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Constancy of ERp29 Expression in Cultured Retinal Pigment Epithelial Cells in the *Ccl2/Cx3cr1* Deficient Mouse Model of Age-Related Macular Degeneration

Varun Verma, Theodor Sauer, Chi-Chao Chan, and Min Zhou

Immunopathology Section, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

Congxiao Zhang

Section on Retinal Diseases and Therapeutics, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

Arvydas Maminishkis

Section on Epithelial and Retinal Physiology and Disease, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

Defen Shen and Jingsheng Tuo

Immunopathology Section, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract

Purpose—Given the critical role of the retinal pigment epithelium (RPE) in the pathogenesis of age-related macular generation (AMD) and the links drawn between chaperone proteins and neurodegenerative disease, we aimed to culture RPE from the *Ccl2*^{−/−}/*Cx3cr1*^{−/−} mouse model of AMD and evaluate expression of chaperone protein ERp29.

Materials and Methods—Murine RPE cells were surgically and chemically isolated and cultured. ERp29 mRNA and protein expression were evaluated by real-time RT-PCR, immunohistochemistry, and Western blot.

Results—*Ccl2*^{−/−}/*Cx3cr1*^{−/−} RPE was successfully isolated and cultured. They presented a decreased but statistically insignificant difference in *ERp29* transcript and protein expression compared to C57B6/L wild type mouse RPE.

Conclusions—The effective murine RPE culture described here enables future investigation into RPE biology and AMD pathogenesis. Although we found a decrease in ERp29 expression in the *Ccl2*^{−/−}/*Cx3cr1*^{−/−} RPE, the difference was less than we previously observed in the whole retina. This suggests that the RPE may not contribute to the greater differential expression and ERp29 might have a more significant role in the neuroretina than in the RPE during AMD pathogenesis.

Keywords

age-related macular degeneration; animal model; cell culture; ERp29; retinal pigment epithelium

Correspondence: Jingsheng Tuo, Bldg. 10, Rm. 10N103, NIH/NEI, 10 Center Drive, Bethesda, MD 20892-1857, USA. E-mail: tuo@nei.nih.gov.

Varun Verma is a HHMI-NIH Research Scholar. Varun Verma and Theodor Sauer contributed equally to this work.

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INTRODUCTION

Age-related macular degeneration (AMD) is a chronic retinal degenerative disorder of indefinite etiology, specifically affecting the macula. It is the leading cause of central vision loss among individuals over 65 years of age in the world.¹ Pathological features include, among others, RPE dysfunction as potentially caused by accumulated oxidative stress, immunological response, lipofuscin, and subretinal drusen.² In previous work, our laboratory generated *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice that developed reproducible retinal lesions, drusen, and heavily lipofuscin laden RPE, mimicking the characteristic features of human AMD.³ At a young age (6 weeks) and with high penetrance, *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice developed retinal histopathological changes, including drusen, RPE alteration with lipofuscin accumulation, and photoreceptor degeneration. Additionally, approximately 15% of animals developed choroidal neovascularization (CNV). Moreover, human AMD-related molecules, such as A2E and complement factors C3d and CD46, were detected in the affected RPE and Bruch's membrane.

The RPE is a monolayer of hexagonal pigmented cells that lies between the neurosensory retina and Bruch's membrane and forms part of the blood-retina barrier. The RPE is responsible for outer rod segment phagocytosis, limiting retinal oxidative damage by absorbing incoming blue light and/or quenching reactive oxygen intermediates, and plays a role in the exchange of nutrients and waste products to and from the neuroretina.⁴ The RPE is believed to play an imperative function in the pathogenesis of AMD.^{5,6} Due to the striking pathology observed in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice, *in vitro* RPE studies are important to further elucidate how these mice mimic human AMD in regards to RPE dysfunction. Although stable cell culture lines for human and rat RPE cells are readily available, stable cell lines for mouse RPE do not exist or are exceedingly difficult to establish.^{7,8}

Many neurodegenerative diseases sharing characteristics with AMD are linked to endoplasmic reticulum (ER) stress and expression of chaperone proteins.^{9,10} As previously reviewed, ER stress may play a fundamental role in AMD pathogenesis, particularly in the RPE.¹¹ Proteomic analyses on whole retinas with RPE of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice found differential expression of four molecules, including the precursor to chaperone protein ERp29. ERp29 is a 29 kDa ubiquitous ER-resident chaperone protein resembling protein disulfide isomerase (PDI).¹² By keeping unfolded proteins in a folding-competent state, ERp29 assists proper protein folding and also functions as a component of the ER-specific protein-degrading apparatus to eliminate denatured proteins.¹³⁻¹⁵ Moreover, oxidative stress has been reported to alter chaperone protein expression and be involved in the pathogenesis of AMD.^{16,17} In this study, we aimed to establish a reliable RPE culturing technique and by doing so examine the expression of ERp29 in RPE cells of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse.

MATERIALS AND METHODS

Animals

Ccl2^{-/-} and *Cx3cr1*^{-/-} mice (obtained from Drs. Bao Lu and Barrett J. Rollins of Children's Hospital, Harvard Medical School, and Dr. Philip Murphy of NI-AID/NIH, respectively) were used as the founder generation (F₀) and cross-bred to obtain animals that were heterozygous (F₁) for both *Ccl2* and *Cx3cr1* alleles, as described previously.³ The heterozygous animals were intercrossed to obtain homozygous *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (F₂). Wild-type (WT) mice were of C57BL/6 background. *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and WT mice were inbred and grown in the National Eye Institute (NEI) animal facility. All animal experiments were performed under protocols approved by the NEI Institutional Animal Care and Use Committee. Mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RPE Cell Isolation and Culture Medium

A dissecting microscope was used following a modified protocol based on existing methods established from rat,^{18,19} mouse,²⁰ and human²¹ RPE isolation. Mice were sacrificed by CO₂ asphyxiation and enucleated by gentle grasping of the ocular muscles with optic nerve and pulling outwards. Globes were kept in growth medium until dissection was performed. In a new tissue culture dish coated with a layer of silicon, globes were secured either by pinning the ocular muscles with 30G1/2 mm sterile needles (Becton Dickinson, Franklin Lakes, NJ, USA) or placing in a dimple in the silicon layer. A limbal puncture was performed with fine-tipped scissors or needle, allowing humor to exit and cornea to be gently grasped with forceps.

Cutting around the limbus with fine-tipped scissors, avoiding pressure on the posterior cup, the cornea, lens, leftover iris, and vitreous were removed. Using a 100- μ l pipette, growth medium was injected gently into the posterior cup to dislodge the neuroretina from the underlying RPE. The neuroretina was subsequently plucked off without disturbing the RPE. Then 100- μ l of 0.25% EDTA-trypsin (Invitrogen, Carlsbad, CA, USA) was added to each posterior eyecup, and the dish placed in a 37°C incubator for 45 min.

The eyecups were then transferred to a new 60 $\times$ 15 mm tissue culture dish, and the RPE cells were dislodged by gently injecting growth media and triturating into a single cell suspension. Growth medium containing serum also served to neutralize trypsin activity. Cells were spun at 1500 rpm for 5 min, resuspended in 5-ml growth medium, and plated onto 25-cm² polystyrene flasks (Corning Life Sciences, Acton, MA, USA) at 5% CO₂/37°C (6 eyes per 25-cm² flask). The first change of growth medium was performed after 72 hr. Thereafter, growth medium was changed every other day and cells split in a 1:3 ratio into new 25-cm² flasks upon reaching 80% confluence.

Growth medium used was based on the modified medium used for culture of fetal human RPE reported by Maminishkis and colleagues,²¹ and contained 500 ml MEM alpha modification (Sigma-Aldrich, St. Louis, MO, USA), 50 ml fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 5 ml of L-glutamine-penicillin-streptomycin (Sigma-Aldrich), 5 ml of 100 $\times$ MEM non-essential amino acids (Invitrogen), and 5 ml of N1 growth medium supplement (Sigma-Aldrich).

Detection of ERp29 Transcripts by Real Time-PCR

One million cells were used for RNA isolation using the TriZOL reagent (Invitrogen) or Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) followed by treatment with RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN, USA). cDNA was synthesized by using the Superscript II reverse transcription kit (Invitrogen) with equal amounts of RNA.

RT-PCR was performed by using the validated and inventoried *ERp29* Taqman gene expression kit, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene (Applied Biosystems, Foster City, CA, USA). The assay was performed according to the manufacturer's instruction. The comparative Ct method was used to establish relative quantification of the fold changes in gene expression according to the user bulletin #2.

Oxidative Stress

Passaged cell cultures (5th and 6th passage) were used to explore ERp29 expression changes after oxidative stress. RPE were grown to 90% confluence and incubated for 4 hr with 1 mM H₂O₂. Afterwards, cells were collected by 5-min incubation at 37°C with 0.25 EDTA-trypsin followed by centrifugation for 5 min at 1500 RPM and immediate RNA isolation, as described.

Immunohistochemistry

RPE from WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice were plated overnight on 8-well glass LabTek chamber slides (Nunc, Rochester, NY, USA) at approximately 10,000 cells/well. RPE were fixed in acetone for 7 min and rinsed in Tris-buffer saline (0.05 M pH 7.4). Endogenous peroxidase activity was blocked in hydrogen peroxide. The slides were immersed in 10% goat serum to specifically block background. Avidin-biotin-complex immunoperoxidase technique was applied. The primary antibody was polyclonal rabbit antibodies against ERp29 (ABR, Golden, CO, USA) at 1:100 concentration. The secondary antibody was biotin conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The substrate was diaminobenzidine (DAB). The RPE cells were counterstained with 1% methyl green.

Western Blot

RPE cell culture flasks were trypsinized and 1×10^6 cells used for protein analysis. Cells were lysed in 1X RIPA lysis buffer (Upstate, Charlottesville, VA, USA) and concentration assessed by Bradford protein assay at 595 nm using Coomassie Plus Bradford Assay Reagent (Pierce Biotechnology, Rockford, IL, USA). Protein was run on 10% TBE polyacrylamide (Bio-rad Laboratories, Hercules, CA, USA) or 4–12% Tris-Bis (Invitrogen) gels and blotted onto a nitrocellulose membrane (Invitrogen). Primary rabbit polyclonal antibody to ERp29 was used at a 1:2500 concentration (ABR) and primary mouse monoclonal to GAPDH at a 1:5000 concentration (Abcam, Cambridge, MA, USA). Secondary antibodies were biotin-labeled goat anti-rabbit IgG (1:2000) and biotin-labeled goat anti-mouse IgG (1:2000) (Vector Laboratories). Membranes were developed with SuperSignal West Dura Extended Duration Substrate per manufacturer's protocol (Pierce Biotechnology).

RESULTS

Characterization of Cultured *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE Cells

Primary culture RPE of both WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} grew as pigmented hexagonal monolayers (Fig. 1). Upon passing the cells, particularly after the third passage, the cells gained a fibroblast-like appearance with a marked decrease in pigmentation. During dissection of both WT mice and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice, we were able to isolate 3×10^4 – 5×10^4 cells per globe. The cell doubling time for the WT RPE primary culture was 4 days, while the doubling time for the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE primary culture was slightly longer at 5 days. Cells appeared to become sub-confluent at 8–10 days post dissection and were ready for splitting at this stage (Table 1). Both the WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE cells in the primary culture were complete with cytofilaments and ribosomes (Fig. 2).

ERp29 Transcript Expression in RPE

There was no significant difference in ERp29 expression between *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and WT in the primary RPE culture. We detected 36% less ERp29 expression in the second passage of the RPE culture in comparison with the WT ($p < 0.05$) (Fig. 3). Hydrogen peroxide treatment did not alter ERp29 expression in either the WT or *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE cells (data not shown).

ERp29 Protein Levels in RPE

Immunostaining against ERp29 on first passage RPE and passaged RPE was consistent and showed ERp29 staining was less intense in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE than WT RPE (Fig. 4). Western blot performed on first-passage and fifth-passage cells did not detect significant change of ERp29 protein expression in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} in comparison to C57BL/6 (data not shown).

DISCUSSION

The recent development of animal models for AMD has allowed researchers to investigate more translational and innovative therapies and pathogenetic studies of AMD.^{3,22, 23} The RPE is known to have a fundamental role in maintaining photoreceptor health and is likely involved in AMD development.^{5,6} Generating a maintainable cell culture was an imperative step to basic functional study of the RPE. Immortalized human and rat RPE cultures have allowed just this, but have limited similarity to AMD-diseased RPE. Additionally, there are limited supplies of RPE samples from AMD eyes and difficulties in passing aged cells.^{19, 21,24} We previously reported the breeding of *Ccl2/Cx3cr1* deficient mice with AMD pathology.³ In the present study, we successfully cultured RPE from these mice, opening the door for future pathological and therapeutic studies.

We previously found ERp29 transcript and protein expression to be significantly depressed in the *Ccl2^{-/-}/Cx3cr1^{-/-}* whole retina, including RPE as compared to age-matched WT mouse.³ Since ERp29 is an ER chaperone protein and AMD exhibits many of the characteristics of other neurodegenerative diseases linked to ER-stress, we hypothesized ERp29 may play a role in the development of AMD in our mouse model and potentially have human AMD implications. In a recent proteomic analysis of human AMD eyes, Ethen and colleagues reported a 33% decrease of ERp29 in the macula with at-onset stage of AMD compared to non-AMD age-matched eyes.²⁵ ERp29 is a major resident protein of the ER and also postulated to play an important molecular role in most animal cells.²⁶ After isolating the RPE from WT and the *Ccl2^{-/-}/Cx3cr1^{-/-}* mice from several different generations, we found ERp29 mRNA transcript and protein expression in the RPE had a lower expression in cultured *Ccl2^{-/-}/Cx3cr1^{-/-}* RPE. Yet, despite the consistent pattern of decreased ERp29 expression from several lines of evidence, the difference was not as substantial as what was observed in the whole retina analysis.³ This contradiction might imply that the difference in ERp29 expression observed in the whole retina was mainly from the neuroretina rather than the RPE alone. This result was surprising, as the RPE must handle many stresses beyond what the neuroretina endures—such as, phagocytosing exhausted photoreceptor segments, lipofuscin accumulation, and oxidative stress from age-related changes in the choroidal vasculature^{16,27}—that might demand a greater ER-stress related protein response. To test this idea, we subjected both RPE stains to hydrogen peroxide and examined ERp29 expression. Interestingly, neither strain exhibited significant ERp29 expression change. Hence, function of chaperone ERp29 alone may not be critical in AMD pathogenesis in the RPE, but perhaps plays a greater role in the neuroretina. The information could be useful for drawing implications between *in vitro* and *in vivo* studies and would be particularly interesting to those investigators who are currently using or planning to use these *Ccl2/Cx3cr1* deficient mice.

In light of these results, one must realize the limitations of our study as well as those of RPE studies in general. First, our AMD mice are generated by the knockout of a chemokine and a cytokine receptor, leading to early development of AMD features. Therefore, development of AMD in this model is likely initiated by and resultant of immunological related responses rather than age-related cumulative factors. Our lack of observance of ERp29 depression may not hold in aged human RPE. Second, although we attempted to maintain a homeostatic environment for RPE cell growth and use early RPE passages for experimentation, it is known that RPE undergo significant adaptive changes in cell culture conditions, and it is possible that our data reflects these adaptations.^{16,27,28} Additionally, removal from the stresses encountered in the AMD model eye into the artificial culture environment may quickly ablate some of the unique expression differences between AMD and normal RPE.

Nonetheless, the successful and maintainable culturing of normal and AMD model RPE unlocks another avenue of AMD investigational research. We expect this reliable method for

the culturing of murine RPE cells, particularly from our *Ccl2*^{-/-}/*Cx3cr1*^{-/-} AMD model, to serve as an important tool for conducting efficient and effective translational research on the pathogenesis and treatment of ocular diseases such as AMD. In summary, the present study reports an effective method to isolate and culture murine RPE and concludes that there may not be biologically meaningful alteration of ERp29 in cultured RPE from the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} AMD model mouse.

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Figure 1.

Inverted light microscopy—primary culture RPE (100 × magnification). The primary culture RPE of both WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} are visualized on chamber slides at three days after dissection and prior to the first medium change. The cells have a pigmented appearance and grow as a monolayer in cell culture flasks.

Figure 2.

Transmission electron microscopy—primary culture RPE. Both the WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} primary culture RPE cells are seen to contain mitochondria (arrows), cytofilaments (open tailed arrows), and ribosomes. There is prominent endoplasmic reticulum (arrow heads) in the WT RPE and several liposomes (asterisks) in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE.

Figure 3. ERp29 mRNA expression in cultured RPE. ERp29 mRNA expression measured by real-time RT-PCR in primary- and secondary-passage cultured RPE from the *Ccl2/Cx3cr1*-deficient and WT mice. Asterisk denotes $p < 0.05$.

Figure 4.

Immunostaining against ERp29—primary culture RPE. Photomicrograph showing expression of ERp29 on both the WT and the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} first-passage culture RPE. The WT RPE display an overall more intense stain to ERp29 (left panel) than the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE (right panel). (Avidin-biotin-complex immunoperoxidase; original magnifications, 100 ×.).

TABLE 1

RPE cell culture characteristics

	C57BL/6 RPE	<i>Ccl2/Cx3cr1</i> deficient RPE
Age of animals at dissection	3 months	3 months
Number of eyes per 25 cm ² cell culture flask	6	6
RPE cells per globe isolated	3×10^5 – 5×10^5	3×10^5 – 5×10^5
Initial plating density	5×10^4 – 1×10^5 cells/cm ² ~30% confluent	5×10^4 – 1×10^5 cells/cm ² ~30% confluent
Doubling time	4 days	5 days
Time to confluence	8 days	10 days
Cell count at time of splitting	5×10^6 – 1×10^7 cells	5×10^5 – 1×10^7 cells