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ARTICLE

Development of a cell line from skeletal trunk muscle of the fish *Labeo rohita*

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Abstract *Labeo rohita* is a widely cultivated tropical freshwater carp and found in rivers of South Asian region. A new cell line, designated LRM, has been developed from the muscle tissue of *L. rohita*. Muscle cells were subcultured up to 38 passages in a Leibovitz's-15 (L-15) supplemented with 10% FBS (Fetal Bovine Serum) and 10 ng/ml bFGF. The LRM cells exhibited fibroblastic morphology with a doubling time of 28 h, and a plating efficiency of 17%. A maximum growth rate was observed for LRM cells at 28 °C, 10% FBS and 10 ng/ml bFGF. A cytochrome C oxidase subunit I (COI) gene sequence was used to authenticate the developed cell line. Chromosome analysis revealed 50 diploid chromosomes. The fibroblastic characteristics of the of the LRM cells was confirmed by immunocytochemistry. The expression of MyoD gene in LRM cells was analyzed by quantitative PCR in comparison with passages 3, 18 and 32. The expression of MyoD was higher at passage 18 compared to the passages 3 and 32. The LRM cells attached properly onto the 2D scaffold and the expression of the F-actin filament protein was confirmed by phalloidin staining followed by counter staining with DAPI to observe the distribution of the muscle cell nuclei and the cytoskeleton protein. A revival rate of 70–80% was achieved when the LRM cells were cryopreserved at − 196 °C using liquid nitrogen. This study would further contribute to understanding the in vitro myogenesis and progress toward cultivated fish meat production.

Keywords Characterization · *Labeo rohita* · Muscle cell line · MyoD · *Scaffold*

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Introduction

The entire world is exploring sustainable, efficient, environment friendly and safe options to feed 10 billion people by 2050 as the world will need 70% more food than the current food production according to the United Nations. Cultivated or in vitro meat is emerging as a promising alternate food production system (Rubio et al. 2019; Post et al. 2020; Bomkamp et al. 2022; Goswami et al. 2022). Currently, a total of $ 1.93 billion has been invested since 2016 for the development of cultivated meat by 107 companies across the globe (2021 State of the Industry Report, GFI). Academic research can play a significant role in accelerating the economic production of cultivated

meat by providing foundational discoveries through open publication of data. Animal cell lines have been central to many significant discoveries for a range of academic disciplines (Bols et al. 2017) and now are critical to in vitro meat production.

Recently, the properties of animal cell lines were shown to consist of two classes: ante factum (before-the-fact) properties and post factum (after-the-fact) properties (Bols et al. 2023). Ante factum properties were features or characteristics of the sample that was used to start the cell line and of the species from which the sample was taken (Bols et al. 2023). Post factum properties were properties revealed during the development, characterization, and use of the cell line (Bols et al. 2023). These two property classes were illustrated with cell lines from the skeletal muscle of ray-finned fishes (Bols et al. 2023). Collectively, these cell lines belong to the skeletal muscle invitrome (Bols et al. 2017). All these cell lines can contribute to studies on in vitro meat production and, both their ante factum properties, such as the species and anatomical site from which the cell line arose, and post factum properties, such as growth factor requirements, are important features to consider.

For these reasons a cell line was sought from the skeletal muscle of *Labeo rohita* and successfully obtained and designated LRM. Commonly called rohu, *L. rohita* contributes significantly to Indian aquaculture and is one of the most imported Indian Major Carp. Rohu accounts for 24.94% of world aquaculture production and remains within top ten aquaculture species (FAO 2022). From the review of the skeletal muscle invitrome (Bols et al. 2023), no skeletal muscle cell lines appear to have been developed from this important species. Skeletal muscle is composed of multiple cell types that express a variety of specific properties, with myoblasts and their ability to proliferate and fuse into myotubes being most prominent (Manneken et al. 2022). Identifying which of these properties are expressed by skeletal muscle cell lines is important for developing the cell lines into tools for studying muscle cell biology and for producing in vitro meat. For example, a myoblast cell line (KFE-5) was developed from the killifish embryo (Gignac et al. 2014); a myofibroblast precursor cell line (CAtmus1PFR) from the tail muscle of Australasian snapper (Chong et al. 2022). However, fish skeletal muscle cell lines are often described simply by their predominate cell shape, which usually has been described as either fibroblast-like, epithelial-like or spindle-like (Bols et al. 2023). Therefore, besides general properties, we have characterized LRM for expression of vimentin and a myogenic regulatory factor, MyoD, and for growth on a mushroom scaffold.

Materials and methods

Ethics statement

The Institutional Animal Ethics Committee (IAEC) and the Board of Studies (BoS) of the ICAR-Central Institute of Fisheries Education, Mumbai, India, approved all experimental protocols in this study. The committee approved all methods conducted in accordance with relevant guidelines and regulations. In order to care for and use fish, all applicable international, national, and/or institutional guidelines were followed.

Experimental animal

The fingerlings of *Labeo rohita* (body weight: 15 to 20 g) were obtained from Crescent Fisheries, Gujrat, and maintained at Wet Laboratory, ICAR-Central Institute of Fisheries Education, Mumbai, India. Fish were kept in 500 L fiberglass tanks at 28 ± 1 °C for a 12 h light/12hr dark cycle. They were fed with commercial pelleted feed daily (GodrejAgrovet-2 mm pellet size). Prior to explant preparation, the donor fish were transported to the Fish Genetics and Biotechnology laboratory and fasted in aerated water for 24 to 36 h.

Development of primary culture from muscle tissue of *L. rohita*

For the preparation of the primary culture, starved fish were exposed to well-aerated water with disinfectant for 30 min to remove the surface microflora. The Fish was euthanized immediately by being exposed to rapid hypothermic shock in the ice-chilled bath for 1 min and wiped with 70% ethanol. Aseptically muscle tissue of the caudal region was dissected and placed in phosphate buffer saline (PBS) solution (Thermo Fisher Scientific). Muscle tissue was further washed thoroughly with PBS containing 500 IU/

mL penicillin, 500 µg/mL streptomycin, and 2.5 µg/mL fungizone. In the last step of washing, the muscle explants were prepared by mincing a tissue to 1 mm^3 size in PBS using sterile scissors and seeded carefully in a cell culture flask (T-25). The excess PBS was removed and fed with 0.2 ml fetal bovine serum (FBS) (Gibco) for the initial adherence of explants in the culture flask. The flasks were kept for incubation at 28 °C overnight. The growth medium, Leibovitz-15 (L-15) (HiMedia) was added by the next day in supplementation with 15% FBS and 10 ng/ml basic fibroblast growth factor (bFGF, Gibco). Once the cell started radiation, half of the medium was replaced with a spent medium in the culture flask.

Subculture and maintenance

For the first subculture, the cells were allowed to attain a confluence of 70–90%. The medium was completely removed and the cells were trypsinized using 1 ml TPVG (0.25% trypsin) (HiMedia) solution. The dislodged cells were resuspended immediately in a growth medium containing 10% FBS and 10 ng/ml bFGF (human basic fibroblast growth factor) containing a family of heparin binding and basic brain-derived growth factors (Catalog Number: 13256-029, Gibco) and seeded in a new 25 cm^2 (T-25) flask with a split ratio of 1:2. For subsequent passages, the serum concentration was dropped to 10% and the flasks were incubated at 28 °C with an optimal pH of 7.4. Once the cell confluency reached 90%, the cells were subcultured.

Growth optimization

The growth optimization of LRM cells was performed at various temperatures, serum, and bFGF concentrations. To establish the ideal growth conditions for LRM cells, the cells were seeded with an initial concentration of 2×10^4 cells/mL in T-25 flask containing 10% FBS in triplicates and incubated at different temperature ranges such as 18, 24, 28, and 32 ℃ for seven days (Sathiyanarayanan et al. 2022, 2023). Similar procedures were carried out with various FBS (5, 10, 15, and 20%) and bFGF (0, 5, and 10 ng/ml) concentrations and incubated for seven days at 28 ℃ with an initial cell density of 2×10^4 cells/mL (Lakra et al. 2010a; Yashwanth et al. 2020). A cell counter was used to calculate the relative number of viable cells for each day (BioRad, USA).

Cell doubling time and colony forming efficiency

Cell doubling time (CDT) is the time taken for a cell population to double in the logarithmic phase of growth and it is calculated by the following formula. The colony forming efficiency is the measure of the number of colonies arise from the desired seeding concentration of cells (Ham and Puck 1962). The LRM cells at 18th passage were seeded at 50, 100, 200, 500, 1000, and 2000 cells per well in a 6-well plate with a growth medium (L-15) containing 10% FBS and incubated at 28 °C. The cells were provided with fresh medium every three days in 14 days intervals. The number of colonies was counted manually under a fluorescence inverted microscope (Nikon, Japan) by fixing with anhydrous methanol and stained with Giemsa stain. The experiment was conducted in triplicates independently and the colony forming efficiency was calculated by the following formula.

Cells/Population Doubling Time (DT)

$$\text{Cell doubling time} = \text{Incubation time} \times \ln \frac{\text{cell number at the end of the incubation time}}{\text{cell number at the beginning of the incubation time}}$$

$$\text{Colony forming effeciency} = \frac{\text{No. of colonies}}{\text{No. of cells seeded}} \times 100$$

Authentication of cell line

To confirm the species of origin, genomic DNA was extracted from LRM cells by following the protocol described by Sambrook et al. (1989) and PCR amplification of mitochondrial gene i.e. Cytochrome oxidase subunit I (COI) was performed. The primers used to amplify the COI gene and 16s rRNA gene were FishF1, FishR1, 16sf1F140 and 16sf1R1524 respectively (Ward et al. 2005; Zhang and Hanner 2012). The primer sequences are given in the Table 1. The master mix was prepared to a total volume of 12.5 µl per reaction containing 0.5 µl of template DNA, 1.25 µl of reaction buffer, 0.5 µl of forward and reverse primers each, 0.25 µl of dNTP's, 0.15 µl of Taq polymerase (5 units/µl) and 9.35 µl of double distilled water to make up the

Table 1 List of primers used (10 pM) for the amplification of mitochondrial genes COI and 16S rRNA

Mitochondrial Region	Primer Name	Primer sequence (5′-3′)	Length (bp)	References
COI	Fish F1	TCAACCAACCACAAAGACATTGGCAC	26	Ward et al. 2005
	FishR1	TAGACTTCTGGGTGGCCAAAGAATCA	26	
16S rRNA	16sf1F140	CGYAAGGGAAHGCTGAAA	18	Zhang and Hanner 2012
	16sf1R1524	CCGGTCTGAACTCAGATCACGTAG	24	

volume. The thermal regime for the PCR amplification consisted of an initial denaturation at 94 °C of 5 min, followed by 35 cycles of denaturaion at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 7 min for COI gene whereas the thermal regime for the PCR amplification of 16s rRNA consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturaion at 95 °C for 40 s, annealing at 53 °C for 60 s, extension at 72 °C for 45 s, and final extension at 72 °C for 7 min. The amplified products were examined and purified using 1% agarose gel stained with ethidium bromide (EtBr) and observed in the gel documentation system (OmegaLum G, Aplegen, USA). The purified COI products were sequenced (Xcelris, Labs, Ltd) and the results were compared with reported sequences using NCBI-BLASTn.

Chromosomal analysis

Chromosomal analysis or karyotyping was performed with LRM cells at the 20th passage in a 25 cm^2 flask by some modification with the protocol described by Kalaiselvi et al. (2019). The cells were treated with 10 µL of 0.5% Colchicine (Sigma-Aldrich, St. Louis, MO) and incubated for 4 h at 28 °C. The colchicine-treated cells were harvested and pelletized by centrifugation followed by resuspended in 0.56% KCl solution and incubated at room temperature for 30 min. The hypotonic effect of pelletized cells was gradually stopped by adding 1 mL of freshly prepared chilled Carnoy's fixative (Methanol: Glacial Acetic acid in 3:1) and incubated for 5 min. The fixation process was repeated 3–4 times until clear suspension was obtained. A small quantity of fixed cell suspension was dropped on a pre-cleaned glass slide and stained with 5% Giemsa for 20–30 min. The metaphase spreads were counted under a microscope (100X).

Immunocytochemistry

The morphology of LRM cells at the 23rd passage was checked through immunotyping using monoclonal antibodies against Cytokeratin (C2931-Clone C-11 Sigma) and Vimentin (V6630-CLONE 9 Sigma) (Dubey et al. 2015). The LRM cells were grown on a coverslip in a 12-well plate and fixed in 4% paraformaldehyde (PFA). The cells were washed trice with PBS and permeabilized with 0.1% Triton X-100 and blocked with 1% bovine serum albumin (BSA) for 45 min at room temperature and washed twice with PBS. The blocked cells were treated with 100 µL anti-Vimentin (1:200 dilution) and anti-pan cytokeratin (1:300 dilution) and incubated for 4 h at room temperature. The excess non-specific antibodies were washed out with PBS and 100 µL of FITC-labelled goat anti-mouse IgG (1:200 dilution) was added. The unbound antibodies were washed with PBS and the coverslip was mounted with 50% glycerol and observed under a fluorescent inverted microscope.

Relative quantification of MyoD

RNA isolation and cDNA preparation from LRM cells A total RNA was extracted from LRM cells at 3, 18 and 32 passages with cell confluency of 80–85%, as well as muscle tissue (control) from *L. rohita* fingerlings was obtained by using TRIzol (Invitrogen) reagent. A cDNA was made from isolated RNA using Thermo Fisher's RevertAid First Strand cDNA Synthesis Kit (#K1622) and stored at − 20 °C. PCR was conducted directly using the product of first strand cDNA synthesis.2.7.3.

qRT-PCR

qRT-PCR was performed using a Roche LightCycler® 480 System and the iQ SYBER Green super mix kit (Bio-Rad). In conventional PCR, primers are annealed at optimum temperatures for optimal PCR conditions. The standard reaction mixture (10 L) was composed of 5 L of iQ SYBER Green super Mix 2x, 0.5 L of 10 pM primers, and 100ng template cDNA. Amplification of the MyoD and beta-actin genes was performed using previously reported primers (Table 2) (Sengupta et al. 2014; Garg et al. 2019).

Statistical analysis

SPSS 22.0 software was used for data analysis, including a one-way ANOVA and Duncan's multiple range test (DMRT). Comparison of the means was conducted at a 5% significance level ($p < 0.05$).

Mycoplasma testing

The presence of mycoplasma in the LRM cells was tested at 25th and 26th passage using MycoFluor™ mycoplasma detection kit (Invitrogen, USA) (Catalogue No. M7006) following manufacturer's instruction with some minor modifcations. MycoFluor™ is a fluorochrome which stains the nuclei of the cells associated with the mycoplasma. Briefly, 1 volume of 20X concentrated MycoFluor reagent was added to 9 volumes of LRM cell suspension (2×10^5 cells/mL) incubated at RT for 10 min and observed using a near-ultraviolet fluorescence filter (excitation at 365 nm) (Nikon, USA).

Growth studies of LRM cells in 2D Scaffold

A proprietary mushroom-based 2D scaffold procured from MyoWorks Pvt Ltd, IIT, Bombay was seeded with LRM cells at 15th passage in order to test adhesion and proliferation (Dr. Ankita Srivastava, Chief Scientist, MyoWorks Pvt. Ltd. Email: admin@myoworks.in). The scaffolds were attached to a coverslip placed on a 12-well plate (Thermo Scientific), disinfected with 70% ethanol, and incubated for 3 h at room temperature. In order to remove excess ethanol from the scaffold, it was dried overnight at room temperature. The next day, the scaffolds were rinsed three times with PBS and sterilized under UV light for 20 min. A sterile scaffolds were used to seed LRM cells. A culture media (L-15 with 10% FBS and 10 ng/ml bFGF) was added to the scaffold after soaking it for 24 h (1 mL per well) followed by seeding LRM cells at a rate of 20,000 cells per scaffold and incubated at 28 °C. The Phalloidin (Thermo Scientific, USA) staining was used to visualize cell proliferation in a scaffold. In addition, the LRM cells were seeded separately in 12-well plates for examination of adhesion and proliferation using DAPI (4′,6-diamidino-2-phenylindole) and Alexa Fluor™ 568 phalloidin (Invitrogen, USA). The staining procedure was carried out as instructed by the product's manufacturer. The fluorescent microscopic images were recorded on days 1, 3, and 4 respectively.

Cryopreservation

The LRM cells were cryopreserved in liquid nitrogen at − 196 °C for 15th, 27th, and 36th passages. The cells were harvested through trypsinization in a confluent monolayer of 1×10^6 cells/mL, and the cell pellet was suspended in a 1 mL freezing medium (L-15) containing 10% DMSO and 20% FBS in cryovials and placed in a freeze control chamber (− 1 °C/min, Thermo Scientific) at − 80 °C ultra-freezer for 24 h followed by placed in liquid nitrogen for long-term

Table 2 List of primers used (10 pM) for relative quantification of MyoD in *L. rohita*

Gene	Primer sequence	Annealing temperature (°C)	Expected amplicon size (bp)
MyoD	Forward-TCCAAGCGCTGCTAAGAAGT	53	109
	Reverse-CATCATGCCATCAGAGCAGT		
Beta-actin	Forward-CACTGCTGCTTCCTCCTCCTCC	60	138
	Reverse-GATACCGCAAGACTCCATACCCAAG		

storage. After 6 months of storage, the cryopreserved cells were retrieved by thawing in water at 28 °C. The cell pellet was resuspended in L-15 media with 10% FBS and examined for revival efficiency.

Results

Primary and subculture of LRM cells

The explants prepared for rohu muscle were firmly attached to the culture flask after the addition of the growth medium. The initial radiation was obtained after 120 h of explant preparation (Fig. 1a) and the first subculture was performed on the 13th day. The subsequent passage was done every 3 days with a split ratio of 1:2. The new muscle cell line of *L. rohita* was designated as LRM and was maintained in an L-15 medium supplemented with 15% FBS for the first five subcultures and 10 ng/ml bFGF. In the subsequent passages, the serum concentration was reduced to 10% and cells have been successfully maintained up to 38 passages (Fig. 1f).

Growth optimization

LRM cells were incubated at different serum concentrations (5, 10, 15, and 20%), temperatures (18, 24, 28, and 32 °C), and bFGF concentrations (0, 5, and 10 ng/ml) for growth studies. The LRM cells grow well at a wide range of temperatures from 24 to 32 °C, 5–20% FBS, and bFGF concentration. However, better growth was recorded in L-15 medium incubated at temperature 28 °C (Fig. 2A)

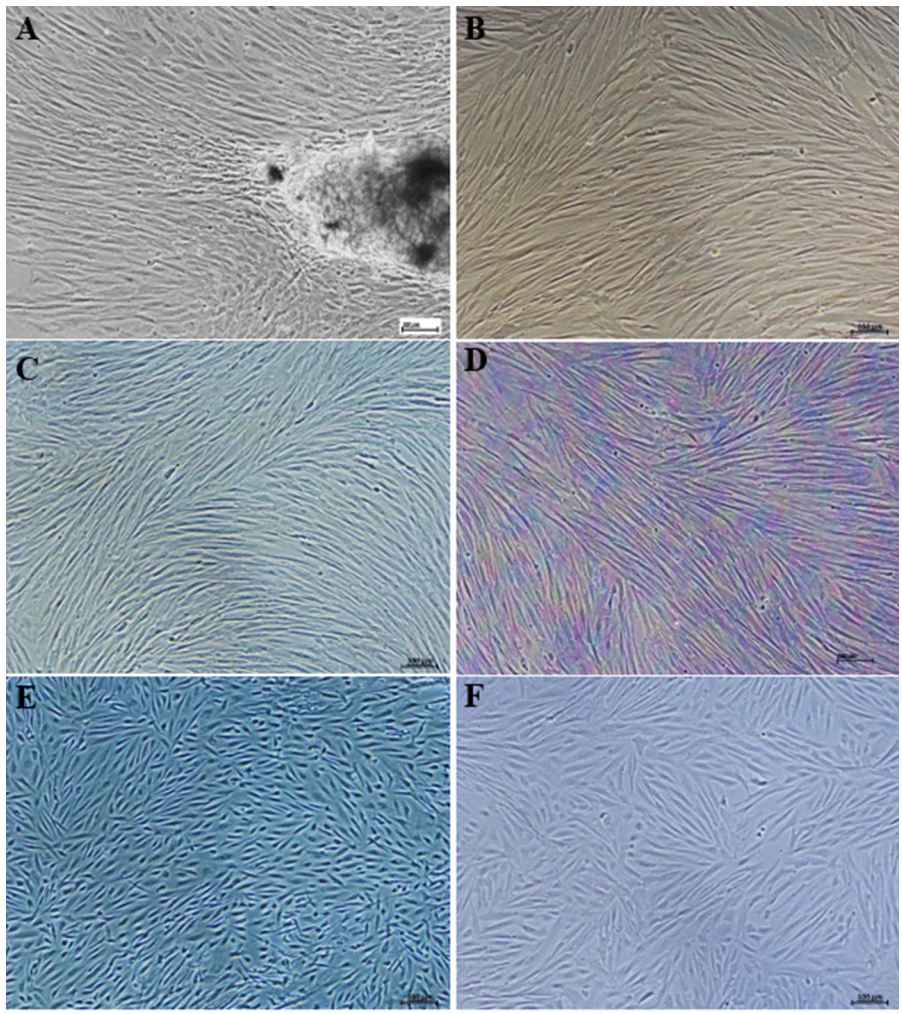

Fig. 1 Photomicrographs in phase contrast of the LRM cell line showing the consistent fibroblast-like morphology as development of the cell line progresses **A** Migration of cells out of an explant after 10 days of explant preparation (10 ×) **B** Confluent monolayer of cells after 4th passage (10 ×) **C** LRM cells at 12th passage (10 ×) **D** LRM cells at 18th passage (10 ×) **E** LRM cells at 35th passage (10 ×) **F** LRM cells at 38th passage (10 ×)

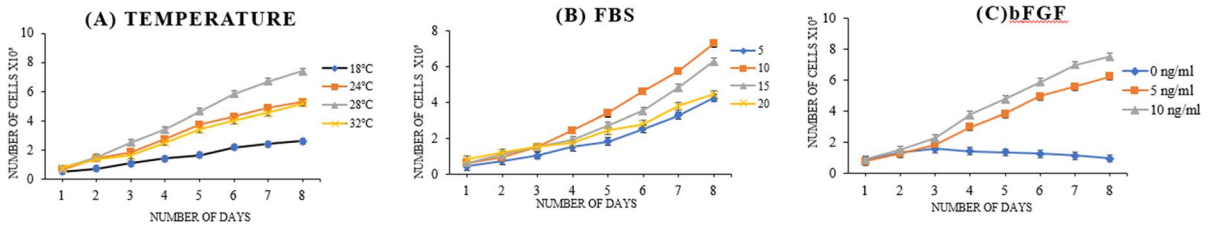

Fig. 2 Growth studies of LRM cells at different **A** Temperatures **B** FBS concentrations **C** bFGF concentration (values are presented as mean ± SD, n = 3)

supplemented with 10% FBS (Fig. 2B), and 10ng/ml bFGF concentration (Fig. 2C).

Cell doubling time and plating efficiency

The estimated cell doubling time of the LRM cell line at 18th passage was 28 h and the plating efficiency was 17%.

Authentication of cell line

The LRM cell line was authenticated for species confirmation. The amplified product of mitochondrial genes confirmed with expected PCR product size ≈ 655 and 1380 bp of COI and 16s rRNA genes respectively (Fig. 3A, C). The sequence alignment was carried out with BLASTn for COI gene sequences which revealed 99–100% similarity with reported voucher specimen of *L. rohita.* The obtained sequences of the two genes COI and 16s rRNA are given in (Fig. 3B and D).

Chromosomal analysis

The LRM cells were arrested at metaphase using colchicine (Fig. 4A). The chromosome frequency distribution of 134 metaphase plates of *L. rohita* muscle cells at 20th passage revealed a diploid chromosome number ranging from 25 to 70 with a modal value of 50 (Fig. 4B).

Immunocytochemistry

The muscle cells of *L. rohita* at 23rd passage revealed the characteristics of fibroblast cell type through the expression of vimentin-FITC a typical intermediate filament protein marker (Fig. 5).

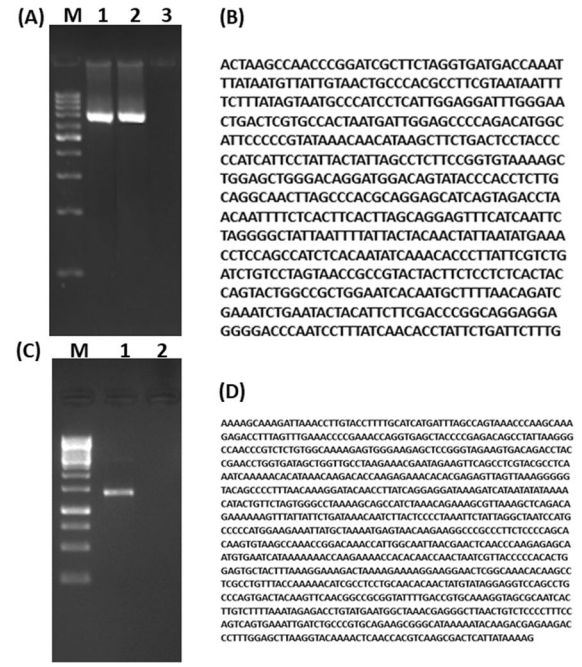

Fig. 3 **A** PCR Amplification of 655 bp fragment of *L. rohita* genome using oligonucleotide primers from the conserved portions of COI (Lane M-generuler express 100 bp DNA ladder (Fermentas) Lane 2-LRM COI, Lane 3-positive control (muscle tissue from Rohu fish), and Lane 4-negative control (Negative control—without DNA template) **B** The partial sequence of COI gene from muscle cells of *L. rohita* **C** PCR Amplification of 1380 bp fragment of *L. rohita* genome using oligonucleotide primers from the conserved portions of 16s rRNA (Lane 1-generuler express 1 kb DNA ladder (Fermentas) Lane 2-LRM 16 S rRNA, and Lane 3-negative control (Negative control—without DNA template) **D** The partial sequence of 16 S rRNA gene from muscle cells of *L. rohita*

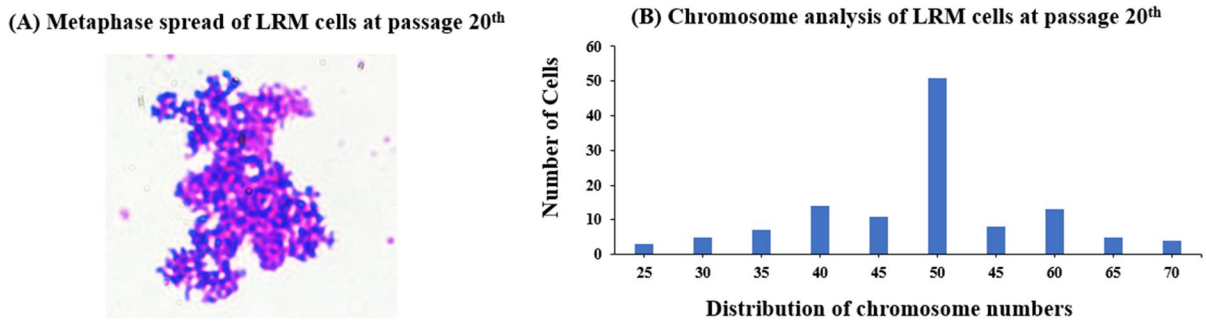

Fig. 4 Chromosome analysis of LRM cells at passage 20th **A** Metaphase spread of LRM cells at passage 20th **B** Distribution of Chromosome number among the metaphase spread (scale bar = 20 µm)

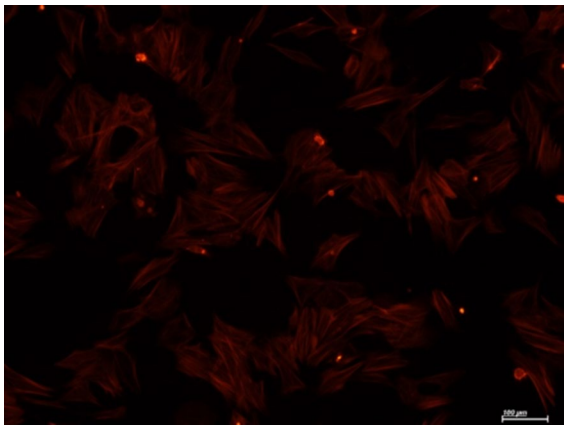

Fig. 5 Photomicrographs of LRM cells showing fluorescence positive for the vimentin-FITC at passage 23 (20X)

Relative quantification of MyoD

The expression of MyoD gene in LRM cells was analysed by quantitative PCR in comparison with passages 3, 18 and 32 (Fig. 6A). Muscle tissue of *L. rohita* fingerlings was used as a control for the expression. Using optimized PCR conditions for annealing temperature, three independent real-time PCR experiments were performed with six replicants. The expression of MyoD was observed at all stages of the cells, but the expression patterns were different w.r.t different passages. The expression of MyoD was higher at passage 18 compared to the passages 3 and 32 (Fig. 6B).

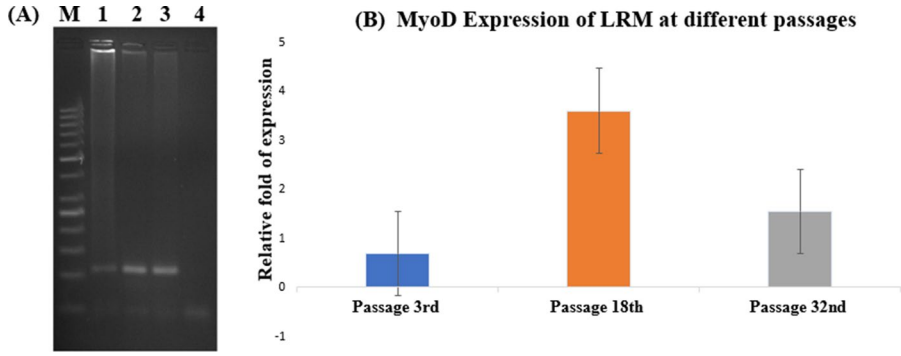

Fig. 6 **A** Agarose gel image of PCR amplification of MyoD gene (Lane M-generuler express 50 bp DNA ladder (Fermentas), Lane 1-LRM 3, Lane 2-LRM 18, Lane 3-LRM 32, and Lane 4-negative control) **B** Relative mRNA expression analysis of myogenic regulatory factor (MRF) MyoD in muscle tissue (control) of *L. rohita* fingerling and LRM cells at passage 3, 18 and 32. All values are presented as mean ± SE ($p \leq 0.05$)

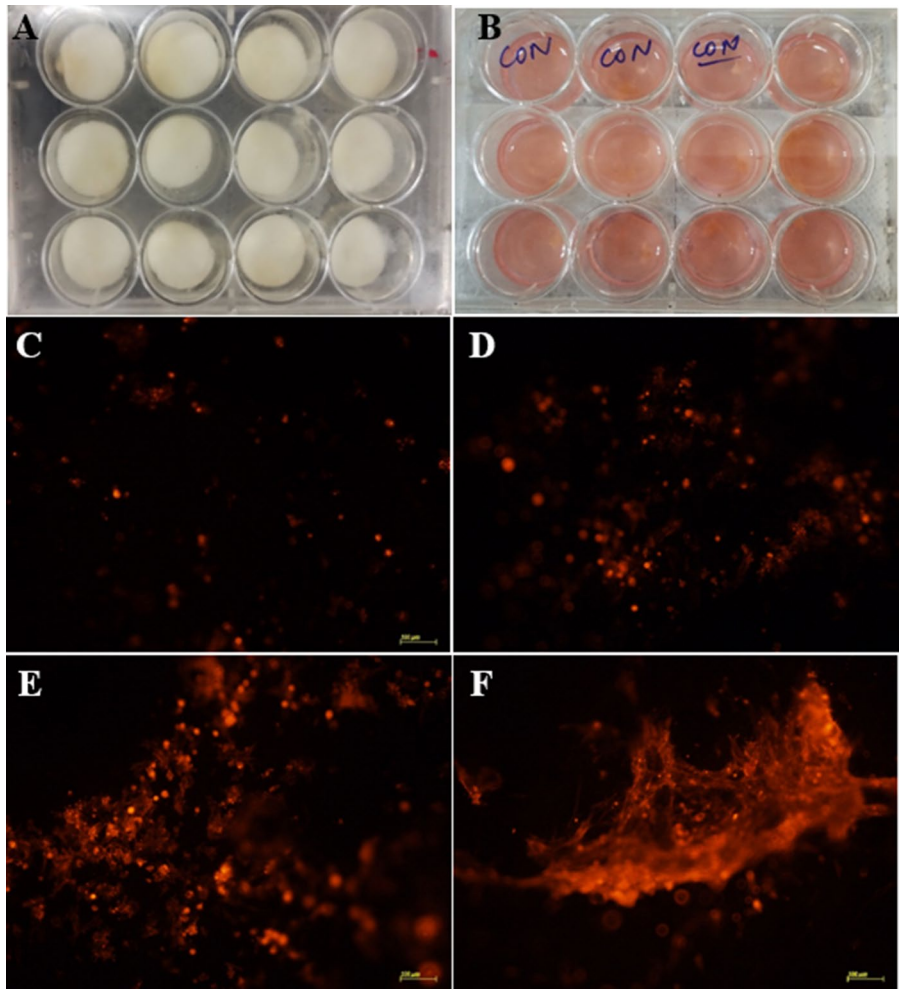

Fig. 7 Photomicrographs of 2D-scaffold **A** scaffolds before cell seeding **B** after cell seeding **C** fluorescent microscopic image of LRM cells stained with phalloidin after 4 h of cell seeding **D** sparsely populated LRM cells at day 1 of cell seeding **E** densely populated cells with uniform distribution at day 3 **F** aggregation of cells over the scaffold's surface showing extended morphology at day 4

Mycoplasma testing

The LRM cells in 25th and 26th passages were observed with no fluorescent signal of stained nuclei associated with cells under a fluorescent microscope. The cells are free from mycoplasma contamination.

Growth of LRM cells in 2D Scaffold

A mushroom-based 2D scaffold was used to validate cell proliferation and adhesion of LRM cells at the 15th passage; the results were further validated by seeding cells in 12-well plates and staining them with DAPI and phalloidin (Thermo Scientific, USA). The staining of cells with phalloidin clearly indicated the expression of cellular F-actin filament protein (Fig. 7C and D). The uniform distribution of cells over the scaffold surface and extended morphology were noticed after staining with phalloidin at Day 4 (Fig. 7C and D). The distribution of the muscle cell nuclei and actin cytoskeleton protein was observed after counter staining with DAPI (images not shown).

Cryopreservation

The LRM cells exhibit 70–80% revival ability after 6 months of cryopreservation with no significant changes observed in morphology and growth rate of cells after thawing.

Discussion

A cell line, LRM, from the caudal muscle of Labeo rohita has been developed and will be discussed around some of its ante factum and post factum properties.

LRM has several interesting ante factum properties. The major ante-factum properties include fish species and cell source. There are only six fish muscle cell lines which are immortalized spontaneously have been reported from the freshwater habitat. Of these, the reports on muscle cell lines from freshwater food fish is very much limited (Rubio et al. 2019a; Potter et al. 2020). Therefore, muscle cell lines from freshwater food fish species with higher consumer preference and global acceptance could lay a foundation for a baseline study of cultivated seafood production. Also, fish muscle comprises of variety of cell types which varies in physiology and metabolic pathway offering a wide range of option to gain insights on myogenesis and its regulation ((Rubio et al. 2019a). Therefore, the isolation and propagation of primary cultures of fish muscle cells could help in research focusing on cell-based lab meat (Saad et al. 2023; Duran et al. 2020). Moreover, fish skeletal muscle cell source possesses unique properties such as low oxygen requirement, pH buffering capacity and adaptation to the wide range of temperatures (Rubio et al. 2019). All these properties make skeletal muscle tissue an ideal target for studying in vitro myogenesis and their applicability in the cultivated seafood production. In some cases, spontaneous immortalization by chromosomal rearrangements and epigenetic changes of fibroblast cell type due to the repeated cell division for the prolonged expansion is also possible (Pasitka et al. 2023).

The post factum properties of LRM are like some other cell lines from other fish trunk skeletal muscle. In order to standardize the process of developing *L. rohita* muscle cell line, different temperatures, serum and bFGF concentrations were optimized, and the cells has been routinely passaged and maintained over 38 times to date. LRM cells grew well at a wide range of temperature and serum concentration and exhibits their maximum growth in L-15 media supplemented with 10% FBS at 28 °C and the results reflect the reported cell lines (Rowlerson et al. 1995; Gao et al. 2019; Kumar et al. 2016). The fetal bovine serum (FBS) contains several growth factors that are essential for optimal cell growth. LRM cells proliferated well even at 5% serum when supplemented with human basic fibroblast growth factor which is in concurrent with the reports of Chen et al. (2004).

The maximum colony forming efficiency was reported from the PCE cell line of 88% when seeded at 1000 cells per well (Goswami et al. 2012). The colony forming efficiency is expressive of genotypic change or transformed characteristic of cells (Freshney et al. 2015). The estimated colony forming efficiency of LRM cells was 17% which is similar to 20% in RF cells, 16% in RH and RSB cell lines (Lakra et al. 2014b).

In this study, we report LRM cells as the fastest growing cells when compared to other reported fish cell lines with an estimated CDT of 28 h, whereas 34 h in the SREM-1 cell line (Murali et al. 2020) and 36 to 48 h in a vertebral cell line of *Sparus aurata* (Marques et al. 2007).

The sequence analysis of COI gene authenticates the muscle cells of *L. rohita.* The COI is considered a universal barcode for species authentication (Hebert et al. 2003). The Karyotyping of LRM cells reveals 2n = 50 which was alike to the modal chromosomal number of *L. rohita* (Lakra et al. 2014b; Abdul et al. 2013).

LRM cell line revealed the fibroblast-like features of LRM muscle cells by the expression of vimentin-FITC a typical intermediate filament protein marker of myoblast cell type (Dubey et al. 2015; Yajing et al. 2018). In Australian snapper cell line (CAtmus1PFR) bFGF and other growth factors such as IGF-I, IGF-II, and TGFβ were used for muscle cell differentiation (Chong et al. 2022). In this study, the expression of vimentin marker could be due to the exposure of cells to the growth factor bFGF. Thus, growth factors were crucial for muscle cell differentiation during myogenesis.

In fish, myoblast cell isolation was reported from the primary culture in the white muscles. During myogenesis, myogenic regulatory factors were important, in the case of zebrafish embryo, the muscle was not formed in the absence of MyoD and Myf5 (Hinits et al. 2011; Froehlich et al. 2013). It has been suggested that MyoD promotes myogenesis by inducing MyoG expression in zebrafish (Hinits et al. 2011). In Killifish, embryonic cells are fibroblastic in nature, however, cells also contain myogenic cells which are differentiated into myocytes (Baroffio et al. 1996;

Gignac et al. 2014). In our study, both fibroblastic and myogenic cells were identified through the expression studies of MyoD gene. The expression of MyoD gene was maximum at 18 passages.

LRM is the first fish skeletal muscle line to be examined for growth on a 2D-scaffold. Zebrafish skeletal Muscle Progenitor Cells (MPC's) were successfully transferred from 2D monolayers onto a collagen-based 3D tissue-engineered scaffold (Vishnolia et al. 2020). Similarly, the LRM cells were transferred to a 2D scaffold constructs and studied for the cell's viability and adhesion. The adhesion of LRM cells to the scaffolds was confirmed by phalloidin staining that resulted in the expression of cellular F-actin filament protein followed by counter staining with DAPI to visualize the distribution of the muscle cell nuclei and actin cytoskeleton protein. In cell-based meat production, the development of proper scaffolds is challenging, especially to customize the meat properties. Among the most used scaffolds, gelatin of salmon, alginate, agarose, and glycerol showed promising results in C2C12 cells, which had large pores (200 m), was biocompatible and allowed myoblast cells to attach (40%) and proliferate in 24 h (Enrione et al. 2017). In our study, myoblasts were suitable for the attachment to the scaffold but in-depth mechanism and proliferation and differentiation are crucial for 3D based scaffolds.

The LRM cells exhibited 70–80% revival ability after 6 months of cryopreservation without noticeable changes in cell morphology, plating efficiency, and CDT. A similar observation has been reported from other cell lines of *L. rohita* such as RH, RF, LRG, and RSB cell lines (Lakra et al. 2014b; Abdul et al. 2013).

The current work offers the fundamental knowledge necessary to comprehend in vitro myogenesis and move toward cultured meat production. In the present study, myoblast cells of rohu provide a baseline study for future fish myoblast differentiation and myogenesis for in-vitro meat production.

Conclusion

LRM cell line was successfully developed from the muscle tissue of *L. rohita*. The maximum growth of LRM cells was achieved by optimizing the growth parameters such as temperatures, FBS concentration and bFGF supplementation. The exogenous gene expression of MyoD, an important myogenic regulation factor was observed in all the passage levels, but the highest expression was noticed at passage 18th. Further studies are required to expand the use of muscle cells towards lab-based fish meat research by a thorough understanding of in vitro differentiation of muscle cells.

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Author contributions MG—Project investigator, conceptualization, overall supervision and guidance; NP—Performed cell line characterization, maintenance, literature, and manuscript preparation; YBS—Cell line development, characterisation, draft preparation; SA—Assisted in cell line maintenance and review literature; RO—Co-Project Investigator, Scaffold work suggested, review, result and discussion on final manuscript.

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Declarations

Competing interests The authors declare no competing interests.

Ethical approval All applicable institutional guidelines for the care and use of animals were followed and approved by Institutional Animal Ethics Committee (IAEC), ICAR- Central Institute of Fisheries Education, Mumbai, Maharashtra, India.

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