Advances in Experimental Medicine and Biology 854

Catherine Bowes Rickman
Matthew M. LaVail
Robert E. Anderson
Christian Grimm
Joe Hollyfield
John Ash *Editors*

Retinal Degenerative Diseases

Mechanisms and Experimental Therapy

Springer

Advances in Experimental Medicine and Biology

Volume 854

Advances in Experimental Medicine and Biology presents multidisciplinary and dynamic findings in the broad fields of experimental medicine and biology. The wide variety in topics it presents offers readers multiple perspectives on a variety of disciplines including neuroscience, microbiology, immunology, biochemistry, biomedical engineering and cancer research. *Advances in Experimental Medicine and Biology* has been publishing exceptional works in the field for over 30 years and is indexed in Medline, Scopus, EMBASE, BIOSIS, Biological Abstracts, CSA, Biological Sciences and Living Resources (ASFA-1), and Biological Sciences. The series also provides scientists with up to date information on emerging topics and techniques.
2013 Impact Factor: 2.012.

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Supplementary material to this book can be accessed at http://link.springer.com/book/10.1007/978-3-319-17121-0

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-17120-3 ISBN 978-3-319-17121-0 (eBook)
DOI 10.1007/978-3-319-17121-0

Library of Congress Control Number: 2015938602

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

Dedication

Holly Jo Whiteside

Holly Whiteside has been an extraordinary RD Symposium Coordinator for 16 years, from RD2000 through the RD2014 meeting. For most of these symposia, she managed all aspects of the meetings, their selection sites, the design and maintenance of the meeting website, all interactions with participants and Travel Awardees, as well as assisting the preparation and submission of the conference grant from the NEI and the proceedings volume. For many, Holly has been the face of the meetings, and she showed remarkable dedication to the meetings and their participants, often giving much of her personal time to be sure the symposia were successful. In so doing, she helped mostly during the period of doubling the size of the biennial meeting. Holly has decided to step down from her involvement with the RD Symposia to devote her time to other aspects of her research and administrative tasks and her personal interests. We will miss her and are honored to dedicate this proceedings volume to her.

Preface

The International Symposia on Retinal Degeneration have been held in conjunction with the biennial meeting of the International Society of Eye Research (ISER) since 1984. These RD Symposia have allowed basic and clinician scientists from around the world to convene and present their new research findings. They have been organized to allow substantial time for discussions and one-on-one interactions in a relaxed atmosphere, where international friendships and collaborations can be fostered.

The XVI International Symposium on Retinal Degeneration (also known as RD2014) was held from July 13–18, 2014 at the Asilomar Conference Center in the beautiful city of Pacific Grove, California, USA. The meeting brought together 272 basic and clinician scientists, retinal specialists in ophthalmology, and trainees in the field from all parts of the world. In the course of the meeting, 43 platform and 159 poster presentations were given, and a majority of these are presented in this proceedings volume. New discoveries and state of the art findings from most research areas in the field of retinal degenerations were presented. This was the largest of all of the RD Symposia, with the greatest number of attendees and presentations.

The RD2014 meeting was highlighted by three special keynote lectures. The first was given by **John Flannery,** PhD, of the University of California, Berkeley, Berkeley, CA. Dr. Flannery discussed "Engineering AAV vectors to target specific functional subclasses of retinal neurons and glia." Dr. Flannery's talk was the first named keynote lecture of the RD Symposia in 32 years, the Edward H. Gollob Lecture, named for the President of the Foundation Fighting Blindness. The second keynote lecture was given by **Sally Temple,** PhD, Director of the Neural Stem Cell Institute, Regenerative Research Foundation, Rensselaer, NY. Dr. Temple discussed "Endogenous RPE stem cells, their surprising plasticity and implications for therapeutic applications." The third keynote lecture was given by **Samuel G. Jacobson,** MD, PhD, of the University of Pennsylvania, Philadelphia, PA. Dr. Jacobson discussed "A treatment trial for an inherited retinal degeneration: what have we learned?"

The scientific meeting ended with a "Welcome to RD2016" by Prof. Nagahisa Yoshimura of Kyoto, Japan, along with the organizers primarily responsible for the meeting, Drs. John Ash and Robert E. Anderson.

We thank the outstanding management and staff of the beautiful Asilomar Conference Center for their assistance in making this an exceptionally smooth-running conference and a truly memorable experience for all of the attendees. These included, in particular, **Suzan Carabarin**, **Vivian Garcia, Sammy Ramos** and **Carlene Miller**. We also thank **Kelly Gilford** and **Jason McIntosh** for providing audio/visual equipment and services that resulted in a flawless flow of platform presentations. We thank **Steve Henry** of Associated Hosts, Inc. for planning and implementing transportation of most of the attendees to and from the Asilomar meeting venue, the memorable whale watching excursion, as well as for providing the dynamic "Beach Boys Band" for the end-of-meeting Gala for a truly California experience. Lastly, we thank **Franz Badura** of Pro Retina Germany for serenading the attendees at the Gala with his beautiful trumpet solos.

The Symposium received international financial support from a number of organizations. We are particularly pleased to thank The Foundation Fighting Blindness, Columbia, Maryland, for its continuing support of this and all previous biennial Symposia, without which we could not have held these important meetings. In addition, for the seventh time, the National Eye Institute of the National Institutes of Health contributed in a major way to the meeting. In the past, funds from these two organizations allowed us to provide 25–35 Travel Awards to young investigators and trainees working in the field of retinal degenerations. However, the response to the Travel Awards program was extraordinary, with 110 applicants, many more than in the past. For this reason, we sought additional support for the Travel Awards program. We are extremely appreciative for the contributions from Pro Retina Germany, the Fritz Tobler Foundation Switzerland and from Ed and Sandy Gollob. In total, we were able to fund 49 Travel Awards, the largest number ever an RD Symposia held in North America. We are grateful to the BrightFocus Foundation, which supported the important poster sessions. Many of the contributing foundations sent members of their organizations to attend the meeting. Their participation and comments in the scientific sessions were instructive to many, offering new perspectives to some of the problems being discussed. The Travel Awardees were selected on the basis of 9 independent scores of their submitted abstracts, 6 from each of the organizers and 3 from the other members of the Travel Awards Committee for RD2014, Drs. Jacque Duncan, Machelle Pardue and XianJie Yang.

We also acknowledge the diligent and outstanding efforts of Ms. **Holly Whiteside,** who along with Dr. **John Ash**, carried out most of the administrative aspects of the RD2014 Symposium, and designed and maintained the meeting website. Holly is the Administrative Manager of Dr. Anderson's laboratory at the University of Oklahoma Health Sciences Center. For this Symposium, Ms. **Melody Marcum**, Director of Development of the Dean McGee Eye Institute, worked closely and extensively in selecting and negotiating the meeting venue, and in planning the meals, entertainment and various events. Melody and Holly were crucial to the success of the RD2014 symposium. Also, Dr. **Michael Matthes** in Dr. LaVail's laboratory

played a major role in all aspects in the production of this volume, along with the assistance of Ms. **Cathy Lau-Villacorta**, also in Dr. LaVail's laboratory.

Finally, we honor the monumental efforts of Holly Whiteside. Holly has been the RD Symposium Coordinator since 2000, and during that time she has been the "face" of the RD Symposia. She has been responsible for virtually all of the administrative aspects of the RD Symposia for 16 years, and most repeat attendees feel a close relationship with Holly. She is now stepping back from the efforts of the RD Symposia to pursue personal and professional avenues. We have valued Holly's efforts enormously over these years, and we are proud to dedicate this volume to her.

Catherine Bowes Rickman
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Travel Awards

We gratefully acknowledge National Eye Institute, NIH, USA; the Foundation Fighting Blindness, USA; Pro Retina Germany; the Fritz Tobler Foundation, Switzerland; and Ed and Sandy Gollob for their generous support of 49 Travel Awards to allow young investigators and trainees to attend this meeting. Eligibility was restricted to graduate students, postdoctoral fellows, instructors and assistant professors actively involved in retinal degeneration research. These awards were based on the quality of the abstract submitted by each application. Catherine Bowes Rickman chaired the Travel Awards Committee of 9 senior retinal degeneration investigators, the 6 organizers and Drs. Jacque Duncan, Machelle Pardue and Xian-Jie Yang. The travel awardees are listed below.

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About the Editors

Catherine Bowes Rickman PhD is a tenured Associate Professor of Ophthalmology and of Cell Biology at Duke University located in Durham, NC. Dr. Bowes Rickman leads a team of researchers focused on developing and using mouse models to understand the pathobiology of age-related macular degeneration (AMD) and on developing and testing therapeutic targets for AMD. Dr. Bowes Rickman received her undergraduate degree at the University of California at Santa Barbara, specializing in Biochemistry/Molecular Biology and Aquatic Biology. She earned a PhD from the University of California at Los Angeles and postdoctoral fellowship at the Jules Stein Eye Institute, California, where she focused on mouse models of retinitis pigmentosa. Dr. Bowes Rickman has a long-standing interest in the molecular and cell biology and pathology of the retina. Amongst her seminal discoveries was the identification of the gene responsible for retinal degeneration in the *rd* mouse. She has applied her expertise in mouse genetics to develop models to study age-related macular degeneration (AMD). Currently, she is using several mouse models developed by her group that faithfully recapitulate many aspects of the human AMD phenotype to provide *in vivo* means to examine the pathogenic contribution of genetic, inflammatory and environmental factors to AMD onset and progression. Recently, she successfully demonstrated therapeutic rescue from dry AMD in one of these models. The last few years has been dedicated towards better understanding the impact of the complement system on the onset and progression of AMD using novel mouse models. Dr. Bowes Rickman's research program has been continually funded by the NIH since 1995 and she has also received support from Research to Prevent Blindness (RPB) Foundation, the Foundation Fighting Blindness, the Macular Degeneration program of the American Health Assistance Foundation, Macula Vision Research Foundation, and The Ruth and Milton Steinbach Fund. Dr. Bowes Rickman has received a RPB Career Development Award, a RPB William and Mary Greve Special Scholars Award and an Edward N. & Della L. Thome Memorial Foundation Award. She has published more than 40 original research and review articles and has edited two books on inherited and environmentally induced retinal degenerations. She currently serves on the Scientific Advisory Boards of the Foundation Fighting Blindness (Owings Mills, Maryland), the Beckman Initiative for Macular Research (Irvine, California) and the Macular Degeneration program of the BrightFocus Foundation (Clarksburg, Maryland) and is a consultant for GlaxoSmithKline and Pfizer.

Matthew M. LaVail PhD is Professor of Anatomy and Ophthalmology at the University of California, San Francisco School of Medicine. He received his PhD degree in Anatomy (1969) from the University of Texas Medical Branch in Galveston and was subsequently a postdoctoral fellow at Harvard Medical School. Dr. LaVail was appointed Assistant Professor of Neurology-Neuropathology at Harvard Medical School in 1973. In 1976, he moved to UCSF, where he was appointed Associate Professor of Anatomy. He was appointed to his current position in 1982, and in 1988, he also became Director of the Retinitis Pigmentosa Research Center at UCSF, later named the Kearn Family Center for the Study of Retinal Degeneration. Dr. LaVail has published extensively in the research areas of photoreceptor-retinal pigment epithelial cell interactions, retinal development, circadian events in the retina, genetics of pigmentation and ocular abnormalities, inherited retinal degenerations, light-induced retinal degeneration, and neuroprotective and gene therapy for retinal degenerative diseases. He has identified several naturally occurring murine models of human retinal degenerations and has developed transgenic mouse and rat models of others. He is the author of more than 180 research publications and has edited 16 books on inherited and environmentally induced retinal degenerations. Dr. LaVail has received the Fight for Sight Citation (1976); the Sundial Award from the Retina Foundation (1976); the Friedenwald Award from the Association for Research in Vision and Ophthalmology (ARVO, 1981); two Senior Scientific Investigators Awards from Research to Prevent Blindness (1988 and 1998); a MERIT Award from the National Eye Institute (1989); an Award for Outstanding Contributions to Vision Research from the Alcon Research Institute (1990); the Award of Merit from the Retina Research Foundation (1990); the first John A. Moran Prize for Vision Research from the University of Utah (1997); the first Trustee Award from The Foundation Fighting Blindness (1998); the fourth Llura Liggett Gund Award from the Foundation Fighting Blindness (2007); and he has received the Distinguished Alumnus Award from both his university (University of North Texas) and his graduate school (University of Texas Medical Branch). He has served on the editorial boards of *Investigative Ophthalmology and Visual Science* and *Experimental Eye Research*. Dr. LaVail has been an active participant in the program committee of ARVO and has served as a Trustee (Retinal Cell Biology Section) of ARVO. In 2009, he was appointed an inaugural ARVO Fellow, Gold, of the 12,000-member organization. Dr. LaVail has been a member of the program committee and a Vice President of the International Society for Eye research. He also served on the Scientific Advisory Board of the Foundation Fighting Blindness from 1973–2011. Dr. LaVail retired from the University of California on July 1, 2014, but continues his laboratory research at UCSF as a Recall Emeritus Professor.

Robert E. Anderson MD, PhD is George Lynn Cross Research Professor, Dean A. McGee Professor of Ophthalmology, Professor of Cell Biology, and Adjunct Professor of Geriatric Medicine at The University of Oklahoma Health Sciences Center in Oklahoma City, Oklahoma. He is also Director of Research at the Dean A. McGee Eye Institute. He received his PhD in Biochemistry (1968) from Texas A&M University and his MD from Baylor College of Medicine in 1975. In 1968, he was a postdoctoral fellow at Oak Ridge Associated Universities. At Baylor, he

was appointed Assistant Professor in 1969, Associate Professor in 1976, and Professor in 1981. He joined the faculty of the University of Oklahoma Health Sciences Center in January of 1995. Dr. Anderson served as director of the Oklahoma Center for Neuroscience (1995–1999) and chairman of the Department of Cell Biology (1998–2007). He has received several honorary appointments including Visiting Professor, West China School of Medicine, Sichuan University, Chengdu, China; Honorary Professorship, Xi'an Jiaotong University, Xi'an, China; and Honorary Professor of Sichuan Medical Science Academy, Sichuan Provincial People's Hospital, Sichuan, China. Dr. Anderson has received the Sam and Bertha Brochstein Award for Outstanding Achievement in Retina Research from the Retina Research Foundation (1980), and the Dolly Green Award (1982) and two Senior Scientific Investigator Awards (1990 and 1997) from Research to Prevent Blindness, Inc. He received an Award for Outstanding Contributions to Vision Research from the Alcon Research Institute (1985), and the Marjorie Margolin Prize (1994). He has served on the editorial boards of Investigative Ophthalmology and Visual Science, Journal of Neuroscience Research, Neurochemistry International, Current Eye Research, and Experimental Eye Research. Dr. Anderson has published extensively in the areas of lipid metabolism in the retina and biochemistry of retinal degenerations. He has edited 17 books, 16 on retinal degenerations and one on the biochemistry of the eye. Dr. Anderson has received grants from the National Institutes of Health, The Retina Research Foundation, the Foundation Fighting Blindness, and Research to Prevent Blindness, Inc. He has been an active participant in the program committees of the Association for Research in Vision and Ophthalmology (ARVO) and was a trustee representing the Biochemistry and Molecular Biology section. He was named a Gold Fellow by ARVO in 2009 and received the Proctor Medal from ARVO in 2011. He received the Llura Liggett Gund Lifetime Achievement Award from the Foundation Fighting Blindness in June 2011. In 2012, he received the Paul A. Kayser International Award, Retina Research Foundation. He has served on the Vision Research Program Committee and Board of Scientific Counselors of the National Eye Institute and the Board of the Basic and Clinical Science Series of The American Academy of Ophthalmology. Dr. Anderson is a past Councilor, Treasurer, and President of the International Society for Eye Research.

Christian Grimm PhD is Professor for Experimental Ophthalmology at the University of Zurich, Switzerland. He received his Ph.D. degree at the Institute for General Microbiology at the University of Berne in 1990. After an initial postdoc position in the field of snRNP maturation, Dr. Grimm conducted research at the University of Wisconsin in Madison, WI, where he studied nucleo-cytoplasmic transport of small RNAs. In 1997 Dr. Grimm moved back to Switzerland where he joined the Lab for Retinal Cell Biology in the department of Ophthalmology at the University of Zurich. Dr. Grimm has led the Lab for Retinal Cell Biology since 2006 and was appointed Professor for Experimental Ophthalmology and joined the medical faculty in 2008. Dr. Grimm has published more than 100 original research and review articles, more than 90 in the field of retinal degeneration. His research focuses on molecular mechanisms of photoreceptor cell death, neuroprotection and hypoxic signaling. Dr. Grimm has received the Alfred Vogt Award (2000), the

Retinitis Pigmentosa Award of Pro Retina Germany (2003) and the Pfizer Research Award in Neuroscience (2004). He serves on the Editorial Boards of *Current Eye Research, Experimental Eye Research* and *Molecular Vision*, is Honorary Board member of *Hypoxic Signaling* and acts as a Scientific Review Associate for the *European Journal of Neuroscience*. Dr. Grimm has received research grants from the Swiss National Science Foundation, the European Union, the University of Zurich and several private funding organizations. He serves on the Scientific Advisory Board of the Foundation Fighting Blindness, ProRetina Germany, Retina Suisse and the Swiss Society of Ophthalmology, is member of the committee of the PhD program in integrative molecular medicine (imMed) and is Vice Chairman of the Center for Integrative Human Physiology, a priority research program of the University of Zurich.

Joe G. Hollyfield PhD is Chairman of Ophthalmic Research and the Llura and Gordon Gund Professor of Ophthalmology Research in the Cole Eye Institute at the Cleveland Clinic, Cleveland, Ohio. He received a PhD from the University of Texas at Austin and did postdoctoral work at the Hubrecht Laboratory in Utrecht, The Netherlands. He has held faculty positions at Columbia University College of Physicians and Surgeons in New York City and at Baylor College of Medicine in Houston, Texas. He was Director of the Retinitis Pigmentosa Research Center in The Cullen Eye Institute at Baylor from 1978 until his move to The Cleveland Clinic Foundation in 1995. He is currently Director of the Foundation Fighting Blindness Research Center at the Cleveland Clinic and oversees activity of the Foundation Fighting Blindness Histopathology Center and Donor Eye Program. He has been an annual Visiting Professor in the Department of Ophthalmology at the University of Puerto Rico, Centro Medico, San Juan, Puerto Rico since 1974, where he and his wife, Mary E. Rayborn, teach the development and anatomy of the eye in the "Guillermo Pico Basic Science Course In Ophthalmology", given for ophthalmology residents in Puerto Rico and 18 other countries in Central and South America. Dr. Hollyfield has published over 200 papers in the area of cell and developmental biology of the retina and retinal pigment epithelium in health and disease. He has edited 17 books, 16 on retinal degenerations and one on the structure of the eye. Dr. Hollyfield received the Marjorie W. Margolin Prize (1981, 1994), the Sam and Bertha Brochstein Award (1985) and the Award of Merit in Retina Research (1998) from the Retina Research Foundation, Houston, Texas; the Olga Keith Weiss Distinguished Scholars' Award (1981) and two Senior Scientific Investigator Awards (1988, 1994) from Research to Prevent Blindness, Inc., New York, New York; an award from the Alcon Research Institute (1987), Fort Worth, Texas; the Distinguished Alumnus Award (1991) from Hendrix College, Conway, Arkansas; the Endre A. Balazs Prize (1994) from the International Society for Eye Research (ISER); the Proctor Medal (2009) from the Association for Research in Vision and Ophthalmology (ARVO), and the 2009 Cless "Best of the Best" Award, given by the University of Illinois Eye and Ear Infirmary, Chicago, Illinois. He was an inaugural Gold Fellow of ARVO when this award was established in 2009. Since 1991 he has been Editor-in-Chief of the journal, *Experimental Eye Research*

published by Elsevier, Amsterdam, The Netherlands. Dr. Hollyfield has been active in ARVO since 1971, serving on the Program Committee (1976), as Trustee (Retinal Cell Biology, 1989–1994), as President (1993–1994) and as Immediate Past President (1994–1995). He also served as President (1988–1991) and Secretary (1984–1987) of the International Society of Eye Research. He is Chairman of the scientific review panel for the Macular Degeneration program of the BrightFocus Foundation (Clarksburg, Maryland), serves on the scientific advisory boards of the Foundation Fighting Blindness (Owings Mills, Maryland), the Helen Keller Eye Research Foundation (Birmingham, Alabama), the South Africa Retinitis Pigmentosa Foundation (Johannesburg, South Africa), is Co-Chairman of the Medical and Scientific Advisory Board of Retina International (Zurich, Switzerland), and is a member of the Board of Trustees of Hendrix College.

John D. Ash PhD Francis M. Bullard Eminent Scholar Chair in Ophthalmological Sciences, Department of Ophthalmology, College of Medicine at the University of Florida. Dr. Ash received his PhD from the Ohio State University Biochemistry Program in 1994, and completed postdoctoral training in the Cell Biology Department at Baylor College of Medicine, in Houston, Texas, and began his faculty career at the University of Oklahoma Health Sciences Center, Oklahoma. Dr. Ash is also a Visiting Professor of the Dalian Medical University, Dalian China. Dr. Ash has written and published 56 manuscripts including research articles, book chapters and invited reviews. He is currently an Executive editor for *Experimental Eye research*, and a Scientific Review Editor for *Molecular Vision*. Dr. Ash is an active reviewer for these journals as well as *Investigative Ophthalmology & Visual Science*. In 2009, Dr. Ash received a research award from Hope for Vision, and in 2010 he received a Lew R. Wasserman Merit award from Research to Prevent Blindness, Inc. Dr. Ash has received grants from the National Institutes of Health, the Foundation Fighting Blindness, Research to Prevent Blindness, Inc., Hope for Vision, and the American Diabetes Association. Dr. Ash has served on the Program and Advocacy committees of the Association for Research in Vision and Ophthalmology. Dr. Ash has served on the scientific review panel for Fight For Sight (2005–2008), and is currently serving on the Scientific Advisory Board of the Foundation Fighting Blindness (Columbia, MD) where he chairs the review committee on Novel Medical Therapies Program. He also serves on the scientific review panel for the Macular Degeneration program of the BrightFocus Foundation (formally the American Health Assistance Foundation, Clarksburg, MD).

Part I
Age-Related Macular Degeneration (AMD)

Chapter 1
Apolipoprotein E Isoforms and AMD

Kimberly A Toops, Li Xuan Tan and Aparna Lakkaraju

Abstract The cholesterol transporting protein apolipoprotein E (ApoE) occurs in three allelic variants in humans unlike in other species. The resulting protein isoforms E2, E3 and E4 exhibit differences in lipid binding, integrating into lipoprotein particles and affinity for lipoprotein receptors. ApoE isoforms confer genetic risk for several diseases of aging including atherosclerosis, Alzheimer's disease, and age-related macular degeneration (AMD). A single E4 allele increases the risk of developing Alzheimer's disease, whereas the E2 allele is protective. Intriguingly, the E4 allele is protective in AMD. Current thinking about different functions of ApoE isoforms comes largely from studies on Alzheimer's disease. These data cannot be directly extrapolated to AMD since the primary cells affected in these diseases (neurons vs. retinal pigment epithelium) are so different. Here, we propose that ApoE serves a fundamentally different purpose in regulating cholesterol homeostasis in the retinal pigment epithelium and this could explain why allelic risk factors are flipped for AMD compared to Alzheimer's disease.

Keywords Apolipoprotein E · ApoE isoforms · Age-related macular degeneration · Retinal pigment epithelium · Cholesterol

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C. Bowes Rickman et al. (eds.), *Retinal Degenerative Diseases,* Advances in Experimental Medicine and Biology 854, DOI 10.1007/978-3-319-17121-0_1

1.1 Introduction

Age-related macular degeneration (AMD), like other multifactorial diseases of aging, has no simple genetic underpinning. A complex mixture of environmental factors, lifestyle choices, and genes influence whether AMD will develop, how rapidly it will advance, and how severe the resulting visual dysfunction will be (Fritsche et al. 2014). Vision loss in AMD results from death of the photoreceptors, particularly in the macula. Photoreceptor loss reflects the terminal step in a cascading pathology whose genesis is in the posterior-most portion of the retina: the RPE, Bruch's membrane (BM) and choroid complex.

The tissue that is the initial site of damage in AMD, the RPE, forms the outer blood-retinal barrier and is responsible for the health and maintenance of the photoreceptors and the choriocapillaris (Toops et al. 2014). One of the many functions of the RPE is to act as the central organizing hub for cholesterol homeostasis for the outer retina (Fliesler and Bretillon 2010; Pikuleva and Curcio 2014). Several independent lines of evidence indicate that cholesterol homeostasis in the RPE and adjacent Bruch's membrane is dysregulated in AMD: one, cholesterol-rich lesions with material at least partly derived from the RPE are found in both sub-retinal and sub-RPE deposits (Bowes Rickman et al. 2013; Pikuleva and Curcio 2014). Two, several critical members of the cholesterol homeostasis pathway including hepatic lipase (LIPC), cholesteryl ester transfer protein (CETP), ATP-binding cassette subfamily A member 1 (ABCA1), and apolipoprotein E (ApoE) have been implicated in modulating AMD susceptibility (Katta et al. 2009; Liu et al. 2012; Fritsche et al. 2014). Of these, how ApoE gene variants alter AMD risk is especially intriguing because of the opposite allele-risk associations between AMD and Alzheimer's disease (AD) (Thakkinstian et al. 2006; McKay et al. 2011; Sivak 2013).

1.2 ApoE Isoforms Structure and Function

The human ApoE gene occurs in three allelic variants E2, E3 and E4 that vary by just two nucleotides resulting in three protein isoforms with amino acid variations at positions 112 and 158. These single amino acid changes profoundly effect protein function because they modify salt bridges within different helices of ApoE leading to altered receptor binding and lipid binding (Mahley and Rall 2000; Huang 2010). Key differences between the three ApoE isoforms are summarized in Table 1.1. The E2 isoform binds poorly to the low-density lipoprotein receptor (LDL-R) compared to E3 or E4 (<2%). E4 associates preferentially with very low-density lipoproteins (VLDL) whereas E2 and E3 associate with high-density lipoproteins (HDL) (Mahley and Rall 2000; Huang 2010). Humans are the only known species that express multiple ApoE isoforms. ApoE expressed by non-human primates and mice is structurally homologous to human ApoE4 with Arg at positions 112 and 158; however, these sequences have Thr at position 61 instead of Arg. This single amino acid switch prevents the formation of an N- and C- terminal domain interaction and results in non-human ApoE functioning more like human ApoE3 (Mahley and Rall 2000; Raffai et al. 2001).

Table 1.1 General properties of the three different human ApoE isoforms are summarized. [a]Population frequency is reported for having at least one allele of a given isoform; total estimated frequencies of the six possible ApoE phenotypes are 55% E3/E3, 25% E3/E4, 15% E3/E2, with E4/E4, E2/2, and E4/E2 being rare phenotypes with 1–2% occurrence (Mahley and Rall 2000). [b]Single polymorphisms lead to alternate amino acids at positions 112 and 158 in the human ApoE isoforms protein primary sequence. [c] ApoE2 has been reported to have less than 2% of the binding capability to LDL-R compared to E3 or E4 (Mahley and Rall 2000)

Properties of human ApoE isoforms				
Isoform	Population frequency (%)[a]	Sequence[b] 112 158	LDL-R affinity	Lipoprotein binding
ApoE2	7	Cys Cys	Very low[c]	HDL
ApoE3	78	Cys Arg	High	HDL
ApoE4	15	Arg Arg	High	VLDL, HDL

1.3 Evidence for ApoE in Human Diseases

1.3.1 Hyperlipidemia

ApoE was first implicated in regulating the balance of serum cholesterol and triglyceride levels (Huang 2010). In this context, ApoE, a component of lipoproteins (primarily chylomicrons, VLDL, and a subset of HDL particles), facilitates entry into cells by acting as a ligand for the low-density lipoprotein receptor (LDL-R), LDL-R like protein (LRP), heparan sulfate proteoglycans, and additional non-canonical receptors (Mahley and Rall 2000; Carlo et al. 2013). E4 is highly enriched in VLDL particles due to its altered lipid-binding region that shows a preference for binding triglyceride-enriched particles. E2 and E3 are more common in HDL particles due to a preference in their lipid-binding regions for phospholipids (Huang 2010). Both E2 and E4 alleles are associated with the development of hyperlipidemia and downstream atherosclerotic lesions, but for different reasons (Mahley and Rall 2000; Huang 2010). Because E2 is a much poorer ligand than E4 for LDL-R, effective uptake of HDL particles is prevented, leading to hyperlipidemia type III in E2 homozygotes. The preferential binding of E4 to VLDL particles leads to a feedback loop of decreased cellular uptake of LDL particles, which can result in hyperlipidemia.

1.3.2 Alzheimer's Disease

In contrast to the above scenario, in individuals with either one or two copies of E4 the risk of developing AD increases by 4- or 12-fold respectively compared to E3 homozygotes (Huang 2010). ApoE4 is the best-characterized risk factor for early-onset familial AD and an estimated 65–80% of AD patients have at least one E4 allele (Carter 2007). Conversely, ApoE2 has been proposed to be mildly protective for AD, although this remains a weak association without a clear mechanism (Maezawa et al. 2004). ApoE4 is thought to contribute to AD mainly by altering how neurons

process the amyloid precursor protein (APP) through a cholesterol-mediated pathway. This pathway results in the accumulation of intra- and extra- neuronal toxic amyloid beta (Aβ) fragments, which eventually kill hippocampal neurons (Carter 2007; de Chaves and Narayanaswami 2008; Huang 2010; Leduc et al. 2010). The mechanism for this is complex and depends on interactions between ApoE, ApoE cell surface receptors, cholesterol, APP and Aβ, within neurons and in the surrounding astrocytes and extracellular space. E4 appears to stabilize toxic Aβ oligomers, which renders them resistant to lysosomal degradation (Cerf et al. 2011). E4 contributes to AD via other mechanisms that are independent of Aβ: one, E4 is a poor supplier of cholesterol for membrane repair in damaged neurons (Rapp et al. 2006; de Chaves and Narayanaswami 2008; Leduc et al. 2010); and two, E4 acts as a pro-inflammatory molecule to exacerbate neuronal damage (Guo et al. 2004).

1.3.3 Age-Related Macular Degeneration

Epidemiological studies suggest that ApoE2 confers risk in AMD, whereas ApoE4 appears to be protective, although the association of E4 with protection is stronger than E2 with risk (McKay et al. 2011). ApoE and its cargo, cholesterol, are abundant components of drusen, the protein- and lipid-rich lesions in the Bruch's membrane characteristic of AMD (Anderson et al. 2001; Curcio et al. 2011; Bowes Rickman et al. 2013; Pikuleva and Curcio 2014). ApoE in drusen could originate from either the retina or the choroidal circulation (or both, since these sources are not mutually exclusive). However, mounting evidence indicates that the material that forms drusen, including ApoE, is secreted from the RPE (even if it is initially transported into the retina from the circulation, as may be the case for certain lipids) (Pikuleva and Curcio 2014). Thus, the retina is an active cholesterol producing and processing tissue and cholesterol efflux mechanisms are critical for maintaining retinal cholesterol homeostasis (Fliesler and Bretillon 2010; Pikuleva and Curcio 2014).

1.4 Cellular Identity and Differential ApoE Function Contributing to Risk

How ApoE4 can be detrimental to neuronal health has been studied extensively in AD. Little is currently known regarding isoform-specific functions of ApoE in the RPE and how these could contribute to AMD. Local sources of ApoE within the retina are the RPE and the Muller glia, indicating that ApoE is a major cholesterol transport in the retina (Anderson et al. 2001; Li et al. 2006; Johnson et al. 2011). RPE cells express the uptake receptors for ApoE (LDL-R and LRP) as well as the machinery for cholesterol efflux (ABCA1 and ABCG1) (Ebrahimi and Handa 2011; Pikuleva and Curcio 2014). Since cholesterol (free, esterified, and oxidized) is a core component of drusen (Curcio et al. 2005), dysregulation of cholesterol

homeostasis seems to be a key player in AMD pathology (Curcio et al. 2011; Ebrahimi and Handa 2011; Pikuleva and Curcio 2014). And it is in this characteristic that hippocampal neurons and RPE cells most likely diverge.

First, whereas RPE have the capacity to synthesize and take up ApoE-containing lipoproteins, neurons are largely at the mercy of the astrocytes for ApoE production and lipid transport (Leduc et al. 2010). This is a critical distinction since very little cholesterol enters the CNS from the circulation and neurons rely on local synthesis and transport of cholesterol to generate and maintain their long membrane-rich axons. As a reflection of this, neuronal plasma membrane has high levels of lipoprotein receptors particularly LRP, which has a strong preference for ApoE2 and E3 (Rapp et al. 2006). On the other hand, although RPE cells express ApoE receptors, they seem to be spatially discreet (i.e., apical vs. basolateral distributions) and with a different abundance (Tserentsoodol et al. 2006a; Tserentsoodol et al. 2006b; Zheng et al. 2012). A comprehensive analysis of this expression remains to be done.

The RPE therefore acts as a hub for ingress and egress of ApoE-cholesterol, while neurons are largely a terminal acceptor. This implies that as far as ApoE is concerned, RPE may be more similar to astrocytes then neurons. Astrocytes are also active producers of ApoE-cholesterol particles and like the RPE, express ABCA1 and ABCG1, which participate in efflux of ApoE rich pseudo-HDL particles (Wu et al. 2010; Johnson et al. 2011; Ito et al. 2014). Astrocytes express LDL-R and LRP but appear to preferentially bind and uptake ApoE4 and E3 containing lipoproteins (Rapp et al. 2006). Astrocytes exposed to ApoE2-, E3- or E4-loaded cholesterol exhibited ApoE isoform-dependent uptake (E4 = E3 > E2) that was exactly opposite to that seen in neurons (E2 = E3 > E4). Further, astrocytes internalized their cholesterol efficiently, whereas in neurons, the cholesterol was retained on the plasma membrane.

1.5 Implications

If the RPE is functionally similar to astrocytes with regard to cholesterol handling, rather than neurons, then the reversed risk alleles for AD and AMD may not be such a puzzle after all. The RPE and astrocytes can preferentially efflux ApoE containing pseudo-HDL particles for efficient intercellular cholesterol transport. In the brain, this becomes problematic for neurons in ApoE4 expressors because poor cholesterol efflux both increases Aβ generation and decreases its degradation. In the retina, a different balance is struck because the RPE is capable of both efflux and re-uptake. This will be more efficient for E4 than E2 due to the presence of LDL-R in RPE, which avidly binds E3 and E4 but has almost no affinity for E2. Experiments aimed at testing how efficiently different ApoE isoforms traffic cholesterol in and out of the RPE will help establish a cellular, mechanistic basis for puzzling epidemiological data.

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Chapter 2
Role of Chemokines in Shaping Macrophage Activity in AMD

Matt Rutar and Jan M Provis

Abstract Age-related macular degeneration (AMD) is a multifactorial disorder that affects millions of individuals worldwide. While the advent of anti-VEGF therapy has allowed for effective treatment of neovascular 'wet' AMD, no treatments are available to mitigate the more prevalent 'dry' forms of the disease. A role for inflammatory processes in the progression of AMD has emerged over a period of many years, particularly the characterisation of leukocyte infiltrates in AMD-affected eyes, as well as in animal models. This review focuses on the burgeoning understanding of chemokines in the retina, and their potential role in shaping the recruitment and activation of macrophages in AMD. Understanding the mechanisms which promote macrophage activity in the degenerating retina may be key to controlling the potentially devastating consequences of inflammation in diseases such as AMD.

Keywords Retinal degenerations · Age-related macular degeneration (AMD) · Inflammation · Macrophages · Microglia · Chemokines

2.1 Introduction

Age-related macular degeneration (AMD) affects millions of individuals worldwide, and is the leading cause of blindness in the industrialised world (Ambati et al. 2003a). AMD is a multifactorial disorder, involving complex interaction between environmental and genetic factors. Evidence for a role of inflammation

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C. Bowes Rickman et al. (eds.), *Retinal Degenerative Diseases,* Advances in Experimental Medicine and Biology 854, DOI 10.1007/978-3-319-17121-0_2

in progression AMD has been accruing over a period of many years, particularly through the observations of leukocyte infiltrates within AMD-affected eyes (Penfold et al. 2001; Forrester 2003).

2.2 Macrophage Recruitment in AMD

The involvement of inflammatory processes in the histopathology of AMD was first noted almost 100 years ago (Hegner 1916), and several histological studies since have established the presence of aggregations of choroidal leukocyte infiltrates in association with disciform macular lesions (Hegner 1916; Paul 1927; Green and Key 1977).

Those early observations were confirmed and extended in a number of electron microscopical investigations which demonstrated the involvement of a number of inflammatory cells—including macrophages, lymphocytes, and mast cells—in RPE atrophy, and breakdown of Bruch's membrane (Penfold et al. 1984, 1985). Macrophages and other leukocytes have also been described in excised neovascular membranes (Lopez et al. 1991; Gehrs et al. 1992; Seregard et al. 1994). Ultrastructural studies also identified a close relationship between macrophages and the formation of choroidal neovascular membranes in wet AMD (Penfold et al. 1987). Multinucleated giant cells—which may form through union of multiple macrophages or microglia (Dickson 1986)—have also been found to correlate spatially with regions of breakdown in Bruch's membrane and with CNV (choroidal neovascularisation) (Penfold et al. 1985). Chronic involvement of macrophages and giant cells has also been shown in atrophic AMD lesions, and on the expanding edges (Penfold et al. 1987; Cherepanoff et al. 2009). Other investigations have shown changes in parenchymal microglia in association with early AMD, including increased MHC-II expression and morphological changes suggestive of activation (Penfold et al. 1997). In advanced AMD, activated amoeboid microglia infiltrate the ONL and subretinal space in the degenerating outer retina, where they are associated with neovascular structures (Combadiere et al. 2007), and appear to have a role in the phagocytosis of photoreceptor debris (Gupta et al. 2003; Combadiere et al. 2007).

2.3 Role of Chemokines

First discovered in 1987 (Walz et al. 1987; Yoshimura et al. 1987), chemokines are a large, growing family comprising more than 50 molecules interacting with at least 20 chemokine receptors, that play an important role in the chemotactic guidance of leukocyte migration and activation (Moser and Loetscher 2001; Bajetto et al. 2002). Chemokines are small molecules grouped according to the relative position of their first N-terminal cysteine residues, comprising C (γ chemokines), CC (β chemokines), CXC (α chemokines), and CX3C (δ chemokines) families (Loetscher et al.

2000; Murphy et al. 2000; Zlotnik and Yoshie 2000; Bajetto et al. 2002). These may be expressed by endothelial cells, resident macrophages (including microglia), as well as infiltrating leukocytes (Crane and Liversidge 2008). Chemokines exert their biological activity through binding cell surface chemokine receptors, which are part of the superfamily of seven transmembrane domain receptors that signal through coupled heterotrimeric G-proteins, consisting of C, CC, CXC, CX3C receptor subclasses (Bajetto et al. 2002). Many of these receptors show a degree of redundancy, as multiple chemokines may bind several receptors; although interactions are mainly restricted to within particular subclasses (Bajetto et al. 2002). Chemokine expression typically generates chemical ligand gradients, which serve as directional cues for guidance of leukocytes bearing the appropriate chemokine receptors to sites of injury, and are also thought to aid in extravasation of leukocytes (Luster 1998).

The expression of chemokines in the guidance and activation of macrophages has garnered considerable interest in AMD. Retinas from human donors show increased expression of both α (Cxcl1, Cxcl1) and β (Ccl2) chemokine genes in 'wet' and 'dry' AMD (Newman et al. 2012), while elevated levels of Ccl2 protein—a potent chemoattractant for monocytes (Matsushima et al. 1989; Yoshimura et al. 1989)—have been detected in aqueous humour samples taken from patients in advanced stages of AMD (Jonas et al. 2010; Kramer et al. 2011). Additionally, elevation in Ccl2 is evident within atrophic 'dry' AMD lesions and is accompanied by influxes of monocytes expressing Ccr2 (Sennlaub et al. 2013), which is the receptor for Ccl2 signalling (Yoshimura and Leonard 1990).

A direct for role of chemokines has been elucidated with animal models of AMD (Patel and Chan 2008). Investigations using laser-induced CNV in mice have focused on the role of β chemokine signalling in neovascular AMD. Ablation of Ccl2 using target gene knockout has been shown to inhibit the infiltration of macrophages and results in reduced lesion size following laser-induced CNV compared to controls (Luhmann et al. 2009). Moreover, a mouse knockout of the receptor Ccr2 exhibits decreased macrophage recruitment and vastly reduced neovascularisation following experimental laser-induced CNV (Tsutsumi et al. 2003). In models of atrophic 'dry' AMD which utilise bright light as a damaging stimulus (Marc et al. 2008; Rutar et al. 2010), the suppression of Ccl2 using either ablation or siRNA-mediated knockdown reduces macrophage recruitment and the extent of cell death (Rutar et al. 2012; Sennlaub et al. 2013). Conversely, other studies suggest that a degree of β chemokine signalling may be necessary for the maintenance retinal homeostasis, and prevention of AMD. An investigation in aged, dual Ccl2/Ccr2 knockout mice showed retinal features similar to AMD including formation of lipofuscin, drusen, photoreceptor degeneration, and neovascularisation (Ambati et al. 2003b), although the AMD-like phenotype in this model has been questioned (Luhmann et al. 2009). Ccl2/Ccr2 knockout results in the accumulation of hypertrophied subretinal macrophages, possibly because of impaired monocyte trafficking (Luhmann et al. 2009).

The only δ chemokine receptor characterised, Cx3cr1, has also been implicated in maintenance of homeostasis and genesis of AMD-like pathology. Cx3cr1 is a chemokine receptor found on microglia, macrophages, astrocytes, and T-cells (Patel

and Chan 2008), whose ligand chemokine Cx3cl1 is constitutively expressed on many cell types in the retina, and together are thought to mediate the trafficking of microglia and macrophages in the clearance of extracellular deposits (Fong et al. 1998; Silverman et al. 2003). Targeted knockout of Cx3cr1 in light-stressed mice induces progressive degeneration of photoreceptors in correlation with an accumulation of engorged subretinal microglia/macrophages and other AMD-like features (Combadiere et al. 2007). Moreover, ablation of Cx3cr1 is associated with an increase in lesion size following experimental neovascularisation (Combadiere et al. 2007).

2.4 Summary

Over a period of many years, the role of inflammation in AMD has gradually emerged as an important factor underpinning its pathogenesis. This is exemplified by traditional histological examinations and electron microscopy identifying macrophage/microglial infiltration in AMD-effected eyes, and more recently through investigations utilising animal models. The expression of chemokine-related genes is prodigious in all forms of AMD pathology, and animal models of both and 'dry' and 'wet' AMD indicate that chemokine expression modulates both the recruitment and activation of macrophages, as well as the extent of retinal degeneration. Reducing inflammation by altering macrophage activity in retina may prove an important therapeutic tool in ameliorating degeneration in AMD.

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Chapter 3
Biology of p62/sequestosome-1 in Age-Related Macular Degeneration (AMD)

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Abstract p62/sequestosome-1 is a multidimensional protein that interacts with many signaling factors, and regulates a variety of cellular functions including inflammation, apoptosis, and autophagy. Our previous work has revealed in the retinal pigment epithelium (RPE) that p62 promotes autophagy and simultaneously enhances an Nrf2-mediated antioxidant response to protect against acute oxidative stress. Several recent studies demonstrated that p62 contributes to NFkB mediated inflammation and inflammasome activation under certain circumstances, raising the question of whether p62 protects against or contributes to tissue injury. Herein, we will review the general characteristics of p62, focusing on its pro- and anti-cell survival roles within different physiological/pathological contexts, and discuss the potential of p62 as a therapeutic target for AMD.

Keywords AMD · RPE · p62 · sqstm1 · Autophagy · Nrf2 · Neurodegeneration · NFκB · PB1

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C. Bowes Rickman et al. (eds.), *Retinal Degenerative Diseases,* Advances in Experimental Medicine and Biology 854, DOI 10.1007/978-3-319-17121-0_3

3.1 Introduction

AMD is the most common cause of blindness among the elderly in western countries (Kaarniranta et al. 2011), and is characterized by dysfunction of the retinal pigment epithelium (RPE). The RPE is under constant oxidative challenge due to phagocytosis and exposure to UV light. Removal of oxidized/misfolded proteins relies on the proteasome and autophagy. We showed that acute stress inhibits the proteasome, but up-regulates anti-oxidant and autophagy related genes, including p62 (Cano et al. 2014). We also confirmed p62's protective role in the RPE, via both autophagic clearance and activation of Nrf2 antioxidant signaling (Wang et al. 2014). As AMD shares pathological and mechanistic features with other adult-onset neurodegenerative diseases (Glass et al. 2010; Kaarniranta et al. 2011), our studies on p62's role in AMD could contribute to the understanding of these diseases.

3.2 Structure and Functions of p62

p62 was initially discovered as an interacting partner of atypical protein kinase C (aPKC) (Puls et al. 1997; Sanchez et al. 1998) via its N-terminal Phox/Bem 1p (PB1) domain, and mediating the activation of NFkB signaling. The following ZZ zinc-finger domain binds receptor interacting protein (RIP), also linking p62 to NFkB signaling. The TRAF6 binding (TB) domain binds TRAF6, which is relevant in osteoclastogenesis, as well as Ras-induced tumorigenesis (Nakamura et al. 2010). Downstream of TB domain, the LC3-interacting region (LIR) interacts with autophagosome protein Atg8/LC3, and the Keap1-interacting region (KIR) is involved with Nrf2 regulation. At the C-terminus, the ubiquitin-associated (UBA) domain regulates p62's interaction with polyubiquitinated proteins targeted for autophagic degradation (Matsumoto et al. 2011). As Table 3.1 shows, p62 is rich in protein-interacting sequences. Its N-terminal region mainly regulates inflammatory responses, and the C-terminal domains mostly contribute to stress reduction. (See Fig. 3.1)

Multiple p62 isoforms have been identified in different species. The rat expresses three p62 protein isoforms (Gong 1999; Croci et al. 2003). The ratio of rat p62

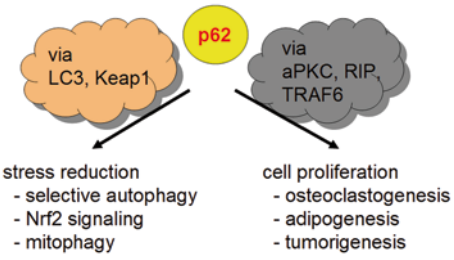

Fig. 3.1 p62 can be either protective or damaging. Its role is determined by its interacting partners, in different pathological context and tissue types

Table 3.1 Studies on p62 functional domains and covalent modifications

References	Studies on individual domain or mutation
(Puls et al. 1997)	p62 interacts with aPKC via the N-terminal PB1 domain
(Bjorkoy et al. 2005)	LC3 interacts with p62
(Jain et al. 2010)	KIR (keap1 interacting region) is mapped
(Linares et al. 2011)	Phosphorylation at T269, S272 influences mitosis and cell proliferation
(Matsumoto et al. 2011)	Phosphorylation at S403 determines its affinity for ubiquitinated cargo
(Ichimura et al. 2013)	Phosphorylation at S351 in an mTORC-1 dependent manner determines its affinity to Keap1
(Shi et al. 2013)	p62 cleavage at TB disrupts autophagy and impairs NFkB signaling

isoform1/isoform2 is tissue specific, and is dynamically regulated in response to stimulation. Humans express two p62 isoforms, of which isoform2 is 84 amino acids shorter at the N-terminus, equivalent to the loss of PB1 domain. Our studies demonstrated that all p62 mRNA species are expressed in cultured human RPE cells, but isoform2 is barely translated (Wang et al. 2014), thus its functional role requires further investigation in AMD patients.

3.3 p62 Protects by Enhancing Autophagic Clearance and Activating Nrf2 Signaling

Aggregates of misfolded/damaged proteins are transported to the autophagy machinery for degradation (Matsumoto et al. 2011). p62 functions as a cargo receptor, binding to polyubiquitinated proteins and guiding them to the autophagosome. Our studies confirmed in RPE cells, that p62 silencing caused cargo loading failure and inefficient autophagy, as demonstrated by a reduced LC3 conversion ratio. Overexpression of p62 gave the opposite results. Interestingly, p62's influence on selective autophagy was observed only when cells were under oxidative stress. We speculate that under basal conditions, RPE cells rely on other protective mechanisms such as the proteasome, and that p62 mediated autophagy is recruited to deal with overwhelming stress.

Along with the p62 mediated autophagic clearance, the antioxidant transcription factor Nrf2 is activated to help maintaining redox homeostasis. Keap1, known to sequester Nrf2 in the cytosol and inhibit its activity, is bound by p62, thus releasing Nrf2 to activate the antioxidant genes (Komatsu et al. 2010). Our studies confirmed in RPE that p62 enhanced Nrf2 activity, and Nrf2 upregulated p62 expression at transcriptional level, thus forming a positive feedback loop. These findings indicate that in response to an acute stress, p62 provides dual cytoprotection to RPE, via autophagic clearance of insoluble proteins and activation of Nrf2 signaling.

3.4 p62, A Double Edged Sword

With aging, the p62 promoter undergoes oxidative damage (Du et al. 2009b; Du et al. 2009a), consistent with our observation of reduced p62 mRNA expression in elderly mouse RPE (unpublished data). We would predict a decline of p62 in the AMD mouse model (Cano et al. 2010) and AMD patients, but p62 accumulation was observed instead (unpublished data). Similar observations were made in neurodegenerative patients (see Table 3.2). This contradiction could result from post-transcriptional up-regulation of p62 to rescue damaged cells, but it is questionable whether p62 can still promote clearance of protein aggregates when the whole autophagy machinery undergoes irreversible failure. It was reported that in autophagy-deficient livers, p62 ablation actually reduced toxicity and prevented cell death (Komatsu et al. 2007).

In vitro studies revealed p62's role in NFkB signaling and inflammasome activation (Takeda-Watanabe et al. 2012; Park et al. 2013). p62 could be a double edged sword - it fights against stress, yet it can promote inflammation, exacerbating cellular crisis. (see Fig. 3.1) Since autophagy failure and a weakened Nrf2 response in the RPE is a component of AMD, the accumulated p62 in disease area possibly exerts a harmful effect by aggravating chronic inflammation, a common feature of neurodegenerative diseases.

Table 3.2 p62 dysregulation is associated with a number of diseases

References	Studies on p62 function	Disease
(Rea et al. 2006)	K378X mutation in p62 is associated with increased NFkB signaling and osteoclast formation	Paget's disease of bone
(Ramesh Babu et al. 2008)	p62 KO leads to accumulation of hyperphosphorylated tau	Alzheimer's disease
(Daroszewska et al. 2011)	p62 mutation (P394L) is associated with bone lesions	Paget's disease of bone
(Braak et al. 2011)	p62 immunostaining in the neurosecretory cells of the paraventricular nucleus	Parkinson's disease
(Salminen et al. 2012)	Lack of p62 provokes the tau pathology; reduced p62 levels were observed in the frontal cortex of AD patients	Alzheimer's disease
(Hirano et al. 2013)	p62 mutations (Ala53Thr, Pro439Leu) are associated with ALS	Amyotrophic lateral sclerosis
(Rue et al. 2013)	p62 accumulation occurs in neuronal nuclei, colocalizing with huntingtin inclusions	Huntington's disease

3.5 Future Experimental Approaches

To evaluate p62's potential as a therapeutic target for AMD, we must elucidate its role under chronic stress (Cano et al. 2010; Wang and Neufeld 2010), to determine:

1) if p62 undergoes posttranscriptional alteration, such as mRNA splicing;
2) if p62 activity is regulated by novel covalent modifications;
3) if p62 has unidentified interacting protein partners under pathological conditions.

A thorough understanding of p62's regulatory mechanism could lead to new therapeutic methods for AMD.

Acknowledgments Funding for this work was provided by NIH EY019904 (JTH), Beckman Foundation AMD Grant (JTH), Thome Foundation (JTH), Research to Prevent Blindness Senior Scientist Award (JTH), NIH P30EY001765 core grant, the Robert Bond Welch Professorship (JTH), a gift from the Merlau family, and an unrestricted grant from RPB to the Wilmer Eye Institute.

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Chapter 4
Gene Structure of the 10q26 Locus: A Clue to Cracking the ARMS2/HTRA1 Riddle?

Elod Kortvely and Marius Ueffing

Abstract Age-related macular degeneration (AMD) is a sight-threatening disorder of the central retina. Being the leading cause of visual impairment in senior citizens, it represents a major public health issue in developed countries. Genetic studies of AMD identified two major susceptibility loci on chromosomes 1 and 10. The high-risk allele of the 10q26 locus encompasses three genes, PLEKHA1, ARMS2, and HTRA1 with high linkage disequilibrium and the individual contribution of the encoded proteins to disease etiology remains controversial. While PLEKHA1 and HTRA1 are highly conserved proteins, ARMS2 is only present in primates and can be detected by using RT-PCR. On the other hand, there is no unequivocal evidence for the existence of the encoded protein. However, it has been reported that risk haplotypes only affect the expression of ARMS2 (but not of HTRA1), making ARMS2 the best candidate for being the genuine AMD gene within this locus. Yet, homozygous carriers of a common haplotype carry a premature stop codon in the ARMS2 gene (R38X) and therefore lack ARMS2, but this variant is not associated with AMD. In this work we aimed at characterizing the diversity of transcripts originating from this locus, in order to find new hints on how to resolve this perplexing paradox. We found chimeric transcripts originating from the PLEKHA1 gene but ending in ARMS2. This finding may give a new explanation as to how variants in this locus contribute to AMD.

Keywords Age-related macular degeneration · HTRA1 · ARMS2 · PLEKHA1 · Chimeric transcripts · Gene transcription · Alternative splicing · rs10490924 · rs11200638 · rs2736911

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C. Bowes Rickman et al. (eds.), *Retinal Degenerative Diseases,* Advances in Experimental Medicine and Biology 854, DOI 10.1007/978-3-319-17121-0_4

4.1 Introduction

Age-related macular degeneration (AMD) is a common blinding disease of the elderly with an exceedingly intricate etiology. An interplay of non-modifiable (i.e. multiple genetic variants) and modifiable (i.e. environmental) factors contribute to disease risk (Seddon and Chen 2004).

The involvement of the complement system had been already proposed in 2001 (Hageman et al. 2001), and four years later genome-wide linkage scans indeed identified complement factor H (CFH) as the first major susceptibility gene for AMD (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005). The second major susceptibility locus was identified shortly after the publication of the above results (Jakobsdottir et al. 2005). This locus on chromosome 10q26 exhibits an even stronger association signal overlying three genes: Pleckstrin Homology Domain Containing, Family A Member 1 (PLEKHA1), Age-Related Maculopathy Susceptibility 2 (ARMS2), and HtrA serine peptidase 1 (HTRA1). Because of the close vicinity of these genes, association studies lack the required discriminative power to determine the causative gene/variant. PLEKHA1 is apparently outside the linkage disequilibrium block exhibiting the peak association. In contrast, there are numerous papers suggesting a role for ARMS2 (Rivera et al. 2005; Fritsche et al. 2008) or for HTRA1 (Dewan et al. 2006; Yang et al. 2006) in AMD. Furthermore, Yang et al. suggests a two-hit model, claiming that both genes are simultaneously affected by the risk haplotype (Yang et al. 2010).

It has been reported in numerous Mendelian diseases that protein products of causal genes tend to physically interact (Brunner and van Driel 2004; Franke et al. 2006). Similarly, growing evidence suggests that products of genes in complex trait-associated loci establish functional protein-protein bindings. The dominance of components belonging to the alternative complement pathway among the proteins implicated in AMD strongly supports this concept. Taking this idea one step further, the sought-after gene within the PLEKHA1/ARMS2/HTRA1 locus should code for a protein that is linked to one of the few disease pathways implicated in AMD (Kortvely and Ueffing 2012). From this vantage point, HTRA1 seems to be the most attracting candidate, because it is involved in the remodeling of the extracellular matrix and participates in TGF beta signaling hinting toward involvement in choroidal neovascularization, a hallmark of the wet form of AMD (Clausen et al. 2011).

In this work we set out to characterize the transcripts generated from the 10q26 locus in order to disentangle the individual effects of these genes on AMD risk. Understanding the regulation of gene expression within this chromosomal region may offer a new explanatory framework to resolve the debate about the AMD gene conferring the highest risk.

4.2 Materials and Methods

4.2.1 Phylogenetic Analysis

To identify the potential homologs/paralogs for the ARMS2 gene and the corresponding putative protein, BLAST searches were performed on the public databases at NIH. Alignments of deduced protein sequences were carried out with the multiple alignment software Geneious (version 7.1). The evolutionary dendrogram (unrooted tree) was calculated by using the Neighbor-Joining method.

4.2.2 RT-PCR

Total RNA was extracted from human term placenta. The RT reaction was performed using 2 ∝g RNA with an oligo(dT) primer using the Omniscript RT kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's manual. The following primers were used to detect chimeric transcripts: 5'-ATAACCTAAGTCGCCATGGTG-3' (PLEKHA1 forward), 5'-CAGTTGAGGCAGCTGGAGGG-3' (ARMS2, reverse). Amplified products were cloned and sequenced.

4.3 Results and Discussion

4.3.1 Phylogeny of ARMS2

While the other two genes (PLEKHA1 and HTRA1) of the 10q26 locus are conserved throughout the vertebrates and beyond, ARMS2 is only found in higher primates (more precisely in simians, Fig. 4.1). Strikingly, the evolutionary appearance of ARMS2 parallels the anatomical specialization of the macula. Most importantly, this specialization represents a tradeoff between performance and vulnerability. The restricted blood supply and the concomitant metabolic stress may even play a role in macular differentiation (Provis et al. 2005; Yu et al. 2010). Like humans, macaque monkeys possess a macula and develop age-related macular pathologies and share risk variants with humans (Francis et al. 2008).

Although the vast majority of genes present in any species descend from a gene present in an ancestor, some genes originate from ancestrally non-genic sequences (Carvunis et al. 2012). In fact, de novo gene birth from a pool of pre-existing open reading frames may be more prevalent than sporadic gene duplication. Accordingly, ARMS2 may be evolved from a placeholder sequence separating PLEKHA1 and

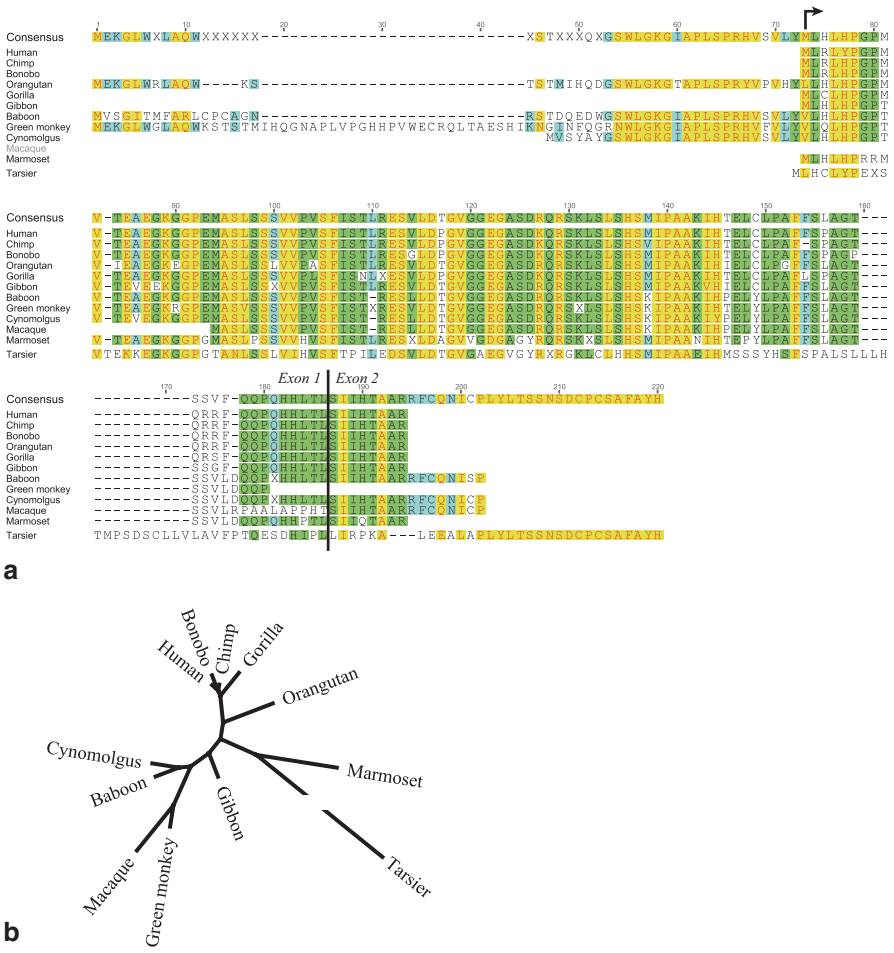

Fig. 4.1 **a** Multiple alignment of predicted ARMS2 amino acid sequences. The putative transcription initiation site in human is marked with a broken *arrow*. Identical residues are indicated by *red* letters on *yellow* background and similar residues are indicated by *green* background. A vertical *line* shows the boundary between the regions encoded by exon 1 and 2. Note that the deduced tarsier sequence (a species not belonging to the simian infraorder) only exhibits a weak similarity to the consensus, thus it is unlikely to exist at protein level. **b** Evolutionary dendrograms of ARMS2 orthologs generated using the Geneious program. Shorter branches indicate larger similarities Tarsier seems to be diverged before the appearance of the functional ARMS2 gene

HTRA1. Primate-specific transcriptional units were found (1) to have transcript lengths comparable with the average length of human cDNAs, and few exons, (2) preferentially expressed in the reproductive system, and (3) to be frequently intercalated in the introns of known protein-coding genes (Tay et al. 2009). To what extent does ARMS2 fit this profile? ARMS2 is indeed composed of only two exons, though the length of the transcript is below the average. Studies suggest that

ARMS2 is primarily expressed in the placenta, being a part of the female reproductive system. Furthermore, we found chimeric transcripts containing exons from both PLEKHA1 and ARMS2 (see below).

4.3.2 Transcript Diversity Originating from the 10q26 Locus

Since it can be easily amplified by RT-PCR, it is generally accepted that ARMS2 exists at RNA level. Beside moderate expression in the placenta, weak expression was detected in the retina (Rivera et al. 2005). Similarly, the transcript was detected in various cell lines (Kanda et al. 2007) and its characteristics fulfill the definition of being a messenger RNA: It possesses a well-defined transcription start site (Fritsche et al. 2008), 5′- and 3′-untranslated regions, two exons separated by a GT-AG intron, and finally a canonical polyadenylation signal and a poly (A) tail. Nevertheless, the detection of the native transcript by Northern analysis still has to be done.

Notably, it has been hypothesized that the defective processing of ARMS2 pre-mRNA due to the removal of the polyadenylation signal by an insertion/deletion in carriers of the risk haplotype is the underlying cause for AMD (Fritsche et al. 2008). Adding to the confusion is the fact that yet another haplotype (R38X) also leads to the failure of ARMS2 synthesis (Fig. 4.2), but this variant is neutral in AMD, thereby contradicting the degradation hypothesis (Allikmets and Dean 2008). Furthermore, in-depth reporter gene assays and the analysis of a large series of human post-mortem retina/RPE samples revealed that the risk haplotype affects ARMS2 but not HTRA1 mRNA expression (Friedrich et al. 2011). Because the lack of ARMS2 does not necessarily leads to AMD and the expression of HTRA1 is not changed in risk vs. non-risk haplotypes, the authors conclude that currently unknown mechanisms mediate the pathogenic effects of the risk-associated variants at the 10q26 AMD locus. It has been also speculated that ARMS2 exists as a non-coding mRNA only. However, antibodies against different epitopes of ARMS2

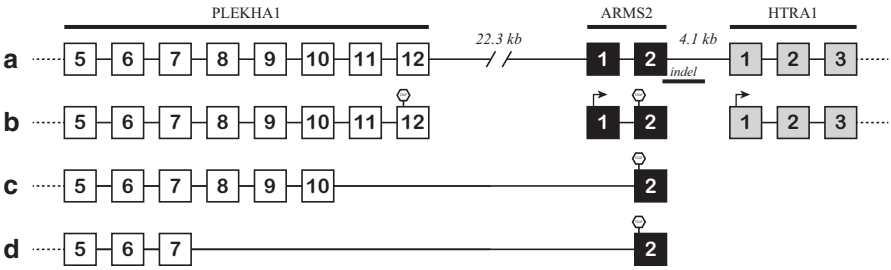

Fig. 4.2 Schematic representation of PLEKHA1/ARMS2 transcript chimerism. Transcription start and stop signals are marked with *broken arrows* and stop signs, respectively. **a** Genomic organization of the 10q26 locus. Only distal exons of PLEKHA1 and proximal exons of HTRA1 are shown. **b** Canonical transcripts of the three genes. **c** and **d** Different spliced isoforms. Note that the indel variant most probably influences the expression of these mRNAs, while the R38X mutation in the first exon of ARMS2 does not

gave rise to identical staining pattern in the choroid layer of human eyes (Kortvely et al. 2010) and Western analyses using the same monoclonals also reveal a single band of the expected size in placental lysates (our unpublished data), supporting the presence of ARMS2 proteins.

Here we propose that the phylogeny of ARMS2 may hold the key to resolve this controversy. Alternative transcript variants have already been described for ARMS2 (Wang et al. 2012). We also examined the exon-intron structure of the transcripts for the entire 10q26 region aimed at finding novel alternative variants also affected by the presence of the risk haplotype. This approach has led to the identification of PLEKHA1/ARMS2 chimeric transcripts (Fig. 4.2). With respect to chimeric proteins, the ENCODE project discovered that gene boundaries extend well beyond the annotated termini in 65 % of cases, often encompassing parts of neighboring genes and at least 4–5 % of the tandem genes in the human genome can be transcribed into a single RNA sequence (Gingeras 2009). Such chimeric mRNAs can augment the number of gene products (Akiva et al. 2006; Parra et al. 2006).

PLEKHA1 and ARMS2 are two adjacent genes in the same orientation that are usually transcribed independently, but occasionally transcribed into a single RNA sequence whose splicing product encodes a protein including coding exons from the two genes. Consequently, the risk variants of the 10q26 locus may also affect the expression of these fusion transcripts, even if the majority of the corresponding gene is outside the linkage block. Since these chimeric RNAs are significantly more tissue-specific than non-chimeric transcripts (Frenkel-Morgenstern et al. 2012), they can exert their biological function restricted, for example, to the eye.

It is of note that we could not detect transcripts containing exons from both ARMS2 and HTRA1, although the intergenic segment is significantly shorter than the one between PLEKHA1 and ARMS2.

In conclusion, the risk variant of the 10q26 locus may influence the expression of these chimeric transcripts and this can exert a pathogenic effect in the eye. Further experiments are warranted to determine the relevance of the corresponding putative chimeric proteins in AMD pathology.

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