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Establishment and characterization of an ovarian cell line from half-smooth tongue sole *Cynoglossus semilaevis*

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A new ovarian cell line, CSO, was established from half-smooth tongue sole *Cynoglossus semilaevis*. Primary culture of CSO cells was initiated from digestion of ovarian tissues pieces by trypsin solution and cultured at 24° C in Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12, 1:1) (pH 7·0), supplemented with 20% foetal bovine serum, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) and human chorionic gonadotropin (HCG). The cultured CSO cells, fibroblastic in morphology, proliferated to 100% confluency 3 days later and had been subcultured to passage 80. Chromosome analyses indicated that the CSO cells exhibited chromosomal aneuploidy with a modal chromosome number of 42, which displayed the normal diploid karyotype of *C. semilaevis* (2n = 42 t, NF = 42). Reverse transcription polymerase chain reaction (RT-PCR) revealed that CSO cells could express ovarian somatic cell functional genes *P450armo*, *Foxl2* and *Sox9a* but not ovary germ cell marker gene *Vasa* and male-specific gene *Dmrt1*. Transfection experiment demonstrated that CSO cells transfected with pEGFP-N3 plasmid could express green fluorescence protein (GFP) with higher transfection efficiency. The CSO cell line might serve as a valuable tool for studies on the mechanism of sex determination and oogenesis of ovary in flatfish.

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Key words: flatfish; gene expression; GFP transfection; karyotype analysis; ovarian granulosa cells.

INTRODUCTION

The main cell types of fish gonad are germ cells and surrounding somatic cells. The major roles of somatic cells are to nourish the developing gonad tissue and to synthesize abundant steroid hormones to regulate either oocyte or spermatocyte development and maturation (Lakra *et al.*, 2011). The interactions between germ cells and adjacent somatic cells may play a role in the development of functional sexes and gonad development in fishes (Devlin & Nagahama, 2002).

In the ovary, thecal layer cells contain all enzymes necessary for the production of testosterone and other precursor androgens such as cytochrome p450 cholesterol-side-chain cleavage enzyme (P450scc), whereas the function of granulosa layer cells is mainly convert testosterone to oestradiol-17h which is necessary for oocyte growth because of the expression of the enzyme aromatase (P450arom) (Nagahama, 1994).

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Gonadal somatic cell lines are of significance to study how the somatic environment controls self-renewal of germ stem cells and their differentiation into gametes, namely oogenesis in the ovary and spermatogenesis in the testis (Hofmann *et al.*, 1994; Feng *et al.*, 2002). The cell lines are also valued tools to study sex determination and differentiation mechanism.

During last 20 years, many teleost cell lines have been developed from a broad range of tissues such as the fin (Béjar *et al.*, 1997, 2005; Imajoh *et al.*, 2007; Wei *et al.*, 2009; Fan *et al.*, 2010; Raja Swaminathan *et al.*, 2010), snout (Zhou *et al.*, 2007), muscle (Zhao *et al.*, 2003), swimbladder (Zhao *et al.*, 2003), heart (Zhao *et al.*, 2003; Williams *et al.*, 2003; Parameswaran *et al.*, 2007; Ku *et al.*, 2009; Wang *et al.*, 2010*b*; Wei *et al.*, 2010), spleen (Qin *et al.*, 2006; Parameswaran *et al.*, 2006; Sun *et al.*, 2011), liver (Williams *et al.*, 2003), kidney (Sahul Hameed *et al.*, 2006; Wang *et al.*, 2010*a*; Zheng *et al.*, 2012), testis (Zhang *et al.*, 2011; Higaki *et al.*, 2013), ovary (Sunil Kumar *et al.*, 2001; Higaki *et al.*, 2013), brain (Wen *et al.*, 2008*a, b*; Ku *et al.*, 2009; Huang *et al.*, 2011) and embryo (Béjar *et al.*, 2002; Chen *et al.*, 2003*a*, *b*, 2004, 2005). One hundred and twenty-four fish cell lines from different fish species have been reported since 1994 and 54 are from marine fishes (Lakra *et al.*, 2011). Interestingly, there are only two ovarian cell lines developed from teleosts in the last 20 years, but both were from freshwater fishes (Sunil Kumar *et al.*, 2001; Higaki *et al.*, 2013).

Half-smooth tongue sole *Cynoglossus semilaevis* Günther 1873 is an important cultured marine fish in China during recent years (Wang *et al.*, 2004). It is also an important model organism that has ZZ/ZW genetic sex determination mechanism (Zhang *et al.*, 2011). Thus, establishment of *C. semilaevis* ovarian cell line will provide a foundation for constructing gene knockout, overexpression and RNA interference models in order to study the function of sex determination- and differentiation-related genes, the interaction mechanism and ovary development mechanism of teleosts.

MATERIALS AND METHODS

PRIMARY CELL CULTURE AND SUBCULTURE

This study was approved by the Animal Care and Use Committee of the Chinese Academy of Fishery Sciences. Healthy *C. semilaevis* (*c.* 250 g) were obtained from the MingBo Fisheries Company. They were maintained in aerated sterile seawater containing 1000 IU ml^{-1} penicillin and 1000 μg ml^{-1} streptomycin at 22–24° C for 24 h.

After being euthanized by etherification, *C. semilaevis* were immersed in 70% ethanol for 1 min. The gonad tissues were collected aseptically, washed three times with phosphate-buffered saline (PBS) and minced into small pieces (*c.* 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 (Invitrogen; www.lifetechnologies.com) (pH 7·2) containing 5% foetal bovine serum (FBS) (Hyclone; www.thermoscientific.com). Tissue pieces were washed again with PBS and then digested with 2 ml of 0·25% trypsin solution (0·25% trypsin and 0·2% EDTA in PBS) for 10 min. Trypsin solution was discarded by centrifugation at 180*g* for 10 min, and the cell pellet was suspended in 1 ml of DMEM/F12 complete medium supplemented with 20% FBS, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 40 ng ml^{-1} epidermal growth factor (EGF), 15 ng ml^{-1} basic fibroblast growth factor (bFGF, Peprotech; www.peprotech.com), 20 ng ml^{-1} insulin-like growth factor-I (IGF-I, Peprotech) and 15 IU ml^{-1} human chorionic gonadotropin (HCG, Sigma-Aldrich; www.sigmaaldrich.com) seeded into six-well cell culture plate.

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The six-well cell culture plate was incubated at 24° C and the medium was supplemented to 2 ml 24 h later. Three days later, cells grew into a confluent monolayer and were subcultured by trypsinization with 0·25% trypsin–EDTA solution (Sigma-Aldrich) at a split ratio of 1:2. Cells grew into confluent monolayer again after 3 days. After passage 20, the medium was changed from 20% FBS-DMEM/F12 to 10% FBS-DMEM/F12 (Invitrogen) without any supplements and the cells were subcultured every 4 days. To date, CSO cell line has been subcultured for more than 70 passages.

GROWTH PROPERTIES

Ovarian cells at passage 60 were trypsinized and resuspended in 20% FBS-DMEM/F12 medium as described above. About 1 ml of CSO cell suspension with a density of $1{\cdot}7 \times 10^5$ cells ml^{-1} was dispensed into each well of two 24-well plates (Corning; www.corning.com) and incubated at 24° C in a 5% CO_2 incubator (Heraeus; www.thermoscientific.com). Three wells of CSO cells were harvested by trypsinization and resuspended in 1 ml of PBS at 12 h intervals. The number of cells in each well was counted with a haemocytometer, and the average value of three wells at each time was used to plot the growth curve. The population doubling time of the cells was calculated.

CHROMOSOME ANALYSIS

The CSO cells at passage 60 at the logarithmic phase were treated with 10 $\mu g\ ml^{-1}$ of colchicine (Fluka; Sigma-Aldrich) for 4 h at 24° C. The cells were harvested by trypsinization and resuspended in 3 ml of 0·075 M KCl hypotonic solution for 30 min, pre-fixed 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 of Carnoy's solution, dropped on cold glass slides, air-dried and then stained with 10% Giemsa for 20 min. The chromosomes were observed and photographed under a Nikon Eclipse 80*i* fluorescence microscope (www.nikoninstruments.com) and 300 metaphase CSO cells were counted.

STORAGE IN LIQUID NITROGEN

For cryopreservation, every five passages of CSO cells at the logarithmic phase were harvested by trypsinization, centrifuged at 1000*g* for 10 min and resuspended in storage protective liquid containing 80% FBS-20% DMEM/F12 complete medium supplemented with 20% dimethyl sulphoxide (DMSO) (Amresco; www.amresco-inc.com). The cell suspensions with a density of $6–7 \times 10^6$ cells ml^{-1} were transferred into sterile plastic freeze-tubes (Corning). Then, the tubes were kept in a Nalgeme Mr Frosty Freezing Containers (Thermo) at −80° C overnight and finally transferred into liquid nitrogen (−196° C).

The CSO cells at passage 8 frozen for 60 days were thawed by immediately incubating the freeze-tube into a 42° C water bath for *c.* 5 min to recover to the optimal temperature. After centrifugation at 1000*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 CSO cells from another freeze-tube were stained with 0·4% trypan blue. The living and dead cells were counted separately with a haemocytometer under a Nikon ECLIPSE TE2000-U fluorescence microscope and the cell viability was calculated.

GENETIC SEX IDENTIFICATION OF CSO CELLS

The genetic sex of CSO cells was identified by a female-specific molecular marker CseF382 (Chen *et al.*, 2007). A pair of polymerase chain reaction (PCR) primers was designed for sex identification according to the sequences of *CseF382* (Table I). A fragment of 270 bp should be amplified from the genome of female *C. semilaevis*. DNA was extracted from male and female *C. semilaevis* as well as from CSO cells at passage 30. PCR was performed in a 20 μl volume with 2·0 μl of 10× PCR buffer, 0·5 μl of Taq DNA polymerase (5 U μl^{-1}), 0·5 μl of each primer,

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Table I. Primers used for reverse transcription polymerase chain reactions (RT-PCRs)

Gene	Primer sequence (5′-3′)	Annealing temperature (° C)
CseF-F	ATTCACTGACCCCTGAGAGC	57
CseF-F	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	
Dmrt1-F	AGGAGGAAGAACTTGGGATTTGT	60
Dmrt1-R	ACGAGATGGTTGGTAGATGTTGTAA	
β-Actin-F	CCAACAGGGAAAAGATGACC	57
β-Actin-R	TTCTCCTTGATGTCACGCAC	

1·5 µl of dNTP mixture, 1 µl of template DNA and 14 µl of sterilized water. The condition of the PCR was as follows: pre-denatured at 94° C (5 min), 33 cycles of denaturation at 94° C (30 s), annealing at 57° C (30s) and extension at 72° C (1 min).

GENE EXPRESSION ANALYSIS

To analyse the gene expression pattern, the gonadal somatic cell marker genes (*P450arom*, *Foxl2*, *Wt1a* and *Sox9a*) and germ cell marker genes (*Vasa* and *Dmrt1*) were examined in CSO cells by reverse transcription PCRs (RT-PCRs), and the expression of these genes in ovarian tissues was also examined. The *β-actin* gene was used as an internal control for RT-PCR.

Total RNA was extracted from CSO cells at passage 35 and ovarian tissues using TRIzol Reagent (Ambion, Life Technologies) according to the manufacturer's instructions. The DNase treatment of the samples and reverse transcription was carried out with PrimeScript RT reagent Kit (Takara Bio; www.takara-bio.com) using 800 ng total RNA. Then, the genes were amplified using primers in Table I as mentioned above. 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 denaturation at 94° C (30 s), annealing at 57 and 60° C (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 (Biotium; www.biotium.com).

TRANSFECTION WITH PEGFP-N3

The pEGFP-N3 plasmid containing a cytomegalo virus (CMV) promoter, an SV40 polyadenylation signal and a neomycin-resistant gene was conserved in the laboratory. The CSO 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 500 µl. After 24 h, the cells were treated by adding a complex of 2·5 µg pEGFP-N3 plasmid and 0·75 µl of Clontech xfect transfection reagent (Clontech; www.clontech.com) diluted separately with 50 µl of reaction buffer to each well. After incubating the cells at 24° C in a 5% CO_2 incubator for 4 h, the old medium was replaced with new 20% FBS-DMEM/F12 medium and cells were cultured at 24° C in 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.

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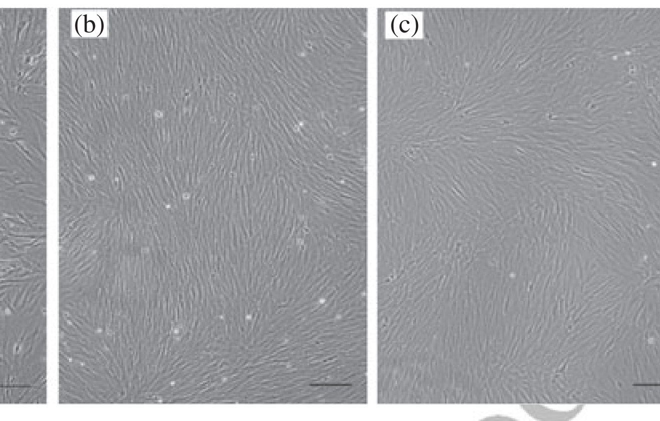

FIG. 1. *In vitro* cultured *Cynoglossus semilaevis* ovarian (CSO) cells. (a) A confluent monolayer formed by the CSO cells after primary culture initiation at 3 days, (b) subcultured CSO cells at passage 20 and (c) subcultured CSO cells at passage 60. Scale bar = 100 μm.

RESULTS

PRIMARY CELL CULTURE AND SUBCULTURE

The CSO cells from *C. semilaevis* were fibroblastic in morphology, uniform and transparent [Fig. 1(b)] and grew to confluency 3 days later in primary culture [Fig. 1(a)]. The cells were subcultured at 3–4 day intervals in 20% FBS-DMEM/F12 medium supplemented with bFGF, EGF, IGF-I and HCG. To date, the CSO cells have been subcultured to passage 80 and are still in a good proliferating state [Fig. 1(c)]. A continuous *C. semilaevis* ovarian cell line (CSO) has been established.

GROWTH PROPERTIES

The growth curve of CSO cells at passage 60 (Fig. 2) showed that the CSO cells were at latent stage on the first day and went into logarithmic stage from day 2·0 to day 3·5. The cell number remained steady between day 3·5 and day 5·0, but began to decline after day 5. The CSO cells grew and proliferated at a steady rate and their doubling time was calculated to be 51·89 h at passage 60.

CHROMOSOME ANALYSIS

The result of chromosome count of 300 metaphase CSO cells at passage 60 revealed that the chromosome numbers varied from 30 to 56 with a modal chromosome number of 42, which accounted for 60% of the metaphase cells [Fig. 3(a)]. The distribution was asymmetrical and both aneuploidy and heteroploidy appeared in the CSO cell line. The metaphase chromosomes [Fig. 3(b)] with a normal diploid number of 42 displayed the normal karyotype morphology, consisting of 21 pairs of telocentric chromosomes (t): 2n = 42t, NF = 42 [Fig. 3(c)], and also exhibited a typical W sex chromosome which is the largest and mainly present in female fish.

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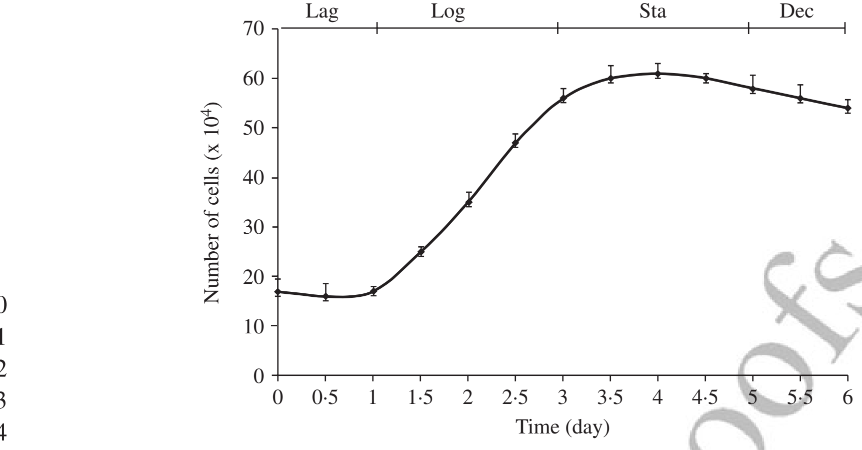

Fig. 2. The growth curve of *Cynoglossus semilaevis* ovarian (CSO) cells at passage 60. The lag phase (Lag), logarithmic phase (Log), stationary phase (Sta) and decline phase (Dec) are shown. Scale bar = 20 μm.

STORAGE IN LIQUID NITROGEN

The cryopreserved CSO cells at passage 8 proliferated to confluency in 4–5 days after thawing. The morphology and proliferation ability of CSO cells were the same before and after cryopreservation. The thawed cell morphology is shown in Fig. 4. The cell viability was calculated to be 90% after trypan blue staining.

GENETIC SEX OF CSO CELLS

The female-specific molecular marker *CseF382* was cloned by genome PCR separately from the DNA of CSO cells at passage 30 and ovary tissues, but was not detected in the testis tissues [Fig. 5 (a)]. Therefore, the genetic sex of CSO cell line was female.

GENE EXPRESSION ANALYSIS

The expression of germ cell and somatic cell marker genes, *Vasa*, *P450arom*, *Foxl2*, *Wt1a*, *Sox9a* and *Dmrt1*, in CSO cell line was analysed at passage 35. As shown in Fig. 5(b), *Vasa* expression was observed in ovarian tissues but not in CSO cells; *P450arom* and *Foxl2* were expressed strongly in CSO cells similar with that in ovary tissues; *Wt1a* expressed weakly in CSO cells but strongly in ovary tissues. *Sox9a* exhibited a weak expression in CSO cells but not expressed in ovary tissues. At last, the expression of *Dmrt1* was not detected in both CSO cells and ovary tissues.

CSO CELLS TRANSFECTED WITH PEGFP-N3 PLASMID

After the CSO cells were transfected with pEGFP-N3 plasmid by Clontech xfect transfection reagent, green fluorescence signals could be detected 24 h later and reached the maximum at 48 h (Fig. 6). The number of CSO cells in (a) was obviously less than that in (c), but the transfection efficiency did not decrease. The transfection efficiency was all calculated to be *c.* 40% at three different cell densities.

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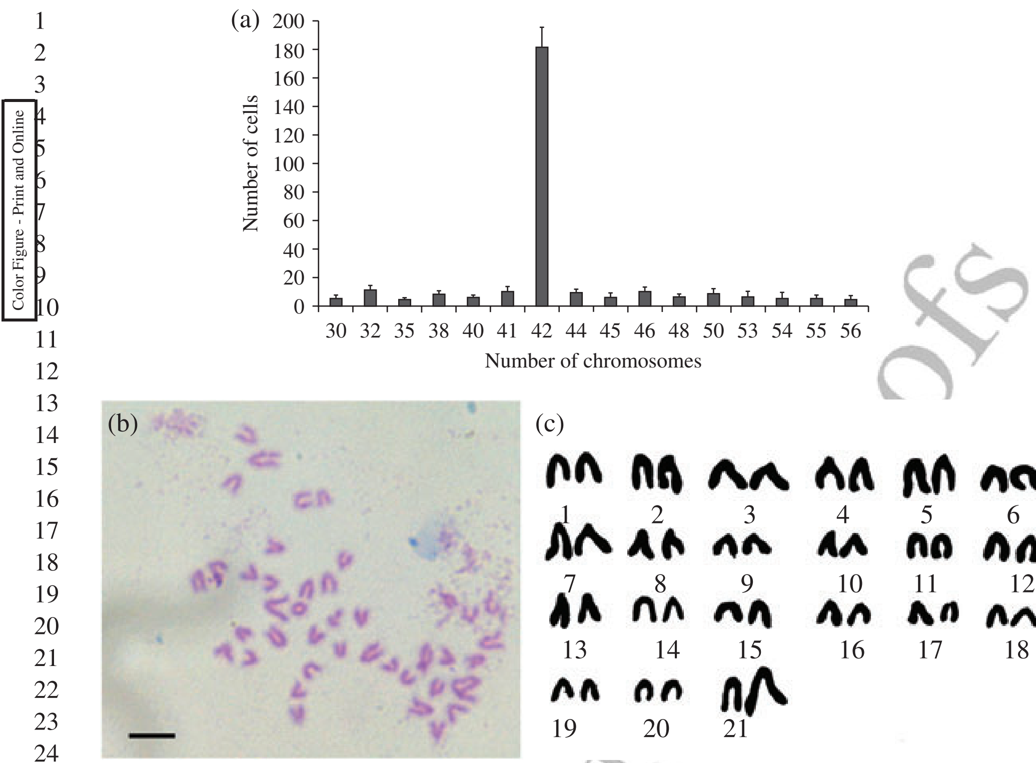

FIG. 3. Chromosome analysis of *Cynoglossus semilaevis* ovarian (CSO) cells at passage 60. (a) Chromosomal aneuploidy of CSO cells with chromosome numbers ranging from 30 to 56 and *c.* 60% of CSO cells have a chromosome number of 42, (b) chromosomes from one CSO cell with a diploid number of 42 and (c) the diploid karyotype of CSO cells, 2n = 42t, NF = 42.

DISCUSSION

For studies of sex determination, sex differentiation and gonad development, a continuous CSO cell line from *C. semilaevis* was established. To date, the cell line had been subcultured to passage 80 and was still in active growth.

PRIMARY CELL CULTURE AND SUBCULTURE

To initiate the primary culture of CSO cells, *C. semilaevis* ovarian tissue pieces were digested with enzyme trypsin. Ovarian tissues are full of fat globules which block cells to migrate out; therefore, trypsin digestion may release cells and discard the fat globules. Similar methods have been reported in the establishment of ovarian cell line from honmoroko *Gnathopogon caerulescens* (Sauvage 1883) (Higaki *et al.*, 2013), but different method was also reported in the establishment of ovarian cell line from African catfish *Clarias gariepinus* (Burchell 1822) (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

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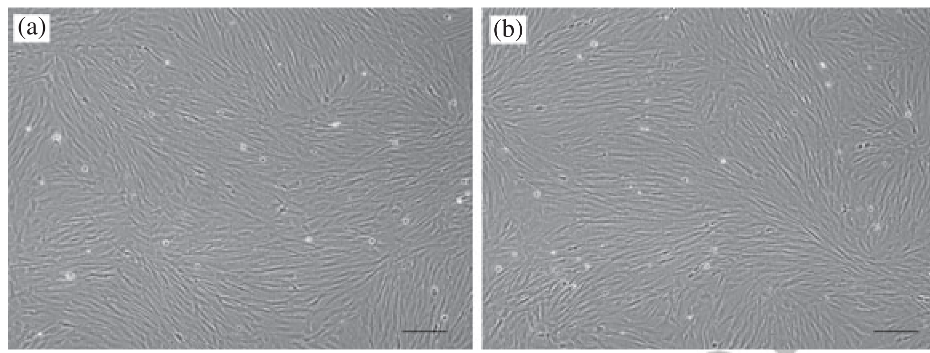

FIG. 4. Cryopreserved *Cynoglossus semilaevis* ovarian (CSO) cells at passage 8 were recovered. (a) The monolayer of passage 8 CSO cells before cryopreservation and (b) the monolayer of passage 8 CSO cells thawed 3 months later. Scale bar =100 μm.

growth were reported in the establishment of fin cell line from brown-marbled grouper *Epinephelus fuscoguttatus* (Forsskål 1775) (Wei *et al.*, 2009), kidney cell line from turbot *Scophthalmus maximus* (L. 1758) (Wang *et al.*, 2010*a*), heart cell line (Zhao *et al.*, 2003), spleen cell line (Sun *et al.*, 2011), ovarian and testicular cell lines from *G. caerulescens* (Higaki *et al.*, 2013), embryonic stem cell line from the sea perch *Lateolabrax japonicus* (Cuvier) (Chen *et al.*, 2003*a*), primary culture of embryonic cells from shrimp *Penaeus chinensis* (Fan *et al.*, 2002) and cartilage cells from Japanese bullhead shark *Heterodortus japonicas* Miklouho-Maclay & Macleay 1884 (Yu *et al.*, 2005). Addition of these supplements in the culture medium was probably the key factor of inducing cell proliferation in primary culture and successful subculture of CSO cells in this study.

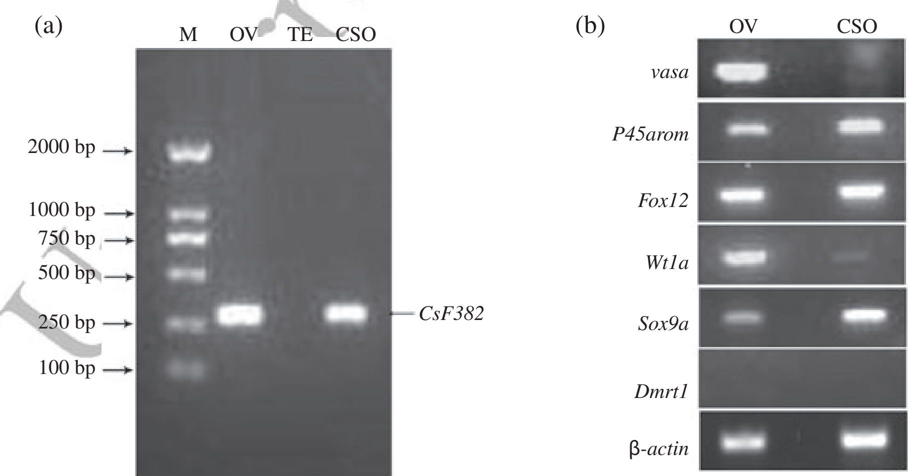

FIG. 5. (a) Genetic sex identification of *Cynoglossus semilaevis* ovarian (CSO) cells. Female-specific molecular marker (M) *CsF382* was detected both in ovary tissue (OV) and CSO cells at passage 30. (b) Gene expression pattern of CSO cells at passage 35. CSO cell line strongly expressed *P450arom* and *Foxl2* strongly, very weakly expressed *Wt1a* and *Sox9a* and did not express *Dmrt1*. TE, testis tissues.

Color Figure - Print and Online

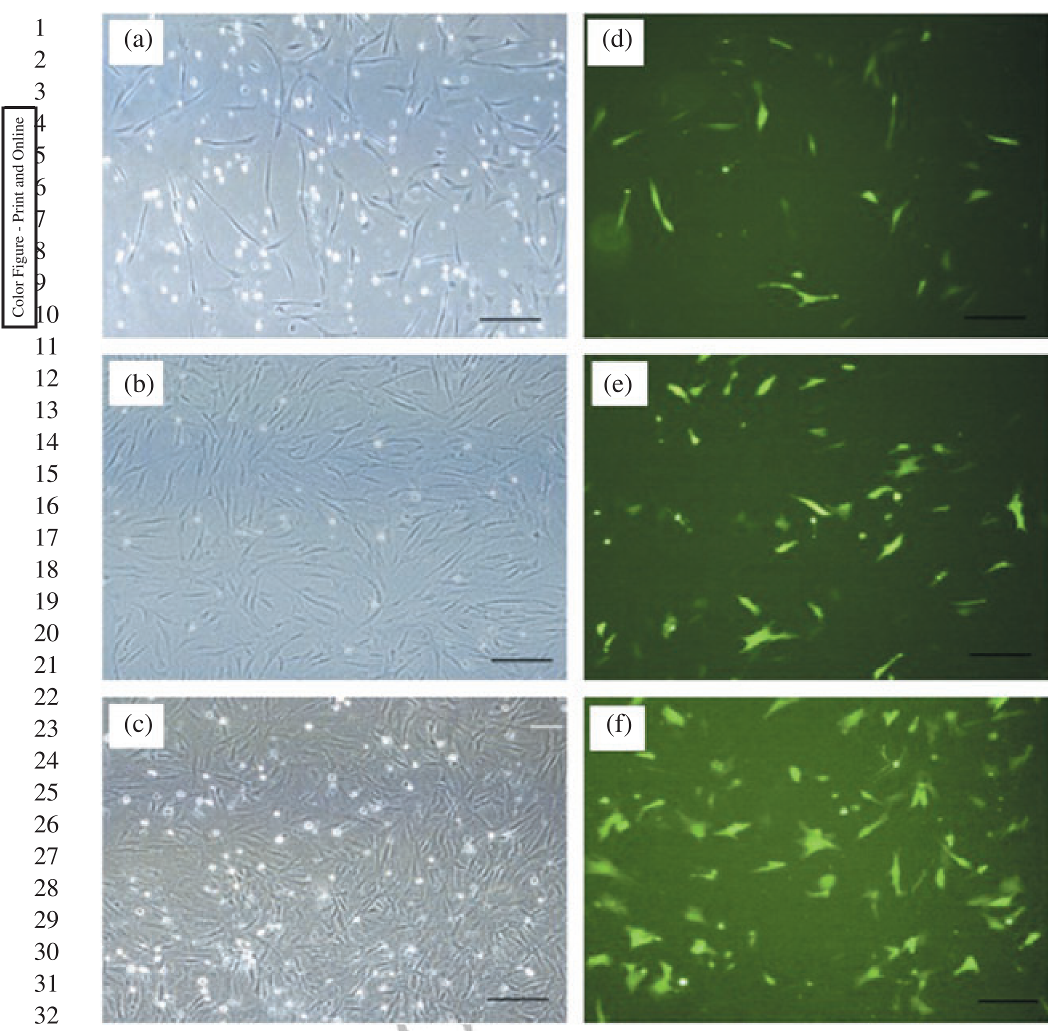

Fig. 6. *Cynoglossus semilaevis* ovarian (CSO) cells transfected with pEGFP-N3 plasmid 48 h later at passage 65. (a, b, c) Optical microscope photographs of CSO cells transfected at three different regions with different cell numbers and (d, e, f) were the same view of (a–c) under fluorescent view. Scale bar =100 μm.

GROWTH PROPERTIES

The fibroblastic cells in CSO cell line proliferated actively during subculture and had a population doubling time of 51·89 h at passage 60. The population doubling time was similar to that of *Epinephelus coioides* (Hamilton 1822) fin cell line (Wei *et al.*, 2009), higher than that of gilthead seabream *Sparus aurata* fin L. 1758 cell line (Béjar *et al.*, 1997; Fan *et al.*, 2010; Wei *et al.*, 2010; Sun *et al.*, 2011) and lower than that of *S. maximus* kidney cell line (Wang *et al.*, 2010*a*). This implies that the CSO cell line still maintains active proliferating ability and could be continuously subcultured.

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CHROMOSOME AND GENETIC SEX ANALYSIS OF CSO CELLS

Karyotype analysis showed that the CSO cells at passage 60, exhibiting chromosomal aneuploidy, still had a modal chromosome number of 42. The diploid karyotype of 2n = 42t of the CSO cells was identical to that of *C. semilaevis* reported earlier (Wang *et al.*, 2010*b*; Zhang *et al.*, 2011; Zheng *et al.*, 2012). The results indicate that the CSO cell line is from *C. semilaevis*. Genetic sex identification revealed that the genetic sex of CSO cell line is female.

GENE EXPRESSION ANALYSIS

The full-length cDNA of *Vasa*, *P450arom*, *Foxl2*, *Wt1a*, *Dmrt1* and *Sox9a* genes have been cloned from *C. semilaevis* (Deng & Cheng, 2008; Deng *et al.*, 2009; Dong *et al.*, 2011; Zhang *et al.*, 2012, 2013). The primers were designed according to the published genes sequences.

Vasa is the marker gene of germ cell (Kobayashi *et al.*, 2002; Hong *et al.*, 2004), *P450arom* (*Cyp19a1*) is granulosa cell marker gene (Devlin & Nagahama, 2002; Rodríguez-Marí *et al.*, 2005) and *Foxl2* is *P450arom*-regulated gene (Yamaguchi *et al.*, 2007; Ijiri *et al.*, 2008; Wen *et al.*, 2010). *Sox9a* and *WT1a* were marker genes of Sertoli cells (Chiang *et al.*, 2001; Sakai, 2002; Kobayashi *et al.*, 2008; Mohapatra *et al.*, 2011), but it was also reported to be expressed in ovarian gonadal epithelium and theca cells (Zhou *et al.*, 2003; Mohapatra *et al.*, 2011). *Dmrt1* was the male germ cell marker gene in zebrafish *Danio rerio* (Hamilton 1822) (Guo *et al.*, 2005) and in *C. semilaevis*.

In this study, CSO cells were found to be highly expressed in *P450arom* and *Foxl2* genes, but weakly expressed in the *Wt1a* gene which was contrary to its expression in ovarian tissues. Taken together, the CSO cell line might be an ovarian granulosa cell line. The germ cells appeared to disappear in the primary culture and the reason might be that they need a special culture medium and additives for division and proliferation *in vitro*.

CSO CELLS TRANSFECTED WITH PEGFP-N3 PLASMID

It had been a problem that fish cell lines were hard to be transfected. This study demonstrated that the transfection efficiency of CSO cell line could reach *c.* 40% which is much higher than reported in other fish cell lines (Chen *et al.*, 2003*a*; Qin *et al.*, 2006; Parameswaran *et al.*, 2006, 2007; Wang *et al.*, 2010*a*, *b*; Sun *et al.*, 2011; Zheng *et al.*, 2012). Thus, an effective method was provided by screening transfection reagent and the ratio of Xfect Polymer:DNA for fish cell line transfection. The transfection by the pEGFP-N3 plasmids containing a CMV promoter, an SV40 polyadenylation signal and neomycin-resistant gene *via* Clontech Xfect transfection reagent is feasible and implies that the CSO cell line can be utilized for transgenic study and gene targeting experiments.

In conclusion, a continuous *C. semilaevis* ovarian cell line, CSO, was established and its transgenic feasibility was evaluated. The CSO cell line could be used to study functions of some important genes in sex determination and oogenesis of ovary in flatfish by gene knockout or gene overexpression in the cell line. The CSO cell line will have some potential applications in cell cloning, cell transfer, virus isolation and cell toxicology in *C. semilaevis*.

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Uncorrected Proofs

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AQ2. Please spell out “NF” (if required).

AQ3. Please check and confirm if the edits made to the sentence “In the ovary, thecal layer…” are appropriate.

AQ4. Please provide complete details for “Wang et al., 2004, Fan *et al*., 2002, Wen *et al*., 2010”.

AQ5. Please provide URL for “MingBo Fisheries Company”.

AQ6. Please check and confirm if the edits made to “80% FBS-20% DMEM/F12” are appropriate.

AQ7. Please check and confirm if the edits made to the sentence “The metaphase chromosomes...” retain the intended meaning.

AQ8. Reference Dong et al. 2008 has not been cited in text. Please check and confirm as to where it has to be cited.

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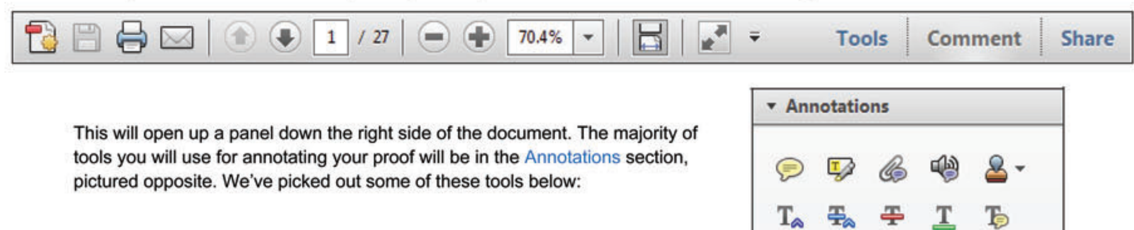

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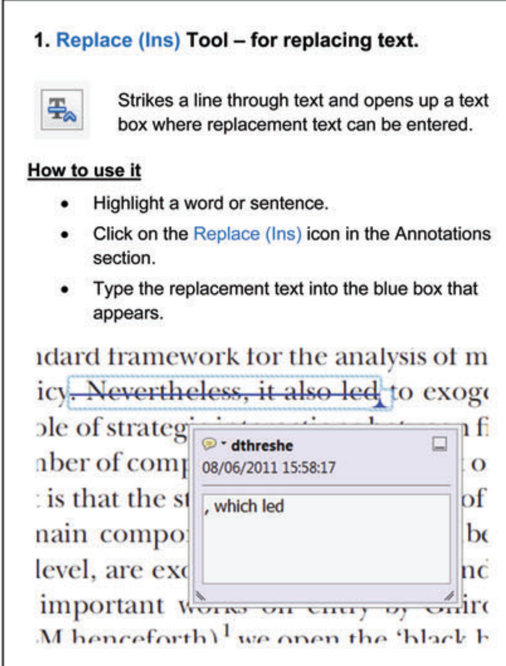

1. Replace (Ins) Tool – for replacing text.

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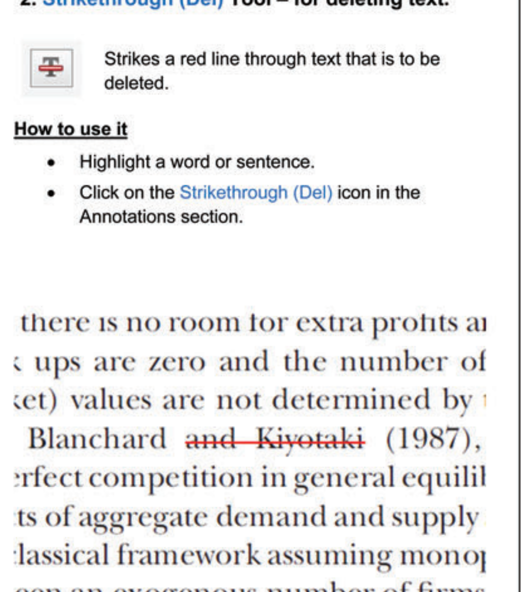
- Type the replacement text into the blue box that appears.



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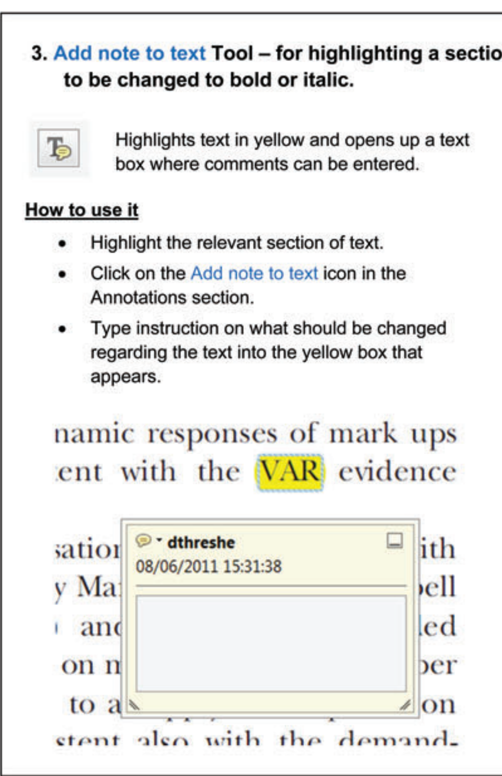

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- Click on the Add note to text icon in the Annotations section.
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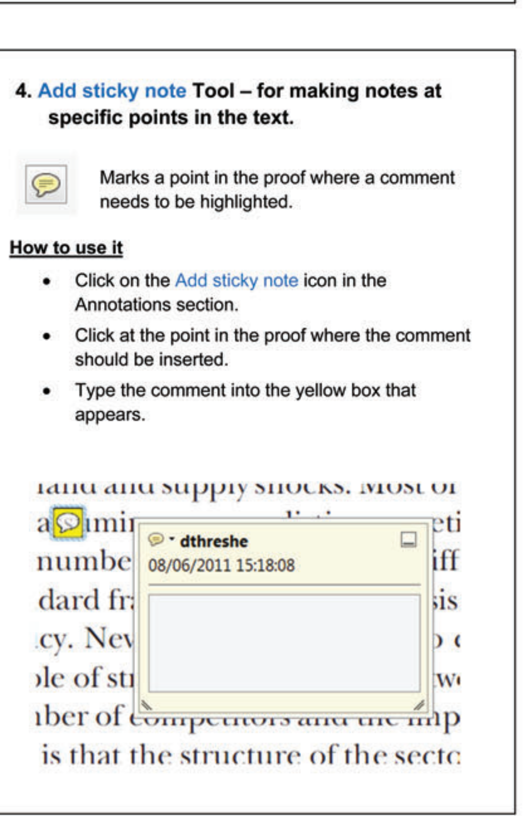

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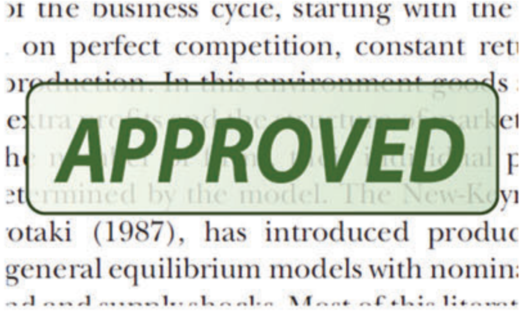

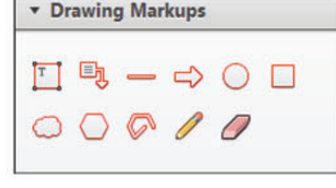

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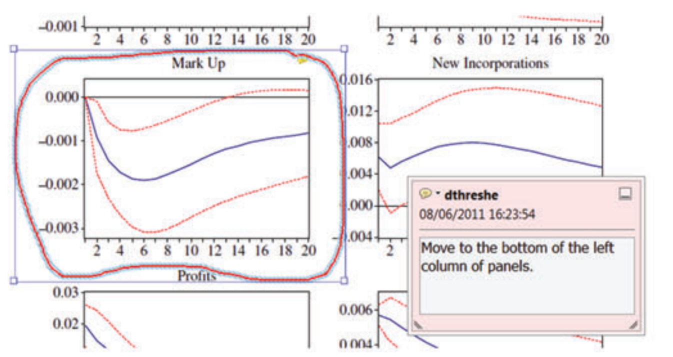

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