Proliferative Vitreoretinopathy in the Swine–A New Model

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Purpose. To develop a large animal model of proliferative vitreoretinopathy (PVR) in the swine to eventually study disease pathophysiology, as well as novel therapies.

Methods. PVR was induced in domestic swine by creation of a posterior vitreous detachment, creation of a retinal detachment by the injection of subretinal fluid, and intravitreal injection of green fluorescent protein-positive retinal pigment epithelial (GFP+ RPE) cells. Control eyes had the same surgical procedures without RPE cell injection. PVR was clinically graded on days 3, 7, and 14. Animals were euthanized on day 14, and enucleated eyes were analyzed by light microscopy and immunohistochemistry.

Results. Injection of GFP+ RPE cells into the vitreous cavity produced localized, traction retinal detachments by day 14 in all eyes (14 of 14); in contrast, the retina spontaneously reattached by day 3 and remained attached in all control eyes (10 of 10). Contractile epiretinal membranes on the inner retinal surface that caused the traction retinal detachments consisted predominantly of GFP+ RPE cells. These cells stained positive for cytokeratin, confirming their epithelial origin, and also expressed α-SMA and fibronectin, markers for myofibroblasts and fibrosis, respectively.

Conclusions. We established a swine PVR model that recapitulates key clinical features found in humans and, thus, can be used to study the pathophysiology of PVR, as well as new novel therapies. GFP+ RPE cells injected into the vitreous cavity formed contractile membranes on the inner retinal surface and caused localized traction retinal detachments. (*Invest Ophthalmol Vis Sci.* 2012;53:4910–4916) DOI: 10.1167/iovs.12-9768

Proliferative vitreoretinopathy (PVR) is the most common cause of failure in the surgical repair of rhegmatogenous retinal detachment (RRD), and is a major complication of posterior segment ocular trauma.[1] Despite recent advances in surgical techniques, nearly 10% of primary RRD repairs fail because of PVR.[2–4] Unfortunately, only 11% to 25% of eyes that have developed PVR can achieve a visual acuity better than 20/100, even with multiple corrective surgeries.[5–7]

PVR is a wound response characterized by the formation of epiretinal and/or subretinal fibrotic membranes. Contraction of these membranes results in the development of localized traction retinal detachments that ultimately progress to a total retinal detachment with reopening of previous retinal holes or the creation of new retinal breaks.[8] Multiple cell types have been identified in the epiretinal membranes associated with PVR, namely, retinal pigment epithelial (RPE) cells, fibroblasts, myofibroblasts, glial cells, and macrophages.[9–11] Although RPE cells are thought to be the major cell type found in epiretinal membranes in the early stage of PVR, the contribution of each cell type is still unclear.[9–11]

RPE cells can undergo epithelial mesenchymal transition (EMT) upon release into the vitreous cavity.[9,10] In this process, RPE cells dedifferentiate and lose their epithelial characteristics and adopt a fibroblastic phenotype; they also become highly migratory and proliferative.[12] Dedifferentiated RPE cells can transform into myofibroblasts in vitro.[13,14] However, there have not been definitive studies confirming that RPE transformation to fibroblasts and myofibroblasts can occur in vivo in epiretinal membranes.

Several different animal models have been used to study the pathogenesis of PVR and to investigate various medical treatments to prevent its development.[15–19] Most models induce PVR in the rabbit or rodent by injecting various cell types into the vitreous cavity or by releasing endogenous cells with proteolytic enzymes. Although these models have been informative, the eye in these species is not like that of humans. The swine eye has become an attractive non-primate model in experimental studies because of its close similarity to the human eye in size, in selective concentration of cone photoreceptors in the visual streak, and its holangiotic retinal vascular pattern.[20–22] In this paper, we present a new model of PVR using the swine. Green fluorescent protein-positive (GFP+) RPE cells were injected intravitreally so that the fate of these cells could be traced during the development of PVR and their contribution to the formation of epiretinal membranes documented. Our results suggest that RPE cells can give rise to both fibroblasts and myofibroblasts in the contractile membranes observed on the inner retinal surface in PVR and can cause localized traction retinal detachments.

Materials and Methods

GFP+ RPE Isolation and Culture

The use of animals was approved by the University of Louisville Institutional Animal Care and Use Committee and adhered to the ARVO statement on the Use of Animals in Ophthalmic and Vision Research. All cell culture supplies were from Invitrogen (Carlsbad, CA), unless stated otherwise. Fetal GFP+ RPE cells were isolated from embryonic week 12 GFP+ porcine eyes using dispase, based on a previously

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Table. Primary Antibodies Used in This Study

Antigen	Ab Class	Source	Catalog No.	Dilution	Sectioning Method
CK 8,18,19	Ms IgG1	Abcam	ab41825	1:50	Cryo/paraffin section
Fibronectin	Rb poly	SCBT	sc9068	1:50	Cryosection
GFAP	Rb poly	Abcam	ab7260	1:1000	Paraffin section
GFP	Rb poly	Invitrogen	A21311	1:100	Paraffin section
Iba-1	Rb poly	Wako Chemicals	019–19741	1:250	Paraffin section
α-SMA	Ms ascites fluid	Sigma-Aldrich	A2547	1:400	Paraffin section
Vimentin	Rb poly	SCBT	sc7557-R	1:100	Paraffin section

α-SMA, alpha smooth muscle actin; CK, cytokeratin; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; Iba-1, ionized calcium binding adaptor molecule 1; Ms, mouse; Rb, rabbit; SCBT, Santa Cruz Biotechnology, Santa Cruz, CA; Abcam, Cambridge, MA; Invitrogen, Grand Island, NY; Wako Chemicals USA, Richmond, VA; Sigma-Aldrich, St. Louis, MO.

published protocol.[23] Harvested RPE cells/partial sheets were cultured in 25 cm^2 tissue culture flasks for 2 to 6 weeks and then plated on permeable support (Transwell, Corning, Tewksbury, MA) and further cultured for 1 to 7 weeks. Growth medium consisting of minimal essential medium alpha (MEM alpha; 500 mL) supplemented with B27 (10 mL), glutamine (5 mL), nonessential amino acids (5 mL), penicillin/streptomycin (5 mL), taurine (125 mg) (Sigma-Aldrich, St. Louis, MO), hydrocortisone (10 μg; Sigma-Aldrich), triiodothyronin (0.0065 μg; Sigma-Aldrich), and fetal bovine serum (FBS; 5% for flask culture and 1% for microplated cultures) were used. Cells isolated and cultured by this method were > 95% cytokeratin-positive.

Preparation of RPE Cells for Intravitreal Injection

Permeable supports with attached GFP+ RPE cells were incubated in 2% dispase for 25 to 30 minutes at 37°C. Resulting RPE sheets were made into small sheets/cell clusters by pipetting up and down through a 200-μL tip. RPE cell clusters were kept on ice in 10% FBS-supplemented Dulbecco's modified Eagle's medium (DMEM) and switched to serum-free DMEM just before use.

PVR Induction Surgery on Swine

Female domestic swine weighing 12 to 16 kg at the time of surgery were obtained from Oak Hill Genetics (Ewing, IL). Anesthesia was initiated with an intramuscular injection of ketamine hydrochloride (5 mg/kg), butorphanol (0.1 mg/kg), atropine (0.025 mg/kg), and dexmedetomidine (0.02 mg/kg). Anesthesia was maintained by isoflurane (1%–3%) inhalation during surgery. After sedation, pupils were dilated and accommodation relaxed with topical applications of 2.5% phenylephrine hydrochloride and 1% tropicamide.

PVR was induced in swine by a three-step procedure, in an attempt to mimic the situation in human patients. Three-port 20-gauge pars plana vitrectomy was performed to complete posterior vitreous detachment after core vitrectomy, followed by shaving of the peripheral vitreous. Then, total retinal detachment was induced by injecting balanced salt solution (Alcon Laboratories, Fort Worth, TX) into the subretinal space, using a 39-gauge angled cannula. Subretinal injections were 1 disc diameter away from the optic disc, and any visible blood vessels were avoided. Four to five subretinal injections, at least one in each quadrant, were made to cause a total retinal detachment. Finally, after closing the eye, GFP+ RPE cells (8×10^4 cells) in 0.1 mL of DMEM or 0.1 mL of DMEM alone (mock surgery control) were injected into the vitreous cavity.

Ophthalmoscopic Examination of the Eye Fundus

Clinical examinations were performed postoperatively on days 3, 7, 10, and 14. PVR grading was based on the Silicone Study Classification System for PVR,[24] using binocular indirect ophthalmoscopy. Fundus color photographs were taken using a Topcon fundus camera (Oakland, NJ).

Histological and Immunohistochemical Procedure

Swine were euthanized with an overdose of intravenous pentobarbital sodium/phenytoin sodium (Beuthanasia D; Merck Animal Health, Summit, NJ) administered under general anesthesia on day 14. Enucleated globes were fixed for 5 minutes in 4% paraformaldehyde (PFA), pH 7.4, and the anterior segment was removed by a circumferential cut just posterior to the ora serrata. The eye cups were further fixed for 2 hours in 4% PFA. Photographs of fixed eye cups were taken using a dissecting microscope equipped with an FITC excitation filter set (Zeiss MicroImaging, Inc., Thornwood, NY) in order to visualize GFP+ cells.

For cryosections, fixed eyes were dehydrated through a grading series of sucrose and embedded in OCT, and 12-μm sections were cut with a cryostat. Sections were dried at room temperature for 1 hour and used for immunohistochemical staining.

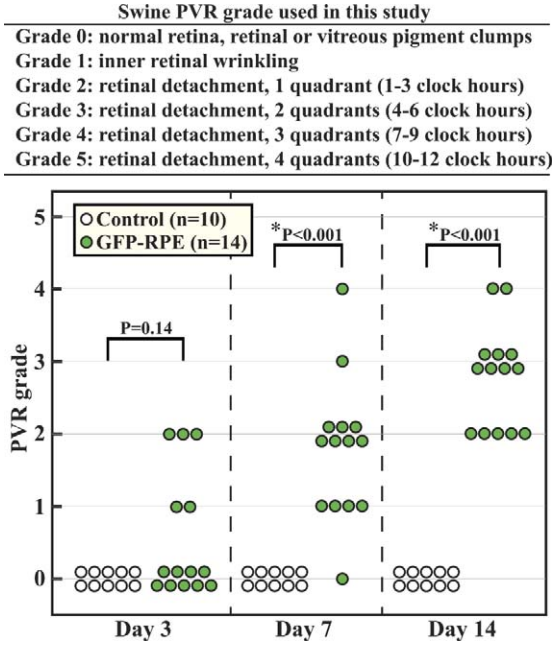

Figure 1. Time course of PVR development in vivo. (*Top*) Classification of PVR grade used in this study. Grading was based on the Silicone Study Classification System (SSCS) for PVR with some modification. Grade 0 included not only normal retina but also vitreous haze as well as pigment clumps on retina or vitreous (SSCS PVR grade A). (*Bottom*) PVR grade of animals on days 3, 7, and 14. No PVR was observed in control eyes (without cell injection, $n = 10$) throughout the 14 days. In contrast, injection of GFP+ RPE ($n = 14$) induced severe PVR (grade ≥2) by day 14 in all animals. *Statistically significant difference between control and experimental eyes.

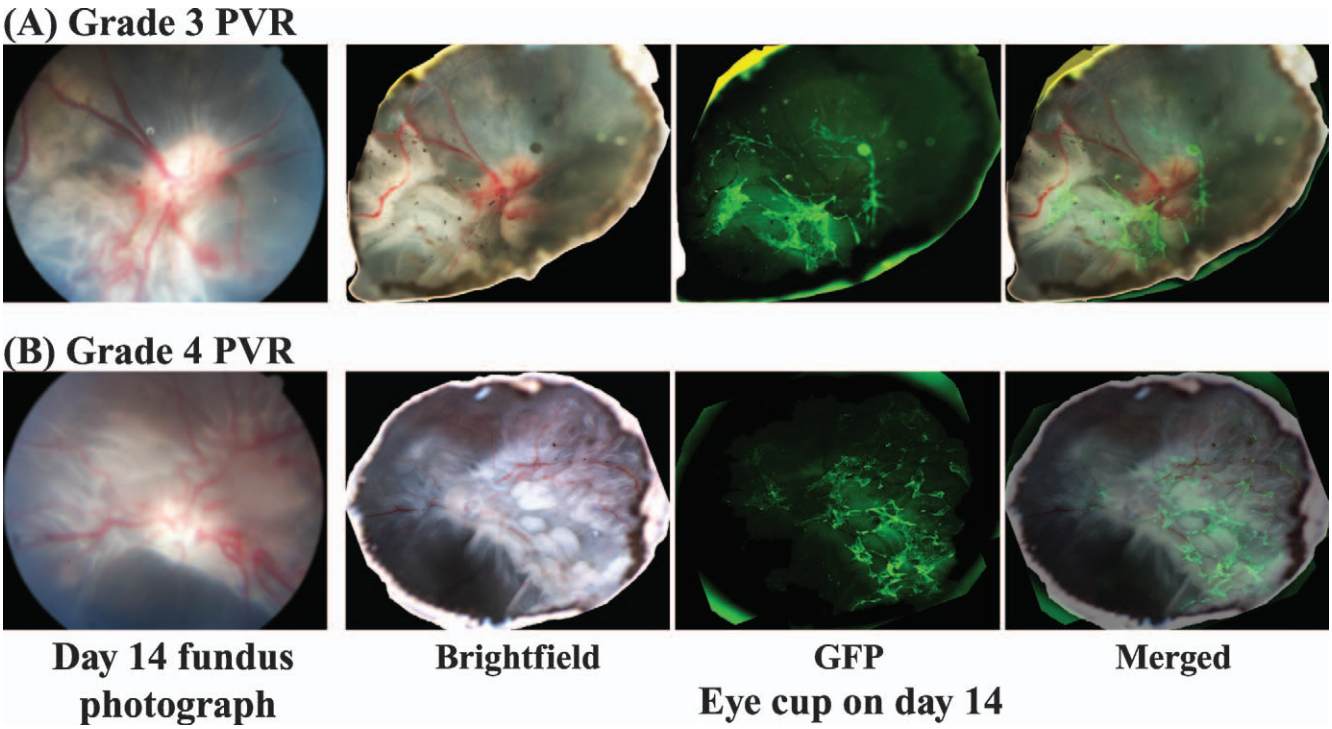

Figure 2. Representative images of PVR on day 14. (**A**) Grade 3 PVR. Fundus photograph and brightfield image show an area of retinal detachment to the left of the optic disc. Eyecups show epiretinal membranes containing GFP+ cells attached to the inner surface of the retina in the area of retinal detachment. (**B**) Grade 4 PVR. Fundus photograph and brightfield images show a more extensive area of retinal detachment with severe wrinkling of the retina. The epiretinal membrane composed of GFP+ RPE-derived cells corresponds to the area of retinal detachment.

Following blocking with 5% normal serum in 0.3% TritonX-100/5% BSA-PBS at room temperature (RT) for 1 hour, sections were incubated with primary antibodies in 5% BSA-PBS at RT for 1 hour. Primary antibodies used are listed in the Table. After being washed three times with PBS, sections were incubated with Alexa Fluor 488 or 555 conjugated secondary antibodies (Invitrogen) in 5% BSA-PBS for 1 hour at RT. Some samples were further counterstained with phalloidin conjugated to Alexa Fluor 647 dye. Following several

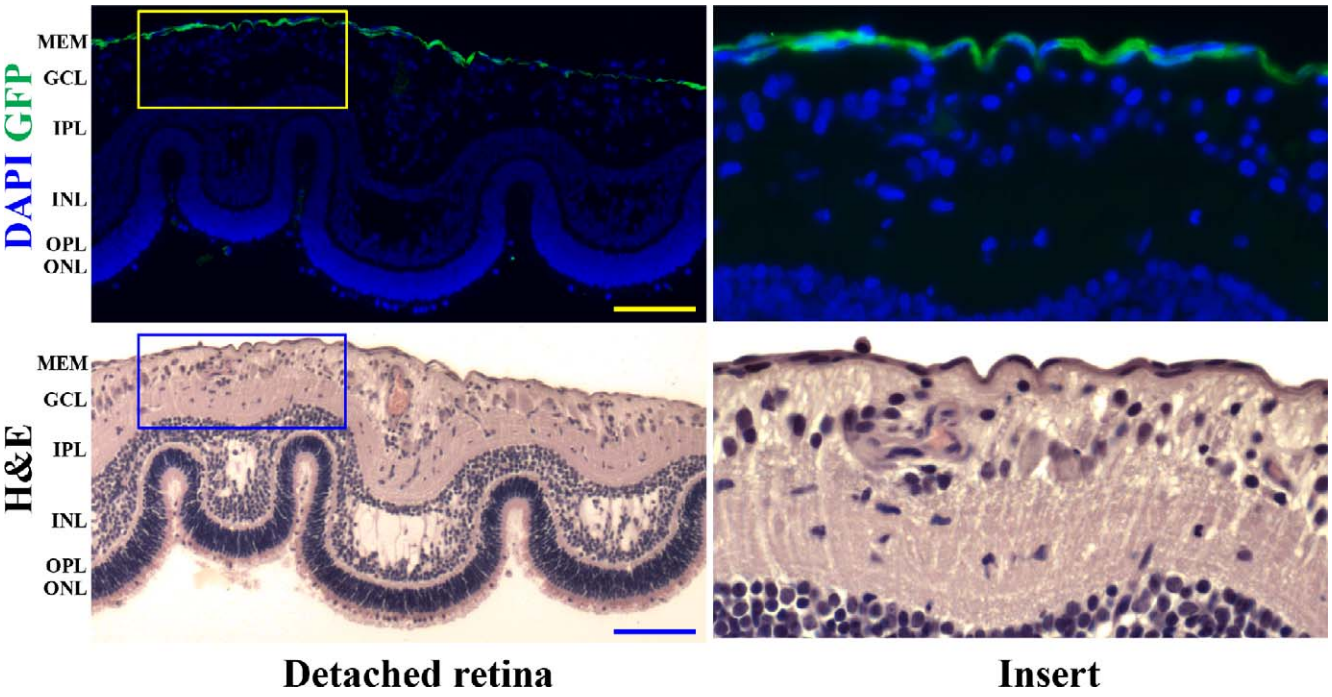

Figure 3. Histologic sections of an epiretinal membrane and detached retina. (*Top*) Immunostaining of a paraffin section with anti-GFP antibody shows GFP+ cells attached to the inner surface of detached retina. (*Inset at right*) shows higher magnification with nuclei stained *blue* with DAPI. (*Bottom*) H&E staining shows stretching of the inner retina and folding of the redundant, detached outer retina. Bars = 100 μm.

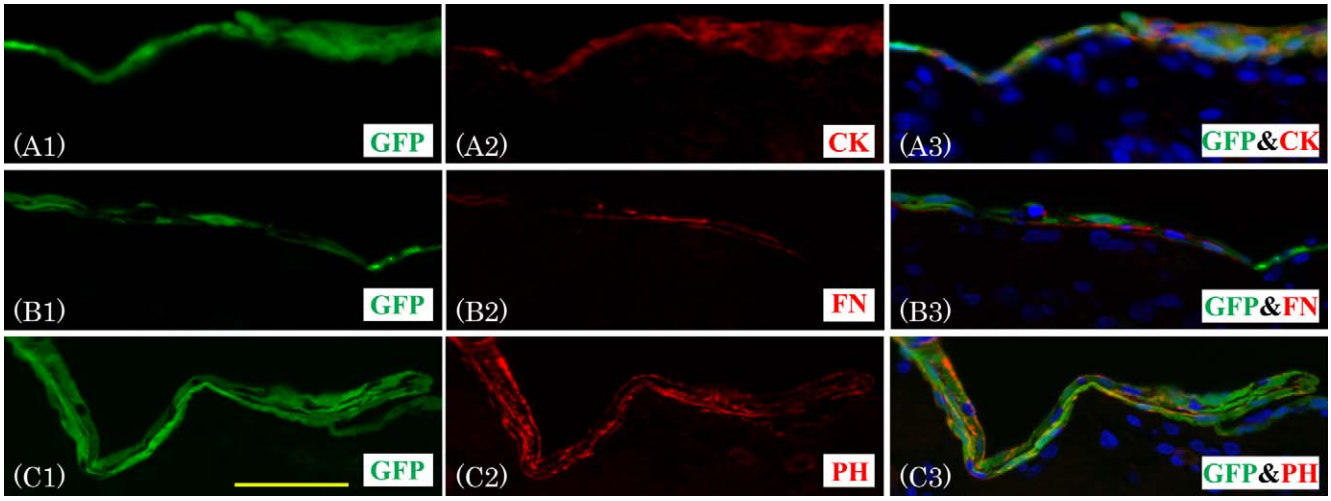

Figure 4. Immunohistochemical staining of cryosections of detached retina from GFP+ RPE injected eyes. (**A1**, **B1**, **C1**) GFP+ cells comprised the epiretinal membrane and attached to the inner surface of the retina. (**A**) All GFP+ cells stained positive for cytokeratin (CK). Merged image (**A3**) clearly shows that GFP- and cytokeratin-positive cells overlap. (**B**) Fibronectin staining (**B2**) was observed beneath some GFP+ cells. (**C**) Actin staining, using phalloidin, showed strong stress-fiber expression in the GFP+ cells. Nuclei are stained *blue* with DAPI. Bar = 100 μm.

washes, samples were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vectashield; Vector Laboratories, Burlingame, CA).

For paraffin sections, fixed eyes were dehydrated through a graded series of ethanol followed by xylene and embedded in paraffin wax. Six-micrometer sections were cut with a microtome. Sections were deparaffinized and rehydrated using a series of incubations with xylene (15 and 10 minutes) and ethanol (2 minutes each in 100%, 100%, 95%, 80%, and 70%), followed by three washes in PBS. Hematoxylin-eosin (H&E) stain was used for routine staining.

For immunohistochemical staining of paraffin-embedded sections, antigen retrieval was performed with deparaffinized sections by heating section in EDTA buffer at 85°C for 30 minutes. Blocking, incubation in primary and secondary antibodies, and mounting procedures were the same as those described above for cryosections, except for staining of cytokeratin. For staining of cytokeratin, biotin-conjugated secondary was followed by Alexa fluor 555-conjugated streptavidin (Invitrogen).

Images of stained sections were captured using fluorescent microscopy and software (Axiovision 4.7; Zeiss MicroImaging). For negative controls, nonimmune mouse or rabbit immunoglobulin G (IgG) in incubation buffer (5% BSA-PBS) was used instead of primary antibodies, and only faint staining was observed.

Statistical Analysis

Differences in PVR grading were analyzed with Mann-Whitney *U* test using MedCalc version 12.1.1 software (MedCalc, Mariakerke, Belgium). A *P* value of less than 0.05 was considered significant.

Results

Clinical Observations

A retinal detachment was created in both experimental and control eyes on day 0 by the subretinal injection of BSS. The detached retina reattached in all control eyes (10/10) by day 3 and remained attached on day 14 (Fig. 1). In contrast, all eyes injected with GFP+ RPE cells (14/14) developed localized traction retinal detachments by day 14 (Figs. 1, 2); in some eyes (3/14), a localized retinal detachment was observed as early as day 3 (Fig. 1). Severity of the retinal detachment on day 14 was variable. The difference in the grade of PVR between experimental and control eyes was significant on both day 7 and day 14.

Morphology

Gross and histologic examinations were performed on eyes enucleated on day 14. Figure 2 shows specimens of grade 3 and grade 4 PVR. Epiretinal membranes on the inner surface of the retina fluoresce green because they are derived from GFP+ RPE cells and colocalized with the area of traction retinal detachment. Light microscopy confirmed that these membranes are composed of GFP+ cells attached to the inner surface of the retina (Fig. 3). H&E staining confirmed traction (i.e., stretching) on the inner retina with folding of the outer retina in the area of the detachment.

Immunohistology

GFP+ cells expressed cytokeratin, a marker for epithelial-derived cells (Fig. 4A) and were distinct from cells expressing glial fibrillary acidic protein (GFAP), a glial cell marker (Fig. 5A). Vimentin, a protein expressed by de-differentiated RPE cells, mesenchymal cells, and glial cells, was observed both in the epiretinal membrane as well as within the retina (Fig. 5B). Alpha smooth muscle actin (α-SMA), a marker for myofibroblasts, was occasionally observed in cytokeratin-positive cells in the epiretinal membrane (Fig. 5C). Fibronectin, a marker for fibrosis, was observed beneath several GFP+ cells (Fig. 4B), and actin staining with phalloidin demonstrated prominent stress fibers in these RPE cells (Fig. 4C). Iba1, which is expressed by macrophages and microglia, was detected within the retina (Fig. 5D) but only rarely in epiretinal membranes.

Discussion

Various models have been used to study PVR in vivo (e.g., see Agrawal et al.[15] for a recent review). Past studies have shown that the intravitreal injection of various cell types found in PVR membranes (i.e., fibroblasts, RPE, glia, or inflammatory cells) can produce PVR-like fibrotic changes in rabbit eyes.[17,19,25–27] Other studies in rabbits and rodents have used proteolytic enzymes that digest the retina and release endogenous cells that result in PVR.[16,18]

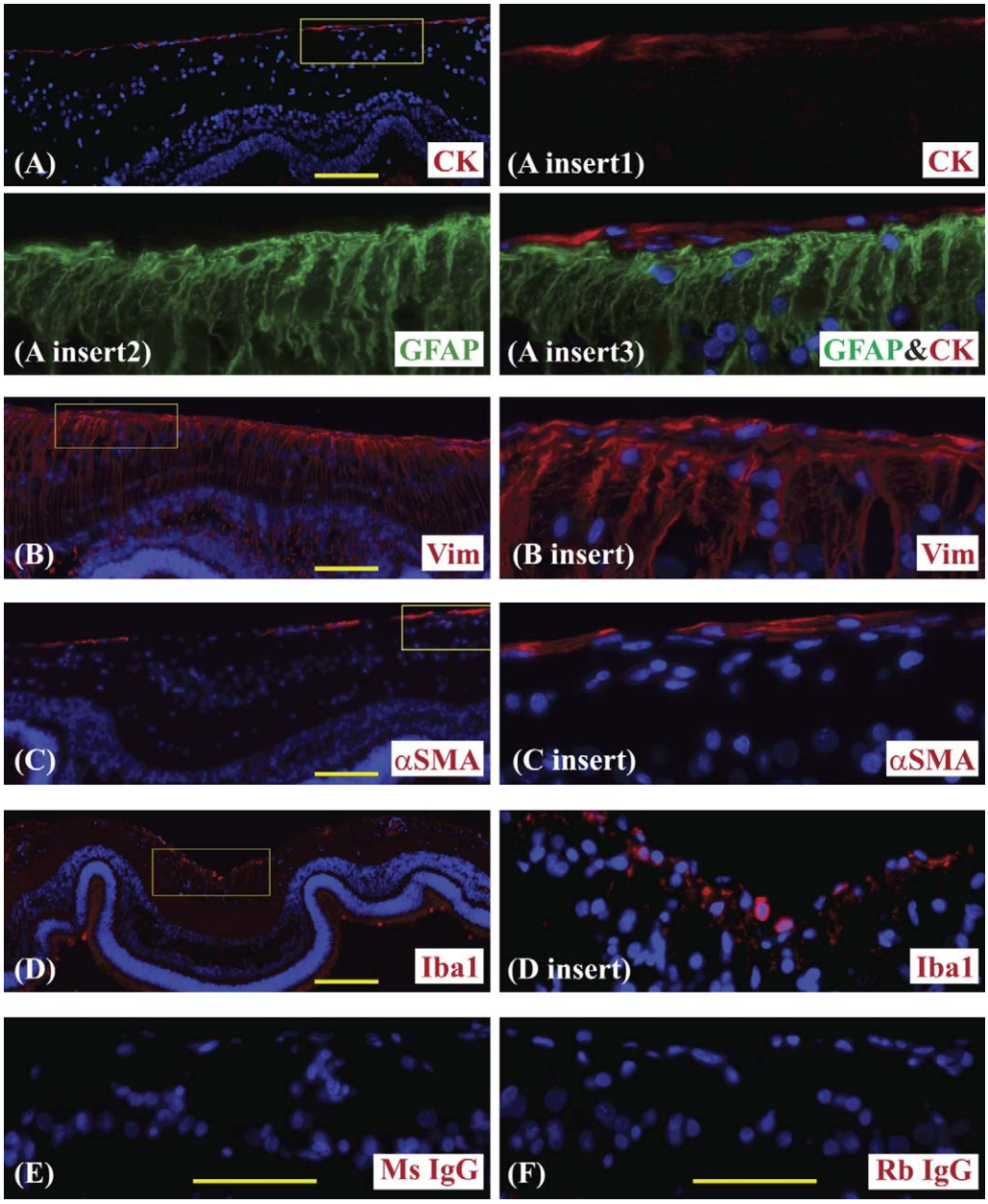

Figure 5. Immunohistochemical staining for cell type-specific markers shows that epiretinal membranes are composed of cells positive for cytokeratin (CK), vimentin (Vim), and α-SMA but not glial fibrillary acidic protein (GFAP). (**A**) CK-positive cells covered the inner surface of the detached retina. Higher magnification (inset) of CK staining (**A**, inset 1), GFAP (**A**, inset 2), and the merged image (**A**, inset 3) show that the epiretinal membrane is composed of CK-positive but not GFAP-positive cells. (**B**) Vimentin staining was observed in cells within the epiretinal membrane, as well as glial cells within the retina. (**C**) Many cells expressed α-SMA within the epiretinal membrane. (**D**) Iba1-positive cells were observed beneath the epiretinal membrane within detached retina. In addition, Iba1-positive mononuclear cells were occasionally observed on the epiretinal membrane. (**E**, **F**) Negative controls in which nonimmune IgG was used instead of primary antibodies showed no staining. Nuclei are stained *blue* with DAPI. Bars = 100 μm.

Although such small animal models have been useful in PVR research, the swine has several advantages when studying this major complication of retinal detachment surgery and severe ocular trauma. The swine eye is similar in size to that of humans, has a holangiotic retinal vasculature, and a cone-enriched area centralis (i.e., the visual streak).[20,28] Despite such advantages, the swine has rarely been used as an animal model for PVR. To the best of our knowledge, there is only one previously published study that used the swine as a model for PVR.[29] That study involved multiple surgical steps including retinectomy, partial vitrectomy, and cryotherapy. In this study, we describe a new model of PVR in the swine, induced by the injection of RPE cells into the vitreous cavity.

We noted the appearance of an epiretinal membrane on the inner surface of the retina following intravitreal injection of RPE cells, with contraction of the membrane causing folding of the neurosensory retina and the eventual development of a localized, traction retinal detachment by day 14, a course similar to that observed in humans.[30]

Epiretinal membranes associated with PVR in patients contain a variety of cell types including RPE cells, glial cells, fibroblasts, myofibroblasts, and macrophages.[10,11] We chose

intravitreal injection of RPE cells because they have been suggested to play an important role in the initiation of PVR. GFP+ RPE cells were used so that we could trace the cells after injection into the vitreous cavity. These cells induced PVR in all (14/14) experimental eyes, whereas none of the control eyes developed PVR. These results clearly establish that injection of RPE cells can result in a clinical picture analogous to PVR.

Gross examination of the enucleated eyes on day 14 confirmed the presence of epiretinal membranes on the inner surface of the retina. These membranes consisted of GFP+ cells and were localized to the areas of traction retinal detachment. The use of GFP+ cells allowed us to follow the cellular phenotype during PVR development in the swine. It also allowed us to distinguish between donor and host cells. Immunohistochemical staining confirmed that the GFP+ cells were of epithelial origin (i.e., cytokeratin-positive). Most of these cells assumed a fibroblastic morphology and strongly expressed vimentin, an intermediate filament protein often used as a marker for EMT of RPE cells. Some GFP+ cells expressed α-SMA, a marker for myofibroblasts, as well as fibronectin, an extracellular matrix protein often used as a marker for fibrosis. α-SMA-positive myofibroblasts are thought to play a role in wound contraction and fibrosis in various organs, including PVR.[31–33] Fibronectin can facilitate cell migration, cell proliferation, and fibrosis.[34] Taken together, our data strongly indicate that in this model RPE cells undergo EMT to assume a fibroblastic phenotype, with some cells further differentiating into myofibroblasts, and that these RPE-derived cells form the epiretinal membrane that eventually contracts and causes localized, traction retinal detachments in vivo.

Iba1 was detected within the detached retina but only rarely in the epiretinal membrane. Past studies have shown that damage to the retina, such as a retinal detachment, can activate microglia, and because Iba1 expression is increased in activated microglia,[35,36] these Iba1-positive cells are most likely activated microglia. Immune cells, specifically macrophages, have been detected in epiretinal membranes from early stage PVR patients,[37] and therefore, it is not surprising that some Iba1-positive cells were detected within epiretinal membranes in this model. Taken together, our data obtained using GFP+ RPE cells and cell type-specific markers demonstrated that epiretinal membranes in this PVR model contain four of the five major cell types found in epiretinal membranes from human PVR patients. Three of these cell types are RPE-derived (RPE, fibroblasts, and myofibroblasts) and constitute the major contractile elements that result in localized retinal detachments in vivo. Very few host immune cells are present, and the absence of GFAP+ glial cells contrasts with past studies that have demonstrated the presence of such cells.[38,39] The absence of a retinal break, which could allow migration of astrocytes/Muller cells into the vitreous cavity and onto the surface of the retina, and the short period of time for PVR development (14 days) in our model could explain the absence of glial cell contribution. Regardless, our results demonstrate that injection of RPE cells into the vitreous cavity can produce contractile cellular epiretinal membranes without astrocyte/Muller cell contribution. Furthermore, contraction of these membranes can lead to traction retinal detachments analogous to that observed in the early phases of human PVR. It will be interesting to know the specificity of RPE cells in inducing PVR, and future studies will examine whether other cell types, such as glial cells and/or macrophages, can replace or augment the PVR effect of RPE cells in the swine model.

In summary, we have created a new animal model of PVR by using the swine, a large animal that has an ocular structure similar to that in humans. The model was created by the intravitreal injection of RPE cells, and our use of GFP+ RPE cells allowed us to trace the contribution of these cells to the epiretinal membranes responsible for PVR. We demonstrated that fibroblastic and myofibroblastic phenotypes can be derived from RPE cells and that they can form a contractile cellular membrane that results in localized, traction retinal detachments. This model may aid in understanding the pathophysiology of PVR, as well as provide a tool to test new therapeutic strategies to prevent the development of PVR in man.

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