JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 103, No. 2, 113–121. 2007
DOI: 10.1263/jbb.103.113

Chemically Fixed Nurse Cells for Culturing Murine or Primate Embryonic Stem Cells

Yoshihiro Ito,[1,2]* Makiko Kawamorita,[1] Toshio Yamabe,[1]
Toru Kiyono,[3] and Kanji Miyamoto[1,4]

Regenerative Medical Bioreactor Project, Kanagawa Academy of Science and Technology, KSP East 309, 3-2-1 Sakado, Takatsu-ku, Kawasaki, Kanagawa 213-0012, Japan,[1] Nano Medical Engineering Laboratory, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan,[2] Cancer Research Center, 5-1-1 Tukiji, Chuo-ku, Tokyo 104-0045, Japan,[3] and Department of Biological Sciences, Tokyo Metropolitan University, 6-6 Asahigaoka, Hino, Tokyo 191-0065, Japan[4]

Received 24 August 2006/Accepted 27 October 2006

In current and past practice, murine or primate embryonic stem (ES) cells are usually cultured on live nurse cells for growth that keeps the cells in an undifferentiated state. It is troublesome, however, to prepare nurse cells for each cell culture and it is difficult to completely remove the nurse cells when they are transferred. In this study, mouse and monkey ES cells were therefore grown on chemically fixed mouse embryonic fibroblast (MEF) or human amniotic epithelial (HAE) cells. MEF cells were fixed by incubation in a glutaraldehyde or formaldehyde solution. HAE cells were immortalized by transfection of *hTERT* and chemically fixed with the same reagents. When mouse ES cells were cultured on these chemically fixed cells, the mouse ES cells grew well and expressed alkaline phosphatase, SSEA-1, and Oct-3/4 as their markers, indicating their undifferentiated state. The monkey ES cells also grew well and expressed alkaline phosphatase, SSEA-4, and Oct-4 as their markers, indicating their undifferentiated state. Freeze-drying HAE or MEF cells did not change their ability to support the undifferentiated growth of ES cells. Additionally, the chemically fixed cells could be utilized repeatedly in the culture of ES cells. These results demonstrate that chemically fixed nurse cells are useful for the maintenance of ES cells in an undifferentiated state in culture.

[**Key words:** chemical fixation, nurse cells, embryonic stem cells, cell culture, undifferentiation]

Embryonic stem (ES) cells were first derived from an inner cell mass of mouse blastocyst in the early 1980s (1). In 1998, human ES cells were also established using similar techniques (2). ES cells have the unique ability to give rise to any type of somatic cell line. Therefore, ES cells are important for the analyses of molecular mechanisms underlying self-renewal and embryogenesis, and are frequently used to generate transgenic mice. Additionally, the implementation of cell-based therapy or regenerative medicine requires ES cells as a renewable source of cells. An efficient *in vitro* method of multiplying ES cells is therefore important for the development of ES cell-based therapeutic strategies.

Recently, it has been shown that gelatin-coated tissue culture plates containing leukemia inhibitory factor (LIF) are useful for supporting the growth of mouse ES cells while maintaining an undifferentiated state (3). In the case of human ES cells, basic fibroblast growth factor (bFGF) has been recently reported as being useful for the growth of human ES cells while maintaining their undifferentiated state (4–7). These supplemental approaches to ES cell maintenance cannot be generalized for all ES cell lines. In the case of mouse ES cells grown in culture, mouse embryonic fibroblast (MEF) cells are commonly used as nurse cells. For primate ES cell culture, (including human ES cells), some types of human nurse cell have also been utilized (8–10). Among these, the human amniotic endothelial (HAE) cells reported by Miyamoto *et al*. (10) are useful owing to the ease of their acquisition. There are, however, two disadvantages of the usage of these nurse cells, including the need for preparation before each ES cell culture prior to use and the inability to completely remove the nurse cells after trypsinization.

We have recently found that chemically fixed nurse cells support the growth of hematopoietic stem cells (11). In this study, nurse cells were chemically fixed to evaluate their ability to support the growth of ES cells while maintaining the ES cells in an undifferentiated state. Glutaraldehyde- or formaldehyde-fixed MEF cells and HAE cells were prepared for the culture of murine and primate ES cells, respectively.

MATERIALS AND METHODS

MEF and HAE cells MEF cells were purchased from Dainippon Pharmaceutical (Osaka) and cultured in accordance

* Corresponding author. e-mail: y-ito@riken.jp
phone: +81-(0)44-819-2044 fax: +81-(0)44-819-2039

with conventional methods as previously reported (12–14). MEF cells were cultured with 10 μg/ml mitomycin-C for 3 h and washed with 10% fetal bovine serum (FBS)-containing Dulbecco's modified minimum essential medium (D-MEM) for ES cell culture.

Human placentas were collected after obtaining the written, informed consent of pregnant women who tested negative for HTLV-I, HIV-I, and hepatitis B virus as previously reported (11). The use of human placenta was recognized as appropriate by the ethical and safety committee of Tokyo Metropolitan Institute of Technology. Human amnion cells were surgically separated from the placenta, incubated in 0.25% trypsin-EDTA (Gibco-Invitrogen, Carlsbad, CA, USA) at 37°C for 30 min, and then washed with phosphate-buffered solution (PBS). Thereafter, the cells were digested with 0.05% collagenase type 1 (Sigma, St. Louis, MO, USA) at 37°C for 30 min and washed three times with PBS. The HAE cells obtained were then placed in 25-cm^2 tissue culture flasks (Falcon BD, San Jose, CA, USA) containing 90% (v/v) D-MEM and 1×penicillin-streptomycin, 10% knockout serum replacement (KSR; Gibco-Invitrogen) or fetal calf serum (Gibco-Invitrogen), and incubated at 37°C in 5% CO_2/95% air for 3 d.

Immortalization of HAE cells The construction of retroviral vector plasmids, namely, pCMSCVpuro-hTERT, pCLXSN-hTERT, and pCLXSN-16E6E7 was described previously (15, 16). Similarly, the entry vectors of 16E6E7 and DsRed were recombined with a lentivirus vector plasmid, CSII-EF-RfA (17), by LR reaction (Invitrogen) according to the manufacturer's instruction to generate CSII-EF-16E6E7 and CSII-EF-DsRed. The production of recombinant retroviruses and lentiviruses has been described before (17, 18). Briefly, retroviral vector plasmids were cotransfected with pCL-10A1, and lentivirus vector plasmids were cotransfected with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev into 293T or 293FT cells (Invitrogen) using TransIT-293 (Mirus, Madison, WI, USA) according to the manufacturer's instruction. The culture medium was harvested between 48 and 72 h post-transfection. The titer of the recombinant retroviruses obtained using HeLa cells was greater than 3×10^5 drug-resistant colony forming units/ml. The titer of the CSII-EF-16E6E7 was approximated using CSII-EF-DsRed ($>7\times10^5$ transducing units/ml). One milliliter of the culture medium was added to HAE cells seeded on gelatin-coated multi-well plates (6 wells per plate), in the presence of polybrene (4 μg/ml). As the mock-infected HAE cells underwent senescence after a few passages, cells with an extended life span were selected by serial passage without drug selection. Subsequently, cells were selected with 1 μg/ml puromycin, and the infected cells were selected with of puromycin and G418. The doubling time of immortalized HAE cells was 27.2 h and the cells could be passaged more than 10 times (over 1 month).

Chemical modification of nurse cells Confluent MEF and HAE cells were washed with PBS. Subsequently, the cells were incubated in 2.5% glutaraldehyde or 2.5% formaldehyde solution at room temperature for 30 min and washed three times with PBS. Some of the treated or nontreated cells were washed with water and freeze-dried for further experiments.

Culture of murine ES (129) and primate ES (CMK6) cells The murine ES cell line 129 was purchased from Dainippon Pharmaceutical, and was passaged on MEF cells. The culture medium consisted of D-MEM containing 15% KSR, 1 mM Na-pyruvate (Sigma), 2 mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (Chemicon, Temecula, CA, USA), 0.1 mM 2-mercaptoethanol (Sigma), 1000 U/ml LIF (Chemicon), and penicillin (25 U/ml)-streptomycin (25 μg/ml) mixture (Gibco). After 0.25% trypsin-EDTA treatment for 5 min at 37°C, dissociated ES cells were cultured with MEF cells.

The cynomolgus monkey CMK6 cell line, which was purchased from Asahi Techno Glass (Chiba), was passaged on MEF feeder cells. The culture medium consisted of 80% (v/v) F-12/D-MEM containing 20% KSR, 0.1 mM 2-mercaptoethanol, 1 mM Na-pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and penicillin (25 U/ml)-streptomycin (25 μg/ml) mixture. After 0.05% trypsin-EDTA treatment for 5 min at 37°C, dissociated ES cells (10–50 cells/clump) were cultured with MEF feeder cells. The number of cells was determined using a WST-1 assay kit purchased from Dojin Chemicals (Kumamoto).

Staining of ES cells ES cells were fixed with 3.8% formaldehyde at room temperature for 10 min, and then washed with PBS. Alkaline phosphatase activity was detected using Vector Red Alkaline Phosphatase Substrate Kit 1 (Vector Laboratories, Burlingame, CA, USA).

To detect the expressions of SSEA-1 and SSEA-4, ES cells were fixed with 2% formaldehyde solution at room temperature for 10 min and then washed with PBS. Subsequently, the fixed ES cells were reacted with a 1:50 diluted mouse anti-SSEA-1 monoclonal antibody and mouse anti-SSEA-4 monoclonal antibody (Chemicon). SSEA-1 and SSEA-4 were detected by immunocytochemical analysis using either an avidin or a biotin horseradish peroxidase complex (Vectastain ABC System; Vector Laboratories).

Examination of Oct-3/4 expression The cultured mouse ES cells were collected using trypsin-EDTA solution and total RNA was extracted using an Rneasy Mini kit (Qiagen Japan, Tokyo). Reverse transcription polymerase chain reaction (RT-PCR) was performed using RT-PCR high-Plus (Toyobo, Osaka). For Oct-3/4, the sequences of the forward and reverse primers were 5′-AGCTG CTGAAGCAGAAGAGG-3′ and 5′-CCTGGGAAAGGTGTCCT GTA-3′, respectively. The length was 468 bp (431–898) and the accession no. NM_013633. For G3PDH, the sequences of the forward and reverse primers were 5′-ACCACAGTCCATGCCATCA C-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, respectively. The length was 452 bp (566–1017) and the accession no. M32599. The PCR product was electrophoresed through a 2% Tris-acetate EDTA (TAE) agarose gel stained with 0.1 mg/ml ethidium bromide. Gels were visualized on a UV transilluminator.

Oct-4 expression was detected RT-PCR. Total RNA was extracted from CMK6 ES cells using an RNeasy Mini kit (Qiagen) and cDNA was synthesized using the Super ScriptTM III First-Strand Synthesis System for RT-PCR and amplified by PCR using Ex Taq (Takara, Otsu). The cynomolgus monkey (*Macaca fascicularis*) Oct-4 gene primers (sense, 5′-GGACACCTGGCTTCGG ATT-3′; antisense, 5′-TTCGCTTTCTCTTTCGGGC-3′) and β-actin internal standard primers (sense, 5′-TGGCACCACACCTTCT AGAATGAGC-3′; antisense, 5′-GCACAGCTTCTCCTTAATGT CACGC-3′) were used.

For mouse ES cells, the PCR cycles consisted of a reverse transcription reaction at 60°C for 30 min, an initial PCR activation step at 94°C for 2 min, then at 94°C for 1 min, 57°C for 30 s, and 72°C for 30 s for 24 cycles, followed by a final PCR step at 72°C for 5 min and then maintained at 4°C. For monkey ES cells, the PCR cycle consisted of a reverse transcription reaction at 50°C for 50 min, an initial PCR activation step at 94°C for 5 min, and then 94°C for 1 min, 65°C for 30 s, and 72°C for 1 min for 25 cycles, followed by a final PCR step at 72°C for 5 min and then maintained at 4°C. The PCR product was electrophoresed through a 2% TAE agarose gel stained with 0.1 μg/ml ethidium bromide. Gels were visualized on a UV transilluminator.

RESULTS AND DISCUSSION

Culture of murine ES cells Figure 1 shows the micrographs of murine ES cells cultured on several materials. Although the colonies of murine ES cells cultured on chemically fixed MEF cells (Fig. 1B, C, E, F) were slightly smaller than that on MEF cells that were treated with mito-