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RESEARCH PAPER

Derivation and Characterization of a New Embryonic Cell Line from the Olive Flounder *Paralichthys olivaceus*

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Abstract

A new embryonic cell line that consisted predominantly of fibroblast cells has been established from embryos at Kupffer's vesicle (KV) stage of the olive flounder *Paralichthys olivaceus*, designated as the PoEKC. PoEKC cells have been subcultured for 61 passages over a period of 18 months. The cells were cultured in DMEM/F-12 medium supplemented with antibiotics, FBS, and growth factors at temperature of 25°C. The growth curve of the PoEKC cells comprised an exponential growth phase and a long plateau phase. Chromosome analysis revealed that the cells possessed the normal flounder diploid karyotype of 2n = 48t. The origin of the cell line was confirmed by testing the partial sequences of cytochrome oxidase c subunit I (*COI*) gene of mtDNA. The pluripotency markers, genes *OCT4-1*, *SOX2*, *KLF4*, and *NANOG* showed positive signals in the PoEKC cells, and the cells also presented high ALP activity. According to the above results, the PoEKC cells might be pluripotency, though the pluripotency needs further confirmation. The cells were also successfully transfected with GFP reporter gene suggesting that it could be utilized for gene function study in the flounder.

Introduction

Fish cell lines offer many advantages over mammalian cell lines, such as valuable, fast, and economic *in vitro* tools for screening toxicity of chemicals and environmental samples (Bols *et al.*, 2005; Davoren *et al.*, 2005; He *et al.*, 2014). Derived from various tissues, including early embryos, they have been providing important contributions in studies relating to immunology, virology, toxicology, genetic regulation, developmental biology, and so on (Pandey, 2013; Pannetier *et al.*, 2019; Futami *et al.*, 2021). In the meantime, development of cell lines from fish species will be also of great importance for aquaculture and conservation of fish germplasm.

There are over 700 fish cell lines in the current Cellosaurus release and the fish invitrome (Bairoch, 2018; Bols *et al.*, 2017). And most of them were developed from tissues, such as caudal fin, heart, eye, gill, brain, intestine, and muscle of ornamental and food fish (Lakra *et al.*, 2011; Soni *et al.*, 2018; Schug *et al.*, 2019; Wang *et al.*, 2020). There are only 94 cell lines from fish embryos in the current Cellosaurus release, and most of them from embryos at blastocyst stage. It is well-known that embryo stage is the early development stage for animals, at which almost all the tissues and organs develop, and then embryonic cell lines can increase our understanding of early development in fish and how the environment influencing the development. Kupffer's vesicle (KV) stage is a critical period of some important organ genesis and development in the fish embryonic development (Ahlstrom *et al.*, 1984). According to the report in the zebrafish *Danio rerio*, as a ciliated organ of asymmetry in embryo, it initiates left-right (LR) development of the organs such as brain, heart, and gut (Essner *et al.*, 2005). So, the cell line at this stage would be a useful tool for the research of these important development issues. However, there is almost no related report.

Olive flounder *Paralichthys olivaceus* is a major mariculture fish in China, Korea, and Japan. Around 7-8 cell lines, including spleen, gill (Kang *et al.*, 2003), brain (Zheng *et al.*, 2015), muscle (Peng *et al.*, 2016a), and testicular (Peng *et al.*, 2016b) cell lines, have been developed, and some of them have been used for study on flounder gene function or fish virus isolation (Liang *et al.*, 2018; Yeh *et al.*, 2018). Similar to other fish, the flounder embryonic cell lines at stages blastula and gastrula were also established (Chen *et al.*, 2004; Kim *et al.*, 2018), and found that they were epithelial-like cells and the fish serum were essential for the primary explant culture. Flounder KV is formed at the 2-somite stage (Hashimoto *et al.*, 2007), and, as other fish embryo, it is essential for organ formation (Niu *et al.*, 2016). There is also no report for the cell line at this stage in the flounder. In this study, a cell line of the flounder embryo at KV stage (PoEKC) was established, and its biological characteristics was analyzed. The efficiency of transfection and expression of foreign DNA were also examined. This new cell line would be useful for biotechnological and toxicological researches in marine fishes as an *in vitro* biological system.

Materials and Methods

Primary Explant Culture

The flounder brooders were cultured in the fish farm of Shenghang, Weihai, China, and fed with nutritional fortified fish flesh twice a day. When spawning behavior was observed, the naturally fertilized eggs were collected from spawning pool with a net of 60-mesh and transferred to the institute lab immediately. The fertilized eggs were washed with filtered sea water, placed into a clean plastic 1 L beaker, and cultured at 15 - 16°C until Kupfferp's vesicle formation was appeared (Figure 1 a1, a2). The embryos at this stage (approximately 30 hrs after fertilization) were harvested and prepared for cell culture. For the primary explant culture, a group of about 50 - 70 embryos were disinfected washed 5 times with sterile seawater for 2 min each time. Then they were transferred to another new sterile dish, rinsed 3 times with sterile seawater, and washed 3 times with PBS containing antibiotics (400 U/mL penicillin and 400 µg/mL streptomycin). After lightly crushed the embryos with a sterile mortar to break the membrane and gently compressed the embryos vertically to separate the cells as much as possible, we added 2 mL of DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Life Technologies, USA, Cat No. 12400024) complete growth medium and mixed well. Around 1 mL supernatant was filtered with 80-mesh sterile sieve and transferred to a 25 cm^2 flask to be incubated at 25°C. After 24 hrs, 1 mL of new DMEM/F-12 complete medium was added. The complete medium (pH 7.2 - 7.4) was DMEM/F-12 supplemented with 23.8 mmol/L $NaHCO_3$, antibiotics (penicillin, 100 U/mL, streptomycin, 100 µg/mL; Gibco BRL, USA), fetal bovine serum (FBS, 20%,

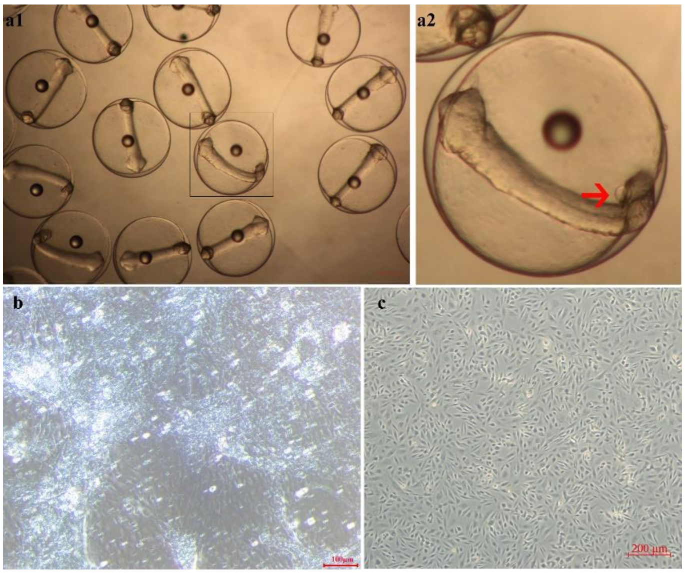

Figure 1. Morphology of embryos and embryo cell culture. a1, flounder embryos used for cell culture; a2, an enlargement of a1, the arrow indicates Kupfferp's vesicle; b and c, confluent monolayer of the PoEKC cells at passages 0 and 42, respectively. Bar, 0.5 mm (a1), 100 µm (b), and 200 µm (c).

BI, Israel), basic fibroblast growth factor (bFGF, 10 ng/mL, Gibco BRL, USA), epidermal growth factor (EGF, 15 ng/mL, Life Technologies, USA), and non-essential amino acid (NEAA, 100 µmol/L, Gibco BRL, USA) referred as Peng *et al.* (2016).

The cells were cultured at 25°C, and the medium was changed every 2 or 3 days. After confluence was reached (90%), the cells were subcultured at a ratio of 1:2 using trypsinization. For cryopreservation, cells were suspended in FBS with 10% dimethyl sulfoxide (DMSO), freezing slowly and stored in liquid nitrogen.

Cell Growth

To assess cell growth, 3.08 × 10^4 cells/mL of passage 50 were inoculated into 12 - well plates and incubated at 25°C. On days 1 to 19, three wells of cells were respectively trypsinized, and cells were counted using a Countess™ II Automated Cell Counter.

Chromosome Analysis

The cells at passage 39 were used for chromosomal analysis. According to our previous study (Zheng *et al.*, 2015), 1.0 × 10^6 cells were seeded into a 25 cm^2 culture flask and incubated at 25°C for 24 hrs. Then the cells were treated with colchicine (1.0 µg/mL, Sigma USA) for 3 hrs, and were trypsinized and harvested in a centrifuge tube by centrifuging at 2200 *g* for 2 min. After removal of supernatant, the cells were suspended in 5 mL hypotonic solution of 0.075 mmol/L KCl for 25 min at 37°C and premixed for 10 min at 4°C with 1 mL cold and fresh Carnoy's fixative (methanol: acetic acid, 3:1), and then centrifugated at 1000 *g* for 10 min. The cell pellet was fixed twice in 3 mL of cold fixative solution, 25 min each time. After the second centrifugation, cells were resuspended in 0.5 mL of cold fixative solution. The cell suspension was dropped onto the clean pre-cooled glass slides and air dried. Chromosomes were stained with 10% Giemsa for 25 min. Thirty photographed cells at metaphase were counted under microscope (Leica DM LB2, Germany).

Authentication of the Cell Line

To confirm whether the cells of the cell line were derived from the flounder, partial sequences of cytochrome oxidase subunit 1 (*COI*) gene of mtDNA were amplified and sequenced. Genomic DNA was isolated from the cells at passage 38 using DNA extraction kit (Tiangen, China). Primers (FishF1: TCAACCAACCACAAAGACATTGGCAC, FishR1: TAGACTTCTGGGTGGCCAAAGAATCA) (Ward *et al.*, 2005) were used for amplification. The 25 µL PCR reaction mix included 1 µL (100 ng) of total genomic DNA as a template, 1 µL of each primer (10 µmol/L), 12.5 µL of 2×Kodaq MasterMix (ABM, China), and 9.5 µL nuclease-free water. The PCR cycling conditions were denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and a final extension of 72°C for 5 min. The products were identified with 1% agarose gel and the purified products were sequenced, then the self-checked sequence was used for comparison with the flounder known sequence in NCBI's GenBank database (Accession No. MH032482.1) using the Basic Local Alignment Search Tool (Nucleotide BLAST).

Immunocytochemical Identification

The PoEKC cells at passage 23 were examined for expression of antibody directed against vimentin (V6630-CLONE 9, mouse monoclonal antibody Sigma), the marker of fibroblast morphology of the cell line (Dubey, *et al.*, 2014; Goswami *et al.*, 2014). The primary antibody was diluted 1:200 in 1% PBST. About 1.1 × 10^5 cells/well were seeded in a 24 - well plate and incubated at 25°C for 24 hrs. Cells were washed 3 times with cold PBS, and fixed for 10 min in paraformaldehyde (PFA, 4.0% in PBS, v/v) at room temperature. After PFA being removed, the cells were washed for 5 min with PBS, perforated for 5 min in 0.2% Triton X-100 PBS, washed twice with PBS, blocked for 30 min in 1% BSA at room temperature, and incubated with primary antibody overnight at 4 °C. After washed 3 times in 0.1% Tween20 PBS, the cells were incubated with the secondary antibody FITC-labeled anti-mouse IgG (Goat anti-Mouse IgG (H + L) antibody, Invitrogen, Waltham, MA, USA, diluted 1:500 1% PBST) at 25°C, washed 3 times in PBS, stained with DAPI for 10 min in dark, then washed in PBS 3 times and observed under the microscope (AxioVert A1, Zeiss, Germany). Negative control (without the primary antibody) was included in the experiment.

Alkaline Phosphatase (ALP) Staining

Cultured cells at passage 41 were washed with PBS, fixed in 4% PFA for 10 min, washed twice with PBS, and then stained 12 hrs in dark using bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT) Alkaline Phosphatase Color Development Kit (Beyotime, China) for ALP activity analysis. The negative control was incubated with PBS. After discarded the BCIP/NPT substrate solution and washed twice with PBS, we observed the cells under microscope (AxioVert A1, Zeiss, Germany). Positive cells were stained from red to purple (Yi *et al.*, 2010; Zhang *et al.*, 2019).

Analysis of Gene Expression Patterns

Total RNA of the cells at passage 61 was isolated by Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan) was used for cDNA synthesis. The RT-PCR was performed to check the expression of pluripotency related genes, *ALP*, octamer-binding transcription factor 4 (*OCT4-1*), sex determining region Y-box 2 (*SOX2*), kruppel-like factor 4 (*KLF4*), and *NANOG*. RT-PCR amplification was conducted using Takara Ex Taq polymerase. Primers of *OCT4*, *SOX2*, and *NANOG* were designed according to the published flounder genes sequences by using Primer-BLAST of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The RT-PCR program was as follows: 95°C for 15 s, 35 cycles of 95°C denaturation for 5 s and annealed and extension at 60°C for 31 s. Relative gene expression data were analyzed with 1% agarose gel, and *β-actin* was used as the reference gene.

Transfection Test with pEGFP-N1 Reporter Gene

The cells at passage 38 were seeded into a 12 - well plate at a density of 4×10^5 cells/well and incubated overnight at 25°C. Sub-confluent cells were transfected with 500 ng/mL pEGFP-N1 express vector (Invitrogen, USA) using 1 µL lipofectamine 2000 (Life Technologies, USA) according to the instructions with modification. In brief, the cells were cultured for 5 hrs at 25°C after DNA-lipid complex was added. And then the supernatant was replaced with fresh complete medium. The cells were observed under a ZEISS AX10 fluorescence microscope (Germany) after being cultured for 48 hrs at 25°C.

Results

Establishment and Morphology of the PoEKC Cells

In around 3 days, the cells migrated from the edges of the embryonic debris tissue. On the fourth day, the cells reached 90% confluence, and the first subculture was performed. Every 7 - 10 days, the cells were subcultured to fresh medium at 1:2 cell suspension based on the observation. The cell line has been subcultured for 61 passages over 18 months and was designated as the PoEKC (Figure 1b, c). Cells of the cell line mainly belonged to small fibroblastic cell according to the results of the immunocytochemical identification with antibody directed against Vimentin, the marker of fibroblast morphology of the cell line (Figure 2). After the cells were cryopreserved at some passages such as P2, P10, P25 and so on, the cells were thawed rapidly, diluted slowly, and reseeded at a high density to optimize recovery. Their survival rates were 60 - 70% when recovered from liquid nitrogen (-196°C) after storage of 6 month, and the cells grew to confluence in 5 - 7 days.

Growth Curve

The PoEKC cells had a long growth cycle, and the number of cells was tested every 2 days until d19. The growth of the cells showed atypical curve, comprising an exponential log phase and a long plateau phase (Figure 3). In the log phase, the cells were in their most reproducible form, and the cell number was increased exponentially. In the plateau phase, cell proliferation ceased almost completely, and dead cells were observed (d 13).

Chromosome Analysis and Molecular Characterization

The chromosome assay showed that the chromosomes were telocentric with 48 chromosomes, which revealed that the metaphases displayed normal karyotype morphology of the flounder, and the ratio was 38.7%. *COI* gene partial sequence was amplified from the PoEKC cell DNA to verify the origin of the cell line. The PCR product of 710 bp was obtained from the cells (Figure 4b). Subsequent blast analysis of the sequences demonstrated that 99% matched with the known flounder mtDNA *COI* sequence (GenBank Accession No. AB028664), which confirmed that the origin of the PoEKC cells (Figure 4b).

Test for the Pluripotency of the Cells

To study the pluripotency of the cells, ALP activity and expression patterns of related genes (*ALP*, *OCT4-1*, *SOX2*, *KLF4*, and *NANOG*) were analyzed. The ALP staining pattern showed that about 50% of cells presented high ALP activity with bluish purple color after incubated for 24 hrs (Figure 5). All tested genes were expressed in the PoEKC cells at passage 61, and *SOX2* expression level was relative higher (Figure 6).

Table 1. Genes and specific primers used for RT-PCR

Gene name		Sequence (5'--3')	Product size (bp)	References/ NCBI Accession No.
NANOG	-F	CGCACACCTCACCAGACTCAT	119	XM_020087232.1
	-R	CCTGTCGCGCACCTCACTTTC		
OCT4-1	-F	TGCGCAGACTTTCTTCCCAT	197	KJ522774.2
	-R	TTGATTTGCTCCCGGGTCTC		
ALP	-F	CAGAAGGGCAACGAGGTCAC	112	Shi *et al.*, 2011
	-R	CATAGCTGGCTGCTGGGGTA		
SOX2	-F	AACGGCTCCCCGACCTACTC	104	XM_020104145.1
	-R	CTGCTGGACTCGGACTTCACC		
KLF4	-F	GCAACTGCACCATCTCACAG	300	Kim *et al.*, 2018
	-R	GGAACTGCATGGAGGATGAC		
β-actin	-F	GGAATCCACGAGACCACCTACA	264	Zheng & Sun, 2011
	-R	CTGCTTGCTGATCCACATCTGC		

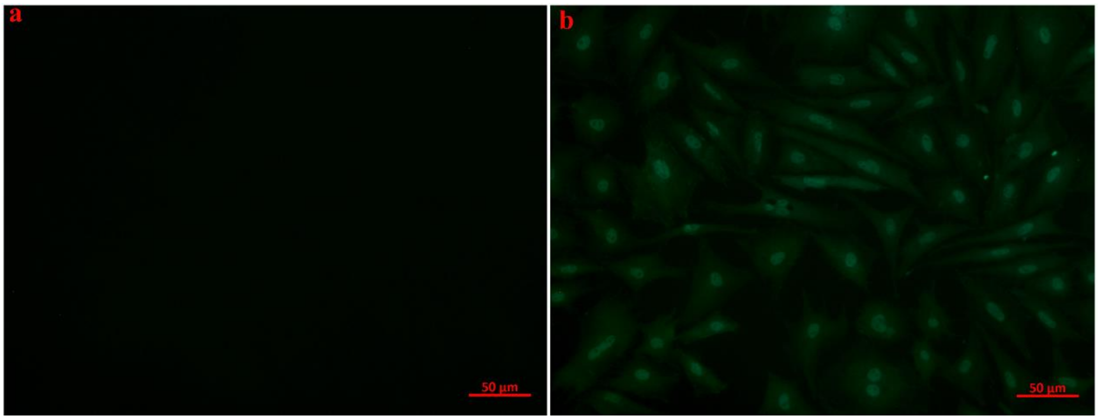

Figure 2. Expression of fibroblastic protein in the PoEKC cells labeled with anti-Vimentin and FITC-conjugated secondary antibody. a, the negative control; b, the expression of Vimentin. Bar, 50 µm.

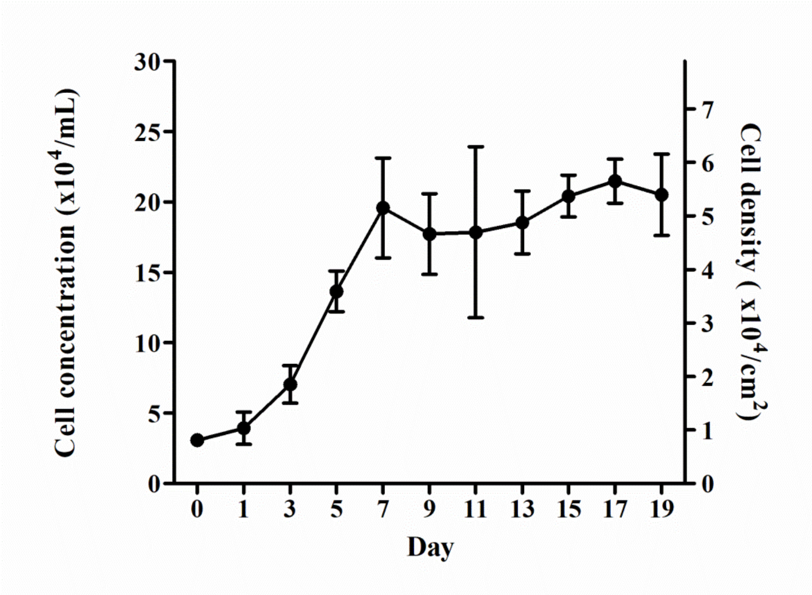

Figure 3. Growth curve of the PoEKC cells showing an exponential growth phase and a long plateau phase. The cells were cultured in DMEM/F-12 medium supplemented with antibiotics, FBS, and growth factors at 25°C. The cell number was counted every 2 days. The starting cell number was 3.08×10^4 cells/mL 24 hrs after seeded. Values are mean ± SE (n =3).

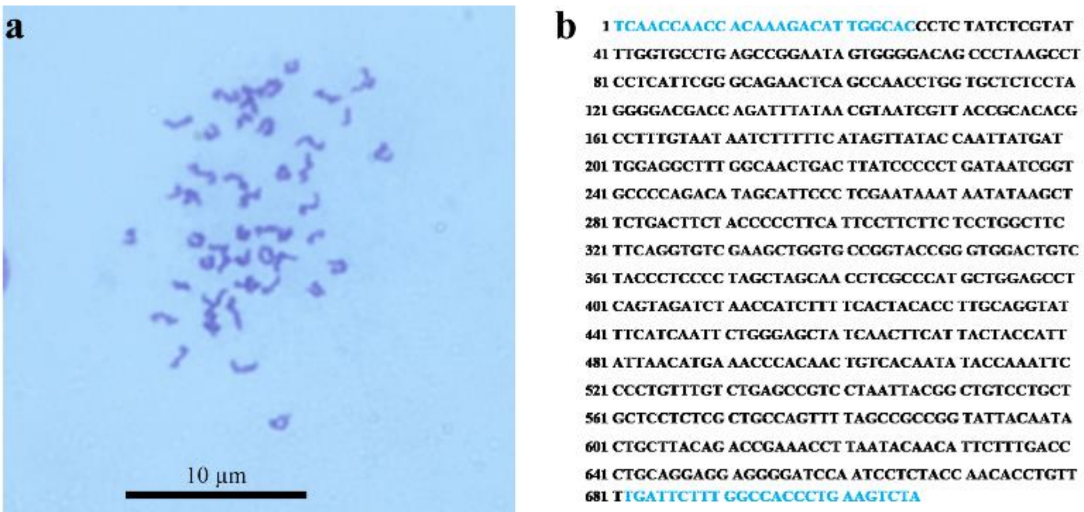

Figure 4. Chromosome and molecular characterization of the PoEKC cells. a, metaphase of the cells at passage 39; b, sequences of *COI* partial region from the cells at passage 38 (the blue font is sequences of the primers). Bar, 10 µm (a).

Transfection Efficiency

After the PoEKC cells at passage 38 were transfected with pEGFP-N1 reporter gene for 24 hrs, clear and strong green fluorescence signals could be detected (Figure 7). The percentages of transfection were 10 - 20%. Results indicated the suitability of the PoEKC cells for transfection.

Discussion

Most of the fish embryos develop outside of their mother's body, which is a big advantage for initiating the embryonic cell culture. What else, embryonic cells at early stages are pluripotent to make their culture *in vitro* much easier (Alvarez *et al.*, 2007), but the previous embryonic stem (ES) cell lines were almost from embryos at stages blastula and gastrula. Kupffer's vesicle, a specific spherical organ attached to the tail region in fish embryo, plays a similar part to the mammalian node during formation of LR axis (Hojo *et al.*, 2007). It is required for fish normal organogenesis and affects the LR axis establishment in fish such as zebrafish (Dasgupta *et al.*, 2018; Navis *et al.*, 2013), medaka (Hojo *et al.*, 2007), plaice *Pleuronectes platessa*, and cod *Gadus morhua* (Nordahl, 2011). And it is also essential for normal organ formation (the internal organs such as the heart, gut, and brain exhibit asymmetry with respect to the LR axis) in the flounder (Ahlstrom *et al.*, 1984; Niu *et al.*, 2016). As far as we know, the PoEKC is the first cell line from fish embryo at this stage with normal flounder diploid karyotype of 2n = 48t and could provide a new tool to research fish gene function, organogenesis and detection or isolation fish virus.

The cultured PoEKC cells were small and their shape looked like fibroblastic under the microscope. And then, the identification of fibroblast morphology of cells of the cell lines was performed by using immunocytochemistry method with Vimentin labelled. Vimentin is a type III intermediate filament (IF) protein that is expressed in mesenchymal cells. IF protein is found in all animal cells (Eriksson *et al.*, 2009) as well as bacteria (Cabeen & Wagner, 2010). Vimentin plays a significant role in supporting and anchoring the position of the organelles in the cytosol, and it is attached to the nucleus, endoplasmic reticulum, and mitochondria, either laterally or terminally (Katsumoto *et al.*, 1990). Vimentin as a marker of fibroblast morphology of the cell line has been used in several fishes, such as *Puntius denisonii*, *Puntius* (*Tor*) *chelynoides*, and *Wallago attu* (Dubey *et al.*, 2014; Goswami *et al.*, 2014; Lakra & Goswami, 2011). After identification, the three cell lines were fibroblastic, fibroblastic, and epithelial cells, respectively, and these cells were also successfully transfected with GFP reporter plasmids. According to these reports and our findings, we implied that the PoEKC cells were also fibroblastic cells.

In the process of primary explant culture, we used a simpler and more convenient method to separate the cells comparing to other method (Barman *et al.*, 2014; Kim *et al.*, 2018), which could retain as much embryonic contents as possible for subsequent cell culture. Previous studies also demonstrated that cell cytoplasmic contents were beneficial to the survival and proliferation of primary culture cells (Chen *et al.*, 2003; Kim *et al.*, 2018). When two other flounder embryonic cell lines at stages blastula and gastrula were primary-cultured, sea perch serum (Chen *et al.*, 2004) or flounder serum and embryo extract (Kim *et al.*, 2018) were added to the complete medium to make cells grow better. Self-prepared fish serum or other additives may carry contaminating substances such as virus and mycoplasma, and the operation process is also complicated. In this study, all used reagents were commercial products, and no special additive, such as target fish serum, was used in the cell culture process. This method could avoid unnecessary contamination and increase the repeatability of the experiment and is also easily to follow.

Stem cells can be differentiated into other types of cells. ES cells are one of the broad types of stem cells, which have the characteristics of pluripotency (Yu & Thomson, 2008) According to Alvarez *et al.*, (2007), ALP activity is one of valuable indicators to test the pluripotency of cell, and it has been used to detect the pluripotency of embryonic stem cell in sea perch, medaka and zebrafish (Chen *et al.*, 2003; Xing *et al.*, 2008; Yi *et al.*, 2010). The PoEKC cells in this study showed high activity of ALP. In addition, the PoEKC cells also expressed pluripotency related genes, *ALP*, *OCT4-1*, *SOX2*, *KLF4* and *NANOG*. NANOG, SOX2, and OCT4 are transcription factors, which are all essential to maintain the pluripotent embryonic stem cell phenotype. Nanog is a homeobox-containing transcription factor with an essential function in maintaining the pluripotent cells of the inner cell mass and in the derivation of embryonic stem cells (ESCs) (Mitsui *et al.*, 2003). The POU domain identified in the transcription factors Pit1, Oct1, Oct2, and Unc86 containing Oct4 and the high mobility group (HMG) domain containing Sox2 are two other transcription factors known to be essential for normal pluripotent cell development and maintenance in mammal (Hart et al., 2004; Malik *et al.*, 2019). Except for OCT4, SOX2, and NANOG, KLF4 is required for ESC self-renewal and pluripotency (Nakatake *et al.*, 2006; Xu *et al.*, 2009). Studies in fish such as medaka haploid ES cells (Yi *et al.*, 2010; Zhang *et al.*, 2019), carp *Labeo rohita* ES cells (Patra *et al.*, 2018), and catfish *Heteropneustes fossilis* ES-like cells (Barman *et al.*, 2014) all indicated that above genes were pluripotency genes (Familari & Selwood, 2006), and these cells were identified to be pluripotency by using above genes. So, the PoEKC cells may be pluripotency cells from the flounder embryo, but a definite answer will require more specific protein markers' tests and induction differentiation of cells in the future.

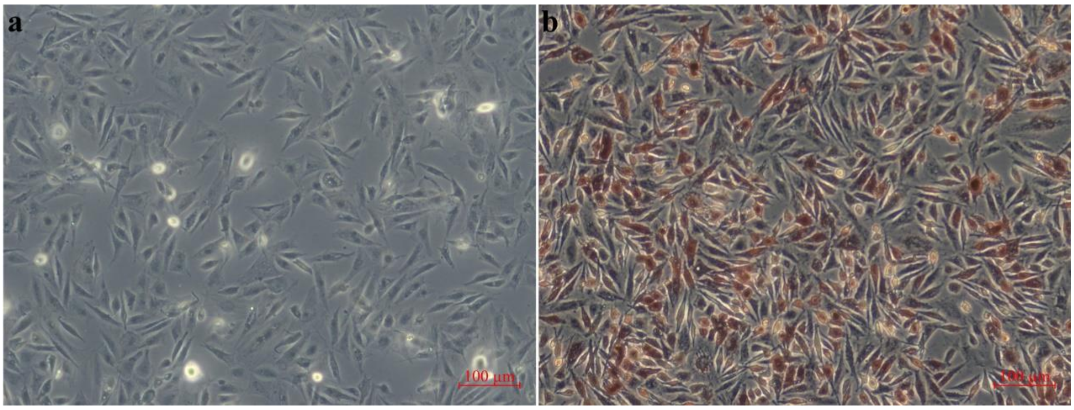

Figure 5. Alkaline phosphatase staining pattern in the PoEKC cells. a, the negative control cells incubated with PBS; b, showing the purple-colored ALP positive PoEKC cells after incubation for 24 hrs. Bar, 100 µm.

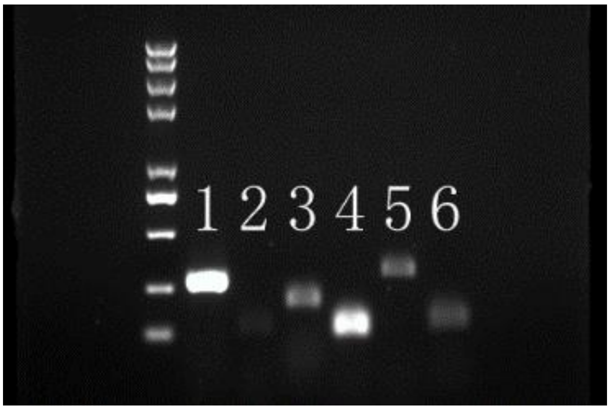

Figure 6. Expression analysis of pluripotency related genes in the PoEKC cells. *β-actin* gene was amplified as a reference gene. 1 - 6, target bands of *β-actin*, *ALP*, *OCT4*, *SOX2*, *KLF4,* and *NANOG*.

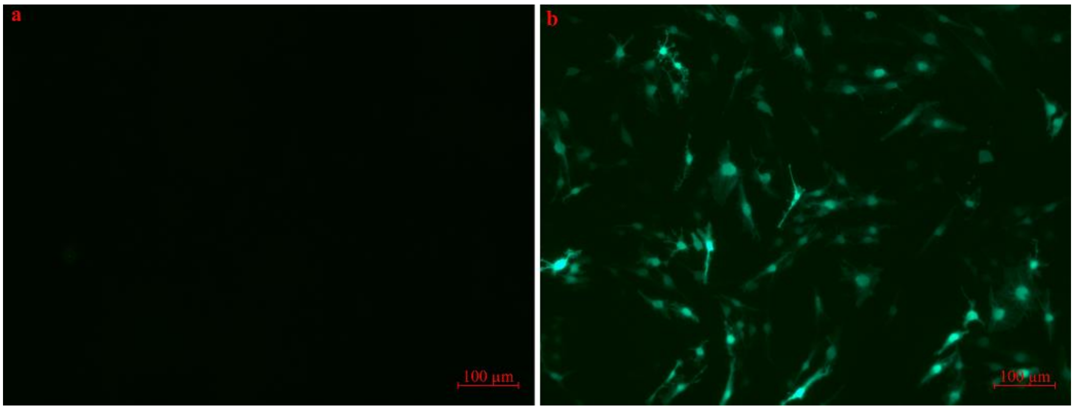

Figure 7. Expression of green fluorescent protein (GFP) in the PoEKC cells after transfection with pEGFP-N1 plasmid. Bar, 100 µm.

Conclusion

In conclusion, a new flounder embryonic cell line at KV stage (PoEKC) was established and characterized in terms of their proliferation, authentication, gene and protein expression, and transfection susceptibility. This is the first report of cell line at this stage in fish. The separate and culture method for embryonic cells is simpler and more convenient. It is capable of prolonging *in vitro* culture and could provide an effective experimental tool to study early development events of organs or virus test in fish.

Ethical Statement

All experiments were performed according to the regulation of local and central government of China and approved by the Institutional Animal Care and Use Committee of Institute of Oceanology, Chinese Academy of Sciences.

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Author Contributions

Miaomiao Nie and Feng You conceived and designed the experiment. Miaomiao Nie performed the experiments. Miaomiao Nie and Zhihao Wu performed data analyses. Zhihao Wu provided ideal embryos. Miaomiao Nie and Feng You wrote and reviewed the manuscript. Miaomiao Nie, Zhihao Wu, and Feng You approved the manuscript. Feng You supervised the study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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