In Vitro Cell.Dev.Biol.—Animal (2011) 47:665–674
DOI 10.1007/s11626-011-9447-y

Misidentification of OLGA-PH-J/92, believed to be the only crustacean cell line

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Received: 13 June 2011 / Accepted: 12 August 2011 / Published online: 22 September 2011 / Editor: T. Okamoto

Abstract Continuous cell lines from aquatic invertebrate species are few and the development of crustacean cell lines remains an elusive goal. Although a crayfish cell line derived from neural ganglia of *Orconectes limosus* was reported in 2000, this cell line OLGA-PH-J/92 failed to be authenticated as such. In this report, we describe our attempts to identify the taxonomic identity of the cell line through immunological and molecular techniques. Immunohistochemical screening for the expression of a suite of invertebrate neuropeptides gave negative results, precluding an invertebrate neural origin. PCR amplification and DNA sequencing for the mitochondrial cytochrome *c* oxydase I, and 18S ribosomal RNA genes that had been widely used to confirm species identity, could not confirm the OLGA-PH-J/92 cells as originating from crayfish. Subsequent attempts to identify the cells provided moderate homology (82%) to *Gephyramoeba* sp. (AF293897) following PCR amplification of an 18S rDNA fragment after a BLAST search. A literature search provided morphological evidence of the similarity of OLGA-PH-J/92 to the *Gephyramoeba* distributed by the American Type Culture Collection as ATCC 50654, which also had been misidentified and was renamed *Acramoeba dendroida* (Smirnov et al., *Eur J Protistol* 44:35–44, 2008). The morphology of the OLGA-PH-J/92 cells which remains identical to the original report (Neumann et al., In Vivo 14:691–698, 2000) and matched corresponding micrographs that were available from the ATCC before the cell line was dropped from their catalog (ATCC CRL 1494) is very similar to *A. dendroida* and could thus belong to the Acramoebidae. These results unequivocally indicate that the OLGA-PH-J/92 cell line is not derived from the crayfish *O. limosus*, and the search for an immortal crustacean cell line continues.

Keywords Aquatic invertebrate cell lines · Crayfish · Cross contamination · DNA bar coding · Species identification

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Introduction

Aquatic invertebrate cell lines have been extremely elusive and only two had been deposited at the American Type Culture Collection (ATCC, Manassas, VA). BGE, a cell line derived from the snail *Biomphalaria glabrata* (ATCC CRL 1494; Hansen 1976), and OLGA-PH-J/92, a cell line reported to have been derived from the neural ganglia of spiny cheek crayfish, *Orconectes limosus* (ATCC CRL 2576; Neumann et al. 2000). The lack of available cell

lines does not reflect the amount of research that has been attempted, especially on crustacean cell cultures. Despite numerous reports, cell line development from commercially important species such as shrimp, using various medium formulations and culture conditions, have been unsuccessful (Toullec 1999; Shimizu et al. 2001; Rinkevich 2005), even through transfection with human cancer genes (Claydon and Owens 2008) or through hybridization attempts with immortal cell lines from fish or insect (Claydon et al. 2010). Thus it appears that this remains the holy grail of cell culturists, and although long-term cultures of shrimp and prawn have been reported (Maeda et al. 2003a, b), no permanent cell line from any marine invertebrate organism appears to have been achieved (Rinkevich 1999, 2005).

The snail cell line BGE has been quite useful for schistosomal research and many reports have been made with their usage in the scientific literature (reviewed by Coustau and Yoshino 2000), whereas OLGA-PH-J/92's usage has been sparse. Recent literature on OLGA-PH-J/92 use was reported by Pino-Figueroa et al. (2010), who evaluated the neuroprotective effects of maca, a Peruvian plant shown to provide neuroprotective effects, in vitro, and based on viability assays, they concluded that maca was neuroprotective, assuming that the OLGA-PH-J/92 cells were of neuronal origin.

The availability of OLGA-PH-J/92 was seen as a positive step towards other crustacean cell line development. However, upon culturing these cells in our labs in North America (Canada and USA) and Ghent (Belgium), we became suspicious that the cells did not behave like other vertebrate or invertebrate cell lines, which prompted molecular evaluation for the true identity of the species of origin. As misidentified cell lines are a serious problem (Nardone 2007, 2008; Rojas et al. 2008; Capes-Davis et al. 2010), careful characterization of the cell line and species authentication was thus necessary.

Cell lines can become misidentified at its initial derivation from wrongly identified tissues or from cross-contamination with other cell lines after its initiation. Cross-contaminated cell lines are a problem that has been widely reported in the mammalian literature (see recent reviews by Nardone 2007, 2008; Lacroix 2008; Rojas et al. 2008; Schweppe et al. 2008; Capes-Davis et al. 2010). Although the cross-contamination of non-mammalian cell lines with other cell types has not been widely reported (Wagg and Lee 2005; Lakra et al. 2011), the recent report by Winton et al. (2010) on the contamination of a widely used fish cell line, EPC, originally derived from *Cyprinus carpio* as Epithelioma Papullosum Cyprini, and distributed by ATCC as CRL-2872 is actually fathead minnow (*Pimephales promelas*), adds to the cell line authentication concerns for all species. EPC is the first aquatic organism-derived cell line to be listed in misidentified cell lines of ATCC in addition to several other cell lines whose origins were not what they were originally described (www.atcc.org/MisidentifiedCellLines/tabid/683/Default.aspx). ATCC has now also included OLGA-PH-J/92 as a misidentified cell line following our inquiries, and removed it from its catalog as its identity could not be determined.

Many invertebrate cell cultures have been reported contaminated with thraustochytrids (Rinkevich 1999). These are poorly characterized organisms belonging to the Labyrinthulomycota (Tsui et al. 2009). The Labyrinthulomycetes are heterotrophic protists with multiple morphologies some of which appeared similar to the morphology depicted by OLGA-PH-J/92, thus, we also probed for its phylogenetic relationship to this group of organisms and to its root phylogeny, the stramenopiles.

In this report, we describe our attempts to characterize and identify the tissue and species of origin for OLGA-PH-J/92 using various molecular and immunological approaches, and for comparison, we authenticated the BGE cell line as being derived from the snail *B. glabrata*.

Materials and Methods

Cell culture. The OLGA-PH-J/92 cell line was obtained directly from the originating scientist, Dr. T. Neumann in Seattle, WA (Ghent group), or propagated from a frozen stock obtained from the American Type Culture Collection, ATCC (CRL-2576) at Mount Desert Island Biological Laboratories (North American group). The cells were originally maintained at 27°C with Eagle's minimum essential medium (EMEM) with Earle's Balanced Salt Solution supplemented with 2 mM L-glutamine, 2.2 g/L sodium bicarbonate, 1.0 mM sodium pyruvate and 40 mM HEPES, and 10% fetal bovine serum (FBS). Initially, slow cell growth was observed, so the medium was gradually changed to Leibovitz L-15 medium (Hyclone, Logan, UT) supplemented with 10% FBS. L-15 medium was chosen after evaluation of several other culture media and optimization. The cells were maintained in 25 cm^2 polystyrene flasks (Falcon, Branchburg, NJ) at room temperature and passaged using sterile cell scrapers approximately every 4 to 5 d. Spent media (conditioned OLGA-PH-J/92 media) was collected at various time points and frozen at −20°C, or filter sterilized and used as conditioned media as growth supplement to the cells.

BGE cells (ATCC, CRL-1494) were grown in 25 cm^2 polystyrene flasks (Falcon, Branchburg, NJ) and maintained in media and conditions as described by Bayne et al. (1977).

Cell growth characteristics. OLGA-PH-J/92 was tested for their ability to survive at various temperatures and various media formulations with changing osmolalities including

water. In their original report, Neumann et al. (2000) indicated that the OLGA-PH-J/92 cells could proliferate in water for 3 wk as well as in the EMEM media, albeit with 6% FBS supplementation. The osmolality of tested solutions were measured three times using a vapor pressure osmometer (Wescor VAPRO 5520). Cells at concentrations around 5×10^5 were seeded into multiwell plates and monitored for morphological changes or for changes in cell number using hemocytometer counts or evaluating viability using Alamar Blue assay as described for fish cell lines (Dayeh et al. 2003). To verify that an increase in fluorescence in the Alamar Blue assay is correlated with increased cell density, the assay was completed on two 96-well tissue culture polystyrene plates (Falcon 353072) that contained various cell concentrations of OLGA-PH-J/92. Various multiwell plates were used for evaluating effects of temperature, osmolality as reported in the "Results" section.

Cell morphology and chitin staining. Neumann et al. (2000) in their original cell line description, noted that the cells grew in "piles" and that their "tops came off." These "floaters" resembled spore fruiting bodies or sporangia, especially in older cultures starved of serum, thus, OLGA-PH-J/92 cells were stained with Calcofluor White (Sigma, St. Louis, MO) which is a commonly used fluorescence stain for chitin (Hageage and Harrington 1984). Calcofluor white selectively binds to cellulose and chitin, and fluoresces white as it is exposed to ultraviolet light. Calcofluor white at 1 mg/ml was added to cells in culture and observed under UV in a fluorescence microscope.

Animals and tissue dissection. For immunohistochemistry, adult *O. limosus* crayfish were collected by hand from streams and ponds in Mount Desert Island, ME. Animals were kept in aerated freshwater aquaria at room temperature (approximately 18°C) until used. To isolate the supraesophageal ganglion (brain), animals were anesthetized on ice for 30–60 min after which the anterior dorsal carapace was removed and the brain dissected free in chilled physiological saline (composition in mM: 200 NaCl, 5.4 KCl, 17.2 $CaCl_2$, 5.5 $MgCl_2$, 22 Tris base, and 4.7 maleic acid; pH 7.2–7.4).

Immunohistochemistry. For immunohistochemistry, cells cultured as described earlier or whole brains were fixed for 12–24 h at 4°C in a solution of 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA; catalog #15710) in 0.1 M sodium phosphate (P) buffer (pH 7.4). After fixation, tissues were rinsed five times at approximately 1-h intervals in a solution of P containing 0.3% Triton X-100 (P-Triton) and then incubated overnight in primary antibody diluted to its final working concentration in P-Triton containing 10% normal donkey serum (NDS; Jackson ImmunoResearch, Inc., West Grove, PA; catalog #017-000-121). After incubation in primary antibody, tissues were again rinsed five times over approximately 5 h in P-Triton and then incubated overnight in secondary antibody diluted to 1:300 in P-Triton containing 10% NDS. After secondary antibody incubation, preparations were rinsed five times over approximately 5 h in P and then mounted between a cover slip and a glass microscope slide using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA; catalog #H-1000). Incubations in both primary and secondary antibody were conducted at 4°C with gentle agitation. All rinses were done at room temperature (approximately 18°C) without agitation. Secondary antibody incubation was conducted in the dark, as was all subsequent processing. Slides were stored in the dark at 4°C until examined.

Antibodies. Primary antibodies. As a marker for members of the FMRFamide family of peptides, a rabbit polyclonal antibody generated against FMRFamide (Immunostar, Hudson, WI; catalog #20091) was used at a final dilution of 1:500. As a marker for the peptide orcokinin, a rabbit polyclonal antibody generated against Asn^{13}-orcokinin (Bungart et al. 1994) was used at a final dilution of 1:1,000. As a marker for the neuropeptide SIFamide, a rabbit polyclonal antibody generated against Val^1-SIFamide was used at a final dilution of 1:1,000 (Christie et al. 2006). As a marker for tachykinin-related peptides, a rat monoclonal antibody (clone NC1/34 HL; Cuello et al. 1979; Abcam, Cambridge, MA) generated against substance P was used at a final dilution of 1:300. As a general marker for synaptic vesicles, a mouse monoclonal antibody (SYNORF1; Klagges et al. 1996) generated against *Drosophila* synapsin was used a final dilution of 1:100.

Secondary antibodies. The secondary antibodies used in our study were a goat anti-mouse IgG conjugated to FITC (Jackson ImmunoResearch; catalog #115-095-146), a donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen-Molecular Probes, Eugene, OR; catalog #A21206), and a donkey anti-rat IgG conjugated to Alexa Fluor 594 (Molecular Probes; catalog #A21209).

Imaging. Following immunohistochemical processing, preparations were viewed using a Zeiss Axiovert 200 inverted fluorescent microscope system (Carl Zeiss MicroImaging Inc., Thornwood, NY), which was equipped with Zeiss EC Plan-Neofluar ×10/0.3 dry and LD Plan-Neofluar ×20/0.4 dry objective lenses, a xenon light source, and standard manufacturer-supplied FITC and rhodamine filter sets.

Molecular characterization. DNA from OLGA-PH-J/92 and from BGE cells were extracted using FTA® cards

(Whatman; North American group) following manufacturers protocol. Logarithmically growing cells at various passages were scraped and approximately 50 µl of a 10^7-cell/ml suspension in phosphate buffered salt solution was spotted onto the FTA® cards which were then sent for assaying using PCR as described below for DNA bar coding at Guelph; DNA extraction and PCR sequencing to Savannah, GA, or to UBC for 18S rRNA genes using a universally targeted eukaryotic primer set (as described in Troedsson et al. 2008).

The Ghent group (Belgium) extracted total RNA from cells and also from a captured whole crayfish of *O. limosus* (lake Ekerse Putten near the city of Antwerp, Belgium), using TRI Reagent® (Sigma, St. Louis, MO) as per manufacturer's instructions. cDNA was synthesized according to the manufacturer's protocol using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). Recombinant Taq DNA polymerase (Invitrogen, Merelbeke, Belgium) was used as polymerase enzyme for all the PCRs. The following primers were designed using Primer3 (Rozen and Skaletsky 2000) to amplify a fragment of cytochrome oxidase subunit I (COI) based on a sequence available in GenBank (DQ882096): 5′-TCAGCCGGGAAGTTTAATTG-3′ (F) and 5′-GAAACCCCTGCTAAATGCAA-3′ (R). PCR amplification conditions were 94°C, 20 s; 52°C, 30 s; and 72°C, 30 s for 30 cycles. Then amplified DNA fragments were purified from the PCR reaction using the E.Z.N.A.® Cycle-Pure Kit (Omega Bio-Tek, Norcross, GA). The purified fragments were ligated into a pGEM®-T vector (Promega, Madison, WI) and transformed into *Escherichia coli*. Transformants were grown on LB agar medium containing 100 µg/ml carbenicillin. PCR was conducted with T7 and SP6 reverse primers to estimate insert size. Plasmids were purified and subsequently sequenced by Agowa (Berlin, Germany).

Besides TRI Reagent®, total RNA was also isolated using the RNeasy Mini Kit (Qiagen), and then cDNA was synthesized according to the manufacturer's protocol using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Same primers and PCR amplification conditions as mentioned above were used to amplify a fragment of COI, but this did not result in any amplified fragment. Lowering the annealing temperature to 46°C resulted in two clearly visible amplified fragments after gel electrophoresis.

DNA from the OLGA-PH-J/92 cell line was isolated using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek). The following universal primers were used to amplify a fragment of the 16S rRNA gene from distantly related invertebrate species: 5′-CGACTGTTTAACAAAAACAT-3′ (F) or 5′-CGACTGTTTATCAAAAACAT-3′ (F) and 5′-GGTCTGAACTCAGATCATGT-3′ (R) (Douris et al. 1998; Soin 2009). A universal set of primers for PCR amplification of nuclear histone H4 genes, 5′-TSCGI GAYAACATYCAGGGIATCAC-3′ (F) and 5′-CKYTTIA GIGCRTAIACCACRTCCAT-3′ (R) were also used (Pineau et al. 2004). Amplified DNA fragments were cloned and sequenced as mentioned above.

DNA bar coding. A 1.2-mm disk was punched out using a Harris Micro-Punch (Whatman). The micro-punch blade was cleaned between samples by punching clean filter paper. Two disks from each card were placed in the same microcentrifuge tube for rinsing. FTA® Reagent (Whatman; 200 µl) was added three times to each microcentrifuge tube and incubated for 5 min at room temperature with moderate manual mixing then the liquid was discarded. Twice, 200 µl 10.1 TE (10 mM Tris, 0.1 mM EDTA, pH 8) was added to each microcentrifuge tube and incubated for 5 min at room temperature then the liquid was discarded. The disks were immediately used in PCR reactions, or stored up to 1 wk at 4°C before use.

The COI bar code region was amplified and sequenced using the primers and thermocycler conditions found in Table 1. The PCR buffer consisted of 6.25 µl 10% trehalose, 4 µl ddH_2O, 1.25 µl 10×-buffer, 0.625 µl 50 mM $MgCl_2$, 0.1 µl 10 µM forward primer, 0.1 µl 10 µM reverse primer, 0.0625 µl 10 mM dNTPs, and 0.06 µl Taq polymerase to make a 12.5-µl total volume. The PCR product was visualized using E-gels (Invitrogen), 2% agarose precast gels stained with ethidium bromide. Samples showing a clean band were sequenced using BigDye 3.1. Sequencing PCR products were sent to the Genomics Facility at the University of Guelph where the products were cleaned using Sephadex plates. Samples were sequenced using Applied Biosystems 3730 DNA Analyzer. Bidirectional sequences were assembled and edited using Sequencher 4.5. Sequences and trace files were uploaded to BOLD project Characterizing Cell Lines from Fish and Shellfish.

DNA extraction, PCR, and sequencing for homology to labyrinthulomycetes. Genomic and mitochondrial DNA was extracted from the Whatman FTA® card following manufacturer's instructions. Small subunit rDNA was amplified with 2 µl DNA, 1.25 µl (10 µmol) of each of the two primer pairs, SR1 and SR12 or SR4 and SR7 (Honda et al. 1999), using puReTaq™ Ready-To-Go™ PCR beads (Amersham Biosciences Corp., Piscataway, NY). The amplification of SSU rRNA genes was carried out in a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA) with the following program: 5 min of denaturation at 95°C and 35 cycles of: 95°C for 30 s, 55°C for 50 s, 72°C for 50 s, with a final extension at 72°C for 7 min. The amplicons were sequenced with the same

Table 1. Immunohistochemical responses of OLGA-PH-J/92 and crayfish brain (control tissue) to five invertebrate neural markers

Marker	1° Ab/2°	Source	Dilution	OLGA (n=3)	Crayfish brain (n=3)
FMRFamides—FMRFamide	R-poly[a]	Immunostar	1:500	–	+++
Orcokinins—Asn^{13}-orcokinin	R-poly[a]	Christie lab	1:1,000	–	+++
SIFamides—Val^{1}-SIFamide	R-poly[a]	Christie lab	1:1,000	–	+++
Tachykinins—substance P	r-mono[b]	AbCam	1:300	–	+++
Synaptic vesicles—synapsin	m-mono[c]	SYNORF1	1:100	–	+++

R-rabbit, r-rat, m-mouse; poly-polyclonal, mono-monoclonal

[a] 2° Ab—donkey anti-R IgG Alexa Fluor488 conjugated (InVitrogen)

[b] 2° Ab—donkey anti-r IgG Alexa Fluor 594 conjugated (Molecular Probes)

[c] 2° Ab—goat anti-m IgG FITC conjugated (Jackson)

primers by BigDye Terminator at the Centre de Recherche du CHUL (CHUQ) in Québec, Canada.

Fragments were assembled using ABI PRISM® AutoAssembler™ v.1.4 (Applied Biosystems, Foster City, CA). The nucleotide sequence of rDNA SSU was submitted to BLAST to verify identity.

Results

Cell culture characteristics. OLGA-PH-J/92 cells, unlike BGE or other aquatic animal cell lines (e.g., fish cell lines), displayed a high rate of cell movement, which resulted in irregular cell shapes with branching dendritic-like processes (Fig. 1*A*). At high densities, achieved under optimal growth conditions (with added conditioned media), cells piled into mounds and formed phase bright clusters (Fig. 1*B*). The dendritic morphology was especially prevalent in the absence of serum supplement in the growth media (Fig. 2). Without added serum supplement, cells changed in shape from an oval or fibroblastic morphology with few projections (Fig. 3*A*) to stellate shaped cells with many projections (Fig. 2*B*), to long elongated tubular shaped cells (Fig. 2*B*) when kept in L-15 media with no serum for prolonged periods. Through phase contrast time lapse observations, the maximum rate of movement was observed at 50 μm/h (data not shown).

The cell shape changed dramatically during the amoeboid-like cell movement, which made it difficult to determine cell size. The average length and width of the cells were approximately 25 and 5 μm, respectively, although elongated cells with greater than 50 μm in length could readily be noted in serum-starved cells.

Under prolonged maintenance in the absence of serum supplement (Fig. 2*B*), cells took an elongated appearance and rounded masses of what appeared as spore bearing fruiting bodies or cysts became apparent. These rounded masses, which could also be noted in cells grown in the presence of serum, as smaller aggregates, stained positively with Calcolfluor white (Fig. 3*B*) which is a common method used to stain chitin covered cysts, or fungal spores. This stain has been shown to have selective binding affinity for chitin (Hageage and Harrington 1984), and other aquatic cell lines like BGE or fish cell lines were negative.

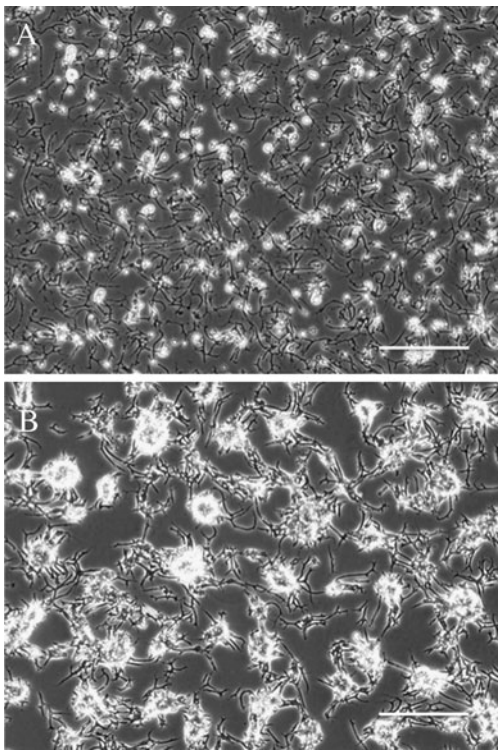

Figure 1. Morphology of OLGA-PH-J/92 cells after 4 d culture in L-15 media with 10% FBS with or without conditioned media. Cells were observed under ×10 magnification using an inverted phase contrast microscope. *Bar*=100 μm. *A*, Confluent OLGA-PH-J/92 cells in L-15 media with 10% FBS after 4 d culture. *B*, OLGA-PH-J/92 cells in 1:1 L-15 media with 10% FBS, and 9-d-old conditioned media after 4 d culture.

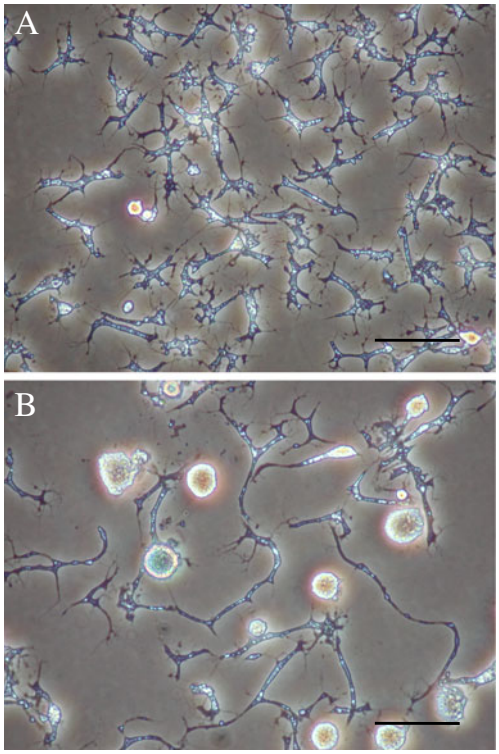

Figure 2. Morphology of OLGA-PH-J/92 cells maintained in the absence of serum. Cells were observed under ×20 magnification using an inverted phase contrast microscope. *Bar*=50 μm. *A*, 24 h after plating in L-15 media no serum. *B*, 7 d after plating in L-15 media no serum. Note *prominent phase-bright rounded masses* of what appeared like sporangia or spore-bearing fruiting bodies.

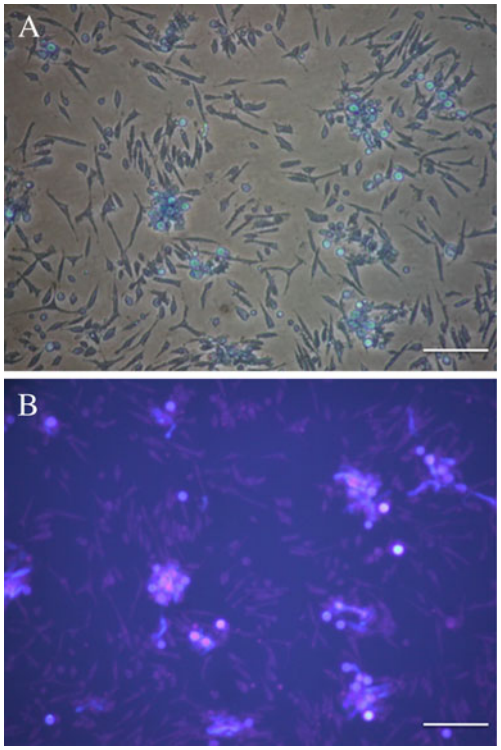

Figure 3. OLGA-PH-J/92 cells grown in L-15 with 5% FBS stained with Calcofluor White. OLGA-PH-J/92 cells were plated in media containing serum and 24 h later, were stained with Calcofluor white and observed through phase contrast (*upper panel*) or with epifluorescence (*lower panel*). Only *rounded sporangia-like structures* appeared bright which indicated presence of chitin. *Bar*=50 μm.

Alamar Blue assay as a measure of cell number. A linear relationship was found between fluorescence intensity and cell number in the Alamar Blue assay for the OL-J/92 cell line. The OL-J/92 cells were plated at 3.52×10^5, 7.05×10^5, 10.58×10^5, and 14.10×10^5 cells/well (Fig. 4). The regression line was derived as follows: $F(c)=2.8153c+3.9317$, $r^2=0.9857$, where F represents the fluorescence units after subtraction of the blanks and c represents the numbers of cells per well.

Effect of temperature on cell growth. Temperature had an effect on the growth rate of OLGA PH-J/92 cells. OL-J/92 cells grew to the highest cell numbers at 24°C and 27°C (Fig. 5). Cell growth at 24°C was significantly greater than cell growth at 22°C and 30°C; $t(78)=2.9$ and 3.5, and $p=0.004$ and $p<0.001$, respectively. Cell growth at 27°C was significantly greater than cell growth at 22°C and 30°C; $t(78)=3.2$ and 3.7, and $p=0.002$ and $p<0.001$, respectively. There was no significant difference in cell growth at 24°C and 27°C; $t(78)=0.35$, $p=0.724$. The low level of cell growth at 22°C and 30°C was not significantly different [$t(78)=0.80$, $p=0.427$].

Effect of medium type on cell growth. Five different medium types were tested: L-15, EMEM, Grace's Insect Medium,

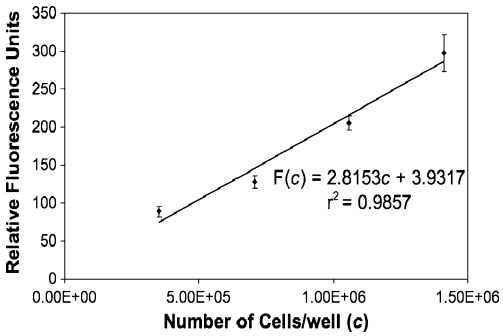

Figure 4. Alamar Blue assay as a measure of cell number. An OLGA-PH-J/92 cell suspension of 7.05×10^5 cells/mL was diluted to 75%, 50%, and 25% of the original solution in L-15 10% FBS and 200 μL of each cell solution was added to six wells of a 96-well tissue culture plate incubated at 28°C. Twenty-four hours after the cells were plated, the Alamar Blue assay was completed. A linear relationship was found between mean fluorescence units and cell number $F(c)=2.8153c+3.9317$, $r^2=0.9857$. F represents the fluorescence units after subtraction of the blanks and c represents the number of cells per well. *Error bars* represent standard error.

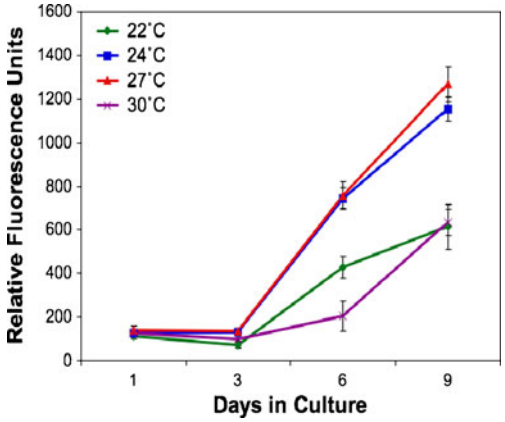

Figure 5. Effect of temperature on cell growth. 200 μL of an OLGA-PH-J/92 cell suspension of 2×10^5 cells/mL in L-15 10% FBS was added to 12 wells of 12 96-well tissue culture plates. After the cells were in culture for 1, 3, 6, and 9 d, the Alamar Blue assay was performed on one plate from each temperature (22°C, 24°C, 27°C, and 30°C). OLGA-PH-J/92 cells showed greatest cell growth when incubated at 27°C. *Error bars* represent standard deviation.

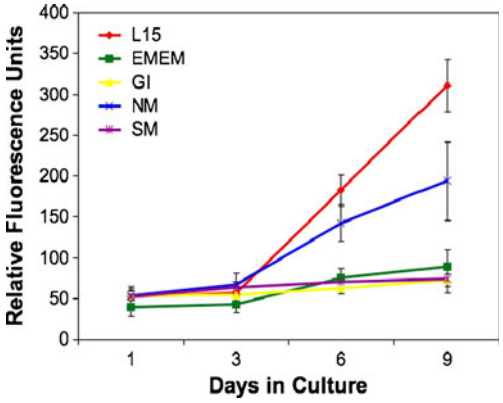

Figure 6. Effect of medium type on cell growth. The medium types tested were L-15, Eagle's Minimal Essential Medium, Grace's Insect Medium, Neurobasal Medium, and Schneider's Medium all supplemented with 10% FBS. An OLGA-PH-J/92 cell suspension of 1.5×10^5 cells/mL in L-15 10% FBS was used to add 200 μL of cells to 40 wells of 4–96-well tissue culture plates. After 24 h of incubation at 27°C, the medium was aspirated from all wells and each medium type was added to 8 wells of each plate. The Alamar Blue assay was completed on one plate at 1, 3, 6, and 9 d after treatment. The OLGA-PH-J/92 cells displayed the most growth when incubated with L-15.

Neurobasal Medium (NM), and Schneider's Medium (SM) all supplemented with 10% FBS. Three measurements using a vapor pressure osmometer were used to determine the osmolality of the various medium types. The osmolalities of the above mediums (with standard deviation) were: 321 (5), 297 (6), 375 (7), 218 (4), and 357 (6)mmol/kg.

The most OL-J/92 cell growth was observed when the cells were incubated in L-15 or NM. Cell growth was significantly greater ($p<0.001$) in L-15 compared to EMEM, SM, and GM; $t(62)=4.4$, 4.3, and 4.6, respectively. Cell growth in NM was also significantly greater ($p<0.001$) compared to EMEM, SM, and GM; $t(62)=4.3$, 4.2, and 4.7, respectively (Fig. 6). Although more cell growth was observed when cells were incubated with L-15 compared with NM, this difference was not significant; $t(62)=1.6$, $p=0.11$. There were no significant differences in the low levels of cell growth when cells were incubated with SM, GM, or EMEM.

Effect of osmolality on cell growth. The osmolality of the original L-15 medium was modified using cell culture grade distilled water. The L-15 medium containing 10% FBS was diluted to 75%, 50%, and 25% of its original osmolality. The osmolality of each solution was measured three times using a vapor pressure osmometer. The osmolality of each solution (with standard deviation) was 321 (5), 235 (2), 154 (3), and 69 (5)mmol/kg, respectively.

OLGA-PH-J/92 cells were able to grow to a similar cell number in the 100%, 75%, and 50% L-15 conditions (Fig. 7). When the 100% condition was compared with the 75% and 50% L-15 condition: $t(62)=1.55$ and $p=0.125$, and $t(62)=0.66$ and $p=0.513$, respectively. When the 75% and 50% condition were compared: $t(62)=1.03$, $p=0.306$. The 100%, 75%, and 50% L-15 conditions supported a significantly greater cell number than the 25% L-15 condition: $t(62)=3.36$, $p=0.001$; $t(62)=3.80$, $p<0.001$; and $t(62)=3.92$, $p<0.001$, respectively.

Immunohistochemistry. In none of the immunohistochemical runs conducted on cultured OLGA-PH-J/92 cells was any

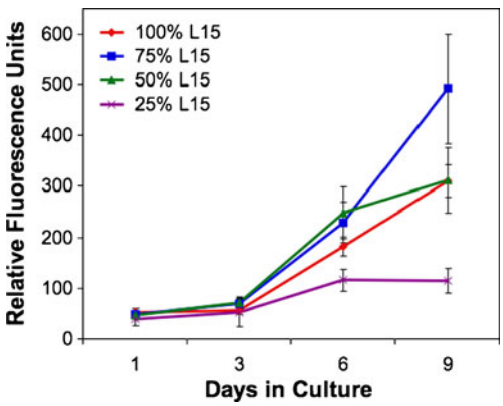

Figure 7. Effect of osmolality on cell growth. The L-15 10% FBS medium was diluted to make final solutions of 75%, 50%, and 25% using cell culture grade distilled water. OLGA-PH-J/92 cell suspension (200 μL) of 1.5×10^5 cells/mL in L-15 10% FBS was added to 32 wells of 4 96-well tissue culture plates. After 24 h of incubation at 27°C, the medium was aspirated from all wells and each medium type was added to 8 wells of each plate. The Alamar Blue assay was completed on one plate 1, 3, 6, and 9 d after treatment. OLGA-PH-J/92 cells displayed the most growth when L-15 10% FBS was diluted to 75%. *Error bars* represent standard deviation.

immunoreactivity evident (n=3 independent runs for each antibody). In contrast, extensive labeling for each of the antibodies was present in brains of *O. limosus* immunoprocessed as whole mounts (n=3 for each antibody; see Table 1).

Identification of the origin of the OLGA-PH-J/92 cell line. Use of the gene-specific primers for COI with the RNA extracts of the OLGA-PH-J/92 cells did not result in any amplified fragment at the routine PCR amplifications. Lowering the annealing temperature until 46°C resulted in two fragments of unexpected size, but after cloning and sequencing, these fragments were apparently not COI (data not shown).

The universal primers for the 16S rRNA gene also did not result in an amplified fragment. The universal primers for the histone H4 genes resulted in the amplification of at least two histone H4 fragments with the OLGA-PH-J/92 cells. However, one fragment showed high similarity with H4 from plants and a marine yeast, and the other fragment showed high similarity with different eukaryotic animals and a fungus.

In parallel, we extracted DNA from five disks from the FTA® cards. Although we were not able to generate any amplicons using primer pair SR1 and SR12, we were able to amplify and sequence 400 bp of 18S rDNA using SR4 and SR7. It grouped with an amoeba *Gephyramoeba* sp. (AF293897) with moderate homology (82%) after a BLAST search. It did not group with any representatives from the stramenopiles from GenBank.

Identification of the crayfish O. limosus. A fragment of COI of the Belgium-captured crayfish was cloned (FJ531639). This cloned sequence showed 100%, 100%, and 99% identity with the following COI sequences from *O. limosus* available in GenBank: DQ882096, AF517105, and AY701199, respectively. The cloned sequence also showed high identities with COI sequences from other *Orconectes* species: *Orconectes obscurus* (AF474355: 98%), *Orconectes jeffersoni* (AF474351: 97%). Because of this result, we can conclude that our captured crayfish was *O. limosus*.

BGE identification. Using a universally targeted eukaryotic primer set (as described in Troedsson et al. 2008), we amplified, cloned, and sequenced a fragment of the 18S rRNA gene from the samples. The clones sequenced for BGE matched with *B. glabrata* 18S sequences in the databases, as did the sequences obtained through COI bar coding (see Table 2).

Discussion

OLGA-PH-J/92 was believed to be the only permanent crustacean cell line and has been used by several researches, including most recently by Nguyen et al. (2009), Capes-Davis et al. (2010), and Pino-Figueroa et al. (2010). However their findings, that are now compromised, are not what they were purported to be. The present report details characterization attempts for identifying the true origins of OLGA-PH-J/92 in comparison with the established aquatic invertebrate cell line, BGE, an embryonic cell line from the snail *B. glabrata* (Hansen 1976). Our results conclusively indicate that OLGA-PH-J/92 is not derived from the spiny cheek crayfish *O. limosus*, as was reported by Neumann et al. (2000). Although, most of the phenotypic characteristics reported for OLGA-PH-J/92 was corroborated in this study, and the cells showed identical morphology and growth characteristics as was originally reported, there were features that made us question the origin of the cell line. In agreement with Neumann et al. (2000), optimal cell growth was noted at 27°C but the cells could proliferate at all tested temperatures, so for convenience, we maintained the cells at room temperature

Table 2. Bar code sequences for BGE cell line

BGE—100% match to *Biomphalaria glabrata* cytochrome *c* oxydase 1 gene

TTATACCTATAATAATTGGTGGTTTTGGTAATTGAATGGTTCCTTTATTAATTGGTGCTCCAGATATAAGTTTTCCTCGGATAAATAATATGTCT
TTTTGATTACTTCCGCCTTCATTTATTTTATTGTTAGTATCTAGTATAGTTGAGGGTGGAGTAGGTACAGGTTGAACTGTATATCCTCCCTT
AAGTGGTCCTATTGCTCATGGTGGTGCTTCTGTGGATTTAGCTATTTTTTCTTTACATTTAGCAGGAATAAGTTCAATTTTAGGTGCTATT
AATTTTATTACTACAATTTTTAATATGCGTGCTCCCGGTATTACAATGGAACGTTTATCATTATTTGTATGATCTGTATTAGTTACAGCATTTT
TACTTTTATTATCTTTACCTGTTTTAGCAGGGGCTATTACAATGTTATTAACAGATCGAAATTTTAATACTAGTTTTTTTGATCCTGCAGGA
GGAGGTGATCCTATTTTATACCAACATTTA

BGE—518 bp sequence for 18S rRNA gene gave 99% match to *Biomphalaria glabrata*

GACGGGCGGTGTGTGCAAAGGGCAGGGACGTAATCAACGCGAGCCTATGACTCGCGCTTACTGGGAATTCCTCGTTCATGGGGAAC
AGTTACAAGCCCCAATCCCTAGCACGAAGGAGATTCAACGGGTTTCCCAACCCTTTCGGGCCAGGGAGGCATCCACGCTGATTCCT
TCAGTGTAGCGCGCGTGCGGCCCCGGACATCTAAGGGCATCACAGACCTGTTATTGCTCAATTTCGTGTGGCTAAACGCCACTCGTC
CCTCTAAGAAGTTGCGCCGGCGCATCGAGGAACCGGCGAACTATTTAATAGGCTAGAGTCTCGTTCGTTATCGGAATTAACCAGACA
AATCGCTCCACCAACTAAGAACGGCCATGCACCACCACCCACCGAATCAAGAAAGAGCTATCAATCTGTCAATCCTTACAGTGTCC
GGACCGGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAAGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTT

without the need of incubators, with L-15 medium as the preferred growth medium since it supported best growth over the originally used EMEM media recommended by ATCC. The cells showed slow motility and were capable of growing under most conditions including absence of serum, although morphological changes were noted including what appeared like spore-bearing fruiting bodies or cyst-like structures under starvation conditions which made us suspect that the cells may not be of crustacean origin. Curiously, Neumann et al. (2000) in 2000 already had noted then that these cells could round up and also proliferate in water supplemented with 6% FBS which is also in agreement with our findings. OLGA-PH-J/92 could proliferate at lower osmolalities in diluted L-15 media and could also be maintained in water for short periods. BGE, on the other hand, do well at low osmolalities but not below 120 mOsm/kg (Bayne et al. 1977). Immunohistochemistry, with a suite of crustacean neuropeptides that were shown to react with *O. limosus*, did not react at all with OLGA-PH-J/92, precluding any neural origin, and our molecular findings could not match the cell line with the putative species of origin. The closest match we could come up with was for *Gephyromoeba* sp. (ATCC 50654) which upon further research, revealed additional misidentification and the Genbank sequence (AF293897) has now been attributed to a new amoeba species named *Acramoeba dendroida* by Smirnov et al. (2008). The photographic images presented by Smirnov et al. (2008) in their Figs. 1, 2, and 3, closely match the morphologies observed with OLGA-PH-J/92, and although not 100% certain, but based on the observed phenotypic characteristics including amoeboid movement of the cells with possible encystation stages that stained positive for chitin (a feature observed with some amoeba life stages), there is high probability that OLGA-PH-J/92 may have been derived from contaminating fresh water amoeba that could have been present in the neural tissue of the originating crayfish. Endosymbionts have been noted to be common contaminants in aquatic invertebrate cell cultures (Villena 2003), among which thraustochytrids had been noted to be common contaminants (Rinkevich 1999; Villena 2003), but molecular testing in this study ruled out any connection with the stramenopiles, the phylogenetic root group to which these contaminants were classified. Further testing is needed to ascertain the true origin of OLGA-PH-J/92, but it is clear that the cells are not derived from *O. limosus*.

OLGA-PH-J/92 cells had been distributed to the scientific community as CRL2576 by ATCC since the early 2000s until early 2009 when they were listed in their misidentified cell line catalog and discontinued the distribution of the cell line (http://www.atcc.org/CulturesandProducts/CellBiology/MisidentifiedCellLines/tabid/683/Default.aspx). OLGA-PH-J/92 along with EPC (Winton et al. 2010) appear to be the only two non-mammalian cell lines that had been misidentified among the many cell lines at the ATCC. Thus, cell line authentication should be mandatory for all cell line work as has been instituted in this journal, such that valuable research findings are not compromised.

Acknowledgments LEJL thanks Wilfrid Laurier University for the 2011 Research Professor Award that allowed finalization of this manuscript. This work was partially funded through a Discovery grant from the Natural Sciences and Engineering Research Council of Canada to LEJL. Support from Mount Desert Island Biological Laboratory in the form of New Investigator grants to LEJL for 3 yr of continuous collaboration with AEC is greatly appreciated. Support by the Special Research Fund of Ghent University, and the Flemish Agency for Innovation by Science and Technology (IWT-Vlaanderen), in Belgium, is also acknowledged.

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