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Derivation of a pluripotent embryonic cell line from red sea bream blastulas

S.-L. Chen*†, H.-Q. Ye*‡, Z.-X. Sha* and Y. Hong§

**Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, Nanjing Road 106, Qingdao 266071, China, ‡Shanghai Fisheries University, Shanghai 200090, China and §Department of Biological Sciences, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260*

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A pluripotent cell line, sea bream embryonic stem-like cells (SBES1), was developed from blastula-stage embryos of the cultured red sea bream, *Chrysophrys major*. The SBES1 cells were cultivated in Dulbecco's modified eagles medium (DMEM) medium supplemented with foetal bovine serum, marine fish serum, fish embryo extract, selenium, basic fibroblast growth factor and leukemia inhibitory factor. They were small and round or polygonal, and grew actively and stabely in culture. The cells exhibited a positive alkaline phosphatase activity upon histochemical staining. When the cells were treated with all-*trans* retinoic acid, they differentiated into various types including neuron-like, neuroglia-like and muscle-like cells, suggesting that the SBES1 cells remained pluripotent in culture. Chromosome analysis revealed that SBES1 cells had a normal diploid karyotype with $2n = 2st + 46t$. At present, SBES1 cells have been cultured for >180 days with more than 60 passages. High survival rate has been obtained after cryopreservation of cell cultures. This embryonic cell line may potentially be used for the production of transgenic red sea bream.

Key words: *Chrysophrys major*; embryonic stem-like cells; *in vitro* differentiation; pluripotency; red sea bream.

INTRODUCTION

Embryonic stem (ES) cells are undifferentiated cell cultures derived from early developing embryos of animals, retaining their full developmental potential and their capability to differentiate (Evans & Kaufman, 1981; Martin, 1981). When introduced into host embryos, ES cells can participate in normal development and contribute to several host tissues including cells of the germline. These characteristics make embryonic stem cells ideal experimental systems for *in vitro* studies of embryo cell development and differentiation, and as a vector for the efficient transfer of foreign DNA into the germline of an organism (Gossler *et al.*, 1986). Mouse ES cell lines have been established and used for

†Author to whom correspondence should be addressed. Tel.: +86 532 5844606; fax: +86 532 5811514; email: chensl@ysfri.ac.cn

research in a variety of areas including targeted gene inactivation and repair (Thomas & Capecchi, 1987; Thompson *et al*., 1989), developmentally-regulated gene expression and cell differentiation and transformation (Mummery *et al*., 1990). Since the development of embryonic stem cell technology in mice in the 1980s (Evans & Kaufman, 1981; Martin, 1981) and the production of the first 'knock out' mice (genes were inactivated) (Thomas & Capecchi, 1987), it has become feasible to generate mice with desired genetic modifications ranging from simple gene mutation to large genomic deletions. In mice, ES cells are routinely used for gene transfer since they are an ideal experimental system for efficient genome manipulation and for the investigation of factors controlling vertebrate development.

Transgenesis has shown an enormous potential for genetic improvement of aquatic organisms (Devlin *et al*., 1994). Up to now, however, transgenic fishes have been generated by direct introduction of foreign genes into fertilized eggs. The foreign genes integrate randomly into the host's genome, and their ultimate fate is neither controllable nor selectable. In most cases, the transgenic animals are highly mosaic for the introduced genes, and the desired integration of the transgene and its proper expression is rarely achieved (Iyengar *et al*., 1996). Transgenic fish research urgently requires development of a novel gene transfer approach for achieving site-specific integration of a transgene into the host genome and regulation of the expression of transgenes (Hong *et al*., 1998*a*). ES cell-mediated gene transfer technique is a promising approach for producing site-mutated transgenic fishes with enhanced growth rate or disease resistance, as well as for analysing the functions of fish genes (Melamed *et al*., 2002; Chen *et al*., 2002*a*).

To develop ES cell lines, and a gene targeting technique in fishes, extensive studies have been done in small model fishes such as zebrafish *Danio rerio* (Hamilton) and medaka *Oryzias latipes* (Temminck & Schlegel). ES-like cell lines have been established in medaka (Wakamatsu *et al*., 1994; Hong *et al*., 1996), zebrafish (Collodi *et al*., 1992; Sun *et al*., 1995), and in one medaka ES-like cell line, MES1, was shown to retain an eudiploid karyotype and have the ability to form viable chimeras (Hong *et al*., 1998*b*). Homologous recombination vectors for medaka p53 gene were constructed on the basis of genomic sequences of the p53 gene (Chen *et al*., 2001, 2002*b*). Also, a positive–negative selection procedure for gene targeting in fish cells has been established (Chen *et al*., 2002*a*). Most importantly, the availability of zebrafish germ-line chimeras from embryo primary cell cultures (Ma *et al*., 2001) and the development of nuclear transplantation technique for medaka embryonic cells (Wakamatsu *et al*., 2001) and for zebrafish long-term cultivated embryonic cells (Lee *et al*., 2002) point to the possibility of developing embryonic stem cell techniques in fishes.

Red sea bream *Chrysophrys major* (Temminck & Schlegel) is a widely cultivated marine fish in China. The farmed fish, however, has often become diseased. Transgenic fish with enhanced resistance to pathogens will be beneficial to aquaculture. With the aim of establishing ES cell-mediated gene transfer technique in this fish, a pluripotent cell line was established, derived from the mid-blastula of this species, and its *in vitro* differentiation and chromosomes analysed.

MATERIALS AND METHODS

RED SEA BREAM EMBRYO CELL CULTURES

Red sea bream embryos at blastula-stage were harvested *c*. 6 h after fertilization and prepared for cell culture. For each culture, a group of *c*. 80–100 embryos were disinfected with 70% ethanol, washed with phosphate-buffered saline (PBS) and the chorions were torn with fine forceps. The cell mass was released and the chorion was removed. Single cells were obtained by gentle pipetting. After several washes with PBS, the cells were transferred into complete medium in gelatin-coated 24-well tissue plates. The complete growth medium was Dulbecco's modified eagles medium (DMEM) supplemented with 20 mM Hepes, pH 7·5, antibiotics (penicillin, 100 U ml^{-1}, streptomycin, 100 μg ml^{-1}; Gibco BRL), foetal bovine serum (FBS, 15%), sea bream serum (1%), embryo extract from sea bream (0·1%), sodium selenite (8 nM, Gibco BRL), basic fibroblast growth factor (bFGF, 5 ng ml^{-1}, Gibco BRL), human recombinant leukemia inhibitory factor (hLIF, 5 ng ml^{-1}; Pepro Tech EC, London U.K.), 2-mecaptoethanol (2-ME, 27·5 μM; Gibco BRL), sodium-pyruvate (1 mM) and nonessential amino acids (1 mM). Cells were cultured at 24° C without CO_2, the medium was changed every 2 or 3 days. When reaching 95% confluence, the cells were subcultured at a ratio of 1 : 2 according to the standard trypsinization method. For cryopreservation, cell cultures were suspended in complete medium with 10% dimethyl sulphoxide (DMSO) and 20% FBS, placed in cryovials and stored in liquid nitrogen.

PREPARATION OF SEA BREAM EMBRYO EXTRACT

For preparation of embryo extract, sea bream embryos were harvested 36 h after fertilization (reared at 15° C) from Maidao Marine Fish Hatchery and rinsed with PBS several times. After removal of PBS, the embryos were stored at −80° C until used. To prepare an extract, *c*. 2000 embryos were thawed at 37° C and homogenized on ice using a glass homogenizer in the absence of extraction buffer. The resulting homogenate was centrifuged for 30 min at 16 400 *g*. The supernatant was collected as the embryo extract, filter-sterilized, and stored at −20° C until used.

INDUCTION OF DIFFERENTIATION *IN VITRO*

To identify the differentiation potential of the pluripotent cell line, sea bream embryonic stem-like cells (SBES1) were inoculated at low density (10^4 cells $well^{-1}$ of a 12-well plate), middle density (10^5 cells $well^{-1}$ of a 12-well plate) or high density (5×10^5 cells $well^{-1}$ of a 12-well plate) and then cultured for 1–4 weeks with regular medium change. Two days after inoculation, the medium was replaced with the leukemia inhibitory factor (LIF)-free medium and supplemented with 0·5–1·0 μM all-*trans* retinoic acid (RA, Sigma). Control cultures were seeded on gelatin-coated wells without retinoic acid. The medium was changed every 2 days. Cell morphology was microscopically examined daily.

ALKALINE PHOSPHATASE STAINING

Cultured cells or embryos were washed with PBS, fixed in glutaraldehyde solution (1%) for 10 min, washed twice with PBS, and then stained in the dark with alkaline phosphatase (AP) staining solution [0·38 mM 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP, Merck) and 0·4 mM nitro blue tetrazolium (NBT, Merck) in a buffer of 100 mM Tris-Cl, pH 9·5, 100 mM NaCl and 5 mM $MgCl_2$].

CHROMOSOME ANALYSIS

Cells of SBES1 at passage 11 were used for chromosome analysis. The cells were inoculated in culture flasks of 25 cm^2 and incubated for 24–48 h until they were in

logarithmic growth. Colchicine was added to the cells to reach a final concentration of 5 μg ml^{-1}. After 4–6 h, the cells were harvested and treated with a hypotonic solution of 0·075 M KCl at 25° C for 30 min, then fixed in a cold and fresh solution of methanol : acetic acid (3 : 1), with three changes of 20 min each, then air-dried. The chromosomes were stained with 5% Giemsa for 20 min. One hundred cells at metaphase were counted, and chromosome karyotype was analysed according to the method of Levan (1964).

RESULTS

DEVELOPMENT OF RED SEA BREAM ES-LIKE CELL LINE

To develop an ES-like cell line in *C. major*, embryos at the mid-blastula stage were used [Fig. 1(a)]. The single blastomeres from 80–100 embryos were isolated and inoculated in one well of 24-well plate. These blastomeres with a diameter

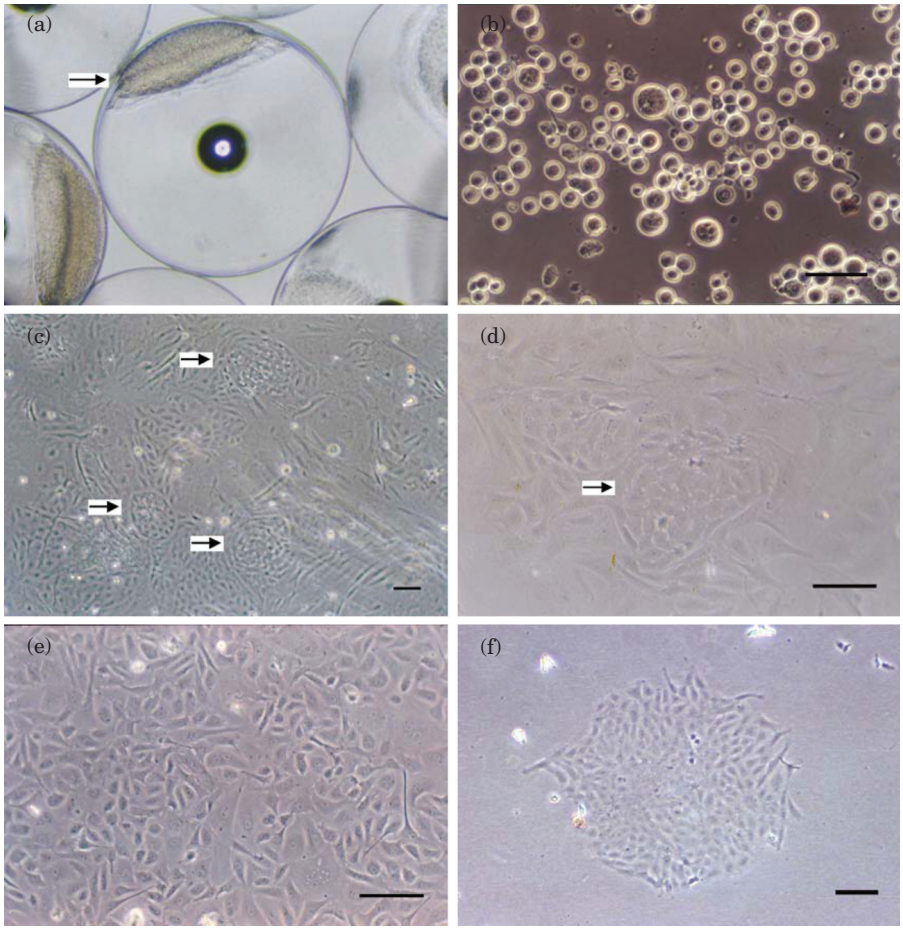

FIG. 1. Morphology and culture of SBES1 cells. (a) Red sea bream embryos at the mid-blastula stage with a blastoderm (⟶). (b) Blastomeres 2 h after inoculation. (c) and (d) ES-like colonies at passage 3 (⟶). (e) SBES1 culture at passage 42, showing small and round or polygonal cells with big nuclei and sparse cytoplasm. (f) Dense, compacted single colony from SBES1 cells at passage 30. Bar, 100 μm.

of *c*. 30–50 μm were first evenly attached to the surface of the well [Fig. 1(b)] and grew to form many dense, homogeneous aggregates within the first 6–12 h after inoculation. The blastomeres in the aggregates began to adhere to the well surface after 1 or 2 days of culture. Two or 3 days later, the cells grew to confluence in the well, and could be subcultured. The cell division occurred very rapidly and could be subcultured every 2 or 3 days. Eight primary cultures were obtained from 35 groups of isolated blastomeres of red sea bream mid-blastula embryos. Six of the eight cultures differentiated and gradually died, the remaining two cultures developed into cell lines that exhibited stable growth and morphology similar to that of mouse ES cells (Evans & Kaufman, 1981), one of which was designated as SBES1. The cells grew actively and stably and formed many dense ES-like colonies [Fig. 1(c), (d)]. The SBES1 cultures at passage 42 are shown in Fig. 1(e). The cells were small (15–30 μm) and round or polygonal with a big nuclei and sparse cytoplasm when their densities were not high. As their densities increased, the cells became smaller (10–20 μm). After culture of >180 days with >60 passages, SBES1 cells displayed stable growth and ES-like morphology. Thus, SBES1 cell culture was considered as a stable cell line. The cryopreservation tests demonstrated that SBES1 cells could undergo cryopreservation in liquid nitrogen (−196° C) with a survival rate of *c*. 50%.

ES-LIKE COLONIES

Colony-forming ability is an important feature of ES-like cells. When plated at a very low cell density (20 to 40 cells cm^{-2}), SBES1 cells at passage 30 were able to grow into dense ES-like cell colonies 7–10 days after plating. Most of the cells in the colonies were ES-like. These colonies were tightly compacted and uniform in morphology. The cells in the colonies were small, round or polygonal in shape [Fig. 1(f)].

DIFFERENTIATION POTENTIAL OF SBES1 *IN VITRO*

Differentiation of SBES1 cells into a variety of cell types could be induced by all-*trans* retinoic acid. When SBES1 cells at up to passages 60 were treated with retinoic acid, most of cells lost their stem cell-like morphology and differentiated into various cell types, including neuron-like, neuroglia-like and muscle-like cells [Fig. 2(a), (b), (c), (d), (e)]. Neuron-like cells began to appear 7–10 days after induction when cells were inoculated at very low densities [Fig. 2(a)]. Neuroglia-like cells occurred between 15–20 days after induction when the cells were grown at middle density [Fig. 2(b)]. Muscle-like cells occurred between 15–20 days after induction when the cells were grown at high density, some of the differentiated muscle cells were very long (up to over 200 μm) and had multiple nuclei [Fig. 2(c), (d), (e)]. Not all cells in a well differentiated into the same cell type, the approximate proportions of neuron-like, neuroglia-like and muscle-like cells were *c*. 60, 85 and 70%, respectively.

ALKALINE PHOSPHATASE ACTIVITY

In mice, AP staining was commonly used to evaluate the pluripotency of the ES cell. In the present study, similar staining was done for identifying SBES1

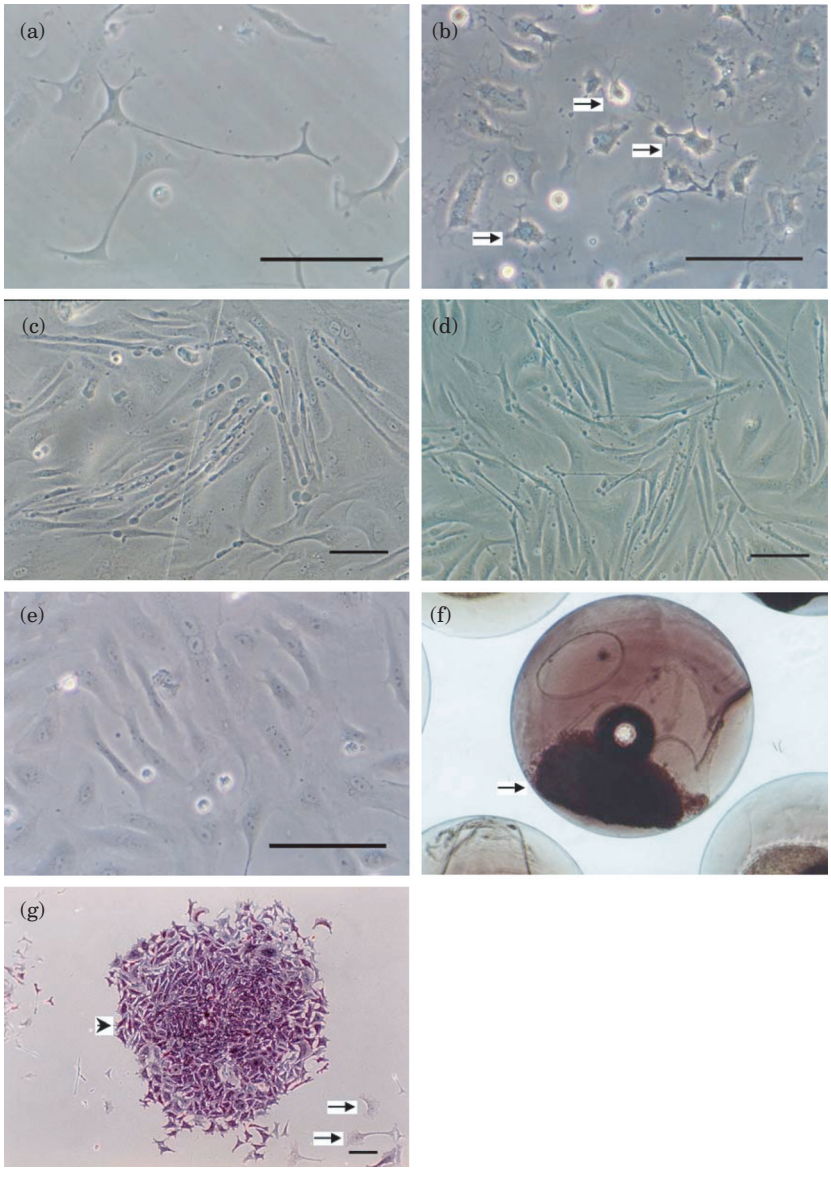

FIG. 2. *In vitro* differentiation and alkaline phosphatase (AP) staining of SBES1 cells. (a) Neuron-like cells appeared in the culture of SBES1 at passage 11, 7 to 10 days after treatment with retinoic acid. (b) Neuroglia cells (⟶) induced at passage 59 of SBES1 cells. (c) and (d) Muscle cells in the culture of SBES1 at passage 60, 2 to 3 weeks after treatment with retinoic acid, showing their length and multiple nuclei structure. (e) Muscle-like cells induced from SBES1 cells at passage 59. (f) AP staining of blastoderm region of sea bream blastula (⟶). (g) AP staining of SBES1 cells at passage 31, exibiting strong (➤) or weak (⟶) AP activity. Bar, 100 μm.

cell cultures. Blastoderms of red sea bream embryos at mid-blastula with a diameter of *c*. 1 mm were strongly stained by BCIP-NBT [Fig. 2(f)]. SBES1 cells at passage 31 also exhibited strong AP activity [Fig. 2(g)], and these cells

are round or polygonal, with a larger nucleus and sparse cytoplasm, whereas those with a larger size and differentiated morphology showed weak activity.

KARYOTYPIC ANALYSIS

The results of chromosome counts of 100 metaphase plates from SBES1 cells at passage 11 revealed that the chromosome numbers varied from 24 to 74. Nevertheless, the modal number of chromosome was 48 [Fig. 3(a)]. The distribution was asymmetrical, most $2n$ values appearing clustered below the modal value. Both aneuploidy and heteroploidy appeared in SBES1 cell line in small proportions. The metaphase [Fig. 3(b)] with normal diploid number displayed the normal diploid karyotype morphology [Fig. 3(c)] consisting of one pair of subtelocentrics and 23 pairs of telocentrics ($2n = 2st + 46t$).

DISCUSSION

Since the establishment of ES cell lines in mice by Evans & Kaufman (1981) and Martin (1981), these totipotent cells have been recognized as powerful experimental material in vertebrate developmental biology. The genetic manipulation of ES cells, *via* homologous recombination, allows the site-specific integration of foreign genes into the genome of the host and the 'knock-out' of specific genes from the host genome. Thus, ES cells provide a promising approach to the generation of transgenic animals with site-specific mutation and to the study of gene function during embryogenesis (Jasin *et al.*, 1996; Muller, 1999).

Although much effort has been made to develop ES cell lines in several species, cultures that are able to contribute to the germ-cell lineage of a host embryo have been successfully reported only for mice (Evans & Kaufman, 1981; Labosky *et al.*, 1994). In fishes, ES-like cells have been cultured in small fishes, zebrafish (Collodi *et al.*, 1992; Sun *et al.*, 1995) and medaka (Wakamatsu *et al.*, 1994; Hong *et al.*, 1996). Also, germ-line chimeras were produced from embryo primary cell cultures of zebrafish (Ma *et al.*, 2001). The ES cell technique has attracted the attention of fish breeders and molecular biologists due to its possible importance in producing transgenic fishes with site-specific integration of foreign gene, and in studying gene function in fishes (Wakamatsu *et al.*, 2001; Ma *et al.*, 2001; Chen *et al.*, 2002*a*). Thus, ES cell lines are urgently needed for use in basic and applied research in teleosts. The present study reports the establishment of an ES-like cell line, SBES1, derived from mid-blastula-stage embryos of red sea bream. The SBES1 cells exhibited typical ES-like cell morphology (Evans & Kaufman, 1981). The cell line maintained stable and rapid growth over 180 days of culture with >60 passages in the absence of feeder layer cells. The ES cells in various species were successfully cultured using a feeder layers technique (Evans & Kaufman, 1981; Wakamatsu *et al.*, 1994; Sun *et al.*, 1995) or a feeder-free system (Nichols *et al.*, 1990; Wurst & Joyner, 1993; Hong *et al.*, 1996). The present study demonstrated that the feeder-free technique was also feasible for the derivation of ES-like cells in red sea bream. This feeder-free system may facilitate the *in vitro* manipulation of ES cells and will be applicable to the cultivation of ES cells from other fish species.

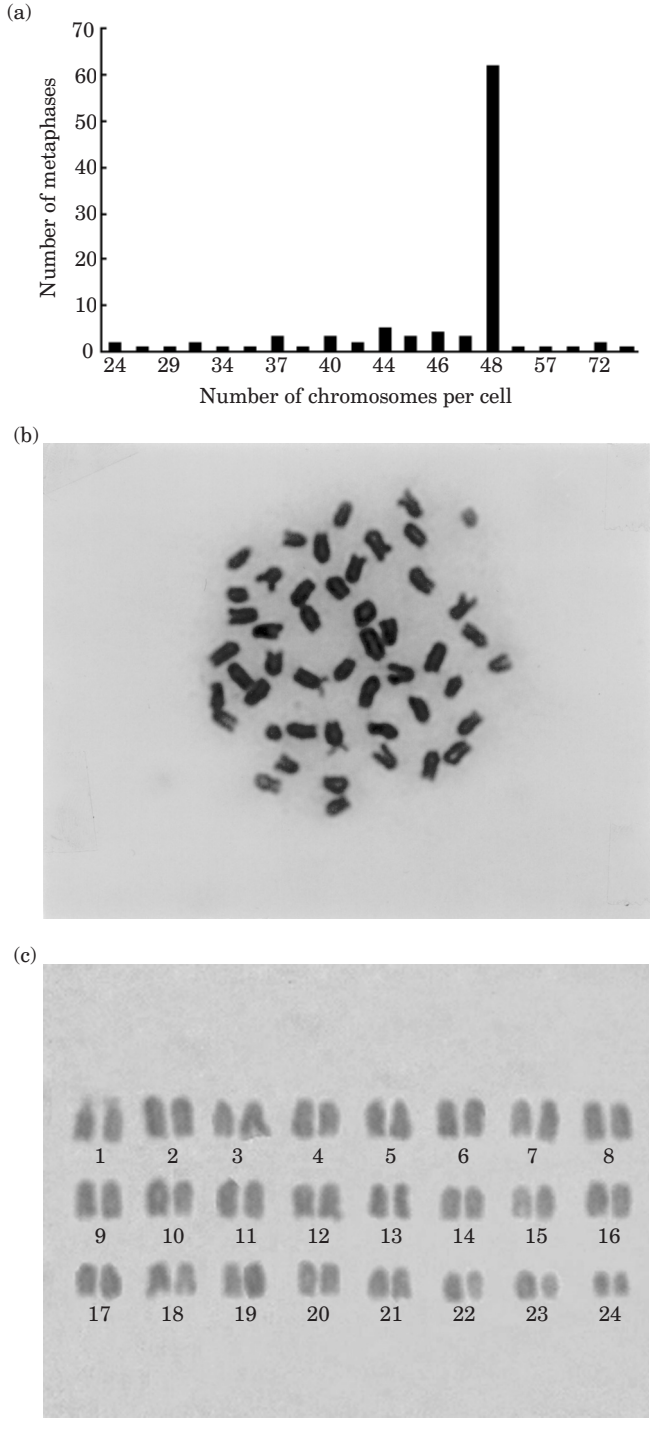

FIG. 3. Chromosome number distribution (a), metaphase (b) and diploid karyotype (c) of SBES1 cells at passage 11. (a) 100 metaphase plates were counted; (b) metaphase of one SBES1 cell (×1000); (c) homologous chromosomes were paired according to their size.

SBES1 cells possessed strong AP activity which is an indication of pluripotency in mouse ES cells (Wobus *et al*., 1984; Pease & Williams, 1990) as well as in early stages of mouse embryos (Mulnard & Huygens, 1978). In SBES1 cultures, most of the cells exhibited intensive AP staining which is similar to other reports (Wakamatsu *et al*., 1994; Hong *et al*., 1996), indicating their undifferentiated state. The heterogeneity in AP staining of SBES1 cells showed that a few SBES1 cells underwent spontaneous differentiation and lost pluripotency during culture.

The *in vitro* differentiation ability is one of important criteria for evaluating the pluripotency of ES cells in other animal species including fishes (Martin, 1981; Thomson *et al*., 1998). The SBES1 cells exhibited the ability to undergo differentiation, *in vitro*, into neuron-like cells and muscles cells in response to all-*trans* retinoic acid induction. The direction of differentiation, however, was only partially controllable by regulating initial plating densities of SBES1 cells. The potential of SBES1 cells to differentiate into the above mentioned cell types under specific conditions suggested that the sea bream ES-like cell line described in the present paper remained pluripotent and in an undifferentiated state. Similar observations were made in mouse and medaka (Nakano *et al*., 1994; Wakamatsu *et al*., 1994; Hong *et al*., 1996). The ultimate proof will have to come from transplantation of SBES1 cells into sea bream blastulae to test their germ line chimera-forming ability.

The euploid karyotype is the most important for ES cells to maintain the ability to form functional germ-line chimeras (Bradley *et al*., 1984). Karyotype analysis revealed that over 60% of the cells possessed a diploid chromosome number of $2n = 48$, which was identical with the modal chromosome number of red sea bream. This diploid rate is similar to or higher than other reports in other ES-like cells (Sun *et al*., 1995; Hong *et al*., 1996).

In summary, a pluripotent embryonic cell line, SBES1, was established from red sea bream blastulae and exhibited *in vitro* differentiation potential. The sea bream ES-like cells will be used to develop gene targeting techniques and as a cellular vehicle for the production of genetically modified stock in this fish.

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