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 manufacture'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 manufacture'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 μL 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 megascope, 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-μg 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 elecrofocusing, 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% idoacetamide 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 QuaNT 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

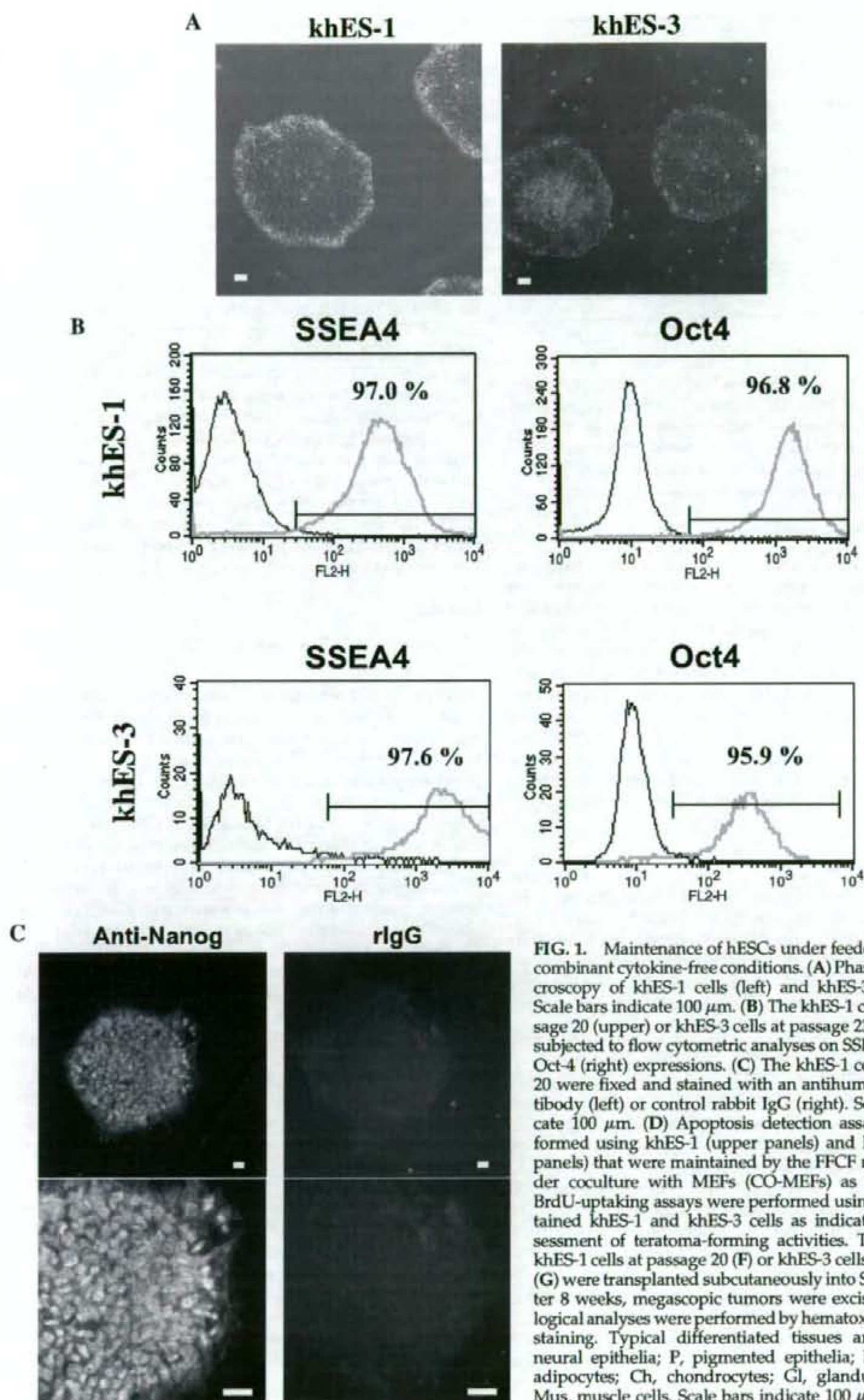
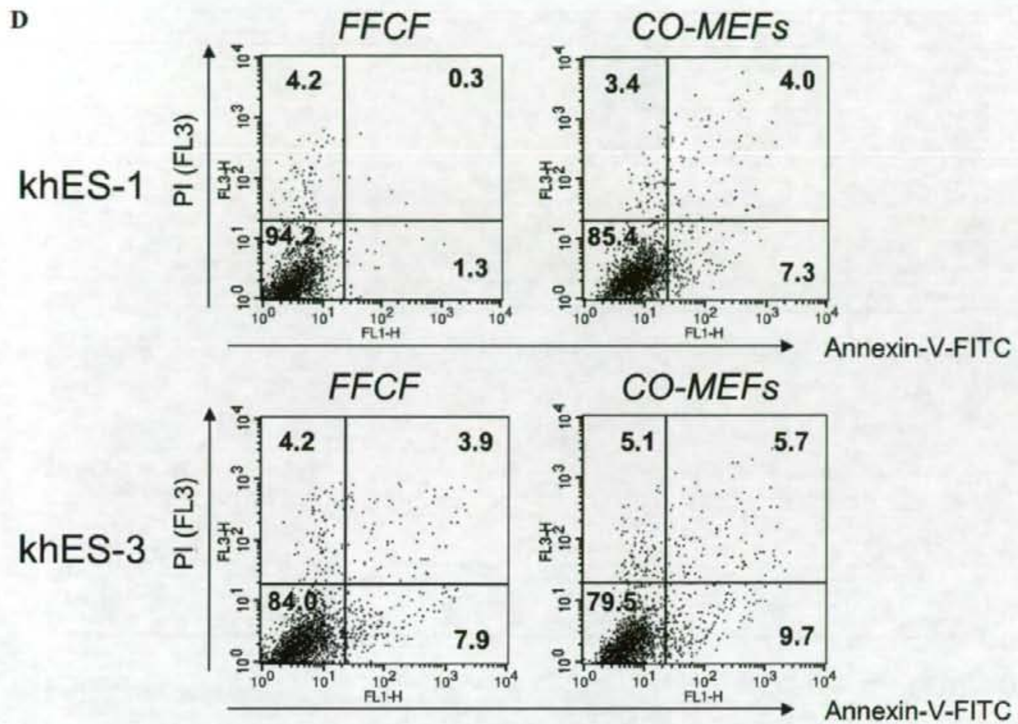


FIG. 1. Maintenance of hESCs under feeder-free and recombinant cytokine-free conditions. **(A)** Phase contrast microscopy of khES-1 cells (left) and khES-3 cells (right). Scale bars indicate 100 μm. **(B)** The khES-1 cells at the passage 20 (upper) or khES-3 cells at passage 23 (lower) were subjected 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 μm. **(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 μm.



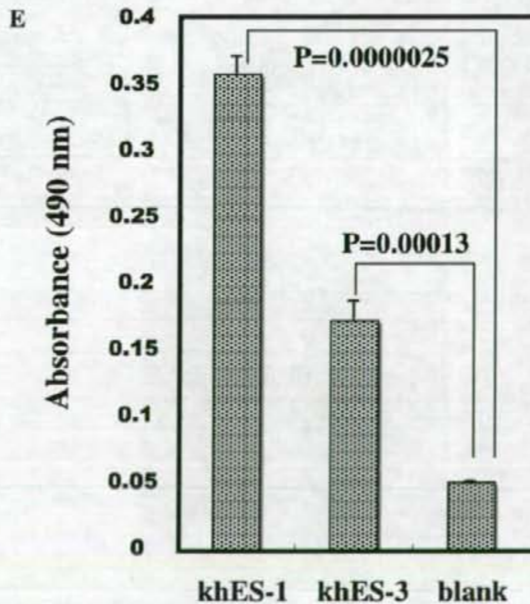
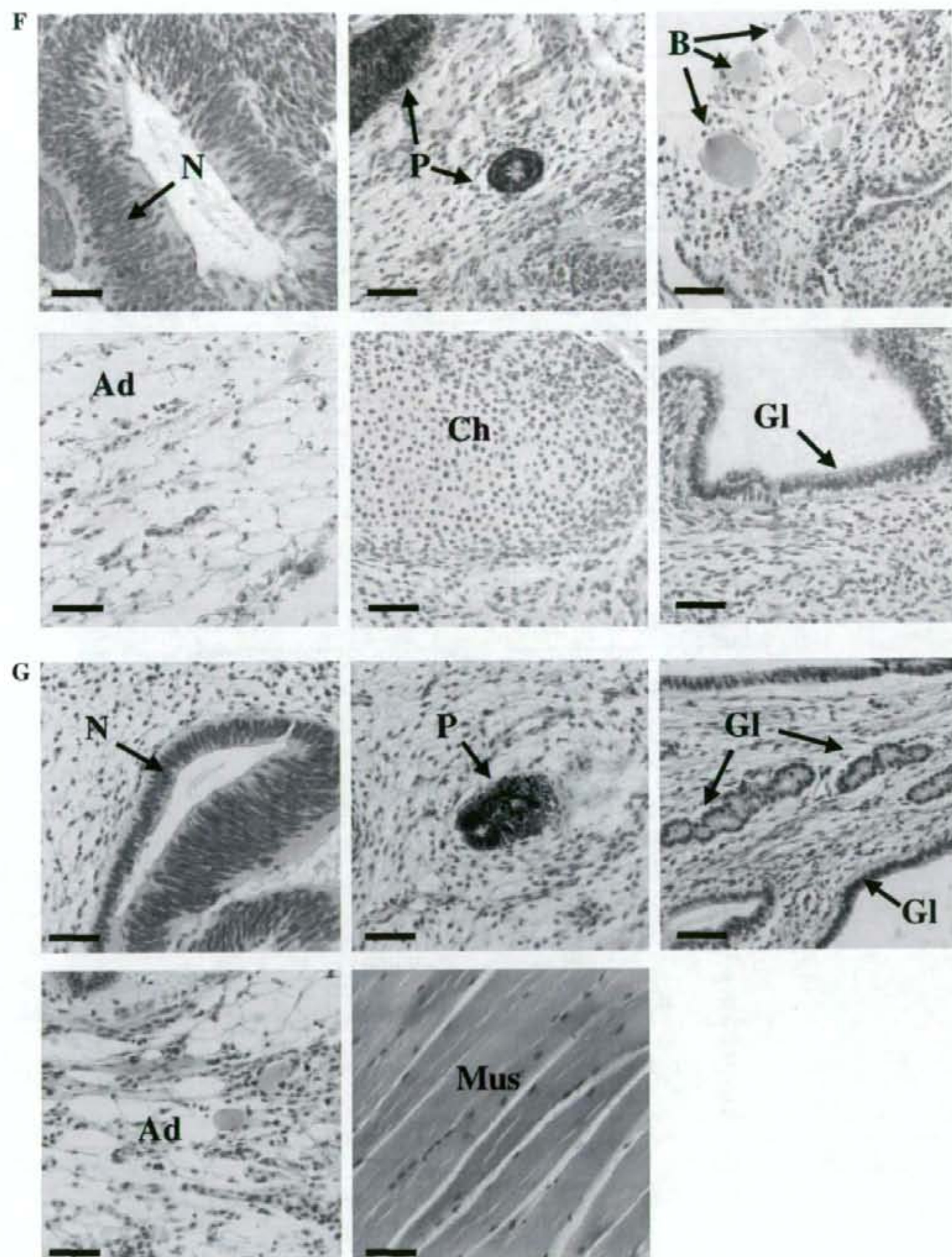


FIG. 1. *Continued.*



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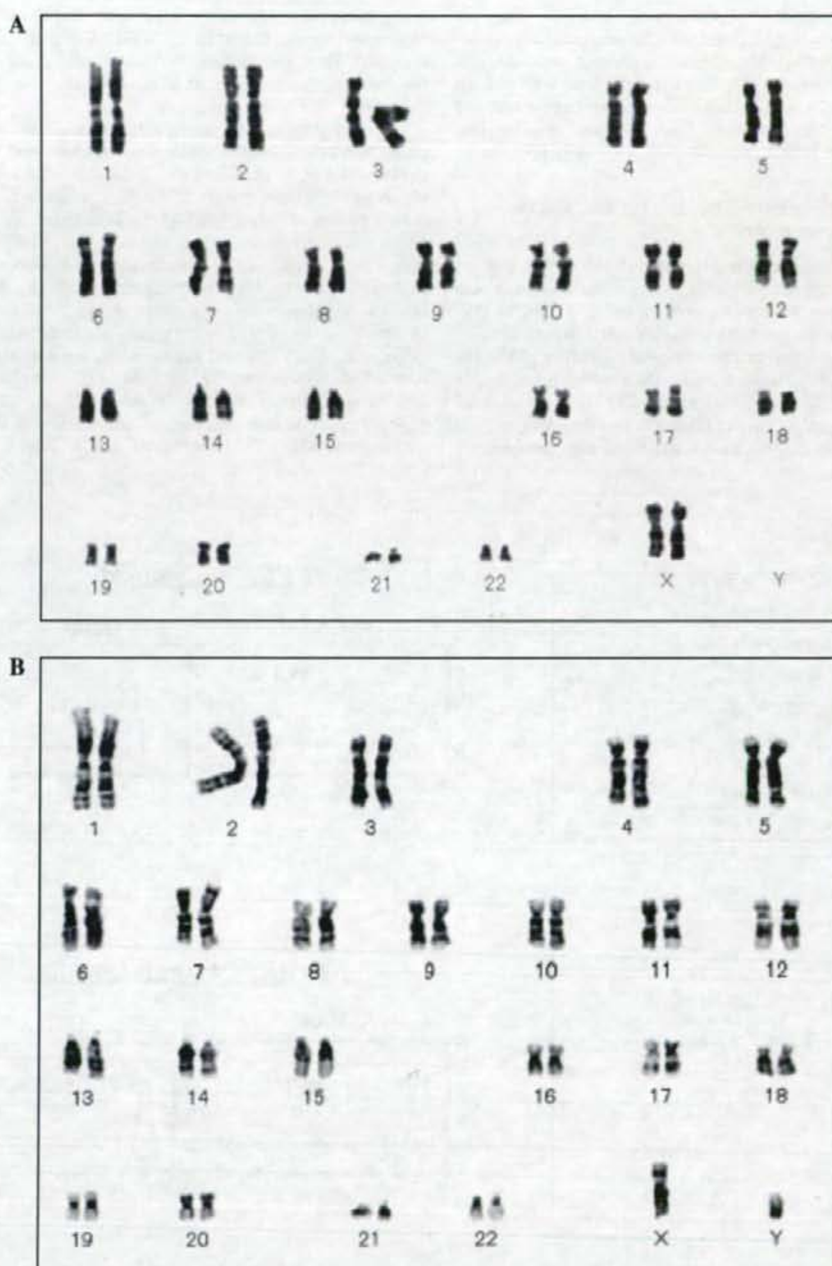

FIG. 2. Karyotyping. Chromosomal analyses with G band staining were performed using khES-1 cells at passage 20 (A) or khES-3 cells at passage 23 (B). The number of chromosomes was 46 for all the cells subjected to the analysis (50 cells for each hESC line). No abnormal G band-staining pattern was detected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

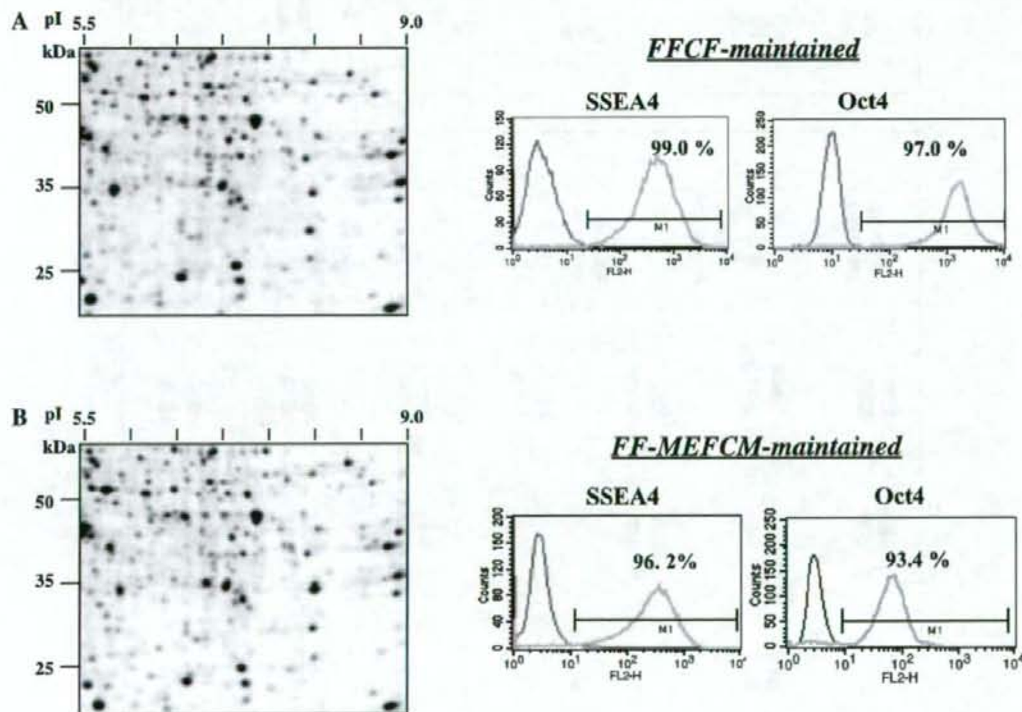

FIG. 3. Evaluation of global protein expressions and signaling molecule activations. (**A–D**) The gel images of 2D electrophoresis of FFCF-maintained khES-1 cells (**A**), FF-MEFCM-maintained khES-1 cells (**B**), CO-MEFs-maintained khES-1 cells (**C**), Co-MEFs/FCS-maintained khES-1 cells (**D**) were 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.

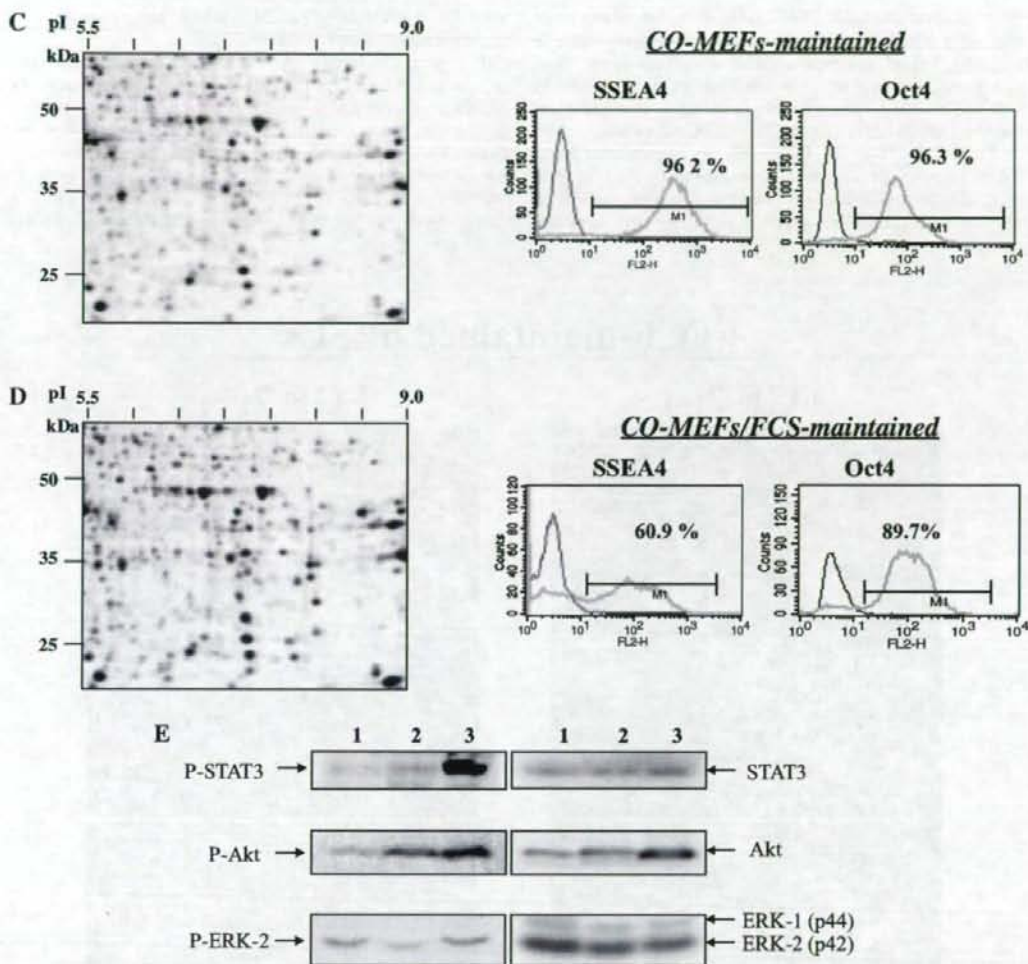

FIG. 3. *Continued.*

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 unfavorable, 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-

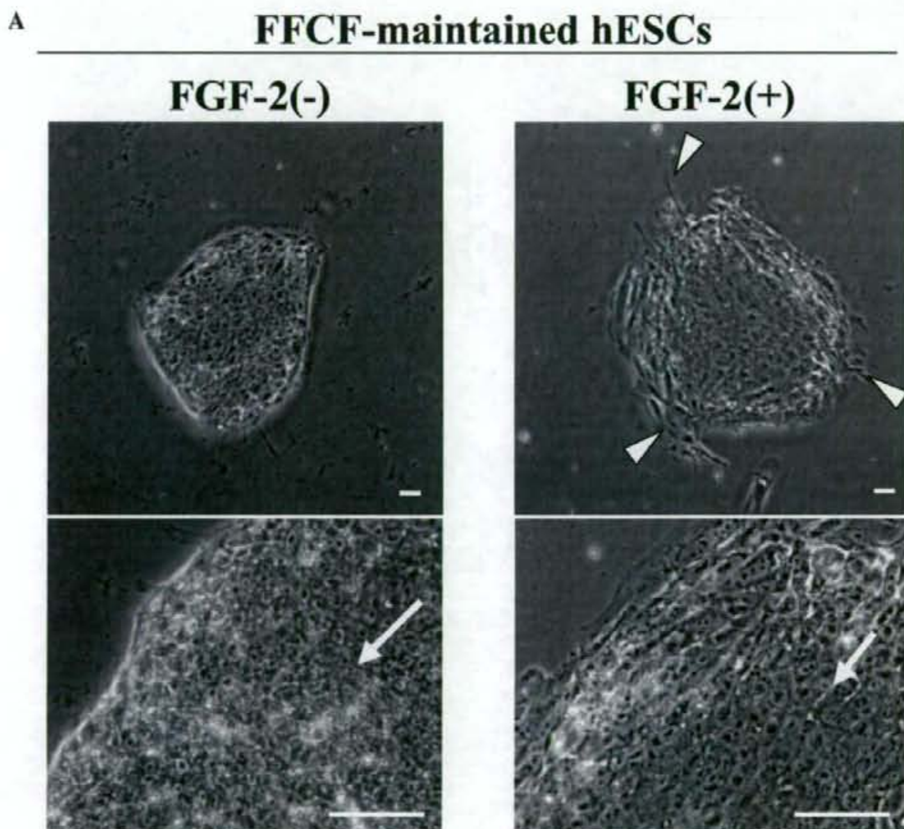

FIG. 4. Effects of FGF-2 supplementation on FFCF-maintained human ES cells. (**A**) Phase contrast microscopy of the FFCF-maintained khES-1 cells (left) and FGF-2-supplemented FFCF-maintained khES-1 cells (right). Scale bar indicates 100 μm. Note the differences in the morphology of the cells lining 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.

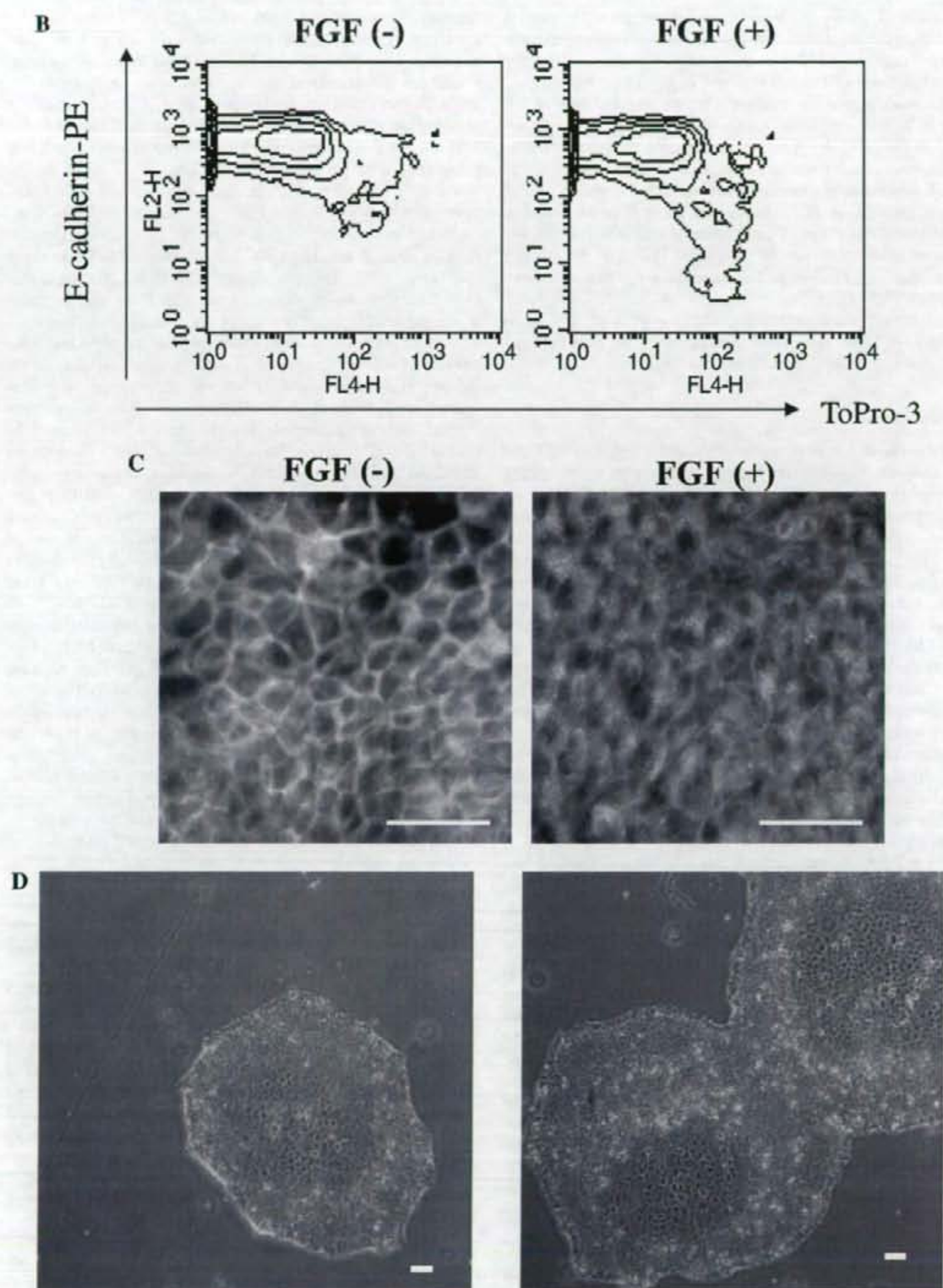

FIG. 4. *Continued.*

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 concerning 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 (Suemori 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 (Buzzard 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 (Buzzard 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.

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Address reprint requests to:
Kumiko Saeki, M.D., Ph.D.
Department of Hematology, Research Institute
International Medical Center of Japan
1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

E-mail: saeki@ri.imcj.go.jp