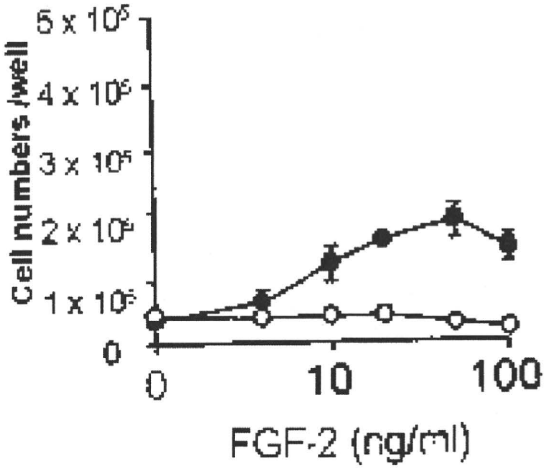


図3　培養条件によるFGF-2の細胞増殖効果の違い
従来の培養条件（□）においては、FGF-2の細胞増殖効果を検出できないが、hESF 9を用いた培養条件（●）においては、FGF-2の細胞増殖効果を検出できた。(Proc. Natl. Acad. Sci. U.S.A. 105, 13409-14, 2008 より)

因子や未知の因子が含まれている。これらに対して、品質の安定性のあるコスト差の少ない合成培地を用いた無血清培養が、望まれている。ヒトES細胞用の無血清培養条件は、市販品を使用したものも含めて十数例が報告されている。筆者は、マウスES細胞をフィーダー細胞なしに未分化性を維持することのできる機能性無血清培養条件ESF 7を開発した。この条件に改良を加え、ヒトES細胞用無血清培地hESF 9を開発した。フィーダー細胞やKSR存在下においては検出できなかった形質が、フィーダーを用いないこの機能性無血清培養条件を用いることにより明らかにできる。ヒトES細胞におけるFGF-2の細胞増殖の影響は、フィーダーあるいはKSR存在下においては検出できなかったが、hESF 9で培養を行うとFGF-2による増殖効果が明らかとなった（図3）。hESF 9を用いることにより、iPS細胞の細胞特性を正確に解析することが可能であり、現在詳細な解析を行っている。

10.5　評価と品質管理

毒性評価などのスクリーニングに用いる細胞の条件として、再現性のある安定した試験が可能なこと、その細胞を十分量安定供給できることなどが重要である。作製されたiPS細胞コレクション、分化細胞コレクションは他の研究機関に配布・分譲する予定である。これらの幹細胞は、体細胞はもとより他の幹細胞にくらべてデリケートで、高品質を維持することは大変難しい。分化しやすく、継代するうちに異なる細胞集団になることも多い。細胞分散法を間違えば、ほとんどの細胞がアポトーシスを起こして死んでしまうこともまれではない。培養維持している間に、細

胞の品質は変化する可能性が高い。標準化されたiPS細胞を他の研究機関に安定的に供給するためには、iPS細胞の品質管理が必要となる。当研究所・厚生労働省細胞バンクにおける20年以上の公的バンクの豊富な経験と知識のもとに、iPS細胞の培養法、保存法、凍結法などを、未分化性、多分化能、染色体安定性といった観点から詳細に検討し、高品質iPS細胞の評価基準を作製中である。また、昨今、世界的に培養細胞のクロスコンタミネーションが問題になってきている[9]。当細胞バンクにおいては、ヒトゲノム内に存在する特異的ローカスのSTR分析（short tandem repeat analysis）によるデータベースを構築して比較解析をすることにより、クロスコンタミネーションの可能性の分析を行っている[10]。これらの問題を克服して高品質の細胞を安定して供給するためには、細胞培養を技術として捉えるだけでなく、体系的な組織培養学を確立していかなくては難しいだろう。細胞培養を再考しなくてはならない時代が来ていると考える。品質のよいヒトiPS細胞を維持するためには、その特性を十分に把握したスタッフが必要であり、当研究所において、培養法を標準化して指導する準備を行っている。

10.6　高効率分化誘導法の開発および分化誘導細胞コレクションの作製

肝細胞は血液細胞や血管内皮細胞などと比較し、幹細胞からの分化誘導が困難であることが知られている。そこで、従来の液性因子を用いた分化誘導法に加え、当研究所・水口らの独自技術である改良型アデノウイルスベクター等を用いた高効率遺伝子導入法を応用することにより、医薬品毒性スクリーニングに適した肝細胞への高効率分化誘導法を開発中である。アデノウイルスベクターは既存の遺伝子導入ベクターの中では最も効率に優れ、染色体への遺伝子組み込み能を有さないため、一過性の遺伝子発現を示すことを特徴とする。従来のアデノウイルスベクターは遺伝子導入がアデノウイルス受容体（CAR）の発現に依存していたが、水口らはウイルス表面タンパク質を改変することで、感染域を任意に制御可能な改良型アデノウイルスベクターを開発し、様々な細胞種に対し、高効率（従来型の100倍）な遺伝子発現が可能となった[11-13]。従来の液性因子添加法と組み合わせて、細胞分化に関連する遺伝子を、これら改良型アデノウイルスベクターを用いてiPS細胞に作用させることで、より効率よく分化細胞が得られると考えられ、遺伝子発現プロファイリングの解析等により肝細胞への分化誘導に必要な導入遺伝子を決定し、分化誘導方法の最適化を行っている。

10.7　トキシコゲノミクス解析による医薬品毒性評価システムの開発

日本の化学物質（薬剤等）の分子毒性解析技術は世界トップクラスであるが、さらに、当研究所は世界最大規模（8億件）の毒性と遺伝子発現に関するトキシコゲノミクス・データベースを有している[14,15]。当研究所の横谷・山田らは、このデータベースを用いてiPS細胞から分化誘導

した組胞に対してトキシコゲノミクス解析を行うことにより，医薬品の毒性に関する新規 in vitro 細胞評価系を確立する。その特徴は，種々の毒性既知化合物を用いて判定基準設定のためのバリデーションが実施可能なことである。当研究所が保有するトキシコゲノミクス・データベースの情報を基にして指向したエンドポイントを in vitro 評価系に設定した後，種々の毒性既知化合物を用いて判定基準設定のためのバリデーションを実施する。種々のiPS細胞由来の分化細胞を使うことにより，個々人の体質の違いによる副作用を事前に予測することが可能になり，将来のテーラーメイド医療に向けた基盤を構築できる。

10.8　おわりに

iPS細胞を利用した安全性・薬効評価系を構築することにより，医薬品の安全性向上，創薬初期段階での正確かつ簡便な毒性評価が可能となる。それによって，これまでの医薬品開発において課題となっていた創薬後期段階での開発中止のリスクが低減するとともに，近年世界的に実施が制限され「種差の壁」の限界を有する動物実験に代わる評価システムが構築されることとなる。新薬開発コストの低減，新薬開発期間の短縮などが実現して数千億円の経済効果が生じるとともに，個々人の体質の違いによる副作用を事前に予測することが可能になるなど，テーラーメイド医療実現に向けての基盤が整備される。新規細胞評価系構築は一つの技術だけでできるものではなく，iPS細胞コレクション作製，品質管理，分化誘導，毒性評価等に関する様々な要素技術を融合させて推進していくことが不可欠である。これらは数千億円の経済効果とともに，合理的な医薬品審査へも寄与し，もって国民の健康の増進に資することになるであろう。

文　献

1) Frank, R. and Hargreaves, R., Clinical biomarkers in drug discovery and development. *Nat. Rev. Drug. Discov.*, 2, 566–80 (2003)
2) 古江　楠田美保，日本におけるES，iPS細胞研究の標準化．Tissue Culture Research Communications (2009)
3) Osafune, K. *et al.*, Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat. Biotechnol.*, 26, 313–5 (2008)
4) Andrews, P. W., *et al.*, The International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. *Nat. Biotechnol.*, 23, 795–7 (2005)
5) Adewumi, O., *et al.*, Characterization of human embryonic stem cell lines by the Interna-

tional Stem Cell Initiative. *Nat. Biotechnol.* (2007)

6) Amit, M., *et al.*, Feeder layer and serum-free culture of human embryonic stem cells. *Biol. Reprod.*, 70, 837-45 (2004)

7) Hayashi, I. and Sato, G. H., Replacement of serum by hormones permits growth of cells in a defined medium, *Nature*, 259, 132-4 (1976)

8) Furue, M., *et al.*, Leukemia inhibitory factor as an anti-apoptotic mitogen for pluripotent mouse embryonic stem cells in a serum-free medium without feeder cells. *In Vitro Cell. Dev. Biol. Anim.*, 41, 19-28 (2005)

9) Furue, M. K., *et al.*, Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc. Natl. Acad. Sci. U S A.*, 105, 13409-14 (2008)

10) Chatterjee, R., Cell biology. Cases of mistaken identity. *Science*, 315, 928-31 (2007)

11) 木澤寿江ほか, STR分析によるヒト培養細胞の迅速同定法, 実験医学, 26, 1395-1403 (2008)

12) 水口裕之ほか, 改良型アデノウイルスベクターを用いた造血幹細胞, 間葉系幹細胞, ES細胞への効率的遺伝子導入. Inflammation and Regeneration, 25, 447-451 (2005)

13) Kawabata, K., *et al.*, Adenovirus vector-mediated gene transfer to stem cells. *Mol. Pharm.*, 3, 95-103 (2006)

14) 堀井郁夫, 山田弘, 非臨床試験―ガイドラインへの対応と新しい試み　創薬段階での非臨床安全性評価・ハイスループット・トキシコロジー, 419-440, エル・アイ・シー (2008)

15) 堀井郁夫, 山田弘, 非臨床試験―ガイドラインへの対応と新しい試み―　創薬段階での非臨床安全性評価―分子毒性学的アプローチ, 441-479, エル・アイ・シー (2005)

In Vitro Cell.Dev.Biol.—Animal
DOI 10.1007/s11626-009-9266-6

BMP4 induction of trophoblast from mouse embryonic stem cells in defined culture conditions on laminin

Yohei Hayashi · Miho Kusuda Furue · Satoshi Tanaka · Michiko Hirose · Noriko Wakisaka · Hiroki Danno · Kiyoshi Ohnuma · Shiho Oeda · Yuko Aihara · Kunio Shiota · Atsuo Ogura · Shoichi Ishiura · Makoto Asashima

Received: 12 October 2009 / Accepted: 16 November 2009 / Editor: J. Denry Sato

Abstract Because mouse embryonic stem cells (mESCs) do not contribute to the formation of extraembryonic placenta when they are injected into blastocysts, it is believed that mESCs do not differentiate into trophoblast whereas human embryonic stem cells (hESCs) can express trophoblast markers when exposed to bone morphogenetic protein 4 (BMP4) in vitro. To test whether mESCs have the potential to differentiate into trophoblast, we assessed the effect of BMP4 on mESCs in a defined monolayer culture condition. The expression of trophoblast-specific transcription factors such as *Cdx2*, *Dlx3*, *Esx1*, *Gata3*, *Hand1*, *Mash2*, and *Plx1* was specifically upregulated in the BMP4-treated differentiated cells, and these cells expressed trophoblast markers. These results suggest that BMP4 treatment in defined culture conditions enabled mESCs to differentiate into trophoblast. This differentiation was inhibited by serum or leukemia inhibitory factor, which are generally used for mESC culture. In addition, we studied the mechanism underlying BMP4-directed mESC differentiation into trophoblast. Our results showed that BMP4 activates the Smad pathway in mESCs inducing *Cdx2* expression, which plays a crucial role in trophoblast differentiation, through the binding of Smad protein to the *Cdx2* genomic enhancer sequence. Our findings imply that there is a common molecular mechanism underlying hESC and mESC differentiation into trophoblast.

Keywords BMP4 · Smad · *Cdx2* · Trophoblast · Mouse embryonic stem cells

Y. Hayashi · H. Danno · K. Ohnuma · S. Oeda · Y. Aihara · S. Ishiura · M. Asashima
Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, Tokyo 153-8902, Japan

S. Tanaka · K. Shiota
Laboratory of Cellular Biochemistry, Animal Resource Sciences/ Veterinary Medical Sciences, The University of Tokyo, Tokyo 113-8657, Japan

M. Hirose · N. Wakisaka · A. Ogura
Bioresource Center, RIKEN, Ibaraki 305-0074, Japan

M. Asashima
Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology, Ibaraki 305-8562, Japan

M. K. Furue (✉) · Y. Aihara
Japanese Collection of Research Bioresources (JCRB) Cell Bank, Laboratory of Cell Culture, Division of Bioresources, National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki, Osaka 567-0085, Japan
e-mail: mkfurue@nibio.go.jp

Introduction

Mouse embryonic stem cells (mESCs) are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst, which differentiate into all the three germ layers in vitro and in vivo (Evans and Kaufman 1981; Martin 1981). However, mESCs are thought to be incapable of differentiating into trophoblast because they do not contribute to placenta in chimeric mouse (Beddington and Robertson 1989). To obtain placental trophoblast from mESCs, genetic manipulations of transcription factors or signaling molecules have been reported, such as the decreased expression of *Oct3/4* (Niwa et al. 2000) or *Sox2* (Masui et al. 2007) or overexpression of *Cdx2* (Niwa et al. 2005; Tolkunova et al. 2006), *Eomes* (Niwa et al. 2005), *Ras* (Lu

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

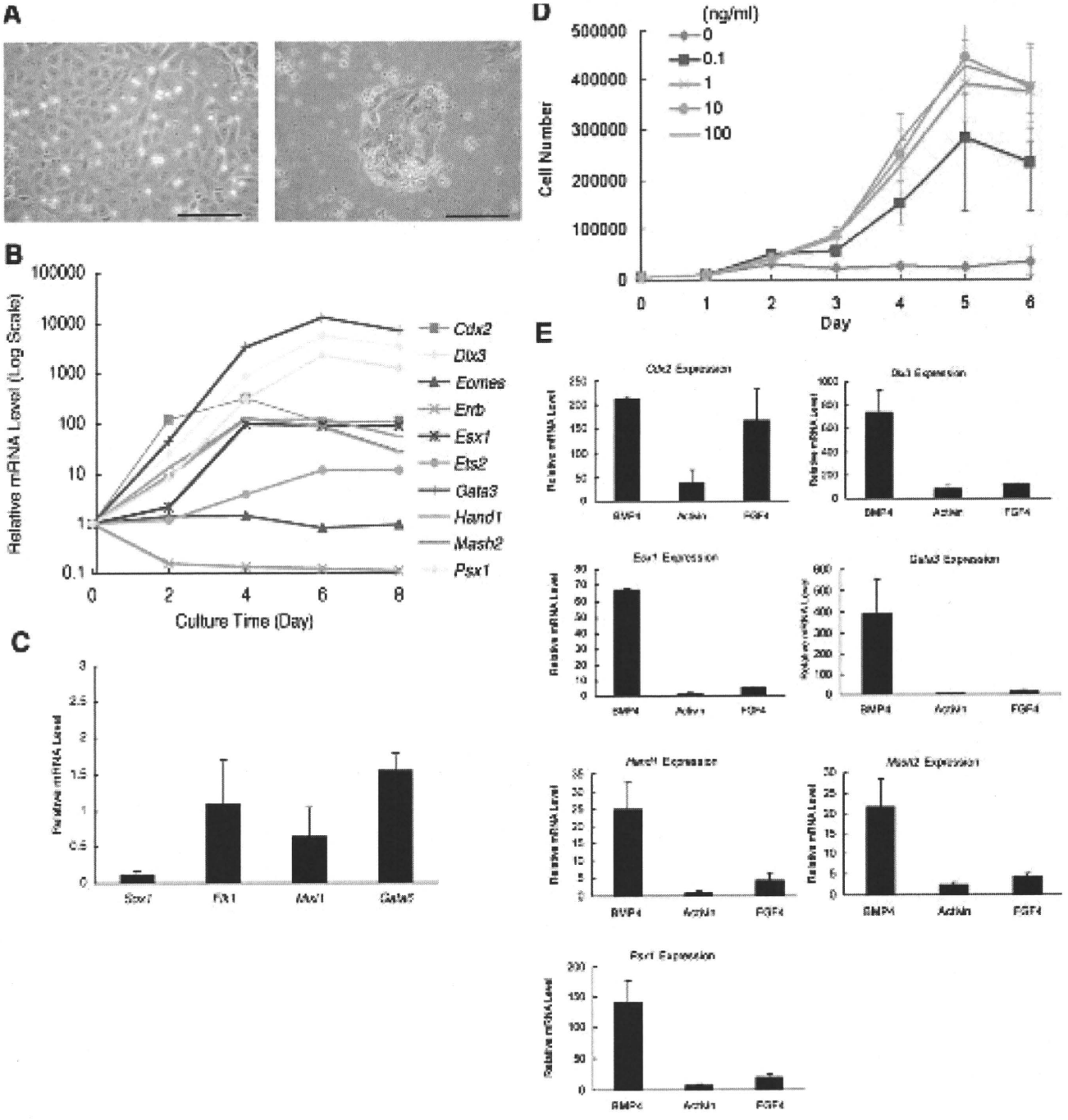


Figure 1. The effect of BMP4 on mESCs in defined culture conditions. (*A*) phase contrast photomicrographs of differentiated mESCs cultured in BMP4-supplemented ESF5 medium for 4 d (*left*) and 8 d (*right*). *Scale bars* are 100 µm. (*B*) quantitative real-time RT–PCR analysis of trophoblast-specific 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).

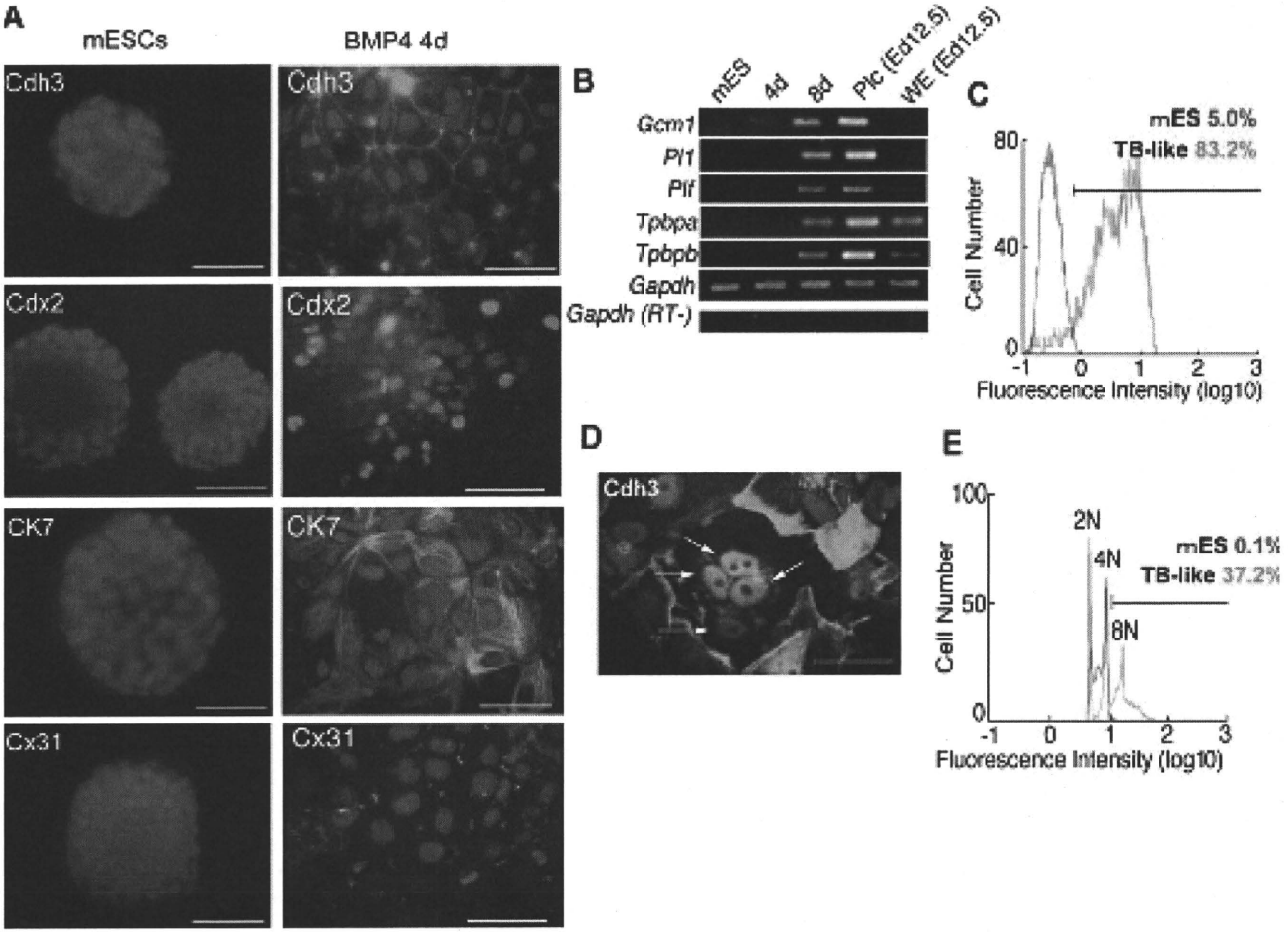


Figure 2. Differentiation into trophoblast from mESCs in vivo. (*A*) Immunocytostaining with trophoblast marker antibodies of the cells cultured in BMP4-supplemented ESF5 medium for 4 d (*right*) or undifferentiated mESCs (*left*). Immunoreactivity of Cdh3 (*upper left*), Cdx2 (*upper right*), CK7 (*lower 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/

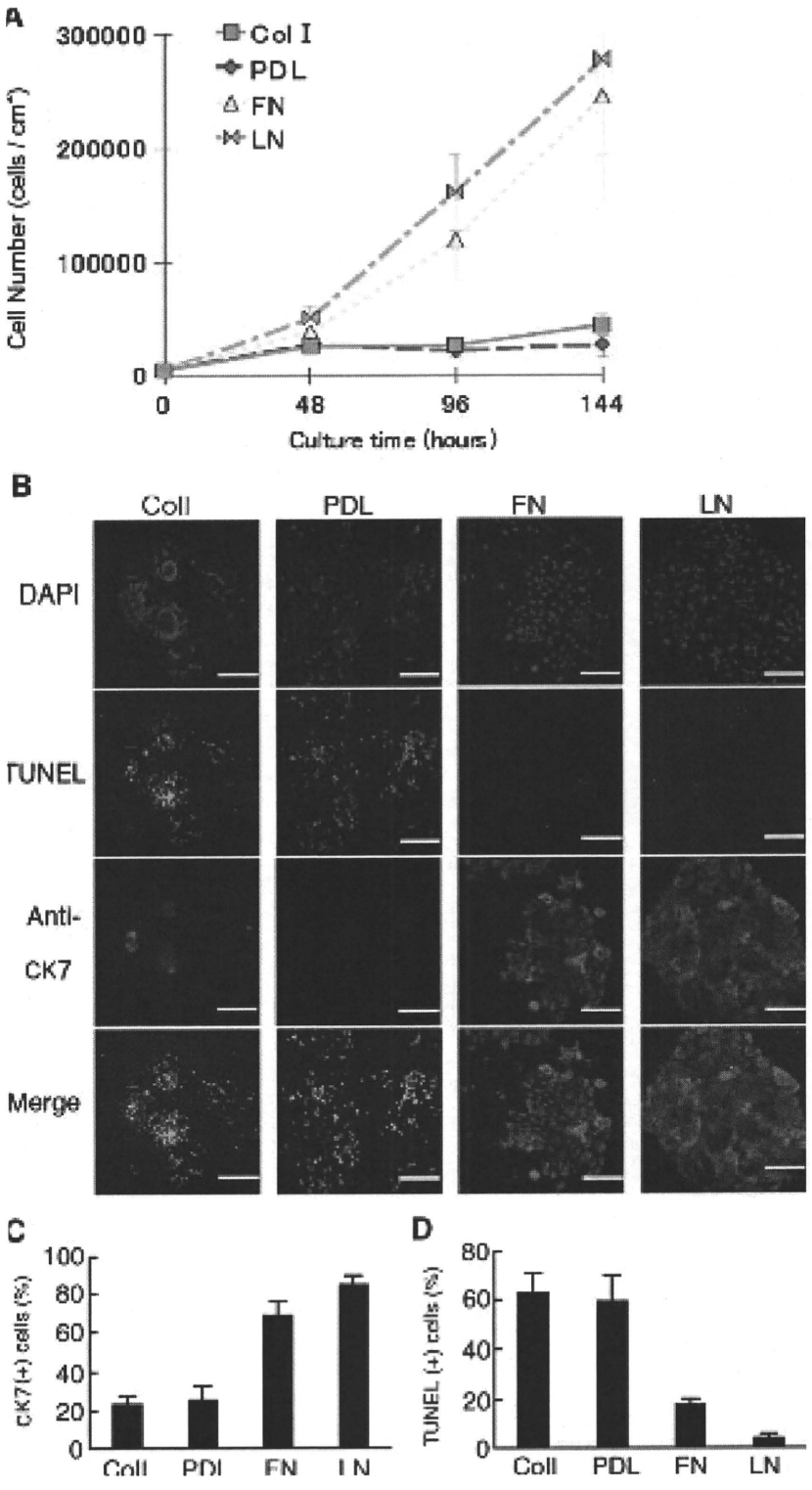


Figure 3. The effect of ECM components on the differentiation into trophoblast from mESCs. (*A*) Proliferation of differentiating mESCs on various ECM components. mESCs were seeded in a 24-well dish at 5×10^3 cells per well on each ECM component in ESF5 medium supplemented with BMP4. Cells were counted 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

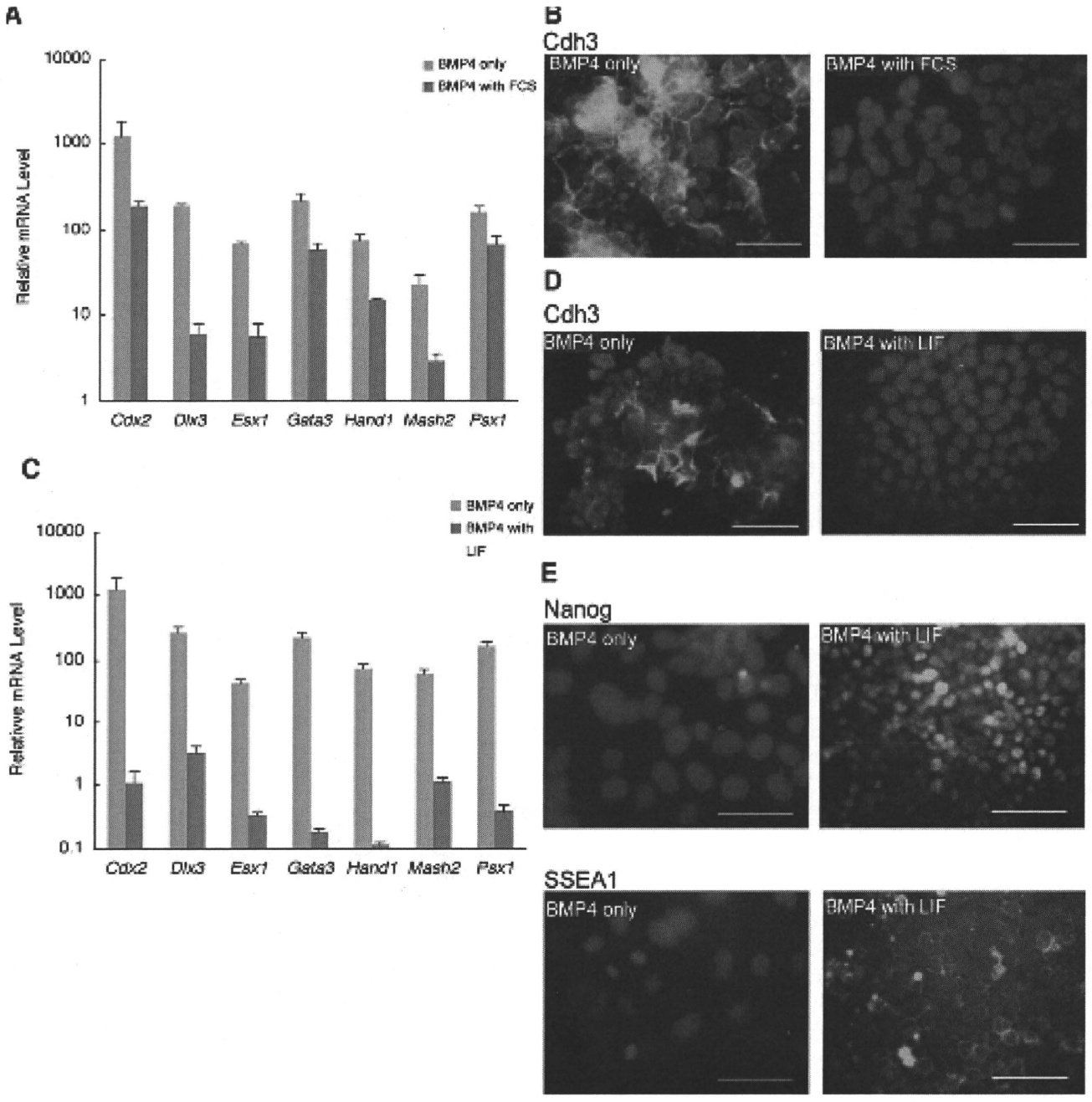


Figure 4. Effect of FCS and LIF on the differentiation into trophoblast. The effect of FCS: the cells were cultured in BMP4-supplemented ESF5 medium with 10% FCS (indicating as "BMP4 with FCS") or without FCS (indicating as "BMP4 only") for 4 d. (*A*) Quantitative real-time RT–PCR analysis of the expression 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

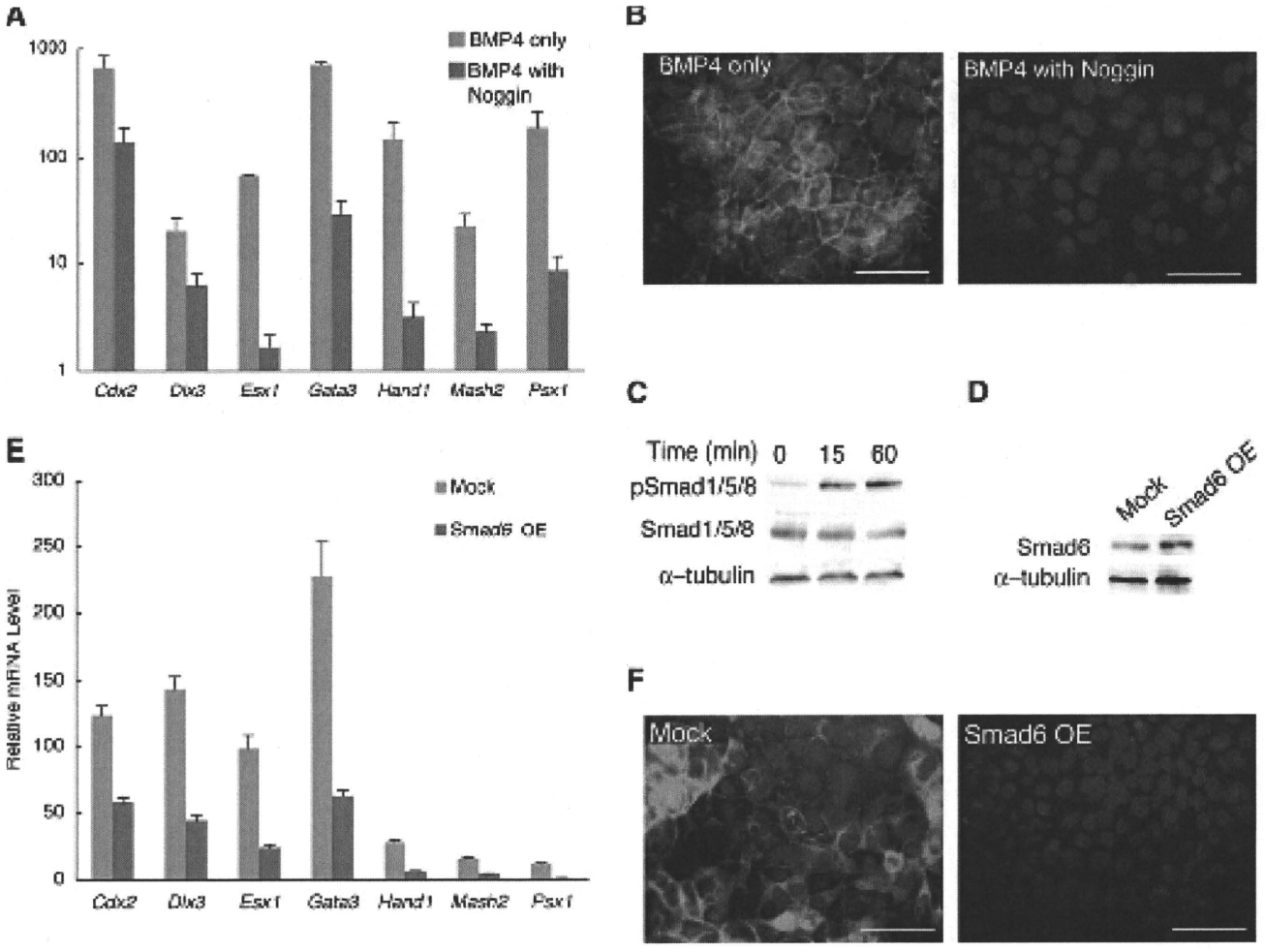


Figure 5. The effects of Noggin, inhibitory Smad6 on the BMP4-induced differentiation of mESCs into trophoblast. The effect of Noggin: the cells were cultured in BMP4-supplemented ESF5 medium with 300 ng/ml of Noggin (indicating as "BMP4 with Noggin") or without Noggin (indicating as "BMP4 only") for 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.

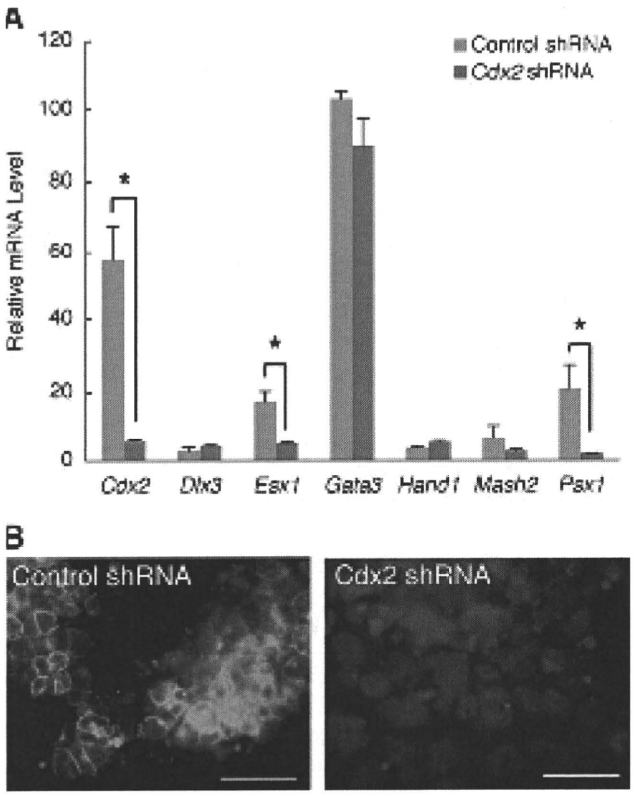


Figure 6. The effects of shRNA against *Cdx2* on the BMP4-induced differentiation of mESCs into trophoblast. The cells carrying shRNA-expressing vectors against *Cdx2* (indicating as "*Cdx2* shRNA") or control shRNA-expressing vectors (indicating as "Control shRNA") were cultured in BMP4-supplemented 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.

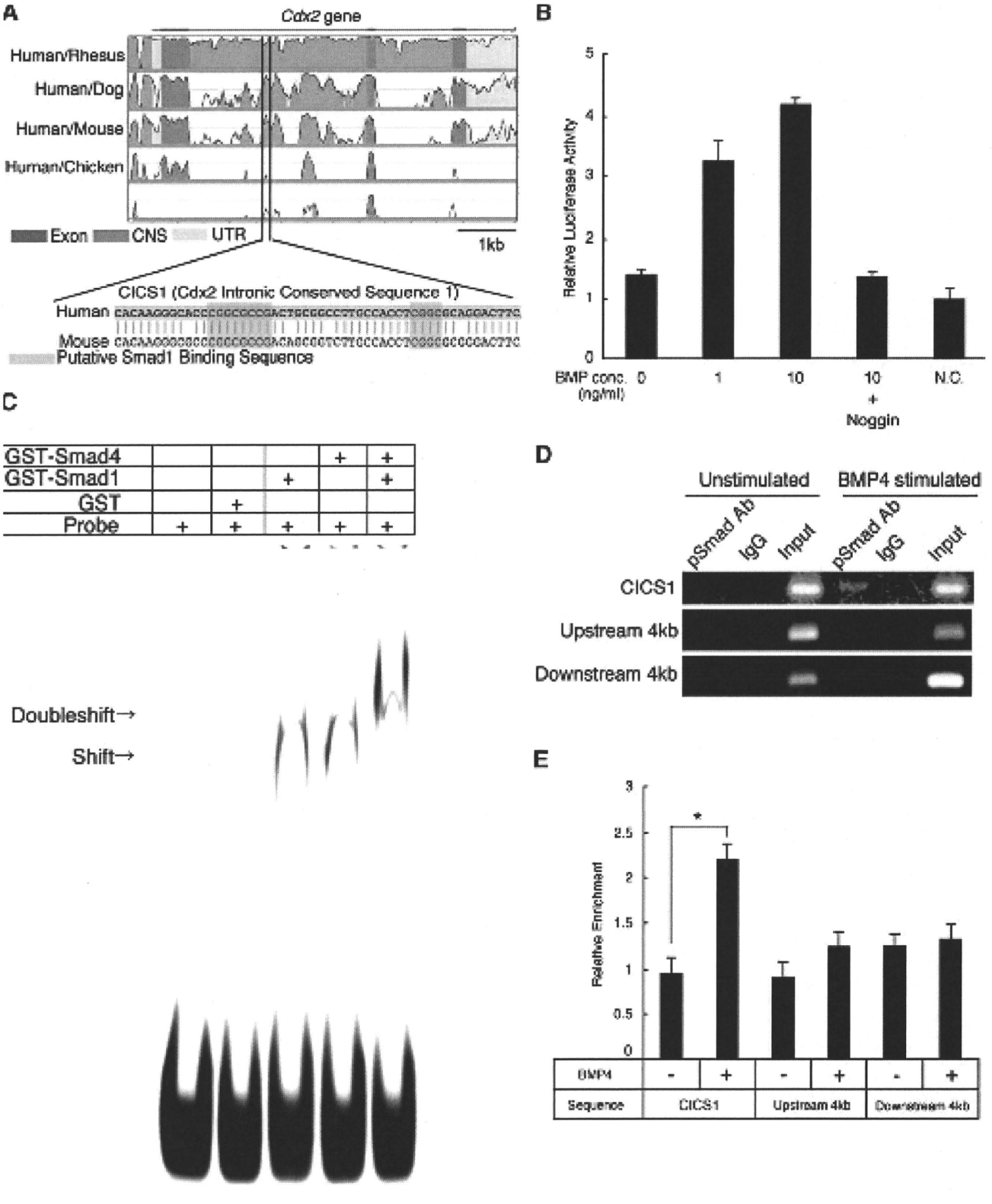
As described above, BMP4 is a key component promoting trophoblast differentiation from both mESCs and hESCs (Xu et al. 2002). These findings contradict previous reports in which BMP4 supported cell self-renewal. However, that was observed with BMP4 in conjunction with LIF (Ying et al. 2003; Qi et al. 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
Mouse
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.

Acknowledgements We would like to thank Prof. J. Rossant for the valuable comments and discussions on the manuscript and Drs. T. Yokota and K. Imamura for generously providing plasmid vectors. This study was supported by the following: Grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to M.K.F. and M.A.; Grants from Wako Pure Chemical Industries, Ltd. to M.A.; Grants-in-aid for scientific research from the Japan Society for the Promotion of Science to Y.H. and H.D.

References

Anson-Cartwright L.; Dawson K.; Holmyard D.; Fisher S. J.; Lazzarini R. A.; Cross J. C. The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. *Nat Genet* 25: 311–314; 2000.

Barros R.; Pereira B.; Duluc I.; Azevedo M.; Mendes N.; Camilo V.; Jacobs R.; Paulo P.; Santos-Silva F.; van Seuningen I.; van den Brink G.; David L.; Freund J. N.; Almeida R. Key elements of the BMP/SMAD pathway co-localize with CDX2 in intestinal metaplasia and regulate CDX2 expression in human gastric cell lines. *J Pathol* 215: 411–420; 2008.

Beddington R. S.; Robertson E. J. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* 105: 733–737; 1989.

Brons I. G.; Smithers L. E.; Trotter M. W.; Rugg-Gunn P.; Sun B.; de Sousa C.; Lopes S. M.; Howlett S. K.; Clarkson A.; Ahrlund-Richter L.; Pedersen R. A.; Vallier L. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448: 191–195; 2007.

Chun J. Y.; Han Y. J.; Ahn K. Y. Psx homeobox gene is X-linked and specifically expressed in trophoblast cells of mouse placenta. *Dev Dyn* 216: 257–266; 1999.

Cross J. C.; Flannery M. L.; Blanar M. A.; Steingrimsson E.; Jenkins N. A.; Copeland N. G.; Rutter W. J.; Werb Z. Hxt encodes a basic helix–loop–helix transcription factor that regulates trophoblast cell development. *Development* 121: 2513–2523; 1995.

Danno H.; Michiue T.; Hitachi K.; Yukita A.; Ishiura S.; Asashima M. Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate Rax expression. *Proc Natl Acad Sci U S A* 105: 5408–5413; 2008.

Evans M. J.; Kaufman M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292: 154–156; 1981.

Faria T. N.; Ogren L.; Talamantes F.; Linzer D. I.; Soares M. J. Localization of placental lactogen-I in trophoblast giant cells of the mouse placenta. *Biol Reprod* 44: 327–331; 1991.

Frazer K. A.; Pachter L.; Poliakov A.; Rubin E. M.; Dubchak I. VISTA: computational tools for comparative genomics. *Nucleic Acids Res* 32: W273–279; 2004.

Furue M.; Okamoto T.; Hayashi Y.; Okochi H.; Fujimoto M.; Myoishi Y.; Abe T.; Ohnuma K.; Sato G. H.; Asashima M.; Sato J. D. Leukemia inhibitory factor as an anti-apoptotic mitogen for pluripotent mouse embryonic stem cells in a serum-free medium without feeder cells. *In Vitro Cell Dev Biol Anim* 41: 19–28; 2005.

Guillemot F.; Caspary T.; Tilghman S. M.; Copeland N. G.; Gilbert D. J.; Jenkins N. A.; Anderson D. J.; Joyner A. L.; Rossant J.; Nagy A. Genomic imprinting of Mash2, a mouse gene required for trophoblast development. *Nat Genet* 9: 235–242; 1995.

Handyside A. H. Time of commitment of inside cells isolated from preimplantation mouse embryos. *J Embryol Exp Morphol* 45: 37–53; 1978.

Hayashi Y.; Furue M. K.; Okamoto T.; Ohnuma K.; Myoishi Y.; Fukuhara Y.; Abe T.; Sato J. D.; Hata R.; Asashima M. Integrins regulate mouse embryonic stem cell self-renewal. *Stem Cells* 25: 3005–3015; 2007.

Hayashi K.; Lopes S. M.; Tang F.; Surani M. A. Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. *Cell Stem Cell* 3: 391–401; 2008.

He S.; Pant D.; Schiffmacher A.; Meece A.; Keefer C. L. Lymphoid enhancer factor 1-mediated wnt signaling promotes the initiation of trophoblast lineage differentiation in mouse embryonic stem cells. *Stem Cells* 26: 842–849; 2008.

Hogan B.; Tilly R. In vitro development of inner cell masses isolated immunosurgically from mouse blastocysts. II. Inner cell masses from 3.5- to 4.0-d p.c. blastocysts. *J Embryol Exp Morphol* 45: 107–121; 1978.

Johansson B. M.; Wiles M. V. Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. *Mol Cell Biol* 15: 141–151; 1995.

Kunath T.; Saba-El-Leil M. K.; Almousailleakh M.; Wray J.; Meloche S.; Smith A. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* 134: 2895–2902; 2007.

Lechleider R. J.; Ryan J. L.; Garrett L.; Eng C.; Deng C.; Wynshaw-Boris A.; Roberts A. B. Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. *Dev Biol* 240: 157–167; 2001.

Lescisin K. R.; Varmuza S.; Rossant J. Isolation and characterization of a novel trophoblast-specific cDNA in the mouse. *Genes Dev* 2: 1639–1646; 1988.

Li Y.; Behringer R. R. Esx1 is an X-chromosome-imprinted regulator of placental development and fetal growth. *Nat Genet* 20: 309–311; 1998.

Lu C. W.; Yabuuchi A.; Chen L.; Viswanathan S.; Kim K.; Daley G. Q. Ras-MAPK signaling promotes trophectoderm formation from embryonic stem cells and mouse embryos. *Nat Genet* 40: 921–926; 2008.

Lu H.; Hesse M.; Peters B.; Magin T. M. Type II keratins precede type I keratins during early embryonic development. *Eur J Cell Biol* 84: 709–718; 2005.

Luo J.; Sladek R.; Bader J. A.; Matthyssen A.; Rossant J.; Giguere V. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. *Nature* 388: 778–782; 1997.

Martin G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78: 7634–7638; 1981.

Masui S.; Nakatake Y.; Toyooka Y.; Shimosato D.; Yagi R.; Takahashi K.; Okochi H.; Okuda A.; Matoba R.; Sharov A. A.; Ko M. S.; Niwa H. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* 9: 625–635; 2007.

Morasso M. I.; Grinberg A.; Robinson G.; Sargent T. D.; Mahon K. A. Placental failure in mice lacking the homeobox gene Dlx3. *Proc Natl Acad Sci U S A* 96: 162–167; 1999.

Ng Y. K.; George K. M.; Engel J. D.; Linzer D. I. GATA factor activity is required for the trophoblast-specific transcriptional regulation of the mouse placental lactogen I gene. *Development* 120: 3257–3266; 1994.

Nishioka N.; Inoue K.; Adachi K.; Kiyonari H.; Ota M.; Ralston A.; Yabuta N.; Hirahara S.; Stephenson R. O.; Ogonuki N.; Makita R.;

Kurihara H.; Morin-Kensicki E. M.; Nojima H.; Rossant J.; Nakao K.; Niwa H.; Sasaki H. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Dev Cell* 16: 398–410; 2009.

Niwa H.; Miyazaki J.; Smith A. G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24: 372–376; 2000.

Niwa H.; Toyooka Y.; Shimosato D.; Strumpf D.; Takahashi K.; Yagi R.; Rossant J. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell* 123: 917–929; 2005.

Nose A.; Takeichi M. A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J Cell Biol* 103: 2649–2658; 1986.

Nostro M. C.; Cheng X.; Keller G. M.; Gadue P. Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. *Cell Stem Cell* 2: 60–71; 2008.

Qi X.; Li T. G.; Hao J.; Hu J.; Wang J.; Simmons H.; Miura S.; Mishina Y.; Zhao G. Q. BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. *Proc Natl Acad Sci U S A* 101: 6027–6032; 2004.

Russ A. P.; Wattler S.; Colledge W. H.; Aparicio S. A.; Carlton M. B.; Pearce J. J.; Barton S. C.; Surani M. A.; Ryan K.; Nehls M. C.; Wilson V.; Evans M. J. Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature* 404: 95–99; 2000.

Schenke-Layland K.; Angelis E.; Rhodes K. E.; Heydarkhan-Hagvall S.; Mikkola H. K.; Maclellan W. R. Collagen IV induces trophectoderm differentiation of mouse embryonic stem cells. *Stem Cells* 25: 1529–1538; 2007.

Simmons D. G.; Cross J. C. Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. *Dev Biol* 284: 12–24; 2005.

Simmons D. G.; Rawn S.; Davies A.; Hughes M.; Cross J. C. Spatial and temporal expression of the 23 murine prolactin/placental lactogen-related genes is not associated with their position in the locus. *BMC Genomics* 9: 352; 2008.

Smith A. G.; Heath J. K.; Donaldson D. D.; Wong G. G.; Moreau J.; Stahl M.; Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336: 688–690; 1988.

Strumpf D.; Mao C. A.; Yamanaka Y.; Ralston A.; Chawengsaksophak K.; Beck F.; Rossant J. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* 132: 2093–2102; 2005.

Sumi T.; Tsuneyoshi N.; Nakatsuji N.; Suemori H. Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, activin/nodal and BMP signaling. *Development* 135: 2969–2979; 2008.

Suzuki A.; Raya A.; Kawakami Y.; Morita M.; Matsui T.; Nakashima K.; Gage F. H.; Rodriguez-Esteban C.; Izpisua Belmonte J. C. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. *Proc Natl Acad Sci U S A* 103: 10294–10299; 2006.

Tanaka S.; Kunath T.; Hadjantonakis A. K.; Nagy A.; Rossant J. Promotion of trophoblast stem cell proliferation by FGF4. *Science* 282: 2072–2075; 1998.

Tesar P. J.; Chenoweth J. G.; Brook F. A.; Davies T. J.; Evans E. P.; Mack D. L.; Gardner R. L.; McKay R. D. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448: 196–199; 2007.

Tolkunova E.; Cavaleri F.; Eckardt S.; Reinbold R.; Christenson L. K.; Scholer H. R.; Tomilin A. The caudal-related protein cdx2 promotes trophoblast differentiation of mouse embryonic stem cells. *Stem Cells* 24: 139–144; 2006.

Toumadje A.; Kusumoto K.; Parton A.; Mericko P.; Dowell L.; Ma G.; Chen L.; Barnes D. W.; Sato J. D. Pluripotent differentiation in vitro of murine ES-D3 embryonic stem cells. *In Vitro Cell Dev Biol Anim* 39: 449–453; 2003.

Tremblay K. D.; Dunn N. R.; Robertson E. J. Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development* 128: 3609–3621; 2001.

Wen F.; Tynan J. A.; Cecena G.; Williams R.; Munera J.; Mavrothalassitis G.; Oshima R. G. Ets2 is required for trophoblast stem cell self-renewal. *Dev Biol* 312: 284–299; 2007.

Wiles M. V.; Johansson B. M. Embryonic stem cell development in a chemically defined medium. *Exp Cell Res* 247: 241–248; 1999.

Williams R. L.; Hilton D. J.; Pease S.; Willson T. A.; Stewart C. L.; Gearing D. P.; Wagner E. F.; Metcalf D.; Nicola N. A.; Gough N. M. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336: 684–687; 1988.

Xu R. H.; Chen X.; Li D. S.; Li R.; Addicks G. C.; Glennon C.; Zwaka T. P.; Thomson J. A. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 20: 1261–1264; 2002.

Yamamoto H.; Flannery M. L.; Kupriyanov S.; Pearce J.; McKercher S. R.; Henkel G. W.; Maki R. A.; Werb Z.; Oshima R. G. Defective trophoblast function in mice with a targeted mutation of Ets2. *Genes Dev* 12: 1315–1326; 1998.

Ying Q. L.; Nichols J.; Chambers I.; Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115: 281–292; 2003.

Ying Q. L.; Wray J.; Nichols J.; Batlle-Morera L.; Doble B.; Woodgett J.; Cohen P.; Smith A. The ground state of embryonic stem cell self-renewal. *Nature* 453: 519–523; 2008.

Zheng-Fischhofer Q.; Kibschull M.; Schnichels M.; Kretz M.; Petrasch-Parwez E.; Strotmann J.; Reucher H.; Lynn B. D.; Nagy J. I.; Lye S. J.; Winterhager E.; Willecke K. Characterization of connexin31.1-deficient mice reveals impaired placental development. *Dev Biol* 312: 258–271; 2007.

1. 培養液

古江 - 楠田　美保

要旨

ヒト胚性幹（ES）細胞や間葉系幹細胞などのヒト幹細胞は臨床用の移植ソースとしてだけでなく，分化細胞を用いての創薬や薬効・毒性評価などにも使用されると予想される。現在，ヒト幹細胞の多くがウシ血清や未知なる物質を含む動物由来成分を用いて培養されている。このような条件では，培養液にロット差が生じ，高品質のヒト幹細胞を安定して維持するのは難しい。添加因子の影響を正確に解析することも困難である。病原体混入の可能性もある。ヒト幹細胞を臨床に応用するためには，すべての組成が明らかにされた合成培地を用いて培養されるべきだろう。本稿では，ヒトES細胞，iPS細胞用培養条件の問題点と，いくつかの培養液を紹介する。

キーワード

ヒト胚性幹細胞，chemically defined serum-free culture，無血清培養法，knockout-serum replacement（KSR），線維芽細胞増殖因子-2（FGF-2），N2サプリメント，B27サプリメント

はじめに

培養細胞では，用いる培養液によって細胞の増殖や分子生物学的性質が大きく変化することがある。特に，ヒト胚性幹（ES）細胞ならびにヒト人工多能性幹（iPS）細胞は，その性質上，体細胞に比べて培養環境の影響を受けやすい。維持するうちに異なる細胞集団になることも多いため，高品質を維持することはたいへん難しい。用いる培養液の性質をよく把握して培養を行う必要がある。

1．一般的なヒトES細胞用培養液

ヒトES細胞やiPS細胞は，現在，一般的にマウス胎児線維芽細胞由来フィーダー細胞上で，ウシ血清，あるいは代替血清knockout-serum replacement[1]（KSR）と線維芽細胞増殖因子-2（FGF-2）[2]を添加した培地を用いて培養されている。国際的に広く使用されている培養条件を**表❶**に記した。また，マトリゲルとフィーダー細胞の培養上清を使用したフィーダー細胞を用いない培養方法もクローニングなどの目的ではよく用いられている[3]。

1．培養液の問題点

表❶の培養条件は，様々な不確定物質を含んだ培養条件となる[4]。さらに，このような条件で培養した細胞にヒト以外の動物の細胞表面に存在するシアル酸・N-グリコリルノイラミン酸（Neu5Gc）が確認され，臨床応用における安全性が懸念されはじめた。従来から治療に使われる細胞は，既知の因子による合成培地を用いた無血清培養か自家血由来の血清を使用して培養されており，ようやくヒト幹細胞においても培養液の重要性が理解されたと言うべきだろう。

癌細胞などにおいては，血清添加の条件で問題なく培養維持できる細胞株は多い。マウスES細胞やヒトES細胞も，血清や代替血清KSRを用いた培養液の中で安定して培養維持されていると考えられがちである。しかし，血清には細胞増殖因子だけでなく，分化促進因子，接着因子やホルモン，また未知の因子が含まれている。適切なタイミングで培地交換や継代を行わねば，分化細胞が現れ，増殖能の高いクローンのみが生き残り，異なる細胞集団となっていく。ロット差があるために，実験結果にもロット差が出てしまう。例えば，ES細胞からembryoid body（胚様体）を作製する際に，