



Isolation of cells from Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* and optimization of culture conditions

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ABSTRACT: We describe a method for fast and easy isolation of cells via trypsin digestion from larvae of Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* resulting in a stable, well-proliferating cell culture. The culture conditions for these cells were optimized with the aim of supporting the production of high amounts of biomass. To enhance cell growth and cell density, 4 different cultivation temperatures as well as commercially available carp serum (CS) and fetal calf serum (FCS) at different concentrations were tested and evaluated. Cell growth was measured via an impedance-based online cell-monitoring system (xCELLigence). These results showed the best cultivation temperature to be at 25°C and a media composition of Dulbecco's modified Eagle's medium (DMEM) supplemented with either 10 or 20% FCS or 5% CS. The cells were stable in the process of long-term cultivation over 33 passages and could be cryo-preserved. Immunocytochemical analysis revealed that the cells expressed proteins of different blastodermic layers. Ectodermic glia fibrillary acid protein, vigilin (mRNA transport protein), and pan cytokeratin were abundant. This fast-growing cell culture provides an important tool for research on Atlantic sturgeon populations.

KEY WORDS: Primary culture · Culture condition · Serum · Sturgeon · Teleost fish

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INTRODUCTION

Aquaculture and therefore fish culture is one of the fastest growing sectors in the food industry because of an increasing world population and excessively fished seas. One of the aims of fish culturing is to breed and cultivate fish to limit impacts on wildlife populations. However, various infectious diseases can occur in intensive stock breeding. In this context, easily manageable tools such as *in vitro* test systems, e.g. for Atlantic sturgeon-specific pathogens, and therefore optimal cell growth parameters are necessary.

The first fish cell line was established by Wolf & Quimby (1962). It originated from gonads of juvenile rainbow trout and was developed for studying fish viruses. During the last 4 decades, research has led to the establishment of cell lines from a wide range of

fish species and by 1980, 61 cell lines originating from 36 fish species from 17 families were reported (Fent 2001). Currently, 283 cell lines have been established from finfish around the world (Lakra et al. 2011). Recently, the Fraunhofer Research Institution for Marine Biotechnology has developed methods for culturing cells from larval fish and also from tissues (e.g. head, kidney, pancreas, brain, muscle, skin, heart and pyloric caeca) of adult fish from 11 mainly commercially important fish species (Ciba et al. 2008, Grunow et al. 2010, Langner et al. 2011).

Fish cell lines have many different applications, e.g. for viral diagnostics, use in ecotoxicology, carcinogenesis, genetic regulation and expression as well as DNA replication and repair (Hightower & Renfro 1988, Babich et al. 1991, Bols & Lee 1991, Bols et al. 2005). For these and other applications, the production of large amounts of cells derived from differ-

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ent fish species is important. These fish cells could also be used to isolate dietary supplements for the food industry, e.g. omega-3-fatty-acids. Furthermore, if fish cells could be produced in high abundance, they would have the biotechnological potential for fish meal production. Hence, this technology might reduce the need for wildlife catches.

Schaeffer (1990) described cell cultures of 2 types independent of the animal source. Primary cultures represent cells taken directly from the animal and are removed prior to any sub-culturing. Cell lines result from sub-culturing or passaging of the developed primary culture. In many cases cell lines can be continuous, namely those which grow indefinitely.

In contrast to cultivation of avian and mammalian cell lines, fish cell lines are usually cultured below 30°C. Many fish cell cultures grow well at room temperature (Wolf & Quimby 1977) and optimal growth for fish cell lines are in most cases well above the optimal temperature of the fish (Bols et al. 2005). Consequently, the amount of supplied carbon dioxide (CO₂) must also be adapted to these differing temperatures and to the individual pH of the prepared tissue of the donor fish. Apart from that, fish cell lines are grown similarly to mammalian cell lines using the same growth media, i.e. basal culture media supplemented with mammalian sera, such as fetal calf serum (FCS). Several researchers have worked on cell and tissue cultures derived from fish to estimate the optimal growth conditions (Bradford et al. 1994, Ghosh & Collodi 1994, Ciba et al. 2008).

In this study, we characterized the cells of Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* (Mitchill, 1815), a teleost of the northern hemisphere. Atlantic sturgeon is threatened globally due to massive overfishing, exploitation in Europe and river damming, regulation and pollution (Kottelat & Freyhof 2007). Therefore, this fish species is cultured in Europe.

The culture conditions of long-term cell lines generated from Atlantic sturgeon larvae have not been reviewed, in contrast to those of cell lines generated from white sturgeon *Acipenser transmontanus* (Wang et al. 2004). The purpose of this study was 2-fold: (1) to optimize the growth of the cells from an economical point of view and (2) to find a substitute for FCS by using a new commercially available serum from fish. Because of the phylogenetic distance between fish and mammals and their different blood compositions, we assumed that serum derived from fish would increase the proliferation of fish cell lines. In this study the dependence of cell proliferation on temperature and sera was evaluated via xCELLigence RTCA

(real fine cell analyzer) SP from Roche. This system monitors cellular events in real time without the incorporation of labels. It measures the electrical impedance and provides information about the biological status of the cells, including cell number, viability and morphology. Furthermore, the cells were characterized by immunochemistry.

MATERIALS AND METHODS

Tissue preparation and cell isolation

Two days after hatching 13 larvae from Atlantic sturgeon, weighing approximately 10 mg with a length of about 15 mm, were obtained from the Research Institute for Agriculture and Fishery in Mecklenburg-Western Pomerania, Born/Darß. The larvae were kept indoors at 10°C in tray incubators with a continuous flow of fresh water (0.15 m s⁻¹) and used the following day for preparation. Protocols for cell isolation have undergone an ethical review process by the German Animal Welfare Law §8a (ethic committee: Ministry for Agriculture, Environment and regions; permit number: 41/A01/09). For anaesthesia, the larvae were incubated for 5 min in a 20% MS222 (methanesulfonic acid salt; Sigma Aldrich) solution dissolved in aquarium water. The whole larvae were dissected with scissors and digested in 100 µl of 0.1% trypsin/EDTA (PAA Laboratories) for 1 to 2 min. Digestion was stopped by addition of a triple volume of DMEM (Dulbecco's modified Eagle medium; Gibco) supplemented with 20% FCS and followed by centrifugation for 5 min at 130 × g. The cell pellets were resuspended in DMEM-20% FCS supplemented with 100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin (Biochrom) and seeded into 6-well culture plates (TPP).

Cell culture

Cells of each larva were cultured in a 6-well plate in 2.5 ml of medium at 20°C and 2.0% CO₂. The medium was changed for the first time after 2 d and later on every third or fourth day. When confluence was reached, cells were sub-cultured at a ratio of 1:2 using 0.1% trypsin/EDTA in PBS (phosphate-buffered saline; PAA Laboratories). After detachment, the cells were centrifuged for 5 min at 130 × g. The achieved cell pellet was resuspended in fresh culture medium and seeded into new culture dishes. The first 5 passages of cells were continuously grown in DMEM-

20% FCS. Then, the medium was changed to DMEM-10% FCS.

In this study, experiments were performed with cells from only 1 larva from Passage 1 to 33. If frozen cells needed to be thawed, cells were sub-cultured at least twice before being used in experiments.

To measure the cell number and size (diameter), cells from Passages 9 and 32 were trypsinized, re-suspended in medium and centrifugated for 5 min at $130 \times g$. The cell pellet was resuspended in 3 ml medium and the measurements were performed following the instruction manual of the Cellometer Vision instrument (peqlab Biotechnology GmbH). Measurements from 3 independent cell cultures for both passages were performed. The mean value and the standard derivation were calculated.

Cryopreservation

For cryoconservation, trypsinized cells were resuspended in ice-cold freezing medium consisting of a 9:1 ratio of FCS:DMSO, transferred into a pre-cooled isopropanol freezing box and stored at -80°C overnight. The vials with the cells were transferred into vials which were stored in a pre-cooled isoproterenol freezing box at -80°C overnight. The vials were transferred into liquid nitrogen atmosphere. For re-seeding, frozen cells were thawed rapidly in 20°C culture medium, spun down for 5 min at $130 \times g$, resuspended and seeded in fresh culture medium. Cells from all 13 prepared larvae are stored in the German Cell Bank for Wild Animals 'Alfred Brehm', also called Cryo-Brehm.

Click-iT® proliferation test (EdU)

Cells from 2 different passages, Passages 10 and 33, were cultured for 2 d in chamber slides (starting cell number: 10.000). Triple replicates were performed for each passage. DMEM-10% FCS was supplemented with $10 \mu\text{M}$ EdU (5-Ethynyl-2-desoxyuridin; Click-iT® Edu Alexa Fluor 488 Proliferation assay; Gibco Invitrogen). EdU is a thymidin analogue and is inserted into the DNA during DNA synthesis instead of thymidin. The detection of EdU is based on the covalent linkage between the alkyne of the nucleotide and a fluorescence azide (Alexa Fluor 488). The staining procedure was carried out according to the manufacturers' instruction. The mean value and the standard derivation of EdU stained nuclei were calculated.

Optimum growth conditions and real-time cell electronic sensing

To investigate the effect of temperature on cell growth, the impedance of the sturgeon cells was determined at 4 different temperatures. Cells from Passage 16 to 22 were incubated at 16°C and 1.9% CO_2 , 20°C and 2.0% CO_2 , 25°C and 2.2% CO_2 or 28°C and 2.5% CO_2 . In these experiments, cell status was measured via impedance using the xCELLigence RTCA SP instrument (Roche Diagnostics GmbH). The impedance contains 3 factors: cell number, cell adhesion and cell size/morphology. It is expressed with the non-dimensional unit cell index (CI) and is defined as $\text{CI} = Z_i - Z_0$, where Z_i is the measured impedance value of each well at any time point i and Z_0 is the background impedance value for medium alone. The increase of the 3 described factors reflects the increase of impedance. In our study, we assumed that the CI can be seen as an indicator for proliferating cells because only healthy cells attach to the bottom, grow and proliferate. If a maximal CI was reached, cells were assumed to be confluent. At first, the xCELLigence RTCA was calibrated, which was followed by incubation with $100 \mu\text{l}$ of DMEM-10% FCS in a special 96-well microplate (E-plate, Roche Diagnostics GmbH) to set the blank value (Z_0). Subsequently, cell numbers were determined using a Nucleocounter® (IUL Instrument). Cells were seeded in $100 \mu\text{l}$ 10% culture medium at densities of 3×10^4 , 1.5×10^4 , 7.5×10^3 , 3.8×10^3 , 1.9×10^3 and 9.4×10^2 cells per 0.31 cm^2 into the E-plate for the first 12 h. This was followed by a medium exchange and the addition of different media compositions. Two different commercially available sera, serum from *Cyprinus carpio* (CS; D.A.P.) and standard FCS (PAA Laboratories) at different concentrations (5%, 10% and 20%) were tested. In this study, serum from *Cyprinus carpio* was used because no commercially available serum from *Acipenser oxyrinchus* could be found. As a control, cells were cultivated without sera supplement. In these trials, cells detached after 1 d, indicating the need for sera in cell proliferation.

In these experiments impedance was measured every 15 min over a period of 7 d. The impedance of each experiment was calculated as a mean value from triple replicates from 3 independent wells.

Immunocytochemistry

For immunocytochemistry, cells from Passage 17 were seeded on chamber slides and cultured for 2 d.

Cells were washed twice with PBS and fixed with 7:3 methanol:acetone containing 1 mg ml⁻¹ DAPI (Roche) for 5 min at room temperature (T_R). Afterwards, cells were washed 3 times in PBS. After incubation with 1.7% normal goat serum at T_R for 20 min, the samples were incubated with interspecies cross-reactive primary antibodies in a humid chamber for 1 h at 37°C.

These antibodies are directed against: (1) pan-cytokeratins containing CK1, CK4, CK5, CK6, CK8, CK10, CK13, CK18, and CK19 (monoclonal mouse, 1:100; catalog number [CN] C2562; Sigma-Aldrich®), which stain epithelial filamentous structures in the cells; (2) glial fibrillary acidic protein (GFAP), a marker for ectodermic structures (rabbit polyclonal, 1:100; CN Z0334; Dako); (3) alpha smooth-muscle actin (alpha-SMA), a marker for mesoderm structures (mouse monoclonal, 1:100; CN M0851; Dako); (4) MF20, a banded muscle marker (mouse monoclonal, 1:50; CN MF20; DSHB); (5) alpha-fetoprotein (AFP), which is a glycoprotein located in the cytoplasm (mouse monoclonal, 1:500; CN sc-8399; Santa Cruz); (6) amylase, an enzyme which catalyses glucan into sugars (rabbit polyclonal, 1:100; CN 171534, Calbiochem), (7) vigilin, a marker which is present during transcription (polyclonal anti-vigilin antiserum FPIII, rabbit, 1:200 Kruse et al. 1996); (8) stage-specific embryonic antigen 1 (SSEA-1), a marker for stem cells and located at the cell surface (monoclonal mouse, 1:50, CN MAB 4301; Chemicon), (9) Ki67, a proliferation marker (rabbit polyclonal 1:500, CN ab 15580; abcam®) and (10) nanog, a stem cell marker (monoclonal mouse, 1:1000, CN AB 5731; Chemicon). GFAP, alpha-SMA, and MF20 are all filamentous structures, and both Ki67 and nanog are located in the nucleus.

After rinsing 3 times in PBS, slides were incubated for 1 h at 37°C with the secondary antibody (FITC-labeled anti-rabbit IgG 1:200 and CY3-labeled anti-mouse IgG 1:400; Dianova). Slides were washed 3 times in PBS, covered in Vectashield mounting medium (Vector) and analyzed with a laser scanning microscope (Zeiss).

RESULTS

The aim of this study was the identification of optimal cultivation conditions such as growth temperature, seeding cell number and sera for larvae cells from Atlantic sturgeon as well as a first characterization of these cells via immunocytochemistry.

Cell culture

Directly after isolation a suspension of cells and some tissue fragments were obtained. Within 2 d, cells and tissue fragments adhered to the bottom of the plastic dish. Cells started to proliferate grow out from the attached tissues during this passage (Fig. 1a). Cells and tissue fragments that did not adhere were removed during the first medium exchange 2 d after preparation. After tissue dissociation, cells showed different shapes and sizes in the first 3 passages. Here, various morphologies could be found, ranging from spindle-shaped to round and very flat cells (Fig. 1a). With increasing numbers of passages the cell morphology became more uniform (Fig. 1b,c). In the first 2 passages 3D cell layers were present, whereas afterwards the cells grew in a monolayer.

No change in the proliferation rate was observed following the changeover from 20% FCS to 10% FCS in Passage 5. The strain of passaging by trypsination and freezing and thawing did not significantly reduce the number of cells or affect the vitality of the cells. To date, the cell population could be passaged and propagated over 3 yr with up to 54 passages.

Cell size and proliferation

The analysis with the Cellometer Vision instrument revealed that the cell size changed over time. In early passages (Passage 9), 87% of the measured cells were between 9 and 19 µm while in later passages (Passage 32) only 60% had a size between 9 and 20 µm. In this passage the cells were more heterogeneous in size (Fig. 2a). The mean cell size (19 µm) and range of sizes (10 to 33 µm) in Passage 32 were both significantly greater than those in Passage 9, where the mean cell size was 14 µm and the range 9 to 28 µm (Fig. 2b). Cells in the first passages displayed smaller sizes than those in higher passages.

The proliferation assay was performed via EdU test in Passages 10 and 33 (Fig. 2c). EdU is a direct method of measuring de novo DNA synthesis or S-phase synthesis of the cell cycle. The staining demonstrated that in Passage 10 27% of the cells divided in 2 d whereas in Passage 33 S-phase synthesis could be determined in 22% of the cells. Statistical analysis demonstrated a significant decrease in the proliferation rate from Passage 10 to 33 ($p < 0.05$; t -test, Graph Prism 5). Cells from Passages 16 to 20 were subjected to further analysis.

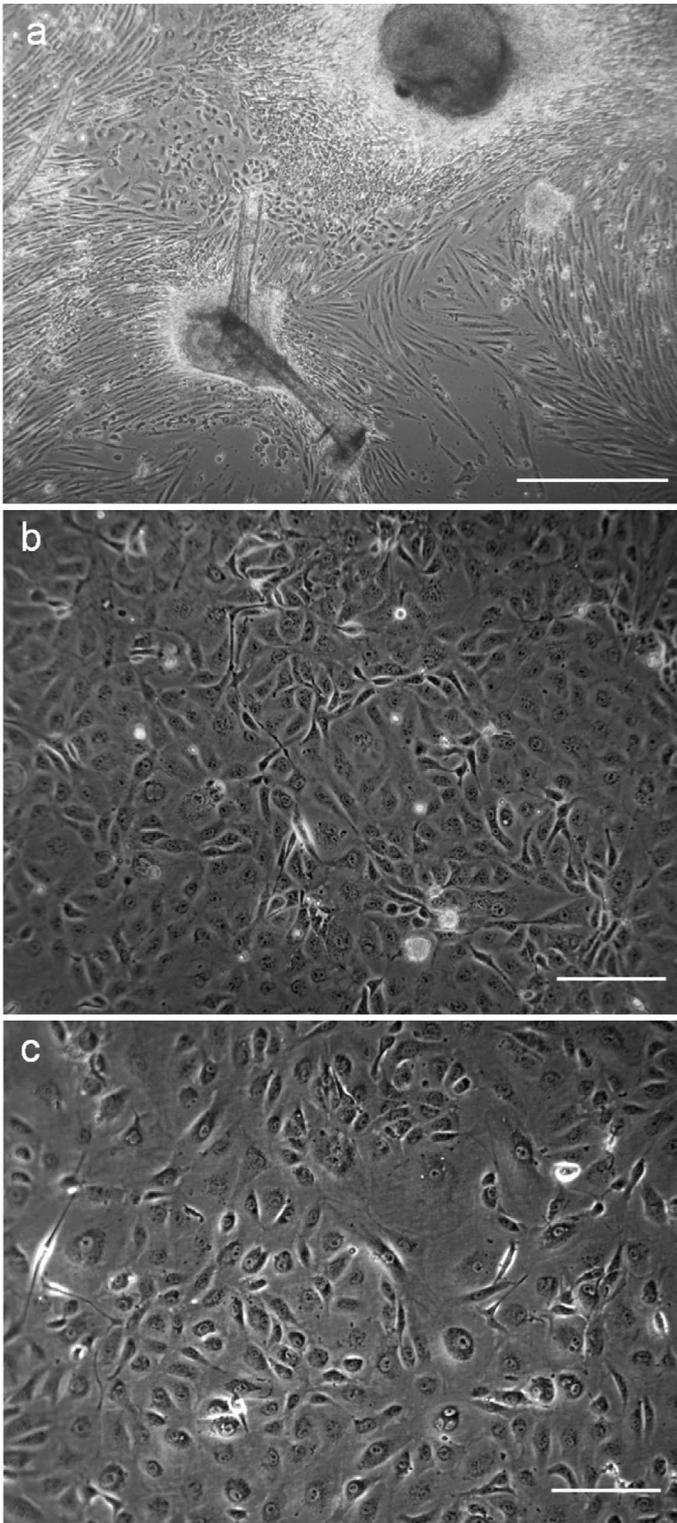


Fig. 1. *Acipenser oxyrinchus oxyrinchus*. Atlantic sturgeon cell cultures from primary culture through passaging. (a) In the primary cell culture different morphologies from spindle-shaped to round cells appeared, while in (b) Passage 9 and (c) Passage 32 cells exhibited more homogenous morphologies. Scale bars = 50 μm

Effects of temperature, cell density and serum on proliferation

The cells kept at 16°C were found to be growing much more slowly (doubling time 382 h) than cells cultured at 20°C (doubling time 110 h) and 25°C (doubling time 100 h). The lowest temperature tested (16°C) is still a suitable temperature for proliferation, but when compared to the nearly 4 times faster proliferation at 20 and 25°C, lower temperatures such as 16°C are not optimal for cell growth. Cells incubated at 28°C died, which was confirmed by measurements of cell impedance and microscopic documentation (data not shown). Thus, we could demonstrate that cells incubated at 28°C were kept above their tolerance limit.

To verify the results of the optimal cultivation temperature for the Atlantic sturgeon cell line, seemingly between 20° and 25°C, we examined the cell impedance via xCELLigence. The initial cell number for optimal growth was surprisingly dependent on the temperature. In total, the results show that at higher temperatures, fewer cells were necessary to achieve the maximal cell index (Fig. 3a). The highest cell indices and therefore the optimal growth conditions could be obtained by seeding 1.9×10^3 cells well^{-1} at 25°C (Fig. 3) with a CI of 19.68 after 35.22 h of incubation. In comparison at 20°C the highest impedance level was achieved with 1.5×10^4 cells well^{-1} , which displayed a CI of 18.7 after 96 h, which was 5.3% lower than at 25°C (Fig. 3a). Additionally, seeding low cell numbers was found to result in non-optimal cell growth. At 25°C and a seeding cell number of 937 cells well^{-1} , achieving the same maximal CI of around 19 took twice as long (75 h) (data not shown).

The analysis of different sera and sera concentrations was performed with 1.9×10^3 cells well^{-1} and revealed that cells incubated with either DMEM-20% FCS or DMEM-10% FCS reached a maximal CI more quickly than those incubated with DMEM-20% CS (Fig. 3b). The incubation with DMEM-5% CS resulted in the second highest maximal CI. In contrast, the approach with 10% CS showed lower CI. In total, optimal cell growth for the sturgeon cell line was achieved with 10 and 20% of FCS as well as 5 and 20% CS, which induced nearly the same proliferation rate, indicating that carp serum could be used as a substitute for FCS.

Immunocytochemistry

As cells have proliferated over a long time period (3 yr and 54 passages up to now) without showing clear signs of senescence, stem cells could have been

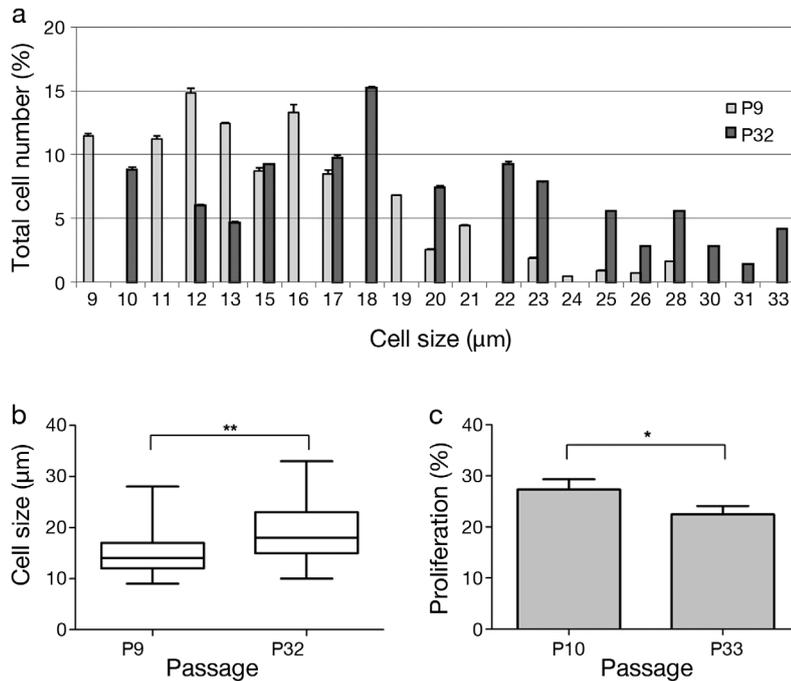


Fig. 2. *Acipenser oxyrinchus oxyrinchus*. (a) Mean (\pm SD) cell size distribution of cultivated Atlantic sturgeon larval cells in Passages 9 and 32 measured via Cellometer Vision for 3 independent cell cultures. Most of the cells in Passage 9 were between 9 and 19 μm , whereas cell distribution in Passage 32 was more heterogeneous. Total mean cell number of the 3 replicates in Passage 9 was between 1.38×10^5 and 1.43×10^5 cells and in Passage 32 between 7.0×10^4 and 7.5×10^4 cells. (b) Mean size of cells of 2 stages in the passage. Minimum and maximum (crossbars at the end), median (line in bar) and upper and lower quartile (borders of the bar) values are illustrated. Cell size in Passage 32 was significantly greater than that in Passage 9 (Graph Prism 5). (c) Mean (\pm SD) proliferation of Atlantic sturgeon cells in Passages 10 and 33 measured via EdU (5-Ethynyl-2-desoxyuridin) test from 3 independent cell cultures. Cells in different passages exhibited significantly different proliferation rates (Graph Prism 5). **p < 0.001, *p < 0.05

isolated from the sturgeon larva. One of the characteristics of stem cells is their ability to differentiate into the 3 blastodermic layers. To test this hypothesis, 2 antibodies for markers of every blastodermic layer as well as stem cell and proliferation markers were chosen. Cells were immunopositive for panCK as ectodermic marker, GFAP (endoderm) and the RNA-binding protein vigilin (Fig. 4). As expected, vigilin, generally involved in translational processes, was

present in the cytoplasm of almost all cells. The glia stem cell marker GFAP showed the typical filamentous structure, interestingly in a high number of cells.

Unfortunately, the other antibodies used (against AFP, amylase, alpha SMA, MF20, SSEA-1, Ki67 and nanog) did not bind or did not bind specifically, as the labeled structures did not coincide with the morphological organization of the protein (data not shown).

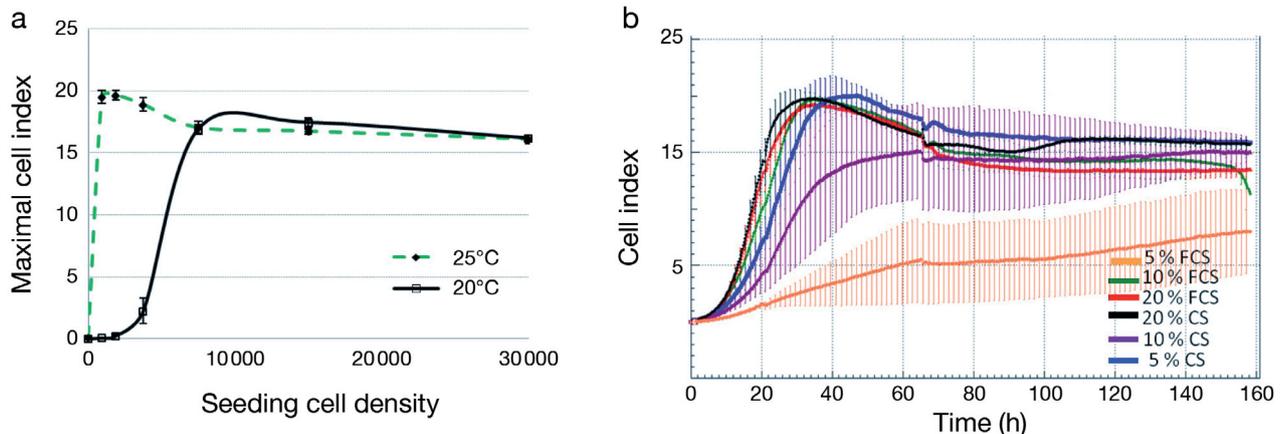


Fig. 3. *Acipenser oxyrinchus oxyrinchus*. Dependency of the maximal cell impedance on different temperatures and seeding cell numbers. Cell index (CI) can be seen as an indicator for cell proliferation. (a) Summarized presentation of the mean (\pm SD) maximal CI from 3 independent cell cultures that used different starting cell numbers (9.4×10^2 , 1.9×10^3 , 3.8×10^3 , 7.5×10^3 , 1.5×10^4 and 3×10^4 cells well^{-1}) cultivated at (green dashed line) 25°C and (black continuous line) 20°C in DMEM-10% fetal calf serum (FCS) at Passage 16. At 25°C fewer cells were necessary to achieve maximal CI. In sum, at 25°C and a starting density of 1.9×10^3 cells well^{-1} , the highest CI was achieved. (b) Cell proliferation assay of the best sera concentrations for achieving the fastest mean (\pm SD) cell proliferation at 25°C with a starting cell density of 1.9×10^3 cells well^{-1} . Cells showed a continuous proliferation after adhesion. Cells cultivated with 10 and 20% FCS as well as those with 5 and 20% carp serum (CS) achieved the highest CI in nearly the same time. A steady state was reached after 90 h (exception: cultivation with 5% FCS)

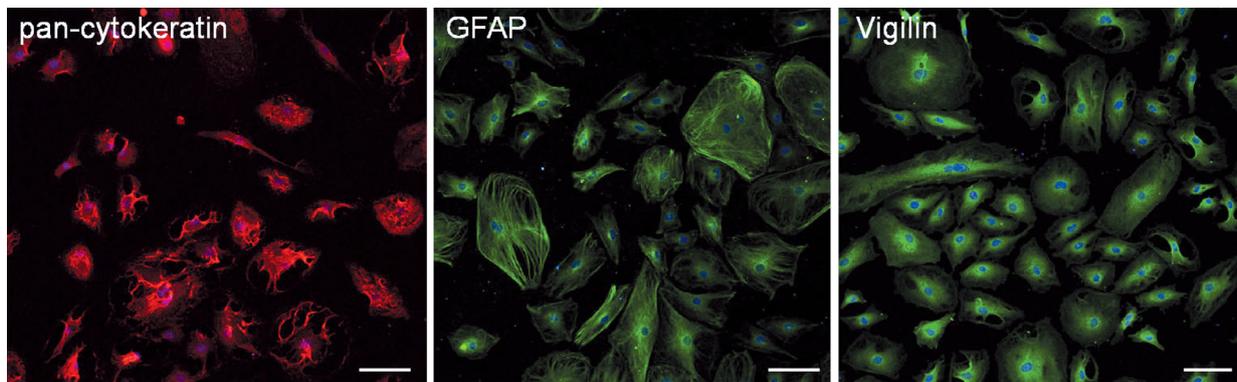


Fig. 4. *Acipenser oxyrinchus oxyrinchus*. Immunocytochemical analysis of isolated sturgeon cells from larval tissue. This cell population showed spontaneous expression of pan cytokeratin, glial fibrillary acidic protein (GFAP) and vigilin. Nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI). Scale bars = 100 μ m

DISCUSSION

In this study, an Atlantic sturgeon cell line that was cultivated in conventional medium (DMEM) and supplemented with mammalian serum (FCS) is described. The cell line was derived from a larva in its yolk-sack stage by trypsin digestion.

Cell size and size range were smaller in an early cell passage (P9) than in a later passage (Passage 33). Furthermore, cells of early passages (Passage 10) showed a significantly higher proliferation rate in comparison to Passage 33. Nevertheless, this cell line was successfully trypsinized up to Passage 54 and is still proliferating, which might be explained by a high constitutive telomerase activity in adult organs in fish. Bols et al. (2005) described this phenomenon and specified this interesting topic for future research in order to understand spontaneous immortalization in fish cell lines.

A comparison of cell proliferation at different temperatures indicated that cells from Atlantic sturgeon have a wide temperature tolerance. The analysis showed that there was an increase in the cell number between 16°C (very low proliferation) and 25°C (optimal growth temperature), while at 28°C the cells detached and died. In further studies, an examination of heat shock proteins should be done to analyze the temperature tolerance and the cell reaction on temperature changes. As already described, several studies in fish cell cultures showed that optimal cell growth occurs at temperatures slightly higher than the temperature of the donor (Ott 2004, Bols et al. 2005). The optimal temperature of the intact fish Atlantic sturgeon is between 18 and 23°C. For that reason, the established cell line was first adapted to 20°C and later to 25°C. Additionally, Bols et al. (2005) and Ott (2004) reported that fish cells cultivated

below their optimal temperature exhibit little to no growth. An increase in the temperature to optimal conditions resulted, after the adaptation of the cells, in an increase in the proliferation rate. Furthermore, cells from Atlantic sturgeon show a higher tolerance for colder than for warmer temperatures. Cells died at 28°C, which is only 3°C more than the measured optimal cultivation temperature.

Our analysis also demonstrates the importance of the optimal cell seeding density. Essential cell-to-cell communication *in vitro* is not possible if too few cells are seeded. Cells secrete cytokines as well as growth factors and hormones that activate the proliferation of other cells. For this reason, low cell densities are associated with low concentrations of growth factors and hence lower cell proliferation rates. In contrast, too many cells have the opposite effect due to shortages of space that result in contact inhibition between cells and accelerated consumption of the medium and its supplements, like sera. In our study, the optimal seeding density was reached with around 1.9×10^3 cells well⁻¹ for a 96-well plate at 25°C.

The next experiment presented the importance of the type and concentration of the serum. Fetal calf serum (FCS) and carp serum (CS) at 5%, 10% and 20% were compared in order to detect the optimal supplement concentration for sturgeon cells. Cells incubated with 20% or 10% FCS were found to have the highest CI. Another effect was observed when using low sera concentration. Here, a high CI was observed with CS, but not with FCS. The poor growth of cell lines in DMEM-5% FCS has already been shown in a previous study (Colyer & Boyle 1985). In total, the measurements showed no clear increase in CI when using fish sera but suggested the possibility of using a lower concentration of serum. The reason for the different growth is speculative,

because no complete information about the sera composition was available. Kondo & Watabe (2006) suggested that carp serum may have especially high densities of lipoprotein, albumin-like proteins and other possibly not yet identified growth factors, which could have an important role in fish cell growth. The FCS used had 73.2% albumin in its total protein amount. In contrast to that of mammals, where no isotypes of albumin are known, 5 different albumin-like proteins were found in carp serum. The low weight albumins seem to be the most important factor in fish cell proliferation. Furthermore, Kondo & Watabe (2006) found that the plasma proteins of fish consist of 30% lipoproteins, which are necessary for the energy metabolism. In the datasheet supplied with the commercially bought FCS, no information about lipoprotein composition was supplied.

Although using carp serum in a lower concentration is possible, the cell index for this serum was not significantly higher than that with 10% FCS. Because the price of CS is considerably higher (~15 times) than that for FCS, FCS would be the recommended medium for the cultivation of Atlantic sturgeon cell lines.

There are further essential parameters for cell propagation, like additional growth factors (Hinsch & Zupanc 2006), and a number of unknown substances in the serum that differ from batch to batch (Sotiropoulou et al. 2006). The growth medium is also an important factor. Three types of media formulations are popular for the propagation of fish cells: Eagle's MEM, Medium 199 and Leibovitz L-15 medium. Eagle's MEM has been the preferred choice of most fish tissue culturists; therefore almost all present cell lines were initially propagated using this medium (Fryer et al. 1965, Fernandez et al. 1993). Hence, in this study Eagle's MEM was chosen for analysis. However, the most substantial factor for cell growth is the cell itself. Cells change their function, morphology and proliferation rate during culturing (Ciba et al. 2008, Neuhuber et al. 2008). These changes require an alteration of culture conditions. Bradford et al. (1994) described that primary cell cultures need higher serum concentrations and seeding densities than cells in later passages. Therefore, determination of the optimal cell growth is always an important factor, because suboptimal conditions may result in apoptosis. Furthermore, the high tolerances of teleost cell lines concerning their culturing temperatures make them very attractive to various applications in biological research (Fernandez et al. 1993).

Cell cultures have been successfully employed as alternatives to animal experiments with whole organisms. Also, fish are used for animal experi-

ments. In 2008, 112000 fishes were officially used for toxicological analysis, safety tests for products in human medicine and fundamental research in Germany. Many of these tests could be performed using cell cultures. Still, only 43 of 283 finfish cell lines are listed in international cell repositories like ATCC (American Type Culture Collection) or ECACC (European Collection of Cell Cultures) (Lakra et al. 2011). For use in biotechnology, a characterization of cell types is necessary. In our study, immunostaining showed that proteins of the ectodermic layer (GFAP and panCK) were present in the cells of our culture from whole larva. Furthermore, vigilin, a protein involved in translational processes, could be detected in all cells. Unfortunately, because no antibodies specifically directed against proteins from Atlantic sturgeon are available, mammalian antibodies had to be used. Only 3 of the chosen antibodies showed a clear cross-reactivity with proteins from sturgeon. With these, even after 17 passages a mixture of several differentiated cell types could be detected. Although some cells, e.g. cardiac cells, once differentiated are no longer capable of dividing, others, especially epithelial cells, expressing a wide array of cytokeratins, are able to proliferate upon injury *in vivo* (Cooper 2000). In another study (Garcia et al. 2004), GFAP-expressing progenitor cells from the mouse forebrain were found to be the main source of adult neurogenesis. Thus, these cell types are possibly stimulated to proliferate after dissociation of the tissue of a whole larva and propagation of this mixed cell culture *in vitro*. However, our study showed that this cell line has an enormous capability to proliferate and other cell types with high proliferation capacity, such as progenitor or stem cells, might be present. To confirm this assumption, markers for these cell types would have to be developed for the commercially important yet phylogenetically old order of the Acipenseriformes, as cross-reactivity of antibodies against mammalian proteins seems to be low.

Further work is also needed in order to characterize the different cell types present in the culture, their individual proliferation capacities, their potential to de-differentiate or perform transitions into other cell types and their karyotypes, as this is an indication towards a possible malignant transformation. Before listing piscine cell lines in an international cell line database and using them commercially, more molecular tools, like antibodies or genomic sequences, for expression analyses are needed in order to render these valuable cell cultures a more useful and common source of experimental material for fish research.

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