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Establishment of testicular and ovarian cell lines from Honmoroko (*Gnathopogon caerulescens*)

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Abstract We succeeded to establish cell lines from endemic fish species Honmoroko *Gnathopogon caerulescens*, which inhabits Lake Biwa, the third oldest lake in the world. Two cell lines designated as RMT1 and RMO1 were established from testis and ovary of *G. caerulescens*, respectively. These cell lines were initially cultured in Leibovitz's L-15 medium supplemented with fetal bovine serum (FBS), fish embryo extract, epidermal growth factor, and basic fibroblast growth factor. Further addition of forskolin and β-mercaptoethanol was required to establish and maintain these cell lines for more than 60 passages. RMT1 and RMO1 cells showed fibroblast- and epithelial-like morphology, respectively. From immunocytochemical staining and gene expression patterns, RMT1 cells showed a characteristic of testicular Sertoli cells and RMO1 cells did that of ovarian theca cells. Both RMT1 and RMO1 cells multiplied well in the medium supplemented with 10 % FBS at 28 °C and their minimum population doubling times were 24.4 and 28.8 h, respectively. At the 45th passage, most of the RMT1 and RMO1 cells had a hyperploid set of chromosomes (67.3 and 96.1 %, respectively). Cells with normal diploid chromosome set were not observed. RMT1 cells were transfected with an enhanced green fluorescent protein (EGFP) expression vector and human elongation factor 1 α promoter worked efficiently to express EGFP. In addition, EGFP-expressing cell lines were also established, suggesting that the cell lines could be utilized as an in vitro monitor system (biosensor) for the evaluation of endocrine disruptors which might affect gonadal function.

Keywords Cell line · Testis · Ovary · Fish

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Introduction

In vitro systems such as cell lines are growing importance in ecotoxicology, because the cell lines are rapid and cost-effective tools for the assessment of adverse effects of chemicals and environmental samples with high reproducibility. The cell lines from

different tissues of various species are valuable for studying tissue- and species-specific responses to chemicals at the cellular level, especially gonadal cells which links to reproductive performance will be useful to validate chemicals as gonad is known to be sensitive and frequently affected by chemicals such as endocrine disruptors (Hotchkiss et al. 2008). More than 280 teleost fish cell lines have been developed from a broad range of species and tissues (Fryer and Lannan 1994; Lakra et al. 2011; Wolf and Mann 1980); however, there is limited number of cell lines (about 20 gonadal cell lines) originating from gonadal tissues in fish (Fryer and Lannan 1994; Lakra et al. 2011; Wolf and Mann 1980; Zhang et al. 2011).

Honmoroko *Gnathopogon caerulescens* is a small cyprinid fish that is endemic to Lake Biwa in Japan. Although the necessity of stock maintenance has been emphasized, the natural stock has rapidly decreased for several decades. In recent years, several endocrine-disrupting chemicals, such as 4-nonylphenols and 4-tert-octylphenol, were found in water and fish from the lake (Tsuda et al. 2000). Endocrine disruptors are believed to have adverse effect on the sexual development and fertility through their multiple hormone-like activities (Scholz and Klüver 2009). However, these effects are poorly understood at the cellar level in fish due to the lack of appropriate cell lines to analyze their mechanism. Since gonadal cell lines of *G. caerulescens* could be used not only as ideal tools for in vitro studies for the mechanism of endocrine disrupters but also as biosensors to detect them, it is necessary to develop the cell lines.

The present study reports two new cell lines from testis and ovary of *G. caerulescens* (RMT1 and RMO1, respectively) and their characterizations. In addition, transfection of foreign DNA and establishment of stable cell line expressing green fluorescent protein were also investigated in RMT1 cells.

Materials and methods

Primary cell culture

Establishment of the testicular and ovarian cell lines was based on the procedure described in male germ cell cultures of zebrafish (Sakai 2006) with slight modifications in cell culture condition. Namely, cells were cultured in growth factors-supplemented medium under feeder cell-free condition.

Matured (approx. 2-year-old) male and female *G. caerulescens* were obtained from Shiga Prefectural Fisheries Experimental Station. They were euthanized by a quick blow to the head and disinfected with 70 % ethanol. The male and female gonads were aseptically dissected out from the fishes and dissociated separately in Leiboviz's L-15 medium supplemented with 500 U ml^{-1} collagenase (GIBCO/Invitrogen, Carlsbad, CA, USA). Following the removal of undissociated fragments by passing through a nylon mesh, the cells in the suspension were washed with HEPES-buffered Leiboviz's L-15 medium (pH 7.9) supplemented with 1 % (w/v) bovine serum albumin fraction V (BSA: Sigma-Aldrich, St. Louis, MO, USA) by 10-min centrifugation at $100\times g$. The cell pellet was suspended in TCCM-FBS (testicular cell culture medium containing 3 % (v/v) fetal bovine serum) (Sakai 2006) supplemented with 10 IU ml^{-1} human chorionic gonadotropin (HCG, Sigma-Aldrich), 10 IU ml^{-1} pregnant mare's serum gonadotropin (PMSG, Sigma-Aldrich), 100 ng ml^{-1} epidermal growth factor (EGF: Peprotech, Rocky Hill, NJ, USA), 100 ng ml^{-1} basic fibroblast growth factor (bFGF: Peprotech), and plated at four paired gonads per 35-mm plastic culture dish coated with 0.1 % (w/v) gelatin. Fish embryo extract, which was contained in TCCM-FBS, was prepared from *Ischikauia steenackeri* embryos according to the procedure as described previously (Westerfield 2007). The dishes were incubated at 28 °C, and the medium was changed every 3 days.

Subculture and maintenance

When the cells reached 90 % confluency, the cells were detached from culture dishes using phosphate-buffered saline (PBS) supplemented with 0.06 % (w/v) trypsin and 1.32 mM ethylenediaminetetraacetic acid. Each culture was examined under an inverted microscope and as soon as the cell shape had changed, the cells were washed with Leibovitz's L-15 medium supplemented with 3 % FBS by 3-min centrifugation at $100\times g$. To establish cell lines, testicular and ovarian cells at the 6th and 3rd subcultures, 10 μM forskolin (Sigma-Aldrich), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 10 % FBS were added in the media described above.

Morphological confirmation by immunocytochemistry

Confirmation of the morphologies of testicular and ovarian cell lines was made by immunocytochemistry with antibodies directed against vimentin and cytokeratin. In brief, cells were grown to confluency in gelatin-coated 4-well dishes and were fixed for 15 min with 4 % (w/v) paraformaldehyde in PBS on the culture dishes. Following the permeabilization with 0.2 % (v/v) Triton X-100 for 10 min, cells were incubated for 30 min in PBS containing 4 % (v/v) goat serum. Then, the cells were incubated with the primary antibodies, mouse anti-vimentin (clone V9: Dako, Glostrup, Denmark; diluted 1:25), and mouse anti-cytokeratin (clone AE3: American Research Products, Waltham, MA, USA; diluted 1:50), overnight at 4 °C. The cells were incubated with secondary antibodies, Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen; diluted 1:500), for 45 min. Nuclei of the cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI: Dojindo Laboratory, Kumamoto, Japan) for 20 min. Fluorescent images were obtained using a confocal microscope (FLUOVIEW FV1000, Olympus, Japan). Appropriate controls for autofluorescence and secondary antibodies were performed.

Gene expression analysis

To verify the origin of the cell lines, reverse transcription polymerase chain reactions (RT-PCR) were performed to examine the gene expression patterns of the testicular and ovarian cell cultures using gonadal somatic (*Sox9a*, *WT1a*, *Cyp11b,* and *Cyp19a1*) and germ cell (*Dmrt1*, *Vasa,* and *Ziwi1*) marker genes. The gene expression patterns of testicular and ovarian tissues were also examined. The *Tuba1* gene was used as an internal control for RT-PCR.

Total RNA was extracted from the cell cultures and gonadal tissues using illustra RNAspin Mini Isolation Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions. After DNase treatment for the samples, reverse transcription was carried out with PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan) using 500 ng total RNA. The cDNAs were amplified using specifically designed primers (Table 1). PCR were carried out using TaKaRa Ex Taq DNA Polymerase (Takara Bio) according to the manufacturer's instructions. The PCR conditions used were: 2 min initial denaturation at 95 °C, 35 cycles of 20 s denaturation at 94 °C, 30 s annealing at 60 or 62 °C, and 1 min extension at 72 °C, followed by 2 min final extension at 72 °C. The RT-PCR products were separated by agarose gel electrophoresis, and the gel was stained with ethidium bromide.

Growth studies

Cell growth was examined at different FBS concentrations and incubation temperatures, using testicular and ovarian cell lines at passages 36–43 and 34–38, respectively. Testicular and ovarian cells were plated in gelatin-coated 24-well plates in triplicate at the concentrations of 8×10^4 cells per well and 6×10^4 cells per well, respectively, and cultured in the subculture medium containing 0, 5, and 10 % FBS at 18 and 28 °C. Cells were harvested daily by trypsinization and counted using a hemocytometer for 5 days. Minimum doubling time was calculated from experimental data during the exponential growth phase (24–48 h of culture) using the algorithm provided by http://www.doubling-time.com.

Chromosome analysis

Testicular and ovarian cell lines at passage 45 were used for chromosome analysis. Cells undergoing exponential growth were treated with 0.05 μg ml^{-1} colcemid for 4 h at 28 °C. Then, the cells were trypsinized and pelleted by centrifugation at $100 \times g$ for 3 min. The cell pellet was suspended in a hypotonic solution (0.075 M KCl) for 10 min, after which the cells were fixed for 20 min by adding four volumes of a 3:1 mixture of methanol and acetic acid. A small volume of the cell suspension was dropped onto clean glass slides, and chromosomes were stained with 10 % (v/v) Giemsa solution (in 10 mM potassium phosphate, pH 6.8) for 10 min and counted under a microscope. Chromosome counts were undertaken in more than 100 metaphase plates.

Transfection with GFP expression vector

The transfection ability of testicular cell line was determined using a plasmid expressing enhanced

Table 1 Primers used for reverse transcription polymerase chain reactions (RT-PCR)

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature (°C)
Sox9a	GACGTGGATATCGGCGAGCT	GGTGTGTACATGGGCCTCTG	60
WT1a	ATGGGGGATCAGCAGTATCC	CTGTGTGTTTACGGCTGTGCAT	60
Cyp11b2	CGTAACCCTGATGTGCAGGA	AGCAGCTGCATCTCGTTCTC	60
Cyp19a1a	AGGCAGTGTGTGTTGGAGATG	GGCTGGAAGAAACGACTCGG	62
Dmrt1	AAGGGCCACAAACGCTTCTG	CCGAGCCCCTGACTCAGGTA	62
Ziwi	CAGATGAACTGCAAAATGGGAGG	CCCTGCCAGTTGTAGTACATGTG	60
Vasa	GCATGCAGTGATGTGGAGCAAAC	CTGTTGTGCCATGAGCACTGAA	62
Tuba1	TCCACCCTGAGCAGCTCATCA	GAAGGCACAGTCAGAGTGCTC	60

green fluorescent protein (EGFP) under the control of human elongation factor 1 α promoter (pCE-EGFP) (Takada et al. 1997). The plasmid was derived from pEGFP-1 (Clontech, Mountain View, CA, USA), which carries neomycin resistance as a selectable marker. Plasmid DNA of this vector was prepared using QIAprep spin Miniprep kit (Qiagen) and transfection was performed using a Lipofectamine 2000 (Invitrogen) according to the supplier's instructions. In brief, the plasmid (480 ng) and the transfection reagent (1.2 μl) were dissolved separately in 30 μl aliquots of Opti-MEM (Invitrogen). The media were mixed and incubated for 20 min at room temperature. The mixture was added dropwise onto 80–90 % confluent testicular cells in 24-well dish, and transfection was carried out for 6 h at 28 °C. After 1-day culture in the subculture medium devoid of antibiotics, presumptive transfected cells were cultured with 400 μg ml^{-1} neomycin (G418: Nacarai Tesuque, Kyoto, Japan). After 10 days of culture, the neomycin-resistant fluorescent colonies were isolated and propagated.

Results

Primary and subsequent culture

In the primary and secondary cultures of the testicular and ovarian cells, mixture of two cell types was observed to coexist in both preparations (Fig. 1). In the testicular cell culture, the first and dominant cell type was fibroblast-like and the other less abundant cells were round-shaped and formed tightly packed colony on the fibroblast-like cells that have attached to the dishes. In contrast, the dominant cell type of ovarian cell culture was epithelial-like and the less abundant cells were rounded-shaped similar to the cells observed in the testicular cell cultures. After the 3rd passage, the cells became uniformly fibroblast- and epithelial-like cells in testicular and ovarian cell cultures, respectively.

At the 6th and 3rd passage of the testicular and ovarian cells, respectively, cell proliferation was almost halted. Supplementation to culture medium with forskolin and β-mercaptoethanol permitted the proliferation of the cells. After the establishment of cell lines, these cell lines can be maintained in reduced concentration of FEE (2 embryos ml^{-1}) with no obvious effect in cell morphology and proliferation.

Cells were subcultured at a ratio of 1:2 every at 2- to 3-day intervals and have been successfully subcultured more than 60 times since their initiation in October of 2010. Cells were cryopreserved at different passages and successfully thawed at high viability with a commercial cell freezing preservation solution (Cell Banker; Nippon Zenyaku Kogyo, Fukushima, Japan) (data not shown). During this period, no change in phenotype was observed and the cell lines from testicular and ovarian tissues were designated as RMT1 and RMO1, respectively.

Morphological confirmation by immunocytochemistry

Immunocytochemical characterization of the RMT1 and RMO1 cells were examined at passage 41 and 43, respectively. Both cell lines showed positive reaction for vimentin and negative reaction for cytokeratin (Fig. 2).

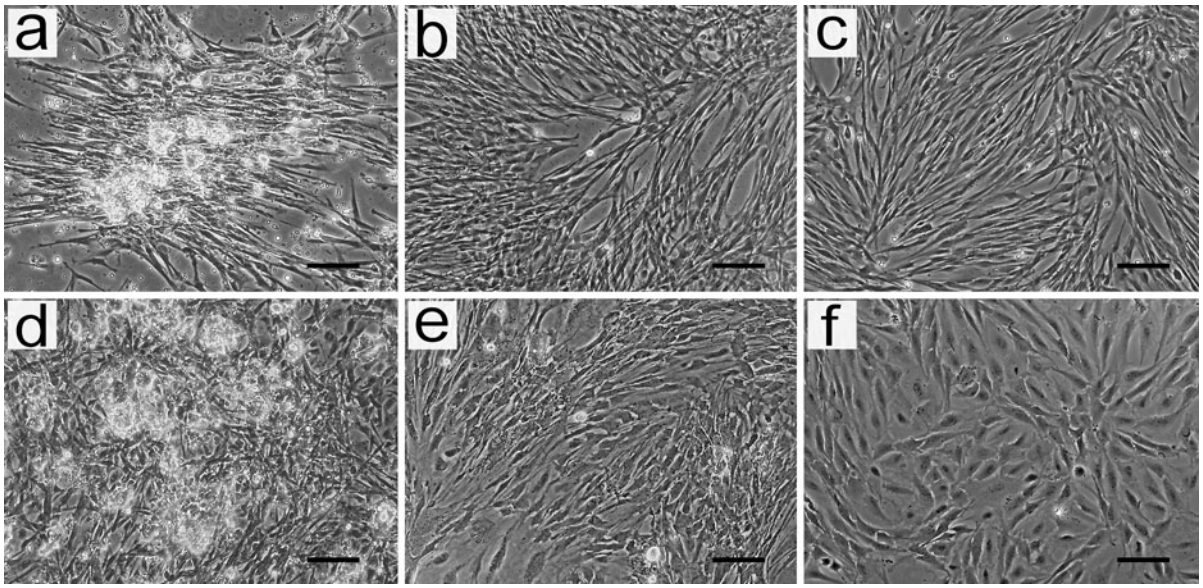

Fig. 1 Morphology of the cell cultures derived from testicular and ovarian tissues of *Gnathopogon caerulescens*. Phase contrast micrographs of testicular cell cultures were taken at primary culture (**a**), at passage 7 (**b**), and at passage 35 (**c**). Phase contrast micrographs of ovarian cell cultures were taken at primary culture (**d**), at passage 5 (**e**), and at passage 20 (**f**). *Bars* = 100 μm (magnification: ×100)

Gene expression analysis

Gene expression of RMT1 and RMO1 cell lines was analyzed at passage 20 and 14, respectively. As shown in Fig. 3, similar in the gonadal samples, both of the *Sox9a* and *WT1a* expressions were observed in RMT1 and RMO1 cells. Although obvious *Cyp11b* expression was detected in testicular tissue, very weak expression was observed in RMT1 and the RMO1 cells and ovarian tissue sample. Ovarian tissue sample expressed *Cyp19a1a* strongly, whereas weak expression was detected in RMT1 cells but not in RMO1 cells and testicular tissue sample. Although expressions of *Vasa*, *Ziwi1,* and *Dmrt1* were detected in both testicular and ovarian tissue samples, expressions of these germ cell markers could not be detected in RMT1 and RMO1 cells.

Growth studies

As shown in Fig. 4, both RMT1 and RMO1 cells did not show obvious growth at 18 °C, regardless of FBS concentrations. The growth rates of the both cell lines increased as the FBS concentration increased from 0 to 10 % at 28 °C. When the cells were cultured in 10 % FBS at 28 °C, both cell lines showed exponential growth during 24–48 h of culture and the minimum population doubling times of RMT1 and RMO1 were calculated to be 24.4 and 28.8 h, respectively. After 5 days of culture in 10 % FBS at 28 °C, cells reached visual confluence and the cell numbers of RMT1 and RMO1 were 5.5×10^5 and 2.5×10^5 in a well of 24-well plate, respectively.

Chromosome analysis

No obvious anomaly such as dicentric or ring chromosomes was observed in chromosomes of RMT1 and RMO1 cells (Fig. 5a, b). Both of the RMT1 and RMO1 cells exhibited aneuploidy in metaphase and diverse chromosome numbers. For RMT1 cells, the chromosome number ranged from 28 to 59 with observed modal chromosome number of 56 (Fig. 5c). The chromosome number of RMO1 cells ranged from 15 to 74 with observed modal chromosome number of 70 (Fig. 5d).

Transfection with GFP reporter gene

RMT1 cells were successfully transfected with pCE-EGFP by means of lipofection. The expression of

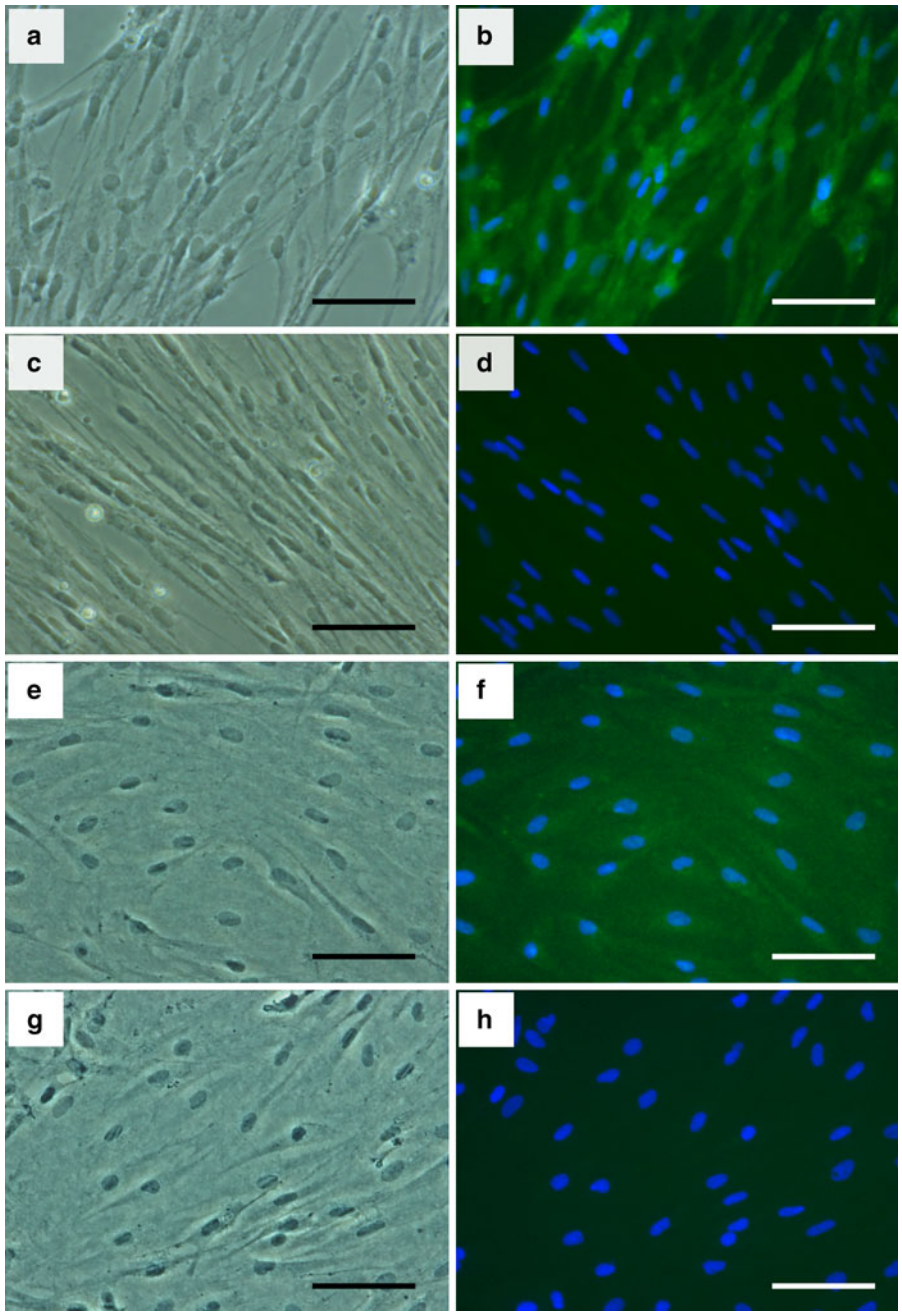

Fig. 2 Immunocytochemical staining for vimentin and cytokeratin in testicular (RMT1) and ovarian (RMO1) cell lines of *Gnathopogon caerulescens*. Phase contrast and fluorescent images of the RMT1 (**a** and **c**, **b** and **d**, respectively) and RMO1 (**e** and **g**, **f** and **h**, respectively) cells were taken at passage 41 and 43, respectively. RMT1 and RMO1 cells were stained with monoclonal anti-vimentin antibody (*green* in **b** and **f**, respectively), but not with anti-cytokeratin (**d** and **h**, respectively). Nuclei were counter stained with DAPI (*blue*). *Bars* = 100 μm (magnification: ×200). (Color figure online)

EGFP in the cells could be detected at 24 h after the transfection. Following the neomycin selection, we obtained a stable population of the EGFP-expressing RMT1 cells (Fig. 6).

Discussion

The fibroblast- and epithelial-like cell populations in testicular and ovarian cell cultures were observed to

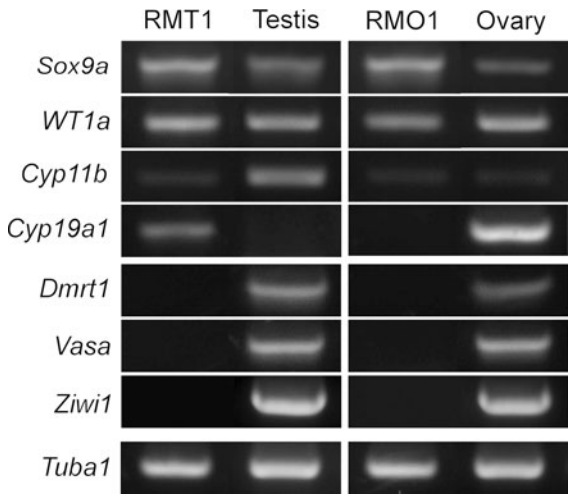

Fig. 3 Gene expression patterns of testicular (RMT1) and ovarian (RMO1) cell lines and male and female gonadal tissues of *Gnathopogon caerulescens*. Gene expression patterns of RMT1 and RMO1 were examined at passage 20 and 14, respectively

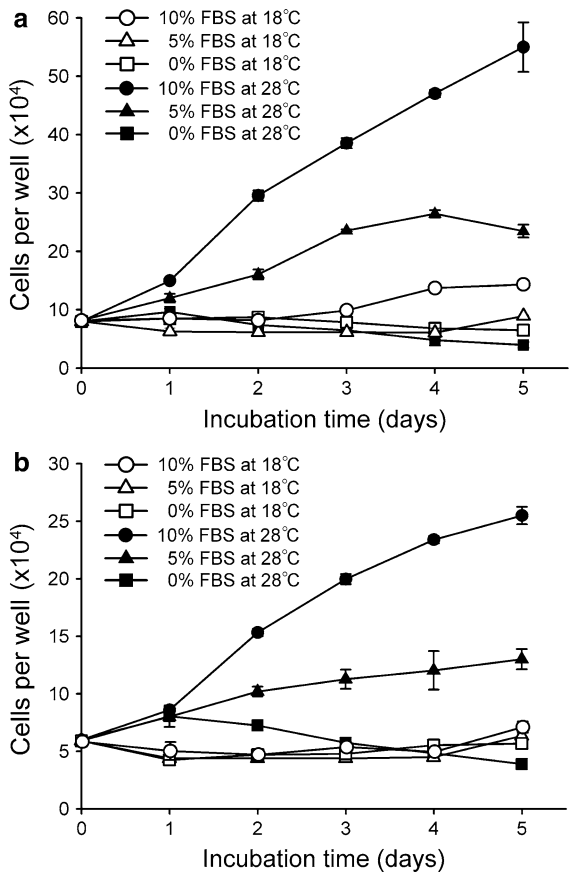

Fig. 4 Growth curves of testicular (RMT1) and ovarian (RMO1) cell lines of *Gnathopogon caerulescens* at different fetal bovine serum (FBS) concentrations and incubation temperatures. Growth rates of RMT1 (passages 36–43) (**a**) and RMO1 (passages 34–38) (**b**) were examined in various FBS concentrations (0, 5, and 10 % v/v) at different incubation temperatures (18 and 28 °C). Values are mean ± SE from triplicate assays

grow more rapidly than the coexisted round-shaped cells. From the previous studies of testicular (Sakai 2002; Song and Gutzeit 2003) and ovarian (Fraser and Hall 1999) primary cell cultures of other species, these round-shaped cells were considered to be germ line cells. Since the round-shaped cells attached on fibroblast- and epithelial-like cells, fibroblast- and epithelial-like cells presumably provides feeder function on maintenance of germ cells at an initial stage of primary culture, same as the culture of spermatogonial stem cells in zebrafish (Kawasaki et al. 2012). However, germ cells were gradually disappeared as passage proceeded. Therefore, culture condition in this study may exert more selective mitogenic effects on the fibroblast- and epithelial-like cells than on germ-line stem cells in the testicular and ovarian cell cultures, respectively.

Both of the RMT1 and RMO1 cells required forskolin and β-mercaptoethanol supplementation for their continuous growth and passage. Although forskolin and β-mercaptoethanol are rarely used in fish cell culture, similar favorable effects have been observed in various mammalian cell cultures and hence they are commonly added to the culture medium (Van Der Valk et al. 2010). Forskolin is considered capable of altering the intracellular signal transduction pathways to enhance the intracellular cAMP levels (Seamon and Daly 1981), thereby promoting the cell proliferation and protecting the survival of the cells. Favorable effects of β-mercaptoethanol supplementation might be resulted from decreasing the oxidative stress (Martindale and Holbrook 2002) and/or providing cysteine to the cells as described previously (Zmuda and Friedenson 1983).

Although the RMT1 and RMO1 cells showed fibroblast- and epithelial-like morphology, respectively, cell shape cannot predict whether cells are from fibroblast or epithelial origin in fish cell culture (Mauger et al. 2009). Despite some differences between fish species, presence of vimentin together with the absence of cytokeratin may indicate the mesenchymal origin of cells (Thompson et al. 1987;

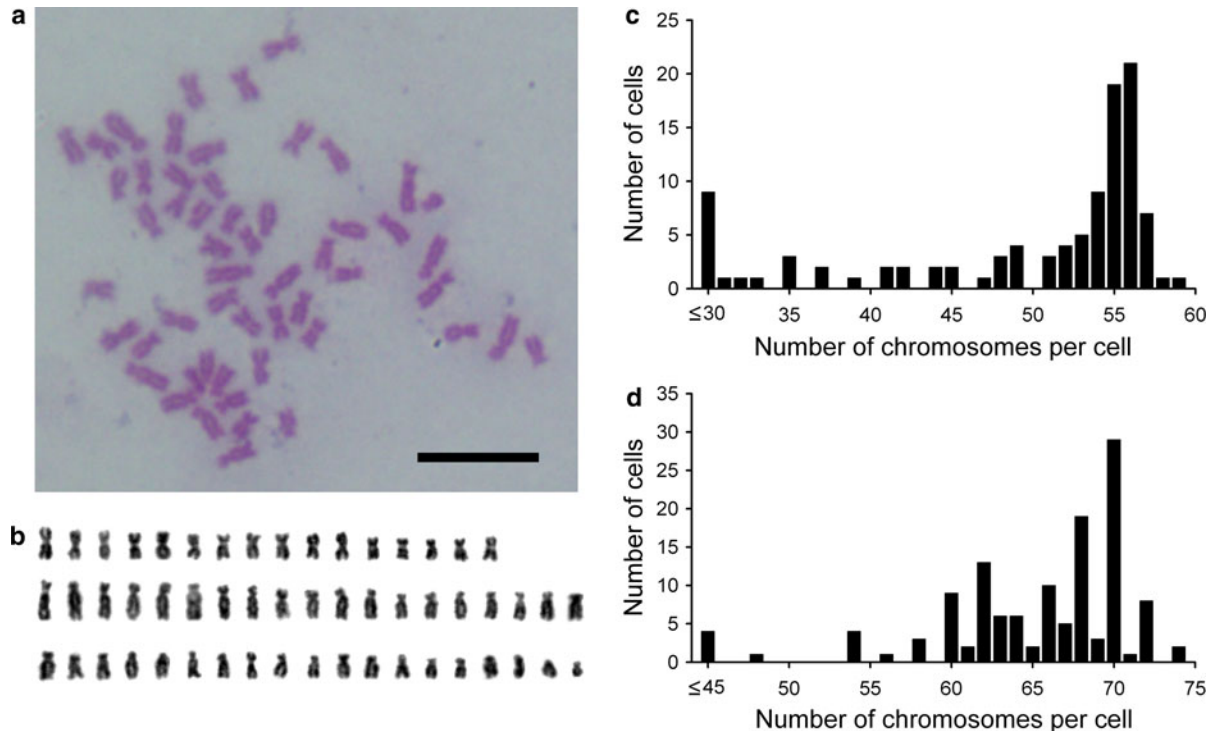

Fig. 5 Chromosome analysis of testicular (RMT1) and ovarian (RMO1) cell lines of *Gnathopogon caerulescens.* Metaphase chromosomes were micrographed after Giemsa staining (**a**) and karyotyped (**b**). *Bar* in **a** = 20 μm (magnification: ×1,000) shown is an example of a metaphase plate of a RMT1 cell with hyperploid (54) chromosomes. Chromosome number distributions of RMT1 (**c**) and RMO1 (**d**) were analyzed at passage 45. In total, 104 and 128 metaphase of RMT1 and RMO1 cells were counted, respectively

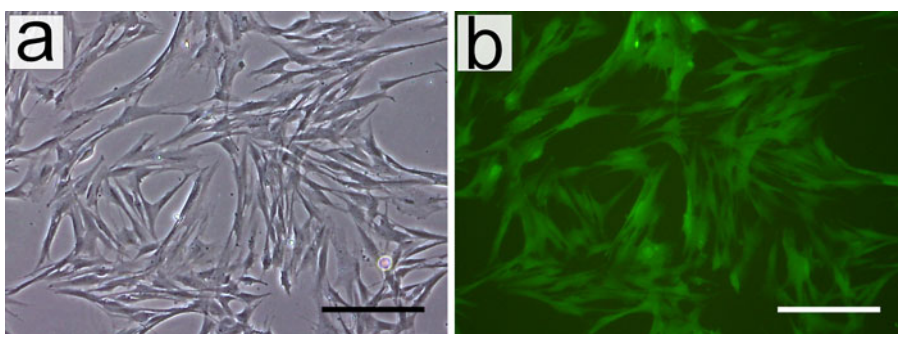

Fig. 6 Testicular cell line (RMT1) of *Gnathopogon caerulescens* transfected with pCE-EGFP plasmid. Phase contrast (**a**) and fluorescent (**b**) images of pCE-EGFP-transfected RMT1 cells were taken after 10 days of selection in neomycin. *Bars* = 100 μm (magnification: ×200)

Bunton 1995). Accordingly, the result of immunocytochemical staining was consistent with the fibroblastic morphology in RMT1 cells. On the other hand, RMO1 cells appeared to be mesenchymal origin, in spite of their epithelial-like morphology.

Due to limited available information about the gene expression patterns in *G. caerulescens* gonads, we used those of other teleost fish species as references. RMT1 cells expressed Sertoli marker genes *Sox9a* (Chiang et al. 2001; Sakai 2002; Kobayashi et al. 2008) and *WT1a* (Mohapatra et al. 2011) but not Leydig marker *Cyp11b* (Wang and Orban 2007) and germ cell marker genes such as *Dmrt1* (Guo et al. 2005), *Vasa* (Kobayashi et al. 2002), and *Ziwi1*

(Houwing et al. 2007). Hence, RMT1 cells might mainly originate from testicular Sertoli cells.

Of interest, RMO1 cells also express *Sox9a* and *WT1a* and expression of these genes were observed in ovary. Although these genes were used as a Sertoli cell marker mentioned above, it has been reported that ovarian gonadal epithelium and theca cells also express *Sox9a* (Zhou et al. 2003) and *WT1a* (Mohapatra et al. 2011) in other fish species, respectively, while no expression of granulosa cell marker *Cyp19a1* (Rodríguez-Marí et al. 2005) and germ cell markers (*Dmrt1*, *Vasa,* and *Ziwi1*) was detected. These results, taken together with the immunocytochemical staining, suggest that RMO1 was mainly derived from the ovarian theca cells.

Both RMT1 and RMO1 cells showed active growth at 28 °C but not at 18 °C, though the water temperature was reported to be 12–14 °C at the beginning and 25–27 °C at the end of the spawning season of *G. caerulescens* (Nakamura 1949). This might indicate that the cell lines were selected, or adapted, to the relatively higher incubation temperature through the establishment and/or subculture at 28 °C as suggested previously (Wolf and Ahne 1982). When the cells were cultured at 28 °C, both cell lines required FBS supplementation in the medium for proliferation. This demonstrated that FBS plays an important role in the proliferation of the present cell lines as generally suggested in the fish cell culture (Bols and Lee 1991).

Within the tested conditions, the highest growth rates of the RMT1 and RMO1 cells were obtained from the cells cultured in the subculture medium supplemented with 10 % FBS at 28 °C. Both of the RMT1 and RMO1 cells exhibited maximum growth rate during 24–48 h of culture and the minimum doubling times were 24.4 and 28.8 h, respectively. These population doubling times were shorter than that of testicular (Ku and Chen 1992; Zhang et al. 2011) and ovarian (Li and Stewart 1965) cell lines of other fish species. This difference could be due to species variance but is more likely related to additives such as EGF, bFGF, forskolin, and β-mercaptoethanol which were not used in these previous studies.

The diploid chromosome number for *G. caerulescens* has been determined as 50 (Ueno et al. 1992). Most of the RMT1 and RMO1 cells had a hyperploid set of chromosomes (67.3 %: 70/104 and 96.1 %: 123/128, respectively) and the cells with normal diploid chromosome set were not observed, suggesting RMT1 and RMO1 cells had low genetic stability. Aneuploidy was commonly observed in fish cell lines and the cells with normal diploid chromosomal number were absent in several fish testicular (Ku and Chen 1992) and ovarian (Bowser and Plumb 1980; Lannan et al. 1984) cell lines. The cause of such high aneuploidy rate in the present study, in both cell lines, remains to be unclear, but may occur as a consequence of chromosome disjoining during cell division.

To examine the feasibility of using RMT1 cells as an in vitro tool such as biosensor by means of gene transfer, transfection with pCE-EGFP plasmid using a commercial lipofection kit, which adapted to the mammalian cells, was performed. In this experiment, RMT1 cell line expressed EGFP efficiently, which implies lipofection is feasible and human elongation factor 1 α promoter efficiently drive reporter genes in fish cell line. We also confirmed that chicken β-actin/rabbit β-globin hybrid promoter (pCAGGS: Niwa et al. 1991) works well in this cell line (data not shown). Although successful GFP transfection was reported in several fish cell lines (Ku et al. 2009; Parameswaran et al. 2007; Parameswaran et al. 2006; Qin et al. 2006; Sun et al. 2011), this is the first testicular cell line which was successfully transfected with the foreign DNA in fish. Our results suggest that gene transfer can be performed like mammalian cells and mammalian ubiquitous promoter works in RMT1 cells.

In conclusion, endemic fish cell lines from testicular and ovarian tissues of *G. caerulescens* were successfully established and designated as RMT1 and RMO1, respectively. Gene expression patterns and immunocytochemical staining of RMT1 and RMO1 cells revealed that these cell lines were originated from testicular Sertoli and ovarian theca cells, respectively. Conventional transfection procedure and ubiquitous promoter adapted to the mammalian cells can be applied to RMT1 cells. This indicated that this cell line could be easily modified by gene transfer and utilized as an in vitro system for the evaluation of direct effect of environmental chemicals on gonadal cells of endemic species and provide an useful biosensors to replace whole animals.

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References

Bols NC, Lee LEJ (1991) Technology and uses of cell cultures from the tissues and organs of bony fish. Cytotechnology 6:163–187. doi:10.1007/BF00624756

Bowser PR, Plumb JA (1980) Fish cell lines: establishment of a line from ovaries of channel catfish. In Vitro Cell Dev Biol 16:365–368. doi:10.1007/BF02618357

Bunton TE (1995) Development of an aquatic bioassay using the medaka (*Oryzias latipes*) to assess human health risk: tumor immunodiagnosis. The defense technical information center (DTIC). http://www.dtic.mil/cgi-bin/GetTRDoc?Location=U2&doc=GetTRDoc.pdf&AD=ADA289837. Accessed 12 Sep 2012

Chiang EFL, Pai CI, Wyatt M, Yan YL, Postlethwait J, Chung B (2001) Two *sox9* genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. Dev Biol 231:149–163. doi:10.1006/dbio.2000.0129

Fraser C, Hall M (1999) Studies on primary cell cultures derived from ovarian tissue of *Penaeus monodon*. Methods Cell Sci 21:213–218. doi:10.1023/A:1009851809288

Fryer J, Lannan C (1994) Three decades of fish cell culture: a current listing of cell lines derived from fishes. Methods Cell Sci 16:87–94. doi:10.1007/BF01404816

Guo Y, Cheng H, Huang X, Gao S, Yu H, Zhou R (2005) Gene structure, multiple alternative splicing, and expression in gonads of zebrafish *Dmrt1*. Biochem Biophys Res Commun 330:950–957. doi:10.1016/j.bbrc.2005.03.066

Hotchkiss AK, Rider CV, Blystone CR, Wilson VS, Hartig PC, Ankley GT, Foster PM, Gray CL, Gray LE (2008) Fifteen years after "wingspread"—environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. Toxicol Sci 105:235–259. doi:10.1093/toxsci/kfn030

Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov DV, Blaser H, Raz E, Moens CB (2007) A role for *Piwi* and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. Cell 129:69–82. doi:10.1016/j.cell.2007.03.026

Kawasaki T, Saito K, Sakai C, Shinya M, Sakai N (2012) Production of zebrafish offspring from cultured spermatogonial stem cells. Genes Cells 17:316–325. doi:10.1111/j.1365-2443.2012.01589.x

Kobayashi T, Kajiura-Kobayashi H, Nagahama Y (2002) Two isoforms of *vasa* homologs in a teleost fish: their differential expression during germ cell differentiation. Mech Dev 111:167–171. doi:10.1016/S0925-4773(01)00613-X

Kobayashi T, Kajiura-Kobayashi H, Guan G, Nagahama Y (2008) Sexual dimorphic expression of *DMRT1* and *Sox9a* during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (*Oreochromis niloticus*). Dev Dyn 237:297–306. doi:10.1002/dvdy.21409

Ku C, Chen SN (1992) Characterization of three cell lines derived from color carp *Cyprinus caprio*. J Tiss Cult Meth 14:63–72. doi:10.1007/BF01404746

Ku C, Teng YC, Wang CS, Lu C (2009) Establishment and characterization of three cell lines derived from the rockfish grouper *Epinephelus quoyanus*: use for transgenic studies and cytotoxicity testing. Aquaculture 294:147–151. doi:10.1016/j.aquaculture.2009.05.010

Lakra W, Swaminathan TR, Joy K (2011) Development, characterization, conservation and storage of fish cell lines: a review. Fish Physiol Biochem 37:1–20. doi:10.1007/s10695-010-9411-x

Lannan CN, Winton JR, Fryer J (1984) Fish cell lines: establishment and characterization of nine cell lines from salmonids. In Vitro Cell Dev Biol 20:671–676. doi:10.1007/BF02618871

Li M, Stewart JE (1965) A quantitative study of the effects of naturally occurring supplements on the growth of rainbow trout (*Salmo gairdneri*) gonadal cells. Can J Microbiol 11:9–14. doi:10.1139/m65-002

Martindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192:1–15. doi:10.1002/jcp.10119

Mauger PE, Labbé C, Bobe J, Cauty C, Leguen L, Baffet G, Le Bail PY (2009) Characterization of goldfish fin cells in culture: some evidence of an epithelial cell profile. Comp Biochem Physiol B Biochem Mol Biol 152:205–215. doi:10.1016/j.cbpb.2008.11.003

Mohapatra S, Liu Z, Zhou L, Zhang Y, Wang D (2011) Molecular cloning of *Wt1a* and *Wt1b* and their possible involvement in fish sex determination and differentiation. In: Joy KP, Inbaraj RM, Kirubagaran R, Chaube R (eds) Proceedings of 9th international symposium on reproductive physiology of fish, 9–14 Aug Cochin, India, pp 85–86

Nakamura M (1949) The life history of a cyprinid fish, *Gnathopogon elongatus caerulescens* (*Sauvage*) in Lake Biwa. Nippon Suisan Gakk 15:88–96

Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199. doi:10.1016/0378-1119(91)90434-D

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

Parameswaran V, Ishaq Ahmed V, Shukla R, Bhonde R, Sahul Hameed A (2007) Development and characterization of two new cell lines from milkfish (*Chanos chanos*) and grouper (*Epinephelus coioides*) for virus isolation. Mar Biotechnol 9:281–291. doi:10.1007/s10126-006-6110-9

Qin Q, Wu T, Jia T, Hegde A, Zhang R (2006) Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus. J Virol Methods 131:58–64. doi:10.1016/j.jviromet.2005.07.009

Rodríguez-Marí A, Yan YL, BreMiller RA, Wilson C, Cañestro C, Postlethwait JH (2005) Characterization and expression pattern of zebrafish *Anti-Müllerian hormone (Amh)* relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad development. Gene Expr Patterns 5:655–667. doi:10.1016/j.modgep.2005.02.008

Sakai N (2002) Transmeiotic differentiation of zebrafish germ cells into functional sperm in culture. Development 129: 3359–3365

Sakai N (2006) In vitro male germ cell cultures of zebrafish. Methods 39:239–245. doi:10.1016/j.ymeth.2005.12.008

Scholz S, Klüver N (2009) Effects of endocrine disrupters on sexual, gonadal development in fish. Sex Dev 3:136–151. doi:10.1159/000223078

Seamon K, Daly J (1981) Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J Cyclic Nucl Res 7: 201–224

Song M, Gutzeit HO (2003) Primary culture of medaka (*Oryzias latipes*) testis: a test system for the analysis of cell proliferation and differentiation. Cell Tissue Res 313:107–115. doi:10.1007/s00441-003-0729-x

Sun A, Fan T, Yang X, Xu B, Xu X, Guo X (2011) Establishment of a spleen cell line from large yellow croaker *Pseudosciaena crocea* and its primitive application in foreign gene transfection. J Ocean Univ China 10:397–401. doi:10.1007/s11802-011-1778-5

Takada T, Iida K, Awaji T, Itoh K, Takahashi R, Shibui A, Yoshida K, Sugano S, Tsujimoto G (1997) Selective production of transgenic mice using green fluorescent protein as a marker. Nat Biotechnol 15:458–461. doi:10.1038/nbt0597-458

Thompson JS, Virtanen I, Lehto VP (1987) Intermediate filaments in normal tissues and lymphomas of northern pike, *Esox lucius* L., from the Åland Islands of Finland. J Comp Pathol 97:257–266. doi:10.1016/0021-9975(87)90089-2

Tsuda T, Takino A, Kojima M, Harada H, Muraki K, Tsuji M (2000) 4-Nonylphenols and 4-tert-octylphenol in water and fish from rivers flowing into Lake Biwa. Chemosphere 41:757–762. doi:10.1016/S0045-6535(99)00465-8

Ueno K, Ye Y, Umeoka T (1992) A comparative study of chromosomes in the cyprinid fish genera *Gnathopogon* and *Squalidus* of Japan. Nippon Suisan Gakk 58:1273–1277

Van Der Valk J, Brunner D, De Smet K, Fex Svenningsen Å, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino M (2010) Optimization of chemically defined cell culture media—replacing fetal bovine serum in mammalian in vitro methods. Toxicol In Vitro 24:1053–1063. doi:10.1016/j.tiv.2010.03.016

Wang X, Orban L (2007) Anti-Müllerian hormone and 11 β-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. Dev Dyn 236:1329–1338. doi:10.1002/dvdy.21129

Westerfield M (2007) Recipes-embryo extract. In: Westerfield M (ed) The zebrafish book, 5th edn. The University of Oregon Press, Eugene, p 10.4

Wolf K, Ahne W (1982) Fish cell culture. In: Maramorosch K (ed) Advances in cell culture, vol. 2. Academic Press, New York, pp 305–328

Wolf K, Mann JA (1980) Poikilotherm vertebrate cell lines and viruses: a current listing for fishes. In Vitro Cell Dev Biol 16:168–179. doi:10.1007/BF02831507

Zhang B, Wang X, Sha Z, Yang C, Liu S, Wang N, Chen SL (2011) Establishment and characterization of a testicular cell line from the half-smooth tongue sole, *Cynoglossus semilaevis*. Int J Biol Sci 7:452–459. doi:10.7150/ijbs.7.452

Zhou R, Liu L, Guo Y, Yu H, Cheng H, Huang X, Tiersch TR, Berta P (2003) Similar gene structure of two *Sox9a* genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (*Monopterus albus*). Mol Reprod Dev 66:211–217. doi:10.1002/mrd.10271

Zmuda J, Friedenson B (1983) Changes in intracellular glutathione levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. J Immunol 130:362–364