DISSERTATIONS IN
HEALTH
SCIENCES

Eliisa Mannermaa

In vitro Model of Retinal Pigment Epithelium for Use in Drug Delivery Studies

Publications of the University of Eastern Finland
Dissertations in Health Sciences

UNIVERSITY OF
EASTERN FINLAND

ELIISA MANNERMAA

In vitro Model of Retinal Pigment Epithelium for Use in Drug Delivery Studies

To be represented by permission of the Faculty of Health Sciences, University of Eastern Finland
for public examination in L1, Canthia building,
University of Eastern Finland
on Friday 19th of March, at 12. noon.

Publications of the University of Eastern Finland
Dissertations in Health Sciences
9

Department of Ophthalmology
Institute of Clinical Medicine
School of Medicine
Faculty of Health Sciences
University of Eastern Finland
Kuopio
2010

Kopijyvä Oy,
Kuopio, 2010

Distribution:

University of Eastern Finland
Kuopio Campus Library
P.O.Box 1627, FI-70211 Kuopio
FINLAND
http://www.uef.fi/kirjasto

ISBN: 978-952-61-0029-6
ISBN: 978-952-61-0030-2 (PDF)
ISSN: 1798-5706
ISSN: 1798-5714 (PDF)
ISSNL: 1798-5706

Author's address: Department of Ophthalmology
Institute of Clinical Medicine
School of Medicine
Faculty of Health Sciences
University of Eastern Finland
P.O.Box 1627, FI-70211 Kuopio, FINLAND
Eliisa.Mannermaa@kuh.fi

Supervisors: Professor Arto Urtti, Ph.D.
Center for Drug Research
University of Helsinki

Professor Kai Kaarniranta, M.D., Ph.D.
Department of Ophthalmology
Institute of Clinical Medicine
School of Medicine
Faculty of Health Sciences
University of Eastern Finland

Lecturer Veli-Pekka Ranta, Ph.D.
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland

Reviewers: Docent Heli Skotman, Ph.D.
Regea, University of Tampere

Professor Maria del Rocio Herrero-Vanrell
Department of Pharmacy and Pharmaceutical Technology
School of Pharmacy, Complutence University, Madrid, Spain

Opponent: Professor Hannu Uusitalo, M.D., Ph.D.
Department of Ophthalmology
University of Tampere

Mannermaa, Eliisa. In vitro Model of Retinal Pigment Epithelium for Use in Drug Delivery Studies.
Publications of the University of Eastern Finland. Dissertations in Health Sciences. 9. 2010. 67 pp.

ISBN: 978-952-61-0029-6
ISBN: 978-952-61-0030-2 (PDF)
ISSN: 1798-5706
ISSN: 1798-5714 (PDF)
ISSNL: 1798-5706

ABSTRACT

The posterior location and the blood-retinal barrier (BRB) make drug delivery in diseases affecting retina and vitreous challenging. The outer part of BRB is composed of retinal pigment epithelium (RPE), which restricts drug entry to the retina from the systemic circulation and from the periocular space. In this study, filter grown ARPE-19 cells have been characterized as a potential *in vitro* model of the human RPE for use in drug delivery studies. ARPE-19 barrier properties were evaluated in different culture conditions. The ARPE-19 model was 3-17 times more permeable than isolated bovine RPE-choroid tissue, but the ARPE-19 model efficiently separated test compounds based on their lipophilicity and molecular sizes. Expression of RPE related genes, *RPE65, CRALBP, TRP1, tyrosinase and Mitf-A and OTX2* transcription factors was greatly enhanced by filter culture. It has become evident that transporters play an important role in pharmacokinetics. The expression of efflux proteins, p-glycoprotein (P-gp), multidrug resistance associated proteins 1-6 (MRP) and breast cancer related protein (BCRP), in various RPE cell lines was studied. As with primary RPE cells, ARPE-19 cells express MRP1, MRP4 and MRP5 efflux proteins. Efflux protein activity was evaluated in cellular uptake studies using calcein-AM and carboxydichorofluorescein as probe molecules, and by bi-directional permeability studies. The studies indicate MRP1 and MRP5 activity in ARPE-19 cell line. Active transport in the ARPE-19 cell model was qualitatively, though not quantitatively similar, with isolated RPE-choroid tissue. Furthermore, non-viral gene transfer was studied in the ARPE-19 cell model. Prolonged gene expression was achieved by liposomal carriers. In conclusion, the ARPE-19 cell model can be used to screen drug molecules with different physicochemical properties and in gene delivery studies. The greater passive permeability may lead to an underestimation of active transport in this model. Several membrane transporters are expressed and active in the ARPE-19 cell model.

National Library of Medicine Classification: QS 525, QU 55.7, QW 38, QZ 52, WW 103

Medical Subject Headings: Blood-Retinal Barrier; Cell Culture Techniques; Drug Delivery Systems; Gene Therapy; Gene Transfer Techniques; Membrane Transport Proteins; Pharmacokinetics; Retinal Pigment Epithelium

Mannermaa, Eliisa. Verkkokalvon pigmenttiepiteelin solumalli lääkekehitystutkimuksiin.
Itä-Suomen yliopiston julkaisuja. Terveystieteiden tiedekunnan väitöskirjat, 9. 2010. 67 pp.

ISBN: 978-952-61-0029-6
ISBN: 978-952-61-0030-2 (PDF)
ISSN: 1798-5706
ISSN: 1798-5714 (PDF)
ISSNL: 1798-5706

TIIVISTELMÄ

Silmän takaosan sairauksien lääkehoito on haasteellista. Veriverkkokalvo-este rajoittaa aineiden siirtymistä verenkierrosta ja silmän viereen annostelussa. Verkkokalvon pigmenttiepiteelisolut muodostavat ulomman osan veriverkkokalvo-esteestä. Tässä tutkimuksessa selvitettiin verkkokalvon pigmenttiepiteelisolulinjan, membraanille kasvatettujan ARPE-19 solujen, soveltumista lääketutkimukseen. Solumaton tiiviyttä tutkittiin eri kasvatusolosuhteissa ja verrattiin naudasta irroitettuun suonikalvo-verkkokalvon pigmenttiepiteelikudokseen. Tulokset osoittivat, että solumalli oli 3-17 kertaa vuotavampi, mutta pystyi erottelemaan aineet molekyylikoon ja rasvaliukoisuuden perusteella. Lisäksi eri kasvatusolosuhteita testatessa havaittiin, että membraanikasvatuksessa pigmenttiepiteelille tyypillisten geenien ekspressoituminen nousee selvästi. Viime vuosikymmenen aikana on tullut ilmeiseksi, että membraanikuljettimilla, jotka aktiivisesti kuljettavat aineita solukalvojen läpi, on tärkeä rooli lääkeaineiden farmakokinetiikassa. Tässä työssä mitattiin aineita solusta ulospäin kuljettavien P-gp:n (p-glycoprotein), MRP:n (multidrug resistance associated proteins 1-6) ja BCRP:n (breast cancer related protein) ilmentymistä useassa verkkokalvon pigmenttiepiteelin solulinjassa. ARPE-19 soluissa, kuten myös ihmisperäisissä primaari pigmenttiepiteelisoluissa ilmentyivät MRP1, MRP4 ja MRP5. Aktiivisuuskokeet osoittivat, että MRP1 ja MRP5 ovat todennäköisesti myös aktiivisia ARPE-19 soluissa. Suuntakokeissa tulokset solumallin ja kudoksen välillä olivat samansuuntaisia, joskin kudoksessa suuntaerot tulivat huomattavasti selvemmin esille. Solumallia käytettiin myös viruksettomien geenikuljettimien testaamiseen. Pigmenttiepiteelin solut ovat normaalitilanteessa jakautumaton soluja, joten voi olla mahdollista, että polarisoitunut solumalli vastaa verkkokalvon pigmenttiepiteelin soluja todellisuudessa paremmin kuin yleensä käytetyt jakautuvat solut. Kun ARPE-19 solut transfektoitiin liposomikantajilla, markkerigeeni ilmentyi yli kahden kuukauden ajan. Yhteenvetona voidaan todeta, että ARPE-19 solumallia voidaan käyttää fysikokemiallisilta ominaisuuksiltaan erilaisten lääkeaineiden testaukseen, mutta solumallin suurempi passiivinen läpäisevyys kudokseen verrattuna voi peittää alleen aktiivisen kuljetuksen. Pigmentin puute rajoittaa käyttöä pigmenttiin sitoutuvien aineiden testaukseen. Useat membraanikuljettimet ilmentyvät ja ovat aktiivisia ARPE-19 solumallissa.

Yleinen suomalainen asiasanasto (YSA): epiteeli, farmakokinetiikka, geenitekniikka, geeniterapia, lääkehoito, proteiinit, verkkokalvo

Even the biggest oak tree was once a little nut.

ACKNOWLEDGEMENTS

This work was carried out at the Department of Pharmaceutics in collaboration with the Department of Ophthalmology during the years 1999-2009. These years have been full of life: bringing up the family and combining research and clinical work.

I am grateful to The Finnish Eye Bank Foundation, The Finnish Tissue and Eye Bank Foundation, The Evald and Hilda Nissi's Foundation, Sokeain Ystävät ry/De Blindas Vänner rf, The Finnish Cultural Foundation of Northern Savo and The Finnish Funding Agency for Technology and Innovation for financial support to this study.

I wish to express my deepest gratitude to my principal supervisor Professor Arto Urtti, whose endless optimism has been a leading power of this work. I would like to thank you for guiding me to the fascinating world of science and giving me the opportunity to test my own ideas. I am also grateful to my other supervisors Professor Kai Kaarniranta and Veli-Pekka Ranta Ph.D. Your expertise has been of outmost importance to this work. It has been a privilege to work with you!

I am honored to have a Professor Hannu Uusitalo as my opponent and Professor Maria del Rocio Herrero-Vanrell and Docent Heli Skotman as reviewers of this thesis. Thank you for your valuable and constructive comments. I also wish to thank Ewen McDonald Ph.D. for revising the language.

Without my co-authors Kati-Sisko Vellonen, M.Sc., Mika Reinisalo, M.Sc. and Marika Ruponen, Ph.D. I would have not been able to accomplish this work. Thank you for your help, support and friendship. With you I have shared both the darkest and the brightest moments during this journey. The discussions with you, both scientific and not so scientific, have been always enjoyable and brought joy to the research.

I am also grateful to Professor Paavo Honkakoski. Your imaging knowledge and all advices have played an important role in this work.

I owe my special thanks to Tuomas Ryhänen, M.Sc. for performing the western blot studies, and thank you for pleasant collaboration. I also wish to thank Giedrus Kalesnykas, Ph.D., Tuomas Paimela, M.D. and Johanna Viiri, M.Sc. and Docent Seppo Rönkkö from the Department of Ophthalmology for your help and friendly co-operation.

I thank Katriina Kokkonen, M.Sc., Anni Saarikko M.Sc. and Heidi Kokki, M.Sc for your contribution to this work. It was nice working with you.

I like to express my gratitude to Professor Jukka Mönkkönen for adopting me to his research group after Professor Urtti moved to Helsinki. It meant a lot to me. Thank you also for your support in the efflux protein project. I also like to thank Professor Kristiina Järvinen for your supportive and friendly attitude. In addition, I thank You both for the excellent working facilities.

I like to thank the personnel at the Department of Pharmaceutics and common staff of the Faculty of Pharmacy, especially Kaarina Pitkänen, Anne Roivainen, Lea Pirskanen, Marja Lappalainen, Leena Lindqvist and Seija Koistinen, and Marko Antikainen, who was always there to help me when I was in trouble with the computer.

I also like to thank the personnel at the clinical genetics laboratory, Kuopio University Hospital, where my scientific career started. I have many joyful and unforgetable memories from those days. Special greetings to Pirkko Jokela and Seppo Helisalmi!

I am also grateful to Head of Department Matti Kontkanen M.D. Ph.D. at Northern Carelia Central Hospital for the flexibility during the last year, which made it possible for me to finish the thesis. I also wish to thank my nice workmates in the Ophthalmology Clinics of Northern Carelia Hospital and Savonlinna central hospital. I enjoyed working with you. Inka, thanks for the creative and refreshing study meetings ☺ I miss them!

I wish to thank vice Head of the Department Iiris Sorri, M.D., Ph.D. at the Kuopio University Hospital for support during the very final weeks.

I have many, many nice memories from these years. All memorable TYKY-events, congresses and wonderful people I have met: our “travel- and DVD-group” Marika, Marja, Elisa, Kati-Sisko, Zanna and Soili, my dear jogging friend, Kaisamari, Marjo M, Jonna, Johanna, Seppo, Eva and all the others. Thank you all.

Without the girls from Southern Carelia, Elina, Pise, Anu and Tiina, I would not be me. I know you are always there. I also wish to thank my dear long lasting friends Maija, Päivi and Elina “in London” for everything.

It has been essential to relax in “Murto” and elsewhere with good company: thank you Anne, Antti, Jukka, Tiina, Laura, Timo, Annaliina, Kimmo, Annakaisa, Mikko, my brother Arto, Iikku and your families. It has been especially enjoyable to spend time with my wonderful godchildren Matilda and Tuomas.

I would like to thank all the “grandparents” Sinikka and Lasse Kajanoja, Ritva and Juho Mannermaa, Maija and Jorma Korhonen and especially the American grandparents my “mom” Marsha England and my “dad” late David England for all your love and support to me and our family.

Thank you Arto for your endless support and patience. My heart is filled with joy every day when I think our children Marjukka, Otso, Aapo and Linnea. You matter the most.

Kuopio, February 2010

Eliisa Mannermaa

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals **I-V**.

I **Mannermaa E**, Reinisalo M, Ranta V-P, Vellonen K-S, Kokki H, Saarikko A, Kaarniranta K, Urtti A: ARPE-19 filter culture as outer blood-retinal barrier model for ocular drug delivery studies. Submitted.

II **Mannermaa E**, Vellonen K-S, Ryhänen T, Kokkonen K, Ranta V-P, Kaarniranta K, Urtti A: Efflux protein expression in human retinal pigment epithelium cell lines. Pharm Res 26:1785-1791, 2009

III Ryhänen T, **Mannermaa E**, Oksala N, Viiri J, Paimela T, Salminen A, Atalay M, Kaarniranta K: Radicicol but not geldanamycin evokes oxidative stress response and efflux protein inhibition in ARPE-19 human retinal pigment epithelial cells. Eur J Pharmacol 584:229-236, 2008

IV **Mannermaa E**, Rönkkö S, Ruponen M, Reinisalo M, Urtti A: Long-lasting secretion of transgene product from differentiated and filter-grown retinal pigment epithelial cells after nonviral gene transfer. Curr Eye Res 30:345-353, 2005

V **Mannermaa E**, Vellonen K-S, Urtti A: Drug transport in corneal epithelium and blood-retinal barrier: Emerging role of transporters in ocular pharmacokinetics. Adv Drug Deliv Rev 58:1136-1163, 2006

Publication **III**: The part concerning efflux proteins interactions with Hsp inhitors is included in this thesis, other Hsp studies are performed by M.Sc. Tuomas Ryhänen and will be included in his thesis.

CONTENTS

ABBREVIATIONS

ADME	absorption, distribution, metabolism and elimination
ARPE-19	human retinal pigment epithelium cell line
AMD	age-related macular degeneration
ATRA	all-trans-retinoic-acid
bFGF	basic fibroblast growth factor
BBB	blood-brain barrier
BCRP	breast cancer related protein
BRB	blood-retinal barrier
BRE	bovine neural retinal extract
bFGF	basic fibroblast growth factor
bRPE	bovine retinal pigment epithelium cells
Calcein-AM	calcein acetoxymethyl ester
CDCF	carboxydichlorofluorescein
CDCFDA	diacetate ester of carboxydichlorofluorescein
CF	carboxyfluorescein
CRALBP	cellular retinal aldehyde binding protein
D407	human retinal pigment epithelium cell line
DHP-12	cationic lipid derivative
DNA	deoxyribonucleic acid
DOPE	1,2-dioleyl-3-phosphatidylethanolamine
DOTAP	N-(1-(2,3-dioleoyloxy)propyl-N, N, N-trimethyl) ammonium methylsulfate
EGF	epidermal growth factor
EMA	ethidium monoazide
FACS	fluorescence assisted cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HEK293	human embryonic kidney cell line 293
h1RPE	immortalized human RPE cell line
HRPEpiC	human primary cells from retinal pigment epithelium
HSP	heat shock protein
hTERT-RPE	telomerase transfected retinal pigment epithelial cells

IBMX	isobutylmethylxanthine
IOP	intraocular pressure
MCT1	monocarboxylate transporter-1
MDCKII	Madin-Darby canine kidney cells
MDR1	multidrug resistance
MerTK	receptor tyrosinase kinase c-mer
MITF-A	microphthalmia-associated transcription factor A
MRP	multidrug resistance protein
MW	molecular weight
OTX2	homeodomain-containing transcription factor
PCR	polymerase chain reaction
PEI	polyethylene imine
P-gp	p-glycoprotein
POS	photoreceptor outer segments
PBS	phosphate buffered saline
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
ROS	rod outer segment
RPE	retinal pigment epithelium
RPE-J	rat derived retinal pigment epithelium cell line
RPE65	retinal pigment epithelium specific gene 65 kDa
RFT-1	folate transporter protein
SEAP	secreted alkaline phosphatase
SLC	solute carrier family
TEP	transepithelial potential
TER	transepithelial resistance
TLR-4	toll like receptor 4
TRP	tyrosinase related protein
VEGF	vascular endothelial growth factor

1 INTRODUCTION

Many vision threatening diseases affect the posterior eye segment. These include age-related macular degeneration (AMD), diabetic retinopathy, glaucoma, retinal venous and arterial occlusive diseases, inherited retinal degenerations and posterior uveitis. In the western world over 60% of visual impairment in the elderly is caused by AMD, whereas in the working age population diabetic retinopathy and inherited retinal degenerations are common causes of visual impairment.

It is estimated that 50 million people all over the world are affected by AMD, and roughly one third of the patients are legally blind (vision in the better eye below 0.3 in Snelle's chart). It is estimated that number of people suffering from AMD will increase significantly during next decades due to the increased aging of population (Rein et al. 2009). The central vision is affected in AMD, which leads to difficulties in reading, writing and recognizing faces. The peripheral vision remains mainly un-affected, but the quality of life is seriously affected by advanced forms of AMD. The pathophysiological mechanisms leading to changes in macular region are still largely unknown. Both environmental (eg. cigarette smoking) and genetic factors play roles in the development of AMD (Ding et al. 2009, Vingerling et al. 1996, Thakkinstian et al. 2006). AMD is divided into atrophic and exudative forms. Approximately 80-90% of the patients suffer from the atrophic form of AMD. Odema and bleeding with rapid change in visual acuity are symptoms of the exudative form of the disease. Increased levels of vascular endothelial growth factor (VEGF) have been associated with the progression of the exudative AMD, and during the last years, VEGF inhibitors have been launched as a new effective treatment in the exudative form of AMD. In contrast, there are no effective treatment options for people suffering from the atrophic form of AMD.

Understanding the pathophysiological and molecular mechanisms of vision threatening diseases is crucial in the discovery of new potential drug molecules. But the drugs must be also efficiently delivered to the posterior eye segment targets. The posterior eye segment is a demanding tissue from the drug delivery point of view because of its posterior location. Furthermore, retina and vitreous are protected by blood-ocular barriers (blood-aqueous and blood-retinal barrier), similarly as brain is protected by blood-brain barrier (Hornof et al. 2005). The blood-retinal barrier is composed of retinal pigment epithelium (RPE) and endothelial cells of the retinal capillaries (Cunha-Vaz 2004). RPE is a tight selective cell monolayer between the retina and choroidal blood circulation. The drug entry to retina and vitreous is restricted by RPE after systemic and periocular drug administration. Furthermore, some drugs are

eliminated from vitreous through RPE. Overall, the RPE plays an essential role in the posterior segment pharmacokinetics, but many aspects remain unknown. For example, the role and expression of membrane transporters in the RPE are poorly understood. The emerging role of membrane transporters in drug delivery and in drug efficacy has become evident during the last decade (Dobson and Kell 2008).

Currently drug delivery to the vitreous and retina is mainly achieved by repeated intravitreal injections. Unfortunetely, many posterior segment diseases require long time periods of medical therapy. Thus, prolonged action drug delivery methods would decrease the number of injections needed. One possibility is gene transfer to ocular cells eg. to RPE cells. The transfected RPE cell could then secrete therapeutic proteins such as growth factors apically (to the retina) or basolaterally (to the choiroid). In this study, polarized, non-dividing RPE cells were transfected with different non-viral gene delivery systems.

Cell models are useful in pathophysiology and molecular studies of diseases. They can be used also to investigate ADME (drug absorption, distribution, metabolism and elimination) properties of drugs and drug delivery systems (Hornof et al. 2005). The limited availability of human ocular tissue emphasizes the value of the ocular cell models. Currently there is no established culture model for human RPE, even though such a model would be a very useful tool in ocular drug delivery studies. In this work, the polarized RPE cell model, ARPE-19, was characterized as potential tool for ocular drug delivery studies.

2 REVIEW OF THE LITERATURE

2.1 Anatomy and physiology of the posterior eye

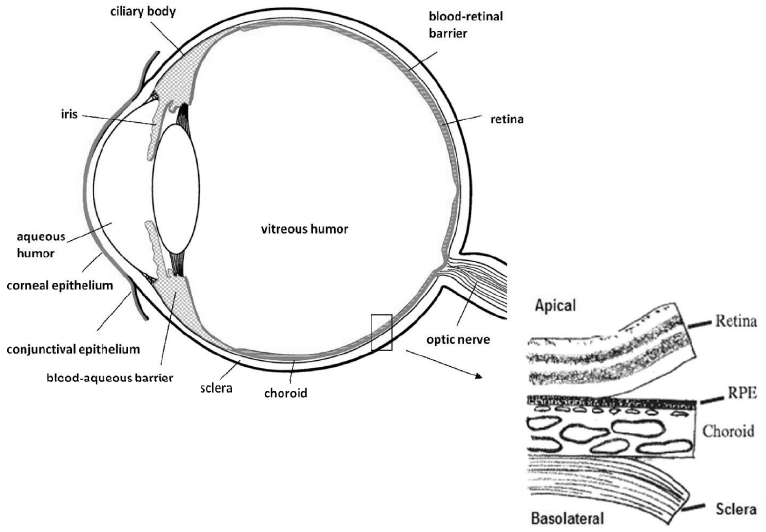

Figure 1. Structure of the eye and tissue layers in the posterior part of the eye. Modified from Hornof et al. 2005 and Steuer et al. 2004.

The retina is protected by inner and outer blood-retinal barriers (BRB). The inner part is composed by the endothelial cell layer of retinal capillaries and the outer part by retinal pigment epithelium, a tight monolayer of epithelial cells. Neural retina has high metabolic activity and this is dependent on the ability of retinal capillaries to deliver nutrients and oxygen to the inner two thirds of the neural retina. The outer third of neural retina and RPE are nourished by the blood supply from choriocapillaries, fenestrated capillaries located underneath the RPE (Fig. 1).Metabolic waste products are transported from the neural retina to retinal and choroidal blood circulation through inner and outer BRB, respectively (Strauss 2005). The higher oncotic pressure in choroid drives water from the neural retina towards the choroid. The pressure inherent in the water flow holds the retinal layers together and in close contact with the choroid. Higher oxygen content in the choroid compared to the retinal vessels ensures that there is an adequate oxygen supply to the retina.

Photoreceptors, rod and cones, are located in the outermost layer of the neural retina. The outer limiting membrane is pierced by the inner segments of the photoreceptors. The outer segments of photoreceptors (POS) are located between apical microvillae of retinal pigment epithelium (RPE) cells.

RPE is a key player in maintaining a healthy retina. POS, which are shed from photoreceptors in a circadian rhythm, are phagocytosed and metabolized by the RPE cells. RPE is constantly exposed to oxidative stress caused by the high oxygen concentration, extensive light exposure, metabolic load of modified lipids from POS and lipofuscin accumulation (Beatty et al. 2000). RPE participates in the visual cycle: vitamin A is recycled back to photoreceptors after being processed in the RPE cells. RPE also produces and releases growth factors including pigment epithelium derived growth factor (PEDF), vascular endothelial growth factor (VEGF) and ciliary neurotrophic factor (CNTF) (Strauss 2005). PEDF is mainly secreted apically towards neural retina and VEGF basolaterally towards choriocapillaris (Becerra et al. 2004, Blaauwgeers et al. 1999). RPE differ from other epithelia in that Na+, K+ ATPase is found in the apical membrane and N-cadherin instead of E-cadherin is located in cell-to-cell junctions (Gundersen et al. 1991, Okami et al., 1990, Youn et al. 2006).

Choroid is composed of suprachoroid, choroidal vessels, choriocapillaries and Bruch's membrane. The suprachoroid is an avascular space containing the extracellular matrix and melanocytes between sclera and choroidal vessels. Large choroidal vessels are found in Haller's layer and smaller and middle sized vessels in Sattler's layer. Choriocapillary layer is composed of lobes, where the artery is located in the middle of the lobe and the veins are situated on the borders of the lobes. In the macula the diameter of choriocapillaries is circa 7 µm and in peripheral retina it is between 10-50 µm (Richard 1992, Spraul et al. 2002). The capillaries are fenestrated with windows of 55-80 nm in diameter on the Bruch's membrane side of the capillaries (Guymer et al. 2004, Torczynski 1995). Bruchs's membrane is located between choriocapillaries and RPE and it is composed of two collagen layers and separated by the middle elastic layer. The innermost layer of Bruch's membrane serves as the basement membrane for RPE cells.

Sclera is an avascular membrane of varying thickness at different locations. In humans, it is thinnest at the equator 0.39±0.17 mm, 0.53±0.14 mm at corneoscleral junction (limbus) and near to the optic nerve its thickness is 0.9-1.0 mm (Olsen et al. 1998). The surface area of human sclera is 16-17 cm^2 (Olsen et al. 1998). It is composed of three layers: episclera, stroma and lamina fuscia. Episclera is continuous with Tenon's capsule which extends from the limbus to the optic nerve. These two fibrous membrane structures are connected by the connective tissue. The periocular space is the potential space between Tenon's capsule and the sclera. Episcleral, conjunctival vessels including lymphatic vessels are found in

episclera. The stroma is composed of collagen (75% of dry weight, mainly type I), proteoglycans, a few fibroblasts and elastic fibers (Geroski and Edelhauser 2001). Melanocytes are found in thin lamina fusca, which is in tight contact with choroid. The eyeball is further surrounded by six eye muscles, fat, connective tissue and bones forming the orbit.

2.2 Drug delivery to retina

Generally it is not possible to achieve adequate drug concentrations in the posterior eye segment by topical administration of eyedrops. Most of the drug is cleared away by drainage of the instilled solution and systemic conjunctival drug absorption even before the drug reaches the cornea or anterior chamber. Low drug diffusion in the lens and the flow of aqueous humor (in the posterior to anterior direction) further hinder drug access to the retina.

Currently, drug acces to retina and vitreous is achieved mainly either by systemic administration or by intravitreal injections (Fig. 2: A and B). With systemic delivery, it is difficult to achieve adequate drug concentrations in the retina and high systemic doses may cause adverse effects (e.g. in the case of per oral carbonic anhydrase inhibitors). Corticosteroids, immunosuppressive agents, and antibiotics in severe infections are used per orally for the treatment of posterior segment eye diseases.

The clinical use of intravitreal injections increased rapidly after the launch of the VEGF-inhibitors for the treatment of exudative form of AMD. These drugs are usually well tolerated and the treatment results with VEGF-antibodies are generally good. The invasive nature of the procedure may lead to retinal detachment, endophthalmitis and vitreous hemorrhage, though fortunately these adverse reactions are rare. In many cases frequent injections for an indefinite period of time are needed in the treatment. The growing number of the injections is a load on physicians in eye clinics and their long term safety is unknown. Prolonged action formulations would decrease the number of injections needed.

Periocular injections, which include subconjunctival, sub-Tenon, peribulbar and retrobulbar injections, are mainly used in the clinics to deliver local anesthetics. Due to its accessibility, relatively high permeability even to macromolecules (mw 70-150 kDa) and the large surface area of sclera (Amaral et al. 2005, Ambati et al. 2000a, Ambati et al. 2000b), the transscleral drug delivery has gained interest as an alternative route of drug delivery (Fig. 2: C). However, transscleral delivery is limited by several membrane barriers (sclera, choroid and RPE) and dynamic factors (episcleral blood flow, choroidal

circulation, membrane transporters, metabolic enzymes, drug binding and pressure related matters) which complicate drug delivery by the transscleral route (Fig. 2: C).

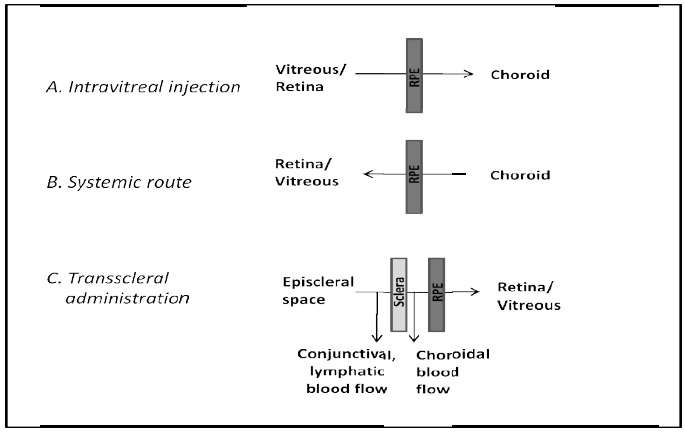

Figure 2. Direction of drug permeation after intravitreal (A), systemic (B) and transscleral (C) drug delivery. RPE plays an important role in drug clearance after intravitreal injections (A) and it is a significant permeation barrier to systemic (B) and in transscleral (C) drug delivery to posterior eye segment.

2.2.1. Passive permeability of sclera, choroid and retinal pigment epithelium

Scleral permeability has been extensively studied (Ambati et al 2000a, Olsen et al 1995, Prausnitz and Noonan 1998) whereas the RPE permeability has been systemically studied only in recent times. An inverse correlation was found in both layers between molecular diameter and permeability (Prausnitz and Noonan 1998, Pitkänen et al. 2005). In sclera, passive diffusion takes place mainly via the interfibrillar aqueous pathway (Geroski and Edelhauser 2001). Rabbit sclera has been observed to be permeable even to 150 kDa IgG molecules and 70 kDa FITC-dextran has been able to diffuse through human sclera (Ambati et al. 2000a, Olsen et al. 1995). Sclera has been shown to be more permeable to negatively than positively charged molecules and this has been suggested to be a consequence of the presence of negatively charged proteoglycans in the sclera (Maurice and Polgar 1977). Pitkänen et al. studied RPE permeability using isolated bovine RPE-choroid tissue. For lipophilic drugs permeability in sclera and RPE was similar but this was not the case for hydrophilic drugs and macromolecules, RPE

being revealed to be 10-100 times less permeable than sclera (Pitkänen et al. 2005). Selected examples are given in Table 1.

Table 1. Permeability of sclera and RPE-choroid

			Permeability*10^{-7}cm/s		
	mw	*log D*	*Sclera*[a]	*RPE-choroid*[b]	*ratio*[c]
6-CF	376	-3.14	100	9.6	10.5
Nadolol	309	-1.06	394	22.4	17.6
Timolol	316	0.09	408	145.0	2.8
FITC-dextran	40 000	n.d.	49	0.5	106.5

mw; molecular weight, log D; logarithm of the apparent distribution coefficient between n-octanol and water at pH 7.4. LogD values from the literature (6-CF (Araie and Maurice 1987), nadolol and timolol (Pitkänen et al. 2005))[a]Prausnitz and Noonan 1998 and Prausnitz 1998 b. [b]Pitkänen et al. 2005 [c]Scleral permeability/RPE-choroid permeability.

Bruch's membrane preparations from young human donors were found to be permeable to proteins exceeding mw of 200 kDa. A decline in permeability was seen in the membranes from older donors but even these membranes were permeable to macromolecules of 100 kDa (Moore and Clover 2001). Compared to RPE Bruch's membrane can be considered to be an insignificant permeation barrier. Interestingly, decreased permeability with increasing lipophilicity was observed in the bovine choroid (including Bruch's membrane) preparations which could be a consequence of binding to melanin and lipids in the choroid (Cheruvu and Kompella 2006, Cheruvu et al. 2008).

2.2.2 *Transscleral drug delivery*

In transscleral delivery the drug is administered by periocular injections, or this can be achieved by placing some kind of controlled release system in the periocular space. Significant drug loss has been observed *in vivo* in animal models due to episcleral, conjunctival blood and lymphatic flow in periocular drug delivery (Kim et al. 2004, Robinson et al. 2006). Kim et al. used a periocular implant containing hydrophilic gadolinium-DTPA (mw 938 Da) in rabbits and measured tracer concentrations in vitreous using magnetic resonance imaging (Kim et al. 2004). Only 0.06% of the released drug was detected in vitreous during 8 hours and a high concentration of gadolinium-DTPA was found in the buccal lymph nodes. The experimental set up was repeated *ex vivo* and the vitreal tracer concentrations were 30 times higher than *in vivo*. Drug loss from the periocular space was also studied by Robinson et al. (2006).

After sub-Tenon injection in rabbits triamcinolone acetonide could be detected in the vitreous only if the lymphatic flow and conjunctival blood flow had been blocked by immediate euthanasia after injection or destruction of conjunctival and lymphatic vessels by 'conjunctival window' incision (Robinson et al. 2006). To overcome the problem of periocular loss, intrascleral implants, or implant with a drug releasing surface towards retina have been tested in animal models and significantly higher vitreal drug concentrations were obtained with these formulations compared to periocular injections (Kato et al. 2004, Okabe et al. 2003, Pontes de Carvalho et al. 2006).

After scleral permeation the drug may be cleared by the choroidal circulation. About 85% of the total blood flow into the eye passes into the choroidal circulation (Bill 1975). From a pulsatile ocular blood flow measurements, it can be estimated that blood flow rate in choroid is on average 1 ml/min/choroid, which if considered in proportion of the size of the choroid, is one of the highest values in the human body (Aydin et al. 2003, Yang et al. 1997). The permeability of fenestrated choriocapillaries has been studied in cats and rabbits (Bill 1968, Tornquist et al. 1979, Bill et al., 1980). The permeability is high for small molecules like sodium and EDTA 70-180*10^{-5} cm/s but less for larger myoglobin (17 kDa), albumin (67 kDa) and gammaglobulin (160 kDa) 0.017-0.54*10^{-5} cm/s (Bill 1968, Tornquist et al. 1979). Small molecules in the choriocapillaries equilibrate rapidly with the extracellular tissue. Thus choriocapillaries may serve as a sink for small molecules and it is believed that the clearance for small molecules is faster than for the larger ones. The contribution of drug clearance by choroidal blood flow in transscleral delivery is unknown. In the study by Robinson et al., inhibition of choroidal blood flow by cryotherapy did not result in detectable drug concentrations in vitreous (Robinson et al. 2006). There are also other flow and pressure related factors that may be relevant to transscleral delivery such as uveoscleral outflow from suprachoroidal space through sclera to the conjunctival lymphatic system (Kim et al. 2007).

2.2.3 *Drug delivery by intravitreal injections*

Intravitreal injections result in high drug concentrations in vitreous and retina. Typical drug molecules (mw below 500 Da) distribute equally in vitreous during the first couple of hours after intravitreal injection (Maurice and Mishima 1984). The main components of vitreous are water (>98%), types II, V/XI and IX collagens and hyaluronan (Bishop 2000). Neural retina including the inner limiting membrane also contains hyaluronan as well as heparan sulfate, chondroitin sulfate and dermatan sulfate, which are

negatively charged (Goes et al. 1999, Inatani and Tanihara 2002). This leads to aggregation and poor penetrations of cationic drugs and particles (Kim et al. 2009, Pitkänen et al. 2003). Jackson et al. found that molecules smaller than 76.5±1.5 kDa are able to freely diffuse through neural retina (Jackson et al. 2003). Drugs are cleared from vitreous by the anterior and by posterior routes usually according to first order kinetics i.e. elimination rate is proportional to remaining drug concentration. All drugs are cleared by the anterior route, from anterior vitreous through the posterior and anterior chambers to Schlemm's canal and aqueous and episcleral veins (Fig. 3). The drugs that are either sufficiently lipophilic or substrates of transporters located in BRB are also cleared by the posterior route (Fig. 3), which shortens their vitreal half-life.

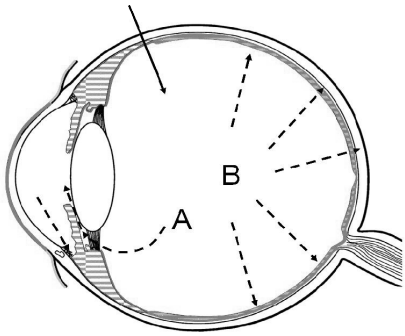

Figure 3. Clearance of drug from vitreous after intravitreal injection at the pars plana (arrow). All drugs are cleared by the anterior route, through posterior and anterior chamber and trabecular meshwork to Schlemm's canal (A). Lipophilic drugs and those drugs which are substrates of transporters in outer BRB are also cleared from the posterior route across the RPE (B). Modified from Hornof et al. 2005.

For example, the vitreal half-life of penicillin and carbenicillin, which are actively transported through RPE, is about 3 hours, whereas gentamycin and streptomycin are cleared only via the anterior route and they have half-lives of 14-18 hours (Maurice and Mishima 1984, Barza et al. 1983). Hydrophilicity and a large molecular weight decrease drug clearance from the vitreous. Mordenti et al. compared the half-lives of full sized human epidermal growth factor receptor 2 antibody (150 kDa) and its fab fragment (48 kDa) in monkeys and reported half-lives of 5.6 and 3.2 days, respectively (Mordenti et al. 1999). These sizes are very similar to currently used VEGF inhibitors, ranibizumab (Fab fragment) and bevasitzumab

(whole antibody). It is also worth noting that the rate of clearance is faster after vitrectomy (Chin et al. 2005, Mason et al. 2004, Moritera et al, 1991).

2.2.4 *Prolonged action drug delivery systems*

Chronic posterior segment diseases may require prolonged medication, perhaps for the rest of the patient's life. Therefore, dosing frequency becomes an important issue, especially in invasive drug administration. Prolonged action drug delivery systems would decrease the frequency of dosing. Tis kinds of drug delivery systems could be placed periocularly, inside the sclera or intravitreally. There are different types of prolonged drug delivery systems e.g. biodegradable and non-biodegradable solid implants, injectable polymers, micro- and nanoparticulates and liposomes. In addition, gene therapy can result in long lasting therapeutic protein secretion.

The rate of drug release can be controlled more precisely with non-biodegradable implants. Initial and final drug burst often seen with biodegradable implants can sometimes cause adverse effects. Both implant types are placed into the vitreous cavity at the pars plana. The implant must be tied with surgical thread to avoid its uncontrolled movements in the vitreous cavity. Biodegradable implants do not need to be removed. Intravitreal non-biodegradable implants containing ganciclovir (Vitrasert) and fluorocinolone acetonide (Retisert) are already in clinical use to treat cytomegalovirus infection and posterior uveitis, respectively (both developed by Bauch & Lomb, Rochester, NY, USA). A biodegradable poly(lactic-co-glycolic acid) (PLGA) based implant that releases dexamethasone (Posurdex, Allergan Inc., Irvine, CA, USA) is in clinical trials to treat uveitis and diabetic macular edema. Estimates for the durations of action are 8, 30 and 1.5-6 months for Vitrasert, Retisert and Posurdex, respectively (Del Amo and Urtti 2008).

Injectable systems, which include injectable polymers, micro- and nanoparticulates, liposomes and gene delivery complexes, are less invasive than implants. Currently these are not in clinical use. In the case of in situ gelling system, the polymer can be injected to the desired site, where changes in temperature, pH or ion concentration may cause a phase transition to semi-solid or solid form (Vehanen et al. 2008) . Particulate systems include nano- and micro sized particles where the drug is either inside the polymer cavity (capsules) or within polymer (spheres) (Bejjani et al. 2005, Bourges et al. 2003, Herrero-Vanrell and Refojo 2001, Moshfeghi and Peyman 2005). In both intravitreal and transscleral administration, the pharmacokinetics of the particulate system is affected by the nature of the polymer and its molecular

weight (Amrite et al. 2008, Sakurai et al. 2001). Studies have shown that intravitreally administered nanoparticles may be taken up by the RPE cells (Bejjani et al. 2005, Bourges et al. 2003, Kimura et al. 1994). The duration of action of these drug delivery systems varies from 24 hours to months (Del Amo and Urtti 2008).

In gene transfer the DNA is transferred into the ocular cells that will produce the protein for as long as the transgene is transcribed and translated to protein. Gene transfer can be accomplished with viral and non-viral gene delivery systems. The natural infectious character of viruses is utilized in viral vectors, while non-viral systems are based on physicochemical interactions between DNA, the carrier material and biological membranes. Viruses for gene-therapy include adeno-, adenoassociated (AAV)-, baculo-, retro- and herpes simplex viruses. The carriers in non-viral gene transfer include cationic liposomes, polymers and peptides. Some viral vectors (AAV, retro- and lentiviral) are capable of integrating DNA permanently into the host genome, but unfortunately the integration is not site-controlled and this may lead to malignancies (Romano et al. 2009). Non-viral vectors are typically less efficient in transfecting than the viral vectors, the duration of transgene expression is short, since they do not generally integrate DNA into genome, but they are less immunogenic, large scale production is possible and there are no strict size limitations for the transgene. Many ophthalmic target cells are differentiated, non-dividing cells, e.g. RPE cells and photoreceptors. With the exception of retroviruses,viruses are capable of transducing non-dividing cells, but the efficacy of non-viral systems is low in non-dividing cells (Magin-Lachmann et al. 2004, Toropainen et al. 2007). This is partly due to lack of cell division and opening of the nuclear envelope in differentiated cells (Ludtke et al. 2002). It has been shown previously that vitreous and neural retina are significant barriers to intravitreal gene delivery of non-viral vectors due to binding of cationic complexes to negative structures of vitreous and neural retina (Pitkänen et al. 2003, Pitkänen et al. 2004, Peeters et al. 2005).

In the cell encapsulation technique, the dividing cells are stably transfected *in vitro* and subsequently they are encapsulated and delivered in this form to the target tissue. Retinal pigment epithelial cell line (ARPE-19) seems to be viable in the microencapsulated form and able to secrete the transfected gene products over prolonged periods (Wikström et al. 2008). NT501 (Neurotech Inc., USA) is a polymer implant that contains CNTF transfected ARPE-19 cells. The product is in clinical trials to treat retinitis pigmentosa patients (Sieving et al. 2006).

2.3 Role of transporters in the outer blood-retinal barrier

Drug transfer through cell membranes and epithelial tissues depends on the passive permeability and active transport processes. Passive drug permeation is determined by the physical and chemical features of the drug (e.g. lipophilicity, hydrogen bonding capacity, charge, molecular weight). Passive permeation is driven by the concentration gradient of the drug while active transport can move the compound even against a concentration gradient. Active transport depends on the affinity of the drug for the transporter, and on the number of the transporters in the cell membrane. The driving force of active transport can be energy from ATP hydrolysis, pH gradient or ion gradient (e.g. Na^+, H^+). Transporters are integral membrane proteins that can be divided into influx and efflux transporters. Influx transporters transfer drugs from the extracellular space into the cell, while efflux transporters act in opposite direction. Currently there are almost 400 genes encoding members of solute carrier (SLC) transporter family (Fredriksson et al. 2008) with these being divided into 46 subfamilies.

Transporters have important role in many tissues like brain, liver, kidney and small intestine. The transporters have their own distinct substrates e.g. nutrients, waste products and xenobiotics which they carry across the cell membranes. Typical substrates include amino acids, peptides, neurotransmitters, monocarboxylates, organic anions and organic cations, various vitamins and metal anions (Fredriksson et al. 2008, Hediger et al. 2004). Due to the large number of transporters and their broad substrate variety, it is evident that membrane transporters are much more relevant in pharmacokinetics than had been previously estimated (Cascorbi 2006, Dobson and Kell 2008, Maeda and Sugiyama 2008, Mizuno et al. 2003).

Depending on the transporter localization and direction of the transporter process, active transport may either enhance or restrict the access of the drug to its site of action. Sometimes competition for the same transporter or induction of the transporter may lead to drug-drug –interactions. Polymorphism is prevalent in the drug transporter genes, and sometimes this can account for inter-individual pharmacokinetic differences (Zhou et al. 2008).

Little is known about which transporters are present in the eye, and their role in ocular drug delivery is poorly understood. Fig. 4 illustrates the possible consequences of the membrane transporter activity in the RPE. The emerging role of transporters in ocular drug delivery has been summarized in publication **V**.

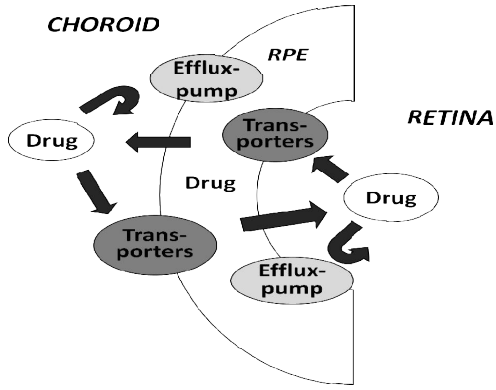

Figure 4. Possible roles of the transporters in RPE. Transporters on the choroidal side of the RPE are expected to facilitate (influx transporters) or restrict (efflux pumps) drug transfer from the choroidal circulation to the retina. From the vitreal side, influx and efflux proteins can either increase or decrease the intravitreal half- life of the drugs, respectively.

2.3.1 *ATP binding cassette efflux transporters*

ATP binding cassette (ABC) efflux proteins transport their substrates from the cell interior across cellular plasma membranes to the extracellular space. Currently, there are 49 human ABC efflux transporter genes subdivided into 8 subfamilies (Vasiliou et al. 2009). The most widely studied efflux transporters include p-glycoprotein (p-gp, ABCG1, encoded by multi drug resistance protein 1 (MDR1)- gene), multidrug resistance associated proteins 1-6 (MRPs, ABCC1-6) and breast cancer resistance protein (BCRP, ABCG2). P-gp consists of 12 transmembrane segments and two nucleotide binding domains (Schinkel and Jonker 2003). The structure of the MRPs is similar with the exception that MRP1-3, 6 and 7 have an additional N-terminal transmembrane domain containing five transmembrane segments (Deeley et al. 2006, Schinkel and Jonker, 2003). BCRP is a half transporter with six transmembrane segments and a single nucleotide binding domain (Haimeur et al. 2004). In order to function as a transporter, two BCRP proteins must dimerize. Information about tissue expression, membrane localization (apical, basolateral or both) and examples of substrates and inhibitors has been summarised in Table 2.

The efflux proteins have a protective role in various tissues (brain, placenta), they are involved in the elimination of many diverse substances (e.g. in kidney, liver) and in many other physiological functions

Table 2. Drug transport related efflux proteins

	Tissue expression	Localisation	Substrate (some representative examples)	Inhibitor (representative examples)
MDR/TAP family				
ABCB1/MDR1/P-gp	Ubiquitously	Apical	Various cancer drugs, corticosteroids, cyclosporin A, HIV-protease inhibitors,tetracycline, fluorokinolones, digoxin, calcein-AM, rhodamine 123	Probenecid, cyclosporin A, verapamil, GF120918, progesterone
MRP/CFTR family				
ABCC1/MRP1	Ubiquitously, High: Lung, testis, kidney	Basolateral	Various glutathione, glucoronide and sulfate conjugated organic compounds, digoxin, calcein-AM, calcein, rhodamine 123 ,fluorescein	Probenecid, MK571, cyclosporin A verapamil, progesterone
ABCC2/MRP2	Ubiquitously, High: Liver, gut, placenta?	Apical	Various glutathione, glucoronide and sulfate conjugated organic compounds, metotrexate, ampicillin, pravastatin, vincristine, calcein, carboxydichlorofluorescein	Probenecid, MK571, cyclosporin A progesterone
ABCC3/MRP3	Ubiquitously, High:. Gut, adrenal gland, placenta	Basolateral	Various glutathione, glucoronide and sulfate conjungated organic compounds, bile acids: cholate, taurocholate and glycolate, carboxyfluorescein, carboxydichlorofluorecein	Probenecid, MK571, verapamil
ABCC4/MRP4	Ubiquitously, High: prostate, kidney, liver, brain, blood vessel endothelium	Apical/basolat	9-(2-phosphonylmethoxyethyl)adenine, ganciclovir, zidovudine, methotrexate, cGMP, cAMP, folate, some prostaglandins: PGE1 and PGE2	(Probenecid), MK571, ibuprofen, sildenefil, dipyridamole,
ABCC5/MRP5	Ubiquitously, High: brain, heart, placenta, smooth muscle cells	Basolateral	9-(2-phosphonylmethoxyethyl)adenine, 5-fluorouracil, methotrexate, folate, cGMP, cAMP, carboxydichlorofluorescein	Probenecid, (MK571), sildenafil, dihydropyridamole
ABCC6/MRP6	High: Liver, kidney; Low: skin, retina	Apical	BQ123 (a cyclopentapeptide antagonist of the endothelin receptor	Probably probenecid and MK571
White-family			some glutathione conjugated organic compounds	
ABCG2/BCRP	Placenta, breast-tissue, kidney, gut, liver, stem cells	Apical	methothrexate, zidovudine, sulfate conjugated compounds, rhodamine 123, Hoechst 33324	GF120918, fumitremorgin C

The data of the table is collected from the following reviews: Schinkel & Jonker 2003, Haimeur et al 2004, Deeley et al. 2005

(Kusuhara and Sugiyama 2009, Nies et al. 2004, Zhang et al. 2004, Young et al., 2003). The substrate specifity between the ABC efflux transporters is partly overlapping, and therefore it is difficult to predict their impact in pharmacokinetics. Furthermore, many cell lines express several efflux transporters, which makes the analysis of the results challenging. In addition, there are often differences between species in expression and functionality. Caution must be exercised in extrapolating results obtained in animal studies to humans with respect to their expression and action. The expression of some of the efflux transporters can be induced by the drug treatment and some efflux transporters have been associated with multi drug resistance, typically found in cancer tissue.

2.3.2 *ATP binding cassette efflux transporters in retinal pigment epithelium*

The results concerning p-gp expression in RPE are partly controversial. In one of the first studies, p-gp expression was detected only after daunomycin exposure (Esser et al. 1998). Later p-gp was found both in apical and basolateral membranes of human RPE (Kennedy and Mangini 2002). The authors suggested that there are different roles for basolaterally and apically located p-gp. In the study of Steuer et al., p-gp was found in porcine RPE (the localization on the plasma membranes was not studied) and in permeability studies with two p-gp substrates, rhodamine 123 and verapamil, the active transport was detected to be in the choroidal direction (Steuer et al. 2005). P-gp expression have been also studied in RPE cell lines: low expression of p-gp was found in h1RPE and D407 cells, but not in ARPE-19 cells (Constable et al. 2006). In the same study cellular uptake of p-gp substrate rhodamine 123 was detected only in the D407 cell line.

MRP1 has been detected in primary human RPE cells, ARPE-19 cells and in porcine RPE tissue (Steuer et al. 2005, Aukunuru et al. 2001). The MRP1 substrate, fluorescein, was transported in porcine RPE-choroid tissue in the choroidal direction (Steuer et al. 2005). MRP5 expression at the mRNA level has been found in ARPE-19 cells (Cai and Del Priore 2006). Stojic et al studied MRP5 splice variant expression and found three shorter splice variants (SV1-3) in addition to the full length MRP5 transcript in human RPE (Stojic et al. 2007). SV2 isoform expression was confirmed by immunohistochemistry in the apical and basolateral surface of mouse RPE. The MRP6 transcript expression was found in patients with pseudoxanthoma elasticum in the retina, but not in RPE (Bergen et al. 2000, Scheffer et al. 2002). The expression of MRP2, MRP3, MRP4 or BCRP have not been studied in RPE.

2.4 *In vitro* models to estimate drug delivery in outer blood-retinal barrier

Differentiated organotypic cell models are important tools in biology. They allow experiments with well-defined conditions and mechanistic studies related to cell biology, physiology, toxicology and pathophysiology. ADME related experiments with differentiated cell models are currently an important part in drug discovery and development programmes (Hornof et al., 2005). In drug development, various ADME cell models (e.g. intestinal, BRB, placenta, skin) are used to screen drug candidates and drug delivery systems.

Despite its importance, there is no established cell model available for the outer BRB. In principle, such models could be based on isolated tissues or cell cultures. Isolated tissue represents the integrity and expression levels of its *in vivo* counterpart, but this approach is tedious and it is not suitable for large-scale routine use. The cell lines express human proteins, they are suitable for routine use, but establishment of the cell model requires detailed characterization of the morphology, barrier properties, and an awareness of gene expression properties.

2.4.1 *Isolated retinal pigment epithelium tissues*

Due to the limited availability of human material, isolated human RPE(-choroid) tissue are rarely used, instead isolated RPE(-choroid) tissues are mainly from porcine, bovine or rabbits (Pitkänen et al. 2005, Steuer et al. 2005, Arndt et al. 2001, Edelman and Miller 1991, Frambach et al. 1988, Kansara and Mitra 2006). During the experimentsseveral electrical parameters eg. transepithelial resistance (TER) and transepithelial potential (TEP) can be monitored to confirm that the membrane is intact and viable. Isolated RPE tissues always contain parts of the choroid (Bruch's membrane) since it is impossible to separate a pure RPE cell layer without disturbing the integrity of the cell layer. However the contribution of Bruch's membrane to TER is negligible (Miller and Edelman 1990). The integrity may be easily compromised in the isolation process and the specimens must be used rapidly after isolation if they are to maintain their viability. There is extensive variation in the measured TER values from different species: bovine 100-260 $\Omega*cm^2$ (Pitkänen et al. 2005, Arndt et al. 2001, Edelman and Miller 1991), rabbits 82-350 $\Omega*cm^2$ (Frambach et al. 1988, Kansara and Mitra 2006), porcine ~260 $\Omega*cm^2$ (Arndt et al. 2001) and human 36-148 $\Omega*cm^2$ (Quinn and Miller 1992).

Isolated RPE-choroid tissues have been used to study passive permeability and active transport. Typically the tissue is located between donor and acceptor compartments in a Ussing type chamber system (Pitkänen et al. 2005, Steuer et al. 2005, Steuer et al. 2004). Pitkänen et al. used bovine RPE-choroid tissues to study the effect of lipophilicity and molecular weight on drug permeability in RPE-choroid (Pitkänen et al. 2005). Asymmetric transport of carboxyfluorescein, sodium fluorescein (organic anion transporter substrate), rhodamine 123 (substrate of p-gp, MRP1 and BCRP) and verapamil (substrate of several transporters including p-gp) have been detected in isolated RPE-choroid tissues in studies by Pitkänen et al. (2005) and Steuer et al. (2005). Recently Kansara and Mitra demonstrated directional folic acid transport in isolated rabbit RPE-choroid tissue (Kansara and Mitra 2006).

2.4.2 *Retinal pigment epithelium cell lines*

In vitro cell models of outer BRB include primary cells and continuous cell lines, which can be immortalized or spontaneously transformed. There are some commonly used continuous cell lines e.g. ARPE-19, D407, hTERT-RPE, h1RPE-7- and h1RPE-116, which are of human origin, and rat RPE-J. Generally, primary cells can be sub-cultured only for a few passages, whereas continuous cell lines can be sub-cultured for several passages. Primary cells may preserve the properties of the original tissue that may be lost when cells are sub-cultured. Melanosomes are seen usually in the primary cells after their extraction, but they disappear during the sub-culturing procedure (Kanuga et al. 2002). The cellular variability of primary cells is higher compared to the continuous cell lines. Continuous cells may have lost some of important features of the original RPE cells. In contrast, continuous cells are easily obtained and their supply is almost unlimited.

Primary cells. Human primary cells are commonly used in countries with eyebanks whereas in other countries, their use is limited by availability. Other commonly used primary RPE cell sources include bovine, pig, rabbit, rat (Chang et al. 1997, Hernandez et al. 1995, Maenpaa et al. 2002, Stanzel et al., 2005). Extraction of primary cells is a laborious process. A commercial primary RPE cell line (HRPEpiC cells) is also available from ScienCell Research Laboratories (CA, USA). Various growth media supplements and growth matrices have been used with primary RPE cells (Capeáns et al. 2003, Hu and Bok 2001, Frambach et al. 1990, Castellarin et al. 1998, Maminishkis et al 2006, Singh et al. 2001, Song and Lui 1990). There are studies demonstrating that the primary cells polarize and form a cell layer with barrier properties (Hornof et al. 2005). The TER values of fetal cell cultures are generally significantly

higher than those of adult primary cells. TER values as high as 500 Ω*cm2 have been measured from fetal RPE cells (Hu and Bok 2001, Rajasekaran et al. 2003).

ARPE-19. During last decade, the ARPE-19 cell line has become a most popular continuous cell line in RPE cell research. Originally the cells were extracted from the eye of an 19-year old male donor and they spontaneously transformed into continuous cell line with the normal number of chromosomes (Dunn et al. 1996). RPE specific markers, retinal pigment epithelia specific protein 65 kDa (RPE65) and cellular retinal aldehyde binding protein (CRALBP), have been shown to be expressed in the ARPE-19 cell line at the mRNA and protein level, respectively (Dunn et al. 1996). In long term culture on laminin coated Transwell membranes, ARPE-19 cells (Fig. 5) were shown to form a polarized monolayer with tight junctions and the tightness could be further increased by culturing cells in a special media designed for RPE cells (Frambach et al. 1990). In addition, some cells exhibited apical microvilli and basal infoldings. The heterogenity of ARPE-19 cells has been reported previously (Luo et al. 2006). Polarized secretion of fibroblast growth factor 5 and interleukins 6 and 8 have been detected in filter grown ARPE-19 cells (Dunn et al. 1998, Holtkamp et al. 1998). ARPE-19 cells have been widely used, in studies on oxidative stress, retinal pathogenesis, signaling pathways as well as in drug and toxicity related studies (Glotin et al. 2008, Haritoglou et al. 2009, Huhtala et al. 2009, Lara-Castillo et al. 2009, Thurman et el. 2009).

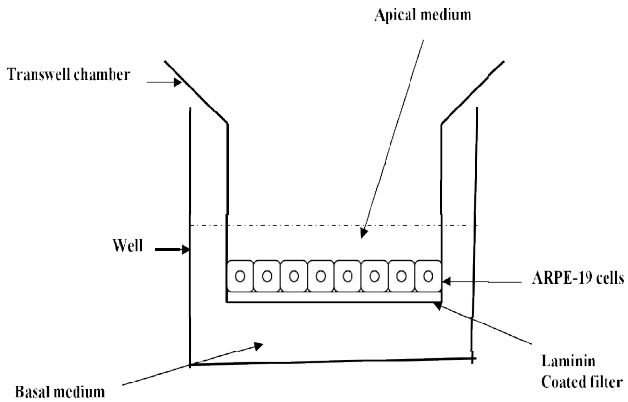

Figure 5. Schematic presentation of the typical ARPE-19 membrane culture. Cells are cultured on membranes with or without coating. Separation of apical and basal compartments allows transepithelial studies in passive diffusion, active transport and, in addition, the direction of protein secretion can be studied using this polarized cell model.

D407. The spontaneously transformed RPE cell line D407 was shown to have typical features of RPE cells including cobblestone appearance, binding of POS, expression of CRALBP protein and expression of cytokeratins typical to RPE (8 and 18) (Davis et al. 1995). The growth potential of these cells is high. This may be related to the nearly perfect chromosomal trisomy observed in these cells. Unfortunately D407 cells do not polarize in filter culture and they do not synthesize pigment.

hTERT-RPE. hTERT-RPE cell line was immortalized by transfecting human telomerase catalytic subunit into human RPE cells (Bodnar et al. 1998). These cells have an extended life span with normal chromosomes and growth behavior (Jiang et al. 1999). Melanization of cells was detected in long-term serum free cultures (Rambhatla et al. 2002) and CRALBP expression has been detected with antibodies (Rambhatla et al. 2002). Alge et al studied differences in protein expression between early passage RPE cells, ARPE-19 and hTERT-RPE, and concluded that a generally similar expression pattern was shared, but some differences were noted in protein expression related to cytoskeleton remodeling, polarization, cell survival, migration and proliferation of the cells (Alge et al. 2006). The polarization and barrier properties of hTERT-RPE cells in membrane culture have not been evaluated.

h1RPE(-7 and -116). hRPE cells have been immortalized by transfecting human adult primary RPE cells with the SV40 large T antigen (Kanuga et al. 2002). These cells have epithelial morphology with apical microvilli, but fail to develop TER above 30-40 $\Omega \cdot cm^2$ in normal culture conditions. This cell line has been used in only a few studies. Sub-retinal transplantation of hRPE cells in Royal College of Surgeons (RCS) rats resulted in rescued photoreceptors for 5 months post-crafting (Lund et al. 2001). P-gp expression was found in these cells, but no activity could be detected (Constable et al. 2006).

RPE-J. The RPE-J cell line was created by infecting primary rat RPE cells with a temperature sensitive SV40 virus. When grown on Matrigel coated nitrocellulose filters these cells developed TER of ~350 $\Omega * cm^2$ (Nabi et al. 1993). The tight junctions were found mostly basally, occasionally also apically, and it was shown that these cells have apical microvilli (Nabi et al. 1993). The cells were capable of phagocytosing latex beads and polarized distribution of viral proteins was observed but they lacked any Na, K-ATPase polarity. The neural cell adhesion molecule (N-CAM) was found in lateral borders. These cells have been used to study RPE phagocytosis (Feng et al. 2002, Hall et al. 2003, Strick et al. 2009), the precense of bestrophin (Marmorstein et al. 2000, Marmorstein et al. 2002) and polarity (Bonilha et al. 1999, Marmorstein et al. 1998). However man must recall that this is a rat cell, and species differences in transport and metabolism may limit its usefulness in ADME studies.

2.4.3 *Cell culture conditions for retinal pigment epithelial cells*

The differentiation of the cell line is affected by the growth conditions such as medium composition and growth substrate. Various culture conditions have been used with RPE cells.

Growth media. A wide variety of media have been used in RPE cultures e.g. Chee's Essential Medium (CEM) replacement medium has been frequently used, since it was described by Hu and Bok (2001). This medium contains zinc, copper, magnesium, selenium, hydrocortisone, linoleic acid, insulin, transferring, L-ascorbic acid, 1% calf serum, bovine retina extract (BRE; 0.5 % v/v) 0.5 % by volume, and several amino acids. TER values over 800 $\Omega*cm^2$ have been measured from fetal primary cells grown in this medium (Hu and Bok, 2001). Promising results have been also achieved by using a calcium switch, where cells are first plated in low calcium media and after reaching confluency, they are changed to a media with normal calcium concentration (Chang et al. 1997, Stanzel et al. 2005, Rak et al. 2006). This method has been shown to synchronize cadherin expression (Nelson, 2008). A low serum concentration is frequently used because serum contains a factor that inhibits the formation of tight junctions (Chang et al. 1997). In primary RPE cell cultures, typical supplements include basic fibroblast growth factor (bFGF) and bovine retina extract (BRE) (Hu and Bok 2001, Frambach et al. 1990, Castellarin et al. 1998, Song and Lui 1990). Different other media supplementations as well as different glucose concentrations have been also tested, but there are no systematic studies nor definitive conclusions about the role of the media supplements in the properness of cultured RPE derived cells (Engelmann and Valtink 2003, Heimsath et al. 2006, Karl et al. 2006, Wood et al. 2004).

Growth substrate. It has been shown that the presence of the basement membrane is essential for the polarization of RPE cells. Since Bruch's membrane contains laminin and collagens, growth matrices with these compounds are often used (Chang et al. 1997). Various biological membranes have been used e.g. amniotic membranes (Stanzel et al. 2005, Capeans et al. 2003, Hamilton et al. 2007, Ohno-Matsui et al. 2005, Ohno-Matsui et al. 2006), donor Bruch's membranes (Castellarin et al. 1998, Del Priore and Tezel 1998), lens capsule (Hartmann et al. 1999, Nicolini et al. 2000, Singh et al., 2001) and extracellular matrix derived by cultured corneal epithelial cells (Song and Lui 1990, Campochiaro and Hackett 1993, Wiencke et al. 2003).

2.4.4 *Differentiation markers of retinal pigment epithelial cells*

RPE related genes and protein. The genes and proteins that are found exclusively or at particularly high quantities in the RPE are often used as differentiation markers of RPE cells. These markers include RPE65, CRALBP and bestrophin (coded by BEST1 formerly VMD2 gene) (Marmorstein and Kinnick 2007, Redmond 2009, Saari and Crabb 2005). RPE65 and CRALBP are both involved in the vitamin-A cycle, in the regeneration of visual pigment (Jin et al. 2005, Moiseyev et al. 2005, Saari et al. 2001). The exact role of bestrophin is not clear, but some studies suggest that bestrophin plays a role in chloride conductance and homeostasis of intracellular calcium and pH in RPE cells (Marmorstein et al. 2009, Qu et al. 2006). Homeodomain containing (OTX2) and microphthalmia-associated (Mitf) transcription factors have important roles in the regulation of RPE differentiation (Martinez-Morales et al. 2003). The polarized expression of some proteins in RPE is known *in vivo*. These have been also used as markers of differentiation and proper polarization. For example, Na/K-ATPase, αVβ5 integrin, monocarboxylate transporter-1 (MCT-1), folate transporter protein (RFT-1), are expressed apically in RPE and basolaterally enriched proteins include monocarboxylate transporter- 3 (MCT-3), folate receptor FRα and bestrophin (Gundersen et al. 1991, Marmorstein et al. 2000, Bridges et al. 2002, Finnemann et al. 1997, Philp et al. 1998).

Phagocytosis. Functional features typical of the derived cell lines such as phagocytosis in the case of RPE cells, provides some indication about the state of differentiation. Phagocytosis in RPE cells is a strictly controlled process involving several proteins. POS phagocytosis is divided into binding and internalization phases. The receptor tyrosinase kinase c-mer (MerTK) and integrin receptor αVβ5 have important roles in POS binding, and MerTK in activation of intracellular signaling cascade (Feng et al. 2002, Finnemann et al. 1997). Macrophage scavenger receptor CD36 and toll like receptor 4 (TLR-4) are also involved in the phagocytosis of POS (Finnemann and Silverstein 2001, Ryeom et al. 1996, Kindzelskii et al. 2004).

3 AIMS OF THE STUDY

The general aim was to evaluate and further develop a filter grown ARPE-19 cell model as an outer blood-retinal barrier model for use in drug and gene delivery studies.

Specific aims of the study were:

1. To compare passive permeability of ARPE-19 cell model with isolated RPE-choroid tissue
2. To enhance the barrier properties of the cell model by modification of the growth conditions
3. To study the influence of the cell culture conditions on RPE specific gene expression
4. To compare active efflux transport of ARPE-19 cell model with isolated RPE-choroid tissue
5. To investigate the expression and functionality of efflux transporters in the cell model
6. To study interactions of Hsp inhibitors with efflux proteins using ARPE-19 cells
7. To investigate the non-viral gene transfer in polarized non-dividing RPE cells
8. To evaluate the potential role of transporters in the blood-retinal barrier by conducting literature search.

4 MATERIALS AND METHODS

4.1 Cell culture

4.1.1 *Cell lines*

Two continuous (ARPE-19 and D407) and two primary RPE cell lines (hRPEpiC and bRPE) were used (**I-IV**). Caco2 and transduced MDCKII and HEK293 cell lines were used as positive controls in efflux protein studies (**II**). Description, source and references of cell lines are summarized in table 3. The cells were cultured as described in the original publications (**I-IV**).

Table 3. Cell lines used in the studies **I-IV**

Cell lines	Description	Source	References
ARPE-19	human secondary RPE cells	ATCC/CRL-2302 and provided by Dr L. Hjelmeland[a]	Dunn et al. 1996
D407	human secondary RPE cells	provided by Dr R. Hunt[b]	Davis et al. 1995
hRPEpiC	human primary RPE cells	ScienCell Research Laboratories	www.sciencellonline.com
bRPE	bovine primary RPE cells	extracted by ourselves	Bejjani et al., 2005)
Caco2	human colorectal adenocarcinoma cells	ATCC/HTB-37	Jumarie et al. 1991
Efflux protein expressing cell lines[c]			
MDCKII-MDR1	Madin-Darby Canine Kidney Cells	Netherlands Cancer Institute	Bakos et al. 1998
MDCKII-MRP1	Madin-Darby Canine Kidney Cells	Netherlands Cancer Institute	Bakos et al. 1998
MDCKII-MRP2	Madin-Darby Canine Kidney Cells	Netherlands Cancer Institute	Evers et al. 1998
MDCKII-MRP3	Madin-Darby Canine Kidney Cells	Netherlands Cancer Institute	Kool et al. 1999)
MDCKII-MRP5	Madin-Darby Canine Kidney Cells	Netherlands Cancer Institute	Wijnholds et al. 2000
HEK293-MRP4	Human Embryonic Kidney Cell Line	Netherlands Cancer Institute	Wielinga et al. 2002

[a]University of California, Davis, CA, USA [b]University of South Carolina, Columbia, SC, USA [c]kindly provided by Dr P.Borst

4.1.2 *Filter culturing of ARPE-19 cells (I, II, IV)*

The basic filter-culture protocol for ARPE-19 described by original authors (Dunn et al. 1996) was used with minor modifications. Dunn et al (2006) used polycarbonate Transwell filters of 6.5 mm diameter, but we used polycarbonate filters of 24 mm diameter (**I**, **II** and **IV**). The cell growth area was increased from 0.33 cm^2 to 4.7 cm^2. The filters were coated with laminin and the cells wree seeded at a density of $1.6x10^6$ cell/cm^2. Filters without coating and collagen coated polyester Transwell filters were tested in preliminary experiments. The basic filter media consisted of DMEM-F12 (1:1) supplemented with 10 % FBS, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Different supplements were added to basic filter media in order to improve the barrier properties of ARPE-19 cell monolayer (Table 4). Most of the supplements were commercially available, but BRE and POS were extracted as previously described (Frambach et al. 1990, Molday et al. 1987). Fresh media with supplements were added to both apical and basolateral compartments twice a week with the media change. POS were added only to the apical compartment, since *in vivo* POS are located only on the apical side of RPE cells. The supplements were tested separately and in addition, media containing BRE, bFGF and taurine in combination (medium III) was tested. ATRA, sodium pyruvate, taurine, bFGF, BRE, ROS and medium III were chosen for further testing based on the first results with TER measurements. FBS from two different suppliers was also tested.

Table 4. Tested media supplements and rationales

Supplement	conc.	Rationale	References
dexamethasone	10 nM	promotes formation of tight junctions	Underwood et al. 1999
insulin	1 µg/ml	integrin expression, viability of RPE cells	Uebersax et al. 2000
EGF	10 ng/ ml	Mitogen, modulates ion transport in diff. RPE	Arrindell et al. 1992
bFGF	10 ng/ ml	promotes proliferation, RPE cell morphology, viability, integrin receptor expression	Kaven et al 2000 Spraul et al. 2004
vitamin-C	40 µg/ml	improves cell growth	Rowe et al. 1977
ATRA	25 nM	promotes epithelial cell type, growth control	Campochiaro et al. 1991
forskolin	10 µM	Increase in intracellular cyclic AMP,	Insel and Ostrom 2003
IBMX	100 µM	Increase in intracellular cyclic AMP	Njie-Mbye et al. 2009
sodium butyrate	1 M	modulates gene expression, differentiation	Cuisset et al. 1997
sodium pyruvate	1 nM	participates cell energy metabolism	Wood et al. 2005
taurine	10 mM	most abundant and essential amino acid in retina	El-Sherbeny et al. 2004
BRE	1 %	unknown composition, promotes differentiation	Frambach et al. 1990
POS	$120x10^6$	ingested by RPE daily *in vivo*	Molday et al. 1987

4.1.3 *Transepithelial resistance measurements (I, II, IV)*

The development of tight junctions was followed by TER-measurements by Endohm chamber (World Precision Instruments) (**I**, **II**, **IV**). The average resistance of laminin coated blank filter was 8 Ωcm^2. The resistance of blank filter was subtracted from the resistance of the filter with cells to obtain the resistance of the cell layer.

4.1.4 *Electron microscopy*

Electron microscopy was used to study morphology of ARPE-19 monolayers in basic filter medium (**V**) and in media supplemented with bFGF and BRE. The preparation of samples is explained in detail in original publication **IV**. Morphology of isolated POS was also studied by electron microscopy. The specimens were inspected with transmission electron microscopy (JEM 1200 EX).

4.1.5 *Photoreceptor outer segment phagocytosis*

POS were isolated from bovine neural retina with sucrose density gradient according to the procedure of Molday et al. (1987). POS were labeled with FITC at room temperature for one hour. FITC-POS were washed four times with 1 x PBS and re-suspended to ARPE-19 culture medium. After counting, FITC-POS were fed to ARPE-19 cells in 6- or 24- well plates. Cell uptake of FITC-POS was measured with flow cytometry (FACScan, Becton Dickinson) using an argon ion laser (488 nm) as the excitation source and fluorescence was detected at 670 nm.

4.2 Expression studies

4.2.1 *Real time quantitative polymerase chain reaction (I, II)*

Expression of efflux proteins (p-gp, MRP1-6, BCRP) and RPE cell related proteins (RPE65, bestrophin, CRALBP, tyrosinase, TRP1, MITF-A (microphthalmia-associated transcription factor) and OTX2 (homeodomain-containing transcription factor) was studied at the RNA level by quantitative real time PCR (RT-qPCR).

The total RNA was extracted using TRI-Reagent®, DNAse treated, quantified using RiboGreen quantification assay and reverse transcribed (**II, III**). The samples were diluted and divided into small aliquots enough for one PCR run in order to avoid repeated thaw and freeze cycles. A total of 40 ng of each cDNA was amplified using ABI Prism 7000 system using either FAM-labeled Assay on Demand TaqMan® Gene Expression Assays (Table 5) or custom made primers and probes (Table 6).

Table 5. Taqman gene expression assays

gene	*primer/probe set*	*gene*	*primer/probe set*
RPE65	Hs00165642_m1	MRP1	Hs00219905_m1
CRALBP	Hs00165632_m1	MRP3	Hs00358656_m1
VMD2	Hs00188249_m1	MRP4	Hs00195260_m1
MITF-A	Hs01115553_m1	MRP5	Hs00981071_m1
OTX-2	Hs00222238_m1	MRP6	Hs00184566_m1

Table 6. Custom made primers and probes for q RT-PCR

gene	*forward primer*	*reverse primer*	*label*
Tyrosinase*	agcaccccacaaatcctaacttac	atggctgttgtactcctccaatc	SYBR green dye
TRP1*	agccttctttctcccttccttactg	gtgcaggaggacaaaaataggatca	SYBR green dye

gene	*forward primer*	*reverse primer*	*Taqman probe*
MDR1**	gatcacaagcccaagacaga	caacaaaataaggccattcagt	ttagtaccaaagaggctctggatgaaagtatacc
MRP2**	acatctgccattcgacatga	ccaggttcacatctcggact	caattttgacaaagccatgcagttttctga
BCRP**	caggactcaatgcaacaggaaa	agatcgatgccctgctttacc	ccttgtaactatgcaacatgtactggcgaagaa

*Primers designed by using Primer express (Applied Biosystems), OLIGO (Molecular Biology Insights, Inc.) and BLAST programs.

**Korjamo et al. 2005

Two approaches were used to analyze gene expression results: Relative gene expression and absolute quantitative approach (Bustin 2002). The relative gene expression allows comparison between one gene

in different conditions, but does not allow comparison of expression levels between different genes. The results were calculated separately from each plate, relative to ARPE-19 cells grown either in flasks or on filters (**I**). The absolute gene expression was calculated with standard curves that were run each time with the samples. The standard curves were generated using restriction fragments of plasmids with the same gene sequences (**II**). In this case, absolute comparison between different genes is possible.

4.2.2 *Western blotting (II)*

Western blotting was used to study MRP1, MRP2, MRP4, MRP5 and BCRP expression at protein levels in different RPE cells. The information about the antibodies is listed in table 7. The western blotting was performed as described in the publication (**II**).

Table 7. Antibodies, dilution rations and positive controls for western blot studies

Efflux protein	***primary antibody***	***secondary antibody***	***positive control cell line***
MRP1	ab3368 (MRPr1) 1:5 000	Rat 1:10 000	MDCKII-MRP1
MRP2	ab3373 (M2 III-6) 1:4 000	Mouse 1:8 000	MDCKII-MRP2
MRP4	ab15602 (M4I-10) 1:5 000	Rat 1:10 000	HEK293-MRP4
MRP5	ab3377 (M5I-1) 1:1 000	Rat 1: 1 000	MDCKII-MRP5
BCRP	ab3380 (BXP-21), 1:2000	Mouse 1:2 000	Caco2

4.3 Permeability experiments (I)

Permeability experiments were used to study both passive and active drug transport across ARPE-19 monolayers (**I**). The permeant molecules included 6-carboxyfluorecein, sodium fluorescein, rhodamin 123, betaxolol, digoxin, cyclosporin A and FITC-dextran 40 kDa. Molecular properties (molecular weight,

logD), donor concentrations and possible transporters of the molecules are shown in table 1 (**I**). The permeant molecules were added to either the apical or basolateral compartments (donor compartment) and samples were taken from the receiver compartment at 15 min intervals up to three hours. Fluorescence of 6-carboxyfluorescein, sodium fluorescein, rhodamine 123 and FITC-dextran 40 kDa samples were measured using Victor 1420 Multilabel Counter. Betaxolol concentrations were measured by HPLC with combined ultraviolet and fluorescence detection as described previously by Ranta et al. (2002). Radioactivity of tritium labelled cyclosporin A and digoxin was detected with liquid scintillation counter. Apparent permeability was calculated according to equation:

$$P_{app} = J/(A*C_0),$$

where A is the surface area of the filter, C_0 the drug concentration in the donor chamber at time zero and J is flux of the permeant molecule from donor to receiver compartment.

4.4 Cellular uptake studies (II, III)

4.4.1 Calcein-AM assay (II, III)

Calcein-AM assay was used to test efflux protein activity of ARPE-19 and primary bovine RPE cells (**II**) and to assess interactions between Hsp90 inhibitors geldanamycin and radicicol with efflux proteins (**III**). Calcein acetoxymethyl ester (calcein-AM) is a cell permeable compound that is metabolized by intracellular esterases to cell impermeable calcein (Fig. 6). Calcein-AM is a substrate of p-gp and MRP1 efflux proteins and calcein is a substrate of MRP1 and MRP2 (Essodaigui et al. 1998, Evers et al. 2000) (Fig. 6). Inhibition of efflux transport by test drugs causes calcein accumulation and an increase in the intracellular fluorescence (Eneroth et al. 2001). The assay was conducted as described earlier (Eneroth et al. 2001, Vellonen et al. 2004). In brief, ARPE-19 cells were cultured for four days prior to the experiment on 96-well plates. The cells were exposed to inhibitors for 15 min before calcein-AM was added for 20 min. The fluorescence from the cells was measured immediately after the experiment using Victor 1420 Multilabel counter.

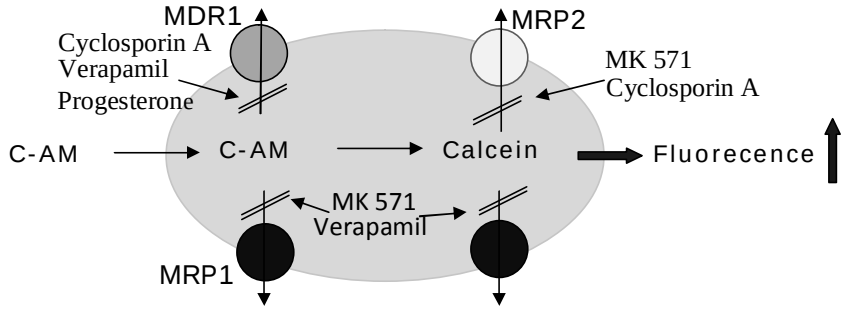

Figure 6. The schematic presentation of calcein-AM assay. Cell permeable calcein-AM is metabolized by esterases to calcein inside the cell. Calcein and calcein-AM are substrates of efflux proteins. Inhibitors of MRP1, MRP2 and MDR1 efflux proteins are shown in the picture. The efflux inhibition can be detected as an increased cellular fluorescence.

4.4.2 *CDCFDA/CDCF assay (III)*

CDCFDA/CDCF assay was used to assess MRP5 activity (**III**). The method is similar to Calcein-AM assay: CDCFDA is a cell permeable compound that is cleaved inside the cell by esterases to relatively the impermeant and fluorescent CDCF. CDCF is a substrate of MRP2, MRP3 or MRP5 (McAleer et al. 1999, Tian et al. 2004, Zamek-Gliszczynski et al. 2003). MRP transport can be inhibited by probenecid, which is a non-specific inhibitor of organic anion transporters. MDCKII cell lines expressing MRP proteins (table 3) and ARPE-19 cells were used for the assay. The cells were cultured for four days prior to the experiments on 96-well plates. The cells were exposed to CDCFDA for 20 min and after two washes the probenecid containing buffer or the plain buffer was added to the cells. The samples were collected from the buffer at 30 and 90 min. The fluorescence was measured with a Victor 1420 Multilabel counter.

4.5 Non-viral gene delivery (IV)

Polarized ARPE-19 cells were used to study non-viral gene delivery and uptake of non-viral gene delivery complexes. The cells were cultured for four weeks prior to the experiments. Polarization was confirmed with TER measurements before and after the experiments. Non-viral gene transfer was accomplished with cationic carriers that form condensed nanoparticulates with plasmid DNA.

4.5.1 *Transfections with non-viral DNA/carrier complexes (IV)*

Plasmid (pCMV-SEAP2) encoding SEAP as a reporter gene under control of CMV promoter was constructed by inserting the CMV enhancer/promoter fragment from p-CL-neo vector to pSEAP2-Basic vector (Clontech). Cationic polymer carriers PEI 25 kDa and lipids DHP-12, DOTAP and DOTAP/DOPE were prepared as described earlier (Boussif et al. 1995, Hyvonen et al. 2000, Ruponen et al. 1999). Charge ratios (carrier:DNA) were optimised in preliminary experiments. Complexes of pCMV-SEAP2 plasmid with the carriers were allowed to form for 20 min before adding them to the apical compartment of polarized ARPE-19 cells. After 5 hours, the complexes were washed off and medium samples from apical and basolateral compartments were collected at different time points. The SEAP concentration was measured using Great EscAPE SEAP Chemiluminescence Detection kit (Clontech).

4.5.2 *Cellular uptake of non-viral DNA/carrier complexes (IV)*

For cellular uptake studies pCMV-SEAP2 plasmid was labelled with fluorescent EMA (Zabner et al. 1995). Transfections with EMA-pCMV-SEAP2 complexed with PEI, DOTAP/DOPE and DOTAP/DOPE/PS were done as described above. The cells were washed with 1 M sodium chloride, detached from filter membranes and fixed with paraformaldehyde for flow cytometric analysis. The results from 10 000 cells were expressed as a percentage of positive cells and the fluorescence intensity of the cells was also monitored.

4.6 Statistical analyses

All statistical analyses were performed using SigmaStat 3.5 software (SSPS Inc., Chicago, IL, USA). Pair-wise comparisons were done using Student's t-test and Mann-Whitney U-test (when a normal distribution was not present) (**I,II, IV**). $P<0.05$ was considered as a significant difference. Kruskal-Wallis analysis was used to compare multiple experimental groups. When the difference was significant ($p<0.05$), the comparisons versus control group (calcein-AM assay) or pair-wise comparisons (CDCFDA/CDCF-assay) were conducted with the Dunn's test (**II**).

5 RESULTS

5.1 Characterization of ARPE-19 cell culture model

5.1.1 *Resistance*

In two weeks, the TER values reached a plateau, typically between 80 and 100 Ω x cm^2 and remained at this level for as long as several months (**I, II, IV**). Higher TER values, even above 150 Ω x cm^2, were detected when either BRE or bFGF was added to the culture media (**I Fig. 2**). In other tested media the resistances were similar or lower than in basic filter medium (**I Fig. 2**).

5.1.2 *Morphology*

In the basic filter culture medium, ARPE-19 cells on Transwell membranes grew mainly as a monolayer (**IV Fig 2A**), but some cell overlapping was observed. Heterogeneity was found in the existence of apical microvillae between cells. Basal infoldings were frequently observed. Cell-to-cell junctions that resembled desmosomes, tight junctions and zonula adherens were found between the cells (**IV Fig. 2B**). in contrast, multilayering of ARPE-19 cells was found when either BRE or bFGF was present in the culture media (pictures not shown).

5.1.3 *Expression of RPE related genes*

ARPE-19 cells expressed all analysed RPE related genes: RPE65, CRALBP, VMD2, tyrosinase,TRP1 and transcription factors (OTX-2, MITF-A), which are responsible for regulating several RPE cell functions (**I**). Expression levels of RPE65, VMD2, CRALBP, TRP1 and tyrosinase were increased 37, 2, 13, 1860 and 550-fold, respectively, when the cells were cultured for prolonged periods on filter membranes (**I Fig. 3A**). The influence of the culture media composition was much smaller (**I** and Fig. 7).

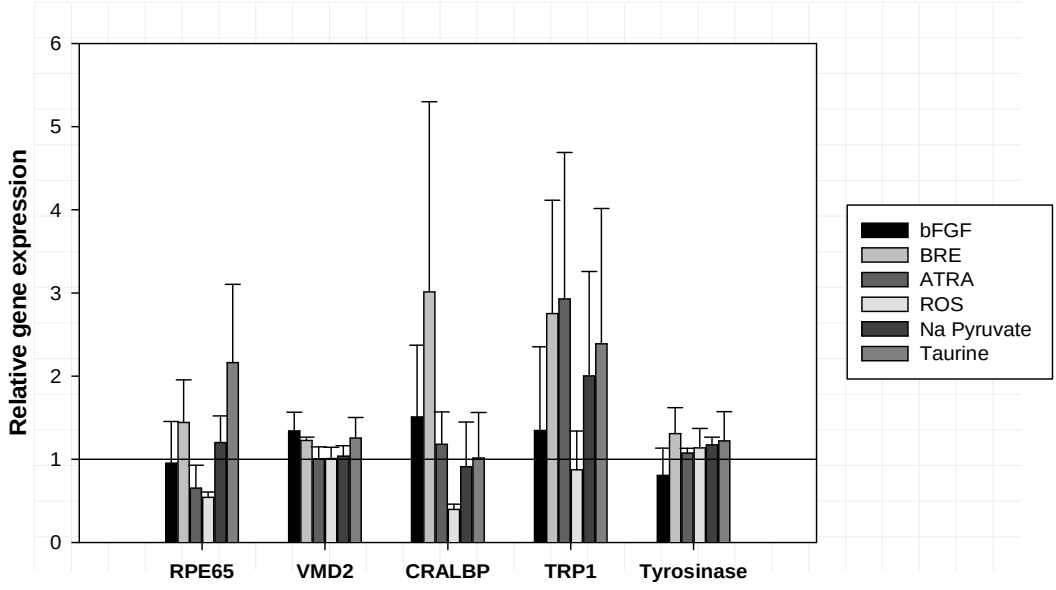

Figure 7. Comparison of RPE65, VMD2, CRALBP, TRP1 and tyrosinase mRNA expression between filter cultured ARPE-19 cells in various media. The results are from two independent cultures each performed in triplicates and presented as the mean normalized expression±SD relative to filter cultured cells in basic filter culture media, which is set to one.

5.1.4 *Passive permeability*

Passive permeability of ARPE-19 monolayer in basic filter culture media was 3-17 times higher than the permeability of the isolated bovine RPE-choroid tissue (**I Fig. 1**). Clear differences in the permeation in ARPE-19 cell model were found among compounds with different molecular sizes and lipophilicities. Permeability values of 6-CF, FD-40 kDa and betaxolol were 7.2, 0.8 and $33.3*10^{-6}$ cm/s respectively. Lower passive permeability of 6-carboxyfluorescein (3.5 and $4.3*10^{-6}$ cm/s) was found when BRE or bFGF or was added to the culture medium (**I Fig. 2**).

5.1.5 *Photoreceptor outersegment phagocytosis*

Typical morphological structures of isolated POS were observed with transmission electronic microscopy (Fig. 8). FACS analysis revealed that ARPE-19 cells ingested FITC-POS by concentration dependent manner and the results were changed by lowering the temperature from 37 °C to 4 °C (Fig. 9) sindicative of an energy dependent process.

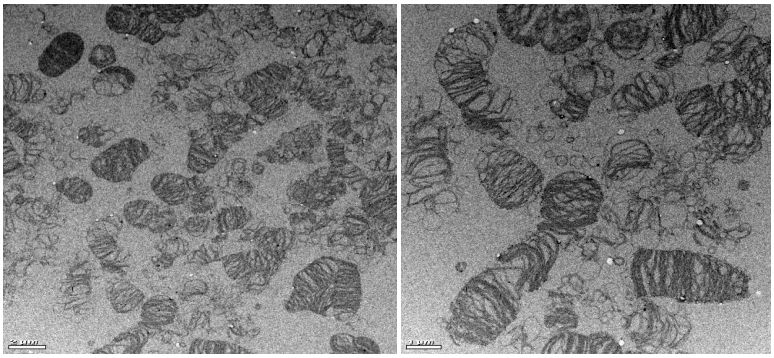

Figure 8: POS isolated from bovine neural retina using a sucrose density gradient. Scale bar 2 µm (left) and 1 µm (right)

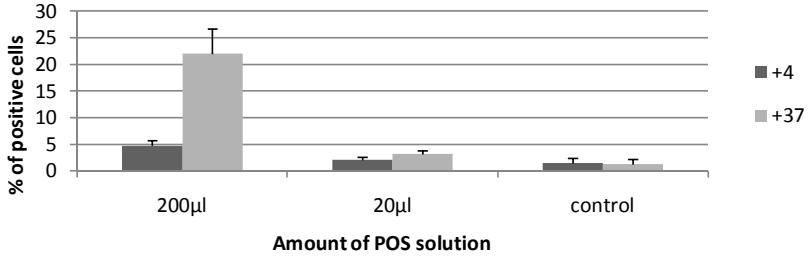

Figure 9: POS ingestion by ARPE-19 cells at +4 and +37 degrees. Cell confluency of 85-90% was reached prior to the experiments. Control: cell culture media. Results are expressed as mean ±sd (n=4).

5.2 Efflux proteins in RPE cell lines

5.2.1 *Expression at the messenger RNA level*

MRP1, MRP4 and MRP5 transcripts were expressed in all the RPE cell lines (ARPE-19, D407 and HRPEpiC) (**II Fig 1**, Fig. 10). MRP2 and BCRP transcripts were present only in D407 cell line (**II Fig 1**). MRP3 was expressed at a much higher level in D407 compared to ARPE-19 and HRPEpiC cell lines (**II Fig 1**). MDR1 mRNA was expressed in D407 and variably in hRPEpiC cells (**II Fig 1**). In ARPE-19 cells filter culturing enhanced MRP1, MRP4 and MRP5 expression, but the same efflux proteins were expressed in different culture culture conditions (**II Fig.2**, Fig 10).

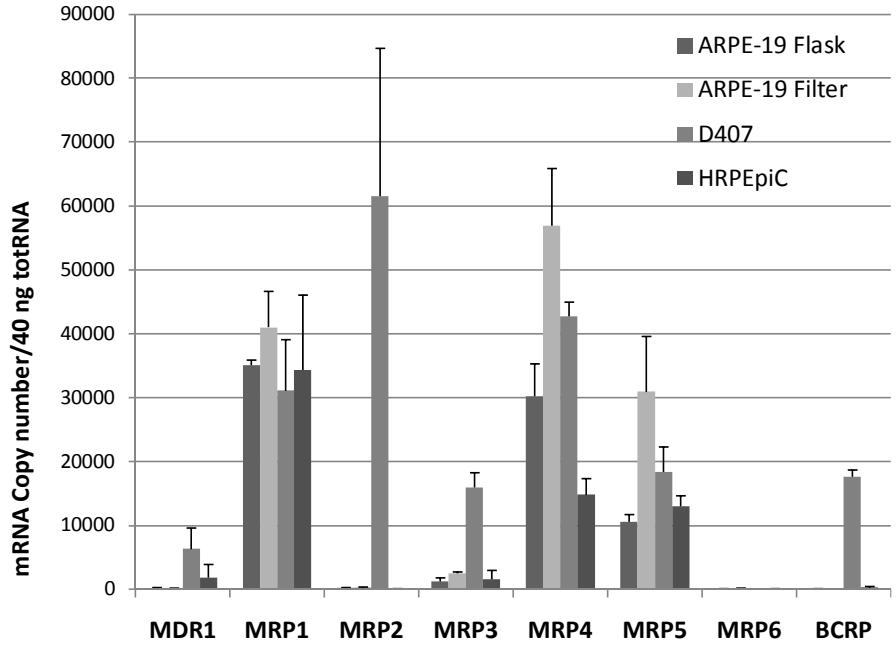

Figure 10. Transcript expression levels of efflux proteins in human RPE cell lines. Data are expressed as transcript copy number/40 ng cDNA from 2 to 3 independent cell cultures measured in triplicate. The data are expressed as the mean±SD.

5.2.2 *Expression at the protein level*

MRP1, MRP4 and MRP5 were expressed also at the protein level in the studied RPE cell lines (**II Fig. 3**). In addition, MRP1 and MRP4 were also detected in the bRPE (**II Fig. 3**). Again MRP2 and BCRP were present only in the D407 cell line (**II Fig.4**)

5.2.3 *Bi-directional permeability*

Similar directionality of transport was seen in ARPE-19 cells and isolated bovine RPE-choroid tissue (**I Table 2**). However, there were quantitative differences between ARPE-19 cells and isolated tissue. AB/BA (apical to basolateral permeability/basolateral to apical permeability) ratios of sodium fluorescein and rhodamine 123 were 1.5 and 1.7 in ARPE-19 cell model and 8.3 and 5.0 in the bovine RPE-choroid, respectively. The AB/BA ratio of digoxin was about the same (~1.2) in the cell model and in the RPE-choroid tissue. The directionality in carboxyfluorescein transport was seen in isolated bovine RPE-choroid tissue (AB/BA~2.4) but not in ARPE-19 monolayers (**I Table 2**).

5.2.3 *Calcein-AM assay*

In the calcein-AM assay, cellular fluorescence of ARPE-19 cells was increased over 100% with all tested inhibitors (cyclosporin A, progesterone, verapamil, MK571) (**II Fig. 5**). In the bRPE cells, progesterone, verapamil and MK571 resulted in >100% increase in the cellular fluorescence, whereas with cyclosporin A, the increase was around 40% (**II Fig. 5**).

The interaction of efflux proteins with two Hsp inhibitors, geldanamycin and radicicol, was evaluated using calcein-AM assay (**III**). Increased cellular fluorescence was detected when the cells were exposed to radicicol (either 1 μM or 5 μM) pointing to an interaction with the efflux proteins (**III Fig. 6**).

5.2.4 *CDCFDA/CDCF assay*

Probenecid inhibited CDCF efflux of ARPE-19 cells indicative of MRP5 activity (**II Fig. 6**). An effect of increasing concentration of probenecid was seen at 90 min, but not at 30 min.

5.3 Non-viral gene transfer

5.3.1 *Marker gene expression in ARPE-19 cell culture model*

Prolonged expression of SEAP for 69 days was seen when the filter grown ARPE-19 cells were transfected with DOTAP/DOPE/PS complexes (**IV Fig. 3 A and B**). In the experiments with DOTAP/DOPE complexes, SEAP expression lasted for 55 days. PEI, DOTAP and DHP-12 complexes resulted in the SEAP expression for 14 days or less. The highest expression peak was detected when DOTAP/DOPE/PS was used as a carrier (**IV Fig. 3 A and B**). The total amount of secreted SEAP was 49 and 143 µg/10^6 cells, with DOTAP/DOPE/PS and DOTAP/DOPE complexes, respectively, whereas other complexes yielded less than 5 µg/10^6 cells (**IV Table 1**). In addition, directionality was observed in the SEAP expression. With DOTAP/DOPE/PS and DOTAP/DOPE complexes, 1.5-fold higher secretion of SEAP was detected on the basolateral side, whereas with PEI/pDNA complexes 1.4 times greater secretion of SEAP was measured on the apical side (**IV Table 1**).

5.3.2 *Cellular uptake of non-viral complexes*

The highest cellular uptake was seen when polarized ARPE-19 cells were transfected with PEI/pDNA complexes (**IV Fig. 4**). Including PS in DOTAP/DOPE/complexes resulted in higher uptake of lipoplexes (**IV Fig. 4**)

6 DISCUSSION

6.1 ARPE-19 filter culture as a model for drug delivery studies

RPE is a polarized epithelium, which is a key feature in its role as drug delivery barrier. An optimal drug screening cell model for outer BRB should mimic the normal *in vivo* tissue, and it should be relatively easy to handle, readily available and suitable for large scale screening. The ARPE-19 cells line is commercially available, the cells can be easily expanded and it has retained many important features of the RPE cells. Importantly, when grown on a filter, it forms a polarized monolayer, which can be maintained for long periods, even for over a year. Very good barrier properties have been achieved with primary cells, but they are too laborious for screening purposes (Hu and Bok 2000, Maminishkis et al. 2006, Sonoda et al.2009). Thus, the ARPE-19 cell line is a candidate model for drug screening in outer BRB, but its drug permeation and transport properties have not been adequately characterized. In addition, it is currently widely used in retinal research.

The barrier properties of ARPE-19 cell filter culture model were studied by TER measurements and permeability studies (**I, II, IV**). Another commonly used method to study barrier properties is to study the expression of cell to cell junctions associated proteins by antibody labelling, with the most commonly used proteins being zonula occludens protein 1 (ZO-1) and occludin. Unfortunately it has been shown that these proteins can be present without the development of tight junctions (Luo et al. 2006). In addition, expression of ZO-1 and occluding has been previously demonstrated in ARPE-19 filter culture (Geisen et al. 2006, Nevala et al. 2008). Taking these facts into account, we chose to study the functionality of the cell to cell junctions by performing TER measurements and permeability studies.

The TER values of ARPE-19 cells on filter were generally between 80-100 $\Omega*cm^2$. These are clearly higher than most of reported TER values (typically between 30 and 40 $\Omega*cm^2$) of ARPE-19 cells cultured on filters without modification of culture medium or growth membrane (Dunn et al. 1996, Luo et al. 2006, Holtkamp et al. 1998, Geisen et al. 2006). Abe et al (2003) reported TER values similar to our values, about 80 $\Omega*cm^2$. The main difference between the previous studies and this present one was that we used filters with a larger growth area (4.7 cm^2) whereas in most studies, smaller filters (growth area 0.33 cm^2) have been used. It is also well known that cell lines can change with time and this may account for some of the observed lab to lab differences. In this study, ARPE-19 cells from two different sources were used and generally the cells behaved in a reproducible manner.

ARPE-19 cells grown on the filter in the standard growth medium formed a more permeable barrier to test compounds than isolated RPE-choroid tissue (**I**). However, the ARPE-19 cell model was able to detect the difference between the permeant molecules based on their size and lipophilicity (**I**). On the other hand, some established drug test cell models (like Caco2 and many brain endothelial cell models) deviate remarkably from their *in vivo* counterparts (small intestine, blood-brain barrier) (Artursson et al. 2001, Deli et al. 2005). Unfortunately the tested culture media supplements did not result in any better barrier properties without disturbing the morphological characteristics of the RPE cells.

The culture media composition can have substantial effects on the cell phenotype. When the culture medium was supplemented with bFGF or BRE, there was multilayering of ARPE-19 cells on the filters. The effect of these compounds were tested, since they have been shown to enhance the differentiation of RPE cells *in vitro* and have been frequently used with primary RPE cells (Hu and Bok 2001, Song and Lui 1990, Campochiaro and Hackett 1993). However, it is possible that different concentrations of bFGF and BRE could have influenced the results. The exact composition of BRE is unknown and its composition is likely to differ between extractions. Similarly, the composition of FBS can also differ between different manufacturers and batches. In general, cells cultured in media containing components extracted from other species are not ideal for human use.

The effect of culture conditions on RPE related gene expression was studied (**I**). All RPE related genes were expressed in ARPE-19 cells and all RPE related genes except Best1/VMD2 were up-regulated in filter cultures compared to the cells grown in flasks. To some extent this up-regulation can be explained as a consequence of the prolonged culture time. Our results are in agreement with Alizadeh et al. (2001) and Pratt et al. (2008) who reported Increasing expression of CRALBP and RPE65 transcripts, respectively, in prolonged culture of ARPE-19 cells. However, it is possible that also filter culturing itself may enhance the gene expression of RPE related genes. Interestingly, differences in gene expression levels were much smaller between different growth media compositions in ARPE-19 filter cultures (**I**). To conclude, our results suggest that prolonged culture on filters enhances RPE related gene expression.

Pigmentation is a landmark feature of RPE cells *in vivo*, and this feature also affects drug delivery. Ophthalmic drugs, particularly lipophilic compounds, may bind to melanin. For example, pilocarpine, timolol and atropine are known to bind to melanin (Atlasik et al. 1980, Nagata et al. 1993). Melanin binding of the drug may lead to its accumulation in the tissue and slow release from the bound depot, which prolongs the drug's action. On the other hand, melanin binding may decrease the access of the drug to its site of action and reduce the peak event. These phenomena have been shown in association

of pilocarpine binding to the pigment in iris and ciliary body (Urtti et al. 1984). In the present study, we detected tyrosinase, TRP1, MITF-A and OTX2, which are important proteins in melanization, from ARPE-19 cells at the mRNA level (**I**). However, we could not detect melanization in normal cell culture of ARPE-19 cells, not even with prolonged culture. The lack of pigmentation is an obvious limitation that affects the intracellular drug accumulation in some cases.

Phagocytosis is one of the most important features of RPE cells. In vivo malfunctioning of phagocytosis leads to visual impairment as seen in Royal College of Surgeons rats, who lack functional MerTK protein (D'Cruz et al. 2000, Nandrot et a. 2000). We have detected the MerTK transcript (data not shown) as well as TLR-4 transcript (Paimela et al. 2007), and αVβ5 integrin (data not shown), all important proteins related to POS phagocytosis, in flask cultured ARPE-19 cells. We also observed that ARPE-19 cells phagocytozed labeled POS in a temperature dependent manner (Fig.9), confirming the previous studies that ARPE-19 cells have retained this important task of the RPE cells *in vivo* (Finnemann et al. 1997).

It is known that cell division affects the access of DNA into the nucleus, and therefore the filter grown ARPE-19 model is probably more relevant than dividing ARPE-19 cells in gene delivery experiments (Ludtke et al. 2002). The filter culture model with secreted marker gene, SEAP, allowed generation of an expression vs time curve. PEI-DNA complexes have been previously shown to be efficient *in vitro* in transfecting dividing cells (Boussif et al. 1995, Toropainen et al. 2007). In this study, lipoplexes were shown to be more efficient than PEI-polyplexes in ARPE-19 filter culture system (**IV**). PEI-polyplexes resulted in short-term SEAP secretion to the medium whereas SEAP expression continued for 2 months after a single DNA lipoplex administration (**IV**). Likewise, Toropainen et al. (2007) showed that the filter grown corneal epithelial cells predict *in vivo* gene transfer much more reliably than dividing corneal epithelial cells. The mechanisms of DNA complex internalization and intracellular trafficing of plasmid DNA in ARPE-19 cells are not fully understood.

6.2 Membrane transporters in retinal pigment epithelium

ABC efflux transporters. Efflux proteins have broad substrate specificity and they affect pharmacokinetics of a large number of drugs (Cascorbi 2006, Dobson and Kell 2008, Maeda and Sugiyama 2008, Mizuno et al. 2003). Therefore, expression of these proteins is a potentially important factor in the RPE related drug delivery. The substrate and inhibitor specificities of ABC efflux proteins overlap, complicating interpretation of the results. In most cases, the functional studies with probe

molecules can only suggest a set of possible transporters. For example, rhodamine 123, a commonly used substrate to study p-gp function, is also a substrate of MRP1 and BCRP (Doyle et al. 1998, Minderman et al. 1996, Twentyman et al. 1994, Zaman et al. 1994).

In this study, efflux protein expression was compared in various RPE cell lines. Previously it was reported by Constable et al (2006) that p-gp was expressed and active only in D407 cell line, whereas ARPE-19 cells lacked the p-gp expression and activity. The present qPCR results are in line with this observation, no p-gp expression was found in ARPE-19, but was consistently found in D407 cells and variably in hRPEpiC cells batches (**I**). In general, there is no consensus in the literature about p-gp expression in the RPE (Esser et al. 1998, Kennedy and Mangini 2002, Steuer et al. 2005). The conflicting results may be explained by different sensitivities of detection methods and also by inducibility of p-gp expression and polymorphism. It is clear that P-gp expression and activity in RPE require further clarification.

MRP1 has been detected in RPE in several studies (Steuer et al. 2005, Aukunuru et al. 2001, Pelis et al. 2009, Zhang et al. 2008). In this study, MRP1 was detected in all studied cells lines and in normal bovine RPE cells (**II**). This is the first report of MRP1 expression in bovine RPE. Several studies have suggested that MRP1 transport takes place from the neural retina to the choroid direction. Active transport of fluorescein from retina to choroid has been known for decades, but the transporter responsible is not known (Barza et al. 1983, Cunha-Vaz and Maurice 1967), though it is likely that MRP1 plays a major role. Calcein-AM studies and bi-directional studies confirmed the activity of MRP1 in ARPE-19 cells (**I**, **II**). Inhibition of efflux activity was detected with the Hsp inhibitor radicicol (**III**). Since neither p-gp nor MRP2 are expressed in ARPE-19 cells, these results indicate that radicicol is interacting with MRP1.

MRP4 and MRP5 were expressed in ARPE-19, D407 and HRPEpiC cells at the mRNA and protein levels (**II**). MRP4 protein was also detected from bovine RPE, but MRP5 protein was not found (**II**). This may be due to an inability of the MRP5 primary antibody to bind to bovine tissue. This was the first study to report MRP4 expression in the RPE. A finding was confirmed recently i.e. MRP4 expression was detected by western blot in the human eye (Pelis et al. 2009). Up-regulated expression of the MRP4 and MRP5 proteins was detected in ARPE-19 filter culture, whereas the level of MRP1 expression was quite constant under different culture conditions (**II Fig 2**). In general, it is possible that cell culture conditions alter the expression of some efflux proteins.

Two papers have been published during 2008-2009 on the expression of efflux transporters in the human eye (Pelis et al. 2009, Zhang et al. 2008). Zhang et al. studied mRNA expression of various

transporters by quantitative RT-PCR and Pelis et al. used western blot to detect protein expression. Zhang et al. had inner and outer BRB in one specimen (retina/choroid) whereas Pelis et al. studied retina (including inner BRB) and RPE (outer BRB) separately. MRP1 mRNA was highly expressed in retina/choroid and MRP1 protein was clearly expressed in RPE. MRP2 was also detected in both studies. MRP3 mRNA was expressed at a low level (not studied at the protein level) whereas MRP4 protein was expressed in the RPE (not studied at the mRNA level). MRP5 and MRP6 were not studied in either study. A faint band was seen in western blot in RPE with P-gp antibody and mdr1 mRNA was expressed at a low level. BCRP was detected at both the RNA and protein levels. These results suggest that also MRP2 and BCRP are expressed in the human RPE. In the present studies, these efflux proteins were detected only in D407 cells (**II Figs. 1** and **4**). Interestingly, these efflux proteins were not detectable from bovine RPE, but one cannot be sure if the primary antibodies are able to detect the bovine efflux protein.

Similar efflux protein expression has been found in other barrier membranes. The expression of P-gp, MRP1, MRP5 and BCRP has been described both in BBB and placenta (Nies et al. 2004, Zhang et al. 2004, Young et al. 2003). MRP4 have been also detected in the BBB and with respect to MRP2 and MRP3 expression, the results are controversial (Berezowski et al. 2004, Nies et al. 2004, Zhang et al. 2004, Young et al. 2003). MRP2 has been found in placenta, whereas expression of MRP3 and MRP4 are uncertain (Pascolo et al. 2003, St-Pierre et al. 2000).

MRP4 and MRP5 may affect the distribution of nucleoside analogue drugs by effluxing the intracellularly formed respective nucleoside monophosphate metabolites from the cells. Some drugs used to treat vitreal and retinal diseases have been shown to interact with MRP4 or MRP5, these include zidovudine, ganciclovir and 5'-fluorouracil (Adachi et al, 2002, Jorajuria et al. 2004, Pratt et al. 2005). Furthermore, it has been shown that vincristine, etoposide and teniposide, which are used to treat retinoblastoma, interact with MRP4 (vincristine) and MRP5 (etoposide and teniposide), however the resistance factor attributable to the presence of the transporter is low (Wijnholds et al. 2000, Norris et al. 2005). In addition MRP4 has an ability to transport prostaglandins and it participates in prostaglandin homeostasis in the BBB (Kis et al. 2006). Active efflux transport for prostaglandins is known to be present also in the eye, where prostaglandins perform several important functions (Bito 1973). Synthetic prostaglandin analogues are used as therapeutics in glaucoma. The clinical significance of MRP4 and MRP5 in drug resistance is unknown.

In summary this study in conjunction with previous reports provides consistent evidence that MRP1, MRP4 and MRP5 are expressed in RPE, but MRP6 is not expressed, and MRP3 is expressed at only a low

level. Details of p-gp expression are unclear. Recent studies have suggested that also MRP2 and BCRP can be expressed in the outer BRB, but these preliminary findings need to be confirmed.

Drug delivery related influx transporters. In the literature review (**V**) 19 SLC membrane transporters that may be involved in drug influx were detected in RPE. Subsequently 7 possible new drug related transporters have been found in RPE. Twenty of these 26 membrane transporters belonging to 10 different SLC subfamilies have been detected in ARPE-19 cell line (Table 8). Some of these transporters have polarized location in confluent or polarized ARPE-19 cell cultures (Table 8). The uptake of adenosine, brimodine, cystine, folate, glutamate, lactate, L-phenylalanine, methylphenyl pyridinium, nicotinate, verapamil, taurine and thiamine by SLC transporters has been observed in ARPE-19 cells (for references see table 8). Interestingly, also several zinc transporters have been found in ARPE-19 cells and active zinc uptake was recently observed (Leung et al. 2008, Rezaei et al. 2008). Zinc transporters may not be relevant from the drug delivery point of view, but may have pathophysiological significance.

Table 8. Drug delivery related influx transporters of ARPE-19 cell line

TRANSPORTER FAMILY/MEMBER	EXPRESSION	FUNCTION	REF.
SLC1 Glutamate/neural amino acid transporter			
SLC1A1/EAAC1/EAAT3	Prot.		Maenpaa et al. 2004
SLC1A5/ASC	RNA		Bridges et al. 2007
SLC1A6/EAAT4	Prot		Maenpaa et al. 2004
SLC5 Sodium glucose cotransporter			
SLC5A6/SMVT	RNA	Uptake of biotin	Janoria et al. 2009
SLC5A8/SMCT1	Prot. basolaterally[1]	Uptake of (^{14}C)nicotinate	Martin et al. 2007
SLC6/Sodium and chloride dependent neurotransmitter			
SLC6A6/TAUT	Prot.	Uptake of taurine	El-Sherbeny et al.2004, Bridges et al. 2001b
SLC6A14/ATB$^{0,+}$	RNA		Bridges et al. 2007
SLC7/Cationic amino acid transporter, y+ system			

SLC7A5/LAT1	Prot.	Uptake of L-leucine	Yamamoto et al. 2009
SLC7A8/LAT2	RNA	Uptake of L-phenylalanine	Gandhi et al. 2004
SLC7A11/xCT system Xc-	RNA	Uptake of cystine and glutamate	Bridges et al. 2001a, Gnanaprakasam et al. 2009
SLC15/Proton oligopeptide cotransporter			
SLC15A4/PHT1	RNA	Activity not found	Ocheltree et al. 2003
SLC16/Monocarboxylate transporter			
SLC16A1/MCT1	Prot apically[2]	Uptake of lactate	Majumdar et al. 2005;Philp et al. 2003
SLC16A3/MCT4	Prot. basolaterally[2]		Majumdar et al. 2005;Philp et al. 2003
SLC16A8/MCT3	Prot. basolaterally[3]		Turowski et al. 2004
SLC19/Folate/thiamine transporter			
SLC19A1/RFT-1	Prot. apically	Uptake and transport of folate[1,4]	Bridges et al. 2002;Chancy et al. 2000;Naggar et al. 2005
SLC19A2/hTHRT-1	Prot.		
SLC19A3/hTHRT-2	Prot.	Uptake of thiamine	Subramanian et al. 2007
SLC22/Organic cation/anion/zwitterion transporter			
SLC22A3/OCT3	RNA	Uptake of 1-methyl-4-phenyl pyridinium	Rajan et al. 2000
Unknown organic cation transpoter		Uptake of verapamil	Han et al. 2001
Unknown organic cation transpoter		Uptake of brimodine	Zhang et al. 2006
SLC28 and SLC29/Nucleoside transporters	not studied	Uptake of adenosine	Majumdar et al.,2004
SLC46/Heme transporter			
SLC46A1/PCFT	RNA		Umapathy et al. 2007

[1]Filter cultured by method by Dunn et al (2006) [2] cultured for 120 days on 100-mm dishes [3]Cultured on porcine lens capsule [4]from basal to apical direction

ARPE-19 cells may be a useful model when one is interested in investigating active transport. However, the *in vitro*/*in vivo* correlation in many cases is not known. No, direct conclusions regarding *in vivo* RPE transporters cannot be drawn based on a cell model. While *in vivo* data (expression and functionality)

represents definitive proof, it is usually lacking and very difficult to obtain. The polarized expression of transporters and directionality in transport have been shown in the ARPE-19 cell model in some cases. However due to the higher permeability compared to isolated RPE-choroid, it is possible that active transport, even when present, may not be always detected in transport studies. Since ARPE-19 cell line is widely used in retinal research, it is important to know which transporters are expressed and present in this cell model.

6.3 Future directions

There are several open questions related to the drug delivery to the posterior eye segment tissues e.g. the effect of aging. Lipid rich deposits (drusens) are found between RPE and Bruch's membrane normally in the elderly and they are also associated with AMD. In addition, RPE atrophy is a main feature of AMD. These changes may affect the transport through BRB. Furthermore, BRB may be compromised after photocoagulation, due to development of choroidal neovascular membranes and may become leaky in an infected eye. The disease state and aging related changes in the RPE may have an influence on ocular pharmacokinetics after intravitreal, transscleral and systemic drug delivery. Therefore further studies on pathophysiology, pharmacology and drug delivery are needed. Human derived cell culture models are important tools especially in the case of the eye since there are such limited possibilities to use the human tissue.

Systematic characterization of the cells in different culture conditions is a prerequisite before the cell model can be established as a drug testing tool. Systems free from biological material from other species like serum free culture media and synthetic growth membranes are more easily controlled than biological material and may avoid problems related to cross species reactions. It may be possible to modify the cell properties also by applying gene transfer technology.

Recently, it has become clear that the intravitreal injections are feasible in clinical practice, but in most cases, frequent injections are needed. Prolonged action drug delivery methods would decrease the number of needed injections. In this study, gene transfer resulted in prolonged protein secretion from the ARPE-19 cells suggesting that gene transfer may be a viable approach for achieving prolongation of the effects of the therapeutic proteins, but this approach requires resolution of other problems inherent to intravitreal gene delivery e.g. DNA complex diffusion in the vitreous and neural retina. Other

possibilities for effect prolongation include implants and the use of particulate systems with slow releasing properties.

There are several ophthalmic drugs that may interact with transporters. The clinical significance of transporter proteins remains to be clarified. Similarly, the roles of transporter polymorphisms and the potential of transporter mediated drug targeting demand further exploration. The well-known species differences in transporter proteins emphasis the need to utilize human derived material.

7 CONCLUSIONS

In this study, filter cultured ARPE-19 cells were characterized as a cell model of outer BRB for use in drug delivery studies.

The specific conclusions of the study are:

1. Filter cultured ARPE-19 model formed a tight monolayer, but passive permeability of the cell model was higher than in the isolated bovine RPE-choroid. The cell model was able to classify the characteristics of several compound based on their lipophilicity and molecular size.
2. The barrier properties of the ARPE-19 cell culture model were not enhanced by growth medium supplementations.
3. RPE related gene expression was up-regulated in filter culture. In filter cultures, the composition of the cell culture medium had only minor effects on RPE related gene expression.
4. The directionality in active transport was quantively, but not quantitatively similar in the ARPE-19 cell model and in isolated bovine RPE/choroid tissue. The higher passive permeability of cell model may lead to relative underestimation of active transport.
5. MRP1, MRP4 and MRP5 efflux proteins were expressed in the ARPE-19 filter culture model. The expression pattern was shared with primary RPE cells. In contrast, D407 cells were found to express also MRP2 and BCRP proteins. The results indicate MRP1 and MRP5 efflux protein activity in the ARPE-19 cells.
6. The calcein-AM assay suggested that Hsp the inhibitor, radicicol, interacted with the MRP1 efflux protein.
7. Prolonged gene expression in the filter grown ARPE-19 cells was achieved with lipoplexes. The filter grown cell model, with non-dividing cells displaying the properties of differentiated RPE cells, resembles *in vivo* RPE.
8. According to the literature, membrane transporters belonging to 11 transporter families are found in the ARPE-19 cell model and some of them are known to transport ophthalmic drugs. Many ophthalmic drugs are known to be transporter substrates, but in very few cases has the expression of these transporters been studied in RPE.

8 REFERENCES

AbeT, Sugano E, Saigo Y, Tamai M: Interleukin-1beta and barrier function of retinal pigment epithelial cells (ARPE-19): aberrant expression of junctional complex molecules. Invest Ophthalmol Vis Sci 2003;44:4097-4104

Adachi M, Sampath J, Lan LB, Sun D, Hargrove P, Flatley R, Tatum A, Edwards MZ, Wezeman M, Matherly L, Drake R, Schuetz J: Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. J Biol Chem 2002;277:38998-39004

Alge CS, Hauck SM, Priglinger SG, Kampik A, Ueffing M: Differential protein profiling of primary versus immortalized human RPE cells identifies expression patterns associated with cytoskeletal remodeling and cell survival. J Proteome Res 2006;5:862-878

Alizadeh M, Wada M, Gelfman CM, Handa JT, Hjelmeland LM: Downregulation of differentiation specific gene expression by oxidative stress in ARPE-19 cells. Invest Ophthalmol Vis Sci 2001;42:2706-2713

Amaral J, Fariss RN, Campos MM, Robison WG, Kim H, Lutz R, Becerra SP: Transscleral-RPE permeability of PEDF and ovalbumin proteins: implications for subconjunctival protein delivery. Invest Ophthalmol Vis Sci 2005;46:4383-4392

Ambati J, Canakis CS, Miller JW, Gragoudas ES, Edwards A, Weissgold DJ, Kim I, Delori FC, Adamis AP: Diffusion of high molecular weight compounds through sclera. Invest Ophthalmol Vis Sci 2000a;41:1181-1185

Ambati J, Gragoudas ES, Miller JW, You TT, Miyamoto K,,, Delori FC Adamis AP: Transscleral delivery of bioactive protein to the choroid and retina. Invest Ophthalmol Vis Sci 2000b;41:1186-1191

Amrite AC, Edelhauser HF, Singh SR, Kompella UB: Effect of circulation on the disposition and ocular tissue distribution of 20 nm nanoparticles after periocular administration. Mol Vis 2008;14:150-160

Araie M, Maurice D: The rate of diffusion of fluorophores through the corneal epithelium and stroma. Exp Eye Res 1987;44:73-87

Arndt C, Sari A, Ferre M, Parrat E, Courtas D, De Seze J, Hache J, Matran R: Electrophysiological effects of corticosteroids on the retinal pigment epithelium. Invest Ophthalmol Vis Sci 2001;42:472-475

Arrindell EL, McKay BS, Jaffe GJ, Burke JM: Modulation of potassium transport in cultured retinal pigment epithelium and retinal glial cells by serum and epidermal growth factor. Exp Cell Res 1992;203:192-197

Arshavsky VY, Lamb TD, Pugh EN: G proteins and phototransduction. Annu Rev Physiol 2002;64:153-187

Artursson P, Palm K, Luthman K: Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev 2001;46:27-43

Atlasik B, Stepien K, Wilczok T: Interaction of drugs with ocular melanin in vitro. Exp Eye Res 1980;30:325-331

Aukunuru JV, Sunkara G, Bandi N, Thoreson WB, Kompella UB: Expression of multidrug resistance-associated protein (MRP) in human retinal pigment epithelial cells and its interaction with BAPSG a novel aldose reductase inhibitor. Pharm Res 2001;18:565-572

Aydin A, Wollstein G, Price LL, Schuman JS: Evaluating pulsatile ocular blood flow analysis in normal and treated glaucomatous eyes. Am J Ophthalmol 2003;136:448-453

Bakos E, Evers R, Szakacs G, Tusnady GE, Welker E, Szabo K, de Haas M, van Deemter L, Borst P, Varadi A, Sarkadi B: Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain. J Biol Chem 1998;273:32167-32175

Barza M, Kane A, Baum J: Pharmacokinetics of intravitreal carbenicillin cefazolin and gentamicin in rhesus monkeys. Invest Ophthalmol Vis Sci 1983;24:1602-1606

Beatty S, Koh H, Phil M, Henson D, Boulton M: The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 2000;45:115-134

Becerra SP, Fariss RN, Wu YQ, Montuenga LM, Wong P, Pfeffer BA: Pigment epithelium-derived factor in the monkey retinal pigment epithelium and interphotoreceptor matrix: apical secretion and distribution. Exp Eye Res 2004;78:223-234

Bejjani RA, BenEzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, Gollomb G, Behar-Cohen FF: Nanoparticles for gene delivery to retinal pigment epithelial cells. Mol Vis 2005;11:124-132

Berezowski V, Landry C, Dehouck MP, Cecchelli R, Fenart L: Contribution of glial cells and pericytes to the mRNA profiles of P-glycoprotein and multidrug resistance-associated proteins in an in vitro model of the blood-brain barrier. Brain Res 2004;1018:1-9

Bergen AA, Plomp AS ,Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J,, Kool M van Soest S, Baas, ten Brink JB, de Jong PT: Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet 2000;25:228-231

Bill A, Tornquist P, Alm A: Permeability of the intraocular blood vessels. Trans Ophthalmol Soc U K 1980;100:332-336

Bill A: Blood circulation and fluid dynamics in the eye. Physiol Rev 1975;55:383-417

Bill A: Capillary permeability to and extravascular dynamics of myoglobin albumin and gammaglobulin in the uvea. Acta Physiol Scand 1968;73:204-219

Bishop PN: Structural macromolecules and supramolecular organisation of the vitreous gel. Prog Retin Eye Res 2000;19:323-344

Bito LZ: Absorptive transport of prostaglandins from intraocular fluids to blood: a review of recent findings. Exp Eye Res 1973;16:299-306

Blaauwgeers HG, Holtkamp GM, Rutten H, Witmer AN, Koolwijk P, Partanen TA, Alitalo K, Kroon ME, Kijlstra A, van Hinsbergh VW, Schlingemann RO: Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris Evidence for a trophic paracrine relation. Am J Pathol 1999;155:421-428

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279:349-352

Bonilha VL ,Finnemann SC, Rodriguez-Boulan E : Ezrin promotes morphogenesis of apical microvilli and basal infoldings in retinal pigment epithelium. J Cell Biol 1999;147:1533-1548

Bourges JL, Gautier SE, Delie F, Bejjani RA, Jeanny JC, Gurny R, BenEzra D, Behar-Cohen FF: Ocular drug delivery targeting the retina and retinal pigment epithelium using polylactide nanoparticles. Invest Ophthalmol Vis Sci 2003;44:3562-3569

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP: A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA 1995;92:7297-7301

Bridges CC, Battle JR, Zalups RK: Transport of thiol-conjugates of inorganic mercury in human retinal pigment epithelial cells. Toxicol Appl Pharmacol 2007;221:251-260

Bridges CC, El-Sherbeny A, Ola MS, Ganapathy V, Smith SB: Transcellular transfer of folate across the retinal pigment epithelium. Curr Eye Res 2002;:4 129-138

Bridges CC, Kekuda R, Wang H, Prasad PD, Mehta P, Huang W, Smith SB, Ganapathy V: Structure function and regulation of human cystine/glutamate transporter in retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2001a;42:47-54

Bridges CC, Ola MS, Prasad PD, El-Sherbeny A, Ganapathy V, Smith SB: Regulation of taurine transporter expression by NO in cultured human retinal pigment epithelial cells. Am J Physiol Cell Physiol 2001b;281: C1825-36

Bustin SA: Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29:23-39

Cai H, Del Priore LV: Bruch membrane aging alters the gene expression profile of human retinal pigment epithelium. Curr Eye Res 2006;31:181-189

Campochiaro PA, Hackett SF: Corneal endothelial cell matrix promotes expression of differentiated features of retinal pigmented epithelial cells: implication of laminin and basic fibroblast growth factor as active components. Exp Eye Res 1993;57:539-547

Campochiaro PA, Hackett SF, Conway BP. Retinoic acid promotes density-dependent growth arrest in human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 1991;32:65-72

Capeans C, Pineiro A, Pardo M, Sueiro-Lopez C, Blanco MJ, Dominguez F, Sanchez-Salorio M: Amniotic membrane as support for human retinal pigment epithelium (RPE) cell growth. Acta Ophthalmol Scand 2003;81:271-277

Cascorbi I: Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. Pharmacol Ther 2006;112:457-473

Castellarin AA, Sugino IK, Vargas JA, Parolini B, Lui GM, Zarbin MA: In vitro transplantation of fetal human retinal pigment epithelial cells onto human cadaver Bruch's membrane. Exp Eye Res 1998;66:49-67

Chancy CD, Kekuda R, Huang W, Prasad PD, Kuhnel JM, Sirotnak FM, Roon P, Ganapathy V, Smith SB: Expression and differential polarization of the reduced-folate transporter-1 and the folate receptor alpha in mammalian retinal pigment epithelium. J Biol Chem 2000;275:20676-20684

Chang CW, Ye L, Defoe DM, Caldwell RB: Serum inhibits tight junction formation in cultured pigment epithelial cells. Invest Ophthalmol Vis Sci 1997;38:1082-1093

Cheruvu NP, Amrite AC, Kompella UB: Effect of eye pigmentation on transscleral drug delivery. Invest Ophthalmol Vis Sci 2008;49:333-341

Cheruvu NP, Kompella UB: Bovine and porcine transscleral solute transport: influence of lipophilicity and the Choroid-Bruch's layer. Invest Ophthalmol Vis Sci 2006;47:4513-4522

Chin HS, Park TS, Moon YS, Oh JH: Difference in clearance of intravitreal triamcinolone acetonide between vitrectomized and nonvitrectomized eyes. Retina 2005;25:556-560

Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG: Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells Cancer Res. 1994;54:5902-5910

Constable PA, Lawrenson JG, Dolman DE, Arden GB ,Abbott NJ: P-Glycoprotein expression in human retinal pigment epithelium cell lines. Exp Eye Res 2006;83:24-30

Cuisset L, Tichonicky L, Jaffray P, Delpech M: The effects of sodium butyrate on transcription are mediated through activation of a protein phosphatase. J Biol Chem 1997;272:24148-24153

Cunha-Vaz JG: The blood-retinal barriers system: Basic concepts and clinical evaluation. Exp Eye Res 2004;78:715-721

Cunha-Vaz JG, Maurice DM: The active transport of fluorescein by the retinal vessels and the retina. J Physiol 1967; 191:467-486

Davis AA, Bernstein PS, Bok D, Turner J, Nachtigal M, Hunt RC: A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture. Invest Ophthalmol Vis Sci 1995;36:955-964

D'Cruz PM, Yasumuru D, Weir J, Matthes MT, Abderrahim H, LaVail MM, Vollrath D: Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rats. Hum Mol Genet 2000; 9:645-651

Deeley RG, Westlake C, Cole SP: Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006;86:849-899

Del Amo EM, Urtti A: Current and future ophthalmic drug delivery systems A shift to the posterior segment. Drug Discov Today 2008;13:135-143

Del Priore LV, Tezel TH: Reattachment rate of human retinal pigment epithelium to layers of human Bruch's membrane. Arch Ophthalmol 1998;116:335-341

Deli MA, Abraham CS, Kataoka Y, Niwa M: Permeability studies on in vitro blood-brain barrier models: physiology pathology and pharmacology. Cell Mol Neurobiol 2005;25:59-127

Ding X, Patel M, Chan CC: Molecular pathology of age-related macular degeneration. Prog Retin Eye Res 2009;28:1-18

Dobson PD, Kell DB: Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat Rev Drug Discov 2008;7:205-220

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD: A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A 1998;95:15665-15670

Dunn KC, Marmorstein AD, Bonilha VL, Rodriguez-Boulan E, Giordano F, Hjelmeland LM: Use of the ARPE-19 cell line as a model of RPE polarity: basolateral secretion of FGF5. Invest Ophthalmol Vis Sci 1998;39:2744-2749

Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM: ARPE-19 a human retinal pigment epithelial cell line with differentiated properties. Exp Eye Res 1996;62:155-169

Edelman JL, Miller SS: Epinephrine stimulates fluid absorption across bovine retinal pigment epithelium. Invest Ophthalmol Vis Sci 1991;32:3033-3040

El-Sherbeny A, Naggar H, Miyauchi S, Ola MS, Maddox DM, Martin PM, Ganapathy V, Smith SB: Osmoregulation of taurine transporter function and expression in retinal pigment epithelial ganglion and muller cells. Invest Ophthalmol Vis Sci 2004;45:694-701

Eneroth A, Astrom E, Hoogstraate J, Schrenk D, Conrad S, Kauffmann HM, Gjellan K: Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. Eur J Pharm Sci 2001;12:205-214

Engelmann K , Valtink M: RPE cell cultivation. Graefes Arch Clin Exp Ophthalmol 2004;242:65-67

Esser P, Tervooren D, Heimann K, Kociok N, Bartz-Schmidt KU, Walter P, Weller M: Intravitreal daunomycin induces multidrug resistance in proliferative vitreoretinopathy. Invest Ophthalmol Vis Sci 1998;39:164-170

Essodaigui M, Broxterman HJ, Garnier-Suillerot A: Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and P-glycoprotein. Biochemistry 1998;37:2243-2250

Evers R, Kool M, Smith AJ, van Deemter L, de Haas M, Borst P: Inhibitory effect of the reversal agents V-104 GF120918 and Pluronic L61 on MDR1 Pgp- MRP1- and MRP2-mediated transport. Br J Cancer 2000;83:366-374

Evers R, Kool M, van Deemter L, Janssen H, Calafat J, Oomen LC, Paulusma CC, Oude Elferink RP, Baas F, Schinkel AH, Borst P: Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. J Clin Invest 1998;101:1310-1319

Feng W, Yasumura D, Matthes MT, LaVail MM, Vollrath D: Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J Biol Chem 2002;277:17016-17022

Finnemann SC, Silverstein RL: Differential roles of CD36 and alphavbeta5 integrin in photoreceptor phagocytosis by the retinal pigment epithelium. J Exp Med 2001;194:1289-1298

Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E : Phagocytosis of rod outer segments by retinal pigment epithelial cells requires alpha(v)beta5 integrin for binding but not for internalization. Proc Natl Acad Sci U S A 1997;94:12932-12937

Frambach DA, Fain GL, Farber DB, Bok D: Beta adrenergic receptors on cultured human retinal pigment epithelium. Invest Ophthalmol Vis Sci 1990;31:1767-1772

Frambach DA, Valentine JL, Weiter JJ: Initial observations of rabbit retinal pigment epithelium-choroid-sclera preparations. Invest Ophthalmol Vis Sci 1988;29:814-817

Fredriksson R, Nordstrom KJ, Stephansson O, Hagglund MG, Schioth HB: The solute carrier (SLC) complement of the human genome: phylogenetic classification reveals four major families. FEBS Lett 2008;582:3811-3816

Gandhi MD, Pal D, Mitra AK: Identification and functional characterization of a Na(+)-independent large neutral amino acid transporter (LAT2) on ARPE-19 cells. Int J Pharm 2004;275:189-200

Geisen P, McColm JR, King BM, Hartnett ME: Characterization of barrier properties and inducible VEGF expression of several types of retinal pigment epithelium in medium-term culture. Curr Eye Res 2006;31:739-748

Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J: The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem Biophys Res Commun 1995;208:345-352

Geroski DH, Edelhauser HF: 1 Transscleral drug delivery for posterior segment disease. Adv Drug Deliv Rev 200;52:37-48

Glotin AL, Debacq.Chainiaux F, Brossas JY, Faussat AM, Treton J, Zubielewicz A, Touissaint O, Mascarelli F: Prematurelly senescent ARPE-19 cells display features of age-related macular degeneration. free Radic Biol Med 2008;44:1348-1361

Gnanaprakasam JP, Thangaraju M, Liu K, Ha Y, Martin PM, Smith SB, Ganapathy V: Absence of iron-regulatory protein Hfe results in hyperproliferation of retinal pigment epithelium: role of cystine/glutamate exchanger Biochem J 2009;69:2826-2832

Goes RM, Laicine EM, Porcionatto MA, Bonciani Nader H, Haddad A: Glycosaminoglycans in components of the rabbit eye: synthesis and characterization. Curr Eye Res 1999;19:146-153

Gundersen D, Orlowski J, Rodriguez-Boulan E: Apical polarity of NaK-ATPase in retinal pigment epithelium is linked to a reversal of the ankyrin-fodrin submembrane cytoskeleton. J Cell Biol 1991;12: 863-872

Guymer RH, Bird AC,: Hageman GS: Cytoarchitecture of choroidal capillary endothelial cells. Invest Ophthalmol Vis Sci 2004;45: 1660-1666

Hackett S, Friedman Z, Campochiaro PA: Cyclic 3'5'-adenosine monophosphate modulates retinal pigment epithelial cell migration in vitro. Arch Ophthalmol 1986;104:1688-1692

Haimeur A, Conseil G, Deeley RG, Cole SP: The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Met 2004;5:21-53

Hall MO, Abrams TA, Burgess BL: Integrin alphavbeta5 is not required for the phagocytosis of photoreceptor outer segments by cultured retinal pigment epithelial cells. Exp Eye Res 2003;77:281-286

Hamilton RD, Foss A, Leach L: Establishment of a human in vitro model of the outer blood-retinal barrier. J Anat 2007;211:707-716

Han YH, Sweet DH, Hu DN, Pritchard JB: Characterization of a novel cationic drug transporter in human retinal pigment epithelial cells. J Pharmacol Exp Ther 2001;296:450-457

Haritoglou C, Priglinger S, Liegl R, May CA, Eibl K, Thaler S, Kampik A, Schuettauf F: Experimental evaluation of aniline and methyl blue for intraocular surgery. Retina 2009;29:1266-1273

Hartmann U, Sistani F, Steinhorst UH: Human and porcine anterior lens capsule as support for growing and grafting retinal pigment epithelium and iris pigment epithelium. Graefes Arch Clin Exp Ophthalmol 1999;237:940-945

Hediger MA, Romero MF, Peng JB, Rolfs A, Takanaga H, Bruford EA: The ABCs of solute carriers: physiological pathological and therapeutic implications of human membrane transport proteins. Introduction. Pflugers Arch 2004; 447:465-468

Heimsath EG, Unda R, Vidro E, Muniz A, Villazana-Espinoza ET, Tsin A: ARPE-19 cell growth and cell functions in euglycemic culture media. Curr Eye Res 2006;31:1073-1080

Hernandez EV, Hu JG, Frambach DA, Gallemore RP: Potassium conductances in cultured bovine and human retinal pigment epithelium. Invest Ophthalmol Vis Sci 1995;36:113-122

Herrero-Vanrell R, Refojo MF: Biodegradable microspheres for vitreoretinal drug delivery Adv Drug Deliv Rev 2001;52:5-16

Hesselink DA, van Hest RM, Mathot RA, Bonthuis F, Weimar W, de Bruin RW, van Gelder T: Cyclosporine interacts with mycophenolic acid by inhibiting the multidrug resistance-associated protein 2 Am J Transplant 2005;5:987-994

Holtkamp GM, Van Rossem M, de Vos AF, Willekens B, Peek R, Kijlstra A: Polarized secretion of IL-6 and IL-8 by human retinal pigment epithelial cells. Clin Exp Immunol 1998;12:34-43

Hornof M, Toropainen E, Urtti A: Cell culture models of the ocular barriers Eur J Pharm Biopharm 2005;60:207-225

Hu J, Bok D: A cell culture medium that supports the differentiation of human retinal pigment epithelium into functionally polarized monolayers. Mol Vis 2001;7:14-19

Huhtala A, Rönkkö S, Teräsvirta M, Puustjärvi T, Sihvola R, Vehanen K, Laukkanen A, Anttila J, Urtti A, Pohjonen T, Uusitalo H: The effects of 5-fluorouracil on ocular tissues in vitro and in vivo after controlled release from a multifunctional implant. Invest Ophthalmol Vis Sci 2009;50:2216-2223

Hyvonen Z, Plotniece A, Reine I, Chekavichus B, Duburs G, Urtti A: Novel cationic amphiphilic 14-dihydropyridine derivatives for DNA delivery. Biochim Biophys Acta 2000;1509:451-466

Inatani M, Tanihara H: Proteoglycans in retina Prog Retin Eye Res 2002;21:429-447

Insel PA, Ostrom RS: Forskolin as a tool for examining adenylyl cyclase expression regulation and G protein signaling. Cell Mol Neurobiol 2003;2:305-314

Jackson TL, Antcliff RJ, Hillenkamp J, Marshall J: Human retinal molecular weight exclusion limit and estimate of species variation. Invest Ophthalmol Vis Sci 2003;44:2141-2146

Janoria KG, Boddu SH, Wang Z, Paturi DK, Samanta S, Pal D, Mitra AK: Vitreal pharmacokinetics of biotinylated ganciclovir: role of sodium-dependent multivitamin transporter expressed on retina. J Ocul Pharmacol Ther 2009;25:39-49

Jiang XR, Jimenez G, Chang E, Frolkis M, Kusler B, Sage M, Beeche M, Bodnar AG, Wahl GM, Tlsty TD, Chiu CP: Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. Nat Genet 1999;21:111-114

Jin M, Li S, Moghrabi WN, Sun H, Travis GH. Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. Cell 2005;122:449-459

Jorajuria S, Dereuddre-Bosquet N, Becher F, Martin S, Porcheray F, Garrigues A, Mabondzo A, Benech H, Grassi J, Orlowski S, Dormont D, Clayette P. ATP binding cassette multidrug transporters limit the anti-HIV activity of zidovudine and indinavir in infected human macrophages. Antivir Ther 2004;9:519-528

Kansara V, Mitra AK: Evaluation of an ex vivo model implication for carrier-mediated retinal drug delivery. Curr Eye Res 2006;31:415-426

Kanuga N, Winton HL, Beauchene L, Koman A, Zerbib A, Halford S, Couraud PO, Keegan D, Coffey P, Lund RD, Adamson P, Greenwood J: Characterization of genetically modified human retinal pigment epithelial cells developed for in vitro and transplantation studies. Invest Ophthalmol Vis Sci 2002;43:546-555

Karl MO, Valtink M, Bednarz J, Engelmann K: Cell culture conditions affect phagocytic function. Graefes Arch Clin Exp Ophthalmol 2007;245:981-991

Kato A, Kimura H, Okabe K, Okabe J, Kunou N, Ogura Y: Feasibility of drug delivery to the posterior pole of the rabbit eye with an episcleral implant. Invest Ophthalmol Vis Sci 2004;45:238-244

Kaven CW, Spraul CW, Zavazava NK, Lang GK, Lang GE: Growth factor combinations modulate human retinal pigment epithelial cell proliferation. Curr Eye Res 2000;20:480-487

Kennedy BG, Mangini NJ: P-glycoprotein expression in human retinal pigment epithelium. Mol Vis 2002;8:422-430

Kim H, Robinson SB, Csaky KG: Investigating the movement of intravitreal human serum albumin nanoparticles in the vitreous and retina. Pharm Res 2009;26:329-337

Kim H, Robinson MR, Lizak MJ, Tansey G, Lutz RJ, Yuan P, Wang NS, Csaky KG: Controlled drug release from an ocular implant: an evaluation using dynamic three-dimensional magnetic resonance imaging. Invest Ophthalmol Vis Sci 2004;45:2722-2731

Kim SH, Lutz RJ, Wang NS, Robinson MR: Transport barriers in transscleral drug delivery for retinal diseases. Ophthalmic Res 2007;39:244-254

Kimura H, Ogura Y, Moritera T, Honda Y, Tabata Y, Ikada Y: In vitro phagocytosis of polylactide microspheres by retinal pigment epithelial cells and intracellular drug release. Curr Eye Res 1994;13:353-360

Kindzelskii AL, Elner VM, Elner SG, Yang D, Hughes BA, Petty HR: Toll-like receptor 4 (TLR4) of retinal pigment epithelial cells participates in transmembrane signaling in response to photoreceptor outer segments. J Gen Physiol 2004;124:139-149

Kis B, Isse T, Snipes JA, Chen L, Yamashita H, Ueta Y, Busija DW: Effects of LPS stimulation on the expression of prostaglandin carriers in the cells of the blood-brain and blood-cerebrospinal fluid barriers. J Appl Physiol 2006;100:1392-1399

Kogishi JI, Akimoto M, Mandai M, Kuriyama S, Hall MO, Honda Y, Yoshimura N: Nitric oxide as a second messenger in phagocytosis by cultured retinal pigment epithelial cells. Ophthalmic Res 2000;32:138-142

Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P: MRP3 an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A 1999;6:6914-6919

Korjamo T, Honkakoski P, Toppinen MR, Niva S, Reinisalo M, Palmgren JJ, Monkkonen J: Absorption properties and P-glycoprotein activity of modified Caco-2 cell lines. Eur J Pharm Sci 2005;26:266-279

Kuntz CA, Crook RB, Dmitriev A, Steinberg RH: Modification by cyclic adenosine monophosphate of basolateral membrane chloride conductance in chick retinal pigment epithelium. Invest Ophthalmol Vis Sci 1994;35:422-433

Kuriyama S, Hall MO, Abrams TA, Mittag TW: Isoproterenol inhibits rod outer segment phagocytosis by both cAMP-dependent and independent pathways. Invest Ophthalmol Vis Sci 1995;36:730-736

Kusuhara H, Sugiyama Y: In vitro-in vivo extrapolation of transporter-mediated clearance in the liver and kidney. Drug Metab Pharmacokinet 2009;24:37-52

Lara-Castillo N, Zandi S, Nakao S, Ito Y, Noda K, She H, Ahmed M, Frimmel S, Ablonczy Z, Hafezi-Moghadam A: Atrial natriuretic peptide reduces vascular leakage and choroidal neovascularization. Am J Pathol 2009;175:2343-2350

Leier I, Hummel-Eisenbeiss J, Cui Y, Keppler D. ATP-dependent para-aminohippurate transport by apical multidrug resistance protein MRP2. Kidney Int 2000;57:1636-1642

Leung KW, Liu M, Xu X, Seiler MJ, Barnstable CJ, Tombran-Tink J: Expression of ZnT and ZIP zinc transporters in the human RPE and their regulation by neurotrophic factors. Invest Ophthalmol Vis Sci 2008;49:1221-1231

Lindenmaier H, Becker M, Haefeli WE, Weiss J: Interaction of progestins with the human multidrug resistance-associated protein 2 (MRP2). Drug Metab Dispos 2005;33:1576-1579

Ludtke JJ, Sebestyen MG, Wolff JA: The effect of cell division on the cellular dynamics of microinjected DNA and dextran. Mol Ther 2002;5:579-588

Lund RD, Adamson P, Sauve Y, Keegan DJ, Girman SV, Wang S, Winton H, Kanuga N, Kwan AS, Beauchene L, Zerbib A, Hetherington L, Couraud PO, Coffey P, Greenwood J: Subretinal transplantation of genetically modified human cell lines attenuates loss of visual function in dystrophic rats. Proc Natl Acad Sci U S A 2001;98:9942-9947

Luo Y, Zhuo Y, Fukuhara M, Rizzolo LJ: Effects of culture conditions on heterogeneity and the apical junctional complex of the ARPE-19 cell line. Invest Ophthalmol Vis Sci 2006;47:3644-3655

Maeda K, Sugiyama Y: Impact of genetic polymorphisms of transporters on the pharmacokinetic pharmacodynamic and toxicological properties of anionic drugs. Drug Metab Pharmacokinet 2008;23:223-235

Magin-Lachmann C, Kotzamanis G, D'Aiuto L, Cooke H, Huxley C, Wagner E: In vitro and in vivo delivery of intact BAC DNA-comparison of different methods. J Gen Med 2004; 6:195-209

Maenpaa H, Gegelashvili G, Tahti H: Expression of glutamate transporter subtypes in cultured retinal pigment epithelial and retinoblastoma cells. Curr Eye Res 2004;28:159-165

Maenpaa H, Mannerstrom M, Toimela T, Salminen L, Saransaari P, Tahti H: Glutamate uptake is inhibited by tamoxifen and toremifene in cultured retinal pigment epithelial cells Pharmacol Toxicol 2002;91:116-122

Majumdar S, Gunda S, Pal D, Mitra AK: Functional activity of a monocarboxylate transporter MCT1 in the human retinal pigmented epithelium cell line ARPE-19. Mol Pharm 2005;2:109-117

Majumdar S, Macha S, Pal D, Mitra AK: Mechanism of ganciclovir uptake by rabbit retina and human retinal pigmented epithelium cell line ARPE-19. Curr Eye Res 2004;29:127-136

Maminishkis A, Chen S, Jalickee S, Banzon T, Shi G, Wang FE, Ehalt T, Hammer JA, Miller SS: Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. Invest Ophthalmol Vis Sci 2006;47:3612-3624

Marmor MF, Negi A: Pharmacologic modification of subretinal fluid absorption in the rabbit eye. Arch Ophthalmol 1986;104:1674-1677

Marmorstein AD, Cross HE, Peachey NS: Functional roles of bestrophins in ocular epithelia Prog Retin Eye Res 2009;28:206-226

Marmorstein AD, Kinnick TR: Focus on molecules: bestrophin (best-1) Exp Eye Res 2007;:5 423-424

Marmorstein AD, Marmorstein LY, Rayborn M, Wang X, Hollyfield JG, Petrukhin K: Bestrophin the product of the Best vitelliform macular dystrophy gene (VMD2) localizes to the basolateral plasma membrane of the retinal pigment epithelium. Proc Natl Acad Sci U S A 2000;7:12758-12763

Marmorstein AD, Gan YC, Bonilha VL, Finnemann SC, Csaky KG, Rodriguez-Boulan E: Apical polarity of N-CAM and EMMPRIN in retinal pigment epithelium resulting from suppression of basolateral signal recognition. J Cell Biol 1998;142:697-710

Marmorstein LY, McLaughlin PJ, Stanton JB, Yan L, Crabb JW, Marmorstein AD: Bestrophin interacts physically and functionally with protein phosphatase 2A. J Biol Chem 2002;277:30591-30597

Martin PM, Dun Y, Mysona B, Ananth S, Roon P, Smith SB, Ganapathy V: Expression of the sodium-coupled monocarboxylate transporters SMCT1 (SLC5A8) and SMCT2 (SLC5A12) in retina. Invest Ophthalmol Vis Sci 2007;48:3356-3363

Martinez-Morales JR, Dolez V, Rodrigo I, Zaccarini R, Leconte L, Bovolenta P, Saule S: OTX2 activates the molecular network underlying retina pigment epithelium differentiation. J Biol Chem 2003;:278 21721-21731

Mason JO, Somaiya MD, Singh RJ: Intravitreal concentration and clearance of triamcinolone acetonide in nonvitrectomized human eyes. Retina 2004;24:900-904

Maurice DM, Mishima S: Ocular pharmacokinetics in: Sears ML (Ed): Handbook of experimental pharmacology. Springer-Verlag Berlin 1984; pp 19-116

Maurice DM, Polgar J: Diffusion across the sclera. Exp Eye Res 1977;25:577-582

McAleer MA, Breen MA, White NL, Matthews N: pABC11 (also known as MOAT-C and MRP5) a member of the ABC family of proteins has anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. J Biol Chem 1999;274:23541-23548

Miller S,S Edelman JL: Active ion transport pathways in the bovine retinal pigment epithelium J Physiol 1990;424:283-300

Minderman H, Vanhoefer U, Toth K, Yin MB, Minderman MD, Wrzosek C, Slovak ML, Rustum YM: DiOC2(3) is not a substrate for multidrug resistance protein (MRP)-mediated drug efflux. Cytometry 1996;25:14-20

Mizuno N, Niwa T, Yotsumoto Y, Sugiyama Y: Impact of drug transporter studies on drug discovery and development. Pharmacol Rev 2003;55:425-461

Moiseyev G, Chen Y, Takahashi Y, Wu BX, Ma JX: RPE65 is the isomerohydrolase in the retinoid visual cycle. Proc Natl Acad Sci U S A 2005;102:12413-12418

Molday RS, Hicks D, Molday L: Peripherin A rim-specific membrane protein of rod outer segment discs. Invest Ophthalmol Vis Sci 1987;28:50-61

Moore DJ, Clover GM: The effect of age on the macromolecular permeability of human Bruch's membrane. Invest Ophthalmol Vis Sci 2001;42:2970-2975

Mordenti ,J Cuthbertson RA, Ferrara N ,Thomsen K, Berleau L, Licko V, Allen PC, Valverde CR, Meng YG, Fei DT, Fourre KM, Ryan AM: Comparisons of the intraocular tissue distribution pharmacokinetics and safety of 125I-labeled full-length and Fab antibodies in rhesus monkeys following intravitreal administration. Toxicol Pathol 1999;27:536-544

Moritera T, Ogura Y, Honda Y, Wada R, Hyon SH, Ikada Y: Microspheres of biodegradable polymers as a drug-delivery system in the vitreous. Invest Ophthalmol Vis Sci 1991;32:1785-1790

Moshfeghi AA, Peyman GA: Micro- and nanoparticulates. Adv Drug Deliv Rev 2005;57:2047-2052

Nabi IR, Mathews AP, Cohen-Gould L, Gundersen D, Rodriguez-Boulan E: Immortalization of polarized rat retinal pigment epithelium J Cell Sci 1993;104:37-49

Nagata A, Mishima HK, Kiuchi Y, Hirota A, Kurokawa T, Ishibashi S: Binding of antiglaucomatous drugs to synthetic melanin and their hypotensive effects on pigmented and nonpigmented rabbit eyes. Jpn J Ophthalmol 1993;37:32-38

Naggar H, Van Ells TK, Ganapathy V, Smith SB: Regulation of reduced-folate transporter-1 in retinal pigment epithelial cells by folate. Curr Eye Res 2005;30:35-44

Naito M, Tsuruo T: Competitive inhibition by verapamil of ATP-dependent high affinity vincristine binding to the plasma membrane of multidrug-resistant K562 cells without calcium ion involvement. Cancer Res 1989;49:1452-1455

Nandrot E, Dufour EM, Provost AC, Pequignot MO, Bonnel S, Gogat K, Marchant D, Rouillac C, Sepulchre de Conde B, Bihoreau MT, Shaver C, Dufier JL, Marsac C, Lathrop M, Menaschhe M, Abitbol MM: Homozygous deletion in the coding sequence of the c-mer gene in RCS rats reveals general mechanism of physiological cell adhesion and apoptosis. Neurobiol Dis 2000; 7:586-599

Nelson WJ: Regulation of cell-cell adhesion by the cadherin-catenin complex. Biochem Soc Trans 2008;36:149-155

Nevala H, Ylikomi T, Tahti H: Evaluation of the selected barrier properties of retinal pigment epithelial cell line ARPE-19 for an in-vitro blood-brain barrier model. Hum Exp Toxicol 2008;27:741-749

Nicolini J, Kiilgaard JF, Wiencke AK, Heegaard S, Scherfig E, Prause JU, la Cour M: The anterior lens capsule used as support material in RPE cell-transplantation. Acta Ophthalmol Scand 2000;78:527-531

Nies AT, Jedlitschky G, Konig J, Herold-Mende C, Steiner HH, Schmitt HP, Keppler D: Expression and immunolocalization of the multidrug resistance proteins MRP1-MRP6 (ABCC1-ABCC6) in human brain. Neuroscience 2004;129:349-360

Njie-Mbye YF, Bongmba OY, Onyema CC, Chitnis A, Kulkarni M, Opere CA, Leday AM, Ohia SE: Effect of Hydrogen Sulfide on Cyclic AMP Production in Isolated Bovine and Porcine Neural Retinae. Neurochem Res 2009 doi:10.1007/s11064-009-0085-7

Norris MD, Smith J, Tanabe K, Tobin P, Flemming C, Scheffer GL, Wielinga P, Cohn SL, London WB, Marshall GM, Allen JD, Haber M: Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan in vitro. Mol Cancer Ther 2005;4:547-553

Ocheltree SM, Keep RF, Shen H, Yang D, Hughes BA, Smith DE: Preliminary investigation into the expression of proton-coupled oligopeptide transporters in neural retina and retinal pigment epithelium (RPE): lack of functional activity in RPE plasma membranes. Pharm Res 2003;20:1364-1372

Ohno-Matsui K, Mori K, Ichinose S, Sato T, Wang J, Shimada N, Kojima A, Mochizuki M, Morita I: In vitro and in vivo characterization of iris pigment epithelial cells cultured on amniotic membranes. Mol Vis 2006;12:1022-1032

Ohno-Matsui K, Ichinose S, Nakahama K ,Yoshida T, Kojima A, Mochizuki M, Morita I: The effects of amniotic membrane on retinal pigment epithelial cell differentiation Mol Vis 2005;11:1-10

Okabe J, Kimura H, Kunou N, Okabe K, Kato A, Ogura Y: Biodegradable intrascleral implant for sustained intraocular delivery of betamethasone phosphate Invest Ophthalmol Vis Sci 2003;44:740-744

Okami T, Yamamoto A, Omori K, Takada T, Uyama M, Tashiro Y: Immunocytochemical localization of Na+K(+)-ATPase in rat retinal pigment epithelial cells. J Histochem Cytochem 1990;38:1267-1275

Olsen TW, Aaberg SY, Geroski DH, Edelhauser HF: Human sclera: thickness and surface area. Am J Ophthalmol 1998;125:237-241

Olsen TW, Edelhauser HF, Lim JI, Geroski DH: Human scleral permeability: Effects of age cryotherapy transscleral diode laser and surgical thinning. Invest Ophthalmol Vis Sci 1995;:6 1893-1903

Paimela T, Ryhanen T, Mannermaa E, Ojala J, Kalesnykas G, Salminen A, Kaarniranta K: The effect of 17beta-estradiol on IL-6 secretion and NF-kappaB DNA-binding activity in human retinal pigment epithelial cells. Immunol Lett 2007;110:139-144

Pascolo L, Fernetti C, Pirulli D, Crovella S, Amoroso A, Tiribelli C: Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells. Biochem Biophys Res Commun 2003;303:259-265

Payen L, Delugin L, Courtois A, Trinquart Y, Guillouzo A, Fardel O: Reversal of MRP-mediated multidrug resistance in human lung cancer cells by the antiprogestatin drug RU486. Biochem Biophys Res Commun 1999;258:513-518

Peeters L, Sanders NN, Braeckmans K, Boussery K, Van de Voorde J, De Smedt SC, Demeester J: Vitreous: a barrier to nonviral ocular gene therapy. Invest Ophthalmol Vis Sci 2005;46:3553-3561

Pelis RM, Shahidullah M, Ghosh S, Coca-Prados M, Wright SH, Delamere NA: Localization of multidrug resistance-associated protein 2 in the nonpigmented ciliary epithelium of the eye. J Pharmacol Exp Ther 2009;329:479-485

Perrotton T, Trompier D, Chang XB, Di Pietro A, Baubichon-Cortay H: S- and R-verapamil differentially modulate the multidrug resistance protein MRP1. J Biol Chem 2007;282:31542-31548

Philp NJ, Wang D, Yoon H, Hjelmeland LM Polarized expression of monocarboxylate transporters in human retinal pigment epithelium and ARPE-19 cells. Invest Ophthalmol Vis Sci : 2003;44:1716-1721

Philp NJ, Yoon H, Grollman EF: Monocarboxylate transporter MCT1 is located in the apical membrane and MCT3 in the basal membrane of rat RPE. Am J Physiol 1998;274:R1824-8

Pitkänen L, Ranta VP, Moilanen H, Urtti A: Permeability of retinal pigment epithelium: effects of permeant molecular weight and lipophilicity. Invest Ophthalmol Vis Sci 2005;46:641-646

Pitkänen L, Pelkonen J, Ruponen M, Ronkko S, Urtti A: Neural retina limits the nonviral gene transfer to retinal pigment epithelium in an in vitro bovine eye model AAPS 2004;6:e25

Pitkänen L, Ruponen M, Nieminen J, Urtti A: Vitreous is a barrier in nonviral gene transfer by cationic lipids and polymers Pharm Res 2003;20:576-583

Pontes de Carvalho RA, Krausse ML, Murphree AL, Schmitt EE, Campochiaro PA, Maumenee IH: Delivery from episcleral exoplants. Invest Ophthalmol Vis Sci 2006;47:4532-4539

Pratt CH, Vadigepalli R, Chakravarthula P, Gonye GE, Philp NJ, Grunwald GB: Transcriptional regulatory network analysis during epithelial-mesenchymal transformation of retinal pigment epithelium. Mol Vis 2008;14:1414-1428

Pratt S, Shepard RL, Kandasamy RA, Johnston PA, Perry W, Dantzig AH: The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. Mol Cancer Ther 2005;4:855-863

Prausnitz MR, Edwards A, Noonan JS, Rudnick DE, Edelhauser HF, Geroski DH: Measurement and prediction of transient transport across sclera for drug delivery to the eye. Ind Eng Chem Res 1998; 37:2903-2907

Prausnitz MR, Noonan JS: Permeability of cornea sclera and conjunctiva: a literature analysis for drug delivery to the eye. J Pharm Sci 1998;87:1479-1488

Qadir M, O'Loughlin KL, Fricke SM, Williamson NA, Greco WR, Minderman H, Baer MR: Cyclosporin A is a broad-spectrum multidrug resistance modulator. Clin Cancer Res 2005;11:2320-2326

Qu Z, Chien LT, Cui Y, Hartzell HC: The anion-selective pore of the bestrophins a family of chloride channels associated with retinal degeneration. J Neurosci 2006;26:5411-5419

Quinn R, Miller SS: Ion transport mechanisms in native human retinal pigment epithelium. Invest Ophthalmol Vis Sci 1992;33:3513-3527

Rajan PD, Kekuda R, Chancy CD, Huang W, Ganapathy V, Smith SB: Expression of the extraneuronal monoamine transporter in RPE and neural retina. Curr Eye Res 2000;20:195-204

Rajasekaran SA, Hu J, Gopal J, Gallemore R, Ryazantsev S, Bok D, Rajasekaran AK: NaK-ATPase inhibition alters tight junction structure and permeability in human retinal pigment epithelial cells. Am J Physiol Cell Physiol 2003;284:C1497-507

Rak DJ, Hardy KM, Jaffe GJ, McKay BS: Ca++-switch induction of RPE differentiation Exp Eye Res 2006;82:648-656

Rambhatla L, Chiu CP, Glickman RD, Rowe-Rendleman C: In vitro differentiation capacity of telomerase immortalized human RPE cells. Invest Ophthalmol Vis Sci 2002;43:1622-1630

Ranta VP, Toropainen E, Talvitie A, Auriola S, Urtti A: Simultaneous determination of eight beta-blockers by gradient high-performance liquid chromatography with combined ultraviolet and fluorescence detection in corneal permeability studies in vitro. J Chromatogr B Analyt Technol Biomed Life Sci 2002;772:81-87

Redmond TM: Focus on Molecules: RPE65 the visual cycle retinol isomerase Exp Eye Res 2009;88:846-847

Reid G, Wielinga P, Zelcer N, De Haas M, Van Deemter L ,Wijnholds J, Balzarini J, Borst P: Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. Mol Pharmacol 2003a;63:1094-1103

Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P: The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs Proc Natl Acad Sci U S A 2003b;100:9244-9249

Rein DB, Wittenborn JS, Zhang X, Honeycutt AA, Lesesne SB, Saaddine J: Vision Health Cost-Effectiveness Study Group 2009 Forecasting age-related macular degeneration through the year 2050: the potential impact of new treatments Arch Ophthalmol 2009;.127:533-540

Rezaei KA, Chen Y, Cai J, Sternberg P: Modulation of Nrf2-dependent antioxidant functions in the RPE by Zip2 a zinc transporter protein. Invest Ophthalmol Vis Sci 2008;49:1665-1670

Richard G: Choroidal circulation. Georg Thieme Verlag, Stuttgart, 1992

Ritter CA, Jedlitschky G, Meyer zu Schwabedissen H, Grube M, Kock K, Kroemer HK: Cellular export of drugs and signaling molecules by the ATP-binding cassette transporters MRP4 (ABCC4) and MRP5 (ABCC5). Drug Metab Rev 2005;37:253-278

Robinson MR, Lee SS, Kim H, Kim S, Lutz RJ, Galban C, Bungay PM, Yuan P, Wang NS, Kim J, Csaky KG: A rabbit model for assessing the ocular barriers to the transscleral delivery of triamcinolone acetonide Exp Eye Res 2006;82:479-487

Romano G, Marino IR, Pentimalli F, Adamo V, Giordano A: Insertional mutagenesis and development of malignancies induced by integrating gene delivery systems: implications for the design of safer gene-based interventions in patients. Drug News Perspect 2009;22:185-196

Rowe DW, Starman BJ, Fujimoto WY, Williams RH:77 Differences in growth response to hydrocortisone and ascorbic acid by human diploid fibroblasts. In Vitro 1997;13:824-830

Ruponen M, Yla-Herttuala S, Urtti A: Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. Biochim Biophys Acta 1999;1415:331-341

Ryeom SW, Sparrow JR, Silverstein RL: CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium. J Cell Sci 1996;109:387-395

Saari JC, Crabb JW: Focus on molecules: cellular retinaldehyde-binding protein (CRALBP). Exp Eye Res 2005;81:245-246

Saari JC, Nawrot M, Kennedy BN, Garwin GG, Hurley JB, Huang J, Possin DE, Crabb JW: Visual cycle impairment in cellular retinaldehyde binding protein (CRALBP) knockout mice results in delayed dark adaptation Neuron 2001;29:739-748

Sakurai E, Ozeki H, Kunou N, Ogura Y: Effect of particle size of polymeric nanospheres on intravitreal kinetics. Ophthalmic Res 2001;33:31-36

Sampath J, Adachi M, Hatse S, Naesens L, Balzarini J, Flatley RM, Matherly LH ,Schuetz JD: Role of MRP4 and MRP5 in biology and chemotherapy. AAPS PharmSci 2002;4 E14 doi: 101208/ps040314 [doi] ?????,

Scheffer GL, Hu X, Pijnenborg AC, Wijnholds J, Bergen AA, Scheper RJ: MRP6 (ABCC6) detection in normal human tissues and tumors. Lab Invest 2002;82;515-518

Schinkel AH, Jonker JW: Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev 2003;55:3-29

Sieving PA, Caruso RC, Tao W, Coleman HR, Thompson DJ, Fullmer KR, Bush RA: Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular . Proc Natl Acad Sci U S A 2006; 103:3896-3901

Singh S, Woerly S, McLaughlin BJ: Natural and artificial substrates for retinal pigment epithelial monolayer transplantation. Biomaterials 2001;22:3337-3343

Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR: A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. Nat Protoc 2009;4:662-673

Song MK, Lui GM: Propagation of fetal human RPE cells: preservation of original culture morphology after serial passage. J Cell Physiol 1990;143:196-203

Spraul CW, Kaven C, Lang GK, Lang GE: Effect of growth factors on bovine retinal pigment epithelial cell migration and proliferation. Ophthalmic Res 2004;36:166-171

Spraul CW, Lang GE, Lang GK, Grossniklaus HE: Morphometric changes of the choriocapillaris and the choroidal vasculature in eyes with advanced glaucomatous changes. Vision Res 2002;42:923-932

Stanzel BV, Espana EM, Grueterich M, Kawakita T, Parel JM, Tseng SC, Binder S: Amniotic membrane maintains the phenotype of rabbit retinal pigment epithelial cells in culture. Exp Eye Res 2005;80:103-112

Steuer H, Jaworski A, Elger B, Kaussmann M, Keldenich J, Schneider H, Stoll D, Schlosshauer B: Functional characterization and comparison of the outer blood-retina barrier and the blood-brain barrier. Invest Ophthalmol Vis Sci 2005;46:1047-1053

Steuer H, Jaworski A, Stoll D, Schlosshauer B. In vitro model of the outer blood-retina barrier. Brain Res Brain Res Protoc 2004;13:26-36

Stojic ,J Stohr H, Weber BH: Three novel ABCC5 splice variants in human retina and their role as regulators of ABCC5 gene expression. BMC Mol Biol 2007;8:42

St-Pierre MV, Serrano MA, Macias RI, Dubs U, Hoechli M, Lauper U, Meier PJ, Marin JJ: Expression of members of the multidrug resistance protein family in human term placenta Am J Physiol Regul Integr Comp Physiol 2000;279:R1495-503

Strauss O: The retinal pigment epithelium in visual function Physiol Rev 2005;85:845-881

Strick DJ, Feng W, Vollrath D: Mertk drives myosin II redistribution during retinal pigment epithelial phagocytosis. Invest Ophthalmol Vis Sci 2009;50:2427-2435

Subramanian VS, Mohammed ZM, Molina A, Marchant JS, Vaziri ND, Said HM: Vitamin B1 (thiamine) uptake by human retinal pigment epithelial (ARPE-19) cells: mechanism and regulation. J Physiol 2007;582:73-85

Thakkinstian A, Han P, McEvoy M, Smith W, Hoh J, Magnusson K, Zhang K, Attia J:Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. Hum Mol Genet 2006;15:2784-2790

Thurman JM, Renner B, Kunchithapautham K, Ferreira VP, Pangburn MK, Ablonczy Z, Tomlinson S, Hollers VM, Rohrer B: Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. J Biol Chem 2009;284:16939-16947

Tian X, Zamek-Gliszczynski MJ, Zhang P, Brouwer KL: Modulation of multidrug resistance-associated protein 2 (Mrp2) and Mrp3 expression and function with small interfering RNA in sandwich-cultured rat hepatocytes. Mol Pharmacol 2004;66:1004-1010

Torczynski E: Choroid and suprachoroid in Duane's foundations of clinical ophthalmology. Tasman W, Jaeger EA (eds) New York 1995; ch. 22

Tornquist P, Alm A, Bill A: Studies on ocular blood flow and retinal capillary permeability to sodium in pigs Acta Physiol Scand 1979;106:343-350

Toropainen E, Hornof M, Kaarniranta K, Johansson P, Urtti A: Corneal epithelium as a platform for secretion of transgene products after transfection with liposomal gene eyedrops J Gene Med 2:07;9 208-216

Turowski P, Adamson P, Sathia J, Zhang JJ, Moss SE, Aylward GW, Hayes MJ, Kanuga N, Greenwood J: Basement membrane-dependent modification of phenotype and gene expression in human retinal pigment epithelial ARPE-19 cells. Invest Ophthalmol Vis Sci 2004;:5 2786-2794

Twentyman PR, Rhodes T, Rayner S: A comparison of rhodamine 123 accumulation and efflux in cells with P-glycoprotein-mediated and MRP-associated multidrug resistance phenotypes. Eur J Cancer 1994;30A:1360-1369

Uebersax ED, Grindstaff RD, Defoe DM: Survival of the retinal pigment epithelium in vitro: comparison of freshly isolated and subcultured cells. Exp Eye Res 2000;70:381-390

Umapathy NS, Gnana-Prakasam JP, Martin PM, Mysona B, Dun Y, Smith SB, Ganapathy V, Prasad PD: Cloning and functional characterization of the proton-coupled electrogenic folate transporter and analysis of its expression in retinal cell types. Invest Ophthalmol Vis Sci 2007;48:5299-5305

Underwood JL, Murphy CG, Chen J, Franse-Carman L, Wood I, Epstein DL, Alvarado JA: Glucocorticoids regulate transendothelial fluid flow resistance and formation of intercellular junction. Am J Physiol 1999;277:C330-42

Urtti A, Salminen L, Kujari H, Jäntti V: Effect of ocular pigmentation on pilocarpine pharmacology in the rabbit eye. II. Drug response. Int J Pharm 1984;19:53-61

Vasiliou V, Vasiliou K, Nebert DW: Human ATP-binding cassette (ABC) transporter family. Hum Genomics 2009;3:281-290

Vehanen K, Hornof M, Urtti A, Uusitalo H: Peribulbar poloxamer for ocular drug delivery. Acta Ophthalmol 2008;86:91-96

Vellonen KS, Honkakoski P, Urtti A: Substrates and inhibitors of efflux proteins interfere with the MTT assay in cells and may lead to underestimation of drug toxicity. Eur J Pharm Sci 2004;23:181-188

Vingerling JR, Hofman A, Grobbee DE, de Jong PT: Age-related macular degeneration and smoking The Rotterdam Study. Arch Ophthalmol 1996;114:1193-1196

Wielinga PR, Reid G, Challa EE, van der Heijden I, van Deemter L, de Haas M, Mol C, Kuil AJ, Groeneveld E, Schuetz JD, Brouwer C, De Abreu RA, Wijnholds J, Beijnen JH, Borst P: Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. Mol Pharmacol 2002;62:1321-1331

Wiencke AK, Kiilgaard JF, Nicolini J, Bundgaard M, Ropke C, La Cour M: Growth of cultured porcine retinal pigment epithelial cells. Acta Ophthalmol Scand 2003;81:170-176

Wijnholds J, Mol CA van Deemter L, de Haas M, Scheffer GL, Baas F, Beijnen JH, Scheper RJ, Hatse S, De Clercq E, Balzarini J, Borst P: Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. Proc Natl Acad Sci U S A 2000;97:7476-7481

Wikström J, Elomaa M, Syvajarvi H, Kuokkanen J ,Yliperttula M, Honkakoski P, Urtti A: Alginate-based microencapsulation of retinal pigment epithelial cell line for cell therapy. Biomaterials 2008;29:869-876

Wood JP, Chidlow G, Graham M, Osborne NN: Energy substrate requirements for survival of rat retinal cells in culture: the importance of glucose and monocarboxylates. J Neurochem 2005;93:686-697

Yamamoto A, Akanuma SI, Tachikawa M, Hosoya KI: Involvement of LAT1 and LAT2 in the high- and low-affinity transport of L-leucine in human retinal pigment epithelial cells (ARPE-19 cells). J Pharm Sci 2009; doi: 101002/jps21991

Yang CP, DePinho SG, Greenberger LM, Arceci RJ, Horwitz SB: Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. J Biol Chem 1989;264:782-788

Yang YC, Hulbert MF, Batterbury M, Clearkin LG:Pulsatile ocular blood flow measurements in healthy eyes: reproducibility and reference values. J Glaucoma 1997;6:175-179

Youn YH, Hong J, Burke JM: Cell phenotype in normal epithelial cell lines with high endogenous N-cadherin: comparison of RPE to an MDCK subclone. Invest Ophthalmol Vis Sci 2006;47:2675-2685

Young AM, Allen CE, Audus KL: Efflux transporters of the human placenta. Adv Drug Deliv Rev 2003;55:125-132

Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ: Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem 1995;270:18997-19007

Zaman GJ, Flens MJ, van Leusden MR, de Haas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ: The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci U S A 1994;91:8822-8826

Zamek-Gliszczynski MJ, Xiong H, Patel NJ, Turncliff RZ, Pollack GM, Brouwer KL : Pharmacokinetics of 5 (and 6)-carboxy-2'7'-dichlorofluorescein and its diacetate promoiety in the liver. J Pharmacol Exp Ther 2003;304:801-809

Zhang N, Kannan R, Okamoto CT, Ryan SJ, Lee VH, Hinton DR: Characterization of brimonidine transport in retinal pigment epithelium. Invest Ophthalmol Vis Sci 2006;47:287-294

Zhang T, Xiang CD, Gale D, Carreiro S, Wu EY, Zhang EY: Drug transporter and cytochrome P450 mRNA expression in human ocular barriers: implications for ocular drug disposition. Drug Metab Dispos 2008;36:1300-1307

Zhang Y, Schuetz JD, Elmquist WF, Miller DW: Plasma membrane localization of multidrug resistance-associated protein homologs in brain capillary endothelial cells. J Pharmacol Exp Ther 2004;311:449-455

Zhou SF, Di YM, Chan E, Du YM, Chow VD, Xue CC, Lai X, Wang JC, Li CG, Tian M, Duan W: Clinical pharmacogenetics and potential application in personalized medicine. Curr Drug Metab 2008;9:738-784

Eliisa Mannermaa

In vitro Model of Retinal Pigment Epithelium for Use in Drug Delivery Studies

Retinal pigment epithelium (RPE) has an essential role in ocular pharmacokinetics. In this study an in vitro model of RPE was characterized. The barrier properties and active transport between the model and ex vivo tissue were comparable. Expression of eight efflux transporters was studied in four different RPE models. Multidrug resistance associated proteins 1, 4 and 5 were constantly expressed. In addition, literature data of influx transporters of RPE models and RPE in vivo is summarized and the role of membrane transporters in ocular pharmacokinetics discussed.

UNIVERSITY OF EASTERN FINLAND

Publications of the University of Eastern Finland
Dissertations in Health Sciences

ISBN 978-952-61-0029-6