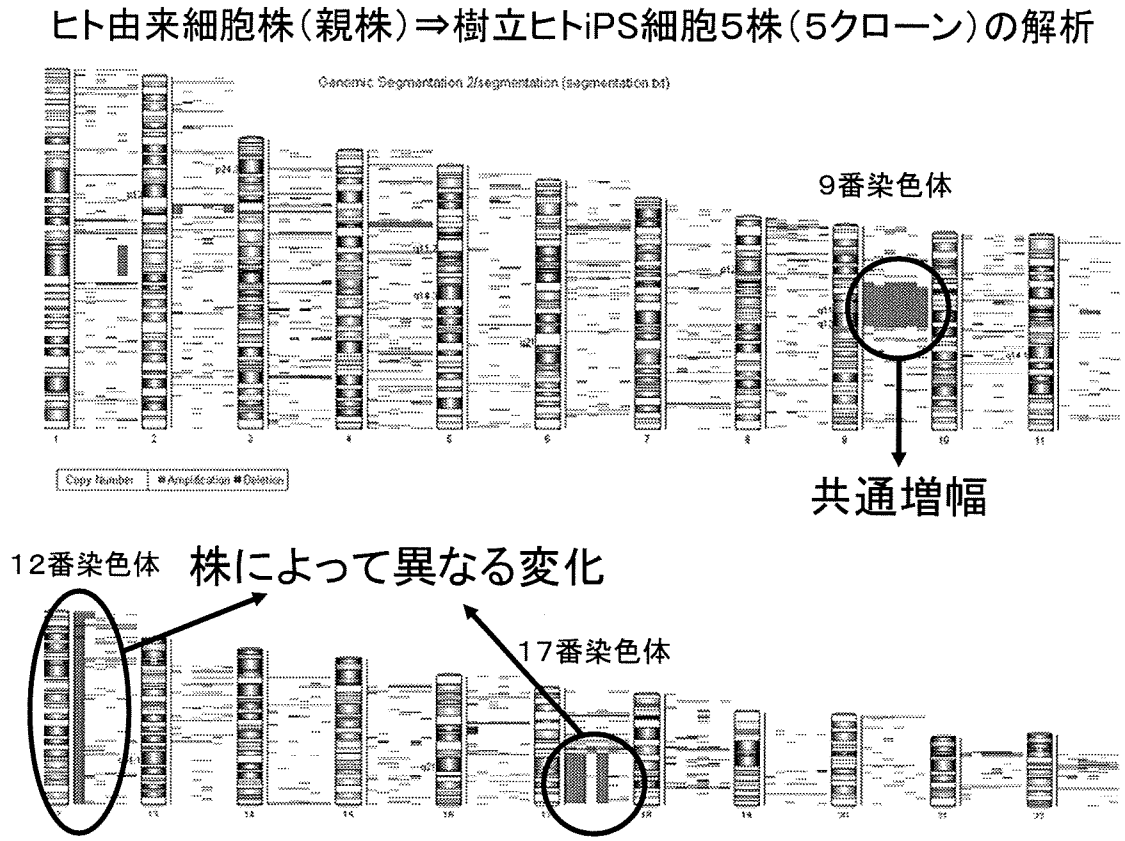

図5．MRC-5 およびヒト iPS 細胞 5 株のアレイ CGH 解析

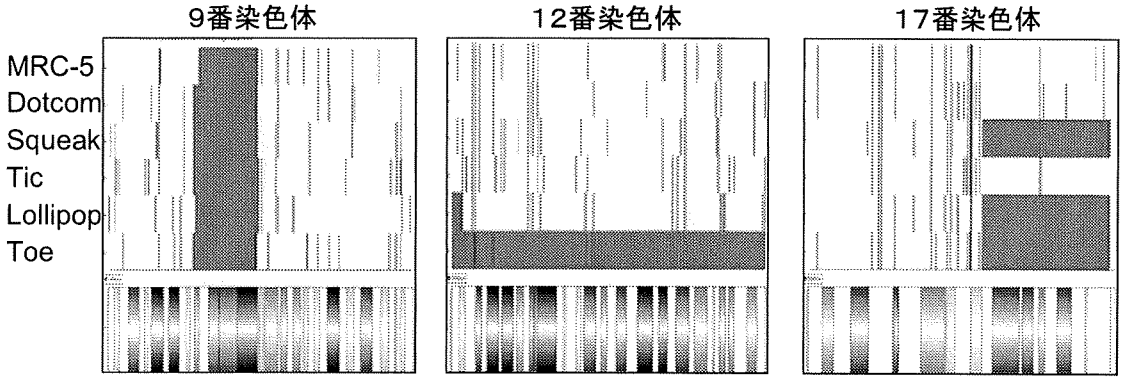

図6．MRC-5 およびヒト iPS 細胞 5 株のアレイ CGH 解析（9 番，12 番，17 番染色体）

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Efficient osteoblast differentiation from mouse bone marrow stromal cells with polylysin-modified adenovirus vectors

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ABSTRACT

Bone marrow stromal cells (BMSCs) are expected to be a source for tissue regeneration because they can differentiate into multiple cell types. Establishment of efficient gene transfer systems for BMSCs is essential for their application to regenerative medicine. In this study, we compared the transduction efficiency in mouse primary BMSCs by using fiber-modified adenovirus (Ad) vectors, and demonstrated that AdK7, which harbors a polylysin (K7) peptide in the C-terminus of the fiber knob, could efficiently express a transgene in BMSCs. Notably, AdK7 robustly drove transgene expression in more than 90% of the BMSCs at 3,000 vector particles/cell. Furthermore, we showed that *in vitro* and *in vivo* osteogenic potential of BMSCs was dramatically promoted by the transduction of Runx2 gene using AdK7. These results indicate that this transduction system could be a powerful tool for therapeutic applications based on BMSCs.

Because bone marrow stromal cells (BMSCs) containing mesenchymal stem cells (MSCs) can be easily isolated from adult tissues and efficiently expanded *in vitro*, and can differentiate into multiple cell types [1,2], BMSCs are expected to be an ideal source of cells for the regeneration of tissues. However, it is difficult to obtain a large amount of pure differentiated cells from BMSCs because of their low differentiation efficiency. The cell transition from stem cells to lineage-committed cells involves many transcription factors that promote or suppress cellular differentiation [3]. Thus, to develop an efficient method for differentiating from BMSCs into specialized cells, we planned to combine the transduction of a functional gene, which promotes cellular differentiation, with stimulation by chemical reagents. To do this procedure, it is essential to develop efficient transduction systems for BMSCs.

Among the various types of gene delivery vectors, adenovirus (Ad) vectors have been widely used for gene transfer studies, since they can achieve high transduction efficiency and transduce both dividing and non-dividing cells [4]. Although Ad vector-mediated transduction into BMSCs has been performed, the transduction efficiency was found to be lower than those of many other cell lines [5,6]. This is due to the low levels of coxsackievirus and adenovirus receptor (CAR), which mediates adenovirus entry, on the cell surface [5,6]. To overcome this problem, we and others have generated several types of fiber-modified Ad vectors, which mediate efficient gene transduction into the cells expressing very low levels of CAR [7,8]. Transduction efficiency was improved in various types of the cells by the insertion of Arg-Gly-Asp (RGD) peptide or 7-tandem lysine residues (KKKKKKK: K7) peptide, which targets αv integrins or heparan sulfates, respectively, on the cell surface, into the fiber knob of the Ad vector [7,8]. In particular, we previously reported that polylysin-modified Ad vector (AdK7) is the most suitable vector for transduction into human bone marrow-derived MSCs (hMSCs) [9].

In this study, we initially investigated the transduction efficiency of mouse primary BMSCs by using fiber-modified Ad vectors. We next examined whether the osteogenic potential of BMSCs was promoted by using Ad vector-mediated transduction of a runt-related transcription factor 2 (Runx2) gene, which is known as a master gene for osteoblastogenesis [10,11].

Materials and methods

Ad vectors. Ad vectors were constructed using an improved *in vitro* ligation method [12,13]. The CA (cytomegalovirus (CMV) enhancer/β-actin promoter) promoter [14]-driven β-galactosidase

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(LacZ)-expressing plasmid, pHMCA-LacZ [15], was digested with I-*Ceu*I/PI-*Sce*I and inserted into I-*Ceu*I/PI-*Sce*I-digested pAdHM15-RGD [16] or pAdHM41-K7 (C) [8], resulting in pAdRGD-CA-LacZ, pAdK7-CA-LacZ, respectively. The CMV or the human elongation factor (EF)-1α promoter-driven LacZ-expressing plasmid, pHMCMV-LacZ [15] or pHMEF-LacZ [15], respectively, was also digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested-pAdHM41-K7 (C), resulting in pAdK7-CMV-LacZ or pAdK7-EF-LacZ, respectively. The CA promoter-driven mouse Runx2-expressing plasmid, pHMCA-Runx2, was generated by inserting a mouse Runx2 cDNA, which is derived from pCMV-Runx2 (a kind gift from Dr. S. Takeda, Tokyo Medical and Dental University, Tokyo, Japan) [17], into pHMCA5. pHMCA-Runx2 was also digested with I-*Ceu*I/PI-*Sce*I, and inserted with pAdHM4 [12] or pAdHM41-K7 (C), resulting in pAd-CA-Runx2 or pAdK7-CA-Runx2, respectively. Ad vectors (Ad-CA-LacZ, AdRGD-CA-LacZ, AdK7-CA-LacZ, AdK7-CMV-LacZ, AdK7-EF-LacZ, Ad-CA-Runx2, and AdK7-CA-Runx2) were generated and purified as described previously [18]. Determination of virus particle (VP) and biological titer were determined using by a spectrophotometrical method [19] and by means of an Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA), respectively. The ratio of the biological-to-particle titer was 1:14 for Ad-CA-LacZ, 1:35 for AdRGD-CA-LacZ, 1:42 for AdK7-CA-LacZ, 1:25 for AdK7-CMV-LacZ, 1:32 for AdK7-EF-LacZ, 1:17 for Ad-CA-Runx2, and 1:28 for AdK7-CA-Runx2.

Mouse primary BMSCs. Primary BMSCs were harvested from female C57BL/6 mice (8 weeks; Nippon SLC, Shizuoka, Japan) as below. Femora and tibias were isolated and placed in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO)/20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin. Bone marrow was obtained by flushing these bones, and cells recovered from the bones of one animal were then seeded into a 150 mm tissue culture plate. Medium was changed every 2 days to remove non-adherent cells, and adherent cells were cultured until reaching confluence. At confluence, BMSCs were passaged after digestion with 0.25% trypsin/1 mM EDTA. BMSCs (passage 4–12) were subsequently used for further analysis.

LacZ assay. BMSCs (1×10^4 cells) were plated in 24-well plates. The next day, they were transduced with the indicated doses of Ad vectors for 1.5 hr. Two days later, X-gal staining and β-gal luminescence assays were performed as described previously [18].

Osteoblasts differentiation. BMSCs (1×10^4 cells) were plated in 24-well plates. Cells were transduced with 3000 VP/cell of Ad vector for 1.5 hr. After aspirating the viral solution, osteogenic differentiation medium, consisting of growth medium (DMEM/20% FBS) containing 50 μg/mL ascorbic acid 2-phosphate (Sigma), 5 mM β-glycerophosphate (Sigma), and 100 nM dexamethasone (Wako, Osaka, Japan), was added. The medium was replaced every 3 days.

von Kossa staining, calcium quantitation. Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) and stained with $AgNO_3$ by the von Kossa method. To measure calcium deposition, cells were washed twice with PBS and decalcified with 0.5 M acetic acid, and cell culture plates were rotated overnight at room temperature (R/T). Insoluble material was removed by centrifugation. The supernatants were then assayed for calcium with the calcium C-test Wako kit (Wako). DNA in pellets was extracted using the DNeasy tissue kit (Qiagen), and calcium content was then normalized to DNA.

ALP assay. Cells were lysed in 10 mM Tris–HCl (pH 7.5) containing 1 mM $MgCl_2$ and 0.1% Triton X-100, and the lysates were then used for assay. Alkaline phosphatase (ALP) activity was measured using the LabAssay ALP kit (Wako) according to the manufacturer's instructions. The protein concentration of the lysates was determined using a Bio-Rad assay kit (Bio-Rad laboratories, Hercules, CA), and ALP activity was then normalized by protein concentration.

RT-PCR. RT-PCR was performed as described previously [18]. The sequences of primers were as follows: Runx2(F), 5′-CCT CTG ACT TCT GCC TCT GG-3′; Runx2(R), 5′-CAG CGT CAA CAC CAT CAT TC-3′; osterix(F), 5′- CTT AAC CCA GCT CCC TAC CC-3′; osterix(R), 5′- TGT GAA TGG GCT TCT TCC TC-3′; bone sialoprotein(F), 5′- AAA GTG AAG GAA AGC GAC GA-3′; bone sialoprotein(R), 5′-GTT CCT TCT GCA CCT GCT TC-3′; osteocalcin(F), 5′-GCG CTC TGT CTC TCT GAC CT -3′; osteocalcin(R), 5′-TTT GTA GGC GGT CTT CAA GC-3′; collagen Iα1(F), 5′-CAC CCT CAA GAG CCT GAG TC-3′; collagen Iα1 (R), 5′-GCT ACG CTG TTC TTG CAG TG-3′, GAPDH(F), 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH(R), 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Western blotting. Western blotting was performed as described previously [18]. Briefly, lysates (20 μg) were subjected to 12.5% polyacrylamide gel and were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking with Immunoblock (DS Pharma Biomedical, Osaka, Japan) at R/T for 1 hr, the membrane was exposed to rabbit anti-Runx2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C overnight, followed by horseradish peroxidase-conjugated secondary antibody at R/T for 1 hr. The band was visualized by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ) and the signals were read using a LAS-3000 imaging system (FUJIFILM, Tokyo, Japan). All blots were stripped and reblotted with antibody against β-actin (Sigma) for normalization.

In vivo heterotopic bone formation. BMSCs (2×10^6 cells, passage 8–9) were transduced with AdK7-CA-LacZ, AdK7-CA-Runx2, or Ad-CA-Runx2, at 3000 VP/cells for 1.5 hr. The next day, cells were collected by trypsin, and resuspended in 150 μl of PBS, and then injected into the hind limb biceps muscle of nude mice (Nippon SLC) (2animal/ group). At 4–5 weeks after injection, mice were anesthetized by isofluorane and bone formation was analyzed with a microcomputed tomography (microCT) system (eXplore Locus CT System; GE Healthcare, London, ON, Canada). Both an X-ray image and a three-dimensional reconstitution image were obtained by using the microCT system.

Results

Optimization of transduction efficiency in BMSCs by using various types of Ad vectors

To optimize Ad vectors for transduction into BMSCs, we prepared three LacZ-expressing Ad vectors, Ad-CA-LacZ, AdRGD-CA-LacZ, and AdK7-CA-LacZ. We investigated the transduction efficiency of these Ad vectors in BMSCs at the indicated vector dose. X-gal staining showed that LacZ-positive cells were less than 10% even at a dose of 3000 vector particles (VP)/cell in Ad-CA-LacZ (Fig. 1A). On the other hand, more than 90% of the cells expressed LacZ at the same dose in AdK7-CA-LacZ. A luminescence assay revealed that, at 3000 VP/cell, the LacZ expression level in the cells transduced with AdRGD-CA-LacZ or AdK7-CA-LacZ was increased by about 5- or 50-fold, respectively, in comparison with that in the cells transduced with Ad-CA-LacZ (Fig. 1B). These results were quite similar to those of our previous report, in which efficient transduction in hMSCs was achieved by using AdK7 [9], and our data clearly demonstrated that AdK7 is a suitable vector for transduction into both mouse BMSCs and hMSCs.

We and others reported that the choice of promoters is important for transduction efficiency, especially in immature cells [15,18,20,21]. Thus, we examined the transduction efficiency by comparing the promoter activities in BMSCs. In addition to the CA promoter, we prepared LacZ-expressing AdK7 under the control of the CMV promoter or the EF-1α promoter (AdK7-CMV-LacZ or AdK7-EF-LacZ, respectively). A luminescent assay showed that the CA promoter represented the highest transgene expression

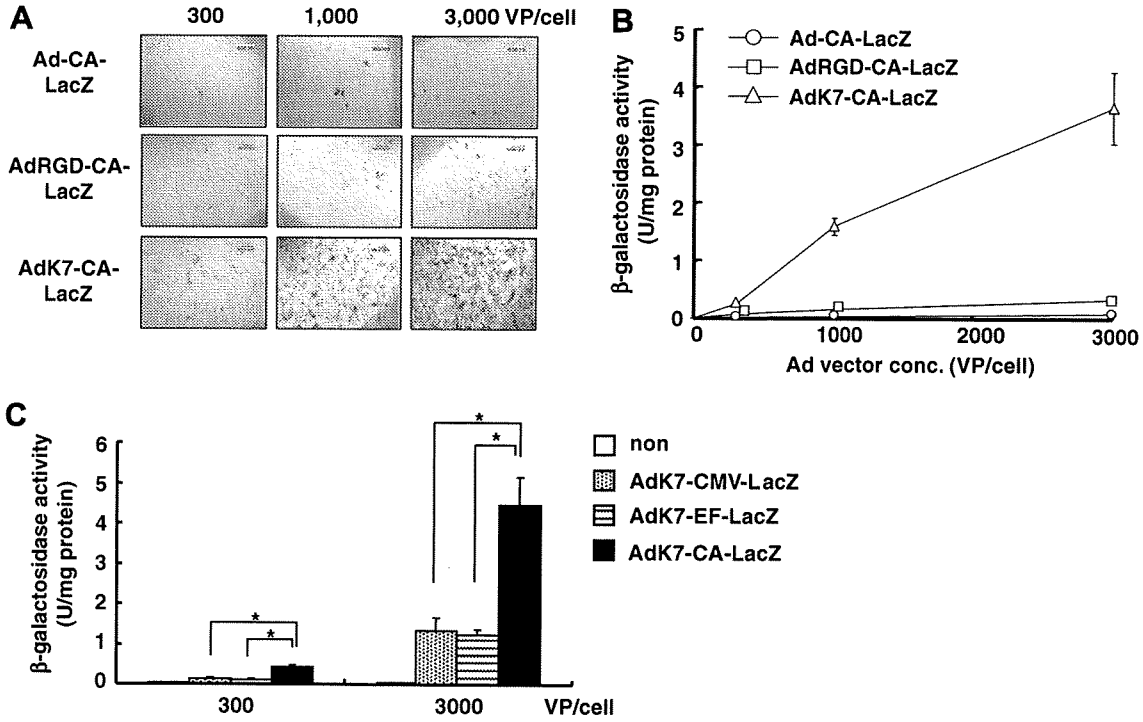

Fig. 1. Gene transduction efficiency in mouse primary BMSCs by various types of Ad vectors. Mouse BMSCs were transduced with the indicated doses of LacZ-expressing Ad vectors. Two days later, (A) X-gal staining and (B) luminescence assay were performed. Similar results of X-gal staining were obtained in three independent experiments. Scale bar indicates 200 μm. (C) Optimization of promoter activity in BMSCs using LacZ-expressing AdK7. BMSCs were transduced with the indicated dose of each Ad vector, and LacZ expression in the cells was measured. The data are expressed as mean ± S.D. ($n = 3$). $^{*}p < 0.01$.

among the three types of the promoters (Fig. 1C). These results demonstrate that AdK7 containing the CA promoter is the most effective at attaining high transduction efficiency in mouse BMSCs.

We also investigated the cytotoxicity in BMSCs transduced with AdK7-CA-LacZ. Almost no difference in cell number between non-transduced cells and AdK7-CA-LacZ-transduced cells was observed on day 2 after transduction (data not shown), indicating that AdK7 is an excellent vector with high transduction activity and low cytotoxicity in BMSCs.

Efficient osteoblast differentiation in vitro and in vivo by fiber-modified Ad vectors

Because an efficient method for transduction into BMSCs could be established by using AdK7 containing the CA promoter, we expected that efficient differentiation into specialized cells from BMSCs might be achieved by using this Ad vector. To test this, we generated mouse Runx2-expressing Ad vectors, AdK7-CA-Runx2 and Ad-CA-Runx2, because a Runx2 gene is both necessary and sufficient for mesenchymal cell differentiation towards osteoblast lineage [3]. Western blot analysis showed that Runx2 protein levels in AdK7-CA-Runx2-transduced cells were quite higher than those in non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells (Fig. 2).

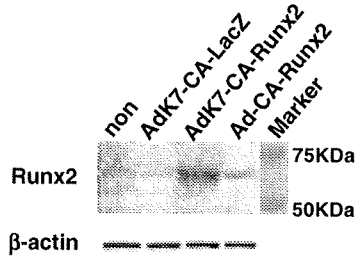

Fig. 2. Runx2 expression in Ad vector-transduced BMSCs. Cell lysates were isolated from BMSCs 2 days after the transduction, and Western blotting was performed.

We next assessed osteoblast differentiation by measuring alkaline phosphatase (ALP) activity, which is a marker of early osteoblast differentiation. After transduction with Ad vector, BMSCs were cultured in osteogenic differentiation medium for the indicated number of days. As shown in Fig. 3A, the ALP activity levels in AdK7-CA-Runx2-transduced cells were extremely increased in comparison with control cells. Notably, AdK7-CA-Runx2 mediated approximately 50-fold higher ALP activity than non-transduction or AdK7-CA-LacZ on day 5 after transduction. These results indicated that early osteoblast differentiation of BMSC was facilitated by AdK7-CA-Runx2. Because mature osteoblasts are known to be specialized in the production of extracellular matrix and the mineralization [22], we next examined the matrix mineralization in BMSCs. von Kossa staining revealed that matrix mineralization in AdK7-CA-Runx2-transduced cells was dramatically increased in comparison with non-, AdK7-CA-LacZ, or Ad-CA-Runx2-transduced cells (Fig. 3B, left). Furthermore, we observed a significant elevation of calcium deposition in AdK7-CA-Runx2-transduced cells even on day 5 after transduction, while neither non-transduced cells nor AdK7-CA-LacZ-transduced cells showed mineralization until day 15 (Fig. 3B, right). Ad-CA-Runx2 mediated slightly higher levels of calcium deposition than non-transduced or AdK7-CA-LacZ-transduced cells, but significantly lower levels than AdK7-CA-Runx2-transduced cells. Additionally, we found that the expression levels of marker genes characteristic of osteoblast differentiation, such as Runx2, osterix, bone sialoprotein, osteocalcin, and type I collagen, were also increased in AdK7-CA-Runx2-transduced cells (Fig. 3C). These results demonstrated that a conventional method using only osteogenic differentiation medium is

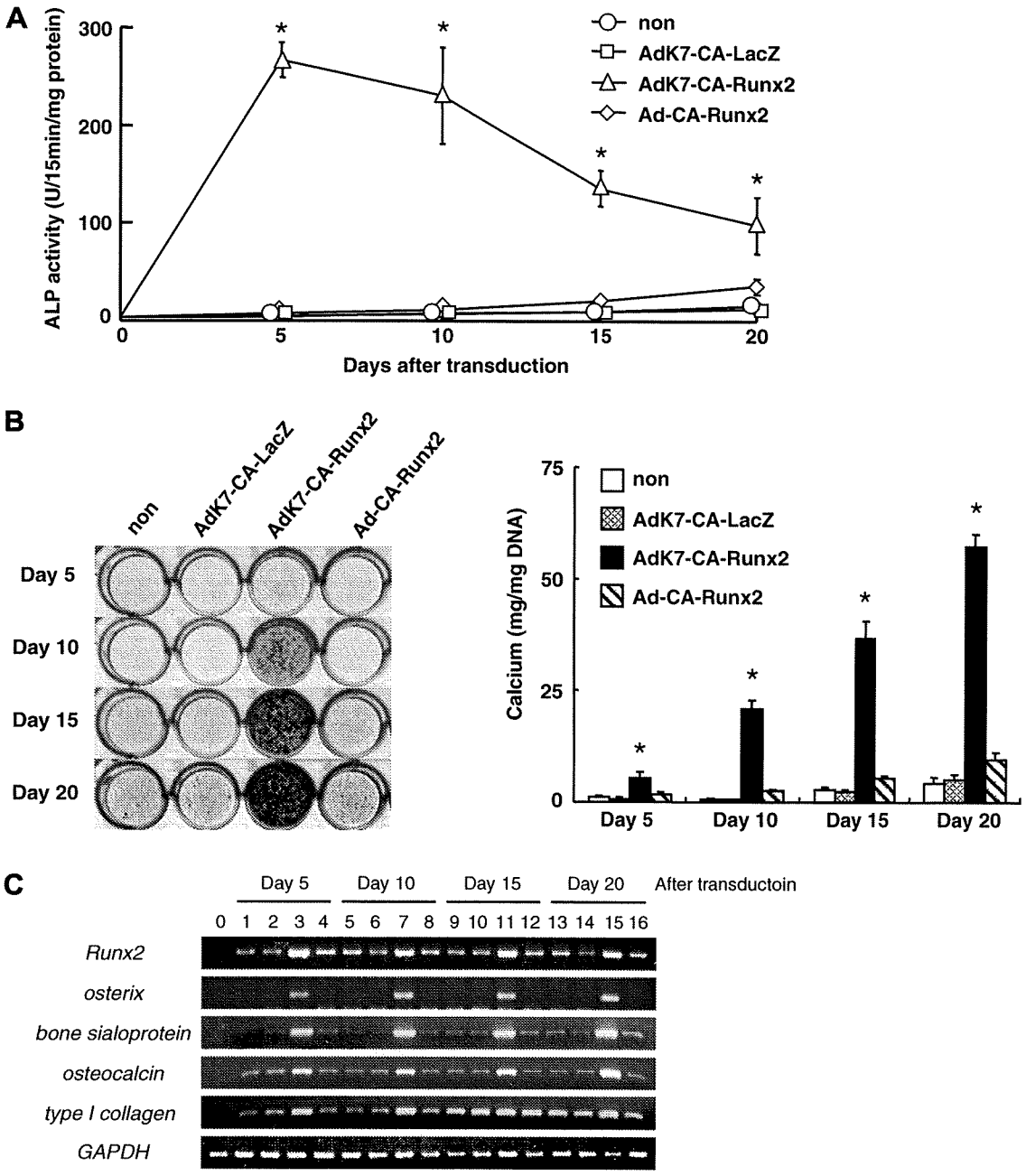

Fig. 3. Promotion of *in vitro* osteoblastic differentiation in AdK7-CA-Runx2-transduced BMSC. After transduction with each Ad vector at 3000 VP/cell for 1.5 hr, BMSCs were cultured for the indicated number of days. (A) ALP activity, (B, left) matrix mineralization, and (B, right) calcium deposition in the cells was determined. The data are expressed as mean ± S.D. ($n = 3$). * $p < 0.01$ as compared with non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells. (C) RT-PCR was performed using primers for Runx2, osterix, bone sialoprotein, osteocalcin, collagen type I, and GAPDH. Lane 0: non-treated BMSCs; lanes 1, 5, 9, and 13: BMSCs with osteogenic supplements (OS); lanes 2, 6, 10, and 14: BMSCs with OS plus AdK7-CA-LacZ; lanes 3, 7, 11, and 15: BMSCs with OS plus AdK7-CA-Runx2; lanes 4, 8, 12, and 16: BMSCs with OS plus Ad-CA-Runx2.

not enough for efficient osteoblast differentiation, and that, by efficient Runx2 transduction using AdK7, osteoblastogenesis of BMSCs could be dramatically accelerated *in vitro*.

Finally, to examine whether the increased levels of Runx2 expression in BMSCs could enhance the osteogenic potential of BMSC *in vivo*, BMSCs transduced with each Ad vector were injected into the hind limb biceps muscle of nude mice. Microcomputed tomography analysis revealed that no bone formation was observed in non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells, while new bone was detected in mice injected with AdK7-CA-Runx2-transduced cells (Fig. 4), indicating that AdK7-CA-Runx2-transduced BMSCs efficiently differentiated into mature osteoblasts *in vivo*. These results clearly showed that AdK7-CA-Runx2 could facilitate the osteogenic potential of BMSCs both *in vitro* and *in vivo*.

Discussion

Because genetic manipulation is considered to be a powerful tool to promote cellular differentiation, it is necessary to establish efficient methods for transduction into BMSCs. Many researchers have reported that transduction efficiency of rat or human MSC was increased by using fiber-modified Ad vectors, such as AdRGD or Ad vectors containing Ad35 fiber knob and

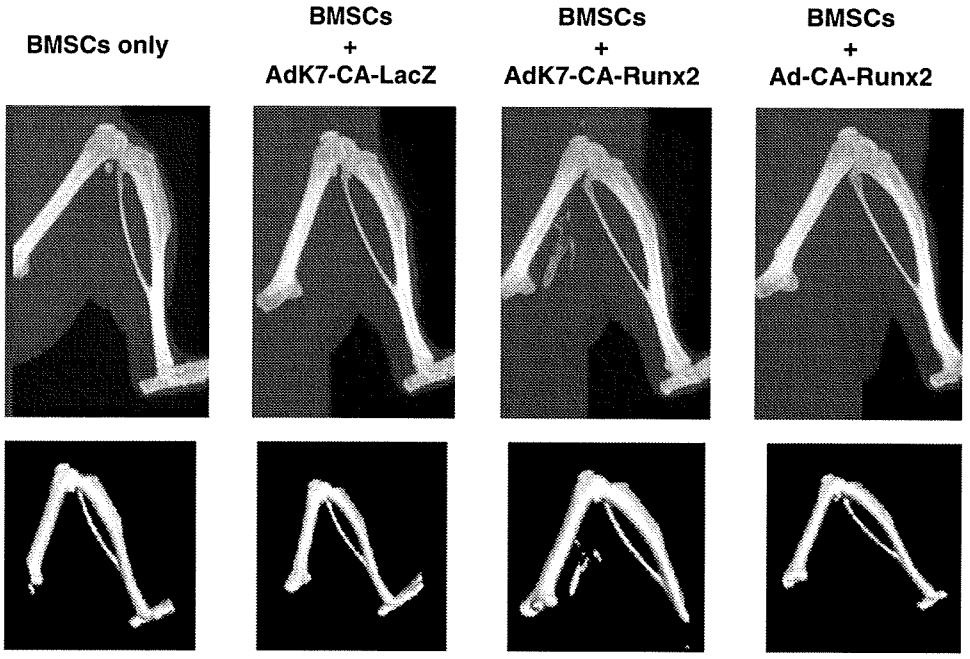

Fig. 4. *In vivo* ectopic bone formation of mouse BMSCs by AdK7-mediated Runx2 gene transduction. BMSCs were transduced with indicated Ad vectors at 3000 VP/cell. On the following day, cells were injected into the hind limb biceps muscle of nude mice. Four weeks later, bone formation was analyzed by the microCT system. Similar results were obtained in two independent experiments. Upper: X-ray images; lower: 3D reconstitution images.

shaft (AdF35) [23–25]. In this study, we demonstrated that AdK7 could express a transgene in BMSCs more efficiently than conventional Ad vector or AdRGD (Fig. 1A and B). Similarly, we have previously shown that the highest transduction efficiency in hMSC could be achieved by using AdK7, but not AdRGD or AdF35 [9]. Therefore, our data indicate that AdK7 is the most appropriate vector for various mesenchymal cells. We also found that the CA promoter showed higher gene expression in BMSCs than did the CMV or EF-1α promoter (Fig. 1C). This appears to be due to the potent activity of the CA promoter in immature cells [18,20]. Hence, we conclude that AdK7 containing the CA promoter is the most suitable vector for transduction into BMSCs.

We demonstrated that osteoblastogenesis of BMSCs was dramatically promoted by using AdK7-mediated Runx2 transduction (Figs. 3 and 4). This is the first study to report the usefulness of AdK7 in the field of stem cell differentiation. Runx2 is known to regulate osteoblastogenesis by controlling the expression of multiple osteoblast marker genes [10]. Because Runx2 protein and mRNA were highly expressed for more than 20 days in AdK7-CA-Runx2-trasduced cells (Figs. 2 and 3C), the expression of marker genes and ALP activity would be increased and would thereby enhance both *in vitro* and *in vivo* osteogenic ability. On the other hand, osteoblast differentiation could not be facilitated by AdK7-CA-Runx2 when osteogenic supplements were removed (data not shown), suggesting that osteogenic supplements were required for matrix mineralization, although differentiation efficiency was low when using only osteogenic supplements. Thus, efficient osteoblast differentiation of BMSCs would be achieved by the synergistic effect of both osteogenic supplements and efficient Runx2 transduction.

Unlike the case with AdK7-CA-Runx2, almost no osteoblast differentiation was seen in Ad-CA-Runx2-transduced cells. However, several groups reported that the osteogenic potential of MSCs was enhanced by Runx2 transduction using the conventional Ad vectors [26,27]. This difference would be attributable to the difference in transduction efficiency in BMSCs using the conventional Ad vector, because they showed that approximately 30–40% of the cells expressed transgenes by conventional Ad vector at 250–500 infectious units (ifu)/cell. Although we could not obtain high transduction efficiency using the conventional Ad vector, we showed that more than 90% of the cells were transduced by using AdK7-CA-LacZ at only 71 ifu/cell (3000 VP/cell) (Fig. 1A), without any decrease in viability (data not shown). Our results indicate that vector doses can be reduced by using AdK7, leading to a decrease in cytotoxicity to the cells. Therefore, AdK7, but not other fiber-modified Ad vectors or conventional Ad vectors, would contribute to safe regenerative medicine procedures.

In summary, we succeeded in developing efficient methods both for transducing mouse BMSCs and differentiating osteoblasts from BMSCs. Recently, many researchers have reported that mesenchymal stem/stromal cells could be isolated from adipose or placental tissues [28,29]. Because these mesenchymal cells are shown to possess mostly the same properties as BMSCs, AdK7 could probably be applied to these cells. Thus, our transduction methods can be a valuable tool for therapeutic applications based on adult mesenchymal stem/stromal cells.

Acknowledgments

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

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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

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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

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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

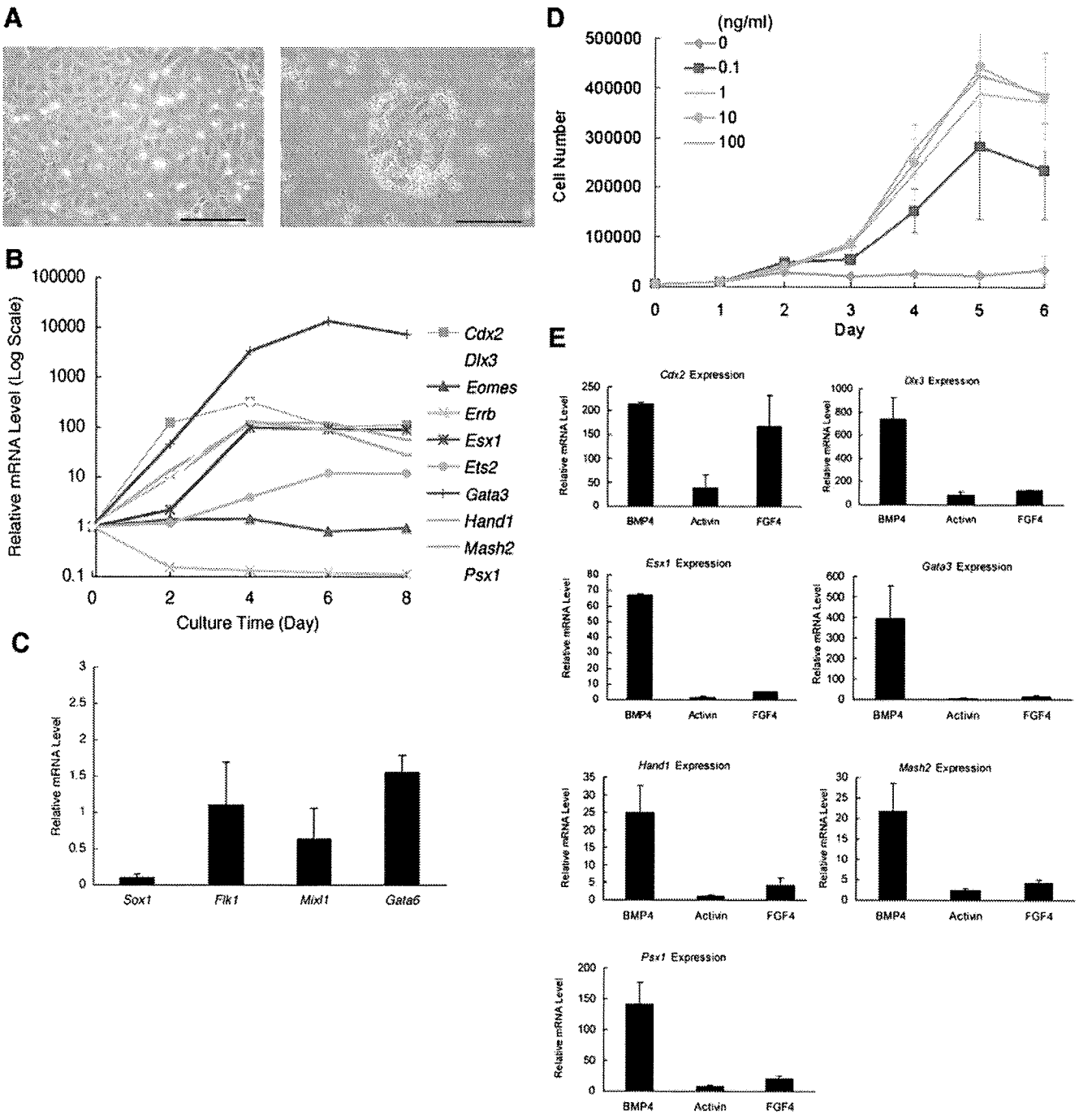

Figure 1. The effect of BMP4 on mESCs in defined culture conditions. (*A*) phase contrast photomicrographs of differentiated mESCs cultured in BMP4-supplemented ESF5 medium for 4 d (*left*) and 8 d (*right*). *Scale bars* are 100 μm. (*B*) quantitative real-time RT–PCR analysis of trophoblast-specific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

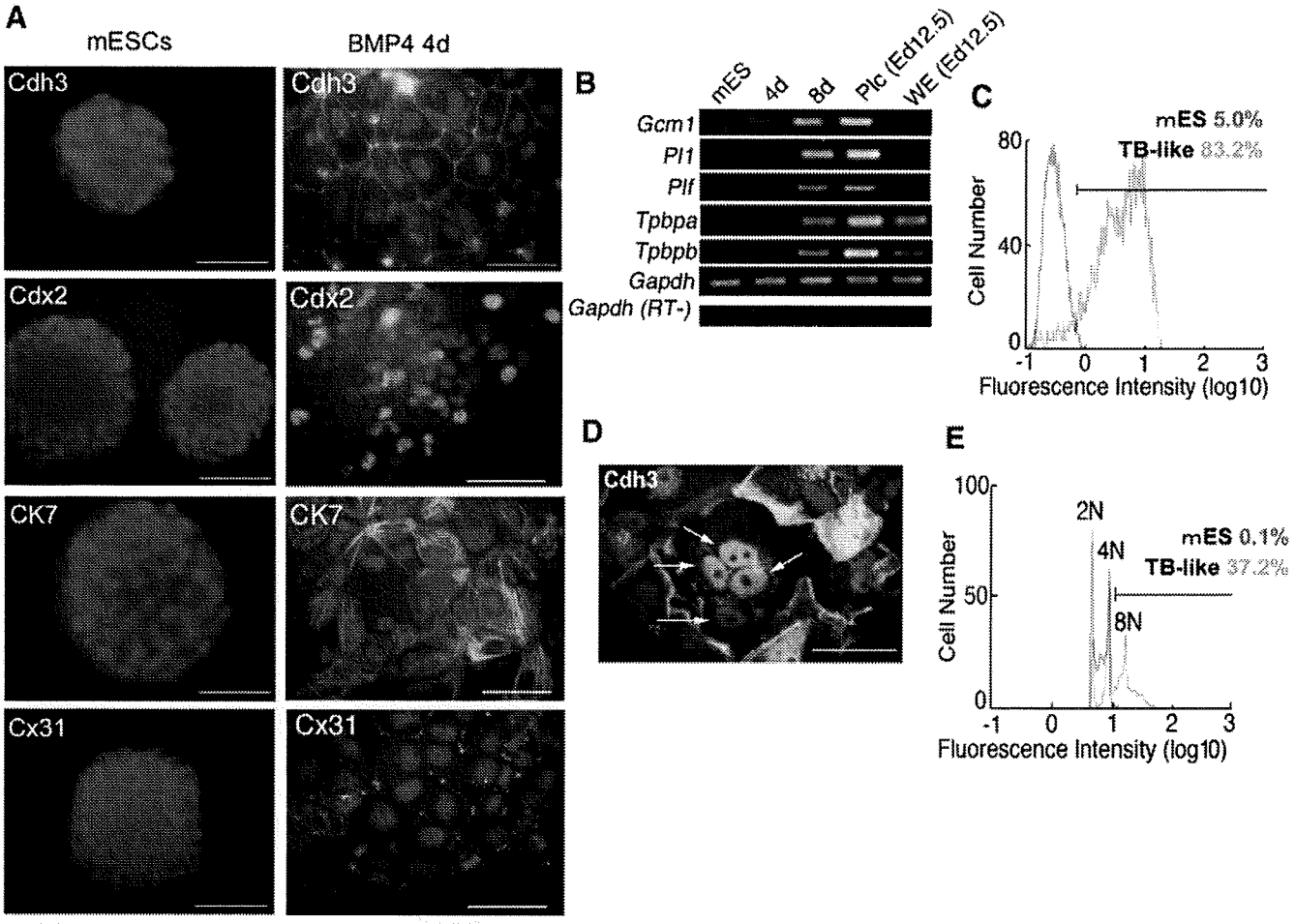

Figure 2. Differentiation into trophoblast from mESCs in vivo. (*A*) Immunocytostaining with trophoblast marker antibodies of the cells cultured in BMP4-supplemented ESF5 medium for 4 d (*right*) or undifferentiated mESCs (*left*). Immunoreactivity of Cdh3 (*upper left*), Cdx2 (*upper right*), CK7 (*lower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

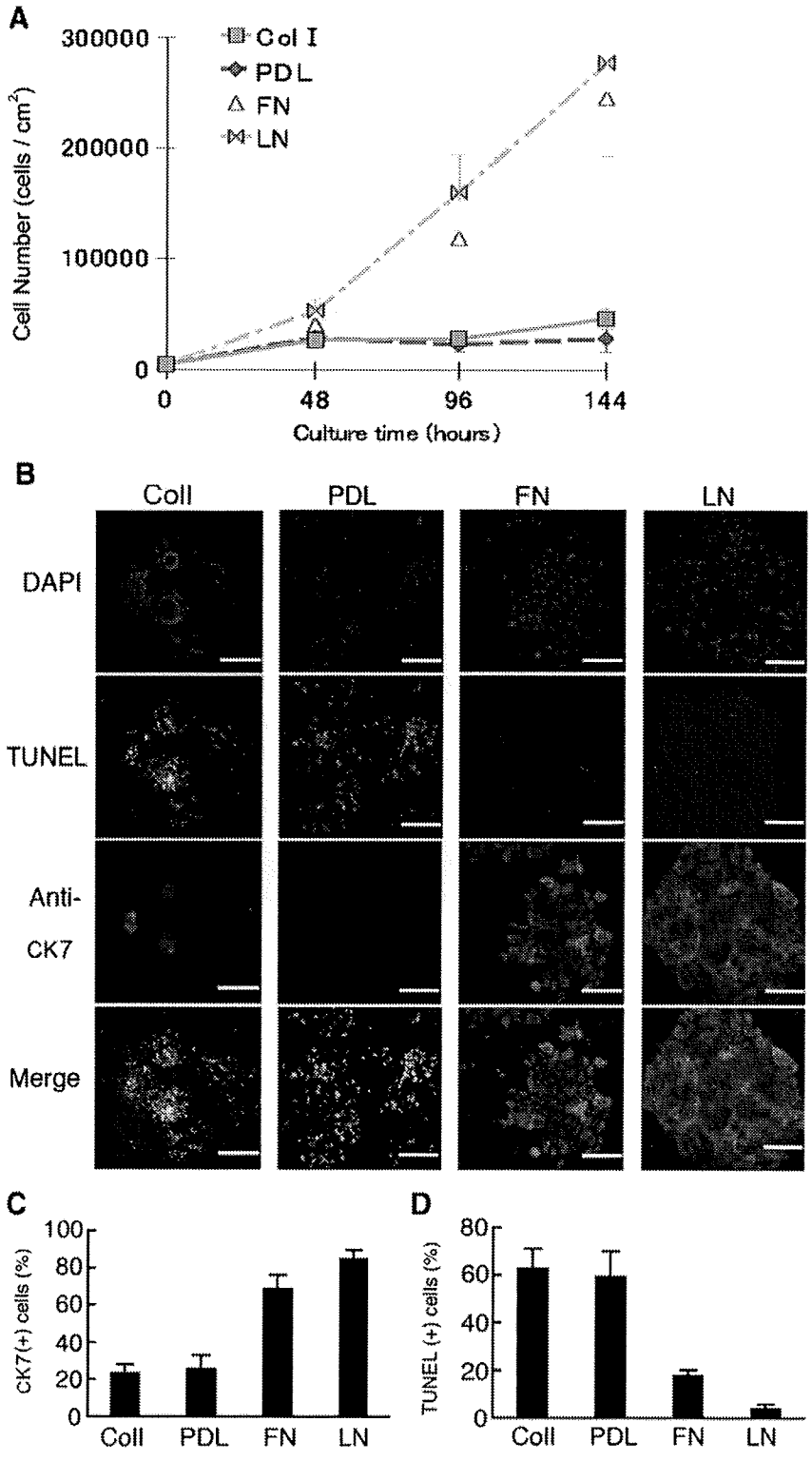

Figure 3. The effect of ECM components on the differentiation into trophoblast from mESCs. (*A*) Proliferation of differentiating mESCs on various ECM components. mESCs were seeded in a 24-well dish at 5×10^3 cells per well on each ECM component in ESF5 medium supplemented with BMP4. Cells were counted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

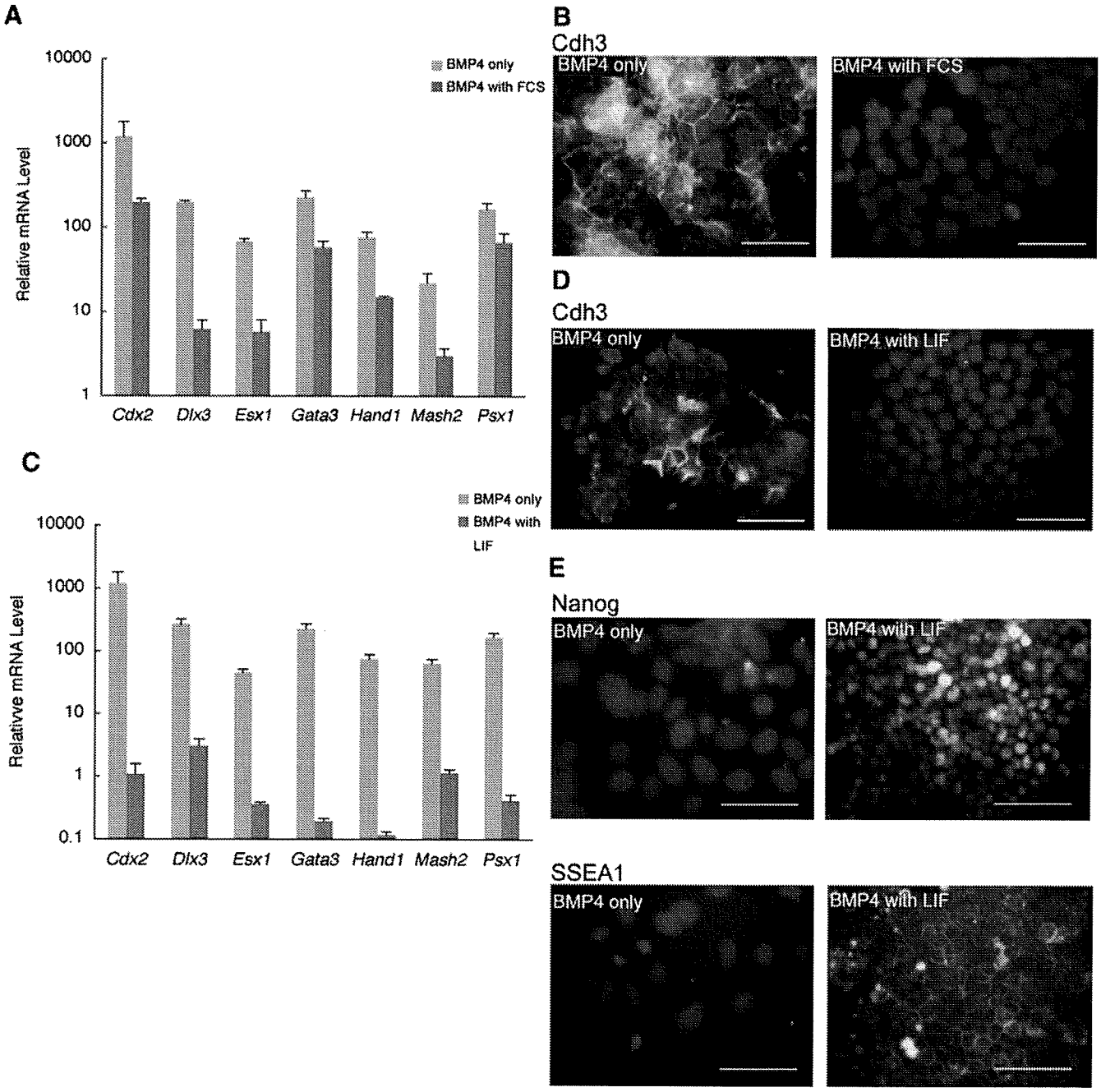

Figure 4. Effect of FCS and LIF on the differentiation into trophoblast. The effect of FCS: the cells were cultured in BMP4-supplemented ESF5 medium with 10% FCS (indicating as "BMP4 with FCS") or without FCS (indicating as "BMP4 only") for 4 d. (*A*) Quantitative real-time RT–PCR analysis of the expression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

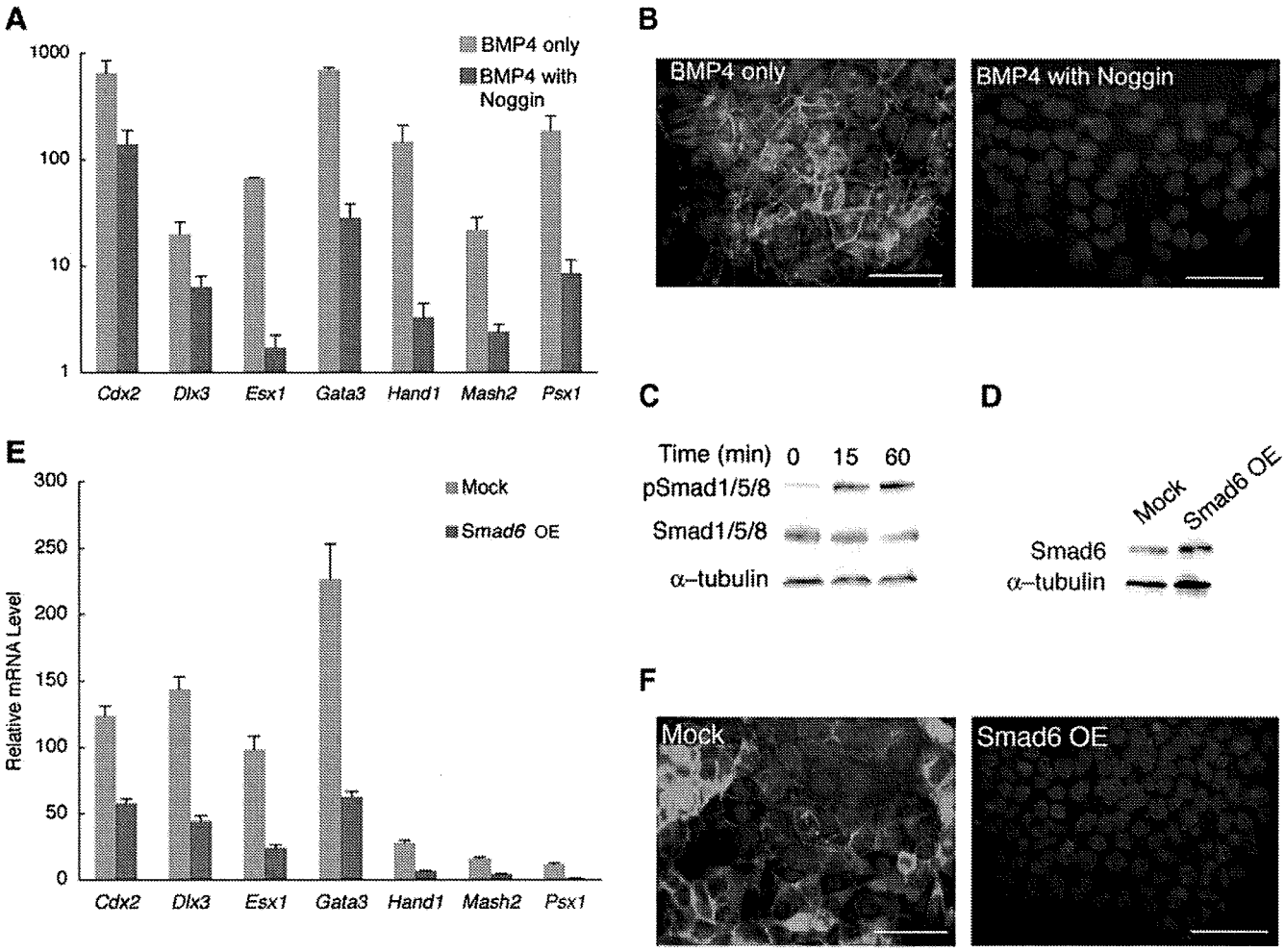

Figure 5. The effects of Noggin, inhibitory Smad6 on the BMP4-induced differentiation of mESCs into trophoblast. The effect of Noggin: the cells were cultured in BMP4-supplemented ESF5 medium with 300 ng/ml of Noggin (indicating as "BMP4 with Noggin") or without Noggin (indicating as "BMP4 only") for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

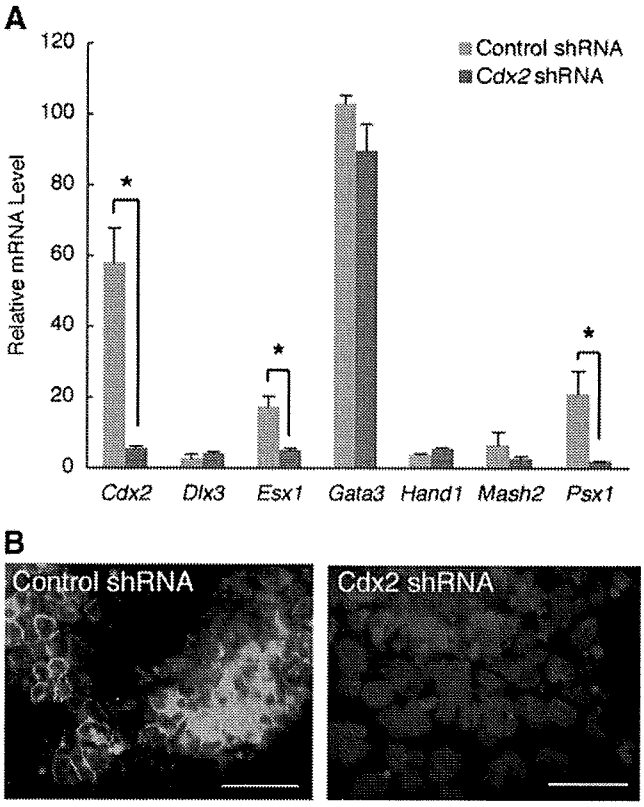

Figure 6. The effects of shRNA against *Cdx2* on the BMP4-induced differentiation of mESCs into trophoblast. The cells carrying shRNA-expressing vectors against *Cdx2* (indicating as "*Cdx2* shRNA") or control shRNA-expressing vectors (indicating as "Control shRNA") were cultured in BMP4-supplemented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