et al. 2008), or *Tead4* (Nishioka et al. 2009). However, the fundamental molecular mechanisms regulating the differentiation of ESCs into trophoblast have not yet been elucidated. Human embryonic stem cells (hESCs) have been reported to express trophoblast markers after treatment with bone morphogenetic protein 4 (BMP4) in vitro (Xu et al. 2002). By contrast, the effect of BMP4 on mESCs is still unclear (Kunath et al. 2007). BMP4 is thought to be involved in activin- or Wnt-induced mesoderm induction or mesodermal tissue specification from ESCs (Johansson and Wiles 1995; Wiles and Johansson 1999; Nostro et al. 2008; Sumi et al. 2008). Coordinating with leukemia inhibitory factor (LIF), BMP4 also supports the mESC self-renewal in defined culture conditions (Ying et al. 2003; Qi et al. 2004).

Recently, Smith and his colleagues have suggested that to elucidate physiologically relevant molecular signals in mESCs, culture conditions with fewer extrinsic stimulators are beneficial (Ying et al. 2008). We previously developed a chemically defined simple serum-free culture condition for mESCs (Furue et al. 2005). Under these culture conditions, the effects of extracellular matrices (ECM) on mESCs were studied, and the results revealed that laminin promoted differentiation into epiblast-like cells (Hayashi et al. 2007). In this study, we assessed the effect of BMP4 on mESC in the defined culture conditions with fewer extrinsic stimulators. Our results show that mESCs can be induced to differentiate into trophoblast by BMP4 in vitro. This differentiation was inhibited by serum or LIF. Furthermore, we also found that BMP4 activates the Smad pathway in mESCs, and in turn, the BMP–Smad pathway directly induces *Cdx2* expression, which plays a crucial role in trophoblast differentiation.

Materials and Methods

Cell culture. The mESC D3 line (CRL-1934, ATCC, Manassas, VA), B6G-2 line (AES0003, RIKEN Cell Bank, Ibaraki, Japan), E14 line, or EB3 line was routinely cultured in 75-cm^2 plastic flasks (Corning, Corning, NY), coated with 15 μg/ml of type I collagen (Nitta gelatin) in a humidified atmosphere of 5% CO_2 at 37°C in a defined ESF7 medium. The ESF7 consisted of ESF basal medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with 10 μg/ml of insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-ethanolamine, 20 nM sodium selenite, 9.4 μg/ml of oleic acid conjugated with 2 mg/ml of fatty-acid-free bovine serum albumin (FAF-BSA; Sigma, St. Louis, MO), and 10 ng/ml of LIF (Chemicon, Billerica, MA), as described previously (Furue et al. 2005; Hayashi et al. 2007). For the differentiation experiments, mESCs were seeded at a density of 1×10^4 cells per square centimeter in the ESF5 medium comprised of ESF basal medium supplemented with 10 μg/ml of insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-ethanolamine, 20 nM sodium selenite added with 0.5 mg/ml FAF-BSA, and 10 ng/ml rhBMP4 (R&D Systems, Minneapolis, MN) on 2 μg/cm^2 laminin-coated (Sigma) dishes. The medium was changed every 2 d. After 4 d of culture under differentiating conditions, the cells reached confluency. Then, in all experiments where cells were differentiated for more than 4 d, the cells cultured for 4 d in BMP4-supplemented ESF5 medium were harvested with 0.2 mg/ml ethylenediaminetetraacetic acid (EDTA)-4Na (Sigma) for 5 min at room temperature and subcultured into BMP4-supplemented ESF5 medium at a density of about 2×10^4 cells per square centimeter. For the examination of the effects of activin and FGF4 on the differentiation of mESCs into trophoblast, the mESCs were cultured in ESF5 medium supplemented with 25 ng/ml of FGF4 (Sigma) or 10 ng/ml of activin A (Ajinomoto, Kawasaki, Japan), respectively. When examining the effect of fetal calf serum (FCS), LIF, and Noggin on the differentiation of mESCs into trophoblast, the mESCs were cultured in ESF5 medium supplemented with 10 ng/ml of LIF (Chemicon), 10% FCS (ES qualified; Gibco, Grand Island, NY), or 300 ng/ml Noggin (R&D Systems), respectively, along with BMP4.

Flow cytometry. Flow cytometry was performed with EPICS ALTRA system (Beckman Coulter) as described previously (Furue et al. 2005; Hayashi et al. 2007). In this study, goat anti-Cdh3 antibody (R&D systems) was visualized with AlexaFluor-488-conjugated mouse antigoat IgG (Invitrogen, Carlsbad, CA). For DNA content analysis, ethanol-fixed samples were stained with propidium iodide (PI).

Immunocytochemistry. Immunocytochemistry was performed as described previously (Furue et al. 2005; Hayashi et al. 2007). Briefly, mESCs were fixed in 4% (*w/v*) paraformaldehyde or ice-cold acetone, permeabilized with 0.1% Triton X-100, and reacted with primary antibodies. The primary antibodies were visualized with AlexaFluor-488-conjugated antirabbit, antimouse, or antigoat IgG or AlexaFluor-594-conjugated donkey antimouse, antirabbit, or antigoat IgG (Invitrogen). The primary antibodies used are as follows: anti-Cdx2 antibody (Biogenex, San Ramon, CA; 1:100), anti-Cdh3 antibody (R&D systems; 1:200), anti-CK7 antibody (Chemicon; 1:100), anti-Cx31 antibody (Chemicon; 1:100), anti-Nanog antibody (ReproCell, Tokyo, Japan; 1:200), and anti-SSEA1 antibody (Kyowa, Tokyo, Japan; 1:100).

Transfection. mESCs were seeded at a density of 5×10^5 cells per well in a six-well plate coated with type I collagen in ESF7. The mESCs were transfected with plasmid DNA

using Lipofectamine 2000 (Invitrogen), according to the supplier's instructions. For inhibitory Smad overexpression experiment, Smad6 expression vector comprising whole Smad6 cDNA under CAG promoter in pCAG-IRES-PURO plasmid (a gift of Dr. Imamura) was used. The pCAG-IRES-PURO-FLAG was used as a mock. Transfected cells were reseeded in ESF5 with 10 ng/ml of BMP4 and 1 μg/ml of Puromycin, 24 h after transfection. These cells were used for immunocytochemistry and reverse transcription–polymerase chain reaction (RT–PCR), 96 h after transfection. For *Cdx2*-knockdown experiment, the shRNA for *Cdx2* expression vector consisted of 29-mer shRNA constructs against Mouse *Cdx2* under U6 promoter in pRS plasmid (purchased from OriGene, Rockville, MD). The pRS plasmid was used as a mock. Transfected cells were reseeded in ESF7 with 0.5 μg/ml of Puromycin, 24 h after transfection. After two passages on this culture conditions, these cells were used for immunofluorescence detection and RT–PCR.

Chromatin immunoprecipitation assay. For chromatin immunoprecipitation (ChIP) assay of endogenous proteins, the mESCs (D3 line) were cultured in ESF5 medium for 48 h on laminin and treated with 100 ng/ml of BMP4 for 1 h. The cells were cross-linked with 1% formaldehyde for 10 min at 37°C. To stop the cross-linking, the samples were washed twice with ice-cold phosphate-buffered saline (PBS) with complete protease inhibitors mixture (Roche). After gentle washing with ChIP lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris–HCl (pH 8.0), complete protease inhibitor mixture], the samples were lysed with 200 μl of ChIP lysis buffer. The lysates were then mixed with 800 μl of ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.0), 167 mM NaCl, complete protease inhibitor mixture] and sonicated four times for 10 s each at the maximum setting (Sonifier 150; Branson, Danbury, CT). Then, 1 ml of ChIP dilution buffer was added, and the samples were centrifuged at 17,000×*g* for 10 min. The supernatants were transferred to a fresh centrifuge tube. An aliquot of 200 μl of the supernatant was stored at 4°C as the input DNA sample. These supernatants were conjugated with anti-phospho Smad1/5/8 antibody (Chemicon) or goat normal IgG (Upstate Biotechnology) bound with Dynabeads Protein G (Invitrogen) through overnight incubation at 4°C with rotation. These antibodies were found to adhere to the Dynabeads Protein G when 5 μg of the antibodies was incubated in 0.1 mg/ml of BSA in PBS for 1 h at room temperature with rotation. The beads were collected with Dynal MPC-S (Invitrogen) and were washed sequentially for 5 min each in low-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl], high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 500 mM NaCl], and LiCl buffer [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris–HCl (pH 8.0)]. The precipitates were then washed twice with ChIP TE buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA] for 5 min. The immunocomplex was extracted twice by incubation for 30 min at room temperature with 200 μl of ChIP elution buffer (1% SDS, 0.1 M $NaHCO_3$, 10 mM DTT). The eluates and input DNA solutions were supplemented with 5 M NaCl to a final concentration of 200 mM and heated at 65°C for 8–12 h to reverse the formaldehyde cross-linking. All the samples were sequentially treated for 30 min with RNase I (Wako) at 37°C and for 1 h with proteinase K (Takara Bio) at 55°C. The DNA fragments were purified using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and analyzed by PCR. The primers, annealing temperature, and number of cycles in the PCR analysis are as follows: for *Cdx2* Intronic Conserved Sequence 1 (CICS1), forward, 5′-GGGCCA CAGCTTCCCTACAT-3′ and reverse, 5′-TGGGTGGTCCGA GACTAGGG-3′, 60°C, 31 cycles; for DS 4 kb, forward, 5′-ATGCCAGAGCCAACCTGGAC-3′ and reverse, 5′-CTCCCGACTTCCCTTCACCA-3′, 60°C, 32 cycles; and for US 4 kb, forward, 5′-AGCCAAG GACCCTTGTTGCT-3′ and reverse, 5′–GGGGACTTGAA CACCCTTCC-3′, 60°C, 32 cycles.

Electrophoretic mobility shift assay. The proteins used in the electrophoretic mobility shift assay (EMSA) were transcribed and translated from the expression vectors pCS2-GST-Smad1 and pCS2-GST-Smad4 in BL21 *E. coli* strain. The probes generated from the sense and the antisense oligonucleotides were labeled with Cy5.5 (Sigma-Aldrich), mixed, and annealed. The DNA–protein binding reaction was performed in the binding buffer [20 mM HEPES (pH 7.8), 45 mM KCl, 10 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.1% Nonidet P-40, 0.2 mg/ml BSA, 1 mM DTT] at 4°C for 1 h. After electrophoresis, the binding reactions were analyzed by using the Odyssey image reader (Li-Cor, Lincoln, NE; ALOKA, Tokyo, Japan) for the Cy5.5-labeled probe. The sense strand sequences of the probes used in EMSA are as follows: 5′-ACAAGGGCGCCCGGCGCCGACAGCGG TCTTGCCACCTCGGCGCGGGACTT-3 ′.

Luciferase assay. The pGL4.74 (Promega) plasmid was used as an internal control. Cultured cells were transfected with the reporter vectors (pGL4.23 with Cdx2-intron1 firefly, 4 μg; Renilla, 100 ng) and were harvested 48 h after transfection. Reporter activities were measured by using the dual-luciferase reporter assay system (Promega). Each assay was performed in duplicate, and all the results presented the mean values (n=4).

RT–PCR. RT–PCR was performed as described previously (Furue et al. 2005; Hayashi et al. 2007). Briefly, total RNA

was extracted from the cultured cells using the total RNA extraction kit (Agilent, Wilmington, DE) and reverse-transcribed using Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed with SYBR Green PCR Master Mix according to the supplier's directions (Applied Biosystems, Foster City, CA) in ABI PRISM 7700 sequence detector or Step One Plus sequence detector (Applied Biosystems). Relative expression of mRNA was calculated and compared with the expression in mouse whole-day 10.5 embryos or 12.5 placenta. Conventional PCR for the detection of trophoblast marker expression or ChIP assay was performed with SYBR Green PCR Master Mix according to the supplier's directions. The sequences of the primers are listed in the Table 1. All the results are given as the mean values (n=4).

Western blot. Western blot was performed as described previously (Furue et al. 2005; Hayashi et al. 2007). Briefly, to detect the phosphorylation of Smads, mESCs were seeded at a density of 6×10^5 cells per square centimeter on six-well plates in the ESF basal medium and were stimulated using 100 ng/ml of BMP4 for 0, 15, or 60 min. The cells were lysed in 200 μl of ice-cold lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1 mM Na_3Vo_4, 0.5% sodium deoxycholate, 5 mM EDTA, 1% NP-40) and 250 μl of PBS. Protein samples (25 or 50 μg) were separated in a 12.5% SDS–polyacrylamide gel and electroblotted to a polyvinylidene fluoride membrane (Amersham, Piscataway, NJ). After incubating in the blocking buffer for 30 min at room temperature, the membrane was incubated with primary antibodies overnight at 4°C. The primary antibodies used are as follows: anti-α-tubulin (Sigma, 1:1,000), anti-Smad1/5/8 (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1,000), anti-phospho Smad1/5/8 (Cell Signaling Technology, Beverly, MA, 1:1,000), and anti-Smad6 (Abcam, Cambridge, UK, 1:1,000). The membranes were then reacted with secondary antibodies followed by horseradish peroxidase substrate, according to the supplier's protocol (Pierce Biotechnology, Rockford, IL). Protein bands on the membranes were visualized with LAS-1000 and PRO-LAS 1000 software (Fujifilm, Tokyo, Japan).

Results

The characterization of BMP4-treated cells in defined conditions. We observed morphological changes of the cells when undifferentiated mES cells at a density of 5×10^3 cells per square centimeter were cultured in ESF5 medium supplemented with 10 ng/ml BMP4 on laminin-coated dishes for 4 d. The morphology of the majority of cells became cobblestone-shaped (Fig. 1*A*, left). Before the cells reached to confluent, we subcultured the cells into the same culture conditions at a density of 2×10^4 cells per square centimeter on culture day 4. After four additional days in culture, multinuclear cells appeared (Fig. 1*A*, right). Trophoblast stem cells (TSCs) exhibit a cobblestone morphology (Tanaka et al. 1998) and subsequently differentiate into multinuclear trophoblast (Simmons and Cross 2005). These findings suggested the possibility that mESCs cultured with BMP4 in ESF5 might differentiate into trophoblast lineages. To explore this possibility further, we examined the gene expression of transcription factors which are expressed in trophoblast, *Cdx2* (Strumpf et al. 2005), *Dlx3* (Morasso et al. 1999), *Eomes* (Russ et al. 2000), *Errb* (Luo et al. 1997), *Esx1* (Li and Behringer 1998), *Ets2* (Yamamoto et al. 1998), *Gata3* (Ng et al. 1994), *Hand1* (Cross et al. 1995), *Mash2* (Guillemot et al. 1995), and *Psx1* (Chun et al. 1999) in the differentiated cells by quantitative real-time RT–PCR. The relative mRNA levels of the majority of these transcription factors were increased over tenfold compared with those of the undifferentiated mESCs (Fig. 1*B*). The mRNA levels of *Eomes*, *Errb*, and *Ets2*, which were involved in self-renewal of undifferentiated TSCs (Luo et al. 1997; Russ et al. 2000; Wen et al. 2007), were not increased. To confirm whether BMP4 specifically activates the expression of trophoblast markers, we examined the transcription of other cell lineage markers in early mammalian development, *Sox1* (ectoderm), *Flk1* (mesoderm), *Mixl1* (definitive endoderm), and *Gata6* (primitive extraembryonic endoderm) in the cells cultured with BMP4 in ESF5 medium on culture day 4 by quantitative real-time RT–PCR. The gene expression of the ectoderm, mesoderm, definitive endoderm, or extraembryonic endoderm cell lineage markers examined here was no higher than that in undifferentiated mESCs (Fig. 1*C*). These results indicate that the differentiated cells specifically upregulated transcription factors for trophoblast.

As the mESCs proliferated in a dose-dependent manner in response to BMP4 concentration in ESF5 medium (Fig. 1*D*) and did not proliferate without addition of BMP4, we could not compare the gene expression with those in the BMP4-untreated cells. To examine whether these genes were specifically induced in BMP4-treated cells or not, we compared the effect of FGF4 or activin with that of BMP4 on mESC gene expression. Gene expression profiles in the cells cultured with BMP4, activin, or FGF4 in ESF5 on laminin for 4 d were analyzed by quantitative RT–PCR. FGF-4 increased the *Cdx2* expression in the cells as BMP4 did, but activin did not increase *Cdx2* expression. Neither FGF-4 nor activin increased the expression of *Dlx3*, *Esx1*, *Gata3*, *Hand1*, *Mash2*, or *Psx1* (Fig. 1*E*). These results indicated that BMP4 specifically upregulated the expression of these trophoblast-specific transcription factors.

Table 1. Primer pairs used in RT–PCR

Names	Sequences	Product size	Cycles
Cdx2	5′-CTGCTGTAGGCGGAATGTATGTCT-3′ 5′-AAGGCTTGTTTGGCTCGTTACAC-3′	146	–
Dlx3	5′-TACTCGCCCAAGTCGGAATA-3′ 5′-AGTAGATCGTTCGCGGCTTT-3′	174	–
Eomes	5′-CGGCAAAGCGGACAATAACA-3′ 5′-ATGTGCAGCCTCGGTTGGTA-3′	195	–
Errb	5′-GCTGTATGCTATGCCTCCCAACG-3′ 5′-ACTCTGCAGCAGGCTCATCTGGT-3′	166	–
Esx1	5′-GAGCTGGAGGCCTTTTTCCA-3′ 5′-ACACCCACAGGGGGACTCAT-3′	194	–
Ets2	5′-CTCGGCTCAACACCGTCAAT-3′ 5′-AGCTGTCCCCACCGTTCTCT-3′	132	–
Flk1	5′-TCCTACAGACCCGGCCAAAC-3′ 5′-ACACGTTGGCAGCTTGGATG-3′	163	–
Gapdh	5′-ACCCAGAAGACTGTGGATGG-3′ 5′-CACATTGGGGGTAGGAACAC-3′	173	–
Gata3	5′-GGGCTACGGTGCAGAGGTAT-3′ 5′-TGGATGGACGTCTTGGAGAA-3′	163	–
Gcm1	5′-TACCTGAGACCCGCCATCTG-3′ 5′-AAGATGAAGCGTCCGTCGTG-3′	152	35
Hand1	5′-TCGCCGAGCTAAATGGAGAA-3′ 5′-TGCTGAGGCAACTCCCTTTT-3′	124	–
Mash2	5′-CGGGATCTGCACTCGAGGAT-3′ 5′-GGTGGGAAGTGGACGTTTGC-3′	183	–
Mixl1	5′-AAGTTGGGGAGTACACAATG-3′ 5′-CACCATACCACACATATGGA-3′	195	–
Pl1	5′-CATTTGGCTGAACTGTCTCA-3′ 5′-GACTTCCTCTCGATTCTCTG-3′	111	35
Plf	5′-AGGAACAAGCCAGGCTCACA-3′ 5′-TTCCGGACTGCGTTGATCTT-3′	178	35
Psx1	5′-CGATGGATGGGTGTGGATGA-3′ 5′-TGACAGGGCTGGCACTCAAG-3′	165	–
Sox1	5′-GTCATGTCCGAGGCCGAGAA-3′ 5′-AGCAGCGTCTTGGTCTTGCG-3′	118	–
Tpbpa	5′-AGTCCCTGAAGCGCAGTTGG-3′ 5′-TTGGAGCCTTCCGTCTCCTG-3′	138	35
Tpbpb	5′-GTCATCCTGTGCCTGGGTGT-3′ 5′-TGCCATCCTTCTCCTGGTCA-3′	163	35

To characterize these putative trophoblast cells, we further analyzed the expression of the trophoblast marker proteins Cdh3 (placental cadherin; Nose and Takeichi 1986), Cdx2, cytokeratin (CK) 7 (Lu et al. 2005), and connexin (Cx) 31 (Zheng-Fischhofer et al. 2007), by immunocytochemistry. The differentiated cells expressed Cdh3, Cdx2, CK7, and Cx31 proteins (Fig. 2*A*). Furthermore, we examined the expression of additional trophoblast-specific genes: *Gcm1* (Anson-Cartwright et al. 2000), *Placental lactogen* (*Pl*)*-1* (Faria et al. 1991), *Plf* (Simmons et al. 2008), *Tpbpa*, and *Tpbpb* (Lescisin et al. 1988) by RT–PCR. These trophoblast markers are expressed at much higher levels in placenta than in whole embryos. The expression of trophoblast marker genes was prominent in the cells cultured with BMP4 in ESF5 for 8 d (Fig. 2*B*). By flow cytometry analysis, 83.2% of cells cultured with BMP4 in ESF5 were positive for Cdh3 expression (Fig. 2*C*). These results indicate that the majority of the differentiated cells exhibit trophoblast characteristics. We observed multinuclear cells surrounded by Cdh3 in this culture conditions, which is a characteristic of differentiated trophoblast (Simmons and Cross 2005; Fig. 2*D*). To examine the frequencies of multinuclear cells in the culture, the intensity of PI in the cells cultured with BMP4 in ESF5 for 8 d was examined. A small population of hyperploid cells (>4 N) was observed in the differentiated cell cultures (Fig. 2*E*). The percentage of hyperploid cells (>4 N) was 37% in the cells, suggesting the presence of multinuclear differentiated trophoblast. We have confirmed that other mESC lines, B6G-2, E14, and EB3, also

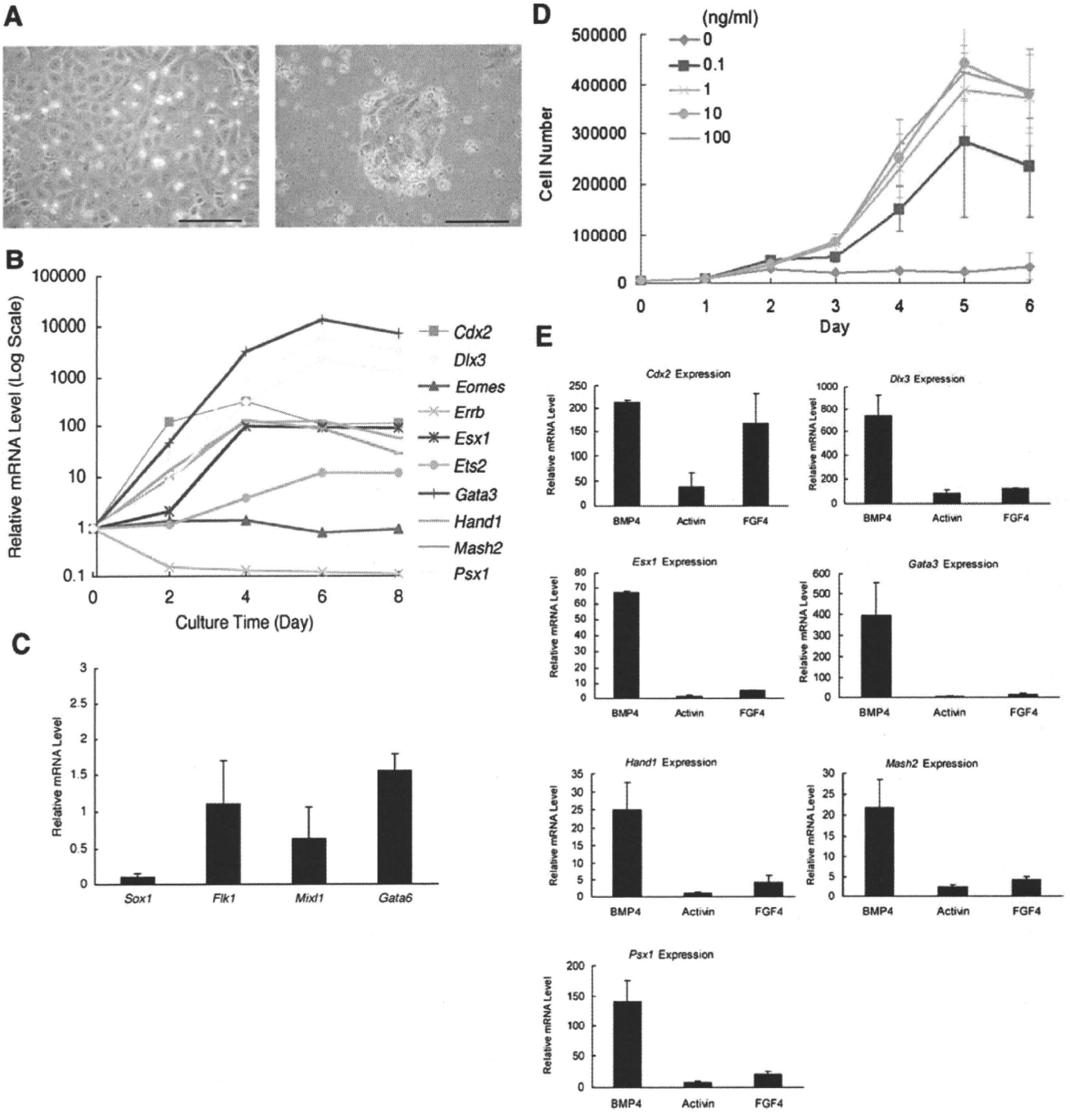


Figure 1. The effect of BMP4 on mESCs in defined culture conditions. (*A*) phase contrast photomicrographs of differentiated mESCs cultured in BMP4-supplemented ESF5 medium for 4 d (*left*) and 8 d (*right*). *Scale bars* are 100 μm. (*B*) quantitative real-time RT–PCR analysis of trophoblast-specific transcription factor expressions. The mESCs cultured in BMP4-supplemented ESF5 medium for 0, 2, 4, 6, and 8 d were analyzed. (*C*) Quantitative RT–PCR analysis of differentiation markers of mESCs. The cells cultured in BMP4-supplemented ESF5 medium for 4 d were analyzed. The amount of undifferentiated mESCs is indicated as 1. (*D*) Proliferation of differentiating mESCs on various BMP4 concentrations. mESCs were seeded in a 24-well dish at 5×10^3 cells per well on each BMP4 concentration in ESF5. Cells were counted every 24 h. The values are the mean ± SEM (n=4). (*E*) quantitative RT–PCR analysis of BMP4-induced trophoblast transcription factors in mESCs cultured in BMP4, activin A (10 ng/ml), or FGF4-supplemented (25 ng/ml) ESF5 medium on laminin for 4 d was analyzed. The gene expressions were normalized by the amount of *Gapdh*. The amount of the undifferentiated mES is indicated as 1. The values are the mean ± SEM (n=4).

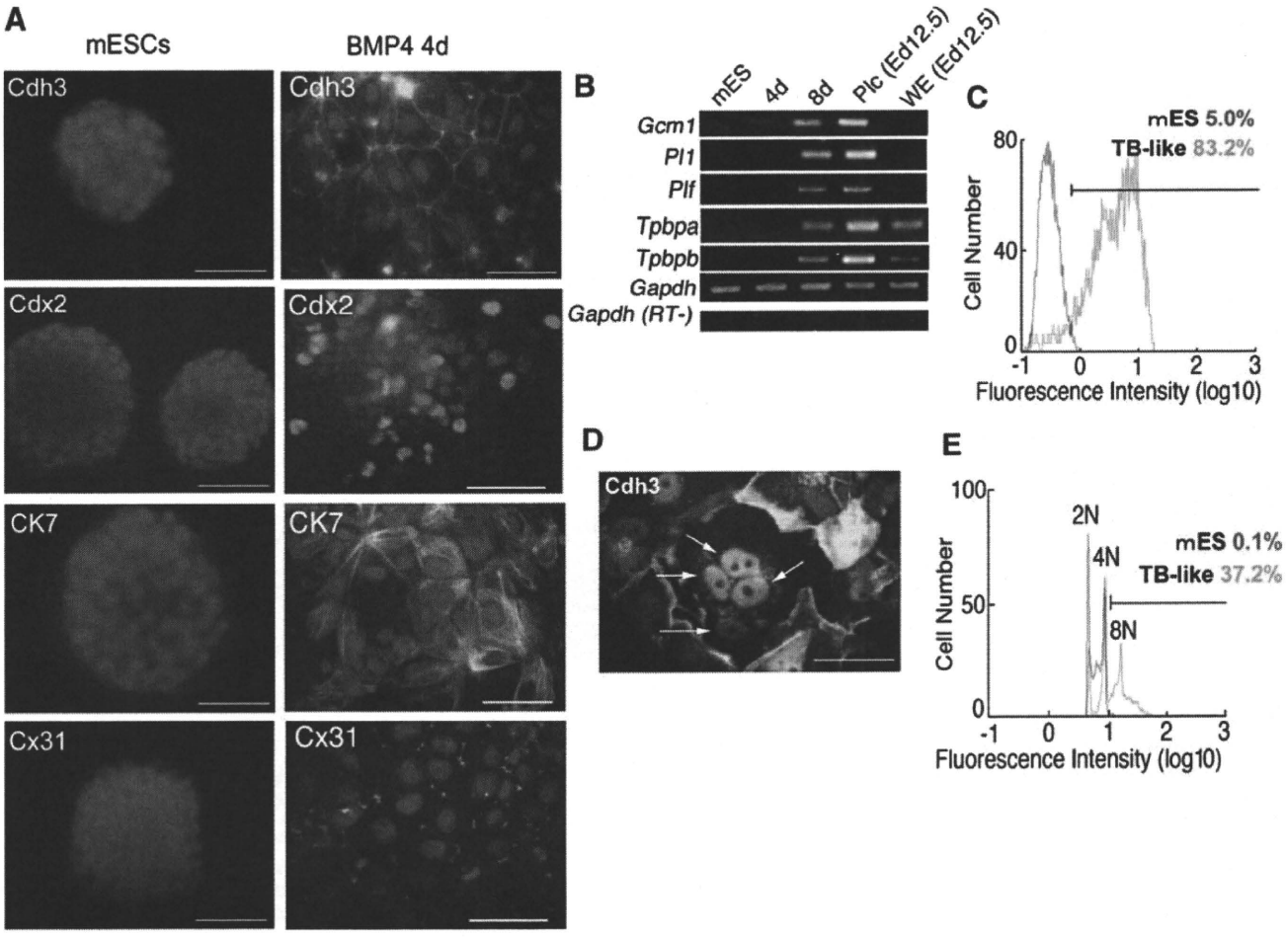


Figure 2. Differentiation into trophoblast from mESCs in vivo. (*A*) Immunocytostaining with trophoblast marker antibodies of the cells cultured in BMP4-supplemented ESF5 medium for 4 d (*right*) or undifferentiated mESCs (*left*). Immunoreactivity of Cdh3 (*upper left*), Cdx2 (*upper right*), CK7 (*lower left*), and Cx31 (*lower right*) was visualized with AlexaFluor-488-conjugated secondary antibodies (*green*). Nuclei were stained with DAPI (*blue*). *Scale bars* were 50 μm. (*B*) RT–PCR analysis of the expression of placental markers in differentiated mESCs. (*C*) Flow cytometric analysis of mESCs (*blue*) and differentiated cells at eight culture days (*red*). (*D*) Immunocytostaining with anti-Cdh3 antibodies of the cells cultured in BMP4-supplemented ESF5 medium for 8 d. *Arrows* indicate the nuclei of hyperploid cells. *Scale bars* were 50 μm. (*E*) Flow cytometric analysis of mESCs (*blue*) and differentiated cells at eight culture days (*red*). The DNA contents were visualized with propidium iodide. Diploid (2N), tetraploid (4N), and octaploid (8N) DNA contents are indicated in the DNA content graph.

differentiated into trophoblast-like cells under these conditions (data not shown).

We examined the effect of extracellular matrix on the differentiation into trophoblast cells from mESCs. We found that the cells steadily proliferated and expressed CK7 when mESC were cultured with BMP4 in ESF5 on fibronectin or laminin (Fig. 3). In contrast, the cells died and failed to express CK7 on type I collagen or PDL. These results indicate that fibronectin and laminin promoted mESC differentiation into trophoblast lineages.

The effect of serum and LIF on mESC differentiation into trophoblast cells. Although it is generally accepted that few mESCs differentiate into trophoblast lineages, we successfully directed differentiation of several mESC lines into trophoblast cells. We presume that this phenotype stems from our use of serum-free culture conditions. To elucidate the effects of serum on mESC differentiation into trophoblast, we compared trophoblast marker expression in the cells cultured with BMP4 in ESF5 medium in the presence or absence of 10% FCS. The addition of FCS decreased BMP4-induced expression of trophoblast transcription factors (Fig. 4*A*) and Cdh3 protein expression (Fig. 4*B*). These results indicate that FCS inhibited the differentiation of mESCs into trophoblast. Another candidate inhibitor of mESC differentiation into trophoblast is LIF, which is known to maintain mESCs in the undifferentiated state (Smith et al. 1988; Williams et al. 1988). To elucidate the effects of LIF on trophoblast differentiation, we examined trophoblast gene expression in cells cultured with BMP4 in the presence or absence of 10 ng/

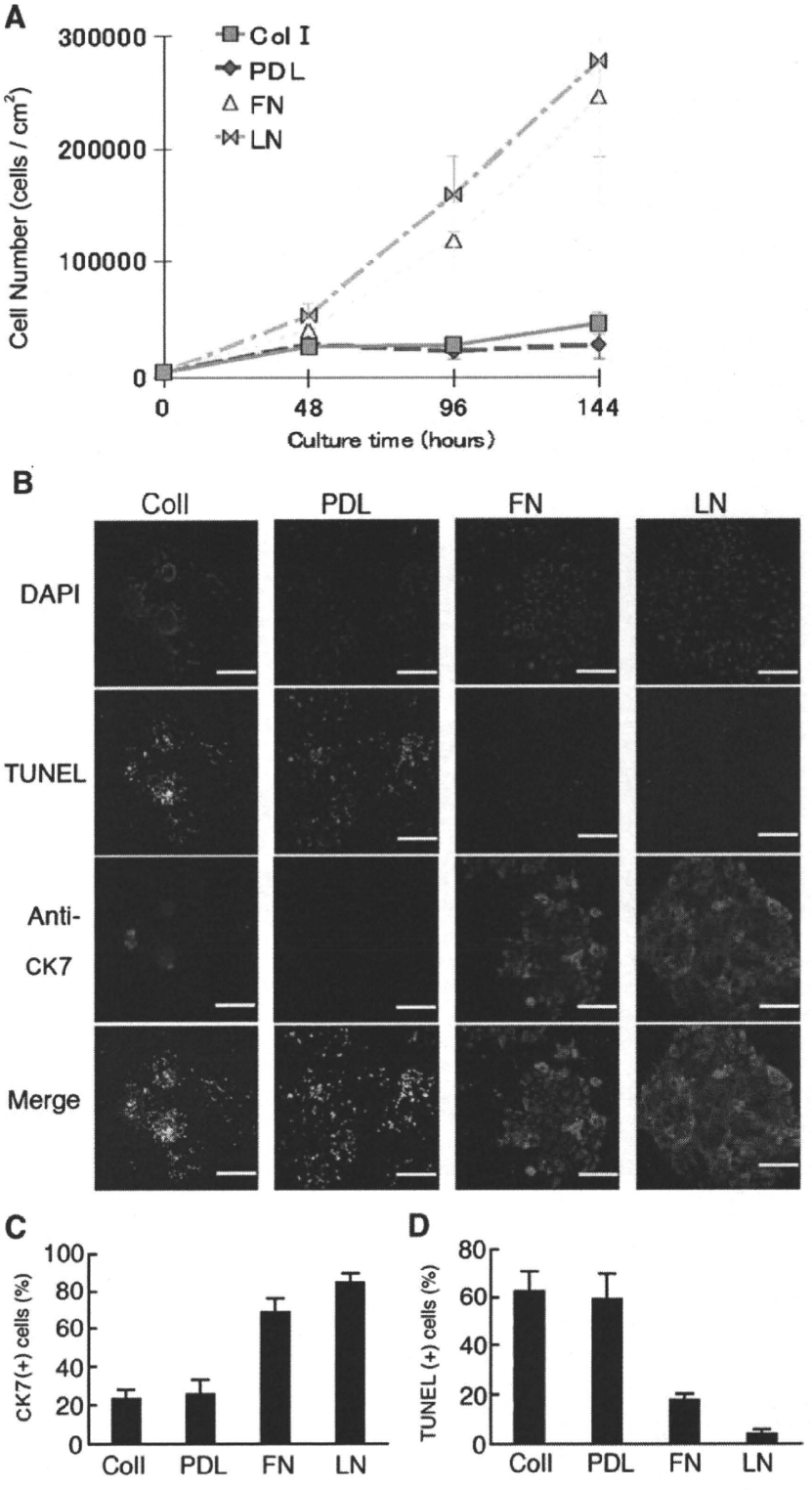


Figure 3. The effect of ECM components on the differentiation into trophoblast from mESCs. (*A*) Proliferation of differentiating mESCs on various ECM components. mESCs were seeded in a 24-well dish at 5×10^3 cells per well on each ECM component in ESF5 medium supplemented with BMP4. Cells were counted every 48 h. The values are the mean ± SEM (n=3). (*B*) Immunocytostaining and TUNEL assay of the differentiated mESCs cultured for 4 d in BMP4-supplemented medium. As trophoblast markers, CK7 was detected with specific antibodies (*red*). TUNEL assay (Chemicon) was performed according to the manufacturers' direction and labeled fixed cells with fluorescence (*green*). Nuclei were stained with DAPI (*blue*). *Scale bars* are 50 μm. (*C*) Percentages of CK7-positive cells. Percentages are calculated from the observation of more than 500 cells for each sample. The values are the mean + SEM (n=3). (*D*) Percentages of TUNEL-positive cells. Percentages are calculated from the observation of more than 500 cells for each sample. The values are the mean + SEM (n=3).

ml of LIF in ESF5. Addition of LIF decreased the BMP4-induced expression of trophoblast-specific transcription factors (Fig. 4*C*) and Cdh3 protein expression (Fig. 4*D*) and enhanced the expression of undifferentiated pluripotent stem cell markers, Nanog and SSEA1 (Fig. 4*E*). These results suggested that LIF inhibited differentiation of mESCs into trophoblast and confirmed that BMP4 in conjunction with LIF functions to maintain the undifferentiated state of mESCs.

Involvement of BMP–Smad pathway in mESC differentiation into trophoblast. To examine whether BMP4 itself may promote the differentiation of mESCs into trophoblast, we

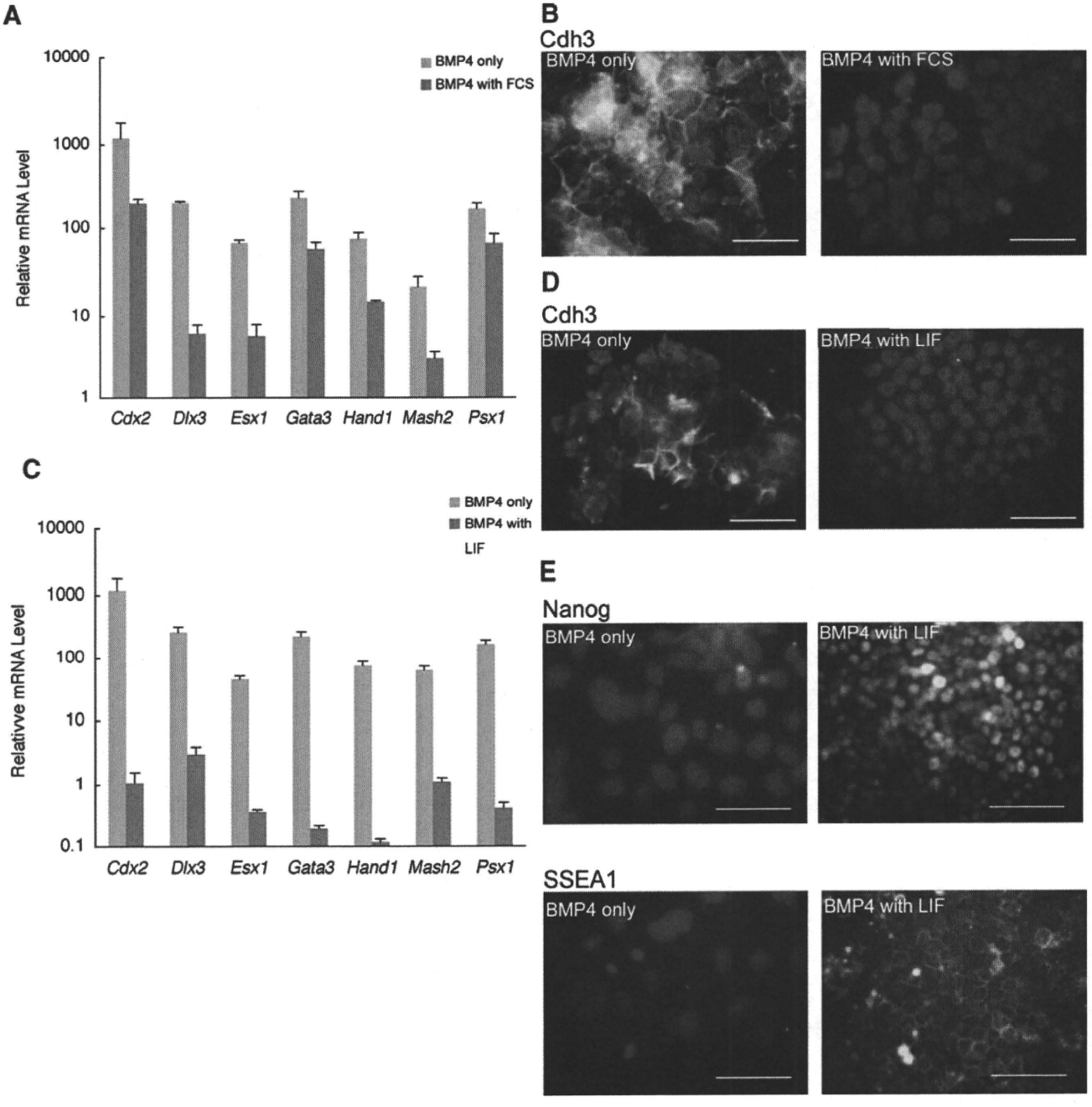


Figure 4. Effect of FCS and LIF on the differentiation into trophoblast. The effect of FCS: the cells were cultured in BMP4-supplemented ESF5 medium with 10% FCS (indicating as "BMP4 with FCS") or without FCS (indicating as "BMP4 only") for 4 d. (*A*) Quantitative real-time RT–PCR analysis of the expression of trophoblast-specific transcription factors. The gene expressions were normalized by the amount of *Gapdh*. The values are the mean ± SEM (n=4). (*B*) Immunocytochemistry with Cdh3 antibodies. Immunopositive reaction of Cdh3 antibody was visualized with AlexaFluor-488-conjugated secondary antibodies (*green*). Nuclei were stained with DAPI (*blue*). *Scale bars* are 50 μm. The effect of LIF: the cells were cultured in BMP4-supplemented ESF5 medium with 10 ng/ml of LIF (indicating as "BMP4 with LIF") or without LIF (indicating as "BMP4 only") for 4 d. (*C*) Quantitative real-time RT–PCR analysis of the expression of trophoblast-specific transcription factors. (*D*) Immunocytochemistry with Cdh3 antibodies. (*E*) Immunocytochemistry with anti-Nanog or anti-SSEA1 antibodies. Immunopositive reaction of anti-Nanog or anti-SSEA1 antibody was visualized with AlexaFluor-488-conjugated secondary antibodies (*green*).

tested the effects of a BMP antagonist, Noggin (300 ng/ml), on the response of mESCs to BMP4. Addition of Noggin decreased the BMP4-induced expression of trophoblast transcription factors (Fig. 5*A*) as well as Cdh3 (Fig. 5*B*) in the differentiated cells. This result indicated that BMP4 itself promoted the mESC differentiation into trophoblast. Next, we examined whether the BMP–Smad pathway was involved in the BMP4-induced differentiation of

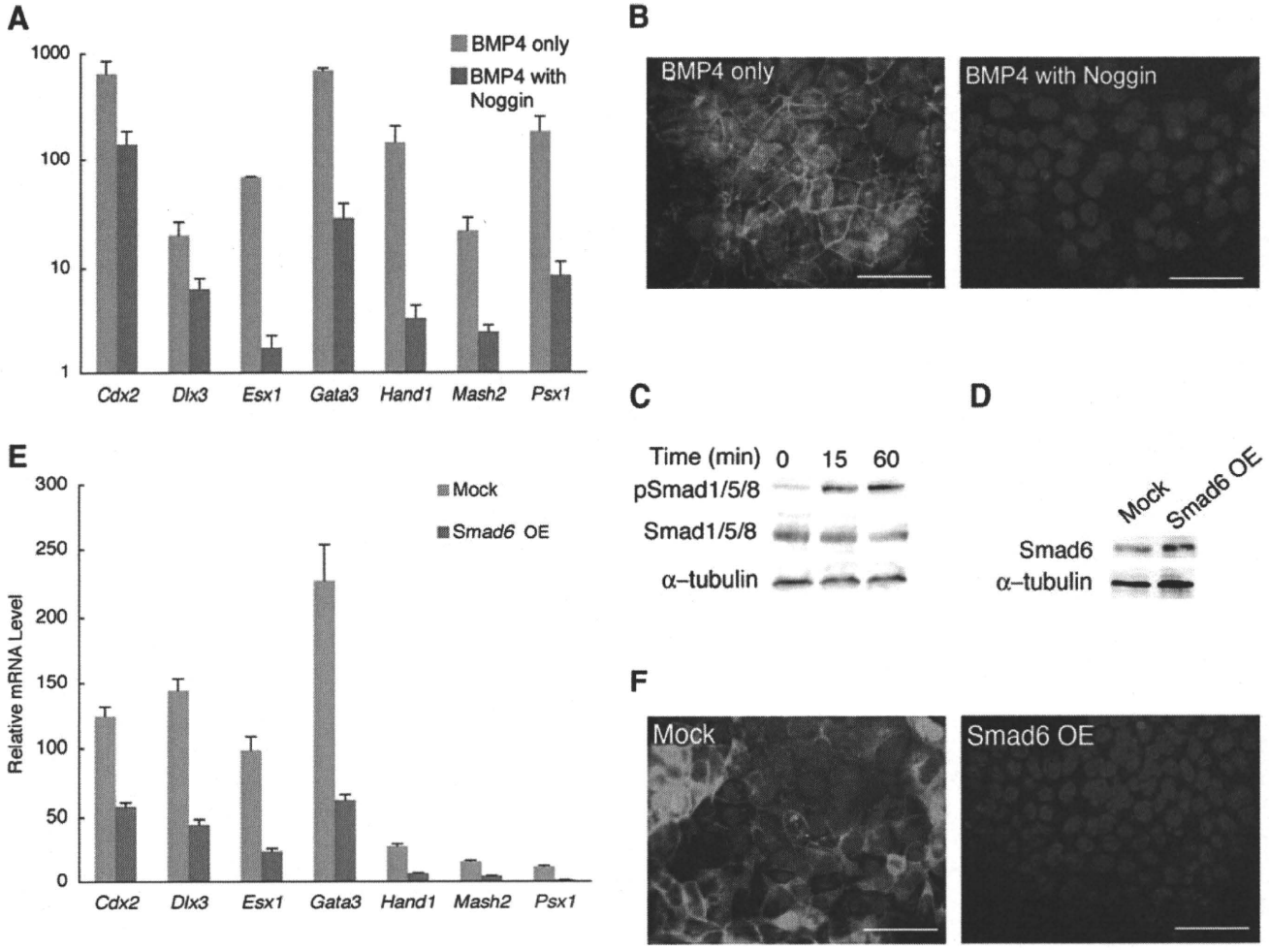


Figure 5. The effects of Noggin, inhibitory Smad6 on the BMP4-induced differentiation of mESCs into trophoblast. The effect of Noggin: the cells were cultured in BMP4-supplemented ESF5 medium with 300 ng/ml of Noggin (indicating as "BMP4 with Noggin") or without Noggin (indicating as "BMP4 only") for 4 d. (*A*) Quantitative real-time RT–PCR analysis of the BMP4-induced expression of trophoblast-specific transcription factors. These gene expressions were normalized by the amount of *Gapdh*. The values are the mean ± SEM (n=4). (*B*) Immunocytostaining with Cdh3 antibodies. Immunoreactivity of Cdh3 antibody was visualized with AlexaFluor-488-conjugated secondary antibodies (*green*). Nuclei were stained with DAPI (*blue*). *Scale bars* were 50 μm. The effect of inhibitory Smad; (*C*) protein samples were lysed from the mESCs stimulated by 100 ng/ml of BMP4 for 0, 15, and 60 min. Phosphorylation level of Smad1/5/8 in mESCs was analyzed by Western blotting using antibodies to phospho Smad1/5/8 proteins or total Smad1/5/8 proteins. Alpha-tubulin was used as the loading control. (*D*) Protein samples were lysed from mESCs transfected with Smad6 plasmids (indicating as "Smad6 OE") or mock plasmids (indicating as "Mock") for 48 h. Protein content of Smad6 in mESCs was analyzed by Western blotting using antibodies to Smad6 proteins. Alpha-tubulin was used as the loading control. (*E*) The cells transfected with Smad6-expressing vectors (indicating as "Smad6 OE") or mock vectors (indicating as "Mock") were cultured in BMP4-supplemented ESF5 medium for 72 h. Quantitative real-time RT–PCR analysis of the BMP4-induced expression of trophoblast-specific transcription factors. (*F*) Immunocytostaining with Cdh3 antibodies.

mESCs into trophoblast. Western blotting analysis showed that the activation by phosphorylation of Smad1/5/8 was observed in cells treated with BMP4 (Fig. 5*C*). Furthermore, the overexpression of Smad6 (Fig. 5*D*), which inhibits the transcriptional activity of Smad proteins, decreased the BMP4-induced expression of trophoblast transcription factors (Fig. 5*E*) as well as Cdh3 (Fig. 5*F*) in the differentiated cells. These results indicated that BMP4-stimulated differentiation of mESCs into trophoblast is mediated through by the Smad pathway.

Identification of Cdx2 as a critical and direct target of BMP–Smad pathway in mESC differentiation into trophoblast. Next, we searched for a crucial gene involved in trophoblast differentiation induced by BMP4. Previous studies have demonstrated that *Cdx2* regulates the induction of trophoblast from mESCs (Niwa et al. 2005; Tolkunova et al. 2006). Thus, we hypothesized that *Cdx2* could be a crucial gene involved in trophoblast differentiation by BMP4. We generated *Cdx2*-knockdown mESCs carrying an shRNA plasmid against *Cdx2* with a puromycin-

resistance gene. The expression of *Esx1* and *Psx1* (Fig. 6*A*) and the expression of Cdh3 protein were decreased in the *Cdx2*-knockdown mESCs cultured with BMP4 in ESF5 (Fig. 6*B*). These results suggested that *Cdx2* was crucial for the BMP4-induced differentiation of mESCs into trophoblast through the regulation of the expression of *Esx1* and *Psx1*.

Finally, we explored the possibility that BMP–Smad pathway could directly regulate the *Cdx2* transcription. We first searched for the putative Smad1/5/8-binding sequence (GCCG) in the noncoding conserved sequences in the genomic region of *Cdx2* using the Vista comparative genomics tool (Frazer et al. 2004; Danno et al. 2008). We found a set of putative Smad1-binding sequences (GCCG) highly conserved among mammals in intron 1 of the *Cdx2* gene, designated as CICS1 (Fig. 7*A*). We cloned a 350-bp sequence containing CICS1 and inserted it into the pGL4.23 luciferase reporter plasmid. To confirm that this sequence has enhancer activity in response to BMP4, a luciferase reporter assay was performed in mESCs transfected with the reporter plasmid or empty plasmid. Transcriptional activity was increased in a BMP4 dose-dependent manner in transfected mESCs, whereas Noggin (300 ng/ml) decreased BMP4-induced transcriptional activity (Fig. 7*B*). These results indicate that CICS1 has BMP4-dependent transcriptional activity. To examine whether Smad proteins can bind to CICS1, we performed EMSA. EMSA revealed that the Smad proteins were bound to CICS1 in vitro (Fig. 7*C*). To confirm whether endogenous Smad proteins were bound to CICS1 in the BMP4-induced trophoblast cells, we performed ChIP assays (Fig. 7*D*, *E*). The CICS1 sequence was specifically contained in the DNA–protein complex immunoprecipitated by the anti-pSmad1 antibodies. These results demonstrate specific binding of the Smad1 proteins to CICS1 in BMP4-treated mESCs. Based on the experimental results obtained here, we conclude that *Cdx2* transcription is directly regulated by the BMP–Smad pathway during BMP4-induced mESC differentiation.

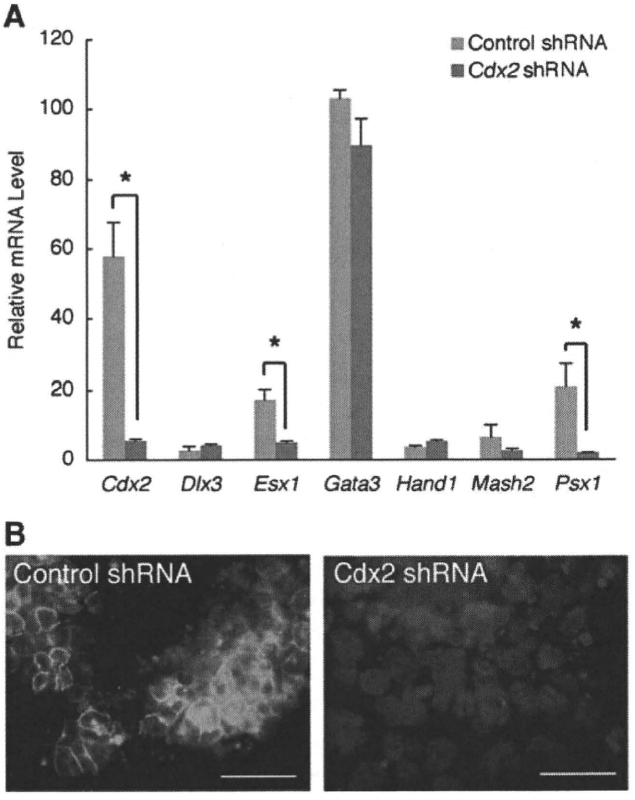


Figure 6. The effects of shRNA against *Cdx2* on the BMP4-induced differentiation of mESCs into trophoblast. The cells carrying shRNA-expressing vectors against *Cdx2* (indicating as "*Cdx2* shRNA") or control shRNA-expressing vectors (indicating as "Control shRNA") were cultured in BMP4-supplemented ESF5 medium for 48 h. (*A*) Quantitative real-time RT–PCR analysis of the BMP4-induced expression of trophoblast-specific transcription factors. These gene expressions were normalized by the amount of *Gapdh*. The values are the mean ± SEM (n=4). *Asterisks* indicate $p<0.05$ by Student's t test. (*B*) Immunocytostaining with Cdh3 antibodies. Immunoreactivity of Cdh3 antibody was visualized with AlexaFluor-488-conjugated secondary antibodies (*green*). Nuclei were stained with DAPI (*blue*). *Scale bars* were 50 μm.

Discussion

Previous studies reported that isolated ICM could partially differentiate into trophoblast (Handyside 1978; Hogan and Tilly 1978) whereas it was reported that mESCs did not contribute to extraembryonic trophoblast in chimeric embryos (Beddington and Robertson 1989). From these findings, we inferred that mESCs under certain conditions have the potential to differentiate into trophoblast lineages as well as all three embryonic germ layers. Toumadje et al. observed spontaneous expression of the trophoblast marker cytokeratin endo-A (CK8) in D3 mESC embryoid bodies cultured in serum in the absence of LIF (Toumadje et al. 2003). A recent study reported that a small subset of trophoblast-like cells appeared when mESCs were cultured on type IV collagen (Schenke-Layland et al. 2007). Another study reported that the addition of Wnt3a induced a small subset of trophoblast lineages from mESCs (He et al. 2008). These findings suggested that mESCs may have the potential to differentiate into trophoblast lineages and that the modulation of culture conditions regulates mESCs to differentiate into trophoblast in vitro.

To induce trophoblast from mESCs, laminin proved to be a key component. We previously reported that laminin or fibronectin promotes cell differentiation in our defined culture conditions (Hayashi et al. 2007). The results in this study indicated that mESC differentiated into trophoblast

lineages only on fibronectin or laminin. Thus, our findings suggested that laminin regulates mESCs differentiation into trophoblast in the defined culture condition. Previous studies using serum or serum replacement which contain a variety of ECM components most likely associated with an ill-defined cocktail of growth factors. We have suggested that serum hampers the elucidation of the biological mechanisms and gives rise to apparently different conclusions on the ability of ESCs to differentiate into trophoblast.

HESCs have been reported to express trophoblast markers after treatment with BMP4 in vitro (Xu et al. 2002). Previous studies reported that mouse epiblast stem cells or a subpopulation of epiblast-like mESCs express trophoblast markers in response to BMP4 (Brons et al. 2007; Hayashi et al. 2008). Mouse epiblast stem cells resemble hESCs more closely than mESCs (Brons et al. 2007; Tesar et al. 2007; Hayashi et al. 2008). We have previously found polarized cell morphology and the upregulation of *Fgf5* expression and downregulation of some self-renewal marker gene expression in mESCs cultured on laminin or fibronectin (Hayashi et al. 2007). These are characteristics of epiblast cells. In this study, cells were treated with BMP4 on laminin in our defined culture conditions. Our findings suggest that the differentiation via an epiblast-like state is important for mESC differentiation into trophoblast, and BMP4 can induce from both human and mouse pluripotent stem cells to differentiate into trophoblast.

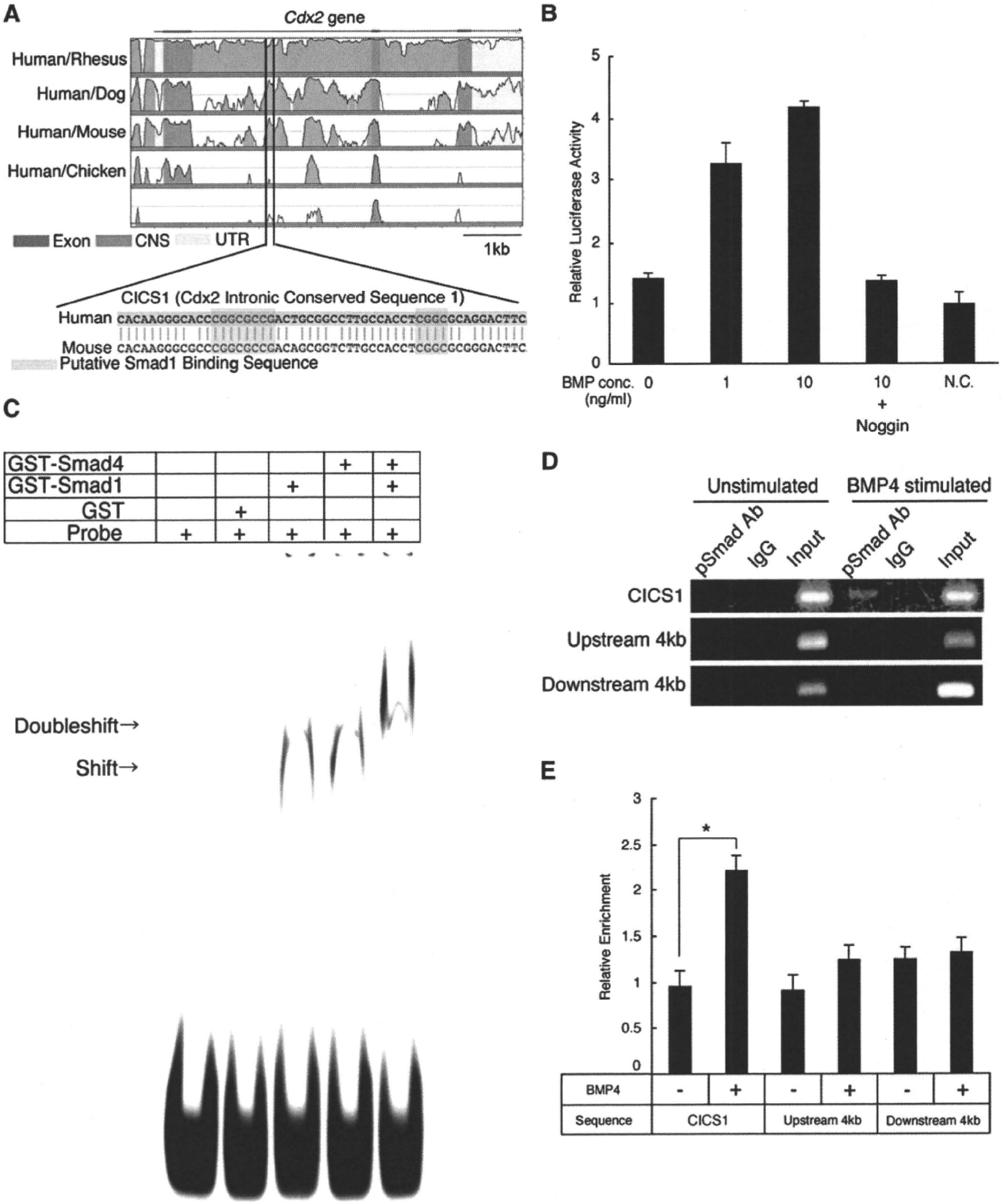
As described above, BMP4 is a key component promoting trophoblast differentiation from both mESCs and hESCs (Xu et al. 2002). These findings contradict previous reports in which BMP4 supported cell self-renewal. However, that was observed with BMP4 in conjunction with LIF (Ying et al. 2003; Qi et al. 2004). BMP4 supports mESC self-renewal by inhibiting neural differentiation which is not inhibited by LIF. We have shown in this study that LIF inhibits trophoblast differentiation by BMP4. These results suggest that LIF and BMP4 reciprocally inhibit differentiation of mESCs into neural or trophoblast lineages and lead to maintain self-renewal of mESCs. Previous studies have also reported that BMP4 is also thought to be involved in mesoderm differentiation from ESCs (Johansson and Wiles 1995; Wiles and Johansson 1999; Nostro et al. 2008; Sumi et al. 2008). These studies employed other signal activation by cytokines or tight cell–cell attachments. Our results suggest that BMP4 induces differentiation into trophoblast in defined conditions in the absence of other activating signals and that differentiation was perturbed by the addition of serum. Thus, BMP4 may function as trophoblast inducer when it acts alone and as mesoderm inducer when it is coordinated with other as yet unidentified signals.

Figure 7. Direct activation of *Cdx2* by BMP–Smad pathway. (*A*) Conserved sequence domain in the genomic region of *Cdx2* gene in vertebrates. Colored peaks (*purple*, coding; *pink*, non-coding) indicate the regions of at least 100 bp and 60% homology. Magnified sequence of CICS1 compared between human and mouse is shown in the bottom part. Putative Smad1 protein binding sequences (GCCG or CGGC) are enclosed in *green box*. (*B*) Dual-luciferase assay of CICS1 transcriptional activity by BMP4. The cells carrying the reporter vectors constructed with the sequence containing CICS1 were cultured in BMP4-supplemented ESF5 medium for 48 h. *N.C.* indicates empty vectors. The values are the mean ± SEM (n=4). (*C*) EMSA of CICS1 bound by Smad1 and the cofactor, Smad4. (*D*) ChIP assays of CICS1 bound by pSmad1. Genomic fragments of CICS1, 4 kb upstream or 4 kb downstream regions bound by pSmad1/5/8 protein, were immunoprecipitated with anti-pSmad1/5/8 antibody and were analyzed by PCR. (*E*) Quantitative graphs of ChIP assays calculated from the ratio of the amount of bound anti-pSmad1 antibodies to the amount of control IgG. The values are the mean ± SEM (n=4). * indicates $p<0.05$ by Student's t test.

We demonstrate that the BMP4–Smad pathway is involved in mESC differentiation into trophoblast. Components of BMP–Smad pathway are expressed in extraembryonic tissues and affect their development (Lechleider et al. 2001; Tremblay et al. 2001). Previous study reported that Nanog protein, which maintains mESC self-renewal, binds to Smad1 protein and inhibits the transcriptional activity of Smad1 protein in mESCs (Suzuki et al. 2006). Our results reveal that the expression of Nanog protein was absent from cells cultured with BMP4 in ESF5 but expressed in cells in the presence of LIF. Thus, *Nanog* expression induced by LIF might inhibit mESC differentiation into trophoblast.

Our knockdown experiment showed that the cells which have reduced *Cdx2* expression did not express *Esx1*, *Psx1*, and *Cdh3*. *Cdx2* is essential for trophoblast lineages development in vivo (Niwa et al. 2005; Strumpf et al. 2005). Thus, our data imply that these cells may differentiate into cell lineages other than trophoblast. Although the characterization of these cell lineages is interesting for further experiment, they may be induced by BMP4 and not express *Cdx2*.

We also demonstrate that BMP–Smad pathway activates the *Cdx2* expression during the differentiation of mESCs into trophoblast and that the *Cdx2* expression is directly regulated by the BMP–Smad pathway through binding of Smad1 protein with evolutionary conserved intronic enhancer in *Cdx2* gene. Correlation between BMP–Smad pathway and *CDX2* expression is reported in human gastric cancer cell lines (Barros et al. 2008). Together with the fact that BMP4 increases the trophoblast marker gene expression in hESCs (Xu et al. 2002), we conclude that common developmental and molecular mechanisms are involved in the differentiation of mammalian pluripotent stem cells into trophoblast. We believe that our methods and findings in this study provide a better understanding of the molecular mechanisms that regulate the differentiation of pluripotent


A
Cdx2 gene
Human/Rhesus
Human/Dog
Human/Mouse
Human/Chicken
Exon
CNS
UTR
1kb
CICS1 (Cdx2 Intronic Conserved Sequence 1)
Human CACAAGGGCACCCGGCGCCGACTGCGGCCTTGCCACCTCGGCGCAGGACTTC
Mouse CACAAGGCGCCCCGGCGCCGACAGCGGTCTTGCCACCTCGGCGCGGGACTTC
Putative Smad1 Binding Sequence
B
Relative Luciferase Activity
BMP conc. (ng/ml)
0
1
10
10 + Noggin
N.C.
C
GST-Smad4
GST-Smad1
GST
Probe
Doubleshift→
Shift→
D
Unstimulated
BMP4 stimulated
pSmad Ab
IgG
Input
CICS1
Upstream 4kb
Downstream 4kb
E
Relative Enrichment
BMP4
Sequence
CICS1
Upstream 4kb
Downstream 4kb

stem cells into trophoblast and placenta during early mammalian development.

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Growth factor-defined culture medium for human mesenchymal stem cells

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ABSTRACT Human bone marrow-derived mesenchymal stem cells (hMSCs) are potential cellular sources of therapeutic stem cells as they have the ability to proliferate and differentiate into a wide array of mesenchymal cell types such as osteoblasts, chondroblasts and adipocytes. hMSCs have been used clinically to treat patients with graft *vs.* host disease, osteogenesis imperfect, or alveolar cleft, suggesting that transplantation of hMSCs is comparatively safe as a stem cell-based therapy. However, conventional culture medium for hMSCs contains fetal bovine serum (FBS). In the present study, we developed a growth factor-defined, serum-free medium for culturing hMSCs. Under these conditions, TGF-β1 promoted proliferation of hMSCs. The expanded hMSC population expressed the human pluripotency markers SSEA-3, -4, *NANOG*, *OCT3/4* and *SOX2*. Furthermore, double positive cells for SSEA-3 and a mesenchymal cell marker, CD105, were detected in the population. The potential to differentiate into osteoblasts and adipocytes was confirmed. This work provides a useful tool to understand the basic biological properties of hMSCs in culture.

KEY WORDS: *mesenchymal stem cell, serum-free culture, TGF-β1*

Introduction

Bone marrow-derived cells can differentiate into osteoblasts *in vitro* and *in vivo* (Friedenstein *et al.*, 1966) and thus are considered a useful source of stem cells for bone regeneration. Recently, many studies have reported that human bone marrow contains a distinct cell fraction referred to as multipotent mesenchymal stem cells (hMSCs) which can give rise to a wide array of mesenchymal cell types, including bone, fat, and cartilage"(Pittenger *et al.*, 1999). However, hMSCs can differentiate along some ectodermal and endodermal cell lineages such as neuronal cells and liver cells (Pittenger *et al.*, 1999; Dezawa *et al.*, 2004; Dezawa *et al.*, 2005). Further, a recent study reported that hMSCs have the ability to generate the multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). It has been estimated that hMSCs comprise about 0.001 to 0.01% of total bone marrow mononuclear cells (Pittenger *et al.*, 1999). For use in cell-based therapies, hMSC populations require extensive *in vitro* expansion to obtain sufficient numbers. The conventional culture medium for hMSCs is composed of a basal nutrient medium supplemented with fetal bovine serum (FBS) (Haynesworth *et al.*, 1992; Lennon DP, 1996). Although these traditional culture conditions provide robust undifferentiated hMSC expansion, the ill-defined components of FBS is undesirable for clinical applications and also hampers analysis of the cell biological mechanisms that control cell behavior.

Abbreviations used in this paper: hES cells, human embryonic stem cells; hMSCs, human mesenchymal stem cells.

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We and others previously described serum-free media consisting of minimum essential components suitable to propagate and accurately analyze the characteristics of differentiated cells (Hayashi and Sato, 1976; Furue and Saito, 1998; Sato *et al.*, 2002; Furue *et al.*, 2005; Furue *et al.*, 2008; Hayashi *et al.*, 2010). One of these media, hESF9, supports the serial cultivation of undifferentiated human embryonic stem (hES) cells in the absence of feeder cells and thus provides an experimental system for elucidating cellular responses to specific environmental stimuli (Furue *et al.*, 2008; Na *et al.*, 2010). For example, either FGF-2 or heparin promotes proliferation of hES cells in a concentration-dependent manner although these effects were not detected under conventional culture conditions. Thus, a defined serum-free medium consisting of minimum essential components should be useful in elucidating hES/iPS cell responses to specific cues that control self-renewal, differentiation, and lineage selection (Furue *et al.*, 2010).

Because hMSCs have multipotent properties similar to hES cells, we speculated that hMSCs should be able to grow in similar culture conditions as hES cells. In the present study, we demonstrated that addition of TGF-β1 to the defined serum-free medium for hES cells supports the robust proliferation of hMSCs. The hMSC population expanded in the absence of serum expressed the mesenchymal cell markers CD44, CD73, CD90, and CD105. Further, they expressed human pluripotency surface markers, SSEA-3, -4, TRA-2-54, and also the transcription factors of *NANOG*, *OCT3/4*, and *SOX2*. We show that the serum-free expanded hMSCs can differentiate into osteoblasts and adipocytes. This work sets the stage for serum-free hMSC cell culture and thereby provides a useful tool to understand the basic biological characteristics of hMSCs.

Results

In this study we used a human bone marrow-derived hMSC line designated UE7T-13 (JCRB 1154). The life span of these cells was prolonged by infecting them with a retrovirus containing human papillomavirus E7 and telomerase reverse transcriptase (hTERT) cDNAs (Mori *et al.*, 2005; Shimomura *et al.*, 2007; Ishii *et al.*, 2008; Takeuchi *et al.*, 2007). We first tested the ability of hESF9 medium, which we had developed for use with hES cells, to support the growth of UE7T-13 cells. The cells were harvested using trypsin/EDTA, from cultures in conventional medium containing 10% FBS (POWERDBY10) and transferred to 0.1% gelatin-coated dishes in hESF9 medium. However, UE7T-13 cell growth was quite slow. We then investigated the effects of various growth factors on proliferation of the cells. UE7T-13 cells were seeded on 0.1% gelatin in hESF9 in the absence of FGF-2 and heparin (hESF9(-/-)), containing increasing concentrations of FGF-1, FGF-2, TGF-β1, activin A, or leukemia inhibitory factor (LIF) (Fig. 1). Both FGF-1 and FGF-2 promoted UE7T-13 proliferation in a dose-dependent manner, and the greatest effect was seen at 10 ng/ml FGF-2. Neither LIF nor activin A affected on UE7T-13 cell proliferation, but TGF-β1 slightly stimulated UE7T-13 proliferation. Next all five factors (FGF-1, FGF-2, TGF-β1, activin A, and LIF) or four factors with increasing concentrations of heparin were added to UE7T-13 cultures (Fig. 2). When either FGF-2 or TGF-β1 was withdrawn from the cultures, the cell numbers decreased significantly. Heparin promoted cell proliferation in a dose-dependent manner. This result suggested that addition of FGF-2 and TGF-β1 to hESF9(-/-) medium, is critical for UE7T-13 proliferation, and heparin also enhanced cell growth. hESF9 medium supplemented with TGF-β1 was designated hESF10.

L-ascorbic acid-2-phosphate (Asc 2-P) in hESF9 medium supported hES cells. However, it is known to promote hMSC cell differentiation into osteoblasts. Therefore, we examined whether the presence of Asc 2-P in hESF10 medium promoted osteoblastic differentiation of UE7T-13 cells. We analyzed the expression of *bone sialoprotein* (*IBSP*), *osteocalcin* (*BGP*), *osteonectin* (*SPOCK2*), and *osteopontin* (*SPP1*) in UE7T-13 cell cultured in hESF10 with or without Asc 2-P and in conventional medium (Fig. 2). These osteoblast genes were expressed at significantly lower levels in cells cultured in the serum-free media than in those cultured in the conventional medium. These results suggest that the serum-free medium is suitable for hMSC maintenance. *IBSP* gene expression was higher in the cells cultured in the

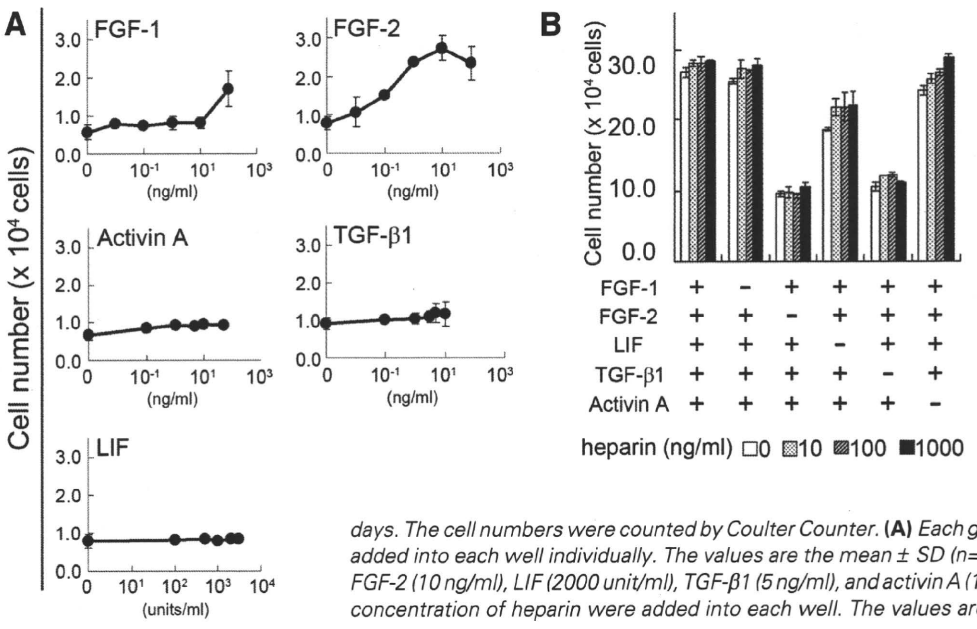


Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. *After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-) at 1 x10⁴ cells per well and cultured for 6 days. The cell numbers were counted by Coulter Counter.* **(A)** *Each growth factor at indicated concentration was added into each well individually. The values are the mean ± SD (n=3).* **(B)** *All five factors of FGF-1 (100 ng/ml), FGF-2 (10 ng/ml), LIF (2000 unit/ml), TGF-β1 (5 ng/ml), and activin A (10 ng/ml) or four factors of them with varying concentration of heparin were added into each well. The values are the mean ± SD (n=3).*

presence of Asc 2-P. These results suggested that Asc 2-P promoted differentiation of UE7T-13 cells into osteoblasts. We removed Asc 2-P from hESF10 medium for hMSCs, and designated the new formulation D-hESF10.

To confirm the characteristics of UE7T-13 cells expanded in the absence of serum, we performed flow cytometry with antibodies to markers for hMSCs and pluripotent cells (Fig. 3A). Cells grown in D-hESF10 medium were positive for CD44, CD73, CD90, CD105, and TRA-2-54 (tissue non-specific alkaline phosphatase antibody), but negative for CD45 (a marker of all hematopoietic cells) and CD56 (a neural cell adhesion molecule). We further stained the cells with antibodies to CD105 and SSEA-3 (Fig. 3B). The immunocytochemical analysis showed that SSEA-3$^+$/CD105$^+$ double positive cells were present in the UE7T-13 population grown in D-hESF10 although cells positive for either CD105 or SSEA-3 were also detected in the population. The cell growth rate in D-hESF10 was comparable to that in conventional culture conditions (Fig. 4).

We subsequently examined the properties of UE7T-13 cells serially passaged in D-hESF10 medium. The morphology of serum-free expanded UE7T-13 cell populations was comparably small, spindle-shaped cells compared with that in conventional medium (Fig. 5A). The expression of hMSC and hES cell pluripotency markers were determined by real-time PCR analysis (Fig. 5B) in UE7T-13 cells cultured for 4 passages in D-hESF10 medium. The expression of hMSC markers, *CD105*, *THY1*, and *integrin β1* (*ITGB1*), and the hES cell pluripotency markers, *OCT3/4* (*POU5F1*) and *NANOG* were similar in the cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. *SOX2* expression was significantly higher in cells cultured in D-hESF10 compared with cells cultured in conventional culture conditions. On the other hand, the expression levels of *IBSP*, *BGP*, *SPOCK2*, and *SPP1* were significantly lower in cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. These results suggest that serum-free expanded UE7T-13 cells retain an undifferentiated phenotype.

We determined the differentiation capacity of the serum-free expanded UE7T-13 cells. After the UE7T-13 cells were cultured in D-hESF10 for 7 passages, the cells were cultured in medium designed to induce differentiation into osteoblasts or adipocytes (Fig. 6). Culturing in osteoblastic differentiation medium induced the formation of nodules that stained positive with Alizarin red, suggesting that the cells had the potential to differentiate into osteoblasts. When the cells were cultured in

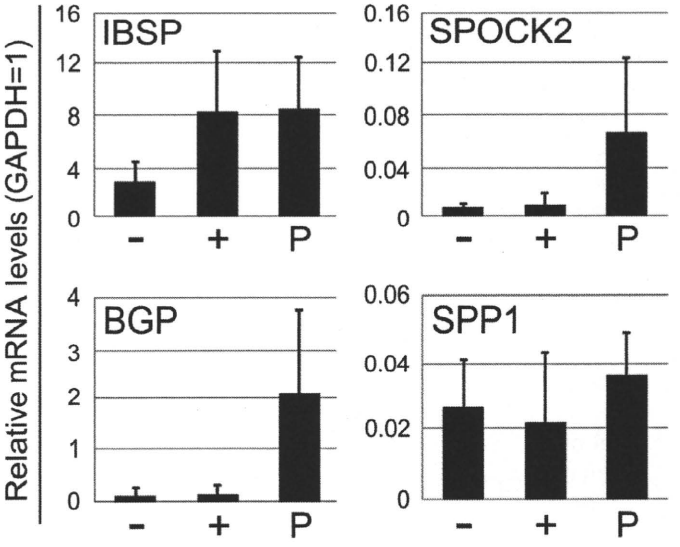


Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. *The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression was normalized by the amount of* GAPDH. *The values are the mean ± SD (n=3).*

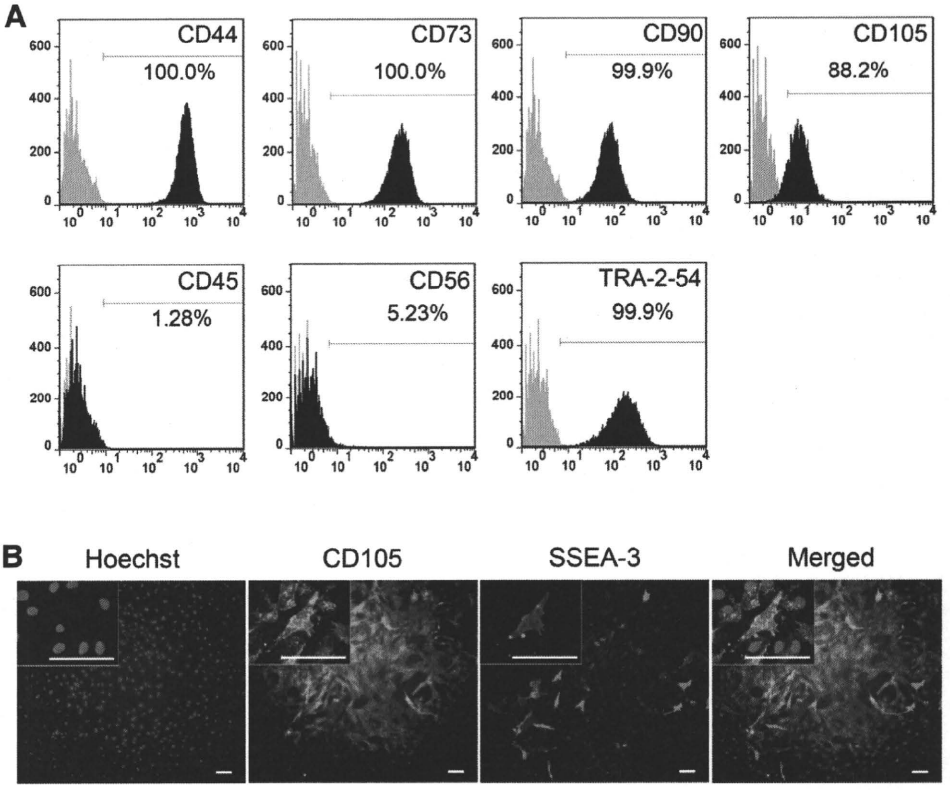
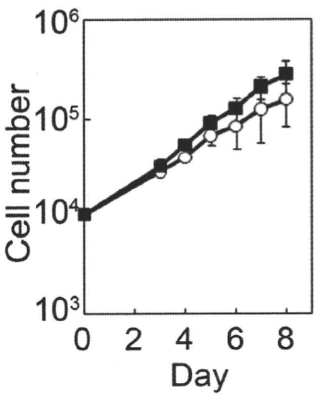


Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) *Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells.* **(B)** *Immunocytochemical analysis of SSEA-3 and CD105 expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days. Scale bars, 100 μm.*



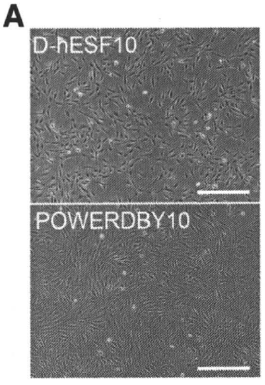


Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. *The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density of $1x10^4$ cells per well. Cell numbers were counted every day. The values are the mean ± SD (n=3).*

adipocytic differentiation medium, Oil red O-positive cells appeared. Taken together these results suggest that the serum-free expanded UE7T-13 cells have maintained the capacity to differentiate into osteoblasts or adipocytes.

Discussion

Developing clinical serum-free media for maintaining and expanding human stem cells is a major research topic in regenerative medicine. Our current results indicate that it is possible to culture hMSCs on gelatin in a defined medium, designated D-hESF10, in which human recombinant insulin, human transferrin, a low concentration of fatty acid-free bovine albumin conjugated with oleic acid, FGF-2, and TGF-β1 are the protein components. The basal medium ESF was developed for mouse ES cells (Furue *et al.*, 2005). For hES cell culturing, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) was removed from ESF but Asc 2-P was added (Furue *et al.*, 2008). For propagating hMSCs, Asc 2-P was removed from the hES cell culture medium because we found that Asc 2-P increased osteoblastic marker expression in hMSCs. These findings indicated that signaling by Asc 2-P in hMSCs is different from that in hES cells.

FGF-2 is a heparin-binding growth factor which stimulates the proliferation of a wide variety of cells. The biological activity of FGF-2 is efficient in the concentration range of 0.1 to 10.0 ng/ml. Addition of FGF-2 has been shown to increase the growth rate and life span of hMSCs from different species (Tsutsumi *et al.*, 2001; Benavente *et al.*, 2003), suggesting that FGF-2 play an important role in self-renewal of hMSCs. In hES cells, FGF-2 is a crucial to maintain the undifferentiated state (Amit *et al.*, 2004; Hoffman and Carpenter, 2005). We previously reported that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hES cells in serum-free without feeders (Furue *et al.*, 2008). In this study, we found that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hMSCs in a serum-free medium. These findings suggest that they share the same signal pathway to support self-renewal. Heparin at 1 mg/ml promoted hMSC cell proliferation, and we previously reported that heparin at 1 mg/ml inhibited hES cell proliferation. Thus the sensitivity to heparin is different between hMSCs and hES cells.

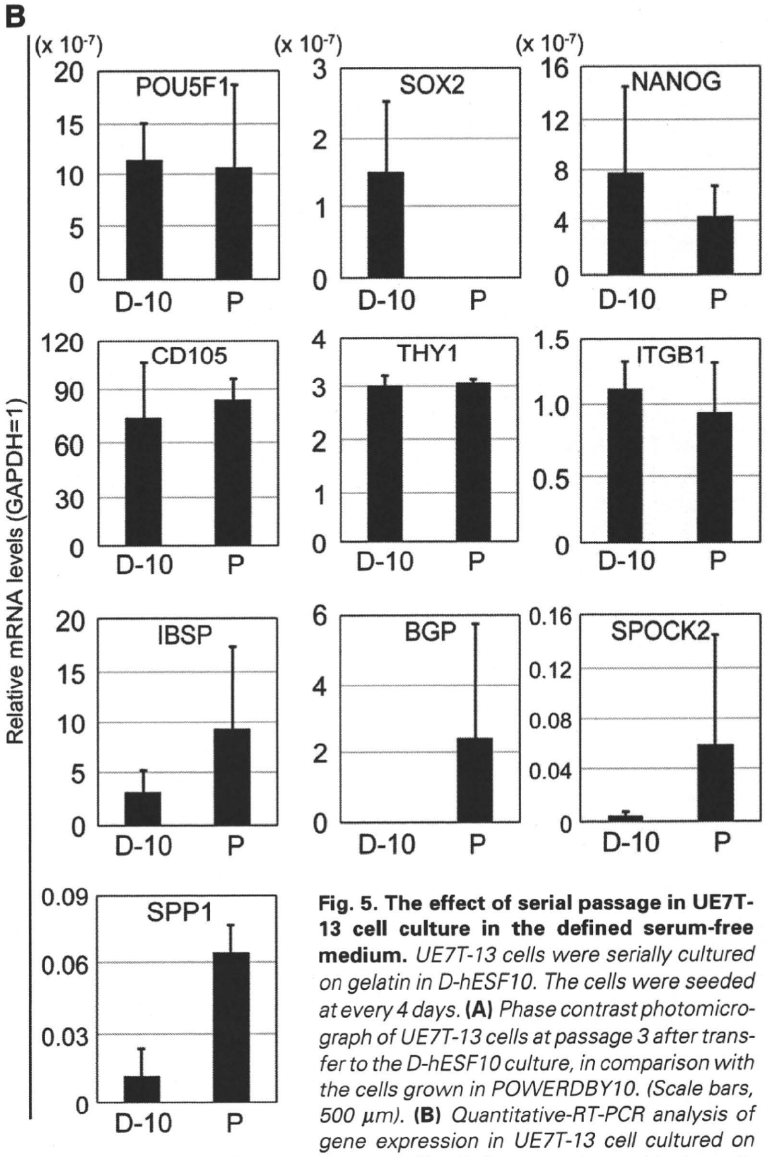


Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. *UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days.* **(A)** *Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture, in comparison with the cells grown in POWERDBY10. (Scale bars, 500 μm).* **(B)** *Quantitative-RT-PCR analysis of gene expression in UE7T-13 cell cultured on gelatin in D-hESF10 at passage 4 (D-10), in comparison with the cells grown in POWERDBY10 (P). The name of each gene is noted in each bar graph. Gene expression was normalized with respect to* GAPDH. *The values are the mean ± SD (n=3).*

The TGF-β1 pathway has been reported to be important in hMSC differentiation into the osteogenic and chondrogenic lineages (Li and Xu, 2005; Kulterer *et al.*, 2007). While we have shown that TGF-β1 alone did not promote cell proliferation of hMSCs, the combination with FGF-2 and heparin enhanced cell proliferation of hMSCs. Chase *et al.* reported the combination of TGF-β1, FGF-2, and PDGF-BB in a commercial serum-free medium for the expansion of hMSCs although the optimal concentrations of these factors were not disclosed. The cell growth rate in D-hESF10 medium was similar with that in the conven-

tional culture conditions suggesting that addition of TGF-β1 and FGF-2 is sufficient to replace serum in supporting hMSC cell growth. A culture medium consisting of the minimum components necessary to support survival and proliferation would be beneficial to understand the characteristics of naïve hMSCs. Therefore, we think that addition of PDGF-BB is not crucial for an hMSC culture medium.

Several studies reported that two distinct cell morphologies are seen in early-passage hMSC cultures: small, spindle-shaped cells that are rapidly self-renewing and large, flat cells that replicate slowly and appear more mature (Mets and Verdonk, 1981; Colter *et al.*, 2001; Sekiya *et al.*, 2002). The morphology of serum-free expanded UE7T-13 cell population contained comparably small, spindle-shaped cells. However, specific undifferentiated markers of hMSCs have not been identified yet (Pochampally *et al.*, 2004). Further, although the cells are cloned, cells within an individual colony are heterogeneous in morphology, growth rates, and efficiency with which they differentiate (Mets and Verdonk, 1981; Bruder *et al.*, 1997; Colter *et al.*, 2001). The International Society for Cellular Therapy (ISCT) has proposed three criteria to define hMSCs (Dominici *et al.*, 2006). hMSC population must be positive at least for several antigens such as CD105, CD73, and CD90, and negative for CD45. CD105 is usually used to identify an hMSC population. Many studies reported that hMSCs also expressed hES cell pluripotency markers, SSEA-3, -4, NANOG, OCT3/4, and alkaline phosphatase (Pochampally *et al.*, 2004; Roubelakis *et al.*, 2007; Battula *et al.*, 2008; Conrad *et al.*, 2008; Pang *et al.*, 2010). We also detected the expression of NANOG, OCT3/4, and SOX2. These findings suggested that hES cell pluripotency markers may be universal stem cell markers in humans. Dezawa's group recently reported that double positive CD105 and SSEA-3 cells have the ability to generate multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). We also confirmed the existence of CD105 and SSEA-3 double positive cells in the hMSC population expanded in D-hESF10. In this study, we confirmed the differentiation potential of hMSCs to generate osteoblasts or adipocytes, but in the future we will examine the ability of hMSCs to generate cells from all three germ layers.

To facilitate the transition of human stem cell biology from basic research to clinical application all the components of maintenance and differentiation media should be publicly disclosed so they can be evaluated by many researchers. A commercial xeno-free serum-free medium for hMSCs was reported recently (Chase *et al.*, 2010). However, the non-disclosure of components is problematic as the medium formulation cannot be usefully modified or improved. Because all the components of D-hESF10 medium are disclosed here, the medium can be modified to study signaling pathways involved in maintaining multipotency and to develop differentiation protocols.

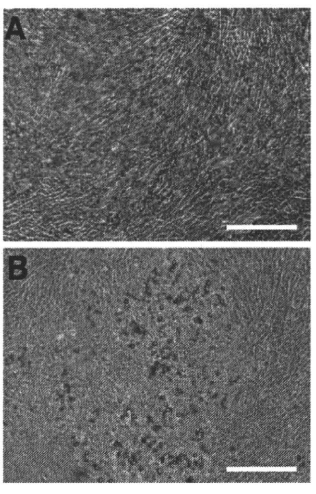


Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. *The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium.* **(A)** *Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were stained with Alizarin Red S (red).* **(B)** *Adipocytic differentiation was induced in adipocytic medium for 24 days. The cells were stained by Oil red O staining (red). Scale bars: 500 μm.*

Materials and Methods

Cell Cultures

An immortalized hMSC line UE7T-13 (Mori *et al.*, 2005) (JCRB 1154, JCRB Cell Bank, Osaka, Japan) was used in this study. Cells were maintained on 100 mm dish (BD Falcon, Oxnard, CA) in POWERDBY10 (MED-SHIROTORI, Tokyo, Japan) that was also used in the experiments as a control medium. The cells were harvested with 0.25% trypsin in 1 mM EDTA-4Na.

Serum-free Cell Culture Media

hESF9 comprises ESF basal medium (Furue *et al.*, 2005) without HEPES supplemented with nine defined factors: Asc 2-P, 6-factors (human recombinant insulin, human transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA)), bovine heparan sulfate sodium salt, and human recombinant FGF-2 (Sigma, St. Louis, MO), as described previously (Furue *et al.*, 2008) (Supplementary Table 1). ESF basal medium without HEPES supplemented with Asc 2-P (hESF-GRO), and ESF basal medium without HEPES and Asc 2-P (hESF-DIF) were purchased by the Cell Science & Technology Institute (CSTI, Sendai, Japan). All other reagents were from Invitrogen (Carlsbad, CA) and Sigma. D-hESF10 medium consists of hESF-DIF medium supplemented with 6-factors, FGF-2, heparin, and TGF-β1 (R&D Systems, Minneapolis, MN). To harvest cells, 0.25% trypsin in 1 mM EDTA-4Na was used and the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). For differentiation into osteoblasts or adipocytes, the cells were cultured according to the instruction by the suppliers (Lonza, Basel, Switzerland). The differentiated cells were stained by Alizarin Red S (Wako Pure Chemical Industries, Osaka, Japan) or Oil Red O (Wako).

Cell proliferation

Before the serum-free experiments, cells grown in POWERDBY10 were incubated by in hESF9 medium without heparin and FGF-2 (hESF9(-/-)) overnight to starve the effect of serum. Cells were replaced at the cell density of $1x10^4$ cells /well on 24-well plate (BD Falcon) coated with 0.1% porcine gelatin solution (Millipore, Billerica, MA) and cultured in hESF9(-/-) medium in the presence of varying growth factors. The cell numbers were counted by Coulter Counter (Beckman Coulter, Hialeah, FL).

Gene expression

A detailed reverse transcription-polymerase chain reaction (RT-PCR) protocol was described previously (Furue, *et al.*, 2005). Total RNA was extracted from hMSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the provider's instructions. Q-RT-PCR was carried out using the TaqMan gene expression Master Mix on in ABI PRISM 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the supplier's instructions (ABI). Specific primers-probe set were listed in Supplementary Table 2. Expression levels were all normalized by the expression level of *GAPDH*. The relative level of each gene in cDNA of undifferentiated hES cells was defined as "1." The KhES-3 cell line was used as a control; the cells were obtained from the Institute for Frontier Medical Science, Kyoto University, and the Review Board of the National Institute of Biomedical Innovation approved this research.

Antigen expression

For *in situ* immunocytochemisty, the cells were immunostained with antibodies, as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, fluorescence images were acquired using by IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, England). Flow cytometry was performed with BD FACS Canto flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, the labeled primary antibodies were used, but the biding of anti-SSEA-3, anti-CD56, and Tra-2-54 antibodies was visualized with RPE-conjugated goat anti-mouse Ig (Dako, Carpinteria, CA) or Alexa Fluor 647 goat anti-rat IgM (Invitrogen). The primary antibodies used are listed in Supplementary Table 3.

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