Characterization of Conditioned Media Collected from Aged versus Young Human Eye Cups

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Purpose. To characterize secretion of in situ retinal pigment epithelium (RPE) from healthy, aged adult, age-related macular degeneration (AMD) adult, and fetal donor eyes and to assess the impact on retinal survival in vitro.

Methods. Conditioned medium (CM) was collected from adult and fetal donor eyes and analyzed for trophic factor composition by multiplex ELISA. Trophic factor receptor occupancy was calculated to evaluate differences in trophic factor concentrations. RPE trophic factor mRNA expression was quantified by real-time PCR. Retina-preserving activity of the collected CM was evaluated using degenerating porcine retina in vitro.

Results. Compared with CM from adult donors, AMD donor CM contained a significantly higher concentration of brain-derived neurotrophic factor (BDNF), whereas fetal donor CM contained significantly higher concentrations of hepatocyte growth factor (HGF) and pigment epithelium-derived factor (PEDF). No consistent correlation was found between trophic factor mRNA expression and protein secretion. Non-RPE components of the RPE-Bruch's membrane-choroid-sclera complex were major contributors of vascular endothelial growth factor-A (VEGF-A). CM of fetal donors was significantly better than CM of adult or AMD donors at improving the survival of degenerating porcine retina.

Conclusions. RPE cells of adult and fetal eyes have significantly different trophic factor production capabilities, which correlated with changes in preservation of porcine retina. Combined with trophic factor receptor occupancy calculations, these data may implicate HGF and PEDF as key factors promoting the preservation of retinal structure and function. (*Invest Ophthalmol Vis Sci.* 2011;52:5963–5972) DOI:10.1167/iovs.10-6440

The retinal pigment epithelium (RPE) is a monolayer of hexagonal, cuboidal, pigmented cells. It is critical for photoreceptor cell and choroid homeostasis,[1,2] as RPE cell degeneration results in abnormal photoreceptor morphology, choriocapillaris degeneration, and alteration of proper retinal function with eventual photoreceptor cell death.[3,4] Maintenance of normal retinal physiology relies on a wide variety of RPE functions,[5] including growth–trophic factor secretion.[6–8] The importance of growth–trophic factor support by RPE in the prevention of photoreceptor cell death was initially proposed in the study of rat chimeras[9] and further substantiated by numerous other investigators (reviewed in Ref. 10).

Trophic factors are endogenously produced substances (either proteins or steroid hormones) that bind to cell surface or nuclear receptors and generally function to promote cell proliferation, maturation, survival, and/or regeneration by activating several downstream pathways.[11,12] Animal studies of retinal degeneration have shown that intravitreal or subretinal administration of select trophic factors (e.g., brain-derived neurotrophic factor [BDNF], ciliary neurotrophic factor [CNTF], basic fibroblast growth factor [bFGF], pigment epithelium-derived factor [PEDF], and others)[13–18] can preserve photoreceptor morphology and physiology, promote a thinner outer segment debris layer and better organized photoreceptor outer segments, ameliorate retinal injury after ischemic- and light-induced damage, and reduce collateral retinal damage from photodynamic therapy. Trophic factors produced by non-RPE cells can prevent retinal degeneration; thus, photoreceptor rescue occurs in rodent models of retinal degeneration after transplantation of non-RPE cells that secrete a variety of trophic factors (e.g., iris pigment epithelial cells, Schwann cells, and stem cells).[19–28]

In this study, we characterized secretion of in situ RPE from healthy, aged, AMD adult, and fetal donor eyes. The study of normal adult and fetal in situ cells was used to identify proteins secreted by native RPE on Bruch's membrane (as well as by subjacent cells in the choroid-sclera). As poor or abnormal RPE secretion could be a contributing factor to photoreceptor degeneration and abnormal retinal morphology that occur in eyes with retinal degenerative diseases, the trophic factor secretion profile of in situ RPE from AMD eyes was elucidated.[29] A cultured porcine retinal assay was used to determine whether differences in trophic factor secretion in the different groups of donor eyes studied might result in differing degrees of retinal preservation.

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 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 and 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) not on a ventilator before death; (3) up to 7 hours from death to enucleation with eyes preserved

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TABLE 1. Adult Donor Eye Information

Eye Cup	A/S/E	Ocular Pathology	D-P (h:m)	D-R (h:m)
1	92/M/C	a: None b: Hard macular drusen	2:44	45:45
2	77/F/C	a: Hard macular and peripheral drusen b: Perimacular drusen	1:57	26:25
3	73/M/C	a, b: Posterior pole RPE clouding	3:15	42:00
4	78/M/C	a: None b: Posterior pole RPE hyperpigmentation	3:09	27:30
5	89/M/C	a, b: Peripheral chorioretinal atrophy	3:39	36:15
6	69/M/C	a, b: Posterior pole RPE clouding	5:39	38:00
7	83/M/C	a, b: None	2:09	35:55
8	71/M/C	a: RPE-choroid hyperpigmentation; b: none	5:21	44:25
9	82/M/C	a: None b: Small, peripapillary RPE hyperpigmentation	5:15	33:00
10	77/M/C	a, b: None	2:21	41:11
11	69/F/C	a, b: None	3:45	45:40
12	101/F/C	a, b: Peripheral chorioretinal atrophy	2:48	26:20

A/S/E, age/sex/ethnicity; M, male; F, female; C, Caucasian; a and b, randomly assigned identity of fellow eyes; D-P, death to preservation; D-R, death to receipt; h:m, hours:minutes.

in a moist chamber and stored on ice immediately after removal; (4) no more than 48 hours from death to receipt; and (5) an 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). At 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 one g/L glucose, L-glutamine, and sodium pyruvate, containing 3.7 g/L sodium bicarbonate; Cellgro-Mediatech, Manasses, VA) supplemented with 250 μg/mL amphotericin B (Gibco-Invitrogen, Carlsbad, CA). Anterior segment and neural retina were removed subsequently, exposing the posterior segment, which contains the RPE, Bruch's membrane, choroid, and sclera posterior to the equator. The latter tissue is referred to as the eye cup.

Collection of Conditioned Medium

Donor Eyes. Eye cups of non-AMD adult (n = 12; mean age, 88.1 years), AMD (n = 10; mean age, 83.0 years), and fetal (n = 23; mean age, 19.6 weeks gestation) eyes were cleaned of extraneous tissue and washed in povidone iodine (Tables 1–3). These preparations were filled with 2 mL (adult eye cups) and ~200 μL (fetal eye cups; volume depending on gestational age) of DMEM and incubated for 6 hours at 37°C, 10% CO_2. The resultant conditioned media (adult-CM, AMD-CM, and fetal-CM, respectively) were collected and centrifuged at 1000 rpm for 5 minutes (model 5415c; Eppendorf, Hauppauge, NY) to remove cellular debris. The supernatant was frozen at −80°C (Bio Freezer; Forma Scientific, Evanston, IL).

Bruch's Membrane-Choroid-Sclera. RPE cells were removed gently from adult (n = 5; mean age, 86.2 years) and fetal (n = 16; mean age, 19.6 weeks gestation) eye cups. Bruch's membrane-choroid-sclera (BrM-C-S) eye cups were washed twice with 200 μL (fetal) or 2 mL (adult) DPBS (Cellgro-Mediatech), filled with 200 μL (fetal) and 1 mL (adult; keeping the medium level below the choroid–sclera separation plane created by mechanical RPE removal) of DMEM, and incubated for 6 hours at 37°C, 10% CO_2. The resultant BrM-C-S-CM was collected, centrifuged, and frozen. BrM-C-S trophic factor levels were expressed as picograms per microgram of BrM-C-S protein and calculated relative to the levels of trophic factors in adult- and fetal-CM. In addition, the expression of bestrophin, an RPE-specific differentiation marker, was quantified by real-time PCR to determine whether there was RPE cell contamination of the BrM-C-S mRNA samples.

TABLE 2. AMD Eye Donor Information

Eye Cup	A/S/E	Ocular Pathology	D-P (h:m)	D-R (h:m)
1	86/F/C	a, b: Soft, confluent macular drusen and hard peripheral drusen	2:40	38:30
2	77/F/C	a, b: Small, soft macular drusen	5:30	41:30
3	78/M/C	a, b: Hard and soft macular and perimacular drusen	2:30	29:00
4	74/M/C	a: Small retinal adhesions; b: hard macular drusen	6:25	46:00
5A	93/F/C	a: Macular adhesion with scar	4:30	26:10
6	84/F/C	a, b: Macular membrane with RPE hyperpigmentation, and intermediate macular and perimacular drusen	3:40	46:20
7	86/M/C	a: Large macular scar and circular, perimacular RPE defect b: Hard and soft macular and perimacular drusen with RPE hyperpigmentation	5:58	20:52
8	82/M/C	a, b: Soft, confluent macular drusen and peripapillary retinal adhesions	5:00	40:20
9	86/F/C	a: Hard and soft macular and peripheral drusen and speckled RPE with peripheral choroidal hyperpigmentation; b: same as A plus macular drusen associated RPE hyperpigmentation	4:45	25:25
10	89/F/C	a, b: Hard and soft macular drusen with RPE hyperpigmentation, a small macular membrane, and hard peripheral drusen with RPE hyperpigmentation	2:55	42:30

Abbreviations are as in Table 1.

TABLE 3. Fetal Eye Donor Information

Eye Cup	Gestational Age (wk)	Sex	Ethnicity
1	19	M	N/A
2	18	M	N/A
3	20	N/A	N/A
4	20	N/A	N/A
5	20	N/A	N/A
6	20	N/A	N/A
7	18	N/A	N/A
8	20	N/A	N/A
9	21	M	Caucasian
10	22	M	Caucasian
11	20	F	N/A
12	18	F	N/A
13	22	F	N/A
14	17	M	N/A
15	19	N/A	N/A
16	22	M	N/A
17	18	F	Caucasian
18	17	N/A	N/A
19	19	N/A	N/A
20	17	N/A	N/A
21	19	M	N/A
22	21	N/A	N/A
23	21	N/A	N/A

All fetal eyes were received within 24 hours after harvest. Exact harvesting times were not provided by the eye banks, to ensure patient confidentiality. None of these eyes had any discernible pathology. N/A, not available.

Trophic Factor Quantification

All preparations of CM were analyzed (in duplicate) via multiplex ELISA (Aushon Biosystems, Woburn, MA) for trophic factors (Table 4). These candidate factors were selected from a literature search using the following criteria: secretion by RPE cells and preservation of photoreceptors and/or the retina. DMEM was analyzed for the same factors as a control for nonspecific binding. The media were quantified in duplicate on three separate occasions to ensure method reliability. CM from eye cups of donor eyes as well as BrM-C-S were corrected for these values. CM from each eye was collected and analyzed separately. Values from each pair of eyes were averaged. An overall mean ± SEM for each trophic factor was calculated and expressed as picograms of trophic factor per microgram of 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. Only trophic factors with a detection frequency ≥85% in adult, AMD, and fetal eye cups were selected for further analysis.

Protein Quantification

Protein was isolated from donor eye cup RPE cells after the 6-hour CM collection period. After the eye cups 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 from 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, 4°C. Protein was also isolated from the BrM-C-S eye cups. The whole eye cup (BrM-C-S tissue) was completely homogenized in 500 μL of 1× lysis buffer using a hard tissue, disposable rotor stator generator probe (Omni TH; Omni International, Marietta, GA), after which the homogenate was centrifuged for 10 minutes at 10,000 rpm, 4°C. The lysates were collected and frozen at −80°C. Protein was quantified using the Bradford reagent (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions.

Real-Time Polymerase Chain Reaction

RNA was isolated from RPE cells of eight adult (average age, 84.0 years), five AMD (average age, 86.6 years), and 16 fetal (average age, 20.1 weeks gestation) eye cups after the 6-hour CM collection period. After the eye cups were washed twice with ice-cold DPBS, ~80 μL (fetal eye cups; depending on gestational age) or 200 μL (adult eye cups) of lysis buffer (RNeasy RNA Mini Kit; Qiagen Valencia, CA) was added. The RPE cells were gently brushed off from the choroid into the lysis buffer and homogenized by running the lysate through a shredder column (QIAshredder; Qiagen Inc.). RNA was washed, bound, and eluted according to the manufacturer's instructions (RNeasy Mini Kit; Qiagen). One microliter of the eluted mRNA was used for quantification with a spectrophotometer (Nanodrop-1000; Thermo-Fisher Scientific, Waltham, MA). The RT-PCR reaction, consisting of 600 ng of mRNA mixed with a high-capacity cDNA reverse transcription kit (10× RT buffer, 100 mM 25× dNTP mix, 10× RT random primers, MultiScribe Reverse Transcriptase, RNase inhibitor, and nuclease-free dH_2O; Applied Biosystems, Inc. [ABI], Foster City, CA), was performed in a thermocycler (MJ Mini Personal Thermo Cycler; Bio-Rad, Hercules, CA) under the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 seconds, and then cooled to 4°C. Real-time

TABLE 4. Candidate Retinal and Photoreceptor-Preserving Trophic Factors Secreted by RPE Cells[14,16,30–33]

Trophic Factor	MW (kDa)	Biological Effect*
Brain-derived neurotrophic factor (BDNF)	14	Neurotrophic
Ciliary neurotrophic factor (CNTF)	24	Neurotrophic
Epidermal growth factor (EGF)	6	Photoreceptor rescue
Basic fibroblast growth factor (bFGF)	18	Proangiogenic, photoreceptor rescue
Glial-derived neurotrophic factor (GDNF)	24	Neurotrophic
Heparin-binding epidermal growth factor (HB-EGF)	23	RPE proliferation, VEGF-A secretion
Hepatocyte growth factor (HGF)	83	RPE survival, neuroprotective
Interleukin-1 beta (IL-1β)	31	Photoreceptor survival
Nerve growth factor (NGF)	13	Neurotrophic, inflammation
Neurotrophin-3 (NT3)	30	Neurotrophic
Pigment epithelium-derived factor (PEDF)	46	Anti-angiogenic, neurotrophic
Vascular endothelium growth factor-A (VEGF-A)	43	Pro-angiogenic, photoreceptor development
Leukemia inhibitory factor (LIF)	20	Photoreceptor rescue, RPE survival
Tumor necrosis factor-alpha (TNF-α)	26	Photoreceptor rescue

* Only trophic effects are listed.

TABLE 5. Trophic Factor Quantification of Adult, AMD, and Fetal Eye Cups

Trophic Factor	Adult (n = 12)			AMD (n = 10)			Fetal (n = 23)		
	Mean	SEM	Range	Mean	SEM	Range	Mean	SEM	Range
HGF	7,950.0	2,488.6	663.8–4,2134.5	7,818.9	2,360.7	829.8–30,752.3	12,440.7*	1,734.1	2,067.9–37,207.8
BDNF	225.1	59.6	31.0–1,516.4	514.1†	112.3	2.5–1,595.7	265.4	35.9	31.6–867.0
EGF	6.6	2.0	1.0–23.8	9.6	1.8	2.4–28.1	8.6	1.5	1.0–32.1
PEDF	1,859,678	692,752	235,122–9,419,625	893,423	229,892	166,538–2,577,187	7,382,019‡	878,926	459,300–22,612,615

Mean, SEM, and range of concentrations of trophic factors (picograms) in adult-, AMD-, and fetal-CM quanified by multiplex ELISA. AMD- and fetal-CM values were compared to adult-CM values for statistical significance ($P < 0.05$) by the nonparametric Mann-Whitney rank sum test.

* $P = 0.018$.

† $P = 0.027$.

‡ $P < 0.001$.

PCR for each trophic factor was performed using 1 μL of cDNA, 1.25 μL of 20× real-time PCR primers (*Taq*Man proprietary sequence; ABI), 12.5 μL of 2× PCR master mix (TaqMan Universal; ABI), 0.2 μL of 20 mg/mL BSA (Sigma-Aldrich), and 1.05 μL of dH_2O on a real-time PCR system (model 7500; ABI), under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 15 seconds at 95°C, and 60°C for 1 minute, and held at 60°C. 18S rRNA transcript served as an endogenous control, whereas bestrophin served as a reference transcript. Trophic factors were then expressed in relation to the arbitrarily chosen epidermal growth factor $(EGF)_{adult}$ transcript, which was assigned a value of 1. The corrections for the various levels of gene expression found in adult, AMD, and fetal samples were made according to the $^{\Delta\Delta}$Ct method.[30] The results were calculated for each group of samples as the mean ± SEM of the level of EGF_{adult}.

Porcine Retina

Porcine Eye Tissue. Eyes from 5- to 9-month, 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 porcine eyes were prepared for dissection by the method outlined previously for human donor eyes. Anterior segment and vitreous were removed, leaving the neural retina in the eye cup. Six-millimeter trephine blades (Storz Ophthalmic-Bausch and Lomb, Manchester, MO) were used to isolate equatorial, full-thickness retina tissue explants (avoiding the peripapillary region) by separating it from the RPE-BrM-C-S. These explants were randomly assigned to different culture conditions to negate the potential effects of selection bias and variability in retinal thickness.[31]

Retinal Cytotoxicity. A 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 the provided lysis buffer by trituration, allowed to react for 30 minutes at room temperature, centrifuged for 5 minutes at 1000 rpm at 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.

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. Our premise was that statistically significant changes in trophic factor concentrations are not necessarily important biologically. We hypothesized that, at minimum, biologically significant (versus statistically significant) changes in trophic factor concentration should be associated with significant changes in relevant receptor occupancy.[32] (For example, if a trophic factor, L, concentration increases from L_1 to L_2, but L_1 already saturates the target receptor, then the change in concentration is not likely to be biologically significant, assuming that the trophic factor effect is mediated via the receptor in question.) Therefore, we used a simplified model of receptor-ligand interactions to predict which trophic factor concentration changes may be important. The following assumptions were made in this model: (1) receptor–

TABLE 6. Trophic Factor mRNA Expression of Adult, AMD, and Fetal RPE Cells

Trophic Factor	Adult RPE (n = 8)	AMD RPE (n = 5)	Fetal RPE (n = 16)	P (Adult vs. AMD)	P (Adult vs. Fetal)	P (AMD vs. fetal)
HGF	0.002 ± 0.0007	0.05 ± 0.05	0.04 ± 0.008	>0.05	<0.001	>0.05
BDNF	0.0007 ± 0.0004*	0.002 ± 0.001	0.002 ± 0.0003	>0.05	<0.001	>0.05
EGF	1.0 ± 0.4	0.4 ± 0.2	0.01 ± 0.002	0.005	<0.001	>0.05
PEDF	408.8 ± 73.6	370.8 ± 56.0	139.5 ± 6.3	>0.05	<0.001	<0.001

Data are mean ± SEM transcript levels (EGF_{adult} = 1). Values were compared for significance ($P < 0.05$) using an unpaired t-test.
* Represents values based on n = 7.

ligand interactions occur according to simple mass-action kinetics; (2) adaptation (e.g., endocytic receptor downregulation or ligand-induced receptor desensitization) is not being considered; (3) receptor occupancy directly results in receptor functionality; and (4) small changes in receptor occupancy might be significant, provided that the ligand concentrations are below saturation. These assumptions may not apply for all trophic factors in complex systems such as the full-thickness retina. Adult-CM was used as a relative control for AMD- and fetal-CM. The basic premise of occupancy theory is that the magnitude of a biological response is directly proportional to the receptor–ligand complex concentration.[33] Thus, increases in trophic factor concentration that lead to significant changes in receptor occupancy may be expected to be biologically relevant. Mathematically, occupancy is defined as the proportion of the concentration of the receptor–ligand complex (i.e., bound receptor) divided by the total concentration of the receptor (i.e., the ligand-bound receptor plus the unbound receptor; equation 1). It is related to the dissociation constant (K_d), which is defined as the product of the concentrations of the free ligand and the free receptor concentration divided by the concentration of the receptor-ligand complex (equation 2). After the equations are rearranged, occupancy equals the concentration of the ligand divided by the quantity K_d, plus the concentration of the ligand (equation 3). K_d values for each trophic factor receptor were identified through the PubMed search engine. Only trophic factor receptors specific to the retina, RPE, and choroid were included. Potential biological activity was only assumed from the calculated changes in trophic factor receptor occupancies and did not mathematically factor into the calculations.

$$\text{Occupancy} = RL/(RL + R) \quad (1)$$

where R is the unbound receptor, L is the ligand, and RL is the receptor–ligand complex.

$$\text{Dissociation constant } (K_d) = (R \cdot L)/RL \quad (2)$$

where R is the receptor, L is the ligand, and RL is the receptor-ligand complex.

$$\text{Receptor occupancy} = L/(K_d + L) \quad (3)$$

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

Statistical Analysis

Significance was accepted at $P < 0.05$ (Sigma Plot 11; Systat Software Inc., San Jose, CA). If the data passed the Shapiro-Wilk normality test and the equal-variance test, an unpaired t-test (two groups) or a one-way analysis of variance (ANOVA) followed by the Holm-Sidak method for multiple pairwise comparisons (multiple groups) was used. However, if the data failed either the normality or the variance test, then the nonparametric Mann-Whitney rank sum test (two groups) or the nonparametric Kruskal-Wallis one-way ANOVA on ranks followed by the Dunn method for pairwise multiple comparisons (multiple groups) was used. Potential correlation of secretion of trophic factors to each other and to death-to-preservation and death-to-receipt times was calculated using the Spearman rank order correlation.

RESULTS

Trophic Factor Protein Secretion

The mean ± SEM micrograms of RPE protein isolated from adult, AMD, and fetal eye cups was 1.47 ± 0.05, 1.38 ± 0.05, and 0.14 ± 0.03, respectively. Four trophic factors were detected in ≥85% of CM samples from adult, AMD, and fetal eye cups: HGF, BDNF, EGF, and CNTF. The secretion of BDNF (expressed as the mean ± SEM picograms per microgram of RPE protein) by AMD eye cups (AMD-CM) was significantly higher than that from non-AMD eye cups (adult-CM; Table 5). The secretion of HGF and PEDF was significantly higher from the fetal eye cups (fetal-CM) than from the adult eye cups (adult-CM; Table 5). For fetal-CM, neither gestational age nor sex consistently affected the trophic factor secretion of all tested factors (data not shown). Neither death-to-receipt nor death-to-preservation time correlated significantly with trophic factor secretion from adult or AMD eye cups. These correla-

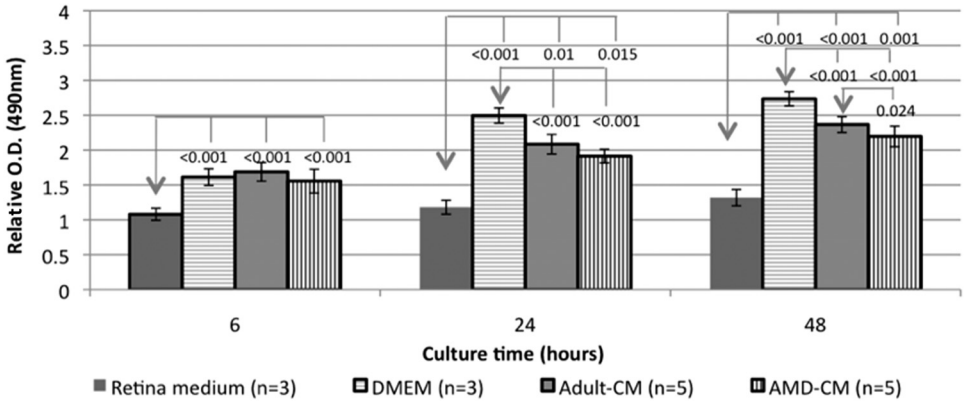

FIGURE 1. Effect of CM collected from adult and AMD eye cup preparations on porcine retinal cytotoxicity. Porcine retina was cultured in CM collected from eye cups of adult (n = 5; adult-CM) and AMD (n = 5; AMD-CM) eyes and in positive (n = 3; retina medium) and negative (n = 3; DMEM) control medium. Data are the mean ± SEM. LDH concentration was compared for statistical significance ($P < 0.05$) by one-way ANOVA followed by the Holm-Sidak method for multiple pairwise comparisons.

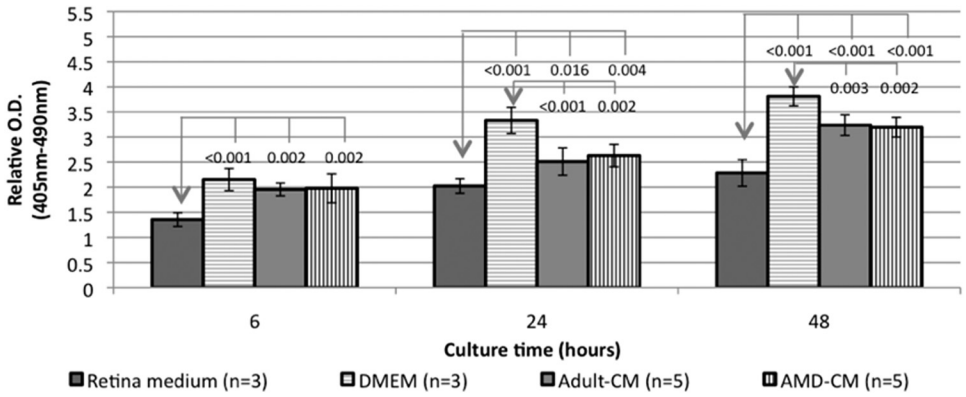

Figure 2. Effect of CM collected from adult and AMD eye cup preparations on porcine retinal apoptosis. Porcine retina was cultured in CM collected from eye cups of adult (n = 5; adult-CM) and AMD (n = 5; AMD-CM) eyes and in positive (n = 3; retina medium) and negative (n = 3; DMEM) control medium. Data are the mean ± SEM. DNA fragmentation was compared for statistical significance ($P < 0.05$) by one-way ANOVA followed by the Holm-Sidak method for multiple pairwise comparisons.

tions were not calculated for the fetal eye cups due to lack of information from the eye banks.

Kruskal-Wallis ANOVA on ranks followed by the Dunn method for pairwise comparison showed that the secretion of BDNF by AMD versus fetal eye cups was significantly different ($P < 0.001$). The change in BDNF secretion represents a disease-specific alteration in trophic factor production, since there were no significant differences in age between adult and AMD donor eyes ($P = 0.243$ by Mann-Whitney rank sum test).

Trophic Factor mRNA Expression

To determine whether there was a correlation between trophic factor mRNA expression and protein secretion, RNA was isolated from normal and AMD adult and fetal RPE cells. Real-time PCR (Table 6) showed that RPE cells from AMD donor eye cups had significantly lower levels of transcripts for EGF than RPE from adult donor eye cups. The levels of all four transcripts (HGF, BDNF, EGF, and PEDF) were significantly different in fetal versus adult RPE cells isolated from donor eyes. In addition, the level of PEDF was significantly higher in AMD versus fetal RPE cells. Transcripts were detected 100% of the time in fetal RPE cells, whereas BDNF was detected in seven of the eight adult samples.

Contribution of BrM-C-S to Trophic Factor Secretion

To determine whether RPE cells (versus other constituents of the Bruch's membrane explants such as choroid-sclera) were the main source of the detected trophic factors in adult- and fetal-CM, RPE cells were gently brushed off Bruch's membrane at time 0, and CM was collected from choroid–sclera eye cups. Three factors (VEGF-A, HGF, and heparin binding-epidermal growth factor [HB-EGF]) identified in adult- and fetal-CM were also identified in BrM-C-S-CM. (VEGF-A and HB-EGF were not included in our study due to <85% identification in adult- or fetal-CM samples.) The relative amount (mean ± SEM) of HGF in BrM-C-S-CM compared to adult- and fetal-CM was 0.47 ± 0.18 and 0.02 ± 0.005, respectively. Of note, the relative levels (mean ± SEM) of VEGF-A in BrM-C-S-CM to those found in 95.8% of adult-CM and 78.1% fetal-CM samples were 3.04 ± 0.66 and 2.07 ± 0.29, respectively. Trophic factor secretion from BrM-C-S of AMD eyes was not studied, since the trophic factors that varied significantly among adult- and AMD-CM (Table 5) were not produced by BrM-C-S of adult eyes. The relative (mean %) bestrophin levels found in adult and fetal BrM-C-S preparations to RPE cell isolations were 0.08% and 1.2%, respectively. These percentages may be due to nonspecific binding of proteins to the Bruch's membrane after RPE cell removal or to a very small number of RPE cells that were not brushed off.

Preservation of Porcine Retina

To determine whether CM from the three different eye cup preparations affect retinal preservation to different degrees, we isolated 6-mm retinal explants from porcine eyes and cultured them from 1 to 48 hours in retina medium (positive control), DMEM (negative control), and eye cup CM (adult-CM, AMD-CM, and fetal-CM). LDH and DNA fragmentation were measured at each time point with the 1-hour results serving as the reference. At each time point, retinas in adult- and AMD-CM showed significantly lower survival than the retinas in retina medium, significantly better survival than retinas in DMEM, and no significant difference from one another (Figs. 1, 2). Except for the 6-hour time point, adult- and fetal-CM induced significantly better retinal survival than DMEM (Figs. 3, 4). In addition, fetal-CM was significantly better than adult-CM at reducing retinal cytotoxicity (at all time points) and apoptosis (at the 48-hour time point only).

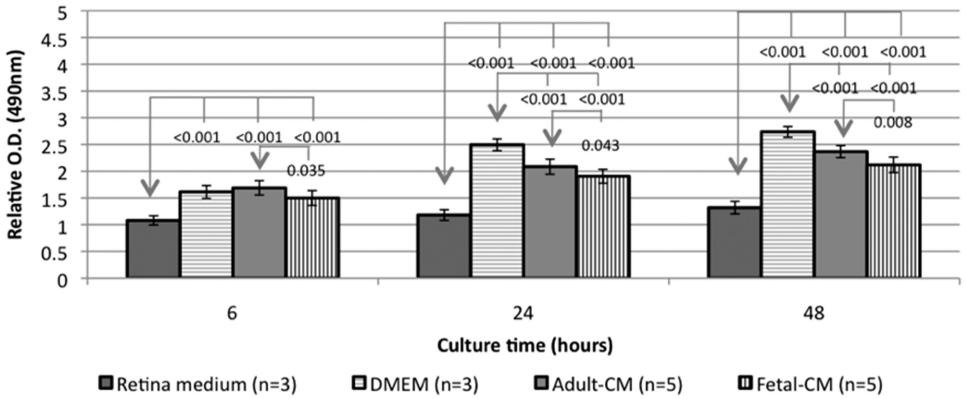

Figure 3. Effect of CM collected from adult and fetal eye cup preparations on porcine retinal cytotoxicity. Porcine retina was cultured in CM collected from eye cups of adult (n = 5; adult-CM) and fetal (n = 5; fetal-CM) eyes and in positive (n = 3; retina medium) and negative (n = 3; DMEM) control medium. Mean ± SEM. LDH concentration was compared for statistical significance ($P < 0.05$) by one-way ANOVA followed by the Holm-Sidak method for multiple pairwise comparisons.

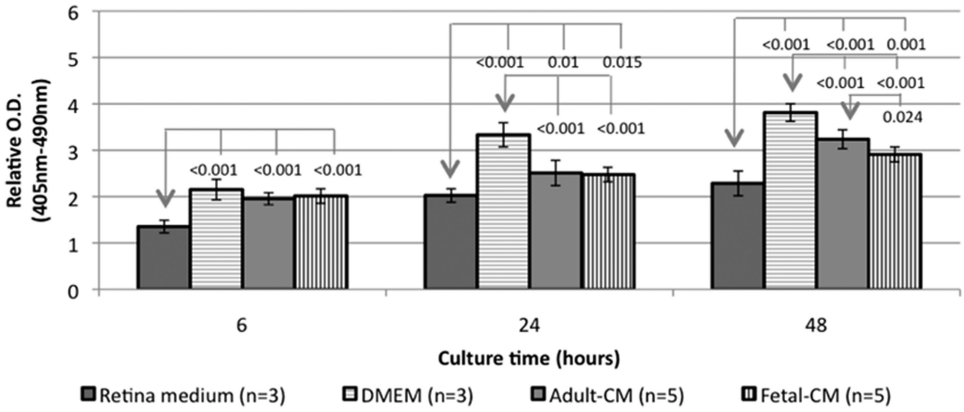

FIGURE 4. Effect of CM collected from adult and fetal eye cup preparations on porcine retinal apoptosis. Porcine retina was cultured in CM collected from eye cups of adult (n = 5; adult-CM) and fetal (n = 5; fetal-CM) eyes and in positive (n = 3; retina medium) and negative (n = 3; DMEM) control medium. Mean ± SEM. DNA fragmentation was compared for statistical significance ($P < 0.05$) by one-way ANOVA followed by the Holm-Sidak method for multiple pairwise comparisons.

Trophic Factor Receptor Occupancy

Compared with CM isolated from adult eyes, concentrations of BDNF and EGF were significantly higher in AMD-CM, and concentrations of HGF, BDNF, and PEDF were significantly higher in fetal-CM. However, the only statistically significant increases in trophic factor receptor occupancies, which corresponded to the significant differences in trophic factor concentrations for fetal-CM versus adult- and AMD-CM, were for HGF and PEDF (Table 7).

DISCUSSION

These data indicate that RPE trophic factor production changes due to cell aging (fetal versus adult) as well as disease progression (adult versus AMD). Some of these changes may result in a biologically significant alteration in the ability of RPE cells to maintain proper retinal homeostasis. Based on multiplex ELISA, biochemical determination of porcine retinal viability, and trophic factor receptor occupancy theory, we have putatively identified HGF and PEDF as key trophic factors for preservation of photoreceptor structure and function.

Although the concentrations of BDNF and EGF for fetal- and/or AMD- versus adult-CM were significantly different, the corresponding calculated changes in trophic factor receptor occupancy have not previously been shown to induce a biologically measurable effect in various neuronal cell and tissue preparations.[34,35] The increase in trophic factor percentage of receptor occupancy of HGF and PEDF for fetal- versus adult- and AMD-CM were statistically significant (Table 7). The dose-dependent effects of these two factors on retinal and photoreceptor homeostasis and protection from injury and degeneration have been described previously.[36,37] In summary, we did not observe changes in trophic factor production that influenced retinal survival to a significant degree without an associated change in receptor occupancy. Significant differences in trophic factor concentration and receptor occupancy that were not associated with changes in retinal survival were below the established biological threshold for those specific factors. Thus, it seems that receptor occupancy theory may be useful for predicting the effects of trophic factors on retinal preservation.

Potential reasons for <100% trophic factor detection frequency (Table 8) include technical (threshold) limitations of the multiplex ELISA method (Van Arsdell S., personal communication, 2010; Table 9), trophic factor hydrolysis, and/or biological variability in regulation of trophic factor production and secretion.[43,44]

The improvement in retinal preservation by CM derived from adult and AMD eye cups versus DMEM alone was approximately 20% to 30%. This difference in improved retinal preservation was most likely due to the presence of numerous trophic factors in the adult- and AMD-CM. Despite the significant difference in BDNF concentration in adult versus AMD eye cups, there seemed to be no difference in the ability to preserve the porcine retina in vitro. This result indicates that the statistically significant difference in BDNF secretion between adult and AMD eye cups did not affect porcine retinal preservation to a large enough degree to enable detection of potentially significant differences in this in vitro bioassay. It is possible that the quantitative methods used lacked sufficient sensitivity for detecting differences in retinal preservation if they were present. On the other hand, based on our calculation of trophic factor receptor occupancy (Table 7), it seems that the compositional differences between the adult- and AMD-CM proteins were not large enough to induce a biologically significant effect on retinal preservation in the in vitro system used.[34,35] Another consideration is that AMD mainly affects the macula, which by area represents a very small

TABLE 7. Trophic Factor Concentrations and Receptor Occupancies[38–42]

Trophic Factor	Receptor* (K_d [pM])	Adult (n = 12)	AMD (n = 10)	Fetal (n = 23)
HGF	c-Met (20–30)†	72.8 ± 22.8 (70.8 ± 5.3)	74.5 ± 24.5 (71.3 ± 5.4)	135.8 ± 18.9‡ (81.9 ± 6.7)
BDNF	TrkB (1000)	11.8 ± 3.1 (1.2 ± 0.2)	32.3 ± 7.1‡ (3.1 ± 0.7)	15.6 ± 2.1§ (1.5 ± 0.2)
	$p75^{NTR}$ (1300)	11.8 ± 3.1 (0.9 ± 0.2)	32.3 ± 7.1‡ (2.4 ± 0.5)	15.6 ± 2.1§ (1.2 ± 0.1)
EGF	EGF-R (700)	0.8 ± 0.1 (0.1 ± 0.02)	1.4 ± 0.3‡ (0.2 ± 0.04)	1.2 ± 0.2 (0.2 ± 0.02)
PEDF	PEDF-R (2500–6500)†	27,491 ± 10,240 (80.9 ± 4.4)	17,261 ± 4,442 (72.6 ± 4.3)	125,671 ± 14,963‡ (95.1 ± 0.5)

Trophic factor concentration (pM), mean ± SEM (% occupancy, mean ± SEM).
* Includes receptors specific to retina, RPE, and choroid only.
† Calculations based on K_d of 30 pM (c-Met) and 6500 pM (PEDF-R), respectively.
‡ $P < 0.001$.
§ $P < 0.05$.

proportion of the posterior pole, and since the collected adult- and AMD-CM mainly originated from nonmacular RPE cells, the potential deleterious effect of abnormal trophic factor secretion by macular RPE on retinal survival may have been difficult to detect in aged versus AMD eyes.

Compared with adult- and AMD-CM, fetal-CM was significantly more efficacious at reducing retinal cytotoxicity (at all time points) and apoptosis (at the 48-hour time point only). This result implies that significantly higher levels of retina-preserving factors were present in fetal-CM in sufficient concentration to induce a significant reduction in retinal cytotoxicity and apoptosis. The improved retinal preservation after fetal-CM culture could be due to effects of unidentified retina-preserving or retina-toxic proteins secreted by these cells or other components of the BrM-C-S portion of the specimens. Based on the trophic factor receptor occupancy calculations (Table 7), the improved retinal preservation after culture in fetal- versus adult-CM could be due to increases in HGF (from 70.8% to 81.9%) and PEDF (from 80.9% to 95.1%) receptor occupancy. In certain models of retinal degeneration, trophic factors in combination can promote significantly more photoreceptor cell survival than individually.[45,46] Application of a trophic factor mixture versus a single factor may result in a larger, biologically significant effect on photoreceptor and/or retinal preservation due to activation of multiple survival pathways (even if by a modest amount) versus saturation of a single receptor-type. Therefore, even subtle differences in trophic factor amounts can have substantial effects on cell activation, survival, and other processes, which makes it difficult to predict which (if any) of the factors underlay retinal preservation based on occupancy considerations alone.

Adult, AMD, and fetal eye cup CM preparations were significantly less efficacious at preserving the retina than the retina medium (positive control) at all time points tested. This result may be due to (1) the presence of taurine, porcine insulin, pyruvate, ascorbic acid, and fetal bovine serum (FBS; contains numerous growth-promoting substances[47,48]) in the retina medium; (2) perhaps lower than optimal levels of trophic factors in the CM; (3) possible presence of some retina-toxic molecules in the CM; or (4) the effects of other unidentified substances in the CM.

Quantification of trophic factor mRNA expression was undertaken to attempt to correlate it with trophic factor protein secretion. In general, a consistent correlation between trophic factor mRNA expression and protein secretion was not identified. This result may mean that some factors are produced as needed, whereas others either are made constitutively or are stored in intracellular vesicles and released on binding of proper extracellular signals.[49] In addition, RNA is particularly sensitive to tissue degradation. In eukaryotes, mRNA is degraded from the 3′ to the 5′ end by cytoplasmic exoribonucleases.[50] Therefore, variability in death-to-preservation and death-to-receipt time for adult donor eyes may have contributed to the experimental variability and discord between mRNA expression and protein secretion data. However, we did not find any statistically significant correlations in this regard.

Table 8. Trophic Factor Detection Frequency in Adult, AMD, and Fetal Eye Cups

Trophic Factor	Adult (n = 12)	AMD (n = 10)	Fetal (n = 23)
HGF	100	100	100
BDNF	95.8	100	88.4
EGF	95.8	94.7	93
PEDF	100	100	100

Data are frequency of occurrence expressed in percent.

Table 9. Trophic Factor Sensitivity and Dynamic Range

Trophic Factor	Sensitivity (pg/mL)	Dynamic Range (pg/mL)
HGF	1.6	1.56–1600
BDNF	3.1	3.13–3200
EGF	0.8	0.4–400
PEDF	58.6	29.3–30000

The data are courtesy of Scott Van Arsdell from Aushon Biosystems (Woburn, MA).

Tian et al.[51] set out to determine the precise relationship between mRNA expression and protein production by comparing steady state levels of mRNA and proteins in two distinct hematopoietic cell lines and to correlate the kinetic changes in liver mRNA and protein levels in a mouse model in response to treatment with different pharmacologic agents. They identified a moderate correlation between mRNA and protein levels with the expression of mRNA reflecting at most 40% of the variation of protein expression. They attributed this level of correlation to inherent biological mechanisms of regulation during protein synthesis, such as posttranscriptional, translational, and/or posttranslational regulation. In another study, Griffin et al.[52] investigated the correlation of induced changes in mRNA and protein expression in yeast cells. They demonstrated discrepancies between mRNA and protein abundance ratios in genes responsible for carbohydrate metabolism, respiration, and energy generation. They attributed these differences to posttranscriptional protein control mechanisms. Additional support for a discord between expression of protein and mRNA comes from studies on Halobacteria,[53] lung adenocarcinoma,[54] and human liver.[55] Overall, the results of these studies indicate that analysis of mRNA expression does not reliably predict or correlate with changes in protein production. Therefore, in addition to the potential explanations listed above, the discrepancy of mRNA expression and protein secretion in our study may reflect the numerous and extensive modifications undergone by mRNA and proteins.

BrM-C-S of adult and fetal donor eyes secreted detectable levels of VEGF-A, HGF, and HB-EGF. Therefore, RPE cells were the most likely contributors of the trophic factors detected in CM collected from the adult and fetal eye cups. Although incomplete RPE removal could have contributed to the identification of HGF and HB-EGF in the CM collected from adult and fetal BrM-C-S components, the levels of VEGF-A produced were approximately two to three times higher after RPE cell removal than in the presence of an intact RPE monolayer. Choroidal endothelial cells (CECs) are the most likely source of the trophic factor contribution of BrM-C-S to the CM.[56,57] Potential reasons for higher VEGF-A concentrations in the BrM-C-S-CM than CM from adult and fetal eye cups include (1) removal of RPE cells that normally downregulate CEC secretion (e.g., via PEDF secretion)[58]; (2) removal of RPE cells that use these trophic factors for cell signaling and homeostasis[59]; (3) removal of RPE cell tight junctions allowing CEC-secreted substances to diffuse into the CM via a paracellular route[60]; and (4) the physical stress of RPE removal potentially damaging the CECs resulting in elaboration of these factors into the BrM-C-S-CM. One may conjecture that RPE disease (and death) in AMD eyes might thus promote VEGF-A production with the attendant complication of choroidal neovascularization.[61–63]

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References

1. Anderson DH, Fisher SK, Steinberg RH. Mammalian cones: disc shedding, phagocytosis, and renewal. *Invest Ophthalmol Vis Sci.* 1978;17:117–133.
2. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev.* 2005;85:845–881.
3. Castellarin AA, Nasir M, Sugino IK, Zarbin MA. Progressive presumed choriocapillaris atrophy after surgery for age-related macular degeneration. *Retina.* 1998;18:143–149.
4. McLeod DS, Grebe R, Bhutto I, Merges C, Baba T, Lutty GA. Relationship between RPE and choriocapillaris in age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2009;50:4982–4991.
5. Boulton M, Dayhaw-Barker P. The role of the retinal pigment epithelium: topographical variation and ageing changes. *Eye (Lond).* 2001;15:384–389.
6. Marneros AG, Fan J, Yokoyama Y, et al. Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function. *Am J Pathol.* 2005;167:1451–1459.
7. Gupta SK, Jollimore CA, McLaren MJ, Inana G, Kelly ME. Mammalian retinal pigment epithelial cells in vitro respond to the neurokines ciliary neurotrophic factor and leukemia inhibitory factor. *Biochem Cell Biol.* 1997;75:119–125.
8. Agarwal N, Martin E, Krishnamoorthy RR, et al. Levobetaxolol-induced Up-regulation of retinal bFGF and CNTF mRNAs and preservation of retinal function against a photic-induced retinopathy. *Exp Eye Res.* 2002;74:445–453.
9. Mullen RJ, LaVail MM. Inherited retinal dystrophy: primary defect in pigment epithelium determined with experimental rat chimeras. *Science.* 1976;192:799–801.
10. Thomas BB, Aramant RB, Sadda SR, Seiler MJ. Retinal transplantation: a treatment strategy for retinal degenerative diseases. *Adv Exp Med Biol.* 2006;572:367–376.
11. Russell DS, Duman, RS. Neurotrophic factors and intracellular signal transduction pathways. Davis KL, Charney D, Coyle JT, Nemeroff C, eds. *Neuropsychopharmacology*: *The Fifth Generation of Progress.* 2002;Chap 16.
12. Talapatra S, Thompson CB. Growth factor signaling in cell survival: implications for cancer treatment. *J Pharmacol Exp Ther.* 2001;298:873–878.
13. Unoki K, LaVail MM. Protection of the rat retina from ischemic injury by brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor. *Invest Ophthalmol Vis Sci.* 1994;35:907–915.
14. LaVail MM, Unoki K, Yasumura D, Matthes MT, Yancopoulos GD, Steinberg RH. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc Natl Acad Sci U S A.* 1992;89:11249–11253.
15. Frasson M, Picaud S, Leveillard T, et al. Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse. *Invest Ophthalmol Vis Sci.* 1999;40:2724–2734.
16. Cayouette M, Smith SB, Becerra SP, Gravel C. Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degenerations. *Neurobiol Dis.* 1999;6:523–532.
17. Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, LaVail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature.* 1990;347:83–86.
18. Cayouette M, Gravel C. Adenovirus-mediated gene transfer of ciliary neurotrophic factor can prevent photoreceptor degeneration in the retinal degeneration (rd) mouse. *Hum Gene Ther.* 1997;8:423–430.
19. Rezai KA, Kohen L, Wiedemann P, Heimann K. Iris pigment epithelium transplantation. *Graefes Arch Clin Exp Ophthalmol.* 1997;235:558–562.
20. Schraermeyer U, Thumann G, Luther T, et al. Subretinally transplanted embryonic stem cells rescue photoreceptor cells from degeneration in the RCS rats. *Cell Transplant.* 2001;10:673–680.
21. Kano T, Abe T, Tomita H, Sakata T, Ishiguro S, Tamai M. Protective effect against ischemia and light damage of iris pigment epithelial cells transfected with the BDNF gene. *Invest Ophthalmol Vis Sci.* 2002;43:3744–3753.
22. Abe T, Yoshida M, Tomita H, et al. Auto iris pigment epithelial cell transplantation in patients with age-related macular degeneration: short-term results. *Tohoku J Exp Med.* 2000;191:7–20.
23. Lawrence JM, Keegan DJ, Muir EM, et al. Transplantation of Schwann cell line clones secreting GDNF or BDNF into the retinas of dystrophic Royal College of Surgeons rats. *Invest Ophthalmol Vis Sci.* 2004;45:267–274.
24. Keegan DJ, Kenna P, Humphries MM, et al. Transplantation of syngeneic Schwann cells to the retina of the rhodopsin knockout (rho(−/−)) mouse. *Invest Ophthalmol Vis Sci.* 2003;44:3526–3532.
25. Haruta M, Sasai Y, Kawasaki H, et al. In vitro and in vivo characterization of pigment epithelial cells differentiated from primate embryonic stem cells. *Invest Ophthalmol Vis Sci.* 2004;45:1020–1025.
26. Arnhold S, Absenger Y, Klein H, Addicks K, Schraermeyer U. Transplantation of bone marrow-derived mesenchymal stem cells rescue photoreceptor cells in the dystrophic retina of the rhodopsin knockout mouse. *Graefes Arch Clin Exp Ophthalmol.* 2007;245:414–422.
27. Lund RD, Wang S, Lu B, et al. Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. *Stem Cells.* 2007;25:602–611.
28. Otani A, Dorrell MI, Kinder K, et al. Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells. *J Clin Invest.* 2004;114:765–774.
29. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci.* 1996;37:855–868.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* 2001;25:402–408.
31. Khodair MA, Zarbin MA, Townes-Anderson E. Synaptic plasticity in mammalian photoreceptors prepared as sheets for retinal transplantation. *Invest Ophthalmol Vis Sci.* 2003;44:4976–4988.
32. Shankaran H, Wiley HS, Resat H. Receptor downregulation and desensitization enhance the information processing ability of signalling receptors. *BMC Syst Biol.* 2007;1:48.
33. Hollenberg MD, ed. *Receptor Models and the Action of Neurotransmitters and Hormones*. New York: Raven Press; 1978:13–39.
34. Harper MM, Adamson L, Blits B, Bunge MB, Grozdanic SD, Sakaguchi DS. Brain-derived neurotrophic factor released from engineered mesenchymal stem cells attenuates glutamate- and hydrogen peroxide-mediated death of staurosporine-differentiated RGC-5 cells. *Exp Eye Res.* 2009;89:538–548.
35. Chen H, Liu B, Neufeld AH. Epidermal growth factor receptor in adult retinal neurons of rat, mouse, and human. *J Comp Neurol.* 2007;500:299–310.
36. Machida S, Tanaka M, Ishii T, Ohtaka K, Takahashi T, Tazawa Y. Neuroprotective effect of hepatocyte growth factor against photoreceptor degeneration in rats. *Invest Ophthalmol Vis Sci.* 2004;45:4174–4182.
37. Cao W, Tombran-Tink J, Chen W, Mrazek D, Elias R, McGinnis JF. Pigment epithelium-derived factor protects cultured retinal neurons against hydrogen peroxide-induced cell death. *J Neurosci Res.* 1999;57:789–800.
38. Nakamura T. Structure and function of hepatocyte growth factor. *Prog Growth Factor Res.* 1991;3:67–85.
39. Rodriguez-Tebar A, Barde YA. Binding characteristics of brain-derived neurotrophic factor to its receptors on neurons from the chick embryo. *J Neurosci.* 1988;8:3337–3342.
40. Dechant G, Biffo S, Okazawa H, Kolbeck R, Pottgiesser J, Barde YA. Expression and binding characteristics of the BDNF receptor chick trkB. *Development.* 1993;119:545–558.
41. Aymerich MS, Alberdi EM, Martinez A, Becerra SP. Evidence for pigment epithelium-derived factor receptors in the neural retina. *Invest Ophthalmol Vis Sci.* 2001;42:3287–3293.

42. Waters CM, Oberg KC, Carpenter G, Overholser KA. Rate constants for binding, dissociation, and internalization of EGF: effect of receptor occupancy and ligand concentration. *Biochemistry.* 1990;29:3563–3569.
43. Persson K, Steers WD, Tuttle JB. Regulation of nerve growth factor secretion in smooth muscle cells cultured from rat bladder body, base and urethra. *J Urol.* 1997;157:2000–2006.
44. Willis L, Quintero EM, Nelson M, Granholm A. Regulation of trophic factor expression by innervating target regions in intraocular double transplants. *Cell Transplant.* 2005;14:21–29.
45. Caffe AR, Soderpalm A, van Veen T. Photoreceptor-specific protein expression of mouse retina in organ culture and retardation of rd degeneration in vitro by a combination of basic fibroblast and nerve growth factors. *Curr Eye Res.* 1993;12:719–726.
46. Ogilvie JM, Speck JD, Lett JM. Growth factors in combination, but not individually, rescue rd mouse photoreceptors in organ culture. *Exp Neurol.* 2000;161:676–685.
47. Baker H, DeAngelis B, Frank O. Vitamins and other metabolites in various sera commonly used for cell culturing. *Experientia.* 1988; 44:1007–1010.
48. Zheng X, Baker H, Hancock WS, Fawaz F, McCaman M, Pungor E Jr. Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. *Biotechnol Prog.* 2006;22:1294–1300.
49. Sheedlo HJ, Turner JE. Immunocytochemical characterisation of proteins secreted by retinal pigment epithelium in retinas of normal and Royal College of Surgeons dystrophic rats. *J Anat.* 1998; 193:223–232.
50. Newbury SF. Control of mRNA stability in eukaryotes. *Biochem Soc Trans.* 2006:34:30–34.
51. Tian Q, Stepaniants SB, Mao M, et al. Integrated genomic and proteomic analyses of gene expression in Mammalian cells. *Mol Cell Proteomics.* 2004;3:960–969.
52. Griffin TJ, Gygi SP, Ideker T, et al. Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae. *Mol Cell Proteomics.* 2002;1:323–333.
53. Baliga NS, Pan M, Goo YA, et al. Coordinate regulation of energy transduction modules in Halobacterium sp. analyzed by a global systems approach. *Proc Natl Acad Sci U S A.* 2002;99:14913–14918.
54. Chen G, Gharib TG, Huang CC, et al. Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics.* 2002;1:304–313.
55. Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis.* 1997;18:533–537.
56. Hoffmann S, Friedrichs U, Eichler W, Rosenthal A, Wiedemann P. Advanced glycation end products induce choroidal endothelial cell proliferation, matrix metalloproteinase-2 and VEGF upregulation in vitro. *Graefes Arch Clin Exp Ophthalmol.* 2002;240:996–1002.
57. Hu W, Criswell MH, Fong SL, et al. Differences in the temporal expression of regulatory growth factors during choroidal neovascular development. *Exp Eye Res.* 2009;88:79–91.
58. Tombran-Tink J, Shivaram SM, Chader GJ, Johnson LV, Bok D. Expression, secretion, and age-related downregulation of pigment epithelium-derived factor, a serpin with neurotrophic activity. *J Neurosci.* 1995;15:4992–5003.
59. Ohno-Matsui K, Yoshida T, Uetama T, Mochizuki M, Morita I. Vascular endothelial growth factor upregulates pigment epithelium-derived factor expression via VEGFR-1 in human retinal pigment epithelial cells. *Biochem Biophys Res Commun.* 2003;303: 962–967.
60. Stern WH, Ernest JT, Steinberg RH, Miller SS. Interrelationships between the retinal pigment epithelium and the neurosensory retina. *Aust J Ophthalmol.* 1980;8:281–288.
61. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med.* 2006;355:1432–1444.
62. Zarbin MA. Current concepts in the pathogenesis of age-related macular degeneration. *Arch Ophthalmol.* 2004;122:598–614.
63. Stepien KE, Rosenfeld PJ, Puliafito CA, et al. Comparison of intravitreal bevacizumab followed by ranibizumab for the treatment of neovascular age-related macular degeneration. *Retina.* 2009;29: 1067–1073.