Derivation and Long-Term Culture of an Embryonic Stem Cell-Like Line from Zebrafish Blastomeres Under Feeder-Free Condition

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Abstract

Existing zebrafish embryonic stem (ES) cell lines are derived and maintained using feeder layers. We describe here the derivation and long-term culture of an ES cell-like line derived from zebrafish blastomeres without the use of feeder cells. This line, designated as ZES1, has been maintained for more than 800 days in defined Dulbecco's modified Eagle's medium supplemented with fetal bovine serum, zebrafish embryo extract, trout serum, and human basic fibroblast growth factor. ZES1 cells possessed a morphology typical of ES cells, being round or polygonal in shape with a large nucleus and sparse cytoplasm and were mostly diploid. The cells formed individual colonies consisting of tightly packed cells that stained positively for alkaline phosphatase. ZES1 cells also formed embryoid bodies when transferred onto uncoated wells. The pluripotent nature of ZES1 cells was confirmed when they could be induced to differentiate *in vitro* into several cell types, through low- or high-density culture conditions. Treatment with retinoic acid also induced the differentiation of ZES1 cells into primarily neuronal cells. Using immunostaining and real-time polymerase chain reaction, we showed that Sox2, a known pluripotent marker in mammalian ES cells, was also present in ZES1 cells. Chimera experiments revealed that fluorescent-labeled ZES1 cells microinjected into zebrafish blastulas participated in the formation of all three germ layers. Using GFP-labeled ZES1 cells, chimera germline transmission was also demonstrated at the F₁ generation. In conclusion, ZES1 cells possess both *in vitro* and *in vivo* pluripotency characteristics, indicating that non-mammalian ES cells can be readily derived and maintained for a long term under feeder-free culture conditions.

Introduction

EMBRYONIC STEM (ES) CELLS are undifferentiated cells derived from early-stage embryos and are capable of retaining their pluripotency after long-term *in vitro* culture. These cells possess the ability to partake in normal development events and can contribute to the germline of recipient embryos to form chimera.¹ The mouse ES cells, which is the first ES cell line to be successfully established, has enabled the generation of gene knockout models through random transgenesis or gene targeting.^{2,3} ES cells are, therefore, ideal experimental tools for the generation of desired animal models for many research areas ranging from development to human health and diseases. Theoretically, ES cells can also be induced to differentiate into a myriad of cell lines for applications in medicine and, most importantly, drug screening.⁴

Attempts to derive ES cells from teleost species were first reported more than two decades ago and have ever since resulted in the establishment of ES-like cell lines from medaka, zebrafish, gilthead seabream, sea perch, red sea bream, Japanese flounder, Asian sea bass, turbot, Indian major carp, and Atlantic cod.^{5–15} A common postulation on the potential application of ES cells from fish species with commercial values is the development of transgenic fish with beneficial traits.^{16,17} This approach has been proposed to be more effective than the direct introduction of foreign transgenes into zygotes, which seldom produces the desired integration of the transgene.¹⁸ In tandem, small teleosts such as medaka and zebrafish, which are important model organisms in developmental biology, could benefit from the ES cell-based gene targeting approach.

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Human and mouse ES cells were initially established and maintained in the presence of feeder layers or supplemented with media conditioned by feeder layers.¹⁹ However, the use of feeder layer poses the problem of contamination, the need to separate ES cells from feeder cells, and the presence of undefined factors, which collectively influence downstream applications. Feeder-free culture methods, which aspire to culture and maintain ES cells in an undifferentiated state without the need for direct contact with feeder cells, were eventually developed.^{19,20} For teleost ES cells, the feeder-free approach has been widely applied, indicating that feeder cells might not be a prerequisite for the derivation of ES cells in teleost or other lower vertebrates.²¹ Surprisingly, long-term feeder-free derivation and culture of zebrafish ES cell lines have not been successfully demonstrated. Despite several attempts, pluripotent zebrafish ES cells could only be maintained for short periods under feeder-free conditions.^{22,23}

To address this issue, we initiated this effort to formulate a feeder-free approach for the derivation and maintenance of zebrafish ES cells. We report the successful establishment of an ES-like cell line and its long-term maintenance under feeder-free conditions. This line, designated as ZES1, possesses cells that remain undifferentiated for long periods and display prominent ES cell characteristics, including positive AP staining and also the ability to form compact cell colonies. ZES1 cells readily formed embryoid bodies (EBs) and under the influence of initial seeding density, differentiated into specific cell types. Fluorescent-labeled ZES1 cells transplanted into zebrafish blastulas resulted in chimerism in the developing embryos. Finally, using GFP-ZES1 cells, which ubiquitously expressed GFP under the influence of the CMV promoter, chimera germline transmission was demonstrated in embryos obtained from the F₁ generation.

Materials and Methods

Isolation of zebrafish blastomeres

Maintenance, breeding, collection, and staging of zebrafish embryos from an established population obtained from a local aquarium shop were carried out as published.²⁴ Embryos were incubated at 28.5°C until blastula stage (3.33 hpf), and 60–70 healthy embryos were used to initiate each primary culture. The embryos were rinsed with phosphate-buffered saline (PBS; Invitrogen) and treated with 0.125% bleach solution (NaOCl). Embryos were treated with Pronase (Sigma) at 28.5°C for 20 min and rinsed with PBS. The dechorionated embryos were exposed to 0.05% Trypsin/EDTA (Invitrogen) for not more than 2 min to dissociate the blastomeres, and 200 μ L of fetal bovine serum (FBS, Invitrogen ES Cell Grade) was added to terminate trypsinization. The cell suspension was centrifuged at 500 *g* for 5 min at 28°C to separate the yolk debris from the dissociated cells. The pelleted cells were seeded into a single well ($\sim 1 \times 10^5$ cells/cm²).

Establishment of zebrafish ES cell-like line

ZES1 cells were cultured using gelatin-coated tissue culture plates and maintained at 28.5°C. Gelatin-coated tissue culture plates were prepared according to Yi *et al.*²⁵ Passaging was carried out when cell confluency reached 80%–90%. Cells were trypsinized with 0.05% Trypsin/

EDTA for 1 to 2 min before addition of 1 mL of ESM2 medium. After trypsinization, the cells were resuspended into single cells and transferred into a new tissue culture well at a split ratio of either 1:2 (6.0×10^4 cells/cm²) or 1:4 (3.0×10^4 cells/cm²). This required ZES1 cells to be subcultured approximately every 2 to 4 days, on reaching confluency of 80%–90%, respectively. After 50 passages, ZES1 cells were subsequently maintained in ESM4 medium, which, similar to ESM2 medium, enabled the maintenance and propagation of pluripotent ZES1 cells but at a reduced cost and labor. For cryopreservation, cells from a single well of a six-well plate were trypsinized, centrifuged and the cell pellet was resuspended in 500 μ L of ESM4 medium. The cell suspension was chilled at 4°C for 10 min before addition of 500 μ L of 2 $\times$ freezing medium (ESM4 medium with 40% FBS and 20% DMSO). Cells were transferred into a cryovial, chilled at 4°C for 20 min, before transferring to a –80°C freezer overnight, and finally kept in liquid nitrogen.

ESM2 medium consisted of high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 20 mM HEPES (Sigma-Aldrich), 15% of ES cell-grade FBS (Invitrogen), 1 embryo/mL of zebrafish embryo extract (self prepared), 1% of trout serum (Caisson), 10 ng/mL of human basic fibroblast growth factor (bFGF; PeproTech), 2 mM of L-glutamine (Invitrogen), 1 mM of nonessential amino acids (Invitrogen), 1 mM of sodium pyruvate (Invitrogen), 100 U/mL of penicillin-streptomycin (Invitrogen), 100 μ M of 2-mercaptoethanol (Sigma-Aldrich), and 2 nM of sodium selenite (Sigma-Aldrich). This medium, with the exception of the zebrafish embryo extract, was originally used for the initiation and maintenance of medaka ES cells.²⁵ The ESM4 medium was similar to ESM2 medium, except for the reduction of zebrafish embryo extract (0.4 embryo/mL) and human basic fibroblast growth factor (2 ng/mL). For preparation of zebrafish embryo extract, a total of 500 embryos (30 hpf) was rinsed with PBS and stored at –20°C until use. Embryos were homogenized gently in cold PBS followed by centrifugation at 3500 *g* for 30 min at 4°C. The supernatant was collected and centrifuged again at 18,000 *g* for 30 min at 4°C. PBS was then added to a final volume of 2.5 μ L/embryo and stored at –20°C. Shortly before addition to either ESM2 or ESM4 media, the embryo extract was thawed and centrifuged at 18,000 *g* for at least 30 min at 4°C to remove any residual debris.

EBs formation

ZES1 cells were inoculated at a density of 1×10^5 cells/mL in a nontreated and uncoated low-adherence six-well plate (NUNC) using ESM4 medium. The ZES1 cells readily formed EBs after 2 days. By Day 4, the EBs were collected and replated onto a gelatin-coated culture well. The ESM4 medium was changed every 2 days.

In vitro differentiation potential at different seeding density

Differentiation potential of ZES1 cells was investigated by culturing the cells in low-density (4.8×10^2 cells/cm²) or high-density (1.2×10^5 cells/cm²) conditions, respectively, with a regular ESM4 medium change every 2 days. For high-density differentiation, the cells were cultured for approximately 8 weeks without passaging. ZES1 cells were also

subjected to retinoic acid (RA; 10 μ M; Sigma) treatment for induction of neuronal differentiation. Changes in cell morphology were observed using a microscope, and the cellular identity of differentiated cells was also confirmed using immunocytochemistry staining.

Immunocytochemistry and alkaline phosphatase staining

Alkaline phosphatase (AP) staining was carried out using an Alkaline Phosphatase Staining Kit II (Stemgent) according to the manufacturer's instructions. Immunocytochemistry was carried out using MAXpack Immunostaining Media Kit (Active Motif) as described²⁶ with slight modifications. The primary antibodies used were rabbit anti-SOX2, mouse anti-SOX17, mouse anti-MAP2 (2A + 2B), mouse anti-Pan Cytokeratin, and mouse anti- α -Actinin (Sigma). Antibodies were diluted in MAXbind Staining Medium (Active Motif). Cells were incubated with primary antibody for 24 h at 4°C, rinsed thrice with MAXwash Washing Medium, and incubated in secondary antibodies anti-Mouse IgG-FITC (Sigma) and anti-Rabbit IgG-FITC (Abcam) for 1 h at 37°C. After rinsing with MAXwash Washing Medium, the cells were exposed to Fluoroshield with DAPI (Sigma) for 15 min and observed under a fluorescence microscope (Olympus IX71).

Fluorescent-activated cell sorting analysis

ZES1 cells were dissociated by 0.125% Trypsin-EDTA and washed twice with DPBS. For fixation, cells were incubated in 4% paraformaldehyde in DPBS for 10 min. For permeabilization, cells were incubated with 0.1% Triton-X-100 in DPBS for 10 min. For blocking, cells were incubated in 1% Blocker™ BSA (Pierce) in DPBS at room temperature for 1 h. Thereafter, cells were incubated with 1:100 (10 μ g/mL) rabbit IgG anti-SOX2 primary antibody (Abcam; cat no. ab97959) at 4°C overnight. Finally, cells were incubated with goat anti-rabbit IgG FITC secondary antibody (Abcam; cat.no. 6717) at room temperature for 1 h. In all these procedures, cells were washed twice with 1%BSA in DPBS after each step. Subsequently, cells were resuspended in 1%BSA/DPBS and processed in a BD LSR Fortessa Flow Cytometry Analyzer (BD Biosciences) adjusted for the detection of FITC fluorescence (Ex 488 nm/Em 515–545 nm). Data were collected for approximately 100,000 events and analyzed by Summit 4.3 software (Beckman Coulter).

RNA extraction and semi-quantitative real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (RT-PCR) was used to evaluate the relative gene expression of pluripotency-associated markers, including *sox2*, *nanog*, *pou5f1*, and *lin28* in ZES1 and a zebrafish liver cell line (ZFL; ATCC). Total RNA was extracted from these cells using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. The primers used for amplification of the candidate pluripotency-associated markers and β -actin are listed in Table 1. PCR amplification was performed using iScript™ One-Step RT-PCR kit on IQ™5 Multicolor Real-time PCR detection system (Bio-Rad). A total of 100 ng of DNase-treated RNA was reverse transcribed into first-strand cDNA at 50°C for 20 min and followed by reverse transcriptase inactivation at 95°C for 5 min. This was followed by PCR amplifications using 40 cycles of denaturation at 95°C for 10 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. Gene expression level was calculated using the IQ™5 optical system software (BioRad), with normalization to β -actin. The experiments were replicated thrice.

Chromosomal analysis of ZES1 cells

Chromosomal analysis was carried out according to Yi *et al.* with slight modifications.²⁷ ZES1 cells at 80% confluency were incubated for 4 h in culture medium containing colchicine (Sigma) at 1 μ g/mL. After trypsinization, the cells were resuspended in 50 μ L of PBS, followed by addition of 40 mM KCl (Sigma) and incubated for 30 min at room temperature. After centrifugation, the cell pellet was resuspended in 1 mL of methanol: glacial acetic acid (3:1) fixative for 1 h with two changes of fixative. Finally, the cell pellet was resuspended in 0.5 mL of fixative and transferred onto cold wet slides. The slides were allowed to air-dry overnight at room temperature and stained with Fluoroshield with DAPI (Sigma). Chromosome numbers were counted and metaphases that contained ± 25 , ± 50 , and ± 100 were grouped as haploid, diploid, and polyploid cells, respectively.

Fluorescent dye-labeling and transplantation of ZES1 cells into zebrafish embryos

Cells from ZES1 and a diploid medaka ES line, MES1, were labeled with fluorescent dyes PHK26 (red; Sigma) and

TABLE 1. PRIMER SEQUENCES FOR REAL-TIME POLYMERASE CHAIN REACTION EXPRESSION ANALYSIS OF *SOX2*, *NANOG*, *POU5F1*, *LIN28*, AND β -ACTIN

Zebrafish gene	Accession number		Primer sequence (5' → 3')
<i>sox2</i>	NM213118	Forward	GTTGACAAGGGCTCTGGCGAGG
		Reverse	AGGTCCTGTTGTGCCACAAACTT
<i>nanog</i>	NM001098392	Forward	CCAAAAGGCCAAAGATGCAG
		Reverse	GGAACCCCTTCTCGACTGCT
<i>lin28</i>	NM201091	Forward	TACCCAAAAGAGGCGGTCAA
		Reverse	CGATTTGCCCTGAGATCCTG
<i>pou5f1</i>	NM131112	Forward	CCGCCGTCACAATATCACCT
		Reverse	CGGAGACAGAGATGGGGATG
β -actin	NM001001831	Forward	GGATTCGCTGGAGATGATGC
		Reverse	CGTGCTCGATGGGGTACTTC

Each sequence is listed starting from the 5'-end with its corresponding GenBank ID. F, forward primer; R, reverse primer.

PHK67 (green; Sigma), respectively.²⁸ Briefly, cells were first trypsinized, before staining with the respective dyes (2×10^{-6} M) for 5 min at a density of 10^7 cells/mL in Diluent C (Sigma). The labeling reaction was terminated by the addition of serum, and unbound dyes were removed by repeated washing with PBS.

Chimera formation was evaluated through co-transplantation of PHK26-labeled ZES1 cells and PHK67-labeled MES1 cells into midblastula zebrafish embryos as described elsewhere.^{29,30} Labeled ZES1 and MES1 cells were suspended in transplantation medium (TM; 100 mM NaCl, 5 mM KCl, 5 mM HEPES, pH 7.1). Zebrafish embryos were treated for 2 min with pronase E (1 mg/mL; Sigma) at 26°C for dechorionation. A total of 200 donor cells (equal ratio of ZES1 and MES1 cells) were injected into the deep cell layer of each midblastula recipient. After injection, the embryos were incubated in zebrafish egg water at 23°C–28°C and regularly monitored under a fluorescent microscope.²⁹

Formation of germline chimera using GFP-ZES1 cell line

ZES1 cells were transfected with the plasmid, pCMV-HygEGFP, using FuGENE HD transfection reagent (Promega), and selected with Hygromycin B (250 μ g/mL) to establish a stable ZES1 cell line (GFP-ZES1) that strongly expressed GFP under the regulation of the CMV promoter.

Zebrafish embryos were rinsed and aligned with their animal pole facing upward on an agarose (1.5%) microinjection plate. Between 50 and 100 single GFP-ZES1 cells, which were prepared by treatment with 0.05% of EDTA-Trypsin for not more than 3 min, were injected into the deep cell layer of zebrafish embryo (sphere stage) using a Nanoliter 2000 Injector (World Precision Instruments) fitted with a borosilicate capillary needle [20 μ m diameter (cat. no.: 4878)]. Injected embryos were allowed to recover in E3 medium for 2 h at 28.5°C before transferring to a petri dish.³¹ GFP fluorescence in zebrafish embryos (48 hpf) was analyzed using a fluorescent microscope (Olympus IX73). Treatment with 0.003% of 1-phenyl 2-thiourea (PTU) to inhibit melanin formation was also carried out. Injected embryos were observed at 48 hpf, and those expressing GFP were separated and raised to adult stage as potential founder chimeras (F₀). Potential F₀ chimeras were outcrossed with wild-type individuals, and the resulting 48 hpf embryos (F₁) were screened for GFP expression using a fluorescent microscope and a confocal microscope (Leica TCS SP5X). Confocal images were processed using ImageJ software (version 1.47).³²

PCR detection of pCMV-HygEGFP in chimera F₁ larvae

For further validation, GFP-positive F₁ chimera embryos were collected for detection of *EGFP* gene.³³ Single F₁ embryos (96 hpf) were submerged in 20 μ L of 50 mM NaOH and heated at 95°C for 10 min until complete lysis. The lysate was cooled on ice, and then, 2 μ L of 1 M Tris-HCl, pH 8.0 was added. PCR amplification to detect the *EGFP* gene was carried out using GoTaq Flexi PCR Kit (Promega) with 1–5 μ L of lysate. The following PCR parameters were used: denaturation at 95°C for 30 s, annealing at 58.5°C for 30 s, and extension at 72°C for 40 s, for 40 cycles. The *EGFP* Forward and Reverse PCR primers were 5'-AGCTG

GACGGCGACGTAAAC-3', and 5'-GTCCATGCCGAGA GTGATCC-3', respectively. The 653 bp *EGFP* PCR product was purified using Expin Gel SV Kit (GeneAll), cloned into pGEMT-Easy cloning vector (Promega), and confirmed by DNA sequencing.

Results

Establishment of zebrafish ES-like cell line

The zebrafish ES-like cell line was derived from blastomeres harvested from blastula-stage embryos (Fig. 1A). These freshly isolated blastomeres had an average diameter of 12 to 25 μ m (Fig. 1B). Homogenous aggregates were formed approximately at 3 to 5 h after seeding, with the majority of the aggregates attaching to the surface after 24 to 30 h (Fig. 1C). Full confluency was achieved at 48 h after initiation of primary culture, and subculture was carried out every 2 days. Of the nine primary cultures that were initiated, one eventually survived, showing stable growth dynamics and, more importantly, ES-like characteristics. The surviving culture was expanded into a stable cell line designated as ZES1.

ZES1 cells were either round or polygonal, with a large nucleus and sparse cytoplasm and to date, they have been continuously cultured for more than 800 days and 400 passages without any phenotypic changes. In addition, the cell line did not display any senescence throughout the entire culture period (Fig. 1D, E). A small percentage of cells (<1%) had undergone spontaneous differentiation into epithelial-like cells (not shown), but since these differentiated cells had a limited life span, they did not affect the majority of the ES-like cells. The majority of the ZES1 cells were positively stained with AP, a widely used marker for pluripotency (Fig. 1F). In addition, ZES1 cells could be cryopreserved in liquid nitrogen without any loss of ES-like characteristics. Single ZES1 cells formed tightly compact and uniform colonies, at usually 10 to 14 days after initial seeding. These colonies could be individually isolated and used to initiate a new cell culture.

In vitro differentiation potential of ZES1 cells is dependent on initial seeding density

When seeded at a low density (4.8×10^2 cells/cm²), ZES1 cells differentiated into neuronal and fibroblast cells (Fig. 2A–C). Conversely, when seeded at a high density (1.2×10^5 cells/cm²) and with only regular media changes for 4 to 8 weeks, ZES1 cells mainly differentiated into muscle or epithelial cells (Fig. 2D–F), which were positively stained with α -Actinin (Fig. 2G) and pan-Cytokeratin (Fig. 2H), respectively. In addition, there were differentiated cells that were positive for SOX17, a marker for endoderm cells (Fig. 2I). Treatment of ZES1 cells with RA mainly induced differentiation into neural cells as indicated by the presence of neuronal marker, MAP2 (Fig. 2J, K). In undifferentiated ZES1 cells, none of the earlier markers could be detected (data not shown). EB formation could also be readily achieved using suspension culture with uncoated tissue culture wells. Individual ZES1 cells reaggregated within 24 h, and these aggregates rapidly increased in size and formed spherical-shaped EBs by the end of Day 4 (Fig. 2L). These EBs were able to differentiate into neuron, fibroblast, and epithelial

FIG. 1. Morphological characteristics of zebrafish blastomeres and ZES1 cells. (A) Blastula stage of zebrafish embryos at 4 hpf. (B) Blastomeres isolated from zebrafish blastula. (C) Formation of cell aggregates at 3 h after seeding of zebrafish blastomeres (scale bar = 50 μ m). (D) Blastula-derived culture at passage 10 showing small and round or polygonal cells. (E) ZES1 cells at 199 days of culture with 81 passages showing large nuclei and sparse cytoplasm. (F) Positive alkaline phosphatase (AP) staining of a colony of ZES1 cells at 415 days.

cells when subsequently replated onto gelatin-coated wells (data not shown).

Immunocytochemistry of SOX2 in ZES1 cells

Immunostaining of ZES1 cells with antibodies against SOX2 confirmed its presence in 100% of the undifferentiated cells (Fig. 3A, B). Treatment of ZES1 with RA significantly reduced the number of SOX2-positive ZES1 cells to around $45.4\% \pm 5.1\%$ (Fig. 3C, D). SOX2 was also no longer expressed when ZES1 cells differentiated into muscle or epithelial cells, as a result of high-density culture conditions (Fig. 3E, F). The percentage of SOX2-positive ZES1 cells was quantitatively determined by employing fluorescent-activated cell sorting technique on ZES1 cells stained with anti-SOX2 and FITC antibodies. Results showed that 99.53% of ZES1 cells expressed SOX2 (Fig. 4).

*Real-time PCR of zebrafish *sox2* and *pou5f1**

Real-time PCR results revealed that ZES1 cells had a significantly higher expression of *sox2* and *lin28* as compared with ZF-L (Fig. 5). As for *nanog* and *pou5f1*, no significant differences in transcript levels of both genes was observed between both cell types, respectively.

Chromosomal analysis of ZES1 cells

Karyotypic analysis on 800 metaphases obtained from ZES1 clones showed that the majority of the modal number of ZES1 chromosomes were within the diploid range of ± 50 (86.25%), while the remaining were of polyploidy nature (Fig. 6 and Table 2).

Chimera formation with ZES1 cells

Fluorescent-labeled ZES1 and MES1 cells transplanted into zebrafish embryos were able to form chimeric embryos (Fig. 7A–C). ZES1 and MES1 cells showed overlapping distribution near the optic primordial of 1 dpf embryo (Fig. 7D–F). Observation of 2 dpf embryos revealed that ZES1 cells were also able to establish chimerism in the eye, heart, and trunk regions (Fig. 8A). Concurrently, some of these regions also showed the presence of MES1 cells (Fig. 8B–F).

A stable ZES1 cell line (GFP-ZES1) expressing GFP under the regulation of the CMV promoter was obtained by liposome transfection of ZES1 cells using the plasmid, pCMV-HyEGFP. The injection of these GFP-ZES1 cells (50 to 100 cells) into zebrafish embryos resulted in colonization of different regions, including the notochord, spinal cord, fin, head,

FIG. 2. *In vitro* differentiation characteristics of ZES1 cells. (A) Low-density culture of ZES1 cells induced differentiation (scale bar = 100 μ m) (B) neuron-like cells and (C) fibroblast-like cells. (D) High-density culture of ZES1 cells (scale bar = 50 μ m) induced differentiation to (E) muscle-like cells and (F) epithelial-like cells that are positively stained with (G) α -Actinin (scale bar = 50 μ m) and (H) pan-Cytokeratin, respectively. (I) High density also resulted in positive staining of SOX17, a marker for endoderm layer. (J) Continuous treatment of ZES1 with all-*trans* retinoic acid (RA) induced differentiation into mostly neural-like cells that are (K) positively stained with MAP2 (scale bar = 20 μ m). (L) 2 day-old embryoid bodies formed from ZES1.

eye, trunk, heart, and extraembryonic lineages such as yolk and yolk sac stripe (Fig. 9A–P).

The outcrossing F_0 founders with wild-type zebrafish were carried out to generate potential chimera F_1 embryos in order to confirm the germline transmission of the *EGFP* gene. A total of 61 F_0 founders were screened, and three individuals

(M1, M3, and M11) that generated GFP-positive germline F_1 chimeras were first identified by fluorescence microscopy, and subsequently verified by PCR amplification of the genomic *EGFP* (Fig. 10A, B). GFP expression at 48 hpf was mainly confined to the head region, eyes, and trunk of the chimera F_1 embryos (Fig. 10C).

FIG. 3. Immunostaining of ZES1 cells and differentiated ZES1 cells with SOX2. Undifferentiated ZES1 cells exhibited positive staining with (A) SOX2 and (B) DAPI. RA-treated ZES1 cells also showed positive (C) SOX2 staining at neural structures and (D) DAPI at nucleus. Immunostaining of ZES1 cells maintained in high density for 60 days showed (E) nonstaining of SOX2 in cells and (F) DAPI in nucleus.

FIG. 4. Overlapped histogram of SOX2 expression in ZES1 cells as analyzed by fluorescent-activated cell sorting. R1=ZES1 immunostained with FITC secondary antibody only, R2=ZES1 immunostained with anti-SOX2 primary antibody, and FITC secondary antibody. SOX2-positive cells lie in the region R2. ZES1 cells are 99.53% SOX2 positive.

FIG. 5. Comparative expression of *sox2*, *nanog*, *lin28*, and *pou5f1* in ZES1 and zebrafish adult liver cell line ZFL using semi-quantitative real-time PCR. Relative expression levels were based on normalization to β -actin level. Results shown here are representative of three independent replicates. *Denotes significance difference in expression ($p < 0.05$).

Discussion

Previously, zebrafish ES cells with self-renewal and pluripotent properties were derived with the aid of zebrafish embryonic fibroblasts or rainbow trout spleen cells as feeder layers.^{10,34} Despite the advantages of a feeder-free culture system, previous efforts to derive and maintain zebrafish ES cells were, however, unsuccessful. For example, primary

cultures of zebrafish blastula cells established without the support of feeder layers rapidly differentiated into melanocytes.²³ A zebrafish ES cell line derived and maintained using rainbow trout spleen cells as feeder layer could not maintain the expression of *pou2* when they were removed from their feeder cells.³⁴ More recently, when feeder layer-derived zebrafish ES-like cell line was cultured using a feeder-free media, it resulted in a potentially differentiated line with

FIG. 6. Chromosomal analysis of ZES1 cells. (A, B) DAPI staining on chromosomes and (C, D) inverse images to emphasize the DAPI staining. (A, C) Metaphase spread of diploid ($2n=50$) ZES1 cells at passage 183 and (B, D) karyogram showing 25 pairs of chromosomes arranged in decreasing lengths (bar = 10 μ m).

TABLE 2. CHROMOSOME COUNTS ON ZES1 CELL LINE

ZES1 clones	Passage number starting from colony isolation	Total count	Chromosome numbers (%)		
			Haploid ± 25	Diploid ± 50	Polyploid ± 100
1	11	100	—	90	10
2	10	100	—	87	13
3	10	100	—	90	10
4	16	100	—	86	14
5	16	100	—	83	17
6	10	100	—	84	16
7	13	100	—	79	21
8	14	100	—	91	9
Total		800	—	690 (86.25)	110 (13.75)

A total of 100 metaphases were counted for each clone.

reduced pluripotency.²² We report for the first time the successful establishment and long-term maintenance of an ES-like cell line termed ZES1, under feeder-free culture condition.

The removal of the feeder layer will require the development of a defined culture medium that could support the maintenance of zebrafish ES cells in their absence.²⁰ The medium used to successfully establish and maintain ZES1 is essentially a DMEM medium supplemented with bFGF, FBS, zebrafish embryo extract, and trout serum. The medium composition was originally used to successfully derive several lineages of medaka ES cells and subsequently extended to the establishment of ES cell lines from other fish species.^{6–9,11,25,35} A key difference between our tissue culture medium as compared with the previous unsuccessful formulations is the supplementation with zebrafish embryo extract.^{22,26} It has been suggested that fish embryo extract contributes to the mitogenic activities of fish ES cell culture in a feeder-free culture system and could be a decisive addition in our present culture medium.^{6,8} The inclusion of embryo extract

was also reported in the medium of the feeder-free ES cells culture protocol of several fish species.^{6,7,15,36}

The attributes of pluripotent mouse ES cells include stable long-term culture, small and polygonal cell morphology, normal karyotype, high AP activity, and the capacity to differentiate into specific cell types under defined conditions.³⁷ Likewise, ZES1 cells have ES-like morphological appearances, display pluripotent properties, and remain undifferentiated after being continuously cultured for almost 30 months. In addition, these cells could be cryopreserved and successfully revived without any damaging effects. The isolated blastomeres formed homogenous aggregates within 3 h of seeding in the well plate before attaching to media surface within 24 h. These events are also reported in the establishment of zebrafish ES cells using a feeder layer.^{38,39} Morphologically, ZES1 cells also share similar characteristics with other fish ES cells, such as having a round or polygonal shape, a large nuclei, and sparse cytoplasm.^{11,35,39} ZES1 colonies were strongly stained with AP, a common marker that has been used to positively identify pluripotency in zebrafish ES cells and other fish ES cell lines.^{6–9,14,38}

Our results also show how low-density culture condition induced the differentiation of ZES1 cells into neural and fibroblast cells, while muscle and epithelial cells appeared exclusively under high-density culture condition. ES cells isolated from several species, including medaka, sea bream, and sea perch, could also be directed to differentiate into neuronal or muscle-like cells when cultured in low and high density, respectively.^{6,7,15} Treatment of ZES1 with RA also induced their differentiation into neuronal cells. This is reminiscent of previous findings that reported differentiation of fish ES cells into neuronal cells after exposure to RA.^{7,9,35} Similarly, ES cells from Asian sea bass differentiated into GFAP-stained oligodendrocytes when treated with RA.¹¹ ZES1 cells could form spherical and tight EBs in suspension culture, similar to observations reported for zebrafish ES cells raised with feeder layer, or medaka ES cells cultured under feeder-free condition.^{6,38} Formation of EBs is also a regular feature of ES cell lines, from sea bass, sea perch, and Indian major carp.^{8,11,35} RA treatment of EBs formed from the ES

FIG. 7. Chimera formation in zebrafish embryos co-transplanted with fluorescent dye labeled ZES1 (red) and MES1 (green) cells during midblastula stage. (A) Bright field image of injected 1 dpf embryos. (B) Green fluorescent image of injected 1 dpf embryos. (C) Red fluorescent image of injected 1 dpf embryos. (D) Bright field image of an injected 1 dpf embryo. (E) Green fluorescent image of injected 1 dpf embryo showing labeled cells. (F) Red fluorescent image of injected 1 dpf embryo showing labeled cells. (op, optic primordia; ys, yolk). Scale bar (A–C 200 μ m; D–F 100 μ m).

FIG. 8. Chimera formation in zebrafish embryos co-transplanted with fluorescent dye labeled ZES1 (red) and MES1 (green) during mid-blastula stage. (A) Merged bright field and red fluorescent image of injected 2 dpf embryo. (B) Merged bright field and green fluorescent image of injected 2 dpf embryo. (C) Merged bright field, red, and green fluorescent image of injected 2 dpf embryo. (D) Merged red and green fluorescent image of injected 2 dpf embryo. (E) Merged bright field, red, and green fluorescent dorsal image of injected 2 dpf embryo. (F) Merged red and green fluorescent dorsal image of injected 2 dpf embryo. *Dotted lines* denote areas showing presence of ZES1 cells (ey, eye; so, somite; ys, yolk). Scale bar (100 μ m).

cells of Indian major carp species also resulted in differentiation into neural cells.⁸ Collectively, our results demonstrate that ZES1 cells possess *in vitro* differentiation capabilities.

Human and mouse ES cells rely on a common core of transcriptional regulators, including NANOG, SOX2, OCT4 (POU5F1), and LIN28, to govern pluripotency and self-renewal pathways.^{40–42} Both immunostaining and real-time PCR results showed that Sox2 was notably present in undifferentiated ZES1 cells. In mouse ES cells, knockdown of SOX2 expression resulted in the loss of pluripotency and a concomitant increase in differentiation rate.⁴³ In addition, SOX2 levels in mouse ES cells are tightly regulated in order to maintain pluripotency.⁴⁴ Similarly, SOX2 is obligatory for both self-renewal and pluripotency in human ES cells.⁴⁵ In tandem, we also detected Sox2 in neuronal cells derived from RA treatment of ZES1. Zebrafish Sox family proteins are important for dorsal patterning, gastrulation, neural differentiation, and patterning, largely in cooperation with Pou5f1.⁴⁶ Robles *et al.* have reported the presence of Sox2 in the region of the blastula which will subsequently give rise to neuronal precursors, leading them to suggest that Sox2 is

more important for neurogenesis rather than for pluripotency in zebrafish.²⁶ Thus, the presence of Sox2 in undifferentiated ZES1 cells and neural cells here is consistent with known roles in their mammalian counterparts.

The transcription factor POU5F1/OCT4 is a pivotal regulator of pluripotency in mouse embryonic inner cell mass cells and ES cell lines of mouse and human.⁴⁷ In zebrafish, *pou2* was first reported as the homolog of the mammalian *POU5F1/OCT4*.⁴⁸ It was proposed that a *POU5F1/POU2* gene syntenic to the zebrafish *pou2* had first undergone duplication and then subsequent selective deletion to give rise to *POU2* in amphibians and avians, and *POU5F1* in mammals, respectively.^{49,50} Subsequently, the zebrafish nomenclature committee has agreed on the use of *pou5f1* instead of *pou2* (RefSeq-ID NM_131112.1), as zebrafish *pou5f1* is considered an ortholog of the mouse *Pou5f1/Oct4*.⁵¹ *Pou5f1* has been reported in the ES cells of carp, gilthead seabream, sea bass, flatfish, and Atlantic cod.^{8,11,12,14,36} In contrast, the level of *pou5f1* was significantly reduced when medaka ES cells were induced to differentiate.⁵² Recent findings also suggest that medaka Pou5f1 could regulate pluripotency in mammalian ES

FIG. 9. Formation of chimera in zebrafish embryos injected with GFP-ZES1 cells. (A–P) Colonization of GFP cells in different regions of zebrafish larvae (indicated by *red arrow*) at 48 hpf by GFP-ZES1. (A, B) spinal cord and notochord, (C, D) fin, (E, F) head, (G, H) trunk, (I, J) heart, (K, L) gonadal region, (M, N) yolk, and (O, P) yolk sac stripe (t, trunk; n, notochord; y, yolk; f, fin; h, head; ht, heart). Scale bar (A–F, I, J) 50 μ m; (G, H, K–P) 100 μ m.

cells.^{53,54} We did not detect any difference between the expression of *pou5f1* in ZES1 and ZFL, which suggest that this gene may not be associated with pluripotency in zebrafish. It has been reported that zebrafish *Pou5f1* could not support pluripotency in mammalian ES cells.⁵⁴ The reason

for this remains to be fully elucidated, although the failure of zebrafish *Pou5f1* in sustaining mammalian ES cell pluripotency has been linked to the role of the mammalian OCT4 in uterine development, which is not represented in lower vertebrate species.⁵⁴

FIG. 10. Analysis of F₁ germline chimeras generated by crossing of GFP-ZES1 transplanted F₀ chimeras with wild-type individuals. (A) Pool of several non-GFP (NG) and GFP 48 hpf F₁ larvae (G), (scale bar = 500 μ m). (B) PCR of 96 hpf F₁ embryos generated by crossing of GFP-ZES1 transplanted F₀ chimeras with wild-type fish for detecting genomic *EGFP* gene. Lane 1 showed nonamplification of *EGFP* gene in single non-GFP expressing F₁ embryo. Lanes 2 and 3 showed amplification of *EGFP* gene in two different GFP expressing F₁ embryos. Lane 4 is positive control using pCMV-HygEGFP as PCR template. Lane 5 is negative control without adding template. 100 bp ladder was used as a marker. (C) Confocal image of a F₁ chimeric larva at 48 hpf with GFP expression in the central nervous system, eye, and trunk region of the larva (scale bar = 100 μ m).

Although *nanog* was detected in ZES1, the level of mRNA was not significantly higher than that in zebrafish liver cell line. In medaka, *nanog* was reported to be unnecessary for early lineage differentiation and pluripotency but crucial for proliferation of medaka ESCs through the regulation of the S phase of the cell cycle.⁵⁵ Elsewhere, the zebrafish *nanog* was able to promote proliferation in mouse ESCs, denoting a possible functional conservation of *nanog* in fish and mammals.⁵⁶ In addition, morpholino-mediated knockdown of *nanog* in zebrafish perturbed gastrulation events in embryos.⁵⁶ The significant presence of Lin28, an RNA-binding protein in ZES1, correlates with the known role of this gene in mammalian ES cells.^{57,58} In zebrafish, *lin28* was shown to be necessary for retinal regeneration, a process that also involves the expression of several other pluripotency factors.⁵⁹ The establishment of a stable zebrafish ES cell line under feeder-free conditions will facilitate efforts to decipher the full spectrum of the pluripotency pathway in this species.

Karyotype stability can be compromised when ES cells are cultured for extended periods in feeder-free conditions, which, in turn, can affect the contribution of ES cells toward chimera formation.⁶⁰ Chromosomal analysis revealed that despite being maintained for extended periods, ZES1 cells had a stable diploid karyotype similar to what has been reported for zebrafish ES cells which were maintained with feeder layers.¹⁰ Equally important, using ZES1 cells which were stained with fluorescent dyes, we showed that they could partake in the development and formation of a wide range of tissues or organs, including the eye, heart, and trunk regions. Therefore, comparable to feeder-layer-derived zebrafish ES cell lines,⁶¹ ZES1 cells not only harbor a normal and stable karyotype but are also able to contribute to the formation of chimerism. The ability of diploid medaka ES cell line to form chimerism in the ZES1-injected embryos further corroborates with the earlier finding reporting the ability of this cell line to embrace the zebrafish developmental program.²⁹

We demonstrated that F₁ germline chimeras could be generated by the transplantation of GFP-ZES1 cells, which overexpressed GFP under the influence of the CMV promoter, into recipient zebrafish embryos. The transplanted GFP-ZES1 cells contributed to normal embryonic development of the recipient embryos, with GFP expression detected in regions such as the brain, eye, epidermis, fin, and heart. This is similar to what has been reported for the medaka ES cells, MES1, which also uses a similar GFP lineage tracing.⁶² Our ability to obtain chimera zebrafish embryos suggests that the ZES1 cells behave in a manner which closely resembles that of pluripotent ES cells. Nevertheless, similar to the zebrafish embryo, cells were previously derived and successfully used for the generation of chimera zebrafish.^{31,34,61} ZES1 cells harbor the potential for further application in cell-mediated gene transfer. Ma *et al.* advocate the use of feeder cells-conditioned medium to maintain zebrafish embryo cells during the processes of introducing targeting vector and selection of successfully transformed cultures, before transferring transformed cells to feeder layers for further expansion.⁶¹ The readiness of ZES1 cells for long-term culture potentially facilitates these processes.

In conclusion, we present here the derivation of an ES-like cell line from the zebrafish blastula stage. The ZES1 cells could be maintained for a long term under feeder-free con-

ditions and retained key morphological and pluripotent characteristics of ES cells such as self-renewal, expression of selected pluripotency markers, capacity for directed differentiation, and ability for formation of germline chimeras. Our protocol has simplified the establishment and maintenance of zebrafish ES cells and will hopefully facilitate additional studies utilizing zebrafish ES cells in the future.

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Disclosure Statement

No competing financial interests exist.

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