New cell line from adipopancreatic tissue of Atlantic herring *Clupea harengus*

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ABSTRACT: We report the isolation, cultivation, and cryopreservation of cells from adipopancreatic tissue of adult Atlantic herring *Clupea harengus*. The cell population was cultured for >1 yr and had a mean doubling time of 2 wk. Immunocytochemical analyses revealed that >50 % of cells contained glial fibrillary acidic protein and pan-cytokeratins. Vigilin, a well-conserved protein related to mRNA transport, was found in only 11 % of cells. Alpha smooth-muscle actin and stage-specific embryonic antigen 1 were even less abundant (<5 %). Although little is known about tissue homeostasis and regeneration in adult *C. harengus*, the heterogeneity and long-term proliferation of the cell population described here suggest that it may contain stem cells or progenitor cells. Before the present study, there was only one *C. harengus* cell line available to researchers. The newly established cell line described in the present study represents an important addition to scientific investigation as well as aquaculture industries and toxicological/virological testing of herring populations.

KEY WORDS: Herring · *Clupea harengus* · Long-term fish cell culture · Fish cell line · Fish · Cryopreservation · Stem cells · Adipopancreatic tissue

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INTRODUCTION

In modern aquaculture, it is a common problem that high population densities and inbreeding promote the spread of fish diseases. New cell lines and model systems are needed, especially for economically important fishes. Another challenge in aquaculture is to find adequate nutrition sources for carnivorous fishes. Currently, common feeding regimes use fish meal, which often leads to the absurd situation that more fish are fed than can be harvested (Naylor et al. 2000). Long-term proliferative cell cultures may offer a solution for both problems: they can be used to establish new cell lines that can be used to investigate diseases and drugs, and prospectively, may be a means to produce high-quality feed in large-scale cell reactors to reduce the use of fish meal. Also, highly unsaturated fatty acids, which are currently added to the diet for the fish, could be provided by fish cell culture, as proposed and demonstrated by Tocher & Dick (1990).

The first fish cell lines were established in the 1960s to study viral diseases of fish (e.g. Gravell & Mals-

berger 1965, Wolf & Vestergard Jorgensen 1970). More recently, fish cells have been used in biomedical research (Hightower & Renfro 1988, Cole et al. 1997), and biotechnology (Bols 1991, Li et al. 1998, Galina et al. 2009), as well as in toxicology (Babich & Borenfreund 1991, Schirmer et al. 1997, Segner 1998, Lee et al. 2009). Endocrine and genetic studies have been carried out using fish cell lines, e.g. to examine changes in the genome caused by viral diseases (Wise et al. 2009).

In the last few years, accumulation of expert knowledge about fish cell culture, along with advances in aquaculture, have led to the establishment of several new cell lines from economically important fishes (Bols 1991, Bejar et al. 1997, Segner 1998, Villena 2003, Ciba et al. 2008, Fan et al. 2010, Lakra et al. in press). Examples are caudal fin cells from gilt-head sea bream *Sparus aurata* (Bejar et al. 1997), neural stem cells from adult seabass *Dicentrarchus labrax* (Servili et al. 2009), muscle and fin cells of bluefin trevally *Caranx melampygus* (Zhao & Lu 2006), gill cells from rainbow trout (Lee et al. 2009), and head kidney cells from Atlantic salmon *Salmo salar* (Dannevig et al. 1995).

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However, there is only one herring cell line described to date (Ganassin et al. 1999), and only one established cell line from fish pancreas (Pilz & Plantikow 1991).

In the present study, we describe the first long-term growable cell line derived from the adipopancreatic tissue of *Clupea harengus*. It has been demonstrated previously by Kruse et al. (2004, 2006) and others (Seaberg et al. 2004, Seeberger et al. 2006, Gorjup et al. 2009, Rapoport et al. 2009) that proliferative cell populations can be obtained from exocrine pancreatic tissue with extraordinarily high yields. The preparation method used by Kruse et al. (2004) to isolate these cells has been subsequently expanded towards other species, such as Burchell's zebra *Equus quagga burchellii*, marine otter *Lontra felina*, and snow leopard *Uncia uncia* (cells of these 3 species were stored in the 'Alfred Brehm' German Cell Bank for Wild Animals, also called Cryo-Brehm) (Lermen et al. 2009, Ciba et al. 2010). From these studies, we concluded that exocrine glandular tissue might generally be a potent source to obtain long-term growable cell cultures, also from fishes.

In herring, the adipopancreatic tissue is located between the appendices pyloricae (Reichenbach-Klinke 1970); the pylorus itself is situated at the transition between stomach and intestinal tract (Reifel & Travill 1978). The pylorus can be easily identified by virtue of its caeca, which appear as finger-like pouches (Buddington & Diamond 1987, Domeneghini et al. 1999, Sanden et al. 2005, Marchetti et al. 2006). Adipopancreatic tissue was removed by dissection of the pylorus. Cells from these dissections were singularized by an enzymatic reaction and were used to establish the stable *in vitro* cultures described in the present study. We characterized the cells by morphology, proliferation rates and immunocytochemistry. We found the cells to be stable in the processes of long-term cultivation and cryopreservation.

MATERIALS AND METHODS

Tissue preparation and cell isolation. Three adult herring *Clupea harengus* from wildlife stock were obtained from the River Trave in Germany, from a local fisherman. Adipopancreatic tissue was isolated and transferred into digestion medium containing HEPES-Eagle medium (pH 7.4), 0.1 mM HEPES buffer (pH 7.6; Roth), 70 % (v/v) modified Eagle medium (Invitrogen), 0.5 % (v/v) Trasylol (Bayer), 1 % (w/v) bovine serum albumin (BSA; PAA Laboratories), 2.4 mM CaCl₂, and collagenase (0.63 U mg⁻¹; Serva). The tissue was minced into small pieces (approx. 1 mm³) using surgical scissors and incubated for 20 min at 37°C with constant shaking (150 cycles min⁻¹). Digestion medium together with fat tissue was removed, and after rinsing

with isolation medium (digestion medium without collagenase), the remaining tissue fragments were dissociated by up- and down-suction (shear forces) through glass pipettes (Roth) with progressively more restrictive openings (20 ml, 10 ml, 5 ml), resulting in a single cell suspension. Afterwards, the cells were centrifuged for 5 min at 20°C and 115 × *g*, then resuspended and seeded in culture medium (Dulbecco's modified Eagle medium [DMEM]; Gibco®, Invitrogen) supplemented with 20 % fetal calf serum (FCS; PAA Laboratories), 100 U ml⁻¹ penicillin (Biochrom), and 0.1 mg ml⁻¹ streptomycin (Biochrom).

Cell culture. Cells were cultured in 25 cm³ culture flasks (TPP) in standard culture medium at 20°C and 2 % CO₂. The medium was changed 2 d after seeding, and subsequently on every 4th day. The cells were subcultured ('passaged') in a 1:1 ratio as soon as confluency was reached using trypsin (0.5 mg ml⁻¹) and EDTA (0.22 mg ml⁻¹) (PAA Laboratories). Total cell number and dead cells were counted using the NucleoCounter® NC-100+ (Chemometec). If needed for seeding, frozen cells were thawed rapidly in 20°C culture medium, spun down for 5 min at 115 × *g*, and resuspended and seeded in culture medium. For immunocytochemical studies, cells were transferred onto 2-well chamber slides (BD Biosciences) 3 d before examination.

Cell proliferation assay. The herring cells were seeded in 100 µl of 20 % culture medium at densities of 2 × 10⁴, 1 × 10⁴, 5 × 10³, and 2.5 × 10³ cells 0.31 cm⁻² into a 96-well microtiter plate with electrodes. These electrode plates are part of a commercially available impedance-based online cell-monitoring system (Xcelligence, E-Plate-96; Roche). In this system, cell status is represented by the relative change in electrical impedance. The arbitrary unit of this system is called the cell index (CI). It is defined as $CI = Z_i - Z_0$, where Z_i is the cell-electrode impedance of the well when it contains cells at an individual time point during the experiment, and Z_0 is the background impedance at the start of the experiment of wells containing medium only (Roche Applied Science 2008). Z_i is a complex parameter, the value of which depends simultaneously on cell number, cell size, and confluency of the cell culture. In this experiment, impedance was measured over a period of 4 to 6 d to monitor cell proliferation. At the beginning of the experiment, 1 measurement was taken as blank. After 30 min, the attachment, spreading, and proliferation of the cells were monitored every 15 min with RTCA 1.2.0.0909 software (Roche). Cells were cultured at 16 and 20°C (2 % CO₂) to investigate the influence of temperature on cell growth.

Cryopreservation. Trypsinated cells were suspended in 4°C freezing medium consisting of 90 % FCS and 10 % dimethyl sulfoxide (DMSO; Roth) and transferred into cryovials. The vials were immediately

conveyed into a precooled isopropanol-freezing box (Nalgene), which, in a -80°C freezer, achieves a cooling rate of $1^{\circ}\text{C min}^{-1}$. After 1 night of cooling/storage at -80°C , the vials were transferred into the liquid nitrogen atmosphere at the Cryo-Brehm, a cell bank for cryopreservation and archiving of proliferative cells obtained from wild animals.

Immunocytochemistry. For immunostaining, cells of Passage 7 cultivated on chamber slides were rinsed with phosphate-buffered saline (PBS), fixed with a mixture of methanol and acetone (7:3) containing $1\text{ }\mu\text{g ml}^{-1}$ DAPI stain (Roche) for 5 min at room temperature, and washed 3 times in PBS. After incubation with 1.7% normal goat serum at room temperature 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: 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 the epithelial filamentous structures in the cells; vimentin, a marker which shows the transcriptional level of the cells (polyclonal anti-vimentin antiserum FPIII, rabbit, 1:200; Kruse et al. 1996); nestin, a stem cell marker as well as a marker for neuro-epithelial cells (rabbit polyclonal, 1:250; CN: ab5968; Abcam®); glial fibrillary acidic protein (GFAP), a marker for ectodermal structures (rabbit polyclonal, 1:100; CN: Z0334; Dako); alpha smooth-muscle actin (alpha-SMA), a marker for mesodermal structures (mouse monoclonal, 1:100; CN: M0851; Dako); Ki-67, a proliferation marker located in the nucleus (rabbit polyclonal, 1:500; CN: ab 15580; Abcam®); octamer-binding transcription factor 4 (Oct-4), a stem cell marker also located in the nucleus (monoclonal mouse, 1:50; CN: MAB 4305; Chemicon); 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); and MF20, a banded muscle marker (mouse monoclonal, 1:50; CN: MF20; DSHB). Nestin, GFAP, alpha-SMA, and MF20 are all filamentous structures. After rinsing 3 times with PBS, the slides were incubated with secondary antibodies in a humid chamber for 1 h at 37°C . The following secondary antibodies were used: cyanine-3 (Cy3)-labeled anti-mouse IgG (1:400; Dianova) and fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (1:200; Dianova). Slides were washed 3 times in PBS, covered in Vectashield® mounting medium (Vector Laboratories), and analyzed under a fluorescence microscope (Axioscope 2; Zeiss). The images were captured using an AxioCam MRC5 (Zeiss) and processed with AxioVision40 software (version 4.7.1). Negative control was carried out with the secondary antibody alone. As a positive control, goat submandibular gland cells,

human parotid gland cells, Schwann cells isolated from rat nervus ischiadicus, and oocyte-like cells (rat) were used. For quantitative analyses, 3 representative micrographs each were analyzed by determining the fraction of marker that was positive out of 100 to 200 cells. This image analysis was carried out 3 times for each marker protein and the average of the 3 values was calculated.

Database-assisted sequence analysis. With the basic local alignment search tool (BLAST, UniProt Knowledgebase, www.expasy.ch) the sequences of *Danio rerio*, *Salmo salar*, *Oryzias latipes* and *Carassius auratus* were compared to those of human and mouse to determine the binding specificity of the antibodies used for immunocytochemistry. The 4 fish species were chosen because no sequences of *Clupea harengus* were available in the BLAST program.

RESULTS

Isolation and cultivation

The isolated primary cells attached to the bottom of the cell culture flask but proliferated initially very slowly. Only 1 cell line reached confluency 6 wk after preparation. The cells were split, and after 3 passages, the cell proliferation rate had accelerated to a doubling time of roughly 2 wk and the cells grew continuously in standard DMEM supplemented with 20% FCS. Trypsinization of the cells for passaging did not cause a significant loss of viability (roughly 10% dead cells). Fig. 1 displays the morphology of the cell population under bright field microscopy. In Fig. 1a, cells of Passage 0 are shown. Polygonal-shaped cells were mingled with large, rather round cells containing filamentous structures (Fig. 1d). In Passage 2 (Fig. 1b), lipid inclusions were observed in some of the cells (Fig. 1c). The quantity of cells with lipid inclusions decreased during cultivation (Fig. 1d).

Cell proliferation assay

At 20°C , high seeding densities (2×10^4 and 10×10^3 0.31 cm^{-2}) led to growth inhibition; after attachment, the CI basically remained constant (Fig. 2a). Cell proliferation was observed at lower seeding densities; after 96 h, the CI reached a plateau. This plateau was almost 2 times higher for the 5×10^3 cells 0.31 cm^{-2} seeding density than for 2.5×10^3 cells 0.31 cm^{-2} . At 16°C , proliferation seemed to stagnate. Regardless of the seeding density, a plateau phase was reached quickly (after 24 h) and no changes in CI occurred thereafter (Fig. 2b).

Fig. 1. *Clupea harengus*. Morphology of subcultured cells from adipopancreatic tissue. (a) Primary cell population in Passage 0. (b,c) Cells of Passage 2. The inset in (c) depicts lipid inclusions; the number of cells with lipid inclusions decreased in the higher passages. (d) Cell population in Passage 7; from this passage on, morphological appearance remained basically unaltered. The inset in (d) displays the typical filamentous structures of the final cell population. Scale bars = 100 μm

Cryopreservation

Freezing and thawing did not lead to a significant loss in cell viability: 85 % of the frozen cells were viable after thawing. Three days after thawing and re-seeding, cells started to proliferate again.

Immunocytochemistry

The glial stem cell marker GFAP showed an interaction in 54 % of cells (Fig. 3e). The RNA-binding protein vigilin was present in the cytoplasm in 11 % of cells (Fig. 3c). Interaction of the alpha-SMA antibody with the typical filamentous structures was found in only a few cells (Fig. 3a). *Clupea harengus* cells were 78 % pan-cytokeratin-positive (Fig. 3g), while only 3 % of cells were SSEA-1-positive (Fig. 3i). The nestin antibody showed no positive interaction (not shown). Also, MF20 (a marker for sarcomeric myosin), the transcription factor Oct-4, and the proliferation marker Ki-67 were not detected (not shown). The fraction of marker-positive cells is shown in Fig. 4.

Database-assisted sequence analysis

Analysis using the basic local alignment search tool (BLAST) revealed that GFAP in *Danio rerio*, alpha-SMA in *D. rerio*, *Salmo salar*, and *Oryzias latipes*, Ki-67 in *S. salar*, and vigilin in *D. rerio* and *S. salar* have a sequence similarity with the human/murine sequence of $\geq 60\%$. For nestin, only a 30 % homology with a nestin fragment from *Carassius auratus* was determined; sequences of nestin in the other 3 fish species were not found.

DISCUSSION

The present study describes the first successful attempt to establish a long-term cell culture from adipopancreatic tissue of Atlantic herring. This cell population was cultivated for >1 yr. The cells were passaged 15 times (roughly 30 cell doublings) with a doubling time of 2 wk.

Initially, the cells proliferated significantly slowly, indicating a transition period during which the cells

Fig. 2. *Clupea harengus*. Cell proliferation assay at 2 different temperatures. Vertical lines are data $\pm$ SD. (a) At 20°C, the seeding densities of 2×10^4 and 1×10^4 cells 0.31 cm^{-2} revealed an initial decline of the cell index (CI). In contrast, densities of 5×10^3 and 2.5×10^3 cells 0.31 cm^{-2} showed a continuous proliferation after adhesion. A seeding density of 5×10^3 cells 0.31 cm^{-2} (roughly 1.6×10^4 cells cm^{-2}) yielded the best proliferation rates. (b) At 16°C, no stable proliferation could be attained at any seeding density. Instead, a steady state was reached after 48 h

adapted to their new environment. Also, it is evident that *in vitro* culturing selects for the fastest-growing cell population, which should accumulate over time. These reasons might explain the initial increase in the proliferation rates.

The fact that the cells proliferate over such long time periods suggests that the *in vitro* cell population does not contain only somatic cells. In mammals, terminally differentiated somatic cells stop dividing after a limited number of population doublings and undergo apoptosis (Holt et al. 1997, Tang et al. 2001, Ohtani et al. 2009). We consider it to be unlikely that oncogenic alterations might account for the observed cell type. The herring cells described in the present study displayed no morphological signs of nuclear aberrations such as polynucleated cells or large nuclei. Moreover, in contrast to

most tumor cell lines, the herring cells did not grow in multiple layers and did slow their proliferation notably when reaching confluency (see proliferation curves in Fig. 2).

Taking into account these considerations, we hypothesize that the cell population described here contains progenitor or adult stem cells. Not only are adult stem cells known to be proliferative over long time periods while keeping their ability for contact inhibition (Pittenger et al. 1999, Poulson et al. 2002), but exocrine pancreatic tissue is a very efficient source for multi- and pluripotent adult stem cells in various species (Kruse et al. 2006, Lermen et al. 2009). In fact, the propensity of exocrine pancreatic tissue to yield high amounts of stem- or progenitor cells has been utilized to establish the Cryo-Brehm cell bank for viable cells of a large variety of endangered vertebrates (Lermen et al. 2009, Ciba et al. 2010).

Compared with mammals, little is known about tissue homeostasis and regeneration in adult *Clupea harengus*. It seems, however, plausible that adult stem cells, regardless of the specific physiological process they are involved in, should continuously give rise to several different cell types. Therefore, we analyzed the heterogeneity of protein expression within the cell population. Using immunocytochemical stainings, we tested for the expression of known stem-cell-specific marker proteins (Oct-4, Nestin, and SSEA-1), as well as of particular differentiation markers (α -SMA, GFAP, MF20, pan-cytokeratin, Ki-67, and vimentin) in different passages.

Unfortunately, there are virtually no antibodies available that are directed specifically against protein epitopes from Atlantic herring. Most available antibodies bind to murine and human proteins. Therefore, we compared the sequences of commonly used human and murine marker proteins (Oct-4, GFAP, α -SMA, nestin, and vimentin) to the homologous proteins from other fish species such as *Danio rerio*, *Salmo salar*, *Oryzias latipes*, and *Oncorhynchus mykiss* using UniProt Knowledgebase (www.expasy.ch). The results indicate that GFAP, α -SMA, Ki-67, and vimentin have a good chance of being detected using anti-human/anti-murine antibodies (>60 % homology).

Another important criterion to assess the specificity of a particular antibody is its spatial binding pattern within the cell. In particular, fibrillary proteins, such as GFAP and cytokeratins, display rather specific structures. Therefore, we infer from the data in Fig. 3 that

Fig. 3. *Clupea harengus*. Immunocytochemical analyses of (a,c,e,g,i) isolated herring cells from adipopancreatic tissue and (b,d,f,h,j) mammalian cells as a positive control. Fluorescence microscopy following incubation with antibodies against (a,b) alpha smooth-muscle actin (alpha-SMA); (c,d) vigilin; (e,f) glial fibrillary acidic protein (GFAP); (g,h) pan-cytokeratin; and (i,j) stage-specific embryonic antigen 1 (SSEA-1). Mammalian cells were from (b) goat submandibular salivary gland, (d,h) human parotid salivary gland, (f) Schwann cells isolated from rat nervus ischiadicus, and (j) oocyte-like cells from rat (Danner et al. (2007). Nuclei are stained with DAPI (blue). Scale bars = 100 μ m

Fig. 4. *Clupea harengus*. Percentage of cells in herring cell cultures expressing specific proteins as detected immunocytochemically. Cytokeratins and glial fibrillary acidic protein (GFAP) were expressed in many cells, while only a few cells (<20 %) expressed vigilin, alpha smooth-muscle actin (alpha-SMA), and stage-specific embryonic antigen 1 (SSEA-1). Values are mean $\pm$ SD of 3 independent stainings

these proteins were abundantly expressed in our cell population (each was detected in ~50 % of the cells). While vigilin (~10 %), alpha-SMA, and SSEA-1 (both $\leq$ 5 %) were found to be present in subpopulations, the herring protein homologs to nestin, MF20, Oct-4, and Ki-67 could not be detected using anti-human/murine/rat antibodies. This might be either due to insufficient epitope homology of the actual target proteins and the herring proteins, but it could also indicate that the respective proteins were not expressed by the cells at all.

Summarizing the protein-expression results, it is apparent that indeed, the cell population studied here was diverse and contained several different cell types. The occurrence of cytokeratin-, alpha-SMA-, and SSEA-1-positive cells can be taken as a corroboration of the hypothesis that the cell population contains adult stem cells. Since terminally differentiated cells (such as alpha-SMA-containing smooth-muscle cells) are known to be not proliferative, their presence in the cell culture after many passages indicates that they were produced by the cultured cells.

Proliferation assays revealed that the Atlantic herring cells proliferated significantly faster at 20°C than at 16°C. This might be attributed to metabolism, which becomes slower at lower temperatures. Ganassin et al. (1999) demonstrated that the best growing temperature for Pacific herring larvae cells is between 18 and 21°C. This is comparable with our data. It should be noted, however, that the optimal temperature for good proliferation needs to be established for every fish species anew. Cells from subtropical fishes like *Oryzias latipes* are best cultured at 33°C (Hirayama et al. 2006),

while the best proliferation temperature for cells of pelagic fishes like haddock *Melanogrammus aeglefinus* is reported to be between 12 and 18°C (Bryson et al. 2006).

In conclusion, a new long-term proliferative cell population from the adipopancreatic tissue of *Clupea harengus* has been established. The proliferative activity described here leads us to the assumption that the cell population contains progenitor or adult stem cells. This hypothesis is supported by the results of immunocytochemical analyses. Another important property of this cell population is its excellent suitability for cryopreservation. The proliferative cells may be used in bioreactors for future mass production of valuable proteins, e.g. for nutrition purposes in aquaculture. There was only 1 available herring cell line prior to the present study; now, long-term culturing can prove to be a useful tool for aquaculture industries, toxicological tests, and genetic analysis of herring populations.

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