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-γ, 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.

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 placentation 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 trophoblast 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 (TGFβ) 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 TGFβ 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 (Fujiiwara 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) possessed 162.3 ± 22.5 (mean ± 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...
(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 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
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, 2010).

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).
Murohashi et al. 2010). BMP4 combined with some other factors stimulates trophoblastic proliferation and differentiation (Fujiiwara 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

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 µm.
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, PPRP1, 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 (3×10⁶/ml) and cultured for 6 h in BO solution containing

![Image](image-url)
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 precipitate 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×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 0.0 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-rBMP4 antibody generation was performed by Qiagen. Briefly, adult Japanese White rabbits were used for immunization. Rabbits were bled before immunization to collect preimmuno-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 antisera 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 antisera was used at 1:1000 dilution for western blotting.

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: ATTATTAAGCGGCGGGTACGCACACCGACAG and a reverse primer: ATCCTCGATCCCTCAGCCGCACCCCCATCCTCTCAG, 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.
**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 × 105 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 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 (a 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, C100 μM 2-mercaptoethanol C5 μg/ml insulin C10 ng/ml EGF C10 ng/ml FGF-1 C10 μg/ml heparin C5 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

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**Table 3 RT-PCR primers.**

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**Table 4 Quantitative RT-PCR primers.**

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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 ATTC protocol in terms of a formula for use for the calculation of population doubling as follows: 

\[ n = 3.32 \left( \log \text{UCY} - \log l \right) + X \]

where \( n \) = the final PDL number at the end of a given subculture, \( \text{UCY} \) =the cell yield at that point, \( l \) =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 \( \chi^2 \) 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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