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Establishment and characterization of a gonad cell line from half-smooth tongue sole *Cynoglossus semilaevis* pseudomale

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Abstract A new cell line was established from half-smooth tongue sole *Cynoglossus semilaevis* pseudomale gonad (CSPMG). Primary culture was initiated from gonad tissues pieces, and the CSPMG cells were cultured at 24 °C in Dulbecco's modified Eagle medium/F12 medium (1:1) (pH7.0), supplemented with 20 % fetal bovine serum, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor-I, 2-mercaptoethanol, penicillin and streptomycin. The cultured CSPMG cells, in fibroblast shape, proliferated to 100 % confluency 10 days later and had been subcultured to passage 109. Chromosome analyses indicated that the CSPMG cells exhibited chromosomal aneuploidy with a modal chromosome number of 42, which displayed the normal diploid karyotype of half-smooth tongue sole ($2n = 42t$, NF = 42). Reverse transcription polymerase chain reaction revealed CSPMG cells could express gonad somatic cell functional genes *Sox9a, Wt1a* and weakly germ cell marker gene *Vasa*, but not male specific gene *Dmrt1*. Transfection experiment demonstrated that CSPMG cells transfected with pEGFP-N3 plasmid and small RNA could express green and red fluorescence signals with high transfection efficiency. In conclusion, a continuous CSPMG cell line has been established successfully. The cell line might serve as a valuable tool for studies on the mechanism of sex determination, sex reversal and gonad development in flatfish.

Keywords *Cynoglossus semilaevis* · Flat fish · Pseudomale · Sertoli cells · GFP reporter gene transfection · RNA transfection

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Introduction

Generally, the sex of genetic sex determination species remain fixed for life, but in some genetic sex determination species, the primary sex can be altered during development and will develop into the opposite sex with their genotype changeless. This phenomenon is known as environmental sex reversal (Stelkens and Wedekind 2010; Shao et al. 2014). Environmental sex reversal mainly happens in hermaphroditic fish such as *Epinephelus akaara, Monopterus albus, Acanthopagrus schlegelii and Lates calcarifer* (Hu et al. 2014). Many factors can trigger sex reversal in fish including abiotic (e.g., temperature, Ph and hormones) and biotic (e.g., crowding and pathogens) factors, of which temperature is the most broadly studied (Kato et al. 2011; Devlin and Nagahama 2002). For example, *Oreochromis niloticus* (Linnaeus 1758) sex change is related to the water temperature (Green and Teichert-Coddington 1993). Currently, sex reversal also can be artificially induced which has been widely used in aquaculture by exposure

of fish to exogenous steroids or different rearing temperatures in order to produce the preferred sex (Stelkens and Wedekind 2010; Shao et al. 2014). When the fish are treated with 17α-methyltestosterone, the gonads can revert from ovaries to testes (Basavaraja et al. 1990; Smith and Phelps 2001; Bhandari et al. 2006; De Bock and Greco 2010; Chakraborty et al. 2011).

Half-smooth tongue sole *Cynoglossus semilaevis* (Günther 1873)is an important cultured marine fish in China in recent years. This species employs a female heterogametic sex determination system (ZW/ZZ) and has clear sexual dimorphism, with females growing much faster and reaching final body sizes that are 2–4 times those of males (Zhuang et al. 2006; Chen et al. 2009; Shao et al. 2014). And more economic benefit will be brought if the ratio of female half-smooth tongue sole can be increased. Half-smooth tongue sole natural sex reversal ration is about 14 % when they are reared under normal conditions (22 °C). But when they are treated with relatively high temperatures (28 °C) during a sensitive developmental period early in life, "pseudomales" ratio of half-smooth tongue sole can reach to 73 %. Furthermore, the offspring of these "pseudomales" and normal females exhibit an extremely high sex reversal rate (∼94 %), even when reared in normal conditions (22 °C) (Chen et al. 2014; Shao et al. 2014). Then, how to explain these phenomenons demanding the knowledge of the sex reversal mechanism of half-smooth tongue sole. And the research to the sex reversal of half-smooth tongue sole also can help to understand the molecular mechanism of sex determination and environmental sex reversal in fishes.

Therefore, establishment of a tongue sole pseudomale gonad cell line will help to study the mechanism of sex determination and environmental sex reversal at cell and molecular levels. The cell line will serve as a model to study the function of sex reversal-related genes and gonad development-related genes by gene knockout, gene over expression and RNA interference technologies. And none of the cell line has been developed from sex reversal fish to date.

Materials and methods

Primary cell culture and subculture

Healthy half-smooth tongue sole (about 250 g) were obtained from the MingBo Fisheries Company in Laizhou, Shandong Province, China. The genotype of selected fish was identified by polymerase chain reaction (PCR) using the CseF382 female-specific molecular marker (Chen et al. 2007). Then, the fish was maintained in aerated sterile seawater containing 1000 IU ml^{-1} penicillin and 1000 μg ml^{-1} streptomycin at 20–22 °C for 24 h.

After euthanized by etherification, half-smooth tongue sole was immersed in 70 % ethanol for 1 min. The gonad tissues were collected aseptically, and one quarter tissues were separated for preparing tissue slice. The remaining gonad tissues were washed for three times with phosphate-buffered saline (PBS) and minced into small pieces (about 1 mm^3 in size) by surgical scissors in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12, 1:1) medium (Gibco) (pH 7.2) containing 5 % fetal bovine serum (FBS) (Gibco). Tissue pieces were washed again with PBS repeatedly and suspended in DMEM/F12 medium without FBS, then seeded into cell culture flask. After 6 h, 1 ml of DMEM/F12 complete medium supplemented with 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 10 ng ml^{-1} basic fibroblast growth factor (bFGF, Gibco), 40 ng ml^{-1} epidermal growth factor (EGF, Gibco), 20 ng ml^{-1} insulin-like growth factor-I (IGF-I, Gibco) and 50 mM 2-mercaptoethanol and added 2-ml complete medium mentioned above 2 days later.

The flask was incubated at 24 °C, and 2-ml old medium was replaced with fresh medium every 3 days. Ten days later, cells migrated and grew into a confluent monolayer and were subcultured by trypsinization with 0.25 % trypsin–EDTA solution (Sigma) at a split ratio of 1:2. Cells grew into confluent monolayer again after 3 days. After passage 15, the medium was changed from 20 % FBS-DMEM/F12 into 10 % FBS-DMEM/F12 (Invitrogen) without any supplements, and the cells were subcultured every 3 days. To date, half-smooth tongue sole *Cynoglossus semilaevis* pseudomale gonad (CSPMG) cell line has been subcultured for more than 109 passages.

Growth properties

Pseudomale gonad cells at passage 20 were trypsinized and resuspended in 20 % FBS-DMEM/F12 medium as described above. About 1 ml of CSPMG cell suspension with a density of 1.0×10^5 cells ml^{-1} was dispensed into each well of two 24-well plates

(Corning) and incubated at 24 °C in a 5 % CO_2 incubator (Heraeus). Three wells of CSPMG cells were harvested by trypsinization and resuspended in 1 ml PBS at 12-h intervals. The number of cells in each well was counted with a hemocytometer, and the average value of 3 wells at each time was used to plot the growth curve. The population doubling time of the cells was calculated.

Chromosome analysis

The CSPMG cells at passage 40 at the logarithmic phase were treated with 10 μg ml^{-1} of colchicine (Fluka) for 4 h at 24 °C. The cells were harvested by trypsinization and resuspended in 3 ml 0.075 M KCl hypotonic solution for 30 min, prefixed with cold Carnoy's solution (3:1, methanol: glacial acetic acid) for 5 min and then fixed with cold Carnoy's solution for 20 min. After centrifugation, cells were resuspended with 0.5 ml Carnoy's solution, and dropped on cold glass slides, air-drying, then stained with 10 % Giemsa (Gibco) for 20 min. The chromosomes were observed and photographed under a Nikon Eclipse 80*i* fluorescence microscope, and 300 metaphase CSPMG cells were counted.

Storage in liquid nitrogen

For cryopreservation, CSPMG cells at the logarithmic phase were harvested by trypsinization, centrifuged at 180 g for 10 min and resuspended in storage protective liquid containing 80 % 20 %FBS-DMEM/F12 complete medium supplemented and 20 % dimethyl sulphoxide (DMSO) (Amresco). The cell suspensions with a density of 6–7 × 10^6 cells ml^{-1} were transferred into sterile plastic freeze tubes (Corning). Then, the tubes were kept in a Nalgeme Mr. Frosty Freezing Containers (Thermo) and −80 °C overnight and finally transferred into liquid nitrogen (−196 °C).

The CSPMG cells at passage 15 frozen for 60 days were thawed by immediately incubating the freeze tube into a 42 °C water bath for about 5 min to recover to the optimal temperature. After centrifugation at 180 g for 10 min, the cells were suspended in 20 % FBS- DMEM/F12 medium and incubated into 25-cm^2 cell culture flasks at 24 °C. In addition, the thawed CSPMG cells from another freeze tube were stained with 0.4 % Trypan blue. The living and dead cells were counted separately with a hemocytometer under a Nikon ECLIPSE TE2000-U fluorescence microscope, and the cell viability was calculated.

Phenotypic sex identification

Tissues collected from the gonad for primary culture were fixed in Bouin's solution for 12 h. After dehydration of gradient ethanol, transparency of dimethylbenzene, paraffin embedding, the tissues were cut into slices which were 5–6 μm thick. Then, the sections were HE stained and observed under a Nikon Eclipse 80*i* fluorescence microscope to assess phenotypic sex of the tongue sole.

Genetic sex identification

The genetic sex of CSPMG cells was also identified by a female-specific molecular marker CseF382 same with that in 2.1. A pair of PCR primers was synthesized according to the sequences of CseF382 (Table 1). A fragment of 270 bp should be amplified from the half-smooth tongue sole pseudomale genome. DNA was extracted from male, female and pseudomale soles as well as from CSPMG cells at passage 20. PCR was performed in a 20 μl volume with 2.0 μl 10× PCR buffer, 0.5 μl Taq DNA polymerase (5U/μl), 0.5 μL each primer, 1.5 μl dNTP mixture, 1 μl template DNA and 14 μl sterilized water. The condition of the PCR was as follows: predenatured at 94 °C (5 min), 33 cycles of denatured at 94 °C (30 s), annealing at 57 °C (30 s) and extension at 72 °C (1 min).

Gene expression analysis

To analyze the gene expression pattern of CSPMG cell line, the gonadal somatic cell marker genes (*P450arom, Foxl2, Wt1a and Sox9a*) and germ cell marker genes (*Vasa, Dmrt1*) were examined in CSPMG cells by reverse transcription polymerase chain reactions (RT-PCR). And the expression of these genes in pseudomale gonad tissues was also detected. The *β-actin* gene was used as an internal control for RT-PCR.

Total RNA was extracted from CSPMG cells at passage 40 and pseudomale gonad tissues using TRIzol Reagent (Ambion by life technology, USA) according to the manufacturer's instructions. The DNase treatment of the samples and reverse transcription were

Table 1 Primers used for polymerase chain reactions (PCR)

Gene	Primer sequence (5′–3′)	Annealing temperature (°C)
CseF-F	ATTCACTGACCCCTGAGAGC	57
CseF-R	TGGCACCATCATTGTAAAACTA	
Vasa-F	CTTGGCTGTCGGAATAGTGGGTG	57
Vasa-R	CATACTCATCAATGCTGCCTGGG	
P450a-F	ACGGGCTGAAATCGCAAG	60
P450a-R	GGTGAGGATGTGACCCAGTGT	
Foxl2-F	TGGTTGGAAGTGCGTGGG	60
Foxl2-R	GAGAGGAAGGGCAACTACTGGA	
Sox9a-F	CAGGCAGGTAATGTTGGGGT	60
Sox9a-R	AAGGAGCCGTAGGTGATGTG	
Wt1a-F	ACCGCCGTTTCCCCTTAC	60
Wt1a-R	GGGCTGGTGGTGATGTGC	
Dmrt1-F	AGGAGGAAGAACTTGGGATTTGT	60
Dmrt1-R	ACGAGATGGTTGGTAGATGTTGTAA	
β-actin-F	CCAACAGGGAAAAGATGACC	60
β-actin-R	TTCTCCTTGATGTCACGCAC	

carried out with PrimeScript RT reagent Kit (Takara, Japan) using 800 ng total RNA. Then, the genes as mentioned above were amplified using primers in Table 1. PCR was performed using TaKaRa Recombinant Taq DNA polymerase according to the manufacturer's instructions. The condition of the PCR was as follows: pre-denatured at 94 °C (5 min), 33 cycles of denatured at 94 °C (30 s), annealing at 57 °C and 60 °C, respectively (30 s), and extension at 72 °C (1 min). The RT-PCR products were separated by 1 % agarose gel electrophoresis, and the gel was stained with GelRed^{TM} (Biotium, CA).

Transfection with PEGFP-N3 plasmid

The pEGFP-N3 plasmid containing a cytomegalovirus (CMV) promoter, a SV40 polyadenylation signal and a neomycin-resistant gene was conserved in our laboratory. The CSPMG cells at passage 60 in 20 % FBS-DMEM/F12 medium were seeded at a density of 5×10^6 cells ml^{-1} into a 12-well plate, and the growth volume was 1000 μl. After 24 h, the cells were treated by adding a complex of 2.5 μg pEGFP-N3 plasmid and 0.75 μl Clontech xfect transfection reagent (Clontech) diluted separately with 50 μl reaction buffer to each well. After incubated at 24 °C in a 5 % CO_2 incubator for 6 h, the old medium was replaced with new 20 % FBS-DMEM/F12 medium, and cells were cultured at 24°Cin CO_2 incubator. The green fluorescence signals were observed every 12 h under a Nikon ECLIPSE T2000-U fluorescence microscope. The transfection efficiency was evaluated by calculating the ratio of cells expressing green fluorescence signals to all cells employed for transfection.

Transfection with small RNA

A small RNA fragment with Alexa Fluor Cy3 was synthesized randomly. The CSPMG cells at passage 60 in 20 % FBS-DMEM/F12 medium were seeded at a density of 1×10^6 cells ml^{-1} into a 12-well plate, and the growth volume was 1000 μl. After 24 h, the cells were treated by adding a complex of 60 pmol RNA and 8 μl xfect polymer transfection reagent (Clontech) diluted separately with 80 μl reaction buffer to each well. After incubated at 24 °C in a 5 % CO_2 incubator for 6 h, the old medium was replaced with fresh 20 % FBS-DMEM/F12 medium, and cells were cultured at 24 °C in CO_2 incubator. The red fluorescence signals were observed every 12 h under a Nikon ECLIPSE T2000-U fluorescence microscope. The transfection efficiency was evaluated by calculating the ratio of cells expressing red fluorescence signals to all cells employed for transfection.

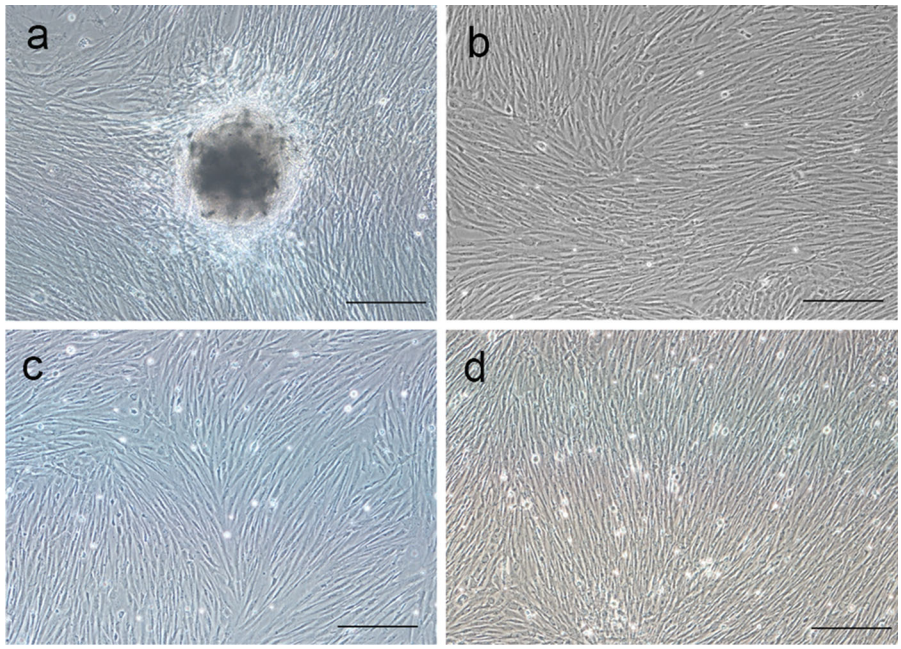

Fig. 1 In vitro cultured half-smooth tongue sole psuedomale gonad (CSPMG) cells. **a** The confluent monolayer formed by the CSPMG cells after primary culture initiation; **b** subcultured CSPMG cells at passage 15; **c** subcultured CSPMG cells at passage 30; **d** subcultured CSPMG cells at passage 60. *Bar* 100 μm

Results

Primary cell culture and subculture

The CSPMG cells from half-smooth tongue sole were fibroblastic morphology, uniform and transparent (Fig. 1b, c) and grew to confluency 10 days later in primary culture (Fig. 1a). The cells were subcultured at 3- to 4-day intervals in 20 % FBS-DMEM/F12 medium supplemented with bFGF, EGF, IGF-I, 2-mercaptoethanol, penicillin and streptomycin. To date, the CSPMG cells have been subcultured to passage 109 and are still in a good proliferating state (Fig. 1d). A continuous half-smooth tongue sole, *Cynoglossus semilaevis* pseudomale gonad cell line (CSPMG), had been established.

Growth properties

The growth curve of CSPMG cells at passage 20 in Fig. 2 showed that the CSPMG cells were at latent stage before day 1.5 and went into logarithmic stage from day 1.5 to day 4. The cell number remained steady between day 4 and day 5.5, but began to decline after day 5.5. The CSPMG cells grew and proliferated at a steady rate, and their doubling time was calculated to be 45.03 h at passage 20.

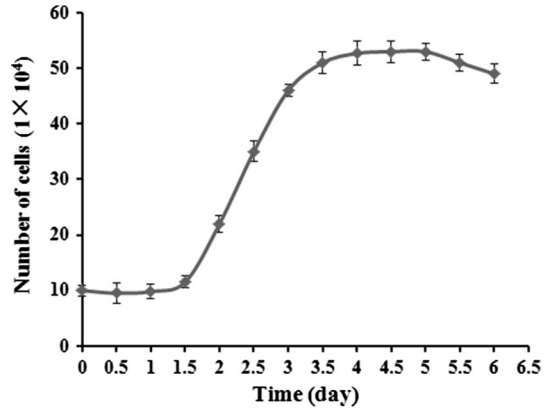

Fig. 2 Growth curve of CSPMG cells at passage 20. The lag phase, logarithmic phase, stationary phase and decline phase are shown

Chromosome analysis

The result of chromosome count of 300 metaphase CSPMG cells at passage 40 revealed that the chromosome numbers varied from 33 to 58 with a modal

chromosome number of 42, which accounted for 50 % of the metaphase cells (Fig. 3a). The distribution was asymmetrical and both aneuploidy and heteroploidy appeared in the CSPMG cell line. The metaphase chromosomes (Fig. 3b) with a normal diploid number of 42 displayed the normal karyotype morphology, consisting of 21 pairs of telocentrics chromosome (*t*): $2n = 42t$, NF = 42 (Fig. 3c) and also exhibited a biggest W sex chromosome belonging to female and pseudomale fish.

Storage in liquid nitrogen

The cryopreserved CSPMG cells at passage 15 proliferated to confluency in 5–6 days after thawed. The morphology and proliferation ability of CSPMG cells were the same with that before cryopreservation. The morphology of thawed cells is shown in Fig. 4, and the cell viability was calculated to be 80 % after Trypan blue staining.

Phenotypic sex identification

Tissue sections and HE staining were made to identify the phenotypic sex of pseudomale gonad tissues which were used for primary culture. The histologic slice results (Fig. 5) displayed that there were a large number of spermatocytes and spermatids in gonad tissues, and the cell morphology was similar with that in male testis, but the cell number is slightly less than that of the normal male. Therefore, it was regarded the phenotypic sex of the gonad tissues was male.

Genetic sex identification

The female-specific molecular marker CseF382 was cloned separately from the DNA of CSPMG cells at passage 20, pseudomale gonad tissues, testis tissues and ovarian tissues by PCR method. As shown in Fig. 6, the molecular marker was detected in CSPMG cells, pseudomale gonad tissues and ovarian tissues, but not detected in testis tissues. Therefore, the genetic sex of CSPMG cell line was considered to be female.

Gene expression analysis

The expression of germ cell and somatic cell marker genes, *Vasa*, *P450arom*, *Foxl2*, *Wt1a*, *Sox9a* and *Dmrt1* in CSPMG cell line was analyzed at passage 40. As shown in Fig. 7, *Sox9a* expressed strongly both in pseudomale gonad tissues and CSPMG cell line; *Wt1a* also expressed in both in pseudomale gonad tissues and

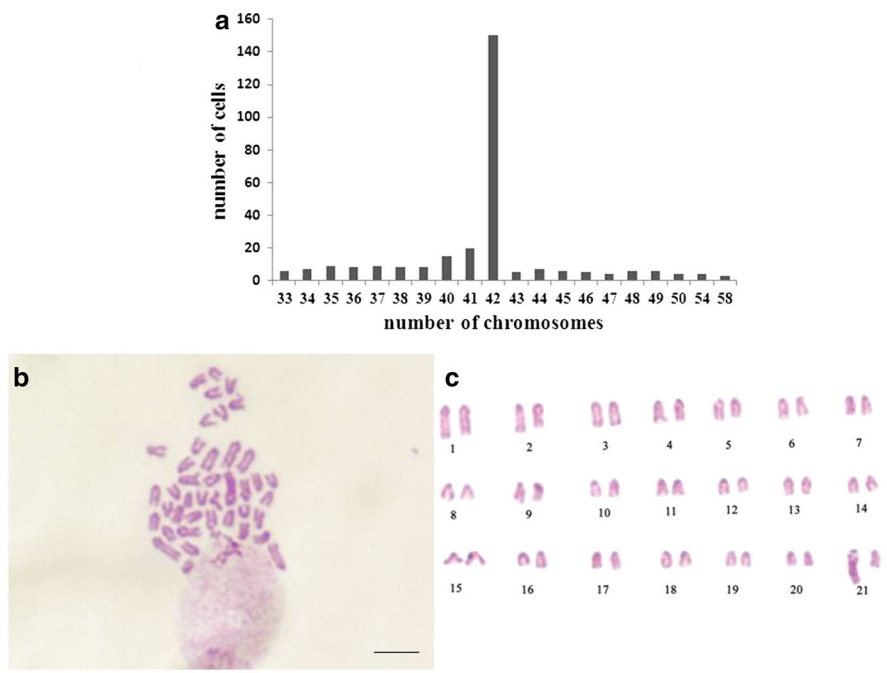

Fig. 3 Chromosome analysis CSPMG cells at passage 40. **a** Chromosomal aneuploidy of CSPMG cells with chromosome numbers ranging from 33 to 58 and about 50 % of CSPMG cells have a chromosome number of 42; **b** chromosomes from one CSPMG cell with a diploid number of 42 which included a big W chromosome, *bar* 20 μm; **c** the diploid karyotype of CSPMG cells, $2n = 42t$, NF = 42

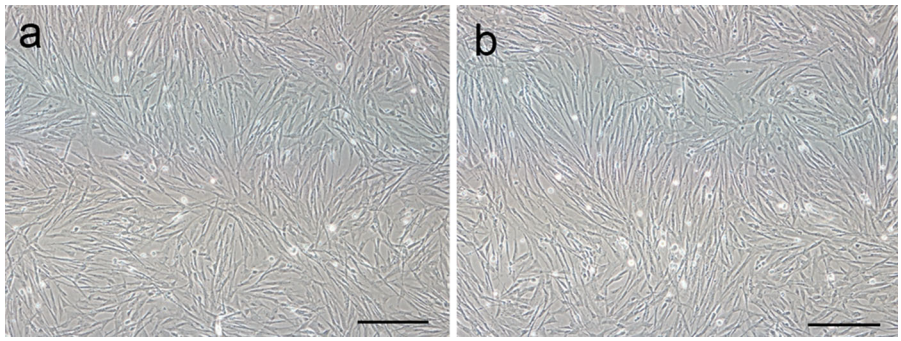

Fig. 4 Cryopreserved CSPMG cells at passage 15 were recovered. **a** The monolayer of CSPMG cells before cryopreservation; **b** the monolayer of CSPMG cells thawed 3 month later. The *bar* 100 μm

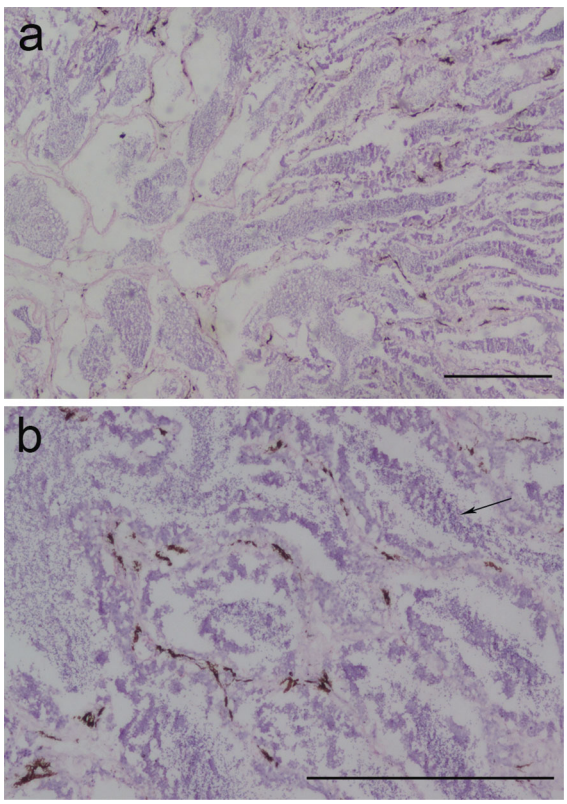

Fig. 5 Phenotypic sex identification. **a** Gonad tissues structure after HE staining; **b** large number of spermatids were observed in gonad tissues. *Arrow* indicated spermatids. *Bar* 100 μm

the CSPMG cell line; *P450arom*, *Dmrt1 and Vasa* expressed in pseudomale gonad tissues but not expressed in the CSPMG cell line; *Foxl2* was not expressed both in pseudomale gonad tissues and CSPMG cell line.

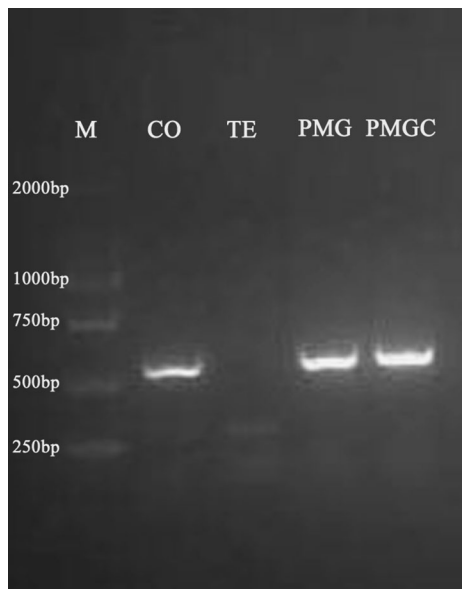

Fig. 6 Genetic sex identificaion. Female-specific molecular marker CseF382 was detected both in OV, PMG and CSPMG cells at passage 20. OV, ovary tissues; TE, testis tissues; PMG, pusedomale gonad tissues; PMGC; and CSPMG cells

CSPMG cells transfection with PEGFP-N3 plasmid

After the CSPMG cells were transfected with pEGFP-N3 plasmid by xfect polymer transfection reagent, green fluorescence signals could be detected 24 h later and reached the maximum at 48 h (Fig. 8). The transfection efficiency was calculated to be about 40 %.

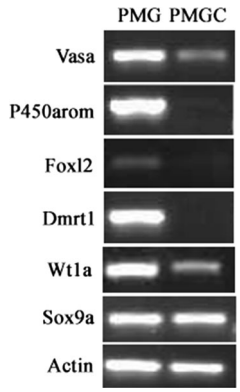

Fig. 7 Gene expression pattern of CSPMG cells at passage 40. CSPMG cell line expressed *Sox9a and Wt1a* strongly, expressed *Vasa* weakly and did not express *Dmrt1*, *P450arom* and *Foxl2*. PMG, pusedomale gonad tissues; PMGC; and CSPMG cells

CSPMG cells transfection with small RNA

After the CSPMG cells were transfected with a small RNA carried Cy3 fluorescent labeling by xfect polymer RNA transfection reagent, red fluorescence signals could be observed 12 h later and reached the maximum at 36 h (Fig. 9). The transfection efficiency was calculated to be above 90 %.

Discussion

For studies of sex determination, sex reversal and gonad development, a continuous CSPMG cell line from half-smooth tongue sole, *Cynoglossus semilaevis,* was established. To date, the cell line had been subcultured to passage 109 and was still in active growth.

To initiate the primary culture of CSPMG cells, half-smooth tongue sole psuedomale gonad tissues pieces were seeded into flask directly without digestion with enzyme trypsin. Similar methods had been reported in the establishment of spleen cell line from large yellow croaker, *Pseudosciaena crocea* (Sun et al. 2011) and testis cell line from half-smooth tongue sole, *Cynoglossus semilaevis* (Zhang et al. 2011)and so on. But different methods were also reported in the establishment of ovarian and testicular cell line from Honmoroko (*Gnathopogon caerulescens*) (Higaki et al. 2013) and ovarian cell line from African catfish (*Clarias gariepinus*) (Sunil Kumar et al. 2001).

To induce in vitro cell proliferation, attempts were made to replenish the culture medium with different supplements. Among them, growth factors such as bFGF, EGF and IGF-I have important regulatory abilities in cell proliferation, migration and differentiation, and similar effects of the supplements on acceleration of cell attachment and growth were reported in the establishment of fin cell line from brown-marbled grouper, *Epinephelus fuscoguttatus* (Wei et al. 2009), kidney cell line from turbot (Wang et al. 2010a), heart cell line (Zhao et al. 2003), spleen cell line (Sun et al. 2011), ovarian and testicular cell lines from Honmoroko (*Gnathopogon caerulescens*) (Higaki et al. 2013), embryonic stem cell line from the sea perch (Chen et al. 2003) and primary culture of embryonic cells from shrimp *Penaeus chinensis,* (Fan and Wang 2002). Addition of these supplements in the culture medium was probably the key factor of inducing cell proliferation in primary culture and successful subculture of CSPMG cells in this study.

The fibroblastic cells in CSPMG cell line proliferated actively during subculture and had a population doubling time of 45.03 h at passage 20. The population doubling time was similar to that of turbot, *Scophthalmus maximus* fin cell line (Fan et al. 2010),

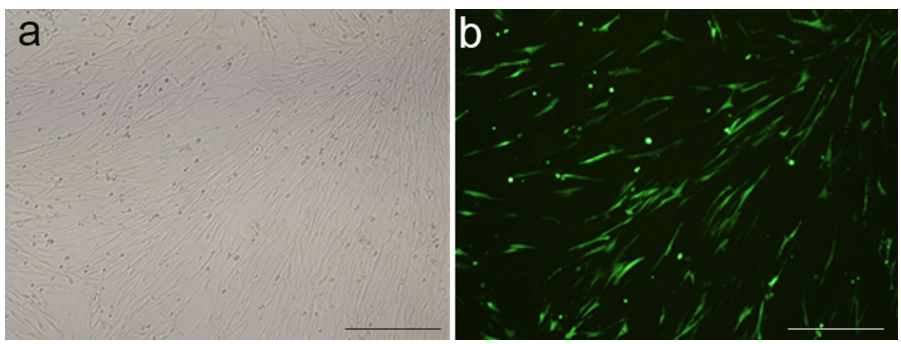

Fig. 8 CSPMG cells transfected with pEGFP-N3 plasmids after 48 h. **a** Optical microscope photographs of CSPMG cells after transfected with pEGFP-N3 plasmids 48 h; **b** the same view of (**a**) under fluorescence view. *Bar* 100 μm

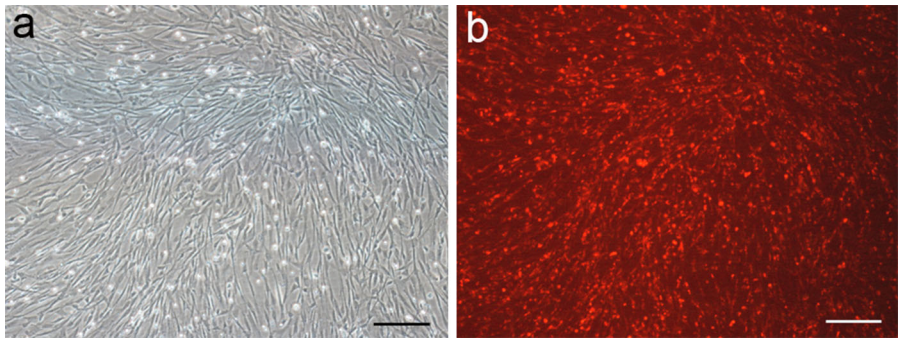

Fig. 9 CSPMG cells transfected with small RNA after 36 h. **a** Optical microscope photographs of CSPMG cells transfected with small RNAs; **b** the same view of (**a**) under fluorescence view. *Bar* 100 μm

higher than that of Honmoroko *Gnathopogon caerulescens* testis, ovary cell line (Higaki et al. 2013), seabream fin cell line (Béjar et al. 1997), brown-marbled grouper *Epinephelus Fuscoguttatus* heart cell line (Wei et al. 2010), and lower than that of grouper fin cell line (Wei et al. 2009), half-smooth tongue sole, *Cynoglossus semilaevis* heart cell line (Wang et al. 2010b), large yellow croaker, *Pseudosciaena crocea* spleen cell line (Sun et al. 2011), sea bass and *Dicentrarchus labrax* neural stem cells (Servili et al. 2009). This implied that the CSPMG cell line still maintained active proliferating ability and could be continuously subcultured.

Chromosome analysis showed that the CSPMG cells at passage 40, exhibiting chromosomal aneuploidy, still had a modal chromosome number of 42. The diploid karyotype of $2n = 42t$ of the CSPMG cells was identical to that of half-smooth tongue sole heart cell (Wang et al. 2010a, b), testis (Zhang et al. 2011) and kidney cells (Zheng et al. 2012) which had been reported. These results indicated that the CSPMG cell line was half-smooth tongue sole origin. The phenotypic sex of the gonad tissue was detected to be male, but genetic sex of CSPMG cell line was identified to be female; therefore, CSPMG cell line was confirmed to be half-smooth tongue sole pseudomale gonad cell line.

The full-length cDNA of *Vasa*, *P450arom*, *Foxl2*, *Dmrt1* and *Sox9a* genes have been cloned from half-smooth tongue sole (Huang et al. 2014; Deng et al. 2009; Dong et al. 2011; Chen et al. 2014). The primers were designed according to the published genes sequences, and the primer of *Wt1a* gene was designed according to the sequence submitted to gene bank (Accession Number: KC261942).

Vasa was the marker gene of germ cell (Kobayashi et al. 2002; Hong et al. 2004) and *P450arom* (Cyp19a1) and *Foxl2* were granulosa cell marker gene (Devlin and Nagahama 2002; Rodríguez-Marí et al. 2005; Ijiri et al. 2008; Yamaguchi et al. 2007). *Sox9a* and *WT1a* were marker genes of sertoli cell (Chiang et al. 2001; Sakai 2002; Kobayashi et al. 2008; She and Yang 2014), but it was reported also expressed in ovarian gonadal epithelium and theca cells (Zhou et al. 2003). And *Dmrt1* was the male germ cell marker gene in zebrafish (Guo et al. 2005).

In this study, it was found that CSPMG cells strongly expressed *Sox9a* gene and *Wt1a* genes, weakly expressed *Vasa* gene, not expressed *P450arom*, *Foxl2* and *Dmrt1* genes compared with pseudomale gonad tissues. Taken together, the CSPMG cell line might be a pseudomale gonad sertoli cell line. The germ cells seemed not to grow and divide in the primary culture period according to the observation to cellular morphology, and the reason might be that they need special medium and additives for division and proliferation in vitro.

It had been a problem that fish cell lines were hard to be transfected by foreign plasmids. The present study demonstrated that the transfection efficiency of CSPMG cell line could reach to 40 % which was higher than reported in other fish cell lines (Chen et al. 2003; Qin et al. 2006; Parameswaran et al. 2006, 2007; Wang et al. 2010a, b; Sun et al. 2011; Zheng et al. 2012). Thus, an effective transfection method for fish cell line was provided by screening right transfection reagent and ratio of transfection reagent to DNA. The transfection with the pEGFP-N3 plasmid via Clontech xfect transfection reagent was feasible and implied

that the CSPMG cell line could be utilized for transgenic and gene targeting research.

Small interference RNA (siRNA) technique was an effective method for studying gene function and cell signal transduction at cell level and was widely used in many research areas. Cell line is the important component of siRNA technology in vitro. Therefore, the siRNA transfection capability of CSPMG cell line was also tested. The results showed CSPMG cell line possessed very high transfection efficiency which would be widely used in the siRNA technology.

In conclusion, a continuous half-smooth tongue sole *Cynoglossus semilaevis* pseudomale gonad cell line, CSPMG, was established. The CSPMG cell line could be used to study functions of important genes took part in sex determination and sex reversal progress of flatfish by gene knockout or gene overexpression methods etc. The CSPMG cell line will also have some potential applications in cell cloning, cell transfer, virus isolation and cell toxicology in half-smooth tongue sole.

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Ethical standard This study was approved by the Animal Care and Use Committee of the Chinese Academy of Fishery Sciences.

References

Basavaraja N, Nandeesha MC, Varghese TJ, Keshavanath P, Srikanth GK (1990) Induction of sex reversal in *Oreochromis mossambicus* by diethylstilbestrol. J Appl Ichthyol 6:46–50

Béjar J, Borrego JJ, Carmen Alvarez M (1997) A continuous cell line from the cultured marine fish gilt-head seabream (*Sparus aurata L.*). Aquaculture 150:143–153

Bhandari RK, Nakamura M, Kobayashi T, Nagahama Y (2006) Suppression of steroidogenic enzyme expression during androgen-induced sex reversal in Nile tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 145:20–24

Chakraborty SB, Banerjee S, Chatterjee S (2011) Increased androgen receptor expression in muscle tissue contributing to growth increase in androgen-treated Nile tilapia. Aquac Int 19:1119–1137

Chen SL, Sha ZX, Ye HQ (2003) Establishment of a pluripotent embryonic cell line from sea perch (*Lateolabrax japonicus*) embryos. Aquaculture 218:141–151

Chen SL, Li J, Deng SP, Tian YS, Wang QY, Zhuang ZM, Sha Z, Xu JY (2007) Isolation of female-specific AFLP markers and molecular identification of genetic sex in half-smooth tongue sole (*Cynoglossus semilaevis*). Mar Biotechnol 9:273–280

Chen SL, Tian YS, Yang JF, Shao CW, Ji XS, Zhai JM, Liao XL, Zhuang ZM, Su PZ, Xu JY, Sha ZX, Wu PF, Wang N (2009) Artificial gynogenesis and sex determination in half-smooth tongue sole (*Cynoglossus semilaevis*). Mar Biotechnol 11:243–251

Chen SL, Zhang GJ, Shao CW, Huang QF, Liu G, Zhang P, Song WT, An N, Chalopin D, Volff JN, Hong YH, Li QY, Sha ZX, Zhou HL, Xie MS, Yu QL, Liu Y, Xiang H, Wang N, Wu K, Yang CG, Zhou Q, Liao XL, Yang LF, Hu QM, Zhang JL, Meng L, Jin LJ, Tian YS, Lian JM, Yang JF, Miao GD, Liu SS, Liang Z, Yan F, Li YZ, Sun B, Zhang H, Zhang J, Zhu Y, Du M, Zhao YW, Schart M, Tang QS, Wang J (2014) Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. Nat Genet 46:253–260

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

De Bock MS, Greco LSL (2010) Sex reversal and growth performance in juvenile females of the freshwater crayfish *Cherax quadricarinatus* (Parastacidae): effect of increasing temperature and androgenic gland extract in the diet. Aquac Int 18:231–243

Deng SP, Chen SLXuJY, Liu BW (2009) Molecular cloning, characterization and expression analysis of gonadal P450aromatase in the half-smooth tongue-sole, *Cynoglossus semilaevis*. Aquaculture 287:211–218

Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genotype, phenotypeal, and environmental influences. Aquaculture 208:191–364

Dong XL, Chen SL, Ji XS, Shao CW (2011) Molecular cloning, characterization and expression analysis of Sox9a and Foxl2 genes in half-smooth tongue sole (*Cynoglossus semilaevis*). Acta Oceanol Sin 30:68–77

Fan TJ, Wang XF (2002) In vitro culture of embryonic cells from shrimp *Penaeus chinensis*. J Exp Mar Biol Ecol 267:175–184

Fan TJ, Ren BX, Geng XF, Yu QT, Wang LY (2010) Establishment of a turbot fin cell line and its susceptibility to turbot reddish body iridovirus. Cytotechnology 62:217–223

Green BW, Teichert-Coddington DR (1993) Production of *Oreochromis niloticus* fry for hormonal sex reversal in relation to water temperature. J Appl Ichthyol 9:230–236

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

Higaki S, Koyama Y, Shirai E, Yokota T, Fujioka Y, Sakai N, Takada T (2013) Establishment of testicular and ovarian cell lines from Honmoroko (*Gnathopogon caerulescens*). Fish Physiol Biochem 39:701–711

Hong YH, Liu TM, Zhao HB, Xu HY, Wang WJ, Liu R, Chen TS, Deng JR, Gui JF (2004) Establishment of a normal

medakafish spermatogonial cell line capable of sperm production in vitro. Proc Natl Acad Sci USA 101: 8011–8016

Hu QM, Chen SL, Gao FT, Li YZ, Liu SS, Liu F, Yang JF, Du M (2014) Differences in sex reversion and growth between normal and neomale stock in half-smooth tongue sole, *Cynoglossus semilaevis*. Aquac Int. doi:10.1007/s10499-014-9757-7

Huang JQ, Chen SL, Liu Y, Shao CW, Lin F, Wang N, Hu QM (2014) Molecular characterization, sexually dimorphic expression, and functional analysis of 3′-untranslated region of vasa gene in half-smooth tongue sole (*Cynoglossus semilaevis*). Theriogenology 82:213–224

Ijiri S, Kaneko H, Kobayashi T, Wang DS, Sakai F, Paul-Prasanth B, Nakamura M, Nagahama Y (2008) Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod 78:333–341

Kato Y, Kobayashi K, Watanabe H, Iguchi T (2011) Environmental sex determination in the branchiopod crustacean Daphnia magna: deep conservation of a Doublesex gene in the sex-determining pathway. PLoS Genet 7:e1001345

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

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

Parameswaran V, Shukla R, Bhonde RR, Sahul Hameed AS (2006) Splenic cell line from sea bass, *Lates calcarifer*: establishment and characterization. Aquaculture 261:43–53

Parameswaran V, Ishaq Ahmed VP, Shukla R, Bhonde RR, Sahul Hameed AS (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

Qin QW, Wu TH, Jia TL, Hegdec A, Zhang RQ (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

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

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

Servili A, Bufalino MR, Nishikawa R, Sanchez de Melo I, Muñoz-Cueto JA, Lee LEJ (2009) Establishment of long term cultures of neural stem cells from adult sea bass, *Dicentrarchus labrax*. Comp Biochem Phys A 152:245–254

Shao CW, Li QY, Chen SL, Zhang P, Lian JM, Hu QM, Sun B, Jin LJ, Liu SS, Wang ZJ, Zhao HM, Jin ZH, Liang Z, Li YZ, Zheng QM, Zhang Y, Wang J, Zhang GJ (2014) Epigenetic modification and inheritance in sexual reversal of fish. Genome Res 24:604–615

She ZY, Yang WX (2014) Molecular mechanisms involved in mammalian primary sex determination. J Mol Endocrinol 53:21–37

Smith ES, Phelps RP (2001) Impact of feed storage conditions on growth and efficacy of sex reversal of Nile tilapia. N Am J Aquac 63:242–245

Stelkens RB, Wedekind C (2010) Environmental sex reversal, Trojan sex genes, and sex ratio adjustment: conditions and population consequences. Mol Ecol 19:627–646

Sun A, Fan TJ, Yang XX, Xu B, Xu XH, Guo XY (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

Sunil Kumar G, Bright Singh IS, Philip R (2001) Development of a cell culture system from the ovarian tissue of African catfish (*Clarias gariepinus*). Aquaculture 194:51–62

Wang N, Wang XL, Sha ZX, Tian YS, Chen SL (2010a) Development and characterization of a new marine fish cell line from turbot (*Scophthalmus maximus*). Fish Physiol Biochem 36:1227–1234

Wang XL, Wang N, Sha ZX, Chen SL (2010b) Establishment, characterization of a new cell line from heart of half smooth tongue sole (*Cynoglossus semilaevis*). Fish Physiol Biochem 36:1181–1189

Wei YB, Fan TJ, Jiang GJ, Sun A, Xu XH, Wang J (2009) Establishment of a novel fin cell line from Brown-marbled grouper, *Epinephelus fuscoguttatus* (Forsskål), and evaluation of its viral susceptibility. Aquac Res 40:1523–1531

Wei YB, Fan TJ, Jiang GJ, Xu XH, Sun A (2010) A novel heart-cell line from brown-marbled grouper *Epinephelus fuscoguttatus* and its susceptibility to iridovirus. J Fish Biol 76:1149–1158

Yamaguchi T, Yamaguchi S, Hirai T, Takeshi K (2007) Follicle stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun 359:935–940

Zhang B, Wang XL, Sha ZX, Yang CG, Liu SS, 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

Zhao ZS, Montgomery-Brock D, Lee CS, Lu YN (2003) Establishment, characterization and viral susceptibility of 3 new cell lines from snakehead, *Channa striatus* (Blooch). Methods Cell Sci 25:155–166

Zheng Y, Wang N, Xie MS, Sha ZX, Chen SL (2012) Establishment and characterization of a new fish cell line from head kidney of half-smooth tongue sole (*Cynoglossus semilaevis*). Fish Physiol Biochem 38:1635–1643

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

Zhuang ZM, Wu D, Zhang SC, Pang QX, Wang CL, Wan RJ (2006) G-banding patterns of the chromosomes of tonguefish *Cynoglossus semilaevis* Günther, 1873. J Appl Ichthyol 22:437–440