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Establishment of long term cultures of neural stem cells from adult sea bass, *Dicentrarchus labrax*

Arianna Servili [a,b], Mary Rose Bufalino [a], Ryuhei Nishikawa [c], Ivan Sanchez de Melo [d], Jose A. Muñoz-Cueto [b], Lucy E.J. Lee [a,c,*]

[a] Department of Biology, Wilfrid Laurier University, Waterloo, ON, Canada
[b] Department of Biology, Faculty of Marine and Environmental Sciences, University of Cadiz, Puerto Real, Spain
[c] Mount Desert Island Biological Laboratory, Salisbury Cove, ME, USA
[d] Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Cadiz, Puerto Real, Spain

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ABSTRACT

Long term cell cultures could be obtained from brains of adult sea bass (*Dicentrarchus labrax*) up to 5 days post mortem. On three different occasions, sea bass brain tissues were dissected, dispersed and cultured in Leibovitz's L-15 media supplemented with 10% fetal bovine serum. The resulting cellular preparations could be passaged within 2 or 3 weeks of growth. The neural cells derived from the first trial (SBB-W1) have now been passaged over 24 times within two years. These cells have been cryopreserved and thawed successfully. SBB-W1 cells are slow growing with doubling times requiring at least 7 days at 22 °C. These long term cell cultures could be grown in suspension as neurospheres that were immunopositive for nestin, a marker for neural stem cells, or grown as adherent monolayers displaying both glial and neural morphologies. Immunostaining with anti-glial fibrillary acidic protein (a glial marker) and anti-neurofilament (a neuronal marker), yielded positive staining in most cells, suggesting their possible identity as neural stem cells. Furthermore, Sox 2, a marker for neural stem cells, could be detected from these cell extracts as well as proliferating cell nuclear antigen, a marker for proliferating cells. SBB-W1 could be transfected using pEGFP-N1 indicating their viability and suitability as convenient models for neurophysiological or neurotoxicological studies.

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1. Introduction

The capacity for self-renewal and the ability to reconstitute a tissue are the classical definitions of a stem cell, and it appears that stem cells are present in most fish tissues, including the brain (Hinsch and Zupanc, 2006; Chapouton et al., 2007; Kaslin et al., 2008). This may explain why fish show "indeterminate growth", or the ability to grow unrestricted given optimal conditions as opposed to the restrictive growth shown by mammals. As fish grow, new cells are generated and this is paralleled throughout all organs including neural tissues. In contrast to mammals, adult fish have the capacity to generate significant amounts of nervous tissue following injury (Nona, 1995; Caminos et al., 1999; Zupanc, 2006; Chapouton et al., 2007; Udvadia, 2008). This replacement is associated with a high degree of functional recovery (Larner et al., 1995), which is attributed to several factors in fish, including the persistence of proliferative zones containing neural stem cells (Zupanc and Clint, 2003; Grandel et al., 2006; Hinsch and Zupanc, 2006). Recently, using a combination of bromodeoxyuridine (BrdU) treatment and immunohistochemical techniques, Pellegrini et al. (2007) demonstrated that radial glial cells have mitotic activity and represent progenitors or precursor cells in adult zebrafish brain. These radial glial cells can divide to generate newborn cells in many brain regions and such newborn cells can further divide, migrate and differentiate into neurons as shown by BrdU, proliferating cell nuclear antigen, Hu, and acetylated tubulin immunostaining (Pellegrini et al., 2007). This is in contrast to that observed in lampreys and amphibians, in which most of the new neurons that are added to the central nervous system appear to be derived from late differentiation of immature neurons (Meeker and Farel, 1997; Farel, 2001; Vidal Pizarro et al., 2004). The persistence of proliferating cells in the central nervous system of fishes may account for the ready establishment of brain derived cell lines from fish including TB2, a neural progenitor cell line from tilapia brain (Wen et al., 2008a), GB cell line, derived from grouper brain (Lai et al., 2003), BB cell line, established from the brain tissue of barramundi (Chi et al., 2005), as well as many short and long term neural cell cultures (De Boni et al., 1976; Anderson and

* Corresponding author. Department of Biology, Wilfrid Laurier University, 75 University Avenue West, Waterloo, ON, Canada N2L 3C5. Tel.: +1 519 884 0710x2252; fax: +1 519 746 0677.
E-mail address: llee@wlu.ca (L.E.J. Lee).

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Waxman, 1985; Anderson, 1993; Hinsch and Zupanc, 2006). It is surprising, however, that so few adult fish brain cell lines exist, as neurogenesis continues at a high rate throughout the adult life in many different areas of the fish CNS (Hinsch and Zupanc, 2006).

Understanding of physiological processes in fish species, especially for those with increasing commercial value in aquaculture such as the sea bass (*D. labrax*) has prompted both applied and basic studies involving fish neural tissues (Cerdá-Reverter et al., 2000; González-Martínez et al., 2002, 2004; Bayarri et al., 2004; Moles et al., 2007), and the need for available cultured neural cells of fish origin. The emergence of neural diseases caused by viruses within the intensive aquaculture practices (Chi et al., 2005; Parameswaran et al., 2006b; Cutrín et al., 2007) also highlights the need for cell cultures from brain tissues of fish as hosts for tissue specific viruses. In this study, we report on the *in vitro* culture of sea bass brain cells derived from various brain regions including telencephalic, diencephalic, mesencephalic, metencephalic and myelencephalic areas as well as from the pituitary and pineal glands. Proliferative cells were obtained from each zone but most consistently from the mesencephalon and metencephalon, and a cell line, SBB-W1, was developed from adult sea bass cerebellar–tegmental areas. These cells were large and irregular in shape with highly euchromatic nuclei and numerous cytoplasmic processes. Immunocytochemical characterization points to a population of neural stem cell progenitors. The cells' transfection capability demonstrate potential for their use to investigate differential responses to toxicants, nutrients and growth factors, and could serve as models for neural regeneration studies as well as for elucidating mechanisms of neurotoxicity, neurophysiology and neuroendocrinology.

2. Materials and methods

2.1. Animals

Adult European sea bass, *D. labrax* (Order Perciformes) were cultured in the Marine Fish Farming laboratory at the University of Cádiz, Puerto Real, Spain. Fish were kept in indoor facilities under natural light environmental conditions, constant temperature and salinity (19±1 °C and 39 ppt, respectively) and were fed at libitum once per day. The fish used for cell cultures were from two different batches of 1 to 1.5 year olds with a mass of 100–250 g. All animals were treated in agreement with the European Union regulation concerning the protection of experimental animals.

2.2. Tissue collection

For the first cell culture attempts, 6 adult sea bass specimens were sacrificed with an overdose of phenoxyethanol and then decapitated. Brains were excised under aseptic conditions and dissected into olfactory bulbs, telencephalon-rostral preoptic area, diencephalon, optic tectum–rostral tegmentum, cerebellum–caudal tegmentum, and medulla oblongata–spinal cord following the cytoarchitectonic sea bass brain atlas elaborated by Cerdá-Reverter et al. (2001a,b, 2008). Pituitary and pineal glands were also collected. All tissues were washed with Hanks' Balanced Salt Solution (HBSS) supplemented with double concentration of antibiotic and antimycotic (2× AB/AM) (containing penicillin, streptomycin and amphotericin, from Invitrogen), and placed in sterile test tubes containing HBSS 1× AB/AM. Immediately after, all tubes were shipped by express courier, on ice packets, to Waterloo, Ontario, Canada. Two months later, the same protocol was repeated processing 6 more sea bass specimens belonging to the same fish stock. Lastly, one year later, another cell culture was initiated with 2 more adult brains from the following generation of animals at Mount Desert Island Biological Laboratory (MDIBL, Salisbury Cove, Maine, USA). For the second and third brain cell culture preparations, fragments of neural tissues were cultured from wider brain regions (anterior, mid and posterior brain) and plated separately.

2.3. Initiation of primary cell cultures and routine maintenance

Tissues took 3 to 5 days post mortem to arrive at the processing labs (Waterloo or MDIBL), and although viability was compromised, live cells could be obtained from all brain fragments regardless of transit time. Each brain tissue sample was mechanically dispersed by pipetting up and down using sterile glass pipettes. Some pieces were enzymatically disrupted using TrypLE, a recombinant form of trypsin, trademarked by Invitrogen. Cells were exposed to the enzyme solution for 5 min and then regular Leibovitz's L-15 (L-15) growth media supplemented with 10% fetal bovine serum (FBS) and with 2× antibiotic and antimycotic was added to stop the enzymatic activity before centrifuging the cell solution at 500 g for 5 min. The pelleted cells were resuspended and plated in the L-15 medium in several 12.5 cm^2 flasks or multi-wells plates. Medium was changed every 3–4 days and when cells became confluent (~10^6 cells per flask), they were passaged and split 1:2. After the first passage, media were replaced weekly and at the third or fourth passage, medium used was L-15 10% FBS 1× AB/AM. All flasks and plates were maintained at 22 °C. The cerebellar–tegmental cultures (SBB-W1) survived past 5 passages, and these were used for growth studies and neural characterization.

2.4. Neurosphere formation

The hanging drop method was used to develop neurospheres from sea bass brain cell cultures. This method has been widely used in embryoid body formation (neurospheres created from embryonic stem cells) and was adapted from Del Duca et al., 2004. Fifteen drops (20 µL per drop) of a cell suspension (~3×10^5 cells/mL) in L-15 10% FBS were placed on the inside of the top cover of a sterile non-tissue culture Petri dish approximately 1 to 2 cm away from each other (Del Duca et al., 2004). The bottom of the Petri dish was filled with cell medium to prevent the drops from drying out and to make it easy to recover neurospheres that could have dropped during plate transfer. The top cover of the Petri plate was then quickly inverted over the bottom and the plate was sealed with Parafilm strip and left undisturbed for 72 h, after which neurospheres were collected for immunostaining.

2.5. Growth studies: growth kinetics, serum concentration and temperature effects

For growth studies, cells were plated at a concentration of 8×10^4 cells/mL in 12–60 mm tissue culture plates in L-15 media supplemented with 10% FBS and maintained at room temperature (22–24 °C). After 24 h, adherent cells were detached with 1 mL of TrypLE for 5 min at room temperature. The enzyme was blocked with 4 mL of L-15 10% FBS media and then cells were counted by hemocytometer (day 0). Every 3 days, 3 plates were processed for cell counting by hemocytometer as described above (days 3, 6 and 9). The same experiment was performed twice after 5 and 10 passages.

The optimal FBS concentration for the growth and proliferation of sea bass brain cells was tested by plating cell suspensions of 6×10^5 cells in five 12 well plates with the regular growth medium (L-15 10% FBS). After 24 h (day 0) cells in one 12 well plate were counted and the medium was changed to the remaining plates at 0, 5, 10 and 15% FBS–L-15, in each of 3 wells per plate. Every 3 days (days 3, 6, 9, 12), cells in one 12 well plate with the various FBS concentrations were counted. For cell counting, cells were fixed in 1:1 ethanol–acetone solution and stained with May-Grunwald-Giemsa. Cells in 10 random fields of view per well were counted under a microscope at 10×.

To investigate the influence of temperature on cell proliferation, the sea bass brain cells (4×10^5 cells) were cultured in five 12 well plates at 5 different temperatures (10, 16, 22, 28 and 37 °C) for 7 days with daily counts by field of view.

2.6. Cryopreservation and storing in liquid nitrogen

Confluent cultures of SBB-W1 sea bass brain cells at passages 11 and 14 that were grown in 75 cm^2 flasks were dissociated and pelleted as indicated above. The cell pellet was resuspended in 1 mL of cryopreservation medium (L-15 10% FBS with 10% dimethyl sulfoxide—DMSO) and quickly frozen in liquid nitrogen. After various time intervals ranging in months to over a year, cells were thawed by resuspending 1 mL of cells with 9 mL of regular growth medium and centrifuging at 500 g for 5 min. The supernatant was discarded and the pellet was resuspended in fresh growth medium and plated in new flasks. Viability was tested using trypan blue dye exclusion.

2.7. Immunocytochemical characterization

For the immunocytochemical characterization of sea bass brain cells in culture, the brain cells were grown on sterile poly-L-Lysine-coated coverslips for 72 h, fixed overnight with 4% paraformaldehyde at 4 °C, washed 5× with PBS containing 0.3% Triton X-100. Antibodies against nestin, neurofilament (NF), glial fibrillary acidic protein (GFAP) obtained in rabbit (Sigma) were used as neural stem cell, neuronal and glial markers respectively. All antisera were diluted (1:500) in 10% normal serum in 0.3% Triton X-100 PBS and incubated overnight at 4 °C. After 5 washes in 0.3% Triton X-100 PBS, cells were incubated with the appropriate secondary antibody (goat anti-rabbit IgG coupled to FITC) at 1:300 dilution overnight at 4 °C. The cells were then washed 5× in PBS and mounted with Gel Mount aqueous Mounting medium (Sigma) and observed under a fluorescence microscope. Before mounting, some coverslips were stained with Hoechst solution at a concentration of 100 ng/mL for 4 min to reveal the cell nucleus, and then they were washed twice with PBS. Furthermore, anti-nestin, anti-NF and anti-GFAP were used to test for immunostaining in sea bass brain cells grown in suspension as neurospheres. After 72 h of growth, neurospheres were plated on poly-L-Lysine-coated coverslips for 24 h and then immunocytochemistry was performed following the same protocol described above for the cells grown in monolayers. Controls were performed by replacing the primary or secondary antibodies appropriately with the corresponding normal serum. The antibodies used in this study have previously been well characterised and used successfully in fish (Forlano et al., 2001; Wen et al., 2008a; Mahler and Driever, 2007).

2.8. Molecular screening for species identity and tissue origin

Approximately, 5×10^5 cells were used to extract total RNA with Trizol (Invitrogen) following the manufacturer's protocol. The RNA samples were treated with amplification Grade DNase I (Invitrogen) to remove any traces of genomic DNA contamination. 1 µg of total RNA was used for cDNA synthesis in a volume of 25 µL using the Clone AMV first strand DNA synthesis kit (Invitrogen). To test that the long term cell cultures actually derived from sea bass, specific primers for *D. labrax* ribosomal protein L17 encoding sequence (GenBank accession no. AF139590) were used (forward primer sequence: 5′TGATACGGCAGCGGAAGTC3′; reverse primer sequence: 5′GACTCCTGCGTTGTCTTCAAA3′). RT-PCR was performed using the following steps: denaturation at 94 °C for 2 min, followed by 38 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and elongation steps at 72 °C for 10 min. The size of the obtained DNA amplicon was 87 bp. The RT-PCR product was analyzed by electrophoresis on a 2% agarose gel and then sequenced (MDIBL Marine DNA Sequencing Center) to verify that the amplicon had the expected sequence. At the same time, parallel RT-PCR reactions were performed on cDNA synthesized from European sea bass brain tissue as positive control, using the same primers, buffers and thermocycler program.

To verify that the long term cell cultures came from brain tissues, specific primers for *D. labrax* HMG box protein Sox 2 encoding sequence (GenBank accession no. AY246992) were designed (forward primer: 5′GAAGATGGCGCAAGAGAATC3′; reverse primer: 5′AGCCTCTTGGCTTCGTCGAT3′). The size of the DNA amplicon was 123 bp. The RT-PCR was performed using cDNA obtained from both the long term cell cultures and fresh sea bass brain tissues as described above but using 57 °C as the temperature for the annealing step. Again, RT-PCR products were analyzed by electrophoresis on a 2% agarose gel and then sequenced to verify the identity of the amplified sequences.

2.9. Evaluation of proliferating activity in long term cell cultures

To test the proliferating activity of sea bass brain cells in culture, RT-PCR was carried out on cDNA synthesized from RNA of cultured cells obtained as indicated above by using specific primers for *D. labrax* PCNA sequence (proliferating cell nuclear antigen). Both forward (5′ATGATCTCCTGCGCCAAGG3′) and reverse (5′CACAGTCTTG-GACAGCGGC3′) primers were designed based on a *D. labrax* PCNA partial sequence cloned by Isorna and Muñoz-Cueto (unpublished data). The PCR protocol was similar to that described above but annealing temperature was 60 °C. The size of the DNA amplicon was 201 bp and the identity of the amplified sequence was confirmed by electrophoresis on a 2% agarose gel and DNA sequencing. The PCNA expression on sea bass long term brain cell cultures was compared to that present in sea bass fresh brain tissue by performing parallel RT-PCR reactions on similar amounts of cDNA templates. The expression of L17 from cell cultures and fresh brain tissue was also determined by RT-PCR on the same cDNA samples using the L17 primers and conditions described above, and used as reference of cDNA quality and concentration. RT-PCR products were analyzed by electrophoresis on 2% agarose gel and sequenced as indicated above.

2.10. Cell transfection with GFP reporter gene

The ability of sea bass brain cells in culture to be transfected was evaluated using pEGFP-N1 plasmid (Biosciences Clontech) which contains a cytomegalovirus (CMV) promoter, a SV40 polyadenylation signal and a gene for the resistance to neomycin. Sea bass cells at 15th passage were seeded at a density of 10^4 cells per coverslip. Monolayers with 60% confluence were transfected using 2 different transfection reagents (according to respective manufacturer's protocols): JetPEI (from Polyplus transfection) and Fugene 6 (from Roche). Briefly, for transfection with JetPEI reagent, 1.8 µg of DNA were diluted into 100 µL of serum-free L-15 medium and 6 µL of JetPEI reagent were added into 100 µL of serum-free L-15 medium per coverslip. Then, the two solutions were mixed together and incubated at room temperature for 30 min.

In the case of transfection with Fugene 6, 6 µL of the transfection reagent was diluted into 100 µL of serum-free L-15 medium and incubated 5 min at room temperature; after that, 1.8 µg of DNA was added to this solution and incubated again at room temperature for 45 min.

Subsequently, each transfecting solution was directly added to different coverslips containing the sea bass brain cell cultures. The reaction was stopped 72 h later by fixation in 4% paraformaldehyde-PBS for 20 min. After several rinses in PBS or 0.1× Triton-PBS and incubation in DAPI solution (1 µg/mL) for 2 min, coverslips were mounted on slides with PBS-glycerol as mounting media. Finally, green (EGFP) and blue (DAPI) fluorescence signals were visualized and photographed under a BH2 Olympus fluorescence microscope using the appropriate filters.

2.11. Statistical analysis

All values shown in the figures are expressed as means ± standard errors. Statistical analyses were performed with StatGraphics Plus 5.1

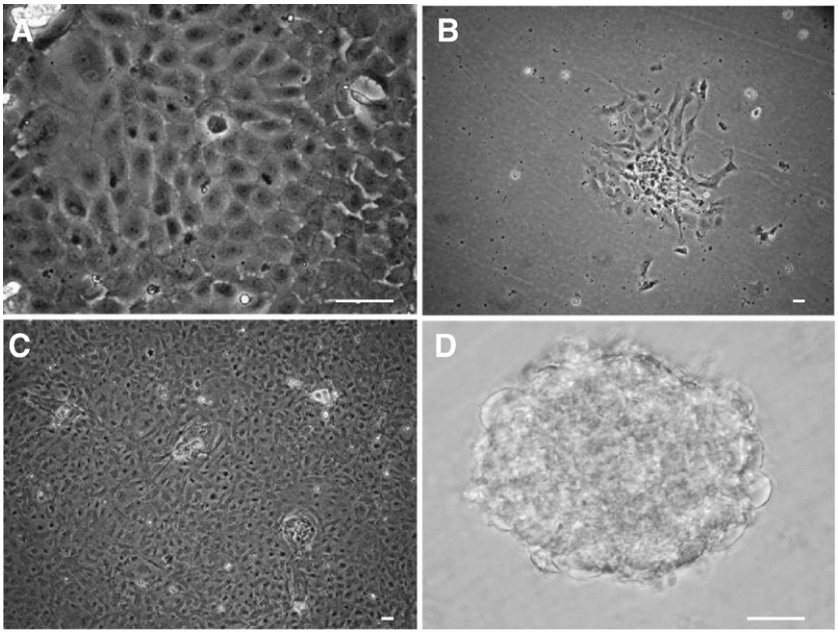

Fig. 1. Phase-contrast micrographs of cells derived from adult sea bass neural tissues. (A) Pituitary cells; (B) Pineal cells; (C) Mid-posterior brain cells; (D) Brain cells grown as neurospheroid body. Scale bars = 100 μm.

software. Differences among means were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Differences were considered significant at $P<0.05$.

3. Results

The aim of this study was to generate long term cell cultures from the brain of adult sea bass *D. labrax*, establishing optimal conditions for their culture and maintenance and describing their main characteristics.

3.1. Early development of cell cultures from sea bass brain

Three different and independent cell culture trials were initiated following similar procedures. In the first trial, cultures were prepared from 6 different sea bass dissected into olfactory bulbs,

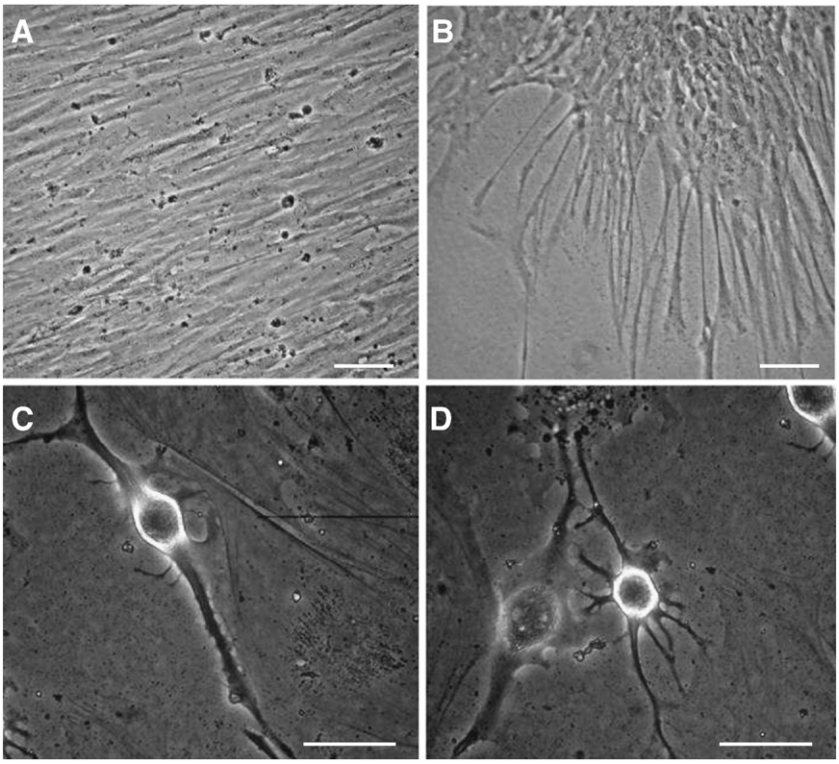

Fig. 2. Morphologies of sea bass brain cells in culture as observed by phase-contrast microscopy. (A) Fibroblast-like cells at confluency. (B) Radial cells extending long processes out from a cell clump. (C) bipolar cell. (D) multipolar cell. Scale bars = 100 μm.

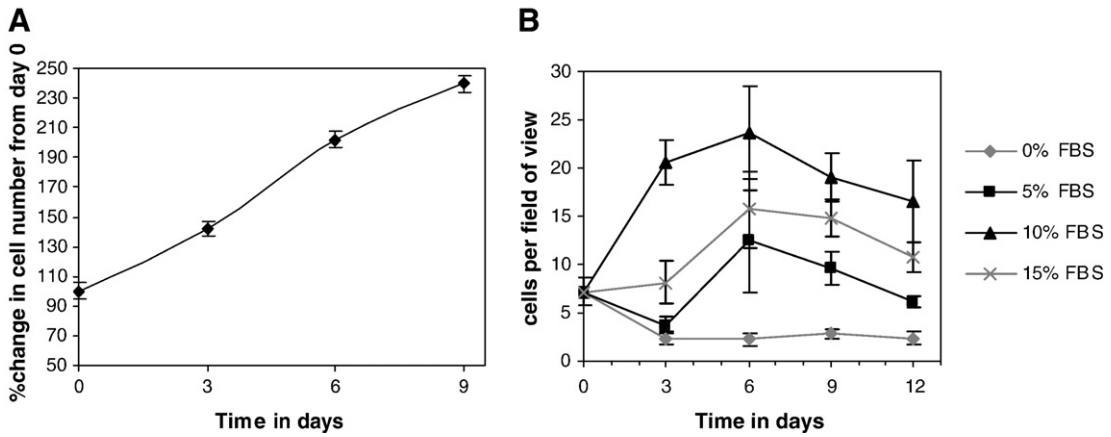

Fig. 3. (A) Growth kinetics of sea bass brain cells cultured in 10% FBS L15 medium at room temperature (22 °C). Cell number is represented as percentage of cells counted compared to the day of plating or day 0 (100%). (B) Effect of different concentrations of FBS on sea bass brain cell growth and proliferation in L15 at room temperature. Values shown represent the mean cell count per field of view at 100× ± standard error. 3 different wells were analyzed per day and concentration and 10 different fields of view were analyzed per well.

telencephalon-rostral preoptic area, diencephalon, optic tectum–rostral tegmentum, cerebellum–caudal tegmentum, and medulla oblongata–spinal cord. Proliferative cells were obtained from each brain region cultured as well as from the pituitary gland (Fig. 1A) and pineal complex (Fig. 1B). However, only the cells of the mid-posterior brain, containing the cerebellum and caudal tegmentum (Fig. 1C), grew continuously. This culture was designated SBB-W1 for sea bass brain Waterloo trial 1.

In the second and third trials, cells derived from the anterior, mid and posterior brain regions were plated without precise differentiation for specific brain regions. Cells from the mid (mesencephalon) and posterior (metencephalon–rhombencephalon) brain gave rise to long term cultures that lasted over a year but were lost due to incubation malfunction.

Proliferative cells obtained in the three separate trials for the corresponding regions were similar in size and morphologies. In general, 48 h after plating, cells were observed adhering to the culture flasks as single cells or in clumps. These cells were able to form neurospheroidal bodies (Fig. 1D). Most of the cells placed in monolayers appeared large and irregular in shape with highly euchromatic nuclei and numerous cytoplasmic processes (Fig. 2). Cell clumps with numerous long fibers extending radially were regularly observed in the primary cultures (Fig. 2B). In addition, bipolar (Fig. 2C) and multipolar cells (Fig. 2D) were observed in primary and subsequent cultures showing neuron-like and also fibroblast-like (Fig. 2A) morphologies. In 25 cm^2 flasks, cells reached confluence in 2–3 weeks of growth at initial seeding concentrations of 10^5 cells/mL of cells in solution. To date, SBB-W1 cells have been grown for over 24 passages in 2 years. SBB-W1 cells were cryopreserved in L-15 with 10% FBS and 10% DMSO and stored in liquid nitrogen. Cells thawed, resuspended in fresh growth medium and plated in new flasks were viable and showed over 80% of survival rate.

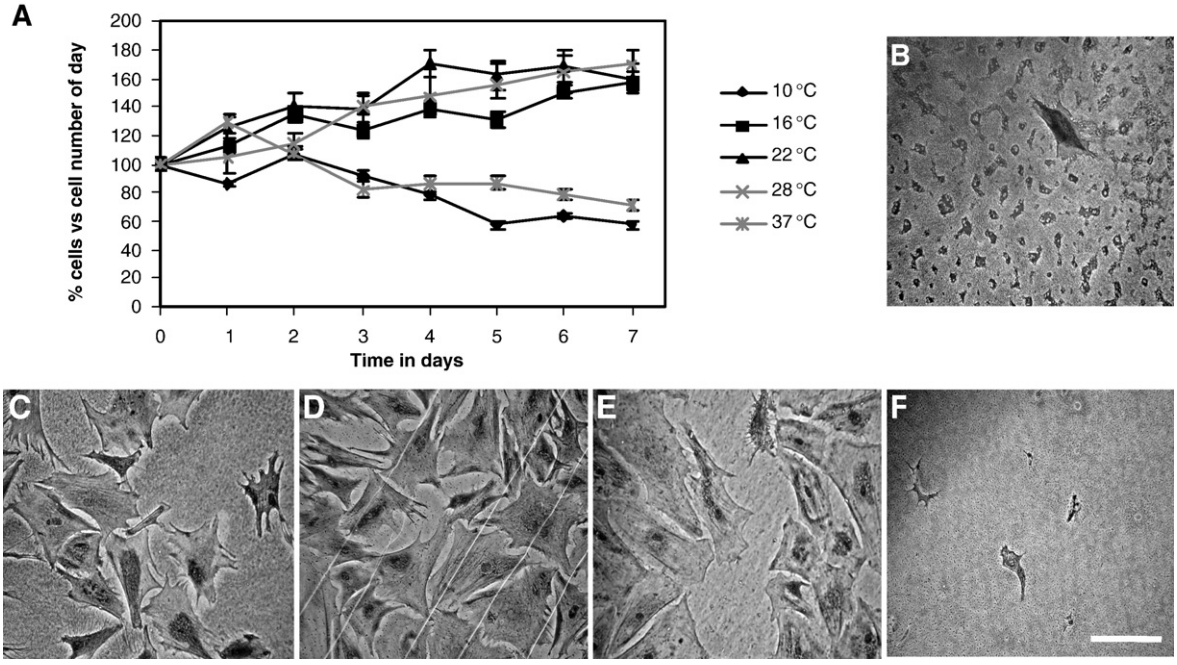

Fig. 4. (A) Effect of temperature on the growth of sea bass brain cells cultured for 7 days in L15 medium supplemented with 10% FBS. Cell number is represented as percentage of cells counted compared to the day of plating or day 0 (100%). Values shown represent the mean cell count per field of view at 100× ± standard error. 3 different wells were analyzed per day and temperature and 10 different fields of view were analyzed per well. (B–F) Photomicrographs of sea bass brain cells after 7 days culture in L15 10% FBS medium at 10 °C (B), 16 °C (C), 22 °C (D), 28 °C (E), 37 °C (F). Scale bar = 100 µm.

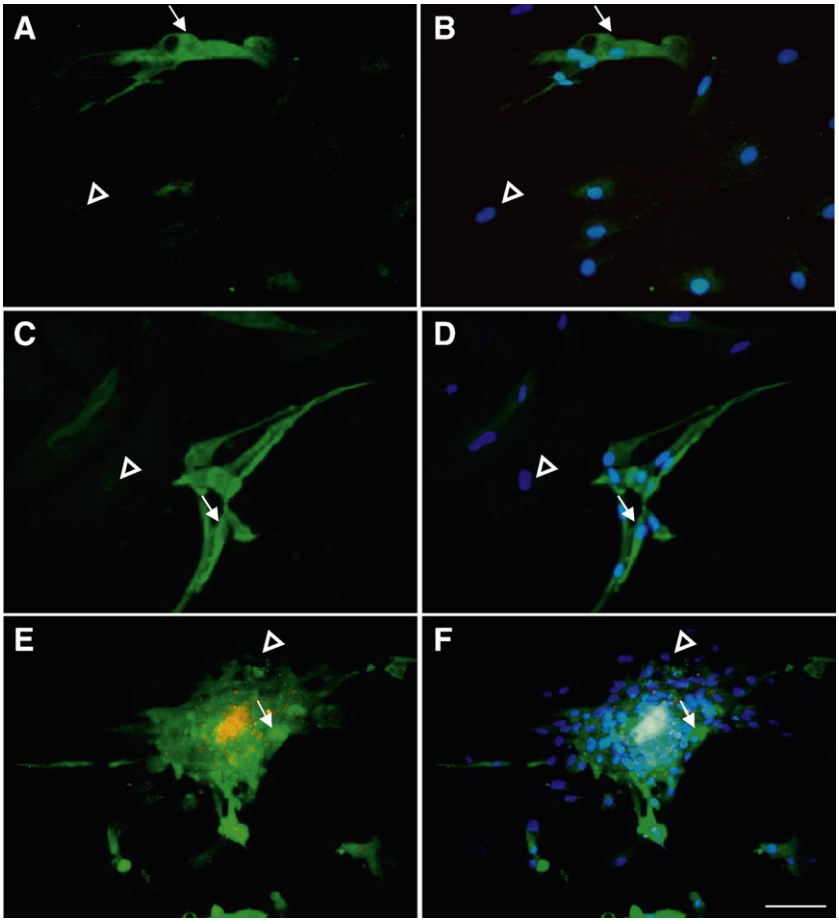

Fig. 5. Immunofluorescence micrographs of sea bass brain cells in long term culture. A, C and E show cells labelled with anti-rabbit monoclonal antibodies for NF (A), GFAP (C) and nestin (E) coupled with FITC. B, D and F present the same micrographs merged with the corresponding images showing the nucleus stained with Hoechst counterstaining. Note that each couple of pictures reveals some immunopositive cells labelled in green (arrows) as well as some cells with no specific labeling (arrowheads). A–D pictures show cells cultured in monolayers, whereas E–F show cells from attached neurospheres. Scale bar = 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Growth studies

Like most cells in culture, growth of sea bass brain cells was dependent on many factors including nutrient availability, substrate accessibility, temperature, initial seeding density, etc. Sea bass brain cells plated at various densities were grown in L-15 10% FBS medium at room temperature for various time points. A representative growth curve for SBB-W1 initially plated at lower densities is shown in Fig. 3A. It presents a continuous linear increase in cell number for 9 days ($y = 15.968x + 99.018$; $R^2 = 0.9924$) and doubling times of 6 to 7 days. The cell number increase was already significant at the third day of culture ($P < 0.05$). This experiment was performed three times, after 5, 10 and 15 passages, and the findings were consistent.

Fetal bovine serum (FBS) supplemented to L-15 medium at various concentrations was tested for growth enhancement of sea bass brain cells (Fig. 3B). 10% FBS in L-15 appeared to be optimal for the proliferation of these cells. At 3 days of growth, cells show a significant increase in cell number only if they are supplemented with 10% FBS in the medium ($P < 0.05$). This enhancement is maintained for 9 days of culture but at day 12 it starts to decrease. When cells are initiated in medium supplemented with 0%, 5% and 15% FBS, cell numbers did not increase significantly ($P > 0.05$) (Fig. 3B). Growth curves obtained at different serum concentration show a decrease in proliferation starting from day 6.

The best temperature for growth of sea bass brain cells appeared to be between 16–28 °C (see Fig. 4A), thus, routine growth of cells was done at 22 °C (room temperature). Cell proliferation at those temperatures was continuous during the 7 days of observation and cellular morphologies were similar (Fig. 4C, D, E). On the other hand, the extreme temperatures, 10° and 37 °C, inhibited cell growth and caused cell shrivelling and cell death (Fig. 4B, F).

3.3. Cell identification

Characterization of sea bass brain cells was performed by immunocytochemistry with various neural antibodies. Immunostaining reveals a co-presence of GFAP and NF positive cells in all the cultures from the 3 trials carried out (Fig. 5A–D). In fact, the same immunocytochemical procedure was performed several times using cells at different passage numbers and no differences in the findings were noted. An important number of cells incubated with anti-NF antibody showed strong staining (40% ± 5%, Table 1) in any one culture (Fig. 5A–B). On the other hand, many cells in culture (80% ± 5%, Table 1)

Table 1
Immunoreactivity of sea bass brain cell cultures to various antibodies

Antibody	Immunoreactivity	% positive cells
NF	+++	40 ± 5
GFAP	+++	80 ± 5
Nestin	++[a]	10 ± 3

Immunoreactivity refers to the intensity of the immunofluorescence staining, representing ++, a moderate intensity and +++, a high intensity. % positive cells refer to relative percentage of cells that appeared immunopositive in each culture.

[a] Positive reaction was only seen when cells were grown as spheroids.

appear to be glial-like cells, as they were mostly GFAP immunoreactive (Fig. 5C–D).

Immunoreactivity was tested on sea bass brain cells grown in monolayers or on cells spread out from neurospheroidal bodies and nestin-positive cells could only be observed when cells had been grown as neurospheres (10%±3, Table 1) (Fig. 5E–F). This suggests that growing cells as neurospheres maintain the cells in their undifferentiated state, since they show immunoreactivity to a neural stem cell marker.

3.4. Molecular identification of sea bass brain cell cultures

Confirmation of the species identity and nervous origin of the developed long term cell culture was determined by performing RT-PCR with sea bass specific pair of primers for *L17* housekeeping gene and HMG box protein *Sox 2* gene, respectively. The analysis of the RT-PCR products by electrophoresis reveals that cells in culture express both *L17* and *Sox 2* genes (Fig. 6A). The amplicons corresponding to the RT-PCR products obtained from the cell's cDNA have the expected size (87 bp for *L17* and 123 bp for *Sox 2* amplicons) and match with those obtained using cDNA template from sea bass brain, which was used as a positive control (Fig. 6A). Sequencing of all PCR products confirmed that what was amplified from cells and from brain tissue was indeed a partial sequence of *D. labrax L17* and *Sox 2* genes.

In order to test the proliferating activity of sea bass brain cells in culture, the expression of PCNA in the long term cell cultures and in the brain tissues was analyzed in comparison with the expression of L17 in the same cDNA templates. Expression of PCNA in the cultured cells was higher than those of the brain tissue as observed by band intensity in the agarose gels (Fig. 6B). Additionally, the difference of intensity between the bands corresponding to PCNA expression in cells and in brain (Fig. 6B) is much more evident than the one between the bands relative to L17 expression (Fig. 6C). Taken together, this indicates higher proliferation rate in cell culture than in brain tissues.

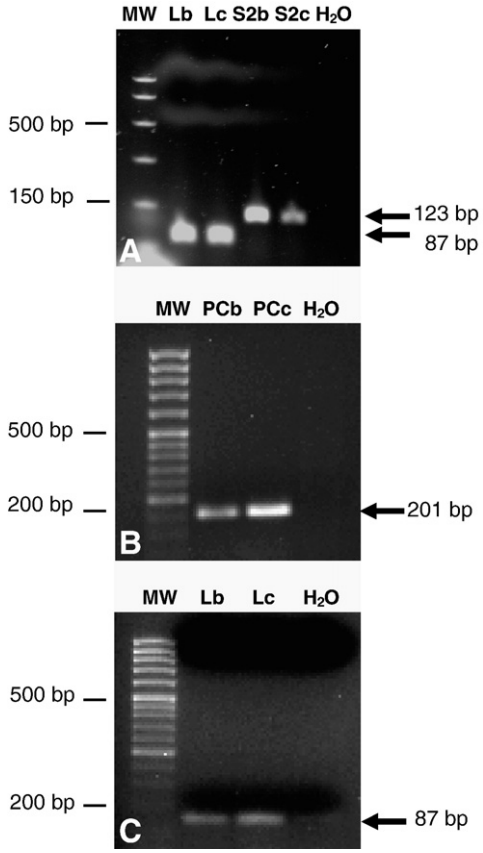

Fig. 6. Electrophoretic profile of amplicons obtained by RT-PCR in 2% agarose gels. (A) Both sea bass brain tissue and cells from the long term cell culture express *Dicentrarchus labrax* L17 (87 bp) and Sox 2 (123 bp). (B) PCNA expression in fresh brain tissue and long term cell cultures. (C) RT-PCR analysis of L17 expression on the same cDNA samples from fresh brain tissue and cell cultures in which PCNA expression was analyzed (B). MW: molecular weight marker; Lb: L17 expression in brain tissue; Lc: L17 expression in cell cultures; S2b: Sox 2 expression in brain tissue; S2c: Sox 2 expression in cell cultures; PCb: PCNA expression in brain tissue; PCc: PCNA expression in cell cultures; H_2O: negative control. Molecular weight markers are indicated.

3.5. Transfection study

Sea bass brain cells were successfully transfected with pEGFP-N1 plasmid by means of the two transfection reagents tested, JetPEI (Fig. 7A) and Fugene 6 (Fig. 7B-D). The expression of EGFP in cells was detected at 72 h after transfection by the observation of a clear green fluorescent signal under a fluorescent microscope (Fig. 7A–D). The transfection efficiency was found to be approximately 80% for both reagents tested. This indicates the suitability of sea bass brain cells for transfection with JetPEI or Fugene 6 and cytomegalovirus promoter as evidenced by the expression of EGFP gene in this long term cell culture.

4. Discussion

Sea bass neural cells can be readily cultured *in vitro* much like many other fish neural cells reported to date (De Boni et al., 1976; Hinsch and Zupanc, 2006; Wen et al., 2008a), including sea bass pituitary cells (Peyon et al., 2001). We were successful in culturing various neural cell preparations from sea bass from three independent trials and have been able to maintain some of these cultures for months at a time. Specifically, cells derived from sea bass cerebellum–caudal tegmentum, dubbed SBB-W1, have been passaged over 24 times since their establishment in 2006, and could be considered a permanent cell line. However, due to technical malfunction in the incubators in one of the laboratories and due to bacterial contamination in the other laboratory, the stock for these cells is currently very low and only two frozen vials are available. Once we recover from this set back, SBB-W1 could be made publicly available and could be useful in many areas of research, including virology where sea bass neural cells susceptible to nodaviruses are being sought. To date, a handful of fish cell lines have been shown to support the growth and isolation of piscine neuropathy nodaviruses which have been detrimental to many aquaculture facilities in Asia and Europe (Cutrín et al., 2007). These include cell lines from striped snakehead, *Channa striatus*: SNN-1 (Frerichs et al., 1996) and Sahul Indian sea bass, *Lates calcarifer*: SISS and SISE (Parameswaran et al., 2006a,b), barramundi brain: BB (Chi et al., 2005), but not from *D. labrax*.

4.1. Long term cell cultures derived from sea bass cerebellum

Although viable populations of cells could be obtained from all tested regions from the adult sea bass brain, long term cultures (lasting past 3 months) could best be obtained from the cerebellum and tegmentum. The cerebellum of fishes has been shown to have high proliferative capacity (Zupanc and Ott, 1999) thus it is not surprising that SBB-W1 arose from cerebellar fragments of sea bass brain. In fact, PCNA immunostaining reveals the existence of an important number of proliferative cells in the cerebellum of adult sole and sea bass (unpublished observation). The cerebellum is thought to retain a population of radial glial cells that are thought to be neural stem cells (Zupanc and Clint, 2003) responsible for neurogenesis in adult fish. SBB-W1 shows characteristics of radial glial cells as they express Sox-2, a neural progenitor cell marker (Brazel et al., 2005), that has been shown to be present in brain tissues of all vertebrates including fish (Hernández et al., 2007). SBB-W1 also express PCNA, a marker for proliferating cells, as well as nestin, GFAP and NF, which radial glial cells have been shown to express (Margota et al., 2007; Hinsch and Zupanc, 2006). It is interesting to note that PCNA expression was higher in SBB-W1 cells than in sea bass

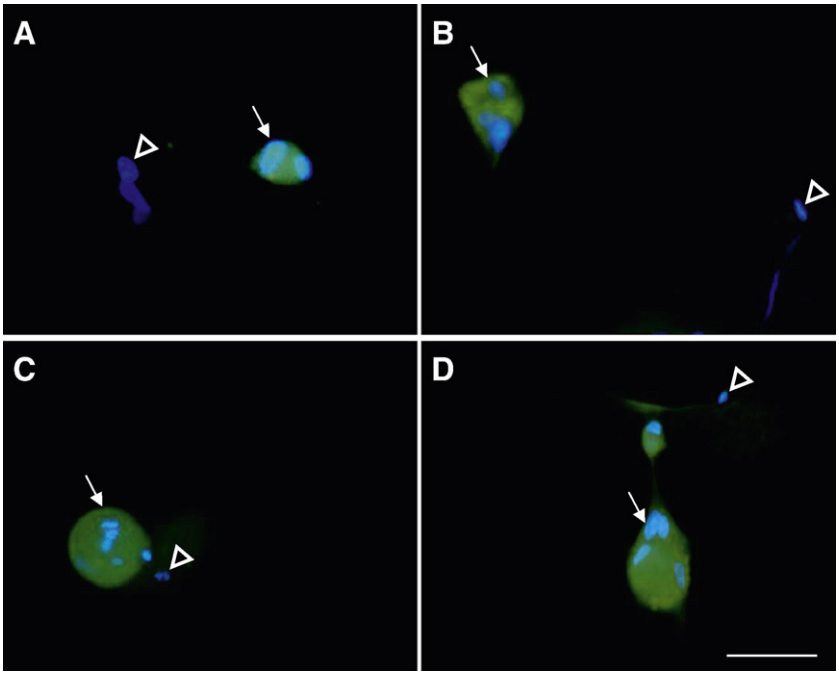

Fig. 7. Expression of GFP in sea bass brain cells in culture at passage 15 transfected with pEGFPN1 using transfection reagents: JetPEI (A) or Fugene 6 (B–D). Fluorescence micrographs show GFP signals merged with the corresponding images showing the nucleus stained with Hoechst counterstaining. Note that each picture reveals some transfected cells in green (arrows) but also some cells with no specific GFP expression (arrowheads). Scale bar: 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

brain tissue as it was determined using L17 expression as a normalization gene. This evidence suggests a higher proliferation rate in cell cultures developed than in adult brain tissues and could reinforce the consideration of SBB-W1 as a permanent cell line.

4.2. Growth characteristics of sea bass brain cell cultures

Optimal media formulations and growth temperatures were investigated for long term growth of sea bass brain cells. Preliminary investigations with Minimum Essential Medium, Neurobasal Medium and Leibovitz's 15 (L-15) medium, determined that L-15 was best at allowing cellular attachment and growth. We then investigated optimal FBS concentrations, and a supplementation of 10% was found to be optimal. The decrease in proliferation observed in growth curves starting from day 6 can be explained taking into account that culture medium was not replaced for the duration of the experiment and probably cells depleted most of the nutrients by day 6. Temperatures of 16, 22 and 28 °C appeared to support growth of sea bass brain cells being 16 to 22 °C within the range of temperatures in which sea bass can be found in the wild. However, SBB-W1 grew optimally at 28 °C, which is a temperature much warmer than their normal temperature range, although this is not unexpected, as juvenile sea bass *in vivo* have been shown to grow even at 29 °C and the estimated temperature for maximal specific growth rate was calculated at 26 °C (Person-Le Ruyet et al., 2004). Additionally, an Asian sea bass cell line derived from *L. calcarifer*, a close species relative to *D. labrax* grew best at 28 °C (Parameswaran et al., 2007). Nevertheless, for convenience, we chose to grow our cells at room temperature (22 °C).

4.3. Characterization of SBB-W1

SBB-W1 joins a short list of cell lines derived from sea bass. Buonocore et al. (2006) reported the development of an embryonic cell line derived from *D. labrax*, the only one available up to date in this species, while Parameswaran et al. (2006a) reported on another embryonic cell line derived from *L. calcarifer*. Another cell line from *L. calcarifer* fry, termed SF for sea bass fry was reported by Chang et al. (2001) and a spleen cell line was reported by Parameswaran et al. (2006b).

Mammalian brain cell cultures have been used for decades in the study of stages in brain development and neurogenesis (Castelli et al., 1992; Yang et al., 1993; Neuhaus and Fedoroff, 1994), neurotoxicology (Walum et al., 1990; Veronesi, 1992; Tang et al., 1996), stem cell isolation (Gregg et al., 2002), response to growth factors (Pitchford et al., 1995) and evaluation of drug delivery systems (Rizk et al., 2004). Fish brain cell cultures, on the other hand, have been rarely used for neurophysiology and have been developed mainly for virological studies. Thus, few neural cell lines have been reported to date. The GB cell line from a diseased brain of grouper, *Epinephelus awoara* (Lai et al., 2003) and the BB cell line from the brain tissue of barramundi, *L. calcarifer* (Chi et al., 2005) have been used for growing nodaviruses. Most recently, TB2 cells, from tilapia brain were reported to have neural cell progenitor characteristics and the cells could be induced to express neural cell characteristics (Wen et al., 2008a). Additionally, Wen et al. (2008b) recently reported the development of two brain derived cell lines from the orange-spotted grouper, *Epinephelus coioides*, again for studying viruses affecting the nervous system of fishes. SBB-W1 can now be added to this short list of neural cell lines, and like the TB2 cell line, shows neural cell progenitor characteristics. These cells formed neurosphere-like bodies when cells were placed in hanging drops or in non-tissue culture plates. The ability of neural cells to form neurospheres has been used in mammalian systems to identify populations of cells that contain stem cell-like characteristics (Bez et al., 2003; Deleyrolle et al., 2008). Adhesion to substrates had been shown to modulate neural cell differentiation in mammals and the recent report by Saha et al. (2008) corroborates previous findings. Although, not extensively investigated, our work also confirms that neurosphere adhesion and cellular spreading in tissue culture substrates may lead to neural differentiation in fish neural cell cultures. This had also been previously observed by Hinsch and Zupanc (2006). Future work with SBB-W1 will involve studies on neural cell differentiation and of factors regulating this, since

a key question arising from the present approach is whether mature neurons could be induced.

Elucidation of cell type in SBB-W1 was aided but not unquestionably determined by immunostaining for nestin, GFAP and NF. SBB-W1 showed positive, fibrillar staining for both GFAP and NF, and positive nestin reaction when grown as neurospheres, suggesting their potential role as neural stem cells (Yang et al., 1993; Wen et al., 2008a). Although GFAP is expressed in the glial cell lineage, neural precursor cells have also been shown to stain with GFAP as was shown by Wen et al. (2008a) and Zhang (2001) who also showed positive GFAP staining in cells with neural stem cell-like properties. Furthermore, to test specificity of antibodies, double staining of NF and GFAP was carried out on brain smears, appearing to label most cells as GFAP-positive, with only presumed axonal tracts labelled as NF-positive. At lower antibody concentrations, anti-GFAP appeared to stain cells considerably stronger than anti-NF, suggesting a greater abundance of GFAP in the cells. It is possible that SBB-W1 cells are similar to the GFAP positive radial glial cells, which are found in several places in the brain of adult fish (Zupanc and Horschke, 1995; Pellegrini et al., 2007).

Because of the important proliferation ability and the high population of neural stem cells present in the adult teleost brain, development of a cell line from a mature sea bass proved to be easier and more rapid than attempts to do so with mammalian cells. SBB-W1 may be applicable for neural regeneration studies, for developing screening batteries for neurotoxins, studying changes in brain cells with senescence, exploring differentiation in response to growth factors, and many other applications in neurobiology that would benefit from an isolated well-defined environment provided by an *in vitro* model. These cells could also serve as models for elucidating mechanisms controlling biological processes such as reproduction, growth, feeding and circadian rhythmicity, in which the brain plays important integrative and effector roles. SSB-W1 cells can be successfully transfected with exogenous plasmids and were functional in the induction of transfected promoters and in the expression of transfected genes, indicating their viability and suitability as models for analyzing gene expression and its regulation.

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