Bone morphogenetic protein 4 accelerates the establishment of bovine trophoblastic cell lines

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Abstract

Trophoblastic cells play a crucial role in implantation and placentogenesis. A large proportion of the failures of conception in cows occur in the peri-implantation period, which are known as early embryo losses. In exploring this critical phenomenon, trophoblastic cell lines can provide substantial information. Unfortunately, there are few cell lines for this purpose in cattle because of the difficulty of raising successive cell stock in the long term. In this study, 12 new cell lines were established using bone morphogenetic protein 4 (BMP4). BMP4 stimulated embryonic cells to enter the trophoblastic cell lineage but there were no significant differences between intact and BMP4-treated groups. Only one out of 49 embryos developed trophoblastic cells in the intact group. Finally, 12 cell lines were maintained for around 30 passages, and they retained trophoblastic characteristics and expressed bovine trophoblastic genes: placental lactogen, interferon- τ , pregnancy-associated glycoprotein 1, and prolactin-related protein 1. Although the gene expression patterns were different among cell lines and depended on the cells, there was no significant relationship between the expression intensities of genes and the treatment dose of BMP4. All of them expressed bovine POU domain class 5 transcription factor 1 and caudal-type homeobox 2. The expression of these genes was confirmed by quantitative RT-PCR and immunohistochemical detection. These results suggest that BMP4 is involved in the raising of trophoblast cell lines from early embryonic cells and the newly developed cell lines can provide different types of bovine trophoblastic cells with different cell lineages. This may constitute a significant new tool for the examination of trophoblastic differentiation.

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Introduction

Trophoblastic cells play a major role in placentogenesis and produce various kinds of biological substances, including cytokines, growth factors, peptides, and steroid hormones (Spencer *et al.* 2008, Marikawa & Alarcón 2009, Tsampalas *et al.* 2010). The physiological functions of trophoblastic cells have been studied; however, their functions have remained unclear because of the lack of definitive information on cell lineages. Trophoblastic cells arise from early embryonic cells, namely, the outerside of the blastomere at the blastocyst stage. They are the first differentiated cells in the embryo and may have the specific potential to act as stem cells. Trophoblastic cells act in the formation of placental villi and direct the exchange of fetal–maternal information. A primitive trophoblastic cell adopts a certain cell lineage for proliferation and differentiation (Hu & Cross 2010).

In ruminants, a mononucleate trophoblast cell (MNC) may directly differentiate into a binucleate cell (BNC) without cytokinesis and the BNC then fuses with an endometrial epithelial cell to form a trinucleate cell (Nakano *et al.* 2002, Fléchon *et al.* 2007, Ullah *et al.* 2009). This event is the start of implantation in ruminants; during the peri-implantation period, the MNC and BNC (or multinucleate) cells produce various molecules for placentogenesis (Spencer *et al.* 2004). The functions and mechanism of differentiation of these trophoblastic cells are obscure. Suitable trophoblastic cell lines are useful tools to clarify the specificity and complex mechanisms of placental formation *in vitro*. However, there are few available bovine trophoblastic cell lines. Various new kinds of cell lines will help in improving the understanding of the functions of trophoblastic cell lineages (Talbot *et al.* 2000, 2008, Shimada *et al.* 2001, Michael *et al.* 2006, Hambruch

et al. 2010). In cattle, trophoblastic cells may have the potential to induce differentiation for different functional features of cells. For example, trophoblastic mononucleate cells have been shown to produce interferon- τ (IFNT), which is a factor that signals recognition of pregnancy in cattle, the same as in other ruminants (Martal *et al.* 1979, Imakawa *et al.* 1987). On the other hand, BNCs produce various molecules – steroid hormones, placental lactogen (PL, also known as CSH1), prolactin-related protein (PRPs), and pregnancy-associated glycoproteins (PAGs), among others (Reimers *et al.* 1985, Hashizume *et al.* 2007) – and take a main role in the fetal–maternal interface (Duello *et al.* 1986). These facts suggest that the establishment of new trophoblast cell lines provide clue as a necessary tools for studying trophoblastic cell lineage. Although we have been developing trophoblastic cell lines from early bovine embryos, the efficiency of generation of these cell lines has been limited (Shimada *et al.* 2001).

Bone morphogenetic protein 4 (BMP4) is a member of the transforming growth factor- β (TGFB) superfamily, which consists of multifunctional growth factors that act through specific signaling pathways, the SMAD pathways. BMP4 has been detected in various reproductive tissues including placenta, ovary, pituitary, uterus, primordial germ cell, etc.; it takes a crucial role in the epiblast-derived tissues and placenta with other TGFB superfamily members (Shimasaki *et al.* 2004). In fact, the protein plays important roles in the development of the early embryo and is a stimulatory factor for trophoblastic cell lineages (Xu *et al.* 2002, Das *et al.* 2007, Wu *et al.* 2008, Hayashi *et al.* 2010); however, its effects are still unclear (Kobayashi *et al.* 2008, Zhang *et al.* 2008). Various factors including BMP4 and transcription factors seem to participate in the differentiation of the trophoblastic cell lineage (Fujiwara *et al.* 2002, Babaie *et al.* 2007, Murohashi *et al.* 2010). These previous reports urged us to examine the roles of BMP4 in the differentiation of early embryonic cells to trophoblastic lineage in cow. In this study, we examined whether BMP4 stimulates raised trophoblastic cell lines in early embryo culture and analyzed the specificity of individual cell lines.

Results

Production of recombinant bovine BMP4 and its biological activity

First, bovine BMP4 cDNA was cloned using the fetal membrane on day 26 of gestation. Then, recombinant bovine BMP4 (rbBMP4) was produced using a cell-free protein expression system. Finally, after 0.45 mg purified and refolded BMP4 protein was collected as shown in Fig. 1a, we examined BMP biological activity using C2C12 myoblasts, which was evaluated histochemically on the basis of ALP activity. rbBMP4 (80 ng/ml)

Figure 1 Features and activity of rbBMP4. (a) Western blotting analysis of rbBMP4. Panel A shows results using monoclonal anti-BMP4 antibody. Lane 1: human BMP4, lane 2: bovine BMP4, and lane 3: molecular weight markers. MAB recognized two bands: a major band at 12 kDa and a minor band at 24 kDa, in human BMP4. In bovine BMP4, there were three bands: a major band at 14 kDa and minor bands at 29 and 11 kDa. Panel B shows polyclonal anti-BMP4 antibody profiles. Lane 1: molecular weight markers, lane 2: human BMP4, and lane 3: bovine BMP4. This antibody recognized components corresponding to 12 and 14 kDa for human and bovine BMP4 respectively. (b) ALP activity of rbBMP4. The assay was repeated three times, and activity is shown as fold change of the value of control (87.4 ± 4.8 nmol pNp/min per mg BMP4 protein, $n=3$).

possessed 162.3 ± 22.5 (mean $\pm$ s.d., $n=3$) ALP activity (nmol pNp/min per mg BMP4 protein), which was 1.8-fold that of non-BMP4-containing medium as a control (87.4 ± 4.8 , $n=3$). The ALP activity of rbBMP4 was increased in a dose-dependant manner. The activity was similar to that of human BMP4 (Fig. 1b).

Cell growth

About ten IVF-derived blastocysts in each group were individually cultured in a well. The culture procedure was repeated four times, and a total of about 40 blastocysts per BMP4 treatment were submitted to the culture for the establishment of cell lines. Then, more than 30 cell clumps were spread out in 2 days after transfer (Table 1) with no significant difference among the BMP4-treated groups, but with the control

Table 1 Effects of bone morphogenetic protein 4 (BMP4) on the development of trophoblastic cell lines.

BMP4 (ng/ml)	Attachment (%)	Spread (%)	Growing ratio (%)					Name of cells
			PDL5	PDL10	PDL15	PDL20	PDL25	
Control (0)	63.2 (31/49)	57.1 (28/49)	57.1 (28/49)	37.6 (18/49)	2 (1/49)	2 (1/49)	2 (1/49)	BT-D
1	85.3 (35/41)	80.4 (33/41)	80.4 (33/41)	46.3 (19/41)	7.3 (3/41)	7.3 (3/41)	7.3 (3/41)	BT-A, BT-E, BT-F
10	80.4 (33/41)	75.6 (31/41)	75.6 (31/41)	46.3 (19/41)	12.2 (5/41)	7.3 (3/41)	7.3 (3/41)	BT-B, BT-C, BT-G
100	78 (32/41)	75.6 (31/41)	75.6 (31/41)	41.5 (17/41)	12.2 (5/41)	12.2 (5/41)	12.2 (5/41)	BT-H, BT-I, BT-J, BT-K, BT-L

Numbers in parentheses indicate no. of developed embryos/no. of embryos used.

(non-BMP4). The non-BMP4 group exhibited significantly lower rates of both attachment and spread ($P < 0.05$). All spread cell clumps were kept to raise cell lines and the survival rates of cell clumps in all groups were gradually decreased to the 25th passage, which were estimated the same as the population doubling level (PDL) stage. In total, 12 cell lines were raised from the different treatment groups. More cell lines were established in higher dose groups; however, there were no significant differences among the groups, including the control. We named the 12 bovine trophoblastic cell lines as BT-A to BT-L in temporal sequence.

Analysis of gene expression profiles

The expression of seven specific genes (bovine *CSH1*, bovine *PRP1*, bovine *PAG1*, bovine *IFNT*, POU class 5 homeobox 1 (*POU5F1*), caudal-type homeobox transcription factor 2 (*CDX2*), and *BMP4*) were analyzed in 12 new cell lines using quantitative PCR (qPCR; Fig. 2a–g). All cell lines expressed *POU5F1*, *CDX2*, and *BMP4* genes, but the intensity depended on the cell line. There were no significant correlations between the rate of establishing cell lines and the dose of BMP4 or the intensity of *BMP4* expression (Table 1 and Fig. 2e). The cell lines were divided into four groups according to the expression of specific genes, *CSH1*, *PRP1*, *PAG1*, and *IFNT*: group 1: no expression of four trophoblastic cell-specific genes (BT-B); group 2: all four trophoblastic cell-specific genes were expressed (BT-C, BT-E, BT-F, BT-H, and BT-J); group 3: BNC-specific genes were expressed, namely, *CSH1*, *PRP1*, and *PAG1*, but not *IFNT* (BT-D, BT-I, BT-K, and BT-L); and group 4: primarily *IFNT* was expressed (BT-A and BT-G).

Expression of trophoblastic cell-specific proteins

Bovine trophoblastic cell-specific proteins, *CSH1* and *IFNT*, were detected by Western blot and/or immunocytochemical methods. Anti-*CSH1* antibody clearly detected the protein in eight cell lines, BT-C, BT-D, BT-E, BT-F, BT-I, BT-J, BT-K, and BT-L (Fig. 3a–c and Table 2). *IFNT* proteins were detected in seven cell lines, BT-A, BT-C, BT-E, BT-F, BT-G, BT-H, and BT-L (Figs 3, 4 and Table 2). *CSH1* protein was found in BNCs and *IFNT*

was found in MNCs. Western blotting data confirmed *IFNT* expression in most of the cell lines, except for BT-E and BT-G (Fig. 4). These results coincided with the gene expression profiles, except for BT-H, BT-J, and BT-L. *POU5F1* and *CDX2* protein expressions were examined using the representative cell line, BT-C. *POU5F1*-positive cells were rare at only about 4% (11/280; positive/examined cells) but about 75% of cells (209/280) expressed *CDX2* protein. Some of them produced both proteins simultaneously (Fig. 5).

Discussion

Trophoblastic cells, MNCs and BNCs, play a crucial role in placental architecture and functions in ruminants; both cells produce a wide range of functional molecules: steroid hormones, *IFNT*, *CSH1*, PRPs, PAGs, cathepsins, bovine secreted protein of Ly-6 domain 1, and cytokines, among others (Reimers *et al.* 1985, Imakawa *et al.* 1987, Gross & Williams 1988, Xie *et al.* 1991, Matamoros *et al.* 1994, Martal *et al.* 1997, Hashizume *et al.* 2007, Ushizawa *et al.* 2009). MNC production of *IFNT* is well known as a factor that signals the recognition of pregnancy; BNCs may play a major role in the establishment of the fetal–maternal interface, namely, BNC fuses with an epithelial cell in endometrium and starts to raise placental villi at around day 20 of gestation (Wooding 1992, Yamada *et al.* 2002). Although cell lines are a convenient tool to analyze and understand the functions and the mechanisms of trophoblastic cell lineages, few bovine trophoblastic cell lines have been available (Talbot *et al.* 2000, Shimada *et al.* 2001). These facts encourage the establishment of new trophoblast cell lines. We have been trying to raise cell lines from early embryo and previously developed a bovine trophoblastic cell line, BT-1. However, the efficiency of raising bovine trophoblastic cell lines is low. In this study, we attempted to improve the efficiency of developing trophoblastic cell lines that have the potential for long-term culture, and to raise various cell lines that have different characteristics in cows.

The cell segregation into trophoblastic cells from embryonic cells at the blastocyst stage is a well-known phenomenon; however, the actual factors responsible are yet to be determined. Various cytokines and transcription factors may be involved; some previous

Figure 2 Gene expression profiles in newly developed trophoblastic cell lines. (a) *CSH1*, (b) *PRP1*, (c) *PAG1*, (d) *IFNT*, (e) *BMP4*, (f) *CDX2*, and (g) *POU5F1*. Data labeled with different letters are significantly different from each other ($P < 0.05$).

reports suggested that the activin/nodal pathway, BMPs, FGF4, FGF2, CDX, and POU5F1 (OCT4) are involved (Pfarrer *et al.* 2006, Ravelich *et al.* 2006, Sakurai *et al.* 2009, Sugawara *et al.* 2010). In a previous study, we used only conditioned medium from fetal fibroblast cells and succeeded in producing only one cell line from about 50 IVF embryos, which has maintained good growth for over 300 passages (PDL; Shimada *et al.* 2001, Hashizume *et al.* 2006). The efficiency was quite low and it generally seems to be difficult to raise trophoblastic cell lines in cattle. In this study, we used BMP4 to raise trophoblastic cell lines because exogenous BMP4-stimulated embryonic stem cells (ES) to become trophoblastic cells and promoted trophoblastic cell lines (Kurihara *et al.* 1993, Murohashi *et al.* 2010). The addition of BMP4 to culture medium at early embryo stages boosted the establishment of trophoblastic cell lines in bovine, but there was no significant difference between untreated and BMP4 groups. Although mechanisms of enhancing BMP4 production of

trophoblastic cells are lacking, some other growth factors/cytokines may be involved in this process because some growth factors play a crucial role for the ES cell lineages (Fujiwara *et al.* 2002, Das *et al.* 2007, Wu *et al.* 2008, Zhang *et al.* 2008, Hayashi *et al.* 2010, Murohashi *et al.* 2010). In this study, we added BMP4 during and after fertilization for 7 days and continuous culture from 6 days. After these treatments of BMP4, no significant effects were found in terms of embryo development, namely, in terms of growth rates up to blastocyst; however, anti-BMP4 antibody suppressed the number of trophoblastic cells on blastocyst (K Imai, T Takahashi, K Kizaki & K Hashizume, unpublished observations). These results suggest that BMP4 can boost entry into the trophoblastic cell lineage; however, critical periods and doses for treatment of BMP4 to determine cell fate remain to be examined. Although BMP4 may be an induction factor for trophoblastic cell lineages, it is not necessary for the development of trophoblastic cells in mice (Kobayashi *et al.* 2008,

Figure 3 Immunohistochemical detection of placental lactogen (CSH1) and interferon- τ (IFNT) in different trophoblastic cell lines. (a–c) Arrow indicates CSH1-positive cell, and arrowhead indicates IFNT-positive cell, bar=50 μ m.

Murohashi *et al.* 2010). BMP4 combined with some other factors stimulates trophoblastic proliferation and differentiation (Fujiwara *et al.* 2002, Das *et al.* 2007, Wu *et al.* 2008, Hayashi *et al.* 2010, Murohashi *et al.* 2010). The other concern for trophoblastic cell lineages from early embryonic cells is that there is a possibility of mixture of epiblast stem cell-like and trophoblast cells; we examined some cell clumps as inner cell mass (ICM) cells, which were condensed and had a dark color under microscopy just after the initiation of culture. The ICM-like cell clumps were eliminated mechanically so they might not be involved as ICM cells. Moreover, even if mixing occurred at an early stage of culture, we think that BMP4 controls the fate of epiblast stem cell-like cells

for trophoblastic cells just like hESC and mESC (Xu *et al.* 2002, Hayashi *et al.* 2010). Considering this evidence, it is difficult to keep ES-like cells under our culture conditions. The various cell lines raised in this study may be trophoblastic cells and constitute new models for studying the trophoblastic cell lineage in bovine placenta.

It is believed that BNCs are derived from MNCs, but the process and mechanisms of formation are still unclear; a certain type of stem cell forms a BNC without cytokinesis. Another expectation for the raising of BNCs is that cell–cell fusion may form BNCs with retroviral envelope proteins (Black *et al.* 2010, Koshi *et al.* 2011). However, these mechanisms are still unclear, and

Table 2 Number of CSH1-positive and interferon- τ (IFNT)-positive cells.

Cell	CSH1-positive	IFNT-positive
BT-A	0/404 (0) ^a	43/264 (16.2)
BT-B	0/544 (0)	0/626 (0)
BT-C	3/266 (1.1)	100/301 (33.2)
BT-D	1/225 (0.4)	0/277 (0)
BT-E	3/470 (0.6)	11/360 (3.1)
BT-F	3/356 (0.8)	21/264 (8.0)
BT-G	0/503 (0)	19/241 (7.9)
BT-H	0/293 (0)	88/333 (26.4)
BT-I	5/258 (1.9)	0/330 (0)
BT-J	14/299 (4.7)	0/381 (0)
BT-K	26/364 (7.1)	0/314 (0)
BT-L	10/344 (2.9)	12/376 (3.2)

^aNo. of immunohistochemical positive cells/no. of counted all cells (%).

trophoblastic cell lines will provide a suitable model for examining this complex feature. In this study, bunches of bovine trophoblastic cell lines were established and they have different features and comprise at least four types, with specific gene expression profiles of *CSH1*, *PRP1*, and *IFNT*.

CSH1 is a representative marker for mature BNCs, and, in this study, four new cell lines expressed this gene intensively and four (BT-C, BT-E, BT-F, and BT-L) of them expressed IFNT, which is an indicator of MNCs, simultaneously. A similar result was confirmed previously in the cell line BT-1 (Shimada *et al.* 2001). Cell lines with different features were established, which were classified in terms of protein expression: cell lines that express only BNC-specific genes (BT-D, BT-I, BT-J, and BT-K), cell lines that express only IFNT (BT-A, BT-G, and BT-H), and a cell line that expresses neither of these features (BT-B). These classifications agreed with gene expressions, but there were some differences. The reason for this is still difficult to identify but protein-based data seem to be reliable.

However, all cell lines established in this study expressed *POU5F1* and *CDX2* genes, and some cells produced both simultaneously, but not all of them. The mechanisms of expression and involvement of these genes are still poorly understood, but they also have been detected in BT-1 (Hashizume *et al.* 2006, Sakurai *et al.* 2009). *POU5F1* is well known as a stem cell marker and plays a crucial role in the maintenance of stem cell properties, including pluripotency in ES cells. Although the repression of *POU5F1* induced ES into the trophoblastic cell lineage in mice (Niwa *et al.* 2005, Yuan *et al.* 2009), these data suggest that the BT cells are involved in stem cell features and are not terminal cells. Another transcription gene, *CDX2*, regulates the trophoblastic cell lineage (Niwa *et al.* 2005, Ealy & Yang 2009, Home *et al.* 2009), and its importance and regulatory mechanisms in trophoblasts may be similar in many species, including cows. The gene may have other specific roles in bovine trophoblasts because it regulates IFNT, which is a specific marker for trophoblastic mononucleate cells in ruminants (Ezashi *et al.* 2008,

Cooke *et al.* 2009, Sakurai *et al.* 2009). The regulatory mechanism of IFNT expression and transcription still remains to be examined (Bai *et al.* 2009). *CSH1* expression is well correlated to the maturation of BNCs among bovine trophoblasts (Nakano *et al.* 2005); however, information on the regulation and transcription factors for *CSH1* is still limited. Some elements, AP2, GATAs, PURA, and SPs, have been reported as activation factors for this gene, and *CSH1* expression was closely related to the implantation process spatiotemporally (Limesand *et al.* 2004, Ushizawa *et al.* 2007, Jeckel *et al.* 2009). GATA may play a regulatory role of *CSH1* expression and differentiation of BNCs (Bai *et al.* 2011).

In conclusion, we developed 12 bovine trophoblast cell lines, including four with different features, with BMP4 supplementation at the early embryo stage. All cell masses expressed *POU5F1* and *CDX2* genes, but they expressed MNC- and/or BNC-specific genes in ways that were dependent on the cell line. These cell lines will provide effective and unique models for the study of trophoblastic cell functions and lineages in ruminants.

Materials and Methods

Production and culture of IVF ova

Ovaries derived from a local slaughterhouse that were brought to the laboratory (National Livestock Breeding Center: NLBC, Fukushima, Japan) were used for IVF as reported previously (Imai *et al.* 2006). Briefly, cumulus-oocyte complexes (COCs) after aspiration from small follicles (2–6 mm in diameter) were cultured for 21–22 h in 5% CO₂ at 38.5 °C in maturation medium: 5% calf serum (CS, Gibco) containing 25 mM HEPES-buffered TCM-199 with Earle's salt (Gibco), and then covered with paraffin oil (Paraffin Liquid, Nacalai Tesque, Kyoto, Japan) in 35 mm Petri dishes (Nunc, Roskilde, Denmark) at 38.5 °C in 5% CO₂ in air with saturated humidity. After maturation culture, COCs were mixed with capacitated spermatozoa (3×10^6 /ml) and cultured for 6 h in BO solution containing

Figure 4 Western blotting analysis of interferon- τ in different trophoblastic cell lines. Recombinant interferon- τ was used as a positive indicator for protein. Arrow indicates a molecular weight of 23 kDa.

Figure 5 Immunocytochemical detection of CDX2 and POU5F1. In this examination, BT-C cell line was used. Arrowhead indicates POU5F1- and CDX2-positive cell, bar = 50 μ m.

2 U/ml heparin (Novo-Heparin Injection 1000, Aventis Pharma Ltd., Tokyo, Japan), 5 mM hypotaurine (Sigma), and 10 mg/ml BSA (crystallized and lyophilized, Sigma) at 38.5 °C in 5% CO₂ in air with saturated humidity. After removal of cumulus cells, putative zygotes were cultured in CR1aa medium containing 5% CS with or without recombinant BMP4 (raised by TT) at 38.5 °C in 5% CO₂ in air with saturated humidity for 7 days. Then, blastocysts were moved into TCM-199 medium containing 20% fetal bovine serum (FBS, Gibco), 100 mM β -mercaptoethanol (2-mercaptoethanol, Sigma) with or without BMP4, and cultured for 6 days at 38.5 °C in 5% CO₂ in air with saturated humidity. Three different concentrations of BMP4 were used: 1, 10, and 100 ng/ml. All animal experiments were individually approved by the Animal Experimental Committee of Iwate University and NBLC.

Raising of rbBMP4 and anti-BMP4 antibody

rbBMP4 was produced using a cell-free protein expression system (Rapid Translation System, Roche Applied Science). In brief, bovine *BMP4* cDNA was cloned using a forward primer: ATTATTAAGCGGCCGCGTAGCCCCAAGCATCACCCACAG and a reverse primer: ATCCTCGGATCCTCAGCGGCACCCACATCCCTCCAC, from the fetal membrane on day 26 of gestation, and put into an expression vector (pIVEX 2.4d, Roche), which was digested with NotI/BamHI, ligated with T4 ligase (Promega). It was transformed into *Escherichia coli* XL-1 Blue Supercompetent cells (Stratagene, La Jolla, CA, USA). The ampicillin-resistant transformants were selected and subcultured with LB broth in the presence of ampicillin. Plasmids were isolated from the culture with centrifugal kit (Mini Prep kit, Qiagen). A plasmid containing the mature protein region of BMP4 with correct nucleotide sequence was used as a template for cell-free protein expression.

The rbBMP4 was produced with the above cell-free protein expression system with RTS500 Proteomaster *E. coli* HY kit (Roche) according to the manufacturer's instructions. During the expression, BMP4 formed an insoluble precipitate in the reaction mixture. After 24 h of expression, the reaction mixture was centrifuged at 22 500 *g* for 15 min at 4 °C. The pellet was dissolved with 5 ml binding buffer (0.1 M PBS, 0.3 M NaCl, 0.02 M imidazole, 6 M urea, pH 8.0), centrifuged at the same conditions as above and the supernatant was incubated with 2 ml Ni-Sepharose (GE Healthcare UK Ltd., Little Chalfont, UK) at 4 °C overnight. Then, it was applied to a disposable column (Bio-Rad). The column was extensively washed with binding buffer to remove unbound proteins. rbBMP4 was eluted with elution buffer (0.1 M PBS, 0.3 M NaCl, 0.5 M imidazole, 6 M urea, pH 8.0). The presence of rbBMP4 in each fraction was determined by western blot analysis with anti-His antibody (Bethyl, Montgomery, TX, USA) because pIVEX 2.4d expression vector resulted in the inclusion of 6 $\times$ His epitope tag at the amino terminal of the recombinant protein. Immunopositive fractions were pooled, utilized for immunization to generate anti-rbBMP4 antibody in rabbits, and some portion was subjected to renaturation. The rbBMP4 was refolded by sequential dialysis with gradual decrease in urea. First, the sample was dialyzed against 0.1 M PBS (pH 7.2) supplemented with 0.1 M NaCl and 3 M urea overnight at 4 °C using a dialysis cassette (Float-a-Lizer, NMCO 8K, Spectrum, Rancho Dominguez, CA, USA), then further dialyzed against 0.1 M PBS (pH 7.2) supplemented with 0.1 M NaCl, 2 M urea, and 0.4 M arginine overnight at 4 °C using the same dialysis cassette. From this point forward, the sample was sequentially dialyzed overnight at 4 °C with gradual decreasing concentration of urea (2.0, 1.0, 0.5, and 0 M) in the presence of 0.4 M arginine. Finally, the sample was dialyzed against the same PBS without urea and arginine. The dialyzed solutions were concentrated in a centrifugal column (MWCO 10K, Amicon, Millipore, Bedford, CA, USA). The protein concentration of the purified product was measured by Bradford dye-binding procedure. The presence of target protein was determined by SDS-PAGE and western blotting with anti-BMP4 antibody (either polyclonal (raised using rbBMP4) or monoclonal anti-human BMP4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, BMP4 (3H2.3) sc-12721)). Anti-rbBMP4 antibody generation was performed by Qiagen. Briefly, adult Japanese White rabbits were used for immunization. Rabbits were bled before immunization to collect preimmune-serum. For primary immunization, 0.2 mg rbBMP4 was injected intradermally into the rabbit with Freund's complete adjuvant. Quintuple booster immunizations with 0.2 mg antigen and incomplete adjuvant were given s.c. every 2 weeks following the initial injection. Animals were bled a week after second and fourth booster injections to determine titers. The titer of antiserum was determined by ELISA as previously reported (Takahashi *et al.* 2004). A week after final booster injection, rabbits were exsanguinated under deep anesthesia. Harvested serum was stored at -30 °C until use. Generated antiserum was used at 1:1000 dilution for western blotting.

Table 3 RT-PCR primers.

Gene names	Annealing temperature (°C)	Cycle number	Accession no.	Primers	Product
<i>GAPDH</i>	60	30	NM_001034034.1	Forward: CCTTCATTGACCTTCACTACATGGTCTA Reverse: GCTGTAGCCAAATTCATTGTCTGTACCA	173–200 1029–1003
<i>CSH1</i>	60	35	NM_181007.2	Forward: CCATCTCCCCATCAGCAGCAGT Reverse: GAGACCCATTACACCCAAACAT	105–126 975–954
<i>PRP1</i>	60	35	NM_174159.2	Forward: CACGGTCAACAGGAGTCTCTACC Reverse: AATTTACAGGTAGCCCGTGTGG	43–65 873–852
<i>PAG1</i>	60	35	NM_174411.2	Forward: CACCATTGGAACACCCCC Reverse: CACTGGGTAGTTGATGCCGTT	245–262 989–969
<i>IFNT</i>	58	30	NM_001015511.2	Forward: TCCCCATGGCCTTCGTGCTCTCTCT Reverse: CTCAAAGTGAGTTGATCTCCACC	75–99 668–644
<i>POU5F1</i>	58	30	NM_174580.2	Forward: GGTCTCTTTGGAAAGGTGTTT Reverse: ACACTCGGACACGTCCTTC	516–537 829–810
<i>CDX2</i>	60	30	XM_871005.3	Forward: TATCACCATCCGGAGGAAAG Reverse: GCTCAGCCTGGAATTGCTAC	778–797 1105–1086
<i>BMP4</i>	60	35	NM_001045877.1	Forward: CGTAGCCCCAAGCATCACCCACAG Reverse: TCAGCGGCACCCACATCCCTCCAC	1075–1098 1428–1405

Assay for BMP4 biological activity

Refolded rbBMP4 was used for cell culture experiments. The biological activity of recombinant BMP4 was determined by ALP assay using the mouse myoblast cell line, C2C12. The cells were purchased from Riken Cell Bank (Tsukuba, Ibaraki, Japan). C2C12 cells were maintained in DMEM (Sigma) containing 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma) supplemented with 15% FBS (HANA-NESCO, Tokyo, Japan; growth medium) at 37 °C in a humidified atmosphere of 5% CO₂ in air. The growth medium was replaced on day 1 with DMEM containing 5% FBS with various concentrations of recombinant BMP4. ALP bioassay was performed following previous reports (Katagiri *et al.* 1994, Kirsch *et al.* 2000) with some modification; in brief, C2C12 cells were stimulated at a density of 1.3×10^5 cells/well in a six-well plate for 120 h with either 20–80 ng/ml bovine recombinant BMP4 or human BMP4 (Sigma). Non-BMP4-containing culture medium was used for a control. Then, cells were washed with 0.9% NaCl and lysed with 200 µl of lysis buffer (1% NP-40, 0.9% NaCl). ALP activity in the cell lysate was assayed at 37 °C in the buffer containing 0.2 M 2-amino-2-methyl-1-propanol and 1 mM MgCl₂, pH 9.5, for 30 min using *p*-nitrophenylphosphate as a

substrate. The enzyme activity was expressed as nanomoles of *p*-nitrophenol produced per min per mg protein. The diluent of protein content was determined using the Bio-Rad Quick Start kit (Bio-Rad) using BSA as the standard. The rbBMP4 exhibited bioactivity the same as human BMP4 (Fig. 1b).

Nursing cell culture

After examining the absence of an ICM-like cell clump by microscopy, spherical cell bodies were transferred into collagen-coated (Cellmatrix Type I-C, Nitta Gelatin, Osaka, Japan) 24-well culture plates (BM, Tokyo, Japan), individually. Each cell body was cultured in ESM-2 medium (α MEM including 8.9 µg/ml L-alanine, 13.2 µg/ml L-asparagine, 13.3 µg/ml L-aspartic acid, 14.7 µg/ml L-glutamic acid, 7.5 µg/ml glycine, 11.5 µg/ml L-proline, and 10.5 µg/ml L-serine, +100 µM 2β-mercaptoethanol +5 µg/ml insulin +10 ng/ml EGF +10 ng/ml FGF-1 +10 µg/ml heparin +5 mg/ml BSA; Research Institute for Functional Peptides, Yamagata, Japan) containing 20% FBS until cells made contact and spread out over the whole well at 37 °C in 5% CO₂. Close to confluence, cells were scaled out and half of them were

Table 4 Quantitative RT-PCR primers.

Gene names	Accession no.	Primers	Product
<i>GAPDH</i>	NM_001034034.1	Forward: AAGGCCATCACCATCTTCCA Reverse: CCACCACATACTCAGCACCAGCAT	280–299 355–332
<i>CSH1</i>	NM_181007.2	Forward: TGCCACACCGAATTCATGAC Reverse: AGGGCTTCGTCTCTGTATTTG	447–466 514–493
<i>PRP1</i>	NM_174159.2	Forward: CACGGAGCTGCAGCATATGA Reverse: CCTTGTGGCGCTTGATAGGA	501–520 558–539
<i>PAG1</i>	NM_174411.2	Forward: TCCACTTTCCGCTTACCAA Reverse: CCTTTCATTCTCCAGATCCAT	375–394 436–415
<i>IFNT</i>	NM_001015511.2	Forward: CTAGGTGCCAGGCAGAACCT Reverse: GGGATGAGGAGAGTCTGTTC	173–192 232–210
<i>POU5F1</i>	NM_174580.2	Forward: AGGAAGCTGACAACAACGAGAAT Reverse: GGCCTGCACAAGGGTCTCT	626–648 687–696
<i>CDX2</i>	XM_871005.3	Forward: CAGAACCCGAGAGCAAAGG Reverse: GTTGCTGCTGCTGTTGCAA	851–869 911–893
<i>BMP4</i>	NM_001045877.1	Forward: CTGTCAATTCAGATATCCCCAAA Reverse: TGGAGATGGCGCTGAGTTC	1289–1311 1348–1330

transferred into a new collagen-coated 24-well culture plate with DMEM (Sigma)/Nutrient Mixture F12 Ham (F12, Sigma) medium that contained 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin (Sigma) at 37 °C in 5% CO₂. Then, the cells were maintained in the same medium and under the same culture conditions as in the previous cell culture method (Shimada *et al.* 2001). The estimation of cell lineage was decided using the PDL following Hayflick's formula with the starting cell number estimated as 3000 at the spherical cell body stage. The estimation followed the ATCC protocol in terms of a formula for use for the calculation of population doubling as follows: $n = 3.32 (\log \text{UCY} - \log I) + X$, where n =the final PDL number at the end of a given subculture, UCY=the cell yield at that point, I =the cell number used as inoculum to begin that subculture, and X =the doubling level of the inoculum used to initiate the subculture being quantitated (ATCC <http://www.atcc.org/CulturesandProducts/Technical-Support/FrequentlyAskedQuestions/tabid/469/Default.aspx>).

In this study, 15- to 25-passage (the same as PDL) cells were used for gene expression, biochemical, and cytochemical analyses.

Conventional and quantitative RT-PCR

After removal of culture medium, 1 ml TRIzol reagent (Invitrogen) was added to a 25 cm² culture bottle (BM) and total RNA was extracted following the manufacturer's instructions. The quality of RNA was confirmed by agarose gel electrophoresis with no degradation of 28S and 18S RNA. The amounts of RNA were measured using Nano Drop (Nano Drop Technologies, Wilmington, DE, USA). Gene expressions and sequences were analyzed as described in previous reports. In brief, conventional RT-PCR was performed by primer sets (Table 3) and amplified with AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA). PCR products were subcloned into pGEM-T Easy Vector Systems in accordance with the manufacturer's instructions and the sequences of cloned plasmids were analyzed with ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The gene expression intensity was determined by quantitative RT-PCR using SYBR Green PCR Master Mix (ABI) and ABI7300 (Applied Biosystems) following a previous report. The primer sets for *GAPDH*, *CSH1*, *PRP1*, *PAG1*, *IFNT*, *BMP4*, *POU5F1*, and *CDX2* were used as shown in Table 4. The amount of expression was estimated using a cloned template of each gene.

Western blotting

Cells close to confluence were collected from the 25 cm² bottle with a cell scraper (Greiner Bio-One Japan, Tokyo, Japan) using the following procedure. First, cells were washed with DMEM/F12 medium without FBS three times and were cultured for 2 h with DMEM/F12 medium without FBS at 37 °C. The medium was replaced with 2 ml fresh DMEM/F12 medium and cells were cultured for 48 h at 37 °C in 5% CO₂. Then, culture medium was transferred into a tube and centrifuged at 3000 *g* for 10 min; supernatants were then collected for western blotting. The proteins in the collected media were precipitated with a fourfold volume of acetone

(Sigma). The precipitates were dissolved in PBS, pH 7.4, and loaded with loading buffer for SDS-PAGE in 2 µg/20 µl protein per lane. After separation, proteins were transferred onto PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA) with semi-dry blotter (Bio-Rad Japan). The transferred membranes were detected with anti-IFNT (×2000) antisera as described in previous reports (Shimada *et al.* 2001, Nakano *et al.* 2002).

Immunocytochemistry

Trophoblast cells were cultured on collagen-coated cover slips. After cells proliferated close to confluence, the slips were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4 °C for 15 min. The cell slips were incubated with one of the following solutions: anti-IFNT (×1000), anti-CSH1 (×1000), anti-POU5F1 (Santa Cruz Biotechnology, ×500), CDX2 (Santa Cruz Biotechnology, ×500), and rabbit IgG for 2 h at room temperature; after washing with PBS, the slips were incubated with secondary antibody: Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen) at a dilution of 1:500 in PBS containing 1% BSA, 0.05% NaN₃, and 0.3% Triton X-100. Slips were stained with Hoechst 33342 (5 µg/ml, Invitrogen) in secondary antibody solution. Then, each protein expression was detected with ECLIPSE E600 (Nikon, Tokyo, Japan), individually. Detailed detection procedures were in accordance with previous reports (Shimada *et al.* 2001, Hashizume *et al.* 2006).

Statistical analysis

Differences in established cell numbers were analyzed by χ^2 test. The gene expression data were analyzed initially by ANOVA and followed by Tukey–Kramer multiple comparison test using JMP software (SAS Institute, Inc., Cary, NC, USA). *P* values of <0.05 were considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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