Methods in Cell Science **25**: 211–220 (2003).
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Establishment, growth, cryopreservation and species of origin identification of three cell lines from white sturgeon, *Acipenser transmontanus*

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Accepted in revised form 29 April 2004

Abstract. Three cell lines derived from fin (WSF), head soft tissue (WSHST) and body muscle (WSBM) were established from white sturgeon (*Acipenser transmontanus*). Characterization included determination of optimal growth kinetics, karyotyping, and mitochondrial ribosomal RNA (rRNA) genotyping. The primary cultures of these cells were generated by the explant technique using the L-15 medium supplemented with 20% fetal bovine serum and epidermal/fibroblast growth factors. The cells grew between 15–30 °C, but optimal growth occurred at 25 °C with a doubling time of 48 hours. The cell lines can be readily maintained *in-vitro* and have been subcultured over 35 times. Following cryopreservation in liquid nitrogen, thawed cells exhibit a viability of > 90% after a 16-month storage period. Chromosomal typing of these cell lines at their 17th passage revealed a chromosomal distribution of 242 to 278 with an apparent peak ranging from 250 to 260. Polymerase chain reaction amplification of mitochondrial 16S ribosomal RNA and sequence analysis indicated 100% identity of the sequences found in the cell lines with those found in the source animal, confirming that the cell lines were of *A. transmontanus* origin.

Key words: *Acipenser transmontanus,* cell culture, white sturgeon

Abbreviations: WSBM – white sturgeon body muscle; WSF – white sturgeon fin; WSHST – white sturgeon head soft tissue

1. Introduction

The culture of white sturgeon (*Acipenser transmontanus)* has increased significantly in the United States, particularly in California, Oregon, Washington and Idaho [15]. This is largely attributed to the success of artificial reproduction which provides a reliable supply of brood-stock, eggs and larvae [2]. White sturgeon aquaculture now encompasses a growing industry and numerous conservation programs involved in the restoration of threatened and endangered species. Sturgeon aquaculture has also been growing as an industry in Europe. White sturgeon was initially introduced into Italy from California in 1989 and since then sturgeon aquaculture has quickly spread to other European countries including France, Spain, Germany and Poland [2]. Sturgeon farming in western and central Europe has steadily grown from 50 tonnes in 1985 to nearly 900 tonnes by 1996, and reached approximately 1300 tonnes by 1999 [2, 25]. White sturgeon, Siberian sturgeon (*A. baerii*) and Adriatic sturgeon (*A. naccarii*) are the three major farmed species and account for 95% of the total production in Europe. Among these animals, white sturgeon represents the most popular species which is now farmed in many parts of the world [2].

The growth of sturgeon aquaculture has also been accompanied by an increased awareness of the adverse impact of diseases, particularly those caused by pathogenic viruses. Three types of viruses including an iridovirus, herpesvirus and adenovirus are presently known to infect white sturgeon [9, 10, 23]. All these viruses can cause acute mortality of cultured white sturgeon [4] and, in fact, viral infections have emerged as a significant obstacle to the development of the white sturgeon industry [9, 10, 26]. For example, infection with the white sturgeon iridovirus (WSIV) can cause a chronic debilitating wasting syndrome which results in severely impaired growth and survival of young fish [10]. In addition, the herpesvirus, WSHV-2, causes serious losses of older fish [11, 23].

To prevent and control infection and spread of sturgeon viruses, establishment of more sensitive cell culture systems are necessary to assist early detection and diagnosis. Cell lines currently established from white sturgeon species, include WSS-2 and WSH-1 derived from spleen and heart tissues of a juvenile animal, respectively [12], and five cell lines established from different organs and tissues of yearling white sturgeon, including WSGO (derived from gonad), WSLV (from liver), WSSB (from swim bladder), WSNV (from brain) and WSHO (from meningeal myeloid organ) [24]. In addition, several cell lines from other sturgeon species were also documented in literature including the SH line which was established from cardiac tissue of Atlantic sturgeon (*A. oxyrhynchus*) [17]. Additionally, three cell lines derived from the connective tissue of the dorsal fin of *Acipenser naccorlii*, *A. ruthenus*, and *A. guldenstaedti* have also been established [6].

The white sturgeon iridovirus and other iridovirus-like agents that have been described in other species of sturgeon have been very difficult to isolate or have only been identified by electron microscopy [16]. There is a need to establish additional cell lines from white sturgeon because of the difficulty in diagnosing some viral infections [15]. Three additional cell lines from fin, body muscle and head soft tissue of young white sturgeon were recently established in our laboratory. The report herein describes the establishment, optimal growth condition, cryopreservation and species of origin identification of these newly established white sturgeon cell lines.

2. Materials

A. Equipment
- Centrifuge, model IEC Centra GP8R.[1]
- GeneAmp PCR System 4800.[2]
- GeneAmp PCR System 9700.[2]
- Hemacytometer, double chamber, No. 02671-5.[3]
- Laminar-flow biological safety cabinet, Class II Type A/B3.[4]
- Liquid nitrogen storage tank, XLC-1110.[5]
- Microscope, inverted, model 1X70.[6]
- Microscope, inverted, model CKX31.[6]
- Mini gel electrophoresis, model Mupid-2.[7]
- Pipet-Aid, No. 13-681-16.[3]
- Refrigerated incubators, model BOD10A14.[8]
- Refrigerator, model TAX6.[9]
- Rocker, Cole Palmer Model 51702-00.[10]
- Slide warmer, No. 12-594.[3]
- Stirrer/hot plate, No. E36559.[3]
- Ultra low freezer, Harris model DLT-17LS-90A14.[11]
- Waterbath, model Isotemp.[3]

B. Culture media, solutions, and chemicals
- L-15 Leibovitz medium, No. L4386.[12]
- Medium 199, No. M3274.[12]
- Minimum essential medium (MEM), No. M0275.[12]
- RPMI-1640 medium, No. R0883.[12]
- Amphotericin B, 250 μg/ml, No. A-2942.[12]
- Chloroform:Isoamyl Alcohol ($CHCl_3$/IAA) 24:1, 100 ml, No. C0549.[12]
- Colcemid, 1.0 mg, D6165.[12]
- Demecolcine, 1 mg, No. D-6165.[12]
- Dimethyl sulfoxide (DMSO), 100 ml, No. D-2650.[12]
- Dulbecco's phosphate buffered saline (PBS), No. D-8537.[12]
- EDTA disodium salt, No. E-5134.[12]
- Epidermal growth factor (EGF), 100 μg, No. E-1264.[12]
- Ethanol, anhydrous, 4 l, No. A405P-4.[3]
- Fetal bovine serum (FBS), 500 ml, No. 7D2078.[13]
- Fibroblast growth factor (FGF), 25 μg, No. F5542.[12]
- Gentamicin sulfate, 5 g, No. G1264.[12]
- Hepes, 100 g, No. H1016.[12]
- L-glutamine–penicillin–streptomycin (GPS), No. G1146.[12]
- Phenol red, 25 g, No. P-3532.[12]
- Phenol:Chloroform:Isoamyl Alcohol (phenol/$CHCl_3$/IAA) 25:24:1 solution, 100 ml, No. P2069.[12]
- Potassium chloride, KCl, No. P-9541.[12]
- Potassium phosphate, KH_2PO_4, No. P-8416.[12]
- Sodium Bicarbonate, 1 kg, No. S-6014.[12]
- Sodium chloride, NaCl, No. S271-3.[3]
- Sodium phosphate, Na_2HPO_4, No. S-5136.[12]
- Trypan blue, 20 ml, No. T-8154.[12]
- Trypsin, 10x, No. T-4549.[12]
- 2-propanol, 4 l, No. A416-4.[3]

C. Glassware and plastics
- Centrifuge tubes, 15 ml, No. 352097.[14]
- Centrifuge tubes, 50 ml, No. 352098.[14]
- Cryogenic controlled-rate freezing container, No. 15350-50.[3]
- CryoStore cell box, No. 12565-182.[3]
- Disposable sterilization filter units, 1000 ml, No. 09740-3A.[3]
- Disposable sterilization filter units, 150 ml, No. 09740-1A.[3]
- Disposable sterilization filter units, 250 ml, No. 09740-2A.[3]
- Disposable sterilization filter units, 500 ml, No. 09740-25A.[3]
- Microscope slides, 75 × 25 mm, No. 12544-7.[3]
- Non-latex exam gloves, No. 6005PF.[14]
- NUNC CryoTube vials, 1.8 ml, No. 12565-171N.[3]

– Premium cover glasses, 24 × 45 mm, No. 12548-5M.[3]
– Serological pipettes, 1 ml, No. 13678-11G.[3]
– Serological pipettes, 10 ml, No. 13678-11E.[3]
– Serological pipettes, 2 ml, No. 13678-11C.[3]
– Serological pipettes, 25 ml, No. 13678-11.[3]
– Serological pipettes, 5 ml, No. 13678-11D.[3]
– Tissue culture flasks, 25 cm, No. 10-126-39.[3]
– Tissue culture flasks, 75 cm, No. 10-126-41.[3]

D. Other reagents
– AmpliTaq DNA Polymerase, 250 U, No. A02060.[2]
– AmpliWax PCR Gem 50, No. N808-0150.[2]
– GeneAmp 10x PCR buffer, 1.5 ml, No. A02905.[2]
– GeneAmp dNTPs set, 10 mM, No. A03613.[2]
– GeneAMp $MgCl_2$, 25 mM, No. A2667.[2]
– Proteinase K, 100 mg, No. P2308.[12]
– RNase, 50 mg, No. R6513.[12]
– Topo TA Clong kit, No. K4500-40.[15]

3. Procedures

A. Preparation of media
1. Fetal bovine serum (FBS)
Divide 500-ml bottle into 10 × 50-ml aliquots in 50-ml sterile conical tubes. Heat inactivate at 56 °C for 30 min in water bath. Store at −30 °C.
2. L-15 medium Leibovitz with additives

– L-15 powder	14.8 g
– GPS (10×)	10 ml
– Amphotericin B, 250 μg/ml	10 ml
– Gentamicin, 50 mg/ml	1 ml
– Sodium bicarbonate	1.5 g
– Double distilled water (ddH_2O) to	900 ml

Dissolve L-15 powder in about 800 ml of ddH_2O. Add sodium bicarbonate and other additives. Dissolve and mix thoroughly. Bring volume to 900 ml with ddH_2O. Transfer to a 1-l filter unit (0.22 μm) and filter sterilize. Store at 4 °C.
3. Medium 199 (M 199) with additives

– M 199 powder	9.87 g
– GPS (10x)	10 ml
– Amphotericin B, 250 μg/ml	10 ml
– Gentamicin, 50 mg/ml	1 ml
– Sodium bicarbonate	1.5 g
– Double distilled water (ddH_2O) to	900 ml

M 199 medium was prepared like L-15.
4. Minimum essential medium with additives

– MEM powder	46.9 g
– GPS (10×)	50 ml
– Amphotericin B, 250 μg/ml	50 ml
– Gentamicin, 50 mg/ml	5 ml
– Sodium bicarbonate	7.5 g
– Double distilled water (ddH_2O) to	4.5 l

Dissolve MEM powder and sodium bicarbonate in 3 l of ddH_2O. While gently stirring, add the remaining solutions and mix thoroughly. Bring volume to 4.5 l with ddH_2O. Dispense into 500 ml sterile, glass serum bottles and sterilize by autoclaving at 121 °C (250 °F) at 15 psi for 15 minutes with validation. Store at 4 °C.
5. RPMI 1640 medium with additives

– RPMI-1640, 10×	100 ml
– GPS, 100×,	10 ml
– Amphotericin B, 250 μg/ml	10 ml
– Gentamicin, 50 mg/ml	1 ml
– double distilled water (ddH_2O) to	900 ml

6. 2X freezing medium

– RMPI 1640 medium with additives	6.0 ml
– FBS	2.0 ml
– Dimethyl sulfoxide	2.0 ml

Mix ingredients together aseptically and prepare fresh mixture for freezing cells.
All the above media were sterilized by either autoclaving or filtering through a 0.22 μm filter before use.
7. Antibiotic-incubation medium (AIM)

– L-15 medium (2×)	100 ml
– GPS (10×)	10 ml
– Amphotericin B (250 μg/ml)	10 ml
– Gentamicin (50 mg/ml)	1.0 ml
– Double distilled water (ddH2O) to	200 ml

Aseptically add ingredients together and mix thoroughly. Store at 4 °C.

B. Preparation of solutions
1. Cell stain-fixative

– Crystal violet	18 g
– Formaldehyde (37%)	1.8 l
– Double distilled water (ddH_2O) to	3.0 l

Mix 3 components thoroughly in a 4-l brown-colored glass bottle and store at room temperature.
2. Phosphate buffered saline (PBS)
– Sterile-filtered Dulbecco's PBS
– Without calcium chloride and magnesium chloride
3. Proteinase K buffer

– 5 M NaCl	15 ml
– 1 M Tris-HCl (pH 7.4)	5 ml
– 0.5 M EDTA (pH 8.0)	10 ml
– 10% SDS	4 ml
– double distilled water (ddH_2O)	466 ml

Combine all ingredients and mix thoroughly. Store at room temperature.
4. Proteinase K/RNase working solution

– Proteinase K buffer	10 ml
– Proteinase K stock solution (10 mg/ml)	0.5 ml
– RNase Stock solution (10 mg/ml)	50 μl

Mix all ingredients well and prepare fresh (before use).
5. Tris–EDTA buffer (TE) (10 mM Tris-HCl, 1 mM EDTA)

– 0.5 M Tris–HCl, pH 8.0	10 ml
– 0.5 M EDTA, pH 8.0	1.0 ml
– double distilled water (ddH_2O)	489 ml

6. Trypsin stock 2.5% Trypsin solution (10×)

100 ml/bottle and store at –30 °C.

7. Versene solution

– NaCl	40.0 g
– KH_2PO_4	1.00 g
– KCl	1.00 g
– Na_2HPO_4	5.75 g
– EDTA–disodium salt	1.00 g
– Phenol red	0.05 g
– double distilled water (ddH_2O) to	5 l

Set up a sterilized 5-l flask containing 3 l of ddH_2O and a magnetic bar. While gently stirring the water, add each of the chemical components into the flask. Stir until dissolved and mix thoroughly. Bring volume to 5 l with ddH_2O. Dispense into 500 ml sterile, glass serum bottles and sterilize by autoclaving at 121 °C (250 °F) at 15 psi for 30 minutes with validation. Store sterilized versene solution at 4 °C.

8. Trypsin-versene solution (TVS)

– Trypsin (2.5% Trypsin in 0.9% NaCl)	10 ml
– Sterilized Versene	90 ml

– Aseptically combine ingredients, mix, adjust pH to 7.5 with 7.5% sodium bicarbonate. Store at 4 °C.

C. Fish sources and tissue collections

Young white sturgeon fish (0.8–1.0 g and 2.0–2.2 cm in length) were obtained from Clear Spring Foods, Inc., in Idaho according to University of Hawaii and the State's biosafety regulations for the importation of fish. Prior to the experiment, donor fish appeared healthy and, following a rinse with 70% alcohol, sacrificed, fish were immediately dissected and head soft tissue, body muscle, and fins from 5 sturgeons were pooled separately. Each pooled tissue was soaked in antibiotic-incubation medium (AIM) solution for 1 hour at room temperature with one change of the AIM solution.

D. Primary cell culture and subculture

Following the AIM incubation, the tissues were minced into 1–2 mm^3 pieces using two sharp sterile scalpels. These tissue fragments were washed twice with AIM, then evenly seeded into Primaria-brand tissue culture flasks (Falcon). These minced tissue pieces were allowed to attach to the flasks for 2.5 hours at room temperature (22–24 °C), then fed with three different media including MEM, L-15 and M199 supplemented with 20% FBS, EGF (30 ng/ml) and FGF (2.5 ng/ml), and various antibiotics. These flasks were then transferred to an 18 °C incubator and the cultures observed daily for cell growth using a phase-contrast inverted microscope. After the formation of partial or entire cell monolayers within the flasks, tissue fragments were dislodged and cell monolayers disrupted using TVS to obtain single cell suspensions. For the first 10 passages the cells were subcultured with the medium containing 50% fresh and 50% conditioned medium. Beginning at passage 11, neither conditioned medium nor growth factors were used. Subcultures were performed every 4–5 days at a split ratio of 1:2–3.

E. Growth and characterization of white sturgeon cells

1. *Growth at different temperatures:* In order to determine the effect of temperature on cell propagation, four different incubation temperatures were used to compare the growth kinetics of WSBM cell line. WSBM cells, at their 22nd passage, were seeded in forty-eight 25-cm^2 tissue culture flasks (2×10^5 cells/flask). The cells were cultured in MEM supplemented with 10% FBS and 12 cultures were incubated at each of the four temperatures: 15, 20, 25 and 30 °C. On alternate days for a total of 12 days, cells from 2 flasks at each temperature were trypsinized, counted 6 times using a hemacytometer (3 times/flask). The mean number of cells and standard deviations (SD) were calculated.

2. *Growth in different FBS concentrations:* The optimal FBS concentration for the growth of the WSBM cell line was determined at 25 °C. In this test, WSBM cells from passage 24 were seeded in 48 flasks (25-cm^2, 9×10^4 cells/flask) of which 12 cultures were incubated in medium supplemented with one of 4 different concentrations of FBS (nil, 5, 10 and 20%). Cells were harvested and counted as described above.

3. *Growth in different culture media:* Growth of WSBM cells in four different media (MEM, Medium 199, L-15 and RPMI 1640) was also compared in replicate 25 cm^2 flasks. The WSBM cell line from pass 24 was incubated in each medium containing 10% FBS at 25 °C.

4. *Cell plating efficiency:* The WSBM cell line at pass 25 was used to determine plating efficiency. Cells diluted in growth medium were added at densities of 200, 500, and 1000 cells per triplicate 25 cm^2 flask and incubated at 25 °C. On day 12, the growth medium was removed and the cells were fixed with 3 ml of cell stain-fixative for 1 hour, then rinsed with tap water and air-dried. Individual cell colonies were counted under a dissecting microscope. Plating efficiencies (PE) were calculated using the formula [7]:

$$PE(\%) = \text{No. of cell colonies}/\text{No. of cells seeded} \times 100$$

F. Cryogenic preservation and stability in liquid nitrogen

The ability of white sturgeon cell lines to be preserved in liquid nitrogen was evaluated according to techniques established previously [18]. Briefly, cells in the exponential phase of growth were

harvested using TVS and suspended in growth medium at a concentration of 2–4 × 10^6 cells/ml. The cell suspension was then mixed slowly with an equal volume of cold 2x freezing medium containing 20% FBS and 20% DMSO. Following very gentle mixing, 1.0 ml aliquots of the cell suspension were dispensed into 1.8 ml sterile plastic vials (Nalgenunc Naperville, IL) and sealed. Vials were then transferred into cryogenic controlled-rate freezing containers, stored at −70 °C for 24 hours then moved to liquid nitrogen. Vials containing cells were removed from N_2 at storage day 480, rapidly thawed at 37 °C, and viable cells were counted using trypan blue exclusion test. Additionally, their ability to attach and grow in tissue culture flasks was assessed.

G. Chromosomal analysis

The WSF cell line at pass 17 was used to determine chromosome distribution by the method of Earley [3]. The cell line was grown in duplicate 75 cm^2 flasks and formed a 70–80% confluent monolayer in MEM containing 20% FBS after 24 hours. The medium in two flasks was replaced with 10 ml of fresh medium and 0.1 ml Colcemid solution (1 μg/ml). The cells were then incubated for an additional 16 hours at 25 °C. The remaining steps, including cell harvesting/fixing, slide preparation, cell staining and chromosome counting, were followed using the standard procedures described by Earley [3].

H. Ribosomal RNA (rRNA) analysis

1. Approximately 1 × 10^7 WSBM cells at passage 22 were harvested using a trypsin-versene solution and placed in a 15-ml polypropylene conical tube. Following washing with 10 ml of PBS by centrifugation, the cell pellet was resuspended in 3 ml of freshly prepared proteinase K/RNase solution that contained RNase (50 μg/ml), Proteinase K (500 μg/ml), NaCl (0.15 M), Tris-HCl (0.01 M, pH 7.4), EDTA (0.01 M, pH 8.0) and 0.1% SDS. The cells were incubated at 56 °C for 3 hours and mixed every 20–30 min.
2. Approximately 3 ml of phenol/$CHCl_3$/IAA (25:24:1) solution was added, mixed gently by inversion and centrifuge at 2000 × g for 10 minutes. This extraction process was repeated once.
3. The aqueous phase was harvested using a pipette and then was extracted once with equal volume of $CHCl_3$/IAA (24:1) by gentle inversion mixing. After centrifuging at 2000 × g for 10 minutes, the aqueous phase was harvested and 300 μl of 10N NH_4-acetate and 6 ml of 100% ethanol was added and slowly and thoroughly mixed.
4. The precipitated DNA was precipitated at −20 °C overnight.
5. The precipitated DNA was collected using a curved glass pipette, rinsed briefly in 70% ethanol by dipping, and air-dried.
6. The DNA was suspended in 400 μl of TE buffer (pH 8.0) and incubated at 37 °C for 1 hour to dissolve the DNA. The DNA concentration was determined with a spectrophotometer and stored at 4 °C.
7. Two sets of oligonucleotide PCR primers were used to amplify the 16S and 18S white sturgeon genes, respectively.

PCR primers for 16S gene (287 bp)
Forward (16sWS/F) 5′-CCGTGCGAAGGTAGC GTAATCA-3′
Reverse (16sWS/R) 5′-CCCGTCGACATGGAG-GTTTTT-3′
PCR primers for 18S gene (251 bp)
Forward (18sWS/F) 5′-GGCGCCCCCTCGATG CTCTTA-3′
Reverse (18sWS/R) 5′-ATGCTTTCGCTTTCGC CCGTCTT-3′

8. PCR reaction mix

PCR buffer (10x)	5.0 μl
DNTPS (2.5 mM)	4.0 μl
$MgCl_2$ (25 mM)	3.6 μl
Forward primer (20 μM)	0.3 μl
Reverse primer (20 μM)	0.3 μl
EX-Taq (5 U/μl)	0.25 μl
Template DNA	(0.2–0.3 μg)
NA-free H_2O to	50 μl

9. PCR cycling conditions

Initial denature at 94 °C for 5 minutes
45 cycles: 94 °C 2 minutes + 60 °C 1/2 minutes + 72 °C 1/2 minutes
Final extension at 72 °C 5 minutes
Hold at 4 °C

10. The amplified PCR product was analyzed by 1% agarose gel electrophoresis.
11. The amplified PCR products were identified by TOPO TA Cloning (Invitrogen). Two positive clones were randomly selected from each amplification and each clone was sequenced in both directions using the vector primer M13 (Invitrogen) and an automated sequencer (Model 373A, Applied Biosystems, Inc.).
12. The amplified sequences were identified by the Editseq and Megalian programs of the DNAstar package.

I. Statistical analysis

The difference in cell growth at different temperatures, concentrations of FBS, and in different media was compared using a two-sample *t*-test. If the *P* value was less than 0.05, the difference between two samples was designated as significant.

4. Results and discussion

All the explanted tissues were readily cultivated *in vitro*. The emergence of new cell growth began

from the edges of seeded tissue explants and was observed the day following tissue explanting. The cells grew quite fast and formed sizable colonies by day 3–4 and partial cell monolayers by day 5–6, after which the first subculture was conducted. The cultures all shared a very similar pattern of growth. The cells were primarily epithelial-shaped during the primary culture phase and then became a mixed population containing both epithelial and spindle-shaped cells after 2–4 passages. After five serial subcultivations the cells appeared uniformly spindle-shaped (Figure 1).

To establish the optimal conditions for *in vitro* cultivation of these newly established white sturgeon cell lines, we tested the growth of the WSBM cells at four different temperatures, ranging from 15 to 30 °C. (Figure 2) As observed for other established anadromous animal cell lines, the growth of white sturgeon cells was temperature-dependent. The optimal temperature for the growth and proliferation of the cells was determined to be 25 °C. At this temperature, the doubling time (dt) of the cell population was about 48 hours. The cells also attached and grew well when incubated at 20 °C, but the doubling time was greater than 72 hours. WSBM cells were also able to spread and grow well at 15 °C, although it took 48 hours for the cells to become well attached. When incubated at 30 °C, the cells proliferated fast during the first 48 hours, but the growth and proliferation slowed dramatically, and the cells appeared to age rapidly. Statistical analysis revealed that the cell line grew significantly better at 25 °C than at other temperatures (*t*-test, $P < 0.05$). At the other temperatures tested, the cells grew better at 20 °C than at 15 and 30 °C ($P < 0.05$). There was no significant difference found between cell growth at 15 °C and 30 °C ($P = 0.25$). These results are consistent with the report by Hedrick and co-workers who documented that both WSS-2 and WSH-1 cells replicated at 15–30 °C with an optimum temperature of 25 °C [12]. Establishment of white sturgeon cell lines was also undertaken at an incubation temperature of 15 °C for the purpose of studying the replication and pathogenesis of WSIV, a virus of cold-water fish [24]. However, this temperature was clearly not the optimal condition for culturing white sturgeon cells as evidenced by the very long incubation time required to establish primary cultures and the slow kinetics of cell proliferation [24].

Growth kinetics of WSBM cells correlated with the concentration of FBS supplemented into the growth media. In general, the cells grew rapidly in media containing FBS, but not in the medium alone (Figure 3). The cells proliferated rapidly in the medium containing 20% FBS, doubling within 48 hours. In comparison, the cell growth was much slower in 10% FBS (dt: approximately 60 hours), which, however, was more rapid than in 5% FBS. Statistical analysis indicated that the growth and proliferation of WSBM cells was significantly different at different concentrations of FBS ($P < 0.05$).

Comparison of WSBM cells in different growth media revealed that all four media could support their *in vitro* propagation (Figure 4). Although the initial propagation of these cell lines was conducted with L-15 medium, it appeared that WSBM and WSF cells preferred to grow in M 199 medium. Statistical analysis indicated that cell propagation was significantly greater in M 199 medium than the other media tested ($P < 0.05$). However, there was no significant difference in cell growth among the other three media ($P > 0.1$). Previous studies indicated that MEM was used successfully for the establishment of two cell lines derived from spleen and heart of white sturgeon [12].

The viability test of WSF, WSBM and WSHST cells demonstrated that all the cells have the ability to survive following storage at −196 °C. Following storage for 16-month in liquid N_2, over 90% white sturgeon cells remained viable (WSF 93.5%, WSHST 92% and WSBM 90.4%). Following thawing, cells attached well when seeded in cell culture flasks and formed cell monolayers within 2–3 days without any apparent morphological change.

The WSBM cell line was also used to determine the plating efficiency (PE). Three groups of 200, 500 and 1000 cells/flask were found to have moderate plating efficiencies of 10.5 (±0.5), 13.07 (±2.53) and 14.37 (±5.66)%, respectively. These data suggested that the cell lines performed better when seeded at a relatively higher density.

White sturgeon fin (WSF) cells at passage 17 were used to determine the chromosome number. Photographs of 50 discrete cell spreads were counted to determine the chromosome number. The cell spreads showed that these cells had a similar karyotype pattern to their host of origin (Figure 5), ranging from 242 to 278 with a major peak between 250 and 260 (66% of the all spreads) chromosomes (Figure 5). White sturgeon is one anadromous species that has a relatively large number of chromosomes with the majority belonging to the microchrosomal group [5, 22]. It has been reported that the karyotype of white sturgeon chromosomes can vary from 240 ± 8 to 271 ± 2.5 [5, 6, 12, 21, 22]. Our results are consistent with the published literature suggesting that the cell lines originated from white sturgeon. However, Hedrick and his coworkers demonstrated the modal chromosomal number for the WSS-2 was 219 while the WSH-1 line had bimodal distribution with peaks at 237 and 243 [12], indicating a lower chromosomal count. In addition, Fontana suggested the diploid number of white sturgeon found in Italy was 248 (±8) [5], which is

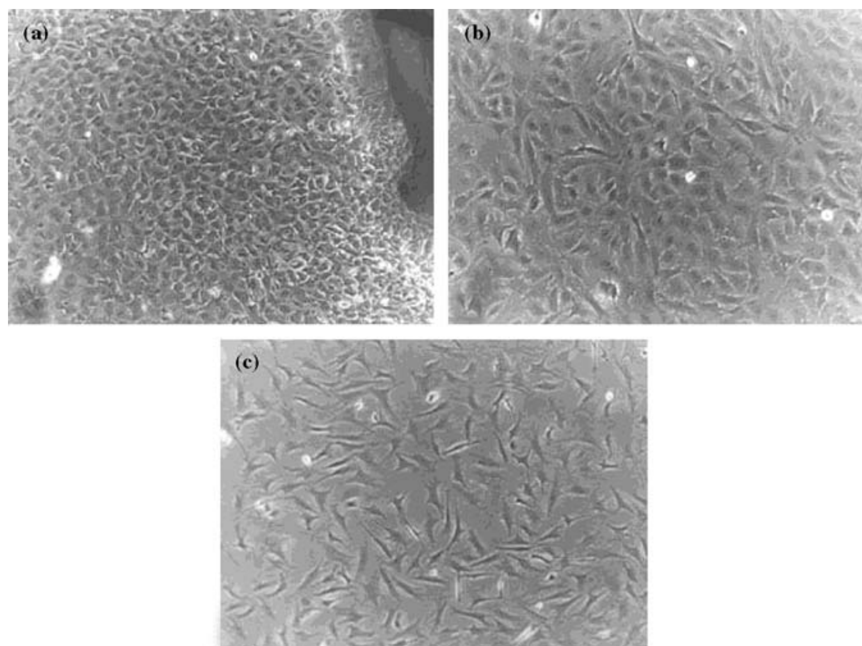

Figure 1. Photomicrographs of cell lines established from white sturgeon fry. Primary cell culture derived from head soft tissue (WSHST) (a), the first cell subcultures derived from fins (WSF) (b) and body muscle (WSBM) (c). Cells were grown in L-15 medium supplemented with 20% FBS, epidermal and fibroblastic growth factors, and various antibiotics. Magnification: 100×.

also lower than the number we counted from the WSBM cells (256 ± 6). The wide range of reported white sturgeon chromosomes could be explained partially by experimental variation and also due to the variable number of entirely heterochromatic chromosomes from different individuals. Since the normal 2n number for white sturgeon in North American is not known, it is hard to determine if the newly established cell lines remained diploid.

Recent studies have indicated that heteroduplex analysis of mitochondrial 16S ribosomal RNA (rRNA) can be employed to determine the conspecificity of established cell lines [8, 14, 19, 20]. To verify the origin of these three cell lines by heteroduplex analysis, DNA was isolated from the

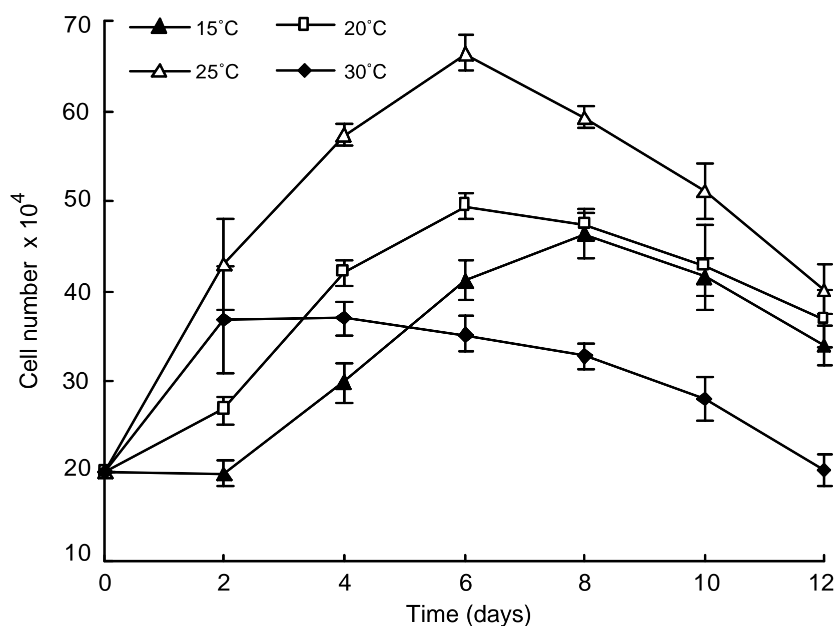

Figure 2. Growth kinetics of white sturgeon body muscle cells (passage 22) in MEM-10 at selected incubation temperatures. Bars indicate standard deviations.

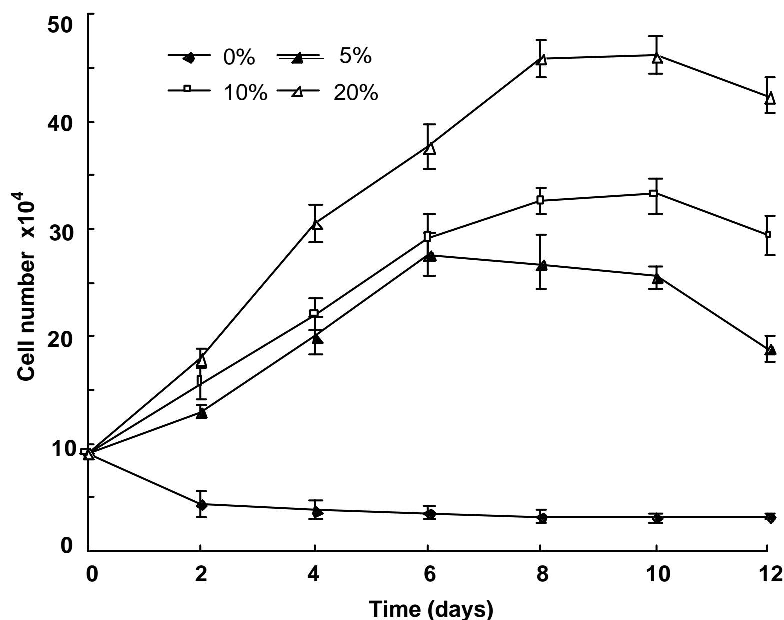

Figure 3. Growth curves of white sturgeon body muscle cells (passage 24) in MEM supplemented with different concentrations of fetal bovine serum. The cells were propagated at 25 °C and the cell number was determined every other day. Bars indicate standard deviations.

WSF cells at their 22nd passage. Amplification of the DNA extracted from the WSF cells using the PCR primers designed from the genomic sequence of 16S ribosomal RNA of white sturgeon revealed the expected PCR product of 288 bp (Figure 6). The amplified PCR fragment was cloned and sequenced and this DNA fragment was determined to match perfectly with the genomic sequence of 16S ribosomal RNA previously reported for white sturgeon [1]. In addition, PCR amplification of the DNA was also conducted using the oligonucleotide primers designed from the nuclear 18S ribosomal RNA and a PCR product of 252 bp was detected (Figure 6). Comparative sequence analysis indicated the identity of the newly detected sequence was the 18S rRNA gene of white sturgeon [13]. These sequence data confirm the newly established cell lines were derived from white sturgeon.

The successful establishment of white sturgeon aquaculture is dependent on strategies focused on growth and disease control. Establishment of permissive cell lines from white sturgeon should provide a sensitive diagnostic method for the identification of viral infections and/or carrier fish in white sturgeon hatcheries. These newly established cell lines are currently being examined for

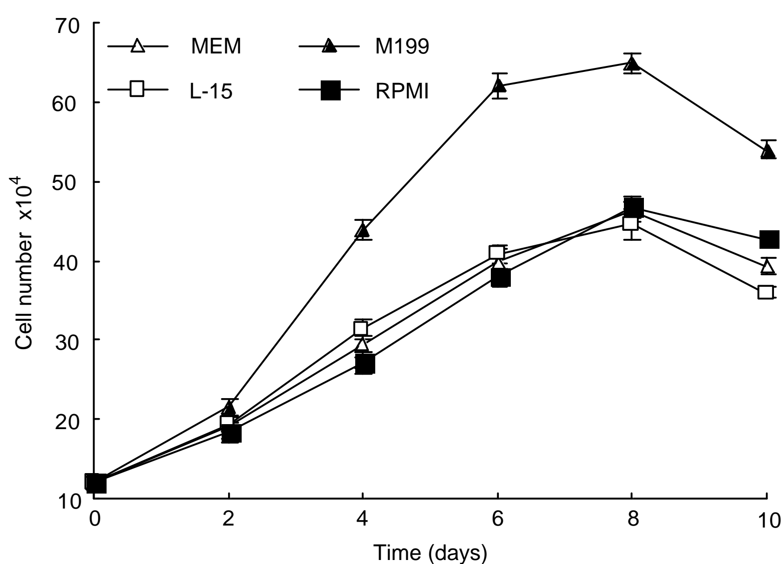

Figure 4. Comparative growth of white sturgeon body muscle cells (WSBM, passage 24) in selected growth media (MEM, M199, L15 and RPMI 1640) supplemented with 10% fetal bovine serum. Bars indicate standard deviations.

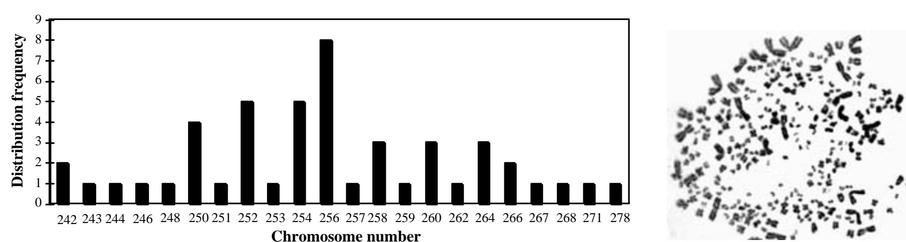

Figure 5. The frequency distribution of chromosomes of white sturgeon cells treated with colcemid at passage 17 (left panel) and phase-contrast photomicrograph of white sturgeon cellular chromosomes arrested in metaphase at passage 17 (Magnification 1000X) (right panel).

Figure 6. (Left panel) PCR amplification of 288-bp and 252-bp sequences of the white sturgeon genome using oligonucleotide primers designed from the conserved portions of the 16S and 18S genes, respectively. 200–300 ng DNA isolated from white sturgeon cells was amplified and then subjected to 2.0% gel electrophoresis. Lanes 1 and 3: WS-DNA and lanes 2 and 4: negative control. M = 100 bp ladder showing the range of 100 bp (bottom) to 500 bp (top). (Right panel) Nucleotide sequences of the 288-bp and 252-bp fragments amplified using oligonucleotide primers designed from the 16S and 18S genes of white sturgeon. Underlined portions represent positions of the PCR primers.

their susceptibility to WSIV and WSHV, and viruses isolated from other anadromous species.

Acknowledgements

This research was supported in part by funding from Kootenai Tribe of Idaho and the CTSA of Oceanic Institute, Honolulu.

Notes on suppliers

1. International Equipment Co., 300 2nd Avenue, Needham Heights, MA 02194, USA
2. Perkin Elmer, 850 Lincoln Center Drive, Foster City, CA 94404, USA
3. Fisher Scientific Co., 200 Park Lane Drive, Pittsburgh, PA 15275, USA
4. The Baker Company., Sanford, ME 04073, USA
5. Pacific Science Inc., 141 Sierra Street, EI Segundo, CA 90245, USA
6. Olympus Corporation, 4 Nevada Drive, Lake Success, NY 11042, USA
7. Cosmo Bio Co., Ltd., 4 Research Court, Rockville, MD 20850, USA
8. Revco, 275 Aiken Road, Asheville, NC 28804, USA
9. General Electric, Jamaica, NY 11423, USA
10. Lab-Line Instrument Inc., Melrose Park, IL 60160, USA
11. Harris Manufacturing, 275 Aiken Road, Asheville, NC 28804, USA
12. Sigma Chemical Co., St. Louis, MO 63178, USA
13. JRH Bioscience, 13804 West 107th Street, Lenexa, KS 66215, USA
14. Becton Dickinson Labware, 1 Becton Drive, Franklin Lakes, NJ 07417, USA
15. Invitrogen Life Technologies, 1600faaday Ave. Calsbad, CA 92008, USA.

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