



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

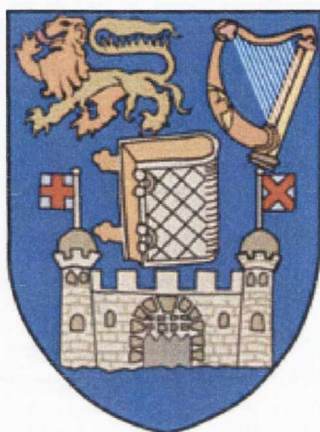
By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Optimisation of a suppression and replacement therapeutic strategy for animal models of rhodopsin-linked autosomal dominant Retinitis Pigmentosa



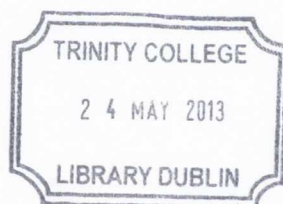
A thesis submitted to the University of Dublin for the Degree of
Doctor of Philosophy

By

Claire Kilty
March 2012

Under the supervision of Professor Jane Farrar

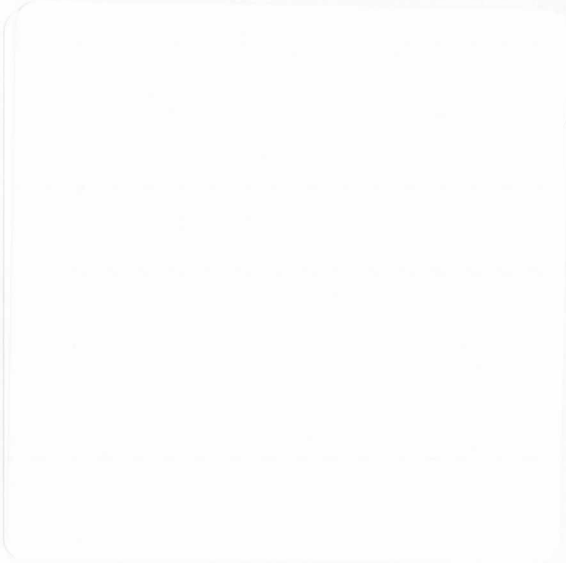
Department of Genetics
Trinity College Dublin



Thesis 10066

Declaration

This thesis has not been previously submitted to this or any other university for the examination of a higher degree. The work presented here is entirely my own except where noted. This thesis may be made available for consultation within the university library. It may be photocopied or lent to other libraries for the purpose of consultation.



Acknowledgments

Many people have been vital in aiding me through my PhD Journey. Firstly my supervisor, Prof. Jane Farrar, thank you for allowing me to work on a worthwhile project and for all the help and advice along the way. To Sophia and Mary I really appreciate all the help and guidance over the last five years, especially for answering all my endless pestering questions. Naomi I am really grateful for all the help and advice with my *in vivo* work, in particular for making all my AAV viruses. To Arpad thanks for helping with the immunohistochemistry and protein work. Thanks to Matthew Campbell for helping me with my western blotting. To Dr. Paul Kenna, thanks a million for the endless subretinal injections and ERGs. Thanks to IRCSET “embark initiative” and SFI for funding this PhD.

To all the PhD students in the laboratory both past and present: Finn, James, Chris, Matthew, Ema, Elif, Sorcha, Carol and Anh thank you for making the laboratory such a great place to work. To Clare and Leanne, I could not have gotten through the last 4 years without you guys. Thanks for being there and for all the laughs (and the cries). I will really miss both of you in the lab.

To all at the animal unit, Caroline, Charlie, Dave and Rebecca, I really appreciate all the help with animal the animal work. To Dr. Alfonso Blanco thanks for performing all the FACS.

To my non-PhD friends: Kate and Nic for all the good times. To the scoobies thanks for being such good friends these past 13 years, a special mention to Ashling for all the weekly coffee meetings.

To my Mam (Monica) and Dad (Finian), I feel really lucky that I have such great parents! Thanks for always being there, for always believing in me and encouraging me. Love you both. To my sister Eithne and my amazing niece Mary-Ella and nephew Aaron thanks for everything. To Nonnie, thanks for being my second mammy and for being there for me and Eithne as we were growing up throughout the years

To my boyfriend Gareth: Thanks for an amazing two years. These past six months have been particularly tough on you – thanks for putting up with all my freak outs and with being second best to the thesis for a while. This time next year it will be my turn! Love you X

Summary

The focus of research presented in this PhD thesis was optimising a therapeutic strategy for an inherited retinal disorder termed Retinitis Pigmentosa (RP). RP is a disease in which the photoreceptors progressively degenerate. This initiates with degeneration of rod cells and as the disease progresses typically the cone photoreceptors are also affected. At the end stage of the disease affected individuals are legally blind. RP can be inherited as an autosomal dominant (adRP), autosomal recessive or x-linked recessive condition, although digenic and mitochondrial inherited forms of the disease have also been characterised. Rhodopsin protein is involved in the initialisation of the rod visual transduction cascade. It is notable that mutations within this gene account for approximately 30-40% of adRP, making it a good target disease for the development of gene therapies. Rhodopsin-linked adRP is mutationally heterogeneous with over 100 mutations identified to date. For this reason a mutation-independent therapeutic strategy (suppression and replacement) was explored during the course of the study presented in this thesis. The suppression agent of choice was RNAi, a post transcriptional gene silencing mechanism by which the introduction of small double stranded RNA molecules direct the sequence specific cleavage of target RNA molecules. In addition as both mutant and wild-type alleles are suppressed with this mutation-independent strategy, a codon-modified replacement rhodopsin gene resistant to suppression by RNAi was designed in an attempt to provide adequate levels of wild type rhodopsin protein in photoreceptor cells.

To evaluate siRNA-based rhodopsin therapeutics in a rhodopsin-linked adRP pig model, the research detailed in Chapter 3 of the thesis was concerned with the initial design of siRNA molecules which could suppress both human and pig rhodopsin. Three molecules were designed and each was co-transfected into HeLa cell culture together with a hRHO plasmid. While significant suppression of rhodopsin by all siRNAs was achieved at the RNA level as measured by real-time RT-PCR, only two of the three efficiently down-regulated hRHO protein as measured by western blotting. It was decided to further evaluate the best suppressor siP3 for *in vivo* analysis

The study presented in Chapter 4 of this thesis involved the optimisation of *in vivo* suppressor molecules based on siP3. One shRNA and two artificial miRNAs (based on miR-30a and miR-155) were cloned and generated into high titre AAV-2/5 vectors and each virus was tested *in vivo* in a transgenic mouse expressing human rhodopsin (NHR mouse). While suppression at the RNA level was obtained with all three AAV vectors, only shP3 consistently suppressed human rhodopsin in these mice at the RNA, protein, histological and functional level (as assessed by electroretinography). Thus it was decided to proceed with *in vivo* analysis of shP3 in a rhodopsin-linked adRP model.

In Chapter 5 a study focused on suppression of pig rhodopsin in addition to the evaluation of the potential therapeutic benefit associated with suppression of mutant porcine rhodopsin is presented. A transgenic mouse model was available in the laboratory that expresses mutant pig rhodopsin on a heterozygous mouse rhodopsin background. The target site for shP3 is not homologous between porcine and mouse rhodopsin. Therefore the rationale behind the work detailed in Chapter 5 involved suppressing the mutant pig rhodopsin in rhodopsin-linked adRP mice, while the non-suppressed mouse rhodopsin allele acted as an endogenous replacement gene and thus therapeutic rescue should in principle be obtained. While efficient suppression of pRHO was observed at the molecular level in terms of RNA, protein and immunohistochemistry, no functional benefit was observed in mice injected with shP3. Additionally *in vitro*, it was found that shP3 may also suppress mouse rhodopsin despite the presence of three mismatches. This latter observation may explain the absence of functional rescue achieved in shP3 injected mice.

Chapter 6 involved the optimisation of a human rhodopsin replacement gene such that transcripts from the replacement gene were resistant to siP3 RNAi-mediated suppression. Initially two codon-modified replacement genes were generated resistant to siP2 or siP3 respectively and evaluated *in vitro*. Notably both replacement genes were found to be refractory to suppression by their respective siRNA counterparts as evaluated at the RNA and protein level. Subsequently the replacement gene rP3 was generated into both AAV-2/5 and AAV-2/8 virus for *in vivo* evaluation. *In vivo* evaluation involved subretinal injection of either serotype into rhodopsin knockout mice to assess the effects of delivery of an AAV-rhodopsin replacement gene in knockout mice. Functional benefit was assessed utilising ERG read outs, histology, western blotting and real-time RT-PCR. In addition to evaluation of the rP3 AAV-2/5 and AAV-2/8 vectors detailed above, a preliminary comparative analysis was undertaken with AAV-2/5, AAV-2/8 and AAV-2/rh10 viral serotypes in order to determine the optimal AAV serotype for subretinal administration of a human rhodopsin replacement gene and expression of the transgene encoded hRHO protein as assessed by western blotting. The work presented in the thesis provides a further impetus to progress this novel dual-component rhodopsin suppression and replacement therapy towards evaluation in large animals models of adRP.

Abbreviations

μl: microlitre
5'GMP: 5' cyclic guanosine monophosphate
7TM: 7 transmembrane
AAV: Adeno-associated virus
adRP: Autosomal Dominant Retinitis Pigmentosa
AGO2: Argonaute 2
AMD: Age related macular degeneration
APS: Ammonium Persulfate
arRP: autosomal recessive Retinitis Pigmentosa
AS: Anti-sense Strand
ATCC: American Type Culture Collection
BCA: Bicinchoninic acid
bp: base pair
BSA: Bovine Serum Albumin
CBA: Chicken β-Actin
C. elegans: *Caenorhabditis elegans*
cDNA: complementary Deoxyribonucleic acid
cGMP PDE: cyclic guanosine monophosphate phosphodiesterase
cGMP: cyclic guanosine monophosphate
CHS: Chalcone synthase
CMV: Cytomegalovirus
CNV: Choroidal Neovascularisation
CNS: Central Nervous System
CS: Cryptic Splice site
CSNB: Congenital Stationary Night Blindness
C_T: threshold cycle
DAPI: 4', 6-diamidine-2-phenylindole-dihydrochloride
ddH₂O: double distilled H₂O
DGCR8: DiGeorge critical region gene eight protein
dH₂O: distilled H₂O
DMEM: Dulbecco's modified Eagle medium
DNA: Deoxyribonucleic acid
dNTPs: Deoxynucleotide Triphosphates
dsRNA: double stranded RNA
E.coli: *Escherichia coli*
EDTA: Ethylenediamine tetra acetic acid
EGFP: enhanced Green Fluorescent Protein
ELISA: Enzyme Linked Immunosorbent Assay
EPO: Erythropoietin
ERG: electroretinogram
ES: Embryonic Stem Cell
EtBr: Ethidium Bromide
F: Forward
FACS: Fluorescent Activated Cell Sorting
FCS: Fetal calf serum
fMRI: Functional Magnetic Resonance Imaging
GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase
GCAP: Guanylate cyclase activating protein

GCL: Ganglion cell layer
 GFP: Green Fluorescent Protein
 GMP: Good Manufacturing Practice
 GPRC: G protein coupled receptor
 HBSS: Hanks Balanced Salt Solution
 HEK: Human embryonic kidney
 hetPP347S: heterozygous porcine P347S mice
 HP347S: human P347S
 hRHO: human rhodopsin
 HRP: Horseradish Peroxidase
 IgG: Immunoglobulin G
 ILM: Inner Limiting Membrane
 INL: inner nuclear layer
 i.p.: intra-peritoneal
 IPL: inner plexiform layer
 IS: inner segment
 ITR: Inverted terminal repeat
 kb: kilobase
 kDA: kiloDaltans
 L: litre
 LB: Luria-Bertani
 LCA: Leber Congenital Amaurosis
 LE: left eye
 MCS: Multiple Cloning Site
 miRNA: micro RNA
 ml: millilitre
 mRHO: mouse rhodopsin
 mRNA: messenger RNA
 NHR: Normal Human Rhodopsin
 NpHR: *Natronomonas pharaonis* Halorhodopsin
 NRL: Neural Retinal Leucine zipper
 OCT: Optimal Cutting Temperature
 ONL: outer nuclear layer
 OPL: outer plexiform layer
 ORF: Open reading frame
 OS: outer segment
 P347S: proline 347 serine
 PBS: Phosphate buffered saline
 PCR: Polymerase Chain Reaction
 PDE: Phosphodiesterase
 PEG: Polyethylene Glycol
 PI: Propidium Iodide
 PLL: Poly-Lysine
 PNA: Peanut Agglutinin
 PolII: Polymerase II
 PolIII: Polymerase III
 PP347S: porcine P347S
 pre-miRNA: precursor miRNA
 pRHO: porcine rhodopsin
 pri-miRNA: primary miRNA precursor

PVDF: Polyvinylidene fluoride
R: Reverse
R*: activated rhodopsin
RE: right eye
RHO: rhodopsin
RHO-adRP: rhodopsin linked autosomal dominant Retinitis Pigmentosa
RI: Refractive Index
RIS: Rod Inner Segments
RISC: RNA-induced silencing complex
RNA: Ribonucleic acid
RNAi: RNA interference
RNase: Ribonuclease
ROS: Rod Outer Segments
RP: Retinitis Pigmentosa
RPE: Retinal Pigment Epithelium
RT-PCR: reverse transcription Polymerase Chain Reaction
SAP: Shrimp Alkaline Phosphatase
SD: Standard Deviation
SDS: Sodium dodecyl sulphate
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
shRNA: short hairpin RNA
siRNA: small interfering RNA
SNALP: Stable Nucleic Acid Lipid Particle
SPF: Specific Pathogen Free
SS: Sense Strand
ssRNA: single-stranded RNA
TAE: Tris Acetate EDTA
TBS: Tris Buffered Saline
TLR: Toll Like Receptor
T_m: Melting Temperature
UNG: Uracil-N-glycosylate
UTR: untranslated region
VEGF: Vascular Endothelial Growth Factor
vp: Viral Particles
XLRP: x-linked recessive Retinitis Pigmentosa
ZFN: Zinc Finger Nuclease

<u>Table of contents</u>	<u>Page</u>
Declaration	i
Acknowledgments	ii
Summary	iii
Abbreviations	v
Table of contents	viii
Figure contents	xviv
Table contents	xvii
 <u>Chapter 1. General Introduction</u>	 <u>1</u>
<u>Section 1: Introduction</u>	<u>2</u>
1.1 Vision and the eye	2
1.2 Retinal structure	3
1.3 Photoreceptor cells	3
1.4 The Rod photoreceptors	4
1.5 Rhodopsin	4
1.6 The phototransduction cascade	5
1.7 Retinitis Pigmentosa	6
1.8 Clinical symptoms of RP	6
1.9 Clinical Assessment of RP	7
1.10 Genetics of RP	7
1.11 Rhodopsin-linked adRP	8
1.12 Animal models	10
1.13 RP and gene therapy	12
1.14 RNA interference-the siRNA pathway	13
1.15 The miRNA pathway	15
1.16 RNAi and gene therapy	17
1.17 Modes of Delivery	19
1.18 Non-viral delivery	20
1.19 Modifications for delivery and stability	20
1.20 Viral delivery	21
1.21 AAV-mediated delivery	21

1.22 AAV in the retina	23
1.23 Gene therapy for LCA using AAV-2/2 in human patients	24
1.24 A mutation-independent suppression and replacement strategy	26
1.25 Objectives	27
<u>Chapter 2 Materials and Methods</u>	39
<u>Section 2.1: Materials</u>	40
2.1.1 Buffers and reagents for general procedures	40
2.1.2 Buffers and reagents for bacterial culturing	42
2.1.3 Cell culturing materials and reagents	42
2.1.4 RNA materials and reagents	43
2.1.5 Protein materials and reagents:	43
2.1.6 Materials and reagents used for western blotting	44
2.1.7 AAV production materials	44
2.1.8 Animal materials	45
2.1.9 Genotyping	45
2.1.10 Anaesthetic and reagents used for animal procedures	45
2.1.11 Materials and reagents used for histology and Immunohistochemistry	46
2.1.12 Antibodies	46
2.1.13 Fluorescent Activated Cell sorting (FACs)	46
<u>Section 2.2: General cloning techniques</u>	48
2.2.1 DNA/RNA spectrophotometry	48
2.2.2 Agarose gel electrophoresis	48
2.2.3 Restriction enzyme digestion	48
2.2.4 Enzyme reaction clean up (Qiagen)	49
2.2.5 Agarose gel extraction (Qiagen)	49
2.2.6 Shrimp Alkaline Phosphatase (SAP) treatment	49
2.2.7 Filling in 5' DNA overhangs (Klenow polymerase reaction)	49
2.2.8 Filling in 3' DNA overhangs (T4 DNA polymerase reaction)	49
2.2.9 Phenol chloroform extraction	49
2.2.10 Ethanol precipitation	50
2.2.11 Ligations	50

2.2.12 XL1-blueMRA competent cells	50
2.2.13 Transformation using XL1-BlueMRA competent cells	51
2.2.14 Transformation using XL10-Gold Ultracompetent Cells	51
2.2.15 DNA mini-preparation (boiling method)	51
2.2.16 DNA mini-preparation (Qiagen kit)	52
2.2.17 DNA maxi-preparation (Qiagen kit)	52
2.2.18 DNA mega-preparation (Qiagen kit)	53
2.2.19 Polymerase chain reaction (PCR)	53
2.2.20 Sequencing (automated capillary method)	53
2.2.21 Sequencing (GATC or Source Bioscience LifeSciences)	54
<u>2.3 Cell culturing procedures</u>	54
2.3.1 Cell growth	54
2.3.2 Cell passaging	54
2.3.3 Cell counting using a Haemocytometer	54
2.3.4 Freezing cells	55
2.3.5 General co-transfection protocol using Lipofectamine® 2000	55
<u>2.4 RNA Methods</u>	55
2.4.1 Ribonuclease (RNase) free conditions	55
2.4.2 RNA isolation from HeLa cells (Using Tri-reagent)	56
2.4.3 RNA isolation from HeLa cells (RNeasy ® Plus mini kit)	56
2.4.4 DNase treatment of HeLa cell RNA	56
2.4.5 RNA isolation from mouse retina (RNeasy mini kit)	56
2.4.6 DNase treatment of mouse retinal RNA (on column digestion)	57
2.4.7 General One Step Real- Time RT-PCR protocol	57
<u>2.5 Protein techniques</u>	59
2.5.1 Protein extraction from HeLa cells	59
2.5.2 Protein extraction from mouse retina	59
2.5.3 BCA protein assay	59
<u>2.6 Western Blot Protocol</u>	60
2.6.1 Preparation of SDS PAGE gels	60
2.6.2 Semi-dry transfer	60
2.6.3 Ponceau S Staining	61
2.6.4 Blocking of immunoblots	61

2.6.5 Antibody probing of blots	61
2.6.7 Chemiluminescent detection of proteins	61
2.6.8 Stripping of immunoblots	62
<u>2.7 Generation of AAV-2/5</u>	62
2.7.1 Triple transfection method	62
2.7.2 AAV-2/5 purification using the caesium chloride purification	63
2.7.3 Determination of viral titre	63
<u>2.8 Animal procedures</u>	
2.8.1 Housing of mice	64
2.8.2 General genotyping procedure	64
2.8.3 Subretinal AAV injection	64
2.8.4 Electroretinograms	65
2.8.5 Fixation of eyes	66
2.8.6 Preparation of the eyes for cryosectioning	66
2.8.7 Cryosectioning	66
2.8.8 Immunohistochemistry	66
2.9 Statistical analysis	67
<u>Chapter 3 RNAi-mediated suppression of rhodopsin <i>in vitro</i></u>	68
<u>3.1 Introduction</u>	69
3.1.2 Mutation-dependent rhodopsin suppression using ribozymes	69
3.1.3 Mutation-dependent approaches for rhodopsin splice site mutations	70
3.1.4 Mutation-dependent siRNA based approach	70
3.1.5 Mutation-independent ribozyme based approaches	71
3.1.6 Mutation-independent Zinc finger nucleases	71
3.1.7 Mutation-independent siRNA based approaches	72
3.1.8 Objectives of chapter 3	72
<u>3.2 Materials and Methods</u>	74
3.2.1 siRNA design	74
3.2.2 Resuspending of annealed siRNAs	74
3.2.3 Co-transfection of hRHO with siRNAs into HeLa cells	74
3.2.4 RNA isolation of HeLa cells co-transfected with hRHO and each siRNA	75
3.2.5 Optimisation of primers for use in subsequent	

real time RT-PCR experiments	75
3.2.6 Real-time RT-PCR of RNA from HeLa cells co-transfected with hRHO and each siRNA	75
3.2.7. Statistical analysis	76
3.2.8 Protein extraction and concentration evaluation	76
3.2.9 Western blotting	77
<u>3.3. Results</u>	78
3.3.1. Design of the siRNAs	78
3.3.2. Co-transfection of siRNA and hRHO into HeLa cells	78
3.3.3 Real-time RT-PCR optimisation	79
3.3.4 siP1 and siP2 RNA suppression data	81
3.3.5 Western blot analysis of hRHO expression	81
3.3.6 siP3 RNA suppression data	82
3.3.7 siP3 protein suppression data	82
<u>3.4 Discussion</u>	84
 <u>Chapter 4. The <i>in vivo</i> evaluation of human rhodopsin suppression by shRNA and artificial miRNA based constructs</u>	 107
 <u>4.1. Introduction</u>	 108
4.1.1. shRNA-mediated toxicity	108
4.1.2. Reducing the shRNA dose	109
4.1.3. Utilising artificial miRNAs	110
4.1.4 Artificial miRNA-30a	110
4.1.5. Artificial miRNA-155	111
4.1.6. Objectives of Chapter 4	112
<u>4.2 Materials & Methods</u>	114
4.2.1 Design and Cloning of shP3 into pSuper.GFP.neo	114
4.2.2 Cloning of shP3 into the pAAV.shNT vector	115
4.2.3. Design and cloning of miRP3-30a into pMA.U6mi30BB	115
4.2.4. Design and cloning of miRP3-30a into pAAV.shNT	116
4.2.5. Design and Cloning of miRP3-155 into pcDNA TM 6.2-GW/ $\pm$ EmGFP-miR vector	117

4.2.6. Cloning of miRP3-155 into pAAV-CMV. EGFP	118
4.2.7. Generation of AAV-2/5	118
4.2.8. NHR transgenic mice	119
4.2.9. Subretinal injection of AAV-2/5 constructs into NHR transgenic mice	119
4.2.10. Fluorescent Activated Cell sorting (FACS)	119
4.2.10.1. Retinal dissociation and preparation of cells for FACS Trypsin method	119
4.2.10.2 Retinal dissociation and preparation of cells for FACS- neural tissue dissociation kit	120
4.2.10.3 FACS analysis	120
4.2.11 RNA isolation of FACS cells	121
4.2.12. Optimisation of real-time RT-PCR for FACS RNA isolated from FACS sorted cells	121
4.2.13 Real-time RT-PCR of FACS RNA for FACS sorted cells	122
4.2.14 Analysis of siP3 expression in NHR retinas	122
4.2.14.1 RNA isolation using a miRNeasy mini kit (Qiagen)	123
4.2.14.2. Reverse transcription of siP3 and sno-202	123
4.2.14.3 qPCR amplification	123
4.2.15. ERG	124
4.2.16 Statistical analysis	124
4.2.17 Protein isolation	125
4.2.18 Western blotting	125
4.2.19 Immunohistochemistry	125
4.3. Results	126
4.3.1 shP3 cloning into pAAV	126
4.3.2 miRP3-30a cloning into pMA.mU6mi30BB	126
4.3.3 miRP3-155 cloning into pcDNA6.3-GW/+EmGFP-miR	127
4.3.4 AAV-2/5 generation	128
4.3.5 NHR transgenic animals	129
4.3.6 Subretinal injections of AAV-2/5 suppressors into NHR mice	129
4.3.7 FACS isolation of EGFP positive retinal cells for hRHO mRNA expression analysis 4 weeks post-injection	129

4.3.8 Optimisation of 18S for real-time RT-PCR analysis of FACS isolated RNA	131
4.3.9 Real-time RT-PCR analysis on FACS isolated retinal RNA 4 weeks post-injection	132
4.3.10 FACS isolation of EGFP positive cells for hRHO mRNA expression analysis 2 weeks post-injection	133
4.3.11 Optimisation of Taqman® Small RNA assay for siP3 expression analysis	134
4.3.12 Analysis of siP3 RNA expression in retinas injected with AAV-2/5.shP3 compared to AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a	135
4.3.13 ERG analysis of suppression by suppressor viruses	135
4.3.14 Analysis of protein expression in NHRs injected with AAV-2/5.NT20 and AAV-2/5.shP3 by western blotting	137
4.3.15 Analysis of protein expression in NHRs injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30a by western blotting	138
4.3.16 Immunohistochemistry on retinal sections from NHR mice injected with AAV-2/5.NT20 and AAV-2/5.shP3	139
4.3.17 Immunohistochemistry on retinal sections from NHR mice injected with AAV-2/5.Luc46 and AAV-2/5.miR-30a	139
<u>Section 4.4 Discussion</u>	141

Chapter 5 Exploration of shRNA based RHO suppression in a rhodopsin-linked adRP mouse

<u>5.1 Introduction</u>	204
5.1.2 Mutations within codon 347 of rhodopsin	204
5.1.3 Transgenic mouse with porcine P347S mutant rhodopsin	205
5.1.4 Transgenic mouse model with the human P347S transgene	205
5.1.5 Transgenic porcine models with P347L and P347S mutations	206
5.1.6 Molecular mechanisms of mutations within the c-terminus of rhodopsin	207
5.1.7 The use of these animal models to study adRP	208

5.18 Objectives of chapter 5	209
<u>Section 5. 2 Materials and Methods</u>	210
5.2.1 Animals	210
5.2.2 Genotyping	210
5.2.3 ERG characterisation	210
5.2.4 Optimisation of the 4D2 antibody for pRHO protein	211
5.2.5 Rhodopsin protein expression over time	211
5.2.6 Immunohistochemistry of hetPP347S retinal sections	212
5.2.7 Sub-retinal injection of hetPP347S mice with AAV-2/5.NT20 and AAV-2/5.shP3	212
5.2.8 Optimisation of real-time RT-PCR for analysis of pRHO expression	212
5.2.9 Optimisation of FACS analysis of EGFP positive regions of hetPP347S retinas	212
5.2.10 Real-time RT-PCR analysis of FACS sorted hetPP347S retinal RNA	213
5.2.11 Real-time RT-PCR analysis of RNA from excised EGFP positive portions of the hetPP347S mouse	213
5.2.12 Real-time RT-PCR analysis of pRHO RNA expression in hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shBB	214
5.2.13 Analysis of pRHO protein suppression by western blotting in hetPP347S animals injected with AAV-2/5.shP3 and AAV-2/5. NT20	214
5.2.14 Functional assessment of therapeutic benefit in hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3	214
5.2.15 Immunohistochemical analysis of suppression of pRHO in hetPP347S mice	215
5.2.16 Analysis of mRHO suppression by siP3	215
5.2.17 Statistical analysis	215
<u>5.3 Results</u>	216
5.3.1 Genotyping the PP347S mice	216
5.3.2 ERG characterisation	216
5.3.3 Optimising of 4D2 for western blotting of pRHO	218
5.3.4 Decrease in RHO protein expression in the hetPP347S mouse over time	218

5.3.5 RHO staining of retinal sections from the hetPP347S mouse at different time-points	219
5.3.6 Lectin-PNA staining of retinal sections from the hetPP347S mouse at different time-points	220
5.3.7 Optimisation of primers for the amplification of pRHO	220
5.3.8 Optimisation of FACS for the hetPP347S mouse	221
5.3.9 Real-time RT-PCR analysis of pRHO from FACS isolated mRNA in the injected hetPP347S mouse	222
5.3.10 Real-time RT-PCR analysis of pRHO mRNA expression from EGFP positive cells excised from the retinas of the hetPP347S mice injected with NT20 & shP3	222
5.3.11 Real-time RT-PCR analysis of pRHO mRNA expression from EGFP positive cells excised from the retinas of the hetPP347S mice injected with NT20 and shBB	223
5.3.12 RHO protein expression in hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 as assessed by western blotting	223
5.3.13 Functional analysis of hetPP347S mice injected with NT20 and shP3	224
5.3.14 Immunohistochemistry of hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3	226
5.3.15 Analysis of mRHO mRNA suppression by siP3 <i>in vitro</i>	227
<u>5.4 Discussion</u>	228
 <u>Chapter 6 optimisation of a human rhodopsin replacement gene both <i>in vitro</i> & <i>in vivo</i> use in a suppression & replacement therapeutic strategy</u>	277
 <u>6.1 Introduction</u>	278
6.1.1 Codon Usage analysis	278
6.1.4 Choice of AAV serotype	279
6.1.5 Choosing the correct promoter	281
6.1.6 RNAi mediated suppression and replacement in the retina for adRP	282
6.1.7 Objectives of chapter 6	283
<u>Section 6.2 Materials and Methods</u>	285

6.2.1. The design of replacement gene resistant to siP2 (rP2) and siP3 (rP3)	285
6.2.2. Generation of the replacement genes by PCR directed mutagenesis	285
6.2.3 Cloning of rP2 and rP3 into the CMV-hRHO plasmid for <i>in-vitro</i> evaluation	286
6.2.4 Co-transfection of rP2 and rP3 with siRNA in HeLa cell culture	286
6.2.5 Real-time RT-PCR analysis of rP2 and rP3 mRNA expression	287
6.2.6. Western blot analysis of rP2 and rP3 protein expression	287
6.2.7 Cloning of rP3	287
6.2.8 Cloning of rP3 into a “replacement only” construct	288
6.2.9 The generation of AAV-2/5.rp3 and AAV-2/8.rP3	288
6.2.10 Determination of the viral titre of AAV-2/5.rP3 and AAV-2/8.rP3	289
6.2.11 Subretinal injection of AAV-2/5 or AAV-2/8 into 129rho ^{-/-}	289
6.2.12 ERG analysis of 129 rho ^{-/-} mice injected with AAV-2/5.rP3	290
6.2.13 ERG analysis of 129rho ^{-/-} mice injected with AAV-2/8.rP3	290
6.2.14 Immunohistochemistry of 129rho ^{-/-} mice injected with AAV-2/5.rP3 and AAV-2/8.rP3	290
6.2.15 Western blot analysis of 129rho ^{-/-} mice injected with AAV-2/5.rP3 and AAV-2/8.rP3	291
6.2.16. Real-time RT-PCR analysis of hRHO mRNA expression from AAV-2/5.rP3 and AAV-2/8.rP3 in 129 wild-type mice	291
6.2.17 Subretinal injections of AAV-2/8.BB24 and AAV-2/8.rP3 at a 3.5 fold lower dose	292
6.2.18 Comparative analysis of hRHO protein expression from AAV-2/5.BB24, AAV-2/8.BB24 and AAV-2/rh10.BB24	292
6.2.19 Statistical analysis	292
<u>6.3. Results</u>	293
6.3.1 Design of the rP2 replacement sequence	293
6.3.2 Design of the rP3 replacement sequence	294
6.3.3 Co-transfection of rP2 and rP3 with hRHO siRNAs in HeLa cells	294
6.3.4 Analysis of rP2 mRNA expression by real-time RT-PCR	294
6.3.5 Analysis of rP3 mRNA expression by real-time RT-PCR	295

6.3.6 Western blot analysis of rP2 protein expression	295
6.3.7 Western blot analysis of rP3 protein expression	296
6.3.8 The preparation of rP3 for <i>in vivo</i> analysis	296
6.3.9 Cloning of rP3 into pAAV.BB32	297
6.3.10 The generation of pAAV.rP3 into AAV-2/5 and AAV-2/8	297
6.3.11 ERG AAV-2/5	298
6.3.12 ERG of 129 rho ^{-/-} subretinally injected with AAV-2/8.rP3	299
6.3.13 Immunohistochemistry on retinal sections from 129 rho ^{-/-} mice injected with AAV-2/5.rP3	300
6.3.14 Immunohistochemistry on retinal sections from 129 rho ^{-/-} mice injected with AAV-2/8.rP3	301
6.3.15 Western blot analysis of hRHO rP3 protein expression by AAV-2/5.rP3	301
6.3.16 Western blot analysis of hRHO rP3 protein expression by AAV-2/8.rP3	302
6.3.17 hRHO mRNA expression in wild-type mice injected with AAV-2/5.rP3 and AAV-2/8.rP3	302
6.3.18 ERG analysis in 129rho ^{-/-} mice injected with AAV-2/8.BB24 and AAV-2/8.rP3 at a lower dose	302
6.3.20 The comparative analysis of AAV-2/5.BB24, AAV-2/8.BB24 and AAV-2/10.BB24 by western blotting	304
<u>6.4 Discussion</u>	306
 <u>Chapter 7: General Discussion</u>	355
<u>Chapter 8 References</u>	365
<u>Chapter 9 Appendix</u>	385

Figure contents	Page
 <u>Chapter 1</u>	
Figure 1.1 The Eye	29
Figure 1.2 Different retinal cell layers	30
Figure 1.4 Rod photoreceptor cell	31
Figure 1.6 The phototransduction cascade	32
Figure 1.7.a The loss of peripheral vision associated with RP	33
Figure 1.7.b Clinical symptoms of RP in the retina	34
Figure 1.9: ERG responses	35
Figure 1.14 The RNA interference pathway	37
 <u>Chapter 3</u>	
Figure 3.3.1.a. hRHO and pRHO siRNA targets and homology	92
Figure 3.3.3.a hRHO standard curve	95
Figure 3.3.3.b Melting curve analysis of hRHO amplicons	96
Figure 3.3.3.c 2% agarose gel analysis of hRHO and h β -actin RT-PCR amplification products	97
Figure 3.3.3.d h β -actin standard curve	98
Figure 3.3.3.e Melting curve analysis of h β -actin amplicons	99
Figure 3.3.4.f Utilising the standard curve method for calculating % hRHO expression in each sample	100
Figure 3.3.4.a. Real-time RT-PCR evaluation of hRHO expression in HeLa cells co-transfected with hRHO and siRNAs	101
Figure 3.3.5.a Optimisation of the 4D2 RHO antibody for western blotting	102
Figure 3.3.5.b Western blot analysis of hRHO protein expression	103
Figure 3.3.6.a Real-time RT-PCR evaluation of hRHO expression in HeLa cells co-transfected with hRHO and siP3	104
Figure 3.3.7.a Western blot analysis of hRHO protein expression in cells co-transfected with hRHO and siP3	105
 <u>Chapter 4</u>	
Figure 4.2.1.a Strategy behind shRNA design within pSUPER (Oligoengine)	153
Figure 4.2.5.a cloning strategy for miR-155 within the pcDNA TM 6.2/GW/EmGFP-miR (Invitrogen)	156

Figure 4.2.7.a EGFP-1 vector (Clontech)	157
Figure 4.3.1.a Cloning of shP3 into pAAV	158
Figure 4.3.1.b sequencing of shP3 into the pSUPER.gfp/neo vector	159
Figure 4.3.2.a Cloning of miRP3-30a into pAAV	160
Figure 4.3.2.b sequencing of miRP-30a into pMA.mU6mi30BB	161
Figure 4.3.3.a Cloning of miRP3-155 into pAAV	162
Figure 4.3.3 b sequencing results for miRP3-155 cloned into pcDNA6.3-GW/+EmGFP-miR	163
Figure 4.3.3.c sequencing results for miRP3-155 cloned into pAAV.EGFP	164
Figure 4.3.5.a NHR Genotyping	166
Figure 4.3.7a The determination of baseline auto fluorescence in EGFP negative retinal cells from an uninjected NHR mouse	167
Figure 4.3.7b The determination of the gate used for EGFP negative cells	167
Figure 4.3.7c The determination of EGFP + cells	168
Figure 4.3.7.d The determination of the gate used to sort EGFP positive cells	168
Figure 4.3.9.a hRHO mRNA expression in NHR mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 1 month post-injection	170
Figure 4.3.9.b hRHO mRNA expression in NHR mice injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30a 1 month post-injection	171
Figure 4.3.9.c hRHO mRNA expression in NHR mice injected with AAV-2/5.miNT and AAV-2/5.miRP3-155 1 month post-injection	172
Figure 4.3.10.a The determination of PI + cells	173
Figure 4.3.10.b The determination of the gate used to sort PI negative and EGFP positive cells	173
Figure 4.3.10 c. The determination of EGFP + cells	174
Figure 4.3.10.d The determination of the gate used to sort EGFP positive cells	174
Figure 4.3.10.e hRHO mRNA expression in NHR mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 14 days post-injection	176
Figure 4.3.12.a siP3 expression in NHR mice injected with AAV-2/5.shP3 versus AAV-2/5.miRP3-30a	177
Figure 4.12.b siP3 expression in NHR mice injected with AAV-2/5.shP3 versus AAV-2/5.miRP3-155	178
Figure 4.3.13.a Rod-isolated ERG for NHRs injected	179

with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE)	
Figure 4.3.13.b mixed rod-cone ERG for NHR mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE)	180
Figure 4.3.13.c cone-isolated ERG for NHR mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE)	181
Figure 4.3.13.d % ERG response in NHR mice injected with AAV-2/5.NT20 versus AAV-2/5.shP3	182
Figure 4.3.13.e % mixed rod-cone ERG response in NHR mice injected with AAV-2/5.NT20 vs AAV-2/5.shP3	183
Figure 4.3.13.f % cone-isolated ERG response in NHR mice injected with AAV-2/5.NT20 vs AAV-2/5.shP3	184
Figure 4.3.13.g Rod-isolated ERG for NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE)	185
Figure 4.3.13.h Mixed rod-cone ERG for NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE)	186
Figure 4.3.13.i Mixed rod-cone ERG for NHRS injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE)	187
Figure 4.3.13.j % rod-isolated ERG response in NHR mice injected with AAV-2/5.Luc46 vs AAV-2/5.miRP3-30a	188
Figure 4.3.13.k % mixed rod-cone ERG response in NHR mice injected with AAV-2/5.Luc46 vs AAV-2/5.miRP3-30a	189
Figure 4.3.13.l % cone-isolated response in NHR mice injected with AAV-2/5.Luc46 vs AAV-2/5.miRP3-30a	190
Figure 4.3.13.m Rod isolated ERG for NHR mice injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155 (RE)	191
Figure 4.3.13.n mixed rod-cone ERG for NHR mice subretinally injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155 (RE)	192
Figure 4.3.13.o Cone-isolated ERG for NHR mice injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155 (RE)	193
Figure 4.3.13.p % rod-isolated ERG response in NHR mice injected with AAV-2/5.miNT vs AAV-2/5.miRP3-155	194
Figure 4.3.13.q. % mixed rod-cone ERG response in NHR mice injected with AAV-2/5.miNT vs AAV-2/5.miRP3-150	195

Figure 4.3.13.r % cone-isolated ERG response in NHR mice injected with AAV-2/5.miNT Vs AAV-2/5.miRP3-155	196
Figure 4.3.14.a Western blot analysis of hRHO protein expression in NHR mice LE-injected eyes (AAV-2/5.NT20) versus RE-injected eyes (AAV-2/5.shP3)	197
Figure 4.3.14.b Densitometric analysis of hRHO protein expression from two NHR mice LE-injected eyes (AAV-2/5.NT20) versus RE-injected eyes (AAV-2/5.shP3)	198
Figure 4.3.15.a Western blot analysis of hRHO protein expression in NHR mice LE-injected (AAV-2/5.Luc46) Vs RE-injected (AAV-2/5.miRP3-30a)	199
Figure 4.3.15.b Densitometric analysis of hRHO protein expression from two NHR mice LE-injected (AAV-2/5.Luc46) Vs RE-injected (AAV-2/5.miRP3-30a)	200
Figure 4.3.16.a Immunohistochemistry of NHR eyes LE-injected (AAV-2/5.NT20) vs RE injected (AAV-2/5.shP3)	201
Figure 4.3.17.a Immunohistochemistry of NHR eyes LE-injected (AAV-2/5.Luc46) Vs RE 205 injected (AAV-2/5.miRP3-30a)	202
<u>Chapter 5</u>	
Figure 5.3.1a PP347S genotyping	239
Figure 5.3.2.a Representative ERG responses for a wild-type 129 mouse (WT)	240
Figure 5.3.2.b A representative ERG response for a heterozygous PP347S animal at 3 weeks	241
Figure 5.3.2.c A representative ERG response for a heterozygous PP347S animal at 8 weeks	242
Figure 5.3.2.d A representative ERG response for a heterozygous PP347S animal at 12 weeks	243
Figure 5.3.2.e A representative ERG response for a heterozygous PP347S animal at 16 weeks	244
Figure 5.3.2.f A representative ERG response for a heterozygous PP347S animal at 6 months	245
Figure 5.3.2.g A representative ERG response for a heterozygous PP347S animal at 9 months	246
Figure 5.3.2.h A representative ERG response for a heterozygous PP347S	247

animal at 1 year	
Figure 5.3.2.i Average rod-isolated b-wave responses in heterozygous PP347S mice at different time-points compared to wild-type	248
Figure 5.3.2.j Average mixed rod-cone b-wave responses at in heterozygous PP347S mice at different time-points compared to wild-type	249
Figure 5.3.2.k Average cone-isolated b-wave responses at in heterozygous PP347S mice at different time-points compared to wild-type	250
Figure 5.3.3.a Optimisation of the 4D2 antibody for porcine rhodopsin	251
Figure 5.3.4.a Reduction in pRHO and mRHO expression overtime	252
Figure 4.3.4.b Densitometric analysis of western blot analysis of hRHO expression in the hetPP347S mice over time	253
Figure 5.3.5.a.Immunohistochemistry of heterozygous PP347S retinas from 3 weeks to 16 weeks	254
Figure 5.3.5.b Immunohistochemistry of heterozygous PP347s retinas from 6months to 1 year	255
Figure 5.3.6.a Immunohistochemistry of PNA staining in heterozygous PP347s retinas from 3 weeks to 16 weeks	256
Figure 5.3.6.a Immunohistochemistry of PNA staining in hetPP347s retinas from 6 months to 1 year	257
Figure 5.3.7.a primer design for pRHO amplification	258
Figure 5.3.8.a The FACS purification of DRAQ5- + cells	259
Figure 5.3.8.b The determination of the gate used to sort DRAQ-5 and EGFP positive cells	259
Figure 5.3.8.c The FACS purification of DRAQ-5 + cells	260
Figure 5.3.8.d The FACS purification of the gate used to sort DRAQ-5+ and EGFP positive cells	260
Figure 5.3.10.a The % pRHO mRNA expression in heterozygous PP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3	262
Figure 5.3.11.a The % pRHO mRNA expression in heterozygous PP347S mice injected with AAV-2/5.NT20 (NT20) and AAV-2/5.shBB (shBB)	263
Figure 5.3.12.a RHO protein expression in the hetPP347S mouse injected with AAV-2/5.NT20 & AAV-2/5.shP3	264
Figure 5.3.12.b Densitometric analysis of RHO protein expression from	265

hetPP347S mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE)	
Figure 5.3.13.a ERG response of the hetPP347 (litter 1) mouse injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)	266
Figure 5.3.13.b ERG response of the hetPP347 (litter 2) mouse injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)	267
Figure 5.3.13.c ERG response of the hetPP347 mouse (litter 1) injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)	268
Figure 5.3.13.d ERG response of the hetPP347 mouse (litter 2) injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)	269
Figure 5.3.13.e Rod-isolated, mixed rod-cone and cone-isolated responses for hetPP347S mice (litter 1) injected with AAV-2/5.NT20 and AAV-2/5.shP3	270
Figure 5.3.13.f Rod-isolated, mixed rod-cone and cone-isolated responses for hetPP347 litter 2 injected with AAV-2/5.NT20 and AAV-2/5.shP3	271
Figure 5.3.13.g ERG response of the hetPP347 mouse (litter 1) injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)	272
Figure 5.3.13.h ERG response of the hetPP347 mouse (litter 2) injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)	273
Figure 5.3.13.i Rod-isolated, mixed rod-cone and cone-isolated responses for hetPP347 litter 3 injected with NT20 and shP3	274
Figure 5.3.14.a Immunohistochemistry of hetPP347S eyes LE-injected (AAV-2/5.NT20) Vs RE injected (AAV-2/5.shP3)	275
Figure 5.3.15.a. Real-time RT-PCR evaluation of mRHO expression in HeLa cells co-transfected with mRHO and each siRNA	276
Chapter 6	
Figure 6.2.2.a PCR directed mutagenesis of hRHO to create rP2 or rP3	316
Figure 6.3.1.a Design of the rP2 replacement gene	318
Figure 6.3.1.b: Sequence of rP2 over the siP2 target site	319
Figure 6.3.2.a Design of the rP2 replacement gene	321
Figure 6.3.2.b: Sequence of rP3 over the siP3 target site	322
Figure 6.3.4.a rP2 RNA expression from HeLa cells transfected with rP3 and siRNAs	323
Figure 6.3.5.a rP3 RNA expression from HeLa cells transfected with rP3 and siRNAs	324

Figure 6.3.6.a Western blot analysis of rP2 protein expression	325
Figure 6.3.7.a Western blot analysis of rP3 protein expression	326
Figure 3.6.9.a sequencing results for rP3 into pAAV.BB32	327
Figure 6.3.9.b sequencing result for pAAV.rP3	328
Figure 6.3.11.a Rod-isolated ERG for 129rho ^{-/-} mice injected with AAV-2/5.rP3 (RE)	
Figure 6.11.b mixed rod-cone ERG for 129rho ^{-/-} mouse injected with AAV-2/5.rP3 (RE)	330
Figure 6.3.11.c Cone-isolated ERG for 129rho ^{-/-} mice injected with AAV-2/5.rP3 (RE)	331
Figure 6.3.11.d The average rod-isolated ERG response in 129rho ^{-/-} mice injected with AAV-2/5.rP3 or uninjected	332
Figure 6.3.11.e The average mixed rod-cone ERG response in 129rho ^{-/-} mice injected with AAV-2/5.rP3 or uninjected	333
Figure 6.3.11.f The average cone-isolated ERG response in 129rho ^{-/-} mice injected with AAV-2/5.rP3 or un-injected	334
Figure 6.3.12.a Rod-isolated ERG for 129rho ^{-/-} mice injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE)	335
Figure 6.3.12.b mixed rod-cone ERG for 129rho ^{-/-} injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE)	336
Figure 6.3.12.c cone-isolated ERG for 129rho ^{-/-} injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE)	337
Figure 6.3.12.d Average rod-isolated ERG response in 129rho ^{-/-} mice injected (AAV-2/8.BB24 (A) or AAV-2/8.rp3 or un-injected	338
Figure 6.3.12.e Average mix rod-cone ERG response in 129rho ^{-/-} injected (AAV-2/8.BB24 (B) or AAV-2/8.rp3) or uninjected	339
Figure 6.3.12.f Average cone-isolated ERG response in 129rho ^{-/-} injected (AAV-2/8.BB24 (A) or AAV-2/8.rp3 or uninjected	340
Figure 6.3.13.a Immunohistochemistry of 129rho ^{-/-} eyes LE uninjected Vs RE injected (AAV-2/5.rP3)	341
Figure 6.3.14.a Immunohistochemistry of 129rho ^{-/-} eyes LE un-injected, or LE-AAV-2/8.BB24 injected with the RE-injected with AAV-2/8.rP3	342
Figure 6.3.15.a Western blot analysis of rP3 protein expression from AAV-2/5.rP3	343

Figure 6.3.16.a Western blot analysis of rP3 protein expression from AAV-2/8.rP3	344
Figure 6.3.17.a The % hRHO mRNA expression from AAV-2/5.rP3 and AAV-2/8.rP3 compared to NHR	345
Figure 6.3.18.a Rod-isolated ERG for 129rho ^{-/-} injected with AAV-2/8.rP3 (LE) and AAV-2/8.BB24 (RE)	346
Figure 6.3.18.b Mixed rod-cone ERG for rho129 ^{-/-} injected with AAV-2/8.rP3 (LE) and AAV-2/8.BB24 (RE)	347
Figure 6.3.18.c Cone-isolated ERG for 129rho ^{-/-} injected with AAV-2/8.rP3 (LE) and AAV-2/8.BB24 (RE)	348
Figure 6.3.18.d The average rod-isolated ERG response in 129rho ^{-/-} injected with AAV-2/8.rP3, AAV-2/8.BB24 or un-injected at a lower dose	349
Figure 6.3.18.e The average mixed rod-cone ERG response in 129rho ^{-/-} injected with AAV-2/8.rP3, AAV-2/8.BB24 or un-injected at lower dose	350
Figure 6.3.18.f The average cone-isolated ERG response in 129rho ^{-/-} injected with AAV-2/8.rP3, AAV-2/8.BB24 or un-injected at a lower dose	351
Figure 6.3.19.a Western blot analysis of rBB protein expression from AAV-2/10.BB24, AAV-2/8.BB24 and AAV-2/5.BB24 14 days post-injection	352
	353

<u>Table Contents</u>	<u>Page</u>
 <u>Chapter1</u>	
Table 1.11 The classification system (I-VI) of human rhodopsin mutations implicated in RP	36
 <u>Chapter 3</u>	
Table 3.2.5 primers used for real-time RT-PCR analysis	91
Table 3.3.1.a Sequence of each siRNA oligonucleotide and the alignment of each siRNA pair	93
Table 3.3.1.b The homology of each siRNA to rhodopsin in different organisms	94
 <u>Chapter 4</u>	
Table 4.2.1a Sequence of oligonucleotides used for generation of RNAi suppressors (Sigma-Aldrich)	154
Table 4.2.1b Primer sequences for Chapter 4	155
Table 4.3.4 The calculation of titres for each suppressor virus	165
Table 4.3.7 EGFP % positive cells and the cell numbers obtained	169
Table 4.3.10 % EGFP positive cells and cell numbers 14 days post-injection	175
 <u>Chapter 5</u>	
Table 5.2.2 Primers used in Chapter 5	238
Table 5.3.8 EGFP positive cells and cell numbers obtained 14 days post-injection of heterozygous PP347S mice	261
 <u>Chapter 6</u>	
Table 6.2.2 The PCR primers used to generate both the rP2 and rP3 replacement genes by PCR directed mutagenesis	315
Table 6. 3.1 Codon usage analysis within the hRHO siP2 target site	317
Table 6. 3.2 Codon usage analysis within the hRHO siP3 target site	320
Table 6.3.10 Calculation of titres for each suppressor virus	329

Chapter 1: General Introduction

Section 1: Introduction

According to the World Health Organisation statistics in 2010, 284 million people world-wide are visually impaired with 39 million people being registered as completely blind. Globally, Cataract, Glaucoma and Age Related Macular Degeneration are the top three causes. Despite these statistics, precise estimates of the number of people suffering from world-wide genetic forms of blindness are not available, however, it is estimated that in industrialised countries, a significant portion of visual impairment is accounted for by genetic diseases (World Health Organisation, 2010). One such genetic disorder that leads to severe visual impairment is Retinitis Pigmentosa (RP). This disorder consists of a collection of hereditary retinal disorders in which photoreceptors are progressively degenerating (Daiger et al, 2007; Hartong et al, 2006). Approximately 1.5 million people are affected worldwide and RP represents the most common form of blindness among the registered blind of working age (Farrar et al, 2002; Farrar et al, 2010; Hartong et al, 2006). RP represents a significant unmet clinical need. With this in mind, in order for one to try to develop therapies for this disease an extensive knowledge of the genetics and molecular mechanisms underlying RP, are essential and will greatly expedite the design and development of novel therapeutics for this disorder.

1.1 Vision and the eye

Vision in mammals involves the input of visual cues, in the form of light energy (photons) from the environment. These light rays are converted and processed into various chemical and electrical signals via a complex signal transduction cascade called phototransduction. These results in the generation of neural impulses that are interpreted by the brain in a process called visual perception (Davidson, 1980; Remington & McGill, 1998). The organ responsible for modulating visual perception is the eye. The eye is made up of three main layers (**Figure 1.1**); the first is the outer protective layer of the eye and consists of the sclera and the cornea (Purves et al, 2008). The second comprises three tissues called the uveal tract and includes the choroid (involved in supplying blood and nutrients to the photoreceptors), the ciliary body (involved in controlling the refraction of the lens as well as producing aqueous humor) and the iris (involved in adjusting the size of the pupils). The final layer of the eye is the neural retina (Davson, 1980; Purves et al, 2008; Remington & McGill, 1998). In mammals, phototransduction occurs in the neural retina (Berg et al, 2002). The eye also contains two kinds of fluid, the aqueous humor, located between the cornea and the lens; is responsible for supplying nourishment to this part of the eye and the vitreous humor, located between the lens and the retina preserves the globe shape of the eye in addition to phagocytosis of any unwanted debris (Purves et al, 2008).

1.2. Retinal structure

The retina comprises three main neural layers; the outer, inner and the ganglion cell layers (Farrar et al, 2002) containing different cell types (**Figure 1.2**), the photoreceptors, bipolar cells, amacrine cells, horizontal cells and ganglion cells (Purves et al, 2008; Sung & Chuang, 2010). The outer layer of the retina contains rod and cone photoreceptors, the primary neurons involved in visual transduction. In the retina, photoreceptor inner and outer segments (IS, OS) are located in the outer layer between the retinal pigment epithelium (RPE) and the outer nuclear layer (ONL). The axons of photoreceptor cells synapse with bipolar cells and horizontal cells in the outer plexiform layer (OPL). The inner layer of the retina contains bipolar cells, horizontal cells, amacrine cells, interplexiform cells and Muller cells. The inner nuclear layer (INL) contains the nuclear bodies of these cells. In the inner plexiform layer (IPL), bipolar cells and amacrine cells interact and form synaptic connections with the output neurons of the retina, retinal ganglion cells. The nuclear bodies of the ganglion cells are located in the ganglion cell layer (GCL) and it is these neurons that relay visual information to the brain. The RPE is located near the outer segments of the photoreceptors cells and separates the outer retina from the choroid (Berg et al, 2002; Farrar et al, 2002; McIlwain, 1996; Rattner et al, 1999).

1.3. Photoreceptor cells

There are two types of photoreceptor cells in the mammalian retina, rods and cones. Each photoreceptor is characterised by an outer segment where phototransduction occurs and an inner segment containing the nucleus and the cellular organelles (Berg et al, 2002). Humans have approximately 125 million rods, the majority of which are located around the periphery of the retina (Campbell & Reece, 2002). Rods are highly sensitive to light and are able to detect just 1 photon of light energy (Bayler et al, 1979; Gomperts et al, 2009; Hecht et al, 1942; Sung & Chuang, 2010). For this reason rods are involved in vision in dim light (scotopic vision, [Berg et al, 2002; Rattner et al, 1999]). Rods are the most abundant photoreceptor cell in the eye, representing approximately 95% of all photoreceptor cells (Rattner et al, 1999). There are approximately six million cones and the bulk of these are found in a region of the retina called the fovea. This part of the retina is the region with the highest visual acuity (Campbell & Reece, 2002; Purves et al, 2008). The cones are involved in controlling vision in bright light (photopic). There are three types of cones; red, green and blue and these cells are responsible for colour vision (Kennan et al, 2005; Rattner et al, 1999).

1.4. The Rod photoreceptors

Rods are long structures containing the rod outer segment (ROS), separated from the rod inner segment (RIS) by a cilium (**Figure 1.4** [Berg et al 2002; Kennan et al, 2005]). At the base of the rods, the cells synapse with the secondary neurons, the bipolar cells (Berg et al, 2002; Hargrave & McDowell, 1992; Kennan et al, 2005). One of the main components of rods is the long tubular structure known as the ROS, which contains approximately 1000 densely stacked rows of ROS discs (Sung & Chuang et al, 2010). These form when the plasma membrane surrounding the ROS discs invaginates and pinches off (Berg et al, 2002; Hargrave & McDowell, 1992). Rhodopsin comprises approximately 90% of the total protein present in the ROS disc membranes and each rod has approximately 10^8 molecules of rhodopsin (Daemen et al, 1973; Palczewski et al, 2006; Purves et al, 2008). Other proteins located on the disc membranes include peripherin 2 and its homolog, rom-1, both of which are important for disc structure, size and stability (Sung & Chuang, 2010). Each ROS protrudes into the RPE as the RPE has a vital role in rod cell maintenance; the older OS discs are constantly being removed and have a life span of approximately 12 days (Purves et al, 2008). New ROS discs are made every day at the bottom of the ROS. When these segments get older they begin to move up along the ROS, eventually reaching the top where they are phagocytosed by the RPE (Purves et al, 2008; Sung & Chuang et al, 2010). The RPE also contains the pigment melanin, which catches scattered light that hits the retina and prevents it from being picked up by sensitive rod photoreceptors (Gomperts et al, 2009).

1.5. Rhodopsin

Rhodopsin (RHO) was first depicted in 1878 by Professor William Kuhne as “visual purple”. This was due to the fact that when found in its dark inactive state, the protein was attached to 11-*cis*-retinal (the source of the purple colour). Upon light hitting “visual purple” the protein gradually transformed from purple to pink as 11-*cis*-retinal isomerised and dissociated from rhodopsin (Gomperts et al, 2009; Kuhne, 1878). Rhodopsin is a transmembrane protein of approximately 40 kiloDaltons in size (kDa [Hargrave & McDowell, 1992]). It is a member of the G-protein coupled receptor (GPCR) family. The amino acid sequence of rhodopsin is organised such that seven blocks of hydrophilic regions are separated by seven blocks of hydrophobic regions of approximately 21-28 amino acids in length, which result in the formation of seven transmembrane domains (7TM [Hargrave & McDowell, 1992]). Rhodopsin is 348 amino acids in length, its amino-terminus is located extracellular to the ROS plasma membrane and its carboxyl terminus is located on the cytoplasmic side (Hargrave & McDowell, 1992; Palczewski et al, 2006). The 7TM helices form a pocket in which the chromophore known as 11-*cis*

retinal is bound (Djmagoz et al 1995; Purves et al, 2008). This binding occurs when lysine 296 within helix VII of rhodopsin is covalently attached to a protonated Schiff base of 11-*cis*-retinal in the inactive form of rhodopsin (Mendes et al, 2005; Ridge & Palczewski, 2007). Rhodopsin is made in the RIS and once the protein has been synthesised and matured it is transported to the ROS (Ridge & Palczewski, 2007). The main role of rhodopsin is in the phototransduction cascade; however it also plays a role in the formation of the ROS disc membranes as rhodopsin knockout mice have no ROS (Humphries et al, 1997; Sung & Chuang, 2010).

1.6. The phototransduction cascade

In the dark inactive state, 3 molecules of cGMP are bound to a cGMP-gated cation channel in the ROS, keeping the channel open and allowing an influx of Na^+ and Ca^{2+} ions (**Figure 1.6** [Gomperts et al, 2009; Kennan et al, 2005]). The exposure to light results in isomerisation of 11-*cis*-retinal to all-*trans*-retinal (Berg et al, 2002; Kennan et al, 2005, Mendes et al, 2005). This changes the inactive conformation of the opsin (R) and within milliseconds leads to its conversion into a series of intermediate states, the last of which is active metarhodopsin II (R^*) (Hargrave & McDowell, 1992; Ridge & Palczewski, 2007). R^* can bind and activate the G protein transducin by allowing the G_α subunit of transducin to catalyse the exchange of GDP for GTP. When GTP is bound, the $\beta\gamma$ transducin subunits dissociate from the complex and the α -subunit activates cGMP-phosphodiesterase (cGMP-PDE) (Berg et al, 2002; Kennan et al, 2005). Active cGMP-PDE hydrolyses cGMP to 5'GMP and cGMP levels decrease. This reduction in cGMP closes the cGMP-gated cation channel in the outer membrane and Na^+ and Ca^{2+} can no longer enter the rod and as K^+ ions flow out of the OS the membrane potential of the cell decreases and the cell becomes hyperpolarised (Berg et al, 2002; Mendes et al, 2005; Purves et al, 2008). The release of the neurotransmitter glutamate is also inhibited and an electrical signal is transmitted through the neurons in the retina, along the optic nerve and to the brain for visual processing (Kennan et al, 2005).

Visual transduction is a rapid signalling process that must quickly be terminated in order to prevent a continuous visual signal being sent to the brain (Berg et al, 2002; Mendes et al, 2005). To inactivate rhodopsin, R^* is phosphorylated on serine and threonine residues (located in the c-terminus) by rhodopsin kinase with milliseconds of activation. A protein called arrestin can then bind to R^* and subsequently prevent it from binding and activating any more transducin. Additionally 11-*cis*-retinal is regenerated from 11-*trans*-retinal (in the RPE) and this can bind R^* , rendering it inactive (Berg et al, 2002; Kennan et al, 2005; Rattner et al, 1999). Thirdly, a calcium feedback loop exists in which a decrease in levels of intracellular Ca^{2+} leads to the derepression of guanylate-cyclase-activating protein

(GCAP). As the name suggests, this protein activates the guanylate cyclase enzyme which is involved in the synthesis of cGMP. As levels of cGMP increase, the cGMP-gated cation channel can reopen and thus the visual cascade can be restarted (Mendes et al, 2005). Finally, active transducin bound to cGMP-PDE is also hydrolysed from GTP to GDP, transducin disassociates from cGMP-PDE and re-associates with $G_{\beta\gamma}$ subunits (Berg et al, 2002).

It should be noted that phototransduction also takes place in the cones and the process is similar to rods (Kennan et al, 2005). The retina clearly mediates a complicated and highly controlled mechanism involving many cell types that result in the generation of visual signals which are perceived and registered by the brain. In recent years the advances in understanding these processes has highlighted the ease at which malfunctions in any of these mechanisms can occur and the impact they can have on vision.

1.7. Retinitis Pigmentosa (RP)

Retinitis Pigmentosa (RP) results in the death of the rod photoreceptor cells by apoptosis leading to a loss in scotopic visual function. As the disease progresses, cone photoreceptors also begin to degenerate. The loss of both rod and cones (i.e. peripheral and central vision) can result in complete loss of vision (Farrar et al, 2002; Rattner et al, 1999).

1.8. Clinical symptoms of RP

The rod photoreceptors are the primary cell type involved in RP (**Figure 1.4.**); therefore clinical symptoms of RP initially involve deterioration of scotopic vision. This is characterised by development of night blindness followed by a progressive loss of peripheral vision, eventually leading to tunnel vision (**Figure 1.7.a** [Daiger et al, 2007; Kennan et al, 2005]). The death of the rods has a negative impact on cones and as the cones begin to die, central day time vision is also lost (Kennan et al, 2005). Changes in the eye include attenuation of retinal vessels and optic nerve head pallor. An additional change to the retina is caused by the degradation of the RPE, resulting in the accumulation of pigmented debris within the retina. The observation of these deposits is the origin of the name RP. The deposition occurs when the cells in the RPE begin to degrade, resulting in migration and accumulation of cellular debris in the retina (**Figure 1.7.b** [Daiger et al, 2007; Hartong et al, 2006; Kennan et al, 2005; Phelan et al, 2000;]).

1.9 Clinical Assessment of RP

There are many ways to characterise the clinical symptoms of RP. One such method is by examining the retina with an ophthalmoscope or a slit-lamp biomicroscope (Hartong & Dryja, 2006; Rattner et al, 1999). Other assessments such as the measurement of decreased visual acuity, the rate of night blindness and measurement of contrast sensitivity are techniques also used to assess RP. Changes in colour vision can also be measured as advanced RP patients often present with diminished function in blue cones (Hartong & Dryja, 2006).

A common and well established technique for measuring retinal functions and used in the diagnosis of RP is electroretinography (ERG [**Figure 1.9**]). When exposed to light, retinal cells produce measurable electric responses. These responses can be recorded by measuring the difference in electrical potential between a reference electrode and one placed on the front of the eye (either on the cornea or the eye lid [Hartong & Dryja, 2006; Rattner et al, 1999]). Each retinal cell produces different electrical waves of activity. The photoreceptors produce an a-wave, the inner retina a b-wave and the RPE a c-wave (Rattner et al, 1999). In 1985 the International Society for Clinical Electrophysiology of vision (ISCEV) released a standard protocol that could be used globally to accurately measure ERG responses (Marmor et al, 2004). The first measurement consists of a rod ERG response and is measured under scotopic conditions. The subject is dark adapted for a minimum of twenty minutes and then presented with a flash of dim blue light with the ERG response usually showing a b-wave and no a-wave. This is followed by a mixed rod-cone response consisting of a bright white flash and usually the response is a negative a-wave followed by a positive b-wave (Hartong & Dryja, 2006; Lanier et al, 2010; Marmor et al, 2004). Following a 10 minute period of light adaption, the general cone response is subsequently measured using a single flash of bright white light. A series of single flashes of 30 Hz is used to measure the response of the cones in the macula (Lanier et al, 2010; Marmor et al, 2004) Patients with RP have reduced amplitudes in these rod and cone responses and also often have a delay in the response of their photoreceptors to the light stimuli (Hartong & Dryja, 2006; Lanier et al, 2010).

1.10 Genetics of RP

RP is a heterogeneous condition with more than 40 genes involved (RetNet, October 31 2011[<http://www.sph.uth.tmc.edu/retnet/sum-dis.htm>]). Most genes can also have many different mutations causing the disorder (Daiger et al, 2007). Fifty percent of RP cases are sporadic but the remainder can be inherited in an autosomal recessive (arRP), autosomal dominant (adRP) or X-linked pattern (Daiger et al, 2007; Farrar et al, 2002). Inherited RP is predominantly monogenic but rarer

forms caused by mitochondrial mutations in addition to digenic forms also exist (Goldberg & Molday, 1996; Mansergh et al, 1999). RP can be a component of various syndromes. One of the most common syndromes associated with RP is Ushers syndrome which in addition to RP involves hearing impairment and occasionally vestibular dysfunction (Millan et al, 2011). A second common syndrome associated with RP is Bardet-Biedl syndrome in which in addition to RP, symptoms include obesity, renal disorders, mental retardation and polydactyly (Ayuso & Millan, 2010). The first genetic studies of RP primarily used linkage mapping in affected families as a way to localise the disease genes (Daiger et al, 2007). Using DNA probes capable of detecting restriction fragment length polymorphisms specific to the X-chromosome, Bhattacharya et al, became the first group to map a form of X-linked RP to the short-arm of the X chromosome (Bhattacharya et al, 1984). Subsequent to this in the late 1980s and early 1990s some genes involved in adRP were elucidated. First a gene encoding rhodopsin was mapped to the long arm of chromosome 3 (Farrar et al, 1990; McWilliam et al, 1989) and a gene encoding RDS-peripherin was mapped to the short arm of chromosome 6 (Farrar et al, 1991; Kajiwarra et al, 1991). To date 18 different genes have been identified for adRP, 25 have been characterised for recessive RP and 6 genes for X-linked RP (RetNet October 31st 2011[<http://www.sph.uth.tmc.edu/retnet/sum-dis.htm>]). RP is caused by mutations in genes involved in a large variety of processes such as visual transduction (e.g. mutations in rhodopsin), photoreceptor structure (e.g. peripherin-2), the retinoid cycle (e.g. RPE-65), retinal transcriptional regulation (e.g. CRX and NRL), pre-mRNA splicing (e.g. PRP31), protein folding and trafficking (e.g. AIPL1), retinal development (CRB1) and disc shedding (MERTK) to name a few. For an extensive list of genes involved in retinal dystrophies see RetNet (<http://www.sph.uth.tmc.edu/retnet/>). Mutations in all of these different genes with different functions highlight the diversity of molecular mechanisms that underlie RP (Farrar et al, 2002; RetNet).

1.11 Rhodopsin-linked adRP

Given that rhodopsin plays a significant role in the phototransduction cascade it is not surprising that mutations in the RHO gene represent the most common cause of RP worldwide. The majority of these mutations are involved in adRP but some autosomal recessive RP rhodopsin mutations have been identified (Azam et al, 2009; Kartasmita et al, 2011; Kumaramanickavel et al, 1994; Rosenfeld et al, 1992). A mutation within RHO (Ala292Glu) causing the constitutive activation of transducin has also been implicated in congenital stationary night blindness (CSNB, Dryja et al, 1993). Over 150 mutations in RHO have been characterised, accounting for approximately 30-40% of adRP (RetNet). Mutations are located throughout the RHO gene, again highlighting the complexity and heterogeneity

of rhodopsin mutations (Al-Magthteh et al, 1993). Cell culture experiments by Sung et al led to classification of RHO mutations into two distinct classes. Mutations belonging to the first class clustered along the c-terminus of RHO (Sung et al, 1993). These mutations lead to wild-type expression levels, the protein does not aggregate on an SDS-PAGE gel, 11-*cis*-retinal is regenerated and mutant protein can migrate to the plasma membrane (Sung et al, 1991; Sung et al 1993). However, in class I, rhodopsin protein cannot translocate to the ROS efficiently (Sung et al, 1994). One example of a class I rhodopsin mutation is P347S, this single base pair change is within the penultimate amino acid of the rhodopsin c-terminus, and is important for proper post-golgi trafficking of the protein to the ROS (Deretic, 1998; Deretic et al, 2006). A transgenic mouse containing the human P347S allele has been generated. Rods in this mouse appear to accumulate vesicles of rhodopsin between the junction of the inner and outer segments (Li et al, 1996). Class II mutations are primarily located in the transmembrane and extracellular loops, are less abundantly expressed than wild-type rhodopsin protein and can aggregate on an SDS-PAGE gel. In addition, 11-*cis*-retinal is capable of binding to mutant protein in this class, but to varying degrees. The mutant RHO protein accumulates predominantly in the endoplasmic reticulum (Sung et al, 1991; Sung et al, 1993; Sung et al, 1994). The pathogenesis of this group of rhodopsin mutations is thought to be due to the protein's inability to fold properly (Kennan et al, 2005). Approximately 10% of adRP in the USA is caused by the class II P23H mutation (RetNet [<http://www.sph.uth.tmc.edu/retnet/>]). The mutant aggregated protein accumulates in aggresomes. Aggresomes are typically highly ubiquitylated and hence targeted for degradation by the ubiquitin proteasome pathway, which may promote apoptosis in cells within this class (Kennan et al, 2005; Illing et al, 2002). Illing et al showed in cell culture that aggregation of P23H mutant protein had a toxic gain-of-function effect on rods by impairing the ubiquitin-proteasome system. This suggests class II RHO mutant protein may function in a similar way to some mutant proteins underlying other important neurodegenerative diseases (Illing et al, 2002). Biochemical characteristics of other rhodopsin mutations have been elucidated that cannot be placed in the first two classes of mutations. For this reason Mendes et al, has proposed an extension of this classification system in order to encompass atypical mutations (**Table 1.11** [Mendes et al, 2005]). Class III mutations were proposed by Chuang et al, who examined the R135L rhodopsin mutation. This mutant protein is constitutively phosphorylated and thus binds to arrestin; arrestin is targeted to the plasma membrane and undergoes abnormal endocytosis. Thus, cells expressing mutant protein in this class undergo anomalous endocytosis (Chuang et al, 2004). Class IV mutant proteins have similar biochemical characteristics to the T4R rhodopsin mutation identified in English Mastiff dogs (a naturally occurring animal model). Proteins in this class have abnormal post-translational modifications and stability (Mendes et al, 2005;

Zhu et al, 2004). Mutant proteins in class V have increased levels of transducin activation (Andres et al, 2003; Mendes et al, 2005). Mutant proteins in class VI are constitutively active even in the dark and in the presence of 11-*cis*-retinal. Rhodopsin proteins with mutations that do not fit into any of these six categories remain as yet uncharacterised (Mendes et al, 2005).

1.12 Animal models

Animal models of a disease are a vital tool for molecular biology, used for identifying disease genes, understanding disease pathology and more recently for testing new treatments. Models for RP are no exception to this and have led to major advances in the retinal field (Chader 2002; Fletcher et al, 2011). There are many models of photoreceptor degeneration available, some have arisen naturally but the majority have been genetically engineered (Li et al, 2001). One commonly used animal to study RP is the mouse. Mice have a short lifespan and generation time making them an attractive species for such studies (Chang et al, 2005). In 1978 Van Nie et al, identified a mouse model (rds mouse) that had a slow retinal degeneration phenotype characterised by lack of formation of ROS and a progressive loss of photoreceptors for up to 1 year (Van Nie et al, 1978). A naturally arising recessive mutation within the coding sequence of the rds-peripherin gene was causative of the retinal pathology. This animal model has since been extensively characterised and utilised in many studies (Connell et al, 1991; Sanyal & Jansen, 1981; Sullivan et al, 2011). In terms of larger animal models, a naturally occurring dog model (the Swedish Briard Beagle dog) of Lebers Congenital Amaurosis (LCA, a severe retinal degeneration presenting in early childhood) has been well characterised and used in many studies. These dogs have a retinal degeneration caused by a 46bp deletion in exon 5 of the RPE65 gene. This model has been extensively utilised for preclinical evaluation of an AAV delivered RPE65 replacement gene facilitating the progression of this therapy to both studies in non-human primates and human clinical trials (Acland et al, 2005; Aguirre et al, 1998; Aguire et al, 2007; Jacobson et al, 2006; Le Meur et al, 2007; Narfstrom et al, 2003; Stein et al, 2011). A naturally occurring dominant rhodopsin mutation has been identified in the English Mastiff dog. This model has a T4R mutation within rhodopsin and the disease pathology appears similar to adRP in humans caused by the same mutation (Kijas et al, 2002). This animal was used to show the effect of light exposure (similar to that used for standard clinical eye examinations in human patients) in accelerating the retinal degeneration in this model in addition to studying the molecular mechanisms causative of this light induced retinal damage (Cideciyan et al, 2005; Gu et al, 2007; Komaromy et al, 2008a). Naturally occurring models of retinal degenerations in feline animal models have also been identified. A recessive mutation in the Cep290 gene in the Abyssinian cat results in a slow retinal degeneration (Narfstrom, 1983; Narfstrom et al,

2011). An autosomal dominant mutation in the CRX gene in a feline Abyssinian cat has also been identified. This mutation causes the photoreceptors to not develop fully and results in a severe retinal degeneration (Barnett et al, 1985; Narfstrom et al, 2011). An example of a naturally occurring model for X-linked RP is the XLPRAl Siberian husky. This dog has a 56bp deletion in ORF15 of the X-linked RP GTPase regulator gene which results in a severe retinal degeneration (Yang et al, 2002a).

A vast number of genetically engineered animal models of retinal degenerations exist and have been invaluable in the study of RP. One such engineered model for rds-peripherin-linked RP is the $\Delta 307$ mouse. In this model a base pair deletion in codon 307 of rds-peripherin (a mutation seen in human RP) was introduced by ES (Embryonic Stem Cell) homologous recombination. This caused a more severe phenotype compared to that observed with the naturally occurring $rds^{-/-}$ mutation suggesting the mutation may act in a dominant-negative fashion (McNally et al, 2002). Other genetically engineered peripherin murine models include the P216L model (Kedzierski et al, 1997) and the C214S model (Stricker et al, 2005) to name but a few. A large number of animal models have been generated for RHO-linked adRP. A rhodopsin knockout ($\rho^{-/-}$) mouse was generated to explore the role of rhodopsin in this disease. Mice with this mutation had no ROS, in addition to a severe retinal degeneration resulting in loss of rod-isolated ERG responses as early as 2 months of age. Furthermore, by three months of age photoreceptors were largely absent (Humphries et al, 1997). In an attempt to mirror RP caused by the P23H mutation in humans, Olsson et al, established various murine transgenic lines with this human mutant transgene. Mice showed a reduction in photoreceptor cell numbers by post-natal day 20 and mirrored the human form of the disease. Interestingly, another model over-expressing a normal human rhodopsin (NHR) transgene by five-fold displayed a retinal degeneration similar to that observed in P23H mutants, suggesting that over-expression of rhodopsin may have a deleterious effect on photoreceptors (Olsson et al, 1992). RHO-adRP models with c-terminus mutations have been generated and used to study the pathogenesis associated with mutations in this region. In particular, mouse models with both the human P347S rhodopsin mutation and a porcine P347S mutation have been characterised (Li et al, 1996; Wong & Hao, 1992). A transgenic mouse with the Q344ter rhodopsin mutation has been described by Sung et al, 1994. This mutation introduces a premature stop codon into amino acid 344 of rhodopsin and results in the abolishment of the last five amino acids of the c-terminal (Sung et al, 1994). The Q344ter mouse has been utilised to study the mislocalisation of mutant rhodopsin in rods (Concepcion & Chen et al, 2010). Given the ongoing progress made in gene therapy trials around the world, an increasing emphasis is being placed on generation of larger animal models for RP. Of particular interest is the transgenic porcine model of RHO-adRP generated by Petters et al. Transgenic pig lines with either the P347L or P347S mutation have been generated. Many

studies have been performed on these models to gain an insight into the pathogenesis associated with rhodopsin mutations in the c-terminus region (Kraft et al, 2005; Li et al, 1998; Ng et al, 2008; Petters et al, 1997; Sommer et al, 2011; Tso et al, 1997).

1.13 RP and gene therapy

Currently no effective treatments for RP exist (Hartong & Dryja, 1992). For this reason, an increasing number of gene-based therapies are being investigated (Verma & Weitzman, 2005). When designing a gene therapy, the different therapeutic approaches employed depend on the type of mutation involved in the disorder. Recessive mutations usually cause a loss-of-function and therefore a therapy based on introducing a wild-type replacement copy of the gene should in principle be beneficial. In dominant disorders the mutant protein often has toxic gain-of-function effects or mutations cause a decrease in the level of wild-type protein (haplo-insufficiency). In order to treat gain-of-function diseases, suppressing the mutant allele may be beneficial (Hartong et al, 2006; Millington-Ward et al, 1997). Some mutant genes may need complete suppression whereas others may only require partial suppression to provide benefit. For this reason understanding the way mutations cause disease and the exact gene expression patterns of the protein is vital.

Various approaches can be used for gene therapy for adRP. Primary therapies may involve suppression of the mutant gene e.g. suppression of mutant rhodopsin. Therapies can also be developed which are independent of the disease causing gene but which modulate secondary biochemical pathways involved in disease pathogenesis e.g. therapies introducing neurotrophic factors to promote photoreceptor survival or anti-apoptotic factors to help prevent photoreceptor cell death (Emerich et al, 2008; Farrar et al, 2002; Farrar et al, 2010; Gregory-Evans et al, 2009; Lawrence et al, 2004; Yang et al, 2009). For example, AAV (Adeno-Associated Virus) vectors containing proteins known as survival factors have been used to promote survival of photoreceptor cells in disease models. In one particular study transgenic rodent models with P23H and S334ter mutations within the rhodopsin gene were used to study neuroprotection. AAV-2/2 with expression cassettes containing fibroblast growth factor family members (FGF-2, FGF-5 and FGF-18) was delivered. The over-expression of these survival factors appeared to prolong photoreceptor cell survival despite no improvement in ERG response (Green et al, 2001; Rolling, 2004). Non erythropoietin (EPO) derivatives are another class of molecule recently implicated in neuroprotection. Both subretinal and systemic delivery of AAV-2/1 or AAV-2/5 EPO to mouse and rat models of retinal degeneration resulted in increased photoreceptor survival in treated animals (Collela et al, 2011; Rex et al, 2009; Sullivan et al, 2011). Clinical trials have been undertaken evaluating a retinal implant which releases ciliary neurotrophic factor (CNTF) to the retina of human

patients and has been shown to be well tolerated. Additionally some patients had an improvement in visual acuity (Emerich et al, 2008; Thanos et al, 2004]. A recent finding has implicated Norgestrel, a common component in the contraceptive pill, in the delay of apoptosis in photoreceptor cells. Studies in transgenic rd10 mice (a severe retinal degeneration) in addition to mice in which a retinal degeneration has been induced by light highlighted the protective effect of this compound. In rd10 mice the delay of onset of photoreceptor degeneration with Norgestrel (in a basic fibroblast growth factor dependent manner) resulted in a significant improvement in ERG function (Doonan et al, 2011). These are just some of the studies out of a large number that are using knowledge of secondary pathways associated with and photoreceptor cell survival to protect the retina from degeneration in RP.

In terms of designing a suppression based therapy for correcting the primary genetic defect in dominant retinopathies, there are various agents and methodologies that can be used for suppression of mutant alleles. Ribozymes are RNA enzymes that can elicit catalytic cleavage of a target RNA (Khan et al, 2006). More recently zinc finger nucleases have emerged as powerful tools for inducing gene correction. This involves utilising a zinc finger binding domain from a zinc finger transcription factor bound to a nuclease domain. The zinc finger nuclease can subsequently be utilised to direct sequence specific double stranded breaks into the target DNA and mediate gene correction by homologous recombination (Urnov et al, 2010). More recently these zinc finger proteins have been utilised alongside a repressor domain to direct sequence specific repression of rhodopsin (Mussolino et al, 2011a). Other molecules used for suppression studies include antisense DNA/RNA, aptamers, antibodies, triple helix DNA, siRNA, artificial miRNA, and exon skipping to name but a few (Akiyama et al, 2006; Baek et al, 2010; Boudreau et al, 2008; Brodin et al, 2011; Koilan et al, 2011; Lv et al, 2011; Millington-Ward et al, 2011; Takayama et al, 1990; Tanner et al, 2009).

1.14 RNA interference-the siRNA pathway

The 1990's was a decade that had a great impact on the world of molecular biology and to the understanding of the complexity of the human genome. Without a doubt one of the most seminal discoveries of this decade was the identification of small regulatory RNAs found to influence gene expression substantially in both plants and animals. These small RNAs have since been used as valuable tools for understanding gene regulation, identifying new genes and for various gene therapy studies (Davidson et al, 2011; McManus & Sharp, 2002).

The presence of these regulatory RNA molecules was first seen in early work by Napoli and colleagues in 1990. They described a process of post-transcriptional gene silencing (PTGS) in the Petunia flower.

In order to study the effect of the enzyme chalcone synthase (CHS) on anthocyanin biosynthesis (important for plant pigmentation) they introduced a chimeric version of the CHS enzyme. However, instead of observing the expected over-expression of CHS and flowers with purple pigmented petals, a novel phenotype with white pigmentation was discovered. It appeared that the introduction of CHS was somehow resulting in the post-transcriptional down-regulation of the recombinant and endogenous CHS mRNA transcripts, a phenomenon they termed “co-suppression” (Napoli et al, 1990). In 1992, in *N.crassa* a study which involved the introduction of exogenous RNA sequences homologous to a particular gene resulted in the “quelling” of the endogenous gene transcript (Romano & Macino, 1992). It wasn't until 1998, in a Nobel Prize winning paper by Andrew Fire and Craig Mello, that the term “RNA interference” (RNAi) was coined for this post-transcriptional silencing mechanism. In this paper, introduction of exogenous double stranded RNA (dsRNA) complementary to *unc-22* mRNA in *C.elegans* led to sequence-specific suppression and a phenotype that mimicked *unc-22* knockout mutants (Fire et al, 1998). This process of RNAi was found to involve small double stranded RNA molecules between 21- 23 nucleotides in length (short interfering RNAs, siRNAs) that could bind to specific mRNA target sequences and mediate suppression of that target mRNA transcript (Elbashir et al, 2001a; Hamilton & Balcombe, 1999; Zamore et al, 2000).

The exogenous siRNA pathway is a process that evolved in invertebrates as a defence mechanism against viral dsRNA that was introduced into a host cell via a viral infection. This dsRNA underwent degradation via the RNAi pathway as part of the innate immune response. It is currently unclear if this response occurs in mammalian cells, but it does suggest that a highly intricate level of protection may exist (Kim & Rossi, 2007). The mechanism of RNAi has been well described (**Figure 1.14**). Exogenous dsRNA molecules are introduced into the cytoplasm and are subsequently cleaved by the RNase III enzyme Dicer complex (containing Dicer bound to TAR-RNA binding protein, TRBP and PACI) in an ATP-dependent manner into small 21-23bp siRNAs (Bernstein et al, 2001; Forstemann et al, 2005; Provost et al, 2002; Saito et al, 2005). Processing of dsRNA by Dicer results in an siRNA with a 3' 2 nucleotide overhang, a 5' monophosphate and a 3' hydroxyl group (Bernstein et al, 2001; Hammond et al, 2000; Ketzinil-Gilad et al, 2006). The strand with the least thermodynamically stable 5' end (the anti-sense strand) is the guide strand of the siRNA duplex that is preferentially loaded into an enzymatic complex consisting of many different proteins known as the RNA-induced silencing complex (RISC [Chu & Rana, 2007; Khvorova et al, 2003]). The endonuclease Argonaute 2 (AGO2) is one of the main components of this complex, responsible for binding the guide RNA strand and cleaving the passenger (sense) RNA strand (Liu et al, 2004). Once this RNA strand is degraded, the RISC complex is guided to an mRNA sequence with complementarity to the antisense siRNA strand.

AGO 2 cleaves the target mRNA between bases 10 & 11 relative to the 5' end of the guide siRNA and the target mRNA becomes degraded by exonucleases, preventing its translation into protein (Chu & Rana, 2007; Kim & Rossi, 2007).

1.15 The miRNA pathway

While the work and discovery of RNAi caused by the introduction of exogenous dsRNA into cells was still ongoing, separate studies in *C.elegans* identified a novel endogenous system in which small RNAs were found to regulate gene expression during development. The *lin-4* gene in *C.elegans* negatively regulates its temporally controlled *lin-14* target during the late stages of larval development. *Lin-14* is a gene involved in the execution of an early larval stage of development known as L1. The negative regulation of *lin-14* in later stages, allows for the progression of larval stages L2-L4 to occur in the nematode. Examination of *lin-4* revealed that rather than coding for a protein, it encoded two small dsRNA species, 22nt and 61nt long respectively (Lee et al, 1993). Both species of RNA contained sequences complementary to various sequence elements in the 3'UTR of the *lin-14* gene. This suggested a mechanism in which the *lin-4* antisense RNA was responsible for *lin-14* suppression by binding to the 3'UTR of the *lin-14* mRNA and causing its translational repression (Lee et al, 1993; Wightman et al, 1993). Seven years later another small RNA was discovered, again using the *C. elegans* model system. This small RNA *let-7* appeared to be responsible for regulating the L4-adulthood stage in the late larval development of the nematode by binding to the 3'UTR of *lin-41* mRNA (Reinhart et al, 2000). Although it wasn't realised at the time, these studies represented the first evidence of small endogenous dsRNA molecules (micro-RNAs) involved in gene regulation. Eventually various studies on both of these systems led to the discovery of the two main RNAi pathways; the exogenous siRNA pathway (described above) and the endogenous micro-RNA (miRNA) pathway.

Hundreds of miRNAs involved in the negative regulation of gene expression via post-transcriptional gene silencing have thus far been identified. miRNAs have vital roles in the regulation of developmental processes and also in multiple pathological processes in many organisms including worms, flies, plants and mammals (Fazi & Nervi, 2008; He & Hannon, 2004 [**Figure 1.14**]). Endogenous miRNA sequences are located in the genome as polycistronic cassettes, within introns and within non-coding DNA (Fazi & Nervi, 2008). The transcription of miRNAs primarily involve RNA polymerase II which facilitates the transcription of these miRNAs into hairpin RNA structures that undergo 5'capping and adenylation (Lee et al, 2004). Studies have also implicated RNA polymerase III in transcription (Borchert et al, 2006). These long hairpin RNA transcripts (primary-miRNA, pri-

miRNA) are cleaved by RNase III endonuclease Drosha in the nucleus. This cleavage results in dsRNA structures of approximately 70 nucleotides consisting of an imperfect stem loop (pre-miRNA [He & Hannon, 2004, Lee et al, 2003]). In order to cleave pri-miRNA correctly, Drosha must form a complex with the dsRNA binding domain protein DGCR8 (Han et al, 2004). The pre-miRNA molecules are exported out of the nucleus and into the cytoplasm by Exportin V in a Ran-GTP dependent manner (Fazi & Nervi, 2008; Yi et al, 2003). Here the pre-miRNA enters the common RNAi pathway utilised by exogenous siRNA molecules. The pre-miRNA is processed by Dicer into a small 21-25nt ds miRNA containing a 3' 2 nucleotide overhang and a 5' phosphate group (Chu & Rana, 2007). As with siRNA, the strand with the less stable 5' end of the miRNA duplex is preferentially chosen to be incorporated into the RISC complex. The mature miRNA guides the RISC to its target site in the 3'UTR of complementary mRNA sequences where it either undergoes AGO-directed cleavage of the target mRNA or translational repression (Filipowicz et al, 2008). In the majority of cases miRNAs show imperfect complementarity to their mRNA targets and the binding of these miRNAs is defined by a specific sequence (the seed sequence) of 2-7bp located on the 5' end of the miRNA. This enables each miRNA to bind to the 3'UTR of many miRNA targets which have this specific seed sequence (Lewis et al, 2003; Lewis et al, 2005; Bartel et al, 2009). Interestingly certain 3'sites in the miRNA have also been predicted to have an effect on the binding and repression of target mRNAs. One site called the 3'supplementary site is predicted to complement the binding of the miRNA seed region via bases 13-16. Additionally a 3' compensatory site may also exist in which complementarity of nucleotides 13-16 in the miRNA to the mRNA target may allow mismatches in the seed region to be tolerated (Bartel, et al, 2009; Grimson et al, 2007). The mechanism of action of miRNA based RNA suppression has been extensively studied and appears dependent on the complementarity between the miRNA and mRNA target. In cases, where there are mismatches between miRNA and the mRNA target (although not fully understood) the mechanism appears to be via translational repression during initiation or elongation, and the targeting of the mRNA to P-bodies where proteins that repress translation are held (Filipowicz et al, 2008; Zimmerman and Wu, 2011). In cases where the sequence between the miRNA and the mRNA target is completely complementary, the miRNA acts similar to an siRNA and transcripts are targeted for AGO-mediated degradation. Many examples of this have been characterised in plants, with fewer examples in mammals (Davis et al, 2005; Jones-Rhoades et al, 2006; Shin et al, 2010; Yekta et al, 2004). Recently Shin et al found that miRNAs that had 11-12 nucleotides of continuous complementarity to the central region of the miRNA (bases 4-16) bound to mRNA targets irrespective of the seed region and 3' regions previously established as important for miRNA function. Indeed this binding resulted in AGO-mediated degradation of the mRNA target in a manner

dependent on the concentration of Mg^{2+} , raising the idea that there still might be a number of binding sites and mechanisms in miRNA-based repression that have yet to be discovered in this complex endogenous system (Shin et al, 2010).

1.16 RNAi and gene therapy

Since that initial discovery that 21nt siRNA duplexes could be introduced into mammalian cells and drive suppression of mRNA targets, along with the first *in vivo* studies of RNAi in the liver and brain of mice, a wave of research has been undertaken in order to optimise this system (Elbashir, 2001b; McCaffrey et al, 2002; McManus & Sharp, 2002; Xia et al, 2002). In particular utilising this RNAi mechanism in mammalian cells for use in suppressing disease causing alleles may in principle offer a lot of promise and hope for patients. However, in terms of siRNA, these molecules are only present transiently, representing a disadvantage for their use in therapy (Kim & Rossi, 2007). To overcome this, stable expression vector systems capable of endogenously transcribing small RNAi molecules known as short hairpin RNAs (shRNA) via polymerase II or polymerase III promoters have been designed. Following transcription, the shRNAs are exported to the cytoplasm via Exportin V and processed via Dicer into siRNA for the RISC complex. RNA polymerase III promoters such as HI or U6 have been utilised to drive long term constitutive expression of these small RNA transcripts, Polymerase II promoters have also been used to drive tissue specific expression (Brummelkamp et al, 2002; Kim & Rossi, 2007; Paddison et al, 2002).

To date utilising this endogenous cellular mechanism for novel gene therapies has been quite successful. 2010 in particular was an exciting year in establishing RNAi mediated suppression in human patients. One study showed at the molecular level, RNAi mediated therapy against solid tumours, while another trial demonstrated the clinical efficacy of an siRNA against keratin 6a (K6a) N171K mutant mRNA in Pachyonychia Congenita, resulting in significant callus regression suggesting that as a therapy for humans this strategy is feasible (Davis et al, 2010; Leachman et al, 2010).

Despite some successes, the use of shRNA in mammals has not been without its setbacks. One hurdle in mammalian systems was the presence of off target effects (OTE). It was found that when the antisense strand of siRNAs has seed sequence matches to the 3'UTR regions of mRNA, they could bind to unintentional targets and possibly function in a similar way to miRNA (Birmingham et al, 2006; Jackson et al, 2006a). In order to avoid OTE and increase specificity of siRNAs, various computer programs have been developed that use sequence alignment algorithms to minimise potential OTE (Ketzinal-Gilad et al, 2006).

Another serious issue when designing RNAi-based therapeutics is ensuring the therapy does not cause an immune response. A large amount of data has indicated that the introduction of dsRNA into a cell can stimulate an interferon response and lead to a global repression of gene expression. Initially it was discovered that long dsRNA (>30nt) could induce the interferon response by binding in a sequence specific manner to dsRNA-activated protein kinase (PKR), by activation of the 2'5'-oligoadenylate synthetase-RNase L mechanism and by binding to and activating various Toll like receptors (TLR). It is now emerging that small siRNAs may also induce an interferon response via TLR3 and TLR7/8 receptors (Aagaard & Rossi, 2007; Davidson et al, 2011). Certain siRNAs have been found to activate the interferon response in a sequence dependent manner via TLR7/8 initiating the innate immune response (Hornung et al, 2005; Judge et al, 2005; Stewart et al, 2011).

In terms of the retina an initial study utilised the RNAi pathway to suppress vascular endothelial growth factor (VEGF) in cell culture and *in vivo* in RPE cells. VEGF is capable of promoting angiogenesis in the retina and plays a role in causing the “wet” form of age related macular degeneration (AMD). siRNA targeting VEGF was tested in mice that had a laser-induced form of choroidal neovascularisation (CNV) similar to that observed in the wet form of AMD. In all cases significant down-regulation of the VEGF protein was observed (Reich et al, 2003a). Various clinical trials for this form of wet AMD and other retinal disorders using RNAi have also been undertaken. In particular a human clinical trial using siRNA against VEGF receptor I (siRNA-207) has found to be well tolerated and efficacious in patients in terms of reducing this CNV (Campochiaro et al, 2006; Kaiser et al, 2010). However, recently siRNAs over 21bp have been shown to cause a decrease in CNV due to activation of TLR3 irrespective of sequence. In particular this study tested two siRNAs (siRNA-027 and Bevasiranib) used in clinical trials to prevent CNV and found that this knockdown was abolished in mice with no TLR3 receptor whereas in wild-type mice the two siRNAs as well as other non-targeting siRNAs suppressed CNV. This indicated that suppression of CNV is a siRNA class effect and not a sequence specific relating to suppression of VEGF or VEGFR1 resulting in decreased CNV (Kleinman et al, 2008; Kaiser et al, 2010). The same group later showed that this suppression of neovascularisation also occurred in mouse models with corneal suture injury and hindlimb ischemia irrespective of RNAi sequence and the target, further confirming this RNAi operates to reduce angiogenesis by activating the innate immune response (Cho et al, 2009). A recent evaluation of siRNA delivery to the retina has implicated siRNAs of over of 21bp in length as causing retinal degeneration via apoptosis in the RPE in a TLR3 dependent manner. Furthermore this toxicity was observed with both unmodified siRNAs and cholesterol conjugated siRNAs irrespective of target (Kleinman et al, 2011). This effect was not observed for siRNA sequences of 16bp in length. This

obviously has implications for clinical trials particularly for the retina. Furthermore it is evident that an understanding of the sequence, length and the mode of action of the RNAi molecule on the immune system is vital to provide an efficacious therapy with no adverse side-effects.

It has been shown in several studies that abundant shRNA expression can be toxic to cells both *in vitro* and *in vivo* (Beer et al, 2010; Boudreau et al, 2009a; Ehlert et al, 2010; Giering et al, 2008; Grimm et al, 2006; Grimm et al, 2010; McBride et al, 2008). One such group examined shRNA expression in mice. Administration of shRNA to the liver resulted in severe hepatotoxicity and in some cases death. This was caused by a down-regulation of important liver miRNAs, suggesting shRNAs may be toxic by saturating the endogenous RNAi machinery. This effect was initially thought to be due to oversaturation of Exportin V, but more recent studies have implicated the four AGO proteins as being involved in this process (Grimm et al, 2006; Grimm et al, 2010). Beverley Davidson's group examined the long term effects of shRNAs targeting Huntington mRNA and SCA-1 mRNA in brains of mice. It was found that the toxicity observed was due to high levels of shRNA (Boudreau et al, 2009a; McBride et al, 2008). Several recent studies have employed artificial miRNAs in an attempt to overcome this problem. To try to mimic endogenous miRNAs, these artificial miRNA-based systems use siRNA sequences cloned into a pri-miRNA backbone (Boudreau et al, 2008). Backbone of miRNA-30a and miR-155 have been utilised with success (Baek et al, 2010; Boden et al, 2004; Boudreau et al, 2009a; Chung et al, 2006; Lagos-Quintana et al, 2002; McBride et al, 2008; Shan et al, 2009a; Shan et al, 2010; Stegmeier et al, 2005; Tam & Dahlberg, 2006; Zeng et al, 2002).

1.17 Modes of Delivery

A significant challenge for gene therapy is optimisation of appropriate delivery systems for use in animal models and subsequently in humans. Unmodified therapeutics such as negatively charged DNA/RNA cannot readily cross the cell membrane unassisted and are often degraded (Ketzinel-Gilad et al, 2006; McManus & Sharp, 2002; Seow & Wood, 2009). In order to design the appropriate delivery vehicle, there are various considerations which must be taken into account, all of which depend on both the molecule being delivered and the target tissue. Vectors should be capable of facilitating the size of the therapeutic transgene and they should be able to target the specific cell type affected by the disease (e.g. photoreceptor cells). In addition, they should be safe with no adverse immune response in the host (Seow & Wood, 2009; Verma & Weitzman, 2005). Several different delivery strategies have been evaluated for use *in vitro* and *in vivo*, either in terms of delivery of the therapeutic directly to cells by techniques such as high pressured injections (e.g. hydrodynamic

injection), electroporation or ultrasound (Seow & Wood, 2009; Yuan et al, 2011) or by vectors using either non-viral delivery or viral delivery (Verma & Weitzman, 2005).

1.18 Non-viral delivery

Non-viral delivery typically involves delivery of nucleic acids either naked or as a plasmid directly into cells (Verma and Weitzman, 2005). This process can involve transfection of therapeutic molecules into cells using various particles, e.g., cationic liposomes, cationic polymers, stable-nucleic-acid-lipid particles (SNALPs), carbon nanotubes etc. (Dykxhoorn & Lieberman, 2006; Guo & Huang, 2011; Kim & Rossi, 2007; Yuan et al, 2011). Liposomes are charged particles capable of carrying nucleic acids across a cell membrane (Dykxhoorn & Lieberman, 2006; Gao et al, 2011; Guo & Huang, 2011; Kim & Rossi, 2006). They were first used *in vivo* in rats delivering preproinsulin I to liver cells (Soriano et al, 1983). Liposomes can transverse the cell membrane by endocytosis (Yuan et al, 2011). Various liposomal complexes are commercially available; one such complex, Lipofectamine® 2000 (Invitrogen), has been used to co-transfect human rhodopsin cDNA and targeting siRNAs with success in HeLa cell culture (Kiang et al, 2005; O'Reilly et al, 2007; O'Reilly et al, 2008). In the retina, both a CMV driven LacZ and a non-histone nuclear protein transgene were successfully and safely delivered to the photoreceptors in a liposome complex (HVJ liposome) by intravitreal or subretinal injection (Hangai et al, 1996). Robust and stable gene expression of peripherin-2 by subretinal injection of a peripherin-2 transgene delivered using compacted DNA nanoparticles was observed in haploinsufficient *rds*^{+/-} mice up to 120 days post-injection (the last time point examined [Cai et al, 2010a]). This resulted in an increase in rod-isolated ERG function and a cone-isolated ERG at levels similar to the wild-type control. In addition, improvement in OS structure was also observed in these mice, suggesting DNA nanoparticles as an efficient delivery system for the retina (Cai et al, 2010a).

1.19 Modifications for delivery and stability

Often therapeutic nucleic acids are chemically modified or conjugated to certain chemical groups to facilitate delivery in a more efficient, stable and targeted manner (Kim and Rossi, 2006). siRNAs can be chemically conjugated to cholesterol groups to aid efficient entry to the cell. In one study, a siRNA targeting apolipoprotein B with a conjugated cholesterol to the 3' sense strand of siRNA via a pyrrolidone linker was systemically administered to mice via tail-vein injection and successfully lowered cholesterol levels in liver and jejunum (Soutschek et al, 2004). A common modification to assist in stable delivery of the nucleic acids is addition of sugars groups to the nucleic acid such as 2' fluoro, 2'-O-methyl, 2'-O halogen, 2'-amine and Locked Nucleic Acids (Gao et al, 2011). Jackson et

al, 2006b reported that addition of 2'-O-methyl ribosyl to the second base of the guide siRNA strand (at the seed region) resulted in silencing of target RNA (Jackson et al, 2006b). Similarly Layzer et al reported a significantly increased half-life in plasma of siRNAs modified with a 2'-fluor-pyridimidine siRNAs (Layzer et al, 2004). Modifications in which the oxygen in the DNA/RNA backbone is replaced with another molecule such that the phosphodiester linkage is altered, has been successful (Behlke et al, 2008). In particular phosphorothioate or boranophosphonate modifications have been reported as effective for increasing the half-life of the therapeutic and with minimal toxicity associated (Behlke et al, 2008; Gao et al, 2011; Hall et al, 2004; Overhoff & Sczakiel, 2005).

1.20 Viral delivery

Even though the delivery systems outlined above are safe and have minimal toxicity they are often inefficient providing only transient expression. The use of viral vectors holds great promise for the future of gene therapy. Viral vectors have an innate ability to deliver genetic material into a host cell. There are several different kinds of viral vectors currently being explored for delivery of gene therapies including RNA-based viruses such as retroviruses and lentiviruses or DNA-based viruses such as adenoviruses and adeno-associated viruses (Verma and Weitzman, 2005). In order to design an efficient therapeutic, the choice of viral vector is particularly dependent on the tropism of the target tissue, the titre that can be achieved with the vector and the safety of the vector (Farrar et al, 2010).

1.21 AAV-mediated delivery

Adeno-associated virus 2 (AAV-2) was initially discovered in 1965 as a DNA containing virus that co-localised with Simian Adenovirus 15 (SV-15). Electron Microscopy images of SV-15 showed the larger adenovirus particles interspersed with a smaller satellite virus serologically different from any known adenovirus (Atchison et al, 1965; Hoggan et al, 1966; Mayor et al, 1965). These AAV viruses were named "defective" as they could only replicate in the presence of adenovirus (Atchison et al, 1965). Subsequently AAV-2 was characterised into the genus *dependovirus* belonging to the *Parvoviridae* family. This viral vector is considered non-pathogenic thus enabling its successful use in various animal models in a safe and stable manner (Le Bec & Douar, 2006; Rolling, 2004). It is a single stranded DNA (ssDNA) virus with a genome size of 4,675 nucleotides and a diameter of between 20-25nm (Mingozzi & High, 2011; Srivastava et al, 1983). It has two coding domains; the 5' domain contains the gene for *rep* which encodes four rep proteins, involved in the replication of the viral genome. The 3' coding domain is the *cap* gene which encodes three capsid proteins (Vp1, Vp2 and Vp3) involved in the assembly of the viral capsid into a 60 sub-unit viron (Wang et al, 2011). Each

open reading frame is surrounded by two inverted terminal repeats (ITRs) of 145nt (Srivasta et al, 1983). The ITRs contain palindromic sequences which can form a t-shaped hairpin and facilitate second strand DNA synthesis and replication via binding sites for the rep proteins. The first 15bp of the ITRs also have a vital function in allowing the excision of AAV DNA contained in recombinant plasmids (McCarthy et al, 2004; Wang et al, 2011).

Many AAV serotypes have been identified; however AAV-2 has been the most comprehensively studied (Le Bec & Douar, 2006). Once delivered to an organism, AAV undergoes five main steps: attachment to cell surface via different receptors (each AAV serotype binds to different cell surface receptors, giving certain serotypes tropisms for specific cell types). The next step is endocytosis, a mechanism by which AAV gets internalized by endosomes to be carried into the cytoplasm. Following this, AAV migrates to the nucleus and uncoats. The ssDNA is subsequently converted into dsDNA (Wang et al, 2011; Choi et al, 2005).

For recombinant AAV (rAAV) vectors used in gene therapy the viral genes (i.e. *rep* and *cap*) have been removed and replaced with an expression cassette (for example an shRNA, a replacement gene or a reporter gene) flanked by the ITRs which are essential in *cis* for efficient packaging of viral DNA into capsids (Le Bec & Douar, 2006; Mingozzi & High, 2011). These rAAV vectors have a small packaging capacity of approximately 5kb and a propensity for transducing non-dividing cells efficiently, making them valuable tools for delivery to cells of the retina, CNS, muscle and hepatocytes amongst others (Mingozzi & High, 2011; Warrington & Herzog, 2006). To produce rAAV, the virus is typically produced using the triple transfection method. This involves co-transfection of the transgene containing plasmid flanked by ITRs, along with an additional plasmid containing the cap and rep genes needed for the AAV capsid formation and replication. A helper plasmid is also transfected which contain adenovirus genes E4, VA and E2A which provide the function of the helper virus. These plasmids typically are transfected into Human embryonic kidney cells (HEK293) and 48-72 hours later the cells are lysed and the cell lysate which contains the viral particles is purified (Liu et al, 2003; Matsushita et al, 1998; Mingozzi & High, 2011; Warrington & Herzog, 2006; Xiao & Salmulski, 1998).

Wild-type AAV2 can remain in the cell episomally, however there have been reports that when helper virus is not present the wild-type AAV can remain latent in the host genome by integrating into chromosome sites with a preference for a site on chromosome 19 (19q 13.4ter [Kotin & Berns, 1989; Kotin et al, 1990; Kotin et al, 1992; McCarty et al, 2004; Samulski et al, 1991]). This region contains a tetranucleotide repeat sequence (AAVS1) that binds to the rep proteins and mediates integration (Kotin

et al, 1991; Linden et al, 1996a; McCarty et al, 2004; Philpott et al, 2002). In contrast to wild-type AAV2, rAAV which lacks rep can integrate non-specifically. This occurs with significantly less frequency than wild-type AAV into areas containing pre existing DNA breaks. (Inagaki et al, 2007; Li et al, 2011; McCarthy et al, 2004; Miller et al, 2004; Miller et al, 2005; Nakai et al, 1999; Nakai et al, 2003; Rutledge & Russell, 1997). Largely these rAAV viruses remain in the cell in an episomal form both *in vitro* and *in vivo* (McCarthy et al, 2004; Nakai et al, 2001; Smith, 2008).

Initially AAV-2 was the rAAV vector of choice, however the AAV-2 capsid binds to only certain cell types limiting its utility (Rabinowitz et al, 2002). For this reason hybrid rAAV serotypes have been explored for the capability of different capsids to transduce various cell types. Chimeric vectors typically contain AAV-2 ITRs within a capsid from one of the other AAV serotypes identified (Rabinowitz et al, 2002; Rolling, 2004). Traditionally the ITRs of AAV-2 are usually used in these hybrid vectors as AAV-2 has been the most extensively studied AAV (Choi et al, 2005). The transduction efficiency of hybrid rAAV containing AAV-2 ITRs with capsids from various other serotypes has been extensively characterised.

1.22 AAV in the retina

The transduction efficiency of various AAV serotypes has been examined in the retina. One main issue when designing an AAV-mediated therapy for the retina is the route of administration. There are two main administration routes; injection into the vitreous or into the subretinal space. Subretinal injection in humans and larger animals typically involves a vitrectomy and induces a retinal detachment at the site of injection to relieve ocular pressure and allow space for the therapeutic to enter the subretinal space. This is therefore a more invasive delivery mechanism compared to intravitreal injection (Liang et al, 2001; Vandenberghe & Auricchio, 2012). However, efficient transduction of retinal cells by unmodified rAAV serotypes has thus far only been reported for AAV-2 by intravitreal injection (Vandenberghe & Auricchio, 2012). Interestingly a recent paper by Dalkara et al, 2009 have implicated the inner limiting membrane (ILM) located between the vitreous and the retina as presenting a barrier to AAV mediated transduction of the retina via intravitreal injection. Various AAV serotypes (AAV-1, 2, 5, 8 & 9) were examined for their ability to attach to the ILM, interestingly while AAV-2, AAV-8 and AAV-9 were able to bind to the vitreoretinal junction, only AAV-2 resulted in GFP expression in the retina. This suggested that certain barriers at the ILM may limit the transduction of other serotypes. Co-injection of all AAVs with pronase, a protease solution which degrades the ILM resulted in

expression from all serotypes within the retina. AAV-2/5 mediated transduction was particularly effective utilising this method (Dalkara et al, 2009).

In terms of subretinal delivery to the retina, AAV-2/2 was the first AAV vector explored and efficient transduction to both the RPE and photoreceptors compared to adenovirus was achieved (Ali et al, 1996). Subsequent studies have optimised and utilised AAV-2 in the retina with success in dogs, non-human primates and human clinical trials (Acland et al, 2001; Auricchio et al, 2001b; Bainbridge et al, 2008; Bennett et al, 1999; Cideciyan et al, 2008; Maguire et al, 2008; Vandenberghe et al, 2011). AAV-2/5 was subsequently identified as outperforming AAV-2/2 in terms of the efficiency and onset of transgene expression in photoreceptors. Subretinal injections into mice with AAV-2/2 or AAV-2/5 vectors were undertaken by Yang et al, to compare the two serotypes. Transgenes expressed from AAV-2/5 were found to be expressed 400-fold higher than from AAV-2/2 vectors. In addition, at 15 weeks post-injection, AAV-2/5 was found at a much higher viral copy number than AAV-2/2. This study highlighted the transducibility of AAV-2/5 in the retina and also outlined the impact that the capsid could have on vector tropism (Yang et al, 2002b). AAV-2/5 has subsequently been utilised in a large array of retinal studies in mice, dogs, pigs and non-human primates (Beltran et al, 2010; Lotery et al, 2003; Millington-Ward et al, 2011; Mussolino et al, 2011b). Interestingly, studies comparing the novel serotypes rAAV-2/7, rAAV-2/8 and rAAV-2/9 to rAAV-2/5 under the control of a proximal fragment of the rhodopsin promoter in the retina suggested that AAV-2/7 and AAV-2/8 were 6-8-fold more efficient than AAV-2/5 at targeting photoreceptors both *in vivo* and *ex vivo* (Allocca et al, 2007). This observation regarding AAV-2/8 compared to AAV-2/5 was supported by studies in mice (Leberz et al, 2008; Natkunarajah et al, 2008). Additional experiments in dogs, pigs and non-human primates have suggested that AAV-2/8 transgene delivery significantly surpasses that of AAV-2/5 and highlight the value of evaluating novel AAV serotypes in retina to optimise gene therapies (Mussolino et al, 2011b; Steiger et al, 2008; Vandenberghe et al, 2011).

1.23 Gene therapy for LCA using AAV-2/2 in human patients

The most exciting studies to date utilising AAV in the retina have probably been the Phase I clinical trials on human LCA patients reported in 2008 and 2009. Following successful therapy in Briard dogs and the analysis of toxicity and safety in non-human primates, three clinical studies set out to test a therapy for human patients deficient in RPE65 (Stein et al, 2011). Three LCA patients with severely impaired vision were used in each clinical study (between 17-26 years). In all three studies a single subretinal injection of AAV-2/2 containing the RPE65 replacement gene was administered to the retina. Despite several differences in these studies, all reported a well tolerated therapy with no vector

dissemination and no adverse immune response to either the vector or the RPE65 transgene (Bainbridge et al, 2008; Cideciyan et al. 2008; Maguire et al, 2008). Efficacy of the therapy was also observed in all studies. In the Maguire et al 2008 study an increased pupillary response was observed in the treated eye of all three patients who received the therapeutic. In addition the injected eye was approximately three times more sensitive to light compared to baseline conditions (testing of that eye prior to the subretinal injection) and the uninjected eye. Patients also showed a reduction in nystagmus compared to the baseline condition. Various visual function tests including the patient's ability to navigate a maze were performed before and after treatment and an improvement in visual acuity was obtained after treatment. These improvements remained stable and persisted for up to 1.5 years post-injection with no adverse effects or immune response observed at this time-point (Maguire et al, 2008; Simonelli et al, 2010). Cideciyan et al, 2008 and Hauswirth et al, 2008 reported an increased visual sensitivity in treated patients as well as an increase in cone and rod-photoreceptor based vision that was maintained for up to a year after treatment (Cideciyan et al, 2008; Cideciyan et al, 2009; Hauswirth et al, 2008). In the Bainbridge et al, 2008 study, patients displayed no clinically significant improvement in visual acuity or in peripheral visual fields as measured by Goldmann perimetry, however, one patient had an improvement in visual function as measured by microperimetry and dark-adapted perimetry. This patient also showed significant improvement in visual mobility under dim light conditions (tested by their ability to navigate a maze) after treatment. Before treatment it took the patient 77 seconds to navigate a maze with the study eye but after vector administration this was reduced to 14 seconds using the treated eye (Bainbridge et al, 2008). Jean Bennett's group undertook a phase 1 clinical trial to assess whether age or dose had an effect on RPE65 gene therapy (Maguire et al, 2009). 12 patients were tested, ranging from 8-44 years, and groups of patients were treated with three different doses of AAV, no adverse effects or immune response was noted in any patients regardless of the dose injected. All 12 patients reported an increase in scotopic vision and improved visual fields and 7 patients had improvements in visual acuity. All patients had improvements in pupillary response, notably the youngest patient (8 years old) had light sensitivity in the injected eye comparable to normal sighted controls. Decreased nystagmus was also observed in patients, in three patients with significantly decreased nystagmus a multifocal ERG was performed and all these patients presented with an ERG signal. The most significant result was that the four children injected with the therapy were successfully able to navigate a maze unassisted when the injected eye was left uncovered. Recovery of function and efficacy remained stable for up to two years (the last time-point monitored). This study highlighted the potency of the gene therapy in the retina at different ages, and suggested that the earliest possible intervention may result in the best outcome, to improve vision in patients

before the retina has undergone irreversible degeneration (Maguire et al, 2009). An interesting question in terms of treating retinopathies is whether severe retinal degenerations from an early age can also impair neuronal connections in the visual cortex and if so does this mean therapeutic efficacy may be limited by this unresponsiveness? An attempt to address this question was undertaken in dogs and human patients of LCA2 (Aguirre et al, 2007) and in a recent follow up to the study described above (Maguire et al, 2009). In this study three of the patients enrolled in the original trial by Maguire et al, 2009 (age 8, 9 and 35 years) were tested two years later for changes in the visual cortex by functional MRI (fMRI). Patients were exposed to a dim light stimulus which they were unable to respond to in the untreated eye and the treated eye pre-injection. It was found that increased cortical activation and improved contrast sensitivity occurred in treated patients, with the areas of activation in the brain corresponding to areas of the retina that received the therapeutic (around the macula for the younger patient and around the periphery in the older patient). Moreover, this study demonstrated that patients with severe retinopathies are still capable of visual cortex stimulation even at a time point where the retinopathy is very severe. This has significant implications for ocular gene therapy as it highlights that even in older patients, visual improvement can be detected and the brain neurons can still be activated (Ashtari et al, 2011). Even though all these studies are for recessive LCA rather than adRP, they all highlight exciting and promising results and pave the way for the use AAV-mediated gene therapies for other retinal disorders.

1.24 A Mutation-independent suppression and replacement strategy

Suppression of the primary defect can be undertaken using various strategies. The first strategy may be undertaken in a mutation-independent manner such that only the mutant allele is suppressed. Various studies have shown that mismatches between siRNA and mRNA target can prevent siRNA from down-regulating the mRNA transcripts. The consequence of this is that with diseases such as RP in which frequently a single point mutation is involved, it may be possible in principle to design an siRNA for some mutations that can suppress a mutant allele, and leave a wild-type allele unsuppressed (Schwarz et al, 2006). The genes involved in RP typically show high levels of mutational heterogeneity and thus designing RNAi therapies for each specific mutation is impractical. Each therapy would need to be optimised and evaluated separately in different animal models. If the therapies progressed to clinical trial stage, they would each have to be produced according to good manufacturing protocols (GMP) and would each undergo clinical trials. Clearly for a heterogeneous disease such as RP this process would take a large amount of time, labour and money and would therefore not be practical (Cashman et al, 2005).

To overcome this hurdle, the use of mutation-independent therapeutic approaches is currently in development for RP. This approach involves the suppression of both mutant and the wild-type alleles (Kiang et al, 2005; Palfi et al, 2006). Using this approach, both alleles are knocked down and therefore a codon-modified replacement gene refractory to suppression would be introduced. This replacement gene can be designed by utilising the degeneracy of the genetic code and modifying bases at wobble positions within the RNAi target site. The gene should thus still be capable of supplying the correct amino acid sequence and functioning as a wild-type allele but should be immune to suppression by the RNAi molecule (Millington-Ward et al, 1997; Millington-Ward et al, 2011; Palfi et al, 2006). This suppression and replacement technology was first investigated using ribozymes as the suppression agents of choice. However more recently RNAi technology has been utilised for this technology (Kiang et al, 2005; Millington-Ward et al, 1997; Millington-ward et al, 2011; O' Neill et al, 2000; Palfi et al, 2006).

1.25 Objectives

The overall objective of the research described in this PhD was the optimisation of novel therapies for RHO-adRP for use in larger animal models using a mutation-independent RNAi-mediated suppression and replacement system. This strategy involved the down-regulation of both mutant and wild-type rhodopsin RNA via RNAi and the simultaneous delivery of a codon-modified rhodopsin replacement gene resistant to suppression. This therapy, although effective in murine RP models has never been tested in larger animal models. Larger animal models are vital components of pre-clinical studies in which both the efficacy and safety of the therapeutic may be assessed. The porcine eye is similar in size to that in humans, and additionally has a more similar rod: cone ratio than mouse retinas. In addition a RHO-linked adRP porcine model is readily available and has been utilised with success to characterise the molecular mechanisms behind rhodopsin mutations. For this reason the main goal of my project was the design of a suppression and replacement therapy for use in the RHO-linked adRP porcine model (Petters et al, 1997). Additionally the study presented in this thesis was concerned with optimising RNAi-mediated suppression of rhodopsin to utilise the most efficient, safe molecule possible. For this reason in addition to AAV-2/5.shRNA based therapeutics it was also decided to evaluate the use of AAV-2/5 driven artificial miRNAs during the course of the study, in order to evaluate if potent suppression of rhodopsin by these constructs could be achieved *in vivo* and indeed if functional effects associated with RNAi suppression were observed in transgenic animal models. The final aim of the study presented in this PhD thesis involved the generation of a rhodopsin replacement gene and the subsequent optimisation of a rhodopsin replacement gene *in vivo* in terms of AAV

production protocols, viral dose and AAV serotype evaluation. In particular one goal involved comparing rhodopsin replacement gene expression from AAV-2/5 compared to AAV-2/8. The data obtained highlight the value of progressing this novel dual-component gene therapy for rhodopsin-linked adRP towards future evaluation in large animals.

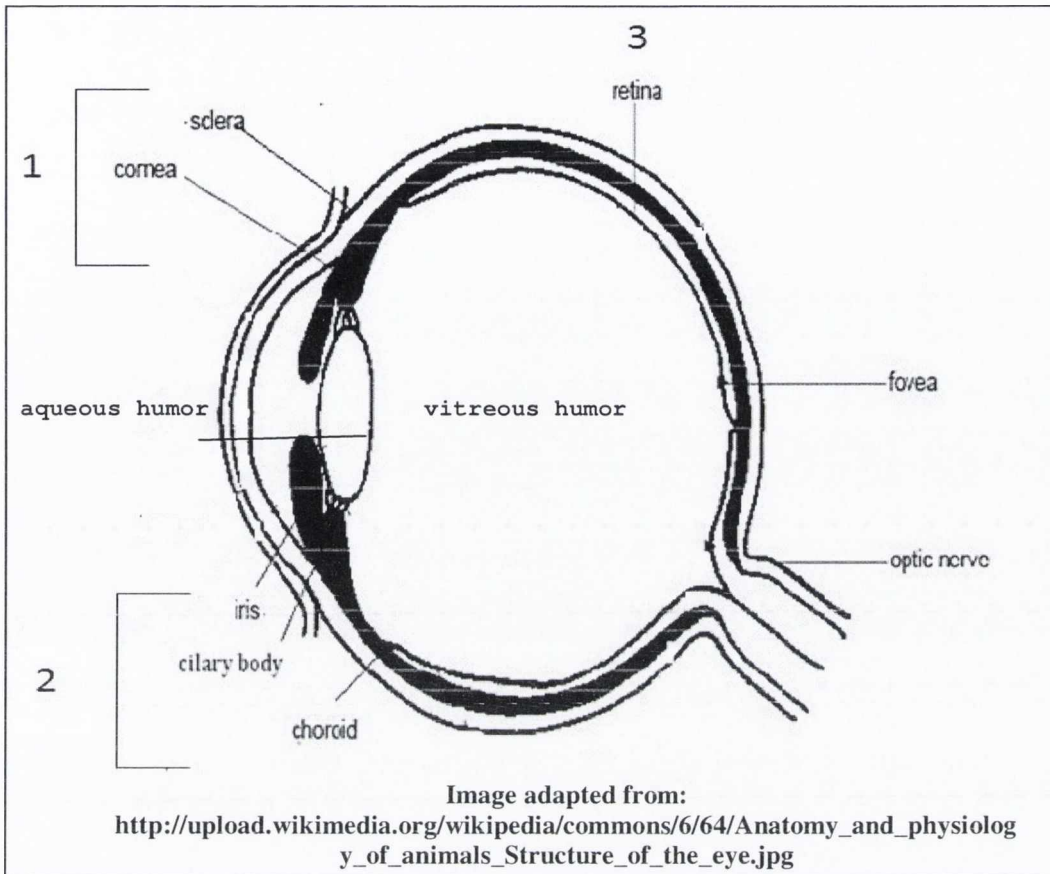


Figure 1.1 The eye

This is a diagrammatic representation of the human eye and its main components. Layer 1 (1) includes the sclera and the cornea; Layer 2 (2) is composed of the uveal tract and includes the choroid, the ciliary body and the iris. Layer 3 is neural retina and includes a cone rich region known as the macula. The highest visual acuity occurs in the fovea. The aqueous humor is fluid located between the cornea and the lens; it provides nourishment to that region of the eye. The vitreous humor is fluid located between the cornea and the retina; it maintains the structure of the eye and is involved in phagocytosis of unwanted debris.

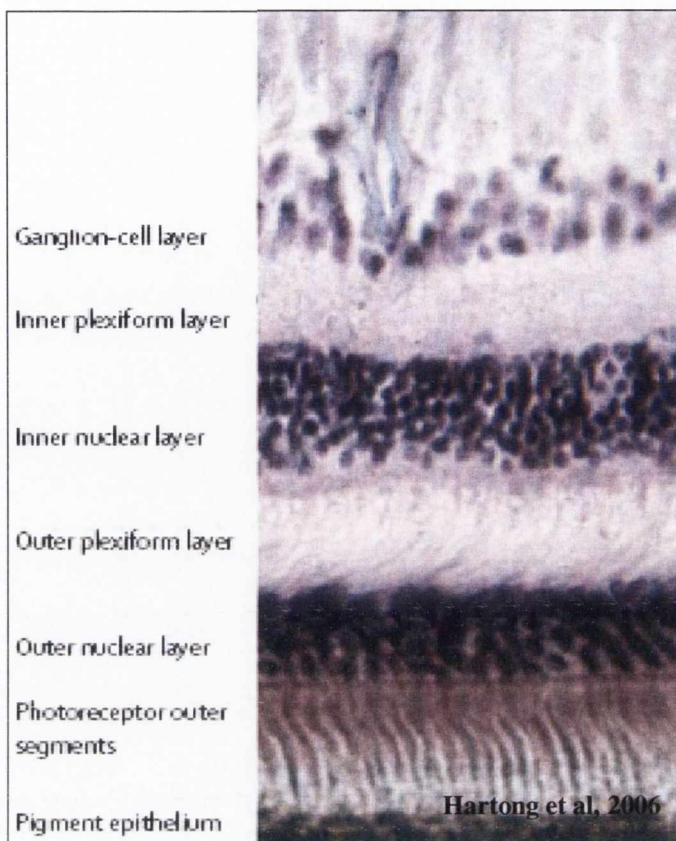


Figure 1.2 Different retinal cell layers.

The first layer of the retina is the outer layer; it comprises the RPE, the photoreceptor OS, the ONL and the OPL. The inner layers of the retina comprise the INL and IPL. The final layer of the retina is the GCL.

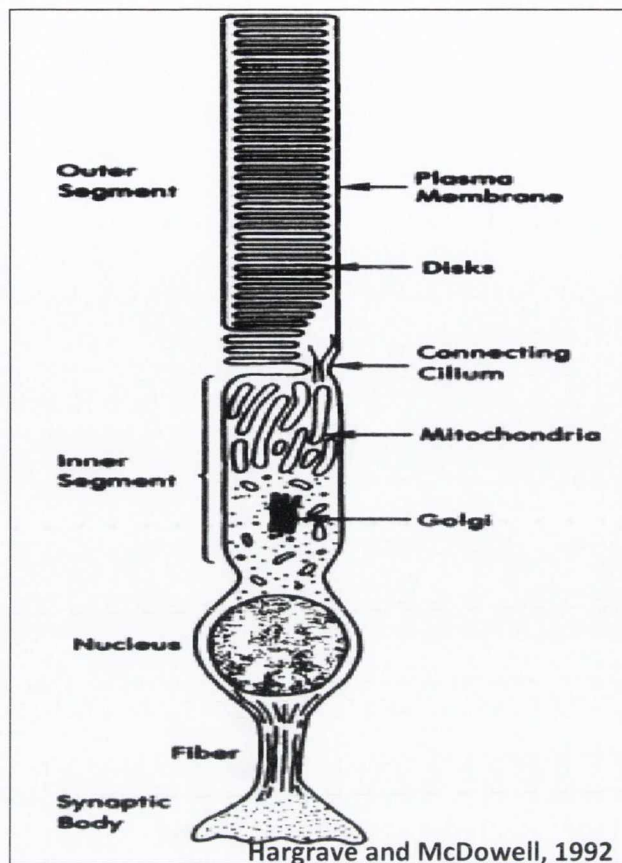


Figure 1.4 Rod photoreceptor cell

The above diagram depicts a rod photoreceptor, the synaptic body synapses with the second order neurons involved in visual transduction. The cell body of the rod houses the nucleus followed by the IS in which the cellular organelles are housed (golgi apparatus, mitochondria). The RIS is connected to the ROS by a connecting cilium. The ROS comprises the ROS disk membranes to which rhodopsin binds.

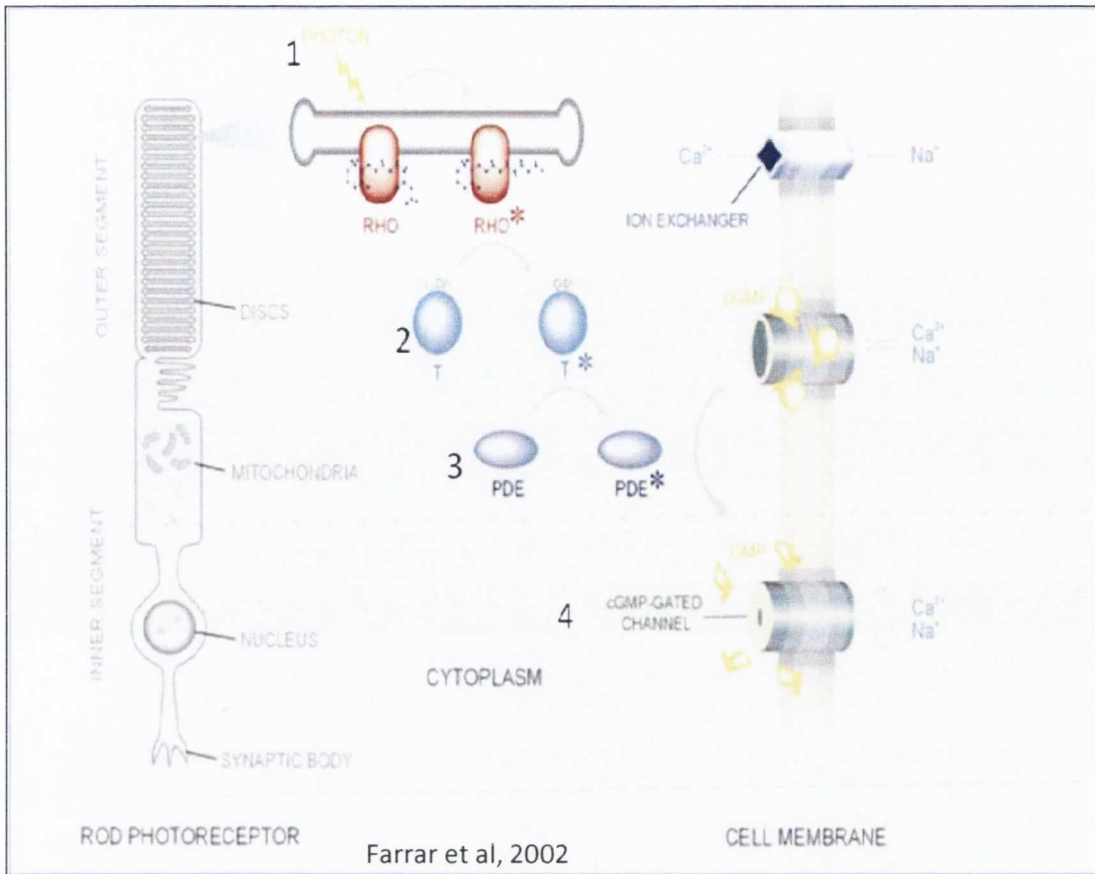
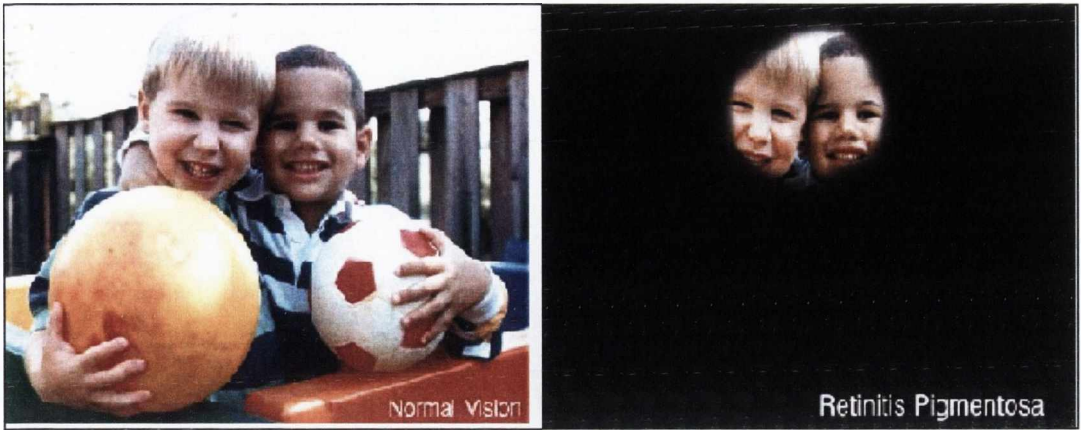


Figure 1.6 The phototransduction cascade

Light hits rhodopsin which is bound to 11-*cis*-retinal, 11-*cis*-retinal becomes isomerated to all-*trans*-retinal and dissociates from rhodopsin (1). This activates rhodopsin. Activated rhodopsin binds to the G protein transducin (T) and GDP is exchanged to GTP (2). This activates transducin, active transducin binds to cGMP-PDE (3.[PDE]). Active cGMP-PDE hydrolyses cGMP to 5' GMP and this reduces cGMP in the rod. This closes the cGMP-gated cation channel in the ROS and Na^+ and Ca^{2+} cannot enter the photoreceptor (4). Glutamate is inhibited and the cell becomes hyperpolarised. This results in the transmission of an electrical signal through the neurons in the retina and sends a signal through the optic nerve to the brain.



Images from NIH National eye institute

Figure 1.7.a The loss of peripheral vision associated with RP

A. Represents a non-diseased retina with normal vision. B. Represents the characteristic symptom of tunnel vision associated with the initial death of the rod photoreceptors in RP.

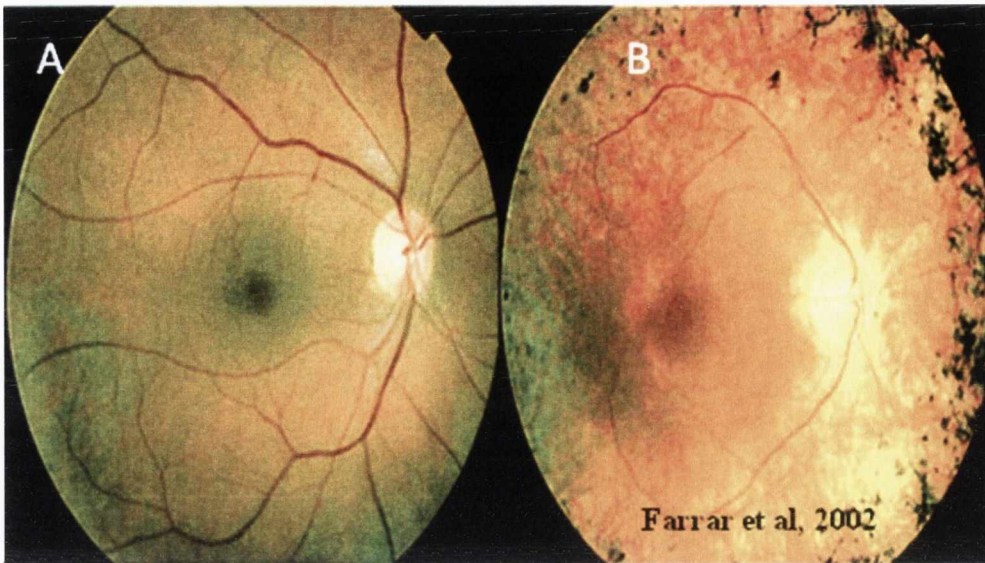


Figure 1.7.b Clinical symptoms of RP in the retina

A. Healthy retina, with well defined blood vessels. B. A degenerating retina from an RP patient. Note the presence of pigmented deposits in addition to fewer and shortened blood vessels

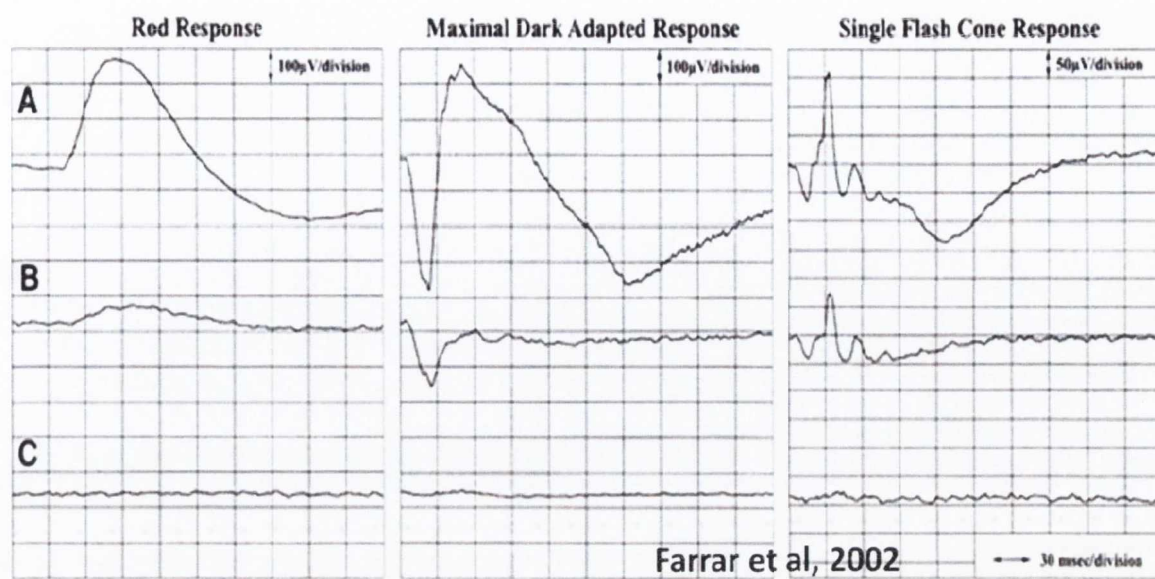


Figure 1.9: ERG responses:

The first ERG panel is the rod response, the second the mixed rod-cone response and the last panel the cone-isolated response. A. represents the ERG of a person with normal vision, B. Represents the ERG of a patient with moderately advanced RP and C. Represents the ERG of a patient with advanced RP

Classification of mutant rhodopsins

Classification	Behaviour	Site of point mutation	Misfolds
Class I	Fold normally but are not transported to the outer segment	L328, T342, Q344, V345, A346, P347	No
Class II	Are retained in the ER and cannot easily reconstitute with 11- <i>cis</i> -retinal	T17, P23, G51, T58, V87, G89, G106, C110, L125, A164, C167, P171, Y178, E181, G182, C187, G188, D190, H211, C222, P267, S270, K296	Yes
Class III	Affect endocytosis	R135	No
Class IV	Do not necessarily affect folding but affect rod-opsin stability and posttranslational modification	T4	No
Class V	Mutations show increased activation rate for transducin	M44, V137	No
Class VI	Show constitutive activation of opsin in the absence of chromophore and in the dark	G90, T94, A292,	No
Unclassified	No observed biochemical or cellular defect or not studied in detail	N15, Q28, L40, F45, L46, P53, G109, G114, S127, L131, Y136, C140, E150, P170, G174, P180, Q184, S186, T193, M207, V209, P215, M216, F220, E249, G284, T289, S297, E341	

Table 1.11 The classification system (I-VI) of human rhodopsin mutations implicated in RP Mutations that do not fit into any of the six categories remain unclassified (Mendes et al, 2005)

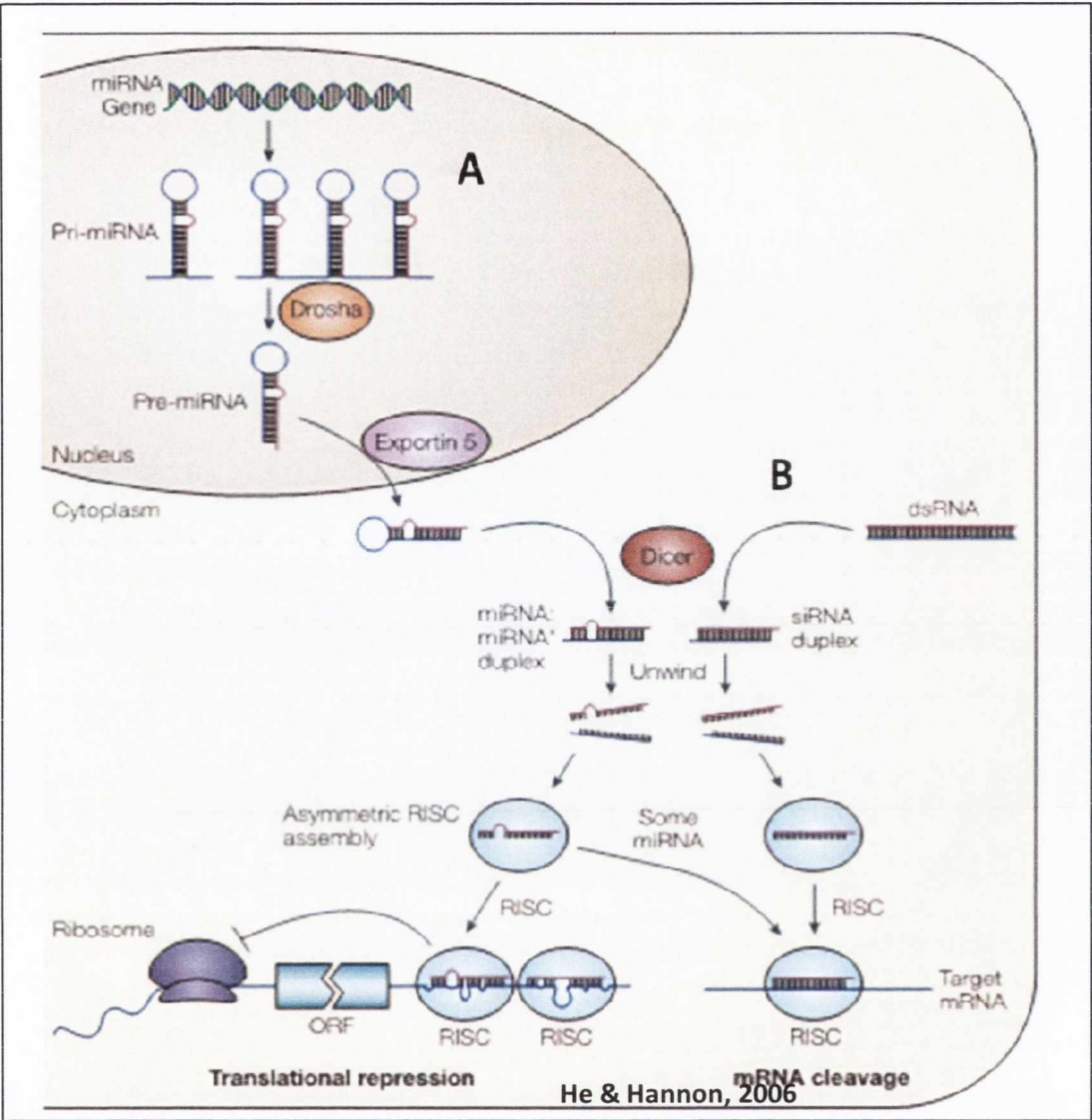


Figure 1.14 The RNA interference pathway

The endogenous miRNA processing pathway (A), miRNAs are transcribed into pri-miRNA and then cleaved by the enzyme Drosha into pre-miRNA, These pre-miRNA (or shRNAs) are than exported from the nucleus by Exportin V where they are cleaved by Dicer into mature dsmiRNA of between 21-23bp in length. It is here that the exogenous siRNAs enter the pathway (B). siRNAs or pre-miRNAs are incorporated into RISC and are guided to target mRNA sequences. Here each target mRNA undergoes AGO-mediated cleavage in the case of siRNAs and some miRNAs, or as is the case with the majority of miRNAs undergo translational repression.

Chapter 2: Materials & Methods

Section 2.1: Materials

Buffers and reagents for general procedures

- Oligonucleotide annealing buffer (Xeragon, pH7.4): 100mM KOAc, 30mM HEPES KOH and 2mM MgOAc
- Tris-acetate-EDTA electrophoresis buffer 10X (TAE, Invitrogen, pH 8.3): 400mM Tris-Acetate and 10mM EDTA (Diluted 1/10 in ddH₂O for a 1X working solution)
- Agarose gel: Agarose powder (Sigma-Aldrich) diluted in the appropriate volume of 1X TAE
- Ethidium Bromide (Sigma-Aldrich): 10mg/ml
- Sybr-Safe (Invitrogen): 10,000X concentrate in DMSO
- Agarose gel loading dyes (Sigma-Aldrich):
 1. Xylene Cyanol (6X)
 2. Bromophenol blue (6X)
- Molecular weight markers: 100bp ladder (New England Biolabs) and 1kb ladder (New England Biolabs) were used as size markers on agarose gels 1µl of marker was diluted in 4µl of 1X TAE with 1µl of loading dye
- Roche enzymes and buffers for cloning (Roche):
 1. Sure Cut enzyme buffer A
 2. Sure Cut enzyme buffer B
 3. Klenow polymerase enzyme 2U/µl
 4. T4 DNA polymerase
 5. T4 Ligase
 6. T4 10X Ligation Buffer pH7 at 20°C: 660mM Tris-HCL, 50mM MgCl₂, 10mM Dithiothreitol, 10mM ATP
- Shrimp Alkaline Phosphatase (SAP, USB Corp)
- SAP 10 X RX Buffer (USB Corp)

- Restriction enzyme buffers (New England Biolabs):

1 X NEBuffer 1	10mM Bis-Tris-Propabe-HCL, 10mM MgCl ₂ and 1mM Dithiothreitol
1X NEBuffer 2 (pH7.9 at 25°C)	50mM NaCl, 10mM Tris-HCl, 10mM MgCl ₂ and 1mM Dithiothreitol
1X NEBuffer 3 (pH7.9 at 25°C)	100mM NaCl, 50mM Tris-HCl, 10mM MgCl ₂ and 1mM Dithiothreitol
1X NEBuffer 4 (pH7.9 at 25°C)	50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate and 1mM Dithiothreitol
Bovine Serum Albumin (BSA [pH7.0 @ 25°C])	BSA, 20mM KPO ₄ , 50mM NaCl, 0.1mM EDTA, 5% Glycerol

- PCR reagents:
 - Crimson *Taq*TM (New England Biolabs)
 - 1X Crimson *Taq*TM reaction PCR buffer pH8.5 at 25°C (New England Biolabs)
12.5mM Tricine, 42.5mM KCl, 1.5mM MgCl₂ and 6% Dextran Red Acid
 - dNTPs (dATP, dTTP, dCTP, dGTP, [Roche])
 - PfuTurbo®DNA polymerase and buffer (Stratagene)
- Buffer-saturated Phenol (pH7.5, Invitrogen)
- Chloroform (Scharlau Chemie)
- Ethanol (Scharlau Chemie)
- Isopropanol (Scharlau Chemie)
- 3M Sodium Acetate (pH5.2 and pH7): 123.045g sodium acetate anhydrous (Sigma-Aldrich) was dissolved in 400ml of ddH₂O. The pH was adjusted to pH5.2 with glacial acetic acid (Sigma-Aldrich)
- Sequencing reagents:
 - BigDye® terminator (Applied Biosystems)X
 - BigDye® sequencing buffer (Applied Biosystems)

3. Formamide (Sigma-Aldrich)

2.1.2 Buffers and reagents for bacterial culturing

- Luria-Bertani (LB) broth: 1% tryptone (Oxoid), 0.5% yeast extract (Oxoid) and 1%NaCl (Sigma-Aldrich) was added to 1 litre of ddH₂O and the LB autoclaved
- LB/Agar: tryptone (1%), 5g yeast extract (0.5%), 10g NaCl (1%) and BBL™Agar Grade A (1.5%) was added to 1 litre of ddH₂O and the LB/agar autoclaved
- LB/Agar plates: 400ml of LB/Agar was melted and cooled to a temperature of approximately 60°C, the appropriate antibody was added and the LB/Agar was subsequently poured immediately into sterile plastic petri dishes and allowed to set
- XL1-BlueMRA Competent Cells (Stratagene)
- XL10-Gold Ultracompetent cells (Agilent Technologies)
- Antibiotics (Sigma-Aldrich):

Antibiotic	Stock Solution	Working Concentration
Ampicillin	20 mg/ml	50µg/ml
Tetracycline	10 mg/ml	25µg/ml
Spectinomycin	10 mg/ml	50µg/ml

0.5M CaCl₂ (Sigma-Aldrich)

- E. Coli Lysis Solution 1: 50mM Tris pH 8 (50mM), 25% sucrose (Sigma-Aldrich) and 2mM EDTA pH8
- Lysozyme (40mg/ml): 40mg Lysozyme (Fluka Biochemika), 250mM Tris pH8
- M-STET: 5% trition X-100 (Sigma-Aldrich), 50mM EDTA pH8. 5% sucrose and 50mM Tris pH8 and was sterilised by autoclaving
- RNase A (1mg/ml, Sigma-Aldrich)

2.1.3 Cell culturing materials and reagents

- Complete DMEM: 10% fetal calf serum (FCS, PAA Laboratories), 2mM L-Glutamine (Cambrex) & 2mM Sodium Pyruvate (Cambrex) in Dulbecco's modified eagle media (DMEM, Cambrex). FCS was first heat inactivated at 50°C for 30 minutes. Complete DMEM was stored at 4°C for up to 1 month

- 1X Sterile Phosphate Buffer Saline (PBS, Cambrex)
- Trypsin-EDTA (Cambrex)
- Trypan-blue (0.4%, Sigma-Aldrich)
- 2X Freezing Media: 25% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich), 25% FCS and 50% complete DMEM
- Lipofectamine[®] 2000 reagent (Invitrogen)
- Opti-MEM (Invitrogen)

2.1.4 RNA materials and reagents

- Nuclease Free water (Ambion)
- RNase Away[®] (Invitrogen)
- Tri-Reagent (Molecular Research Centre)
- β -mercaptoethanol (BDH chemicals LTD)
- RQ1 DNase (Promega)
- RQ1 DNase 10X Buffer (Promega): 400mM Tris-HCl (pH8), 100mM MgSO₄ and 10mM CaCl₂
- RQ1 Stop solution (Promega): 20mM EGTA (pH8)
- Hanks Balanced Salt Solution without Ca, Mg and Phenol red (HBSS [Lonza BioWhittaker[®]])
- DNase I, RNase-Free (lyophilised, Qiagen)
- Buffer RDD (Qiagen)
- QuantiTect SYBR Green reverse transcriptase (RT)-PCR Master Mix (Qiagen- Xeragon): HotStarTaq[®] DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer, dNTP mix, including dUTP, SYBR Green 1, ROX passive reference dye, 5mM MgCl₂
- QuantiTect reverse transcriptase RT) Mix: Omniscript[®] Reverse Transcriptase, Sensiscript[®] Reverse Transcriptase

2.1.5 Protein materials and reagents

- 1% Triton X-100
- 0.5M EDTA (pH8)
- 10X TBS (pH7.5): 30.3g Tris (Sigma-Aldrich), 80g NaCl (Sigma-Aldrich) and 2g KCl (Sigma-Aldrich)
- Roche protease inhibitor mix: 1 complete mini-protease inhibitor cocktail tablet (Roche) in 1.5 ml of ddH₂O

- Protein lysis buffer (6ml): 1% triton X-100, 1mM EDTA (pH8), 10X TBS (pH7.5) and 900µl of Roche protease inhibitor mix in ddH₂O
- 1 X PBS (Sigma-Aldrich): 2 PBS tablets in 400ml of ddH₂O.

2.1.6 Materials and reagents used for western blotting

- 10% Sodium Dioecyl Sulphate pH7.2 (SDS [Sigma-Aldrich])
- 1.5M Tris-HCl pH8.8
- 0.5M Tris-HCl pH6.8
- 10% Ammonium Persulphate (APS [Sigma-Aldrich])
- 5X Lane Marker Reducing Sample Buffer (Thermo Scientific): 0.3M Tris-HCL, 5% SDS, 50% glycerol, 100mM Dithiothreitol (DTT) and proprietary pink tracing dye
- Prestained protein loading marker-broad range (New England Biolabs)
- Resolving gel for SDS-PAGE electrophoresis (12%): 5ml 1.5M Tris-HCl pH8.8, 6.6ml ddH₂O, 8ml bis-acrylamide (30%, Sigma-Aldrich), 200µl 10% SDS, 200µl APS and 20µl of TEMED
- Stacking gel for SDS-Polyacrylamide gel electrophoresis (SDS-PAGE [4%]): 2.5ml 0.5M Tris-HCl pH6.8, 6.8ml ddH₂O, 1.33ml bis-acrylamide (30%, Sigma-Aldrich), 100µl 10% SDS, 100µl APS and 10µl of TEMED (Sigma-Aldrich)
- 10X Running buffer (pH8.6): 30.3g Tris, 144.2g Glycine (Sigma-Aldrich) and 1% SDS
- Transfer buffer: 3.03g Tris, 14.42g Glycine and 20% methanol (Scharlau Chemie)
- Ponceau S (Sigma-Aldrich): 0.1% PonceauS (w/v) in 5% (v/v) acetic acid.
- 5% blocking solution: 5% Marvel original dries skimmed milk in 1X TBS
- Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare)
- Developer (Kodak GBX developer and replenisher (Sigma-Aldrich)
- Fixer (Kodak GBX fixer and replenisher (Sigma-Aldrich)
- Restore™ Western Blotting Stripping Buffer (Thermo Scientific)

2.1.7 AAV production materials:

- Plasmids:
 1. pAAV-MCS (Stratagene)
 2. pRep2/Cap521 (Hildinger et al, 2001)
 3. pHelper (Stratagene)

- HEK293 cells were obtained from ATCC (accession number CRL-1573).
- 1 M NaCl (pH5):
- Polyethylenimine (Sigma-Aldrich): 0.1g/L 150mM NaCl, pH5
- Deoxycholic acid (Sigma-Aldrich)
- Bensonaze (Sigma-Aldrich)
- Polyethylene glycol (PEG8000, Sigma-Aldrich)
- Cesium Chloride (Sigma-Aldrich)
- Slide-a-lisers (MSC/ Fischer)

2.1.8 Animal materials

- Strains used:
 1. Wild-type 129/Sv mice (Charles River Laboratories [129])
 2. Normal Human Rhodopsin (NHR) mice (Ollson et al, 1992)
 3. 129 rhodopsin knockout (129rho^{-/-}) mice (Humphries et al, 1997)
 4. RHO-M (RHO-M^{+/-}rho^{-/-}) mice (O'Reilly et al, 2008)
 5. Pig P347S rhodopsin (PP347S) mice (kindly provided by Prof. Tiansen Li; Wong & Hao, 1992)
 6. Human P347S rhodopsin mice (Li et al, 1996)

2.1.9 Genotyping

- Tissue Lysis Buffer: 50mM Tris pH8, 100mM EDTA pH8, 100mM NaCl and 10% SDS
- Proteinase K: proteinase K (10mg/ml) was used to digest tissue samples (Sigma-Aldrich)
- Glycogen (Sigma-Aldrich)

2.1.10 Anaesthetic and reagents used for animal procedures

- Anaesthetic for subretinal injections: Medetomidine (10mg/10g body weight) and Ketamine (750mg/10g body weight) was administered via intraperitoneal (i.p) injection
- Anaesthetic for ERGs: Ketamine (16mg/10g body weight)
- and Xylazine (1.6mg/10g body weight)
- For dilating eyes: 1% Cyclopentolate and 2.5% Phenylephrine was administered
- Analgesia: 1% Amethocaine and Vidisic gel
- Reversal agent: Atipamezole Hydrochloride was administered at a dose of 100mg/10g body weight via i.p injection

2.1.11 Materials and reagents used for histology and immunohistochemistry

- 4% Paraformaldehyde (PFA [Sigma-Aldrich]): 4% PFA was dissolved in IL 1X PBS by heating at 64°C on a hot stir plate. The pH 7.4 was obtained using NaOH and HCl.
- 10, 20, 30% sucrose (Sigma-Aldrich) was made by in 1X PBS
- Optimal cutting temperature compound (OCT compound, Lennox Laboratory Supplies)
- Polylysine® (PLL) slides (Sigma-Aldrich)
- Donkey Serum (Sigma-Aldrich)
- 10% Triton-PBS solution: 10% Triton X 100 in 1X PBS
- Blocking solution: 10% donkey serum, 0.3% Triton-PBS in 1X PBS
- Antibody solution: 2% donkey serum, 0.1% Triton-PBS in 1X PBS
- 4',6-diamidine-2-phenylindole-dihydrochloride (DAPI, Sigma-Aldrich): 1/10,000 dilution in 1X PB
- Aqueous mounting media (Aqua PolyMount, Polysciences)

2.1.12 Antibodies

- Anti-mouse rhodopsin primary antibody (4D2) obtained from Prof. RS Molday (Hicks & Molday, 1986)
- Anti-rabbit polyclonal β -actin (Abcam)
- Anti-mouse secondary IgG (Fc-specific) peroxidase conjugate (Sigma-Aldrich)
- Anti-mouse light chain only secondary antibody (Millipore)
- Anti-rabbit secondary antibody (Abcam)
- Anti-mouse Lectin PNA antibody conjugated to Alexa Fluor 568 (Molecular Probes)

2.1.13 Fluorescent Activated Cell sorting (FACs)

- HBSS
- Trypsin-EDTA 10mg/ml (Sigma-Aldrich)
- Trypsin Inhibitor 20mg/ml (Roche)
- DNase I 10ug/ μ l in DNase buffer (Sigma-Aldrich)
- Neural tissue dissociation kit (Macs Miltenyi Biotech):

- I. Solution 1
- II. Solution 2
- III. Solution 3
- IV. Solution 4
- V. Enzyme mix 1: Solution 1 (50µl) + solution 2 (1900µl)
- VI. Enzyme mix 2: (Solution 3 (20µl) + solution 4 (10µl))

- Propidium Iodide 1mg/ml (Sigma-Aldrich)
- DRAQ5 1mg/ml (Biostatus)

Section 2.2: General cloning techniques

2.2.1 DNA/RNA spectrophotometry

DNA and RNA concentration was calculated from absorbance at a wavelength (λ) of 260nm (A₂₆₀) using the NanoDropTM1000 spectrophotometer (Thermo Scientific). The Beer-Lambert Law states that the concentration of double stranded DNA and RNA is directly proportional to their absorption of light at 260nm. This equation is based on the different extinction coefficients of each nucleic acids (DNA=50 μ g/OD₂₆₀, RNA=40 μ g/OD₂₆₀). 2 μ l of nuclease free water was added to the optical surface of the nanodrop in order to obtain a blank measurement. The software was either set to DNA50 or RNA40 depending on the nucleic acid being measured. 2 μ l of each sample was loaded onto the optical surface and a concentration for each was calculated. Purity of nucleic acids was measured by examining A₂₆₀/A₂₈₀ ratios measured on the nanodrop.

2.2.2 Agarose gel electrophoresis

Agarose gels were made by adding the appropriate amount of agarose extract to 1X TAE buffer and heating in a microwave oven until all the agarose had melted. Depending on the type of gel needed either 2 μ l of SyberSafe or 1 μ l EtBr was added to 30ml of melted agarose. This was poured into a gel cast (Bio-Rad) with a comb and the gel was left to set for at least twenty minutes. Loading dye was added to each DNA sample at a dilution of 1 in 6 and each sample was loaded onto the gel along with a DNA size ladder. The gel was electrophoresed at a constant voltage of 100V and DNA was visualised on a UV transilluminator (Vilber Lourmat). Photographs were taken using a Mini Bis PRO (DNR, Bio-Imaging Systems) with Gel capture software (DNR, Bio-Imaging Systems).

2.2.3 Restriction enzyme digestion

Restriction enzyme digestion of DNA was performed as follows: A 1X concentration of the recommended enzyme buffer and bovine serum albumin (BSA) was added to each reaction. The amount of units of restriction enzyme was recommended by the manufactures. Reactions were typically performed in a 50 μ l volume in ddH₂O. Unless otherwise stated all reactions were carried out for 2 hours at 37°C. The enzyme activity was inactivated by heating to 65°C for 20 minutes, by a reaction enzyme clean up kit (**Section 2.2.4**) or agarose gel extraction (**Section 2.2.5**).

2.2.4 Enzyme reaction clean up (Qiagen)

Purification of PCR products and restriction digests was carried out using the QIAquick PCR purification kit (Qiagen). A 5:1 volume of buffer PB to DNA was added and this was transferred to a QIAquick spin column. The remainder of the protocol was carried out according to the manufacturer's instructions. DNA was eluted in 50µl of ddH₂O.

2.2.5 Agarose gel extraction (Qiagen)

DNA was extracted from 1% agarose gels using the QIAquick gel extraction kit (Qiagen). DNA on agarose gels were visualised under the UV transilluminator (Vilber Lourmat) and using a sterile blade, gel sections containing DNA of interest was excised. The gel slice was weighed and 3 volumes of Buffer QG was added per 100mg of gel. The remainder of the protocol was carried out according to the manufacturer's instructions. DNA was eluted in 50µl of ddH₂O.

2.2.6 Shrimp Alkaline Phosphatase (SAP) treatment

5µl of 10X SAP and 5µl SAP buffer was added to linearised vector solution in a 50µl final volume with ddH₂O. The reaction was incubated for 2 hours at 37°C and subsequently heat inactivated at 65°C for 20 minutes in a Thermocycler (Biometa T300 Thermocyclers, Biosciences).

2.2.7 Filling in 5' DNA overhangs (Klenow polymerase reaction)

Klenow polymerase was used to fill in 5'DNA overhangs. 2µl of Klenow polymerase, 2µl dNTPs (2mM) and 6µl buffer A was added to 1µg digested DNA in a final volume of 60µl. This reaction was incubated at 37°C for 30 minutes.

2.2.8 Filling in 3' DNA overhangs (T4 DNA polymerase reaction)

The T4 DNA polymerase was used to fill in 3'DNA overhangs. 2µl T4 DNA polymerase, 4µl buffer A, 2µl dNTPs (2mM) and 1µg DNA in a 100µl reaction volume was incubated at 12°C for 10 minutes and heat inactivated at 75°C for 10 minutes.

2.2.9 Phenol-chloroform extraction

Buffer-saturated phenol was added to an equal volume of DNA solution, the sample was vortexed and centrifuged at 13,000rpm (IEC Micromax Microcentrifuge, Thermo-Electron) for 1 minute. The top aqueous layer (containing the DNA) was taken and again an equal amount of phenol was added,

vortexed and centrifuged at 13,000rpm for 1 minute. The upper aqueous layer was removed and mixed with a 1:1 mixture of phenol chloroform, was vortexed and centrifuged as above. Again the top phase was taken, mixed with an equal volume of chloroform, vortexed and centrifuged at 13,000rpm. The upper aqueous phase containing the DNA was removed and the DNA was precipitated with by ethanol precipitation (see below 2.2.10).

2.2.10 Ethanol precipitation

250% of ethanol and 10% 3M NaAc pH5.4 were added to the DNA solution. The sample was vortexed and incubated at -20°C for 30 minutes. This was centrifuged at 13,000rpm (IEC Micromax Microcentrifuge, Thermo-Electron) for 20 minutes and the supernatant was carefully removed. The pellet was washed with 70% ethanol, centrifuged for 5 minutes, the supernatant was removed and the DNA pellet was air dried. DNA was resuspended in 50µl of ddH₂O.

2.2.11 Ligations

Ligations were carried out in 10µl reaction volumes. The amount of vector and insert DNA was estimated by visualisation (of various concentrations of vector and insert) on a 1% agarose gel. Vector: insert ratios of approximately 1:3 and 1:10 were typically used in ligation reactions. 1µl of T4 DNA ligase and 1µl of 10X DNA ligase buffer was added to the appropriate amount of vector and insert. The reaction volume was made to 10µl with ddH₂O. Ligation controls were included with all cloning reactions, typically this involved two controls; the first was a ligase control containing digested vector and ligase and the second was a vector digestion control containing digested vector.

2.2.12 XL1-blueMRA competent cells

A colony of XL1- blueMRA *E.coli* was streaked onto an LB/agar plate containing tetracycline. This was incubated overnight at 37°C. Using a toothpick a single colony from this plate was used to inoculate 10ml LB broth with tetracycline, which was and incubated overnight in an orbital incubator, shaking at 220 rpm (Innova 4300) at 37°C. 250µl of the overnight culture was added to a conical flask containing 25ml of LB broth and tetracycline which was grown in an orbital incubator for approximately 2 hours until the bacterial cells were in their early exponential growth phase (OD₆₀₀ of 0.3 to 0.5). Cells were centrifuged at 3,000rpm (Centrifuge 5810, Eppendorf) for 5 minutes and the supernatant was removed. 25ml of ice cold, sterile 50mM CaCl₂ was added to the cells and the pellet was gently resuspended. The cell suspension was left on ice for 15 minutes and centrifuged at

3,000rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in 5ml ice cold, sterile 50mM CaCl₂. The competent cells were stored on ice and were used within 2 days.

2.2.13 Transformation using XL1-BlueMRA competent cells

5µl of ligated DNA was added to 200µl of XL1-BlueMRA competent cells and placed on ice for 20 minutes. Cells were heat shocked at 42°C for 5 minutes in a water bath. 1 ml of LB was added to each tube and this was incubated at 37°C for 1 hour. 200µl of each sample was spread on an LB/agar plate and left to dry. Each LB/Agar plate contained the appropriate antibiotic for selecting transformed bacterial colonies (unless otherwise stated the antibiotics used were Ampicillin and Tetracycline). Each plate was incubated at 37°C overnight. Transformation controls were also included in this experiment. A circular plasmid was transformed to confirm the transformation process was efficient. In addition non-transformed competent cells were plated, to ensure the competent cells and the LB/agar plates were not contaminated.

2.2.14 Transformation using XL10-Gold®Ultracompetent cells

XL10-Gold®Ultracompetant cells (Stratagene) were stored at -80°C until needed. 6µl of β-mercaptoethanol (Stratagene) were added to each 150µl vial of cells which were placed on ice for 10 minutes, gently tapping the eppendorf tubes at two minute intervals. 50µl of cells were transferred to universal tubes and the DNA to be transformed was added. Each tube was incubated on ice for 30 minutes and then heat shocked at 42°C for 30 seconds. The samples were returned to ice for a further 2 minutes. 900µl of warm LB was added to each tube which were incubated for 60 minutes at 37°C in an orbital incubator, shaking at 110rpm (Innova 4300). 10µl of each transformation mixture were spread on an LB/agar plate supplemented with appropriate antibiotics which were incubated overnight at 37°C.

2.2.15 DNA mini-preparation (boiling method)

10ml LB broth containing the appropriate selection antibiotic were inoculated with each transformation colony and grown overnight at 37°C in the orbital incubator. The overnight cultures were centrifuged for 10 minutes at 3,000rpm (Centrifuge 5810, Eppendorf) and the supernatant was removed. Pellets were resuspended in 60µl of lysis solution 1 by pipetting up and down; and this suspension was transferred to an eppendorf. 20µl of lysozyme (10mg/ml) was added and mixed by pipetting. Samples were left at room temperature for 10 minutes. 550µl of MSTET solution was subsequently added to each sample, mixed by pipetting and the samples incubated for a further 10 minutes. Samples were

boiled for 90 seconds and centrifuged at 13,000rpm (IEC Micromax Microcentrifuge, Thermo-Electron) for 15 minutes. Bacterial DNA pellets were discarded using a toothpick; 1µl of RNase A was added to each tube and samples were incubated at 37°C for 15 minutes. A phenol extraction was then carried out; 500µl of buffer-saturated phenol were added to each tube and all samples were vortexed. Tubes were centrifuged at 13,000rpm for 5 minutes. The top organic layer was transferred to a fresh eppendorf (approximately 400µl) and 240µl of isopropanol (0.6 of the total volume) were added. Samples were vortexed for a few seconds. These were then incubated for 15 minutes at room temperature and subsequently centrifuged at 13,000rpm for 15 minutes. The supernatant was removed. DNA pellets were washed with 70% ethanol. Pellets in ethanol were left to stand for ten minutes and the ethanol was then discarded. Samples were pulse spun and the remainder of the ethanol was removed using a pipette. Pellets were left to air dry for 30 minutes and resuspended in 100µl of ddH₂O. DNA was stored at -20°C.

2.2.16 DNA mini-preparation (Qiagen kit)

DNA mini-preparation was also carried out using the QIAprep® miniprep kit (Qiagen). Cells from single colonies were grown in 5ml of LB supplemented with appropriate antibiotics overnight at 37°C in an orbital incubator (Innova 4300). The overnight culture was centrifuged at 3,000rpm (Centrifuge 5810, Eppendorf) for 10 minutes and the supernatant was removed. A mini-preparation was carried out on the pellet according to the QIAprep® miniprep handbook (Qiagen). DNA was resuspended in 50µl of ddH₂O and stored at -20° C.

2.2.17 DNA maxi-preparation (Qiagen kit)

DNA maxi-preparation (to get high quality DNA of up to 1mg) was carried out using the HiSpeed® Plasmid Midi and Maxi kit (Qiagen). 5ml of LB broth with the appropriate antibiotic was inoculated with the colony to be prepared. This starter culture was grown for 8 hours at 37°C in an orbital incubator, shaking at 220rpm (Innova 4300). Following this, 250µl (1/1000 dilution) of this starter culture was used to inoculate 250 ml LB broth containing the appropriate antibiotic. Cultures were grown at 37°C overnight, shaking at 220rpm in an orbital incubator. The next day the cultures were centrifuged at 3000rpm for 15 minutes (Centrifuge 5810, Eppendorf) and the supernatant was removed. The maxi-preparation was carried out on bacterial pellets according to the HiSpeed® Plasmid Purification handbook. Pellets were re-suspended in 1ml ddH₂O and stored at -20°C.

2.2.18 DNA mega-preparation (Qiagen kit)

DNA mega-preparation (to get high quality DNA of up to 2.5mg) was carried out using the Qiafilter™ Plasmid Megakit (Qiagen). 2ml of LB broth with the appropriate antibiotic was inoculated with cells from the colony to be prepared. This starter culture was grown for 8 hours at 37°C in an orbital incubator, shaking at 220rpm (Innova 4300). Following this, the 2 ml starter culture (1/300 dilution) was used to inoculate into 600ml LB broth (in a 2000ml conical flask) containing the appropriate antibiotic. Cultures were grown at 37°C overnight, shaking at 220rpm in an orbital shaker. The next day the bacteria were harvested by centrifugation at 9000rpm (Sorvall RC-5B refrigerated superspeed centrifuge-rotor GSA). The mega-preparation was carried out on bacterial pellets according to the Qiafilter™ Plasmid Purification handbook. Following step 16 (the washing of the DNA pellet with 70% ethanol), the remainder of the protocol was carried out in a laminar flow cabinet (Holten LaminAir Model 1.2, Thermo Electron Corporation), in order to prevent contamination of the DNA. The pellet was air dried (for approximately 30 minutes) and resuspended in 1.5ml ddH₂O.

2.2.19 Polymerase chain reaction (PCR)

All PCRs were carried out in a final volume of 20µl containing 250ng DNA template, 0.5pmoles of forward and reverse primers, 4mM dATP, dTTP, dCTP and dGTP, 4µl 5X Crimson *Taq*™ reaction PCR buffer and 1 unit of Crimson *Taq*™ DNA polymerase. Samples were vortexed and placed on a thermocycler (Biometra T300 Thermocycler, Biosciences) typically programmed with a standard PCR protocol: 94°C for 5 minutes, 35cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.2 minutes. Finally samples were incubated at 72°C for 10 minutes.

2.2.20 Sequencing (automated capillary method)

DNA samples to be sequenced were amplified using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). PCR reaction mixtures contained: 500ng of plasmid DNA, 2.5pmoles of primer, 2µl of BigDye® terminator and 2µl of the 5X BigDye® sequencing buffer. PCRS were performed in 10µl volumes. PCR reactions were performed using a thermocycler (Biometra T300 Thermocycler, Biosciences) as follows: 96°C for 2 minutes, 26 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes and finally 15 minutes at 72°C. DNA was subsequently precipitated by the addition of 62.5µl of 96% ethanol, 3µl 3M sodium acetate (pH5.2) and 24.5µl ddH₂O. Samples were left at room temperature for 20 minutes and centrifuged for an additional 20 minutes at 13,200rpm (IEC Micromax Microcentrifuge, Thermo Scientific). The supernatant was removed and the pellet was washed with 250µl of 70% ethanol. The samples were centrifuged at

13,200rpm for 10 minutes, the ethanol was removed and the pellet was air dried. Each pellet was resuspended in 25µl formamide and transferred to sequencing tubes. The samples were denatured at 95°C for 5 minutes and immediately transferred to ice. Sequencing was performed using an ABI 373A DNA sequencing machine as per the manufacturer's protocol by Alex McKee. Sequencing was analysed using ABI data collection and analysis software.

2.2.21 Sequencing (GATC or Source Bioscience LifeSciences)

Sequencing reactions were outsourced to GATC Biotech or Source Bioscience LifeSciences. Plasmid DNA samples were sent at a concentration of approximately 30-100ng/µl along with 3.2 pmoles/µl of the sequencing primer. Sequencing was performed on a 7x ABI 3730xl sequencer. Results were viewed using GATC viewer v1.00 (GATC Biotech).

2.3 Cell culturing procedures

2.3.1 Cell growth

HeLa cells (America type culture collection [ATCC] no. CCL-2) were maintained in complete DMEM and grown at 37°C in a humidified Steri Cycle CO₂ incubator (Hepa Class 100, Thermo Scientific) in the presence of 5% CO₂. All cell culture procedures were carried out in a laminar flow cabinet (Holten LaminAir Model 1.2, Thermo Electron Corporation). Cells were visualised using a microscope (Olympus CK30).

2.3.2 Cell passaging

Cells were passaged routinely on average every 2-3 days. Complete DMEM was removed from cells. Cells were then washed with PBS and removed from tissue culture dishes by incubation with 1-2ml of Trypsin-EDTA at 37°C for 5 minutes. Once the cells had been removed from the tissue culture plates a 5-fold excess of complete DMEM was added to the cell suspension in order to inactivate the Trypsin. 1/6 of the total cell suspension was then added to new 10cm tissue culture dishes and grown in complete DMEM.

2.3.3 Cell counting using a Haemocytometer

Cells were prepared for counting by trypsinising, adding a 5-fold excess of complete DMEM and centrifuging at 1000rpm for 10 minutes (IEC-Centra-3C centrifuge). The resulting cell pellet was resuspended in 10ml complete DMEM. A 1:1 dilution of cell suspension with 0.4% trypan blue in was

performed and cells were counted using a Haemocytometer. The Haemocytometer was washed with 70% ethanol and dried. A clean coverslip was placed over the counting area and the cell suspension was applied beneath the coverslip by capillary action. The number of cells per grid of 16 squares was counted. Cells were counted in 8 different grids and the average taken. In order to obtain the number of cells per ml this average was multiplied by 2×10^4 .

2.3.4 Freezing cells

Cells were frozen at low passage numbers in order to maintain stocks. 500 μ l of cells were placed in a Nunc Cryo TubeTM (Nunc) along with 500 μ l of 2X freezing media. These were placed in a prechilled cryo freezing container (Nalgene) and frozen at -80°C.

2.3.5 General co-transfection protocol using Lipofectamine® 2000

HeLa cells were seeded in complete DMEM at a density of 2×10^5 on 24 well plates and transfected when they were approximately 90-95% confluent using Lipofectamine® 2000 reagent. Each siRNA was co-transfected with a rhodopsin expression plasmid into HeLa cells. All rhodopsin expression plasmids expressed rhodopsin cDNA from a CMV promoter. A non-silencing negative control siNT (5'-UUCUCCGAACGUGUCACGU-3', [Qiagen]) and a positive control siBB targeting nucleotides 254-274 of hRHO (5'-TCAACTTCCTCACGCTCTACGT-3, [Proligo]) were used in all co-transfection experiments (O'Reilly et al, 2007). The optimal amounts of each siRNA (usually 16picomoles) and rhodopsin plasmid DNA (usually 400ng) were first diluted with Opti-MEM. Lipofectamine® 2000 was also diluted 1/25 with Opti-MEM and incubated for 5 minutes at room temperature. Once the incubation period was complete the DNA/RNA solution was mixed with the Lipofectamine® 2000 solution and liposomal complexes were allowed to form for 25 minutes. 100 μ l of this co-transfection complex was subsequently added to each well of HeLa cells, mixed well with the culture medium and incubated. RNA and protein were extracted for analysis 24 hours post-transfection. Transfections were typically performed in either triplicate or quadruplicate.

2.4 RNA Methods

2.4.1 Ribonuclease (RNase) free conditions

In order to prevent RNA degradation by RNases certain precautions were taken. Nuclease-free eppendorf tubes (Axygen Scientific Inc.), nuclease-free filtered pipette tips (Axygen Scientific Inc.) and nuclease-free water (Ambion) were used at all times. RNA samples were kept on ice during experiments. The benches were wiped with 70% ethanol and RNase AWAY® (Invitrogen).

2.4.2 RNA isolation from HeLa cells (Using Tri-reagent)

RNA was extracted from cells 24 hours post-transfection. The medium was removed from each well and the cells were washed gently with 500µl PBS. Cells were lysed by pipetting 250µl of Tri-reagent into each well of a 24 well plate and mixing gently. The plate was incubated at room temperature (RT) for 5 minutes. Quadruplicate transfection samples were pooled and 200µl of chloroform was added. Each tube was shaken vigorously for 2 minutes and left for 2-3 minutes at RT. The samples were centrifuged at 12,000rpm for 15 minutes at 4°C (Beckman GS-6 centrifuge). 300µl of the upper phase of each sample was transferred to a new eppendorf tube. RNA was precipitated with 200µl of isopropanol at RT for 10 minutes. Tubes were subsequently centrifuged at 4°C for 10 minutes at 12,000rpm. The supernatant was removed and RNA pellets were washed with 75% ethanol. Tubes were centrifuged for 5 minutes at 12,000rpm at 4°C. RNA pellets were first air dried then resuspended in 50µl nuclease-free H₂O. RNA samples were stored at -20°C.

2.4.3 RNA isolation from HeLa cells (RNeasy® Plus mini kit)

RNA was isolated using the RNeasy®Plus mini-kit (Qiagen). 87.5µl of Buffer RLT plus (0.88µl β-mercaptoethanol) was added to each well and quadruplicate transfection samples were pooled. The pooled cell lysates were then homogenised by placing them onto a QIAshredder spin column placed in a 2ml collection tube. Samples were centrifuged at 13,000rpm (IEC Micromax Microcentrifuge, Thermo-Electron) for 2 minutes. The remainder of the isolation was carried out according to the manufacturer's instructions. Samples were resuspended in 50µl of nuclease-free water.

2.4.4 DNase treatment of HeLa cell RNA

In order to remove contaminating DNA from HeLa cell RNA samples, 5µl of RQ1 DNase and 6µl RQ1 DNase Buffer was added to each sample. Samples were incubated at 37°C for 1 hour and the DNase was deactivated by adding 6.5µl of RQ1 stop solution and by heating samples to 80°C for 10 minutes. Each RNA sample was frozen and stored at -20°C.

2.4.5 RNA isolation from mouse retina (RNeasy mini kit)

Unless otherwise stated all *in vivo* RNA isolations were carried out using the RNeasy mini kit (Qiagen). Retinas were extracted from mice and immediately washed in 1 ml ice cold HBSS in a 24 well plate. The lens and surrounding RPE was removed from the retina, and each retina was placed into 350µl buffer RLT (containing 3.5µl β-mercaptoethanol). The retina was homogenised by pipetting

the sample up and down a number of times. Retinal RNA extraction following addition of buffer RLT was carried according to the manufacturer's protocol. RNA was eluted in 50µl nuclease free H₂O.

2.4.6 DNase treatment of mouse retinal RNA (on column digestion)

During the above protocol (using the RNeasy mini kit), an on column DNase digestion step was performed. The RNeasy spin column was washed with 350µl Buffer RW1 and centrifuged for 15 seconds at 10000rpm (IEC Micromax Microcentrifuge, Thermo-Electron), the flow through was discarded. Following this, 80µl of DNase 1 in Buffer RDD (10µl DNase 1 + 70µl Buffer RDD) was added to the centre of each column and incubated for 2 hours at 37°C. The column was then briefly centrifuged to remove the DNase in RDD Buffer mixture and subsequently washed with 350µl of Buffer RW1. The sample was centrifuged at 10000rpm for 15 seconds and the flow through discarded.

2.4.7 General One Step Real- Time RT-PCR protocol

Real-time reverse transcriptase-PCR (RT-PCR) was used to measured levels of RNA. In all one-step real-time RT-PCR experiments in this thesis, a relative standard curve method for quantification was used. This method determines the ratio between the amount of rhodopsin RNA and an internal control housekeeping gene (Applied Biosystems Real-Time PCR systems chemistry guide, 2005). The mRNA transcript of a housekeeping gene is ubiquitously expressed, therefore by comparing the ratio of rhodopsin RNA expression and the housekeeper RNA levels (either β-actin RNA or 18S rRNA), expression of rhodopsin across different samples can accurately be compared.

A one step real-time RT-PCR kit, the QuantiTect®SYBR® Green RT-PCR kit (Qiagen-Xerogon) was used in all one-step RT-PCR reactions which were carried out on an Applied Biosystems 7300 Real-Time PCR system. This system uses the Sybr green 1 fluorescent dye. Sybr green 1 binds to double stranded DNA and fluoresces. During the PCR reaction the more DNA that has been amplified, the more Sybr Green 1 will bind to it and the higher the fluorescent intensity (Applied Biosystems Real-Time PCR systems chemistry guide, 2005). The 7300 real-time RT-PCR machine measures the fluorescence after each PCR cycle and records this.

Each reaction was carried out in a final volume of 20µl containing 10µl of the supplied Sybr Green master mix, 0.147µl of the RT Mix (containing reverse transcriptase enzymes), 10 pmoles of the forward and reverse primer, 4.35µl Nuclease-free H₂O and 4.5µl of each RNA sample. These solutions were kept on ice at all times. Each reaction was performed in triplicate in a MicroAMP™Optical 96-

well reaction plate (Applied Biosystems). A negative control (water blank) to which no RNA was added was included. The real-time RT-PCR program was as follows:

- **Reverse transcription:** 50°C for 20 minutes
- **Activation of DNA polymerase:** 95°C for 15 minutes.
- **cDNA amplification:** 37 cycles at 95°C for 15 seconds, 60°C for 1 minute.
- **Dissociation:** 95°C for 15 seconds, 60°C for 30 seconds and 95°C for 15 seconds.

Analysis was carried out using sequence detection software v1.4 (Applied Biosystems). Initially in order to verify the presence of each RT-PCR product a dissociation curve analysis was performed. Each product has a particular melting temperature (T_m); therefore examining the temperature at which the PCR product DNA dissociates (melts) helps to confirm that the correct product of the correct size is present. Dissociation curves also help identify contaminations in water blanks and primer dimers (recognised by dissociation peaks with low T_m , typically in the region of 70-75°C). It was always critical to check for the presence of these peaks after every real-time RT-PCR.

In order to measure relative gene expression levels (typically rhodopsin) in samples and to compare levels in different samples, expression levels were normalised using a housekeeping gene (typically β -actin or 18S RNA). Hence two standard curves were generated using rhodopsin and housekeeping gene primers and relative gene expression levels of both genes determined in each sample. Each standard curve was analysed using the auto analysis function in the software (auto CT). For both the rhodopsin and the housekeeping standard curves a line of best fit was generated based on the known dilution factors of the standards. This compares cycle no. to the log of the dilution value. The slope and R^2 value of each standard curve was checked to make sure the assay was efficient (the slope should be -3.33 for 100% efficiency and the R^2 value should be as close to 1 as possible). Each sample to be tested was then compared to this line and a value of relative expression for each was determined. This was automatically done with the software package. The quantity value for each sample was taken for use in the relative quantification calculation. For each sample, the relative rhodopsin quantity was normalised to the relative quantity of the house keeping in the same sample.

2.5 Protein techniques

2.5.1 Protein extraction from HeLa cells

Protein was extracted from HeLa cells by adding 150-200µl of protein lysis buffer to each well containing cells (work was done on ice at all times). The cells were dislodged from the base of each well using a plastic cell scraper, quadruplicate wells were pooled and each cell solution was transferred to an eppendorf. These were stirred for 1 hour at 4°C using small magnetic stir-bars. The contents of each eppendorf was transferred to a fresh eppendorf tube and centrifuged at 12,000rpm for 30 minutes at 4°C (Beckman GS-6 centrifuge). The supernatant containing the protein was retained and frozen at -20°C.

2.5.2 Protein extraction from mouse retina

Retinas were extracted from mice eyes and immediately placed in ice cold PBS in a 24 well plate. The lens and RPE was removed from the retina and each retina was added to 200µl of protein lysis buffer. Retinas were homogenised by pipetting up and down a number of times with a P1000 pipette tip. In the case of retinal samples that were particularly viscous, the extract was sonicated (Labsonic U, B. Braun Biotech International). Samples were stirred on ice for 1 hour at 4°C using small magnetic stir-bars. The contents of each eppendorf was transferred to a fresh eppendorf tube and centrifuged at 12,000rpm for 30 minutes at 4°C (Beckman GS-6 centrifuge). The supernatant containing the protein was retained and frozen at -20°C.

2.5.3 BCA protein assay

The protein concentration of each sample was quantified using the Pierce BCA protein assay kit (Pierce). This method uses bichinochonic acid (BCA) for the colourmetric detection of protein. In alkaline medium Cu^{2+} is reduced to Cu^{1+} by protein. When one molecule of Cu^{1+} is present it chelates with two molecules of BCA to give a purple coloured product. This absorbs light of 562 nm. The more protein is present (20-2000µg/ml), the more molecules of Cu^{1+} are generated, the more intense the purple colour and the higher the absorbance. In this kit BSA (Pierce) of known concentration (2mg/ml) was used to generate the following standards curve; 0mg/ml, 0.25mg/ml, 0.75mg/ml, 1.0mg/ml, 1.0mg/ml, 1.5mg/ml and 2 mg/ml. Protein samples were diluted 1:5 with protein lysis buffer. 10µl of each BSA standard, protein dilution and lysis buffer blank control were loaded in triplicate onto a 96 well reaction plate (Nunc™). The BCA reagent was prepared [50 parts BCA A (alkaline solution containing BCA):1 part BCA B (4% cupric sulphate)]. 200µl of this solution was added to each well.

This was covered in tinfoil and incubated at 37°C for 30 minutes. Absorbance was read on a plate reader at 595nm (Rosys Anthos). The absorbance values of each standard (y-axis) were plotted against the known concentration of each standard (x-axis). A trend-line was generated and the slope of this line calculated. The concentration of protein present in each unknown protein sample was calculated using the equation: $y=mx+c$, where $y=OD$, m =slope of the line, x = concentration of protein and c is the intercept (0).

2.6 Western Blot Protocol

2.6.1 Preparation of SDS PAGE gels

SDS-PAGE gels were cast using the AE-6450 Dual Mini Slab Kit (ATTO Bioscience and Biotechnology Corporation). All gel cast was assembled according to the manufacturer's instructions. A 12% resolving polyacrylamide gel was made and approximately 8ml of this was poured into the gel cast leaving a space at the top for the Teflon comb. The resolving gel was left to polymerise for 30 minutes and then the ddH₂O overlay was removed. A 4% stacking polyacrylamide gel was prepared and poured on top of the resolving gel and a Teflon comb was added to the stacking gel. This was left to set for 15 minutes.

The gel electrophoresis apparatus was assembled according to the manufacturer. Following quantification with a BCA assay, 40µl of each protein sample was added to 10µl Lane Marker Sample Buffer (1/5 dilution). Samples were denatured by heating to 65°C for 30 minutes and loaded directly onto the western gel. A broad range prestained protein marker was heated for 5 minutes and 10µl was added to the outer most lane of the gel. The SDS-PAGE electrophoresis was carried out at 30mV per gel for 90 minutes (Consort EV261).

2.6.2 Semi-dry transfer

The semi dry transfer rig (AE-6688 HorizBlot) was assembled according to the operating instructions (ATTO Bioscience and Biotechnology Corporation) with the cathode locked at the highest position. 4 pieces of filter paper were soaked in transfer buffer and stacked one after the other onto the anode of the HorizBlot apparatus. Transfer membrane (Nitrocellulose, Protran BA 83, Whatman or PVDF, Immobilon™) was placed on the filter paper. In the case of PVDF, the membrane was first activated with methanol then, washed with ddH₂O and transfer buffer. The membrane was gently placed onto the stacked filter paper and all bubbles removed. The resolving gel (washed in transfer buffer) was gently placed onto transfer membrane. 4 pieces of filter paper were soaked in transfer buffer and placed on

top of the gel. All bubbles were removed. The transfer rig was closed and the cathode gently lowered until it pressed down on the stacked materials. The cathode was then locked. The transfer was carried out at 80mV for 2 hours (Consort EV261).

2.6.3 Ponceau S Staining

Following electroblotting, the membrane was stained with Ponceau S protein stain (Sigma Aldrich), in order to ensure that protein bands had been transferred correctly. Once the presence of protein has been verified, the membrane was washed four times for 15 minutes (4 X 15 minutes) with 1X TBS on an orbital shaker (Bellco Biotechnology).

2.6.4 Blocking of immunoblots

The membrane was blocked with 30ml of 5% blocking solution for 1 hour at room temperature on an orbital shaker. The blocking solution should bind to all non-specific protein binding sites on the membrane, and ensured that non-specific binding of the primary antibody does not occur.

2.6.5 Antibody probing of blots

The membrane was probed with 10ml of the appropriate dilution of primary antibody (diluted in 5% blocking solution) specific for the protein to be examined. The membrane was covered in tinfoil to prevent evaporation and subsequently incubated overnight at 4°C.

The primary antibody was removed and the membrane was washed 4 X 15 minutes with 1X TBS on an orbital shaker (Bellco Biotechnology). The blot was probed with the appropriate dilution (in 5% blocking solution) of the corresponding secondary antibody. The membrane was covered in tinfoil and incubated at room temperature for 2 hour. Following this incubation the membrane was washed for 4 X 15 minutes with 1X TBS.

2.6.7 Chemiluminescent detection of proteins

The rest of the western blot protocol was performed under dark conditions. Chemiluminescence was carried out using the Amersham ECL Western blotting detection reagents. A 1:1 dilution of detection reagent 1: detection reagent 2 was made and added to each immunoblots. Horseradish-peroxidase (HRP) substrate, which is located on the secondary antibody will oxidise the luminal in the detection solution and this will cause the luminal to be in an excited state and it will emit light. It is this light emission that can be detected by exposure of the blot on a blue light sensitive autoradiography film.

The amount of light emitted, correlates to the amount of HRP which in turn is dependent on the amount of primary antibody present and the amount of protein expressed. After 1 minute the detection solution was removed from the membrane and the excess removed. The membrane was positioned between two transparent sheets (acetates) and placed in a film cassette. Autoradiography film (Super RX medical X-ray film, FUJIFILM) was put on top of the membrane and the cassette was closed. After the appropriate amount of exposure time the film was developed. After developing the autoradiography film it was washed with ddH₂O and then fixed.

2.6.8 Stripping of immunoblots

In order to reprobe membranes with different antibodies, membranes were stripped with Western Blot Stripping Buffer for 30 minutes with gentle agitation. After stripping the membrane was washed 4 X 15 minutes with 1X TBS and then reprobed overnight with the appropriate antibody.

2.7 Generation of AAV-2/5

2.7.1 Triple transfection method

AAV was generated using the triple transfection method according to Xiao et al, 1998. This involves the transfection of the plasmid containing the transgene cloned between AAV-2 ITRs (pAAV-MCS), a pHelper plasmid and a pRep2/CAP5 plasmid (AAV-2 Rep proteins and Capsid 5 proteins [Hildinger et al, 2001]) into HEK293 cells (ATCC accession number: CRL-1573). The AAV-2/5 production was carried out in house by Dr. Naomi Chadderton according to the protocol outlined by O'Reilly et al, 2007.

To generate AAV-2/5, 5 X 10⁶ cells/ml of HEK 293 cells were seeded onto fifty 150mM tissue culture dishes and grown to 90% confluency in 20ml DMEM containing 10% FCS. 625µg of the pAAV-MCS, 625µg pRep2/CAP5 and 1250µg pHelper (a ratio of 1: 1:2) was added to 50ml of 150mM NaCl pH5.1. An equal volume of the transfection reagent polyethlenimine was added and this transfection solution was incubated for 8 minutes at room temperature (Reed et al, 2006). 1ml of this transfection solution was added to each plate of HEK293 cells such that each plate was transfected with 12.5µg pAAV-MCS, 12.5µg pRep2/CAP5 and 25µg pHelper. 7 hours post-transfection, the medium in each plate of transfected cells was replaced and cells were maintained for a further 48 hours.

2.7.2 AAV-2/5 purification using the Caesium chloride purification method (Aurricchio et al, 2001a)

Forty eight hours post-transfection, transfected HEK293 cells were dislodged from tissue culture dishes using a cell scraper, and the cells were centrifuged at 1000rpm for 30 minutes (SL-16, Thermo Scientific). Cell pellets were resuspended in 10ml 1X PBS and underwent three rounds of freeze thawing (by heating to 37°C in water bath, followed by cooling to -80°C) in order to lyse the cells. 10mg RNase A and 10,000U DNase I were added to lysed cells and this was incubated for 30 minutes at 37°C. This was subsequently centrifuged at 1000rpm (SL-16, thermo Scientific) for 15 minutes and the supernatant was removed. Deoxycholic acid was added to the supernatant, to act as a detergent (0.5% final concentration) and samples were incubated for 30 minutes at 37°C. CsCl₂ (0.454g/ml) was then added to the supernatant which was incubated on ice for 5 minutes. A CsCl₂ Density gradient was generated (Polyallomer Beckman tubes) by adding 9ml 1.4 density CsCl₂ (548.3mg/ml) on top of 9ml 1.6 CsCl (816.5mg/ml). The viral supernatant was placed on the top of this CsCl₂ gradient. Each tube was equilibrated and centrifuged at 25,000rpm for 18 hours at 4°C in an ultracentrifuge with a Beckman SW28 rotor (Beckman Coulter Optima™ L-100 XP ultracentrifuge). Using a 16G needle, the gradient tubes were gently pierced, and 1ml fractions were collected from each. The refractive index (RI) of each fraction was taken using a refractometer (Thermo Electron Corporation). Fractions of between 1.373-1.368 were collected, pooled and equilibrated with the 1.4 density CsCl₂. These were centrifuged at 59,000rpm for 18 hours at 4°C in an ultracentrifuge with a Beckman fixed angle rotor VTi65. The RI of 500µl fractions was obtained and samples with a RI of between 1.373-1.368 were pooled and centrifuged as above. 500µl fractions with a RI of 1.373-1.368 were collected (usually about three fractions) and dialysed in Slide-a-Lysers with 10 changes of 1X PBS.

2.7.3 Determination of viral titre (Rohr et al, 2002)

Before determining viral titre, 1µl from each viral fraction was treated with 7U DNase I for 30 minutes at 37°C and this was incubated for 10 minutes at 70°C. Proteinase K (7µg) was added and the reaction was incubated at 50°C for 1 hour and inactivated at 95°C for 20 minutes. qPCR analysis was carried out with transgene specific primers. A serial dilution of a plasmid containing the viral transgene was made consisting of between 10⁴-10⁸ copies of plasmid. These dilutions were used to generate a standard curve for determining viral titre. qPCR was performed using the two-step RT-PCR Sybr green PCR kit (Qiagen) and reaction were carried out as follows:

- **Activation of DNA polymerase:** 95°C for 10 minutes

- **cDNA amplification:** 37 cycles at 95°C for 15 seconds, 67°C for 15 seconds and 72°C for 10 seconds.

2.8 Animal procedures

2.8.1 Housing of mice

All mice were kept under specific pathogen free (SPF) conditions in individually ventilated racks with 12 hours light and 12 hours darkness at a temperature of 21°C±2°C. All work was carried out in compliance with the European Communities Regulations 2002 and 2005 (Cruelty to Animals Act) and the Association for Research in Vision and Ophthalmology (ARVO) statement regarding animal procedures in ophthalmic and vision research. All young mice were kept with the mother until weaning at 21 days. After weaning, males and females were separated from each other. No more than four female weaned mice were kept together in each cage and no more than three male weaned mice were kept together in a cage. For breeding purposes females were always placed into the males cage. Mice were sacrificed by CO₂ asphyxiation.

2.8.2 General genotyping procedure

Ear clips or tail tips were taken from mice to be genotyped and placed in 400µl tissue lysis buffer along with 30µl of proteinase K (10mg/ml). Samples were incubated overnight at 55°C with occasional vortexing. The tissue was centrifuged at 7500rpm (IEC Micromax Microcentrifuge, Thermo-Electron) to remove particulate matter. Two phenol/chloroform extractions were performed, followed by one chloroform extraction. Two volumes of ethanol to one volume of DNA solution were added in order to precipitate DNA. Prior to centrifuging the ethanol-DNA mixture, 1µl of glycogen was added. DNA pellets were resuspended in 100µl of ddH₂O. Unless otherwise stated 1µl of each DNA sample was used in a standard 50µl PCR reaction (**Section 2.2.16**). Transgene specific primer pairs were used in PCR reactions to confirm the presence of the transgene. PCR products were sized on an agarose gel (**Section 2.2.2**) in order to amplify up the gene of interest. For genotyping it was important to ensure the proper positive controls (a DNA sample containing the gene of interest) and a negative control (water blank or a sample known to be negative for the gene of interest) were included.

2.8.3 Subretinal AAV injection

All subretinal injections were carried out by Dr. Paul F Kenna, a registered ophthalmologist according to O'Reilly et al, 2007 (for adult mice). Adult mice were anaesthetised and the pupils were dilated.

AAV contained in a 10µl syringe (usually 3µl of AAV unless otherwise stated) was injected using a blunt-ended microneedle (34-gauge, Hamilton) into the subretinal space via a small incision made under local anaesthetic and a retinal detachment was induced. A reversing agent was administered to the mouse and body temperature was maintained using a homoeothermic heating device. Mice were carefully monitored until recovery. Sub-retinal injections on new born mice were carried out in accordance with Matsuda and Cepko as follows (Matsuda and Cepko, 2004). New born mice (P5) were chilled on ice for six minutes in order to anaesthetise them. A small incision was made into the eye lid and the eye was exposed. The eyes of new born mice are not clearly visible, therefore all sub-retinal injections were performed by touch, rather than visualisation of the needle into the sub-retinal space (The eye was gently touched until a slight movement and resistance was felt at the far wall of the eye). The eye was punctured at the sclera at the edge of the lens with a 30-gauge needle and AAV was then injected into the sub-retinal space of the eye using a blunt ended microneedle (33-gauge, Hamilton). Mice were warmed for 30 minutes at 33°C. Upon recovery they were returned to their mother.

2.8.4 ERGS

ERGS were carried out by Dr. Paul F Kenna a registered ophthalmologist according to Chadderton et al, 2009. This was based on a protocol approved by the International Clinical Standards Committee for human electroretinography. The mice were dark-adapted for 12 hours and all preparations were subsequently carried out under dim red light. The pupils of the mice were first dilated and the mice were anaesthetised and the eyes fixed in place in a proptosed position for the majority of the procedure. In order to record the ERG response simultaneously in both eyes, small contact lens electrodes (Goldwire electrodes, Roland Consulting) were placed on the cornea of both eyes using 1% Amethocaine as a topical anaesthetic. To prevent the cornea from drying out and also to maintain conductivity a small amount of Vidisic gel was used. A gold reference electrode was placed subcutaneously 1mm from the temporal canthus of the eye followed by a reference electrode placed subcutaneously 1mm to the anterior of the tail. To ensure uniform retinal illumination, full field standardised flashes of light were presented to the mouse and analysed using a RetiScan RetiPort electrophysiology unit. Rod-isolated responses were recorded under dim white light conditions (a flash of -25dB maximal intensity where maximal flash intensity was 3 candelas/m²/s), followed by a mixed rod-cone response to the maximal intensity flash. Mice were subjected to 10 minutes of light (30 candelas/m²/s) and then cone-isolated responses were measured. The a-wave is measured from the baseline to the trough and the b-wave from the baseline (for rod-only responses) or the a-wave trough.

2.8.5 Fixation of eyes

The eyes and the optic nerve was removed from the eye cup of each mouse by proptosing the eye using a forceps and gently pulling upward to detach the eye. Eyes were washed in PBS and subsequently fixed overnight in 4% Paraformaldehyde at 4°C with gentle rotation. Following this, the eye was washed with 1X PBS and the lens were removed under a light source (Intralux® 6000-1, Volpi) using a dissection microscope. Eyes were then washed three times with 1X PBS

2.8.6 Preparation of the eyes for cryosectioning

Eyes were cryoprotected in a series of sucrose gradients. They were initially placed in 10% sucrose for 30 minutes, 20% sucrose for 1 hour and 30% sucrose overnight at 4°C. The following day, the same volume of OCT to 30% sucrose was added to each vial of eyes and gently rotated for 30 minutes at room temperature. Each eye was placed in plastic moulds containing OCT, and the mould was placed in a container with isopropanol, this container was then submerged in liquid nitrogen and the eyes were frozen. Snap frozen eyes were stored at -20°C and sectioned as soon as possible.

2.8.7 Cryosectioning

Frozen eye blocks were cryo-sectioned on a cryostat (Leica CM 1900 Cryostat, Leica Biosciences) with a head temperature of -25°C and a unit temperature of -20°C. Each eye was cut into sections of 12µM thickness and the sections were collected on Polysine® slides (Thermo Scientific). Approximately 14 sections were collected on each slide, with on average of 13 slides/ eye. All slides were stored in a slide box at 20°C.

2.8.8 Immunohistochemistry

Where possible the middle slides (5-7) of each eye were used for the staining protocol so as to ensure the visualisation of the central part of the retina. Each slide was removed from the freezer and left to dry at room temperature for 20 minutes. Using a PAP pen a mark was drawn around the section on each slide and the PAP stain was left to dry. The slides were then washed for 5 minutes with 1X PBS. Each slide was blocked with blocking solution (120µl/slide) for 1 hour in a humidified chamber. The blocking solution was removed by gently tapping the slide to tissue paper, and the primary antibody was applied at the appropriate dilution. Slides were incubated over-night in a humidified chamber. The following day, the slides were washed 3 X 5 minutes with 1X PBS and the anti-mouse CY-3 secondary antibody (Sigma-Aldrich) was subsequently applied for two hours at room temperature in a humidified chamber (1/500 dilution). The secondary antibody was removed and the slides were washed 1 X 5

minutes with 1X PBS. Nuclei were counterstained by adding 4', 6-diamidine-2-phenylindole-dihydrochloride (DAPI, 1/10,000 dilutions) for 5 minutes at room temperature. Slides were then washed 2 X 5 minutes with 1 X PBS and mounted using aqueous mounting media. Sections were analysed using the fluorescent microscope (Axiophot microscope, Carl Zeiss LTD). DAPI staining was visualised under the '02' filter, EGFP under the '10' filter and rhodopsin was visualised under the '00' filter of the microscope. Photographs were taken of sections at 20X magnification using analysis B software. Composite images were produced using Adobe Photoshop (Adobe Systems Europe).

2.9 Statistical analysis

Statistical analysis was performed on results using Data Desk 6.0. The averages and standard deviation (SD) were calculated using Microsoft Excel. Unless otherwise stated a two sample t-test was utilised in order to determine the significance. $P < 0.05$ was statistically significant.

Chapter 3: RNAi-mediated suppression **of rhodopsin *in vitro***

3.1 Introduction

As mentioned in the introduction (**Section 1.10**), the most common genetic mutations associated with adRP are those found within the rhodopsin gene (RetNet). This form of RHO-adRP is a highly heterogeneous condition, with more than 100 mutations, located in various regions of the rhodopsin genomic sequence (Al-Magthteh et al, 1993; RetNet). Typically dominant rhodopsin mutations do not appear to result in haploinsufficiency, owing to the fact that the presence of one allele of rhodopsin appears to be sufficient for a functioning retina and results in only a very mild retinopathy (Humphries et al, 1997; Lem et al, 1999; Millington-ward et al, 1997; Rosenfield et al, 1992). With this in mind a gene therapy resulting in the suppression of mutant rhodopsin would in principle be a highly feasible therapeutic strategy. Owing to the fact that both significant knock-out of the rho gene and over-expression of wild-type rhodopsin results in a retinal degeneration, a suppression strategy that maintains the correct balance in terms of levels of rhodopsin expression needed for normal retinal function is essential (Humphries et al, 1997; Ollson et al, 1992; Millington-ward et al, 1997). There are many ways to design therapies for suppressing the dominant mutations of RHO-adRP and several different groups have displayed successful suppression of rhodopsin, in various transgenic models of adRP. Mechanisms for suppression of gene expression have traditionally involved both mutation-dependent and mutation-independent approaches (**Section 1.13**) using various molecular tools such as ribozymes, exon-skipping, zinc finger proteins and RNAi technology (Gorbatyuk et al, 2007a; Millington-Ward et al, 2011; Mussolino et al, 2011a; Tanner et al, 2009).

3.1.2 Mutation-dependent rhodopsin suppression using ribozymes

Drenser et al, 1998 designed both hammerhead and hairpin ribozymes (**Section 1.13**) targeting two nucleotide changes in both mutant P23H and S334ter rhodopsin transgenes, not present in wild-type rat rhodopsin. This group demonstrated efficient cleavage of both mutant transgenes *in vitro* using their corresponding ribozymes compared to controls; the wild-type rhodopsin transcripts remained uncleaved. More importantly the P23H hammerhead ribozyme resulted in cleavage of 20% of mutant rhodopsin RNA after 10 minutes and 80% cleavage after 5 hours (Drenser et al, 1998). AAV mediated delivery of this ribozyme to a transgenic line of P23H-3 rat (containing the 2 nucleotide changes that the ribozyme targets) resulted in a the down-regulation of P23H RNA but not wild-type RNA, additionally an increase in the photoreceptor cell number and increased ERG responses were also demonstrated. This efficacy was maintained for up to 8 months, the longest time-point evaluated (LaVail et al 2000; Lewin et al, 1998). Shaw et al, 2001 successfully designed hammerhead ribozymes to cleave the P347S porcine rhodopsin mutation efficiently *in vitro*, this ribozyme did not cleave wild-

type porcine rhodopsin making it an attractive mutation-dependent model. However a similar ribozyme designed to specifically cleave the human P347S rhodopsin mutation was not efficient. Furthermore as the porcine P347S hammerhead ribozyme was designed specifically for porcine rhodopsin mutations, the evaluation of its efficacy for human's patients was therefore not possible (Shaw et al, 2001).

3.1.3 Mutation-dependent approaches for rhodopsin splice site mutations

A novel a mutation-dependent approach for RHO-adRP was proposed by Tanner et al, 2009. A rhodopsin splice mutation was characterised within exon 4. Transfection of a mini mutant rhodopsin construct (containing the exon 4 mutation) into cells and mouse retinal explants resulted in an increased level of mutant rhodopsin with exon 4 skipping in addition to the activation of a cryptic splice site (cs). The activation of the cs led to a 4bp deletion at the end of the exon. Rescue of this phenotype was achieved in cells by altering both the U1 snRNA (to match the mutation and the surrounding exon 4 sequence) and by inactivating the cs site. This led to an 89% reduction in incorrectly spliced rhodopsin transcripts compared to non-rescued mutant rhodopsin (Tanner et al, 2009). In addition, the same group later utilised this approach to correct a splice site mutation in BBS1, a gene involved in Bardet-Biedl syndrome (Schmid et al, 2011). Another group, designed a therapy for the dominant splice mutations c.531-2a>g and c. 937-1G>T. siRNAs that specifically target the aberrant splice transcripts were made. In particular, an siRNA targeting the c.937-1G>T mutant transcript suppressed only mutant rhodopsin spliced RNA and not wild-type spliced RNA significantly in cell culture (Hernan et al, 2011a). All the above examples describe a system in which a mutation-dependent approach is harnessed to target rhodopsin mutations involved in aberrant splicing.

3.1.4 Mutation-dependent siRNA based approach

Other mutation-dependent RNAi (Section 1.23) based approaches have been tested for rhodopsin. Both siRNAs and shRNAs targeting the mouse P23H mutation were designed and found to efficiently suppress mouse P23H RHO but not mouse wild-type RHO *in cellulo*. AAV mediated *in vivo* RNAi-based suppression was achieved at an RNA level. AAV-2/5 expressing mouse shP23H was subretinally injected into transgenic rats that have normal wild-type rhodopsin alleles and a mutant mouse P23H allele (the sequence between the mutant and wild-type rhodopsin differs by 4bp at the RNAi target site). However therapeutic efficacy was not achieved in terms of functional or histological improvement. Rhodopsin is the most abundant protein in the eye therefore it may be that 62% suppression of mouse P23H RNA may not be adequate to confer efficacy (Tessitore et al, 2006).

3.1.5 Mutation-Independent ribozyme based approaches

Given the mutational heterogeneity present in RHO-adRP, it would be of value to develop a strategy which corrects the primary defect but in a mutation-independent manner. The initial approach to mutation-independent strategies for rhodopsin suppression, involved a strategy in which ribozymes would be designed to target both the mutant and the wild-type rhodopsin sequence, following this rhodopsin expression would be rescued by the simultaneous introduction of a rhodopsin transgene, engineered as resistant to the ribozyme by the creation of silent mutations (Millington-Ward et al, 1997). Originally, different hammerhead ribozymes were designed to target mouse rhodopsin *in vitro*. Ribozymes targeted the 5'UTR of mouse wild-type rhodopsin efficiently, whereas rhodopsin constructs in which the 5'UTR had been altered (by replacing it with the 5'UTR of mouse peripherin or by altering the ribozyme site from GUC to GGC) remained intact. Both monomeric and multimeric ribozymes also achieved efficient cleavage of both mouse and human rhodopsin open reading frames (ORFs) *in vitro*. Rhodopsin transcripts in which the wobble base of the codon at the ribozyme site was altered by taking advantage of the degeneracy of the genetic code were resistant (Millington-ward et al, 1997; O'Neill et al, 2000). Sullivan et al, 2002 designed ribozymes to successfully target and cleave full length wild-type and mutant human rhodopsin *in vitro* in human cells (Abdelmaksoud et al, 2009; Sullivan et al, 2002). *In vivo* mutation-independent ribozymes have been a somewhat successful. Ribozymes homologous to both mouse and dog rhodopsin transcripts were generated; *in vitro* these ribozymes significantly suppressed both endogenous and mutant rhodopsin (Gorbatyuk et al, 2005; Gorbatyuk et al, 2007a). Furthermore ribozyme-based suppression in a rodent model of adRP, the P23H rat containing a mouse P23H allele, resulted in significant suppression of mouse RNA and an improvement in both ERG response and ONL thickness (Gorbatyuk et al, 2007a).

3.16 Mutation-independent Zinc finger nucleases

A recent mutation-independent suppression approach may offer an alternative method to correcting rhodopsin mutations based on inducing homologous recombination (**Section 1.13**). Zinc finger nucleases (ZFN) targeting the human rhodopsin sequence were designed, and *in vitro* induced double stranded breaks into rhodopsin after 48 hours with minimal toxicity, furthermore when rhodopsin targeting ZFN were co-transfected with linear donor DNA into a cell line stably expressing human P23H rhodopsin, an increase in homologous recombination was observed (Greenwald, et al 2010). Mussolino et al, 2011a used a zinc finger protein bound to transcriptional repressors to specifically target human rhodopsin for repression both *in vitro* and *in vivo*. Moreover AAV-2/8 subretinal delivery of this zinc finger repressor resulted in a 26% reduction in human P347S transcript in a mouse model

of adRP, this reduction resulted in both an improvement of ERG response and in photoreceptor cell survival.

3.17 Mutation-independent siRNA based approaches

Various groups have reported mutation-independent siRNA based approaches for rhodopsin suppression both *in vitro* and *in vivo* (Cashman et al, 2005; Gorbatyuk et al, 2007b; Kiang et al, 2005; O'Reilly et al, 2007). Cashman et al, 2005 demonstrated efficient silencing of both wild-type human rhodopsin and P23H rhodopsin *in vitro* at the protein level in human embryonic retinoblasts, a codon modified replacement gene was also assessed *in vivo* and found to be resistant to siRNA mediated suppression (Cashman et al, 2005). Gorbatyuk et al, 2007b established AAV-2/5 mediated suppression of rhodopsin in hemizygous rhodopsin mice. This resulted in the significant suppression of rhodopsin RNA and protein and caused a significant reduction in ERG response and ONL thickness (Gorbatyuk et al, 2007b). The team in TCD have been involved in the development of mutation-dependent RNAi-based rhodopsin therapies. siRNAs and shRNAs assessed in cell culture reduced human rhodopsin RNA and protein significantly, *in vitro* (cell culture), *ex-vivo* (in retinal explants) and *in vivo* (mouse retinas), furthermore efficacy was also achieved in terms of increased ONL thickness and improved ERG (Chadderton et al, 2009; O'Reilly et al, 2007). Subretinal injection of a shRNA (shQ1) which only targets human rhodopsin into RHO-adRP P347S transgenic mice demonstrated suppression of mutant human P347S. However, the endogenous mouse rhodopsin alleles remained resistant. This resulted in rescue of the P347S phenotype in terms of improved ONL thickness and ERG responses (Chadderton et al, 2009). Replacement rhodopsin constructs resistant to rhodopsin suppression have also successfully been generated and rescue of the phenotype in rhodopsin null mice (129rho^{-/-}) obtained (Palfi et al, 2010). The two component suppression and replacement therapy has subsequently been evaluated *in vivo* in mouse RHO-adRP models and of note significant benefit was obtained using histological and electrophysiological readouts (Millington-Ward et al, 2011; O'Reilly et al, 2007; O'Reilly et al, 2008; Palfi et al, 2010).

3.18 Objectives of chapter 3

siRNA mediated suppression of target RNA is an effective approach to achieve *in vivo* suppression of rhodopsin for RHO-adRP. In this regard it is of note that no clinical trials correcting the primary defect for this form of adRP currently exist (www.clinicaltrials.gov). For a gene such as rhodopsin in which so many mutations are implicated in the disease pathology, a tailor-made therapy for each individual mutation is not realistic. With this in mind, a more feasible and economically viable therapy may be a

mutation-independent strategy (Cashman et al, 2005; Millington-Ward et al, 1997). Of the suppression approaches described above, the most well established and efficacious therapy *in vitro* and *in vivo* was the siRNA based therapy. This approach resulted in the reduction of over 90% of rhodopsin *in vivo*, potency in terms suppression which was not observed for any of the alternative mutation-independent approaches described in the sections above (**Section 3.1**). Furthermore the RNAi-based suppression and replacement approach has been evaluated in disease in the models of adRP and therapeutic benefit has been demonstrated (Millington-Ward et al, 2011). Thus far no gene therapy for RHO-adRP has been evaluated in a larger animal model. In order to proceed towards human clinical trial, studies in larger animal models which better represent the human condition are vital (Ellinwood & Clay, 2009). This path towards clinical trial has been demonstrated in the RPE65 clinical trials, in which efficacy in both dogs and toxicity and biodistribution in non-human primates was demonstrated prior to testing in human patients (Stein et al, 2011). The pig represents a valuable animal model for the evaluation of adRP therapies owing to the fact that the pig eye is similar to the human eye and in addition RHO-adRP porcine models are available for such evaluations (Petters et al, 1997). With this in mind the aim of the study described in this chapter of the thesis was to design siRNAs homologous to both porcine and human rhodopsin for the eventual evaluation of a suitable therapy for use in RHO-adRP pig models. However before an *in vivo* based siRNA approach can be evaluated, initial testing *in vitro* needs to be undertaken. Therefore the overall aim of the work presented in this chapter involved generating siRNA molecules and testing their efficacy *in vitro* in HeLa cell culture as detailed below.

3.2 Materials and Methods:

3.2.1 siRNA design

All siRNAs were designed using online software by either the GenScript Corporation (siP1 & siP2) or by Invitrogen BLOCK-iT™ RNAi Designer (siP3). These software programs use specific criteria and advanced algorithms to generate siRNA targets. The human rhodopsin mRNA (hRHO [GenBank accession number NM_000539]) was submitted to these programs and siRNA targets of between 30-60% GC content and of 19bp were recommended. The siRNAs were subsequently aligned with human, mouse (mRHO [GenBank accession number NM_145383]) and porcine rhodopsin (pRHO [GenBank accession number NM_214221]) mRNA sequences and targets with 100% homology between human and pig were selected (**Figure 3.3.1.a**). The program used to perform the sequence homology between human, mouse and porcine rhodopsin was clustalw and can be found at <http://www.genome.jp/tools/clustalw/>. All custom made siRNA oligonucleotides were ordered pre-annealed and HPLC purified (Ambion).

3.2.2 Resuspending of annealed siRNAs

Vials containing the siRNA oligonucleotides (40nmoles) were briefly centrifuged and resuspended to a stock concentration of 100μM using nuclease-free H₂O, each resuspended siRNA was then aliquot and stored at - 20°C. For a working concentration of siRNA, an aliquot of 100μM was thawed and diluted to 20μM (20pmoles/μl).

3.2.3 Co-transfection of hRHO with siRNAs into HeLa cells

Each siRNA was co-transfected with a human rhodopsin cDNA plasmid (hRHO, a kind gift from Prof. W. Baehr, University of Utah, USA) into HeLa cell culture (**Section 2.3.5**). Initially a number of controls were tested in order to check for toxicity associated with the hRHO or siRNAs. The following three controls were included. A Lipofectamine® 2000 only control where no DNA or RNA was included to ensure the concentration of Lipofectamine® 2000 was not toxic. A hRHO plasmid control where no siRNA was included was performed to ensure the DNA plasmid was not toxic. Finally, a siRNA only control was included to ensure that the siRNA was not toxic to the cells. After 24 hours each well of cells was viewed under a microscope and the amount of cell death observed. Following this analysis, 400ng of hRHO was co-transfected with 16 pmoles of each siRNA to be evaluated (siNT, siBB, siP1, siP2 or siP3) in separate wells and this was performed in quadruplicate such that each

siRNA was co-transfected with hRHO in four wells. An untransfected cells control was also included on every plate. Each co-transfection experiment was carried out at least four times.

3.2.4 RNA isolation of HeLa cells co-transfected with hRHO and each siRNA

RNA isolations were carried out using Tri-Reagent or the RNeasy plus mini kit (**Section 2.4.2 & Section 2.4.3.**); quadruplicate wells containing hRHO and each siRNA were pooled. Following extraction of RNA the OD₄₀ of each RNA sample was measured in order to assess the quality of the RNA. A DNase digestion was undertaken on every RNA sample (**Section 2.4.4**).

3.2.5 Optimisation of primers for use in subsequent real-time RT-PCR experiments

When first using real-time RT-PCR it is vital to optimise all primer sets, to identify a consistent highly expressed housekeeping gene as well as to evaluate efficient primer pairs. Initially hRHO primers and human β -actin primers were evaluated (**Table 3.2.5**). A standard curve for both rhodopsin and β -actin was made using RNA samples from HeLa cells co-transfected with hRHO RNA and a non-silencing siRNA (siNT). This sample was diluted 1/100 in nuclease free H₂O and 5-fold serial dilutions were generated and given the arbitrary numbers 625, 125, 25, 5 and 1 respectively. A Sybr green master mix containing hRHO primers (forward and reverse 10pmoles/ μ l) was made along with a second mix containing the β -actin primers. Two different sets of the 5 standard curve RNA samples were pipette in triplicate into the real-time plate, one each for each set of primer mixes. The real-time RT-PCR was set up as described (**Section 2.4.7**). When the real-time RT-PCR had concluded, two standard curves were generated, one for the hRHO primer pairs—which amplified hRHO in the RNA samples and the other one for the β -actin primer pairs which amplified human β -actin (h β -actin) in the RNA samples. The slope and R² value for both hRHO and h β -actin was determined, and a melting curve analysis was undertaken to ensure that the primers were specifically amplifying the correct products with no contamination. A sample of the hRHO amplicon and the h β -actin amplicon were removed from the plate and run on a 2% agarose gel (**Section 2.2.2**), in order to determine the correctly sized products had been amplified.

3.2.6 Real-time RT-PCR of RNA from HeLa cells co-transfected with hRHO and each siRNA

Following the successful optimisation of both hRHO and h β -actin primers for real-time RT-PCR, hRHO RNA expression from each co-transfection experiment was analysed. As with the primer evaluation, the HeLa RNA samples from cells co-transfected with hRHO and siNT were used to generate the two 5X standard curves for hRHO and h β -actin. Each standard curve sample was used in

duplicate for both hRHO and h β -actin. Each RNA sample to be evaluated (siNT, siBB, siP1, siP2 and siP3) was diluted 1/500 in nuclease-free H₂O and used in triplicate on each plate. A water blank sample was used for each Sybr green mix to ensure the water used to dilute all RNA samples was not contaminated. The real-time RT-PCR was analysed as described previously (**Section 2.4.7**). A melting curve analysis was undertaken on each RNA sample to ensure the amplification in each well was specific. Each RNA sample was measured against the standard curve generated for hRHO and h β -actin, and a value was calculated for each. Each RNA sample was evaluated in triplicate therefore the average quantity of hRHO and h β -actin per sample was obtained. Following this, each RNA hRHO value was normalised to its corresponding h β -actin value in order to obtain a true comparison of relative hRHO expression in each sample. The normalised value for hRHO expression in the control sample (RNA sample from HeLa cells co-transfected with hRHO and siNT) was set at 100% hRHO expression, and all other samples (RNA samples from HeLa cells co-transfected with hRHO and siBB, siP1, siP2 and siP3) were expressed as a percentage of this value. Each real-time RT-PCR was carried out at least twice for each set of transfections.

3.2.7 Statistical analysis

The average raw data and standard deviation of normalised rhodopsin (rhodopsin/h β -actin) of each sample (averaged for at least four different co-transfections) was calculated using Microsoft Excel. A two sample t-test was performed to analyse the significance between rhodopsin expression in the control sample (RNA samples from HeLa cells co-transfected with hRHO and siNT) compared to the siRNA samples to be tested (RNA samples from HeLa samples co-transfected with hRHO and siBB, siP1, siP2 or siP3) using Datadesk 6. The statistical significance between each sample to be tested (i.e. between siBB, siP1, siP2 and siP3) was also analysed using a two-sample t-test to determine if any siRNAs performed statistically better than others.

3.2.8 Protein extraction and concentration evaluation

Protein isolations from HeLa cells co-transfected with hRHO and each siRNA was carried out as described previously (**Section 2.5.1**). An important negative control was the inclusion of protein from untransfected cells. The protein concentration was determined for each protein sample using a BCA assay (**Section 2.5.3**). Each sample from a particular transfection experiment was analysed at the same time on the same plate, in order to accurately normalise the levels of protein in samples to each other. The concentration of each protein was calculated in $\mu\text{g/ml}$.

3.2.9 Western Blotting

Western blotting was carried out according to **Section 2.6**. Between 10 μ l-20 μ l of the protein sample with the lowest concentration was added to the appropriate well and the amount of protein added for all other samples was normalised to this value. This was to ensure that in each gel, each protein sample would have the same concentration of protein. In an attempt to limit the oligomerisation of rhodopsin protein, the protein samples were prepared for western blotting according to Vihtelic et al, 1999 and boiled at 65°C for 30 minutes. Nitrocellulose was the transfer membrane used for the western blotting described in this chapter and each membrane was probed only twice (for rhodopsin and h β -actin). The primary anti-mouse 4D2 rhodopsin antibody (1/1000 dilution [Hicks & Molday, 1986]) and the anti-mouse secondary antibody (1/1000 dilution) were used. For h β -actin, the primary anti-rabbit antibody (1/1000 dilution) and the anti-rabbit secondary antibody (1/1000 dilution) were used. Initially a western blot was undertaken on a hRHO positive sample (HeLa protein from cells transfected with hRHO and siNT) and a hRHO negative control (protein from untransfected HeLa cells). This was undertaken to ensure the 4D2 antibody was working efficiently. Following this preliminary experiment, evaluation of suppression of hRHO by each siRNA at the protein level was undertaken.

3.3. Results

3.3.1. Design of the siRNAs

In order to evaluate suppression of rhodopsin in the porcine animal model it was important to design siRNAs with complete homology to both hRHO and pRHO. A pairwise sequence alignment was performed between the human, porcine and mouse RHO ORFs (1047bp, **Section 9.0**). It was found that human and pig RHO sequences are 91% identical, human and mouse RHO 87% identical and mouse and pig RHO sequences are 88% identical (**Section 9.1**). Using online software the hRHO ORF was used to generate siRNAs that target hRHO. The siRNAs chosen were 19 nucleotides in length as this is in accordance with other siRNAs used in the laboratory as well as the positive and negative controls that were used in all subsequent experiments (O'Reilly et al, 2007). GenScript Corporation ranked the top ten siRNAs predicted to suppress hRHO; of the ten, two were 100% homologous to pRHO (**Figure 3.3.1.a**). The first, named siP1 (ranked no.7) had a GC content of 47.37% and targets position 289-308 of the hRHO ORF. The second was siP2 (ranked no.10) had a GC content of 52.63% and targets 618-637 of the hRHO ORF. Subsequently a third siRNA (siP3) was designed by BLOCK-IT™ RNAi designer. siP3 (ranked no.3) had a GC content of 53.64% and targets 404-423 of the hRHO ORF (**Figure 3.3.1a**). These three siRNA sequences were chosen as they were the only three homologous to both hRHO and pRHO. All siRNA oligonucleotides were ordered with high purity and with 3'dTdT ends according to the design by Elbashir et al 2001c (**Table 3.3.1.a**). A homology check was undertaken between each siRNA and rhodopsin RNA sequence from other species. siP2 is the only siRNA homologous to mouse rhodopsin (**Table 3.3.1.b**).

3.3.2. Co-transfection of siRNA and hRHO into HeLa cells

Co-transfection experiments of siRNA and hRHO cDNA in HeLa cells have previously been described and optimised in the laboratory (O'Reilly et al, 2007; O'Reilly et al, 2008) using Lipofectamine® 2000 as the transfection reagent of choice. In order to assess toxicity, wells of cells containing a hRHO plasmid only control, an siRNA only control and a Lipofectamine® 2000 only control were initially included in experiments. After 24 hours each well of cells was viewed under a microscope and the amount of cell death observed. Cells from untransfected wells appeared to display less cell death than the cells from all transfected wells suggesting that the co-transfecting of rhodopsin and siRNA by Lipofectamine® 2000 may have a negative effect on cell survival. However, this assessment of cell death was purely observational and no cell counts or statistical analysis was undertaken to confirm this

result. When the dead cells were washed with 1X PBS, healthy living cells were still adhered to the cell culture dish and were used for analysis.

3.3.3 Real-time RT-PCR optimisation

When first using real-time RT-PCR it is vital to optimise the use of all primer sets and to identify a consistent highly expressed housekeeping gene. The hRHO and h β -actin primers used (**Table 3.2.5**) were previously designed in the laboratory and are known to be both specific and efficient (O'Reilly et al, 2007). Analysis of a typical hRHO standard curve is shown (**Figure 3.3.3.a**). A 5X standard curve was generated by using RNA from HeLa cells transfected with hRHO and siNT and arbitrary concentrations of 1, 5, 25, 125 and 625 was set for each standard from low to high respectively. In an efficient assay each different standard is amplified at a particular cycle number, the most concentrated is amplified at the earliest cycle number and as it is a 5X curve, the second highest standard should be amplified 2 cycles later and so on. Following the real-time RT-PCR, the cycle number at which each standard is amplified is graphed with the log of the concentration and a line of best fit is generated. The slope of the hRHO standard curve was -3.4 which is within the optimal slope range for this assay (-3.1 to -3.6 i.e. 100 $\pm$ 10% efficiency) and the R² value was 0.9998 which means there is an almost perfect correlation between cycle number and concentration. This is indicative of a good standard curve for hRHO using this RNA and these primers. The dissociation curve (**Figure 3.3.3.b**) indicates that the T_m of all amplified products is approximately 82°C, which is the correct predicted T_m for hRHO amplification with these primer pairs. No unspecific amplification had occurred. In addition no primer dimers formed. A low insignificant amount of hRHO amplification did occur in water blank samples but this was at such a low level therefore it was deemed not to affect results. hRHO amplification products were run on a 2% agarose gel and a band at approximately 108bp was generated. This represents the correct amplification size for hRHO amplified with the hRHO primer set used in the experiment (**Figure 3.3.3.c**).

For previous real-time RT-PCR studies analysing hRHO expression a human β -actin housekeeper (h β -actin) was used to normalise rhodopsin expression levels (O' Reilly et al, 2007). As observed in (**Figure 3.3.3.d**) the h β -actin standard curve was as efficient as the hRHO standard curve with a slope of -3.2 and an R² value of 0.999. A dissociation curve with a T_m of approximately 81°C was consistently observed for h β -actin amplification products and this was also consistent with the predicted T_m for h β -actin amplified by this primer pair. The water blank was free from contamination and no primer dimers were observed in standard samples (**Figure 3.3.3.e**). Primer dimers were present in water blank controls, this is however not uncommon as primers can often dimerise in samples were

there is very little or no cDNA present. h β -actin amplification products were 103bp, which represent the correct amplification size predicted for this primer pair. This indicates that these h β -actin primers are specifically amplifying h β -actin with high efficiency (**Figure 3.3.3.c**).

Samples were analysed using the standard curve method as this was used in the laboratory previously to assess rhodopsin expression (O'Reilly et al, 2007). Additionally, use of the comparative CT method is dependent on the target efficiencies for both rhodopsin and β -actin being equal and having assays of between 90-110% efficiency (slope of between - 3.1 and -3.6). Since this may not always be the case for rhodopsin and h β -actin the relative standard curve method was used (Applied Biosystems training manual 7300; Livak & Schmittgen, 2001). For all real-time RT-PCR quantification, the relative RHO expression levels in treated samples (RNA from cells transfected with hRHO and each hRHO siRNA) were expressed as percentages of the relative RHO expression levels in a control sample (RNA from HeLa cells co-transfected with hRHO and a non-targeting negative control).

For clarity regarding the analysis used to calculate the level of hRHO expression in each sample, a working example is presented (**Figure 3.3.4.f**). The average quantity of hRHO and h β -actin in each sample (samples were in triplicate) was obtained by comparison to both the hRHO and h β -actin standard curves. In the working example siNT (RNA from cells co-transfected with hRHO and siNT control) had an average rhodopsin quantity of 132.5 (the average was obtained from each sample in triplicate) and an average h β -actin quantity of 158.3. The quantity in each sample is an arbitrary value obtained by comparing the Ct value of each "unknown" sample to the Ct values of either the 5X hRHO or 5X h β -actin standard curve (in which 5X arbitrary values of 1, 5, 25, 125 & 625 have been assigned) and the relative quantity (concentration) compared to the arbitrary standard curve quantities is obtained. The siBB sample (RNA from cells co-transfected with hRHO and siBB, the positive control) had an average hRHO quantity of 21.8 and an average h β -actin quantity of 177.9. The hRHO value in each sample was normalised to h β -actin by dividing the average hRHO quantity by the average h β -actin quantity ($132/158.3 = 0.84$ for siNT and $21.8/177.92 = 0.12$ for siBB). When these values were obtained the relative hRHO expression in siBB compared to siNT was calculated. As siNT is the sample in which cells are transfected with hRHO and a non-targeting siRNA, the level of hRHO is set at 100% expression, in order to calculate relative hRHO levels in siBB the normalised hRHO value of siNT (0.84) is divided by the normalised hRHO value of siBB (0.12), and multiplied by 100 to get a percentage value (15%). Thus in the working example for relative hRHO expression, hRHO in cells transfected with siBB is suppressed to 15%.

3.3.4 siP1 and siP2 RNA suppression data

Originally two siRNAs (siP1 and siP2) were co-transfected into HeLa cells with hRHO. Levels of hRHO RNA expression in RNA samples from HeLa cells transfected with hRHO and each siRNA were compared to levels of hRHO expression obtained in HeLa cells co-transfected with siNT (**Section 2.3.5**) and hRHO. The hRHO RNA suppression results are graphed (**Figure 3.3.4.a**). The positive control siBB (**Section 2.3.5**) significantly reduced hRHO as expected to $15\pm3\%$, $P<0.001$. siP2 efficiently reduced hRHO RNA levels to $22\pm7\%$, $P<0.0005$. siP1 also significantly reduced hRHO RNA levels to $36\pm13\%$, $P<0.001$. The statistical significance between test siRNAs was also evaluated (between siBB, siP1 and siP2). hRHO suppression by siBB and siP2 was significantly similar ($P=0.0970$). Additionally, hRHO suppression by siP1 and siP2 was statistically similar ($P=0.1167$). siBB was a more potent suppressor of hRHO when compared to siP1 ($P<0.05$). This indicated that both siP1 and siP2 were efficient suppressors of hRHO but siP1 was not as efficient when compared to siBB *in vitro*.

3.3.5 Western blot analysis of hRHO expression

Suppression of hRHO RNA by siRNAs should theoretically result in suppression of hRHO protein. It was decided to examine hRHO protein expression in HeLa cells co-transfected with siRNAs (siNT, siBB, siP1 and siP2) and hRHO cDNA using western blotting. Initially in order to test the 4D2 antibody, a pilot western with protein from HeLa cells co-transfected with hRHO and the siNT (the positive hRHO control) and with untransfected cells (the negative hRHO control) was undertaken. hRHO bands should be present in the positive control sample but absent in the negative control. This preliminary experiment should determine if any unspecific binding of the 4D2 antibody to HeLa cell protein was occurring. The representative western blot is shown in **Figure 3.3.5.a** hRHO protein was only observed in the positive control (lane 1) and not the negative control (lane 2). The hRHO monomer was present at 39kDA, which is the correct predicted size for RHO protein. RHO bands at higher molecular weights were also visible, these bands represent dimers and various oligomers of hRHO. Following this analysis western blots were established for protein samples from HeLa cells co-transfected with hRHO and each siRNA. The protein concentration of HeLa protein from each transfection was normalised to each other in order to ensure equal loading. **Figure 3.3.5.b** represents a typical western blot obtained from this analysis. Bands were visible at 39kDA representing the hRHO monomer in the siNT sample (lane 1), as well as higher molecular weight forms of hRHO; this pattern of RHO immunoreactivity was in accordance with that observed in the pilot study (**Fig 3.3.5.a**). There

was no visible hRHO protein in samples transfected with either siBB (lane 2) or siP2 (lane 4) and these lanes were comparable to the negative control lane (lane 5). This corresponds to the RNA result and suggests that 78% suppression of hRHO RNA is sufficient to result in a significant reduction in hRHO protein from the cell. A somewhat surprising result was that siP1 did not seem to be efficient at suppressing hRHO at the protein level (lane3). β -actin was used to probe for equal loading, and this was obtained in all cases, suggesting that the suppression of hRHO with siBB and siP2 was a valid observation. Western blotting was carried out on 4 separate co-transfection experiments and all western blots gave the same result.

3.3.6 siP3 RNA suppression data

As siP1 was not as efficient at suppressing hRHO at the protein level compared to siBB and siP2, a third RHO-targeting siRNA (siP3) was designed and tested *in vitro*. hRHO RNA levels analysed from HeLa cells co-transfected with wild-type hRHO and siP3 (and controls siNT and siBB) are shown (**Figure 3.3.6.a**). siBB reduced hRHO RNA as expected to $15 \pm 2\%$, $P < 0.0001$. siP3 reduced hRHO RNA the most efficiently of all three siRNAs designed (to $8 \pm 6\%$, $P < 0.0001$) making it a good candidate for future use *in vivo*. siP3 and siBB were statistically similar in terms of potency of suppression achieved ($P = 0.2877$). In contrast siP2 and siP3 were statistically different from each other ($P < 0.05$). This suggested that siBB and siP3 were the most efficient at suppressing hRHO RNA *in vitro*.

3.3.7 siP3 protein suppression data

To confirm that suppression of hRHO by siP3 at the RNA level is mirrored at the protein level, western blotting was also performed on protein samples from HeLa cells co-transfected with hRHO and siP3 (**Figure 3.3.7.a**). Rhodopsin protein expression was undetected in cells transfected with siP3 (lane 3) compared to siNT (lane 1). The lack of hRHO protein in this lane was comparable to lane 4 (untransfected cells) in which no hRHO is present. hRHO protein suppression achieved with siBB was similar to that observed in the previous analysis (**Fig.3.3.5.b**). The concentration of protein added to each well was the same and this correlated with the observation that equal levels of human β -actin were visible in each lane of the western blot. Western blotting was carried out seven times, using protein from seven separate co-transfection experiments for siP3 and of note the same result was obtained every time.

Overall these results indicate that two out of the three RNAi based suppressors designed suppress hRHO efficiently at both the RNA and protein levels. As evaluated in cell culture by real-time RT-PCR and western blotting.

3.4 Discussion

Various therapeutic strategies exist for genetic disorders, all of which rely on a firm knowledge of the gene implicated in the disease, the mode of inheritance and the type of mutation (den Hollander et al, 2010; Farrar et al, 2002; Farrar et al, 2010). For heterozygous autosomal dominant mutations, such as those found in RHO-adRP specific suppression of the mutant allele may be beneficial. This is particularly relevant for rhodopsin given that mice with only one wild-type allele of rhodopsin maintain sufficient visual function (Humphries et al, 1997; Rosenfeld et al, 1992). Strategies involving suppression of the mutant rhodopsin allele but not the wild-type allele have been described in the introduction in terms of using ribozymes, splicing approaches and RNAi to elicit suppression. However, these strategies, although innovative each have their limitations. The mutation-specific approaches using ribozymes have mainly focused on two well known and common RHO mutations (P23H and P347S, RetNet). For a P23H ribozyme, while the authors achieved efficacy *in vitro* and *in vivo* in transgenic rat models, this precise ribozyme therapeutic would not be applicable to human patients. The P23H mutation in human's results in a 1 nucleotide mismatch, which does not specifically create a ribozyme cleavage site, thus a specific ribozyme for this mutation in human patients is not feasible (Drenser et al, 1998). In terms of specific ribozymes for the P347S mutation, while efficient cleavage of the porcine P347S allele was achieved, the ribozyme designed to cleave the human equivalent, mutant P347S hRHO, failed to function efficiently. The porcine ribozyme was specific for pRHO and thus is not an approach that can be used in human patients (Shaw et al, 2001). In terms of using molecular tools which alter aberrant splicing of hRHO in RP patients with splice site mutations, this approach, while efficient *in vitro* has yet to be tested *in vivo* (Hernan et al, 2011a; Tanner et al, 2009). This mutation-dependent approach would only be capable of specifically targeting splice site mutations which unfortunately do not account for a large number of known hRHO mutations (RetNet). One mutation-dependent siRNA approach evaluated provided 62% suppression of the mutant allele. Unfortunately this suppression was not sufficient to rescue the disease phenotype or provide therapeutic benefit in this study (Tessitore et al, 2006). Dominant mutations in RHO are separated into different classifications based on their different biochemical properties which lead to retinal degeneration, thus while 62% suppression was not sufficient to prevent pathogenesis by the P23H allele, rescue of the disease phenotype caused by rhodopsin mutations in other classes with this level of suppression may still be achievable (Mendes et al, 2005; Tessitore et al, 2006).

An alternative approach is the optimisation of a strategy in which both the mutant and wild-type allele are significantly suppressed. This therapeutic strategy would include the simultaneous delivery of a

replacement rhodopsin allele refractory to suppression. This approach has the significant advantage in that it is mutation-independent. Thus in principle a single therapy could be used to treat a wide range of rhodopsin mutations (Millington-Ward et al, 1997; Millington-ward et al, 2011). In terms of mutation-independent approaches, while ribozymes and correction using zinc finger transcription factors have been described and represent interesting suppression molecules for rhodopsin (Greenwald et al, 2010; Mussolino et al, 2011a). The most efficient therapeutic to date was that of mutation-independent suppression using RNA interference. This resulted in high levels of hRHO suppression at the RNA and protein levels in adRP models along with the achievement of therapeutic benefit (Chadderton et al, 2009; Millington-ward et al, 2011; O'Reilly et al, 2007). In addition, RNAi based suppression and replacement could be considered for the treatment of other autosomal dominant heterogeneous disorders such as dominant dystrophic collagen-linked Epidermolysis Bullosa (MIM # 131750) and collagen-linked autosomal dominant Osteogenesis Imperfecta (MIM # 166200). Indeed this approach has recently been utilised *in vivo* in mice for the suppression and replacement of SOD-1, a gene involved in dominant forms of Amyotrophic Lateral Sclerosis (Kubodera et al, 2011). In addition *in vitro* suppression and replacement strategies have been explored in the retina for other genes implicated in adRP such as NRL and peripherin (Hernan et al, 2011b; Palfi et al, 2006). Thus it was felt that further investigation of this approach should yield promising results.

One objective of this study was the optimisation of suppression and replacement based therapy for RHO-adRP in larger animal models. To the author's knowledge, this is the first study in which siRNAs have been designed to specifically target both human and porcine rhodopsin. The 9% nucleotide sequence differences between the human and porcine rhodopsin genes limited the choice of target sites for siRNAs to three sites as predicted by software from GeneScript Corporation siRNA target finder or Invitrogen BLOCK-IT RNAi designer (**Figure 3.3.1a**). However, one siRNA (siBB) which has previously been evaluated in the laboratory and is an efficient suppressor of hRHO both *in vitro* and *in vivo* is also homologous to pRHO (O'Reilly et al, 2007; Millington-Ward et al, 2011). It was critical to have a range of therapeutics for evaluation of suppression in the porcine RHO-adRP model. Therefore with this in mind two additional siRNAs were initially designed for *in vitro* assessment of hRHO suppression.

siRNA design is a crucial feature in generating potent RNAi mediated therapeutic strategy. A thorough knowledge of the elements needed to make the most safe, efficient and functional siRNA molecule is key to an efficacious gene therapy (Walton et al, 2010). Initially complementarity, siRNA length and the presence of 3'overhangs were believed to be the main features vital for RNAi (Chang et al, 2011;

Walton et al, 2010). All siRNAs designed in this study were 19bp in length with two dTdT overhangs on the 3' end, this classic format was proposed to bring about efficient incorporation of the siRNA into RISC. Even though various studies have recently been published suggesting there is more flexibility on the target length of siRNAs and the presence of the 2nt 3' overhangs than previously evaluated, it was decided to remain with the classic siRNA format proposed by Elbashir et al, 2001c (Chang et al, 2011; Elbashir et al, 2001c). In addition all other siRNAs previously designed in the laboratory as well as the two control siRNAs (siNT and siBB) had been previously designed according to this strategy (O'Reilly et al, 2007).

More recently various algorithms have evolved to aid in the identification and prediction of functional siRNAs. These algorithms are frequently updated and gaining complexity as various criteria regarding what makes an efficient siRNA is constantly being discovered (Walton et al, 2010). The siRNA prediction tools used in this study for rhodopsin were GenScript Corporation siRNA target finder and Invitrogen BLOCK-IT RNAi designer. These programs use a wide range of conditions, published from the literature in order to predict functional siRNA binding sites. The criteria these two programs take into account include; analysis of thermodynamic stability, sequence composition, the nucleotides present at the siRNA termini, GC content and OTE etc. One important feature of designing siRNAs is ensuring the siRNA candidate has a low internal stability at the 5' termini of the antisense strand (AS). It has been validated that siRNAs in which the 5' AS termini has low thermodynamic stability are more likely to be functional and are more likely to be loaded into RISC (Khvorova et al, 2003), with this in mind siRNAs which have an A/U as the last base on the 5' end of the AS and which have a significant number of AU's in the sequence of this region are favourable (Birmingham et al, 2007; Reynolds et al, 2004; Shabalina et al, 2006a; Ui tei et al, 2004). This prediction has been confirmed with the finding that the middle (MID) domain of human AGO2 preferentially binds to AMP or UMP as opposed to CMP or GMP (Frank et al, 2010). Of the three siRNAs chosen, siP1 and siP3 had a U/A as the last base on the 5' AS strand, siP2 had a G. All siRNAs designed had a favourable number of A/U nucleotides in the 5' AS region. siP1 and siP3 had 4/5 A/Us in this region and siP2 had 3/5 A/Us. A G/C at the 5' termini of the sense strand (SS) was also predicted to be favourable (Ui-Tei et al, 2004). Both siP2 and siP3 had G/C in this region. In addition, a GC content of between 30-60% is essential for ensuring efficient separation of the sense and antisense strands within RISC (Birmingham et al, 2007; Reynolds et al, 2004). All siRNAs had % GC content within this range. Other sequence effects such as internal repeats, palindrome sequences, a GC stretch bigger than 7 nucleotides and a repeat run of single nucleotides are unfavourable (Khvorova et al, 2003; Ui Tei et al, 2004). None of the siRNAs selected had any of these features. Taking all the above optimal parameters into account

siP3 was predicted as the most efficient siRNA out of the three. This result correlated with the fact that it was ranked in the top three of the BLOCK-IT list of favourable hRHO targets as opposed to siP1 and siP2 which was seventh and tenth respectively with the GeneScript Corporation algorithm.

A limiting factor of *in vitro* rhodopsin experiments is the lack of an immortal photoreceptor cell line endogenously producing sustained consistent levels of the rhodopsin protein. Rhodopsin is only expressed in rod photoreceptors and the pineal gland, i.e., in cells that are post-mitotic and difficult to culture. One retinoblastoma cell line, Y79 has been shown to express rod specific genes including low levels of rhodopsin RNA (Di Polo et al, 1995). Furthermore Y79 cells have subsequently been used for evaluating the transcriptional regulation of various rod specific genes including rds-peripherin, rhodopsin and cGMP-PDE β (Cai et al, 2010b; Rehemtulla et al, 1996; Verado et al, 2009). Thus it was proposed that Y79 cells may be beneficial for the analysis of therapeutic strategies for rhodopsin *in vitro*. However in the laboratory, Y79 cells stably expressing RHO soon lost their initial RHO expression levels, possibly due to the innate toxicity of the protein, excluding this cells line from future studies (Dr. Sophia Millington-Ward, unpublished data). Another method for the analysis of endogenous rhodopsin expression has been successfully employed by culturing retinal murine explants *ex vivo* and transfecting these with particular therapeutics (Palfi et al, 2006). However, this method is rather cumbersome, so for a quick and reliable *in cellulo* method for testing suppression, an alternative approach was adopted where a hRHO plasmid and siRNAs were co-transfected into HeLa cells. HeLa cells were chosen as these cells are amenable to efficient transfection by Liposomes (McManus and Sharp, 2002). One difficulty with these rhodopsin co-transfection experiments using Lipofectamine2000® has been toxicity resulting in cell death. Ensuring the cells were of the proper confluency at the time of transfection and that the passage numbers of HeLa cells were optimal or at least did not differ too much between transfections (p12-p18 was always used in these experiments) was of the utmost importance. Furthermore, in cases where cell death following transfection was observed, there were also a number of healthy cells adhered to each well when the dead cells had been washed away with PBS. The proportion of cell death in each co-transfection well was approximately equal as evaluated at the observational level, allowing for consistent measurement of RNA and protein expression between wells and siRNAs. It could be argued that gene expression profiles may change as cells begin to die. The level of cell death was variable at all times and as previously mentioned was dependent on cell confluency and passage number. Yet despite this variability between experiments, consistent results in terms of levels of suppression achieved with a particular siRNA were always obtained.

Suppression of rhodopsin in HeLa cells by the first two siRNAs, siP1 and siP2, was evaluated at the RNA level using real-time RT-PCR (**Figure 3.3.4.a**). Both siRNAs significantly suppressed rhodopsin, with siP2 achieving 78% knock-down of RHO. These results mirrored other studies utilising siRNA against rhodopsin *in vitro*. Gorbatyuk et al, reported a RHO suppression level of up to 80% evaluated in HEK 293 cells as analysed by real-time RT-PCR (Gorbatyuk et al, 2007b). Interestingly previous studies from our laboratory have obtained up to 87% suppression *in vitro* in HeLa cell culture with amongst others siBB (Chadderton et al, 2009; O'Reilly et al, 2007). This siRNA represents the positive control used in the current study and a significant down-regulation of rhodopsin RNA by approximately 85% was observed using siBB in these experiments. This level of suppression is consistent with O'Reilly et al, 2007, thus indicating the validity of our experimental control and the co-transfection procedure.

Down-regulation of RNA should theoretically result in down-regulation of protein expression. Therefore in order to minimise variability, every time a co-transfection experiment was undertaken, one set of wells was used for RNA analysis and another set of wells was used for protein analysis. This ensured that all cells tested, whether for RNA or protein, were subject to the same experimental procedures. In order to quantify rhodopsin protein expression in the laboratory, previous studies typically relied on enzyme-linked immunosorbent assays (ELISAs) and immunohistochemical techniques. While these techniques are valid, it was felt that optimising the western blotting procedure for rhodopsin protein would be of benefit for both this study and for future analysis involving rhodopsin. The western blotting procedure was readily optimised owing to the fact that a good anti-mouse rhodopsin antibody (4D2) was available in the laboratory which cross-reacts well with human rhodopsin. This antibody was designed to target the n-terminus of the bovine rhodopsin protein (Hicks & Molday, 1986). Rhodopsin protein in all western blotting experiments was visible as multiple bands. While the 39kDA monomer of rhodopsin protein was always observed, very often bands of higher molecular weight were also present (**Figure 3.3.5.a**). These bands were thought to represent various dimers, trimers and oligomers of the rhodopsin protein. This is a known characteristic of this protein (Fotiadis et al, 2003; Illing et al, 2002).

At the protein level, bands of hRHO in the positive control sample controlling for RNAi suppression (HeLa cells co-transfected with hRHO & siBB) were always absent (**Figure 3.3.5.b [lane 2]**). In fact this lane of the gel looked similar to the lane with protein from untransfected control HeLa cells (where no rhodopsin is expressed in HeLa cells). This result supports the ELISA and immunohistochemistry results observed by O'Reilly et al, 2007. siP2 also efficiently suppressed hRHO protein, indicating that

like siBB, it is indeed a potent RNAi based suppressor *in vitro* (**Figure 3.3.5.b [lane 4]**). Somewhat surprisingly, potent suppression of hRHO by siP1 was not observed at the protein level (**Figure 3.3.5.b [lane 3]**). Currently it is not known why this was found to be the case. However, siP1 suppressed rhodopsin RNA the least efficiently and it was therefore not unexpected that the level of protein suppression may be lower. The estimated half life of rhodopsin is 30 hours. Thus a possible reason for the lack of RHO protein suppression observed by siP1 may be that by extracting the cellular protein 24 hour post-transfection, enough time has not passed between RNA suppression and degradation of the existing rhodopsin protein. There are two pieces of evidence that do not support this hypothesis. The first is that none of the other siRNAs tested in the same way showed a reduction in suppression efficiency at the protein level. Secondly all studies undertaken were co-transfection experiments, transfecting in wild-type rhodopsin cDNA at the same time as the siRNA into a rhodopsin-deficient cell line. There is, therefore no pre-existing store of rhodopsin protein present before siRNA-based suppression occurs. One possibility is that siP1 suppresses RHO more slowly than the hRHO plasmid produces mRNA, allowing rhodopsin protein to accumulate. There may be a threshold present to the level of hRHO RNA that must be suppressed in order to correspond to a change in the protein level. If this is the case, siP2 suppresses hRHO RNA by 78% and siP1 suppresses by 64% this threshold may possibly lie somewhere between these two points, however this is just speculation. In terms of *in vivo* rhodopsin expression, hRHO rhodopsin is a highly expressed protein, accounting for 90% of the protein in the membranes of the ROS (Palczewski et al, 2006). Therefore it is likely that a potent suppressor would be required in order to suppress such high quantities of rhodopsin and achieve therapeutic benefit. Thus, the fact that siP1 does not suppress hRHO RNA or protein as efficiently as siP2 would preclude its further *in vivo* evaluation.

As siP1 was inefficient at suppressing rhodopsin protein expression in HeLa cells after 24 hours, it was decided a third siRNA (siP3) would be designed and evaluated. siP3 suppressed hRHO RNA the most efficiently (92%) of all three siRNAs tested (**Figure 3.3.6.a**). In addition siP3 was predicted to be the most favorable and efficient siRNA based on various siRNA design rules incorporated into the online programs used for siRNA target selection, further strengthening the validity of these online programs. In terms of the most efficient siRNA at suppressing hRHO at the RNA level, siP3 and siBB were statistically the same. In contrast siP3 was found to be significantly more potent than either siP2 or siP1. Therefore based on these *in vitro* studies siP3 and siBB would seem to be statistically the best hRHO siRNAs at the RNA level.

Protein analysis of siP3-based suppression was undertaken, again using western blotting (**Figure 3.3.7.a**). In all cases rhodopsin expression appeared completely obliterated in cells transfected with siP3 and hRHO (**Figure 3.3.7.a [lane 3]**). No bands were observed in these wells and results were similar to those obtained for the untransfected HeLa cell control (**Figure 3.3.7.a [lane 4]**). This result was comparable to that observed with siBB (**Figure 3.3.7.a [lane 2]**). Consequently in terms of efficacy, as with the RNA results siBB and siP3 may be the most potent suppressor molecules for future use in animal models such as the porcine RP models.

Overall the aim of the research described in this chapter of the thesis involved designing and evaluating a suitable siRNA homologous to both pRHO and hRHO, which could subsequently be used for *in vivo* evaluation. *In vivo* evaluation for an RNAi mutation-independent strategy has never before been explored in larger animal models for RHO-adRP. Owing to the availability of a RHO-adRP porcine model, it was decided to generate a siRNA based gene therapy for use in both pigs and humans (Petters et al, 1997). Typically, the evaluation of an RNAi based *in vivo* therapeutic, is initially evaluated using siRNAs *in vitro*. The most efficient siRNAs will then subsequently be generated into shRNA constructs and evaluated *in vivo*. The least efficient siRNAs will not proceed to *in vivo* evaluation. During the course of the study outlined in this chapter of the thesis, three siRNAs were designed and evaluated. Two of the three siRNAs (siP2 and siP3) were found to significantly suppress hRHO RNA and protein expression in cell culture. siP3 potently suppressed expression of hRHO at a level comparable to siBB previously evaluated in the laboratory (O'Reilly et al, 2007). Furthermore, unlike both siP2 and siBB, siP3 is not homologous to mRHO mRNA, therefore in principle this siRNA could be ideally utilised in a RHO-adRP mouse model for evaluating therapeutic benefit associated with suppression of the mutant hRHO while maintaining expression of mRHO. Chadderton et al, 2009 demonstrated therapeutic efficacy in an adRP mouse model containing a mutant transgene by AAV-mediated subretinal delivery of an siRNA (shQ1) targeting the hRHO transgene but containing mismatches to the endogenous mRHO alleles (Chadderton et al, 2009). Thus it was proposed that like shQ1, siP3 may be useful in a RHO-adRP transgenic mouse for suppressing a mutant pRHO or hRHO, while leaving the endogenous mRHO alleles protected. These endogenous wild-type alleles could therefore act as a natural replacement and the therapeutic benefit of siP3 could be assessed. For the reason outlined above it was decided to further evaluate siP3 based suppressors *in vivo*.

hRHO F	5'-CTTTCCTGATCTGCTGGGTG-3'
hRHO R	5'-GGCAAAGAACGCTGGGATG-3'
β -actin F	5'-AGAGCAAGAGAGGCATCC-3'
β -actin R	5'-TCATTGTAGAAGGTGTGGTGCC-3'

Table 3.2.5 primers used for real-time RT-PCR analysis

Both hRHO and β -actin primers pairs were optimised using RNA from HeLa cells co-transfected with hRHO and siNT. hRHO amplifies hRHO products with a T_m of 81°C and that are 108bp in length. The β -actin primers amplify β -actin cDNA transcripts with a T_m of 81°C and these products are 103bp in length. All primers were obtained from Sigma-Aldrich

siP1 target

Human RHO 289 ACCTCTCTGCATGGATACT

|||||

Pig RHO 289 ACCTCTCTGCATGGATACT

siP2 target

Human RHO 618 CATGTTTCGTGGTCCACTTC

|||||

Pig RHO 618 CATGTTTCGTGGTCCACTTC

siP3 target

Human RHO 404 GGTACGTGGTGGTGTGTAA

|||||

Pig RHO 404 GGTACGTGGTGGTGTGTAA

Figure 3.3.1.a. hRHO and pRHO siRNA targets and homology

siP1 targets nucleotide 289 to 308 of hRHO and pRHO, siP2 targets 618 to 637 of hRHO and pRHO and siP3 targets position 404 to 423 of hRHO and pRHO.

<u>siRNA</u>	<u>Homologous organisms</u>
siP1	human, pig, chimpanzee, dog Human ACCTCTCTGCATGGATACT Pig ACCTCTCTGCATGGATACT Chimp ACCTCTCTGCATGGATACT Dog ACCTCTCTGCATGGATACT Cow ACCTCTCTGCACGGGTACT Mouse ACATCACTCCATGGCTACT Rat ACCTCACTGCATGGCTACT *****
siP2	human, pig, chimpanzee, dog, cow, mouse, rat Human CATGTTCGTGGTCCACTTC Pig CATGTTCGTGGTCCACTTC Chimp CATGTTCGTGGTCCACTTC Dog CATGTTCGTGGTCCACTTC Cow CATGTTCGTGGTCCACTTC Mouse CATGTTCGTGGTCCACTTC Rat CATGTTCGTGGTCCACTTC *****
siP3	human, pig, chimpanzee, dog Human GGTACGTGGTGGTGTGTAA Pig GGTACGTGGTGGTGTGTAA Chimp GGTACGTGGTGGTGTGTAA Dog GGTACGTGGTGGTGTGTAA Cow GGTACGTGGTGGTGTGCAA Mouse GGTACGTGGTGGTGTGCAA Rat GGTACGTGGTGGTGTGCAA * ***** *

Table 3.3.1.b The homology of each siRNA to rhodopsin in different organisms

Of all the three siRNAs designed, only the siP2 target site is homologous to the sequences of all organisms examined including mRHO. siP1 and siP3 are not homologous to the mRHO sequence (yellow nucleotides). In addition these siRNAs also contain mismatches to the cow rhodopsin sequence and the rat rhodopsin sequence (yellow nucleotides). The fact that siP1 & siP3 are not homologous to mRHO is of particular relevance for future *in vivo* applications of siRNAs, as the initial testing *in vivo* typically involves transgenic mice.

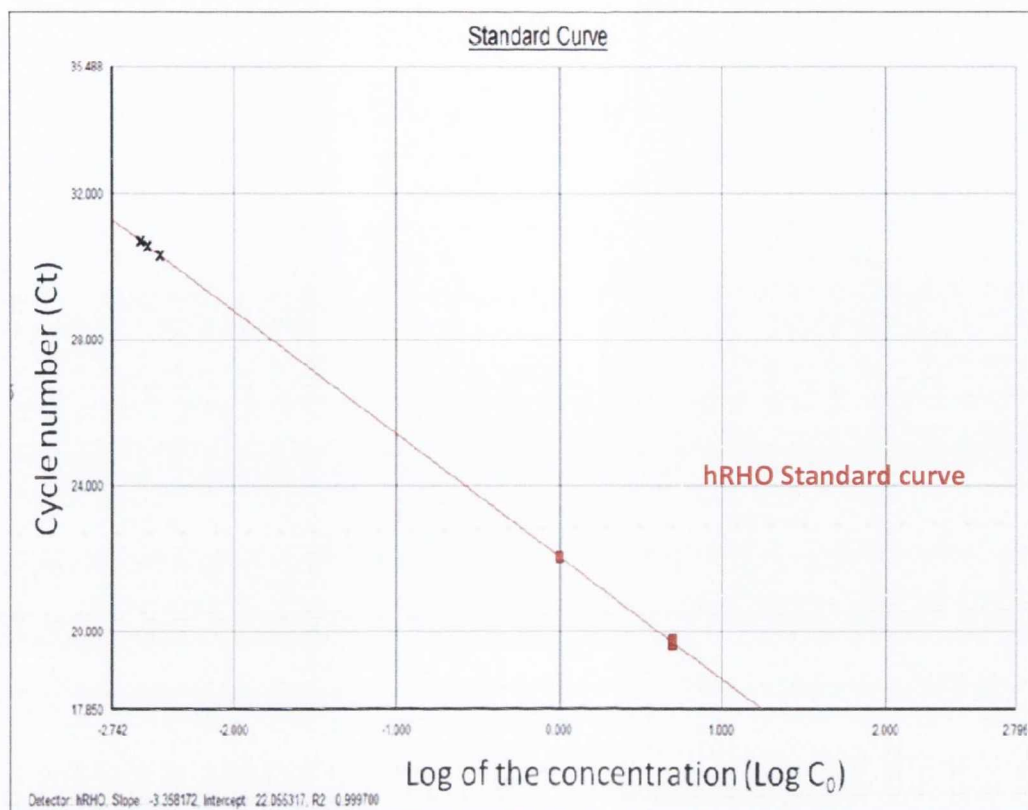


Figure 3.3.3.a hRHO standard curve.

This standard curve was generated using RNA from the HeLa cell sample co-transfected with hRHO and siNT. Five, 5X serial dilutions were used to generate the standard curve. The cycle threshold (C_t) is the point at which fluorescence from each accumulating amplicon in each standard curve crosses the threshold value (assigned at a point during which the rate of amplification is exponentially increasing relative to the baseline). This number is then plot against the log of the known concentration of values (each 5X standard was arbitrarily assigned 1, 5, 25, 125 and 625) and a line of best fit is generated. Providing the slope and R² values are in the right range the C_t of each hRHO sample can be measured against this line and the relative concentration calculated. In this case the slope of the hRHO standards is -3.4 which is in the right range (-3.1 to -3.6 i.e. 100±10% efficiency) and the R² value is 0.9997 which is very close to 1.

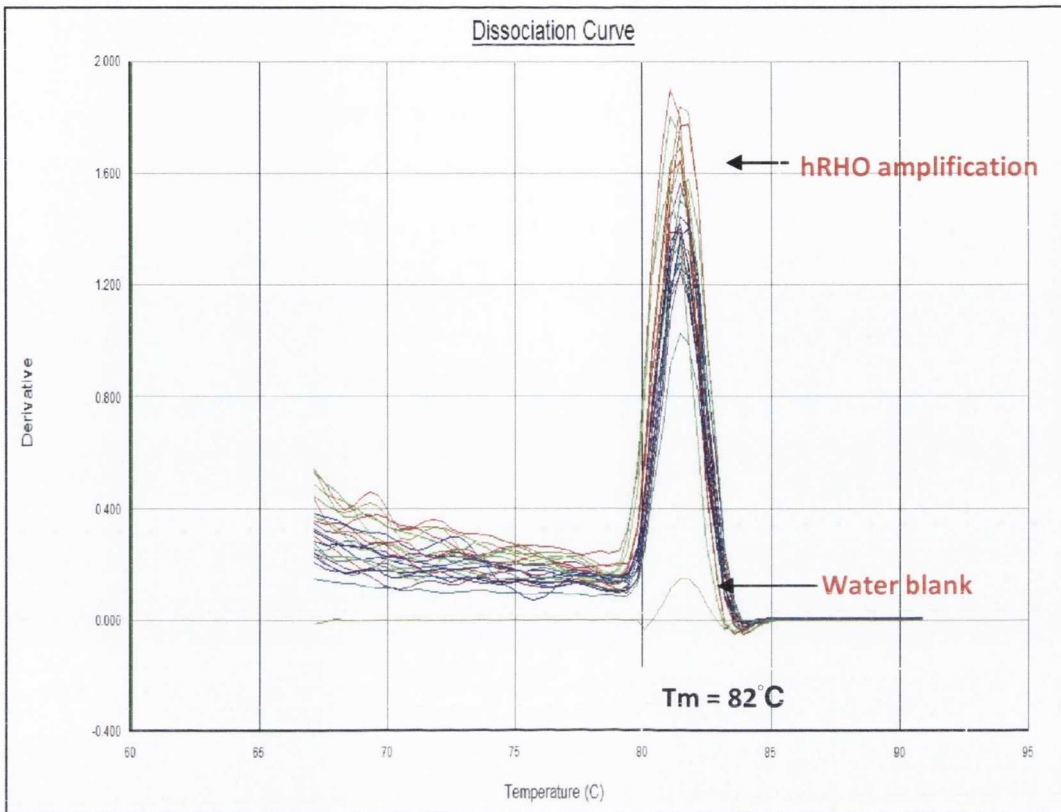


Figure 3.3.3.b Melting curve analysis of hRHO amplicons

This figure describes the dissociation curve generated from the hRHO amplification that results from the two hRHO primers used. Each product has a specific melting temperature (T_m), this is the temperature at which the double stranded DNA is denatured (Temperature C), and is correlated to the change in the fluorescence produced by Sybr green at this T_m (Derivative). As can be observed from the above figure, the only peak present is that at 82°C, this is the correct predicted T_m for the hRHO amplicon. There is a small peak present in the water blank sample, which suggests a small amount of hRHO contamination; this however is at such a small value that it is not deemed to have an impact on the analysis of hRHO expression in each sample

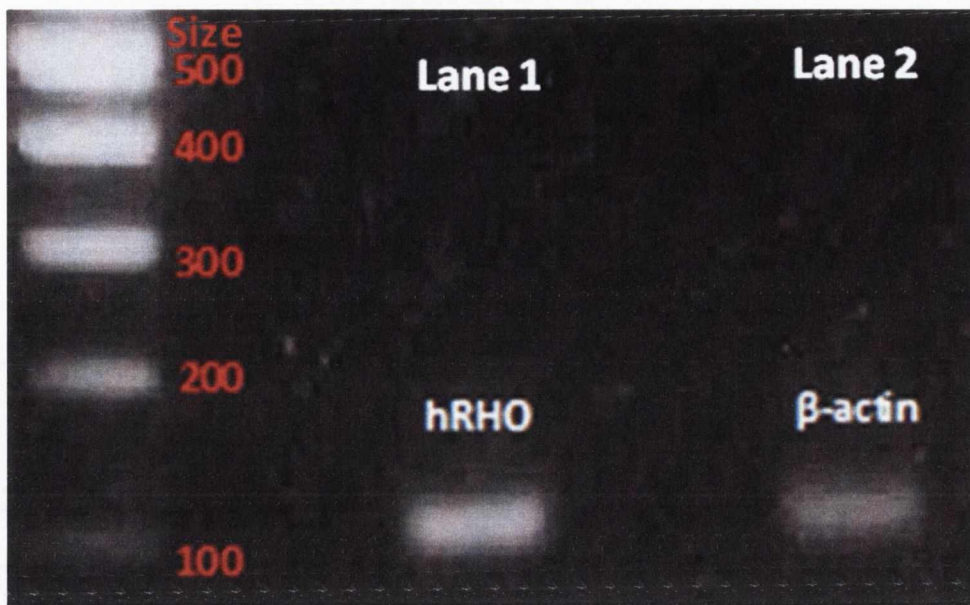


Figure 3.3.3.c 2% agarose gel analysis of hRHO and hβ-actin RT-PCR amplification products.

From the PCR plate used to optimise hRHO and hβ-actin, a sample from each primer pair was taken from two different wells. One well containing cDNA amplified by hRHO and another well containing cDNA amplified by hβ-actin primers was used. These products were run on a 2% agarose gel. A band of approximately 108bp was amplified for hRHO primers (lane 1) and a band of 103bp was amplified for the hβ-actin primers (lane 2).

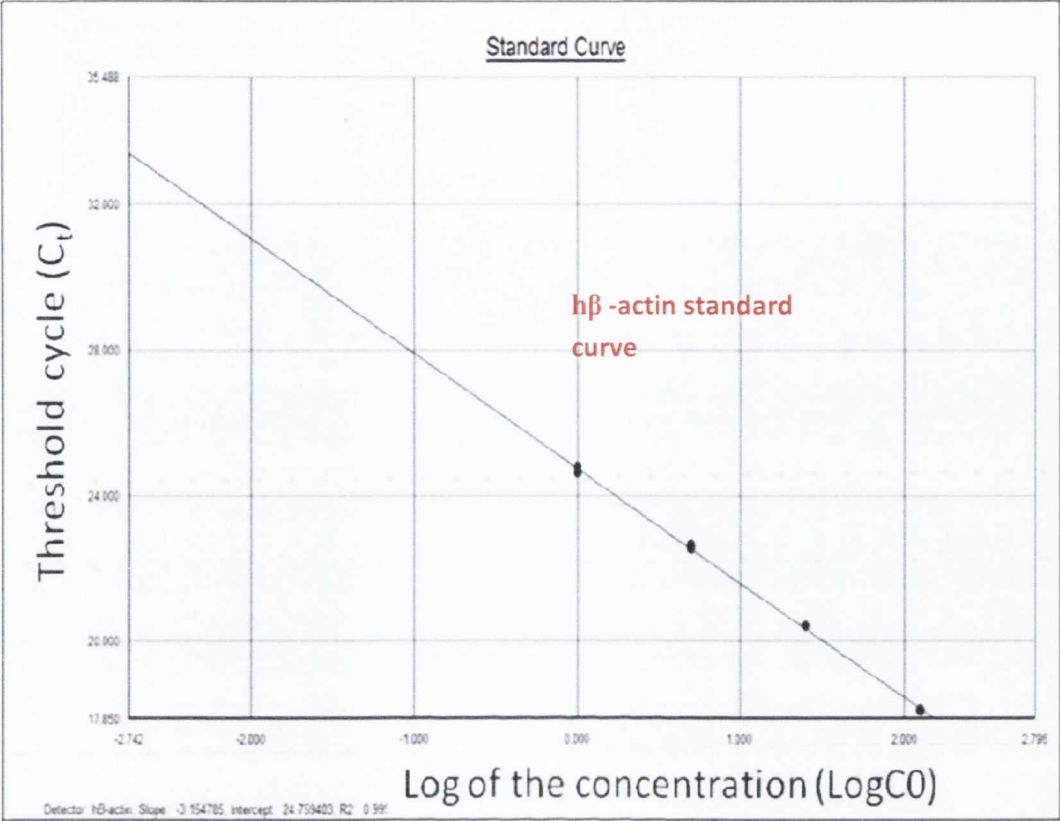


Figure 3.3.3.d hβ-actin standard curve.

This hβ-actin standard curve was generated using RNA from the HeLa cell sample co-transfected with hRHO and siNT. Five, 5X serial dilutions were used to generate the standard curve. The cycle threshold (C_t) is the point at which fluorescence from each accumulating amplicon in each standard curve crosses the threshold value (assigned at a point during which the rate of amplification is exponentially increasing relative to the baseline). This number is then plot against the log of the known concentration of values (each 5X standard was arbitrarily assigned 1, 5, 25, 125 and 625) and a line of best fit is generated. Providing the slope and R^2 values are in the right range the C_t of each hRHO sample can be measured against this line and the relative concentration calculated. In this case the slope of the hβ-actin standards is -3.2 which is in the right range (-3.1 to - 3.6 i.e. -100±10 efficiency), and the R^2 value is 0.9999 which is very close to 1.

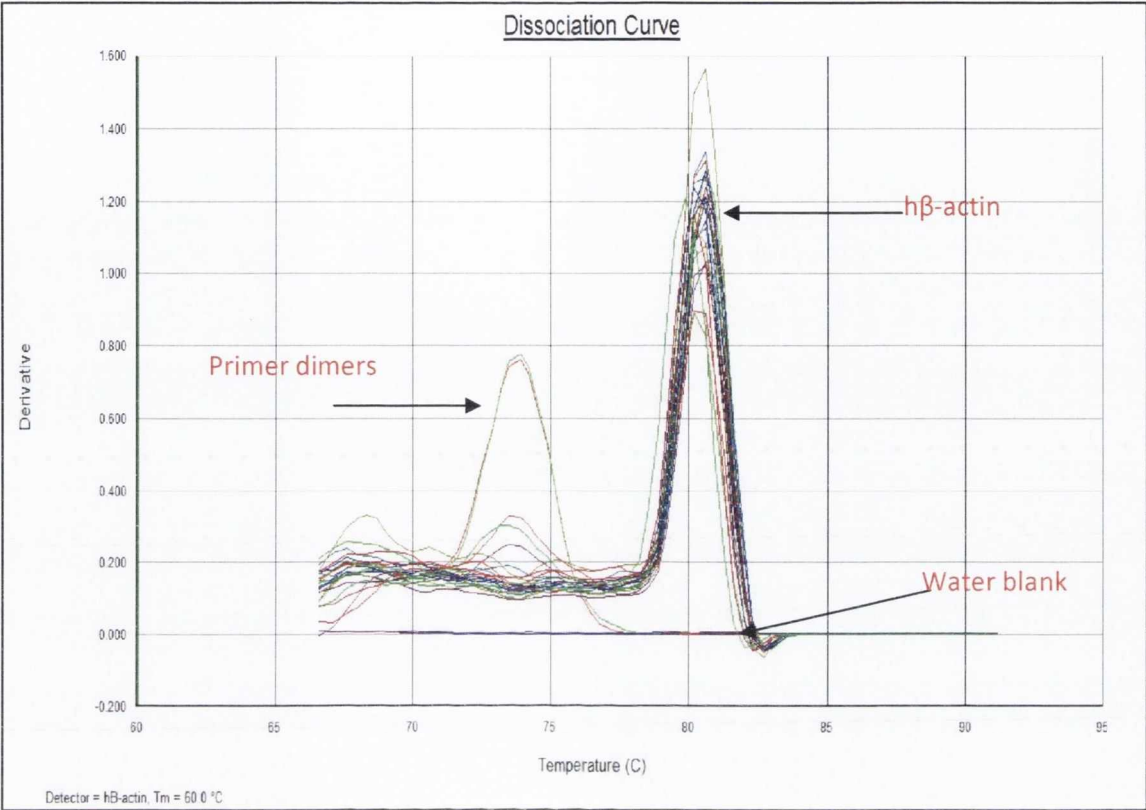


Figure 3.3.3.e Melting curve analysis of hβ-actin amplicons

This figure describes the dissociation curve generated from the hβ-actin amplification that results from the two hβ-actin primers used. hβ-actin has a specific melting temperature (T_m), this is the temperature at which the double stranded DNA is denatured (Temperature C), and is correlated to the change in the amount of fluorescence produced by Sybr green at this T_m (Derivative). The highest peak present is that at 81°C, this is the correct predicted T_m for the hβ-actin amplicon. There is clearly no amplification of hβ-actin in the water blank, as no amplification is occurring at 81°C. The presence of a peak at 73°C in the blank sample is representative of primer dimers. The formation of these dimers often occurs in a sample with little or no DNA present.

RHODOPSIN ANALYSIS

β-ACTIN ANALYSIS

Sample	Primer	Quantity	AVERAGE
siNTa	hRHO	140.82	
siNTb	hRHO	129.669	132.519
siNTc	hRHO	127.067	
siBBa	hRHO	20.6927	
siBB	hRHO	20.3135	21.8329
siBBc	hRHO	24.4927	

Sample	Primer	Quantity	AVERAGE
siNTa	mβ-actin	164.7	
siNTb	mβ-actin	147.1	158.3
siNTc	mβ-actin	163.1	
siBBa	mβ-actin	165.7	
siBBb	mβ-actin	184.8	177.92
siBBc	mβ-actin	183.3	

Divide the average hRHO by the average hβ-actin for each sample (siNT and siBB)

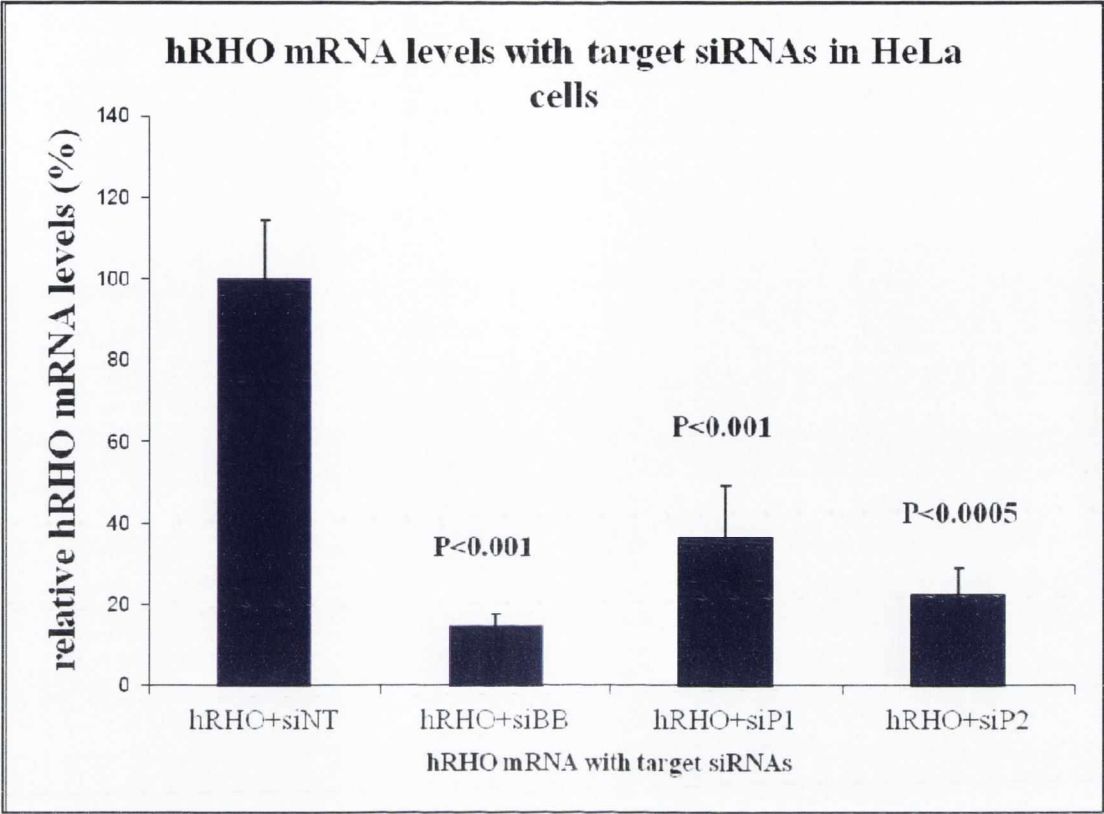
Average sample	hRHO/ mB-actin
siNT	0.837085465
siBB	0.122711893

Set siNT (0.837085465) as 100% hRHO expression and express siBB (0.122711893) as a percentage of the siNT value (0.122711893/0.837085465 X 100)

Sample	% hRHO
siNT	100 %
siBB	15 %

Figure 3.3.4.f A working example of the calculation the % hRHO expression in each sample using for standard curve method

This is an example of typical calculations utilised to obtain the % hRHO RNA expression in RNA samples co-transfected with hRHO and each siRNA compared to the % RNA expression in RNA samples co-transfected with hRHO & siNT as assessed by real-time RT-PCR. Each standard curve sample was assigned 5 arbitrary quantities (1, 5, 25, 125 & 625), thus by comparing the C_t values of “unknown” samples to the C_t values of the standard curve samples the relative concentration (quantity) in each could be assigned. The average of each set of RNA triplicates (siNT a-c and siBB a-c) was obtained for hRHO and hβ-actin and the average hRHO value was normalised to hβ-actin (0.8371 for siNT & 0.1227 for siBB). hRHO RNA expression from samples transfected with hRHO & siNT was set at 100 % and the hRHO RNA expression from samples transfected with hRHO & siBB was expressed as a percentage of this value (15%)



Sample	hRHO +siNT	hRHO + siBB	hRHO +siP1	hRHO +siP2
% of siNT	100	15	36	22
% SD	15	3	13	7

Figure 3.3.4.a. Real-time RT-PCR evaluation of hRHO expression in HeLa cells co-transfected with hRHO and siRNAs

Relative hRHO RNA levels in HeLa cells following co-transfection with hRHO and siNT (a non-targeting siRNA), siBB (a positive control), siP1 and siP2 in HeLa cells. The data are presented as percentages of rhodopsin RNA levels in RNA samples co-transfected with wild-type hRHO and the non-targeting siRNA (siNT, 100%). The average and standard deviation of each sample was calculated using Microsoft Excel. Statistical significance was assessed using a two-sample t-test (DataDesk 6.0). Error bars represent % SD values



SAMPLE NAME	hRHO +	hRHO -
BCA (µg/ml)	2840	3650
protein/ well (µl)	8.45	6.58
[protein]/well (µg)	24	24

Figure 3.3.5.a Optimisation of the 4D2 RHO antibody for western blotting

This pilot study was undertaken in order to evaluate that the 4D2 antibody was working efficiently and specifically binding to hRHO. HeLa protein from cells co-transfected with hRHO and siNT was used as the positive hRHO control (hRHO + cells, lane 1) and untransfected cells were used as the negative hRHO control (hRHO – cells, lane 2). A hRHO monomer is visible at 39kDA in lane 1 as well as various bands at higher molecular weights representing multimeric hRHO. In lane 2 no hRHO is present. This indicates that 4D2 bind specifically to hRHO only in HeLa cells. The table represents the concentration of total protein in each sample added to each well in order to ensure equal loading. As this was an initial assessment of the 4D2 anti-mouse hRHO antibody hβ-actin was not utilised for this particular western blot to evaluate equal loading

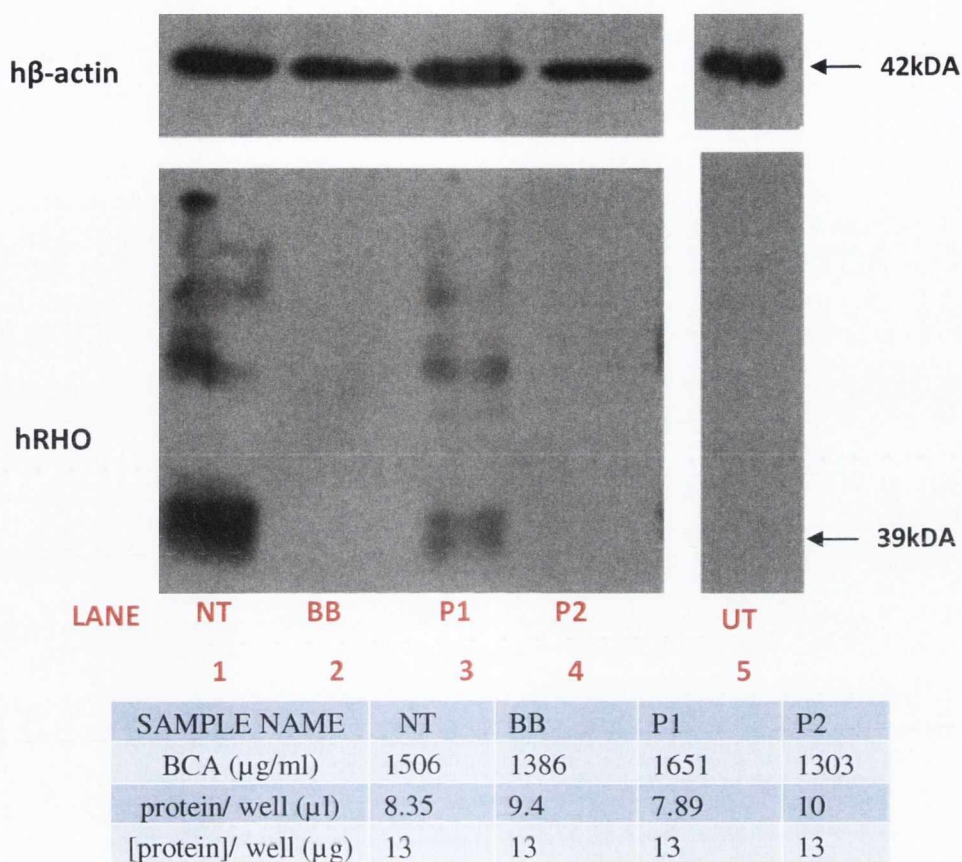
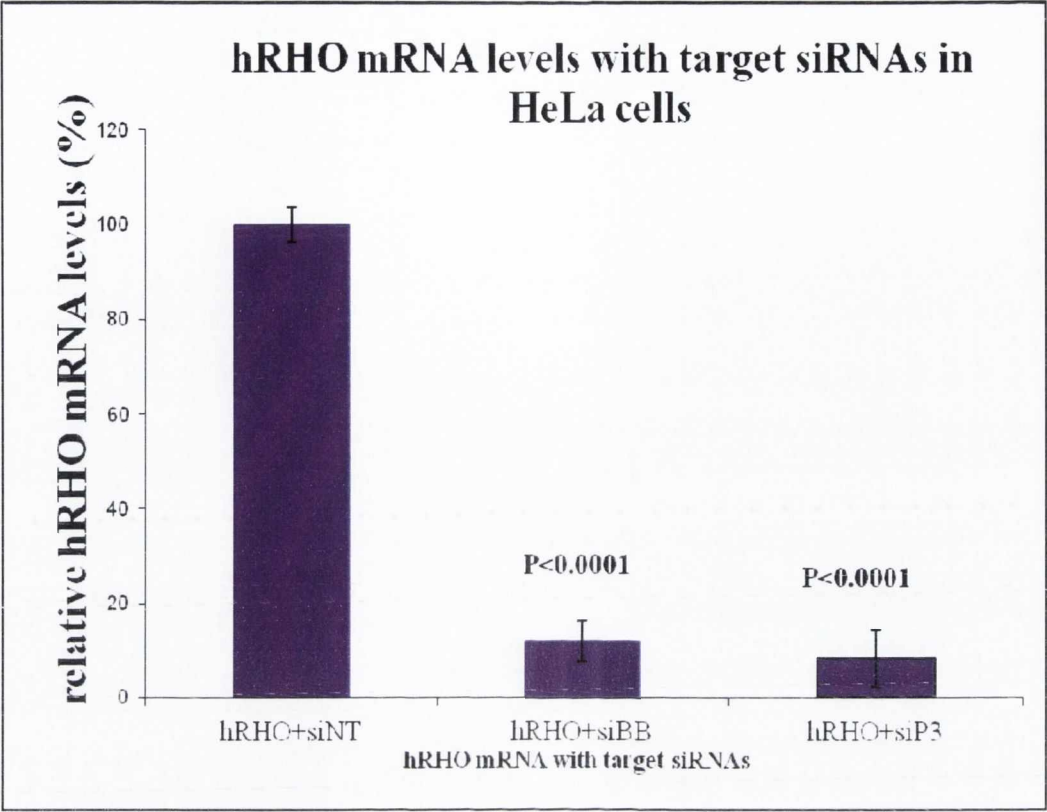


Figure 3.3.5.b Western blot analysis of hRHO protein expression.

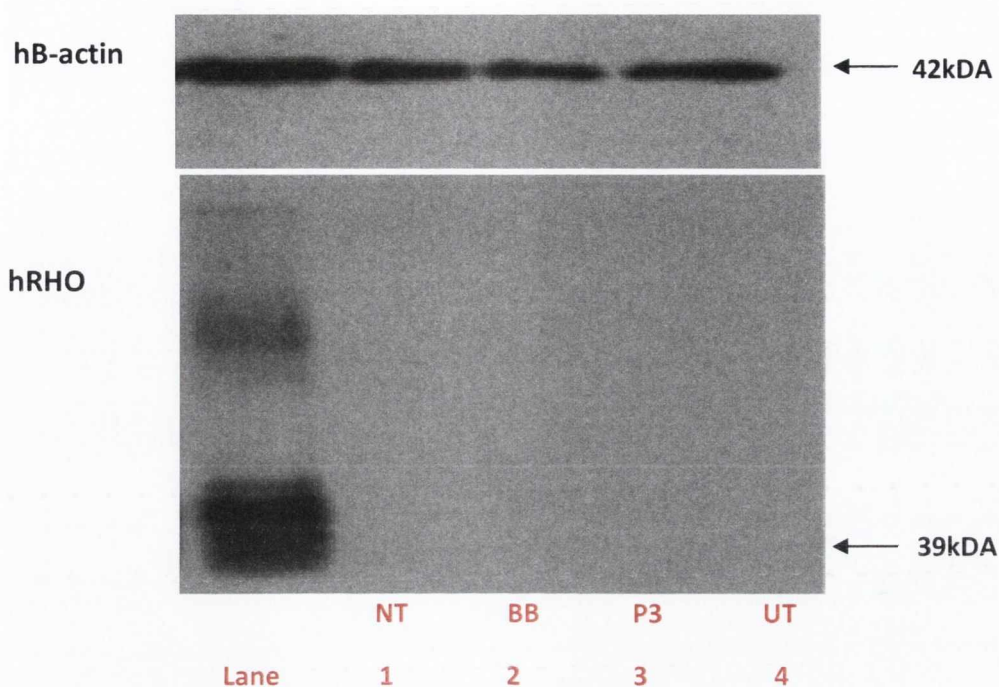
Lane 1 is protein from cells transfected with hRHO and siNT (NT, the negative non-targeting siRNA control). Lane 2 contains protein from cells transfected with hRHO and siBB (BB, a positive control siRNA). Lanes 3 & 4 contains protein from cells transfected with hRHO and siP1 or siP2 respectively (P1 & P2). Lane 5 is protein from untransfected HeLa cells (UT). Human β -actin (42kDA) was used as an equal loading control. The presence of hRHO monomers (39kDA) as well as multimeric forms of hRHO is clearly observed in lane 1 and 3 and completely absent in lane 2, 4 and 5 (untransfected cells). This indicates that siBB and siP2 are causing a significant reduction in hRHO protein with no visible protein remaining. siP1 is not as efficient at suppressing hRHO at the protein level. The table represents the concentration of total cytoplasmic protein in each sample added to each well in order to ensure equal loading. This is a representative blot of one transfection experiment. Additional western blotting was carried out with three more transfection experiments and the same result was obtained in all cases.



sample	hRHO+siNT	hRHO + siBB	hRHO+ siP3
% of siNT	100	15	8
% SD	9	2	6

Figure 3.3.6.a Real-time RT-PCR evaluation of hRHO expression in HeLa cells co-transfected with hRHO and siP3

Relative hRHO RNA levels in HeLa cells following co-transfection with hRHO and siNT (a non-targeting siRNA), siBB (a positive control) and siP3 in HeLa cells. The data are presented as percentages of rhodopsin RNA levels in RNA samples co-transfected with wild-type hRHO and the non-targeting siRNA (siNT, 100%). The average and standard deviation of each sample set was calculated by Microsoft Excel. Statistical significance was assessed using a two sample t-test (DataDesk 6.0). Error bars represent SD values



SAMPLE NAME	NT	BB	P3	UT
BCA (μg/ml)	423	678.2	698.4	698.4
protein/ well (μl)	20	12.5	12.11	12.11
[protein]/ well (μg)	8.46	8.46	8.46	8.46

Figure 3.3.7.a Western blot analysis of hRHO protein expression in cells co-transfected with hRHO and siP3

Lane 1 contains protein from cells transfected with hRHO and siNT (NT, the negative non-targeting siRNA control). Lane 2 is protein from cells transfected with hRHO and siBB (BB, a positive control siRNA). Lane 3 contains protein from cells transfected with hRHO and siP3 (P3). Lane 4 is protein from untransfected HeLa cells (UT). Human β -actin (42 kDA) was used as an equal loading control. The presence of hRHO monomers (39kDA) as well as multimeric forms of hRHO is clearly observed in lane 1 and completely absent in lane 2, 3 and 5 (untransfected cells). This indicates that siBB and siP3 are causing a significant reduction in hRHO with no visible protein remaining. The table represents the concentration of total cytoplasmic protein in each sample added to each well in order to ensure equal loading.

Chapter 4: The *in vivo* evaluation of human rhodopsin suppression by shRNA and artificial miRNA based constructs

4.1. Introduction

The screening of potential RNAi-based suppressors to efficiently knock-down mRNA expression of target genes often initially involves the design, generation and evaluation of siRNA based suppressors *in vitro* as described in Chapter three for rhodopsin. However suppression by siRNAs is only temporary, owing to their short half life and rapid degradation (Kim & Rossi, 2007; McManus & Sharp 2002; Sibley et al, 2010). An efficient therapeutic for human clinical trials, ideally must be capable of long-term sustained expression. In 2002 this siRNA limitation was overcome by the design of shRNA molecules (**Section 1.16**), capable of expression from different vectors and different promoters (Brummelkamp et al, 2002; Paddison et al, 2002). These shRNAs are composed of the sense and antisense siRNA duplexes connected by a stem loop, and are designed to resemble pre-miRNAs (Sibley et al, 2010). shRNAs have been used with success in a large range of *in vivo* studies and are currently being investigated for use in human clinical trials (Davidson et al, 2011). However, despite the excitement surrounding RNAi-based therapeutics, recent advances particularly in the liver and central nervous system (CNS) have indicated that achieving high therapeutic efficacy with low toxicity using shRNAs may not be as simple as previously envisioned (Boudreau et al, 2008; Boudreau et al, 2009a; Ehler et al, 2010; Grimm et al, 2006; Grimm et al, 2010; McBride et al, 2008).

4.1.1 shRNA mediated toxicity

A major indication that shRNAs may result in toxicity came from studies by Grimm et al, 2006, who discovered high dose systemic shRNA administration to the mouse liver resulted in severe hepatotoxicity and death in mice irrelevant of the shRNA target (Grimm et al, 2006; Grimm et al, 2010). Furthermore these high dose shRNAs caused down-regulation of important liver miRNAs (*in vitro* and *in vivo*), suggesting that the shRNAs may be saturating the endogenous RNAi machinery. Lowering the shRNA dose tenfold alleviated this liver toxicity; further indicating high dose shRNA delivery may have detrimental effects (Grimm et al, 2006; Yi et al, 2005). Over expression of Exportin V appeared to improve liver cell survival, suggesting that competition between shRNA and miRNAs may result in the saturation of RNAi machinery, in particular Exportin V (Grimm et al, 2006). Recently the saturation of the AGO proteins by shRNAs has also been implicated as being involved in this process (Grimm et al, 2010; Vickers et al, 2007). Furthermore Beer et al, 2010 established that even low doses of shRNAs irrespective of the target may induce a subtle level of liver damage promoting liver cell division. While this did not result in severe toxicity in wild-type mice, in mice expressing an oncogene MYC it resulted in an increase of tumorigenesis. This highlights the need to regulate shRNAs in models where oncogenesis may be promoted (Beer et al, 2010). Beverley Davidson

and colleagues have explored the effects of AAV-1 delivered, U6 driven shRNAs targeting Huntington mRNA and SCA-1 mRNA in the brains of transgenic mice. Striatal toxicity and Purkinje cell death was obtained in these mice due to high levels of unprocessed shRNA transcripts (Boudreau et al, 2008; Boudreau et al, 2009a; McBride et al, 2008). Neuronal toxicity was also observed in the red nuclei of AAV-1 delivered CMV driven shRNAs targeting Class 3 Semaphorin receptors in rats. This resulted in severe toxicity in rat red nuclei, independent of AAV or GFP (Ehlert et al, 2010). In addition, Martin et al, 2011 obtained shRNA mediated striatal toxicity in mouse brains. A shRNA was administered to both transgenic (a human DYT1 dystonia model) and wild-type mouse via intrastriatal injections, severe toxicity and death was observed in these mice. Of particular note, the level of toxicity was dependent on the genetic background. An earlier onset, more severe effect occurred with mice with a 129/SvEV background compared to C57BL/6 background. This is an important finding and must be taken into account for future *in vivo* studies (Martin et al, 2011).

4.1.2. Reducing the shRNA dose

Clearly shRNA dose can have significant effect on tissue toxicity, particularly in liver and brain. This is clearly an unwanted side effect of shRNAs, and ways to circumvent this need to be explored. While Grimm et al, 2006 found that lowering shRNA dose tenfold, alleviated toxicity and still resulted in suppression in the liver, McBride et al, 2008 established that lowering the AAV titre by a full log, did not lessen toxicity, furthermore, decreasing the titre any lower than this abolished therapeutic efficacy in the striatum (McBride et al, 2008). Similarly, Ehlert et al, 2010 switched from using AAV-1 based vectors to AAV-2 based vectors in the rat red nuclei as AAV-2, has lower transduction rate than AAV-1 in this tissue. While toxicity was not observed, efficacy of the shRNA was eradicated (Ehlert et al, 2010). Consequently in terms of designing a therapy for clinical trial, while lowering the viral titre or transduction may reduce toxicity, it does so potentially at the cost of therapeutic benefit.

Other methods to overcome abundant expression of unprocessed shRNAs, resulting in the saturation of the RNAi machinery could include over-expressing RNAi proteins which become saturated. This was validated by Grimm et al, 2010 who relieved hepatotoxicity *in vivo* by over-expressing Exportin V and AGO2 while still maintaining efficacy (Grimm et al, 2010). However, for human clinical trials, over-expressing such proteins may not always be a feasible option. One possibility could involve utilising weaker promoters to drive shRNA expression. Highly expressing promoters such as the polymerase III promoter U6 can be more toxic than weaker promoters in certain tissues. In primary human T lymphocytes U6 driven shRNAs (U6-shRNAs) for CCR5 were much more potent than H1-driven shRNAs (H1-shRNAs), however, U6-shRNAs produced much more shRNA compared to H1 which

was most likely the reason for this difference. Both *ex vivo* and *in vivo* administration of U6-shRNAs resulted in a loss of cell number and a reduction in EGFP positive cells compared to administration of H1-shRNAs. Furthermore H1-shCCR5 was still capable of significant suppression *in vivo*, making it a more desirable candidate for reducing toxicity and maintaining efficacy (An et al, 2006). Moreover, Grimm et al, 2010 utilised weaker polymerase III H1 or 75K promoters to express shRNA in the liver. This resulted in reduced hepatotoxicity *in vivo* compared to U6 and conferred significant therapeutic efficacy. shRNA expression is driven primarily by polymerase III promoters, as they are easy to clone and have well defined transcription and termination signals. Giering et al, 2008 set about optimising shRNAs under the control of polymerase II promoters. One such APoE promoter driven shRNA had no toxicity associated with it, even at high doses, compared to U6. Furthermore a significant level of suppression was maintained despite a 40X decrease in shRNA transcripts from ApoE compared to U6. Additionally the expression of the shRNA was confined to the liver, which is not a trait of constitutive polymerase III promoters (Giering et al, 2008).

4.1.3. Utilising artificial miRNAs

An approach, currently gaining popularity is the use of artificial miRNAs. This involves, removing endogenous mature miRNA duplex sequences from endogenously expressed miRNAs and replacing with siRNA sequences within these pri-miRNA backbones (Boudreau et al, 2008; Sibley et al, 2010). These artificial miRNAs would resemble and be processed in a similar manner to natural miRNAs upstream of Drosha, and thus may improve efficacy. Furthermore miRNA are more flexible than shRNAs in that they are more amenable to incorporation of polymerase II promoters, thus these artificial miRNA based constructs could in principle be expressed in a controlled, tissue specific manner (Sibley et al, 2010).

4.1.4 Artificial miRNA-30a

Artificial miRNA based systems initially evolved from the exploration of miR-30a. This miRNA was first isolated from HeLa cells as an endogenously expressed HeLa miRNA (Lagos-Quintana et al, 2001). Experiments expressing a 71 nucleotide miR-30a within various mRNAs sequences, introns or 3' UTRs in cell culture, resulted in its efficient processing of a 22 nucleotide functional miRNA, capable of knocking down target gene expression (Zeng & Cullen, 2005). Furthermore, replacing the mature miR-30a duplex sequence with siRNA sequence resulted in efficient siRNA processing and suppression of the siRNA target via mRNA cleavage (Zeng et al, 2002; Zeng et al, 2003). Following on from this innovative artificial miRNA design, several studies utilised artificial miRNA based on

miR-30a under the control of both polymerase II and polymerase III promoters to efficiently suppress target genes (Boden et al, 2004; Silva et al, 2005). Despite the initial excitement surrounding these artificial miRNA based suppressors, Li et al, 2007 demonstrated that the efficacy of both polymerase II CMV driven and polymerase III U6 driven artificial miRNAs, were significantly reduced compared to their shRNA counterparts *in vitro* (Li et al, 2007). However, evaluation of transgenic mice brains have at times revealed toxicity associated with shRNA molecules (Boudreau et al, 2008; Boudreau et al, 2009a; Martin et al, 2011; McBride et al, 2008). Notably by expressing shRNAs in the backbone of miRNA-30a, this toxicity issue was drastically alleviated. Furthermore these artificial miRNAs suppressed target transcripts at comparable levels of shRNA equivalents (McBride et al, 2008). Boudreau et al, 2008 set about reducing the variability between these two constructs (shRNA and artificial miRNA), in order to gain an accurate comparison of efficacy. This involved designing shRNA and artificial miRNAs with optimal overhangs and processing sites such that processed siRNA sequences from shRNAs and miRNAs would have similar strand bias. Evaluation of these optimal constructs *in vitro* and *in vivo*, resulted in more potent suppression by the shRNA based construct, as described by Li et al 2007 (Boudreau et al, 2008; Li et al, 2007). Additional exploration revealed that while unprocessed shRNA transcripts can compete with endogenous miRNA biogenesis, artificial miRNAs do not, even when used at a tenfold higher dose than shRNAs (Boudreau et al, 2009a). *In vivo* AAV-2/1 injection of shRNAs or miRNAs designed to target human spinocerebellar ataxin-1 (SCA-1) into the cerebellum of SCA-1 transgenic mice resulted in efficient silencing of SCA-1 in addition to histological improvement with the miRNA. The AAV-delivered shRNA construct in contrast resulted in severe neuronal death (Boudreau et al, 2009a). Furthermore an artificial miRNA targeting Huntington mRNA resulted in an enhanced rotarod performance in transgenic mice with Huntington's disease, demonstrating that therapeutic benefit with artificial miRNAs can be achieved in the central nervous system (CNS [Boudreau et al, 2009b]). This artificial miRNA-30a has been used in other studies involving many target tissues including the retina (Georgiadis et al, 2010). Efficient artificial miRNA-based knock down of peripherin-2 was achieved with a miR-30a peripherin-2 construct, which indicated the retina may be amenable to miRNA-based suppressors (Georgiadis, et al, 2010).

4.1.5. Artificial miRNA-155

The endogenous human miR-155 is expressed from the third exon of a conserved non-coding RNA known as BIC, located on chromosome 21 (Lagos-Quintana et al, 2001; Lagos-Quintana et al, 2002; Tam, 2001). The BIC gene was first identified in chickens, activated by retroviral insertion at the BIC locus in B cell lymphomas caused by Avian Leukosis Virus or by BIC over-expression in a retrovirus

vector (Tam et al, 1997; Tam & Dahlberg, 2006). The miRNAs ability to knockdown target sequences was experimentally validated by Chung et al, 2006, who established a 108 nucleotide region containing the pri-miRNA-155 with the relevant flanking regions driven by a polymerase II CMV promoter was enough for efficient processing of the miRNA in mammalian cells. Furthermore, a luciferase reporter gene, containing a miR-155 target site within the 3'UTR, was efficiently down-regulated following transfection with this miR-155 construct. An artificial miRNA was generated by replacing the miR-155 duplex with siRNA sequences of a target gene, and these artificial miRNAs could efficiently suppress these target mRNA sequences (Chung et al, 2006). Following on from this study, an optimised artificial miRNA based on murine-miR-155 became commercially available from Invitrogen (The BLOCK-iTTM Pol II miR RNAi system, Invitrogen). This system generated engineered pre-miRNA sequences within an optimised miR-155 backbone, under the control of a CMV promoter. Some of the first studies utilising this vector focused on suppressing targets involved in cancer metastases. Studies using, artificial-miRNA-155 vectors to significantly reduce cancer cell migration and invasion in addition to decreasing both peritoneal and breast cancer metastases *in vitro* and *in vivo* were successful (Li et al, 2006; Liang et al, 2007). Another group generated an artificial miRNA-155 targeting the neurokinin-1 receptor in mice brains; this resulted in a decrease in the voluntary alcohol consumption in mice and presented a novel example of treating alcohol addiction (Baek et al, 2010). A comparison of shRNA and miRNA-155 suppression, established CMV driven artificial miRNAs were significantly more efficient at silencing GFP and LacZ target sequences than U6-driven shRNAs (Anesti et al, 2008). An interesting comparison between CMV driven shRNA, CMV driven artificial miRNA-155 and CMV driven artificial miRNA-30 was undertaken *in vitro*. At the RNA and protein level both the artificial miRNAs were significantly better at suppressing the target gene compared to the shRNA. Furthermore miR-155 was more superior to miR-30a at suppressing both GFP and GAPDH RNA and protein in cell culture as assessed by semi-quantitative RT-PCR, Immunocytochemistry and western blotting (Shan et al, 2009b).

4.1.6. Objectives of Chapter 4

While toxicity with shRNAs in the retina has never been observed in any studies in our laboratory, it was felt that artificial miRNA-based constructs for RHO-adRP would nevertheless be worth exploring in the context of the mammalian retina. However, ensuring efficient silencing of rhodopsin by artificial miRNAs at comparable levels to that previously obtained by shRNAs *in vivo* would be critical (Chadderton et al, 2009; O'Reilly et al, 2007). This is of particular importance as rhodopsin accounts for such a large proportion of the protein found in ROS disc membranes (Palczewski et al, 2006). It has

been previously shown that a 62% decrease in rhodopsin mRNA is likely not sufficient to improve retinal function in a RHO-adRP animal model (Tessitore et al, 2006), thus if artificial miRNAs reduce efficacy, they may not be an appropriate tool for rhodopsin mediated suppression. With this in mind the best *in vitro* suppressor (siP3) characterised as a result of the study described in Chapter three, was generated into two artificial miRNAs, one expressed within the miR-155 backbone under the control of a CMV promoter and one expressed within a miR-30a backbone, under the control of a U6 promoter in addition to a H1-driven shRNA. All three constructs were engineered into AAV-2/5 and recombinant AAV-2/5 suppressor vectors were tested for efficacy *in vivo*, in a mouse model with a wild-type hRHO transgene.

4.2 Materials & Methods

4.2.1 Design and Cloning of shP3 into pSuper.GFP.neo

An shRNA for siP3 (shP3) was designed and generated according to Brummelkamp et al, 2002 and by using a protocol obtained from Oligoengine (**Figure 4.2.1.a**). A pSuper.GFP.neo vector (Oligoengine; **Section 9.2**) containing a H1 promoter to drive the shRNA and an EGFP gene under the control of the phosphoglycerate kinase (PGK) promoter was linearised by restriction enzyme digest with *BglIII* and *HindIII* (**Section 2.2.3**). The linearised vector was isolated using agarose gel extraction method (**Section 2.2.5**). In order to prevent the linearised end of the vector from reannealing they were dephosphorylated by treatment with SAP (**Section 2.2.6**). The SAP treated reaction was cleaned using the Qiagen enzymatic reaction clean up kit (**Section 2.2.4**).

Two oligonucleotides (shP3F & shP3R) were designed according to Brummelkamp et al, 2002 and used to generate the shRNA sequence (**Table 4.2.1.a**). shP3F had a 5'15 nucleotide sequence incorporating a *BamHI* site, the 19 nucleotide sense P3 siRNA sequence, a 9 nucleotide spacer followed by the 19 nucleotide antisense P3 sequence. A poly T sequence provided a poly A transcription terminator signal and this was followed by a 3' sequence containing a *HindIII* site. The *BamHI* and *HindIII* ensured that when oligonucleotides were annealed and digested with *BamHI* and *HindIII*, the 5'*BamHI* overhang and the 3'*HindIII* overhang would facilitate directional cloning into the vector. These desalted oligonucleotides were supplied by Sigma-Aldrich and each strand was resuspended to a concentration of 100µM in nuclease free H₂O. 1µl of each F and R oligonucleotide was mixed with 48µl of oligonucleotide annealing buffer, heat inactivated at 94°C for 5 minutes and gradually cooled to 18°C overnight. The annealed oligonucleotides were purified using the Qiagen enzyme reaction clean up kit (**Section 2.2.4**) and digested with *HindIII* and *BamHI* (**Section 2.2.3**).

The *BamHI* and *HindIII* digested annealed oligonucleotides were ligated (**Section 2.2.11**) to *BglIII* and *HindIII* sites of the linearised SAP treated pSuper.GFP.neo vector downstream of the H1 TATA box (**Section 9.3**). Following transformation into XL1-BlueMRA *E.coli* competent cells (**Section 2.2.12**), positive clones containing the shRNA were identified by restriction enzyme analysis using *HindIII* and *EcoRI* enzymes (**Section 2.2.3**). Plasmid DNA from putative positive clones was purified using the plasmid mini-preparation method (**Section 2.2.16**) and verified by sequencing (**Section 2.2.21**) using the pSuperR primer (**Table 4.2.1b**).

4.2.2 Cloning of shP3 into the pAAV.shNT vector

In order to generate AAV for subretinal delivery of shP3 to the eye, the H1-shP3 construct must be located between AAV-2 ITRS into a pAAV-MCS vector. A two step cloning process was used. pSuper.shP3 (**Section 9.3**) was digested with *EcoRI* and *HindIII* (**Section 2.2.3**) and the H1-shP3 fragment isolated using agarose gel extraction (**Section 2.2.5**). In order to facilitate blunt-end cloning, the *EcoRI* and *HindIII* overhangs were removed by Klenow polymerase (**Section 2.2.7**). This reaction was cleaned by the Qiagen enzyme reaction clean up kit (**Section 2.2.4**). A pAAV plasmid (pAAV.rp3) was linearised by *XbaI* (**Section 2.2.3**). The overhanging ends of the vector were filled by Klenow polymerase and the vector then purified by phenol chloroform extraction followed by ethanol precipitation (**Section 2.2.9 & Section 2.2.10**). The H1-shP3 fragment was subsequently ligated (**Section 2.2.11**) into the blunt sites of the pAAV.rp3 vector such that H1-shP3 was situated between two spacers (SRSR and SRSF) in the vector. Ligated DNA samples were transformed using XL10-Gold Ultracompetent cells (**Section 2.2.14**). Plasmid mini-preparations (**Section 2.2.15**) of positive clones were identified by sequencing (**Section 2.2.20**) using the SRSR primer (**Table 4.2.1.b**). A *NdeI/SfiI* fragment containing the spacers and H1-shP3 fragment was isolated using agarose gel extraction (**Section 2.2.5**). The 5' and 3' overhangs were filled using Klenow polymerase (**Section 2.2.7**) and T4 DNA polymerase respectively (**Section 2.2.8**) and the DNA was isolated from the reaction mixture using the Qiagen enzyme reaction clean up method (**Section 2.2.4**). A pAAV.CMV.EGFP vector [**Section 9.4**] was digested with *NsiI* and *HindIII* and purified by phenol chloroform extraction and ethanol precipitation (**Section 2.2.3, Section 2.2.9 & Section 2.2.10 respectively**). The 3' and 5' overhanging ends were filled using Klenow polymerase (**Section 2.2.7**) and T4 DNA polymerase respectively (**Section 2.2.8**) and a Qiagen enzyme reaction clean up was performed (**Section 2.2.4**). The H1-shNT spacer fragment was ligated (**Section 2.2.11**) between the blunt sites of the pAAV.shNT.CMV.EGFP vector and transformed into XL10-Gold Ultracompetent cells (**Section 2.2.14**). Positive clones of pAAV.shP3.CMV.EGFP (**Section 9.5**) were verified by sequencing with the SRSR primer (**Table 4.2.1b**).

4.2.3. Design and cloning of miRP3-30a into pMA.U6mi30BB

miRP3-30a was designed according to Boudreau et al, 2008. miRP3-30a was cloned into pMA.U6mi30BB (this was made by and obtained from Dr. Mary O'Reilly). This plasmid contains the mouse U6 (mU6) promoter with mU6 flanking regions (Boudreau et al, 2008). Downstream of this promoter are the miRNA flanking regions (miV2) which were designed to provide optimal silencing by Boudreau et al, 2008. Within this miV2 site are *XhoI* and *NotI* sites to facilitate cloning of miRP3-

30a. The pMA.U6mi30BB vector was digested with *XhoI* and *NotI* (**Section 2.2.3**) to replace mi30BB (an artificial miRNA previously evaluated in the laboratory) with miRP3-30a and the backbone was isolated by agarose gel extraction (**Section 2.2.5**).

The miRP3-30a oligonucleotides were designed according to Boudreau et al, 2008 (**Table 4.2.1.a**). They contain a *XhoI* site followed by miR30-a 5'flanking regions, the sense siP3 sequence and the endogenous 19 nucleotide miR-30a loop. Following the loop is the antisense siP3 sequence, some 3'miR-30a sequence and a *NotI* site. As the full length oligonucleotide is long (109 nucleotides), oligonucleotides were designed such that the F oligonucleotide contained 56 nucleotides of the 5' region finishing at the end of the loop sequence and the R oligonucleotide contained the reverse complement starting at the start of the loop sequence and continuing until the end of the 3' region. This ensured that once annealed, both strands would bind to each other in the region of complementarity (19bp) and could then be extended to produce a full length annealed oligonucleotide. The F and R miRP3-30a oligonucleotides each at a concentration of 2.5 μ M in 25 μ M NaCl were annealed at 94°C for 5 minutes and cooled to 18°C overnight. The DNA reaction was purified using the enzyme reaction clean up kit (**Section 2.2.4**). Annealed oligonucleotides were extended using Klenow polymerase (**Section 2.2.7**) which filled in the 5'overhangs on each strand generating a double stranded molecule. This reaction was purified by using the Qiagen enzyme reaction clean up kit (**Section 2.2.4**). The miRP3-30a DNA was digested by *XhoI* and *NotI* and the fragment was purified by the Qiagen enzyme reaction clean up kit (**Section 2.2.4**). miRP3-30a was subsequently ligated into the *XhoI* and *NotI* sites of the pMA.U6 vector (**Section 2.2.11**) and transformed into XL-1 BlueMRA competent cells (**Section 2.2.13**). Plasmid DNA was extracted from cells using mini-preparation (**Section 2.2.16**) and positive clones were identified and verified by sequencing (**Section 2.2.21**) with the U6R3 primer (**Table 4.2.1.b**).

4.2.4. Design and cloning of miRP3-30a into pAAV.shNT

Similar to shP3, miRP3-30a was cloned into the pAAV.CMV.EGFP vector (**Section 9.4**) using a two step cloning process. pMA.U6miRP3-30a was digested with *XbaI* in order to release the mU6-miRP3-30a fragment (**Section 2.2.3**); this was isolated by agarose gel extraction (**Section 2.2.5**). The U6-miRP3-30a fragment was ligated (**Section 2.2.11**) into the *XbaI* site of pAAV.rp3 between spacers SRSF and SRSR. Ligations of DNA samples were transformed onto XL10-Gold Ultracompetent cells (**Section 2.2.14**). Positive clones were identified by sequencing (**Section 2.2.21**) using the SRSF primer (**Table 4.2.1.b**). The fragment containing the spacers, and U6-miRP3-30a was excised from the pAAV.rp3 vector using *NdeI* and *SfiI* (**Section 2.2.3**) and isolated by agarose gel extraction (**Section**

2.2.5). The 5' *NdeI* overhang was filled by Klenow polymerase and the 3' *SfiI* overhang was filled by T4 DNA polymerase (**Section 2.2.7 & Section 2.2.8**) and the DNA was cleaned using the Qiagen enzyme reaction clean up kit (**Section 2.2.4**). The fragment was blunt-end ligated (**Section 2.4.11**) to the pAAV.CMV.EGFP vector (**Section 9.6**) at *HindIII* and *NsiI* which were filled with Klenow polymerase (**Section 2.2.7**) and T4 DNA polymerase (**Section 2.4.8**) and transformed using XL10-Gold Ultracompetent cells (**Section 2.2.14**). Positive clones were verified by sequencing (**Section 2.2.21**) with the SRSF primer (**Table 4.2.1b**).

4.2.5. Design and Cloning of miRP3-155 into pcDNATM6.2-GW/±EmGFP-miR vector

siP3 was cloned into the miR-155 backbone based on the protocol by Invitrogen. The pcDNATM6.2-GW/±EmGFP-miR plasmid (Invitrogen, [**Section 9.7**]), contains a CMV promoter which controls the expression of an EmGFP gene. Directly downstream of this gene, are the 5' and 3' miR-155 flanking regions and the double stranded oligonucleotide is inserted between these two sites (**Figure 4.2.5.a**). This miRNA plasmid was digested with *BsaI* (**Section 2.2.3**). This cleaved two *BsaI* sites facilitating replacement of a 26bp stuffer sequence with the artificial miRNA. The linearised vector was separated from the stuffer sequence by agarose gel extraction (**Section 2.2.5**).

A pair of F and R miRP3-155 oligonucleotides was obtained from Sigma-Aldrich according to the design recommended by Invitrogen (**Table 4.2.1.a**). The oligonucleotides have 5 nucleotides from the 5' endogenous miR-155 sequence and this forms a 4 nucleotide 5'F overhang to facilitate cloning into the *BsaI* site of the linearised miR vector. This is followed by the antisense siP3 sequence and a 19 nucleotide loop from miR-155. After this loop the oligonucleotide had nucleotides 1-8 and 11-21 of the sense siP3 sequence incorporated. Nucleotides 9 and 10 were removed to form a 2 nucleotide internal loop in the mature miRNA. 4 more nucleotides from the 3' miR-155 flanking region follow this loop. An important feature of this artificial miR-155 design is that it was optimised in the literature to generate a mature miRNA sequence of 21 nucleotides in length (Chung et al, 2006). For this reason it was decided to increase the length of the siP3 sequence to 21 nucleotides. This was done by incorporating an extra nucleotide at each end of the siRNA sequence (3' and 5'), homology between hRHO and pRHO was still maintained. The miRP3-155 oligonucleotides were resuspended to 100µM with nuclease-free H₂O. The oligonucleotides were annealed by adding 2µl of each to 46µl of annealing buffer. The reaction incubated at 95°C for 4 minutes and allowed to cool slowly to room temperature. The annealed miRP3-155 was ligated (**Section 2.2.11**) into the linearised miRNA vector and transformed into XL1 blueMRA *E.coli* competent cells (**Section 2.2.13**). Plasmid DNA mini-

preparations were performed (Section 2.2.15). The successful insertion of miRP3-155 was verified by sequencing (Section 2.2.21) using the EmGFP F primer (Figure 4.2.1b).

4.2.6. Cloning of miRP3-155 into pAAV-CMV. EGFP

In order to facilitate the generation of miRP3-155 into an AAV based construct, miRP3-155 (Section 9.8) was cloned into pAAV.CMV.EGFP.b ([Section 9.9]). pAAV.CMV.EGFP.b was linearised with *BglIII* (Section 2.2.3.) and purified by phenol chloroform extraction and an ethanol precipitation (Section 2.2.9 & Section 2.2.10). The miRP3-155 plasmid (Section 9.8) was digested with *BamHI* and *BglIII* to excise miRP3-155 and both the 5' and 3' miR-155 flanking regions from the original vector. The miRP3-155 fragment was ligated (Section 2.2.11) into the *BglIII* site of pAAV-CMV.EGFPb and transformed into XL-10 Gold Ultracompetant cells (Section 2.2.14). Positive clones were identified by restriction enzyme digest using *BglIII* and *XhoI* of plasmid (Section 2.2.3). The presence of the miRP3-155 fragment was verified by sequencing (Section 2.2.21) with miRNA R primer (Table 4.3.1.b).

4.2.7. Generation of AAV-2/5

shP3, miRP3-155 and miRP3-30a have each been cloned into a pAAV vector and can be used to generate AAV-2/5 virus for *in vivo* evaluation in the retina. All three transgene constructs (pAAV.shP3, pAAV.miRP3-155 and pAAV.miRP3-30a) were used to generate virus in house by Dr. Naomi Chadderton (Section 2.7.1 & 2.7.2). Each of the three RNAi constructs were triple transfected into separate batches of HEK-293 cells and generated into AAV-2/5 at the same time so as to minimise experimental variation between them.

In terms of determining the viral titre (Section 2.7.3), a 5 log standard curve was generated using pEGFP-1 plasmid DNA (Figure 4.2.7.a). The primers used in this qPCR reaction were designed to amplify a fragment of the EGFP transgene within each AAV-2/5 (Table 4.2.1.b). Two controls, of known titre were used in this reaction. They were AAV-2/5.CMV-EGFP (1×10^{12} vp/ml) and AAV-2/5. NT20 (2×10^{13} vp/ml). The copy number of all viral fractions from the three viruses (AAV-2/5.shP3, AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a) was determined and this allowed the viral titre to be calculated. The fraction from each virus with the highest titre was aliquot and stored at -80°C.

The non-targeting control viruses for each construct had previously been generated and evaluated in the laboratory. The control for AAV-2/5.shP3 is AAV-2/5.NT20 containing a non-targeting control

shRNA (titre= 2×10^{13} vp/ ml). The control for AAV-2/5.miRP3-155 was AAV-2/5.miNT, this was made in house by Dr. Naomi Chadderton and contained a non-targeting siRNA within the miR-155 backbone (titre= 2×10^{12} vp/ml). The control for miRP3-30a was AAV-2/5.Luc46. This was a luciferase targeting siRNA within the miR-30a backbone made by Dr. Mary O'Reilly and generated into AAV-2/5 by Dr. Naomi Chadderton (titre= 1×10^{12} vp/ml).

4.2.8. NHR transgenic mice

The mouse model used to test the efficacy of the three AAV-2/5 viruses against hRHO was a model known as NHR (Section 2.1.8; Ollsen et al, 1992). This model has no mRHO alleles and one hRHO allele (NHR^{+/-}129rho^{+/-}). For breeding purposes NHR^{+/-}129rho^{+/-} were mated with 129rho^{+/-} (Humphries et al, 1997) such that 50% will be positive for the NHR transgene. It was necessary to genotype mice prior to injection. Following weaning, they were genotyped (Section 2.8.2) using P23HF and P23HR primers (Table 4.2.1.b). Mice positive for the NHR transgene were subsequently used for *in vivo* evaluation of hRHO suppression.

4.2.9. Subretinal injection of AAV-2/5 constructs into NHR transgenic mice

The three AAV-2/5 viruses (AAV-2/5.shP3, AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a) were titre matched by diluting with PBS. The control viruses were titre matched to their respective constructs where possible. Adult NHR mice were subretinally injected with approximately 3µl of virus/eye (Section 2.8.3.) The left eye (LE) was injected with the control virus and the right eye (RE) with the suppressor virus.

4.2.10 Fluorescent Activated cell sorting (FACS);

4.2.10.1. Retinal dissociation and preparation of cells for FACS – Trypsin method

In order to accurately assess suppression, the retinal cells that have received virus need to be separated from cells that did not receive the virus. Each AAV-2/5 virus contained an EGFP gene. This enables EGFP positive cells (cells in which has been transduced) to be detected and sorted from EGFP negative cells by FACS. Injected mice were sacrificed (Section 2.8.1), and retinas were extracted (Section 2.4.5). Each retina was added to 1ml Trypsin/HBSS (1mg/ml). This was incubated at 37°C for 20 minutes. During this incubation, the cells were dissociated by pipetting up and down at 5 minute intervals. 5µl DNase I was added after the first five minute interval. 100µl Trypsin inhibitor was added to the dissociated mixture and the cells were centrifuged at 2500rpm for 5 minutes (IEC MicroMax Microcentrifuge). Following centrifugation, half the supernatant was discarded and the remaining cells

were centrifuged at 2500rpm for 5 minutes. The remaining supernatant was carefully removed and discarded and the cell pellet was resuspended in 1ml HBSS. 10µl DNaseI was added and the mixture was incubated for 10 minutes at 37°C. 1ml of HBSS was added to the cell solution, and the cells were placed on ice.

4.2.10.2 Retinal dissociation and preparation of cells for FACS- neural tissue dissociation kit

During the course of FACS analysis, occasionally, there was a large volume of cell debris. It was thought that the trypsinising protocol might be too harsh on the cells. For this reason, a dissociation kit using Papain instead of Trypsin was utilised (**Section 2.1.13**). The kit used was the neural tissue dissociation kit P (NTDK P, MACS Miltenyi Biotec). The retinal dissociation was carried out according to the manufacturer's instructions. Four retinas were processed at a time. Briefly, the first two retina's were placed in the C Tubes containing 1950µl of preheated enzyme mix 1, placed in the gentleMACS dissociator and ran under program "m_brain_01". Two retinas were then incubated at 37°C for 10 minutes, gently rotating at 4rpm using a MACSmix tube rotator. When the first pair of retinas had started this incubation, the next pair of retinas were then placed in enzyme mix 1 and underwent step one of this protocol. This staggering of retinas was to ensure that all retinal cells underwent the same incubation times. After 15 minutes, the retinas were placed on the gentleMACS dissociator and the "m_brain_02" program was selected. 30µl enzyme mix 2 was added to each C tube and incubated with rotation for 10 minutes at 37°C. The retinas were placed under the "m_brain_03" program on the gentleMACS dissociator and incubated at 37°C for 10 minutes with gentle rotation. The C-tubes containing the retinas were centrifuged at 2500rpm (Centrifuge 5810, Eppendorf) for 5 minutes, half the supernatant was discarded and the cells were centrifuged again for a further 5 minutes. The supernatant was gently removed, and the cells were resuspended in 2ml HBSS. The cells were incubated on ice and transported to FACS facility. 1mg/ml Propidium Iodide (PI) was added to each sample prior to FACS in order to distinguish the proportion of dead cells within the mixture.

4.2.10.3 FACS analysis

FACS analysis was undertaken in the UCD Cytometry core facility by Dr. Alfonso Blanco. Prior to sorting, dissociated retinal cells were filtered through a 50µm filter (Filcon, DakoCytomation) into small polystyrene round bottom Falcon tubes (BD falcon) to remove debris which may interfere with the FACS machine. Each filtered retinal cell suspension was analysed using a BD FACSaria cell sorting system (BD Biosciences) which detected the EGFP fluorescence. This sorting system uses three lasers, A blue laser, detects light at 488nm, a red laser detects light at 635nm and a violet laser

detects light at 405nm. The cells suspension is injected into a flow stream, which compresses the cells into single file. The cells are passed through a focussed laser beam, and at the point in which the cells and the laser beam meet, light scatter and fluorescence is measured. Flow cytometry enables the detection of two signals. The first, called forward scatter measures light in the forward direction and is proportional to cells size. The second, is side scatter, this is a measure of the shape and granularity of a cell. The blue laser at 488nm is the beam used to detect EGFP fluorescence, a FITC optical filter was used to detect and filter EGFP fluorescence from the cell. Initially, a uninjected retinal sample (EGFP negative cells) was used as a negative EGFP control, to determine the limits of autofluorescence and act as a baseline for EGFP positive cells. A measure of fluorescence and autofluorescence was presented for each sample, in the form of two peaks. The first peak on the left hand side is a measure of auto fluorescence (**Figure 4.3.7.b**), the second peak on the right is a measure of fluorescence (EGFP positive cells [**Figure 4.3.7.d**]). In order to ensure that every cell chosen was EGFP positive, a gate from a GFP signal of $-10^{3.5}$ was generated and EGFP positive cells with an EGFP signal greater than $10^{3.5}$ were chosen and collected for further analysis. For all three viruses evaluated (AAV-2/5.shP3, AAV-2/5.miRP3-155 and AAV-2/5.miR-30a) the same gate was set in order to ensure that every sample contained the same gated population of EGFP positive cells. The ideal EGFP positive cells numbers chosen were between 50,000-100,000 cells. However in samples with EGFP positive cells lower than this, the maximum numbers of EGFP positive cells were obtained. Cells were immediately concentrated on an Ultrafree-MC filter unit (Millipore) by centrifugation at 1500rpm for 1 minute. These cells were placed on dry ice for transportation and subsequently stored at -20°C .

4.2.11 RNA isolation of FACS cells

350 μl of ice cold buffer RLT (containing 3.5 μl β -mercaptoethanol) was added to the Ultrafree-MC filter unit of each cell sample. This was centrifuged at 6500rpm for 2 minutes (IEC Micromax Microcentrifuge, Thermo-Electron). This was done four times, passing the same supernatant from each sample through its corresponding filter. Following this, the RNA isolation was carried out (**Section 2.4.5**). RNA was eluted in 30 μl of nuclease free H_2O .

4.2.12. Optimisation of real-time RT-PCR for FACS RNA isolated from FACS sorted cells

In a whole retina, hRHO is confined to photoreceptors; β -Actin is expressed in every cell of the retina. Thus the level of hRHO expression is similar to the level of β -actin. FACS isolated retinal cells, containing AAV-2/5.EGFP represent a small population of retinal cells, the majority of which are photoreceptors. Thus in these samples the hRHO expression is much more concentrated compared to

whole retina RNA, and the β -actin expression compared to concentrated hRHO is much lower. 18S is however, much more highly expressed than β -Actin, and was therefore chosen as the housekeeper to be used in this study to normalise hRHO. Optimisation of the 18S primer pair (**Table 4.2.1.b**) was carried out in a similar manner to the optimisation of hRHO and β -actin (**Section 3.2.5**). The RNA used to generate the 18S standard curve was FACS retinal RNA from NHR mice sub-retinally injected with AAV-2/5.NT20.

4.2.13 Real-time RT-PCR of FACS RNA for FACS sorted cells

Real-time RT-PCR analysis was undertaken on FACS isolated retinal RNA from mice subretinally injected with AAV-2/5.shP3, AAV-2/5miRP3-155 and AAV-2/5.miR-P3-30a and their corresponding controls. As the amount of RNA present in FACS cells is too low, to enable the determination of RNA concentration by spectrophotometry, the concentration of RNA used in each set of real-time RT-PCR was determined by FACS cell number. RNA from the three samples with the highest cell numbers from control injected retinas were pooled and used to generate the 5X standard curve. Each RNA sample to be tested was diluted, such that they all represented an RNA concentration from the same cell number. The cell number for dilution was the average number present in the middle of the standard curve. The real-time RT-PCR was carried out and analysed as previously described (**Section 2.4.7 & 3.2.6**). hRHO expression in each sample was normalised to 18S expression in each sample. The hRHO expression in retinal RNA from mice sub-retinally injected with the AAV-2/5 controls was set at 100% and each AAV-2/5 suppressors were compared to that of its respective control.

4.2.14 Analysis of siP3 expression in NHR retinas

To quantify mature siP3 expression generated from shP3 compared to the two miRNAs (miRP3-155 and miRP3-30a) a Custom Taqman®Small RNA assay (Applied Biosystems) was undertaken. This assay is used to quantify small RNA sequences such as mature miRNA or processed shRNA. The Custom Taqman®Small RNA assay design tool was used to design an assay for the siP3 sequence (Applied Biosystems). A stem-loop RT-primer specific to siP3, a mixture of siP3 specific F & R primers and a Taqman®MGB probe specific to siP3 were supplied. This was a two-step reaction, in the first step all siP3 RNA is converted to cDNA, this is done using the siP3 specific stem loop primer. The second reaction is the qPCR reaction for the siP3 cDNA. A control miRNA known as sno-202 was used to normalise the expression of siP3 in the retina. This control miRNA was available as a pre-designed assay (Applied Biosystems). In order to evaluate the expression of siP3, a siP3 control sample of known concentration was used to generate the standard curve. The control used in this instance was

the synthetic siP3 (20 μ M) used previously for *in vitro* studies in Chapter 3 and obtained from Ambion (**Section 3.2.1**). RNA from an uninjected NHR retina was used to generate a standard curve for sno-202.

4.2.14.1 RNA isolation using a miRNeasy mini kit (Qiagen)

To isolate small RNA molecules of fewer than 100 nucleotides, the miRNeasy mini isolation kit was used [Qiagen]. Retinas from NHR mice were extracted (**Section. 2.4.5**). The RNA extraction protocol was carried out according to the manufacturer's protocol. RNA was eluted in 50 μ l of nuclease-free H₂O.

4.2.14.2. Reverse transcription of siP3 and sno-202

An RT reaction was carried out twice for each set of retinal RNA samples using the TaqMan®MicroRNA RT kit (Applied Biosystems). The first was carried out specifically for siP3 and the second was carried out for sno-202. Each RNA sample was reverse transcribed using both siP3 and sno-202 primers. siP3 was utilised such that in the siP3 assay it acted as a positive control for siP3 analysis (siP3+) and a negative control for sno-202 analysis (sno-202-) and vice versa for RNA from an uninjected retina in the sno-202 assay (sno-202+ & siP3-) Each RNA sample was diluted to approximately 4.7 ng/ μ l. In order to denature the double stranded siP3 and sno-202 duplex 5 μ l of the diluted RNA was mixed with 3 μ l of the specific RT primer (siP3 or sno-202 RT primer). This was heated at 85°C for 5 minutes followed by 60°C for 5 minutes. The reactions were then cooled on ice for 5 minutes. This reaction mixture was then combined with 7 μ l of the RT master mix and incubated on ice for 5 minutes. Using a Thermocycler (Biometra T300 Thermocycler, Biosciences), the RT reaction was carried out as follows: 30 minutes at 16°C, followed by 30 minutes and 42°C and 5 minutes at 85°C. The RT product was stored at -20°C.

4.2.14.3 qPCR amplification

The qPCR reaction was carried out using the TaqMan®Universal PCR Master Mix II with no Uracil-N-glycosylate (UNG [Applied Biosystems]) and undertaken on an Applied Biosystems 7300 real time PCR system. A small pilot qPCR was undertaken to optimise primers similar to that previously described for hRHO and h β -actin (**Section 3.2.5**). Two standard curves were generated, one using the siP3 and the other the sno-202 control. For the siP3 standard curve, the siP3 cDNA was initially diluted 1/600 in nuclease free H₂O and a 10X standard curve was generated. For the sno-202 standard curve, sno-202 cDNA from an NHR mouse retina was used at a dilution of 1/10 and a 5X standard curve was

generated. For siP3 analysis the two controls (siP3 + and siP3-) were included and for sno-202 analysis the two controls (sno-202+ and sno-202 -). All control samples were diluted 1/60. Two master mixes were made, one for siP3 and one for sno-202. For each siP3 and sno-202 reaction, 1.33µl of cDNA from the RT reaction was mixed with 1µl of 20X Taqman® Small RNA assay (containing siP3 (or sno-202) F & R primers and the siP3 (or sno-202) Taqman® MGB probes). This was mixed with 10µl Taqman® Universal PCR Master Mix II (2X), no UNG and 7.67µl nuclease-free H₂O. Each sample was pipette in duplicate onto a PCR plate, and the reaction was carried out as follows:

- **Activation of DNA polymerase:** 95°C for 10 minutes.
- **cDNA amplification:** 40 cycles at 95°C for 15 seconds, 60°C for 1 minute.

Following the qPCR, the RT-PCR reaction was analysed according to (**Section 2.4.7**) and standard curves were generated for both siP3 and sno-202. Optimisation using the pilot study allowed analysis of siP3 in retinal RNA samples to proceed. The reaction was carried out identical to the pilot study, except every sample was loaded onto the plate in triplicate. Each sample for siP3 or sno-202 was compared to the siP3 or sno-202 standard curves respectively and a value for each was obtained. siP3 expression was then normalised to sno-202 expression in each sample in order to obtain the relative amount of siP3 species. siP3 expression from retinas injected with shP3 was set as 100% and siP3 expression from retinas injected with either of the two miRNAs were compared to this value.

4.2.15 ERG

ERGs were carried out on NHR mice subretinally injected with each suppressor construct (AAV-2/5.shP3, AAV-2/5.miRP3-155 or AAV-2/5.miRP3-30a) in the right eye (RE) versus each corresponding control (AAV-2/5.shNT20, AAV-2/5.miNT or AAV-2/5.Luc-46) in the left eye (LE). These ERGs were performed 6 weeks post-injection and carried out by Dr. Paul Kenna (**Section 2.8.4**). A rod-isolated, mixed rod-cone and cone-isolated response was obtained for each eye. The average b-wave of each ERG response in the LE or RE were combined. For each response the average LE b-wave was set at 100% and the average RE b-wave (suppressor injected eyes) was expressed as a percentage of this value.

4.2.16 Statistical analysis

The mean and standard deviation of data sets was calculated using Microsoft Excel. For RNA analysis statistical significance was calculated using a two-sample t-test, for ERG analysis a paired two-sample

t-test was utilised to evaluate statistical significance. All statistical analysis was carried out using DataDesk 6.0.

4.2.17 Protein isolation

6 weeks post-injection (following ERG) retinas from two NHR mice injected with AAV-2/5.shP3 (or the control virus AAV-2/5.shNT20) and two mice injected with AAV-2/5.miRP3-30a (or the control virus AAV-2/5.Luc-46) were extracted and placed in HBSS on a glass microscope slide. The retinas were visualised using the fluorescent microscope (Axiophot microscope, Carl Zeiss LTD) and under the blue filter “10”, EGFP positive regions of the retina were excised by Dr. Arpad Palfi using a dissection scissors. This EGFP positive portion of the retina was placed in protein lysis buffer, the protein was isolated and the protein concentration determined (**Section 2.5.2 and Section 2.5.3**).

4.2.18 Western blotting

Western blotting was undertaken to analyse protein suppression in NHR mice injected with AAV-2/5.shP3 (RE) and AAV-2/5.NT20 (LE) or AAV-2/5.miRP3-30 (RE) and AAV-2/5.Luc46 (LE). The protocol was undertaken as described (**Section 2.6**). PVDF membrane was used for transfer, as this is more amenable to repeated probing for different proteins. A negative hRHO control from a rho knockout mouse (129rho^{-/-}, Humphries et al, 1997) was included for each western. The 4D2 antibody (Hicks & Molday, 1986) was used to detect hRHO protein expression (1/1000 dilution). The anti-mouse light chain only secondary antibody was used (1/2000 dilution). The anti-rabbit h β -actin antibody (at a dilution of 1/500) was used to ensure equal loading. The anti-rabbit secondary antibody (at a dilution of 1/1000) was used in this case

4.2.19 Immunohistochemistry

Immediately following ERG analyses at 6 weeks post-injection immunohistochemistry was undertaken on retinal samples from three NHR mice injected with AAV-2/5.shP3 (and the control virus AAV-2/5.shNT20) and on three mice injected with AAV-2/5.miRP3-30a (and the control AAV-2/5.Luc46). This was done 6 weeks post-injection (**Section 2.8.5-Section 2.8.8**). The 4D2 rhodopsin antibody (Hicks & Molday, 1986) at a dilution of 1/100 was used to stain all sections for hRHO. The anti-mouse Cy2 antibody was used at a dilution of 1/500. EGFP positive regions were examined, and once identified the presence of hRHO staining within this region was evaluated, hRHO staining in EGFP positive regions of the LE was compared to hRHO staining in EGFP positive regions of the RE. hRHO staining in uninjected regions (EGFP negative) were also used as a control in LEs and REs.

4.3. Results

4.3.1 shP3 cloning into pAAV

The first *in vivo* siP3 based suppressor designed was generated in a shRNA form (shP3). Oligonucleotides were designed according to Brummelkamp et al, 2002 and the Oligoengine protocol as described in **Section 4.2.1 (Figure 4.3.1.a [step a])**. The oligonucleotides were designed so that the sense and antisense siP3 sequence were located between a nine nucleotide spacer (Brummelkamp et al, 2002), this was to ensure that once transcribed by the H1 promoter the shP3 would form a unpaired loop (9 nucleotide spacer) attached to a stem of the siP3 sense and antisense sequence annealed to each other. The 5 thymidines in a row at the 3' end of the oligonucleotide signified a well defined transcriptional termination site was present in which cleavage occurs after the second U. This ensures the transcript resembles the 2 nt 3' overhang associated with synthesised siP3. The oligonucleotide was cloned into pSuper.gfp/neo (**Section 9.2**) such that the 5' *Bam*HI overhang of the shP3 oligonucleotide was inserted into the *Bgl*II site of the pSuper.gfp/neo vector (**Section 9.3**), destroying both the *Bgl*II and *Bam*HI sites. The 3' *Hind*III restriction site was however maintained (**Figure 4.3.1.a [step b]**). The presence of the shP3 sequence in positive clones was verified by sequencing (**Figure 4.3.1.b**).

In order to express the H1 driven shP3 between AAV-2 ITRs (for generation into AAV-2/5), the 281 nucleotide fragment containing the H1 promoter and shP3 was blunt cloned into pAAV.rp3 (**Figure 4.3.1.a [step c]**) such that the H1-shP3 sequence was situated between two spacers SRSF and SRSR. Positive clones were identified and verified by sequencing (**Section 9.11**). The 5' spacer is SRSR and the 3' spacer was SRSF. The 870 nucleotide fragment containing the H1-shP3 construct between the two SRSR and SRSF spacers was blunt cloned into the pAAV.CMV.EGFP vector (**Figure 4.3.1a [step d]**). shP3 was placed between the two AAV-2 ITRs and upstream of a CMV driven EGFP reporter gene (**Section 9.5**). This was verified by sequencing (**Section 9.12**).

4.3.2 miRP3-30a cloning into pMA.mU6mi30BB

miRP3-30a was designed and cloned according to Boudreau et al, 2008. This miRNA was generated to contain the 19 nucleotide terminal loop of miRP3-30a separating the sense and antisense sequence of siP3; additionally nucleotides were incorporated into the base of the stem and prior to the stem loop in order to facilitate optimal processing of the miRNA by Drosha and Dicer respectively. The miV2 flanking region was used, as described in Boudreau et al, 2008 and was found to drive miRNA based suppression 20% more efficiently than the endogenous miR30-flanking regions. The full length 109bp oligonucleotide was generated by Klenow polymerase extension of the 19bp complementary region

between the F and R strands (**Figure 4.3.2.a [step a]**). This miRP3-30a insert was subsequently cloned into the linearised MA.U6mi30BB (**Figure 4.3.2.b [step b]**). The sequence results (**Figure 4.3.2.b**) confirmed that miRP3-30a was located downstream of the mU6 promoter.

In order to facilitate the generation of miRP3-30a into AAV-2/5 the miRP3-30a needed to be expressed within an AAV vector between AAV-2 ITRs. For this reason the fragment containing the miRP3-30a sequence, the miRNA flanking regions and the mU6 promoter was blunt cloned into the pAAV.rP3 vector, such that the mU6-miRP3-30a sequence was situated between the SRSF and SRSR spacers (**Figure 4.3.2.a[step c]**). Positive clones were identified, and the location of miRP3-30a between the two spacers was verified by sequencing (**Section 9.13**). The 870bp fragment containing the mU6-miRP3-30a sequence situated between the two SRSR and SRSF spacers was excised and blunt-end cloned into pAAV.CMV.EGFP (**Figure 4.3.2.a [step d]**). This placed miRP3-30a and the mU6 promoter between the two AAV-2 ITRs and upstream of a CMV driven EGFP reporter gene (**Section 9.6**). The mU6 promoter was situated such that it was transcribed in the opposite direction to the CMV driven EGFP gene. This was verified by sequencing (**Section 9.14**). Boudreau et al, 2008 recommend the use of a polymerase III terminator signal with miRP3-30a, while this was not included in the original MA.U6mi30BB vector, a string of 5 thymidines are present approximately 80bp downstream of miRNA-30a vector located in the SRSF spacer in pAAV.miP3-30a.rP3 and pAAV.miRP3.CMV.EGFP and likely acts as the termination signal for this sequence.

4.3.3 miRP3-155 cloning into pcDNA6.3-GW/+EmGFP-miR

miRP3-155 was designed according to Invitrogen and based on the miRP3-155 analysis undertaken by Chung et al, 2006 (**Figure 4.3.3.a [step a]**) The siP3 oligonucleotide comprised the stem of the miRP3-155 miRNA with the siP3 antisense and the siP3 sense sequence separated by the endogenous miRP3-155 19 nucleotide terminal loop. The sequences at the base of the stem loop were the 3' and 5' flanking regions of the miRP3-155 sequence such as to aid efficient cleavage and processing of this miRNA by Drosha and Dicer in a strategy which mimics the endogenous processing of miR-155. As all analysis in the literature incorporated a 21bp siRNA sequence containing a 2 nucleotide deletion in the centre of the sense strand, it was decided to incorporate an extra two nucleotides into siP3. An extra nucleotide from each end of the siP3 target site was inserted into the oligonucleotide such that homology between hRHO and pRHO was still maintained. The presence of a 2 nucleotide deletion of nucleotides 9 and 10 of the sense siP3 strand was to facilitate the formation of a 2 nt short terminal loop which has been shown to result in more efficient suppression (Chung et al, 2006).

The annealed miRP3-155 oligonucleotides had been ligated into the pcDNA6.3-GW/+EmGFP-miR (Section 9.8) (Figure 4.3.3.a [step b]). Positive clones were sequence verified (Figure 4.3.3.b). To express miRP3-155 between AAV-2 ITRs, the fragment containing the endogenous 5' and 3' miR-155 sequence from the vector along with the miRP3-155 sequence (a 150 nucleotide fragment) was cloned into a pAAV-CMV.EGFPb vector (Section 9.9), such that miRP3-155 was expressed downstream of EGFP from the same CMV promoter (Figure 4.3.3.a [step c]). Sequencing verified the presence of the 150 nucleotide miRP3-155 fragment with the miR-155 flanking regions (Figure 4.3.3.c).

4.3.4 AAV-2/5 generation

All suppressors' shP3, miRP3-155 and miRP3-30a were generated as high titre AAV-2/5 vectors using the triple transfection method. When cells were harvested 48 hours post transfection, the DMEM was visibly tinged with green for each suppressor owing to the expression of the EGFP gene which is contained on each construct as a reporter gene. This was a good initial indicator that the suppressor plasmids may be functional and expressing protein. As the cells were lysed and further processed through the AAV-2/5 generation protocol, the green colour of the cell lysate gradually faded as the virus was purified. After dialysis, each fraction of virus for each suppressor (three fractions for shP3, three fractions from miRP3-155 and two fractions for miP3-30a) underwent qPCR with EGFP primers in order to evaluate viral titre. The copy number obtained during qPCR analysis of each suppressor fraction was compared to that of the two controls of known titre, thus allowing the titre in each suppressor fraction to be calculated (Table 4.3.4). Fraction 5 of AAV-2/5.shP3 (AAV-2/5.shP3-5) had the highest copy number of the shP3 fractions (2.51235×10^6) and an estimated titre of 5.6×10^{12} vp/ml. Fraction 4 of AAV-2/5.miRP3-155 (AAV-2/5.miRP3-155-4) had the highest copy number for miRP3-155 (1.48388×10^6) and an estimated titre of 3.3×10^{12} vp/ml. Fraction 3 of AAV-2/5.miRP3-30a had the highest copy number for miRP3-30a (1.52966×10^6) and an estimated titre of 3.4×10^{12} vp/ml. The controls for each suppressor virus had previously been generated and titres calculated by Dr. Naomi Chadderton against the AAV-2/5.CMV.EGFP controls. The titre of the miRP3-155 control (AAV-2/5.miNT) was 2×10^{12} vp/ml and the titre of the miRP3-30a control (AAV-2/5.Luc46) was 1×10^{12} vp/ml. Thus an important decision had to be undertaken regarding the dilutions to use *in vivo*. If all suppressors and their corresponding controls were diluted to be titre matched (so as to ensure an accurate comparison between each) then they all would be diluted to 1×10^{12} vp/ml (the titre of AAV-2/5.Luc46). However, by lowering the titre, the efficacy of suppressor becomes compromised, thus it was decided to titre match all suppressors to each other (AAV-2/5.shP3, AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a) to 3.3×10^{12} vp/ml. In terms of the controls AAV-2/5.NT20 (NT20) was also

diluted to match AAV-2/5.shP3. Both the titres of AAV-2/5.miNT and AAV-2/5.Luc46 controls were lower than the titres of the suppressors.

4.3.5 NHR transgenic animals

To evaluate AAV delivered RNAi mediated suppression of hRHO *in vivo* the NHR transgenic mouse, which carries the wild-type hRHO gene was employed (Ollson et al, 1992). In the laboratory the NHR mice are maintained on a 129rho^{-/-} background (Humphries et al, 1997) such that the genotype of all mice is NHR^{+/-}rho^{-/-}. When the NHR mice are bred (with 129rho^{-/-}), only 50% of the resulting progeny have the NHR transgene and the remaining 50% are negative for both hRHO and mRHO (129rho^{-/-}). Genotyping was therefore undertaken on DNA from every mouse used for evaluation. If the mice were positive for the NHR transgene, a hRHO amplification product of approximately 0.5Kb would be evident on a 1% gel (**Figure 4.3.5.a**). If the mice were negative for the NHR transgene, no hRHO amplification would be visible. In all cases the positive control (a known positive NHR mouse) yielded a hRHO product of 0.5kb and the negative control (dH₂O) sample never yielded any amplification products, therefore validating the PCR results.

4.3.6 Subretinal injections of AAV-2/5 suppressors into NHR mice

For the evaluation of AAV-2/5.shP3; AAV-2/5.NT20 (9.9 X 10⁹vp) was subretinally injected into the left eye (LE) and AAV-2/5.shP3 (9.9 X 10⁹vp) into the right eye (RE) of each NHR mouse. For AAV-2/5.miRP3-155 (miRP3-155); AAV-2/5.miNT (6 X 10⁹vp) was subretinally injected into the LE and AAV-2/5.miRP3-155 (9.9 X 10⁹vp) into the RE of each mouse. For miRP3-30a analysis; AAV-2/5.Luc46 (3 X 10⁹vp) was subretinally injected into the LE and AAV-2/5.miRP3-30a (1.02 X 10¹⁰vp) into the RE of NHR mice. Any bad injections were noted, as they could affect analysis.

4.3.7 FACS isolation of EGFP positive retinal cells for hRHO mRNA expression analysis 4 weeks post -injection

It is of note that every AAV-2/5 control and suppressor virus had a CMV driven EGFP transgene, thus in order to quantify suppression of hRHO at the RNA level with each suppressor virus, the EGFP positive retinal cells from each mouse were purified using FACS. Typically only about a third of a non-degenerating retina is transduced, after subretinal injection (Chadderton et al, 2009). Therefore the EGFP positive portion of the retina representing transduced cells is the population of cells that should be evaluated for suppression (suppression may be masked if whole retina RNA was used). Initially each suppressor virus and its corresponding control were subretinally injected into adult NHR mice

and cells dissociated four weeks post-injection. This time point was chosen to allow sufficient time for high levels of EGFP expression in each injected retina. An EGFP negative (uninjected) NHR retina was used to set the baseline parameters for EGFP fluorescence (**Figure 4.3.7a**). The same baseline parameters and gating parameters were used in this study and that of O'Reilly et al, 2007 to ensure that similar populations of cells in each study were being compared (**Figure 4.3.7.b**). A background GFP signal from 10^2 to 10^3 was observed representing EGFP negative cells, thus from 10^3 onwards (the threshold at which no cells are present in the negative control) represents the region in transduced samples that EGFP positive cells would be present. An EGFP positive sample (NHR mouse retina injected with AAV-2/5.NT20 incorporating EGFP) was used to check these parameters (**Figure 4.3.7.c**). At a GFP signal from 10^3 onwards (M1) a peak representing EGFP positive cells was present (**Figure 4.3.7.d**), this was not observed in the EGFP negative control cell population. A gate in which the GFP signal was $10^{3.5}$ was chosen as the threshold for which all EGFP positive cells would be collected.

The first mice to undergo FACS were four NHR mice injected with AAV-2/5.NT20 (LE) eye and AAV-2/5.shP3 (RE). The number of EGFP positive cells obtained from this FACS ranged between 50,000-120,000 cells (**Table 4.3.7**). The average % EGFP positive cells for retinas injected with AAV-2/5.NT20 was 34% compared to 16% for retinas injected with AAV-2/5.shP3. The lower percentage of EGFP positive cells in the sample containing the suppressor was not surprising. If shP3 is suppressing hRHO in the NHR mouse, this may lead to retinal degeneration, thus the EGFP cells may begin to die. The average mean fluorescence intensity (Media, MFI) in retinal cells with AAV-2/5.shP3 was 7008MFI compared to 3232MFI for AAV-2/5.NT20 injected retinas. The lower EGFP intensity for NT20 has been observed previously (Dr. Naomi Chadderton [personal communication]). One mouse failed to have any EGFP positive cells in either eye. This mouse however, was noted to have had a bad subretinal injection which may be the reason for this result.

For FACS analysis with NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE), lower cells numbers were obtained than with shP3 analysis. The cell numbers from each retina ranged from 18,000-100,000 EGFP positive cells (**Table 4.3.7**). AAV-2/5.Luc46 injected retinas had between 18,000-50,000 EGFP positive cells with an average of 36,000 cells. In the retina with 18,000 EGFP positive cells, it had been noted that the mouse did not receive a good injection in that eye. EGFP positive cell numbers for AAV-2/5.miRP3-30a injected retinas ranged from 40,000-100,000 EGFP positive cells with an average cell number of 65,000. The average % EGFP positive cells for AAV-2/5.Luc46 versus AAV-2/5.miRP3-30a treated eyes was 5.65% compared to 11.6%. There was

twofold less EGFP positive cells in the control eye; this may possibly due to titre difference. AAV-2/5.Luc46 was used at a 3 fold lower titre than AAV-2/5.miRP3-30a. The % of EGFP positive cells correlated well with the EGFP positive cells number obtained during FACS, the lowest number of AAV-2/5.miRP3-30a injected EGFP positive cells collected was 40,000 which corresponded with 3.4% EGFP positive cells in the whole retinal sample. Furthermore this correlated with the fact that the mouse had a subretinal haemorrhage in this eye; this highlights the importance of noting any significant changes to the retina during subretinal injection. Fluorescent intensity was also lower in AAV-2/5.Luc 46 injected retinas (3875MFI) compared to miRP3-30a injected retinas (10544MFI); again this may likely be due to titre difference.

For FACS analysis with 5 NHR mice injected with AAV-2/5.miNT(LE) and AAV-2/5.miRP3-155(RE), the EGFP positive cell number ranged from 4,900-75,000 (**Table 4.3.7**). Mouse eyes injected with AAV-2/5.miNT had between 23,000-75,000 EGFP positive cells (50,000 average) and approximately 13.6% of retinal cells were EGFP positive. Mouse eyes injected with AAV-2/5.miRP3-155 had an EGFP positive cell number ranging from 4,900-55,000 (26,980), 7.88% of all cells were EGFP positive. The reason for this difference may be due to the suppressor activity of miRP3-155. The intensity of fluorescence in cells injected with AAV-2/5.miRP3-155 was lower (6632 MFI) than cells injected with AAV-2/5.miNT (14162). One NHR mouse injected with miNT failed to generate any EGFP positive cells from the FACS analysis, which corresponded to a low % of EGFP positive cells (0.5%), again due to a bad subretinal injection.

4.3.8 Optimisation of 18S for real-time RT-PCR analysis of FACS isolated RNA

Initially, prior to evaluation of hRHO RNA expression *in vivo*, assays for assessing the house keeping gene to be used for real-time RT-PCR was optimised. Real-time RT-PCR assays for hRHO and human β -actin had previously been optimised as described in chapter 3 (**Section 3.3.3**). 18S primers were previously designed in the laboratory and were used to generate a 5X 18S standard curve using RNA from FACS purified EGFP positive cells from an NHR mouse subretinally injected with AAV-2/5.NT20. Similar to the results obtained with hRHO and human β -actin, an efficient slope (-3.1) and an R^2 value (0.98) was obtained. In addition all 18S products had the correct predicted T_m (81°C) and size (101bp). Thus this 18S primer set is appropriate for use on FACS isolated retinal RNA.

4.3.9 Real-time RT-PCR analysis on FACS isolated retinal RNA 4 weeks post-injection

Real-time RT-PCR analysis to evaluate hRHO expression in retinas injected with either AAV-2/5.NT20 versus AAV-2/5.shP3 was undertaken. The RNA concentration obtained from EGFP positive retinal cells collected during FACS is too low to be accurately quantified using a nanodrop spectrophotometer. Therefore each RNA sample was diluted according to the EGFP positive cell number obtained for that sample during FACS. RNA from three AAV-2/5.NT20 injected eyes in which the highest cell numbers were obtained was used to generate a standard curve. RNA from an average of 5,833 cells was used as the first standard and a 5X standard curve was generated. RNA from approximately 2,000 cells was used as the input RNA concentration for each sample. Each sample was compared to the two curves (hRHO & 18S) and a quantity of hRHO RNA and 18S in AAV-2/5.NT20 versus AAV-2/5.shP3 injected retinas was obtained. hRHO expression in each sample was normalised to 18S and was analysed as described (**Section 4.2.13**). The level of hRHO expression in NHR retinas injected with AAV-2/5.shNT was $49.7 \pm 27.5\%$ compared to AAV-2/5.NT20 (**Figure 4.3.9.a** [$P=0.07$]). One of the animals (316) showed no hRHO suppression in the AAV-2/5.shP3 injected eye; this appeared to be an outlier and is not uncommon in animal studies. In the other two mice (301 and 286), suppression was obtained in AAV-2/5.shP3 treated eyes (35% and 24% of AAV-2/5.NT20 respectively). However in the 301 mouse the 18S value from NHR mice injected with AAV-2/5.shP3 was almost half that obtained for the 18S RNA in AAV-2/5.NT20 injected mice. This may be indicative that suppression of NHR by shP3 could be resulting in the degeneration of the retina and may cause the 18S RNA levels to decrease. As this could not be confirmed owing to the small n numbers used, it was decided to repeat this analysis with a fresh set of mice and evaluate these mice at an earlier time point of 14 days (**Section 4.3.10 below**).

In a similar manner to that described above, real-time RT-PCR analysis to evaluate hRHO expression in retinas subretinally injected with either AAV-2/5.Luc46 or AAV-2/5.miRP3-30a was undertaken (**Section 4.2.13**). RNA from the AAV-2/5Luc46 treated samples in which the highest cell numbers was obtained was used to generate a standard curve. This was undertaken such that RNA from an average of 1,775 cells was used as the first standard of the 5X curve. Approximately 1,000 cells from each RNA sample was used as the input RNA dilution. Following real-time RT-PCR, the relative concentration of hRHO RNA and 18S in AAV-2/5.Luc46 injected retinas versus AAV-2/5.miRP3-30a injected retinas was obtained (As outlined above for the shP3 analysis.). Compared to Luc46 injected eyes, miRP3-30a suppressed hRHO to $11.2 \pm 6.85\%$, $P < 0.05$ ($n=4$ [**Figure 4.3.9.b**]).

Real-time RT-PCR analysis to evaluate hRHO expression in eyes injected with either AAV-2/5.miNT or AAV-2/5.miRP3-155 was undertaken. EGFP positive cell numbers in miRP3-155 treated eyes were low, therefore only a 1/5 dilution of RNA from 3 eyes injected with AAV-2/5.miNT in which the highest EGFP positive cell numbers were obtained was used to generate the standard curve. An average of 2,373 cells was used to generate the first standard of the 5X standard curve. Approximately 1,500 cells from each RNA sample was used as the input RNA concentration. Following the real-time RT-PCR, the quantity of hRHO mRNA and 18S in AAV-2/5.miNT vs. AAV-2/5.miRP3-155 injected retinas was obtained (as outlined for the shP3 analysis). In miRP3-155-injected mice the average 18S values were lower than in AAV-2/5.miNT injected mouse; this also corresponded to a lower percentage of EGFP positive cells in these retinas. This result may be due to the nature of hRHO suppression in the litter of AAV-2/5.miRP3-155 treated NHR mice, which at 4 weeks post-injection was beginning to cause a retinopathy. Compared to AAV-2/5.miNT injected eyes, AAV-2/5.miRP3-155 suppressed hRHO to $25 \pm 13\%$, $P < 0.05$ (**Figure 4.3.9.c**).

4.3.10 FACS isolation of EGFP positive cells for hRHO mRNA expression analysis 2 weeks post – injection

As significant suppression had been obtained for AAV-2/5-delivered miRP3-30a and miRP3-155, and owing to a shortage of NHR mice, it was decided to only analyse AAV-2/5 delivered shP3 at 14 days post-injection. The retinal dissociation protocol was altered (**Section 4.2.10.2**) such that Papain instead of Trypsin was used to dissociate cells. It was suspected that the low cell numbers obtained previously may have been due to the Trypsin dissociation method being too harsh. Prior to FACS analysis PI solution was added to each sample, when excited by light at a wave length of 488nm, PI fluoresces red. PI cannot enter the cell membrane of viable cells, thus the appearance of red fluorescence is indicative of dead cells and can be used to sort healthy cells from non-healthy cells. Retinal cells from an uninjected EGFP negative control mouse were used to set the PI baseline (**Figure 4.3.10.a**). PI negative cells (live cells, red arrow) were clearly separated from PI positive cells (dead cells/debris). No EGFP positive healthy cells were obtained in this control as expected (**Figure 4.3.10.b**). An EGFP positive and PI positive control was used to assess the efficiency of the assay. This was an NHR mouse injected with AAV-2/5.NT20, cells that were dissociated 14 days post-injection. The PI positive cells were distinguished from PI negative cells (**Figure 4.3.10.c**), indicating that there was a larger population of healthy cells compared to non-viable cells. From this population of viable cells, EGFP positive healthy cells were isolated (**Figure 4.3.10.d**) such that only PI negative, EGFP positive cells were chosen for FACS (black cells).

The cell numbers obtained for NHR mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 and analysed 14 days post-injection were between 21,000-97,000 PI negative and EGFP positive cells (**Table 4.3.10.e**). No difference in cell number was obtained between AAV-2/5.NT20 or AAV-2/5.shP3 treated mice and the average % EGFP positive cells for AAV-2/5.NT20 versus AAV-2/5.shP3 was 15.6% and 20.2% respectively. The higher percentage of EGFP positive cells obtained for AAV-2/5.shP3 compared to the last AAV-2/5.shP3 FACS experiment may be due to the shorter incubation time of 14 days, i.e., retinal cells in which hRHO was suppressed were not yet undergoing degeneration. AAV-2/5.NT20 was only slightly less fluorescent than AAV-2/5.shP3 (1198MFI and 1814MFI respectively).

Real-time RT-PCR analysis to evaluate hRHO expression in retinas injected with either AAV-2/5.NT20 or AAV-2/5.shP3 14 days post-injection was undertaken. An average of RNA from 7,746 cells was used as the first standard of the 5X standard curve. 833 cells from each RNA sample was used as the input RNA concentration. Real-time RT-PCR analysis for AAV-2/5.NT20 versus AAV-2/5.shP3 was undertaken as previously described for the 1 month shP3 analysis (**Section 4.9**). Compared to AAV-2/5.NT20 injected eyes, AAV-2/5.shP3 suppressed hRHO to $22\pm 8\%$, $P<0.05$ (**Figure 4.3.10.e**)

4.3.11. Optimisation of Taqman®Small RNA assay for siP3 expression analysis

To evaluate the level of mature siP3 species produced from AAV-2/5.miRP3-30a and AAV-2/5.miRP3-155 compared to AAV-2/5.shP3 it was decided to use the custom Taqman®Small RNA assay designed by Applied Biosystems. The first part of this assay involved the specific reverse transcription of mature siP3; this was achieved using a stem loop RT primer specific for the siP3 sequence. This stem loop primer cannot bind to pri-miRNA or pre-miRNA (shRNA) siP3 species as these sequences already contained a hairpin, thus this ensured only mature siP3 species were evaluated. In the qPCR amplification step, specific primers amplified siP3, an siP3 specific Taqman®MGB probe was bound to the siP3 sequence of the siP3 cDNA, DNA polymerase cleaved the probe once it was hybridised to the target DNA. This released a quencher which separated from the reporter dye. This separation caused the reporter dye to fluoresce. The level of fluorescence was proportional to the specific amount of siP3 being amplified. As with all real-time RT-PCR evaluation, the relative level of siP3 in each sample was calculated by normalising to a housekeeping sequence. For this analysis, sno-202 was chosen as this is known to have consistent expression across retinal samples (Loscher et al, 2008).

Standard curves for siP3 and sno-202 were optimised as previously described (**Section 3.3.4**). For siP3, the slope was -3.09 and the R^2 value was 0.998 indicating that this siP3 curve was efficient. For sno-202, the slope was -3.5 and the R^2 value was 0.9996, indicating that the sno-202 primer set was efficient.

4.3.12 Analysis of siP3 RNA expression in retinas injected with AAV-2/5.shP3 compared to AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a

Having optimised the assays for siP3 and sno-202, these assays were used to evaluate expression of suppressors in AAV-suppressor treated NHR mouse eyes. NHR mice were subretinally injected with AAV-2/5.shP3 (LE) and AAV-2/5.miRP3-30a (RE) and RNA was extracted 10 day post injection (n=4). All samples were converted to siP3 and sno-202 cDNA and underwent qPCR using the siP3 and sno-202 standard curves described above (**Section 4.3.11**). siP3 RNA expression in retinas injected with AAV-2/5.shP3 was set as 100% and siP3 expression in retinas injected with AAV-2/5.miRP3-30a was expressed as a percentage of this value (**Figure 4.3.12.a**). siP3 expression from AAV-2/5.miRP3-30a injected retinas compared to AAV-2/5.shP3 was $8.8 \pm 4.4\%$, $P < 0.005$. Thus siP3 was 11 fold less expressed using the miRP3-30a backbone compared to siP3 expression from shP3.

NHR mice were subretinally injected with AAV-2/5.shP3 (LE) and AAV-2/5.miRP3-155 (RE) and RNA was extracted 10 day post injection (n=6). All samples were converted to siP3 and sno-202 cDNA and underwent qPCR and were analysed using the siP3 and sno-202 standard curves as described above (**Section 4.3.11**). siP3 expression from AAV-2/5.miRP3-155 compared to shP3 was $2 \pm 1.63\%$, $P < 0.005$ (**Figure 4.3.12.b**). Thus siP3 was expressed from the AAV-2/5.miRP3-155 backbone approximately 50 fold lower compared to siP3 expression from AAV-2/5.shP3. In summary the results suggest that significantly lower levels of siP3 were obtained *in vivo* using constructs with miRNA backbones compared to short hairpin RNA constructs.

4.3.13 ERG analysis of suppression by suppressor viruses

ERG analysis was undertaken on NHR mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE), n=9. Six weeks post injection ERG responses (b-wave values) were measured for rod-isolated responses (**Figure 4.3.13.a**), mixed rod-cone responses (**Figure 4.3.13.b**) and cone-isolated responses (**Figure 4.3.13.c**). The average b-wave values for each of the three ERG tests were combined and the ERG response in mice injected with AAV-2/5.NT20 was set at 100%, the average b-wave response in the eyes injected with AAV-2/5.shP3 were compared to this value. The average rod isolated response in mice injected with AAV-2/5.NT20 was $582 \pm 165 \mu V$ and AAV-2/5.shP3 was $256 \pm 94 \mu V$,

($P < 0.0005$). This corresponded to a rod-isolated decrease in ERG function to $44 \pm 16\%$ (**Figure 4.3.13.d**). The average mixed rod-cone response was $852 \pm 229 \mu\text{V}$ for AAV-2/5.NT20 injected eyes and $379 \pm 119 \mu\text{V}$ for AAV-2/5.shP3 injected eyes ($P < 0.001$) similarly this resulted in an average decrease in ERG response to $45 \pm 14\%$ in AAV-2/5.shP3 injected eyes (**Figure 4.3.13.e**). The cone-isolated ERG was also significantly decreased in AAV-2/5.shP3 injected mice. The ERG response was $78 \pm 26 \mu\text{V}$ for AAV-2/5.NT20 injected mice and $38 \pm 19 \mu\text{V}$ for AAV-2/5.shP3 injected mice ($P < 0.05$), which was a decrease to $49 \pm 25\%$ compared to AAV-2/5.NT20 injected eyes (**Figure 4.3.13.f**).

ERG analysis was undertaken on NHR mice subretinally injected with AAV-2/5.rP3.Luc46 (LE) and AAV-2/5.miRP3-30a (RE [$n=8$]). Six weeks post-injection the ERG responses in both eyes were measured for rod-isolated responses (**Figure 4.3.13.g**), mixed rod-cone responses (**Figure 4.3.13.h**) and cone-isolated responses (**Figure 4.3.13.i**). The average b-wave values for each ERG test were combined and the ERG response in mice injected with AAV-2/5.Luc46 was set at 100%, the average b-wave response in eyes injected with AAV-2/5.miRP3-30a were compared to this value. The average rod isolated response in AAV-2/5.Luc46-injected eyes was $496 \pm 196 \mu\text{V}$ and in the AAV-2/5.miRP3-30a-injected eyes was $404 \pm 157 \mu\text{V}$, $P = 0.2460$ (**Figure 4.3.13.j**). This indicated that despite a highly significant down regulation of hRHO RNA, miRP3-30a was not significantly suppressing the rod-isolated ERG function. Additionally this was mirrored with the mixed rod-cone response with an average b-wave amplitude of $763 \pm 337 \mu\text{V}$ in AAV-2/5.Luc46 injected eyes compared to $637 \pm 224 \mu\text{V}$ in AAV-2/5.miRP3-30a injected eyes, $P = 0.3322$ (**Figure 4.3.13.k**). Likewise in the cone-isolated response AAV-2/5.miRP3-30a did not significantly influence function. The average b-wave amplitude was $71 \pm 45.4 \mu\text{V}$ for AAV-2/5.Luc46 and $70 \pm 22 \mu\text{V}$ for AAV-2/5.miRP3-30a, $P = 0.9593$ (**Figure 4.3.13.l**). Of note, of the 8 mice that underwent ERG, 3 mice appeared to have a reduced ERG function in the AAV-2/5.miRP3-30a injected eye compared to the AAV-2/5.Luc46 injected eye, however when all the results were combined together overall not significance was not achieved.

ERG analysis was undertaken on NHR mice subretinally injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155(RE), $n=5$. Six weeks post injection ERG responses in both eyes were measured for rod-isolated responses (**Figure 4.3.13.m**), mixed rod-cone responses (**Figure 4.3.13.n**) and cone-isolated responses (**Figure 4.3.13.o**). The average b-wave values for each ERG test were correlated and the ERG response in mice injected with AAV-2/5.miNT was set at 100%, the average b-wave response in the eyes injected with AAV-2/5.miRP3-155 were compared to this value. The average rod-isolated b-wave amplitude for the AAV-2/5.miNT injected eye was $254 \pm 39 \mu\text{V}$ and in the AAV-2/5.miRP3-155 injected eye was $341.8 \pm 116 \mu\text{V}$, $P = 0.2088$. This resulted in a modest but not significant increase to

135±46% in the AAV-2/5.miRP3-155 injected eye compared to the AAV-2/5.miNT injected eye (**Figure 4.3.12.p**). The mixed rod-cone ERG correlated to the rod-isolated result with average b-wave amplitude of 404±56µV in AAV-2/5.miNT injected eyes compared to 551±215µV in AAV-2/5.miRP3-155 injected eyes, $P=0.2154$. This resulted in a modest but not significant increase in the mixed rod-cone ERG function to 136.5±53% (**Figure 4.3.12.q**). In the cone only response miRP3-155 also did not significantly influence function, the average b-wave amplitude is 35.9±4.7µV for AAV-2/5.miNT and was increased to 62±24.9µV (171±69%) for AAV-2/5.miRP3-155, $P=0.1013$ (**Figure 4.3.12.r**). It was clear that the hRHO RNA suppression obtained by AAV-2/5.miRP3-155 was not sufficient to confer a functional effect on the NHR retina. Thus it was decided not to pursue the AAV-2/5.miRP3-155 suppressor for hRHO any further.

4.3.14 Analysis of protein expression in NHRs injected with AAV-2/5.NT20 and AAV-2/5.shP3 by western blotting

In order to obtain protein for western blotting, EGFP positive cells in the retina must be separated from EGFP negative cells or RNAi mediated suppression may potentially be masked by the presence of total retinal protein. For protein expression analysis evaluated by a western blotting, EGFP positive cells should not undergo FACS analysis, as photoreceptor OS are often lost or damaged during the retinal dissociation FACS protocol. Although this is not an issue for RNA analysis (hRHO mRNA is in the cell body and not the OS), this is an issue for protein. For this reason the EGFP positive portion of the retina was physically excised and separated from the EGFP negative portion of the retina. This was delicate work and at times it was difficult to excise all EGFP negative cells from the EGFP positive cells. Western blot analysis was undertaken on samples from two NHR mice that had previously undergone ERG. These mice were subretinally injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE). Eyes were extracted 6 weeks post-injection (**Figure 4.3.14.a**). In the retinal protein samples from the two mice injected with AAV-2/5.NT20 (LE), abundant hRHO expression at 39kDA was present (lanes 1 & 3). In the retinal protein sample from two mice injected with AAV-2/5.shP3 (RE [lane 2 & 4]) hRHO protein expression was lower than that of the corresponding controls (lanes 1 & 3). Retinal protein from a 129rho^{-/-} mouse was used as a negative hRHO control (lane 5). Only a small amount of hRHO at higher molecular weights was observed in the retina protein samples containing AAV-2/5.NT20 but not in the AAV-2/5.shP3 injected samples and this was in accordance with Illing et al, 2002 in which the absence of multimeric hRHO at lower expression levels was observed (Illing et al, 2002). Mouse β-actin was used to assess equal loading however as only a small portion of the retina (the EGFP positive portion) was used, β-actin reactivity was lower than would usually be obtained in

HeLa cell protein (**Section 3.3.11.a**) or for whole retina. For this reason longer exposure times and a higher concentration of primary antibody was used. In this case an overnight exposure for β -actin was performed. As equal loading in some samples was not always clear, densitometry was undertaken in order to calculate the relative % of hRHO protein expression in AAV-2/5.shP3 injected retinas (**Figure 4.3.14.b**). The mean pixel intensity of hRHO in protein samples from retinas injected with AAV-2/5.NT20 was set at 100% expression and the mean pixel intensity of hRHO from protein samples of retinas injected with AAV-2/5.shP3 was compared to this value. It was found that shP3 suppressed hRHO protein by $68\% \pm 7.4\%$. As only $n=2$ animals were used for western blot analysis, evaluation of statistical significance was not undertaken.

4.3.15 Analysis of protein expression in NHRs injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30a by western blotting

Despite the fact that AAV-2/5.miRP3-30a did not significantly suppress ERG function in NHR mice, a highly significant suppression of hRHO by miRP3-30a was achieved at the RNA level. For this reason, it was decided to evaluate hRHO protein expression in NHR mice injected with AAV-2/5.miRP3-30a. Two of the mice that had undergone ERG analysis were used to evaluate hRHO protein expression (**Figure 4.3.15.a**). Abundant hRHO protein was visible at 39kDA in the retinal protein samples from mice injected with AAV-2/5.Luc46 (lanes 1 & 3), compared to $129\rho^{-/-}$ in which no hRHO protein was observed (lane 5). hRHO protein was also observed in retina protein samples from mice injected with AAV-2/5.miRP3-30a (lanes 2 and 4). β -actin was used to assess equal loading at an exposure time of twenty minutes. Even though it does appear that hRHO expression was lower in AAV-2/5.miRP3-30a injected eyes compared to AAV-2/5.Luc46, it was unclear if this result was definitive. For this reason densitometry was undertaken on this western blot (**Figure 4.3.15.b**). The % hRHO protein expression in AAV-2/5.miRP3-30a injected eyes compared to the hRHO protein expression in AAV-2/5.Luc46 was undertaken (**Section 4.3.14**). It was established that miRP3-30a suppressed hRHO protein by $40 \pm 13\%$. The number of animals used in the study was $n=2$ therefore evaluation of statistical significance was not undertaken. This suppression by miRP3-30a was lower than that obtained at the mRNA level, which may be the reason that the ERG function in NHR mice injected with miRP3-30a was not significantly altered compared to the control eye. It must be emphasised that n numbers are small for these experiments.

4.3.16 Immunohistochemistry on retinal sections from NHR mice injected with AAV-2/5.NT20 and AAV-2/5.shP3

Immunohistochemistry was undertaken on retinal sections from n=3 mice injected with AAV-2/5.NT20 and AAV-2/5.shP3. These mice had previously undergone ERG analysis at 6 weeks and significant suppression of ERG responses had been obtained. Thus it was decided to evaluate if the suppression of ERG responses by shP3 translated to changes in the retinal histology. Histology was performed at 6 weeks post-injection using the mouse 4D2 antibody, which should specifically bind the hRHO protein in the NHR photoreceptors. All three mice examined showed the same result and a representative retinal section is presented (**Figure 4.3.16.a**). The first two columns depict the control NHR mouse injected in AAV-2/5.NT20, column 1 is the EGFP positive region of the control retina in which the AAV-2/5.NT20 had transduced. Heavy hRHO staining was observed in this eye (1A) and this staining was comparable to the hRHO staining present in the EGFP negative region of the same retina (2A), thus AAV-2/5.NT20 was not negatively effecting hRHO immunoreactivity. In the EGFP positive portion of this retina (1B), a strong EGFP signal was observed; this suggests that by 6 weeks the NT20 EGFP vector was expressing EGFP efficiently compared to earlier time points. Additionally an overlay of RHO, EGFP and DAPI showed numerous healthy green IS. This was consistent with the finding that AAV-2/5.NT20 does not negatively impact the NHR retina. In the untransduced portion of the AAV-2/5.shP3 injected retinas hRHO was present (3B), however in contrast no hRHO staining was observed in regions of the retina transduced by AAV-2/5.shP3 (4A). Furthermore when the overlay of RHO, EGFP and DAPI (4C) was examined no EGFP positive IS or OS were present, and the EGFP signal was confined to the ONL. This suggested that suppression of hRHO in the NHR mice probably had a negative impact on photoreceptor cell survival and initiated degeneration of the photoreceptors. This finding was supported by the visible reduction photoreceptor nuclei rows in the ONL compared to the AAV-2/5.NT20 control and the EGFP negative region of the same retina.

4.3.17 Immunohistochemistry on retinal sections from NHR mice injected with AAV-2/5.Luc46 and AAV-2/5.miR-30a

It was decided to examine retinal sections from three of the NHR mice previously injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30a that had undergone ERG at 6 weeks. Despite significant suppression of hRHO RNA by AAV-2/5.miRP3-30a, overall no significant change in ERG function in the NHR animals injected with AAV-2/5.miRP3-30a was observed. Moreover in two of the animals that had previously undergone ERGS, only a 40% reduction in hRHO protein was obtained. It should be mentioned that three of the 8 animals that had undergone ERG showed a decrease in ERG

responses, however when responses from all 8 mice were combined this reduction was insignificant. It was decided to evaluate the histology of two of the mice in which no change in ERG function was obtained and compare it to one mouse that had a significant reduction in b-wave responses. Surprisingly, in the retina of the mouse that did show suppression of ERG function (mouse 361), no change in RHO immunoreactivity or DAPI staining was observed in this mouse compared to controls. This suggests that the reason for the lowered ERG response in the AAV-2/5.miRP3-30a injected eye may not have been attributable to any changes in histology. It may be that an inefficient ERG analysis was undertaken in this injected eye (suboptimal contact between electrode and cornea) or that this RE had a slower recovery rate than its corresponding LE. A similar result was obtained for the second mouse (mouse 356) in which no change in ERG was obtained, however the staining pattern was difficult to visualise owing to only a small EGFP positive area in this retina. However, the most surprising result was from the third mouse (mouse 358 [Figure 4.3.17.a]). In the AAV-2/5.Luc46 injected eye the RHO staining and DAPI cell layers were similar in the EGFP positive region (1A and 1C) and in the EGFP negative region of the same retina (2A and 2C) which suggests that like AAV-2/5.NT20, AAV-2/5.Luc46 does not negatively impact on the health of the photoreceptor cells. This was further supported by the presence of EGFP positive healthy inner segments (1C). However in the AAV-2/5.miRP3-30a injected eye, while RHO immunoreactivity and DAPI staining (3A and 3C) were similar in the EGFP negative regions (3B) to that observed in the AAV-2/5.NT20 injected eye, a reduction in RHO immunoreactivity (4A) was clearly observed in the EGFP positive region of the same retina (4C). This was surprising given the negative result from the two other mice and the fact that a negative ERG was obtained for this mouse. When the overlay of RHO, EGFP and DAPI was examined (4D), EGFP staining was clearly observed in the IS, indicating that despite the lack of RHO the photoreceptor cells had not yet degenerated. Overall it appears that AAV-2/5.miRP3-30a-based suppression was somewhat variable in these mice and therefore it was decided not to proceed with miRP3-30a at this point in time.

Section 4.4 Discussion

For degenerating retinopathies such as those caused by RHO-adRP, an effective therapeutic conferring long term stable expression *in vivo* in the retina would likely be the most advantageous. As siRNA-based suppressors provide only a short term solution for the down-regulation of hRHO, efficient therapeutic efficacy would require re-administration of the siRNA via subretinal injection, which is not a particularly feasible option for human patients. For this reason, the design of siRNAs, modified to allow stable suppression from an efficient promoter within the context of the degenerating retina would be beneficial. Previous RNAi based studies in the retina have traditionally used shRNA molecules delivered by viral vectors to achieve potent therapeutic efficacy (Millington-ward et al, 2011; O'Reilly et al, 2007). Various groups are now seeking to optimise artificial miRNA based systems, such that siRNAs can be cloned within the backbone of an endogenous pri-miRNA. Consequently, the siRNA can then act as a mimic for an endogenous miRNA and undergo processing through the miRNA pathway (Boudreau et al, 2007; Boudreau et al, 2009a; Davidson et al, 2011). Various artificial miRNA-based systems have been employed for use to date, miRNA-based shuttles appear particularly efficient at processing exogenous siRNA sequences provided the correct secondary structure of the endogenous miRNA is retained (Sibley et al, 2010). In particular ensuring the endogenous miRNA terminal stem loop, the Drosha and Dicer recognition sequences and maintaining mismatches present in the endogenous miRNA sequence appears particularly important (Feng et al, 2011; Han et al, 2006; Zeng & Cullen, 2005 ; Zhou et al, 2005). Additionally conventional shRNA-based vectors have depended on the use of polymerase III promoters where as artificial miRNAs can be transcribed from polymerase II promoters. This allows for a better control on both the timing and location of siRNA expression. Recently miRP3-30a-based artificial miRNA shuttles have been utilised with tissue specific promoters in various target cells and tissues such as the striatum and vascular smooth muscle, to deliver potent suppression of target mRNA transcripts (Nielsen et al, 2009; Rhee et al, 2009; Sibley et al, 2010; Zeng et al, 2007). Many groups have found high dose shRNAs cause toxicity, particularly in liver (Grimm et al, 2006; Grimm et al, 2010) and in brain (Boudreau et al, 2008; McBride et al, 2008). While this shRNA mediated toxicity has not to the authors knowledge thus far been reported within the retina, artificial miRNAs which imitate their endogenous counterparts may be more beneficial as a gene therapy targeting the delicate degenerating retinal tissue. However, aside from safety, an important factor to be considered when designing a therapeutic strategy for a disorder such as RHO-adRP is also therapeutic efficacy. It has previously been established that a high level of hRHO suppression is needed *in vivo* to provide therapeutic benefit in a RHO-adRP mouse model (Tessitore et

al, 2006). Thus only artificial miRNAs and indeed shRNAs that suppress hRHO to sufficient levels, such that functional benefit in transgenic mice is obtained will be considered for further evaluation.

Given that efficient suppression of hRHO RNA and protein by siP3 in HeLa cell culture was achieved (Chapter 3), it was decided to evaluate this siRNA *in vivo*. This involved the generation of siP3 into a shRNA construct (shP3) in addition to artificial miRNA constructs. Two artificial miRNA constructs have successfully been optimised and evaluated in the literature; the first is based on the endogenous miR-155 backbone (Chung et al, 2006) and is commercially available from Invitrogen (miRP3-155). The second is an artificial miRNA based on the endogenous miR-30a backbone (miRP3-30a); this has previously been optimised to mediate high suppression of target transcripts in the CNS and to provide therapeutic efficacy in transgenic mice with diseases such Huntington's disease and Alzheimer's disease (Boudreau et al, 2008; Boudreau et al, 2009; Piedrahita et al, 2010). Efficient delivery and efficacy of artificial miRNAs to the brain is of particular relevance to this study given that the retina is part of the CNS (Purves et al, 2001). Each construct (shP3, miRP30-155 and miRP3-30a) was placed under the control of their recommended promoter (H1 for shP3 [Brummelkamp et al, 2002], CMV for miR-155 [Chung et al, 2006] and U6 for miRNA-30a [Boudreau et al, 2008]), thus an accurate comparative analysis of hRHO suppression between each construct could not be obtained. In order to enable a comparison between each suppressor, each construct would need to be under the control of the same promoter. However, it was decided to leave each promoter unaltered in order to ensure the efficacy and safety of the optimised suppressors was not be compromised Therefore each suppressor was evaluated individually with its individual optimised parameters.

In order to assess the *in vivo* suppression of hRHO an animal model which expresses hRHO was needed. The NHR mouse was an ideal candidate as it has one hRHO transgene, and displays a wild-type phenotype (Ollson et al, 1992). The NHR mouse was placed on a mRHO null background (129rho^{-/-} [Humphries et al, 1997]). The absence of the mRHO alleles is of particular benefit for this study as the 4D2 antibody typically used for protein analysis targets mRHO and would otherwise mask any results obtained (Hicks & Molday, 1986). Additionally the absence of endogenous mouse alleles, allowed the evaluation of hRHO suppression by siP3 based suppressors to be undertaken at the functional level. Furthermore, this NHR mouse has been used previously with success to evaluate hRHO suppression by other shRNAs *in vivo* (Chadderton et al, 2009; O' Reilly et al, 2007).

Initially a four week time point was chosen in order to examine hRHO suppression at the RNA level, previous studies in the laboratory has established that the negative control construct for shP3 evaluation (AAV-2/5.NT20) has lower EGFP expression than AAV-2/5.shP3 and this can make it

difficult to collect significant high numbers of cells by FACS. Owing to the limited availability of NHR mice, only four mice were used for shP3 analysis. Of these one had bad injections in both eyes and AAV-2/5 transduction did not occur (**Table 4.3.7**). This result highlights how important it is to keep injection records to explain anomalies which may occur during later analysis. Of the three mice for which EGFP positive cells were collected, no significant suppression of hRHO was achieved (**Figure 4.3.9.a**), however, 2/3 mice did suppress hRHO mRNA efficiently and the low overall insignificant suppression was most likely masked by the lack of suppression observed in a third outlier. Additionally, one of the mice in which hRHO suppression by AAV-2/5.shP3 was achieved had half the level of 18S mRNA within the retinal sample injected with shP3 compared to AAV-2/5.NT20. This could further mask the relative hRHO expression value obtained for this sample by altering the hRHO to 18S ratio. This 18S anomaly indicated that AAV-2/5.shP3 may be suppressing hRHO to such levels that may cause the death of the rods and result in a decreased level of 18S. There was half as much % EGFP positive cells in AAV-2/5.shP3 treated retinas compared to AAV-2/5.NT20 treated retinas which suggests that EGFP positive cells may be dying as the retinal cells containing the suppressor degenerate. A shorter time-point of 14 days was therefore chosen for the shRNA analysis in order to accurately assess hRHO RNA expression in AAV-2/5.shP3 injected samples before a retinal degeneration phenotype was induced. This obviously had consequences for the AAV-2/5.NT20 treated samples in which EGFP expression at this time point may not have reached adequate levels to enable FACS. For this reason, it was decided to use a gentler retinal dissociation protocol in order to get the maximum amount of EGFP positive cells possible despite low fluorescent intensity. In the previous analysis using the Trypsin dissociation protocol a large number of cells were damaged and thus no longer EGFP positive. PI was added to cells prior to FACS in order to ensure only healthy EGFP positive cells were collected. This was done with the premise that if hRHO suppression was causing retinal degeneration than by getting rid of any dead cells, normal 18S expression would be preserved and thus an accurate analysis of hRHO suppression could be undertaken. Two weeks post-injected AAV-2/5.shP3 was found to suppress hRHO *in vivo* by 78% (**Figure 4.3.10.e**) This suppression of hRHO by an shRNA was higher than obtained in a previous study for shRNAs targeting mRHO in wild-type mice (Gorbatyuk et al, 2007b). In terms of hRHO, previous hRHO RNA suppression with a different shRNA in NHR mice also achieved significant suppression by approximately 95% (O'Reilly et al, 2007; Chadderton et al, 2009).

To date no analysis of artificial miRNA-155-based suppressors in the retina have been published, however, high levels of suppression of target mRNA by artificial miRNA-155 have been achieved at the mRNA level in other tissues such as the brain (Baek et al, 2010). For the current study the design

used is based on Chung et al, 2006 who generated an artificial miRNA targeting an area of 21bp. The endogenous miRNA-155 antisense strand is 2 nucleotides longer than the complimentary strand; therefore in designing the artificial miRNAs, Chung et al removed 2 nucleotides from the sense miRNA strand to create a 2 nucleotide internal loop similar to the endogenous counterpart. In addition artificial miRNAs with this 2 nucleotide deletion were more functional than those without it (Chung et al, 2006). For this reason it was decided to design siP3 within the miR-155 backbone to be 21bp, in order to maintain a similar structure to both published artificial miRNAs and the endogenous miR-155. In this study AAV-2/5.miRP3-155 was found to significantly reduce hRHO mRNA by 75%, which indicates the extra two nucleotides added to either side of the original siP3 target site does not abolish siP3 efficacy (**Figure 4.3.9.c**). Lower 18S levels and lower EGFP positive cell percentages in retinal cells injected with miRP3-155 suggested that like shP3, at 4 weeks AAV-2/5.miRP3-155 may be resulting in retinal degeneration, owing to potent hRHO suppression. Analysis at 2 weeks post-injection of vector in NHR mice was not undertaken as the AAV-2/5.miRP3-155 did not result in changes in ERG function at 4 weeks and was thus precluded from further analysis. The miRP3-155 based suppression achieved in this study was better than reported previously using the artificial miRNA-155 construct delivered to mouse brains via lentivirus, where the target mRNA (neurokinin receptor -1) was only suppressed by 40-50% *in vivo* as assessed by real-time RT-PCR (Baek et al, 2010). Interestingly a study by Liu et al, 2010 exploring the use of artificial miRNA-155 constructs using the lentiviral system established that the CMV promoter results in a reduced lentiviral titre (Liu et al, 2010). No effect on titre was observed for AAV-2/5.CMVmiRP3-155 generation compared to other artificial miRNA or shRNA constructs driven by U6 or H1 respectively, indicating that this is not the case for the production of AAV-2/5 virus

miRP3-30a was designed similar to the optimised parameters described by Boudreau et al, 2008. The experimentally validated Drosha processing and Dicer cleavage sites were maintained as were any mismatches in the endogenous stem-loop, so as to aid in efficient production of miRP3 in the retina (Feng et al, 2011; Lee et al, 2003; Silva et al, 2005; Zeng & Cullen et al, 2003). One of the main changes to this construct involved altering the flanking region at the base of the stem loop to a sequence miV2. This sequence originated from flanking regions within the MCS of the vector used by Boudreau et al, 2008 which had been altered to a sequence predicted to form RNA secondary structures. Boudreau et al, 2008 found 20% more suppression of target RNA using this region compared to the endogenous miR-30a flanking region. The authors hypothesised that this was due to a sequence within miV2 which is single stranded and therefore may be more amenable to the binding of the Drosha-DGCR8 RNAi complex, as the substrate for Drosha-mediated cleavage appears to be an

extended stem loop followed by a region of single stranded RNA (Boudreau et al, 2008; Zeng & Cullen, 2005). The highest level of hRHO suppression was achieved with AAV-2/5.miRNA-30a compared to AAV-2/5.shP3 and AAV-2/5.miRP3-155 at the RNA level (89% hRHO suppression [Figure 4.3.9.b]). This is comparable to that achieved using artificial miRNA-30a constructs targeting peripherin-2 RNA in the retina, in which an AAV-2/8 delivered construct resulted in an 83% suppression of endogenous peripherin-2 mRNA as assessed by real-time RT-PCR (Georgiadis et al, 2010).

Despite the differences in backbone and promoters, all constructs (AAV-2/5.shP3, AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a) significantly suppressed hRHO RNA *in vivo*. It was decided to examine the expression of mature siP3 species processed from each vector. Boudreau and colleagues established via northern blotting that mature siRNAs from an shRNA-based backbone are expressed more abundantly than siRNAs from artificial miRNA-30a based constructs, even though therapeutic efficacy can be achieved with miRNA-30a based suppressors (Boudreau et al, 2008; Boudreau et al, 2009a). Therefore it was proposed that AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a may yield lower quantities of siP3 species than AAV-2/5.shP3. Previous studies in the laboratory examining the expression of siRNA from shRNA shuttles utilised RNase protection assays with a P³²-based siRNA probe (O'Reilly et al, 2007). Northern blot analysis has been used by Boudreau et al, in order to quantify both processed and unprocessed shRNAs and artificial miRNAs (Boudreau et al, 2008; Boudreau et al, 2009a). In this study a highly quantitative two step real-time RT-PCR approach was undertaken, using a probe designed specifically for mature siP3. This approach has been used previously with success in the retina to evaluate the relative expression of various miRNAs (Loscher et al, 2008). The results of this experiment mirror those found in the literature, i.e. shRNA based RNAi produce more siRNA species than miRNAs. Of note the most efficient suppressor AAV-2/5.miRP3-30a produced 11 times less siP3 than AAV-2/5.shP3 (Figure 4.3.12.a) supporting findings that high efficacy with artificial miRNAs can be achieved, despite lower levels of processed miRNA compared to shRNAs (Boudreau et al, 2009a). AAV-2/5.miRP3-155 produced low levels of mature siP3 (50 times less siP3 than shP3), which was not surprising given that the level of suppression of hRHO by AAV-2/5.miRP3-155 was the lowest of the three constructs (Figure 4.3.12.b). Previous studies comparing mature siRNA expression from different shuttles have used the same promoter in all constructs for a direct comparison, however this was not the case in this instance, therefore it is impossible to conclude if the reason for mature siP3 expression differences is due to miRNA processing or differences in promoter. The finding that CMV driven miRP3-155 was the least efficient suppressor is interesting given the fact that when CMV driven shRNA, CMV driven miR-30a and

CMV driven miR-155-based constructs targeting both GAPDH and GFP were compared *in vitro*, miR-155-based suppression was more potent (Shan et al, 2009b). This suggests that placing shP3 and miRP3-30a under the control of CMV may possibly reduce the efficacy of these suppressors, and thus the CMV promoter may not be as efficient as U6. An interesting experiment would entail evaluating miRP3-155 driven by the mU6 promoter and measuring if hRHO suppression may be as efficient as with mU6 driven miRP3-30a.

Previous data in the laboratory has been undertaken comparing shBB, an shRNA known to be a potent hRHO suppressor *in vivo* (O'Reilly et al, 2007), to artificial miRNAs based on miR-155 and miR-30a in an evaluation similar to the current study (Millington-Ward, personal communication [unpublished data]). No significant hRHO RNA suppression was achieved from either of the artificial miRNAs. Furthermore analysis of mature siBB expression from shBB compared to miRBB-30a, revealed a 200 fold difference in siBB expression between the two suppressors. As miRBB-155 had two extra nucleotides incorporated into its siRNA duplex as recommended by Chung et al, 2006, it is possible that these extra nucleotides abolished the efficacy with which siBB binds to its target site. However as the miR-30a-based suppressor for siBB exactly mirrored the miRP3-30a suppressor, this suggests that at least for miR-30a the siP3 target sequence within the miRNA vector must be favourable to miRNA-mediated suppression compared to the siBB sequence. Secondary structure and the flanking sequences surrounding miRNA target sites have been identified as important for recruiting the miRNA machinery. In one particular study U rich regions within target mRNA promoted the binding of dead end 1 (Dnd-1) and negatively regulated miRNA based suppression by preventing the miRNA machinery from binding (Ameres et al, 2007; Kedde et al, 2007; Kertesz et al, 2007). It is possible that the BB target sequence is within a region of the RHO ORF less favourable for miRNA mediated regulation compared to the P3 target site, however, presumably siRNA based suppression may also have been inhibited if this was the case and shBB based suppression is very efficient *in vivo*. Differences in the siRNA processing machinery and the miRNA processing machinery could account for this change in efficacy such as processing of the miRNA-BB by Drosha. miRNA target sites near the 3' end of the mRNA ORF are much more efficient target sites compared to sites further upstream (Forman et al, 2010). Despite the siP3 target site not being near the 3' end of the RHO ORF, it is more 3' than the siBB site and thus might be more amenable to the miRNA machinery. This finding highlights the importance of screening RNAi targets in various different locations within an mRNA sequence and suggests that efficacious shRNAs may not necessarily translate into efficacious artificial miRNAs.

ERGs are one of the most predominant techniques used to assess visual function in clinical settings (Reynolds et al, 2008). This technique can also be used to assess changes in retinal function in mice. ERG responses were assessed in mice 6 weeks post-injection, this was the time-point chosen in order to allow the mice to sufficiently recover from the injection. Even though all suppressors significantly suppressed hRHO, it was important to establish if this down-regulation of hRHO was sufficient to provide a functional effect in the NHR mouse. The rod-isolated ERG response which is a measure of the rod cell function (where hRHO is expressed) was reduced significantly by 56% with AAV-2/5.shP3 compared to AAV-2/5.NT20 (**Figure 3.13.d**). Thus 78% suppression of hRHO RNA by shP3 was sufficient to alter rod function, moreover both mixed rod-cone and cone-isolated ERG responses were reduced to similar levels (**Figure 3.13.e & Figure 3.13.f**). This corresponds to the literature, where prior findings established that degenerating rod photoreceptor cells have the secondary effect of being detrimental to cone function (Chadderton et al, 2009; Humphries et al, 2001; Leveillard et al, 2004).

For mice injected with AAV-2/5.miRP3-155 no significant effect on ERG function in the NHR mouse was achieved when compared with that of AAV-2/5.miNT (**Figure 4.3.13.p, Figure 4.3.13.q & Figure 4.3.13.r**) this may be due to the fact that AAV-2/5.miRP3-155 was the least efficient suppressor of hRHO. Additionally 62% reduction in mutant RHO RNA was not sufficient to provide functional benefit to a RHO-adRP mouse model (Tessitore et al, 2006). In an NHR mouse injected with 2×10^{12} vp/ml of AAV-2/5, the virus has been found to transduce about 30-40% of the retina (Chadderton et al, 2009). If only a third of the retina is receiving the therapeutic, then 75% suppression of EGFP positive cells, would translate to approximately 25% suppression of overall retinal hRHO therefore it could be speculated that a threshold may be present in which there is a minimum amount of hRHO needed overall in order to confer a functional change. As AAV-2/5.miRP3-155 did not confer adequate suppression to disrupting ERG function, it was decided not to proceed further with this construct.

Interestingly no significant changes in ERG responses in NHR mice injected with AAV-2/5.miRP3-30a compared to the AAV-2/5.Luc46 control were achieved (**Figure 4.3.13.j, Figure 4.3.13.k & Figure 4.3.13.i**) This was surprising given that AAV2/5.miRP3-30a resulted in the most potent hRHO suppression at the RNA level. The reason for this discrepancy is unknown. In general potent hRHO suppression at the RNA level has been found to correlate well with hRHO protein suppression (Chadderton et al, 2009; Millington-Ward et al, 2011; O'Reilly et al, 2007). It is of note that even though significant alterations in ERG function were not achieved overall, 3 of the 8 mice tested did show a decreased ERG response in AAV-2/5.miRP3-30a injected eyes compared to AAV-2/5.Luc46

injected eyes, which was masked by the five mice in which no changes in ERG response were noted. It is possible that for these three mice, the electrode on the shP3-injected eye was not functioning as efficiently (e.g. not attached to the cornea properly etc) and this may be the reasons for the anomalies. Increasing the n numbers of animals tested at this time was not possible due to the limited number of NHR mice available.

Now that AAV-2/5.shP3 was confirmed as successfully suppressing hRHO RNA to such an extent as to alter ERG function, it was decided to examine the down-regulation of hRHO protein both at the molecular level and at the histological level in order to ensure that hRHO protein suppression was being achieved. Even though AV-2/5.miRP3-30a based hRHO mRNA suppression did not result in significant alterations in ERG responses compared to control, it was decided to also further evaluate this construct at the protein level so as to elucidate a reason for this anomaly. Initially the hRHO protein expression was examined in the retinas of injected NHR mice by western blotting. The FACS analysis dissociation protocol can be harsh on cells, and rather than risk losing the delicate outer segments containing hRHO protein, it was decided to physically excise the EGFP positive region of the NHR retina so as to obtain hRHO protein only from cells which had been transduced. Bands representing hRHO monomeric protein were successfully obtained despite the small amount of tissue isolated; this is owing to the high sensitivity of the 4D2 antibody. During *in vitro* analysis (Chapter 3), hRHO over expression resulted in the presence of hRHO at high molecular weights. At lower expression levels, wild-type rhodopsin is present only as a monomer, therefore by only using the EGFP portion of the retina for analysis, less rhodopsin was present and consequently no higher molecular weight forms of rhodopsin were observed (Illing et al, 2002).

hRHO protein expression in NHR samples injected with AAV-2/5.shP3 compared to AAV-2/5.NT20 were lower, densitometry was undertaken and a 68% hRHO protein reduction was achieved with shP3, correlating with the RNA result (**Figure 4.3.14.a & Figure 4.3.14.b**). Interestingly the level of hRHO protein suppression achieved by AAV-2/5.miRP3-30a was not as obvious, and it was clear that a large amount of hRHO immunoreactivity were still present in lanes containing retinal protein from mice injected with miRP3-30a (**Figure 4.3.15.a**). Using densitometry analysis it was established that only a 40% reduction in hRHO protein was observed with AAV-2/5.miRP3-30a (**Figure 4.3.15.b**). This may however, explain the discrepancy observed with the ERG result in which no significant change in ERG amplitudes were noted in AAV-2/5.miRP3-30a injected retinas. The reason that the 89% hRHO mRNA suppression obtained did not correspond to protein suppression is not known. However, owing

to limitations with animal numbers only two mice for each construct shP3 and miRP3-30a was used for western blot evaluation, thus no statistical analysis could be undertaken.

It was decided to examine the exact effects of both AAV-2/5.shP3 and AAV-2/5miRP3-30a on the retinal structure by performing immunohistochemistry, 6 weeks post-injection on NHR mice injected with both suppressors and their corresponding controls (**Figure 4.3.16.a**). In all case AAV-2/5.shP3 transduced eyes displayed a decrease in hRHO immunoreactivity in the EGFP positive sections of the retina compared to the EGFP negative sections of the retina and to the AAV-2/5.NT20 injected retinas. Furthermore intact, healthy IS were clearly observed in the EGFP positive regions of the AAV-2/5.NT20 injected retinal sections, whereas with the EGFP positive regions of the shP3 retinal sections no IS were observed. A similar result was obtained with shQ1, which suppresses hRHO by up to 95% *in vivo*, in which a reduction in hRHO reactivity led to the loss of healthy IS and OS within the retina, compared to the control eyes (Chadderton et al, 2009). This supports the RNA analysis at 4 weeks in which hRHO RNA suppression was hypothesised to cause the degeneration of the retina and lowering the levels of 18S in the AAV-2/5.shP3 injected samples.

From the three NHR animals injected with AAV-2/5.miRP3-30a and AAV-2/5.Luc46, the results obtained were more variable. In one of the animals a clear decrease in hRHO immunoreactivity, EGFP positive IS and ONL cell nuclei was observed, suggesting that, corresponding to the RNA results efficient hRHO suppressions was occurring (**Figure 4.3.17.a**) In one of the animals, the result was less clear due to inefficient transduction of the AAV resulting in only a small region EGFP reactivity. In the third animal examined hRHO immunoreactivity was visible in EGFP positive regions of mice injected with miRP3-30a compared to controls. Furthermore healthy IS were also present. This result is very surprising given that the ERG performed on this animal prior to histology, was one of the three ERG responses to yield decreased amplitude in the AAV-2/5.miRP3-30a treated eye. One possibility that cannot be excluded was that during the fixation of eyes, AAV-2/5.miRP3-30a injected eyes from the first and the third mouse were accidentally swapped, which would explain why the results for each eye were opposite to what was expected. Another explanation is the possibility that some error occurred during ERG analysis so as to not yield a proper result in the AAV-2/5.miRP3-30a injected NHR 361. This would not explain the suppression observed in the NHR 358 mouse, however it is evident from the RNA, protein and ERG results that variation in the assessment of miRP3-30a based suppression, seems to be occurring, given this variation any further analysis of miRP3-30a in the retina was not considered at this time.

Throughout the course of the study described in this chapter, it became apparent that siP3 may possibly contain sequence characteristics unfavourable to the innate immune response (Judge et al, 2005). The inclusion of these sequences in the siP3 sequence was surprising given the stringent criteria Invitrogen claim to utilise to select efficient siRNA sequences. Systemic delivery of siRNAs containing amongst other motifs the sequence 5'UGUGU3' were found to stimulate the immune response in blood immune cells when systemically delivered via liposomes irrespective of the mRNA target site (Gantier et al, 2010; Judge et al, 2005). Delivery of these immunostimulatory siRNAs by this method required the incorporation of the siRNAs into endocytotic compartments, where they stimulated TLR7/8 receptors (Heil et al, 2004; Hornung et al, 2005; Sioud et al, 2005). While this may be a cause for concern for the siRNA-based study delivered to HeLa cells using Lipofectamine® 2000 (Chapter 3), no adverse reaction such as toxicity or altered house keeper gene expression was observed with siP3 compared to any of the other siRNAs evaluated. Furthermore, evaluation of a replacement gene for siP3 which was undertaken in parallel to this study (Chapter 5), confirmed hRHO based suppression was target site dependent and the efficacy achieved both *in vitro* and *in vivo* was clearly a result of an efficient RNAi molecule. The main goal of this thesis was the design of a therapeutic for use *in vivo* for delivery specifically to the retina, an immunocompromised tissue. This therapeutic would therefore not be delivered systemically with liposomal based complexes, but by sub-retinal delivery of recombinant AAV. Robbins et al, 2006 explored delivery of shRNAs containing "immunostimulatory motifs" and found these shRNAs stimulated the immune system if delivered by cationic liposomes. Of note stable expression of these shRNAs from a lentivirus vector resulted in minimal stimulation of the innate immune response (Robbins et al, 2006). The shRNAs in this study and all subsequent studies were expressed stably by AAV-2/5 and this should in theory evade detection by the TLR7/8 receptors present within endosomal compartments. A recent publication established that tagging this 5'UGUGU3' sequence to the 5' end of an siRNA was much more potent at up regulating an immune response compared to the tagging of this sequence to the 3' end of an siRNA, the 5'UGUGU3' sequence within siP3 is at the 3' end of the sequence and therefore it is possible that even when systemically delivered via liposomal complexes may not be stimulatory to the immune system (Stewart et al, 2011). Taking all these points into account, and the fact that AAV-2/5.shP3 was the most potent suppressor of hRHO, it was decided to continue with siP3-based suppression studies. However, in the future the immune response of this shRNA and all other shRNAs in the laboratory utilised *in vivo* should be evaluated thoroughly to rule out any adverse reactions.

In conclusion, AAV-2/5.shP3 is the most consistent, efficient suppressor of hRHO *in vivo* at the mRNA, protein, functional and histological level evaluated in the current study. Notably significant

RNA suppression was obtained with all suppressors assessed. AAV-2/5.miRP3-155, the least efficient suppressor at the RNA level was not a sufficient suppressor in terms of providing changes in ERG amplitudes and was therefore excluded from further evaluation. The activity of AAV-2/5.miRP3-30a represents the most surprising result obtained in this study. On one hand the most efficient hRHO suppression at the RNA level was achieved by this suppressor, but in terms of all other readouts of suppression efficiency, the results obtained were highly variable and less than positive. It is possible that the AAV-2/5.miR-30a backbone may not be optimal for the retina. However in studies using a miR-30a based suppressor of peripherin -2 within the retina, efficient suppression was achieved (Georgiadis et al, 2010). The mature siRNA species was never quantified in this instance nor was any functional assessment undertaken. It is possible that the suppression of peripherin-2 observed in this study does not correspond to a functional change; furthermore peripherin-2 expression in the retina is lower than hRHO and therefore may possibly have a lower threshold in which suppression can lead to benefit. Additionally AAV-2/8 was used in the peripherin-2 study in contrast to the AAV-2/5 used in this chapter. AAV-2/8 seems to be better at transducing photoreceptors compared to AAV-2/5 (Allocca et al, 2007; Georgiadis et al, 2010; Leberherz et al, 2008; Mussolino et al, 2011b) and thus may explain why such potent suppression was achieved. As the AAV-2/5.miRP3-30a results were quite variable and AAV-2/5.shP3 achieved efficient suppression of hRHO mRNA and protein in addition to a decrease in ERG responses and RHO immunoreactivity it was decided to focus on shP3 for further evaluation of RNAi-based AAV-delivered *in vivo* suppression of hRHO.

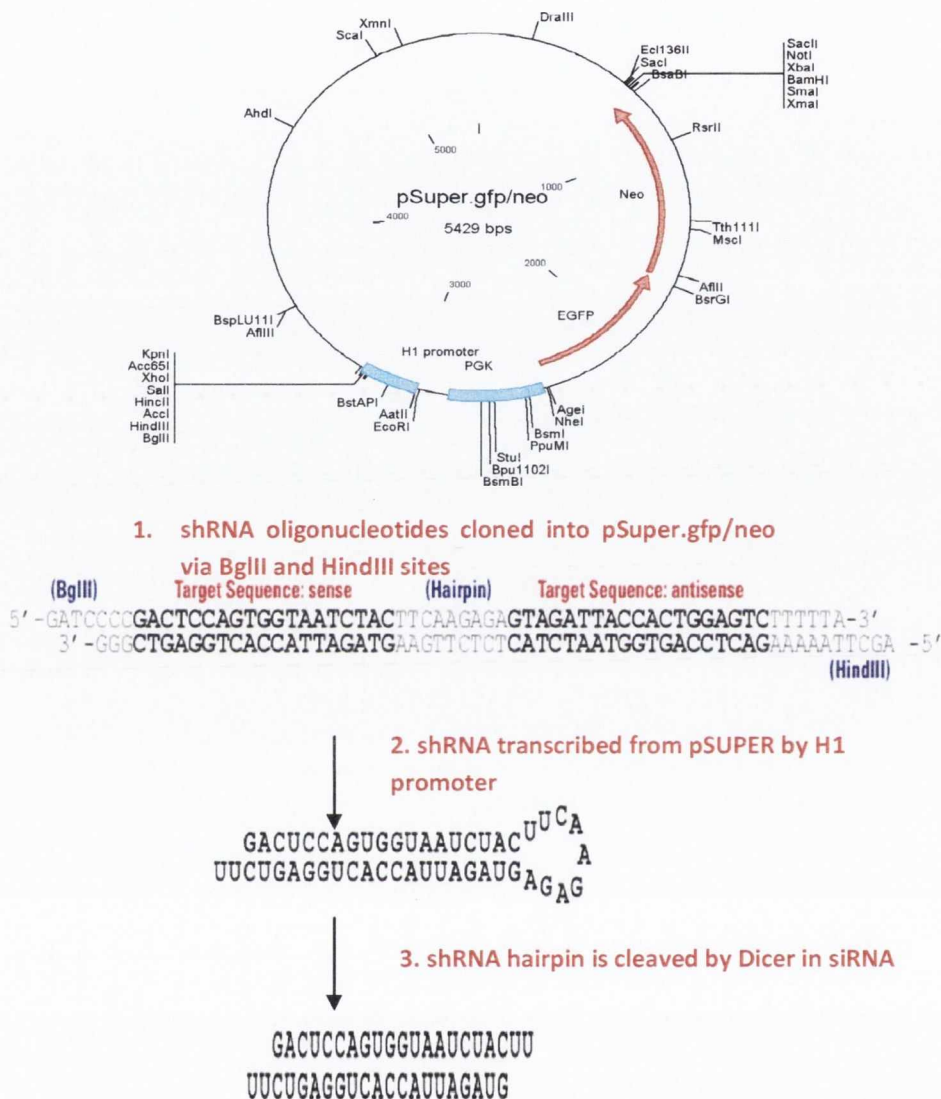


Figure 4.2.1.a Strategy behind shRNA design within pSUPER (Oligoengine)

1. The shRNA oligonucleotide is designed such that the 5' *Bam*HI overhang and the *Hind*III overhang were easily inserted into the *Bgl*III and *Hind*III restriction enzyme sites in the linearised pSUPER vector such that the shRNA is located downstream of the H1 promoter.
2. The shRNAs are efficiently transcribed from the polymerase III H1 promoter into short hairpin RNA structures.
3. Dicer cleaves the hairpin such that an 19 nucleotide siRNA is released which can enter RISC

Shp3 F	5'-GAGTACAGGATCCCGGTACGTGGTGTGTAATTC AAGAGATTACACACCACCGTACCTTTTAAAGCTTGAGTACA
shp3 R	5'-TGTACTCAAGCTTAAAAAGGTACGTGGTGGTGTGTAATCTCTTGAATTACACACCACCGTACCGGGGATCCTGTACTC
miRP3-155 F	5'-TGTGCTTACACACCACCGTACCGGTTTGGCCACTGACTGACCGGTACGTTGGTGTAAAG
miRP3-155 R	5'-CCTGCTTACACACCAACGTACCGGTCAGTGAGTGCCCAAAACCGGTACGTTGGTGTGTAAAGC
miRP3-30a F	5'-AAAACTCGAGTGAGCGCGGTACGTGGTGGTGTGTAACTGTAAAGCCACAGATGGG
miRP3-30a R	5'-AAAAAGCGCCGCTCTAGTAGGCGAGGTACGTGGTGGTGTGTAAACCATCTGTGGCTTTACAG

Table 4.2.1.a Sequences of oligonucleotides used for generation of RN*Ai* suppressors (Sigma-Aldrich)

The first two sequences are the oligonucleotides for shp3 with *Bam*HI sites (G/GATCC) and *Hind*III sites (A/AGCTT) to facilitate cloning into pSuper.gfp/neo. The third and fourth oligonucleotides are the sequence for miRP3-155 with overhangs that allow cloning into the pcDNATM6.2-GW/±EmGFP-miR vector. The fifth and sixth oligonucleotide sequences are the sequences used to generate miRP3-30a. 19 nucleotides (red) are homologous within each oligonucleotide and Klenow extension from this site facilitates the generation of the full length oligonucleotide.

pSuper F	5'-CTTGCGACTGCAGTAGTTGG-3'
pSuper R	5- AACAGCTATGACCATG-3'
SRSF	5'-GTGCCTGTGACTTAAC-3'
SRSR	5'-AGTAAGTGTGACCACAGAAGGTA-3'
EmGFP F	5'-GGCATGGACGAGCTGTACAA-3'
miRNA R	5'-CTCTAGATCAACCACTTTGT-3'
U6F	5'-TGGCGGAAGGCCGTCAG-3'
U6R	5'-CTCATGGGCCTTCCGCTC-3'
3'155R	5'-CCCACCAGCCTTGTCTAAT-3'
EGFPR	5'-AAACGCCAGCAACGCGGCC
EGFPF	5'-CCGTTTACGTCGCCGTCAG-3'
18S F	5'-CAAGAACGAAAGTCGGAGGT
18S R	5'-CGGGTCATGGGAATAACG-3'
P23H R	5'-CATTCTTGGGTGGGAGCAG-3'
P23H F	5'-GGACAGGAGAAGGGAGAAGG-3'

Table 4.2.1b Primer sequences for Chapter 4

All primers were ordered from Sigma-Aldrich. pSuper forward and reverse primers (pSuper F and pSuper R) were used to screen for the successful cloning of an shRNA into pSuper.gfp/neo. SRS forward and reverse primers (SRSF and SRSR) were used to determine the successful cloning of shP3 and miRP3-30a into both the pAAV.rP3 and pAAV.CMV.EGFP vectors. EmGFP forward and miRNA reverse primers (EMGFP F and miRNA R) were used to detect positive clones of miRP3-155. U6 forward and reverse (U6F and U6R) determined if miRP3-30a was successfully cloned downstream of the mU6 vector in pMA.mi30BB.EGFP. Forward and reverse primers (EGFPF and EGFPR) were used to determine the titre of the AAV-2/5 suppressor viruses. P23H forward and reverse primers (P23HF & P23HR) were used to genotype the NHRs. 18S forward and reverse primers (18SF and 18SR) were used as primers to amplify the 18S housekeeper in all real-time RT-PCR experiments.

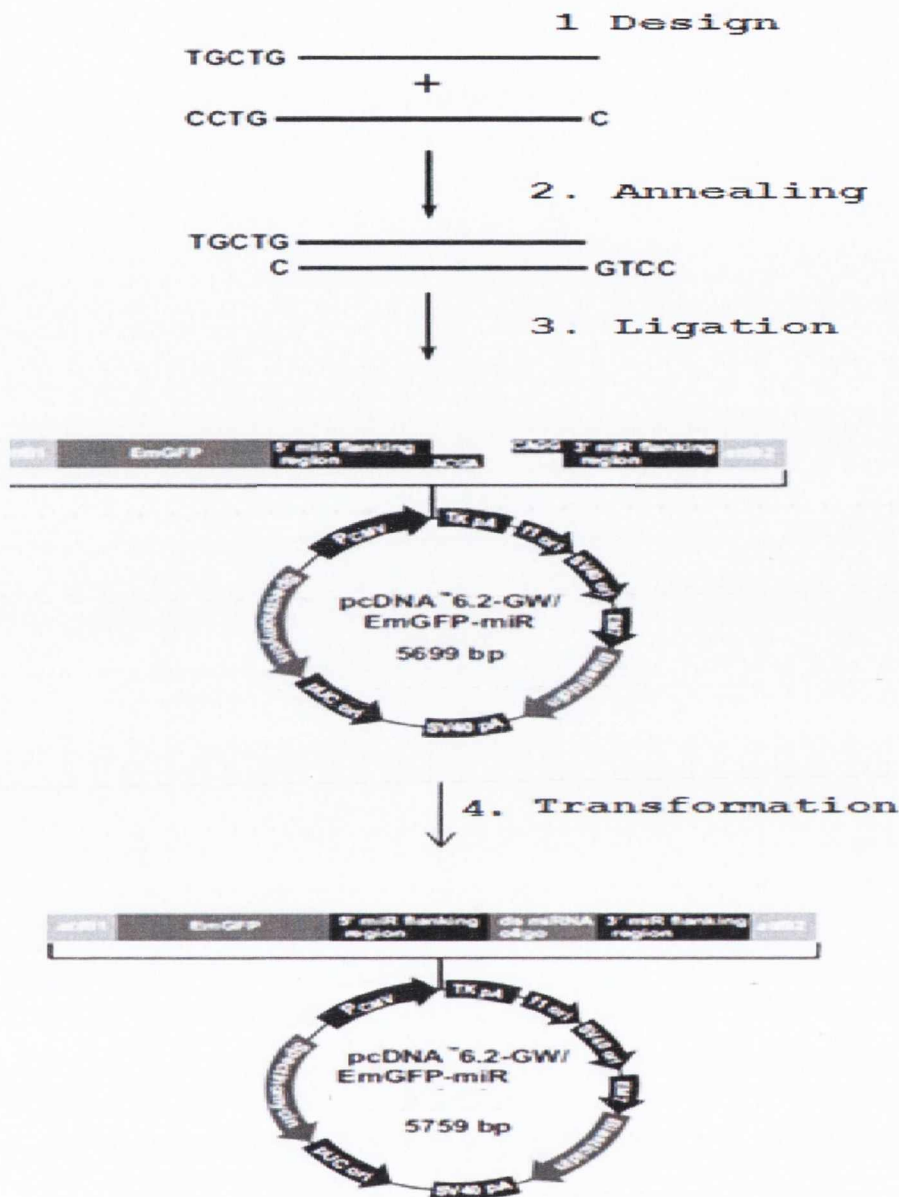


Figure 4.2.5.a cloning strategy for miR-155 within the pcDNA™6.2/GW/EmGFP-miR (Invitrogen)

1. Oligonucleotides were designed with the appropriate overhangs to facilitate directional cloning,
2. The two oligonucleotides were annealed to generate a double stranded miRNA oligo,
3. The double stranded oligonucleotide is ligated into the miR vector via the 5' and 3' overhangs. This is to ensure the miRNA is inserted down-stream of the CMV-EmGFP promoter.
4. The ligation reactions are cloned and colonies screened for positive clones.

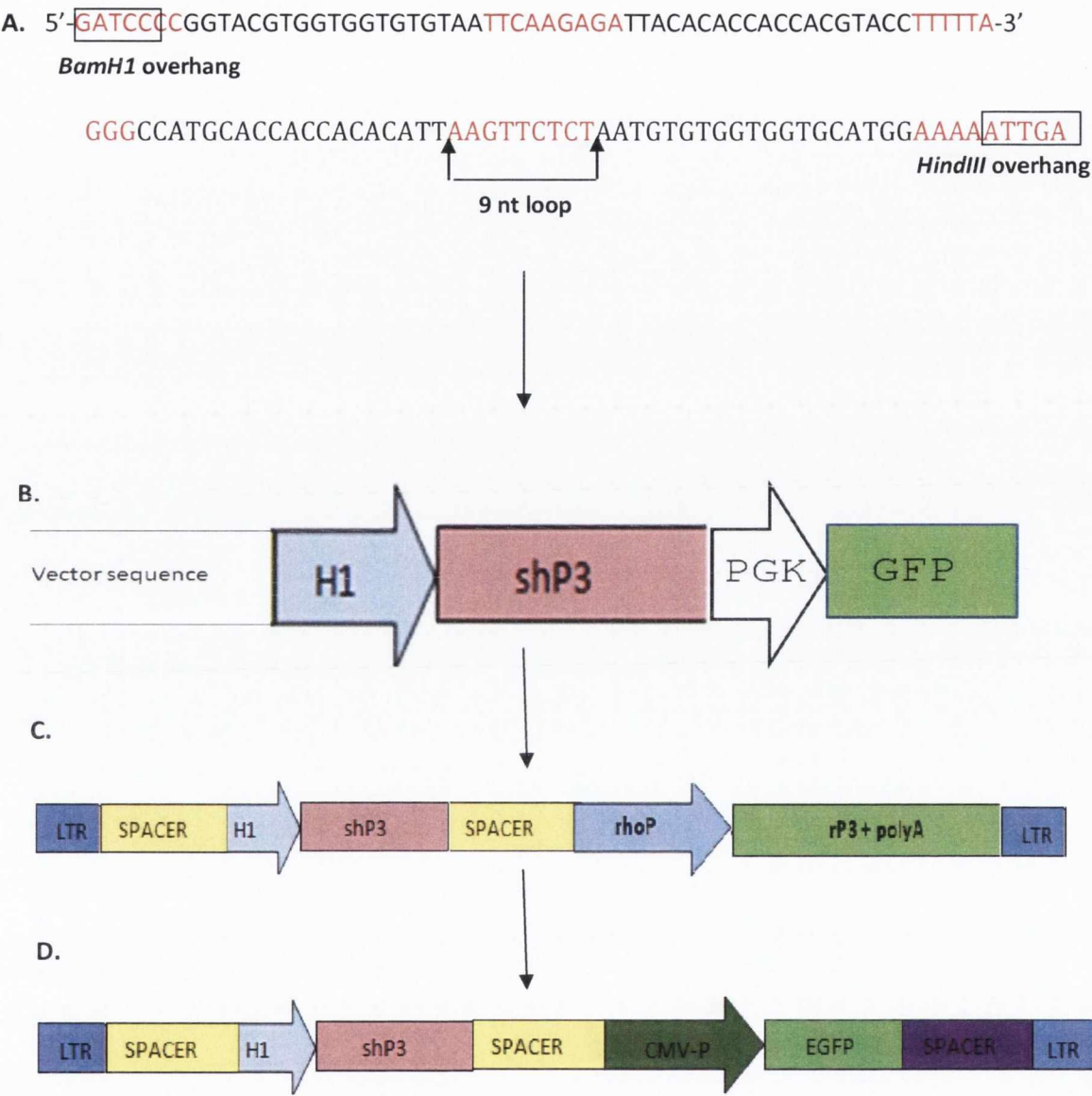


Figure 4.3.1.a Cloning of shP3 into pAAV.

Oligonucleotides were designed according to Brummelkamp, 2002 (step A) and cloned into the *Bgl*III and *Hind*III overhangs of pSuper.GFP.neo/neo under the control of the H1 promoter (step B). The H1 promoter and shP3 was excised from B using *Eco*RI and *Hind*III and blunt cloned into the blunt *Xba*I sites of pAAV.rp3 (step C). The final cloning step involved excising the spacers and shP3 from C with *Nde*I and *Sfi*I and blunt end cloning into the blunt end *Hind*III and *Nsi*I sites of pAAV.NT20 to form pAAV.shP3 (step D).

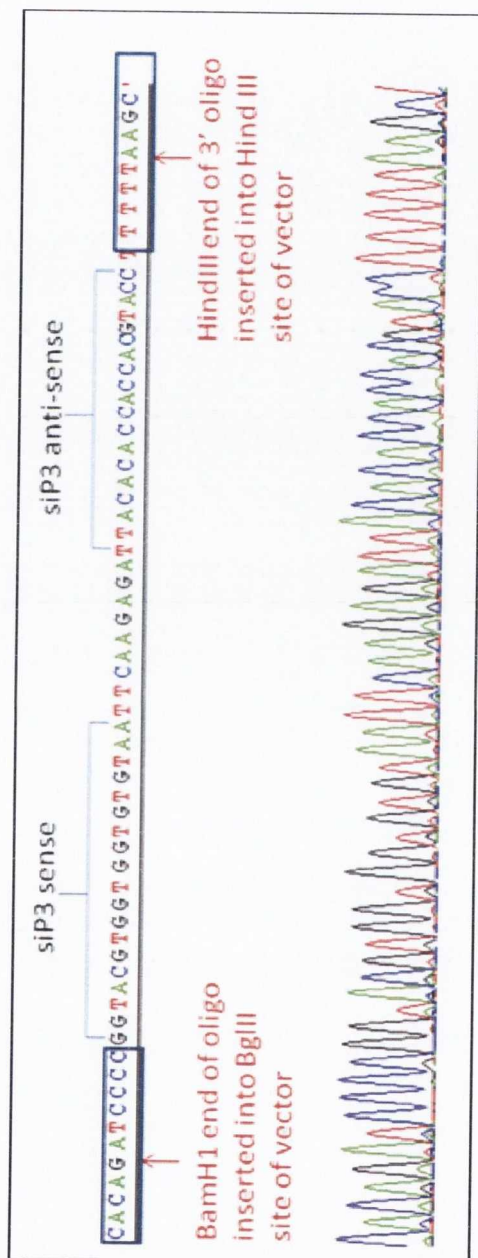


Figure 4.3.1.b sequencing of shP3 into the pSUPER.gfp/neo vector

The sequencing reaction was carried out by the pSuper F primer. The *Bam*HI site on the 5' end of the shP3 oligo was successfully inserted into the *Bgl*II site of the vector. Similarly the *Hind*III site at the 3' end of the shP3 oligo was successfully cloned into the *Hind*III site of the vector. The correct sequence for the siP3 sense strand, the 9 nucleotide loop and siP3 anti-sense strand was obtained, verifying that this shP3 had successfully been generated

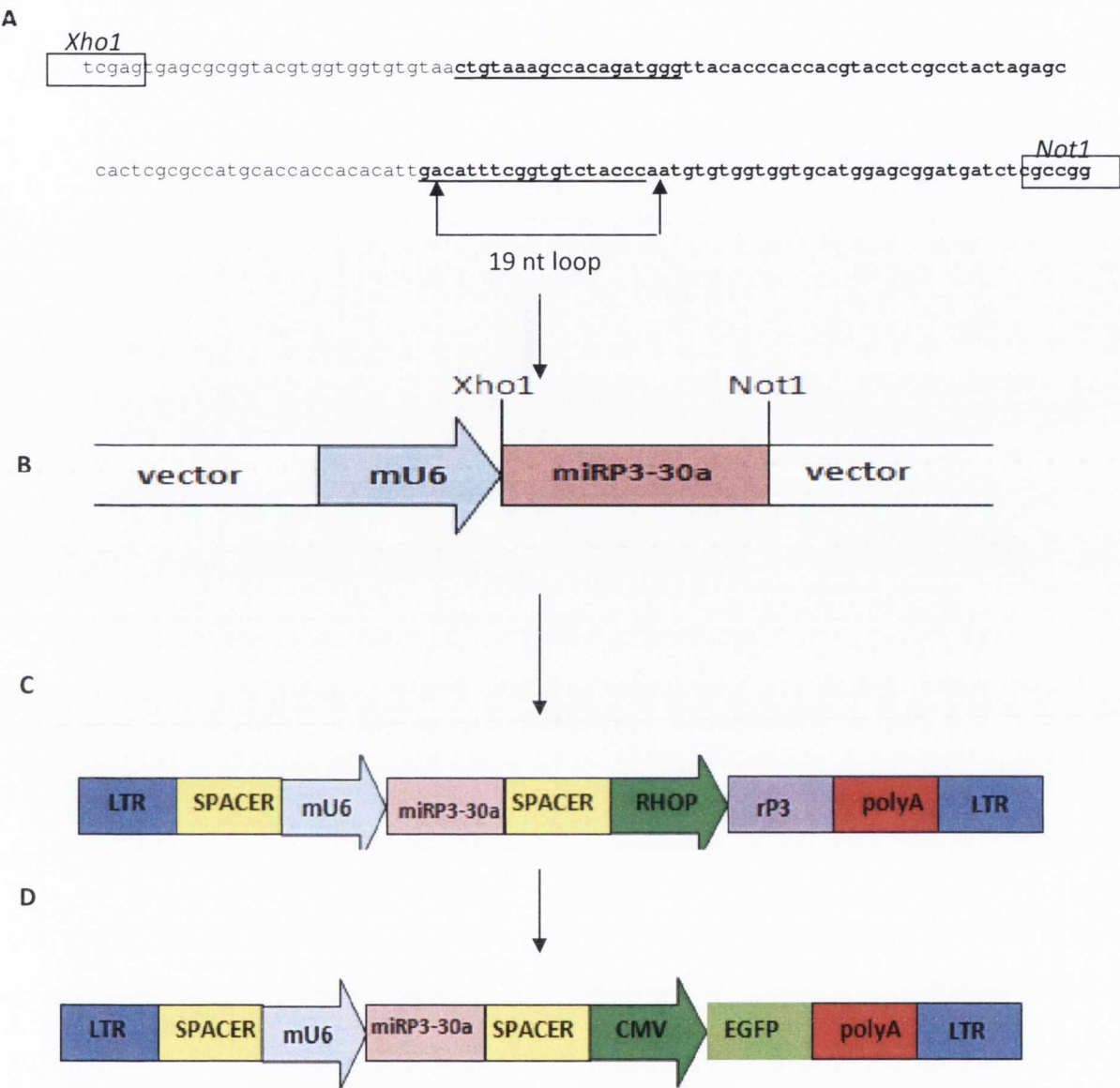


Figure 4.3.2.a Cloning of miRP3-30a into pAAV.

Oligonucleotides were designed according to Boudreau et al, 2008 (A) and cloned into the *Xho1* and *Not1* overhangs of pmU6 under the control of the mU6 promoter (B). The mU6 promoter and miRP3-30a was excised from B using *Xba1* and blunt cloned into the blunt *Xba1* sites of pAAV.rp3 (C). The final cloning step involved excising the spacers and miRP3-30a from C (*Nde1* and *Sfi1*) and blunt cloning into pAAV.NT20 to form pAAV.miRP3-30a (D).

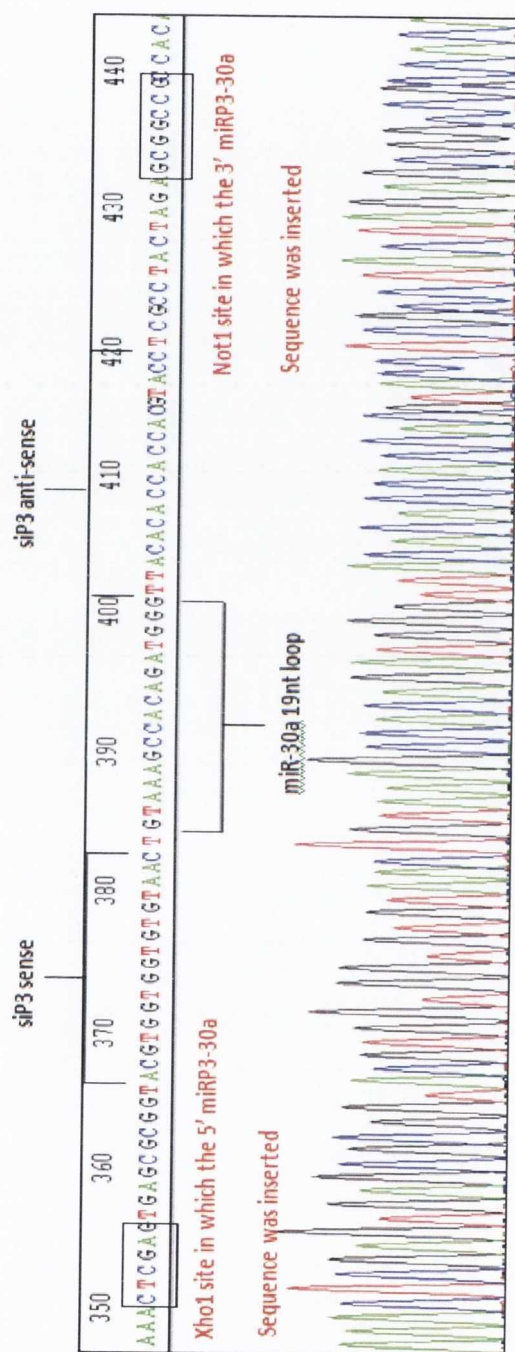


Figure 4.3.2.b sequencing of miRP-30a into pMA.mU6mi30BB

The sequencing reaction was undertaken using the U6F primer. The *XhoI* overhang on the 5' end of the miRP5-30a sequence (boxed) was successfully inserted into the *XhoI* site of the vector. Similarly the *NotI* overhang at the 3' end of miRP3-30a was successfully cloned into the *NotI* site of the vector. This ensured that miRP3-30a was cloned directly downstream of the mu6 promoter. The correct sequence for the miRP3-30a sense strand, the 19 nucleotide miR-30a loop and miRP3-30a anti-sense strand was obtained, verifying that miRP3-30a had successfully been generated. Sequencing was outsourced to GATC biotech.

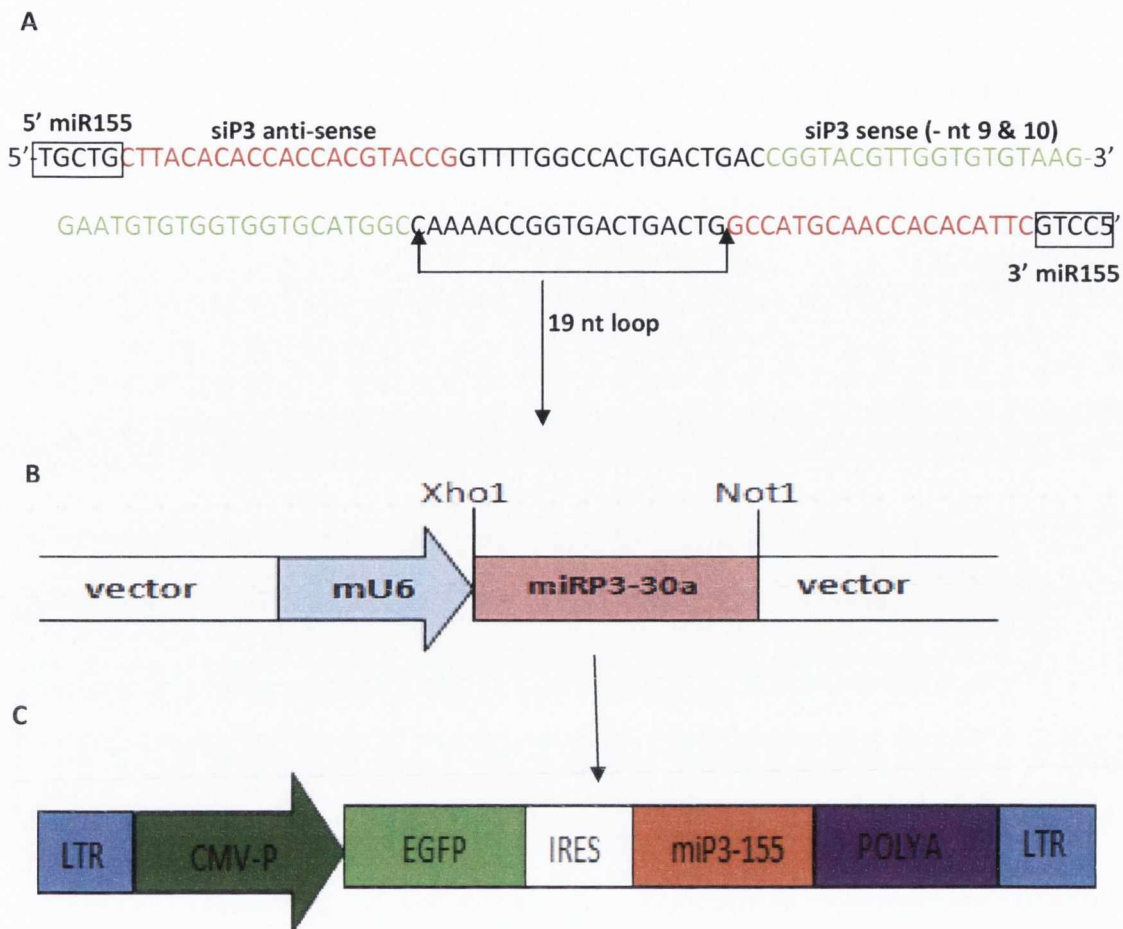


Figure 4.3.5.a Cloning of miRP3-155 into pAAV

Oligonucleotides were designed according to Boudreau et al, 2008 (A) and cloned into the *Xho1* and *Not1* overhangs of pmU6 under the control of the mU6 promoter (B). The mU6 promoter and miRP3-30a was excised from B using *Xba1* and blunt cloned into the blunt *Xba1* sites of pAAV.rp3 (C). The final cloning step involved excising the spacers and miRP3-30a from C. (*NdeI* and *SfiI*) and blunt cloning into pAAV.NT20 to form pAAV.miRP3-30a (D).

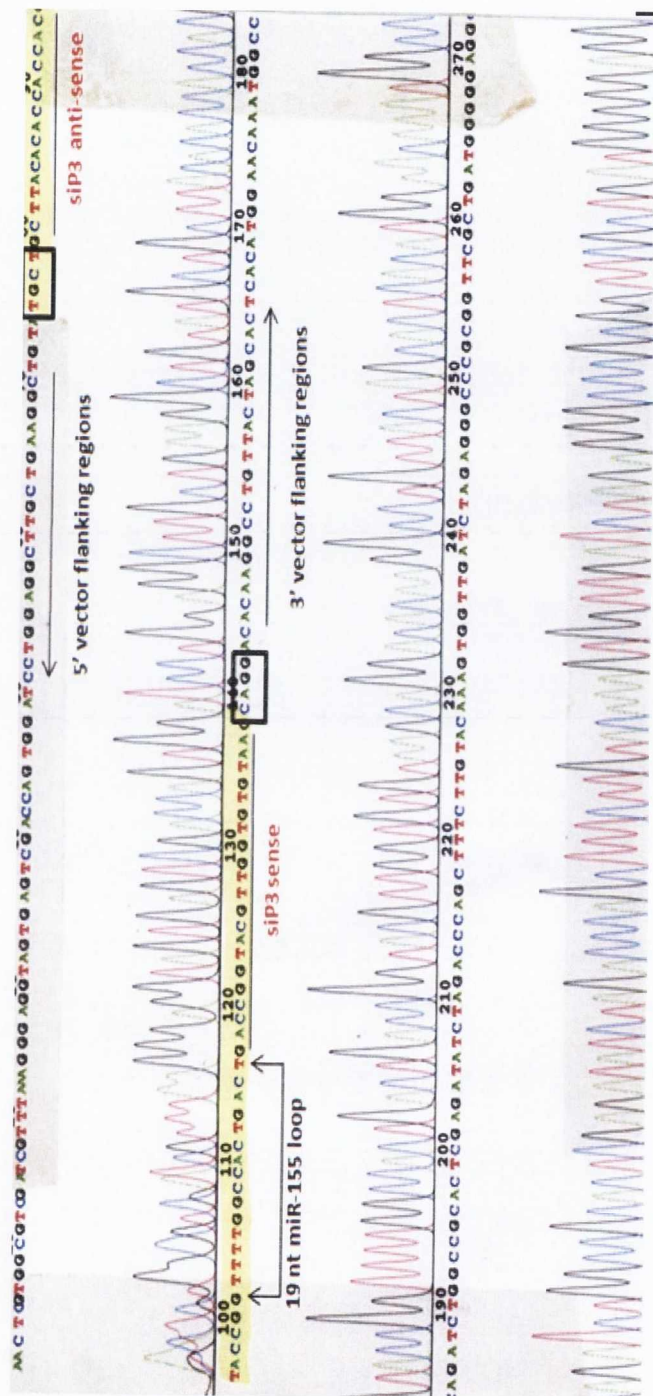


Figure 4.3.3 b sequencing results for miRP3-155 cloned into pcDNA6.3-GW/+EmGFP-miR

The sequencing reaction was carried out by the EGFP R primer. The miRP3-155 overhangs regions were successfully inserted into the flanking regions of the pcDNA6.3-GW/+EmGFP-miR (boxed). The 21 nt anti-sense sip3 sequence and the 19 nt miR-155 loop and the sense sip3 sequence with nt 9 & 10 deleted were also present. The presence 5' and 3' flanking regions of the pcDNA6.3-GW/+EmGFP-miR vector indicates the miRNA was cloned into the correct location.

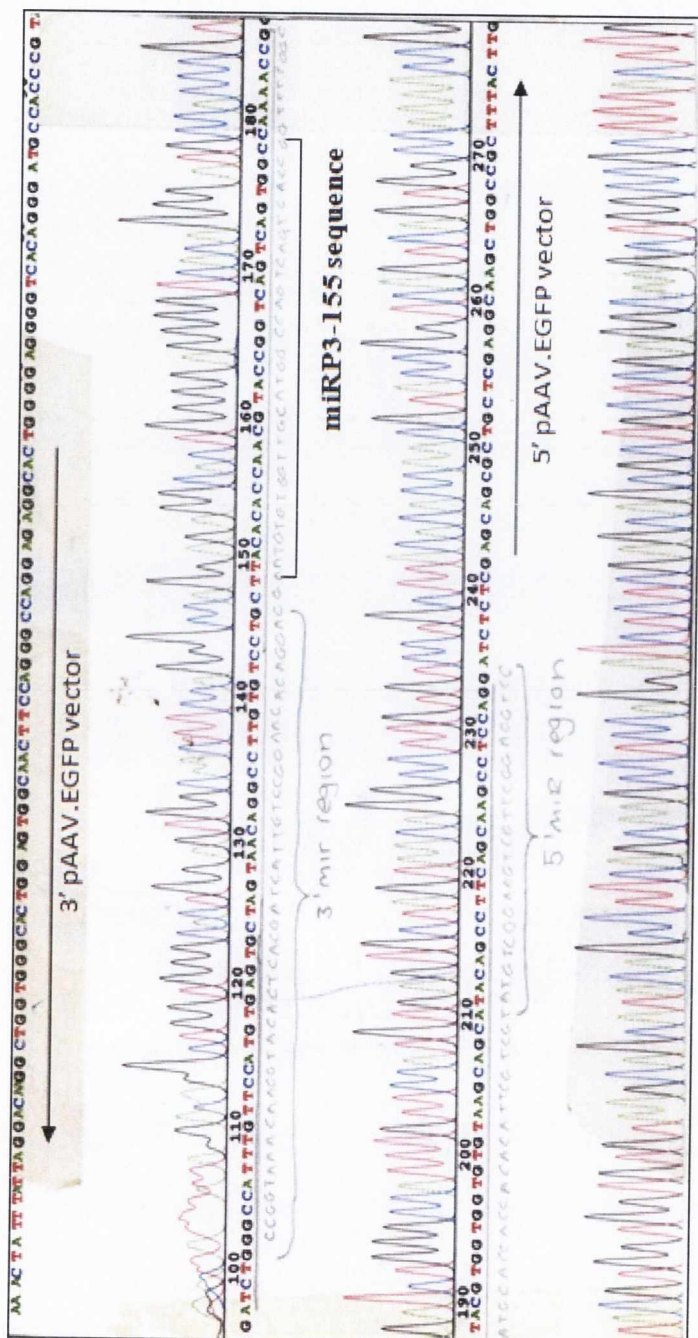


Figure 4.3.2.c sequencing results for miRP3-155 cloned into pAAV.EGFP

AAV-2/5.shP3 fractions	Copy number
AAV-2/5.shP3- 4	8.32622×10^5
AAV-2/5.shP3-5	2.51235×10^6
AAV-2/5.shP3-6	1.06418×10^6

AAV-2/5.miRP3-155 fractions	Copy number
AAV-2/5.miRP3-155- 3	1.558×10^6
AAV-2/5.miRP3-155- 4	1.48388×10^6
AAV-2/5.miRP3-155-5	5.89687×10^5

AAV-2/5.miRP3-30a fractions	Copy number
AAV-2/5.miRP3-30a- 3	1.52966×10^5
AAV-2/5.miRP3-30a- 4	9.64454×10^5

Controls	Copy number	Known titre
AAV-2/5.CMV.EGFP	4.61396×10^5	1×10^{12} vp/ml
AAV-2/5.NT20 (JB)	9.84516×10^6	2×10^{13} vp /ml

$$\frac{\text{Copy no each suppressor}}{\text{Copy number of control}} \times \text{Control titre}$$

+

Obtain average for each control

AAV-2/5 suppressors	Assumed Titres
AAV-2/5.shP3-5	5.6×10^{12} vp/ml(dilute 1 in 1.7)
AAV-2/5.miRP3-155- 4	3.3×10^{12} vp/ml
AAV-2/5.miRP3-30a-3	3.4×10^{12} vp/ml

Table 4.3.4 The calculation of titres for each suppressor virus

The copy number for each fraction was obtained based on the standard curve. For each suppressor (AAV-2/5.shP3, AAV-2/5.miRP3-155 and AAV-2/5.miRP3-30a), the fraction with the highest copy number was taken (red). The copy number of each suppressor was compared to the copy number of the two controls and multiplied by the known titre of each control. The average of the two titre values obtained from the two controls was taken as the estimated titre for each suppressor virus. The titre from the control viruses had previously been estimated against the AAV-2/5.CMV.EGFP control.

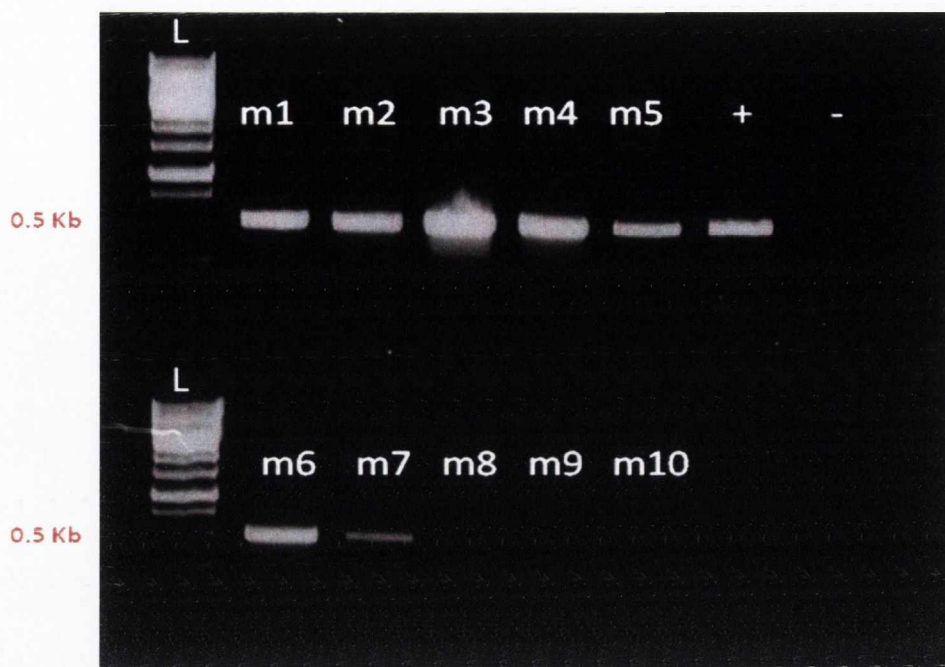


Figure 4.3.5.a NHR Genotyping

The above is an example of a typical genotyping result for the NHR litters. The positive control is DNA from a mouse known to be positive for the NHR transgene, and has a positive band at 0.5Kb, the correct PCR product size for the P23HF and P23HR primers. The negative control is water blank and demonstrates that there was no DNA contamination during the PCR reaction. M1-M7 have a band at 0.5kb therefore they are NHR+. M8-M10 have no bands at 0.5Kb hence they are NHR - .

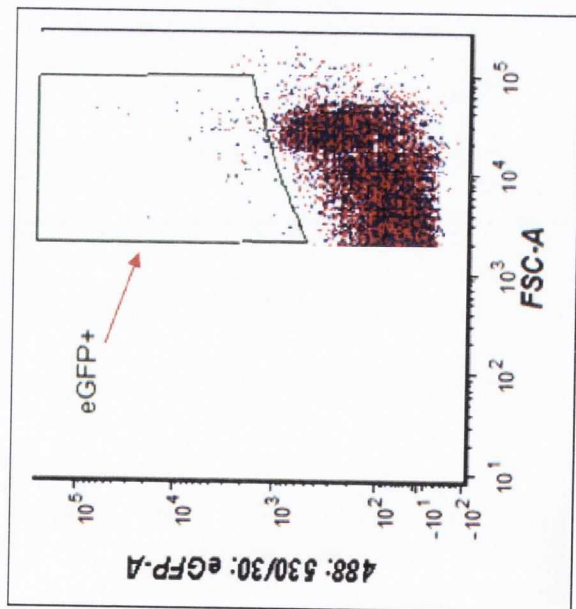


Figure 4.3.7.a The determination of baseline auto fluorescence in EGFP negative retinal cells from an uninjected NHR mouse.

The above diagram is retinal cells from an uninjected NHR mouse. There are clearly very few cells present in the EGFP positive region (red arrow) compared to the majority of cells present in the EGFP negative region. The x-axis represents forward scatter (FSC-A) and the y-axis represents the EGFP signal (488: 530/30)

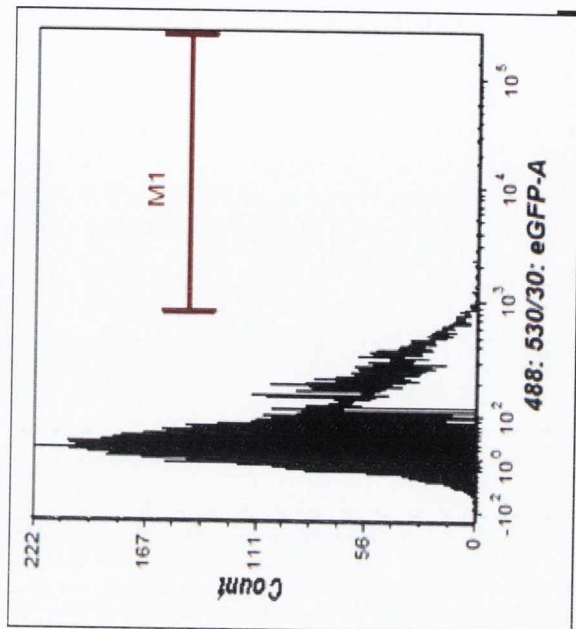


Figure 4.3.7.b The determination of the gate used for EGFP negative cells.

The above diagram is retinal cells from an uninjected NHR mouse. An EGFP signal at 10^2 to 10^3 represents cells that are EGFP negative. M1 is the region in which EGFP positive cells would be present. Note there is no peak at a GFP signal higher than 10^3 , which indicates that as expected the negative control has no EGFP positive cells.

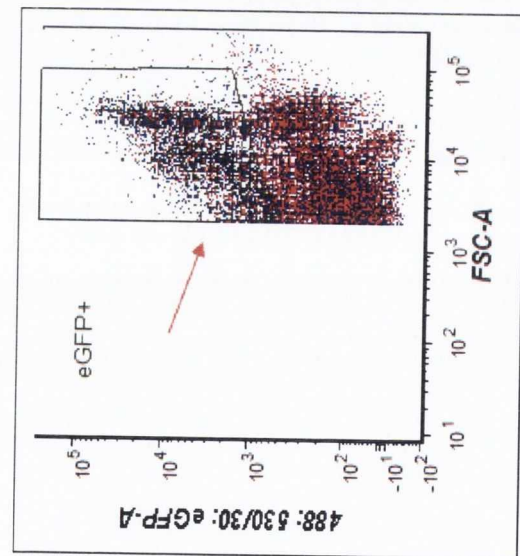


Figure 4.3.7.c The determination of EGFP + cells

The above diagram is retinal cells from a NHR mouse injected with AAV-2/5 NT20. There are clearly EGFP positive cells in the EGFP positive region (red arrow) compared to cells in the EGFP negative region. The x-axis represents forward scatter (FSC-A) and the y-axis represents the EGFP signal (488: 530/30).

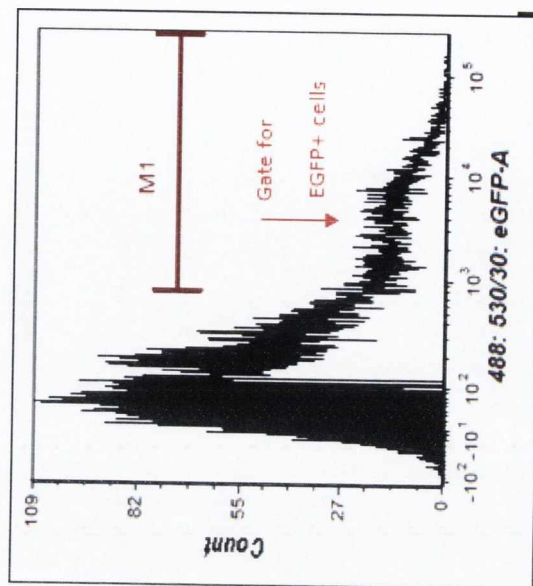


Figure 4.3.7.d The determination of the gate used to sort EGFP positive cells

The above diagram is retinal cells from a NHR mouse injected with AAV-2/5 NT20. M1 is the region in which EGFP positive cells are present. A gate at an EGFP signal of $10^{3.5}$ was used to sort EGFP positive cells from background EGFP fluorescence. The x-axis represents the EGFP signal (488: 530/30) and the y-axis represents the cell count (count).

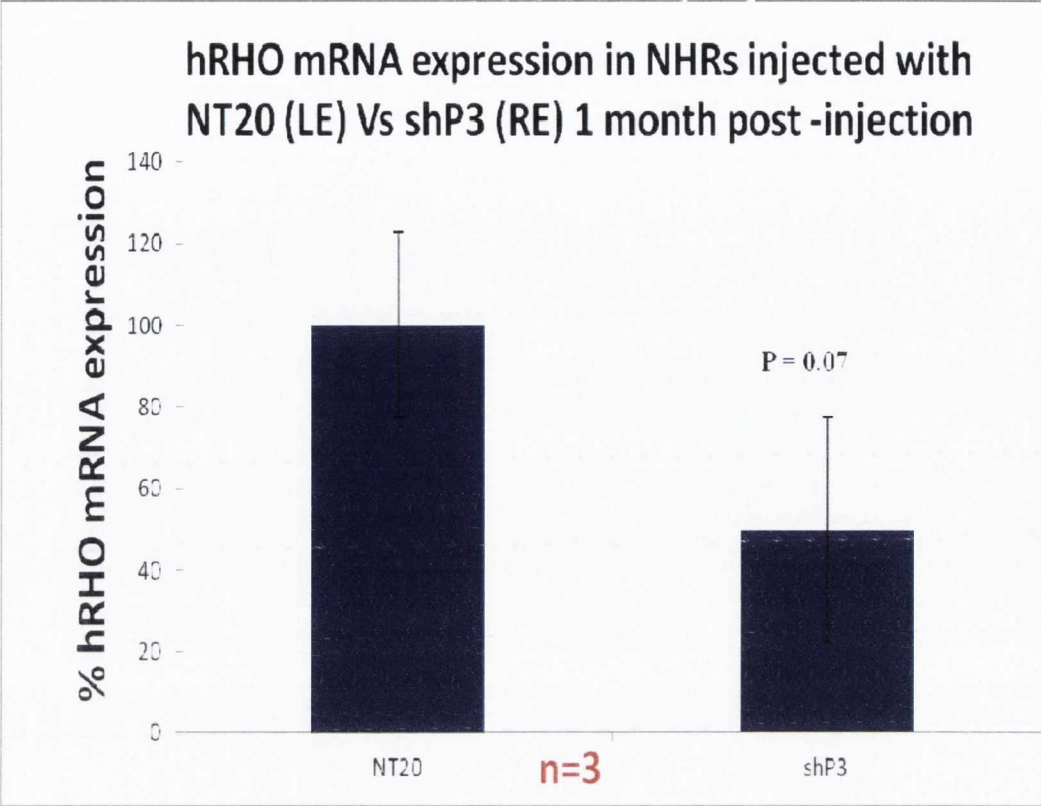
NHR mouse	% green cells	Mean Fluorescence Intensity (Media, MFI)	Cell number obtained
301 NT20 (LE)	63	2696	120,000
301 shP3 (RE)	24.1	3352	50,000
286 NT20 (LE)	23	4106	100,000
286 shP3 (RE)	17.4	12144	121,000
288 NT20 (LE)	0.4	2036	0
288 shP3 (RE)	3.9	6181	0
316 NT20 (LE)	17.3	2894	60,000
316 shp3 (RE)	6.4	5527	120,000

NHR mouse	% green cells	Mean Fluorescence Intensity (Media, MFI)	Cell number obtained
292 Luc46 (LE)	6.7	3104	49,000
292 miRP3-30a	16.6	15326	100,000
294 Luc46 (LE)	5.9	3119	25,000
294 miRP3-30a	14.9	7256	70,000
310 Luc46 (LE)	7.5	7105	50,000
310 miRP3-30a	3.7	11462	40,000
320 Luc46 (LE)	2.5	2171	18,000
320 miRP3-30a	11.3	8133	50,000

NHR mouse	% green cells	Mean Fluorescence Intensity (Media, MFI)	Cell number obtained
304 miNT (LE)	0.5	6995	0
304 miRP3-155 (RE)	14.3	12386	55,000
305 miNT (LE)	17.7	14477	75,000
305 miRP3-155 (RE)	3.9	3150	4,900
306 miNT(LE)	20.9	11963	53,000
306 miRP3-155 (RE)	10.6	7698	50,000
308 miNT(LE)	8.8	15345	50,000
308 miRP3-155 (RE)	6.7	7108	10,000
326 miNT(LE)	7.3	14864	23,000
326 miRP3-155 (RE)	3.9	2817	15,000

Table 4.3.7. EGFP positive % cells and the cell numbers obtained

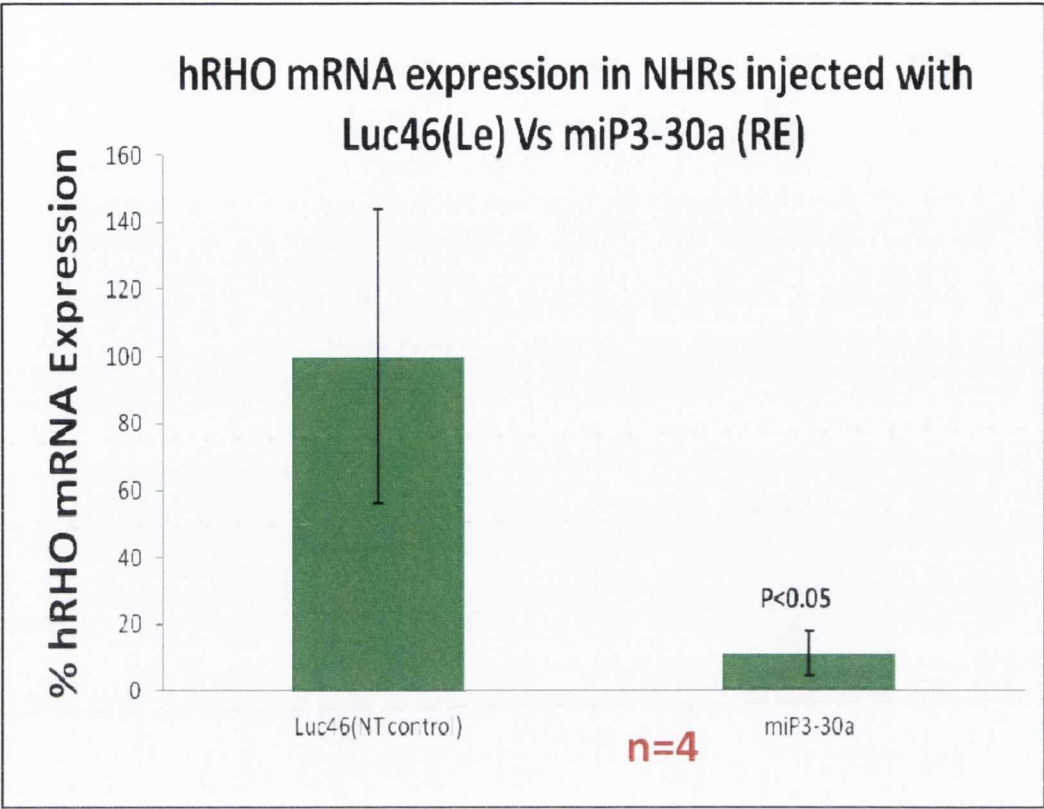
Each retina cell sample was analysed 1 month post-injection for the % EGFP positive cells present in the sample compared to % non-green. The optimal cell numbers obtained were between 50,000-150,000 EGFP positive cells. Samples marked in red represented retinal cell samples in which a very insignificant amount of EGFP positive cells were present due to bad injections.



Sample	NHRs with NT20	NHRs with shP3
% of NT20	100	49
% of SD	23	28

Figure 4.3.9.a hRHO mRNA expression in NHR mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 1 month post-injection.

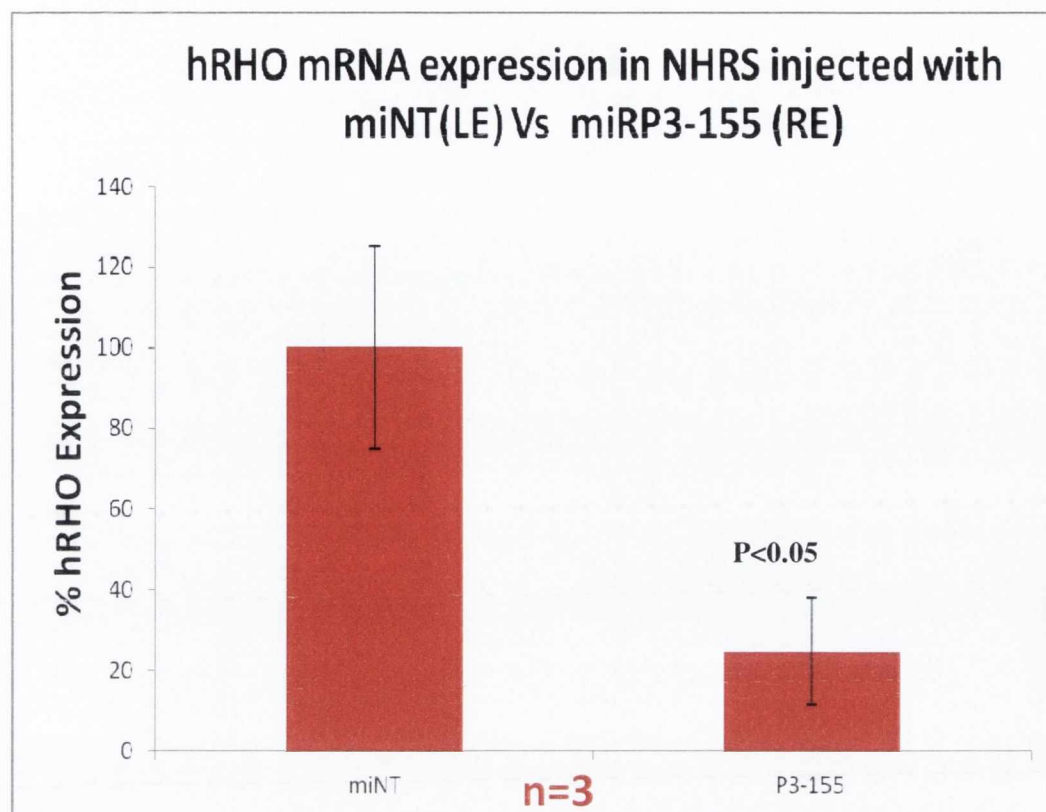
Three NHR mice were subretinally injected with 3µl AAV-2/5.NT20 and AAV-2/5.shP3 (9 X 10⁹vp) and 1 month post-injection, retinal cells were dissociated and the EGFP positive cells were purified by FACS. The average hRHO, average hβ-actin in each sample and the standard deviation was calculated using Microsoft Excel. hRHO mRNA expression from the retinal RNA of mice injected with AAV-2/5.NT20 was set at 100% and the hRHO mRNA expression from the retinal RNA of mice injected with AAV-2/5.shP3 was expressed as a % of this value. Statistical significance was assessed using the normalised rhodopsin expression values by a two sample t-test (DataDesk6.0). Error bars represent % SD values.



sample	NHR with Luc46	NHR with miRP3-30a
% of Luc46	100	11
% of SD	44	7

Figure 4.3.9.b hRHO mRNA expression in NHR mice injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30a 1 month post-injection.

Four NHR mice were subretinally injected with 3µl AAV-2/5.Luc46 (3 X 10⁹vp) and AAV-2/5.miRP3-30a (1.02 X 10¹⁰vp) and 1 month post-injection, retinal cells were dissociated and the EGFP positive cells were purified by FACS. hRHO mRNA expression from the retinal RNA of mice injected with AAV-2/5.Luc46 was set at 100% and the hRHO mRNA expression from the retinal RNA of mice injected with AAV-2/5.miRP3-30a was expressed as a % of this value. Statistical significance in the form of a two sample t-test was assessed using normalised hRHO values (DataDesk 6.0). The average and standard deviation of each normalised hRHO value was calculated by Microsoft Excel. Error bars represent % SD values.



sample	NHR with miNT	NHR with miRP3-155
% of miNT	100	25
% of SD	25	13

Figure 4. 3.9. c hRHO mRNA expression in NHR mice injected with AAV-2/5.miNT and AAV-2/5.miRP3-155 1 month post-injection.

Four NHR mice were subretinally injected with 3µl AAV-2/5.miNT (6×10^9 vp) and AAV-2/5.miRP3-155 (9.9×10^9 vp) and 1 month post-injection, retinal cells were dissociated and the EGFP positive cells were purified by FACS. The average and standard deviation was calculated for normalised hRHO values using Microsoft Excel, in addition a two-sample t-test was utilised on normalised hRHO data sets to assess statistical significance (DataDesk6.0). Normalised hRHO mRNA expression from the retinal RNA of mice injected with AAV-2/5.miNT was set at 100% and the hRHO mRNA expression from the retinal RNA of mice injected with AAV-2/5.miRP3-155 was expressed as a % of this value. Error bars represent % SD values.

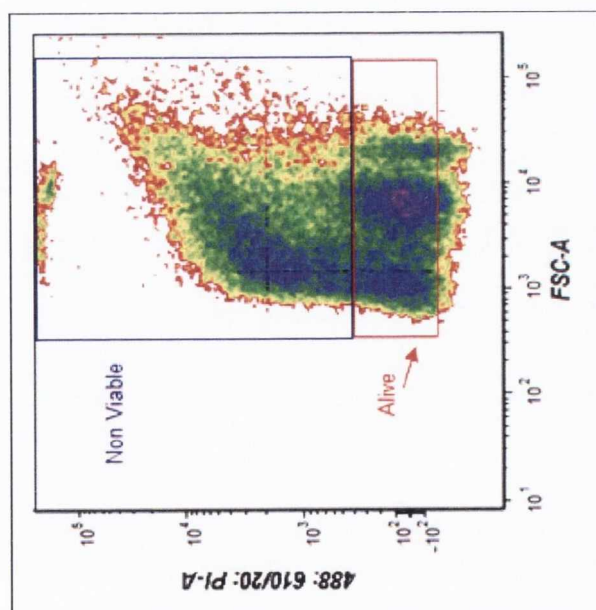


Figure 4.3.10.a The determination of PI + cells

The above diagram is retinal cells from an un-injected NHR mouse. The live cells (red arrow) can clearly be separated from the non-viable cells. The x -axis represents forward scatter (FSC-A) and the y axis represents the propidium iodide (PI) signal (488:610/20)

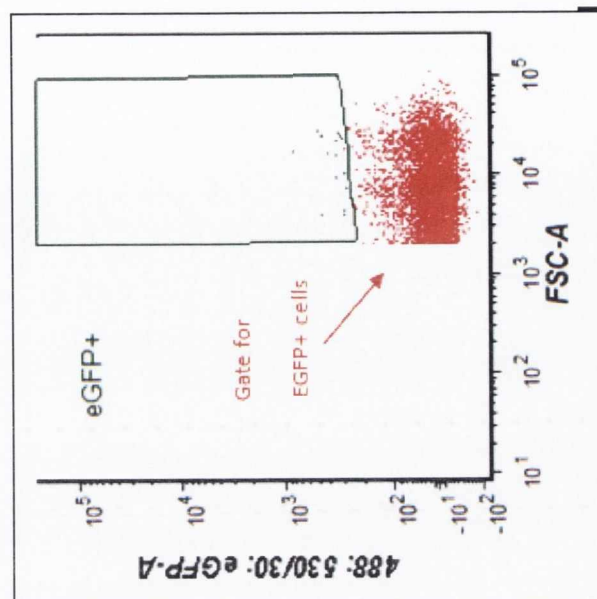


Figure 4.3.10.b The determination of the gate used to sort PI negative and EGFP positive cells

The above diagram is retinal cells from an un-injected NHR mouse. The PI negative healthy cells were taken and assessed for EGFP. No EGFP positive cells were obtained in this sample owing to the fact it is an EGFP negative control. The x-axis represents forward scatter and the y axis represents the EGFP signal (488:530/30)

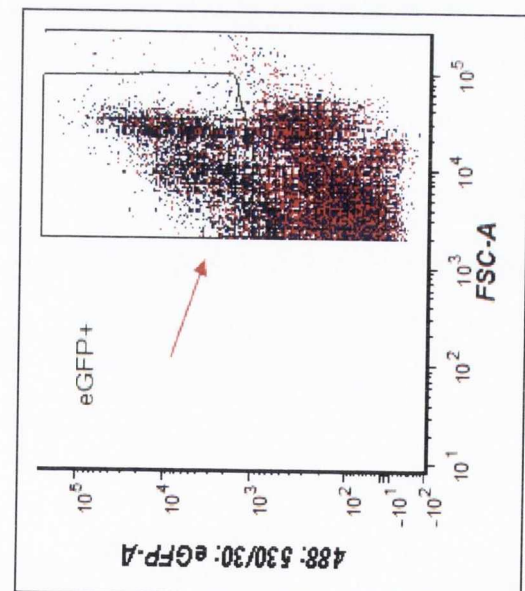


Figure 4.3.10.c The determination of EGFP + cells

The above diagram is retinal cells from a NHR mouse injected with AAV-2/5.NT20 in which PI has been used to sort the healthy (PI positive) population of cells. Within this PI positive cell population, there are clearly EGFP positive cells in the EGFP positive region (red arrow) compared to cells in the EGFP negative region. The x-axis represent forward scatter (FSC-A) and the y-axis represents the EGFP signal (488: 530/30).

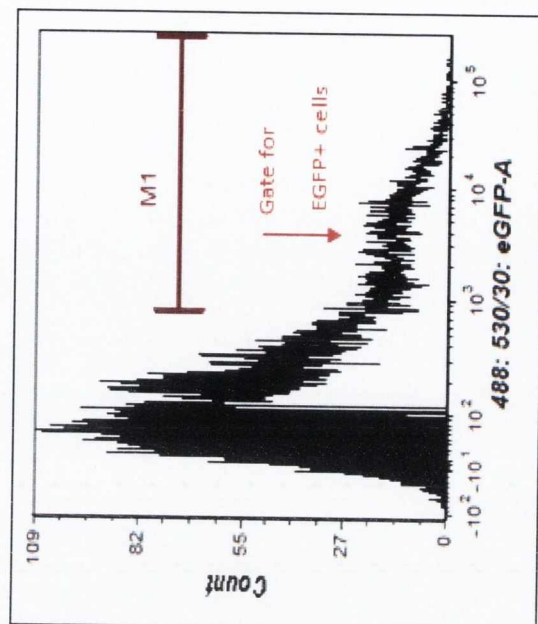


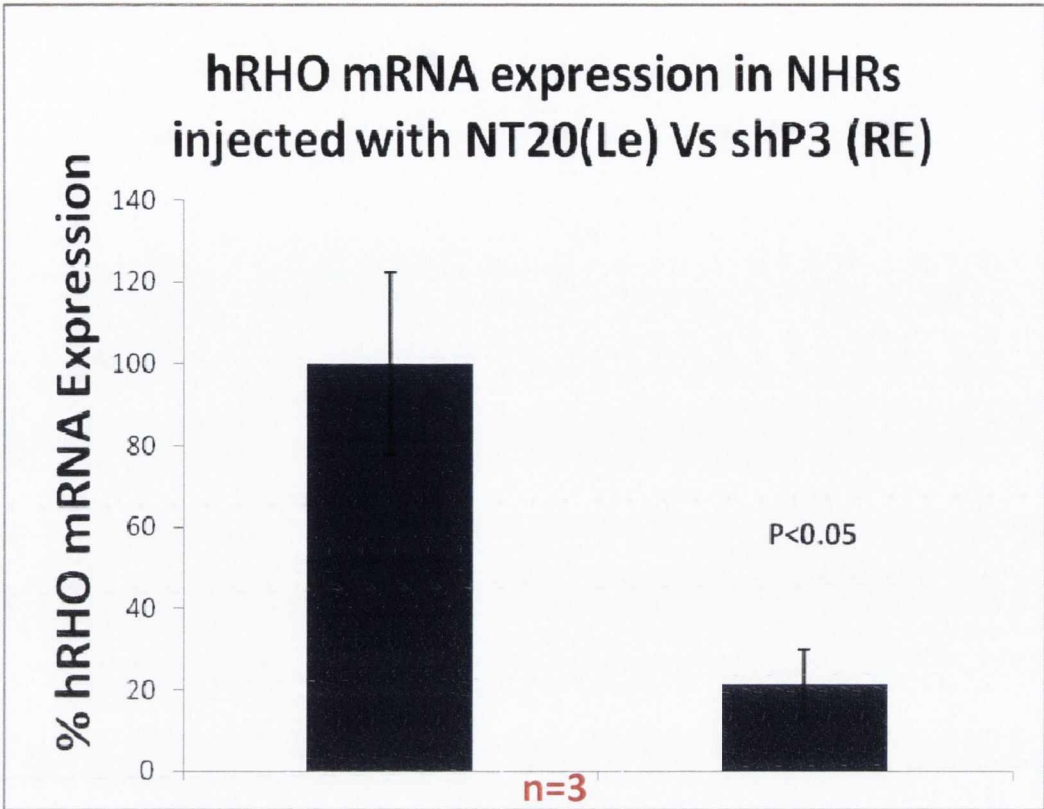
Figure 4.3.10.d The determination of the gate used to sort EGFP positive cells

The above diagram is PI positive retinal cells from a NHR mouse injected with AAV-2/5.NT20. M1 is the region in which EGFP positive cells are present. A gate at $10^{3.5}$ was used to sort EGFP positive cells. The x-axis represents the EGFP signal (488: 530/30) and the y-axis represents the cell count (count).

NHR mouse	% green cells	Mean Fluorescence Intensity (Media, MFI)	Cell number obtained
1 NT20 (LE)	20.8	1498	50,000
1 shP3 (RE)	3.8	860	21,000
2 NT20 (LE)	12.1	1075	39,000
2 shP3 (RE)	15.2	1732	65,000
3 NT20 (LE)	0.9	399	0
3 shP3 (RE)	37.8	3475	58,000
4 NT20 (LE)	8.2	750	30,000
4 shp3 (RE)	34.1	1600	97,000
5 NT20 (LE)	21.4	1470	60,000
5 shP3 (RE)	10.4	1404	43,000

Table 4.3.10. % EGFP positive cells and cell numbers 14 days post-injection

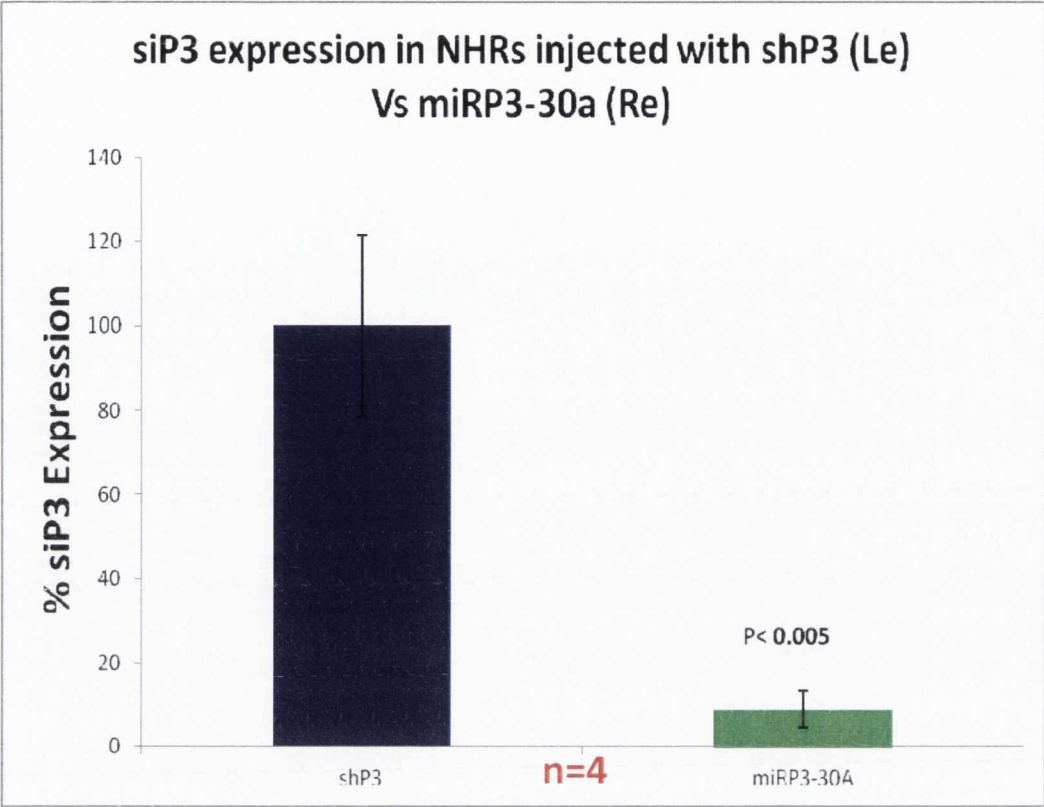
Each retinal cell sample was analysed 14 days post-injection for the % of EGFP positive cells present in the sample compared to % of EGFP negative cells. The optimal cell numbers obtained were between 50,000-150,000 EGFP positive cells. Samples marked in red represented retinal cell samples in which an insignificant amount of EGFP positive cells were present due to bad injections.



Sample	NHR with NT20	NHR with shP3
% of NT20	100	22
% of shP3	22	8

Figure 4.3.10.e hRHO mRNA expression in NHR mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 14 days post-injection.

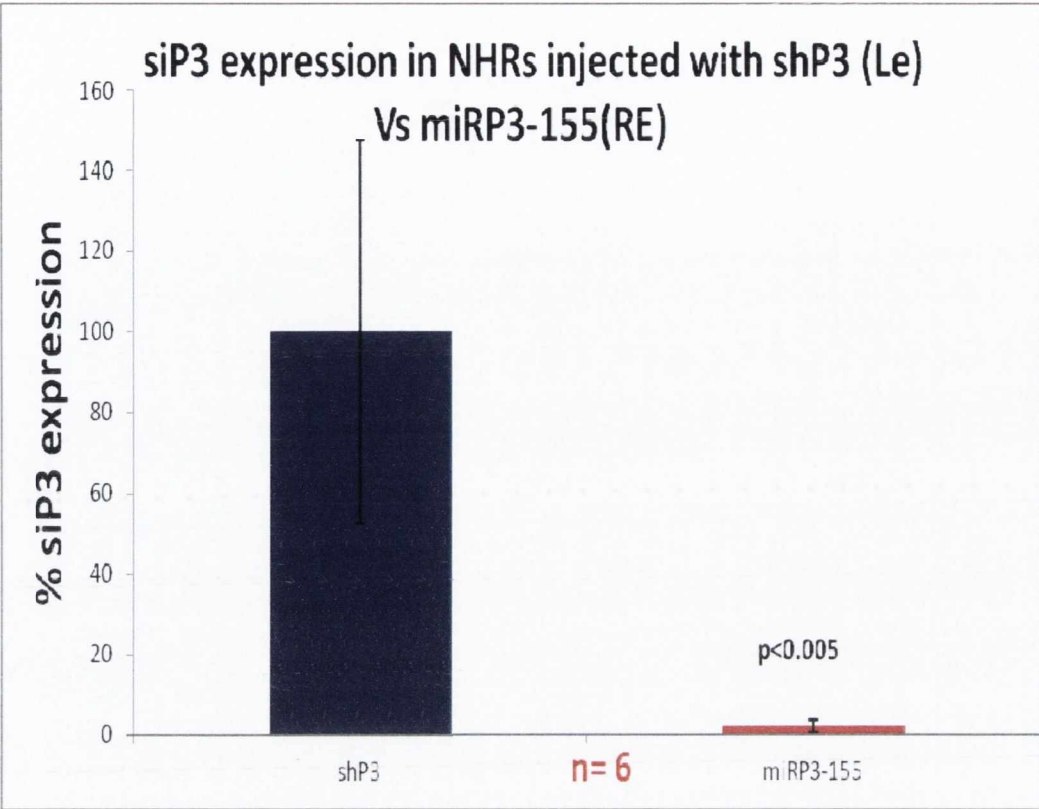
Three NHR mice were subretinally injected with 3µl AAV-2/5.NT20 and AAV-2/5.shP3 (9.9×10^9 vp) and 14 days post-injection, retinal cells were dissociated and the EGFP positive cells were purified by FACS. The average and standard deviation for normalised hRHO expression values in each data set were calculated using Microsoft Excel. Statistical significance was assessed using a two-sample t-test (DataDesk 6.0). HRHO RNA expression from the retinal RNA of mice injected with AAV-2/5.NT20 was set at 100% and the hRHO mRNA expression from the retinal RNA of mice injected with AAV-2/5.shP3 was expressed as a % of this value. Error bars represent % SD values.



Sample	NHRs with shP3	NHRs with miRP3-30a
% siP3 expression	100	9
% SD	22	4

Figure 4.3.12.a siP3 expression in NHR mice injected with AAV-2/5.shP3 versus AAV-2/5.miRP3-30a

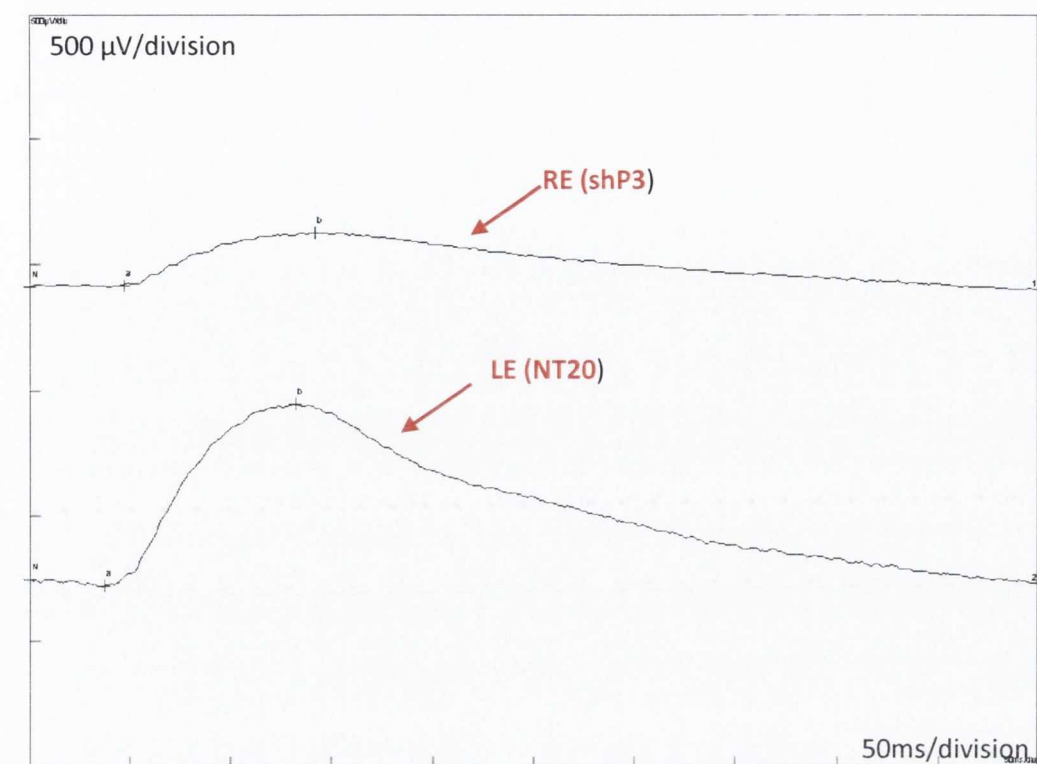
Analysis was undertaken in order to determine the level of mature siP3 species processed and expressed from the shRNA backbone compared to the miR-30a backbone. All normalised siP3 values were averaged and the standard deviation obtained by Microsoft Excel. Normalised siP3 values in each data set (AAV-2/5.shP3 or AAV-2/5.miRP3-30a) were assessed for statistical significance using a two sample t-test (DataDesk 6.0). As efficient shRNAs typically have abundant mature siRNA expression, siP3 expression from shP3 was set at 100% and siP3 expression from miRP3-30a was compared to this value (8.8%). siP3 is expressed 11 fold less from the miRP3-30a backbone. Error bars represent SD values.



Sample	NHRs with shP3	NHRs with miRP3-155
% siP3 expression	100	2
% SD	47	2

Figure 4.12.b siP3 expression in NHR mice injected with AAV-2/5.shP3 versus AAV-2/5.miRP3-155

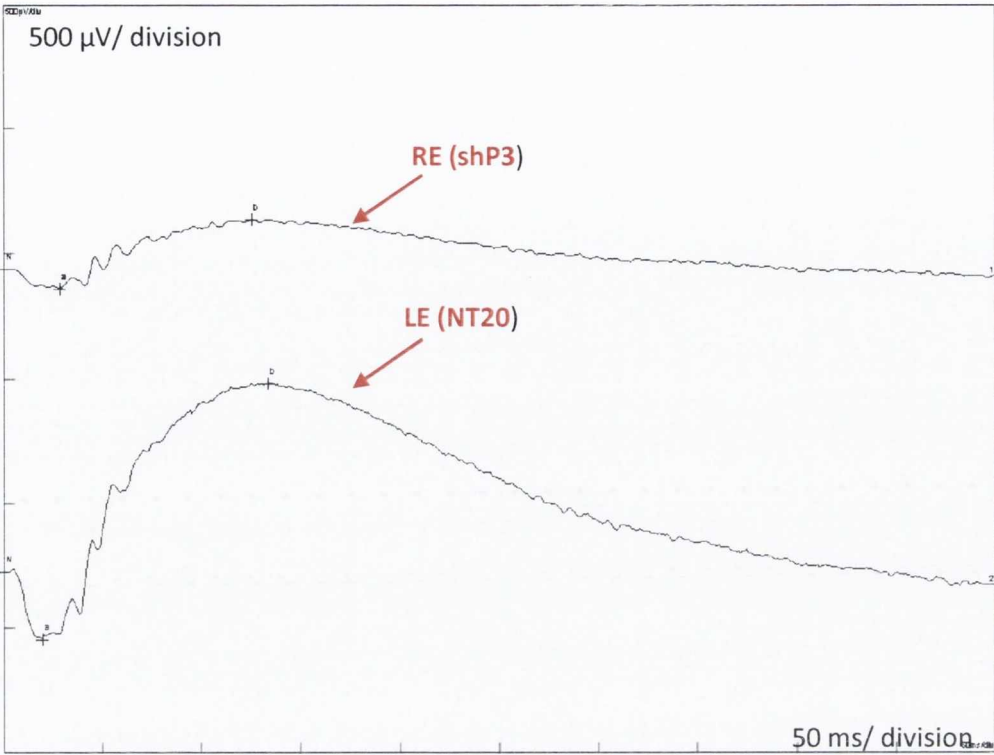
Analysis was undertaken in order to determine the level of mature siP3 species processed and expressed from the shRNA backbone compared to the miR-155 backbone. Averages and standard deviations on normalized siP3 datasets were calculated using Microsoft Excel. Statistical significance was assessed with a two sample t-test (DataDesk6.0). As efficient shRNAs typically have abundant mature siRNA expression, siP3 expression from AAV-2/5.shP3 was set at 100% and siP3 expression from AAV-2/5.miRP3-155 was compared to this value (2%). siP3 is expressed 50 fold less from the miRP3-155 backbone. Error bars represent SD values.



Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	47	142	3.87μV	209μV
2 left Max	37	132	25.4μV	726μV

Figure 4.3.13.a Rod-isolated ERG for NHR mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE).

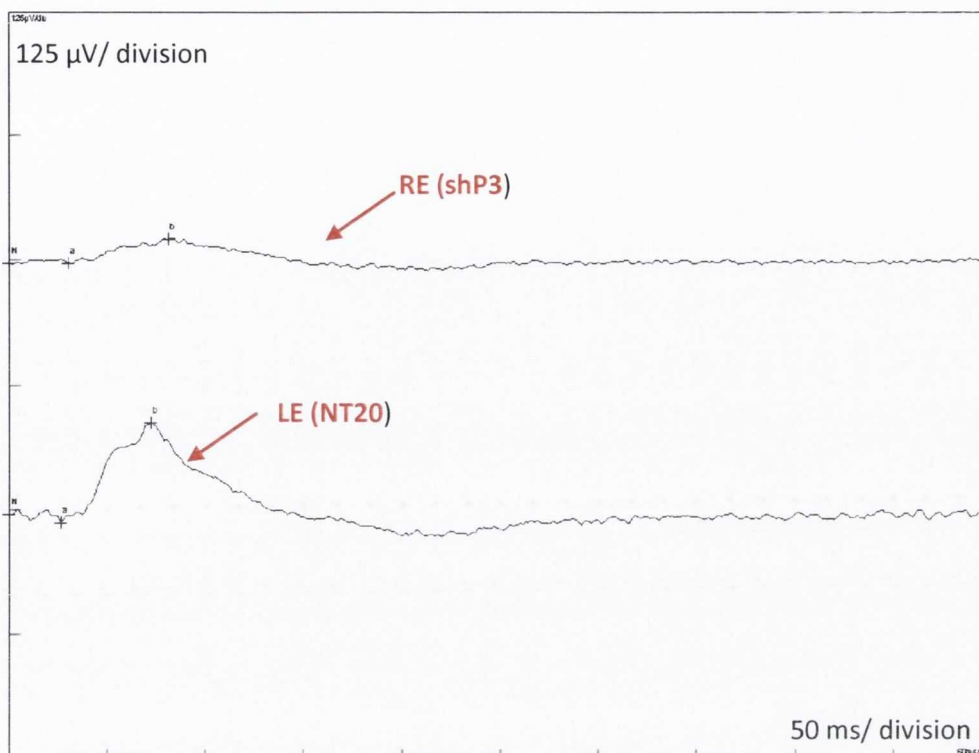
This is a representative rod-isolated ERG response from an NHR mice subretinally injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE). This was the most significant result obtained out of all 9 mice analysed.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	28	125	81.2 μ V	281 μ V
2 left Max	20	133	272 μ V	1.02mV

Figure 4.3.13.b mixed rod-cone ERG for NHR mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE).

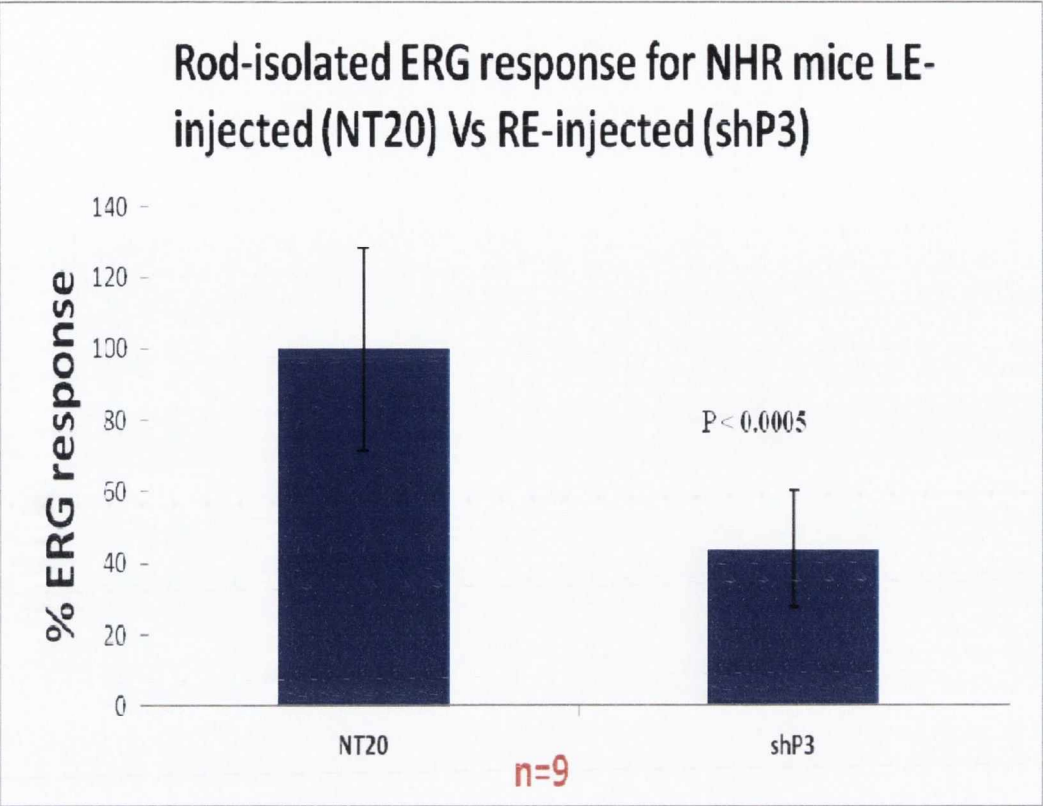
This is a representative mixed rod-cone ERG result from an NHR mice subretinally injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE). This was the most significant result obtained out of all 9 mice analysed.



	Diagnostic Data				
Channel	N[ms]	a[ms]	b[ms]	a-wave	b-wave
1 right cone SF	0	30	81	381nV	24 μ V
2 left cone SF	0	26	72	8.64 μ V	99.6 μ V

Figure 4.3.13.c cone-isolated ERG for NHR mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE).

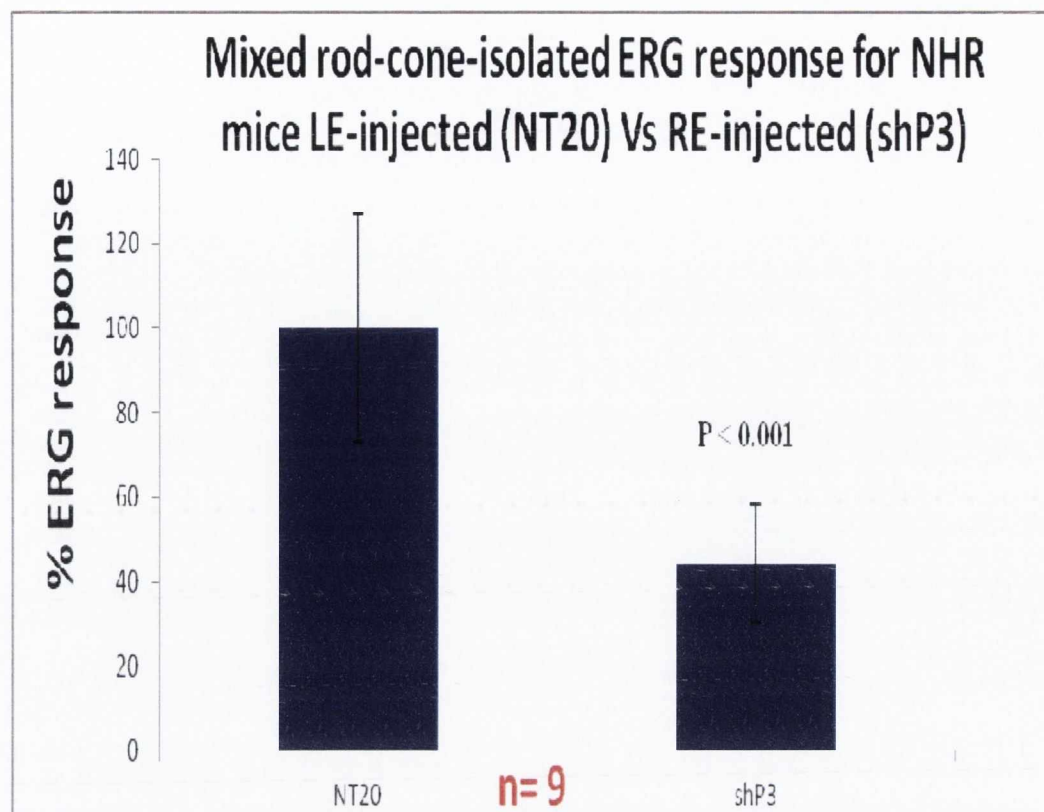
This is a representative cone isolated ERG result from an NHR mice subretinally injected with AAV2/5.NT20 (LE) and AAV-2/5.shP3 (RE). This was the most significant result obtained out of all 9 mice analysed.



	NHR with NT20	NHR with shP3
% of NT20	100	44
% of SD	28	16

Figure 4.3.13.d % ERG response in NHR mice injected with AAV-2/5.NT20 versus AAV-2/5.shP3

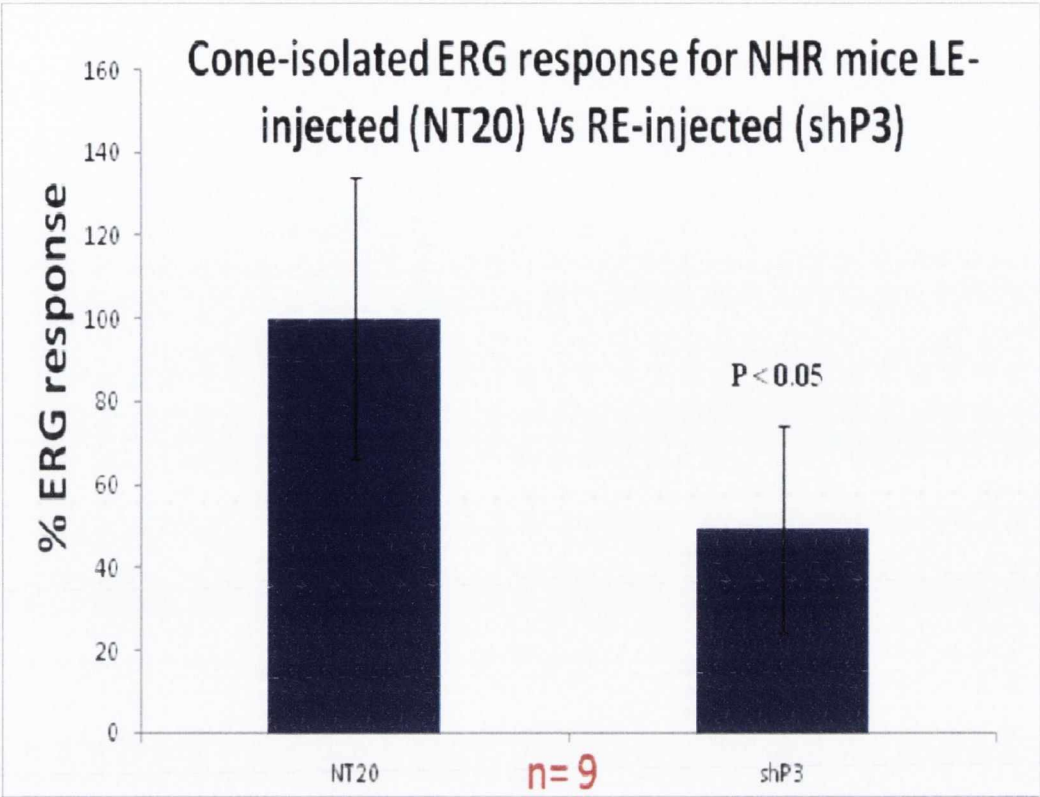
The rod-isolated b-waves of all mice subretinally injected with AAV-2/5.NT20 and AAV-2/5.shP3 were combined for each construct. Averages and the standard deviation of each data set were calculated using Microsoft Excel. A paired t-test was undertaken to assess statistical significance (DataDesk 6.0). The average b-wave value for AAV-2/5.NT20 injected eyes was set at 100% and the rod-isolated b-wave for AAV-2/5.shP3 injected eyes was expressed as a % of this value. AAV-2/5.shP3 suppressed the rod-isolated b-wave response significantly to 44%. Error bars represent % SD values



	NHR with NT20	NHR with shP3
% ERG response	100	45
% SD	27	14

Figure 4.3.13.e % mixed rod-cone ERG response in NHR mice injected with AAV-2/5.NT20 vs. AAV-2/5.shP3

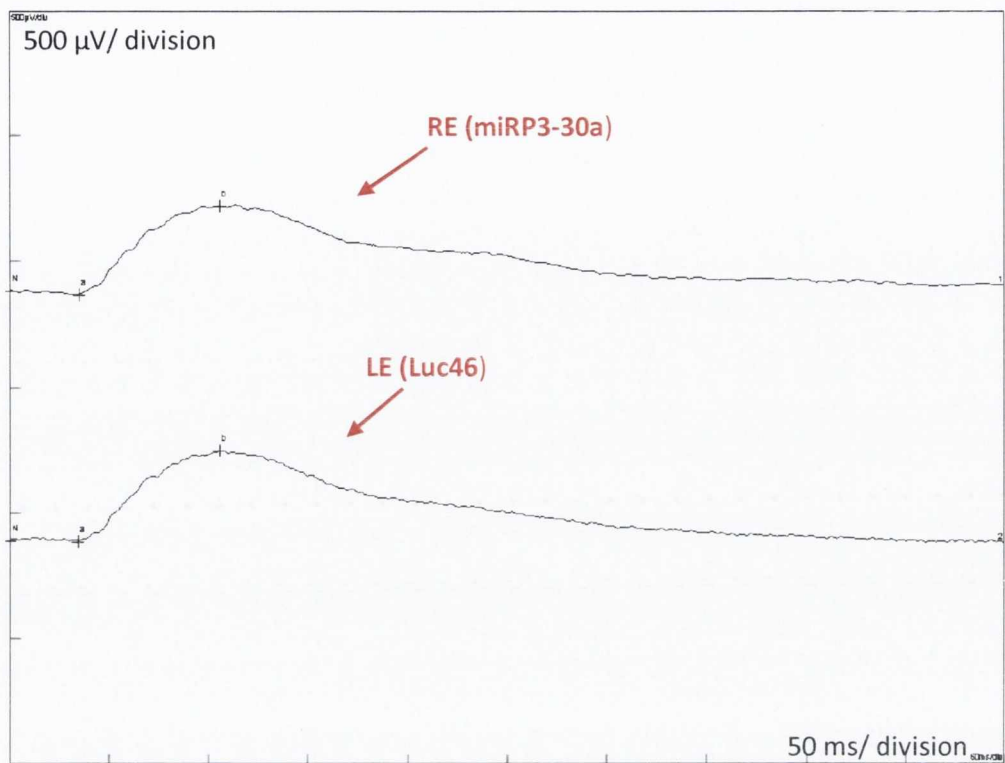
The mixed rod-cone b-waves of all mice subretinally injected with AAV-2/5.NT20 and AAV-2/5.shP3 were combined for each construct. Averages and the standard deviation of each data set were calculated using Microsoft Excel. A paired t-test was undertaken to assess statistical significance (DataDesk 6.0). The average b-wave value for AAV-2/5.NT20 injected eyes was set at 100% and the mixed rod-cone b-wave for AAV-2/5.shP3 injected eyes was expressed as a % of this value. AAV-2/5.shP3 suppressed the mixed rod-cone b-wave response significantly to 45%. Error bars represent % SD values



	NHR with NT20	NHR with shP3
% of NT20	100	49
% of SD	34	25

Figure 4.3.13.f % cone-isolated ERG response in NHR mice injected with AAV-2/5.NT20 vs. AAV-2/5.shP3

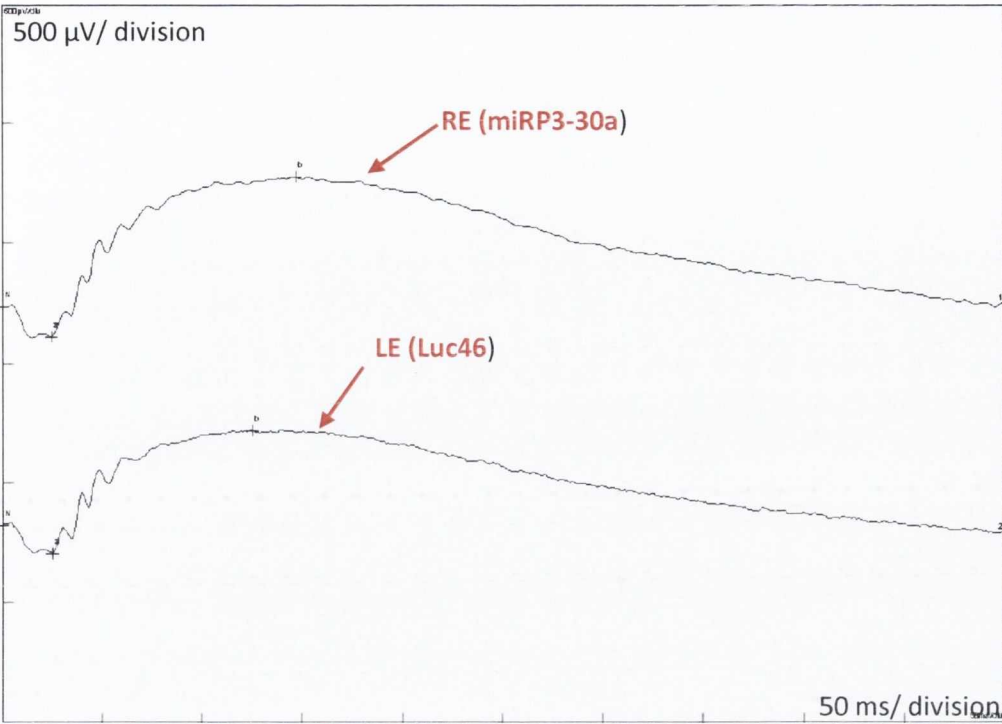
The cone-isolated b-waves of all mice subretinally injected with AAV-2/5.NT20 and AAV-2/5.shP3 were correlated for each construct. Averages and the standard deviation of each data set were calculated using Microsoft Excel. A paired t-test was undertaken to assess statistical significance (DataDesk 6.0). The average b-wave value for AAV-2/5.NT20 injected eyes was set at 100% and the cone-isolated b-wave for AAV-2/5.shP injected eyes was expressed as a % of this value. AAV-2/5.shP3 suppressed the cone-isolated b-wave response significantly to 49%. Error bars represent % SD values



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	35	105	15 μ V	353 μ V
2 left Max	34	105	4.67 μ V	360 μ V

Figure 4.3.13.g Rod-isolated ERG for NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE).

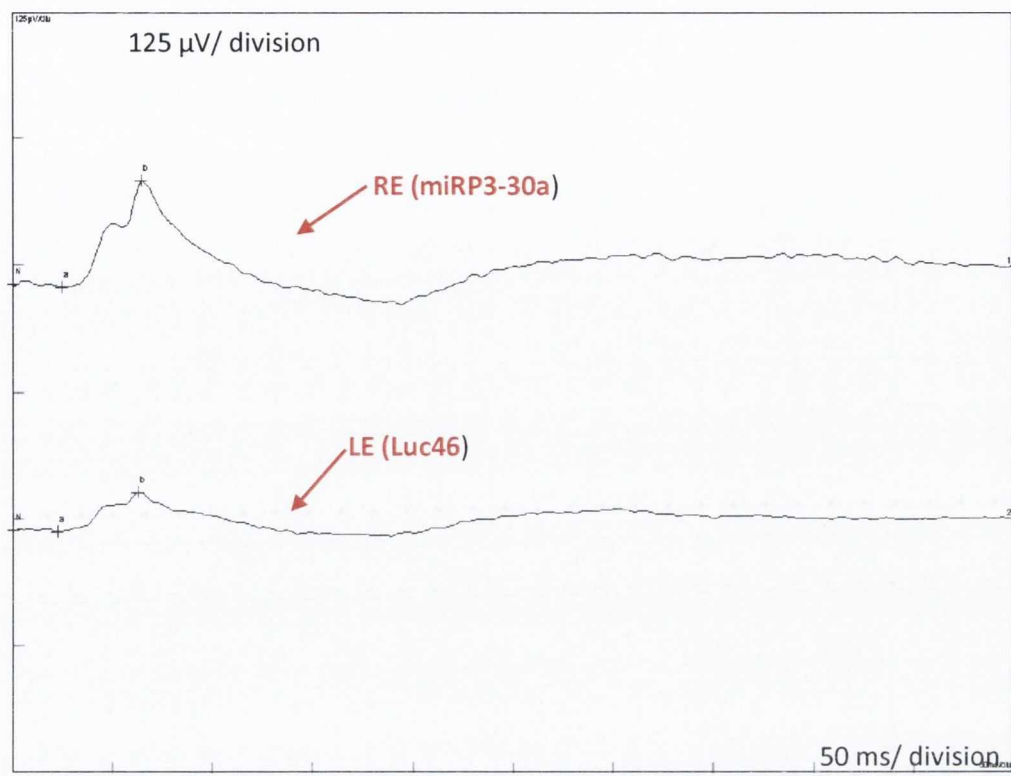
This is a representative rod-isolated ERG result from an NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE). Of the 8 mice analysed, five of the mice tested had no change in ERG function in AAV-2/5.miRP3-30a injected eyes compared to AAV-2/5.Luc46 injected eyes. This typical ERG is representative of the 5 mice with no ERG change



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	24	146	128 μV	670 μV
2 left Max	25	125	119 μV	516 μV

Figure 4.3.13.h Mixed rod-cone ERG for NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE).

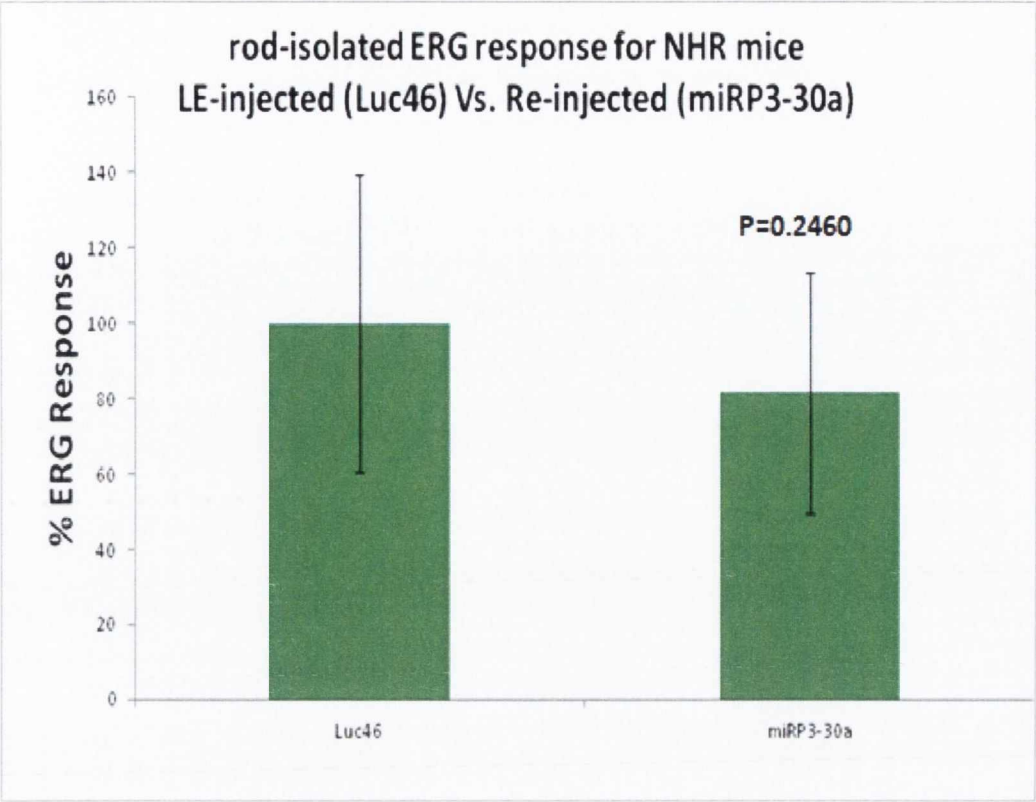
This is a representative mixed rod-cone ERG result from an NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE).



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	24	64	395 μ V	105 μ V
2 left Max	22	62	212 μ V	39.2 μ V

Figure 4.3.13.i Cone-isolated ERG for NHRS injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE).

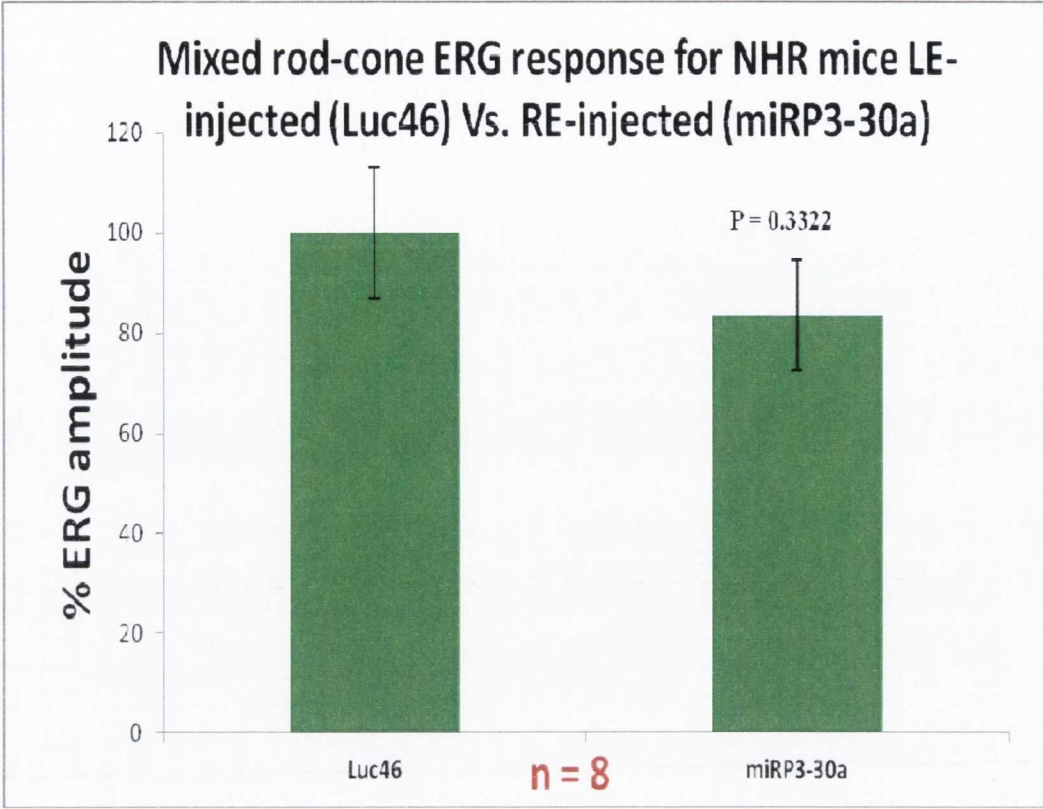
This is a representative cone-isolated ERG result from an NHR mice injected with AAV-2/5.Luc46 (LE) and AAV-2/5.miRP3-30a (RE). This is a typical ERG



	NHR with Luc46	NHR with miRP3-30a
% on Luc46	100	82
% SD	40	32

Figure 4.3.13.j % rod-isolated ERG response in NHR mice injected with AAV-2/5.Luc46 vs. AAV-2/5.miRP3-30a

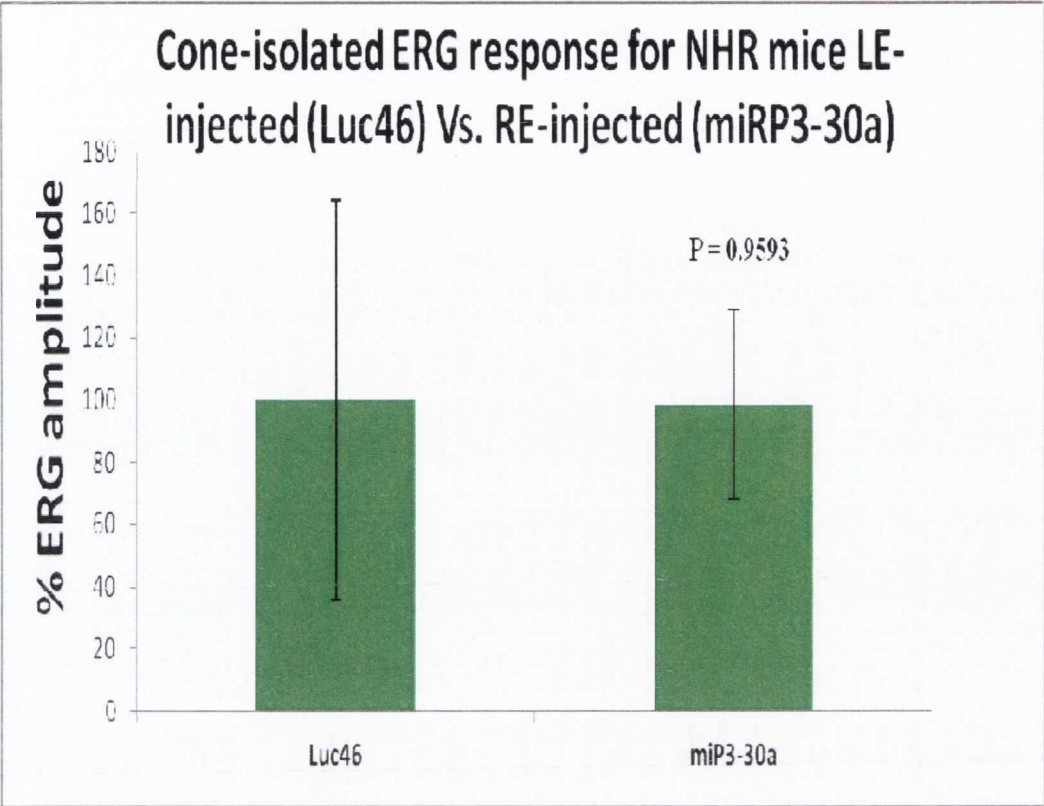
The rod-isolated b-waves of all mice subretinally injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30 were combined for each construct. The average and standard deviation was calculated using Microsoft Excel and statistical significance was calculated by a paired t-test (DataDesk6.0). The average b-wave value for AAV-2/5.Luc46 injected eyes was set at 100% and the rod-isolated b-wave for AAV-2/5.miRP3-30a injected eyes was expressed as a% of this value. AAV-2/5.miRP3-30a is suppressed to 82% of AAV-2/5.Luc 46 and this is not significant. Error bars represent % SD



	NHR with Luc46	NHR with miRP3-30a
% on Luc46	100	83
% SD	13	11

Figure 4.3.13.k % mixed rod-cone ERG response in NHR mice injected with AAV-2/5.Luc46 vs. AAV-2/5.miRP3-30a

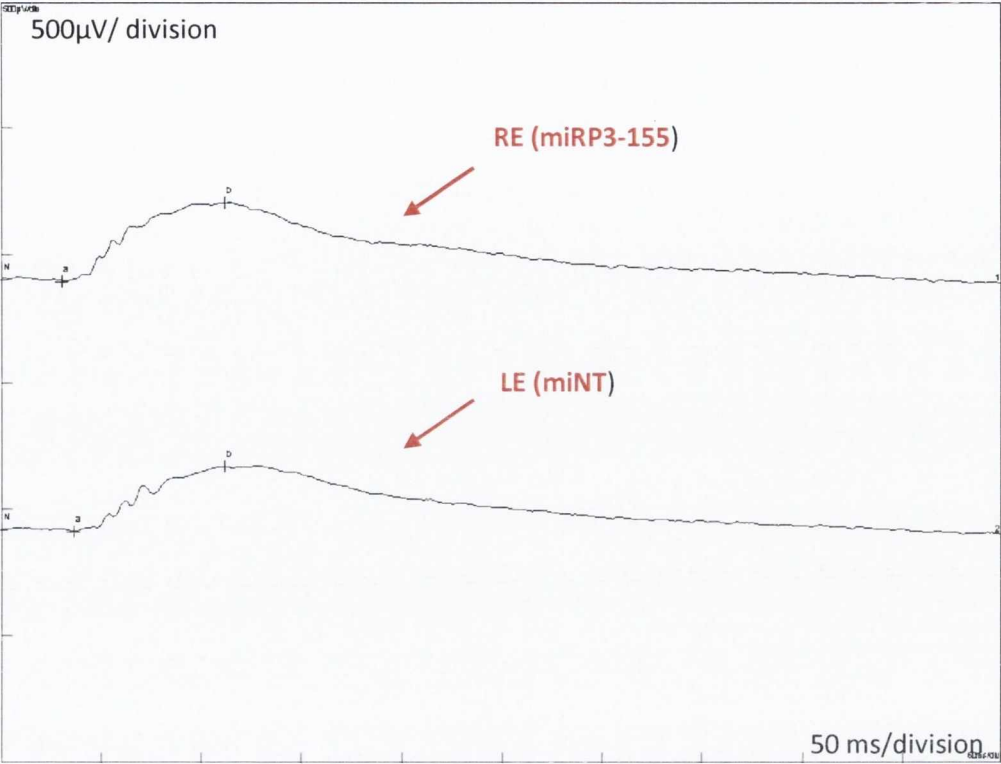
The mixed rod-cone b-waves of all mice injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30 were combined for each construct. The average and standard deviation was calculated by Microsoft Excel. Statistical significance was assessed using a paired t-test (DataDesk 6.0). The average b-wave value for AAV-2/5.Luc 46 injected eyes was set at 100% and the mixed rod-cone b-wave for AAV-2/5.miRP3-30a injected eyes was expressed as a % of this value. AAV-2/5.miRP3-30a is suppressed to 83% of AAV-2/5.Luc 46 and this was not significant. Error bars represent % SD values.



	NHR with Luc46	NHR with miRP3-30a
% on Luc46	100	99
% SD	64	31

Figure 4.3.13.1 % cone-isolated response in NHR mice injected with AAV-2/5.Luc46 vs. AAV-2/5.miRP3-30a

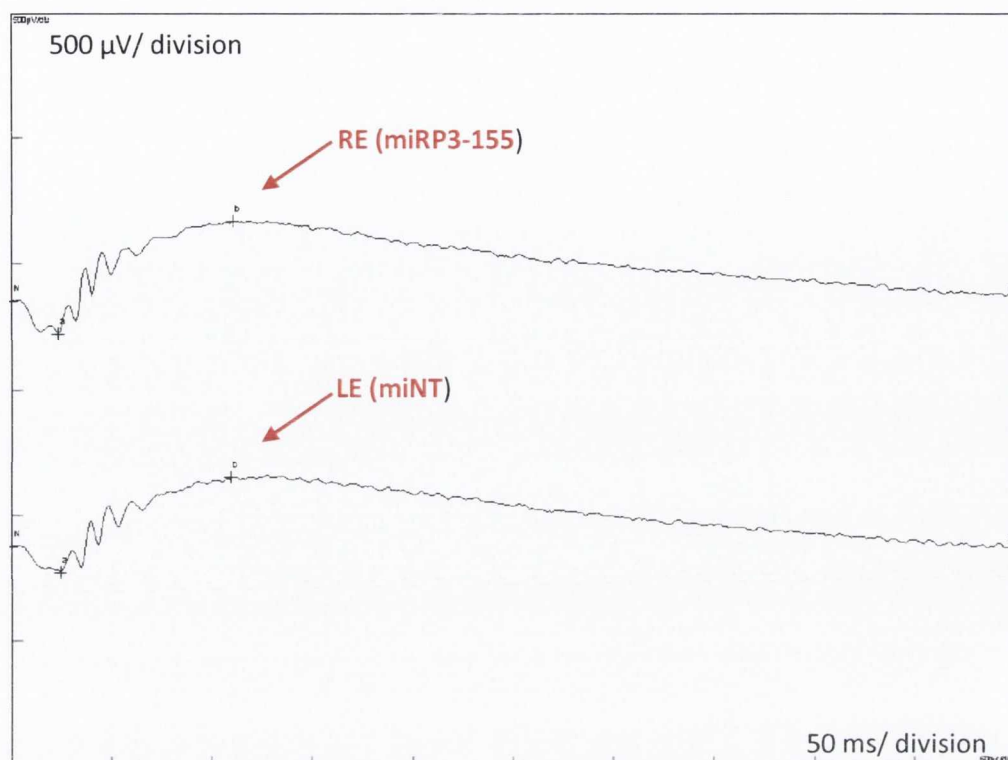
The cone-isolated b-waves of all mice injected with AAV-2/5.Luc46 and AAV-2/5.miRP3-30 were combined for each construct. The average and standard deviation was calculated using Microsoft Excel and statistical significance was assessed by a paired t-test (Datadesk6.0). The average b-wave value for AAV-2/5.Luc 46 injected eyes was set at 100% and the cone-isolated b-wave for AAV-2/5.miRP3-30a injected eyes was expressed as a % of this value. AAV-2/5.miRP3-30a is 99% compared to 100% of AAV-2/5.Luc 46 and this is not significant. Error bars represent % SD



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	30	111	9.44 μ V	310 μ V
2 left Max	36	111	9.56 μ V	257mV

Figure 4.3.13.m Rod isolated ERG for NHR mice injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155(RE).

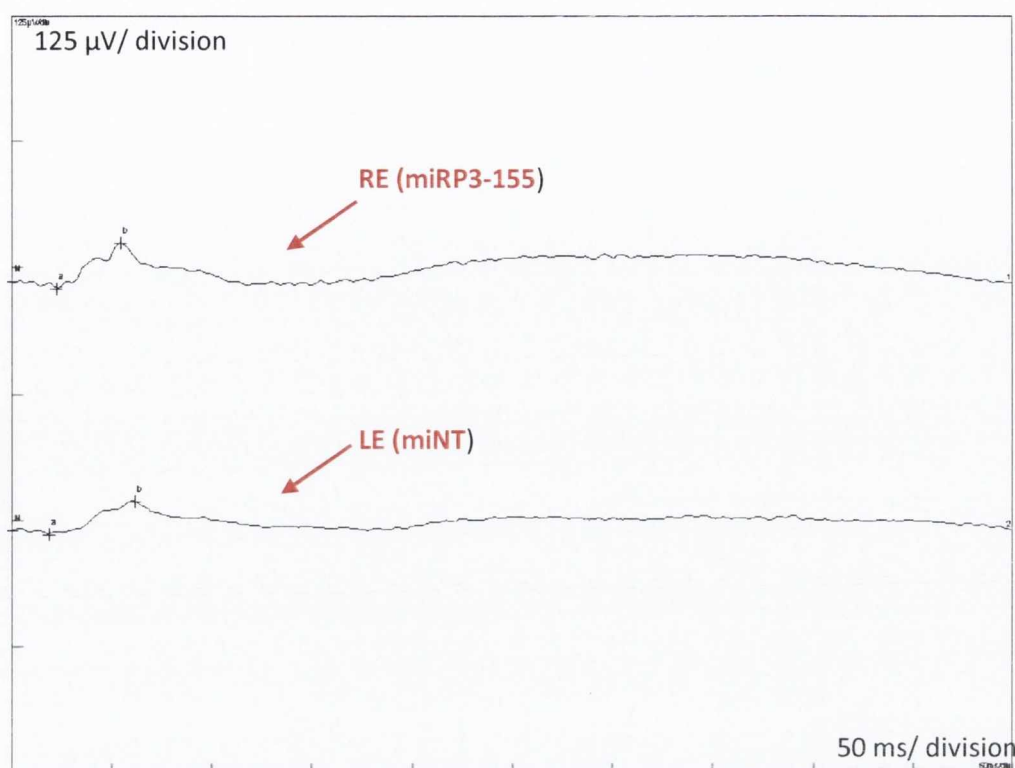
This is a representative rod-isolated ERG result from an NHR mice subretinally injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155 (RE).



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	23	110	129 μ V	443 μ V
2 left Max	24	109	102 μ V	374 μ V

Figure 4.3.13.n mixed rod-cone ERG for NHR mice subretinally injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155(RE).

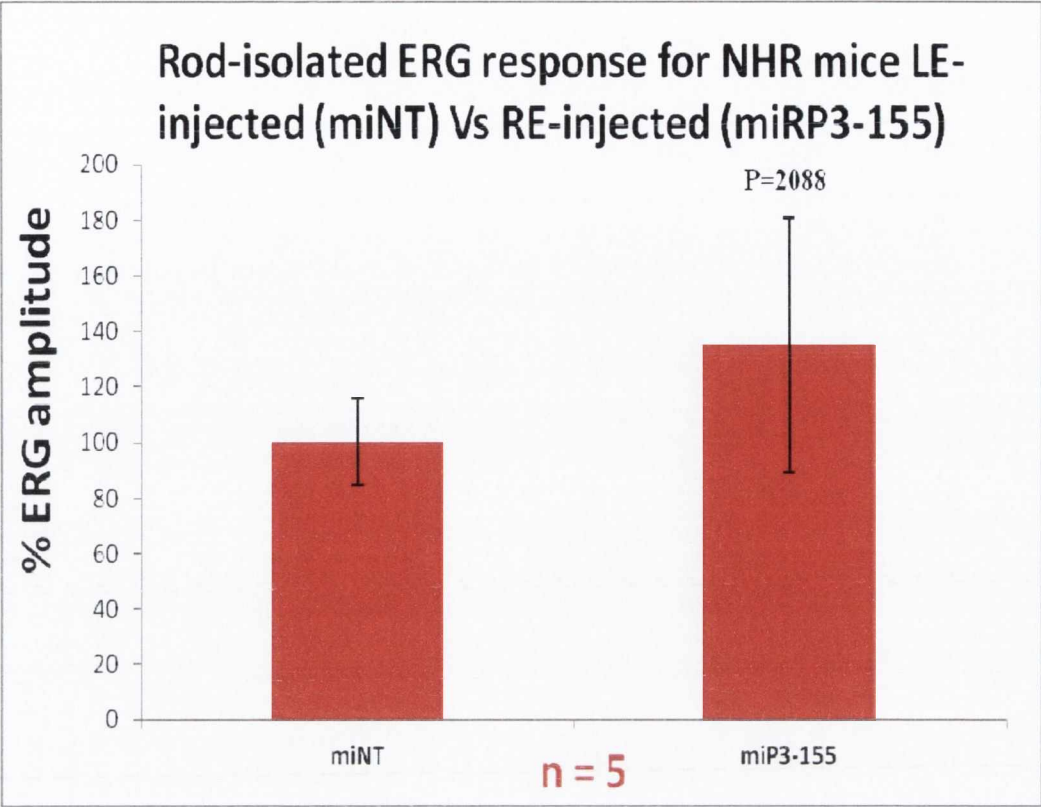
This is a representative mixed rod-cone ERG result from an NHR mice subretinally injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155 (RE).



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	22	55	7.33 μ V	45.7 μ V
2 left Max	19	62	3.59 μ V	32.1 μ V

Figure 4.3.13.o Cone-isolated ERG for NHR mice injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155(RE).

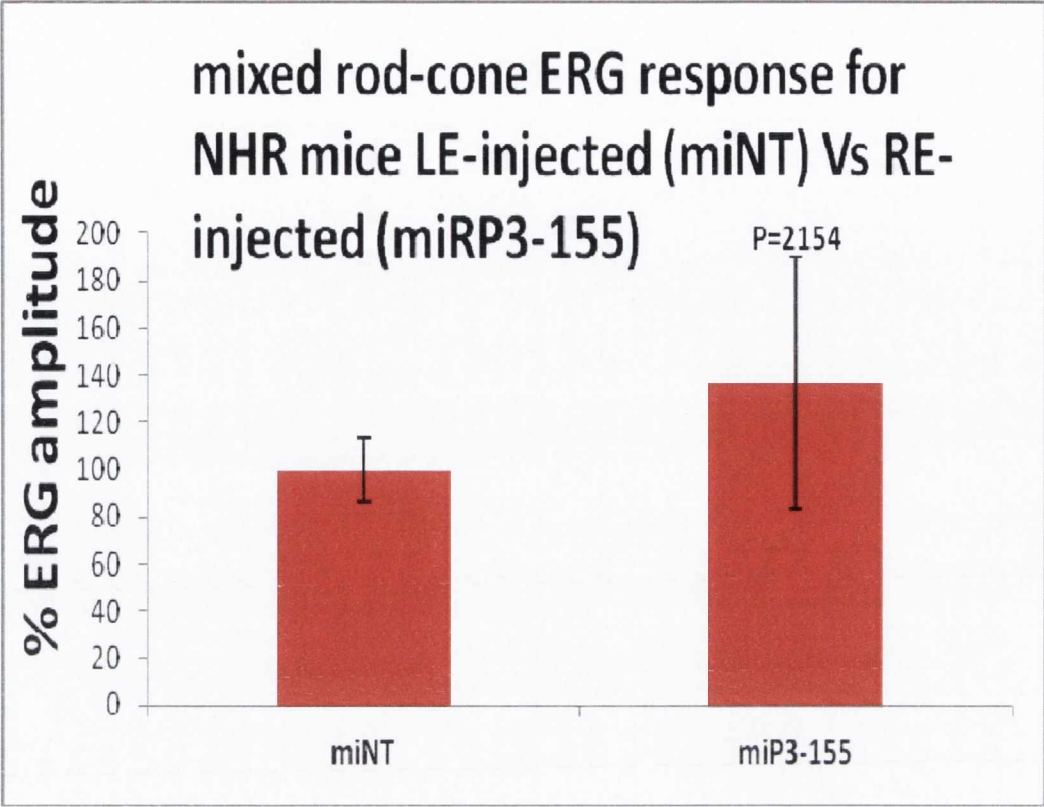
This is a representative atypical cone-isolated ERG result from an NHR mice subretinally injected with AAV-2/5.miNT (LE) and AAV-2/5.miRP3-155 (RE)/



	NHR with miNT	NHR with miRP3-155
% of miNT	100	135
% of SD	15	46

Figure 4.3.13.p % rod-isolated ERG response in NHR mice injected with AAV-2/5.miNT vs. AAV-2/5.miRP3-155

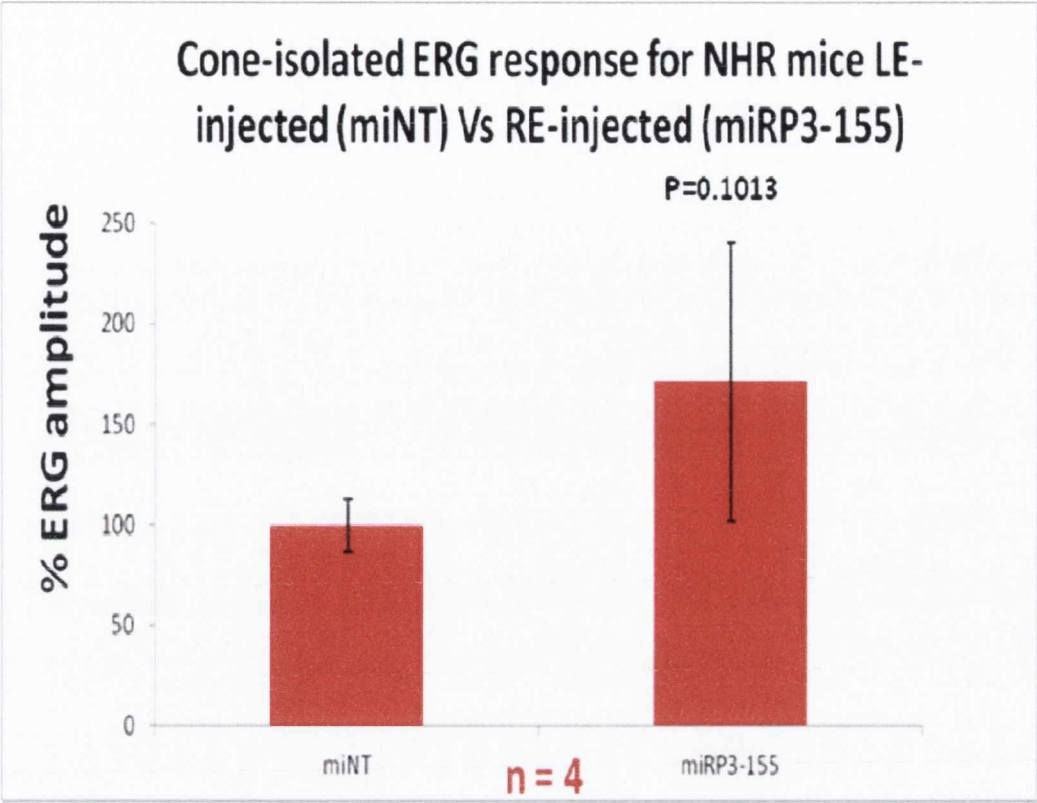
The rod-isolated b-waves of all mice subretinally injected with AAV-2/5.miNT and AAV-2/5.miRP3-155 were combined for each construct. The average and standard deviation was calculated using Microsoft Excel. The average b-wave value for AAV-2/5.miNT injected eyes was set at 100% and the rod-isolated b-wave for AAV-2/5.miRP3-155 injected eyes was expressed as a % of this value. AAV-2/5.miRP3-155 is 135% of AAV-2/5.miNT and this was not significant as assessed by paired t-test (DataDesk 6.0). Error bars represent % SD values.



	NHR with miNT	NHR with miRP3-155
% of miNT	100	136.5
% of SD	14	53

Figure 4.3.13.q. % mixed rod-cone ERG response in NHR mice injected with AAV-2/5.miNT vs. AAV-2/5.miRP3-155

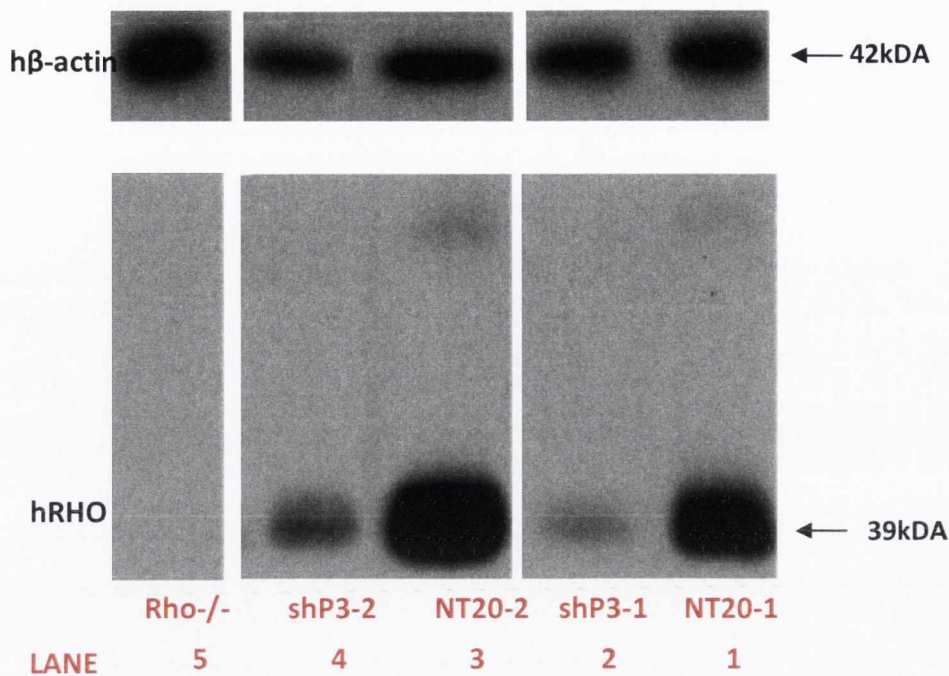
The mixed rod-cone b-waves of all mice subretinally injected with AAV-2/5.miNT and AAV-2/5.miRP3-155 were combined for each construct. The average and standard deviation was calculated using Microsoft Excel. The average b-wave value for AAV-2/5.miNT injected eyes was set at 100% and the mixed rod-cone b-wave for AAV-2/5.miRP3-155 injected eyes was expressed as a% of this value. AAV-2/5.miRP3-155 is 136% of AAV-2/5.miNT and this was not significant as assessed by paired t-test (DataDesk 6.0). Error bars represent% SD values.



	NHR with miNT	NHR with miRP3-155
% of NT20	100	171
% of SD	13	69

Figure 4.3.13.r % cone-isolated ERG response in NHR mice injected with AAV-2/5.miNT Vs AAV-2/5.miRP3-155

The cone-isolated b-waves of all mice subretinally injected with AAV-2/5.miNT and AAV-2/5.miRP3-155 were combined for each construct. The average and standard deviation was calculated using Microsoft Excel. The average b-wave value for AAV-2/5.miNT injected eyes was set at 100% and the cone-isolated b-wave for AAV-2/5.miRP3-155 injected eyes was expressed as a % of this value. AAV-2/5.miRP3-155 is 171% of AAV-2/5.miNT and this was not significant as assessed by a paired t-test (DataDesk 6.0). Error bars represent % SD values.



SAMPLE NAME	Rho-/-	shP3-2	NT-20-1	shP3-1	NT20-1
BCA (μg/ml)	2506	1271.2	659.1	604.2	2014
protein/ well (μl)	2.63	5	10	10.9	3.27
[protein]/ well (μg)	6.6	6.6	6.6	6.6	6.6

Figure 4.3.14.a Western blot analysis of hRHO protein expression in NHR mice LE-injected eyes (AAV-2/5.NT20) versus RE-injected eyes (AAV-2/5.shP3)

Lane 1 contains protein from the first NHR mouse LE injected with AAV-2/5. NT20 (NT20-1). Lane 2 is hRHO protein from the first NHR RE injected with AAV-2/5.shp3 (shP3-1). Lane 3 is hRHO protein expression from the second mouse LE injected with NT20 (NT20-2) and Lane 4 is hRHO expression in the second NHR mouse RE injected with shP3 (shP3-2). Lane 5 contains protein from cells a 129rho^{-/-} mouse (the negative control). The hRHO monomer is clearly present at 39kDA, in the lanes in which had the highest protein expression (NT20 lanes) some hRHO oligomerisation at higher molecular weights was observed. hRHO expression in eyes injected with AAV-2/5.NT20 is clearly higher than in eyes injected with AAV-2/5.shP3. There is no hRHO present in the 129rho^{-/-} sample, validating the experimental control used. hβ-actin was used to probe for equal loading. Due to the low amount of retinal protein obtained, an overnight exposure was used to obtain adequate β-actin expression. The table represents the concentration of protein added to each well as determined by BCA assay,

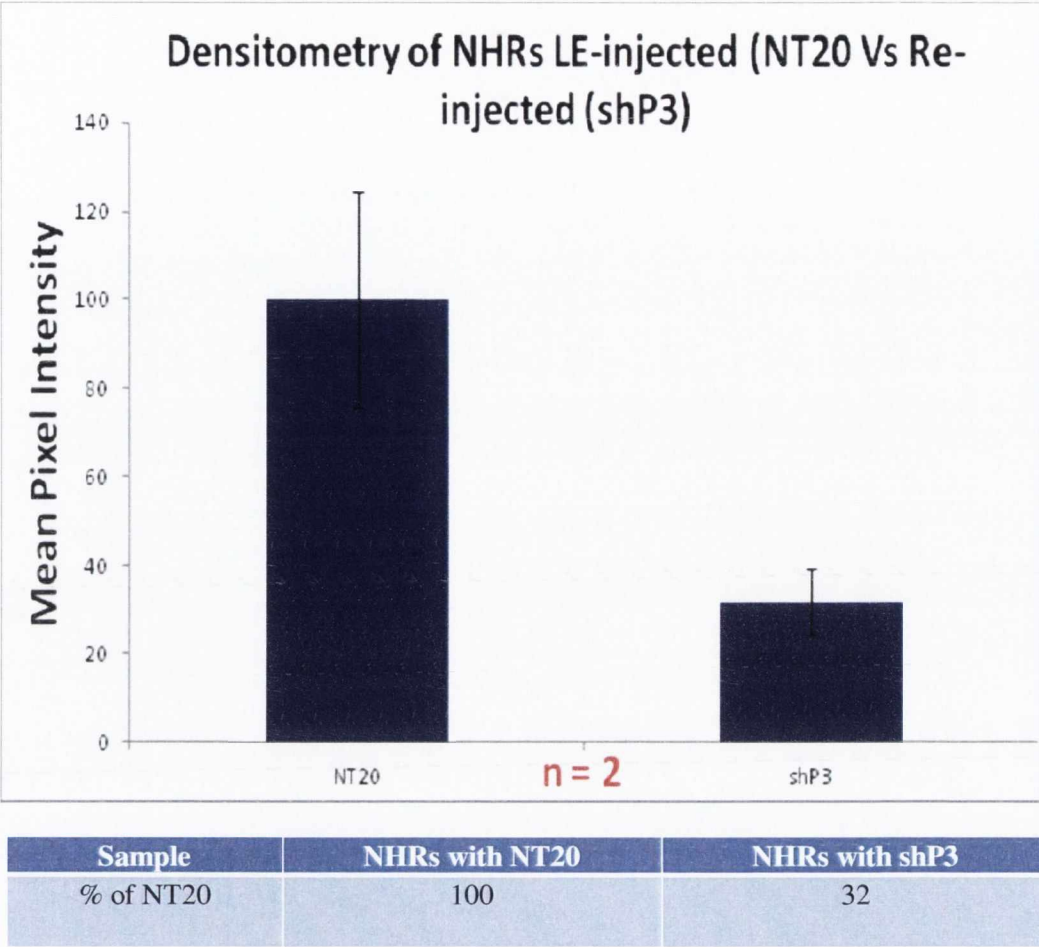
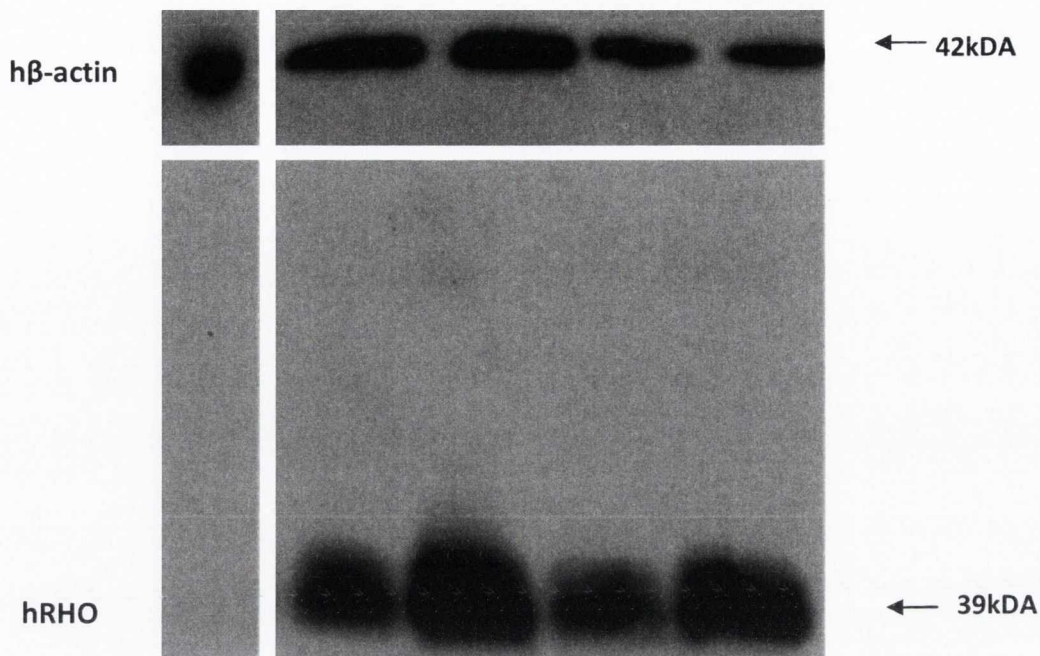


Figure 4.3.14.b Densitometric analysis of hRHO protein expression from two NHR mice LE-injected eyes (AAV-2/5.NT20) versus RE-injected eyes (AAV-2/5.shP3).

The mean pixel intensity of hRHO protein bands was calculated using image J and normalised to the mean pixel intensity of hβ-actin protein bands from the same western blot. The average and standard deviation was assessed by Microsoft Excel. hRHO protein expression for AAV-2/5.NT20 injected eyes was set at 100% expression and hRHO protein from AAV-2/5.shP3 eyes was compared to this value (68% suppression). n=2, therefore no statistical analysis was undertaken.



LANE	Rho-/- 5	30a-2 4	Luc46-2 3	30a-1 2	luc46-1 1
SAMPLE NAME	Rho-/-	30a-2	Luc46-2	30a-1	Luc46-1
BCA (μg/ml)	5133	1905	1107	491	918
protein/ well (μl)	1.9	5.14	8.86	20	10.67
[protein]/ well (μg)	9.8	9.8	9.8	9.8	9.8

Figure 4.3.15.a Western blot analysis of hRHO protein expression in NHR mice LE-injected (AAV-2/5.Luc46) Vs RE-injected (AAV-2/5.miRP3-30a)

Lane 1 contains protein from the first NHR mouse LE injected with AAV-2/5.Luc46 (Luc46-1). Lane 2 is hRHO protein from the first NHR RE injected with AAV-2/5.miRP3-30a (30a-1). Lane 3 is hRHO protein from the second mouse LE injected with Luc46 (Luc46-2) and lane 4 is hRHO expression in the second NHR mouse RE injected with shP3 (30a -2). Lane 5 contains protein from a 129rho^{-/-} mouse (the negative control, no hRHO is present). The hRHO monomer is clearly present at 39 kDA. There does appear to be some difference in hRHO expression in eyes injected with AAV-2/5.Luc46 compared to AAV-2/5.miRP3-30a however n is only 2. Human β-actin was used to probe for equal loading (20 minute exposure). The table represents the concentration of protein added to each well as assessed by BCA assay.

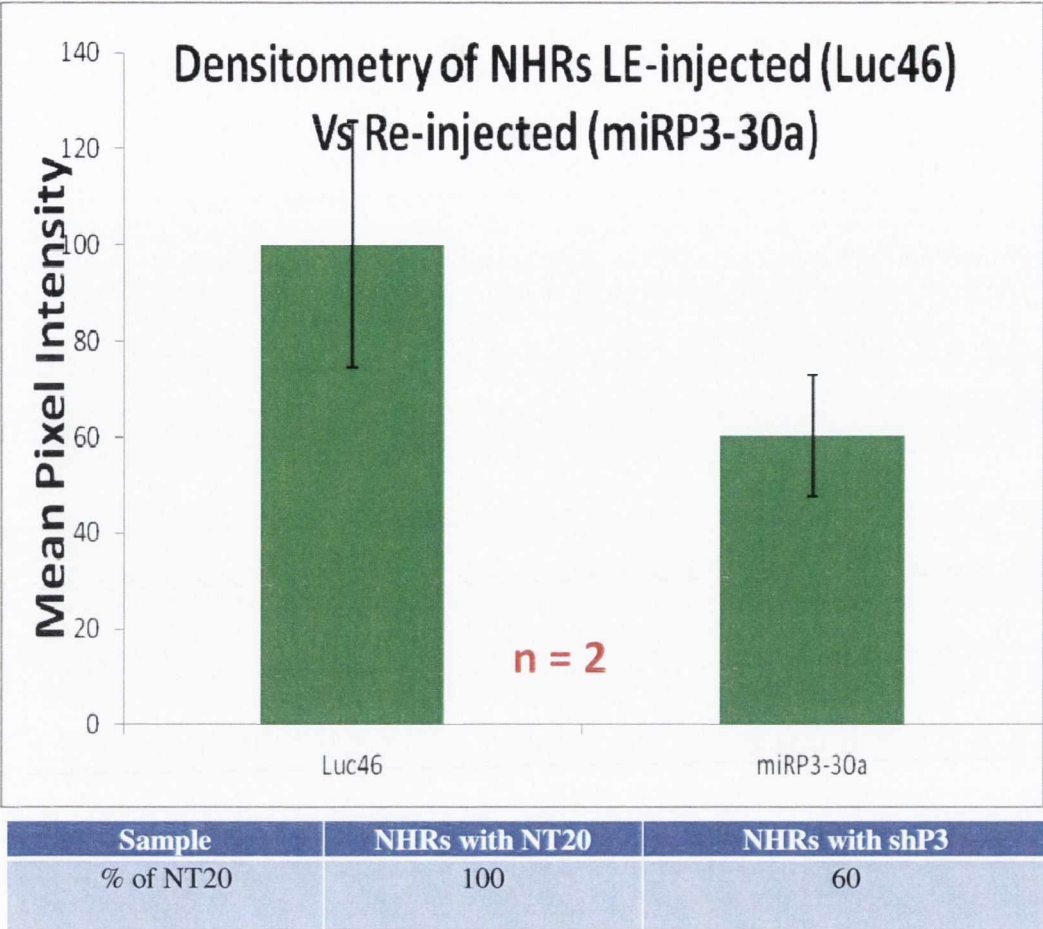


Figure 4.3.15.b Densitometric analysis of hRHO protein expression from two NHR mice LE-injected (AAV-2/5.Luc46) Vs RE-injected (AAV-2/5.miRP3-30a)

The mean pixel intensity of hRHO protein bands was calculated using image J and normalised to the mean pixel intensity of human β -actin protein bands from the same western blot. The average and standard deviation was calculated by Microsoft Excel. hRHO Protein expression for AAV-2/5.Luc46 injected eyes was set at 100% expression and hRHO protein from AAV-2/5.miRP3-30a eyes was compared to this value (40% suppression). However additional n numbers would need to be used to assess for statistical significance (n=2, in this case).

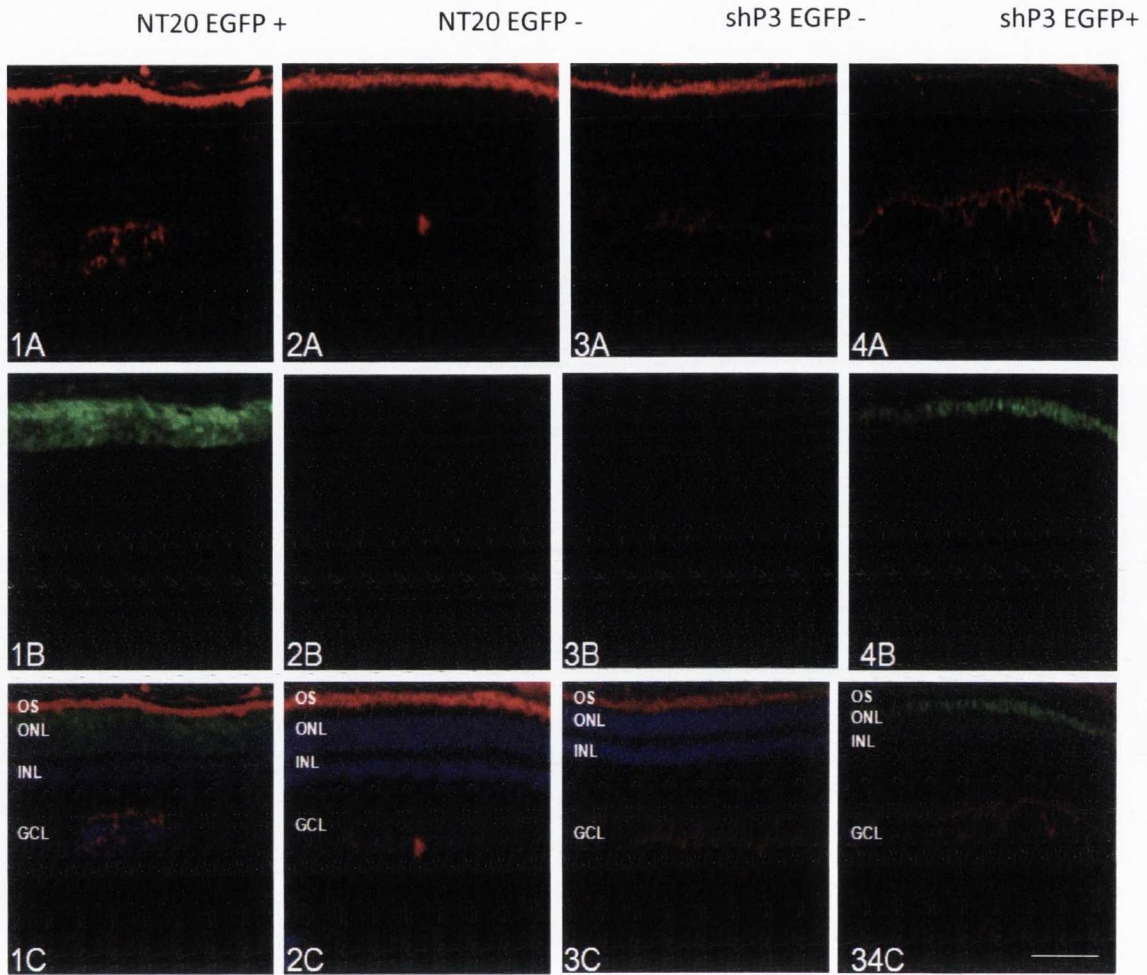


Figure 4.3.16.a Immunohistochemistry of NHR eyes LE-injected (AAV-2/5.NT20) Vs RE injected (AAV-2/5.shP3)

Immunohistochemistry analysis was performed on three NHR mice LE-injected with AAV-2/5.NT20 and RE-injected with AAV-2/5.shP3. All figures depicted above are from the mouse which gave the best ERG response. The Immunohistochemistry was performed on three mice and similar results were obtained each time. hRHO staining was carried out using the 4D2 RHO antibody (1/100 dilution). The first column of diagrams is from an EGFP positive region of an NHR injected with NT20 (1A, 1B & 1C), hRHO is clearly present (red staining) and overlaps with the EGFP staining in the outer segments (1A & 1B). Column 2 is the same retina from column 1 (NHR injected with NT20) but in an EGFP negative region (2A, 2B & 2C). hRHO staining is clearly visible in the outer segments, comparable to column 1 (2A). Column 3 is an EGFP negative region from the retina of an NHR injected with shP3 (3A, 3B & 3C), hRHO is clearly labeled in the outer segments (3A). Column 4 represents the same retina as column 3 but in an EGFP positive region injected with shP3 (4A, 4B & 4C). hRHO immunostaining is negative in the outer segments compared to the NT20 figures and the shP3 EGFP negative control of the same retina (4A). Row A is hRHO staining only, row B is EGFP only and row C represents hRHO, EGFP and DAPI staining overlaid. OS= outer segments, ONL = outer nuclear layer, INL = inner nuclear layer and GCL = ganglion cell layer. Scale bar (L) represents 116µM.

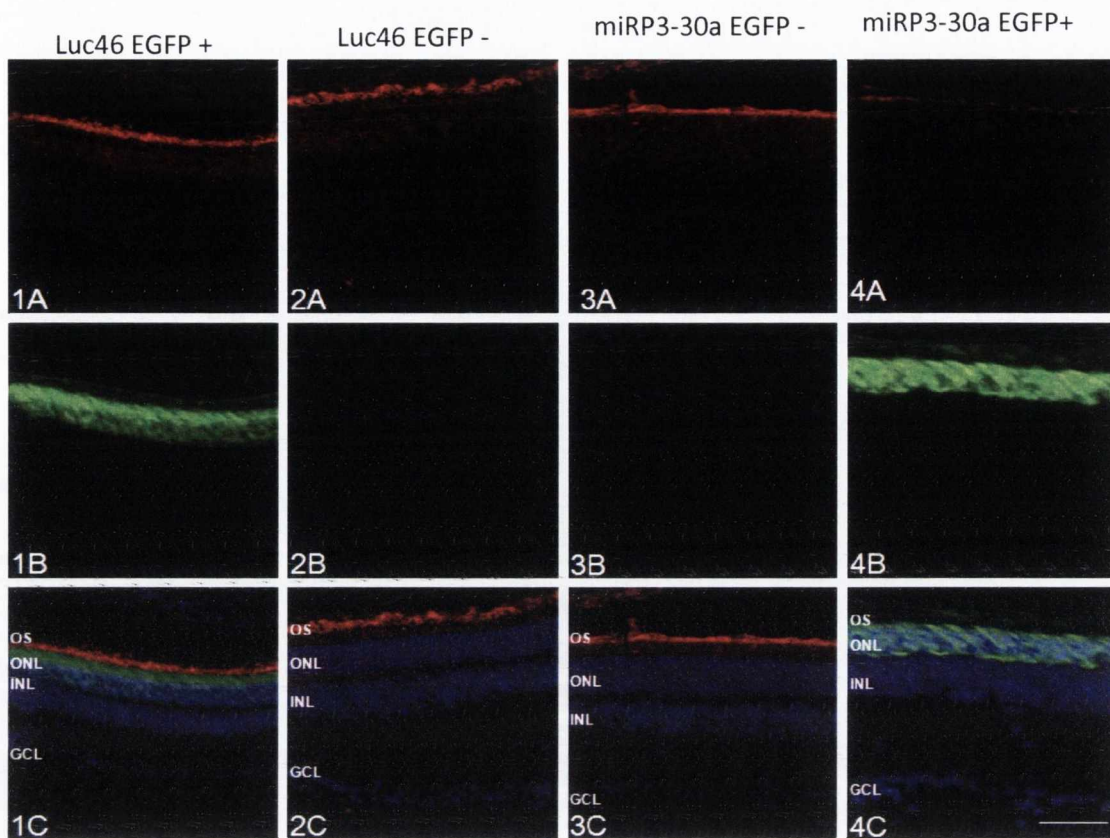


Figure 4.3.17. a Immunohistochemistry of NHR eyes LE-injected (AAV-2/5.Luc46) Vs RE injected (AAV-2/5.miRP3-30a)

Immunohistochemistry analysis was performed on three NHR mice LE-injected with AAV-2/5 Luc46 and RE-injected with AAV-2/5.miRP30-a. Of all the three mice observed, this was the only mouse in which a decrease in hrHO staining was observed in the miRP3-30a injected eye. hrHO staining was carried out using the 4D2 RHO antibody (1/100 dilution). The first column of diagrams is from an EGFP positive region of an NHR injected with Luc46 (1A, 1B & 1C), hrHO is clearly present (red staining) and overlaps with the EGFP staining in the outer segments (1A & 1B). Column 2 is the same retina from column 1 (NHR injected with Luc46) but in an EGFP negative region (2A, 2B & 2C). hrHO staining is clearly visible in the outer segments, comparable to column 1 (2A). Column 3 is an EGFP negative region from the retina of an NHR injected with miRP3-30a (3A, 3B & 3C), hrHO is clearly labeled in the outer segments (3A). Column 4 represents the same retina as column 3 but in an EGFP positive region injected with miRP3-30a (4A, 4B & 4C). hrHO immunostaining is negative in the outer segments compared to the Luc46 figures and the miRP3-30a EGFP negative control of the same retina (4A). Row A is hrHO staining only, row B is EGFP only and row C represents hrHO, EGFP and DAPI staining overlaid. OS= outer segments, ONL = outer nuclear layer, INL = inner nuclear layer and GCL = ganglion cell layer. Scale bar (L) represents 116µM.

Chapter 5: Exploration of shRNA-based **RHO suppression in a rhodopsin-linked** **adRP mouse model**

5.1 Introduction

In chapter 4 the role of RNAi-based suppressors for hRHO was explored *in vivo* and AAV-2/5.shP3 was found to be particularly efficient at suppressing hRHO. While the initial results from the animal *in vivo* suppression studies are promising it is however vital to explore the use of such suppressors in models of the disease to determine if gene suppression translates to therapeutic benefit. Animal models of retinal disease are very valuable as many develop retinal degeneration with a similar pathology to human patients. This allows not only for the dissection of the molecular mechanisms behind these retinal diseases but also for the evaluation of efficacy and for toxicity studies of the possible therapeutics (Fletcher et al, 2011; Penha et al, 2010). Previous studies in which suppression of rhodopsin has been achieved have examined the mutation-independent suppression of both the wild-type and mutant forms of rhodopsin *in vitro* and *in vivo* using RNAi technology (Cashman et al, 2005; Chadderton et al, 2009; Gorbutyuk et al, 2007a). O'Reilly et al, 2007 initially evaluated therapeutic benefit using an RNAi-based rhodopsin suppression and replacement strategy in a mouse model with the P23H hRHO mutation. While an improvement in ONL thickness was obtained in eyes injected with a suppression and replacement construct, an improvement in visual function could not be determined due to the fast rate of retinal degeneration in the P23H mouse model (O'Reilly et al, 2007). An animal model in which the disease pathogenesis caused by the mutation is well characterised and in which the rate of disease progression is sufficient to allow the evaluation of therapeutic efficacy is vital. One set of hRHO mutations that have been well characterised and that have been incorporated into various animal models are mutations within the c-terminal of rhodopsin as detailed below.

5.1.2 Mutations within codon 347 of rhodopsin

Dryja et al, 1990 examined a subgroup of 28 patients from separate families with RP. Of the 28, 2 patients were heterozygous for a C to T nucleotide mutation within the second nucleotide of codon 347. Mutations within this codon disrupt an *MspI* restriction site within rhodopsin, which provided a method for screening all 150 patients. This confirmed that 9/150 patients had mutations within codon 347. Sequencing of these patients revealed 8 with a CCG to CTG mutation in codon 347 which substituted proline for leucine (P347L) and 1 had a CCG to TCG mutation resulting in a proline to serine mutation (P347S). Furthermore patients with these mutations displayed reduced (P347L) or undetectable (P347S) rod and cone ERG responses (Dryja et al, 1990). In cell culture mutant P347S mutants were found to belong to Class I rhodopsin mutant proteins. This class of mutant rhodopsin can regenerate 11-*cis*-retinal, and have similar expression levels to wild-type hRHO. In addition, these Class I mutations cluster all along the c-terminus region (Sung et al, 1993). This c-terminus has

subsequently been found to be highly conserved and a 'hot-spot' for adRP mutations (Sung & Tai, 2000). Elucidating the molecular mechanisms behind these c-terminus mutations has involved the use of both *in vitro* assays and *in vivo* studies using animal models.

5.1.3 Transgenic mouse with porcine P347S mutant rhodopsin

The first animal model generated to have a P347S mutation was a mouse containing a porcine mutant rhodopsin gene with the P347S mutation (Wong & Hao et al, 1992). Several transgenic lines were created with the Serine 6 (Ser 6) line having been the most characterised. The photoreceptor cells develop as normal until 3 weeks of age and by 7 weeks 50% of the nuclei in the ONL are abolished, by one year there are few photoreceptors remaining and this represents the end stage of the degeneration (Chang et al, 1993; Huang et al, 1993; Wong and Hao, 1992). The mutant porcine rhodopsin mouse model displays increased levels of cAMP in light and dark conditions which is indicative of cell death and is consistent with the fact that this model has been shown to have an increased levels of cell death by apoptosis (Chang et al, 1993; Weiss et al, 1995). Additionally, abnormal mutant rhodopsin is ectopically expressed within the IS of rods. Western blot analysis of mutant retinal porcine rhodopsin compared to wild-type porcine rhodopsin indicates that the proteins are identical in banding pattern and size, thus the mutant rhodopsin appears to undergo normal post-translational modifications and expression. *In vitro* assays of mutant porcine P347S in HEK293 cells revealed that this protein was similar to wild-type in terms of activating transducin, additionally the mutant protein was able to undergo phosphorylation by protein kinase and thus bind as normal to arrestin (Weiss et al, 1995). An interesting study generated a chimeric mouse model by aggregating the embryos from the Ser 6 mutant rhodopsin mouse and a wild-type mouse. The resulting mice had patches of porcine transgenic photoreceptors and patches of wild-type mouse photoreceptors within the retina. This model was used to elucidate the interactions between each photoreceptor. In all chimeric mice generated, retinal degeneration occurred in all photoreceptors, not just the cells expressing the transgene. Furthermore the rate of degeneration was proportional to the extent of the chimerism. Retinas with more wild-type patches displayed a phenotype less severe than retinas with large amounts of transgene patches. The fact that patches of normal retinal cells displayed a retinal degeneration phenotype was an important indicator that photoreceptors may interact with each other (Huang et al, 1993).

5.1.4 Transgenic mouse model with the human P347S transgene

A mouse model with the human P347S transgene has also been generated by Li et al, 1996. Six transgenic lines were made with varying degrees of transgene expression and the rate of degeneration

in each line corresponded to the transgene expression (i.e. the higher the mutant hRHO RNA expression, the faster and more severe the phenotype). Mice with intermediate levels of transgene expression compared to endogenous rhodopsin expression had 1 to 2 rows of nuclei at 4 months. In contrast, at 4 months one transgenic line in which the expression of the transgene was low, displayed a phenotype in which only half the photoreceptors were remaining. Furthermore, altered ERG responses were observed in all animals at rates corresponding to the level of degeneration. The majority of mutant rhodopsin was observed in the OS as expected, with only a small level of rhodopsin immunoreactivity observed in the IS and ONL. Upon examining the photoreceptor ultrastructure, mutant rhodopsin was present in ectopic vesicles near the base of the OS. Additionally, the number of these vesicles corresponded to the level of the mutant rhodopsin transgene. The results from this study therefore suggested that the P347S mutation may lead to abnormal transport of rhodopsin containing post-golgi vesicles (Li et al, 1996)

5.1.5 Transgenic porcine models with P347L and P347S mutations

While mice are valuable animal models for use in retinal research, often the use of larger animal models may be more advantageous. This is particularly relevant in terms of retinal studies using the porcine model. Pigs have eyes that are of comparable size and morphology to human eyes and that have a similar retinal vasculature (Beauchemin et al, 1974; De Schaepdrijver et al, 1992; Fletcher et al, 2011; Simoens et al, 1992). Additionally, despite the fact that pigs do not have a cone rich fovea, which is a distinct characteristic of the human retina, they do have a similar rod and cone ratio (Chandler et al, 1999). This makes them an attractive model to use for studies of eye disease. A porcine transgenic line was generated by pronuclear microinjection in which the pigs had a porcine RHO transgene with either the P347L or P347S mutation (Petters et al, 1997). The degeneration in the P347L transgenic pig has subsequently been well characterised. Initially the rods undergo degeneration such that by 3 weeks, two thirds of the retina has degenerated and by 6 weeks only 50% of the ONL remain. By 8 weeks no rods remain. An ERG at 4 weeks shows a normal cone-isolated response when compared to wild-type pigs, however, following the initial stages of rod cell death the cones undergo severe degeneration with only 50% of the cone cells remaining from 6-12 weeks. After this point the remaining cones slowly degenerate until 20 months, when there is only one cone photoreceptor layer. This cone degeneration is thus highlighted at 20 months when a significant decrease in the cone-isolated ERG response is obtained compared to wild-type mice (Petters et al, 1997; Tso et al, 1997). Further characterisation of this animal model of adRP established that at birth, rhodopsin immunoreactivity was observed in the IS, OS & ONL and that within this region rhodopsin containing

membranes (similar to that reported by Li et al, 1996) were present. It has been established that rod photoreceptors in transgenic pigs with the P347L mutation do not synapse with rod bipolar neurons in the OPL, a finding that has been observed in human patients with P347L mutations (Peng et al, 2000). This prompted the exploration of the expression of specific synaptic markers in these mutant porcine models. The expression of one protein, PSD-95, which is usually localised to the rod and cone synapses in wild-type retinas was examined; interestingly this protein was absent from the rod synapses (but not the cone synapses) from an early stage in transgenic pigs. This study therefore highlighted the affect the P347L mutant rhodopsin may have on important synaptic proteins within the degenerating retina (Blackmon et al, 2000; Li et al, 1998). Additional studies focused on elucidation of the mechanisms of cone cell dysfunction and resulted in the observation that an increased level of oxidative stress was observed in cones in transgenic pigs harbouring the P347L mutation (Shen et al, 2005). Recent assessment of this porcine adRP model was undertaken in order to assess the effect of breeding this transgenic pig for nine generations on a heterogeneous background (Sommer et al, 2011). Importantly, the retinal degeneration phenotype originally observed by Petters et al, 1997 remained stable thus highlighting the value of this model for use in preclinical studies, for example, for potential gene therapies.

5.1.6 Molecular mechanisms of mutations within the c-terminus of rhodopsin.

Studies using the various animal models described above elucidated the fact that adRP mutations located within the last four amino acids of the rhodopsin c-terminal do not result in altered phototransduction (Li et al, 1996; Osawa and Weiss, 1994; Weiss et al, 1995). Therefore mutant rhodopsin with mutations in the 347 codon must have an alternative mode of pathogenesis. Deretic & Papermaster, 1991 characterised post-golgi vesicles involved in the transport of rhodopsin to the ONL using an *in vitro* retinal free model system. Furthermore Deretic & Papermaster, established that the addition of an antibody which targets the last 9 amino acids of the rhodopsin c-terminal abolished rhodopsin localization to the ONL (Deretic & Papermaster, 1991). In addition, Sung et al showed that rhodopsin with a stop codon mutation eliminating the last five amino acids of the rhodopsin c-terminal abolishes rhodopsin expression in the ROS (Sung et al, 1994). Subsequently using the retinal free system originally described by Deretic et al, it was established that the introduction of either missense rhodopsin mutations within this c-terminal region or a truncated rhodopsin protein results in the mislocalisation of rhodopsin (Deretic & Papermaster, 1991; Deretic et al, 1998; Deretic et al, 2006). The proposal that mutations within the VXPX c-terminal domain of rhodopsin (the last four amino acids) caused its altered subcellular location was further supported by the evidence that transgenic mice

with the human P347S rhodopsin mutation accumulated ectopically expressed rhodopsin vesicles at the base of the ROS, in a manner which correlated with the amount of mutant transgene expression (Li et al, 1996). All these *in vitro* and *in vivo* studies established that mutations within codon 347 (P347S or P347L) or indeed within any of the codons in the VXPX c-terminus result in the targeting signal of rhodopsin to the ROS being abolished (Sung & Chuang, 2010).

5.1.7 The use of these animal models to study adRP

While no studies have yet utilised the mouse with mutant pig rhodopsin transgene to test potential treatments for RHO-adRP, this model was vital for understanding the P347S disease pathology (Wong & Hao, 1992). In contrast, the transgenic mouse with the human P347S mutation has been extensively used to evaluate therapeutic strategies for RHO-adRP. Chadderton et al, 2009 bred this transgenic mouse onto a 129 wild-type background to evaluate RNAi-mediated suppression of the human rhodopsin mutant. Additionally the therapeutic efficacy provided by the presence of two endogenous wild-type alleles (acting as natural shRNA resistant replacement genes) was examined (Chadderton et al, 2009). Further elucidation of an shRNA which suppresses both mRHO and hRHO established that co-administration of this shRNA along with a resistant hRHO replacement gene conferred significant therapeutic benefit in this RP model and highlighted the value of testing this therapy in larger animal models (Millington-ward et al, 2011). P347S or P347L porcine models of RHO-adRP have not thus far been used to evaluate a gene therapy. However, this model has been used to test other novel treatments for photoreceptor degeneration. Neurotrophic factors have previously been shown to promote photoreceptor cell survival. It was proposed that a lensectomy and a vitrectomy may release these factors and preserve a degenerating retina. These procedures were performed on the P347L transgenic pig and 8 weeks post-surgery an increased number of cell nuclei were observed compared to the unoperated eye; suggesting that these procedures resulted in the delay of photoreceptor cell death in this transgenic model (Mahmoud et al, 2003). Another treatment involved surgically placing a normal fetal neonatal retina graft within the host of a severely degenerated transgenic pig eye which resulted in the grafts forming rods, cones and inner retinal cells. An increased number of surviving rods in treated eyes compared to controls was observed. In addition, an eventual improvement in the cone ERG response in operated retinas (once the eyes had recovered from surgery) compared to control eyes was obtained (Ghosh et al, 2004; Ghosh et al, 2007).

Objective of Chapter 5

The premise for designing siRNAs for the studies detailed in this thesis was to enable the evaluation of a suppression and replacement therapeutic strategy in larger animal models of RHO-adRP. One such model available for this assessment is the transgenic pig model described above (**Section 5.1.5**). The pigs have a P347L RHO mutation and display a severe rod degeneration followed by a much slower degeneration of the cones (Petters et al, 1997). Prior to testing such therapies in these larger animal models of RHO-adRP, two important parameters need evaluation in smaller animal models: 1. that the shRNAs designed suppress rhodopsin sufficiently to confer therapeutic benefit in RHO-adRP models and 2. In addition to human rhodopsin, the shRNAs suppress porcine rhodopsin. These two questions could be simultaneously addressed given the presence in the TCD laboratory of the transgenic mouse expressing the mutant porcine P347S mutation (Wong & Hao et al, 1992). Of note the P347S and P347L pigs (Petters et al, 1997) were generated using porcine rhodopsin sequence. It would therefore be of significant value to have an RNAi suppressor targeting porcine rhodopsin which could directly be evaluated in this pig model in the future. Ideally this suppressor should be homologous in its target site to human rhodopsin such that it could in principle be directly relevant to the treatment of human patients in the future. Of note siP3/shP3 has these features and so continued assessment of this suppressor *in vivo* was deemed of significant value. In this regard the RHO-adRP mouse with the porcine rhodopsin transgene provided an opportunity to simultaneously assess specific RNAi mediated suppression of porcine rhodopsin together with the potential therapeutic benefit of RNAi mediated suppression of porcine rhodopsin. The latter could be evaluated as siP3/shP3 does not target the endogenous mouse rhodopsin gene and hence could in principle act as an RNAi resistant rhodopsin replacement gene. With this in mind, the porcine RHO-adRP transgenic mouse bred onto the 129 background first needed to be characterised in order to establish baseline degeneration conditions. In addition, confirmation was required that the degeneration on this genetic background was not too rapid to preclude it from use. The main goal of the research presented in this chapter was to establish if both potent suppression of porcine rhodopsin and therapeutic efficacy could be achieved by shP3 *in vivo* in a RHO-adRP mouse.

Section 5. 2 Materials and Methods

5.2.1 Animals

The animal model used in this chapter was a mouse model, containing a porcine rhodopsin gene, with the porcine P347S (PP347S) autosomal dominant rhodopsin mutation first identified in human subjects (Dryja et al, 1990). This mouse had previously been generated by Wong & Hao, 1992. The original transgenic line was hemizygous for the PP347S mutation on a C57BL/6 background (Jackson Laboratories). The original line was maintained by crossing hemizygous females with C57BL/6 mice such that 50% of the mice were transgenic (Huang et al, 1993). This model was obtained in our laboratory in 2004, and bred on to a pure 129rho^{-/-} background for 10-12 generations (Humphries et al, 1997). A homozygous PP347S^{+/+} rho^{-/-} line was then established. For the analysis in this chapter it was decided to use the mouse with the heterozygous genotype PP347S^{+/-} rho^{+/-} by breeding homozygotic transgenic males with female wild-type 129rho^{+/+} mice such that 100% of the progeny would have the PP347S^{+/-} rho^{+/-} genotype.

5.2.2 Genotyping

Random spot checks were performed so as to ascertain the genotype of the mice. The genotyping procedure was carried out (**Section 2.8.2**) using the primers P347SF and PP347SR (**Table 5.2.2**) in order to specifically amplify the rhodopsin pig transgene. The P347SF primer target site is at the 3' end of exon 4 of pRHO and the PP347SR primer targets the 3'UTR of the pRHO sequence. A positive control (a mouse homozygous for the PP347S allele) was used in the PCR reaction. A PCR fragment of 1093bp indicated the presence of the P347S allele.

5.2.3 ERG characterisation

In order to characterise the degeneration of the heterozygous PP347S mouse (hetPP347S) at a functional level, ERG analysis was undertaken (**Section 2.8.4**) on different hetPP347S litters at 3, 8, 12, and 16 weeks, 6 months, 9 months and 1 year. Rod-isolated, mixed rod-cone and cone-isolated responses were measured at each time point. For each animal the eye that gave the highest b-wave at each timepoint was the one used for analysis. The b-wave responses for each ERG test at each timepoint were combined and the average for each obtained.

5.2.4 Optimisation of the 4D2 antibody for pRHO protein

The initial western blotting undertaken was to optimise the 4D2 antibody for use with pRHO. A pig eye was obtained from an abattoir by Dr. Sophia Millington-Ward, the retina was isolated from the eye using a blade and placed into 10ml of protein lysis buffer. The retinal sample was placed on ice for thirty minutes and then sonicated. The protein extraction and the evaluation of protein concentration were carried out according to **Section 2.5.2 & Section 2.5.3**. Approximately 20µg of porcine retinal protein was used for western blotting. The western blotting protocol was carried out according to **Section 2.6**. The transfer membrane used was PVDF. 4D2 (Hicks & Molday, 1986 [1/1000] dilution) and the secondary anti-mouse antibody (1/2000 dilution) were used. The membrane was also probed for pig β -actin using the anti- β -actin antibody (1/2000 dilution) and the anti-rabbit secondary antibody (1/2000 dilution).

5.2.5 Rhodopsin protein expression over time

As the retina in the hetPP347S mouse degenerates, the level of both mRHO and pRHO protein should also decrease over time as the photoreceptors die. No specific pRHO antibody exists therefore it was decided to use the 4D2 rhodopsin antibody. This antibody also detects mRHO such that western blotting of retinal protein for the hetPP347S mouse would show both mRHO and pRHO protein expression.

When it was established that the 4D2 antibody could be used to detect pig rhodopsin, retina protein from the hetPP347S mouse at the following time points; 3 weeks, 16 weeks, 6 months, 9 month and 1 year was isolated (**Section 2.5.2**) and the protein concentration estimated (**Section 2.5.3**). The number of protein samples for each time point was at least 3. Not all samples could be analysed on the one blot. The initial western blot undertaken evaluated the rhodopsin protein expression in the hetPP347S mouse at 3 weeks, 16 weeks and 6 months. The second set of western blots evaluated protein expression at the time points of 6 months, 9 months and 1 year. One sample from a 3 week PP347S animal was also included in this analysis to ensure the same result was obtained as with the previous sets of western gels. Similarly the 6 month time point protein sample had been analysed in previous set of westerns and was used to check the same result was obtained. Retinal protein from each of the time-points was loaded onto a western gel in addition to a positive control (retinal protein from an NHR or RHO-M mouse) and a negative control (retinal protein from a 129rho^{-/-} mouse). Densitometry was carried out by scanning in western blots and analysed using image J software.

5.2.6 Immunohistochemistry of hetPP347S retinal sections

Retinal sections from hetPP347S mouse eyes at 3 weeks, 16 weeks, 6 months, 9 months and 1 year were prepared (**Section 2.8.5-Section 2.8.8**). Sections from each mouse (n=3 for each time point) were taken and stained for rhodopsin (4D2, 1 in 100 dilution [Hicks & Molday, 1986]) in order to assess the decrease in rhodopsin (mRHO and pRHO) immunoreactivity over time as the retina degenerates. DAPI staining was also undertaken in order to assess the decrease in ONL cell nuclei over time as the retina degenerates. In order to evaluate if degeneration of the cone photoreceptor cells was taking place, sections were stained for Lectin Peanut Agglutinin (PNA [1/500 dilution]). This protein is only present within the cone cells of the retina. It is conjugated to an Alexa Fluor 568 and does therefore not require incubation with a secondary antibody. PNA staining was analysed using the green channel '10' of the fluorescent microscope (Axiophot microscope, Carl Zeiss LTD).

5.2.7 Sub-retinal injection of hetPP347S mice with AAV-2/5.NT20 and AAV-2/5.shP3

AAV-2/5.shP3 was tested in the hetPP347S animal model to determine if pRHO was efficiently suppressed and to evaluate if therapeutic benefit in a RHO-adRP model was achieved. Each animal was injected at P10 with 5×10^9 vp of AAV-2/5.NT20 (LE) and 5.6×10^9 vp of AAV-2/5.shP3 (RE) for all injections undertaken in this chapter. These injections were undertaken by Dr. Paul Kenna (**Section 2.8.3**).

5.2.8 Optimisation of real-time RT-PCR for analysis of pRHO expression

In order to analyse if shP3 specifically suppresses pRHO, three primer sets were designed (**Table 5.2.2** [P1, P2 and P3]) targeting pRHO and not mRHO. These primers were optimised similarly to previously described (**Section 3.25**). A 5X pRHO RNA standard curve was generated using RNA from 3 week old hetPP347S mice in order to evaluate if each primer pair could efficiently amplify pRHO. Additionally a 5X standard curve was generated using RNA from a wild-type 129^{+/+} mouse in order to ensure no mRHO amplification was occurring with these primers.

5.2.9 Optimisation of FACS analysis of EGFP positive regions of hetPP347S retinas

The quantity of pRHO RNA obtained from AAV-2/5 injected hetPP347S retinas following FACS analysis was determined. Six hetPP347S mice were injected with AAV-2/5.NT20(LE) and AAV-2/5.shP3(RE) and retinal cells were dissociated (**Section 4.2.10.2**) using the Papain method. As the animal model used is a retinal degeneration model, DRAQ5 was added to each sample prior to FACS analysis; this was to ensure that only live and healthy cells were obtained. An uninjected hetPP347S

mouse was used to establish baseline gating parameters for both EGFP and DRAQ-5. FACS analysis was undertaken on these cells and the highest possible number of healthy EGFP positive retinal cells was obtained and the RNA was isolated (**Section 2.4.5 & Section 4.2.10.3**).

5.2.10 Real-time RT-PCR analysis of FACS sorted hetPP347S retinal RNA

As so few EGFP positive cells were obtained for each retinal sample, a real-time RT-PCR was undertaken using half the RNA from each sample isolated (a 2X dilution in nuclease free H₂O). A 10X standard curve was generated using mRNA from an un-injected 3 week old hetPP347S mouse. The real-time RT-PCR was carried out (**Section 2.4.7**) using the P1 primers to amplify pRHO and the 18S primer set previously optimised (**Section 4.2.12**). The number of cycles in the qPCR reaction was increased to 50. Following the real-time RT-PCR, the amplification plots were analysed for both pRHO and 18S in order to ensure that all samples were amplified within the range of the standard curve samples.

5.2.11 Real-time RT-PCR analysis of RNA from excised EGFP positive portions of the hetPP347S mouse

As no pRHO could efficiently be amplified from FACS isolated RNA from hetPP347S mice injected with AAV-2/5.shP3 and AAV-2/5.NT20, it was decided to attempt extracting RNA by excising the EGFP positive regions of each retina using a sterile blade as described previously (**Section 4.2.17**). This was undertaken by Dr. Arpad Palfi. Mice were injected with AAV-2/5.NT20 and AAV-2/5.shP3 and two weeks post-injection the EGFP positive portion of each retina was placed in 350µl buffer RLT (containing 3.5µl β-mercaptoethanol) and pipetted up and down until the retinal cells were dissociated. The RNA was isolated (**Section 2.4.5 & Section 2.4.6**) and the RNA concentration determined (**Section 2.2.1**). Retinal RNA from an uninjected 3 week hetPP347S (PP347S UI) was used to generate the 5X standard curve for pRHO and 18S. All samples to be tested were diluted to 0.01ng and two master mixes were made; one for pRHO using the P1 primer pair and the other for the 18S primer pair. The real-time RT-PCR was undertaken (**Section 2.4.7**) and the results analysed. All samples in which pRHO was amplified were checked to confirm they fell within the range of the pRHO standard curve. Following this, each sample was compared to that of the pRHO or the 18S standard curves and a relative value for pRHO and 18S expression was obtained in each. pRHO expression was normalised to 18S. pRHO expression from retinal RNA samples of mice injected with AAV-2/5.NT20 was set at 100% and pRHO expression from retinal RNA samples of mice injected with AAV-2/5.shP3 was compared to this value.

5.2.12 Real-time RT-PCR analysis of pRHO RNA expression in hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shBB

AAV-2/5.shBB has previously been established in the laboratory as a potent suppressor of hRHO *in vivo* in NHR mice (O'Reilly et al, 2007). As the target site for shBB was 100% homologous to pRHO it was decided to evaluate shBB mediated suppression of pRHO *in vivo* in the hetPP347S mouse. Five mice were injected with AAV-2/5.NT20 and AAV-2/5.shBB (2×10^9 vp) and 2 weeks post-injection, the EGFP positive regions of each retina were excised. The real-time RT-PCR was undertaken by Dr. Sophia Millington-Ward. A 5X standard curve was generated using RNA from AAV-2/5.NT20 injected mice. Each RNA sample to be tested was diluted 1/600 in nuclease free H₂O. Two master mixes were made; one for pRHO using the P1 primers and the other for the 18S primers pair. The real-time RT-PCR was undertaken (Section 2.4.7) and the results analysed similar to Section 5.2.11.

5.2.13 Analysis of pRHO protein suppression by western blotting in hetPP347S animals injected with AAV-2/5.shP3 and AAV-2/5. NT20

In order to assess if suppression of pRHO RNA corresponds to a change in pRHO protein expression, it was decided to carry out a western blot. HetPP347S mice were subretinally injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE), 2 weeks post-injection the EGFP positive region of the retina was excised (Section 4.2.17), the protein was isolated (Section 2.5.2) and the concentration of each sample determined (Section 2.5.3). Western blotting was carried out using the 4D2 antibody (Hicks & Molday, 1986[1/1000 dilution]) and the anti-mouse light chain only secondary antibody (1/2000 dilution). As there was only a small amount of retinal tissue being analysed, the β -actin antibody and the anti-rabbit secondary antibody were used at a dilution of 1/500 & 1/1000 respectively. Longer exposure times than usual were used for β -actin, these were 10, 30 minutes and an overnight exposure. It was decided to also probe for EGFP in order to assess if this could be used as a measure of equal loading for AAV injected animals. The anti-rabbit EGFP and the anti-rabbit secondary antibody were each used at a dilution of 1 in 2000

5.2.14 Functional assessment of therapeutic benefit in hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3

Eleven hetPP347S mice (2 litters) were injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE) and ERGS were undertaken (Section 2.8.4) at 6 and 12 weeks in order to assess if any functional ERG change was taking place. Another 6 mice (1 litter) were injected with AAV-2/5NT20 (LE) and AAV-2/5.shP3 (RE) and ERGS were undertaken at 10 and 14 weeks. The rod-isolated, mixed rod-cone and

cone-isolated responses at each time-point were measured and the b-wave for each of these tests was combined and the average b-wave taken for each litter.

5.2.15 Immunohistochemical analysis of suppression of pRHO in hetPP347S mice

Retinal sections of injected hetPP347S mice were taken at 12 weeks following ERG analysis (**Section 5.2.14**). Sections from each mouse retina were taken and stained for rhodopsin (4D2, 1/100 dilution [Hicks & Molday, 1986]) to assess if there was a decrease in pRHO and mRHO in the EGFP positive sections injected with AAV-2/5.shP3 compared to the EGFP positive regions injected with AAV-2/5.NT20. DAPI staining was also undertaken in order to visualise the nuclei in the ONL. This protocol was undertaken according to **Section 2.8.5-2.8.8**.

5.2.16 Analysis of mRHO suppression by siP3

During the course of the analysis of shP3 suppression in the hetPP347S mouse, it appeared that siP3, despite having three mismatches to the mRHO target sequence, might target mRHO mRNA. It was decided to check this *in vitro* in cell culture. mRHO and siNT, siBB, siP2 or siP3 were co-transfected into HeLa cells (**Section 3.2.4**) and 24 hours later the RNA was extracted (**Section 2.4.3**). A real-time RT-PCR assay was performed to analyse mRHO RNA from HeLa cells co-transfected with mRHO and each siRNA (**Section 3.2.7**). mRHO mRNA expression in HeLa cells transfected with siNT was set at 100% and mRHO mRNA expression in HeLa cells transfected with each siRNA was compared to this value. siBB was used as the positive RNAi control in this instance as it is 100% homologous to mRHO.

5.2.17 Statistical analysis

The average and standard deviation of all ERG, real-time PCR and densitometry data sets were undertaken using Microsoft Excel. For experiments in which the hetPP347S mouse was characterised, two-sample t-tests were undertaken for each data set (ERG and western blotting) in order to obtain statistical significance. For the analysis of suppression in mice injected with AAV-2/5.NT20 versus AAV-2/5.shP3 paired t-tests were carried out on RNA and western blotting data sets. All statistical analysis was performed using Datadesk 6.0

5.3 Results

5.3.1 Genotyping the PP347S mice

Initially homozygous mice (PP347S^{+/+}129rho^{-/-}) were genotyped in order to confirm the porcine P347S (PP347S) transgene was present. A positive control, which was DNA from a known homozygous PP347S mouse, was used to confirm the genotyping PCR. A negative H₂O blank control was used to ensure the H₂O used was not contaminated. The P347S F targets the 3' region of exon 4 of pRHO and the PP347S R primer targets the 3'UTR of pRHO. This ensures amplification of a portion of exon 4, intron 4, exon 5 and 103bp of the 3'UTR such that positive bands of 1093bp confirmed the presence of the PP347S gene in the homozygous mice and the positive control. There were no bands present in the negative control sample (**Figure 5.3.1.a**). The homozygous PP347S mice were mated to wild-type 129^{+/+} mice and all the offspring were heterozygous PP347^{+/-}129rho^{+/-}.

5.3.2 ERG characterisation

ERGs were undertaken at 3 week, 16 week, 6 month, 9 month and 1 year time points on different hetPP347S mice. At least n=3 were used per time-point. For each mouse an ERG response (b-wave value) in the right eye (RE) and the left eye (LE) was obtained. For analysis of each mouse it was decided to use the eye (RE or LE) with the highest b-wave value. In a sub-optimal ERG (due for example to contact with the electrode) the response is going to decrease with respect to the correct value, thus it was felt that by taking the higher ERG value this would better represent the b-wave for this animal. ERGs at all time-points were compared to that of 129 wild-type mice (4 weeks, n = 3). A representative ERG from a wild-type mouse for rod-isolated, mixed rod-cone and cone isolated responses showing typical a-wave and b-wave values is presented (**Figure 5.3.2.a**). For all wild-type mice analysed there was little variance between mice and eyes. In all the hetPP347S mice variance in ERG responses were obtained, likely due to the fact that this model has a degenerating retina and degeneration was occurring at different rates depending on the mouse. A representative ERG from hetPP347S mice at 3 weeks is presented (**Figure 5.3.2.b**). A rod-isolated b-wave, a mixed-rod-cone and a cone-isolated b-wave was obtained, similar to the high wild-type values. The degeneration was more apparent at the 8 week time-point (**Figure 5.3.2.c**), with the b-waves and a-wave having decreased in the rod-isolated and mixed rod-cone responses from that observed in the wild-type and 3 week hetPP347S mice. The cone-isolated a-wave and b-wave ERG appeared unchanged. This pattern of decreasing rod-isolated and mixed rod-cone a-wave and b-wave continue at 12 (**Figure 5.3.2.d**) and 16 weeks (**Figure 5.3.2.e**) for hetPP347S mice. By 6 months (**Figure 5.3.2.f**) the a-wave and b-wave

amplitudes appeared severely reduced for rod-isolated and mixed rod-cone responses, the cone-isolated responses also appear reduced. This reduction in all three ERG responses was observed at 9 months (**Figure 5.3.2.g**) until finally at 1 year the a-wave and b-waves appear flat (**Figure 5.3.2.h**).

Rod-isolated ERG responses for all animals at all time-points were combined and the average b-wave at each time-point obtained. The wild-type rod-isolated b-wave was set at 100% and all of the hetPP347S rod-isolated ERG responses were compared to this value (**Figure 5.3.2.i**). The hetPP347S rod-isolated b-wave at 3 weeks was of similar levels to wild-type ($75\pm19\%$, [$P=0.1502$]), by 8 weeks only a $35\pm14\%$ b-wave response remained compared to wild-type ($P<0.0001$). A modest b-wave decrease was observed between 12 ($31\pm11\%$ [$P<0.01$]) and 16 weeks ($25\pm6\%$, [$P<0.0001$]). By 6 months only $7.5\pm4.8\%$ ($P<0.0001$) b-wave function remained and this decreased further at 9 months ($3\pm3.5\%$, [$P<0.0001$]) until at 1 year no rod-isolated b-wave remained ($P<0.0001$).

The average mixed-rod b-wave responses for each time-point compared to the wild-type followed a similar pattern to that observed for rod-isolated responses (**Figure 5.3.2.j**). The hetPP347S mice at 3 weeks had a b-wave response statistically similar to the wild-type b-wave ($87\pm18\%$, [$P=0.3$]). At 8 weeks only 41% of the mixed rod-cone response remained compared wild-type mice ($P<0.0001$). This significant reduction in mixed rod-cone responses was observed at 12 ($52\pm18\%$, [$P<0.05$]) and 16 weeks ($30\pm6\%$, [$P<0.0001$]) in transgenic mice compared to wild-type controls. No statistical significance was observed between the 8, 12 and 16 week time-points. By 6 months only $9\pm6\%$, $P<0.0001$ of the ERG response remained and continued to decrease at 9 months ($3\pm3\%$, [$P<0.0001$]) until at 1 year the mixed rod and cone b-wave ERG was $0.2\pm0.4\%$, $P<0.0005$.

The average cone-isolated b-wave response for each time-point compared to wild-type was different than observed for the rod-isolated and mixed rod-cone response (**Figure 5.3.2.k**), with the cone degeneration occurring much slower than the rod degeneration. At 3 weeks $98.9\pm15\%$ ($P=0.9$) of the hetPP347S cone-isolated b-wave compared to wild-type mice remained. At 8 weeks $71\pm26.5\%$ ($P<0.05$) of the b-wave function remained. Surprisingly at 12 weeks $97\pm18.5\%$ remained, $P=0.8$. The reason for this discrepancy was most likely due to the large standard deviation obtained with the 8 week time-point. By 16 weeks $75\pm12\%$ of the b-wave cone function was present ($P<0.05$). At 6 months a sharp decrease to $12\pm8\%$ in the cone-isolated response was observed ($P<0.0001$). By 9 months $9\pm5\%$ remained ($P<0.0001$) and by 1 year only $1.6\pm1\%$ of a cone-only b-wave amplitude was present ($P<0.0001$).

5.3.3 Optimising of 4D2 for western blotting of pRHO

To evaluate the hetPP347S mouse at the protein level, an efficient antibody would be needed that reacted with pRHO. It was decided to examine if the RHO 4D2 antibody could react with pRHO. The hetPP347S mouse has 1 mouse allele and thus by using the 4D2 antibody (which definitely reacts with mRHO) in this model it would be impossible to assess if 4D2 was reacting with pRHO. Therefore retinal protein from a pig was obtained and the 4D2 antibody was tested with this sample (**Figure 5.3.3.a**). Rhodopsin staining was present in protein samples from porcine retinas. A band at 39kDA represented the pRHO monomer. Bands of higher molecular weight were also observed representing the RHO dimers and trimers. It was decided to assess if the anti-rabbit β -actin antibody used in the laboratory was reactive to pig β -actin for use in future studies. β -actin was present at 42kDA. Thus when this study eventually proceeds to the porcine animal model both the 4D2 and β -actin antibodies will be available for use.

5.3.4 Decrease in RHO protein expression in the hetPP347S mouse over time

An evaluation of the decrease of total RHO protein in the retinas of the hetPP347S mice at different time-points was undertaken. As the retina degenerates and the OS begin to die, RHO protein expression should decrease. It was decided to indirectly assess the degeneration of the OS by examining the decline in RHO protein. The 4D2 antibody reacts with both pRHO (**Section 5.3.3**) and mRHO, therefore any decrease observed is that of the total RHO protein expression within the retina. The time-points of 3 week, 16 week, 6 month, 9 month and 1 year were chosen for analysis. Later time-points were selected as this was proposed to be when the rods would have severely degenerated and thus these time-points should have the least amount of RHO. Not all time-points could fit on the one western gel so each western was carried out in two parts. The first was protein from hetPP347S mice at 3 weeks, 16 weeks and 6 months (**Figure 5.3.4.a1**) together with a positive (NHR rhodopsin retinal protein) and negative control (129rho^{-/-} retinal protein). In all 3 week hetPP347S samples had RHO monomers at 39kDA in addition to the higher molecular weight forms of RHO, this pattern of expression was similar to that obtained for wild-type RHO (lane 9). At 16 weeks, RHO monomers were present with less intensity than at 3 weeks. There were no higher molecular weight forms of RHO. Faint monomers of RHO were present at 6 months and there were no higher molecular weight forms. Mouse β -actin was used to assess equal loading. As these hetPP347S retinas were degenerating it was difficult to obtain equal loading, therefore densitometry was also performed (**Figure 5.3.4.b1**). As no rhodopsin was present in the negative control 129rho^{-/-} sample this was used as the experimental blank sample. The pixel intensity value obtained for RHO in the 129rho^{-/-} sample for each western was

subsequently subtracted from the RHO pixel intensities obtained from all other samples of the same western blot. All time-points were compared to the average normalised RHO pixel intensity expression in the 3 week hetPP347 mice which was set at 100%. RHO protein was expressed at $40\pm 11\%$, $P=0.07$ in the 16 week samples and at $5\pm 2\%$, $P<0.0005$ at 6 months compared to the 3 week time-point. In the second set of westerns one sample from a 3 week hetPP347S was used, to act as a comparison between westerns. RHO protein expression from 6 month, 9 month and 1 year old mice was examined (**Figure 5.3.4.a2**). The 6 month samples were included to ensure the same RHO expression in these samples compared to the 3 week sample was similar to previously obtained with the first set of western blots. In the 3 week sample a RHO monomer at 39 kDa was observed, as was a band at 80kDa, representing RHO dimers. Only faint RHO monomers were visible in the 6 month sample, which was similar to that obtained in the first set of westerns. No RHO bands were visible at 9 months or 1 year and this was comparable to the 129rho^{-/-} sample. It was very difficult to obtain β -actin at the later time-points due to the degenerating retina. Thus an overnight exposure was needed; this had the disadvantage of overexposing some of the earlier time-point β -actin bands (**Figure 5.3.3.a2 [3f]**). Densitometry was performed to evaluate the % RHO expression of each timepoint compared to the 3 week hetPP347S sample (**Figure 5.3.4.b2**). RHO was expressed to $7\pm 5\%$, $P<0.005$ at the 6 month hetPP347S time-point compared to the 3 week time-point and this was similar to that obtained in the first set of westerns. At 9 months the majority of RHO protein expression was gone from the retina ($0\pm 1\%$, $P<0.0001$) and by 1 year no RHO protein remains as assessed by western blotting ($0\pm 1\%$, $P<0.0001$) compared to the 3 week time-point respectively. No significant difference was obtained between the 6 and 9 month time-points ($P=0.1679$) or the 6 month and 1 year time-points ($P=0.1658$), similarly no significant difference in RHO expression was occurring between the 9 month and 1 year time-points ($P=0.8138$). Thus it can be concluded that as the hetPP347S retina degenerates, RHO levels begin to reduce to the extent that by 6 months little RHO protein is remaining.

5.3.5 RHO staining of retinal sections from the hetPP347S mouse at different time-points

Retinal sections from hetPP347S mice were stained for RHO and DAPI at 3 week, 16 week, 6 month, 9 month and 1 year time-points compared to a control retina. This was undertaken to assess the staining patterns of RHO in this RHO-adRP model (**Figure 5.3.5.a & Figure 5.3.5.b**). A unique RHO staining pattern was present in hetPP347S mice. RHO appeared predominantly in the ONL, IS and OS in all sections (A). At 3 weeks to 6 months this RHO mislocalisation was also observed in the OPL (2A, 3A & 4A). In contrast in the control mouse, RHO (1A) was typically localised to the OS. At three weeks the retina was heavily stained for RHO (2A) but by 16 weeks (3A & 3B) both RHO and DAPI

staining have decreased. By 6 months (4B) only 4-5 rows of DAPI stained nuclei remained and this corresponded to a significant decrease in RHO expression (4A). By 9 months (5B) only 1-2 rows of nuclei were visible in the ONL and this correlated to a very small amount of RHO staining (5A). By 1 year the ONL had completely degenerated (6B) and no RHO staining was visible (6A).

5.3.6 Lectin-PNA staining of retinal sections from the hetPP347S mouse at different time-points

In the retina, PNA lectin protein is specific to the cone photoreceptor cells and the cone domains in the interphotoreceptor matrix; therefore it was an ideal antibody for charting the degeneration of cone cells in the hetPP347S model at different time-points (**Figure 5.3.6.b & Figure 5.3.6.b**). At 3 (2A & 2B) and 16 (3A & 3B) weeks, efficient staining of the cone cells was clearly observed and this was comparable to the cone staining pattern observed in the control eye (1A & 1B). By 6 months (4A & 4B) there were fewer, shorter cones. This corresponded to the point at which a significant decrease in the cone-isolated ERG was observed. This cone cell degeneration continued at 9 months (5A & 5B) when only 1-2 nuclei rows remained. At 1 year (6A & 6B) no ONL cell nuclei were present and staining for cones was completely abolished. Thus cones in hetPP347S mice appear relatively similar to control mice until 6 months when a significant decline in number and morphology was observed.

5.3.7 Optimisation of primers for the amplification of pRHO

To assess suppression of pRHO by AAV-2/5.shP3, primers that amplify pRHO and not wild-type mRHO were designed. Three primer pairs were generated that contained mismatches to mRHO but were 100% homologous to pRHO (**Figure 5.3.7.a**). The first primer set was P1, the P1 forward primer (P1 forward) had 6 mismatches to mRHO, located all along the primer. The P1 reverse primer (P1 reverse) also had 6 mismatches to mRHO, 4 of these mismatches were within the last 4 nucleotides of the 3' end of the primer. The second primer set (P2), the P2 forward primer (P2 forward) was the P1 reverse primer but in the opposite orientation, the P2 reverse primer (P2 reverse) had 2 mismatches located on the 3' end of the primer. The last primer set designed was the P3 set, the P3 forward primer (P3 forward) contained 4 mismatches to mRHO, situated all along the primer. The P3 reverse primer contained 6 mismatches to mRHO, again these were located all along the primer (**Figure 5.2.2.a**).

A real-time RT-PCR was carried out with each primer set on RNA from the hetPP347S mouse and on RNA from a 129 wild-type mouse to ensure that pRHO was specifically and efficiently amplified but not mRHO. All primers amplified pRHO and the standard curve and melting temperature analysis for each set were examined similar to previously described (**Section 3.3.4**). The P1 set of primers was the most efficient at amplifying pRHO with a slope of -3.3 and an R^2 value of 0.99. Additionally the P1

primers had a similar T_m to that predicted (84°C) and the correct sized amplification product of 241bp was also obtained. None of the primers amplified mRHO significantly, a small level of unspecific amplification was detected with the P1 primers but this was lower than the water blank. It was decided to utilise the P1 primer for future experiments as this set had the most efficient standard curve.

5.3.8 Optimisation of FACS for the hetPP347S mouse

As the retina of the hetPP347S mouse was degenerating, it was unknown if FACS analysis could be undertaken in order to isolate sufficient EGFP positive cells for subsequent analysis. Six mice were injected with AAV-2/5.NT20 and AAV-2/5.shP3 at P10 and 3 weeks post-injection dissociated retinal cells underwent FACS analysis. This time-point was chosen in order to allow sufficient time for EGFP expression from the AAV-2/5.NT20 vector. DRAQ-5 was used in these samples in order to distinguish the unhealthy or dead cells from the viable EGFP positive cells, as DRAQ-5 only binds to live cells. An uninjected hetPP347S control was used to establish the baseline parameters of DRAQ-5 in the absence of EGFP fluorescence (**Figure 5.3.8.a**). High levels of dead cells were present in this sample, this was not surprising given that the retina in this mouse was degenerating and undergoing cell death. Analysis of the gate for EGFP positive cells (**Figure 5.3.8.b**) in this mouse revealed that as expected only DRAQ-5 positive, EGFP negative cells could be obtained. A hetPP347S mouse injected with AAV-2/5.NT20 was used to assess the presence of DRAQ-5 positive cells in an injected EGFP positive retina (**Figure 5.3.8.c**). A large number of dead cells similar to that observed in **Figure 5.3.8.a** was present in this sample, of the DRAQ-5 positive healthy cells, EGFP positive cells were clearly distinguished from the EGFP negative, DRAQ-5 positive cell population (**Figure 5.3.8.d**).

The EGFP positive cell numbers obtained with hetPP347S mice were between 2,500-29,000 cells (**Table 5.3.8**). This was substantially lower than previous cell numbers obtained in the NHR mouse using the same FACS protocol (**Section 4.3.10**). The average % EGFP positive cells obtained from the hetPP347S mice injected with AAV-2/5.NT20 was 8% compared to a 14.5% average obtained from AAV-2/5.shP3 injected eyes. If shP3 was suppressing mutant pRHO, the AAV-2/5.shP3 injected eyes may be protected from degeneration, thus a higher % of EGFP positive cells would be expected in these eyes. In terms of the mean fluorescence intensity, an average intensity of 5334MFI was obtained for AAV-2/5.NT20 injected eyes and an average intensity of 5553MFI for AAV-2/5.shP3 injected eyes. Therefore in this hetPP347S model both AAV-2/5.NT20 and AAV-2/5.shP3 were expressing EGFP to comparable levels. Using this FACS protocol in the NHR mouse, AAV-2/5.NT20 and AAV-2/5.shP3 were expressed to comparable levels in the mice that underwent FACS at 14 days. At 1 month however, the AAV-2/5.shP3 EGFP expression was much higher in the NHR mouse than AAV-

2/5.NT20. The reasons for these differences were most likely attributable to the different post-injection time-points used and the different genotype of the mice (healthy retina versus degenerating retina).

5.3.9 Real-time RT-PCR analysis of pRHO from FACS isolated RNA in the injected hetPP347S mouse

As such low cell numbers were obtained from the hetPP347S mouse by FACS it was unknown if adequate pRHO RNA could be obtained for real-time RT-PCR analysis. 50% of the RNA extracted from the EGFP positive cells was used for the real-time RT-PCR with the P1 primers. High 18S amplification in all samples was obtained as expected, however only low pRHO expression was obtained within each sample compared to 18S. This amplification was not sufficient to assess suppression of pRHO in AAV-2/5.shP3 versus AAV-2/5.NT20 injected retinas as it was below the pRHO standard curve samples. Thus FACS-isolated RNA from hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 was precluded from further analysis.

5.3.10 Real-time RT-PCR analysis of pRHO mRNA expression from EGFP positive cells excised from the retinas of the hetPP347S mice injected with NT20 & shP3

As too little pRHO RNA was obtained from EGFP positive retinal cells that had undergone FACS, it was decided to physically excise the EGFP positive region of each injected hetPP347S retina. HetPP347S mice were subretinally injected with AAV-2/5.NT20 and AAV-2/5.shP3 and 14 days post-injection (the same time-point used for analysis of shP3 in the NHRs), the EGFP portion of the retina was taken. Compared to the NHR mice, the EGFP portion of the retina was much smaller in the hetPP347S mouse. This was not surprising given that this is a model with retinal degeneration. Furthermore, AAV-2/5.NT20 was expressing at lower levels than the AAV-2/5.shP3 vector. Real-time RT-PCR analysis was carried out on RNA from the EGFP positive regions of each mouse retina using the P1 primer set to specifically amplify pRHO and the 18S primer set to amplify mouse 18S. pRHO expression in the hetPP347S mice injected with AAV-2/5.shP3 was $33 \pm 18\%$ compared to AAV-2/5.NT20 (**Figure 5.3.10.a**). As each animal had a retinal degeneration, and thus would degenerate with some variability, a paired t-test was undertaken to analyse the statistical significance between AAV-2/5.NT20 and AAV-2/5.shP3 injected eyes and significant suppression with shP3 was established ($P < 0.05$).

5.3.11 Real-time RT-PCR analysis of pRHO mRNA expression from EGFP positive cells excised from the retinas of the hetPP347S mice injected with NT20 and shBB

A previous shRNA successfully designed in the laboratory to suppress hRHO expression *in vitro* and *in vivo* was shBB; additionally therapeutic efficacy has been demonstrated with this shRNA in the HP347S mouse model (O'Reilly et al, 2007; Millington-Ward et al, 2011). shBB is also 100% homologous to pRHO and therefore it was decided to establish if potent pRHO RNA suppression with shBB could be obtained. 4 hetPP347S mice were subretinally injected with AAV-2/5.NT20 (LE) and AAV-2/5.shBB (RE). 14 days post-injection, RNA from EGFP positive regions of the retina was extracted and pRHO RNA expression was analysed by real-time RT-PCR identical to **Section 5.3.10**. This real-time RT-PCR analysis was undertaken by Dr. Sophia Millington-Ward. AAV-2/5.shBB suppressed pRHO to $33 \pm 15\%$, $P < 0.01$ compared to AAV-2/5.NT20 (**Figure 5.3.11.a**). Analysis of pRHO suppression by AAV-2/5.shP3 compared to AAV-2/5.shBB established that there was no statistical difference between pRHO suppression by AAV-2/5.shBB or AAV-2/5.shP3 ($P = 0.2937$).

5.3.12 RHO protein expression in hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 as assessed by western blotting

Suppression of pRHO RNA by shP3 should correlate with suppression of pRHO protein. To assess if this was the case, western blotting was undertaken. The 4D2 antibody reacts with both mRHO and pRHO in the hetPP347S mouse, therefore analysis of specific pRHO protein expression could not be undertaken. However, if sufficient pRHO suppression was occurring then a decrease in overall RHO content of each retina should be observed. HetPP347S mice were injected with AAV-2/5.NT20 and AAV-2/5.shP3 and 14 days post-injection protein was extracted from the EGFP positive portion of each retina. A western blot was carried out (**Figure 5.3.12.a**) using the 4D2 antibody (Hicks & Molday, 1986). In protein samples from AAV-2/5.NT20 injected eyes, RHO was visible at 39kDa (all three AAV-2/5.NT20 samples) and at 80kDa (for 1NT [lane 1] and 2NT [lane 3]). For protein samples from two of the AAV-2/5.shP3 injected eyes (1shP3 [lane 2] and 3shP3 [lane 6]), fainter RHO monomers were visible compared to AAV-2/5.NT20, no multimeric RHO was observed. One AAV-2/5.shP3 injected sample (2shP3 [lane 4]) had no RHO protein similar to that observed in the 129rho^{-/-} negative control (lane 7). Mouse β -actin was evident, however equal loading was somewhat difficult to obtain as each retina was degenerating at different rates and thus the level of β -actin protein in each retina may be different. It was decided to determine if EGFP expression could be analysed as a marker of equal loading. An anti-rabbit EGFP antibody was used to probe for EGFP protein; however EGFP protein expression was higher in AAV-2/5.shP3-injected samples than in AAV-2/5.NT20 injected

samples. This is in accordance with the observation that AAV-2/5.NT20 produces less EGFP than AAV-2/5.shP3. As equal loading was not clearly obtained, densitometry was carried out (**Figure 5.3.12.b**). AAV-2/5.shP3 suppressed RHO protein to $56\pm0.2\%$ compared to RHO expression in AAV-2/5.NT20 injected eyes ($P<0.05$).

5.3.13. Functional analysis of hetPP347S mice injected with NT20 and shP3

Three litters of hetPP347S mice were injected with AAV-2/5.NT20 and AAV-2/5.shP3 to evaluate if significant suppression of pRHO RNA and protein corresponded to a significant change in ERG response. If the mutant dominant allele was being suppressed to sufficient levels, one would expect to see an improvement in function due to the presence of the RNAi resistant endogenous mouse allele. Two litters of mice underwent ERG analysis at 6 and 12 weeks and one litter underwent ERG at 10 and 14 weeks. In the two litters in which ERG analyses was undertaken, at 6 and 12 weeks, it appeared that the litters gave different results. This was surprising given the mice are of the same genotype and have been bred on a pure background for many generations. The average litter 1 rod-isolated, mixed rod-cone and cone-isolated b-wave responses appeared lower in the AAV-2/5.shP3 injected eyes than in the AAV-2/5.NT20 injected eyes at both 6 (**Figure 5.3.13.a**) and 12 weeks (**Figure 5.3.13.b**) suggesting that suppression of retinal function was taking place. However in litter 2 the average b-waves for rod-isolated, mixed rod-cone and cone-isolated responses appeared higher in AAV-2/5.shP3 injected eyes than in AAV-2/5.NT20 injected eyes at 6 (**Figure 5.3.13.c**) and 12 weeks (**Figure 5.3.13.d**). Rod-isolated responses at 6 and 12 weeks were combined for each litter and the average b-wave response was obtained for each. The average b-wave value for AAV-2/5.NT20 injected eyes was set at 100% and the average b-wave value for mice injected with AAV-2/5.shP3 was expressed as a percentage of AAV-2/5.NT20. The litter 1 b-wave response in AAV-2/5.shP3 injected eyes was reduced to $80\pm13\%$ of the AAV-2/5.NT20 injected b-value at 6 and 12 weeks was reduced to $84\pm27\%$ of the AAV-2/5.NT20 injected b-value (**Figure 5.3.13.e[a]**). However AAV-2/5.shP3 does not appear to suppress rod-isolated ERG function significantly in AAV-2/5.shP3 injected eyes with respect to AAV-2/5.NT20 injected eyes at either time-point ($P=0.1959$ and $P=0.1638$) indicating no significant ERG change was recorded in this litter with AAV-2/5.shP3. There was significant differences between the AAV-2/5.NT20 injected eyes at 6 and 12 weeks ($P<0.05$) and also between the shP3 injected eyes at these time points ($P<0.05$). This was not surprising given that the retinas were degenerating. In litter 2 the average rod-isolated b-wave increased to $132\pm24\%$ in AAV-2/5.shP3 injected eyes compared to AAV-2/5.NT20 injected eyes at 6 weeks and was $115\pm29\%$ at 12 weeks (**Figure 5.3.13.f[a]**). In terms of statistical significance the 32% increase of rod function in AAV-2/5.shP3 injected eyes was significant

when compared to the b-wave in AAV-2/5.NT20 injected eyes at 6 ($P<0.05$) but not at 12 weeks ($P=0.2205$). There was no significant difference between AAV-2/5.NT20 injected eyes at different time-points ($P=0.2736$) but there was between AAV-2/5.shP3 injected eyes at the different time-points ($P<0.01$). This was likely a function of the degeneration or of the rate of recovery from injection. In terms of the mixed rod-cone-response, in litter 1 the average b-wave value was $75\pm7\%$ in AAV-2/5.shP3 injected eyes compared to AAV-2/5.NT20 at 6 weeks and was $79\pm11\%$ at 12 weeks (**Figure 5.3.13.e[b]**). Similar to the rod-isolated response this decrease was not significant at either time-point ($P=0.1053$ and $P=0.1010$). There was no significant decrease observed for each eye injected with AAV-2/5.NT20 at the different time-points ($P=0.06$) but there was for AAV-2/5.shP3-injected eyes ($P<0.05$). For litter 2, the average b-wave response in AAV-2/5.shP3 injected eyes was increased to $135\pm26\%$ compared to AAV-2/5.NT20 injected eyes and to $113\pm28\%$ at 12 weeks (**Figure 5.3.13.f[b]**). Unlike the rod-isolated response the result at 6 or 12 weeks was not significant ($P=0.0701$ and $P=0.3713$). Similar to the rod-isolated response no significant difference was obtained in AAV-2/5.NT20 eyes at the different time-points ($P=0.4934$). The AAV-2/5.shP3 injected ERG function between 6 and 12 weeks was significantly different ($P<0.01$). At 6 weeks the cone-isolated response (**Figure 5.3.13.e[c]**) for litter 1 was reduced to $73\pm10\%$ with AAV-2/5.shP3 compared to the AAV-2/5.NT20, this was not significant ($P=0.2742$) and at 12 weeks was $84\pm19\%$ ($P=0.1146$). There was no significant difference in AAV-2/5.NT20 ERG responses or AAV-2/5.shP3 ERG responses at the two different time-points ($P=0.5318$ and $P=0.8122$). In litter 2 the cone-isolated b-wave at 6 weeks in AAV-2/5.shP3 injected eyes significantly increased to $151\pm32\%$ ($P<0.05$) compared to AAV-2/5.NT20 injected eyes and at 12 weeks had increased to $119\pm30\%$ in AAV-2/5.shP3 injected eyes compared to AAV-2/5.NT20 eyes (**Figure 5.3.13.f[c]**), this later increase was not significant ($P=0.369$). There was no difference between AAV-2/5.NT20 ERG responses or AAV-2/5.shP3 ERG responses at 6 or 12 weeks ($P=0.4375$ and $P=0.4966$). For litter 1, it appeared that despite a slight decrease in ERG function, overall no significant change was apparent for all three ERG tests undertaken, in contrast some significant changes were observed for litter 2. Litter 3 was analysed at 10 (**Figure 5.3.13.g**) and 14 weeks respectively (**Figure 5.3.13.h**). When AAV-2/5.shP3 injected eyes were compared to AAV-2/5.NT20 injected eyes the rod-isolated response was reduced to $95\pm25\%$ ($P=0.6970$) at 10 weeks and to $70\pm15\%$ ($P<0.05$) at 14 weeks (**Figure 5.3.13.i[a]**). There was a significant difference in AAV-2/5.NT20 for each time-point ($P<0.01$). There was however no significance between ERG response with AAV-2/5.shP3 at different time-points ($P=0.6418$). The mixed rod-cone isolated response was reduced to $91\pm20\%$ and $63\pm10\%$ at 10 and 14 weeks respectively (**Figure 5.3.13.i[b]**). Similar to the rod-isolated response this was not significant at 10

weeks ($P = 0.4926$) but was at 14 weeks ($P < 0.01$). Again as with the rod-isolated response the ERG responses in AAV-2/5.NT20 injected eyes at the two different time-points assessed were significantly different ($P < 0.005$) but not in the AAV-2/5.shP3 injected eyes between time points ($P = 0.099$). The cone isolated response at 10 and 14 weeks was reduced to $87 \pm 29\%$ and $63 \pm 12\%$ respectively (**Figure 5.3.13.i [c]**). This was not significant at 10 weeks ($P = 0.5417$) but was at the later time-point of 14 weeks ($P < 0.05$). Both the AAV-2/5.NT20 and AAV-2/5.shP3 injected eyes between time-points had a significant change in ERG response ($P < 0.01$ & $P < 0.05$). These three litters gave conflicting results; on one hand with litter 1, no significant change had occurred in all ERG tests performed suggesting that no therapeutic benefit was derived. On the other hand in litter 2 increases in ERG function at all time-points was observed. This was however only significant for the rod-isolated and cone-isolated b-wave responses at 6 weeks. Statistical analysis using a two sample t-test was performed on the ERG responses from AAV-2/5.NT20 injected eyes at 6 and 12 weeks between litter 1 and 2 and from AAV-2/5.shP3 injected eyes at the same time-points for all three ERG tests. Surprisingly a significance difference between the two litters was only observed between the two AAV-2/5.NT20 injected eyes examined at 6 weeks and only for the rod-isolated response ($P < 0.05$). No other significance differences between the two litters were observed. Thus it appears that overall there was no significant difference in ERG function between AAV-2/5.NT20 or AAV-2/5.shP3 injected eyes or between each litter. The differences observed with litter 2 in which there was a significant difference in the rod-isolated and cone-isolated b-wave response between AAV-2/5.NT20 and AAV-2/5.shP3 was likely attributable to differences in injection or animals. As a modest decrease in ERG function, while not significant, was observed for litter 1 one explanation for the data could be that in addition to pRHO, AAV-2/5.shP3 may also suppress mRHO (despite 3 mismatches). If this was the case, then the decrease in levels of mRHO in the region of viral transduction may counteract the expected therapeutic efficacy achieved by suppressing pRHO. In Litter 3, significant differences in ERG function only occurred at 14 weeks and not 10 weeks. This suggested that timing of analysis may be important. In future, long time points may be needed to fully assess suppression in this model. Additionally use of larger n numbers of mice would also be valuable.

5.3.14. Immunohistochemistry of hetPP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3

It was decided to stain retinal sections for RHO in mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 that had undergone ERG at 12 weeks. Eyes from both litter 1 and 2 were analysed at 12 weeks (**Figure 5.3.14.a**). The diagram represents the retina in which the best result was achieved. In all eyes examined, approximately 3-4 slides out of 13 were EGFP positive, in comparison to the 6-7

EGFP positive slides usually obtained from injected NHR eyes. This indicated that in this degenerated transgenic model the viral spread may not have been as extensive as wild-type. In AAV-2/5.NT20 injected retinas, the EGFP expression was often low and difficult to visualise. In the AAV-2/5.NT20 injected eyes, RHO staining (1A) in EGFP positive regions (2A) was comparable to that of RHO (1B) staining in the EGFP negative region of the same retina (2B). In the AAV-2/5.shP3 injected retinas RHO staining (1C) in the EGFP negative region (2C) was comparable to that of the AAV-2/5.NT20 eye i.e. RHO was expressed in the OPL, ONL IS and OS. In contrast, in the EGFP positive region of the same shP3 injected retina (2D), RHO staining was reduced and was mostly confined to the OS (1D). This suggested that AAV-2/5.shP3 was efficiently suppressing RHO protein at the histological level. Interestingly little if any RHO staining remains in the ONL and IS, which are the regions in which RHO was found to be ectopically expressed in the hetPP347S mouse suggesting that RHO, when expressed at lower levels (due to the presence of AAV-2/5.shP3), was not mislocalised (at least to some extent). Despite the differences in ERG between litter 1 and litter 2, a similar RHO staining pattern was observed.

5.3.15 Analysis of mRHO mRNA suppression by siP3 *in vitro*

There are three mismatches between the siP3 target site and mRHO, however the ERG and immunohistochemistry results from mice injected with AAV2/5.NT20 and AAV-2/5.shP3 indicated that in addition to pRHO, mRHO may also be suppressed. Hence it was decided to examine at the RNA level if siP3 was capable of targeting and suppressing mRHO. As with previous co-transfection experiments a mouse mRHO plasmid (provided by Dr. Sophie Kiang), was co-transfected along with siNT, siBB, siP2 or siP3 into HeLa cells. The real-time RT-PCR result is shown in (**Figure. 5.3.15. a**). siBB suppressed mRHO to 13.2% which was similar to that achieved with hRHO and siBB (**Section 3.3.5**). siP2 suppressed mRHO to 17.4%, again this was similar to the level of suppression achieved for hRHO and siP2 (**Section 3.3.5**). siP3 suppressed mRHO to 53.7% which was somewhat surprising given the presence of three mismatches at the siP3 target site in mRHO. It should be noted that this transfection experiment was only carried out once, however the values obtained for suppression by siBB and siP2 were in accordance with suppression values previously obtained from hRHO analysis. While this result should be repeated, overall the *in vitro* results in HeLa cells suggest that siP3 may suppress mRHO (to some extent). Whether this *in vitro* result in HeLa cells translated to suppression *in vivo* using subretinally injected AAV-shP3 would need to be evaluated.

5.4 Discussion

Previous analysis of therapeutic benefit in the laboratory in RHO-adRP mouse models have utilised the P23H mouse model (Ollson et al, 1992) or the HP347S mouse (Li et al, 1996). However, despite the fact that efficacy of a suppression and replacement strategy for RHO-adRP was observed using a histological read out in the P32H mouse, ERG benefit could not be evaluated due to the speed of the retinal degeneration observed in these mice (Ollson et al, 1992; O'Reilly et al 2007). This problem has since been circumvented by utilising the HP347S model, which presents with a slower retinal degeneration. Photoreceptor cell death is not complete until 4-5 months, which increases the therapeutic window for therapeutic intervention. This allowed therapeutic benefit to be analysed in terms of both histology and ERG analyses (Chadderton et al, 2009). In the study presented in this chapter it was decided to simultaneously examine RNAi-based suppression of pRHO by shP3 at a molecular level in addition to evaluating potential therapeutic benefit. This was undertaken using the PP347S model previously described by Wong and Hao, 1992. This mouse model was bred onto a heterozygous wild-type 129 background such that the transgenic mouse had one pRHO PP347S allele and one wild-type mRHO allele. In comparison, a transgenic model on a homozygous wild-type RHO background was used by Chadderton et al, 2009. This heterozygous genotype was chosen as it was felt it would more accurately mirror the genotype of human patients with RHO-adRP which have one mutant and one wild-type allele. Additionally, as siP3 has three mismatches over the siP3 target site in mRHO and should therefore not suppress mRHO, the presence of the wild-type mRHO allele in the PP347S model should act as a natural replacement gene and allow evaluation of therapeutic efficacy to be undertaken.

To evaluate shP3 in the hetPP347S transgenic mouse, the degeneration needed to be characterised in this model in order to establish the progression of the degeneration over time. The original transgenic PP347S mouse generated (Ser 6 line) was maintained on a wild-type C57BL/6 ^{+/+} background. Since obtaining these mice in 2004 the model has since been bred to homozygosity on a 129rho^{-/-} background (PP347S^{+/+} 129rho^{-/-}). Therefore it was felt that the degeneration of the hetPP347S^{+/+}129rho^{+/+} mice utilised in this study should be charted. Furthermore, to our knowledge ERG analysis characterising the degeneration of photoreceptor function overtime has not been reported previously for this transgenic PP347S mouse. A feature of RP is that the rods degenerate first and this has a secondary effect of causing cones to die. This cone photoreceptor degeneration happens late in the course of the retinal degeneration (Farrar et al, 2002). The rod and cone ERG function in the hetPP347S was consistent with this observation. At 3 weeks the rod-isolated response (**Figure 5.3.2.i**) and mixed rod-

cone (**Figure 5.3.2.j**) response was modestly lower but statistically similar to wild-type levels, however, a dramatic decrease in ERG function occurred between 3 to 8 weeks with only a third of the rod-isolated ERG function remaining at 8 weeks. This decrease in function remained relatively stable from 8 to 16 weeks, at 6 months less than 10% of rod-isolated ERG and mixed rod-cone response remained. By 1 year the ERG response was flat as degeneration of the rods was complete (**Figure 5.3.2.1 & Figure 5.3.2.j [1y]**). In contrast to the rod response and the mixed rod-cone response, reduction in the cone-isolated response was not apparent until 6 months when a decrease in cone-isolated ERG function to 12% was observed (**Figure 5.3.2.k**). This diminution in response continued until 1 year (the final time-point measured) when the average cone isolated b-wave had been reduced to less than 10%.

Western blot analysis was subsequently undertaken as it was proposed that as the rods degenerate, both levels of mutant and wild-type rhodopsin should theoretically be reduced. In principle this reduction in RHO immunoreactivity could also be used as a measure of the photoreceptor degeneration. Establishing the time-point at which very little RHO protein remains in photoreceptor cells was an important baseline for future studies in which western blot analysis of RHO suppression would be undertaken in mice injected with AAV-2/5.shP3. As no pRHO specific antibody was available, it was decided to optimise an antibody currently available in the laboratory for these studies. The 4D2 antibody (Hick & Molday, 1986) was found to react with pRHO extracted from a porcine retina and pRHO extracted from the hetPP347S mouse (**Figure 5.3.3.a**). This will be particularly useful when analysis of this therapy progresses into the porcine RHO-adRP model, as an antibody will already have been optimised for use in these studies. The pattern of RHO staining in the hetPP347S mouse was the same as that observed both in the original PP347S mouse generated by Weiss et al, 1995. This was consistent with the observation that mutant P347S RHO protein does not undergo any post-translational abnormalities (Weiss et al, 1995). In terms of the reduction in total RHO protein overtime, a significant decrease was observed between 3 and 16 weeks, however RHO depletion was particularly evident from 6 months (**Figure 5.3.3.a1 & 5.3.3.b1**). The level of rhodopsin present in all samples from 6 months to 1 year was comparable to that observed in a 129rho^{-/-} mouse (**Figure 5.3.3.a2 & 5.3.3.b2**). This indicated that by 6 months very little RHO containing rod photoreceptors must remain and this correlates well with the rod-isolated and mixed rod-cone responses in which less than 10% ERG function remains at this time (**Figure 5.3.2.1 & Figure 5.3.2.j**).

At all time-points examined in the hetPP347S retinal sections (identical time-points to the western blot analysis), RHO was localised not only to the OS but also strong RHO staining was visible in the IS,

ONL and OPL (**Figure 5.3.5.a**). While RHO staining in the ONL has been observed in wild-type mice up until approximately three weeks, at later time-points wild-type RHO is confined to OS (O'Reilly et al, 2008). Consistent with this staining pattern, in the original studies with this PP347S transgenic mouse on C57BL/6 ^{+/+} background (Weiss et al, 1995), RHO was localised to the IS in addition to the OS. No mention of RHO staining in the ONL was noted, however while the RHO localisation pattern in this paper was mentioned, no data to confirm the location of mutant RHO was presented (Weiss et al, 1995). In contrast, the original report of the transgenic HP347S mouse demonstrated mutant hRHO localisation to the OS with little staining in the ONL and IS (Li et al, 1996). The reason for the different staining pattern between these transgenic mice models (HP347S and PP347S) is unknown, however ultrastructure analysis did reveal the mislocalisation of RHO into ectopically expressed rhodopsin containing extracellular vesicles between the junction of the IS and OS (Li et al, 1996). This suggested that mislocalisation of RHO into these vesicles may possibly be involved in the degeneration. The aberrant staining pattern of RHO observed with the transgenic PP347S mice would be consistent with this hypothesis. It has now been established that mutations in the c-terminal domain of rhodopsin (including P347S) result in mislocalisation of RHO throughout photoreceptors, this c-terminal region appears to be vital for the interaction of rhodopsin with various proteins involved in the post-golgi transportation of RHO to the OS disc membranes (Deretic et al, 1998; Deretic et al, 2006, Mazelova et al, 2009). Interestingly a mutation in codon 334 of rhodopsin exists in which a stop codon (Q334ter) is generated; this results in the last five amino acids (including codon 347) of rhodopsin being deleted. Examination of transgenic mice with this mutation reveals that mutant RHO has a similar staining pattern to that observed in the hetPP347S mice used in this study (Concepcion & Chen et al, 2010; Lee & Flannery, 2007; Sung et al, 1994). In addition the transgenic porcine model with the P347L pig transgene that simulates RHO-adRP has a similar pattern of rhodopsin staining in the IS, ONL and synaptic terminals (Blackmon et al, 2000; Li et al, 1998). This further highlights the importance of the c-terminal amino acids of rhodopsin for adequate trafficking to the OS to confer function and confirms that the aberrant staining observed in hetPP347S transgenic mice is consistent with that reported in transgenic mice with other mutations in the c-terminal region of RHO.

At a histological level, hetPP347S mice (at the different time-points) presented with a moderate retinal degeneration, slower than that in transgenic HP347S mice. In the HP347S transgenic mouse utilised in previous studies in the TCD laboratory, rhodopsin staining was significantly reduced by 10 weeks with 1-2 ONL nuclei remaining. At 20 weeks no ONL nuclei remain and there was no rhodopsin staining. Rod-isolated ERG function at this time was completely abolished (Chadderton et al, 2009; Millingtonward et al, 2011). In contrast the ONL cell nuclei did not decrease to 1-2 rows until 9 months in the

hetPP347S retinas and furthermore this degeneration was only complete at 1 year (compared to 5 months in HP347S mice). In the original PP347S characterisation studies (Chang et al, 1993; Weiss & Hao et al, 1992), half the photoreceptor cell nuclei were abolished as early as 8 weeks (4-5 rows of cell nuclei remain in the hetPP347S mouse at 6 months) however the degeneration slowed pace and was complete by 1 year (Chang et al, 1993; Wang & Hao, 1992). The decrease in RHO over the time-course of this degeneration was most evident from 6 months onwards, which was consistent with the ERG and western results previously obtained in the study described in this thesis in which only a 10% and 6% reduction in function and RHO expression respectively was observed at this time-point.

It was decided to examine the degeneration of the cones at the histological level to ensure the change in cone-isolated ERG response observed at 6 months was consistent with a significant decrease in cone photoreceptors (**Figure 5.3.6.a**). Peanut Agglutinin protein (PNA) is a 110 kDA plant lectin protein which selectively binds to the inter photoreceptor matrix surrounding the cone inner and outer segments and thus allows visualisation of the cones in the mammalian retina (Blanks & Johnson et al, 1983; Johnson et al, 1986; Krishnamoorthy et al, 2008). No difference in PNA staining was observed at early time-points between the hetPP347S and wild-type control retinas and this was consistent with the cone-isolated ERG results for these time-points. However a significant reduction in PNA staining was clearly visible at 6 months, this corresponds to the point at which a significant decrease in the cone-isolated ERG function occurs. As with the ERG analysis, this PNA staining continued to decrease until 1 year when no staining was visible, corresponding to the end point of the retinal degeneration (**Figure 5.3.6.b**). This is consistent with prior findings in this transgenic mouse in which by 1 year the retinal degeneration was virtually complete (Weiss et al, 1995).

Having characterised the hetP347S transgenic mouse in terms of ERG, protein expression and histology the results suggested that the suppression strategy detailed in Chapter 4 may be amenable to evaluation in this mouse model given to the large window available for therapeutic intervention. Initially retinas from mice injected with AAV-2/5.NT20 and AAV-2/5.shP3 underwent FACS analysis in order to examine if sufficient EGFP positive cell numbers could be obtained for further analysis in this model using this method. However an insufficient number of EGFP positive cells for real-time RT-PCR analysis were collected from these injected mice (**Table 5.3.8.a**). Obtaining EGFP positive cells from the H347S mouse had also been difficult using FACS (Dr. Naomi Chadderton [personal communication]). There were several likely reasons for this observation: The mice were injected at a young age (P10) before the photoreceptors have completed development, as the eyes of these mice are smaller only 1µl of virus (instead of the 3µl usually administered) was subretinally injected into these

mice, therefore a lower number of cells were transduced. Additionally previous analysis has established that typically only 10-20% of the retina was transduced in the HP347S mouse compared to 30-40% observed in an aged and titre matched injected NHR mouse (Chadderton et al, 2009), thus the viral spread in the PP347S transgenic mouse was likely to be similar. The reduced viral spread and lower viral volume injected would clearly contribute to a lower percentage of EGFP positive cells. Moreover the AAV-2/5.NT20 vector has previously been shown in the laboratory to be inefficient at expressing EGFP. The retina of the PP347S mouse was degenerating thus more cell death was observed in these retinas, the retinal cells undergo dissociation prior to FACS and this process was likely to cause the loss of some cells from the retina as evidenced by the high number of DRAQ-5 negative cells in the hetPP347S retinal sample. While a loss of viable cells may be tolerated in the wild-type mouse retina which had significantly more EGFP positive cells to begin with, the death of cells in the hetPP347S may have a significant impact on the overall EGFP cell numbers obtained in this model. Therefore an altered protocol in which the EGFP positive regions of the retina were physically excised and the RNA immediately extracted was undertaken to avoid disrupting the delicate retinal cells. Using this protocol it was established that as expected both the viral spread and the expression of AAV-2/5.NT20 were lower in the hetPP347S mouse.

Real-time RT-PCR was undertaken on mice injected with AAV-2/5.NT20 and AAV-2/5.shP3. shP3 significantly reduced pRHO RNA by 67% in the hetPP347S transgenic mouse (**Figure 5.3.10.a**). This level of suppression was comparable to that obtained with shBB in the H347S model. Furthermore this level of hRHO suppression by shBB was sufficient to provide therapeutic benefit in the HP347S mouse when a replacement gene refractory to shBB mediated suppression was simultaneously delivered to this transgenic mouse (Millington-Ward et al, 2011). Therefore in the context of the hetPP347S transgenic mouse model and shP3, it was hoped that similar therapeutic efficacy could be achieved. shBB is 100% homologous to both hRHO and pRHO, therefore one would assume that a potent suppression of hRHO by shBB in previous studies would correspond to a potent suppression of pRHO by shBB, this however had not been evaluated (Millington-Ward et al, 2011; O'Reilly et al, 2007). Therefore the suppression of pRHO by shBB at the mRNA level in these hetPP347S mice was assessed (**Figure 5.3.11.a**). A suppression level statistically similar to shP3 of 67% ($P=0.2937$) was obtained using shBB. As shBB and shP3 suppress pRHO to similar levels *in vivo* in the hetPP347S mouse and shBB suppresses hRHO to similar levels in the HP347S mouse, this suggested that shP3 may be a potent suppressor of RHO in RHO-adRP models and be as efficient as the best suppressor characterised previously in the TCD laboratory (Millington-Ward et al, 2011; O'Reilly et al, 2007).

As suppression of pRHO mRNA by shP3 should correlate with suppression of pRHO protein, it was not surprising that a decrease in RHO protein by shP3 was achieved (**Figure 5.3.12.a**). No previous studies in the TCD laboratory have examined the suppression of RHO in a RHO-adRP model by western blotting. The 4D2 antibody reacts with both pRHO and mRHO therefore as mRHO expression would mask the suppression result, only a modest change in RHO protein expression was expected. Evidently a significant decrease in RHO protein was observed, this suggested that despite three mismatches over the target site for shP3, suppression of the endogenous mouse allele RHO may be taking place (**Figure 5.3.12.b**).

The functional effects of pRHO suppression by shP3 was analysed at 6 and 12 weeks post-injection for two litters of n=10 mice and 10 and 14 weeks for one litter for n=6 mice, however some variability in results between litters were observed. For litter 1, examined at 6 and 12 weeks, no significant functional benefit was obtained for this time-point (**Figure 5.3.13.e [a-c]**). In contrast for litter 2, evaluated at 6 and 12 weeks, an increase in retinal function was observed suggesting therapeutic benefit could be achieved in these mice (**Figure 5.3.13.f [a-c]**). When this litter was assessed for significance, only increases in rod-isolated and cone-isolated responses at 6 weeks were significant. Furthermore, comparative analysis between the b-wave values in the AAV-2/5.NT20 injected retinas versus the b-wave values in the AAV-2/5.shP3 injected retinas between litter 1 and litter 2 revealed that no significant difference between AAV-2/5.shP3 injected eyes at 6 weeks for the two litters was observed, nor for the AAV-2/5.NT20 injected or AAV-2/5.shP3 injected eyes at 12 weeks. The only significant change occurred in the AAV-2/5.NT20 ERG responses at 6 weeks between the two litters. This suggests that overall, no significant benefit was taking place in these injected mice. For litter 3, examined at 10 and 14 weeks, a significant decrease in ERG function only at 14 weeks was noted (**Figure 5.3.13.k [a-c]**). The reason for the variability among litters could be attributed differences in injection; the second litter of mice were born on a weekend and thus the exact age of injection may not have correctly been determined. All three litters were injected on different days, with different aliquots of virus which could also lead to variability. The fact that the third litter only showed significant suppression of the ERG function at 14 weeks suggests that perhaps a longer post-injection evaluation period may be required in these mice. Mussilino et al, 2011a noted that despite identical transgene levels in the HP347S transgenic mice on a C57BJ/6 background, there was variability in ERG responses between different litter mates, thus they contextually measured baseline ERG parameters of each animal prior to injection (Mussilino et al, 2011a). This may be an approach to employ for future suppression studies in these mice to overcome variability. It is also of note that increases in n numbers of mice used might lead to the generation of more robust results.

As no robust effects at the ERG level were found in the majority of animals injected with AAV-2/5.shP3 in this study, this suggested that 67% suppression of pRHO observed in transduced cells (approximately 10-20% of the retina [Chadderton et al, 2009]) was not sufficient to provide efficacy in this model. This could be due to lower viral transduction obtained in these mice compared to NHR mice. Interestingly, when an shRNA alone (shBB), which suppresses both hRHO and mRHO, was subretinally injected into the HP347S model no significant change in rod-isolated ERG function was obtained (Millington-Ward et al, 2011). This was similar to the ERG analysis observed in this study suggesting that mRHO in the hetPP347S mouse may be suppressed in part by shP3 and that this may be the reason no overall benefit was obtained.

Immunohistochemistry was undertaken on mice from litter 1 and 2 that had previously undergone ERG analysis at 12 weeks in order to assess if any differences in RHO staining were observed (**Figure 5.3.14.a**). A decrease in RHO immunoreactivity was evident in both mouse litters, of note RHO staining previously observed in the ONL, INL and IPL in hetPP347S appeared to be reduced. This indicated that levels of mutant pRHO are reduced due to shP3-mediated suppression. A decrease in overall RHO was evident, and this would correspond with the ERG and western blotting results to suggesting mRHO may also be suppressed by shP3 in the retina.

In order to evaluate if siP3 may also suppress mRHO, despite the presence of 3 mismatches, an *in vitro* experiment co-transfecting hRHO and each siRNA into HeLa cells was undertaken (**Figure 5.3.15.a**). siBB and siP2 suppressed mouse rhodopsin to a similar extent as hRHO as expected, given there was 100% homology for these target sites in both species. Surprisingly, siP3 was found to suppress mRHO albeit to a lesser extent (46%). This was an almost 50% reduction in silencing efficiency compared to hRHO. Kiang et al, 2005 found that an mRHO targeting shRNA with 2 mismatches to hRHO was still capable of suppressing hRHO. Similarly, mutating nucleotide 10 within the shRNA target site in mRHO still resulted in mRHO suppression (20 % lower than the wild-type mRHO mRNA). These results indicated that certain mismatches between siRNA and rhodopsin can be tolerated (Kiang et al, 2005). Moreover, it has been experimentally validated in the literature that the number of mismatches, the position of these mismatches and type of nucleotide substituted has an effect on silencing efficacy (Dahlgren et al, 2008; Du et al, 2005; Huang et al, 2009). Studies have indicated that mismatches between the 5' end of the antisense guide strand are more tolerated than mismatches at the 3' end, in particular mutations in positions 5-11 appear to be least tolerated to confer efficient suppression (Dahlgren et al, 2008; Du et al, 2005). One study examined the effect of different nucleotide mismatches at different positions along the target sequence, relative to the siRNA guide

strand. In general it was found that a purine to a pyrimidine was the most well tolerated mismatch compared to purine to purine or pyrimidine to pyrimidine (Huang et al, 2009). mRHO has three mismatches with the siP3 antisense strand. The 1st is an A: C mismatch at position 3, which is the best tolerated at this position (pyrimidine: purine). The other two mismatches were at position 6 and position 18 and the mismatch was a C: C. This mismatch (purine: purine) is the least tolerated at this position. However cytosine mismatches in these regions were well tolerated compared to cytosine based mismatches in most of the 17 other positions examined (Huang et al, 2009). Most studies have taken into account the effect of a single mismatch between siRNAs and target whereas in this case there are three mismatches, the position and type of mismatches relative to each other is likely to influence efficacy. Dahlgren et al characterised the position and type of double mismatches between a particular target sequence and siRNAs. The majority of these double mismatches provided efficacy of between 20-60% compared to the 92% achieved with a wild-type sequence. Furthermore the 3' end and central region of the antisense strand appeared to be the most sensitive to both single and double mutations (Dahlgren et al, 2008; Du et al, 2005). Two of the mRHO mutations are in the 3' of the target site (5' to AS siP3) and as such should be more tolerated than if they were in the 5' target region. The third mutation is in position 18 relative to the 3' AS of siP3, however mutations in nucleotide position 18-19 have been found generally to be well tolerated (Dahlgren et al, 2008; Du et al, 2005).

In terms of mRHO, 46% suppression mRHO is not efficient compared to the activity of siP3 targeting pRHO. siP1 which suppressed pRHO RNA by 64% (**Section 3.3.4**) was not efficient at completely eliminating hRHO protein, therefore it is likely that there would still be significant levels of mRHO protein present after siP3 suppression. The transgenic model utilised in this study had only one mRHO allele, therefore a 46% reduction in this allele would reduce the total wild-type RHO in this mouse to approximately 27%. Despite the fact that one endogenous RHO allele is sufficient for photoreceptor function, a very mild retinopathy is observed in heterozygous RHO^{+/-} knockout mice (Humphries et al, 1997), therefore by reducing RHO levels even further in AAV-2/5.shP3 treated hetPP347S mice, one might not expect to rescue photoreceptor function. In this model a balance may exist where suppression of mutant RHO rescues the disease phenotype only when sufficient levels of endogenous wild-type RHO are also present. There is most likely a certain threshold of RHO expression that is needed in the retina for function. If this is the case, stabilising the balance between the suppression of mutant RHO and the expression overall RHO in the retina to a point where rescue is obtained is vital. Rhodopsin expression in the shP3 treated hetPP347S mice may be on this threshold, which may account for the variations of ERG function observed between the different mice litters evaluated.

In conclusion, the study presented in this thesis involved the evaluation of a RHO-adRP with a porcine rhodopsin mutant transgene model and the utilisation of this model for examining suppression of mutant RHO. The hetPP347S mouse used had a slow degeneration as evaluated by ERG, western blot analysis and histology which provided a larger therapeutic window for testing the suppression strategy than for example the P23H or the HP347S mouse models. Furthermore the aberrant location of mutant P347S RHO was identified as a characteristic of mutations within the c-terminal region of RHO. Mutations in this region abolish the signal used for transporting RHO efficiently to the OS of the photoreceptors (Deretic et al, 2006), therefore it was not surprising that RHO is mislocalised in these transgenic mice. The evaluation of suppression of pRHO by shP3 was successfully undertaken as assessed by real-time RT-PCR and western blot analysis. However, despite potent suppression, overall no rescue of photoreceptor function was observed. Further analysis determined that siP3 also suppresses mRHO albeit to a lesser extent than pRHO. As the transgenic mouse used for the research described in this chapter was placed on a background in which only one mouse allele is present, the lack of rescue may be due to the reduction in wild-type mRHO that accompanied shP3-mediated suppression of the mutant porcine RHO. Two strategies could be utilised to overcome this, firstly the suppression of mRHO was only evaluated *in vitro* in this study. In order to evaluate suppression *in vivo* AAV-2/5.shP3 should be subretinally injected into wild-type mice and suppression subsequently analysed. Secondly, Chadderton, et al, utilised the HP347S transgenic mouse on a wildtype^{+/+} background to confirm therapeutic efficacy. If like siP3 *in vitro*, shP3 only suppresses mRHO by less than 50% then evaluating the PP347S mouse on a wild-type^{+/+} background should in theory achieve rescue (Chadderton et al, 2009). Furthermore, the hetPP347S mouse could subsequently be utilised for studies involving the simultaneous delivery of both a shP3 suppressor and replacement gene resistant to suppression, similar to those undertaken by Millington-Ward et al, 2011. The development of such RNAi resistant replacement gene is detailed in Chapter 6.

The evaluation of suppression *in vivo* in this laboratory to date has utilised AAV-2/5 as the delivery vehicle to the retina (Chadderton et al, 2009; Millington-Ward et al, 2011; O'Reilly et al, 2007). Recently however, AAV-2/8 has been established as an efficient delivery vehicle for *in vivo* suppression of target genes in the retina. Georgiatis et al, 2009 utilised AAV-2/8 to achieve potent suppression of peripherin-2 in the photoreceptors (Georgiatis et al, 2009). An AAV-2/8 Zinc finger based suppressor of rhodopsin was found to successfully transduce the OS of the HP347S transgenic mouse, providing both significant suppression of hRHO and therapeutic rescue of photoreceptors (Mussilino et al, 2011a). Of all the recombinant AAV viruses thus far identified, it has been suggested that AAV-2/8 may be most efficient delivery vehicle to photoreceptors identified to date (Allocca et al,

2007; Leberherz et al, 2008; Natkunavajeh et al, 2008; Steiger et al, 2008). Specifically AAV-2/8 was established to transduce the porcine photoreceptors more efficiently than the AAV-2/5 (Mussilino et al, 2011b). This is of particular interest for the current study given the therapeutic is being developed for its eventual use in the pig. Therefore a future avenue for the analysis of therapeutic benefit derived from shP3 could be the evaluation of AAV-2/8.shP3-based constructs in the retinas in the PP347S model of RHO-adRP.

P347SF	5'CCATCTACAACCCTGTCAT-3'
PP347SR	5'-TTTGGTTCTGACAGGTGCG-3'
P1F	5'-AGAAGGCCTGCAGTGCTCG-3'
P1R	5'-AGCCACGACCATGATGATG-3'
P2F	5'-CATCATCATGGTCGTGGCT-3'
P2R	5'-GGCAAAGAAGGCTGGGTG-3'
P3F	5'-CTACGTGCCTTTCTCCAACAA-3'
P3R	5'-TCCTCCAAGTTGCAT-3'
MRHOF	5'-CTGAGGGCATGCAATGTTCA-3'
MRHOR	5'-CATAGCAGAAGAAGATGACG-3'

Table 5.2.2 Primers used in Chapter 5

P347S forward and PP347S reverse (P347S F and PP347S R) were used for genotyping mutant pRHO in the transgenic mouse with pRHO. P1-P3 forward and reverse (F & R) were used to analyse pRHO suppression by shP3. MRHO forward and reverse primers (MRHO F & R) were used to analyse mRHO expression.

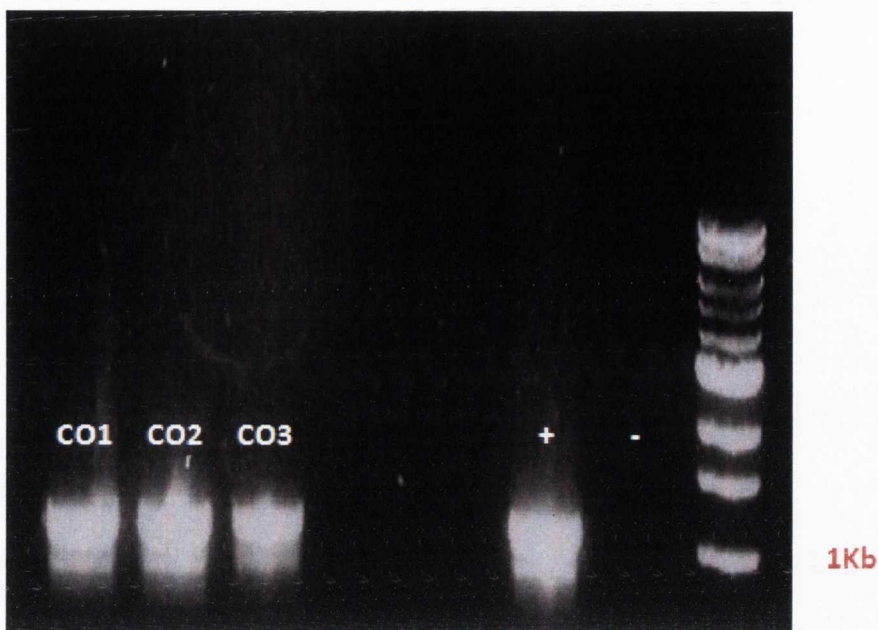


Figure 5.3.1a PP347S genotyping

This is an example of a typical genotyping result for the homozygous PP347S mice. The positive control is DNA from a mouse known to be positive for the PP347S transgene, and has a positive band at 1.1kb which is the correct size in which the P347SF and PP347SR primers amplify pRHO. The negative control is water blank; it is negative for any bands. This demonstrates that there was no DNA contamination during the PCR reaction. C01-C03 have a band at 1.1kb

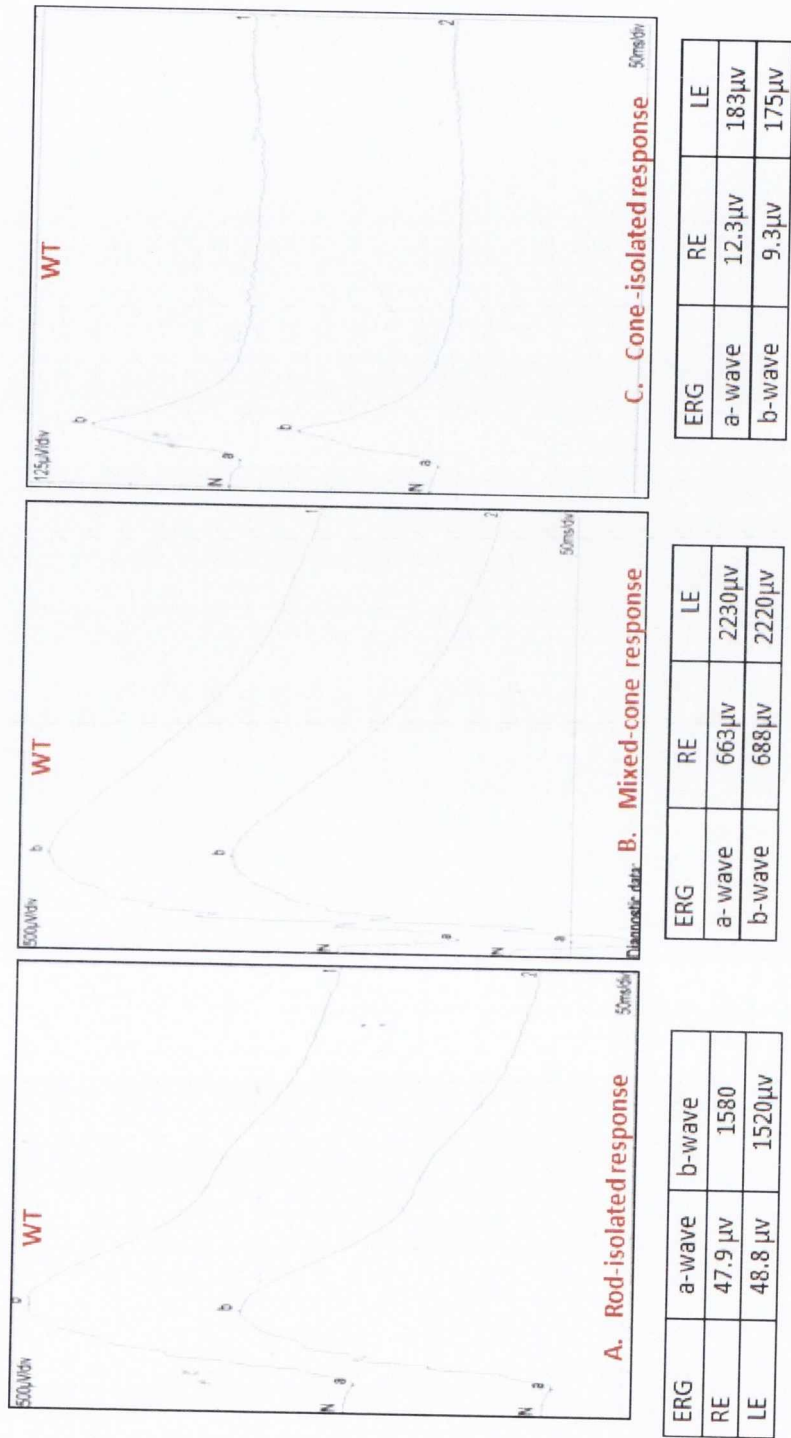


Figure 5.3.2.a Representative ERG response for a wild-type 129 mouse (WT)

A is the rod-isolated response, B is the mixed-cone response and C is the cone-isolated response. The a-wave and b-wave values are a-typical for a wild-type non-degenerating mouse retina.

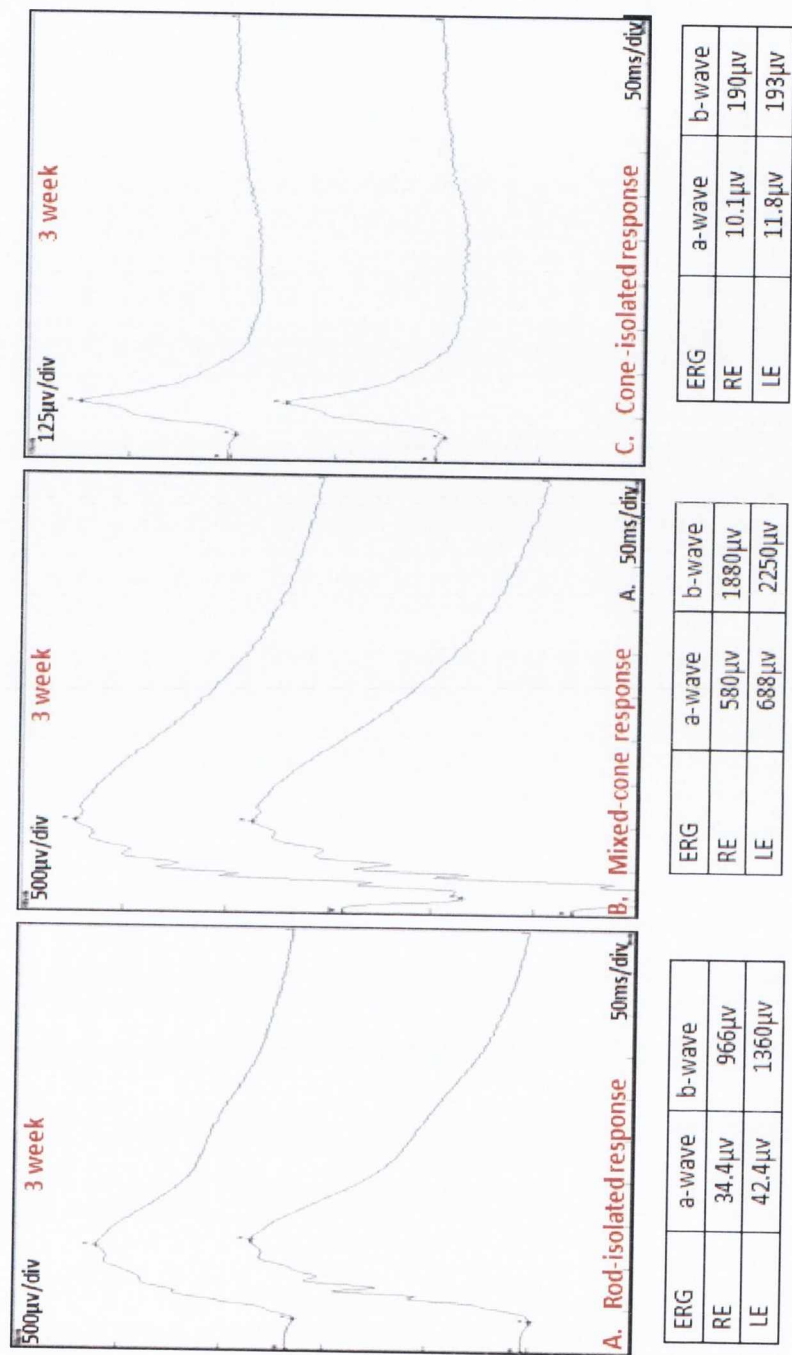


Figure 5.3.2.b A representative ERG response for the heterozygous PP347S animal at 3 weeks

A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response. The highest value was obtained for the LE in which a rod-isolated b-wave of 1360 µV, a mixed-rod-cone b-wave of 2250 µV and a cone-isolated b-wave of 193 µV was obtained which is similar to the high wild-type values

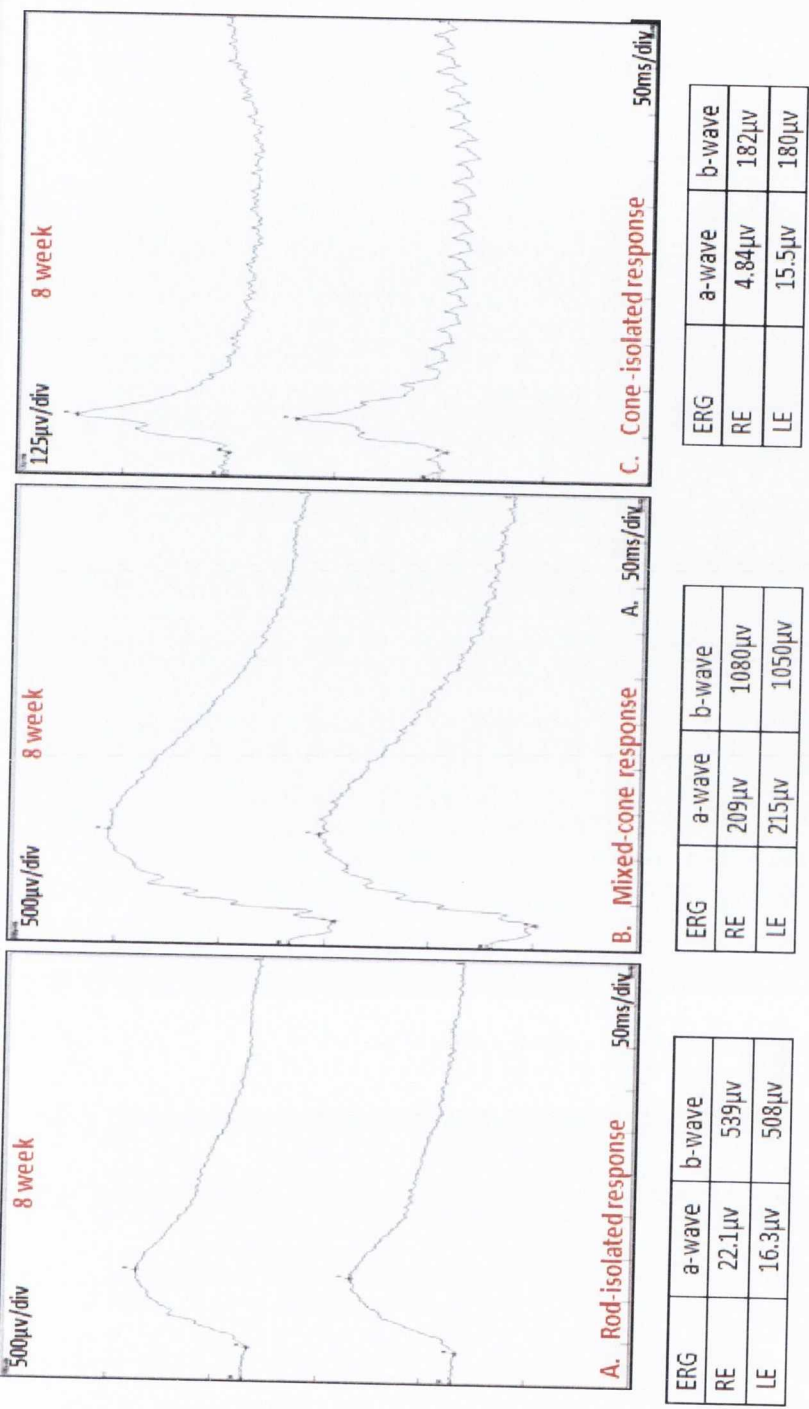


Figure 5.3.2.c A representative ERG response for a heterozygous PP347S animal at 8 weeks

A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response. The degeneration is more apparent in the 8 week mice compared to the 3 week mice.

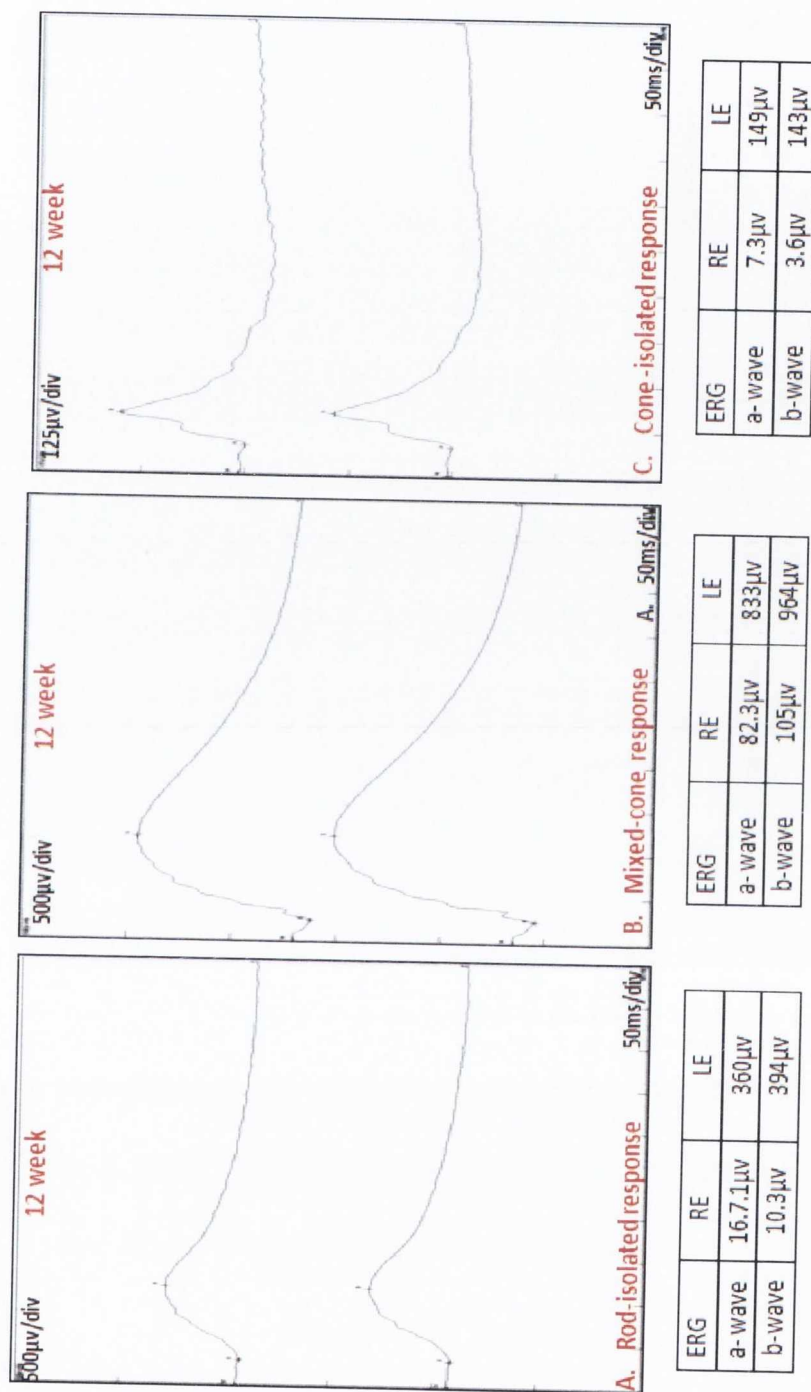


Figure 5.3.2.d Representative ERG response for the heterozygous PP347S animal at 12 weeks

A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response.

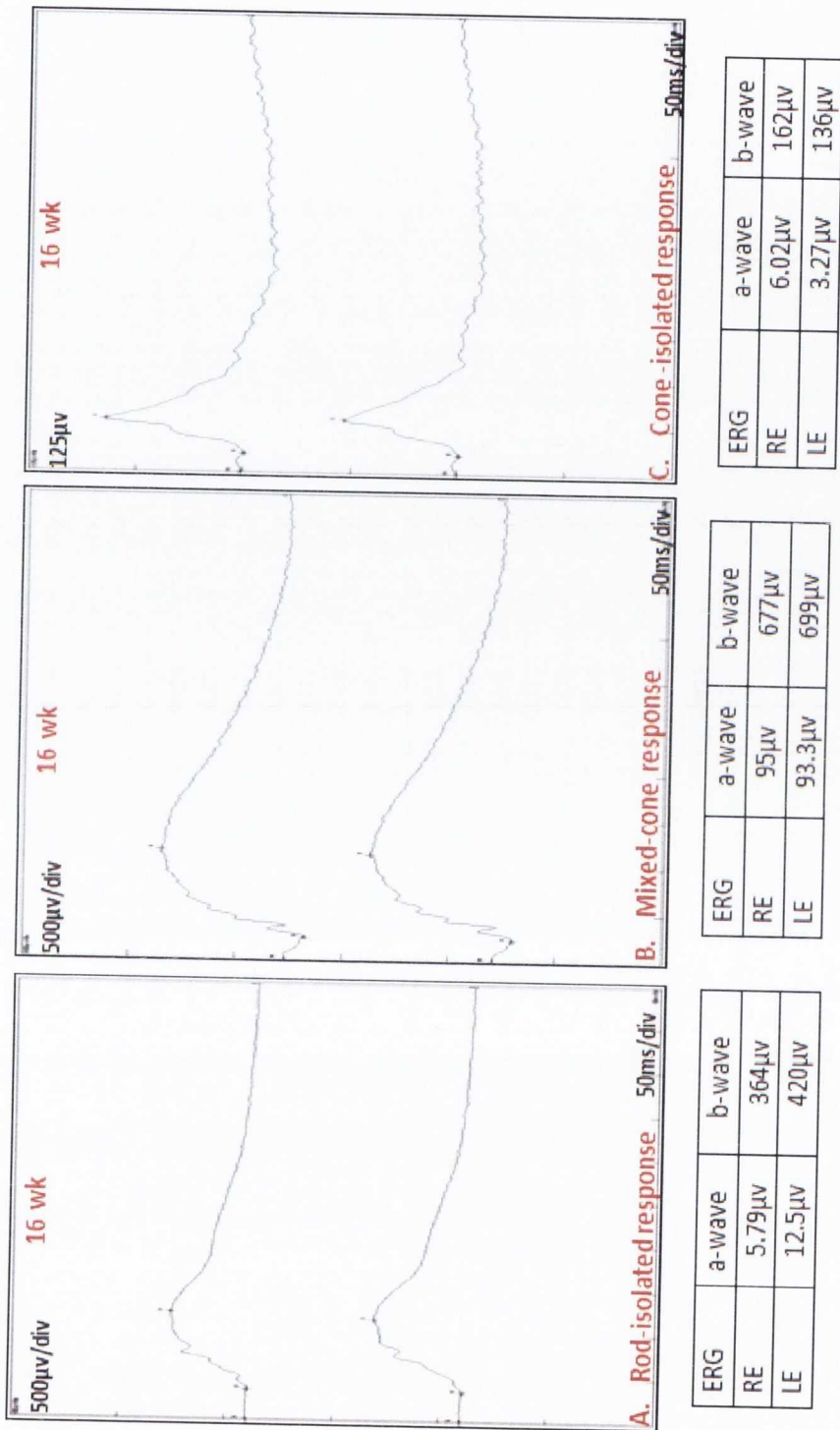


Figure 5.3.2.e A representative ERG response for the heterozygous PP347S animal at 16 weeks.

A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response.

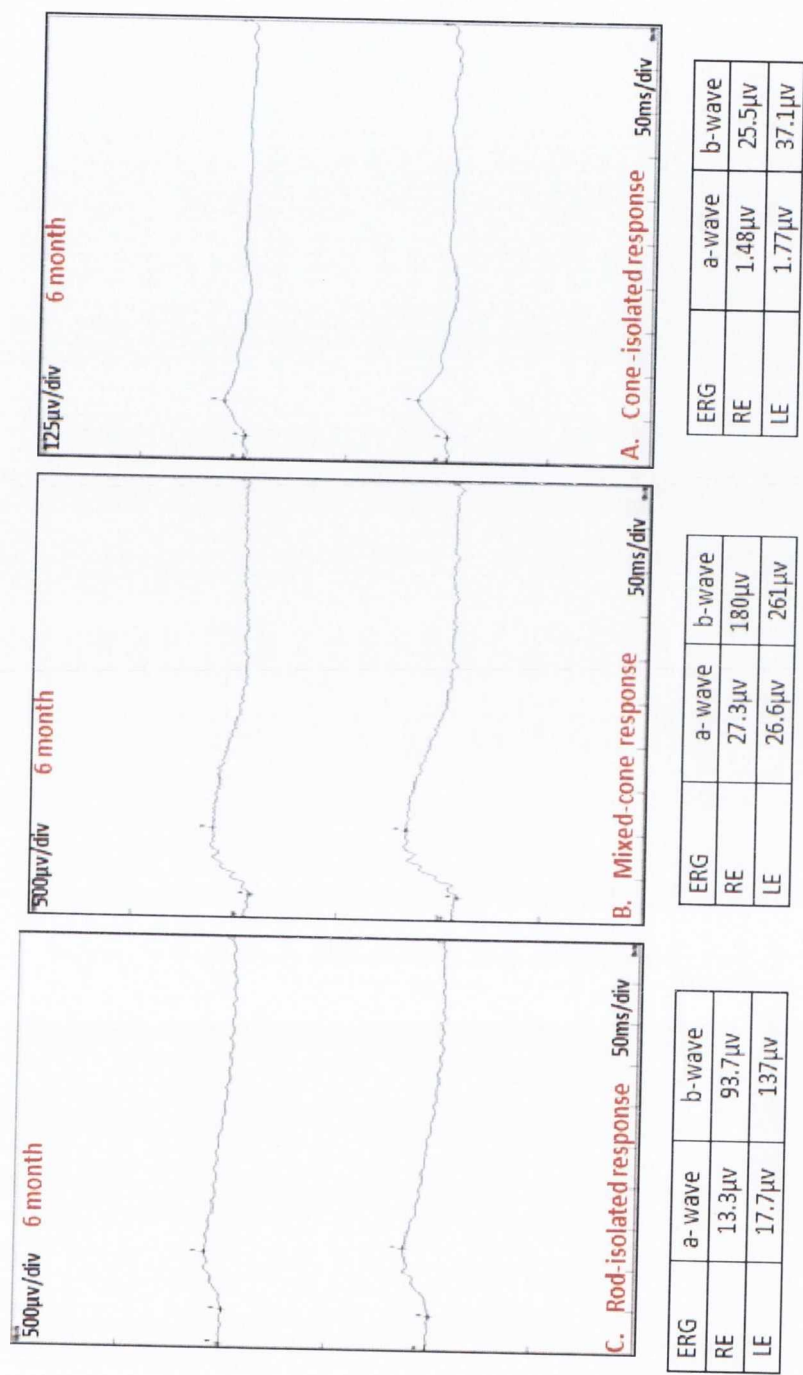


Figure 5.3.2.f A representative ERG response for a heterozygous PP347S animal at 6 months.

A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response. The cone-isolated response highlights the cone cell degeneration at this time point.

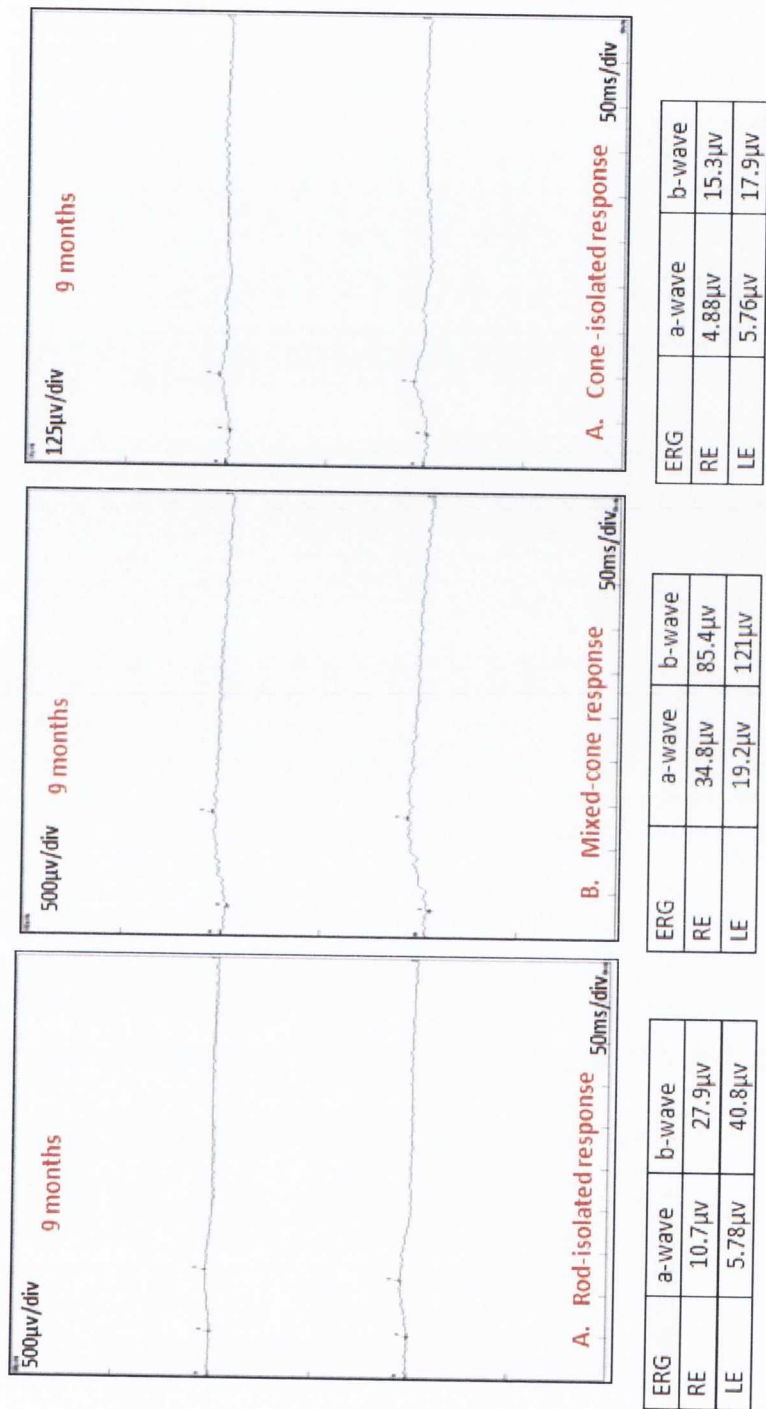


Figure 5.3.2.g. A representative ERG response for a heterozygous PP347S animal at 9 months

A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response. All responses signify a severe rod and cone cell degeneration has occurred in these mice.

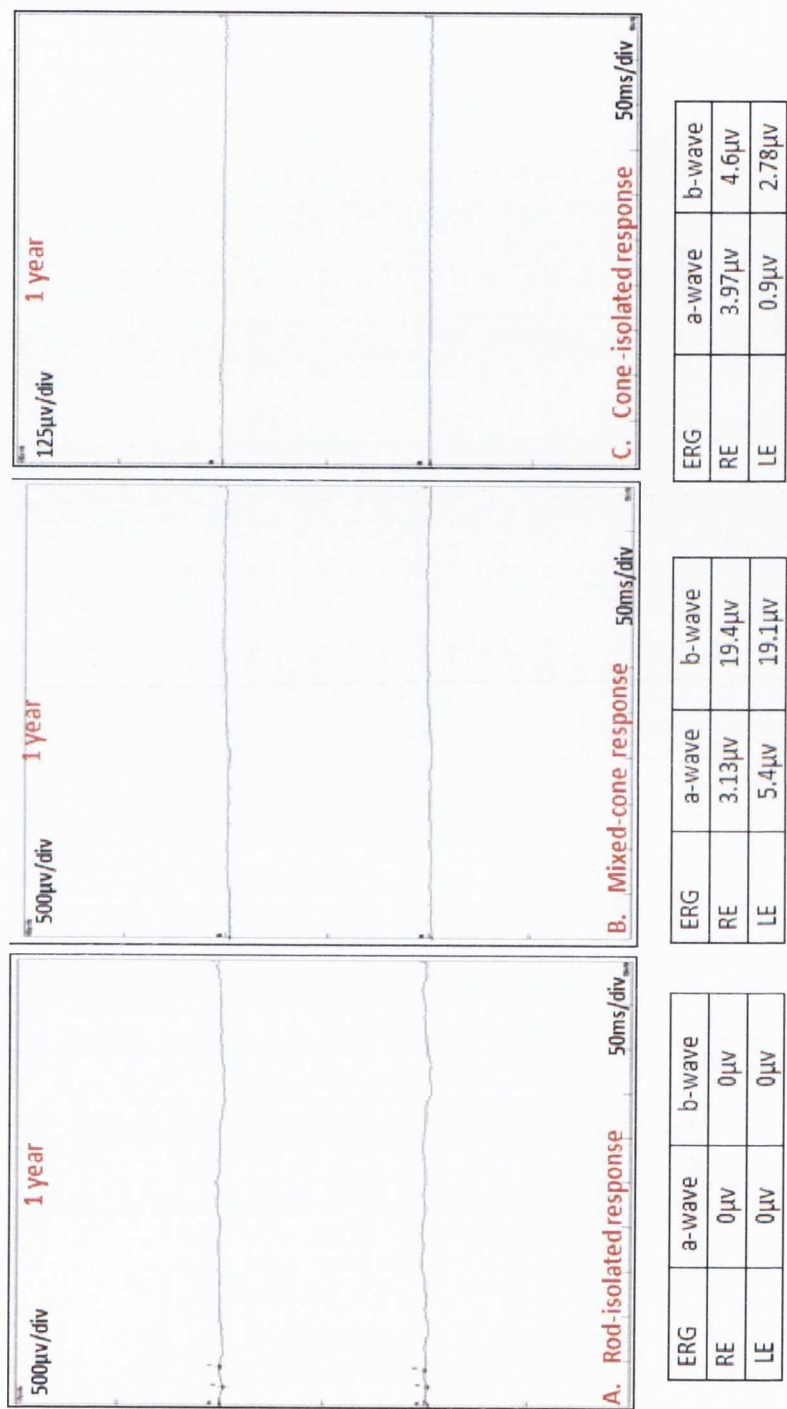
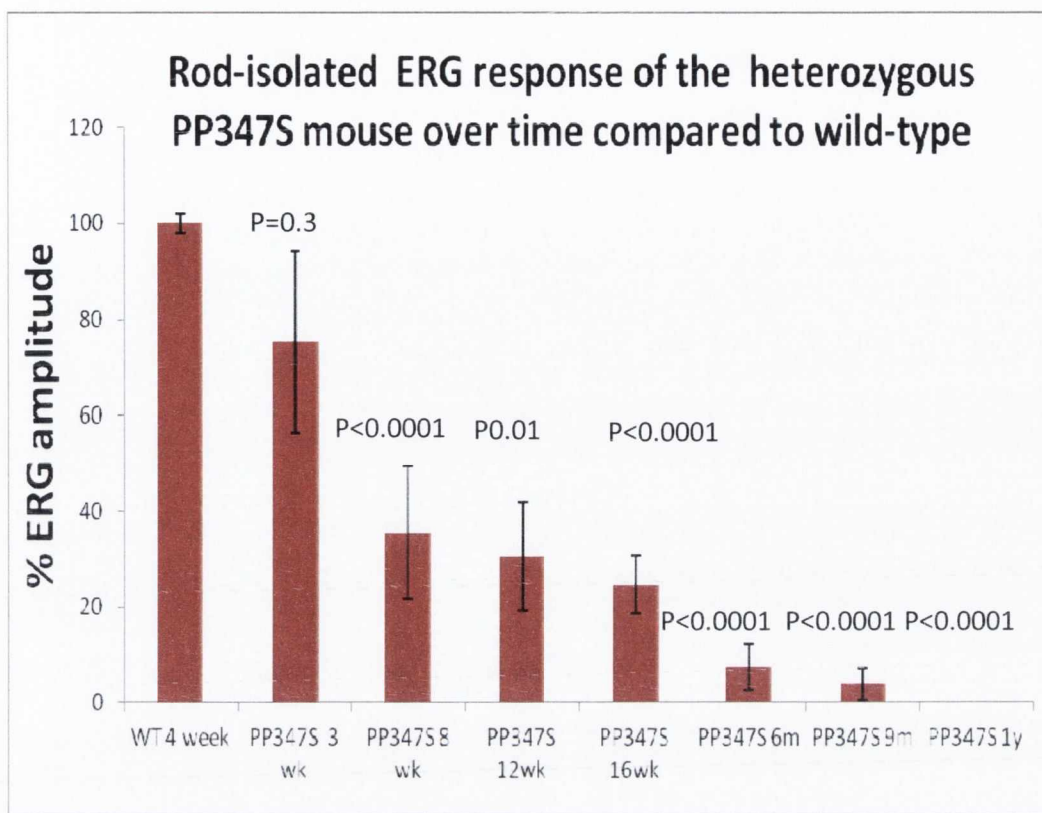


Figure 5.3.2.h Representative ERG response for a heterozygous PP347S animal at 1 year.

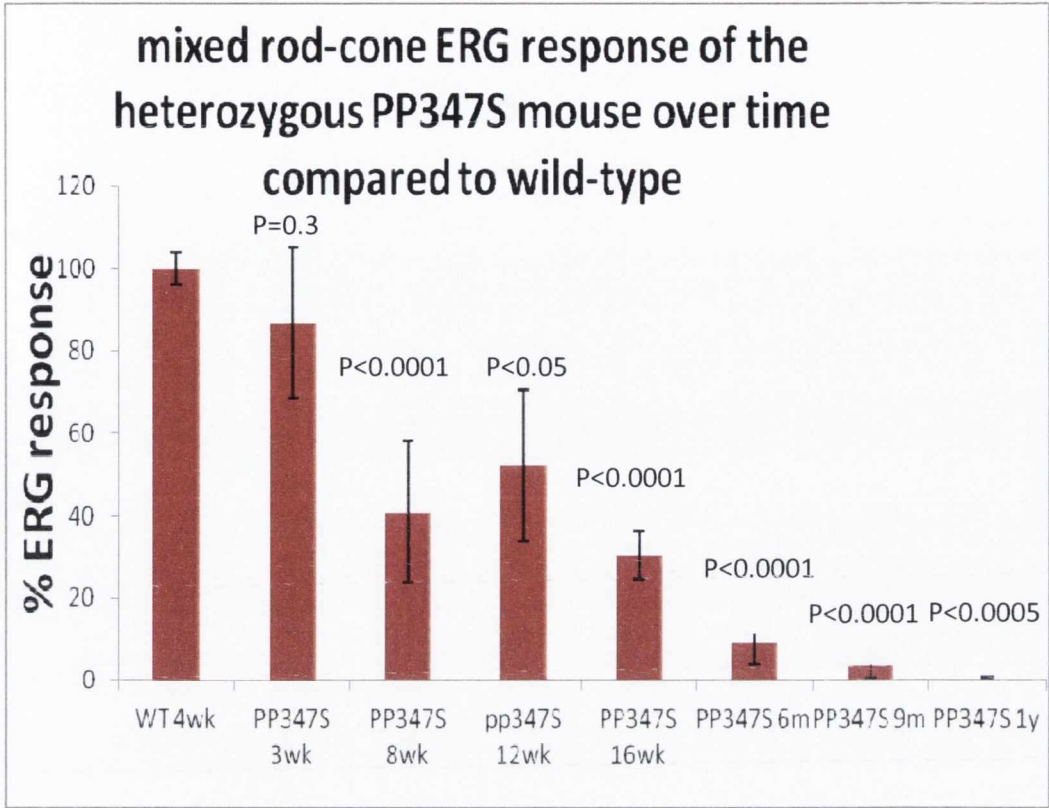
A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response. The rod-isolated response is completely flat indicating the complete degeneration of the rod photoreceptors. A minimal response remains for the cone-photoreceptors. Overall the degeneration of the hetPP347S mouse appears complete by 1 year.



rod-isolated ERG responses								
Timepoint	WT 4wk	PP347S 3wk	PP347S 8wk	pp347S 12wk	PP347S 16wk	PP347S 6m	PP347S 9m	PP347S 1y
% of WT	100	75.4	35.4	30.6	24.7	7.5	3.9	0
% SD	2	19	14	11	6	4.8	3.5	0

Figure 5.3.2.i Average rod-isolated b-wave responses in heterozygous PP347S mice at different time-points compared to wild-type.

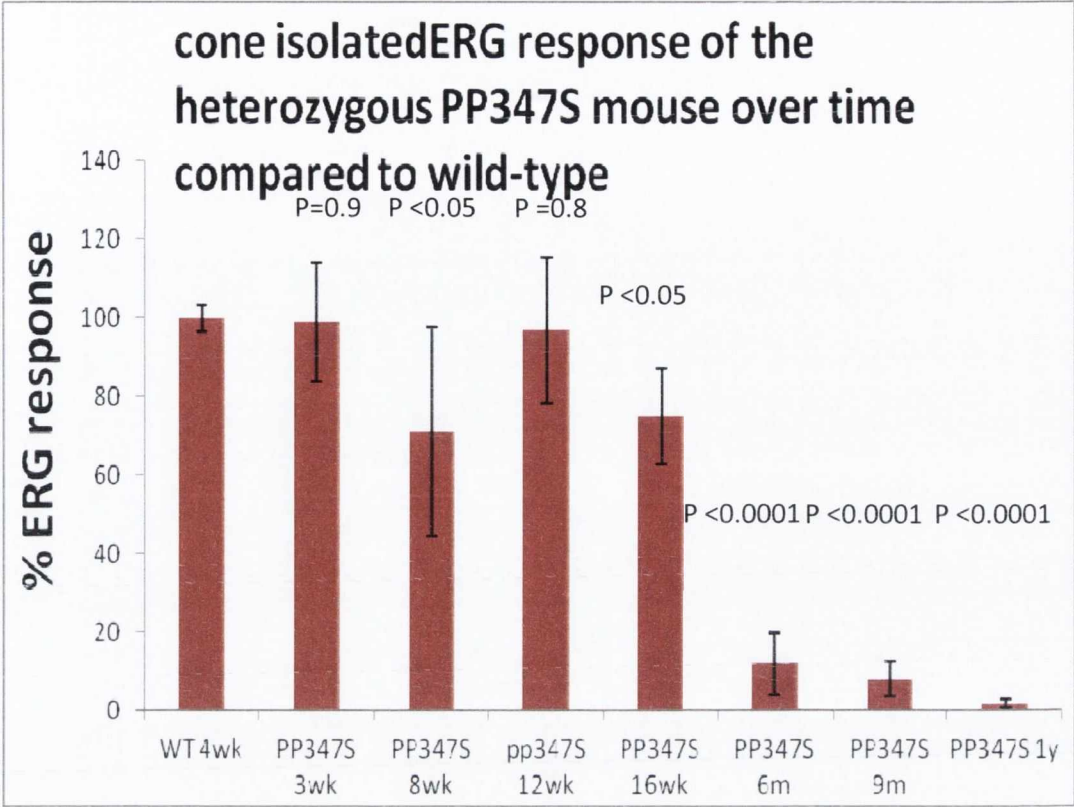
The average rod-isolated b-wave responses for 4 week old wild-type mice and hetPP347S mice at 3 week (wk), 8wk, 12wk, 16wk, 6month (m), 9m and 1 year (y) was combined for each time-point. The average and standard deviation was calculated using Microsoft Excel. Statistical significance was assessed by a two sample t-test (DataDesk6.0). The rod-isolated response in the wild-type was set at 100% and all other hetPP347S b-wave values at different time-points were expressed as percentages of this value. Error bars represent % SD.



Mixed rod-cone isolated ERG responses								
Timepoint	WT 4wk	PP347S 3wk	PP347S 8wk	PP347S 12wk	PP347S 16wk	PP347S 6m	PP347S 9m	PP347S 1y
% of WT	100	86.9	40.9	52	30.4	9.1	3.4	0.2
% of SD	4	18	17	18	6	6	3	0.4

Figure 5.3.2.j Average mixed rod-cone b-wave responses at in heterozygous PP347S mice at different time-points compared to wild-type.

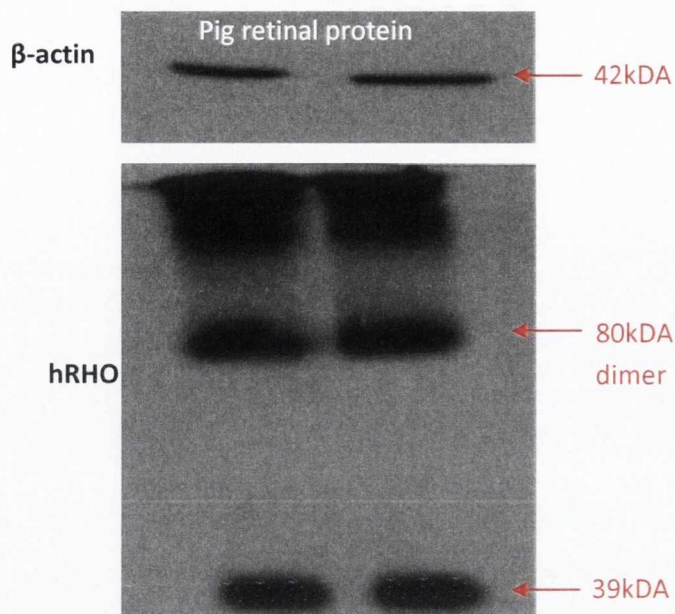
The average mixed rod-cone b-wave responses for 4 week old wild-type mice and hetPP347S mice at 3 week (wk), 8wk, 12wk, 16wk, 6month (m), 9m and 1 year (y) was combined for each time-point. The average and standard deviation were calculated using Microsoft Excel. Statistical significance was analysed by a two sample t-test (DataDesk6.0). The mixed rod-cone response in the wild-type was set at 100% and all other hetPP347S b-wave values at different time-points were expressed as percentages of this value. Error bars represent % SD.



Cone isolated ERG responses								
Timepoint	WT 4wk	PP347S 3wk	PP347S 8wk	pp347S 12wk	PP347S 16wk	PP347S 6m	PP347S 9m	PP347S 1y
% of WT	100	98.9	71	96.8	75.1	12	9	1.6
% of SD	3	15	26	19	12	8	5	1

Figure 5.3.2.k Average cone-isolated b-wave responses at in heterozygous PP347S mice at different time-points compared to wild-type.

The average cone-isolated b-wave responses for 4 week old wild-type mice and hetPP347S mice at 3 week (wk), 8wk, 12wk, 16wk, 6month (m), 9m and 1 year (y) was combined for each time-point and the standard deviation obtained using Microsoft Excel. Statistical significance was assessed by a two sample t-test (DataDesk6.6). The cone-isolated response in the wild-type was set at 100% and all other hetPP347S b-wave values at different time-points were expressed as percentages of this value. Error bars represent % SD.



SAMPLE NAME	pRHO
BCA ($\mu\text{g}/\text{ml}$)	1035
protein/ well (μl)	20
[protein]/ well (μg)	21

Figure 5.3.3.a Optimisation of the 4D2 antibody for porcine rhodopsin.

Retinal protein from a porcine retina was loaded in duplicate and probed with 4D2. The pRHO monomer at 39kDA was clearly observed in both samples as was the presence of pRHO at higher molecular weights. The porcine protein was probed for β -actin to check the β -actin antibody was reactive to pig. Positive bands at 42 kDA indicated that this antibody could react with pig β -actin.

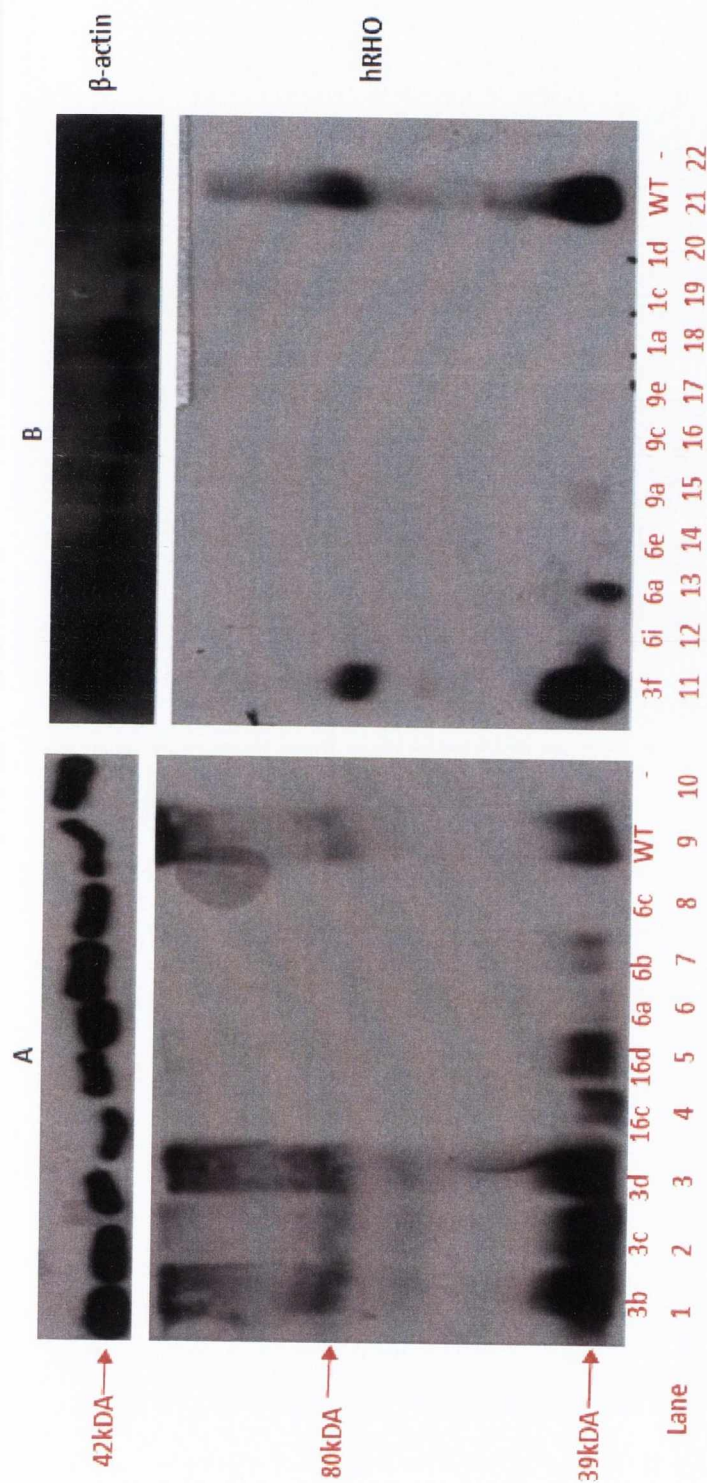
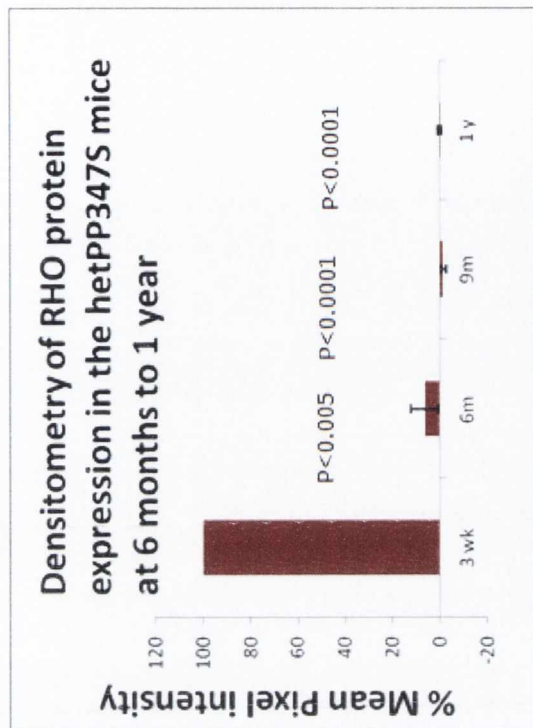
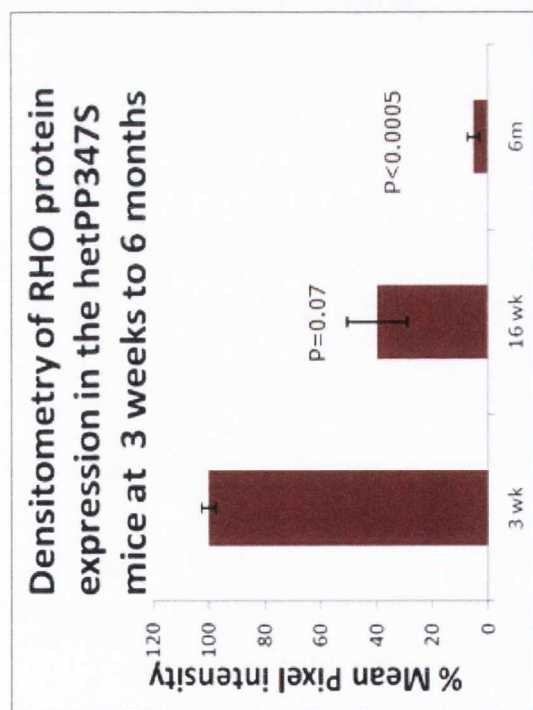


Figure 5.3.4.a Reduction in pRHO and mRHO expression overtime

This is representative of two sets of western blots characterising the loss of RHO from photoreceptor cells of the *hetPP347S* mouse. Both western blots include a positive (wild-type, WT) and negative (*129rho^{-/-}*) control to ensure the 4D2 antibody was working correctly. A is representative of the reduction in rhodopsin from 3 weeks (3b, 3c and 3f) to 16 weeks (16c and 16d) to 6 months (6a, 6b and 6c). There is high RHO expression at 3 weeks with RHO monomers at 39kDA and other higher molecular weight RHO oligomers. With increasing age mice produced less RHO monomers and the presence of higher molecular weight forms of RHO were absent. B represents RHO protein expression in mice at 6 months (6i, 6a & 6e), 9 months (9 a, 9c & 9e) and 1 year (1a, 1c & 1d). RHO monomers and dimers are abundant in the 3 week sample (3f) which is consistent with A. There is low expression of RHO monomers at 6 months, again consistent with A. No RHO immunoreactivity was visible at 9 months or a 1 year. Mouse β-actin was used for equal loading. This was difficult to obtain as when the retinal begins to degenerate levels of β-actin decrease. Over exposure of β-actin allowed observation of mouse β-actin bands.



hetPP347S	3 wk	16 wk	6 m
% of 3 week	100	40	5
% SD	2	11	2

hetPP347S	3 wk	6m	9m	1 y
% of 3 week	100	6	0	0
% SD	0	6	1	1

Figure 4.3.4.b Densitometry of western blot analysis of hRHO expression in the hetPP347S mice over time

The first graph represents densitometry of the first set of western blotting studies. The pixel intensity for RHO monomers in every sample was obtained using image J. For each western (western 1 or western 2), the pixel intensity obtained in the uninjected rho-/- control of each gel was used as a blank and this value was subsequently subtracted from all RHO values for that western. The hRHO pixel intensity was normalised to the corresponding β-actin pixel intensity and the average of each time-point was obtained. All calculations were undertaken using Microsoft Excel. The mean pixel intensity of RHO protein at 3 week (wk) was set to 100 % and the 16 wk, 6 month (m) and negative control (rho129^{-/-}) samples was expressed as a percentage of this. Similarly the second graph represents densitometry of the second set of westerns, the mean pixel intensity of protein from the 3 wk time point was set as 100 % and the pixel intensities at 6 m, 9m, 1 year (y) and of the 129rho^{-/-} sample was expressed as a percentage of this value. All statistical analysis was undertaken using a two sample t-test (Datadesk 6.1). Standard error represents % SD values.

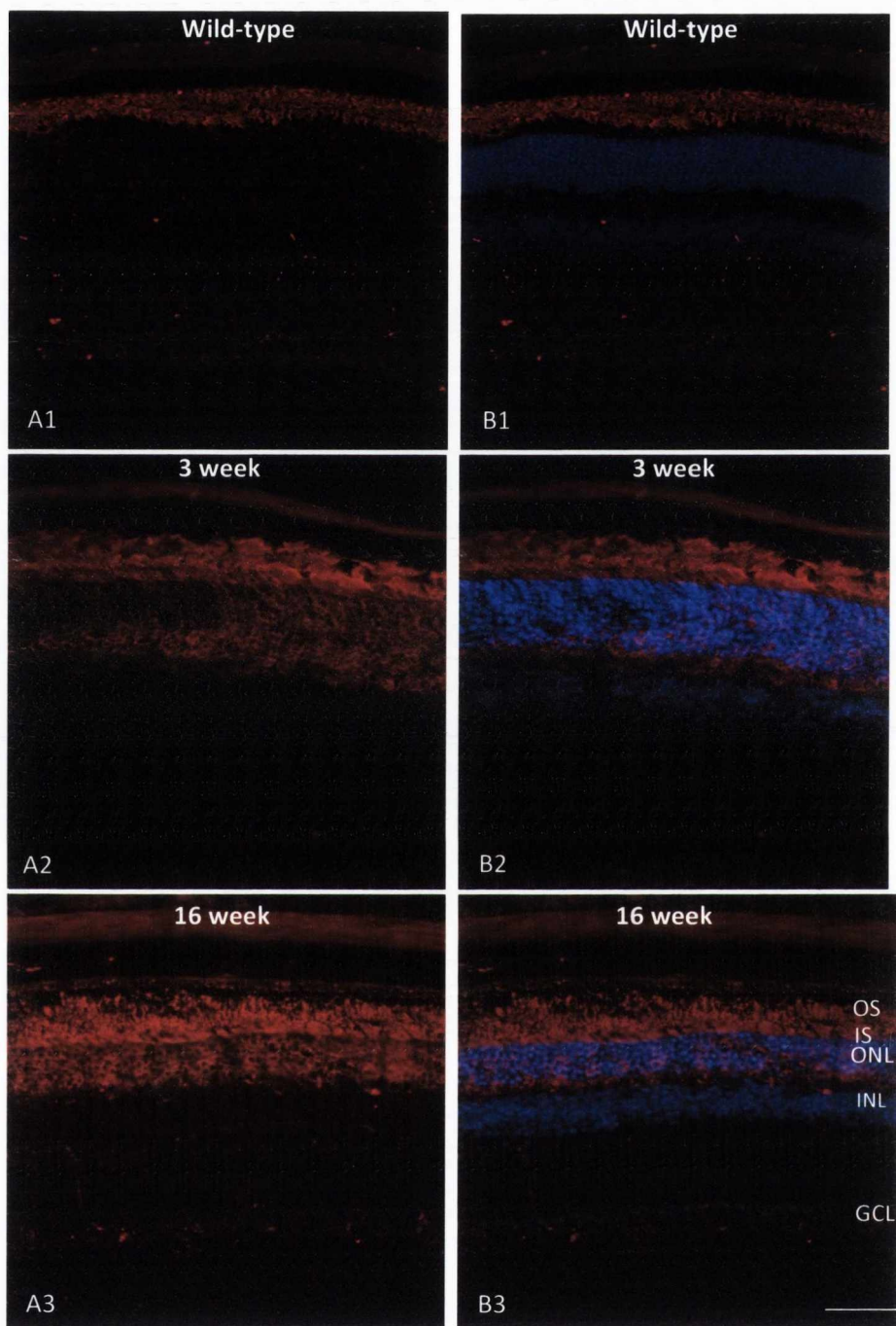


Figure 5.3.5.a. Immunohistochemistry of heterozygous PP347S retinas from 3 weeks to 16 weeks

A1-A6 represents RHO staining with 4D2, B1-B6 represents the RHO staining overlaid with DAPI. OS=outer segments, IS=inner segments, ONL=outer nuclear layer, INL= inner nuclear layer and GCL=ganglion cell layer. Scale bar (L) represents 116 μ m

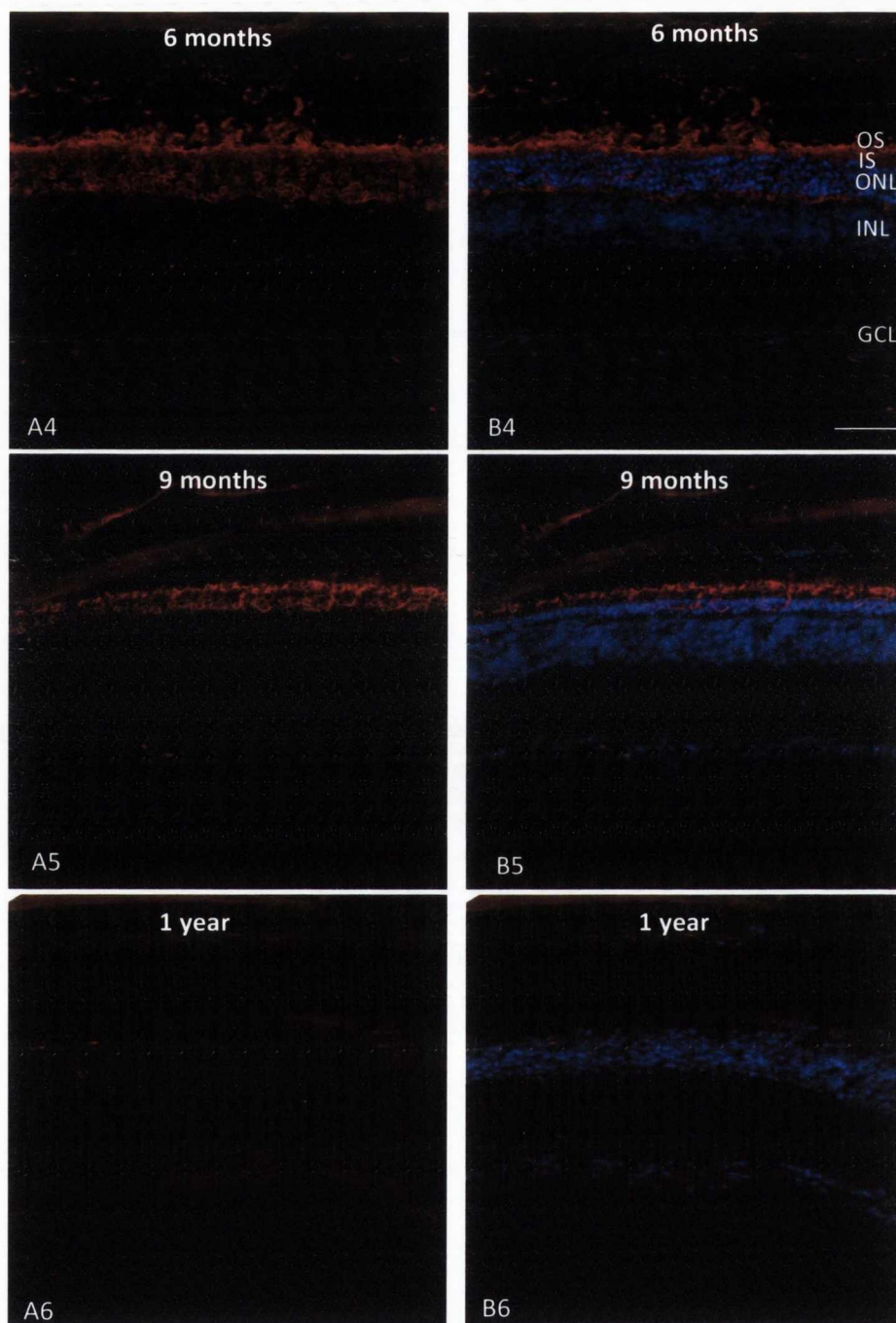


Figure 5.3.5.b Immunohistochemistry of heterozygous PP347s retinas from 6 months to 1 year.

A1-A6 represents RHO staining with 4D2, B1-B6 represents the RHO staining overlaid with DAPI. OS=outer segments, IS=inner segments, ONL=outer nuclear layer, IN = inner nuclear layer and GCL=ganglion cell layer. Scale bar (L) represents 116 μ M

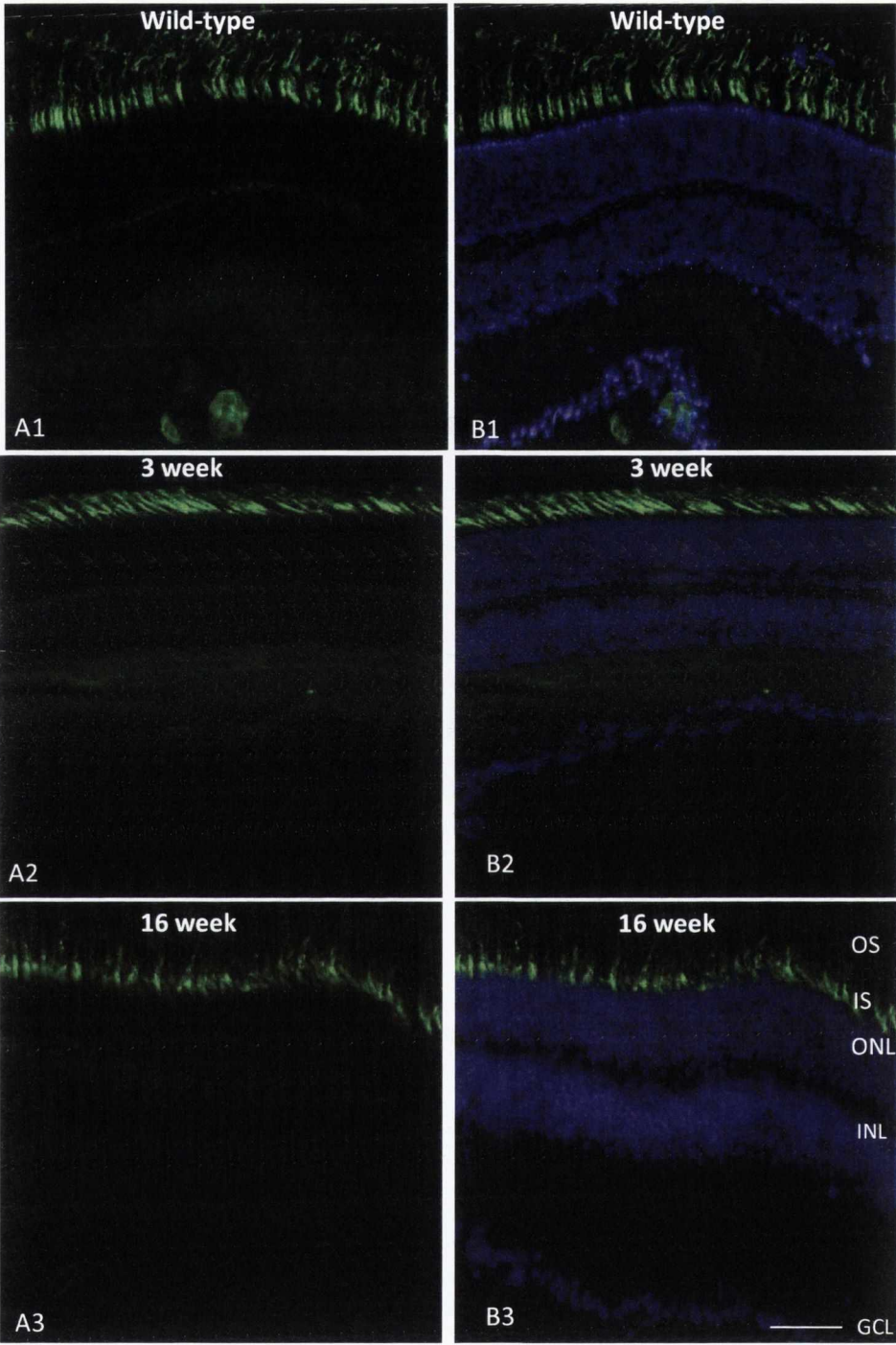


Figure 5.3.6.a Immunohistochemistry of PNA staining in heterozygous PP347s retinas from 3 weeks to 16 weeks

A1-A6 represents PNA staining of the cone cells, B1-B6 represents the PNA staining overlaid with DAPI. OS=outer segments, IS=inner segments, ONL=outer nuclear layer, INL=inner nuclear layer and GCL=ganglion cell layer. Scale bar (L) represents 116 μ M

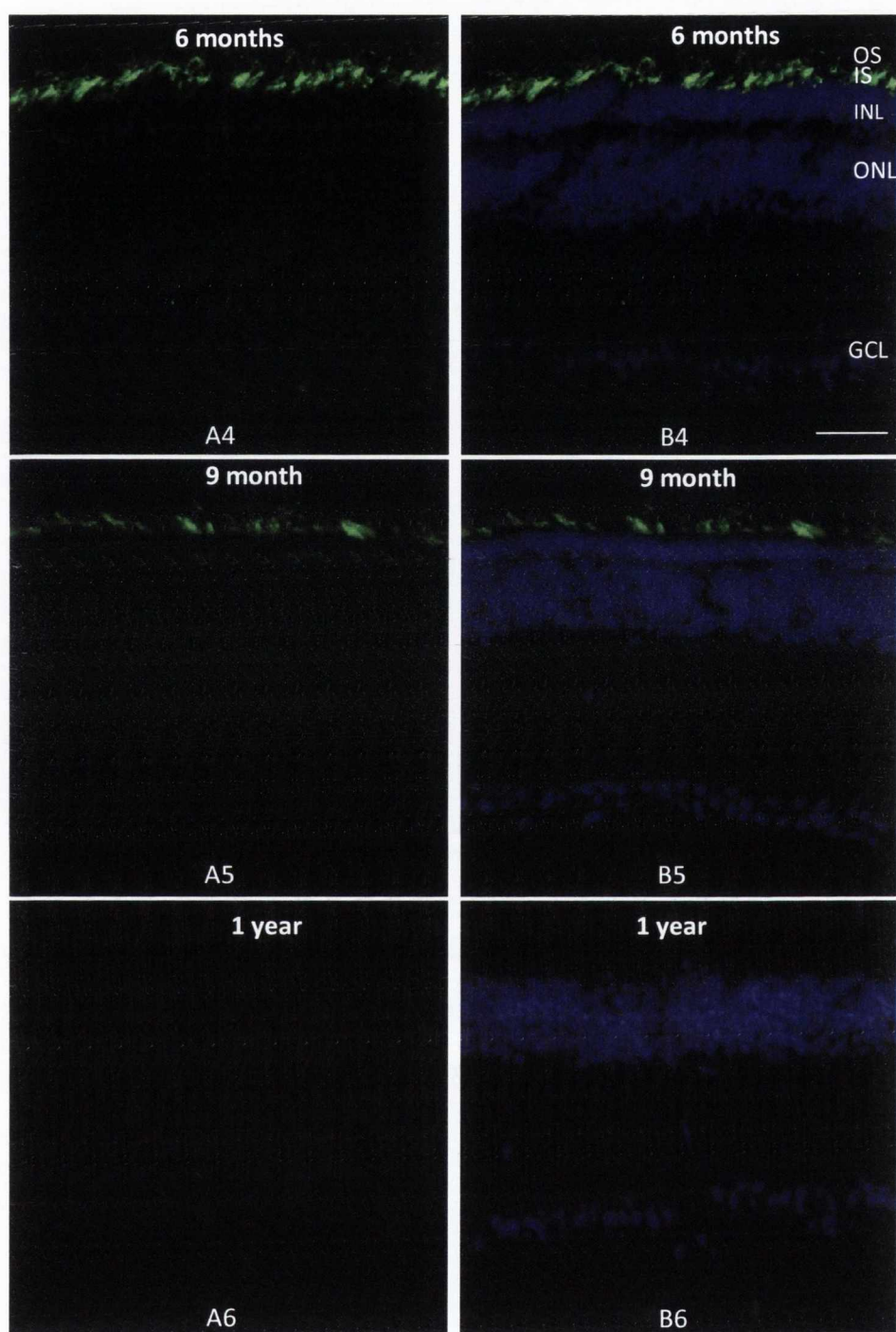


Figure 5.3.6.a Immunohistochemistry of PNA staining in hetPP347s retinas from 6 months to 1 year.

A1-A6 represents PNA staining of the cone cells, B1-B6 represents the PNA staining overlaid with DAPI. OS=outer segments, IS=inner segments, ONL=outer nuclear layer, INL=inner nuclear layer and GCL=ganglion cell layer. Scale bar (L) represents 116 μ M

P1 Forward	PIG	AGAAGGCCTGCAGTGCTCG
	Mouse	TGAGGGCATGCAATGTTCA
P1 Reverse	PIG	CATCATCATGGTCGTGGCT
	Mouse	TATCATCATGGTCATCTTC
P2 Forward	PIG	CATCATCATGGTCGTGGCT
	Mouse	TATCATCATGGTCATCTTC
P2 Reverse	PIG	CACCCACCAGGGCTCCGACTTTGG
	Mouse	CACCCACCAGGGCTCCAACCTCGG
P3 Forward	PIG	CTACGTGCCTTTCTCCAACA
	Mouse	TTATGTGCCCTTCTCCAACG
P3 Reverse	PIG	ATGCAACTTGGAGGGA
	Mouse	CTGTAATCTCGAGGGC

Figure 5.3.7.a primer design for pRHO amplification

Three primer sets were designed that were 100% homologous to pRHO but contained mismatches to mRHO. This was to ensure that only pRHO mRNA would be amplified from the heterozygous PP347S mouse.

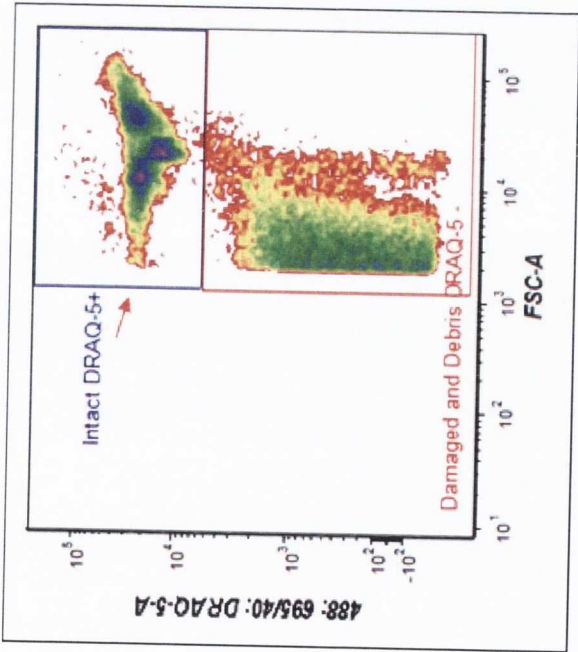


Figure 5.3.8.a The FACS purification of DRAQ5^{-/-} cells

The above diagram represents retinal cells from an un-injected hetPP347S. Live cells (red arrow) were clearly separated from the non-viable cells. The x-axis measures forward scatter (FSC-A) and the y-axis is the measure of DRAQ5 signal (488: 695/40)

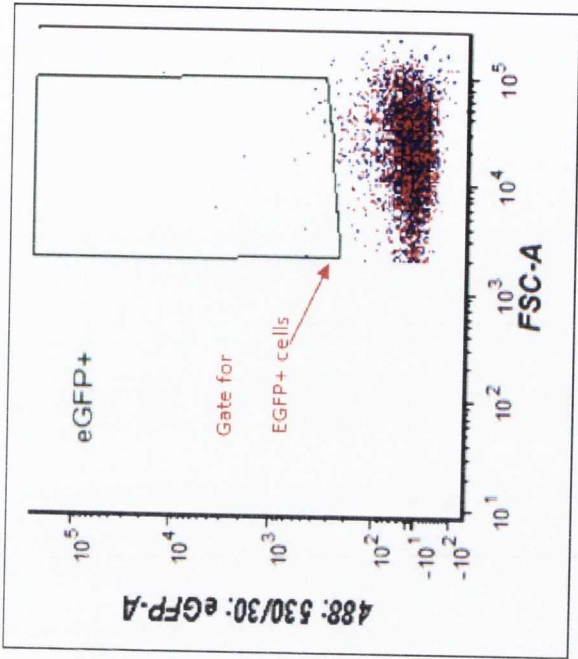


Figure 5.3.8.b The determination of the gate used to sort DRAQ5^{-/-} and EGFP positive cells

The above diagram represents retinal cells from an un-injected hetPP347S mouse. The DRAQ5 positive healthy cells were taken and assessed for EGFP. No EGFP positive cells were obtained is an EGFP negative control. The x-axis is a measure of forward scatter (FSC-A) and the y-axis is a measure of the GFP signal (488: 530/30)

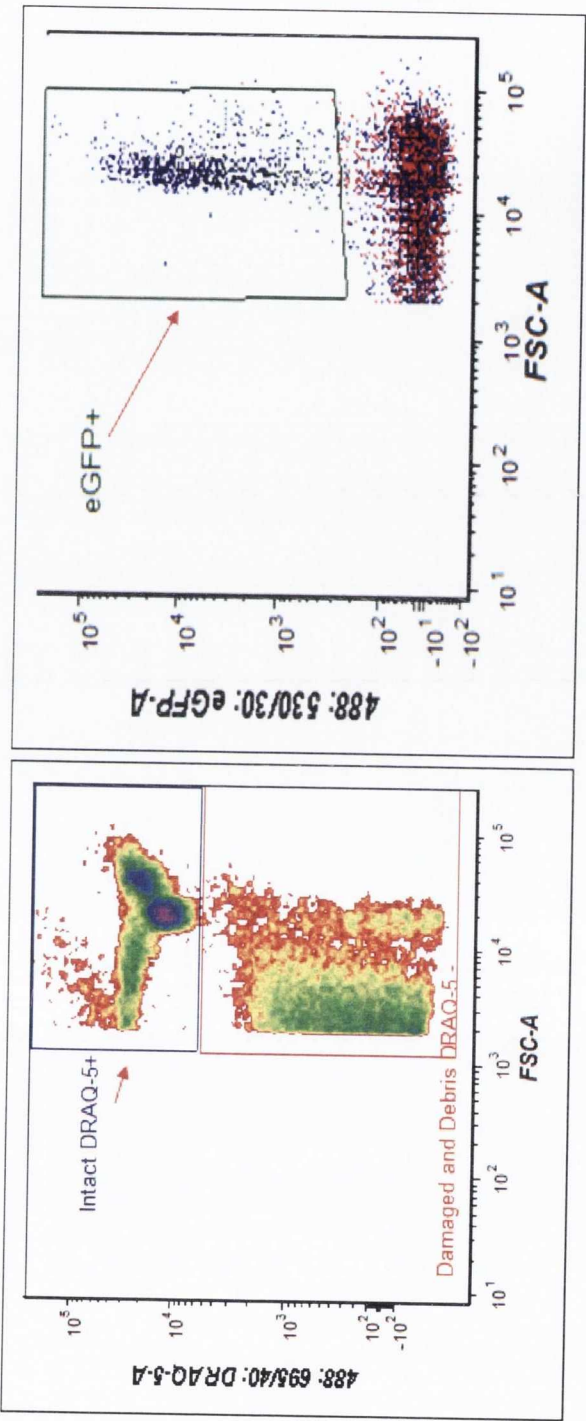


Figure 5.3.8.c The determination of DRAQ-5 + cells

The above diagram was retinal cells from a transgenic hetPP347S mouse injected with AAV-2/5.NT20. The live cells (red arrow, DRAQ-5 +) were separated from the non-viable cells.

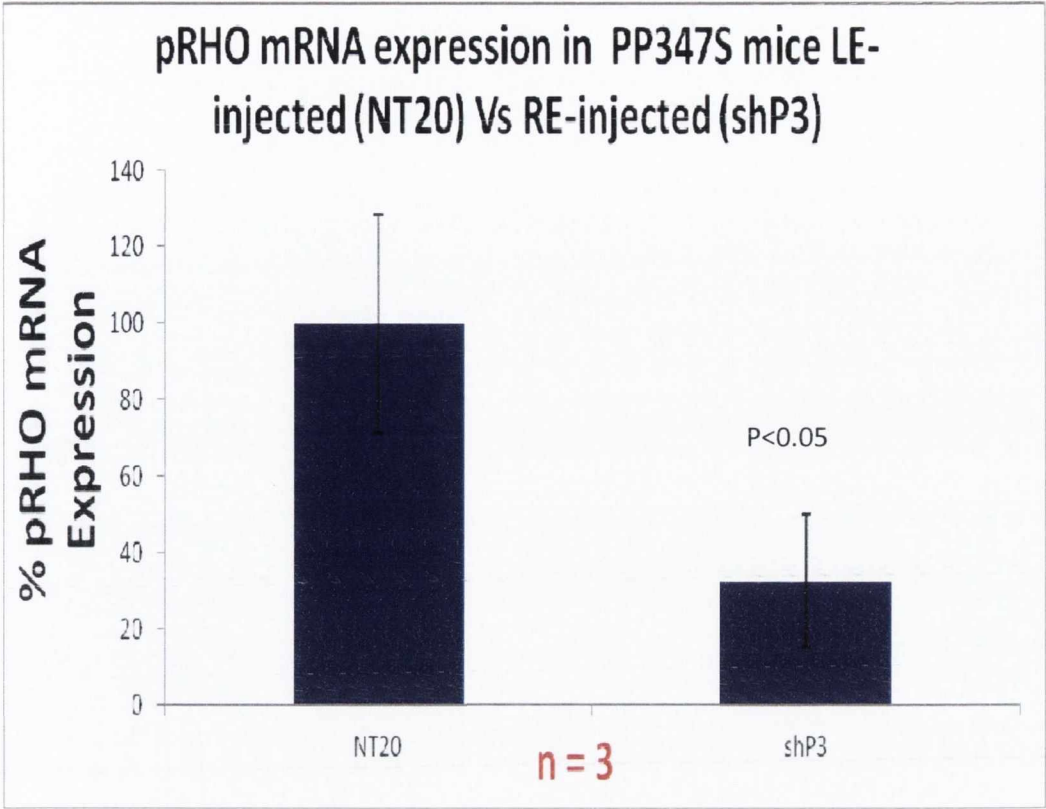
Figure 5.3.8.d The determination of the gate used to sort DRAQ-5 + and EGFP positive cells

The above diagram was retinal cells from a transgenic hetPP347S mouse injected with AAV-2/5.NT20. DRAQ-5 positive healthy cells were taken and assessed for EGFP. Lots of EGFP positive cells were obtained (red arrow) in this sample.

PP347S mouse	% green cells	Mean Fluorescence Intensity (Media, MFI)	Cell number obtained
P1 NT20 (LE)	6.3	4971	4,808
P1 shP3 (RE)	5.8	5035	7,198
P2 NT20 (LE)	8.4	5559	10,000
P2 shP3 (RE)	4.8	3440	2858
P3 NT20 (LE)	1.3	3609	9833
P3 shP3 (RE)	4.3	4598	7801
P4 NT20 (LE)	13.5	5440	7205
P4 shp3 (RE)	36.2	7496	28,589
P5 NT20 (LE)	11	7137	7186
P5 shP3 (RE)	21.6	6751	15500
P6 NT20 (LE)	10.1	5288	6360
P6 shP3 (RE)	14.5	5995	6280

Table 5.3.8 EGFP positive cells and cell numbers obtained 14 days post-injection of heterozygous PP347S mice

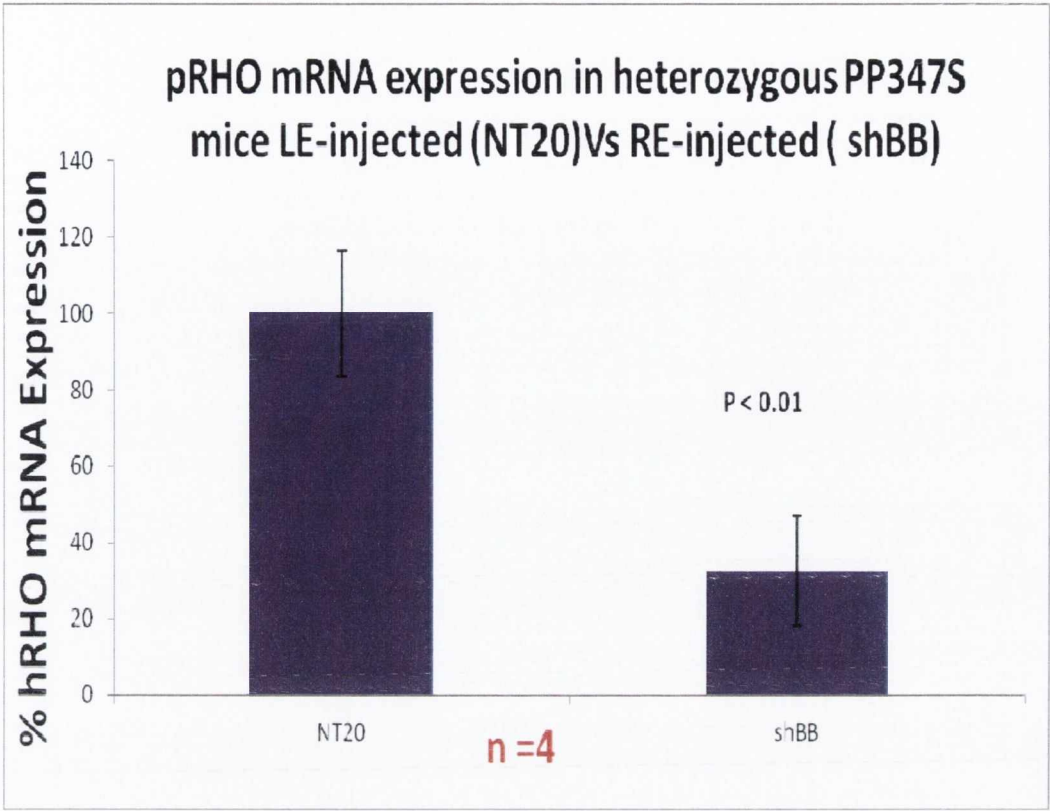
Each retina sample was analysed 14 days post-injection for the % of EGFP positive cells present in each sample compared to the % of EGFP negative cells. The cell numbers obtained were between 2,500-29,000 EGFP positive cells.



Sample	hetPP347S with NT20	hetPP347S with shP3
%NT20	100	33
%shP3	27	18

Figure 5.3.10.a The % pRHO mRNA expression in heterozygous PP347S mice injected with AAV-2/5.NT20 and AAV-2/5.shP3.

pRHO (P1) specific primers were used to amplify pRHO RNA from each retina samples. pRHO expression from mice -injected with AAV-2/5. NT20 (LE) was set at 100% and the pRHO expression from mice with AAV-2/5.shP3 (RE) was expressed as a % of this value. Averages and standard deviations were calculated using Microsoft Excel. A paired t-test was used to assess statistical significance (DataDesk 6.0) Error bars represent % SD values.



Sample	Heterozygous PP347S with NT20	Heterozygous PP347S with shP3
% of NT20	100	33
	16	15

Figure 5.3.11.a The % pRHO mRNA expression in heterozygous PP347S mice injected with AAV-2/5.NT20 (NT20) and AAV-2/5.shBB (shBB)

pRHO (P1) specific primers were used in each reaction to amplify pRHO RNA only in each retina. The average and standard deviation of each data set were calculated using Microsoft Excel. pRHO expression from mice LE-injected with NT20 was set at 100% and the pRHO expression from mice RE-injected with shBB was expressed as a % of this value. Statistical significance was assessed using a paired t-test (Datadesk 6.0) Error bars represent % SD values.

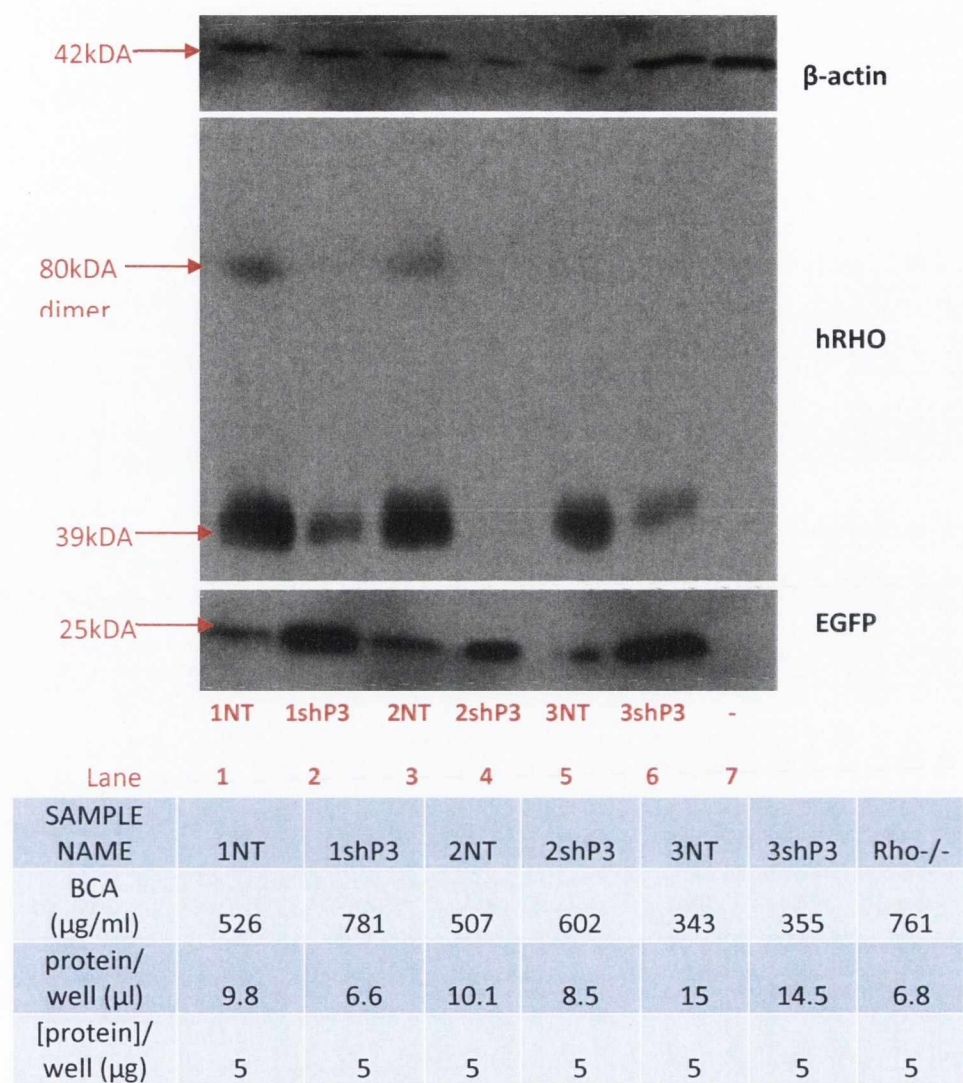


Figure 5.3.12.a RHO protein expression in the hetPP347S mouse injected with AAV-2/5.NT20 & AAV-2/5.shP3

Three hetPP347S mice were subretinally injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE) and 14 days post-injection RHO protein expression was analysed using 4D2. Abundant RHO expression was present in the NT20 injected eyes (1NT [lane 1], 2NT [lane 3] & 3NT [lane5]). In contrast lower levels of RHO immunoreactivity was visible in shP3 injected eyes (1shP3 [lane2], 2shP3 [lane4] & 3shP3 [lane 6]). Retinal protein from a 129rho^{-/-} mouse (Rho^{-/-}[lane 7]) was used as a negative control. Both β-actin and EGFP were used to assess equal loading.

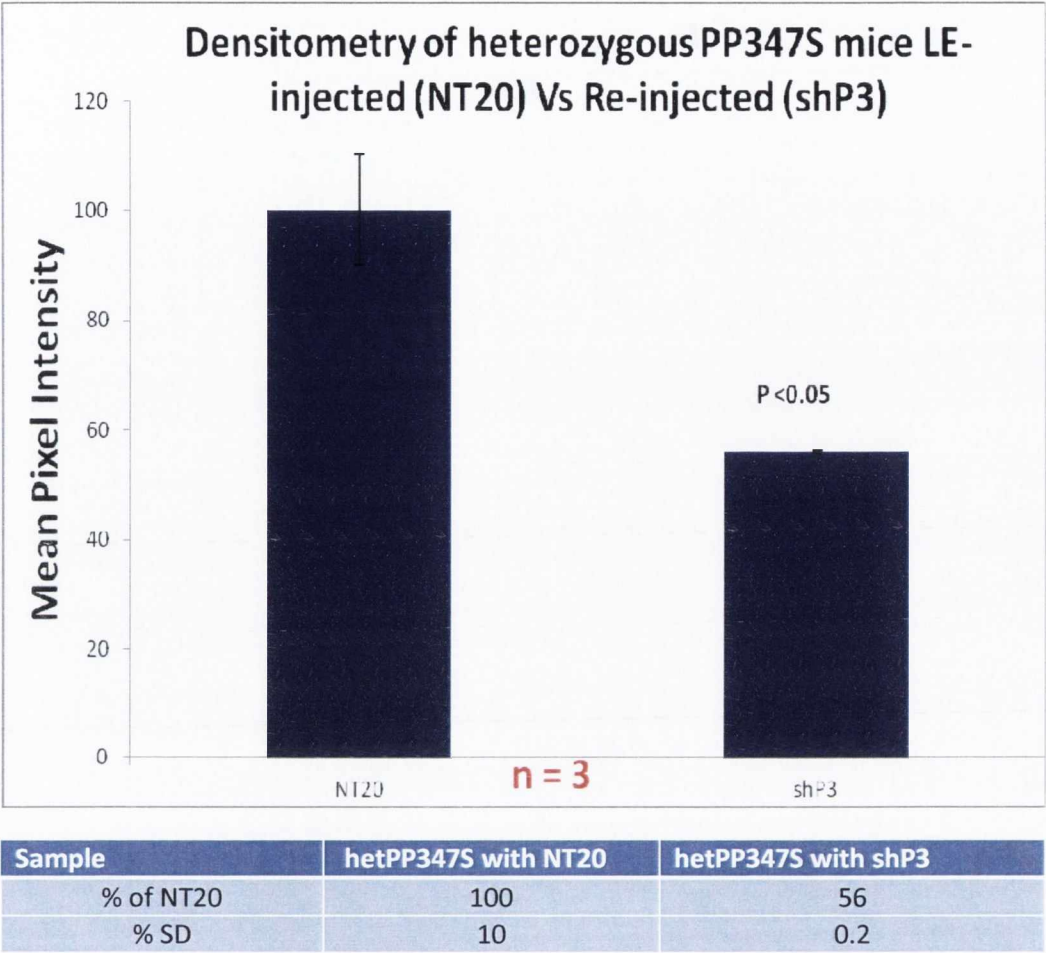


Figure 5.3.12.b Densitometric analysis of RHO protein expression from hetPP347S mice injected with AAV-2/5.NT20 (LE) and AAV-2/5.shP3 (RE)

The mean pixel intensity of RHO protein in each sample was calculated using image J and normalised to the mean pixel intensity of mouse β -actin protein from the same western blot. RHO protein expression for AAV-2/5.NT20 injected eyes was set at 100% expression and RHO protein exp using Microsoft Excel. RHO protein expression from AAV-2/5.shP3 injected eyes was compared to this value. Error bars represent % SD values.

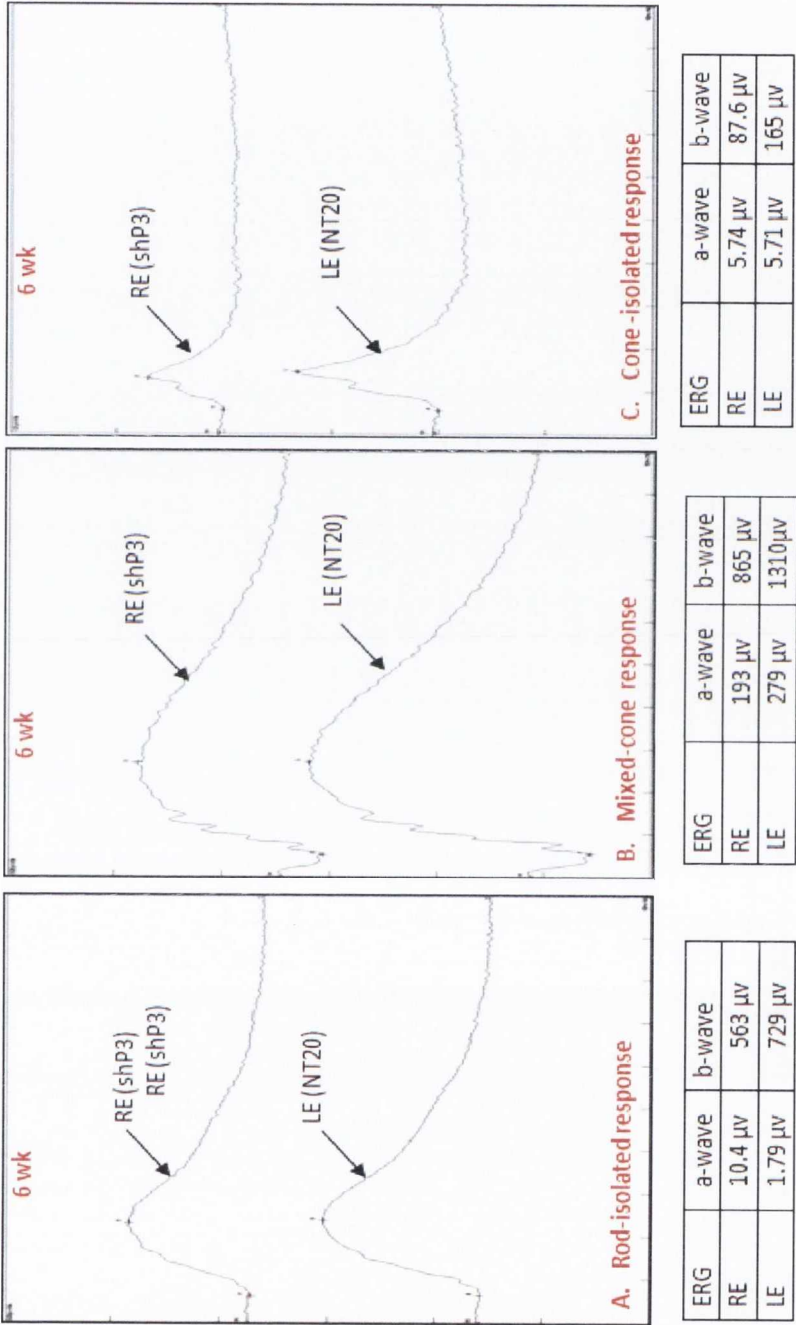


Figure 5.3.13.a ERG response of the hetPP347 (litter 1) mouse injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE) at 6 weeks. The a-wave and b-wave values are typical for this litter.

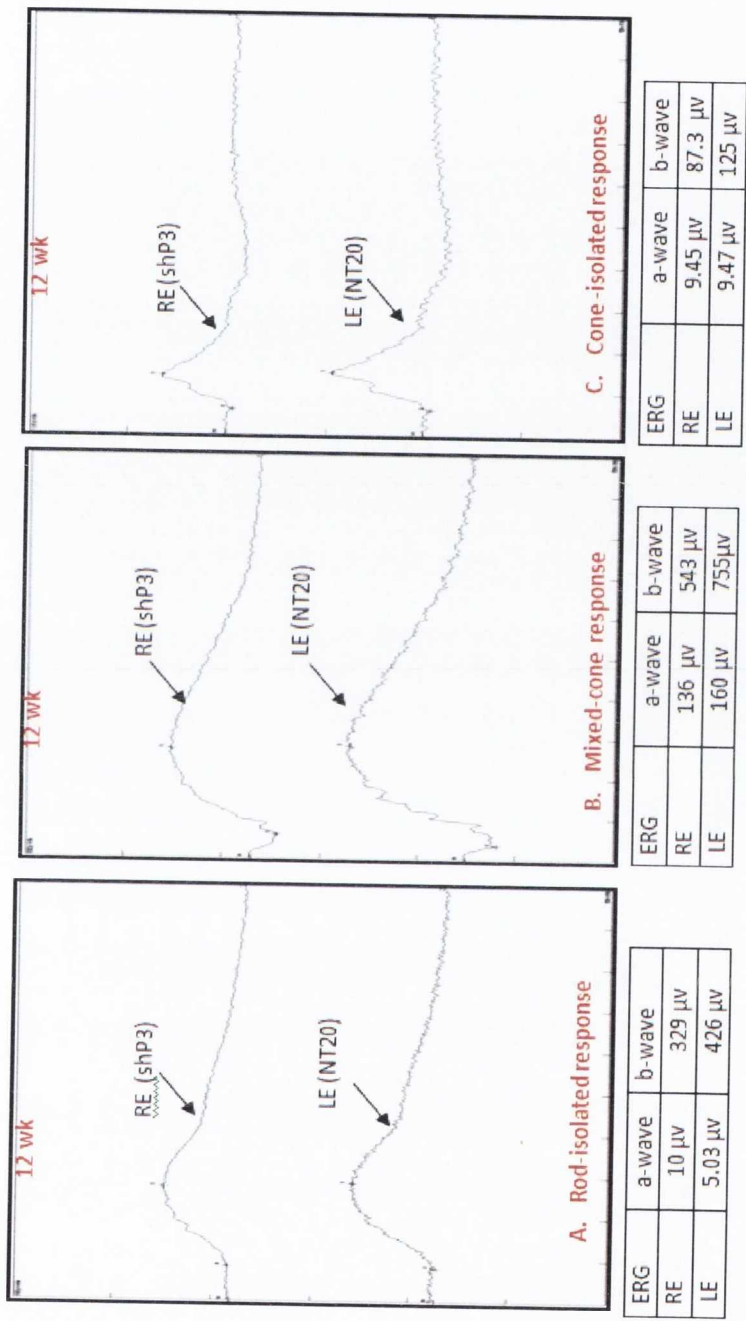


Figure 5.3.13.b Representative ERG response of the hetPP347 (litter 1) mouse injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE) Rod-isolated response, B. Mixed-cone response C. Cone-isolated response. The hetPP347S mice were injected with AAV-2/5.shP3 and AAV-2/5.NT20 and ERG analysis was undertaken at 6 weeks and again at 12 weeks (shown here). The a-wave and b-wave values are typical for this litter.

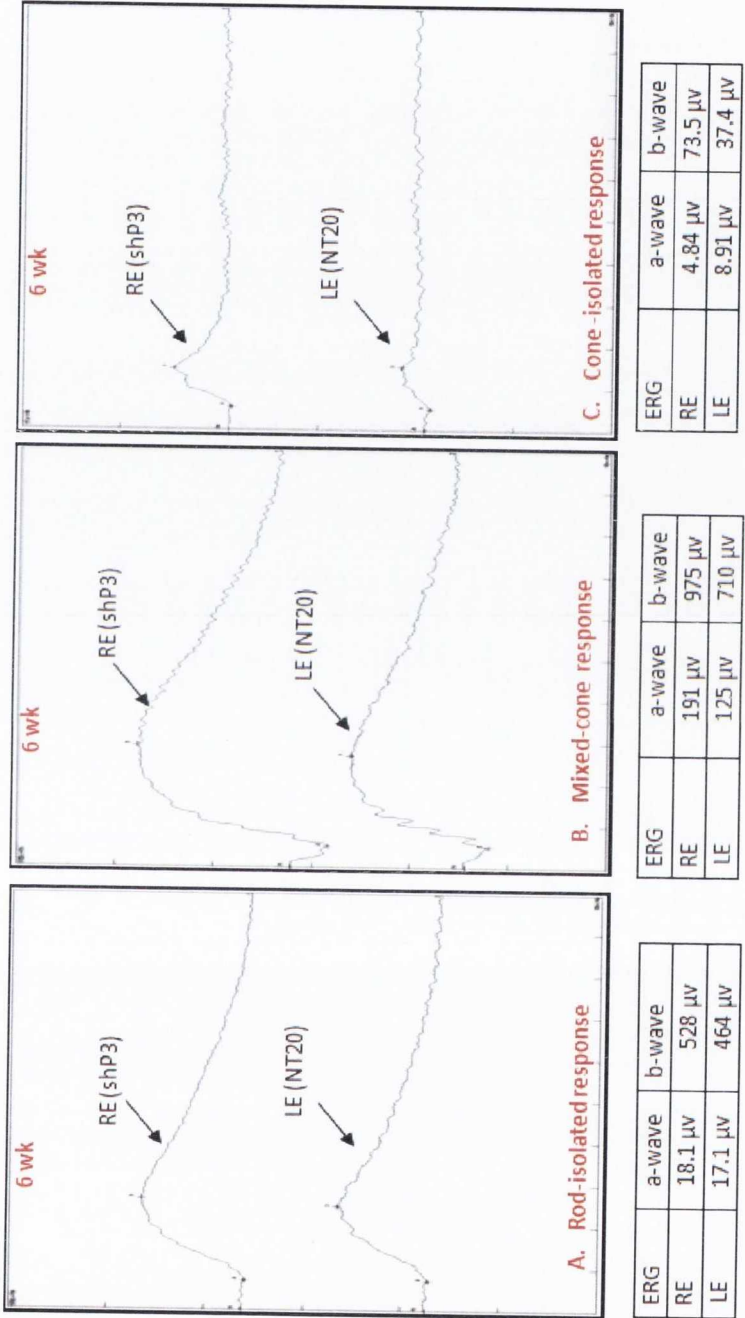


Figure 5.3.13.c ERG response of the hetPP347 mouse (litter 2) injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)

A. Rod-isolated response, B. Mixed-cone response C. Cone-isolated response. The hetPP347S mice were injected with AAV-2/5.shP3 and AAV-2/5.NT20 and ERG analysis was undertaken at 6 weeks (shown here) and again at 12 weeks (shown here). The a-wave and b-wave values are typical for this litter.

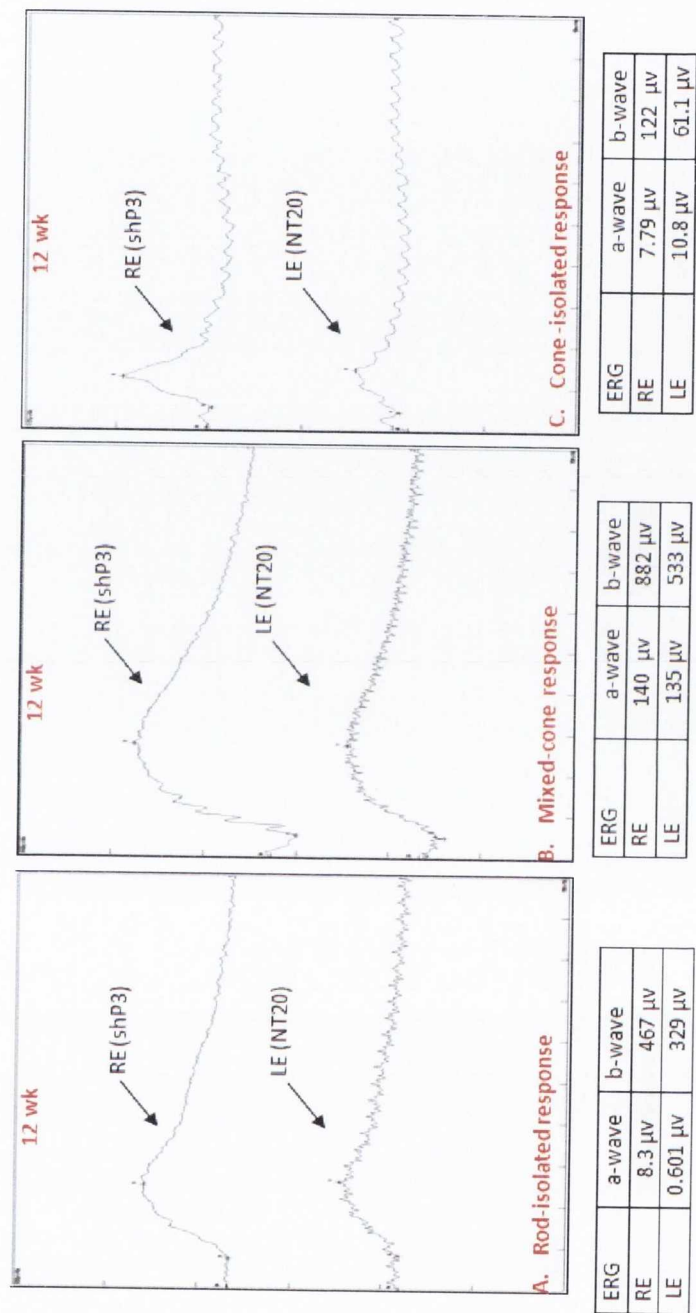


Figure 5.3.13.d A representative ERG response litter 2 of the hetPP347 mouse injected with NT20 (LE) & shP3 (RE).

A. is the rod-isolated response, B. is the mixed-cone response and C. is the cone-isolated response. The hetPP34&s was injected with shP3 and NT20 and ERG analysis was undertaken at 6 weeks and again at 12 weeks (shown here). The a-wave and b-wave values are a typical for this litter.

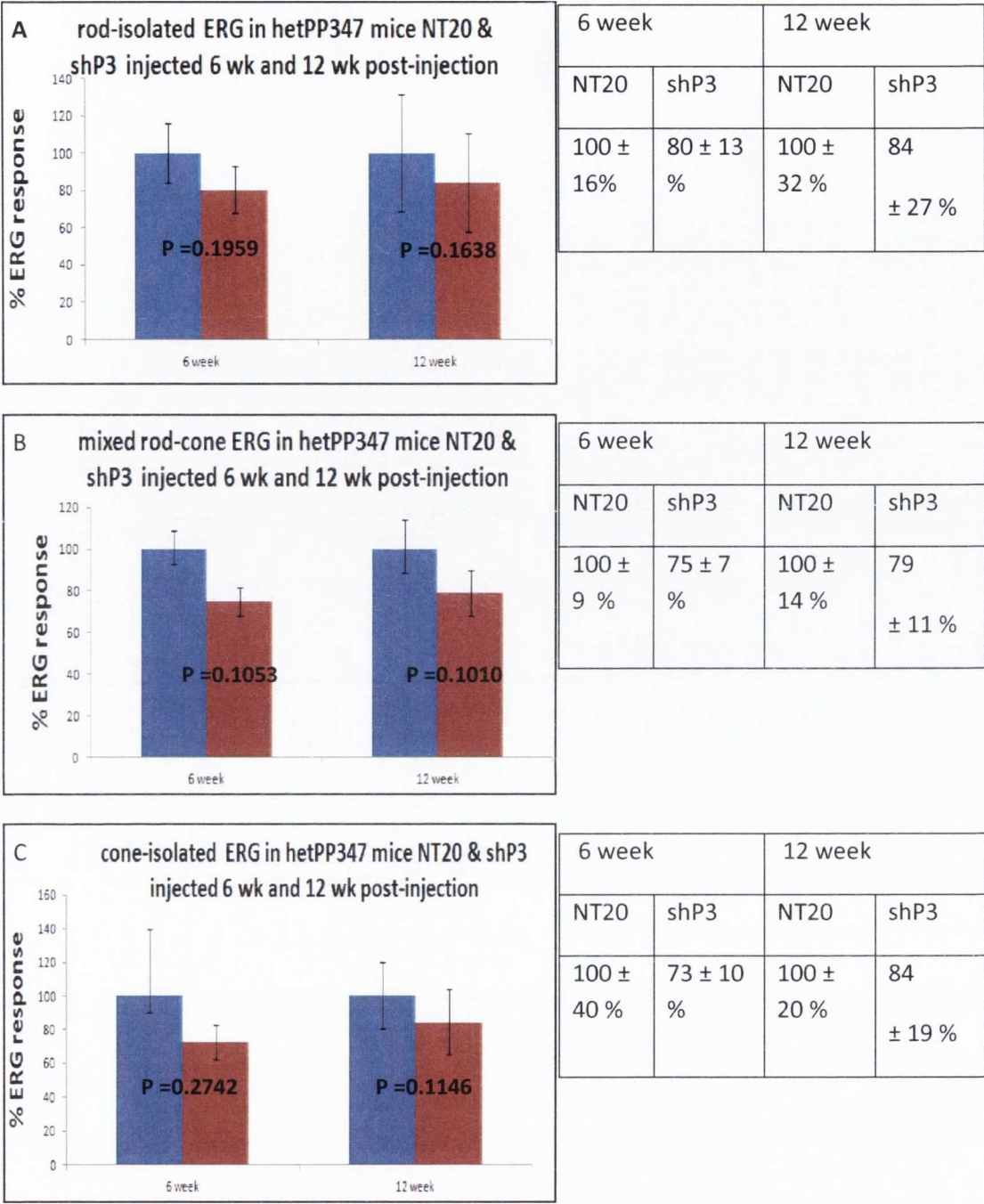


Figure 5.3.13.e Rod-isolated, mixed rod-cone and cone-isolated responses for hetPP347S mice (litter 1) injected with AAV-2/5.NT20 and AAV-2/5.shP3

The b-wave responses for each ERG test (rod-isolated [A], mixed rod-cone [B] and cone-isolated[c]) for each construct were combined , the average and standard deviation calculated for both 6 wk and 12 wk time-points using Microsoft Excel. For mice injected with AAV-2/5.NT20 (blue) the average b-wave for each response was set at 100%. The average b-wave responses for mice injected with AAV-2/5.shP3 (red) were expressed as a percentage of this value. The statistical significance was assessed using a paired t-test (Datadesk 6.0) Error bars represent % SD

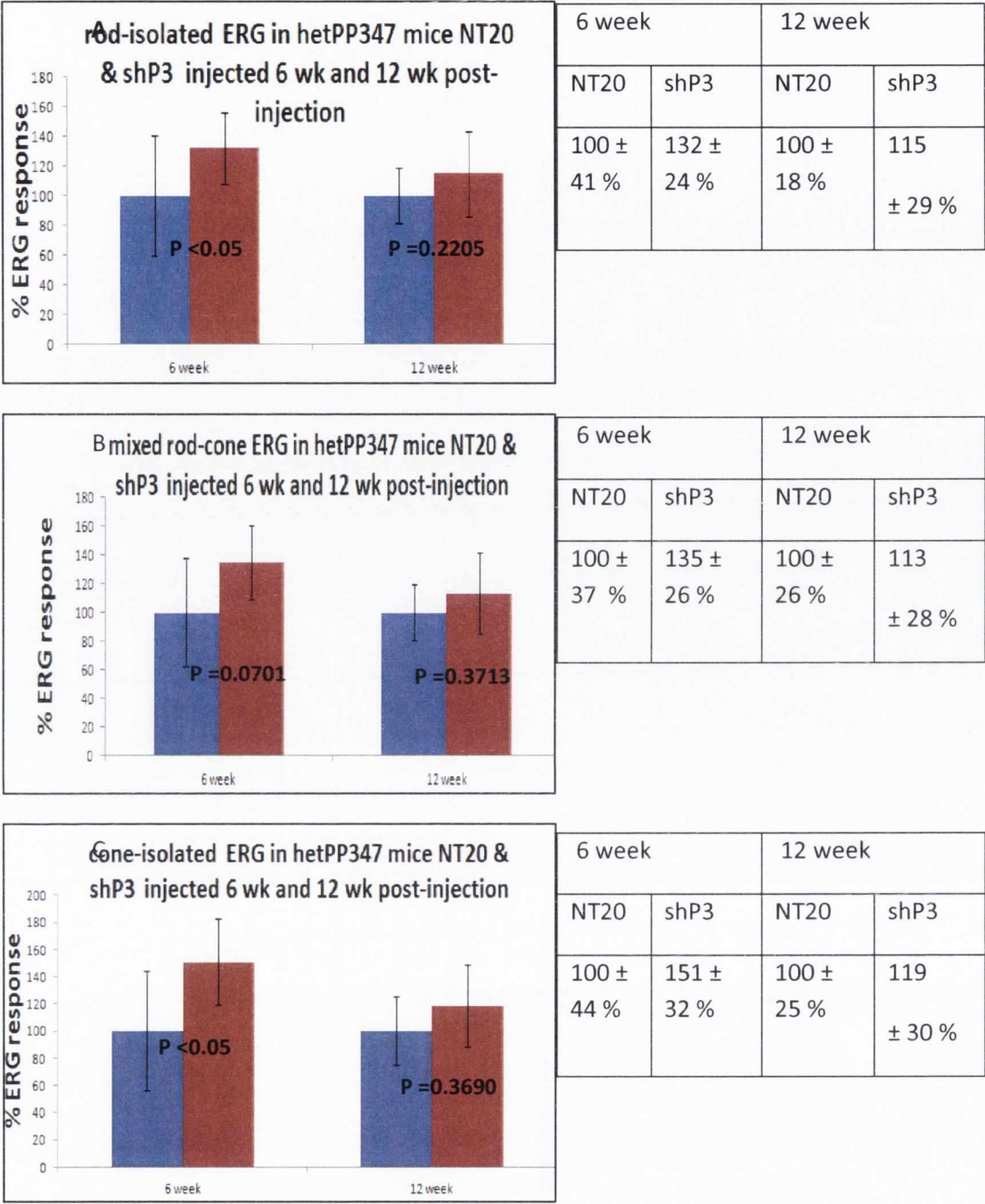


Figure 5.3.13.f Rod-isolated, mixed rod-cone and cone-isolated responses for hetPP347 litter 2 injected with AAV-2/5.NT20 and AAV-2/5.shP3

The b-wave responses for each ERG test (rod-isolated [A], mixed rod-cone [B] and cone-isolated [C]) for each construct were combined and the average and standard deviation calculated for both 6 wk and 12 wk time-points using Microsoft Excel. For mice injected with NT20 (blue) the average b-wave for each response was set at 100 %. The average b-wave responses for mice injected with shP3 (red) were expressed as a percentage of this value. The statistical significance was assessed using a paired t-test (Datadesk 6.0) Error bars represent % SD

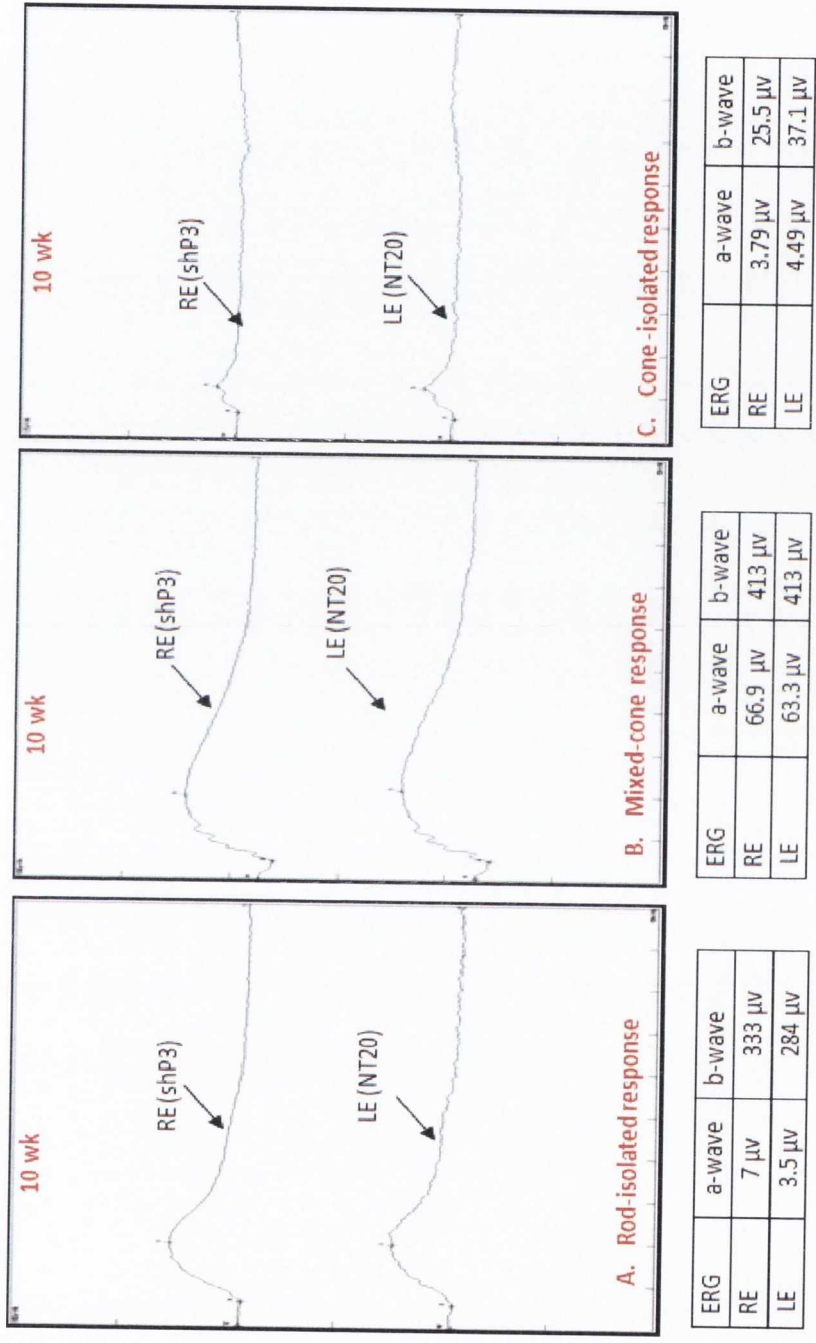


Figure 5.3.12.g ERG response of the hetPP347 mouse (litter 3) injected with AAV-2/5.NT20 (LE) & AAV-2/5.shp3 (RE)

A. Rod-isolated response, B. Mixed rod-cone response C. Cone-isolated response. The hetPP347S mice were injected with AAV-2/5.shp3 and AAV-2/5.NT20 and ERG analysis was undertaken at 10 weeks (shown here) and again at 14 weeks. The a-wave and b-wave values are typical for this litter.

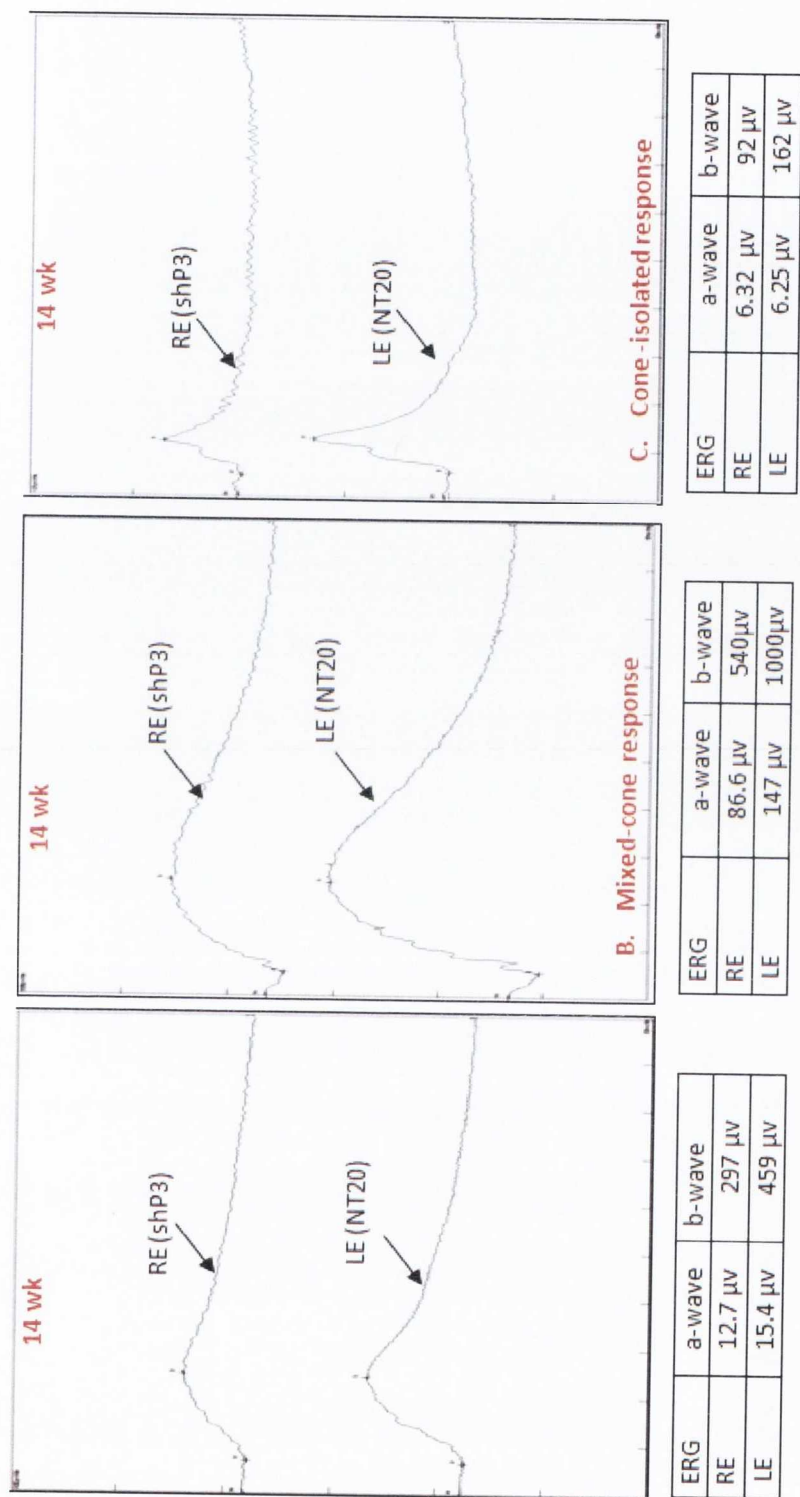


Figure 5.3.13.h ERG response of the hetPP347 mouse (litter 3) injected with AAV-2/5.NT20 (LE) & AAV-2/5.shP3 (RE)

A. Rod-isolated response, B. Mixed rod-cone response C. Cone-isolated response. The hetPP347S mouse was injected with AAV-2/5.shP3 and AAV-2/5.NT20 and ERG analysis was undertaken at 10 weeks and again at 14 weeks. (shown here). The a-wave and b-wave values are typical for this litter. At 14 weeks all b-wave responses are lower in the shP3 injected eye. This is particularly apparent in the rod-isolated and mixed rod-cone responses.

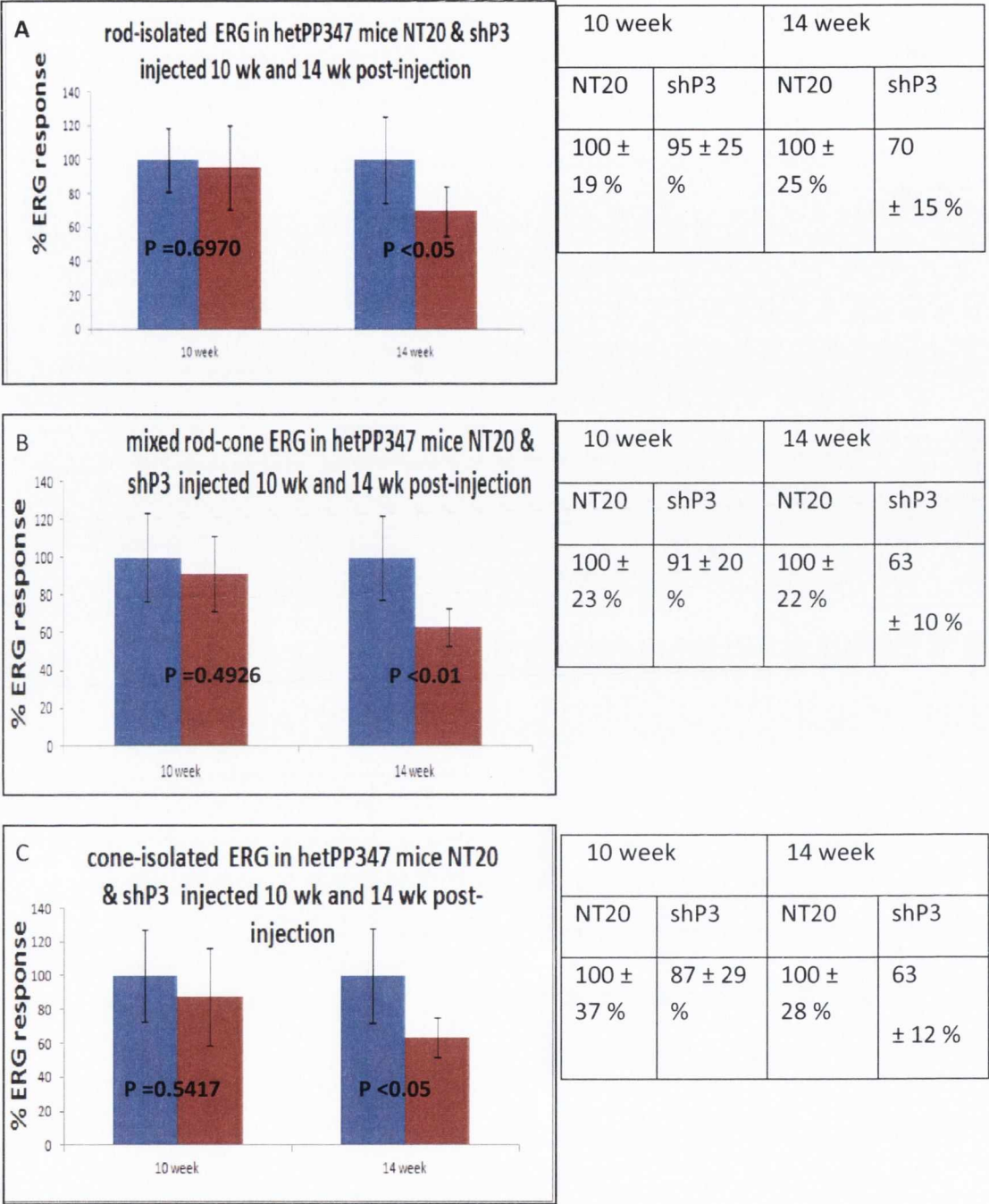


Figure 5.3.13.i Rod-isolated, mixed rod-cone and cone-isolated responses for hetPP347 litter 3 injected with NT20 and shP3

The b-wave responses for each ERG test (rod-isolated [A], mixed rod-cone [B] and cone-isolated [C]) for each construct were combined and the average and standard deviation calculated for both 10 wk and 14 wk time-points using Microsoft Excel. For mice injected with AAV-2/5.NT20 (blue) the average b-wave for each response was set at 100%. The average b-wave responses for mice injected with AAV-2/5.shP3 (red) were expressed as a percentage of this value. The statistical significance was assessed using a paired t-test (Datadesk 6.0) Error bars represent % SD

AAV-2/5.NT20 EGFP + AAV-2/5.NT20 EGFP - AAV.2/5.shP3 EGFP - AAV-2.5.shP3 EGFP+

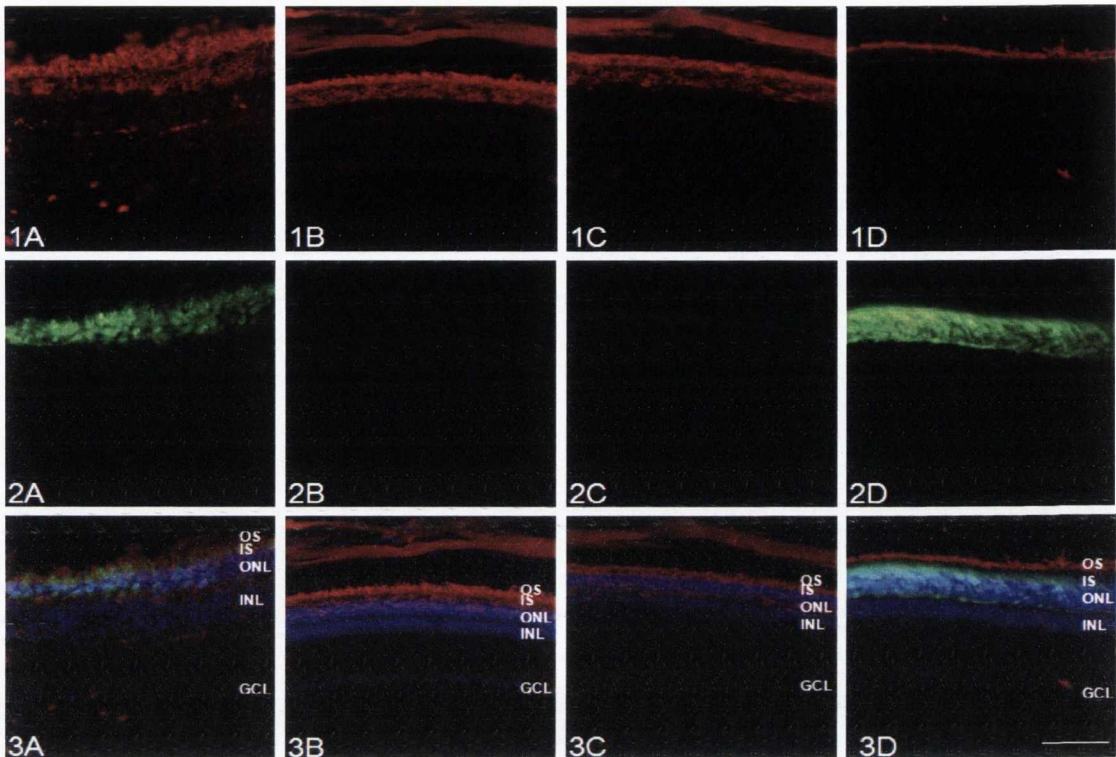
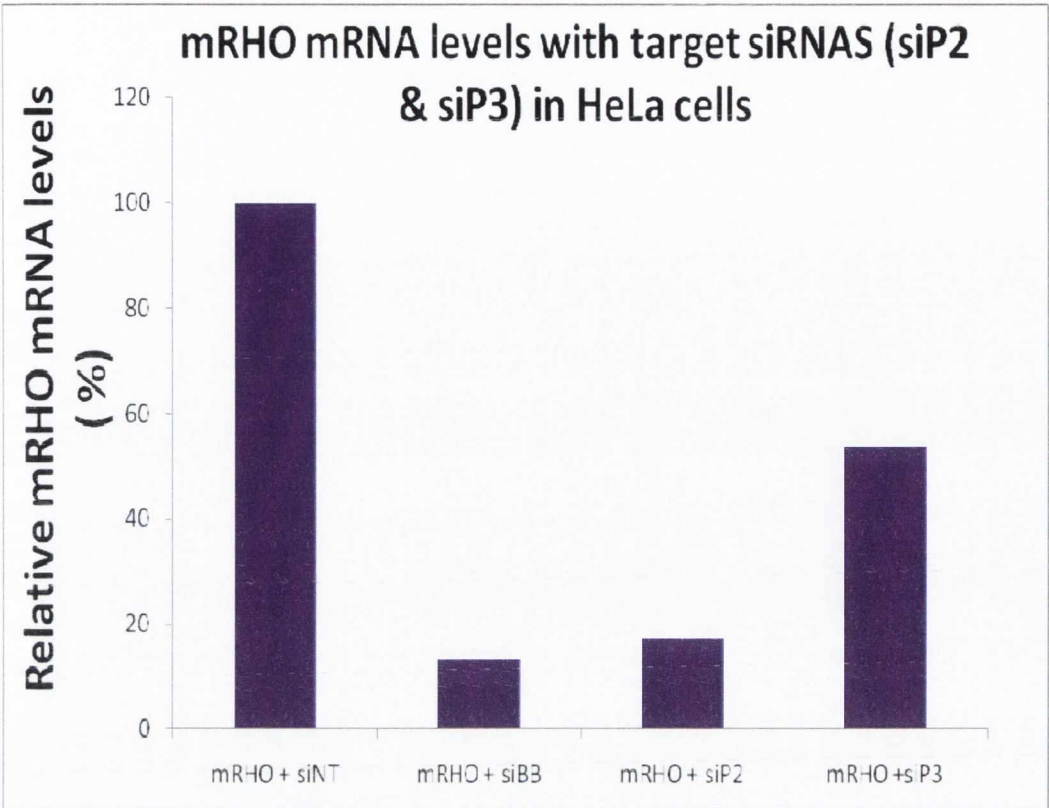


Figure 5.3.14.a Immunohistochemistry of hetPP347S eyes LE-injected (AAV-2/5.NT20) Vs RE injected (AAV-2/5.shP3)

The above animal was taken at 12 weeks post-injection. Row 1 represents RHO staining, row 2 represents EGFP and row 3 is an overlay of RHO, EGFP and DAPI from each section. Column A (1A, 2A & 3A) is from an EGFP positive region of the heterozygous PP347S retina injected with NT20. , RHO is clearly present (1A) in the ONL, IS and OS and overlaps with both EGFP (2A) staining and DAPI staining (3A). Column 2 is the same retina from column 1 (PP347S injected with NT20) but in an EGFP negative region (2A, 2B & 2C). RHO staining is clearly visible in the ONL, IS and OS, comparable to column 1. Column 3 is an EGFP negative region from the retina of a heterozygous PP347S mouse injected with AAV-2/5.shP3 (3A, 3B& 3C), RHO is clearly labeled in the ONL, IS and OS. Column 4 represents the same retina as column 3 but in an EGFP positive region injected with shP3 (4A, 4 B & 4C). RHO immunostaining is less than observed in controls (1, 2 & 3) and is present in the outer segments only compared to the NT20 figures and the shP3 EGFP negative figures of the same retina. OS=outer segments, IS=inner segment, ONL=outer nuclear layer, INL=inner nuclear layer and GCL=ganglion cell layer. Scale bar (L) represents 116 μM.



sample	hRHO +siNT	hRHO + siBB	hRHO + siP2	hRHO + siP3
% of siNT	100	13.1	17.4	53.7

Figure 5.3.15.a. Real-time RT-PCR evaluation of mRHO expression in HeLa cells co-transfected with mRHO and each siRNA

Relative mRHO mRNA levels in HeLa cells following co-transfection with mRHO and siNT (a non-targeting siRNA), siBB (a positive control), siP2 and siP3 in HeLa cells. The average and standard deviation was calculated by Microsoft Excel. The data are presented as percentages of rhodopsin mRNA levels in RNA samples co-transfected with wild-type mRHO and the non-targeting siRNA (siNT, 100%). Statistical significance was assessed using a two-sample t-test (Datadesk6.0). Error bars represent SD values.

**Chapter 6: Optimisation of a hRHO
replacement gene both *in vitro* and *in vivo*
for use in a suppression and replacement
therapeutic strategy**

6.1 Introduction

One major issue when developing a mutation-independent therapy for autosomal dominant disorders such as RHO-adRP or peripherin-linked adRP is suppression abolishes expression from both the mutant and wild-type alleles (Millington-Ward et al, 1997). This is of particular concern for rhodopsin in which the total depletion of RHO in transgenic mice results in a severe retinal degeneration phenotype (Humphries et al, 1997). However, this problem can be alleviated by the simultaneous delivery of a replacement gene refractory to suppression by the RNAi molecule. Historically for RNAi this has involved utilising of the degeneracy of the genetic code to alter synonymous nucleotides within each codon such that although the nucleotide sequence has been altered the amino acid code remains unchanged (Millington-ward et al, 1997; O'Reilly et al, 2007). This suppression and replacement therapy has been particularly efficient in the retina in which successful suppression and replacement both *in vitro* and *in vivo* has been undertaken with genes including rhodopsin (Millington-ward et al, 2007; Millington –ward et al, 2011; O'Reilly et al, 2007; O'Reilly et al, 2008; Palfi et al, 2010). A critical factor in designing a replacement gene is obtaining appropriate levels of replacement gene expression. On one hand, suppression of the mutant allele (in addition to the wild-type allele), followed by an insufficient level of expression of the replacement gene may result in too little rhodopsin in the photoreceptors. This in itself may have a negative impact on the treated cell type. Therefore ways of increasing gene expression to close to wild-type levels are needed (Millington-Ward et al, 1997). On the other hand, over-expression of the replacement allele may also have a negative impact on phenotype. As mentioned previously, in studies by Olsson et al, 1992, 5-fold over-expression of normal human rhodopsin, resulted in a retinopathy in mice similar to that seen in P23H models of RP (Olsson et al, 1992). This indicates maintaining correct balance between having too little and too much replacement gene expression is crucial to any RHO-based suppression and replacement therapy. Therefore a detailed analysis of factors which may influence gene expression such as codon usage, promoter, enhancers and viral delivery are required.

6.1.1 Codon Usage analysis

As nucleotide changes are only incorporated into the degenerative position of each codon within the siRNA target site such that the protein sequence is still maintained, it would not be expected that any impact on protein function would be observed from these synonymous mutations. However since the 1980's unequivocal evidence has been building suggesting changes to degenerative nucleotides can have significant effects on gene expression, protein folding and function (Angov et al, 2011). Initial studies with prokaryotes established that highly expressed genes, show codon bias and incorporate

preferred codons. This correlates with the fact that more abundant isoacceptor tRNAs for these preferred codons were available within the cell and supports a model in which highly expressed genes are more likely to utilise a codon if the tRNA for that codon is abundant in order to increase translational efficiency (Gouy & Gautier 1982; Ikemura et al, 1981; Ikemura et al, 1985). Furthermore evidence of this codon bias has been shown in *C.elegans*, *D.melanogaster* and *X.laavis* (Duret, 2000; Duret, 2002; Musto et al, 2001). There has been controversy surrounding codon bias in mammals that cannot be explained by translational efficiency alone, it is now emerging that factors such as GC content in isochore structures, mRNA secondary structure and stability are also subject to codon usage bias (Angov et al, 2011; Bernardi, 2000; Chamary & Hurst, 2005; Shabalina et al, 2006b). Recently a study has demonstrated in humans the importance of maintaining preferred codon usage. A synonymous mutation within the human MDR1 gene, which codes for a P glycoprotein (P-gp), was found to result in altered substrate specificity when expressed in various cell lines. This is an important protein involved in drug metabolism and has been implicated in the multidrug resistance of cancer cells. Further investigations established that while this protein was of similar length and had similar expression levels compared to the wild-type, it had an altered protein conformation. The authors hypothesised that the likely reason for this altered protein product was due to the timing of translation. As the cell produces more P-gp, the t-RNA species for preferred codons become depleted. The incorporation of a rarer codon caused translation to slow down; this may allow time for the region of the peptide chain that has already been translated to fold into an alternative structure, thus altering protein conformation (Kimchi-Sarfaty et al, 2007). Thus minimising the potential for codon bias by altering the degenerative nucleotides within siRNA target sites for rhodopsin with preferred codons would be beneficial and has been undertaken in previous studies in which rhodopsin replacement genes were generated (O' Reilly et al, 2007).

6.1.4 Choice of AAV serotype

A major consideration for gene therapy to the retina using AAV is ensuring the correct and most efficient serotype is chosen for the specific cell type and species chosen for evaluation (Vandenberghe & Auricchio, 2012). Previously for hRHO suppression and hRHO replacement vectors AAV-2/5 has been used (Chadderton et al, 2009; Millington-ward et al, 2011; O'Reilly et al, 2007; O'Reilly et al, 2008; Palfi et al, 2010). However, as outlined in Section 1.22 since the initiation of these hRHO based suppression studies, a novel serotype AAV-2/8 has emerged as a potent transducer of the photoreceptor cells in mice, conferring high transgene expression compared to AAV-2/5 (Allocca et al, 2007; Lebherz et al, 2008 Natkunarajah et al, 2008).

In dogs subretinal injections of AAV-2/8 was found to efficiently transduce the RPE, the photoreceptors, the inner retina, the ganglion cell layer and the brain (Stieger et al, 2008). Of particular relevance to the current study, was a recent evaluation of subretinal injection of both AAV-2/5 and AAV-2/8 in the porcine retina. Robust AAV-2/5 and AAV-2/8 transgene expression was achieved in the RPE and photoreceptors with both serotypes. Furthermore cells in the inner retina were also efficiently transduced. In contrast to the rodent retina, the pig retina is cone-rich. PNA staining of the cones in this model demonstrated that in addition to the rods, the cones were efficiently transduced. Overall AAV-2/8 transgene expression was approximately five times higher compared to AAV-2/5, indicating that similar to observations in mice, for the porcine retina AAV-2/8 may be the vector of choice. No aberrant immunological responses or toxicity was observed. In terms of biodistribution AAV-2/8 was observed in the retinas and optic nerves but not the brain (Mussolino et al, 2011b). AAV-2/2 transduction compared to AAV-2/8 has also been evaluated in cynomolgus macaques; this was of particular interest as macaques have a fovea, a feature of the retina confined to primates. AAV-2/8 was found to transduce the RPE and photoreceptors efficiently at lower doses compared to AAV-2/2 (transduction was only observed at a tenfold higher dose). While typically transduction was restricted to rods, however, at high doses of both AAV-2/5 & AAV-2/2 cone transduction occurred, particularly in the cone rich fovea (Vandenberghe et al, 2011). This is in contrast to dogs and pigs in which efficient cone transduction occurred and highlights the importance of testing each AAV serotype in a wide range of species. In addition, increased anti-AAV neutralising antibodies were present both locally and systemically in this study. A systemic t-cell response was also observed in response to the AAV transgene in animals injected with high dose AAV and inflammation in the retina was visible in these animals due to either high doses of AAV and/or high doses of the EGFP transgene. The authors noted however, that no toxicity has ever been reported for the LCA clinical trials with AAV-2/2. Furthermore the EGFP transgene is foreign to the host thus is different to a clinical setting in which an endogenous gene would most likely be administered. As AAV-2/8 can target both the RPE and photoreceptors it represents an attractive gene vehicle for future *in vivo* analysis

An AAV based transduction strategy, delivering an enzyme involved in lysosomal storage disease (GUSB) to the brain of mice established that in addition to AAV-2/8 and AAV-2/9, AAV-2/rh10 virus was efficient at targeting the brain (Cearley & Wolff, 2006). Furthermore AAV-2/rh10 has been found to confer widespread luciferase transgene expression to both mitotic and non-mitotic cells when administered to neonatal mice at P2, compared to AAV1-AAV-9. This suggested that AAV-2/rh10 might be an efficient viral vector for gene therapy studies to the CNS (Hu et al, 2010). This finding was further supported in studies comparing AAV-2/1, AAV-2/2, AAV-2/rh8, AAV-2/9 and AAV-

2/rh10 EGFP mediated delivery to the striatum of rats and pigs via convection enhanced delivery (White et al, 2011). In this study efficient AAV-2/rh10 based transgene expression was observed in the rat striatum, however in the pig striatum compared to AAV-2/rh8, AAV-2/1 and AAV-2/9 transduction was relatively poor. The discrepancy in the rat versus pig brain demonstrates the need to evaluate viral transduction in various species (White et al, 2011). In terms of AAV-2/rh10 in the eye, two recent studies established efficient transduction of AAV-2/rh10 to the retina. Giove et al delivered AAV-2/rh10 via intravitreal injection to the mouse retina and efficient transduction of the ONL and RPE was observed. Delivery to the inner retinal cells including the bipolar and horizontal cells in the outer plexiform layer was also achieved (Giove et al, 2010). Mao et al 2011, intravitreally delivered an AAV-2/rh10 virus expressing Bevacizumab (an anti-VEGF monoclonal antibody used in therapy for AMD or diabetic retinopathy) to provide long term stable expression which was confined to the RPE. Furthermore this therapy provided a 90% decrease in neovascularisation in a transgenic mouse model, demonstrating that AAV-2/rh10 can provide therapeutic efficacy in the retina. The discrepancy between RPE only transduction in this study and the widespread retinal transduction observed by Giove et al, 2010 may possibly be due to the use of a secreted transgene in the Mao et al, study as opposed to intracellular GFP in the Giove et al, study. In addition, differing mouse strains or the fact that the AAV-2/rh10 receptors are unknown may also have a role (Giove et al, 2010; Mao et al, 2011). These studies suggest that in addition to AAV-2/8, AAV-2/rh10 may be a likely candidate for efficient photoreceptor transduction.

6.1.5 Choosing the correct promoter

While many AAV serotypes drive robust transgene expression in photoreceptors there has to date been no photoreceptor specific AAV serotype, an important issue in ensuring transgene expression is restricted to the correct target when designing human clinical trials. While selecting various AAV serotypes with different tropisms can aid in this restriction, the choice of promoter or other regulatory elements can offer a significant level of control in terms of transgene expression (Karali et al 2011). Constitutive promoters such as the CMV/CBA promoters have been utilised with success in the retina to drive transgene expression in the RPE, the photoreceptors and the inner retina for AAV serotypes such as AAV-2/2, AAV-2/5 or AAV-2/8 in species such as mouse, rats, dogs, pig and monkey (Acland, 2005; Allocca et al, 2007; Beltran et al, 2010; Leberherz et al, 2008; Mussolino et al, 2011a; Pang et al, 2008; Vandenberge et al, 2011a; Yang, 2002b). Additionally the CBA promoter has been utilised for AAV-2/2 delivery of RPE65 to human LCA patients with no toxicity reported (Maguire et al, 2008). Palfi et al, 2010 utilised the CMV promoter to drive robust expression of EGFP in

photoreceptors, however, the authors noted that utilising the mRHO proximal promoter resulted in a five times higher expression of EGFP, thus the mRHO promoter was utilised for subsequent studies (Palfi et al, 2010).

The rhodopsin or rhodopsin kinase promoter has been utilised to drive photoreceptor specific transgene expression in the retina with various AAV serotypes (Allocca et al, 2007; Mussolino et al, 2011b; O'Reilly et al, 2008; Palfi et al, 2010; Young et al, 2003). For hRHO, transgene expression has been particularly effective using a region of the hybrid rhodopsin promoter to drive a hRHO transgene with AAV-2/5. Furthermore hRHO expression was sufficient to provide therapeutic benefit in murine models of RHO-adRP (Millington-ward et al, 2011; Palfi et al, 2010). However some of the promoters outlined in the various studies above result in transduction of not only rods but also cones, as additional regulatory elements may be needed to restrict expression to rods (Allocca et al, 2007; Glushakova et al, 2006; Karali et al, 2011; Mussolino et al, 2011b). A recent study in canines utilising the mouse opsin promoter (mOP) and human G coupled receptor protein kinase 1 promoter (hGRK1) with AAV-2/5 resulted in rod-only restricted transgene expression except at higher viral titres. In contrast studies in mice with the hGRK1 promoter resulted in expression in rods and cones irrespective of titre; this again highlights the need for evaluating therapies in different species prior to human clinical trials (Beltran et al, 2010; Khani et al, 2007). Several studies have used cone-specific promoters to restrict transgene expression to cones, in particular the cone red opsin promoter has been used with success in mice (Komaromy et al, 2008b; Komaromy et al, 2010). A recent paper utilised a clever mechanism of specific miRNA targets sites to restrict transgene expression. In this study incorporating RPE specific miRNA target sites within a CMV driven EGFP construct resulted in restricted expression of the AAV-2/5 driven EGFP to photoreceptors but not to the RPE in mice and pigs. Similarly incorporating a miRNA expressed in all retinal layers except the RPE into this construct resulted in RPE only restricted expression in mouse and pig. This represents a novel approach utilising the RNAi machinery to alter and confine AAV mediated delivery to specific cell types (Karali et al, 2011).

6.16 RNAi mediated suppression and replacement in the retina for adRP

The suppression and replacement strategy has been successfully employed both *in vitro* and *in vivo* for several retinal genes (Millington-Ward et al, 1997). Suppression of both mouse and human peripherin was demonstrated *in vitro* in COS-7 and *ex vivo* in mouse retinal explants by Palfi et al, 2006. In addition, generation of a replacement peripherin gene using the degeneracy of the genetic code resulted in protection of the peripherin replacement gene from RNAi based suppression (Palfi et al, 2006).

adRP mutations in the neural leucine zipper gene (NRL), an important photoreceptor specific transcription factor have been identified (Bessant et al, 1999; Hernan et al, 2011b). Subsequently shRNAs which suppressed NRL RNA in co-transfection experiments in COS-7 cells were characterised. Furthermore a codon-modified NRL replacement gene was designed (Hernan et al, 2011b). A suppression and replacement based strategy has been utilised with success for hRHO both *in vitro* and *in vivo*. Similar to the experiments described by Palfi et al, 2006 and Hernan et al, 2011b shRNAs for hRHO were designed and this was followed by generation of codon-modified replacement genes refractory to hRHO mediated suppression *in vitro* (Kiang et al, 2005; O'Reilly et al, 2007; O'Reilly et al, 2008). Additionally optimisation of the hRHO replacement to obtain adequate levels of functional RHO protein has been undertaken (Palfi et al, 2010). A transgenic mouse was generated on a mouse rhodopsin null background in which the hRHO transgene contained mismatches to a particular hRHO shRNA sequence. Of note the replacement transgene expressed hRHO to 70% wild-type levels and was fully functional, providing a benchmark level of expression against which future replacement constructs could be measured (O'Reilly et al, 2008). Furthermore, the hRHO gene in this transgenic mouse was resistant to suppression with its corresponding shRNA compared to wild-type hRHO mice (O'Reilly et al, 2007; O'Reilly et al, 2008). In addition AAV-2/5-mediated delivery of a hRHO replacement gene achieved high levels of hRHO expression and provided functional improvement in the 129rho^{-/-} mouse (Palfi et al, 2010). More recently significant suppression of rhodopsin in a transgenic mouse of RHO-adRP in addition to effective replacement of hRHO has been evaluated. Moreover this dual component strategy resulted in therapeutic benefit as measured by histology and ERG analysis (Millington-Ward et al, 2011).

6.1.17 Objectives of chapter six

The research described in Chapters 3-5 focused on optimising efficient suppression strategies for both human and porcine rhodopsin *in vitro* in cell culture (Chapter 3), *in vivo* for wild-type hRHO (Chapter 4) and for mutant pRHO (Chapter 5). However, this suppression strategy represents the first part of a two component therapy for RHO-adRP patients. Therefore the work outlined in this chapter focuses on the optimisation of the second component for this suppression and replacement therapeutic. Replacement genes for hRHO have previously been generated for the most efficient hRHO suppressor (shBB) previously evaluated (Millington-Ward et al, 2011; O'Reilly et al, 2007; Palfi et al, 2010). In the current study siRNAs 100% homologous to hRHO and pRHO have been identified as efficient suppressors of hRHO *in vitro* in HeLa cells. Therefore it was decided to design replacement RHO genes refractory to suppression by siP2 and siP3 respectively and evaluate *in vitro* if they are protected

from RNAi-based suppression. The current best *in vivo* suppressor evaluated as part of the study in this thesis is shP3, thus the main aim of the study described in this chapter was to generate a siP3-based replacement gene and evaluate it *in vivo*. Traditionally AAV-2/5 has been utilised in the TCD laboratory for RHO replacement gene studies, however, more recently AAV-2/8 has emerged as a potent delivery vehicle for photoreceptors. Therefore it was decided to undertake a comparative analysis of AAV-2/5 and AAV-2/8-mediated delivery of the siP3-based replacement gene *in vivo* in rhodopsin mice. The final aim of this study was to initialise preliminary comparative evaluation of hRHO protein expression from AAV-2/5, AAV-2/8 and AAV-2/rh10 based viral vectors with a hRHO replacement gene subretinally injected into wild-type and 129rho^{-/-} mice.

Section 6.2 Materials and Methods

6.2.1. The design of replacement gene resistant to siP2 (rP2) and siP3 (rP3)

In order to design a hRHO replacement gene resistant to siP2 or siP3- mediated cleavage (rP2), the third nucleotide of each codon (the wobble position) within the siP2 or siP3 target site was altered such that while the mRNA sequence was changed, the amino acid sequence encoded by the P2 or P3 target site remained identical. This ensured that the replacement protein with the rP2 or rP3 changes were identical to the endogenous wild-type hRHO protein. A maximum of six nucleotide changes could be made within both the rP2 and rP3 target sites. In order to maintain similar codon usage patterns in the rP2 and rP3 genes to hRHO, codon usage in rhodopsin was analysed using the sequence manipulation suite (http://www.ualberta.ca/~stothard/javascript/codon_usage.html) [Section 9.15]. Where possible, the six nucleotide changes to the third nucleotide of each codon were generated such that similar codon usage patterns between the replacement genes and hRHO were maintained. When the rP2 and rP3 changes had been designed, the replacement genes were generated using PCR directed mutagenesis.

6.2.2. Generation of the replacement genes by PCR directed mutagenesis

PCR directed mutagenesis uses mismatches in a primer sequence to introduce mutations in a PCR product. With this premise, this technique was used to generate the six changes for rP2 or rP3 within a hRHO PCR product. For each replacement gene (rP2 and rP3) two PCR mutagenesis replacement primers (F & R) containing the rP2 or rP3 mismatches were designed to introduce silent mutations in the replacement genes target regions (Table 6.2.2). Additionally two primers which amplify regions of wild-type hRHO the forward 5' and the reverse 3' primers located either side of each siRNA target site were designed (Table 6.2.2). Each contained a unique restriction site to facilitate the cloning of the rP2 and rP3 gene. For both replacement genes these primers were identical, the F primer contained a unique *HindIII* site and was located in the 5' UTR of hRHO and the R primer contained a unique *BstEII* restriction site and was located within the coding sequence near the 3' end of hRHO. Oligonucleotides were obtained from Sigma-Genosys. The wild-type hRHO plasmid (hRHO, Section 3.2.3) was used as the initial template for PCR-directed mutagenesis. The generation of the replacement rhodopsin regions was carried using three different PCR reactions. The first PCR reaction using the hRHO DNA as template contained the reverse primer (for rP2 or rP3) with the 6 directed mutations and the forward wild-type hRHO 5' *HindIII* primer (Figure 6.2.2.a [step 1]). This yielded a PCR product (PCR1) of the 5' region of hRHO and the replacement changes (for rP2 or rP3). The second PCR reaction using the hRHO DNA template contained the forward primer with the directed mutations (for rP2 or rP3) and

the reverse wild-type hRHO 3' *BstEII* primer (**Figure 6.2.2.a [step 2]**). This yielded a PCR product (PCR2) containing the 3' region of wild-type hRHO and the replacement changes (for rP2 or rP3). PCR1 and PCR2 rP2 or rP3 amplified products were gel isolated in order to remove primers from the reactions (**Section 2.2.5**). The purified products were then used as template DNA for a third PCR using the 5' *HindIII* F and 3' *BstEII* R primers (**Figure 6.2.2.a [step 3]**). Since the PCR 1 and PCR 2 amplified PCR products overlap, they anneal together, therefore PCR 3 yields one long PCR fragment containing the rP2 or rP3 mutations and the *HindIII* and *BstEII* unique restriction enzyme sites on either end. These PCR3 fragments were subsequently cloned into a rhodopsin vector (**Figure 6.2.2.a [step 4]**). All PCRs were carried out using a proofreading *PfuTurbo* DNA polymerase kit (Stratagene [**Section 2.1.1**]) and contained: 100ng DNA template, 10pmoles of each primer, 2.5nmols of each dNTP (dGTP, dCTP, dTTP, and dATP), 2.5U *PfuTurbo* DNA polymerase and 10X *PfuTurbo* Buffer. The PCR amplification protocol was identical to the standard PCR protocol (**Section 2.2.19**) with the exception that it consisted of 30 cycles rather than the usual 35 as recommended by the manufacturer's instructions.

6.2.3 Cloning of rP2 and rP3 into the CMV-hRHO plasmid for *in-vitro* evaluation

Each replacement PCR3 product (rP2 or rP3) and the wild-type hRHO plasmid underwent sequential restriction digestion with *HindIII* and *BstEII* (**Section 2.2.3** and **Section 2.2.4**). Following restriction digestion the linearised hRHO vector was purified by phenol chloroform extraction, followed by an ethanol precipitation (**Section 2.2.9; 2.2.10**). Each replacement PCR3 product was subsequently ligated into the *HindIII* and *BstEII* sites of the hRHO vector such that the wild-type hRHO ORF was essentially swapped for the replacement hRHO ORF (**Section 2.2.11**). Ligations were transformed into XL1-BlueMRA E.Coli competent cells and the colonies obtained were purified and plasmid mini-preparation was performed (**Section 2.2.15**). Positive clones containing the 6 nucleotide changes of either rP2 or rP3 were identified by sequencing (**Section 2.2.20 for rP2 and Section 2.2.21 for rP3**) with the 3' *BstEII* R primer (**Table 6.2.2**).

6.2.4 Co-transfection of rP2 and rP3 with siRNA in HeLa cell culture

rP2 or rP3 plasmids were co-transfected with siRNAs into HeLa cell culture using Lipofectamine® 2000. 400ng of the rP2 or rP3 plasmid was co-transfected with 16pmoles of each siRNA (siNT, siBB, siP2 and siP3) in quadruplicate in a 24 well plate, in an identical method previously described (**Section 2.3.5 and Section 3.2.3**).

6.2.5 Real-time RT-PCR analysis of rP2 and rP3 mRNA expression

Prior to real-time RT-PCR analysis the RNA from each co-transfection was isolated using the RNeasy® Plus mini kit (Section 2.4.3) and the concentration of each RNA sample was evaluated (Section 2.2.1). The primers for this real-time RT-PCR analysis were hRHO and h β -actin and had previously been optimised (Section 3.2.5). For rP2 and rP3 analysis an RNA sample from HeLa cells co-transfected with hRHO and siNT was used to generate the 5X standard curves and the real-time RT-PCR was undertaken (Section 3.2.6). The relative hRHO expression from HeLa cells co-transfected with the replacement gene (rP2 or rP3) and siNT was set as 100%, the RNA from all other samples was expressed as a percentage of this value.

6.2.6. Western blot analysis of rP2 and rP3 protein expression

Protein from each co-transfection was extracted according to Section 2.5.1 and the protein concentration of each sample was obtained (Section 2.5.3). For rP2 a western blot was undertaken with five samples. These consisted of protein from HeLa cells co-transfected with rP2 and siNT, siBB or siP2, the fourth was protein from HeLa cells co-transfected with wild-type hRHO and siNT and the final sample was untransfected cells. For rP3 six samples were analysed, protein from HeLa cells co-transfected with siNT, siBB, siP2 or siP3, protein from HeLa cells co-transfected with hRHO and siNT and protein from untransfected cells. The protein from HeLa cells transfected with hRHO and siNT represented the negative RNAi control and protein from untransfected cells represented the negative hRHO control. Western blot analysis was carried out (Section 2.6) using nitrocellulose transfer membrane. The hRHO 4D2 antibody (1/1000 dilution [Hicks & Molday, 1986]) and the anti-mouse secondary antibody (1/1000 dilution) was used. The Anti-rabbit β -actin (1/2000 dilution) and the anti-rabbit secondary antibody (1/2000 dilution) were used to probe for β -actin.

6.2.7 Cloning of rP3

In order to evaluate the rP3 replacement construct *in vivo* with AAV it was cloned in a pAAV-based vector between the two AAV-2 ITRs. Initially the rP3 construct was cloned into the pAAV.BB32 vector. This pAAV.BB32 vector contains a bicistronic construct with a shRNA (shBB) and a hRHO replacement gene (rBB). It was decided to clone rP3 in this vector to facilitate eventual generation of a shP3-based suppression and replacement system for use in future studies. This vector has the hybrid rhodopsin promoter with various conserved sequences as detailed in Palfi et al, 2010. The rP3 RHO fragment was excised from the hRHO vector by a sequential restriction enzyme digest with *HindIII* and *BstEII* (Section 2.2.3). The rP3 gene fragment was separated from the linearised hRHO vector by

agarose gel extraction (**Section 2.2.5**). Similarly the BB32 vector was linearised by *HindIII* and *BstEII* digestion in an identical manner (**Section 2.2.3**) such that the rBB ORF within this vector was excised. Linearised BB32 was purified by phenol chloroform extraction followed by an ethanol precipitation (**Section 2.2.9 and Section 2.2.10**). The rP3 fragment was ligated into the linearised BB32 plasmid, such that rP3 RHO replaced rBB RHO (**Section 2.2.11**). Ligations were transformed using XL10-Gold Ultracompetent cells (**Section 2.2.14**). Colonies were purified and plasmids extracted using mini-preparation (**Section 2.2.15**) and positive clones were identified by sequencing (**Section 2.2.21**) with the 3' *BstEII* R primer (**Table 6.2.2**).

6.2.8 Cloning of rP3 into a “replacement only” construct

The pAAV.rP3 plasmid generated was subsequently utilised to clone the siP3 based suppressors into a pAAV based vector (**Section 4.2.2 & Section 4.2.4**). This was undertaken to place each suppressor within the correct spacers, in addition to having a dual suppression and replacement construct readily available for future evaluation. Therefore in order to generate an AAV-based vector containing only the rP3 replacement, the suppressor sequence was removed. pAAV.shP3.rP3 underwent restriction enzyme digestion with *XbaI* to remove the shP3 fragment (**Section 2.2.3**). The rP3 fragment was separated from the shP3 fragment by agarose gel isolation (**Section 2.2.5**). The pAAV.rp3 fragment was subsequently ligated (**Section 2.2.11**). Clones were purified by mini-preparation (**Section 2.2.15**) and the absence of the shP3 in positive clones was determined by a restriction enzyme digest with *XbaI* (**Section 2.2.3**). This was verified by sequencing using the SRSF primer (**Table 4.2.1**)

6.2.9 The generation of AAV-2/5.rp3 and AAV-2/8.rp3

During the *in vivo* suppression studies (Chapter 4 and Chapter 5) it became apparent that AAV-2/8 would be worth evaluating as a delivery vector. As efficient expression of the replacement gene in a suppression and replacement based therapy is vital for obtaining therapeutic efficacy, it was decided to generate the replacement only construct with both AAV-2/5 and AAV-2/8 and undertake a comparative analysis between these two AAV serotypes. The AAV protocol undertaken by Dr. Naomi Chadderton was a second generation CsCl gradient protocol, this was very similar to the 1st generation CsCl gradient AAV protocol previously used (**Section 2.7**). However some modifications were undertaken to increase vector purity and viral titre. The major change involved precipitating the AAV particles by polyethylene glycol (PEG 800). The protocol was undertaken according to Ayuso et al, 2010. The triple transfection protocol was carried out as described previously (**Section 2.7.1**). A master mix containing 1250µg of pAAV.rP3, 1250µg pReP2/CAP5 or pREP2CAP8 and 2500µg of pHelper

plasmids in 50ml of 150mM NaCl pH5.1 was generated. This ensured that each plate of cells was transfected with 25µg pAAV.rP3, 25µg of pRep2/CAP5 (or pRep2/CAP8) and 50µg of pHelper. The cells were harvested and 48 hours later centrifuged at 1000rpm for 30 minutes (SL-16, Thermo Scientific). The supernatant was collected and left on ice overnight. The cell pellet was lysed in 200ml lysis buffer and underwent three rounds of freeze thawing. The supernatant and the clarified cell lysates were pooled and PEG800 was added to a final volume of 8%. This was incubated at 4°C overnight and then centrifuged at 2500rpm for 30 minutes at 4°C and the supernatant was discarded (SL-16, Thermo Scientific). The cell pellet was resuspended in 200ml of PBS and benzonase (2.5U/µl) was added. This was incubated at 37°C for 1 hour. The clarified cell lysate was overlaid onto a CsCl gradient previously described (Section 2.7.2).

6.2.10 Determination of the viral titre of AAV-2/5.rP3 and AAV-2/8.rp3

The viral titre for each virus was calculated (Section 2.7.3) using a hRHO plasmid to generate the standard curve. The primers used in the qPCR reaction were the hRHO F & R primer pair (Table 3.2.1) and were used such that RHO expression from each replacement gene would be amplified from each of the viruses during the qPCR reaction. Two controls of known titre were used in this reaction. Two individual preparations of AAV-2/5.BB24 (A) one titre was 6×10^{13} vp/ ml and the second (AAV-2/.BB24 [B]) had a titre of 1×10^{13} vp/ml. The copy number of all viral fractions from each virus was obtained and was used to determine viral titre (vp/ml). The fraction of AAV-2/5.rP3 and AAV-2/8.rP3 with the highest titre was aliquot and stored at -80°C.

6.2.11 Subretinal injection of AAV-2/5 or AAV-2/8 into 129rho^{-/-}

In order to evaluate expression of rP3, a mouse model is required in which no rhodopsin is expressed. Therefore if rhodopsin expression is achieved, it is definitively attributed to the rhodopsin replacement. The mouse model used in this study was the 129rho^{-/-} mouse (Humphries et al, 1997). Subretinal injections were carried out by Dr. Paul Kenna (Section 2.8.3). The 129rho^{-/-} mice were subretinally injected at P5 with 0.8µl of virus per eye. For eyes injected with virus a 1/10 dilution of AAV-2/5.RHO-EGFP (EGFP under the control of the rhodopsin promoter) was also included in the viral prep. This acted as a tracer in order to visualise the delivery of AAV to the photoreceptors. For AAV-2/5.rP3 analysis, three litters (15 mice) were used; the LE was uninjected and the RE was injected with AAV-2/5.rP33 (4.8×10^{10} vp). For one litter (7 mice) the LE was injected with AAV-2/5.BB24 (A) and the RE with AAV-2/5.rP3 (4.8×10^{10} vp). For AAV-2/8.rP3 analysis two litters of 129rho^{-/-} mice (5 mice) were LE-uninjected and RE-injected with AAV-2/8.rP3 (3.2×10^{10} vp). Additionally three litters

(14 mice) were injected with AAV-2/8.BB24 (JB) in the LE (3.2×10^{10} vp) and AAV-2/8.rP3 in the RE (3.2×10^{10} vp).

6.2.12 ERG analysis of 129rho^{-/-} mice injected with AAV-2/5.rP3

The initial replacement virus evaluated in this study was AAV-2/5.rP3. ERGs represent a good indication that a replacement gene is functional and thus it was decided to first analyse the replacement gene in terms of ERG response. If the replacement rhodopsin is expressed at sufficient levels an ERG response should be detected in these 129rho^{-/-} mice (uninjected mice have a flat rod-isolated ERG). All mice underwent ERG at 6 weeks post-injection by Dr. Paul Kenna (**Section 2.8.4**). The rod-isolated, mixed rod-cone and cone-isolated responses were examined and the presence of any increased amplitudes in rod-only responses were recorded as a positive ERG response.

6.2.13 ERG analysis of 129rho^{-/-} mice injected with AAV-2/8.rP3

The 14 mice injected with AAV-2/8.BB24 (LE) and AAV-2/8.rP3 (RE) underwent ERG at approximately 6 weeks post injection. Two litters in which the LE was uninjected and the RE was injected with AAV-2/8.rp3 additionally underwent ERG at approximately 6 weeks. ERGs were measured by Dr. Paul Kenna (**Section 2.8.4**).

6.2.14 Immunohistochemistry of 129rho^{-/-} mice injected with AAV-2/5.rP3 and AAV-2/8.rP3

Immediately following ERG the mice that showed an increased ERG response in the AAV-2/5.rP3 or AAV-2/8.rP3 injected eyes (n=3 for each construct) were processed for immunohistochemistry (**Section 2.8.5-Section 2.8.8**). Retinal sections from each eye (LE and RE of each mouse) were stained for hRHO using the 4D2 antibody (1/100 dilution [Hicks & Molday, 1986]). A DAPI stain (1/10,000 dilution) was also performed for each section in order to visualise the retinal cell nuclei. The absence of RHO immunostaining in uninjected eyes was used to define the base line parameters for eyes injected with AAV-2/5.rP3. For AAV-2/8.rP3 two of the eyes evaluated had an uninjected LE and represented the negative control for EGFP expression and hRHO immunoreactivity. One mouse was injected with AAV-2/5.BB24 in the left eye and this represented the positive control eye for AAV-2/8.rP3 in which the level of RHO in the EGFP positive region could be used for comparison.

6.2.15 Western blot analysis of 129rho^{-/-} mice injected with AAV-2/5.rP3 and AAV-2/8.rP3

In order to establish if hRHO expression was present even in the absence of an ERG response it was decided to undertake western blotting on retinal protein from eyes in which no change in the baseline

ERG was recorded for both AAV-2/5.rP3 and AAV-2/8.rP3. Total retinal protein was isolated from mouse and protein concentrations obtained (**Section 2.5.2 and Section 2.5.3**). Western blot analysis was undertaken according to **Section 2.6** using PVDF as the transfer membrane. The 4D2 antibody (Hicks & Molday, 1986[1/1000 dilution]) was used for all westerns in addition to the anti-mouse light chain only antibody (1/2000 dilution). For AAV-2/5.rP3 analysis n=3 mice LE uninjected and RE injected with AAV-2/5.rP3 were analysed for hRHO immunoreactivity. For AAV-2/8.rP3 three mice with LE injected with AAV-2/5.BB24 and RE injected with AAV-2/5.rP3 was analysed for hRHO immunoreactivity. Protein from RHO-M mouse (O' Reilly et al, 2008) retinas was used as a positive control and protein from a 129rho^{-/-} mouse retina was used as a negative control. The anti-rabbit β -actin antibody was used (1/1000 dilution) to assess for equal loading. The anti-rabbit secondary antibody (1/2000 dilution) was used. As the retina was degenerating it was often difficult to obtain h β -actin, therefore longer exposure times of 30 minutes and overnight were needed.

6.2.16. Real-time RT-PCR analysis of hRHO mRNA expression from AAV-2/5.rP3 and AAV-2/8.rP3 in 129 wild-type mice

To assess the hRHO replacement RNA expression from AAV-2/5.rP3 and AAV-2/8.rP3, six adult 129 wild-type mice were LE-injected with AAV-2/5.rP3 (1.8×10^{11} vp/eye) and RE-injected with AAV-2/8.rP3 (1.2×10^{11} vp). 10 days post-injection RNA was extracted (**Section 2.4.5**) and real-time RT-PCR was undertaken (**Section 2.4.7**). RNA from an NHR retina was used to generate the 5X standard curve. Each RNA sample from the injected wild-type retinas was diluted to 0.08ng/ μ l. RNA from NHR retinas and RNA from RHO-M retinas was included for each analysis and acted as control samples (Ollson et al, 1992; O'Reilly et al, 2008). The hRHO primers were used to amplify rP3 rhodopsin mRNA expressed from each virus (**Table 3.2.1**). hRHO expression in each sample was normalised to mouse β -actin. The average relative hRHO expression from the NHR mouse was set at 100% and the relative hRHO expression from each RNA sample in wild-type mice injected with AAV-2/5.rP3 or AAV-2/8.rP3 was expressed as a percentage of this value. Relative hRHO expression from RHO-M RNA samples were also expressed as a percentage of NHR.

6.2.17 Subretinal injections of AAV-2/8.BB24 and AAV-2/8.rP3 at a 3.5 fold lower dose

It was decided to assess if lowering the AAV-2/8.rP3 injection dose 3.5-fold in 129rho^{-/-} mice would improve the numbers of mice with an improved ERG result. At the same time as this analysis, an AAV-2/8.BB24 (B) virus was generated in the laboratory according to the new second generation protocol (**Section 6.2.10**). Therefore it was decided to evaluate this virus in the LE (6.9×10^{10} vp/ml)

and a low dose of AAV-2/8.rP3 in the RE (9×10^9 vp). Seven 129rho^{-/-} mice were injected with these doses of virus and 6 weeks post-injection ERG analysis was undertaken. The rod-isolated, the mixed rod-cone and cone-isolated responses were measured (Section 2.8.4)

6.2.18 Comparative analysis of hRHO protein expression from AAV-2/5.BB24, AAV-2/8.BB24 and AAV-2/rh10.BB24

A preliminary experiment was undertaken to assess the level of hRHO protein expression from AAV-2/5.BB24, AAV-2/8.BB24 and AAV-2/rh10.BB24. n=3 mice (129rho^{-/-}) were injected with AAV-2/5.BB24 (LE) and AAV-2/8.BB24 (RE), an additional three mice were injected with AAV-2/5.RHOP-EGFP (1/10 dilution [LE]) and AAV-2/rh10.BB24 (RE). All BB24 injections were undertaken with 4.8×10^{10} vp/eye. A 1/10 dilution of AAV-2/5.RHOP-EGFP was included in each injected eye to utilise the EGFP protein as a measure of equal loading. Mice LE-injected with AAV-2/5.RHOP-EGFP served as the negative hRHO control. Protein was extracted 14 days post-injection (Section 2.5.2 & Section 2.5.3). Western blotting analysis (Section 2.6) was undertaken on all protein samples using the 4D2 antibody (1/1000 dilution) and the anti-mouse light chain only antibody (1/2000 dilution). The anti-rabbit EGFP antibody (1/2000 dilution) and the anti-rabbit secondary antibody (1/2000 dilution) was used to probe for equal loading.

6.2.19 Statistical analysis

For all *in vitro* real-time RT-PCR data, the mean and standard deviation was obtained for relative hRHO expression in samples co-transfected with hRHO and each siRNA (siBB, siP2 or siP3). For each transfection (a minimum of four separate transfections was carried out for rP2 or rP3 analysis) and a student t-test was used to assess significance in the NT control and samples transfected with siRNAs. For *in vivo* real-time RT-PCR data the mean and the standard deviation of the relative hRHO expression in wild-type animals injected with either AAV-2/5.rP3 or AAV-2/8.rP3 was obtained and compared to the mean hRHO expression in NHR and RHO-M retinas. Statistical significance was undertaken using a two-sample t-test. For all ERG data the average rod-isolated, mixed rod-cone and cone-isolated ERG responses were combined for both injected and uninjected eyes and the standard deviation for each data set was obtained. Statistical significance was assessed using a two-sample t-test. All statistical analysis was carried out using DataDesk 6.0.

6.3. Results

6.3.1 Design of the rP2 replacement sequence

A replacement gene for each siRNA was generated based on the degeneracy of the genetic code where only nucleotides at wobble positions within the siRNA target sequence were altered. This ensures replacement genes should express a wild-type rhodopsin protein. The siP2 target sequence was examined in order to determine the maximum number of nucleotide changes that were possible within this target site without changing the amino acid sequence. Despite no change occurring to the amino acid sequence of the hRHO gene, interfering with codon usage may have an effect on mRNA secondary structure or half-life thus affecting gene expression (O'Reilly et al, 2007; Ren et al, 2007; Shabalina et al, 2006b). For this reason analysis of codon usage in the rhodopsin cDNA was undertaken. Nucleotides at third base positions of each codon within these sites were altered so that possible changes in codon usage within the hRHO gene were minimised (**Table 6.3.1**). A detailed analysis of codon frequencies with hRHO is presented in **Section 9.15**. In the siP2 site the first amino acid within the siP2 site is tyrosine (Try) and the codon sequence utilised is TAC, this was changed to TAT. The second codon is Metionine (Met) and this is the start codon and therefore no flexibility for codon changes is tolerated. The third codon is phenylalanine (Phe) and the sequence used is TTC which was converted to TTT. The fourth and fifth codon within the siP2 target site was valine (Val) and the codon sequence was GTG and GTC, for these two codons, GTG was changed to GTC and GTC was changed to GTG. The sixth codon was histidine (His) and the codon sequence was CAC and the only possible change that could be made to this sequence was CAT. The final codon change made was within Phe, this was the same sequence as codon three in the siP2 target site.

When these changes had been designed, rP2F & R PCR mutagenesis primers were generated which incorporated these mutations, and which aided in the creation rP2 replacement gene sites (**Figure 6.3.1.a**). Two primers with unique restrictions enzyme sites (*HindIII* and *BstEII*) either side of the siP2 target sequence were also generated (**Table 6.2.1**). The forward primer was located 718bp upstream of the siP2 target sequence within the 5'UTR hRHO sequence and included a *HindIII* restriction site. The reverse primer was located 123bp downstream of the siP2 target site and included a *BstEII* restriction site. The first PCR for the generation of the rP2 sequence amplified a PCR fragment of 746bp with the 5' *HindIII* F primer and rP2 R primer. PCR 2 yielded a 147bp product with the rP3F and 3' *BstEII*R primers. The third PCR product generated from using the amplification products from PCR1 and PCR2 was approximately 860bp. This fragment was cloned into pCMV.hRHO and the rP2 changes were verified by sequencing (**Figure 6.3.1.b**).

6.3.2 Design of the rP3 replacement sequence

Similar to the rP2 gene, codon usage analysis was undertaken within the siP3 sequence and all possible codon changes were minimised (**Table 6.3.2**). A detailed analysis of the codon usage of hRHO can be obtained from **Section 9.15**. The first amino acid within the siP3 target site was arginine (Arg) and the codon sequence utilised was CGG, this was altered to CGC, the most common Arg codon within hRHO. The second codon within the siP3 target site was Tyr, and the only change available for Tyr was TAT. The third, fourth and fifth codons all code for Val, and the sequence used within the siP3 region was GTG, and this sequence was altered to GTC. The final amino acid within the siP3 site was cystine (Cys), the sequence was TGT and this was changed to the only other nucleotide sequence available for this amino acid TGC.

rP3F & R PCR mutagenesis primers were generated which incorporated these sequence alterations and which aided in creation of the rP3 replacement gene (**Figure 6.3.2.a**). Primers with unique restrictions enzyme sites used previously for the generation of rP2 were utilised (5' *HindIII*F & 3' *BstEII*R, [**Section 6.2.1.b**]). PCR with the 5' *HindIII*F primer and rP3R primer yielded a 537bp product. PCR2 utilised the rP3F and 3' *BstEII*R primers and resulted in an amplification product of 309bp. The final PCR used the amplification products from PCR1 and PCR2 and generated a rP3 amplification product of 846bp. This rP3 fragment was cloned into pCMV.hRHO and the rP3 changes were verified by sequencing with the 3' *BstEII*R primer (**Figure 6.3.2.b**).

6.3.3 Co-transfection of rP2 and rP3 with hRHO siRNAs in HeLa cells

Co-transfection experiments were carried out using the same protocol as for the original hRHO and hRHO siRNAs in Chapter 3. However, in this study instead of wild-type hRHO, replacement hRHO was co-transfected with each siRNA, to analyse its resistance to its respective siRNA. rP2 (or rP3) was transfected with siNT (negative control) or siBB (positive control). In addition, rP2 was co-transfected with siP2 and rP3 was co-transfected with siP3 in order to verify that each replacement gene was resistant to its respective siRNA. All co-transfection experiments undertaken out a minimum of four times

6.3.4 Analysis of rP2 RNA expression by real-time RT-PCR

Initially rP2 RNA expression in HeLa cells transfected with siNT, siBB or siP2 was evaluated by real-time RT-PCR as described (**Section 6.2.5**). siBB was found to suppress rP2 to $11.8 \pm 3\%$, confirming that similar to the wild-type hRHO rP2 is capable of being significantly suppressed by siBB

($P < 0.001$ [Figure 6.3.4.a]). In contrast to the previous results obtained with wild-type hRHO and siP2 in which hRHO was reduced to 22% expression levels (Section 3.3.5), rP2 was expressed to $83.5 \pm 23\%$ with siP2 and this was comparable to rP2 expression with siNT ($P = 0.3273$). Therefore this suggested that unlike wild-type hRHO rP2 is resistant to suppression by siP2 at the RNA level.

6.3.5 Analysis of rP3 mRNA expression by real-time RT-PCR

Analysis of resistance of rP3 RNA to siP3 was carried out in a similar manner to Section 4.3.4. As expected siBB reduced rP3 RNA to $14 \pm 6\%$ ($P < 0.005$), this is consistent with previous analysis in which siBB reduced wild-type hRHO to 15% and confirms that similar to wild-type hRHO, rP3 is amenable to suppression by RHO siRNAs (Section 3.3.5 [Figure 6.3.5.a]). rP3 expression with siP3 was not significantly reduced ($92 \pm 26\%$, $P = 0.6338$) when compared to rP3 expression with siNT. This is in contrast to wild-type hRHO in which hRHO RNA is reduced to 8% (Section 3.3.8) and indicates that the six nucleotide changes within the rP3 target site are sufficient to render hRHO resistant to siP3-mediated suppression.

6.3.6 Western blot analysis of rP2 protein expression

In addition to evaluating the resistance of rP2 RNA to siP2 suppression it was important to assess if this correlated with the protection of the rP2 protein. Western blotting was carried out on protein from HeLa cells co-transfected with rP2 and each siRNA (siNT, siBB and siP2). Additionally protein from HeLa cells co-transfected with hRHO and each siRNA (siNT, siBB, siP2 and siP3) was included in the analysis. This set of protein samples acted as the positive control and the protein sample from HeLa cells co-transfected with hRHO and siNT acted as a control to ensure similar expression levels of rP2 with siNT were obtained (Figure 6.3.6.a). hRHO immunoreactivity was visible in lane 1 which was protein from HeLa cells transfected with rP2 and siNT; this hRHO expression was similar to that obtained from HeLa cells transfected with wild-type hRHO and siNT. This suggests that the sequence alterations in rP2 are not conferring any significant change in hRHO protein expression. No hRHO immunoreactivity is present in the lane containing protein from HeLa cells transfected with rP2 and siBB (lane 2); this was comparable to lane 5 which contains HeLa cell protein from cells transfected with hRHO and siBB. This confirms that both hRHO and rP2 protein are significantly suppressed by siBB. rP2 protein was visible in lane 3 (cells co-transfected with rP2 and siP2). This level of immunoreactivity was comparable to both lane 1 (rP2 and siNT) and lane 4 (hRHO and siNT) and suggests that similar to the RNA result, rP2 was resistant to suppression by siP2. In contrast no hRHO protein was present in lane 6 which is protein from HeLa cells with wild-type hRHO and siP2,

supporting the view that six nucleotide changes in rP2 was sufficient for protection of hRHO from siP2 mediated suppression. In contrast as expected hRHO was not protected from suppression. Protein from HeLa cells co-transfected with hRHO and siP3 were included in the analysis (lane 7) as an additional control for hRHO suppression. Untransfected cells acted as the negative control (lane 8). The expression of the h β -actin protein was used to evaluate equal loading, this was obtained in all cases and further validates the results from the western blot experiments.

6.3.7 Western blot analysis of rP3 protein expression

In order to ensure that rP3 RNA resistance to siP3 translated to abundant rP3 protein expression in HeLa cells co-transfected with rP3 and siP3, a western blot was undertaken similar to the rP2 analysis (**Figure 6.3.7.a**). Abundant monomeric and multimeric forms of hRHO were visible in protein samples transfected with rP3 and siNT (lane 1); this was comparable to the hRHO protein expression observed in samples from HeLa cells transfected with hRHO and siNT and suggests that the P3 target sequence may not be conferring any negative changes on hRHO protein expression. Owing to the absence of hRHO immunoreactivity in lane 2 and 5, in which protein from HeLa cells co-transfected with either rP3 and siBB or hRHO & siBB, it was determined that as expected siBB efficiently suppressed both rP3 and hRHO protein. In lane 3 (protein from cells transfected with rP3 and siP3), hRHO immunoreactivity was present at similar levels to rP3 and siNT or hRHO and siNT. In contrast in protein samples from HeLa cells co-transfected with hRHO and siP3 no hRHO immunoreactivity was observed (lane 6). This supports a view that transcripts from rP3 are resistant to siP3-mediated suppression. Untransfected cells acted as the negative control (lane 7). Human β -actin immunoreactivity was used to assess equal loading.

6.3.8 The preparation of rP3 for *in vivo* analysis

Throughout the course of these *in vitro* replacement gene experiments, analysis of shP3-based suppression of hRHO *in vivo* was being undertaken in parallel using AAV as the vector for subretinal delivery (Chapter 4). Efficient suppression by shP3 was consistently being observed, therefore it was decided to design a replacement construct based on the rP3 sequence for the *in vivo* analysis of replacement gene expression. A major consideration which had to be undertaken for *in vivo* investigation was the design of the replacement construct. Previous evaluation of various replacement hRHO genes *in vivo* had been undertaken in the TCD laboratory (Palfi et al, 2010). Various elements such as the length of the promoter or the presence of various enhancer elements were assessed in order to obtain a hRHO gene which expressed sufficient levels of hRHO to provide benefit in 129rho-/-

mice. Due to the size constraints of AAV it was impossible to include a larger RHO promoter, e.g., the entire 5kb sequence which precedes the hRHO ORF. Therefore a comparative analysis was undertaken by Dr. Mary O'Reilly examining the 5'UTR RHO sequence for multiple species to evaluate conserved regions which may be important for hRHO expression. Regions that were deemed important in the two species were included in the gene construct. These promoter sequences have previously been described by Palfi et al, 2010. It was decided to clone the rP3 fragment into the rBB rhodopsin sequence within the BB32 vector such that rP3 would undergo the same regulation as the replacement gene in Palfi et al, 2010.

6.3.9 Cloning of rP3 into pAAV.BB32

The BB32 plasmid was generated in the TCD laboratory by Dr. Mary O'Reilly. It contains a H1 driven shBB suppressor construct followed by the BB24 replacement gene. The rP3 RHO sequence was cloned into this plasmid to facilitate the future evaluation of a dual shP3-based suppression and replacement construct (pAAV.rP3). A region of 854 nucleotides containing the rBB region of the BB32 plasmid was excised and replaced with the 854 nucleotide rP3 fragment, such that rP3 was expressed downstream of the hybrid RHO promoter (**Figure 6.3.9.a**). In order to generate a construct that was "replacement only", the 300 nucleotide shP3 suppressor was excised from the pAAV.rP3 plasmid by Xba1 digestion. Positive clones were identified by the absence of this 300 nucleotide fragment and this was verified by sequencing (**Figure 6.3.9b**).

6.3.10 The generation of pAAV.rP3 into AAV-2/5 and AAV-2/8

Traditionally in the laboratory AAV-2/5 was utilised as the vector for delivery to photoreceptors (Millington-ward et al, 2011; Palfi et al, 2010). However as stated above AAV-2/8 has recently been described as a highly efficient viral vector in the retina (Allocca et al, 2007; Leberherz et al, 2008; Mussolino et al, 2011b; Steiger et al, 2008; Vandenberghe et al, 2011). Several groups have employed AAV-2/8 for gene delivery to several mouse models of recessive retinal dystrophies with success (Carvalho et al, 2011; Mihelec et al, 2011; Tan et al, 2009). In addition AAV-2/8 has been utilised to provide efficient suppression of target RNA within the retina including rhodopsin and peripherin-2 (Georgiadis et al, 2010; Mussolino et al, 2011a). For this reason it was decided to generate the rP3 replacement gene in both AAV-2/5 and AAV-2/8 to undertake a comparative analysis between the two viruses. A new second generation protocol was undertaken for generating AAV-2/5 and AAV-2/8; this was based on the method outlined by Ayuso et al, 2010. This protocol used the precipitation of the AAV particles with PEG800 prior to CsCl purification for a better purification yield (Ayuso et al,

2010). Producing high titre, pure AAV virus for the rhodopsin replacement gene was particularly important to obtain sufficient hRHO expression. Previously in the laboratory efficient hRHO expression was achieved with the BB24 replacement in 129rho^{-/-} with 1.3×10^{13} vp/ml (Palfi et al, 2010). AAV-2/5.rP3 and AAV-2/8.rP3 was generated utilising the new protocol with viral titres in the region of 6×10^{13} vp/ml and 4×10^{13} vp/ml respectively (**Table 6.3.10**).

6.3.11 ERG AAV-2/5

Initial experiments were undertaken using AAV-2/5.rP3. In order to evaluate if the presence of the hRHO rP3 replacement gene could sufficiently improve ERG function in a 129rho^{-/-} mouse, 15 129rho^{-/-} mice were subretinally injected with 4.8×10^{10} vp of AAV-2/5.rP3 (RE). The LE was uninjected acting as the negative control. Mice were allowed to recover from injection for approximately six weeks before ERG responses were measured. Of the 15 mice evaluated (3 litters), a rod-isolated response was observed in only four mice. This was somewhat surprising given that when 129rho^{-/-} mice were injected with BB24 approximately half of the mice had improved retinal function (Dr Paul Kenna [personal communication]). An additional 7 mice were injected with AAV-2/5.BB24 (4.8×10^{10} vp) in the LE and AAV-2/5.rP3 in the RE (4.8×10^{10} vp); AAV-2/5.BB24 acting as the positive control. However due to a bad batch of AAV-2/5.BB24 virus the LEs of these mice could not be included in the analysis. Six weeks post-injection only 1/7 mice had an improved rod-isolated ERG, in total this corresponds to only 5/22 mice showing any sufficient improvement in ERG (< 22%). Representative rod-isolated (**Figure 6.3.11.a**), mixed rod-cone (**Figure 6.3.11.b**) and cone-isolated (**Figure 6.3.11.c**) ERG responses are presented here. This ERG was representative of the mouse with the highest rod-isolated ERG response in the AAV-2/5.rP3 injected eye recorded for any of the mice. The highest rod-isolated ERG b-wave was 125µV in AAV-2/5.rP3 injected retinas compared to the uninjected 129rho^{-/-} control (0 µV). The mixed rod-cone response in the same AAV-2/5.rP3 injected eye was 314µV compared to 204µV in the uninjected eye. For the cone-isolated response from this mouse, the b-wave value was 6.98µV in the AAV-2/5.rP3 injected eye compared to the uninjected eye (83.5µV). Of the 22 mice evaluated the results from the five in which a rod-isolated b-wave was obtained were combined in order to determine if improvement in rod function was significant (**Figure 6.3.11.d**). The average rod response was significantly increased to $87 \pm 39 \mu V$, $P < 0.01$ in the eye injected with AAV-2/5.rP3 compared to the uninjected eye in which a rod response of 0µV was always obtained. This suggested that rP3 was expressing hRHO in the retina in these mice and resulting in an increase in the rod cell function. Similarly, the mixed rod-cone response was increased to $231 \pm 102 \mu V$ in the AAV-2/5.rP3 injected eye compared to $147 \pm 51 \mu V$ in the uninjected retina, this increase in the

mixed rod-cone response was however not significant ($P=0.1493$ [Figure 6.3.11.e]). In contrast, the cone-isolated response in AAV-2/5.rP3 injected retinas decreased to $19\pm 18\mu\text{V}$ compared to $52\pm 25\mu\text{V}$ in the uninjected cones (Figure 6.3.11.f). The decrease in cone function was consistently observed in the injected eyes, this could be as a result of mechanical damage to the cones by the injection process. While damage would likely also occur in the rods cells, the expression of hRHO may be sufficient to mask this in the rod-isolated or mixed-rod cone ERG. This cone response was not significant ($P=0.0786$). Cone damage was observed in the study by Palfi et al, 2010 when AAV-2/5.Rho-EGFP or PBS was injected implicating the injection process as the cause. Overall it does appear that despite the low ratio of mice with an increased ERG response, hRHO must clearly be expressed in these five mice at sufficient levels to result in improvement in the rod photoreceptor cells.

6.3.12 ERG of 129 rho^{-/-} subretinally injected with AAV-2/8.rP3

As only low numbers of mice with an improved ERG response were obtained for AAV-2/5.rP3 it was decided to include an AAV-2/8.BB24 positive control for future analysis. AAV-2/8.BB24 had been previously established to provide potent hRHO expression in 129rho^{-/-} retinas [Chadderton et al, [unpublished data]]. Therefore it was expected that when injected into 129rho^{-/-} mice in this study, it should act as a potent positive control. 13 129rho^{-/-} mice (3 litters) were injected with 3.2×10^{10} vp of AAV-2/8.BB24 (LE) and AAV-2/8.rP3 (RE). 10 /13 of the mice had an improved rod-isolated response in the eye injected with AAV-2/8.BB24 and the highest rod-isolated response recorded for this construct was $371\mu\text{V}$. In contrast, only 2/13 mice injected with AAV-2/8.rP3 had any increase in the rod-isolated ERG function. Four rho129^{-/-} mice were injected with 3.2×10^{10} vp of AAV-2/8.rP3 and remained uninjected in the LE. 2/4 of the mice had an increased rod function compared to the uninjected mouse. This brought the overall total of mice in which an increased ERG response was obtained to 4/17 mice (24%). A rod-isolated ERG (Figure 6.3.12.a) is presented from the mouse in which the highest rod-isolated response with AAV-2/8.rP3 was achieved. The b-wave in this mouse was $82\mu\text{V}$ for the AAV-2/8.rP3 injected eye and this was compared to $148\mu\text{V}$ in the eye with AAV-2/8.BB24. For the mixed rod-cone response the b-wave in this mice was $194\mu\text{V}$ with AAV-2/8.rP3 compared to $379\mu\text{V}$ with AAV-2/8.BB24 (Figure 6.3.12.b). The cone-isolated response with AAV-2/8.rP3 was 0 and was $28.8\mu\text{V}$ with AAV-2/8.BB24 (Figure 6.3.12.c). Of the 17 mice evaluated the results from the four in which a rod-isolated b-wave was obtained were combined in order to determine if the improvement in rod function was significant (Figure 6.3.12.d). Compared to uninjected mice (rod response of $0\mu\text{V}$), the average rod b-wave was significantly increased to $47.2\pm 28\mu\text{V}$, $P<0.05$ in the eye injected with AAV-2/5. This suggested that rP3 was expressing hRHO in the retina of these

mice and resulting in an increase in rod cell function. For BB24 the average b-wave was $239 \pm 85 \mu\text{V}$ ($P < 0.005$). When the rod-isolated response of BB24 and rp3 were compared it was determined that these two viruses were performing significantly different to each other ($P < 0.005$), which suggested that AAV-2/8.rp3 may not be as efficient as AAV-2/8.BB24. For the rod-cone mixed response (**Figure 6.3.12.e**), the b-wave for the AAV-2/8.rp3 injected retina ($122 \pm 53 \mu\text{V}$) was comparable to the uninjected retina ($143 \pm 18 \mu\text{V}$ [$P = 0.5039$]). The average b-wave response was $433 \pm 127 \mu\text{V}$ in mice injected with AAV-2/8.BB24 and this was significantly different to both AAV-2/8.rp3 injected and uninjected eyes ($P < 0.005$ and $P < 0.01$). Similar to that observed with AAV-2/5.rp3, the average cone-isolated response in AAV-2/8.rp3 injected retinas decreased to $5.6 \pm 11.2 \mu\text{V}$ compared to uninjected mice ($52 \pm 9 \mu\text{V}$, $P < 0.005$). The cone isolated response in AAV-2/8.BB24 injected eyes was modestly improved compared to AAV-2/8.rp3 injected retinas ($27 \pm 15 \mu\text{V}$ [$P < 0.05$]) (**Figure 6.3.12.f**). Overall these results suggest that AAV-2/8.rp3 may not be as efficient at expressing hRHO compared to the titre matched BB24 control. When AAV-2/5.rp3 at a titre of $4.8 \times 10^{10} \text{vp}$ was compared to AAV-2/8.rp3 ($3.2 \times 10^{10} \text{vp}$) no significant difference in the rod-isolated ($P = 0.1198$), mixed rod-cone ($P = 0.0835$) or cone-isolated responses ($P = 0.2559$) was obtained.

6.3.13 Immunohistochemistry on retinal sections from 129 rho^{-/-} mice injected with AAV-2/5.rp3

Three 12 129rho^{-/-} mice in which improved ERG responses were obtained in eyes injected with AAV-2/5.rp3 underwent immunohistochemistry with the 4D2 antibody six weeks post injection. For all mice examined hRHO immunoreactivity was present in the EGFP positive portions of the retina injected with AAV-2/5.rp3 but absent in the EGFP negative regions of the same retina. The animal in which the best hRHO staining was achieved is presented (**Figure 6.3.13.a**). An aged matched 7 week RHO-M transgenic mouse was used as the positive control. RHO-M is a mouse with a transgenic hRHO replacement gene that expresses hRHO to 70% wild-type levels and provides a rod-isolated response of approximately $800 \mu\text{V}$ (O'Reilly et al, 2008). Compared to RHO-M, no hRHO staining was observed in uninjected 129rho^{-/-} retinas. Similar to that observed in uninjected mice, no hRHO immunoreactivity was observed in the non transduced retinal regions from 129rho^{-/-} mice injected with AAV-2/5.rp3. However, hRHO staining was evident in the ROS of the transduced (EGFP positive) region of these animals, albeit at lower levels than the hRHO expression in the RHO-M mouse. Furthermore regions of EGFP expression in the IS indicate that short photoreceptor OS were formed in this region. Overall this result verifies the ERG result, confirming that hRHO expression with AAV-2/5.rp3 is present in these mice in which a positive ERG was obtained.

6.3.14 Immunohistochemistry on retinal sections from 129 rho^{-/-} mice injected with AAV-2/8.rP3

Two mice in which a positive ERG result was obtained with AAV-2/8.rP3 were analysed for hRHO expression. These retinal sections were compared to AAV-2/8.BB24 and uninjected retinas. Similar to AAV-2/5.rP3, RHO-M was used as a hRHO control. The results presented are from the mouse in which the best hRHO staining was observed (**Figure 6.3.14.a**). In general hRHO staining for AAV-2/8.rP3 was not as good as that obtained with AAV-2/5.rP3. While no RHO staining was visible in the the non transduced region of retinal sections from 129rho^{-/-} injected with AAV-2/8.BB24, abundant hRHO staining was visible in the transduced region of this retina and this was comparable to hRHO staining in the RHO-M mouse. Additionally abundant healthy EGFP IS were present, verifying that AAV-2/8.BB24 was capable of very efficient hRHO expression. The EGFP positive portion of the retina transduced with AAV-2/8.rP3 showed very few, short EGFP positive IS, with EGFP positivity mainly confined to the ONL. hRHO staining was visible but at low levels.

6.3.15 Western blot analysis of hRHO rP3 protein expression by AAV-2/5.rP3

While few animals had an improved rod-isolated response when injected with AAV-2/5.rP3 or AAV-2/8.rP3, it was decided to evaluate if hRHO expression was present in animals in which a negative ERG was obtained. Retinal protein from three mice in which the LE remained uninjected and the RE had been subretinally injected with AAV-2/5.rP3 was extracted and a western blot was undertaken using the 4D2 antibody (**Figure 6.3.15**). RHO-M was used as the positive control for this experiment and in this lane (lane 8) abundant hRHO immunoreactivity was observed. Low hRHO immunoreactivity was observed in two of the three mice injected with AAV-2/5.rP3 (lane 1 & 3) compared to the uninjected left eye (lane 2 & lane 4) when blots were overexposed for forty minutes. This suggested that hRHO expression from AAV-2/5.rP3 was present but most likely at low levels such that no ERG function would be recorded. However this western was carried out twice and the second time the presence of the second band (lane 3) was not detected. An interesting observation was the presence of immunoreactivity at approximately 25kDA in all 129rho^{-/-} animals (injected and uninjected) used for the AAV-2/5 study. This may represent the mouse light chain IgG that the anti-mouse light chain only antibody is binding to, however no immunoreactivity at 25kDA was ever previously observed in any western blotting experiments. The anti-rabbit β -actin antibody was used to ensure that actin protein was present in the lanes in which no hRHO immunoreactivity was observed, in order to rule out protein quality as a reason for no hRHO in these retinas.

6.3.16 Western blot analysis of hRHO rP3 protein expression by AAV-2/8.rP3

In an experiment similar to the AAV-2/5.rP3 protein expression study, protein from three retinas injected with AAV-2/8.BB24 (LE) and AAV-2/8.rP3 (RE) and in which no ERG response was obtained within the AAV-2/8.rP3 injected eyes were extracted for western blotting (**Figure 6.3.16.a**). Abundant hRHO immunoreactivity was visible at 39kDA for all eyes injected with AAV-2/8.BB24 (lane 1, 3 & 5) this correlates to a positive rod-isolated ERG response in these animals and further supports AAV-2/5.BB24 as an efficient *in vivo* replacement gene. In contrast only one animal injected with AAV-2/8.rP3 expressed hRHO (lane 6). No immunoreactivity at 25kDA was observed in any of these samples. β -actin was used to ensure the presence of protein in the samples in which there was no hRHO immunoreactivity. It therefore appears that hRHO is being expressed at very low levels in these mice which is the likely reason for the unaltered rod-isolated b-wave. Western blotting was performed on two different occasions

6.3.17 hRHO RNA expression in wild-type mice injected with AAV-2/5.rP3 and AAV-2/8.rP3

In order to evaluate the level of hRHO RNA expression from AAV-2/5.rP3 and AAV-2/8.rP3, 5 adult 129 wild-type mice were injected with AAV-2/5.rP3 (1.8×10^{11} vp) and AAV-2/8.rP3 (1.2×10^{11} vp) and RNA was extracted 10 days post-injection. The availability of hRHO specific primers allowed this evaluation to be undertaken in wild-type mice rather than 129rho^{-/-} mice (**Figure 6.3.17.a**). RNA from RHO-M was also included in the analysis, and was expressed to $44 \pm 14\%$ NHR levels. AAV-2/5.rP3 expression was significantly different to hRHO expression in NHR ($70 \pm 12\%$, $P < 0.01$). AAV-2/8.rP3 expressed hRHO to similar levels as NHR ($54 \pm 45\%$, $P = 0.09$). The reason that the % expression was lower in the AAV-2/8.rP3 injected eyes compared to AAV-2/5.rP3, yet is still statistically significant can be explained by the high standard deviation between the AAV-2/8.rP3 injected eyes. Two samples within this group express hRHO only at 14 and 16 % of NHR levels, when these eyes are omitted from the analysis; the hRHO expression was 79% of NHR. The fact that both AAV-2/5.rP3 and AAV-2/8.rP3 was expressed similar to RHO-M is interesting given that RHO-M RHO expression is from the whole retina. However in the AAV-2/5.rP3 and AAV-2/8.rP3 129rho^{-/-} injected mice, only approximately 30-40% of the retina is transduced with hRHO (Palfi et al, 2010).

6.3.18 ERG analysis in 129rho^{-/-} mice injected with AAV-2/8.BB24 and AAV-2/8.rP3 at a lower dose

AAV-2/8.BB24 (B) has been produced in the TCD laboratory using the new protocol (Dr. Naomi Chadderton) at a titre of 3×10^{14} vp/ml. It was decided that during the analysis of AAV-2/8.BB24 (B),

AAV2/8.rP3 would be injected into the opposite eye at a lower dose previously used as a final evaluation for this construct. For AAV-2/5.rP3 the dose was reduced 3.5-fold to 9×10^9 and injected into 7 mice. For AAV-2/8.BB24 (B) the dose was 6.9×10^{10} vp. 6 weeks post-injection these mice underwent ERG analysis. Of the 7 mice injected, 1 mouse had a subretinal hemorrhage in the AAV-2/8.rP3 injected eye and thus was excluded from analysis. 4/6 mice however had an increased rod-isolated b-wave compared to uninjected mice. Similarly 4/6 mice showed an increased rod-isolated response in the AAV-2/8.BB24 injected eyes. The rod-isolated (**Figure 6.3.18.a**), mixed rod-cone (**Figure 6.3.18.b**) and cone-isolated (**Figure 6.3.18.c**) responses from the mouse in which the best rod-isolated ERG response was achieved with AAV-2/8.rP3 is presented here. The rod-isolated response in the LE injected with AAV-2/8.BB24 was 239 μ V and in the RE injected with AAV.rP3 was 234 μ V. This was in contrast to the high dose AAV-2/8.rP3 in which the highest rod-isolated response was 85 μ V. For the mixed rod-cone response in this mouse the b-wave was 544 μ V in AAV-2/8.BB24 injected eye compared to 659 μ V in the AAV-2/8.rP3 eye. This was compared to a response of only 122 μ V in the high dose AAV-2/8.rP3 mouse. The cone-isolated response in the eye injected with AAV-2/8.BB24 was 15.1 μ V compared to 56.3 μ V in the eye injected with AAV-2/8.rP3, again this is in stark contrast to the high dose AAV-2/8.rP3 in which a response of only 5.6 μ V was recorded. The average b-wave responses for all ERG tests in mice injected with either AAV-2/8.BB24 or AAV-2/8.rP3 were combined and compared with the average b-wave responses for aged matched uninjected mice (**Figure 6.3.18.d**) For the rod-isolated response AAV-2/8.BB24 had a statistically similar response ($109 \pm 97 \mu\text{V}$) compared to AAV-2/8.rP3 ($132 \pm 70 \mu\text{V}$, $[P=0.7212]$). The rod-isolated response for AAV-2/8.rP3 was significantly different to the uninjected eyes ($P<0.05$), confirming that AAV-2/8.rP3 was efficiently improving the rod cell function. In contrast the average b-wave response with the old dose of AAV-2/8.rP3 was 47 μ V. The rod-isolated b-wave in AAV-2/8.BB24 eyes was however not significant compared to uninjected ($P=0.1099$). This was probably due to the high standard deviation between samples; the lowest ERG was 22.3 μ V for this construct. The mixed rod cone responses for AAV-2/8.BB24 was $227 \pm 230 \mu\text{V}$ compared to $403 \pm 180 \mu\text{V}$, $P=0.2772$ for AAV-2/8.rP3. Unsurprisingly, compared to the uninjected eye, AAV-2/8.BB24 was not significantly altering the mixed rod-cone response ($P=0.5208$). Additionally AAV-2/8.rP3 appeared to have no significant effect on the mixed rod cone response compared to the uninjected mice $P=0.06$. The cone-isolated (**Figure 6.3.18.f**) response in AAV-2/8.BB24 injected retinas ($11 \pm 9 \mu\text{V}$) was significantly different to AAV-2/8.rP3 ($52 \pm 19 \mu\text{V}$ [$P<0.05$]). This implies that despite any damage to cones which may have been caused by the injection process the lower dose AAV-2/8.rP3 may be having a positive impact on cone-cell function. The cone-isolated response in AAV-2/8.rP3 and uninjected mice was statistically similar

($P=0.9764$) supporting the view that some protection of cone cells maybe taking place in order to increase the cone-isolated ERG compared to uninjected mice. This may be the reason for the non-significant result obtained with the mixed rod-cone response between AAV-2/8.rP3 and uninjected eyes. The increased cone response in the AAV-2/8.rP3 to levels similar to uninjected mice eyes was probably sufficient enough to mask the significant rod-isolated contribution to this response. Overall it appears that the lower dose AAV-2/8.rP3 is efficient at rescuing the ERG response in the 129rho^{-/-} mice. The injection process has been established as being damaging to the photoreceptors at this age (Palfi et al, 2010), the fact that an improved cone response compared to other injected mice was obtained with this construct suggests that benefit to the cones may be achieved. Statistical analysis was performed between the old-dose AAV-2/8.rP3 and the new dose AAV-2/8.rP3, no significant difference was obtained between the rod isolated responses of these two doses ($P=0.09$). However the experimental variation is quite high in these mice, the highest b-wave response in the mice injected with the old dose of AAV-2/8.rP3 was 87.9 μ V whereas the lowest ERG response in the mice injected with the low-dose AAV-2/8.rP3 was 86.3 μ V and this is most likely reason for statistical similarity. The improved ERG response with the old dose was better reflected in the mixed rod-cone ($P<0.05$) and cone-isolated responses ($P<0.01$). It has to be noted that all of these results have been generated with relatively small n numbers of mice. Furthermore given significant variation in the quality of subretinal injection in mice, caution must be taken not to over-interpret data. Therefore the data thus far obtained promotes trends that would have to be validated with later experiments including larger n numbers of mice.

6.3.19 The comparative analysis of AAV-2/5.BB24, AAV-2/8.BB24 and AAV-2/10.BB24 by western blotting

AAV-2/5 and AAV-2/8 have previously been shown to be efficient transducers of the mouse photoreceptors (Allocca et al, 2007). AAV-2/rh10 has recently been shown in the literature to be efficient at transducing the brain and retina (Mao et al, 2011; White et al, 2011). Therefore research currently ongoing in the TCD laboratory has been to establish hRHO expression from BB24 with the AAV-2/5, AAV-2/8 or AAV-2/rh10 serotype. One of the preliminary studies undertaken was to determine by western blotting which AAV serotype was capable of producing the most robust hRHO expression. Titre matched AAV-2/5.BB24 (LE), AAV-2/8.BB24 (RE) and AAV-2/8.AAV-2/rh10 (RE) were each injected into a three aged matched 129rho^{-/-} mice eyes. Three additional eyes (LE) were injected with a 1/10 dilution of AAV-2/5.RHO-P-EGFP (RE). Each AAV virus was injected alongside a 1/10 dilution of AAV-2/5 RHO-P-EGFP to utilise the expression of EGFP as an equal loading

control for western blotting. 14 days post-injection protein was extracted from each retina and western blot analysis was undertaken using the 4D2 antibody (**Figure 6.3.19.a**). Protein from RHO-M was used as a positive control for 4D2 (lane 1). hRHO immunoreactivity was visible with AAV-2/rh10.BB24 (lanes 2-4) and AAV-2/5.BB24 (lanes 8-10) at 39kDA. However in samples from AAV-2/8.BB24 injected mice abundant monomeric and multimeric forms of hRHO was observed (lanes 5-7). It seemed that AAV-2/8.BB24 was expressing hRHO 14 days post-injection more efficiently than AAV-2/5 or AAV-2/rh10. Animals injected with AAV-2/5. RHO-P.EGFP, which is a vector containing EGFP and no hRHO represented the negative control. In previous western blotting experiments equal loading with β -actin was somewhat difficult to obtain in 129rho^{-/-} mice, therefore it was decided to utilise EGFP as an equal loading control, EGFP at 25kDA was clearly present in all lanes of protein from mice injected AAV and absent from the RHO-M mouse. Overall EGFP appeared to be an appropriate loading control for these experiments. As this was only one preliminary western blot experiment, in future this evaluation would need to be repeated to verify AAV-2/8.BB24 is definitely the most efficient serotype in the photoreceptor cells. In addition evaluating different time-points would be beneficial in order to assess the onset of hRHO expression from these viral serotypes.

6.4 Discussion

Mutation-independent suppression and replacement strategies for autosomal dominant disorders involving suppression of both the mutant and wild-type alleles can be challenging (Millington-ward et al, 1997). Thus for RHO-adRP in which absence of hRHO results in a severe degeneration an approach to circumvent this rhodopsin loss is needed (Humphries et al, 1997). The suppression and replacement strategy for rhodopsin was first described by Millington-Ward et al, 1997 utilising ribozymes as the suppression agent. RNAi based mutation-independent suppression followed by the simultaneous delivery of a codon-modified human rhodopsin replacement gene has been explored *in vitro* and *in vivo* (Kiang et al, 2005; Millington-ward et al, 2011; O' Reilly et al, 2007; O'Reilly et al, 2008). RNAi-mediated mutation-independent suppression of wild-type hRHO and mutant pRHO has been successfully obtained as described in Chapters 3-Chapter 5 of this thesis. Therefore the focus of work presented in the current chapter was the optimisation of the second component of this therapy, codon-modified replacement, both *in vitro* and *in vivo*.

It was initially decided to design replacement genes two potent suppressors for siP2 and siP3. It was important to evaluate each replacement gene in cell culture as the sequence changes in each modified codon may not confer resistance to the corresponding siRNA. Therefore prior to *in vivo* analysis, replacement genes were designed and cloned into CMV-driven RHO plasmids to evaluate resistance to siRNA targets in HeLa cell culture similar to the experiments described in Chapter 3. An important feature of replacement gene design was ensuring an adequate amount of sequence changes have been introduced into the specific siRNA target site such that no suppression would take place. It has previously been established in chapter 5 that three mismatches between mRHO and the siP3 target site was sufficient to significantly suppress mRHO by 46% (**Section 5.3.14**). In addition Kiang et al established that mRHO with one mismatch to the siRNA and hRHO with 2 mismatches still resulted in rhodopsin suppression (Kiang et al, 2005). Thus it was decided to maximise the number of changes over the siP2 and siP3 target sites in hRHO. For siP2 the maximum number of changes that could be generated was 6, other replacement rhodopsin genes have been designed with between 4 to 10 mismatches which suggested that 6 changes should be sufficient (Cashman et al, 2005; O'Reilly et al, 2007; O'Reilly et al, 2008). However, it has previously been established that a siRNA (siC) with 5 mismatches to replacement hRHO was not sufficient to confer resistance, in contrast siCC a siRNA whose target site overlaps with siC had 4 mismatches to the replacement gene and did not suppress hRHO. This highlights the importance of how slight differences in target region can have an impact on siRNA mediated suppression and confirmed the need to evaluate every replacement gene *in vitro*

(O'Reilly et al, 2008). When designing replacement genes, some amino acids allow changes of more than one nucleotide within a codon such as leucine, arginine or serine in which nucleotide changes in the two positions of each codon are tolerated. O'Reilly et al utilised this concept to design replacement genes for two shRNA target sites shBB and shQ1. The siP2 target site had a codon for methionine within its sequence, which contains no redundancy and thus could not be altered. In terms of minimising codon usage, the number of nucleotide changes which could be introduced into siP2 without altering the codon usage frequency of the replacement gene was limited (**Table 6.3.1**). One codon for the amino acid tyrosine and two codons for phenylalanine were within the siP2 target site. Only two sequence variations exist for these amino acids, therefore only a choice of one different codon was available. These alternate codons had low codon usage within wild-type rhodopsin, which may not be ideal. There were fewer restrictions within the siP3 target site (**Table 6.3.2**) Three of the codons in this region coded for valine, therefore three possible choices for an alternative codon were possible, enabling conservative changes in the codons to be used. This allowed codon usage to be optimised. As previously mentioned the arginine within the siP3 site had five possible changes, however only one of these changes was present with high frequency in hRHO. The only codon which limited codon usage in the siP3 target site was phenylalanine.

Each replacement rhodopsin gene (rP2 & rP3) was co-transfected with its corresponding siRNA (siP2 & siP3) or the negative or positive control siRNAs (siNT or siBB). In theory, a replacement gene should be resistant to suppression by its corresponding siRNA but not resistant to other siRNAs. In principle the level of rhodopsin expression in HeLa cells transfected with the replacement gene and a non-targeting siRNA should therefore be similar to the level in HeLa cells transfected with the replacement gene and its corresponding siRNA. At the RNA level, rhodopsin replacement expression levels in cells co-transfected with siP2 and rP2 (82% rP2 expression [**Figure 6.3.4.a**]) and siP3 and rP3 (92% rP3 expression [**Figure 6.3.5.a**]) did not significantly differ from cells co-transfected with siNT and hRHO. However, significant suppression of the replacement genes was consistently observed when they were co-transfected with a non-corresponding siRNA (siBB).

It was important to establish if, despite the six nucleotide changes, each replacement gene was capable of adequate translation and protein expression *in vitro* (**Figure 6.3.6.a** & **Figure 6.3.7.a**). The amount of rhodopsin protein in samples transfected with the replacement gene and a non-targeting control was approximately equal to the amount of protein in samples transfected with wild-type rhodopsin and a non-targeting control. This suggests that in HeLa cells the expression levels of the rhodopsin replacement genes rP2 and rP3 are not significantly different from wild-type rhodopsin levels when

expressed from CMV promoters. Furthermore, each replacement rhodopsin gene appeared to be fully resistant to suppression by the corresponding siRNAs and demonstrated expression levels similar to when it was transfected with the non-targeting control.

The *in vivo* evaluation of shP3 in chapter 4 and chapter 5 established that this construct provided potent suppression the retina of both wild-type and rhodopsin linked adRP mice, therefore it was decided to choose the siP3 replacement gene rP3 for *in vivo* evaluation. In order to generate an *in vivo* replacement construct one of the most important considerations for rhodopsin is achieving appropriate levels of gene expression. In RHO knockout mice, the absence of RHO results in retinal degeneration as does over-expression, thus maintaining the correct balance of expression for a healthy retina is of extreme importance (Humphries et al, 1997; Millington-ward et al, 1997; Ollson et al, 1992). The creation of a transgenic mouse which expresses a hRHO replacement transgene *in vivo* was vital in providing proof of principle evidence that a replacement rhodopsin gene could provide the functional equivalent of its endogenous counterpart (O'Reilly et al, 2008). A vital understanding of the components needed for adequate hRHO expression from an AAV delivered rhodopsin replacement was achieved by comparative analysis of the hRHO replacement constructs with various promoters, promoter length and different transgene regulatory elements. The most efficient construct "BB24" contained a hybrid rhodopsin promoter as described in Palfi et al, 2010. Functional rescue both in terms of histology and ERG read-outs was achieved using this construct. It was therefore decided to model the rP3 replacement gene on the BB24 construct in order to obtain robust rP3 expression in the retina similar to that achieved with rBB *in vivo* (Millington-ward et al, 2011; Palfi et al, 2010).

As AAV-2/5 has been the vector of choice for all *in vivo* based studies in this laboratory previously and is an efficient delivery vehicle of human rhodopsin to the retina it was decided to generate AAV-2/5 mediated rP3 replacement (Millington-ward et al, 2011; Palfi et al, 2010). Recent studies however, have explored the use AAV-2/8 based delivery to the photoreceptors with success. In particular Mussolino et al utilised AAV-2/8 for RHO-based suppression in the photoreceptors (Mussolino et al, 2011a). Thus it was decided to generate AAV-2/8.rP3 and perform a comparative analysis of rP3 transgene expression in the retina from AAV-2/5 and AAV-2/8 based constructs. Previously in the laboratory, the first generation CsCl method was used to purify AAV-2/5, in this protocol the supernatant of the clarified cell lysates following HEK293 transfection is typically discarded (Auricchio et al, 2001b). This is a disadvantage given that the supernatant has been shown to have high titre AAV infectivity (Vandenberghe et al, 2010). For human clinical trials, a high titre, purified virus made in accordance with good manufacturing practices is needed. Ideally this virus must achieve

potent transduction, resulting in sufficient transgene expression without causing immunogenicity in human patients (Wright, 2011).

Recently a second generation AAV CsCl purification protocol has been published. This method involves precipitation of DNA and protein impurities from AAV particles present not only in the cell pellet but also in the supernatant by PEG800. This was done prior to the first ultracentrifugation step and resulted in significantly reduced DNA and protein contamination compared to the standard protocol. Furthermore, this high titre purified AAV virus was comparable to scaled up AAV production methods currently being used in human clinical studies. In addition, when this optimised virus was compared in mice and dogs, an improved viral transduction was observed to the target tissues compared to virus made by the standard protocol. This indicated that low doses of potent AAV may be both favorable and achievable at reducing the immune response and conferring significant therapeutic benefit in human patients (Ayuso et al, 2010). As a result of this study it was decided to generate AAV-2/5.rP3 and AAV-2/8.rP3 using this optimised protocol. In accordance with that described by Ayuso et al, 2010 viral titres in the region of 6×10^{13} vp/ml or 4×10^{13} vp/ml for AAV-2/5 and AAV-2/8 respectively were achieved.

High titre AAV-2/5.rP3 and AAV-2/8.rP3 were subretinally injected into 129rho^{-/-} mice. The main goal of this experiment was to evaluate if rP3 rhodopsin could produce hRHO in the ROS of these mice and have functional effects. The second aim of this experiment was to explore if there were differences in hRHO expression between AAV-2/5.rP3 and AAV-2/8.rP3. The initial evaluation of rP3 expression involved assessing if rP3 could provide functional benefit in 129rho^{-/-} mice by ERG. The highest titre possible for both AAV-2/5.rP3 and AAV-2/8.rP3 was injected. It has previously been reported that at P0 the injection process may damage photoreceptors, while at P10 this damage is greatly reduced (Palfi et al, 2010). In addition P4 is the age at which endogenous rhodopsin begins to be expressed in wild-type mice (McNally et al, 1999). For this reason a time-point of P5 was chosen for injection to prevent as much damage as possible while still providing early therapeutic intervention. The rod-isolated response obtained from uninjected mice was consistently 0μV which is consistent with that described for this model (Humphries et al, 1997). For AAV-2/5.rP3 out of 22 mice only 22% showed a rod-isolated ERG this was surprising as previously in the laboratory utilising AAV-2/5.BB24 or AAV-2/8.BB24 approximately half of 129rho^{-/-} mice have improved rod-isolated b-waves (Dr. Paul Kenna[personal communication]). Furthermore when the positive rod-isolated b-wave responses were combined at a dose of 4.8×10^{10} vp an average b-wave response of 87μV was recorded (**Figure 6.3.11.d**). While this represented a significant difference compared to the uninjected eye, it was lower

than that previously obtained with AAV-2/5.BB24 at a lower dose of 6.5×10^9 vp in 129rho^{-/-} mice (135μV[Palfi et al, 2010]). The highest b-wave for AAV-2/5.rP3 was 125μV and the standard deviation was high at 49μV, it may be that this difference is due to the lack of n numbers showing an improved rod-isolated response. In order to rule out any anomalies with the 129rho^{-/-} mice, a titre matched AAV-2/5.BB24 was included as a positive control in 7 mice. Unfortunately this control virus was from a bad batch of virus and thus was subsequently excluded from the analysis. For AAV-2/8.rP3 a titre matched AAV-2/8.BB24 control was injected into the fellow eye as a control. This AAV-2/5.BB24 control was made by Jean Bennett's laboratory and had previously been shown as very efficient. As expected of the 13 eyes injected with AAV-2/8.BB24, 10 had a significantly increased rod-isolated response. In contrast similar to AAV-2/5.rP3 only 24% of mice gave an increase in the rod-isolated ERG response when injected with AAV-2/8.rP3. AAV-2/8.BB24 elicited potent rod-isolated responses in the region of 239μV, in comparison AAV-2/8.rP3 resulted in an average rod-isolated response of 47μV (**Figure 6.3.12.d**). While no significant differences were recorded between AAV-2/5.rP3 and AAV-2/8.rP3 overall the trend was towards lower rod responses with AAV-2/8.rP3. It should be noted that in all cases, the cone-isolated responses with AAV-2/5.rP3 (**Figure 6.3.11.f**) and AAV-2/8.rP3 (**Figure 6.3.13.f**) injected eyes were lower than the uninjected eye. This has previously been noted by Palfi et al, in which delivery of an AAV-2/5.BB24 replacement gene resulted in alteration of cone function. Furthermore in this study it was established that both AAV-2/5 RHO-P.EGFP and PBS injections resulted in the same phenomenon, suggesting this may be a result of mechanical injury to the retina. At P10 the cone damage is not observed, however, by increasing the age of injection to P10, one may not achieve as much therapeutic efficacy in 129rho^{-/-} mice (Palfi et al, 2010). A recent study by Beltran et al, 2010 in dogs established high dose EGFP delivered by AAV-2/5 and driven by the CBA or hGRK1 promoter resulted in damage to the cones as assessed by histology. While it is possible that cone damage could be occurring as a result of the promoter used or the AAV vector, it is likely that high dose EGFP expression was causing cone mediated toxicity in this case (Beltran et al, 2010).

Retinal sections of mice in which increased rod-isolated ERG responses were observed when injected with AAV-2/5.rP3 (**Figure 6.3.13.a**) and AAV-2/8.rP3 (**Figure 6.3.13.b**) were taken and hRHO and DAPI staining were performed. Similar to that observed in Palfi et al, 2010 hRHO staining was clearly evident in the retinal sections from mice injected with AAV-2/5.rP3 compared to uninjected mice. Furthermore hRHO staining was only observed in the EGFP positive region which confirmed that AAV-2/5 mediated rP3 expression had occurred in these mice. Furthermore EGFP positive inner

segments were present, indicating that some improvement in histology in transduced areas of the retina. The aged matched RHO-M transgenic mouse had abundant hRHO expression as expected validating the use of this model as an experimental control (O'Reilly et al, 2008). For AAV-2/8.BB24 the transduced area of the retina had nice healthy EGFP positive inner segments and robust staining of the OS with hRHO, similar to that observed in RHO-M mice. This is not surprising given the rod-isolated significant ERG responses recorded in these animals. Histological examination of AAV-2/8.rP3 treated eyes demonstrated that in transduced of the retina only minimal, shortened inner segments with a small amount of hRHO staining was present and indicated that AAV-2/8.rP3 may not be efficient at providing sufficient hRHO to 129rho^{-/-} mice

A western blot was undertaken with mice injected with AAV-2/5.rP3 (**Figure 6.3.15.a**) or AAV-2/8.rP3 (**Figure 6.3.16.a**) in which no improved ERG response was recorded. This was undertaken in order to establish if despite no functionality, if these vectors were still producing hRHO protein but just at lower levels. For AAV-2/5.rP3 2/3 mice had low levels of hRHO. Similarly for AAV-2/8.rP3, 1/3 mice had low hRHO expression; this appeared to indicate that hRHO in some of these animals was being produced. In order to confer functionality it is important to obtain sufficient levels of hRHO. It may be that the level of hRHO rP3 expression from AAV-2/5 or AAV-2/8 is just below this threshold and thus would explain why only a small number of animals demonstrated a minimal rod-isolated ERG increase in this set of subretinal injections. AAV-2/8.BB24 injected animals had robust hRHO expression, which was consistent with the high rod-isolated ERG responses and abundant hRHO outer segment staining obtained in each of these mice.

In order to check if the AAV-2/5.rP3 and AAV-2/8 rP3 dose administered may have been too high and therefore not tolerated in these mice, real-time RT-PCR analysis was undertaken examining hRHO rP3 mRNA expression in adult wild-type mice injected with the equivalent dose of either AAV-2/5.rP3 or AAV-2/8.rP3 and compared RHO expression in both NHR and RHO-M mice (**Figure 6.3.17.a**). While a significant difference in expression was achieved with AAV-2/5.rP3 compared to NHR, no significant difference was achieved with AAV-2/8.rP3. In addition there was no significant difference between RHO-M and either AAV-2/5.rP3 or AAV-2/8.rP3. However there was high variability between the AAV-2/8.rP3 samples, with two mice in particular only expressing hRHO at 16 & 14% of NHR respectively. It may be possible that the variance observed with AAV-2/8.rP3 is the reason that only some mice yielded an ERG read-out. In this regard it is notable that in a 129rho^{-/-} mouse, typically the viral spread is approximately 30-40% after subretinal injection (Palfi et al, 2010). Thus in the AAV-2/8.rP3 mice (where expression was comparable to NHR and RHO-M) or indeed in the AAV-

2/5.rP3 in which high hRHO expression was obtained (comparable to RHO expression in RHO-M in which the entire retina expresses RHO) it may be possible that too much hRHO in the injected region of the retina may have a negative impact on the transduced cells. In order to evaluate if lowering the dose had any difference on functionality, diluted virus may be employed.

Towards the end of the study presented in this thesis, a high titre AAV-2/8.BB24 was produced by Dr. Naomi Chadderton using the new second generation CsCl gradient protocol (Ayuso et al, 2010). It was decided that during the evaluation of this newly generated vector in 129rho^{-/-} mice, a lower dose of AAV-2/8.rP3 would also be tested in order to confirm if lowering viral titre rescues hRHO functionality. At a lower dose of 9×10^9 vp, 4/6 mice had a significantly higher rod-isolated ERG when injected with AAV-2/8.rP3 compared to uninjected mice (**Figure 6.3.18.d**). This represented 66% of the mice showing an improved response with the lower dose vector compared to 24% with the high dose AAV-2/8. The average rod-isolated response was in the region of 132μV, with the highest achieved being 234μV, suggesting that lowering dose improved the functional benefit obtained. Previous analysis had shown an impaired cone response in AAV injected eye compared to the uninjected eye. This however was not the case for the lower dose AAV-2/8.rP3 vector in which a cone response similar to the uninjected eyes was observed (**Figure 6.3.18.f**). Thus in the context of subretinal surgery at P5 into the young mouse eye delivery of a high dose AAV-2.8BB24 or AAV-2/8.rP3 may have an impact on cone photoreceptors. It may be that while the injection may damage both rods and cones, any improvement in rod-isolated function in the 129rho^{-/-} mouse in which the rod function was zero may be sufficient to mask the damage to rod-cells and provide improvement in some rod responses. In 129 rho^{-/-} the cones cells still function therefore damage due to injection may have a negative impact on cell function (Humphries et al, 1997). Overall lowering the titre of AAV-2/8.rP3 successfully improved the rod-isolated responses in a 129rho^{-/-} mice. This supports the further optimisation of low dose AAV-2/8.rP3 for use in a suppression and replacement strategy for rP3. In addition this highlights the need for studies prior to proceeding to human clinical trial, evaluating different doses not only in mice but also larger animal models to establish the dose limiting toxicity of each individual therapeutic (Vandenberghe & Auricchio, 2012). For retinal human clinical trials the highest AAV dose administered to the retina of human patients was 1.5×10^{11} vp of AAV-2/2, no adverse effects were noted with this dose (Maguire et al, 2009). In addition for larger animal models such as non-human primates the highest dose evaluated to date has been 2.7×10^{13} vp for a therapeutic strategy for red green color blindness and 6.4×10^{13} vp was used in dogs in order to rescue cone function in a congenital achromatopsia model (Komaromy et al, 2010; Mancuso et al, 2009;

Vandenberghe & Auricchio, 2012). However despite efficacy being achieved in these models no thorough dose toxicity studies were specifically undertaken (Vandenberghe & Auricchio, 2012). In other studies assessing AAV delivered transgenes to the retina in non-human primate using high dose AAV-2/2 (10^{10} vp) and AAV-2/8 (10^{11} vp) the authors noted a dose related increase in neutralising antibodies to the vector capsid. In addition evidence of t-cell responses to the GFP transgene product was obtained in two animals that had received the highest dose of AAV-2 and AAV-8. This highlights the need to evaluate high dose AAV-based therapies fully in terms of immune responses to the AAV capsid and/or the transgene (Vandenberghe et al, 2011).

One of the main questions to be answered in response to the above result is why was high dose AAV-2/8.rP3 or AAV-2/5.rP3 toxic to photoreceptors? The second generation CsCl protocol utilised in this laboratory to make these vectors is only in the early stages of optimisation. AAV-2/2 has been shown to be prone to aggregation (Wright et al, 2003; Wright et al, 2005). However the serotypes used in this study were AAV-2/5 and AAV-2/8. No aggregation of AAV-2/5 has thus far been obtained in the TCD laboratory. As AAV-2/8 is not a virus that has traditionally been generated in the laboratory it is possible that AAV-2/8 may be aggregating, and thus by diluting out the aggregating virus 3.5 fold adverse effects on the retina are being prevented. One of the differences between the protocol used in this study and the Ayuso et al, 2010 protocol is that in the original method two ultracentrifugation steps are performed, in contrast three ultracentrifugation steps are undertaken in our laboratory. It could be that by performing an extra ultracentrifugation step the amount of time in which the virus would have an opportunity to aggregate is being increased (Dr. Naomi Chadderton [personal communication]). In future studies with this protocol, aggregation of both AAV-2/8 and AAV-2/5 viruses will be tested using a dynamic light scattering device. In addition each qPCR reaction is only performed twice in our laboratory therefore one cannot rule out slight under estimations of viral titre as reasons for discrepancies between “titre matched” viruses. Further analysis into the low dose AAV-2/8.rP3 virus is warranted in order to fully confirm the extent of hRHO rescue in 129rho^{-/-} mice. Due to the time constraints it was not possible to further evaluate this lower dose. However in future analysis both low dose AAV-2/5.rP3 and AAV-2/8.rP3 in terms of ERG (with increased animal numbers), immunohistochemistry, western blotting and ultrastructure analyses will be performed to optimise this replacement component. In addition, the generation of alternate viral preparations may be needed to rule out any impurities or abnormalities that may have occurred during the generation of AAV-2/5.rP3 and AAV-2/8.rP3 in this study.

The final experiment undertaken in this study was a comparative analysis of protein expression from AAV-2/5.BB24, AAV-2/8.BB24 and AAV-2/rh10.BB24 (**Figure 6.3.19.a**). It has been shown that both AAV-2/8 and AAV-2/rh10 are efficient at producing high levels of transgene expression (Allocca et al, 2007; Mao et al, 2011a). In order to assess rhodopsin expression from these three vectors a comparative analysis is currently ongoing in the TCD laboratory utilising the BB24 rhodopsin construct. Three titre matched (6×10^{13} vp/ml) are currently being evaluated in terms of ERG, transduction efficiencies, distribution and expression. The first time-point chosen was 14 days as this is the time-point at which AAV1, 4, 5, 7, 8, 9 and rh10 reach their peak in transduction efficiency (Lebherz et al, 2008; Mao et al, 2011a). Western blot analysis with protein samples from three mouse eyes injected with each construct and assessed at 14 days suggests that AAV-2/8 efficiently expresses hRHO compared to AAV-2/5 and AAV-2/rh10. This study also demonstrated the use of EGFP as an equal loading control for western blotting for these experiments. This western blot experiment was only undertaken once, clearly in order to confirm the result this analysis needs to be repeated. Furthermore assessing AAV-2/5, -2/8 and -2/rh10 at earlier and later time points would be of value to determine the rhodopsin transgene expression pattern from all serotypes.

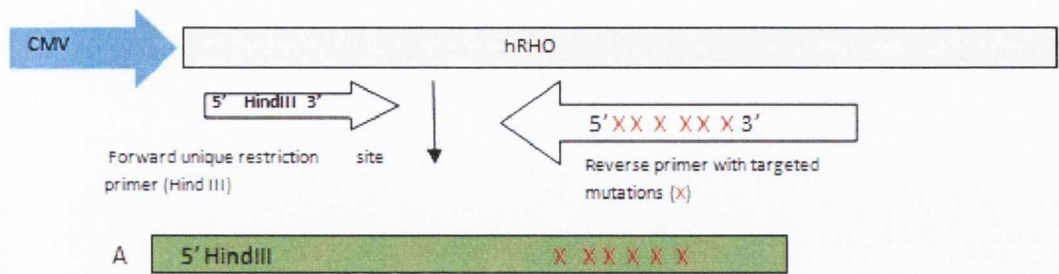
In conclusion the research presented in this chapter has involved the optimisation of a replacement strategy for rhodopsin for use as part of a two-component suppression and replacement gene therapy for RHO-adRP. Replacement genes made for the siP2 and siP3 target sites within hRHO were successfully generated and tested *in vitro* in order to confirm resistance from siRNA-mediated suppression. In addition, optimising the gene expression of the rP3 replacement gene *in vivo* was explored. A previous replacement gene construct BB24 had been designed using a hybrid rhodopsin promoter to drive replacement hRHO expression. This promoter has been utilised with success *in vivo* therefore rP3 was also expressed from this promoter (Millington-Ward et al, 2011; Palfi et al, 2010). While high dose AAV-2/5.rP3 or AAV-2/8.rP3 was not found to be as efficient as previous replacement genes generated in the laboratory, lowering the dose injected had a significant impact on rod-isolated ERG function. In one study recently therapeutic benefit in terms of improved histology and ERG function when a wild-type replacement gene was delivered to a mouse model with the P23H mutant transgene was achieved (Mao et al, 2011b). This was somewhat surprising given the dominant nature of the P23H mutation. In addition delivery of a hRHO replacement gene in the absence of a suppressor molecule to the HP347S mouse was not sufficient to rescue the retinal degeneration in studies in this laboratory (Millington-Ward et al, 2011). The results from these studies highlight the need for evaluating each therapeutic approach in a wide range of animal models with different forms of RHO-adRP to obtain a complete and accurate analysis of each therapy.

rP2F	5'-CATCTATATGTTTGTCTGTCGTCATTTTACCATC-5'
rP2R	5'-GATGGTAAAATGCACGACAAACATATAGATG-3'
rP3 F	5'-CGAGCGCTATGTCGTCGTCTGCAAGCCCATG- 3'
rP3 R	5'-CATGGGCTTGCAGACGACGACATAGCGCTCG- 3'
5' HindIII F	5'-GATAAGCTTAGAGTCATCCAGC-3'
3' BstEII R	5'-ATGCGGGTGACCTCCTTC-3'

Table 6.2.2 The PCR primers used to generate both the rP2 and rP3 replacement genes by PCR directed mutagenesis.

The rP2 forward and reverse primer (rP2 F and rP2 R) were designed such that they introduce 6 nucleotide alterations into the rP2 target site. The rP3 forward and reverse primers (rP3 F and rP3 R) were designed such that they introduce the 6 nucleotide changes into the rP3 target site. The 5'*HindIII* Forward and 3'*BstEII* reverse (5' *HindIII* F and 3'*BstEII* R) were used that each replacement RHO PCR product was flanked by *HindIII* and *BstEII* restriction sites, in order to facilitate cloning.

Step 1: PCR 1



Step 2: PCR 2

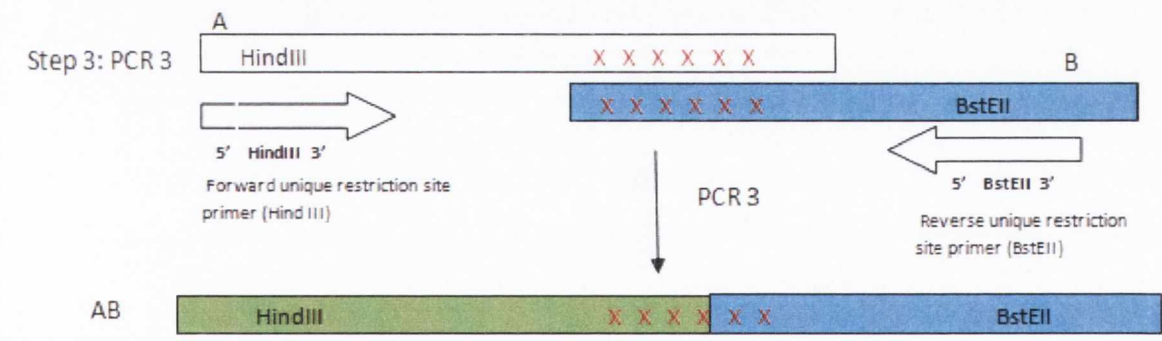
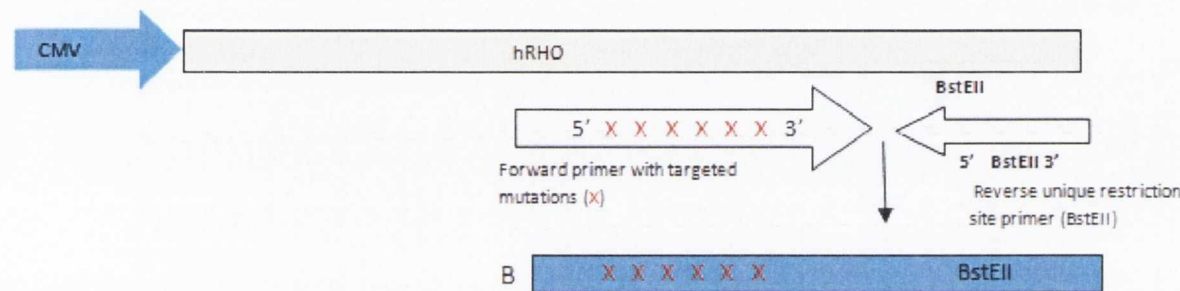


Figure 6.2.2.a PCR directed mutagenesis of hRHO to create rP2 or rP3

1. Using hRHO as a DNA template the 5' *HindIII* F and R replacement primer with the directed mutations in used for PCR 1
2. PCR 2 is carried out using the hRHO template and the F replacement primer with the directed mutations
3. The third PCR ins carried out using the amplification products from PCR1 and PCR2 as the template at the 5' *HindIII* and 3' *BstEII* primers, such that an overall replacement hRHO is generated flanked by *HindIII* and *BstEII*

Codon usage in wild-type <i>RHO</i> and the replacement <i>RHO</i> gene for siP2, <i>rP2</i>							
RHO amino acid	Y	M	F	V	V	H	F
<i>RHO</i> nucleotide sequence	TAC	ATG	TTC	GTG	GTC	CAC	TTC
RHO Codon Usage (No. of codons in hRHO)	17	15	25	13	15	3	25
rP2 amino acid	Y	M	F	V	V	H	F
<i>rP2</i> nucleotide sequence	TAT	ATG	TTT	GTC	GTG	CAT	TTT
rP2 Codon usage (No. of codons in rP2)	2	15	5	15	13	2	5

Table 6. 3.1 Codon usage analysis within the hRHO siP2 target site

Rows 1 to 3 depict codon usage of the siP2 target site within wild-type hRHO. Six changes were made to the seven codons within this sequence; all changes minimised changes to codon usage were possible. Rows 4-6 are the nucleotide changes present within each of the six codons of the siP2 target sequence, for Tyr (Y), Phe (F) and His (H); a choice of only one additional codon was available. For Val (V) the most common codon following the original codon was utilised. No changes were made to Met (M); this is the start codon for transcription; there is no degeneracy of this codon.

ATG AAT GGC ACA GAA GGC CCT AAC TTC TAC GTG CCC TTC TCC AAT GCG ACG
 GGT GTG GTA CGC AGC CCC TTC GAG TAC CCA CAG TAC TAC CTG GCT GAG CCA
 TGG CAG TTC TCC ATG CTG GCC GCC TAC ATG TTT CTG CTG ATC GTG CTG GGC
 TTC CCC ATC AAC TTC CTC ACG CTC TAC GTC ACC GTC CAG CAC AAG AAG CTG
 CGC ACG CCT CTC AAC TAC ATC CTG CTC AAC CTA GCC GTG GCT GAC CTC TTC
 ATG GTC CTA GGT GGC TTC ACC AGC ACC CTC TAC ACC TCT CTG CAT GGA TAC
 TTC GTC TTC GGG CCC ACA GGA TGC AAT TTG GAG GGC TTC TTT GCC ACC CTG
 GGC GGT GAA ATT GCC CTG TGG TCC TTG GTG GTC CTG GCC ATC GAG CGG TAC
 GTG GTG GTG TGT AAG CCC ATG AGC AAC TTC CGC TTC GGG GAG AAC CAT GCC
 ATC ATG GGC GTT GCC TTC ACC TGG GTC ATG GCG CTG GCC TGC GCC GCA CCC
 CCA CTC GCC GGC TGG TCC AGG TAC ATC CCC GAG GGC CTG CAG TGC TCG TGT
 GGA ATC GAC TAC TAC ACG CTC AAG CCG GAG GTC AAC AAC GAG TCT TTT GTC
 ATC **TAC ATG TTC GTG**
GTC CAC TTC ACC ATC CCC ATG ATT ATC ATC TTT TTC TGC TAT GGG CAG CTC
 GTC TTC ACC GTC AAG GAG GCC GCT GCC CAG CAG CAG TCA GCC ACC ACA
 CAG AAG GCA GAG AAG GAG GTC ACC CGC ATG GTC ATC ATC ATG GTC ATC GCT
 TTC CTG ATC TGC TGG GTG CCC TAC GCC AGC GTG GCA TTC TAC ATC TTC ACC
 CAC CAG GGC TCC AAC TTC GGT CCC ATC TTC ATG ACC ATC CCA GCG TTC TTT
 GCC AAG AGC GCC GCC ATC TAC AAC CCT GTC ATC TAT ATC ATG ATG AAC AAG
 CAG TTC CGG AAC TGC ATG CTC ACC ACC ATC TGC TGC GGC AAG AAC CCA CTG
 GGT GAC GAT GAG GCC TCT GCT ACC GTG TCC AAG ACG GAG ACG AGC CAG GTG
 GCC CCG GCC TAA

B
 TCGACGGTATCGAT**AAGCTT**AGAGTCATCCAGCTGGAGCCCTGAGTGGCTGAGCTCAGGCCTTCGCAGC
 ATTCTTG **5'HindIII F primer**
 GGTGGGAGCAGCCACGGGTCAGCCACAAGGGCCACAGCC ATG AAT GGC ACA GAA GGC CCT
 AAC TTC TAC GTG CCC TTC TCC AAT GCG ACG GGT GTG GTA CGC AGC CCC TTC
 GAG TAC CCA CAG TAC TAC CTG GCT GAG CCA TGG CAG TTC TCC ATG CTG GCC
 GCC TAC ATG TTT CTG CTG ATC GTG CTG GGC TTC CCC ATC AAC TTC CTC ACG
 CTC TAC GTC ACC GTC CAG CAC AAG AAG CTG CGC ACG CCT CTC AAC TAC ATC
 CTG CTC AAC CTA GCC GTG GCT GAC CTC TTC ATG GTC CTA GGT GGC TTC ACC
 AGC ACC CTC TAC ACC TCT CTG CAT GGA TAC TTC GTC TTC GGG CCC ACA GGA
 TGC AAT TTG GAG GGC TTC TTT GCC ACC CTG GGC GGT GAA ATT GCC CTG TGG
 TCC TTG GTG GTC CTG GCC ATC GAG CGG TAC GTG GTG GTG TGT AAG CCC ATG
 AGC AAC TTC CGC TTC GGG GAG AAC CAT GCC ATC ATG GGC GTT GCC TTC ACC
 TGG GTC ATG GCG CTG GCC TGC GCC GCA CCC CCA CTC GCC GGC TGG TCC AGG
 TAC ATC CCC GAG GGC CTG CAG TGC TCG TGT GGA ATC **GAC TAC TAC ACG CTC**
AAG CCG GAG GTC AAC AAC GAG TCT TTT GTC ATC TAT ATG TTT GTC GTG CAT
TTT ACC ATC CCC ATG ATT ATC ATC TTT TTC TGC TAT GGG CAG CTC GTC TTC
 ACC GTC AAG GAG GCC GCT GCC CAG CAG CAG GAG TCA GCC ACC ACA CAG AAG
 GCA GAG AAG GAG **GTC ACC** CGC ATG GTC

3'BstEII R primer
 ATC ATC ATG GTC ATC GCT TTC CTG ATC TGC TGG GTG CCC TAC GCC AGC GTG
 GCA TTC TAC ATC TTC ACC CAC CAG GGC TCC AAC TTC GGT CCC ATC TTC ATG
 ACC ATC CCA GCG TTC TTT GCC AAG AGC GCC GCC ATC TAC AAC CCT GTC ATC
 TAT ATC ATG ATG AAC AAG CAG TTC CGG AAC TGC ATG CTC ACC ACC ATC TGC
 TGC GGC AAG AAC CCA CTG GGT GAC GAT GAG GCC TCT GCT ACC GTG TCC AAG
 ACG GAG ACG AGC CAG GTG GCC CCG GCC TAA

Figure 6.3.1.a Design of the rP2 replacement gene

The first sequence (A) with wild-type hRHO with the siP2 target site marked (red). The second sequence (B) is the rP2 replacement gene (rP2) with the 6 changes within the siP2 target site (red) and with the two primers 5' and 3' to the target site with the *HindIII* and *BstEII* restriction sites marked (yellow)

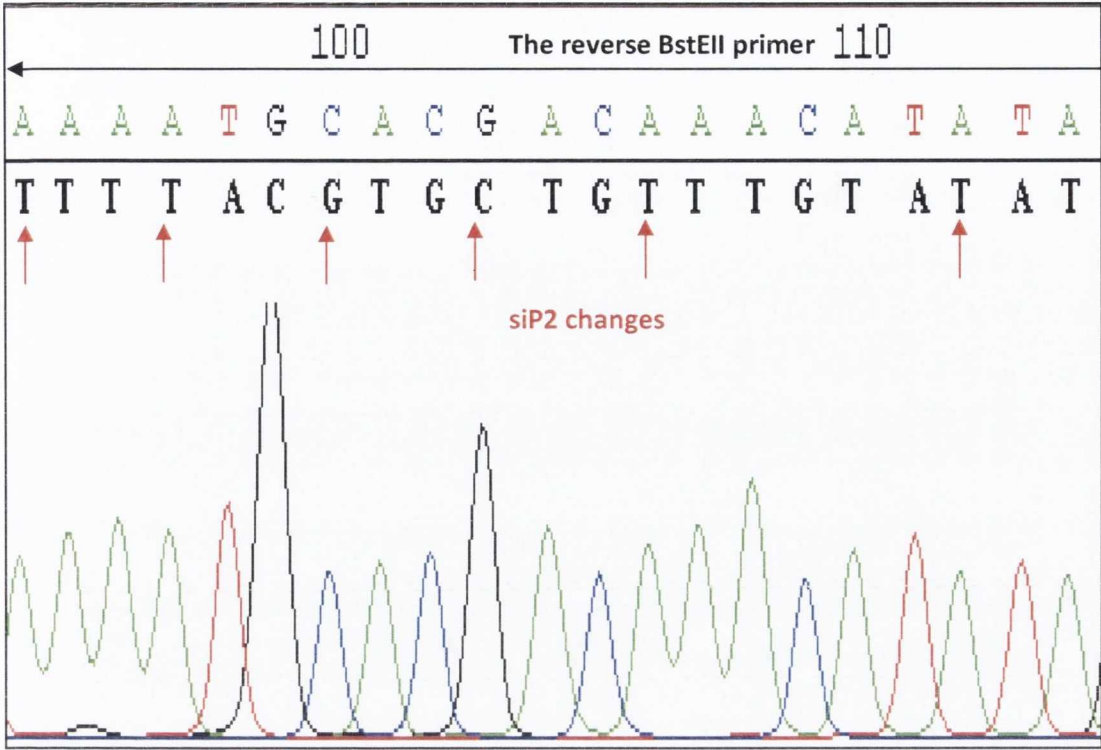


Figure 6.3.1.b: Sequence of rP2 over the siP2 target site
 The reverse *BstEII* primer was used for sequencing of rP2 over the siP2 site; the six changes (TACATGTTCGTGGTCCACTTC to TATATGTTTGTGCGTCATTTT) are indicated by the red arrows.

Codon usage in wild-type <i>RHO</i> and the replacement <i>RHO</i> gene for siP3, <i>rP3</i>						
	R	Y	V	V	V	C
<i>RHO</i> nucleotide sequence	CGG	TAC	GTG	GTG	GTG	TGT
No. codons in hRHO						
<i>RHO</i> Codon Usage	4	17	13	13	13	2
rP3 amino acid	Y	M	F	V	V	H
<i>rP3</i> nucleotide sequence	CGC	TAT	GTC	GTC	GTC	TGC
rP3 Codon usage	4	2	15	15	15	8
No. of codons in rP3						

Table 6. 3.2 Codon usage analysis within the hRHO siP3 target site

Rows 1 to 3 depict codon usage of the siP3 target site within wild-type hRHO. Six changes were made to all six codons within this sequence; all changes minimised changes to codon usage were possible. Rows 4-6 are the nucleotide changes present within each of the six codons of the siP3 target sequence, for Tyr (Y) and Cys (C), a choice of only one additional codon was available. For Arg (R) and Val (V) the most common codon following the original was utilised.

A

ATG AAT GGC ACA GAA GGC CCT AAC TTC TAC GTG CCC TTC TCC AAT GCG ACG GGT GTG
GTA CGC AGC CCC TTC GAG TAC CCA CAG TAC TAC CTG GCT GAG CCA TGG CAG TTC TCC
ATG CTG GCC GCC TAC ATG TTT CTG CTG ATC GTG CTG GGC TTC CCC ATC AAC TTC CTC
ACG CTC TAC GTC ACC GTC CAG CAC AAG AAG CTG CGC ACG CCT CTC AAC TAC ATC CTG
CTC AAC CTA GCC GTG GCT GAC CTC TTC ATG GTC CTA GGT GGC TTC ACC AGC ACC CTC
TAC ACC TCT CTG CAT GGA TAC TTC GTC TTC GGG CCC ACA GGA TGC AAT TTG GAG GGC
TTC TTT GCC ACC CTG GGC GGT GAA ATT GCC CTG TGG TCC TTG GTG GTC CTG GCC ATC
GAG CGG TAC GTG GTG GTG TGT AAG CCC ATG AGC AAC TTC CGC TTC GGG GAG AAC CAT
GCC ATC ATG GGC GTT GCC TTC ACC TGG GTC ATG GCG CTG GCC TGC GCC GCA CCC CCA
CTC GCC GGC TGG TCC AGG TAC ATC CCC GAG GGC CTG CAG TGC TCG TGT GGA ATC GAC
TAC TAC ACG CTC AAG CCG GAG GTC AAC AAC GAG TCT TTT GTC ATC TAC ATG TTC GTG
GTC CAC TTC ACC ATC CCC ATG ATT ATC ATC TTT TTC TGC TAT GGG CAG CTC GTC TTC
AAC GTC AAG GAG GCC GCT GCC CAG CAG CAG GAG TCA GCC ACC ACA CAG AAG GCA GAG
AAG GAG GTC AAG CCG ATG GTG ATC ATC ATG GTC ATC GCT TTC CTG ATC TGC TGG GTG
CCC TAC GCC AGC GTG GCA TTC TAC ATC TTC ACC CAC CAG GGC TCC AAC TTC GGT CCC
ATC TTC ATG ACC ATC CCA GCG TTC TTT GCC AAG AGC GCC GCC ATC TAC AAC CCT GTC
ATC TAT ATC ATG ATG AAC AAG CAG TTC CGG AAC TGC ATG CTC ACC ACC ATC TGC TGC
GGC AAG AAC CCA CTG GGT GAC GAT GAG GCC TCT GCT ACC GTG TCC AAG ACG GAG ACG
AGC CAG GTG GCC CCG GCC TAA

B

TCGACGGTATCGATAAGCTTAGAGTCATCCAGCTGGAGCCTGAGTGGCTGAGCTCAGGCCTTCGCAGCATTCTTG

5'*HindIII* F primer

GTA CGC AGC CCC TTC GAG TAC CCA CAG TAC TAC CTG GCT GAG CCA TGG CAG TTC TCC
ATG CTG GCC GCC TAC ATG TTT CTG CTG ATC GTG CTG GGC TTC CCC ATC AAC TTC CTC
ACG CTC TAC GTC ACC GTC CAG CAC AAG AAG CTG CGC ACG
CCT CTC AAC TAC ATC CTG CTC AAC CTA GCC GTG GCT GAC CTC TTC ATG GTC CTA GGT
GGC TTC ACC AGC ACC CTC TAC ACC TCT CTG CAT GGA TAC TTC GTC TTC GGG CCC ACA
GGA TGC AAT TTG GAG GGC TTC TTT GCC ACC CTG GGC GGT GAA ATT GCC CTG TGG TCC
TTG GTG GTC CTG GCC ATC GAG CGC TAT GTC GTC GTC TGC
AAG G CCC ATG AGC AAC TTC CGC TTC GGG GAG AAC CAT GCC ATC ATG GGC GTT GCC
TTC ACC TGG GTC ATG GCG CTG GCC TGC GCC GCA CCC CCA CTC GCC GGC TGG TCC AGG
TAC ATC CCC GAG GGC CTG CAG TGC TCG TGT GGA ATC GAC TAC TAC ACG CTC AAG CCG
GAG GTC AAC AAC GAG TCT TTT GTC ATC TAT ATG TTT GTC GTG CAT TTT ACC ATC CCC
ATG ATT ATC ATC TTT TTC TGC TAT GGG CAG CTC GTC TTC ACC GTC AAG GAG GCC GCT
GCC CAG CAG CAG GAG TCA GCC ACC ACA CAG AAG GCA GAG AAG GAG GTC ACC CGC ATG
GTC

3'*BstEII* R primer

ATC ATC ATG GTC ATC GCT TTC CTG ATC TGC TGG GTG CCC TAC GCC AGC GTG GCA
TTC TAC ATC TTC ACC CAC CAG GGC TCC AAC TTC GGT CCC ATC TTC ATG ACC ATC CCA
GCG TTC TTT GCC AAG AGC GCC GCC ATC TAC AAC CCT GTC ATC TAT ATC ATG ATG AAC
AAG CAG TTC CGG AAC TGC ATG CTC ACC ACC ATC TGC TGC GGC AAG AAC CCA CTG GGT
GAC GAT GAG GCC TCT GACT ACC GTG TCC AAG ACG GAG ACG AGC CAG GTG GCC CCG
GCC TAA

Figure 6.3.2.a Design of the rP3 replacement gene

The first sequence (A) with wild-type hRHO with the siP2 target site marked (red). The second sequence (B) is the rP2 replacement gene (rP2) with the 6 changes within the siP2 target site (red) and with the two primers 5' and 3' to the target site with the *HindIII* and *BstEII* restriction sites marked (yellow).

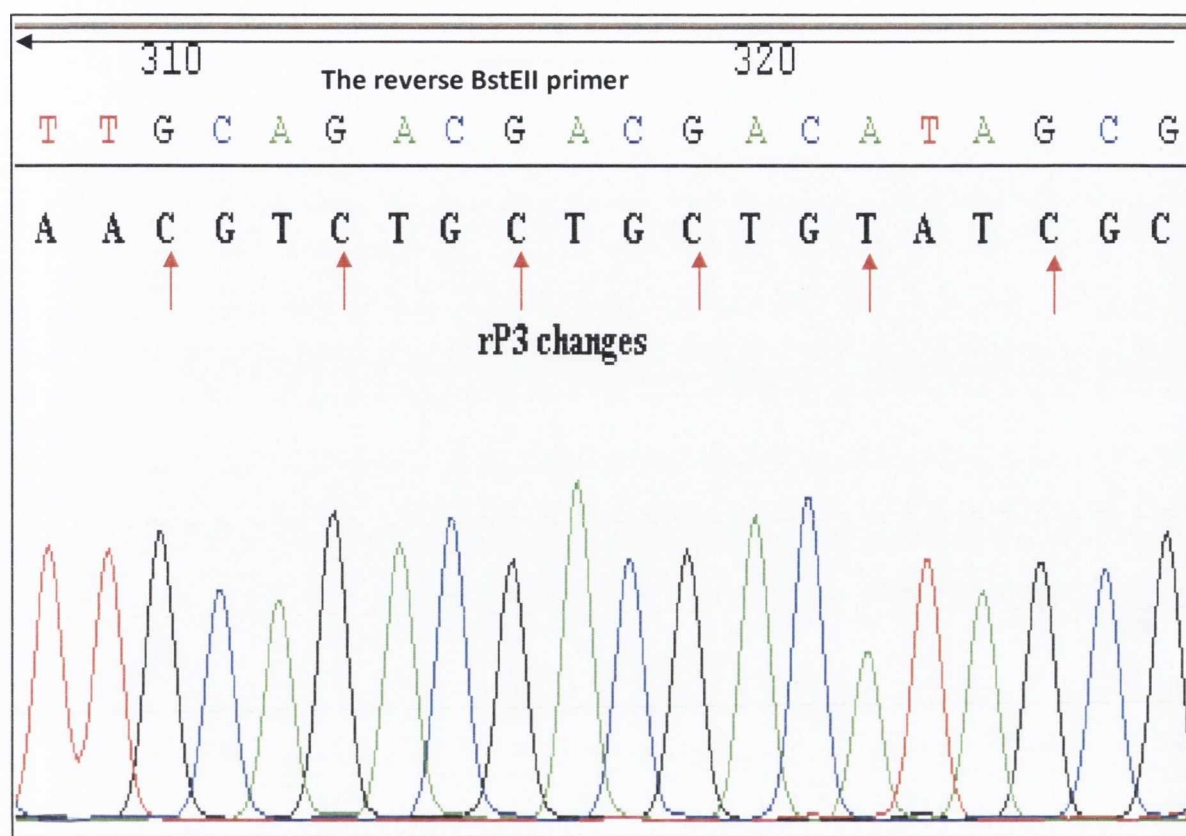
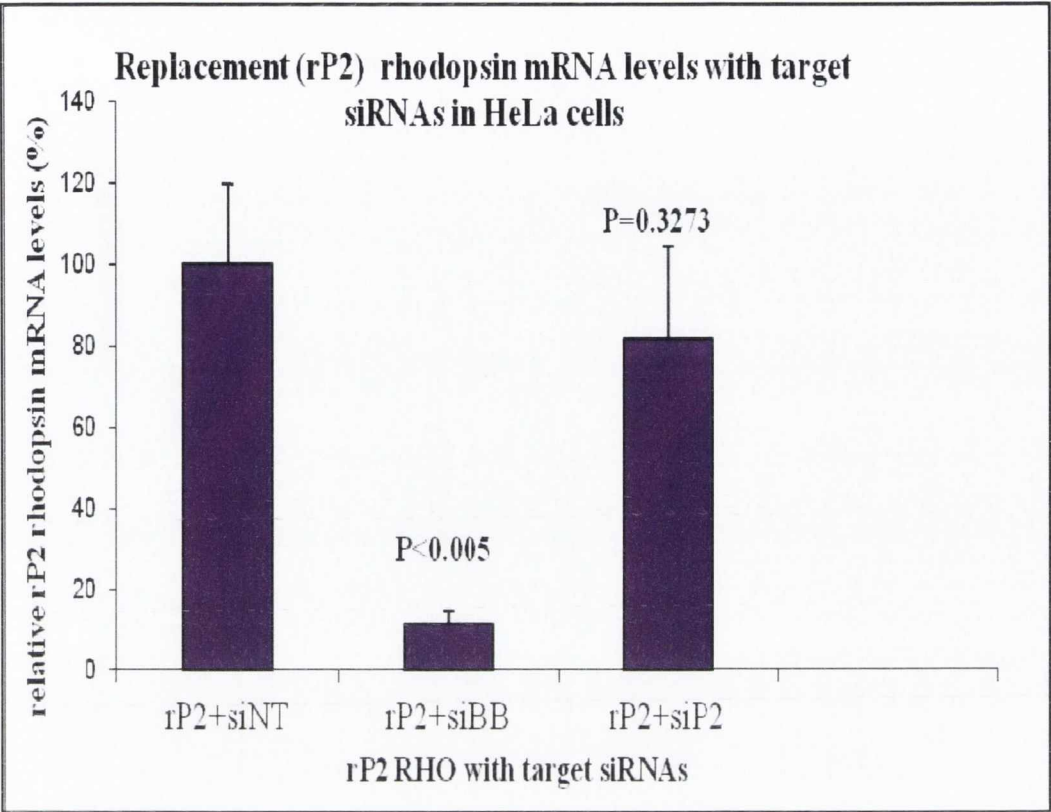


Figure 6.3.2.b: Sequence of rP3 over the siP3 target site

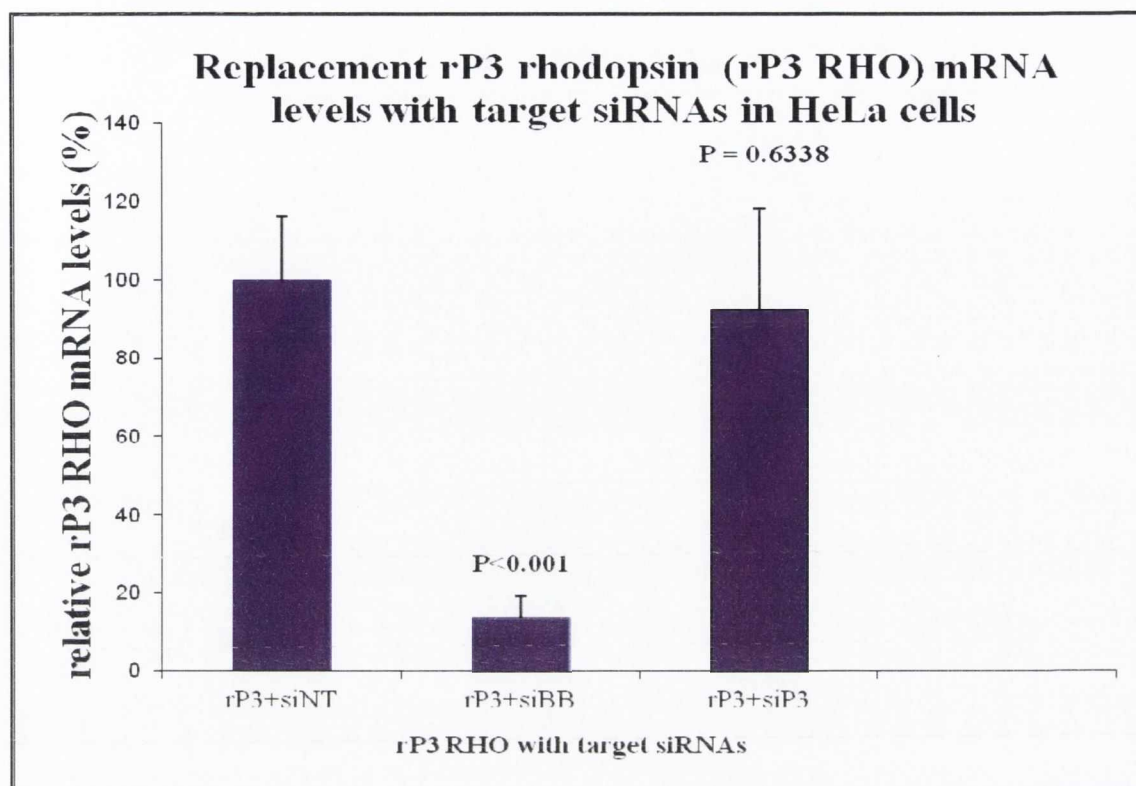
The reverse *BstEII* primer was used for sequencing of rP3 over the siP3 site; the six changes (CGGTACGTGGTGGTGTGT to CGCTATGTCGTCTGTC) are indicated by the red arrows.



Sample	rP3 + siNT	rP3 +siBB	rP3 + siP3
% of rP3 with NT	100	11.8	83.5
% SD	20.8	2.9	22.9

Figure. 6.3.4.a rP2 RNA expression from HeLa cells transfected with rP3 and siRNAs

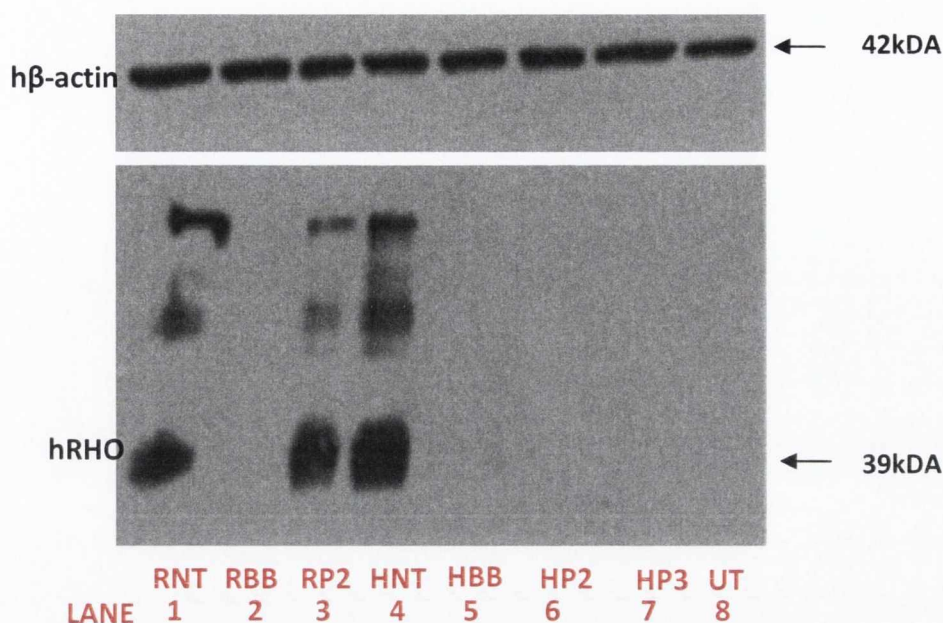
Relative replacement (rP2) human rhodopsin RNA levels in HeLa cells co-transfected with siP2, siNT siBB and rP2. The data are given as percentages of rhodopsin RNA levels in samples transfected with rP2 and a non-targeting negative control (siNT, 100%). The positive control siRNA used was siBB. The average and standard deviation (SD) for each sample was calculated using Microsoft Excel. A two-sample t-test was used to assess statistical significance of data sets using DataDesk 6.0 software. Error bars represent % SD values.



Sample	rP3 + siNT	rP3 +siBB	rP3 + siP3
% of rP3 with NT	100	14	92
% SD	16	6	26

Figure. 6.3.5.a rP3 RNA expression from HeLa cells transfected with rP3 and siRNAs

Relative replacement (rP3) human rhodopsin RNA levels in HeLa cells co-transfected with siP3, siNT siBB and rP3. The data are given as percentages of rhodopsin RNA levels in samples transfected with rP3 and a non-targeting negative control (siNT, 100%). The positive control siRNA used was siBB. The average and standard deviation (SD) for each sample was calculated using Microsoft Excel. A two-sample t-test was used to assess statistical significance of data sets using DataDesk 6.0 software. Error bars represent % SD values.



SAMPLE NAME	RNT	RBB	RP2	HNT	HBB	HP2	HP3	UT
BCA (μg/ml)	1161	933	1031	895	972	928	847	1568
protein/ well (μl)	15	18	16	19	17	18	20	11
[protein]/ well (μg)	17	17	17	17	17	17	17	17

Figure 6.3.6.a Western blot analysis of rP2 protein expression.

Lane 1 contains protein from cells transfected with rP2 and siNT (rNT) and this representing the control for rP2 expression in this experiment. Lane 2 contains protein from cells transfected with rP2 and siBB (rBB), rP3 is not resistant to siBB as can be observed by absence of hRHO immunoreactivity. Lane 3 is protein from HeLa cells transfected with rP2 and siP2 (rP2). hRHO protein expression is visible and comparable to Lane 1, thus rP2 is resistant to siP2. Lane 4 contains protein from cells transfected with wild-type hRHO and siNT (HNT), hRHO expression in this lane is comparable to hRHO expression in both RNT and RP2. Lane 5, 6 lane 7 represents protein from cells transfected with wild-type hRHO and siBB (HBB), siP2 (HP2) and siP3 (HP3) respectively and represents the controls to ensure suppression with each was still occurring with wild-type hRHO. Lane 8 is protein from un-transfected cells and represents the 4D2 antibody negative control (UT). Human β-actin was used as a control for equal loading. The table represents the concentration of total cytoplasmic protein in each sample added to each well in order to ensure equal loading.

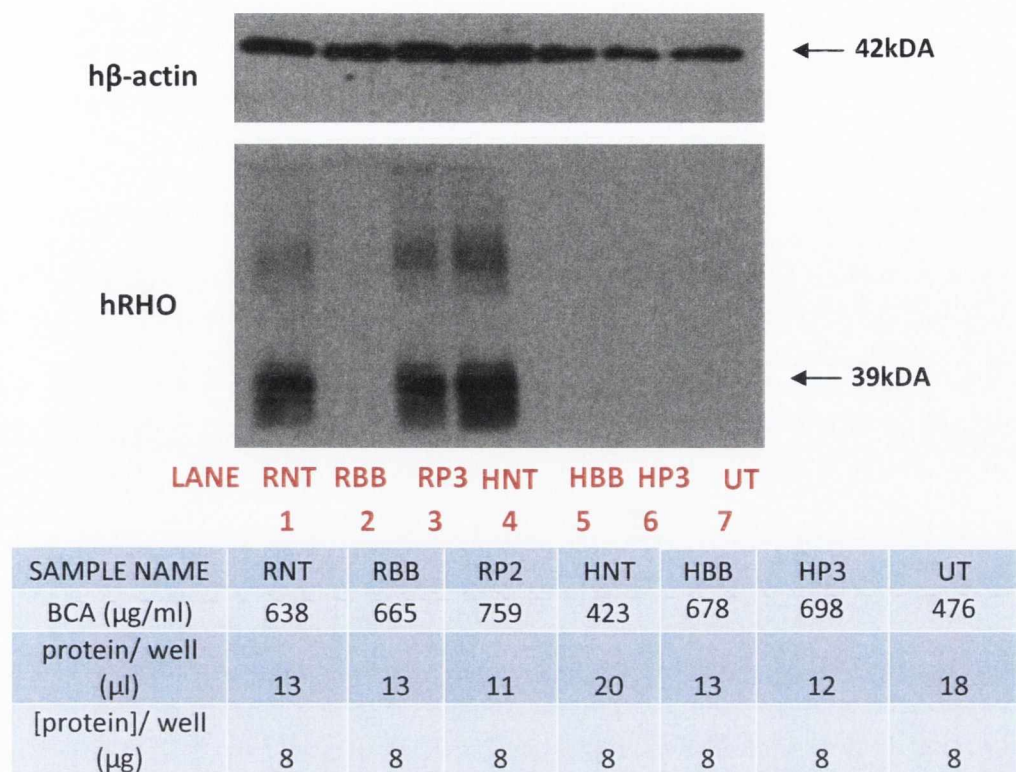


Figure 6.3.7.a Western blot analysis of rP3 protein expression.

Lane 1 contains protein from cells transfected with rP3 and siNT (rNT) representing the control for rP3 expression. Lane 2 contains protein from cells transfected with rP3 and siBB (rBB), rP3 is not resistant to siBB as can be observed by absence of hRHO immunoreactivity. Lane 3 is protein from HeLa cells transfected with rP3 and siP3 (rP3). hRHO protein expression is visible and comparable to Lane 1, thus rP3 is resistant to siP3. Lane 4 contains protein from cells transfected with wild-type hRHO and siNT (HNT), hRHO expression in this lane is comparable to hRHO expression in both RNT and RP3. Lane 5 and 6 represents protein from cells transfected with wild-type hRHO and siBB (HBB) and siP3 (HP3), this represents the controls to ensure suppression with each siRNA was still occurring with wild-type hRHO. Lane 7 is protein from un-transfected cells and represents the 4D2 antibody negative control (UT). Human β -actin was used as a control for equal loading. The table represents the concentration of total cytoplasmic protein in each sample added to each well in order to ensure equal loading.

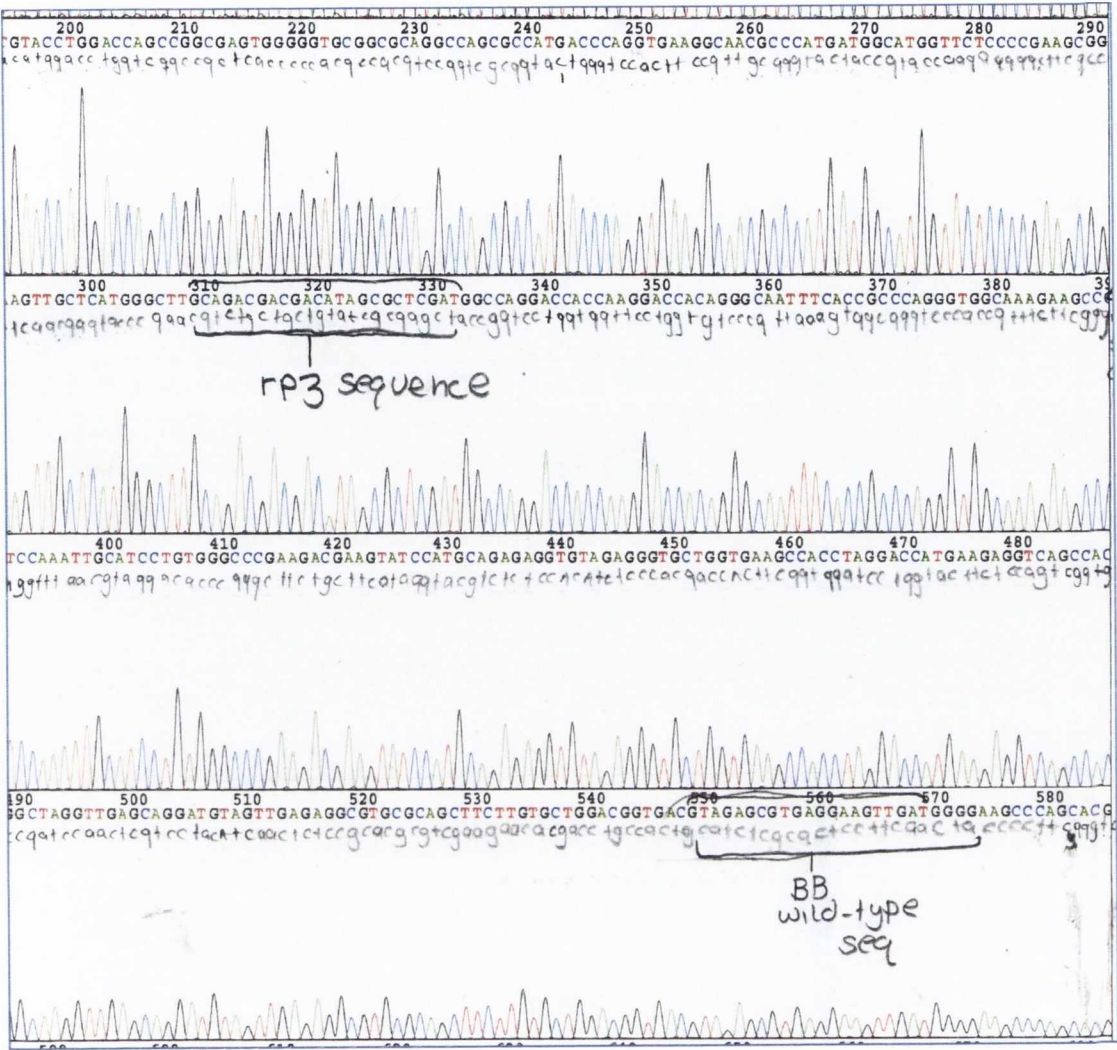


Figure 3.6.9.a sequencing results for rP3 into pAAV.BB32.

Sequencing was verified using the 3'*BstEII* R primer, the rP3 sequence changes are present. The siBB target site was checked to ensure the rBB sequence had been swapped with the wild-type siBB target sequence.

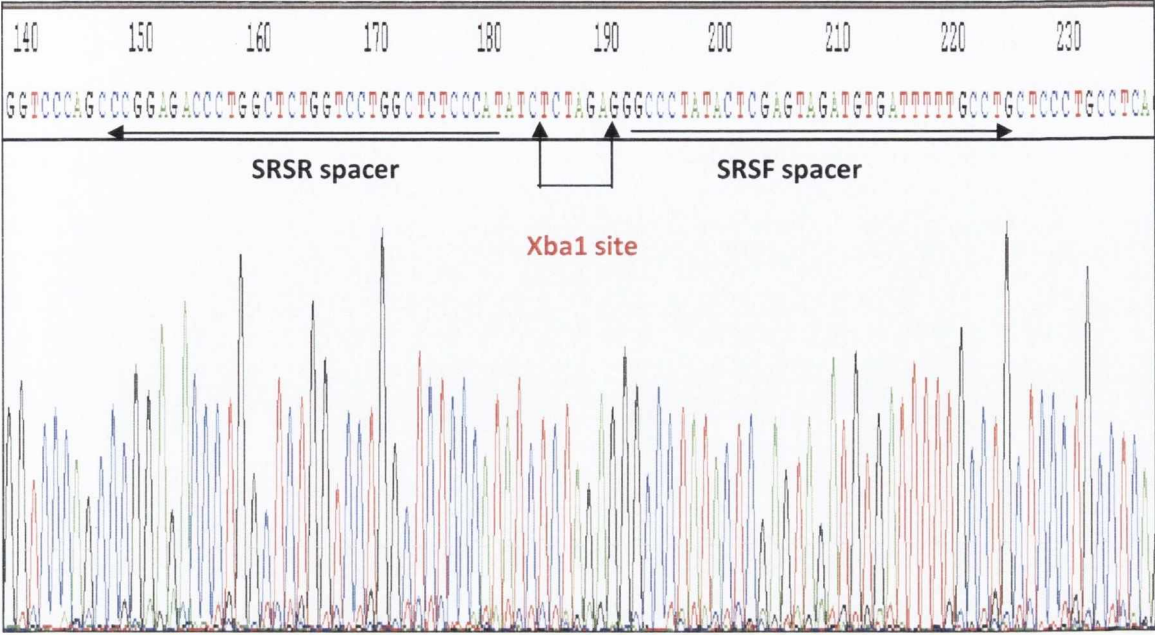


Figure 6.3.9.b sequencing result for pAAV.rP3

The sequencing of the pAAV.rP3 plasmid was undertaken using the SRSR primer. The absence of the shP3 was verified by checking that only one *XbaI* site was present in the sequence. This *XbaI* site was flanked by the SRSR and SRSF spacers.

AAV-2/5.rP3 fractions	Copy number
AAV-2/5.rP3-3	6.61819 x 10 ⁶
AAV-2/5.rP3-4	9.98627 x 10 ⁶
AAV-2/5.rP3-5	8.25069 x 10 ⁷

Controls for AAV 2/5.rP3 fractions	Copy number	Known titre
AAV-2/5.BB24 (A)	9.68044 x 10 ⁷	6 x 10 ¹³ vp/ml
AAV-2/5.BB24 (B)	1.11182 x 10 ⁷	1 x 10 ¹³ vp /ml

AAV-2/8.rP3 fractions	Copy number
AAV-2/8.rP3 -4	4.76014 X 10 ⁶
AAV-2/8.rP3-5	2.21393 x 10 ⁷
AAV-2/8.rP3-7	1.06487 x 10 ⁸

Controls for AAV-2/8 fractions	Copy number	Known titre
AAV-2/5.BB24 (A)	2.71287 X10 ⁷	6 x 10 ¹³ vp/ml
AAV-2/5.BB24 (B)	3.576893 x 10 ⁶	1x 10 ¹³ vp /ml

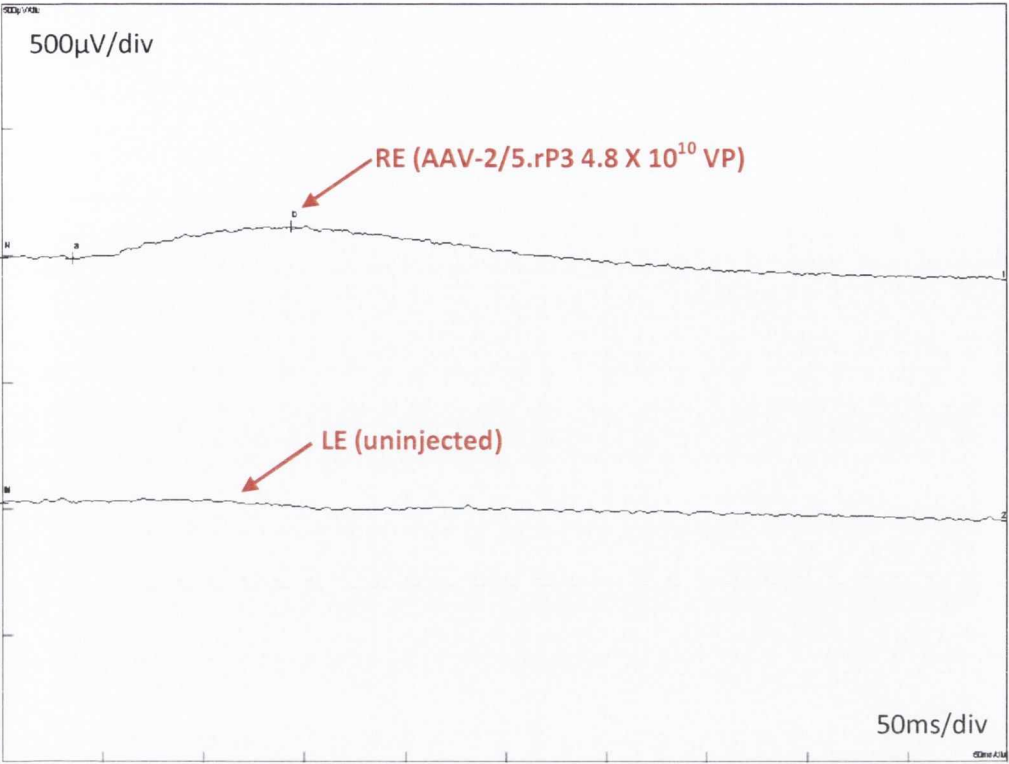


AAV replacement genes	Assumed Titres
AAV-2/5.rP3-4	6 x 10 ¹³ vp/ml (use neat)
AAV-2/8.rP3-7	4 X 10 ¹³ vp/ml (use neat)

Controls	Assumed Titres
AAV-2/5.BB24	6 x 10 ¹³ vp/ml
AAV-2/8.BB24(A)	4 x 10 ¹³ vp/ml
AAV-2/8.BB24(B)	3 X 10 ¹⁴ vp/ml

Table 6.3.10 Calculation of titres for each suppressor virus

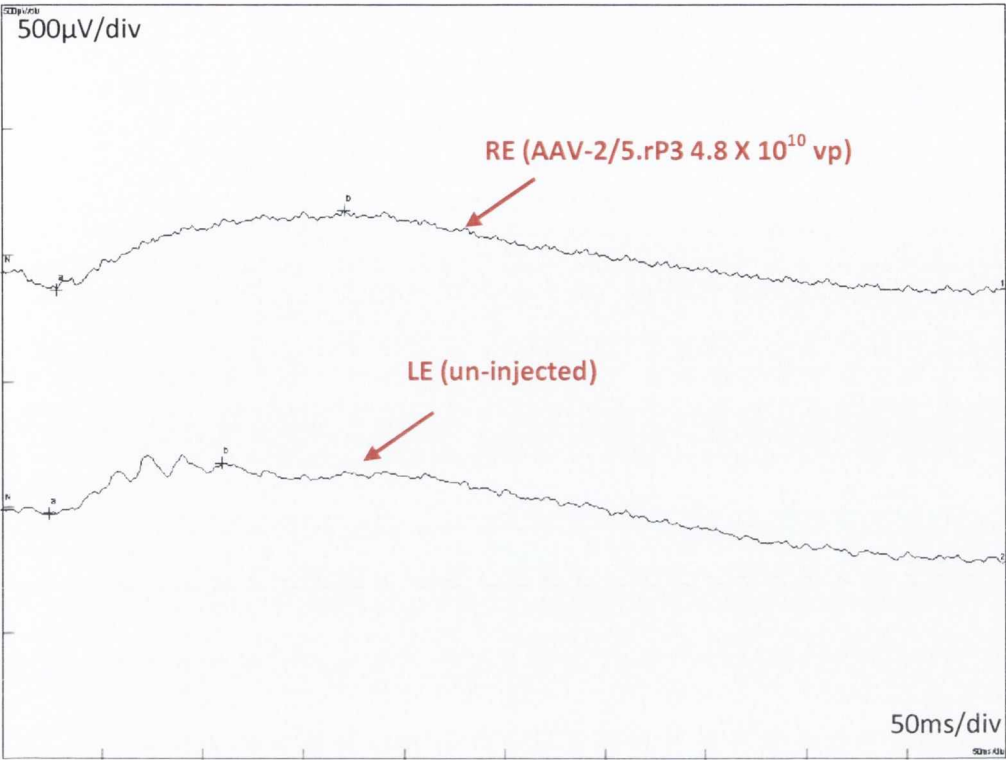
The copy number for each fraction was obtained based on the standard curve. For each replacement rhodopsin virus (AAV-2/5.rp3 and AAV-2/8.) the fraction with the highest copy number was taken (red). The copy number of each suppressor was compared to the copy number of the two controls and multiplied by the known titre of each control. The average of the two titre values obtained from the two controls was taken as the estimated titre for each suppressor virus. The titre from the control viruses had previously been estimated.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	35	143	305 µV	125 µV
2 left Max	0	0	0 µV	0 µV

Figure 6.3.11.a Rod-isolated ERG for 129rho^{-/-} mice injected with AAV-2/5.rP3 (RE)

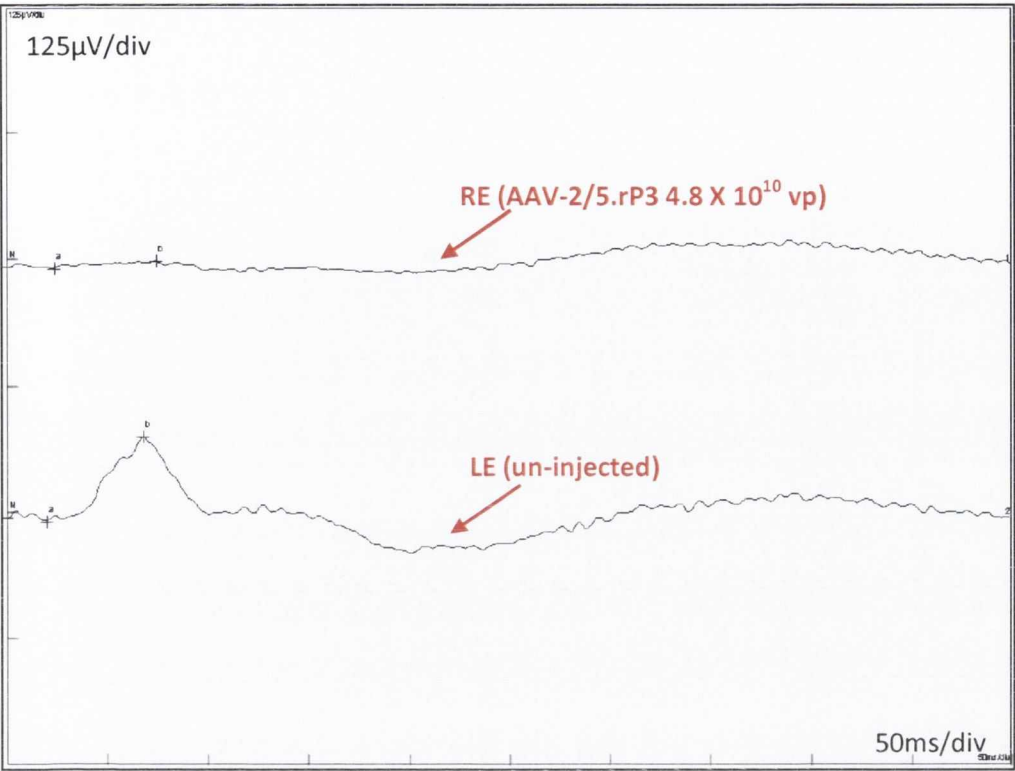
The highest rod-isolated ERG result obtained from 129rho^{-/-} mice injected with AAV-2/5.rP3 (RE) and analysed 6 weeks post-injection is presented.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	27	170	70.8 μV	314μV
2 left Max	23	109	18.6μV	204μV

Figure 6.11.b mixed rod-cone ERG for 129rho^{-/-} mouse injected with AAV-2/5.rP3 (RE)

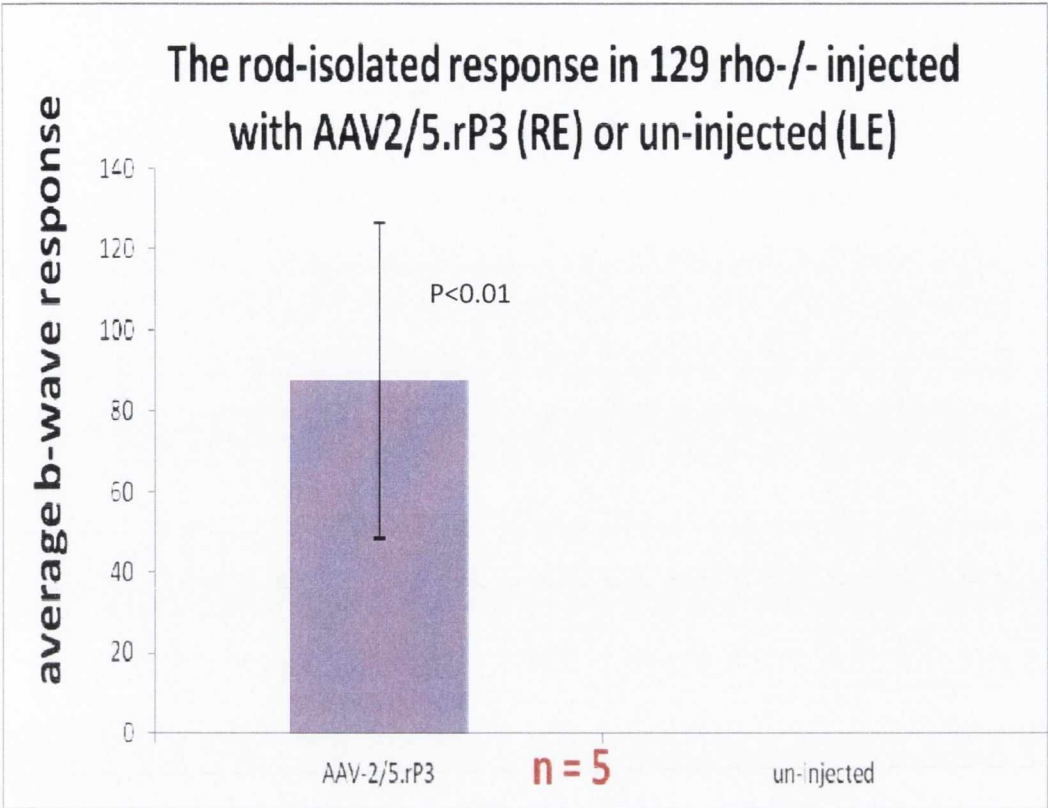
The mixed rod-cone response from the mouse in which the highest rod-isolated ERG result was obtained from 129rho^{-/-} mice injected with AAV-2/5.rP3 (RE) and analysed 6 weeks.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	23	74	2.31μV	6.98μV
2 left Max	20	67	3.31μV	83.5μV

Figure 6.3.11.c Cone-isolated ERG for 129rho^{-/-} mice injected with AAV-2/5.rP3 (RE)

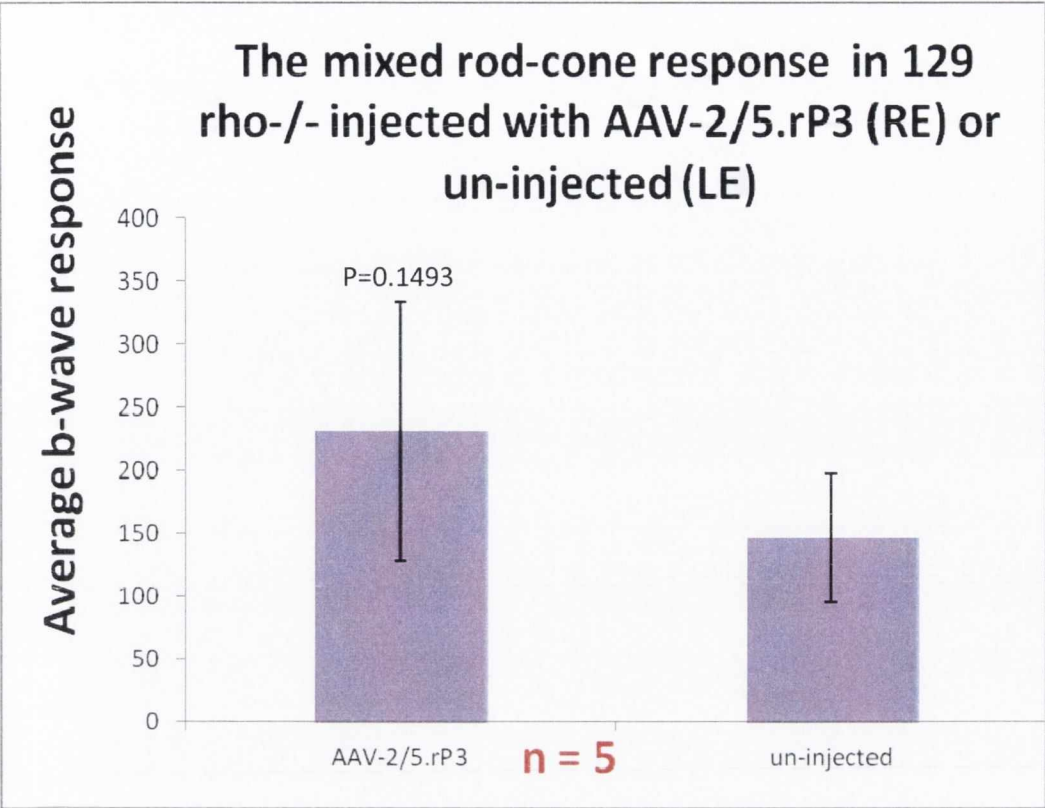
The cone-isolated ERG result obtained from 129rho^{-/-} mice with the best rod-isolated ERG response injected with AAV2/5. rP3 (RE) and analysed 6 weeks post-injection is presented.



129 rho ^{-/-}	AAV-2/5.rP3	Un-injected
Average b-wave	87	0
SD	39	0

Figure 6.3.11.d The average rod-isolated ERG response in 129rho^{-/-} mice injected with AAV-2/5.rP3 or uninjected

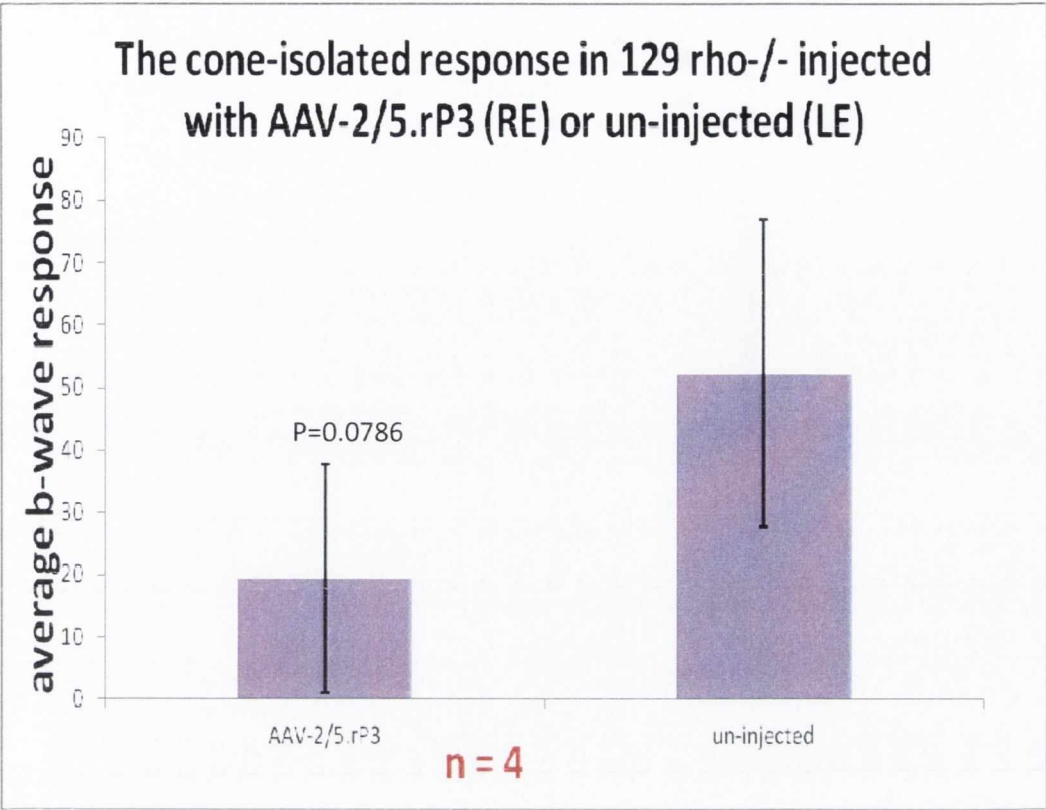
The rod-isolated b-wave value for the 129rho^{-/-} mice injected with AAV-2/5.rP3 at a dose of 4.8 x 10¹⁰ vp were combined and the average and standard deviation (SD) obtained using Microsoft Excel. Only the b-wave values from mice in which a positive ERG response was obtained were used for this analysis. Two sample t-tests were undertaken to determine statistical significance between data sets (DataDesk 6.0). The average b-wave value for aged matched uninjected mice that underwent ERG analysis at the same time was used as the control.



129 rho-/-	AAV-2/5.rP3	Un-injected
Average b-wave	231	147
SD	102	51

Figure 6.3.11.e The average mixed rod-cone ERG response in 129rho^{-/-} mice injected with AAV-2/5.rP3 or uninjected

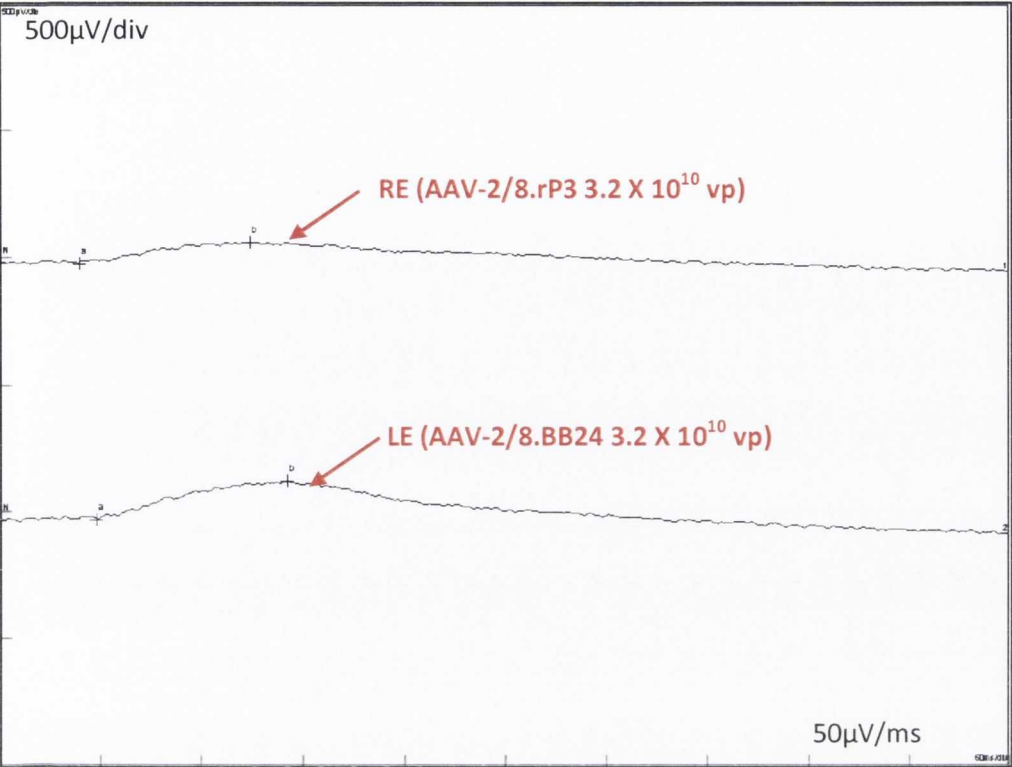
The mixed rod-cone b-wave value for the 129rho^{-/-} mice injected with AAV-2/5.rP3 at a dose of 4.8 x 10¹⁰ vp were combined and the average and standard deviation (SD) obtained using Microsoft Excel. Only the b-wave values from mice in which a positive ERG response was obtained were used for this analysis. Two sample t-tests were undertaken to determine statistical significance between data sets (DataDesk 6.0). The average b-wave value for aged matched uninjected mice that underwent ERG analysis at the same time were used as the control for negative rhodopsin expression.



129 rho ^{-/-}	AAV-2/5.rP3	Un-injected
Average b-wave	19	52
SD	18	25

Figure 6.3.11.f The average cone-isolated ERG response in 129rho^{-/-} mice injected with AAV-2/5.rP3 or un-injected

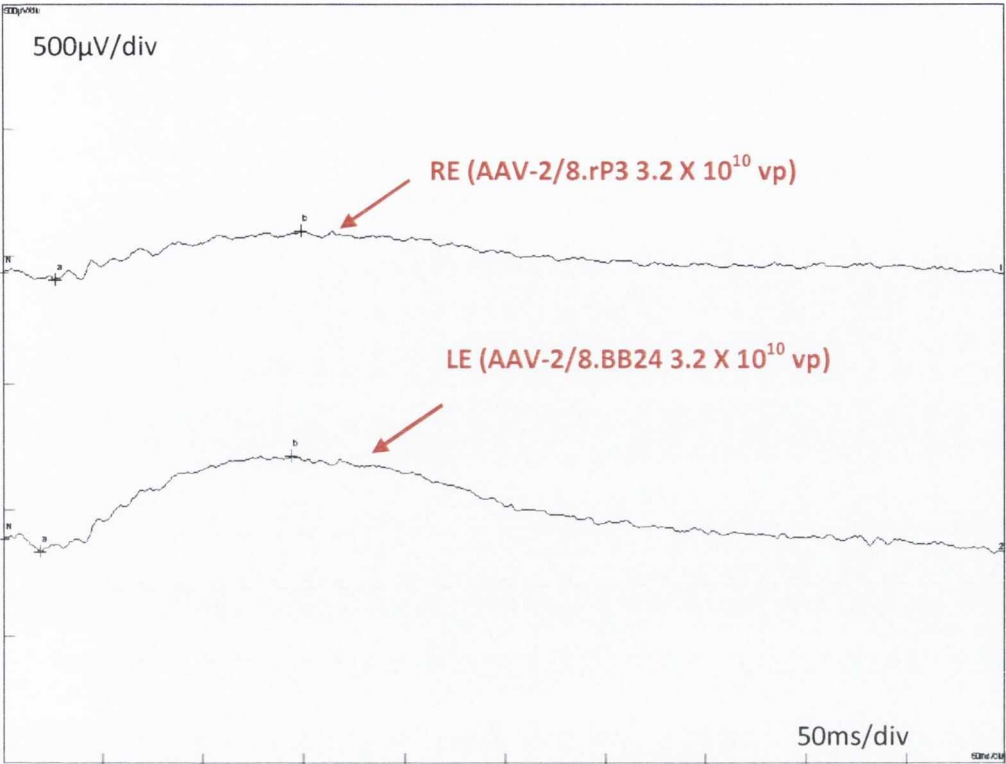
The cone-isolated b-wave value for the 129rho^{-/-} mice injected with AAV-2/5.rP3 at a dose of 4.8 x 10¹⁰ vp were combined and the average and standard deviation (SD) obtained using Microsoft Excel. Only the b-wave values from mice in which a positive ERG response was obtained were used for this analysis. Two sample t-tests were undertaken to determine statistical significance between data sets (DataDesk 6.0). The average b-wave value for aged matched uninjected mice that underwent ERG analysis at the same time were used as the control for negative rhodopsin expression.



Diagnostic Data				
Chan nel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	39	123	2.52 μV	81.7 μV
2 left Max	48	142	4.8 μV	148 μV

Figure 6.3.12.a Rod-isolated ERG for 129rho^{-/-} mice injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE)

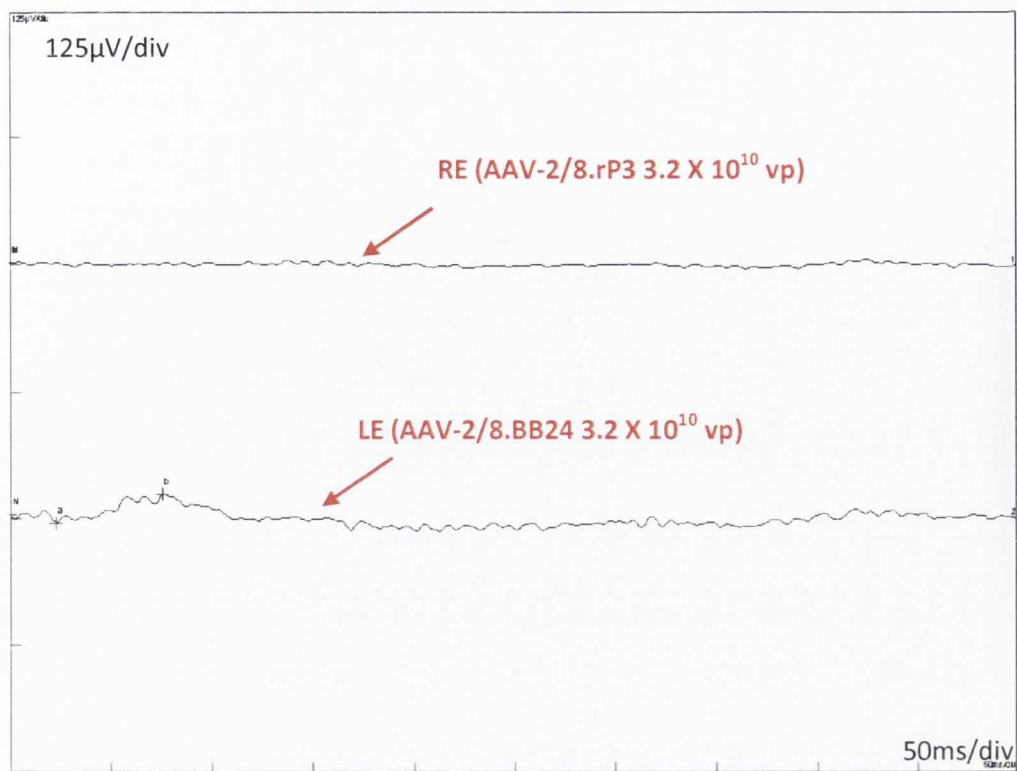
The highest rod-isolated ERG result obtained from 129rho^{-/-} mice injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE) is injected.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	26	148	31.1µV	194µV
2 left Max	19	144	51.8µV	379µV

Figure 6.3.12.b mixed rod-cone ERG for 129rho^{-/-} injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE)

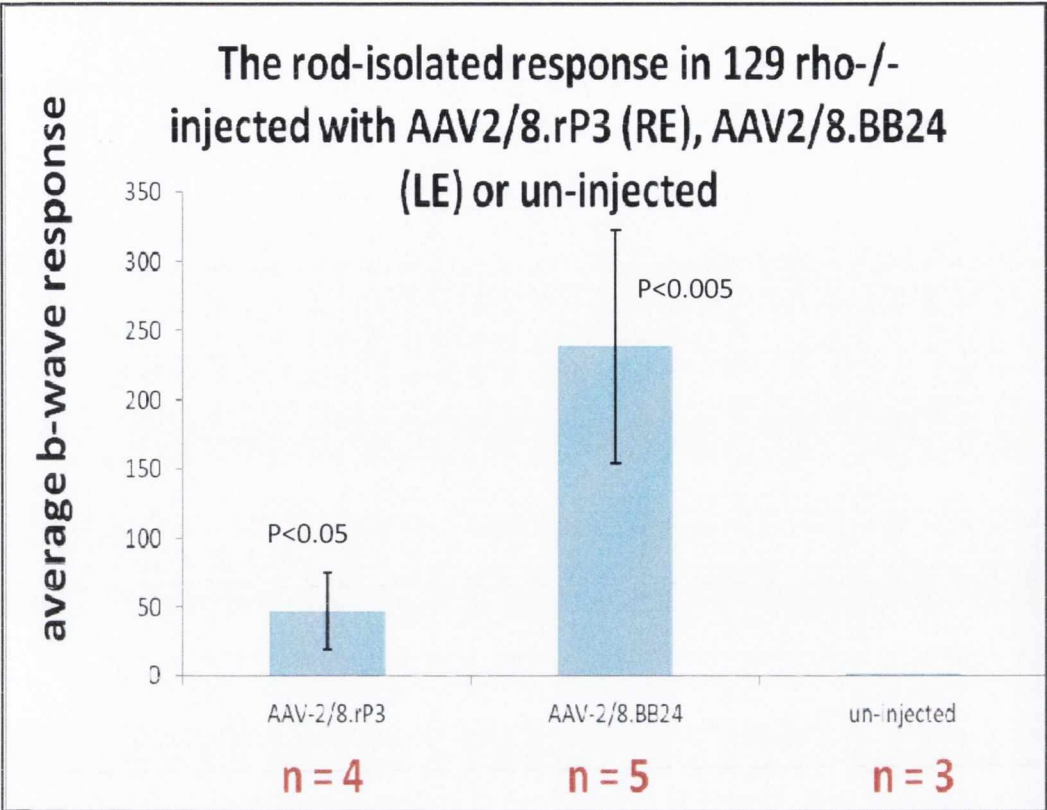
The highest mixed rod-cone ERG result obtained from 129rho^{-/-} mice injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE) is presented.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	0	0	0 µV	0 µV
2 left Max	22	75	8.54 µV	28.8 µV

Figure 6.3.12.c cone-isolated ERG for 129rho^{-/-} injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE)

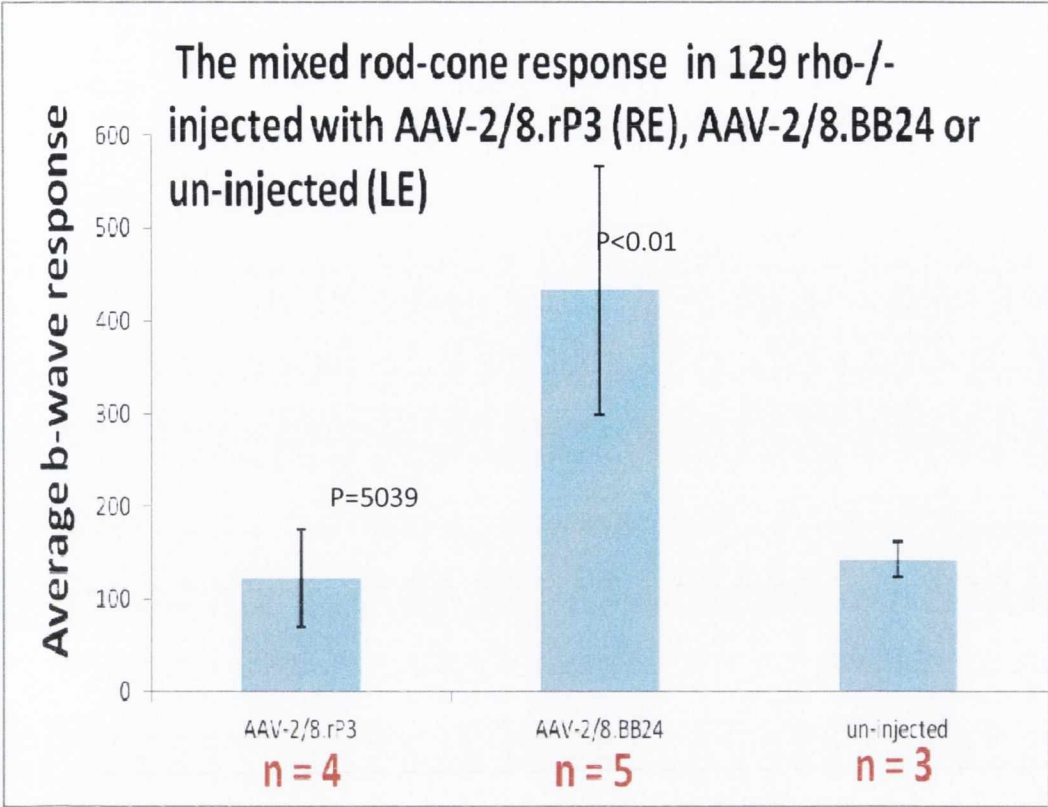
The highest cone-isolated ERG result obtained from 129rho^{-/-} mice injected with AAV-2/8.rP3 (RE) and AAV-2/8.BB24 (LE) is presented.



129 rho ^{-/-}	AAV-2/8.rp3	AAV-2/8.BB24	Un-injected
Average b-wave	47	239	0
SD	28	85	0

Figure 6.3.12.d The average rod-isolated ERG response in 129rho^{-/-} injected with AAV-2/8.rP3, AAV-2/8.BB24 or un-injected

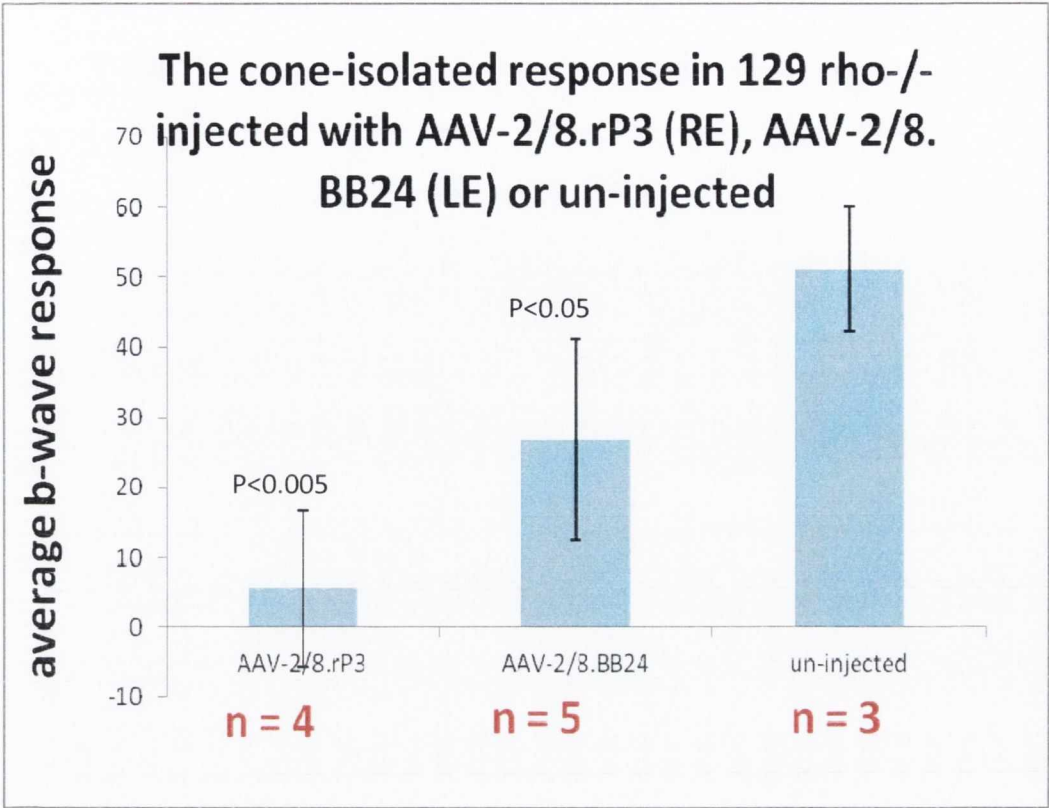
Rod-isolated b-wave values for the 129rho^{-/-} mice injected with AAV-2/8.rP3 or AAV-2/8.BB24 at a dose of 3.2 x 10¹⁰ vp were combined and the average and standard deviation obtained using Microsoft Excel. Only the b-wave values from mice in which a positive ERG response was obtained was used for this analysis. The average b-wave value for aged matched uninjected mice that underwent ERG analysis at the same time were used as the negative control for rhodopsin expression. Statistical significance was assessed by a two sample t-test between data sets (DataDesk 6.0). Error bars represent SD values.



129 rho ^{-/-}	AAV-2/8.rp3	AAV-2/8.BB24	Un-injected
Average b-wave	122	433	143
SD	53	127	18

Figure 6.3.12.e Average mix rod-cone ERG response in 129rho^{-/-} injected (AAV-2/8.rP3 or AAV-2/8.BB24) or uninjected

Mixed rod cone b-wave values for 129rho^{-/-} mice injected with AAV-2/8.BB24 or AAV-2/8.rP3 at a dose of 3.2 x 10¹⁰ were combined and the average and standard deviation obtained using Microsoft Excel. Only the b-wave values from mice in which a positive ERG response was obtained were used for this analysis. The average b-wave value for aged matched uninjected mice that underwent ERG analysis at the same time were used as the control for negative rhodopsin expression. Statistical significance was assessed by a two-sample t-test between data sets (DataDesk 6.0). Error bars represent SD values.



129 rho-/-	AAV-2/8.rP3	AAV2/8.BB24	Un-injected
Average b-wave	6	27	51
SD	11	15	9

Figure 6.3.12.f Average cone-isolated ERG response in 129rho-/- injected (AAV-2/8.BB24 (A) or AAV-2/8.rp3 or uninjected

Cone-isolated b-wave values for 129rho^{-/-} mice injected with AAV-2/8.BB24 or AAV-2/8.rP3 at a dose of 3.2 x 10¹⁰ vp were combined and the average and standard deviation obtained using Microsoft Excel. Only the b-wave values from mice in which a positive ERG response was obtained were used for this analysis. The average b-wave value for aged matched un-injected mice that underwent ERG analysis at the same time were used as the control for negative rhodopsin expression. Statistical significance was assessed by a two-sample t-test between data sets (DataDesk 6.0). Error bars represent SD values.

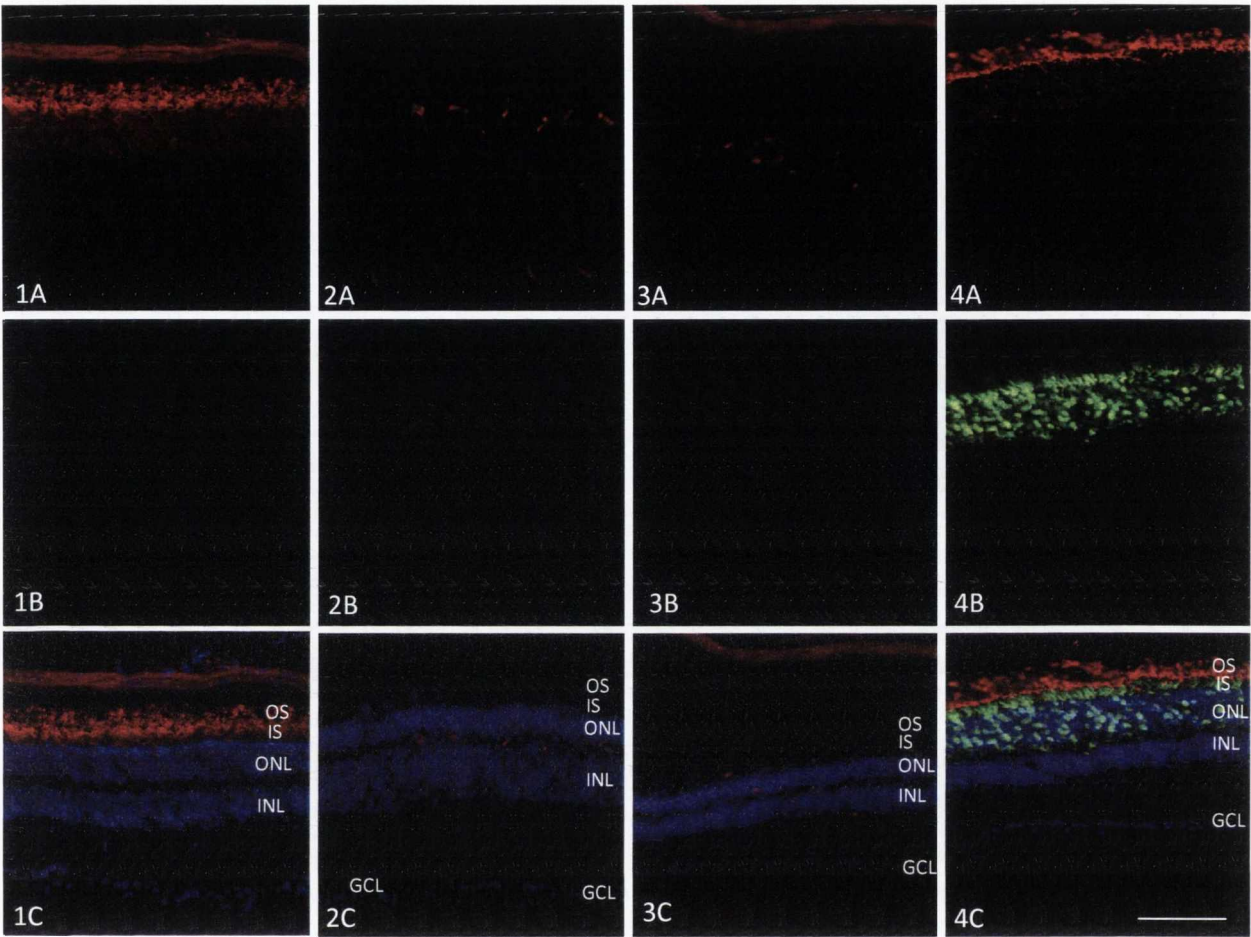


Figure 6.3.13.a Immunohistochemistry of 129rho^{-/-} eyes LE uninjected Vs RE injected (AAV-2/5.rP3)

Immunohistochemistry analysis was performed on three 129rho^{-/-} mice. The LE was uninjected and served as the experimental control. The RE was injected with AAV-2/5.rP3. hRHO staining was carried out using the 4D2 RHO antibody (1/100 dilution). The first column of diagrams is retinal sections from a RHO-M mouse (1A, 1B & 1C), hRHO is clearly present (red staining) in the OS layer (1A). This mice is an un-injected animal, therefore is EGFP negative (1B). Column 2 represents an uninjected LE from a 129rho^{-/-} mouse. These mice do not express RHO (2A) Column 3 is an EGFP negative region from the retina of a 129rho^{-/-} injected with AAV-2/5.rP3 (3A, 3B & 3C) hRHO is clearly absent in the outer segments (3A) in addition to EGFP expression (3B). Column 4 represents the same retina as column 3 but in an EGFP positive region injected with AAV-2/5.rP3 (4A, 4B & 4C). hRHO immunostaining is positive in the outer segments (4A). The EGFP expression depicts the formation of inner segments in this retina (4B). Row 1 is hRHO staining only, row 2 is EGFP only and row 3 represents hRHO, EGFP and DAPI staining overlaid. OS= outer segments, ONL = outer nuclear layer, INL = inner nuclear layer and GCL = ganglion cell layer. Scale bar (L) represents 116 μM.

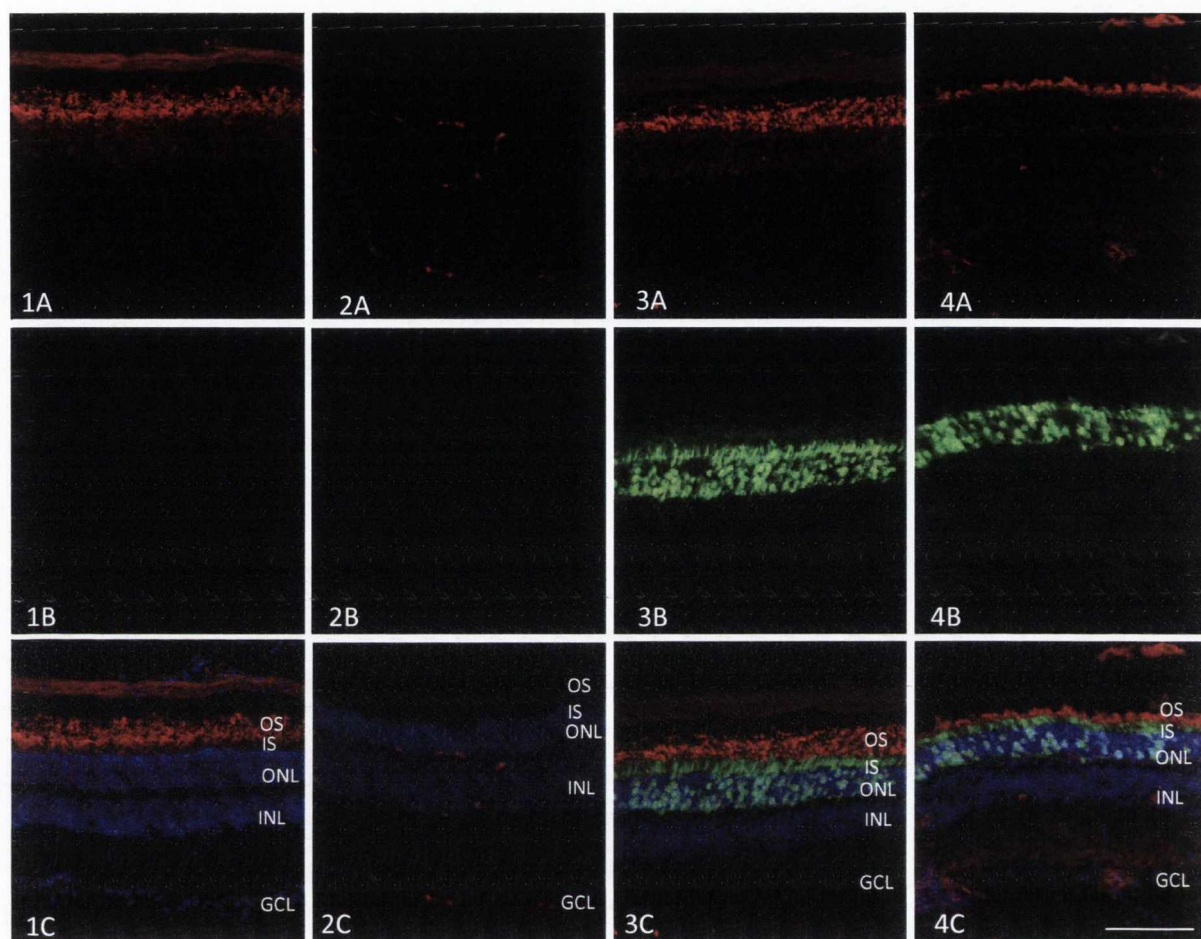
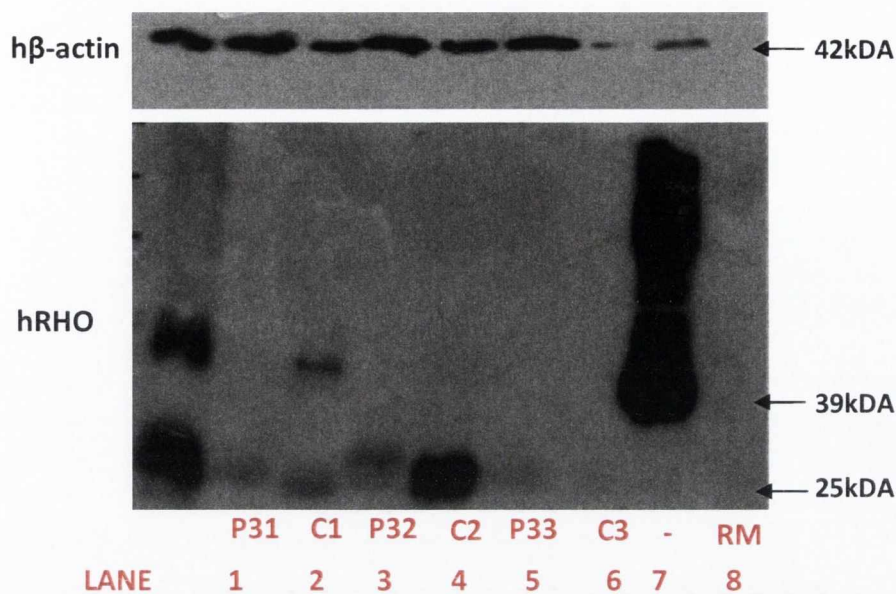


Figure 6.3.14.a Immunohistochemistry of 129rho^{-/-} eyes LE un-injected, or LE-AAV-2/8.BB24 injected with the RE-injected with AAV-2/8.rP3

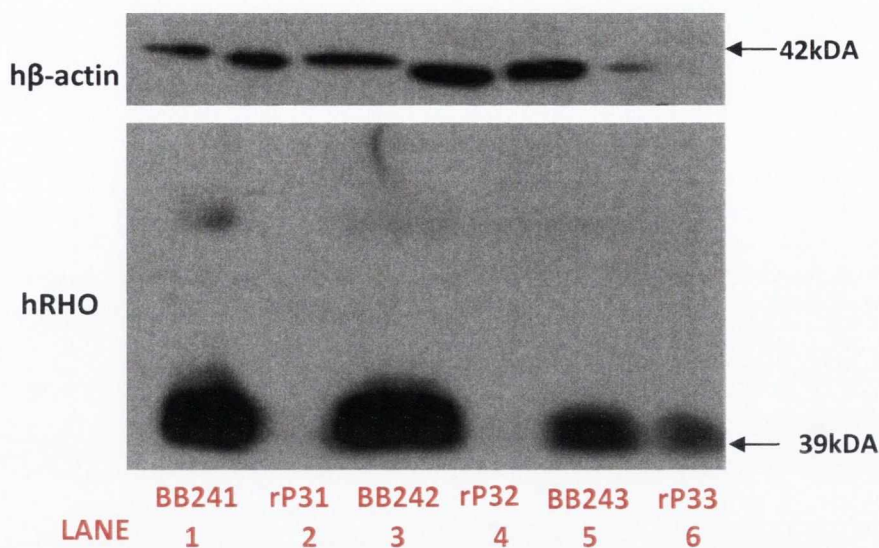
In one mouse the LE was un-injected and served as the control. In the second mouse the LE was injected with AAV-2/8.BB24 and was the positive control. The RE was injected with AAV-2/8.rP3. hrHO staining was carried out using the 4D2 RHO antibody (1/100 dilution). Column 1 is retinal sections from RHO-M mouse (1A, 1B & 1C), hrHO is clearly present (red staining) in the OS layer (1A). This mice was an un-injected animal, therefore was EGFP negative (1B). Column 2 represents an un-injected LE from a 129rho^{-/-} mouse. These mice to not express RHO (2A) Column 3 is the LE of the EGFP positive region from the retina of a 129rho^{-/-} injected with AAV-2/8.BB24 (3A, 3B & 3C) hrHO is clearly visible in the outer segments (3A) in addition to EGFP expression (3B).The overlaid figured (3C) portray healthy EGFP positive IS and abundant hrHO staining. Column 4 represents an EGFP positive region of the RE injected with AAV-2/8.rP3 (4A, 4B & 4C). hrHO immunostaining can be detected at a low level (4A). EGFP expression is detected (4B) in the ONL, very few, short EGFP positive IS and OS can be detected compared to BB24. Row 1 is hrHO staining only, row 2 is EGFP only and row 3 represents hrHO, EGFP and DAPI staining overlaid. OS=outer segments, ONL=outer nuclear layer, INL=inner nuclear layer and GCL=ganglion cell layer. Scale bar (L) represents 116 μ M.



SAMPLE NAME	P31	C1	P32	C2	P33	C3	-	RM
BCA (μg/ml)	1220	838	8422	908	3317	851	3069	2782
protein/well (μl)	14	20	2	19	5	20	5	6
[protein]/well (μg)	17	17	17	17	17	17	17	17

Figure 6.3.15.a Western blot analysis of rP3 protein expression from AAV-2/5.rP3

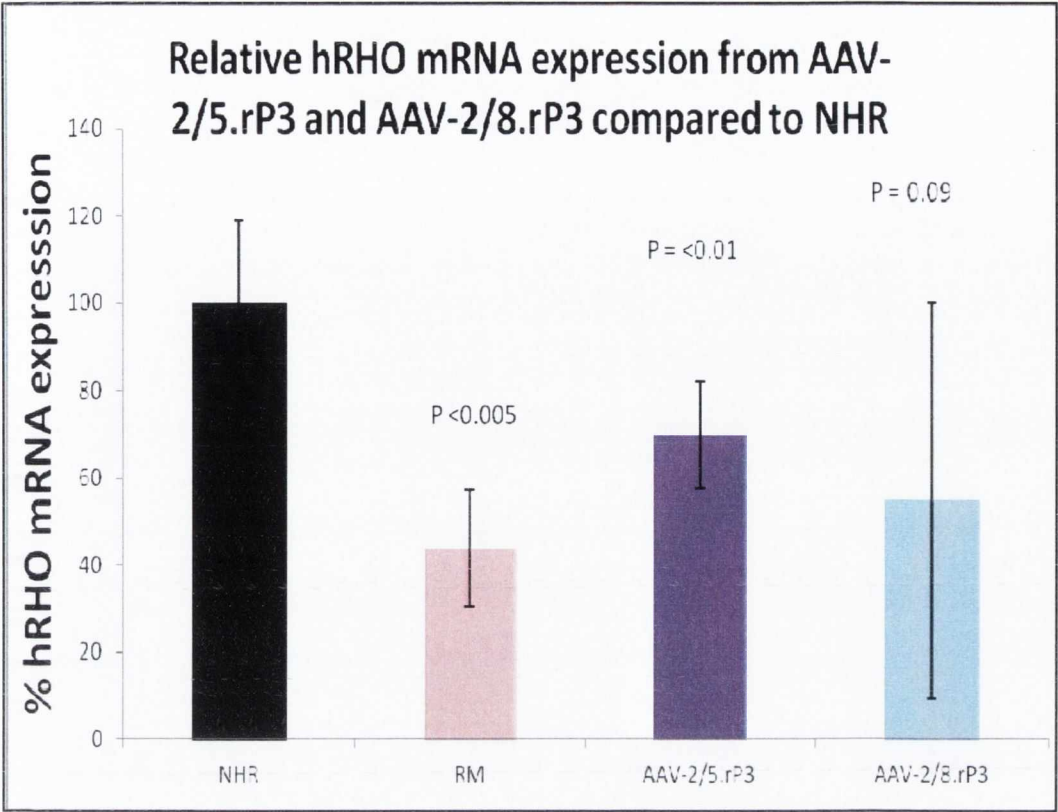
In order to detect if hRHO protein was being expressed from AAV-2/5.rP3 in mice in which no ERG response was recorded a western blot was undertaken. Protein from three mice in which a rod-isolated response of 0 in the AAV-2/5.rP3 injected eye was taken for analysis. Lanes 1, 3 & 5 represent the 3 AAV-2/5.rP3 injected eyes (P31, P32 & P33) Lane 2, 4 & 6 represent their respective un-injected controls (C1, C2 and C3). At high exposure times (30 minutes) the presence of hRHO was detected in 2/3 mice injected with AAV-2/5.rP3 compared to the un-injected eyes. Thus hRHO protein is being expressed in these retinas from AAV-2/5.rP3 just at low expression levels. Lane 7 represents the negative rho^{-/-} control for this experiment. RHO-M (lane 8) represents the positive control, as the western blot was exposed for 30 minute; the hRHO expression in RHO-M is overexposed. Interesting in all samples of 129rho^{-/-} protein, extra bands at 25 kDA were present. Human β-actin was used as a control for equal loading and to ensure that protein in the samples negative for hRHO was present. The table represents the concentration of total cytoplasmic protein in each sample added to each well in order to ensure equal loading



SAMPLE NAME	BB241	rP31	BB322	rP32	BB243	rP33
BCA (μg/ml)	568	2723	418	1598	1214	6000
protein/ well (μl)	14.7	3.1	20	5.23	6.88	1.64
[protein]/ well (μg)	8	8	8	8	8	8

Figure 6.3.16.a Western blot analysis of rP3 protein expression from AAV-2/8.rP3

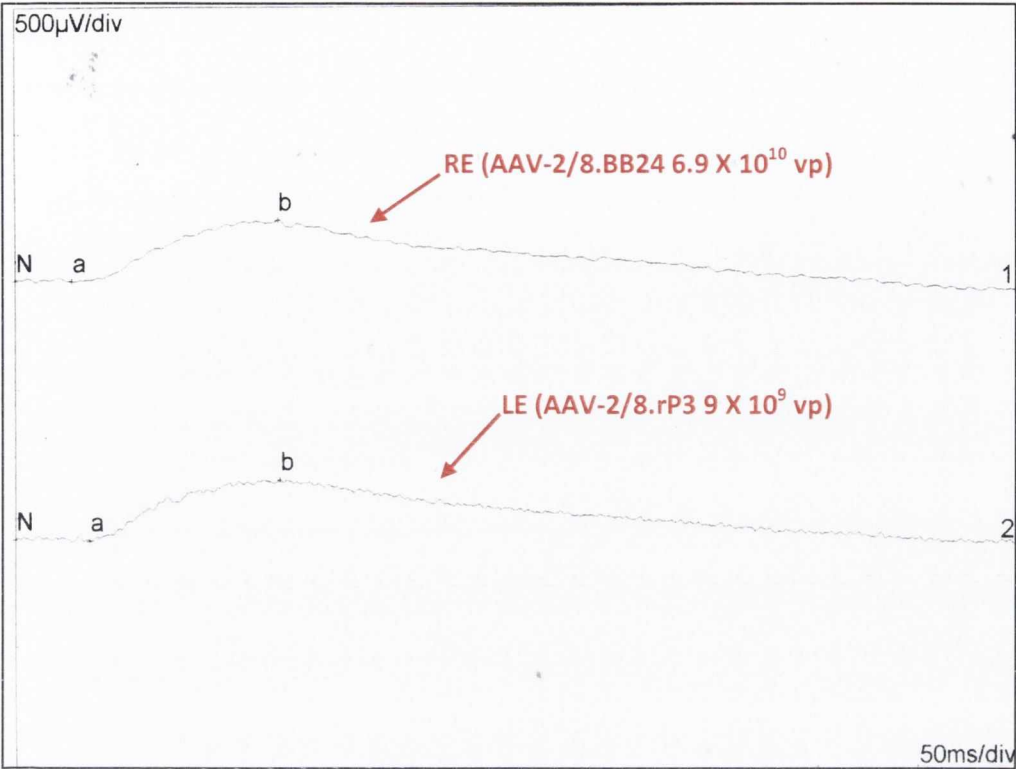
In order to detect if hRHO protein was being expressed from AAV-2/5.rP3 in mice in which no ERG response was recorded in the AAV-2/8.rP3 injected eye, a western blot was undertaken. Protein from three mice in which a rod-isolated response of 0μV in the AAV-2/8.rP3 injected eye was taken for analysis. Lanes 1, 3 & 5 represent the 3 AAV-2/6.BB24 injected eyes (BB241, BB242 & BB243). This represents the positive control for rP3. AAV-2/8.BB24 yielded high ERG rod-isolated responses and would therefore be expected to produce sufficient hRHO. Lane 2, 4 & 6 represent mice injected with AAV-2/8.rP3 (rP31, rP32 and rP33). The presence of hRHO was detected in only 1/3 mice injected with AAV-2/8.rP3 compared to the abundant hRHO expression produced from all 3 AAV-2/8.BB24 injected eyes. Unlike AAV-2/5.rP3 no immunoreactivity at 25 kDA was observed. Human β-actin was used as a control for equal loading and to ensure that protein in the samples negative for hRHO was present. Equal loading was difficult to obtain in these mice owing to the fact that the protein samples are from mice with a retinal degeneration. The table represents the concentration of total cytoplasmic protein in each sample added to each well in order to ensure equal loading.



Sample	NHR	RHO-M	AAV-2.5.rP3	AAV-2/8.rp3
% of NHR	100	44	70	54
% SD	12	14	12	45

Figure 6.3.17.a The % hRHO RNA expression from AAV-2/5.rP3 and AAV-2/8.rP3 compared to NHR

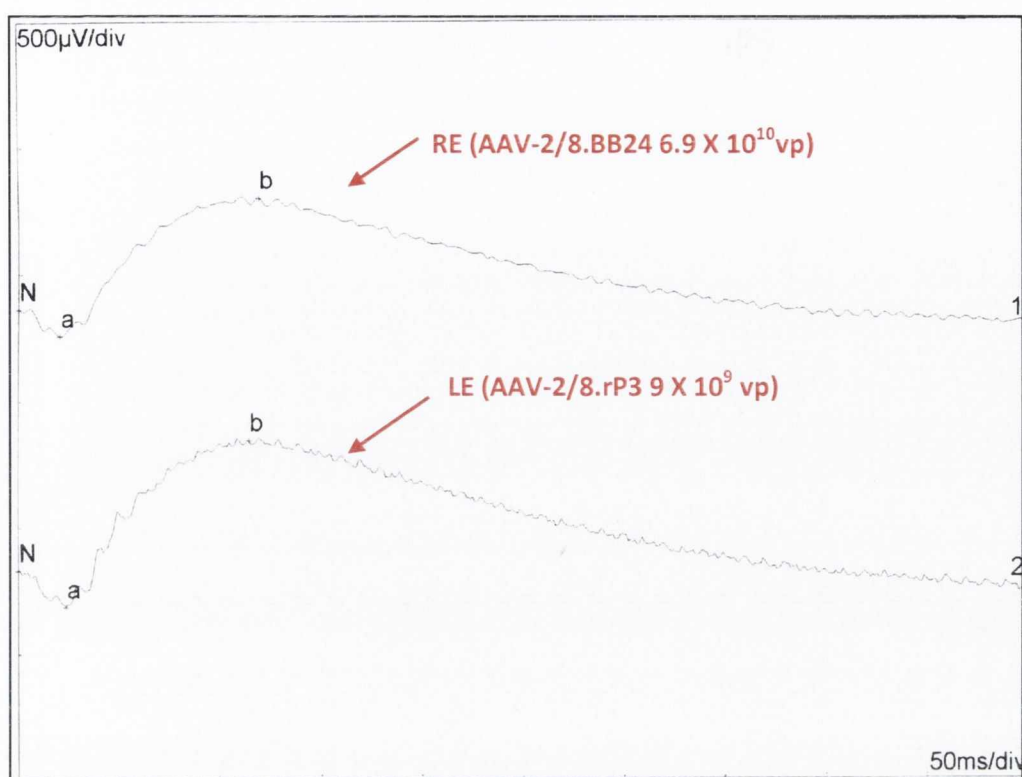
Five wild-type 129 mice were injected with 1.8×10^{11} vp of AAV-2/5.rP3 (LE) and 1.2×10^{11} vp of AAV-2/8.rP3 (RE) in order to evaluate the relative hRHO expression from each virus compared to the hRHO expression in the NHR mouse by real-time RT-PCR. hRHO specific primers ensured the specific amplification of hRHO expression only. RHO-M which was used as an experimental control, it is known that this mouse expresses hRHO 70% the level of NHR. The mean and standard deviation was calculated with Microsoft Excel. Two-sample t-tests were performed on the data set to assess statistical significance (DataDesk 6.0). Error bars represent % SD



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	28	131	2.28μV	239μV
2 left Max	37	132	6.35μV	234μV

Figure 6.3.18.a Rod-isolated ERG for 129rho^{-/-} injected with AAV-2/8.rP3 (LE) and AAV-2/8.BB24 (RE).

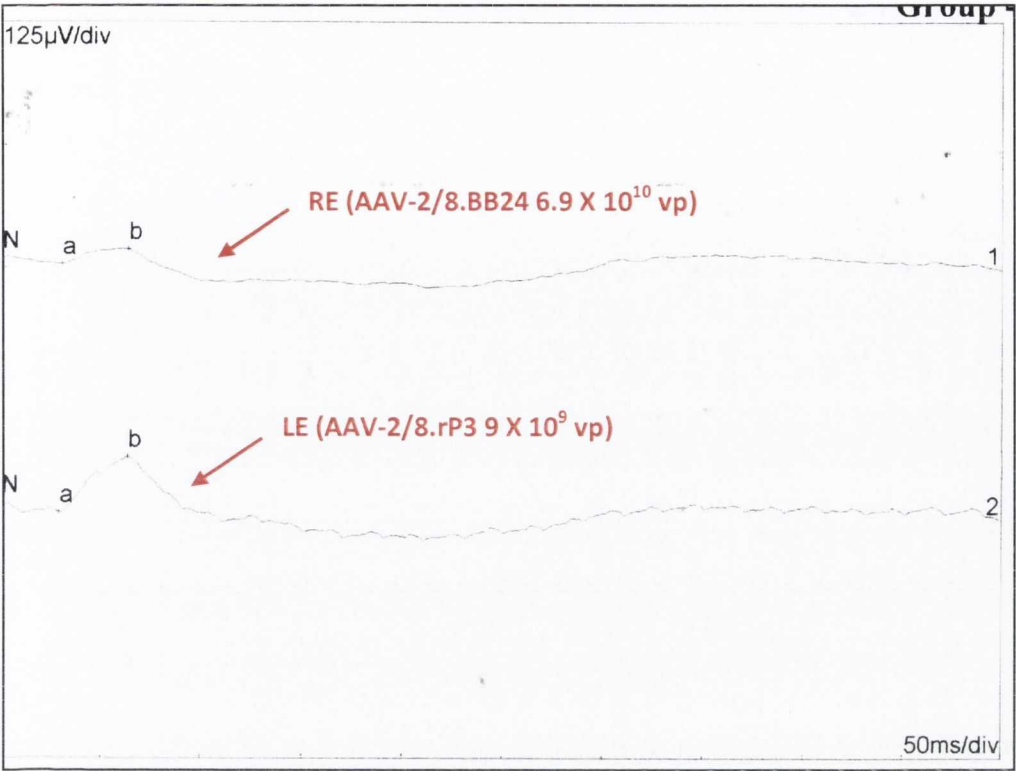
The highest rod-isolated ERG result obtained from 129rho^{-/-} mice injected with AAV-2/8.rP3 (LE) and AAV-2/8.BB24 (RE) is presented.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	21	119	107 μV	544 μV
2 left Max	23	114	145 μV	659 μV

Figure 6.3.18.b Mixed rod-cone ERG for $\rho 129^{-/-}$ injected with AAV-2/8.rP3 (LE) and AAV- 2/8.BB24 (RE).

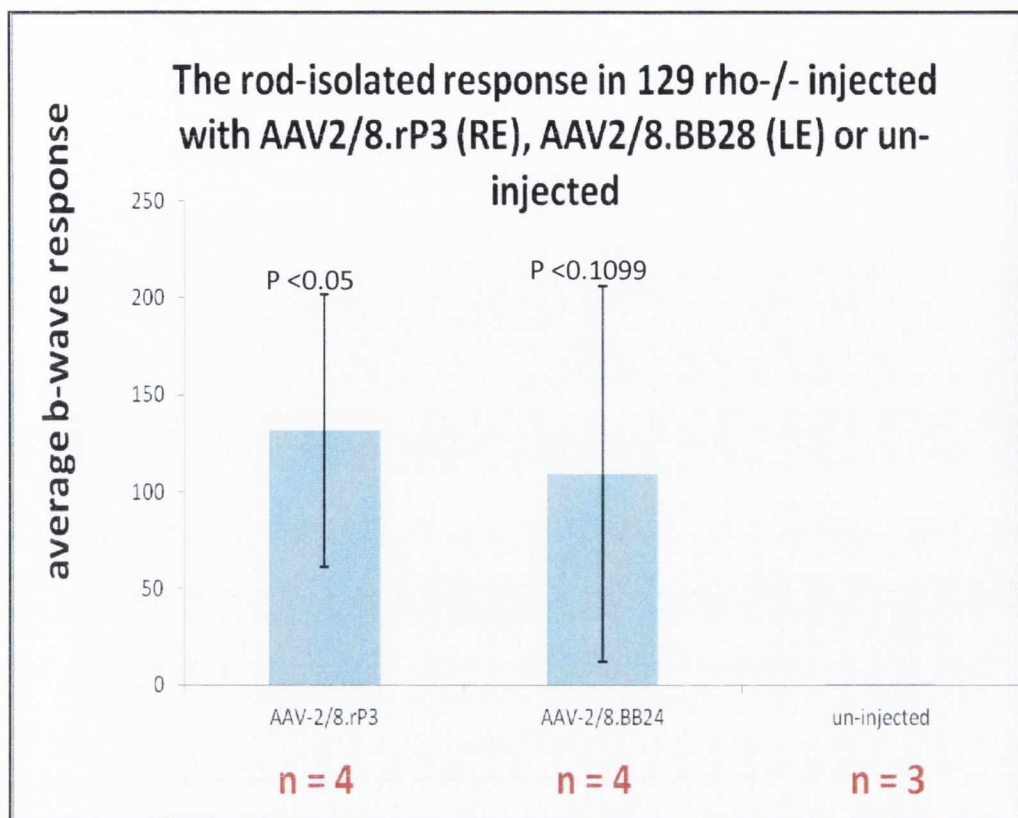
The highest mixed rod-cone ERG result obtained from $129\rho^{-/-}$ mice injected with AAV-2/8.rP3 (LE) and AAV-2/8.BB24 (RE) is presented.



Diagnostic Data				
Channel	a[ms]	b[ms]	a-wave	b-wave
1 right Max	30	63	6.12μV	15.1μV
2 left Max	29	63	9.71μV	56.3μV

Figure 6.3.18.c cone-isolated ERG for 129rho^{-/-} injected with AAV-2/8.rP3 (LE) and AAV-2/8.BB24 (RE).

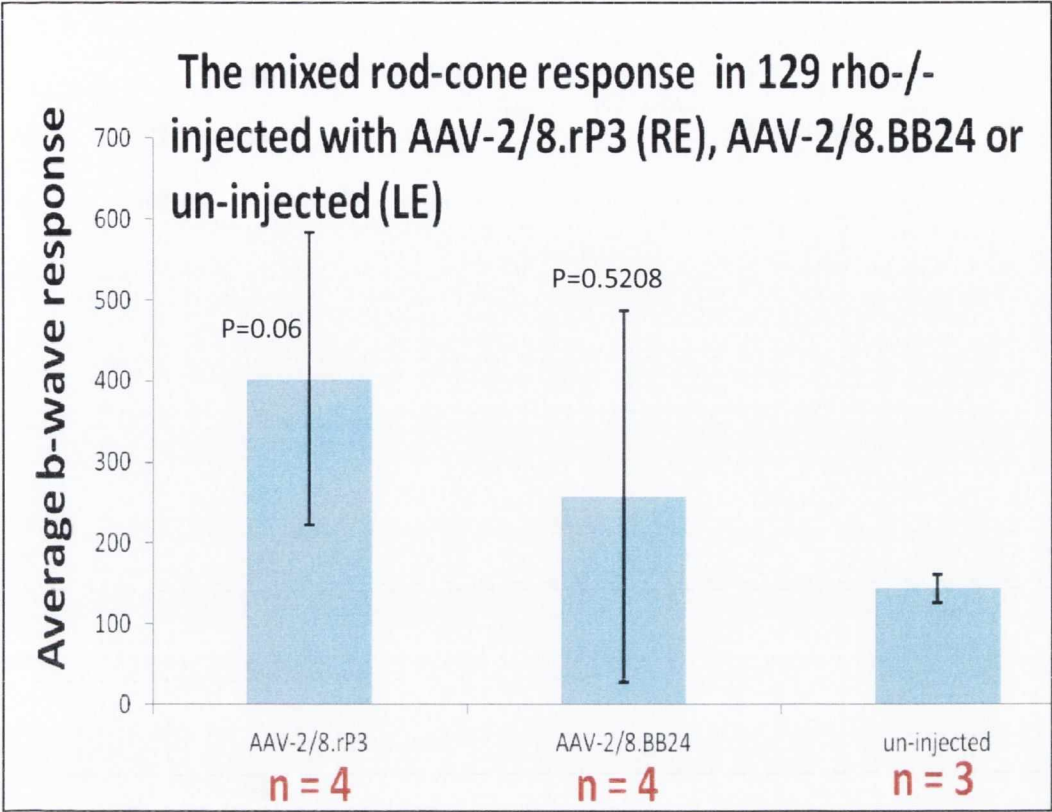
The highest cone-isolated ERG result obtained from 129rho^{-/-} mice injected with AAV2/8. rP3 (LE) and AAV-2/8.BB24 (RE) is presented.



129 rho ^{-/-}	AAV-2/8.rp3	AAV-2/8.BB24	Un-injected
Average b-wave	132	109	0
SD	70	97	0

Figure 6.3.18.d The average rod-isolated ERG response in 129rho^{-/-} injected with AAV-2/8.rP3, AAV-2/8.BB24 or un-injected at a lower dose

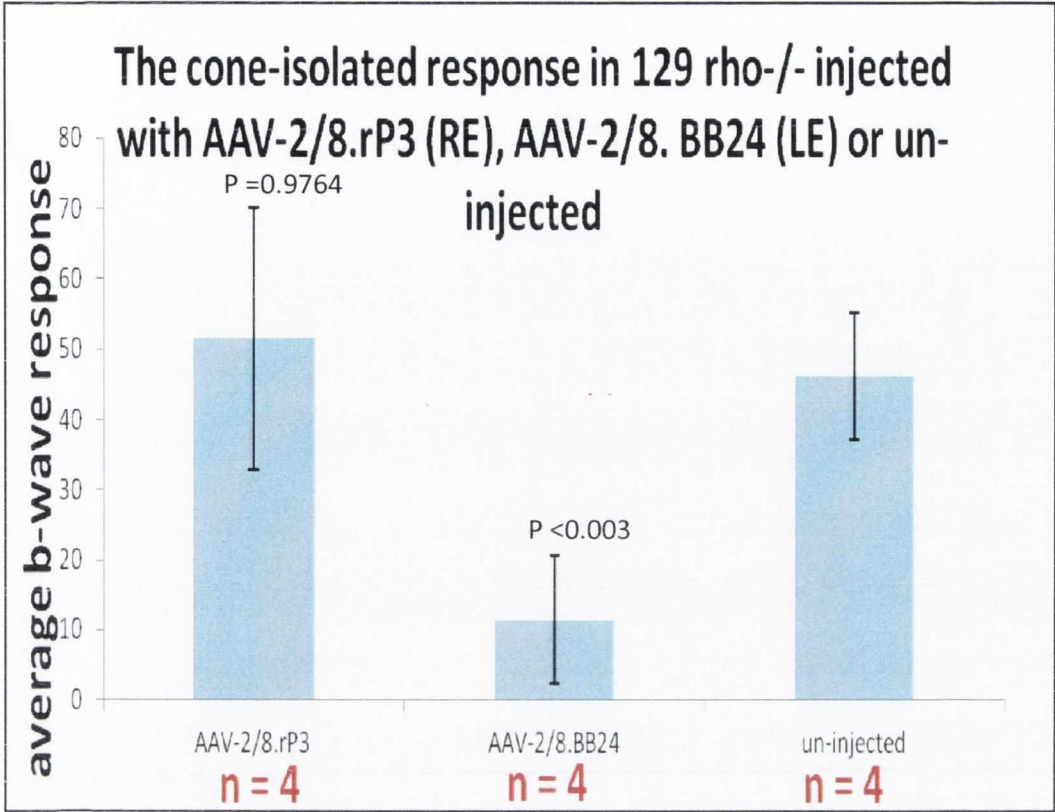
The average rod-isolated b-wave value for the 129rho^{-/-} mice injected with AAV-2/8.rP3 (9×10^9) or AAV-2/8.BB24 (6.9×10^{10} vp), in addition to age matched un-injected mice were combined and the average and standard deviation obtained using Microsoft Excel. In the AAV-2/8.rP3 injected mice, only the b-wave values from mice in which a positive ERG response was obtained was used for this analysis. Statistical significance between injected and uninjected mice was evaluated using two sample t-test (DataDesk 6.0).



129 rho ^{-/-}	AAV-2/8.rp3	AAV-2/8.BB24	Un-injected
Average b-wave	403	227	143
SD	180	230	18

Figure 6.3.18.e The average mixed rod-cone ERG response in 129rho^{-/-} injected with AAV-2/8.rP3, AAV-2/8.BB24 or un-injected at a lower dose

The average mixed rod-cone b-wave value for the 129rho^{-/-} mice injected with AAV-2/8.rP3 (9 X 10⁹vp) or AAV-2/8.BB24 (6. 9 x 10¹⁰ vp), in addition to age matched un-injected mice were combined and the average and standard deviation obtained using Microsoft Excel. In the AAV-2/8.rP3 injected mice, only the b-wave values from mice in which a positive ERG response was obtained was used for this analysis. Statistical significance between injected and uninjected mice was evaluated using two sample t-test (DataDesk 6.0).



129 rho ^{-/-}	AAV-2/8.rp3	AAV-2/8.BB24	Un-injected
Average b-wave	52	12	46
SD	19	9	9

Figure 6.3.18.f The average cone-isolated ERG response in 129rho^{-/-} injected with AAV-2/8.rP3, AAV-2/8.BB24 or un-injected at a lower dose

The average cone-isolated b-wave value for the 129rho^{-/-} mice injected with AAV-2/8.rP3 or AAV-2/8.BB24 at a dose of 9 x 10⁹ vp, in addition to aged matched un-injected mice were correlated and the average and standard deviation obtained using Microsoft Excel. In the AAV-2/8.rP3 injected mice, only the b-wave values from mice in which a positive ERG response was obtained was used for this analysis. Statistical significance between injected and uninjected mice was evaluated using two sample t-test (DataDesk 6.0).

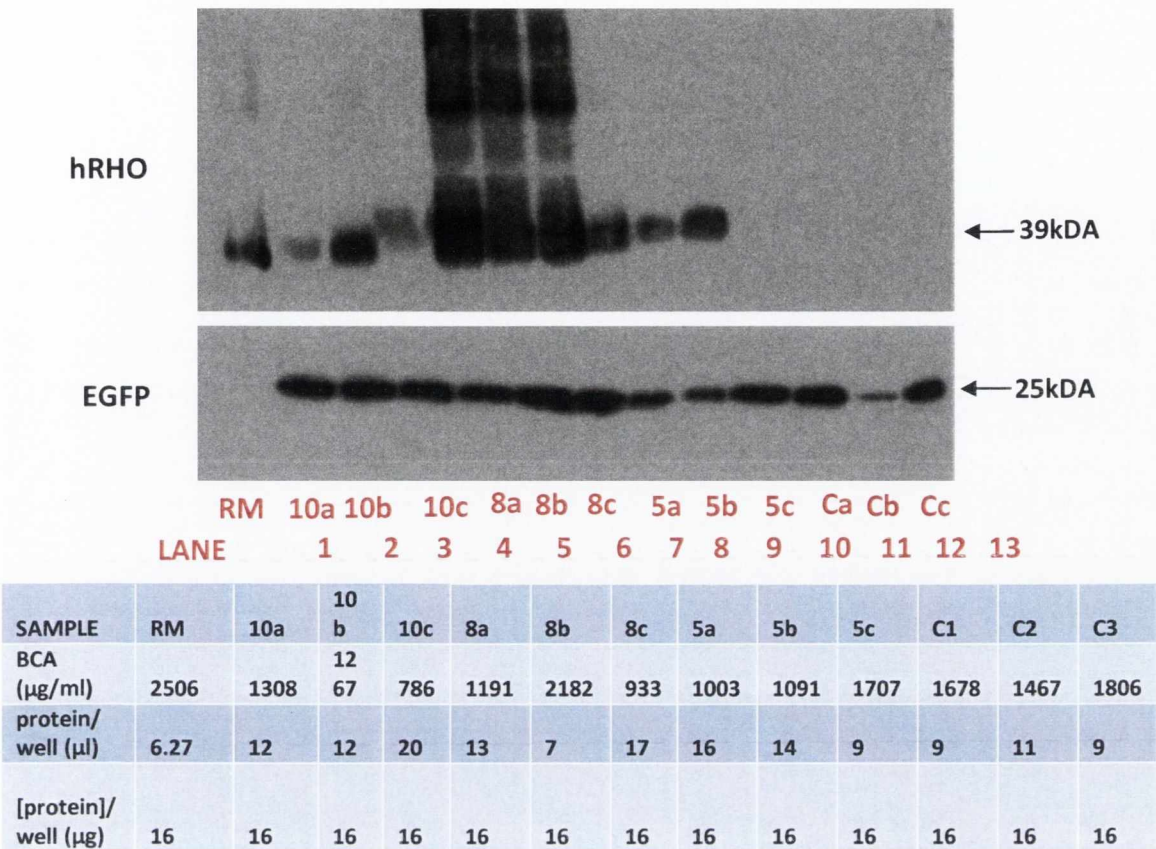


Figure 6.3.19.a Western blot analysis of rBB protein expression from AAV-2/10.BB24, AAV-2/8.BB24 and AAV-2/5.BB24 14 days post-injection

In order to detect which AAV serotype was the most efficient at expressing hRHO protein a comparative analysis was undertaken by injecting 129rho^{-/-} mice with each serotype and analysing by western blotting 14 days post-injection. 3 mice were injected with AAV-2/5.BB24 in the LE and AAV-2/8 in the REBB24. An additional 3 mice were injected with AA-2/10.BB24 in the LE and 1/10 dilution of AAV-2/5RHO-EGFP in the RE. RHO EGFP does not express rhodopsin and acts as the negative control in this experiment. 1/10 the volume of AAV-2/5.RHO-EGFP was added to each viral prep in order to allow EGFP to be utilised as an equal loading control. Lane 1 is protein from a RHO-M mouse and it the positive control for 4D2. Lanes 2-4 is protein from mice injected with AAV-2/10.BB24. Lanes 5-7 is protein from mice injected with AAV-2/8.BB24, Lanes 8-10 is protein from mice injected with AAV-2/5.BB24 and Lanes 11-13 is protein from retinas injected with EGFP (AAV-2/5.RHO-EGFP 1/10 dilution). Clearly at 14 days post-injection AAV-2/8.BB24 is the most efficient at expressing hRHO compared to AAV-2/5 or AAV-2/10. The anti-rabbit EGFP antibody was used to probe for equal loading

Chapter 7: General discussion

Given the dominant nature of mutations in RHO-adRP, a therapeutic strategy which suppresses the effects of the mutant allele may be the most feasible approach for correcting the primary defect in rhodopsin. Two methods for rhodopsin suppression have been described (**Section 3.1**), the first involves the specific suppression of the mutant rhodopsin allele in a mutation-dependent manner and has been undertaken utilising ribozymes and siRNA *in vitro* and *in vivo* (Gorbutyuk et al, 2007b; Tessitore et al, 2006). While the second approach involves the mutation-independent suppression of both the mutant and wild-type rhodopsin alleles in addition to simultaneous delivery of a codon-modified replacement gene refractory to suppression (Millington-Ward et al, 1997). Given the mutational heterogeneity in the rhodopsin gene a mutation-dependent approach may not be feasible. In addition, the demonstration of therapeutic benefit in a RHO-adRP model in previous studies using suppression and replacement technology supports the further exploration of this approach (Millington-Ward et al, 2011). Thus, it was decided that aspects of a mutation-independent suppression and replacement therapeutic approach for RHO-adRP using the porcine RHO gene in a mouse model would be explored as part of the research undertaken during the course this PhD study.

A mutation-independent RNAi-based suppression approach was explored for use in the porcine model of RHO-adRP such that siRNA molecules homologous to both hRHO and pRHO were designed (Chapter 3). The *in vitro* evaluation of these molecules was successfully undertaken by co-transfection of hRHO and each siRNA into HeLa cells. At the RNA and protein level two out of the three siRNAs were established as potent hRHO suppressors *in vitro*. The siP3 molecule in particular was found to result in a 92% downregulation of hRHO RNA and was thus chosen for further analysis *in vivo*.

While potent suppression by siRNAs *in vitro* provides proof of principle for the evaluation of RNAi molecules for rhodopsin, *in vivo* assessment of these molecules in the context of the retina is vital. Rhodopsin comprises 90% of the ROS disc membrane protein and therefore while adequate suppression of rhodopsin may be achieved in a laboratory setting, achieving sufficient potent suppression in photoreceptors may be challenging (Palczewski, 2006). With this in mind siP3-based suppression detailed in Chapter 4 involved the use of a transgenic mouse model expressing human rhodopsin (the NHR mouse [**section 4.3.7**]). This NHR model represents a valuable tool in the laboratory for *in vivo* assessment of mutation-independent strategies applicable to RHO- adRP patients (Ollson et al, 1992). It was decided to generate siP3, the most potent siRNA into a shRNA-based form and evaluate shRNA based suppression *in vivo* (Chadderton et al, 2009; Millington-ward et al, 2011; O' Reilly et al, 2007). Additionally due to recent concern with regards to potential shRNA-mediated

toxicity in tissues such as liver and brain, it was decided to evaluate artificial miRNAs as potential rhodopsin suppressors *in vivo* (Boudreau et al, 2008; Boudreau et al, 2009a; Elhert et al, 2010; Grimm et al, 2010). Two artificial miRNA backbones were chosen for this evaluation miR-30a and miR-155 (Boudreau et al, 2008; Chung et al, 2006). Of note, despite AAV-2/5 mediated delivery of these constructs into the NHR mouse, potent suppression in terms of RNA, protein, ERG function and immunohistochemistry was only consistently achieved with the shP3 molecule, thus no further evaluation with artificial miRNAs was undertaken during the course of the studies described in this thesis. The reason for less robust and less consistent suppression with miRNA-based vectors currently remains undetermined.

The pathway for development of a novel two-component gene therapy involving RNAi-mediated suppression of hRHO in conjunction with provision of an RNAi-resistant hRHO replacement gene will involve preclinical evaluation in rodents, followed by the same in large animals e.g. pigs. Additionally, in larger animal models, toxicity and biodistribution studies will be undertaken eventually leading to clinical trial. An essential element of this is studies in larger animals such as pig. Indeed the pig has been chosen in the current study as the animal model for further R&D associated with hRHO suppression and replacement. Here a key objective of the current study was the evaluation of the therapeutic approach in an animal model relevant to pig as a prelude to further studies in the porcine animal model. In this regard another murine model of hRHO-adRP was characterised as part of the research in this thesis, a transgenic mouse engineered to express a mutant porcine RHO gene (P347S mutation [Chapter 5]). While the initial evaluation of hRHO suppression in NHR mice provided a valuable demonstration of the potency of shP3, the need to evaluate both suppression of pig rhodopsin by shP3 in addition to therapeutic efficacy was of the utmost importance. Thus the study presented in Chapter 5 was concerned with these two parameters. It was decided that given the 3 nucleotide difference between shP3 and the mouse rhodopsin sequence the utilisation of a transgenic mouse with mutant porcine rhodopsin on a heterozygous mouse rhodopsin background may be of value for the purpose outlined above (hetPP37S mouse). Thus suppression of mutant pRHO could be evaluated in addition to the potential benefit conferred by the remaining mouse allele. While significant suppression of pRHO RNA and protein was achieved with shP3 *in vivo* in this animal model, in addition to a reduction of RHO immunoreactivity in mouse retinal sections significant therapeutic benefit was achieved in terms of improved ERG functionality. Additionally preliminary testing in cell culture revealed shP3 was capable of suppressing mRHO RNA by 46%. The suppression of mRHO may potentially aid in interpreting the absence of therapeutic benefit derived from AAV-delivered shP3 in

the PP347S mouse model of RHO-adRP. It may be that while significant suppression of the mutant pRHO allele is beneficial, the one remaining wild-type mRHO allele may reduce levels of wild-type rhodopsin protein to such an extent that detrimental effects are present. In contrast, previous shRNA-mediated suppression of hRHO has been found to provide benefit when mRHO alleles were resistant to RNAi-mediated suppression (Chadderton et al, 2009). Thus in future utilising a model in which mutant pRHO is expressed on a homozygous mouse rhodopsin background may be more amenable to achieving therapeutic efficacy at a functional level.

The analysis of RNAi-based suppression undertaken in Chapters 3-5 established shP3 as a potent suppressor of both human and porcine rhodopsin, thus it was decided to initialise the generation and evaluation of a shP3-resistant replacement gene (Chapter 6). Initially the design and examination of replacement genes resistant to siP2 and siP3 suppression at the RNA and protein level was successfully undertaken by utilising the degeneracy of the genetic code to introduce nucleotide sequence changes over the target sites of RNAi suppression, while maintaining the encoded wild-type amino-acid sequence. The replacement gene (rP3) resistant to siP3-based suppression was subsequently generated in AAV for *in vivo* evaluation. The promoter sequence employed for this replacement gene had previously been shown to provide sufficient levels of rhodopsin to achieve functional benefit in rhodopsin knockout mice (Palfi et al, 2010). Recent explorations in mice, dogs, pigs and non-human primates have established that significantly higher levels of transgene expression may be achieved using AAV-2/8 compared to AAV-2/5 (Allocca et al, 2007; Lebherz et al, 2008; Mussolino et al, 2011b; Steiger et al, 2008; Vandenberghe et al, 2011). For this reason the generation of rP3 in both AAV-2/5 and AAV-2/8 vectors was undertaken to evaluate if superior transgene expression could be achieved with AAV-2/8. A novel second generation AAV production protocol was used to generate the AAV vectors described in Chapter 6, to increase vector purity and titre (Ayuso et al, 2010). Of note, when the highest dose possible was utilised for both AAV-2/5 and AAV-2/8 in rhodopsin knockout mice, less than 25% of the mice showed an improvement in ERG function. This was in contrast to previous studies in the laboratory in which approximately half of the mice had an improved rod-isolated ERG (Dr Paul Kenna, [personal communication]). The results in particular were disappointing for AAV-2/8.rP3 especially given the fact that a control AAV-2/8.BB24 construct significantly out-performed AAV-2/8.rP3 in terms of ERG benefit. Additionally AAV-2/8.BB24 hRHO protein was abundantly expressed when analysed by western blotting or immunohistochemistry compared to AAV-2/8.rP3. This prompted the evaluation of hRHO RNA expression from AAV-2/5.rP3 and AAV-2/8.rP3 injected wild-type mice to assess the level of transgene expression compared

to levels in NHR and RHO-M mice. While AAV-2/5 was expressing hRHO levels statistically lower than hRHO in the NHR retina, surprisingly there was no significant difference between AAV-2/8.rP3 compared to hRHO expression in the NHR retina. However a high variability of hRHO AAV-2/8.rP3 between samples was observed which prompted the view that this virus may be at a dose which causes retinal toxicity. This speculation was confirmed when lowering the dose of AAV-2/8.rP3 delivery 3.5X increased the number of animals with a rod-isolated ERG response and improved cone function. Thus while this newer protocol achieved high AAV titre it possibly did so at the cost of therapeutic efficacy. A thorough evaluation of AAV-2/5.rP3 or AAV-2/8.rP3 doses using this protocol would be needed to choose the most beneficial replacement gene for future evaluations.

Despite the initial optimisation of suppression and replacement for porcine models of adRP described in this thesis there are still a lot of avenues that need exploring. The next step for this project would involve assessing therapeutic benefit with AAV-shP3 using the appropriate animal model. Initially AAV-shP3 would be subretinally injected into wild-type mice to evaluate if mRHO is suppressed *in vivo* by shP3. If shP3 is suppressing mRHO by less than 50 % (as reported in preliminary *in vitro* studies) than the therapeutic benefit associated with AAV-shP3 would be examined in the homPP34S mouse with two wild-type mRHO alleles similar to previous studies (Chadderton et al, 2009). If however shP3 suppresses mRHO *in vivo* by more than 50% it is likely that evaluation of efficacy would proceed directly to utilising a suppression and replacement therapeutic.

Another parameter which should be further examined is the possible immunogenicity of shP3. Although it is unlikely that AAV-mediated shP3 is immunogenic due to the reasons outlined in **Section 4.4**. Although no adverse effects *in vivo* have so far been observed for AAV-shP3, the fact that the siRNA sequence contains possible immunostimulatory motifs would suggest that activation of TLR7/TLR8 and increases in interferon α *in vivo* with this shRNA should be monitored (Judge et al, 2005). Due to time constraints no such analysis was carried out, however, prior to testing AAV-shP3 in larger animal models, this would be undertaken. A recent report has suggested that siRNAs over 21 base pairs (19bp+2nt overhang) result in the sequence independent activation of TLR3 both intracellularly and extracellularly in the RPE. Of particular note, this activation was reported to lead to degeneration of the RPE (Kleinman et al, 2011). Additionally preliminary findings have reported this may also be the case for AAV delivered siRNAs to the retina (Luo et al, 2011). Clearly this has significant implications for all siRNA mediated studies within the retina. However, shRNA based delivery to the retina has been well established and evaluated in this and other laboratories with various

different shRNA molecules for many years and no such degeneration has ever been observed (Chadderton et al, 2009; Millington-ward et al, 2011; O'Reilly et al 2007; O'Reilly et al, 2008). Moreover in several models of retinal degeneration, siRNA-based therapies have provided rescue, a result one would not expect to achieve if the shRNA was in itself inherently toxic to the retinal tissue (Chadderton et al, 2009; Gorbatyuk et al, 2007b; Jiang et al, 2011; Millington-Ward et al, 2011; O'Reilly et al, 2007).

As mentioned previously AAV-2/8 mediated transgene delivery thus far reported in the retina has led to higher levels of transgene expression compared to AAV-2/5 (Allocca et al, 2007 ; Leberherz et al 2008; Mussolino et al, 2011b; Steiger et al, 2008; Vandenberghe et al, 2011). In addition low dose AAV-2/8.rP3 in this study achieved significant rescue of rod function (and cone function) in the rhodopsin knock out mouse. Furthermore preliminary evaluations undertaken at the end of the study presented in Chapter 6 indicate that AAV-2/8 might be superior to AAV-2/5 and AAV-2/rh10 in terms of rhodopsin protein expression. Therefore the future focus for the replacement gene optimisation described in this study would likely involve further evaluation of low dose AAV-2/8.rP3 in rhodopsin knockout mice in terms of increasing the n numbers of animals for ERG analysis, evaluating rhodopsin immunoreactivity and histological benefit in these mice, in addition to ultrastructure analysis. Moreover a dosage study utilising a range of low to high viral doses may be beneficial in order to determine the optimal AAV titre required rescue RHO-adRP.

For the eventual evaluation of the dual-component suppression and replacement strategy two methods of delivery need to be taken into account. The first method involves the utilisation of a bicistronic construct containing both the shP3 suppressor and the rP3 replacement gene. A bicistronic suppression and replacement vector has been utilised previously in the laboratory to evaluate therapeutic efficacy in the P23H mouse model of rhodopsin linked adRP, however, while improvement in terms of ONL thickness was obtained utilising this strategy, no ERG benefit was observed. This was likely due the rapid retinal degeneration in the P23H mouse necessitating P0 subretinal injection and excluding ERG and the low levels of replacement gene expression (Millington-ward et al, 2011; O'Reilly et al, 2007). Subsequent RHO replacement constructs have been optimised (Palfi et al, 2010) and used in separate suppression and replacement vectors. Co-delivery of these two vectors has been explored and benefit observed (Millington-Ward et al, 2011). Furthermore co-delivery of these vectors enabled greater control on the dose of suppression and replacement administered. Thus in terms of the future direction

for suppression and replacement utilising AAV-shP3, co-delivery of an AAV-2/5-shP3 in addition to delivery of AAV-2/8.rP3 based replacement gene may be explored.

As mentioned, a particular limitation of AAV-mediated delivery is the packaging capacity of 4.5kb for the majority of AAV serotypes (Farrar et al, 2010). This issue is particularly vital when delivering large transgenes to the retina involving multiple components e.g. for suppression and replacement or for gene supplementation of larger retinal transgenes implicated in RP such as APCA4, Cep290, Myosin VII or USH2a all of which are larger than 4.5kb (den Hollander et al, 2010). There have been several conflicting reports regarding the ability of AAV to package genomes larger than 4.5kb. In particular Allocca et al 2008 evaluated several rAAV serotypes and found that AAV-2/5 could package large genomes of up to 8.9kb more efficiently than other serotypes. Moreover, this packaging of larger genomes resulted in only an eight fold lower titre compared to packaging of smaller transgenes. Furthermore southern blot analysis confirmed that the full length genes were packaged by this virus. Additionally expression of these transgenes (ABCA-4 and MYO 7a) was detected *in vitro* and *in vivo*. The administration of AAV-2/5.ABCA-4 to ABCA-4 knockout mice resulted in the rescue of the disease phenotype, suggesting that AAV-2/5 was efficient at packaging larger transgenes and capable of conferring therapeutic efficacy (Allocca et al, 2008). Despite the initial excitement, several studies have attempted to repeat this without success. Three groups independently attempted to package large transgenes in various AAV serotypes, however, using southern blot analysis they found that no vector sequence larger than 5kb was ever retrieved from the AAV (Dong et al, 2010; Lai et al, 2010; Wu et al, 2010). Additionally Wu et al, established that these AAV serotypes packaged transgenes with heterologous sequence lengths and truncated 5' ends (Wu et al, 2010). Despite these observations however all three groups noted either *in vitro* or *in vivo* that the full length transgene was capable of expression (Dong et al, 2010; Lai et al, 2010; Wu et al, 2010). One hypothesis to explain these data is that the partial packaging of transgene segments may complement each other and by homologous recombination may result in generation of the longer transgenes (Hirsch et al, 2010). Thus alternative methods may need to be examined for AAV-based therapies in which transgene size is the limiting factor. One such alternative method may be to employ a transplicing approach. This involves separating the transgenes across two separate AAV vectors, and utilising engineered intronic sequences on both half of the transgene to ensure that within the target cell the two transgene components will be spliced together into one full length sequence (den Hollander et al, 2010). Proof of principle of this approach in the retina was demonstrated by Reich et al 2003b in which a LacZ gene was split, the 5' half was engineered to have a splice donor site and the 3' half was engineered to half a splice acceptor site. Expression of the transgene was achieved both *in vitro* and *in vivo* in the mouse retina when the

AAV vectors were delivered either with AAV-2/2 (expression was achieved in RPE) or more efficiently with AAV-2/5-mediated delivery (expression in both photoreceptors and RPE). Furthermore when either half of the LacZ was delivered alone no transgene expression was observed (Reich et al, 2003b). The recent optimisation of a dual AAV-2/5 vector strategy for the delivery of suppression and a replacement gene might be amenable to the AAV transsplicing approach (Millington-Ward et al, 2011).

Given the success of the LCA clinical trials and the establishment of long term safe expression of the AAV-2 delivered RPE65 transgene to human patients, the future for AAV-mediated ocular gene therapy is bright (Bainbridge et al, 2008; Cidecyian et al, 2008; Maguire et al, 2008; Maguire et al, 2009). The research discussed in this thesis has preferentially focused on correction of the primary genetic mutation associated with RHO-adRP. While correcting the primary defect may be a rationale approach for adRP or indeed any genetically inherited disorders, this may not always be the most feasible option. For example some patients with cases of advanced RP may not have sufficient numbers of photoreceptors remaining. Thus the primary defect cannot be corrected, if the cells expressing the mutant gene no longer remain. Indeed the importance of patients receiving therapeutic intervention as early as possible has been demonstrated by the LCA phase I clinical trials in which delivery of the RPE65 therapeutic was reported as more efficacious in younger patients (Maguire et al, 2009). Focusing on therapies that can benefit patients with advanced RP is vital. Strategies being explored for such patients currently include cell transplantation, the use of electronic prosthesis or microchips and optogenetics (Ahuja et al, 2011; Busskamp et al, 2012; Chader et al, 2009; Cronin & Bennett, 2011; Gias et al, 2007; Keegan et al, 2003; Lawrence et al, 2004; Stern & Temple, 2011).

The electronic prosthesis, such as Argus II involves a video camera attached to the patient's glasses, which records external images and transmits the information to a microprocessor. The images are then converted into electrical stimuli and conveyed to an electrode array implanted into the retina. This signal is thus passed onto the retinal ganglion cells and transmitted to the visual cortex (Chader et al, 2009). This device has undergone phase I and phase II/III clinical trials and involves an epiretinal implant of the electrode array. Argus II has been utilised with success in human patients where improvements in both spatial and temporal motor task have been observed (Ahuja et al, 2011). A pilot study with three blind human patients utilised a microchip placed subretinally to restore visual function has been undertaken. This light sensing microchip detects light from the environment and transmits the electrical signals to the retinal ganglion cells for visual processing. In patients with this implant, improvements in vision such as the ability to recognise and locate illuminated objects placed on a dark

table were demonstrated Furthermore one patient had the ability to read large letters as complete words (Zrenner et al, 2011).

A novel advance in designing a therapeutic for patients with RP had been the use of optogenetics, this involves delivering certain light inducible channels or receptors into the remaining inner cells of the retina in order to convert these cells into artificial photoreceptors and mediate light induced visual responses to the brain independent of the rods and cones (Busskamp et al, 2012; Cronin & Bennett, 2011). Several of these optogenetic tools have been explored in the retina, channel-rhodopsin-2 from *Chlamydomonas reinhardtii* (chR2) is a light gated cation channel which causes depolarisation of neurons and has been utilised with success in mouse and rat RP models targeting “ON” bipolar and retinal ganglion cells (Busskamp et al, 2012). One of the first studies utilising this approach involved the intravitreal delivery of an AAV-2 cassette with chR2 driven by a constitutively expressed CAG promoter to a mouse model with severe photoreceptor degeneration (the rd1 mouse). This resulted in the light induced depolarisation of the retinal ganglion cells and the transmission of this light induced signals to the visual cortex in these mice (Bi et al, 2006). In addition chR2 has been specifically delivered to the “ON” bipolar cells by electroporation or AAV-8 delivery utilising an “ON” bipolar cell specific promoter to mice models of retinal degeneration. This enabled not only the light induced depolarisation of these bipolar cells but also activation of amacrine and retinal ganglion cells, in addition to the transmission of this signal to the visual cortex. Furthermore increases in light induced behavior were also observed in these studies (Doroudchi et al, 2011; Lagali et al, 2008). In one study an optical switch consisting of an ionotropic glutamate receptor (LiGluR), covalently attached to maleimide linked to azobenzene (a photoisomerable molecule [MAG]) has been created. When light at 380nm isomerises MAG, glutamate is moved such that the ion channel opens; at other wavelengths glutamate is in a conformation which keeps the ion channels closed. This molecule has been utilised in mice with retinal degeneration. AAV-2-LiGluR was delivered to the retinal ganglion cells of rd1 mice and resulted in stimulation of electrical responses in retinal ganglion cells and transmission of these signals to the visual cortex. Furthermore the pupillary light reflex in these mice was restored in addition to restoration of light avoidance behaviour (Caporale et al, 2011; Cronin & Bennett, 2011). An alternative approach has utilised a light activated chloride pump isolated from Archea *Natronomonas pharaonis* called halorhodopsin (NpHR) to induce hyperpolarisation of “OFF” retinal ganglion cells in a rd1 mouse model. Furthermore, co-delivery of two AAV-2 CMV driven constructs containing chR2 and NpHR to retinal ganglion cells resulted in generation of light induced ON, OFF and ON-OFF retinal responses (Zhang et al, 2009). The AAV-2 mediated delivery of NpHR specifically to the cone IS of rd1 mice resulted in activation of retinal ganglion cells and transmission

of these responses to the visual cortex. Furthermore it resulted in restoration of light induced retinal responses in these mice and suggested a means of recuing the phototransduction response further upstream of bipolar, amacrine and retinal ganglion cells. In addition, when this NpHR was delivered *ex vivo* to human retinal explants photocurrents in the cone photoreceptor cells were generated, suggesting that human patients may be amenable to this therapy (Busskamp et al, 2010).

The novel therapeutic strategies described above highlight just how rapidly the ocular gene therapy field is changing. Indeed since the beginning of this PhD in 2007 a vast number of advances have been made and many clinical trials for RP have been initiated, the most well described for the retina being the AAV-RPE65 clinical trials for LCA (Stein et al, 2011). A future in which gene-based medicines are available as standard treatments for human patients would be an achievement for genetic research and indeed the medical field. Furthermore a scenario in which each patient with a retinal degeneration can be individually assessed such that a particular therapeutic is administered to suit the needs of that patient would be the ultimate objective. For patients with early onset retinal degeneration that may involve correction of the primary mutation using for example, the suppression and replacement approach described in this thesis, or approaches that increase photoreceptor cell survival such as the use of growth factors, or anti-apoptotic agents or indeed any combination of these methods may be of benefit. For patients with advanced RP in which very little photoreceptors are remaining, a therapy involving cell transplantation, retinal prosthesis or implants may be more viable. Additionally utilisation of novel optogenetic approaches may also be of benefit to these patients. The recent designation of a RHO-adRP therapy as an orphan disease represents a small step in driving the suppression and replacement therapies toward human clinical trial (European Medicines Agency, December 2010). A prerequisite prior to human clinical trial is the evaluation of the efficacy of AAV-delivered suppression and replacement therapies in larger animal models such as pig. Hence the work undertaken as part of this thesis in which AAV-shP3 suppression of porcine rhodopsin was demonstrated represents a valuable step towards progressing this novel therapeutic for RHO-adRP towards clinical trials. Transgenic pigs generated previously by Petters et al, 1997 will provide a vital resource to undertake such proposed studies in larger animals. The demonstration of efficacy in large animals and subsequent progression to clinical trial for a AAV-delivered suppression and replacement therapy for RHO-adRP would represent the first in man demonstration of a mutation-independent method for correcting a dominantly inherited disorder while still targeting the primary genetic defect and would pave the way for such a technology to be applied to other dominantly inherited conditions.

Chapter 8: References

Section 8: References

- Aagaard L, Rossi J (2007) RNAi therapeutics: principles, prospects and challenges. *Adv Drug Deliv Rev* **59**: 75-86
- Abdelmaksoud HE, Yau EH, Zuker M, Sullivan JM (2009) Development of lead hammerhead ribozyme candidates against human rod opsin mRNA for retinal degeneration therapy. *Exp Eye Res* **88**: 859-79
- Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J, Dejneka NS, Pearce-Kelling SE, Maguire AM, Palczewski K, Hauswirth WW, Jacobson SG (2005) Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol Ther* **12**: 1072-1082
- Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J, Dejneka NS, Pearce-Kelling SE, Maguire AM, Palczewski K, Hauswirth WW, Jacobson SG (2005) Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol Ther* **12**: 1072-82
- Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, Hauswirth WW, Bennett J (2001) Gene therapy restores vision in a canine model of childhood blindness. *Nat Genet* **28**: 92-95
- Aguirre G, Baldwin V, Pearce-Kelling S, Narfström K, Ray K, Acland G (1998) congenital stationary night blindness in the dog: common mutation in the RPE65 gene indicates founder effect. *Mol Vis* **4**: 23
- Aguirre GK, Komáromy AM, Cideciyan AV, Brainard DH, Aleman TS, Roman AJ, Avants BB, Gee JC, Korczykowski M, Hauswirth WW, Acland GM, Aguirre GD, Jacobson SG (2007) Canine and human visual cortex intact and responsive despite early retinal blindness from RPE65 mutation. *PLoS Med* **4**: e230
- Ahuja AK, Dorn JD, Caspi A, McMahon MJ, Dagnelie G, Dacruz L, Stanga P, Humayun MS, Greenberg RJ, Argus II Study Group (2011) Blind subjects implanted with the Argus II retinal prosthesis are able to improve performance in a spatial-motor task. *Br J Ophthalmol* **95**: 539
- Akiyama H, Kachi S, Silva RL, Umeda N, Hackett SF, McCauley D, McCauley T, Zoltoski A, Epstein DM, Campochiaro PA (2006) Intraocular injection of an aptamer that binds PDGF-B: a potential treatment for proliferative retinopathies. *J Cell Physiol* **207**: 407-12
- Ali RR, Reichel MB, Thrasher AJ, Levinsky RJ, Kinnon C, Kanuga N, Hunt DM, Bhattacharya SS (1996) Gene transfer into the mouse retina mediated by an adeno-associated viral vector. *Hum Mol Genet* **5**: 591-4
- Allocca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D, Kim SR, Maguire A, Rex TS, Di Vicino U, Cuttillo L, Sparrow JR, Williams DS, Bennett J, Auricchio A (2008) Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest* **118**: 1955-64
- Allocca M, Mussolino C, Garcia-Hoyos M, Sanges D, Iodice C, Petrillo M, Vandenberghe L, Wilson J, Marigo V, Surace E, Auricchio A (2007) Novel adeno-associated virus serotypes efficiently transduce murine photoreceptors. *J Virol* **81**: 11372-11380
- Al-Maghtheh M, Gregory C, Inglehearn C, Hardcastle A, Bhattacharya S (1993) Rhodopsin mutations in autosomal dominant retinitis pigmentosa. *Hum Mutat* **2**: 249-255
- Ameres SL, Martinez J, Schroeder R (2007) Molecular basis for target RNA recognition and cleavage by human RISC. *Cell* **130**: 101-12
- An DS, Qin FX, Auyeung VC, Mao SH, Kung SK, Baltimore D, Chen IS (2006) Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther* **14**: 494-504
- Andrés A, Garriga P, Manyosa J (2003) Altered functionality in rhodopsin point mutants associated with retinitis pigmentosa. *Biochem Biophys Res Commun* **303**: 294-301
- Anesti AM, Peeters PJ, Royaux I, Coffin RS (2008) Efficient delivery of RNA Interference to peripheral neurons in vivo using herpes simplex virus. *Nucleic Acids Res* **36**: e86
- Angov E (2011) Codon usage: nature's roadmap to expression and folding of proteins. *Biotechnol J* **6**: 650-9
- Applied Biosystems (2005) Real-Time PCR systems, Chemistry guide
- Applied Biosystems training manual 7300
- Ashtari M, Cyckowski LL, Monroe JF, Marshall KA, Chung DC, Auricchio A, Simonelli F, Leroy BP, Maguire AM, Shindler KS, Bennett J (2011) The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J Clin Invest* **121**: 2160-8

Atchison RW, Casto BC, Hammon WM (1965) Adenovirus-associated defective virus particles. *Science* **149**: 754-6

Auricchio A, Kobinger G, Anand V, Hildinger M, O'Connor E, Maguire AM, Wilson JM, Bennett J (2001a) Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: the retina as a model. *Hum Mol Genet* **10**: 3075-81

Auricchio A, O'Connor E, Hildinger M, Wilson JM (2001b) A single-step affinity column for purification of serotype-5 based adeno-associated viral vectors. *Mol Ther* **4**: 372-4

Ayuso C, Millan JM (2010) Retinitis pigmentosa and allied conditions today: a paradigm of translational research. *Genome Med* **2**: 34

Ayuso E, Mingozzi F, Montane J, Leon X, Anguela XM, Haurigot V, Edmonson SA, Africa L, Zhou S, High KA, Bosch F, Wright JF (2010) High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. *Gene Ther* **17**: 503-10

Azam M, Khan MI, Gal A, Hussain A, Shah ST, Khan MS, Sadeque A, Bokhari H, Collin RW, Orth U, van Genderen MM, den Hollander AI, Cremers FP, Qamar R (2009) A homozygous p.Glu150Lys mutation in the opsin gene of two Pakistani families with autosomal recessive retinitis pigmentosa. *Mol Vis* **15**: 2526-34

Baek MN, Jung KH, Halder D, Choi MR, Lee BH, Lee BC, Jung MH, Choi IG, Chung MK, Oh DY, Chai YG (2010) Artificial microRNA-based neurokinin-1 receptor gene silencing reduces alcohol consumption in mice. *Neurosci Lett* **475**: 124-8

Bainbridge J, Smith A, Barker S, Robbie S, Henderson R, Balaggan K, Viswanathan A, Holder G, Stockman A, Tyler N, Petersen-Jones S, Bhattacharya S, Thrasher A, Fitzke F, Carter B, Rubin G, Moore A, Ali R (2008) Effect of gene therapy on visual function in Leber's congenital amaurosis. *N Engl J Med* **358**: 2231-2239

Barnett KC, Curtis R. (1985) Autosomal dominant progressive retinal atrophy in Abyssinian cats. *J Hered.* **76**: 168-70

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* **136**: 215-33

Baylor DA, Lamb TD, Yau KW (1979) Responses of retinal rods to single photons. *J Physiol* **288**: 613-34

Beauchemin ML, Albrecht Von Graefes (1974) The fine structure of the pig's retina. *Arch Klin Exp Ophthalmol* **190**: 127-45

Beer S, Bellovin DI, Lee JS, Komatsubara K, Wang LS, Koh H, Börner K, Storm TA, Davis CR, Kay MA, Felsher DW, Grimm D (2010) Low-level shRNA cytotoxicity can contribute to MYC-induced hepatocellular carcinoma in adult mice. *Mol Ther* **18**: 161-70

Behlke MA (2008) Chemical modification of siRNAs for in vivo use. *Oligonucleotides* **18**: 305-19

Beltran WA, Boye SL, Boye SE, Chiodo VA, Lewin AS, Hauswirth WW, Aguirre GD (2010) rAAV2/5 gene-targeting to rods: dose-dependent efficiency and complications associated with different promoters. *Gene Ther* **17**: 1162-7

Bennett J, Maguire AM, Cideciyan AV, Schnell M, Glover E, Anand V, Aleman TS, Chirmule N, Gupta AR, Huang Y, Gao GP, Nyberg WC, Tazelaar J, Hughes J, Wilson JM, Jacobson SG (1999) Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proc Natl Acad Sci U S A* **96**: 9920-5

Berg JM, Tymoczko JL, Stryer L (2002) Biochemistry, 5th edition. New York: W. H. Freeman and Co.

Bernardi G (2000) Isochores and the evolutionary genomics of vertebrates. *Gene* **241**: 3-17

Bernstein E, Caudy A, Hammond S, Hannon G (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**: 363-366

Bessant DAR, Payne AM, Mitton KP, Wang Q-L, Swain PK, Plant C, Bird AC, Zack DJ, Swaroop A, Bhattacharya SS (1999) A mutation in NRL is associated with autosomal dominant retinitis pigmentosa. *Nat Genet* **21**: 355-356

Bhattacharya SS, Wright AF, Clayton JF, Price WH, Phillips CI, McKeown CM, Jay M, Bird AC, Pearson PL, Southern EM et al. (1984) Close genetic linkage between X-linked retinitis pigmentosa and a restriction fragment length polymorphism identified by recombinant DNA probe L1.28 *Nature* **309**: 253-255

Bi A, Cui J, Ma YP, Olshevskaya E, Pu M, Dizhoor AM, Pan ZH (2006) Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. *Neuron* **50**: 23-33

Birmingham A, Anderson E, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y, Baskerville S, Maksimova E, Robinson K, Karpilow J, Marshall W, Khvorova A (2006) 3'UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods* **3**: 199-204

Birmingham A, Anderson E, Sullivan K, Reynolds A, Boese Q, Leake D, Karpilow J, Khvorova A (2007) A protocol for designing siRNAs with high functionality and specificity. *Nat Protoc* **2**: 2068-78

- Blackmon SM, Peng YW, Hao Y, Moon SJ, Oliveira LB, Tatebayashi M, Petters RM, Wong F (2000) Early loss of synaptic protein PSD-95 from rod terminals of rhodopsin P347L transgenic porcine retina. *Brain Res* **885**: 53-61
- Blanks JC, Johnson LV (1983) Selective lectin binding of the developing mouse retina. *J Comp Neurol* **221**: 31-41
- Boden D, Pusch O, Silbermann R, Lee F, Tucker L, Ramratnam B (2004) Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. *Nucleic Acids Res* **32**: 1154-1158
- Borchert G, Lanier W, Davidson B (2006) RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* **13**: 1097-1101
- Boudreau R, Monteys A, Davidson B (2008) Minimizing variables among hairpin-based RNAi vectors reveals the potency of shRNAs. *RNA* **14**: 1834-1844
- Boudreau RL, Martins I, Davidson BL (2009a) Artificial microRNAs as siRNA shuttles: Improved safety as compared to shRNAs in vitro and in vivo. *Mol Ther* **17**: 169-175
- Boudreau RL, McBride JL, Martins I, Shen S, Xing Y, Carter BJ, Davidson BL (2009b) Nonallele-specific silencing of mutant and wild-type huntington demonstrates therapeutic efficacy in Huntington's disease mice. *Mol Ther* **17**: 1053-63
- Brolin C, Shiraishi T (2011) Antisense mediated exon skipping therapy for duchenne muscular dystrophy (DMD). *Artif DNA PNA XNA* **2**: 6-15
- Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**: 550-553
- Busskamp V, Duebel J, Balya D, Fradot M, Viney TJ, Siebert S, Groner AC, Cabuy E, Forster V, Seeliger M, Biel M, Humphries P, Paques M, Mohand-Said S, Trono D, Deisseroth K, Sahel JA, Picaud S, Roska B (2010) Genetic reactivation of cone photoreceptors restores visual responses in retinitis pigmentosa. *Science* **329**: 413-7
- Busskamp V, Picaud S, Sahel JA, Roska B (2012) Optogenetic therapy for retinitis pigmentosa. *Gene Ther* **19**: 169-75
- Cai X, Conley SM, Cheng T, Al-Ubaidi MR, Naash MI (2010b) A 350 bp region of the proximal promoter of Rds drives cell-type specific gene expression. *Exp Eye Res* **91**: 186-94
- Cai X, Conley SM, Nash Z, Fliesler SJ, Cooper MJ, Naash MI (2010a) Gene delivery to mitotic and postmitotic photoreceptors via compacted DNA nanoparticles results in improved phenotype in a mouse model of retinitis pigmentosa. *FASEB J* **24**: 1178-91
- Campbell NA, Reece JB (2002) Biology. Sixth edition. San Francisco. Benjamin Cummings
- Campochiaro PA, Nguyen QD, Shah SM, Klein ML, Holz E, Frank RN, Saperstein DA, Gupta A, Stout JT, Macko J, DiBartolomeo R, Wei LL (2006) Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. *Hum Gene Ther* **17**: 167-76
- Caporale N, Kolstad KD, Lee T, Tochitsky I, Dalkara D, Trauner D, Kramer R, Dan Y, Isacoff EY, Flannery JG (2011) LiGluR restores visual responses in rodent models of inherited blindness. *Mol Ther* **19**: 1212-9
- Carvalho LS, Xu J, Pearson RA, Smith AJ, Bainbridge JW, Morris LM, Fliesler SJ, Ding XQ, Ali RR (2011) Long-term and age-dependent restoration of visual function in a mouse model of CNGB3-associated achromatopsia following gene therapy *Hum Mol Genet* **20**: 3161-75
- Cashman SM, Binkley EA, Kumar-Singh R (2005) Towards mutation-independent silencing of genes involved in retinal degeneration by RNA interference. *Gene Ther* **12**: 1223-1228
- Cearley CN, Wolfe JH (2006) Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Mol Ther* **13**: 528-37
- Chadderton N, Millington-Ward S, Palfi A, O'Reilly M, Tuohy G, Humphries MM, Li T, Humphries P, Kenna PF, Farrar GJ (2009) Improved retinal function in a mouse model of dominant Retinitis Pigmentosa following AAV-delivered gene therapy. *Mol Ther* **17**: 593-599
- Chader G (2002) Animal models in research on retinal degenerations: past progress and future hope. *Vision Res* **42**: 393-399
- Chader GJ, Weiland J, Humayun MS. (2009) Artificial vision: needs, functioning, and testing of a retinal electronic prosthesis. *Prog Brain Res* **175**: 317-32
- Chamary JV, Hurst LD (2005) Biased codon usage near intron-exon junctions: selection on splicing enhancers, splice-site recognition or something else? *Trends Genet* **21**: 256-9
- Chandler MJ, Smith PJ, Samuelson DA, MacKay EO (1999) Photoreceptor density of the domestic pig retina. *Vet Ophthalmol* **2**: 179-184

Chang B, Hawes N, Hurd R, Wang J, Howell D, Davisson M, Roderick T, Nusinowitz S, Heckenlively (2005) J Mouse models of ocular diseases. *Vis Neurosci* **22**: 587-593

Chang CI, Kim HA, Dua P, Kim S, Li CJ, Lee DK (2011) Structural diversity repertoire of gene silencing small interfering RNAs. *Nucleic Acid Ther* **21**:125-31

Chang GQ, Hao Y, Wong F (1993) Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. *Neuron* **11**: 595-605

Cho WG, Albuquerque RJ, Kleinman ME, Tarallo V, Greco A, Nozaki M, Green MG, Baffi JZ, Ambati BK, De Falco M, Alexander JS, Brunetti A, De Falco S, Ambati J (2009) Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. *Proc Natl Acad Sci U S A* **106**: 7137-42

Choi VW, McCarty DM, Samulski RJ (2005) AAV hybrid serotypes: improved vectors for gene delivery. *Curr Gene Ther* **5**: 299-310

Chu C, Rana T (2007) Small RNAs: regulators and guardians of the genome. *J Cell Physiol* **213**: 412-419

Chuang JZ, Vega C, Jun W, Sung CH (2004) Structural and functional impairment of endocytotic pathways by retinitis pigmentosa mutant rhodopsin-arrestin complexes *J. Clin Invest* **114**: 131-140

Chung KH, Hart CC, Al-Bassam S, Avery A, Taylor J, Patel PD, Vojtek AB, Turner DL (2006) Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res* **34**: e53

Cideciyan A, Aleman T, Boye S, Schwartz S, Kaushal S, Roman A, Pang J, Sumaroka A, Windsor E, Wilson J, Flotte T, Fishman G, Heon E, Stone E, Byrne B, Jacobson S, Hauswirth W (2008) Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc Natl Acad Sci U S A* **105**: 15112-15117

Cideciyan AV, Hauswirth WW, Aleman TS, Kaushal S, Schwartz SB, Boye SL, Windsor EA, Conlon TJ, Sumaroka A, Roman AJ, Byrne BJ, Jacobson SG (2009) Vision 1 year after gene therapy for Leber's congenital amaurosis. *N Engl J Med* **361**: 725-7

Cideciyan AV, Jacobson SG, Aleman TS, Gu D, Pearce-Kelling SE, Sumaroka A, Acland GM, Aguirre GD (2005) In vivo dynamics of retinal injury and repair in the rhodopsin mutant dog model of human retinitis Pigmentosa *Proc Natl Acad Sci U S A* **102**: 5233-8

Colella P, Iodice C, Di Vicino U, Annunziata I, Surace EM, Auricchio A (2011) Non-erythropoietic erythropoietin derivatives protect from light-induced and genetic photoreceptor degeneration. *Hum Mol Genet* **20**: 2251-62

Concepcion F, Chen J. (2010) Q344ter mutation causes mislocalization of rhodopsin molecules that are catalytically active: a mouse model of Q344ter-induced retinal degeneration. *PLoS One* **5**: e10904

Connell G, Bascom R, Molday L, Reid D, McInnes R, Molday R (1991) Photoreceptor peripherin is the normal product of the gene responsible for retinal degeneration in the rds mouse. *Proc Natl Acad Sci U S A* **88**: 723-726

Cronin T, Bennett J (2011) Switching on the lights: the use of optogenetics to advance retinal gene therapy. *Mol Ther* **19**: 1190-2

Daemen FJ (1973) Vertebrate rod outer segment membranes. *Biochim Biophys Acta* **300**: 255-788

Dahlgren C, Zhang HY, Du Q, Grahn M, Norstedt G, Wahlestedt C, Liang Z (2008) Analysis of siRNA specificity on targets with double-nucleotide mismatches. *Nucleic Acids Res* **36**: e53

Daiger SP, Bowne SJ, Sullivan SL (2007) Perspective on genes and mutations causing retinitis pigmentosa. *Arch Ophthalmol* **125**: 151-158

Dalkara D, Kolstad KD, Caporale N, Visel M, Klimczak RR, Schaffer DV Flannery JG (2009). Inner limiting membrane barriers to AAV-mediated retinal transduction from the vitreous. *Mol Ther* **17**: 2096-2102

Davidson BL, McCray PB Jr (2011) Current prospects for RNA interference-based therapies. *Nat Rev Genet* **12**: 329-40

Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A (2010) Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* **464**: 1067-70

Davson H (1980) Physiology of the eye, 4th edition. Edinburgh. Churchill Livingstone

De Schaepe-drijver L, Simoons P, Pollet L, Lauwers H, De Laey JJ (1992) Morphologic and clinical study of the retinal circulation in the miniature pig. B: Fluorescein angiography of the retina. *Exp Eye Res* **54**: 975-85

den Hollander AI, Black A, Bennett J, Cremers FP (2010) Lighting a candle in the dark: advances in genetics and gene therapy of recessive retinal dystrophies *Clin Invest* **120**: 3042-3053

Deretic D (2006) A role for rhodopsin in a signal transduction cascade that regulates membrane trafficking and photoreceptor polarity. *Vision Res* **46**: 4427-33

- Deretic D, Papermaster DS (1991) Polarized sorting of rhodopsin on post-Golgi membranes in frog retinal photoreceptor cells *J Cell Biol* **113**: 1281-93
- Deretic D, Schmerl S, Hargrave PA, Arendt A, McDowell JH (1998) Regulation of sorting and post-Golgi trafficking of rhodopsin by its C-terminal sequence QVS(A)PA. *Proc Natl Acad Sci U S A*. **95**: 10620-5
- Di Polo A, Farber DB.(1995) Rod photoreceptor-specific gene expression in human retinoblastoma cells. *Proc Natl Acad Sci U S A* **92**: 4016-20
- Djmagoz MBA, Archer SN, Vallerger S (1995) Neurobiology and clinical aspects of the outer retina. 1st edition. Chapman & Hall
- Dong B, Nakai H, Xiao W (2010) Characterization of genome integrity for oversized recombinant AAV vector *Mol Ther* **18**: 87-92
- Doonan F, O'Driscoll C, Kenna P, Cotter TG (2011) Enhancing survival of photoreceptor cells in vivo using the synthetic progestin Norgestrel. *J Neurochem* **118**: 915-27
- Doroudchi MM, Greenberg KP, Liu J, Silka KA, Boyden ES, Lockridge JA, Arman AC, Janani R, Boye SE, Boye SL, Gordon GM, Matteo BC, Sampath AP, Hauswirth WW, Horsager A (2011) Virally delivered channelrhodopsin-2 safely and effectively restores visual function in multiple mouse models of blindness. *Mol Ther* **19**: 1220-9
- Drenser KA, Timmers AM, Hauswirth WW, Lewin AS (1998) Ribozyme-targeted destruction of RNA associated with autosomal-dominant retinitis pigmentosa. *Invest Ophthalmol Vis Sci* **39**: 681-9
- Dryja T, Berson E, Rao V, Oprian D (1993) Heterozygous missense mutation in the rhodopsin gene as a cause of congenital stationary night blindness. *Nat Genet* **4**: 280-283
- Dryja TP, McGee TL, Hahn LB, Cowley GS, Olsson JE, Reichel E, Sandberg MA, Berson EL (1990) Mutations within the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa. *N Engl J Med* **19**: 1302-7
- Du Q, Thonberg H, Wang J, Wahlestedt C, Liang Z (2005) A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. *Nucleic Acids Res* **33**: 1671-7
- Duret L (2000) tRNA gene number and codon usage in the *C. elegans* genome are co-adapted for optimal translation of highly expressed genes. *Trends Genet* **16**: 287-9
- Duret L (2002) Evolution of synonymous codon usage in metazoans. *Curr Opin Genet Dev* **12**: 640-9
- Dykxhoorn DM & Lieberman J (2006) Knocking down disease with siRNAs. *Cell* **126**: 231-235
- Ehlert EM, Eggers R, Niclou SP, Verhaagen J (2010) Cellular toxicity following application of adeno-associated viral vector-mediated RNA interference in the nervous system. *BMC Neurosci* **11**: 20
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001b) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 428-429
- Elbashir SM, Lendeckel W, Tuschl T (2001a) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15**: 188-200
- Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T (2001c) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J* **20**: 6877-88
- Ellinwood NM, Clay CM (2009) Large animal models of genetic disease: pertinent IACUC issues. *ILAR J* **50**: 225-8
- Emerich DF, Thanos CG (2008) NT-501: an ophthalmic implant of polymer-encapsulated ciliary neurotrophic factor-producing cells. *Curr Opin Mol Ther* **10**: 506-15
- Farrar G, Jordan S, Kenna P, Humphries M, Kumar-Singh R, McWilliam P, Allamand V, Sharp E, Humphries P (1991) Autosomal dominant retinitis pigmentosa: localization of a disease gene (RP6) to the short arm of chromosome 6. *Genomics* **11**: 870-87
- Farrar G, McWilliam P, Bradley D, Kenna P, Lawler M, Sharp E, Humphries M, Eiberg H, Connelly P, Trofatter J (1990) Autosomal dominant retinitis pigmentosa: linkage to rhodopsin and evidence for genetic heterogeneity. *Genomics* **8**: 35-40
- Farrar GJ, Kenna PF, Humphries P (2002) On the genetics of retinitis pigmentosa and on mutation-independent approaches to therapeutic intervention. *EMBO J* **21**: 857-864
- Farrar GJ, Palfi A, O' Reilly M (2010) Gene-based therapies for Dominant Retinopathies *Curr Gene Rev.* **10**: 381-388
- Fazi F, Nervi C (2008) MicroRNA: basic mechanisms and transcriptional regulatory networks for cell fate determination. *Cardiovasc Res* **79**: 553-561
- Feng Y, Zhang X, Song Q, Li T, Zeng Y (2011) Drosha processing controls the specificity and efficiency of global microRNA expression. *Biochim Biophys Acta* **1809**: 700-7

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**: 102-14

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806-811

Fletcher EL, Jobling AI, Vessey KA, Luu C, Guymer RH, Baird PN (2011). Animal models of retinal disease. *Prog Mol Biol Transl Sci* **100**: 211-86

Forman JJ, Collier HA (2010) The code within the code: microRNAs target coding regions. *Cell Cycle* **9**: 1533-41

Förstemann K, Tomari Y, Du T, Vagin VV, Denli AM, Bratu DP, Klattenhoff C, Theurkauf WE, Zamore PD (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol* **3**: e236

Fotiadis D, Liang Y, Filipek S, Saperstein DA, Engel A, Palczewski K (2003) Atomic-force microscopy: Rhodopsin dimers in native disc membranes. *Nature* **421**: 127-8

Frank F, Sonenberg N, Nagar B (2010) Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature* **465**: 818-22

Gantier MP, Williams BR (2010) Monitoring innate immune recruitment by siRNAs in mammalian cells. *Methods Mol Biol* **623**: 21-33

Gao Y, Liu XL, Li XR (2011) Research progress on siRNA delivery with nonviral carriers.. *Int J Nanomedicine* **6**: 1017-25

Georgiadis A, Tschernutter M, Bainbridge JW, Robbie SJ, McIntosh J, Nathwani AC, Smith AJ, Ali RR. (2010) AAV-mediated knockdown of peripherin-2 in vivo using miRNA-based hairpins. *Gene Ther* **17**: 486-93

Ghosh F, Engelsberg K, English RV, Petters RM (2007) Long-term neuroretinal full-thickness transplants in a large animal model of severe retinitis pigmentosa. *Graefes Arch Clin Exp Ophthalmol* **245**: 835-46

Ghosh F, Wong F, Johansson K, Bruun A, Petters RM (2004) Transplantation of full-thickness retina in the rhodopsin transgenic pig *Retina* **24**: 98-109

Gias C, Jones M, Keegan D, Adamson P, Greenwood J, Lund R, Martindale J, Johnston D, Berwick J, Mayhew J, Coffey P (2007) Preservation of visual cortical function following retinal pigment epithelium transplantation in the RCS rat using optical imaging techniques. *Eur J Neurosci.* **25**: 1940-8

Giering JC, Grimm D, Storm TA, Kay MA (2008) Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. *Mol Ther* **16**: 1630-6

Giove TJ, Sena-Esteves M, Eldred WD (2010) Transduction of the inner mouse retina using AAVrh8 and AAVrh10 via intravitreal injection. *Exp Eye Res* **91**: 652-9

Glushakova LG, Timmers AM, Issa TM, Cortez NG, Pang J, Teusner JT, Hauswirth WW (2006) Does recombinant adeno-associated virus-vectored proximal region of mouse rhodopsin promoter support only rod-type specific expression in vivo? *Mol Vis* **12**: 298-309

Goldberg AF & Molday RS (1996) Defective subunit assembly underlies a digenic form of retinitis pigmentosa linked to mutations in peripherin/rds and rom-1. *Proc Natl Acad Sci USA* **93**: 13726-13730

Gomperts BD, Tatham PE, Kramer I (2009) Signal transduction. 2nd edition. San Diego. Academic Press

Gorbatyuk M, Justilien V, Liu J, Hauswirth W, Lewin A (2007b) Suppression of mouse rhodopsin expression in vivo by AAV mediated siRNA delivery. *Vision Res* **47**: 1202-1208

Gorbatyuk M, Justilien V, Liu J, Hauswirth WW, Lewin AS (2007a) Preservation of photoreceptor morphology and function in P23H rats using an allele independent ribozyme. *Exp Eye Res* **84**: 44-52

Gorbatyuk MS, Pang JJ, Thomas J Jr, Hauswirth WW, Lewin AS (2005) Knockdown of wild-type mouse rhodopsin using an AAV vectored ribozyme as part of an RNA replacement approach. *Mol Vis* **11**: 648-56

Gouy M, Gautier C. (1982) Codon usage in bacteria: correlation with gene expressivity. *Nucleic Acids Res* **10**: 7055-74

Green ES, Rendahl KG, Zhou S, Ladner M, Coyne M, Srivastava R, Manning WC, Flannery JG (2001). Two animal models of retinal degeneration are rescued by recombinant adeno-associated virus-mediated production of FGF-5 and FGF-18. *Mol Ther* **3**: 507-515

Greenwald DL, Cashman SM, Kumar-Singh R (2010) Engineered zinc finger nuclease-mediated homologous recombination of the human rhodopsin gene. *Invest Ophthalmol Vis Sci* **51**: 6374-80

Gregory-Evans K, Chang F, Hodges MD, Gregory-Evans CY (2008) Ex vivo gene therapy using intravitreal injection of GDNF-secreting mouse embryonic stem cells in a rat model of retinal degeneration. *Curr Opin Mol Ther* **10**: 506-1

Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA (2006) Fatality in mice due to oversaturation on cellular microRNA/short hairpin RNA pathways. *Nature* **44**: 537-541

Grimm D, Wang L, Lee JS, Schürmann N, Gu S, Börner K, Storm TA, Kay MA (2010) Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. *J Clin Invest* **120**: 3106-19

Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* **27**: 91-105

Gu D, Beltran WA, Li Z, Acland GM, Aguirre GD (2007) Clinical light exposure, photoreceptor degeneration, and AP-1 activation: a cell death or cell survival signal in the rhodopsin mutant retina? *Invest Ophthalmol Vis Sci* **48**: 4907-4918

Guo X, Huang L (2011) Recent Advances in Nonviral Vectors for Gene Delivery. *Acc Chem Res* PMID: **21870813**

Hall AH, Wan J, Shaughnessy EE, Ramsay Shaw B, Alexander KA (2004) RNA interference using boranophosphate siRNAs: structure-activity relationships. *Nucleic Acids Res* **32**: 5991-6000

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**: 950-2

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**: 293-6

Han J, Lee Y, Yeom K, Kim Y, Jin H, Kim V (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* **18**: 3016-3027

Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, Sohn SY, Cho Y, Zhang BT, Kim VN (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* **125**: 887-901

Hangai M, Kaneda Y, Tanihara H, Honda Y (1996) In vivo gene transfer into the retina mediated by a novel liposome system. *Invest Ophthalmol Vis Sci* **37**: 2678-85

Hargrave PA and McDowell JH (1992) Rhodopsin and phototransduction: a model system for G protein-linked receptors. *FASEB J* **6**: 2323-31

Hartong DT, Berson EL, Drya TP (2006) Retinitis Pigmentosa. *Lancet* **368**: 1795-1809

Hauswirth WW, Aleman TS, Kaushal S, Cideciyan AV, Schwartz SB, Wang L, Conlon TJ, Boye SL, Flotte TR, Byrne BJ, Jacobson SG (2008) Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. *Hum Gene Ther* **19**: 979-90

He L & Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5**: 522-532

Hecht S, Schlaer S, Pirenne MH (1942) Energy, quanta and vision. *J. Gen Physiol* **25**: 819-814

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S (2001) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**: 1526-9

Hernan I, Gamundi MJ, Borràs E, Maseras M, García-Sandoval B, Blanco-Kelly F, Ayuso C, Carballo M (2011b) Novel p.M96T variant of NRL and shRNA-based suppression and replacement of NRL mutants associated with autosomal dominant retinitis Pigmentosa. *Clin Genet* PMID: **21981118**

Hernan I, Gamundi MJ, Planas E, Borràs E, Maseras M, Carballo (2011a) Cellular expression and siRNA-mediated interference of rhodopsin cis-acting splicing mutants associated with autosomal dominant retinitis pigmentosa. *Invest Ophthalmol Vis Sci* **52**: 3723-9

Hicks D & Molday RS (1986) Differential immunogold-dextran labeling of bovine and frog rod and cone cells using monoclonal antibodies against bovine rhodopsin. *Exp Eye Res* **42**: 55-71

Hildinger M, Auricchio A, Gao G, Wang L, Chirmule N, Wilson JM (2001) Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. *J Virol* **75**: 6199-203

Hirsch ML, Agbandje-McKenna M, Samulski RJ (2010) Little vector, big gene transduction: fragmented genome reassembly of adeno-associated virus. *Mol Ther* **18**: 6-8

Hoggan MD, Blacklow NR, Rowe WP (1966) Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proc Natl Acad Sci USA* **55**: 1467-74

Hornung V, Guenther-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougerolles A, Endres S, Hartmann G (2005) Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* **11**: 263-70

Hu C, Busuttil RW, Lipshutz GS (2010) RH10 provides superior transgene expression in mice when compared with natural AAV serotypes for neonatal gene therapy. *J Gene Med* **12**: 766-78

- Huang H, Qiao R, Zhao D, Zhang T, Li Y, Yi F, Lai F, Hong J, Ding X, Yang Z, Zhang L, Du Q, Liang Z (2009) Profiling of mismatch discrimination in RNAi enabled rational design of allele-specific siRNAs. *Nucleic Acids Res* **37**: 7560-9
- Huang PC, Gaitan AE, Hao Y, Petters RM, Wong F (1993) Cellular interactions implicated in the mechanism of photoreceptor degeneration in transgenic mice expressing a mutant rhodopsin gene. *Proc Natl Acad Sci U S A* **90**: 8484-8
- Humphries MM, Kiang S, McNally N, Donovan MA, Sieving PA, Bush RA, Machida S, Cotter T, Hobson A, Farrar J, Humphries P, Kenna P (2001) Comparative structural and functional analysis of photoreceptor neurons of Rho^{-/-} mice reveal increased survival on C57BL/6J in comparison to 129Sv genetic background. *Vis Neurosci* **18**: 437-43
- Humphries MM, Rancourt D, Farrar GJ, Kenna P, Hazel M, Bush RA, Sieving PA, Sheils DM, McNally N, Creighton P, Erven A, Boros A, Gulya K, Capecchi MR, Humphries P (1997) Retinopathy induced in mice by targeted disruption of the rhodopsin gene. *Nat Genet* **15**: 216-219
- Ikemura T (1981) Correlation between the abundance of Escherichia coli transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the E. coli translational system. *J Mol Biol* **151**: 389-409
- Ikemura T (1985) Codon usage and tRNA content in unicellular and multicellular organisms. *Mol Biol Evol* **2**: 13-34
- Illing ME, Rajan RS, Bence NF, Kopito RR (2002) A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interact with the ubiquitin proteasome system. *J Biol Chem* **277**: 34150-34160
- Inagaki K, Ma C, Storm TA, Kay MA, Nakai H (2007) The role of DNA-PKcs and artemis in opening viral DNA hairpin termini in various tissues in mice. *J Virol* **81**: 11304-21
- Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, Johnson JM, Lim L, Karpilow J, Nichols K, Marshall W, Khvorova A, Linsley PS (2006b) Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* **12**: 1197-205
- Jackson AL, Burchard j, Schelter J, Nelson Chau B, Cleary M, Lim L, Linsley PS (2006a) Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA* **12**: 1179-1187
- Jacobson SG, Acland GM, Aguirre GD, Aleman TS, Schwartz SB, Cideciyan AV, Zeiss CJ, Komaromy AM, Kaushal S, Roman AJ, Windsor EA, Sumaroka A, Pearce-Kelling SE, Conlon TJ, Chiodo VA, Boye SL, Flotte TR, Maguire AM, Bennett J, Hauswirth WW (2006) Safety of recombinant adeno-associated virus type 2-RPE65 vector delivered by ocular subretinal injection. *Mol Ther* **13**: 1074-1084
- Jiang L, Zhang H, Dizhoor AM, Boye SE, Hauswirth WW, Frederick JM, Baehr W (2011) Long-term RNA interference gene therapy in a dominant retinitis pigmentosa mouse model *Proc Natl Acad Sci U S A* **108**: 18476-81
- Johnson LV, Hageman GS, Blanks JC (1986) Interphotoreceptor matrix domains ensheath vertebrate cone photoreceptor cells. *Invest Ophthalmol Vis Sci* **27**: 129-35
- Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. *Annu Rev Plant Biol* **57**: 19-53
- Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol* **23**: 457-62
- Kaiser PK, Symons RC, Shah SM, Quinlan EJ, Tabandeh H, Do DV, Reisen G, Lockridge JA, Short B, Gueriolini R, Nguyen QD; Sirna-027 Study Investigators (2010) RNAi-based treatment for neovascular age-related macular degeneration by Sirna-027. *Am J Ophthalmol* **150**: 33-39.e2
- Kajiwarra K, Hahn L, Mukai S, Travis G, Berson E, Dryja T (1991) Mutations in the human retinal degeneration slow gene in autosomal dominant retinitis pigmentosa. *Nature* **354**: 480-483
- Karali M, Manfredi A, Puppo A, Marrocco E, Gargiulo A, Allocca M, Corte MD, Rossi S, Giunti M, Bacci ML, Simonelli F, Surace EM, Banfi S, Auricchio A (2011) MicroRNA-restricted transgene expression in the retina. *Gene Ther* **18**: 637-45
- Kartasmita A, Fujiki K, Iskandar E, Sovani I, Fujimaki T, Murakami A (2011) A novel nonsense mutation in rhodopsin gene in two Indonesian families with autosomal recessive retinitis Pigmentosa. *Ophthalmic Genet* **32**: 57-63
- Kedde M, Strasser MJ, Boldajipour B, 3-86. Oude Vrielink JA, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Ørom UA, Lund AH, Perrakis A, Raz E, Agami R (2007) RNA-binding protein Dnd1 inhibits microRNA access to target mRNA localization. *J Mol Biol* **348**: 1079-90. *Cell* **131**: 127

- Kedzierski W, Lloyd M, Birch D, Bok D, Travis G (1997) Generation and analysis of transgenic mice expressing P216L-substituted rds/peripherin in rod photoreceptors. *Invest Ophthalmol Vis Sci* **38**: 498-509
- Keegan DJ, Kenna P, Humphries MM, Humphries P, Flitcroft DI, Coffey PJ, Lund RD, Lawrence JM (2003) Transplantation of syngeneic Schwann cells to the retina of the rhodopsin knockout (rho(-/-)) mouse. *Invest Ophthalmol Vis Sci* **44**: 3526-32
- Kennan A, Aherne A, Humphries P (2005) Light in retinitis pigmentosa. *Trends Genet* **21**: 103-110
- Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E (2007) The role of site accessibility in microRNA target recognition. *Nat Genet* **39**: 1278-84
- Ketzinel-Gilad M, Shaul Y, Galun E (2006) RNA interference for antiviral therapy. *J Gene Med* **8**: 933-950
- Khan AU (2006) Ribozyme: a clinical tool. *Clin Chim Acta*. **367**: 20-7
- Khani SC, Pawlyk BS, Bulgakov OV, Kasperek E, Young JE, Adamian M, Sun X, Smith AJ, Ali RR, Li T (2007) AAV-mediated expression targeting of rod and cone photoreceptors with a human rhodopsin kinase promoter. *Invest Ophthalmol Vis Sci* **48**: 3954-61
- Khvorova A, Reynolds A, Jayasena S (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**: 209-216
- Kiang AS, Palfi A, Ader M, Kenna PF, Millington-Ward S, Clark G, Kennan A, O'Reilly M, Tam LC, Aherne A, McNally N, Humphries P, Farrar GJ (2005) Toward a gene therapy for dominant disease: validation of an RNA interference-based mutation-independent approach. *Mol Ther* **12**: 555-561
- Kijas JW, Cideciyan AV, Aleman TS, Pianta MJ, Pearce-Kelling SE, Miller BJ, Jacobson SG, Aguirre GD, Acland GM (2002) Naturally occurring rhodopsin mutation in the dog causes retinal dysfunction and degeneration mimicking human dominant retinitis pigmentosa. *Proc Nat Acad Sci USA* **99**: 6328-6333
- Kim DH & Rossi JJ (2007) Strategies for silencing human disease using RNA interference. *Nat Rev Genet* **8**: 173-184
- Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, Gottesman MM (2007) A "silent" polymorphism in the MDR1 gene changes substrate specificity. *Science* **315**: 525-8
- Kleinman ME, Kaneko H, Cho WG, Dridi S, Fowler BJ, Blandford AD, Albuquerque RJ, Hirano Y, Terasaki H, Kondo M, Fujita T, Ambati BK, Tarallo V, Gelfand BD, Bogdanovich S, Baffi JZ, Ambati J (2011) Short-interfering RNAs Induce Retinal Degeneration via TLR3 and IRF3. *Mol Ther* PMID **21988875**
- Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ, Albuquerque RJ, Yamasaki S, Itaya M, Pan Y, Appukuttan B, Gibbs D, Yang Z, Karikó K, Ambati BK, Wilgus TA, DiPietro LA, Sakurai E, Zhang K, Smith JR, Taylor EW, Ambati J (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* **452**: 591-7
- Koilan S, Hamilton D, Baburyan N, Padala MK, Weber KT, Guntaka RV (2010) Prevention of liver fibrosis by triple helix-forming oligodeoxyribonucleotides targeted to the promoter region of type I collagen gene. *Oligonucleotides* **20**: 231-7
- Komáromy AM, Acland GM, Aguirre GD (2008a) Operating in the dark: a night-vision system for surgery in retinas susceptible to light damage. *Arch Ophthalmol* **126**: 714-7
- Komáromy AM, Alexander JJ, Cooper AE, Chiodo VA, Glushakova LG, Acland GM, Hauswirth WW, Aguirre GD (2008b) Targeting gene expression to cones with human cone opsin promoters in recombinant AAV. *Gene Ther* **15**: 1049-55
- Komáromy AM, Alexander JJ, Rowlan JS, Garcia MM, Chiodo VA, Kaya A, Tanaka JC, Acland GM, Hauswirth WW, Aguirre GD (2010) Gene therapy rescues cone function in congenital achromatopsia *Hum Mol Genet* **19**: 2581-93
- Kotin RM, Berns KI (1989) Organization of adeno-associated virus DNA in latently infected Detroit 6 cells. *Virology* **170**: 460-7
- Kotin RM, Linden RM, Berns KI (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *EMBO J* **11**: 5071-8
- Kotin RM, Menninger JC, Ward DC, Berns KI (1991) Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. *Genomics* **10**: 831-4
- Kotin RM, Siniscalco M, Samulski RJ, Zhu XD, Hunter L, Laughlin CA, McLaughlin S, Muzyczka N, Rocchi M, Berns K (1990) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A* **87**: 2211-5
- Kraft T, Allen D, Petters R, Hao Y, Peng Y, Wong F (2005) Altered light responses of single rod photoreceptors in transgenic pigs expressing P347L or P347S rhodopsin. *Mol Vis* **11**: 1246-1256

Krishnamoorthy V, Jain V, Cherukuri P, Baloni S, Dhingra NK (2008) Intravitreal injection of fluorochrome-conjugated peanut agglutinin results in specific and reversible labeling of mammalian cones in vivo. *Invest Ophthalmol Vis Sci* **49**: 2643-50

Kubodera T, Yamada H, Anzai M, Ohira S, Yokota S, Hirai Y, Mochizuki H, Shimada T, Mitani T, Mizusawa H, Yokota T (2011) In vivo application of an RNAi strategy for the selective suppression of a mutant allele. *Hum Gene Ther* **22**: 27-34

Kühne W (1878) On the Stable Colours of the Retina. *J Physiol.* **1**: 109-212.5

Kumaramanickavel G, Maw M, Denton MJ, John S, Srikumari CR, Orth U, Oehlmann R, Gal A (1994) Missense rhodopsin mutation in a family with recessive RP. *Nat Genet* **8**: 10-11

Lagali PS, Balya D, Awatramani GB, Münch TA, Kim DS, Busskamp V, Cepko CL, Roska B (2008) Light-activated channels targeted to ON bipolar cells restore visual function in retinal degeneration. *Nat Neurosci* **11**: 667-75

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. *Science* **294**: 853-8

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T (2002) Identification of tissue-specific microRNAs from mouse. *Curr Biol* **12**: 735-9

Lai Y, Yue Y, Duan D (2010) Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. *Mol Ther* **18**: 75-9

Lanier KT, Joy JT, Morris RW (2010) Nonclassic retinitis pigmentosa: A challenging clinical diagnosis solved by pedigree analysis and electrodiagnostic testing. *Optometry* **81**: 181-187

LaVail MM, Yasumura D, Matthes MT, Drenser KA, Flannery JG, Lewin AS, Hauswirth WW (2000) Ribozyme rescue of photoreceptor cells in P23H transgenic rats: long-term survival and late-stage therapy. *Proc Natl Acad Sci U S A* **97**: 11488-93

Lawrence JM, Keegan DJ, Muir EM, Coffey PJ, Rogers JH, Wilby MJ, Fawcett JW, Lund RD (2004) Transplantation of Schwann cell line clones secreting GDNF or BDNF into the retinas of dystrophic Royal College of Surgeons rats. *Invest Ophthalmol Vis Sci* **45**: 267-74

Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA (2004) In vivo activity of nuclease-resistant siRNAs. *RNA* **10**: 766-71

Le Bec C & Douar AM (2006) Gene therapy progress and prospects-vecterology: design and production of expression cassettes in AAV vectors. *Gene Ther* **13**: 805-813

Le Meur G, Stieger K, Smith AJ, Weber M, Deschamps JY, Nivard D, Mendes-Madeira A, Provost N, Péréon Y, Cherel Y, Ali RR, Hamel C, Moullier P, Rolling F (2007) Restoration of vision in RPE65-deficient Briard dogs using an AAV serotype 4 vector that specifically targets the retinal pigmented epithelium. *Gene Ther* **14**: 292-303

Leachman SA, Hickerson RP, Schwartz ME, Bullough EE, Hutcherson SL, Boucher KM, Hansen CD, Eliason MJ, Srivatsa GS, Kornbrust DJ, Smith FJ, McLean WI, Milstone LM, Kaspar RL (2010). First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder. *Mol Ther* **18**: 442-6

Lebherz C, Maguire A, Tang W, Bennett J, Wilson JM (2008) Novel AAV serotypes for improved ocular gene transfer. *J Gene Med* **10**: 375-82

Lee ES, Flannery JG (2007) Transport of truncated rhodopsin and its effects on rod function and degeneration. *Invest Ophthalmol Vis Sci* **48**: 2868-76

Lee R, Feinbaum R, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**: 843-854

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S, Kim V (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**: 415-419

Lee Y, Kim M, Han J, Yeom K, Lee S, Baek S, Kim V (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* **23**: 4051-4060

Lem J, Krasnoperova NV, Calvert PD, Kosaras B, Cameron DA, Nicolò M, Makino CL, Sidman RL (1999) Morphological, physiological, and biochemical changes in rhodopsin knockout mice. *Proc Natl Acad Sci U S A* **96**: 736-41

Léveillard T, Mohand-Saïd S, Lorentz O, Hicks D, Fintz AC, Clérin E, Simonutti M, Forster V, Cavusoglu N, Chalmel F, Dollé P, Poch O, Lambrou G, Sahel JA (2004) Identification and characterization of rod-derived cone viability factor. *Nat Genet* **36**: 755-9

- Lewin AS, Drenser KA, Hauswirth WW, Nishikawa S, Yasumura D, Flannery JG, LaVail MM (1998) Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. *Nat Med* **4**: 967-71
- Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**: 15-20
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell* **115**: 787-98
- Li H, Malani N, Hamilton SR, Schlachterman A, Bussadori G, Edmonson SE, Shah R, Arruda VR, Mingozzi F, Wright JF, Bushman FD, High KA (2011) Assessing the potential for AAV vector genotoxicity in a murine model. *Blood* **117**: 3311-9
- Li L, Lin X, Khvorova A, Fesik SW, Shen Y. (2007) Defining the optimal parameters for hairpin-based knockdown constructs. *RNA* **13**: 1765-1774
- Li T (2001) Disease model: photoreceptor degenerations. *Trends Mol Med* **7**: 133-135
- Li T, Snyder WK, Olsson JE, Dryja TP (1996) transgenic mice carrying the dominant rhodopsin mutation P347S: evidence for defective vectorial transport of rhodopsin to the outer segments. *Proc. Natl. Acad. Sci. USA* **93**: 14176-14181
- Li Z, Wong F, Chang J, Possin D, Hao Y, Petters R, Milam A (1998) Rhodopsin transgenic pigs as a model for human retinitis pigmentosa. *Invest Ophthalmol Vis Sci* **39**: 808-819
- Li Z, Zhan W, Wang Z, Zhu B, He Y, Peng J, Cai S, Ma J (2006) Inhibition of PRL-3 gene expression in gastric cancer cell line SGC7901 via microRNA suppressed reduces peritoneal metastasis. *Biochem Biophys Res Commun* **348**: 229-37
- Liang FQ, Anand V, Maguire AM, Bennett J (2001) Intraocular delivery of recombinant virus. *Methods Mol Med* **47**: 125-139
- Liang Z, Wu H, Reddy S, Zhu A, Wang S, Blevins D, Yoon Y, Zhang Y, Shim H (2007) Blockade of invasion and metastasis of breast cancer cells via targeting CXCR4 with an artificial microRNA. *Biochem Biophys Res Commun* **363**: 542-6
- Linden RM, Ward P, Giraud C, Winocour E, Berns KI (1996) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A* **93**: 11288-94
- Liu J, Carmell M, Rivas F, Marsden C, Thomson J, Song J, Hammond S, Joshua-Tor L, Hannon G (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**: 1437-1441
- Liu YL, Wagner K, Robinson N, Sabatino D, Margaritis P, Xiao W, Herzog RW (2003) Optimized production of high-titer recombinant adeno-associated virus in roller bottles. *Biotechniques* **34**: 184-9
- Liu YP, Vink MA, Westerink JT, Ramirez de Arellano E, Konstantinova P, Ter Brake O, Berkhout B (2010) Titers of lentiviral vectors encoding shRNAs and miRNAs are reduced by different mechanisms that require distinct repair strategies. *RNA* **16**: 1328-39
- Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻($\Delta\Delta C_T$) Method. *Methods* **25**: 402-408
- Loscher CJ, Hokamp K, Wilson JH, Li T, Humphries P, Farrar GJ, Palfi A (2008) A common microRNA signature in mouse models of retinal degeneration. *Exp Eye Res* **87**: 529-34
- Lotery AJ, Yang GS, Mullins RF, Russell SR, Schmidt M, Stone EM, Lindbloom JD, Chiorini JA, Kotin RM, Davidson BL (2003) Adeno-associated virus type 5: transduction efficiency and cell-type specificity in the primate retina. *Hum Gene Ther* **14**: 1663-71
- Luo L, Uehara H, Das SK, Archer B, Simonis JM, Singh N, Liu Y, Olsen T, Cahoon J, Ambati BK (2011) Short Hairpin RNA Delivered By Adeno associated Virus Vectors (aav.shRNA) Induces Retinal Degeneration Via Extracellular And Intracellular TLR3. **10/A1** (Abstract at ARVO 2011)
- Maguire A, Simonelli F, Pierce E, Pugh EJ, Mingozzi F, Bennicelli J, Banfi S, Marshall K, Testa F, Surace E, Rossi S, Lyubarsky A, Arruda V, Konkle B, Stone E, Sun J, Jacobs J, Dell'Osso L, Hertle R, Ma J, Redmond T, Zhu X, Hauck B, Zelenia O, Shindler K, Maguire M, Wright J, Volpe N, McDonnell J, Auricchio A, High K, Bennett J (2008) Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* **358**: 2240-2248
- Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F, Mingozzi F, Bennicelli JL, Ying GS, Rossi S, Fulton A, Marshall KA, Banfi S, Chung DC, Morgan JJ, Hauck B, Zelenia O, Zhu X, Raffini L, Coppieters F, De Baere E, Shindler KS, Volpe NJ, Surace EM, Acerra C, Lyubarsky A, Redmond TM, Stone E, Sun J, McDonnell JW, Leroy BP, Simonelli F, Bennett J (2009) Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. *Lancet* **374**: 1597-605

Mahmoud TH, McCuen BW 2nd, Hao Y, Moon SJ, Tatebayashi M, Stinnett S, Petters RM, Wong F (2003) Lensectomy and vitrectomy decrease the rate of photoreceptor loss in rhodopsin P347L transgenic pigs. *Graefes Arch Clin Exp Ophthalmol* **241**: 298-308

Mancuso K, Hauswirth WW, Li Q, Connor TB, Kuchenbecker JA, Mauck MC, Neitz J, Neitz M (2009) Gene therapy for red-green colour blindness in adult primates. *Nature* **461**: 784-7

Mansergh FC, Millington-Ward S, Kennan A, Kiang AS, Humphries M, Farrar GJ, Humphries P, Kenna PF (1999) Retinitis pigmentosa and progressive sensorineural hearing loss caused by a C12258A mutation the mitochondria MTTS2 gene. *Am J Hum Genet* **64**: 971-985

Mao H, James T Jr, Schwein A, Shabashvili AE, Hauswirth WW, Gorbatyuk MS, Lewin AS (2011b) AAV delivery of wild-type rhodopsin preserves retinal function in a mouse model of autosomal dominant retinitis pigmentosa. *Hum Gene Ther* **22**: 567-75

Mao Y, Kiss S, Boyer JL, Hackett NR, Qiu J, Carbone A, Mezey JG, Kaminsky SM, D'Amico DJ, Crystal RG (2011a) Persistent Suppression of Ocular Neovascularization with Intravitreal Administration of AAVrh.10 Coding for Bevacizumab. *Hum Gene Ther* **22**: 1525-35

Marmor MF, Holder GE, Seeliger MW, Yamamoto S (2004) Standard for clinical electroretinography (2004 update). *Doc Ophthalmol* **108**: 107-114

Martin JN, Wolken N, Brown T, Dauer WT, Ehrlich ME, Gonzalez-Alegre P (2011) Lethal toxicity caused by expression of shRNA in the mouse striatum: implications for therapeutic design. *Gene Ther* **7**: 666-73

Matsuda T & Cepko CL (2004) Electroporation and RNA interference in the rodent retina in vivo and in vitro. *Proc. Natl. Acad. Sci USA* **101**: 16-22

Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, Iwaki Y, Colosi P (1998) Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* **5**: 938-45

Mayor HD, Jamison RM, Jordan LE, Melnick JL (1965) Structure and Composition of a Small Particle Prepared from a Simian Adenovirus. *J Bacteriol* **90**: 235-42

Mazelova J, Astuto-Gribble L, Inoue H, Tam BM, Schonteich E, Prekeris R, Moritz OL, Randazzo PA, Deretic D (2009) Ciliary targeting motif VxPx directs assembly of a trafficking. *EMBO J* **28**: 183-192

McBride JL, Boudreau RL, Harper SQ, Staber PD, Mas Montey A, Martins I, Gilmore BL, Burstein H, Peluso RW, Polisky B, Carter BJ, Davidson BL (2008) Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: Implications for the therapeutic development of RNAi. *Proc Natl Acad Sci USA* **105**: 5868-5873

McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA (2002). RNA interference in adult mice. *Nature* **418**: 38-9

McCarty DM, Young SM Jr, Samulski RJ (2004) Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annu Rev Genet* **38**: 819-45

McIlwain JT (1996) An introduction to the biology of vision, Cambridge: Cambridge University Press.

McManus MT & Sharp PA (2002) Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* **3**: 737-747

McNally N, Kenna P, Humphries MM, Hobson AH, Khan NW, Bush RA, Sieving PA, Humphries P, Farrar GJ (1999) Structural and functional rescue of murine rod photoreceptors by human rhodopsin transgene. *Hum Mol Genet* **8**: 1309-12

McNally N, Kenna P, Rancourt D, Ahmed T, Stitt A, Colledge W, Lloyd D, Palfi A, O'Neill B, Humphries M, Humphries P, Farrar G (2002) Murine model of autosomal dominant retinitis pigmentosa generated by targeted deletion at codon 307 of the rds-peripherin gene. *Hum Mol Genet* **11**: 1005-1016

McWilliam P, Farrar G, Kenna P, Bradley D, Humphries M, Sharp E, McConnell D, Lawler M, Sheils D, Ryan C (1989) Autosomal dominant retinitis pigmentosa (ADRP): localization of an ADRP gene to the long arm of chromosome 3. *Genomics* **5**: 619-622

Mendes HF, van der Spuy J, Chapple JP, Cheetham ME (2005) Mechanisms of cell death in rhodopsin retinitis pigmentosa; implications for therapy. *Trends Mol Med* **11**: 177-185

Mihelec M, Pearson RA, Robbie SJ, Buch PK, Azam SA, Bainbridge JW, Smith AJ, Ali RR (2011) Long-term preservation of cones and improvement in visual function following gene therapy in a mouse model of leber congenital amaurosis caused by guanylate cyclase-1 deficiency. *Hum Gene Ther* **22**: 1179-90

Millán JM, Aller E, Jaijo T, Blanco-Kelly F, Gimenez-Pardo A, Ayuso C (2011) An update on the genetics of usher syndrome. *J Ophthalmol* **2011**: 417217

Miller DG, Petek LM, Russell DW (2004) Adeno-associated virus vectors integrate at chromosome breakage sites. *Nat Genet* **36**: 767-73

Miller DG, Trobridge GD, Petek LM, Jacobs MA, Kaul R, Russell DW (2005) Large-scale analysis of adeno-associated virus vector integration sites in normal human cells. *J Virol* **79**: 11434-42

Millington-Ward S, Chadderton N, O'Reilly M, Palfi A, Goldmann T, Kilty C, Humphries M, Wolfrum U, Bennett J, Humphries P, Kenna PF, Farrar GJ (2011) Suppression and replacement gene therapy for autosomal dominant disease in a murine model of dominant retinitis pigmentosa. *Mol Ther* **19**: 642-9.

Millington-Ward S, O'Neill B, Tuohy G, Al-Jandal N, Kiang AS, Kenna PF, Palfi A, Hayden P, Mansergh F, Kennan A, Humphries P, Farrar GJ (1997) Stratagems in vitro for gene therapies directed to dominant mutations. *Hum Mol Genet*. **6**: 1415-1426

Mingozi F, High KA (2011) Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* **12**: 341-55

Mussolino C, della Corte M, Rossi S, Viola F, Di Vicino U, Marrocco E, Neglia S, Doria M, Testa F, Giovannoni R, Crasta M, Giunti M, Villani E, Lavitrano M, Bacci ML, Ratiglia R, Simonelli F, Auricchio A, Surace EM (2011b) AAV-mediated photoreceptor transduction of the pig cone-enriched retina. *Gene Ther* **18**: 637-45

Mussolino C, Sanges D, Bonetti C, Marigo V, Meroni G, Surace EM (2011a) Transcriptional repression of a human rhodopsin gene by artificial zinc finger protein recovers retinal function in a model of autosomal dominant Retinitis Pigmentosa. *Mol Ther* **17**: S287

Musto H, Cruveiller S, D'Onofrio G, Romero H, Bernardi G (2001) Translational selection on codon usage in *Xenopus laevis*. *Mol Biol Evol* **18**: 1703-7

Nakai H, Iwaki Y, Kay MA, Couto LB (1999) Isolation of recombinant adeno-associated virus vector-cellular DNA junctions from mouse liver. *J Virol* **73**: 5438-47

Nakai H, Montini E, Fuess S, Storm TA, Grompe M, Kay MA (2003) AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat Genet* **34**: 297-302

Nakai H, Yant SR, Storm TA, Fuess S, Meuse L, Kay MA (2001) Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *J Virol* **75**: 6969-76

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**: 279-289

Narfström K (1983) Hereditary progressive retinal atrophy in the Abyssinian cat. *J Hered* **74**: 273-6

Narfström K, Holland Deckman K, Menotti-Raymond M (2011) The domestic cat as a large animal model for characterization of disease and therapeutic intervention in hereditary retinal blindness. *J Ophthalmol*. **2011**: 906943

Narfström K, Katz ML, Bragadottir R, Seeliger M, Boulanger A, Redmond TM, Caro L, Lai CM, Rakoczy PE (2003) Functional and structural recovery of the retina after gene therapy in RPE65 null mutation dog. *Invest Ophthalmol Vis Sci* **44**: 1663-1672

Natkunarahaj M, Trittibach P, McIntosh J, Duran Y, Barker SE, Smith AJ, Nathwani AC, Ali RR (2008) Assessment of ocular transduction using single-stranded and self-complementary recombinant adeno-associated virus serotype 2/8. *Gene Ther* **15**: 463-7

Ng YF, Chan HH, Chu PH, To CH, Gilger BC, Petters RM, Wong F (2008) Multifocal electroretinogram in rhodopsin P347L transgenic pigs. *Invest Ophthalmol Vis* **49**: 2208-15

Nielsen TT, Marion I, Hasholt L, Lundberg C (2009) Neuron-specific RNA interference using lentiviral vectors. *J Gene Med* **11**: 559-69

O'Neill B, Millington-Ward S, O'Reilly M, Tuohy G, Kiang AS, Kenna PF, Humphries P, Farrar GJ (2000) Ribozyme-based therapeutic approaches for autosomal dominant retinitis pigmentosa. *Invest Ophthalmol Vis Sci* **41**: 2863-2869

Ollson JE, Gorden JW, Pawlyk BS, Roof D, Hayes A, Molday RS, Mukai S, Cowley GS, Berson EL, Dryja TP (1992) Transgenic mice with rhodopsin mutation (Pro23His): A mouse model of autosomal dominant retinitis pigmentosa. *Neuron* **9**: 815-830

O'Reilly M, Millington-Ward S, Palfi A, Chadderton N, Cronin T, McNally N, Humphries M, Humphries P, Kenna P, Farrar G (2008) A transgenic mouse model for gene therapy of rhodopsin-linked Retinitis Pigmentosa. *Vision Res* **48**: 386-391

O'Reilly M, Palfi A, Chadderton N, Millington-Ward S, Ader M, Cronin T, Tuohy T, Auricchio A, Hildinger M, Tivnan A, McNally N, Humphries MM, Kiang AS, Humphries P, Kenna PF, Farrar GJ (2007) RNA interference-mediated suppression and replacement of human rhodopsin in vivo. *Am J Hum Genet*. **81**: 127-135

Osawa S, Weiss ER (1994) The carboxyl terminus of bovine rhodopsin is not required for G protein activation. *Mol Pharmacol* **46**: 1036-40

Overhoff M, Sczakiel G (2005) Phosphorothioate-stimulated uptake of short interfering RNA by human cells. *EMBO Rep* **6**: 1176-81

Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16**: 948-958

Palczewski K (2006) G protein-coupled receptor rhodopsin. *Annu Rev Biochem* **75**:743-767

Palfi A, Ader M, Kiang AS, Millington-Ward S, Clark G, O'Reilly M, McMahon HP, Kenna PF, Humphries P, Farrar GJ (2006) RNAi-based suppression and replacement of rds-peripherin in retinal organotypic culture. *Hum Mutat* **27**: 260-268

Palfi A, Millington-Ward S, Chadderton N, O'Reilly M, Goldmann T, Humphries MM, Li T, Wolfrum U, Humphries P, Kenna PF, Farrar GJ (2010) Adeno-associated virus-mediated rhodopsin replacement provides therapeutic benefit in mice with a targeted disruption of the rhodopsin gene. *Hum Gene Ther* **21**: 311-23

Pang JJ, Boye SL, Kumar A, Dinculescu A, Deng W, Li J, Li Q, Rani A, Foster TC, Chang B, Hawes NL, Boatright JH, Hauswirth WW (2008) AAV-mediated gene therapy for retinal degeneration in the rd10 mouse containing a recessive PDEbeta mutation. *Invest Ophthalmol Vis Sci* **49**: 4278-83

Peng YW, Hao Y, Petters RM, Wong F (2000) Ectopic synaptogenesis in the mammalian retina caused by rod photoreceptor-specific mutations. *Nat Neurosci* **3**: 1121-7

Penha FM, Rodrigues EB, Maia M, Dib E, Fiod Costa E, Furlani BA, Nunes Moraes Filho M, Dreyfuss JL, Bottós J, Farah ME (2010) Retinal and ocular toxicity in ocular application of drugs and chemicals--part I: animal models and toxicity assays. *Ophthalmic Res* **44**: 82-104

Petters RM, Alexander CA, Wells KD, Collins EB, Sommer JR, Blanton MR, Rojas G, Hao Y, Flowers WL, Banin E, Cideciyan AV, Jacobson SG, Wong F (1997). Genetically engineered large animal model for studying cone photoreceptor survival and degeneration in retinitis pigmentosa. *Nat Biotechnol* **15**: 965-970

Phelan JK & Bok D (2000) A brief view of retinitis pigmentosa and the identified retinitis pigmentosa genes. *Mol Vis* **6**: 116-124

Philpott NJ, Gomos J, Berns KI, Falck-Pedersen E (2002) A p5 integration efficiency element mediates Rep-dependent integration into AAVS1 at chromosome 19. *Proc Natl Acad Sci U S A* **99**: 12381-5

Piedrahita D, Hernández I, López-Tobón A, Fedorov D, Obara B, Manjunath BS, Boudreau RL, Davidson B, Laferla F, Gallego-Gómez JC, Kosik KS, Cardona-Gómez GP (2010) Silencing of CDK5 reduces neurofibrillary tangles in transgenic alzheimer's mice. *J Neurosci* **30**: 13966-76

Provost P, Dishart D, Doucet J, Frendewey D, Samuelsson B, Rådmark O (2002) Ribonuclease activity and RNA binding of recombinant human Dicer. *EMBO J* **21**: 5864-74

Purves D, Augustine GS, Fitzpatrick D, Hall WC, La Mantia AS, McNamara JO, White LE (2008) Neuroscience, 4th edition. Sunderland Massachusetts. Sinauer Associates Inc.

Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ.(2002) Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* **76**: 791-801

Rattner A, Sun H, Nathans J (1999) Molecular genetics of human retinal disease. *Annu Rev Genet* **33**: 89-131

Reed SE, Staley EM, Mayginnes JP, Pintel DJ, Tullis GE (2006) Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *J Virol Methods* **138**: 85-98

Rehmtulla A, Warwar R, Kumar R, Ji X, Zack DJ, Swaroop A (1996) The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. *Proc Natl Acad Sci U S A* **93**: 191-5

Reich SJ, Auricchio A, Hildinger M, Glover E, Maguire AM, Wilson JM, Bennett J (2003b) Efficient trans-splicing in the retina expands the utility of adeno-associated virus as a vector for gene therapy. *Hum Gene Ther*. 2003 **14**: 37-44

Reich SJ, Fosnot J, Kuroki A, Tang W, Yang X, Maguire AM, Bennett J, Tolentino MJ (2003a) Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol Vis* **9**: 210-216

Reinhart B, Slack F, Basson M, Pasquinelli A, Bettinger J, Rougvie A, Horvitz H, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403**: 901-906

Remington LA, McGill EC (1998) Clinical anatomy of the visual system, Boston; Oxford: Butterworth-Heinemann.

Ren L, Gao G, Zhao D, Ding M, Luo J, Deng H (2007) Developmental stage related patterns of codon usage and genomic GC content: searching for evolutionary fingerprints with models of stem cell differentiation. *Genome Biol* **8**: R35

Rex TS, Wong Y, Kodali K, Merry S (2009) Neuroprotection of photoreceptors by direct delivery of erythropoietin to the retina of the retinal degeneration slow mouse. *Exp Eye Res* **89**: 735-40

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorovova A (2004) Rational siRNA design for RNA interference. *Nat Biotechnol* **22**: 326-30

Reynolds AL, Danciger M, Farrar GJ, Humphries P, Kenna PF (2008) Influence of a quantitative trait locus on mouse chromosome 19 to the light-adapted electroretinogram. *Invest Ophthalmol Vis Sci*. **49**: 4058-63

Rhee SW, Stimers JR, Wang W, Pang L (2009) Vascular smooth muscle-specific knockdown of the noncardiac form of the L-type calcium channel by microRNA-based short hairpin RNA as a potential antihypertensive therapy. *J Pharmacol Exp Ther* **329**: 775-782

Ridge KD & Palczewski K (2007) Visual rhodopsin sees the light: structure and mechanism of g protein signaling. *J Biol Chem* **282**: 9293-9301

Robbins MA, Li M, Leung I, Li H, Boyer DV, Song Y, Behlke MA, Rossi JJ (2006) Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs in vitro. *Nat Biotechnol* **24**: 566-71

Rohr UP, Wulf MA, Stahn S, Steidl U, Haas R, Kronenwett R (2002) Fast and reliable titration of recombinant adeno-associated virus type-2 using quantitative real-time PCR. *J Virol Methods* **106**: 81-8

Rolling F (2004) Recombinant AAV-mediated gene transfer to the retina: gene therapy perspectives. *Gene Ther* **11**: S26-S32

Romano N, Macino G (1992) Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol Microbiol* **6**: 3343-53

Rosenfeld P, Cowley G, McGee T, Sandberg M, Berson E, Dryja T (1992) A null mutation in the rhodopsin gene causes rod photoreceptor dysfunction and autosomal recessive retinitis pigmentosa. *Nat Genet* **1**: 209-213

Rutledge EA, Russell DW (1997) Adeno-associated virus vector integration junctions. *J Virol* **71**: 8429-36

Saito K, Ishizuka A, Siomi H, Siomi MC (2005) Processing of pre-microRNAs by the Dicer-1-Loquacious complex in *Drosophila* cells. *PLoS Biol* **3**: e235

Samulski RJ, Zhu X, Xiao X, Brook JD, Housman DE, Epstein N, Hunter LA (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J* **10**: 3941-50

Sanyal S, Jansen H (1981) Absence of receptor outer segments in the retina of rds mutant mice. *Neurosci Lett* **21**: 23-26

Schmid F, Glaus E, Barthelmes D, Fliegau M, Gaspar H, Nürnberg G, Nürnberg P, Omran H, Berger W, Neidhardt J (2011) U1 snRNA-mediated gene therapeutic correction of splice defects caused by an exceptionally mild BBS mutation. *Hum Mutat* **32**: 815-24

Schwarz D, Ding H, Kennington L, Moore J, Schelter J, Burchard J, Linsley P, Aronin N, Xu Z, Zamore P (2006) Designing siRNA that distinguish between genes that differ by a single nucleotide. *PLoS Genet* **2**: e140

Seow Y, Wood MJ (2009) Biological gene delivery vehicles: beyond viral vectors. *Mol Ther* **17**: 767-77

Shabalina S, Ogurtsov A, Spiridonov N (2006b) A periodic pattern of mRNA secondary structure created by the genetic code. *Nucleic Acids Res* **34**: 2428-2437

Shabalina SA, Spiridonov AN, Ogurtsov AY (2006a) Computational models with thermodynamic and composition features improve siRNA design. *BMC Bioinformatics* **7**: 65

Shan Z, Lin Q, Deng C, Li, Xiaohong Li, Huang, W, Tan H, Fu, Y, Yang, Min, Yu XY (2009b) An efficient method to enhance gene silencing by using precursor MicroRNA designed small hairpin RNAs. *Mol Biol Rep* **36**: 1438-9

Shan ZX, Lin QX, Deng CY, Zhou ZL, Tan HH, Fu YH, Li XH, Zhu JN, Mai LP, Kuang SJ, Lin SG, Yu XY (2010) Comparison of approaches for efficient gene silencing induced by microRNA-based short hairpin RNA and indicator gene expression. *Mol Biol Rep* **37**: 1831-9

Shan ZX, Lin QX, Yang M, Deng CY, Kuang SJ, Zhou ZL, Xiao DZ, Liu XY, Lin SG, Yu XY (2009a) A quick and efficient approach for gene silencing by using triple putative microRNA-based short hairpin RNAs. *Mol Cell Biochem* **323**: 81-9

Shaw LC, Skold A, Wong F, Petters R, Hauswirth WW, Lewin AS (2001) An allele-specific hammerhead ribozyme gene therapy for a porcine model of autosomal dominant retinitis Pigmentosa. *Mol Vis* **7**: 6-13

Shen J, Yang X, Dong A, Petters RM, Peng YW, Wong F, Campochiaro PA (2005) Oxidative damage is a potential cause of cone cell death in retinitis pigmentosa. *J Cell Physiol* **203**: 457-64

Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP (2010) Expanding the microRNA targeting code: functional sites with centered pairing. *Mol Cell* **38**: 789-802

Sibley CR, Seow Y, Wood MJ (2010) Novel RNA-based strategies for therapeutic silencing. *Mol Ther* **18**: 466-76

Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, Sheth N, Bradshaw J, Burchard J, Kulkarni A, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, Hannon GJ (2005) Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet* **37**: 1281-8

Simoens P, De Schaepdrijver L, Lauwers H (1992) Morphologic and clinical study of the retinal circulation in the miniature pig. A: Morphology of the retinal microvasculature. *Exp Eye Res* **54**: 965-73

Simonelli F, Maguire AM, Testa F, Pierce EA, Mingozzi F, Bennicelli JL, Rossi S, Marshall K, Banfi S, Surace EM, Sun J, Redmond TM, Zhu X, Shindler KS, Ying GS, Ziviello C, Acerra C, Wright JF, McDonnell JW, High KA, Bennett J, Auricchio A (2010) Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. *Mol Ther* **18**: 643-50

Sioud M (2005) Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J Mol Biol* **348**: 1079-90

Smith RH (2008) Adeno-associated virus integration: virus versus vector. *Gene Ther* **15**: 817-22

Sommer JR, Wong F, Petters RM (2011) Phenotypic stability of Pro347Leu rhodopsin transgenic pigs as indicated by photoreceptor cell degeneration. *Transgenic Res* **20**: 1391-5

Soriano P, Dijkstra J, Legrand A, Spanjer H, Londos-Gagliardi D, Roerdink F, Scherphof G, Nicolau C (1983) Targeted and nontargeted liposomes for in vivo transfer to rat liver cells of a plasmid containing the preproinsulin I gene. *Proc Natl Acad Sci U S A* **80**: 7128-31

Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Röhl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliensky V, Limmer S, Manoharan M, Vornlocher HP (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**: 173-8

Srivastava A, Lusby EW, Berns KI (1983) Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J Virol* **45**: 555-64

Stegmeier F, Hu G, Rickles R, Hannon G, Elledge S (2005) A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci U S A* **102**: 13212-13217

Stein L, Roy K, Lei L, Kaushal S (2011) Clinical gene therapy for the treatment of RPE65-associated Leber congenital amaurosis. *Expert Opin Biol Ther* **11**: 429-39

Stern JH, Temple S (2011) Stem Cells for Retinal Replacement Therapy *Neurotherapeutics* **8**: 736-43

Stewart CR, Karpala AJ, Lowther S, Lowenthal JW, Bean AG (2011) Immunostimulatory motifs enhance antiviral siRNAs targeting highly pathogenic avian influenza H5N1. *PLoS One* **6**: e21552

Stieger K, Colle MA, Dubreil L, Mendes-Madeira A, Weber M, Le Meur G, Deschamps JY, Provost N, Nivard D, Cherel Y, Moullier P, Rolling F (2008) Subretinal delivery of recombinant AAV serotype 8 vector in dogs results in gene transfer to neurons in the brain. *Mol Ther* **16**: 916-23

Stricker H, Ding X, Quiambao A, Fliesler S, Naash M (2005) The Cys214-->Ser mutation in peripherin/rds causes a loss-of-function phenotype in transgenic mice. *Biochem J* **388**: 605-613

Sullivan JM, Pietras KM, Shin BJ, Misasi JN (2002) Hammerhead ribozymes designed to cleave all human rod opsin mRNAs which cause autosomal dominant retinitis pigmentosa. *Mol Vis* **8**: 102-13

Sullivan T, Kodali K, Rex TS (2011) Systemic gene delivery protects the photoreceptors in the retinal degeneration slow mouse. *Neurochem Res* **36**: 613-8

Sung C, Davenport C, Hennessey J, Maumenee I, Jacobson S, Heckenlively J, Nowakowski R, Fishman G, Gouras P, Nathans J (1991) Rhodopsin mutations in autosomal dominant retinitis pigmentosa. *Proc Natl Acad Sci U S A* **88**: 6481-6485

Sung C, Makino C, Baylor D, Nathans J (1994) A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment. *J Neurosci* **14**: 5818-5833

Sung CH, Chuang JZ (2010) The cell biology of vision. *J Cell Biol* **190**: 953-63

Sung CH, Davenport CM, Nathans J (1993) Rhodopsin mutations responsible for autosomal dominant Retinitis Pigmentosa. *J Biol Chem* **268**: 26645-26649

Sung CH, Tai AW (2000) Rhodopsin trafficking and its role in retinal dystrophies. *Int Rev Cytol* **195**: 215-67

Takayama KM, Inouye M (1990) Antisense RNA. *Crit Rev Biochem Mol Biol* **25**: 155-84

Tam W (2001) Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA *Gene* **274**: 157-67

- Tam W, Ben-Yehuda D, Hayward WS (1997) bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol Cell Biol* **17**: 1490-502
- Tam W, Dahlberg JE (2006) miR-155/BIC as an oncogenic microRNA. *Genes Chromosomes Cancer* **42**: 211-2
- Tan MH, Smith AJ, Pawlyk B, Xu X, Liu X, Bainbridge JB, Basche M, McIntosh J, Tran HV, Nathwani A, Li T, Ali RR (2009) Gene therapy for retinitis pigmentosa and Leber congenital amaurosis caused by defects in AIPL1: effective rescue of mouse models of partial and complete Aipl1 deficiency using AAV2/2 and AAV2/8 vectors. *Hum Mol Genet*. **19**: 735
- Tanner G, Glaus E, Barthelmes D, Ader M, Fleischhauer J, Pagani F, Berger W, Neidhardt J (2009) Therapeutic strategy to rescue mutation-induced exon skipping in rhodopsin by adaptation of U1 snRNA. *Hum Mutat* **30**: 255-63
- Tessitore A, Parisi F, Denti M, Allocca M, Di Vicino U, Domenici L, Bozzoni I, Auricchio A (2006) Preferential silencing of a common dominant rhodopsin mutation does not inhibit retinal degeneration in a transgenic model. *Mol Ther* **14**: 692-699
- Thanos CG, Bell WJ, O'Rourke P, Kauper K, Sherman S, Stabila P, Tao W (2004) Sustained secretion of ciliary neurotrophic factor to the vitreous, using the encapsulated cell therapy-based NT-501 intraocular device. *Tissue Eng* **10**: 1617-22
- Tso M, Li W, Zhang C, Lam T, Hao Y, Petters R, Wong F (1997) A pathologic study of degeneration of the rod and cone populations of the rhodopsin Pro347Leu transgenic pigs. *Trans Am Ophthalmol Soc* **95**: 467-483
- Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res* **32**: 936-48
- Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD (2010) Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* **11**: 636-46
- van Nie R, Iványi D, Démant P (1978) A new H-2-linked mutation, rds, causing retinal degeneration in the mouse. *Tissue Antigens* **12**: 106-108
- Vandenberghe LH, Auricchio A (2012) Novel adeno-associated viral vectors for retinal gene therapy. *Gene Ther* **19**: 236
- Vandenberghe LH, Bell P, Maguire AM, Cearley CN, Xiao R, Calcedo R et al (2011) Dosage thresholds for AAV2 and AAV8 photoreceptor gene therapy in monkey. *Sci Transl Med* **3**: 88ra54
- Vandenberghe LH, Xiao R, Lock M, Lin J, Korn M, Wilson JM (2010) Efficient serotype-dependent release of functional vector into the culture medium during adeno-associated virus manufacturing. *Hum Mol Genet* **8**: 1309-12
- Verardo MR, Viczian A, Piri N, Akhmedov NB, Knox BE, Farber DB (2009) Regulatory sequences in the 3' untranslated region of the human cGMP-phosphodiesterase beta-subunit gene. *Invest Ophthalmol Vis Sci* **50**: 2591-8
- Verma I, Weitzman M (2005) Gene therapy: twenty-first century medicine. *Annu Rev Biochem* **74**: 711-738
- Vickers TA, Lima WF, Nichols JG, Crooke ST (2007) Reduced levels of Ago2 expression result in increased siRNA competition in mammalian cells. *Nucleic Acids Res* **35**: 6598-610
- Vihetic TS, Doro CJ, Hyde DR (1999) Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. *Vis Neurosci* **16**: 571-85
- Walton SP, Wu M, Gredell JA, Chan C (2010) Designing highly active siRNAs for therapeutic applications. *FEBS J* **277**: 4806-13
- Wang S, Liu P, Song L, Lu L, Zhang W, Wu Y (2011) Adeno-associated virus (AAV) based gene therapy for eye diseases. *Cell Tissue Bank* **12**: 105-10
- Warrington KH Jr, Herzog RW (2006) Treatment of human disease by adeno-associated viral gene transfer. *Hum Genet* **119**: 571-603
- Weiss ER, Hao Y, Dickerson CD, Osawa S, Shi W, Zhang L, Wong F (1995) Altered cAMP levels in retinas from transgenic mice expressing a rhodopsin mutant. *Biochem Biophys Res Commun* **216**: 755-61
- White E, Bienemann A, Sena-Esteves M, Taylor H, Bunnun C, Castrique E, Gill S (2011) Evaluation and optimization of the administration of recombinant adeno-associated viral vectors (serotypes 2/1, 2/2, 2/rh8, 2/9, and 2/rh10) by convection-enhanced delivery to the striatum. *Hum Gene Ther* **22**: 237-51
- Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75**: 855-862
- Wong, F. & Hao, Y (1992) *Invest Ophthalmol Vis Sci* **33**: 944 (abstr.)

Wright JF (2011) Adeno-associated viral vector manufacturing: keeping pace with accelerating clinical development. *Hum Gene Ther* **22**: 913-4

Wright JF, Le T, Prado J, Bahr-Davidson J, Smith PH, Zhen Z, Sommer JM, Pierce GF, Qu G (2005) Identification of factors that contribute to recombinant AAV2 particle aggregation and methods to prevent its occurrence during vector purification and formulation *Mol Ther* **12**: 171-8

Wright JF, Qu G, Tang C, Sommer JM (2003) Recombinant adeno-associated virus: formulation challenges and strategies for a gene therapy vector. *Curr Opin Drug Discov Devel* **6**: 174-8

Wu Z, Yang H, Colosi P (2010) Effect of genome size on AAV vector packaging. *Mol Ther*. **18**: 80-6

Xia H, Mao Q, Paulson HL, Davidson BL (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* **20**: 1006-10

Xiao X, Li J, Samulski RJ (1998) Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**: 2224-32

Yang G, Schmidt M, Yan Z, Lindbloom J, Harding T, Donahue B, Engelhardt J, Kotin R, Davidson B (2002b) Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. *J Virol* **76**: 7651-7660

Yang Y, Mohand-Said S, Danan A, Simonutti M, Fontaine V, Clerin E, Picaud S, Léveillard T, Sahel JA (2009) Functional cone rescue by RdCVF protein in a dominant model of retinitis pigmentosa. *Mol Ther* **17**: 787-95

Yang Z, Peachey NS, Moshfeghi DM, Thirumalaichary S, Chorich L, Shugart YY, Fan K, Zhang K (2002a) Mutations in the RPGR gene caused x-linked cone dystrophy. *Hum Mol Genet* **1**: 605-611

Yekta S, Shih IH, Bartel DP (2004) MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**: 594-596

Yi R, Doehle BP, Qin Y, Macara IG, Cullen BR (2005) Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *RNA* **11**: 220-6

Yi R, Qin Y, Macara I, Cullen B (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**: 3011-3016

Young JE, Vogt T, Gross KW, Khani SC (2003) A short, highly active photoreceptor-specific enhancer/promoter region upstream of the human rhodopsin kinase gene. *Invest Ophthalmol Vis Sci*. **44**: 4076-85

Yuan X, Naguib S, Wu Z (2011) Recent advances of siRNA delivery by nanoparticles. *Expert Opin Drug Deliv* **8**: 521-36

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**: 25-33

Zeng Y, Cai X, Cullen BR (2007) Use of RNA polymerase II to transcribe artificial microRNAs. *Cell* **130**: 101-12

Zeng Y, Cullen BR (2003) Sequence requirements for micro RNA processing and function in human cells. The nuclear RNase III Drosha initiates microRNA processing. *RNA* **9**: 112-23

Zeng Y, Cullen BR (2005) Efficient processing of primary microRNA hairpins by Drosha requires flanking nonstructured RNA sequences. *J Biol Chem*. **280**: 27595-603

Zeng Y, Wagner E, Cullen B (2002) Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell* **9**: 1327-1333

Zeng Y, Yi R, Cullen BR (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci U S A* **100**: 9779-84

Zhang Y, Ivanova E, Bi A, Pan ZH (2009) Ectopic expression of multiple microbial rhodopsins restores ON and OFF light responses in retinas with photoreceptor degeneration. *J Neurosci* **29**: 9186-96

Zhou H, Xia XG, Xu Z An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi (2005) *Nucleic Acids Res*. **33**: e62

Zhu L, Jang G, Jastrzebska B, Filipek S, Pearce-Kelling S, Aguirre G, Stenkamp R, Acland G, Palczewski K (2004) A naturally occurring mutation of the opsin gene (T4R) in dogs affects glycosylation and stability of the G protein-coupled receptor. *J Biol Chem* **279**: 53828-53839

Zimmerman AL, Wu S (2011) MicroRNAs, cancer and cancer stem cells. *Cancer Lett* **300**: 10-9

Zrenner E, Bartz-Schmidt KU, Benav H, Besch D, Bruckmann A, Gabel VP, Gekeler F, Greppmaier U, Harscher A, Kibbel S, Koch J, Kusnyerik A, Peters T, Stingl K, Sachs H, Stett A, Szurman P, Wilhelm B, Wilke R (2011) Subretinal electronic chips allow blind patients to read letters and combine them to words. *Proc Biol Sci* **278**:1489-97

Web resources

1. European Medicines Agency (www.ema.europa.eu)
2. <http://www.clinicaltrials.gov>
3. <http://www.ncbi.nlm.nih.gov/>
4. http://www.ualberta.ca/~stothard/javascript/codon_usage.html
5. RetNet (<http://www.sph.uth.tmc.edu/retnet/>)
6. World health organization (<http://www.euro.who.int/en/home>)
7. www.ebi.ac.uk/Tools/msa/clustalw2/

Chapter 9:Appendix

Section 9.0 Rhodopsin mRNA for human (*Homo sapiens*), pig (*Sus scrofa*) and mouse (*Mus musculus*)

> gi|169808383:96-1142 *Homo sapiens* rhodopsin (RHO), mRNA

ATGAATGGCACAGAAGGCCCTAACTTCTACGTGCCCTTCTCCAATGCGACGGGTGTGGTACGCAGCCCCCTTCGAG
TACCCACAGTACTACCTGGCTGAGCCATGGCAGTTCTCCATGCTGGCCGCTACATGTTTCTGCTGATCGTGCTG
GGCTTCCCCATCAACTTCCTCACGCTCTACGTACCGTCCAGCACAGAAGCTGCGCACGCCTCTCAACTACATC
CTGCTCAACCTAGCCGTGGCTGACCTCTTCATGGTCTTAGGTGGCTTCACCAGCACCCCTCTACACCTCTCTGCAT
GGATACTTCGTCTTTCGGGCCCCACAGGATGCAATTTGGAGGGCTTCTTTGCCACCCTGGGCGGTGAAATTGCCCTG
TGGTCCTTGGTGGTCTTGGCCATCGAGCGGTACGTGGTGGTGTGTAAGCCCATGAGCAACTTCCGCTTCGGGGAG
AACCATGCCATCATGGGCGTTGCCTTCACCTGGGTCTAGGCGTGGCCTGCGCCGACCCCCACTCGCCGGCTGG
TCCAGGTACATCCCCGAGGGCCTGCAGTGCTCGTGTGGAATCGACTACTACAGCTCAAGCCGGAGGTCAACAAC
GAGTCTTTTGTCTATCTACATGTTCTGTGGTCCACTTCACCATCCCCATGATTATCATCTTTTTCTGCTATGGGCAG
CTCGTCTTCACCGTCAAGGAGGCGCTGCCAGCAGCAGGAGTCAGCCACCACACAGAAGGCAGAGAAGGAGGTC
ACCCGCATGGTCTATCATGGTCTATCGCTTTCCTGATCTGCTGGGTGCCCTACGCCAGCGTGGCATTCTACATC
TTCACCCACCAGGGCTCCAACCTTCGGTCCCCTCTTCATGACCATCCCAGCGTTCTTTGCCAAGAGCGCCGCCATC
TACAACCCTGTCTATCTATATCATGATGAACAAGCAGTTCCGGAAGTGCATGCTCACCACCATCTGCTGCGGCAAG
AACCCACTGGGTGACGATGAGGCCTCTGCTACCGTGTCCAAGACGGAGACGAGCCAGGTGGCCCCGGCCTAA

>gi|47523517|ref|NM_214221.1| *Sus scrofa* rhodopsin (RHO), mRNA

ATGAATGGGACGGAGGGCCCCAACTTCTACGTGCCCTTCTCCAACAAGACGGGCGTGGTGCAGCCCCCTTTGAG
TATCCGCAGTACTACCTGGCTGAGCCATGGCAGTTCTCCATGCTGGCTGCCTACATGTTTCATGCTGATTGTGCTT
GGCTTCCCCATCAACTTCCTCACGCTCTACGTACCGTCCAGCACAGAAGCTGCGCACACCCCTCAACTACATC
CTTCTCAACCTGGCAGTGGCTGACCTCTTCATGGTCTTCGGAGGCTTCACCACCACCCTCTACACCTCTCTGCAT
GGATACTTCGTCTTTGGACCCACAGGATGCAACTTGGAGGGATTCTTTGCCACCCTGGGCGGTGAAATCGCCCTG
TGGTCCTTGGTGGTCTTGGCCATTGAACGGTACGTGGTGGTGTGTAAGCCTATGAGCAACTTCCGCTTCGGGGAG
AACCACGCCATCATGGGCTTGGCCCTCACCTGGGTCTAGGCTCTGGCCTGCGCTGCGCCCCCTCTCGTCGGCTGG
TCCAGGTACATCCCAGAAGGCCTGCAGTGCTCGTGCGGGATTGACTACTACACACTCAAGCCGGAGGTCAACAAT
GAGTCCTTCGTCTATCTACATGTTCTGTGGTCCACTTCTCCATCCCCCTTGGTCTATCATCTTCTTCTGCTATGGGCAG
CTGGTCTTCACAGTCAAGGAGGCTGCCGCCAGCAGCAGGAGTCAGCCACCACACAGAAGGCCGAGAAGGAGGTC
ACTCGCATGGTCTATCATGGTCTGTGGCTTTCCTGATCTGCTGGCTGCCCTACGCCAGTGTGGCATTCTACATC
TTCACCCACCAGGGCTCCGACTTTGGCCCCATCTTCATGACCATCCCAGCCTTCTTTGCCAAGAGCGCCTCCATC
TACAACCCCGTCTATCTATATCATGATGAACAAGCAGTTCCGGAAGTGCATGCTCACCACGCTCTGCTGTGGCAAG
AACCCACTGGGTGATGACGAGGCCTCCACCACCACTTCCAAGACAGAGACCAGCCAGGTGGCACCAGCCCTAAGCC
CCGCCAAGGACTCTGCGGCTCTGCGGCTCTGTAGGAGTCTCCCCACCCCTGCCCATCCCAGCCACAGCCACCCCA
CCAGGAGCCGCACCTGTCTAGAACCA

>gi|21717804:79-1125 *Mus musculus* rhodopsin (Rho), mRNA

ATGAACGGCACAGAGGGCCCCAATTTTTATGTGCCCTTCTCCAACGTACAGGCGTGGTGGGAGCCCCCTTCGAG
CAGCCGCAGTACTACCTGGCGGAACCATGGCAGTTCTCCATGCTGGCAGCGTACATGTTCTGCTCATCGTGCTG
GGCTTCCCCATCAACTTCCTCACGCTCTACGTACCGTACAGCACAGAAGCTGCGCACACCCCTCAACTACATC
CTGCTCAACTTGGCCGTGGCTGACCTCTTCATGGTCTTCGGAGGATTACCACCACCCTCTACACATCACTCCAT
GGCTACTTCGTCTTTGGGCCCCACAGGCTGTAATCTCGAGGGCTTCTTTGCCACACTTGGAGGTGAAATCGCCCTG
TGGTCCCTGGTGGTCTTGGCCATTGAGCGCTACGTGGTGGTCTGCAAGCCGATGAGCAACTTCCGCTTCGGGGAG
AATCACGCTATCATGGGTGTGGTCTTCACCTGGATCATGGCGTTGGCCTGTGCTGCTCCCCACTCGTTGGCTGG
TCCAGGTACATCCCTGAGGGCATGCAATGTTTCATGCGGGATTGACTACTACACACTCAAGCCTGAGGTCAACAAC
GAATCCTTTGTCTATCTACATGTTCTGTGGTCCACTTCACCATTCCATGATCGTCTATCTTCTTCTGCTATGGGCAG
CTGGTCTTCACAGTCAAGGAGGCGGCTGCCAGCAGCAGGAGTCAGCCACCCTCAGAAGGCAGAGAAGGAAGTC
ACCCGCATGGTTATCATCATGGTCTATCTTCTTCCTGATCTGCTGGCTTCCCTACGCCAGTGTGGCCTTCTACATC
TTCACCCACCAGGGCTCCAACCTTCGGCCCCATCTTCATGACTCTGCCAGCTTCTTTTGCTAAGAGCTCTTCCATC
TATAACCCGGTCTATCTACATCATGTTGAACAAGCAGTTCCGGAAGTGTATGCTCACCACGCTGTGCTGCGGCAAG
AATCCACTGGGAGATGACGACGCCTCTGCCACCGCTTCCAAGACGGAGACCAGCCAGGTGGTCCAGCCTAA

Section 9.1 Clustalw multiple sequence alignment between human, pig and mouse rhodopsin

Human RHO	ATGAATGGCACAGAAGGCCCTAACTTCTACGTGCCCTTCTCCAATGCGAC	
Pig RHO	ATGAATGGGACGGAGGGCCCCAACTTCTACGTGCCCTTCTCCAACAAGAC	
Mouse RHO	ATGAACGGCACAGAGGGCCCCAATTTTTATGTGCCCTTCTCCAACGTCAC	
	***** ** ** * ***** ** * * * ***** ***** **	
Human RHO	GGGTGTGGTACGCAGCCCCCTTCGAGTACCCACAGTACTACCTGGCTGAGC	
Pig RHO	GGGCGTGGTGCAGCCCCCTTTGAGTATCCGCAGTACTACCTGGCTGAGC	
Mouse RHO	AGGCGTGGTGGGAGCCCCCTTCGAGCAGCCGCAGTACTACCTGGCGGAAC	
	** ***** ** ***** ** * * * ***** ***** ** *	
Human RHO	CATGGCAGTTTCTCCATGCTGGCCGCCTACATGTTTCTGCTGATCGTGCTG	
Pig RHO	CATGGCAGTTTCTCCATGCTGGCTGCCTACATGTTTCTGCTGATTGTGCTT	
Mouse RHO	CATGGCAGTTTCTCCATGCTGGCAGCGTACATGTTTCTGCTCATCGTGCTG	
	***** ***** ** ***** **** * * *****	
Human RHO	GGCTTCCCCATCAACTTCCTCACGCTCTACGTACCGTCCAGCACAAGAA	
Pig RHO	GGCTTCCCCATCAACTTCCTCACGCTCTACGTACCGTCCAGCACAAGAA	
Mouse RHO	GGCTTCCCCATCAACTTCCTCACGCTCTACGTACCGTACAGCACAAGAA	
	***** ***** ** ***** *****	
Human RHO	GCTGCGCACGCCTCTCAACTACATCCTGCTCAACCTAGCCGTGGCTGACC	
Pig RHO	GCTGCGCACACCCCTCAACTACATCCTTCTCAACCTGGCAGTGGCTGACC	
Mouse RHO	GCTGCGCACACCCCTCAACTACATCCTGCTCAACTTGGCCGTGGCTGACC	
	***** ** ***** ***** * * * *****	
Human RHO	TCTTCATGGTCTTAGGTGGCTTCACCAGCACCTCTACACCTCTCTGCAT	siP1 site
(289-307)		
Pig RHO	TCTTCATGGTCTTCGGAGGCTTCACCACCACCTCTACACCTCTCTGCAT	
Mouse RHO	TCTTCATGGTCTTCGGAGGATTACACCACCCTCTACACATCACTCCAT	
	***** * * * * ***** ***** ** * * *	
Human RHO	GGATACTTCGTCTTCGGGCCACAGGATGCAATTGGAGGGCTTCTTTGC	
Pig RHO	GGATACTTCGTCTTTGGACCCACAGGATGCAACTGGAGGGATTCTTTGC	
Mouse RHO	GGCTACTTCGTCTTTGGGCCACAGGCTGTAATCTCGAGGGCTTCTTTGC	
	** ***** ** ***** ** * * * ***** *****	
Human RHO	CACCCTGGGCGGTGAAATTGCCCTGTGGTCCTTGGTGGTCCTGGCCATCG	
Pig RHO	CACCCTGGGCGGTGAAATCGCCCTGTGGTCCTTGGTGGTCCTGGCCATTG	
Mouse RHO	CACACTTGGAGGTGAAATCGCCCTGTGGTCCTTGGTGGTCCTGGCCATTG	
	*** ** * ***** ***** ***** ***** *	
Human RHO	AGCGGTACGTGGTGGTGTGTAAAGCCCATGAGCAACTTCGCTTCGGGGAG	
Pig RHO	AACGGTACGTGGTGGTGTGTAAAGCCTATGAGCAACTTCGCTTCGGGGAG	siP3 site
(499-518)		
Mouse RHO	AGCGCTACGTGGTGGTCTGCAAGCCGATGAGCAACTTCGCTTCGGGGAG	
	* * * ***** ** ***** ***** *****	
Human RHO	AACCAATGCCATCATGGGCGTTGCCTTCACCTGGGTTCATGGCGCTGGCCTG	
Pig RHO	AACCACGCCATCATGGGCGTTGGCCCTCACCTGGGTTCATGGCTCTGGCCTG	
Mouse RHO	AATCACGCTATCATGGGTGTGGTCTTCACCTGGATCATGGCGTTGGCCTG	
	** * * * ***** * * * ***** ***** *****	
Human RHO	CGCCGCACCCCCACTCGCCGGCTGGTCCAGGTACATCCCCGAGGGCCTGC	
Pig RHO	CGCTGCGCCCCCTCTCGTCGGCTGGTCCAGGTACATCCAGAAGGCCTGC	
Mouse RHO	TGCTGCTCCCCACTCGTTGGCTGGTCCAGGTACATCCCTGAGGGCATGC	
	** * * ***** ***** ***** ***** ** * * *	
Human RHO	AGTGCTCGTGTGGAATCGACTACTACAGCTCAAGCCGGAGGTCAACAAC	

Pig RHO AGTGCTCGTGCGGGATTGACTACTACACACTCAAGCCGGAGGTCAACAAT
Mouse RHO AATGTTTCATGCGGGATTGACTACTACACACTCAAGCCTGAGGTCAACAAC
* * * * *

Human RHO GAGTCTTTTGTCTATCTACATGTTTCGTGGTCCACTTCCACCATCCCCATGAT
Pig RHO GAGTCCTTCGTCTATCTACAIGTTTCGTGGTCCACTTTCATCCCCCTTGGT
site (618-636)
Mouse RHO GAATCCTTTTGTCTATCTACATGTTTCGTGGTCCACTTCCACCATTCCTATGAT
* * * * *

siP2

Human RHO TATCATCTTTTTCTGCTATGGGCAGCTCGTCTTCCACCGTCAAGGAGGCCG
Pig RHO CATCATCTTCTTCTGCTATGGGCAGCTGGTCTTCCACAGTCAAGGAGGCTG
Mouse RHO CGTCATCTTCTTCTGCTATGGGCAGCTGGTCTTCCACAGTCAAGGAGGCCG
* * * * *

Human RHO CTGCCCAGCAGCAGGAGTCAGCCACCACACAGAAGGCAGAGAAGGAGGTC
Pig RHO CCGCCCAGCAGCAGGAGTCAGCCACCACACAGAAGGCCGAGAAGGAGGTC
Mouse RHO CTGCCCAGCAGCAGGAGTCAGCCACCCTCAGAAGGCAGAGAAGGAAGTC
* * * * *

Human RHO ACCCGCATGGTCATCATCATGGTCATCGCTTTCCTGATCTGCTGGGTGCC
Pig RHO ACTCGCATGGTCATCATCATGGTCGTGGCTTTCCTGATCTGCTGGCTGCC
Mouse RHO ACCCGCATGGTTATCATCATGGTCATCTTCTTCTGATCTGCTGGCTTCC
* * * * *

Human RHO CTACGCCAGCGTGGCATTCTACATCTTCAACCACCAGGGCTCCAATTTCG
Pig RHO CTACGCCAGTGTGGCATTCTACATCTTCAACCACCAGGGCTCCGACTTTG
Mouse RHO CTACGCCAGTGTGGCCTTCTACATCTTCAACCACCAGGGCTCCAATTTCG
* * * * *

Human RHO GTCCCATCTTCATGACCATCCCAGCGTTCTTTGCCAAGAGCGCCGCCATC
Pig RHO GCCCCATCTTCATGACCATCCCAGCCTTCTTTGCCAAGAGCGCCTCCATC
Mouse RHO GCCCCATCTTCATGACTCTGCCAGCTTCTTTGCTAAGAGCTCTTCCATC
* * * * *

Human RHO TACAACCCTGTCATCTATATCATGATGAACAAGCAGTTCCGGAAGTGCAT
Pig RHO TACAACCCCGTCATCTATATCATGATGAACAAGCAGTTCCGGAAGTGCAT
Mouse RHO TATAACCCCGTCATCTACATCATGTTGAACAAGCAGTTCCGGAAGTGTAT
* * * * *

Human RHO GCTCACCACCATCTGCTGCGGCAAGAACCCACTGGGTGACGATGAGGCCT
Pig RHO GCTCACCACGCTCTGCTGTGGCAAGAACCCACTGGGTGATGACGAGGCCT
Mouse RHO GCTCACCACGCTGTGCTGCGGCAAGAATCCACTGGGAGATGACGACGCCT
* * * * *

Human RHO CTGCTACCGTGTCCAAGACGGAGACGAGCCAGGTGGCCCCGGCCTAA---
Pig RHO CCACCACCACTTCCAAGACAGAGACCAGCCAGGTGGCACCAGCCTAAGCC
Mouse RHO CTGCCACCGCTTCCAAGACGGAGACCAGCCAGGTGGCTCCAGCCTAA---

Section 9.2 The region of pSuper.GFP/Neo sequence used to clone shP3 (Starting from the H1 promoter)

EcoR1 site (Green), H1 promoter (Red), BglII site (Blue), HindIII site (Purple)

ACCCGGTGAATTCGAACGCTGACGTCATCAACCCGCTCCAAGGAATCGCGGGCCAGTGTCTACTA
GGCGGGAACACCCAGCGCGCGTGCCTTGGCAGGAAGATGGCTGTGAGGGACAGGGGAGTGGCGC
CCTGCAATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAATGTCTTTGGATT
TGGGAATCTTATAAGTTCTGTATGAGACCACAGATCCCCAGCTATCGATACCGTCGACCTCGAG
GGGGGGCCCGGTACCCAGCTTTTGTTCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATG
GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAG
CATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACT
GCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAG
AGGCGGTTTTCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTGTTTCG
GCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAA
CGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGCT
GGCGTTTTTCCATAGGCTCCGCCCTTACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTG
GCGAAACCCGACAGGACTATAAAGATACCAAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCCTCTCC
TGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGGCGCTTTC
TCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTGCTTCGCTCCAAGCTGGGCTGTGTGCA
CGAACCCCCCGTTTACGCCCCGACCGCTGCGCCTTATCCGGTAACCTATCGTCTTGAGTCCAACCCGGT
AAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGG
CGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTAT
CTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAAC
CACCGCTGGTAGCGGTGGTTTTTTTTTGTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCA
AGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAACTCACGTTAAGGGAT
TTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAA
ATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACC
TATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTAC
GATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGC
TCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTT
ATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCCGCCAGTTAATAG
TTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTC
ATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGT
TAGCTCCTTCGGTCTCCGATCGTTGTGAGAAGTAAGTTGGCCGAGTGTTATCACTCATGGTTAT
GGCAGCACTGCATAATTCTTACTGTATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTA
CTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACG
GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAACCGTTCTTCGGGGCG
AAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTG
ATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC
AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTG
AAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACA
AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

Section 9.3 Region of pSuper with shP3

EcoR1 site (Green)

H1 promoter (Red)

BglII site (Blue)

shP3 sequence (Yellow)

HindIII site (Purple)

shP3 is cloned between *BglII* and *Hind III*

CTTGGGAAAAGCGCCTCCCCTACCCGGTAGAATTCGAACGCTGACGTCATCAACCCGCTCCAAGGA
ATCGCGGGCCCAAGTGTCACTAGGCGGGAACACCCAGCGCGCGTGCGCCCTGGCAGGAAGATGGCTG
TGAGGGACAGGGGAGTGGCGCCCTGCAATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATA
AACGTGAAATGTCTTTGGATTTGGGAATCTTATAAGTTCTGTATGAGACCACAGATCCCCGGTACG
TGGTGGTGTGTAATCAAGAGATTACACACCACCACGTACCTTTTTAGCTTATCGATACCGTCGA
CCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGT
AATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCACACAACATACGAG
CCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGC
GCTCACTGCCCCGCTTTCCAGTCGGGAAACCTGTGCGTCCAGCTGCATTAATGAATCGGCCAACGCG
CGGGGAGAGGCGGTTTGCCTATTGGGCGCTCTTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTGCGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAG
GGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTC
AGAGGTGGCGAAACCCGACAGGACTATAAAGATAACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC
GCTCTCCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGG
CGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCCGTTAGGTGCTTCGCTCCAAGCTGGGCT
GTGTGCACGAACCCCCCGTTTACGCCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCA
ACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTAT
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCA
AACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTGTTGCAAGCAGCAGATTACGCGCAGAAAAAAG
GATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTT
AAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAA
GTTTTAAATCAATCTAAAGTATATATGAGTAA

Section 9.4 pAAV.CMV.EGFP

AAV-2 ITRS (Red), SRSR-H1-shP3-SRSF fragment, CMV promoter (Purple), EGFP (Green), HindIII (yellow), NsiI (Blue)

CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCGGGCAAAG
CCCCGGGCGTCGGGCGACCTTTGGTCGCCCCGGCCTCAGTGAGCGAGCGAGC
GCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCTGCGGCCGAT
CTCGAGCTCAAGCTTATGCAATTAGTTATT
GAAATAGTAATCAATTACGGGGTCATTAGTTTCATAGCCCATATATGGAG
TTCCGCGTTACATAACTTACGGTAAATGGCCCCGCTGGCTGACCGCCCAA
CGACCCCCCGCCCATTTGACGTCAATAATGACGTATGTTCCCATAGTAACGG
CAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACT
GCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTAT
TGACGTCAATGACGGTAAATGGCCCCGCTGGCATTATGCCCAGTACATGA
CCTTATGGGACTTTCTTACTTGGCAGTACATCTAOGTATTAGTCATCGCT
ATTACCATGGTGTATGUGGTTTTTGGCAGTACATCAATGGGCGTGGATAGCG
GTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTTGACGTCAATGGCA
GTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCTGAACAA
TCCGCCCATTTGACGCAAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTA
TATAAGCAGAGGTGGTTTTAGTGAACCGTCAATCCGCTAGCGGATCCACC
GGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGC
CCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTACGCGTG
TCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTT
CATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCCACCCTCGTGACCA
CCCTGACCTACGGCGTGCAGTGCTTACGCCGTACCCCGACCACATGAAG
CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCG
CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGA
AGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC
TTCAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACCTACAA
CAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGG
TGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCC
GACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCC
CGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACG
AGAAGCGCGATCACATGGTCTGCTGGAGTTCGTGACCGCCGCCGGGATC
ACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCGACTCTAGATC
ATAATCAGCCATACCACATTTGTAGAGTTTTTACTTGCTTTAAAAAACCT
CCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTG
TTAACTTGTTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATC
ACAAATTTACAAATAAAGCATTTTTTTTCACTGCATTCTAGTTGTGGTTT
GTCCAAACTCATCAATGTATCTTAAGGCCGAGGAACCCC TAGTGATGGA
GTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGAC
CAAAGGTCGCCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAG
CGAGCGCGCAGCTGCCTGCAGGGGCGCCTGATGCGGTATTTTCTCCTTAC
GCATCTGTGCGGTATTTACACCGCATACGTCAAAGCAACCATAGTACGC
GCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCG

Section 9.5 pAAV.shP3.CMV.EGFP

AAV-2 ITRS (Red), SRSR-H1-shP3-SRSF fragment (Light Blue [H1-shP3 underlined]), CMV promoter (Purple), EGFP (Green)

CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCG
GCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACCTCCATCACTAGGGGTTCTCTGCGGCCGATCTCGAGCTCA
AGCTGGGGCCCTCTAGTAAGTGTGACCACAGAAGGTAACAGAGATGCCAACAGTGCTACCAAGGCAGTACCCTGGACACG
GGGAGATAAGCAAGCTCGACCCACCCATCACCCCTGGCTTTGGGCTTTGCTACACCCACTTCTGTTTCCTCCTGTATCCAC
TGGAACATCTCTAGGTCCCAGCCCGGAGACCCTGGCTCTGGTCTGGCTCTCCCATATCTCTAGAATTGGAACGCTGAC
GTCATCAACCCGCTCCAAGGAATCGCGGGCCAGTGTCCTAGGCGGGGAACACCCAGCGCGCGTGGCCCTGGCAGGAAG
ATGGCTGTGAGGGACAGGGGAGTGGCGCCCTGCATATTTGCATGTGCGTATGTGTTCTGGGAAATCACCATAAACGTGAA
ATGTCTTTGGATTGGAATCTTATAAGTTCTGTATGAGACCACAGATCCCCGGTACGTGGTGGTGTGTAATTCAAGAGA
TTACACACCACCACGTACCTTTTTAAGCTCTAGAGGGCCCTATACTCGAGTAGATGTGATTTTTGCCTGCTCCCTGCCTC
AGTTTTCTCAGCTAACAGGCTCTGTTGCTTCTCGAAAGAGCCTCATTGACCTTCCAGTTAAGGGAGAGAAAATGTTTGG
AAACACCTGGCGTAGCATAGTGGTCCGTGCCGTGACTTAACTAGACATTAGATTATTGAAAGTAATCAATTACGGGGT
CATTAGTTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCTGGCTGACCCGCCAACGAG
CCCCGCCCATTTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGA
GTATTTACGGTAAACTGCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACG
GTAAATGGCCCGCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCTACTTGGCAGTACATCTACGTATTAGTC
ATCGCTATTACCATGGTIGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCGA
TCTCCACCCCATTTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTGCTAACAACTCC
GCCCATTTGACGCAATGGGCGGTAGGCGTGTACGGTGGGAGGTCATATATAAGCAGAGCTGGTTTAGTGAACCGTCAATC
CGCTAGCGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTTCGAG
CTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCC
GAAGTTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCACCCCTCGTGACCACCCCTGACCTACGGCGTGCAGTGCT
TCAGCCGCTACCCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCAAC
ATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCG
GCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACATAACAGCCACAACGCTC
ATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAG
CTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCA
GTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCAGATGGTCTGCTGGAGTTGCTGACCGCCGCCGGGATCACTC
TCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTAC
TTGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATT
GCAGCTTATAATGTTTACAAATAAAGCAATAGCATC
ACAAATTTACAAATAAAGCATTTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACCTCATCAATGTATCTTAA
GGCCGCAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGAC
CAAAGGTCGCCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAG
CGAGCGCGCAGCTGCCTGCAGGGGCGCTGATGCGGTATTTTCTCCTTAC

Section 9.6 pAAV.miRP3-30a.CMV.EGFP
AAV-2 ITRS (Red), SRSF-miRP3-30a-mU6-SRSR fragment (Light Blue [mU6-miRP3-30a underlined]), CMV promoter (Purple), EGFP (Green)

CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTC
GCCCCGCCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCTCTGCGGCCG
GGCCGATCTCGAGCTCAAGCTTATGTCTAGTTAAGTCACAGGCACGGACCCTATGCTACGCCAGGTGTTTCCAA
ACATTTTCTCTCCCTTAACTGGAAGGTCAATGAGGCTCTTTTCGAGAAGCAACAGAGCCTGTTTAGCTGAGAAAAC
TGAGGCAGGGAGCAGGCAAAAATCACATCTACTCGAGTATAGGGCCCC**TCTAGATTATCATGTCTGGATCTCCCCG**
CTGTGGCGGCCGCTCTAGTAGGCGAGGTACGTGGTGGTGTGTAACCCATCTGTGGCTTTACAGTTACACACCACC
ACGTACCGCGCTCACTCGAGTTTAAACGGTACCATCTGACGGTTCATAAACGCAAAACAAGGCTTTTCTCCAAGG
GATATTTATAGTCTCAAAACACACAATTACTTTACAGTTAGGGTGAGTTTCCTTTTGTGCTGTTTTTTAAATAA
TAATTTAGTATTTGTATCTCTTATAGAAATCCAAGCCTATCATGTAAAATGTAGCTAGTATTAAGAAGACAGAT
TATCTGTCTTTTATCGCACATTAAGCCTCTATAGTTACTAGGAAATATTATATGCAAATTAACCGGGGCAGGGGA
GTAGCCGAGCTTCTCCCAAGTCTGTGCGAGGGGGCGCGCGGCCCTAGAGATG**TCTAGAG**ATATGGGAGAGC
CAGGACCAGAGCCAGGGTCTCCGGGCTGGGACCTAGAGATGTTTCCAGTGGATACAGGAGGAAACAGAAGTGGGT
GTAGCAAAGCCCCAAAGCCAGGGTGATGGGTGGTTCGAGCTTGCTTATCTCCCCGTGTCCAGGGTACTGCCTTGGT
AGCACTGTTGGGCATCTCTGTTACCTTCTGTGGTCACACTTACTAGAGGGCCCCTTAGTTATTAA**AGTAATCAAT**
TACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCGGCGTTACATAACTTACGGTAAATGGCCCCGCTGGCTG
ACCGCCCCAAGCAGCCCGGCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCCAATAGGGACTTTCCA
TTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTAG
GCCCCCTATTGACGTCAATGACGGTAAATGGCCCCGCTGGCATTATGCCCAGTACATGAUCTTATGGGACTTTCC
TACTTGGCAGTACATCTACGTATTAGTTCATCGCTATTACCATGGTGATGCGGTTTTTGGCAGTACATCAATGGGG
TGGATAGCGGTTTGACTCAGGGGGATTTCCAAAGTCTCCACGCCAATTGACGTCAATGSGAGTTTGTTTTGGCAOCC
AAATCAACGGGACTTTCCAAAATGTGTAACAACCTCCGCCCATTTGACGCCAATGGCGGCTAGGCGGTGACGGT
GGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACGGTCA**ATCCGCTAGCGGATCCACCGGTGCGCCACC**ATGGTG
AGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAG
TTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGC
AAGCTGCCCCGTGCCCTGGCCACCCTCGTGACCACCCTGACCTACGGCGTGCACTGCTTCAGCCGCTACCCCGAC
CACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCAACCATCTTCTTCAAG
GACGACGGCAACTACAAGACCCGCGCCGAGGTGA
AGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGG
GGCACAAGCTGGAGTACAACACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGG
TGAATTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCA
TCGGCGACGGCCCCGTGCTGCTGCTGCCCCACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACG
AGAAGCGCGATCACATGGTCCTGCTGGAGTTCTGTGACCGCCGCCGGGATC
ACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAG
AGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTG
TTAATTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATT
TTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGGCCGAGGAACCC**TAGTGATGGA**
GTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGAC
CAAAGGTCGCCCGACGCCCGGCTTTGCCGGGGCGGCCCTCAGTGAGCGAG
CGAGCGCGCAGCTGCCTGCAG

Section 9. 7 MCS of the pcDNA6.2-GW/EmGFP-miR validated vector from (invitrogen.com)

Note this is NOT the full sequence, it is shown from the CMV promoter through the MCS

CMV promoter (Purple)

EmGFP (Green)

5' miR-155 flanking region (Yellow)

3' miR-155 flanking region (Red)

```
GTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATA
TGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCGCCCAT
TGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGG
AGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTG
ACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAOCTTATGGGACTTTCTACTTT
GGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGTATGCGGTTTTGGCAGTACATCAATGGGC
GTGGATAGCGGTTTTGACTCAAGGGGATTTCCAAATGTCTGAACAACTCCGCCCCATTGACGCAAATGGGC
GGCACCAAAATCAACGGGACTTTCCAAATGTCTGAACAACTCCGCCCCATTGACGCAAATGGGC
GGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGC
TTATCGAAATTAATACGACTCACTATAGGGAGTCCCAAGCTGGCTAGTTAAGCTATCAACAAGTTTGTA
CAAAAAAGCAGGCTTTAAACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCT
GGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCCACCCTCGT
GACCACCTTCACCTACGGCGTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCAGCACTTCTT
CAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAA
GACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTT
CAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACCTACAACAGCCACAAGGTCTATATCAC
CGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTCAAGACCCGCCACAACATCGAGGACGGCAGCGT
GCAGCTCGCCGACCCTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCA
CTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCTGCTGGA
GTTCTGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAGCTAAGCACTTCGTGGC
CGTCGATCGTTTTAAAGGGAGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGCTGATGCT
CAGGACACAAGGCCTGTACTAGCACTCACATGGAACAAATGGCCAGATCTGGCCGCACTCGAGATAT
CTAGACCCAGCTTTCTTGTAACAAGTGGTTGATCTAGAGGGCCCGCGTTTCGTGATGGGGGAGGCTAA
CTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGGCAATAAAAAGACAGAATAAAA
CGCACGGGTGTTGGGTGCTTTGTTTATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTGATACC
CCACCGTGACCCCAATTGGGGCCAATA
CGCCCGCGTTTTCTTCTTTTCCCCACCCCAACCCCAAGTTCGGGTGAAGGC
CCAGGGCTCGCAGCCAAC
```

Section 9.8 Region of pcDNA6.2-GW/EmGFP with miRP3-155

CMV promoter (Purple), EmGFP (Green), 5' miR-155 flanking region (Yellow), miRP3-155 (Grey)
3' miR-155 flanking region (Red)

GTGACATTGATTATTGACTAGTTATTAAATAGTAATCAATTACGGGGTCATTAGTTTCATAGCCCAT
ATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGOCCAACGACCCCG
GCCCATTTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTC
AATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA
CGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCAGTACATGACCTTAT
GGGACTTTCTTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTT
GGCAGTACATCAATGGGCGTGGATAGCGTTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTG
ACGTCAATGGGAGTTTTGTTTTGGCACCA
AAATCAACGGGGACTTTCCAAAATGTGTAACAACCTCCGCCCATTTGACGCAATGGGCGGTAGGGG
TGTACGTTGGGAGSTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCT
TATCGAAATTAATACGACTCACTATAGGGAGTCCCAAGCTGGCTAGTTAAGCTATCAACAAGTTTG
TACAAAAAAGCAGGCTTTAAACCATGGTGAGCAAGGGCGAGGAGCTGTTACCCGGGGTGGTGCCC
ATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTTACGCGTGTCCGGCGAGGGCGAGGGC
GATGCCACCTACGGCAAGCTGACCTGAAGTTTATCTGCACCACCGGCAAGCTGCCCGTGCCCTGG
CCACCCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAG
CAGCACGACTTCTTCAAGTCCGCCATGC
CCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCGG
AGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG
ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACATAACAGCCACAAGGTCTATATCACCGCCG
ACAAGCAGAAGAACGGCATCAAGGTGAACCTCAAGACCCGCCACAACATCGAGGACGGCAGCGTGC
AGCTCGCCGACCACTACCAGCAGAACACCCCATCGGGACGGCCCCGTGCTGCTGCCCGACAACC
ACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGC
TGGAGTTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAGCTAAGCACT
TCGTGGCCGTCGATCGTTTTAAAGGGAGG
TAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGCTGTATGCTGCTTACACACCACCACGT
ACCGTTTTTGGCCACTGACTGACCGGTACGTTGGTGTGTAAGCAGGACACAAGGCCTGTTACTAGC
ACTCACATGGAACAAATGGCCAGATCTGGCCGCACTCGAGATATCTAGACCCAGCTTTCTTGTAC
AAAGTGGTTGATCTAGAGGGCCCGCGTTTCGTGATGGGGGAGGCTAACTGAAACACGGAAGGAGA
CAATACCGGAAGGAACCCGCGCTATGACGGCAATAAAAAGACAGAATAAAACGCACGGGTGTTGGG
TCGTTTGTTT
ATAAACGCGGGGTTTCGGTCCCAGGGCTGGCACTCTGTGATACCCACCGTGACCCATTGG

Section 9.9 pAAV. CMV. EGFPb

AAV-2 ITR (Red), CMV promoter (Purple), EGFP (Green), *Bgl*III (Blue)

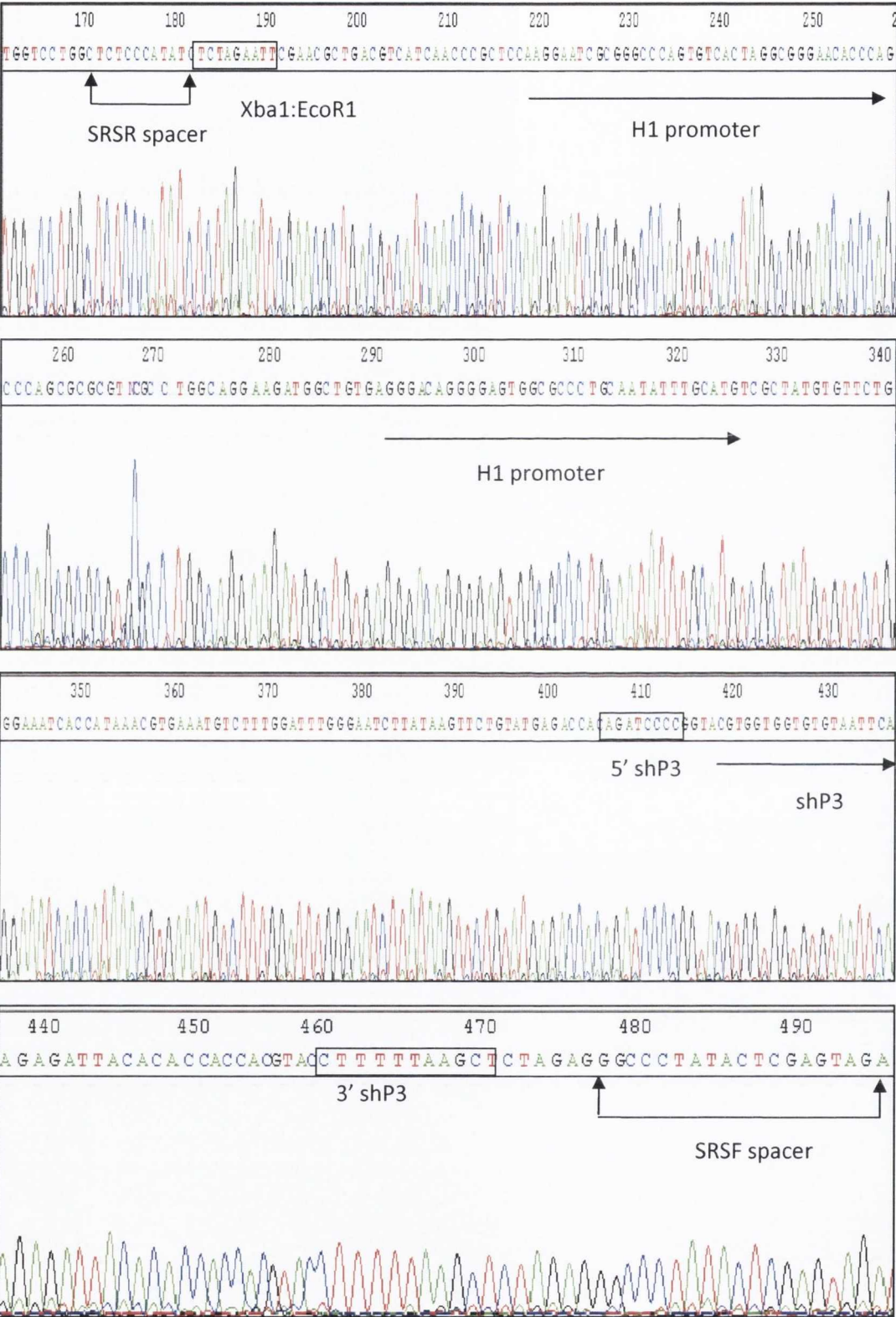
CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAG
CCCCGGCGTCGGGCGACCTTTGGTCGCCCCGGCCTCAGTGAGCGAGCGAGC
GCGCAGAGAGGGAGTGGCCAACCTCCATCACTAGGGGTTCTGCGGCCGC
CGCGTGGAGCTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTTCAT
AGGCCATATATGGAGTTCCGGCTTACATAACTTACGGTAAATGGCCCGCC
TGGCTGACCGGCCAACGACCCCGGCCATTGACGTCAATAATGACGTATG
TTCCCATAGTAACGTCAATAGGGACTTTCCATTGACGTCAATGGGTGGAG
TATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCC
AAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATT
ATGCCCAGTACATGACCTTATGGGACTTTCTTACTTGGCAGTACATCTAC
GTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTTGGCAGTACATCAA
TGGGCGTGGATAGCGGTTTTGACTCACGGGGATTTCGAAGTCTCCACCCCA
TTGACGTCAATGGGAGTTTGTGTTTGACCAAAAATCAACGGGACTTTCCAA
AATGTGTAACAACCTCCGCCCATTTGACGCAATGGGCGGTAGGCGTGT
CGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGC
CTGGAGAGGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGAC
EATCCAGCCTCCGCGGATTCTCGAACATCGATTGAATTCCCGGGGATCG
TCTAGAGTCGACCTGCAGAAgctGATCCACCGGTGCCACCatgGTGAGC
AAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGA
CGGCGACGTAAACGGCCACAAGTTTACGCGTGTCCGGCGAGGGCGAGGGCG
ATGCCACCTACGGCAAGCTGACCCTGAAGTTTATCTGCACCACCGGCAAG
CTGCCCCGTGCCCTGGCCCAACCTCGTGACCACCTGACCTACGGCGTGCA
GTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAGACTTCTTCAAGT
CCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC
GACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCT
GGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACA
TCTTGGGGCACAAAGCTGGAGTACAACACAACAGCCACAACGTCTATATC
ATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTTCAAGATCCGCCA
CAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACA
CCCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGC
ACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGT
CCTGCTGGAGTTCTGTGACCGCCGCGGGATCAACTCTCGGCATGGACGAG
CTGTACAagtaaaGCggccAGCTTGCCTCGAGCAGCGCTGCTCGAGATCT
ACGGGTGGCA
TCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTC
CAGTGCCCAACAGCCTTGTCTTAATAAAATTAAGTTGCATCATTTTGTCT
GACTAGGTGTCTTCTATAATATTATGGGGTGGAGGGGGGTGGTATGGAG
CAAGGGGCAAGTTGGGAAGACAACCTGTAGGGCCTGCGGGGTCTATTGGG
AACCAAGCTGGAGTGCAGTGGCACAATCTTGGCTCACTGCAATCTCCGCC
TCTTGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTTGTTGGGATT
CCAGGCATGCATGACCAGGCTCAGCTAATTTTTGTTTTTTGGTAGAGAC
GGGTTTTACCATATTGGCCAGGCTGGTCTCCAACCTCTAATCTCAGGTG
ATCTACCCACCTTGGCCTCCCAAATTGCTGGGATTACAGGCGTGAACCAC
TGCTCCCTTCCCTGTCTTCTGATTTTGTAGGTAACCACGTGCGGACCGA
GCGGCCGAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCG
CTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCGCGGGC
TTTGCCCGGGCGGCCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGGG
G

Section 9.10 pAAV.miRP3-155

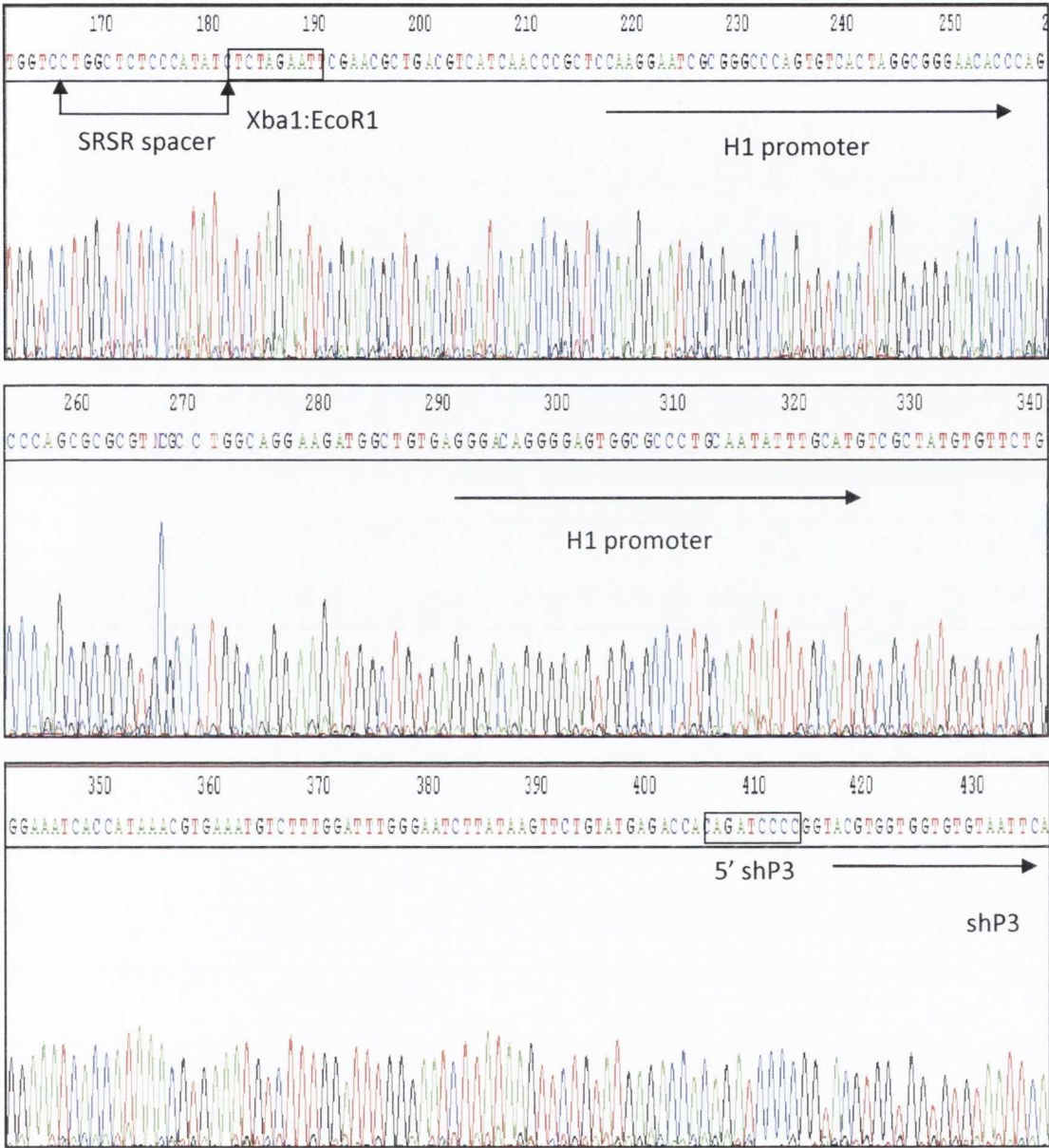
AAV-2 ITR (Red), CMV promoter (Purple), EGFP (Green), BglIII-BamHI destroyed site (Yellow), miRP3-155 (Grey), BglIII (Blue)

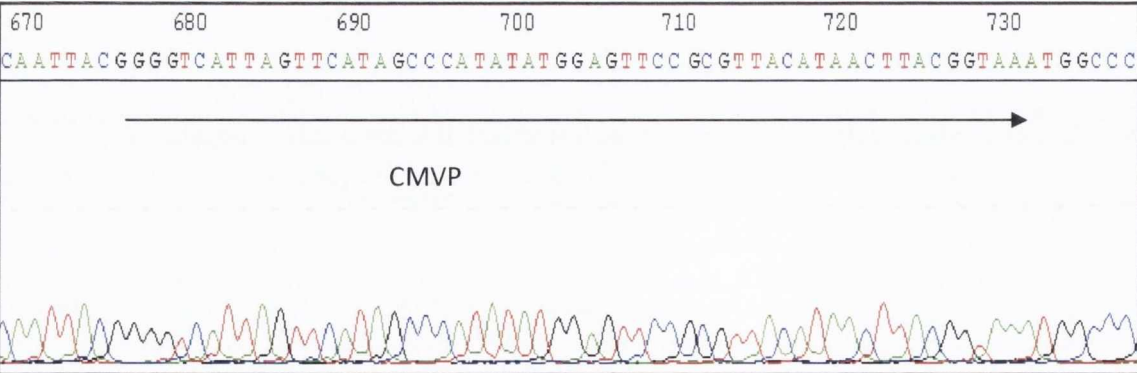
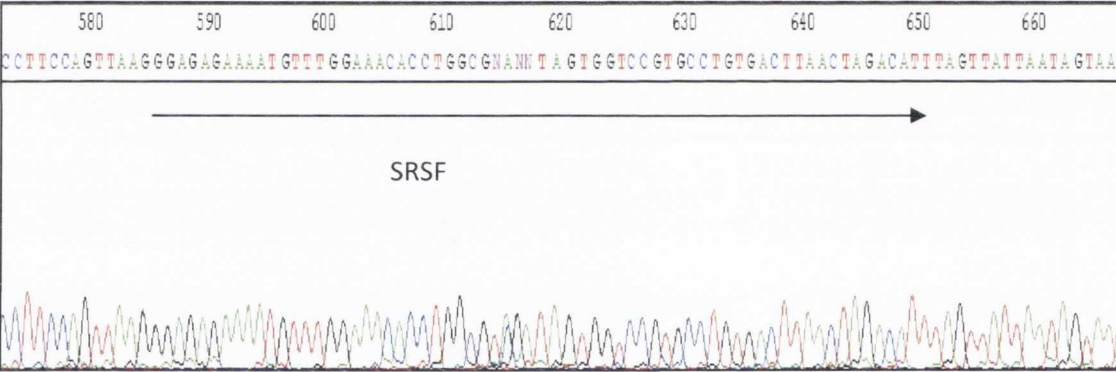
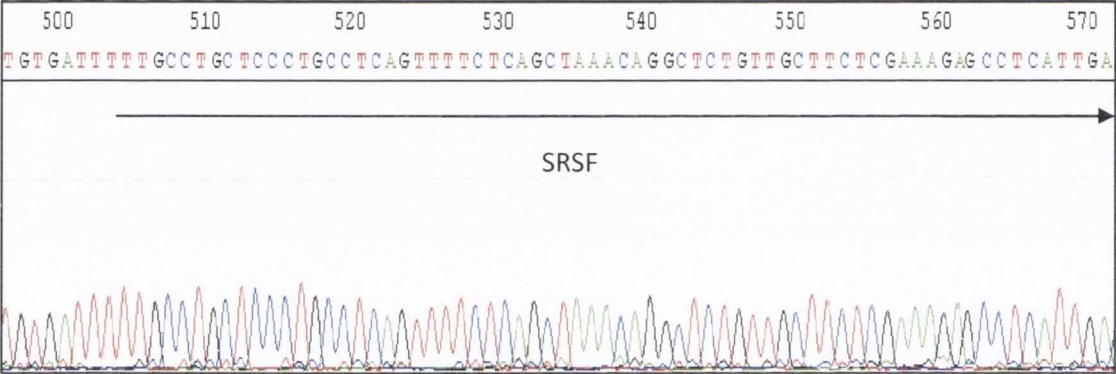
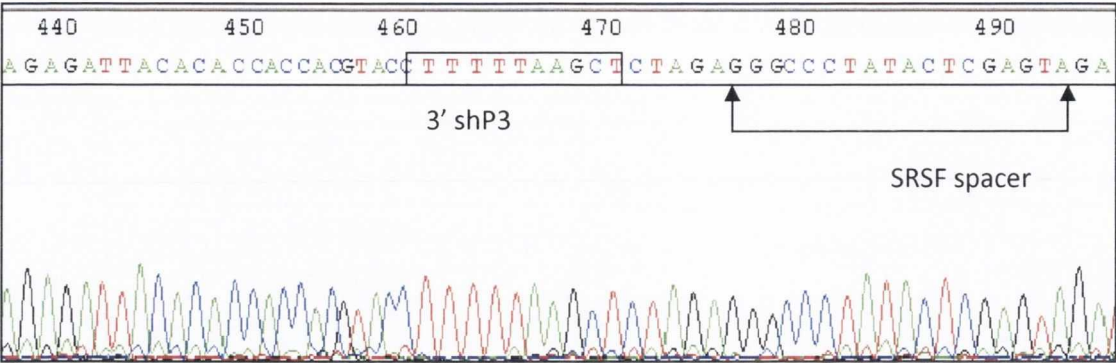
CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCGGGCAAAG
CCCCGGGCGTCGGGCGACCTTTGGTCGCCCCGGCCTCAGTGAGCGAGCGAGC
GCGCAGAGAGGGAGTGGCCAACCTCCATCACTAGGGGTTCTGCGGCCGC
CGCGTGGAGCTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTTCA
AGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCC
TGGCTGACCGCCCAACGACCCCGGCCATTGACGTCAATAATGACGTATG
TTCCCATAGTAACGTCAATAGGGACTTTCCATTGACGTCAATGGGTGGAG
TATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCC
AAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATT
ATGCCCAGTACATGACCTTATGGGACTTTCCCTACTTGGCAGTACATCTAC
GTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAA
TGGGCGTGGATAGCGGTTTTGACTCACGGGGATTTCCTAAGTCTCCACCCCA
TTGACGTCAATGGGAGTTTTGTTTTGCACCAAAATCAACGGGACTTTCCAA
AATGTCGTAACCACTCCGCCCATTTGACGCAAAATGGGCGGTAGGCGTGTA
CGTGGGAGGCTCTATATAAGCAGAGCTCGTTTTAGTGAACCGTCAGATCGC
CTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACC
GATCCAGCCTCCGCGGATTTCGGAACATCGATTGAATTCGCCGGGGATCC
TCTAGAGTCGACCTGCAGAagctGATCCACCGGTGCGCACCATgGTGAGC
AAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGA
CGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCG
ATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAG
CTGCCCCTGCCCCTGGCCCAACCCTCGTGACCACCCTGACCTACGGCGTGCA
GTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGT
CCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC
GACGGCAACTACAAGACCCGCGCGGAGGTGAAGTTCGAGGGCGACACCCT
GGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACA
TCTGGGGCACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATC
ATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAGTTCAGATCCGCCA
CAACATCGAGGACGGCAGCTGCAGCTGCCGACCCTACCGAGCAGAACA
CCCCATCGGGCAGCGGCCCGTGTGCTGCTGCCGACAACCCTACCTGAGC
ACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGT
CCTGCTGGAGTTCGTGACCGCCCGGGATCAACTCTCGGCATGGACGAG
CTGTACAagtaaaGCggccAGCTTGCCTCGAGCAGCGCTGCTCGAGAGAT
CCTGGAGGCTTGCTGAAGGCTGTATGCTGCTTACACACCACCACGTACCG
GTTTTGGCCACTGACTGACCGGTACGTTGGTGTGTAAGCAGGACACAAGG
CCTGTTACTAGCACTCACATGGAACAAATGGCCCAGATCTACGGGTGGCA
TCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTC
CAGTGCCCACCAGCCTTGTCTAATAAAATTAAGTTGCATCATTTTGTCT
GACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGTGGTATGGAG
CAAGGGGCAAGTTGGGAAGACAACCTGTAGGGCCTGCGGGGTCTATTGGG
AACCAAGCTGGAGTGCAGTGGCACAATCTTGGCTCACTGCAATCTCCGCC
TCTGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTTGTTGGGATT
CCAGGCATGCATGACCAGGCTCAGCTAATTTTTGTTTTTTGGTAGAGAC
GGGTTTTACCATAATTGGCCAGGCTGGTCTCCAACCTCTAATCTCAGGTG
ATCTACCCACCTTGGCCTCCCAAATTGCTGGGATTACAGGCGTGAACCAC
TGCTCCCTTCCCTGCTTCTGATTTTGTAGGTAACCACGTGCGGACCGA
GCGGCCGAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCG
CTCGCTCGCTCACTGAGGCCGGGCGACCAAAAGGTGCGCCGACGCCCGGGC
TTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG

Section 9.11 Sequencing confirming the successful cloning of shP3 into pAAV.rP3 between the correct spacers SRSR and SRSF

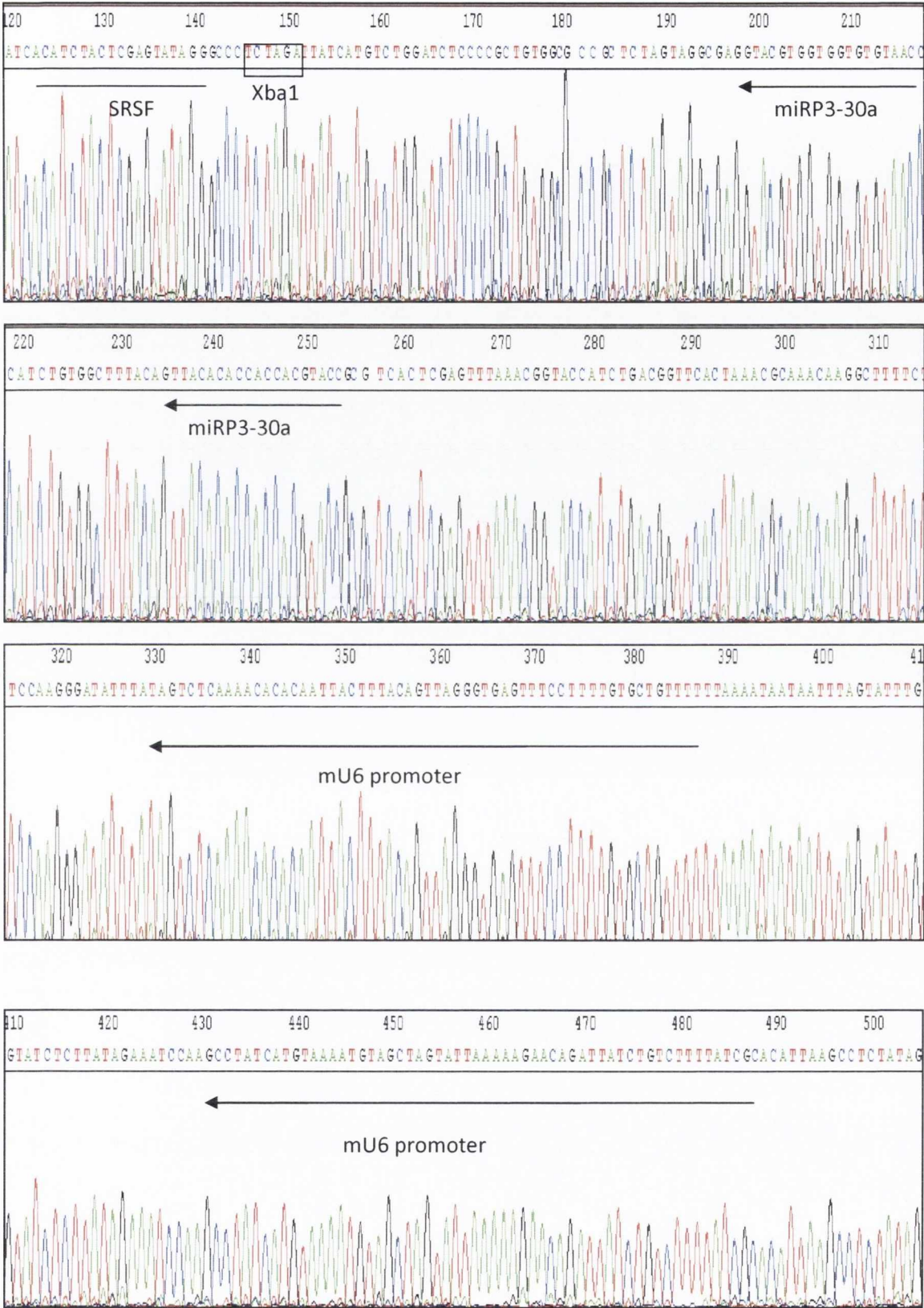


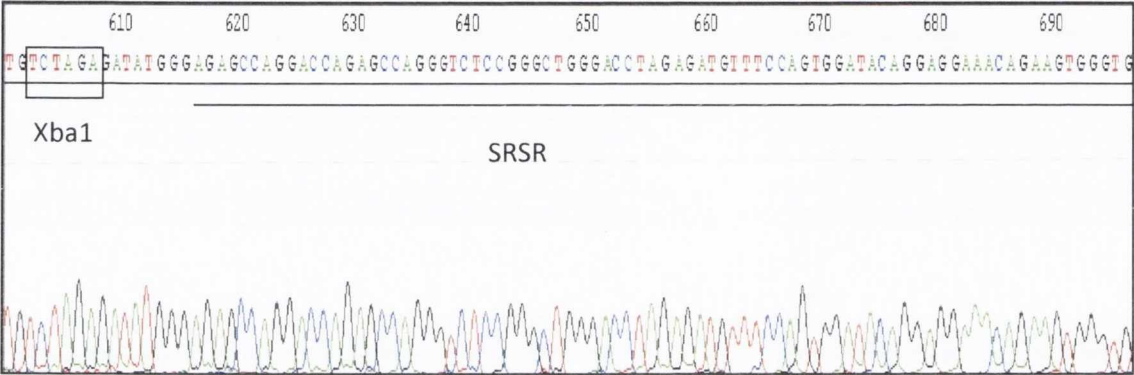
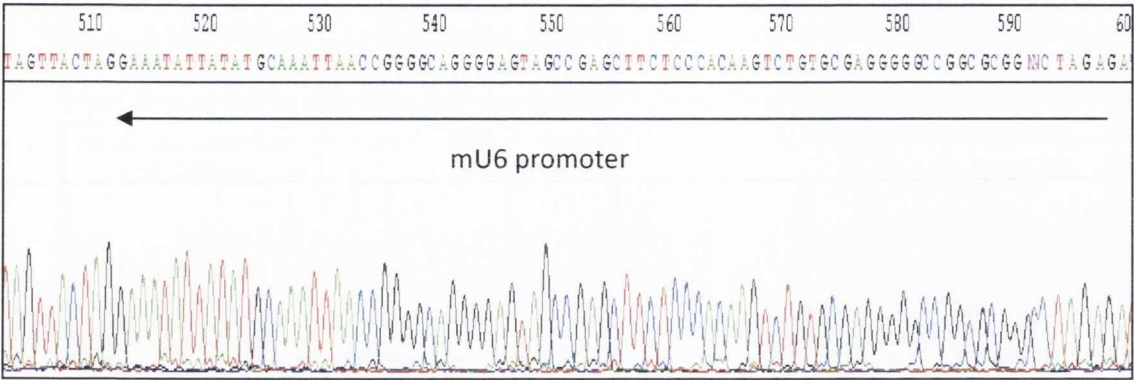
Section 9.12 Sequencing confirming the successful cloning of shP3 into pAAV.CMV.EGFP between the correct spacers SRSRR and SRSF



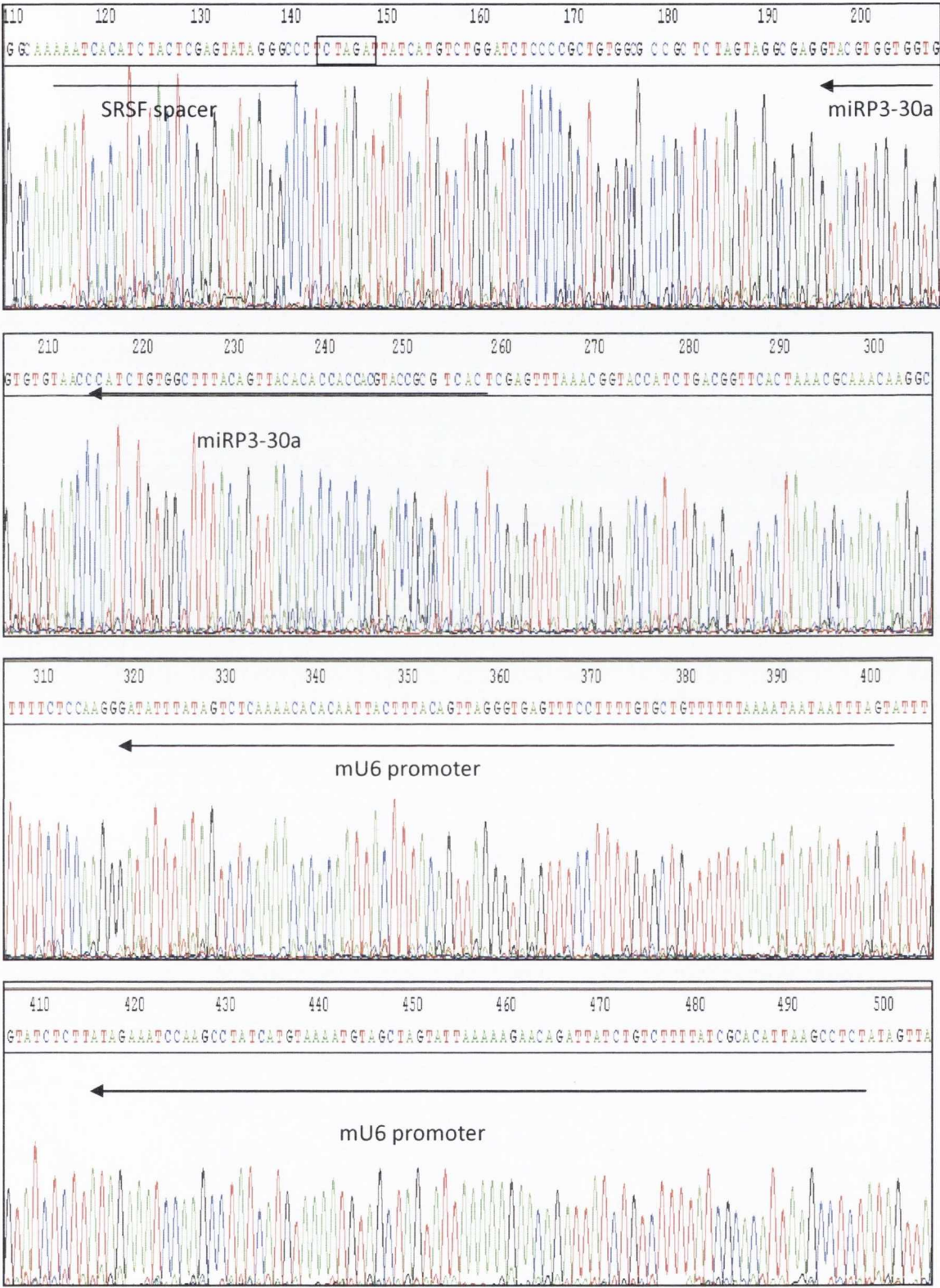


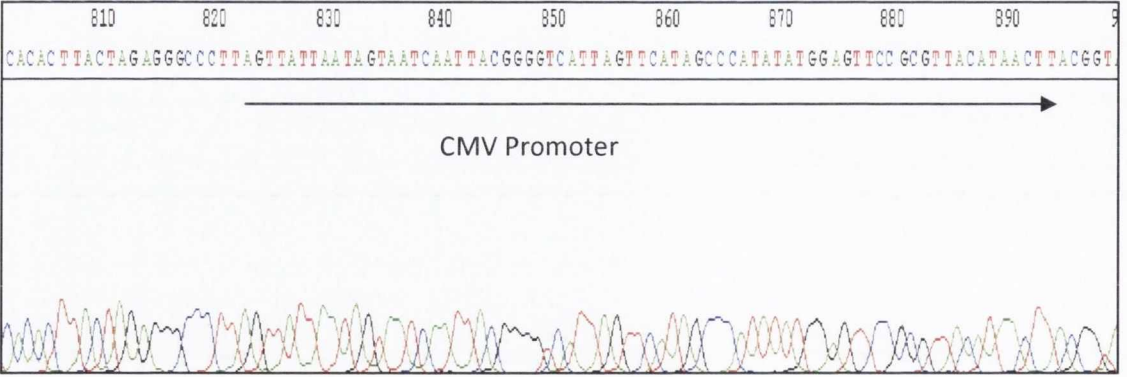
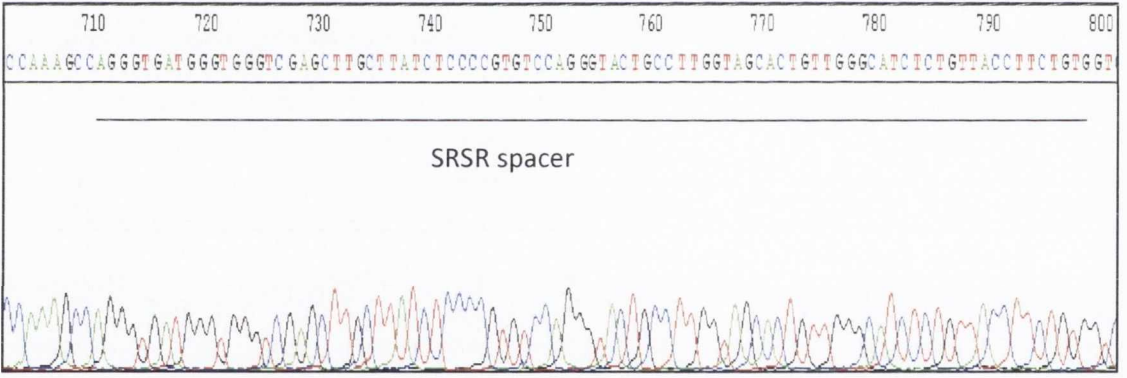
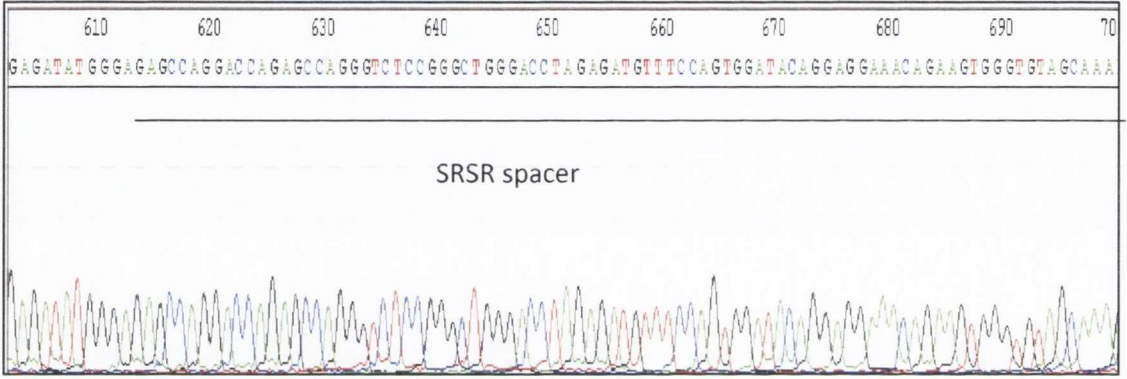
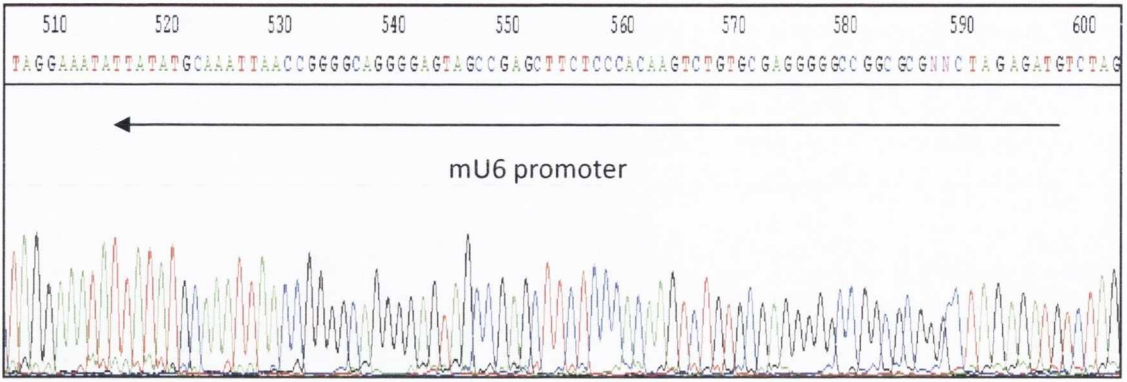
Section 9.13 sequencing of miRP3 within the pAAV.miRP3.rP3 vector





Section 9.14 Sequencing of pAAV.miRP3-30a with the SRSF primer





Section 9.15 Codon usage results for wild-type human rhodopsin
http://www.ualberta.ca/~stothard/javascript/codon_usage.html

A= Amino acid
 B= Codon Sequence
 C= Frequency of codon within hRHO
 D= % of that codon in hRHO
 E= % of that codon for that amino acid within hRHO

AmAcid	A Codon	B No.	C % of codon within hRHO	D % codon representing that amino acid in hRHO	E
Ala	GCG	3	0.86	9	
Ala	GCA	3	0.86	9	
Ala	GCT	5	1.4	16	
Ala	GCC	21	6.17	66	
Cys	TGT	2	0.573	20	
Cys	TGC	8	2.29	80	
Asp	GAT	1	0.287	25	
Asp	GAC	3	0.860	75	
Glu	GAG	14	4.11	88	
Glu	GAA	2	0.573	13	
Phe	TTT	5	1.433	17	
Phe	TTC	25	7.163	83	
Gly	GGG	3	0.86	14	
Gly	GGA	3	0.86	14	
Gly	GGT	5	1.43323		
Gly	GGC	11	3.52	50	
His	CAT	2	0.573	40	
His	CAC	3	0.860	60	
Ile	ATA	0	0	0	
Ile	ATT	2	0.573	8	
Ile	ATC	22	6.304	92	
Lys	AAG	11	3.152	1	
Lys	AAA	0	0	0	
Leu	TTG	2	0.573	7	
Leu	TTA	0	0	0	
Leu	CTG	15	4.298	52	
Leu	CTA	2	0.573	7	
Leu	CTT	0	0	0	
Leu	CTC	10	2.865	34	
Met	ATG	15	4.298	1	
Asn	AAT	3	0.860	19	

Asn	AAC	13	3.725	81
Pro	CCG	2	0.57310	10
Pro	CCA	5	1.43325	25
Pro	CCT	3	0.860	15
Pro	CCC	10	2.865	50
Gln	CAG	12	3.438	1
Gln	CAA	0	0	0
Arg	AGG	1	0.287	14
Arg	AGA	0	0	0
Arg	CGG	2	0.57329	29
Arg	CGA	0	0	0
Arg	CGT	0	0	0
Arg	CGC	4	1.146	57
Ser	AGT	0	0	0
Ser	AGC	6	1.719	35
Ser	TCG	1	0.287	6
Ser	TCA	1	0.287	6
Ser	TCT	3	0.860	18
Ser	TCC	6	1.719	35
Thr	ACG	6	1.719	25
Thr	ACA	3	0.860	13
Thr	ACT	0	0	0
Thr	ACC	15	4.298	63
Val	GTG	13	3.725	43
Val	GTA	1	0.287	3
Val	GTT	1	0.287	3
Val	GTC	15	4.298	50
Trp	TGG	5	1.4331	
Tyr	TAT	2	0.57311	11
Tyr	TAC	17	4.871	89
End	TGA	0	0	0
End	TAG	0	0	0
End	TAA	1	0.287	1

