Current Research in Food Science 9 (2024) 100841

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Advances in development of long-term embryonic stem cell-like cultures from a marine fish, *Sciaenops ocellatus*

Catherine J. Walsh [a,*], Nicole Rhody [b], Kevan L. Main [c], Jessica Restivo [d], Andrea M. Tarnecki [a,e]

[a] Marine Immunology Program, Mote Marine Laboratory, Sarasota, FL, 34236, USA
[b] Mote Aquaculture Research Park, Sarasota, FL, 34240, USA
[c] Mote Aquaculture Research Park, Sarasota, FL, 34250, USA
[d] Department of Neurology, Washington University School of Medicine, St. Louis, MO, 63110, USA
[e] Auburn University Shellfish Lab, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, Dauphin Island, AL, USA

ARTICLE INFO

Handling Editor: Professor A.G. Marangoni

Keywords:
Red drum
Cell-based seafood
Stem cells
Marine finfish
Cultivated seafood

ABSTRACT

The overall goal of our research was to develop an embryonic stem cell line from red drum, *Sciaenops ocellatus*. These experiments were conducted to support future production of cell-based cultivated seafood products as a means towards meeting the growing global demand for sustainable seafood. Our hypothesis was that characteristics of embryonic stem cells, such as high proliferation and pluripotency, would facilitate development of a continuous cell line that could eventually be directed toward a muscle cell phenotype. We isolated embryonic stem cells from fertilized red drum eggs at the blastomere stage. These cells were seeded into culture wells at 50,000 cells/well. We tested various media, supplements, growth factors, and plate coatings to achieve growth of red drum embryonic cells. Cells at isolation reacted positively with the stem cell markers, OCT4, Nanog, and Sox2. Our cells had a fibroblast-like appearance and were maintained in culture for more than 43 days before senescence. Over time, most of the cultures showed extensive differentiation or died. The establishment of *in vitro* cultures of embryonic stem cell-like cells derived from red drum embryos represents progress towards developing cultured seafood products from marine fish.

1. Introduction

Red drum (*Sciaenops ocellatus*) production has grown into a global aquaculture industry with the U.S. producing only a small fraction of the total fish produced (Monterey Bay Seafood Watch, March). Worldwide production of red drum grew from 53,000 metric tons in 2010 to 84,300 metric tons in 2020. This represents a 54.6% increase in production over the past 10 years (FAO, 2022). In the U.S., 3 producers in Texas farmed all the red drum intended for the food market, with most of this production by one company on a 200-acre farm. U.S. production of red drum in 2014 was 1,500 metric tons (Data from FAO on red drum production from 2010 to 2014). In recent years, deep freezes in Texas have drastically reduced domestically produced product availability. The U.S. also imports farmed red drum from Taiwan and China, which are the major producers of red drum.

Cell-based seafood is rapidly emerging as a new approach for alternate protein sources to meet growing global protein needs (Goswami et al., 2022) and potentially mitigate ecological damage and animal welfare concerns associated with agriculture (Bonkamp et al., 2023). This effort requires development of continuous cell lines from seafood-relevant species with high commercial potential – techniques have not been determined for many species (Bonkamp et al., 2023). Cell culture research on aquatic species is relatively understudied when compared to that on mammalian cells, with zebrafish as a model organism being an exception. Consequently, establishing cell lines useful in cellular agriculture has encountered significant challenges (Soice and Johnston, 2021). To create cell lines useful for cellular agriculture, scientists must determine ideal culture conditions, establish methods to characterize the cells of interest, and induce myogenesis (Thorley et al., 2016) – a sizable undertaking. An early publication on cultured seafood

Abbreviations: FBS, fetal bovine serum; ES, embryonic stem cell; PBS, phosphate-buffered saline; TBST, (Tris-buffered Saline-Tween 20; FGF, fibroblast growth factor; DHA, docosahexaenoic acid; LIF, leukemia inhibitory factor; OCT4, octamer transcription factor.

* Corresponding author. Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, FL, 34236, USA.
E-mail addresses: cjwalsh@mote.org (C.J. Walsh), nrhody@mote.org (N. Rhody), kmain@mote.org (K.L. Main), jrestivo@wustl.edu (J. Restivo), atarnecki@auburn.edu (A.M. Tarnecki).

https://doi.org/10.1016/j.crfs.2024.100841
Received 31 May 2024; Received in revised form 29 August 2024; Accepted 7 September 2024
Available online 13 September 2024

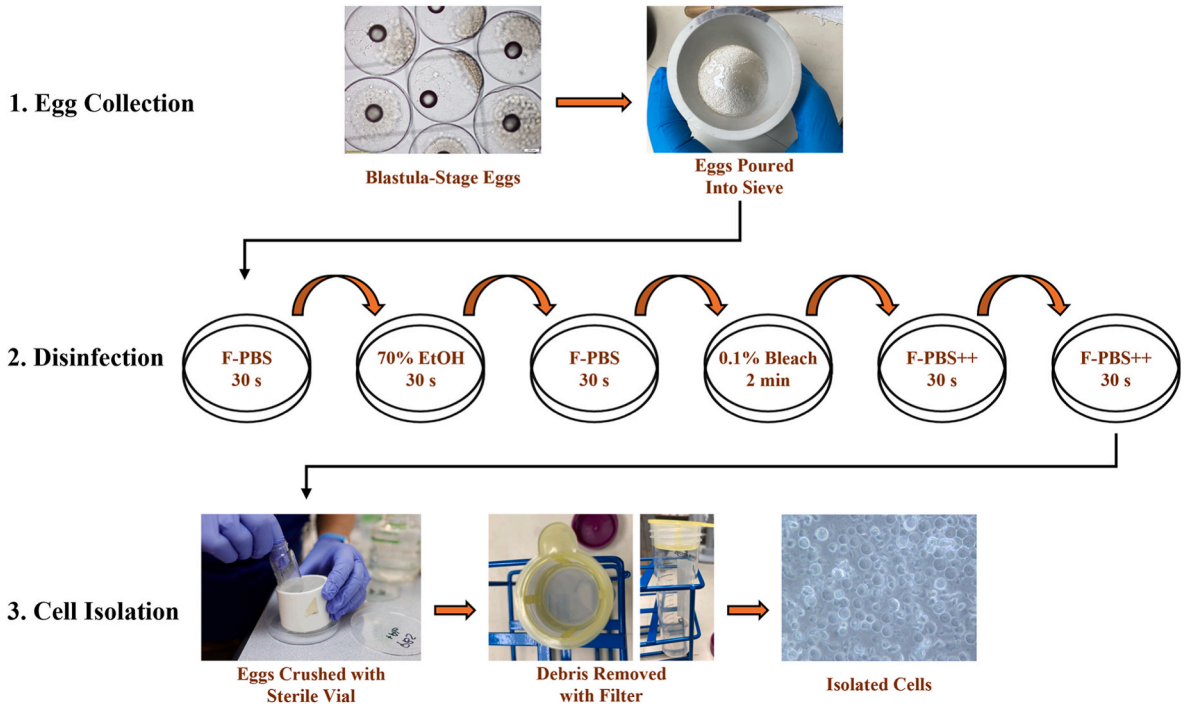


Fig. 1. Flow chart depicting the series of steps comprising the protocol for isolating embryonic stem cells from fertilized embryos of red drum, *S. ocellatus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. Fertilized red drum egg showing area of cell isolation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

described production of a goldfish filet *in vitro* with the goal of generating a protein source for long-term space travel (Benjaminson et al., 2002). Saad et al. (2023) report the first spontaneously immortalized fish muscle cell line from a marine species. However, most fish cell lines have been established as a promising tool for studying key issues of aquaculture such as growth, disease, genetics, reproduction, biotechnology, toxicology, and immunology (Goswami et al., 2022).

To further develop and refine stem cell technology from marine fish, we have established embryonic stem cell cultures from a marine fish, *S. ocellatus*. Culturing pluripotent embryonic stem cells represents a unique model system for *in vitro* cell growth and differentiation. Stem cells are distinctive cell populations with the ability to undergo both self-renewal and differentiation. Embryonic stem (ES) cells originate from

Table 1
Cell culture media used with red drum ES-like cell culture. AA = amino acids; ATCC = American Type Culture Collection; R&D = R&D Systems; MML = Mote Marine Laboratory.

Component	M1 (C2003; D2-2023)[a]	M2 (D3A-2023)[b]	M3 (F5-L)	M4 (ZF)[c]
Base media (Sigma)	DMEM	DMEM	L-15	L-15
FBS (ATCC)	15%	10%	15%	15%
Penicillin/ streptomycin (Sigma)	100 U/mL/ 10 μg/mL	100 U/mL/ 10 μg/mL	100 U/mL/ 10 μg/mL	100 U/mL/ 10 μg/mL
AmpB (Sigma)	250 ng/mL	250 ng/mL	250 ng/mL	250 ng/mL
L-glutamine (Sigma)		20 mM	2 mM	
HEPES (Sigma)	25 mM	25 mM	20 mM	
Nonessential AA (Sigma)		1 nM	1%	
Glucose (Sigma)		4.5 g/L		
LIF (Millipore)	5 ng/mL	100 U/mL		
bFGF (R&D)	5 ng/mL		10 ng/mL	
Sodium selenite (Sigma)	8 nM	2 mM	2 nM	
Sodium pyruvate (Sigma)	1 mM	10 mM	1 mM	
β-ME (BioRad)	27.5 μM	50 μM	0.34 μL[d] per 100 mL	
Glutamax (Gibco)				2 mM
$CaCl_2$ (Sigma)				0.8 mM
Gentamycin (Gibco)				10 μg/mL
Red drum embryo extract (MML)		1%		
Osmolarity	310–320 mOsm	310–320 mOsm	310–320 mOsm	310–320 mOsm
Adjust pH	7.4	7.4	7.4	7.4

[a] Chen et al. (2002), 2003.
[b] Holen and Hamre, (2003).
[c] Choorapoikayil et al, (2013).
[d] 2-ME.

Table 2
Summary of culture duration and cell characteristics with each media type.

	M1	M1+2XFGF (M1b)	M2	M3	M4
Days in Culture	43	28	9	9	15
Morphology descriptions	Round, nonadherent cells with adherent cells visible underneath	Round, nonadherent cells with adherent cells visible underneath	Initially adherent cells underneath, but these did not persist	Initially, adherent cells underneath, but these did not persist	Adherent cells growing underneath
Passages	2 (15 days)	2 (11 days)	n/a	n/a	n/a
Passaged cells description	Initially, round adherent morphology, with elongated morphology appearing later which initially increased in cell number. With trypsinization, cell numbers decreased dramatically.	Initially, round adherent morphology, with elongated morphology appearing later which initially increased in cell number. With trypsinization, cell numbers decreased dramatically.	n/a	n/a	n/a

Table 3
Expression of pluripotent proteins relative to β-actin.

Antibody Designation	Full Name	Role in pluripotency	Company and Catalog #	Target Species	Concentration on blot	Bands detected (kDa)
OCT4	Octamer binding transcription factor 4	Transcription factor with key role in embryonic development and stem cell pluripotency	Invitrogen (PA5-85098)	Human/ mouse	1:1,000	40, 60
SSEA-1	Stage specific embryonic antigen 1	Expressed in stem cells	Invitrogen (620–470)	Human	1:2,500	58
NANOG	Nanog homeobox	Transcription factor involved in embryonic stem (ES) cell proliferation, renewal, and pluripotency	Biotechne (AF272)	Mouse	1:2,000[a]	38
Sox2	SRY-box transcription factor 2	Transcription factor involved in regulation of embryonic development and determination of cell fate	Biotechne (AF2018)	Human/ mouse/rat	1 μg/mL	36, 28
ALPL	Alkaline phosphatase	Expressed in stem cells	Biotechne (AF2909)	Human/ mouse	0.5 μg/mL	30, 21. 5, 14.6
PCNA	Proliferating nuclear antigen	DNA replication	Santa Cruz (sc-56)	Mouse	1:300	38

[a] Yang et al, (2014).

pluripotent cells derived from the inner mass of fertilized eggs up to midblastula stage. These cells can be induced to generate muscle, fat, and other cell types that might be found in a fish filet.

Attempts to culture ES cells from other fish species have been reported. For example, authors report on ES-like cells from zebrafish (*Danio rerio*) (Collidi et al., 1992; Sun et al., 1995), medaka (*Oryzias latipes*) (Wakamutsu and Ozato, 1994), gilt-head sea bream (*Sparus aurata*) (Bejar et al., 2002), Japanese sea bass (*Lateolabrax japonicus*) (Chen et al., 2002), turbot (*Scophthalmus maximus*) (Holen and Hamre, 2003), and Atlantic cod (*Gadus morhua*) (Holen et al., 2010). A gene-targeting approach has been used in zebrafish and medaka (Hong et al., 1996; Chen et al., 2003) to develop ES-like cell lines. Several olive flounder (*Paralichthys olivaceus*) cell lines have been developed (Chen et al., 2004), with Kim et al. (2018) developing a primary cell culture of olive flounder cells from the blastula stage of embryos. A pluripotent cell line was derived from blastula-stage embryos of a cultured marine fish, Japanese sea bass (*Lateolabrax japonicas*) (Chen et al., 2002). A pluripotent ES cell-like cell line from blastula stage embryos of Asian sea bass (*Lates calcarifer*) was reported (Parameswaran et al., 2007). Despite these examples, publicly available cell lines for use in cultured seafood research are lacking, and generation of ES cell lines from fishes is an important step for advancing cell-based seafood.

Complications in research efforts related to marine finfish cell line development are likely directly related to a deficit in species-specific data on the biochemical and physiological properties of relevant species (Soice and Johnston, 2021). Spontaneous differentiation is frequent in marine finfish cultures, and critical conditions and components for immortalization in these species are unknown (Soice and Johnston, 2021). Cell culture media design is a significant challenge in cellular agriculture research, and multiple cell types likely have quite different nutrient needs (O'Neill et al., 2022).

The overall goal of our research was to develop an ES-like cell line from fertilized red drum eggs to support future production of cell-based cultivated seafood products. Our hypothesis was that cells isolated from blastula-stage embryos can be developed into a continuously growing embryonic cell line. Data in this paper describes the process for collecting ES-like cells from red drum which prevents contamination from other aquatic organisms and provides insight into media formulations that can be optimized in future experiments. This information is vital to advance development of such a cell line, to include inhibition of spontaneous differentiation and direction of cells towards myogenesis. Embryonic cells have high potential for active proliferation and pluripotency and appear to be optimal sources from which to establish continuous cell lines. In this paper, we describe the establishment of red drum derived ES-like cells.

2. Materials and methods

2.1. Red drum reagents

Fish PBS (F-PBS) was prepared by adding 0.21% NaCl to normal PBS (Sigma P3813), for a final osmolarity of ~320 mOsm. Osmolarity was measured using an osmometer (Advanced Instruments, Model 3320). F-PBS containing antibiotics/antimycotics (F-PBS++) was formulated with F-PBS, Penicillin/Streptomycin (100 U/mL/10 μg/mL, Sigma P4333), and amphotericin B (250 ng/mL, Sigma A2942). Reagents were sterilized using 0.2 μM filtration.

2.2. Cell isolation

Fertilized red drum eggs were collected at the blastula stage (Fig. 2), approximately 5 h after spawning. Approximately 50,000 eggs were collected for cell isolation by pouring eggs suspended in seawater into a sieve constructed of 100 μM mesh and PVC pipe. The sieve was disinfected in 70% ethanol and rinsed in sterile PBS prior to use. To prevent contamination of the culture, eggs were cleaned using the following

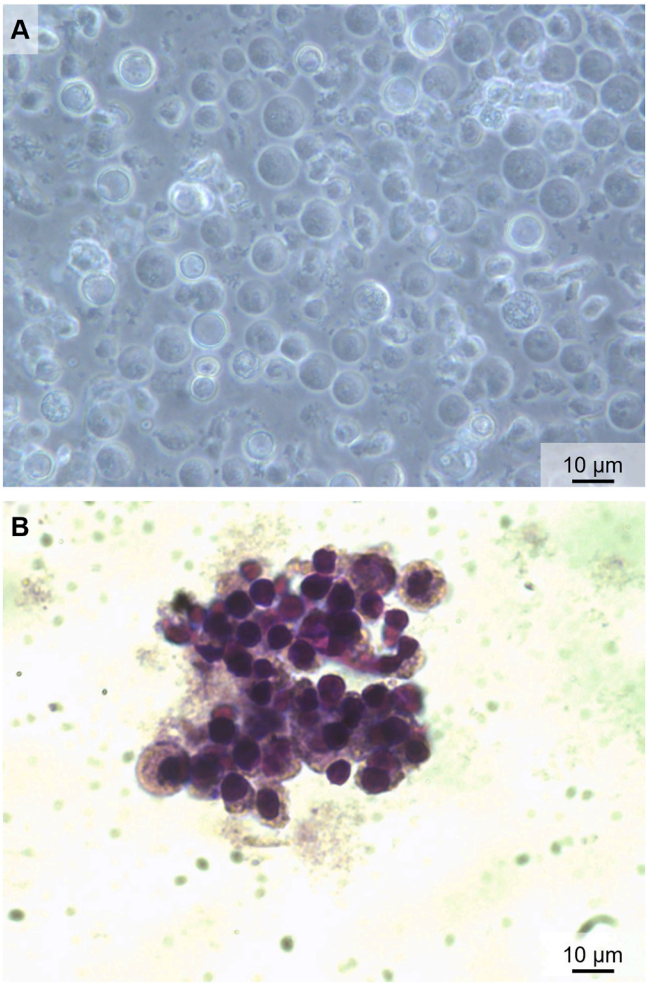


Fig. 3. Red drum embryonic stem cells. A) **Day 0**, at time of isolation; magnification = 40x. B) **Day 2**, Wright-Giemsa stain; magnification = 40x. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

procedure. The sieve containing the eggs was placed into a 100 mm sterile Petri dish containing F-PBS and swirled for 30 s to rinse the eggs thoroughly. The sieve was then transferred to another Petri dish containing 70% EtOH, swirled for 30 s, and then transferred to another Petri dish containing F-PBS to remove the ethanol. After removing the ethanol, the sieve was transferred to a Petri dish containing 0.1% bleach, and swirled for 2 min. The sieve was then transferred to a Petri dish containing F-PBS++ and swirled for 30 s to remove bleach. The eggs were transferred to a final Petri dish containing F-PBS++, and the eggs were crushed using the bottom of a sterile borosilicate vial. This process released cells from the egg into the F-PBS++, which was collected and transferred to a 50 mL sterile centrifuge tube by passing through a 40 μm sterile centrifuge top filter (Falcon) to remove egg debris. The procedure was repeated until sufficient cells were obtained. A flow chart depicting this protocol is shown in Fig. 1. Cells were enumerated using a hemacytometer and viability determined using trypan blue (Sigma; T8154) exclusion.

2.3. Cell culture reagents and cell culture medium

Cell culture media were prepared by modifying two commercially available base formulations, Dulbecco's Modified Eagle Medium (DMEM; Sigma D6429) and L-15 (Sigma L4836). A primary difference in base media formulations is that L-15 contains galactose as the carbohydrate source whereas DMEM contains glucose (Supplementary Table 1). Four media formulations were tested (Table 1). Media 1 (M1) and Media 2 (M2) were DMEM-based and were modifications of media used in Chen et al. (2002, 2003), Holen and Hamre (2003) and Holen et al. (2010). Media 3 (M3) and Media 4 (M4) were L-15 based. M3 was similar to the DMEM-based M2, in that it did not contain added glucose, or supplements such as recombinant leukemia inhibitory factor (LIF; Millipore Cat. #LIF1010-K) or basic fibroblast growth factors (bFGF; R&D Systems 233-FB; Table 1). M4 was based on a zebrafish growth media (Choorapoikayil et al., 2013). Fetal bovine serum (FBS) was heat-inactivated (56 °C, 30 min), and added to cultures at 10%. Red drum embryo extract, used as a supplement for M2, was prepared by homogenizing approximately 300 fertilized eggs in 3 mL F-PBS. The homogenate was centrifuged at 15,000×*g* for 10 min at 4 °C to pellet debris. The supernatant was frozen at −20 °C until use. The osmolarity of all media was adjusted to 320 mOsm, pH 7.4. Cell culture media were sterilized by filtration through 0.2 μm filters.

Cells (50,000) were added to 3 wells of a 24-well tissue culture plate (Corning) for each media type in 500 μL volumes and incubated at 26 °C (Benchmark). Depending on media type, cells were cultured either with or without 5% CO_2 atmosphere. For determining cell adherence, three plate coatings were tested: untreated, laminin (Sigma L2020) and fibronectin (Sigma F2006). Adherence was tested after 48 h by removing media and rinsing with F-PBS. For cryopreservation, cells ($1x10^6$ cells per vial) were cryopreserved in 10% DMSO, 10% FBS, and 80% cell culture media using a CoolCell® (Corning) which freezes cells at a rate of −1 °C/min. After 24 h in CoolCell®, vials were transferred to vapor phase of liquid nitrogen storage.

2.4. Wright stain protocol

Cells in culture were stained using Wright-Giemsa (Sigma WG16). Cells (>$1x10^6$) were transferred to a microcentrifuge tube and centrifuged at 500×*g*, 5 min. The supernatant was removed, 500 μL ice cold methanol added to the pellet, the pellet gently dispersed, and the mixture incubated at room temperature for 5 min. Cells were centrifuged at 500×*g*, 5 min to pellet fixed cells. Wright-Giemsa stain (200 μL) was added to Table 2. the suspension and allowed to remain for 5 min. Tap water (200 μL) was added and the cells were pelleted at 500×*g*, 5 min. Cells were rinsed again with tap water and centrifuged again. The cell pellet was resuspended in ~10 μL water. A wet mount with a coverslip was prepared for examining cells. For oil immersion, 10 μL cells were mixed with 10 μL aqueous mounting media (Sigma F4680) before adding the cover slip.

2.5. Western blotting

At Day 0, octamer transcription factor 4 (OCT4), a marker for stem cells in other species (Li et al., 2022; Liu et al., 2015), was evaluated in cell lysates. Other antibodies used in detecting pluripotent cells included Nanog (Camp et al., 2009; Hong et al., 2011; Hatano et al., 2005), Sox2 (Robles et al., 2011), and alkaline phosphatase (Štelkova et al., 2015). PCNA (proliferating cell nuclear antigen) was also used to detect proliferation (Strzalka and Ziemienowicz, 2011). The antibodies used, source, target specificity and catalog numbers are listed in Table 3. Isolated red drum cells ($5x10^6$ cells) were lysed on ice in 100 μL Western Lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate) with a protease inhibitor cocktail (Sigma P8340). Cell debris was pelleted at 12,500×*g* for 15 min at 4 °C. The supernatant was transferred and protein was determined using the BioRad DC assay (Cat # 5000111). Cell lysates were run on a 12% Mini-PROTEAN TGX gel (BioRad Cat. No. 4561044) using 20 μg protein/well. Precision Plus Dual Color Standard (BioRad 161-0374) was run on the gel to confirm protein size. Protein was separated at 200V for 25 min and then the gel was transferred to nitrocellulose at 35V overnight at 4 °C. The blot was

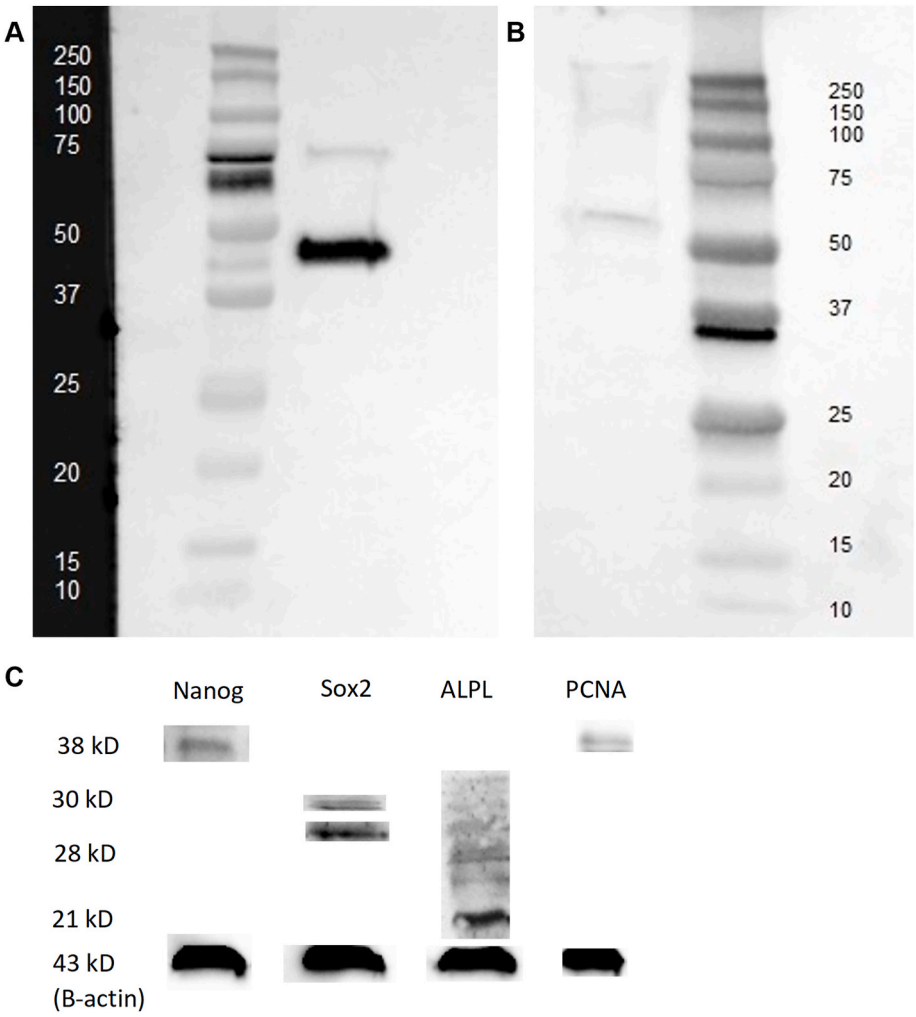


Fig. 4. Western blots of **Day 0** cell lysate from freshly isolated embryonic cells stained with antibodies against pluripotent targets. 4A) Transcription factor Octamer-4 (OCT4). Bands with molecular sizes of ~40 kDa and ~60 kDa were detected; B) SSEA-1. A band with molecular size of 58 kDa was detected. C) Multiple antibodies are shown in this image. Nanog detected a band at molecular size of ~38 kDa; Sox2 detected two bands with molecular sizes of ~28 and 36 kDa; ALPL detected multiple bands at 30, 21.5, and 14.6 kDa; PCNA detected a band with molecular size of 38 kDa.

blocked (3% Non-Fat Dry Milk (NFDM, Quality Biological Cat. # A614-1005), 2% BSA, 1X TBST (TBS-Tween20) for 1 h at room temperature. Primary antibody, OCT4 polyclonal antibody (ThermoFisher, Cat. # PA5-85098), was diluted 1:4,000 in antibody dilution buffer (1% BSA, 1X TBST) and incubated overnight at 4 °C. Following incubation, the blot was washed three times with TBST. Secondary antibody, goat anti-rabbit IgG H + L HRP (ThermoFisher Cat. # 65–6120), was diluted 1:5,000 in antibody dilution buffer and incubated for 1 h at room temperature. For the other antibodies tested, a secondary antibody anti-goat HRP (R&D Systems, Cat. # HAF017) was used. Another round of TBS-T washes was performed following the secondary antibody incubations. Bands were visualized by adding Clarity Western ECL Substrate (BioRad 170-5060) and imaged on ChemiDoc XRS+ (BioRad) using the Image Lab software. β-actin was used as protein loading control.

2.6. Cell passages

Red drum embryonic stem cell cultures were subcultured by first aspirating media and nonadherent cells, then adding 500 μL 0.25% trypsin (Sigma T4549/0.3% EDTA to wells and immediately removing the supernatant. Wells were incubated with residual trypsin/EDTA for 3 min at 26 °C. Detached cells were removed by adding 1 mL cell culture media. Cells were centrifuged (500×g, 5 min) and resuspended in fresh cell culture media before inoculating new 24-well plates.

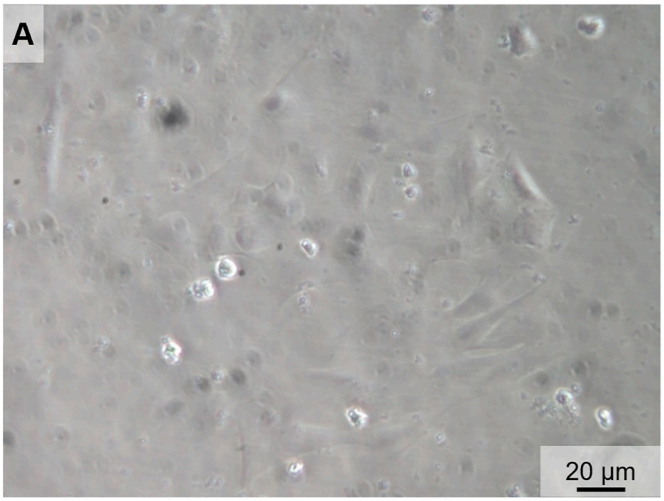
3. Results

3.1. Cell isolation

An image of a fertilized red drum egg at the blastomere stage is shown in Fig. 2. This is the stage at which cells were isolated for these experiments. A batch of 50,000 fertilized red drum eggs yielded approximately 1.4×10^7 cells using the described protocols. Viability at cell isolation was greater than 90%, as measured using trypan blue exclusion. Immediately after isolation (Day 0), cells were round with a large nucleus and a small cytoplasm (Fig. 3A), and were approximately 10–15 μm in diameter. The cells were a heterogeneous population of agranular and granular cells. On Day 2, a Wright's stain on cell cultures (Fig. 3B) showed primarily large granulocytic cells with eosinic granules and blue nuclei.

3.2. Characterization of cells using antibodies for pluripotency

Western blot images of red drum cell lysate proteins are shown in Fig. 4. The red drum cell lysate proteins were probed with several antibodies used to characterize pluripotent cells in other species. Antibodies tested include OCT4, SSEA-1, Nanog, SOX2, ALPL, and PCNA. Approximate molecular sizes of bands detected with all antibodies are listed in Table 3. A Western blot with OCT4 antibody showed positive



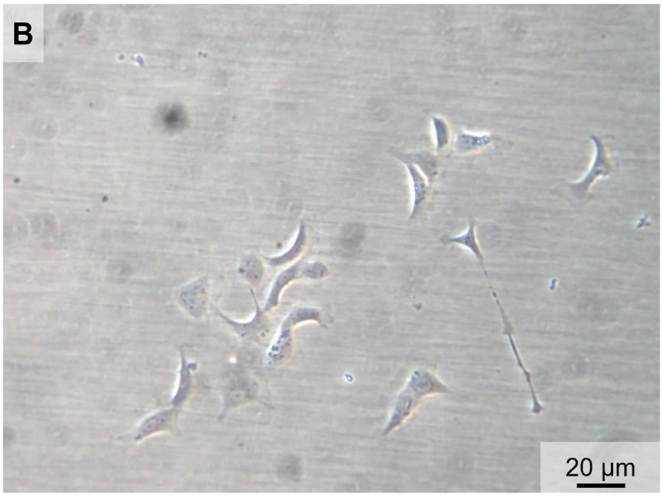
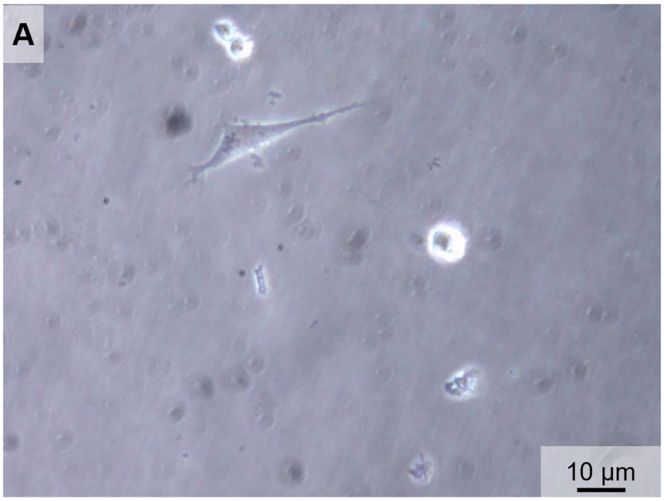


Fig. 5. Day 7 image of red drum ES-like cells. A) Prior to media exchange, showing nonadherent cells; B) after media exchange showing elongated, adherent cells with fibroblast-like appearance. Cells were cultured in Media 1 on uncoated 24-well cluster plates; magnification = 20x.



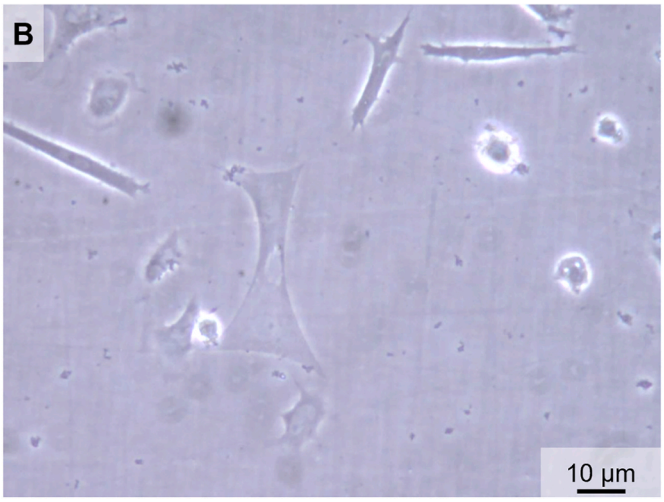


Fig. 6. Day 2 images of adherent ES-like cells on untreated plastic (A) vs. fibronectin-coated (B) plates. For both images, wells were washed gently with PBS to remove nonadherent cells. Cells were cultured with Media 1, magnification = 40x.

bands reacting at ~40K MW and ~60K MW (Fig. 4A). Results with SSEA-1 antibody are shown in Fig. 4B, and show a band with an approximate molecular size of 58 kDa. Results with Nanog, Sox2, ALPL, and PCNA are shown in Fig. 4C. Using a mouse Nanog antibody, a band of ~38 kDa was detected in red drum cell lysate. With a Sox2 antibody with specificity for human/mouse/rat Sox2 detected bands of approximately 28 and 36 kDa in red drum cell lysate. With mouse alkaline phosphatase, multiple bands were detected at 14.6, 21.5, and 30 kDa. These represent much lower molecular sizes than the 75 kDa expected with this antibody. Red drum cell lysate showed a positive reaction with mouse PCNA at ~38 kDa.

3.3. Cell culture media and plate coatings

After 8 days in culture, a fifth media type – M1 with 2Xb FGF included (M1b), was added to encourage the growth of fibroblast-like cells that were appearing in cell cultures. Duration of cells in culture with each media type are listed in. Media types M2 and M3 lasted less than 10 days. Media type M4 lasted 15 days. M1 with 2X bFGF lasted 28 days. In all cases, adherent cells were growing underneath nonadherent cells and were often not visible until the nonadherent cell population was removed (Fig. 5). Morphology of cells changed from rounded cells to adherent cells with a fibroblast-like appearance. The adherent cell population did not persist with media formulations M2 and M3 but persisted for 15 days in media formulation M4. M1 resulted in longest duration of culture at > 43 days initial culture. Supplementation of M1 with 2X bFGF did not alter cell morphology (Fig. 8G) or improve culture duration. Over time, most cell cultures became predominated by nonadherent, round, granular cells, indicating cell differentiation was likely occurring. A major observation was an increase in round nonadherent cells as length of time in culture increased. Table 2 shows cell culture durations in various media types.

At Day 2, coatings with fibronectin (Fig. 6B) initially showed greater adherence than plastic alone (Fig. 6A). Although adherent cells were observed in uncoated plates, they were fewer in number and not the predominant cell morphology. Fibronectin coated plates were used for the duration of the cell cultures. Images from adherent cells on fibronectin coated plates on Day 5 are shown in Fig. 7. Photomicrographs of adherent red drum ES-like cells grown in M1 in fibronectin-coated 24-well cluster well plates are shown in Fig. 8. Fibroblast-like cells appeared at Day 9 of culture (Fig. 8A) and remained through Day 15 (Fig. 8A–E). During this time, nonadherent cells gave the appearance of a mixed cell culture; these cells were removed when plates were rinsed with F-PBS.

3.4. Passaging and longer-term cultures

A closely packed cell population with a cobblestone appearance was

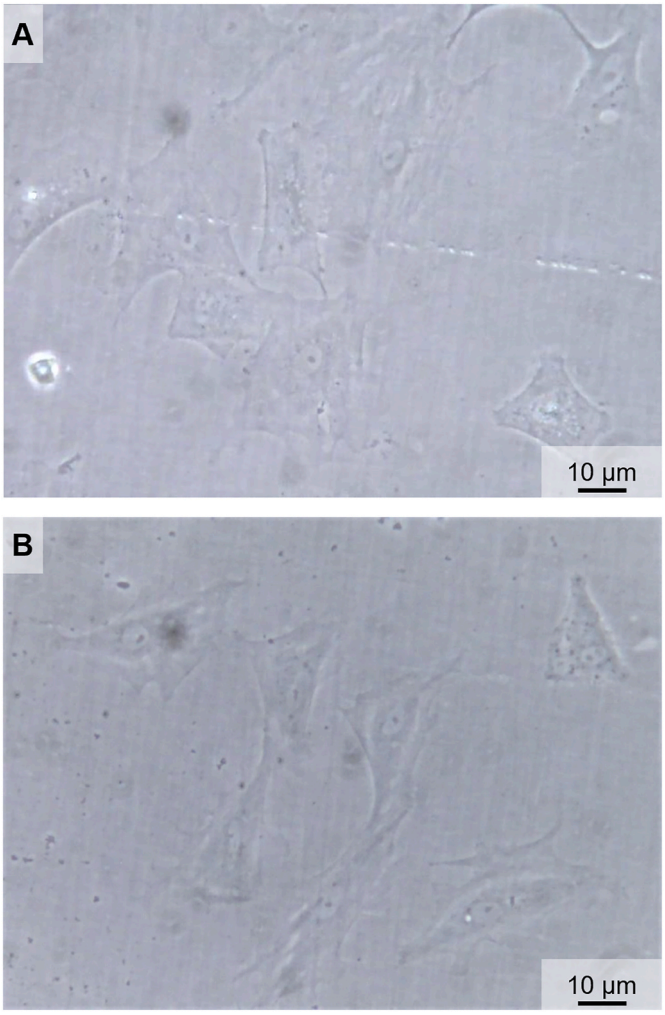


Fig. 7. Day 5 images (A and B) of adherent ES-like cells on fibronectin-coated plates using Media 1. Wells were washed gently with PBS to remove non-adherent cells at 48 h; magnification = 40x.

seen at Day 21 (Fig. 8F). At Day 27, nonadherent cells again formed in the cultures (Fig. 8H), presumably from differentiation of adherent cells. After media exchange on Day 34, adherent cells have a fibroblast-like morphology (Fig. 8I). The longest duration of culture was >43 days from initial start of the culture. However, this culture only survived one passage after subculture with trypsinization. This subculture lasted for 15 days before cells senesced.

On Day 4, adherent cells were trypsinized to passage them (Fig. 8J). At 12 days post-trypsinization (Fig. 8K), cells were not thriving well, indicating a negative impact on cell viability. Morphologies on Days 2 and 4 of passage showed some adherent cells and some fibroblast-like adherent cells (Fig. 9A-B). On Day 5, many cells with round morphologies were observed and remained following trypsinization and removal of nonadherent cells (Fig. 9C and D). On Day 6, cells showed fibroblast-like morphology (Fig. 9E). On Day 8 of subculture, cells decreased in number as evidenced by reduced confluence (Fig. 9F). Cultures were passaged on Day 15, Day 33, and Day 41. No cultures thrived beyond the second passage. The longest Passage 2 culture lasted 15 days.

4. Discussion

We report progress towards an ES-like cell line isolated from red drum embryos before reaching the mid-blastula stage. Cells grew successfully in cell culture for about 43 days, and then senesced. Attempts to passage cells failed to produce continued cultures beyond about Day 15 after subculture. We observed spontaneous differentiation in our cultures, with development of round, granulocytic cells. Spontaneous differentiation is a challenge in developing embryonic stem cell cultures with fish and contributes to the limited number of stem cell lines available (Goswami et al., 2022; Hong et al., 2000). Although we were not successful in inhibiting spontaneous differentiation, the ability to do so is a critical component to move forward with cultivated seafood using ES-like cells as a source. Future studies would explore approaches to inhibiting spontaneous differentiation potentially through the use of feeder layers (Wakamutsu et al., 1994) or conditioned media (Collodi et al., 1992). Feeder-free approaches have been successful in medaka (Hong et al., 1996), and use gelatin, embryo extracts, or growth factors such as bFGF and/or leukemia inhibitory factor (LIF; Hong et al., 2000). In our study, we tested various plate coatings, including gelatin, with only fibronectin showing promise for supporting growth of red drum cells long term. We also tested red drum embryo extract, bFGF, and LIF, with limited success. Efforts to optimize concentrations and combinations might improve the success of these approaches.

The concept of producing cell-based seafood has emerged as a new approach to producing alternate animal protein (Rubio et al., 2019). Fish are especially attractive model organisms in ES cell research due to the cost effectiveness of maintaining animals, external fertilization, high fecundity, large transparent embryos, and rapid development (Alvarez et al., 2007). The inner cell mass of a blastocyst is a source of embryonic stem cells, which grows into derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm (Lakhan et al., 2018). Several ES-like cells have been developed from other marine fish species using the approach of isolating cells from blastula-stage embryos (Chen et al., 2002, 2003; Parameswaran et al., 2006, 2007; Holen and Hamre, 2003). In these reports, the description of cell morphology was generally similar, and consistent with observations regarding cell morphology described in this paper. Cells were most often described as small (10–30 μM), round or polygonal cells, and were adherent and grew actively. These cells also generally had large nuclei and sparse cytoplasm. In Fig. 8F, adherent polygonal cells can be seen. Such cells were described in embryonic stem cells derived from red sea bream (Chen et al., 2003) and Japanese sea bass (Chen et al., 2002). Holen and Hamre (2003) describe cells growing in a monolayer and showing typical morphology of undifferentiated ES-like cells, namely small in size, round or polygonal in shape and tightly compacted in culture. With freshwater fish, medaka ES-like cells (Hong et al., 1996) were also small, round or polygonal in shape, with sparse cytoplasm and large nuclei. Many authors also reported positive reactivity with OCT4 in ES-like stem cells from marine fish, including Japanese sea bass (Chen et al., 2002), Asian sea bass (*Lates calcarifer*, Parameswaran et al., 2006, 2007), gilt-head sea bream (Bejar et al., 2002), marine flatfish (*Scopthalmus maximus*; Holen and Hamre, 2003) and medaka (Hong et al., 1996). Most media types used in other studies included DMEM and L-15, with FBS as supplementation. Other common supplements were embryo extract (Chen et al., 2002, 2003; Bejar et al., 2002), HEPES, non-essential amino acids, sodium pyruvate, selenium, and β-ME. Most investigators cited bFGF (Chen et al., 2002, 2003, 2004; Bejar et al., 2002) to have a positive impact on cell growth, without inducing cell differentiation. Some investigators included LIF (Chen et al., 2002, 2003), but did not observe its presence to have a significant impact on cell growth. Chen et al. (2004) concluded that bFGF stimulates proliferation of flounder cells and can be used as a potent mitogenic factor in embryonic cell cultures. In medaka, initial cell cultures included LIF, but as cultures progressed, it was removed without decreasing cell culture growth (Hong et al., 1996). The most common plate coatings used was gelatin, however other coatings were tested without reporting if they made a significant impact on cell growth or survival. In our studies, coating cell culture plates with fibronectin enhanced adherence.

We found DMEM-based media was most successful in supporting growth of red drum embryonic cells long term. The carbohydrate source

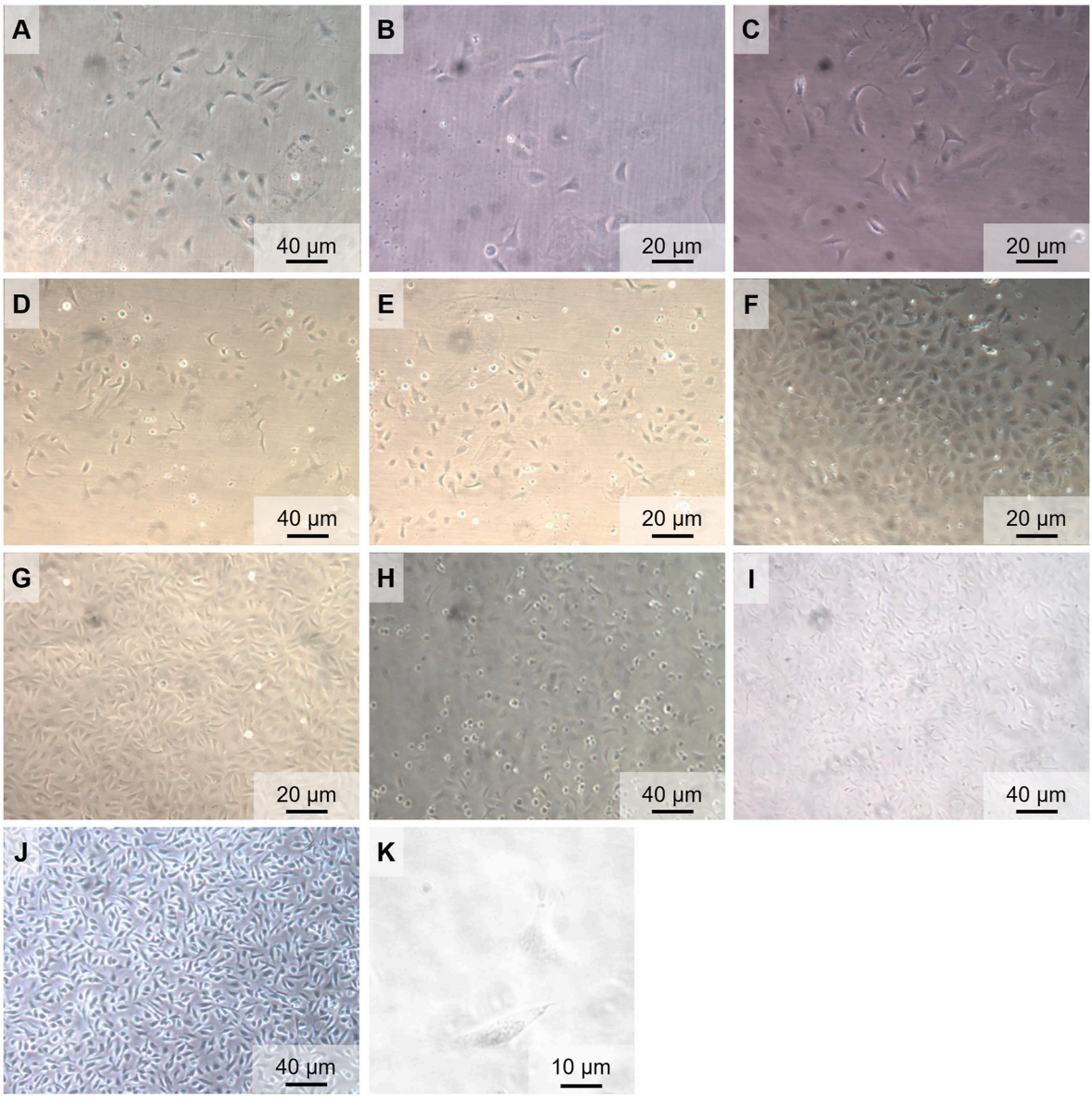


Fig. 8. Photomicrographs of adherent red drum ES-like cells grown in fibronectin-coated 24-well plates and cultured in Media 1. A) **Day 9**, magnification = 10x; B), **Day 12**, magnification = 20x; C) **Day 13**, after PBS wash to remove nonadherent cells. Media supplemented with 2×bFGF; magnification = 20x; D) **Day 14**, adherent cells discovered underneath mixed cell culture after 14 days. Magnification = 20X; E) **Day 15**, adherent cells discovered underneath mixed cell culture after 15 days, magnification = 10x; F) **Day 21** of culture, from mixed cell population, Media supplemented with 2X FGF; magnification = 10x; G) **Day 23** of culture, Media supplemented with 2X FGF; Magnification = 10x; H) **Day 27** of cell culture from mixed cell culture, prior to media exchange. Magnification = 20x; I) **Day 34** of cell culture from mixed cell culture, after media exchange. Magnification = 10x; J) **Day 41** of cell culture, during trypsinization, magnification = 10x; K) **Day 12** after trypsin, magnification = 40x. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in L-15 is galactose whereas in DMEM it is glucose (Supplementary Table 1), potentially indicating glucose has a higher potential to support red drum cell growth. Embryo extract has been reported to have mitogenic activity, but we did not observe this with our cultures. Other studies have reported species-specific serum promoted growth of cells in culture (Kim et al., 2018); we did not test different sera sources in our cultures. Various growth factors such as bFGF (Chen et al., 2004) have been used to stimulate growth of fish cell lines. bFGF is a potent mitogen for ES cells derived from Japanese rice fish (*Oryzias latipes*) and sea perch and embryonic cells from olive flounder (Chen et al., 2004). We used bFGF in our cell cultures, which appeared to support growth of red drum cells to some extent. Inclusion of LIF is very common in fish cell culture media (Holen and Hamre; 2003; Chen et al., 2003; Holen et al., 2010), but we did not detect a benefit of including this in our media formulations. Addition of vitamin E to cell cultures helped prevent against oxidative stress in southern bluefin tuna (*Thunnus maccoyii*; Scholefield and Schuller, 2014). Adding DHA reduced cell proliferation, despite high concentrations of DHA in cells (Scholefield and Schuller, 2014).

Based on the results obtained, M1 supported growth for the longest period of time, and allowed for some passages. M1 was DMEM-based and supplemented with 15% FBS, LIF and bFGF. Increasing the concentration of FGF (M1b) did not increase the culture duration.

We used the mammalian stem cell marker, OCT4, in freshly isolated cells to see if it could be useful to detect stem cells from red drum. OCT4 showed strong binding at the initiation of cell cultures; however, we were not able to test this in cells further into culture due to limited cell numbers. Li et al. (2022) found that OCT4 is one of the important markers of cellular pluripotency in fish. It plays a central role in maintaining the self-renewal and differentiation of embryonic stem cells into

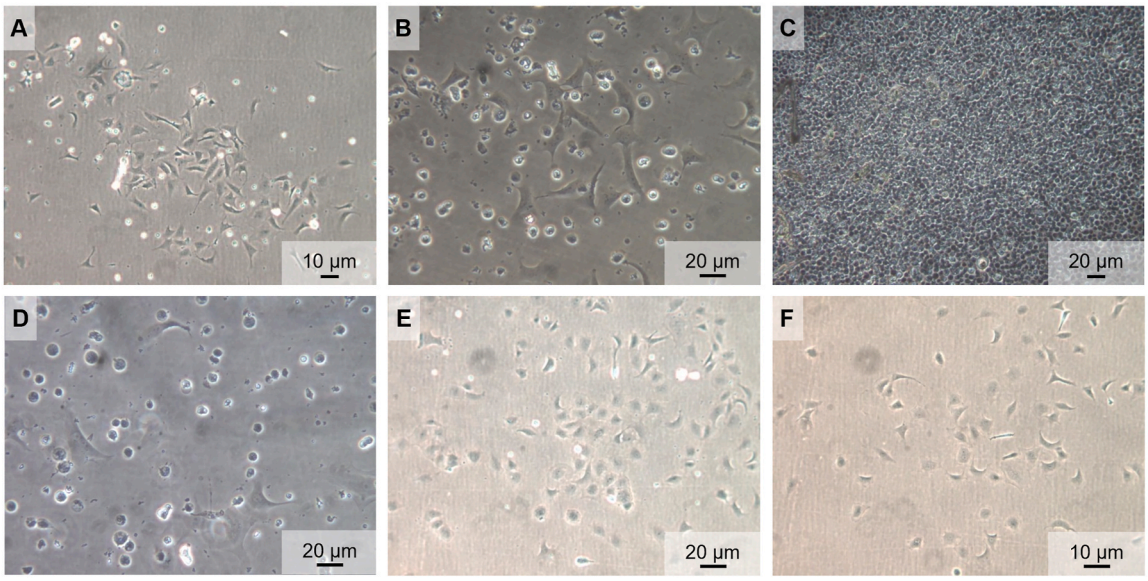


Fig. 9. Passaged cells from **Day 15** cell culture in Media 1. A) **Day 2** of passage; magnification = 10x; B) **Day 4**, magnification = 20X; C) **Day 5**, magnification = 10x; D) **Day 5**, after nonadherent cells removed; magnification = 20x; E) **Day 6** of passage, Media supplemented with 2×-FGF; magnification = 10X; F) **Day 8** of subculture, Media supplemented with 2×-FGF; magnification = 10X.

specific cell lines. This study suggests that this protein may act as a biomarker for ES-like cells in red drum. In an effort to characterize pluripotent potential of our cells, we tested other antibody markers as well. These include Nanog, Sox2, and alkaline phosphatase. The positive reaction of PCNA indicated cells were proliferating. In the data presented here, mouse (Nanog, Sox2) and human (Sox2) antibodies were shown to react to proteins in red drum lysate at approximate molecular weights found in other species. A mouse alkaline phosphatase antibody, however, did not react with red drum cell lysate proteins at the expected molecular size of 75 kDa. Exploration of different antibodies for alkaline phosphatase might have yielded a more positive result, as may have assessing cells for alkaline phosphatase enzyme activity at isolation. Alkaline phosphatase enzyme activity was shown to be active in freshly isolated shrimp embryonic cells (Walsh et al., unpublished data). A challenge with development of fish cell lines is lack of appropriate markers for specific cells types. Existing antibodies used in other species may or may not react with cell proteins in meaningful ways, depending on degree of homology. Some of the important transcription factors in mammalian species are not characterized in fish species, and also may not play the same roles. As an example, Nanog has been documented not to play the same roles in zebrafish development as in other species (Gagnon et al., 2018).

A common method to evaluate pluripotency is through transplantation of ES cells into developing embryos for chimera formation, and determine the ability to differentiation into cell types of all three germ layer lineages (Tripathy and Mohanty, 2017). This approach has been used in several marine fish species (Chen et al., 2003, 2007; Bejar et al., 2002; Holen and Hamre, 2003). Such experiments were not part of these studies. Therefore, despite the positive results with several pluripotent markers, the lack of a continued cell line and lack of definitive experiments leaves the pluripotency of the isolated cells in question. Future studies could build off the data presented here into chimera experiments, full characterization of the ES-like cells generated, and varied directed approaches to inhibit spontaneous differentiation.

The potential combinations of media supplements and growth factors can be quite expansive. In fact, many complications in research efforts related to marine finfish cell line development are very likely directly related to the lack of information regarding species-specific data on nutrient requirements of cells (Soice and Johnston, 2021). Studies designed to understand specific nutrients requirements of cells, such as spent media analysis, and characterization of species-specific growth factors, will help move the field forward.

5. Conclusions

To provide progress in stem cell technology for marine fish, we established cultures of ES cells isolated from fertilized embryos of red drum. The red drum embryonic cells were adherent, and after initial culture, grew with a fibroblast-like morphology. This is promising for the overall goal of cultivated seafood, as fibroblasts are versatile and can be differentiated into other cell types (Alberts et al., 2002). Red drum embryonic cells were grown for a total of 41 days before becoming senescent and had limited survival after subculture using trypsin. These efforts improve the knowledge of culture of embryonic stem cells from a marine fish, red drum (*S. ocellatus*).

Funding

This work was supported by the Good Food Institute.

CRediT authorship contribution statement

Catherine J. Walsh: Conceptualization, Methodology, Investigation, Funding acquisition, Project administration, Writing – original draft. **Nicole Rhody:** Resources, Conceptualization, Supervision. **Kevan L. Main:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Jessica Restivo:** Investigation, Data curation. **Andrea M. Tarnecki:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Catherine Walsh reports financial support was provided by The Good Food Institute. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We are grateful for the assistance of Mote Aquaculture Research Park staff members who assisted in maintaining red drum broodstock and collecting fertilized embryos. We also acknowledge Mote Marine Laboratory for use of facilities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100841.

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Fibroblasts and their transformation: the connective-tissue cell family. Molecular Biology of the Cell, fourth ed. Garland Science, New York.

Alvarez, M.C., Bejar, J., Chen, S., Hong, Y., 2007. Fish ES cells and applications to biotechnology. Mar. Biotechnol. 9, 177. https://doi.org/10.1007/s10126-006060344-4, 127.

Bejar, J., Hong, Y., Alvarez, M.C., 2002. An ES-like cell line from the marine fish *Sparus aurata*: characterization and chimaera production. Transgenic Res. 11, 279–289.

Benjaminson, M.A., Gilchriest, J.A., Lorenz, M., 2002. *In vitro* edible muscle protein production system (MPPS): stage 1, fish. Acta Astronaut. 51, 879–889. https://doi.org/10.1016/S0094-5765(02)00033-4.

Bonkamp, C., Musgrove, L., Marques, D.M., Fernando, G.F., Ferreira, F.C., Specht, E.A., 2023. Differentiation and maturation of muscle and fat cells in cultivated seafood: lessons from development biology. Mar. Biotechnol. 25, 1–29.

Camp, E., Sánchez-Sánchez, A.V., Garcia-España, A., DeSalle, R., Odqvist, L., O'Connor, J.E., Mullor, J.L., 2009. Nanog regulates proliferation during early fish development. Stem Cell. 27 (9), 2081, 2019.

Chen, S.L., Sha, Z.X., Ya, H.Q., 2002. Establishment of a pluripotent embryonic cell line from sea perch (*Lateolabrax japonicas*) embryos. Aquaculture 218, 141–151.

Chen, S.L., Ye, H.Q., Sha, Z.X., Hong, Y., 2003. Derivation of a pluripotent embryonic cell line from red sea bream blastulas. J. Fish. Biol. 63, 795–805. https://doi.org/10.1046/j.1095-8649.2003.00192.x.

Chen, S.L., Ren, G.C., Sha, Z.X., Shi, C.Y., 2004. Establishment of a continuous embryonic cell line from Japanese flounder *Paralichthys olivaceus* for virus isolation. Dis. Aquat. Org. 60, 241–246.

Choorapoikayil, S., Overvoorde, J., den Hertog, J., 2013. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 10 (3), 316–325. https://doi.org/10.1089/zeb.2103.0866.

Collodi, P., Kamei, Y., Sharps, A., Weber, D., Barnes, D., 1992. Fish embryo cell cultures for derivation of stem cells and transgenic chimeras. Mol. Mar. Biol. Biotechnol. 4–5, 257–265.

FAO, 2022. The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. FAO, Rome. https://doi.org/10.4060/cc0461en.

Gagnon, J.A., Obbad, K., Schier, A.F., 2018. The primary role of zebrafish nanog is in extra-embryonic tissue. Development 145 (1), dev147793. https://doi.org/10.1242/dev.147793.

Goswami, M., Yashwanth, B.S., Trudeau, V., Lakra, W.S., 2022. Role and relevance of fish cell lines in advanced *in vitro* research. Mol. Biol. Rep. 49, 2393–2411.

Hatano, S.-Y., Tada, M., Kimura, H., Yamaguchi, S., Kono, T., Nakano, T., Suemori, H., Nakatsuji, N., Tada, T., 2005. Pluripotential competence of cells associated with *Nanog* activity, 122 (1), 676–679.

Holen, E., Hamre, K., 2003. Towards obtaining long term embryonic stem cell like cultures from a marine flatfish, *Scophtalmus maximus*. Fish Physiol. Biochem. 29, 245–252.

Holen, E., Kausland, A., Skjaerven, K., 2010. Embryonic stem cells isolated from Atlantic cod (*Gadus morhua*) and the developmental expression of a stage-specific transcription factor *ac-Pou2*. Fish Physiol. Biochem. 36, 1029–1039.

Hong, Y., Winkler, C., Schartl, M., 1996. Pluripotency and differentiation of embryonic stem cell lines from the medakfish (*Oryzias latipes*). Mech. Dev. 60, 33–44.

Hong, Y., Chen, S., Schartl, M., 2000. Embryonic stem cells in fish: current status and perspectives. Fish Physiol. Biochem. 22, 165–170.

Hong, N., Li, Z., Hong, Y., 2011. Fish stem cell cultures. Int. J. Biol. Sci. 7 (4), 392–402.

Kim, J.W., Oh, B.G., Kim, J., Kim, D.G., Nam, B.H., Kim, Y.O., Park, J.Y., Cheong, J.H., Kong, H.J., 2018. Development and characterization of a new cell line from olive flounder *Paralichthys olivaceus*. Dev. Reprod. 22 (3), 225–234.

Lakhan, L.M., Chandan, H., Ram, R., 2018. Fish embryonic stem cells: culture types, characterization and applications: a review, 2018 Examines Mar. Biol. Oceanogr 2 (4), 000542. https://doi.org/10.31031/EIMBO.2018.02.0054. EIMBO.

Li, H., Xu, W., Xiang, S., Tao, L., Fu, W., Liu, J., Liu, W., Xiao, Y., Peng, L., 2022. Defining the pluripotent marker genes for identification of teleost fish cell pluripotency during reprogramming. Front. Genet. 13, 819682.

Liu, R., Li, M., Li, Z., Hong, N., Xu, H., Hong, Y., 2015. Medak OCT4 is essential for pluripotency in blastual formation and ES cell derivation. Stem Cell Rev. and Rep. 11, 11–23.

Monterey Bay Seafood Watch, 2023. Monterey Bay Seafood Watch, red drum (Sciaenops ocellatus). Report ID 27822. https://www.seafoodwatch.org/globalassets/sfw-data-blocks/reports/d/seafood-watch-red-drum-27822.pdf.

O'Neill, E.N., Ansel, J.C., Kwong, G.A., Plastino, M.E., Nelson, J., Baar, K., Block, D.E., 2022. Spent media analysis suggests cultivated meat media will requires species and cell type optimization. npj Sci. Food 6, 46. https://doi.org/10.1038/s41538-022-00157-z.

Parameswaran, V., Shukla, R., Bhonde, R., Hameed, A.S., 2006. Establishment of embryonic cell line from sea bass (*Lates calcarifer*) for virus isolation. J. Virol. Methods 137 (2), 309–316. https://doi.org/10.1016/jviromet.2006.07.006.

Parameswaran, V., Shukla, R., Bhonde, R., Hameed, A.S., 2007. Development of a pluripotent ES-like cell line from Asian sea bass (*Lates calcarifer*) – an oviparous stem cell line mimicking viviparous ES cells. Mar. Biotechnol. 9, 766–775. https://doi.org/10.1007/s10126-007-9028-y.

Robles, V., Marti, M., Belmonte, J.C.I., 2011. Study of pluripotency markers in zebrafish embryos and transient embryonic stem cell cultures. Zebrafish 8 (2). https://doi.org/10.1089/zeb.2010.0684.

Rubio, N., Data, I., Stachura, D., Kaplan, D., Krueger, K., 2019. Cell-based fish: a novel approach to seafood production and an opportunity for cellular agriculture. Front. Sustain. Food Syst. 3 https://doi.org/10.3389/fsufs.2019.00043.

Saad, M.K., Yuen, J.S.K., Joyce, C.M., Li, X., Lim, T., Wolfson, T.L., Wu, J., Laird, J., Vissapragada, S., Calkins, O.P., Ali, A., Kaplan, D.L., 2023. Continuous fish muscle cell line with capacity for myogenic and adipogenic-like phenotypes. Sci. Rep. 13, 5098. https://doi.org/10.1038/s41598-023-31822-2.

Scholefield, A.M., Schuller, K.A., 2014. Cell proliferation and long chain polyunsaturated fatty acid metabolism in a cell line from southern Bluefin tuna. Lipids 49, 703–714.

Soice, E., Johnston, J., 2021. Immortalizing cells for human consumption. Int. J. Mol. Sci. 22, 11660 https://doi.org/10.3390/ijms222111660.

Strzalka, W., Ziemienowicz, A., 2011. Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. Ann. Bot. 107 (7), 1127–1140.

Sun, L., Bradfort, C.S., Ghosh, C., Collodi, P., Barnes, D.W., 1995. ES-like cell cultures derived from early zebrafish embryos. Mol. Mar. Biol. Biotechnol. 4 (3), 93–199.

Thorley, M., Duguez, S., Mazza, E.M.C., Valsoni, S., Bigor, A., Mamchaoui, K., Harmon, B., Voit, T., Mouly, V., Duddy, W., 2016. Skeletal muscle characteristics are preserved in hTERT/cdk4 human myogenic cell lines. Skeletal Muscle 6, 1–12.

Tripathy, S., Mohanty, P.K., 2017. Embyonic stem (ES) cells of fish: an innovative approach in stem cell science. Advances in Aquaculture 209–229.

Wakamutsu, Y., Ozato, K., Sasado, T., 1994. Establishment of a pluripotent cell line derived from a medaka (Oryzias latipes) blastula embryo. Mol. Mar. Biol. Biotechnol. 3 (4), 185–191.

Yang, W., Lee, Y.H., Jones, A.E., Woolnough, J.L., Zhou, D., Dai, Q., Wu, Q., Giles, K.E., Townes, T.M., Wang, H., 2014. The histone H2A deubiquitinase Usp16 regulates embryonic stem cell gene expression and lineage commitment. Nat. Commun. 5 (1), 3818.