-stimulated Genes (ISGs) within the nucleus (Haque and Williams, 1998; Stark *et al.*, 1998). The dsRNA recognition protein that alerts this system is the dsRNA-dependent protein kinase, PKR and has also been identified as a component of signal transduction pathways that regulate events such as cell growth and stress responses (Kumar *et al.*, 1994). A dsRNA signal will cause PKR to autophosphorylate and cause the subsequent phosphorylation of the eukaryotic initiation factor 2 α (eIF2 α) subunit resulting in the general inhibition of protein synthesis (Srivastava *et al.*, 1998).

Before *in vivo* trials, any early indication of the potential for an immune response to siRNA treatment in a cell was examined *in vitro*. The choice of cell line for monitoring immune response to siRNA is critical, as many commonly used tumour cell lines have been shown to have a defective immune response (Stojdl *et al.*, 2000). Kim *et al.* showed the presence of interferon α and β in the serum of HEK293 cells infected with siRNA synthesised by T7 phage polymerase (2004). However, other investigators have shown that HEK293 cells lack an anti-viral immune response (Stojdl *et al.*, 2000). Activation of interferon pathway as a consequence of RNAi distinct from a vector response is of interest in this regard. For example, Pebernard *et al.* observed that IF-induction may be based on cellular response to lentiviral infection, the vector from which the siRNA was transcribed, rather than activation of the RNAi machinery itself (2004).

5.1.5 Alternative methods retinal gene knockdown: Hammerhead ribozymes and antisense

Alternative strategies to siRNA suppression may also be of value, in light of the potential risk of dsRNA-induced immune responses. Therefore, it is worth continuing with research into suppression tools that have shown promise in the past for down-regulation of mutant transcripts linked to ADRP. Hammerhead ribozymes are small RNA molecules, which possess sequence-specific RNA cleavage activity. These molecules can occur naturally, recognising and cleaving the sequence motif, 5'-NUX (where N is any nucleotide; X is A, C, or U), and also may be created artificially to target sequence in *cis* or *trans* (Ruffner *et al.*, 1989). Ribozymes do not require endogenous nucleases as they possess a Mg^{2+} -dependent catalytic domain flanked by two substrate-binding arms and can independently cleave specific phosphodiester linkages of target mRNAs (Ruffer *et al*, 1990).

Much of the research using ribozymes to date has focused on the structure and function of the hammerhead ribozyme and it is this type of molecule that is used in results section 5.3.4 of this thesis. Ribozyme specificity to the target site is necessary to achieve inhibition of gene expression. This specificity is dependent on the homology of the binding arms of the ribozyme which hybridise to the sequences flanking the cleavage site of the target mRNA. It is this feature that allows for the design of a replacement gene that can provide corrected transcripts coding for functional protein. Thus, the replacement gene can be modified at any redundant sequence within the region covered by the ribozyme stems at the site flanking the NUX target motif allowing the gene to escape ribozyme-mediated suppression.

Hammerhead ribozymes have been shown to be effective in delaying retinal degenerations in animal models of dominant RP. Delivery of a ribozyme targeting pro23his mutated rhodopsin mRNA delayed apoptotic death of the photoreceptors of pro23his transgenic rats (Lavail and Hauswirth, 2000). Recently combined with a replacement approach, a hammerhead ribozyme targeting human rhodopsin has been used to demonstrate the potential of these molecules when incorporated into a suppression and replacement gene therapy (Gorbatyuk *et al.*, 2005). Hammerhead ribozymes have also been applied to a porcine model of ADRP (Shaw *et al.*, 2001). Notably these studies demonstrate that reduction in levels of mutant rhodopsin can

provide therapeutic benefit in animal models. This view is further supported by work with human rhodopsin transgenic mice in which a direct correlation between severity of disease pathology and level of transgene expression was obtained (Olsson *et al.*, 1992).

While siRNAs have proven to be potent molecular tools for suppression of gene expression, ribozymes prior to the discovery of RNAi, represented one of the most promising tools available for gene suppression. For this reason, the ability of hammerhead ribozymes to target human peripherin/*rds* cDNA is assessed in the work described in this chapter. As such, it is hoped that the comparable specificities and efficiencies of parallel strategies involving suppression agents such as siRNAs and ribozymes for treating genes implicated in ADRP may be evaluated.

5.2 METHODS

5.2.1 Constructing *pH1-Mir23C*

Forward and reverse complement oligonucleotides (65-nt in length) were used to generate the Mir23C hairpin loop (fig. 5.2) and were ordered as desalted primers from Sigma_genosys. These sequences are listed in appendix 1A (Mi-23C F and Mi-23C R). The design of the oligos included the incorporation of the sense and antisense 19-nt sequence derived from the target site (for silencing); a BglII restriction site at the 5' end of one strand and a HindIII site at the 5' end of the homologous strand; and a 2-nt bulge at positions 9 and 10. The oligos were annealed and cloned into the pSuper vector backbone as described for the H1-vectors in section 3.2.3.

5.2.2 Cloning *pIRES-EGFP-HumRDS*

The full-length human peripherin cDNA including 5'UTR, kindly provided by Dr. Gabriel Travis, was cloned into the EcoRI site of pIRES2-EGFP (Clontech). (The general cloning techniques used are described in section 2.2.2).

5.2.3 Cloning *pcZeoRz8* and *pcZeoRz9*

Forward and reverse oligonucleotides encoding ribozyme 8 and ribozyme 9, 55-bp and 56-bp in length respectively, were ordered as desalted primers from Sigma_genosys. These ribozymes targeting the human peripherin transcript were selected following radiolabelling of test-tube cleavage reactions by Dr. Gearoid Tuohy. The ribozyme oligos were designed with XbaI and BamHI overhanging restriction sites to facilitate cloning (primers listed in appendix 3A). The oligos were reconstituted in ddH₂O to a concentration of 500 pMol and 1 µl of each the forward and reverse strands was added to 47 µl of ddH₂O to which 1 µl of 1 M NaCl was added. The mixture was heated to 94°C for 5 mins and then left to anneal overnight at room temperature. The pcDNA3.1/Zeo (-) (Invitrogen) vector was digested at the Bam HI and Xba I sites in the polylinker and 100 ng of the cut vector used in a 15 µl ligation reaction with a 1 µl volume of the annealed ribozymes. The ligation products were used to transform XL1B, colonies carrying recombinant plasmids identified by PCR and the plasmids purified using a Qiagen maxiprep kit according to manufacturer's instructions as described in section 2.2.2.

5.2.4 Generating double stable Ribozyme/humRDS COS7 cell line:

6 µg of plasmid pIRES-EGFP-HumRDS was used to transfect 1×10^6 COS7 cells using lipofectamine 2000 (Invitrogen) as described in section 2.2.5.4. At 48 hours post-transfection, cell medium was supplemented with 600 µg/ml G418 (Invitrogen). By growing the cells in G418-medium for three weeks a stable plasmid-expressing cell-line could be generated. Colony selection of clonal cell populations was made using cloning cylinders, (Sigma) as described in section 2.2.5.6. The peripherin transgene was deemed to be stably expressed based on real-time rtPCR results derived from three consecutive passage numbers of the chosen clonal cell line. This cell line was selected based on the expression level in real-time rtPCR analysis, whereby the RDS gene was amplified at the same cycle number as a GAPDH control gene. 6 µg of the plasmids pc3.1ZeoRz8 or pc3.1ZeoRz9 were subsequently used to transfect this peripherin line. A concentration of 400 µg/ml zeocin (Invitrogen) antibiotic was used to select for ribozyme expressing cells over 3–4 weeks. In this case cells were not viable for double-stable selection (discussed further in section 5.4.4) and a transient transfection protocol into the stable peripherin line (2.2.5.4) was subsequently used to analyse ribozyme activity.

5.2.5 Animal matings

The pro23his +/- mouse (Dryja *et al.*, 1992) was mated to a mouse knockout background, (rho/-). The pro23his+/--(MouRho/-) genotypes were selected by PCR analysis tail-tip DNA from the offspring (as outlined in materials and methods chapter, 2.2.6.1). In this way the transgene target level was kept constant between all animals tested for siRNA-based suppression.

5.2.6 Protein Analysis

Protein was extracted from mammalian cell culture as described in materials and methods chapter 2, section 2.2.4. Biorad assay to quantify total protein and Western blot analysis to quantify rhodopsin protein level is described in section 2.2.4.2 and 2.2.4.4.

All explant techniques including the dissociation, FACS, immunostaining and sectioning of explant tissue is described in chapter 2, section 2.2.6.

5.3 RESULTS

5.3.1 Treatment of the pro23his degeneration with siRNA

5.3.1.1 *In vitro* suppression of the pro23his rhodopsin cDNA with siC:

The pro23his mutation involves a C-to-A transversion in codon 23, encoding an amino acid located in the intradiscal region of the rhodopsin protein. Plasmid expressing pro23his human rhodopsin cDNA and driven by the CMV promoter was cotransfected with the plasmid pEGFP-siC in HeLa cells. Thus, prior to being evaluated in retinal explants, it was confirmed that the siC construct was still capable of silencing rhodopsin when the transcript carries the pro23his mutation to the same degree as achieved against the HR cDNA *in vitro* in chapter 3 (data not shown).

5.3.1.2 FACS analysis of the pro23his explant model 14 days post-electroporation:

The pro23his animal model carrying a human rhodopsin allele containing the pro23his missense mutation was originally developed by insertion and over-expression of this allele in transgenic mice, (Olsson *et al*, 1992). The pro23his model has been bred onto a L129 background and the offspring genotyped for the mutant allele. Genotyping involved using human-rhodopsin specific primers designed to amplify exon 2 of the gene by PCR (primer sequence in Appendix 1). The transgene segregates as an autosomal dominant allele and produces a severe retinal degeneration that progresses with increasing level of expression of the transgene in different transgenic lines (Olsson *et al*, 1992). The rapidity of the degeneration in this transgene line in-house in TCD, is such that photoreceptors are largely devastated by post-natal day 15, (P15). Retinal explants from the pro23his mouse were electroporated with pEGFP-siC at P0 in an attempt to down-regulate the level of pathogenic pro23his rhodopsin in early development and thus conserve enough transfected photoreceptor cells to allow analysis of the knockdown. While transfected müller glia cells were present as larger fluorescent cells, (indicating that electroporation had been successful at P0), transfected photoreceptor cells were not evident. The cultured retinas from the pro23his model were obtained from Dr. Marius Ader in the research team, (retinas were cultured in selective media for 14 days as

described in section 2.2.6). The two-week-old retinal explants were dissociated into individual cells, and FACS sorted according to size and granularity as described in section 3.1 (fig. 5.7).

While cells of the size and granularity typical of photoreceptors could not be detected in the 14-day pro23his retina, a large amount of cellular fragments were evident. This P4 population (as shown in fig 5.7) has not been observed in the FACS sorted populations of NHR (fig. 3.18) or HR5m (fig. 4.16) retinas described in chapter 3 and 4, respectively. Immunostained vibratome-sections (50 μ m) of these explants revealed the extent of photoreceptor cell loss. A representative section can be seen in the figure 5.8.

Retinal explants were therefore shown to have too low a level of rhodopsin for an effect on the message levels by siRNA to be assessed (figure 5.8). It may however be possible to determine whether a siRNA-based knockdown may be occurring at an earlier time-point. Suppression may thus possibly be assessed before the pathogenic effect of the pro23his mutation causes excessive loss of photoreceptor cells.

5.3.1.2 11 Days: Immunostaining pro23his retinas

Pro23his explant electroporated with pEGFP-siNT and pEGFP-siC at p0:

At an earlier time-point of 11 days, cells of the pro23his explants were dissociated and immunostained to determine if knockdown of rhodopsin is evident. Figure 5.9 shows cells following electroporation of a non-targeting siRNA plasmid, pEGFP-siNT, into the explants at P0. Arrows highlighting localised areas of fluorescence indicate delivery of the non-targeting construct pEGFP-nt, to the pro23his retina (fig. 5.9). These areas continue to show rhodopsin-specific immunostaining (fig. 5.9d). In contrast, figure 5.10 shows the effects of the targeting siRNA plasmid, pEGFP-siC on the pro23his retina at day 11 following electroporation at day 0. Arrows to localised areas of fluorescence indicate delivery of the targeting construct pEGFP-siC, to the pro23his retina. Overall rhodopsin staining is reduced as demonstrated in the areas indicated.

Four retinas for each construct (pEGFP-siNT and pEGFP-siC) were electroporated and cultured with selective medium for the 11-day period. At the end of this time, the cells of the explants were trypsinised and used to overlay poly-L-lysine-coated covers-slips for immunostaining. However there were very few cells

remaining from these degenerating retinas that were capable of fixing to the coverslips. The loss of photoreceptors is too great to determine the level of rhodopsin even at this earlier 11-day time-point. Typically the photoreceptor cells that survived carrying the mutant rhodopsin were preserved in tight clusters at the edge of the ONL. As illustrated in the dapi-stained pictures of the immunostained figures (fig. 5.9d), these cells, following dissociation remain as smears on the coverslips, potentially in multiple layers and do not offer a suitable spread for an accurate cell count of rhodopsin-positive and -negative cells. Therefore, despite some qualitative evidence that the construct pEGFP-siC may be specifically down-regulating rhodopsin in the degenerating pro23his retina, the loss of photoreceptors is too great to determine the level of rhodopsin suppression even at the earlier 11-day time-point for pro23his explants.

Given the rapid degeneration present in pro23his mice, it may be difficult to obtain a fully quantitative assessment of the effect of siRNA in the degenerating pro23his retina. Unfortunately, it was not possible, thus far, to quantify rhodopsin RNA or protein levels in the pro23his mouse, by applying the approaches used for the NHR and HR5m mouse models in chapters 3 and 4, respectively. However the above qualitative data (of fig. 5.9 and 5.10) adds support to the RNA and protein data derived from the NHR mouse. In this model significant down-regulation of the target gene was obtained with pEGFP-siC (fig. 3.19 and 3.21-22). RNA levels in the pro23his mouse are currently being generated for day 7, (subsequent to electroporation of pEGFP-siNT and pEGFP-siC into P0 explants). This may be the earliest time-point considered feasible for analysing the effect of siC silencing on rhodopsin expression (given that rhodopsin is expressed only from day 4, post-natally). By this 7-day time frame, it is hoped that in this very early stage of the degeneration, sufficient photoreceptor cells with rhodopsin expression will be present to distinguish if siRNA-mediated knockdown of rhodopsin message has occurred. The pro23his data shown in figs. 5.7–5.10 is further discussed in the discussion section (5.4.2).

5.3.2 A comparison of the suppression of human rhodopsin cDNA by siRNA and miRNA-based agents.

5.3.2.1 RNA analysis of miR23C cotransfection with pIRES-EGFP-NHR construct.

This study involves assessing the effect on HR cDNA expression of a modified version of siC, in which the NHR-targeting siC sequences include the loop sequence from the human *miR-23* miRNA. The resulting mir23-C construct includes a central mismatch in the sense sequence (fig. 5.2). Quantitative rtPCR analysis was used to measure human rhodopsin cDNA following cotransfection with the construct into HeLa cells. The cotransfection conditions of pIRES-NHR and the suppressor were kept at the same concentrations as used for the study of siC activity in chapters 3 and 4; 400ng of each of the suppressor and target plasmids were cotransfected into 5×10^4 HeLa cells. The cotransfections were carried out three times and the RNA for each extraction analysed twice by real-time rtPCR. The target human rhodopsin message was found to be upregulated compared to the non-targeting control when miR-23C was introduced (fig. 5.11). The upregulation was not, however, statistically significant ($t=0.06$) (Tables 5.1a and 5.1b). In summary, no suppression of the target human rhodopsin cDNA was obtained.

Given that a siRNA sequence had been engineered into a microRNA hairpin loop structure, it remained possible that translational inhibition may be occurring in the absence of post-transcriptional suppression. Although it was acknowledged that translational inhibition was a remote possibility, the unexpected result that RNA suppression should have been lost in this modified mir23C construct encouraged further investigation at the protein level. Targets for translation inhibition in mammals have only been identified in the 3'UTRs of genes. However, in the early days of this research, it was as yet unknown whether a microRNA-inhibition effect might be targeted in protein coding gene regions. Moreover, so-called trans-acting siRNAs (tasiRNAs) have been identified in plants, which are capable of exerting effects at both protein coding and untranslated regions of a transcript (Vaucharet *et al.*, 2005). Message may still be stably produced and may be detected prior to reaching the polysomes (Davidson *et al.*, 2004). In this case the down-regulation may only become obvious at the protein level; therefore a western blot was carried out to examine the effect of mi23-C on levels of the target rhodopsin protein.

5.3.2.2 Western blot analysis of protein expression from human rhodopsin cDNA in HeLa cells post-treatment with siC and mi23C:

To determine whether inhibition of translation of the rhodopsin cDNA may possibly be occurring, we used the monoclonal rhodopsin antibody, 4D2 (Hicks and Molday, 1986), to probe for rhodopsin protein in cells transfected in the same manner as for the RNA analysis described in 5.3.2.1. The target gene (pIRES-EGFP-HR) and either a plasmid expressing siRNA-C (pH1-siC) or a plasmid expressing siRNA-C modified with a microRNA loop from mi23 (pH1-mi23C) were transfected into HeLa cells (1×10^6 cells/transfection). Protein was extracted from cells 72 hours post-transfection, using methods described in section 2.2.4.1. The protein was initially quantified (2.2.4.2) and equivalent concentrations of each sample loaded on SDS-PAGE gels for analysis by western blot. The western blot of fig. 5.12 shows down-regulation of the rhodopsin target gene treated with pH1-siC at the protein level, (lane 3) however the rhodopsin protein is clearly present in cells treated with pH1-mi23C (lane 4). Strong rhodopsin signal was also detected for the two controls - protein samples from cells transfected with NHR alone (lane 1) and from cells cotransfected with non-targeting siRNA and NHR cDNA (lane 2). Although, these results are to be considered in the context that the protein experiment was only performed once, the findings would suggest that translational inhibition on NHR cDNA was not occurring using the miR23C construct and hence, support the findings at the RNA level of fig. 5.11.

In summary, the data presented in figs. 5.11 and 5.12 would suggest that the construct pH1-miR-23C is not capable of repressing rhodopsin mRNA or protein in HeLa cell culture. The results suggest that the down-regulation of rhodopsin mRNA and protein levels observed in chapter 3 is indeed caused by siRNA target transcript cleavage and is destroyed rather than enhanced by incorporating a microRNA-based loop into the hairpin and a mismatch in the sense arm.

5.3.3 Immune Response to RNAi

5.3.3.1 Examining interferon levels induced by siRNA targeting human rhodopsin:

RNA extracted from HeLa cells transfected with siRNAs siNT and siC was assessed to establish if an immune response had been activated in transfected cells. In mammalian cells, dsRNAs longer than 30bp can activate 1) the dsRNA-dependent kinase PKR, (leading to the inhibition of general translation by the eukaryotic factor EIF2 α); and 2) the enzyme 2'-5'-oligoadenylate synthetase, OAS-1 (causing non-specific degradation of mRNA via activation of RNase L) (Stark *et al.*, 1998). Both of these proteins are normally induced by interferon, however, by virtue of the small size of the siRNA molecules used, the dsRNAs used in this research, (siC and siB) are expected to avoid activation of the Interferon (IFN) response.

In spite of this, the potential for induction of an immune response by siRNAs or the vehicles in which siRNAs are delivered may still need to be monitored in the cell and mouse models used. Two distinct pathways are potentially provoked, one involving PKR, and leading to activation of the interferon cascade, and a second involving 2'-5'-oligoadenylate synthetase. It was therefore decided to test for expression from a gene that appears early in the interferon cascade, interferon-inducible protein α (Ifi- α), and to test directly for expression of OAS-1. The expression levels in each case were assessed by quantitative rt-PCR, using HPLC-purified primers specific for the genes implicated in the immune response, Ifi- α and OAS-1 (Appendix 1A).

5.3.3.2 Interferon in HeLa and HEK293 cell culture

Cotransfections of presynthesised siRNA, siC (Qiagen), and of plasmid-driven siRNA, H1-siNT and H1-siC into HeLa cells and HEK293 cells along with human rhodopsin cDNA were described in chapter 3 (see section 3.3.2). RNA extracted from these transfections, where rhodopsin-specific knockdown was observed, was analysed for the activation of a dsRNA-stimulated immune response. Primers specific for the genes encoding the two protein products induced by the interferon cascade— IFi α (encoding protein α) and OAS1 (encoding 2'-5'-oligoadenylate synthetase) were used in the real-time rtPCR analysis (primer sequences listed in section 1A). As a positive control for induction of an immune response, a long dsRNA, 60 bp in length, targeting mouse rhodopsin was transfected (courtesy of Dr. Sophie Kiang, TCD) into

cells and RNA extracted and amplified in real-time-rtPCR reaction using the Ifia and OAS1 primers as for the test RNA samples. No amplification of either interferon-response related transcripts were detected in the mammalian cell RNA analysed from the cotransfections undertaken in section 3.3.2. However, the long dsRNA construct used as a positive control for PKR activation also failed to cause an immune response (fig. 5.13). This lack of interferon induction for the positive control may not be unexpected in the embryonic line HEK293, where innate and independent functional immune activity may be lacking. However, HeLa cells, (where interferon induction has previously been shown by dsRNA – Yang *et al.*, 2002), similarly failed to show expression of immune response genes following cotransfection of the long dsRNA (60-bp) targeting the NHR transcript. Serving as a positive control, the long dsRNA molecule should, in principle, be capable of initiating a global shutdown of gene expression. It may be possible that the effects of the interferon-induced gene upregulation may precipitate cell death before the response can be measured, however as extensive cell death was not evident in these cell cultures this is considered unlikely.

Ultimately, there was no detectable expression of the immune response genes evident in the transformed cells where silencing of rhodopsin was detected. However, refinement of *in vitro* assays to monitor immune response due to dsRNA may be necessary for future RNAi experiments.

5.3.4 Alternative methods of knocking down retinal genes

Ribozymes targeting human peripherin, RDS.

The ability of hammerhead ribozymes to knockdown expression from the human peripherin/*rds* photoreceptor gene, (which, similar to the rhodopsin gene, is a significant source of ADRP-causing mutations – Kajiwara *et al.*, 1994), was assessed by transfection of plasmid carrying the CMV-driven ribozymes into a COS7 cell line stably expressing human peripherin.

The ribozymes tested were originally proven to possess catalytic activity when assessed in test-tube cleavage reactions (Tuohy, 2001). The primary aim of the ribozyme section of this chapter is to further assess the potential of these catalytic RNAs, termed rz8 and rz9, in gene therapy strategies for ADRP. Rz 8 and 9 (fig. 5.14a-b) were designed to target the 5'UTR of the peripherin transcript at NUX and GUX sites (CUA and GUU) at positions 234-236 and 190-192, respectively. The peripherin/*rds* cDNA was cloned as the first cistron in the bicistronic pIRES2-EGFP plasmid (Clontech) to generate pIRES-EGFP-HumRDS, as illustrated in fig 5.4.

Cotransfection of either ribozyme 8 or ribozyme 9 with peripherin/*rds* in COS7 cells showed no evidence by microscope analysis and by real-time rtPCR of down-regulation of the peripherin transcript by the ribozyme. However, transient co-expression of suppressor and target plasmid in cells (the approach used to analyse siRNA-mediated suppression in chapter 3) may not be optimal for analysis of ribozyme-mediated suppression. The kinetics of the ribozyme-mediated reaction is dependent on each individual ribozyme and overall levels of down-regulation are likely to be less than for RNAi suppression. Moreover, controls for transfection efficiency using a plasmid carrying a β -gal gene (methods section 2.2.5.5) showed 62% transfection when stained cells were counted under the microscope. A varying number of plasmids carrying ribozyme may be transfected into each cell, with the possibility that some cells may have many episomal plasmids; in contrast other cells may not be transfected with any ribozyme-carrying plasmid. Therefore to account for inaccuracies in the experimental design that may be introduced by the co-transfection of two plasmids, a COS7 line stably expressing human peripherin/*rds* was established (described in section 5.2.4). As mentioned previously, the IRES element in the peripherin construct used to make the stable peripherin cell line allows for

transcription of EGFP in tandem with peripherin/*rds* (figs. 5.4). The peripherin gene is therefore expressed on a transcript linked to EGFP, allowing, in principle, for a visual read-out of peripherin down-regulation at the protein level. However, no suppression of peripherin was detected following transfection of either ribozyme 8 or 9 into COS7- *rds*/peripherin cells compared to mock-transfected control of COS7-humRDS cells at the mRNA level (by real-time rtPCR) or protein level (by EGFP emission).

The COS7-HumRDS line was established by G418-selection of cells over a two-week period. Isolated colonies were selected and grown to confluency. Two cell lines showing consistent peripherin expression across three passages (as assessed by real-time rtPCR) were selected for this assay. Cell line A, showed on average 50% expression when compared to internal GAPDH control, while cell line B, showed on average 80% expression compared to GAPDH. Both lines A and B were used for transfection of ribozymes and the data was ultimately pooled to obtain the graph of fig. 5.15.

In this study it appears that hammerhead rz8 and rz9 failed to silence human peripherin/*rds* expression in a stably expressing peripherin cell line. It is possible that the level of suppression that would be achieved with the hammerhead ribozymes, rz8 and rz9, may be too low to determine accurately by transient transfection. A varying number of plasmids carrying ribozymes may be transfected into each cell, with the possibility that some cells may have many episomal plasmids; in contrast other cells may not receive any ribozyme-carrying plasmid. For a full assessment of suppression effects in cell culture, it may be necessary for the ribozymes to be stably integrated in the genome, (as is the target gene), and the effect of the ribozyme on suppression of the target gene monitored over time.

Therefore attempts were made to generate doubly stable COS7 cells. This cell line would be maintained by double-antibiotic selection — the ribozyme-carrying plasmid conferring zeocin-resistance, and the peripherin-carrying plasmid conferring neomycin-resistance. However, over a three-week period this selection process proved too aggressive for the COS7 cells. The peripherin/*rds* stable cells could be generated with three weeks of neomycin selection, however the cells continually failed to survive the second zeocin selection bottleneck. Further investigation of any Rz8/9-suppression effect in transformed cells has therefore been put on hold while alternative peripherin/*rds* expression systems are investigated.

5.4 DISCUSSION

5.4.1 Pro23his rhodopsin as a target for siRNA C

The pro23his mutation is the most common mutation causing ADRP, accounting for 15% of all cases in the US (estimate obtained from the RETNET database). As such, animal models are crucial for the investigation of the mechanism of degeneration and for the development and validation of potential therapies. As with many transgenic mouse models, the pro23his model was engineered based on the assumption that the sequences directing photoreceptor-specific expression of rod opsin are within the transcriptional unit or the flanking up- or downstream sequences in the opsin gene (Olsson *et al.*, 1992). Moreover it was hoped that the xenogenous nature of expression of the human pro23his opsin gene in the mouse would not compromise the processing of the human pro23his mutant protein. However the exaggerated degenerative phenotype in this transgenic model suggests that the mutant opsin derived from humans may be overexpressed or may be misdirected to a greater degree by chaperoning proteins in the mouse than it is in the human (Roof *et al.*, 1994). Paradoxically this phenotype makes the pro23his mouse less, rather than more, suitable as a model of retinal degeneration as the rapid spread of the degeneration affords little time to undertake intervention.

As for chapters 3 and 4 of this thesis a retinal explant model system was used for assessing the effect of siC-based suppression on endogenous rhodopsin transcripts. However in this case, the rhodopsin gene carries a mutation conferring a degeneration that has consequence for the experimental design. Testing the retinal explant system as to whether it represents a suitable platform for exploring therapies for the pro23his-degenerating retina became a key issue in this study.

Primary retinal cell cultures can be somewhat difficult to work with on account of limited supply, and finite life span. However retinal explants represent a suitable *ex vivo* method of assessing potential therapeutics in a retinal context. Efforts have been made, though with limited success, to develop appropriate model systems using proliferative cultures from retinoblastoma tumours (Reid *et al.*, 1974; McFall *et al.*, 1977; Seshedri *et al.*, 1986; Fournier *et al.*, 1987; Sery *et al.*, 1990). Many such cell lines have been developed, with the common feature of forming tumours *in vivo*, and have been used to help elucidate the role of the retinoblastoma susceptibility gene, *Rb*,

in tumour formation (Yi and Jie, 1990). Due to the transformed nature of these cell lines, the expression characteristics of such cells have been significantly altered and therefore do not accurately reflect the retinal environment. Retinal explants, on the other hand, retain the highest level of tissue preservation of all retinal cell culture systems and are therefore the material of choice for the assays. Explant cultures of whole or partial retina preserve the overall tissue architecture of the retina *in situ*. This is shown in the wild-type retinal explant presented in fig.5.8 (left panel).

The retinas from the pro23his mouse were removed and electroporated at p0 with shRNA constructs (pEGFP-siNT and pEGFP-siC) as outlined for the NHR and HR5m animal models in chapters 3 and 4. However, it was found that photoreceptor cell loss from the time of retinal development is too rapid to demonstrate successful therapeutic intervention in the explants from the pro23his mouse model. This is clear from the FACS analysis undertaken after two weeks of culturing the pro23his explanted retina. Figs. 5.7a and 5.7b, show a cell population profile significantly altered from that obtained from the NHR retina (chapter 3, fig. 3.18). Immunostaining of vibratome sections from these pro23his explants showed a reduced signal for rhodopsin in the outer segments (compare the stained pro23his retinal section with the wild-type mouse rhodopsin retinal section in figure 5.8).

Wu *et al.* similarly performed immunocytochemical studies on the pro23his mouse and have suggested that the protein is also present, though at a reduced level, in the ROS (1998). Moreover they found no evidence of rhodopsin trapped in the Golgi apparatus, implying that the pathology is not caused simply by retention of the molecule prior to trafficking. This group instead observed a loss of polarity in the rhodopsin molecule with the movement of the pro23his rhodopsin protein following exit from the golgi apparatus both to and away from the ROS. They suggest that the rate of delivery of the opsin molecule to the outer segment may therefore be too slow to facilitate disc formation at a rate required to compensate for the ongoing disc loss.

Subsequent to this finding, it was hoped that electroporation of newborn animals with the siRNAs at P0 and culturing for 11 days rather than 14 days would allow a sufficient time-frame for the siRNA, siC to achieve suppression while some pro23his rhodopsin-expressing cells still survive. The immunostaining of dissociated cells from the 11-day explants showed preliminary evidence of a siC-induced down-regulation of rhodopsin expression (fig. 5.9 and 5.10). However, a far reduced rhodopsin signal from the antibody label was obtained in these explants compared to

the NHR immunostainings of chapter 3 (fig. 3.20 and 3.21). The extent of photoreceptor loss in the siNT-treated retina suggests that a statistically relevant cell count would not be possible in this model, even at very early time-points such as day 11.

Recent evidence that reduction in rhodopsin gene expression does not need to be complete to achieve a significant improvement in the light-elicited responses of the eye is particularly encouraging for our pro23his studies. Geng *et al.* have shown how an autosomal dominant mutation Trp^{P365} in the *trp Drosophila* gene (transient receptor potential), a gene predominantly expressed in the fly photoreceptors, may be suppressed by RNAi (2004). The neuronal degeneration in the fly that results from Trp^{P365} expression can thus be prevented. It is worth noting that a 7-day pro23his trial is currently underway that may allow us to observe the effect of siRNA-based silencing of rhodopsin in 1-week-old pro23his retinal explants before embarking on exploration of the replacement aspect of the suppression and replacement strategy in this animal model.

5.4.2 MicroRNA analysis

The aim of the microRNA study described in this chapter is to investigate the potential of miRNA-based constructs targeting rhodopsin in loop 14 (the same region targeted in chapter 3) as a potential gene therapy strategy for ADRP. The common plasmid-based hairpin siRNA expression systems used to date are designed to activate RISC by mimicking the structure of endogenous regulatory dsRNA precursor species such as microRNAs (Brummelkamp *et al.*, 2002). These hairpin constructs may also serve as a more favourable substrate for exportin-5, the export factor for delivery of microRNA precursors from the nucleus to the cytosol (Lund *et al.*, 2003). The miRNA targets identified to date are conserved to the 3'UTR of genes. Some siRNAs have been designed to target known miRNA target sites (Doench *et al.*, 2003), however, known siRNA target sites have not been reported as candidates for miRNA-based inhibition. The miR23C molecule incorporating a microRNA loop structure was designed with the intention of testing whether miRNA structural and sequence-specific features might further reduce the production of rhodopsin protein compared to the reduction achieved by the purely siRNA-based molecule, siC, (chapter 3) targeting the same region of the mRNA.

5.4.2.1 The activity of H1-MiR-23C:

An important breakthrough in miRNA research has been the identification of the time-point in the transcription/translation machinery of a cell in which the miRNA is thought to intervene. The suppression effect of a microRNA is achieved at a later stage in the processing of an mRNA transcript (just prior to translation) than the stage at which siRNA-mediated gene silencing occurs (immediately post-transcription) (Doench *et al.*, 2003). As such, an indicator of a miRNA distinct from a siRNA effect is in the extent of reduction in protein rather than mRNA level. Davidson *et al.* recently reported the effect of vector-delivered siRNA on transcript and protein levels in primary neuronal cultures (Davidson *et al.*, 2004). While the siRNA showed specific effects on protein levels and function within 24 hours, there was no correlation with the effect on mRNA level. In one case mRNA decline was preceded by protein knockdown. While there was complete downregulation of protein at 6 hours, the mRNA level did not drop until the 24-hour mark. This they suggest may reflect a negative feedback loop derived from translational repression due to a miRNA effect on the targeted gene. To assess whether miR23C down-regulates expression of

the target gene rhodopsin, and to probe the nature of that down-regulation, RNA and protein levels were examined at 48 and 72 hours, respectively, post-transfection with miR-23C.

The silencing of human rhodopsin cDNA achieved using siC sequence was lost when incorporated into a mi23-based molecule with a central mismatch in the sense sequence typical of endogenous microRNA found enriched in cells. No change in rhodopsin mRNA level was detected when the pIRES-HR plasmid (expressing rhodopsin) was cotransfected with H1-miR23C or with H1-siNT (fig. 5.11). Similarly, cotransfection of H1- miR23C with pIRES-HR failed to achieve rhodopsin down-regulation at the protein level, where H1-siC was used as a positive control, and H1-siNT as a negative control for suppression (fig. 5.12). The relaxed requirements for the stem sequence of certain natural microRNAs may not extend to all endogenous miRNAs. The activity of miR23 may be more critically linked to its natural target site, and miRNA processing may not occur if alternative sequences (such as the sequence targeting loop 14 of human rhodopsin) are introduced.

5.4.2.2 Use of a 'pri-miRNA'-based shRNA construct for gene silencing

A number of miRNAs are encoded in the introns of primary mRNA transcripts. In many cases these miRNAs are encoded in the same orientation and may be driven by the same mRNA promoter as the parent transcript (Lagos-Quintana, 2003). This feature offers the possibility of adapting these miRNA motifs for pol-II-based tissue-specific expression of shRNA, by engineering pri-miRNA constructs incorporating the target sequence of interest. Using a native promoter to drive a pri-miRNA precursor it may be possible to efficiently generate siRNA through the endogenous miRNA processing system. Miyagishi *et al.* (2004) compared results using a simple adaptation of the miR30 loop in the shRNA hairpin construct (as discussed for miR23C above), to a (Pol II)-promoter driven 'pri-miR30' construct. The 'pri-miR30' expression vector was made up of the long precursor transcript of the miR30 microRNA (including much of the surrounding intronic sequence) with the miR30 sequence replaced by the target sequence for the luciferase gene. However the suppression observed for the (Pol II)-pri-miR30 design was less than achieved where the miR30 loop alone had been recruited in an shRNA molecule. In spite of this, the possibility of using pol-II promoters to drive expression of a pri-miRNA-based shRNA molecule broadens the potential application of gene silencing. For example,

the use of pol-II promoters provides an opportunity to achieve tissue-specific gene silencing. The identification of microRNA species specific to the tissue where the target gene is expressed would be preferable for use of pri-miRNA constructs in such gene silencing experiments (Krek *et al.*, 2005). The microRNA used in the experiments outlined in section 5.3.2, mir-23, was previously implicated in neural specification (Kawasaki *et al.*, 2003). It has been shown, since this experiment, that miR23 is in fact restricted in expression to astrocytes (Smirnova *et al.*, 2005). Conditional and tissue specific gene silencing in the photoreceptor cell may, in time, be achieved as retinal miRNAs come to be identified.

5.4.2.3 MicroRNAs and off-target suppression by siRNA.

Results from many relatively early studies on RNAi suggested that a single mismatch between siRNA and target mRNA was all that was necessary to abrogate silencing (Elbashir *et al.*, 2001). However in recent years microarray analyses have sketched a somewhat different picture of the effect dsRNA may be having in the cell (Jackson *et al.*, 2003; Semizarov *et al.*, 2003; Chi *et al.*, 2003). In one study the authors reported reductions in the level of RNA transcript from genes with as few as 11 contiguous matches to the siRNA (Jackson *et al.*, 2003). In a more recent study a 7-nucleotide motif in a siRNA was shown to be responsible for off-target effects (Lin *et al.*, 2005). Such a non-specific effect is typical of miRNA-based translational inhibition. It is clear that the sequence-complementarity requirements for translational repression are far less stringent than for the inhibition achieved by siRNA molecules. Such stringency has been illustrated in model systems (Doench *et al.*, 2003), and algorithms have been designed to search for low stringency sequence interactions between siRNAs and the 3'UTR of known human mRNAs (Scacheri *et al.*, 2004). As a result some transcripts at risk of translational repression from off-target miRNA-effects of siRNA molecules have been identified. We have observed a marginal level of silencing by an siRNA, 'siC' of a replacement rhodopsin cDNA with 5 nucleotide changes from the siC sequence (see section 4.3.8). The possibility of off-target miRNA effects for the rhodopsin gene should be considered in future studies involving suppression of the human rhodopsin gene and clearly need to be evaluated in the context of siRNAs which may be considered as potential gene medicines for human clinical trial.

5.4.3 Immune response to siRNA treatment *in vitro* and *in vivo*

It is becoming clear that siRNAs have broad and multiple effects beyond the selective silencing of target genes when introduced into cells. Early attempts to harness RNAi for study of gene function in mammals and for potential therapeutic value were ineffective. A robust antiviral defence system is triggered in mammalian systems upon exposure to dsRNA (reviewed in Williams, 1997). The breakthrough for RNAi technology occurred first of all with studies by Fire *et al.* in *C. elegans* and later with work in mammalian cell lines (Fire *et al.*, 1998; Elbashir *et al.* (2001a-c), as discussed in the review of RNAi in chapter 3 – section 3.1.3). These groups demonstrated how RNAi could be provoked to specific activity by using siRNAs that, by virtue of their small size, could bypass the dsRNA-dependent protein kinase (PKR) response. However even with the use of short 21-22nt siRNAs, there is still no certainty that this evolutionary immune response is overcome. Sledz *et al.*, for example, have reported an immune response caused by PKR-mediated activation of the interferon system, (IFN) upon introduction of 21-bp siRNAs (Sledz *et al.*, 2003). This group immunoprecipitated the dsRNA recognition protein, PKR, from siRNA-transfected cells and found that the 21-mer siRNA caused an increase in kinase activity similar to levels obtained in a positive control of transfected long dsRNA (>60 bp). In addition, Bridge *et al.* reported activation of this response when the mammalian cell was challenged with DNA vector-based dsRNA (Bridge *et al.*, 2003). The PKR-mediated induction of interferon was consistent for lentiviral vectors as well standard bluescript vectors. The magnitude of induction was dose-dependent; it was therefore suggested that saturation of the RNAi processing machinery and the accumulation of unprocessed Pol III transcripts might be triggering the interferon response (Bridge *et al.*, 2003). This dose-dependence may similarly be relevant to the immune response *in vivo*.

The IFN α/β protein as a component of the innate immune response provides a signal for initiation of adaptive immune responses (reviewed by Bonjardim , 2005). There is also evidence that IFN α/β may promote the differentiation of human peripheral blood monocytes into dendritic cells, which in turn have the ability to stimulate T-cell proliferation (Le Bon , 2002). The discovery of a TLR3-mediated dsRNA-responsive pathway in mammalian cells suggests that the extent of the

immune response to siRNA may not be simply limited to PKR (Alexopoulou *et al.*, 2001).

5.4.3.1 Immune response *in vitro*:

The transformed lines tested, (HEK293 and HeLa), showed no detectable activation of the candidate gene products involved in an immune response, interferon-inducible protein α , (IF- α), and oligoadenylate synthetase 1, (OAS-1) following transfection with siRNA. However, the positive control of a longer dsRNA (60 bp) failed to induce an immune response suggesting that the cell lines may be unsuitable cell types for assessing RNAi-induced immune responses. It is important to note that, as this control was synthesised in a different manner to the siRNAs being tested, i.e. by T4 phage polymerase rather than from a DNA-based vector, it may not in any case be the optimal control for this experiment. It is believed that the induction of interferon in HEK293 cells is less likely because of its embryonic immature status. However, Kim *et al.* have found that as little as 1 nM of *in vitro* transcribed siRNA is sufficient to induce an immune response in these cells (Kim *et al.*, 2004). Despite this finding, questions remain as to whether these cell lines fully reconstitute the endogenous signalling pathways active in innate immune cells, with other investigators suggesting that innate immunity is absent in HEK293 cells (Heil *et al.*, 2004).

Before further development of siC *in vivo*, assessing possible side effects of siC delivery to the retina *ex vivo* may be of use. Potential immune responses in the explanted retinal tissue from the mouse NHR model (as described in chapter 3, section 3.1.12) may therefore be analysed in future explant studies using siRNA. In particular, a measure of cytokine levels by ELISA is necessary to determine the extent and the nature of this response. The eye is traditionally considered to be an immune-privileged tissue and immune response gene products may be expressed at extremely low steady-state levels, therefore any increase in these levels may have potential phenotypic effects and hence such a study is worthwhile undertaking.

Distinct from a pure dsRNA-induced immune response, the H1 vector itself requires further investigation for future *in vivo* experiments concerned with immune response to shRNA-based RNAi. Delivery vectors are potential immune-stimulators in gene therapy, with CpG DNA in the plasmid from bacterial DNA provoking T-cell-

mediated inflammatory responses. The frequency of unmethylated CpGs in bacterial DNA is 3–4 times greater than methylated CpGs in vertebrate DNA. Therefore the vectors for gene therapy based on plasmids derived from bacterial DNA also have higher frequency of unmethylated CpGs (Krieg *et al.*, 1995). Such sites act as danger signals recognised by toll-like receptors on the surface of immune cells, such as B-lymphocytes, natural killer cells, macrophages and dendritic cells (Hemmi *et al.*, 2000). Sledz *et al.* found that IFN-mediated activation of the Jak-Stat pathway is in turn mediated by the dsRNA-dependent protein kinase, (PKR) which is activated by 21-bp siRNAs (2003). This result may suggest that a two-pronged immune attack may occur following H1-driven dsRNA transfection to mammalian cells: a response to the plasmid DNA and a response specific to siRNA. The latter immune response is hypothesised to be the core function of RNAi as a host defence pathway against foreign nucleic acids such as viruses and transposons (Finnegan and Matzke, 2003). The RNA-based silencing mechanism has been proposed as the “immune system” of the genome (Plasterk, 2002), hence it may be inevitable that siRNAs have broad effects beyond the selective silencing of homologous target genes when introduced into cells. A microarray analysis of both the transcriptome and proteome from siRNA-transfected cells may be necessary to determine such effects.

The risk of provoking immune responses following RNAi in mammalian cells has led to a renewed interest in alternative methods of suppression. While other suppression agents may be less potent than siRNA for targeted down-regulation of gene expression they may possibly be less likely to provoke a dsRNA-based inflammatory response in the cell and therefore may be more appropriate for some therapeutic applications. Hence, a ribozyme-based suppression strategy, in this case for peripherin/*rds*-linked ADRP, was investigated in parallel with the rhodopsin-based siRNA study and is discussed in the following section.

5.4.4 Hammerhead Ribozymes as tools for knockdown of the ADRP gene, human *rds*/peripherin.

Two hammerhead ribozymes, rz 8 and rz 9 (fig. 5.5), designed to cleave the 5'UTR of the human RDS/peripherin mRNA were expressed from the CMV promoter of the carrier plasmid pcDNA3.1(-)Zeo (Invitrogen). The peripherin target cloned into the pIRES2-EGFP vector was similarly expressed from a CMV promoter (fig. 5.4). Each ribozyme and the target cDNA were cotransfected into mammalian cell culture and the levels of peripherin expression compared to levels in cells transfected with the peripherin target alone. Real-time rtPCR was used to measure transcription from the pIRES-EGFP-HumRDS construct (using human peripherin and GAPDH primers). In addition, microscopy comparing the levels of fluorescence generated from the peripherin transcript (linked by an IRES site to EGFP) was used to provide an estimate of peripherin/RDS protein expression. There was no evidence of a change in peripherin expression following cotransfection of the pIRES-EGFP-HumRDS plasmid with ribozyme plasmids in these experiments (fig. 5.15). It is of note, that obtaining the optimal concentration of ribozyme in the correct sub-cellular location is vital when determining the potential of ribozyme-based strategies. In a subsequent analysis a COS7 line stably expressing human peripherin/RDS was generated (as described in section 5.2.4). However, no suppression of peripherin was detected following transfection of either ribozyme 8 or 9 into COS7- *rds*/peripherin cells compared to mock-transfected control of COS7-humRDS cells at the mRNA level (by real-time rtPCR) or protein level (by EGFP emission).

Ideally, in order to minimise the variation that may be introduced by the transfection procedure in a ribozyme suppression assay, analysis of a double stable cell line, expressing both the target gene and ribozyme may be necessary to identify any suppression. Notably, for ribozyme suppression, the host cell's machinery is not recruited to target the RNA. Unlike antisense or double-stranded RNA, a ribozyme must find its target and achieve its effect in isolation. However it is clear that the cellular environment has a profound effect on the kinetics of ribozyme-target RNA interaction; *in vitro* assays therefore need to be interpreted with caution, as they may at times inaccurately reflect ribozyme *in vivo* potency (Nedbal *et al.*, 1997). For example, ssRNA binding proteins of the nucleus (including the heterogeneous nuclear ribonucleoproteins, hnRNPs) have been shown to reduce the free energy of unwound

complementary RNAs and allow greater rates of ribozyme-target RNA hybridisation than would be seen in protein-free conditions. Moreover, the half-life of unmodified ribozymes in cells is unpredictable, (unmodified RNA has a half-life of seconds in bodily fluids). It is for these reasons that a cell line stably expressing both the ribozyme and the ribozyme target may offer a more accurate picture of ribozyme activity over time in a mammalian cell. Unfortunately, it was not possible to establish this line under the aggressive double antibiotic selection protocol used. Despite attempts involving varying concentrations of cycling antibiotics, double stable cells expressing the neomycin-resistant vector carrying peripherin/RDS as well as the zeocin-resistant vector carrying ribozyme could not be induced to survive long enough for analysis. Therefore no results for ribozyme effect on peripherin/RDS expression in double stable cell lines over several passages have been obtained.

However, the application of ribozyme suppression of ADRP-linked genes remains an attractive therapeutic strategy. This is particularly relevant in light of concerns regarding a potential siRNA-linked immune response (as discussed in section 5.4.3). A 2001 review by Soeth *et al.* states that of all the specific ribozymes delivered to mammalian cells and animals, there appear to have been no reports of non-specific RNA cleavage or unexpected side-effects, despite global gene screening analyses (2001). This appears to hold true to date, at least within the pubmed literature. Literature searches would also suggest that hammerhead ribozymes have proven their specificity. The hammerhead ribozyme tools combine the antisense methods with catalytic activity but do not recruit any endogenous cellular factors and therefore, (distinct from specific-target downregulation) they may be less inclined to cause disruption to a cell than RNAi-based methods. Ribozymes can be stabilized to improve their activity with various base substitutions and 3', 5' modifications (Burlina *et al.*, 1997; Earnshaw *et al.*, 1997; Eckstein *et al.*, 1997). For these reasons ribozymes remain an attractive tool for gene therapy strategies and hence continue to be explored in the development of suppression and replacement-based therapeutics for dominant disease.

The study described in this section of the thesis is distinct from previous sections of this thesis as it involves a different suppression and replacement approach for genes implicated in ADRP. The use of an alternative target, (peripherin/*rds*) an alternative method of suppression, (ribozymes) and an alternative

replacement strategy, (targeting the 5'UTR allows for modification of the UTR to produce an unaltered replacement protein) has been explored. While the aim of the strategy was to demonstrate the flexibility of the suppression and replacement approach, results from the study to date suggest that siRNAs may be more potent than ribozymes for the suppression aspect of the suppression and replacement strategy. Others in this laboratory (Palfi *et al.*, 2005) have demonstrated how siRNA can be effectively applied to the silencing of human peripherin/*rds*. While ribozymes, as artificial agents, may arguably be less disruptive to the regulatory circuits of the mammalian genome, the RNAi pathway offers greater opportunity for knocking down endogenous genes and hence achieving potential therapeutic effects. Overall, current evidence suggests that the use of siRNA for targeted suppression and replacement therapies may be the more effective therapeutic strategy for ADRP, assuming that potential non-specific and immune responses can be closely monitored.

5. FIGURES AND TABLES

Alternative pathways of RNA suppression

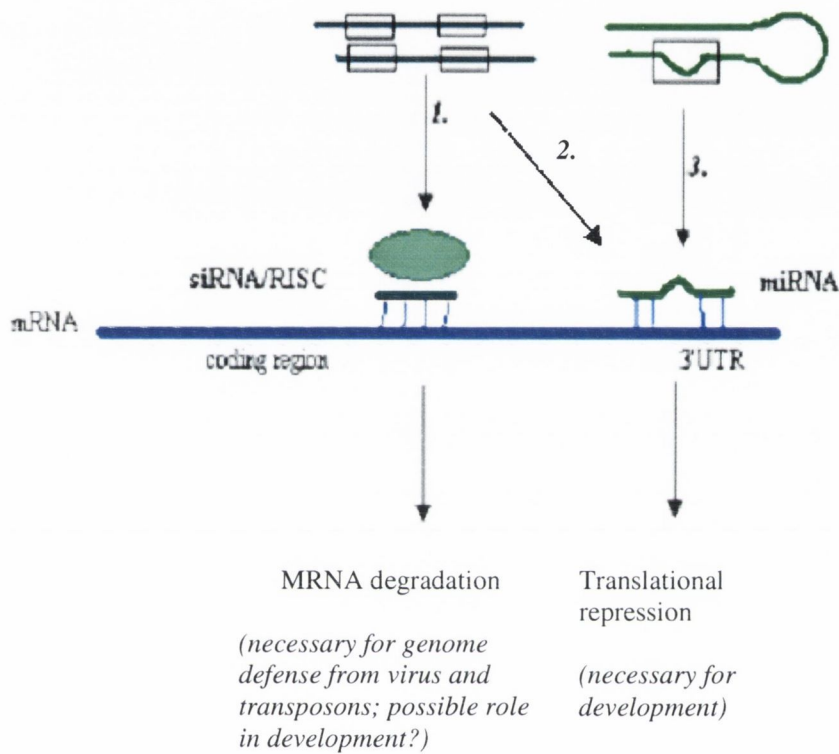


Fig. 5.1:

1. RNAi in plants; Post-transcriptional gene silencing in plants; quelling in *Neurospora* achieved with perfect sequence complementarity between antisense siRNA and target mRNA.
2. SiRNAs acting as microRNAs in off-target suppression of gene expression.
3. Translational arrest by imperfect base-pairing of miRNA to target, usually in the 3'UTR of the mRNA.

RISC: RNA-induced silencing complex; siRNA: small interfering RNA; miRNA: microRNA; 3'UTR: 3' Untranslated region.

Mir23C construct: Investigation of a microRNA-based siRNA molecule

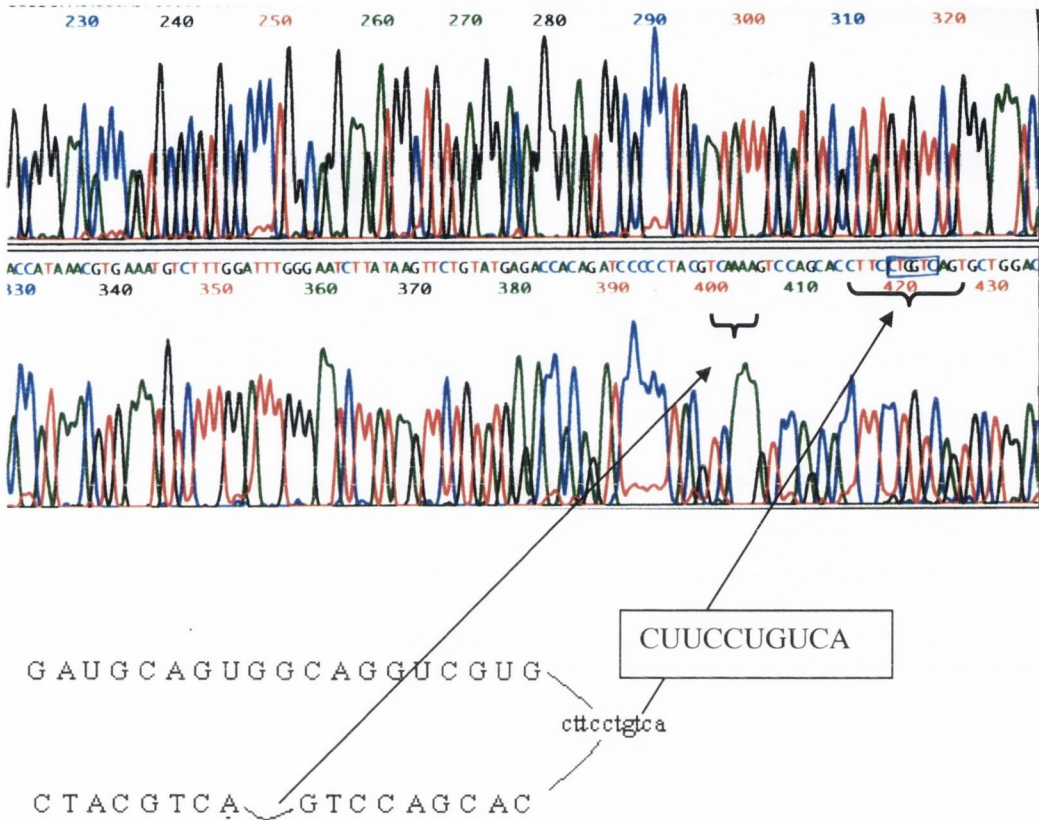


Fig. 5.2:

Electropherogram presentation of the miR23C sequence and the accompanying design of the miR23-based dsRNA molecule. The sequence shown is complementary to the loop-14 region of the human rhodopsin gene. This construct has been used to assess whether a suppression effect (conferred by the microRNA-based loop and the mismatch in the sense sequence of this construct) may be applied to a region of rhodopsin coding sequence shown in chapter 3 to be susceptible to post-transcriptional gene silencing.

Vector maps

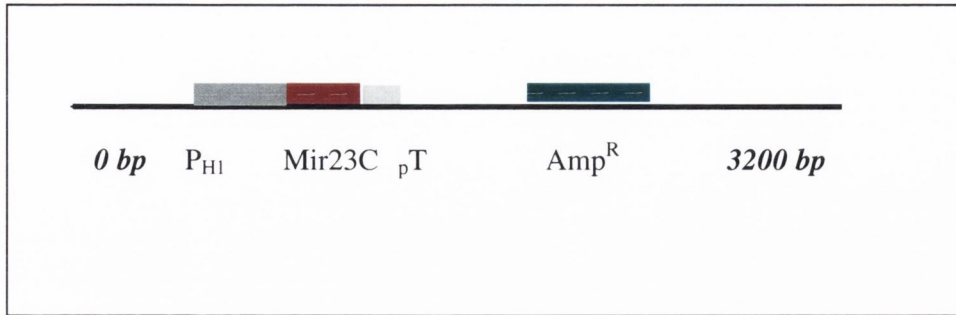


Fig. 5.3:

Vector map for the pH1-Mir23C construct. P_{H1}: H1 promoter; Mir23C: sequence as shown in fig. 5.2. The Mir23C oligonucleotide sequence contains the 19-nt target, in both sense and antisense orientation, separated by a 10-nt spacer sequence derived from the miR-23 loop (primers listed in appendix 1A). The resulting transcript can thus fold back on itself to form the 19-base pair stem-loop structure; Amp^R: Ampicillin-resistance gene; pT: poly-T tail.

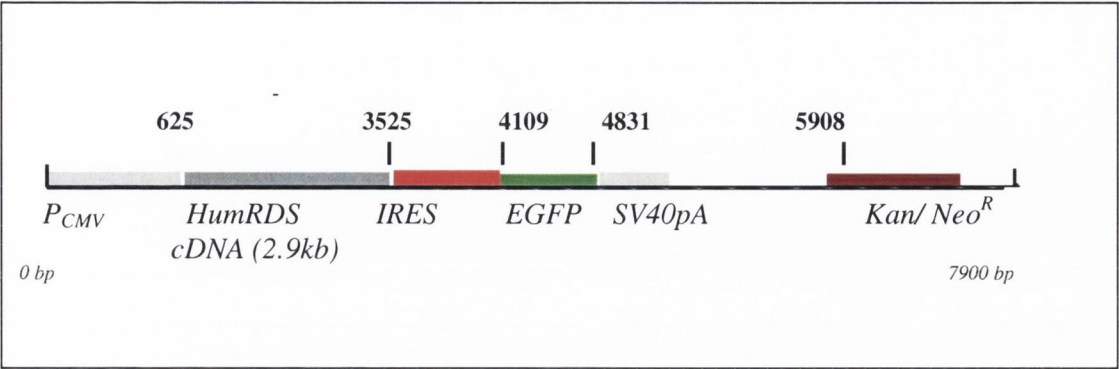


Fig. 5.4:

Vector map for the pIRES2-HumRDS construct, carrying the human *rds*/peripherin cDNA target (for experiments involving siRNA-mediated silencing). *P_{CMV}*: Cytomegalovirus promoter; HumRDS: 2.9 kb of the human peripherin/RDS cDNA; IRES: Internal Ribosome Entry Site; EGFP: Enhanced Green Fluorescent Protein; SV40pA: poly A from Simian virus-40 genome; Kan/ Neo^R: Kanamycin and Neomycin resistance gene.

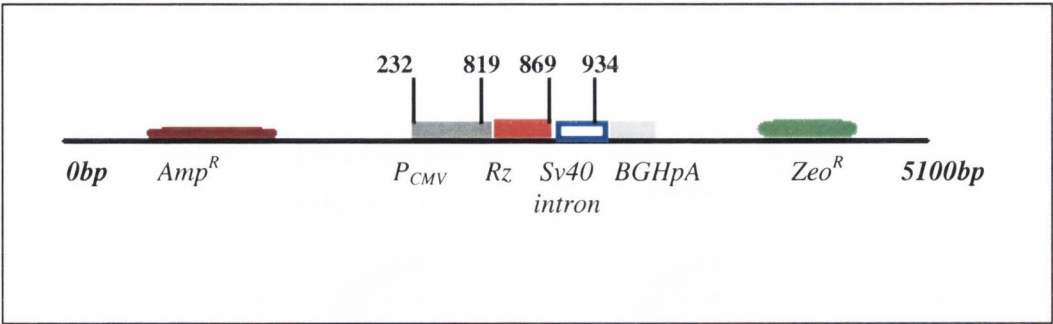


Fig. 5.5:

Vector map for pcZeoRz8/9. The ribozymes 8 and 9 targeting the 5'UTR of human peripherin/RDS were cloned into the pcDNA3.1/Zeo(-) vector (Invitrogen). This plasmid carries the zeocin resistant gene, allowing for dual selection of this construct with neomycin resistant plasmid in cell culture. P_{CMV} : Cytomegalovirus promoter; Rz: ribozyme oligonucleotides; Intron from Simian virus-40 genome; BGHpA: poly A from the Bovine growth hormone gene; Zeo^R : Zeocin-resistance gene; Amp^R : Ampicillin-resistance gene.

Animal Matings

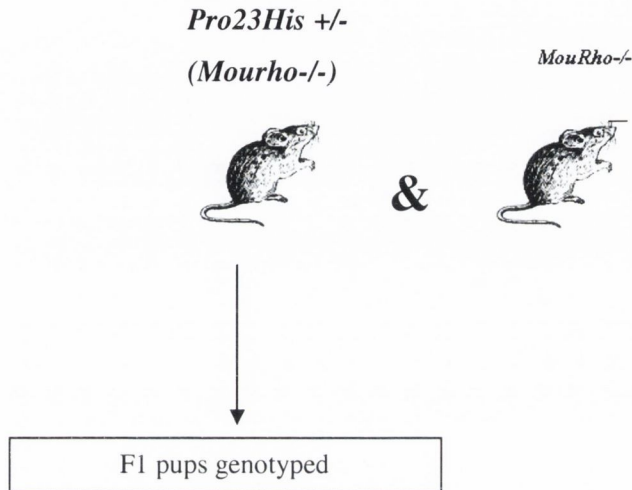


Fig. 5.6:

Figure showing the mouse matings set up for the retinal explant experiment described in section 5.3.1. Animals positive for the *pro23his* transgene and negative for endogenous mouse rhodopsin were identified by PCR genotyping of tail-tip DNA (as described in section 2.2.6.1) and were used in the *ex vivo* experiments outlined in this chapter.

Analysis of H1-siC-mediated silencing in the pro23his-rhodopsin degenerating mouse retina

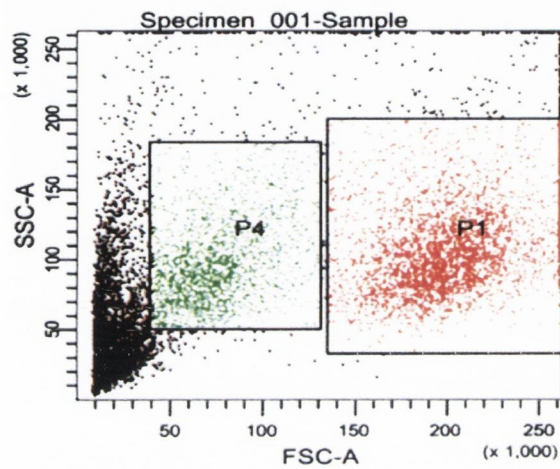


Fig. 5.7a:
The presence of a cell population that matched the scatter parameter set out for the P2 population of the NHR retina could not be detected in the 14-day pro23his retina (see chapter 3, fig. 3.18). In addition to the intact glial cells and remaining photoreceptor cells (P4) a large pool of cell fragments and debris (P1) was detected.

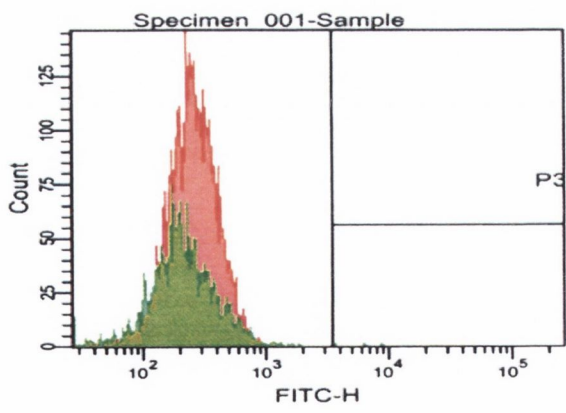


Fig 5.7b:
The P3 population is selected for size, granularity, and fluorescence and determined in section 3.3.8 to be derived from electroporation of the pEGFP-shRNA construct into photoreceptor cells. The P3 population could not be detected in the explanted pro23his retina when electroporated with the rhodopsin-targeting construct at P0 and sorted at 14 days.

Wild-type mouse explant

Pro23His explant

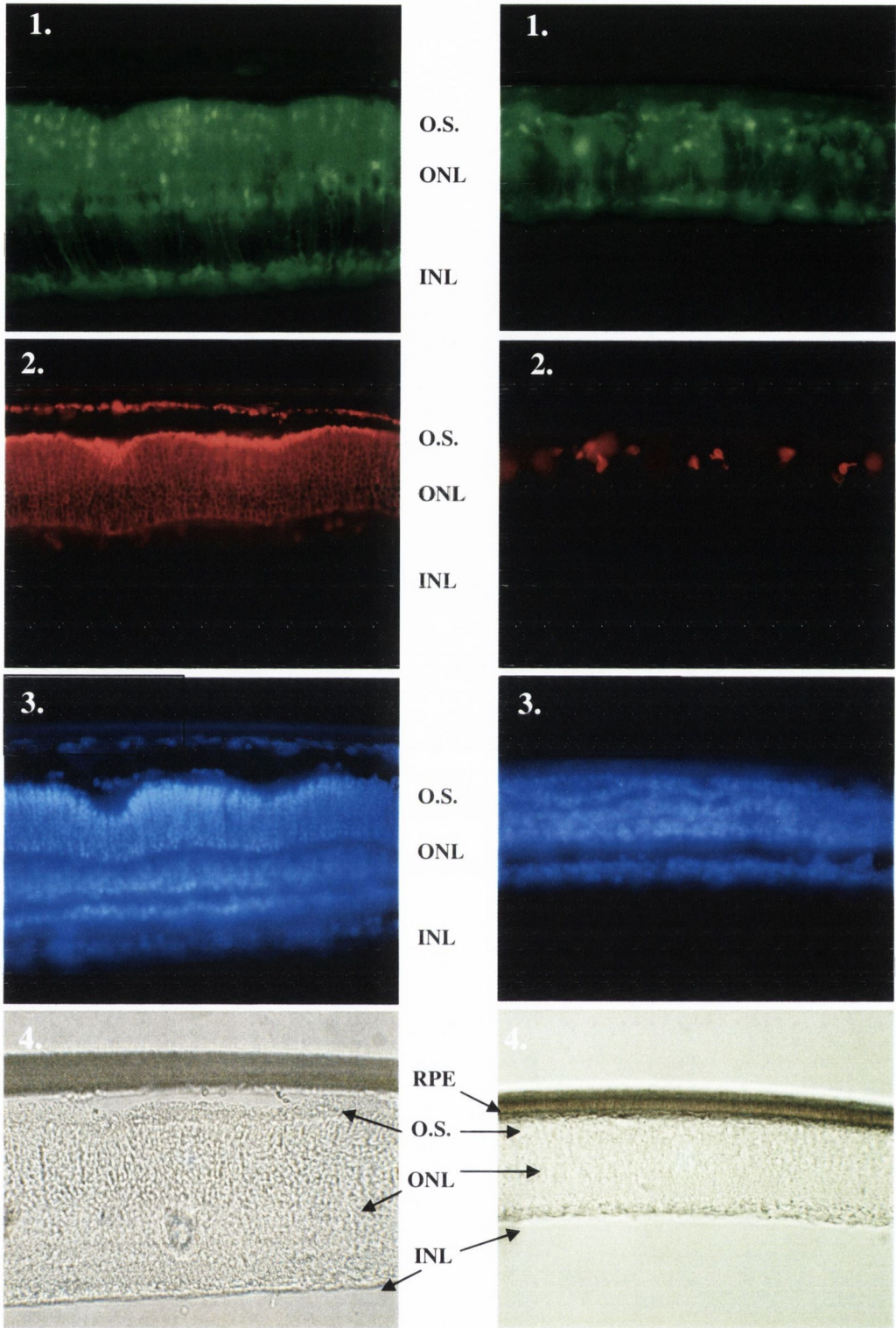


Fig. 5.8 Vibratome sections (50 μ m) of explants (2-weeks) electroporated with a pEGFP vector (Clontech). RPE: Retinal pigment epithelium; OS: Outer segment; ONL: Outer Nuclear Layer; INL: Inner Nuclear Layer. Microscope views: 1: EGFP; 2: 4D2-Rho antibody immunostain; 3: Dapi-nuclear stain; 4: White light.

Pro23his explant electroporated with pEGFP-siNT at p0

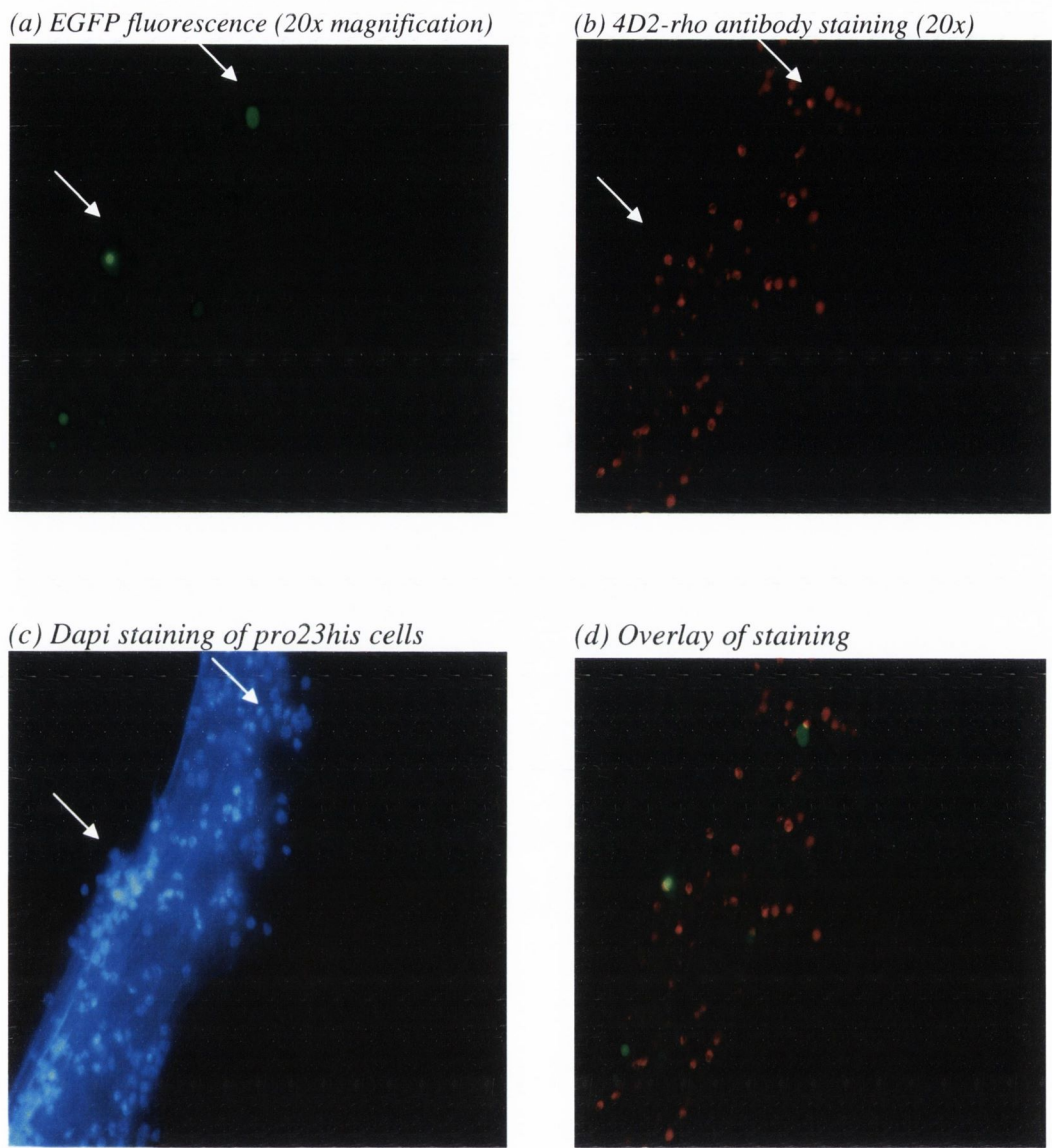
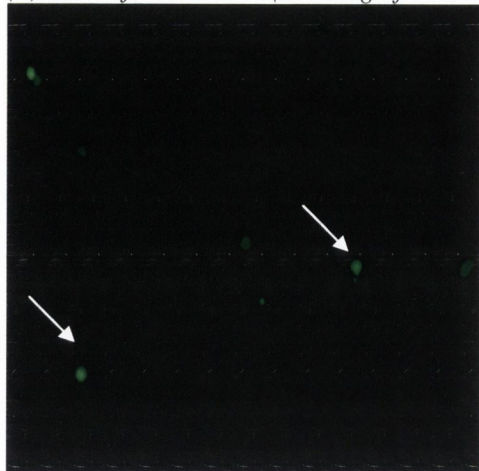


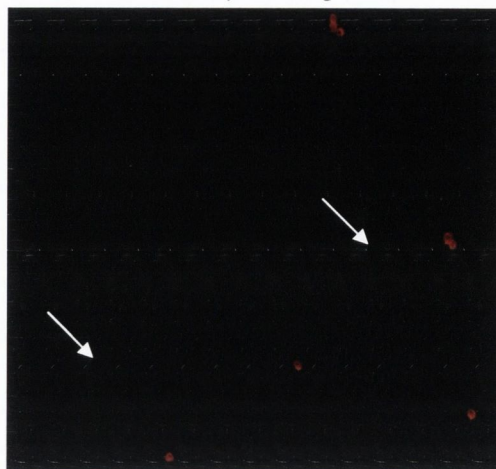
Fig 5.9: 20X microscope images of coverslips coated with pro23his retinal cells electroporated with pEGFP-siNT, (the non-targeting siRNA). Eight coverslips were examined coated with cells from four retinas; The EGFP picture shows electroporated cells (a); the red alexa-fluor labelled cells shows those cells positive for pro23his rhodopsin (4D2 antibody) (b); the dapi-nuclear stain shows the total cells in view (c); and an overlay of the green and the red shows the cells transfected with pEGFP- siNT and still expressing pro23his rhodopsin (d).

Pro23his explant electroporated with pEGFP-siC at p0

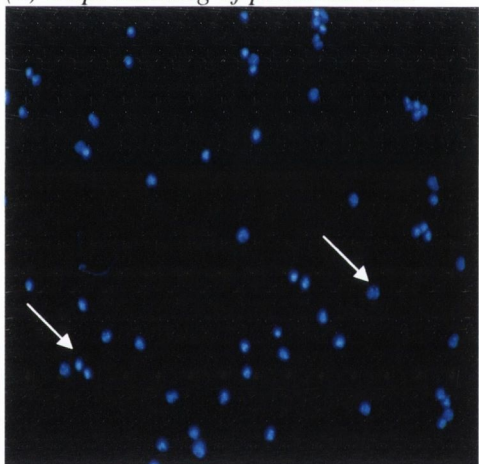
(a) EGFP fluorescence (20x magnification)



(b) 4D2-rho antibody staining (20x)



(c) Dapi staining of pro23his cells



(d) Overlay

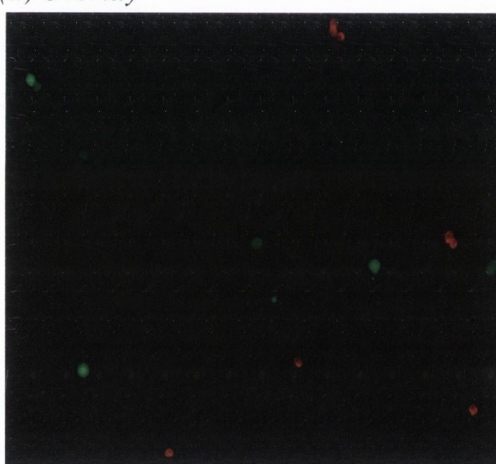


Fig 5.10:

20X microscope images of coverslips coated with pro23his retinal cells electroporated with pEGFP-siC (rhodopsin-targeting siRNA). Eight coverslips were examined coated with cells from four retinas; The EGFP picture shows electroporated cells (a); the red alexa-fluor labelled cells shows those cells positive for pro23his rhodopsin (4D2 antibody) (b); the dapi-nuclear stain shows the total cells in view (c); and an overlay of the green and the red shows the cells transfected with pEGFP- siNT and still expressing pro23his rhodopsin (d).

Effect of mir23C-shRNA on rhodopsin RNA levels *in vitro*

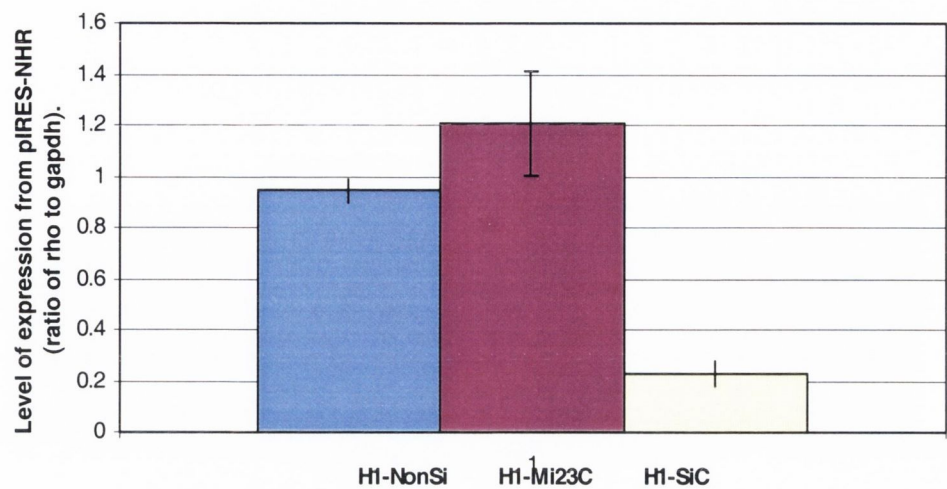


Fig 5.11: Graph of rhodopsin expression in HeLa cells following treatment with H1-dsRNA constructs.

Average rho expression ratios		Std Deviation
H1-dsRNA	Rho/Gap	
H1-siNT	0.945	0.093338
H1-Mi23C	1.21	0.406; $t = 0.061$
H1-SiC	0.227	0.151; $t = 0.02$

Table 5.1a: Table showing the raw data for fig. 5.11. Rhodopsin expression levels normalised to cellular GAPDH levels following targeted siRNA and miRNA-based suppression. Cotransfection of H1-dsRNA constructs and pIRES-NHR was carried out in HeLa cells. While the p -value for the difference between H1-siNT and H1-Mir23C was not statistically significant ($p = 0.061$), the p -value for H1-siNT compared to H1-siC was significant ($p = 0.02$).

SiRNA	Rho expression
Non	100%
SiC	24.02%
MiR-23C	128.04%

Table 5.1b: Table showing the level of rhodopsin expression from pIRES-EGFP-HR where suppression by H1-construct with non-targeting siRNA is fixed at 100%.

Effect of mir23C-shRNA on rhodopsin protein expression *in vitro*

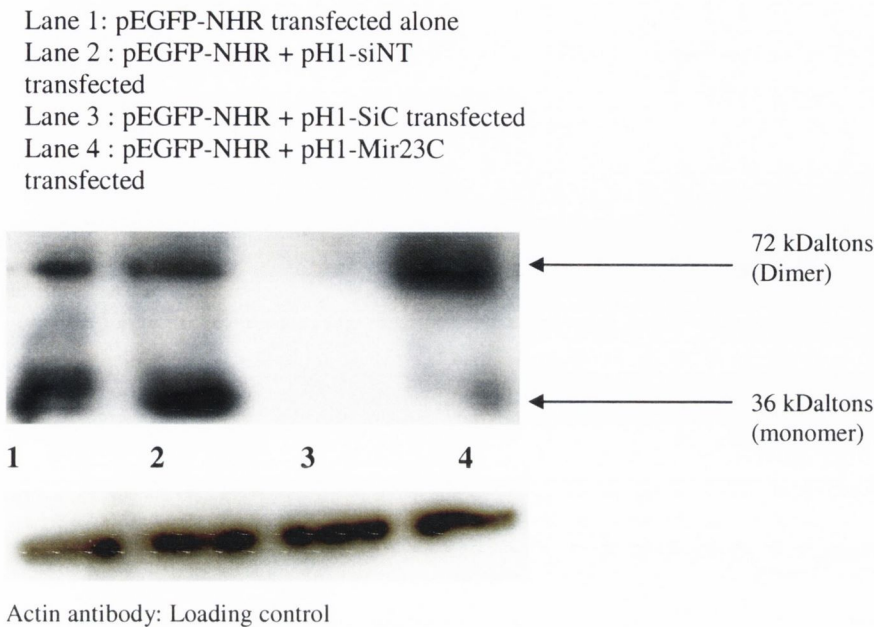
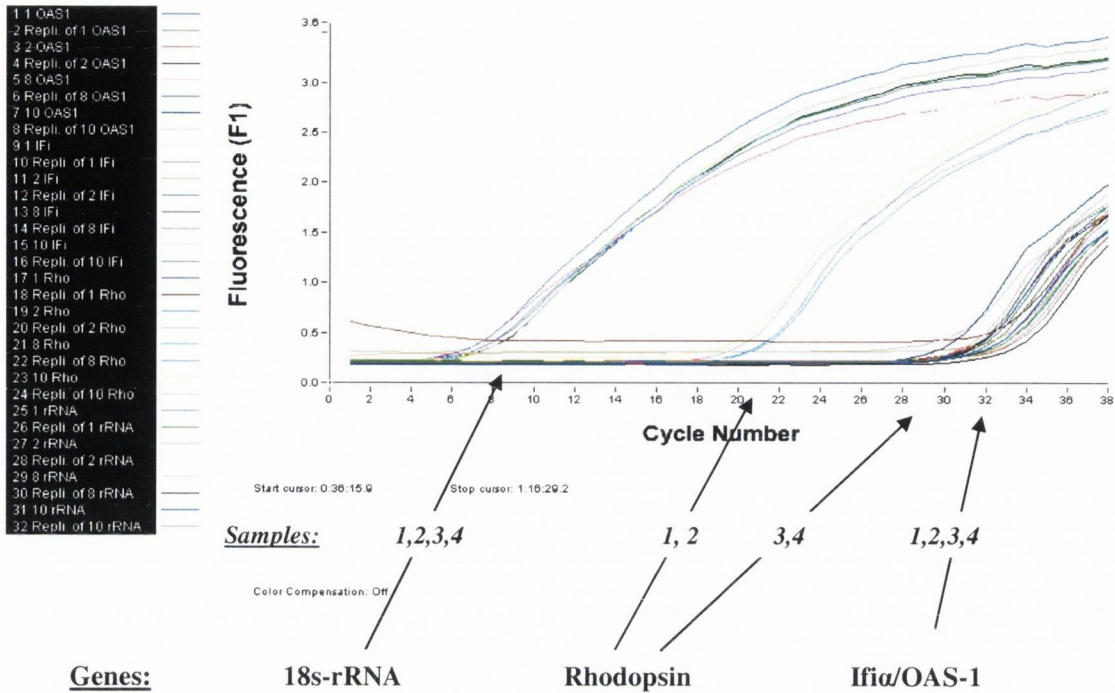


Fig.5.12:

Western Blot showing the protein expression from HeLa cells transfected with siRNA and miRNA constructs. Protein samples from the cells were run on polyacrylamide gels, transferred to PVDF membranes before probing for rhodopsin or β -actin expression (anti-Rho4D2, 1:300; anti-actin, 1:4000) and visualising by chemiluminescence as described in section 2.4.4.4). Lanes 1 and 2 show the NHR target transfected alone or with pH1-siNT, respectively. Lanes 3 and 4 show NHR target transfected with pH1-siC or pH1-Mir23C, respectively. Lane 3 shows a far reduced rhodopsin signal while lane 4 shows no reduction in rhodopsin signal, with much of the protein banding as a dimerised molecule. The rhodopsin protein has a tendency to dimerise following the denaturation step and during the heating of the purified protein.

Immune response to siRNA and shRNA agents *in vitro*



Genes: 18s-rRNA Rhodopsin Ifia/OAS-1

Samples: 1, 2, 3, 4 1, 2 3, 4 1, 2, 3, 4

Sample 1: *Hela* cells transfected with pIRES-EGFP-HR and long dsRNA (60 bp)
Sample 2: *Hela* cells transfected with pIRES-EGFP-HR and siNT
Sample 3: *Hela* cells transfected with pIRES-EGFP-HR and siC
Sample 4: *Hela* cells transfected with pIRES-EGFP-HR and shRNA-C

Fig. 5.13:

Report from a quantitative rtPCR run on RNA extracted from *Hela* cells. The cells were cotransfected with rhodopsin-expressing plasmid (pIRES-EGFP-HR), and either long dsRNA (sample 1), siRNA molecules (samples 2 and 3) or shRNA vector (sample 4). Three sets of transfections and RNA extractions were performed and the RNA samples were tested in duplicate PCR reactions. The individual graphs shows the level of fluorescence emitted by the amplified gene product at a given PCR cycle number for each primer tested. At later rounds of amplification, (>30 cycles), background amplification through non-specific binding of the primers may occur. All samples 1, 2, 3 and 4 show high levels of 18S ribosomal RNA gene expression (amplified at cycle number 5); samples 1 and 2 show intermediate levels of rhodopsin expression from the pIRES-EGFP-HR plasmid cotransfected with non-targeting siRNA (amplified at cycle number 20), while samples 3 and 4 which were cotransfected with pIRES-EGFP-HR and siRNA agents targeting rhodopsin show very low or background rhodopsin expression (cycle number >30); the immune response genes do not appear to be amplified in any of the samples (cycle number >30).

Ribozyme-mediated silencing of ADRP-linked gene expression

Ribozyme 9

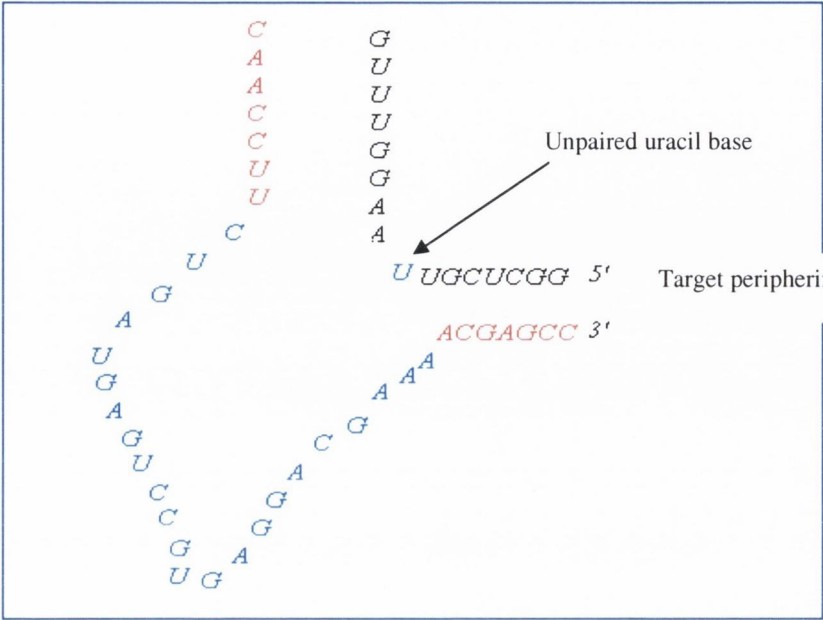


Fig 5.14a: Hammerhead Ribozyme 9

Ribozyme 8

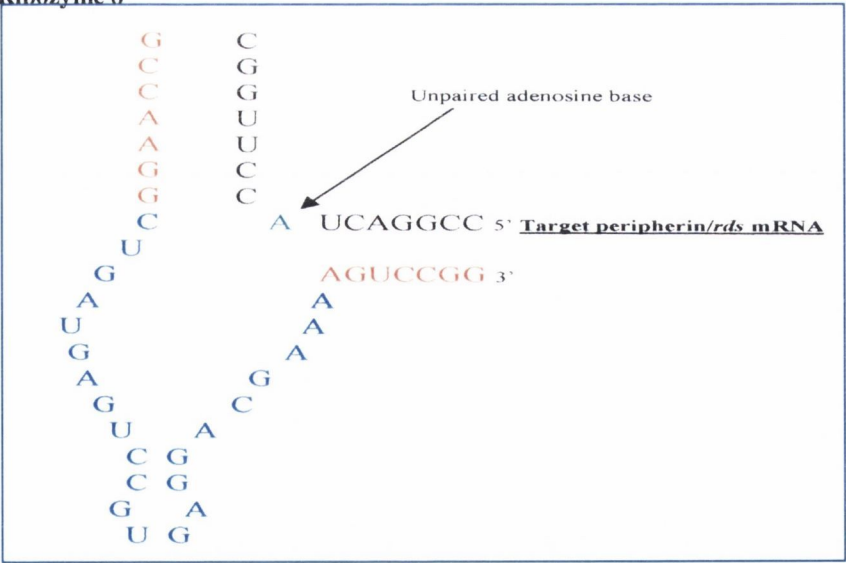


Fig. 5.14b: Hammerhead Ribozyme 8

Expression level of human *rds*/peripherin in COS7-humRDS cells following transfection with hammerhead ribozymes

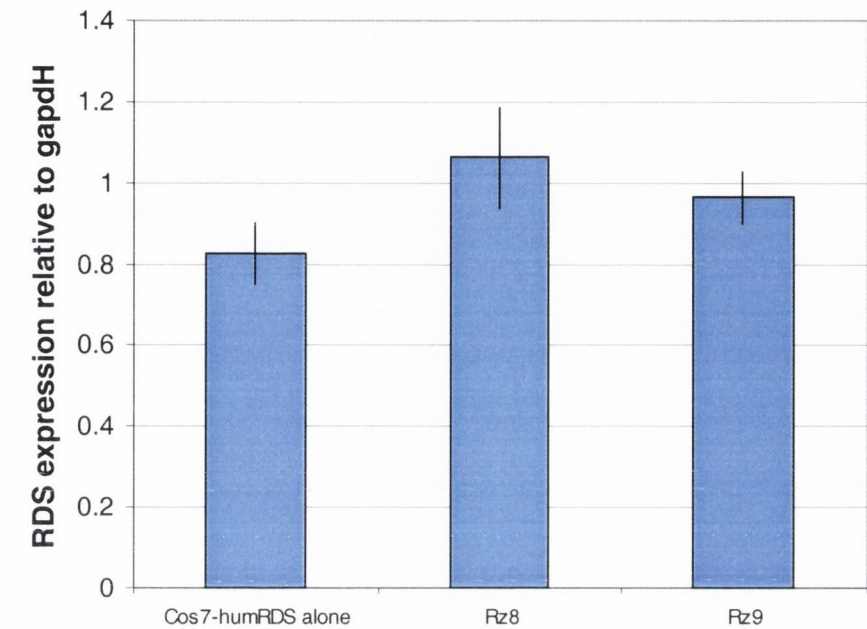


Fig.5.15: Chart showing peripherin/*rds* expression in a COS7-humRDS stable line. The level of expression was analysed from RNA extracted from the cells at 48 hours post-transfection.

Transfection into COS7-RDS cells	Cells alone	Ribozyme 8	Ribozyme 9
humRDS/GAPDH	0.83	1.06	0.96
S.d.	0.167	0.246	0.126
t-value	rz8: $t = 0.32$; rz9: $t = 0.41$.		

Table 5.3: Peripherin gene expression normalised to GAPDH expression in COS7-humRDS cells following transfection with ribozymes targeting the 5'UTR of peripherin. Three sets of transfections of each ribozyme was carried out in the cells and the results from real-time rtPCR analysis performed on each RNA extraction pooled to obtain the average values shown in table 5.3.

Chapter 6

Gene delivery to the mammalian retina

CHAPTER 6

6.1 INTRODUCTION

6.1.1 Objectives of chapter 6

In this chapter initial data from attempts to achieve long-term silencing of the human rhodopsin gene will be presented. Progress in gene therapy for ADRP, will require retinal-specific vectors that stably express a therapeutic combined with efficient delivery methodologies. Efficient gene transfer into postmitotic somatic cells, in principle, would enable analysis of the therapeutic effect of the encoded protein for many disease states. In this study, a non-viral and a viral approach for gene delivery shall be compared for their suitability as vehicles for stable expression of siRNA for therapeutic purposes.

Inevitably, there are problems associated with achieving stable and long-term gene expression in most cell types, including those of the retina. Irrespective of the stable expression of a transgene, the mechanical aspect of gene delivery to photoreceptors, even for short-term assessment, is technically difficult. Many research efforts are currently directed towards bypassing the RPE and targeting expression to the photoreceptor cell layer. This has led, in clinical trials, to the optimisation of iontophoresis (Behar-Cohen, 2004), while in ongoing mouse studies, electroporation as well as intra-vitreous and sub-retinal injection amongst other methodologies have been employed. Approaches based on electroporation are typically effective *in vitro* but are limited, in part, by the ability to generate the electrical field *in vivo*. Bioimplants containing growth factor transgenes have also been employed and have been shown to have a therapeutic effect in retinal degenerations (Thanos *et al.*, 2001; Tao *et al.*, 2001; Uteza *et al.*, 1999). Combining the skills and expertise of research groups enables an assessment of the suitability of most current delivery techniques for different diseases. An initial evaluation of a number of methods for delivery to the retina forms the focus of the research described in this chapter.

When choosing a gene transfer vehicle for the retina, requirements may include: a large transgene capacity; appropriate regulators or inducible promoters; the ability to generate a high viral titre for delivery; stability of transgene expression; a

targeting strategy for the appropriate cell type; and a lack of toxicity and immune response. Although the eye is considered to have immune privilege, inflammatory responses do occur and damage to healthy ocular tissue is a potential risk.

6.1.2 Non-Viral Gene Transfer

Many researchers believe that nonviral vectors may be the future for gene transfer following the reported limitations of viral vectors due to their potential pathogenicity associated with random integration and immune responses. Non-viral gene delivery systems include naked DNA, cationic lipids and polycationic polymers amongst others. Naked DNA typically consists of a plasmid DNA expression cassette delivered into the tissue by injection. Cationic polypeptides, such as polylysine, may be recruited to condense the DNA (Jiao *et al.*, 1993). Cationic lipids encapsulate DNA in positively charged complexes. Liposomes can enter the cell by endocytosis and often carry protective fusion proteins to avoid degradation by the endocytic pathway. These complexes may also be equipped with nuclear localisation signals to aid transfer through the cytoplasm to the nucleus (Aronsohn *et al.*, 1998). Zhang *et al.* have engineered liposomes that have been modified specifically for retinal uptake (Zhang *et al.*, 2003). The surfaces of these liposomes are conjugated with many strands of polyethylene glycol that may minimise uptake by the reticulo-endothelial system. Specialised targeting ligands on the liposome may trigger receptor-mediated endocytosis into cells and facilitate transcytosis across the blood-brain barrier.

6.1.2.1 Site-selective non-viral gene transfer:

Selectivity may be achieved when integration occurs by homologous recombination between the transfer vehicle and the host genome. The efficiency of recombination is generally too low for use in gene therapy although a technique called small fragment homologous recombination, (SFHR), has been contemplated in this regard (Weber *et al.*, 2003). Site-specific recombinases, such as Cre recombinase, derived from *E. coli* phage P1 may perform integration and excision efficiently without the need for co-factors in mammalian systems (Sauer, 1994). The net integration frequency is low, estimated at merely 0.03% for the Cre recombinase system (O’Gorman *et al.*, 1991).

Most recombinases also perform both integration and excision events using the same target site, (Weber *et al.*, 2003). The net integration frequency is therefore further reduced by repeated excision in a back reaction at the integration site. Recombinases that perform only the integration reaction and require accessory factors for the reverse reaction typically offer higher integration rates (Vega *et al.*, 1991). Some phage integrases have this property, e.g. the integrase from the *Streptomyces* phage ϕ c31 (Sauer, 1994). These integrases carry out recombination between attachment sites on the phage, known as attP sites and attachment sites on the bacterial genome, attB sites (Thorpe *et al.*, 1998). In 2000 Calos's research team *et al.* determined the minimal sizes of these sites with the aim of harnessing their function in mammalian cells (Groth *et al.*, 2000). The team demonstrated that by using these compact integration sites, ϕ c31 integrase could function in 293T cells. The minimal consensus sequence size was 34 base pairs for attB and 39 base pairs for attP, making it statistically feasible that there would be endogenous chromosomal targets with significant sequence homology to the attachment sites in human and mouse genomes. Such sites are referred to as ψ att sites. The expected rarity of suitable ψ att sites in the human genome, in principle, should ensure specific gene targeting. Calos *et al.*, 2002, however found no more than 56% identity between the closest fit ψ attP human site and the consensus attP site. The team are currently pursuing a directed evolution strategy on the recombinase to achieve higher sequence and integration specificity.

6.1.2.2 ϕ c31 integrase in gene therapy:

ϕ c31 integrase was found to be fully functional in human cells implying a lack of requirement for streptomyces-specific cofactors (O'Gorman *et al.*, 1991). Calos *et al.* achieved a specific integration frequency more than two orders of magnitude higher than that of Cre recombinase. This may in part be a consequence of the uni-directional nature of this integration with two distinct sites, attP and attB as opposed to the identical dual loxP sites of the Cre recombinase system. The frequency of integration at ψ att sites competed favourably with the background of random integration with 80–90% integration above background reported (Thorpe *et al.*, 1998). This specificity of integration is in spite of the low sequence homology between the human pseudo-attP site and the phage consensus sequence for

integration. By sequencing ψ att-genome junctions of rescued plasmids from human cells, five sites of integration were found. One site, ψ A on chromosome 8 accounted for 32 out of 67 integration events observed. The remaining events were divided equally between another 4 sites. The result obtained in this study was significant – in essence, out of the 3×10^9 bp of the human genome, the team had managed to restrict integration to approximately 10^2 bp. The integrase system was first put to the test in a disease setting for the recessive form of the skin disorder *Epidermolysis bullosa* (Ortiz-Urda *et al*, 2002). Primary RDEB epidermal cells were cotransfected with a plasmid carrying both a replacement col7A1 gene, the gene mutated in RDEB, an attB site, and an integrase-expressing plasmid. Stably integrated cells were enriched by a short course of selection. As a result normal skin tissue could be produced. In a second paper, Calos *et al.* (2001) used a high-pressure tail-vein injection to deliver and integrate the hFIX gene permanently into the ψ A site and indeed other sites of the mouse genome. The benefit of delivery could be seen in histological analysis of liver sections. No immune response to integrase in mouse was reported.

Stimulated by the promising results obtained in these and other studies, we have chosen to explore this non-viral vector system as a potentially promising way of delivering stable expression of nucleotide-based therapeutic to human retinal cells (see figure 6.2).

6.1.3 Viral Gene Transfer

To date, the poor efficiency of gene transfer *in vivo* has been a major limitation of non-viral vectors. Viruses have evolved very efficient mechanisms to introduce their DNA into recipient cells. Therefore viral vectors have become important research tools in somatic gene therapy. Several animal models have been used to demonstrate the potential of viral vector mediated gene transfer in the nervous system. It is considered easier to extrapolate viral dosage according to the size of the eye from animals to humans, than it is for most organs as the comparative size of the eye has been well characterised and remains relatively fixed between animals (Bennett *et al.*, 1999). A number of viral vector systems have thus far been evaluated for introduction of foreign genes to the eye and CNS. These include herpes simplex virus (HSV), retroviruses such as lentivirus, adenovirus (AD), and adeno-associated virus (AAV). Shortcomings in all these systems have impeded progress, and certain

viruses may be better suited than others. We considered these viral vectors in terms of their ability to transduce retinal cells and their ability to deliver suppressing agents, in particular dsRNA. AAV was chosen for the experiments that are described in this chapter of the thesis and a description of the AAV system will follow a more general introduction to the alternative viral vector system which have been used for delivery to the mammalian retina.

6.1.3.1 Herpes Simplex Virus:

Replication-defective HSV vectors have been shown to have several potential advantages for gene transfer to the nervous system (Chattopadhyay *et al.*, 2004). Such a vector has been engineered such that it is non-pathogenic but retains the ability of the wild-type virus to efficiently enter neuronal cells. The efficiency of this transduction means that in principle relatively low titres are necessary to produce therapeutic effects. This minimises the risk of immune response to virus and the risk of introducing contaminating wild-type virus. Furthermore, manufacturing processes to scale up such vectors for human clinical trials should be more readily achieved for HSV than for other viral vectors. The utility of HSV has been established in preclinical models of brain tumour, Parkinson's disease and peripheral neuropathy among others (Glorioso and Fink , 2004). In addition to the deletion of genes necessary for viral replication in the recombinant vector, accessory genes may also be deleted to create extra capacity for insertion of exogenous therapeutic gene sequences (Krisky *et al.*, 1998). However, the HSV genome is too large to be manipulated by conventional cloning techniques and transgene insertion may only be accomplished by homologous recombination using a shuttle plasmid containing the transgene. Moreover, the recombination rate is low (typically 0.1% to 1%) and acyclovir selection is necessary to identify virus carrying the transgene targeted to, (and thus disrupting) the viral thymidine kinase gene. Although alternative reporter gene systems for screening are being developed (Krisky *et al.*, 1997), the low level of recombinant positive plaques is a drawback associated with the use of this system. However, the HSV is naturally neurotrophic and insertional mutagenesis is unlikely to be a concern as this virus remains episomal for its entire life-cycle (Glorioso *et al*, 2004). Therefore this virus clearly has therapeutic potential and human clinical trials

for HSV gene transfer by direct inoculation for treatment of glioblastoma are ongoing (Rampling *et al.*, 2000;Niranjan *et al.*, 2003, Pulkannen and Yla-Herttuala, 2005).

6.1.3.2 Retrovirus:

Retrovirus has been studied extensively for retinal gene therapy. However as this vector can only transduce dividing cells interest in this vector for retinal research has lessened. Despite this fact, retroviruses have been used somewhat successfully in photoreceptor cell targeting and long-lasting gene expression can be achieved upon integration. For example, Kido *et al.* reported that the internal opsin promoter fragment directed gene expression to photoreceptor cells following retroviral-mediated gene transfer (Kido *et al.*, 1996).

Lentivirus is an RNA virus with a reverse transcriptase belonging to a subfamily of the retrovirus (Lau *et al.*, 2000). This virus is distinct in the retrovirus family in that it is capable of transducing senescent as well as dividing cells. Lentivirus can thus potentially maintain stable transgene expression in host retinal cells. In 1997, Verma *et al.* reported the first photoreceptor specific gene transfer using a lentiviral vector (Miyoshi *et al.*, 1997). Photoreceptor rescue of *rd* mice was soon reported using the same vector system (Takahashi *et al.*, 1999). Tiscornia *et al.* meanwhile, have reported the use of lentiviral vectors expressing siRNAs for knockdown of specific targets *in vitro* and *in vivo* (Tiscornia *et al.*, 2003). Another group used lentivirus to achieve a stable knockdown of EGFP expression in mouse brain (Den Haute *et al.*, 2003). However the issue of safety is a serious concern for this vector in clinical gene therapy. Random integration of the lentiviral genome into the host genome presents an unpredictable risk, the tragic results of which have already been seen with other viral vectors in human gene therapy trials (Schmidt *et al.*, 2003) and are further discussed in chapter 7.

6.1.3.3 Adenovirus:

Production of adenoviral vectors at a high titre of 10^{11} to 10^{12} pfu/ml is easier to achieve than for most viral vectors. The first generation Ad vectors have been used in the CNS by four laboratories in 1993 (Akli *et al.*, 1993). At least three more generations of the virus have since been engineered. In particular, defective Ad vectors termed 'gutless' vectors in which all Ad genes are deleted were generated

(Fisher *et al.*, 1996). Ad is very efficient at transferring genes to cells and has been extensively investigated for retinal gene therapy (Li *et al.*, 1994; Bennet *et al.*, 1994). Bennett *et al.* showed the beneficial effect of expression of wild-type beta phosphodiesterase in an RP mouse model, the *rd* mouse (Bennett *et al.*, 1996). In other studies, the gene transfer of ciliary neurotrophic factor and fibroblast growth factor-2 to the retina retarded photoreceptor degeneration (Cayouette and Gravel, 1997; Akimoto *et al.*, 1999). Such findings may be crucial in halting the progression of diseases like RP. Apart from the transience of gene expression, a shortcoming of Ad specific to retinal gene therapy is the immune reaction it provokes. The inflammation is mild but consistent in all eyes that have received this vector. Ad-mediated gene silencing using dsRNA has recently been shown in cell culture (Shen *et al.*, 2003). However, given the inflammatory response, an alternative vector system may be more suited to the eye. Moreover, However, some reports have found the duration of gene expression mediated by Ad to be extremely limited (Fisher *et al.*, 1996).

There are several requirements that make a viral gene transfer agent suitable for neurons: 1) ability to transduce non-dividing cells; 2) absence of replication, thus avoiding viral spread; 3) non-toxicity of the viral envelope and of viral gene expression; 4) the ability to generate a purified high titre, free of contaminating wt or helper virus; 5) once in the nucleus the virus should be capable of expressing the therapeutic gene. The AAV parvovirus meets many of these specific requirements and therefore has been used as the vector of choice to develop a delivery system for the siRNA molecules targeting human rhodopsin identified in chapter 3.

6.1.4 Adeno-associated virus, AAV

AAV is a single-stranded DNA parvovirus currently popular in viral gene therapy research. The reason for this popularity is based on the fact that AAV does not appear to cause any disease in humans, and none of its zootropic relatives appear to cause disease in animals. In addition the virus tends to be inactive without the support of helper virus, usually adenovirus (Richardson *et al.*, 1981). However for these same reasons, AAV remained in relative obscurity in the early days of virus research and an understanding of the biology of AAV in terms of its epidemiology and persistence is far from complete.

6.1.4.1 The Biology of AAV:

AAV is packaged as a 4.7 kb single-stranded DNA molecule containing two sets of open reading frames in between T-shaped palindromic elements, the inverted terminal repeats (ITRs). The genes encode replication (*rep*) and structural (*cap*) proteins (McLaughlin *et al.*, 1988). In the absence of helper virus AAV enters the lytic stage of its life-cycle by integrating into the host cell genome. In human cells integration occurs in a site-specific manner on chromosome 19q13.3 (AAVS1) (Kotin *et al.*, 1992). This site-specificity is conferred upon the virus by the *rep* gene, as recombinant virus lacking this gene appears to integrate randomly in the absence of helper virus. At least 18 other potential *rep*-binding sites have been identified flanking or within human genes, however no secondary preferred integration sites have been found (Wonderling and Owens, 1997). Factors such as chromatin accessibility may be crucial to *rep* binding. Furthermore the AAVS1 sequence has only been found in higher primates and AAV appears to integrate randomly in rodent cells (Bakowska *et al.*, 2003). The first 125 bps of each 145 bp ITR at either end of the AAV genome is capable of folding into a T-shaped hairpin conformation (Lusby *et al.*, 1980). This structure is crucial for productive AAV infections. As well as serving as a suitable substrate for *rep* binding, the hairpin fold provides a primer for replication initiation terminating with a free 3'-OH, (Berns, 1990). To date 8 different serotypes (AAV1-8) of the virus have been isolated from the genome of different mammalian tissues (Rabinowitz *et al.*, 2002). While most were isolated as contaminants of adenoviral cultures, AAV 5, 7, and 8 were found in tissue through selection for their own unique properties (Bantel-Schaal *et al.*, 1984). Of these AAV5 shows the most divergent sequence (Bantel-Schaal *et al.*, 1999; Chiorini *et al.*, 1999).

6.1.4.2 Recombinant AAV, (rAAV):

The recombinant AAV virus can reach titers of 10^{12} to 10^{13} infectious units per ml, and can be purified and concentrated on a cesium chloride gradient. The single-stranded genome was initially manipulated to make a double stranded copy of the AAV2 genome (Samulski *et al.*, 1982). Transfection of this plasmid into adenovirus-infected human cells allowed for the wild-type AAV genome to be retrieved from the plasmid and packaged as wild-type particles. In the absence of helper adenovirus the rAAV genome was capable of integrating into the host cell DNA. Upon adenovirus infection, rAAV could, however, be rescued from the host cell (Hermonat *et al.*, 1984). The first constructs used in AAV gene transfer contained the *rep* gene and the neomycin phosphotransferase gene (McLaughlin *et al.*, 1988). Later constructs retained only the two viral ITRs with the *cap* genes delivered independently and led to the generation of the first AAV 'defective' vector stocks (Samulski *et al.*, 1989). Most AAV-based vectors do not include the *rep* gene, and thus integration is rare and occurs randomly, however the possibility of retrieving site-specific integration remains. Recently researchers have begun to create hybrid systems that include the *rep* gene and the AAV ITRs, which are delivered using non-viral constructs (Pieroni *et al.*, 1998). These vectors may ultimately be safer to use in a clinical trial setting, however the efficiency of transgene expression is typically reduced and transgene size restricted (fig. 6.3).

Unfortunately, the packaging capacity of AAV has been a limitation where delivery of a transgene that exceeds the capacity of the vector is an issue. In many cases viruses with large transgenes have shown a loss of infectivity (Dong *et al.*, 1996). The usual packaging limit for AAV appears to be in the region of 4.7 kb, although it may be even less for certain serotypes (Hermonat *et al.*, 1997). However the rearrangements that occur to the AAV genome in the host nucleus immediately following transduction have been exploited to overcome the limited packaging capacity of AAV. After second strand synthesis, intermolecular rearrangements result in formation of head-to-tail, head-to-head, and tail-to-head concatamers (Duan *et al.*, 1997). Transgenes larger than 4.7 Kb can be split between two different AAV vectors. While in one virus the 5' end of the gene possesses a splice donor site, in the other virus the 3' end of the gene possesses a splice acceptor site. When both viruses

transduce the same cell, the head-to-tail concatemers formed between the 5' and 3' AAVs leads to transcription of the entire gene (Duan *et al.*, 2003a).

6.1.4.3 AAV for retinal gene transfer:

Recombinant AAV has been shown to efficiently transduce rod photoreceptors and to a lesser extent retinal pigment epithelial cells after a single sub-retinal administration (Ali *et al.*, 1996). Grant *et al.* reported AAV-mediated transfer of a β -gal reporter gene to three major cell types in the mouse retina: the RPE, ganglion cells, and photoreceptor cells (Grant *et al.*, 1997). Gene expression was reported to be stable for the three months of this experiment. However the level of gene expression is particularly important for the development of therapies and treatment of retinal disease. Hence, the rhodopsin promoter has been used to restrict AAV-transgene expression to rod photoreceptors (Flannery *et al.*, 1997). AAV-mediated gene delivery has since been shown to successfully slow photoreceptor loss in rodent models of primary photoreceptor disease (Jomary *et al.*, 1997; Sarra *et al.*, 2001). For example photoreceptor structure and function has been restored in *rd*s mice, (as discussed in section 1.3, chapter 1) through subretinal injection with AAV-prph2 (Ali *et al.*, 2000). La Vail *et al.* have used AAV-delivered ribozymes to enhance the survival of photoreceptors in the P23H rodent model (LaVail *et al.*, 2000), highlighting the potential of this gene delivery system for correction of dominant mutations in the retina. Regardless of promoter used, subretinal injection of serotype AAV2/5 in the non-human primate retina has resulted in efficient and specific targeting of rod photoreceptors (Bennett *et al.*, 1999). These findings suggest that AAV is a particularly suitable vector for the treatment of photoreceptor abnormalities. The isolation of new serotypes has furthered the development of improved AAV delivery vehicles.

The first report of long-term success for the treatment of a retinal disease has been described in a canine model of Leber's congenital amaurosis (LCA) (Acland *et al.*, 2001). This model possesses a null mutation in RPE65, a protein primarily expressed in the RPE and in cone photoreceptors. AAV-RPE65 delivery to the briard dog resulted in stable restoration of visual function with little or no toxic effects.

An understanding of the AAV vector in the retina is not yet complete. Questions remain regarding mechanisms of cellular transduction, intracellular

trafficking and expression of the virus when remaining episomal and when integrated into the genome. Although we have chosen initially to use the AAV2/2 serotype to assay for delivery to the target tissue, siRNA and replacement rhodopsin genes will ultimately be delivered to rod photoreceptors using AAV2 pseudotyped with the capsid protein of AAV5, i.e. the AAV2/5 serotype. The reasons for this choice of hybrid serotype will be discussed in section 6.4.3.

6.1.4.4 AAV and siRNA delivery:

Xia *et al.* injected rAAV vectors expressing short hairpin RNAs targeting mutant ataxin-1 into the cerebellum of transgenic mice presenting with spinocerebellar ataxia-1 (SCA-1), the underlying pathogenesis in this mouse model being a polyglutamine expansion in the ataxin-1 protein resulting in neurodegeneration (Xia H, 2004). The shRNA treatment improved motor coordination and restored cerebellar morphology. Results from the study demonstrated the potential use of an AAV-RNAi vector for therapeutic intervention for dominant neurodegenerative disease. It is notable that over the past couple of years, rAAV has been used for delivery of shRNAs targeting a variety of genes *in vivo* and in each case phenotypic effects have been observed. For example, Babcock *et al.* recently demonstrated learning and behaviour changes associated with an AAV delivered shRNA targeting hippocampal alpha-Ca2+/calmodulin-dependent protein kinase II in rats (Babcock *et al.*, 2005). In the current study, we chose to assess and compare a viral and non-viral system for delivery of siC to the mammalian retina. Ultimately it is hoped that the chosen method should enable efficient delivery of a cassette expressing both a suppressor and replacement gene to rod photoreceptor cells.

In the following sections results from the *in vitro* exploration of both non-viral and viral vectors for delivery of shRNA are presented. As the viral AAV approach showed more initial promise *in vitro*, the delivery of viral cassettes was further explored *in vivo*. The results achieved show that it should, in principle, be possible to achieve retinal delivery of a therapeutic shRNA vector and hopefully observe corresponding changes in retinal function over time. In an initial investigation of a non-viral approach, the Φ c31 integrase was adapted for stable expression of the control siRNA, siEGFP (Qiagen, as listed in appendix 4A) over 24 days in a stably-expressing EGFP cell-line.

Following on from this initial work with the Φ c31 integrase system, a viral approach was favoured due to the increasing quantity of reports highlighting the benefits of rAAV as a means of delivery to retinal tissues. Hence, the ability of the construct AAV-EGFP-siC to achieve specific targeting of human rhodopsin *in vitro* was assessed. However, this viral construct was generated using the AAV 2/2 capsid which typically takes approximately two weeks to achieve optimal transgene expression in retina (Sarraf *et al.*, 2002). The slow kinetics of serotype 2 is a drawback where the aim is to achieve therapeutic effect as rapidly as possible in a retina, which may be degenerating. Therefore the serotype-5 capsid of AAV was assessed for ability to achieve early and specific transgene expression in a mouse retina. The effects of various viral purification methods were evaluated for resulting transgene expression and delivery. As such, the level of transgene expression that can ultimately be achieved by use of a caesium-chloride purified AAV2/5 vector for photoreceptor-cell-targeting is demonstrated with the sub-retinal injection of an rAAV2/5 vector expressing EGFP in mouse retina. The EGFP gene is driven by a 1.7 Kb mouse rhodopsin promoter in this virus. Notably, strong and specific expression of EGFP in the photoreceptor cell layer of the retina should demonstrate the efficacy of this vector, rAAV2/5, for future *in vivo* experiments with RNAi molecules such as siC.

6.2 METHODS

6.2.1 Constructing *pAttB-siNT*, *pAttB-siEGFP* and *pAttB-siB*.

The pH1-siRNA cassettes were extracted from the pH1-siNT and pH1-siEGFP constructs using XhoI and Not-I restriction sites. The 400 bp fragments were then cloned directly into the equivalent sites in the pTOPO-attB vector (kindly donated by Michele Calos, Stanford, CA).

6.2.2 Generating a *HeLa* cell line stably expressing *EGFP*

HeLa cells were plated at a density of 1×10^6 in 100 mm tissue culture dish. Approximately 5 μ g of pEGFP plasmid (Clontech, Palo Alto) was transfected into the cells using 12 μ l of lipofectamine 2000 (Invitrogen) as per manufacturer's instructions and as described in methods section 2.2.5.4. Cells were selected using G418 (GibcoBrl, 600 μ g/ml) over a 3-week period. This cell line was not clonally selected and the heterogeneous nature of the EGFP expression within the surviving cell population was taken into account during subsequent experiments.

6.2.3 Adeno-Associated Virus

6.2.3.1 Constructing *pAAVsiC-EGFP*

The siRNA and EGFP construct was cloned into pAAV-MCS (Stratagene), flanked by the inverted terminal repeats of AAV2. The viral vector, pAAV (Stratagene, La Jolla, CA) was digested at the Not-I site and the cut vector end-filled using Klenow enzyme (Stratagene). The pEGFP-siC cassette (extracted away from the original backbone using AflIII digestion and blunt-ended by klenow reaction) was blunt ligated to the cut pAAV2/2 vector. Sequencing could not be performed across the ITRs of this vector; therefore, the EGFP-siC and the AAV backbone were checked in separate sequencing reactions. Not I digests were also performed on all miniprep constructs to check for recombinant plasmids.

6.2.3.2 Producing AAV:

(All virus was produced in the unit with the help of Dr. Naomi Chadderton using the AAV helper-free system. A triple transfection methodology was used with viral plasmids from stratagene - the original reference for the system is Xiao X, 1998).

Recombinant AAVs are generated using the helper virus free, triple transfection method of Xiao et al (1998). The suppression and replacement construct was cloned into pAAV-MCS (Stratagene), flanked by the inverted terminal repeats of AAV2. The adenoviral helper

plasmid pHelper (Stratagene) and a second helper expressing plasmid AAV-2 Rep and serotype 5 capsid proteins (pRep/Cap5) were used to generate rAAV 2/5.

AAV HEK293 cells were seeded in 50 × 150 mm plates (un-coated) at 5×10^6 cells/plate and grown to confluency in 20 ml DMEM containing 10% FCS. For each plate, 12.5 µg pAAV-MCS, 12.5 µg pRep/Cap5 and 25 µg pHelper (quantitated by spectrophotometer OD 260/280 readings in TE solution) was prepared in a 1 ml volume of 150 mM NaCl, pH5. This was mixed with 1ml polyethylenimine (0.1 g/litre 150mM NaCl, pH5) and left to stand at room temperature for 8 mins before adding to the cells (10% final volume – 2 mls added to 18 mls). 7 hours post-transfection the media was replaced with 20 mls DMEM containing 10% FCS and the cultures incubated for 48 hrs.

6.2.3.3 Preparing viral stocks

Three viral purification methods were used in this thesis:

1. VIRAPUR (ViraKit™ AAV)

The virus was harvested according to manufacturers' instructions. Briefly, cells were dislodged from the plate by gently pipetting up and down, thus resuspending the virus-containing cells in the 5 mls growth media. All the cell lysate and media were collected in 50 ml blue cap tubes and subjected to 3 rounds of freeze and thaw cycles (-80°C, 37°C), followed by 30 min incubation at 37°C with 25 U/ml benzonase nuclease (recombinant DNase/RNase, Novagen). The samples were spun at 3000 rpm for 30 min and the supernatant collected in fresh blue cap tubes. The bottle top filter and pre-filter supplied were assembled and pre-wet with 25 ml PBS to adhere the pre-filter to the bottle top filter. The virus containing supernatant was then applied to the filter. The filtered supernatant was diluted with dilution buffer 1 (1 part dilution buffer to 9 parts filtered supernatant) and mixed thoroughly. The purification filter assembly was attached to a syringe filled with sterile PBS and 5 ml passed through. The syringe was detached and the tubing assembly attached to the purification assembly and the viral supernatant pulled through at a flow rate of 20 ml/min. The large filter was discarded and the small filter washed with 30 ml wash buffer 2 at a flow rate of 20 ml/min. The small filter was connected to two 5 ml syringes and the virus eluted with 0.5 ml elution buffer by passing the buffer slowly through one syringe and back through the other, followed by a little air to collect all the virus particles in one syringe. The virus was aliquoted in 10 µl volumes and stored at -80°C. Virus titre was ascertained by an AAV-2 titration ELISA (Progen). However ELISA doesn't distinguish full capsids from empty and thus over estimates titre. Subsequently viral titre was determined by the method of Rohr *et al*, 2002.

2. HEPARIN affinity purification (specific to AAV2/2, native ability to bind heparin receptor) (Auricchio *et al* 2001).

Cells were dislodged from the plate by gently pipetting up and down, thus resuspending the virus containing cells in the 5mls growth media. The cell containing media from each plate was pooled in 50 ml blue cap tubes and centrifuged at 3,000 rpm for 30 min. The resulting supernatant was discarded and the pellet resuspended in 5ml PBS-MK (2.5mM KCl, 1mM MgCl). The resuspended cell pellet underwent 2 freeze-thaw cycles to liberate the virus from the cells (-80°C, 37°C), followed by 30min incubation at 37°C with 25U/ml benzonase nuclease (recombinant DNase/RNase, Novagen) and 0.5% by volume deoxycholate (detergent). The virus containing solution was then filtered through 5µm and 0.8µm filters before loading onto the heparin column (sigma, 5ml pre-loaded heparin affinity column). The sample was loaded and once eluted the column was washed with 2 column volumes (10 ml) 0.1M NaCl in PBS-MK. The sample was eluted with 0.4M NaCl in PBS-MK. The first 2 ml, representing the column void, was discarded. The following 4ml collected in 1ml volumes and titered using the method of Rohr *et al*; 2002. Analysis of the individual fractions showed the first ml collected to have the highest titre and this was used in subsequent experiments. To further concentrate the virus and thus further increase the titre the volume was applied to a centricon centrifugal filter device (YM-30, Millipore). This led to a 5x concentration of virus but resulted in a 50% loss of total virus. This approach was therefore deemed unsuitable for *in vivo* work.

3. Caesium chloride purification, (Zolotukhin *et al*. 1999).

Vector-containing cells were dislodged by using a cell scraper and collected by centrifugation at 1000 g for 30 mins. The cell pellet was resuspended in 10 mls PBS and subjected to 3 rounds of freeze (-80°C) and thaw (37°C) and incubated with 10 mg RNaseA and DNase I for 30 min at 37°C. Following 15 min centrifugation at 1000 g, the 10 ml supernatant was transferred to a new tube and incubated with 0.5% (final concentration) deoxycholic acid for 30 min at 37°C. Generally, three days post-transfection was found to be the optimal time to prepare the AAV stocks described in this thesis. At this stage, the virus could be collected by caesium chloride purification. Briefly, CsCl₂ 0.454 g / ml, was added to the sample (4.54 g CsCl to the 10ml supernatant) and incubated on ice for 5 min. Meanwhile a density gradient was prepared in polyallomer Beckman tubes (89 mm height x 24 mm diameter) by layering 9 ml of 1.4 density CsCl₂ (548.3 mg/ml) over 9 ml of 1.6 density CsCl₂ (816.5 mg/ml) pipetted below. The sample was then applied to the top of the gradient. The tubes equilibrated and spun at 25,000 rpm in a Beckman SW28 rotor for 18 hrs at 4°C. 1ml fractions were collected using a 16G syringe needle and the refractive index read on a

refractometer. Fractions within the range 1.373–1.368 were collected and pooled in polyallomer Beckman tubes (6 ml volume, 13 mm height × 51 mm diameter), equilibrated with the 1.4 density CsCl₂ and spun at 59,000 rpm in a Beckman fixed angle rotor VTi65 for 18 hrs at 4°C. 0.5 ml fractions were collected and those within the refractive index (1.373–1.368) were pooled into a single polyallomer Beckman tube and centrifuged as above. Following collection, 0.5 ml fractions from within the range 1.373–1.368 were dialysed against 10 litres of PBS over 40 hrs (1 litre/4 hrs) using 0.5 ml on a Slide-aLysers (Pierce).

6.2.3.4 Determining infective viral titre

A 2 µl viral sample from the preparations of 6.2.3.3 was used to perform an infectivity assay on HEK293 or HeLa cells over a 24 hour period. The cells were seeded in a 96-well plate at 5×10^3 cells/well and infected with the 2µl viral suspension after 1hr (uninfected HEK293 cells were used as a negative control). After 24 hrs, cells were trypsinised, pellets washed 2X with PBS and resuspended in 50 µl PBS (without Ca or Mg). 10 µg ProteinaseK was added and the cells were left at 50°C for 60 min, before the proteinase K was inactivated at 95°C for 15 min. The lysate was spun for 10 min at 16,000 g and the supernatant transferred to a fresh tube. 10 µl lysate was digested with 10u S1 nuclease (Sigma), this was left for 30 min at 37°C, and the nuclease reaction inactivated at 95°C for 5 min. The mixture was diluted 1:100 in order to prevent inhibition of the PCR by S1 nuclease. Q-PCR was performed as described Rohr *et al* (2002) using 2µl of viral template. The 2 µl (DNaseI and PoteinaseK treated)-sample was subjected to 36 cycles of amplification (10 min pre-incubation at 95°C followed by 36 cycles of 15 s at 95°C, 5 s at 67°C and 10s at 72°C) in a 7300 Q-PCR (Applied Biosystems) using primers specific for the transgene. In order to determine the copy number of the transgene-containing viral DNA in whole cell lysates of AAV infected cells, a standard curve was generated using transgene-containing plasmid (standard curve of 10^5 – 10^9).

Calculation to determine infectious particles/ml:

(copy number in qPCR) × diln whole cell lysate (100) × final volume of whole cell lysate (55) × factor to yield infectious particles/ml (500). Total divided by template volume in qPCR (2).

6.2.3.5 Transduction of HeLa-HR cells with AAV

To confirm that pAAV-EGFP-siC was capable of expressing functional siRNA molecules, a HeLa cell line stably expressing human rhodopsin cDNA (HeLa-HR) was transduced with the virus. A 30- μ l volume of virus with an estimated number of 10^7 particles/ml was diluted in 200 μ l of serum-free DMEM. Following two PBS washes and one wash with serum-free DMEM media, the cells were covered with the virus solution for 3 hours. After this time the viral solution was supplemented with 800 μ l of DMEM containing 10% fetal calf serum. Rhodopsin expression was analysed in RNA extracted from the cells after 72 hours incubation at 37°C using real-time rtPCR methodologies outlined in chapter 2 sections 2.2.3.

6.2.4 In Vivo techniques

All animal studies were performed in accordance with the FELASA category B & C guidelines.

6.2.4.1 Sub-retinal injections

The stock anesthetic was 1 ml Ketamine (Vetalar) and 0.5 ml Xylazine (Rompum). 0.5 mls of this solution was added to 2 mls of water for injection. A 20 g mouse was anaesthetized with 166.7- μ l volume of ketamine/xylazine. The dosage was delivered intraperitoneally. Under microscopic control, a Hamilton 33 gauge needle delivered the 30- μ l volume of therapeutic to the subretinal space underlying the central retina. A registered ophthalmologist performed all procedures in a barrier facility with sterile instruments and solutions.

6.2.4.3 Electroretinography:

Dr. Paul Kenna carried out all ERGs.

After over-night dark adaptation, mice were anaesthetized as above, and pupils dilated with topical 1% atropine sulfate. Contact-lens electrodes were used for recording bilateral simultaneous full-field electroretinograms, (ERGs). Reference electrode in the mouth and a needle-ground electrode in the tail were used. Short wavelength flashes were shone in the eyes and responses measured. B-wave amplitude and timing were measured graphically. Rod and cone photoreceptor responses were modeled using a computer-based system (Retiport, Germany).

6.2.4.4 Vibratome sectioning of retinal tissue:

Before sectioning, the eyes were fixed in 1 ml of 4% paraformaldehyde and incubated at 37°C for 2–3 hours. Following removal from the 4% paraformaldehyde, the eyes were washed

twice for five minutes at a time in PBS. The retinas were removed from the eyes under a microscope using a sterile blade and forceps. To obtain a front cross-section the retina was embedded as a whole piece into the agar. To obtain a side section, the retina was split in two and flattened before being embedded in the agar (see fig. 6.1 below). The optimal shape of the agar to ensure straight sectioning by the vibratome blade is illustrated in figure 6.1. To make a solution of 6% agar for the sectioning, 6 grams of Select agar (Sigma) was mixed with 100 mls of PBS. This was melted over a period of approximately 40 minutes on a hot plate with a magnetic stirbar and was then poured into a 60 mm tissue-culture dish (Nunc). While the agar was still molten, the retinal tissue (removed from the eye) was embedded into the agar using a sterile forceps.

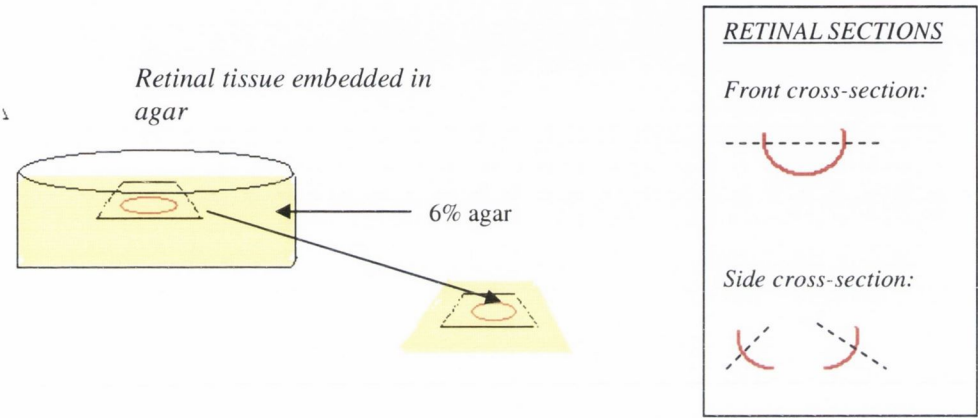


Fig. 6.1: Preparing retinal tissue for vibratome sectioning.

The agar blocks were sectioned in a bath of PBS using a Leica VT 1000S vibratome. Sections of 50 μm in width were made with this vibratome.

6.2.4.5 Retinal RNA extraction:

The rAAV-transduced mouse retinas were vortexed in a solution of 500 μl Guanidinium Thiocyanate and 7.1 $\mu\text{l/ml}$ β -mercaptoethanol and left overnight at room temperature.

50 μl of 2 M Sodium Acetate (pH4.0), 500 μl DEPC-treated H_2O saturated Phenol and 200 μl chloroform/Isoamyl alcohol (49:1) were added to the lysate and mixed gently by inversion. The solution was left on ice for 30 minutes and centrifuged at 13,200 RPM for 20 minutes. The supernatant was transferred to a new eppendorf. 1 μl glycogen and 1ml of cold isopropanol was added and mixed by inversion before being left at -20°C for 2 hours. The supernatant from a 30-minute spin was discarded and the pellet washed in 500 μl of 75% ethanol. Pellets were dried at 80°C for 3 minutes. The RNA was resuspended in 30 μl depe-

treated H₂O and stored immediately at -70°C . The quality of the RNA was assessed by spectrophotometric reading of $\text{OD}_{260}/\text{OD}_{280}$ and also by examining 28S, 18S, and 5S bands on a 2% agarose gel.

Additional techniques used in this chapter are described in the materials and methods chapter 2, including MTT assay (section 2.2.5.7); cell culture (section 2.2.5); RNA extraction and real time rtPCR analysis (section 2.2.3).

6.3 RESULTS

6.3.1 Use of ϕ c31 integrase in mammalian cell culture

The ϕ c31 integrase can be used to integrate attB-plasmid DNA into multiple sites in the genome of HEK293 cells (Groth *et al.*, 2000). As well as achieving long-term stable integration, this integrase system reduces random integration by 10-fold thus minimising the risks associated with random integration of a therapeutic. This selective integration is achieved through a unidirectional recombination event between an attB sequence on the plasmid and an attP sequence in the host genome, as outlined in the introduction (section 6.1.1).

HEK293 cells were stably transformed to express the pEGFP plasmid (Clontech), using selection over a two-week period with Geneticin (Sigma Aldrich). The DNA-based cassette carrying dsRNA-targeting EGFP, H1-siEGFP, was cloned into a plasmid containing the attB sequence, producing pAttB-siEGFP (figure 6.4). As a silencing control, the H1-siNT cassette was also cloned into this vector. The attB-containing plasmids can then be cotransfected with the integrase gene on plasmid pCMV-int (Calos *et al.*, 2001). For each transfection 600 ng of suppressor plasmid and/or 1000 ng of integrase plasmid was delivered to 1×10^5 HEK293-EGFP stable cells using lipofectamine 2000 (Invitrogen). The ability of ϕ c31 integrase to stably integrate plasmid DNA carrying siRNA was tested by comparing the level of target EGFP mRNA from stable cells receiving suppressor plasmid alone (pattB-siEGFP) or integrase-expressing plasmid alone, (pInt) to EGFP expression from cells receiving suppressor plasmid and plasmid carrying the integrase gene (attB-siEGFP & pInt). Transfected cells were split every three days and RNA extractions taken from 5×10^4 cell aliquots at intervals of 2, 10 and 24 days. Integrase expression was also determined at 3 days to establish that the cells were actively transcribing the enzyme. In addition this 20-day experiment was repeated and the results from RNA extractions for the time-points between the two experiments pooled to obtain figure 6.8.

Expression levels from the cells receiving integrase alone represent the basal EGFP expression level at the time points analysed. The transfection procedure alone appeared to have a significant effect on EGFP expression values in this stable cell line, as can be seen in the 50% drop in expression in the integrase control RNA samples compared to RNA from mock transfected cells (i.e. cells treated with

lipofectamine reagent alone). In the (attB-siEGFP & integrase) cotransfected cells a minimal percentage of the silencing achieved at 48 hours remained when analysed by real-time rtPCR at 24 days. However, the difference in EGFP level between cells transfected with both pAttB-siEGFP and pInt compared to those cells receiving the pAttB-siEGFP plasmid alone, was not found to be significant ($t=0.76$). It appears from this initial data, that while the EGFP expression in cells receiving siRNA alone increases by 100% at each time-point, the expression increase in cells that received integrase as well as silencer is closer to 65%. This suggests that the loss of silencing may be slower in the cells receiving both plasmids, and it is therefore possible that some preservation may be occurring as a result of integration in a small number of cells. Real time rtPCR was also performed using primers specific for the integrase enzyme, demonstrating that the integrase message is actively transcribed in the cell (results not shown). In this regard it is worth noting that integrase protein can be efficiently produced in mammalian cells as has been demonstrated by Calos *et al.*, 2003.

The result presented here represents, in essence, only a preliminary evaluation of the integrase system and no attempt was made to identify by PCR the sites where any potential integration event may have occurred. However, in this experiment siRNA-based silencing of EGFP expression was not maintained to a significant degree in mammalian cells. Viral delivery systems were therefore investigated as an alternative option to achieve long-term gene silencing. Considering the ultimate goal for gene therapy, adeno-associated virus may offer the safest viral vector option. Some success has already been reported in the use of this virus for retinal delivery (Ali *et al.*, 1996, 2000; Grant *et al.*, 1997; Jomary *et al.*, 1997; Bennett *et al.*, 1999; Sarra *et al.*, 2001). The initial investigation of this viral vector as a vehicle to deliver suppression and replacement therapeutics to the retina has therefore been carried out and results from the initial investigation are presented in the following sections of this chapter.

6.3.2 Adeno-associated viral delivery of siRNA

6.3.2.1 Adeno-associated viral delivery of siRNA to HeLa cells

The siRNA siC was cloned between the inverted terminal repeats of the viral expression cassette as described in the methods section of this chapter, section 6.2.3.1. An EGFP gene was included in this expression cassette thus enabling the identification of those transduced cells positive for viral gene expression (fig. 6.9). The CMV-driven EGFP gene was cloned in the opposite orientation to the H1-siC gene with a 300 bp spacer separating the two components of the cassette (as illustrated in fig. 6.6). It was decided, for an initial assay, to deliver this virus to a HeLa cell line stably transformed with human rhodopsin, (HeLa-HR) for analysis of viral-mediated rhodopsin knockdown. The serotype used was AAV2/2 and the initial AAV stock for cell culture analysis was isolated using a virapur column (Virapur, LLC). The virus was packaged by cotransfection with helper plasmid into a HEK293 cell line carrying the necessary accessory elements for packaging. Dr. Naomi Chadderton (TCD) performed the manufacture and harvesting of the AAV titred particles. A quantitative PCR assay was used in retrospect to determine the infective titre of the virus. This assay is described in section 6.2.3.4.

In the first experiment 10^7 viral particles were delivered to 5×10^4 HeLa-HR cells as described in section 6.2.4. Relative to control rAAV2/2 carrying non-targeting siRNA (AAV2/2-EGFP-siNT), cells transduced with the AAV2/2-EGFP-siC showed approximately 32% HR gene expression, i.e. over 67% suppression of human rhodopsin, as assessed by real time rtPCR (fig. 6.10). Primers for the EGFP gene were also used in these real-time rtPCR analyses as a reference for gene expression within the inverted terminal repeats from the AAV expression cassette and hence for transduction. Rhodopsin expression in the cell line could therefore be calculated relative to viral gene expression (EGFP), as well as relative to cellular house-keeping gene expression (GAPDH and 18S rRNA). In this case rhodopsin gene expression was similarly reduced by over 65% in the AAV2/2-EGFP-siC compared to AAV2/2-EGFP-siNT cells (data normalised to EGFP not shown).

The level of suppression, in HeLa cells, achieved using AAV-EGFP-siC is slightly less than that achieved using cotransfections of H1-siC plasmid and target into cells (chapter 3, fig. 3.12). Some variation in levels of suppression achieved may be caused by transduction of a stable rhodopsin line (in the rAAV experiment outlined

in this section) compared to cotransfections of rhodopsin and shRNA (in chapter 3). In addition, techniques such as real-time rtPCR introduce a degree of variation due to the nature of the methodology – the error associated with the real-time rtPCR assay in our lab is estimated in the order of approximately 10%. It is also worth noting that the overall infective viral titre of rAAV used in this experiment (purified by virapur methods) was quite low at this point. A high titre AAV-EGFP-siC virus should be readily achievable (discussed in section 6.3.4).

6.3.2.2: MTT assay to assess toxicity of AAV-siRNA to HeLa cells

The MTT Cell Proliferation Assay measures cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the assay measures reduction in cell viability (Mosmann, 1983). The assay is described in more detail in section 3.2.5.

We chose the number of cells to seed for the MTT assay based on a plot of absorbance readings from cells seeded at a range of densities and treated with the MTT reagent vs cell number. The cell density of 5×10^4 and corresponding to values lying in the linear range of the plot, which yielded an absorbance of 1–1.25 was chosen for the assay. The MTT assay was carried out twice on HeLa cells in a 96-well plate, with three wells analysed per transfection. The cells transduced with virus are not significantly less viable than those receiving plasmid-carrying siRNA (fig. 6.11). There is a 20% reduction in the level of the formazan metabolite detected by the assay, between three concentrations of H1-siC and the three concentrations of pAAV-EGFP-siC. It may be that the transduction as opposed to the transfection procedure is responsible for this reduction as the former involves subjecting the cells to serum-free media for two hours, while the more sophisticated transfection reagents currently available allow for transfection to occur in the presence of serum. Therefore a drop in cell proliferation may result from the protocols used for preparing the cells for transfection/transduction rather than from viral-induced toxicity. Values outside of the linear range of the curve for cell density (between 0.75 and 1.75) may not yield an accurate quantification of changes in the rate of cell proliferation, however the assay does reveal that cell viability is not significantly affected in these experiments.

6.3.2.3 Interferon response in HeLa-rhodopsin stable cell line transduced with AAV-siRNA

In section 5.3.3 preliminary results suggested that no significant immune response was provoked *in vitro* by the H1-driven siRNA plasmids used in this thesis were described. It was decided to investigate whether any immune response would occur stimulated by the same shRNA cassettes when delivered by AAV. The interferon response was assessed by monitoring alterations in expression levels of the protein- α (Ifi- α) and oligo-adenylate synthetase 1 (OAS1) genes in cellular RNA treated with AAV. These immune response gene products were described previously in chapter 5, section 5.1.4.

RNA was extracted from cells at 72 hours post-transduction of a HeLa cell line stably expressing human rhodopsin cDNA, as used in the previous section. The AAV cassettes investigated were AAV-e (empty cassette), AAV-EGFP-siNT (carrying siNT, a non-targeting siRNA as used as control in all previous experiments), and AAV-EGFP-siC (carrying siRNA-C, targeting human rhodopsin cDNA). Interferon-induced protein- α , (Ifi- α) expression appeared to be upregulated by the presence of an AAV capsid in the cell, with greater expression levels for the empty virus, AAV-e, than for either of the shRNA-carrying viruses. The increase in oligoadenylate-synthetase-1 expression, however, may have a significant siRNA-dependent component. There was greater expression for this gene in the cells transfected with siRNA-carrying vehicles rather than empty viral vector alone. Levels of expression for both genes in RNA extracted following mock transfection of the stable cells were negligible. While the RNA data for this experiment was based on data pooled from three real-time rtPCR runs, the transfection of cells was only carried out once. This preliminary result may require further investigation of any AAV-shRNA-induced immune response in future studies.

6.3.2.4 Adeno-associated viral delivery of siRNA to explants

A difficulty associated with AAV involves the preparation of sufficiently high viral titres. The overall infectivity of rAAV may be only one hundredth of the number of viral particles prepared (Feudner *et al.*, 2001). The experiments of section 6.3.2.1 involved rAAV purified using Virapur methods (described in methods section 6.2.3.3 of this chapter). Ultimately, infective viral titre may be too low to achieve retinal transduction when purified by this approach.

An alternative method for rAAV preparation, involves the use of a Heparin column, which carries a proteoglycan receptor for AAV type 2 virions (Summerford & Samulski, 1998). Dr. Chadderton carried out this second rAAV purification method for the retinal explant experiment undertaken for the current study in accordance with an established protocol (Auricchio *et al.*, 2001). AAV2/2-EGFP-siC or AAV2/2-EGFP-siNT, (figure 6.6) was used to overlay a retinal explant immediately after seeding at P0. At two weeks the cells of the explant were dissociated and immunostained as described in section 2.2.6. However no EGFP fluorescence was observed in dissociated cells suggesting that the virus may have failed to transduce the explanted retina. An overall loss of infectivity was considered the most likely reason for the lack of EGFP positive retinal cells. The initial titre protocol using ELISA, doesn't distinguish full capsids from empty capsids and thus over-estimates titre. Indeed, when aliquots of the viral stock were further analysed in a quantitative PCR run using EGFP primers to titre for genome copy number, it was found that the genome titre was no more than 10^7 particles/ml. This was considerably less than the titre of a positive control virus (AAV-EGFP obtained from Dr. Robin Ali), which was purified using caesium chloride gradient. Using an increased volume of this diluted heparin preparation; again significant transduction of the retinal explant was not achieved. This experiment emphasised the disparity between the infectious titre and overall particle titre of the AAV virus. It was concluded that viral purification methods would require further optimisation before rAAV carrying shRNA sequences could be used for *in vivo* experiments.

With the overall infective viral titre too low at this point, the results from this initial study suggested need for alternative purification methods to obtain a sufficient number of viable infective particles for *in vivo* assay. As such attempts to optimise viral delivery using a control EGFP-carrying AAV vector are further discussed in section 6.3.3.

6.3.3 Optimising delivery of AAV to ocular tissue for future AAV-shRNA retinal experiments - Use of caesium chloride purification techniques and testing the AAV serotype 2/5

A viral construct has been designed to test the specificity of delivery mediated by AAV serotype 2/5 to the photoreceptor cell layer of the mouse eye. The viral construct in this case carries the EGFP reporter gene driven by a 1.7 kb mouse

rhodopsin promoter (fig. 6.7). The 1.7 kb rhodopsin promoter is smaller than the 3.2 kb mouse rhodopsin promoter (used in the HR5m model of chapter 4 and designed by Dr. Mary O'Reilly). It is hoped that this cassette may offer a high level of EGFP expression while minimising the transgene size so that it can be packaged into AAV. The rAAV2/5-EGFP vector is assessed in terms of localisation and strength of transgene expression in this analysis. The aim of the experiment is to determine the possibility of achieving retinal-specific expression of AAV-shRNA constructs (such as AAV-EGFP-siC described above) in future experiments using a different hybrid serotype, that is the rAAV2/5 pseudotyped vector rather than rAAV2/2 (as used previously in section 6.3.2). In addition, a higher titre rAAV preparation (achieved using caesium-chloride purification) than previously obtained (virapur/heparin purification) was assessed for the level of transduction that may be achieved *in vivo*.

Dr. Paul Kenna injected a 1 μ l volume of a 1×10^{12} vpu/ml genomic titre of the control viruses, AAV-1.7P_{MuRHO}-EGFP into rho-/- (L129Sv) mice at post-natal day 7 (P7). Animals were sacrificed at a range of ages and their retinas vibratome-sectioned to determine the level of EGFP expression achieved in the retina. Fig. 6.13–6.16 shows representative retinal sections evaluated using fluorescent microscopy from eight mice sacrificed at a range of ages following injection rAAV2/5-EGFP at P7. In the course of extracting the retinas from these animals, the portion of the retina where EGFP expression had occurred was evident even to the naked eye and typically encompassed approximately 40–50% of the retina. The spread of AAV infection and EGFP expression is particularly evident in the cross-section of whole retinas as shown in fig. 6.13. Strong EGFP fluorescence is clearly evident in these retinal sections. Moreover, the fluorescence typically increases over time as shown in figs. 6.15a–c of retinal sections from mice at 21 days compared to sections from mice at 42 days post-injection. Notably, the EGFP fluorescence is largely confined to the photoreceptor cell layer compared to the inner nuclear layer as is evident in the confocal micrograph images of section 6.16.

It is notable that in the study described here, rAAV2/5-EGFP was subretinally injected into rho-/- mice, who clearly have compromised retinal tissues. Rho-/- mice do not elaborate rod outer segments and the photoreceptor nuclei die over a 3–6 month period (Humphries *et al.*, 1997). Recombinant AAV2/5 transduction may be more efficient in a degenerating vs wild-type retina where cellular borders are breaking down.

The strength and specificity of EGFP expression subsequent to a single subretinal injection of high titre rAAV(2/5)-EGFP vector is clearly demonstrated in this study. The use of AAV serotype 2 with the AAV serotype 5 capsid enables efficient targeting of photoreceptor cells as the AAV capsid 5 protein seems to result in preferential transduction of photoreceptor cells as has been borne out in the current study (fig. 6.16). These preliminary results also suggest that while rAAV2/5 results in significant EGFP expression 14 days post-injection (fig. 6.15b), significantly higher levels of expression were obtained three weeks post-injection (fig. 6.15c). In summary, the results of this section support the idea that an AAV2/5 virus may be the most suitable vector for delivery of a coupled siRNA and replacement rhodopsin transgene to the ADRP retina.

6.4 DISCUSSION

6.4.1 øc31 bacteriophage integrase-based delivery

The øc31 bacteriophage integrase delivery system has been shown to be useful for stable integration of large DNA sequences (Ortiz-Urda *et al.*, 2002). We wished to determine whether a stable long-term knock down of gene expression could be achieved *in vitro* using this integrase approach. The EGFP gene was chosen as a target for down-regulation as it offered a relatively fast and reliable protein read-out, which could be monitored over time. Moreover it was hoped that, as the suppression of this gene using Qiagen's positive control siRNA was shown to be in excess of 80% (3.3.1), the extent of the siRNA effect might still be observed, even when factors such as transfection and integration efficiencies are taken into account. We examined the maintenance of an siRNA effect targeting EGFP in HEK293 cells stably expressing EGFP over a 24 day period. Following the initial silencing event evaluated at 48 hours, approximately 100% increases in EGFP expression were estimated over 24 days in cells receiving shRNA plasmid without integrase plasmid. While in cells that received integrase as well as shRNA plasmid, the expression was found to increase by approximately 65% at each time-point (fig. 6.8). This trend may reflect an integrase-mediated integration of the siRNA and thus, a drop in the rate at which EGFP transgene expression levels were able to recover following RNAi. However the differences in the data sets from integrase and non-integrase expressing-cells are not statistically significant ($p = 0.76$).

Ultimately, any maintenance of suppression achieved in cells using the attB-siEGFP and integrase constructs is minimal by day 24 (Table 6.1). Integration, if it occurs in the mammalian cells, may be at too low a level to achieve a significant effect. A selectable marker for integration may be necessary before integration can be monitored. However, this may cause too high a background of non-specific integration *in vitro*, which may mask øc31-integrase mediated events.

The study using the integration system represents simply a first attempt to evaluate the vector as a means of achieving non-viral site-specific gene delivery. Additional work-up would be required and detailed analysis of potential sites of integration using, for example, PCR-based assays should be undertaken. In this regard an initial PCR screen was undertaken of DNA extracted from HeLa cells

transfected as for fig. 6.8 using primers Ψ attBf and Ψ attBr as listed in the appendix at the back of this thesis (section 1A). This was an initial search to discover if integration was occurring at a common integration site (Ψ A on chromosome 8) identified by Calos team (Thyagarajan *et al.*, 2001). In this case there was no evidence of integration at this particular candidate site of the HeLa cell genome. Soon after Prof. Calos kindly donated the plasmids required to explore this integrase system for suppression and replacement therapeutic delivery to the retina, recombinant Adeno-associated virus technology was established in the laboratory in TCD. Given a number of reports suggesting rAAV may represent an efficient and safe vector for retinal delivery of nucleotide-based therapeutics (Bennett *et al.*, 1999; Bainbridge *et al.*, 2003) the focus of the vector aspect of the research project evolved towards evaluation of rAAV *in vitro* and *in vivo*. Hence, the following sections of the discussion are focused on the work-up of rAAV as a means of delivery of suppression-and -replacement-based therapeutics to the retina.

6.4.2 AAV-EGFP-siC

Adeno-associated virus may be particularly suitable for the delivery of agents to treat photoreceptor degenerations (LaVail *et al.*, 2000). The pAAV(2/2)-EGFP-siC vector was constructed as an shRNA delivery vehicle with EGFP expressed from the CMV promoter in reverse orientation from the H1-driven siRNA 'siC'. Prior to production of the virus, the plasmid vector was shown to be fully functional by analysing levels of rhodopsin mRNA downregulation in HeLa cells following lipid-mediated transfection (lipofectamine 2000) and determining that they matched those of other shRNA vectors carrying siC (data not shown). HeLa cells stably expressing human rhodopsin from a CMV promoter were treated with pAAV(2/2)-EGFP-siC at a M.O.I. of approximately 1000 (total number of particles/number of cells seeded). The efficient transduction of cells was assessed by microscopy in HEK293 cells (fig. 6.9). The results in cells stably expressing target showed significant levels of suppression when treated with AAV(2/2)-EGFP-siC compared with a non-targeting control, AAV(2/2)-EGFP-siNT (Fig 6.10).

However it is notable that suppression of the target gene rhodopsin was not achieved when the virus was used on an explanted retina. There were no fluorescent cells visible when the cells of the explant were dissociated suggesting that AAV(2/2)-EGFP-siC had failed to transduce the explant tissue. This may be due to the low titre

(10^7 vpu) of the viral preparation (Heparin-purified) that was used for this study. The relationship between AAV titre and transgene expression has, in the past, been clearly established with expression demonstrated to increase in a dose-dependent manner over a 3.3-log range of viral concentrations (Klein *et al.*, 2002). Sarra *et al.* have shown that titres on a low scale of $2 \times 10^6 - 2 \times 10^8$ infective U/ml reach a maximum transduction rate of 15% of photoreceptors *in vivo* compared to a transduction rate of 85% of photoreceptors achieved with viral titres of 2×10^{10} I.U./ml (Sarra GM, 2002). The aim with AAV-EGFP-siC is therefore to improve the viral titre assessed not only by genome copy number but also that high titres of infectious viral particles are achieved. In addition hybrid serotypes, which may be better suited to photoreceptor delivery, were investigated and are discussed in section 6.4.4. Given the low titre of the rAAV(2/2)-EGFP-siC further exploration of siC-based knockdown in retinal explants or indeed *in vivo* was not be possible and will be undertaken in the future when a sufficiently high titre is produced.

It is notable that CsCl₂ gradient purification is still the most commonly used viral preparation method. Typically it would seem that a higher titre of rAAV2 may be achievable using a CsCl₂ gradient purification approach and indeed the method has been used to produce the high titre AAV2/5-EGFP virus that is used in section 6.3.3 of this chapter. However a considerable amount of contaminating non-viral proteins and a high ratio of genome copies versus infectious units may result from the CsCl₂ preparation. Therefore suitable assays to titre the final yield for infective titre as well as genome copy number is vital. Techniques to purify and concentrate recombinant AAV continue to evolve and improve, with the result that in principle the production of very high titres can now be achieved.

6.4.3 Toxicity and Immune Response to AAV(2/2)-EGFP-siC

MTT assay used to detect overall levels of cell viability between cells transduced with virus-transduced shRNA compared to lipid-based delivery of shRNA, showed no significant variation in the level of cell survival (estimated across six wells of HeLa cells seeded at 5×10^4 /ml – fig. 6.11).

The levels of expression genes associated with immune response were assessed in cells transduced with AAV-EGFP-siRNA and the results presented in fig. 6.12. The immune response was detected in RNA extracted from cells shown to have active transgene expression, i.e. active silencing from the shRNA cassette. An

increase in expression was found for both Ifi α and OAS-1 transcripts in the AAV-transduced cells compared to mock transduced cells (i.e. receiving medium alone). Bessis and Boissier noted that while expression of inflammatory cytokines following vector transfer is high for adenovirus-mediated delivery, it is almost null for AAV-mediated delivery (2004). This facilitates sustained AAV-mediated transgene expression *in vivo*. However capsid antigens may cause specific immunity toward AAV. This may be the cause of the increase in interferon expression that was observed in HeLa cells transduced with AAV in section 6.3.4. OAS1 expression is upregulated in cells transduced with virus carrying siRNA distinct from empty virus, possibly suggesting that it is the transgene in this case rather than virus provoking the OAS-1 response. The type of promoter used to drive transgene expression has been shown to influence immune response, and it may be promoter activity (rather than RNAi) that caused the increase in OAS1 transcript levels (Cordier *et al.*, 2001).

These preliminary results are based on *in vitro* findings and firstly should be repeated and moreover require an analysis of cytokine levels in cell lysates as well as *ex vivo* or *in vivo* support.

6.4.4 AAV serotypes

There are currently at least 8 known serotypes of AAV (Rabinowitz JE, 2002). The isolation of new AAV serotypes has led to the design of therapeutic-specific vectors. Different serotypes possess distinct AAV virion capsid proteins, which vary in their ability to bind to and infect depending on host cell types. Optimised promoters and regulatory elements are of little value if the AAV fails to bind a target cell and be efficiently internalised. The ITRs of AAV virion 2 have been extensively studied and are used in conjunction with many other virion capsids to create hybrid AAV serotypes. While the ITRs of AAV2 are well studied and have been shown to be effective for retinal transgene expression, different AAV capsid proteins confer a new tropism to the virus and faster kinetics of transgene expression (Auricchio *et al.*, 2002). This is of particular relevance for the assessment of therapies in the Pro23His mouse model of human ADRP. The degeneration within the retina occurs early in development. Subsequent to subretinal injection of AAV2/2 in the murine retina, the onset of gene expression is estimated to occur approximately 2–4 weeks after vector administration (Bennett *et al.* 1997, Auricchio *et al.*, 2002, Sarra *et al.*, 2002). By this time extensive photoreceptor loss will already have occurred in the murine pro23his

retina (as illustrated in figure 5.8 of chapter 5). The rAAV-2/5 vector carries the therapeutic transgene flanked by AAV-2 ITRs encapsidated in an AAV-5 shell. Subretinal administration of AAV2/5 results in strong and early expression in photoreceptors (Yang *et al.*, 2002). However in addition to the kinetics of transgene expression, the biodistribution of the transgene is affected by the AAV serotype used for delivery. Vector DNA was detected in the brain and optic nerve of dogs following intravitreal delivery of rAAV-2/2, suggesting transit from the vitreous and through the optic nerve (Provost *et al.*, 2005). Although these vector sequences are not believed to be transcriptionally active, the need for restriction of transgene expression to the targeted tissue is of relevance in choosing the viral serotype and the injection method used for delivery. Subretinal administration of rAAV-2/5 did not result in detectable levels of vector DNA in the brain, although it was detected in the optic nerve. When developing future AAV delivery vehicles for siRNA targeting human rhodopsin, we propose to optimise the AAV-2/5 hybrid serotype, which it is hoped shall provide a faster kinetics of transgene expression allowing for rescue before an autosomal dominant mutation takes effect. It may also be ultimately safer in terms of a restricted distribution within ocular tissue.

In this regard, the AAV serotype 5 capsid has been used in the current study. Capsid 5 was used in conjunction with the ITRs of AAV serotype 2 to express an EGFP reporter gene driven by the mouse rhodopsin promoter (fig. 6.7). The virus was purified to a high titre of 1×10^{12} particles per ml by caesium chloride purification (in collaboration with Dr. Chadderton). A 1µl volume of this rAAV2/5-EGFP virus was sub-retinally injected into mice with a retinopathy induced by disruption of the endogenous mouse rhodopsin gene (Rho-/-L129Sv) at P7 by the ophthalmic surgeon on the team, Dr. Paul Kenna. The animals recovered from the anaesthetic and typically showed no aberrant retinal effects from the injection (as assessed by ERG measurements on the mice, undertaken by Dr. Paul Kenna). Before applying rAAV technology to exploring if RNAi-based can be achieved in retina *in vitro*, it was important to explore if the vector system of choice did indeed provide efficient and safe gene delivery to the retina. Hence, the initial work-up of the rAAV2/5 hybrid serotype was undertaken using an EGFP reporter gene. Thus, it was possible to monitor the levels of viral transduction and transgene expression. Mice, subretinally injected with rAAV2/5-EGFP were sacrificed at 21- and 42-days and the

retinas vibratome-sectioned for microscope analysis (fig. 6.13-16). In total the retinas from 8 mice were examined. Strong fluorescence was found to be localised to the photoreceptor cell layers of the retina and can be observed in fig. 6.14. The level of EGFP expression at a 30-day time-point can be seen in the cross-section of whole retina provided in fig. 6.13. EGFP fluorescence is clearly evident when compared to an untreated control retina (fig. 6.15a) and the specificity of the EGFP expression to the photoreceptor cell layer is unequivocal in a confocal micrograph image of the same section (fig. 6.16). This strong and specific level of expression achieved in photoreceptors demonstrates how AAV(2/5) may be an appropriate vector for the future testing of therapeutics, such as the suppression and replacement constructs described in this thesis. The faster kinetics of transgene expression achieved using the serotype-5-capsid also makes this AAV more applicable to testing in animal models of retinal degeneration particularly where there is early-onset of the degeneration. This may include models such as the pro23his mouse, the degeneration which proved difficult to test as described in chapter 5 of this thesis, as onset of a severe degeneration from the time of rhodopsin gene expression (day 5 post-natally) proved too rapid to demonstrate RNAi-based suppression of rhodopsin (fig. 5.7–5.10).

Given that clearly the results from this study demonstrate that rAAV2/5 as prepared in the lab in TCD can result in efficient delivery to the target tissue, the retina, future work will involve the analysis of AAV(2/5)-EGFP-siC *in vivo*. Notably, the cassette (within an AAV2/2 vector) carrying an EGFP reporter gene and siC was shown to be functional in initial *in vitro* assay (fig. 6.10). In addition, the viral serotype has been optimised using a hybrid vector with the capsid from AAV5. Moreover higher purified titre preparations (in the order of 1×10^{12} – 1×10^{13} vpu) have now been achieved for *in vivo* experiments. Preliminary study of the AAV2/5 titre suggests that efficient transduction of photoreceptor cells can now be achieved. Hence, it is proposed that siC is engineered into a rAAV2/5 vector and that subsequently suppression of a target gene, rhodopsin is evaluated *in vivo*. Subsequently viruses carrying both elements of the proposed therapeutic, both the suppression part (dsRNA) and the replacement gene (rhodopsin) be engineered and administered to mice presenting with rhodopsin-linked retinal degenerations (see chapter 7 for discussion of this construct).

6. FIGURES and TABLES

ϕ c31 Integrase-mediated gene delivery:

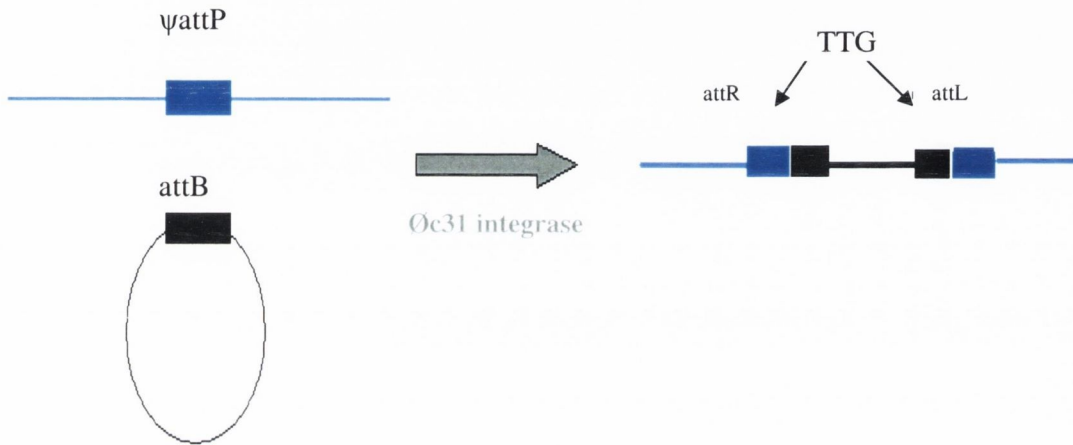


Fig. 6.2

The integrase enzyme derived from the *Streptomyces* c31 phage, is used to mediate integration of a gene of interest into the mammalian genome. A plasmid bearing the attB attachment sequence and the transgene is transfected with the recombinant ϕ c31 Integrase plasmid into the mammalian cells. The integrase is capable of mediating integration between the att-B sequence on the plasmid and endogenous genomic pseudo-attP sites. The attR and attL sites refer to the hybrid sequence comprising half of attP and half of the attB sequence produced following an integration event (primers to identify such a hybrid site are listed in appendix 1A). Thus, integration is restricted to a specific number of sites in the genome (Groth *et al.*, 2000).

AAV-mediated gene delivery:

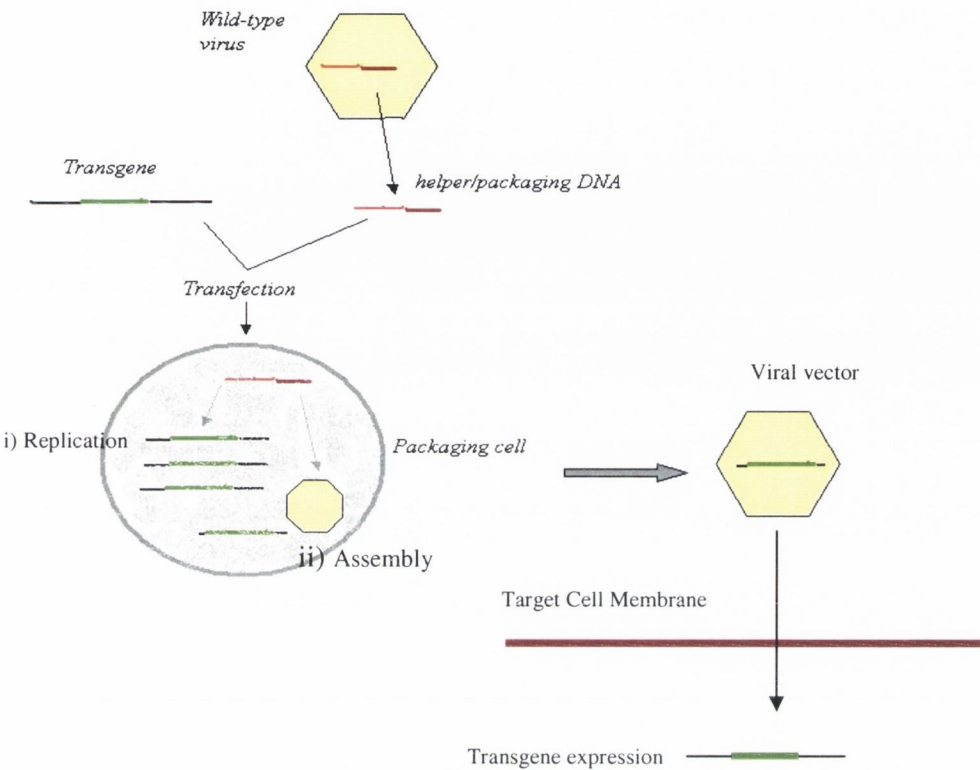
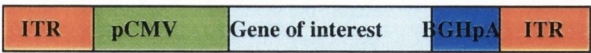


Fig. 6.3a:

The generic strategy to generate a replication-defective vector from a replication competent virus. Transgene on vector DNA is transfected into cells expressing i) helper functions, to enable transgene replication and ii) packaging functions, to enable viral particle assembly.

Recombinant AAV production:

pAAV MCS (Stratagene)



ITR sequences provide all of the *cis*-acting elements necessary for replication and packaging.

pAAV-RC



AAV-2 replication proteins and viral capsid structural proteins (confers serotype)

phelper



Subset of adenovirus genes, VA, E2A and E4

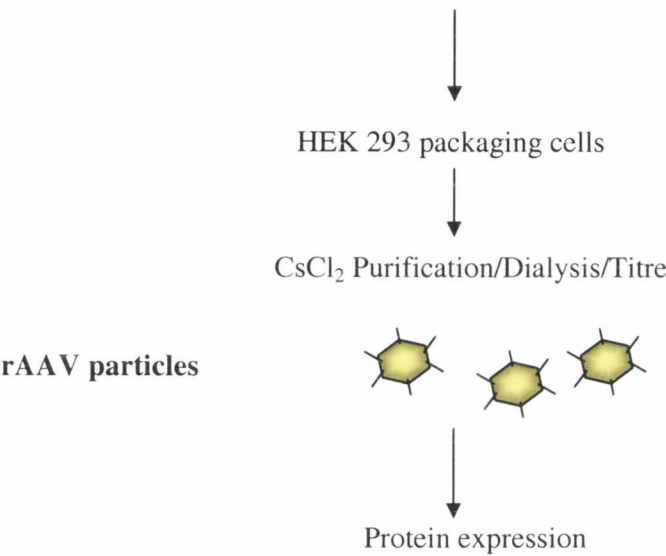


Fig. 6.3b:

pAAV-RC and phelper together supply all of the trans-acting factors required for AAV replication and packaging. Adenovirus proteins E1A and E1B are stably expressed in the HEK 293 packaging cells. ITR: Inverted Terminal Repeat; P_{CMV}: Cytomegalovirus promoter; BGHpA: bovine growth hormone poly A.

Øc31-Integrase constructs

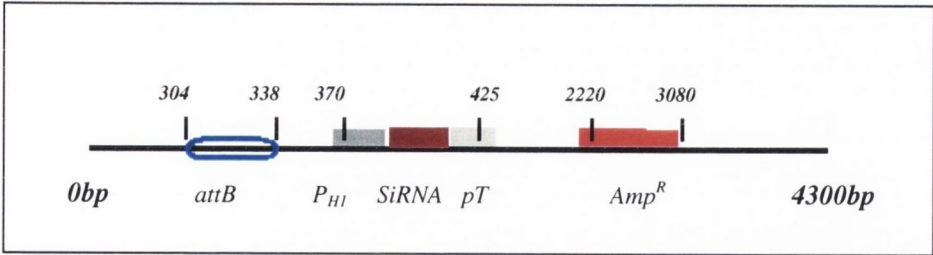


Fig 6.4:

pAttB-siRNA. In addition to the siC sequence used for suppression and replacement in chapters 3 and 4, the construct shown here includes an attB site. This sequence is necessary to allow Øc31-integrase-mediated homologous recombination with a ψ attP site in the human genome. The vector backbone was originally obtained from Dr. Michele Calos. The construct is used in results section 6.3.1. P_{H1} : promoter derived from the H1 gene; pT : poly T termination signal; Amp^R : ampicillin resistance gene; attB: attachment site for Øc31-integrase-mediated recombination with a ψ attP site;

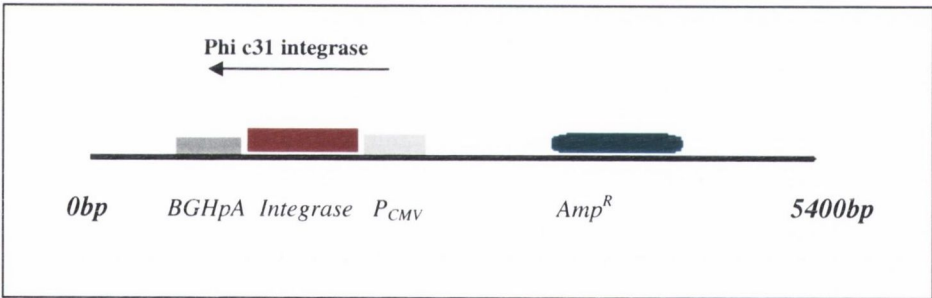


Fig. 6.5:

pInt vector, which carries the Øc31 integrase enzyme from the Øc31 phage of Streptomyces. This enzyme is used in results section 6.3.1 for nonviral-mediated integration into Hela cells. The Øc31 integrase plasmid pCMV-int, (as described by Chalberg *et al.*, 2001), was obtained from Michele Calos. P_{CMV} : Cytomegalovirus promoter; BGHpA: bovine growth hormone poly A; Amp^R : ampicillin resistance gene.

Adeno-associated viral vector constructs

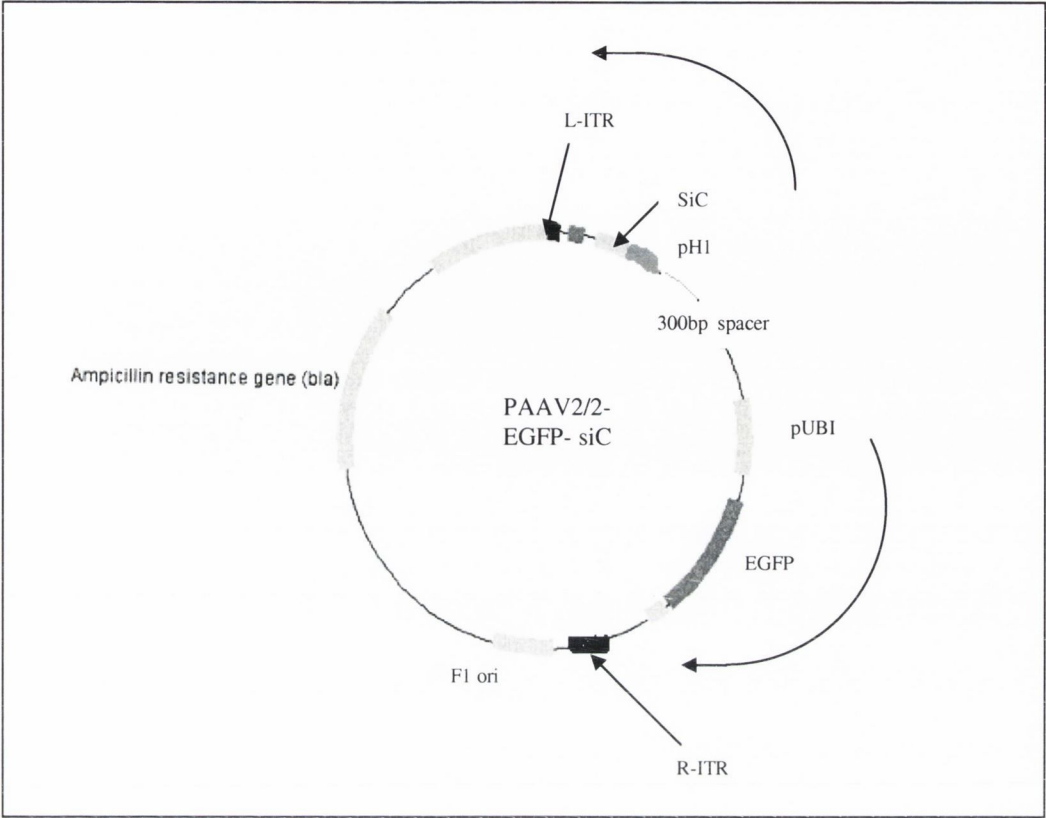


Fig. 6.6: pAAV(2/2)-EGFP-siRNA

The AAV2/2 transgene vector expresses an siRNA targeting human rhodopsin in reverse orientation to a marker EGFP gene, allowing for identification of cells transduced with siRNAs. The AAV vector used in this case is AAV2/2. R-ITR: Right inverted terminal repeat; L-ITR: Left inverted terminal repeat; pUBI: Ubiquitin promoter; pH1: H1 promoter.

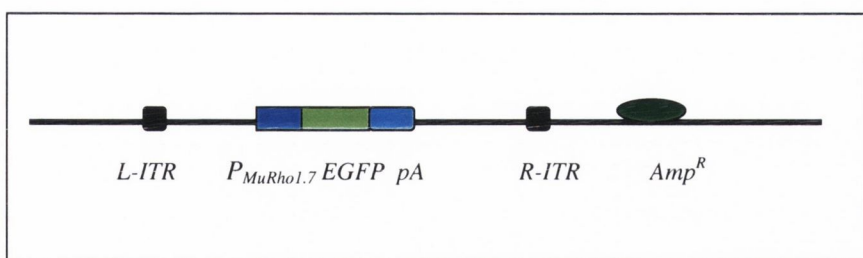


Fig. 6.7:

Diagram of the pAAV(2/5)-P_{MuRho1.7}EGFP vector, which carries the EGFP gene and is used for assessing transgene deliver to the retina. P_{MuRho1.7}: 1.7 kb mouse rhodopsin promoter; L-ITR: Left inverted terminal repeat; R-ITR: right inverted terminal repeat; Amp^R: Ampicillin resistance gene; P_{MuRho1.7}: 1.7 kb mouse rhodopsin promoter; EGFP: Enhanced green fluorescent protein. This viral vector has a serotype 5 capsid to allow early and photoreceptor-specific transgene expression.

Integrase-mediated RNAi in mammalian cell culture

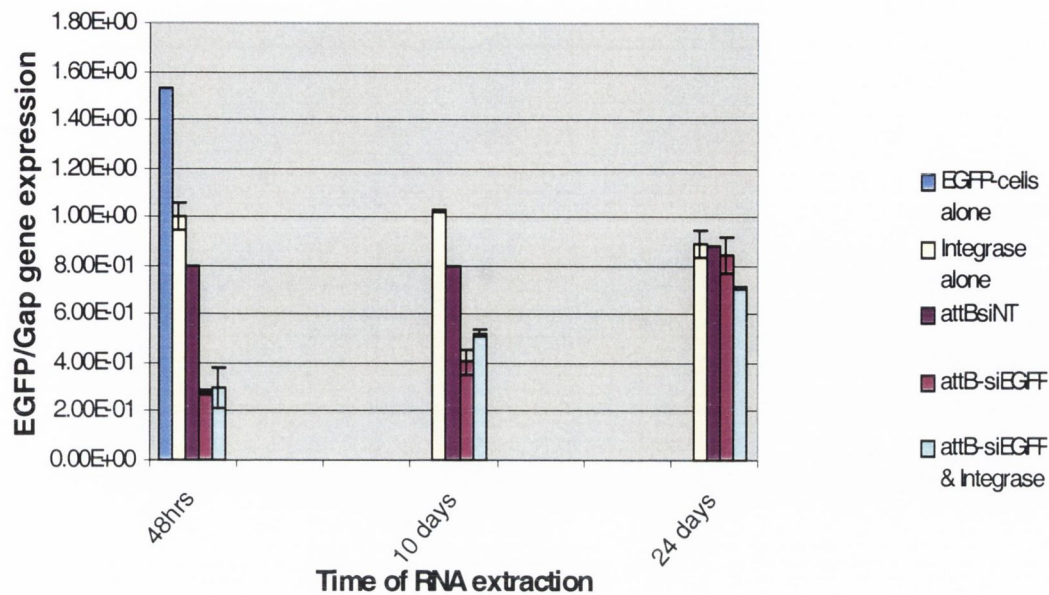


Fig 6.8: Long-term gene silencing in HEK293-EGFP cells using phage c31 Integrase system. RNA was extracted from EGFP-expressing cells treated with silencer targeted to the EGFP transgene with and without the recombinant integrase enzyme. The RNA was extracted at 3 time points over 24 days and expression levels compared by real time rtPCR. The results of three real time rtPCR runs from two separate experiments were averaged to determine expression levels in the cells.

Transfection	Ratio of EGFP expression to GAPDH expression:		
	48 hours	10 days	24 days
Mock	1.53	n/a	n/a
AttB-siNT	0.8	0.8	0.886
AttB-siEGFP	0.279	0.404	0.846
Integrase	1	1.027	0.892
AttB-siEGFP & Integrase	0.3	0.524	0.71

Table 6.1: EGFP expression normalised to GAPDH expression following integrase-mediated siRNA delivery to HEK293-EGFP cells. ‘Mock’ refers to cells receiving lipofectamine alone; ‘attB-siNT’ refers to non-targeting siRNA on a vector carrying the attB attachment site; ‘attB-siEGFP’ refers to EGFP-targeting siRNA on the attB vector. The probability of a statistically significant difference in the rate of increase of EGFP expression (over 24-days) between siRNA with integrase and siRNA without integrase was not found to be significant. P-value of array set for (attBsiEGFP) vs data-set for (attB-siEGFP + int) = 0.76.

AAV-mediated delivery of siRNA to HeLa-HR cells

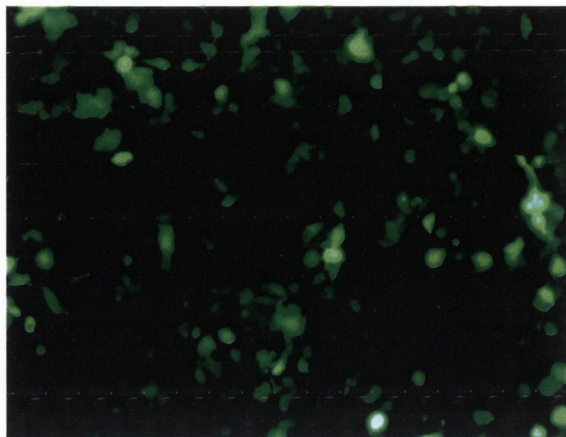


Fig. 6.9:

AAV(2/2)-EGFP-siC expression in HEK-293 cells, visualised by fluorescent microscopy 72-hours post-transduction of cells.

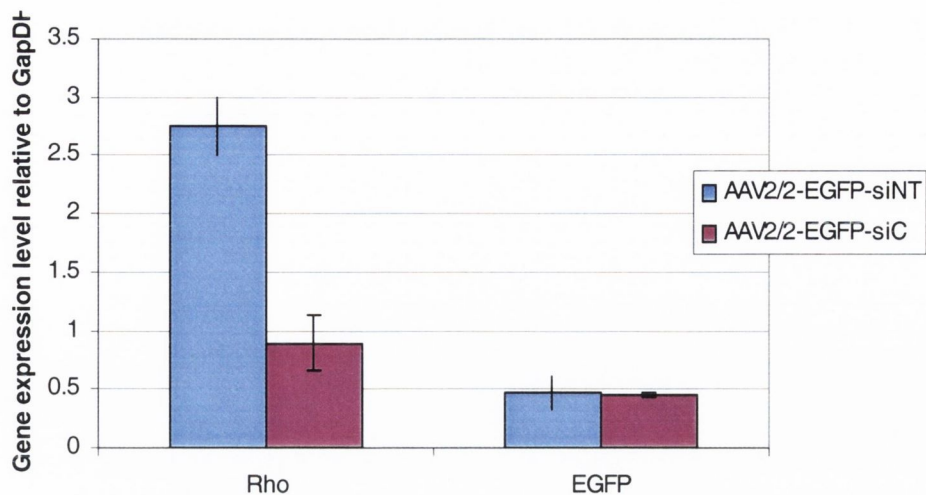


Fig 6.10:

Rhodopsin and EGFP gene expression relative to GAPDH expression following AAV(2/2)-mediated delivery of siRNA targeting human rhodopsin in HeLa-HR cells. EGFP expression is equivalent in the AAV- EGFP-siNT and AAV- EGFP-siC treated samples, implying equivalent levels of transduction of the cells for both constructs. Rhodopsin expression in the AAV-EGFP-siC RNA sample is 32.5% that of the AAV-EGFP-siNT RNA sample.

AAV-siRNA	Rhodopsin/GAPDH	EGFP/GAPDH
AAV-siNT	2.75	0.463
AAV-EGFP-siC	0.895 (t-value = 0.04)	0.45 (t-value = 0.998)

Table 6.2:

Data for fig.6.10. Gene expression in HeLa-HR cells transduced with AAV(2/2)-EGFP-siC.

MTT analysis of AAV-transduced mammalian cells

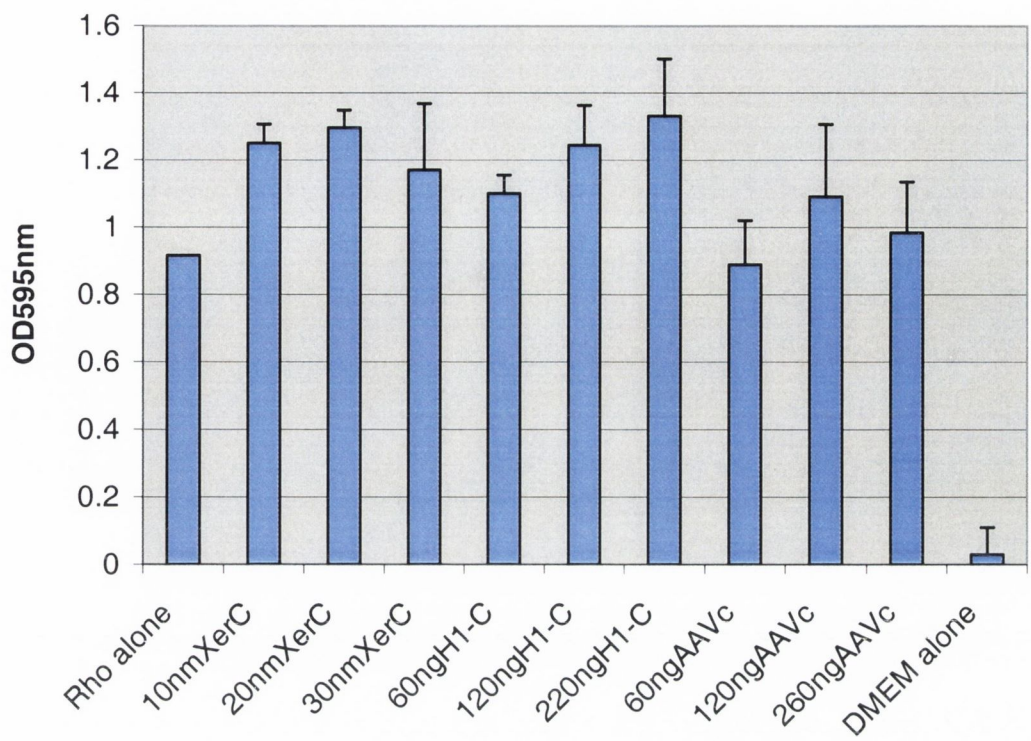


Fig 6.11: Optical density readings showing the level of formazan metabolite formed in cells treating with silencing reagents. “XerC” denotes the synthesized siRNA C, “H1-C” the hairpin plasmid, and “AAVc” represents the AAV vector as used in figs.6.8-9. The readings have been normalised to the reading from a well of mock transfected control cells treated with lipofectamine 2000 (invitrogen) alone. “DMEM alone” refers to wells that have medium but no cells. The experiment was carried out twice with the read-out for three wells of a 96-well tissue-culture plate seeded per sample in each experiment. The average of the readings was used to obtain the final values.

Immune response in HELA-HR cells of AAV-shRNA transduction:

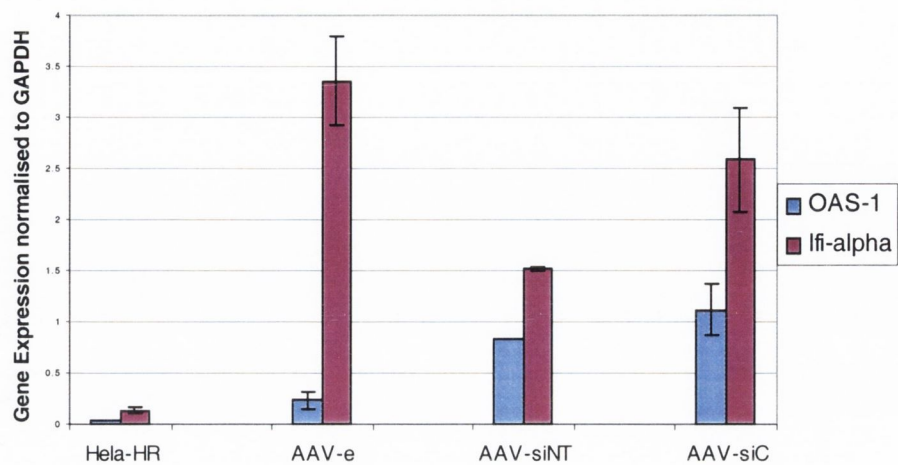


Fig 6.12:

Interferon response in HeLa-HR transduced with AAV-siRNA. HeLa-HR: HeLa cells stably expressing human rhodopsin; AAV-e: AAV2/2 virus without transgene; AAV-siNT: AAV2/2 virus expressing non-targeting siRNA; AAV2/2-siC: AAV2/2 virus expressing siRNA targeting the human rhodopsin gene. The dsRNA-responsive ISGs (immune-stimulated genes), OAS-1 and Ifi- α were chosen for analysis of potential immune responses to the AAV-shRNA cassettes.

	HeLa-HR alone	AAV-e	AAV-siNT	AAV-EGFP- siC
OAS-1	0.045	0.233	0.825	1.12
Ifi- α	0.132	3.36	1.52	2.59

Table 6.3:

Table showing the data used for fig. 6.12. Expression levels, relative to GAPDH, of immune-response genes following viral transduction of HeLa-HR cells. These results are derived from three real-time rtPCR runs on RNA extracted from a single transfection of the HeLa-HR cells.

Sub-retinal injection of AAV-P_{MuRho}EGFP into the rho^{-/-} retina of mice at post-natal day 7:

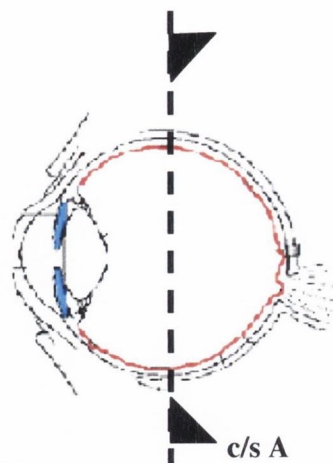
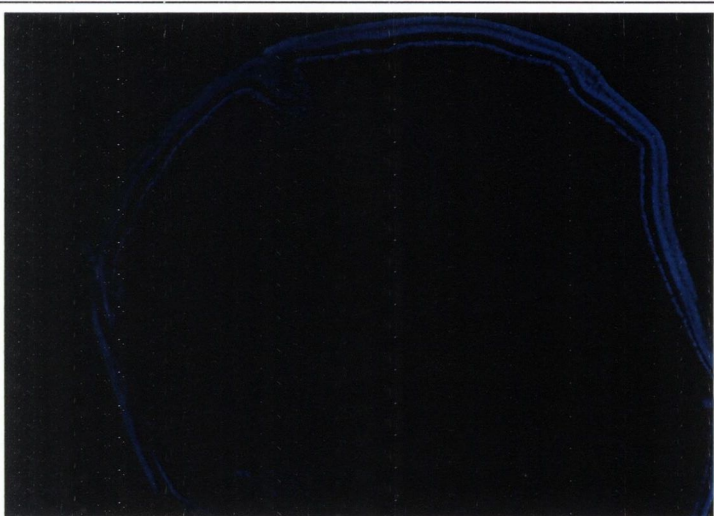


Fig. 6.13: Microscope images (10X magnification) of the 50 μ m vibratome sections from a rho^{-/-} mouse retina. The mouse was sub-retinally injected with 1 μ l of 1×10^{12} vpu/ml of AAV-1.7pMuRho-EGFP virus at post-natal day (P7). The animal was sacrificed at P42 and the retina isolated for sectioning (removing the RPE or surrounding tissue). This image shows a cross-section orientated from the front of the eye (c/s A). Dapi-staining of the retina is shown in image (i) while EGFP fluorescence of the same section is shown under UV light in image (ii).

(i).



(ii)



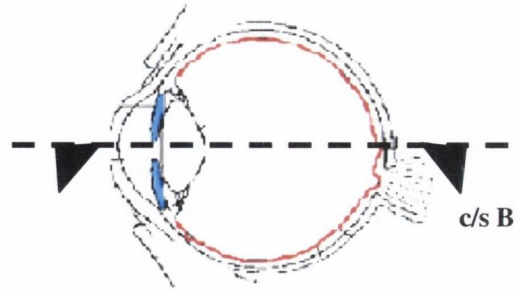
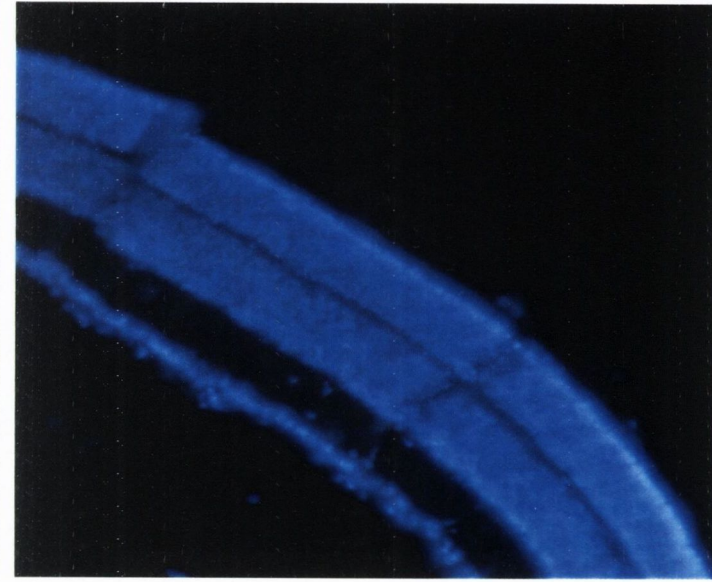


Fig. 6.14:

Microscope images (20X magnification) of the 50 μm vibratome sections from a $\text{rho}^{-/-}$ mouse retina. The mouse was sub-retinally injected with $1\mu\text{l}$ of 1×10^{12} vpu/ml of AAV-1.7pMuRho-EGFP virus at P7. The mouse was sub-retinally injected with 1×10^{12} vpu/ml of AAV-1.7pMuRho-EGFP virus at P7. The animal was sacrificed at P42 and the retinas removed for sectioning. The cross-section is of the width of the retina (c/s B). Dapi-staining of the retina is shown in image (i) while EGFP fluorescence of the same section is shown under UV light in image (ii).

(i)



(ii)



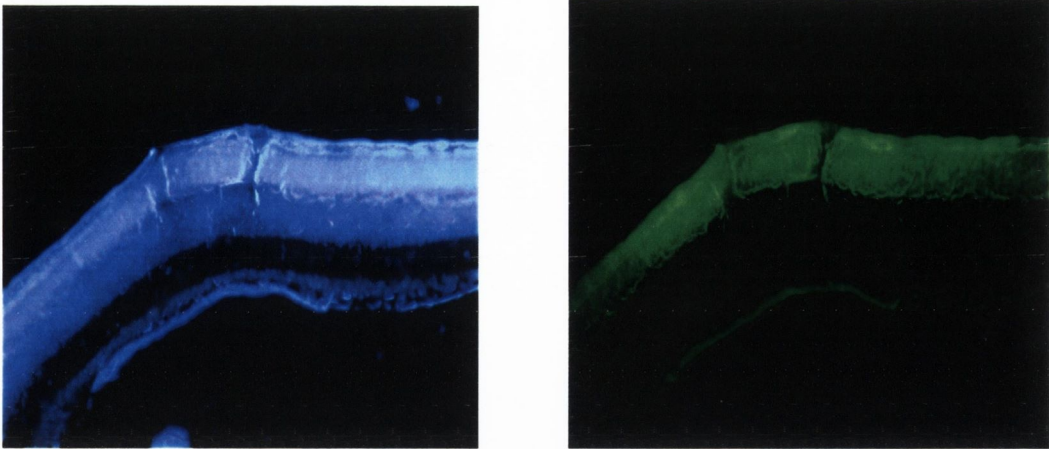


Fig. 6.15a: Vibratome sections of retinas from Rho^{-/-} mice electroporated with pAAV(2/5)-P_{MouRHO}EGFP construct at P7 and analysed at P21 (40X magnification).

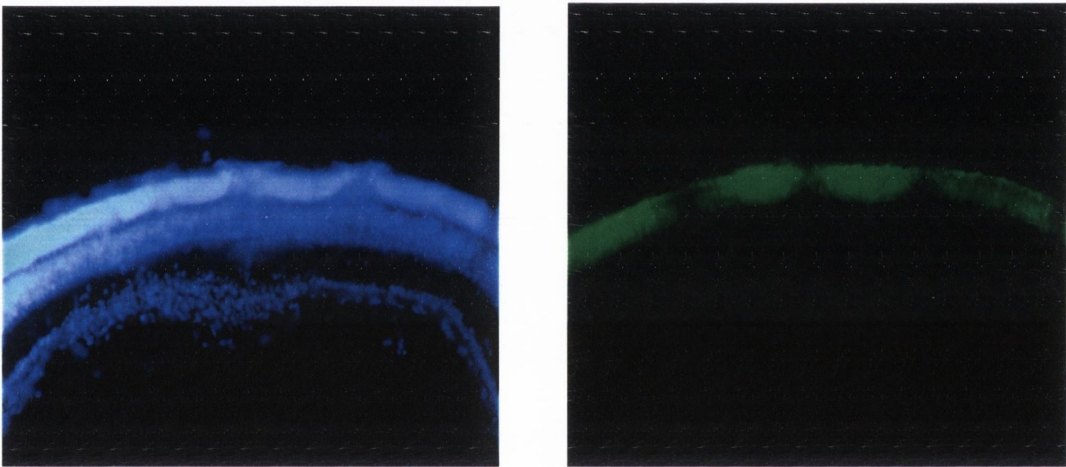


Fig 6.15b: Vibratome sections of retinas from Rho^{-/-} mice electroporated with pAAV(2/5)-P_{MouRHO}EGFP construct at P7 and analysed at P21 (20X magnification).

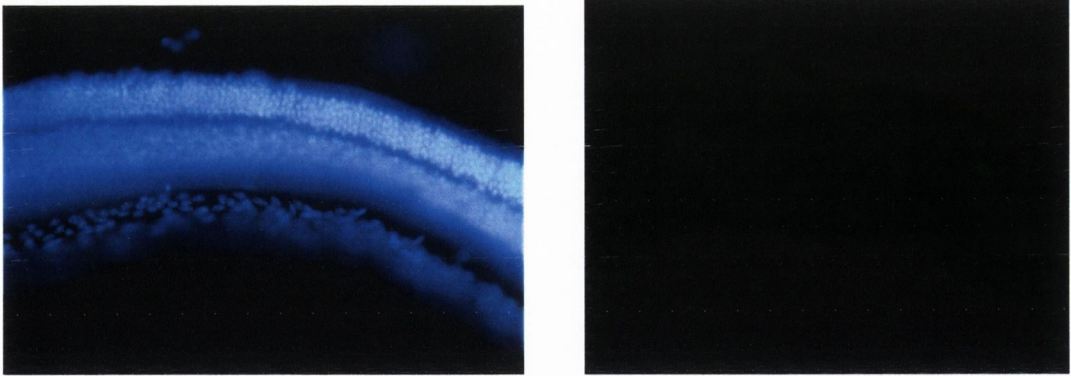


Fig. 6.16a: Negative control showing the background level of fluorescence from the rho-/- retina. The dapi-stained image is shown on the left and the same section under UV light is shown on the right.

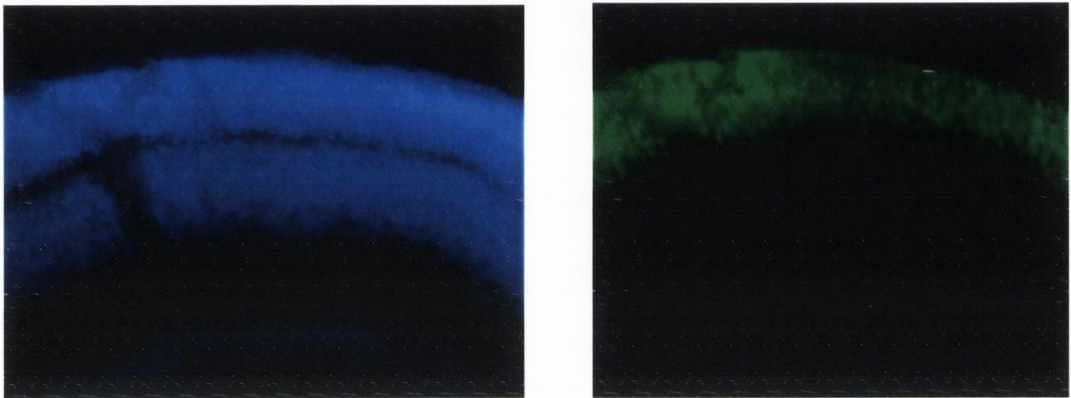


Fig. 6.16b: Post-natal day 21, (P21) rho-/- retinas showing EGFP fluorescence from the pAAV(2/5)-P_{MouRHO}EGFP construct. EGFP expression is specific to the photoreceptor cell layer. Six mice were assessed at this age.

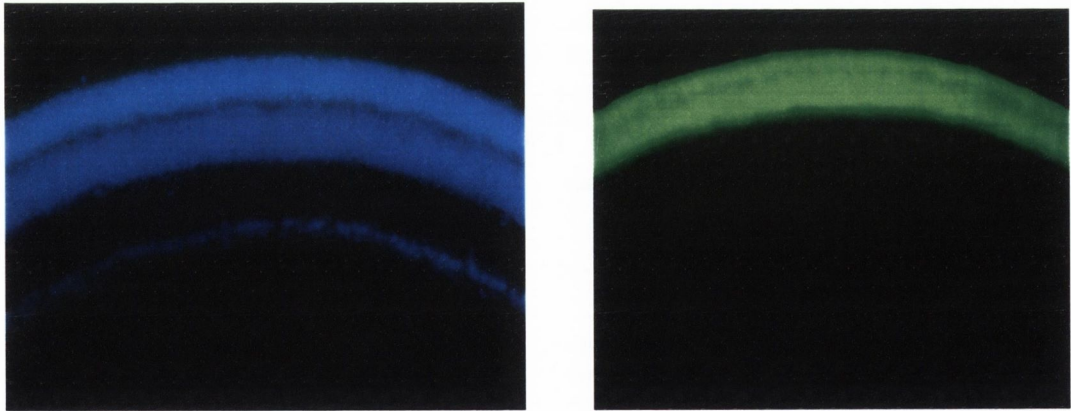


Fig. 6.16c: P42 retinas showing EGFP fluorescence from the pAAV(2/5)-P_{MouRHO}EGFP construct. The dapi-stained image is shown on the left and the same section under UV light is shown on the right. EGFP expression appears to increase over time but remains specific to the photoreceptor cell layer

Fig. 6.17: Confocal pictures of the pAAV(2/5)-P_{MouRHO}EGFP- injected retinas.

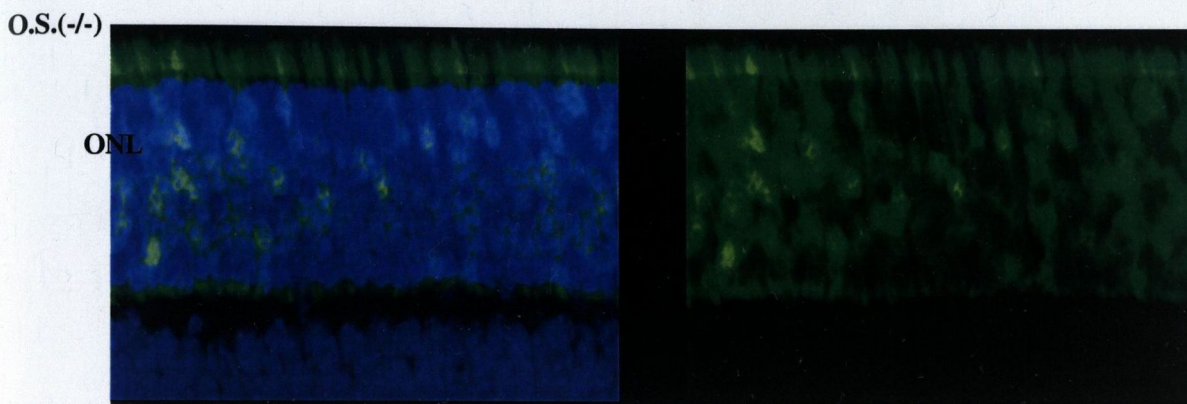


Fig. 6.17a: Section from the confocal microscope imaging of the pAAV(2/5)-P_{MouRHO}EGFP-injected retina at post-natal day 20. The lateral cross-section corresponding to the microscope image of fig. 6.13 is shown under 20X magnification. OS(-/-): Region where outer segments would be identified in the wild-type mouse retina. These outer segments are not elaborated in the rho-/- retina used in these studies; ONL: Outer Nuclear Layer. The cells of the ONL are shown to be expressing the EGFP transgene carried by the AAV2/5 virus.

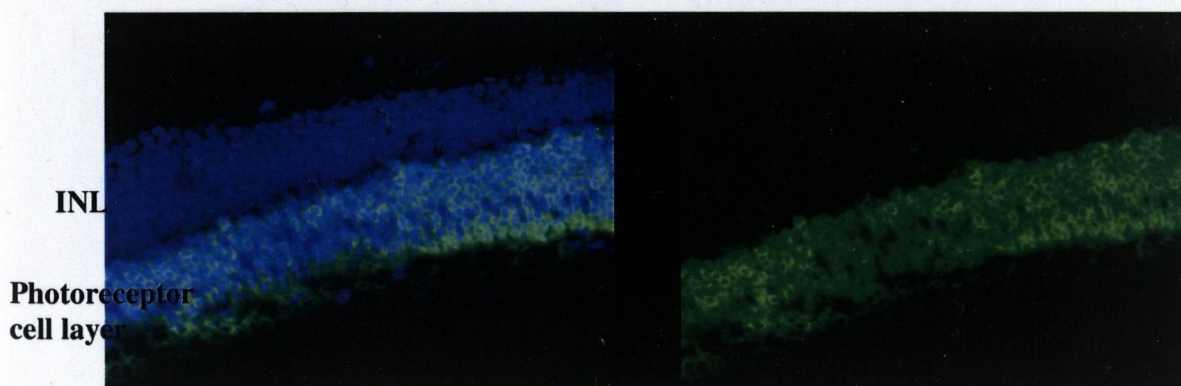


Fig. 6.17b: Confocal microscopy on a 50-μm vibratome retinal section of a PN30 rho-/- mouse injected with pAAV(2/5)-P_{MouRHO}EGFP. The front cross-section of this retina (corresponding to fig. 6.12) was analysed under 20X magnification and a single display view is shown from the imaging. The fluorescence is specific to the photoreceptor cell layer.

The microscope images of figs. 6.13–6.17 show the potential for the AAV(2/5) viral vector in retinal gene therapy. The viral capsid, of serotype 5, results in a more specific transduction of the photoreceptor layer. The faster kinetics of transgene expression from this pseudotyped virus allows for earlier onset of expression of this virus compared to the AAV(2/2) viral vector (see fig. 6.10 for results using the viral transgene cassette from AAV2/2). This result suggests that the AAV2/5 viral vector may be appropriate for future testing of novel therapeutics in animal models of retinal degeneration, such as the pro23his mouse, which was used in chapter 5 of this thesis.

Chapter 7

Concluding remarks

CHAPTER 7

7.1 Introduction

Retinitis pigmentosa may be considered a suitable disease for exploration of gene-based medicines as many of the genomic mutations causative of RP have been fully characterised. Moreover, the large number of RP patients, the severity of the disease, and the lack of effective treatment highlight the need for therapeutic solutions for this debilitating condition. This thesis is concerned with exploration of a suppression and replacement therapeutic approach for ADRP and the principle results from the work are summarised below. The current state of the art in retinal gene therapy and the possible future directions for such research are discussed. In addition the application of suppression and replacement technologies to other inherited conditions is discussed.

Mutations in two retinal genes, rhodopsin and peripherin/RDS, account for around 35% of all dominant cases of RP (Sohocki *et al.*, 2001), therefore, these genes are of particular relevance for suppression and replacement studies. Mutations causative of ADRP are especially prevalent in the rhodopsin gene, with over 150 currently documented in the retnet database - <http://www.sph.uth.tmc.edu/RetNet/>. A study focused on the down-regulation of rhodopsin gene expression that can be achieved using a series of siRNA molecules targeting a specific region of the is described in chapter 3 of this thesis (section 3.3.1). Rhodopsin RNA and protein suppression was assessed in three transformed cell culture lines (COS7, HeLa and HEK293 lines) following delivery of siRNAs and shRNA vectors (sections 3.3.2, 3.3.5 and 3.3.6). Dose curves of RNAi activity and toxicity levels within the cells following siRNA delivery were also examined *in vitro* (sections 3.3.3 and 3.3.4). Based on this analysis a selected shRNA construct expressing an siRNA termed 'siC' was further investigated in a transgenic animal model. Primary retinal cell culture, (retinal explants) derived from the retinas of neonatal mice expressing the normal human rhodopsin transgene on an endogenous mouse rhodopsin knockout background (the NHR+/--(MouRho-/-) mouse – Olsson *et al.*, 1992), was used as an experimental system in which to test siC-based silencing of human rhodopsin transcripts in the retina. FACS analysis combined with real-time rtPCR, as well as immunostaining of

dissociated retinal explant cells and explant sections were the techniques employed to determine suppression levels by siC in this animal model (section 3.3.7-3.3.9). In summary using the approach a number of RNAi molecules, which elicit suppression of human rhodopsin gene expression, were identified.

Based on the results of chapter 3 the most potent siRNAs, siB and siC, were carried forward for assessment of their effects on the replacement rhodopsin gene in chapter 4. This replacement gene was altered using the degeneracy of the genetic code at two nucleotides covered by siB and at five nucleotides covered by siC. In essence in the study, the level of protection from siRNA-mediated suppression afforded to the replacement rhodopsin transgene was being assessed. Full protection of the modified rhodopsin transcript against siRNA-based suppression in transformed cell cultures was not complete using the vector-driven forms of siB (covering only two changes to the replacement rhodopsin gene, section 4.3.4). However protection of rhodopsin expression was demonstrated for both the presynthesized siC molecule and the hairpin-vector-expressed form of siC (covering all 5 nucleotide changes – section 4.3.3–4.3.5).

In chapter 4, suppression was evaluated using RNAi assays. The ability of siC to suppress expression of the modified human rhodopsin replacement gene was also assessed in retinal explants from a replacement rhodopsin transgenic mouse model, the 'HR5m' mouse. This mouse model carries the five-codon-modified rhodopsin transgene on an endogenous mouse rhodopsin knockout background, (HR5m(MouRho-/-)). The same techniques as used in chapter 3 were utilised for this study. In cell culture (using RNA assays) siC-mediated suppression of human rhodopsin was abolished using the HR5m replacement gene. However, complete protection of rhodopsin expression was not initially achieved following electroporation of replacement rhodopsin retinal explants with the rhodopsin-targeting siC vector (section 4.3.7 – 4.3.8) – some residual suppression of the expression of the HR5m gene was obtained. Further analysis suggested that the disrupted endogenous mouse rhodopsin gene in this model was still capable of producing rhodopsin transcript (fig. 4.19). As such, mouse rhodopsin mRNA as well as replacement rhodopsin transcripts could in principle be detected in the real-time rtPCR assay used to determine expression levels. This mouse rhodopsin transcript is a possible target for cleavage by siC. Therefore, the residual level of rhodopsin suppression estimated

in HR5m retinal explants may be due to siC targeting endogenous mouse rhodopsin transcript whilst the human rhodopsin replacement transgene transcript may possibly be left largely intact.

Furthermore, an apparent suppression of rhodopsin at the protein level in retinal explants from the HR5m mouse (section 4.3.9) may be attributed to the considerably reduced level of transgene expression in the HR5m replacement rhodopsin mouse relative to the level of transgene expression in the normal human rhodopsin (NHR) mouse studied in chapter 3. Preliminary RNA results would suggest that relatively small amount of replacement rhodopsin transcript may be produced by the replacement mouse (fig. 4.18). As a result the non-quantitative immunolabelling assay used to measure protein levels may not be sensitive enough to detect the rhodopsin replacement protein if a reduced amount (relative to the NHR mouse) is indeed being produced. A quantitative ELISA may have been the best option for this protein analysis of shRNA in retinal explant cells. However, until methods of introducing shRNA constructs to these retinal cells were improved such analysis may have been uninformative (electroporation typically allowed for delivery of shRNA constructs to 10-15% of the explant cells). An alternative delivery method, which enables efficient transfection of photoreceptors, would significantly enhance the resolution of this experiment and in this regard approaches for retinal delivery of these constructs were explored in chapter 6.

Prior to this delivery study however, some additional exploration of the suppression element of the suppression and replacement strategy was undertaken. In the study described in chapter 5, the pro23his rhodopsin mouse was used as a model in which to test the shRNA, siC. However, the early onset of degeneration in this model made it difficult to obtain data from studies using a cultured retinal explant of the pro23his mouse. The study outlined in chapter 5 was also focused on attempts to modify the shRNA constructs used in chapters 3 and 4 by altering the loop sequence of the hairpin vector to one found in naturally-occurring microRNAs. In addition, work was presented on an alternative suppression agent, the hammerhead ribozyme, targeting the *peripherin/rds* gene. These approaches were not successful in achieving suppression *in vitro* and research was resumed on the shRNA construct design currently under investigation and described in chapters 3 and 4 based on Brummelkamp's pSuper design.

The research described in chapter 6 was concerned with the delivery of the shRNA cassette carrying siC for future *in vivo* studies. A non-viral approach for delivery was initially investigated (based on the Φ c31-integrase from Prof. Calos). While a preliminary evaluation of this non-viral was undertaken (fig. 6.8, chapter 6), further evaluation of the approach was put on hold in favour of adeno-associated viral delivery methods. Results from a number of studies have shown how AAV can be successfully used as a vector for retinal-specific delivery (Bennett *et al.*, 1999; Ali *et al.*, 2000; Bainbridge *et al.*, 2003). In particular the serotype AAV(2/5) has shown promise for photoreceptor-specific transduction (Yang *et al.*, 2002; Provost *et al.*, 2005). In this part of the thesis, an *in vivo* analysis of AAV(2/5) vector carrying an EGFP reporter gene subretinally injected into mice showed strong and specific fluorescence in the photoreceptor cell layer of vibratome-sectioned retinas. The EGFP expression was clearly localised to the outer nuclear layer of retinas from mice carrying a targeted disruption of the rhodopsin gene (section 6.3.3). Notably, when in addition the shRNA cassette expressing siC was cloned into an AAV vector, suppression of rhodopsin was achieved *in vitro* (section 6.3.2). A preliminary immune response study and a measure of toxicity levels in the AAV-treated cells was also carried out at this point. The most suitable purification method (caesium chloride) and the most appropriate AAV serotype (2/5) have since been optimised in the TCD lab, it should therefore now be possible to commence preclinical evaluation of AAV-delivered shRNA-replacement therapeutic constructs for rhodopsin-linked ADRP in transgenic mouse models of ADRP.

7.2 RNA-based Therapeutics for retinal disease

It is 28 years since Paul Zamecnik and Mary Stephenson first suggested therapeutic intervention in a disease at the point where disease-causing genes are transcribed from DNA into RNA (Stephenson and Zamecnik, 1978). The possibility of pursuing this approach was presented, when, in 1983 Green *et al.* demonstrated that RNA is itself an antisense molecule used by nature to control messages from genes. Thomas Cech later earned a nobel prize for the discovery that RNA can act as an enzyme, by binding and cleaving a target mRNA, and recycling itself to destroy another target (Kruger *et al.*, 1982). As a result of this finding, Cech founded a

company, Ribozyme Pharmaceuticals, (now siRNA inc.) thus furthering the ambitions to put RNA-based therapeutics into clinical practice.

It has since been demonstrated that synthetic RNAs of 21 and 22 nucleotides in length are capable of mediating cleavage of target homologous RNA without provoking PKR activation (Fire *et al.*, 1998; Elbashir *et al.*, 2001a). This led to the exploration of a direct therapeutic application for siRNA targeting mutant transcripts in animal models (Song *et al.*, 2003; Yokota *et al.*, 2003; Xia *et al.*, 2004). It appears likely that as the field of gene-therapy evolves it shall include the use of RNA-based therapies. In fact, the RNA therapeutic approach is already applied clinically with the antisense oligodinucleotide drug, Fomivirsen (VitraveneTM) currently in use to treat *Cytomegalovirus Retinitis* infections in AIDS patients (Isis Pharmaceuticals, Carlsbad, CA, US).

7.3 Delivery of transgenes to the retina

In this thesis sub-retinal injection (carried out by the ophthalmic surgeon in the unit, Dr. Kenna) was used effectively to deliver an AAV vector carrying a transgene to the retina (section 6.3.3). Challenges unique to delivery to retinal tissue have to be overcome before the expression of any transgene can be achieved in the retina. The Blood Retinal Barrier (BRB) in the posterior segment of the eye, and the Blood Aqueous Barrier (BAB) in the anterior section isolate the retina and prevent the entry of many drug molecules. While the choroid is rich in blood vessels and capable of rapid drug uptake from systemic circulation, the BRB prevents free passage from the choroid to the retina and vitreous (fig.1.1, chapter 1). The increase in retinal pathologies associated with conditions such as AIDS and diabetes, has meant that the development of methods enabling drug delivery to the posterior segment of the eye has become the focus of an increasing number of research groups and pharmaceutical companies. Thus far, only surgical methods, such as intra-vitreous implants and intra-vitreous injections have been used in the clinic and with only limited success. A recent and promising approach has involved *ex vivo* gene therapy. Through the use of genetically engineered and encapsulated cells implanted directly into the vitreous, long-term delivery of a transgene (which may be switched on or off using regulatory elements) may be possible (Uteza *et al.*, 1999; Tao and Aguirre, 2002). Leveillard *et al.* have reported their intention to use this technology in human clinical trials to test

the recently identified rod-dependent cone viability factors, RdCVFs (Leveillard *et al.*, 2004.)

7.3.1 Possible routes of retinal delivery:

Systemic Administration: Intravenous therapy will result in only 1–2% of the plasma drug concentration being achieved in the vitreous. Therefore to be effective the drug will often require concentrations in great excess of the therapeutic dose and may have toxic effects due to its general circulation (Costabile, 1998).

Topical Administration: For a drug to reach the posterior segment upon topical application using drops or an ointment, it must traverse through the cornea, aqueous humor and lens. Loss of the therapeutic through tears, drainage and conjunctival absorption mean that the agent is unlikely to reach minimum therapeutic levels by this route (Cekic *et al.*, 1998).

Intravitreal injections: Ocular antivirals and antibiotics have been delivered by injection directly into the vitreous to treat conditions such as CMV Retinitis (Lopez-Cortes *et al.*, 2001; Waga *et al.*, 1999). This approach avoids the risk of systemic side-effects. However the need for repeated injections means that the risk of infection is high and retinal detachments or cataracts may occur upon each injection.

Transporter-mediated drug delivery: Effective passage across the cell membrane may be easier to achieve by targeting drugs to transporters on the RPE following systemic administration. The drug is coupled to a moiety that is similar to the endogenous substrate of that transporter (Smith, 1993). Intracellular enzymatic cleavage of the ligand-drug bond will release the therapeutic agent; as the ligand typically is a nutrient toxicity concerns are unlikely to arise. Peptide, glucose, and amino acid transporters have been used in attempts to enhance retinal drug delivery (Berger and Hediger, 1999; Ban and Rizzolo, 2000). Various transporters have been identified and characterised for the retina (Pow, 2001). These transporters could be administered by various modes of administration including systemic and intravitreal injection and may limit the side effects associated with such delivery methods. Even small increases in bioavailability of drug molecules can result in a significant increase in therapeutic response.

Sub-retinal injection and Electroporation: A relatively new technique for delivery of DNA into neonatal mouse retina is called electroporation. One version of this method combines delivery by sub-retinal injection with electric pulses applied by tweezer-

type electrodes. Electroporation to introduce dsRNA has achieved a high level of cellular transduction in neonatal mouse pups (Matsuda and Cepko, 2004). With a voltage of 80V being applied to the eye, this approach is clearly too aggressive to have a clinical application, however, an allied technique, termed iontophoresis, is already routinely used in retinal drug delivery in humans. This involves a lower potential generated across the cornea and while it is not powerful enough to enable delivery to photoreceptors, the treatment is open to further optimization with, for example, a sub-retinal injection as used with electroporation. In the meantime Cepko's team have demonstrated that electroporation can deliver dsRNA to mouse retina. This technique was used in this thesis to generate results of chapters 3, 4 and 5.

7.4 AAV-mediated retinal transgene expression

Although initial attempts at human gene therapy were dominated by retrovirus vector-mediated gene delivery, gene carriers based on the DNA viruses, adenovirus and adeno-associated virus were also developed in the mid 1980s (Van Doren *et al.*, 1984; Hermonat and Muzycka, 1984). AAV possesses fewer of the advantages, which make helper-independent adenovirus so convenient as a carrier vector: AAV has, in the past, been difficult to produce; it has a limited cloning capacity; and is relatively slow at producing transgene expression. However while transgene expression tends to be short-lived from adenovirus, long-term expression (i.e. years) has been achieved using AAV in immunocompetent animals (Lai *et al.*, 2002). Unlike adenoviral vectors, AAV do not encode any potential immunogenic viral products (although a generation of 'gutless' adenovirus devoid of viral genes has been engineered – reviewed by Thomas and Kay, 2003). Therefore, while the early burst of transgene expression from recombinant adenovirus makes it ideally suited to the short-term production of therapeutic proteins, AAV is becoming specialised for the targeting of disease conditions requiring long-term transgene expression (Schmidt-Wolf, 2003) such as RP, the subject of this PhD thesis. The AAV(2/5) serotype has particular relevance to retinal gene therapy as this viral capsid exhibits strong preferential transgene expression in photoreceptor cells (Yang *et al.*, 2002). It is for these reasons that the AAV vector system was recruited in the study described in chapter 6 for *in vivo* evaluation of siRNAs targeting the rhodopsin gene.

7.5 Immune responses in gene therapy

Immune response to AAV-shRNA constructs:

Results presented in this study indicate that AAV serotype 2 vectors are capable of delivering active shRNA molecules targeting rhodopsin to HeLa cells (section 6.3.2). However, while this virus did not appear to have a significant effect on the proliferation of HeLa cells, an increase in expression of interferon protein alpha was observed when the cells were transduced with both the empty viral shell and the shRNA-carrier virus. In chapter 6, it was of interest to investigate the expression levels of genes encoding OAS-1 and Ifi- α , as these proteins have been linked to siRNA-induced immune reactions. However it would appear from the comparison of the preliminary data from chapters 5 and 6 that a virus-specific, rather than shRNA-specific, increase in Ifi- α level may have occurred in HeLa cells (comparing the results of section 5.3.3 and section 6.3.2). This demonstrates how systemic effects of the viral capsid may be of greater concern than the expressed shRNA transgene in provoking an immune response. Capsid antigens have been associated with immune responses to AAV in the past (Bessis and Boissier, 2004). In particular, humoral immunity arising from early infections of wild-type AAV may have an effect on the efficiency of gene transfer with recombinant vectors. While alterations to capsid structure may enhance vector safety, the best chance of circumventing the immune response may come from the immunology and pharmacology field. This may involve the use of immunosuppression drugs and has been applied alongside a current retinal therapy with some success, (Di Polo *et al.*, 1998).

Bennett *et al.* have found that while AAV could induce a systemic humoral immune response, the virus did not induce neutralizing antibodies after delivery to the subretinal space (Anand *et al.*, 1998). Notably, the immune response in the eye to foreign antigen exposure may vary substantially from that of a typical systemic response (Niederhorn, 1990). This may ultimately be advantageous to the development of ocular gene therapy. Information regarding the potential immune response to AAV administration *in vivo* may require the measurement of antibody IgG levels in serum following administration of the vector.

7.6 Prospects for gene therapy for ADRP

Mutations in virtually every gene whose expression is restricted to rod photoreceptor outer segments have been found associated with RP phenotypes (Hims

et al., 2003). Therefore gene-mediated therapy strategies may be the only potential way to “cure” RP. However, there are over 102 genes responsible for RP cloned and many more already mapped (<http://www.sph.uth.tmc.edu/RetNet/>), moreover within some of these genes, such as rhodopsin, there are multiple mutations. Therefore it may not be possible for RP gene therapy to remain mutation-specific, hence the need for a suppression and replacement approach as adopted in this thesis and as used by other studies into ADRP-based retinopathies (Palfi *et al.*, 2006; Kiang *et al.*, 2005; O’Neill *et al.*, 2000). However, such approaches are, in principle, applicable to any of the approximately 1500 human autosomal dominant mendelian diseases recorded in humans (Farrar *et al.*, 2002).

The ‘gene-exchange’ technology used in this thesis involved replacement of a rhodopsin gene with an engineered replacement version of the gene following suppression targeted to a specific region of the endogenous rhodopsin transcript. The alteration of the redundant nucleotides of this targeted 5-codon region ensured protection of the replacement rhodopsin gene from suppression. A second approach, involving suppression targeted to the UTR of a gene, offers similar scope for intervention by delivery of a UTR-modified replacement gene (the approach was adopted in the peripherin suppression study carried out in chapter 5). A third approach is to target a known SNP of a mutant transcript for suppression, and replace it with the alternative allele of the gene. In cases where the animal is a heterozygote for this SNP a replacement therapy may not even be necessary, assuming sufficient transcript levels can be produced from the second endogenous allele (in chapter four, this approach was considered unfeasible for the rhodopsin gene at the present time, due to the lack of known SNPs with a significant heterozygosity level in the rhodopsin coding sequence). As mentioned, suppression and replacement has been used by a number of research groups as a therapeutic strategy to overcome the mutational heterogeneity displayed by many hereditary disease states. Yokota *et al.* have, for example, applied the strategy to the treatment of familial ALS caused by mutations in the SOD-1 gene (2003). The strategy has also been investigated for intervention in the debilitating bone disease, Osteogenesis imperfecta (Millington-Ward *et al.*, 2004).

Insights into secondary cone photoreceptor loss have led to a link between dominant mutations of the rhodopsin gene and developmental abnormalities in the postsynaptic bipolar cells of the cone pathway. A key to preventing the vision loss

associated with cone photoreceptor degeneration is likely therefore to involve early therapeutic gene delivery to rod cells (Zack *et al.*, 1999). In addition, the ability of protective pharmacological molecules to slow down the progression of photoreceptor degeneration has been well documented. Such agents include vitamin A supplementation, trophic factors or endogenous viability factors, (Li *et al.*, 1998; Liang *et al.*, 2001; Leveillard *et al.*, 2004). Although outside of the scope of this thesis, it is this pharmacological approach that may be most applicable to diseases of the retina in the medium term. Ultimately, neuroprotective molecules may be used to slow down the degeneration, allowing time for a gene-mediated therapy to take effect. Therefore, a two-pronged somatic gene therapy approach may be required, such as, for example, an anti-apoptotic therapeutic in conjunction with an RNA knockdown therapy.

Many researchers in the field of gene therapy agree that the goal of 'curing' a genetic disease by gene transfer techniques has technically been achieved. This is due to the research of Cavazzana-Calvo *et al.* involving the use of a Mo-MuLV vector to deliver, *ex vivo*, a gene encoding the cytokine receptor γ chain to CD34+ cells from children with X-linked SCID (2000). Early encouraging results also emerged from an ADA-SCID clinical trial, (a disease phenotype more challenging than X-SCID) and carried out in haemopoietic stem cells as opposed to mature T lymphocytes (Aiuti *et al.*, 2002). Since this trial the random nature of retroviral integration has been put into question, and the risk of insertional oncogenesis by a retrovirus is no longer considered to be negligible. Genome-wide scans of the recently available full human genome sequence show the high prevalence of provirus-chromosomal DNA junctions in the vicinity of promoters (for Mo-MuLV) or within transcription units (for HIV) (Wu and Burgess, 2004.) The overgrowth of T-cell clones resulting in leukaemia-like symptoms in two patients on ADA-SCID trials have subsequently been attributed to retroviral vector insertion near to and deregulation of the proto-oncogene *LMO-2* (Hacein-Bey-Abina *et al.*, 2003.) In particular, one analysis of the human genome has suggested that an adverse event is less random than believed and possibly inevitable as one in every 10,000 modified cells might harbour a dangerous insertion (Baum *et al.*, 2003). More than a decade of clinical trials in humans has highlighted to researchers and the general public the complexities of clinical gene therapy.

In terms of gene therapy for inherited retinopathies it is probable that much shall be learnt from the anticipated AAV retinal trials in the US (Bennet *et al.*) and the

UK (Ali *et al.*). In the meantime, efforts are on-going in cell culture and preclinical animal experiments within many research groups, to determine the efficiency and possible side-effects of current gene transfer techniques.

7.7 Future directions

7.7.1 Transgene expression levels in animal models:

The results obtained for siRNA-treatment of the normal human rhodopsin mouse (NHR mouse) and the replacement rhodopsin mouse model (HR5m mouse) are presented in chapters 3 and 4 respectively. The data suggests that relative transgene levels need to be tightly controlled to allow for direct comparisons in a suppression and replacement study involving these models. In particular, a full assessment of RNA and protein expression levels may be necessary in the HR5m model over a number of generations to facilitate the comparison of data derived from this model with the NHR mouse model. However, it is encouraging that the results from the replacement rhodopsin mouse model suggest that a relatively low level of rhodopsin expression may be sufficient on a rho-/- background, (fig. 4.18) to rescue the rho-/- phenotype and result in development of rod outer segments and normal ERG function when assessed in mice at three months of age, (determined by M. O'Reilly – unpublished data). This may be of benefit in the continued use of replacement-modified rhodopsin transgenes in rhodopsin-linked ADRP.

7.7.2 Design of a therapeutic construct, pAAV-HR7m-siC:

A therapeutic vector carrying the replacement rhodopsin gene bearing 7 changes (fig 4.12) in tandem with the siRNA, siC, will be assessed in a mouse model of ADRP. The HR7m rather than HR5m gene will likely be incorporated as the replacement gene in this vector, so as to optimise the level of protection afforded to replacement rhodopsin transcripts from siC-based suppression. The pro23his explant was used as a disease model in chapter 5 and it is anticipated that the expression of a replacement rhodopsin gene combined with suppression of the dominant pro23his transgene should in principle enable survival of the photoreceptor cells in the retina of this mouse. However, an ADRP mouse model carrying the pro347ser mutation (derived from the porcine pro347ser transgene – Dryja *et al.*, 1990) is now available in the lab and may be used to assess siRNA-based suppression and replacement

constructs. Notably, pro347ser mice show slower rate of disease progression than that observed in the pro23his mouse and hence in principle enables a greater timeframe in which to explore therapeutic intervention. As discussed above, the AAV vector of serotype 2/5 may be used as a vehicle to achieve early photoreceptor-specific expression of the nucleotide-based therapeutics under development in the retina. Based on the results from this PhD thesis it is hoped that constructs such as HR7m-siC might offer therapeutic potential for rhodopsin pro23his or pro347ser-linked ADRP and allied retinal degenerative diseases caused by rhodopsin-based mutations. Given the enormous mutational heterogeneity present in disorders such as rhodopsin-linked ADRP where over 100 different mutations are causative of this retinopathy, there is a clear need for innovative therapeutic solutions which are not mutation-specific but instead are mutation-independent such as the suppression and replacement approach under investigation during the above course of the study described in this PhD thesis.

APPENDIX

1A Oligonucleotides

(i) Primers

PRIMER	SEQUENCE	
Rho1037F	CTTTCCTGATCTGCTGGGTG	
Rho1179R	GGCAAAGAACGCTGGGATG	
Rho124F	CCTACATGTTTCTGCTGATCG	
Rho258R	CATGAAGAGGTCAGCCACG	
GapdH F	CAGCCTCAAGATCATCAGCA	
GapdH R	CATGAGTCCTTCCACGATAC	
NeoF	CCTGAATGAACTGCAAGACG	
NeoR	CGTCTTGCAGTTCATTCAGG	
mou18SrRNAF	CAAGAACGAAAGTCGGAGGT	
mou18SrRNAR	CGGGTCATGGGAATAACG	
NHR F	CTG GTC CAG GTA ATG GCA CT	
NHR R	CCA GCC CTT GTA GCA ACA TT	
Pro23His F	TCA GAA CCC AGA GTC ATC CA	
Pro23His R	GCC ACC TAG GAC CAT GAA GA	
OAS1 F	ACG CTG ACC TGG TTG TCT TC	
OAS1 R	GAC CTC AAA CTT CAC GGA AAA	

Ifi-α F	ACG CCA CCC ACA AGTATC TC
Ifi-α R	GAG GCT AGG AGT TGG AGG C
HRho+1f(NHE1)	CTAGCTAGCATGAATGGCACAGAAGGCCC
MP2f	GTGCAACATAAGAAGCTGCGC
MP1r	GTGCGCAGCTTCTTATGTTGC
Hrho852r	ATGCGGGTGACCTCCTTC
Snprho2441-2460	GAAATTGCCCTGTGGTCCTT
snpRs2855558FpcrP23H	CCCTGACTCAAGCCTCTTGCC
snpRs2855558RpcrP23H	TGTGGGCGGGTGAGCATGGCTTC
snpRs2855558FseqP23H	GTCACAGGAATGCAGGATGCAG
snpRs2269736FP23H	GAGTACCTCTCCTCCCTGACC
snpRs2269736RP23H	GAAGGCCTCTCAGCCACCACCG
snpRs2269736RpcrREP	AGGGTGGCAAGAAGCCCTCCC
RhoPF1	AAGCAGCCTTGGTCTC
(HINDIII)HR+1F	GATAAGCTTAGAGTCTCCAGC
pTOPO1201f	AAC AGC TAT GAC CAT G
PTOPO1224r	CGC CAG GGT TTT CCC AGT CAC GAC
ΨattBf	CGA TGT AGG TCA CGG TCT CGA
ΨattPr	TTG CAT GGC CTC ATT TCC GTC
PINTEGRASEf	CGT CTA CTG ACG CGC GCC CCC
PINTEGRASer	GCA GCA AGG GGC GGA AGA GC
PAAVrepf	CTA CCA GCG GTG GTT TGT TT
PAAVrepr	GCA GAG CGA GGT ATG TAG GC

(ii) Oligos for cloning of shRNA constructs

H1-siRNA F	GATCCCC...(sense)...CAAGAGA...(antisense)...TTTTTGGAAA
H1-siRNA R	AGCTTTTCCAAAAA...(antisense)...TCTCTTG...(sense)...GGG
Mi-23C F	GAT CCC CCT ACG TCA AAG TCC AGC ACC TTC CTG TCA GTG CTG GAC TTT GAC GTA GTT TTT GGA AA
Mi-23 C R	AGC TTT TCC AAA AAC TAC GTC AAA GTC CAG CAC TGA CAG GAA GGT GCT GGA CTT TGA CGT AGG GG

(iii) Oligos for cloning Ribozyme constructs

Ribozyme	Sequence
Rz8F	GAACGCTAGCCCAAGTGCTGATGAGTCCGTGAGGACGAAAGTCCGGGGAT CCGCA
RzR	TGCGGATCCCCGGACTTTCGTCCTCACGGACTCATCAGCACTTGGGCTAGC GTTC
Rz9F	GAACGCTAGCCAAACCTTCTGATGAGTCCGTGAGGACGAAACGAGCCGGA TCCGCA
Rz9R	TGCGGATCCGGCTCGTTTCGTCCTCACGGACTCATCAGAAGGTTTGGCTAG CGTTC

2A RNAi target sites

siRNA	SEQUENCE	
Silencer A	DNA target	AAGTCCAGCACAGAAGCTGCGC
	Sense strand	GUCCAGCACAGAAGCUGCGCUU
	Anti-sense	GCGCAGCUUCUUGUGCUGGACUU
SilencerB	DNA target	ATCAACTTCCTCACGCTCTACGT
	Sense strand	CAACUUCCUCACGCUCUACdGT
	Anti-sense	GUAGAGCGUGAGGAAGUUGdAT
SilencerC	DNA target	CTCTACGTCACCGTCCAGCACAA
	Sense strand	CUACGUCACCGUCCAGCACdAA
	Anti-sense	GUGCUGGACGGUGACGUAGdAG
SilencerD	DNA target	AAACTTCCTCACGCTCTACGT
	Sense strand	ACUUCCUCACGCUCUACGU
	Anti-sense	ACGUAGAGCGUGAGGAAGU
SilencerE	DNA target	GCTCTACGTCACCGTCCAG
	Sense strand	GCUCUACGUCACCGUCCAdGdT
	Anti-sense	CUGGACGGUGACGUAGAGCdGdT
SilencerUTR (Rs7984)	DNA target	GGTCAGCCACAAGGGCCAC
	Sense strand	UCAGCCACAAGGGCCACUU
	Anti-sense	GUGGCCCCUUGUGGCUGACC

Control siRNA	SEQUENCE	
Silencer NT: Negative control (www.qiagen.com/siRNA)	DNA target	AATTCTCCGAACGTGTCACGT
	Sense strand	UUCUCCGAACGUGUCACGUdTdT
	Anti-sense	ACGUGACACGUUCGGAGAAdTdT
SilencerEGFP: “gfp-22” (www.qiagen.com/siRNA)	DNA target	CGGCAAGCTGACCCTGAAGTTCAT
	Sense strand	GCAAGCUGACCCUGAAGUUCAU
	Anti-sense	GAACUUCAGGGUCAGCUUGCCG

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