Recharacterization of the RGC-5 Retinal Ganglion Cell Line

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Purpose. The transformed RGC-5 retinal ganglion cell line is used widely in glaucoma research. Increased resistance to glutamate was noted in published literature and led to the recharacterization of the RGC-5 cell line.

Methods. Characterization of the RGC-5 cell line was performed by sequencing of a region of the nuclear Thy1 gene and mitochondrial DNA sequencing of a region of the d-loop and tRNA^{Phe} gene. Marker expression was examined in undifferentiated cells, and cells differentiated with 50 μg/mL succinyl concanavalin A (S Con A) for 3 days. Glutamate sensitivity was examined in undifferentiated and S Con A differentiated cells by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24-hours of glutamate treatment.

Results. RGC-5 cells were found to be of mouse (*Mus musculus*), not rat (*Rattus norvegicus*), origin by mitochondrial and nuclear DNA analyses. RGC-5 DNA sequenced in a second laboratory was subsequently found to be of *M. musculus* origin. Cells stained positively for the neuronal markers β-tubulin and PGP9.5 and for the microtubule-associated protein tau, but not for known markers of ganglion cells such as neurofilaments or Thy1.2, suggesting that they likely represented a lineage of mouse neuronal precursor cells. Differentiation with S Con A did not increase RGC-5 sensitivity to glutamate excitotoxicity or increase the expression of retinal or ganglion cell marker proteins.

Conclusions. Investigators using cells designated as RGC-5 should confirm the species to be of rat origin and retinal-specific marker expression before considering their use as retinal ganglion-like cells. (*Invest Ophthalmol Vis Sci.* 2009;50: 4267–4272) DOI:10.1167/iovs.09-3484

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The in vitro study of optic nerve diseases was limited to primary retinal ganglion cell culture before the establishment of a transformed rat retinal ganglion cell line (RGC-5) derived by transforming postnatal day 1 rat retinal cells with ψ_2 EA1 virus.[1] This line was designated positive for certain characteristics of retinal ganglion cells, including Thy-1 and Brn-3C expression, and for sensitivity to glutamate excitotoxicity and neurotrophin withdrawal.[1] RGC-5 cells also expressed brain-derived neurotrophic factor, nerve growth factor neurotrophin-3, neurotrophin-4, and receptors TrkA, p75, low levels of TrkB, and minimal, if any, expression of TrkC.[2] On differentiation with succinyl concanavalin A (S con A), RGC-5 cells became sensitive to glutamate toxicity,[1] with 1 mM glutamate sufficient to cause approximately 50% cell death in differentiated RGC-5 cells.[3]

Cell lines are vital tools in biochemical research and have aided in the development of drugs, vaccines, and other therapeutics and in allowing further understanding of genetics and cellular processes involved in diseases. The wide use of cell lines mandates verification of their origin and characteristics before their use.

We have become aware, from recent publications, of increased resistance to glutamate using the RGC-5 line. Earlier studies showed greater sensitivity of these cells to glutamate, with only 35% cell survival after 24 hours of incubation in 5 mM glutamate.[4] Later publications show survival levels at 75%[5] and 85%.[6] This indicated a potential change in the characteristics of this cell line. The purpose of this study, therefore, was to recharacterize the RGC-5 cell line.

Methods

Cell Lines

The rat retinal ganglion cell line (RGC-5) was provided by Neeraj Agarwal (University of North Texas Health Science Center, Fort Worth, TX) at passage 19 in February 2007. A second batch was provided in June 2007. RGC-5 cells were cultured in 1 mg/mL glucose DMEM (Sigma-Aldrich, St. Louis, MO), 10% fetal calf serum (Sigma-Aldrich), and 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) and were grown in a humidified 37°C/5% CO$_2$ incubator.

Other cell lines used as controls were the *Rattus norvegicus* line RN1T (American Type Culture Collection [ATCC]), *Mus musculus*, *Mus dunni*, and *Mus pahari* primary fibroblast cells,[7] and a xenomitochondrial (*M. dunni* mtDNA) mouse.[8]

DNA Amplification, Restriction Digest Analysis, and DNA Sequencing

Cell line genomic DNA was isolated by phenol chloroform extraction (Sigma-Aldrich) and DNA quantified using a spectrophotometer (Nanodrop; Nanodrop Technologies, Wilmington, DE). PCR amplification of a region of nuclear DNA was achieved with primers internal to the Thy1 gene. A region that showed sequence variation between *M. musculus* (GI:134152684) and *R. norvegicus* (GI:6981653) was amplified with primers (forward, 5'-ccatccagcatgagttcagcct-3'; reverse, 5'-accagtcacagagaaatgaagtcc-3') and heating to 94°C for 5 minutes, then

35 cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, followed by 72°C for 5 minutes.

A region of mitochondrial DNA (mtDNA), including the d-loop and part of the tRNA-phe gene, was chosen because it showed sequence variation between *M. musculus* (GI:34538597) and *R. norvegicus* (GI:110189714).[9] Amplification was performed with forward (5′-ctc aac ata gcc gtc aag gc-3′; nucleotides 15934–15953) and reverse (5′-acc aaa cct ttg tgt tta tgg g-3′; nucleotides 80–59) primers by first heating to 94°C for 5 minutes, before 35 cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, followed by 72°C for 5 minutes. Restriction digest analysis of the amplified mtDNA used Sca1 (New England Biolabs, Beverly, MA), which cuts the PCR products of only *M. musculus* and *M. parhai*. Bands were separated on 1% TAE agarose gel.

For DNA sequencing, unincorporated nucleotides and primers were removed from the PCR reaction with a PCR cleanup kit (Qiagen, Valencia, CA), and DNA was sent for sequencing (Australian Genome Research Facility, Brisbane, Australia) using the forward primer for each PCR reaction. Sequences were aligned with the use of multiple sequence alignment (ClustalX).[9]

Glutamate Sensitivity Assays

RGC-5 cells were plated at 5000 cells per well into 96-well plates (Greiner, Longwood, FL) and were allowed to attach overnight. The following day increasing concentrations of L-glutamate, pH 7.4 (Sigma), were added to media and incubated with cells for 24 hours. Numbers of viable cells were quantified with the MTT kit (Roche Diagnostics, Indianapolis, IN), as described by the manufacturer.

Succinyl Concanavalin A Differentiation of RGC-5 Cells

Differentiation of RGC-5 cells was performed as described previously,[1] with a minor modification of decreased differentiation time, as used previously.[10] Briefly, attached cells were cultured in the absence of serum for 24 hours, after which the cells were cultivated in growth media supplemented with serum and S Con A (50 μg/mL; Sigma) for 3 days.

Analysis of Selected Protein Expression by Immunoblot

RGC-5 cells for immunoblot analysis were seeded in 75-cm^2 culture flasks at an approximate density of 25% growth area and were left to grow for 6 hours in normal growth medium before putative induction of differentiation was established, as described with S Con A. After treatment, RGC-5 cells were harvested by scraping into phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH_2PO_4, 7 mM Na_2HPO_4; pH 7.4), and then cell pellets were sonicated in freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 2 mM EDTA, 0.5 mM ethylene-glycol-tetraacetic acid, 1 mM dithiothreitol, and the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (50 μg/mL), aprotinin (50 μg/mL), and pepstatin A (50 μg/mL). An equal volume of sample buffer (62.5 mM Tris/HCl, pH 7.4, containing 4% sodium dodecyl sulfate, 10% glycerol, 10% β-mercaptoethanol, and

0.002% bromophenol blue) was added, and samples were boiled for 3 minutes. An aliquot was taken at this stage for the determination of protein content. Electrophoresis of samples was performed using 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Samples were then transferred onto polyvinylidene difluoride (PVDF) membranes overnight. PVDF blots were probed with antisera raised against neuron- or RGC-specific proteins (β-tubulin, PGP9.5, neurofilament species, tau) and cytoskeletal proteins to act as reference (β-actin, α-tubulin). Primary antibody incubations (detailed in Table 1) were carried out overnight at 4°C on a spiral roller. Appropriate secondary antibodies conjugated to horseradish peroxidase were subsequently used (90 minutes, room temperature). Nitrocellulose blots were developed with a 0.016% solution of 3-amino-9-ethylcarbazole in 50 mM sodium acetate (pH 5.0) containing 0.05% (vol/vol) Tween-20 and 0.03% (vol/vol) H_2O_2. Images were acquired from labeled blots (2000R ImageStation; Eastman Kodak, Rochester, NY) and were analyzed for densitometry using image analysis software (Eastman Kodak). Rat retina and rat optic nerve were used as controls for immunoblotting.

Immunohistochemical Analysis for Neuronal and RGC-5 Cell Markers

RGC-5 cells grown on borosilicate glass coverslips were fixed for 10 minutes in 10% neutral-buffered formalin with additional methanol (1% vol/vol). After they were washed with PBS, cells were permeabilized in PBS plus 0.1% (vol/vol) Tween-20 and then blocked in PBS containing 3% normal horse serum. Coverslips were incubated with primary antibodies overnight at room temperature (diluted in PBS containing 3% normal horse serum; see Table 1 for details of antibodies used), followed by consecutive incubations with the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and streptavidin-Alexa Fluor 488 conjugate for visualization. After subsequent washing with PBS, coverslips were incubated in PBS containing 500 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) for 15 minutes at room temperature before final washing in PBS. Coverslips were mounted using antifade mounting medium (ProLong Gold; Invitrogen) and were examined under a fluorescence microscope (Zeiss, Thornwood, NY).

RESULTS

RGC-5 Cell Line Containing *Mus musculus* DNA, Not *Rattus norvegicus*, DNA

Results from cells received in February 2007 indicate the *M. musculus* origin of the RGC-5 cell line. Results from cells received in June 2007 were identical with those from February 2007. Amplification of a region of the Thy1 gene containing 84.3% sequence variation between *M. musculus* (Mm) and *R. norvegicus* (Rn) revealed that RGC-5 cells share 97.8% sequence identity with Mm (Fig. 1A). Further analysis of a region of the mtDNA, including the d-loop and part of the tRNA-phe gene, with 67.2% homology between Mm and Rn, confirmed 99.4% homology between RGC-5 and Mm (Fig. 1B). Comparison of mtDNA fragments after restriction digest also indicated

TABLE 1. Antibody Sourcing and Optimized Dilutions

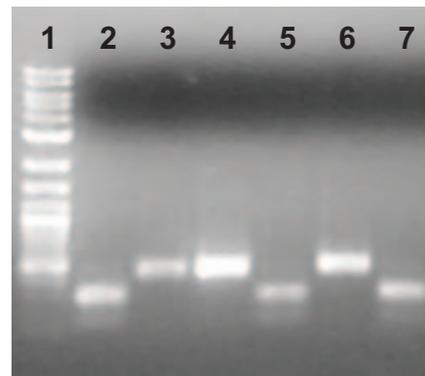| Antibody	Company	Species	Blot Dilution	ICC Dilution
β-Actin	Sigma	Mouse	1:100000	—
α-Tubulin	BioGenex	Mouse	1:10000	1:10000
β-Tubulin	Chemicon	Mouse	1:5000	1:5000
NF-H	Sigma	Mouse	1:20000	1:10000
NF-M	Zymed	Mouse	1:50000	1:10000
NF-L	Sigma	Mouse	1:50000	1:10000
PGP9.5	Ultraclone	Mouse	1:10000	1:10000
Tau	Santa Cruz	Goat	1:1000	1:1000
Thy 1.2	Biocore Pty	Mouse	—	1:400

A.
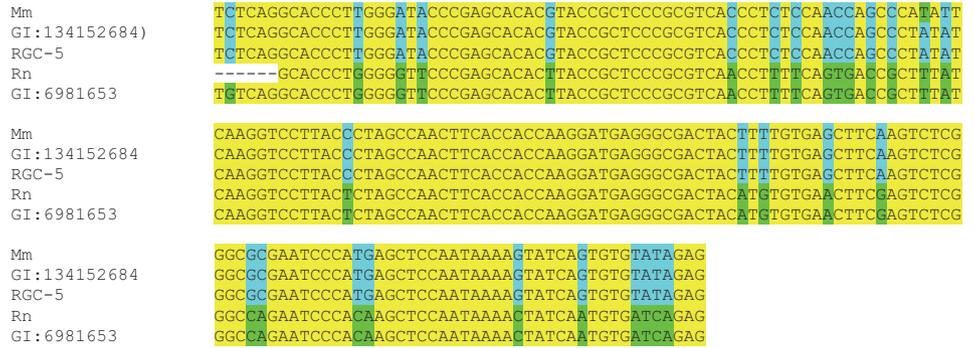
B.
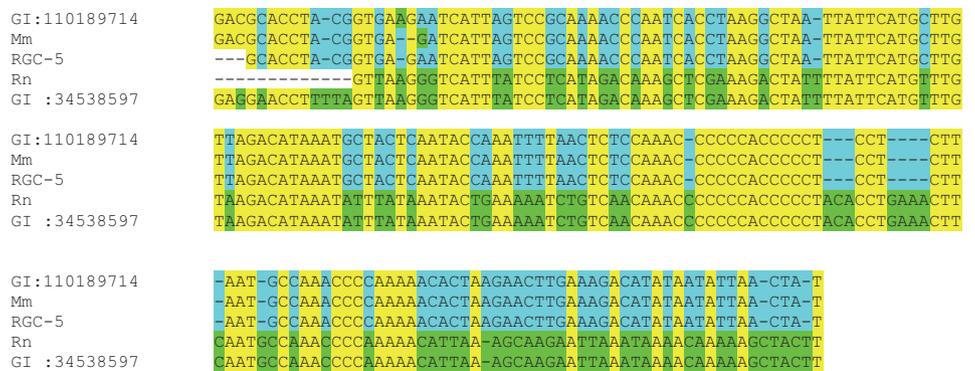
C.

FIGURE 1. DNA alignment of RGC-5 against *M. musculus* and *R. norvegicus.* PCR products of *M. musculus* (Mm), *R. norvegicus* (Rn), and RGC-5 were amplified, sequenced, and aligned. Alignments for (**A**) Thy1 nDNA gene and (**B**) mtDNA d-loop and part of the tRNA-phe gene both confirm the *M. musculus* origin of the RGC-5 cell line. Included are alignments against the following published genes: *M. musculus* Thy1 (GI:134152684), *R. norvegicus* Thy1 (GI:6981653), *M. musculus* mtDNA (GI:34538597), and *R. norvegicus* mtDNA (GI:110189714). (**C**) Restriction digestion of mtDNA provides confirmation of the mouse origin of the RGC-5 cells. *Lane 1*: 2-log molecular weight ladder; *lane 2*: *M. musculus* fibroblast; *lane 3*: xenomitochondrial mouse (*M. dunni* mtDNA); *lane 4*: *M. dunni* fibroblast; *lane 5*: *M. pahari* fibroblast; *lane 6*: *R. norvegicus* fibroblast; *lane 7*: RGC-5 from University of North Texas Health Science Center.

RGC-5 cells were of mouse origin (Fig. 1C) because the RGC-5 PCR fragment was cut with Sca1, along with positive controls of Mm and *M. pahari.*

Glutamate Sensitivity of RGC-5 from Recent Publications

Recent publications have shown that the glutamate sensitivity of the RGC-5 cell line has declined since generation of the cell line in 2001 (Table 2). All conditions of treatment were the same: 24 hours incubation in the presence of 5 mM glutamate. Our data showed approximately 95% cell survival with 5 mM glutamate (Fig. 2D).

Previous reports indicate increased sensitivity of RGC-5 cells on differentiation with S Con A,[1] with 1 mM glutamate causing 50% cell death in S Con A–differentiated cells.[3] Our data showed approximately 98% survival in differentiated cells treated with 1 mM glutamate. We also found that RGC-5 cells

TABLE 2. Glutamate Sensitivity of Undifferentiated RGC-5 Cells to L-Glutamate Toxicity

Authors	Cell Survival (%)
Aoun et al.[4]	35
Fan et al.[5]	75
Maher et al.[6]	85
Current study	95

kDa (neurofilament light; NF-L), neurofilament-160 kDa (neurofilament medium; NF-M), or neurofilament-200 kDa (neurofilament heavy; NF-H) compared with rat retina (lane 1) and rat optic nerve (lane 2) extracts. Clearly detectable levels of β-tubulin and tau were observed in treated and untreated RGC-5 cell extracts, possibly along with trace amounts of the neuronal marker protein PGP9.5 (protein gene product 9.5; UCH-L1). The cytoskeletal proteins actin and α-tubulin were present in all extracts examined. Wherever protein expression was detected, no significant difference in levels was detected between untreated and S Con A–treated cells.

Expression of Neuronal and RGC-Specific Protein Markers in RGC-5 Cells by Immunocytochemistry

The neuronal protein β-tubulin was expressed in treated (Fig. 4D) and untreated (Fig. 4C) RGC-5 cells and mouse retinal sections (Fig. 4E). NF-H and PGP9.5, however, were not detected in either cell type (Figs. 4F, G, I, J) but were detected in mouse retinal sections (Figs. 4E, H). The cytoskeletal protein α-tubulin was also detected. No difference was observed in the labeling of α- and β-tubulin between treated and untreated cells (Figs. 4A–D). Similarly, there was no evidence of Thy 1.2 labeling in treated or untreated cells (Figs. 4L, M), but positive labeling was detectable in mouse retinal sections.
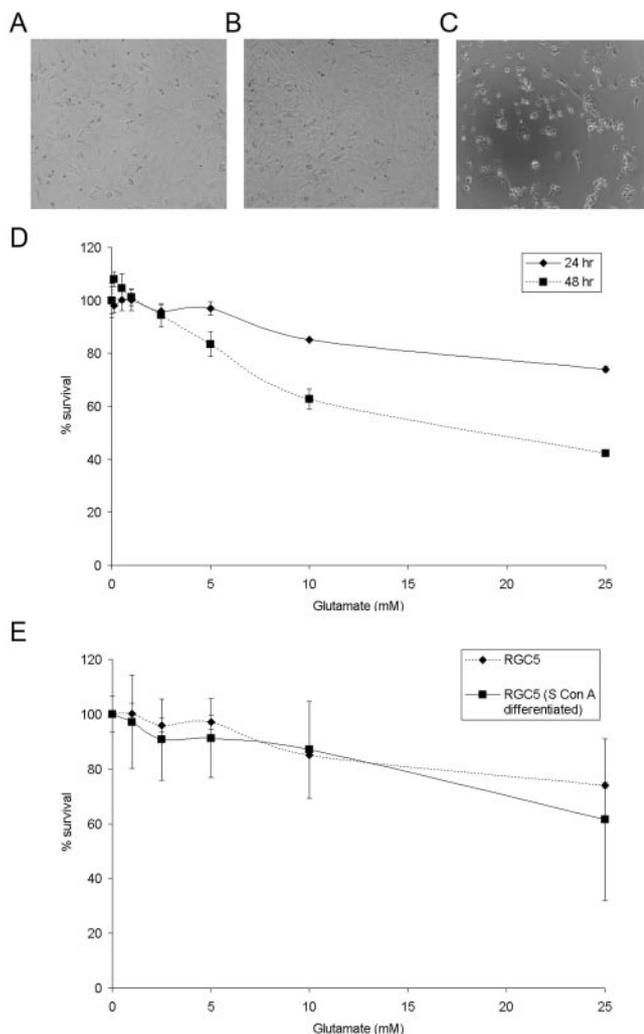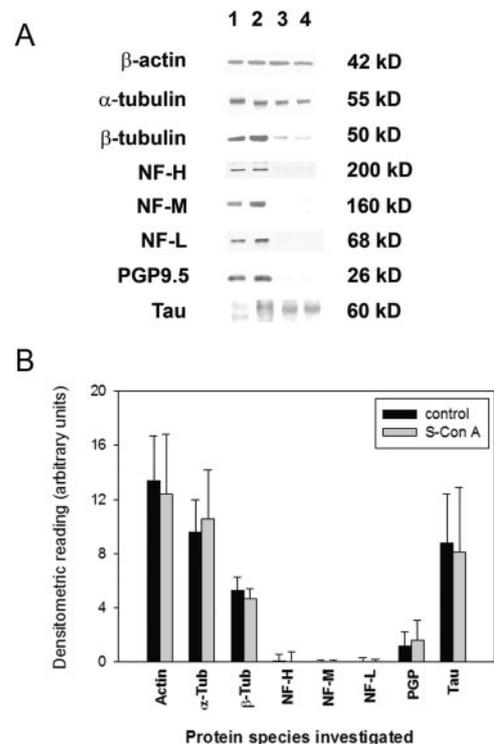

FIGURE 2. Effects of L-glutamate on RGC-5 cell viability and S Con A differentiation on L-glutamate survival of RGC-5. RGC-5 cells were plated, incubated overnight, and treated with various concentrations of glutamate. Increased cell death was apparent at higher glutamate concentrations. Phase-contrast images were captured (100× magnification) after 48 hours with (**A**) no glutamate, (**B**) 25 mM glutamate, or (**C**) 50 mM glutamate. (**D**) Cell viability 24 or 48 hours later was determined using MTT assay, and data were presented as the percentage survival compared with controls (non–glutamate-treated cultures). (**E**) RGC-5 cells were differentiated with 50 μM S Con A for 3 days before they were treated with various concentrations of glutamate. Increased cell death was apparent at higher glutamate concentrations. However, S Con A differentiation did not increase RGC-5 cell sensitivity to glutamate toxicity. Cell viability 24 hours later was determined using MTT assay, and data were presented as the percentage of survival compared to controls (non–glutamate-treated cultures). Data are mean ± SD; $n \geq 3$ independent experiments.

differentiated with 50 μg/mL S Con A for 3 days did not show increased glutamate sensitivity (Fig. 2E) to undifferentiated cells. Significant reductions in viable cell numbers were observed at higher glutamate concentrations, and these values were similar for undifferentiated and differentiated cells.

Expression of Neuronal and RGC-Specific Protein Markers in RGC-5 Cells by Immunoblot

The immunoblot data shown in Figure 3 clearly demonstrated that neither untreated (lane 3) nor S Con A–treated (lane 4) RGC-5 cells expressed detectable levels of neurofilament-68

FIGURE 3. Immunoblot analysis of selected protein expression in cultured RGC-5 cells. (**A**) Example of protein expression in control RGC-5 cells (*lane 3*) and in cells treated for 3 days with 50 μM S Con A (*lane 4*) compared with extracts of rat retina (*lane 1*) and rat optic nerve (*lane 2*). There is trace β-tubulin expression in control and treated cells, but levels of neurofilaments and the neuronal marker PGP9.5 are essentially undetectable. (**B**) Quantification of immunoblot analysis of RGC-5 protein expression as exemplified in (**A**). Data points represent mean ± SEM for $n = 8$ samples for each treatment. NB α-tub, α-tubulin; β-tub, β-tubulin; NF-H, heavy neurofilament (200 kDa); NF-M, medium neurofilament (160 kDa); NF-L, light neurofilament (68 kDa); PGP9.5, protein gene product 9.5 (UCH-L1).
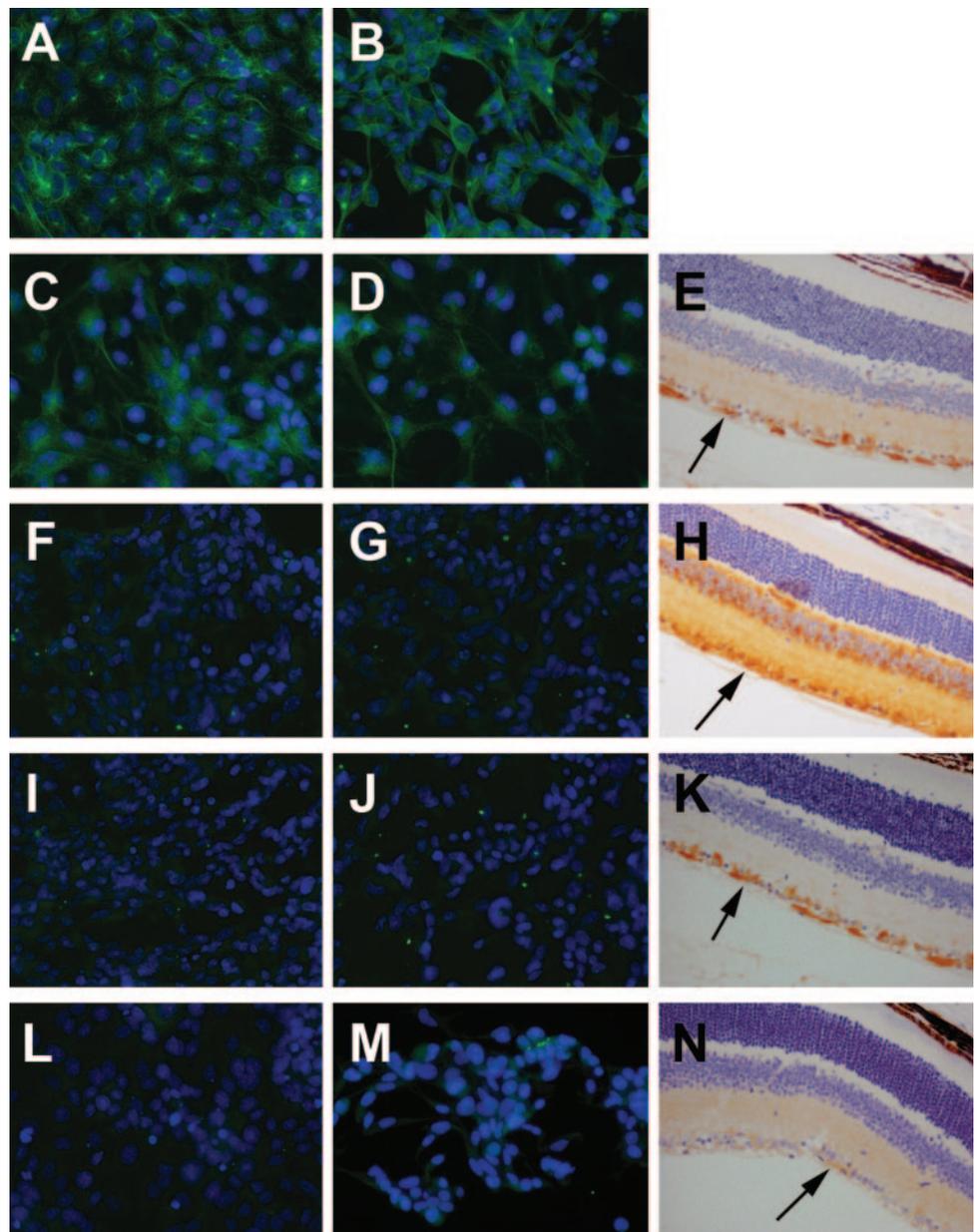
FIGURE 4. Analysis of selected neuronal protein expression in cultured RGC-5 cells by immunocytochemistry. Selected expression is shown for control RGC-5 cells (**A, C, F, I, L**) and for cells treated for 3 days with 50 μM S Con A (**B, D, G, J, M**). Positive labeling for selected antisera in mouse retinal sections is also shown (**E, H, K, N**) to prove labeling specificity. (**A, B**) α-Tubulin, (**C-E**) β-tubulin, (**F-H**) PGP 9.5, (**I-K**) heavy neurofilament (200 kDa), NF-H, (**L-N**) Thy1.2. There was evident labeling in treated and untreated RGC-5 cells for both tubulins but not for NF-H, PGP9.5, or Thy1.2, though positive labeling for each was detectable in ganglion cells in mouse retinal sections (*arrows*). Treatment with S Con A had no apparent effect on the expression of any of these markers. Images shown at 200× magnification. Cultured cell nuclei labeled with DAPI for visualization.

DISCUSSION

DNA from RGC-5 cells sourced twice from the University of North Texas Health Science Center at Fort Worth was found to be of mouse (*M. musculus*), not rat (*R. norvegicus*), origin by mitochondrial and nuclear DNA sequence analyses. Further confirmation was obtained recently from a second laboratory that independently sequenced portions of the COX-1 and mitochondrial D-loop genes from RGC-5 cells carried since 2004. This investigation found 97% to 99% identity with the mouse sequences (L. A. Levin, personal communication, November 2008).

Since generation of the cell line in 2001,[1] a decline in glutamate sensitivity has been observed in more recent studies[5,6] reporting glutamate sensitivity similar to that of our data. Cells stained positively for the control cell label α-tubulin and the neuronal markers PGP9.5, β-tubulin, and tau, but they did not express the neurofilaments (NF-L, NF-M, NF-H) even after treatment with S Con A, which was originally described to lead to the differentiation of RGC-5 cells into ganglion cell-like neurons.[1] Cells were also negative for the neuronal marker PGP, which labels ganglion cells in retina, and Thy1.2, which labels ganglion cells in mouse retina. The present data suggest that the RGC-5 cell line represents a lineage of mouse neuronal precursor cells. Differentiation with S Con A using an established protocol[1] with decreased S Con A exposure time[10] did not increase RGC-5 sensitivity to glutamate excitotoxicity or alter the expression of any of the proteins examined, suggesting that this compound was not able to induce the differentiation of RGC-5 cells; however, differentiation may be dependent on cell culture conditions. Previous studies have suggested other means to differentiate RGC-5 cells into ganglion cells using either histone deacetylase inhibitors[11] or the nonselective protein kinase inhibitor staurosporine.[12]

Differentiation of RGC-5 cells with staurosporine arrested cell growth, upregulated neuronal markers, and established outward rectifying channels, giving them many of the characteristics of mature RGCs,[12] including Thy1 and NMDAR1.[12] According to the literature, staurosporine may be better at

differentiating RGC-5 cells than S Con A. The aim of our present study, however, was to highlight the potential mouse origin of RGC-5 rather than to redefine the expression of retinal markers and differentiation techniques.

Accumulating evidence from large cell repositories—including the ATCC, the Japanese Collection of Research Resources, the European Collection of Cell Cultures, Deutsche Sammlung von Mikrorganismem und Zellkulturen, and the Coriell Institute for Medical Research—describes misidentified cell lines submitted for deposit. It has been suggested that up to 20% of studies using cell lines have used misidentified cells.[13] Persistent use of such cell lines appears to be largely ignored by many researchers,[14] and a collaborative approach between institutes and funding bodies may be a means of reducing this major problem.

There are many examples of misidentification of cell lines. The NCI-60 multidrug resistant breast cancer line, distributed by the National Cancer Institute, was ultimately identified to be an ovarian cancer cell line[15,16]; the authors Liscovitch and Rabin[15] estimated more than 300 publications with this incorrect cell line. Human tumor cells placed in animals in human metastases studies frequently turn out to be non-human mouse cells or recruited host cells.[17,18] The LCC15-MB breast bone metastasis cell line was genetically linked to the MDA-MB-435 cell line from the Lombardi Cancer Centre, and this prompted a review of commonly misidentified breast and prostrate lines and resulted in the creation of a register of misclassified cell lines.[19] More than 80 publications with RGC-5 cells had been listed by the time of submission.

Numerous methods are readily available to mainstream cell biologists for identifying newly arrived cell lines, including genotyping, karyotyping, and examination of cell markers. There have been successes in the biopharmaceutical production industry in eliminating mischaracterized cell lines, and only two cell lines in more than 900 analyzed were of cross-species contamination between 1996 and 2006.[20]

Data presented in this study indicate the mouse origin of the RGC-5 cell line by direct sequencing of nuclear and mitochondrial DNA. We recommend other researchers reidentify their RGC-5 cell lines by direct sequencing, as described in this study, to confirm species origin. We also recommend examining the expression of retinal markers to confirm the ganglion-like phenotype of the RGC-5 line.

Acknowledgments

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