

A Feeder-Free and Efficient Production of Functional Neutrophils from Human Embryonic Stem Cells

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Human Embryonic Stem Cells with Maintenance under a Feeder-Free and Recombinant Cytokine-Free Condition

Masako Nakahara,¹ Kumiko Saeki,¹ Naoko Nakamura,¹ Satoko Matsuyama,¹ Yoshiko Yogiashi,¹ Kazuki Yasuda,² Yasushi Kondo,³ and Akira Yuo¹

Abstract

We previously reported that cynomolgus monkey embryonic stem (ES) cells could be maintained under a feeder-free condition without using recombinant cytokines if sizes and numbers of ES colonies were kept within an appropriate range. Here we show that this finding is also true with human ES cells (hESCs). The two lines of hESCs, khES-1 and khES-3, were appropriately maintained in the absence of feeder layers or exogenous cytokines such as fibroblast growth factors, Noggin, transforming growth factor β , and Activin by closely controlling the size and number of hESC colonies. High-level expressions of immature markers including SSEA-4, Oct-4, and Nanog were detected in feeder-free and cytokine-free hESCs, and they formed teratomas when implanted into severe combined immunodeficiency (SCID) mice. No chromosomal abnormalities were observed over 20 passages, ruling out the possibility that special clones with growth advantages had been selected. Global protein expression profiles were quite similar among the hESCs maintained by our feeder- and cytokine-free method, by coculture with mouse embryonic fibroblasts (MEFs) and by a feeder-free method using conditioned media of MEFs. However, the activation level of Akt, an important player for the maintenance of ES cells, was highest and the activation level of extracellular signal-regulated kinase, a critical player for differentiation of ES cells, was lowest in the hESCs maintained by our cytokine-free method. Our results not only show a technical improvement for the maintenance of hESCs but also open a new avenue for the understanding of autocrine signaling networks of hESCs.

Introduction

HUMAN EMBRYONIC STEM (ES) cells (hESCs), which were established from inner cell mass of blastocysts via coculture with mouse embryonic fibroblasts (MEF), are generally maintained by coculture with MEFs. In aim of clinical application, however, usage of xenogenic cells should completely be avoided. The first success in performing the feeder-free culture of hESCs was reported by Xu et al. (2001), where hESCs were cultured on MatrigelTM Matrix-coated vessels using conditioned media (CM) of MEFs, namely, MEF-CM. Afterward, several MEF-CM-free methods were reported (Amit et al., 2004; Beattie et al., 2005; Chen et al., 2006; Ludwig et al., 2006; Sato et al., 2004; Wang et al., 2005; C Xu et al., 2005; RH Xu et al., 2005; Yao et al., 2006). In those methods, either small-molecular-weight chemical substances

of yet undetermined safety or nonphysiological doses of cytokines including fibroblast growth factor (FGF)-2, Noggin, transforming growth factor (TGF)- β and Activin were used. Although hESCs were effectively maintained by using these substances in each experimental condition, the molecular bases for the self-renewal of hESCs remain controversial. For example, one report interpreted the roles of FGF-2 for the maintenance of hESCs as follows: it stimulates autologously derived human ES fibroblast-like cells (hdFs) to secrete insulin-like growth factor (IGF)-II, which in turn, stimulate self-renewal of hESCs (Bendall et al., 2007). However, it is well known that KnockoutTM Serum Replacement (KSR), which was added to the culture medium, has a sufficient IGF-like activity (Wang et al., 2007). Thus, it may be possible that KSR per se contributed to the maintenance of hESCs rather than the IGF-II produced by the FGF2-stimulated

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hdFs. Moreover, the existence of hdFs per se seems quite controversial because a few reports showed the absence of hdFs around the well-maintained human ES colonies (Ludwig et al., 2006; Wang et al., 2005; RH Xu et al., 2005). Recently, a very interesting finding on self-renewal pathways of murine ES cells was reported. It was shown that extrinsic stimuli were dispensable for the maintenance of murine ES cells as long as their spontaneous differentiation was blocked by using three categories of intracellular signaling inhibitors: FGF receptor inhibitor, glycogen synthase kinase (GSK)-3 inhibitor and extracellular signal-regulated kinase (ERK) inhibitor (Ying et al., 2008). Authors concluded that murine ES cells have an innate program for self-replication that does not require extrinsic instruction, which may account for their latent tumorigenicity. Considering culture technique, it is noteworthy that the cell density was reportedly a critical factor in the feeder-free maintenance of hESCs (Xu et al., 2001). Thus, we hypothesized that, if human ES colonies were carefully cultured at appropriate densities to inhibit spontaneous differentiation, they could be maintained even in the absence of exogenous stimuli.

We recently succeeded in maintaining the cynomolgus monkey ES cells without feeder layers or exogenous cytokines by closely controlling the size and number of ES colonies (Nakahara et al., 2008). To assess the effectiveness of this culture technique in the maintenance hESCs, we cultured hESCs without feeder layers or exogenous cytokines by a controlling of the size and number of colonies.

Here we show that two lines of hESCs, both of which have normal karyotype, can be maintained without feeder layers or recombinant cytokines by controlling the size and number of ES colonies. Over 20 passages, no chromosomal abnormality was detected. Our method proposes the most natural condition for the maintenance of hESCs, which is free from possible disturbing side effects of exogenously added cytokines and also provides ideal conditions for autocrine signaling networks in hESCs.

Materials and Methods

Cells and reagents

khES-1 and khES-3 (Suemori et al., 2001) were cultured on Matrigel™ Matrix (BD Biosciences, San Jose, CA)-coated culture dishes using DMEM/F12 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 20% Knockout Serum Replacement (KSR™) (Invitrogen Corp.), 1% nonessential amino acids solution (Invitrogen Corp.), 1 mM Sodium Pyruvate Solution (Invitrogen Corp.), 2 mM L-glutamin (Invitrogen Corp.), and 0.1 mM β -mercaptoethanol (Sigma Chemical Co., St. Louis, MO) without using recombinant cytokines. The hESCs were maintained as a crop of colonies of similar sizes with diameters between 200 μ m and 1000 μ m at densities less than eight colonies per one cm^2 . The hESCs were passed every 4 days. For detachment from the culture dish, cells were treated with a minimum volume of dissociation liquid including 0.25% trypsin (Invitrogen Corp.), 1 mg/mL collagenase IV (WAKO Pure Chemical Industries, Osaka, Japan), 20%KSR™, 1 mM CaCl_2 at 37°C for 5 to 15 min. Cells were gently collected without pipetting to avoid cell damage. They were seeded at split ratios of 1:2–1:4 on newly Matrigel™ Matrix-coated dishes, avoiding fusion between colonies. Culture medium was changed

every day. In some experiments, hESCs were cultured on the layer of 40 Gy-irradiated MEFs (i.e., coculture with MEFs) using the previously described media or on Matrigel™ Matrix-coated dishes using conditioned media (CM) of MEF supplemented with 4 ng/mL of FGF-2 (PeproTech Inc., Rocky Hill, NJ). MEF-CM was prepared as reported by elsewhere (Xu et al., 2001).

Flow cytometry

Cell surface markers of hESCs were analyzed by flow cytometric analysis. Cells were collected by treatment with trypsin/EDTA (Invitrogen Corp.) and, after a wash in phosphate-buffered saline (PBS), 1×10^6 cells were treated with primary antibodies on ice for 30 min. The expression level of each protein was analyzed using a FACSCalibur (BD Biosciences, San Jose, CA). The antibodies used were a mouse antihuman SSEA-4-phycoerythrin (PE) (clone MA813-7) (R&D Systems Inc., Minneapolis, MN), a mouse antihuman E-cadherin-PE (clone #180224) (R&D Systems Inc.) and a rat antihuman Oct-4-PE (clone 240408) (R&D Systems Inc.). For studies on SSEA-4 and E-cadherin expressions, cells were further stained with TO-PRO3 fluorescent dye (Invitrogen Corp.) for 10 min to gate out the dead cell populations as FLA-higher fractions during analyses. For studies on Oct-4 expression, cells had been fixed and permeabilized by FIX & PERM® reagents (Invitrogen Corp.) according to the manufacturer's guidelines before the antibody reaction.

Immunostaining

Cells were fixed on slide glasses by using a cytospin apparatus (Cytospin 2) along with further fixation with acetone/methanol solution (1:3). The immunostaining procedure was performed as previously described (Saeki et al., 2003). The first antibody reactions were performed using a mouse monoclonal antihuman Oct-3/4 antibody (C-10, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a rabbit polyclonal antihuman Nanog antibody (ReproCELL Inc., Tokyo, Japan). The second antibody reactions were performed using Alexa Fluor® 488 chicken antimouse IgG (H+L), Alexa Fluor® 568 goat antirabbit IgG (H+L) (Invitrogen Corp.).

Western blotting

Western blotting was performed as previously described (Saeki et al., 2003) using an antihuman extracellular signal-regulated kinase (ERK) (p44/p42 MAPK) antibody (Cell Signaling Technology Inc., Beverly, MA), antihuman phosphorylated ERK (phospho-p44/p42 MAPK (T202/Y204)) antibody (Cell Signaling Technology Inc.), a mouse monoclonal antihuman signal transducer and activator of transcription 3 (Stat3) (BD Biosciences), antihuman phospho-STAT3 (Tyr705) antibody (Cell Signaling Technology, Inc.), antihuman phospho-Akt (Ser473) antibody (Cell Signaling Technology Inc.), an antihuman Akt antibody (Cell Signaling Technology Inc.), a rabbit polyclonal antihuman β -tubulin (H-235) antibody (Santa Cruz Biotechnology Inc.). The second antibody reaction was performed using a horseradish peroxidase-conjugated antirabbit or antimouse IgG (Cell Signaling Technology, Inc.). The final detection procedure was performed using ECL Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

Karyotype analyses

The metaphase hESCs were collected after 16-h incubation of hESCs with 0.06 mg/mL of colcemid. Then the cells were treated by hypotonic buffer and fixed by methanol/acetic acid (3:1). Chromosomal analyses with G band staining were performed by SRL Inc. (Tokyo, Japan) by a standard method.

Morphological examinations

Viable cells were directly observed under an inverted phase contrast light microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Evaluation of cell viability

Cell viability was assessed via flow cytometric analyses by using Annexin V-FITC Kit (Beckman Coulter Inc., Fullerton, CA), which is an apoptosis detection kit based on the binding properties of annexin V to phosphatidylserine and on the DNA-intercalating capacities of propidium iodide (PI), according to the manufacturer's guidelines.

Assessment of cell proliferation

Cell proliferation activities were assessed after 3 days from cell passage by measuring the 5-bromo-2'-deoxyuridine (BrdU)-uptaking activities by using BrdU Cell Proliferation Assay (Calbiochem Co., La Jolla, CA) according to the manufacturer's guidelines. Briefly, ES cells were incubated in six-well multiwell plates (5×10^4 cells/well) in the presence of BrdU for 17 h, fixed and stained by anti-BrdU primary antibody along with a horseradish peroxidase (HRP)-conjugated secondary antibody. After substrate reactions, 200 μ L supernatants were collected into 96-well plates and the absorbance at 490 nm was measured by Model 550 Microplate Reader (Bio-Rad Lab. Alfred Nobel Drive Hercules, CA).

Teratoma formation

hESCs (1×10^5) maintained by our novel feeder-free method were subcutaneously injected into adult male severe combined immunodeficient (SCID) mice (Stojkovic et al., 2005). After 8 weeks, mice were sacrificed and testes were removed. After tumor formation was checked by megascopy, tumor tissues were fixed, sliced, and stained by hematoxylin and eosin solutions for subsequent histological examinations.

Two-dimensional gel electrophoresis (2-DE)

The hESCs were collected by 0.2% EDTA treatment. After washing the cells with washing buffer (10 mM Tris-HCl buffer, pH 8.0, 5 mM magnesium acetate), 4×10^7 cells were suspended with 7 volumes of lysis buffer containing 2 M thiourea, 7 M urea, 4% (w/v) CHAPS and 1 mM Pefablc SC PLUS (Roche Diagnostics GmbH, Mannheim, Germany). The cell suspensions were kept for 10 min on ice, sonicated intermittently and centrifuged at $12,000 \times g$ for 10 min at 4°C, and then the supernatant fractions were collected. The protein concentration was determined in the lysis solution with a dye reagent from Amersham Biosciences (Piscataway, NJ) using bovine serum albumin as a standard. The lysate was alkylated with Ready Prep™ Reduction-Alkylation Kit (Bio Rad Laboratories, Hercules, CA). The 100- μ g protein lysate

per gel were subjected to 2-DE. The first-dimensional isoelectric focusing was carried out using Immobiline dry strip (18-cm long, pH 3–10 with linear gradient) in a horizontal electrophoresis system, Ettan IPGphor (Amersham Biosciences) according to the manufacturer's instructions. After the first-dimensional electrofocusing, IPG gels were equilibrated with buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 0.01% BPB, and 0.5% dithiothreitol, followed by alkylation with equilibration buffer containing 4.5% iodoacetamide instead of 0.5% dithiothreitol at room temperature for 15 min. The gels were subjected to the second-dimensional SDS polyacrylamide gel electrophoresis using 9–18% gradient gels. Proteins were visualized in the gels by staining with SYPRO Ruby Protein Gel Stain (Bio Rad Laboratories) overnight. The fluorescence intensity of each protein spot was digitally recorded by FluorImager 595 (Amersham Biosciences) using Image QuanNT software. Gel images were attached with a horizontal axis of pI and a vertical axis of molecular weights (MW). For brief comparisons, a typical area with high resolution and separation (pI 5.5–9.0, MW 20–75 kDa) was shown for each sample. The regions that were rich in cytoskeletal proteins including actins and tubulins were also trimmed out.

Results

Maintenance of hESCs without using recombinant cytokines

We previously reported that cynomolgus monkey ES cells could be maintained on feeder-free, Matrigel™ Matrix-coated dishes without using recombinant cytokines if the sizes and numbers of ES colonies were kept within appropriate ranges during subculture processes (Nakahara et al., 2008). To determine the generality of this finding among primate ES cells, two lines of hESCs (khES-1 and khES-3) were cultured in the absence of feeder layers and exogenous cytokines. Cells were observed daily and passed at an appropriate timing before signs of spontaneous differentiation became apparent. After trial-and-error processes, we determined the optimal protocol as described in Materials and Methods. The hESCs maintained under a feeder-free and cytokine-free condition showed immature compact morphologies over 20 passages (Fig. 1A). Flow cytometric analyses indicated that high percentages of the cells expressed undifferentiated hESC markers of SSEA-4, of which the positivity was 97.0% in khES-1 at passage 20 (Fig. 1B, upper panel) and 97.6% in khES-3 at passage 23 (Fig. 1B, lower panel), and Oct-4, of which the positivity was 96.8% in khES-1 at passage 20 (Fig. 1B, upper panel) and 95.9% in khES-3 at passage 23 (Fig. 1B, upper panel). Immunostaining studies further demonstrated that each nuclei of the cells was stained by anti-Nanog in khES-1 (Fig. 1C) and khES-3 (data not shown). Viabilities of the hESCs maintained by this "feeder-free and cytokine-free (FFCF)" method were evaluated by checking apoptosis-inducing states in comparison with the hESCs maintained by the conventional method of coculture with MEFs (CO-MEFs). As shown in Figure 1D, viabilities of the FFCF-maintained hESCs were comparable to those of CO-MEFs-maintained hESCs (Fig. 1D). Active cell proliferation was also confirmed by positive incorporations of BrdU into FFCF-maintained hESCs (Fig. 1E). Pluripotency

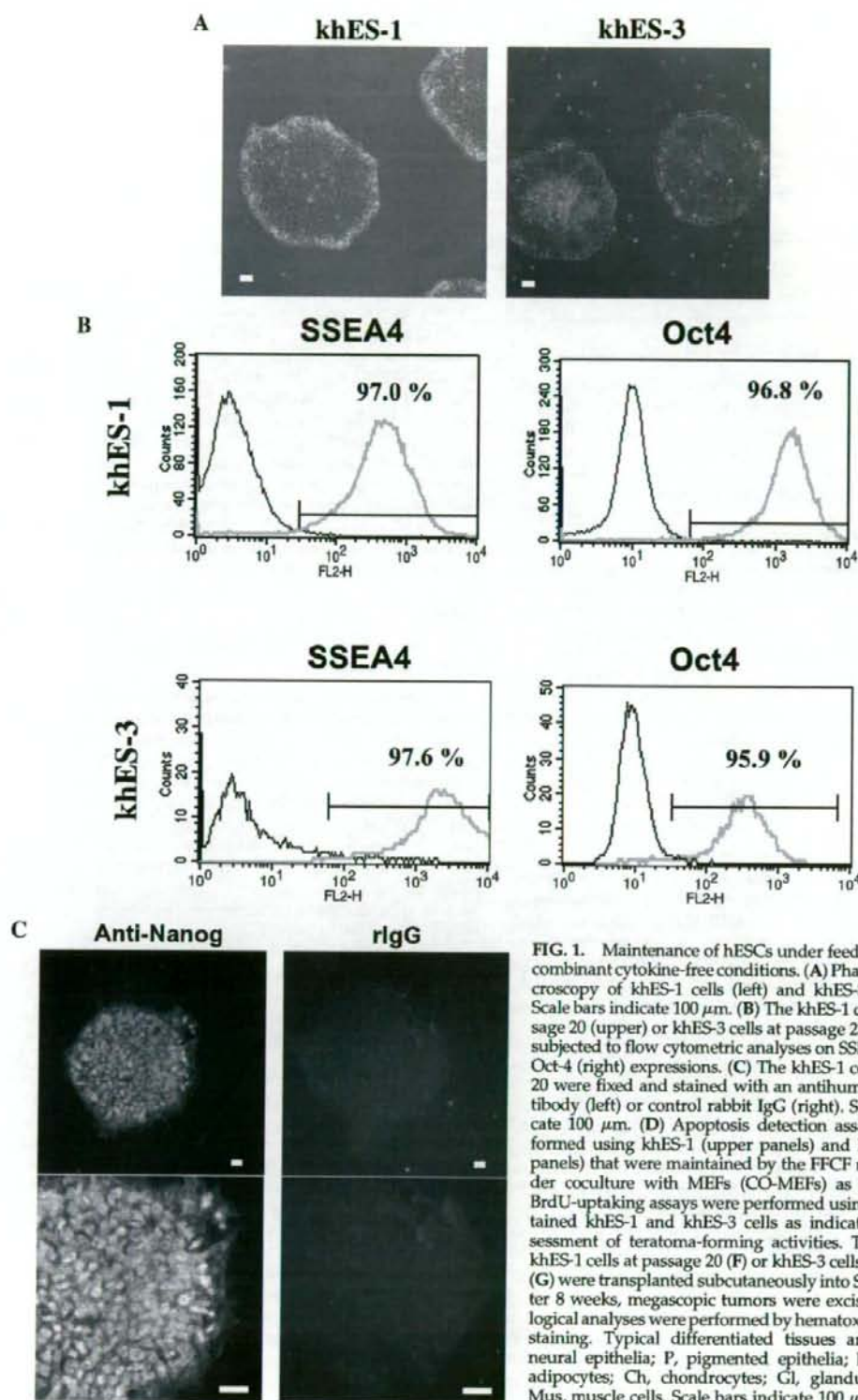


FIG. 1. Maintenance of hESCs under feeder-free and recombinant cytokine-free conditions. (A) Phase contrast microscopy of khES-1 cells (left) and khES-3 cells (right). Scale bars indicate 100 μ m. (B) The khES-1 cells at the passage 20 (upper) or khES-3 cells at passage 23 (lower) were subjected to flow cytometric analyses on SSEA-4 (left) and Oct-4 (right) expressions. (C) The khES-1 cells at passage 20 were fixed and stained with an antihuman Nanog antibody (left) or control rabbit IgG (right). Scale bars indicate 100 μ m. (D) Apoptosis detection assays were performed using khES-1 (upper panels) and khES-3 (lower panels) that were maintained by the FFCF method or under coculture with MEFs (CO-MEFs) as indicated. (E) BrdU-uptaking assays were performed using FFCF-maintained khES-1 and khES-3 cells as indicated. (F,G) Assessment of teratoma-forming activities. The 3×10^6 of khES-1 cells at passage 20 (F) or khES-3 cells at passage 23 (G) were transplanted subcutaneously into SCID mice. After 8 weeks, megascopic tumors were excised and histological analyses were performed by hematoxylin and eosin staining. Typical differentiated tissues are shown. N, neural epithelia; P, pigmented epithelia; B, bones; Ad, adipocytes; Ch, chondrocytes; Gl, glandular epithelia; Mus, muscle cells. Scale bars indicate 100 μ m.

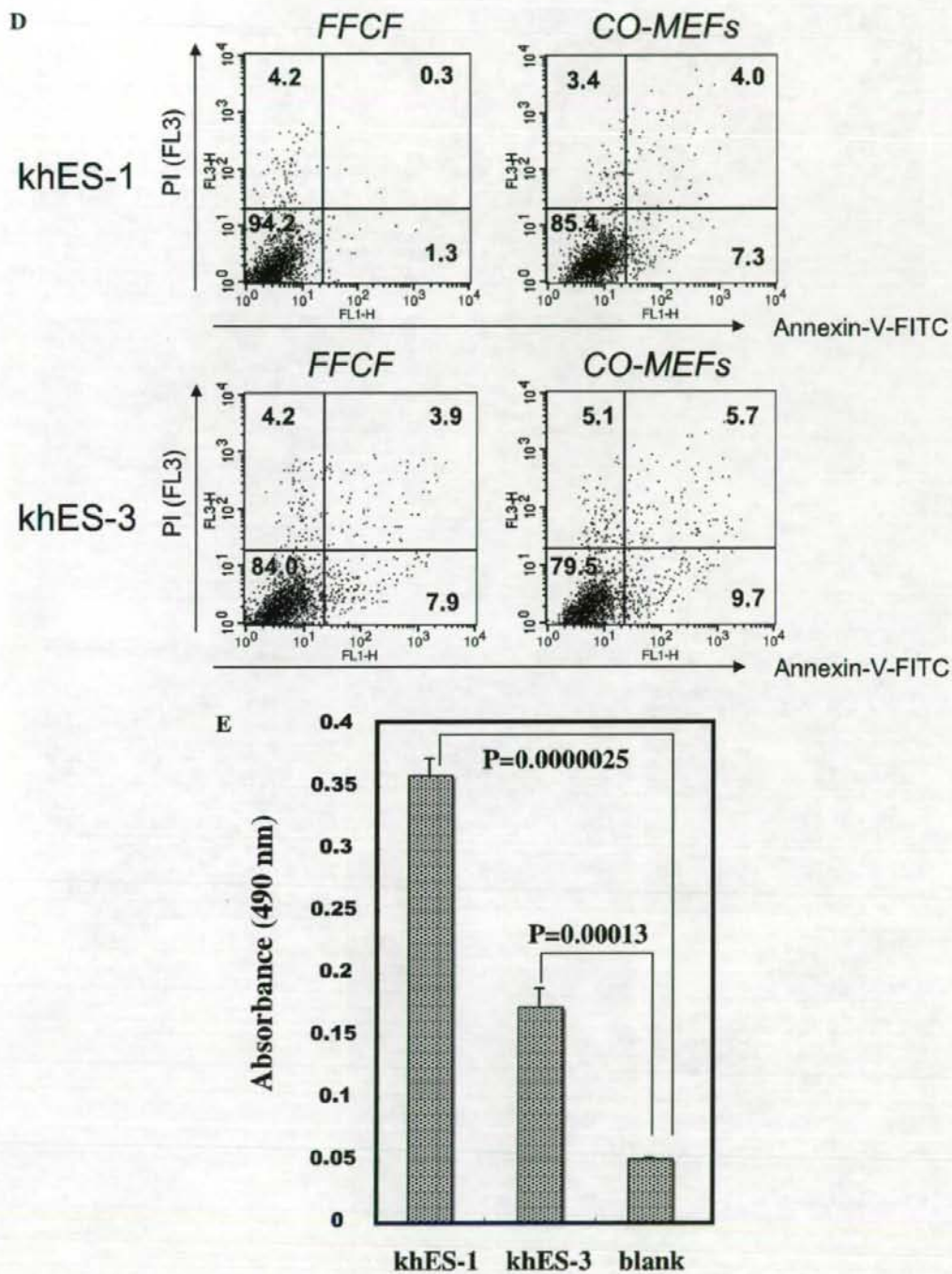
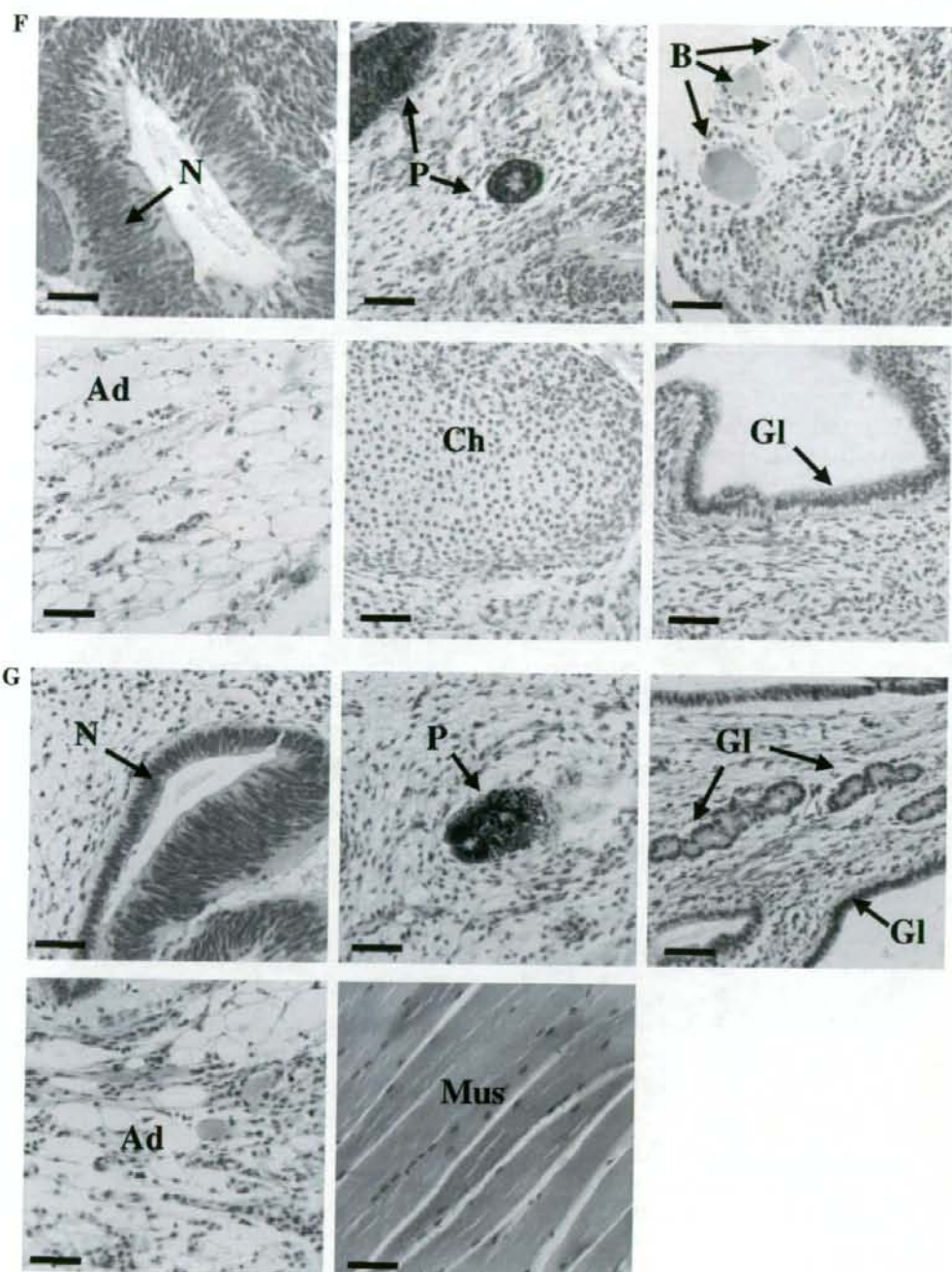


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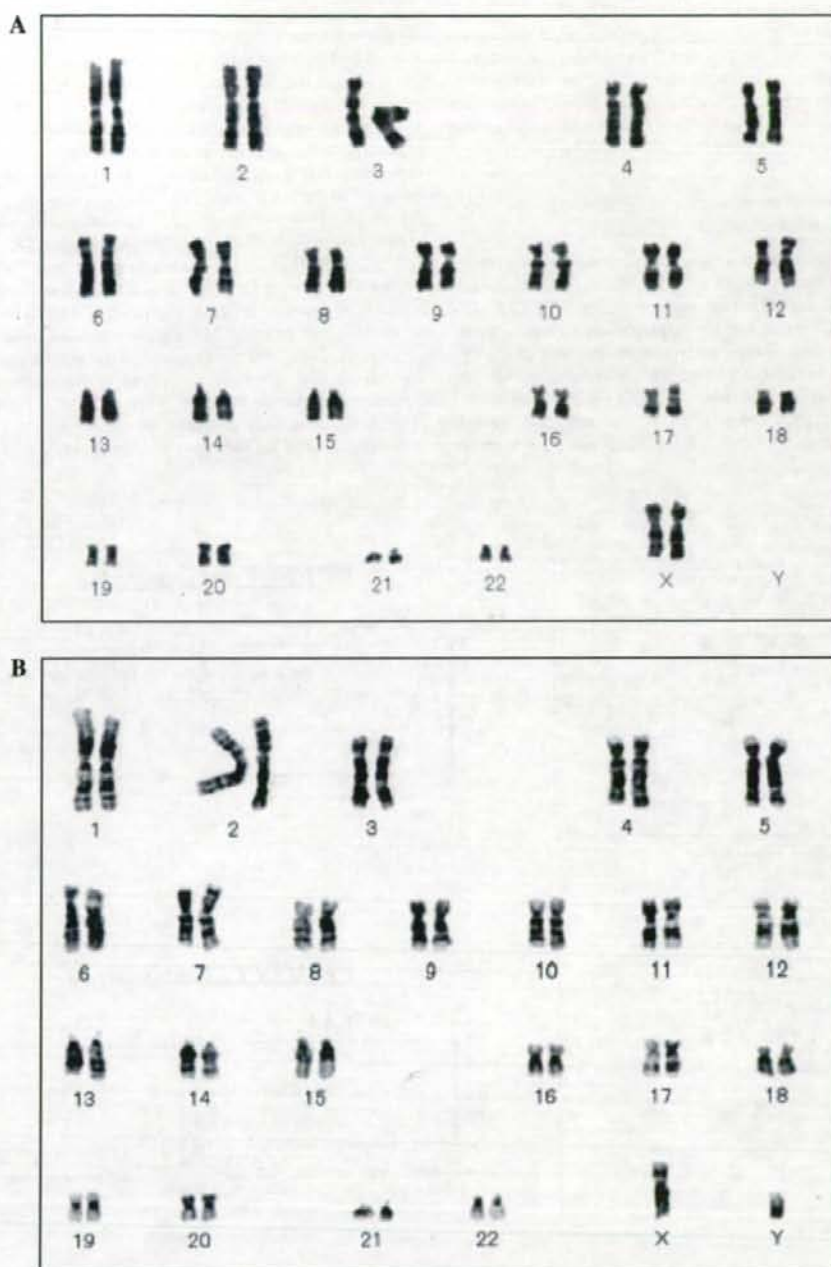


FIG. 2. Karyotyping. Chromosomal analyses with G band staining were performed using khES-1 cells at passage 20 (A) or khES-3 cells at passage 23 (B). The number of chromosomes was 46 for all the cells subjected to the analysis (50 cells for each hESC line). No abnormal G band-staining pattern was detected for all the cells subjected to the analysis (10 cells for each hESC line).

of the FFCF-maintained hESCs was confirmed by teratoma formation assays (Figs. 1F and G). Chromosomal abnormality was not detected throughout the process (Figs. 2A and B), excluding the possibility that a special clone with growth advantages was selected. Thus, a close control of the size and number of colonies enables the feeder-free and cytokine-free maintenance of hESCs as in the case of monkey ES cells.

Characterization of the feeder- and cytokine-free method for the maintenance of hESCs

We next evaluated the property of our novel culture method by comparison with the two conventional methods: CO-MEFs and a feeder-free culture using MEF-CM (FF-MEFCM). First, we checked the major protein expression patterns by 2-DE. Global protein expression profiles of the FF-MEFCM- and FFCF-maintained hESCs were quite similar (Figs. 3A and B), while that of the CO-MEFs-maintained hESCs was slightly different (Fig. 3C). Nevertheless, overall protein expression patterns of the three samples seemed

fairly analogous when compared with the ill-maintained hESCs that were cultured in the presence of fetal calf serum (Fig. 3D). Thus, our culture method is quite comparable to the conventional methods, at least where the global protein expression profile was concerned.

We then performed a more focused analysis. By using phosphorylation-specific antibodies, we examined the activation states of the signaling molecules that were reportedly involved in the maintenance or the differentiation of ES cells. A high phosphorylation of STAT-3 was detected only in CO-MEFs-maintained hESCs (Fig. 3E). Although STAT3 activation was involved in the maintenance of inhibitory factor (LIF)-added murine ES cells, it was dispensable for the maintenance of primate ES cells (Sumi et al., 2004) as well as murine ES cells cultured in the presence of inhibitor cocktail (Ying et al., 2008). We next examined the activation states of Akt, which was reportedly involved in the maintenance of murine and primate ES cells (Watanabe et al., 2006). Akt phosphorylation was detected at similar levels in FFCF-maintained and CO-MEFs-maintained hESCs (Fig. 3E).

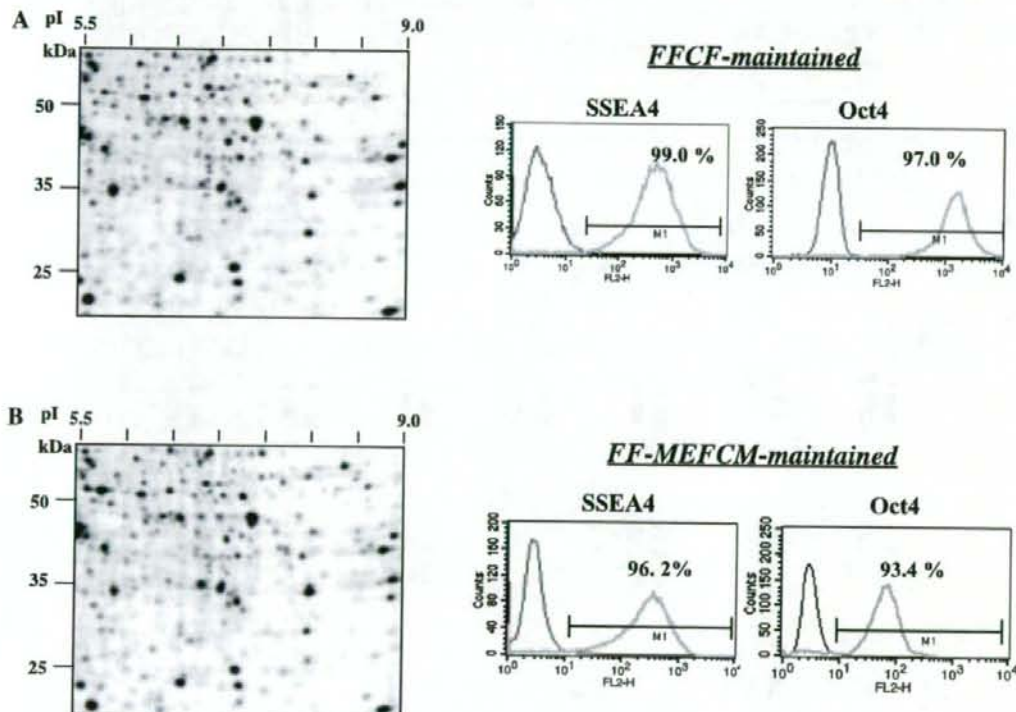
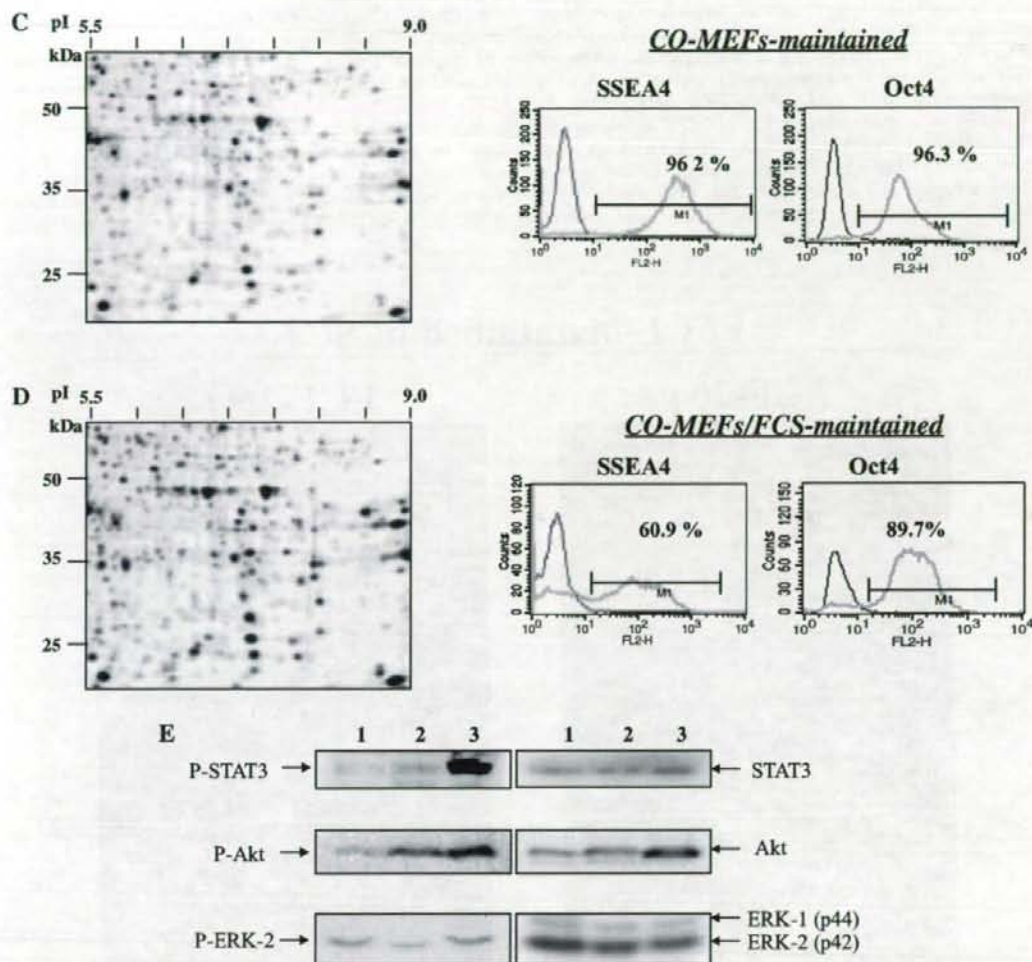


FIG. 3. Evaluation of global protein expressions and signaling molecule activations. (A–D) The gel images of 2D electrophoresis of FFCF-maintained khES-1 cells (A), FF-MEFCM-maintained khES-1 cells (B), CO-MEFs-maintained khES-1 cells (C), Co-MEFs/FCS-maintained khES-1 cells (D) were shown in the left panels. The typical areas with high separation were shown as described in Materials and Methods. Right panels demonstrate the results of flow cytometric analyses on SSEA4 and Oct4 expressions. (E) Activations of the signaling molecules. The FF-MEFCM-maintained khES-1 cells (lane 1), FFCF-maintained khES-1 cells (lane 2) and CO-MEFs-maintained khES-1 cells (lane 3) were subjected to Western blotting using indicated antibodies.

FIG. 3. *Continued.*

However, normalization of the signal intensity of phosphorylated Akt (P-Akt) by that of total Akt protein indicated that relative activation levels of Akt was highest in FFCF-maintained hESCs (the intensity ratio of P-Akt to Akt were 0.56, 0.96, and 0.60 in FF-MEFCM-, FFCF-, and CO-MEFs-maintained hESCs, respectively). Finally, we examined the activation states of ERKs, which induces spontaneous differentiation of ES cells (Burdon et al., 1999; Ying et al., 2008). As shown in Figure 3E, the activation level of ERK was lowest in FFCF-maintained hESCs compared to CO-MEFs- and FF-MEFCM-maintained hESCs (the intensity ratio of P-ERKs to ERKs were 0.35, 0.18, and 0.55 in FF-MEFCM-, FFCF-, and CO-MEFs-maintained hESCs, respectively). Thus, our novel culture method for the maintenance of hESCs has no obvious disadvantages compared to the conventional methods,

at least as far as the phosphorylation states of the signaling molecules are concerned.

Effects of exogenously added FGF-2 on FFCF-maintained hESCs

We finally examined the effects of exogenously added FGF-2, which is commonly used in the feeder-free maintenance of hESCs (Bendall et al., 2007; Ludwig et al., 2006; Wang et al., 2005; C Xu et al., 2005; RH Xu et al., 2005). Although the presence of recombinant FGF-2 did not affect the expression levels of immature ES markers including SSEA-4, Oct-4, and Nanog (data not shown), careful examinations revealed its minor, rather unfavorable, effects. First, FGF-2 supplementation promoted the differentiation-associated

morphological changes: a reduced compaction of the inner cells along with cytoplasmic extension of the marginal cells (Fig. 4A). Second, the expression of an epithelial marker of E-cadherin, which is expressed in ES cells and involved in their self-renewal (Nagaoka et al., 2006), was slightly reduced by an addition of recombinant FGF-2 as assessed by flow cytometry (Fig. 4B). Eventually, its intercellular localization became obscure by the presence of FGF-2 (Fig. 4C). Thus, although FGF-2 might exert beneficial effects in certain culture conditions, it is not required, or even unfavor-

able, for the culture of hESCs that has been maintained in the absence of recombinant cytokines.

It is generally recognized that a daily medium changing is crucial for the good performance of the maintenance of hESCs irrespective of the culture method. Although the molecular mechanism of the spontaneous differentiation induced by skipping of medium changing is not known, it seems that one study described a good suggestion for it. It was shown that, during a feeder-free maintenance of hESCs using high doses of Noggin and FGF-2, the absence of Nog-

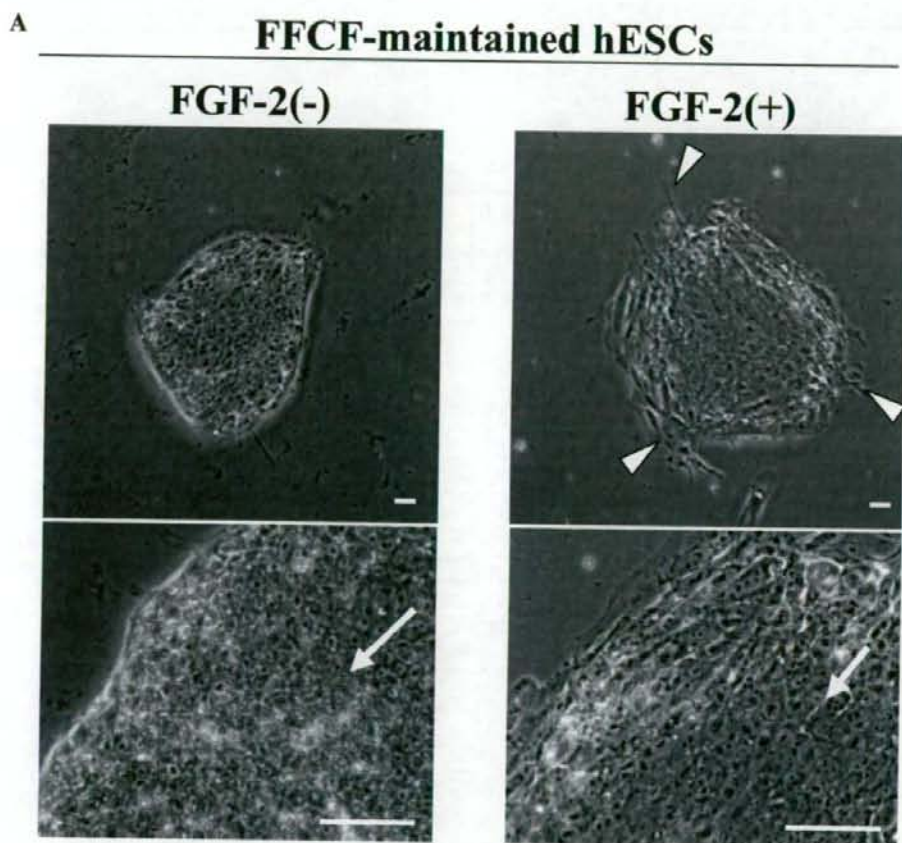
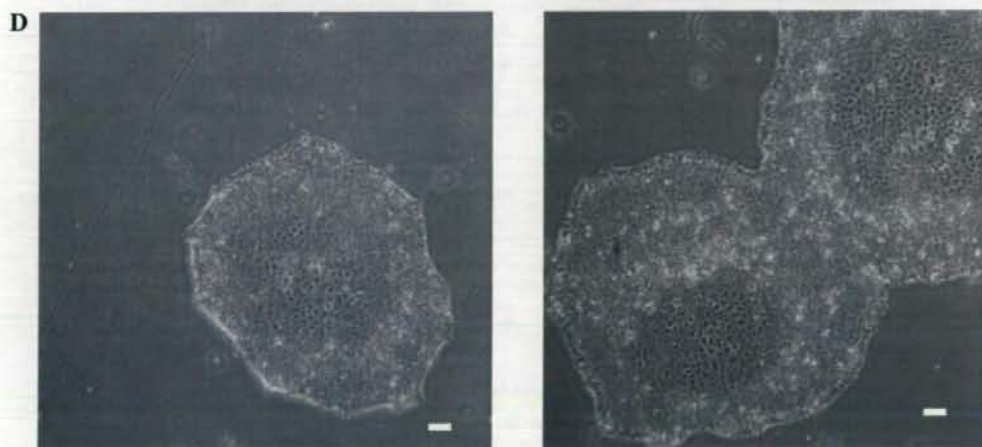
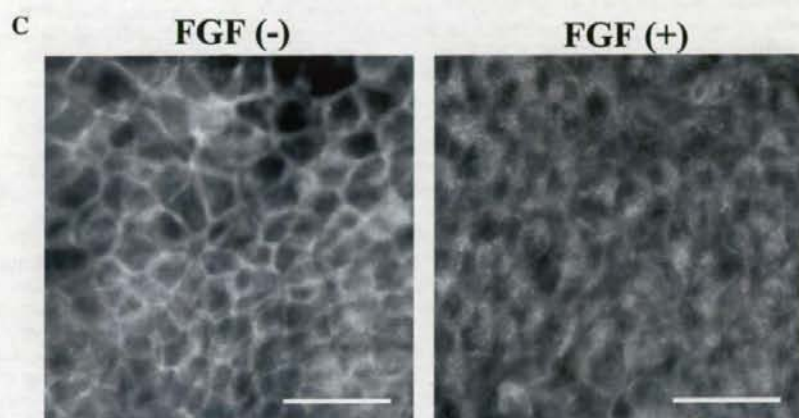
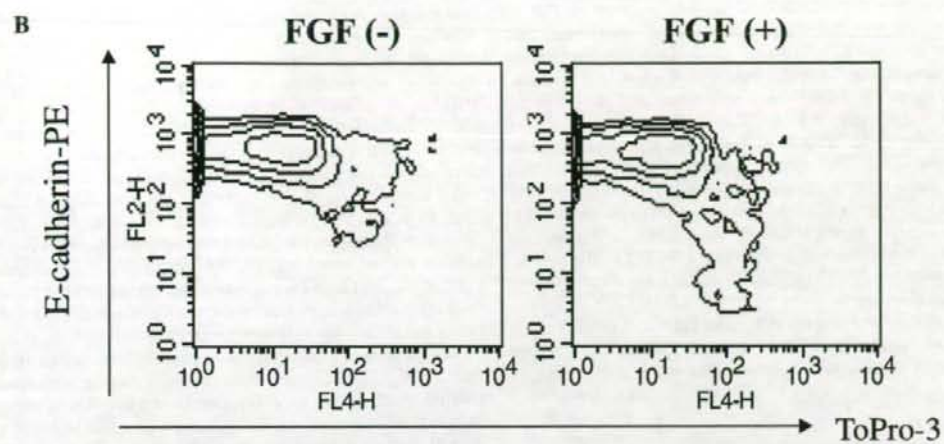


FIG. 4. Effects of FGF-2 supplementation on FFCF-maintained human ES cells. (A) Phase contrast microscopy of the FFCF-maintained khES-1 cells (left) and FGF-2-supplemented FFCF-maintained khES-1 cells (right). Scale bar indicates 100 μm . Note the differences in the morphology of the cells lining at marginal regions of colonies (arrow heads) and the size of the cells located in inner parts of colonies (arrows) between the two samples. (B) The FFCF-maintained khES-1 cells (left) and FGF-2-supplemented FFCF-maintained khES-1 cells (right) were subjected to flow cytometry using anti-E-cadherin antibody. (C) The FFCF-maintained khES-1 cells (left) and 10 ng/mL of FGF-2-supplemented FFCF-maintained khES-1 cells (right) were subjected to immunostaining studies using anti-E-cadherin antibody. Clear intercellular localization of E-cadherin was detected in FFCF-maintained hESCs while its localization became obscure in FGF-2-supplemented hESCs. Scale bar indicates 50 μm . (D) The effects of medium change skipping and colony fusing on the maintenance of hESCs were determined by morphological observation. FFCF-maintained khES-1 cells were cultured without changing the medium, and after 4 days, microscopic observations were performed (left). As in the case of high density cultures, where hESC colonies were fused with one another (right), cells located in inner portions of the colonies became flattened and enlarged.

FIG. 4. *Continued.*

gin induced differentiation at central regions of the ES colonies, whereas that of FGF-2 induced differentiation at peripheral regions (RH Xu et al., 2005). To acquire a hint for the mechanism of the self-renewal of the FFCF-maintained hESCs, we skipped the medium changing and observed cell morphologies over time. As shown in Figure 4D, cells located at the central region of the colonies showed differentiation-associated flattened morphological changes. Thus, certain bone morphogenetic proteins (BMPs)-antagonizing signals, rather than FGF-like signals, seem to be involved in the self-renewal of the FFCF-maintained hESCs. Indeed, we detected no immunoreactivity against FGF-2 in the culture supernatant of FFCF-maintained hESCs by ELISA assays (data not shown).

Collectively, the autocrine signaling network of hESCs sufficiently works for their self-renewal if they are kept in crops of colonies of an appropriate size and number.

Discussion

In the present study, we confirmed the generality of our previous finding obtained from the experiments using cynomolgus monkey ES cells in the case of hESCs: we showed that two lines of hESCs could appropriately be maintained in the absence of feeder layers and exogenous cytokines by closely controlling the size and number of their colonies. They showed high-level expressions of immature ES markers and bore teratoma-forming capacities. Although global protein expression profiles of human ES cells maintained by this novel technique were highly similar to those maintained by the conventional methods, studies on activation states of Akt and ERK indicates that our method is no way inferior, but rather superior, to the conventional methods. Eventually, we successfully induced a high-efficiency vascular endothelial differentiation of FFCF-maintained hESCs (manuscript in preparation) by applying our recently established method for a directed differentiation of vascular endothelial cells from cynomolgus monkey ES cells maintained on MEF layers (Saeki et al., 2008).

Our unique culture method has two merits. First, it has cost- and labor-saving benefits by cutting the expense to purchase recombinant cytokines and the labor to prepare MEFs. Second, it provides the most appropriate way to analyze the autocrine signaling networks of hESCs, eliminating the effects of MEFs and the nonphysiological doses of recombinant cytokines. MEFs are heterogeneous materials that may produce unidentified unfavorable substances as well as the identified favorable substances. Moreover, usage of high-dose cytokines may possibly destroy the self-directed maintenance systems of human ES cells. By contrast, our system is free from those disturbances. Although it still uses a crude xenogenic Matrigel™ Matrix as a dish-coating material, our preliminary studies show that Matrigel™ Matrix can be substituted by more purified human materials such as type IV collagen plus laminin. Thus, our system will be able to be upgraded to meet the requirement for the more refined basic analyses as well as clinical applications in the future.

The discrepancy between our results and other groups' reports, where high doses of recombinant cytokines were used for the maintenance of hESCs, may come from the difference in cell lines, or at least in part, in degrees of technical maturity. Eventually, we found that the optimal condition con-

cerning the sizes and numbers of ES colonies differ depending on the lines, and thus, trial-and-error processes are required for optimization. Nevertheless, once an optimal condition is determined for each line, it can be transferable without modification. In support of this, it was recently shown that self-renewal of murine ES cells could be achieved without extrinsic signals when spontaneously differentiating properties were properly blocked (Ying et al., 2008). At the same time, we admit that there may be a number of hESC lines to which our method cannot be applied. Indeed, we have not yet succeeded in determining the optimal condition for khES-2, another hESC line established in Japan (Sue-mori et al., 2001), and we still use high doses of noggin and FGF-2 for their feeder-free maintenance. It seems that there is a considerably large heterogeneity among hESC lines in a variety of senses including chromosomal instabilities (Buz-zard et al., 2004; Draper et al., 2004), genomic alterations (Maitra et al., 2005), and directional propensities of differentiation (Osafune et al., 2008). Moreover, there is a controversial finding concerning the existence of ES-derived fibroblastic cells. Some groups showed the existence of hESC-derived fibroblastic cells around immature hESC colonies (Bendall et al., 2007; Xu et al., 2001) and their significance for the self-renewal of hESCs were even insisted (Bendall et al., 2007), while other groups did not show the presence of such hESC-derived fibroblastic cells (Ludwig et al., 2006; Nakahara et al., 2008; Wang et al., 2005; RH Xu et al., 2005). The "heterogeneity among the hESC lines" may possibly give account for a number of controversial results reported on hESCs including chromosomal instability (Buz-zard et al., 2004; Draper et al., 2004) and cytokine requirement (Bendall et al., 2007; Nakahara et al., 2008). Finally, it should be noted that the majority of hESCs were established by coculture with MEFs, and thus, the existing populations of hESCs should be biased towards MEF dependency rather than self-dependency. In this sense, a trial to establish hESCs, or human iPS cells, under a feeder-free and exogenous cytokine-free condition may possibly increase the number of high-quality, self-dependent human ES or iPS lines.

References

- Amit, M., Shariki, C., Margulets, V., et al. (2004). Feeder layer- and serum-free culture of human embryonic stem cells. *Biol. Reprod.* 70, 837–845.
- Beattie, G.M., Lopez, A.D., Bucay, N., et al. (2005). Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells* 23, 489–495.
- Bendall, S.C., Stewart, M.H., Menendez, P., et al. (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature* 448, 1015–1021.
- Burdon, T., Stracey, C., Chambers, I., et al. (1999). Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Dev. Biol.* 210, 30–43.
- Buzzard, J.J., Gough, N.M., Crook, J.M., et al. (2004). Karyotype of human ES cells during extended culture. *Nat. Biotechnol.* 22, 381–382.
- Chen, S., Do, J.T., Zhang, Q., et al. (2006). Self-renewal of embryonic stem cells by a small molecule. *Proc. Natl. Acad. Sci. USA* 103, 17266–17271.
- Draper, J.S., Smith, K., Gokhale, P., et al. (2004). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat. Biotechnol.* 22, 53–54.

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- Ezashi, T., Das, P., and Roberts, R.M. (2005). Low O₂ tensions and the prevention of differentiation of hES cells. *Proc. Natl. Acad. Sci. USA* 102, 4783–4788.
- Fujioka, T., Yasuchika, K., Nakamura, Y., et al. (2004). A simple and efficient cryopreservation method for primate embryonic stem cells. *Int J Dev. Biol.* 48, 1149–1154.
- Ludwig, T.E., Levenstein, M.E., Jones, J.M., et al. (2006). Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* 24, 185–187.
- Maitra, A., Arking, D.E., Shivapurkar, N., et al. (2005). Genomic alterations in cultured human embryonic stem cells. *Nat. Genet.* 37, 1099–1103.
- Nagaoka, M., Koshimizu, U., Yuasa, S., et al. (2006). E-cadherin-coated plates maintain pluripotent ES cells without colony formation. *PLoS ONE* 1, e15.
- Nakahara, M., Matsuyama, S., Saeki, K., et al. (2008). A feeder-free hematopoietic differentiation system with generation of functional neutrophils from feeder- and cytokine-free primate embryonic stem cells. *Cloning Stem Cells* (in press).
- Niwa, H., Ogawa, K., and Kitajima, H. (2004). Molecular mechanism to maintain self-renewal of human ES cells. *Cell Technology* 23, 1260–1263.
- Osafune, K., Caron, L., Borowiak, M., et al. (2008). Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat. Biotechnol.* 26, 313–315.
- Saeki, K., Hong, Z., Nakatsu, M., et al. (2003). Insulin-dependent signaling regulates azurophilic granule-selective macroautophagy in human myeloblastic cells. *J. Leukoc. Biol.* 74, 1108–1116.
- Saeki, K., Yogiashi, Y., Nakahara, M., et al. (2008). Highly efficient and feeder-free production of subculturable vascular endothelial cells from primate embryonic stem cells. *J. Cell Physiol.* 217, 261–280.
- Sato, N., Meijer, L., Skaltsounis, L., et al. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* 10, 55–63.
- Stojkovic, P., Lako, M., Przyborski, S., et al. (2005). Human-serum matrix supports undifferentiated growth of human embryonic stem cells. *Stem Cells* 23, 895–902.
- Suemori, H., Tada, T., Torii, R., et al. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Dev. Dyn.* 222, 273–279.
- Sumi, T., Fujimoto, Y., Nakatsuji, N., et al. (2004). STAT3 is dispensable for maintenance of self-renewal in nonhuman primate embryonic stem cells. *Stem Cells* 22, 861–872.
- Takahashi, K., Tanabe, K., Ohnuki, M., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872.
- Wang, G., Zhang, H., Zhao, Y., et al. (2005). Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem. Biophys. Res. Commun.* 330, 934–942.
- Wang, L., Schulz, T.C., Sherrer, E.S., et al. (2007). Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. *Blood* 110, 4111–4119.
- Watanabe, S., Umehara, H., Murayama, K., et al. (2006). Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. *Oncogene* 25, 2697–2707.
- Xu, C., Inokuma, M.S., Denham, J., et al. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* 19, 971–974.
- Xu, C., Rosler, E., Jiang, J., et al. (2005). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells* 23, 315–323.
- Xu, R.H., Peck, R.M., Li, D.S., et al. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat. Methods* 2, 185–190.
- Yao, S., Chen, S., Clark, J., et al. (2006). Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc. Natl. Acad. Sci. USA* 103, 6907–6912.
- Ying, Q.L., Wray, J., Nichols, J., et al. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453, 519–523.

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