Characterization of Conditioned Media Collected from Cultured Adult versus Fetal Retinal Pigment Epithelial Cells

Anton M. Kolomeyer, Ilene K. Sugino, and Marco A. Zarbin

Purpose. To characterize trophic factor secretion of cultured adult and fetal retinal pigment epithelial (RPE) cells and to assess the impact on porcine retinal survival in vitro.

Methods. Conditioned media (CM) were collected from cultured adult and fetal RPE cells and analyzed for trophic factor composition and concentration by multiplex ELISA. Trophic factor receptor occupancy was calculated to evaluate the potential biological effectiveness of the differences in trophic factor concentrations. Retina-preserving activity of the collected CM was evaluated using an in vitro model of degenerating porcine retina. Isobaric tag for relative and absolute quantification (iTRAQ) was used to identify additional proteins with a potential effect on porcine retinal survival.

Results. The overall trophic factor secretion profile of cultured fetal RPE cells remained stable over multiple passages and extended culture duration. Compared with CM from adult RPE cells, fetal RPE-CM had significantly higher concentrations of vascular endothelial growth factor-A (VEGF-A), brain-derived neurotrophic factor (BDNF), and pigment epithelium-derived factor (PEDF) and significantly lower concentrations of leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and nerve growth factor (NGF). Fetal RPE-CM was significantly better than adult RPE-CM at improving degenerating porcine retina survival. iTRAQ analysis identified insulin-like growth factor binding protein-3 (IGFBP-3), semaphorin-3B, transforming growth factor (TGF)-β, hepatoma-derived growth factor (HDGF), and gelsolin as factors potentially contributing to this activity. Co-culture of fetal RPE and porcine retina was significantly better than fetal RPE-CM at preserving porcine retinal survival.

Conclusions. Adult and fetal RPE cells have significantly different trophic factor secretion profiles, which correlate with differences in their ability to support porcine retina survival. Combined with trophic factor receptor occupancy calculations, these data implicate VEGF-A and PEDF as key RPE-derived factors promoting preservation of retinal structure and function. (*Invest Ophthalmol Vis Sci.* 2011;52:5973–5986) DOI:10.1167/iovs.10-6965

Retinal degenerative diseases constitute the leading causes of blindness in the industrialized world.[1] Age-related macular degeneration (AMD), the most prevalent of these, can be treated pharmacologically, although at this time most patients do not recover lost vision.[2] Cell-based therapy may be sight-preserving and/or restoring for patients with these diseases.[3] Transplanted cells can serve as a source of many trophic factors that rescue dying nerve cells[4] and may actually replace the dying cells that are the target of the degenerative condition (e.g., retinal pigment epithelial [RPE] cells and photoreceptors).[5] The existence of RPE-derived trophic factors that rescue photoreceptors was first deduced by Mullen and LaVail,[6] on the basis of experiments involving RCS chimeras. The first successful treatment of retinal degeneration by cellular transplantation was reported by Li and Turner,[7] who described the prevention of photoreceptor cell degeneration by RPE transplantation in the Royal College of Surgeons (RCS) rat. Subsequently, photoreceptor rescue was observed, not only at the RPE transplantation site, but also in the nontransplanted areas, implying trophic factor diffusion.[5,8] Similar findings were observed in transplantation studies of iris pigment epithelial and Schwann cells.[4,9]

Maintenance of normal retinal morphology and physiology relies, in part, on a wide variety of RPE functions, including production of trophic/growth factors.[10] These secreted substances are responsible for regulation of angiogenesis (e.g., vascular endothelial growth factor [VEGF] and pigment epithelium-derived factor [PEDF]),[11,12] apoptosis (e.g., basic fibroblast growth factor [bFGF]),[13] prevention or promotion of cell differentiation and maturation (e.g., platelet-derived growth factor [PDGF]),[14] and provision of trophic support to photoreceptor cells and the choroid (e.g., PEDF, ciliary neurotrophic factor [CNTF], brain-derived neurotrophic factor [BDNF], neurotrophin-3 [NT-3], and others).[15–18]

In this study, we characterized trophic factor secretion of cultured adult and fetal RPE cells. The study of cultured fetal RPE cells enabled assessment of the changes in secretory potential of these cells under different culturing conditions. As transplantation of adult RPE cells has been considered a potential therapeutic option for select retinal degenerative diseases (reviewed in Ref. 19), the trophic factor secretion profile of these cells was also elucidated. A degenerating porcine retina in vitro assay was used to ascertain the ability of various conditioned media to support retinal survival. These results were compared to a co-culture of fetal RPE cells and porcine retina, to assess whether RPE-conditioned media could support retinal survival in vitro to the same degree as direct RPE–retina interactions.

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METHODS

Human Donor Eye Tissue

This research complied with the Declaration of Helsinki and was approved by the Institutional Review Board of UMDNJ-New Jersey Medical School. Eyes from non-AMD and AMD Caucasian donors of 55 years of age or older were obtained through various eye banks or suppliers (the National Disease Resource Interchange, Inc., Philadelphia, PA; Vision Share, Apex, NC; Midwest Eye banks, Ann Arbor, MI; the Tampa Lions Eye Institute for Transplant & Research, Tampa, FL; and the San Diego Eye bank, San Diego, CA) in the United States and Canada. The tissue acceptance criteria included (1) no recent history of chemotherapy or radiation to the head; (2) no mechanical ventilation before death; (3) an interval of up to 7 hours between death and enucleation, with eyes preserved in a moist chamber and stored on ice immediately after removal; (4) an interval of no more than 48 hours between death and receipt; and (5) intact, bright, not opaque, orange-colored RPE monolayer as visualized through a dissecting microscope. Fetal eyes (17–22 weeks gestation) were obtained through Advanced Bioscience Resources, Inc. (ABR; Alameda, CA). By these gestational ages, the RPE cell monolayer is formed completely. On arrival, the eyes were cleaned of extraneous tissue; dipped in povidone-iodine solution (10% Betadine; The Purdue Frederique Company, Stamford, CT), which was immediately rinsed off with balanced salt solution; and washed twice for 10 minutes at 4°C in Dulbecco's modified Eagle's medium (DMEM with 1 g/L glucose, L-glutamine, and sodium pyruvate, containing 3.7 g/L sodium bicarbonate (Cellgro-Meditech, Inc., Manassas, VA), supplemented with 250 μg/mL amphotericin B (Gibco-Invitrogen, Carlsbad, CA).

RPE Cell Isolation

The anterior segment and neural retina were removed, exposing the posterior segment. The RPE-choroid was separated from the sclera and incubated in 0.8 mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37°C, 10% CO_2 (fetal posterior segments) or 0.4 mg/mL collagenase type IV for 30 minutes at 37°C, 10% CO_2 (adult posterior segments). The RPE sheets were separated carefully from the choroid with a 22-gauge needle, rinsed in Dulbecco's phosphate-buffered saline (DPBS; Cellgro-Mediatech, Inc.), cut into small pieces (fetal RPE) or triturated with a 200-μL pipette (adult nonmacular RPE), and plated on 35-mm tissue culture dishes (treated by vacuum glass plasma) (TCTP) coated with bovine corneal endothelium-extracellular matrix (BCE-ECM)[20–22] (adult and fetal RPE) or uncoated TCTP dishes (fetal RPE only) (Falcon; BD Biosciences-Falcon Labware, New Bedford, MA). The homogenous RPE cell population was verified by morphology and cytokeratin staining according to the manufacturer's instructions (Sigma-Aldrich). BCE-ECM-coated TCTP dishes were prepared according to previously established methods.[23] The cells were cultured in DMEM supplemented with 15% fetal bovine serum (FBS; Gibco-Invitrogen), one ng/mL of human recombinant bFGF (Gibco-Invitrogen), 2 mM L-glutamine (Gibco-Invitrogen), 2.50 μg/mL amphotericin B (Gibco-Invitrogen), and 0.05 mg/mL gentamicin sulfate (Cellgro-Mediatech, Inc.). This medium, hereafter known as RPE medium, was changed three times a week. On reaching confluence (12–16 days after plating), the fetal RPE cells were detached from dishes with 0.25% trypsin-EDTA treatment for ~7 minutes at 37°C, 10% CO_2. Viable cells were counted (trypan blue solution; Cellgro-Mediatech, Inc.) under a light microscope (Standard 20; Carl Zeiss Meditec, Oberkochen, Germany), and 5×10^5 cells were seeded on either BCE-ECM–coated or uncoated TCTP dishes (passage-1 fetal RPE cells). On confluence and before subsequent passaging, passage-1 fetal RPE cells were cultured in DMEM for 24 hours to synchronize the cell cycle.[24] Cultured passage-2 to passage-6 fetal RPE cells were used for all experiments. Adult RPE cells were not passaged (primary culture).

Conditioned Medium Collection

Primary adult RPE cells ($n = 7$; average age, 81.7 years) were isolated from donor eyes without subretinal pathology (based on biomicroscopy with a dissecting microscope) and cultured in 35-mm dishes for 12 to 16 days (until visually determined cessation of cell division). Passage-2, -4, and -6 fetal RPE cells ($n = 5$; average age, 19.0 weeks gestation) were seeded at 1.2×10^6 cells per well (one half of in situ RPE density)[25] in 12-well BCE-ECM–coated or uncoated TCTP plates (Costar; Corning Inc., Corning, NY) and allowed to grow for 6 or 29 days. Confluence was reached after 3 to 4 days. The cells were photographed before media collection. RPE medium was changed three times a week. Quiescent adult RPE and passage-2, -4, and -6 fetal RPE cells after 6 and 29 days in culture were washed twice with DPBS to remove any remnants of FBS and bFGF found in the RPE medium and cultured in 0.21 mL/cm^2 (adult RPE) or 0.39 mL/cm^2 (fetal RPE) of DMEM for 24 hours at 37°C, 10% CO_2. The resultant CM (cultured adult RPE-CM; and passage-2, day-7; passage-2, day-30; passage-4, day-7; passage-4, day-30; passage-6, day-7; and passage-6 day-30 cultured fetal RPE-CM, respectively) were collected, centrifuged at 1000 rpm for 5 minutes (model 5415c; Eppendorf, Hauppauge, NY) to remove cellular debris, and frozen at −80°C (Bio Freezer, Forma Scientific, Evanston, IL).

Trophic Factor Quantification

Preliminary studies testing secretion of trophic factors (previously shown to possess photoreceptor and/or retina-preserving functions)[26–31] determined that nine trophic factors (Table 1) were consistently secreted by the fetal RPE cells at a concentration >10 pg/μg RPE protein (epidermal growth factor [EGF], glial-derived neurotrophic factor [GDNF], PDGF-β, NT-3, and interleukin 1- [IL-1β] were excluded from quantification). Preparations of CM were analyzed for these nine factors in duplicate via multiplex ELISA (Aushon Biosystems, Woburn, MA). DMEM was analyzed for the same factors as a control for nonspecific binding (quantified in duplicate on three separate occasions to ensure method reliability). CM from cultured RPE cells were corrected for these values. Mean ± SEM for each trophic

TABLE 1. Retinal and Photoreceptor-Preserving Trophic Factors Quantified by Multiplex ELISA[26–31]

Trophic Factor	MW (kDa)	Biological Effect*
Brain-derived neurotrophic factor (BDNF)	14	Neurotrophic
Ciliary neurotrophic factor (CNTF)	24	Neurotrophic
Basic fibroblast growth factor (bFGF)	18	Proangiogenic, photoreceptor rescue
Heparin-binding epidermal growth factor (HB-EGF)	23	RPE proliferation, VEGF secretion
Hepatocyte growth factor (HGF)	83	RPE survival, neuroprotection
Nerve growth factor (NGF)	13	Neurotrophic, inflammation
Pigment epithelium-derived factor (PEDF)	46	Antiangiogenic, neurotrophic
Vascular endothelial growth factor-A (VEGF-A)	43	Proangiogenic, photoreceptor development
Leukemia inhibitory factor (LIF)	20	Photoreceptor rescue, RPE survival

* Only trophic effects are listed.

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factor with a level higher than the threshold detection level was expressed as picograms of factor per microgram of RPE protein. Trophic factor detection frequencies, defined as the number of times a trophic factor was successfully identified by multiplex ELISA divided by the total number of CM samples analyzed, were calculated.

Protein Quantification

Protein was isolated from RPE cells after the 24-hour CM collection period. After the 35-mm culture dishes (cultured adult RPE cells) and 12-well plates (cultured fetal RPE cells) were washed twice with ice-cold DPBS, 200 μL of 1× lysis buffer (10 mM Tris, 500 μM EDTA, 75 mM NaCl, 0.5% Triton X-100, 5% glycerol, and 1% 100× protease inhibitor cocktail; Pierce-Thermo Fischer Scientific, Rockford, IL) prepared in dH_2O was added. RPE cells were gently brushed off the choroid into the lysis buffer, triturated on ice, sonicated three times for 10 seconds at 4°C (Branson Sonifier 250; VWR Scientific, West Chester, PA), and centrifuged for 10 minutes at 10,000 rpm and 4°C. The lysates were collected and frozen at −80°C. Protein was quantified with the Bradford reagent (Sigma-Aldrich), according to the manufacturer's instructions.

Porcine Retina

Porcine Eye Tissue. Eyes from 5- to 9-month-old, 150 to 230 lb, male and female American Yorkshire pigs were obtained from a local abattoir within 3 hours of enucleation (transported on ice). The eyes were prepared for dissection by the method outlined previously for the human donor eyes. The anterior segment and vitreous were removed, leaving the neural retina in the eye cup. Six millimeter trephine blades (Storz Ophthalmic-Bausch & Lomb, Manchester, MO) were used to isolate equatorial, full-thickness retina tissue explants (avoiding the peripapillary region) by separating the retina from the underlying RPE-choroid-sclera.[32] These explants were randomly assigned to different culture conditions to negate the potential effects of selection bias and variability in retinal thickness.[33]

Retinal Cytotoxicity. The lactate dehydrogenase (LDH) in vitro toxicology assay (TOX-7; Sigma-Aldrich) was used to assess the effects of various CM on retinal membrane integrity. Media from retinal explants collected at 1, 6, 24, and 48 hours of culture were centrifuged for 5 minutes at 1000 rpm to remove cellular debris. The supernatant was frozen at −20°C and processed per the manufacturer's instructions. Colorimetric absorbances were assessed by a microplate reader (ELx800; BioTek, Winooski, VT) at 490 nm. The data are expressed relative to the 1-hour levels.

Retinal Apoptosis. A cell-death detection ELISA kit (Roche Diagnostics, Piscataway, NJ) was used to quantify the effects of various CM on the amount of retinal DNA fragmentation. After 1, 6, 24, or 48 hours of culture, the explants were homogenized in 200 μL of provided lysis buffer by trituration, allowed to react for 30 minutes at room temperature and centrifuged for 5 minutes at 1000 rpm, 4°C, and the supernatant was frozen at −20°C. The specimens were processed per the manufacturer's instructions. Absorbances were measured at 405 nm with reference wavelength at 490 nm (ELx800, BioTek). A DNA-histone complex (included) served as the positive control. The data are expressed relative to the 1-hour levels.

Real-Time Polymerase Chain Reaction. RNA was isolated from cultured porcine retina and quantified for trophic factor mRNA expression. Briefly, RNA was washed, bound, and eluted according to manufacturer's instructions (RNeasy Mini Kit; Qiagen). It was quantified using a spectrophotometer (Nanodrop-1000; Thermo Fisher Scientific, Waltham, MA). cDNA was prepared using an RT-PCR reaction mixture (Applied Biosystems, Inc. [ABI], Foster City, CA) in a thermocycler (MJ Mini Personal Thermo Cycler; Bio-Rad, Hercules, CA). Real-time PCR for each trophic factor was performed (model 7500 real-time PCR system; ABI). 18S rRNA transcript served as an endogenous control. Trophic factors were expressed in relation to the arbitrarily chosen heparin-binding epidermal growth factor $(HB\text{-}EGF)_{time\ 0}$ transcript, which was assigned a value of 1. The results were calculated for each group of samples as the mean ± SEM of the level of $HB\text{-}EGF_{time\ 0}$.

Fetal RPE–Porcine Retina Co-culture

Passage-2 and -6 fetal RPE cells were isolated as described previously and seeded in 96-well TCTP plates (Falcon; BD Biosciences-Falcon Labware) at 3.26×10^4 cells per well. RPE medium was changed three times a week. On day 7, the cells were washed twice with DPBS, and 100 μL of DMEM, and one porcine retinal explant (6 mm in diameter) was added to each well. Control wells included cultured fetal RPE cells with no retina in DMEM or porcine retina in DMEM (no fetal RPE). Retinal cytotoxicity and apoptosis were measured at 1, 6, 24, and 48 hours. Cytotoxicity was corrected by subtracting the fetal RPE ($n = 5$) contribution (control wells) from the total measurements. The 1-hour time point served as an internal control of retinal preservation. CM was collected 24 and 48 hours after culture from wells containing fetal RPE cells and porcine retina (fetal RPE-retina-CM), wells with fetal RPE cells only (fetal RPE-CM), and wells with porcine retina only (retina-CM). CM was centrifuged, frozen, and analyzed for selected trophic factors (Table 1). To determine whether alterations of porcine retinal trophic factor mRNA expression correlated with changes in protein secretion (i.e., retinal trophic factor contribution to fetal RPE-retina-CM), retinas were harvested after co-culture with the fetal RPE cells at time 0 and 1, 6, 24, and 48 hours for mRNA quantification. 18S rRNA transcript served as an endogenous control with the VEGF-A transcript arbitrarily set to equal 1 ($^{\Delta}$Ct method).

Isobaric Tag for Relative and Absolute Quantification

The isobaric tag for relative and absolute quantification (iTRAQ) multiplex global protein analysis allows for simultaneous identification and quantification of proteins from different sources in one experiment (reviewed in Ref. 34). iTRAQ analysis was performed to determine whether factors, in addition to those tested, that possess the potential to affect photoreceptor and retinal preservation could be identified in the CM. Four different preparations of primary cultured adult RPE-CM and passage-2, day-7 cultured fetal RPE-CM (10 mL of each medium) were submitted to and processed by the Center for Advanced Proteomics Research at UMDNJ (University of Medicine and Dentistry of New Jersey). A detailed description of the iTRAQ analysis has been published.[35] Briefly, after acetone precipitation of the proteins, the samples were desalted (2D gel Clean up Kit; Bio-Rad). The protein pellets were resuspended in 60 μL of a lysis buffer containing 150 mM triethylammonium bicarbonate (TEAB), 1% NP40 (Igepal CA-630; Sigma-Aldrich), 1% Triton X-100, and 3 μL of phosphatase inhibitor cocktails I and II. Protein concentrations were measured using the bicinchoninic acid (BCA) method (BCA Protein Assay Kit; Thermo–Fisher Scientific) according to the manufacturer's instructions. The iTRAQ labeling procedures (using amine-specific, stable isotope reagents) were performed according to the manufacturer's instructions (ABI), with equal volumes of each medium. Protein enzymatic digestion was performed by addition of 8 mg of trypsin (Promega Corp., Madison, WI) to each of the eight samples at 37°C, overnight. Peptides derived from four cultured adult RPE-CM were labeled with iTRAQ tags 113, 114, 115, and 116 whereas samples obtained from four cultured fetal RPE-CM were labeled with tags 117, 118, 119, and 121. The labeled peptides were mixed, loaded onto a strong cation exchange column (BioCAD Perfusion Chromatography System; ABI), and detergents and free iTRAQ reagents were washed out. Labeled peptides were fractionated and analyzed on a tandem mass spectrometer (4800 Proteomics Analyzer MALDI-TOF-TOF; ABI). To reduce the probability of false identification, only proteins with at least two peptides with confidence values ≥95% were reported. Relative quantification of peptides in each sample was calculated from the areas under the peaks. Average change ratios between the adult and fetal RPE-CM were calculated. Scaffold Q+ software (Proteome Software Inc., Portland, OR) was used to visualize peptide change across samples and sort differentially expressed proteins. Pathway analysis software (Ingenuity ver. 8.7; Ingenuity Systems, Inc., Redwood City, CA) was used to

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Table 2. Trophic Factor Composition of CM Collected from Cultured Fetal RPE Cells of Different Passages (Passage-2, -4, or -6) and Times in Culture (Day-7 or -30 Grown on TCTP Dishes

Fetal RPE Cell Culture	LIF	bFGF	HB-EGF	HGF	VEGF-A	NGF	BDNF	CNTF	PEDF
P2D7									
Mean ± SEM	9 ± 4.1	86.8 ± 14.9*†	78.3 ± 17.1	807.8 ± 92.6	21,518.8 ± 2,666.2‡‡	13 ± 2.6	246.8 ± 35.5	34.5 ± 6.7	84,5334 ± 89,237
Range	4.6–17.2	55.5–137.7	33–122	628.7–938.1	15,390–27,596.9	9.9–18.3	146.1–298.8	27.8–41.1	627,720–992,325
P2D30									
Mean ± SEM	6.6 ± 2.2	65.4 ± 6.2	95.6 ± 17.3	557 ± 66.9	16,673.7 ± 3,456.2	15.1 ± 2.1	263.5 ± 31.3	37.2 ± 6.5	1,045,309 ± 119,245§§,‖‖
Range	2.4–9.6	48.6–79.4	42.3–137.3	489.5–690.8	9,780–24,296.8	11–22.7	184.1–356.9	29–50	820,748–1,373,697
P4D7									
Mean ± SEM	8.9 ± 2.6	171.4 ± 19.2‡§‖	75.3 ± 8.5	500 ± 82.2	10,045.7 ± 1,664.5	11.5 ± 2.3	171.3 ± 36.8	58.2 ± 11.3	483,253 ± 59,223
Range	2.8–14.4	132.8–238.7	45.6–98.8	405.3–663.8	4,829.8–14850	7.5–17.5	84.9–263.1	35.7–70.3	469,635–608,933
P4D30									
Mean ± SEM	7 ± 1.5	57.3 ± 10	102.2 ± 16.6	725.5 ± 87.2	13,732.2 ± 1,892.5	9.2 ± 1.8	248.2 ± 45.8	50.7 ± 10.4	721,518 ± 87,148
Range	2.9–9.8	37.2–94.3	45.9–131.2	638.2–812.7	8,423.9–18,652.5	4.5–12.9	133.2–348	32–67.8	528,855–887,213
P6D7									
Mean ± SEM	10.7 ± 2.9	160.4 ± 20.4#**††	81.3 ± 12.9	617.8 ± 44.9	8,563.6 ± 1,368.6	12.2 ± 2.5	138.8 ± 31.1	50.4 ± 3.1	483,256 ± 63,146
Range	4.1–18.4	110.9–200.7	46.8–106.8	533.5–712.1	5,334–12,171	6–17.4	49.5–182.4	44.1–56.3	383383–666180
P6D30									
Mean ± SEM	8.5 ± 3.4	70.3 ± 10.4	102.2 ± 13.4	771.5 ± 71.4	11,691.7 ± 2,221.4	17.6 ± 2.1	210.7 ± 38.2	54.4 ± 5.2	725,030 ± 12,4978
Range	2.3–17.1	38.7–102.8	62.7–138.9	629.4–855.1	4,014.5–17,439.2	13.5–19.7	127.2–312	45.1–63	522,720–1,08,6026

Differences in trophic factor production with respect to passage number and duration of culture were evaluated for statistical significance ($P < 0.05$) by one-way ANOVA followed by the Holm-Sidak pairwise multiple comparison. P2D7, passage-2, day-7; P2D30, passage-2, day-30; P4D7, passage-4, day-7; P4D30, passage-4, day-30; P6D7, passage-6, day-7; P6D30, passage-6, day-30.

The following comparisons were statistically significant: *passage-2 day-7 vs. passage-4 day-7 ($P < 0.001$); †passage-2 day-7 vs. passage-6 day-7 ($P = 0.002$); ‡§‖passage-4 day-7 vs. passage-2 day-30, passage-4 day-30, and passage-6 day-30 ($P < 0.001$ for all); #**††passage-6 day-7 vs. passage-2 day-30, passage-4 day-30, and passage-6 day-30 ($P < 0.001$ for all); ‡‡passage-2 day-7 vs. passage-6 day-7 ($P < 0.05$); §§passage-2 day-30 vs. passage-4 day-7 ($P < 0.001$); and ‖‖passage-2 day-30 vs. passage-6 day-7 ($P < 0.001$).

* Passage-2 day-7 vs. passage-4 day-7 ($P < 0.001$).
† Passage-2 day-7 vs. passage-6 day-7 ($P = 0.002$).
‡ Passage-4 day-7 vs. passage-2 day-30 ($P < 0.001$).
§ Passage-4 day-7 vs. passage-4 day-30 ($P < 0.001$).
‖ Passage-4 day-7 vs. passage-6 day-30 ($P < 0.001$).
Passage-6 day-7 vs. passage-2 day-30 ($P < 0.001$).
** Passage-6 day-7 vs. passage-4 day-30 ($P < 0.001$).
†† Passage-6 day-7 vs. passage-6 day-30 ($P < 0.001$).
‡‡ Passage-2 day-7 vs. passage-6 day-7 ($P < 0.05$).
§§ Passage-2 day-30 vs. passage-4 day-7 ($P < 0.001$).
‖‖ Passage-2 day-30 vs. passage-6 day-7 ($P < 0.001$).

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Table 3. Trophic Factor Composition of CM Collected from Cultured Fetal RPE Cells of Different Passages (Passage 2, 4, or 6) and Times in Culture (Day 7 or 30) Grown on BCE-ECM-Coated Dishes

Fetal RPE Cell Culture	LIF	bFGF	HB-EGF	HGF	VEGF-A	NGF	BDNF	CNTF	PEDF
P2d7									
Mean ± SEM	8.2 ± 2.9	92.8 ± 18.9	149 ± 16.3	521.4 ± 63.2	19,719.5 ± 1,540.8	14.6 ± 3.4	321.9 ± 37	35 ± 5.4	803,526 ± 121,280
Range	1.6–16.9	38.6–119.9	110.6–193.8	340.2–610.9	15,309.1–24,367.5	4.4–24.5	258.5–386.7	24.7–42.9	526,845–1,101,413
P2D30									
Mean ± SEM	9.6 ± 1.8	60.4 ± 9.9	176.7 ± 26.1	760.7 ± 126.3	19,390 ± 3,651.1	22.3 ± 3.1	185.9 ± 31.5	41.9 ± 6.7	1,033,486 ± 190,309
Range	3.3–13.4	31.7–93.5	113.5–270.6	412.8–1015	8,452.5–24,832.8	14–27	154.4–217.4	28.9–60.5	657,218–1,544,275
P4D7									
Mean ± SEM	6.5 ± 2.1	177.6 ± 25*†‡§	136.8 ± 25.5	488.3 ± 95.4	13,256.4 ± 3,010.5	22.7 ± 5	282 ± 52.8	56.2 ± 5.5‡‡§§||||	671,511.6 ± 49,339
Range	3.2–12.6	94.2–248.1	75–196	242.2–617.4	5,101.5–20Z475	13.7–32.4	229.2–334.8	42–74.7	579,244–809,888
P4D30									
Mean ± SEM	9.7 ± 2.8	67 ± 16.8	177.2 ± 21.5	628.6 ± 83.2	18,346.4 ± 2,440.9	28.2 ± 3.4	244.6 ± 28	33.8 ± 5.2	935,137 ± 94,989
Range	3.6–17.2	16.5–119.1	135–252.6	411.7–909.9	12,318.3–26,985	20–35.1	200.7–296.8	28.6–39	695,988–1,095,681
P6D7									
Mean ± SEM	12.3 ± 2.9	216.8 ± 25||#**††	118.7 ± 18.5	448.5 ± 92	9,032 ± 1,446.6	25.6 ± 5.5	206.5 ± 30.8	58.2 ± 3.6##***†††	440,924 ± 64,603
Range	8.8–20.9	168.9–312	90–184.5	225.4–775.2	60,29.4–13,822.5	14.9–38.2	175.7–237.4	54.6–61.8	323,910–632,376
P6D30									
Mean ± SEM	10.2 ± 2.3	82.7 ± 26.1	157.4 ± 29.6	545.4 ± 65.1	17,715.7 ± 3,817	32.6 ± 6	218.5 ± 32.5	26 ± 4.4	765,632 ± 178,870
Range	3.6–14.2	40.4–185.5	86.1–240.4	355.4–698.2	7,926.6–24,982.5	19.9–48.8	186–251	21.1–39.2	264,750–1,334,755

Differences in trophic factor production with respect to passage number and duration of culture were evaluated for statistical significance ($P < 0.05$), by one-way ANOVA followed by the Holm-Sidak pairwise multiple comparison. P2D7, passage-2, day-7; P2D30, passage-2, day-30; P4D7, passage-4, day-7; P4D30, passage-4, day-30; P6D7, passage-6, day-7; P6D30, passage-6, day-30.

The following comparisons were statistically significant: *†‡§passage-4 day-7 vs. passage-2 day-7 ($P = 0.009$), passage-2 day-30 ($P < 0.001$), passage-4 day-30 ($P = 0.001$), and passage-6 day-30 ($P = 0.004$); ||#**††passage-6 day-7 vs. passage-2 day-7 ($P < 0.001$), passage-2 day-30 ($p < 0.001$), passage-4 day-30 ($P < 0.001$), and passage-6 day-30 ($P < 0.001$); ‡‡§§||||passage-4 day-7 vs. passage-2 day-7 ($P = 0.018$), passage-4 day-30 ($P = 0.027$), and passage-6 day-30 ($P < 0.001$); and ##***†††passage-6 day-7 vs. passage-2 day-7 ($P = 0.034$), passage-4 day-30 ($P = 0.041$), and passage-6 day-30 ($P = 0.004$).

* Passage-4 day-7 vs. passage-2 day-7 ($P = 0.009$).
† Passage-4 day-7 vs. passage-2 day-30 ($P < 0.001$).
‡ Passage-4 day-7 vs. passage-4 day-30 ($P = 0.001$).
§ Passage-4 day-7 vs. passage-6 day-30 ($P = 0.004$).
|| Passage-6 day-7 vs. passage-2 day-7 ($P < 0.001$).
Passage-6 day-7 vs. passage-2 day-30 ($P < 0.001$).
** Passage-6 day-7 vs. passage-4 day-30 ($P < 0.001$).
†† Passage-6 day-7 vs. passage-6 day-30 ($P < 0.001$).
‡‡ Passage-4 day-7 vs. passage-2 day-7 ($P = 0.018$).
§§ Passage-4 day-7 vs. passage-4 day-30 ($P = 0.027$).
|||| Passage-4 day-7 vs. passage-6 day-30 ($P < 0.001$).
Passage-6 day-7 vs. passage-2 day-7 ($P = 0.034$).
*** Passage-6 day-7 vs. passage-4 day-30 ($P = 0.041$).
††† Passage-6 day-7 vs. passage-6 day-30 ($P = 0.004$).

Table 4. Detection Frequencies of Trophic Factors Identified in CM Collected from Cultured Adult and Fetal Cells

	Fetal RPE		Adult RPE
	TCTP	BCE-ECM	BCE-ECM
LIF	73	87	100
bFGF	97	100	100
HB-EGF	100	100	86
HGF	57	87	86
VEGF-A	100	100	100
NGF	77	83	100
BDNF	83	47	71
CNTF	57	67	57
PEDF	80	87	100

Data are expressed as the percentage detection frequency.

identify the functional location (i.e., intracellular versus secreted) of the identified proteins.

Trophic Factor Receptor Occupancy

To relate the differences in the measured concentrations of secreted trophic factors in various CM to potential biological activity (defined as the ability of the CM to improve the survival of degenerating porcine retina), we calculated the mean (±SEM) percentage of receptor occupancy for each trophic factor and its primary receptor. Passage-6, day-7 cultured fetal RPE-CM and cultured adult RPE-CM trophic factor receptor occupancies were compared with those of passage-2, day-7 cultured fetal RPE-CM in a pairwise manner, to elucidate statistically significant differences. The mathematical theory and equations required to calculate trophic factor receptor occupancy have been described.[36] Briefly, in occupancy theory, the magnitude of a biological response is posited to be directly proportional to the receptor-ligand complex concentration.[36] It is also a function of the dissociation constant (K_d). Occupancy equals the concentration of the ligand divided by the quantity K_d plus the concentration of the ligand (equation 1). K_d values for each trophic factor receptor were identified through the PubMed search engine.

$$\text{Receptor occupancy} = L/K_d + L$$

where L is the ligand concentration, and K_d is the dissociation constant.

Statistical Analysis

Trophic factors found to be below the factor-specific detection threshold were not included in the statistical analyses (all CM analyses [except that of the iTRAQ data] performed with Sigma Plot 11; Systat Software. Inc., San Jose, CA). Significance was accepted at $P < 0.05$. After the data passed the Shapiro-Wilks normality test and the equal-variance test, an unpaired *t*-test (two groups) or a one-way analysis of variance (ANOVA) was used followed by the Holm-Sidak pairwise multiple comparison (multiple groups). Potential correlation of secretion of trophic factors to each other and to death-to-preservation and death-to-receipt times were calculated with the Spearman rank order correlation. Comparison of adult versus fetal iTRAQ data was performed with the two-tailed *t*-test for each peptide (Excel; Microsoft Corp., Redmond, WA).

Results

Trophic Factor Protein Secretion

The mean ± SEM micrograms of RPE protein isolated from fetal RPE cells of passage 2, 4, and 6 on days 7 and 30, grown on uncoated (TCTP) and BCE-ECM–coated dishes was 0.23 ± 0.027 and 0.25 ± 0.023, respectively. Fetal RPE cell culture purity was confirmed by cytokeratin staining of passage-2, day-7 cells grown in 12-well TCTP plates (not shown). Trophic factor secretion for different passages and times in culture is shown for fetal RPE grown on uncoated (Table 2) and BCE-ECM–coated (Table 3) TCTP dishes. For cells grown on TCTP dishes (Table 2), passage number and time in culture affected the secretion of some trophic factors including: (1) bFGF (passage-4, day-7 and passage-6, day-7 were significantly higher than other passages); (2) VEGF-A (passage-2, day-7 was significantly higher than passage-6, day-7); and (3) PEDF (passage-2, day-30 was significantly higher than that of passage-4, day-7 and passage-6, day-7). For cells grown on BCE-ECM-coated dishes (Table 3), passage number and time in culture affected the secretion of the following trophic factors: (1) bFGF (passage-4, day-7 and passage-6, day-7 were significantly higher than other passages) and (2) CNTF (passage-4, day-7 and passage-6, day-7 were significantly higher than passage-2, day-7, passage-4, day-30, and passage-6, day-30). A paired *t*-test was used to compare fetal RPE trophic factor secretion within each passage and duration of culture as a function of the underlying substrate. The secretion of the following factors was significantly higher for fetal RPE on TCTP versus BCE-ECM–coated dishes: (1) hepatocyte growth factor (HGF) ($P = 0.045$) for passage-2, day-7 cells and (2) CNTF ($P = 0.009$) for passage-6, day-30 cells. The secretion of the following factors was significantly higher for fetal RPE cells on BCE-ECM-coated versus TCTP dishes: (1) HB-EGF ($P = 0.017$) for passage-2, day-7 cells; (2) HB-EGF ($P = 0.032$) for passage-2, day-30 cells; (3) PEDF ($P = 0.05$) for passage-4, day-7 cells; (4) HB-EGF ($P = 0.024$) for passage-4, day-30 cells; and (5) nerve growth factor (NGF; $P = 0.003$) for passage-4, day-30 cells. The trophic factor detection frequencies for cells grown on TCTP and BCE-ECM–coated dishes are listed in Table 4.

Secretion by cultured primary adult RPE cells was significantly higher for leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and NGF and significantly lower for VEGF-A, BDNF, and PEDF compared with passage-2, day-7 cultured fetal RPE cells (Table 5). This comparison was undertaken for the cell subtypes cultured on BCE-ECM-coated dishes only, since adult RPE cells were not cultured on TCTP dishes due to poor attachment and growth on this substrate. The

Table 5. Trophic Factor Composition of CM Collected from Cultured Primary Adult RPE Cells and Passage-2, Day-7 Cultured Fetal RPE Cells Grown on BCE-ECM–Coated Dishes

	LIF	bFGF	HB-EGF	HGF	VEGF-A	NGF	BDNF	CNTF	PEDF
P2D7-fRPE ($n = 5$)	8.2 (2.9)	92.8 (18.9)	149 (16.3)	521.4 (63.2)	19,719.5 (1,540.8)	14.6 (3.4)	321.9 (37.0)	35 (5.4)	803,526 (121,280)
Adult RPE ($n = 7$)	135.9 (17.7)	308.3 (34.2)	366 (156.1)	605.9 (154.7)	9,434.4 (2,147.6)	93.8 (14.8)	60.2 (5.6)	307.2 (118.0)	180,381 (77,460)
P*	0.003	<0.001	>0.05	>0.05	0.005	0.003	0.036	>0.05	0.001

Data are expressed as the mean (SEM) picograms per milligram RPE protein. P2D7-fRPE, passage-2 day-7 fetal RPE.
* Unpaired *t*-test.

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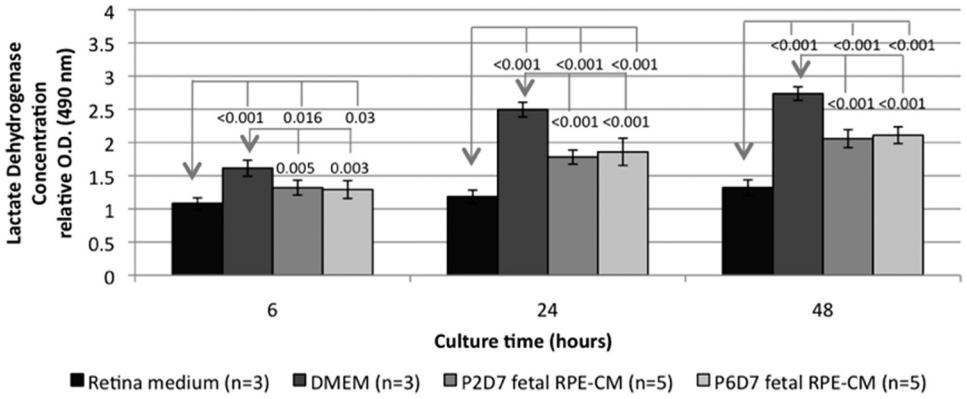

FIGURE 1. Effect of CM collected from passage-2, day-7 and passage-6, day-7 cultured fetal RPE cells on porcine retinal cytotoxicity. Porcine retina was cultured in CM collected from passage-2, day-7 ($n = 5$; P2D7 fetal RPE-CM) and passage-6, day-7 ($n = 5$; P6D7 fetal RPE-CM) cultured fetal RPE cells and in positive ($n = 3$; retina medium) and negative ($n = 3$; DMEM) control medium. Mean ± SEM LDH concentration was compared for statistical significance ($P < 0.05$) by one-way ANOVA followed by the Holm-Sidak pairwise multiple comparison.

overall detection frequencies of the secreted trophic factors in adult versus fetal RPE-CM were similar; therefore, we do not believe that the significant differences identified between cultured adult and fetal RPE cells were biased by detection frequencies (Table 4).

Preservation of Porcine Retina

To determine whether CM from cultured adult and fetal preparations affect retinal preservation to different degrees, 6 mm in diameter retinal explants were isolated from porcine eyes and cultured from 1 to 48 hours in retina medium (positive control), DMEM (negative control), and cultured RPE-CM (passage-2, day-7 and passage-6, day-7 cultured fetal RPE-CM and primary adult RPE-CM grown on BCE-ECM-coated dishes). LDH and DNA fragmentation were measured at each time point, with the 1-hour results serving as the reference time point. At each time point, retinas in passage-2, day-7 and passage-6, day-7 cultured fetal RPE-CM showed significantly lower survival than the retinas in retina medium, significantly better survival than retinas in DMEM, and did not differ significantly from one another (Figs. 1, 2). Passage-2, day-7 cultured fetal RPE-CM was significantly better than primary cultured adult RPE-CM at reducing retinal cytotoxicity and apoptosis at the 24- and 48-hour time points (Figs. 3, 4). In addition, by the 48-hour time point, retinal cytotoxicity and apoptosis in primary cultured adult RPE-CM did not differ significantly from that of DMEM.

Cultured Fetal RPE: Retina Co-culture

Effect on Preservation of Porcine Retina. Porcine retinal cytotoxicity and apoptosis were compared after a 6-, 24- or 48-hour culture in one of two conditions: (1) passage-2, day-7 cultured fetal RPE-CM versus co-culture with passage-2, day-7 fetal RPE cells and (2) passage-6, day-7 cultured fetal RPE-CM versus co-culture with passage-6, day-7 fetal RPE cells (Table 6). A significantly higher level of retinal cytotoxicity was identified in passage-2, day-7 and passage-6, day-7 cultured fetal RPE-CM than in the corresponding co-cultures at 24 and 48 hours. In addition, significantly more retinal apoptosis was identified in passage-2, day-7 cultured fetal RPE-CM than passage-2, day-7 cultured fetal RPE-retina co-culture at the 6-hour time point and in passage-6, day-7 cultured fetal RPE-CM than passage-6, day-7 cultured fetal RPE-retina co-culture at the 6- and 48-hour time points (Table 6).

Effect on Trophic Factor Production. In addition to quantifying retinal cytotoxicity and apoptosis, trophic factor composition of passage-2, day-7 fetal RPE-retina-CM (co-culture) was analyzed at the 24- and 48-hour time points via multiplex ELISA. This analysis was performed on three fetal RPE cell lines (mean age, 20.7 weeks' gestation) grown on BCE-ECM– coated dishes. In comparison to cultured fetal RPE-CM, at the 24-hour time point, the fetal RPE-retina co-culture CM had significantly higher levels of bFGF and HGF and, at the 48-hour time point, significantly higher levels of bFGF, HB-EGF, and HGF (Table 7). There was also a strong trend for increased BDNF production by the fetal RPE-retina co-culture at the 24-hour ($P = 0.057$) and 48-hour ($P = 0.059$) time points.

Retinal Trophic Factor Production. To determine whether trophic factor secretion by the porcine retina could have accounted for the significant differences in CM collected from fetal RPE cells versus fetal RPE-retina co-culture (Table 7), porcine retinal trophic factor secretion was quantified by multiplex ELISA. After nonspecific binding was accounted for, the only factors identified in the retina-CM were bFGF, HB-EGF, and VEGF-A. After 48 hours of culture in DMEM, the porcine retina produced 21 to 30 pg of VEGF-A and 25 to 36 pg of HB-EGF per explant. The amount of bFGF increased from 98 pg/explant at 1 hour to 128 pg/explant at the 6-hour time point and remained relatively constant thereafter throughout the

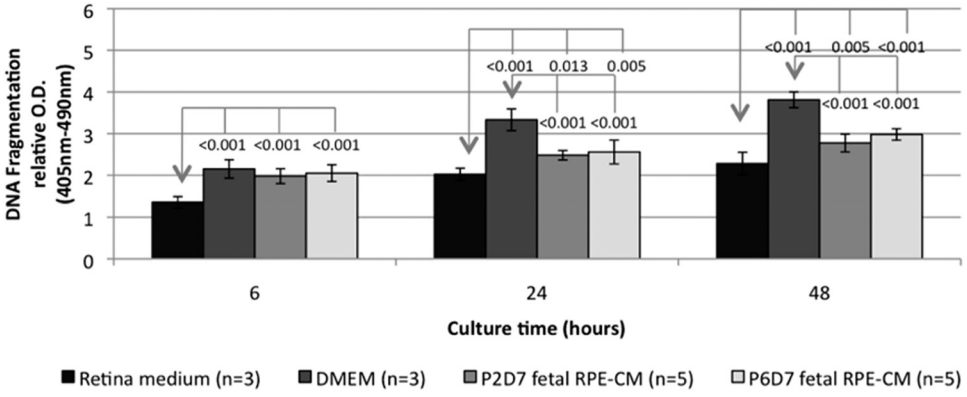

FIGURE 2. Effect of CM collected from passage-2, day-7 and passage-6, day-7 cultured fetal RPE cells on porcine retinal apoptosis. Porcine retina was cultured in CM collected from passage-2, day-7 ($n = 5$; P2D7 fetal RPE-CM) and passage-6, day-7 ($n = 5$; P6D7 fetal RPE-CM) cultured fetal RPE cells and in positive ($n = 3$; retina medium) and negative ($n = 3$; DMEM) control medium. Mean ± SEM DNA fragmentation was compared for statistical significance ($P < 0.05$), by one-way ANOVA followed by the Holm-Sidak pairwise multiple comparison.

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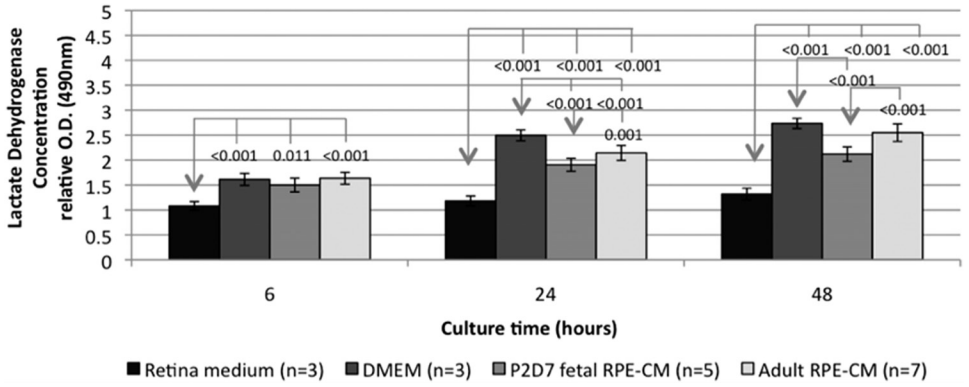

Figure 3. Effect of CM collected from passage-2, day-7 cultured fetal RPE cells and primary cultured adult RPE cells on porcine retinal cytotoxicity. Porcine retina was cultured in CM collected from passage-2, day-7 ($n = 5$; P2D7 fetal RPE-CM) and cultured adult RPE cells ($n = 7$; adult RPE-CM) and in positive ($n = 3$; retina medium) and negative ($n = 3$; DMEM) control medium. Mean ± SEM LDH concentration was compared for statistical significance ($P < 0.05$) by one-way ANOVA followed by the Holm-Sidak pairwise multiple comparison.

48-hour culture period. The relative contribution of retinal VEGF-A, HB-EGF, and bFGF to the amounts found in the fetal RPE-retina co-culture CM after 24 and 48 hours of culture were approximately 0%, 4% to 8%, and 4% to 9%, respectively.

Retinal Trophic Factor mRNA Expression. Compared with time 0, there was no transcript upregulation after 24 or 48 hours of culture that would correspond to the significantly increased levels of selected proteins identified in co-culture CM (data not shown). In addition, although retinal VEGF-A protein levels were almost identical with HB-EGF and were three to five times lower than those of bFGF, the amount of VEGF-A mRNA transcript was approximately three orders of magnitude higher than those of HB-EGF and bFGF.

Isobaric TAG for Relative and Absolute Quantification

A total of 381 proteins were identified with at least two peptides with a confidence interval of ≥95%. The false-discovery rate of the peptides was estimated at 4.7%. Of these 381 proteins, 95 (25%) were significantly ($P < 0.05$) different between cultured adult RPE-CM and passage-2, day-7 cultured fetal RPE-CM (cells grown on BCE-ECM-coated dishes). Forty-seven (49%) of these significantly different proteins, most of which were secreted proteins, previously have been shown to regulate apoptosis, affect cellular response to oxidative stress, or involve the complement cascade (Table 8). Candidate proteins that seem most likely to have contributed to the differential effect of cultured adult RPE-CM and passage-2, day-7 cultured fetal RPE-CM on retinal preservation are listed in Table 9. These proteins were identified as candidate proteins based on their function and the degree of significance of the difference between adult and fetal CM. Fetal RPE cells secreted significantly less IGFBP-3 (relative ratio = 0.3), semaphorin-3B (relative ratio = 0.5), and TGF-β (relative-ratio = 0.7), and significantly more hepatoma-derived growth factor (HDGF; relative ratio = 2.1) and gelsolin (relative ratio = 2.1). iTRAQ confirmed significantly higher production of PEDF (relative ratio = 2.5) by fetal RPE cells, which was previously shown by multiplex ELISA (Table 4).

Trophic Factor Receptor Occupancy

Compared with the CM isolated from passage-2, day-7 fetal RPE cells, the concentrations as well as the corresponding trophic factor receptor occupancies for VEGF-A and PEDF were significantly lower in the CM isolated from cultured adult RPE cells and passage-6, day-7 fetal RPE cells (Table 10).

Discussion

Although RPE cells grown on TCTP plates have been shown to exhibit a gene expression profile closest to that of native RPE cells,[45] we were interested in determining how BCE-ECM (an excellent substrate for RPE cell growth and differentiation) influenced RPE trophic factor secretion.[21] Various underlying substrates have been shown to affect RPE cell secretion.[46,47] The secretion of bFGF, VEGF-A, CNTF, and PEDF was significantly affected by passage number and/or duration of culture, whereas the secretion of HB-EGF, HGF, CNTF, and PEDF was significantly affected by culture surface. Knowledge of the effects of culturing surface as well as cell passage number and duration of time in culture on trophic factor production could be used in engineering an RPE cell line with the highest degree of potential photoreceptor/retina preservation.

The Spearman rank order correlation was used to identify potential correlations in trophic factor secretion. The following high-degree ($\rho > 0.7$) correlations were identified: (1) bFGF and VEGF-A in CM isolated from passage-2, day-7 fetal RPE cells grown on TCTP dishes (inverse correlation); (2) bFGF and HB-EGF in CM isolated from passage-6, day-30 fetal RPE

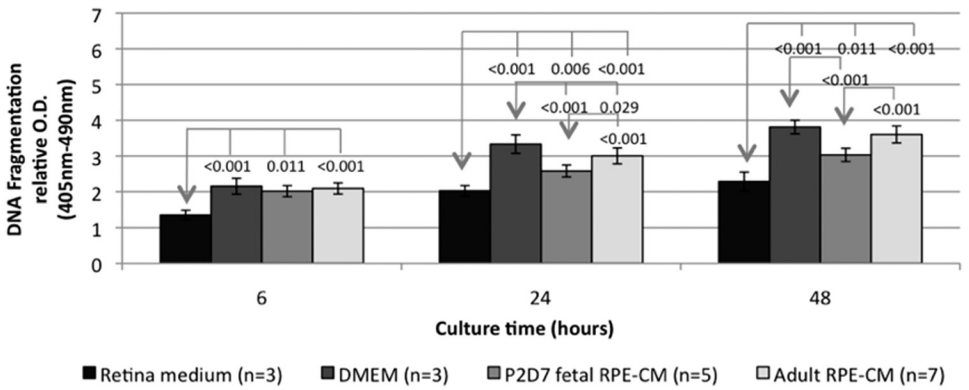

Figure 4. Effect of CM collected from passage-2, day-7 cultured fetal RPE cells and primary cultured adult RPE cells on porcine retinal apoptosis. Porcine retina was cultured in CM collected from passage-2, day-7 ($n = 5$; P2D7 fetal RPE-CM) and cultured adult RPE cells ($n = 7$; adult RPE-CM) and in positive ($n = 3$; retina medium) and negative ($n = 3$; DMEM) control medium. Mean ± SEM DNA fragmentation was compared for statistical significance ($P < 0.05$) using a one-way ANOVA followed by the Holm-Sidak pairwise multiple comparison.

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TABLE 6. Effect of CM Collected from Cultured Fetal RPE Cells or Fetal RPE-Retina Co-cultures on Porcine Retinal Cytotoxicity and Apoptosis

	Retinal Cytotoxicity			Retinal Apoptosis		
Type of Conditioned Medium (n = 5)	**6 h**	**24 h**	**48 h**	**6 h**	**24 h**	**48 h**
Passage-2, day-7 cultured fetal RPE	122.2 ± 6.0	150.8 ± 5.2*	156.1 ± 5.9†	146.2 ± 7.6‖	122.6 ± 3.2	121.5 ± 5.4
Passage-2, day-7 fetal RPE-retina co-culture	118.8 ± 8.0	113.9 ± 5.9	111.6 ± 3.0	123.9 ± 6.9	118.1 ± 3.5	114.0 ± 2.1
Passage-6, day-7 cultured fetal RPE	119.5 ± 7.1	157.4 ± 10.0‡	160.0 ± 5.5§	151.5 ± 8.5#	126.4 ± 8.1	130.5 ± 3.5**
Passage-6, day-7 fetal RPE-retina co-culture	123.2 ± 5.8	118.5 ± 4.9	114.5 ± 3.7	128.3 ± 5.6	117.7 ± 4.0	116.1 ± 4.9

Data are represented as amount (mean ± SEM) (%) of extracellular LDH (retinal cytotoxicity) and retinal DNA fragmentation (retinal apoptosis) compared to levels found after retinal culture in the retina medium at the corresponding time points. Corresponding pairs (passage-2, day-7 fetal cells vs. retina co-culture and passage-6, day-7 fetal cells vs. retina co-culture) were evaluated for statistical significance ($P < 0.05$) at each time point by unpaired *t*-test.

The following comparisons were statistically significant:

* $P = 0.046$.
† $P = 0.005$.
‡ $P = 0.02$.
§ $P = 0.05$.
‖ $P = 0.01$.
$P = 0.009$.
** $P = 0.037$.

cells grown on TCTP dishes; and (3) LIF and HB-EGF in CM isolated from passage-2, day-30 fetal RPE cells grown on BCE-ECM–coated dishes. Basic FGF and VEGF-A previously have been shown to induce each others secretion and stimulate parallel intracellular signaling cascades[48,49]; however, in our experimental system, their secretion was inversely related. Since both factors are proangiogenic, this interaction may represent a regulation strategy used by RPE cells to keep angiogenesis under control.[50] Correlation of increased PEDF secretion to counteract rising VEGF-A levels was not observed in our system.[51] Another interesting interaction is that of bFGF-induced upregulation of HB-EGF secretion, which has been shown to upregulate VEGF-A secretion.[30,52] This induced VEGF-A secretion could act as a check-point mechanism for bFGF production, thereby creating feedback control between the proangiogenic factors. Although, the direct correlation between LIF and HB-EGF on photoreceptor–retinal preservation has not been established, these factors previously have been shown to induce RPE cell proliferation and survival, which could in turn improve photoreceptor survival.[15,30]

The effects of CM collected from passage-2, day-7 and passage-6, day-7 cells grown on TCTP dishes on porcine retina were pursued for the following considerations: (1) large differences in trophic factor secretion were identified between these two CM preparations for VEGF-A, BDNF, CNTF, and PEDF; (2) the passage-2, day-7 cells were considerably more like in situ fetal RPE cells based on size, shape, and organization than are passage-6, day-7 cells; therefore, transplanting passage-2, day-7 cells may result in better cell integration and function; and (3) since exponentially more retina-preserving CM can be collected from passage-6, day-7 than passage-2, day-7 cells due to cell propagation, if CM from passage-6, day-7 cells was as good as CM from passage-2, day-7 cells at preserving the retina, the use of passage-6, day-7 cells would dramatically facilitate CM preparation from limited donor tissue. Both of these CM significantly decreased retinal cytotoxicity and apoptosis compared with DMEM treatment alone. They did not differ from one another at any of the tested time points, despite differences in trophic factor secretion that we measured. Therefore, either our quantitative methods are too crude to detect a difference in retinal preservation due to the significantly different amounts of trophic factors between these two CM, or, although significantly different, the differences in trophic factor levels are not biologically significant. Assessment of porcine retinal viability at longer time points might yield a better biological separation. One possible limitation in the approach used here is reflected in the <100% trophic factor detection frequency (Table 4) of the proteins studied, which could be related to technical (threshold) limitations of the multiplex ELISA method, trophic factor hydrolysis, RPE cell degeneration, and/or biological variability in regulation of trophic factor production and secretion.[53,54]

Compared with cultured adult RPE-CM, passage-2, day-7 cultured fetal RPE-CM was significantly more efficacious at reducing retinal cytotoxicity and apoptosis. These results imply that differences in trophic factors in these CM were sufficient to induce a change in retinal preservation. The most profound differences with regard to trophic factor receptor occupancy between passage-2, day-7 cultured fetal RPE-CM and cultured adult RPE-CM included significant decreases in VEGF (26.7% vs. 4.6%, respectively) and PEDF (90.0% vs. 17.9%, respectively; Table 10).[55,56] In addition to significant differences in trophic factor composition of these CM, the diminished ability to improve retinal preservation could be due

TABLE 7. Comparison of Trophic Factor Composition of CM Collected from Passage-2, Day-7 Fetal RPE Cells and a Co-culture of Passage-2, Day-7 Fetal RPE Cells with Porcine Retina after 24 and 48 Hours of Culture

	24-h Culture		48-h Culture		
	bFGF	**HGF**	**bFGF**	**HGF**	**HB-EGF**
−Retina (n = 3)	139.1 (5.0)	45.4 (10.6)	74.2 (38.3)	46.5 (23.1)	332 (9.8)
+retina (n = 3)	3036.1 (317.5)	154 (11.3)	1383.9 (254.4)	230 (36.4)	419.7 (20.5)
P*	<0.001	0.002	0.007	0.013	0.018

Data are expressed as the mean (SEM) picograms per milligram RPE protein.

* Unpaired *t*-test.

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TABLE 8. List of Secreted Proteins Found in Significantly Different Quantities from Cultured Adult versus Fetal RPE Cells

Identified Protein Name	Accession Number	Size (kDa)	General Functions	Ratio (Fetal/Adult)	*P**
Cathepsin B	IPI00295741.4	38	Regulation of apoptosis	0.2	0
Alpha-crystallin B chain	IPI00021369.1	20	Negative regulation of cell growth; anti-apoptosis	0.2	0
Retinoid isomerohydrolase (RPE-65)	IPI00029250	65	Implicated in some types of Leber Congenital Amaurosis and retinitis pigmentosa; regulation of rhodopsin gene expression	0.2	0
Serpin peptidase inhibitor	IPI00550991.3	51	Increases cortical neuron apoptosis	0.3	0
Superoxide dismutase	IPI00022314.1	25	Negative regulation of neuron apoptosis; affects neuronal development	0.3	0
Prosaposin	IPI00012503.1	58	Increases cell apoptosis (caspase-dependent)	0.3	0.02
Metalloproteinase inhibitor 1 (TIMP)	IPI00032292.1	23	Anti-apoptotic; regulates cell proliferation	0.3	0
Secretogranin-2	IPI00009362.2	71	Negative regulation of cell apoptosis; MAPKKK cascade; cell proliferation, migration	0.3	0
Granulin (isoform 1)	IPI00296713.4	64	Cytokine/growth factor activity; positive regulation of epithelial cell proliferation	0.3	0.02
C3 Complement (fragment)	IPI00783987.2	188	Complement system activation (classical and alternative pathways)	0.3	0
Alpha-2-macroglobulin	IPI00478003.2	163	Protease inhibitor and cytokine transporter; negative regulation of complement activation	0.3	0
NAD(P)H dehydrogenase	IPI00012069	31	Positive regulation of neuron apoptosis; oxidation reduction	0.3	0
Insulin-like growth factor-binding protein-3	IPI00018305	32	Cell growth, proliferation, differentiation, survival; positive regulation of apoptosis	0.3	0.01
Retinaldehyde-binding protein-1	IPI00218633.5	36	Implicated in retinitis pigmentosa and rod-cone dystrophies; 11-cis-retinal transport; visual cycle	0.3	0
Galectin-1	IPI00219219.3	15	Autocrine negative growth factor; modulates cell-cell, cell-matrix interactions; regulation of apoptosis; positive regulation of I-kappa-B kinase/NF-kappa-B cascade	0.3	0
Inter-alpha (globulin) inhibitor H4 (isoform 2)	IPI00218192	101	Acute phase response; cell apoptosis	0.3	0
Ceruloplasmin	IPI00017601.1	122	Copper ion transport; cellular iron ion homeostasis; oxidation reduction	0.4	0
Collagen alpha-2(I) chain	IPI00304962	129	Transforming growth factor beta receptor signaling pathway; Rho protein signal transduction; cell migration, proliferation, and growth	0.4	0
Latent-transforming growth factor beta-binding protein-2	IPI00292150.4	195	Transforming growth factor beta receptor signaling pathway; protein secretion	0.4	0
Extracellular matrix protein-1 (isoform 1)	IPI00003351.2	61	Positive regulation of I-kappa-B kinase/NF-kappa-B cascade; cell growth, apoptosis, and proliferation	0.4	0.02
Glutathione peroxidase-3	IPI00026199.2	26	Cell damage, survival, viability, growth, and apoptosis; response to oxidative stress	0.4	0
Thrombospondin-1	IPI00296099.6	129	Activation of MAPK activity; fibronectin, integrin, bFGF, and TGF-beta binding; increases neurite outgrowth	0.5	0
Fibronectin (isoform 1)	IPI00022418.1	263	Cell adhesion, migration, spreading, and apoptosis	0.5	0
Inter-alpha (globulin) inhibitor H2	IPI00305461	106	Cell apoptosis; extracellular matrix stabilization	0.5	0
Low-density lipoprotein related protein-1 (alpha-2-macroglobulin receptor)	IPI00020557.1	505	Positive regulation of anti-apoptosis; cell proliferation, attachment, and death	0.5	0
EGF-containing fibulin-like extracellular matrix protein-1 (isoform 1)	IPI00029658.1	55	Visual perception; VEGF-A regulation	0.5	0.01
Glutathione S-transferase omega-1	IPI00019755.3	28	Cell redox homeostasis; stress response	0.5	0.02
Semaphorin-3B (isoform 1)	IPI00012283.2	83	Axonal (growth cone) guidance; apoptosis inducer	0.5	0.01
Laminin subunit alpha-5	IPI00783665.4	400	Cell adhesion, survival, apoptosis, migration, and proliferation	0.5	0
Fibulin-1 (isoform B)	IPI00218803	77	Cell apoptosis; extracellular matrix structural constituent; Ca^{+2} ion binding; implicated in AMD	0.6	0.01

(*continues*)

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TABLE 8 (*continued*). List of Secreted Proteins Found in Significantly Different Quantities from Cultured Adult versus Fetal RPE Cells

Identified Protein Name	Accession Number	Size (kDa)	General Functions	Ratio (Fetal/Adult)	*P**
Superoxide dismutase (Cu-Zn)	IPI00027827	26	Caspase-dependent apoptosis; decreases apoptosis induced by NGF-depletion	0.6	0.02
Protein kinase C-binding protein NELL2	IPI00015260.1	91	Regulation of neural cell growth and differentiation; cell adhesion	0.6	0.01
Heat shock protein beta-1 (cytoplasm/nucleus)	IPI00025512.2	23	Anti-apoptotic (regulates CASP3, 8, 9, P38 MAPK, Jnk, NFkB, Erk, Akt, IKBKB)	0.6	0.03
Insulin-like growth factor-binding protein complex (acid labile subunit)	IPI00020996.5	66	Insulin-like growth factor binding	0.6	0.03
Tumor protein (translationally-controlled-1)	IPI00009943	21	Cell proliferation; apoptosis	0.7	0.05
Transforming growth factor (beta-induced)	IPI00018219.1	68	Negative regulation of cell adhesion; cell proliferation; extracellular matrix organization	0.7	0.01
Complement C4 (acidic form)	IPI00032258.4	193	Classical activation pathway	0.7	0.01
Galectin-3-binding protein	IPI00023673.1	65	Cell adhesion and apoptosis	1.5	0.03
Collagen alpha-1(VI) chain	IPI00291136	109	Cell growth and apoptosis; platelet-derived growth factor binding	1.6	0.02
Collagen alpha-1(XI) chain (isoform b)	IPI00218539	182	Cell adhesion, organization, and development	1.8	0.02
Inter-alpha (globulin) inhibitor H5	IPI00328829	106	Extracellular matrix stabilization; cell apoptosis	2.1	0.02
Hepatoma-derived growth factor	IPI00020956.1	27	Cell proliferation; signal transduction; heparin binding	2.1	0.04
Gelsolin (isoform 1)	IPI00026314.1	86	Cell adhesion and apoptosis	2.1	0.01
Pigment epithelium-derived factor	IPI00006114.4	46	Neurotrophic; affects NPD1 synthesis, secretion; increases NGF, GDNF, and BDNF mRNA expression (in rats)	2.5	0
Collectin-12 (isoform 1) (transmembrane receptor)	IPI00414467.6	82	Removes oxidized or apoptotic cells by recognizing oxidized phospholipids	2.5	0.01
Cathepsin L2 (lysosome)	IPI00000013.1	37	Regulation of cell apoptosis	2.6	0
Collagen alpha-1(XVIII) chain (isoform 2)	IPI00022822.5	154	Cell adhesion, proliferation, and migration; extracellular matrix organization; positive regulation of apoptosis	2.8	0

* Two-tailed *t*-test.

to the presence of retina-toxic substances in the cultured adult RPE-CM in concentrations higher than those found in passage-2, day-7 cultured fetal RPE-CM or the effect of some unidentified trophic factors. The differences in the trophic factor production by adult versus fetal RPE cells may be one reason that cultured adult RPE cells have been shown to be inferior to cultured fetal RPE cells for transplantation purposes (reviewed in Ref. 19). Additional studies on the effects of conditioned media from adult and fetal RPE cells on in vitro preparations of retina from animals with inherited retinal degenerative diseases may provide further information regarding the effectiveness (or lack thereof) of RPE-CM that is not available from studying normal retina in vitro.

TABLE 9. iTRAQ-Identified Proteins with a Potential to Affect Retinal Preservation[37–44]

Protein Name	kDa	Mean Ratio (Fetal/Adult)	*P**
Insulin-like growth factor-binding protein-3	32	0.3	0.01
Semaphorin-3B (isoform 1)	83	0.5	0.01
Transforming growth factor (beta-induced)	68	0.7	0.01
Hepatoma-derived growth factor	27	2.1	0.04
Gelsolin (isoform 1)	86	2.1	0.01
Pigment epithelium-derived factor	46	2.5	<0.01

* Two-tailed *t*-test.

Judging by the calculations of trophic factor receptor occupancy (Table 10), if one assumes that the total biological effect on degenerating retina was due to the nine quantified trophic factors, one would not expect to see large differences between passage-2, day-7 and passage-6, day-7 fetal RPE-CM. The one significant difference in receptor occupancy (VEGF receptor occupancy was 26.7% vs. 11.7% for passage-2, day-7 vs. passage-6, day-7 fetal RPE cells, respectively), although previously shown to be of potential biological significance in terms of retinal neuronal preservation,[55] did not result in significant differences in porcine retinal survival in our in vitro system. Differences between the aforementioned study showing the neurotrophic effects of VEGF and our work include (1) use of rat retinal ganglion cells versus full-thickness porcine retina, (2) administration of isolated VEGF versus complex mixture (CM), and (3) ischemic injury versus retinal detachment. Therefore, the increase in trophic factor percent receptor occupancy of VEGF-A and PEDF for passage-2, day-7 vs. passage-6, day-7 cultured fetal RPE-CM was statistically but not biologically significant, whereas the much higher increase versus cultured adult RPE-CM was statistically and biologically significant.[55–58]

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TABLE 10. Trophic Factor Concentrations and Receptor Occupancies

Trophic Factor (K_d [pM])	Adult (n = 7)	P2D7 Fetal (n = 5)	P6D7 Fetal (n = 5)
LIF (2000)	2.1 ± 0.5 (0.1 ± 0.02)	0.7 ± 0.5 (0.04 ± 0.02)	0.4 ± 0.1 (0.02 ± 0.005)
bFGF (1000)	5.1 ± 1.0 (0.5 ± 0.1)	3.1 ± 0.9 (0.3 ± 0.09)	8.2 ± 2.3 (0.8 ± 0.2)
HB-EGF (200)	4.3 ± 1.5 (2.1 ± 0.7)	4.3 ± 2.2 (2.1 ± 1.1)	2.7 ± 1.0 (1.3 ± 0.5)
HGF (100)	1.8 ± 0.5 (1.7 ± 0.5)	4.3 ± 1.9 (4.1 ± 1.9)	1.7 ± 1.0 (1.7 ± 1.0)
VEGF-A (1000)	47.7 ± 4.5* (4.6 ± 0.4)	364.3 ± 64.4 (26.7 ± 6.0)	132.5 ± 19.1† (11.7 ± 1.9)
NGF (300)	2.3 ± 0.6 (0.8 ± 0.2)	0.4 ± 0.1 (0.1 ± 0.04)	1.6 ± 0.7 (0.5 ± 0.2)
BDNF (1000)	1.5 ± 0.2 (0.2 ± 0.02)	7.1 ± 4.1 (0.7 ± 0.4)	3.8 ± 1.4 (0.4 ± 0.1)
CNTF (100)	5.0 ± 2.1 (0.3 ± 0.1)	3.4 ± 2.5 (3.3 ± 2.5)	2.8 ± 1.8 (2.7 ± 1.8)
PEDF (3000)	656.2 ± 154.3‡ (17.9 ± 4.9)	27012 ± 14810 (90.0 ± 83.2)	14061 ± 11792§ (82.4 ± 79.7)

Data are expressed as the mean trophic factor concentration ± SEM (in picomoles) with the mean percentage of receptor occupancy ± SEM in parentheses. P2D7, passage-2 day-7; P6D7, passage-6 day-7.

*†‡§ Significantly different (*t*-test) from passage-2 day-7 fetal RPE at $P < 0.05$.

A comparison (mean relative ratio) between four different preparations of CM isolated from passage-2, day-7 fetal RPE cells and primary cultured adult RPE cells identified five significantly ($P < 0.05$) different proteins with a potential to affect retinal preservation that were not previously quantified by multiplex ELISA, and one previously identified, PEDF. These included insulin-like growth factor binding protein-3 (IGFBP-3), isoform 1 of semaphorin-3B, TGF-β, hepatoma-derived growth factor (HDGF), and isoform 1 of gelsolin (Table 9). IGFBP-3 increases caspase 3 activation and the relative ratio of proapoptotic to antiapoptotic proteins, and induces growth inhibition and activation of apoptosis by stimulating p53 expression.[37,38] Semaphorin-3B is also an apoptosis inducer such that when applied as a soluble agent it blocks Akt, increases cytochrome *c* release, caspase cleavage and activation, and phosphorylation of proapoptotic factors.[39,40] Exogenous application of TGF-β, increases RPE cell death and death of large retinal ganglion cells (RGCs), but not necessarily death of small RGCs or photoreceptors.[41] Therefore, a significantly lower relative ratio of IGFBP-3, semaphorin-3B, and TGF-β, in fetal RPE-CM could be of benefit to retinal preservation. HDGF is structurally related to bFGF; it stimulates spinal motor neuron outgrowth, and it is expressed in neuronal nuclei. On exogenous application, it localizes to the nucleus and prevents neuronal cell death.[42,43] Gelsolin regulates the activity and activation of caspase-3, and it inhibits activation of apoptosis by preventing the loss of mitochondrial membrane potential.[44] Finally, PEDF is a neurotrophic factor that promotes photoreceptor survival.[27] In addition to serving antiangiogenic functions, PEDF increases the expression of mRNA for other neurotrophic factors such as NGF, BDNF, and GDNF in rat cerebellar neurons, although the effects on protein secretion of this upregulation is not known.[59] (We did not identify a strong correlation between PEDF and NGF, BDNF, or GDNF protein secretion.) Therefore, a significantly higher relative ratio of HDGF, gelsolin, and PEDF in fetal RPE-CM could be of benefit to retinal preservation. Validation of these results as well as characterization of the effects of these newly identified proteins on porcine retinal preservation will be pursued in future experiments.

The results outlined in Tables 6 and 7 may mean that continuous production and/or replenishment of trophic factors by an active fetal RPE cell culture is better than a finite, starting amount of exogenously applied trophic factors in the fetal RPE-CM for retinal preservation. Loss of activity by trophic factors due to their relatively short in vivo half-life is consistent with this hypothesis.[60] Another potential contributing factor is a possible loss of protein activity due to CM freezing and extended storage. Thus, we may not have identified all the factors (not necessarily proteins) that the RPE cells provide to the retina that help maintain its proper structure and function. In fact, in addition to secreting numerous trophic factors, the RPE cells regulate nutrient (e.g., vitamin A) and waste transport, phagocytize photoreceptor outer segments, quench reactive oxygen species and high-energy light, and have additional retina-preserving functions (reviewed in Ref. 10). Furthermore, the addition of retinal explants to a fetal RPE culture significantly changed the composition of the analyzed CM such that the amounts of bFGF and HGF were significantly upregulated at the 24-hour time point, and the amounts of bFGF, HB-EGF, and HGF were significantly upregulated at the 48-hour time point versus cultured fetal RPE cells alone. Fetal RPE cells appear to be the main source of the statistically significant increases in trophic factor secretion observed with the retina co-culture since the porcine retina alone does not produce HGF in the in vitro system used, and the retinal contribution of HB-EGF and bFGF is less than 10% of the amount of these substances in the fetal RPE-retina co-culture CM. This result implies the existence of an interaction between the retina and the RPE cells that, in addition to maintaining proper photoreceptor organization and differentiation, may be responsible for regulating and modifying trophic factor secretion.[61,62] However, based on calculations of trophic factor receptor occupancy, one would not expect differences of these magnitudes to be large enough to induce a change in the degree of retinal survival. Although not significantly different after the addition of porcine retinal explants to the fetal RPE culture, the detected levels of VEGF-A and PEDF in the co-culture CM were such that a receptor occupancy (13.2%–39.0% and 47.4%–79.5%, respectively) of potential, biological significance would be reached in both the 24- and 48-hour co-cultures.[55–58]

In summary, the results of this study show that cultured fetal RPE trophic factor secretion is maintained in long-term culture conditions. Differences in the ability of CM from cultured adult and fetal RPE cells to preserve degenerating retina may be due to differences in trophic factor secretion as shown by multiplex ELISA and iTRAQ analysis. In addition, co-culturing porcine retina with an active RPE cell culture versus CM collected from cultured fetal RPE cells resulted in a higher degree of retinal rescue. Thus, the RPE-CM, as produced by the methods described here, does not fully replicate direct RPE–retina interactions with regard to retina preservation.

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