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Original Research Report

Proteomic Differentiation Between Murine Retinal and Brain-Derived Progenitor Cells

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ABSTRACT

Previous studies have transplanted a variety of neural stem cells (NSCs) to the eye in hopes of developing a therapy to replace retinal neurons lost to disease. Successful integration, survival, and differentiation of the cell types has been variably successful. At the moment, little is known about the fundamental biological differences between stem cell or progenitor cell types. Characterization of these differences will not only increase our general understanding of this broadly characterized group of cells, but also lead to development of criteria for sorting cells, evaluating their differentiation, and predicting their suitability for transplantation. We have used two-dimensional gel electrophoresis protein expression profiles to characterize the molecular differences between two populations of murine progenitor cells—retinal progenitor cells (RPCs) and brain progenitor cells (BPCs) isolated from mice of the same age and same genetic background. Our protein expression profiling identified 22 proteins that are differentially expressed in RPCs when compared to BPCs. Four of the differentially expressed proteins correspond to proteins known to be involved in a cellular response to stress, and analysis of potential transcription factor binding sites in the promoter regions of their genes suggests these proteins could be co-regulated at the transcriptional level. On the basis of this discovery, we tested the hypothesis that the addition of the antioxidant vitamin E would decrease the expression of the stress-response proteins and influence differentiation of RPCs. Further investigation of differences between multiple populations of RPCs and BPCs during their maintenance and differentiation will further identify fundamental differences that define 'retinal-like' characteristics and provide tools to assay the success of efforts to influence many populations of stem cells to adapt a retinal cell fate.

INTRODUCTION

BLINDING DEGENERATIVE RETINAL DISEASES, including retinitis pigmentosa and macular degeneration, are characterized by a loss of photoreceptors. At this time, there is no way to replace retinal cell loss due to disease or injury because differentiated retinal cells are unable to regenerate. As a potential approach for treating retinal disease, neural progenitor cells (NPCs) have been proposed as a unique source of transplantable cells to replace lost cells in the damaged eye. Various NPC types have been transplanted into the retina including retinal progenitor cells (RPCs) [1,2] brain progenitor cells (BPCs) [3–5], adult hippocampal progenitor cells (AHPCs) [6–8], and iris-derived/ciliary body cells [9–11]. Although transplantation of different progenitor cell types has been variably successful, retinal and ciliary body derived cells have shown the most promise.

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Little is known, however, about the fundamental biology of these cell types and how that may influence their successful transplantation.

In this study, we used protein expression profiling to compare RPCs with BPCs isolated from neonatal mice. Both of the cell populations chosen have been transplanted into developing and degenerate retinal environments [1,3–5,12–15]. Characterizing the molecular differences between these cells using a proteomics approach is important because different populations of progenitor cells display differential differentiation, integration, and migration after transplantation. It is generally accepted that progenitor cells derived from the retina are more appropriate for retinal transplantation; however, beyond the expression of a few basic transcription factors, fundamental differences between RPCs and BPCs have not been well characterized. A powerful aspect of this study is that protein expression patterns were compared in RPCs and BPCs isolated from mice of the same age, in the same laboratory, and from the same genetic background [3,16]. Thus, differences in protein expression profiles are more likely to represent actual differences between the cell populations as opposed to artifacts introduced by differences in culture methods.

In this analysis, significant differences in expression levels between RPC and BPC protein spots were identified. Here, we focus on 22 proteins that were successfully identified by tandem mass spectrometry and manually verified by known or predicted molecular weights and pI values. Notably, 4 out of the 22 proteins were stress-response proteins encoded by genes that share at least 11 potential transcription factor-binding sites in common, suggesting that their expression could be coregulated. We also tested the hypothesis that addition of the antioxidant vitamin E (α-tocopherol) to differentiating RPCs could decrease their expression of stress-response proteins and alter their differentiation profile. This is the first study in which protein expression profiling has identified differences between two populations of murine progenitor cells (BPCs, and RPCs) isolated from genetically similar mice. Our results suggest that protein expression profiling offers a useful approach to understanding the fundamental differences between different populations of stem cells.

MATERIALS AND METHODS

Cell culture

RPCs and BPCs used in this study were isolated and expanded in vitro as previously reported [16] from postnatal (0–1 days postnatal) enhanced green fluorescent protein (eGFP)-expressing transgenic mice [TgN β-act-eGFP] 040bs). Both BPCs and RPCs have been reported to express Ki-67, a marker of proliferation, as well as other neurodevelopmental markers [1,4]. The progenitor cells were maintained as neurospheres in neurobasal medium (Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine (Invitrogen), and 10,000 units/ml of penicillin/streptomycin solution (Sigma, St Louis, MO), 1% B-27 supplement (Invitrogen), 20 ng/ml of human recombinant epidermal growth factor (EGF; Invitrogen), and 10,000 units/ml of Nystatin (Sigma, St. Louis, MO). Cells were fed every 2 days and maintained in a 37°C incubator containing 95% CO_2 and 5% O_2.

Cell fractionation

For protein extraction, RPCs and BPCs were rinsed in Earl's balanced salt solution (EBSS; Invitrogen), resuspended in EBSS, and placed on ice. The cells were rinsed with phosphate-buffered saline (PBS, 0.14 M NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2), resuspended in 1 ml of hypo buffer (1 mM Tris, 1 mM $MgCl_2$) with Complete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland), and placed on ice for 20 min. Cells were sonicated for 30 sec and centrifuged at 4,000 rpm for 10 min at 4°C. The protein extract was acetone precipitated from the supernatant overnight at −20°C. The protein sample was resuspended in 1× sample rehydration buffer containing urea (8 M), CHAPS (2%), and bromophenol blue (0.002%) and stored at −80°C. A protein assay was done using the EZQ Protein Quantitation Kit (Invitrogen) to determine the total protein concentration. The final concentration of the diluted sample was 35 μg/165 μl (0.212 μg/μl).

Two-dimensional separation of proteins

For two-dimensional gel electrophoresis (2D), isoelectric focusing (IEF) Zoom strips (Invitrogen; pH 3-10NL 7.7 cm) were rehydrated by adding protein sample, dithiothreitol (DTT; Invitrogen; 20 mM), ampholytes (Bio-Rad, Hercules, CA; 0.5% vol/vol), and iodoacetamide (Bio-Rad; 116 mg) for 35 μg of total protein per strip. The strips rehydrated overnight (8–16 h) at room temperature. The voltage protocol used for IEF was 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2,000 V for 45 min.

After IEF proteins were separated by molecular weight using precast Nupage 4–12% Bis-Tris Zoom®Gels (Invitrogen). The voltage used for the gels was continuous at 200 V for 50 min. Proteins were detected with an overnight incubation of SYPRO Ruby fluorescent protein stain (Molecular Probes). After destaining for 3 h in 10% methanol and 7% acetic acid, gels were imaged at the proteomics facility at Iowa State University using a Typhoon 9410 fluorescent scanner (GE Biosciences, Piscataway, NJ). Once imaged, the gels were stained with Simply Blue Comassie (Invitrogen) protein stain overnight.

Protein spot analysis and identification

Gels were analyzed for significant changes in expression levels using Phoretix 2D software (Nonlinear Dynamics, Durham, North Carolina). Phoretix 2D Expression (Nonlinear Dynamics, North Carolina) software was used for analysis. Data from three replicates were combined to generate an average gel for each cell type. After matching like protein spots between average gels, normalized expression levels were determined for each

protein spot. Proteins that were more highly expressed in RPCs were hand picked from the gels. Trypsin digestion and deposit to a target for matrix-assisted laser desorption/ionization (MALDI) were performed using an Ettan Spot Handling Workstation (Amersham Biosciences, Newark, NJ). For MALDI analysis, the tryptic peptides dissolved in 50% CH_3CN/0.1% trifluoroacetic acid (TFA) were mixed with a matrix solution (CHCA 10 mg/mL in 50% CH_3CN/0.1% TFA) and applied on a target plate. For electrospray ionization (ESI), protein digest solution was taken out after trypsin digestion, extracted and dried to needed volume.

MALDI-time-of-flight mass spectrometry (TOF MS/MS) analyses were performed using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an MALDI ion source. The mass spectrometer was operated in the positive ion mode. Mass spectra for MS analysis were acquired over *m/z* 500 to 4000. After every regular MS acquisition, MS/MS acquisition was performed against most intensive ions. The molecular ions were selected by information-dependent acquiring in the quadrupole analyzer and fragmented in the collision cell. For ESI MS, the peptide digest samples were introduced to the QSTAR XL quadrupole TOF mass spectrometer with a Switchos LC pump and a FAMOS autosampler (LC Packings, San Francisco, CA). Other parameters of the mass spectrometer were the same as MALDI analysis.

All spectra were processed by MASCOT (MatrixScience, London, UK) database search. Peak lists used for MS/MS ion searches were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada). The search parameters were as follows: Max-missing cleavage of one, fixed modification of carboxyamidomethyl cysteine, variable modification oxidation of methionine. Peptide mass tolerances were set to ± 100 ppm and fragment mass tolerances were set to ± 1 Da. No restrictions on protein molecular weight were applied. Protein identification was based on the probability-based Mowse Score [17]. The significance threshold p was set to less than 0.05.

For each identified protein, gene ontology (GO) annotations were manually retrieved from Swiss-Prot (http://ca.expasy.org) [18].

Promoter region analysis

For each gene analyzed, DNA sequences for a 1-kb promoter region (from 700 bp upstream to 300 bp downstream from the transcript start site) were retrieved from Cold Spring Harbor Laboratory *Mus musculus* Promoter Database version 2.33 (MmPD; http://rulai.cshl.edu/cgi-bin/CSHLmpd2/mmpd.pl) [19].

Potential transcription factor binding sites (TFBS) were identified within each promoter region using TRANSFAC version 7.0 (http://www.gene-regulation.com) [20] P-Match version 1.0, (21), a tool that combines pattern matching and weight matrix approaches.

Differentiation of RPCs

To induce differentiation, cells were plated in eight well chamber slides (Fisher Scientific, Pittsburgh, PA). Slides were double coated with 0.1% poly-L-lysine (Sigma) and 1 mg/ml of laminin (mouse, BD Biosciences, Bedford, MA). Poly-L-lysine was added to 0.1 M borate buffer (0.1 M H_3BO_3, 0.1 M $Na_2B_40_7$, pH 8.5) and sterile filtered with a 0.22- μm millipore filter (Millipore Corporation, Billerica, MA). The solution was added to each chamber and incubated at room temperature. After 3 h, the slides were washed with sterile tissue culture water (Sigma) and stored in the refrigerator. Prior to use, the sterile water was removed from the slides and allowed to dry for 2 h, after which laminin was added to $1\times$ of sterile phosphate-buffered saline (PBS, 0.14 M NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2), pipetted into each slide and incubated at 4°C overnight. The next day, the laminin was aspirated off the slides and washed with sterile PBS. After the last wash, the slides were washed two times with culture medium just prior to cell plating.

Cells growing as neurospheres were dissociated before plating. Medium containing retinal neurospheres was extracted from flasks (Fisher) and centrifuged to form a pellet. The pellet was washed in EBSS (Invitrogen) containing a penicillin/streptomycin solution. The pellet was then incubated in EBSS containing 0.25% trypsin (Invitrogen) for 15 min and centrifuged for 1 min. The supernatant was aspirated off, and the pellet was washed with EBSS and centrifuged. After the wash, the pellet was incubated in EBSS containing 0.0025% trypsin inhibitor (Invitrogen) for 5 min and centrifuged. The pellet was washed with EBSS and centrifuged. After the last wash, culture medium was added to the conical tube. The cells were then dissociated by triturating with a fire-polished 1-ml pipette until the suspension became cloudy. Dissociated retinal cells were counted on a hemacytometer and plated at a density of 45,000 cells/cm^3 or 80,000 cells/cm^3on coated eight-well chamber slides.

RPCs in eight-well chamber slides were allowed to differentiate for 10 days. Cells were fed by replacement of half of the culture medium consisting of Ultraculture media (Cambrex, East Rutherford, NJ) containing 2 mM of L-glutamine (Gibco; Invitrogen), 10,000 units/ml of Nystatin (Sigma), and 10 ng/ml of gentamicin (Gibco; Invitrogen) every other day. Vitamin E (in the form of α-tocopherol; Sigma) was diluted in ethanol and added to ultraculture media at 5 μM, 25 μM, or 50 μM. For experiments with vitamin E, controls included both no treatment and vehicle (EtOH). No differences were observed between the no-treatment and the vehicle control cultures.

Immunocytochemistry

Immunocytochemistry was used to assay protein expression and cellular differentiation. After 10 days in culture the cells were fixed in 4% paraformaldehyde in PBS. Cells were rinsed and incubated in blocking solution consisting of potassium phosphate-buffered saline (KPBS; 0.15 M NaCl, 0.034 M K_2HPO_4, 0.017 M KH_2PO_4, pH 7.4), 1% bovine serum albumin (BSA; Fisher), 0.4% Triton- X 100 (TrX-100, Sigma, St. Louis, MO), and 1.5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratory, West Grove, PA) at room temperature for 2 h. Cells were incubated in primary antibodies at room temperature overnight. The following day the cells were washed in KPBS containing 0.02% TrX-100 and incubated in secondary antibody for 2 h. After being washed in KPBS containing TrX-100, the slides were incubated for 5 min in 300

μM DAPI (100 mM) diluted in KPBS at room temperature. Again the slides were washed in KPBS and coverslipped with Vectashield fluorescence mounting medium (Vector Laboratories, Burlingame, CA).

Antibodies

Primary antibodies used were anti-nestin (mouse monoclonal immunoglobulin G, IgG; Chemicon, Temecula, CA; 1:10), anti-glutamine synthetase (GS; rabbit polyclonal IgG; Sigma; 1:10,000) , anti-protein kinase C α (PKC-α; rabbit polyclonal IgG; Sigma; 1:10,000) , anti-heat shock protein 60 (Hsp60, mouse monoclonal IgG; Chemicon; 1:500), anti-heat shock protein 70 (Hsp70, rabbit polyclonal IgG; Chemicon; 1:200), anti-catalase (CAT, mouse monoclonal IgG; Sigma; 1:1,000), and anti-copper-zinc superoxide dismutase (Cu-Zn SOD, rabbit polyclonal IgG; Stressgen, BC, Canada; 1:1,000). Secondary antibodies (Alexa Fluor 594 donkey anti-mouse or donkey anti-rabbit, Molecular Probes; Invitrogen; 1:500) were diluted in KPBS with 1% BSA, 1.5% NDS, and 0.4% TrX-100.

Imaging

A Nikon E800 (Melville, NY) microscope equipped with a Retiga 1300 digital camera (Qimaging Burnaby, BC, Canda) was used to capture images of cells. Adobe Photoshop version 9 (Adobe Systems Incorporated, San Jose, CA) was used to crop images and Macromedia Freehand 8 (Macromedia Incorporated, San Francisco, CA) software was used to prepare figures.

Cell counting

Quantification of cells expressing immunocytochemical markers were done by counting 10 random fields using a 20× objective for each chamber. Total cells in one field were counted using the nuclear counter stain 4′, 6-diamidino-2-phenylindole (DAPI). The number of immunopositive cells for a given antibody was also counted for each field. Cell differentiation was expressed as percentage of total cells and compared to the untreated control.

Statistics

Values were given as the means ± standard error mean (SEM) and, where appropriate, significance of differences between mean values were determined by analysis of variance (ANOVA; Super ANOVA, ABACUS, Berkeley, CA). p values of less than 0.05 were considered significant.

RESULTS

Proteomics analyses were used to examine differences in protein expression between RPCs and BPCs, isolated from mice of the same age, in the same laboratory, and from the same genetic background. The samples were separated first by their isoelectric point (pI) and second by their molecular weight. Fig. 1 shows representative gels from RPCs and BPCs.

A total of 323 distinct protein spots were separated from RPC samples and 233 distinct protein spots from BPC samples. There were significant differences in the expression levels of 136 distinct protein spots. Ninety out of these 136 spots were unique to RPC gels; 32 showed an increase of at least two-fold in RPC gels and 14 showed a decrease of at least two-fold in RPC gels. On the basis of their differential expression in RPCs, these protein spots were hand picked and analyzed using tandem-mass spectrometry (MALDI or ESI MS/MS). Protein identifications were manually verified by comparing known or predicted molecular weights and pIs with their approximate molecular weight and pI on gels. Twenty-two proteins (16%) were confirmed using these criteria. Table 1 shows a list of protein accession numbers along with their protein identifications and their expression in RPCs.

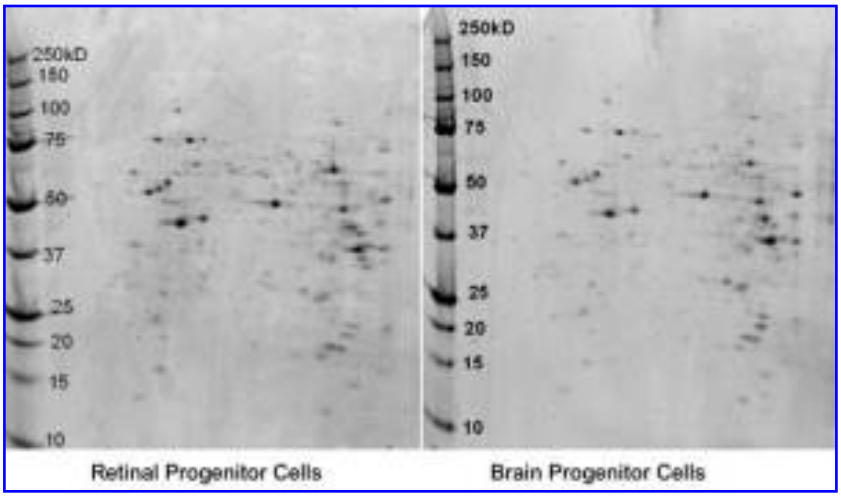

FIG. 1. Representative gels after two-dimensional gel electrophoresis of samples from RPCs (*left*) and BPCs (*right*).

TABLE 1. LIST OF PROTEIN IDENTIFICATIONS AND EXPRESSION LEVELS IN RPCs COMPARED TO BPCs

Identified protein spots overexpressed in RPCs when compared to BPCs

Accession number	*Protein identifications*	*Expression fold increased in RPCs compared to BPCs*	*Accession number*	*Protein identifications*	*Expression fold increased in RPCs compared to BPCs*
P63017	Heat shock cognate 71-kD protein	Unique		mitochondrial precursor	
P14733	Lamin B1	+2.408	O35737	Heterogeneous nuclear ribonucleoprotein H (hnRNP H)	+2.108
P63038	60-kD heat shock protein, mitochondrial precursor (Hsp60)	+2.015	O70251	Elongation factor 1-β (EF-1-β)	+2.371
P61979	Heterogeneous nuclear ribonucleoprotein K	Unique	P29758	Ornithine aminotransferase, mitochondrial precursor (EC 2.6.1.13)	Unique
P47753	F-actin capping protein α-1 subunit	Unique			
Q99PF4	Cadherin-23 precursor (otocadherin)	Unique			
P57776	Elongation factor 1-δ	Unique	P56959	RNA-binding protein FUS (Pigpen protein)	+2.287
P67778	Prohibitin (B-cell receptor asociated protein 32) (BAP 32)	+2.093	P60710	Actin, cytoplasmic 1 β-actin)	Unique
P10639	Thioredoxin	+2.802	P31786	Acyl-CoA-binding protein (ACBP) (Diazepam binding inhibitor) (DBI) (Endozepine)	Unique
P16045	Galectin-1 (β-galactoside-binding lectin L-14-I)	+2.973			
P08228	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	+3.313	Q9D051	Pyruvate dehydrogenase E1 component β subunit, mitochondrial precursor	Unique
P62962	Profilin-1	+2.459			
P49312	Heterogeneous nuclear ribonucleoprotein A1	+2.451			
P08249	Malate dehydrogenase,	+2.711	Q9DBJ1	Phosphoglycerate mutase 1 (EC 5.4.2.1)	+5.498

As a first step in characterizing these RPC proteins, gene ontology (GO) annotations were retrieved for each protein. Table 2 lists GO annotations for biological process, molecular function, and cellular localization, respectively. Analysis of the biological processes annotations (Table 2) indicated that 4 out of the 22 proteins were involved in transport, 2 were involved in protein folding, 2 were involved in actin cytoskeleton organization, 2 were cell adhesion proteins, 1 in glycolysis, 3 in protein biosynthesis, and 3 in metabolism. Biological process annotations were not available for five proteins. The GO molecular function annotations (Table 2) showed that 13 out of the 22 proteins identified were involved in protein, actin, nucleic, lipid, RNA, or DNA binding whereas 8 were identified as to being involved in catalytic, elongation, or structural activity. One protein had an unknown molecular function. GO cellular localization annotations revealed that 7 out of the 22 proteins localized to the nucleus, 8 were localized to the mitochondria, 2 were localized to the cytosol, 2 were translation elongation factors, 1 F-actin capping protein, and 1 membrane protein. There was 1 protein with no GO localization annotation. This initial, relatively high-level GO annotation analysis failed to reveal any obvious functional relationships among the identified proteins.

Analysis of annotations at a more detailed level of the GO hierarchy revealed that 4 of the differentially expressed proteins were known to be involved in the cellular response to stress. These 4 proteins were: Hsp60, Hsp70, thioredoxin, and Cu-Zn superoxide dismutase. Hsp60, thioredoxin, and Cu-Zn superoxide dismutase showed at least a two-fold or greater increase in RPCs relative to BPCs whereas, the protein spot identified for Hsp70 was unique to RPC cells.

To investigate the mechanism of this observed differential expression, we examined the genes encoding all

Table 2. Identified Proteins with Their Corresponding GO Annotations for Biological Process, Molecular Function, and Cellular Localization

Protein	*GO: Biological process*	*GO: Molecular function*	*GO: Cellular localization*
Phosphoglycerate mutase 1	1. Glycolysis 2. Metabolism	1. Bisphosphoglycerate mutase activity 2. Catalytic activity 3. Hydrolase activity 4. Intramolecular transerase activity, phosphotransferases 5. Isomerase activity 6. Phosphoglycerate mutase activity	1. Cytosol
RNA-binding protein FUS (Pigpen protein)	1. Positive regulation of transcription from RNA polymerase II promoter	1. Transcriptional activator activity 2. DNA binding 3. Nucleic acid binding 4. RNA binding 5. Zinc ion binding	1. Cytoplasm 2. Nucleus
60-kD heat shock protein, mitochondrial precursor (Hsp60)	1. Cellular protein metabolism 2. Protein folding	1. ATP binding 2. Protein binding 3. Unfolded protein binding	1. Mitochondrion
Malate dehydrogenase, mitochondrial precursor	1. Glycolysis 2. Malate metabolism 3. Tricarboxylic acid cycle intermediate metabolism	1. L-malate dehydrogenase activity 2. Malate dehydrogenase activity 3. Oxidoreductase activity	1. Mitochondrion
Heterogeneous nuclear ribonucleoprotein A1 (Helix-destabilizing	1. mRNA processing 2. mRNA-nucleus export 3. RNA processing 4. Transport	1. Nucleic acid binding 2. RNA binding	1. Nucleus 2. Ribonucleoprotein complex
Acyl-CoA-binding protein (ACBP) (diazepam-binding inhibitor) (DBI) (Endozepine)	1. Transport	1. Acyl-CoA binding 2. Lipid binding	1. Mitochondrion
Lamin B1		1. Structural molecule activity	1. Lamin filament 2. Nucleus 3. Intermediate filament
Prohibitin (B-cell receptor associated protein 32) (BAP32)	1. DNA metabolism		1. Mitochondrion
Thioredoxin	1. Electron transport 2. Transport	1. Electron transporter activity	1. Mitochondrion
Superoxide dismutase	1. Activation of MAPK 2. DNA fragmentation during apoptosis 3. Response to oxidative stress 4. Removal of superoxide radicals 5. Superoxide metabolism	1. Copper, zinc, superoxide 2. Antioxidant activity 3. Metal ion binding 4. Oxidoreductase activity 5. Superoxide dismutase activity	1. Mitochondrion 2. Cytoplasm
Profilin-1	1. Neural tube closure 2. Regulation of transcription from RNA polymerase II promoter	1. Protein binding 2. Actin binding	1. Cytoplasm 2. Nucleus 3. Actin cytoskeleton 4. Cytoskeleton

Table 2. Identified Proteins with Their Corresponding GO Annotations for Biological Process, Molecular Function, and Cellular Localization (Cont'd)

Protein	*GO: Biological process*	*GO: Molecular function*	*GO: Cellular localization*
	3. Regulation of actin polymerization and/or depolymerization 4. Sequestering of actin monomers 5. Actin cytoskeleton organization and biogenesis 6. Cytoskeleton organization and biogenesis		
Heterogeneous nuclear ribonucleoprotein H (hnRNP H)		1. Nucleic acid binding 2. RNA binding	1. Nucleus 2. Ribonucleoprotein complex
Heat shock cognate 71-kD protein (Heat shock 70-kD protein 8)	1. Chaperone cofactor dependent protein folding 2. Protein folding 3. Regulation of cell cycle 4. Response to unfolded protein	1. ATPase activity, coupled 2. Protein binding 3. Unfolded protein binding 4. ATP binding unfolded protein binding	1. Nucleus
Actin, cytoplasmic 1 (β-actin)		1. Motor activity 2. Structural constituent of cytoskeleton 3. Structural molecule activity	1. Actin cytoskeleton 2. Actin filament 3. Cytoskeleton 4. Cytosol 5. Soluble fraction
Ornithine aminotransferase, mitochondrial precursor		1. Ornithine-oxo-acid transaminase activity 2. Pyridoxal phosphate binding 3. Transaminase activity 4. Transferase activity	1. Mitochondrion
Pyruvate dehydrogenase E1 component beta subunit, mitochondrial precursor	1. Glycolysis	1. Oxidoreductase activity 2. Pyruvate dehydrogenase (acetyl-transferring) activity	1. Mitochondrion
Elongation factor 1-β (EF-1-β)	1. Protein biosynthesis 2. Translational elongation	1. Translation elongation factor activity	1. Translation elongation factor 1 complex
Heterogeneous nuclear ribonucleoprotein K		1. Protein binding 2. DNA binding 3. Nucleic acid binding 4. RNA binding	1. Nucleus 2. Ribonucleoprotein complex
Galectin-1 (β-galactoside-binding lectin L-14-I) (lactose-binding lectin 1)	1. Myoblast differentiation 2. Heterophilic cell adhesion 3. Biological–process unknown	1. Galactose binding 2. Sugar binding	1. Extracellular space 2. Cellular_component unknown
F-actin capping protein α-1 subunit	1. Actin cytoskeleton organization and biogenesis	1. Actin binding	1. F-actin capping protein complex
Cadherin-23 precursor (octocadherin)	1. Cell adhesion 2. homophilic cell adhesion 3. Perception of sound.	1. Calcium ion binding 2. Protein binding	1. Integral to membrane 2. Membrane
Elongation factor 1-δ	1. Protein biosynthesis 2. Translational elongation	1. Translation elongation factor activity	1. Translation elongation factor activity

four proteins and compared the DNA sequences of their promoter regions. Sequences within a region including 700 bp upstream and 300 bp downstream from the transcript start site for each gene were queried for the presence of known TFBS. This analysis revealed that potential TFBS for 11 transcription factors are located within the promoters regions of all 4 genes (Table 3). Potential binding sites for several additional transcription factors were shared among two or three, but not all four genes (data not shown). Most of the shared TBFS common to all four genes were present in multiple copies (e.g., all had 8–10 copies of the CREB motif). Although the significance of this finding requires further investigation, the presence of sets of shared TFBS in the promoters of all 4 genes suggests that they could be co-regulated at the transcriptional level.

Differentiation in vitamin E decrease expression of stress-response proteins

We used immunocytochemistry to determine if expression of stress-response proteins was decreased by addition of the antioxidant vitamin E to culture medium. To induce differentiation of RPCs, spheres were dissociated, plated on coated slides, and cultured in medium without EGF for 10 days, after which they were fixed and labeled with antibodies against the stress response proteins Hsp60, Hsp70, Cu-Zn SOD, and catalase (CAT). Two blinded investigators then independently sorted the images of 10 fields of cells from each condition (captured with identical exposure times) based on perceived intensity of immunoreactivity with each antibody. Images of cells were easily sorted into vitamin E-treated and control by both observers based on immunoreactivity for each of the four stress-response proteins. Figure 2 demonstrates immunoreactivity for Hsp60, Hsp70, Cu-Zn SOD, and CAT in cells differentiated with vitamin E (B, D, F, H) or without vitamin E (A, C, E, G). Expression of all 4 proteins was subjectively decreased when 50 μM vitamin E was added compared to control treatments.

Vitamin E influences differentiation profiles of RPCs

To investigate the effects of the antioxidant vitamin E on differentiation of RPCs, cells were differentiated in media containing 5 μM, 25 μM, or 50 μM of α-tocopherol (vitamin E). Figure 3 shows the antibody labeling profiles of RPCs differentiated in media with three differing concentrations of vitamin E (5 μM, 25 μM, or 50 μM) compared to controls. The number of cells immunopositive for nestin was significantly increased with the addition of 50 μM vitamin E (48% $\pm$ 2.5) compared to the control (33% $\pm$ 2.5), but lower concentrations of vitamin E did not have a significant effect. The percentage of cells expressing glutamine synthetase (a marker for Müller glia) was significantly increased with 25 μM (56% $\pm$ 2.4) and 50 μM (51% $\pm$ 2.6) vitamin E compared to the control cultures (42% $\pm$ 2.2). Finally, the number of cells immunopositive for PKC-α (a marker of rod bipolar cells in the retina) was increased at all three vitamin E concentrations but the increase only reached significance at 5 μM (48% $\pm$ 2.8) and 50 μM (44% $\pm$ 3.1) compared to the control cultures (19% $\pm$ 4) (Fig. 3).

TABLE 3. TRANSCRIPTION FACTOR-BINDING SITES AND THE NUMBER OF TIMES THEY ARE SEEN IN THE PROMOTER REGION OF EACH STRESS-RESPONSE GENE

	Number of times each transcription factor-binding site was identified in the promoter region of the gene			
Transcription factors	*Hsp60*	*Hsp70*	*Thioredoxin*	*Superoxide dismutase [Cu-Zn]*
AP-4	4	7	3	1
BSAP	8	3	7	1
c-Rel[a]	107	86	91	95
CREB	9	7	8	8
Elk-1[a]	5	10	10	9
Evi-1[a]	43	38	50	50
Myogenin/NF-1	4	1	3	4
NF-κB	1	4	3	2
NF-κ (p50)	2	2	3	1
NRF-2	3	6	5	3
Zid	3	1	4	2

[a]Represents the three transcription factor-binding sites that were identified more than 10 times in their promoter region.

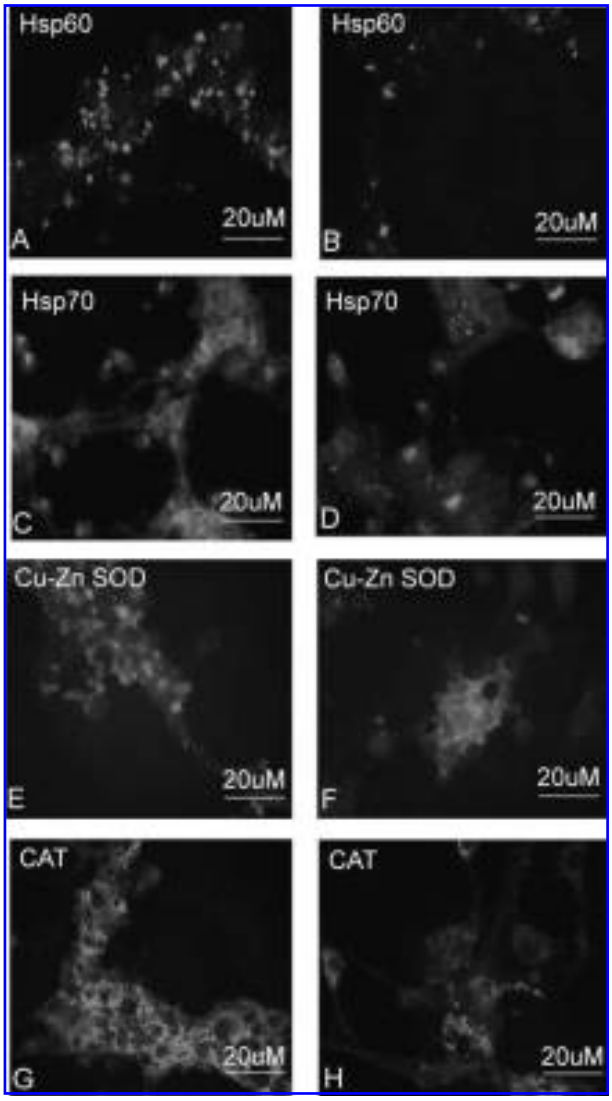

FIG. 2. Immunoreactivity of Hsp60, Hsp70, Cu-Zn SOD, and CAT in RPCs differentiated for 10 days without (**A,C,E,G**) or with (**B,D,F,H**) vitamin E.

DISCUSSION

Our protein expression profiling identified 22 proteins that are differentially expressed in RPCs when compared to BPCs isolated from mice of the same strain. Four of the differentially expressed proteins correspond to proteins known to be involved in a cellular response to stress, and analysis of potential transcription factor binding sites in the promoter regions of their genes suggests these proteins could be co-regulated at the transcriptional level.

Furthermore, we have shown that the addition of the antioxidant vitamin E to differentiating RPCs decreases expression of stress-response proteins (3 that we identified as more highly expressed in RPCs, Hsp60, Hsp70 and Cu-Zn SOD, and a fourth stress response protein, Catalase). The RPCs used in this study were isolated from early postnatal mice (0–1 days postnatal). Endogenous retinal progenitors at this age would be expected to differentiate primarily into rod photoreceptors, bipolar cells, and Müller glia [22]. Addition of vitamin E also alters the differentiation profile of RPCs when added to medium. There were significant increases in cells immunopositive for nestin, a marker for NPCs glutamine synthetase, a marker for Müller glia and PKC-α, a marker for rod bipolar cells when 50 μM of vitamin E was added to the medium. A previous study by Zahir and colleagues [23] also reported biasing differentiation of expanded RPCs toward PKC-α-expressing cells. They reported that ciliary neurotrophic factor (CNTF) increases the percentage of cells expressing PKC-α and induces changes in the morphology and rate of proliferation of RPCs. They observed an increase in expression of PKC-α, but also most of the PKC-α-positive cells exhibited bipolar morphology once treated with CNTF [23]. RPCs in our study also exhibited a bipolar morphology when differentiated in the presence of vitamin E.

In addition, we saw a decrease in the stress-response proteins Hsp60, Hsp70, Cu-Zn SOD, and CAT when vitamin E was added to the medium compared to control conditions, suggesting that their expression was at least in part in response to the oxidative load they were experiencing in culture. However, despite these effects of vitamin E treatment, we were still not able to detect recoverin immunoreactivity (a marker for photoreceptors), demonstrating that we were still unable to bias cells toward a photoreceptor fate.

Potential developmental role of stress-response proteins expressed by RPCs

In addition to the possibility that expression of stress-response proteins in RPCs was in response to oxidative stress, expression of at least some of these proteins may also be developmentally regulated [24]. Heat shock proteins are a group of proteins that are present in all cells at all biological levels. These proteins are needed for normal cell growth and maintenance and have been detected during embryogenesis in various organisms [25]. Different developmental profiles of heat shock proteins have suggested that these proteins have a role in neural cell differentiation [25].

Hsp70 is found in the cytosol, nucleus, and endoplasmic reticulum, and its expression has been detected in numerous cell populations within the nervous system, including neurons, glia, and endothelial cells [25,26]. By embryonic day (E) 15.5 in mice, Hsp70 is detectable in the central nervous system and at E17.5 all heat shock proteins are expressed in the hippocampus [25,27]. Developmental analysis in the postnatal rat brain has shown that basal levels increased in the cerebral hemisphere un-

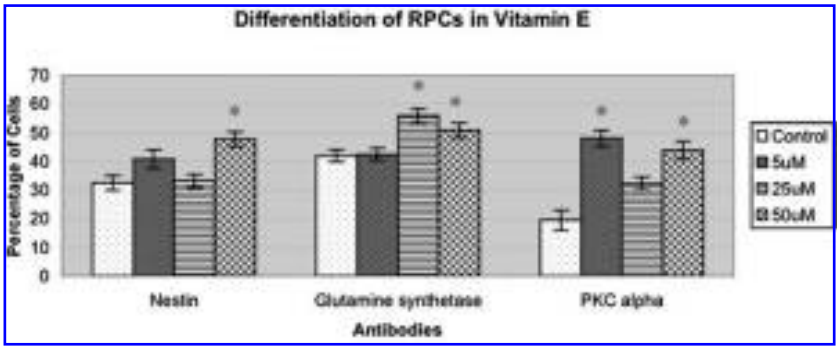

FIG. 3. Percentage of RPCs immunopositive for nestin (NPC marker), glutamine synthetase (marker for Müller glia), and PKC-α (in the retina is a marker for rod bipolar cells) after 10 days *in vitro* in differentiation conditions with the addition of vitamin E (5 μM, 25 μM, and 50 μM). The bars represent the standard error. * $p < 0.05$.

til postnatal (P) day 20 and then decreased in the adult, whereas there was little change observed in the cerebellum during postnatal development [25]. Hsp60 has also been described in neurons [25]. Developmental profiles have shown that Hsp60 increases during postnatal development in the rat brainstem and the cerebral hemispheres; therefore, Hsp60 levels are significantly higher at P20 and in the adult compared to P1 in these regions [25].

Thioredoxin (TRX) is small protein (12–13 kDa) known to function as an antioxidant protein. TRX appears to play biologically important roles in hormone secretion, cell signaling, regulation of the intracellular apoptotic pathway, and cell proliferation [29,30,31]. TRX has also been shown to regulate the DNA binding activity of various transcriptional factors such as nuclear factor (NF-κB), activator protein 1 (AP-1), myb, redox factor 1, and mitogen-activated kinase [32,33]. Although many of the functions attributed to TRX are known to be important developmental processes, we are not aware of any specific studies of the role of TRX during neural or retinal development.

Copper-zinc superoxide dismutase (Cu-Zn SOD1) is an antioxidant enzyme found in the nucleus, mitochondria, and cytosol. It is expressed abundantly in all vertebrate tissue and catalyzes the conversion of superoxide anion to hydrogen peroxide [34,35]. SOD1 is considered a key enzyme to helping a cell protect itself against oxygen free radicals [34,36]. A mutation in the SOD1 gene causes amyotrophic lateral sclerosis (ALS), a neurodegenerative disease. To our knowledge, however, there are no published reports suggesting a potential role for SOD1 during neural or retinal development.

CAT is a common enzyme located in the peroxisome. Its function includes catalyzing the decomposition of hydrogen peroxide to water [37,38]. This catalytic reaction protects the cell from the toxic effects of hydrogen peroxide [24]. Developmental changes in embryonic and postnatal brains of mice showed that CAT protein levels were high at E18 and remained elevated throughout development [37].

It is not yet clear what developmental role these stress-response proteins may have in RPCs. However, expression of these proteins does clearly differentiate RPCs from BPCs that were generated and maintained under the same conditions and may well represent fundamental biological differences between these two cell populations.

The increased expression of stress-response proteins in RPCs, and the subsequent decrease in expression of stress-response proteins after vitamin E treatment, suggests RPCs are sensitive to the oxidative load in their environment. Glaucoma and retinitis pigmentosa are two diseases in which oxidative stress is a component of the pathogenesis. Glaucoma is a degenerative disease that can lead to blindness. Vision is lost because of damage to the optic nerve, which carries information from the eye to the brain [39]. Retinitis pigmentosa is a genetic condition of the eye that affects the photoreceptors (rods) and can lead to progressive vision loss. Once the rods begin to die, the cones will follow progressively. Rods consume a high level of oxygen, therefore, as the rods die, the tissue level of oxygen in the retina increases [40]. This oxygen increase is very damaging to the photoreceptors and results in the generation of reactive oxygen species, which causes the cells to die [40]. Degenerative retinal environments may very well impart a significant oxidative burden. The results of our studies suggest that changing the oxidative environment (in our example, decreasing the oxidative load by the addition of antioxidant to the culture medium) can affect RPC differentiation. Thus, if cells are to be transplanted into an oxidative environment we must understand how it will affect their differentiation.

Shared transcriptional regulatory motifs in stress-response genes expressed in RPCs

Co-regulation of genes by sets of shared transcription factors is a common theme in the developmental regulation of eukaryotic gene expression [41]. Analysis of the promoter regions of the genes encoding the four stress-response proteins that were over-expressed in RPCs relative to BPCs revealed 11 transcription factor-binding site motifs in common. At least 10 copies of the motifs for three of these (c-Rel, Evi-1, and Elk-1) were found within a 1-kb promoter region of each gene. Thus, transcriptional co-regulation of the genes for these four stress-related proteins is potentially responsible for their elevated expression in RPCs relative to BPCs.

The c-Rel transcription factor is a member of the NF-κB transcription factor family. These transcription factors play an important role in cell proliferation, apoptosis, stress response, and inflammation. Evi-1 is a transcription factor that encodes a 145-kD nuclear protein of the zinc finger family, which contains two domains of zinc fingers [42,43]. Evi-1 is thought to promote growth and cell proliferation or block differentiation in some cell types [44]. Elk-1 is an ETS-domain transcription factor involved in the mitogen-activated protein (MAP) kinase pathway [45,46]. Recently, it was demonstrated that Elk-1 did not fulfill an essential function in mouse development, although in rats Elk-1 was expressed selectively in neurons [47].

Co-regulation of preferentially RPC-expressed cellular stress-response proteins by c-Rel, Evi-1, or Elk-1 would be consistent with a role during proliferation of RPCs (a neuronal cell population). Future studies to assay the expression of these transcription factors in RPCs compared to BPCs may address this question. Certainly identification of transcription factors that underlie the differential expression of proteins between populations of cells is an important aspect of understanding fundamental biological differences between them.

We also characterized the differentiation of RPCs and discovered that addition of the antioxidant vitamin E affects their expression of the GS and PKC-α, markers of differentiated retinal cells. We observed an increase in expression of nestin, GS, and PKC-α. Furthermore, we observed a decrease in expression of stress-response proteins that many not directly affect differentiation but suggests that the environmental oxidative load does influence RPC differentiation. This is the first study to examine the effects of the oxidative environment on RPC differentiation. Our results suggest that the oxidative environment can significantly affect RPC differentiation and should be considered in the context of developing cell replacement therapies to treat retinal degenerative diseases.

Proteomics is a powerful approach for identifying differences in protein expression and post-translational modification between cell types [48,49]. Profiling the expression of proteins in tandem with differential RNA expression is critical for understanding the many post-transcriptional mechanisms that regulate the ultimate function of proteins in cellular processes because changes in cellular mRNA levels often do not directly correlate with changes in their protein levels [49]. Furthermore, protein expression profiling allows detection of many protein post-translational modifications and can provide valuable snapshots of cellular metabolism that complement the results of RNA-based assays.

We have provided the first systematic proteomics comparison of expression patterns in RPCs and BPCs from mice of the same strain. The identification of four stress-response proteins among those differentially expressed in RPCs relative to BPCs is intriguing. Perhaps proliferation or differentiation of RPCs is inherently more stressful for RPCs than for BPCs, or perhaps RPCs normally have a higher baseline level of these proteins. Although there are no direct data to support either of these possibilities at present, two of the four stress-response proteins have been implicated in neural development by other studies, leading us to propose that they are somehow involved in RPC development. Finally, the shared potential transcriptional regulatory sites in the genes for these four stress response proteins, suggesting their transcriptional co-regulation in RPCs makes these cells potentially useful as biomarkers to differentiate 'retinal-like' progenitor cells from 'brain-like' progenitor cells. Further investigation of differences between multiple populations of RPCs and BPCs during their maintenance and differentiation will further identify fundamental differences that define 'retinal-like' characteristics and provide tools to assay the success of efforts to bias many populations of stem cells to adopt a retinal cell fate.

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