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Long-term culture of a cell population from Siberian sturgeon (*Acipenser baerii*) head kidney

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Abstract In vitro cultures of native fish cell lines are of great importance, both for basic research and applied science. In particular, there is strong demand for long-term growable cell lines from breeding fish, like sturgeon. Here, we describe the culture of cells from Siberian sturgeon (*Acipenser baerii*) head kidney. The cells have so far been cultured over a period of 12 months (24 passages). Cytochemical and immunocytochemical examination suggests that, in vitro, the cells exhibit markers that are indicative for different cell types. In particular, fat storing cells (adipocytes) were observed, and the expression of cytokeratins and glial fibrilar acidic protein (GFAP) can be concluded on the basis of immuncytochemical analysis. The observation of different morphologies additionally underlines the heterogeneity of the cell population and matches the typical behaviour of in vitro cultures of stem/progenitor cells. Different applications can be imagined.

Keywords Aquaculture · Cell culture · Fish · In vitro · Progenitor cells · Pronephros · Stem cells · Test system

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Introduction

We describe the culture of cells from Siberian sturgeon (*Acipenser baerii*) head kidney. Head kidney is a haematopoietic organ in several fish species, analogous to bone marrow in mammals (Catton 1951; Zuasti and Ferrer 1989; Zapata et al. 1997; Zapata and Amemiya 2000), and therefore contains proliferative and plastic cells, i.e. cells that bear the capabilities to divide and finally differentiate. Preparation of sturgeon head kidney yielded a population of cells that have so far been cultured and propagated over a period of 12 months (24 passages). To the best of our knowledge, the culture of a suchlike proliferative cell population from head kidney has not been described before. Cytochemical analysis evidenced cells with adipocyte phenotype, and immunostains performed in different passages revealed that mammal-specific, yet cross-reactive, antibodies bind to fish proteins that are organized in structures resembling their mammalian counterparts in detail. While a number of cell lines can be applied for research from common model

organisms, like zebrafish (*Danio rerio*), little is known about long-term culturable cells from fish grown in aquaculture, like sturgeon. In any case, fish breeding aims to spare wildlife populations and, because a considerable amount of fish products is acquired by means of aquaculture, has become an important economic sector. Since aquacultures are prone to become infested with various infectious diseases, it requires easily manageable tools to monitor the health status of these populations. In this context, the cells could be applied as an in vitro test system for sturgeon specific pathogens. Further possible uses are apparent, for example the appliance in ecotoxicological research.

Material and methods

Tissue preparation and cell isolation

A one-year-old Siberian sturgeon bred in aquaculture was obtained from the research institute for agriculture and fishery in Mecklenburg-Vorpommern, Born/Darß. Head kidney tissue was transferred in digestion medium containing HEPES-Eagle medium (pH 7.4), 0.1 mM HEPES-buffer (pH 7.6), 70% (v/v) modified Eagle-medium, 0.5% (v/v) Trasylol (Bayer, Leverkusen, Germany), 1% (w/v) bovine serum albumin, 2.4 mM $CaCl_2$ and collagenase (0.63 PZ/mg; Serva, Heidelberg, Germany). The tissue was reduced to small pieces using small scissors and incubated for 20 min at 37°C with constant shaking (200 cycles/min). Then the digestion medium was removed and the tissue was rinsed with culture medium (formulation: see below) and dissociated by up-and-down suction through a 2-ml pipette with restrictive openings. Afterwards, the cells were centrifuged for 4 min at 800 *g* and resuspended and seeded in culture medium.

Cell culture

Cells were cultured in 25-cm^3 culture flasks in Dulbecco's modified Eagle's medium (DMEM; Gibco, Germany) supplemented with 20% FCS, 1 U/ml penicillin (Biochrom, Berlin, Germany) and 10 mg/ml streptomycin (Biochrom) at 18°C in 2.5% CO_2. Medium was changed every 3–4 days. When confluence was reached, the cells were subcultured at ratio 1:2 using 0.1% trypsin (PAA Laboratories, Austria). For cytochemical and immunocytochemical studies cells were transferred onto 2-well chamber slides (BD Biosciences, Belgium) 5–7 days before examination. For cryoconservation, trypsinized cells were suspended in ice-cold freezing medium consisting of FCS:DMSO (9:1) and transferred into cryovials. The vials were immediately put into a precooled isopropanol-freezing box and stored at −80°C over night. Then the vials were transferred into liquid nitrogen atmosphere. For re-seeding, frozen cells were thawed rapidly in 18°C culture medium, spun down for 4 min at 800 *g* and resuspended and seeded in culture medium.

Cytochemistry

For immunostaining, cells cultivated on chamber slides were rinsed three times in PBS, fixed with methanol:aceton (7:3) containing 1 g/ml DAPI (Roche, Switzerland) for 5 min at −20°C and washed three 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 directed against pan-Cytokeratin (mouse monoclonal mix, 1:100; Sigma), vigilin (polyclonal anti-vigilin antiserum FPIII, rabbit, 1:200) and GFAP (rabbit polyclonal, 1:100; DAKO, Denmark) in a humid chamber for 1 h at 37°C. After rinsing three times with PBS, the slides were incubated with secondary antibodies in a humid chamber for 1 h at 37°C. The following secondary antibodies have been used: Cy3-labelled anti-mouse IgG (1:400; Dianova), FITC-labelled anti-rabbit IgG (1:200, Dianova). Slides were washed three times in PBS, covered in Vectashield mounting medium (Vector, USA) and analysed with a fluorescence microscope (Axioscope 2 Zeiss, Germany).

Lipid stain (Oil red O; Sigma) was performed according to the manufacturers instructions.

Results

The cell preparation yielded single cells and small tissue pieces that both attached to the culture dish. The cell colonies developing from this primary material predominantly consisted of cells with a fibroblast-like shape (Fig. 1). However, along with

first subcultures and accompanied removal of remaining tissue pieces the fibroblastoid cells were increasingly replaced by cells with different morphologies. These included polygonal-shaped cells with tight cell contacts, cells with processes of varying number, shape and size, and giant cells up to 1,000 µm with pronounced stress fibers (Fig. 2). Frequently, cells appeared to be polynucleated (Fig. 3).

The cells grew continuously in standard DMEM culture medium supplemented with 20% FCS and were subcultured at ratio 1:2 every 10–14 days on confluency. Passaging by trypsination did not significantly reduce the number of cells, and also subsequent freezing and thawing did not impair viability to a notable degree. So far, the cell population could be propagated and passaged over a period of 12 months with 24 passages. A histological analysis performed in passages 10 and 14 revealed the existence of lipid drops located around the nuclei of a number of cells (Fig. 4).

In passage 4 and passage 10, cells were stained with antibodies directed against Vigilin (Fig. 3), pan-Cytokeratin (Figs. 3 and 5) and GFAP (Fig. 6). Vigilin, a RNA-binding protein generally involved in translational processes, was present in the cytoplasm of almost all cells, as expected, whereas reactivity of anti-cytokeratin antibody was detectable in few cells only. There, pronounced filamentous structures were labelled. Also, the GFAP antibody clearly reacted with filament proteins. Neither stains showed any differences compared to immunostains of mammalian adult stem cells expressing cytokeratines or GFAP. Other mammal specific antibodies tested (against α-smooth muscle actin, neurofilaments and the stem cell marker oct-4) apparently did not bind specifically, as either all cells showed strong fluorescence signal or the labelled structures did not coincide with the morphological organisation of the protein (data not shown).

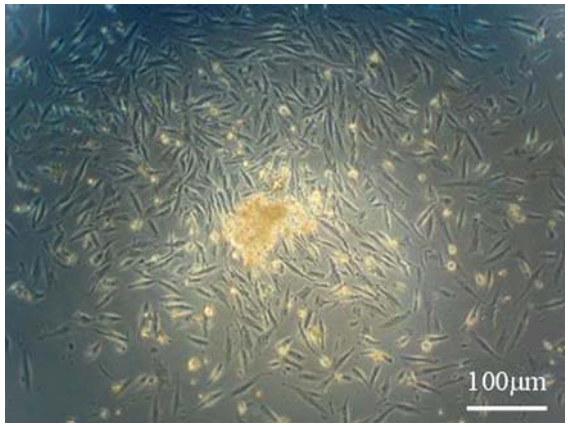

Fig. 1 Primary material obtained through digestive cell preparation: Single cells and tissue pieces obtained from head kidney attached to the dish on the first day of culture and gave rise to a population of fibroblastoid shaped cells (picture taken in passage 0, day 6 of culture)

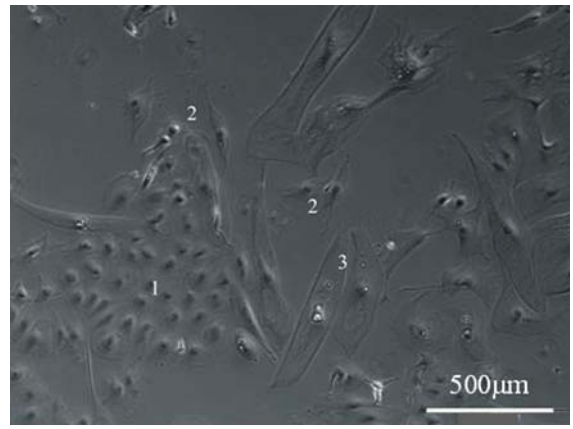

Fig. 2 Different morphologies of long-term cultured cells: Along with first subcultures the cell population increasingly exhibited cells with striking morphologies. In particular, polygonal cells with tight cell contacts (1), cells with processes (2) and giant cells (3) were present. Fibroblastoid cells were no longer observed (picture taken in passage 9)

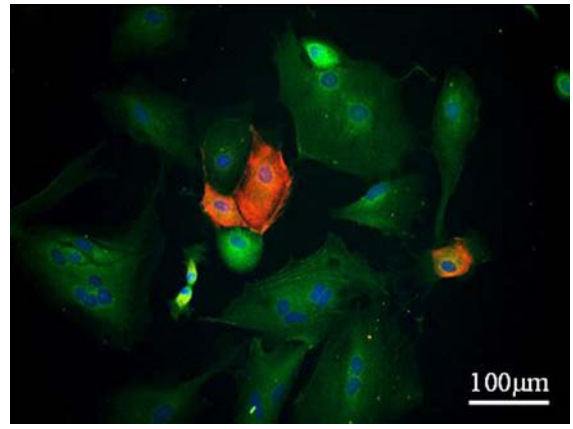

Fig. 3 Co-imunostain with cross reactive antibodies against Vigilin (*green*) and Cytokeratines (*red*): Reactivity of Vigilin antibody was detectable in all cells, as expected. In contrast, reactivity of the anti-cytokeratin antibody was observed only in some of the polygonal cells, indicating specific antibody binding. Nuclear stain with DAPI (*blue*) revealed the existence of polynucleated cells (picture taken in passage 4)

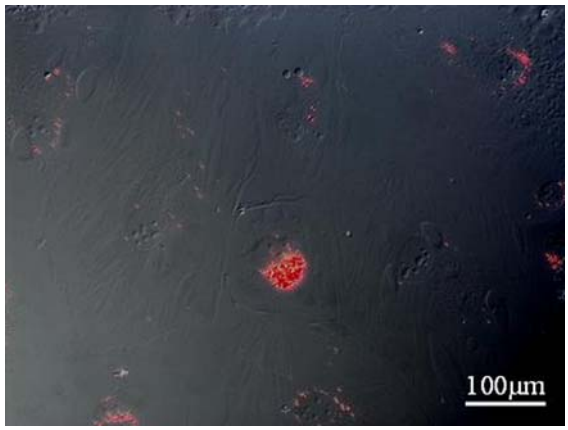

Fig. 4 Detection of lipid storing cells (adipocytes): Oil red stain revealed the existence of lipid-drops located around the nuclei of a great number of cells. Some cells contained large fat deposits (picture taken in passage 10)

Discussion

Head kidney serves as a haematopoetic organ in several fish species, including sturgeon (Fänge 1986), and therefore has analogous functions to bone marrow in mammals. The mammalian bone marrow holds different types of adult stem/progenitor cells (Lemischka et al. 1986; Friedenstein 1991), and it is to be expected that head kidney also contains proliferative and plastic cells. While the haematopoietic and immunological properties of head kidney

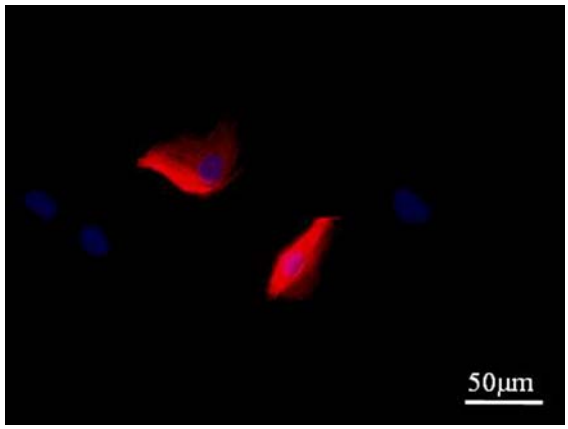

Fig. 5 Immunostain against cytokeratines: the antibody labelled structures that match the morphology of the cytoskeleton protein. Only few cells were positive for cytokeratines, while in the prevailing number of cells no signal was detected, suggesting specific binding of the antibody (picture taken in passage 10)

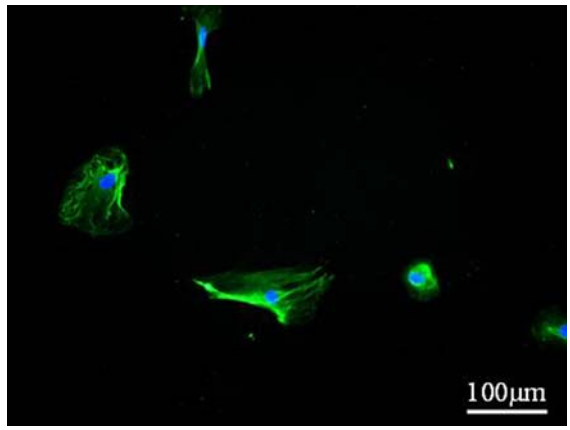

Fig. 6 The anti-GFAP antibody recognized pronounced filaments, suggesting the expression of the glial protein in cells obtained from head kidney

cells from various species have been investigated (e.g. Braun-Nesje et al. 1982; Siegl et al. 1993; Joerink et al. 2006), the aim of this work was to explore the possibility of long-term propagation of cells from Siberian sturgeon and to evaluate cross-reactivity of antibodies that specifically bind to mammalian variants of prominent proteins.

Primary culture of cells obtained from Siberian sturgeon head kidney yielded a population of cells that could be long-term cultured and passaged without a loss of viability. The cells described here differ from other cultured head kidney cells by an astonishing capability to proliferate. To the best of our knowledge, no such cell population from head kidney or other fish organs is reported in the literature. Among the cell population cells with different morphologies were present. Our observations resemble previously performed investigations on trout head kidney cells in many details, which exhibited cells with morphologies identical to those observed here (Siegl et al. 1993). Cytochemical and immunocytochemical examinations performed repeatedly in different passages revealed the existence of cells with adipocyte-like phenotype and suggested the expression of further markers which are indicative for different cell types. No divergence was observed between cytochemical stains performed in early and late passages, respectively. The immunodetection of Vigilin, GFAP and pan-Cytokeratin was performed using mammal specific antibodies. Yet, the known properties of the antibodies as well as the results suggest specific binding to sturgeon proteins.

The Vigilin antibody has been approved to cross-react with, for example, trout Vigilin (Kruse et al. 1996). The anti pan-Cytokeratin antibody according to the manufacturers information exhibits a wide interspecies cross-reactivity, down to *Xenopus laevis* and the GFAP antibody, though not explicitly approved on lower vertebrate protein, due to strong conservation of GFAP structure, is also predicted high cross-reactivity. However, the antibodies recognized cellular structures that were morphological identical to their mammalian counterparts, suggesting specific binding to sturgeon proteins. Besides, mammal specific antibodies have already been used for immunocytochemical examinations on cells from different fish species by others (e.g. Holen and Hamre 2004, Raymond et al. 2006). Raymond and colleagues successfully used the same GFAP antibody we applied for sturgeon cells on retinal cells from zebrafish in situ. Nevertheless, for further detailed characterization specific antibodies would be preferable.

The characteristics of the described cell population match circumstances frequently observed in cultures of stem/progenitor cells. Among the population, a cell type or a pool of cells is present that is capable of constantly undergoing cell divisions. At the same time, the population contains cells with distinctive morphologies, exhibiting different cell-type markers. Indeed, gene expression and cell shape under in vitro conditions do not need to be related to the cells physiological properties in vivo. This behaviour is a characteristic in mammalian stem/progenitor cell cultures. There, certain types of adult stem cell are considered pluripotent, as in vitro markers for all three germ layers are detectable (Kruse et al. 2004; Serafini and Verfaillie 2006; Jiang et al. 2007). Thus, the expression of a molecular marker indicative for a cell type of progeny different from the tissue from which the cells were obtained is not an unusual phenomenon. Also, the detection of certain gene products does not necessarily imply the existence of maturated and fully functional cells, but rather indicates the potential of cells to activate different lineage expression patterns under in vitro conditions. However, their plasticity makes the cells highly appropriate to be applied for different purposes.

For instance, long-term growable cells can help to address questions in ichthyology, such as phylogenetic research, infection and disease, virological studies and ecotoxicological or xenobiotical investigations. Morphological examination and expression analysis, as well as proliferation or survival assays, can provide reliable criteria to assess the tolerability or cytotoxicity of certain environmental or pharmaceutical compounds. PCR techniques, applied on sturgeon cells, can be used to analyse the dependency of transcriptional patterns on environmental factors. Importantly, in aquacultures, the cells can serve as a diagnostic tool for infectious (viral) diseases. Exposed to smears or dropping samples from sturgeon (Dishon et al. 2005), the cells can be used to amplify the infectious agents in vitro. Subsequently, pathogen antigens can be detected by means of ELISA or gene sequences identified by PCR. Such protocols can contribute to an improvement of the health management in sturgeon aquacultures.

Conclusions

The cells from sturgeon head kidney described here exhibit an enormous capability to proliferate and express genes from different lineages. The cell population is therefore suitable for a wide range of applications. It can be applied as a test system for pathogens, in ecotoxicology research and pharmacological testings.

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