Characterization of three cell lines established from medaka embryos

メダカ胚より樹立した細胞株の解析

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We established three cell lines from medaka embryos. In the present study, we investigated the optimal conditions for growth of these cells with regard to the basal culture medium and concentration of fetal bovine serum (FBS). The best cell growth for all three cell lines was achieved with a 1:1 mixture of L15 medium and 199 medium with a relatively high concentration (20%) of FBS. The growth curves for the cell lines revealed that their doubling times ranged from 48 to 93 h. We also analyzed the numbers of chromosomes in the cell lines and found that their numbers varied from near-diploid to hyper-tetraploid. The results of the present study should be helpful for research in fish cell culture.

Key words : Medaka, cell culture, growth conditions, chromosome number

I. INTRODUCTION

Small fresh-water fish such as the medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) are important animal models for research in many fields of biology. Medaka can be maintained easily in the laboratory and have a short generation time of about 3 months. Many eggs are obtained daily, and these eggs are relatively large (about 1mm in diameter) and transparent, making it easy to observe or manipulate them under a low-magnification microscope. Thus, medaka are useful experimental animals. Many cell lines have been established from several fish species, including the medaka, and are utilized in various fields of research.^{1,2}

Mammalian pluripotent embryonic stem (ES) cell lines have been established from the inner cell masses of blastocysts. These cell lines provide an experimental system for genetic manipulations that create transgenic animals. In addition, these cell lines are expected to be useful for tissue engineering by producing specifically differentiated cells during *in vitro* cultures. Although attempts to obtain such ES cells from fish embryos have been reported,^{3,4}

success remains limited at present. To advance the technology for fish cell culture, additional information about the optimal culture conditions is indispensable. We previously established three cell lines from medaka embryos in our laboratory. However, we did not analyze the best growth conditions or characterize these cells in detail. To clarify the optimal conditions for culture of these fish cell lines, we focused on the basal culture medium, concentration of FBS and growth rate. We also investigated the chromosome numbers in these cells.

II. METHODS AND MATERIALS

II-A. Cell culture

The three medaka cell lines (MR-1, MIC-3 and Mib) used in this study were established in our laboratory. The MR-1 cell line was derived from 5-day-old embryos, while the MIC-3 and Mib cell lines were derived from blastula-stage embryos. Fertilized eggs were sterilized in a neutralized 0.4% NaClO solution for 1 min, immersed in 70% ethanol for 10 s and then washed in a sterile medaka saline solution (0.75% NaCl, 0.02% KCl, 0.02% CaCl₂, 0.02%

NaHCO₃)⁵ containing 100 µg/ml of ampicillin and 50 µg/ml of streptomycin. The embryos were removed from the eggs and dissected into small pieces using a pair of fine scissors. The cells were dissociated by using a trypsin solution (0.1% trypsin and 0.05% EDTA in phosphate-buffered saline) or by simply pipetting in case of the blastula-stage embryos. The dissociated cells were incubated in culture flasks (25 cm²; NUNC) containing 5 ml of 199 medium supplemented with 15% fetal bovine serum (FBS; HyClone Lab. Inc.). The incubation temperature was set at 28°C since the cells were found to grow well at this temperature. The cells were serially transferred every 8-10 days at split ratios of 1:2 to 1:5 and continuously cultured for more than 5 years.

II-B. Culture media

In the present study, four basal media (199, DMEM, F12 and L15) were used for cell culture. Medium 199 was originally developed by Morgan *et al.* (1950)⁶ for culturing chick embryo fibroblasts, but is now used for culturing a wide variety of cells, including mammalian cells. DMEM (or DME; Dulbecco's modified Eagle's medium) is a modified version of Eagle's medium (Eagle 1955)⁷ created by Dulbecco and Freeman (1959)⁸ to achieve high-density growth of cultured human or hamster cells. In contrast to these media, F12 was designed by Ham (1965)⁹ for clonal growth of low-density hamster cells. These three media were purchased from Nissui Seiyaku Co. L15 medium was developed by Leibovitz (1963)¹⁰ and contains galactose and Na-pyruvate instead of glucose. L15 medium was purchased from COSMO Bio Co. All the basal media were prepared in 20 mM HEPES (pH 7.3) with 1.7 g/l NaHCO₃.

II-C Analysis of cell growth

For analysis of cell growth, aliquots containing 3×10⁵ cells were plated in culture flasks (25 cm²) and incubated for 8 days. After the incubation, the cells were dissociated into single cells with a trypsin solution and the cell numbers were determined using a Coulter counter (Beckman Coulter Inc.).

II-D. Karyotype analysis

For determination of the numbers of chromosomes, log-phase cells were treated with 50 ng/ml of

colcemid for 8 h. The cells were then collected, incubated in 75 mM KCl at 37°C for 10 min and then fixed in acetic acid-ethanol (1:3) for 30 min at room temperature. The cells were collected by centrifugation, suspended in a small volume of the fixative and applied to a glass slide. The glass slide was flamed to achieve better spreading of the mitotic cells. The cells were stained with Giemsa's solution (MERCK Ltd.). Metaphase cells were photographed and recorded using a micrographic digital camera.

III. RESULTS AND DISCUSSION

The cell lines used in this study were originally established using 199 medium supplemented with 15% FBS and serially transferred under the same conditions. During the extended period of cell culture, the growth rates did not alter and we never experienced senescence of the cells. Thus, these cells were considered to be immortal. In general, fish cells are known to be immortal,^{1,11,12} unlike normal mammalian somatic cells that usually show senescence over about 50 cell generations.¹³ Fig. 1 shows the morphologies of the cells used in this study. All three cell lines showed fibroblastic or spindle-shaped appearances.

First, we compared the cell growth rates in four basal media (199, DMEM, F12 and L15) to find out the optimal medium for these cell lines. All the culture media were supplemented with 15% FBS. In each flask, 3×10⁵ cells were plated in 5 ml of culture medium and incubated for 8 days. The results are shown in Fig. 2. The growth rates of the cells varied among the cell line. For this reason, the highest yield of cells for each cell line was indicated as 100% and the relative cell numbers are shown in Fig. 2. When each basal medium was used independently, L15 gave the best or second-best results for all three cell lines. Fig. 2 also shows that the effectiveness of each culture medium differed among the cell lines. For example, Mib cells showed relatively slow growth in F12 medium compared to the other two cell lines. We expected that 199 medium would give the best results since all the cell lines were established and maintained in this medium as mentioned above. During the long-term culture, cells that grew well in

199 medium may have been selected in the cell population while those cells that did not show suitable growth may have been eliminated. However, the results indicated that 199 medium was not the best medium for all three cell lines. Thus, we tried a 1:1 mixture of L15 medium and 199 medium. The results showed that this mixture gave the best cell growth rates (indicated as 100% in Fig.2).

Next, we analyzed the serum concentrations (1-20%) added to the culture medium. Collodi *et al.* (1992)¹⁴ reported that 10% FBS was inhibitory for growth of cells derived from zebrafish embryos. For this reason, they used 1% FBS together with trout serum and an embryo extract. As shown in Fig. 3, the growth rates of all three medaka cells were dependent on FBS, and 20% FBS was the best

concentration for growth. These findings may also be interpreted as a result of the cells having been selected to grow in the presence of FBS over a long

Fig. 2. Growth rates of the three cell lines in different basal media.

Fig. 3. Growth rates of the three cell lines in different FBS concentrations. Open column; MR-1 cell, gray column; MIC-3 cells and hatched column; Mib cells.

Fig. 1. Morphology of the cultured medaka embryo cells. A; MR-1. B; MIC-3. C; Mib. Bar = 100 μ m.

period of time. However, our preliminary study showed that the growth of cells in primary cultures starting from early-stage embryos was also strongly dependent on FBS (data not shown), suggesting that FBS is an essential factor for growth of these cells.

The growth rates of the three cell lines were determined. In this study, a 1:1 mixture of L15 medium and 199 medium was used as the culture

medium and supplemented with 15% FBS. As shown in Fig. 4, the growth rates differed among the cell lines. The doubling time of MR-1 cells, which exhibited the fastest growth among the cell lines, was about 48 h. The doubling time of Mib cells was 62 h, while that of MIC-3 cells was 93 h. Fig. 4 also shows that the cells continued to grow for 15 or more days.

We analyzed the chromosome numbers in the three cell lines. The normal chromosome number of medaka somatic cells is 48.¹⁵ As shown in Fig. 5, the numbers of chromosomes were quite variable among the cell lines. The modal peak was found to be near-tetraploid (90-99) in the MR-1 (Fig. 5A) and MIC-3 (Fig. 5C) cell lines. However, in both cell lines, there were considerable numbers of cells containing more than 100 chromosomes. There were two peaks in the case of Mib cells (Fig. 5B), one at near-diploid (40-49) and the other in the range of 60-69. This cell line also contained cells with

Fig. 4. Growth curves of the three cell lines cultured at 28°C. ■; MR-1 cells. ●; Mib cells. ▲; MIC-3 cells.

tetraploid or more chromosomes. These results indicate that the chromosome numbers in the medaka cells were not stable and changed during serial

transfer over a long time. These results will help to establish new cell lines, and possibly ES cell lines, or to understand the nature of cultured medaka cells.

Fig.5 Frequency distribution of chromosomes of the cultured cells. A; MR-1 cells, B; MIC-3 cells, C; Mib cells.

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