In Vitro Cell.Dev.Biol.—Animal (2015) 51:763–768
DOI 10.1007/s11626-015-9894-y

REPORT

# Development and characterization of an embryonic cell line from endangered endemic cyprinid Honmoroko *Gnathopogon caerulescens* (Sauvage, 1883)

**Shogo Higaki[1] · Manami Shimada[2] · Yoshie Koyama[1] · Yasuhiro Fujioka[3] · Noriyoshi Sakai[4] · Tatsuyuki Takada[1,2,5]**

Received: 5 January 2015 / Accepted: 15 March 2015 / Published online: 2 April 2015 / Editor: T. Okamoto


**Abstract** Establishing a cell line from endemic species facilitates the cell biological research of these species in the laboratory. In this study, an epithelium-like cell line RME1 was established from the blastula-stage embryos of the critically endangered cyprinid Honmoroko *Gnathopogon caerulescens*, which is endemic to ancient Lake Biwa in Japan. To the best of our knowledge, this is the first embryonic cell line from an endangered fish species. This cell line is well adapted to grow at 28°C in the culture medium, which was successfully used for establishing testicular and ovarian cell lines of *G. caerulescens*, and has displayed stable growth over 60 passages since its initiation in June 2011. Although RME1 did not express the genes detected in blastula-stage embryos, such as *oct4*, *sox2*, *nanog*, and *klf4*, it showed a high euploidy rate (2*n*=50; 67.2%) with normal diploid karyotype morphology, suggesting that RME1 retains the genomic organization of *G. caerulescens* and can prove to be a useful tool to investigate the unique properties of endangered endemic fishes at cellular level.

**Keywords** Cell line · Embryo · Endangered species · Fish

✉ Tatsuyuki Takada
ttakada@ph.ritsumei.ac.jp

Shogo Higaki
shogohigaki@gmail.com

Manami Shimada
sb005082@ed.ritsumei.ac.jp

Yoshie Koyama
koyama55111@yahoo.co.jp

Yasuhiro Fujioka
yasuhiro@lbm.go.jp

Noriyoshi Sakai
nosakai@nig.ac.jp

[1] Ritsumeikan Global Innovation Research Organization, Ritsumeikan University, Nojihigashi 1-1-1, Kusatsu, Shiga 525-8577, Japan

[2] Laboratory of Cell Engineering, Graduate School of Life Sciences, Ritsumeikan University, Nojihigashi 1-1-1, Kusatsu, Shiga 525-8577, Japan

[3] Lake Biwa Museum, Oroshimo 1091, Kusatsu, Shiga 525-0001, Japan

[4] Genetic Strains Research Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan

[5] Laboratory of Cell Engineering, Department of Pharmaceutical Sciences, Ritsumeikan University, Nojihigashi 1-1-1, Kusatsu, Shiga 525-8577, Japan

Fish cell lines provide a useful tool for biological studies in the fields of physiology, toxicology, and virology (Hightower and Renfro 1988; Villena 2003). Establishing cell lines is important and critical, particularly for studying endangered fish species owing to difficulties in the routine collection of cell samples. Although only a few cell lines have been developed from endangered fish species (Lakra *et al.* 2006; Ciba *et al.* 2008), hundreds of fish cell lines have been established from a broad range of species (Wolf and Mann 1980; Fryer and Lannan 1994; Lakra *et al.* 2011).

Honmoroko *Gnathopogon caerulescens* (Sauvage, 1883) is a critically endangered small cyprinid endemic to ancient Lake Biwa in Japan (Ministry of the Environment, Japan 2014). Recently, we established testicular and ovarian cell lines of *G. caerulescens* (Higaki *et al.* 2013b) and demonstrated the antiandrogenic effects of nonylphenol, one of the potent endocrine disruptors, as well as the response to fish-specific hormones, using a testicular cell line (Higaki *et al.* 2013a). Cell lines derived from various tissues, including early embryos, will be valuable for studying physiological responses to

endogenous and exogenous molecules and viral infection at the cellular level. In this study, we focused on early development, establishing a cell line from blastula-stage embryos of *G. caerulescens* and performing the characterization of the cell line.

The brood stock of *G. caerulescens* used in this study was reared in outdoor ponds at the Shiga Prefectural Fisheries Experimental Station. Naturally spawned blastula-stage embryos were collected into a 40-μm mesh cell strainer (BD Biosciences, San Jose, CA). A group of about 20 embryos was disinfected by 2-min immersion in sterile phosphate-buffered saline (PBS) supplemented with 0.5% (*v*/*v*) commercial bleach solution. Following three washes in sterile PBS for 2 min each, three to four embryos were transferred to one well of a 0.1% (*w*/*v*) gelatin-coated four-well plate (Thermo Scientific, Waltham, MA) filled with 300 μl of culture medium. The embryos were then ruptured with stainless steel needles, and scattered blastomeres were cultured at 28°C in humidified air. When the cells reached confluence, they were passaged by trypsinization using PBS supplemented with 0.06% (*w*/*v*) trypsin and 1.32 mM ethylene diamine tetraacetic acid. From passage 3, cells were cultured in a gelatin-coated 35-mm plastic tissue culture dish (Asahi Glass, Tokyo, Japan) filled with 1.3 ml of the culture medium. The cells were passaged at 1:2 or 1:3 cell dilutions every 2–3 days.

Culture medium was the same as that used for establishing testicular and ovarian cell lines of *G. caerulescens* (Higaki *et al.* 2013b), namely, testicular cell culture medium containing 10% (v/v) fetal bovine serum (TCCM-FBS) (Sakai 2006) supplemented with 10 IU $ml^{-1}$ human chorionic gonadotropin (ASKA Pharmaceutical, Tokyo, Japan), 10 IU $ml^{-1}$ pregnant mare's serum gonadotropin (ASKA Pharmaceutical), 100 ng $ml^{-1}$ epidermal growth factor (Peprotech, Rocky Hill, NJ), 100 ng $ml^{-1}$ basic fibroblast growth factor (Peprotech), 10 μM forskolin, and 0.1 mM β-mercaptoethanol. Fish embryo extract (FEE) was prepared from crucian carp (*Carassius carassius*) embryos according to a previously described procedure (Westerfield 2007).

The expression of genes known to be expressed in the blastula-stage embryos of the closely related zebrafish, such as *oct4* (Burgess *et al.* 2002), *sox2* (Okuda *et al.* 2006), *nanog* (Xu *et al.* 2012), and *klf4* (Onichtchouk *et al.* 2010), were examined in the embryonic cell line and embryos of *G. caerulescens*. For gene expression analyses, total RNA was extracted from the cell cultures at passage 28 and blastula-stage embryos using illustra RNAspin Mini Isolation Kit (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. After the DNase treatment of the samples, reverse transcription was carried out with PrimeScript RT regent Kit (Takara Bio, Shiga, Japan) using 500 ng total RNA. The complementary DNA (cDNA) was amplified using primers designed for *G. caerulescens* zebrafish orthologue *oct4*, *sox2*, *nanog*, and *klf4* (Table 1). The *tubulin-alpha 1* (*tuba1*) gene was used as an internal control. PCR was performed using TaKaRa ExTaq DNA Polymerase (Takara Bio) according to the manufacturer's instructions. The PCR conditions were as follows: initial denaturation for 2 min at 95°C, 35 cycles of denaturation for 20 s at 94°C, annealing for 30 s at the temperatures indicated in Table 1, and extension for 1 min at 72°C, followed by a final extension for 2 min at 72°C. The RT-PCR products were separated by 2% (*w*/*v*) agarose gel electrophoresis, and the gel was stained with ethidium bromide.

The effects of incubation temperature and concentrations of FBS and FEE on the growth of the embryonic cell line were investigated at passages 30–34. Cells were plated in gelatin-coated 24-well plates in triplicate at $5\times10^4$ cells per well and incubated at 18°C, 23°C, or 28°C. Cells were cultured for 6 days, and cell number was counted daily using a hemocytometer. Minimum doubling time was calculated from experimental data during the exponential growth phase using the algorithm provided by http://www.doubling-time.com. Growth responses to different concentrations of FBS (1%,

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

| Gene | Primer (5′–3′) | Sequence | Annealing temperature (°C) | Expected product size (bp) |
|---|---|---|---|---|
| *oct4* | Forward | GATGTGGGACTCGCGCTTGG | 64 | 442 |
| | Reverse | GGGGTGGAGGTGGTGGACTCT | | |
| *sox2* | Forward | AGCCAAACGCCTTCGGGCTC | 60 | 304 |
| | Reverse | CATCTGCGCCGTGTTGTGCG | | |
| *nanog* | Forward | CAGGTAAAAACATGGTTTCAGAACCG | 60 | 323 |
| | Reverse | GGGTTGGGGAACTCATAGTG | | |
| *klf4* | Forward | CGCAAAAGGACCGCGACGCA | 64 | 236 |
| | Reverse | GCGGGAGAAAGCCCTCTCGC | | |
| *tuba1* | Forward | TCCACCCTGAGCAGCTCATCA | 60 | 346 |
| | Reverse | GAAGGCACAGTCAGAGTGCTC | | |

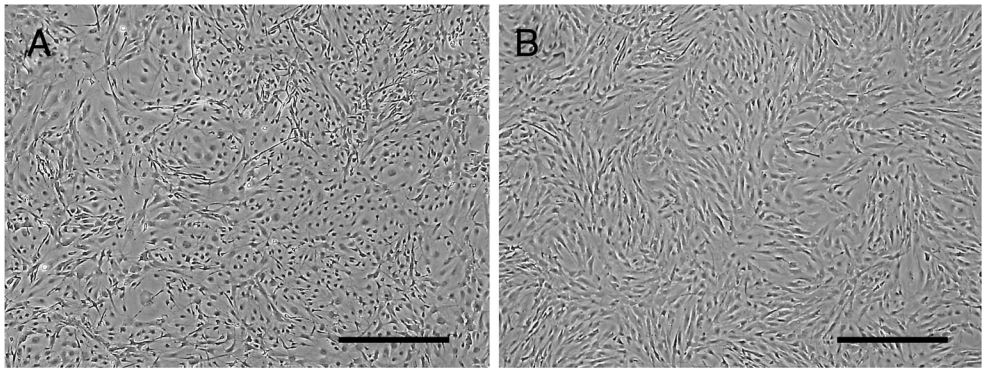


**Figure 1.** The morphology of the cell cultures derived from blastula-stage embryos of *Gnathopogon caerulescens*. Phase contrast micrographs of embryonic cell cultures were taken at passage 2 (*A*) and 30 (*B*). RME1 cells were grown in testicular cell culture medium containing 10% (*v*/*v*) fetal bovine serum (TCCM-FBS) supplemented with 10 IU $ml^{-1}$ human chorionic gonadotropin, 10 IU $ml^{-1}$ pregnant mare's serum gonadotropin, 100 ng $ml^{-1}$ epidermal growth factor, 100 ng $ml^{-1}$ basic fibroblast growth factor, 10 μM forskolin, and 0.1 mM β-mercaptoethanol. *Bars*=500 μm (magnification, ×100).

5%, and 10%) and FEE (0, 1, and 2 embryos $ml^{-1}$) were determined using the procedures described above with the culture medium containing 2 embryos $ml^{-1}$ FEE and 10% FBS, respectively, at 28°C. The effects of culture temperatures, concentrations of FBS or of FEE, and culture periods on the number of cells were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference as a post hoc test using a computer program (SPSS for Windows, version 12.0, SPSS Inc., IL).

Cells at passage 29 were used for chromosome analysis. Cells undergoing exponential growth were treated with 0.05 μg $ml^{-1}$ colcemid for 4 h at 28°C. Cells were then harvested and centrifuged at 120×*g* for 3 min, and the pellet was gently resuspended in 0.75 M KCl and incubated at 28°C for 10 min. Following incubation, cells were centrifuged again, and the pelleted cells were fixed with a mixture of methanol and acetic acid (3:1) for 20 min. A small volume of the cell suspension was dropped on clean glass slides, air dried, and stained with 10% (*v*/*v*) Giemsa solution (in 10 mM potassium phosphate, pH 6.8) for 10 min. Chromosome counts were determined in 137 metaphase plates.

In the early passages, cell culture was heterogeneous and epithelium- and fibroblast-like cells were noticed; however, at the later passages, only epithelium-like cells were observed (Fig. 1). Embryonic cells grew rapidly without growth stunting and have been successfully subcultured at a ratio of 1:2 or 1:3 at 2- to 3-d intervals for more than 60 passages since their initiation in June 2011. The 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 phenotypic change was observed, and the cell line was designated as RME1, which did not express the genes detected in blastula-stage embryos, such as *oct4*, *sox2*, *nanog*, and *klf4* (Fig. 2). Although many embryonic cell lines have been established from more than 30 fish species (Wolf and Mann 1980; Fryer and Lannan 1994; Lakra *et al.* 2011), to the best of our knowledge, this is the first embryonic cell line established from an endangered fish species.

There was an interaction between the effects of the culture temperatures and the culture periods on the number of cells ($P<0.01$). Within the tested temperatures, RME1 cells grew optimally at 28°C (Fig. 3*A*), which is higher than the upper limit of the water temperature during the spawning season (12–27°C) of *G. caerulescens* (Nakamura 1949). The cells grew slowly at 23°C, whereas they did not grow at all at 18°C. These results may imply that the cell line was adapted and/or selected to the relatively higher incubation temperature through the establishment and/or subculture at 28°C, as suggested previously (Wolf and Ahne 1982). The growth rate of the cell line increased as the FBS

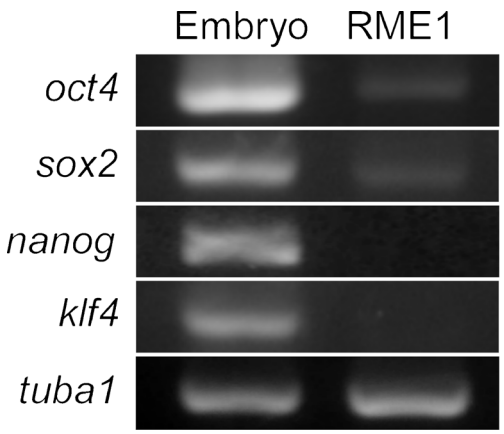


**Figure 2.** Gene expression patterns of embryonic cell line (RME1) and blastula stage embryos of *Gnathopogon caerulescens*. Total RNAs for RT-PCR were extracted from RME1 cells at passage 28 and *G. caerulescens* embryos at blastula stage. Following reverse transcription, cDNA was amplified using primers designed for *G. caerulescens* zebrafish orthologues *oct4*, *sox2*, *nanog*, and *klf4*. The *tubulin-alpha 1* (*tuba1*) gene was used as an internal control. The RT-PCR products were separated by 2% (*w*/*v*) agarose gel electrophoresis, and the gel was stained with ethidium bromide.

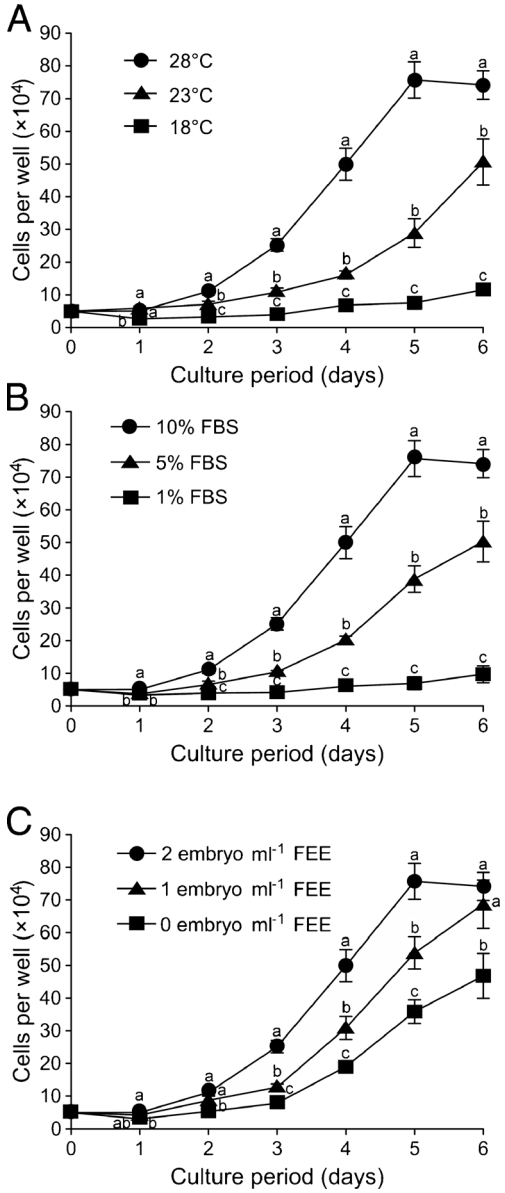


**Figure 3.** Growth rate of an embryonic cell line (RME1) of *Gnathopogon caerulescens* at different incubation temperatures and concentrations of fetal bovine serum (*FBS*) and fish embryo extract (*FEE*). Growth rates of RMT1 cells were examined at passages 30–34. Cells were cultured in a medium containing 10% FBS (*v*/*v*) and 2 embryos $ml^{-1}$ FEE at 18°C, 23°C, or 28°C (*A*). Cells were cultured in a medium containing 1, 5, or 10% FBS and 2 embryos $ml^{-1}$ FEE at 28°C (*B*). Cells were cultured in the medium containing 10% FBS and 0, 1, or 2 embryos $ml^{-1}$ FEE at 28°C (*C*). Cells were plated in gelatin-coated 24-well plates in triplicate at $5\times10^4$ cells per well. Cells were trypsinized and counted daily using a hemocytometer. *Superscripts a–c* Values (mean± standard error of three replicates) with different *letters* within the same culture periods differed significantly ($P<0.05$).

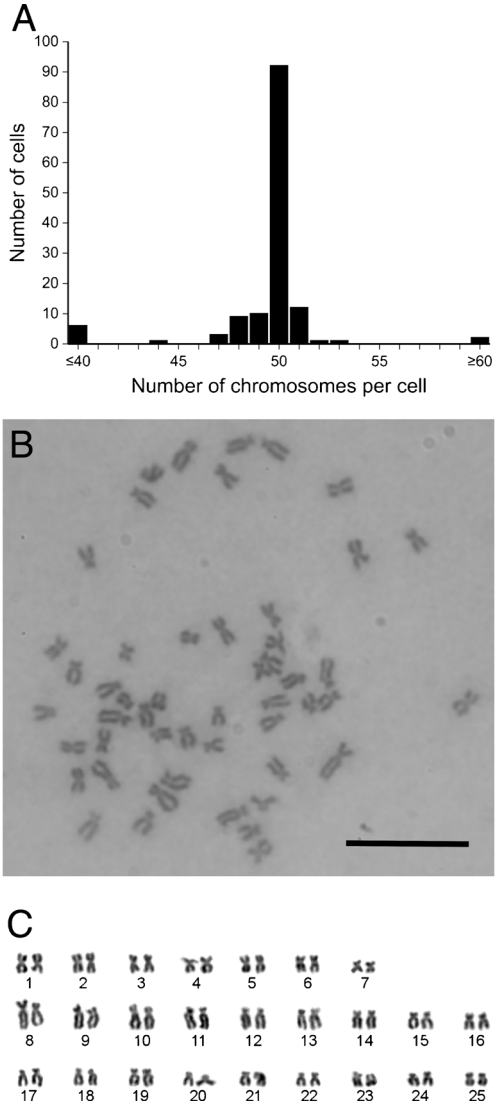


**Figure 4.** Chromosome analysis of embryonic cell line (RME1) of *Gnathopogon caerulescens*. Chromosome number distribution of RME1 cells was analyzed at passage 29 according to counts from 137 metaphase preparations (*A*). Metaphase chromosomes were micrographed after Giemsa staining (magnification, ×1000) (*B*). Giemsa-stained chromosomes attentively arranged into a karyogram (*C*). Chromosomes grouped in the *upper row* are metacentric chromosomes; the *middle and bottom rows* are submetacentric–subtelocentric chromosomes, respectively. An example of a metaphase plate of RME1 cells with euploid ($2n=50$) chromosomes is shown. *Bar*= 10 μm.

concentrations increased from 1% to 10% (Fig. 3*B*) ($P<0.01$), indicating that FBS plays an important role in the proliferation of the present cell line, as generally suggested in fish cell culture (Bols and Lee 1991). Conversely, the minimum population doubling times of the RME1 cells cultured with 0, 1, and 2 embryos $ml^{-1}$ FEE were similar to each other: 18.73 h (days 3–4), 18.78 h (days 3–4), and 20.32 h (days 2–3), respectively (Fig. 3*C*). These doubling times were shorter than those of the embryonic cell lines established in other fish species (Collodi *et al.* 1992; Hong *et al.* 1996; Ristow *et al.* 1998; Shimizu *et al.* 2003; Parameswaran *et al.* 2007), indicating that the RME1 cells are capable of rapid growth in FEE-reduced or FEE-devoid culture medium.

Interestingly, RME1 cells showed a normal diploid number ($2n=50$; Ueno *et al.* 1992) in 67.2% (92/137) of the cells (Fig. 4*A*). In addition, metaphase with a euploid set of chromosomes displayed the normal diploid karyotype morphology (Fig. 4*B*) consisting of seven pairs of metacentric chromosomes and 18 pairs of submetacentric–subtelocentric chromosomes (Fig. 4*C*), as reported in *G. caerulescens* (Ueno *et al.* 1992). This euploid rate was similar to or even higher than other fish embryonic cell lines at similar passage levels (Ristow and de Avila 1994; Parameswaran *et al.* 2007; Dash *et al.* 2010). Accordingly, RME1 cells presumably have less genomic rearrangement, and therefore, the cell line can be a useful tool to study cell biology in *G. caerulescens*. In addition, the euploidy of RME1 cells imply the potential use for somatic cell nuclear transfer (SCNT) and induced pluripotent stem (iPS) cell generation in the future. Many technical obstacles such as poor yields, developmental anomalies, and different nuclear–mitochondrial interactions in SCNT (Loi *et al.* 2011) remain to be solved, and developmentally competent iPS cells have not yet been accomplished in fish (Rosselló *et al.* 2013).

In conclusion, we have succeeded in developing an epithelium-like cell line RME1 from blastula-stage embryos of a critically endangered cyprinid *G. caerulescens*. This cell line is well adapted to grow at 28°C in the culture medium, which was successfully used for establishing testicular and ovarian cell lines of *G. caerulescens*, and displayed stable growth over 60 passages with euploidy. RME1 cells can be cultured in the absence of FEE, which simplifies medium preparation and expands its use to any laboratory. In addition, RME1 cells can be a useful tool as a model of the embryonic cell and provides new insights into the research of endemic fish *G. caerulescens*, particularly emphasizing cell biology.

**Acknowledgments** The authors thank personnel of the Shiga Prefectural Fisheries Experimental Station, for providing *G. caerulescens* embryos. This work was funded in part by a Grant-in-Aid for challenging Exploratory Research (23651248 to T.T.) and Scientific Research on Priority Area (21028021 to T.T.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. This study was also supported by NIG Collaborative Research Program (2010-A41, 2011-A36, and 2012-A28 2013-A47 to T.T.), Ritsumeikan Global Innovation Research Organization (R-GIRO) program (to T.T.) and Center of Innovation Trial Program from Japan Science and Technology Agency, JST (to T.T.).

## References

Bols N, Lee L (1991) Technology and uses of cell cultures from the tissues and organs of bony fish. Cytotechnology 6:163–187

Burgess S, Reim G, Chen W, Hopkins N, Brand M (2002) The zebrafish spiel-ohne-grenzen (*spg*) gene encodes the POU domain protein Pou2 related to mammalian *Oct4* and is essential for formation of the midbrain and hindbrain, and for pre-gastrula morphogenesis. Development 129:905–916

Ciba P, Schicktanz S, Anders E, Siegl E, Stielow A, Klink E, Kruse C (2008) Long-term culture of a cell population from Siberian sturgeon (*Acipenser baerii*) head kidney. Fish Physiol Biochem 34:367–372

Collodi P, Kame Y, Ernst T, Miranda C, Buhler D, Barnes D (1992) Culture of cells from zebrafish (*Brachydanio rerio*) embryo and adult tissues. Cell Biol Toxicol 8:43–61

Dash C, Routray P, Tripathy S, Verma D, Guru B, Meher P, Nandi S, Eknath A (2010) Derivation and characterization of embryonic stem-like cells of Indian major carp *Catla catla*. J Fish Biol 77: 1096–1113

Fryer JL, Lannan C (1994) Three decades of fish cell culture: a current listing of cell lines derived from fishes. J Tissue Cult Method 16:87–94

Higaki S, Koyama Y, Shimada M, Ono Y, Tooyama I, Fujioka Y, Sakai N, Ikeuchi T, Takada T (2013a) Response to fish specific reproductive hormones and endocrine disrupting chemicals of a Sertoli cell line expressing endogenous receptors from an endemic cyprinid *Gnathopogon caerulescens*. Gen Comp Endocr 191:65–73

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

Hightower EL, Renfro LJ (1988) Recent applications of fish cell culture to biomedical research. J Exp Zool 248:290–302

Hong Y, Winkler C, Schartl M (1996) Pluripotency and differentiation of embryonic stem cell lines from the medakafish (*Oryzias latipes*). Mech Dev 60:33–44

Lakra W, Bhonde RR, Sivakumar N, Ayyappan S (2006) A new fibroblast like cell line from the fry of golden mahseer *Tor putitora* (Ham). Aquaculture 253:238–243

Lakra W, Swaminathan TR, Joy K (2011) Development, characterization, conservation and storage of fish cell lines: a review. Fish Physiol Biochem 37:1–20

Loi P, Modlinski AJ, Ptak G (2011) Interspecies somatic cell nuclear transfer: a salvage tool seeking first aid. Theriogenology 76:217–228

Ministry of the Environment, Japan (2014) Red list of Japanese brackish and freshwater fishes. http://www.env.go.jp/press/file_view.php?serial=21437&hou_id=16264. Accessed 28 Aug 2014

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

Okuda Y, Yoda H, Uchikawa M, Furutani-Seki M, Takeda H, Kondoh H, Kamachi Y (2006) Comparative genomic and expression analysis of group B1 sox genes in zebrafish indicates their diversification during vertebrate evolution. Dev Dyn 235:811–825

Onichtchouk D, Geier F, Polok B, Messerschmidt MD, Mössner R, Wendik B, Song S, Taylor V, Timmer J, Driever W (2010) Zebrafish Pou5f1-dependent transcriptional networks in temporal control of early development. Mol Syst Biol 6:354

Parameswaran V, Shukla R, Bhonde R, Hameed AS (2007) Development of a pluripotent ES-like cell line from Asian sea bass (*Lates calcarifer*)—an oviparous stem cell line mimicking viviparous ES cells. Mar Biotechnol 9:766–775

Ristow SS, de Avila J (1994) Susceptibility of four new salmonid cell lines to infectious hematopoietic necrosis virus. J Aquat Anim Health 6:260–265

Ristow SS, Grabowski LD, Ostberg C, Robison B, Thorgaard GH (1998) Development of long-term cell lines from homozygous clones of rainbow trout. J Aquat Anim Health 10:75–82

Rosselló AR, Chen C, Dai R, Howard TJ, Hochgeschwender U, Jarvis DE (2013) Mammalian genes induce partially reprogrammed pluripotent stem cells in non-mammalian vertebrate and invertebrate species. eLife 2:e00036

Sakai N (2006) *In vitro* male germ cell cultures of zebrafish. Methods 39: 239–245

Shimizu C, Shike H, Malicki MD, Breisch E, Westerman M, Buchanan J, Ligman RH, Phillips BR, Carlberg MJ, van Olst J, Burns CJ (2003) Characterization of a White bass (*Morone chrysops*) embryonic cell line with epithelial features. In Vitro Cell Dev Biol Anim 39:29–35

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

Villena JA (2003) Applications and needs of fish and shellfish cell culture for disease control in aquaculture. Rev Fish Biol Fish 13:111–140

Westerfield M (2007) The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*). University of Oregon Press, Eugene

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

Wolf K, Mann JA (1980) Poikilotherm vertebrate cell lines and viruses: a current listing for fishes. In Vitro 16:168–179

Xu C, Fan PZ, Müller P, Fogley R, DiBiase A, Trompouki E, Unternaehrer J, Xiong F, Torregroza I, Evans T, Megason GS, Daley QG, Schier FA, Young AR, Zon IL (2012) Nanog-like regulates endoderm formation through the Mxtx2-nodal pathway. Dev Cell 22:625–638