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METHOD IN CELL SCIENCE

Unidentified cells reside in fish skeletal muscle

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Abstract Cell cultures were established from the skeletal muscle tissue of 6–13 months old rainbow trout and 12–14 months old yellow perch. Approximately 27,000 ± 5,000 cells/g (trout; $N = 5$) and 5,000 ± 1,200 cells/g of tissue (perch; $N = 4$) were obtained. Isolation and propagation were qualitatively greater for both species when the cells (younger fish producer more cells than older fish) were exposed to DMEM + 15% FBS, rather than L-15 + 15% FBS, at 20 °C (trout) and at 24 °C (yellow perch). Two morphologically distinct cell types were observed in cultures of both species, some of which eventually formed very small myotubes, which displayed immunocytological reactivity for myogenin, myosin heavy chain, and α-actinin; the second population of cells remained unstained. Successful cryopreservation was achieved using a 5% DMSO and 95% serum mixture, but post-thawing viabilities were low 5–27% (trout) and 14–30% (perch). Further research is needed in order to determine cell type specificity of isolated cells.

Keywords Fish cells · Yellow perch · Rainbow trout · Cell markers

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Introduction

Much has been published regarding the possibility of stem cells residing in skeletal muscle of mammals, and for the possible use of such cells in tissue engineering applications. Most of these stem cells are derived from similar isolation methods as one uses to acquire myogenic satellite cells. Other than satellite cells, stem-like cells have not been identified associated with the satellite cell fraction from any fish species. Consequently, fish are a viable animal model for both elucidating the satellite cell involvement in myofiber hyperplasia, and for the potential identification of other (stem) cells co-isolating with the satellite cell fraction.

Myogenic satellite cells have been isolated from a variety of animal and fish species (Powell et al. 1989; Greenlee et al. 1995; Venkateswaran et al. 1995; partially reviewed in Dodson et al. 1996; Burton et al. 2000). In the present study we used rainbow trout (*Oncorhynchus mykiss*), which are fast growth fish (Thorgaard et al. 2002), and yellow perch (*Perca flavescens*), which are slow growth fish (Malison 2000; Manci 2000). We compared these fish with

respect to the variables of cell isolation, attachment to cell cultureware, and initial development/growth of the cells in vitro. Our results demonstrate that the two cell populations that we co-isolated from rainbow trout and yellow perch, by previously defined methods, do not display efficient growth and development in vitro. However, continued/future studies with the isolated cells may show that many of these cells are reminiscent of resting stem cells, rather than committed myogenic stem cells.

Materials and methods

Reagents

Dulbecco's modified Eagle medium (DMEM), Leibovitz's L-15 medium (L-15), fetal bovine serum (FBS), horse serum, gentamicin, penicillin/streptomycin (P/S), Fungizone™, collagenase, trypsin and an Image-iT™ FX kit were purchased from Invitrogen Life Technologies (Carlsbad, California). Protease, 3-aminobenzoic acid ethyl ester (methanesulfonate salt, MS-222), giemsa stain, pig skin gelatin, Nonidet P-40, anti-desmin, anti-α actinin and the components of phosphate buffered saline (PBS: 10 g/L NaCl, 0.25 g/L KCL, 1.44 g/L Na_2HPO_4, 0.25 g/L KH_2PO_4, pH 7.3) were procured from Sigma-Aldrich (St. Louis, Missouri). Triton-X-100 was purchased from Fisher Scientific (Pittsburgh, Pennsylvania). Normal goat serum (NGS), Vectastain™ ABC reagent, an avidin-biotin blocking kit, and goat anti-mouse biotinylated IgG were purchased from Vector Laboratories (Burlingame, California). The NA4 and F5D antibodies were purchased from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa. Cell cultureware were acquired either from BD Biosciences (Bedford, Massachusetts) or Greiner-Bio-One Inc. (Longwood, Florida).

Fish

The Washington State University (WSU) Institutional Animal Care and Use Committee approved the use of fish in these experiments. Upon arrival at WSU, fish were processed immediately for cell isolation. The rainbow trout (*Oncorhynchus mykiss*; $N = 39$ isolations) ranged from 6 to 13 months in age and 20–113 g in weight and were reared in 12 feet circular fiberglass tanks at the University of Idaho Aquaculture Research Institute. They were fed twice daily with extruded floating feed and were transported live in a cooler to WSU. The yellow perch (*Perca flavescens*; $N = 15$ isolations) were reared in ponds (at temperatures between 20 and 24 °C) at the Ohio State University Center for Aquaculture Development as two different groups: the first group (used in four isolations) contained fish of approximately 15–19 months in age and from 41 to 103 g in weight. The fish from the second group were less than 12 months of age and weighed from 15 to 29 g. The perch were fed twice daily with commercial sinking feed and were shipped live to WSU via commercial carrier.

Isolation of cells

We evaluated many different combinations of isolation procedures in order to maximize cells obtained; for each isolation attempt, one item was changed from the previous effort. The final cell isolation procedure that we used for obtaining cells from both fish species is outlined in Fig. 1. Each fish was anesthetized by immersion in a solution of MS-222 (0.03%), killed by a blow to the head, weighed and measured, rinsed in a beaker containing 70% ethanol and finally rinsed in a beaker of PBS. Next the fish was placed on a cutting board, and the white lateralis muscle was excised from both sides, using a filleting knife. The muscle tissue was then placed in a beaker containing PBS with P/S, gentamicin and Fungizone™ and rinsed well. Antibiotics were added to all subsequent solutions, but the Fungizone™ was only used in the initial PBS solutions and not in the growth media. All cell cultureware and instruments were sterile.

In a biocontainment hood, the rinsed muscle was placed in a 150 mm preweighed cell culture dish containing 20 mL of a mixture of PBS + P/S, gentamicin and Fungizone™ to keep the tissue moist, and the weight of the dish (tissue plus fluid) was recorded. Next, approximately 10 g (10 mL) of tissue was placed into a centrifuge tube that already contained 25 mL of PBS (bringing the total volume to 35 mL per tube) and mixed well. The number of tubes used depended on the amount of muscle collected. The tissue in each tube was then minced with scissors, and the tubes were centrifuged for 3 min at 1,500*g*. The 150 mm dish with any

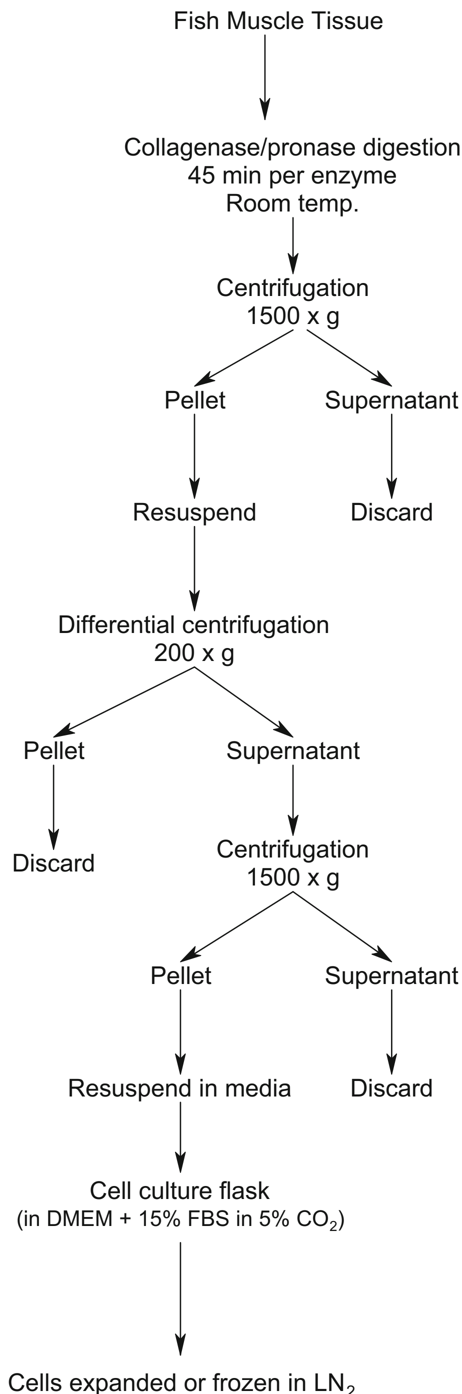

Fig. 1 Flow diagram of the general isolation protocol for obtaining cells from rainbow trout and yellow perch muscle tissue

remaining tissue and liquid was weighed to determine the actual amount of tissue used in the isolation procedure.

After centrifugation the supernatant from each tube was discarded and replaced with 20 mL of a sterile collagenase solution (1 mg collagenase/mL PBS). The tubes were then vortexed to mix the tissue thoroughly with the collagenase and placed on a platform rocker at medium speed and at room temperature for 45 min. Next the tubes were centrifuged for 3 min at 1,500*g*, and the supernatants were again discarded. A second enzyme solution (protease, 1 mg/mL PBS) was added (20 mL per tube). The tubes were vortexed to mix the tissue with the enzyme and placed on a platform rocker for 45 min at medium speed and at room temperature. The tubes were then centrifuged for 3 min at 1,500*g*, and the supernatants were discarded. Twenty milliliter of PBS were added to each tube, and the tubes were vortexed and centrifuged at 200*g* for 10 min (this step collected the total cell fraction in the supernatant fraction). The supernatants were pooled, transferred into clean tubes and centrifuged at 1,500*g* for 3 min to pellet the cells. The supernatants were then discarded, and the pellets were resuspended in DMEM + 15% FBS plus antibiotics (P/S and gentamicin were added to the media for the first two subpassages).

Establishment of cell cultures

The cell suspensions were then plated into cell culture flasks that had been coated with PSG (0.1%). The flasks were placed into incubator culture chambers (C.B.S. Scientific Co, Del Mar, California) and gassed with a 5% CO_2 95% air mixture. The chambers were then placed within low temperature incubators and held at either 20 °C for trout or 24 °C for yellow perch. After the cells had attached in the initial isolation flasks (18–24 h), representative fields of cells (40×, phase) were counted in each flask (100 fields per 25 cm^2 flask) in order to calculate the number of cells isolated per gram of muscle tissue. Also, cultures of cells from both trout and perch were trypsinized, counted, and replated in a series of subpassage cultures (Venkateswaran et al. 1995). The morphologies of the passaged cells were observed and photographed using a Nikon Diaphot inverted phase microscope, a Sony CCD/RGB color video camera (Sony Corporation of America, Montvale, NJ), and Image-Pro® Plus software (Media Cybernetics®, Silver Spring, Maryland).

Evaluation of the ability of cells to express myogenic proteins

If all of the cells that we isolated were myogenic satellite cells, then all cells should express myogenic markers. Complete fish cell fractions were fixed in 4% paraformaldehyde for 30 min and then rinsed in PBS three times (3×). Next, the cells were permeabolized in 3% Nonidet P-40 for 30 min and rinsed in PBS 3×. The cells were blocked with 3% hydrogen peroxide in PBS for 5 min, followed by rinsing in PBS 3×. The cells were then treated with avidin-biotin blocking reagents following the instructions in the kit. Next the cells were blocked with NGS (2.5% in 0.1% BSA in PBS) for 20 min and rinsed in PBS 3×. The cells were incubated with the primary antibody (diluted in 2.5% NGS) for 1 h. The primary antibodies used were F5D (myogenin), NA4 (myosin heavy chain), α-actinin, and desmin, all generated in the mouse. The cells were then rinsed 3× with PBS and incubated with the secondary biotinylated antibody (goat anti-mouse) for 1 h. Following rinses 3× with PBS, the cells were incubated with Vectastain Elite ABC Reagent for 30 min. After a subsequent rinse in PBS 3×, the cells were reacted with Nova Red for 7–14 min., followed by rinsing in water for 2 min. Each primary antibody reaction required a set of four negative controls: no primary antibody, no secondary antibody, no primary or secondary antibody and color substrate alone. An Image-iT™ FX kit (Invitrogen, Grand Island, New York) was used to fluorescently stain primary cultures of trout cells (only). In brief, isolated cells were plated into the chambers of Lab-Tek II slides (Nalgene Nunc, Rochester, New York) and grown for 7 days. The cells were then rinsed in warm PBS, fixed in warm 3.7% formaldehyde, and incubated 10–15 min at room temperature. Next the cells were rinsed in PBS 3× for 1 min/time. The cells were permeabolized in 0.2% Triton X-100 (diluted in PBS) for 5 min and rinsed in PBS 3×. Then sufficient Image-iT™ FX signal enhancer was added to cover each chamber well, and the chamber slides were incubated for 30 min at room temperature in a humid environment and rinsed thoroughly with PBS.

Cryopreservation and recoverability

During the logarithmic phase of growth in vitro, the cells in propagation flasks were trypsinized, resuspended in a medium consisting of 95% serum and 5% dimethylsulfoxide (DMSO), and aliquoted into cryovials. The vials were frozen at a −1 °C/min cooling rate using a nonmechanical freezing device (Nalgene) and finally stored in liquid nitrogen. Seven cryovials of rainbow trout cells and four vials of yellow perch cells were thawed to check recoverability. A vial of cells was recovered by thawing it in 37 °C water and then slowly adding media over several minutes to dilute out the DMSO and reduce osmotic damage to the cells. The diluted cells were then plated into flasks for attachment and propagation. Cell counts (100 fields per 25 cm^2 flask) were done at 18–24 h to determine the percent attachment of cells (i.e. plating efficiency).

Results

Rainbow trout consistently yielded greater cell numbers per gram of tissue than the yellow perch (Table 1). Of the 54 cell isolation attempts, by three different individuals, we determined that no

Table 1 Tissue amounts and cell numbers isolated from rainbow trout and yellow perch

Rainbow trout					
Age (months)	7.8	8.6	9.5	10.6	13.1
Tissue wt (g)	30.5	40.3	78.8	63.4	72.5
Total cells isolated	3.01×10^5	1.58×10^6	2.07×10^6	1.95×10^6	2.2×10^6
Cells/g tissue	9,869	39,200	26,269	30,757	30,345
Yellow perch					
Age (months)	15	17	18	12	
Tissue wt (g)	27.8	32.4	32.8	28.4	
Total cells isolated	No counts	1.2×10^5	2.7×10^5	1.44×10^5	
Cells/g tissue	No counts	3,703	8,232	5,070	

established method was satisfactory in obtaining maximum fish-derived cells to insure that adequate representatives of all cell populations were present. Scissors proved to be more effective at disrupting the muscle tissue than using a meat grinder. Collagenase and pronase, used for 45 min each in succession, resulted in the greatest number of cells being isolated. Passing the cell suspension through filters to remove tissue debris decreased the final number of cells, so the filtration step was discontinued. Moreover, the cell counts per gram of tissue decreased as the size of the fish increased. The initial cell numbers isolated,

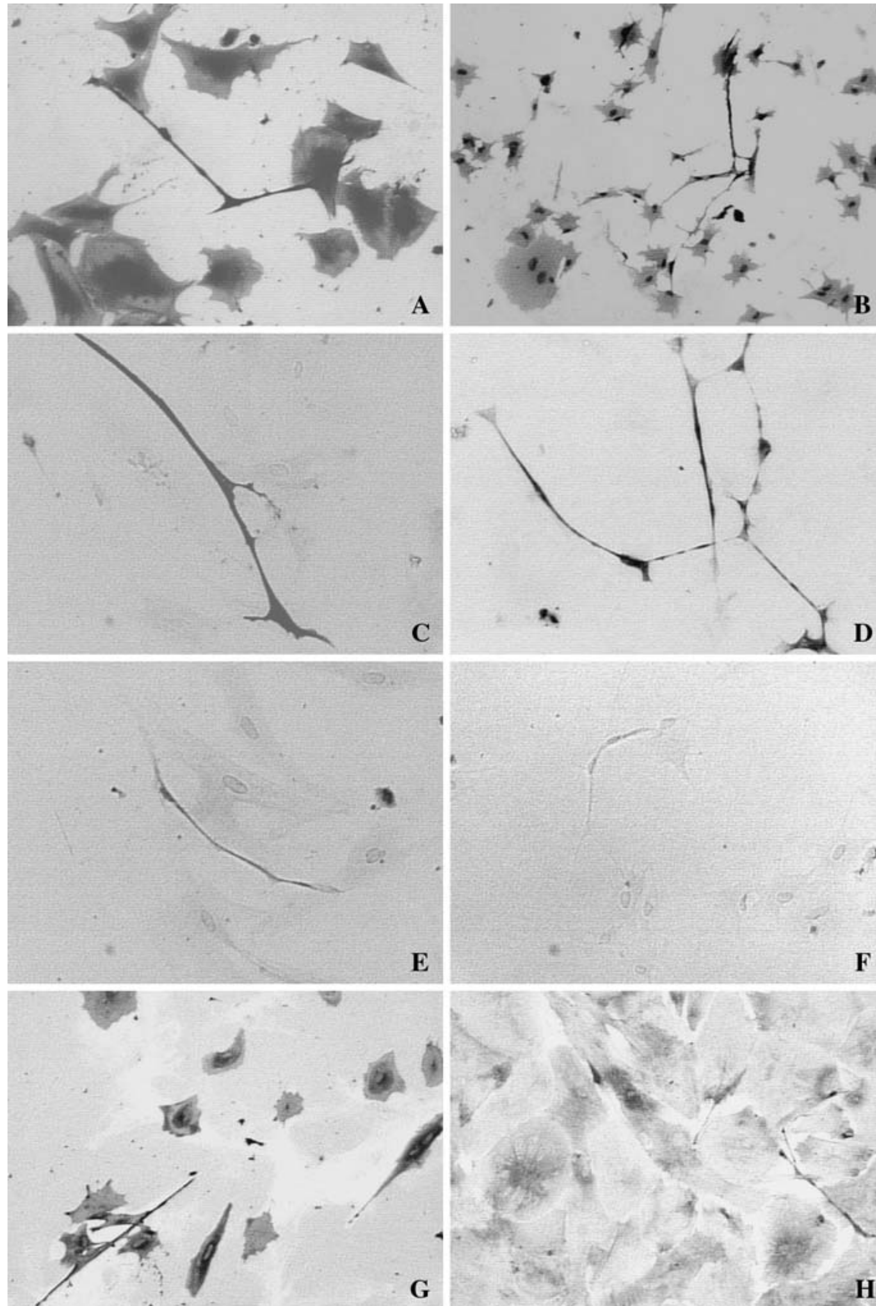

Fig. 2 Immunocytochemical staining of rainbow trout (**a**, **c**, **e**, **g**) and yellow perch (**b**, **d**, **f**, **h**) cells. (**a**, **b**) Anti-F5D (anti-myogenin), 100×; (**c**, **d**) anti-NA4 (anti-myosin heavy chain), 200×; (**e**, **f**) anti-PAX7, 100×; (**g**, **h**) anti-α actinin, 100×

and cell numbers over time in culture, were greater in DMEM + 15% FBS than in L-15 + 15% FBS for both species of fish, so DMEM became the basal medium of choice. Fetal bovine serum proved to be the optimal choice of serum in both attachment and growth. Cells died when exposed to fish serum and grew slowly in horse serum. Of the four substrata investigated, both fibronectin and pig skin gelatin gave satisfactory attachment and growth. Pig skin gelatin was selected because of its lower cost. The optimal growth temperature for the rainbow trout satellite cells was 20 °C. The yellow perch cells were always cultured at 24 °C

A fraction of the cells from both species of fish stained strongly positive for NA4 (myosin heavy chain), F5D (myogenin) and α-actinin (Fig. 2), and thus were considered to be satellite cells. All negative controls used in the antibody staining experiments were negative. The cells that were reacted with fluorescently tagged F5D antibody fluoresced green (Fig. 3). However, by passage number four, the growth of cells of both species slowed down and displayed similar morphologies over time. The primary cultures contained a mixture of slender, elongated cells and a smaller population of large epithelial-like cells. By passage number four the larger cell morphology predominated (Fig. 4).

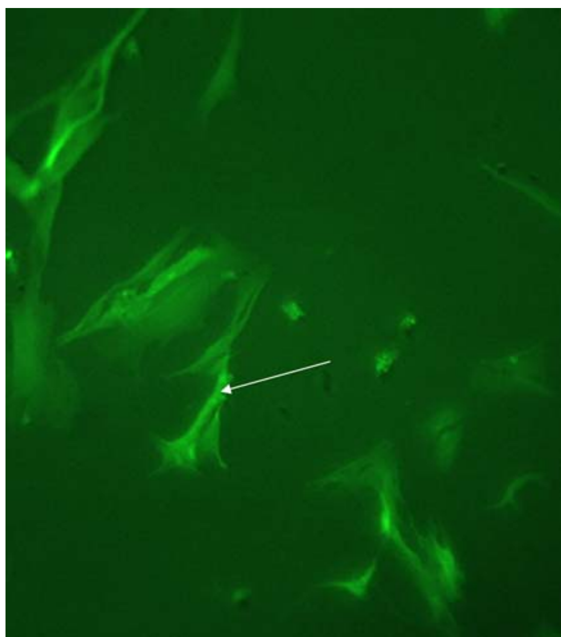

Fig. 3 Fluorescent micrograph of trout cells reacted with F5D antibody (anti-myogenin). The white arrow indicates a cell that has reacted with anti-myogenin

Following cryopreservation, the percent recoverability of trout satellite cells ranged from 5% to 27%. Similarly, satellite cells from yellow perch yielded a percent recovery of 14–30%. The initial health of the recovered cells from both species appeared to be satisfactory.

Discussion

The focus of this paper is directed to a potential remnant mesodermal cell fraction of *Oncorhynchus mykiss* and *Perca flavescens*. However, our data provide more information about the myogenic satellite cell fraction: First, we have documented procedures to isolate myogenic satellite cells from both species of fish. Second, we have provided information about the ability of the isolated cells to develop in vitro. Considering that both types of fish are popular aquaculture species and that efforts are underway to develop more efficient growth conditions for these animals in the aquaculture setting, these data will build knowledge in this effort. Third, we have provided knowledge about the viability of isolated cells through sustained passage, in vitro, and through cryopreservation of the cells. If the cells cannot be propagated or preserved, few subsequent studies may be performed without conducting another lengthy cell isolation procedure.

Many of the earliest efforts to isolate fish satellite cells utilized juvenile fish of small size and required large sample numbers to gather enough tissue for culturing (Matschak and Stickland 1995; Fauconneau and Paboeuf 2001; Castillo et al. 2004). Mulvany and Cyrino (1995) did attempt to culture satellite cells from catfish ranging from 1.9 to 330 g in weight, but they only propagated the cultures to 48 h and experienced poor replating efficiency of the cells. One major difference with our studies is that we have used older fish, and in the case of trout, fish of larger size. Previous work with larger fish showed somewhat similar results (Powell et al. 1989; Greenlee et al. 1995; Venkateswaran et al. 1995), but quite an extensive period of time has elapsed between those studies and the present one. During that lag time, we know of no scientific report in which satellite cells from any fish species have been cultured for long

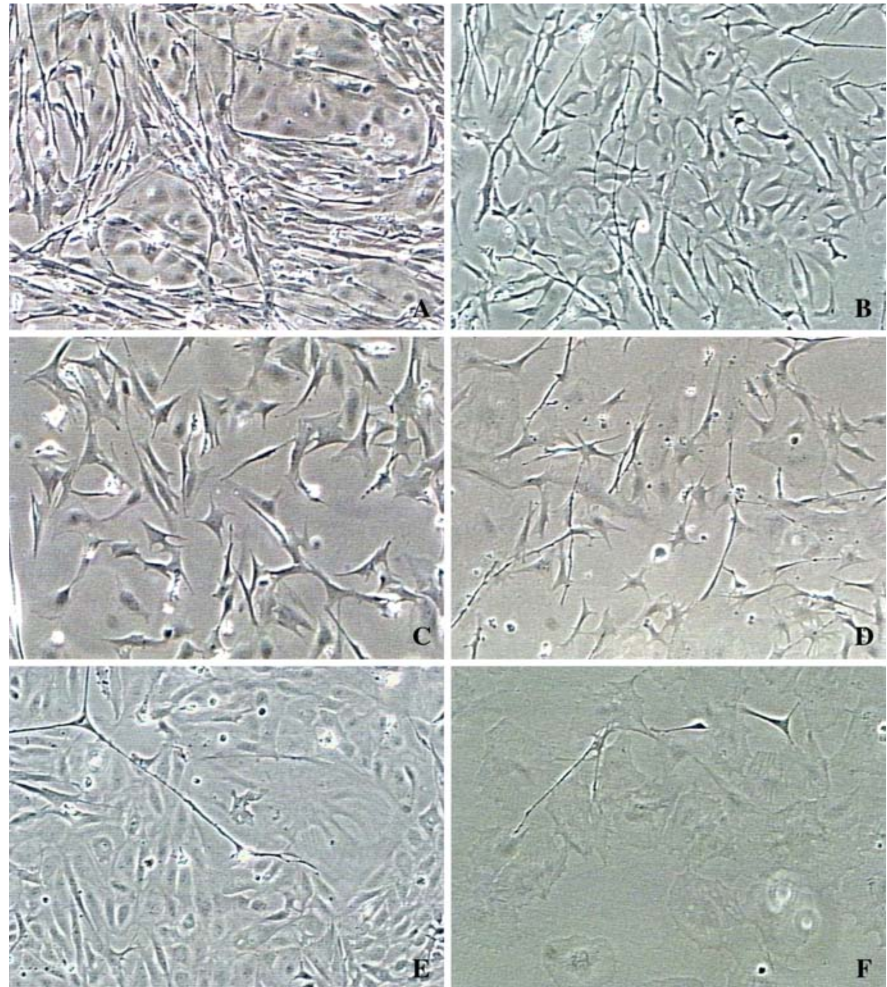

Fig. 4 Phase photomicrographs depicting the change in morphology in cultures of cells with ascending passage numbers. **a**, **c**, **e**: Trout cells as (**a**) primaries, (**c**) PN 3, and (**e**) PN4. **b**, **d**, **f**: Yellow perch cells as (**b**) primaries, (**d**) PN3, and (**f**) PN4. All photomicrographs are at 100× magnification

durations in vitro. Such a long-term culture system might facilitate numerous types of study in this scientific arena. Our report, in part, documents new efforts to optimize the isolation and culture of such cells from two different fish species.

The presence of the two morphologically distinct types of cells in both species of fish is confounding, and suggests a cell type (other than satellite cells) also resides in fish skeletal muscle. We have not observed these phenomena in cultures of satellite cells from sheep, cows, horses, dogs or elk. Immunocytochemical staining of the two types of cells demonstrated that both the long slender cells and the larger cells reacted with the myogenin antibody (Fig. 2a, b). These findings support the premise that we are isolating some myogenic satellite cells from the two species, as myogenin is a member of a family of myogenic regulatory factors and is expressed in satellite cells during myoblast differentiation (Pownall et al. 2002). Both types of the trout and perch satellite cells reacted with the α-actinin antibody (Fig. 2g, h), which was raised against a sarcomeric protein, and is expressed by both proliferating satellite cells and mature myotubes in culture (Van der Ven et al. 1992). However, only the slender, more tube-like cells were reactive with anti-myosin heavy chain (MHC) (Fig. 2c, d), another sarcomeric protein found in skeletal muscle (Rice and Leinwand 2003). The lack of dark staining of the larger cell type by anti-MHC suggests that these cells might be another population of cells that is not as fully differentiated as the MHC+ cells.

Working with satellite cells from rainbow trout and yellow perch has proved to be challenging (Funkenstein et al. 2006). The quantities of satellite cells isolated and the proliferation rates of these cells are lower than those we have obtained in other animal species. Both the trout and perch satellite cells are more difficult to cryopreserve, thaw, and replate (with any high percentages of recovery) than similar cells from cows, sheep and rats, which often display greater than 80% recovery (data not shown) from freezing. Repeated sub passage of the fish satellite cells is also more limited than in other species.

In this paper we report on our findings when comparing rainbow trout and yellow perch in the areas of satellite cell isolation, cell attachment, and development/growth of the cells in vitro. Our results reiterate the presence of a second population of cells that are co-isolatable with the satellite cells. Future studies will determine the cellularity of the second population of "unidentified" cell.

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