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Derivation of a continuous myogenic cell culture from an embryo of common killifish, *Fundulus heteroclitus*

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ABSTRACT

The common killifish or mummichog (*Fundulus heteroclitus*) is an estuarine teleost increasingly used in comparative physiology, toxicology and embryology. Their ability to withstand extreme environmental conditions and ease of maintenance has made them popular aquatic research organisms. Scientific advances with most popular model organisms have been assisted with the availability of continuous cell lines; however, cell lines from *F. heteroclitus* appear to be unavailable. The development of a killifish cell line, KFE-5, derived from the mid trunk region of a late stage embryo is described here. KFE-5 grows well in Leibovitz's L-15 media with 10% fetal bovine serum (FBS). This cell line has been passaged over 60 times in a span of three years, and cells at various passages have been successfully cryopreserved and thawed. The cells are mostly fibroblastic but contain myogenic cells that differentiate into mono-, bi- and multi-nucleated striated myocytes. Immunofluorescence detection of muscle specific antigens such as α-actinin, desmin, and myosin confirms KFE-5 as a myogenic cell line. KFE-5 has a temperature preference for 26–28 °C and has been shown to withstand temperatures up to 37 °C. The cell line responds to chemical signals including growth factors, hormones and extracellular matrix components. KFE-5 could thus be useful not only for mummichog's thermobiology but also for studies in fish muscle physiology and development.

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1. Introduction

Fundulus heteroclitus is a small teleost that commonly resides in euryhaline environments mainly in estuaries along eastern North America, from southwestern Newfoundland to northeastern Florida (Samaritan and Schmidt, 1982; Able and Felley, 1986). *F. heteroclitus* is a non-migratory fish that thrives best at warmer temperatures from 25 to 30 °C (Fangue et al., 2009) and can also tolerate higher temperatures of up to 35 °C (Fangue et al., 2009; Healy et al., 2010). Their relatively sedentary nature and their ability to withstand extreme temperatures, salinity changes and hypoxic conditions have made them popular aquatic model organisms to study environmental impacts (Burnett et al., 2007). Furthermore, *F. heteroclitus* has been a popular lab organism since the 19th century and is also notable for having been the first fish in space (Atz, 1986); thus, mummichogs have been proposed as key aquatic vertebrate models for toxicological, genetic and physiological studies (Burnett et al., 2007). However, unlike most other model organisms, cell cultures derived from *F. heteroclitus* are not available, even though studies using primary cell cultures date back to the 1920s (Dederer, 1921; Lewis, 1921). A convenient, manipulable and readily available cell culture model derived from this organism is desirable to complement whole organismal studies and elucidate mechanisms of thermotolerance, effects of salinity, hypoxic conditions, pathogen interactions, contaminant effects, etc.

Many fish cell lines are currently available from model organisms such as trout, zebrafish, and fathead minnow. These make the bulk of fish species cell lines reported to date and are prominent among the combined 45 different fish cell lines that are available from the main cell culture repositories worldwide: the American Type Culture Collection, ATCC and the European Collection of Animal Cell Cultures, ECACC (Dayeh et al., 2013). No *F. heteroclitus* cell lines appear to have been developed that were described in the scientific literature. Here we report on the first continuous cell line, KFE-5 (KilliFish Embryo-5), derived from a 7 d embryo mid-trunk explant. The cells have mesodermal origin characteristics, and myogenic cells are abundant. This cell line appears

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to be the first continuous fish myogenic cell line, and since fish muscle unlike mammalian muscle, grows continuously through both hypertrophy and hyperplasia, and demonstrates environmental plasticity (Johnston et al., 2011), the KFE-5 cell line could be useful for muscle physiology studies as well as for their capacity to survive environmental extremes.

Myogenic cell cultures and cell lines are useful to study myogenesis and factors associated with its diseased/dysfunctional states (Yaffe, 1968; Richler and Yaffe, 1970; Yaffe and Saxel, 1977; Blau et al., 1985). Serving as convenient tools for medical and basic research, immortal myogenic cell lines have been established from several mammalian and avian species. Two model examples of widely used continuous myogenic cell lines are C2C12 from mouse (Yaffe and Saxel, 1977; Blau et al., 1985) and L6 from rat (Yaffe, 1968; Richler and Yaffe, 1970). In fish, even though myoblasts or myosatellite cells from white muscles could be isolated and primary-cultures prepared from a variety of fish species (Powell et al., 1989; Koumans et al., 1990; Greenlee et al., 1995; Matschak and Stickland, 1995; Mulvaney and Cyrino, 1995), these cultures did not give rise to continuous cell lines (Funkenstein et al., 2006). The lack of fish muscle cell lines (Johnston et al., 2011) makes studies on differentiation of myoblasts or exploration on mechanistic pathways difficult. It also impedes the investigation of in vitro testing or the characterization of specific muscle growth factors such as myostatin or insulin-like growth factors (IGFs) in fish. The availability of the KFE-5 cell line may now facilitate or open up more avenues of research.

2. Materials and methods

2.1. Mummichog embryos

Mummichog (*F. heteroclitus*) broodstock were collected from estuaries near Shediac, NB, Canada, transported to Wilfrid Laurier University and induced to spawn as described by Maclatchy et al. (2003). Fertilized eggs were collected and transferred to Petri plates in 16 ppt saline water (half strength seawater) at 26 °C with daily water renewal. Cell cultures were initiated from these embryos, at various stages of development, following similar protocols to those used for establishing a haddock embryo cell line as per Bryson et al. (2006) but adapted to the warmer euryhaline species.

2.2. Cell line development

Unless specified otherwise, tissue culture (TC) supplies and chemicals were purchased from Sigma (St. Louis, MO, USA). Embryos were aseptically isolated from the chorion and the yolk sac was excised. Individual embryos (numbered 1 to 13, processed at various times) were placed in Hank's buffered salt solution (HBSS) and mechanically cut into smaller pieces on sterile Petri dishes using sterile needles. Specific embryonic tissue fragments (cephalic, mid or posterior parts) were transferred into several multi-well TC plates. Viable cultures were obtained from all 13 embryos processed, but only ten primary cultures contained proliferating cells that could be further subcultured. Commercially available TrypLE (Gibco, Life Technologies, Burlington, ON, Canada) was used to dissociate adherent cells and early proliferating cells were transferred into 12.5-cm^2 TC flask. Cells were maintained at 26–28 °C in a growth medium consisting of Leibovitz's L-15 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (regular media). Over approximately a month, the cells grew into confluent monolayers in several flasks. From one of these flasks (derived from the mid trunk of embryo #5), the cells were passaged into a 75-cm^2 flask, where the cells grew to confluency in approximately two weeks. This flask was split 1:2 and the passaged cells grew rapidly to confluency (within 6 d) and subsequent passaging gave rise to the KFE-5 cell line. Striated myocyte-like cells were present from the start and subsequent subculturing did not diminish their appearance. The embryo #5 was at stage 33 of Armstrong and Child (1965) who reported detailed descriptions of the mummichog embryo development.

KFE-5 were cryopreserved at various passage levels. Cryopreservation was achieved by freezing vials of cells (5 to 10×10^6 cells) in 1 mL of cryopreservation medium consisting of 10% dimethyl sulfoxide (DMSO) in L-15 with 10% FBS, into liquid nitrogen. Viability post-thawing was evaluated using trypan blue exclusion test. Cells were routinely monitored for mycoplasma contamination by Hoechst staining as described previously for other fish cell lines (Lee et al., 1993, 1997; Bryson et al., 2006; Xing et al., 2008; Servili et al., 2009).

2.3. Other cell lines

The KFE-5 cell line was compared in various aspects with several other fish cell lines established in our laboratory. These were: RTL-W1, a liver cell line from rainbow trout (Lee et al., 1993); GFSk-S1, a goldfish skin cell line (Lee et al., 1997); Zeb2J, a zebrafish embryo cell line (Xing et al., 2008); and EelB, an eel brain cell line (unpublished). RTL-W1 were grown at 18 °C in Leibovitz's L-15 supplemented with 5% FBS, GFSk-S1 and EelB were grown at room temperature (~21 °C ± 2 °C) in L-15 with 10% FBS, while Zeb2J were grown at 24 °C in L-15 with 10% FBS.

2.4. Cell line authentication

Cells in flasks, frozen pellets or blotted onto FTA cards (Whatman) were submitted to the Biodiversity Institute of Ontario (Guelph, ON, Canada) for species authentication (as per Cooper et al., 2007). Cytochrome c oxidase subunit 1 (CO1) gene sequences were analyzed to match the cell lines to their originating species. KFE-5 cells at passage 28 were blotted onto FTA cards, DNA was extracted, CO1 sequence was amplified using PCR primer cocktail and procedures were developed for teleost fish (Cooper et al., 2007; Ivanova et al., 2007).

2.5. Antigen detection by immunocytochemistry and Western blotting

KFE-5 cultures were analyzed by immunocytochemistry for the presence of characteristic myogenic markers (see Table 1). KFE-5 and other cells were seeded at 250,000 cells/well in 4-chamber tissue culture slides (Lab-Tek), and incubated overnight at appropriate temperatures (26 °C for KFE-5). After 24 h, cells were fixed with ice-cold absolute methanol and primary antibodies (at the dilutions indicated in Table 1) added for 2 h at room temperature. The appropriate secondary antibodies were then added (indicated also in Table 1), and incubated for 1 h in the dark. Fluoroshield with DAPI (Sigma) as a counterstain to label the nuclei of the cells was then added before fluorescence microscopy. Slides with appropriate controls including normal sera and

Table 1
Antibodies tested for immunocytochemistry.

Marker	Supplier	Source	1° Ab dilution	2° Ab	2° Ab Dilution
Desmin	Sigma D8281	Rabbit polyclonal	1:20	Goat anti-rabbit, FITC label (Sigma 0382)	1:100
α-Actinin	Sigma A7811	Mouse monoclonal	1:500	Goat anti-mouse, Alexa flour 488-conjugated (Invitrogen A11001)	1:1000
Myosin	Sigma M7523	Rabbit polyclonal	1:200	Goat anti-rabbit, Alexa flour 488-conjugated (Invitrogen A11008)	1:300

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