BIOLOGY OF REPRODUCTION **68,** 2150–2156 (2003)
Published online before print 22 January 2003.
DOI 10.1095/biolreprod.102.012583

Human Feeder Layers for Human Embryonic Stem Cells[1]

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ABSTRACT

Human embryonic stem (hES) cells hold great promise for future use in various research areas, such as human developmental biology and cell-based therapies. Traditionally, these cells have been cultured on mouse embryonic fibroblast (MEF) feeder layers, which permit continuous growth in an undifferentiated stage. To use these unique cells in human therapy, an animal-free culture system must be used, which will prevent exposure to mouse retroviruses. Animal-free culture systems for hES cells enjoy three major advantages in the basic culture conditions: 1) the ability to grow these cells under serum-free conditions, 2) maintenance of the cells in an undifferentiated state on Matrigel matrix with 100% MEF-conditioned medium, and 3) the use of either human embryonic fibroblasts or adult fallopian tube epithelial cells as feeder layers. In the present study, we describe an additional animal-free culture system for hES cells, based on a feeder layer derived from foreskin and a serum-free medium. In this culture condition, hES cells maintain all embryonic stem cell features (i.e., pluripotency, immortality, unlimited undifferentiated proliferation capability, and maintenance of normal karyotypes) after prolonged culture of 70 passages (>250 doublings). The major advantage of foreskin feeders is their ability to be continuously cultured for more than 42 passages, thus enabling proper analysis for foreign agents, genetic modification such as antibiotic resistance, and reduction of the enormous workload involved in the continuous preparation of new feeder lines.

developmental biology, embryo

INTRODUCTION

Embryonic stem (ES) cells are cells derived from the inner cell mass of the mammalian blastocyst, initially derived from the mouse blastocyst [1, 2]. These cells are pluripotent, immortal, and can be continuously cultured in an undifferentiated state. They retain their developmental potential after prolonged culture and maintain normal karyotypes after continuous culture. Thomson et al. [3] reported the first derivation of human ES cell lines. Given the outstanding potential of human ES cell lines, their availability offers a unique and novel research tool with prospective widespread clinical applications. Human ES cells may be utilized in the future in various research areas, such as human developmental biology, teratology, and cell-based therapies.

The first reports on the derivation of human ES cells described the necessity for a mouse embryonic fibroblast (MEF) feeder layer to grow continuously in an undifferentiated stage in culture [3, 4]. Contrary to human ES, mouse ES cells can be grown directly on gelatin-coated plates with the addition of leukemia inhibitory factor [5]. Handling the simultaneous growth of both ES cells and MEF requires meticulous care and may prove to be rather expensive. In addition, the dual growth of these cells exposes the human ES cells to mouse retroviruses, which may prevent their future use in cell-based therapy.

One of the first improvements in the basic culture methods used for human ES cell cultures has come from the ability to grow human ES cells under serum-free conditions, using serum replacement (SR) supplemented with basic fibroblast growth factor (bFGF). These better-defined culture conditions support the continuous culture of human ES cells while maintaining all ES cell characteristics of primates [6].

Further improvement was reported by Xu et al. [7], who were able to maintain human ES cells on Matrigel matrix (Becton, Dickinson & Co., Bedford, MA) with 100% MEF-conditioned medium supplemented with 20% SR and bFGF. Although we confirmed this finding, this culture condition still requires massive growth of MEF for the production of a conditioned medium. In addition, the use of an MEF-conditioned medium may still expose the human ES cells to mouse retroviruses. Therefore, additional solutions were needed to create an entirely animal-free culture system for the human ES cells.

Another suggested solution is a culture system based on a human feeder layer. Recently, Richards et al. [8] reported the possibility of growing human ES cells on human embryonic fibroblasts or adult fallopian tube epithelial feeder layers. Cultured with these human feeder layers in medium supplemented with human serum, human ES cells were found to maintain ES cell features, including pluripotency, morphology, and expression of cell-surface markers, for at least 20 passages. This condition was also found to support the derivation of a line similar to human ES cell.

In the present study, we offer an alternative and completely animal-free culture condition for human ES cells based on foreskin feeders and a serum-free medium.

MATERIALS AND METHODS

Preparation of Foreskin Lines

Foreskins were obtained from newborn babies after circumcision and were donated by their parents. The foreskins were washed, minced by scissors, and dissociated to single cells by trypsinization. The resulting

[1]The research done in our laboratory was partly supported by the Fund for Medical Research and Development of Infrastructure and Health Services, Rambam Medical Center, and the Technion Development and Research Fund (TDRF).
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Received: 24 October 2002.
First decision: 13 November 2002.
Accepted: 8 January 2003.

ISSN: 0006-3363. http://www.biolreprod.org

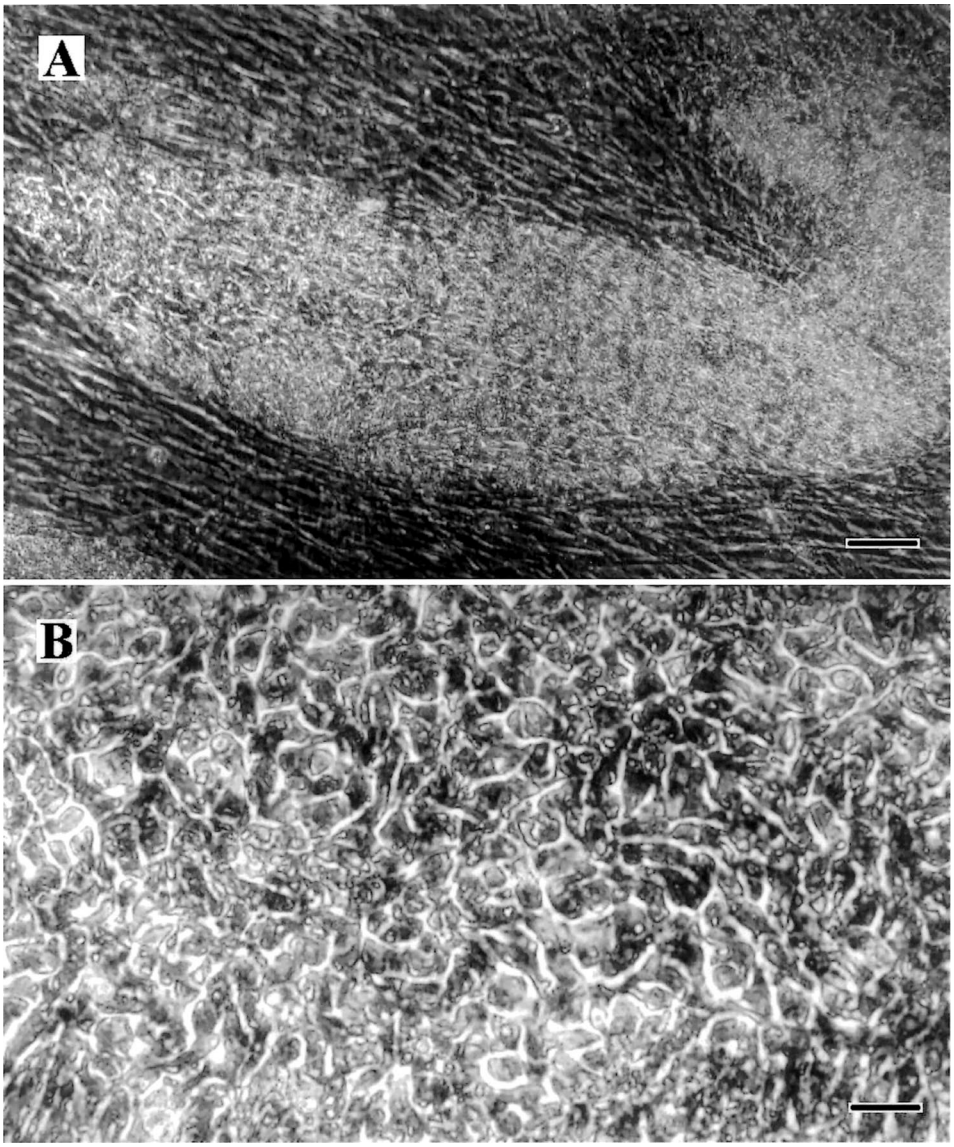

FIG. 1. Human ES cells grown on foreskin feeder layers. **A**) Human ES cell colony from I-6 line after 61 passages with foreskin feeder layers. Note the colony is organized in a long and elliptic manner. **B**) Individual human ES cells, from the I-3 line, retain their typical ES cell morphology (i.e., high nucleus:cytoplasm ratio, presence of nucleoli, and spaces between cells) when cultured on human feeder layers for 37 consecutive passages. Bar = 100 μm (**A**) and 38 μm (**B**).

cells were grown in a culture medium consisting of 80% Dulbecco modified Eagle medium (DMEM; no pyruvate, high-glucose formulation) supplemented with either 20% fetal bovine serum (FBS; Hyclone, Logan, UT); 20% SR; or 20% human serum (Chemicon International, Temecula, CA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acid stock (Gibco Invitrogen Corporation, Carlsbad, CA). The foreskin cells were split using trypsin-EDTA (0.5% trypsin and 0.25% EDTA; Gibco Invitrogen) every 5–7 days. Three foreskin lines (F3, F7, and F8) were derived using FBS, three (F2, F4, and F6) using SR, and two (F1 and F5) using human serum. The foreskin lines were maintained using the derivation medium; shortly before use, feeder layers were transferred to the human ES cell medium supplemented with 20% SR as described be-

TABLE 1. PCR primers used in the present study.

Gene product	Primer	Reaction condition	Size (base pairs)
Oct-4	GAGAACAATGAGAACCTTCAGGAGA TTCTGGCGCCGGTTACAGAACCA	30 Cycles at 60°C in 1.5 mM $MgCl_2$	219
GAPDH[a]	AGCCACATCGCTCAGACACC GTACTCAGCGGCCAGCATCG	30 Cycles at 60°C in 1.5 mM $MgCl_2$	302
Albumin	TGCTTGAATGTGCTGATGACAGGG AAGGCAAGTCAGCAGCCATCTCAT	35 Cycles at 60°C in 1.5 mM $MgCl_2$	157
α-fetoprotein	GCTGGATTGTCTGCAGGATGGGGAA TCCCCTGAAGAAAATTGGTTAAAAT	30 Cycles at 60°C in 1.5 mM $MgCl_2$	216
Glut2	AGGACTTCTGTGGACCTTATGTG GTTCATGTCAAAAAGCAGGG	35 Cycles at 55°C in 1.5 mM $MgCl_2$	231
NF-68KD	GAGTGAAATGGCACGATACCTA TTCCTCTCCTTCTTCACCTTC	30 Cycles at 60°C in 2 mM $MgCl_2$	473
α-cardiac actin	GGAGTTATGGTGGGTATGGGTC AGTGGTGACAAAGGAGTAGCCA	35 Cycles at 65°C in 2 mM $MgCl_2$	486

[a] GAPDH: Glyceraldehyde phosphate dehydrogenase.

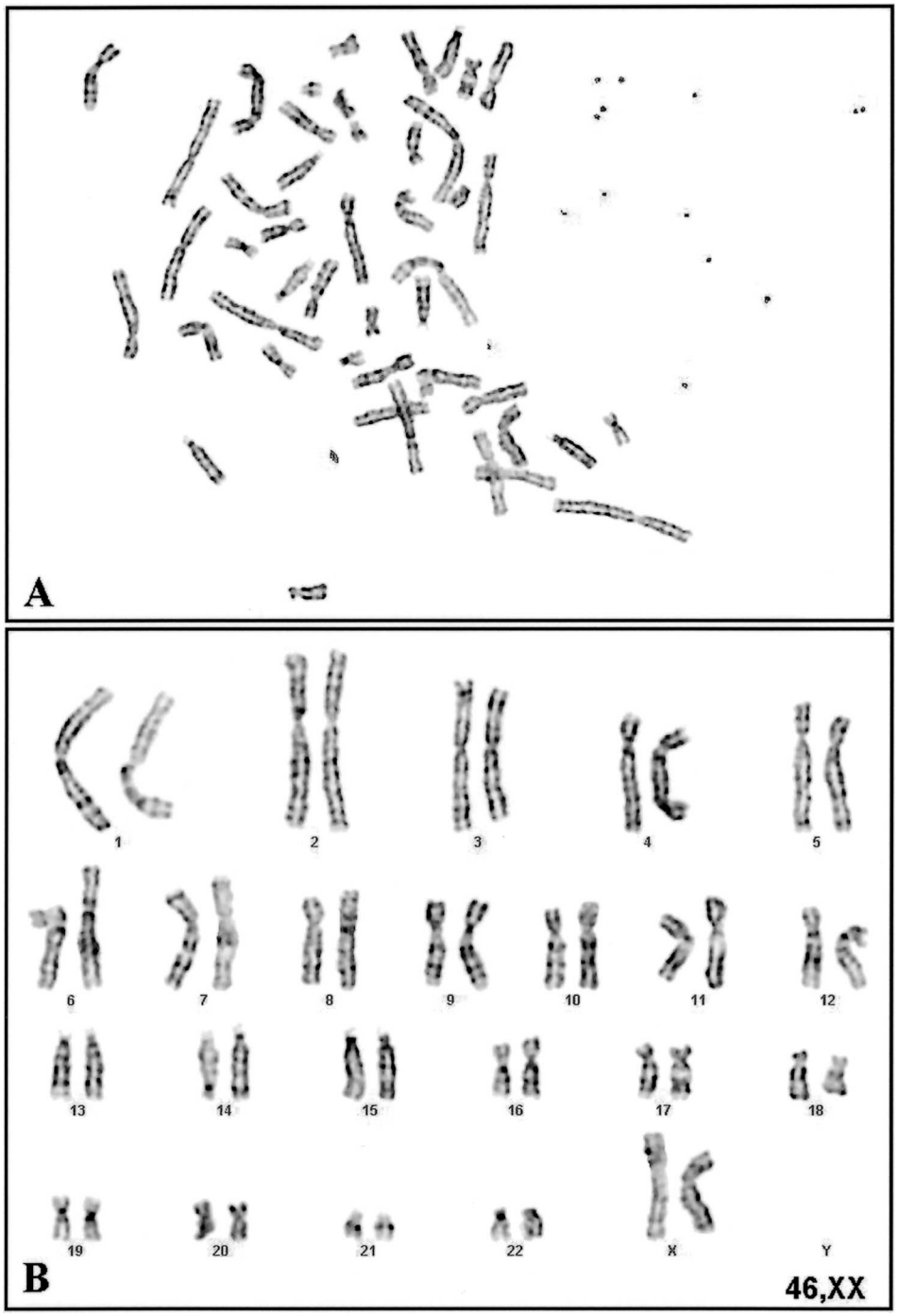

FIG. 2. Karyotype analysis of I-3 cells using the G-band method. The karyotype was examined after 29 passages of continuous culture with the foreskin lines, at passage 107 from derivation. The karyotype was found to be 46,XX normal female. **A**) Captured metaphase preparation from I-3 cell. **B**) The 46 separated chromosomes from the metaphase demonstrated at **A**.

low. The cells were frozen in liquid nitrogen using freezing solution consisting of 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO), 10% FBS or human serum or SR (according to the medium used for derivation), and 80% Ko-DMEM.

ES Cell Culture

Human ES cell lines I-6, I-3 [9], and H-9 [3] were initially cultured with MEF and then transferred to the foreskin lines. The cells were grown in a culture medium consisting of 85% Ko-DMEM supplemented with 15% SR, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acid stock, and 4 ng/ml bFGF (all from Gibco Invitrogen). They were passaged every 4–6 days using 1 mg/ml of type IV collagenase (Gibco Invitrogen). According to the freezing protocol, the cells are frozen in liquid nitrogen using freezing solution consisting of 10% DMSO, 10% FBS, and 80% Ko-DMEM.

Formation of Teratomas

The potential to form derivatives of all three embryonic germ layers was examined in teratomas. After more than 20 passages in culture with foreskin lines, the ES cells drawn from four to six confluent wells (10 cm^2 each) were injected into the rear leg muscle of 4-wk-old, male, SCID-beige mice (Harlan, Israel). At least 10 wk after the injection, the resulting teratomas were examined histologically.

Embryoid Body Formation

For the formation of embryoid bodies (EBs), we used one confluent, six-well plate (60 cm^2). The ES cells were passaged using 1 mg/ml of type IV collagenase, further broken into small clumps using 1000 μl of Gilson pipette tips, and cultured in suspension in 58-mm Petri dishes (Greiner, Germany). The EBs were grown in medium consisting of 80%

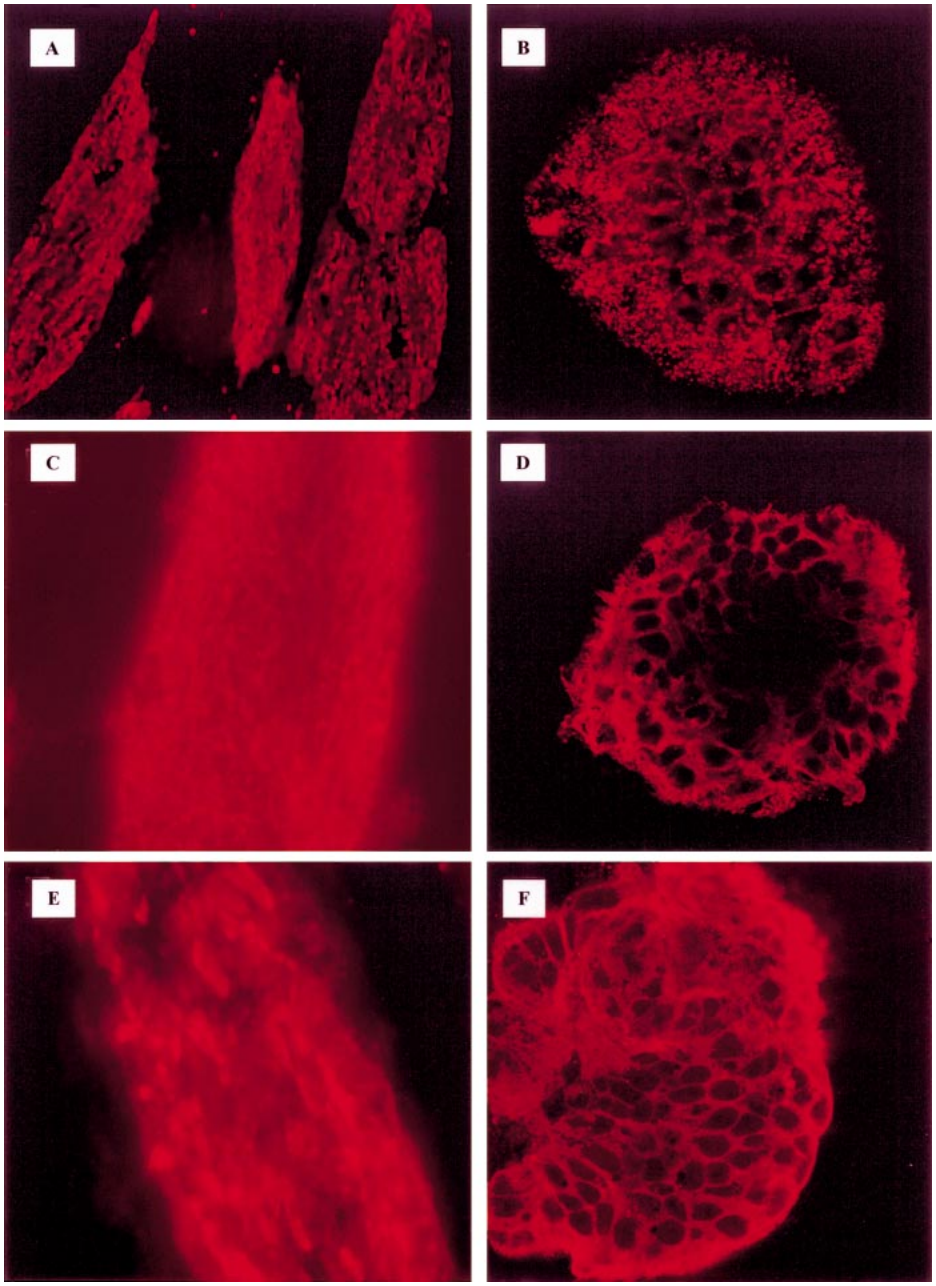

FIG. 3. Fluorescent immunostaining of human ES cell line I-6 after being cultured 57 passages (**A**–**C**) or 75 passages (**D**–**F**) with foreskin lines. **A**) Immunostaining of I-6 human ES cell colony with anti-TRA-1-60 antibodies. **B**) Immunostaining of I-6 human ES cell colony with anti-SSEA4 antibodies. **C**) Immunostaining of I-6 human ES cell colony with anti-TRA-1-81. **D**–**F**) Confocal imaging of human ES cell line I-6 after a total of 75 passages on foreskin lines; the last three passages on line F5. Note the darkness of the cytoplasm and the clearly stained cell surface. **D**) Immunostaining of I-6 human ES cell colony with anti-SSEA4 antibodies. **E**) Immunostaining of I-6 human ES cell colony with anti-TRA-1-60 antibodies. **F**) Immunostaining of I-6 human ES cell colony with anti-TRA-1-81 antibodies. Magnification ×5 (**A**), ×20 (**B** and **C**), and ×63 (**D**–**F**).

Ko-DMEM supplemented with 20% FBS (HyClone), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acid stock (all but marked from Gibco Invitrogen).

Immunohistochemistry

The human ES cells were fixed with 4% paraformaldehyde and exposed to the primary antibodies (1:50) overnight at 4°C. As secondary antibodies (1:100), we used Cys-3-conjugated antibodies (Chemicon International). The primary antibodies, stage-specific embryonic antigen (SSEA) 1, SSEA3, and SSEA4, as well as the TRA1-60 and TRA1-81 were kindly provided by Prof. P. Andrews (University of Sheffield, U.K.).

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from undifferentiated or 1-mo-old EBs from cells grown on foreskin using Tri-Reagent (Sigma), according to the manufacturer's recommended protocol. The cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase RNase H minus (Promega, Madison, WI). The polymerase chain reaction (PCR) included denaturation for 5 min at 94°C, followed by repeated cycles of 94°C for 30 sec, annealing at the temperature as in Table 1 for 30 sec, and extension at 72°C for 30 sec. The PCR primers and the reaction conditions used are described in Table 1. The PCR products were size fractionated using 2% agarose gel electrophoresis. As negative controls, we used reverse transcription (RT) mixes (see Fig. 5) and RT reaction from the foreskin lines (data not shown). Total RNA was collected from undifferentiated human ES cells grown for more than 30 passages on the foreskin lines and from 1-mo-old EBs formed by ES cells grown for more than 30 passages on the foreskin lines.

Karyotype

Cell division was blocked in mitotic metaphase using colcemid-spindle formation inhibitor (karyoMax colcemid solution; Gibco Invitrogen). Nuclear membranes were broken after hypotonic treatment. For the chromosome visualization, we used G-band standard staining (Giemsa; Merck, Darmstadt, Germany). The karyotypes were analyzed and reported according to the International System for Human Cytogenetic Nomenclature. At least 50 cells were examined from each sample.

RESULTS

All foreskin lines tested gave rise to fibroblast-like lines, which were grown and split for more than 25 consecutive

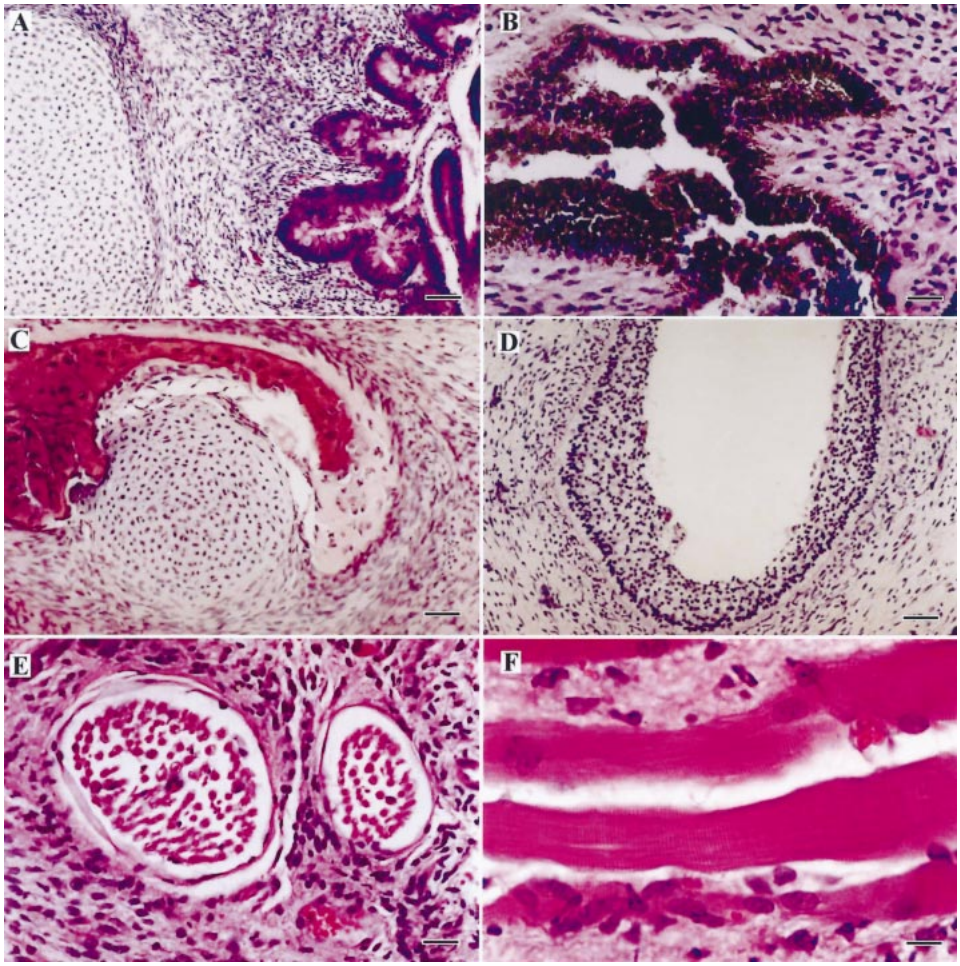

FIG. 4. In vivo differentiation in teratomas of human ES cells grown on human newborn fibroblast feeder. **A**) Cartilage and mucus-secreting epithelium, from I-3, after 20 passages with foreskin lines. **B**) Epithelium with cells containing melanin, from H-9, after 34 passages with the foreskin feeder layers. **C**) Calcified cartilage tissue, from I-3, after 20 passages with foreskin lines. **D**) Stratified epithelium, from I-3, after 20 passages with foreskin lines. **E**) Transverse section of myelinated nerves, from H-9, after 34 passages with the foreskin feeder layers. **F**) Developing striated muscle, from I-6, after 50 passages with the foreskin lines. Bar = 50 μm (**A**, **C**, and **D**), 25 μm (**B** and **E**), and 10 μm (**F**). Hematoxylin and eosin stain.

passages. The foreskin lines are still continuously cultured. No reduction in the rate of growth or the ability to support human ES growth was noted after using high-passage foreskin cells or after freeze-and-thaw cycles. When in culture, foreskin fibroblasts are known to grow at least 42 passages before they go through senescence (American Type Culture Collection [ATCC] cell lines Hs27 and Hs68). The additional foreskin lines were derived using SR or human serum (F1, F2, F4, F5, and F6) and have been continuously growing for more than 3 mo; their growth rate and morphology are similar to the lines derived with FBS.

Human ES cell lines I-3, I-6, and H-9 were easily transferred to the foreskin feeders. Each transferred line continued to proliferate and maintained normal ES features. The morphology of ES cell colonies that grew on the human feeder layers are slightly different from the ones cultured on MEF. It seems that the cells are organized according to the direction of the foreskin feeder layers (Fig. 1A). Consequently, the colonies are not as round as those grown on MEF. However, the individual human ES cell morphology remained the same as that of cells cultured on MEF. The cells remained round and small, with a high nucleus:cytoplasm ratio, notable presence of one to three nucleoli, and typical spacing between the cells (Fig. 1B).

Initially, each human ES line was grown on one different foreskin line only. Approximately 20 passages later, each line was grown on one of the three foreskin feeders, chosen randomly. No difference was noted between the ability of the F3, F7, or F8 foreskin lines to support the human ES cell growth or between the different human ES cell lines to adjust to the human feeders. Human ES cells from lines I-6 and I-3 were transferred to foreskin lines derived using either SR (F2 and F4) or human serum (F1 and F5). Having been grown for six and eight passages, respectively, the cells maintained the typical morphology and expression of undifferentiated surface markers, typical of human ES cells.

Karyotype analysis was performed on the XX lines only to avoid examining the foreskin cells instead of the ES cells. Two separate batches of I-3 and one of the H-9 cell lines were tested after 22, 29, and 47 passages of continuous culture with the human feeder layers, respectively. At least 50 cells from each line were examined; each one was found to possess normal human 46,XX karyotype. An example of the examined metaphase and chromosomes is illustrated in Figure 2.

Several surface markers, typical of primate ES cells, were examined using immunofluorescent staining [4, 10, 11]. The human ES cells grown with foreskin cells for 57 passages were strongly positive for the surface markers SSEA4, TRA-1-60, and TRA-1-81 (Fig. 3), with weakly positive staining for SSEA3 and negative staining for SSEA1. The same expression pattern was found in the other two human ES cell lines (I-3 and H-9) grown on the foreskin feeders for more than 20 passages.

The developmental potential of the cells after long culture on the foreskin feeders was examined in vivo using the teratoma model. The three human ES cell lines used in this research (I-3, H-9, and I-6) formed teratomas following injection into SCID-beige mice after 20, 34, and 50 passages of prolonged growth on the foreskin lines, respec-

tively. Each teratoma contained representative tissues of the three embryonic germ layers, including cartilage tissue, smooth muscle, stratified epithelium, melanin-containing cells, connective tissue, gut-like epithelium, etc. Representative tissues formed in these teratomas are demonstrated in Figure 4.

In vitro, human ES cells cultured with foreskin lines, similar to ES cells grown on MEF, formed EBs when cultured in suspension [12]. Within these EBs, the stem cells differentiated into representative cells of the three embryonic germ layers. Whereas undifferentiated cells grown on the foreskin lines expressed Oct-4, cells harvested from 1-mo-old EBs expressed genes such as neurofilament (ectoderm) and albumin (endoderm), as demonstrated by RT-PCR in Figure 5. α-Cardiac actin (mesoderm) expression was found in undifferentiated ES cells. This early gene was also found to be expressed in ES cells in other reports [13]. The expression of Oct-4 decreased after differentiation into EBs.

DISCUSSION

Traditionally, human ES cells have been cultured using MEF as feeder layers and media supplemented with FBS [3, 4]. The dual growth of the cells with MEF exposes the human ES cells to mouse retroviruses, which may prevent the future use of these cells in cell-based therapy. The culture system described in the present study offers completely animal-free growth conditions for human ES cells.

Under these conditions, the human ES cells maintained all the characteristics of ES cells. After prolonged culture of more than 57 passages of growth with the foreskin feeders, the cells remained undifferentiated, as demonstrated by the expression of surface markers typical of undifferentiated primate ES cells [3, 4, 10, 11]. The lines cultured with the human foreskin feeders were strongly positive for surface markers that are also typical of undifferentiated primate ES cells (SSEA4, TRA-1-60, and TRA-1-81), weakly positive staining for SSEA3, and negative staining for SSEA1.

When the undifferentiated ES cells grown with the human feeder layers were removed from the coculture conditions and grown in suspension, they spontaneously differentiated into various cell types, including representative cells from the three embryonic germ layers. The developmental potential of the cells after continuous culture with the foreskin feeder layers was also examined in vivo in teratomas. The three cell lines grown on the foreskin lines formed teratomas following injection into SCID beige mice. These teratomas contained tissues from all three embryonic germ layers. Thus, the human ES cells cultured for prolonged periods on the foreskin lines possess the same developmental potential as human ES cells grown on MEF.

Similar to human ES cells cultured with MEF, the karyotypes of the cells remained 46,XX normal during the continuous culture of the cells in the animal-free culture condition. The karyotype analysis was carried out on ES cells at relatively high passages: 87 (H-9), 104 (I-3), and 107 (I-3) after line derivation. None of the examined cells ($n = 50$ from each line) had karyotype abnormalities, which emphasizes the human ES cell karyotype stability.

The first ES line transferred to the animal-free conditions (I-6) is still being cultured with the foreskin line. After 70 passages (>250 doublings) in this culture system, no difference has been observed in the rate of passaging (every 4–6 days), background differentiation (≤5%), or in the survival rates during passages with collagenase (>95%) between I-6 grown with foreskin line and I-6 cultured with MEF.

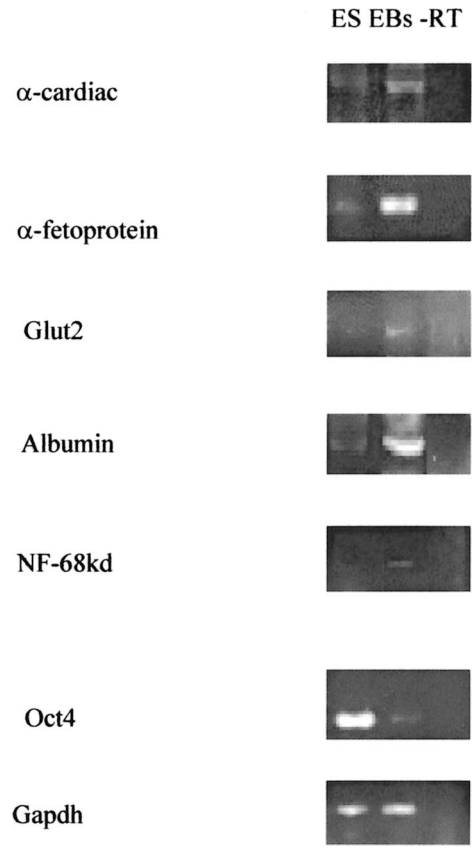

FIG. 5. RT-PCR analysis of human ES cell lines I-3 and I-6 after more than 30 passages of continuous culture with the foreskin lines as undifferentiated ES cells (ES) or after 1 mo (after 30 passages as undifferentiated cells on foreskin lines) of growth as EBs in suspension (EBs).

Taken overall, these data demonstrate that human ES cells maintain all ES cell features after continuous culture on human feeder layers (i.e., pluripotency, immortality, and unlimited undifferentiated proliferation capability), which has no effect on their remarkable developmental potential, and they also maintain normal karyotypes [3].

Another advantage of the foreskin lines as feeder layers is the ability to grow them for more than 42 passages. In contrast, MEF go through senescence five to six passages after derivation. Therefore, a continuous need exists for MEF formation. Different batches of MEF may vary in their growth rates and ability to support ES cells. Because of their short life span, MEF could not be analyzed for retroviruses before use and could not be genetically modified. On the other hand, the long culture ability of the foreskin lines allows testing for retroviruses or the insertion of an antibiotic-resistance gene and the creation of antibiotic-resistant clones to use them for antibiotic selection. No difference was found between the ability of various foreskin lines to support human ES cell cultures as with various MEF batches.

In our experience, some of the alternative human lines, such as commercially available or self-made human embryonic fibroblasts [8], and commercial whole-embryo human fibroblasts have short life span similar to that of MEF (unpublished data). Because the derivation of these lines involves the consent of patients undergoing abortions, the need to recreate lines may raise technical and ethical prob-

lems. In addition, the availability of aborted human embryos and human fallopian tubes is relatively low, although their medical records are easier to obtain. On the other hand, the foreskin lines' prolonged culture and the ability to freeze and thaw them at any passage without losing their ability to support human ES cells enables us to use cell lines derived from one foreskin for more than a year. Furthermore, foreskin lines that can undergo 42 passages are commercially available (ATCC C.N. CRL-1634 and CRL-1635).

Richards et al. [8] used mechanical cutting followed by enzymatic digestion with dispase to passage the ES cell colonies, which limits the amount of cells available for use. On the other hand, the ability to use collagenase to passage the cells cultured on the foreskin lines ($>10^7$ cells at once) with high survival rates allowed animal-free, large-scale growth of human ES cells.

The ability of foreskin lines to support human ES cell line derivation was not tested. However, the long-term use of these lines as feeders without notable differences from MEF suggests that possibility.

ACKNOWLEDGMENTS

The authors thank Mrs. Ludmilla Mazor for technical assistance and Mrs. Hadas O'Neill for editing the manuscript.

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