A novel coculture model of porcine central neuroretina explants and retinal pigment epithelium cells

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Purpose: To develop and standardize a novel organ culture model using porcine central neuroretina explants and RPE cells separated by a cell culture membrane.

Methods: RPE cells were isolated from porcine eyes, expanded, and seeded on the bottom of cell culture inserts. Neuroretina explants were obtained from the area centralis and cultured alone (controls) on cell culture membranes or supplemented with RPE cells in the same wells but physically separated by the culture membrane. Finally, cellular and tissue specimens were processed for phase contrast, cyto-/histological, and immunochemical evaluation. Neuroretina thickness was also determined.

Results: Compared to the neuroretinas cultured alone, the neuroretinas cocultured with RPE cells maintained better tissue structure and cellular organization, displayed better preservation of photoreceptors containing rhodopsin, lower levels of glial fibrillary acidic protein immunoexpression, and preservation of cellular retinaldehyde binding protein both markers of reactive gliosis. Neuroretina thickness was significantly greater in the cocultures.

Conclusions: A coculture model of central porcine neuroretina and RPE cells was successfully developed and standardized. This model mimics a subretinal space and will be useful in studying interactions between the RPE and the neuroretina and to preclinically test potential therapies.

In 1989, Caffe et al. [1] published a method for organ culture in which the neuroretina was placed with the photoreceptor layer facing downward on rafts made of nitrocellulose filters and polyamide gauze grids. Since then, mammalian retinal organ cultures have been commonly used to research retinal physiology and pathobiology. Retinal cell dynamics have shown that organotypic models can be comparable with in vivo conditions, especially those of the outer retina [2]. Thus, retinal cultures are still used in electrophysiological studies to monitor drug effects on retinal cell functionality [3], to evaluate neurotrophic factors or physical tension on retinal cells [4], and to study the molecular basis of potential therapies for photoreceptor death [5]. Our group has previously used neuroretina explant cultures to evaluate modifications induced by exogenous cells such as blood-derived macrophages or by cytokines such as tumor necrosis factor alpha (TNF-α) that are implicated in the pathobiology of proliferative vitreoretinopathy [6,7]. We have also established a mixed coculture model composed of three cellular layers, the neuroretina, the RPE, and adipose tissue-derived stem cells, to evaluate the neuroprotective effects of stem cells [8].

There are some obvious limitations of these culture systems, such as the axotomy of ganglion cells as part of the dissection procedure and the absence of a blood supply. Thus, degenerative changes in retinal cells, especially at the inner retina, could differ from in vivo conditions [9]. In addition, a major limitation is the absence of the RPE. The RPE is a monolayer of pigmented, cuboidal epithelial cells that are closely associated with the photoreceptor outer segments. The most important functions of the RPE are the synthesis and maintenance of the interphotoreceptor matrix, photoreceptor membrane turnover, and retinoid metabolism [10]. The importance of the neuroretina–RPE association is supported by the fact that neuroretinas adjoined to RPE monolayer cultures have better-preserved tissue architecture in culture studies [11]. The RPE is also considered a key element in the development of some retinal diseases and, importantly, in the physiopathology of retinal detachment and central serous chorioretinopathy. In these pathologies, the neuroretina and the RPE become physically separated. Furthermore, the RPE secretes across the apical membrane neuroprotective factors...
that helps to maintain retinal homeostasis [10]. Thus, an adequate ex vivo tool for studying retinal degeneration and the importance of molecular signaling across the subretinal space after physical separation of the neuroretina and the RPE would be inherent to overcome the complexities that are inherent in vivo studies.

In this study, we developed and characterized a novel organotypic coculture system in which the central cone-dominated porcine neuroretina was cocultured with RPE cells that were maintained physically separated from the neuroretina by the culture medium and a porous membrane. This arrangement prevented any physical contact between the cocultured cells but allowed signal molecules to pass between them. This model could be useful for studying in vitro the interactions between the neuroretina and the RPE when they lose their natural contact, as happens in several retinal diseases such as retinal detachment and central serous chorioretinopathy. Further, this ex vivo model will allow study of the role of RPE-secreted factors and the evaluation of potential therapies for reducing the progression of neuroretina degeneration.

**METHODS**

Fifteen porcine eyes from animals aged 6–8 months were obtained from the local slaughterhouse and processed within 2 h of death. Immediately after enucleation, the eyes were immersed in ice-cold transport medium composed of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with a 10% antibiotic-antimycotic mixture containing penicillin, streptomycin, and amphotericin B (Gibco, Invitrogen, Paisley, UK) and transported on ice to the laboratory. Under aseptic conditions in a laminar airflow hood, the eyes were dissected free from all periocular tissue. After full immersion of the eyeballs in 70% ethanol during 2 min, the eyes were washed in clean DMEM supplemented with 10% antibiotic-antimycotic at room temperature.

**RPE cell isolation and culture:** RPE cells from the porcine eyes (n=5) were obtained as previously described [12]. Briefly, the eyes were dissected at the ora serrata to exclude the iris and lens. The vitreous was then removed from the posterior eye cup with cotton swabs, and then the neuroretina was detached and discarded. The remaining eye cup was covered with 0.05% trypsin-tetrasodium ethylene diamine tetra-acetate (Trypsin-EDTA, Gibco) for 30 min at 37 °C. RPE cells were removed by filling the eye cup with complete DMEM, the cells were plated in 25 cm² flasks (Nunc, Roskilde, Denmark). The RPE cells were maintained in complete DMEM under standard culture conditions of 37 °C in an atmosphere of 5% CO₂, with 95% humidity. The medium was renewed at 2–3 day intervals. RPE cell morphology was evaluated with a Nikon Eclipse TS100 inverted-phase contrast microscope (Nikon Instruments Inc., Tokyo, Japan) during culture. Trypan blue exclusion assay (Sigma-Aldrich, St. Louis, MO) was used to determine viability and cell numbers. After reaching >90% confluence, the cells were trypsinized with 0.05% trypsin-EDTA, washed, and resuspended in PBS (Gibco).

Passage 2 RPE cells were seeded (30,000 cells/cm²) [8] on the bottom of Transwell® culture plates (Corning Inc., Corning, NY) and grown for 24 h in complete DMEM to allow cellular adhesion before coculturing with neuroretina explants.

**Central neuroretina explant preparation and culture:** Twenty neuroretina explants were obtained from ten eyes as previously described by our group [6]. The porcine cone-enriched visual streak was identified, as described by Hendrickson and Hicks [13], and two 5×5 mm adjacent explants from each eye were obtained with Castroviejo corneal scissors (John Weiss & Son Ltd., Milton Keynes, UK) from the temporal area 1 mm superior to the optic disc (Figure 1). The neuroretina explants were laid over the Transwell® membranes (24-mm diameter with 0.4-µm pore polycarbonate membrane insert; Product #3412, Corning Inc.) with the photoreceptor layer facing the membrane. Nine neuroretina explants were cocultured with RPE cells but physically separated from them by the culture medium overlying the RPE cells and the Transwell® membrane (Figure 2). Another nine explants were cultured as controls without RPE cells. Cultures were maintained in 1:1 Neurobasal-A/DMEM supplemented with 10% fetal bovine serum, 2% B-27 (Gibco), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO), and 1% antibiotic-antimycotic mixture and maintained in standard culture conditions. The culture medium level (1.5 ml as suggested by the manufacturer) was maintained in contact with the support membrane beneath the explants and changed with freshly prepared, warmed medium every day. The explants were harvested for analysis after 9 days of culture, as previously described by our group [14], and were cut into two halves for subsequent processing. Two fresh central neuroretina specimens were used as culture day 0 samples and processed in parallel.

**Histological and immunochemical processing:** RPE cells that were cocultured with neuroretina explants for 9 days were fixed in the Transwell® culture plates with ice-cold methanol (Panreac Quimica S.A.U., Barcelona, Spain) for 15 min at
4 °C. The RPE cells were immunostained for phenotype-specific marker proteins. Primary antibodies (Table 1) for zonula occludens protein 1 (ZO-1), RPE-specific 65 kDa protein (RPE65), and cellular retinaldehyde binding protein (CRALBP) were diluted in PBS containing 0.5% Triton X-100, 10% goat serum, and 1% bovine serum albumin (BSA; all Sigma-Aldrich). The antibodies were applied directly to the cultures and incubated overnight at 4 °C. After washing in PBS, the corresponding species-specific secondary antibodies to immunoglobulin gamma conjugated to Alexa Fluor 488 or 568 (green and red; Molecular Probes, Eugene, OR) were applied at a 1:200 dilution for 1 h. Nuclei were stained with 10 µg/ml 4',6-diamino-2-phenylindole dihydrochloride (DAPI, Molecular Probes). Finally, the cells were washed in PBS, mounted in fluorescent mounting medium (DakoCyto- mation Inc., Carpinteria, CA), and coverslipped.

Neuroretina samples were fixed overnight in 1% paraformaldehyde and 1% glutaraldehyde (both Panreac Quimica S.A.U.) in phosphate buffer (PB, Gibco). After gradual dehydration in an ethanol series, the pieces were embedded in low-viscosity epoxy resin (Spurr, TAAB, Aldermaston, UK). Semithin sections (1 µm) were obtained with an ultramicrotome (LKB Bromma 8800 Ultratome III, Freiburg, Germany) and stained with 1% toluidine blue in 3% sodium tetraborate (both Panreac Quimica S.A.U.).

For immunohistochemistry, the other half of the samples were fixed in 4% paraformaldehyde in PB, pH 7.4, for 2 h and then subjected to sucrose (Panreac Quimica S.A.U.) cryo-protection [15]. On the following day, they were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe B.V., Alphen, the Netherlands). Sections (5 µm) were cut on a cryostat (Leica Instruments, Nussloch, Germany) and mounted on glass slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany). The neuroretinas were immunostained for the phenotype-specific markers (Table 1) rhodopsin (RHO), calbindin D-28K (CB), glial fibrillary acidic protein (GFAP), and CRALBP. Specific combinations of antibodies were diluted in PBS containing 0.5% Triton X-100 and incubated overnight at 4 °C. The next day, the sections were washed in PBS. Thereafter, the corresponding species-specific secondary antibodies conjugated to Alexa Fluor 488 and/or 568 were applied at a 1:200 dilution for 1 h. Nuclei were stained with 10 µg/ml DAPI. Finally, the sections were washed in PBS, mounted in fluorescent mounting medium, and coverslipped.

The primary antibodies used in this work have been used in previous studies and have been well characterized by our group and other authors regarding specific cell-type immunostaining in whole fixed porcine eyes and in porcine neuroretina samples [6-8,16]. Furthermore, control samples in which primary antibodies were omitted were processed in parallel, and no immunoreactivity was found in any case. Samples were analyzed with a Leica DM4000B...
light microscope (Leica Microsystems, Wetzlar, Germany) equipped for epifluorescence, Leica HCX PL FLUOTAR PH2 20x/0.50 and 40x/0.75 and Leica N PLAN 63x/0.8 objectives (Leica Microsystems) were used, and images were obtained with a Leica DFC490 digital camera (Leica Microsystems). Brightness and contrast were minimally adjusted and final figures composed with Pixelmator 3.4 Twist (Apple, Cupertino, CA).

Neuroretina thickness quantification: The thickness of the neuroretina and the thickness of the outer nuclear layer (ONL) and the inner nuclear layer (INL) were measured on toluidine blue histological images with Image J 1.47v (NIH Image, National Institute of Health, Bethesda, MD). Measurements were performed in representative serial sections. Neuroretina thickness was determined between the outer and the inner limiting membranes. In each case, six measurements were performed in 20X images from each of the neuroretina explants (n=20).

Statistical analysis: Statistical analysis was performed using R Statistical Software version 3.1.0 (Foundation for Statistical Computing, Vienna, Austria). The means and standard deviations were calculated for continuous variables. The statistical significance level was set at 5%.

To assess preservation of the RPE morphology and phenotype during coculture with the neuroretina explants, the RPE cells were examined with antibodies against ZO-1, which is a peripheral adaptor protein of tight junction structure between RPE cells. The expression of the RPE65 protein, which is located in the cytoplasm and involved in the production of 11-cis-retinal and in visual pigment regeneration, and CRALBP, a retinoid-binding protein implicated in vitamin A metabolism and found in the RPE apical microvilli, was also detected with immunohistochemistry. Nine days after coculture with the neuroretina explants, the RPE cells showed variable ZO-1 immunoexpression at the polygonal cell periphery (Figure 3D) and maintained the expression of cytoplasmic

### Results

**RPE cells**: Isolated porcine RPE cells started to adhere on culture day 1 and to form clusters of cells at day 4 in flasks (Figure 3A). These cells were polygonal and contained pigment. At day 7 of culture in flasks, the RPE cells were almost confluent in a monostratified epithelium, had a polygonal shape, and had pigmented cytoplasmic granules (Figure 3B). However, some cells had dedifferentiated morphology, with increased size and reduced pigment content (Figure 3B, arrowheads). Cellular characteristics were maintained until passage 2, when the RPE cells were cocultured with neuroretina explants. At coculture day 9 (Figure 3C), the RPE cells formed a confluent monolayer in the bottom of the cell culture inserts, retained the polygonal shape, and still had some pigment content (Figure 3C, insert); some cells looked dedifferentiated (Figure 3C, arrowheads).

| Molecular marker                        | Antibody                        | Source                                      | Working dilution |
|----------------------------------------|---------------------------------|---------------------------------------------|------------------|
| Zonula occludens protein 1 (ZO-1)      | Mouse monoclonal [ZO1–1A12]    | Invitrogen, #33–9100 (Camarillo, CA)       | 1:50             |
| Retinal pigment epithelium-specific 65 kDa protein (RPE65) | Mouse monoclonal               | Novus Biologicals, #NB100–355 (Cambridge, UK) | 1:50             |
| Cellular retinaldehyde-binding protein (CRALBP) | Mouse monoclonal [B2]          | Abcaml, #Ab15051 (Cambridge, UK)            | 1:50 (RPE) 1:1000 (NR) |
| Calbindin D-28K (CB)                   | Rabbit polyclonal               | Swant, #CB-38a (Bellinzona, Switzerland)    | 1:500            |
| Rhodopsin (RHO)                        | Rabbit polyclonal               | Chemicon-Millipore, #AB9279 (Temecula, CA) | 1:200            |
| Glial fibrillary acidic protein (GFAP)  | Rabbit polyclonal               | DakoCytomation, Inc., #N1506 (Glostrup, Denmark) | 1:500            |

RPE: retinal pigment epithelium cells; NR: neuroretina explant
RPE65 (Figure 3E). CRALBP was not detectable in the RPE coculture (Figure 3F).

Neuroretina explants: Neuroretina explants stained with toluidine blue showed the general overview of all layers of the porcine retina (Figure 4). In the freshly isolated neuroretina explants (Figure 4A,D), the characteristic neuroretina architecture was apparent, with the notable presence of cone photoreceptors corresponding to the porcine visual streak. The delicate structures of the photoreceptor outer and inner segments (OS and IS) were well preserved before culturing. In the absence of cocultured RPE cells, the retina structure at 9 days of culture (Figure 4B,E,F) was disorganized, and cellular vacuolization was present throughout the retina layers. Photoreceptor OS were lost while the remaining IS appeared shorter and swollen compared to the freshly isolated neuroretina explants. In the ONL, photoreceptor cell bodies were oriented to form degenerative rosette-like structures (Figure 4B,E, asterisks). The plexiform layers were partially disrupted and unstructured. The Müller cells were hypertrophied, the nuclei were translocated to the ONL, and cytoplasmic pigmented granules were present (Figure 4F, open arrow and open arrowheads). Furthermore, cellular extensions of Müller cells covered the photoreceptor (Figure 4B,F, arrows). At 9 days of coculture with RPE cells (Figure 4C,G), the cellular architecture of the neuroretina explants was better preserved, and the retina layers were more easily discerned than those in the neuroretina explants cultured alone. Photoreceptor IS were condensed and swollen (Figure 4G, arrowheads); no rosette-like formation was evident. The integrity of the plexiform layers and the INL were maintained. Cellular extensions over the photoreceptors were present (Figure 4C,G, arrows).

To further assess photoreceptor integrity and retina cell morphology during culture, the neuroretina explants were examined with antibodies against RHO, an opsin protein present in rod OS, and CB, a calcium-binding protein present in cone photoreceptors. Additionally, to assess the degree of glial cell activation, the neuroretina explants were immunostained with antibodies against GFAP, an intermediate filament protein present in glial cells, and against CRALBP, found in Müller cells.

In the freshly fixed neuroretina explants, the rods maintained normal long and straight OS morphology expressing
RHO (Figure 5A). At 9 days of culture without the RPE, partial displacement of RHO immunoexpression was detected at the swollen rod IS (Figure 5B). Additionally, photoreceptor nuclei were displaced to the IS region (Figure 5B asterisks). After 9 days of coculture with RPE, RHO expression (Figure 5C) was evident at the ONL (Figure 5D arrows) and was displaced to shorter and swollen rod IS.

In the freshly fixed neuroretina explants, cones were immunoreactive for CB and had a normal morphology (Figure 5D). At 9 days of culture without the RPE, the CB immunostained cones showed markedly altered morphology (Figure 5E). At 9 days of coculture with the RPE, the cones underwent morphological degenerative changes (Figure 5F).

In the freshly fixed neuroretina explants, CRALBP immunoeexpression revealed Müller cells with normal morphology (Figure 5G). GFAP immunostaining was limited to the innermost layers of the neuroretinal tissue. At 9 days of culture without RPE, GFAP was clearly upregulated in the cytoplasm of the glial cells (Figure 5H). GFAP cellular processes extended outside the retinal tissue and formed layered structures (Figure 5H, arrows). At 9 days of coculture with RPE, CRALBP was scarcely present, and glial cell

Figure 4. Neuroretina morphology during the culture and coculture period. In the freshly isolated retina explants (A and D), the porcine central retina showed the characteristic highly organized layered structure. At 9 days of culture alone (B, E, and F), the retinal architecture was disorganized. Photoreceptor OS were lost, and the cell bodies formed degenerative rosette-like structures (B and E, asterisks). Müller cells were hypertrophied, with nuclei translocated to the outer nuclear layer (F, open arrow) and cell cytoplasm pigmented granules (F, open arrowheads). Cellular extensions over photoreceptors were also present (B and F, arrows). At 9 days of coculture with RPE cells (C and G), the layered retinal morphology was preserved with condensed photoreceptor IS still present (G, arrowheads). Cellular prolongations were observed over the photoreceptors (C and G, arrows). OS=outer segments; IS=inner segments; OLM=outer limiting membrane; ONL=outer nuclear layer; OPL=outer plexiform layer; INL=inner nuclear layer; IPL=inner plexiform layer; GCL=ganglion cell layer. Scale bars=20 µm.
immunoexpression of GFAP was reduced compared with cultures without RPE (Figure 5I). GFAP processes were also present over the outer limiting membrane; the thickness of the cellular extensions was reduced (Figure 5I, arrows).

**Neuroretina thickness quantification:** The thickness of the neuroretina, the INL, and the ONL changed over time during the culture period (Figure 6). The thickness of freshly isolated neuroretinas (n=2) was 135.29±17.02 µm. After 9 days of culture alone, the thickness of the neuroretinas (n=9) was not provided.
92.28±14.94 µm, while that of the RPE-cocultured neuroretinas (n=9) was 109.35±4.25 µm (p<0.0001). The ONL thickness of the freshly isolated samples was 34.64±1.43 µm. After 9 days of culture alone, the ONL thickness was 21.7±3.57 µm, while that of the RPE-cocultured neuroretinas was 30.82±3.16 µm (p<0.0001). The INL thickness of the freshly isolated samples was 35.54±1.81 µm. After 9 days of culture alone, the INL thickness was 21.89±2.68 µm, while that of the RPE-cocultured neuroretinas was 27.88±2.35 µm (p<0.0001).

**DISCUSSION**

The present study described the development and characterization of a novel coculture model of cone-dominated porcine central retina and RPE cells to closely simulate ex vivo the subretinal space microenvironment. Retinal degeneration is a common finding of many retinal diseases, and in some cases, such as retinal detachment and central serous chorioretinopathy, it is in part the consequence of physical separation between the neuroretina and the RPE. This separation starts a cascade of events that result in cellular changes throughout the retina. These events are in part responsible for the poor functional results that can occur even after successful reattachment surgery [18]. Currently, research in retinal degeneration is largely limited by the difficulty in obtaining specimens from freshly detached human retinas and the limitations of experimental animal models [19]. In this sense, simple neuroretina cultures represent a useful tool for studying retinal modifications. These models are inexpensive and easy to develop. Importantly, they closely simulate in vivo retinal cellular and molecular dynamics and have been the source of improved knowledge of retinal physiopathology in recent decades [1]. Nevertheless, these previously reported culture models lack the influence of the RPE cells that are necessary for maintaining the viability and functionality of the outermost retinal layers [10]. Furthermore, RPE cells secrete various growth and trophic factors that act on the neuroretina and the choroidal endothelium [10]. The RPE also plays an important role in the retinal degeneration process and is considered a key element in retinal detachment physiopathology [19].

There are some obvious limitations of these culture systems, such as the absence of choroidal and retinal blood flow, the lack of vitreous, and the axotomy of ganglion cells, that may considerably limit the study of the inner retina modifications [9]. However, neuroretina organotypic cultures are still considered an adequate tool for improving knowledge of retinal physiopathology [3]. Other authors have previously described models in which RPE monolayers were cocultured in contact with the neuroretina [11]. Those models are complex to develop and do not adequately mimic a retinal pathology related to the separation between the neuroretina and the RPE. Our purpose was to study and characterize the differences between neuroretinas cultured alone and those cocultured with RPE cells while physically separated from each other. This novel model could be the basis for studying...
the interaction between the neuroretina and RPE cells when they are separated in vivo because no such studies currently exist. In this model, neuroretina explants were cultured over cell culture membranes that physically separated the RPE cells cultured on the bottom of the cell culture inserts. Thus, the porous cell culture membranes inhibit RPE cell migration into the retinal tissue and direct contact with neuroretina cells; while molecular exchange among the different cell types can occur. In this scenario, RPE-secreted factors can diffuse through the cell culture membrane pores and influence neuroretina cell dynamics, thus recreating the subretinal space milieu.

The porcine retina possesses some characteristics that make this species particularly useful in retinal research, such as retinal extent, ultrastructure, and the lack of a tapetum [13]. The retinal parenchyma is quite similar to that of humans, with a double circulation system and a central zone, the visual streak. This zone is a broad horizontal retinal streak above the optic disc, with high cone density and without direct vascularization [13]. Unlike the human fovea, no depression is present throughout the pig area centralis [13]. The porcine retina has a rod density that is similar to that of humans, and the cellular dynamics after neuroretina–RPE separation are comparable in both species, especially those of key cells such as Müller and RPE cells [20]. Therefore, in the coculture model that we have developed, we used explants from the porcine cone-dominated area centralis because of the similarity with the human central retina.

As shown with phase contrast microscopy, RPE cells largely maintained their morphological characteristics throughout the coculture period. Furthermore, these cells maintained the expression of ZO-1 outlining the polygonal shape of the RPE cells within the monolayer, and RPE65 supporting the preservation of the RPE phenotype, without dedifferentiation to form other cell types or undergoing an epithelial–mesenchymal transition [21]. As previously described in primary RPE cell cultures, a few clusters of cells became partially dedifferentiated [22]. CRALBP immunoexpression was not observed in RPE cells cocultured with neuroretinas, probably due to the loss of normal cell polarization, especially seen as the disappearance of the apical microvilli [21]. The ability of RPE in culture to secrete neurotrophic factors has been described by other authors [23].

In the cultures in the present study, the neuroretina explants without the RPE showed degenerative alterations in the tissue and retina cells as previously described [14]. However, the presence of degenerative rosette-like structures reported here were not observed by our group in previous work in which the central retina was not used [14]. Degenerative rosettes commonly occur in abnormally developing retinas [24]. Our immunohistochemical studies revealed retinal cell degenerative modifications as we [6,7] and others previously described in organotypic porcine retina cultures [9]. Although RHO and CRALBP immunoexpression was better preserved and lower levels of GFAP were detected in the neuroretinas cocultured with RPE cells, we did not quantify the GFAP or CRALBP labeling, and this will be part of future experiments. The thickness of the neuroretinas was greater in the cocultures. However, it is not clear if that is related to cellular swelling or to better cellular viability. In this sense, it will be useful to explore cell death and survival in future studies. In any case, the neuroretina explants cocultured with RPE cells maintained better preserved tissue and cellular characteristics and significantly better conserved tissue and nuclear layer thicknesses. These findings were consistent through the complete 5-mm neuroretina explants and were observed in all the experiments performed.

Based on the morphological and immunohistochemical results found in this study, neuroretina preservation in cocultures with RPE cells may be partially linked to neuroprotective factors secreted by RPE cells. The secretion of neurotrophic/neuroprotective factors by RPE cells is stimulated during retinal damage [10].

**Conclusions:** Our group has developed and standardized a novel coculture model of central cone–dominated porcine neuroretina that was supplemented with RPE cells maintained separately within the same culture wells. This coculture system mimics ex vivo the subretinal space that develops during retinal detachment (RD) and other disease conditions of the retina. Compared to the neuroretina explant cultures alone, the presence of cocultured RPE provided improved neuroretina architecture and thickness, better preservation of RHO and CRALBP immunoexpression, and lower levels of GFAP. These data suggest that the cocultured RPE, while not in direct contact with the neuroretina explant, had a neuroprotective role. This effect may be linked to the beneficial effects of neurotrophic factors secreted or induced by RPE cells during coculture. Furthermore, the proposed model will be useful to better study interactions between the RPE and the neuroretina and to test neuroprotective and/or anti-inflammatory drugs for retinal degenerative diseases.

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REFERENCES

1. Caffe AR, Visser H, Jansen HG, Sanyal S. Histotypic differentiation of neonatal mouse retina in organ culture. Curr Eye Res 1989; 8:1083-92. [PMID: 2612197].

2. Ogilvie JM, Speck JD, Lett JM, Fleming TT. A reliable method for organ culture of neonatal mouse retina with long-term survival. J Neurosci Methods 1999; 87:57-65. [PMID: 1065994].

3. Alt A, Hilgers RD, Tura A, Nassar K, Schneider T, Hueber A, Januschowski K, Grisanti S, Luke J, Luke M. The neuroprotective potential of Rho-kinase inhibition in promoting cell survival and reducing reactive gliosis in response to hypoxia in isolated bovine retina. Cell Physiol Biochem 2013; 32:218-34. [PMID: 23898844].

4. Ghosh F, Taylor L, Arner K. Exogenous glutamate modulates porcine retinal development in vitro. Dev Neurosci 2012; 34:428-39. [PMID: 23147515].

5. Martinez-Fernandez de la Camara C, Sequedo MD, Gomez-Pinedo U, Jaijo T, Aller E, Garcia-Tarraga P, Garcia-Verdugo JM, Millan JM, Rodrigo R. Phosphodiesterase inhibition induces retinal degeneration, oxidative stress and inflammation in cone-enriched cultures of porcine retina. Exp Eye Res 2013; 111:122-33. [PMID: 23578797].

6. Fernandez-Bueno I, Pastor JC, Gayoso MJ, Alcalde I, Garcia MT. Muller and macrophage-like cell interactions in an organotypic culture of porcine neuroretina. Mol Vis 2008; 14:2148-56. [PMID: 19052655].

7. Fernandez-Bueno I, Garcia-Gutierrez MT, Srivastava GK, Gayoso MJ, Gonzalez-Orden JM, Pastor JC. Adalimumab (tumor necrosis factor-blocker) reduces the expression of glial fibrillary acidic protein immunoreactivity increased by exogenous tumor necrosis factor alpha in an organotypic culture of porcine neuroretina. Mol Vis 2013; 19:894-903. [PMID: 23687426].

8. Rodriguez-Crespo D, Di Lauro S, Singh AK, Garcia-Gutierrez MT, Garrosa M, Pastor JC, Fernandez-Bueno I, Srivastava GK. Triple-layered mixed co-culture model of RPE cells with neuroretina for evaluating the neuroprotective effects of adipose-MSCs. Cell Tissue Res 2014; 358:705-16. [PMID: 25213807].

9. Winkler J, Hagelstein S, Rohde M, Laqua H. Cellular and cytoskeletal dynamics within organ cultures of porcine neuroretina. Exp Eye Res 2002; 74:777-88. [PMID: 12126951].

10. Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005; 85:845-81. [PMID: 15987797].

11. Kaempf S, Walter P, Salz AK, Thomann G. Novel organotypic culture model of adult mammalian neurosensory retina in co-culture with retinal pigment epithelium. J Neurosci Methods 2008; 173:47-58. [PMID: 18632159].

12. Dintelmann TS, Heinmann K, Kayatz P, Schraermeyer U. Comparative study of ROS degradation by IPE and RPE cells in vitro. Graefes Arch Clin Exp Ophthalmol 1999; 237:830-9. [PMID: 10502058].

13. Hendrickson A, Hicks D. Distribution and density of medium- and short-wavelength selective cones in the domestic pig retina. Exp Eye Res 2002; 74:435-44. [PMID: 12076087].

14. Fernandez-Bueno I, Fernandez-Sanchez L, Gayoso MJ, Garcia-Gutierrez MT, Pastor JC, Cuenca N. Time course modifications in organotypic culture of human neuroretina. Exp Eye Res 2012; 104:26-38. [PMID: 23022403].

15. Cuenca N, Kolb H. Circuitry and role of substance P-immuno-reactive neurons in the primate retina. J Comp Neurol 1998; 393:439-56. [PMID: 9550150].

16. Fernandez-Bueno I, Rodriguez de la Rua E, Hileeto D, Parrado ML, Regueiro-Purinos M, Sala-Puigdollers A, Srivastava GK, Gonzalez-Orden JM, Pastor JC. Histology and immunochernistry evaluation of autologous transplantation of retinal pigment epithelium-choroid graft in porcine eyes. Acta Ophthalmol 2013; 91:e125-32. [PMID: 23256869].

17. Gastwirth JL, Gel WL, Wallace Hui WL, Lyubchich V, Miao W, Noguchi K. Lawstat: An R package for biostatistics, public policy and law. R package version 2.4. http://CRAN-R-project/package=lawstat 2013; Accessed January 2015.

18. Fisher SK, Lewis GP, Linberg KA, Barawid E, Verardo MR. Cellular Remodeling in Mammalian Retina Induced by Retinal Detachment. 1995.

19. Pastor JC, de la Rua ER, Martin F. Proliferative vitreoretinopathy: risk factors and pathobiology. Prog Retin Eye Res 2002; 21:127-44. [PMID: 11906814].

20. Chandler MJ, Smith PJ, Samuelson DA, MacKay EO. Photoreceptor density of the domestic pig retina. Vet Ophthalmol 1999; 2:179-84. [PMID: 11397262].

21. Alge CS, Suppmann S, Priglinger SG, Neubauer AS, May CA, Hauck S, Welge-Lussen U, Ueffing M, Kampik A. Comparative proteome analysis of native differentiated and cultured dedifferentiated human RPE cells. Invest Ophthalmol Vis Sci 2003; 44:3629-41. [PMID: 12882817].

22. Srivastava GK, Reinoso R, Singh AK, Fernandez-Bueno I, Martino M, Garcia-Gutierrez MT, Pastor JC, Corell A. Flow cytometry assessment of the purity of human retinal pigment epithelial primary cell cultures. J Immunol Methods 2013; 389:61-8. [PMID: 23318778].

23. Slomiany MG, Rosenzweig SA. Autocrine effects of IGF-I-induced VEGF and IGFBP-3 secretion in retinal pigment epithelial cell line ARPE-19. Am J Physiol Cell Physiol 2004; 287:C746-53. [PMID: 15140752].
24. Engelsberg K, Johansson K, Ghosh F. Development of the embryonic porcine neuroretina in vitro. Ophthalmic Res 2005; 37:104–11. [PMID: 15746566].