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UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

MULTIFACTORIAL APPROACH TO NON-VIRAL GENE THERAPY: DEVELOPMENT OF AN EFFICIENT SYSTEM FOR THE RETINA

Sofia de Amaral Melo Calado

Tese para obtenção do grau de Doutor em Ciências Biomédicas

Trabalho efetuado sob a orientação de:

Doutora Gabriela Araújo da Silva

Co-orientação: Doutora Inês Maria Pombinho de Araújo

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.”

Sofia Calado

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A todos os que me tornaram mais forte.

“We don’t know how strong we are until being strong is the only choice!”
(Bob Marley)

“I’m not ill…My pancreas is just lazy.”
(Anonymous)

Agradecimentos/Acknowledgements

Em primeiro lugar gostaria de agradecer à Doutora Gabriela Silva por me ter aceite no seu laboratório e me ter (des)orientado nestes últimos seis anos. Por confiar em mim e no meu trabalho. Por fazer sempre os possíveis e impossíveis para que nada nos faltasse, nem que para isso tivesse que "chatear" meio mundo e, acima de tudo, ser mais do que uma chefe, ser uma amiga. Muito obrigada por tudo!

I would also like to thank to Doctor Shomi Bhattacharya for accepting me in his lab, allowing me to work with his amazing group.

Não podia deixar de agradecer também à Professora Doutora Leonor Cancela, diretora da Licenciatura e Programa Doutoral em Ciências Biomédicas, por todo o apoio nos últimos nove anos em que frequentei a Universidade do Algarve. Por nos ouvir e apoiar, e pelos sábios conselhos.

À Doutora Inês Araújo e ao Doutor Luís Almeida, membros da minha comissão de acompanhamento, por todos os conselhos e discussões científicas e por introduzirem uma visão diferente ao meu trabalho.

Ao Doutor José Belo e todo o seu grupo, por toda a ajuda e "empréstimos" de material e reagentes que nos fizeram e continuam a fazer.

Gostaria também de agradecer a todos os meus colegas e amigos do lab 2.33, Andreia, Vanessa, Sónia, Susana, Daniela, Liliana e Bitoque por me terem ajudado em tudo o que precisei, não só profissionalmente como também pessoalmente. À Mónica e à Marinella que, apesar de não fazerem parte do lab 2.33, são como se fizessem. Obrigada por todos os cafezinhos, almoços e momentos de parvoíce pegada que fizeram com que a ciência se tornasse ainda mais divertida.

To all my Sevillian friends from CABIMER, Ana Belén, Ana Artero, Rossio, Dani, Andrea, Lourdes, Berta and specially Fran, THANK YOU SO MUCH for receiving me with open arms and integrating me in your *desayunos*, for sharing with me your gastronomic specialties and for teaching me to be a better scientist. I loved working with you and I hope our paths cross again in a near future.

Aos meus queridos amigos de Faro, principalmente à Teresa, Joanas, Iolanda e Ricardo, por terem feito do Algarve a minha segunda casa, por fazerem com que o meu *Erasmus* para o Continente fosse bem mais simples, por todas as Semanas Académicas, noitadas, jantaradas e afins.

Às fifinhas mais fifinhas de sempre, Margarida e Inês, por me fazerem ter a certeza de que mudar é bom! Mudar traz coisas boas! Neste caso trouxe-vos à minha vida! Nunca pensei vir a gostar tanto de duas pessoas em tão pouco tempo.

A todos os membros do CEDOC, por me terem recebido de forma tão simpática e sempre com um sorriso no rosto. Fizeram, sem dúvida, com que os meus últimos três meses de trabalho fossem os melhores.

À Anokas, Kisinha, Ninês, Isabel e Patrícia por estes mais de 15 anos de amizade, por estarem sempre comigo. Por me mostrarem que a amizade é imutável mesmo estando a um Oceano Atlântico de distância.

À minha Iris, por ser a "minha pessoa", minha partner in crime e partner in wine! Por todos os brainstormings, chats e SMS quando ambas estávamos longe de casa. Por

todas as gargalhadas, lágrimas, abraços e discussões… Por ter estado sempre comigo nos maus mas, principalmente, nos bons momentos! Uma vez disseste-me que os amigos que fazemos quando estamos na universidade são aqueles que ficam para a vida…Contigo isto é verdade! Obrigada por fazeres parte da minha vida!

Aos meus pais, por apoiarem todas as minhas decisões e mostrarem que mesmo longe estão sempre perto. Por acreditarem em mim e me mostrarem que tudo é possível, mesmo quando o Mundo à nossa volta está a desabar. Por me darem força e terem sempre algo sábio para me dizer, nem que seja “Eu avisei-te!”. Obrigada por terem feito de mim, aquilo que sou hoje. Obrigada Mãeínha e Paínho.

À minha irmã, Mónica, por partilhar tudo comigo, até mesmo os momentos de doença. Ao meu cunhado, por convencer a famelga toda a vir passar férias ao Algarve para vir ter com a Ti Bi (apesar de eu saber que vocês só vêm por causa do sol…ou pensavas que me enganavas?!).

E como não podia deixar de ser, ao meu sobrinho e padrinho Gongas, por ser o Bé mais chato de todos (para mim vais ser sempre bebé), preencher o meu coração e fazer de mim a sua Ti Bi.

Finalmente gostaria de agradecer ao Rafa, simplesmente por existir. Pela paciência, amor, dedicação, carinho, apoio, força, já tinha dito paciência? PACIÊNCIA, etc, etc, etc. Por partilhar comigo as minhas lágrimas de dor, alegria, frustração e aguentar isto tudo, sempre com um sorriso no rosto. Por me ter feito a primeira injecção de insulina, por cuidar de mim e ajudar-me em todas as picadas, medições de glicémia, nódoas negras, mudanças alimentares e, ainda assim, continuar a gostar de mim e da minha nova bagagem. Sei que para ti também não foi fácil, estes últimos quatro anos trouxeram imensas mudanças, mas juntos conseguimos superar! Obrigada por caminhares de mão dada comigo pela Serra Algarvia…”Because we are Better Together”…Até aos 80! Obrigada!

Este trabalho não teria sido possível sem o apoio de todos os que me acompanharam nestes últimos quatro anos e é para eles que vai o meu grande **OBRIGADA**!

A produção deste trabalho teve o financiamento da Fundação para a Ciência e Tecnologia, à qual expresso os meus agradecimentos. Referência da bolsa SFRH/BD/76873/2011.

List of Contents

Resumo .... **xv**

Abstract .... **xix**

List of abbreviations and acronyms .... **xxi**

Chapter 1 – General Introduction **1**

1.1 Gene Therapy 3
1.2. The eye as target for gene therapy 4
1.3. Retinal diseases 8
1.3.1. Retinitis Pigmentosa 9
1.3.1.1. Vitamin Therapy 10
1.3.1.2. Retinal Implant 10
1.3.1.3. Stem cells and retinal transplantation 10
1.3.2. Diabetic retinopathy 11
1.3.2.1. Laser photocoagulation 13
1.3.2.2. Vitrectomy 13
1.3.2.3. Anti-VEGF Therapy 14
1.4. Barriers to Gene Therapy 16
1.4.1. Extracellular barriers 16
1.4.2. Extracellular barriers in the eye 16
Blood-Aqueous Barrier (BAB) 17
Blood-Retinal Barrier (BRB) 17
Corneal barrier 18
Vitreal barrier 18
1.4.3. Intracellular barriers of gene delivery 19
1.5. Gene Delivery Systems 21
1.5.1. Viral-based Gene Delivery Systems 22
1.5.1.1. Adenoviruses 23
1.5.1.2. Adeno-Associated Virus 23
1.5.1.3. Retroviral vectors 24
1.5.1.4. Herpes Simplex Virus 25
1.5.2. Non-viral Gene Delivery 25
1.5.2.1. Physical methods for gene delivery 26
Needle injection 26
Gene gun 26
Electroporation 27
Sonoporation 28

Photoporation ... 28
Magnetofection ... 28
Hydroporation ... 29
1.5.2.2. Chemical methods of gene delivery ... 29
Inorganic particles ... 30
Cationic Lipids ... 30
Cationic polymers ... 31
Peptide-mediated gene transfer ... 32
1.6. Optimization of expression vectors for long-term gene therapy ... 32
1.6.1. Minicircle: a plasmid devoid of bacterial backbone ... 34
1.6.2. MIDGE vectors ... 37
1.6.3. Plasmids Free of Antibiotic Resistance Gene (pFARs) ... 39
1.6.4. Self-replicating episomal plasmids ... 39
1.6.4.1. Self-replicating viral-based vectors ... 40
1.6.4.2. Chromosome-based vectors ... 40
1.6.4.3. Episomal vectors containing S/MARs ... 41
1.7. Specific Aims ... 44

Chapter 2 – Identification of Molecular Targets for Gene Therapy for *Retinitis Pigmentosa* ... **45**
2.1. Abstract ... 49
2.2. Introduction ... 50
2.3. Materials and Methods: ... 52
2.3.1. Antibodies ... 52
2.3.2. Cell Lines ... 52
2.3.3. Primary cilium induction ... 52
2.3.4. Immunofluorescence ... 52
2.3.5. Western blot ... 53
2.3.6. ATR and γ-tubulin immunoprecipitation ... 53
2.3.7. ATR inhibition and cilia length measurement ... 54
2.3.8. Statistical Analysis ... 54
2.4. Results and Discussion ... 55
2.4.1. ATR colocalizes with the centrosome of the ciliated cells ... 55
2.4.2. Inhibition of ATR (by caffeine) affects ciliary length ... 58
2.5. Conclusion ... 63

Chapter 3 – Identification of Molecular Targets for Gene Therapy for *Diabetic Retinopathy* ... **65**
3.1. Abstract ... 69
3.2. Introduction ... 70
3.3. Materials and Methods ... 72

3.3.1. Cell lines 72
3.3.2. Detection of GLUT1 expression by Western blot 72
3.3.3. Cellular localization of GLUT1 by immunocytochemistry 73
3.3.4. Glucose consumption assay 73
3.3.5. PEDF detection in the culture medium of the RPE cells 74
3.3.6. Animals 74
3.3.7. Statistical analysis 75
3.4. Results 76
3.4.1. Hypoxia induces overexpression of GLUT1 in RPE cells 76
3.4.2. GLUT1 translocation to the cell membrane of RPE cells increases in response to hypoxia 77
3.4.3. Glucose consumption is affected by hypoxia 78
3.4.4. Secretory function of RPE cells is impaired by high glucose and hypoxia 79
3.4.5. GLUT1 and PEDF expression is altered in the RPE of diabetic mice .. 80
3.5. Discussion 82
3.6. Conclusion .... **Erro! Marcador não definido.**

Chapter 4 – Expression Systems for Retinal Gene Therapy 87
4.1. Abstract 91
4.2. Introduction 92
4.3. Materials and Methods 94
4.3.1. pEPI-1 and pEPito-based vectors 94
4.3.2. Transfection Efficiency Assay 95
4.3.3. Colony-forming Assay 95
4.3.4. Injection of pEPito vectors in C57Bl6 mice and eGFP expression 96
4.3.5. Statistical analysis 97
4.4. Results 98
4.4.2. pEPito plasmids express GFP for three months in mitotically active human RPE cells 99
4.4.3. pEPI and pEPito gene transfer to mouse retinas: 102
4.5. Discussion 105

Chapter 5 – Gene Therapy for Diabetic Retinopathy 109
5.1. Abstract 113
5.2. Introduction 114
5.3. Materials and Methods 116
5.3.1. Vector construction 116
5.3.2. Evaluation of PEDF expression driven by the pEPito vector in vitro .. 116
5.3.3. Evaluation of PEDF expression driven by the pEPito vector *in vivo* ... 117
5.3.4. Immunofluorescence for PEDF expression in vivo 117

5.3.5. PEDF expression by Western blot analysis 118
5.3.6. Statistical analysis 119
5.4. Results 120
5.4.1. Characterization of PEDF in the retina of $Ins2^{Akita}$ diabetic mice 120
5.4.2. pEPito enables long-term expression of PEDF in mitotic RPE cells... 121
5.4.3. Restoration of PEDF protein levels in the retina of $Ins2^{Akita}$ mice 121
5.5. Discussion 125

Chapter 6 – General Discussion 126

Chapter 7 – References 126

Resumo

A terapia génica é uma estratégia terapêutica que se caracteriza pela entrega de material genético a uma célula alvo. A terapia génica tem sido vastamente utilizada no combate a diversas doenças genéticas e adquiridas, tais como as doenças cardiovasculares, cancro e doenças oculares, entre outras.

Devido às suas características únicas, como tamanho reduzido, relativo isolamento da circulação sistémica e fácil acessibilidade a diferentes tipos celulares e tecidos, o olho é considerado o órgão ideal para o desenvolvimento de estratégias de terapia génica. A Retinite Pigmentosa e a Retinopatia Diabética são exemplos de doenças oculares genéticas e adquiridas, respetivamente, que afetam a retina e conduzem a uma perda irreversível de visão. Apesar da sua etiologia ser diferente, ambas as patologias partilham o facto de, atualmente, não existir um tratamento eficaz, o que faz destas bons alvos para terapia génica.

Atualmente, cerca de 80% das estratégias de terapia génica usadas para doenças oculares são baseadas na utilização de vírus como vetores de entrega do material genético. Apesar da sua eficácia, esta abordagem acarreta uma série de desvantagens, principalmente do ponto de vista de segurança a nível imunológico e mutagénico. Assim, os vetores não-virais aparecem como estratégia alternativa aos vetores virais, pela sua fácil utilização, ilimitada capacidade de empacotamento de genes e ausência de resposta imunitária. Porém, a sua aplicação clínica encontra-se limitada, não só pela sua reduzida eficiência de transfeção como pela expressão genética transiente conferida pelos, até agora utilizados, sistemas de expressão.

Neste sentido, nas últimas décadas têm sido desenvolvidos sistemas de expressão baseados em plasmídeos de ADN, que visam uma expressão prolongada, como os minicírculos, os vetores MIDGE, os pFARs e os plasmídeos epissomais com capacidade de auto-replicação. Exemplos de plasmídeos com capacidade de auto-replicação são os pEPI-1 e pEPito. Nestes plasmídeos, a capacidade de auto-replicação é conferida pela presença de S/MARs (*Scaffold/Matrix Attachment Regions*)

no corpo do plasmídeo. As S/MARs são sequências de ADN ricas em nucleótidos Adenina (A) e Timina (T) capazes de ancorar a cromatina à matriz nuclear. As S/MARs parecem estar envolvidas na destabilização e abertura da dupla hélice de ADN, sugerindo um envolvimento na replicação e expressão do mesmo, uma vez que a transição de cadeia dupla para cadeia simples é necessária para a replicação e transcrição genética. Além disso, vetores com S/MARs são capazes de prevenir o silenciamento epigenético, protegendo o transgene das sequências regulatórias adjacentes e da heterocromatinização, mantendo o vetor num estado transcricionalmente ativo e conferindo, assim, estabilidade mitótica. As S/MARs têm, também, a capacidade de mediar a associação da partícula episomal aos cromossomas metafásicos, permitindo, assim uma distribuição igualitária dos epissomas para as células filhas, após a mitose.

O objetivo deste trabalho foi desenvolver uma estratégia de terapia génica não-viral para doenças da retina, recorrendo ao uso de sistemas de expressão epissomais com capacidade de auto-replicação (pEPito) e de um sistema de entrega eficaz (eletroporação), de modo a conseguir uma expressão prolongada dos genes terapêuticos. Experimentalmente é possível dividir este trabalho em três secções: *i)* identificação de genes com potencial terapêutico em patologias da retina, como RP e RD; *ii)* clonagem dos genes em questão em vectores epissomais, pEPito e *iii)* administração dos vetores desenvolvidos *in vivo*, na retina de ratinhos, utilizando eletroporação como método de entrega dos sistemas de expressão.

Foi recentemente descrito em ratinhos que mutações no gene *ATR* (*Ataxia telangiectasia and Rad3 related*) induzem uma acumulação de pigmento na retina, com consequente degeneração dos fotorrecetores (bastonetes e cones), semelhante à que acontece em pacientes com Retinite Pigmentosa. Nos animais *Wild-Type* esta proteína está localizada nos cílios dos fotorrecetores. Nos mutantes, a presença da proteína mutada induz um encurtamento dos cílios, originando uma degeneração dos bastonetes e, posteriormente, dos cones. Neste contexto, tentámos investigar qual a

função desta proteína na formação e alongamento dos cílios retinianos. Os nossos estudos *in vitro* demonstraram que esta proteína está associada ao centrossoma das células ciliadas. Nestas células, o centrossoma é o local onde o cílio se forma e por onde começa a alongar. A inibição de ATR (pela cafeína) originou uma diminuição da expressão proteica e, como consequência, verificou-se uma diminuição no comprimento dos cílios das células tratadas, demonstrando uma relação direta entre a expressão de ATR e a função ciliar. Assim, podemos inferir que mutações no gene *ATR* podem ser responsáveis por alguns dos casos de RP que não estão associados aos genes normalmente implicados na doença.

A Retinopatia Diabética é uma das principais complicações da Diabetes *Mellitus*. É considerada uma doença das barreiras hematorretinianas, na qual a hiperglicemia e isquémia são as principais responsáveis pelo desequilíbrio entre os fatores pro- e anti-angiogénicos que conduzem à neovascularização e consequente perda de visão. Neste estudo avaliámos os efeitos da hiperglicemia e isquémia na barreira hematorretiniana externa, *in vitro*, em culturas de células do epitélio pigmentar da retina, sujeitas a glicose elevada e *in vivo,* no epitélio pigmentar da retina de ratinhos diabéticos $Ins2^{Akita}$. Os nossos resultados mostraram um aumento do transportador de glicose (GLUT1) nos nossos modelos diabéticos. Este aumento está associado, não só, a um aumento no número de transportadores na membrana das células do epitélio pigmentar da retina, como também a um aumento da sua atividade, aumentando o consumo de glicose. Este aumento no consumo de glicose induz uma diminuição na produção e secreção de factores anti-angiogénicos, como o PEDF (*Pigment Epithelium-Derived Factor*) por estas células. Esta diminuição contribui para o desequilíbrio entre os fatores pro- e anti-angiogénicos, contribuindo assim para o desenvolvimento da neovascularização.

Baseado nos resultados anteriores, decidimos clonar o gene *PEDF* no vetor de expressão pEPito e, através de injeção subretiniana, administrá-lo nas células do epitélio pigmentar da retina de ratinhos $Ins2^{Akita}$. Os nossos resultados mostraram que

os nossos vetores foram capazes de sobre-expressar PEDF até três meses após a injeção, em níveis semelhantes aos dos animais controlo (não diabéticos). Esta sobre-expressão foi associada a uma diminuição de marcadores inflamatórios e angiogénicos, associados à doença. Estes resultados mostram que a sobre-expressão de PEDF pode constituir uma nova estratégia para o tratamento da RD.

Os nossos resultados indicam que esta abordagem baseada em sistemas de expressão com capacidade de auto-replicação e menos susceptíveis ao silenciamento epigenético, como os pEPito, aliada a um método de entrega de ADN eficaz, como a eletroporação, pode ultrapassar as limitações associadas à utilização de vetores virais, conferindo um padrão de expressão génica prolongado e mantendo um elevado perfil de segurança, consistindo assim numa alternativa eficaz para a terapia génica retiniana.

Palavras-chave: Terapia génica; pEPito; S/MARs, Retinite Pigmentosa; Retinopatia Diabética

Abstract

Retinitis Pigmentosa (RP) and Diabetic retinopathy (DR) are blinding disorders that contribute to 25 to 30% of blindness cases in work-age people. The ineffectiveness of current treatments makes them ideal targets for gene therapy. The gene therapy strategy should include the main advantages of viral vectors – long-term expression and high transduction capacity – without presenting its disadvantages – immune response, random integration and tumor formation. This can be achieved by using self-replicating episomal vectors, such as pEPito.

The goal of this project was to develop new strategies using pEPito vectors encoding therapeutic genes for retinal diseases, such as DR and RP.

RP is an inherited degenerative disease, characterized by loss of photoreceptors. Recently, depletion of ATR in the mouse retina was associated to a RP-like phenotype. Our results show that, in ciliated cells, ATR is localized in the centrosome, corresponding to the connecting cilium. The inhibition of ATR induced a decrease in cilia length, showing a direct relationship between ATR and cilia formation and elongation. These findings suggest ATR as a new therapeutic target for RP.

DR is the main complication of diabetes. Several studies have shown an imbalance between pro- and anti-angiogenic factors, due to ischemia and hyperglycemia. In this work we describe an impairment of the oBRB due to an increase GLUT1 expression and function, in diabetic models. As a consequence, we detected a decrease in PEDF production and secretion. We decided to clone *PEDF* into pEPito backbone and overexpressed it in Ins2^{Akita} diabetic mice, using subretinal injection and electroporation. Our system showed sustained gene expression for up to three months in levels comparable to WT mice. The overexpression of PEDF was associated with a decrease in several inflammatory and angiogenic factors associated with DR.

Our results indicate that the pEPito-based approach, combined with electroporation may overcome some limitations found in viral-mediated gene transfer,

while maintaining a high safety profile and sustained gene expression, thus constituting an alternative for retinal gene delivery.

Keywords: Gene Therapy, pEPito, S/MARs, *Retinitis Pigmentosa*, Diabetic Retinopathy

List of abbreviations and acronyms

A
AC – Auxotrophy Complementation
Ad – Adenovirus
ADA – Adenosine Deaminase
adRP – Autossomal Dominant *Retinitis Pigmentosa*
AGEs – Advanced Glycation End-products
Am – Amacrine cell
AMD – Age-related Macular Degeneration
arRP – Autossomal Recessive *Retinitis Pigmentosa*
ARVO – Association for Research in Vision and Ophthalmology
ATM – Ataxia Telangiectasia Mutated
ATR – Ataxia Telangiectasia and Rad-3
B
BAB – Blood-Aqueous Barrier
BC – Bipolar Cell
BRB – Blood-Retinal Barrier
BSD – Blasticidin
C
CAR – Coxsackievirus and Adenovirus Receptor
CDK2 – Cyclin-Dependent Kinase 2
CEP290 – Centrosomal Protein 290
CHM - Choroideremia
CMV – Cytomegalovirus
CsCl – Cesium Cloride
D
DAPI – 4'-6-diamidino-2-phenylindole
DDR – DNA Damage Response
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic Acid
DPI – Days Post Injection
DR – Diabetic Retinopathy
dsDNA – Double-Stranded DNA
E
EBNA1 – Epstein Barr Virus Nuclear Antigen 1
EBV – Epstein Barr Virus
ECL – Enhanced Chemiluminescence
eGFP – Enhanced Green Fluorescent Protein
ERG – Electroretinogram
F
FBS – Fetal Bovine Serum
FELASA – Federation for Laboratory Animal Science Association
G
GAG – Glycosaminoglycans
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GC – Ganglion Cell
GCL – Ganglion Cell Layer
GFAP – Glial Fibrillary Acidic Protein
GLUT1 – Glucose Transporter 1
H
hCMV/EF1 – Human Cytomegalovirus/Elongation Factor alpha 1
HIV – Human Immunodeficiency Virus
HRP – Horseradish Peroxidase
HSV – *Herpes Simplex* Virus

I
Iba1 – Ionized Calcium-Binding Adapter molecule 1
iBRB – Inner Blood-Retinal Barrier
INL – Inner Nuclear Layer
iPS – Induced-Pluripotent Stem Cell
IRES – Internal Ribosomal Entry Sequence
IU – International Units
K
kb – Kilo base
M
mcDNA – Minicircle DNA
MIDGE – Minimalistic, Immunogenically Defined Gene Expression
MP – Miniplasmid
mRNA – Messenger RNA
N
NFDM – Non-fat Dry Milk
NLS – Nuclear Localization Signals
NPDR – Non-Proliferative Diabetic Retinopathy
NPs – Nanoparticles
O
oBRB – Outer Blood-Retinal Barrier
OCT – Optic Coherence Tomography
ONL – Outer Nuclear Layer
Ori – Origin of Replication
ORT – Operator-Repressor Titration
P
PBS – Phosphate Buffered Saline
PBS-T – Phosphate Buffered Saline-Triton
pDMAEMA - Poly(2-(dimethylamino)ethylmethacrylate
pDNA – Plasmid DNA
PDR – Proliferative Diabetic Retinopathy
PEDF – Pigment Epithelium-Derived Factor
PEG – Polyethylene Glycol
PEI – Polyethylenimine
PFA – Paraformaldehyde
pFARs – Plasmids Free of Antibiotic Resistance
PKC – Protein Kinase C
PP – Parental Plasmid
PR – Photoreceptor
PSK – Post-Segregational Killing
PVDF – Polyvinylidene Fluoride
R
RAS – Renin-Agiotensin System
RIPA buffer – Radioimmunoprecipitation assay buffer
RNA – Ribonucleic Acid
ROS – Reactive Oxigen Species
RP – *Retinitis Pigmentosa*
RPE – Retinal Pigment Epithelium
RPGR – Retinitis Pigmentosa GTPase Regulator
S
S/MARs – Scaffold/Matrix Attachment Regions
SCID – Severe Combined Immunodeficiency
SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
siRNA – Small Interfering RNA
ssDNA – Single-Stranded DNA

ssRNA – Single-Stranded RNA
STZ – Streptozotocin
SV40 – Simian Vacuolating 40 Virus
T
TLR4 – Toll-Like Receptor 4
tRNA – Transfer RNA
V
VEGF – Vascular Endothelium Growth Factor
W
WT – Wild-Type

Chapter 1 – General Introduction

1.1 Gene Therapy

Gene therapy is a therapeutic strategy that involves intracellular delivery of exogenous genetic material (DNA or RNA) to correct or modify the expression of genes responsible for a certain genetic or acquired disease (Naik *et al.*, 2009, Ramamoorth *et al.*, 2015). Depending on the disease, this can be achieved by:

- Replacement of a mutated gene that causes a disease by a correct copy of the gene (Kaufmann *et al.*, 2013, Ramamoorth *et al.*, 2015);
- Inactivation of a malfunctioning mutated gene by using antisense RNA that binds to the messenger RNA (mRNA) blocking it, or by using the siRNA (small interfering) technology, a class of double-stranded RNA molecules that neutralizes the mRNA transcript, resulting in no translation into protein (Kaufmann *et al.*, 2013, Ramamoorth *et al.*, 2015);
- Introduction of new genes, a strategy that is being widely used in cancer gene therapy in which the insertion of specific genes induces the host cell to enter apoptosis (Kaufmann *et al.*, 2013, Ramamoorth *et al.*, 2015).

The ideal gene therapy strategy includes four basic prerequisites: *i)* it should be efficient and nontoxic, *ii)* the targeted disease should be well characterized in terms of genetic defect and the tissue/ cell types affected, *iii)* the expression levels of the therapeutic gene must be tightly controlled, and *iv)* an adequate animal model should be available for pre-clinical studies and proof-of-concept (Hauswirth *et al.*, 2000).

Since the first approved clinical trial in 1990 for the treatment of SCID (Severe Combined Immunodeficiency), using ADA (*Adenosine Deaminase*) gene (Blaese *et al.*, 1995), several disorders, either genetic or acquired, have been targeted using gene therapy, namely cancer, cardiovascular diseases and ocular diseases (Figure 1.1) (Gascón *et al.*, 2013, Kaufmann *et al.*, 2013, Ramamoorth *et al.*, 2015).

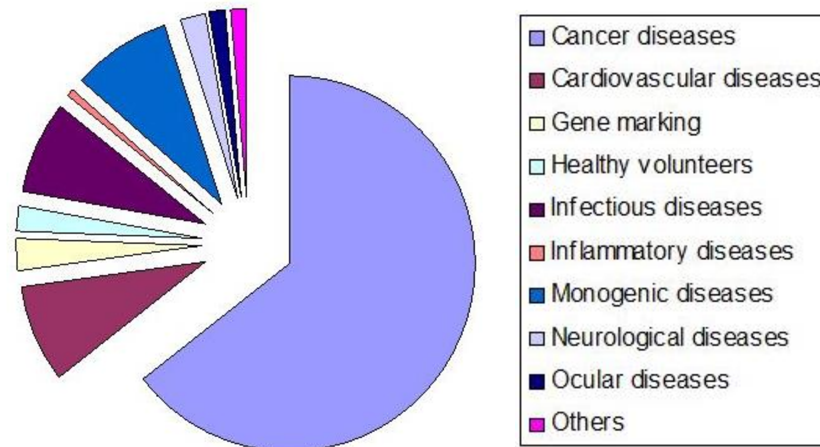

Figure 1.1: Targeted diseases in gene therapy clinical trials. Cancer is the most representative disorder in gene therapy applications, but cardiovascular, infectious and monogenic diseases are also noteworthy. Adapted from (Gascón *et al.*, 2013).

The ideal vehicle for gene therapy should penetrate the cell membrane and efficiently deliver genes specifically into target cells, without being toxic or immunogenic. The genes must be directed to the nucleus and integrate the host genome in a non-mutagenic way or be maintained as an episome for a long time period. It is also desirable the therapeutic gene to be delivered independently of the mitotic status of the recipient target cell and the expression level should be constant over time (Chaum *et al.*, 2002, Gascón *et al.*, 2013).

1.2. The eye as target for gene therapy

The eye is the organ responsible for the vision. It consists of two distinct anatomic regions: the anterior cavity and the posterior cavity (Figure 1.2) (de la Fuente *et al.*, 2010).

The anterior cavity is the segment between the cornea and the lens, including the conjunctiva. These structures are responsible for focusing the light on the photoreceptor cells of the retina, in the posterior cavity of the eyeball, allowing vision. The cornea is a non-vascularized tissue composed by five to seven layers, conferring

high resistance to passive diffusion of ions and molecules. It is also responsible for enduring the intraocular pressure (de la Fuente *et al.*, 2010).

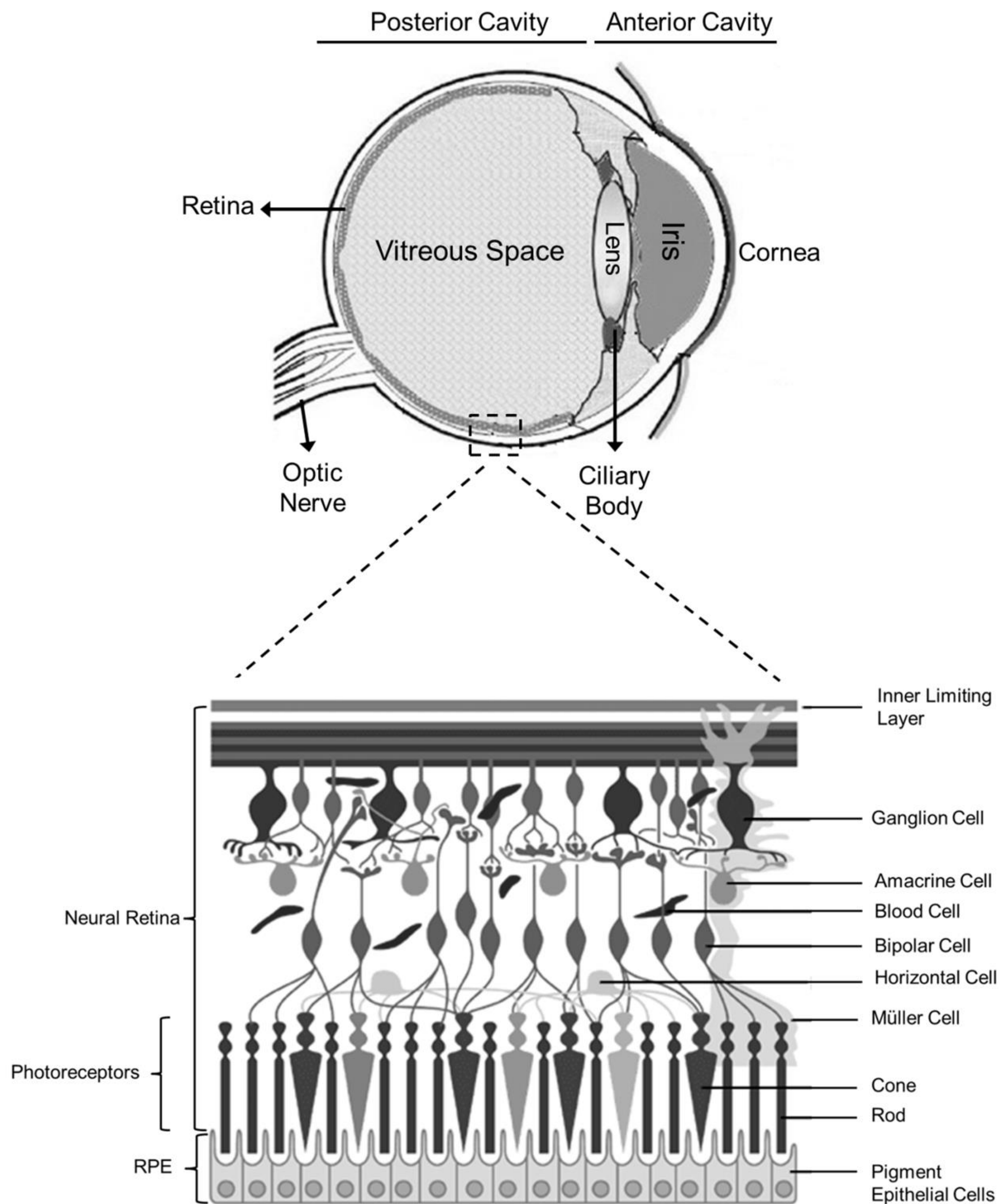

Figure 1.2: Schematic representation of the eye with an enlargement of the retina, showing its main cells types. Based on (Farjo *et al.*, 2010).

The posterior cavity is the space between the lens and the sclera, including the retina and choroid. The posterior cavity contains the vitreous humor, a gel-like substance composed of water, collagen, hyaluronic acid and proteoglycans. The

vitreous humor acts as a shock absorber, supports the shape of the eye, and is in contact with the retina, keeping it in place by pressing it into the choroid. The sclera is a hard tissue composed mainly by connective tissue. It is also responsible for maintaining the structure of the eye, by resisting intraocular pressure. The choroid is a vascular layer responsible for providing blood supply to retinal cells (Colthurst *et al.*, 2000).

The retina is the most metabolically active tissue of the human body, with a fast rate of glucose and oxygen consumption. It is the sensory tissue that lines the inner surface of the posterior segment of the eye and is organized in seven major layers composed mainly by three cell types (Naik *et al.*, 2009):

- Neural cells (ganglion cells, bipolar neurons and amacrine cells): The ganglion cells (GC) are the output neurons. Its axons constitute the optic nerve and transmit the visual information from the retina to the brain. The bipolar cells (BC) are involved in the synaptic transmission from the photoreceptors to the ganglion cells. Amacrine cells (Am) are interneurons that affect the output from bipolar cells, interacting with ganglion cells (Naik *et al.*, 2009, Hoon *et al.*, 2014).
- Photoreceptors (cones and rods): Photoreceptors (PRs) are responsible for the conversion of light energy through changes in the membrane potential that alters neurotransmitter release. There are two classes of PR, rods and cones. Rods are responsible for the low-light vision and are located throughout the peripheral retina. By contrast, cones are located at the central part of the retina (macula) and are responsible for the central and color vision (Colthurst *et al.*, 2000, Naik *et al.*, 2009).
- Retinal pigment epithelium (RPE): The RPE is located between the outer segments of the PR and the choroid. It is a monolayer composed by hexagonal cells connected by tight-junctions, containing pigment granules. The apical membrane faces the subretinal space and interacts with the outer segment of

the PRs. The basolateral membrane of the RPE is in contact with the Bruch membrane that is in direct contact with blood in fenestrated vessels of the choroid (Strauss, 1995). Figure 1.3 outlines some of the RPE functions.

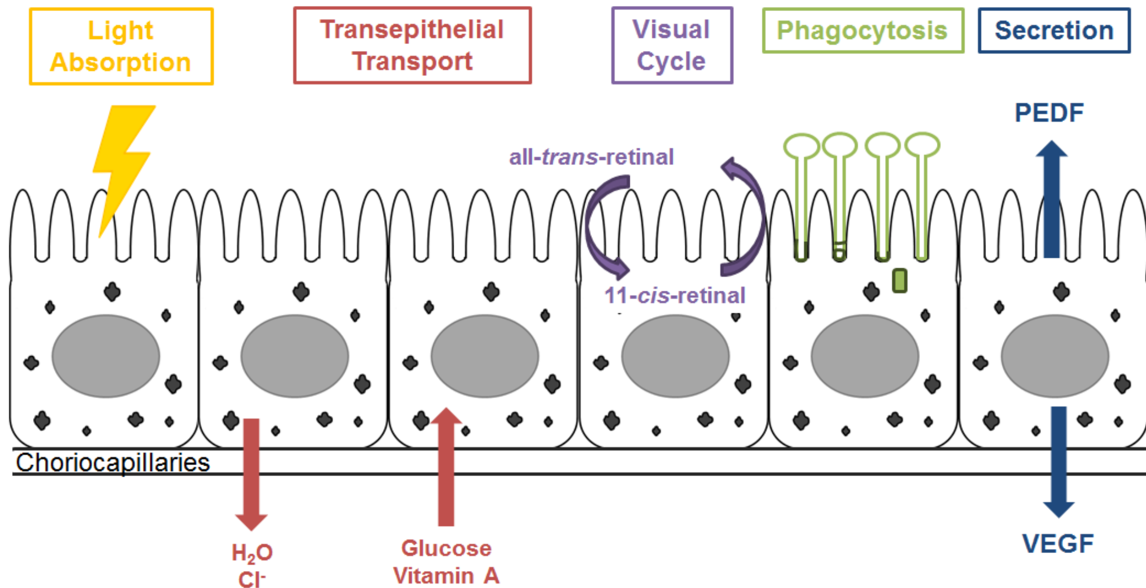

Figure 1.3: The retinal pigment epithelium (RPE) and its main functions: light absorption, transepithelial transport, reisomeriztion of all-*trans*-retinal, phagocytosis and secretion of growth factors, such as Vascular Endothelial Growt Factor (VEGF) and neurotrophic factor, like Pigment Epithelium-Derived Factor (PEDF). Adapted from (Strauss, 2005).

RPE is responsible for light absorption, transport of ions, water, and metabolic end products from the subretinal space to blood. RPE takes up nutrients, such as glucose, retinol, and fatty acids, essential for the maintenance of photoreceptors. Another important function of RPE cells is the reisomerization of all-*trans*-retinal into 11-*cis*-retinal, a key element of the visual cycle that is further transported back to photoreceptors to be incorporated in the visual cycle pathway. To ensure the excitability of PRs, RPE phagocytes and digests their shed outer segments, where recycling of retinal occurs to be returned and rebuild the outer segments of photoreceptors. Thus, the RPE needs to maintain the structural integrity of the retina by defending it efficiently from free radicals, photo-oxidative exposure and light energy. Furthermore, RPE produces and

secretes a wide range of growth and neurotrophic factors to support and maintain the structural basis of photoreceptors and choriocapillaris endothelium. A failure in any of these functions can induce retinal degeneration, loss of visual function, and irreversible blindness (Strauss, 2005, Simo *et al.*, 2010).

The eye is an attractive target for gene therapy. Due to its relative small size, it is only necessary a small amount of a drug to obtain a significant therapeutic effect. The eye also contains different cell types that can be specifically accessed by different delivery methods, enabling a targeted therapy when combined with the vector's tropism (Bloquel *et al.*, 2006). Moreover, its immune-privileged status due to the presence of the blood-retinal barrier (BRB) limits inflammatory reactions towards the vector (Bloquel *et al.*, 2006, Farjo *et al.*, 2010). Besides, therapy outcomes can be easily monitored by non-invasive methods, such as electroretinography (ERG) and optical coherence tomography (OCT), to complement patient input (Petrs-Silva *et al.*, 2014). In particular, retinal disorders are good targets of ocular gene therapy because in most cases the genetic etiology is known and several animal models exist or have been generated to help design new therapeutic strategies. Moreover, PR and RPE cells, which are more often affected by these mutations, can be easily accessed by subretinal injection (Cheung *et al.*, 2010).

1.3. Retinal diseases

Diseases affecting the retina are blinding disorders influenced by genetic and environmental factors, altogether contributing to more than 25% of blindness cases (Naik *et al.*, 2009).

The fact that current treatments for these diseases are ineffective makes them ideal candidates for gene therapy (Naik *et al.*, 2009).

Retinal disorders can be classified as inherited or acquired diseases. Most of inherited retinal diseases are caused by mutations in genes expressed in the PRs and

RPE cells. There are more than 200 identified genes responsible for these diseases (http://www.sph.yth.tmc.edu/RetNet/) (McClements *et al.*, 2013). Examples of inherited retinal diseases are Leber Congenital Amaurosis (LCA), Choroideremia (CHM), *Retinitis Pigmentosa* (RP), among others. Acquired retinal disorders include, among others, Age-related Macular Degeneration (AMD) and Diabetic Retinopathy (DR) that results from a combination of aging, environmental, and genetic factors that damage the retina and RPE (Chaum *et al.*, 2002). In the following sections a genetic and an acquired retinal disease will be used as examples of potential therapeutic approaches, mostly focused on gene therapy.

1.3.1. Retinitis Pigmentosa

Retinal degeneration is characterized as a deterioration of the retina, usually caused by a massive cell death. *Retinitis Pigmentosa* (RP) is one of the most common types of inherited retinal degeneration that can result from defects in more than 60 genes, displaying all three types of Mendelian inheritance: autosomal dominant (30%-40% of cases), autosomal recessive (50%-60%) or X-linked (5%-15%), but it can also occur in combination with other systemic disorders such as Usher syndrome, Refsum disease, Bassen-Kornzweig syndrome, Bardet-Biedl syndrome, and Batten disease. There are still 30%-35% of RP patients whose mutations cannot be identified (Shintani *et al.*, 2009, Petrs-Silva *et al.*, 2014).

Despite the genetic heterogeneity of this disease, RP patients present several common features, such as pigment accumulation, dysfunction of PRs and/or RPE, massive loss of PRs, abnormal ERG, night blindness and loss of peripheral field vision (Petrs-Silva *et al.*, 2014).

To date there is no cure available for RP, however the effectiveness and safety of several potential treatments are being evaluated (Musarella *et al.*, 2011). A brief description of each of these is presented below.

1.3.1.1. Vitamin Therapy

Vitamin A may protect PRs through its trophic and antioxidant properties. Berson *et al.* have demonstrated a delay in PRs death after daily ingestion of 15000 IU of Vitamin A (Berson *et al.*, 1993). More recently, the same group showed that the intake of Vitamin A can retard blindness up to 10 years (Berson, 2007). The administration of Vitamin A in combination with a diet rich in omega-3 fatty acids also show slight improvements (Musarella *et al.*, 2011).

Other studies showed a delay in the progression of the disease with the combination of Vitamin A with the antioxidant lutein (Berson *et al.*, 2010).

1.3.1.2. Retinal Implant

Epiretinal and subretinal implants have been performed in animal models, showing that cortical activity can be induced. This is currently being tested in humans, but the long-term effect of the implants has yet to be evaluated, as well as the effect on the retinal function of the electrodes placed between the neuroretina and the RPE layer (Musarella *et al.*, 2011).

1.3.1.3. Stem cells and retinal transplantation

Some studies showed an improvement in visual acuity and pupillary light response after the implant of fetal retina and PR precursors, respectively. The use of stem cells is advantageous because it is possible to prepare unlimited numbers of progenitor cells. The main disadvantages are the need to predict the ideal number of cells for engraftment and most of time the engrafted cells does not develop synaptic connections (Musarella *et al.*, 2011) and the need of lifelong immunosuppressive therapy due to allograft transplantation (Li *et al.*, 2012). The development of autografts from patient-specific induced pluripotent stem (iPS) cells has shown great potential to solve this problem and has been used as cell therapy in pre-clinical trials (Li *et al.*,

2012), as well as an *in vitro* model to study the signaling pathways involved in the pathophysiology of the disease (Lukovic *et al.*, 2015).

1.3.2. Diabetic retinopathy

Diabetes *mellitus* is a group of metabolic diseases characterized by high blood glucose that leads to several complications, including diabetic retinopathy (DR). With the increased survival of individuals with diabetes, DR remains the major cause of vision loss in developed countries, affecting mostly working-age adults, as it develops approximately 20 years after the onset of diabetes (Cheung *et al.*, 2010).

DR is classically regarded as a microvascular complication of diabetes that leads to the neovascularization within the retina (Cheung *et al.*, 2010, Farjo *et al.*, 2010). The pathophysiology underlying diabetic retinopathy is still unknown, but is believed that the chronic exposure to hyperglycemia and other risk factors, such as hypertension, triggers a cascade of biochemical and physiological changes that originate microvascular damage and retinal dysfunction (Cheung *et al.*, 2010). Several biochemical mechanisms have been proposed to be responsible for the pathophysiology of the disease (Figure 1.4) including the accumulation of sorbitol and advanced glycation end-products (AGEs), reactive oxygen species (ROS) production, protein kinase C activation (PKC), inflammation, upregulation of the renin-angiotensin system (RAS) and of vascular endothelial growth factor (VEGF) (Frank, 1995, Antonetti *et al.*, 2006, Cheung *et al.*, 2010).

Altogether, these biochemical mechanisms contribute to structural and physiological changes, including retinal capillary basement membrane thickening that induces pericyte and endothelial cell death, causing inner BRB (iBRB) breakdown. This loss of retinal capillary function leads to vascular wall leakage, inflammation, and ischemia, contributing to retinal neovascularization, formation of microaneurysms, edema, and hemorrhages leading to irreversible blindness (Frank, 1995, Cheung *et al.*, 2010).

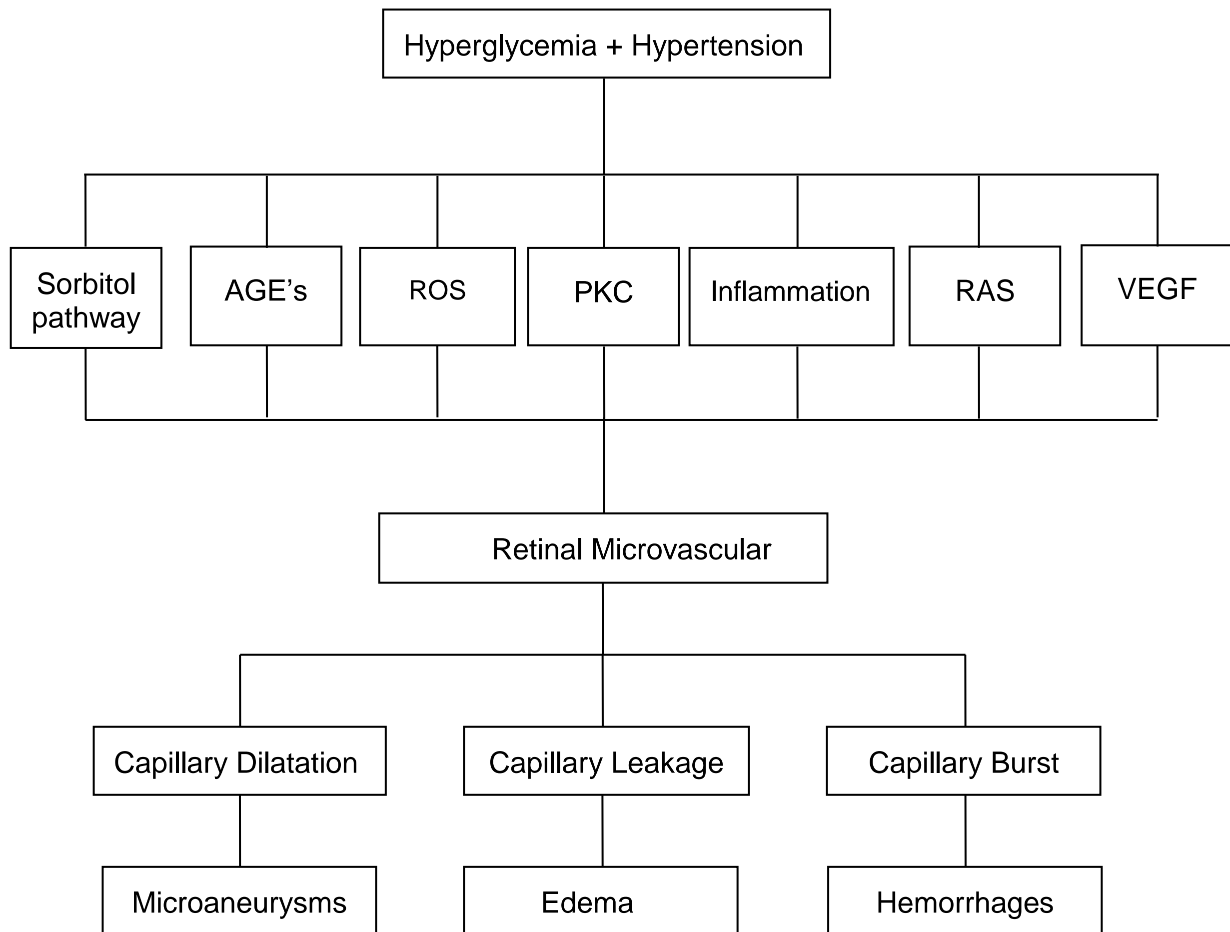

Figure 1.4: Pathophysiology of diabetic retinopathy. Hyperglycemia and hypertension initiates a cascade of biochemical events that leads to microvascular damage and ultimately vision loss. Adapted from (Cheung *et al.*, 2010).

Retinal ischemia is an important component of the pathogenesis of retinal neovascularization. Ischemia induces cellular hypoxia that in turn is responsible for a broad-range of signaling pathways to overexpress angiogenic stimulators, such as VEGF, a potent pro-angiogenic factor. In a normal retina, RPE expresses a small amount of vascular endothelial growth factor (VEGF) and high levels of pigment epithelium-derived factor (PEDF), a potent anti-angiogenic molecule. Due to hypoxia during diabetes, the balance between pro- and anti-angiogenic molecules is disrupted, leading to up-regulation of VEGF and down-regulation of PEDF. As a consequence of this imbalance, there is promotion of neovascularization and subsequent vision loss (Farjo *et al.*, 2010).

Regarding its progression, DR can be clinically classified as non-proliferative DR (NPDR), characterized by microaneurisms and damage in the retinal capillaries, and proliferative DR (PDR), which is recognizable by the presence of ischemia, neovascularization and hemorrhages, causing irreversible vision loss (Cheung *et al.*, 2010).

At present, there is no effective treatment for DR. Tight glycemic control is the most efficient therapy to slow progression of DR. Blood pressure should also be taken in consideration since hypertension aggravates the disease by increasing blood flow, and causing mechanical damage to the vascular endothelial cells, thus stimulating the production of VEGF (Fong *et al.*, 2004, Cheung *et al.*, 2010).

For advanced stages, more invasive strategies are clinically applied an attempt to block progression of the disease, such as laser photocoagulation, vitrectomy and anti-VEGF therapy.

1.3.2.1. Laser photocoagulation

Laser photocoagulation is the most widely used technique to treat proliferative DR. The aim of photocoagulation is to cause laser burns all-over the retina to help regression and slow progression of retinal neovascularization, by reducing ischemia induced by VEGF production. However, despite its efficiency preventing visual loss, the damaging nature of laser carries several ocular side-effects, including poor light-dark adaptation and decrease in visual acuity. Moreover, if the therapy is not started timely, halting of vision loss cannot be guaranteed (Cheung *et al.*, 2010).

1.3.2.2. Vitrectomy

Vitrectomy is the most effective surgical treatment for complications of advanced retinopathy, such as vitreous hemorrhage and retinal detachment. However, despite its efficiency reducing the risk of retinal neovascularization development, it markedly

increases the risk of iris neovascularization and cataract formation (Cheung *et al.*, 2010).

1.3.2.3. Anti-VEGF Therapy

VEGF is an important mediator of angiogenesis and is the main responsible for abnormal neovascularization, leakage and BRB breakdown. Inhibition of VEGF may play an important role the DR prevention. Three VEGF antagonists have been assessed in clinical trials as anti-VEGF therapy: *i)* Pegaptanib, *ii)* Ranibizumab and *iii)* Bevacizumab. These agents are delivered by intravitreal injection, to ensure maximum local efficiency with minimal systemic side effects (Shah, 2008, Cheung *et al.*, 2010).

Pegaptanib (Macugen®) is a RNA aptamer that acts by binding $VEGF_{165}$ isoform, the prevalent isoform in the human eye and principal responsible for normal and pathologic neovascularization. The binding of Pegaptanib to $VEGF_{165}$ leads to its deactivation and ultimately inhibition of vascular permeability and neovascularization (Praidou *et al.*, 2010).

Ranibizumab (Lucentis®) is a humanized recombinant monoclonal antibody fragment that recognizes all known human VEGFA isoforms (i.e. $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$) and possesses one antigen binding epitope (Shah, 2008, Praidou *et al.*, 2010).

Similarly to Ranibizumab, Bevacizumab (Avastin®) is also a humanized recombinant monoclonal antibody that binds and blocks the action of all VEGF-A isoforms but displays two antigen binding epitopes (Shah, 2008, Praidou *et al.*, 2010).

Despite its efficacy inhibiting VEGF and reducing neovascularization and its complications, the major disadvantage of these drugs is its short half-life that varies from 3 days for Ranibizumab (Bakri *et al.*, 2007), 10 days for Pagaptanib (Vinores, 2006) and 21 days for Bevacizumab (Praidou *et al.*, 2010), increasing the need of repeated injections and severe side-effects, such as cataract formation, retinal

detachment, vitreous hemorrhage, infection and loss of neural retinal cells (Cheung *et al.*, 2010).

Other strategies using VEGF receptor blockers and siRNA against VEGF mRNA have also being used in order to prevent the interaction of VEGF with its receptor, thus stopping the subsequent signaling pathway, and the production of VEGF protein (Praidou *et al.*, 2010). However, these approaches still need recurrent administrations, halting the importance of an efficient and prolonged strategy to target VEGF.

Regardless of the acquired or genetic nature of the disease, it is clear that conventional therapies are insufficient for retinal pathologies, either because they do not prevent the progression of the disease or because they treat the symptoms, not the cause. In this regard, gene therapy is a very promising approach to treat retinal pathologies, as shown by clinical trials for LCA (Maguire *et al.*, 2009) and CHM (MacLaren *et al.*, 2014). For RP, gene therapy currently represents the most promising strategy. AAVs are the most widely used vector for this purpose, due to tropism for retinal cells, lack of toxicity and absence of immune response, all features that will be later discussed (Petrs-Silva *et al.*, 2014). In the autosomal dominant forms of RP (adRP) both normal and abnormal outer segment proteins are produced in the PRs but the abnormal form of the protein is toxic, causing progressive apoptosis of PRs. Because there are several mutations associated with adRP, targeted gene elimination or repair for each separate mutation is difficult to achieve. An alternative would be to stimulate cell survival, by the use of growth factors, and anti-apoptotic therapies to delay the progression of the disease (Chaum *et al.*, 2002, Petrs-Silva *et al.*, 2014).

In autosomal recessive RP (arRP), the patient has two mutated copies of the gene. In this cases gene replacement of the mutated copies by the correct gene is the most effective strategy (Chaum *et al.*, 2002). X-linked RP is the most severe form of the disease and similarly to arRP, gene replacement is the most viable approach (Chaum

et al., 2002, Petrs-Silva *et al.*, 2014). However, for neither two forms exist yet a proposed gene therapy approach under trial.

For diabetic retinopathy, there are gene therapy strategies being studied (Igarashi *et al.*, 2003, Zhang *et al.*, 2015) that have yet to reach clinical trials. This is caused by the fact that the etiology is still not fully elucidated.

For a gene therapy strategy to be successful in treating retinal diseases, it needs to reach the therapeutic target, and for that, it needs to overcome several barriers.

1.4. Barriers to Gene Therapy

Gene delivery is considered the most challenging issue in gene therapy, as the delivery vector needs to overcome a series of extra and intracellular barriers. The next section focuses on these barriers that are common to all administration routes.

1.4.1. Extracellular barriers

Regardless of the route by which the vector is administered, it will interact with a wide-range of different molecules. Vectors administrated intravenously can easily suffer degradation by endo- and exo-nucleases present in the serum. The serum itself is composed by several proteins that can bind to vectors, resulting in aggregation, degradation and removal from the circulation by the reticuloendothelial system, affecting the stability and bioavailability of the vectors. The administration of vectors can also stimulate the immune system resulting in inflammation and complement activation, contributing for the clearance of the vectors (Gottfried *et al.*, 2013).

1.4.2. Extracellular barriers in the eye

Gene delivery through intravenous administration is often associated with reduced bioavailability due to removal from blood circulation. Apart from the blood circulation, retinal gene therapy needs to overcome various barriers present at the anterior and posterior segments of the eye that restrict the entry of naked DNA, or viral and non-

viral vectors, such as the blood-aqueous barrier, the blood-retinal barrier, the corneal barrier, and the vitreal barrier (Figure 1.5).

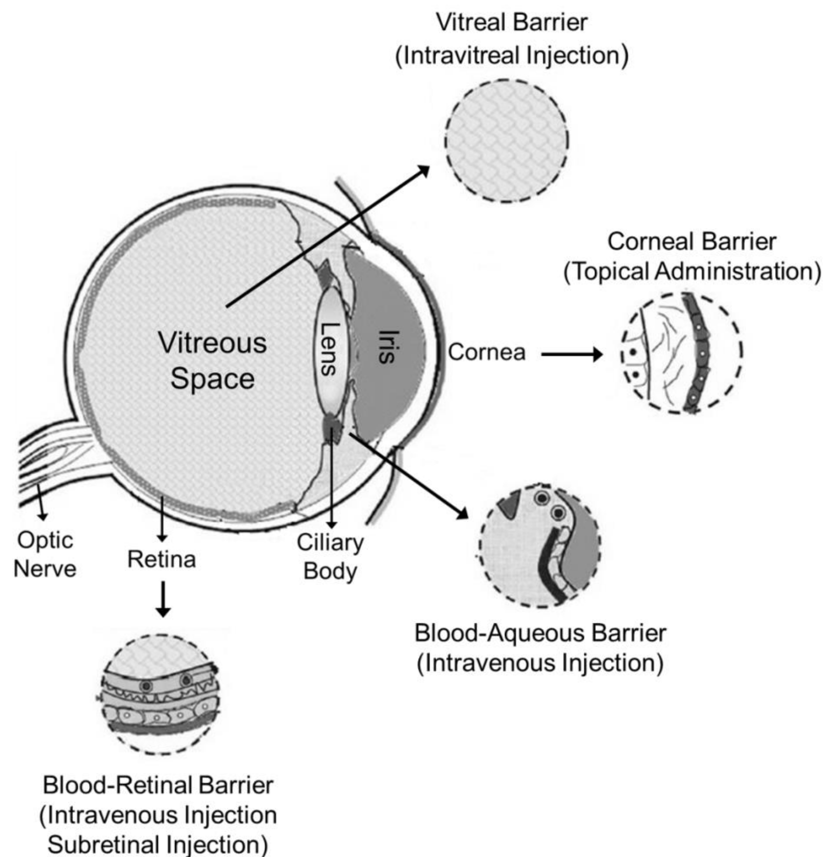

Figure 1.5: Scheme of the human eye, showing the main barriers for retinal gene delivery. The blood-aqueous barrier and blood-retinal barrier represent an obstacle to intravenous and subretinal injection. Corneal barrier is a barrier to topic administration and vitreal barrier represents a barrier after intravitreous injection. Adapted from (Naik *et al.*, 2009).

Blood-Aqueous Barrier (BAB)

The BAB is responsible for the nutrition of the cornea and lens, and its main components are the ciliary body and the iris. BAB regulates nonspecific entry of potential dangerous substances from systemic circulation to the aqueous humor. However, the BAB is less powerful than the blood-retinal barrier (Chen *et al.*, 2008, Tamboli *et al.*, 2011).

Blood-Retinal Barrier (BRB)

The BRB is responsible for the homeostasis of the neuroretina and is divided into:

i) the inner BRB (iBRB) – formed by tight junctions between endothelial cells of the retinal vessels. This barrier allows the transport of small molecules such as glucose, by facilitated diffusion, while preventing widespread diffusion of substances into the retina (Cunha-Vaz, 1979, Farjo *et al.*, 2010, Gottfried *et al.*, 2013);
ii) the outer BRB (oBRB) – formed by intracellular tight junctions in the RPE monolayer. This barrier allows bidirectional flow of essential metabolites, such as glucose, through the Na^+/K^+-ATPase pump, but restricts the passage of more complex molecules from the choroidal blood supply into the neural retina (Cunha-Vaz, 1979, Farjo *et al.*, 2010, Gottfried *et al.*, 2013).

Corneal barrier

An alternative to intravenous administration would be topical administration. However, this is not an effective way for retinal gene delivery since it comprises penetration of the cornea and diffusion from the vitreous to the retina, against the natural flow of the aqueous humour.

The cornea consists of five to seven layers, in which the outermost one is composed of six to seven layers of stratified epithelium connected by tight junctions, which act as a barrier for vectors entry, limiting the amount of vector that reaches the retina. Moreover, a great portion of the vehicle is returned to the systemic blood circulation through the conjunctival and nasal blood vessels (Naik *et al.*, 2009, Tamboli *et al.*, 2011)

Vitreal barrier

In order to overcome these barriers, administration can be local. This is achieved by subretinal and intravitreal injections, which have become the most common administration procedures for *in vivo* retinal gene delivery.

Direct injection in the subretinal space, between the retina and choroid, increases contact between the vector and the retinal layers; the contact area is however restricted to the injection site. Additionally, this type of injection can induce lesions in RPE cells and is technically challenging, restricting its clinical application.

Intravitreal injection is more acceptable for clinical applications, because it is a less invasive technique. But it is not as effective as subretinal injection, since the vectors can still be cleared by fluid flow, thus needing repeated intraocular administration to achieve therapeutic levels in the retina, which might lead to lens injury and retinal detachment. Moreover, the passage through the vitreous itself represents a barrier due to its gel-like three-dimensional net of collagen, proteoglycans, glycosaminoglycans (GAG) and serum components that are known to interact with nonviral vectors by immobilizing them in the proteoglycan mesh, binding to negative-charged GAG, causing its aggregation and blocking cellular uptake and/or intracellular trafficking (Naik *et al.*, 2009).

1.4.3. Intracellular barriers of gene delivery

The vectors that overcome the extracellular barriers and reach the target cells need to be internalized in order to ensure the genetic material is directed to the nucleus. In Figure 1.6 the intracellular barriers are represented: cellular membrane, endocytic trafficking, cytoplasmic transport, nuclear penetration, and gene expression.

The cellular membrane represents the first barrier for the vectors. DNA is an anionic molecule and cannot cross the also negatively-charged cell membrane. The use of positively-charged carriers can minimize this problem by neutralizing the negative charge of DNA, thus promoting cellular uptake by receptor-mediated endocytosis or non-specific endocytosis (Wiethoff *et al.*, 2003, Wong *et al.*, 2007).

Once inside the cell, the endocytic vesicles containing the vectors can be recycled back to the cell surface, converted into acidic vesicles (such as lysosome and phagosome), or delivered to an intracellular organelle (such as Golgi apparatus or

endoplasmic reticulum), depending on the internalization path. There are several proposed mechanisms by which the vectors can perform the endosomal escape, including membrane fusion, the proton-sponge effect, and integration of fusogenic peptides (Wong *et al.*, 2007).

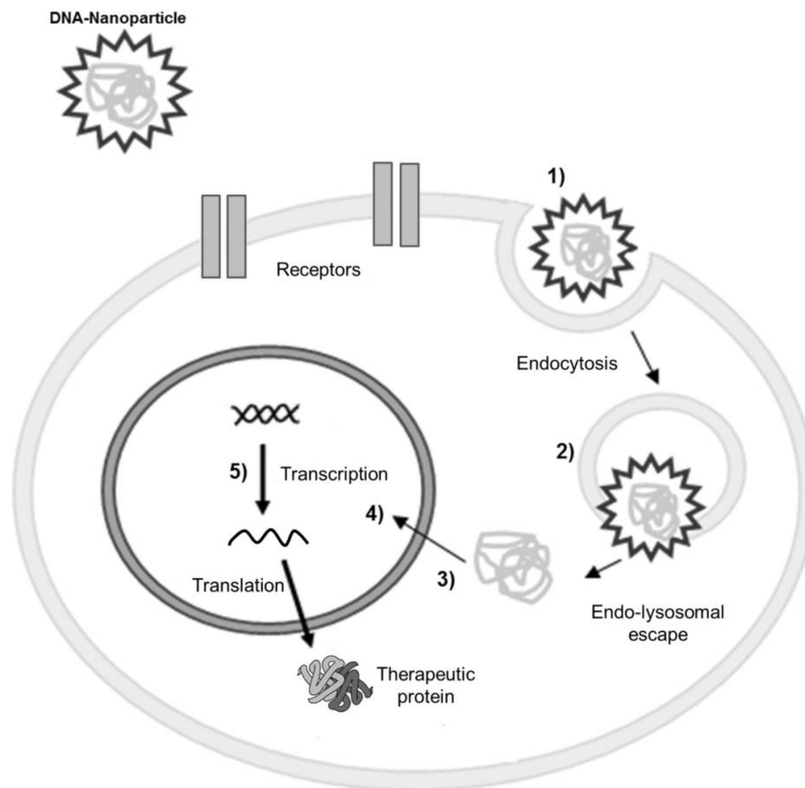

Figure 1.6: Cellular barriers to gene transfer: DNA protected by a cationic nanoparticle enters into the cell by endocytosis (1). Inside the cell, the DNA/nanoparticle complex should escape lysosomal degradation (2), cross the cytoplasm (3) and the nuclear envelope (4) and finally access to the cellular transcription machinery (5). Adapted from (Wong *et al.*, 2007).

Vectors based on cationic lipids are able to escape the endosome through fusion with the endosome membrane. In contrast, cationic polymers accomplish endosomal escape through a proton-sponge mechanism. The proton-sponge effect is based on the protonation of the amine groups of the cationic polymers, leading to an accumulation of protons. This accumulation causes influx of chloride ions that induces water entry, resulting ultimately in endosome swelling and rupture (Gottfried *et al.*, 2013).

After the DNA is successfully released, it must be directed to the nucleus. The cytosol represents another significant barrier to gene delivery. It has been postulated

that only 1-10% of the transfected plasmid reaches the nucleus (Gottfried *et al.*, 2013). This is due to the presence of nucleases in the cytosolic milieu that can degrade the naked DNA and the mechanical resistance exerted by the cytoskeleton network that impedes the diffusion of the unprotected DNA to the nucleus (Wiethoff *et al.*, 2003, Wong *et al.*, 2007). However, it is believed that several vectors interact with the cytoskeleton proteins to reach the nuclear envelope (Wiethoff *et al.*, 2003, Vaughan *et al.*, 2006).

There are at least three possible pathways for nucleic acids to enter the nucleus. The simplest way is during mitosis, when there is disruption of the nuclear envelop. If smaller enough, nucleic acids can also cross the nuclear membrane through nuclear pore complexes by facilitated diffusion or it could transverse the nuclear envelope by simple diffusion. DNA transport through nuclear pores can be even facilitated by incorporation of specific sequences recognized by nuclear pore complexes, called nuclear localization signals (NLS) (Wiethoff *et al.*, 2003, Gottfried *et al.*, 2013).

Gene therapy can constitute an efficient therapeutic strategy for the retinal pathologies described previously. In order to develop such a therapy, several points need to be taken into consideration, such as the delivery vector and gene expression system. These will be dealt with in greater detail in the following sections of this chapter.

1.5. Gene Delivery Systems

For overcoming the barriers mentioned in section 1.4 of this chapter and guarantee the success of gene therapy, the gene of interest should be efficiently delivered to the target cells. Since naked DNA is not usually internalized by the cells due to their hydrophilic nature, large size and negative charge (conferred by the phosphate groups), delivery systems have been developed to guarantee efficient gene transfer (Cevher *et al.*, 2012).

Gene delivery systems can be categorized in two major groups: viral and non-viral. Non-viral methods can be further divided into physical and chemical delivery methods (Chaum *et al.*, 2002, Cevher *et al.*, 2012).

Each method was developed and optimized to achieve therapeutic gene expression levels based on the nature of the gene to be delivered, target cells and route of administration (Chaum *et al.*, 2002). Both viral and non-viral vectors currently used possess not all characteristics of an ideal vector. Their characteristics, advantages and disadvantages for retinal use will be discussed in this section.

1.5.1. Viral-based Gene Delivery Systems

Viral vectors represent currently more than 50% of the gene therapy vehicles used in clinical trials (Figure 1.7) (Gascón *et al.*, 2013). In the retina, the most widely used viral vectors are Adenovirus (Ad), Adeno-Associated virus (AAV), Retrovirus, Lentivirus, and *Herpes Simplex* virus (HSV) (Chaum *et al.*, 2002, Cevher *et al.*, 2012).

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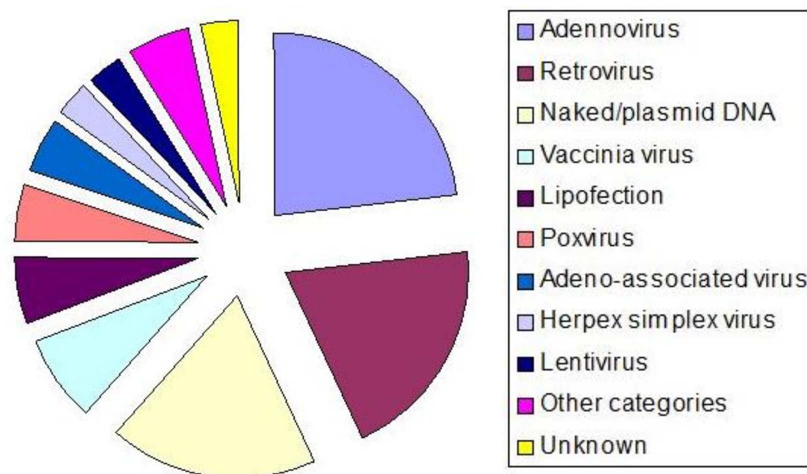


Figure 1.7: Gene delivery systems used in clinical trials. The use of naked/ plasmid DNA is one of the most widely used gene delivery systems, preceded only by Adenoviruses and Retroviruses. Adapted from (Gascón *et al.*, 2013).

The main advantage of viruses as gene vectors is their natural capacity to transduce cells, efficiently delivering their cargo into host cells, originating high levels of

gene expression (Atkinson *et al.*, 2010, Cevher *et al.*, 2012). Moreover, some viruses display cellular tropism, such as HSV for neuronal cells (Chaum *et al.*, 2002) and some AAV serotypes for retinal cells (Rabinowitz *et al.*, 2002), allowing for a targeted therapy. Disadvantages associated with viral vectors are their immunogenicity, toxicity, tumorigenicity, limited packaging capacity, and difficulties in large-scale production (Naik *et al.*, 2009, Cevher *et al.*, 2012)

1.5.1.1. Adenoviruses

Adenoviruses are well characterized, non-enveloped, non-integrative, and linear double stranded DNA (dsDNA) viruses that are able to transduce a huge variety of human and non-human cells by binding to specific receptors, such as Coxsackievirus and Adenovirus Receptor (CAR) and Toll-Like Receptor-4 (TLR4) (Chaum *et al.*, 2002, Cevher *et al.*, 2012). In the retina, Adenovirus vectors have been efficiently used to transduce PR (Anglade *et al.*, 1998, Bennett *et al.*, 1998, Von Seggern *et al.*, 2003), GC (Bennett *et al.*, 1994, Cayouette *et al.*, 1997), Müller cells (Di Polo *et al.*, 1998, Fukuhara *et al.*, 1998, Zhou *et al.*, 2014) and RPE cells (da Cruz *et al.*, 1996, da Cruz *et al.*, 1998, Lam *et al.*, 2014).

These viruses are capable of incorporating DNA fragments up to 36 kb (Cevher *et al.*, 2012) but it remains as an episomal particle (since it is not integrated into the cell genome), being eventually silenced or lost, with gene expression decaying in few weeks (Bennett *et al.*, 1994, Jomary *et al.*, 1994). Repeated administrations lead to the most serious problem associated in using Adenovirus vectors: the strong immune and inflammatory response that hampers gene expression (Chaum *et al.*, 2002, Cevher *et al.*, 2012).

1.5.1.2. Adeno-Associated Virus

Adeno-Associated Virus (AAV) vectors are non-enveloped, single stranded DNA (ssDNA) viruses that, similarly to Adenovirus, are able to infect both mitotic and post-

mitotic cells persisting as an episomal particle, without integration into host cell genome. However, the presence of the *rep* protein in 10% of transduction events enables AAVs to integrate into host genome in a specific *locus* of chromosome 19, allowing a stable transgene expression and, simultaneously decreasing the possibility of random insertional mutagenesis (Kay *et al.*, 2001). The main disadvantage of using AAV is however its limited packaging capacity of 5 kb (Chaum *et al.*, 2002).

In the retina, AAVs have found widespread use, being used to transduce PR, GC and RPE cells and are currently being used in more than one clinical trial for congenital blindness (Bainbridge *et al.*, 2008, Hauswirth *et al.*, 2008, Maguire *et al.*, 2009, MacLaren *et al.*, 2014).

1.5.1.3. Retroviral vectors

Retroviruses are a family of diploid ssRNA that have the ability to infect and randomly integrate its genome into the host cells' genome to produce new viral particles, being associated with insertional mutagenesis and tumor formation (Kay *et al.*, 2001, Chaum *et al.*, 2002, Cevher *et al.*, 2012). The possibility of generating wild-type (WT) HIV virus during titer production or by viral recombination are also disadvantages of these vectors (Chaum *et al.*, 2002).

The retroviral family is divided into gammaretrovirus and lentivirus, which differ in their capacity to infect only post-mitotic or any cell, respectively.

Retroviral and lentiviral vectors can both pack up to 8 kb of exogenous DNA, but retroviral vectors require cell division to transduce the host cell. Since most retinal cells are post-mitotic, these vectors are not considered the ideal vector for retinal gene therapy, where most of the cells are post-mitotic (Kay *et al.*, 2001, Chaum *et al.*, 2002, Cevher *et al.*, 2012).

Unlike retrovirus, lentiviruses can infect post-mitotic cells. Depending on the proteins present in the viral envelope, it is possible to construct "pseudo-typed" lentivirus to obtain a targeted gene transfer (Chaum *et al.*, 2002, Cevher *et al.*, 2012,

Nayerossadat *et al.*, 2012). These pseudo-typed lentiviruses have been used to transduce a broad range of retinal cells (Miyoshi *et al.*, 1997, Bainbridge *et al.*, 2001, Bemelmans *et al.*, 2005).

1.5.1.4. Herpes Simplex Virus

Herpes Simplex Viruses (HSV) are DNA virus with a natural tropism for neuronal cells, representing a great advantage for gene therapy of diseases affecting the nervous system, including the retina (Nayerossadat *et al.*, 2012). Besides neurons, HSV virus is also able to infect other cell types and does not need nuclear envelope disruption to reach the nucleus, where its DNA recircularizes and is maintained as an episome (Chaum *et al.*, 2002).

This viral class is the one with the highest packaging capacity, being able to carry up to 150 kb of transgenic DNA (Nayerossadat *et al.*, 2012).

However, infection with HSV is usually associated with inflammation, cytotoxicity and cell death. For this, it has been mostly used for suicide gene therapy (Chaum *et al.*, 2002).

1.5.2. Non-viral Gene Delivery

Despite the advantages in terms of transduction efficiency and gene expression, safety issues of viral vectors led the continuous search and development of alternative delivery systems. Non-viral vectors emerge as an alternative to viral vectors, due the lack of specific immune response, no limitation in transgene size, ease of large-scale and low cost of production (Niidome *et al.*, 2002, Nayerossadat *et al.*, 2012). However, the major limitation of using non-viral systems is the low transfection efficiency, precluding transgene expression at therapeutic levels (Cevher *et al.*, 2012, Nayerossadat *et al.*, 2012).

Non-viral gene therapy can be classified as physical and chemical depending on how the transgene is delivered.

1.5.2.1. Physical methods for gene delivery

The most basic form of gene therapy is the administration of naked DNA. However, it is easily degraded by nucleases in the serum and cleared by the monocytes and macrophages. Moreover, naked DNA alone is not able to cross the cell membrane, resulting in low expression and limited transfected area. The use of physical delivery methods can increase the transfection efficiency of naked DNA (Niidome *et al.*, 2002, Cevher *et al.*, 2012).

Gene delivery using physical methods takes advantage of a physical force to disturb the cell membrane allowing intracellular gene transfer (Cevher *et al.*, 2012, Ramamoorth *et al.*, 2015). These methods include needle injection, gene gun, electroporation, sonoporation, photoporation, magnetofection, and hydroporation (Figure 1.8) (Gascón *et al.*, 2013).

Needle injection

DNA is locally administered using a needle system. This technique has been used to transfect tissues such as muscle, skin, liver, cardiac muscle and tumours but the efficiency of needle injection is limited to the injection area and the DNA is often degraded by the mononuclear phagocytic system (Gascón *et al.*, 2013, Ramamoorth *et al.*, 2015).

Gene gun

Gene gun is an alternative to DNA injection, developed for gene delivery to plant cells, which possess a cell wall. In gene gun procedures, DNA-coated gold, silver or tungsten particles are accelerated to high speed to penetrate the target cells of the tissues, skipping the endocytosis step and lisossomal degradation. This technique allows the administration of precise amounts of DNA. The main disadvantages of this method are high cost of the system, the transient, and low gene expression and the superficial penetration of the DNA into the tissue, only transfecting cells at the surface

(Niidome *et al.*, 2002, Nayerossadat *et al.*, 2012, Gascón *et al.*, 2013, Ramamoorth *et al.*, 2015).

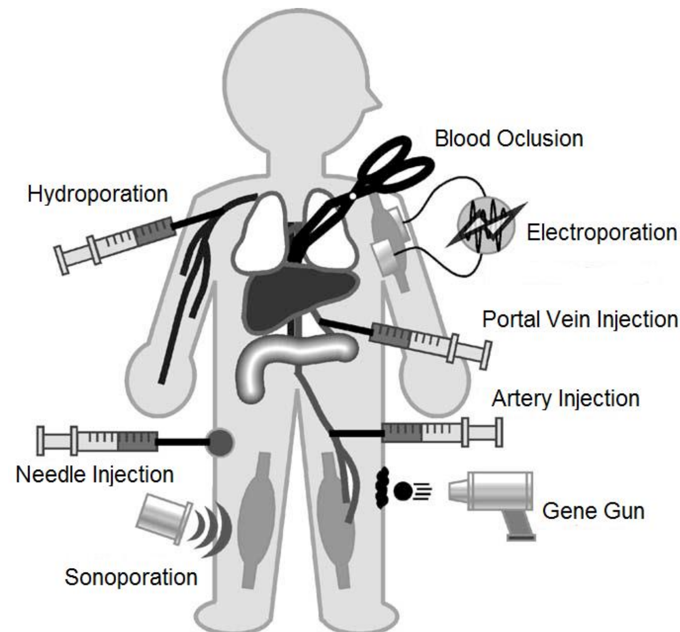


Figure 1.8: Overview of physical methods for gene delivery. Adapted from (Niidome *et al.*, 2002).

Electroporation

Electroporation is used to enhance gene uptake after local naked DNA injection. It acts by generating a transient permeability on the cell membrane through electric pulses that create pores. Pore formation occurs in approximately 10 nanoseconds and their size is estimated to be smaller than 10 nm in diameter. Small molecules such as nucleic acids can pass through these pores and travel from the cytoplasm towards the nucleus (Niidome *et al.*, 2002, Ramamoorth *et al.*, 2015).

In vivo, electroporation has been extensively used for skin (Denet *et al.*, 2004), muscle (Schertzer *et al.*, 2008), lung (Zhou *et al.*, 2008), corneal (Zhou *et al.*, 2007) and retinal gene therapy (Matsuda *et al.*, 2004, de Melo *et al.*, 2011), as well as vaccination (Sardesai *et al.*, 2011) and tumor treatment (Al-Sakere *et al.*, 2007).

Electroporation is a reliable and efficient non-viral method for gene delivery, showing transfection levels comparable to viral vectors (Cevher *et al.*, 2012, Ramamoorth *et al.*, 2015). The drawbacks associated with this method rely on the

depth of penetration of the electric pulse and potential tissue damage associated with the technical procedure, as well as some discomfort to the patient (Nayerossadat *et al.*, 2012).

Sonoporation

In sonoporation, ultrasounds are used to permeabilize the cell membrane for DNA uptake in a non-invasive and site-specific way (Gascón *et al.*, 2013).

The transfection efficiency of sonoporation is dependent on the frequency, time of treatment, size, and concentration of DNA, but it has been reported a 10-fold increase in gene expression after ultrasound irradiation, compared with naked DNA. The transfection efficiency can even be improved by the combination of ultrasounds with microbubbles. This system is usually used in brain, cornea, kidney, skeletal and heart muscle (Niidome *et al.*, 2002, Cevher *et al.*, 2012, Gascón *et al.*, 2013). The major disadvantage of this technique is its low efficiency *in vivo* (Nayerossadat *et al.*, 2012).

Photoporation

Photoporation uses a single laser pulse to generate transitory pores on the cell membrane, leading to DNA entry. The efficiency of the system is controlled by the size of the focal point and pulse frequency of the laser. The gene expression is described to be similar to that of electroporation but an in depth study on its efficiency has yet to be performed (Nayerossadat *et al.*, 2012, Gascón *et al.*, 2013).

Magnetofection

Gene transfer by magnetic force that uses a magnetic field to accomplish gene transfer. DNA is complexed with magnetic nanoparticles (NPs) of iron oxide and coated with cationic lipids or polymers. This technique produces a rapid and efficient transfection, even in cells that are known to be difficult to transfect by other means, such as primary cells (Nayerossadat *et al.*, 2012, Gascón *et al.*, 2013). *In vivo*,

magnetofection has been used for gene and drug delivery to the brain, blood vessel endothelium, lung, liver and several carcinoma xenografts in mice (Plank *et al.*, 2011).

Hydroporation

Also known as hydrodynamic gene transfer, hydroporation is a technique that uses hydrodynamic pressure of the injection to increase the permeability of the cell membrane. It allows the injection of a large amount of DNA in few seconds (Niidome *et al.*, 2002). This technique is usually applied in tail vein injection of rodents and is very effective for liver cells transfection, due to direct entry of the DNA into the inferior vena cava that stretches the myocardial fibers, inducing cardiac congestion, and driving the injected DNA solution into the liver in retrograde (Suda *et al.*, 2007). The disadvantage of this method is the high injection volume that can cause damage to the tissues (Nayerossadat *et al.*, 2012, Gascón *et al.*, 2013).

1.5.2.2. Chemical methods of gene delivery

For gene delivery, chemical methods are more commonly used than physical ones (Nayerossadat *et al.*, 2012). Gene carriers can be designed to simultaneously *i)* protect the therapeutic gene from blood components and nucleases, *ii)* to target a specific cell type, *iii)* to increase the delivery of the DNA to the cytoplasm or to the nucleus, *iv)* to be able to dissociate from the DNA in the cytosol, and also *v)* to release the cargo in the tissue in a continuous and controlled way (Niidome *et al.*, 2002).

For these purposes, different chemical systems have been developed, such as inorganic particles, cationic lipid-based particles, polymer-based particles, and synthetic peptides. Among these, polymer- and lipid-based systems are the best studied and characterized (Nayerossadat *et al.*, 2012, Ramamoorth *et al.*, 2015) and will be briefly addressed in this section.

Inorganic particles

Inorganic particles are nanostructures that can be obtained from metals (such as gold, silver and iron), inorganic salts, and ceramics (like calcium-phosphate and silica). Calcium phosphate nanoparticles were the first inorganic particles used for gene therapy (Ramamoorth *et al.*, 2015). These particles have the advantage of being easily engineered in size, shape, porosity, and surface-functionalized to escape the reticuloendothelial system and to protect the genetic material from degradation or denaturation (Gascón *et al.*, 2013).

Cationic Lipids

Cationic lipids are amphiphilic molecules containing a hydrophilic positive-charged head and a hydrophobic tail. They promote DNA complexation by electrostatic interactions between the negative charge of DNA (conferred by the phosphate groups) and the positive charge of the positive head (that are usually amine groups). The complexation of a positive-charged lipid with the negative-charged DNA is denominated lipoplex, in which DNA is encircled by the positive lipid (Gascón *et al.*, 2013, Ramamoorth *et al.*, 2015).

The transfection efficiency of the lipoplex depends on the shape, number of charged groups per molecule, and the charge ratio to produce the lipoplexes. Due to its positive charge, lipoplexes can interact electrostatically with negative-charged proteins and proteoglycans present in the membrane of the cells, thus promoting the uptake of the complex. The complex is then internalized into the cell by the vesicular path, followed by the release of DNA from the lipoplex into the cytosol that hopefully will reach the nucleus to be transcribed and translated into a therapeutic protein (Cevher *et al.*, 2012).

The limitations of using these molecules for gene therapy are the structural instability of the lipoplexes, aggregation, low transfection efficiency, poor targeting ability, toxicity, and fast clearance from the circulation. Some strategies have been

used to avoid some of this disadvantages caused by the excessive charge-charge interaction (Cevher *et al.*, 2012, Gascón *et al.*, 2013, Ramamoorth *et al.*, 2015). These include combination of the neutral polymer polyethylene glycol (PEG) that acts like a shield from the immune system, prolonging the half-life of the circulating lipoplexes (Gascón *et al.*, 2013).

Cationic polymers

Polymers are long-chain structures composed by small repeating molecules, named monomers. Polymers composed by the same monomer are denominated homopolymers and polymers composed by different monomers are called copolymers (Cevher *et al.*, 2012).

According to its origin, polymers can be classified as natural or synthetic polymers. Regarding the mechanism of release, polymers can either be classified as degradable or non-degradable. Biodegradable polymers (such as chitosan, starch, polyamides, and dextran, among others) suffer chemical or physical reactions in biological environments, leading to chain shortening. In contrast, non-biodegradable polymers (like PEG, Polyethylenimine (PEI), and Poly(2-(dimethylamino)ethylmethacrylate) (pDMAEMA)) are not metabolized in biological environments, and therefore remain unchanged (Cevher *et al.*, 2012, Ramamoorth *et al.*, 2015).

For the success of gene transfer, the polymer-based delivery system must protect the negative-charged DNA backbone from repulsion from anionic cell surface, condense the DNA as much as possible to facilitate endocytosis and phagocytosis, and should also protect DNA from nuclease degradation (Niidome *et al.*, 2002, Cevher *et al.*, 2012). While attempting to improve the performance of those systems, a direct relationship between the length of the polymer, gene transfer efficiency, and toxicity was found: as the length of the polymer increases, the transfection efficiency increases, but so does toxicity (Cevher *et al.*, 2012).

It has recently been shown that these systems can be used as gene delivery systems for ocular gene delivery. No toxic effects due to the particles were observed and the retinal structure and function was maintained. The well-tolerated nature of these polyplexes suggests they are promising candidates for gene transfer to the retina and RPE (de la Fuente *et al.*, 2008, Han *et al.*, 2012). The only obstacle remaining to be overcome is the relative low transfection efficiency and transient gene expression, due to epigenetic silencing of the expression vector (Bloquel *et al.*, 2006). On the former, there are several groups working towards optimized delivery vectors. The latter will be addressed later in this chapter.

Peptide-mediated gene transfer

Cationic peptides, such as lysine and arginine have also been widely used for gene therapy and some studies showed they can even be more efficient that polyplexes and lypoplexes in protecting the cargo, targeting cell-specific receptors, disrupting endosomal membrane and delivering the genetic material. They can also be used to functionalize polyplexes and lypoplexes enabling a targeted therapy or promoting the transport of genetic material into the nucleus, known as nuclear localization signal (NLS) peptides. (Niidome *et al.*, 2002, Ramamoorth *et al.*, 2015). Despite all the advantages, the use of synthetic peptides for gene delivery is limited by high cost production and purification. Moreover there are no studies showing the ability of these systems to deliver large genes.

1.6. Optimization of expression vectors for long-term gene therapy

Besides the gene carrier, another factor that has to be taken into account for the success of gene therapy is the long-term and sustained transgene expression.

Plasmids are widely used as expression vectors for non-viral gene therapy. They are easy to construct and allow large-scale propagation. Since its genomic integration is negligible, they display an excellent safety profile. More importantly, plasmids have a

virtual unlimited packaging capacity and can accommodate large fragments of DNA (Williams *et al.*, 2011).

A plasmid is a circular dsDNA molecule that can be found in almost all bacteria and is usually responsible for antibiotic resistance (Williams *et al.*, 2011). Two distinct parts compose plasmid DNA (pDNA): *i)* the transcription cassette, and *ii)* the bacterial backbone (Figure 1.9).

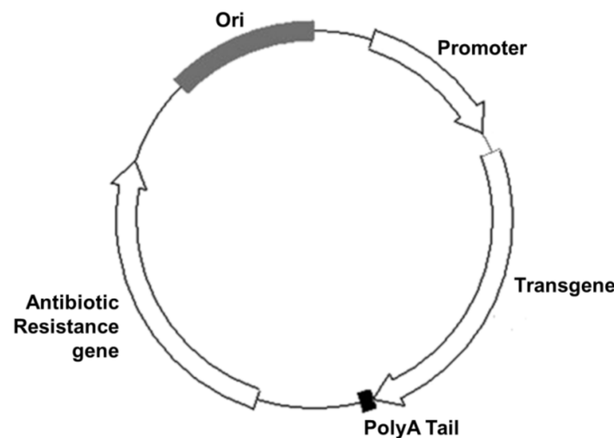


Figure 1.9: pDNA for gene therapy and its main components: origin of replication (Ori), promoter, transgene, polyA tail and antibiotic resistance gene. Adapted from (Mayrhofer *et al.*, 2009).

The transcription cassette carries the target gene and its regulatory elements, like the promoter and enhancers. The bacterial backbone contains all the machinery required for bacterial propagation, such as origin of replication (Ori) and antibiotic resistance gene (Mayrhofer *et al.*, 2009).

The effectiveness of gene therapy is compromised by the transient expression of genes due to pDNA loss during mitosis and gene silencing by epigenetic silencing events (Yew *et al.*, 2002, Jackson *et al.*, 2006, Hyde *et al.*, 2008, Gill *et al.*, 2009). The mechanism by which pDNA vectors are often silenced is still not fully understood. However, several studies point to immunostimulatory properties of pDNA, in particular its bacterial backbone. Bacterial DNA contains a high percentage of unmethylated CpG motifs responsible for the activation of innate immune response. The recognition of these unmethylated motifs by the mammalian host leads an inflammatory response,

including activation of B cells, monocytes, macrophages, dendritic cells and natural killer cells. This contrasts with eukaryotic cells, where the CpG content is much lower and these islands are usually methylated. Therefore, the removal of bacterial elements can improve the safety and efficiency of pDNA for gene therapy (Mayrhofer *et al.*, 2009), and this led to the development of minicircles, MIDGE, and pFAR expression vectors, which will be further described.

1.6.1. Minicircle: a plasmid devoid of bacterial backbone

Minicircle DNA (mcDNA) is a supercoiled recombinant DNA molecule that only contains the expression cassette with the promoter, the transgene and the polyA tail (Mayrhofer *et al.*, 2009). Minicircles were firstly described in 1997 by Darquet *et al* (Darquet *et al.*, 1997) and have since been extensively used as vector for gene therapy (Darquet *et al.*, 1999, Chen *et al.*, 2003, Vaysse *et al.*, 2006, Wu *et al.*, 2006, Schuttrumpf *et al.*, 2008, Zhang *et al.*, 2008, Huang *et al.*, 2009, Stenler *et al.*, 2009, Yoon *et al.*, 2009, Jia *et al.*, 2010, Osborn *et al.*, 2011, Viecelli *et al.*, 2014). In all studies, minicircles displayed greater and longer transgene expression than the parental plasmid.

mcDNA is originated from recombination of parental plasmid (PP). Conversion of PP into mcDNA is a complex process that implies an external stimulus to induce bacteria to express the genes encoding the recombination machinery, followed by the recombination step (Gaspar *et al.*, 2015). The expression cassette present in the PP is flanked by two recombinase recognition sequences that after recombination originate two supercoiled DNA molecules derived from PP: *i)* a mcDNA molecule, containing the therapeutic expression cassette and *ii)* a miniplasmid (MP) containing the undesired bacterial backbone (Figure 1.10) (Mayrhofer *et al.*, 2009).

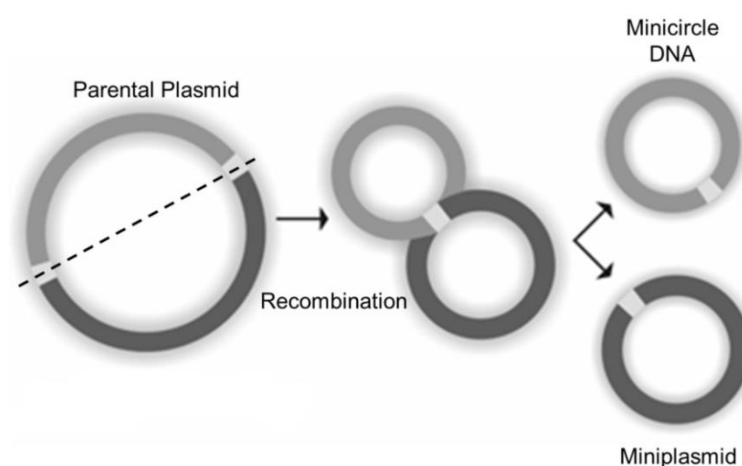


Figure 1.10: Minicircle production: after the recombination process, the parental plasmid originates a mcDNA molecule and a miniplasmid (MP) molecule. Adapted from (Mayrhofer *et al.*, 2009).

The yield of mcDNA is highly affected by the recombination step that, ideally, should convert 100% of the PP to mcDNA molecules. However, this is not observed and the contamination of the mcDNA preparation with PP and MP vectors is very inconvenient from a therapeutic point of view. Therefore, after recombination a purification step is required to eliminate the remaining PP and the MP originated from the recombination, thus isolating the mcDNA molecules. Different integrases have been used to increase the effectiveness of the recombination process, such as Phage λ integrase, Phage P1 Cre recombinase, ParA resolvase and PhiC31-integrase/I-SceI homing endonuclease system (Mayrhofer *et al.*, 2009, Gaspar *et al.*, 2015).

1.6.1.1. Phage λ Integrase

Phage λ integrase is a tyrosine recombinase that catalyzes conservative recombination. Its natural function is to perform integration of the λ phage into host cell genome (Gaspar *et al.*, 2015).

In the mcDNA production context, λ integrase was firstly used by Darquet *et al.* in where the recombinase was expressed endogenously by the bacteria, with the PP containing the hybrid *attP/attB* inserts flanking transcription cassette (Darquet *et al.*, 1997, Gaspar *et al.*, 2015).

The major disadvantage of this system was the low yield of PP converted to mcDNA (40-70% recombination) due to the intrinsic toxicity of the λ integrase system. Therefore, the final mcDNA preparation was significantly contaminated with the PP template (Gaspar *et al.*, 2015).

1.6.1.2. Phage P1 Cre Recombinase

Phage P1 Cre recombinase promotes bidirectional and site-specific recombination while it binds to its *loxP* recombination sites (Mayrhofer *et al.*, 2009, Gaspar *et al.*, 2015).

Its major disadvantage lies on the bidirectional and reversible recombination that can lead to the production of unwanted PP, MP, and concatamers (Mayrhofer *et al.*, 2009, Gaspar *et al.*, 2015). This can be avoided by adding a mutated recombination site ensuring a unidirectional recombination (Gaspar *et al.*, 2015).

Even after this improvement, the conversion yield is not 100% and PP and MP are still present. An additional purification step was developed involving restriction endonuclease digestion of PP and MP after recombination and (CsCl)-ethidium bromide density centrifugation. However, due to its high cost, low yield, and poor scalability for pharmaceutical applications, this option was discarded (Gaspar *et al.*, 2015).

1.6.1.3. ParA Resolvase

ParA is a serine recombinase that catalyzes a unidirectional, conservative, site-specific intramolecular recombination by binding to a target sequence containing two identical hybrid binding sites. With this system, no concatemerization was observed and the yield of recombination was about 99.5% (Gaspar *et al.*, 2015).

After recombination, the minicircle was purified using an affinity chromatography based on the recognition between LacO (present in the mcDNA) and its repressor LacI (present in the chromatography matrix). Only mcDNA containing these recognition sites

can bind to the matrix, making this system a promising platform for the large scale manufacture, and purification of clinical-grade mcDNA (Mayrhofer *et al.*, 2009, Gaspar *et al.*, 2015).

1.6.1.4. PhiC31-integrase/I-SceI Homing System

The bacteriophage ΦC31 integrase is a serine recombinase that promotes irreversible recombination between its *attP/attB* recombination sites, using catalytic and DNA-binding domains to control the procedure (Gaspar *et al.*, 2015).

With this system, the recombination sites in the PP are under tight control of the inducible pBAD/AraC protomer, allowing a conversion to mcDNA higher than 97% (Gaspar *et al.*, 2015). The purification step is performed by co-expression of an endonuclease (I-SceI) that degrades both PP and MP remaining after recombination (Mayrhofer *et al.*, 2009).

Overall, mcDNA have shown great promise improving both expression levels and duration of expression. The elimination of the immunostimulatory CpG motifs present in the bacterial backbone together with its small size, known to facilitate the diffusion of the DNA through the cytoplasm and enhance its passage through the nuclear pores, seems to have a significant impact on mcDNA performance. However, further manipulation and monitoring of the process concerning the purification step is fundamental for clinical application (Gaspar *et al.*, 2015).

1.6.2. MIDGE vectors

Minimalistic, Immunogenically Defined Gene Expression (MIDGE) vectors are linear molecules containing just the transcription unit (promoter, transgene and polyA tail) flanked by two hairpin oligonucleotide sequences, arranged in a covalently closed dumbbell-shaped molecule (Figure 1.11) (Schakowski *et al.*, 2001, Machelska *et al.*, 2009).

These systems are obtained from the enzymatic digestion of a template plasmid and subsequent ligation of the resulting fragments to hairpin oligonucleotides. Afterwards, MIDGE vectors are purified by anionic exchange column chromatography (Schakowski *et al.*, 2001, Machelska *et al.*, 2009). MIDGE vectors have been used mostly for prophylactic genetic vaccination purposes (Moreno *et al.*, 2004, Endmann *et al.*, 2010).

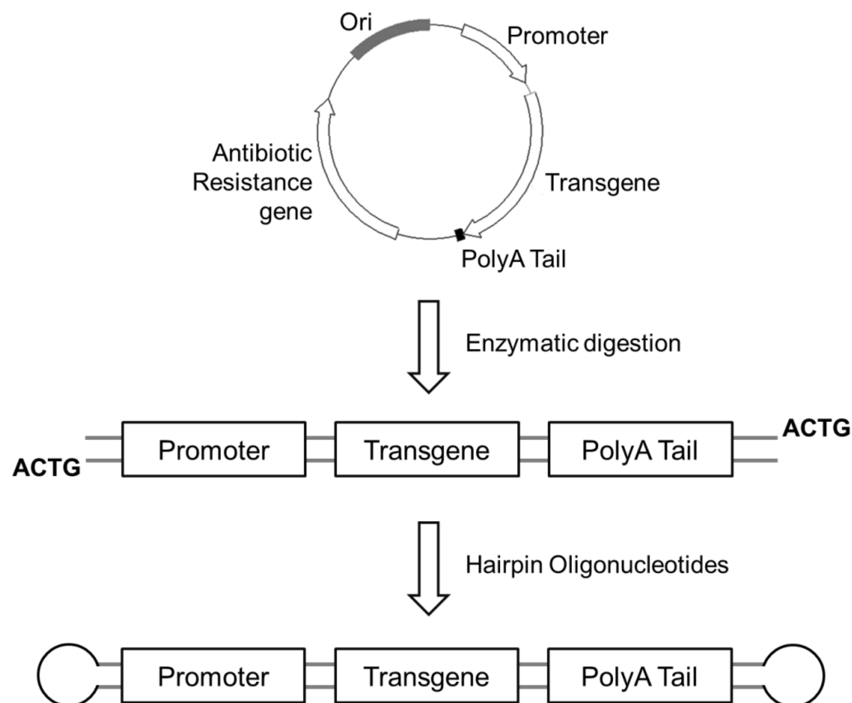


Figure 1.11: MIDGE vectors production. MIDGE vectors are obtained from enzymatic digestion of the template plasmid following by hairpin oligonucleotide ligation. Adapted from (Machelska *et al.*, 2009).

Like mcDNA vectors, MIDGE vectors display several advantages over traditional plasmids such as their small size and low content in CpG motifs, due to the absence of bacterial backbone. Hairpin oligonucleotides present in MIDGE vectors can be functionalized by anchoring molecules such as peptides, proteins or sugars (Moreno *et al.*, 2004). Examples of molecules that can be added are NLS peptides to target DNA to the nucleus (Machelska *et al.*, 2009).

1.6.3. Plasmids Free of Antibiotic Resistance Gene (pFARs)

Although mcDNA and MIDGE vectors lack antibiotic resistance gene, the PP is produced by dividing bacteria in the presence of antibiotics for selection. Besides unmethylated CpG motifs, the use of antibiotic resistance genes can contribute to the emergence of multidrug-resistance organisms, thus compromising the safety of the gene therapy (Marie *et al.*, 2010). For that reason, plasmids free of antibiotic resistance genes (pFAR) were developed. There are several systems to replace antibiotic resistance gene, like auxotrophy complementation (AC), post-segregational killing (PSK) and operator-repressor titration (ORT) (Dong *et al.*, 2010).

The AC system uses an auxotrophic strain mutated for a crucial metabolite. In the absence of the specific metabolite, only strains having the plasmid encoding a tRNA allowing the translation of the metabolite can grow (Marie *et al.*, 2010).

The PSK system is dependent of the equilibrium between toxin and antitoxin encoded by genome and plasmid, respectively. If a cell loses the plasmid, the antitoxin will be degraded and the cell killed by the toxin encoded by its genome. However, this type of system is unable to maintain the plasmid during long-term bacterial culture (Dong *et al.*, 2010).

Finally, the OTR system uses plasmids with *lac* operator sequences that encode a repressor bound to an essential promoter or operator region (Marie *et al.*, 2010). In case of plasmid loss, there is no expression of the repressor leading to bacteria death (Dong *et al.*, 2010).

1.6.4. Self-replicating episomal plasmids

One of the major disadvantages of the non-viral gene therapy systems is their loss during mitosis, due to their inability to replicate in mammalian cells. Therefore, self-replicating extrachromosomal systems represent several advantages over other non-viral and also viral vectors.

Episomal vectors can be categorized in three general groups: *i)* self-replicating viral-based vectors, *ii)* chromosome-based vectors and *iii)* vectors containing S/MARs.

1.6.4.1. Self-replicating viral-based vectors

Epstein Barr virus (EBV) is a human herpes virus that can be maintained episomally in dividing cells. The maintenance is achieved by the expression of viral proteins, such as EBV nuclear antigen 1 (EBNA1) that allows the replication of the viral genome and its retention in the nucleus (Conese *et al.*, 2004).

Simian Vacuolating 40 (SV40) virus has the ability to replicate its genome episomally in eukaryotic cells through the interaction with the virally encoded *trans*-acting factor large T-antigen (Piechaczek *et al.*, 1999).

Inclusion of these sequences in common plasmids allows them to replicate and be maintained in dividing cells. However, the presence of these viral elements can induce immortalization and tumor formation in transfected cells and have therefore reduced therapeutic usefulness (Piechaczek *et al.*, 1999, Conese *et al.*, 2004).

1.6.4.2. Chromosome-based vectors

Unlike self-replicating viral-based vectors previously described, chromosome-based vectors do not require viral proteins for their functioning. They behave like natural chromosomes and are able to be stably maintained at a low and defined copy number in the cell in the absence of selection. The cloning capacity of these expression systems is infinite, allowing not only the insertion of the gene of interest but also promoters and all the elements required for a regulated and cell specific expression of the transgene (Lipps *et al.*, 2003, Conese *et al.*, 2004). The major drawback related with this system relates to the difficulty of finding a carrier to efficiently deliver such large artificial DNA chromosomes (Conese *et al.*, 2004).

1.6.4.3. Episomal vectors containing S/MARs

Scaffold/Matrix Attachment Regions (S/MARs) are AT-rich DNA sequences that anchor chromosomal loops to the nuclear matrix (Bode *et al.*, 2000, Giannakopoulos *et al.*, 2009). They are usually found at the borders of chromatin domains, either in non-transcribed regions or within transcription units (close to promoters, enhancers and ORIs), but not in coding regions, suggesting they may act to link those regions to matrix-bound DNA/RNA enzymatic machineries (Bode *et al.*, 2000, Giannakopoulos *et al.*, 2009).

S/MARs seem to be involved in DNA duplex destabilization and strand opening, suggesting an involvement in DNA replication and gene expression (Giannakopoulos *et al.*, 2009), since the transition from the double stranded state to open stranded is required for replication and transcription (Giannakopoulos *et al.*, 2009).

Some viruses, such as the SV40 virus, contain S/MARs in their genome, which are part of the large T-antigen coding region. Without S/MARs, the virus is unable to maintain the episomal status for a prolonged period of time (Bode *et al.*, 2000).

Additionally, S/MARs-containing vectors are able to prevent epigenetic silencing by shielding the transgene sequence from adjacent regulatory sequences and heterochromatinization. This feature allows the maintenance of the vector in a transcriptional active state, conferring mitotic stability (Wong *et al.*, 2011). S/MARs also mediates the association of the episome with the metaphase scaffold and facilitate the use of the centromere from the chromosome of host cells (Figure 1.12) (Bode *et al.*, 2000).

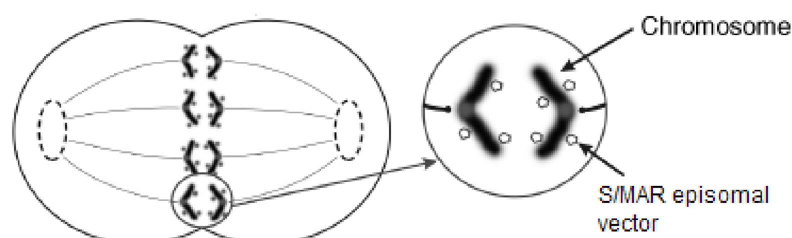


Figure 1.12: Mechanism of retention of S/MAR-based vectors in mitotic cells. Adapted from (Lufino *et al.*, 2007).

pEPI-1 was created by Piechaczek and coworkers and seems to be an alternative for gene transfer in mammals, allowing a stable maintenance in cells for about 100 generations (Piechaczek *et al.*, 1999). In pEPI-1, the gene coding for the SV40 T-antigen was replaced by the S/MAR from the 5'-region of the human interferon β-gene (Piechaczek *et al.*, 1999). The original pEPI-1 vector contains two mammalian transcription units and a total of 305 CpG motifs, most of them located in the elements of the vector required for bacterial propagation. In order to reduce the CpG content in the bacterial backbone of pEPI-1and achieve increased transgene expression *in vitro* and *in vivo*, a new S/MAR-based vector, named pEPito, was created (Haase *et al.*, 2010). pEPito was constructed by cloning the pEPI-1 plasmid replicon in a plasmid backbone lacking CpG motifs and excluding the second transcription unit (Haase *et al.*, 2010). Thus, pEPito contains only 37 CpG motifs (Haase *et al.*, 2010). These plasmids, containing a CpG-rich transcription unit in a CpG-depleted bacterial backbone, seem to show similar expression patterns to minicircles and other CpG-free plasmids (Haase *et al.*, 2010). Several other modifications have been made in order to try to improve S/MAR-containing plasmids, as depicted in table 1.

The replacement of the CMV promoter by CAG promoter, hCMV/EF1 or tissue-specific promoters were found to be crucial to avoid the epigenetic silencing of the promoter due to cytosine methylation (Hagedorn *et al.*, 2012). Moreover, plasmids containing less CpG motifs in the backbone, such as pEPito vector, showed improved results compared with the original pEPI-1 vector (Haase *et al.*, 2010).

Likewise, minicircles containing S/MARs presented an increased and sustained expression, being maintained episomally with high establishment rates both *in vitro* and *in vivo* (Argyros *et al.*, 2011).

Additionally, plasmids containing S/MARs are currently being used to improve viral vectors, such as lentiviral vectors (Verghese *et al.*, 2014) and adenoviral vectors (Voigtlander *et al.*, 2013) in order to achieve a safe and efficient expression system for gene therapy.

Table 1: Modifications of the original pEPI-1 applied to the promoter, backbone and transgene

Plasmid	Backbone	Promoter	Transgene	Expression Time	Reference
pEPI-1	pEPI-1	CMV	eGFP	2 months 1 month	(Piechaczek *et al.*, 1999), (Papapetrou *et al.*, 2006)
pEPI-Luc	pEPI-1	CMV	Luciferase	6 months	(Argyros *et al.*, 2008)
pLucA1	pEPI-1	AAT	Luciferase	6 months	(Argyros *et al.*, 2008, Wong *et al.*, 2011)
pBcLucA1	pEPI-1	AAT	Bcl2-Luciferase	3 months	(Wong *et al.*, 2011)
pEPito-CMV	pEPito	CMV	eGFP	1 month	(Haase *et al.*, 2010)
pEPito-hCMV	pEPito	hCMV/EF1P	eGFP	1 month	(Haase *et al.*, 2010, Calado *et al.*, 2014)
pEPito-hCMV/RPE65	pEPito	hCMV enhancer/ RPE65	eGFP	1 month	(Calado *et al.*, 2014)
pUbC-Luc-S/MAR	pEPI-1	UbC	Luciferase	5 months	(Wong *et al.*, 2013)
pUbC-FLCN-S/MAR	pEPI-1	UbC	FLCN	5 months	(Wong *et al.*, 2013)
pEPI-TetON	pEPI-1	TRE-Tight	eGFP	2 months	(Rupprecht *et al.*, 2010)
Mini-AAT-S/MAR	mcDNA	AAT	Luciferase	3 months	(Argyros *et al.*, 2011)
Mini-pUbC-S/MAR	mcDNA	Ubc	Luciferase	3 months	(Argyros *et al.*, 2011)
iBAC-S/MAR	pEPI-1	CMV	LDLR-eGFP	3 months	(Lufino *et al.*, 2007)
hβ-S/MAR	pEPI-1	βLCR	HBB	3 months	(Sgourou *et al.*, 2009)
pEPI-b3a2	pEPI-1	hU6	bcr-abl shRNA	4 months	(Jenke *et al.*, 2005)
pEPI-RNAi	pEPI-1	hU6	HBV shRNA	8 months	(Jenke *et al.*, 2008)
VMD2-hRPE65-S/MAR	pEPI-1	VMD2	hRPE65	6 months	(Koirala *et al.*, 2013)

As S/MARs-based vectors consist of extra-chromosomal elements, their episomal status gives an advantage over the safety risks associated with viral vectors. Moreover, their mitotic stability, long term expression, and high cloning capacity make them ideal systems for therapeutic purposes and future clinical applications.

1.7. Specific Aims

Despite the potential of non-viral vectors, their application in ocular gene therapy is still hindered by disadvantages such as low transfection efficiency and transient expression. Therefore, in this work we aimed to develop a multifactorial approach by combining long-term episomal expression systems, such as pEPitos with therapeutic genes and an efficient physical delivery method to enhance retinal gene therapy. With this combinatorial approach we intend to achieve expression of therapeutic levels of a gene of interest for a significant time period, therefore avoiding frequent administration and associated drawbacks. To achieve our goal we have divided our work-plan in three different tasks:

i) Identify molecular targets of genetic and acquired retinal pathologies, such as *Retinitis Pigmentosa* (RP) and Diabetic Retinopathy (DR), respectively.

ii) Test the efficacy of pEPito episomal vectors in long-term gene expression in retinal cells, both *in vitro* and *in vivo*.

iii) Use the molecular targets identified in the first task combined with pEPito expression systems and test their ability of long-term gene expression and rescue of animal models of RP and DR, using electroporation to enhance transfection.

Chapter 2 – Identification of Molecular Targets for Gene Therapy for *Retinitis Pigmentosa*

This chapter is based on the following manuscript:

The role of ATR for retinal cilium formation and elongation in *Retinitis Pigmentosa*

Sofia M. Calado[1,2,3], Berta de la Cerda[3], Francisco Diaz-Corrales[3], Gabriela A. Silva[2], Shomi Bhattacharya[3,4]

[1]Doctoral Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, Portugal

[2]CEDOC, NOVA Medical School | Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Portugal

[3]Department of Cell Therapy and Regenerative Medicine, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain

[4]UCL-Institute of Ophthalmology, London, UK

Manuscript In Preparation

2.1. Abstract

Ataxia telangiectasia and rad-3 (ATR) is a DNA damage response (DDR) enzyme that is usually associated with DNA damage checkpoint and cell cycle arrest. In humans, mutations in the ATR gene cause Seckel Syndrome, a rare disease characterized by intra-uterine growth retardation, microcephaly, facial and osteoskeletal abnormalities, as well as retinal degeneration. We have recently shown that the ATR protein is localized in the connecting cilium of photoreceptors (PR), and the mutant mice ($ATR^{+/s}$) exhibit early-onset degeneration of rods and cones, and accumulation of pigment in the retina, suggesting a *Retinitis Pigmentosa*-like degeneration. To better understand the role of ATR in cilia formation and elongation, we decided to use an *in vitro* model of ciliated cells. Our hypothesis is that ATR inhibition should affect ciliary length. RPE-1 cells were cultured under serum-starvation conditions to induce primary cilia formation. ATR inhibition was performed by adding different concentrations of caffeine and ETP46464 to the culture medium. Our results show that under serum-starvation cultures, ATR is located in the base of the primary cilia and co-localizes with the centrosome of the ciliated cells. Moreover we found that ATR co-immunoprecipitates with γ-tubulin, a centrosome marker in the cells under serum-starvation conditions. After caffeine treatment, ATR expression decreases and this decrease is associated to a marked decrease in the cilia length, showing a direct effect of ATR in cilia elongation. Our results show that similarly to other DNA damage respose proteins, ATR is interacting with the centrosome of the ciliated cells and its inhibition is associated with shortened cilia, indicating a different role of ATR that might be involved in retinal ciliopathies.

2.2. Introduction

Ataxia telangiectasia and rad-3 (*ATR*) is a gene that encodes for a serine-threonine protein kinase. This protein and its partner ataxia telangiectasia mutated (ATM) display an important role in DNA damage response (DDR) (Zhang *et al.*, 2007). While ATM is activated in the presence of double strand breaks, ATR is recruited during single strand breaks, due to the collapse of the replication forks (Sarkaria *et al.*, 1999, Zhang *et al.*, 2007). In humans, mutations in *ATR* gene are associated with Seckel syndrome, a rare disease characterized by intra-uterine growth retardation, microcephaly, facial and osteoskeletal abnormalities as well as retinal degeneration (Guirgis *et al.*, 2001).

Our group has previously characterized a Seckel syndrome mouse model ($ATR^{+/s}$) regarding its retinal phenotype. We found that the mutant animals displayed a massive destruction of the photoreceptor (PR) layer (Valdes-Sanchez *et al.*, 2013). We also described that in WT mice ATR is present in the connecting cilia of PR and $ATR^{+/s}$ mice presented shorter cilia, suggesting that PR degeneration results from a cilia defect in those animals (Valdes-Sanchez *et al.*, 2013).

The primary cilium is an organelle that comes from the cellular membrane of most mammalian cells. It consists of an axoneme of nine doublet microtubules, extended from the basal body and derived from the centrosome (Satir *et al.*, 2010). This organelle is present in a vast number of cells, including neuronal and retinal cells, including PRs (Satir *et al.*, 2010, Drivas *et al.*, 2013, Valdes-Sanchez *et al.*, 2013). It is now known that this structure is involved in multiple cellular events, such as sensory mechanisms and inhibition of cell cycle by recruitment of the centriole (Satir *et al.*, 2010, Drivas *et al.*, 2013). It is easy to understand that mutations that affect primary cilia are responsible for a selection of human diseases and developmental disorders, named ciliopathies (Drivas *et al.*, 2013). There are several mutations associated with retinal ciliopathies already described (Wheway *et al.*, 2014), but the most common are the ones in *CEP290* gene that is associated with 20% of Leber Congenital Amaurosis

(LCA) cases (Drivas *et al.*, 2013) and mutations in *RPGR* gene that is associated with X-linked *Retinitis Pigmentosa* (Hildebrandt *et al.*, 2011, Wheway *et al.*, 2014). Emerging evidences have described the involvement of other DDR genes with retinal ciliopathies, such as *MRE11A*, *CEP164* and *ZNF423*, suggesting a close relationship between DDR proteins and functional cilium maintenance (Yuan *et al.*, 2013, Wheway *et al.*, 2014).

In this work we aimed to understand if ATR is involved in ciliary function and therefore has a role in RP. Herein we present further data about the ciliary function of ATR besides its known function related with DNA damage checkpoint. Furthermore, we show that ATR seems to have a 60 kDa isoform responsible for ciliary function.

2.3. Materials and Methods:

2.3.1. Antibodies

Primary antibodies

Anti-ATR (rabbit, Abcam); Anti-acetylated α-tubulin (mouse, Sigma); Anti-γ-tubulin (mouse, Sigma); Anti-CDK2 (rabbit, Santa Cruz Biotechnology)

Secondary antibodies

Alexafluor donkey anti-rabbit 594 (1: 500, Molecular Probes); Alexafluor donkey anti-mouse 488 (1: 500 Molecular Probes); HRP-conjugated anti-rabbit (1:20 000, Sigma); HRP-conjugated anti-mouse (1:20 000, Sigma)

2.3.2. Cell Lines

In this study, the human immortalized retinal pigment epithelial cell line (hTERT RPE-1 ATCC CRL4000)) was used as model of ciliated cells. It is known that RPE-1 cells are able to develop primary cilia under serum-starvation conditions (Spalluto *et al.*, 2013).

2.3.3. Primary cilium induction

For primary cilia induction, RPE-1 cells were cultured with Dulbecco's Modified Eagle Medium:F12 Mix (DMEM:F12, Sigma) supplemented with 1% of L-Glutamin (Sigma) and 1% of Penincillin/Streptomycin (Sigma), and were kept without Fetal Bovine Serum (FBS) for 48h, at 37° C and 10% CO_2. Then, RPE-1 cells were either fixed for immunofluorescence, or collected for protein extraction and western blot analysis.

2.3.4. Immunofluorescence

RPE-1 cells were fixed in ice-cold methanol for 10 minutes. Coverslips were washed twice in PBS and permeabilized in 0.1% Triton X100/PBS for 15 minutes. The coverslips were blocked in 1% goat serum/PBS at room temperature for 1h. Double immunocytochemistry against ATR (1:100) and acetylated α-tubulin (1:500) or ATR and

γ-tubulin (1:500) were performed for 1h at room temperature. Cells were washed then 3 times in PBS, and incubated with the secondary antibody (1:500) at room temperature for 1 h. After a final 3 washes, coverslips were mounted on glass slides with Vectashield containing 4', 6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories Ltd., Peterborough, United Kingdom). Images were obtained using a Leica confocal microscope with a 63 × objective, using the appropriate filter sets.

2.3.5. Western blot

Proteins were extracted in cold RIPA buffer containing protease and phosphatase inhibitor cocktails. The protein content was measured by the Bradford assay and the samples stored at -80 ºC. Thirty micrograms of each extract were separated in a denaturing 10% SDS–PAGE gel and the proteins transferred to a PVDF membrane and blocked using 5% NFDM or Superblock (Thermo) for 1 h. The ATR antibody was incubated overnight at 4 ºC (1:1000 dilution). Acetylated α-tubulin and γ-tubulin were incubated for 1h at room temperature (1:1000 dilution). Membrane was probed with an HRP-conjugated secondary antibody for 1 h at room temperature and the immunoreactive bands were detected by chemiluminescence, using an ECL plus kit (Amersham).

2.3.6. ATR and γ-tubulin immunoprecipitation

The immunoprecipitation was performed using IgG magnetic beads (Millipore), according with the manufacturer’s instructions. 20 µg of ATR and γ-tubulin antibodies were used to immunoprecipitate ATR and γ-tubulin, respectively. Equal amounts of IgG were used as control antibodies. The antibody-linked beads were incubated,overnight at 4º C, with 500 µg of whole cell extract of RPE-1 cultured with FBS (FBS+) or without serum (FBS-), washed three times with PBS-T (0.1%) and eluted with glycine buffer, pH 2, for western blot analysis.

2.3.7. ATR inhibition and cilia length measurement

For ATR inhibition, two known inhibitors were tested: caffeine (Sigma) and ETP-46464 (Millipore). Caffeine was dissolved in distilled water at 72 mM. The caffeine solution was then added to the culture media in a final concentration of 2.5, 5 and 10 mM. ETP-46464 was resuspended in DMSO at 21 mM and was added to the culture media in a final concentration of 10 nM. After an overnight incubation, the cells were either fixed (for immunofluorescence) or collected for protein extraction and western blot analysis. The cilia length was measured using ImageJ software.

2.3.8. Statistical Analysis

Statistical analysis was performed using the SPSS software, version 20.

The values were expressed as mean ± the standard error of the mean (SEM). The differences between groups were examined for statistical significance using the t-test and one-way ANOVA test. A P-value of 0.05 denoted the presence of a statistically significant difference.

2.4. Results and Discussion

2.4.1. ATR colocalizes with the centrosome of the ciliated cells

Our prior studies using $ATR^{+/s}$ mouse model have shown that in the retina, ATR is located in the cilia of PRs and the mutant mice display shortened cilia, suggesting that the PR degeneration results from a cilia defect in those animals (Valdes-Sanchez *et al.*, 2013). To better understand the role of ATR in cilia formation and elongation we decided to use an *in vitro* model of ciliated cells. It was previously described that RPE-1 cells are able to develop primary cilia under serum-starvation conditions (Spalluto *et al.*, 2013). The RPE-1 cells were maintained under serum-starvation (FBS-) conditions for 48h and primary cilia formation was analyzed by immunocytochemistry. Our results show that, under FBS- conditions approximately 45% of the cells developed primary cilia, while under with serum conditions (FBS+) only about 5% of the cells developed this organelle (Figure 2.1).

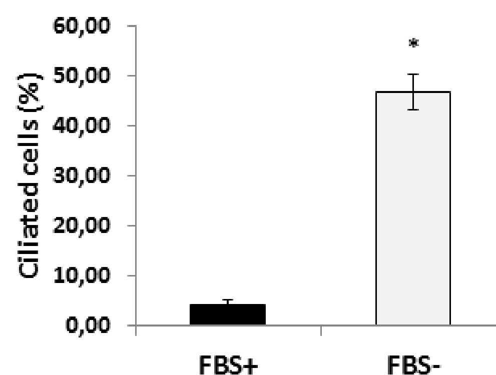


Figure 2.1: Percentage of RPE-1 cells presenting cilia after being cultured with (FBS+) or without (FBS-) serum. Result represents the mean of ciliated cells ± SEM of ≥ 100 individual cells. $*P<0.05$, represents significant result obtained by unpaired two-sample t-tests.

As expected, in FBS+ cultures ATR is located all over the nucleus of the cells. However, in FBS- cultures there is a decrease in the ATR staining in the nucleus and an accumulation of ATR signal in the base of the primary cilia (Figure 2.2).

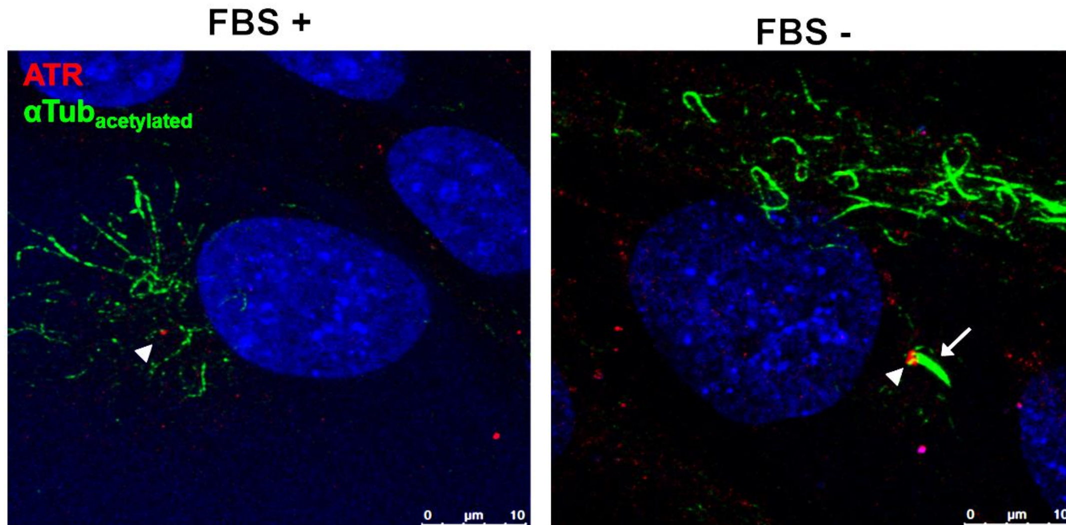


Figure 2.2: Immunofluorescence staining of methanol fixed RPE-1 cells cultured with (FBS +) and without serum (FBS -). Under serum starvation RPE-1 cells develop primary cilia (arrow) and ATR is located at the base of the primary cilia (arrow head). DAPI (blue) stains nuclei, ATR is stained in red and green represents acetylated α-tubulin. Magnification: 630x

This result corroborate with our previous *in vivo* results, in which we described ATR to be located in the primary cilia of PR cells (Valdes-Sanchez *et al.*, 2013), showing that in post-mitotic cells, ATR seems to play a different role.

It was previously described by Zhang and co-workers that there is a strong co-localization of ATR with the centrosome of the interphase non-ciliated HeLa cells (Zhang *et al.*, 2007). Moreover, it is also known that the primary cilia start growing from the basal body which is, in fact, derived from the mother centriole of the centrosome (Satir *et al.*, 2010). Based on these findings, we decided to confirm if the accumulation of ATR found in the base of the primary cilia of RPE-1 cells corresponds to the centrosome. We found that in the FBS- condition there is a total co-localization of the ATR signal with γ-tubulin signal (Figure 2.3 - lower panel). However, this was not evident in the cells under FBS+ cultures (Figure 2.3 - upper panel). This means that ATR is co-localizing with the centrosome of the cells that develop primary cilia.

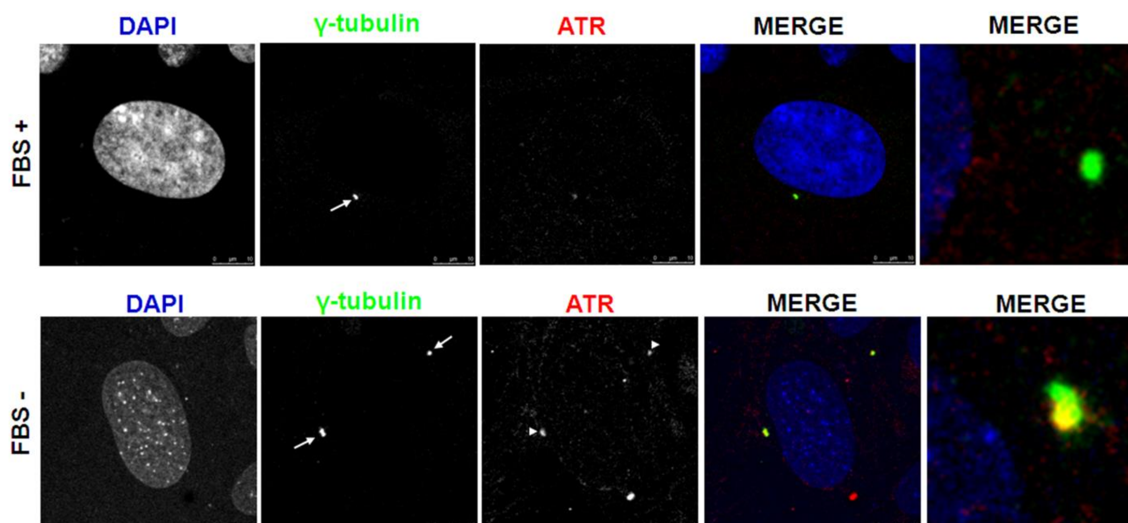


Figure 2.3: Immunofluorescence staining of methanol fixed RPE-1 cells cultured with (FBS +) and without serum (FBS -).γ-tubulin (arrow) and ATR (arrow head) are co-localized at the centrosome. DAPI (blue) stains nuclei, ATR is stained in red and green represents γ-tubulin. Magnification: 630x

To confirm this result we decided to perform an immunoprecipitation of γ-tubulin to prove its interaction with ATR in the centrosome. Indeed we found that ATR is co-immunoprecipitating with γ-tubulin in the cells cultured under serum-starvation conditions (Figure 2.4). However we were not able to detect ATR signal in the whole extracts of RPE cells cultured with serum (Figure 2.4). This result corroborates our immunofluorescence results in which we were only able to find co-localization of γ-tubulin and ATR in FBS- condition, in which the cells develop primary cilia (Figure 2.3).

Surprisingly, the co-immunoprecipitation result was not a 300 kDa fragment corresponding to the full length ATR. The co-immunoprecipitated fragment presents a molecular weight of approximately 60 kDa. This result can be explained by the presence of an isoform of the ATR protein that can probably be associated with cilia function, formation and elongation.

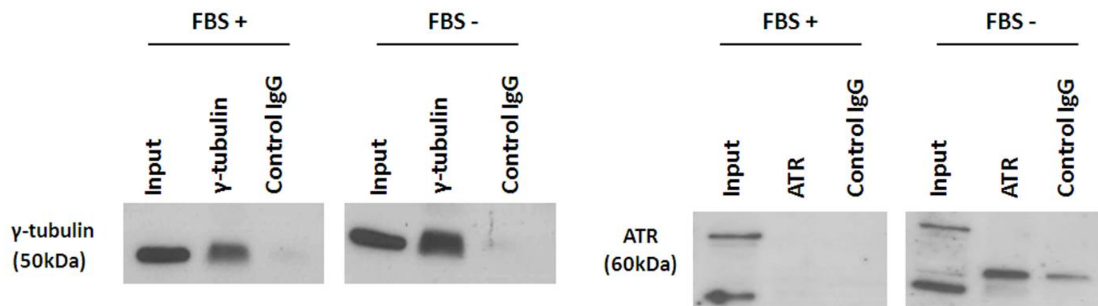


Figure 2.4: Immunoprecipitation of γ-tubulin and co-immunoprecipitation of ATR showing that ATR is only detected in the celular extracts cultured under serum-starvation conditions (FBS-). (FBS +: cells cultured with serum; FBS -: cells cultured without serum).

2.4.2. Inhibition of ATR (by caffeine) affects ciliary length

To test the effect of ATR inhibition in cilia formation, we decided to test two different agents that were described to inhibit ATR function: caffeine (Hall-Jackson *et al.*, 1999) and ETP-46464 (Toledo *et al.*, 2011).

ATR is known to be implicated in both S/M and G2/M checkpoints (Hall-Jackson *et al.*, 1999). However, it was proved that in the presence of caffeine, cell cycle arrest induced by this checkpoints was showed to be suppressed (Hall-Jackson *et al.*, 1999) suggesting that caffeine inhibits the catalytic activity of ATR (Sarkaria *et al.*, 1999). We decided to test three different concentrations of caffeine (2.5, 5 and 10 mM) and evaluate the effect in ATR expression and cilia length. The western blot analysis of the RPE-1 cells cultured under FBS+ showed no significant changes with the addition of caffeine to the culture medium of the cells (Figure 2.5). Unexpectedly, we were not able to detect the 300 kDa fragment corresponding to the full length ATR. On the other hand, we observed a decrease in the full length ATR protein in a caffeine-dependent manner, as well as a reduction in the 60 kDa fragment (Figure 2.5), the fragment size that is co-immunoprecipitating with γ-tubulin, in the cells cultured in FBS - conditions. However, the caffeine treatment did not have effect on the mitotic CDK2 kinase,

showing that the effect of caffeine in the levels of ATR was not due to cell cycle re-entrance (Figure 2.5).

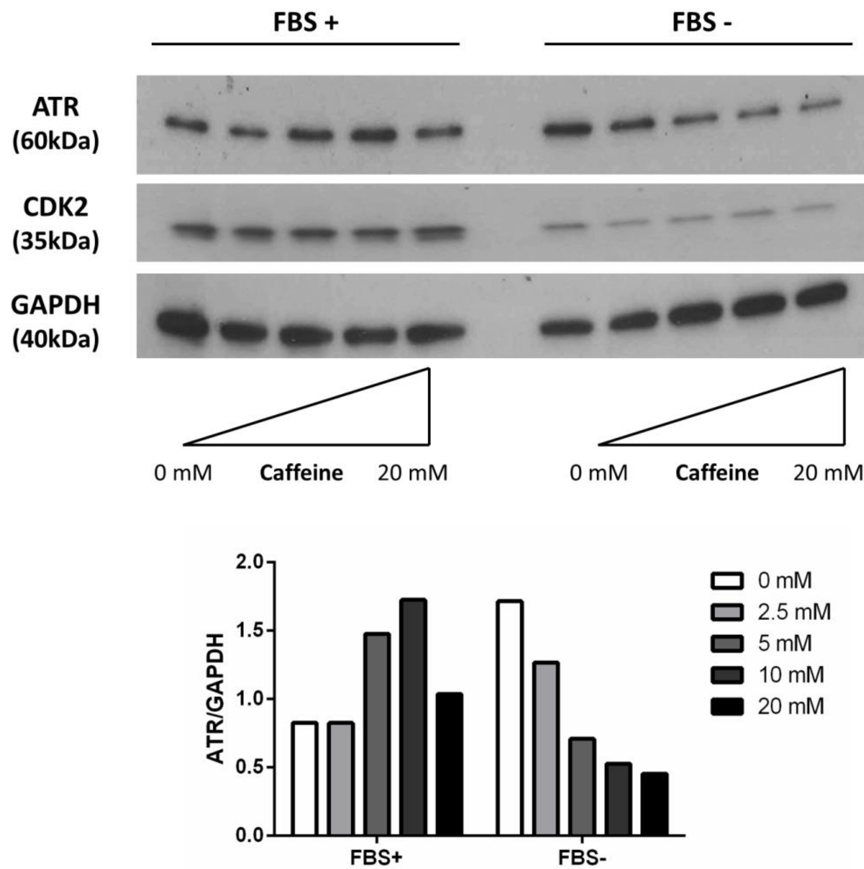


Figure 2.5: Western blot analysis of RPE-1 cells cultured with (FBS+) or without (FBS-) serum and treated with increased concentrations of caffeine. In the FBS- cells treated with caffeine is possible to observe a decrease in ATR expression in a caffeine-dependent manner.

In order to evaluate the effect of caffeine in cilia length we performed a double immunocytochemistry against ATR and acetylated α-tubulin. Our results show that after the addition of caffeine to the culture medium of the cells cultured with FBS- there was a marked decrease in the cilia length in a caffeine-dependent manner (Figure 2.6).

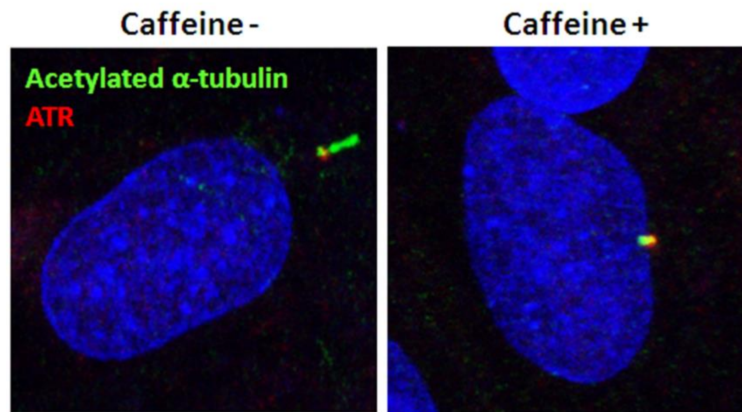


Figure 2.6: Immunofluorescence staining of methanol fixed RPE-1 cells cultured in FBS - showing a decrease in the ciliary length after treatment with 5 mM of caffeine. DAPI (blue) stains nuclei, ATR is stained in red and green represents acetylated α-tubulin. Magnification: 630x

Figure 2.7 shows that 5 mM of caffeine was the concentration that induced a stronger effect in the ciliary length, showing approximately 80% shorter cilia than the ones without treatment with caffeine.

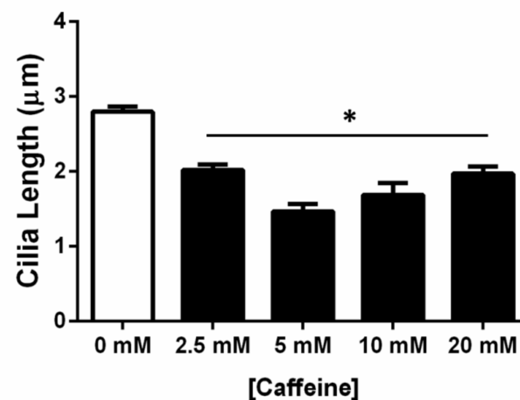


Figure 2.7: Cilia measurement of the RPE-1 cells cultured without serum and treated with different concentrations of caffeine. It is possible to observe a significant reduction in the length of the cilia after culturing the cells with caffeine. Result represents the mean of cilia length ± SEM of ≥ 100 individual cilia. ∗P<0.05, one-way ANOVA.

Those results were also confirmed by immunoprecipitation of ATR in the cytosolic fraction of RPE-1 cultured without FBS and treated with caffeine. Figure 2.8 shows that after the caffeine treatment there is a decrease in the amount of 60 kDa ATR fragment being immunoprecipitated.

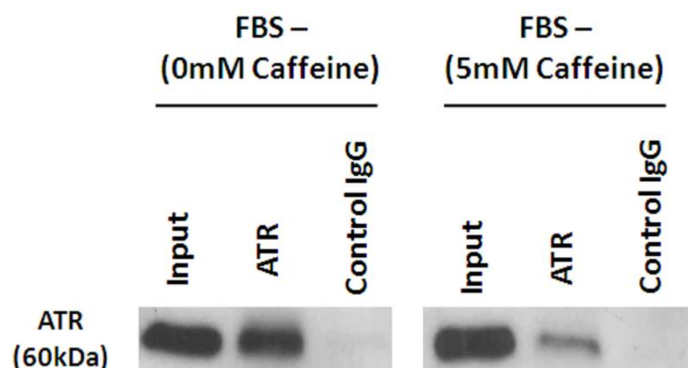


Figure 2.8: Immunoprecipitation of ATR from citosolic extracts of RPE-1 cells under serum-starvation conditions (FBS -) showing a decrease in ATR after caffeine treatment (5 mM Caffeine).

These finding leads us to believe that the 60 kDa isoform of ATR that is highly affected by caffeine treatment is involved in ciliary function.

ETP-46464 was previously described to be a potent inhibitor of ATR an IC_{50} = 10 nM (Toledo *et al.*, 2011). We tested the effect of ETP-46464 in the cilia length of RPE-1 cells. We found no differences regarding the cilia length (Figure 2.9).

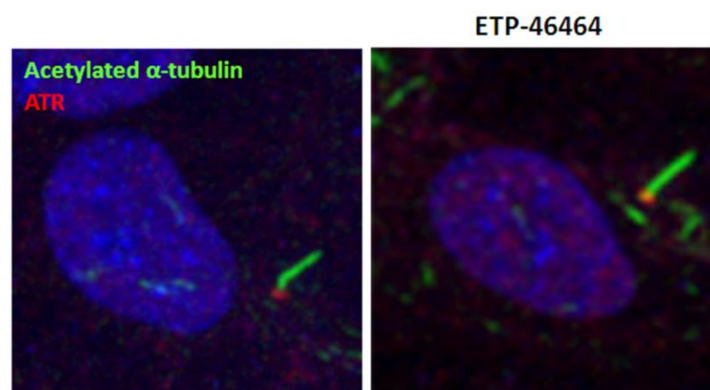


Figure 2.9: Immunofluorescence staining of methanol fixed RPE-1 cells cultured in FBS - showing no differences in the ciliary length after treatment with 10 nM of ETP-46464. DAPI (blue) stains nuclei, ATR is stained in red and green represents acetylated α-tubulin. Magnification: 630x

The western blot analysis revealed a slightly decrease in ATR expression not only in the 300 kDa full ATR protein, as well as in the 60 kDa fragment, which we expect to be involved in ciliary function (Figure 2.10). However, the decrease in the 60 kDa

fragment was not as evident as we observed after caffeine treatment (Figure 2.5). This can probably be the reason why there was not a visible reduction in the cilia length.

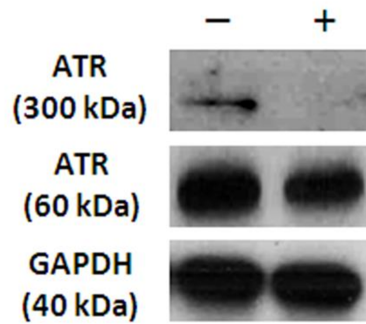


Figure 2.10: Western blot analysis of RPE-1 cells cultured under serum-starvation conditions and treated (+) or not (-) with the inhibitor agent ETP-46464. In the treated cells is possible to observe a decrease in the full length ATR fragment (300 kDa) but this reduction is not so evident in the 60 kDa fragment.

2.5. Conclusion

ATR is a DDR protein recruited during single strand breaks, leading to cell cycle arrest (Cimprich *et al.*, 2008). Several animal studies have shown that complete depletion of *ATR* originates early embryonic lethality, showing its importance in maintaining genome integrity during DNA synthesis (Brown *et al.*, 2000, Murga *et al.*, 2009). Its deletion in adult mice has been associated to age-related phenotypes, such as premature hair loss and graying, kyphosis, osteoporosis, and defects in spermatogenesis, due to stem and progenitor cell degeneration (Ruzankina *et al.*, 2007).

In humans, mutations in *ATR* gene are responsible for Seckel Syndrome, a disease characterized by severe intra-uterine growth retardation, microcephaly, facial and osteoskeletal abnormalities (O'Driscoll *et al.*, 2003). In our previous study using $ATR^{+/s}$ mice we found that ATR is essential for the postnatal development of the PR layer. Moreover we found that the mutant mice display shorter cilia compared with the WT mice (Valdes-Sanchez *et al.*, 2013). A study performed in three siblings with Seckel Syndrome have found severe, early onset retinal degeneration, showing no rod and cone responses in the ERG (Guirgis *et al.*, 2001).

To better understand the retinal degeneration presented by $ATR^{+/s}$ mice we decided to evaluate the role for ATR in retinal cilium formation and elongation.

In this work we show for the first time that ATR is associated with the centrosome of G_0 RPE-1 ciliated cells, cultured under serum-starvation conditions. This result was confirmed by immunoprecipitation of the centrosomal marker γ-tubulin and co-immunoprecipitation of ATR. Interestingly we found that the ATR fragment that was co-immunoprecipitated with γ-tubulin was a fragment of approximately 60 kDa and not a 300 kDa fragment corresponding to the full length ATR. This 60 kDa fragment can correspond to an alternative splicing isoform from ATR. According to the *AceView* database there are 12 ATR variants described so far (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&c=Gene&l=AT

R) and one of them (cataloged as ATR.cAug10) have the predicted size of our fragment. Further studies are being currently performed to understand if this variant corresponds to the 60 kDa fragment that we identified.

The inhibition of ATR by caffeine induced not only a decrease in the 300 kDa full length ATR but also in the 60 kDa ATR fragment, in a caffeine dependent manner. With the inhibition of ATR by caffeine we observed a concomitant reduction in the cilia length. The immunoprecipitation of ATR in the cytosolic extracts of RPE-1 cells treated with caffeine, compared with the untreated ones showed a decrease in the 60 kDa ATR fragment. In humans, 95% of multi-exon genes suffer alternative splicing, generating proteins with potential different functions (Eksi *et al.*, 2013). Examples of well-known splicing variants with different functions are the splicing variants of *BCLX* that are proapoptotic and anti-apoptotic (Revil *et al.*, 2007). In these work, our results lead us to believe that the 60 kDa ATR isoform is involved in the ciliary function despite its well know DNA damage repair role.

Chapter 3 – Identification of Molecular Targets for Gene Therapy for *Diabetic Retinopathy*

This chapter is based on the following manuscript:

GLUT1 activity contributes to the impairment of PEDF secretion by the RPE

Sofia M. Calado[1,4], Liliana S. Alves[2,3], Sónia Simão[3,4] and Gabriela A. Silva[4,#]

[1]PhD Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, Campus Gambelas, Faro, Portugal

[2]Department of Biomedical Sciences and Medicine, University of Algarve, Campus Gambelas, Faro, Portugal

[3]Centre for Biomedical Research (CBMR), University of Algarve, Campus Gambelas, Faro, Portugal

[4]CEDOC, NOVA Medical School | Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal

Manuscript Submitted to the journal *Molecular Vision, 2016*

3.1. Abstract

In this study we aimed to understand if glucose transporter 1 (GLUT1) activity affects the secretion capacity of anti-angiogenic factor pigment epithelium-derived factor (PEDF) by the retinal pigment epithelium (RPE) cells, thus explaining the reduction of PEDF levels observed in patients with diabetic retinopathy (DR).

Analysis of GLUT1 expression, localization and function was performed *in vitro* in RPE cells (D407) cultured with different glucose concentrations, corresponding to non-diabetic (5 mM of glucose) and diabetic conditions (25 mM of glucose), further subjected to normoxia or hypoxia. The expression of PEDF was also evaluated in the secretome of the cells cultured in these conditions. Analysis of GLUT1 and PEDF expression was also performed *in vivo* in the RPE of $Ins2^{Akita}$ diabetic mice and age-matched wild-type (WT) controls.

We have observed an increase in GLUT1 under hypoxia in a glucose-dependent manner, which we found to be directly associated to the translocation and stabilization of GLUT1 in the cell membrane. This stabilization led to an increase in glucose uptake by RPE cells. This increase was followed by a decrease in PEDF expression in RPE cells cultured in conditions simulating DR. Compared with non-diabetic WT mice, the RPE of $Ins2^{Akita}$ mice showed increased GLUT1 overexpression with a concomitant decrease in PEDF expression.

Collectively, our data shows that expression of GLUT1 is stimulated by hyperglycemia and low oxygen supply, and this overexpression was associated with increased activity of GLUT1 in the cell membrane that contributes to the impairment of RPE secretory function of PEDF.

3.2. Introduction

Diabetic retinopathy (DR), a blood-retinal barrier disorder, is the main complication of diabetes and the leading cause of blindness in working-age adults (Cheung *et al.*, 2010). The major pathological features at advanced stages of the disease are the abnormal neovascularization due to hypoxia, and blood leakage as a result of inner blood-retinal barrier breakdown (Cheung *et al.*, 2010, Simo *et al.*, 2010). The blood-retinal barrier (BRB) is responsible for the homeostasis of the neuroretina and is composed by two different structures: the inner BRB (iBRB), formed by tight junctions between the endothelial cells of the retinal vessels, and the outer BRB (oBRB), formed by intracellular tight junctions in the retinal pigment epithelium (RPE) monolayer (Cunha-Vaz, 1979, Farjo *et al.*, 2010, Gottfried *et al.*, 2013).

Most of the studies on the pathophysiology of DR focused their research in the iBRB breakdown and neuroretina damage (Kumagai *et al.*, 1995, Knott *et al.*, 1996, Badr *et al.*, 2000, Duffy *et al.*, 2006), with little attention on the effects of diabetes on the oBRB and RPE cells. Since the RPE is responsible, among others, for the transport of nutrients, such as glucose, ions and water, and secretion of factors crucial for the homeostasis of the neuroretina such as the pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) (Simo *et al.*, 2010, Xu *et al.*, 2011), its role on DR is worth investigating. The healthy eye is characterized by low levels of angiogenic VEGF and high levels of anti-angiogenic factors, such as PEDF (Farjo *et al.*, 2010). This balance is disrupted by ischemia during the pathogenesis of DR, increasing the ratio of angiogenic to anti-angiogenic factors and promoting abnormal neovascularization in the retina (Farjo *et al.*, 2010). During ischemia, increasing levels of the heterodimeric hypoxia-inducible factor-1 (HIF-1) are detected (Catrina *et al.*, 2004, Hughes *et al.*, 2010). Both HIF-1 subunits are constitutively expressed, but in normoxia conditions the HIF-1α subunit is rapidly degraded by an oxygen-dependent mechanism (Wang *et al.*, 1995). However, in a hypoxic environment both HIF-1 subunits are able to form dimers and translocate to the nucleus, where they can induce

the transcription of a wide range of genes (Huang *et al.*, 1996, Kallio *et al.*, 1997, Ke *et al.*, 2006) including VEGF (Levy *et al.*, 1995), EPO (Semenza *et al.*, 1991), and the glucose transporter 1 (GLUT1) (Chen *et al.*, 2001).

GLUT1 is one of the 12 GLUT isoforms described (Shah *et al.*, 2012), composed by a glycoprotein with 12 transmembrane domains and a single N-glycosylation site. GLUT1 is able to transport glucose bi-directionally across the cell membrane based on a concentration gradient (Bell *et al.*, 1990, Shah *et al.*, 2012). In the retina, as in the brain, glucose is the only fuel source for cells, and in both tissues glucose is transported to cells exclusively through the GLUT1 transporter (Sone *et al.*, 2000, Shah *et al.*, 2012).

In this study we aimed to establish a correlation between RPE, GLUT1 and their role in diabetic retinopathy. Our hypothesis is that under diabetic conditions, where there is an increase in glucose and local hypoxia the expression of GLUT1 in RPE cells is increased, and that can affect the secretory function of RPE, namely their production of PEDF. This change in PEDF secretion can lead to an imbalance between VEGF and PEDF, contributing to diabetic retinopathy. To test our hypothesis, we studied the effect of both hyperglycemia and hypoxia on the cellular localization and expression of GLUT1 and PEDF expression on RPE cells, and further confirmed our findings in a mouse model of diabetic retinopathy.

3.3. Materials and Methods

3.3.1. Cell lines

D407, a human retinal pigment epithelial (RPE) cell line (Davis *et al.*, 1995) used in the *in vitro* experiments was kindly provided by Dr. Jean Bennett from the University of Pennsylvania (USA). Cells were kept in culture in a humid chamber with 5% CO_2 at 37ºC and were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 1% Penicillin/Streptomycin (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich) and 5% foetal bovine serum (Sigma-Aldrich). Culture medium was changed every 2 days. For the experiments on the glucose effect, cells were cultured in 22.1 cm^2 plates (TPP, Switzerland) for 3 days either in DMEM containing 5 mM D-Glucose (to simulate normoglycemia) or in DMEM with 25 mM of D-Glucose (to simulate hyperglycemia). Cells were also grown in DMEM containing 5 mM of D-Glucose in which mannitol was added up to a final concentration of 25 mM. Mannitol was chosen as an osmotic control because it is a carbohydrate with no biological activity and cannot be used by the cells as source of energy (Ellis *et al.*, 1941, Duffy *et al.*, 2006). Hypoxia was induced by addition of desferrioxamine (Aprelikova *et al.*, 2004, Triantafyllou *et al.*, 2006) (DFO, Sigma-Aldrich) to the culture media at a final concentration of 100 µM. After 16h of incubation with DFO, cells were collected for Western blot and immunocytochemistry for GLUT1.

3.3.2. Detection of GLUT1 expression by Western blot

Whole cell proteins were extracted in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM EDTA) supplemented with protease inhibitor cocktail (Roche, Germany). For isolation of the membrane/soluble fractions, cells were extracted in cold homogenization buffer (20 mM HEPES pH 7.4; 1 mM EDTA; 250 mM sucrose) containing a protease inhibitor cocktail. The lysate was cleared by centrifugation at 4 ºC for 10 minutes at 3500 rpm. The supernatant was

centrifuged at 35 000 rpm for 1 hour at 4ºC, and the pellet corresponding to the membrane fraction, was suspended in buffer (10 mM HEPES pH 7.4; 250 mM sucrose) supplemented with a protease inhibitor cocktail. Protein content was measured by the Bradford assay and samples stored at -80 ºC. Thirty micrograms of each extract were separated in a denaturing 12% SDS–PAGE gel, the proteins transferred to a PVDF membrane (Amersham, UK), and blocked using Superblock Blocking buffer (Thermo Scientific) containing 0.1% of Tween-20 (Sigma-Aldrich, USA) for 1h at room temperature. GLUT1 antibody (ab32551; Abcam, UK) was incubated overnight at 4 ºC (1:2000 dilution) and β-Actin (A5441, Sigma-Aldrich) was incubated for 1h at room temperature (1:10000 dilution). The membrane was probed with an HRP-conjugated secondary antibody for 1 h at room temperature and the immunoreactive bands were detected by chemiluminescence, using an ECL Plus kit (Amersham).

3.3.3. Cellular localization of GLUT1 by immunocytochemistry

The cells were fixed in ice-cold methanol for 10 minutes, washed twice in PBS, and blocked in 1% goat serum/PBS at room temperature for 1h. Incubation with the GLUT1 antibody (1:250) was performed for 1h at room temperature, followed by a wash step and incubation with the secondary antibody (Alexa Fluor® 594; 1:500; Life Technologies, USA) at room temperature for 1 h. Coverslips were mounted on glass slides with Fluoromount G (SouthernBiotech, USA) containing DAPI. Images were obtained using an AxioVision microscope with a 63× objective, using the appropriate filter sets (Axio Observer Z2, Zeiss).

3.3.4. Glucose consumption assay

For determining glucose consumption by glucose depletion from the culture medium, D407 cells were seeded at a density of 7.0×10^5 cells/well in 6-well flat-bottom tissue culture plates and maintained for 72h in culture medium containing 25 mM of D-Glucose. A sample of culture medium was collected from each well and glucose

concentration was determined spectrophotometrically using the Glucose (GO) Assay Kit (Sigma-Aldrich), following the manufacturer's instructions. Culture medium with 25 mM of D-Glucose was used as control.

3.3.5. PEDF detection in the culture medium of the RPE cells

The cells were grown as previously described and PEDF levels were detected by Western blot in the culture medium. Briefly, the supernatant was collected and four volumes of ice-cold acetone were added. After an incubation of 30 min at -20 ºC, the supernatant was centrifuged for 10 minutes at 13000g and decanted. The pellet containing the precipitated proteins was re-suspended in 1x Sample Buffer. Protein content was measured by the Bradford assay and thirty micrograms of each extract were separated in a denaturing 12% SDS–PAGE gel and transferred to a nitrocellulose membrane (Biorad). Equal amounts of protein were loaded in the gel, determined by *Ponceau S* staining of the membranes before blocking. Western blot was performed as described previously, using a PEDF antibody (07-280, Merck Millipore; 1:1000).

3.3.6. Animals

For the *in vivo* experiments, male C57BL/6 (wild-type) and C57Bl/6 Ins2^{Akita} (diabetic) mice (The Jackson Laboratory, USA) 2, 4, 7, and 10 months after the onset of hyperglycemia (two months after birth) were used. The animals were housed under controlled temperature, a 12 h light/dark cycle with food and water *ad libitum*. Diabetic phenotype was confirmed 2 months after birth by measuring blood glucose levels in a drop of blood from a tail puncture (Freestyle Precision, Abbot, USA), with animals used in this study exhibiting blood glucose ≥ 500 mg/dl. All experimental procedures were carried out according to the Portuguese and European Laboratory Animal Science Association (FELASA) Guide for the Care and Use of Laboratory Animals, the European Union Council Directive 2010/63/EU for the use of animals in research and the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) for

the use of animals in ophthalmic and vision research. Animals were humanely sacrificed by cervical dislocation and the eyes enucleated. The RPE was isolated by dissection of the eyeball and homogenized in ice-cold RIPA buffer.

3.3.7. Statistical analysis

All experiments were performed in triplicate and the results expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism software. A value of $P<0.05$ was considered to be statistically significant.

3.4. Results

3.4.1. Hypoxia induces overexpression of GLUT1 in RPE cells

To evaluate the effects of glucose and ischemia in GLUT1 expression within the oBRB, we have used an *in vitro* setup using human RPE cells. D407 cells were cultured either with 5 mM of D-Glucose (corresponding to normoglycemia) or 25 mM of D-Glucose (corresponding to hyperglycemia) and further subjected to hypoxia by the addition of DFO, a chelating agent that induces hypoxia by inhibiting HIF-1α degradation at the proteasome (Aprelikova *et al.*, 2004, Triantafyllou *et al.*, 2006). We have confirmed that DFO does not induce cell death at the final used concentration of 100 µM (data not shown).

Figure 3.1 shows no differences at the protein level in cells in normoglycemic conditions under normoxia (N), when compared with hypoxia (H). On the other hand, there is a significant increase in GLUT1 protein in the cells cultured with 25 mM of D-Glucose in hypoxia when compared with the cells cultured in normoxia, showing a direct effect of glucose concentration in GLUT1 expression.

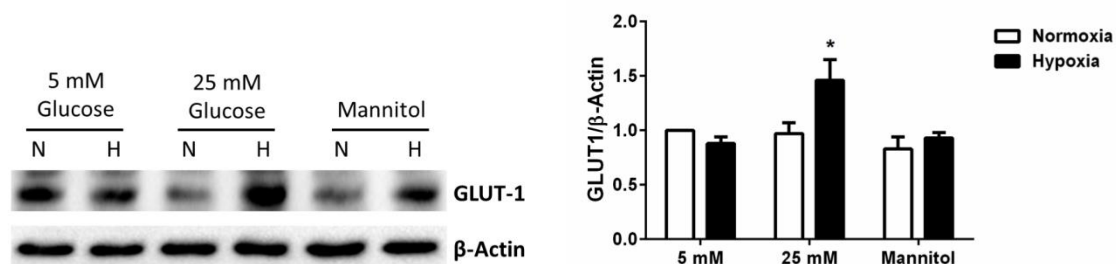


Figure 3.1: Effect of glucose and hypoxia in GLUT1 expression. Western blot analysis of GLUT1 in D407 RPE cells cultured under normoxia (N) and hypoxia (H) conditions and different concentrations of glucose in the culture medium: 5 mM of D-Glucose (corresponding to normoglycemia), 25 mM of D-Glucose (corresponding to hyperglycemia) and 25 mM of mannitol (osmolarity control). Quantitative data was obtained by normalization with β-Actin bands. N=4. *P<0.05 represents a significant difference in GLUT1 levels in cells cultured under hypoxia with high glucose concentration medium, determined by Tukey's multiple comparisons test.

3.4.2. GLUT1 translocation to the cell membrane of RPE cells increases in response to hypoxia

To determine if the increase in GLUT1 expression observed in figure 1 corresponded to an increase in the transport of glucose, we have first determined the cellular localization of GLUT1 in human RPE cells by immunocytochemistry. The results show a significant increase in the accumulation of GLUT1 in the membrane of cells under hypoxia compared with cells under normoxia (Figure 3.2). Again, we observed GLUT1 staining to be stronger in cells in hypoxia and hyperglycemia compared with cells in hypoxia and normoglycemia.

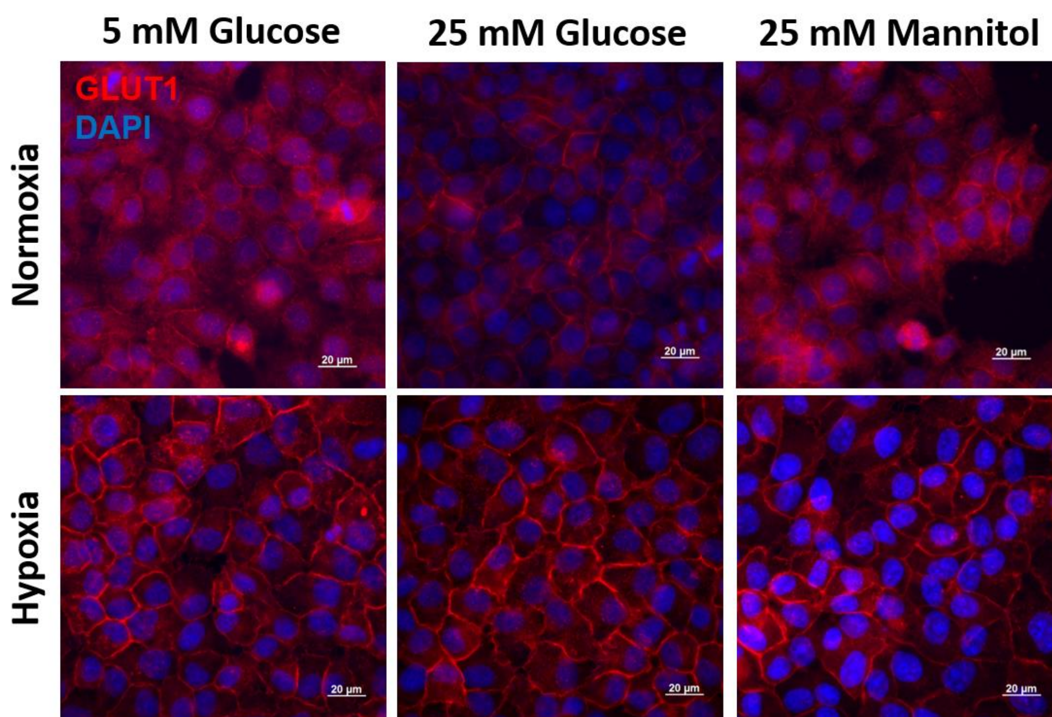


Figure 3.2: Immunocytochemistry for GLUT1 in RPE cells. D407 cells were cultured with different concentrations of glucose and subjected to hypoxia and normoxia. Staining for GLUT1 (red) shows higher intensity in the cell membrane of cells subjected to hypoxia. DAPI (blue) represents the nuclei. Magnification: 630X, scale bar: 20 µM

We have confirmed these results by Western blot analysis of the membrane and soluble fractions of RPE cells cultured in high glucose either in normoxia or hypoxia (Figure 3.3). In Figure 3.3, it is clear the marked increase of the GLUT1 transporter in the membrane of cells in hypoxia compared to normoxia, which correlates with the

results in Figure 3.2. We could not observe an increase of GLUT1 in the soluble fraction of cells in hypoxia, which was expected since GLUT1 is a membrane transporter.

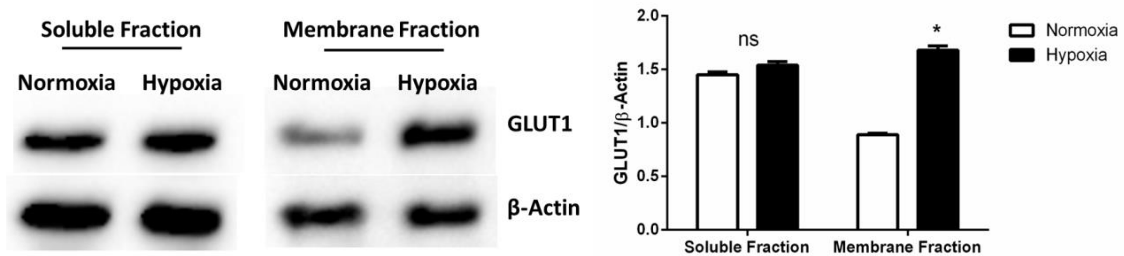


Figure 3.3: GLUT1 expression is stabilized in the cell membrane in response to hypoxia. GLUT1 protein levels in the soluble and membrane fraction of D407 cells cultured with 25 mM of D-Glucose and subjected to normoxia and hypoxia, show a marked increase in GLUT1 expression in the membrane of cells cultured in hypoxic conditions. N=3. *P<0.05 symbolizes a significant increase in GLUT1 expression in the membrane fraction of the cells cultured under hypoxia with high glucose concentration medium, determined by Sidak's multiple comparisons test.

3.4.3. Glucose consumption is affected by hypoxia

Based on the finding that GLUT1 expression is increased in the cell membrane of the RPE cells subjected to hypoxia, it was necessary to determine if this translates into increased glucose uptake by RPE cells. GLUT1 activity was measured by glucose consumption through glucose depletion in the culture medium. We have found a significant increase in glucose consumption induced by hypoxia (Figure 3.4), with cells consuming 60% of the glucose present in the culture medium compared to the 40% consumption in normoxia. This shows a marked effect of hypoxia on glucose consumption in diabetic retinopathy conditions.

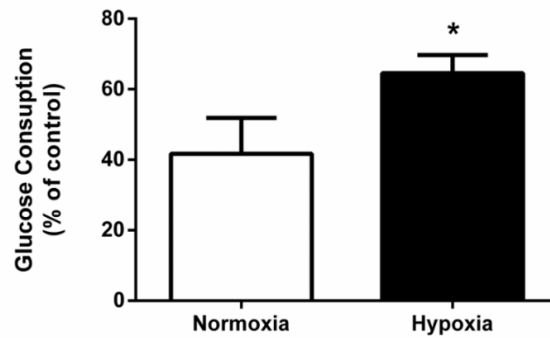


Figure 3.4: Glucose consumption by RPE cells. Glucose depletion from the culture medium was increased for cells cultured under hypoxia, compared with cells in normoxia. The results are expressed as percentage of the control (culture medium). N=6. *$P<0.05$ denotes a significant difference in glucose consumption by cells in hypoxia compared with cells in normoxia, determined by the two-tailed t-test.

3.4.4. Secretory function of RPE cells is impaired by high glucose and hypoxia

One of the main functions of RPE cells is the secretion of multiple trophic factors essential for the maintenance and integrity of the neuroretina and choriocapillaries (Simo *et al.*, 2010). One of these factors is PEDF, a neurotrophic and anti-angiogenic factor responsible for protecting neurons from ischemia-induced apoptosis (Takita *et al.*, 2003) and inhibiting endothelial cell proliferation caused by VEGF (Hutchings *et al.*, 2002). We have evaluated the expression of PEDF in RPE cells cultured as described previously and found a significant decrease in PEDF levels for conditions where cells were cultured in hyperglycemia (25 mM glucose) and hypoxia (H) (Figure 3.5).

This result shows that in diabetic conditions there is a decrease in the secretion of PEDF, which contributes to the disruption of the balance between the anti-angiogenic and angiogenic factors, as observed in human diabetic retinas (Boehm *et al.*, 2003, Funatsu *et al.*, 2006).

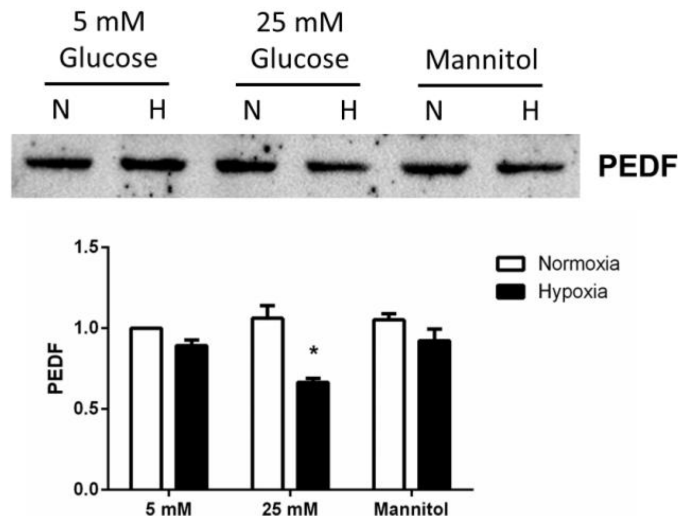


Figure 3.5: Effects of glucose and hypoxia in PEDF secretion by RPE cells. Western blot analysis of PEDF secretion in D407 cells cultured under normoxia (N) and hypoxia (H) conditions and different concentrations of glucose in the culture medium: 5 mM of D-Glucose (corresponding to normoglycemia), 25 mM of D-Glucose (corresponding to hyperglycemia) and Mannitol (osmolarity control). N=4. *P<0.05 represents a significant decrease in PEDF secretion by the RPE cells cultured under hypoxia with high glucose concentration medium, determined by Tukey's multiple comparisons test.

3.4.5. GLUT1 and PEDF expression is altered in the RPE of diabetic mice

To confirm the validity of our in vitro findings, we have analyzed the expression of GLUT1 and PEDF in the RPE of wild-type and Ins2^{Akita} diabetic mice (Figure 3.6). For all timepoints – 2, 4, 7 and 10 months after the onset of hyperglycemia – expression of GLUT1 was significantly increased in the RPE of diabetic mice compared with age-matched wild-type animals. Additionally, we have found a marked decrease in PEDF levels in the RPE of the diabetic mice, especially at later ages. These *in vivo* results corroborate our *in vitro* results in which we found an increase in GLUT1 (Figure 3.1) and a decrease in PEDF secretion by RPE culls cultured in conditions simulating DR (Figure 3.5).

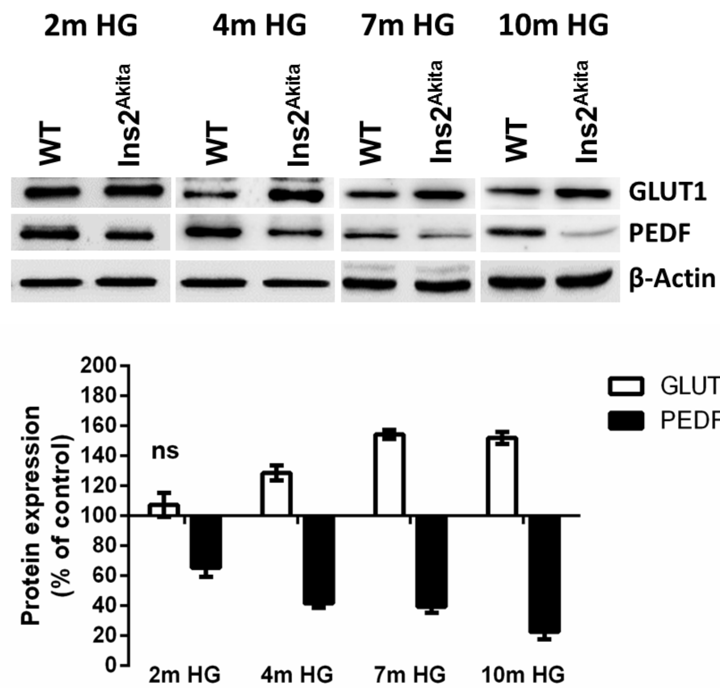


Figure 3.6: Secretory function of RPE is impaired in Ins2^{Akita} mice. PEDF and GLUT1 protein expression was assessed by Western blot of RPE tissue from diabetic mice with 2, 4, 7 and 10 months of hyperglycemia (HG) and compared with age-matched WT controls. Quantitative data was obtained by normalization of β-Actin bands and represents GLUT1 and PEDF expression in Ins2^{Akita} related to WT (control). N=6. ns represents non-significant data determined by Sidak's multiple comparisons test, $P < 0.05$.

3.5. Discussion

Diabetic retinopathy (DR) is one of the most frequent complications of *Diabetes mellitus*, affecting about 90% of patients with type 1 diabetes (Garg *et al.*, 2009). It is known that hyperglycemia and ischemia are key factors for the progression of the disease (Farjo *et al.*, 2010); however the mechanism by which hyperglycemia contributes for the development of the disease remains unclear (Cheung *et al.*, 2010). DR is traditionally characterized as a blood-retinal barrier disorder, in which the leakage of blood content, due to pathological neovascularization, is the main feature of the disease (Cheung *et al.*, 2010). While the iBRB breakdown has been extensively investigated, the effects of diabetes on the RPE cells composing the oBRB are still not well known (Simo *et al.*, 2010). The RPE monolayer is extremely important to maintain the homeostasis of the neural retina (Simo *et al.*, 2010) suggesting that its impairment can compromise the retinal function.

The retina is one of the most metabolically active tissues in the human body and glucose is its only source of energy (Chiu *et al.*, 2011). In the retina, glucose transport is exclusively mediated by the glucose transporter 1 (GLUT1) (Kumagai *et al.*, 1995). To better characterize GLUT1 expression in conditions of DR, we have devised a series of *in vitro* experiments using D407 cells, a spontaneously transformed human retinal pigment epithelial (RPE) cell line derived from a primary culture of human RPE cells (Davis *et al.*, 1995). These cells are extensively used as *in vitro* models and are suitable to study molecular mechanisms of the RPE (Slomiany *et al.*, 2004, Reinisalo *et al.*, 2012). RPE cells were exposed to different concentrations of glucose to mimic normoglycemia (5 mM of D-Glucose) and hyperglycemia (25 mM D-Glucose). In addition, cells were also exposed to hypoxia to simulate retinal ischemia observed in DR patients (Farjo *et al.*, 2010). It was previously shown that hypoxia induces overexpression of GLUT1 in mouse fibroblasts in response to metabolic adaptation (Chen *et al.*, 2001), but there was no evidence regarding changes in GLUT1 expression due to hypoxia in RPE cells. Our Western blot analysis showed no

differences in GLUT1 protein expression in the cells cultured with normoglycemic medium (Figure 3.1). In contrast, it is possible to observe that hypoxia induces a significant increase in GLUT1 protein levels in the cells cultured with 25 mM of D-Glucose compared with the control cells cultured in normoglycemic (5mM of D-Glucose) medium. This suggests that the diabetic environment induced by high glucose and hypoxia most likely contribute to the overexpression of GLUT1. Analysis of protein expression by immunocytochemistry shows an increase of GLUT1 staining in the cell membrane of the RPE cells in response to hypoxia (Figure 3.2). Similarly to the previous Western Blot results shown in Figure 3.1, the staining is stronger in cells cultured with 25 mM of D-glucose, which further supports the contribution of high glucose levels for the expression of GLUT1. To confirm this finding we have isolated both the membrane and soluble fractions of D407 cells cultured with 25 mM of D-glucose. We have found a marked increase in GLUT1 expression in the membrane fraction of cells in hypoxic conditions when compared with cells cultured with the same glucose concentration in normoxia (Figure 3.3). Interestingly, the soluble concentration of GLUT1 is similar in both conditions, suggesting that the increase in GLUT1 expression observed in the whole cell lysates (Figure 3.1) is due to an increase in GLUT1 expression in the cell membrane (figure 3.3). These results are in accordance to what was previously shown for GLUT4 in cardiomyocytes, where hypoxia induces a translocation of this protein to the cell membrane (Sun *et al.*, 1994). It is known that in response to low levels of oxygen cells shift their glucose metabolism to anaerobic respiration, which is much less efficient (Wenger, 2002). The translocation of GLUT1 to the cell membrane can be a response to achieve a more effective glucose uptake in low oxygen conditions. To test this we have performed a glucose consumption assay in which the glucose remaining in the culture medium was measured in cells cultured under normoxia and hypoxia. Our results show that cells under hypoxia display higher glucose consumption compared with cells cultured under normoxia (Figure 3.4), showing an increase of glucose uptake by GLUT1 under hypoxia.

As previously stated one of the main functions of RPE cells is their secretory capacity, being responsible for producing and secreting a wide range of factors that support photoreceptors and guaranteeing optimal circulation and supply of nutrients (Strauss, 2005, Simo *et al.*, 2010). One of those factors is PEDF, a neurotrophic and anti-angiogenic factor secreted by RPE cells that acts by inhibiting retinal endothelial cell growth and migration (Barnstable *et al.*, 2004).

Several studies have shown an imbalance between VEGF and PEDF levels in the vitreous of diabetic patients, showing that secretory function of RPE might be impaired (Ogata *et al.*, 2002, Boehm *et al.*, 2003, Funatsu *et al.*, 2006). A significant decrease in PEDF secreted by cells cultured with high glucose and subjected to hypoxia is observed in Figure 3.5, showing that the increase in glucose uptake by GLUT1 has a negative impact in the PEDF expression.

Our *in vitro* findings were also confirmed *in vivo* in the RPE of the diabetic $Ins2^{Akita}$ mice, comparing with non-diabetic WT mice. The $Ins2^{Akita}$ mouse is a non-obese model of type 1 diabetes that has been widely used as a model of DR (Barber *et al.*, 2005, Gastinger *et al.*, 2006, Han *et al.*, 2013).This mouse model is considered a more reliable model to study DM complications as it develops spontaneously the disease (Barber *et al.*, 2005) in contrast with STZ models. In $Ins2^{Akita}$ animals, hyperglycemia starts approximately at 4 weeks after birth, with retinal complications visible approximately 3 months after the onset of hyperglycemia, including vascular leakage, loss of pericytes, thickening of the inner retinal layers, (Barber *et al.*, 2005) and increase of angiogenic markers such as VEGF (Han *et al.*, 2013).

In our study we have found a marked increase in GLUT1 expression in the RPE of diabetic mice, 4, 7 and 10 months after the onset of hyperglycemia, when compared with non-diabetic WT animals (Figure 3.6). This is consistent with the findings of Badr and co-workers in STZ-induced diabetic mice, where the fraction of glucose entering the retina is higher across the RPE than across the iBRB (Badr *et al.*, 2000).

Diabetic retinopathy is usually characterized as a disease affecting primarily the retinal microvasculature, but its etiology is still not fully elucidated. We have found a significant decrease in PEDF expression in the RPE of diabetic mice, showing a significant impairment of the neurotrophic secretory function of RPE immediately after the onset of the disease. These results together with our *in vitro* results and work of others (Han *et al.*, 2013), confirm the imbalance between pro- and anti-angiogenic factors in the retina of this diabetic mouse model. Although they observed no significant decrease in PEDF levels in the neuroretina (which excludes RPE) of diabetic mice before 7 months of hyperglycemia (corresponding to 9 months of age), we found a significant decrease in PEDF expression by RPE cells two months after the onset of hyperglycemia. This result points to the possibility that in DR the oBRB is affected before the iBRB.

3.6. Conclusion

Our data with RPE cells show an increase in GLUT1 expression in a glucose-dependent manner under hypoxic conditions. This increase is associated with increased translocation, stabilization, and function of GLUT1 in the cell membrane of cells cultured under hypoxia compared with cells under normoxia. The increase of GLUT1 was associated to a decrease in PEDF production, showing an impairment of RPE secretory capacity. This was also confirmed *in vivo*, in which with an increase in GLUT1 and decrease in PEDF expression was also observed in the RPE of $Ins2^{Akita}$ diabetic mice.

Further studies will focus on determining if this increase in GLUT1 expression and activity can explain the increase in levels of reactive oxygen species (ROS) (Kowluru *et al.*, 2007) and advanced glycation end products (AGEs) (Singh *et al.*, 2014). Additionally, our lab is studying if the well-studied up-regulation of VEGF by ROS,

AGEs (Lu *et al.*, 1998, Sasaki *et al.*, 2002) and hypoxia itself can contribute to the decrease in PEDF secretion by RPE cells, thus promoting the imbalance between PEDF and VEGF that is visible in patients suffering from DR.

Chapter 4 – Expression Systems for Retinal Gene Therapy

This chapter is based on the following manuscript:

Sustained Gene Expression to the Retina by Episomal Vectors

Sofia M. Calado[1,2,3], Ana V. Oliveira[1,2,3], Susana C. Machado[3], Rudolf Haase[4], Gabriela A. Silva[2,3]

[1]Doctoral Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, Portugal

[2]Department of Biomedical Sciences and Medicine, University of Algarve, Portugal

[3]Center for Molecular and Structural Biomedicine/Institute for Biotechnology and Bioengineering (CBME/IBB) LA, University of Algarve, Portugal

[4]Department of Pharmacy, University of Munich, Germany

*Published in Tissue Engineering – Part A, **20**(19-20): 2692-2698, March 2014*

DOI: 10.1089/ten.tea.2013.0672

4.1. Abstract

Gene and cellular therapies are nowadays part of therapeutic strategies for the treatment of diverse pathologies. The drawbacks associated with gene therapy—low levels of transgene expression, vector loss during mitosis, and gene silencing—need to be addressed. The pEPI-1 and pEPito family of vectors was developed to overcome these limitations. It contains a scaffold/matrix attachment region, which anchors its replication to cell division in eukaryotic cells while in an extrachromosomal state and is less prone to silencing, due to a lower number of CpG motifs.

Recent success showed that ocular gene therapy is an important tool for the treatment of several diseases, pending the overcome of the aforementioned limitations. To achieve sustained gene delivery in the retina, we evaluated several vectors based on pEPito and pEPI-1 for their ability to sustain transgene expression in retinal cells. These vectors stably transfected and replicated in retinal pigment epithelial (RPE) cells. Expression levels were promoter dependent with constitutive promoters cytomegalovirus immediate early promoter (CMV) and human CMV enhancer/human elongation factor 1 alpha promoter yielding the highest levels of transgene expression compared with the retina-specific RPE65 promoter. When injected in C57Bl6 mice, transgene expression was sustained for at least 32 days. Furthermore, the retina-specific RPE65 promoter showed higher efficiency *in vivo* compared to *in vitro*.

In this study, we demonstrate that by combining tissue-specific promoters with a mitotic stable system, less susceptible to epigenetic silencing such as pEPito-based plasmids, we can achieve prolonged gene expression and a sustained therapeutic effect.

4.2. Introduction

Over the last decades, gene therapy has been a driving force for the development of strategies to treat genetic and acquired diseases. The major disadvantages of DNA-based gene therapy are the epigenetic silencing due to unmethylated CpG motifs present in the bacterial backbone of plasmids grown in bacteria (Yew *et al.*, 2002), and vector loss during mitosis, due to their incapacity to integrate the genome and replicate in the host cell (Conese *et al.*, 2004, Jackson *et al.*, 2006, Gill *et al.*, 2009). The use of plasmids with the capacity to replicate in mammalian cells, less frequent silencing events and species/tissue-specific promoters could lead to sustained gene expression (Conese *et al.*, 2004). To address these issues, Peichaczek and co-workers developed a new vector, named pEPI-1, which contains a scaffold/matrix attachment region (S/MAR), derived from the human interferon β-gene (Piechaczek *et al.*, 1999). Other authors have shown the involvement of S/MARs in DNA duplex destabilization and strand opening, suggesting these sequences to be involved in DNA replication and gene expression (Bode *et al.*, 2006, Giannakopoulos *et al.*, 2009). Additionally, it was shown that S/MARs-containing vectors prevent epigenetic silencing of gene expression by shielding the transgene sequence from adjacent regulatory sequences and heterochromatinization (Argyros *et al.*, 2008), maintaining the vector in a transcriptionally active state (Wong *et al.*, 2011). The original pEPI-1 vector contains two mammalian transcription units and a total of 305 CpG motifs, most of them located in the elements of the vector required for bacterial propagation. To reduce the CpG content in bacterial backbone of pEPI-1 and obtain increased transgene expression *in vitro* and *in vivo*, a new non-viral vector, pEPito, was created by Haase and colleagues (Haase *et al.*, 2010). pEPito was constructed by cloning the pEPI-1 plasmid replicon in a plasmid backbone containing 60% less CpG motifs and excluding the second transcription unit. A comparative study between pEPito and pEPI-1 constructs showed pEPito-based vectors to be more efficient, both *in vitro* and *in vivo* (Haase *et al.*, 2010), explained by CpG depletion of the bacterial backbone, which exhibits a strong

influence on epigenetic silencing events (Yew *et al.*, 2002, Hyde *et al.*, 2008, Bauer *et al.*, 2010, Haase *et al.*, 2010).

The eye is an attractive target for gene therapy because it is an accessible and immune-privileged organ (Bloquel *et al.*, 2006, Conley *et al.*, 2008). Moreover, due to its small size, only a small amount of a therapeutic agent is necessary to observe a therapeutic effect (Bloquel *et al.*, 2006). Diseases like glaucoma, retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are blinding disorders that contribute to more than 25% of blindness cases, worldwide (Naik *et al.*, 2009). The ineffectiveness of current treatments makes these diseases ideal targets for gene therapy (Bloquel *et al.*, 2006, Naik *et al.*, 2009). Moreover, in most of cases its genetic etiology is well known and there is easy access to the photoreceptors or RPE by localized injection (Musarella *et al.*, 2011). Studies with viral vectors, based on adeno-associated virus (AAVs), have as major limitation the small size of the therapeutic gene that can be incorporated into the viral vector (Kumar-Singh, 2008). Therefore, our goal is to combine a non-viral vector with an efficient gene expression system both in duration and expression levels, to overcome the need of repeated injections, thus increasing patient compliance.

In this study, pEPI-1 and pEPito-based vectors were evaluated for their ability of sustained transgene expression both *in vitro*, in retinal pigment epithelial (RPE) cells and *in vivo* in the mouse retina. We found pEPito to be an excellent gene expression system, adequate for sustained gene expression.

4.3. Materials and Methods

4.3.1. pEPI-1 and pEPito-based vectors

Table 2 illustrates the five different plasmids tested in this study, which are based on pEPI-1 and pEPito vectors.

Table 2 – pEPI and pEPito vectors used in this study

Plasmid	Size	Backbone	Promoter	Transgene	CpG motifs (backbone)
pEPi-1	6.7 kb	pEPi	CMV	eGFP	206
pEPito-CMV-eGFP	5.3 kb	pEPito	CMV	BSD-eGFP	37
pEPito-hCMV-eGFP	5.2 kb	pEPito	hCMV	BSD-eGFP	37
pEPito-RPE65-eGFP	7.5 kb	pEPito	RPE65	BSD-eGFP	37
pEPito-hCMV/RPE65-eGFP	7.8 kb	pEPito	hCMV/RPE65	BSD-eGFP	37

The pEPI-1 vector contains a S/MAR sequence, derived from the human interferon β-gene, two mammalian transcription units, and a CMV constitutive promoter. The derivative pEPito vectors contain a S/MAR sequence, and four different promoters: CMV promoter, human CMV enhancer/human elongation factor 1 alpha promoter (hCMV), human CMV enhancer/RPE65 (hCMV/RPE65), and RPE65, a RPE tissue-specific promoter (Le Meur *et al.*, 2007). pEPito vectors also contain an eGFP-BSD cassette in which eGFP (enhanced green fluorescent protein) and BSD (blasticidin) are expressed through an IRES (internal ribosomal entry site). In addition to the promoter, the backbones of the vectors vary in CpG content and size (between 5.2 and 7.8 Kb).

Chemically competent *E. coli* GT115 bacteria (Invivogen) were transformed using 30 ng of each plasmid. Transformed bacteria were selected on LB-plates containing either ampicillin (Sigma) or kanamycin (Sigma). After bacterial propagation, pDNA was isolated using the QIAGEN® Plasmid Maxi Kit (QIAGEN), according to the

manufacturer's instructions. The restriction map of the plasmids was confirmed using endonuclease digestion and subsequent gel electrophoresis.

4.3.2. Transfection Efficiency Assay

In this study, the transfection efficiency of the plasmids was evaluated in a human RPE cell line, D407. This cell line was derived from an eyeball of a 12 year-old child (Davis *et al.*, 1995). These cells have been extensively used as a model of the retina pigmented epithelium (Slomiany *et al.*, 2004, Reinisalo *et al.*, 2012).

D407 cells were cultured in Dulbecco's Modified Eagle Medium (Sigma) supplemented with 5% FBS (PAA), 1% of L-Glutamine (Sigma) and 1% Penicillin/Streptomycin (Sigma).

For the transfection assay, $2x10^5$ cells were seeded in a 6-well tissue culture plate (Orange Scientific). Twenty-four hours after plating, the cells were transfected with 1 µg of DNA and FuGENE® HD (Promega) as transfection reagent using a 3:1 (µL of FuGENE® HD:µg of DNA) ratio, according to the manufacturer's instructions.

Forty-eight hours after transfection, cells were suspended, washed three times with phosphate buffered saline (PBS) and GFP expression analyzed using a FACSCalibur (Becton Dickinson) device and JDS Uniphase® laser, with 100 000 events recorded. Controls included non-transfected cells cultured in the same conditions.

4.3.3. Colony-forming Assay

Fourty-eight hours post transfection, D407 cells were transferred from the 6-well tissue culture plate to 10 cm Petri dishes (BD Biosciences) and selected in the presence of blasticidin (Sigma). Due to conflicting reports in the literature regarding the blasticidin concentration for selection of stably transfected cells (Notari *et al.*, 2006, Zhu *et al.*, 2009), we have determined the adequate blasticidin concentration to select D407 transformed cells by a MTT assay where six different concentrations of blasticidin were

tested - 0.01, 0.1, 1, 3, 5 and 10 µg/mL. The selected concentration of blasticidin was 1 µg/mL (please see supplementary data).

After 32 days of selection with blasticidin, three colonies of each plasmid were extracted and maintained in culture for two additional months under selection (up to three months in total), with eGFP positive cells quantified by flow cytometry as described in the previous section.

The remaining colonies were fixed with 4% paraformaldehyde (PFA, Sigma) in PBS, stained with 2% methylene blue in methanol (VWR) and counted.

4.3.4. Injection of pEPito vectors in C57Bl6 mice and eGFP expression

To test the *in vivo* efficiency of the pEPito-derived vectors, FuGENE® HD-plasmid complexes were injected into the eye of C57Bl6 mice pups. All methods involving animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Litters and nursing dams were housed in plastic cages in a pathogen-free environment, with continuous access to food and water on a 12-hour light-dark schedule.

The DNA-FuGENE complex used in vivo were prepared by mixing 2 µg of plasmid DNA and 1µL of FuGENE® HD in a total volume of 20 µL of PBS. Pups at postnatal age of 5 days (P5) were anesthetized on ice. Using a needle, the sclera was punched in the temporal side and 1 µL of the DNA-FuGENE complex was injected into the vitreous cavity. After the injection a topical ointment consisting of gentamicin and prednisolone acetate was applied to reduce the pain and the risk of infection. The contralateral non-injected eye was used as control.

At 3, 7, 14, 21 and 32 days post injection (dpi), 8 mice per plasmid DNA were humanely sacrificed, the eyes enucleated and fixed in ice-cold 4% PFA in PBS, for 24 hours. This was followed by overnight immersion in 30% sucrose (Sigma) in PBS and embedded in OCT (Tissue-Tek), for cryosection. 10 µm thick serial sections were

performed, counterstained with DAPI and mounted with Fluoromount G (Electron Microscopy Sciences).

In vivo transfection was evaluated by eGFP expression using an AxioImager Z2 Fluorescence microscope (Zeiss), with AxioCam HRm and magnifications of 50x and 200x. The transfected area was quantified using ImageJ software, by quantifying both the GFP-positive and the ganglion cell layer areas, and calculating the percentage of GFP-positive area relative to the ganglion cell layer area.

4.3.5. Statistical analysis

Statistical analyses were performed using the SPSS Software, version 19.0. One-way ANOVA was used to compare the *in vitro* transfection efficiency of the different plasmids and the *in vitro* colony-forming efficiency of stably-selected D407 cells. In the event of a significant ANOVA, Tukey test was used for post hoc analysis.

Data are expressed as means ± standard deviation and $^{*}p<0.05$

4.4. Results

4.4.1. Transfection efficiency of pEPI-1 and pEPito vectors in RPE cells is promoter-dependent

In vitro experiments were performed in human RPE cells, D407. The transfection efficiency was analyzed by flow cytometry of GFP-positive cells, 48 h post transfection.

Our results for D407 cells showed that the plasmids pEPito-CMV, pEPito-hCMV and pEPI-1, containing either the CMV and hCMV constitutive promoters, presented transfection efficiencies in the range of 30% (Figure 4.1). The plasmid containing the hCMV enhancer element and the RPE65 promoter (pEPito-hCMV/RPE65) had the lowest transfection efficiency (6%), compared to the plasmids containing either the CMV or hCMV promoters (Figure 4.1). No GFP-positive cells were detected in cells transfected with pEPito-RPE65 (Figure 4.1).

Due to the low transfection efficiency observed with pEPito-RPE65, this vector was not further tested.

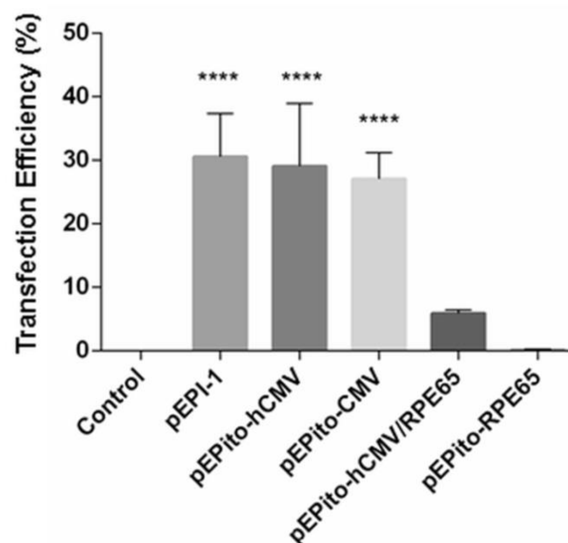


Figure 4.1: Transfection efficiencies for transiently transfected D407 cells with the 5 different plasmids. Mean values are derived from four independent experiments and statistical significance was determined with One-Way Anova followed by a post hoc Tukey Test. The statistical difference is indicated by a star (*) symbol (*p<0.05).

4.4.2. pEPito plasmids express GFP for three months in mitotically active human RPE cells

As previously stated, one of the major advantages of the S/MAR-containing plasmids is their ability to be maintained as an episome and to be replicated during mitosis (Wong *et al.*, 2011), therefore avoiding one of the major drawbacks of plasmid gene therapy, which is the vector dilution effect, as cells divide, lesser number of cells will contain the plasmid with the gene of interest. To test the capacity of these plasmids to transfect cells and to be replicated during cell division, hence forming a stable transfected colony, D407 cells were transfected with pEPito-CMV, pEPito-hCMV and pEPito-hCMV/RPE containing the blasticidin resistance gene (BSD), for mammalian cell selection.

Due to conflicting reports in the literature regarding the BSD concentration for colony selection, we have performed a MTT assay to determine the minimal BSD concentration to eliminate non-transfected cells and therefore select for pEPito-modified cells.

The ideal concentration of BSD to eliminate all untransfected cells, in one week, was 1 µg/mL (Figure 4.2).

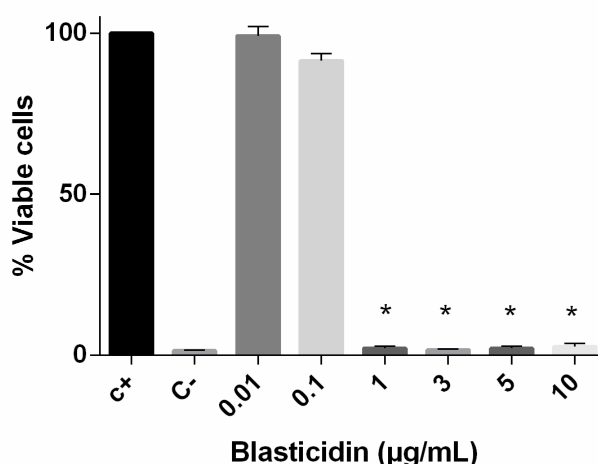


Figure 4.2: Relative percentage of D407 viable cells using different concentrations of BSD. C + is the positive control, without BSD; C - is the negative control, latex extract. Statistical significance was determined with one-way ANOVA followed by a post hoc Tukey test. The statistical difference is indicated by a star (*) symbol (*$p < 0.05$). BSD, blasticidin.

These results were further used for all selection experiments. All plasmids were able to originate stably transfected colonies of D407 cells (Figure 4.2).

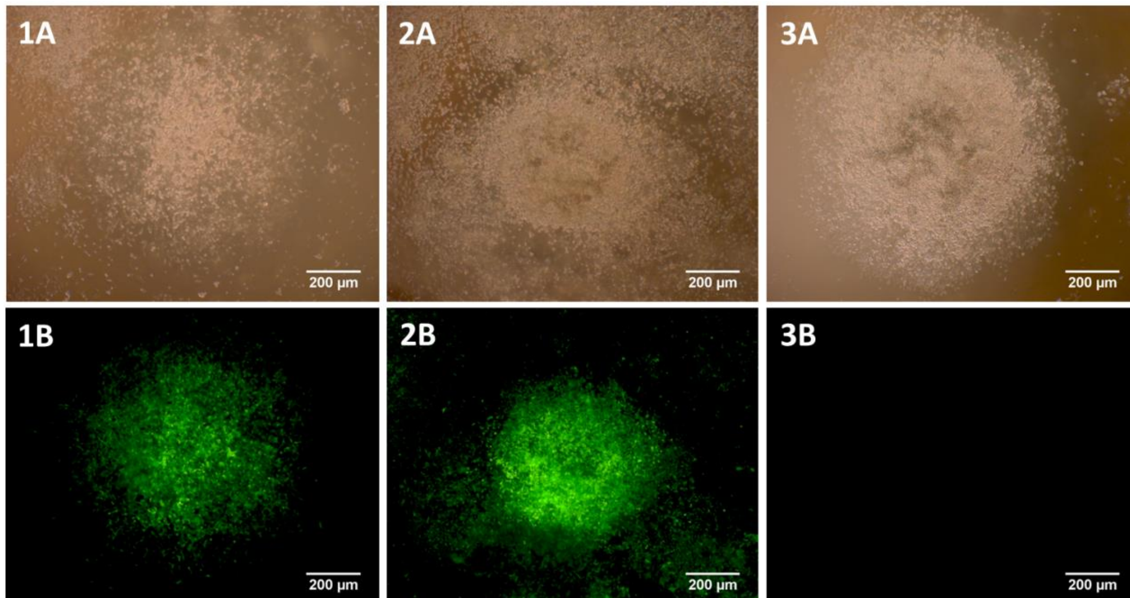


Figure 4.2: Bright field (A) and Fluorescence (B) microscopy of a stably transfected D407 cell line colony with pEPito-CMV-eGFP-BSD (1); pEPito-hCMV-eGFP-BSD (2) and pEPito-hCMV/RPE65-eGFP-BDS (3) 32 days post-transfection.

The plasmid that led to a higher number of colonies after 32 days of selection was pEPito-hCMV, resulting in approximately 510 colonies (Figure 4.3). pEPito-CMV originated 360 colonies (Figure 4.3) and for pEPito-hCMV/RPE65, the number of colonies formed was markedly lower, resulting in about 200 colonies (Figure 4.3). This result was not surprising, as because pEPito-hCMV/RPE65 had a lower transfection efficiency, compared with the plasmids containing either the CMV or hCMV promoter.

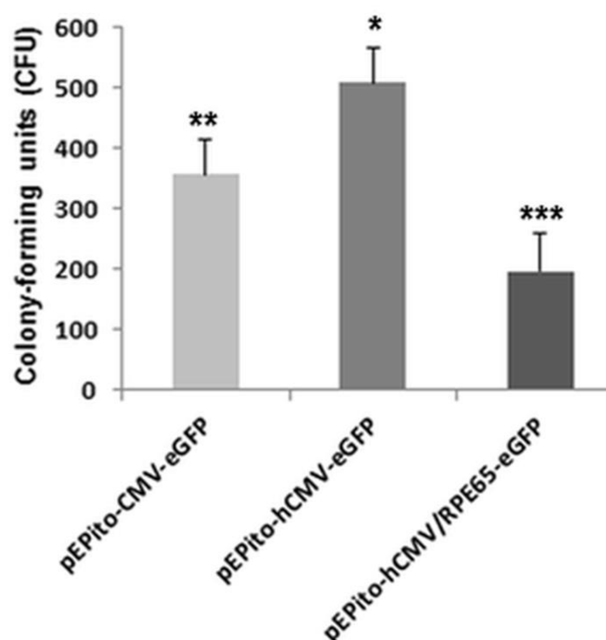
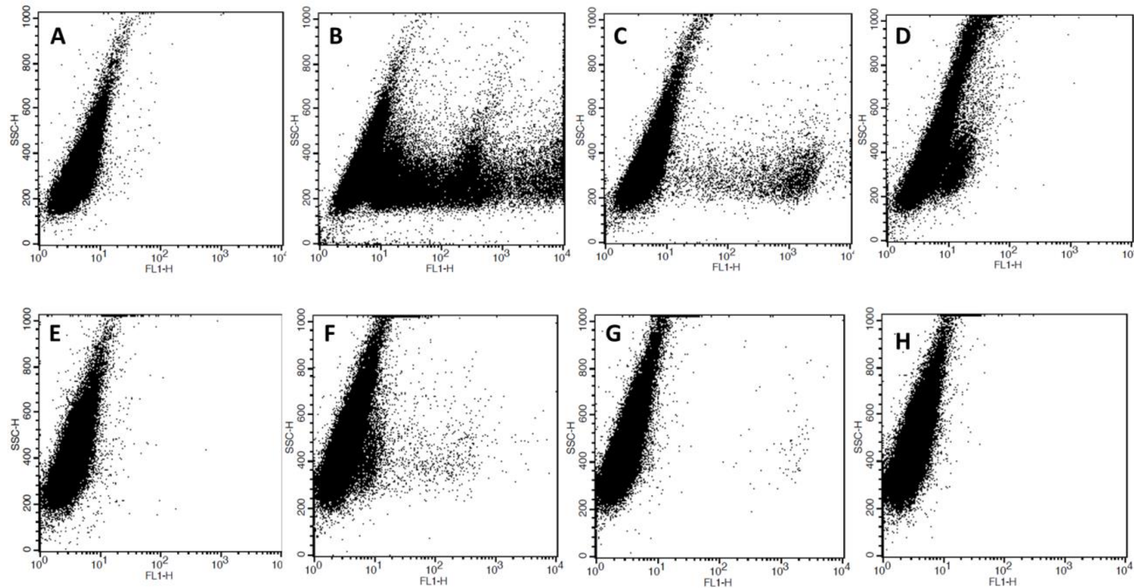


Figure 4.3: Colony-forming efficiency of D407 cells selected with blasticidin, for each of the pEPito plasmids. Mean values are derived from three independent experiments and statistical significance was determined with One-Way Anova followed by a post hoc Tukey Test. Bars labeled with * indicate statistical difference ($p<0.05$).

After one month of selection, the fluorescence of the colonies, observed by fluorescence microscopy, shows that the intensity of the fluorescence decreased over time in the colonies transfected with pEPito-CMV. On the other hand, in the colonies transfected with pEPito-hCMV, the intensity of fluorescence remained constant and high. These results were further confirmed by flow cytometry. After two months of selection, using a selection medium with BSD, about 19% of the cells transfected with pEPito-hCMV presented fluorescence (Figure 4.4 B). On the other hand, only 3% of the cells transfected with pEPito-CMV presented fluorescence (Figure 4.4 C) and in the cells transfected with pEPito-hCMV/RPE65 the eGFP expression was only detected in 1% of the cells (Figure 4.4 D). After three months of selection, almost no eGFP positive cells were detected, in cells transfected with both pEPito-CMV and pEPito-hCMV/RPE65 (Figure 4.4 G;H). In contrast, 2% of the cells transfected with pEPito-hCMV remain fluorescent (Figure 4.4 F).



E F G H

Figure 4.4: Flow cytometry of D407-colonies stably-transfected with pEPito-hCMV (B;F); pEPito-CMV (C;G) and pEPito-hCMV/RPE65 (D;H). Upper panels represent cells after two months of selection with BSD and lower panels corresponds to cells after three months of selection. A and E are the control non-transfected cells.

4.4.3. pEPI and pEPito gene transfer to mouse retinas:

An intravitreal injection of 1 µL of each plasmid using FuGENE® HD was performed in 5 days post-natal (P5) C57Bl6 mice. These results show absence of an inflammatory in the injected eyes, compared with the controls (non-injected contralateral eye) and a normal appearance.

At 3, 7, 14, 21 and 32 dpi the GFP expression was analyzed in eye sections. Since the administration method was intravitreal injection, it was expected that the retinal ganglion cell layer was the one to be transfected, because it is in direct contact with the vitreous cavity we have indeed observed that the transfected cells expressing GFP were visible in clusters on the border of the ganglion cell layer (Figure 4.5).

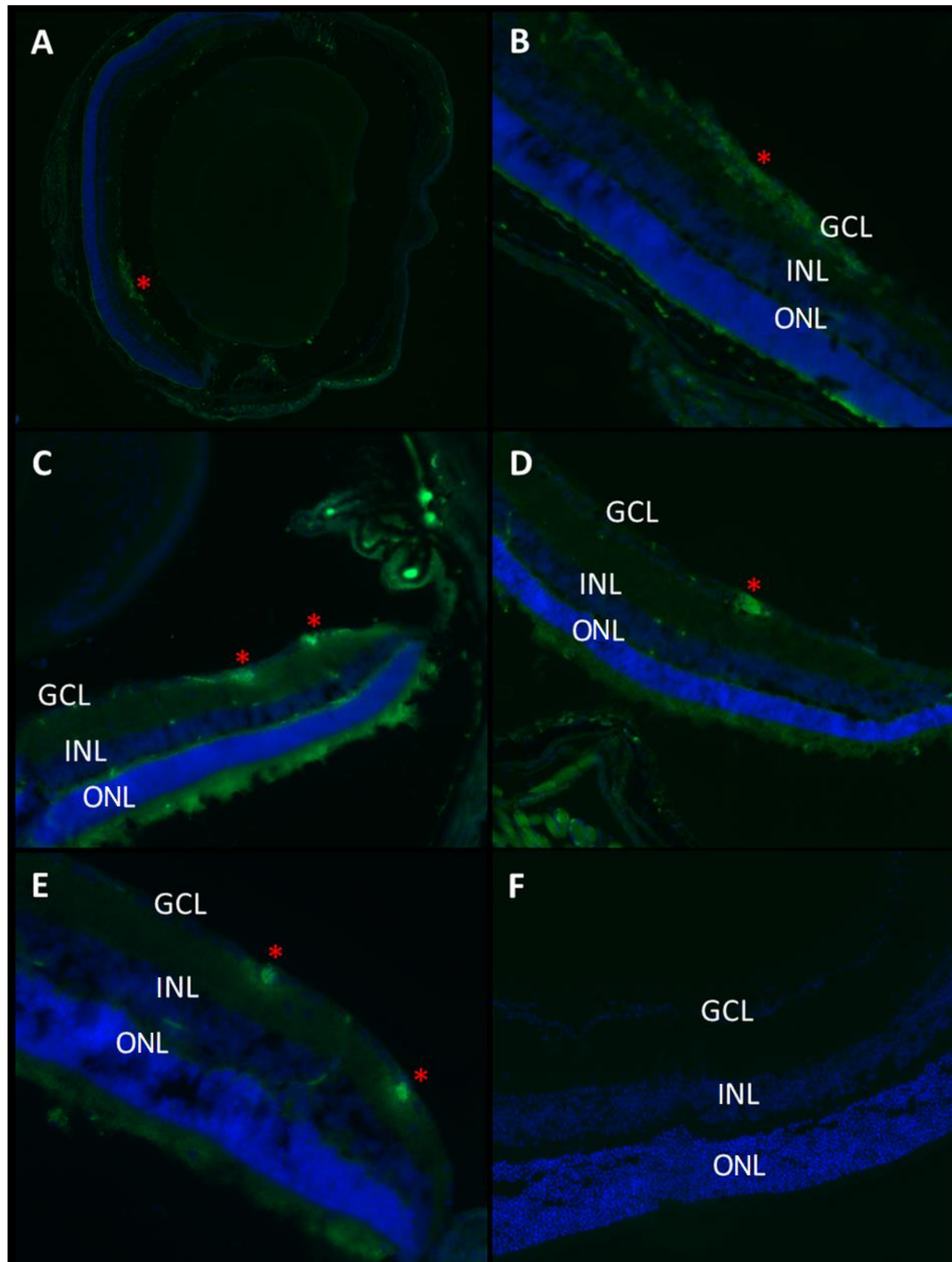


Figure 4.5: Transversal sections of a mouse retina injected with pEPI-1 and pEPito-based vectors, sacrificed 32 days post injection. * indicates clusters of eGFP-expressing ganglion cells transfected with pEPito-hCMV (A) (Magnification: 50x); (B) corresponds to an amplification of image (A) (Magnification: 200x); pEPito-CMV (C) (Magnification: 200x); pEPI-1 (D) and pEPito-hCMV/RPE65 (E). F illustrates a non-injected retina, without GFP expression. DAPI (blue) stains nuclei. GCL – Ganglion Cell Layer; INL – Inner Nuclear Layer; ONL – Outer Nuclear Layer.

The overall analysis of the retinal sections showed that all the plasmids were able to express *in vivo*, up to 32 days after injection. Table 3 shows the number of retinas, per plasmid and per time-point, in which was possible to detect GFP expression. Since

it was not possible to follow the same animal along time, the results are shown as number of retinas (animals) with GFP expression.

Table 3 – Summary of the number of GFP-positive retinas. (N=8)

DPI	pEPi-1	pEPito-CMV	pEPito-hCMV	pEPito-hCMV/RPE65
3	1	0	4	3
7	3	3	6	4
14	4	4	5	5
21	4	4	5	5
32	3	3	5	4

DPI means days post-injection

Overall, 3 dpi was the time-point in which less retinas exhibited GFP expression, as expected since it is a very short time period for the cell machinery to start the transcription of the genetic material within the plasmid. The number of retinas where it was possible to detect eGFP expression was reduced for pEPI-1 compared with the number of retinas injected either with pEPito-CMV or pEPito-hCMV. On the other hand, we observed a higher number of retinas displaying fluorescence in the eyes injected with pEPito-hCMV than with pEPito-CMV. Even in spite of the *in vitro* experiments using pEPito-hCMV/RPE65 had lower transfection efficiencies than those containing either CMV or hCMV promoter, this result was reversed *in vivo,* since the number of retinas expressing eGFP was comparable to the one observed for pEPito-hCMV. As expected, no fluorescence was detected in the retinas of the contralateral non-injected eye (Figure 4.5- F).

After quantification, we conclude that the transfected area was about 1.5–2% of the GCL area. This area was not affected by the plasmid used.

4.5. Discussion

The major disadvantages of pDNA-based gene therapy are the low transfection efficiency, the epigenetic silencing, due to the unmethylated CpG motifs present into its bacterial backbone (Yew *et al.*, 2002), and the loss of the vector during mitosis, due to the incapacity of these vectors to integrate the genome of the host cell (Conese *et al.*, 2004, Jackson *et al.*, 2006, Gill *et al.*, 2009). The use of plasmids containing sequences that enable them to replicate in mammalian cells, such as plasmids containing S/MARs, with a minimum of unmethylated CpG motifs and species specific tissue promoters could lead to gene expression for longer periods of time.

In this study, the potential of plasmids containing S/MARs, pEPI-1 and its derivative pEPito, as expression systems for gene transfer to the retina was tested *in vitro* and *in vivo*. The aim was to assess if these expression systems could display, in the retina, a prolonged and sustained expression, as observed for other tissues such as the liver (Argyros *et al.*, 2008, Haase *et al.*, 2010, Argyros *et al.*, 2011, Wong *et al.*, 2011).

Our transfection assay showed that for retinal cells D407, the constructs containing the RPE-specific were less effective than all the other constructs tested with either CMV or hCMV promoter, which presented a transfection efficiency of about 30%. All *in vitro* experiments were performed by transfecting equal amounts of plasmid (and not equimolar mass of DNA), this can account for differences regarding the increased transient transfection efficiencies observed for the smaller plasmids.

To test the ability of these vectors to be replicated during mitosis, a colony-forming assay was performed, and all the plasmids were able to originate stably transfected colonies for at least 32 days post transfection. pEPito-hCMV originated the highest number of colonies and pEPito-hCMV/RPE65 the lowest. This result was expected because pEPito-hCMV/RPE65 had a lower transfection efficiency compared with the plasmids containing either CMV or hCMV promoter. Moreover, the cells extracted from the colonies transfected with pEPito-hCMV were able to maintain the GFP expression

for further two months, whereas in the colonies transfected with pEPito-CMV or pEPito-hCMV/RPE65 no GFP positive cells were detected by flow citometry after this period. This result emphasizes the importance of the promoter in the expression profile of a plasmid, showing that the CMV promoter is more susceptible to silencing by epigenetic events than the hCMV promoter. Moreover, the plasmids are capable of stably replicate for over 100 cell divisions *in vitro*, which indicates that, *in vivo*, in retinal cells, mostly post-mitotic, gene expression will be maintained for long periods of time.

In the *in vivo* assay, our results demonstrate that all the plasmids were able to express eGFP in mouse retinas for at least 32 dpi. The number of retinas expressing eGFP was lower for pEPI-1 than for pEPito-CMV or pEPito-hCMV. It was demonstrated that plasmids with a higher CpG content are more susceptible to epigenetic silencing events (Yew *et al.*, 2002, Hyde *et al.*, 2008, Bauer *et al.*, 2010). Since the pEPI-1 backbone has more CpG motifs than the pEPito backbone (Haase *et al.*, 2010), our results are in agreement with the literature for other organs emphasizing the importance of a CpG-depleted vector for a sustained expression. On the other hand, we observed a higher number of retinas displaying fluorescence in the eyes injected with pEPito-hCMV than with pEPito-CMV. This is an accordance to what was described by Haase *et al (Haase et al., 2010),* which has shown these plasmids to originate the strongest luciferase expression *in vivo*, due to the fact that the hCMV promoter is less affected by epigenetic silencing events than CMV promoter (Hyde *et al.*, 2008).

Finally, although in our *in vitro* experiments pEPito-hCMV/RPE65 had lower transfection efficiency, the plasmid originates better results *in vivo,* highlighting the relevance of a tissue-specific promoter. Moreover, the expression of the pEPito-hCMV/RPE65, containing a RPE-specific promoter observed in ganglion cells can be explained by the presence of the hCMV enhancer, which has been described as strongly potentiating gene expression.

Our *in vivo* proof-of-principle study has shown lower efficiency than other studies where these systems were complexed with chitosan nanoparticles for corneal gene

delivery (Klausner *et al.*, 2012), or using AAVs for retinal transduction (Wang *et al.*, 2011), it is important to highlight that the amount of DNA used in this study is much less than in the other studies. Our ultimate goal is to combine these very efficient systems with new nonviral vectors being developed in our lab.

With this study we prove that these improved episomal vectors containing S/MARs and less CpGs - pEPito vectors - can be used for efficient gene transfer to the retina. In addition, the combination of an enhancer that is less affected by epigenetic silencing effects with a tissue-specific promoter, such as the hCMV-RPE65 promoter, is crucial to extend the transgene expression, *in vivo*, and contribute for an efficient gene therapy.

Chapter 5 – Gene Therapy for Diabetic Retinopathy

This chapter is based on the following manuscript:

pEPito-driven PEDF expression ameliorates Diabetic Retinopathy hallmarks

Sofia M. Calado[1,2], Francisco Diaz-Corrales[3] and Gabriela A. Silva[2]

[1]Doctoral Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, Faro, Portugal

[2]CEDOC, NOVA Medical School | Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal

[3]Department of Cell Therapy and Regenerative Medicine, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain

Manuscript under revision in the journal *Human Gene Therapy – Methods*

5.1. Abstract

Diabetic retinopathy (DR) is one of the major complications of Diabetes *mellitus.* It is characterized by retinal microvascular changes caused by chronic exposure to hyperglycemia, leading to low tissue oxygenation and ultimately to neovascularization. Laser photocoagulation and vitrectomy are the most efficient treatments for DR, but display severe side effects such as the destruction of the healthy retina. Another clinical approach uses anti-angiogenic agents to prevent and delay progression of neovascularization, but these require recurrent local administrations which increase the possibility of retinal detachment, vitreous hemorrhage, and cataract formation.

Studies in human diabetic retinas have revealed an imbalance between pro-angiogenic factors such as the vascular endothelial growth factor (VEGF) and anti-angiogenic factors, like pigment epithelial-derived factor (PEDF). This imbalance favors pathological angiogenesis contributing to DR, and can constitute a therapeutic target. Gene therapy was recently shown to be an adequate intervention for long-term treatment of several retinal pathologies. We have previously shown the newly engineered episomal vector pEPito to be able of sustained gene expression in the mouse retina. We here show that pEPito was able to overexpress PEDF for up to three months, both in *in vitro* cultures of human RPE cells, as well as in the retina of diabetic mice after a single subretinal injection. In vivo, in parallel with the increase in PEDF we observed a decrease in VEGF levels in injected compared with non-injected eyes and a significant effect on two hallmarks of DR: reduction of glucose transport (by glucose transporter GLUT1), and reduction of inflammation by decreased reactivity of microglia. Jointly, these results point to a significant therapeutic potential of gene therapy with pEPito-PEDF for the treatment of DR.

Key words: Gene therapy; Diabetic Retinopathy; pEPito; PEDF; microglia;

5.2. Introduction

Diabetes *mellitus* (DM) is a group of metabolic diseases characterized by high blood glucose that leads to several complications, including diabetic retinopathy (DR). DR is a progressive disease that features microvascular dysfunction due to chronic exposure to hyperglycemia (Cheung *et al.*, 2010). Hyperglycemia is associated with a wide-range of biochemical events that contribute for the progression of the disease, such as accumulation of sorbitol, advanced glycation end-products (AGE), oxidative stress, protein kinase C activation, inflammation, up-regulation of the renin-angiotensin system and vascular endothelial growth factor (VEGF) (Cheung *et al.*, 2010). Altogether, these mechanisms contribute to physiological changes in the retina, including capillary basement membrane thickening that induces pericyte and endothelial cell death, causing inner blood retinal barrier (BRB) breakdown. This loss of retinal capillary function leads to vascular wall leakage, inflammation and ischemia, contributing to retinal neovascularization, formation of microaneurysms, edema, and hemorrhages, which lead to irreversible blindness (Frank, 1995, Cheung *et al.*, 2010, Chiu *et al.*, 2011).

Attempts to revert pathological neovascularization have focused on the inhibition of VEGF, since the increase in its levels causes changes in the BRB, resulting in retinal macular edema and neovascularization (Chiu *et al.*, 2011). However, altered VEGF levels are not the single event associated with neovascularization: during DR, the balance between pro- and anti-angiogenic factors that inhibits pathological angiogenesis.is disrupted. This is caused by an up-regulation of angiogenic VEGF and down-regulation of the anti-angiogenic and neurotrophic factor pigment epithelial-derived factor (PEDF), secreted by retinal pigment epithelial (RPE) cells (Broadhead *et al.*, 2010, Farjo *et al.*, 2010). PEDF has been shown to reduce neovascularization when administered as a protein to a mouse model of neovascularization (Yoshida *et al.*, 2009, Liu *et al.*, 2012).

Common treatments for DR are laser photocoagulation and vitrectomy; they can however cause retinal damage and permanent impaired vision, with the success of the treatment highly influenced by the disease progression stage (Fong *et al.*, 2004). Recently, anti-VEGF agents, such as pegaptanib, ranibizumab and bevacizumab, have been shown to be more effective inhibiting NV than surgical approaches. The major disadvantage of these agents is their short half-life, which requires repeated intravitreal injections in order to obtain a sustained therapeutic effect (Vinores, 2006, Bakri *et al.*, 2007, Farjo *et al.*, 2010, Praidou *et al.*, 2010). To achieve a sustained therapeutic effect, one can turn to gene therapy, which has already been tested as a treatment for several retinal pathologies (Bainbridge *et al.*, 2008, Maguire *et al.*, 2009, MacLaren *et al.*, 2014), including ocular neovascularization (Igarashi *et al.*, 2003). Viral vectors, with overall limitations such limited size of the inserted gene, immune response, toxicity, and possible oncogenicity, are widely used for gene therapy. Alternative approaches encompass both non-viral vehicles and expression systems that can efficiently deliver and provide sustained gene expression. We have previously described the effectiveness of the self-replicating episomal vector, pEPito, in long-term gene expression in the mouse retina (Calado *et al.*, 2014). Based on this study, we have used pEPito as an expression vector for PEDF to assess its potential for treatment of diabetic retinopathy. Using a two-fold approach - *in vitro* using human RPE cells and *in vivo*, using the $Ins2^{Akita}$ mouse model of DR – we aimed to determine if PEDF can constitute a therapeutic alternative for diabetic retinopathy.

5.3. Materials and Methods

5.3.1. Vector construction

pEPito-hCMV-eGFP-IRES-IB containing the human CMV enhancer/human elongation factor 1 alpha promoter was used as backbone, as described before (Haase *et al.*, 2010, Calado *et al.*, 2014). Human *PEDF* was amplified with specific primers containing Nhe I (5') and Bgl II (3') restriction sites. The amplified fragment was digested with Nhe I and Bgl II and cloned in the pEPito-hCMV, digested with the same enzimes. The resulting construct was confirmed by restriction enzyme digestion and sequencing. The new plasmid pEPito-hCMV-PEDF with a 6.2 kb size was propagated in *E. coli* GT115.

5.3.2. Evaluation of PEDF expression driven by the pEPito vector in vitro

Human retinal pigment epithelial (RPE) cell line D407, used in the *in vitro* experiments, was kindly provided by Dr. Jean Bennett from the University of Pennsylvania (USA). Cells were kept in culture at 37 ºC in a humid chamber with 5% CO_2 and were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 1% Penicillin/Streptomycin (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich) and 5% foetal bovine serum (Sigma-Aldrich). Culture medium was changed every 2 days.

Transfection was performed using FuGENE® HD (Promega) with a 3:1 (µL of FuGENE® HD:µg of DNA) ratio, according to the manufacturer's instructions. Briefly, $2x10^5$ cells were seeded in a 6-well tissue culture plate (Orange Scientific) and 24h after seeding, cells were transfected with 1 µg of DNA. 24 h post transfection cells were transferred from the 6-well tissue culture plate to 6 cm Petri dishes. Once a week, for up to three months, the cells were subcultured and the samples of cells and culture medium collected for Western blot analysis.

5.3.3. Evaluation of PEDF expression driven by the pEPito vector *in vivo*

Four-month old $Ins2^{Akita}$ (diabetic) mice (The Jackson Laboratory) housed under controlled temperature and a 12 h light/dark cycle with food and water *ad libitum* were used for the *in vivo* experiments. Diabetic phenotype was confirmed 2-months after birth by measuring blood glucose levels from a tail puncture (Freestyle Precision, Abbot), with animals used in this study exhibiting blood glucose ≥ 500 mg/dl. All experimental procedures were carried out according to the Portuguese and European Laboratory Animal Science Association (FELASA) Guide for the Care and Use of Laboratory Animals, the European Union Council Directive 2010/63/EU for the use of animals in research and the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and vision research. Mice were profoundly anesthetized by intraperitoneal injection of Avertin®. 1uL of DNA (1 ug/uL) was injected in the subretinal space of the left eye by using an automatic pump injector (WPI) and electroporated using 7 mm tweezer electrodes (tweezertodes, Harvard Apparatus) connected to a BTX ECM 830 (Harvard Apparatus), as previously described (Matsuda *et al.*, 2004).

At 3-months post injection the animals were humanely sacrificed by cervical dislocation, the eyes enucleated, and processed depending on the experiment.

5.3.4. Immunofluorescence for PEDF expression in vivo

For immunohistochemistry, eyes were fixed with ice-cold 4% paraformaldehyde (4% PFA) in PBS overnight at 4 ºC, cryoprotected in 30% sucrose in PBS and embedded in OCT mounting medium (Tissue-Tek). Immunohistochemistry was performed either in 10 µm thick serial sections or whole retinas. Briefly, the samples were washed in 0.1% Triton X100/PBS and blocked in 1% goat serum/PBS at room temperature for 1h. Incubation with the primary antibodies - rabbit polyclonal anti-PEDF 1:100; (Merck Millipore) and rabbit polyclonal anti-Iba1 (1:500; Wako) - was performed overnight at 4ºC.

After incubation samples were washed 3 times in 0.1% Triton X100/PBS, and incubated with the secondary antibody (Alexa Fluor® 594; 1:500; Life Technologies, USA) at room temperature for 1 h. After 3 washes, slides were mounted with Fluoromount G (SouthernBiotech, USA) containing 4',6'-diamidino-2-phenylindole (DAPI). Images were obtained using an AxioVision microscope, using appropriate filter sets (Axio Observer Z2, Zeiss).

5.3.5. PEDF expression by Western blot analysis

Since PEDF is a secreted protein and PEDF-pEPito administration was performed subretinally, we evaluated its expression by Western blot in whole retinas and RPE cells. The latter were isolated by dissection of the eyeball and homogenized in ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA) containing a protease inhibitor cocktail (Roche, Germany).

Whole protein extract of D407 cells was also prepared using cold RIPA buffer supplemented with protease inhibitor cocktail. Protein content was measured by the Bradford assay and samples stored at -80 ºC. Thirty micrograms of protein extract were separated in a denaturing 12% SDS–PAGE gel, the proteins transferred to a PVDF membrane (Amersham, UK), and blocked using Superblock Blocking buffer (Thermo Scientific) containing 0.1% of Tween-20 (Sigma-Aldrich, USA) for 1h at room temperature. The primary antibodies used were rabbit polyclonal anti-PEDF (1:1000; Merck Millipore), rabbit polyclonal anti-VEGF (1:1000; Abcam), rabbit polyclonal anti-GLUT1 (1:3000; Abcam), goat polyclonal anti-Iba1 (1:1000; Sigma-Aldrich), and rabbit polyclonal anti-GFAP (1:1000; Abcam), and were incubated overnight at 4 ºC. β-Actin was incubated for 1h at room temperature (1:10000; Sigma-Aldrich). The membrane was probed with an HRP-conjugated secondary antibody for 1 h at room temperature and the immunoreactive bands were detected by chemiluminescence, using an ECL Plus kit (Amersham).

Secreted PEDF was isolated from the culture medium by protein precipitation using 4 volumes of ice-cold acetone for 1h, at -20ºC. After precipitation samples were centrifuged for 10 minutes at 13 000g, the supernatant decanted, and the pellet air-dried for 30 minutes at room temperature. The pellet containing precipitated proteins was then re-suspended in 1x sample buffer and analyzed by Western blot.

5.3.6. Statistical analysis

All experiments were performed in triplicate and the results expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism software. A value of $P<0.05$ was considered to be statistically significant.

5.4. Results

5.4.1. Characterization of PEDF in the retina of Ins2^{Akita} diabetic mice

Clinical studies have demonstrated a negative correlation between PEDF levels and the development of proliferative diabetic retinopathy (Zhu *et al.*, 2012). To establish if Ins2^{Akita} diabetic mice mimic what was observed in humans and are a valid animal model to evaluate PEDF effects, PEDF expression was evaluated in the retina of 4-month old Ins2Akita diabetic and compared to age-matched wild-type mice. As can be observed in Figures 5.1A and B, there is a significant decrease in PEDF expression in the retina of diabetic animals compared with wild-type ones, similar to what was observed in humans. This decrease is much more evident in the retinal pigment epithelium (RPE) of diabetic mice (Figure 5.1 C and D, compared with the expression in the neural retina.

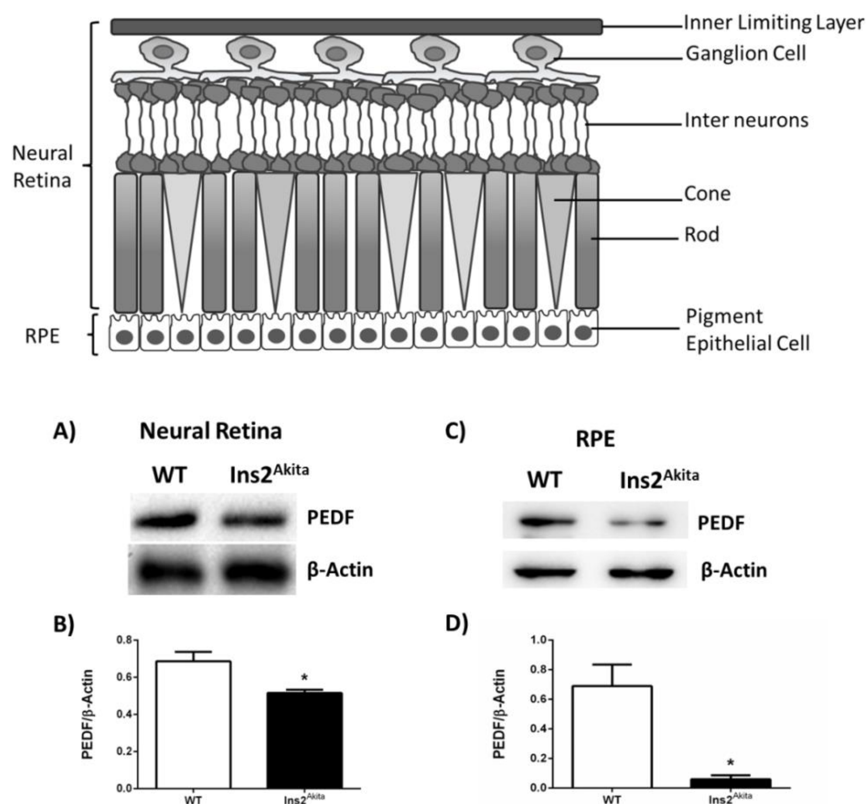


Figure 5.1: PEDF expression is significantly reduced in the retina of diabetic mice. PEDF protein expression was assessed by Western blot of inner retina **(A)** and RPE **(C)** tissue from diabetic and wild type (WT) mice. **B** and **C** represent quantitative data

normalized by the intensity of β-Actin bands. N=5, *P<0.05 represents significant differences in PEDF expression in the retina of Ins2^{Akita} mice compared with the age-matched WT mice, determined by the two-tailed t-test.

5.4.2. pEPito enables long-term expression of PEDF in mitotic RPE cells

For an efficient gene therapy strategy, the expression vector should be able of long-term and sustained levels of transgene expression. In our previous study we have described the effectiveness of the pEPito episomal vectors in sustained gene expression in retinal cells (Calado *et al.*, 2014). We have also shown that plasmids containing the humanized CMV promoter (hCMV), known to be less affected by epigenetic silencing effects, were more efficient in promoting gene expression in retinal cells both *in vitro* and *in vivo* (Calado *et al.*, 2014). Based on these results, we have chosen the pEPito-hCMV backbone to clone PEDF. We have found that our new construct was able to overexpress PEDF for almost three months in actively dividing human RPE cells (Figure 5.2).

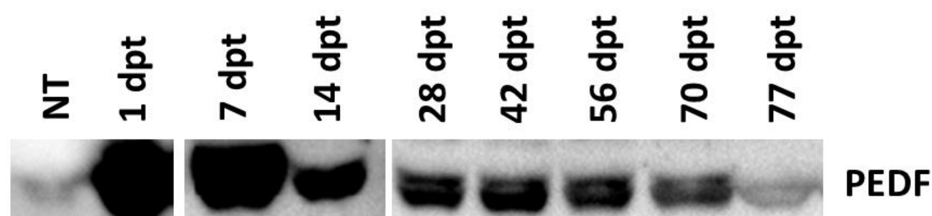


Figure 5.2: Long-term expression of PEDF by D407 cells transfected with pEPito-hCMV-PEDF. pEPito-hCMV-PEDF was able to overexpress PEDF for up to 77 dpt (days post-transfection) in mitotically active cells in levels significantly higher than those observed for non-transfected cells (NT).

5.4.3. Restoration of PEDF protein levels in the retina of Ins2^{Akita} mice

Subretinal injection of a 1 µL solution of pEPito-hCMV-PEDF was performed into one eye of 4-month old Ins2^{Akita} mice, with the contralateral eye remaining non-injected, as a control. Immediately after injection the eyes were electroporated to facilitate entry of the expression vector into the cell (Matsuda *et al.*, 2004). After the procedure and

until sacrifice of the animal, the injected eyes had a normal appearance and absence of inflammation, similar to the non-injected, contralateral eye.

The effect of PEDF overexpression was evaluated in the retina of the Ins2^{Akita} diabetic mouse, a model of DR. Three months post injection, PEDF expression was analyzed in 10 micron eye sections, and immunohistochemistry shows an increase in PEDF detected in the injected eye, when compared with the contralateral non-injected one. Moreover, the intensity of PEDF expression was comparable to one observed in the WT mice (Figure 5.3).

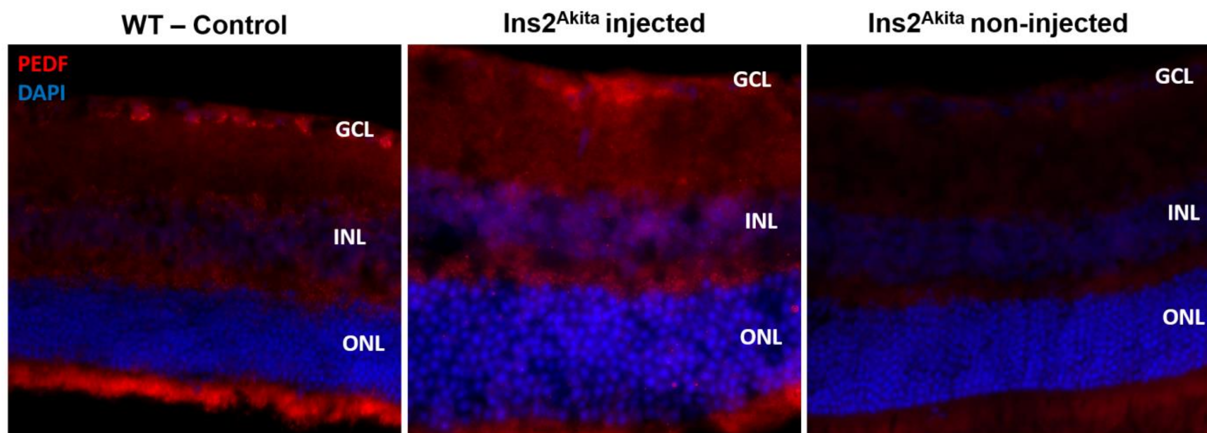


Figure 5.3: PEDF protein levels are restored after injection of pEPito-hCMV-PEDF. Retinal section of an Ins2^{Akita} mouse eye injected with pEPito-hCMV-PEDF-IB three months post injection, showing PEDF levels comparable to age-matched WT mice. The contralateral, non-injected eye, exhibits a significant reduction in PEDF expression. DAPI (blue) stains nuclei and PEDF is marked red. Magnification: 400x

These results were confirmed by Western Blot, with an increase in PEDF expression in injected compared with non-injected eyes. Moreover, the overexpression of PEDF in the retina of Ins2^{Akita} diabetic mice was also associated with a decrease in other hallmarks of diabetic retinopathy, such as VEGF and glucose transporter 1 (GLUT1) in the retina, as well as in the RPE (Figure 5.4).

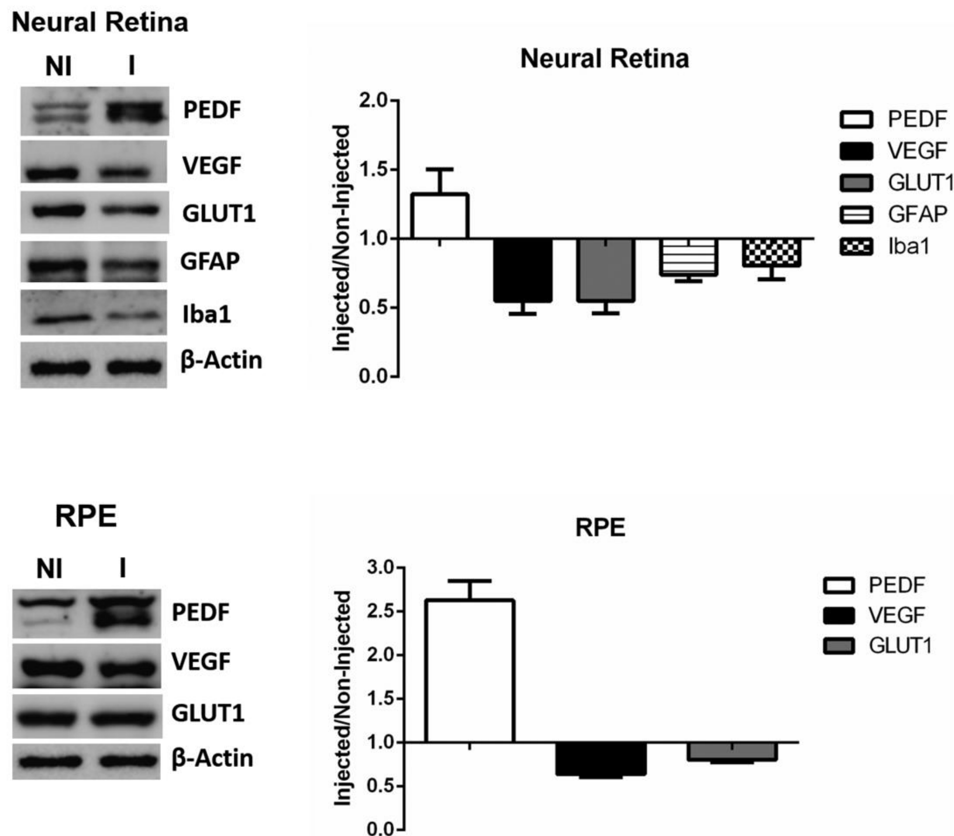


Figure 5.4: Overexpression of PEDF driven by pEPito-hCMV-PEDF three months post subretinal injection in Ins2^{Akita} mice. Expression of several markers relevant for the DR phenotype was analyzed in whole retinas and RPE cells. PEDF expression was significantly higher in injected animals, while VEGF (angiogenic factor), GLUT1 (responsible for glucose uptake to the cell), Iba1 and GFAP (marker of microglia reactivity and indicator of inflammation, respectively) show reduction of their levels.

One of the features observed in the Ins2^{Akita} diabetic mouse is the activation of microglia evidenced by retracted and swollen projections (Zhu *et al.*, 2009), which correlates with inflammation in the retina.

To analyze for microglia activation as a marker of inflammation, we have performed immunohistochemistry for Iba1 (ionized calcium-binding adaptor molecule, Figure 5.5), specific for microglia and macrophages (Zhu *et al.*, 2009). When compared with injected retinas (panel B), the microglia of non-injected retinas (panel A) has more retracted and swollen projections, a feature of reactive morphology of microglia. Iba1 expression is decreased in the injected eyes (panel D) when compared with non-injected (panel C), indicating that overexpression of PEDF has anti-inflammatory properties.

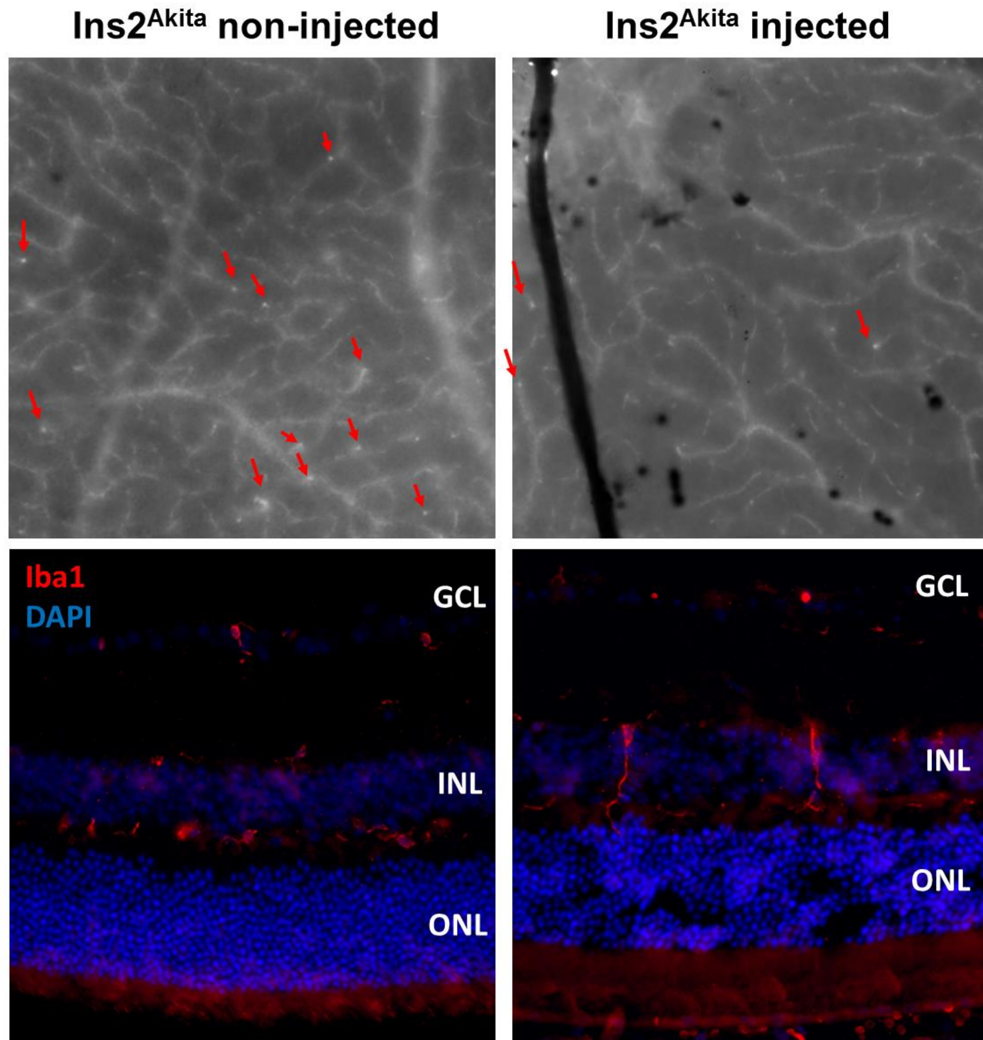


Figure 5.5: Overexpression of PEDF reduces microglia reactivity in the retina of diabetic mice. Whole mount retinas (upper panels, A and B) and section of an Ins2^{Akita} mouse eye (lower panels, C and D) staining with Iba1. Panels of pEPito-hCMV-PEDF injected retinas three months post injection show migroglia with non-reactive morphology. Non-injected retinas display retracted and swollen projections, typical of reactive microglia. DAPI (blue) stains nuclei and Iba1 is stained in red. Magnification: 400x.

5.5. Discussion

Diabetic Retinopathy is a severely handicapping disease that affects 90% of type 1 DM patients and 60% of type 2 DM patients,(Garg *et al.*, 2009) and the leading cause of blindness in developed countries. Anti-VEGF agents used to treat neovascularization have shown to be effective in delaying the progression of the disease; however, due to their short half-life there is a need of repeated injections, with severe side-effects (Cheung *et al.*, 2010).

Neovascularization has been associated with an imbalance between VEGF, an angiogenic factor, and PEDF, an anti-angiogenic factor known to counteract VEGF function (Farjo *et al.*, 2010). Targeting this imbalance can constitute an innovative therapeutic approach. To be an effective therapeutic strategy, the balance should be restored in the long-term, and gene therapy can achieve long-term expression. In the last decade, non-viral gene therapy vectors emerged as an alternative to viral vectors (Nayerossadat *et al.*, 2012). A self-replicating episomal vector, pEPito, has been shown to replicate in mitotic cells while promoting long term gene expression (Haase *et al.*, 2010). More recently, our group has shown its effectiveness in transgene expression in the mouse retina (Calado *et al.*, 2014).

Based on our previous results with pEPito, in this study we have cloned the *PEDF* gene into the pEPito-hCMV backbone and tested the effectiveness of this vector both *in vitro* in human RPE cells, and *in vivo* in the Ins2^{Akita} mouse model of diabetic retinopathy. The Ins2^{Akita} mouse is a spontaneous non-obese model of type 1 diabetes that has been used to study DR (Barber *et al.*, 2005, Gastinger *et al.*, 2006, Han *et al.*, 2013). In these animals, hyperglycemia starts roughly at 8 weeks after birth and retinal complications are visible approximately 12 weeks after the onset of hyperglycemia, including vascular leakage, loss of pericytes, thickening of the inner retinal layers, inflammation (Barber *et al.*, 2005), and increase of angiogenic markers such as VEGF (Han *et al.*, 2013).

To test the therapeutic potential of our approach, we first needed to assess if the PEDF expression profile in diabetic Ins2^{Akita} mice reproduces what is observed in humans. Our results show a significant decrease in PEDF expression levels in the inner retina of Ins2^{Akita} mice compared with age-matched WT controls (Figure 5.1A-B). This was even more pronounced in the RPE of the diabetic mice (Figure 5.1C-D), showing that the secretion of PEDF by the retinal pigment epithelium (RPE) is affected in the early stages of the disease. This correlates well to what was described for humans (Boehm *et al.*, 2003, Funatsu *et al.*, 2006)

Our *in vitro* results show that pEPito was able to overexpress PEDF for up to three months in mitotically active RPE cells, proving the mitotic stability of this episomal vector, and its long-term expression. These results were confirmed by Western blot of total cell lysates (data not shown) and secreted proteins present in the culture medium (Figure 5.2). Having shown that pEPito is capable of driving long-term PEDF expression, we aimed to determine if this can be achieved in vivo and consequently attenuate the DR phenotype. In the Ins2^{Akita} diabetic mouse, a model of DR, our results show that the expression system was able to express PEDF for up to three months. As expected, PEDF overexpression was detected in the RPE layer of the retina of injected eyes, where the plasmid was delivered, and in the inner retina (Figure 5.3), since PEDF is a secreted protein.

It was previously described that angiogenic VEGF is overexpressed in the retina of 6-month old Ins2^{Akita} diabetic mice (Han *et al.*, 2013). Moreover, it was also described that intravitreal injection of PEDF was responsible for down-regulation of VEGF and inhibition of VEGF-VEGFR2 binding in the retina of the oxygen-induced retinopathy mouse model (Zhang *et al.*, 2006). Similarly, our results show that overexpression of PEDF in the retina of Ins2^{Akita} diabetic mice markedly decreases the expression of VEGF (Figure 5.4), compared with non-injected eyes, where VEGF levels are higher.

Recent studies suggest that changes in the microglia, such as increased reactivity, are the early feature of DR (Grigsby *et al.*, 2014). In with Ins2^{Akita} diabetic mice, an

increase in retinal microglia immune reactivity was detected as early as 8 weeks after the development of hyperglycemia (Barber *et al.*, 2005). This activation of microglia involves a series of proliferative, morphological, immunoreactive and migratory changes and can be used as a measure of the inflammatory status of the retina (Grigsby *et al.*, 2014). We have evaluated microglia reactivity after injection of the pEPito-hCMV-PEDF and we found that the overexpression of PEDF strongly reduces microglial activity, visualized by the typical nonreactive morphology of microglia of long and thin cellular projections. In contrast, in the non-injected eye we have found the morphology typical of reactive microglia, characterized by retracted and swollen projections (Figure 5.5). These results are in accordance with a previous study, in which topical administration of PEDF peptide (PEDF78-121) in Ins2^{Akita} mice prevented microglia activation (Liu *et al.*, 2012). We have also found a decrease in GFAP expression in the injected eye, compared with the non-injected one (Figure 5.4). GFAP is an intermediate filament protein expressed by astrocytes, Müller and glial cells. In normal conditions glial cells express small amounts of GFAP; however after retinal injury, such as ischemia, glaucoma (Chang *et al.*, 2007) and diabetes its expression increases (Barber *et al.*, 2000). Our results show that treatment with PEDF decreases GFAP expression, which can be associated with a reversal in the retinal injury.

Overall, this study demonstrates the beneficial effect of long-term expression of PEDF in the retina of diabetic mice, through the anti-inflammatory and anti-angiogenic potential of PEDF in preventing retinal complications of diabetes.

Chapter 6 – General Discussion

6. General Discussion

Retinitis Pigmentosa (RP) and Diabetic Retinopathy (DR) are highly incapacitating genetic and acquired retinal disorders, respectively, affecting working-age adults (Cheung *et al.*, 2010, Petrs-Silva *et al.*, 2014). Despite the different etiology, they have in common the fact that there is no cure for either of them and the current treatments are ineffective and transient (Barnstable *et al.*, 2004, Shintani *et al.*, 2009, Cheung *et al.*, 2010, Petrs-Silva *et al.*, 2014). As such, gene therapy can constitute a therapeutic alternative for these diseases.

Gene therapy strategies have been successfully applied in clinical trials for a wide-range of retinal disorders, such as Leber's Congenital Amaurosis (LCA), Choroideremia (CHM), and Age-related Macular Degeneration (AMD). In all these studies Adeno-Associated Viruses (AAV) are being used as vectors for gene delivery (Bainbridge *et al.*, 2008, Maguire *et al.*, 2009, Maclachlan *et al.*, 2011, MacLaren *et al.*, 2014). However, in case of AAVs, their limited capacity of the inserted DNA limits their widespread use (Hauswirth *et al.*, 2000, Cevher *et al.*, 2012). Alternative approaches, including both non-viral vehicles and improved expression systems, that can efficiently deliver and provide sustained gene expression, are being investigated (Conese *et al.*, 2004, Gascón *et al.*, 2013).

The effectiveness of gene therapy is highly associated with the delivery method, which should be able to protect the gene of interest from the extra- and intracellular barriers, and the expression system that might provide prolonged gene expression without being integrated into host cell genome (Conese *et al.*, 2004, Gascón *et al.*, 2013).

In this project we aimed to develop a multifactorial strategy for efficient non-viral gene transfer to the retina. We intended to do this by *i)* identifying target genes for RP and DR, *ii)* cloning them in optimized self-replicating episomal expression systems

(pEPito), and *iii)* enhancing delivery to retinal cells by using a physical method, such as electroporation.

In Chapter 2 we described ATR, a DNA damage response (DDR) protein, as being involved in cilia formation and elongation. Previous studies from the group of Shomi Bhattacharya demonstrated massive rod-cone photoreceptor (PR) degeneration, with pigment accumulation in mutants of ATR ($ATR^{+/s}$), similar to the one observed in *Retinitis Pigmentosa* patients (Valdes-Sanchez *et al.*, 2013). They also found that ATR is localized in the connecting cilium, corresponding to the centrosome of photoreceptors (Valdes-Sanchez *et al.*, 2013). This unusual localization of ATR signal in PRs have been described also in other DDR proteins such as MRE11A, CEP164 and ZNF423, which are associated with syndromic retinal degeneration (Anders *et al.*, 2011, Wheway *et al.*, 2014). Thus, these results suggest that ATR, as well as other DDR proteins, might have a different role in post-mitotic cells, such as PRs. Herein we demonstrate that ATR is localized in the basal body (centrosome) of ciliated retinal pigment cells (Figure 2.3). This result was confirmed by immunoprecipitation of γ-tubulin (centrosomal marker) with concomitant co-immunoprecipitation of ATR in the ciliated cells (Figure 2.4).

Surprisingly the molecular weight of the protein co-immunoprecipitated with γ-tubulin does not correspond to the full length protein (60 kDa and 300 kDa, respectively), pointing to a potential ATR isoform, specifically related with cilia function. The inhibition of ATR by caffeine induced a marked decrease in the full length ATR protein level, but especially in the 60 kDa fragment co-immunoprecipitated with γ-tubulin (Figure 2.5 and 2.8). These results were followed by a 80% reduction in cilia length (Figure 2.6 and 2.7), proving that alterations in ATR expression influence cilia maintenance and function. In the future, it would be relevant to identify the 60 kDa fragment and, since it is co-immunoprecipitated with a centrosomal marker, try to understand its role in cell division and cilia formation and elongation.

According to the AceView database there is one isoform, named ATR.cAug10, which can correspond to our unidentified 60 kDa fragment (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&c=Gene&l=ATR). This isoform has 1664 bp long, one missing exon (Figure 6.1) and match with the antigen binding-site of the antibody used for the immunoprecipitation experiments (ab2905, Abcam). We are currently trying to identify the presence of this isoform in our samples. Our preliminary results show that the isoform is present in our ciliated cells in culture. We will try to clone and overexpress the isoform in retinal cells to track its cellular localization. In case it localizes in the cilium, we will confirm the size of the protein and attempt immunoprecipitation, sequencing and checking for interacting partners.

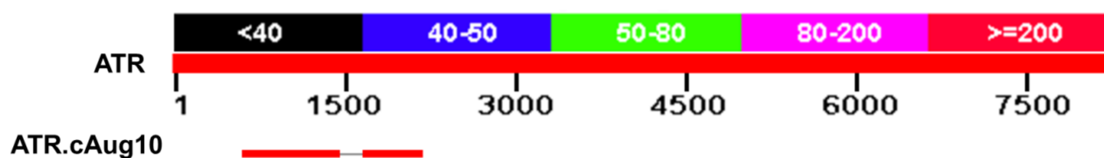


Figure 6.1: Nucleotide Blast between ATR and ATR.cAug10 variant, showing its localization related to the full length gene and the missing exon.

In a gene therapy context, it is desirable to overexpress both the full-length and the 60 kDa ATR fragment in the retina of ATR$^{+/s}$ mutants to analyze the rescue of the retinal phenotype. In rescued animals, PRs degeneration should be halted or reversed.

Chapter 3 is focus on molecular targets for treatment of DR. DR is characterized as a BRB disease, but while the effects of hyperglycemia and ischemia have been widely explored in the iBRB, its effects in the oBRB remain unclear. The oBRB is composed by the intracellular tight junction of the RPE cells (Cunha-Vaz, 1979). As previously described in section 1.2, one of the main functions of RPE is the production and secretion of neurotrophic and anti-angiogenic factors to maintain the homeostasis of the neuroretina. One of such factors is PEDF. Studies performed in PEDF KO mice

have shown that decreased levels of PEDF are associated with massive retinal neovascularization (Huang *et al.*, 2008).

It is well known that the key trigger for the development and progression of DR is the high blood glucose present in patients with Diabetes *Mellitus* (Cheung *et al.*, 2010). In the retina, glucose uptake is exclusively performed by GLUT1 (Sone *et al.*, 2000, Shah *et al.*, 2012). Here we evaluated the direct effect of hyperglycemia and also of hypoxia, a consequence of hyperglycemia visible in diabetic patients (Bresnick *et al.*, 1975), in the transepithelial transport and the secretory capacity of RPE cells. Our results show that, under conditions simulating DR, there is an increase in GLUT1 expression (Figure 3.1 and 3.6) and translocation to the cell membrane (Figure 3.3), contributing to an increase in glucose consumption (Figure 3.4). This was also associated with a decrease in PEDF production and secretion by the RPE cells, showing an impairment of RPE secretory function (Figure 3.5 and 3.6). In figure 6.2 we propose a mechanism by which diabetes can contribute to the loss of function of RPE cells.

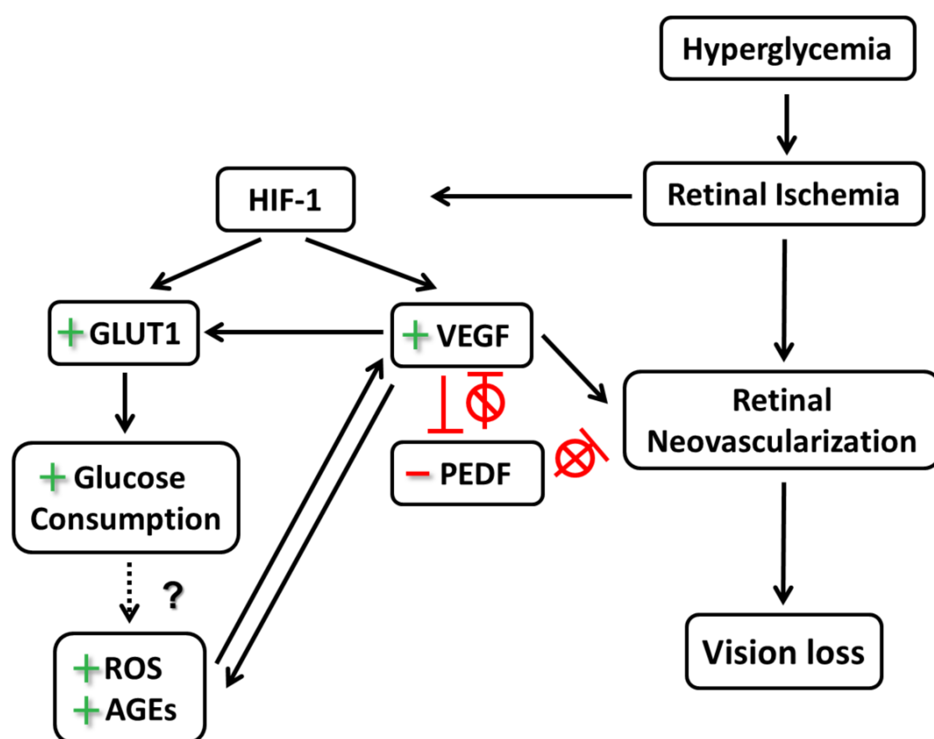


Figure 6.2: Proposed mechanism by which the increase of GLUT1 can contribute for RPE impairment and progression of DR.

With the chronic exposure to hyperglycemia, there is increased microvascular damage, reducing retinal blood supply. This contributes to retinal ischemia, increasing the expression and stabilization of Hypoxia Inducible Factor-1 (HIF-1) (Catrina *et al.*, 2004), which acts as a transcription factor for several genes, including GLUT1 (Chen *et al.*, 2001) and VEGF (Levy *et al.*, 1995). The increase of VEGF (Sone *et al.*, 2000) and GLUT1 (as shown in this work) contribute to an increase in intracellular glucose which, in turn, might contribute to the increase of ROS (Kim *et al.*, 2015) and AGE's (Singh *et al.*, 2014), upregulating VEGF expression (Lu *et al.*, 1998, Sasaki *et al.*, 2002), thus contributing to PEDF downregulation. The downregulation of PEDF aggravates the imbalance between PEDF and VEGF towards VEGF expression, inducing retinal neovascularization and later on, vision loss.

Considering these results, we have identified PEDF as a therapeutic target for gene therapy to treat DR, further explored in Chapter 5.

As described in Chapter 1, the major limitation of non-viral vector is its transient gene expression and, due to that, low transgene levels are expressed (Cevher *et al.*, 2012, Nayerossadat *et al.*, 2012). Efforts have been undertaken to improve expression systems to overcome these drawbacks (Conese *et al.*, 2004, Gill *et al.*, 2009). Examples are the development of minicircle DNA (mcDNA), Minimalistic Immunogenically Defined Gene Expression (MIDGE) vectors, plasmids free of antibiotic resistance gene (pFARs) and self-replicating episomal vectors, such as pEPito. Chapter 4 describes the use of pEPito vectors for long-term expression to the retina. We have tested several promoters combined with pEPito backbone, such as CVM promoter, hCMV promoter (a humanized CMV promoter containing less CpG motifs), and RPE tissue-specific promoter (pEPito-hCMV/RPE65) for a targeted therapy. We have found that the pEPito containing the hCMV promoter had higher mitotic stability (Figure 4.3) and a more prolonged expression, both *in vitro* (Figure 4.4) and *in vivo* (Table 3) when compared with the other promoter-pEPito constructs. We also found that, despite the low transfection efficiency *in vitro* of the plasmid containing

the tissue-specific promoter (pEPito-hCMV/RPE65), this was partially reversed *in vivo*. These results highlight the relevance of using low CpG containing promoters (such as hCMV and RPE65 promoter) for a sustained gene expression profile. As RPE65 promoter is tissue-specific promoter for retinal pigment epithelium (RPE) cells (Boulanger *et al.*, 2000, Boulanger *et al.*, 2002), it would be most interesting to test the feasibility of these systems in rescuing models of RPE degeneration, such as forms of RP and DR.

In Chapter 5 we combine the findings from Chapter 3 and 4 to develop an efficient non-viral gene therapy for DR. In Chapter 3 we described an increase of GLUT1 with a concomitant decrease in PEDF expression in the RPE of $Ins2^{Akita}$ diabetic mice. Based on the results obtained in Chapter 4, in Chapter 5 we have cloned the *PEDF* gene into pEPito-hCMV backbone. Our *in vitro* results confirmed the mitotic stability and long-term expression of PEDF driven by pEPito-hCMV backbone in human RPE cells (Figure 5.2). *In vivo*, pEPito-PEDF was able to overexpress PEDF for up to three months in the retina of diabetic mice at levels comparable to age-match WT controls (Figure 5.3). The overexpression of PEDF in the retina and RPE of diabetic mice was followed by a decrease in the expression of several molecular markers of DR, such as GLUT1, vascular endothelial growth factor (VEGF), glial fibrillary acidic protein (GFAP), and ionized calcium-binding adapter molecule 1 (Iba1) (Figure 5.4 and 5.5), confirming the anti-inflammatory and anti-angiogenic capacity of PEDF in ameliorating complications of DR. Moreover, these results show that electroporation is an efficient and safe method for retinal gene delivery. In the future we intend to determine the amount of pEPito plasmids that are present in each retinal cell. Altogether, the results of this thesis will pave the way for a preclinical study, in which we will perform physiological as well as functional analysis of the rescue of the DR phenotype. These include eletroretinography (ERG), since $Ins2^{Akita}$ are characterized by having defects in scotopic a-wave, b-wave, and photopic b-wave amplitudes (Han *et al.*, 2013), and optical coherence tomography (OCT) to measure retinal thickness, considering that the

diabetic mice display a significant reduction of the inner layers of the retina (Barber *et al.*, 2005).

Although much remains to be done, some issues that are currently a drawback for the success of non-viral gene therapy were addressed:

i) Short-term/transient gene expression: the presence of S/MARs into pEPito backbone allows mitotic stability and long-term expression, in an episomal state.

ii) Gene silencing: the removal of CpG content in pEPito backbone and promoter reduces epigenetic silencing events, thus promoting a prolonged gene expression profile.

iii) Low transfection efficiency: using electroporation as a delivery method, we obtained high transfection efficiency in vivo, originating expression levels of therapeutic genes comparable to WT animals.

Altogether, the multifactorial approach presented in this work supports the potential of non-viral gene therapy as a clinical alternative to viral gene therapy.

Chapter 7 – References

7. References

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