ERK Activation by Retinal Progenitor Cells Protect RPE Cells from Oxidative Damage

Zhaohui Wang, Thomas J. Bartosh, Jr.[2] and Rouel S. Roque[3]

Abstract **–** Age-related macular degeneration (AMD) results from oxidative damage to the retinal pigment epithelium (RPE) with concomitant photoreceptor cell death. The effects of oxidative stress were investigated on a human RPE cell line (ARPE-19) in the presence of 48h-conditioned media from human retinal progenitor cells (RPC). Freshly isolated RPCs rapidly formed spherical clusters (neurospheres) and expressed markers of undifferentiated retinal progenitor cells such as nestin and pax6. Conditioned media from RPC neurospheres inhibited RPE cell death and upregulated ERK 1/2 activity. The protective effect of RPC-CM was blocked by U0126, a selective inhibitor of MEK1/2. Intravitreal injections of RPC-CM suppressed photoreceptor cell death in dystrophic rat retinas. Our study shows that adult human retinal progenitor cells secrete putative prosurvival molecules that protect RPE cells from oxidative stress and suppress photoreceptor cell death in vivo. Trophic factors secreted by human RPCs may prove useful in the treatment of degenerative retinal diseases such as AMD.

Keywords **–** dystrophic retinas, Erk1/2, macular degeneration, oxidative stress, photoreceptor cells, reactive oxygen species, retinal pigment epithelium, retinal progenitor cells.

I. Introduction

AMD, the most common cause of irreversible vision loss in the elderly, is an ocular disease characterized by progressive degeneration of the retinal pigmented epithelium (RPE) and neighboring photoreceptors in the macula of the retina [1]. This often leads to severe visual loss and even permanent blindness. Although the visual loss results from degeneration loss of RPE cells. The progressive RPE dysfunction and eventual cell death lead to subsequent degeneration of of photoreceptors, the initial pathology involves injury and photoreceptor cells, perhaps due to loss of trophic support. Although the genetic and molecular mechanisms of AMD remain vague, studies suggest that it is multifactorial and photo-oxidative stress is strongly implicated [2].

Antioxidants have been utilized in the clinic and elevated concentrations of zinc, vitamin C, vitamin E, or beta-carotene have all been used in clinical trials to protect the RPE from oxidative damage, but their beneficial effects remain controversial [3,4].

Stem cell therapy has emerged as a promising experimental approach to restore retinal function and vision. RPE and human photoreceptor transplantation have been used to treat AMD and retinitis pigmentosa patients [5-7] although the results are less gratifying and beneficial. The use of neuronal/retinal stem cells to treat ocular disease lead to similar results. For example, undifferentiated rat retinal progenitor cells expanded *in vitro* and grafted into RCS rats close to the optic disc differentiated into glia but not neurons [8].

Although successful differentiation of stem cells into functional cells remains a tantalizing though challenging goal, numerous studies have demonstrated the therapeutic potential of stem cell-secreted growth factors in various disease conditions [9-11]. Thus, in the following studies, the effects of retinal progenitor cell-derived trophic factors were tested on the survival of RPE cells following oxidative stress.

II. Materials And Methods

A. Reagents

Calcein AM, ethidium homodimer-1, Hoechst 33258, H_2DCFH-DA, human recombinant FGF2 and EGF proteins, and Alexa-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Cell Titer 96 Aqueous Cell Proliferation Assay (MTS) kit were obtained from Promega (Madison, WI). Monoclonal antibodies against Erk 1/2 and phospho-Erk1/2 were from Cell Signaling (Danvers, MA) while polyclonal antibodies against Pax6, GFAP, and neurofilament 68 were obtained from Abcam (Cambridge, MA). Glucose oxidase, D-glucose, and N-Acetyl Cysteine and other cell culture media and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

B. Human Retinal Progenitor Cells

Retinas were isolated from human donor eyes (0-44 yrs. of age) as described [12]. Briefly retinas were enucleated, rinsed 3X in basal medium (DMEM with 2 mM L-glutamine, 100units/ml Penicillin, 100mg/ml Streptomycin, and 15mM HEPES) then incubated in basal medium containing 0.1% collagenase/0.1% trypsin for 1h at 37°C. After digestion, tissues were rinsed and plated in 6-well plates in growth medium (basal medium containing 10% fetal bovine serum) supplemented with FGF2 20ng/ml. Retinal progenitor cells (RPC) derived from the cultures

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were collected and expanded in growth medium containing 10% serum, FGF2 20 ng/ml and EGF 20 ng/ml.

Conditioned medium was collected from semi-confluent cultures of RPCs maintained for 48h in serum-free growth factor-free basal medium, centrifuged at 210xg for 10min, sterile-filtered, and frozen at -20°C prior to use.

C. In Vitro Model for Oxidative Stress

To investigate the effects of oxidative stress on the RPE, a spontaneously transformed human retinal pigment epithelial cell line, ARPE-19 cells, was used in this study. ARPE-19 cells maintain the major characteristics of RPE cells including expression of specific cell markers, tight junction formation, and retinoid metabolism [13]. ARPE-19 cells were maintained in growth medium (basal medium supplemented with 10% fetal bovine serum) at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

ARPE-19 cells were exposed to a glucose-based oxidant-generating system catalyzed by glucose oxidase (GO/G) as previously described [14]. Glucose oxidase catalyzes the oxidation of β-D-glucose to D-glucono-1,5-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. Briefly, ARPE-19 cells were plated in growth medium then transferred to glucose-free medium for 24h. The following day, cells were exposed to 25mU/ml glucose oxidase in the presence of 1g/L glucose for 5h. The generation of intracellular reactive oxygen species (ROS) was measured using the fluorescent dye H_2DCFH-DA (data not shown).

D. Cell Survival Assays

ARPE-19 cells plated on 96-well plates at $4X10^3$ cells/well, kept in basal medium for 24h, then treated with GO/G for 5hr were washed with serum-free DMEM and incubated with 2mM calceinAM and 4mM ethidium homodimer, or Hoechst 33258 at 37°C for 30min. Cells were observed and images captured under a fluorescence microscope.

To measure the amount of cell death, ARPE-19 cells were incubated with MTS reagent (3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethosyphenyl)-2-(4sulfophenyl)-2H-tetrazolium) for 1h and the number of surviving cells was determined using MTS assay (Promega, Madison, WI). Absorbance was read on a microplate reader at 490 nm after 1h and converted to cell counts using a standard curve was prepared for each set of experiments using 1.25-50×10^3cells/well. Experiments were done at least three times. Data was subjected to statistical analyses using *Student's t-test*.

E. Western Blots

Human RPCs and ARPE-19 cells were grown in 6-well dishes and processed for immunoblotting. Lysates were separated by SDS-PAGE, then transferred to nitrocellulose membrane at 100V. Membranes were blocked with 5% non-fat dried milk for 1h and and incubated with primary antibodies overnight, followed by secondary antibodies for 1h. Reactions were visualized using luminol reagent.

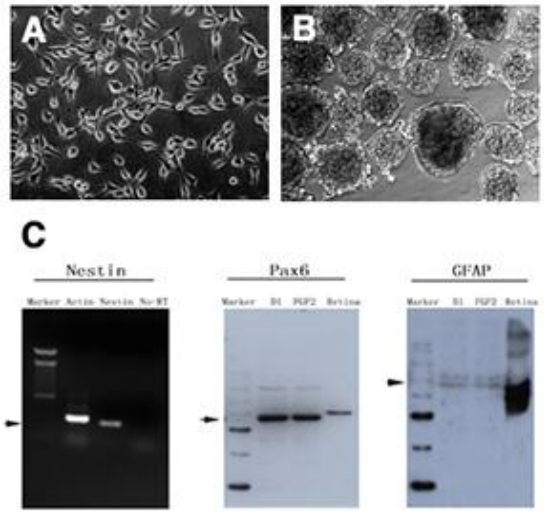

Fig.1. Isolation and characterization of human retinal progenitor cells.
Retinal progenitor cells proliferated **[A]** and formed neurospheres in growth medium containing 10% serum, FGF2 20ng/ml and EGF 20ng/ml. The neurospheres continued to increase in size and eventually floated in the medium **[B].** RT-PCR or Western blots **[C]** showed neurospheres grown in basal medium containing 1% serum (D1) only or with FGF2 expressed nestin and pax6 but not glial fibrillary acidic protein (GFAP; marker for Müller glia or astrocytes).

F. Reverse Transcription Polymerase Chain Reaction

Total RNA from RPC cells was extracted using RNAzol B (Biotecx Laboratories, Houston TX) and used for cDNA synthesis by random priming. PCR analysis for human nestin was performed using the PCR primer pairs 5'–AGGTGGCCACGTACAGGAC–3' and 5'–GGAGCAGAG AGAGAGGAGCA–3'. β-actin was used as internal control.

G. Intravitreal Injections

Conditioned media from human RPCs were pooled together and concentrated 100X and injected intravitreally in 6 6-week-old Royal College of Surgeons dystrophic rats. Briefly, rats were anesthetized with sodium pentobarbital, the upper eyelid was retracted, and 5ul of 100X concentrated RPC-CM was injected intravitreally at the ora serrata with a 32 G. needle; the other eye either received intravitreal basal media or no injection at all. After 4-6 weeks, animals were sacrificed using sodium pentobarbital overdose followed by intracardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Tissues were embedded in paraffin, section, and stained for microscopy.

Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Texas HSC in accordance with guidelines outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication 85- 23, revised 1996).

III. RESULTS

A. Human retinal progenitor cells form neurospheres.

Human retinal progenitor cells (RPC) were isolated

from donor retinas (0-44 years of age) and expanded in vitro in the presence of serum and mitogenic factors such as basic fibroblast growth factor (FGF2) and epidermal growth factor (EGF) [Fig.1A]. The cells rapidly proliferated and formed ball-like clusters (neurospheres) when grown in the presence of serum and growth factors [Fig.1B].

RT-PCR and immunoblotting verified the expression of retinal stem cell markers in the human RPCs such as nestin, and pax6 [Fig.1C]. RPCs also labeled for other progenitor cell markers such as p75NTR and neurofilament 68 [data not shown]. However, consistent with their undifferentiated phenotype, RPCs expressed low levels of mature retinal cell markers such as Rho4D2 (rod photoreceptors), GFAP (Müller glia), or glutamine synthase (astrocytes).

B. GO/G induces RPE cell death

To characterize the effects of oxidative injury on nuclear DNA, ARPE-19 cells were exposed to 25mU/ml GO/G for 5h and incubated with calcein AM and ethidium homodimer or Hoechst 33258 to determine cell viability as previously published [14]. Nuclei of ARPE-19 cells exposed to oxidative stress (GO/G) labeled for both ethidium homodimer and Hoechst 33258, but not the untreated cells (CTR), consistent with the increased plasma membrane permeability to the dyes of damaged cells [Fig.2]. Images of GO/G-treated cells showed nuclear DNA shrinkage, but not DNA fragmentation consistent with apoptotic cells.

C. RPC-CM protect RPE cells from oxidative damage

To test the hypothesis that RPC may secrete trophic factors that could rescue RPE cells from oxidative damage, ARPE-19 cells were pre-treated with conditioned media from RPC prior to exposure to 25mU/ml glucose oxidase plus 1g/L glucose for 5h. The protective effects of RPC-CM were determined using MTS assay or uptake of fluorescent dyes calcein AM and ethidium homodimer. Diminished ethidium homodimer staining and increased calcein AM labeling in cells pre-treated with RPC-CM indicate the significant inhibition of RPE degeneration induced by GO/G [Fig.3A].

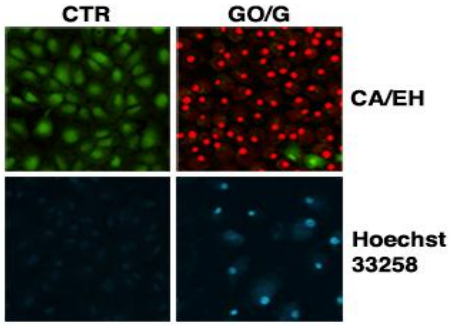

Fig.2. Prolonged exposure to oxidative stress induces cell death. Nuclei of ARPE-19 cells exposed to 25mU/ml glucose oxidase plus 1g/L glucose for 5h stained intensely with ethidium homodimer (red) or Hoechst 33258 (blue) but not calcein AM (green) consistent with damaged cells. Untreated ARPE-19 cells (CTR) exhibited intense cytoplasmic staining for calcein AM but not for ethidium homodimer or Hoechst 33258.

MTS assay verified the protective effect of RPC- CM, which was comparable to the anti-oxidant effect of N-Acetyl Cysteine [Fig.3B].

D. Specificity of RPC-CM Activity on RPE cells

To establish the specificity of the protective effects of RPC-CM against oxidative stress on ARPE-19 cells, RPC-CM was compared to conditioned media from human Müller glia and human RPE cells. ARPE-19 cells were pretreated with the various conditioned media prior to administration of oxidative stress. Cell viability was measured using MTS assay. As shown in Fig.4, RPC-CM, but not CM from Müller glia or RPE cells promoted RPE cell viability following exposure to GO/G for 5h. CM from the three cell types did not exhibit any toxicity to the ARPE-19 cells in the absence of GO/G.

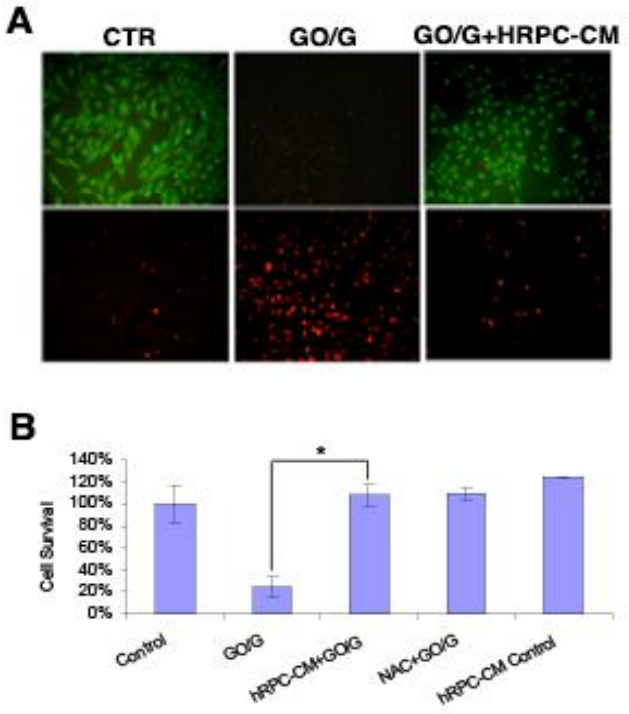

Fig.3. RPC-CM protects RPE cells from oxidative damage**.** Conditioned media were collected from RPCs cultured in serum-free DMEM for 48h. ARPE-19 cells were exposed to 25mU/ml glucose oxidase plus 1g/L glucose for 5h in the presence or absence of RPC-CM. After overnight recovery, calcein AM/ethidium homodimer were used to assay live/dead cells **[A].** MTS cell proliferation assay was used to measure the viability of ARPE-19 cells following oxidative stress in the presence or absence of RPC-CM or N-Acetyl Cysteine (NAC; anti-oxidant) **[B].** Experiments were done in triplicates (n=3) and analyzed by Student's t-test.

E. RPC-CM inhibits RPE cell death by activating Erk1/2

To further establish the specificity of the RPC-derived trophic factors, the putative molecular mechanisms involved in the protective effect of RPC-derived factors on RPE cells exposed to oxidative stress were investigated. Previous studies in the lab implicate the downregulation of ERK 1/2 in oxidative stress-induced RPE cell death [14]. Hence, ARPE-19 cells were incubated in RPC-CM or serum-free basal medium (BM) for 12h and assayed for ERK 1/2 activity using immunoblotting for phospho-Erk 1/2. RPC-CM-treated cells upregulated their expression of phospho-ERK 1/2, especially ERK2, following 12h treatment with

RPC-CM [Fig.5A]. The specificity of this phosphorylation event was verified by the addition of U0126, a specific inhibitor of MEK 1/2. ARPE-19 cells were pretreated with 20uM U0126 for 2h prior to exposure to GO/G for 5h. Fig.5B shows suppression of the protective effect of RPC-CM against oxidative stress by U0126. These findings suggest that RPC-CM may protect RPE cells from oxidative damage by activating ERK 1/2 signaling.

F. RPC-CM suppress retinal degeneration in rat retinas

The progressive RPE dysfunction and cell death in the aging retina lead to subsequent degeneration of photoreceptor cells due to loss of trophic support. In the Royal College of Surgeons (RCS) dystrophic rat retina, RPE changes similarly result in photoreceptor cell loss [15]. Moreover, subretinal injections of normal rat RPE cells in dystrophic retinas rescued photoreceptor cells for as long as one year [16-17].

Intravitreal injections of RPC-CM in young dystrophic rats resulted in thicker photoreceptor cell layers (blue arrows) [Fig.6B] as compared with either those injected with basal medium [Fig.6A] or those that were not injected. Moreover, the RPE cell layer in the RPC-CM injected retinas appeared more regular and uniform (red arrows).

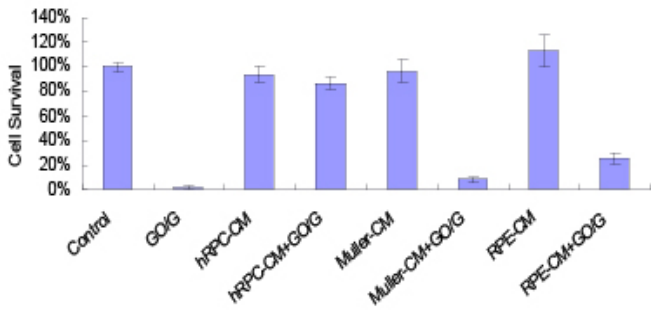

Fig.4. Specificity of RPC-CM on ARPE-19 cells. ARPE-19 cells were treated with 25mU/ml glucose oxidase plus 1g/L glucose for 5h in the presence of conditioned media of human RPC, human Muller cells or human RPE cells. Cell viability was measured using MTS assay. Experiments were done in triplicates (n=3) and analyzed by Student's t-test.

IV. Discussion

Stem cell research has been focused on expansion and purification of stem cells and repopulation of diseased organs with differentiated stem cells. However, stem cells have also been shown to secrete trophic factors that can promote the survival of various cell types. Embryonic stem cells were found to release pro-survival growth factors (IGF-1 and WNT5a) to completely reverse cardiac defects in transgenic mice through either local or long-range action [9]. Adult hippocampal neural stem/progenitor cells could secrete autocrine/paracrine factors such as SDNSF (stem-cell derived neural stem/progenitor cell supporting factor) in order to provide trophic support for neural stem/progenitor cells [10]. Moreover, it was reported that bone marrow-derived mesenchymal stem cells protected cultured cardiomyocytes from hypoxia. The concentrated medium also rescued acute myocardial infarction in an animal model [11]. These findings suggest that the therapeutic potential of stem cells can be expanded to include their secretion of growth factors.

Although stem cell transplantation has been employed in the treatment of retinal degenerations in animal models, the effects have not been satisfactory and the therapeutic potential of stem cells have not been well documented. Whereas stem cells can serve as a reservoir of trophic factors for the protection and/or regeneration of diseased organs, this prompted us to investigate whether human retinal progenitor cells secrete growth factors with the potential to treat retinal diseases such as AMD. Our present study clearly demonstrates that retinal progenitor cells secrete pro-survival molecules that could protect human RPE cells from oxidative damage *in vitro*. Moreover, these molecules possess specificity and activate molecular pathways involved in cell survival. Moreover, the therapeutic potential of these molecules were made evident by the intravitreal injection studies showing neuroprotection of the RPE and the retinal photoreceptors in the dystrophic rat retinas. These findings suggests that adult retinal stem cells could serve as a source and reservoir of trophic factors for the treatment of retinal diseases such as AMD.

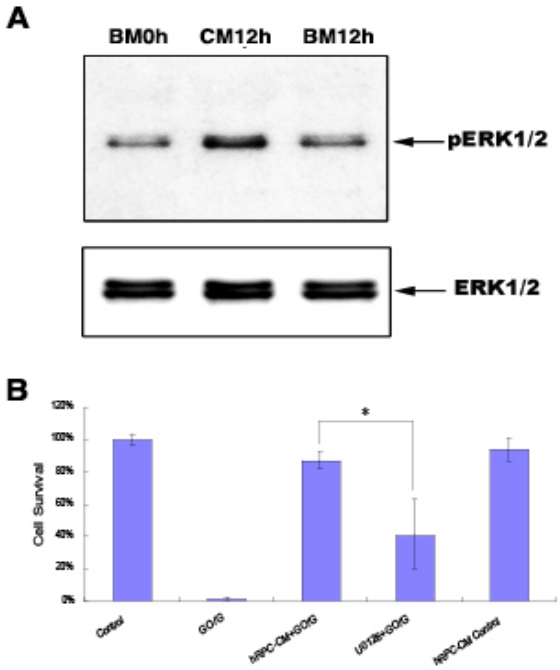

Fig.5. RPC-CM inhibits RPE cell death by activating Erk 1/2. ARPE-19 cells were treated with RPC-CM for 12h and the expression of phospho-Erk 1/2 was examined using immunoblotting **[A].** Total Erk 1/2 was used as loading control. ARPE-19 cells were also exposed to 25mU/ml glucose oxidase plus 1g/L glucose for 5h in the presence of RPC-CM alone or RPC-CM with U0126 (MEK 1/2 specific inhibitor). Cell viability was measured using MTS assay **[B].** Experiments were done in triplicates (n=3) and analyzed by Student's t-test.

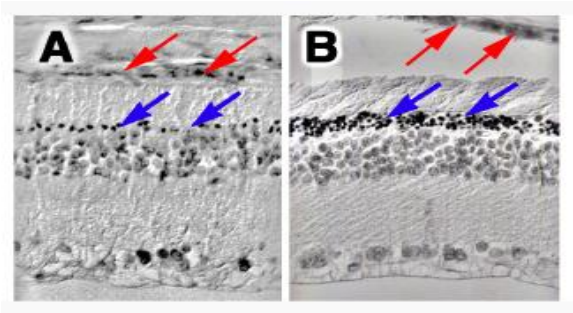

Fig.6. RPC-CM inhibits photoreceptor cell death in dystrophic rat retinas. Dystrophic rats, 6 weeks of age, received intravitreal injections of 5ul RPC-CM in one eye; the other eye either received intravitreal basal media or no injection at all. After 4-6 weeks, animals were sacrificed and retinas were embedded in paraffin for morphological studies. Dystrophic retinas injected with basal media [A] continued to degenerate resulting in a single layer of photoreceptor cells (blue arrows). The other eye of the same animal injected with RPC-CM [B] showed a much thicker photoreceptor cell layer (blue arrows) and normal-looking RPE monolayer (red arrows).

Retinal progenitor cells have the potential for treating retinal degeneration; substantial efforts are now being expended in isolating stem cells or progenitor cells from the human retina [18-20]. Adult human retinal stem cells have been isolated from the pars plicata and pars plana of the retinal ciliary margin from early postnatal human retinae to seventh-decade human retinae. Similar to mouse retinal stem cells, human retinal stem cells could self-renew, proliferate rapidly *in vitro* and possess the potential to give rise to retinal cells. They could survive, migrate, integrate and differentiate in the postnatal diseased mouse eyes and embryonic chick eye after transplantation.

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