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Differentiation of Human Embryonic Stem Cells and Human Induced Pluripotent Stem Cells into Steroid-Producing Cells

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Although there have been reports of the differentiation of mesenchymal stem cells and mouse embryonic stem (ES) cells into steroid-producing cells, the differentiation of human ES/induced pluripotent stem (iPS) cells into steroid-producing cells has not been reported. The purpose of our present study was to establish a method for inducing differentiation of human ES/iPS cells into steroid-producing cells. The first approach we tried was embryoid body formation and further culture on adherent plates. The resultant differentiated cells expressed mRNA encoding the steroidogenic enzymes steroidogenic acute regulatory protein, 3β-hydroxysteroid dehydrogenase, cytochrome P450-containing enzyme (CYP)-11A1, CYP17A1, and CYP19, and secreted progesterone was detected in the cell medium. However, expression of human chorionic gonadotropin was also detected, suggesting the differentiated cells were trophoblast like. We next tried a multistep approach. As a first step, human ES/iPS cells were induced to differentiate into the mesodermal lineage. After 7 d of differentiation induced by 6-bromoindirubin-3′-oxime (a glycogen synthase kinase-3β inhibitor), the human ES/iPS cells had differentiated into fetal liver kinase-1- and platelet derived growth factor receptor-α-expressing mesodermal lineage cells. As a second step, plasmid DNA encoding steroidogenic factor-1, a master regulator of steroidogenesis, was introduced into these mesodermal cells. The forced expression of steroidogenic factor-1 and subsequent addition of 8-bromoadenosine 3′,5′-cyclic monophosphate induced the mesodermal cells to differentiate into the steroidogenic cell lineage, and expression of CYP21A2 and CYP11B1, in addition to steroidogenic acute regulatory protein, 3β-hydroxysteroid dehydrogenase, CYP11A1, and CYP17A1, was detected. Moreover, secreted cortisol was detected in the medium, but human chorionic gonadotropin was not. These findings indicate that the steroid-producing cells obtained through the described multistep method are not trophoblast like; instead, they exhibit characteristics of adrenal cortical cells. **(*Endocrinology* 153: 4336–4345, 2012)**

Stem cells are gaining attention as promising tools in the field of regenerative medicine and developmental biology especially after the establishment of human embryonic stem (ES) cells in 1998 (1) and human induced pluripotens stem (iPS) cells in 2007 (2). Human iPS cells are considered to possess similar characteristics to human ES cells (2). Because they overcome the immunological and ethical problems associated with human ES cells, human iPS cells represent a powerful new tool with which to investigate disease-specific disorders of organ development/differentiation, and represent another potentially effective approach to cell transplantation-based regenerative medicine.

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Abbreviations: BIO, 6-Bromoindirubin-3′-oxime; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; CYP, cytochrome P450-containing enzyme; DHEA, dehydroepiandrosterone; EB, embryoid body; ES, embryonic stem; Flk1, fetal liver kinase-1; GSK-3β, glycogen synthase kinase-3β; hCG, human chorionic gonadotropin; 3β-HSD, 3β-hydroxyl steroid dehydrogenase; HSD3B, 3β-hydroxysteroid dehydrogenase; iPS, induced pluripotens stem; MSC, mesenchymal stem cell; PDGFRα, platelet-derived growth factor receptor-α; SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory protein; TRA 1-60, tumor rejection antigen 1-60.

Among the classic endocrine cells, the differentiation of pancreatic β-cells from ES/iPS cells has been most intensively investigated (3, 4). The differentiation of stem cells into steroid-producing cells has also been investigated, and in 1997 Crawford *et al.* reported that the forced expression of steroidogenic factor-1 (SF-1), a transcriptional factor belonging to the nuclear receptor superfamily, directed mouse ES cells into the steroidogenic cell lineage (5), although the steroidogenic capacity of these cells was very limited because progesterone was the only steroid hormone produced in the presence of an exogenous substrate, 20α-hydroxycholesterol. More recently several groups have reported that both mouse and human mesenchymal stem cells (MSC) can be induced to differentiate into steroid-producing cells through forced expression of SF-1 and that the resultant steroid-producing cells produce a wider variety of steroid hormones (6–9), but the MSC-derived steroid-producing cells have not been well characterized because there is no evidence that the steroid-producing cells naturally develop from the MSC. In 2011 Yazawa *et al.* (10) reported a method for differentiating mouse ES cells into steroid-producing cells through tetracycline-controlled transcriptional activation of SF-1. However, human ES cells possess a number of characteristics distinct from those of mouse ES cells, such as surface antigens, leukemia inhibitory factor independency, and long doubling time (11), and no investigation of steroidogenic differentiation using human ES or iPS cells has yet been reported.

Adrenal insufficiency, which is caused by Addison's disease, congenital adrenal hyperplasia, hypopituitarism, and other diseases, is a condition in which the adrenal glands do not produce adequate amounts of the steroid hormones cortisol, aldosterone, and adrenal androgen (12). Hormone replacement therapy is currently the best treatment strategy for steroid insufficiency (12, 13), and most of these patients, especially those with adrenal insufficiency, require therapy for their entire lives and are thus always at risk from side effects, which sometimes can be life threatening (12). Therefore, an innovative therapy that could solve these problems would be desirable. Establishing a method for differentiating human ES/iPS cells into steroid-producing cells could potentially lead to a cell therapy for adrenal insufficiency in the foreseeable future. Moreover, through the use of comprehensive high-throughput screening of the actions of small molecules during the differentiation of human ES/iPS cells into steroidogenic cells, it may be possible to identify new agents that promote the regeneration of steroidogenic organs from somatic stem cells. In addition, it could also help in the elucidation of the molecular mechanisms underlying the development and the differentiation of the adrenal cortex and the gonad. We previously characterized the process of human ES/iPS cell differentiation into vascular cells (14) and adipocytes (15). The purpose of our present study was to establish a method for inducing differentiation of human ES/iPS cells into steroid-producing cells.

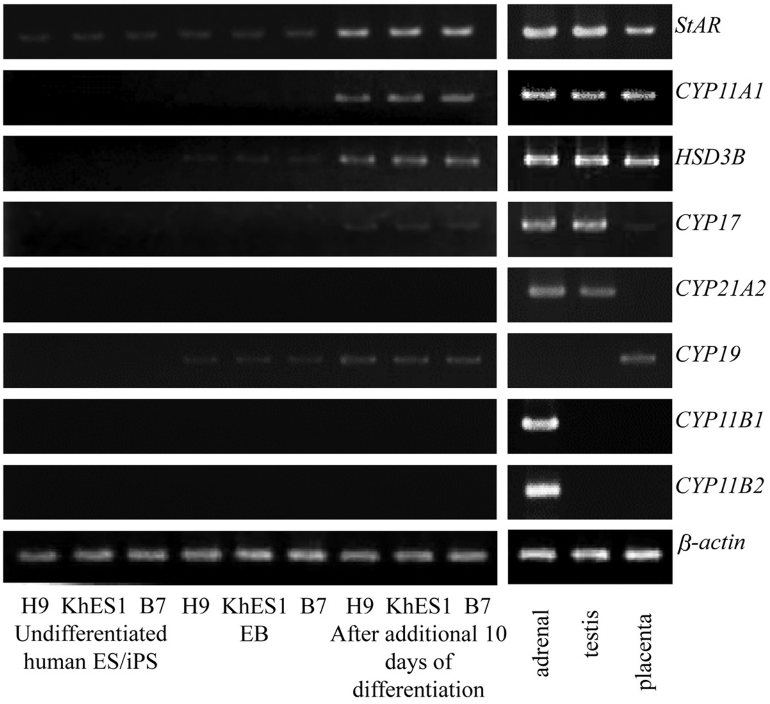

FIG. 1. RT-PCR analysis of the indicated cell lines (H9, KhES1, and B7) before differentiation (undifferentiated), during EB formation (EB), and after 10 d of culture on gelatin-coated dishes. Results obtained with human adrenal gland, testis, and placenta are shown for comparison.

Materials and Methods

Cells and culture

Two human ES cell lines (H9 and KhES1) and one human iPS cell line (201B7) were investigated. The iPS 201B7 line was generated by introducing four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) into human skin fibroblasts. Undifferentiated human ES and iPS cells were grown on mitomycin C-treated mouse embryonic fibroblast feeders in primate ES medium (ReproCELL, Tokyo, Japan) supplemented with 4 ng/ml recombinant human basic fibroblast growth factor (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Routine maintenance of human ES cell cultures was performed according to the protocol recommended by Kyoto University (16). All research on human ES cells was conducted in conformity with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells (2009) published by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

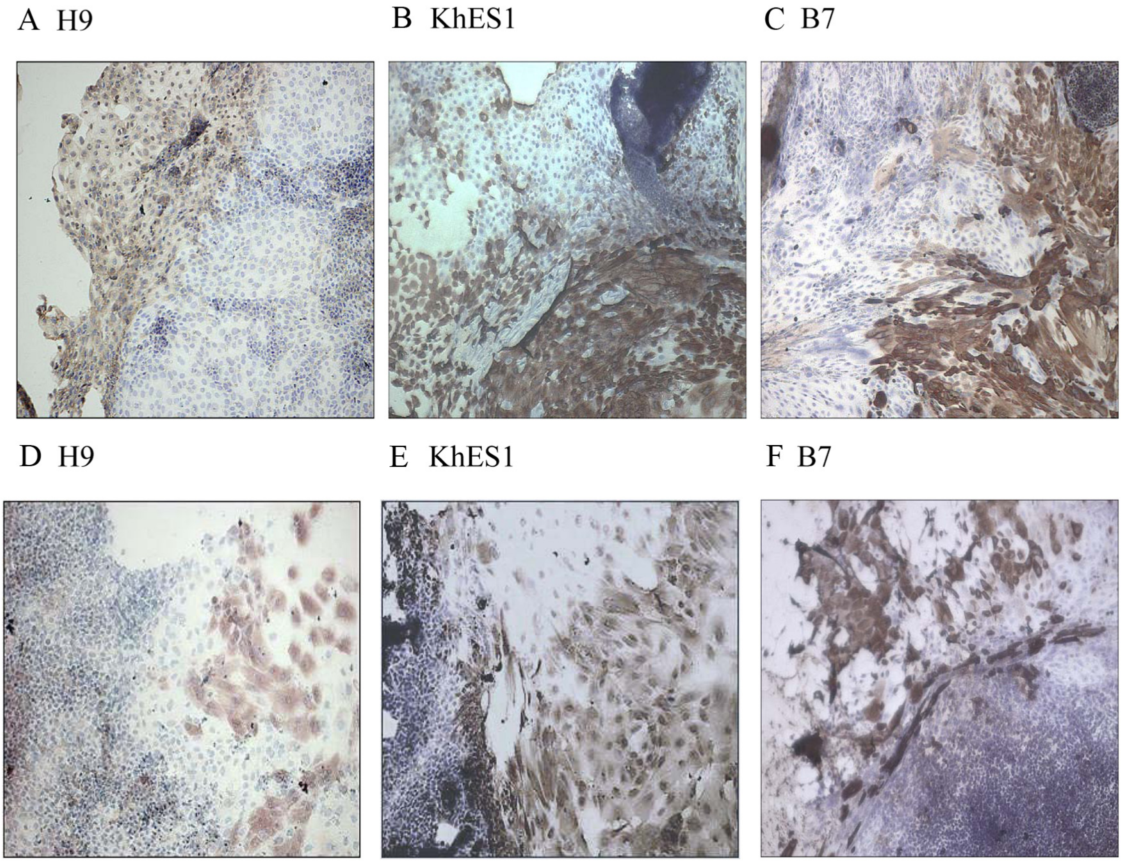

FIG. 2. Immunocytochemical analysis of 3β-HSD (A–C) and hCG (D–F) expression in the indicated cell lines (H9, KhES1, and B7) after 10 d of culture on gelatin-coated dishes after EB formation.

Embryoid body formation

For embryoid body (EB) formation, human ES/iPS cell colonies were digested with 1 mg/ml collagenase type IV (Gibco, Carlsbad, CA) and plated onto nonadherent culture dishes, in which they were allowed to aggregate in maintenance medium [77% DMEM/F12 medium, 20% knockout serum replacement, 1% nonessential amino acids, 1% penicillin/streptomycin, 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol] without basic fibroblast growth factor for 14 d. About 100 of the resultant EB were lysed for RNA extraction. Also, about 40 of the resultant EB were transferred to each well of 12-well plates coated with gelatin. The transferred EB were grown in maintenance medium for further differentiation.

Mesodermal differentiation of human ES and iPS cells

Mesodermal differentiation of human ES/iPS cells was conducted as described previously with a little modification (17). Briefly, undifferentiated human ES/iPS cells were detached using PBS containing 1 mg/ml collagenase type IV, 0.25% trypsin, and 20% knockout serum replacement, dissociated into small cell

TABLE 1. Hormone levels in medium conditioned by undifferentiated ES/iPS cells and differentiated cells obtained via EB formation

	Undifferentiated			Differentiated		
	H9	KhES1	B7	H9	KhES1	B7
Progesterone (pg/ml)	N.D.	N.D.	N.D.	4320 ± 510	3450 ± 1290	10800 ± 2030
Corticosterone (pg/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Cortisol (pg/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Aldosterone (pg/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DHEA (pg/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Estradiol (pg/ml)	N.D.	N.D.	N.D.	110 ± 30	110 ± 40	350 ± 40
hCG (mIU/ml)	N.D.	N.D.	N.D.	120 ± 30	280 ± 60	1300 ± 110

Data are means ± SEM of triplicate experiments. Differentiated cells were cultured on gelatin-coated dishes for 10 d after EB formation on nonadherent dishes. N.D., Not detectable.

clumps, and plated onto type I collagen-coated dishes. The cells were then cultured for 1 d in maintenance medium, after which the medium was changed to maintenance medium containing 5 μM 6-bromoindirubin-3′-oxime (BIO), a glycogen synthase kinase-3β (GSK-3β) inhibitor, and the cells were cultured for 3 d. The medium was then changed to maintenance medium, and the cells were cultured for an additional 3 d.

Flow cytometry

Cells were detached and harvested using 0.05% trypsin/EDTA (Gibco) and were neutralized with DMEM containing 10% fetal bovine serum for 30 min at 37 C. The cells were then washed twice and stained with the following monoclonal antibodies: fluorescein isothiocyanate-conjugated antihuman tumor rejection antigen 1–60 (TRA 1–60) (BD Biosciences, Franklin Lakes, NJ), phycoerythrin-conjugated antihuman platelet-derived growth factor receptor-α (PDGFRα) (BD Biosciences), and Alexa Flour 647 (Invitrogen, Carlsbad, CA)-labeled anti-fetal liver kinase-1 (Flk1). After the antibody reaction, the cells were washed twice and filtered through a nylon screen (pore size, 35 μm; BD Biosciences). Flow cytometric analysis and cell sorting were performed using fluorescence-activated cell sorter Aria II (BD Biosciences), following the manufacturer's instructions.

DNA transfection

About 1×10^6 cells were transfected with 5 μg of expression plasmid encoding SF-1 driven by the cytomegalovirus promoter (pCMFlag-hsNR5A1, obtained from Riken BRC, Saitama, Japan). The transfection was accomplished using Nucleofector (Lonza, Gaithersburg, MD) according to the manufacturer's instructions. Nucleofector technology is a nonviral approach to transferring nucleic acids into cells and is based on the method of electroporation. The transfection efficiency was about 40–60%.

RT-PCR and quantitative real-time PCR

Total RNA was extracted using an RNeasy minikit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions. Aliquots of total RNA were then reverse transcribed into cDNA using PrimeScript reverse transcriptase reagent (Takara Bio, Shiga, Japan); the reaction was carried out at 42 C for 15 min and terminated by heating at 70 C for 2 min. For RT-PCR, 1 μg of total RNA was reverse transcribed, and one 50th of the reaction mixture was subjected to PCR using a thermal cycler. The reaction protocol entailed denaturation at 94 C for 30 sec, annealing at 58 C for 30 sec, and extension at 72 C for 30 sec. Ten microliters of the PCR products were electrophoresed on a 1.5% agarose gel and then visualized by ethidium bromide staining. Levels of mRNA expression were quantified by real-time RT-PCR using an ABI PRISM 7300 sequence detection system (Applied Biosystems, Carlsbad, CA) and a SYBR Premix Ex Taq kit (Takara Bio). To calculate the copy number of each mRNA, standard curves were generated using synthesized oligo DNA fragments containing the PCR amplicon region (Sigma-Aldrich Japan, Tokyo, Japan). The PCR protocol entailed an initial denaturation at 95 C for 10 min, followed by 40 cycles of 95 C for 10 sec and 60 C for 31 sec. The levels of each mRNA were normalized with that of a housekeeping gene, β-actin.

Western blot analysis

For protein extraction, cultured cells were homogenized in ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing protease inhibitor cocktails (Roche Applied Science, Indianapolis, IN). After centrifugation at 15,000 × *g* for 10 min, lysates were separated by SDS-PAGE, followed by electrophoretic transfer to an Immobilion-P membrane (Millipore, Bisley, UK). Immunoblotting was performed with the following primary antibodies: β-catenin (Abcam, Cambridge, UK) and β-actin (Abcam). The immunoblot was incubated with horseradish peroxidase-conjugated secondary antibody, and the chemiluminescent signals were developed by ECL-Prime (Amersham, Aylesbury, UK). The signals were quantified with an ImageQuant LAS-4010 system (GE Healthcare, Tokyo, Japan).

Immunocytochemistry

For immunocytochemical analyses, cells were fixed in either PBS containing 4% paraformaldehyde for 10 min at room temperature or 70% ethanol for 30 min at 4 C. After washing the fixed cells with PBS, they were incubated with mouse antihuman 3β-hydroxyl steroid dehydrogenase (3β-HSD) antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse antihuman chorionic gonadotropin (hCG) antibody (1:500; Dako, Glostrup, Denmark). Following the primary antibody reaction, the cells were treated according to the manufacturer's instruc-

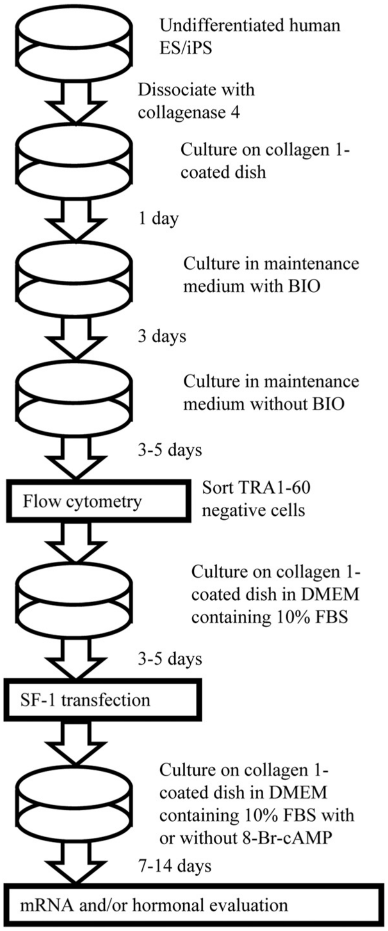

FIG. 3. Schematic flow diagram of the multistep method. FBS, Fetal bovine serum.

tions with a Dako Envision+ kit, mouse/horseradish peroxidase (Dako), which is a peroxidase-labeled polymer kit conjugated to goat antimouse immunoglobulin. Thereafter the cells were stained with 3,3′-diaminobenzidine (DAB) using DAB+ Liquid (Dako). Finally, the nuclei were stained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

Steroid hormone measurement

Steroid hormones and hCG in conditioned cell medium were measured 24 h after the prior medium change. Levels of progesterone, corticosterone, cortisol, aldosterone, dehydroepiandrosterone (DHEA), and estradiol in the medium were all measured using commercially available enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI). hCG levels were similarly measured using a commercially available enzyme immunoassay kit (Cayman Chemical).

Statistical analysis

All data are expressed as the means ± SEM. Comparison of means between two groups was done using Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

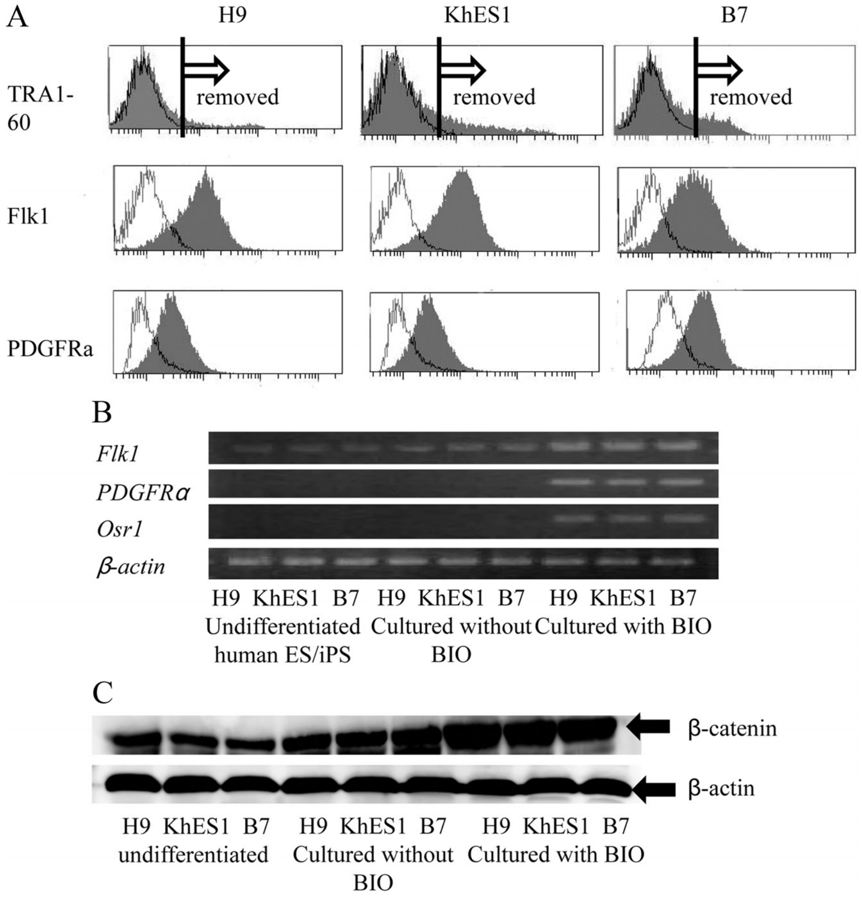

FIG. 4. Mesodermal differentiation of human ES/iPS cells using BIO. A, Flow cytometric analysis of the indicated cell lines after treatment with BIO. TRA1–60-negative, differentiated cells were sorted, and expression of the mesodermal cell surface markers Flk1 and PDGFRα was evaluated. B, RT-PCR analysis of the indicated cell lines before differentiation (undifferentiated), after differentiation induced without BIO, and after differentiation induced with BIO. C, Western blot analysis for β-catenin and β-actin of the indicated cell lines before differentiation (undifferentiated), after differentiation induced without BIO, and after differentiation induced with BIO.

Results

Induction of steroid-producing cells via EB formation

An EB is a three-dimensional aggregate of ES or iPS cells formed in suspension culture; it is thought to mimic some of the early stages of embryonic development and to differentiate into the three primary germ layers: endoderm, mesoderm, and ectoderm. Human ES cells have been shown to differentiate into many cell types, including β-cells (18), hepatocytes (19), adipocytes (15), cardiomyocytes (20), bone (21), and neurons (22) via EB formation. For that reason, we initially tried to use the EB formation approach to induce differentiation of human ES/iPS cells into steroidogenic cells. RT-PCR and quantitative real-time RT-PCR analyses showed that, in undifferentiated human ES or iPS cells, mRNA expression of steroidogenic genes were not detectable or hardly detectable except steroidogenic acute regulatory protein (StAR) (Fig. 1 and Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). After EB formation during 14 d of suspension culture, the cells expressed mRNA encoding the steroidogenic enzymes StAR, cytochrome P450-containing enzyme (CYP)-11A1, 3β-hydroxysteroid dehydrogenase (HSD3B), CYP17A1, and CYP19, but they did not express CYP21A2, CYP11B1, or CYP11B2 mRNA, whose translation products are required for the synthesis of both adrenocortical and gonadal steroid hormones (Fig. 1 and Supplemental Fig. 1).

The EB were next plated on gelatin-coated dishes and cultured for an additional 10 d, which up-regulated the expression of StAR, CYP11A1, HSD3B, CYP17A1, and CYP19 mRNA. However, expression of CYP21A2, CYP11B1, and CYP11B2 mRNA was still not detected (Fig. 1 and Supplemental Fig. 1). In immunocytochemical analyses, some of the cells stained positively for 3β-HSD (Fig. 2). The 3β-HSD-positive cells were present on the periphery of the EB and had large amounts of cytoplasm and a large nucleus, and some were multinucleate (Fig. 2). When we then measured steroid hormones secreted into the medium, we found that the medium contained progesterone and estradiol but not cortisol, corticosterone, aldosterone, or DHEA (Table 1). These results indicate that EB formation induced human ES/iPS cells to become steroidogenic but did not induce their differentiation into the adrenocortical lineage.

One major source of progesterone is trophoblasts. We therefore next measured hCG secretion from the cells because hCG production is specific to trophoblasts. We found that the conditioned medium also contained hCG (Table 1); moreover, immunocytochemical analysis showed that the differentiated cells included hCG-positive cells, which appeared morphologically identical to the 3β-HSD-positive cells (Fig. 2). This indicated that the steroidogenic cells obtained via EB formation were trophoblast-like cells, not adrenocortical lineage cells.

Induction of steroid-producing cells using the multistep method

To differentiate human ES/iPS cells into steroid-producing cells that are not trophoblast like but instead exhibit the characteristics of adrenal cortical cells, we used the multistep method shown schematically in Fig. 3. The adrenal cortex develops from the adrenogenital primodium, which originates from mesoderm. Therefore, as a first step, human ES/iPS cells were induced to differentiate into the mesodermal lineage cells using BIO, a GSK-3β inhibitor that activates the Wnt signaling pathway (Fig. 3). Then, as a second step, the mesodermal cells were transfected with plasmid DNA encoding SF-1, a master regulator of the differentiation/development of steroid-producing cells, and the transfectants were then plated on type I collagen-coated dishes and further differentiation was induced under the addition of 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; a membrane-permeable cAMP analog) (Fig. 3) because cAMP is known to induce steroidogenesis in a number of steroidogenic cell lines.

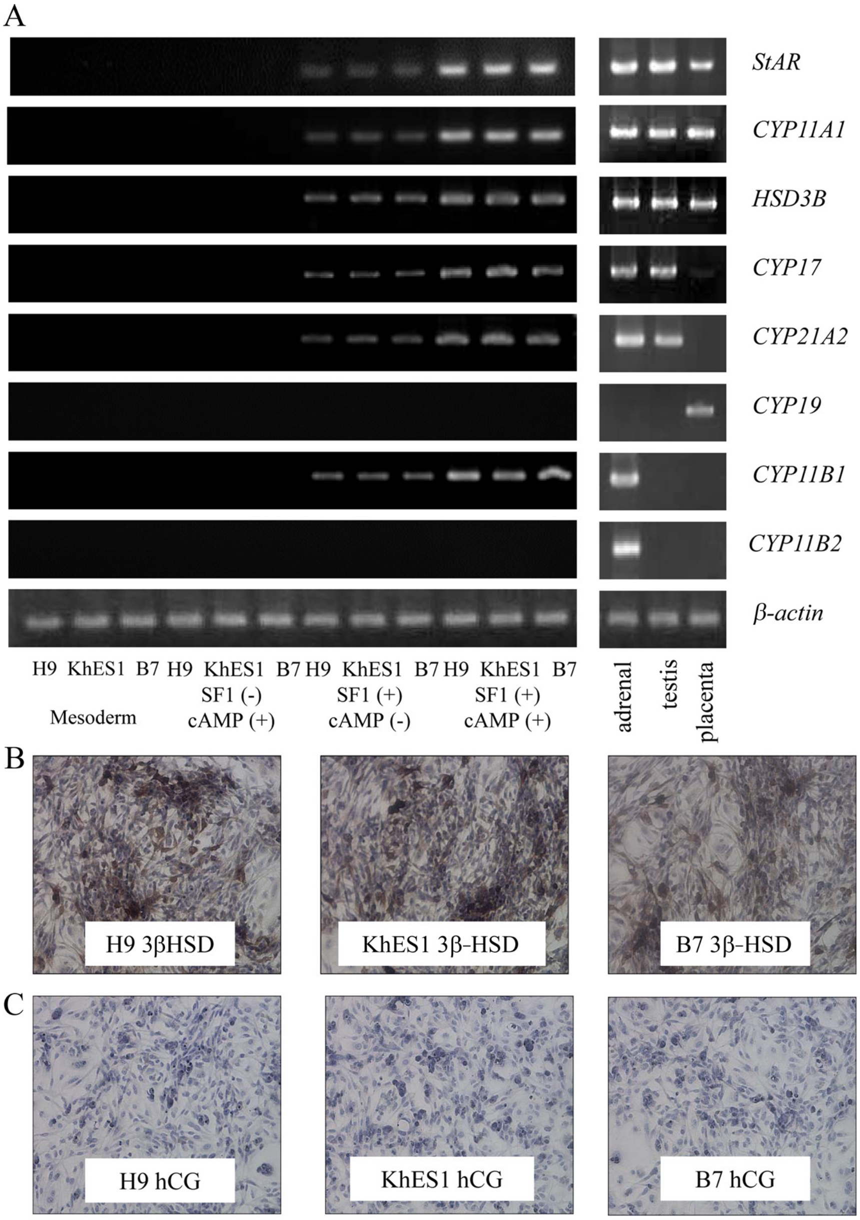

FIG. 5. Differentiation of human ES/iPS cells using the multistep method. A, RT-PCR analysis of the indicated cell lines before SF-1 transfection (mesoderm), cultured with 8-Br-cAMP without SF-1 transfection [SF-1(−)cAMP(+)], after SF-1 transfection followed by culture without 8-Br-cAMP [SF-1(+)cAMP(−)] and after SF-1 transfection followed by culture with 8-Br-cAMP [SF-1(+)cAMP(+)]. Results obtained with human adrenal gland, testis, and placenta are shown for comparison. B and C, Immunocytochemical analysis of 3β-HSD (B) and hCG (C) expression in the indicated cell lines after SF-1 transfection followed by 7 d of culture with 8-Br-cAMP.

In undifferentiated human ES/iPS cells, mRNA expression of PDGFRα or steroidogenic enzymes were not detectable or hardly detectable (Figs. 1 and Fig. 4 and Supplemental Fig. 2), although slight expression of Flk1 was detected. After 7 d of differentiation with 5 μM BIO, flow cytometric analysis revealed that TRA1–60-negative non-ES/iPS cells had appeared, and these cells were positive for the mesodermal markers Flk1 and PDGFRα (Fig. 4). Also, these cells had higher levels of β-catenin, indicating the activation of canonical Wnt signaling pathway (Fig. 4). Then the TRA1–60-negative cells were sorted by flow cytometry to remove undifferentiated cells, and RT-PCR and quantitative real-time RT-PCR analyses of the sorted cells confirmed that they expressed Flk1 and PDGFRα mRNA as well as mRNA encoding the intermediate mesodermal marker odd-skipped related 1 (Osr1) (Fig. 4). RT-PCR and quantitative real-time RT-PCR analyses of the sorted cells also revealed that the mRNA expression of steroidogenic genes were not detectable or hardly detectable (Fig. 5 and Supplemental Fig. 3).

The mesoderm lineage cells, which were sorted by flow cytometry, were then replated on type I collagen-coated dishes and cultured for 3–5 d in DMEM containing 10% fetal bovine serum, after which they were transfected with an expression plasmid encoding SF-1 driven by the cytomegalovirus promoter. The transfectants were then cultured in DMEM containing 10% fetal bovine serum and 1 mM 8-Br-cAMP. After 7 d of culture, RT-PCR and quantitative real-time RT-PCR analysis revealed the mRNA expression of the steroidogenic enzymes CYP21A1 and CYP11B1 as well as StAR, CYP11A1, HSD3B, and CYP17A1 (Fig. 5 and Supplemental Fig. 3). Low levels of mRNA expression of these steroidogenic enzymes were also detected in cells cultured in the absence 8-Br-cAMP (Fig. 5 and Supplemental Fig. 3). In addition, measurement of hormones secreted into the medium during 7 d of culture with 8-Br-cAMP revealed the presence of progesterone, corticosterone, and cortisol but not hCG (Table 2). In immunocytochemical analyses, the differentiated cells stained positively for 3β-HSD (Fig. 5) but were negative for hCG (Fig. 5). These results indicated that the steroidogenic cells obtained using the multistep method were not trophoblast like but were adrenocortical lineage cells. Moreover, real-time RT-PCR analysis showed that the levels of CYP11A1, HSD3B, and CYP17A1 mRNA in the cells after 14 d of culture with 8-Br-cAMP were not lower than the levels in the cells cultured with 8-Br-cAMP for only 7 d (Fig. 6).

Discussion

In this study, we demonstrated that human ES/iPS cells can be differentiated into steroid-producing cells by first inducing them to differentiate into the mesodermal lineage and then introducing SF-1. The steroidogenic cells thus obtained expressed mRNA encoding adrenal cortical or gonad-specific steroidogenic enzymes, such as CYP17A1, CYP21A2, and CYP11B1 and produced steroid hormones such as progesterone, corticosterone, and cortisol.

We first assessed whether human ES/iPS cells could differentiate into steroidogenic cells via EB formation. After EB formation in suspension and further culture on gelatin-coated dishes, the cells spontaneously differentiated into steroidogenic cells that expressed StAR, CYP11A1, HSD3B, and CYP17A1 mRNA and secreted progesterone. However, these cells also expressed hCG, which is specific to trophoblasts. Consequently, we considered the steroidogenic cells obtained via EB formation to be trophoblast like. This finding is consistent with the earlier report by Gerami-Naini *et al.* (23), who showed that human ES cells can spontaneously differentiate into trophoblast-like cells via EB formation

In the present study, we used the GSK-3β inhibitor BIO to induce differentiation of human ES/iPS cells into the mesodermal lineage. GSK-3β is a serine/threonine kinase that phosphorylates and promotes the degradation of β-catenin. Wnt signaling functions by regulating the translocation of β-catenin to the nucleus. GSK-3β inhibitors, including BIO, induce the accumulation of β-catenin (24, 25), and the activation of the canonical Wnt/β-catenin signaling pathway in ES cells results in mesodermal differentiation (24–27). Moreover, knockout mice deficient in β-catenin die *in utero* due to a complete lack mesoderm (28). As shown in this study, human ES/iPS cells treated with BIO, in which the canonical Wnt signaling pathway was activated, expressed mesodermal markers, including

TABLE 2. Hormone levels in medium conditioned by undifferentiated ES/iPS cells and differentiated cells obtained using a multistep method

	Undifferentiated			Differentiated		
	H9	**KhES1**	**B7**	**H9**	**KhES1**	**B7**
Progesterone (pg/ml)	N.D.	N.D.	N.D.	1730 ± 460	3080 ± 1180	2480 ± 590
Corticosterone (pg/ml)	N.D.	N.D.	N.D.	460 ± 57	605 ± 55	661 ± 59
Cortisol (pg/ml)	N.D.	N.D.	N.D.	10510 ± 1530	10030 ± 1650	8540 ± 820
Aldosterone (pg/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DHEA (pg/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Estradiol (pg/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
hCG (mIU/ml)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Data are means ± SEM of triplicate experiments. Differentiated cells were cultured with 8-Br-cAMP for 7 d after transfection of SF-1 into mesodermal lineage cells derived from human ES/iPS cells. N.D., Not detectable.

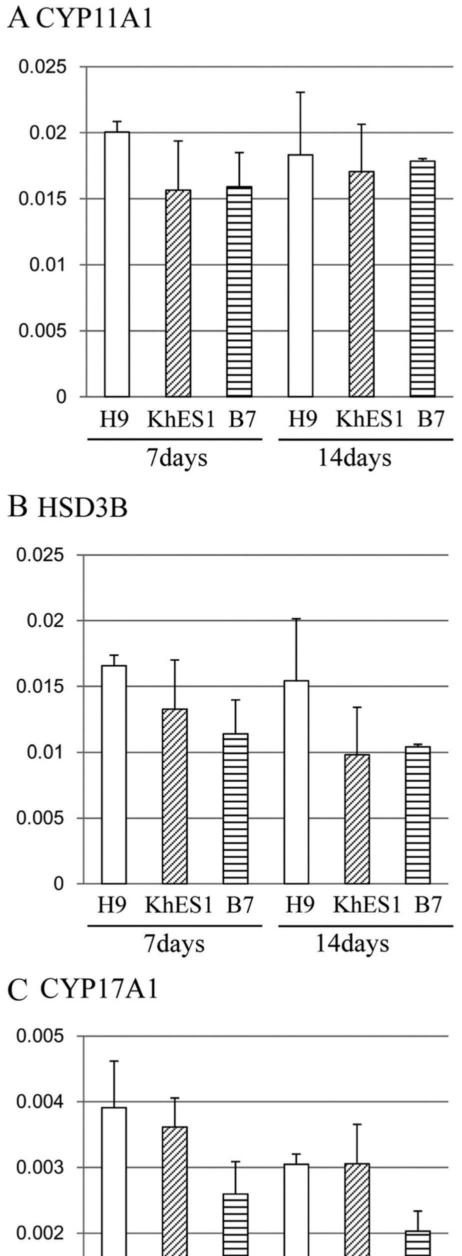

FIG. 6. Real-time RT-PCR analysis of CYP11A1 (A), HSD3B (B), and CYP17A1 (C) mRNA expression in the indicated cell lines after 7 or 14 d of culture after SF-1 transfection. The copy number of mRNA of each steroidogenic enzymes was normalized with that of a housekeeping gene, β-actin. Data are means and SEM of three triplicate experiments.

Flk1 and PDGFRα (29). In addition, the mesodermal cells expressed mRNA encoding the intermediate mesodermal marker odd-skipped related 1 (Osr1), which is known to be essential for development of the heart and intermediate mesoderm, including the adrenal cortex (30). However, the mesodermal cells that we induced from human ES/iPS cells through Wnt/β-catenin activation by BIO did not express mRNA encoding the steroidogenic enzymes themselves. We therefore introduced SF-1 into the mesodermal cells as a next step.

SF-1 is an orphan nuclear receptor that belongs to the NR5A subfamily of the nuclear receptor superfamily. SF-1 is essential for sexual differentiation and formation of the primary steroidogenic tissues (31); SF-1 knockout mice completely lack adrenal glands and gonads and die soon after birth (32). In humans, heterozygous SF-1 mutations can cause XY sex reversal (*i.e.* testicular failure), ovarian failure, and occasionally adrenal insufficiency (33).

In an earlier study, Crawford *et al.* (5) showed that exogenous expression of SF-1 directed mouse ES cells into the steroidogenic lineage, although the steroidogenic capacity of the differentiated cells was very limited. Recently two other groups reported that exogenous expression of SF-1 induced differentiation of mouse and human MSC into steroidogenic cells that produced a variety of steroid hormones in addition to progesterone (6–9). On the other hand, forced expression of SF-1 in already differentiated cells, such as preadipocytes, fibroblasts, and human embryonic kidney 293 cells, did not induce their further differentiation into the steroidogenic lineage (6). Moreover, Gondo *et al.* (6) reported that steroid hormone production was observed for more than 112 d when bone marrow-derived MSC were transfected with SF-1 by means of an adenoviral vector (mean half-life is usually 2–3 wk). These findings indicated that SF-1 can serve as a differentiating factor for the steroidogenic differentiation of multipotent stem cells. In the present study, we used an expression plasmid encoding SF-1 in part because transiently increased expression of SF-1 is reportedly crucial for differentiation of the adrenal cortex and gonads (34). Another reason was that persistently elevated levels of SF-1 are reportedly associated with adrenal tumorigenesis (35), which would be an obstacle to clinical application. Our study showed that transiently expressed SF-1 functioned as a differentiating factor in the steroidogenic differentiation of mesodermal cells and that the expression of steroidogenic enzymes persisted for much longer than the half-life of the plasmid (mean half-life is usually 2–3 d).

One of the limitations of our study is that the differentiated cells do not proliferate as well as undifferentiated cells do, making it difficult to obtain large numbers of cells. Another limitation is that their *in vivo* functionality was not evaluated. Clearly, further studies will be required before clinical application of the steroidogenic cells we developed is possible.

In conclusion, we showed in this study that human ES/iPS cells can be induced to differentiate into mesodermal lineage cells using a GSK-3β inhibitor and that subsequent introduction of SF-1 can induce the mesodermal lineage cells to differentiate into steroid-producing cells with the characteristics of adrenal cortical cells for the first time to our knowledge. Although further studies are required, our method will open a new avenue to the elucidation of the molecular mechanisms underlying the development/differentiation of the adrenal cortex and to the future possibility of cell therapies for patients with adrenal insufficiency.

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