cell contact-dependent AKT activation may be able to compensate for the detrimental effect caused by ERK1/2 inhibition in human ES cells once they reach a certain density.

Based on our results, we propose that ERK1/2 plays multiple important roles in human ES cells (Fig. 7): it is required for neural and mesendoderm induction and essential for cell attachment to the substrate; however, it can suppress BMP signaling and prevent extraembryonic differentiation. Our model could explain the discrepancies regarding the roles of FGF2 and ERK1/2 in human ES cells and mouse ES cell self-renewal. Further dissecting the "balancing action" among FGF, BMP, and activin A/TGFβ pathways under chemically defined conditions will offer insights into how these developmental signals regulate mammalian ES cell fate decisions.

Materials and methods

Cell lines and maintenance

HUES1 (normal and adapted), SHEF4, SHEF5 and SHEF6 human ES cells were used in this study. SHEF4, SHEF5, and SHEF6 cells were maintained on feeders in an ES medium containing KO-DMEM (Invitrogen) supplemented with nonessential amino acids, 0.1 μM 2-mercaptoethanol, 1 mM glutamine, 20% serum replacement (Invitrogen), and 4 ng/ml FGF2 (Peprotech) (Amit et al., 2000). HUES1 cells were maintained on feeders in an HUES1 medium containing KO-DMEM supplemented with nonessential amino acids, 0.1 mM 2-mercaptoethanol, glutamine, 8% serum replacement, 8% plasminate (Bayer), and 4 ng/ml FGF2 (Cowan et al., 2004). In both cases, the cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were passaged with 1 mg/ml collagenase IV (Invitrogen) (for SHEF lines) and 0.05% trpsin/0.04% EDTA (Sigma) (for HUES1), respectively. The cells were seeded onto a 25 cm^2 flask (NUNC) that had been previously coated with 0.1% porcine gelatin (Sigma) and mitomycin C (Sigma)-inactivated MEF as feeder cells.

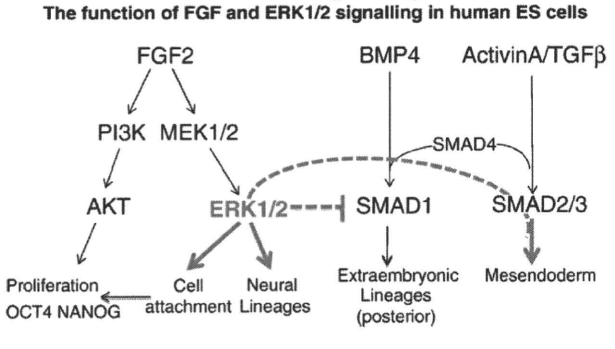

Figure 7 Model depicting multiple roles of ERK1/2 in human ES cells. FGF2 activated both AKT and ERK1/2. ERK1/2 promotes neural and mesendodermal (together with a high dosage of activin A/TGFβ) differentiation from ES cells while it suppresses BMP-induced extraembryonic lineage differentiation. It is also needed for human ES cell attachment to the substrate, which is essential for ES cell survival and proliferation.

Human ES cell culture in serum-free, feeder-free, and chemically defined medium

Prior to growth-factor-treatment experiments, human ES cells were grown for more than 3 passages in the absence of feeders in a serum-free and chemically defined medium on type I collagen gel (Nita Gelatin, Japan) (Furue et al., 2008). The hESF basal medium was purchased from the Cell Science & Technology Institute Inc., Sendai, Japan. The components of hESF8, 9, and 9A are listed in Supplementary Table 1. Because cells are very sensitive to the passaging strategy used, including the size of colonies that are passaged, the addition of activin A permits the maintenance of more robust and reproducible cultures of undifferentiated cells. Thus, hESF9A containing 10 ng/ml of FGF2 and activin A each was used for regular serum-free culture. Before the start of each series of experiments, when necessary, manual passaging was used to ensure a uniform undifferentiated starting cell population for all treatment groups.

Growth-factor treatment and differentiation protocols

Serum-free, feeder-free cultured human ES cells were seeded in 12-well plates at a density of 4×10^4/well. One day after seeding, the cells were washed with an hESF8 medium, and then fed with hESF8 and with activin A (R&D systems), BMP4, and FGF2 (Peprotech) added at the desired concentrations. The medium was changed every 2 days with freshly added growth factors. Cells were collected after 5 days and analysed. For neural differentiation, we used a protocol modified from Zhang et al. (2001). Briefly, SHEF4 and SHEF6 cells were first cultured as floating EBs in hESF8 with 4 ng/ml of FGF2 for 1 week. These EBs were then plated in 6-well plates coated with poly-L-ornithine hydrobromide (PLOH) and 2 μg/cm^2 Laminin (LN) (Sigma) and fed with hESF8 and 20 ng/ml of FGF for 1 week. Afterward, colonies with neural rosette morphology were manually picked and cultured in suspension in the same medium for another 7 days. These rosettes were replated on a PLOH/LN surface for 7–10 days. Rosettes with extensively long neurites were picked and dissociated to obtain a relatively pure population of neuronal cells. To initiate mesendodermal or extraembryonic differentiation, cells were treated with 100 ng/ml activin A plus 10 ng/ml FGF2 or 10 ng/ml of BMP4 on 2 μg/cm^2 FN-coated surface for 7 days.

Antibodies and chemical inhibitors

The following antibodies were used in this study: SMAD4, OCT4, ERK1/2, and BRACHYURY (Santa Cruz); CDX2, FOXA2, NANOG, and SOX17 (R&D systems); SIX3 (a rabbit polyclonal antibody raised against polypeptide RLQ HQA IGP SGM RSL AEP GC which corresponds to the C terminus of mouse SIX3; the same sequence was present in human SIX3); PAX6 and FORSE1 (Developmental Studies Hybridoma Bank, IA, USA); phospho-ERK1/2, phospho-AKT, phospho-GSK3β, total GSK3 β (Cell Signaling); SOX2 and actin (Abcam, Cambridge UK). The monoclonal antibody to SSEA4 was produced from a culture of hybridoma 813-70 (Kannagi et al., 1983). The chemical inhibitors U0126, LY294002, and PD0325901 were

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purchased from Promega, Merck, and AXON Medchem (Groningen, the Netherlands), respectively.

Western blot

For Western blot analysis, HUES1 cells were first cultured in hESF9A for 48 h and then washed briefly with PBS and cultured in hESF8 (without FGF and activin A) for 48 h. Afterward, 10 ng/ml of FGF2 and chemical inhibitors (U0126 10 μM, LY294002 10 μM, PD0325901 0.3 μM, or DMSO only) were added, while 30 min later cells were detached and lysed on ice in a sample-loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, PhosSTOP (Roche), and 0.002% bromophenol blue). The amount of 2×10^5 cells-equivalent lysate was loaded per lane.

Quantitative PCR

Total RNA was extracted with TRIZOL (Invitrogen). The amount of 1 μg RNA of each sample was used for reverse transcription (20 μl) using Superscript III (Invitrogen). Q-PCRs were performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in an iCycler (Bio-Rad). Each gene was done in triplicate (Fig. 1) or duplicate (Figs. 2 and 3). The sample input was normalised against the C_t (critical threshold) value of the housekeeping gene GAPDH. Relative quantification of each gene was performed using the iCycler Excel program. The error bar is the standard deviation of the C_t value. Primer sequences are listed in Supplementary Table 2.

Immunostaining, FACS, and high-content screen

For in situ immunostaining and high-content screen, cells were fixed in 6-well plates with 4% PFA in PBS. OCT4 and SSEA4 staining was scanned in an In Cell Analyser 1000 (Amersham Biosciences) and analysed using the Developer's Toolbox. OCT4, SIX3, and PAX6 staining was imaged with an Olympus FV1000 confocal microscope. For FACS analysis, cells were incubated with 0.05% trypsin in 0.2% EDTA in 37 °C, 10% CO_2 incubator until most dissociated into single cells. Trypsin inhibitor (Sigma T6522) (1 mg/ml in DMEM) was immediately added to inactivate the trypsin. Cells were subsequently rinsed off the flask or 6-well plate with an hESF8 medium, centrifuged down, and washed 3 times in PBS containing 4 mg/ml polyvinylpyrrolidone (Sigma) to prevent them from sticking together. They were fixed in 4% PFA in PBS 0.1% Tween 20 (PBST) for 20 min at room temperature. For intracellular staining of Oct4, 0.2% Triton X-100 was included in the fixative. Afterward, cells were washed 3 times in PBST, blocked with 5% FBS–PBST for 1 h, and incubated with the desired primary and secondary antibodies. FACS was done using a DAKO Cytomation $CyAn_{ADP}$ machine.

Y27632 treatment and clonogenicity assay

SHEF6 cells were dissociated into single cells as described above and seeded at 10,000 cells/cm^2 in FN (2 μg/cm^2) coated 6-well plates in hESF9A. Y27632 (10 μM) or U0126 (10 μM) was added 2 h prior to passaging and overnight during seeding as described in Watanabe et al. (2007). The medium was replaced the next day. Cells were grown in hESF9A for another 10 days, then fixed briefly, and stained with alkaline phosphatase (AP) Red Substrate Solution (Sigma). AP-positive colonies were photographed and counted.

Uncited reference

Harb et al., 2008

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.scr.2010.06.002.

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LETTER

Advantages and difficulties in culturing human pluripotent stem cells in growth factor-defined serum-free medium

Miho Kusuda Furue · Daiki Tateyama · Masaki Kinehara · Jie Na · Tetsuji Okamoto · J. Denry Sato

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Dear Editor,

Serum- and feeder-free culture conditions have received a great deal of attention for culturing human embryonic stem (hES) cells or induced pluripotent stem (iPS) cells although hES/iPS cells are still most commonly maintained on inactivated mouse embryonic fibroblast feeders (MEF) in medium supplemented with FBS (Thomson et al. 1998; Reubinoff et al. 2000), or proprietary replacements, such as knockout serum replacement (KSR) together with fibroblast growth factor-2 (FGF-2) (Amit et al. 2000; Draper et al. 2004). Use of culture media containing undefined or undisclosed components has limited the development of applications for pluripotent cells because of our lack of knowledge of their responses to specific cues that control self-renewal, differentiation, and lineage selection. Therefore, a defined serum-free medium consisting of minimum essential components for culturing hES/iPS cells could contribute to advances in the field.

Previously, we have developed a defined growth factor-supplemented serum-free medium, hESF9, for the culture of human ES cells on a type I collagen substrate without feeders (Furue et al. 2008). This medium consists of hESF basal medium supplemented with heparin and only four protein components: insulin, transferrin, albumin conjugated with oleic acid, and FGF-2 (10 ng/ml). Under these culture conditions, FGF-2 promotes proliferation of hES cells in a concentration-dependent manner. Heparin, which is known to enhance the activity of FGF, also promotes proliferation of hES cells in a concentration-dependent manner in the absence of FGF-2 suggesting that endogenous FGF-2 is produced by hES cells. In conventional cultures with KSR-based medium, the proliferative effects of FGF-2 or heparin are not detectable although it is well known that FGF-2 supports hES cell growth. Thus, a defined serum-free medium consisting of minimum essential components could aid in elucidating hES/iPS cell responses to specific cues that control self-renewal, differentiation, and lineage selection.

The modern era of serum-free growth began in the mid-1970s when Izumi Hayashi and Gordon Sato (1976) defined conditions for growth of a rat pituitary cell line in a hormonally defined serum-free medium that did not alter cell phenotypes or cell growth. N2 supplements for neural

M. Kusuda Furue · D. Tateyama · M. Kinehara
Laboratory of Cell Cultures, Division of Bioresources,
National Institute of Biomedical Innovations,
Osaka 567-0085, Japan

M. Kusuda Furue (✉)
Laboratory of Cell Processing,
Institute for Frontier Medical Sciences, Kyoto University,
Kyoto 606-8507, Japan
e-mail: mkfurue@nibio.go.jp

J. Na
School of Medicine, Tsinghua University,
Beijing, China

T. Okamoto
Department of Molecular Oral Medicine and Maxillofacial Surgery, Division of Frontier Medical Sciences,
Graduate Science School of Biomedical Sciences,
Hiroshima University,
Hiroshima 734-8553, Japan

J. D. Sato
Mount Desert Island Biological Laboratory,
Salisbury Cove, ME 04672, USA

J. Na
The Centre for Stem Cell Biology,
The University of Sheffield,
Sheffield, S10 2TN,
United Kingdom

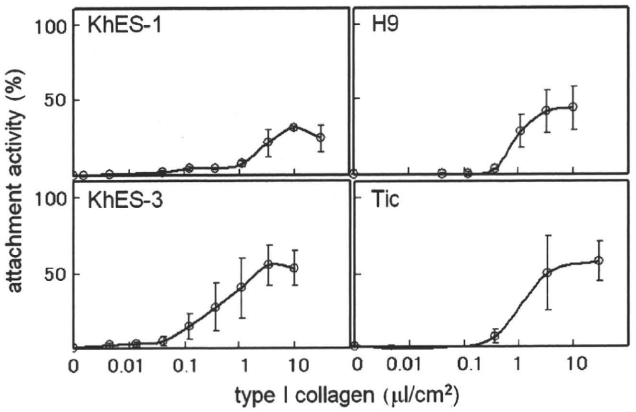

Figure 1. Attachment and proliferation of hES and iPS cells on type I collagen. The attachment of hES/iPS cells to ECM components was measured by the procedures followed by Fassler and Meyer (1995). Briefly, a 96-well microplate (Corning Costar, Corning, NY) was coated with each adhesion molecule at 37°C for 3 h. hES/iPS cells were seeded at confluent density (3×10^6cells cm^{-2}) on type I collagen (Nitta Gelatin, Inc.) coated plates in hESF9 (Cell Science & Technology Institute, Inc.). After 3 d, the attached cells were fixed and stained for 30 min with 0.4% crystal violet (Sigma) in methanol. After the plate was washed and dried, a solution (1% acetic acid and 30% ethanol in water) was added to the wells to dissolve the crystal violet. The absorbance of 595 nm, which indicated the concentration of the dissolved crystal violet, was measured with a microplate reader (model 550; Bio-Rad, Hercules, CA). Each graph shows the percentage of the attached cells on type I collagen in hESF9 relative to the attached cells on fibronectin (Sigma) as 100% as all the cell lines attached to fibronectin in hESF9. *Bar*=SE (n=3).

cells were developed by Bottenstein and Sato (1979). Later, we described a variety of serum-free media that can be used to propagate and analyze differentiated cells (Barnes and Sato 1980; Sato 1987; Furue et al. 1994; Okamoto et al. 1996; Sato et al. 2002). Serum contains variable amounts of hormones, soluble growth and differentiation factors, attachment factors, and undefined components. Also MEFs secrete a variety of nutrients or growth factors most of which have not been identified. The precise formulation of KSR is not in the public domain, and although "serum-free," it is likely to contain a variety of animal products (PCT/US98/00467; WO98/30679). The formulations of several other products for serum-free culture from several companies are also proprietary. These undefined or disclosed components of the culture conditions hamper analysis of the mechanisms that control cell behavior.

However, there are several difficulties in culturing hES/iPS cells in defined conditions without MEFs. First, propagation of pluripotent hES/iPS cells has been difficult to achieve even in conventional culture media due to their propensity to differentiate (Skottman and Hovatta 2006; Adewumi et al. 2007) as most researchers working with hES/iPS cells know. When cells grown to confluence are split, many differentiated cells appear in the next passage. The variability of batches of MEFs or KSR affects the tendency of cells to differentiate. The dissociation method is the most problematic. Even in conventional cell culture, imprecise handling during dissociation decreases cell survival as single hES/iPS cells cannot survive. Centrifugation at high speed decreases cell survival or promotes cell aggregation. Unless the cells are handled with care for several passages on MEFs in KSR-based medium, the cells do not survive or differentiate when transferred to feeder-free cultures. We had used EDTA to dissociate hES cell colonies in the feeder-free culture method using hESF9 medium. As the attachment activity of undifferentiated hES cells is different from that of differentiated cells, the undifferentiated hES cells were able to be transferred to the next passage, which led to the successful continuous culture of undifferentiated cells. However, this method requires previous practical experience with human primary

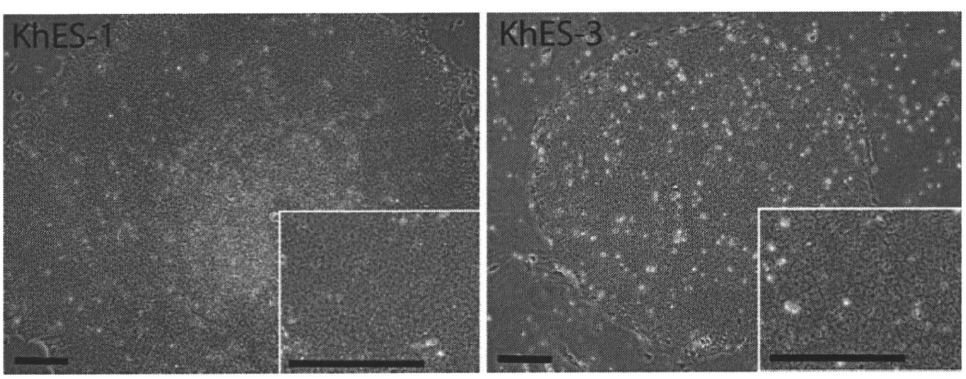

Figure 2. hES cells cultured on fibronectin in hESF9 medium. Phase-contrast microphotographs of KhES-1 at passage 16 and KhES-3 at passage 6 cultured on fibronectin in hESF9 medium. After 2 d, the KhES-1 cells and KhES-3 cells (from Kyoto University) cultured on MEF in KSR-based medium were passaged by the routine procedure onto the new MEF; the medium was changed to hESF9 medium, and then the cells were cultured on MEF for more than 4 d. Previously, we dissociated the hES cells with EDTA, but some of cell lines could not survive after the dissociation with EDTA. Then, we have utilized 1 U/ml dispase (Roche Applied Science) for approximately 1 min at 37°C to dissociate the hES/iPS colonies into the small clumps. The cells were washed twice at 20 g and then seeded on fibronectin-coated flask (Corning Costar) in hESF9 medium.

cell cultures. As hESF9 medium consists of minimum essential components, improper handling greatly affects cell viability and culture outcome.

Second, there are variations in the characteristics of hES/iPS cell lines as most of the researchers working on hES/iPS cells have come to realize. hES cell lines, Shef1, Shef5, and HUES1 cultured in the University of Sheffield were able to attach to type I collagen and grow well in hESF9 medium (Furue et al. 2008). However, we found that attachment activity of the KhES-1 line (Nakatsuji 2005), which was established in Kyoto University, to type I collagen seemed low, suggesting that there is a difference between the cell lines in their attachment ability. Owing to regulatory issues for hES cell importation into Japan, we were unable to directly compare KhES-1 cells with Shef1 or HUES1. We have examined the attachment and growth activity of other hES lines (KhES-3 from Kyoto University, Kyoto, Japan; H9 from National Stem Cell Bank, WiCell, Madison, WI) and the MRC-5-derived iPS cell line Tic (JCRB 1331, JCRB Cell Bank, Osaka, Japan), which was established in the National Center for Child Health and Development, on type I collagen (Nitta Gelatin, Osaka, Japan) in hESF9 medium (Cell Science & Technology Institute, Sendai, Japan). The results show that there is a difference between hES/iPS cell lines in attachment activity to type I collagen. This result suggests that there is a difference among cell lines in integrin signaling (Fig. 1).

Based on these findings, we have modified the culture protocol and tried to culture KhES-1 and KhES-3 cell lines on fibronectin (Sigma, St. Louis, MO) in hESF9 medium without feeders (Fig. 2). Matrigel, a basement membrane preparation from the Engelbreth–Holm–Swarm mouse tumor, is often used for feeder-free culture for hES/iPS cells with MEF-conditioned medium (Draper et al. 2004). However, it contains a complex and ill-defined mixture of fibronectin, laminin, type IV collagen, entactin, and heparan sulfate proteoglycans, and various growth factors such as FGF-2, EGF, PDGF, and NGF (Yang et al. 2003). Ludwig et al. (2006) have reported that in place of matrigel, a combination of collagen IV, fibronectin, laminin, and vitronectin supported robust, long-term proliferation of human ES cells in their chemically defined medium TeSR1. We have previously reported by using defined serum-free culture conditions for mouse embryonic stem (mES) cell that integrins regulate mES cell self-renewal. mES cells remained undifferentiated when cultured on type I and type IV collagen or poly-D-lysine whereas they differentiated when cultured on laminin or fibronectin where LIF-induced self-renewal signaling was decreased (Hayashi et al. 2007). Now, we are investigating the role of integrins in the pluripotency of hES/iPS cells.

For robust cultures, we have further modified the culture protocol. We have used 1 U/ml dispase (Roche Applied Science, Indianapolis, IN) to dissociate the cell colonies and washed the dispase with the medium supplemented with recombinant human albumin (1 mg/ml, Millipore, Bedford, MA). If differentiated cells appear in the culture, addition of low concentration of activin (2~10 ng/ml, R&D Systems, Minneapolis, MN) or middle concentration of noggin (10~20 ng/ml, R&D Systems) seems also to inhibit the differentiated cell growth as previously reported (Beattie et al. 2005; James et al. 2005; Vallier et al. 2005; Wang et al. 2005). However, addition of these growth factors confounds the analysis of the actions of other exogenous factors. We are using hESF9 medium to develop a drug screening test.

It would be convenient if the cell culture novice could propagate and passage any type of cell without difficulty. Unfortunately, this is currently not the case for undifferentiated hES/iPS cell lines. Although serum has proved to be a universal medium supplement that allowed the isolation and characterization of a few normal diploid cell lines and numerous abnormal transformed cell lines over the years, the use of serum or other undefined medium components impedes our ability to understand cell responses to controlled environmental stimuli. There are advantages and disadvantages to culturing hES/iPS cells under defined serum-free culture conditions, and the suitability of any particular medium depends on the purpose of the experiment.

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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

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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

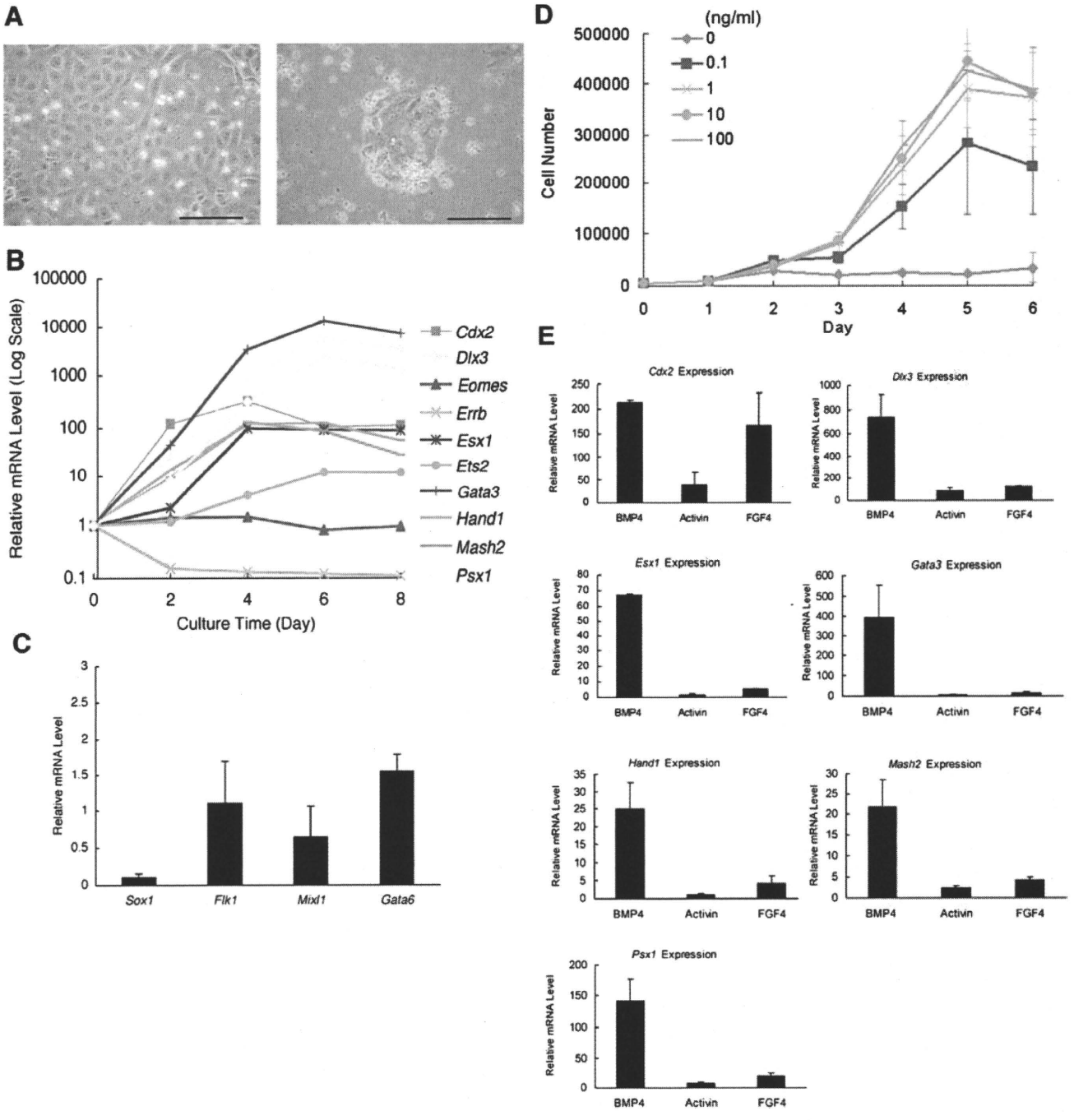


Figure 1. The effect of BMP4 on mESCs in defined culture conditions. (*A*) phase contrast photomicrographs of differentiated mESCs cultured in BMP4-supplemented ESF5 medium for 4 d (*left*) and 8 d (*right*). *Scale bars* are 100 μm. (*B*) quantitative real-time RT–PCR analysis of trophoblast-specific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

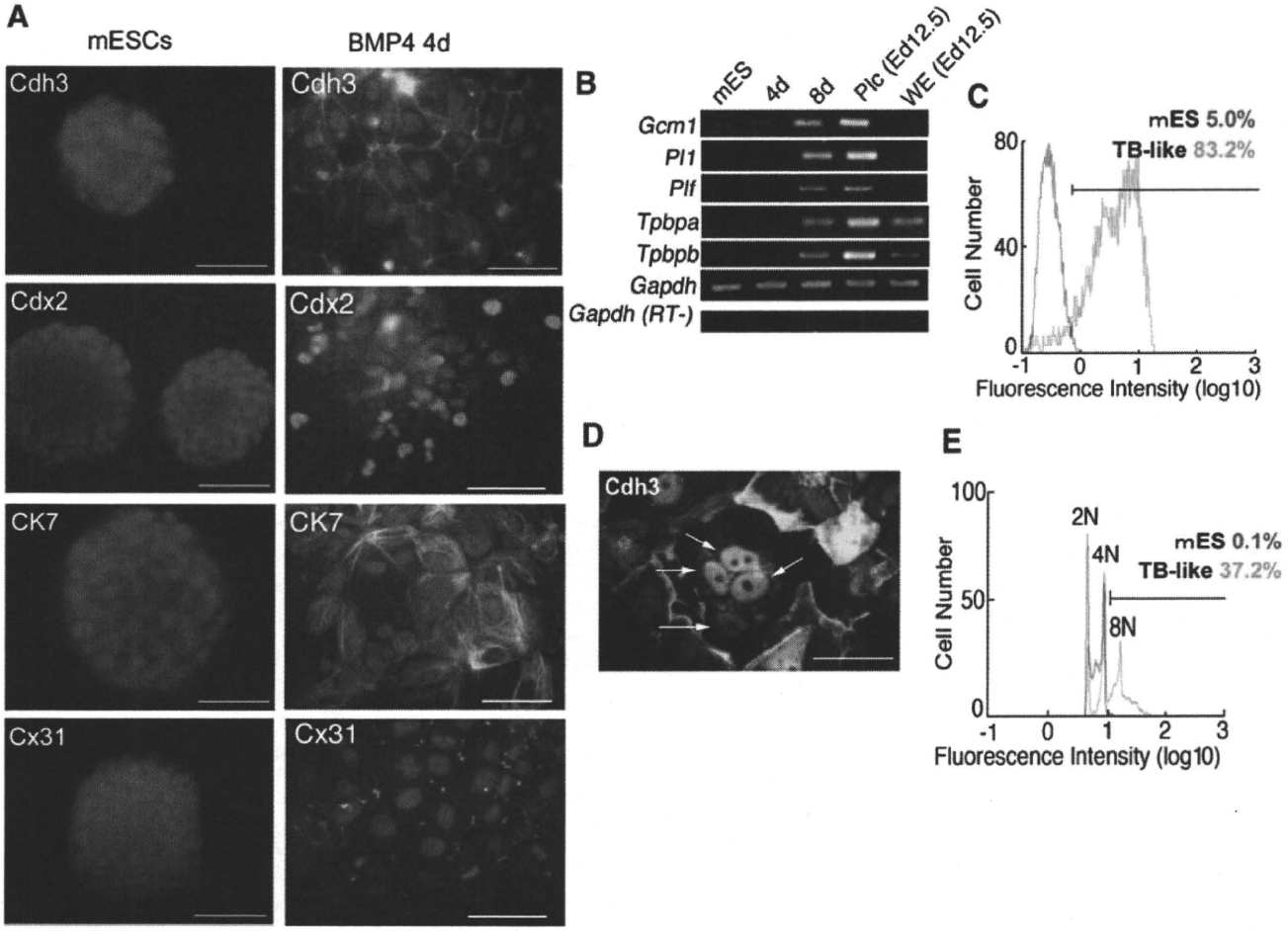


Figure 2. Differentiation into trophoblast from mESCs in vivo. (*A*) Immunocytostaining with trophoblast marker antibodies of the cells cultured in BMP4-supplemented ESF5 medium for 4 d (*right*) or undifferentiated mESCs (*left*). Immunoreactivity of Cdh3 (*upper left*), Cdx2 (*upper right*), CK7 (*lower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

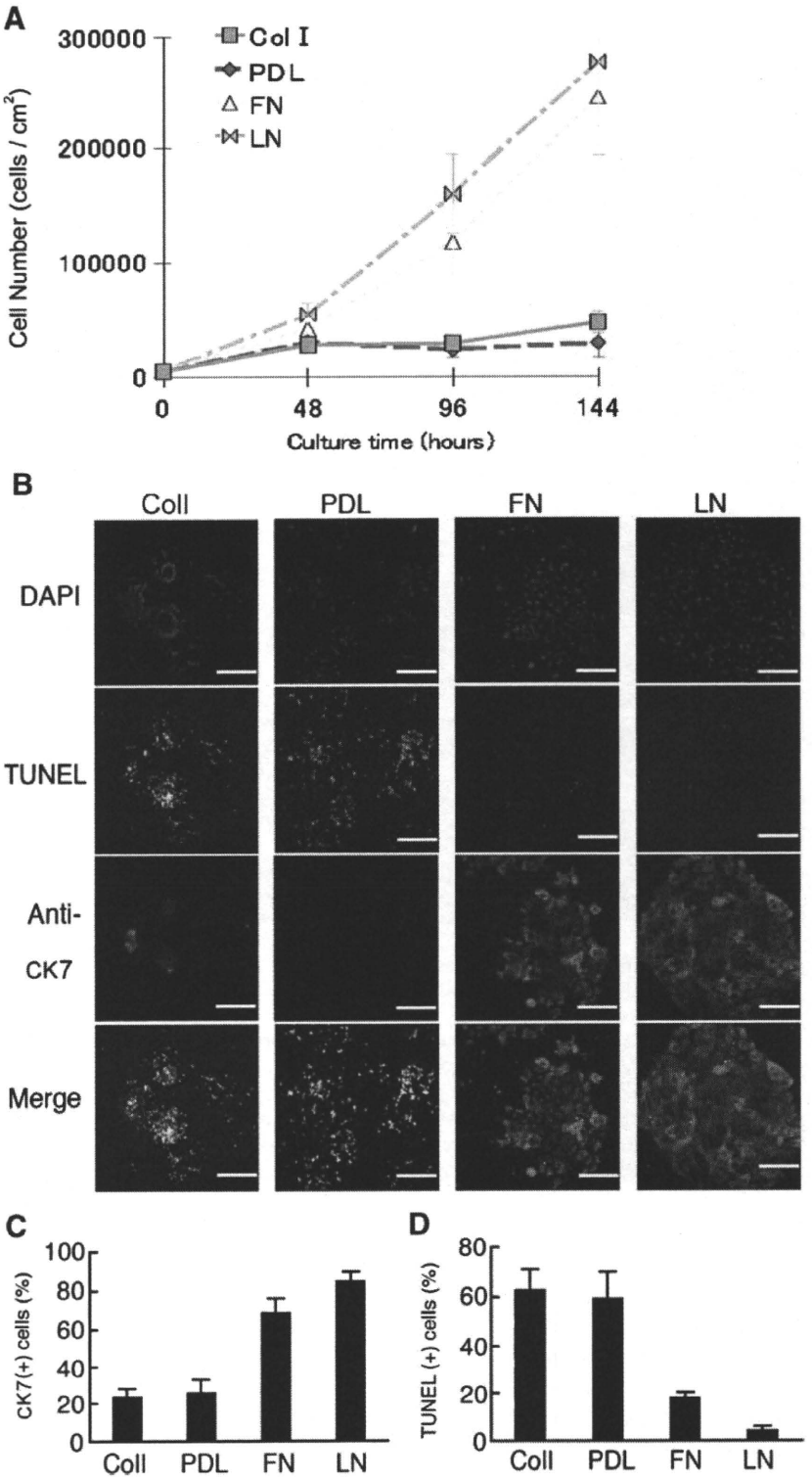


Figure 3. The effect of ECM components on the differentiation into trophoblast from mESCs. (*A*) Proliferation of differentiating mESCs on various ECM components. mESCs were seeded in a 24-well dish at 5×10^3 cells per well on each ECM component in ESF5 medium supplemented with BMP4. Cells were counted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

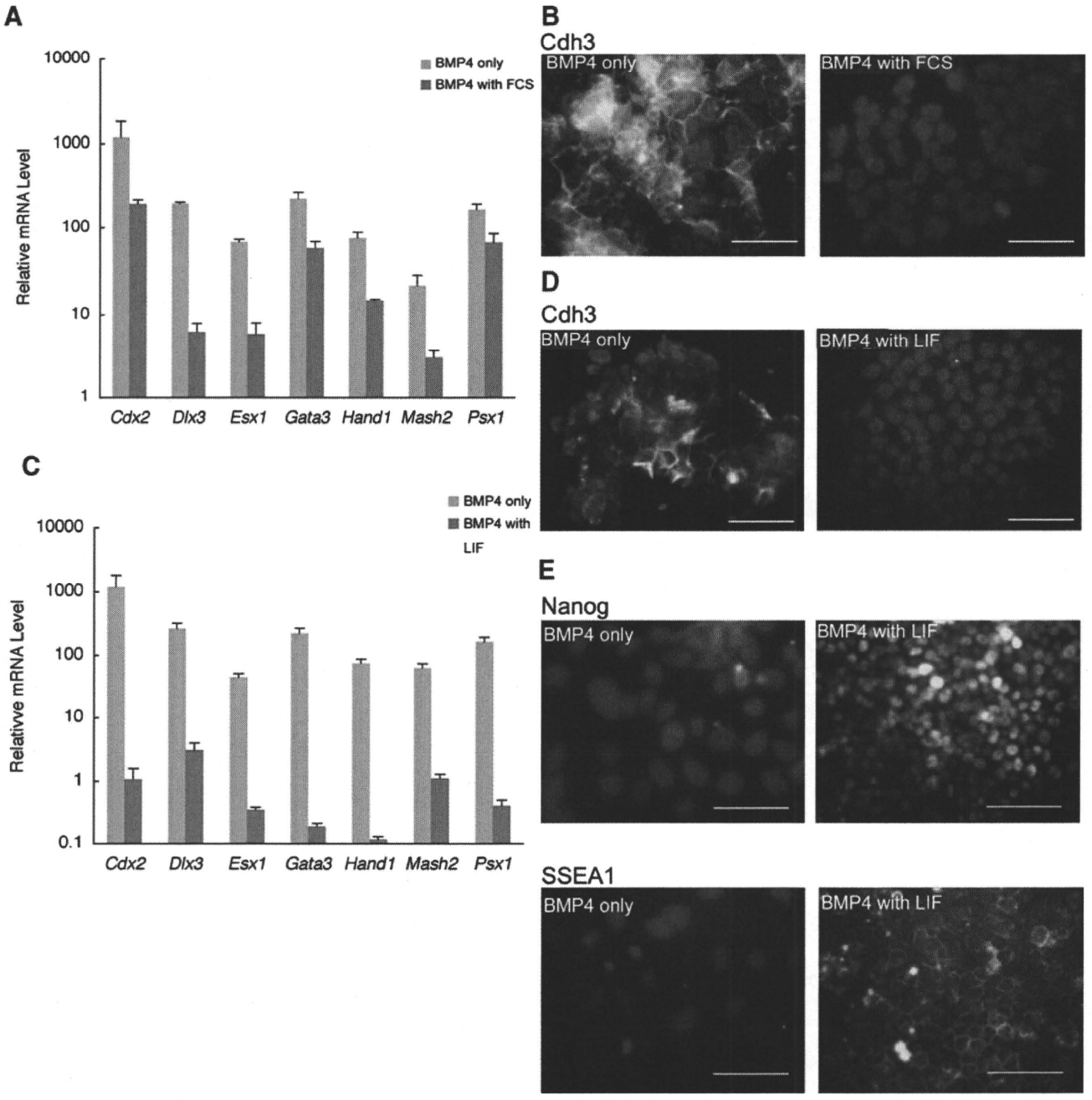


Figure 4. Effect of FCS and LIF on the differentiation into trophoblast. The effect of FCS: the cells were cultured in BMP4-supplemented ESF5 medium with 10% FCS (indicating as "BMP4 with FCS") or without FCS (indicating as "BMP4 only") for 4 d. (*A*) Quantitative real-time RT–PCR analysis of the expression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

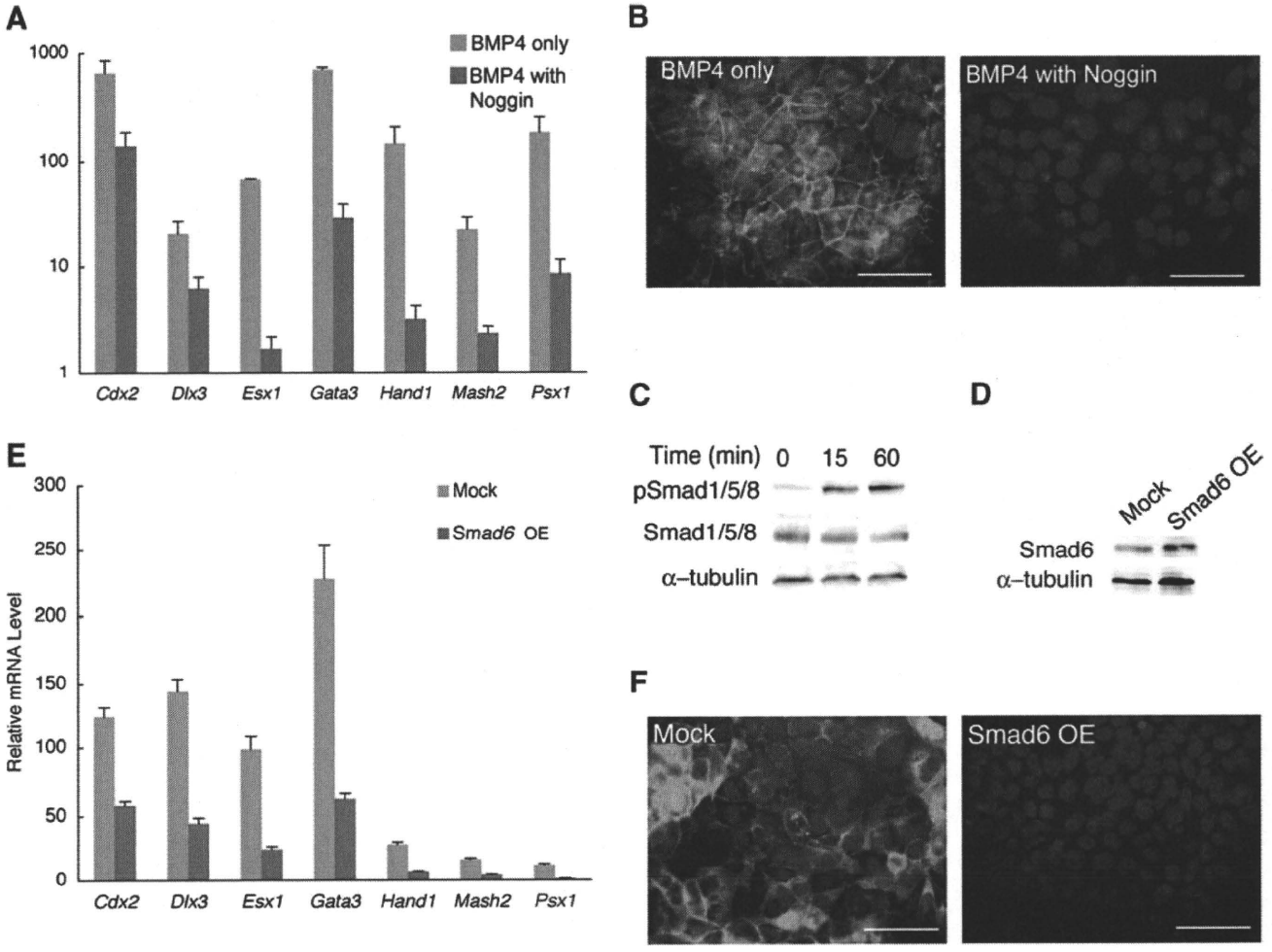


Figure 5. The effects of Noggin, inhibitory Smad6 on the BMP4-induced differentiation of mESCs into trophoblast. The effect of Noggin: the cells were cultured in BMP4-supplemented ESF5 medium with 300 ng/ml of Noggin (indicating as "BMP4 with Noggin") or without Noggin (indicating as "BMP4 only") for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

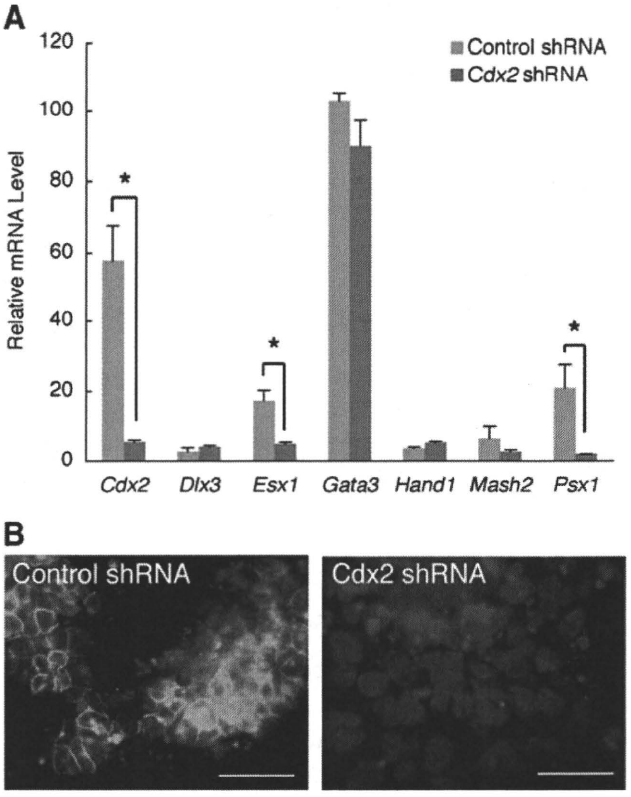


Figure 6. The effects of shRNA against *Cdx2* on the BMP4-induced differentiation of mESCs into trophoblast. The cells carrying shRNA-expressing vectors against *Cdx2* (indicating as "*Cdx2* shRNA") or control shRNA-expressing vectors (indicating as "Control shRNA") were cultured in BMP4-supplemented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

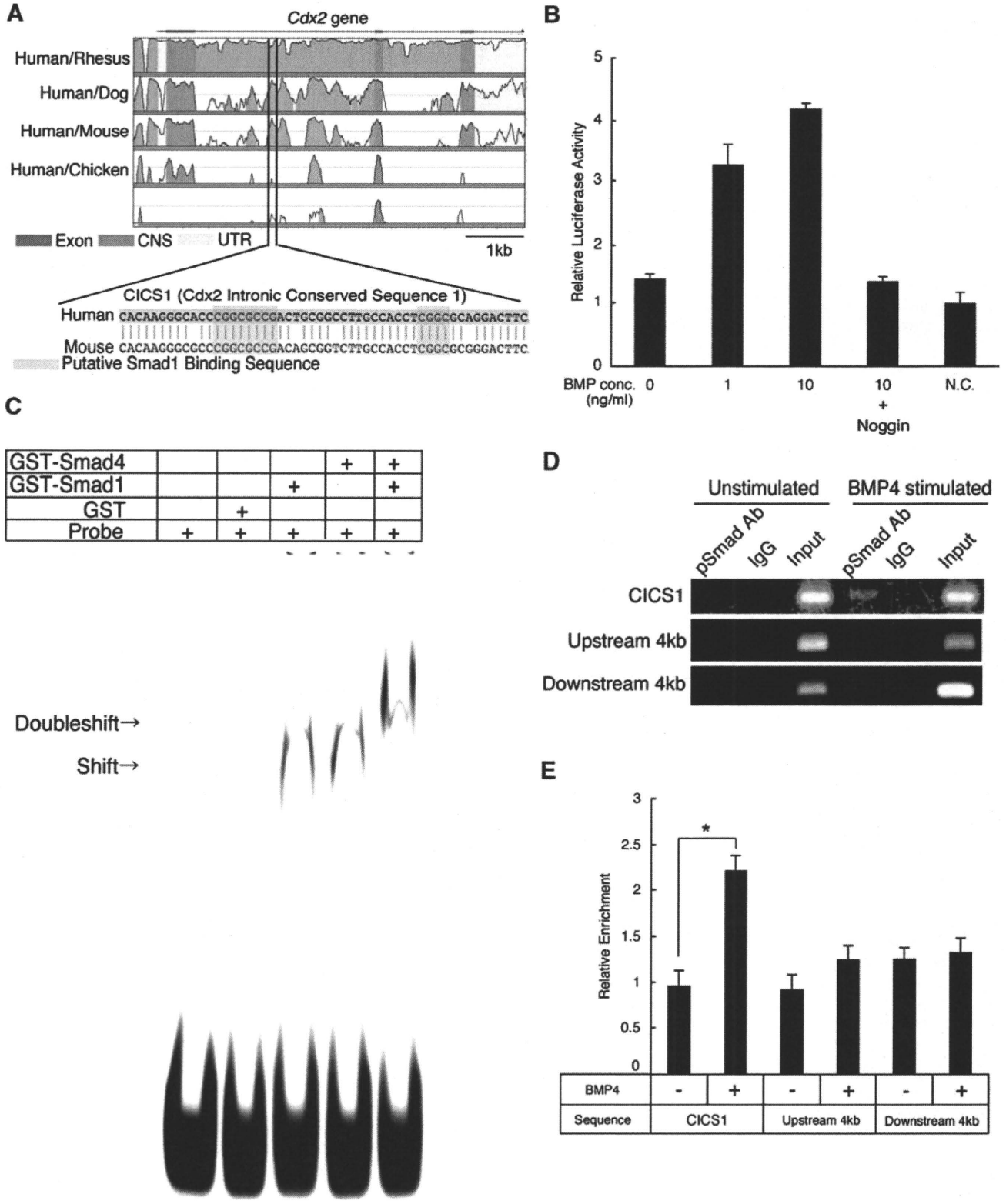
As described above, BMP4 is a key component promoting trophoblast differentiation from both mESCs and hESCs (Xu et al. 2002). These findings contradict previous reports in which BMP4 supported cell self-renewal. However, that was observed with BMP4 in conjunction with LIF (Ying et al. 2003; Qi et al.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