

Establishment and Characterization of Clonal Cell Lines from Zebrafish, *Danio rerio*

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ABSTRACT Three types of clonal cell lines were isolated according to their size and phenotype from the adherent cell populations in long-term liquid cultures from the embryonic fibroblast cells of Zebrafish, *Danio rerio*. All kind of cell lines were well proliferated. The size and number of clonal cell lines derived colonies from stable embryonic cells were significantly increased in the presence of NAC and A2P conditioned medium from the cell lines. The stable cell lines and clonal cell lines were capable of well proliferation *in vitro*. These cell lines have been maintained in continuous culture without change in characteristics. A majority of the clonal cells (80%) was shown a normal chromosomal complement (50 chromosomes, 2N) in according with FACs analysis. Majority of cells were positive to vimentin staining and none of them were positive for nestin and Oct-4 by immunocytochemistry. These results indicate that the clonal cell lines obtained from cultured cells are fibroblasts and may be extremely useful in genetic manipulation for further nuclear transfer and fish cloning.

Key words : Zebrafish, clonal cell line, embryonic cell

INTRODUCTION

Once the scheduled zebrafish genome project is complete, targeted genetic manipulations in zebrafish will become even more desirable. Zebrafish has become an important organism for the vertebrate development because of its ease of use in forward genetics, embryonic manipulation, and transgenic analysis (Haffter *et al.*, 1996; Amsterdam *et al.*, 1999; Murphey and Zon, 2002). Transparent zebrafish embryos are well suited to manipulations involving DNA or mRNA injection, cell labeling, and transplantation. Although zebrafish cell cultures exhibiting some characteristics of embryonic stem cells have been described, only short-term cell cultures, which must be maintained in the presence of cells from the rainbow trout, have produced germline chimeras (Ma *et al.*, 2001). It remains to be determined if these cells will contribute to germline transmission after long-term culture.

As an alternative to embryonic stem cells, cultured somatic cells offer the possibility of producing cloned animals with targeted genetic manipulations (McCreath *et al.*, 2000; Lai *et al.*, 2002; Lee *et al.*, 2002).

Since zebrafish was cloned first in 2002, the efficiency has remained low which must be used individual nonclonal donor cells (Lee *et al.*, 2002), hindering the broad adoption of the technique. Although the benefits of *in vitro* fish cell cultures are well established, most of cells are mixed with several kind of cell types as its cell size and shape. Nobody has been established the clonal cell line from fish, and nonclonal cell lines have been used for its application such as animal cloning or understandings of cellular reprogramming, and it leads to the low efficiency (Lee *et al.*, 2002).

Therefore this study focused on the establishment of clonal cell line and the characterization of its cell lines isolated from embryonic cell lines of zebrafish. Moreover, the long term goal of our study is to improve the efficiency of cell nuclear transfer using the established clonal cell lines.

MATERIALS AND METHODS

Zebrafish strain

The wild-type Tubingen strain was used through the experiments.

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Culture media (prewarmed)

Synchronously developing eggs are decontaminated by washing them several times with 0.1% bleach in water and sterile Holtfreter's buffer. Cells were derived from zebrafish embryos (15~25 hours post-fertilization) and cultured in K-NAC; Keratinocyte SFM supplemented with 5% FBS, NAC, 1% Trout serum, 0.1% embryonic extracts, 10 ng/mL Bovine insulin and A2P (Lin *et al.*, 2005). First 2 weeks, 25 ng/mL basic fibroblast growth factor (Invitrogen) was added to media to inhibit melanocyte formation (Bradford *et al.*, 1994).

Embryonic fibroblast cell culture

The primary fibroblast cells were obtained from 15 hour old embryos (Tubingen). Briefly, the embryos were dechorionized using 3 mg/mL pronase in Holtfreter's solution. The embryos were washed with Holtfreter's solution following with 0.9X PBS with antibiotics. For further disinfection, the embryos were incubated in 0.04% bleach (Sigma) for 3 minutes, and a bleach residue was washed away using 0.9X PBS. To disassociate the embryos, we mechanically homogenize the embryos following with trypsinized in 0.25% trypsin at 28°C, 10 minutes. The cells were washed and resuspended in DMEM supplemented (5% FBS, 1% trout serum, embryonic extracts, 0.1 mg/mL bovine insulin and antibiotics). For first two weeks of culture, human basic fibroblast growth factor (25 ng/mL) were added to the culture medium. The prepared cells were incubated at 28°C with 5% CO₂. After two days, It was changed one third of media everyday.

Cell characterization and cell cycle analysis

Cell types presented in culture were investigated by immunocytochemistry to detect vimentin, nestin and

Oct-4 (Pou2). Cell cultures were grown to confluence under either serum-starved (0.5% FBS) or nonstarved (5% FBS) conditions for the cell cycle analysis. Cells were trypsinized, washed with DMEM, and resuspended to a concentration of 1×10^5 cells/mL in ice-cold ethanol. Cells were fixed for at least 1 h in ethanol at 4°C. For nuclear staining, 1 mL aliquot (1×10^5 cells/mL) was resuspended in PBS and passed through a 25G5/8 syringe. Next, 2 μ L of RNase A (10 mg/mL, Sigma) and 10 μ L of propidium iodide (5 mg/mL, Sigma) were added. Cells were incubated for 30 min at room temperature in the dark and then analyzed by FACS.

Chromosome analysis

To examine the ploidy status of the cultured cells, chromosome numbers were determined by using the standard method for chromosome preparation. Briefly, rapidly growing cultures were incubated in 2 drops of colcemid (μ g/mL, GIBCOBRL) to the culture dish (P100 Plate) and replaced in 37°C incubator for 30 minutes to 1 hour. Cells were then trypsinized, centrifuged, and the pellet gently resuspended in hypotonic KCl (0.075 M). Treated cells were fixed in acetic acid/methanol (vol/vol=1:3), and drops of the cell suspension were spread on clean microscopic slides. The chromosome were stained with the Giemsa stain stock solution (0.4%, Sigma) for 10 min at room temperature. The number of well-spread chromosomes was counted under a light microscope at 1,000 \times magnification under oil.

RESULTS

The diploid gastrula-derived cells were maintained continuously for more than 16 weeks (14 passages) in

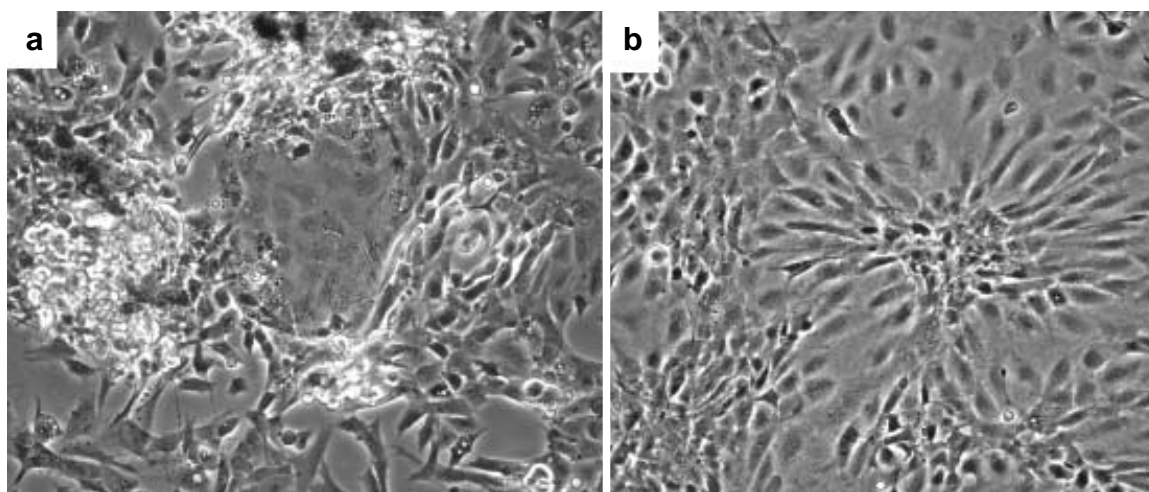


Fig. 1. (a) the primary cell, 4 day-old embryonic cells after plating ($\times 200$), (b) Ezfb cell line, 4 weeks old, from primary cell ($\times 200$).

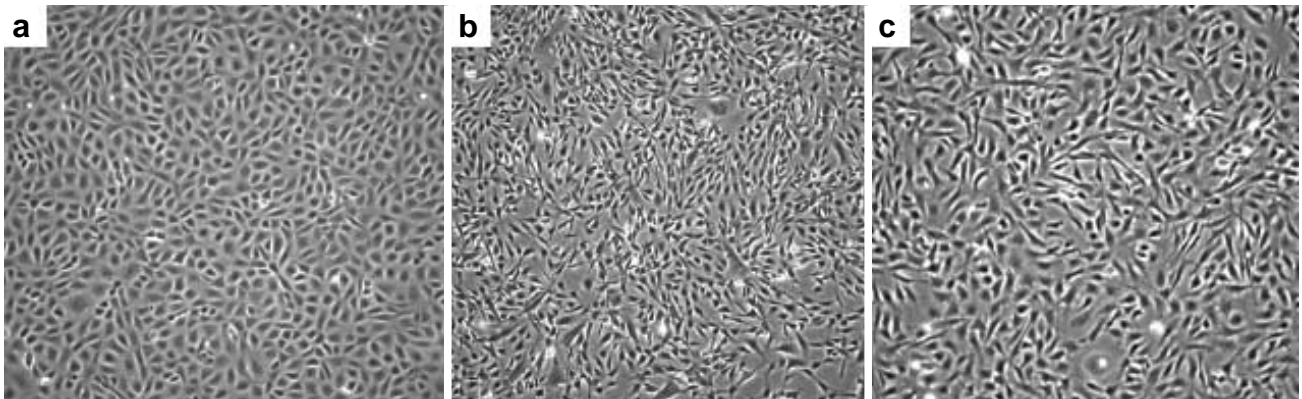


Fig. 2. Three kinds of clonal cell lines ($\times 100$) viewed with an inverted microscope. (a) round shape and large size cell line, CZF1 (b) fibroblast-like shape and smaller size, CZF6 (c) fibroblast-like shape and larger size, CZF7.

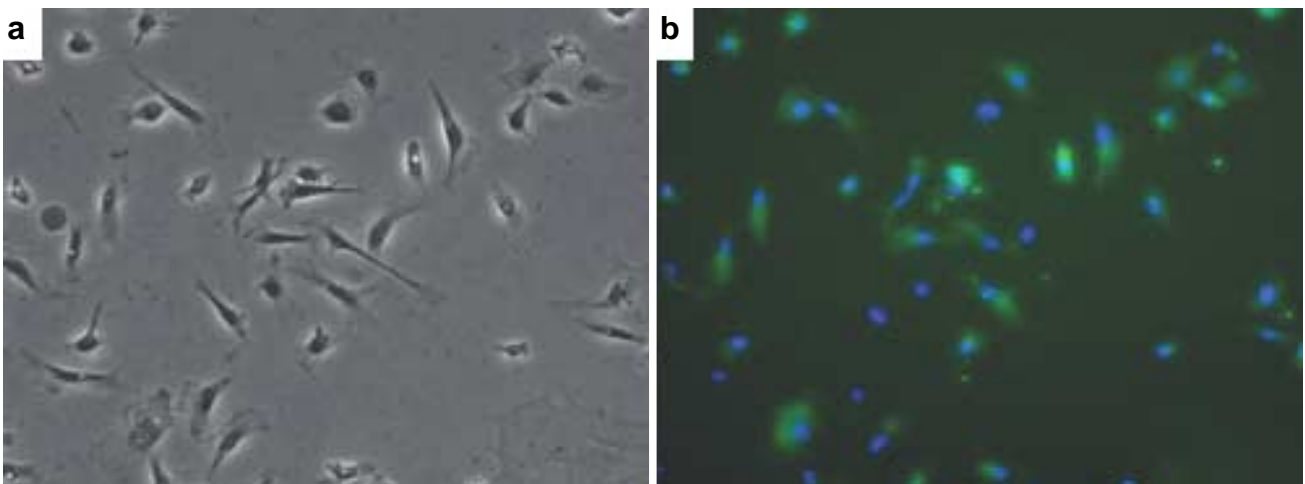


Fig. 3. (a) the bright field image, (b) positive cells for Vimentin (green) and nucleus stained with Hoechst 33342 (blue) from the same field ($\times 200$).

basal nutrient medium. The doubling time of the embryo cells under these conditions was approximately 5 days by first 3 weeks for primary cell and 3 days from the 3rd week for cell line. Growth of the cell lines was superior to the primary cell. Fig. 1-a shows the primary cell, 4 day-old fibroblast, from diploid gastrula-stage embryos (5~20 somite stage) and Fig. 1-b is 4-week-old Ezfb cell line from primary cell.

Some black spots known as melanocytes were shown during the initial primary cell culture under the light microscope, although other pigmented cells exist in the fish that also appeared in dense cultures and represented less than 1% of the total cells population. However, these pigmented cells were inhibited after adding the bovine fibroblast growth factor (bFGF). The growth rate of primary cells was low (doubling time more than 5 days) in the primary stage, but stable cell lines and clonal cell lines were capable of well proliferation *in vitro*.

The cells have been maintained in continuous culture without change in characteristics.

After establishing the stable cell line, Ezfb, the cell clones were selected using the cloning ring and placed in 96-well plates, and cultured in 90% of confluence. And then this high confluence cells were subcultured in 24-well plates. A total of 18 clonal lines were obtained from the stable cell line; 3 out of 18 clones could be defined by its shape and size (Fig. 2).

Karyotype analysis of clonal cell line, CZF6 revealed that after approximately 22 doublings in culture, a majority of the cultured cells (80%) showed a normal chromosomal complement (50 chromosomes, 2N). About 20% of cells was aneuploid which is the same percentage with FACS analysis.

Cell line, CZF6, were tested for the effect of serum starvation on DNA content by FACS depicting serum-starved and non-serum-starved cells. In the nonstarved

cells, 59% were at G0+G1 stage, whereas serum starvation for four days increased the proportion of G0+G1 stage cells to 80%.

Cell types presented in culture were investigated by immunocytochemistry to detect vimentin, nestin and Oct-4 (Pou2) for the cell characterization. Majority of cells were positive to vimentin staining (Fig. 3). None of them were positive for nestin and Oct-4.

DISCUSSION

Previous studies successfully cultured embryonic cells (Fan *et al.*, 2004) and primary cells derived from embryos and adult tissues (Collodi *et al.*, 1992; Driever and Rangini, 1993; Ghosh and Collodi, 1994; Lee *et al.*, 2002). Stable cell lines from a variety of fish, such as carp, blugill sunfish (Bradford *et al.*, 1994) have been reported. Stable cell lines derived from embryonic zebrafish (Collodi *et al.*, 1992; Driever and Rangini, 1993; Sun *et al.*, 1995) as well as from caudal fin. Although stably maintained in the standard Dulbecco's modified Eagle's medium (DMEM) media widely used in mammalian tissue culture, the ZF4 line derived from 1-day-old zebrafish embryos exhibits transformed features such as the loss of contact inhibition, formation of foci, and hyperdiploid karyotype (Driever and Rangini, 1993). Therefore the establishment of these embryonic lines required complex growth media supplements, such as bovine basic fibroblast growth factor (bFGF) (Sun *et al.*, 1995). bFGF stimulates cell growth at concentrations as low (Bradford *et al.*, 1994). In the absence of bFGF, pigmented cells appear in the primary cultures on the second day. In this study, first 2 weeks, 25 ng/mL basic fibroblast growth factor was added to media to inhibit melanocyte formation. Some black spots known as melanocytes were shown during the initial primary cell culture under the light microscope. However, these pigmented cells were inhibited after adding bFGF. That was coincident with Collodi *et al.* (1992). All of the cultures required zebrafish embryo extract and bFGF in addition to trout embryo extracts during the initial passages in our culture (Collodi and Barnes, 1990).

The growth rate of primary cells was low, but stable cell lines were capable of well proliferation *in vitro* like Collodi *et al.* (1992). The cells have been maintained more than 16 weeks in continuous culture without change in characteristics. After establishing the stable cell line, as shown in Fig. 2, three out of 18 clones could be defined and Karyotype analysis of this clonal cell line, CZF6, revealed that 80% of the cultured cells were maintained a modal chromosome number of 50 (diploid) after approximately 22 doublings in culture and maintained for more than 2 months of continuous proliferation *in vitro*. Similarly, Enho and Ingalls (1968) re-

ported that diploid chromosome number for *Danio rerio* is 50. The wide range in chromosome number in the Ezfb culture may be due to a gradual diploidization of the cell line as it is grown in culture. Alternatively, the range in chromosome number may deflect that the culture was derived from a pool of dissociated cells from several embryos.

Although cloned calves have been produced from fetal fibroblast without serum starvation (Cibelli *et al.*, 1998), serum starvation has been employed in most of the recently reported mammalian nuclear transfer experiments. A study in sheep suggests that arrest in G0 by serum starvation allows donor somatic nuclei to better support embryonic development (Wilmot *et al.*, 1997). Kasinathan *et al.* (2001) have showed that nuclei from G1 cells are important in supporting late fetal development. We chose to include a starvation step because it seemed to push more cells into G0+G1, which should be beneficial in zebrafish nuclear transfer. Clonal cell line, CZF6 were tested for the effect of serum starvation on DNA content by FACS depicting serum-starved and non-serum-starved cells. Serum starvation for four days increased the proportion of G0+G1 stage cells to 80%. This results indicate clonal cell lines can be used for fish cloning.

Cell types were investigated by immunocytochemistry to detect vimentin, nestin and Oct-4 (Pou2) for the cell characterization. As shown in Fig. 3, majority of cells were positive to vimentin staining. None of them were positive for nestin and Oct-4. This means that clonal cell lines established from the embryos are fibroblast cells.

As the results, we address that established clonal cell lines will contribute to understanding of knock-in/-out genetic studies by zebrafish cloning as well as basic understandings of cellular reprogramming.

ACKNOWLEDGMENTS

This study was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (R01-2007-000-11941-0). The author also thank Coastal Research Center, Kunsan National University for supporting the paper.

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제브라피쉬 (*Danio rerio*) 배아로부터 동형세포주 확립

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요 약 : 제브라피쉬 배아로부터 확보한 세포주로부터 외부형태와 세포 크기에 따라 3종류의 동형세포주를 확립하였다. 활발하게 증식하는 안정된 세포주 및 이들로부터 확립된 동형세포주의 세포특성은 변하지 않고 지속적으로 유지되었으며, 안정된 세포주로부터 총 18개의 콜로니를 확보하여 배양한 다음 3종류의 동형세포주를 선별하여 세포 특성을 분석하였다. 대부분의 동형세포주는 약 80% 정도 정상적인 염색체 (2N=50)를 가지고 있었으며 FACs 분석과 일치하였다. 배아로부터 확립된 동형세포주에 항체테스트 결과, vimentin에서 양성을 보이는 결과로 볼 때 확립된 세포주는 분화된 섬유세포임이 확인되었다. 이러한 결과는, 확립된 동형세포주를 이용한 유전자조작과 어류복제의 활용도를 높일 수 있음을 시사한다.

찾아보기 낱말 : 제브라피쉬, 동형세포주, 어류복제