ISOLATION AND CULTIVATION OF STERLET MYOBLASTS

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Abstract. Over the past 20 years, significant advances have been made in the understanding of cell and tissue culture in the fields of bioengineering, cell biology, and genetics; however, these achievements have largely focused on mammalian systems. In vitro studies on fish cells and tissues have been relatively limited, but the use of fish cell lines as in vitro models for environmental toxicology, particularly cytotoxicity analysis, has been significant. In addition, cultured cells have been used to study fish parasites and as in vitro models for screening immunostimulants. Skeletal muscle is the most important edible tissue in fish and rapid muscle growth determines the advantages of in vivo aquaculture. The generation of fish muscle cell lines can help to provide a reliable platform for deciphering the mechanism of fish skeletal muscle growth both in vivo and in vitro. In addition, cultured fish meat is a promising technology for animal protein-based foods and the concept of cell biomass meat from fish needs to be further developed. Our data demonstrated the feasibility of obtaining and culturing sterlet muscle cells in vitro. This study highlights the potential advantages of cell aquaculture over traditional fishing and aquaculture, and the potential applications of fish muscle cell lines in the study of fish skeletal muscle growth and the production of edible cultured fish meat products.

Keywords: myoblast, cell culture, myotubes, proliferation, differentiation, starlet.

List of Abbreviations

FBS – fetal bovine serum

DMEM – Dulbecco's Modified Eagle Medium

MYH ½ – Myosin Heavy Chain 1/2

FGF2 – Fibroblast growth factor 2

EDTA – Ethylenediaminetetraacetic acid, organic compound, a quaternary carboxylic acid

Dapi-4′,6-diamidino-2-phenylindole, fluorescent dye in vitro – technology for carrying out experiments or other works under artificial conditions

Ki67 – protein, marker of proliferative activity

PAX7 – transcription factor, regulating cell proliferation muscle precursor cells

Introduction

Over the past 20 years, bioengineering, cell biology and genetics have made significant advances in understanding cell and tissue culture - but these advances have mostly focused on mammalian systems. Fish cells and tissues have not been extensively studied in vitro (Rubio *et al.*, 2019). However, the use of fish cell lines as in vitro models for environmental toxicology studies, particularly cytotoxicity analysis, is considerable (Aarattuthodi & Dharan, 2021). Cell cultures have also been used to study parasites that infect fish (Shaw *et al.*, 2001) and as in vitro models for screening immunostimulants (Fierro-Castro *et al.*, 2013).

Skeletal muscle is a basic edible tissue in fish. Rapid muscle growth determines the benefits of aquaculture in vivo. The establishment of fish muscle cell lines could help to provide a reliable platform for deciphering the growth mechanism of fish skeletal muscle both in vivo and in vitro (Kong *et al.*, 2021). The use of cultured myoblasts could help in understanding the regeneration and recapitulation of the myogenic programme of different fish species and the differences between them (Froehlich *et al.*, 2014). In addition, cultured meat from fish is a promising technology animal-based proteins for nutrition. In this respect, the concept of meat from biomass fish cells still needs further development (Rischer *et al.*, 2020).

The sterlet (Acipenser ruthenus) is an understudied cartilaginous fish species, with few in vivo research studies and no in vitro cell studies published. It is a relatively small fish found in Eurasian rivers. The sterlet is considered to be an economically important species. However,

during the twentieth century, all wild sterlet populations declined due to anthropogenic factors such as poaching, overfishing, pollution and dam construction (Pobedintseva *et al.*, 2018). Sterlet (Acipenser ruthenus) has been included in the Red List of Threatened Animals. As an alternative to fished sterlet, isolation and propagation of its cells (muscle, fat) for making cell-cultured tissue offers an option to address the above concerns.

Our data showed that it is possible to obtain and cultivate sterlet muscle cells in vitro.

Ethics statement: The work was undertaken in accordance with a permission from the Ethical Committee of Kazan (Volga Region) Federal University (Permit Number 3, 5 May 2015).

Materials and Methods

Myoblast cell culture

One month old sterlet juveniles were collected from the fish farm "Biosphere fish" (RF) and transported to the Kazan Federal University (RF). The sterlet was humanely killed by head dislocation followed by destruction of the brain with a scalpel. The whole fish was then immersed in 70% ethanol for 10 seconds before being transferred to the laminar. Pieces of dorsal muscles were mechanically dissociated with scalpels and enzymatically digested with 0.2% type IV collagenase (Worthington, USA) in a shaker within 1 h at room temperature. The tissue was then centrifuged twice and the supernatant decanted. The pellet was resuspended in 0.1% trypsin-EDTA (PanEco, RF). Trypsin-EDTA was warmed to 37 °C. The suspension was incubated at room temperature for 20 min. The cells were then filtered through 100 and 40 μm cell strainers (Thermo Fisher Scientific, USA) to remove any debris. After several washes, the cells were resuspended in L-15 medium containing 5% fetal bovine serum (FBS) and 1% antibiotic mixture, 2 mM L-glutamine (all PanEco, RF). The suspension was filtered through 20 μm cell sieves. Cells were then seeded into 6-well plates pretreated with 0.1% gelatin. The cells were then incubated for 30 minutes at 18°C to separate the satellite cells from the fibroblasts. The cell suspension was then decanted and non-adherent cells removed by washing. The remaining cells were cultured in L-15 medium containing 20% FBS and 1% antibiotic mixture, 2 mM L-glutamine, 2.5 ng/mL FGF2 (Sigma, USA), 200 μM ascorbic acid (Sigma, USA). Cells were incubated at 18°C. The culture medium was changed every 3d. Cell culture images were captured using an Axio Observer Z1 microscope (Carl Zeiss, Germany). Fish primary muscle-derived cells were continuously maintained in culture by subculturing. We used 0.25% trypsin-EDTA to detach the cells from the surfaces (PanEco, RF).

Determination viability myoblasts fish

Apoptosis was investigated using a commercial Annexin V Apoptosis Detection Kit (Santa Crus, sc-4252 AK, USA) according to the previously described method (Zakirova *et al.*, 2019). The stained cell suspension was analyzed using flow cytometry (Guava 8T, Millipore, USA).

Flow cytofluorometry

To estimate the percentage of myogenic cells contained in the culture, we used an antibody against MYH ½ (SANTA CRUZ, USA, sc 53088), Myogenin (SANTA CRUZ, USA, sc 52903), PAX7 (CUSABIO TECHNOLOGY, USA, CSB-PA891015), Desmin (SANTA CRUZ, USA, sc 14026). Cells mitotic activity was detected by immunostaining cultures with the antibody against Ki 67 (ABCAM, USA, ab 15580). The cells were analyzed using flow cytometry.

Formation of myotubes in myoblast cultures in vitro

To assess myoblast differentiation into myotubes, cells were cultured in growth medium. After monolayer formation, the culture medium was changed every week. Myotube formation was detected after 20 days (Aimaletdinov *et al.*, 2022).

The cell monolayer was fixed in 4% formalin for 1 hour at room temperature and then washed with PBS. Cells were then stained with hematoxylin-eosin (JSC Lenreaktiv, Russia),

phalloidin Alexa Fluor™ 488 (Invitrogen™, A12379) and Dapi (Dia-M, Russia) according to the manufacturer's recommendations. Results were evaluated using an Axio Observer Z1 microscope.

Differentiation fish myoblasts

Differentiation of the cultured cells was performed in three directions: osteogenic, adipogenic and chondrogenic. Differentiation in the chondrogenic direction was performed according to a previously published method (Zakirova *et al.*, 2014). To investigate the ability of the obtained cell culture to undergo adipogenic and osteogenic differentiation, cells from the third passage were seeded in 12-well plates at 30,000 cells per well and incubated in growth medium until a monolayer was obtained. The cells were then cultured in specific adipo- and osteo-differentiation media (Gibco, USA) to induce differentiation. Other samples were grown to 100% confluence to induce lipid accumulation towards an adipogenic phenotype. The cells were then fed with adipogenic medium three times a week, consisting of growth medium DMEM high glucose (PanEco, RF) supplemented with 10% FBS, 10 μg/mL insulin (Sigma Aldrich, USA), 100 μg/mL streptomycin, 500 мкМ 3-isobutyl-1-methylxanthine (IBMX, Sigma, USA) and 10 μg/mL insulin (Sigma, USA), 1 μM dexamethasone, 2 mM L-glutamine, 100 μM indomethacin (Sigma, USA). The control samples were grown in medium DMEM high glucose with 10% FBS, 100 μg/mL streptomycin, 2 mM L-glutamine. To visualise neutral lipids after 21 days of lipid accumulation, cells were fixed, stained with Nile Red (Sigma, USA) and imaged by fluorescence microscopy.

To determine the presence of mineralisation, a sign of osteogenic differentiation, Von Kossa staining reactions were used according to previously described methods and imaged by microscopy (Zakirova *et al*., 2021).

All differentiation was maintained for 21d in a CO_2 incubator at 25 °C, 2% CO_2.

Results

Myoblasts from sterlet muscle tissue were adherent and actively proliferating for 24 h (Fig. 1).

The cells had a fusiform shape with a central nucleus. When cultured in poor medium, myotube formation occurred after 20-25 days (Fig. 2).

Chondrogenic medium caused myoblast death in fish in vitro. However, osteogenic medium induced calcium deposition in the samples (Fig. 3).

Adipocytic differentiation, the red color, indicates lipid vacuoles stained with Nile Red (arrow). Von Kossa staining of osteoclasts differentiated from fish myoblasts. The optimisation of fish myoblast differentiation involved the investigation of differentiation regimes chosen on the basis of previous literature studies showing efficacy in different mammalian cells (Zakirova *et al*., 2022).

The cell viability study showed that 2% of the cells in the population were undergoing apoptosis, while 1% were in necrosis. In total, 71% of the cells expressed Ki 67, a marker found in nuclei during all active phases of the cell cycle: G1, S, G2 and M (Sun *et al*., 2018). Thus, our results showed high proliferative activity and viability of fish cells.

Fish cells expressed 62% Desmin, 67% Pax7, 25% Myogenin, 63% MYH1/2. Desmin is the major subunit of muscle-type intermediate filaments and is expressed by skeletal, cardiac and most types of smooth muscle cells in both embryonic and adult tissues (Shahini *et al*., 2018).

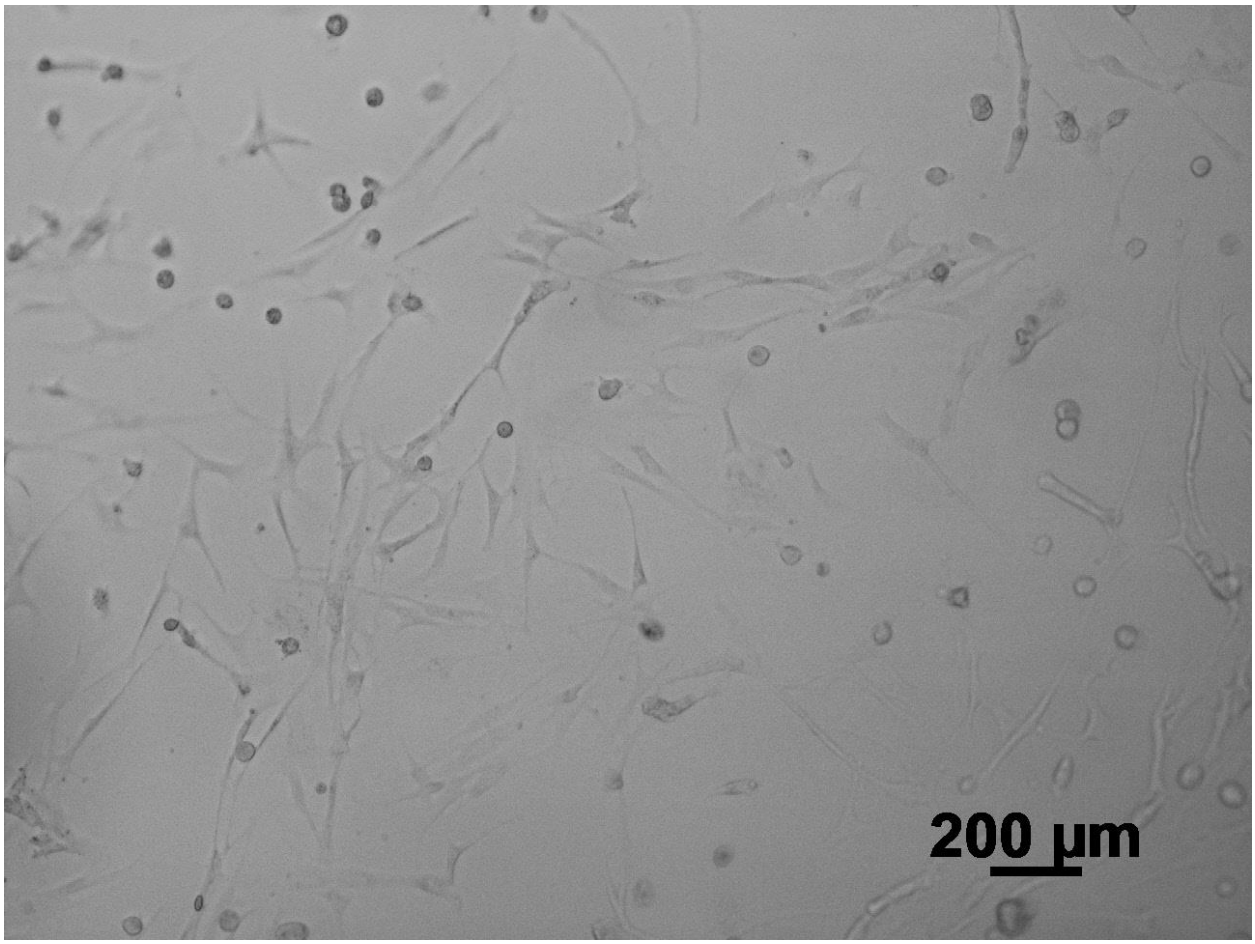

Fig. 1. Primary fish myoblast

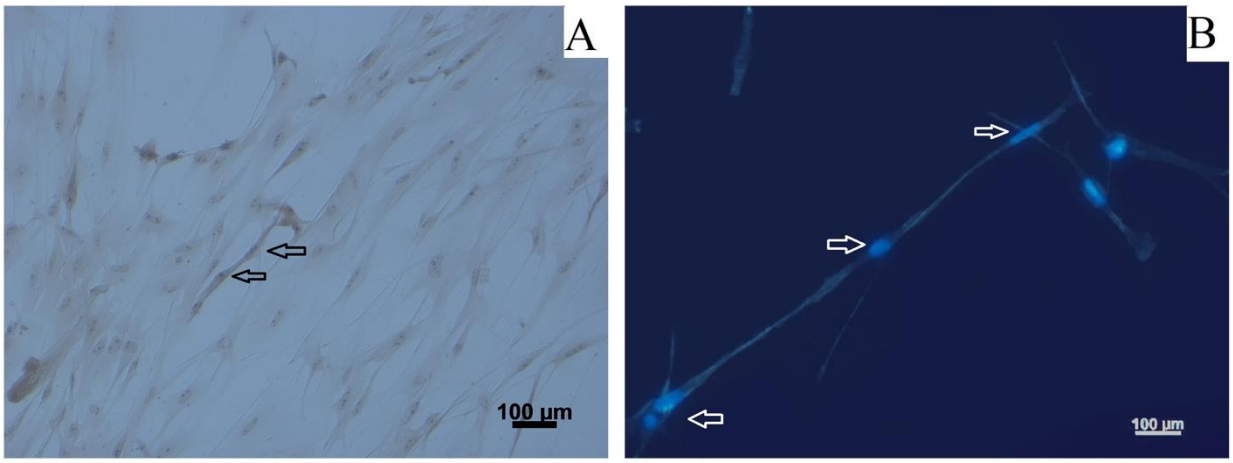

Fig. 2. Primary fish myoblasts. A. Staining of fish myotubes with haematoxylin-eosin showed the multinucleated character of myotubes (arrows); B. Staining of cells with phalloidin (green staining of actin fibres) and Dapi (blue staining of nuclei) showed the multinucleated character of myotubes (arrows)

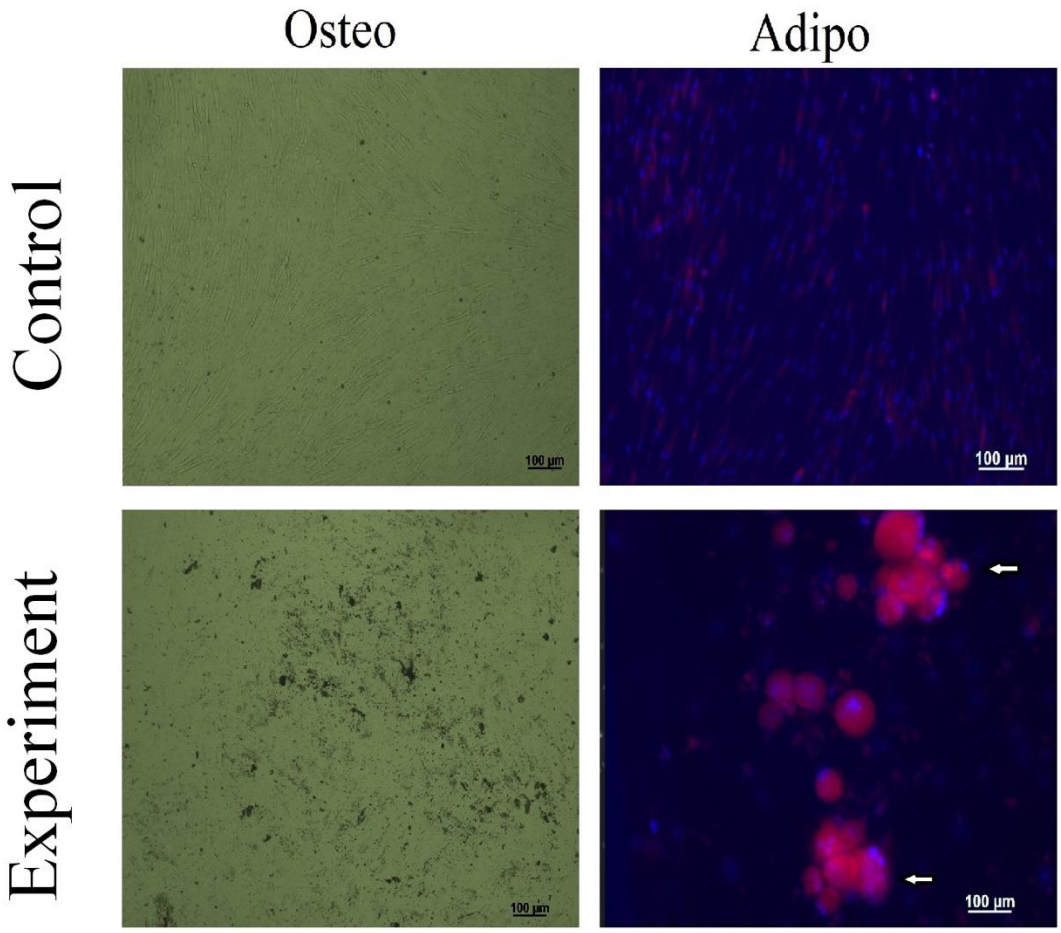

Fig. 3. Myoblasts were cultured in appropriate differentiation media and assessed for differentiation using specific staining methods

Discussion

Pax7 is currently the most important marker for the identification of myosatellite cells. Available literature suggests that PAX7 is expressed in both activated and quiescent satellite cells (Dumont *et al*., 2015). PAX7 is a recognised marker of self-renewing cells and is an important factor in maintaining the stemness of satellite cells (Furuichi *et al*., 2021). PAX7 expression levels are known to be rapidly downregulated during satellite cell activation (Machado *et al*., 2017).

MYH is one of the most abundant proteins in adult myofibers and a major determinant of myofibre contraction velocity (Matthieu *et al*., 2021). Our results showed that MYH1/2 is expressed at low levels in the cells. However, its levels gradually increase during the muscle differentiation process and peak during the later stages of myogenesis (Choi *et al*., 2020).

Myogenin is an important marker of activated myosatellitocytes. It stimulates the proliferation and subsequent differentiation of myoblasts. Myogenin is a late marker of myogenic differentiation that accompanies the exit of myoblasts from the cell cycle and their terminal differentiation. The timing of myogenin detection in the nucleus coincides with the period of terminal differentiation of myosatellites, synthesis of proteins in the muscle fibre contractile apparatus (desmin, MYHs) and further formation of muscle tubules and young muscle fibres (Furuichi *et al*., 2021). Given the reported context, the muscle phenotype of the muscle-derived cells was confirmed.

To investigate the potential of sterlet muscle-derived cells to accumulate lipids, the induction towards an adipogenic-like phenotype by media supplementation was investigated. Adipogenic medium developed in the laboratory stimulated lipid accumulation in the cells. Commercial adipodifferentiation medium was cytotoxic and induced myoblast death. Available literature describes a skeletal muscle cell line from fish with an adipocyte-like phenotype. This line was isolated from muscle biopsies of freshly caught Atlantic mackerel (Scomber scombrus). A muscle phenotype of this cell line was confirmed by characterising muscle stemness and differentiation by PAX7 and myosin heavy chain MHC immunostaining and qPCR HPRT, PAX3B, MYOD1, MYOG, TNNT3A and PPARG used to characterise mackerel cell genotypes respectively (Saad *et al*., 2022).

Conclusion

Cellular aquaculture offers many potential advantages over conventional fisheries and aquaculture. Myosatellite cells have been isolated and cultured in vitro from a variety of teleost fish species, including carp, trout, sea bream and salmon (Gabillard *et al*., 2010). However, no information is available on isolated and cultured in vitro myoblasts from cartilaginous fish, and the differential potential of these cells in culture remains largely unknown. Our studies have demonstrated peculiarities cultivation myoblasts sterlet. Our data showed that 60–70% of adherent cells from sterlet muscle tissue are myogenic based on their immunoreactivity. We confirmed the formation of myotubes by immunocytochemistry for phalloidin in cell cultures. The characterisation of these extracted muscle cells thus validates the use of this in vitro myogenesis system for further studies of myogenic activity in sterlet.

Myoblasts are a population of self-renewing cells that provide a source of cells to maintain the homeostasis of muscle development (Kong *et al*, 2021). Our results showed that sterlet myoblasts can differentiate towards adipose and osseous tissue. The possibility of chondrodifferentiation myoblasts of sterlet in vitro required the use of another medium. Our data demonstrating myogenic and adipogenic-like phenotypes cells of sterlet underline ability its future use to create food from a type of cells.

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