Studies on induction of pluripotency in bovine somatic cells and generation of induced pluripotent stem cells

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Chapter 1

General Introduction

Fertilized eggs develop into pre-implantation embryos at the blastocyst stage after the first cellular differentiation and are divided into two distinct cell types: inner cell mass (ICM) and trophectoderm (TE). From the ICM, which gives rise to the latter embryo proper, embryonic stem cells (ESCs) are established and proven to have the ability to differentiate into any cell type (pluripotency) and to self-renew without senescence (Evans & Kaufman, 1981). On the other hand, from the TE, which gives rise to the latter placenta, trophoblast stem cells (TSCs) are established and proven to retain the ability to differentiate into all trophoblast derivatives proceeding to the direction toward placenta (Tanaka et al., 1998).

The differentiating ability of ESCs is associated with an important source for producing healthy therapeutic cells for regenerative medicine in human (Thomson et al., 1998). However, due to the necessity for sacrificing human embryos in the process of derivation of human ESCs, the production and the use of human ESCs raise profound ethical concerns. Therefore, numerous attempts have been made to obtain patient-distinct pluripotent stem cells by reprogramming of somatic cells using recent biotechnology such as somatic cell nuclear transfer (Surani, 2001, Wilmut et al., 1997), cell fusion (Do & Schöler, 2004, Do & Schöler, 2006, Do & Schöler, 2010), culture with cell extracts (Hansis et al., 2004, Miyamoto et al., 2011). In 2006 and 2007, Takahashi and Yamanaka demonstrated that mouse and human somatic cells can be reprogrammed via introduction of four transcriptional factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) and leading to the derivation of induced

pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006, Takahashi et al., 2007). iPSCs have a similar capacity to ESCs in terms of the ability of self-renewal and pluripotency, thereby become a more attractive tool for regenerative medicine. In addition to these cell characteristics, iPSCs and ESCs also have the potentials to induce homologous recombination applied by gene targeting procedure (Buecker et al., 2010), and to repopulate into the ICM region after aggregation or injection into host blastocysts ; these features allow them to develop into chimeras that, in turn, can transmit mutated or modified genes to subsequent generations via the germline cell linage (Nichols & Smith, 2009, Silva et al., 2009). Thus, iPSCs could be applied to a genetic preservation of endangered species, genetic improvement of domestic animals as well as preclinical models for human regenerative medicine and disease. In order to achieve these applications, iPSCs are required to have the ability to develop into chimeras. However, the generation of iPSCs in large domestic animals has achieved only limited success, and only mouse iPSCs show the capacity to produce chimeric offspring to date (Hanna et al., 2010b, Nichols & Smith, 2009).

In this study, we attempted to generate bovine iPSCs that have a high pluripotency to contribute to produce chimeric offspring. Bovine amnion-derived cells were introduced with doxycycline-inducible piggyBac transposon of reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*). The properties of resultant cell lines were characterized both in vitro and in vivo.

Chapter 2

Generation of naïve bovine induced pluripotent stem cells using piggyBac transposition of doxycycline-inducible transcription factors

Abstract

Generation of pluripotent stem cells (PSCs) in large domestic animals has achieved only limited success; most of the PSCs obtained to date have been classified as primed PSCs, which possess very little capacity to produce chimeric offspring. By contrast, mouse PSCs have been classified as naïve PSCs that can contribute to most of the tissues of chimeras, including germ cells. Here, we describe the generation of two different types of bovine induced pluripotent stem cells (biPSCs) from amnion cells, achieved through introduction of piggyBac vectors containing doxycycline-inducible transcription factors (*Oct3/4*, *Sox2*, *Klf4,* and *c-Myc*). One type of biPSCs, cultured in medium supplemented with knockout serum replacement (KSR), FGF2, and bovine leukemia inhibitory factor (bLIF), had a flattened morphology like human PSCs; these were classified as primed-type. The other type biPSCs, cultured in KSR, bLIF, Mek/Erk inhibitor, GSK3 inhibitor and forskolin, had a compact morphology like mouse PSCs; these were classified as naïve-type. Cells could easily be switched between these two types of biPSCs by changing the culture conditions. Both types of biPSCs had strong alkaline phosphatase activity, expressed pluripotent markers (*OCT3/4*, *NANOG*, *REX1*, *ESRRβ*, *STELLA*, and *SOCS3*), and formed embryoid bodies that gave rise to differentiated cells from all three embryonic germ layers. However, only naïve-type biPSCs showed the hallmarks of naïve mouse PSCs, such as LIF-dependent proliferation, lack of FGF5 expression,

and active *XIST* expression with two active X chromosomes. Furthermore, naïve-type biPSCs could contribute to the inner cell mass (ICM) of host blastocysts and most tissues within chimeric embryos. This is the first report of generation of biPSCs with several characteristics similar to those of naïve mouse PSCs and a demonstrated potential to contribute to chimeras.

Introduction

Somatic cells can be reprogrammed to a pluripotent state via ectopic expression of the transcription factors Oct4, Sox2, Klf4, and c-Myc, thereby generating induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007, Takahashi & Yamanaka, 2006). Pluripotent stem cells (PSCs), including iPSCs and embryonic stem cells (ESCs), can be classified into two categories: 'naïve' and 'primed' PSCs (Hanna et al., 2010b, Kinoshita, 2014, Nichols & Smith, 2009). Naïve PSCs correspond to ICM of blastocysts, and are similar to mouse ES cells (mESCs), whereas primed PSCs correspond to the epiblast at the postimplantation stage, as represented by mouse epiblast stem cells (mEpiSCs) and human ES cells (hESCs). Naïve PSCs exhibit some distinctive characteristics, such as a compact and dome-shaped morphology, the ability to be passaged as a single cell, dependence of proliferation on the leukemia inhibitory factor (LIF)–Jak/Stat signaling pathway, two active X chromosomes (X_aX_a), and specific expression of REX1, ESRR β, and STELLA (Hanna et al., 2010b, Nichols & Smith, 2009). In addition, naïve PSCs are more frequent in homologous recombination (Buecker et al., 2010), and more efficient in repopulating the ICM region after aggregation or injection into host blastocysts; this feature allows them to develop into chimeras that, in turn, can transmit mutated or modified genes to subsequent generations via the germline (Nichols & Smith, 2009, Silva et al., 2009). On the other hand, primed PSCs have a flattened morphology, basic fibroblast growth factor (bFGF)-dependent proliferation, an inactivated X chromosome (X_aX_i), and a relatively limited capacity to produce chimeras (Tesar et al., 2007). Recent studies demonstrated that primed human PSCs can be converted into a naïve state using certain chemical compounds (a GSK3 β inhibitor and a MEK/ERK inhibitor (2i), and the protein kinase A pathway agonist forskolin) (Hanna et al., 2010a). Thus, naïve PSCs are a feasible potential source of material for production of PSC-derived offspring

in domestic species.

Naïve iPSCs and PSCs in large animals, such as cattle, could be applied to genetic improvement of domestic animals as well as preclinical models for human regenerative medicine and disease. To date, however, it has been quite difficult to establish naïve iPSCs and PSCs in mammals other than rodents; most of the iPSCs reported in monkeys (Liu et al., 2008, Tomioka et al., 2010), pigs (Esteban et al., 2009, Ezashi et al., 2009, Montserrat et al., 2011, Wu et al., 2009), and rabbits (Honda et al., 2010) have been of the primed type. In cattle, Sumer et al (2011) reported the generation of bovine iPSCs (biPSCs), but these cells exhibited characteristics of primed iPSCs. The major reason for this is that the optimal culture conditions for generation and maintenance of iPSCs in various mammalian species have not been determined. Recently, Tsukiyama et al (2011) proposed an efficient system using piggyBac (PB) transposition of doxycycline (Dox)-inducible transcription factors, which allows the expression of introduced reprogramming factors to be controlled by the presence or absence of Dox, for screening of culture conditions for generation of iPSCs. The PB transposon vector (Kaji et al., 2009, Woltjen et al., 2009, Yusa et al., 2009) is a non-viral and safe vector system, in which integrated constructs (including reprogramming factors) can be removed by the re-expression of transposase (PBase). On the other hand, the source of somatic cells for iPSCs significantly affects reprogramming efficiency (Aasen et al., 2008, Kim et al., 2008). For example, mouse neural progenitor cells can be more efficiently reprogrammed than fibroblasts, and iPSCs can be established from these cells by expression of only one or two exogenous factors, due to their endogenous expression of Sox2 and c-Myc (Kim et al., 2008). However, neural progenitor cells are not easily accessible, and are not available in large quantities. By contrast, amnion-derived cells (ADCs) can be readily obtained from the placental tissue after delivery. The amnion, derived from the epiblast as early as the eighth day after fertilization, is a thin membrane-lined cavity filled with fluid that protects

the developing fetus. Moreover, in both human and mouse, reprogramming of ADCs into iPSCs is more efficient and faster than that of fibroblasts (Li et al., 2009, Nagata et al., 2009, Zhao et al., 2010). Therefore, ADCs represent an ideal cell source for the generation of iPSCs.

In this experiment, we attempted to establish biPSCs from bovine amnion-derived cells by introducing Dox-inducible PB vectors expressing the mouse reprogramming factors (*Oct3/4, Klf4, Sox2,* and *c-Myc*) and culturing cells in the presence of certain chemical compounds (2i and forskolin). Two different types (primed and naïve) of biPSCs appeared under different culture conditions, and we characterized the pluripotent properties of the resultant biPSCs both *in vitro* and *in vivo*.

Materials and Methods

Ethics statements

All cattle were fed grass silage-based diet *ad libitum*. All procedures involving the care and use of animals were approved by the Animal Research Committee of NARO institute of Livestock and Grassland Science.

Isolation of bovine amnion-derived cells (bADCs)

Bovine amnion layer was harvested from a female Japanese black cattle fetus at 50 days of gestation at the National Institute of Livestock and Grassland Science, Japan. The amnion was mechanically peeled away from the chorion and the allantois, divided into small pieces with fine surgical scissors, and dissociated by incubating for 2 hours at 37°C with 0.3% collagenase (Wako) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, JRH Biosciences). After collagenase digestion, the cell suspension was kept at room temperature for 5 min, and then poured through a cell strainer; the filtered suspension was then centrifuged at 200 *g* for 5 min. The precipitated cells were cultured in DMEM (Sigma) containing 10% FBS, penicillin (Sigma), and streptomycin (Sigma). When the cells reached confluence, they were cryopreserved in liquid nitrogen until use.

Preparation of bovine LIF

Total RNA was isolated from bovine fetal fibroblasts (bFFs) using TRIzol RNA Isolation Reagents (Invitrogen). DNase I (Takara) was added to the RNA preparation to avoid genomic DNA contamination. For reverse transcription, ReverTra Ace (Toyobo) and Random Primer (Invitrogen)

were used. Polymerase chain reaction (PCR) was then performed to amplify the bovine LIF (bLIF) cDNA sequence (GenBank accession no. NM_173931.1) using the KOD -Plus- Neo kit (TOYOBO) and specific primers: sense, 5'- GGA GAG CTC CAC CAT GAA GGT CTT GGC GGC AGG -3'; reverse, 5'- AAG GCT AGC CTA GAA GGC CTG GGC CAG CA -3'. The amplified cDNA fragment (630 bp) was inserted into the pCAGGS vector (Niwa et al., 1991). Specifically, after digestion of the vector with *Sac*I and *Nhe*I (Takara Bio), the cDNA was inserted downstream of the chicken β-actin–based promoter (CAG) in pCAGGS to create the bLIF expression vector. This vector was transfected into COS-7 cells using Lipofectamine LTX (Invitrogen), and the conditioned medium was collected, filtered, and stored at -20°C until use. The conditioned medium was diluted 1:1000 for biPSC culture.

Culture of bADCs and biPSCs

bADCs were maintained on collagen-coated (Nitta Gelatin) dishes in somatic cell medium consisting of DMEM containing 10% FBS, 50 ng/ml epidermal growth factor (EGF, Calbiochem), penicillin, and streptomycin. The cells were dissociated enzymatically with TrypLE Select (Invitrogen) for further propagation.

Primed- and naïve-type biPSCs were generated from bADCs as described below. Primed-type biPSCs resembling hESCs were maintained in primed iPSC medium (piPSC medium) consisting of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Invitrogen) containing 20% Knockout Serum Replacement (KSR, Invitrogen), 2 mM L-glutamine (MP Biomedicals), 1× MEM nonessential amino acids (NEAA, Invitrogen), 0.1 mM 2-mercaptoethanol (2-ME, Wako), penicillin, and streptomycin supplemented with 2.0 μg/mL doxycycline (Dox, Sigma), 5 ng/mL human basic fibroblast growth factor (bFGF, Wako or ReproCELL), and bLIF-conditioned

medium (1:1000 dilution), prepared as described above. Cells were subcultured every 7 days by physical splitting using a pulled Pasteur pipette, and maintained on 35-mm-diameter cell culture dishes (IWAKI) on a feeder layer of 3–5 × 10^5 cells SNL cells (McMahon & Bradley, 1990, Okita et al., 2007) inactivated with mitomycin C (Sigma).

Naïve-type biPSCs were maintained in naïve iPSC medium (niPSC medium), which consisted of piPSC medium supplemented with 1 μM MEK/ERK inhibitor (PD) (PD0325901, REAGENTS DIRECT), 3 μM GSK3β inhibitor (CH) (CHIR99021, REAGENTS DIRECT), and 10 μM forskolin (FK) (REAGENTS DIRECT) in the absence of bFGF. Cells were subcultured every 4 days by enzymatic dissociation using TrypLE Select and maintained on an SNL feeder layer.

Medium lacking bLIF and containing 20 μM JAK Inhibitor I (Calbiochem #420099) was used to test the influence of the LIF signal pathway on self-renewal and survival of biPSCs. Cells were maintained on an SNL feeder layer, and cell number was counted.

All the cultures were maintained in a humidified incubator at 38.5°C with 5% CO_2 in air.

Reprogramming of bADCs

bADCs were plated at 1.25 × 10^5 cells per 35-mm dish in culture medium without antibiotics and incubated overnight. Cells were then transfected using Lipofectamine LTX. Briefly, equal amounts (0.4 μg) of hyPBase vector (pCAG-hyPBase) (Yusa et al., 2011), PB vectors with reprogramming factors (PB-TET-OKS and pPB-TET-c-Myc) (Okita et al., 2008, Tsukiyama et al., 2011, Woltjen et al., 2009), rtTA PB vector (PB-CAG-rtTA Adv, Addgene), and/or TagRFP PB vector (pPBCAG-TagRFP-IH) (Tsukiyama et al., 2011), 2 μL of Plus reagent (Invitrogen), and 10 μL of Lipofectamine LTX transfection reagent were diluted and mixed in 400 μL of Opti-MEM medium (Invitrogen). DNA–lipid complex was then added to the culture dish. The culture medium was

changed 6 hours after transfection. One day after transfection, the culture was supplemented with 2.0 μg/mL Dox. Four days after Dox addition, cells were dissociated with TrypLE Select, 1×10^5 cells were reseeded on an SNL feeder layer, and the medium was replaced with piPSC or niPSC medium lacking 2i and forskolin. Eight days after the addition of Dox, 2i and forskolin were added to niPSC medium. Fourteen days after the addition of Dox, primary colonies were mechanically picked and isolated (in the case of primed-type biPSCs) or chemically dissociated (in the case of naïve-type biPSCs) and transferred onto an SNL feeder layer in 48-well or 4-well plates. The medium was changed every 1–2 days, depending on cell growth (Fig. 1).

The original vectors (PB-TET-MKOS, PB-CAG-rtTA Adv, and pCX-OKS-2A) were obtained from Addgene (plasmids 20959, 20910, and 19771, respectively) (Okita et al., 2008, Woltjen et al., 2009). The empty PB vector and the c-Myc PB vector (pPB-TET-c-Myc) were kind gifts from Dr. Hitoshi Niwa at the RIKEN Center for Developmental Biology. The hyPBase vector (pCMV-hyPBase) was a kind gift from Dr. Keisuke Yusa at Sanger Institute. To generate pCAG-hyPBase, pCMV-hyPBase was cloned into blunt-ended pCAGGS.

Alkaline phosphatase and immunofluorescence staining

Alkaline phosphatase staining was performed using the Vector Alkaline Phosphatase Substrate kit (Vector). For immunofluorescence analysis, cells were fixed with PBS containing 3.7% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were blocked with 5% bovine serum albumin (Sigma) and 0.1% Triton X-100 (Sigma) for 45 min at room temperature, and then incubated overnight at 4°C with primary antibodies against OCT3/4 (1:25, SC-5279, Santa Cruz Biotechnology), NANOG (1:250, AB5731, Millipore), glial fibrillary acidic protein (GFAP, 1:100, Z0334, DAKO), actin smooth muscle (ASM, 1:1000, MS-113-P0, Thermo), or

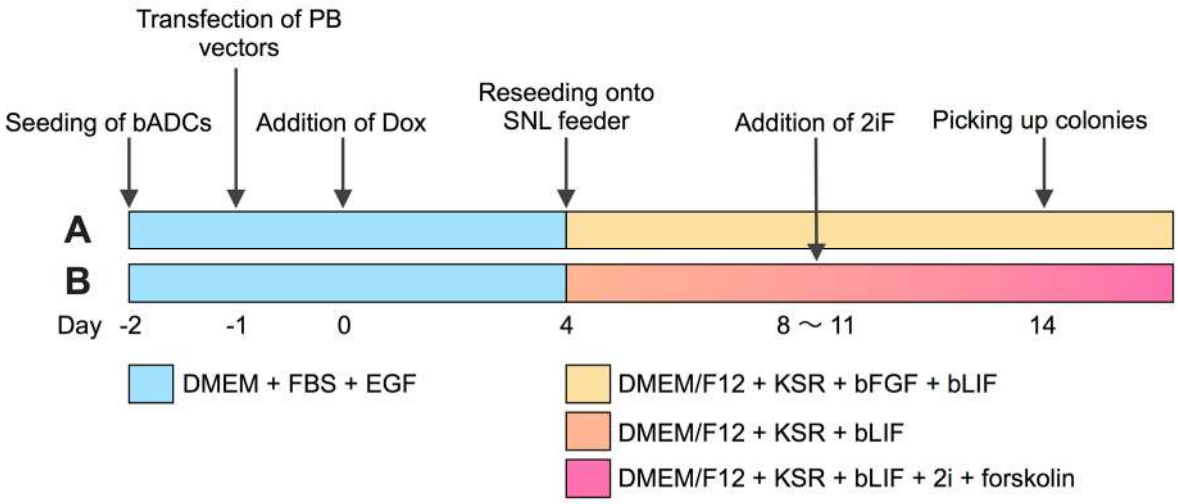

Fig. 1. Reprogramming of bovine amnion-derived cells (bADCs) into iPSCs using Dox-inducible PB vectors.

(A) Timeline for the establishment of primed-type biPSC lines. (B) Timeline for the establishment of naïve-type biPSC lines.

alpha-fetoprotein (AFP, 1:100, MAB1368, R&D Systems). Alexa Fluor 594 goat anti-mouse IgG or IgM (1:500, Invitrogen), Alexa Fluor 594 goat anti-rabbit IgG (1:500, Invitrogen), Alexa Fluor 488 goat anti-mouse IgG or IgM (1:500, Invitrogen), and Alexa Fluor 488 goat anti-rabbit IgG (1:500, Invitrogen) were used as secondary antibodies. Nuclei were stained with 1 μg/mL Hoechst 33342 (Sigma).

Reverse transcription PCR

Total RNAs of cells were prepared using TRIzol reagent. DNase I was added to preparations to avoid genomic DNA contamination. For reverse transcription, ReverTra Ace and Random Primer were used. PCR was carried out with ExTaq (Takara). An example PCR condition was as follows: 94°C for 2 min, followed by 35 amplification cycles (94°C, 20 s; 60°C, 30 s; 72°C, 30 s). The reaction was terminated by an elongation step at 72°C for 7 min. Primer sequences are shown in Table 1.

Differentiation of biPSCs *in vitro*

To produce embryoid bodies (EBs), established biPSCs were harvested by trypsinization (in case of naïve-type biPSCs) or physical splitting (in case of primed-type biPSCs), and then transferred to MPC-treated round-bottom dishes (Nunc) in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) containing 15% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× NEAA, 0.1 mM 2-mercaptoethanol, penicillin, and streptomycin supplemented with 2.0 μg/mL Dox. After 3 days of culture, the medium was changed to fresh medium without Dox. After an additional 3 days of culture, floating cell masses were transferred onto gelatin-coated dishes and cultured in 10% FBS DMEM for another 6 days. The resulting cell culture was analyzed by immunocytochemistry and reverse-transcription PCR, as described above.

Table 1. Primers for RT-PCR

Gene	Forward	Reverce
O-2A-K (Tg)	GCTCTCCCATGCATTCAAA	ACGCAGTGTCTTCTCCCTTC
c-Myc (Tg)	CTGTCCATTCAAGCAGACGA	ATCAGCCTCGACTGTGCCT
OCT3/4 (Endo)	AGCCAAACGACTATCTGCCG	ACACTCGGACCACGTCTTTC
SOX2 (Endo)	TTACCTCTTCTTCCCACTCC	TTCTTGCTGTCCTCCATTTC
KLF4 (Endo)	CTGCTCACGACTTTCCCTTG	GCAAACTTCCACCCACAACC
c-MYC (Endo)	AGCGACTCTGAGGAAGAACA	CAAGAGTTCCGTATCTGTTCAAG
NANOG	GACACCCTCGACACGGACAC	CTTGACCGGGACCGTCTCTT
REX1	GCAGAATGTGGGAAAGCCT	GACTGAATAAACTTCTTGC
ESRRβ	CCAACGGTCTGGACTCGCC	GCACACCTTCCTTCAGCAT
STELLA	TGCAAGTTGCCACTCAACTC	TTCCTTTGGCATAGCGAAGT
CDH1	CCGGGACAATGTGTATTACT	GTACTGGGGCACACTCAT
LIFR	AGACATGCCCTTGGAGTGTG	TCCCGCAAAAACAACCGTTC
SOCS3	GAAGCGAACTTGCTGCTGAG	CAGCTGGGTGACTTTCTCGT
FGF5	CTCCCACGAAGCCAATATGT	GGCTTTTTCTTTTCGGGAAC
OTX2	GGAGAGGACGACATTCACCC	AAATGGCTGGGACTGAGGTG
CDX2	GCCACCATGTACGTGAGCTAC	ACATGGTATCCGCCGTAGTC
VIM	AAGTTTGCTGACCTCTCTGAAGCT	CAGAGACTCGGTTAGTCCCTTTGA
BMP4	CAGCATGTCAGGATTAGCCG	GGCTTTGGGGATACTGGAAT
AFP	AAGGCACCCTGTCCTGTATG	AGACACTCCAGCACGTTTCC
bACT	GCGGCATTCACGAAACTACC	CGGACTCATCGTACTCCTGC
Uni-ACT	CCGATCCACACAGAGTACTTGCG	CGAGCGTGGCTACAGTTCACC

O-2A-K (Tg) primers amplify Oct3/4-2A-Klf4 sequences in the PB-TET-OKS vector. They were also used for genomic PCR. VIM, VIMENTIN; bACT, bovine β-ACTIN specific for cattle; Uni-ACT, universal β-ACTIN that reacts with both cattle and mice.

Karyotype analysis

Karyotype analysis of the established biPSC lines (pbiPSCs at 65 passages and nbiPSCs at 10 passages) was performed using KaryoMAX Colcemid Solution (Invitrogen) at the time of subculture. For each cell line, 20 metaphase plates were counted.

Generation of chimeric fetuses from naïve-type biPSCs

RFP-expressing naïve-type biPSCs were generated for the analysis of chimera production. bADCs were transfected with a constitutive TagRFP-expressing PB vector (pPBCAG-TagRFP-IH) simultaneously with pCAG-hyPBase PB-TET-OKS, pPB-TET-c-Myc, and PB-CAG-rtTA Adv, and RFP-expressing colonies were picked and propagated.

For aggregation of biPSCs with bovine embryos, *in vitro* fertilized host embryos were prepared. *In vitro* maturation, fertilization, and culture were performed as described previously (Booth et al., 2001). Sixty hours after fertilization, 8- to 16-cell stage embryos were collected and transferred to acid Tyrode solution (pH 1.5) until the zona pellucida was completely dissolved. Microwells (WOWs; Vajta et al., 2000) were prepared in four-well dishes (Nunc) by making microspots with an aggregation needle (BLS) filled with 50 mL of modified SOF medium containing 1.5 mM glucose and 10% KSR and supplemented with 2.0 μg/mL Dox, bLIF-conditioned medium (1:1000 dilution), 1 μM PD0325901, 3 μM CHIR99021, and 10 mM forskolin, and then covered with 400 μL of paraffin oil. Subsequently, the zona-free 8- to 16-cell stage embryos were transferred into the WOWs, and 20–30 naïve-type biPSCs were also transferred and co-cultured at 38.5°C in an atmosphere consisting of 5% CO_2, 5% O_2, and 90% N_2. Five days after initiation of co-cultivation, the aggregated embryos were collected. Three aggregated embryos were then transplanted into each uterine horn of a Japanese black cow at 7 days after heat. Transplantation was performed using a catheter (Misawa Medical Industry).

Pregnancy was diagnosed by rectal palpation or ultrasonography. At 90 days after transplantation, the pregnant cow was sacrificed by an overdose of sodium pentobarbital; the uteri were isolated, dissected, and rinsed with PBS. Fetuses were then isolated, rinsed with PBS, and separated by individual organs/tissues including the brain, skin, lung, liver, stomach, small intestine, large intestine, heart, kidney, spleen, muscle, gonad, amnion, and placenta. Genomic DNA (gDNA) was isolated from the tissues by using TRIzol reagent. Genomically integrated Oct3/4-2A-Klf4 sequences in these samples were detected by PCR analysis of isolated gDNA using O-2A-K (Tg) primers (Table 1). PCR products from all tissues were confirmed by DNA sequencing. Frozen tissue sections isolated from the chimera were prepared using OCT compounds, and the tissue sections were immunostained with anti-Vasa (1:200, ab13840, Abcam) and/or anti-RFP (1:500, MM-0172-P, MédiMabs) primary antibodies, and Alexa Fluor 488 goat anti-rabbit IgG (1:500, Invitrogen) and/or Alexa Fluor 594 goat anti-mouse IgG (1:500, Invitrogen) secondary antibodies.

Statistical analysis

Each experiment was repeated at least three times. The values were analyzed using a t-test. p values < 0.05 were considered to be statistically significant.

Results

Generation of bovine iPSCs

bADCs in culture originally exhibited a heterogeneous population consisting of epithelial and fibroblastic cells (Fig. 2A). After transfection with Dox-inducible piggyBac vectors containing reprogramming factors, the bADCS were cultured in piPSC medium, which was similar to medium generally used for maintenance of human ES cells. Around 8 days after Dox addition, primary colonies appeared, and flattened colonies (human ES cell-like) emerged at day 14 (Fig. 2B). The efficiency of colony appearance at day 14, relative to the total number of cells reseeded onto the SNL feeder layer at day 5, was 0.01%. These colonies (pbiPSCs) were then picked mechanically and transferred onto a fresh SNL feeder layer. pbiPSCs could be stably propagated and subcultured every 7 days over 70 passages in piPSC medium (Fig. 2C).

Addition of 2i and forskolin to culture medium can support naïve characteristics of human iPSCs (Hanna et al., 2010a). In this study, pbiPSCs were dissociated enzymatically and transferred to niPSC medium containing KSR, bLIF, 2i, and forskolin. These cells proliferated and formed mouse ES cell-like colonies with 3-dimensional morphology (Fig. 2D). Cells converted from the primed to the naïve state (pnbiPSCs) were maintained in niPSC medium by at least 15 rounds of trypsinization and single-cell dissociation. Furthermore, when the transfected cells were directly cultured in niPSC medium from 8 days after Dox addition, when primary colonies appeared, nbiPSCs colonies with dome-shape and compact morphology emerged at day 14 (Fig. 2E). The efficiency of colony appearance at day 14 against the total number of cells, which were reseeded on the SNL feeder layer at day 4, was 0.01%. After passage of nbiPSCs by trypsinization and reseeding onto a fresh SNL feeder layer, cells could be maintained for at least 15 passages by single-cell dissociation every 4 days (Fig.

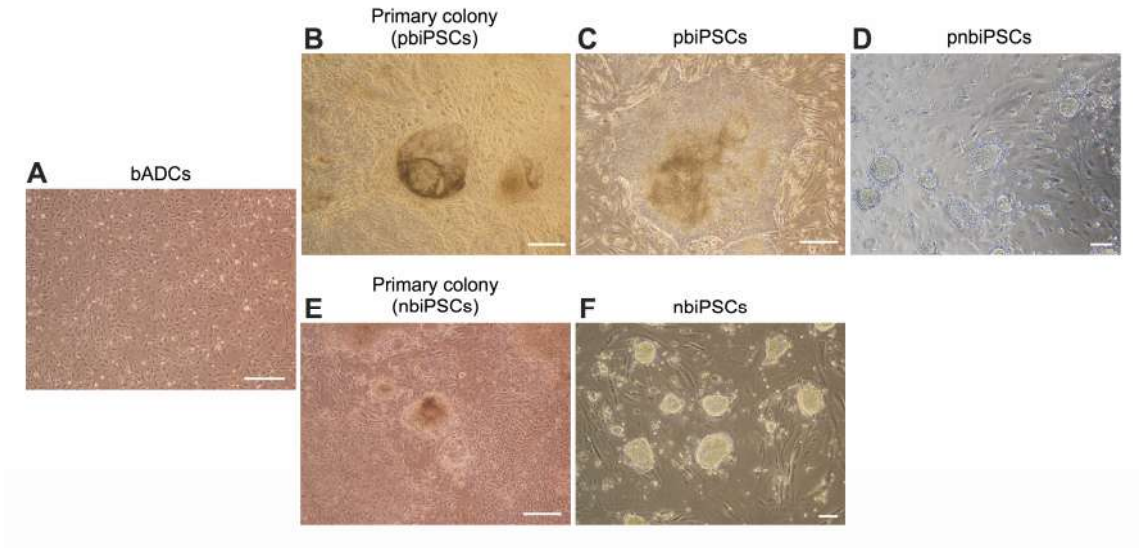

Fig. 2. Phase-contrast images of biPSCs established in two different culture conditions.

(A) bADCs. (B) Primary colonies appearing in primed cell-culture medium. (C) Established primed-type biPSCs. (D) Colonies converted from the primed to naïve state. (E) Primary colonies appearing in niPSCs medium. (F) Established naïve-type biPSCs. (A)–(C), (E), scale bars = 500 μm. (D), (F), scale bars = 100 μm.

2F). When naïve-type biPSCs (pnbiPSCs and nbiPSCs) were transferred to piPSC medium, they reverted to flattened hESC-like colony morphology, and could be maintained in this medium (retaining the flattened morphology) for over 10 passages.

To test which culture compounds were essential for maintaining naïve characteristics, pnbiPSCs were cultured in different culture conditions, in the presence or absence of GSK3 inhibitor (CH), Mek/Erk inhibitor (PD), and/or forskolin (FK) (Fig. 3.). In the absence of all of these compounds (Fig. 3A.), or in the presence of FK alone (Fig. 3D.), colonies became more flattened. In the presence of PD, a subset of colonies retained the three-dimensional morphology (Fig. 3B.), whereas in the presence of CH, most of the colonies had the naïve-type morphology (Fig. 3C.). Cell number was elevated in the presence of FK (Fig. 3I.).

Characterization of primed-type and naïve-type biPSCs

Both primed-type and naïve-type biPSCs were positive for alkaline phosphatase (AP) activity (Fig. 4A, B and Fig. 5A). In the karyotype analysis, 19 out of 20 (95%) pbiPSCs or nbiPSCs with metaphase plates had the normal number ($2n = 60$) of chromosomes, even after they were propagated over 65 passages (Fig. 4C–E); one pbiPSC had 59 chromosomes, and one nbiPSC had 61 chromosomes.

Immunocytochemistry analysis revealed that biPSCs expressed exogenous and/or endogenous OCT3/4 and NANOG (Fig. 4F–I, J–M, and Fig. 5B-G). RT-PCR analysis also revealed that these cells expressed the pluripotency-related genes including *OCT3/4*, *SOX2*, *NANOG*, *E-CADHERIN* (*CDH1*), as well as naïve mouse iPSC–specific genes such as *REX1*, *ESRRβ*, *STELLA*, *LIFR*, and *SOCS3* (Fig. 6). On the other hand, *FGF5*, and *OTX2*, a marker gene for primed human iPSCs, was only detected in pbiPSCs (Fig. 6).

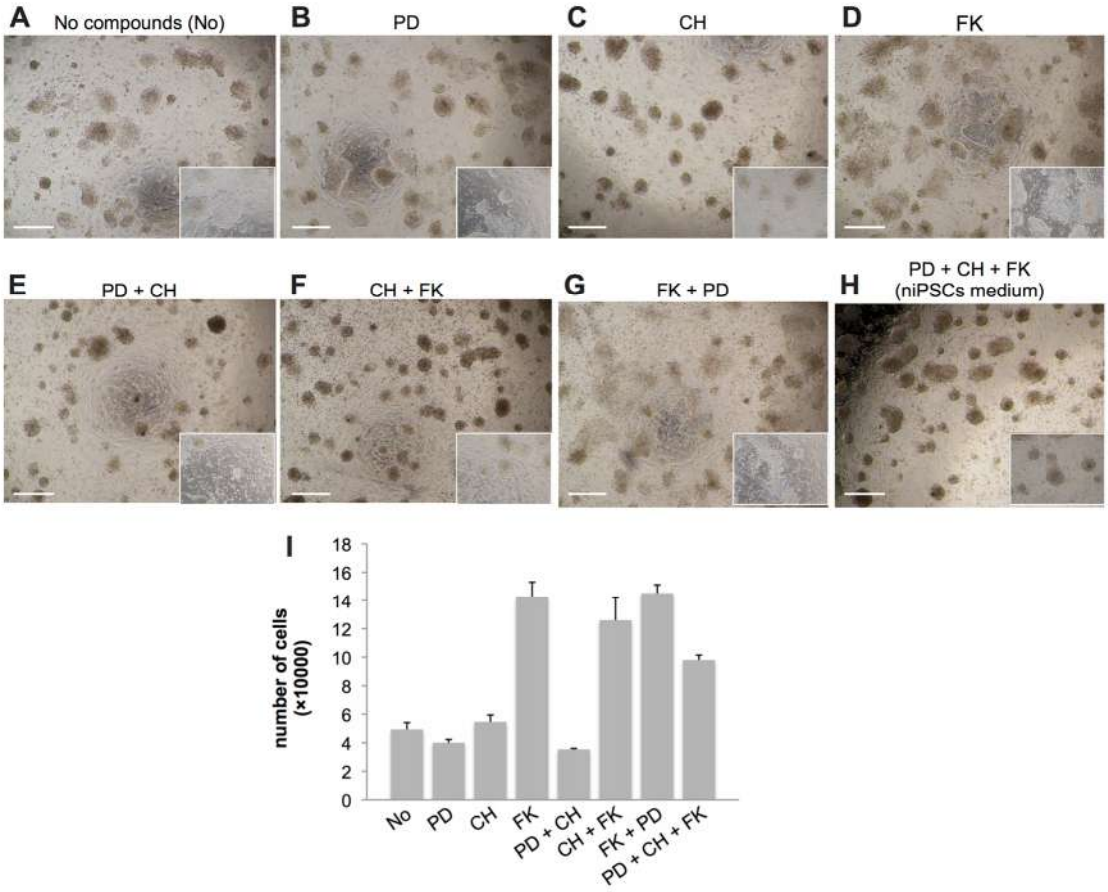

Fig. 3. Appearance of naïve-type biPSCs cultured under different conditions.

pnbiPSCs cultured only with bLIF (A), bLIF+CH (B), bLIF+PD (C), bLIF+FK (D), LIF+CH+PD (E), bLIF+PD+FK (F), bLIF+FK+CH (G), and bLIF+FK+CH+FK (H). (I) Numbers of growing cells after cultivation in different conditions for 4 days. (A)–(H), scale bars = 500 μm.

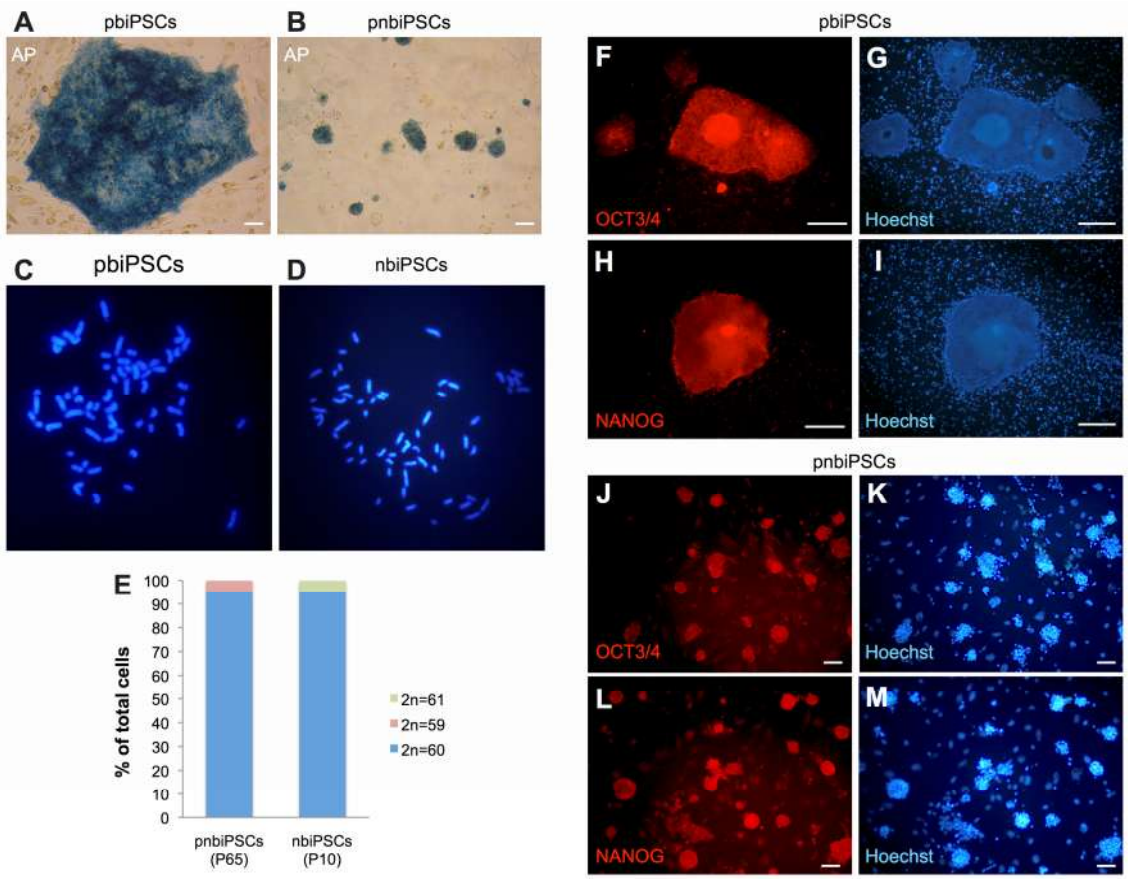

Fig. 4. Characterization of biPSCs.

(A) Alkaline phosphatase activity in primed-type iPSCs (pbiPSCs). (B) Alkaline phosphatase activity in naïve-type iPSCs derived from pbiPSCs (pnbiPSCs). (C) Karyotyping image of pbiPSCs at passage 65. (D) Karyotyping image of nbiPSCs at passage 10. (E) Proportion of cells with the indicated number of chromosomes (n = 20). (F)–(I) OCT3⁄4 (F, OCT3⁄4 staining; G, Hoechst staining) and NANOG (H, NANOG staining; I, Hoechst staining) expression in pbiPSCs. (J)–(M) OCT3⁄4 (J, OCT3⁄4 staining; K, Hoechst staining) and NANOG (L, NANOG staining; M, Hoechst staining) expression in pnbiPSCs. (A), (B), (J)–(M), scale bars = 100 μm. (F)–(G), scale bars = 500 μm.

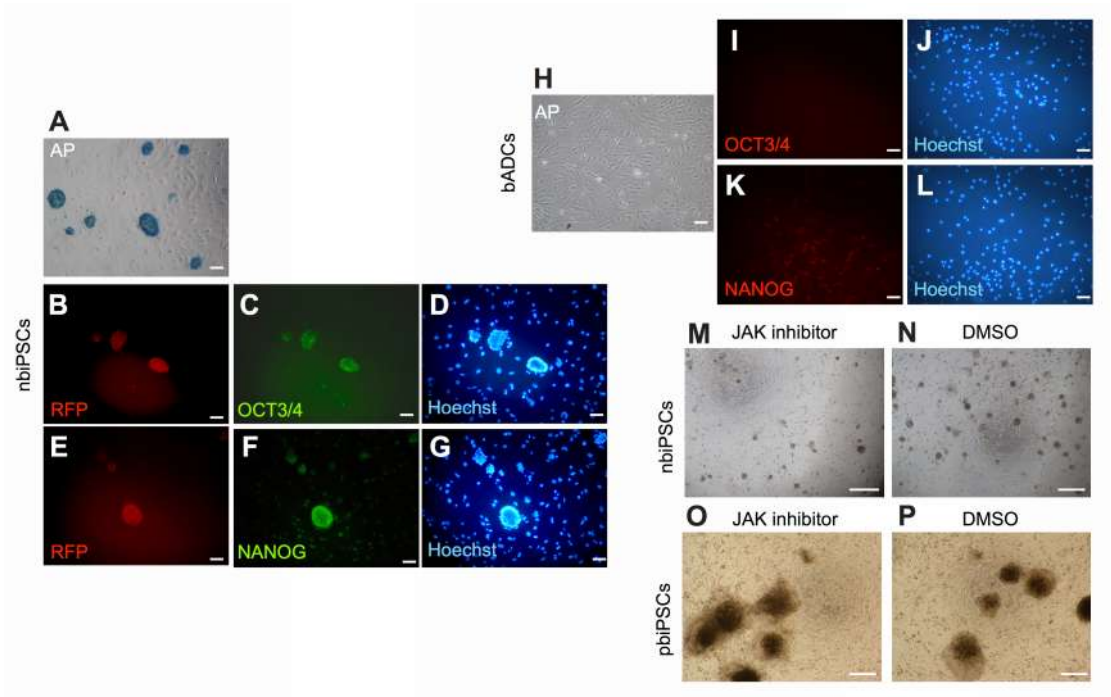

Fig. 5. Characterization of biPSCs.

(A) Alkaline phosphatase activity in nbiPSCs. OCT3⁄4 (B, RFP-positive image; C, OCT3⁄4 staining; D, Hoechst staining) and NANOG (E, RFP-positive image; F, NANOG staining; G, Hoechst staining) expression in nbiPSCs expressing RFP. (H) nbiPSCs cultured in the presence of JAK inhibitor for 4 days. (I) nbiPSCs cultured in the presence of DMSO. (J) pbiPSCs cultured in the presence of JAK inhibitor for 4 days. (K) pbiPSCs cultured in the presence of DMSO. (A)–(G) scale bars = 100 μm. (H)–(K) scale bars = 500 μm.

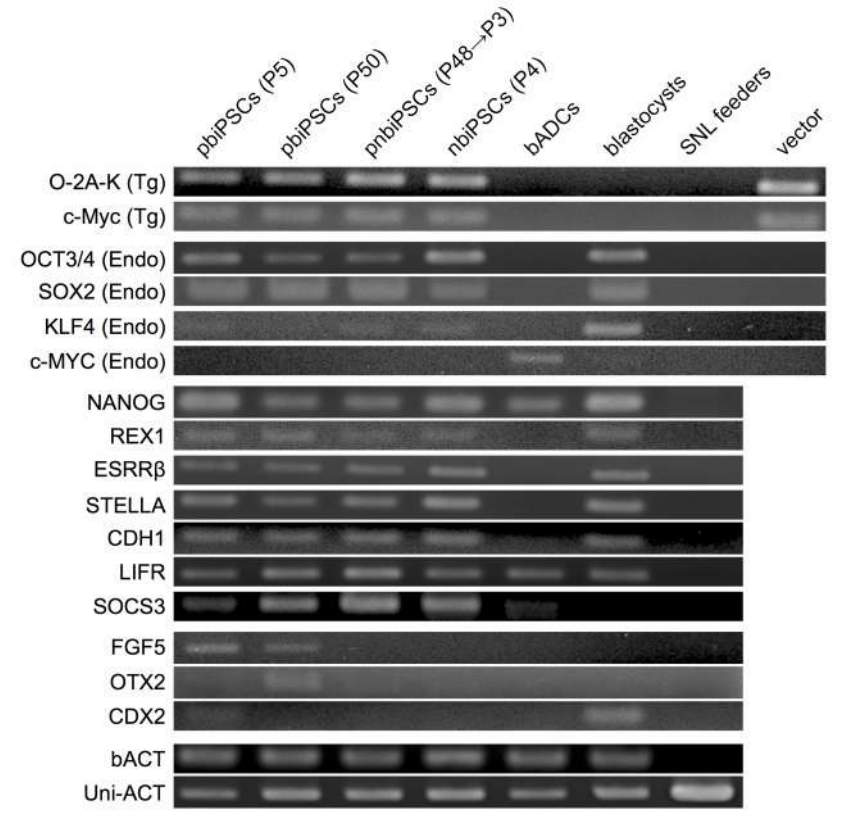

Fig. 6. Endogenous and exogenous expression of genes specific to undifferentiated ESCs in biPSCs.

mRNA expression was evaluated by reverse-transcription polymerase chain reaction (RT-PCR). pbiPSCs (P5), primed-type iPSCs at passage 5; pbiPSCs (P50), primed-type iPSCs at passage 50; pnbiPSCs, naïve-type iPSCs at passage 3 converted from primed-type iPSCs at passage 48; nbiPSCs, naïve-type iPSCs cultured under naïve medium from primary culture; bADCs, bovine amnion-derived cells; SNL feeder, SNL feeder cells; vector, plasmid DNA of PB vectors; bACT, bovine β-ACTIN specific for cattle; Uni-ACT, universal β-ACTIN that reacts with both cattle and mice.

To investigate the signaling pathways required for the maintenance of self-renewal of biPSCs, naïve-type biPSCs were cultured with JAK inhibitor in the absence of bLIF for 4 days. Under these culture conditions, the number of cells was significantly reduced, by more than 60% in pnbiPSCs (Fig. 7A, B, C) and 50% in nbiPSCs (Fig. 5H, I. and Fig. 7C). By contrast, judging by their appearance, the proliferation of pbiPSCs was not affected in this culture condition (Fig. 5J and K.).

Next, we examined X-chromosome inactivation states in the established biPSC cell lines. RT-PCR analysis revealed that *XIST* mRNA was not expressed in pnbiPSCs, but it was expressed in pbiPSCs and female bADCs (used as a positive control) (Fig. 7D). On the other hand, histone H3K27 trimethylation signals associated with X chromosomes inactivation were observed in pbiPSCs, but not in pnbiPSCs (Fig. 7E–H).

When biPSCs were cultured in the absence of Dox, they readily changed their morphology and differentiated (Fig. 8A and B.). RT-PCR analysis revealed that in the absence of Dox, pbiPSCs and pnbiPSCs no longer expressed exogenous transgenes and endogenous pluripotent genes, such as *OCT3/4*, *ESRR β*, and *STELLA*, but instead began to express endogenous *c-MYC* (Fig. 8C.).

Differentiation potential of biPSCs

When biPSCs were induced to differentiate in low-adhesion culture dishes for 6 days, they formed embryoid bodies (Fig. 9A, E and Fig. 10A, B). After they were cultured for another 6 days, they differentiated into all three germ layers, as assessed by immunocytochemistry using specific markers for each layer: GFAP for the ectoderm, ASM for the mesoderm, and AFP for the endoderm (Fig. 9B–D, F–H and Fig. 10C–K). We also assessed the *in vitro* differentiation potential of pnbiPSCs by RT-PCR using specific markers such as *VIMENTIN* (*VIM*) for ectoderm, *BMP4* for mesoderm, and AFP for endoderm (Fig. 10 L.).

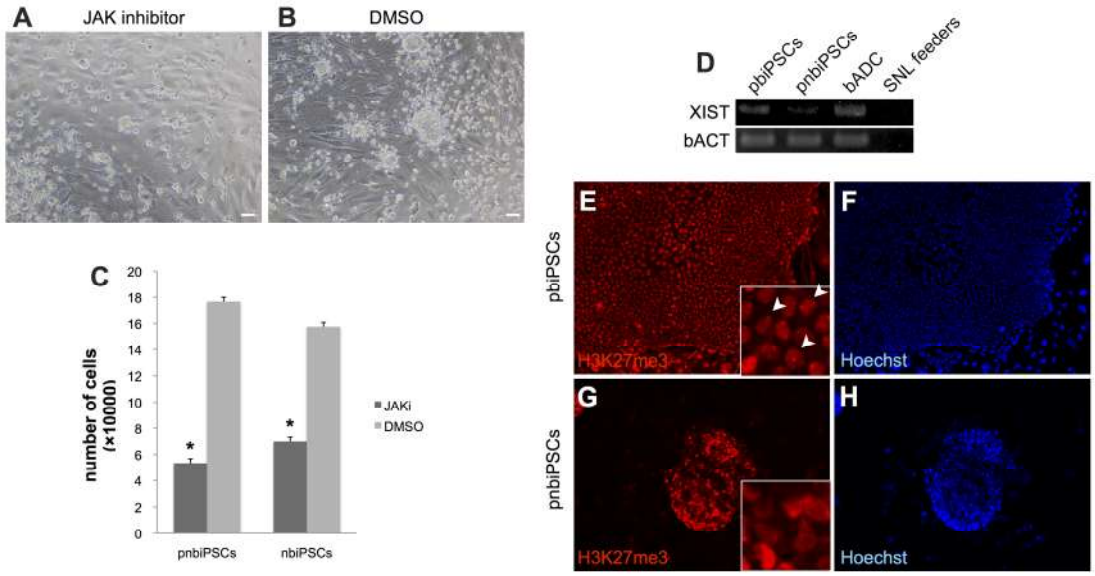

Fig. 7. Naïve-type features of iPSCs.

(A) pnbiPSCs cultured for 4 days in niPSC medium in the presence of JAK inhibitor. (B) pnbiPSCs cultured in the presence of DMSO. (C) The number of cells cultured in the presence of JAK inhibitor or DMSO (*$p < 0.05$). (D) *XIST* expression evaluated in pbiPSCs, but not in pnbiPSCs. Immunocytochemistry images of methylation status at H3K27me3 sites (E, pbiPSCs; F, Hoechst staining; G, pnbiPSCs; H, Hoechst staining). Arrowheads indicate puncta of H3K27me3. (A), (B), scale bars = 500 μm.

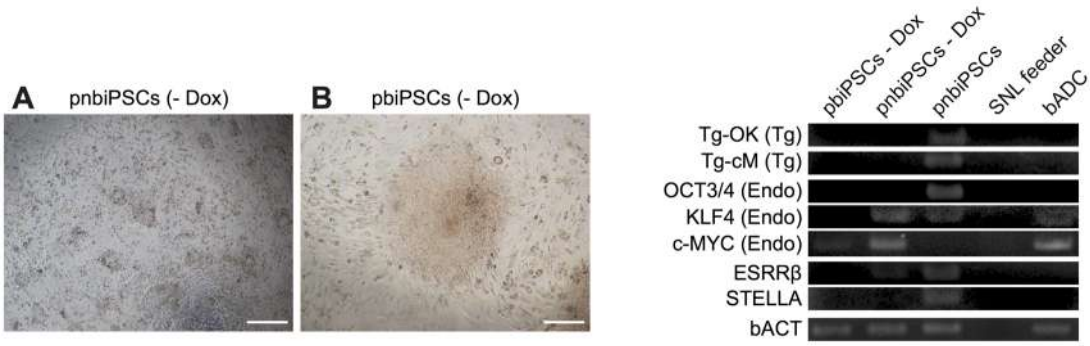

Fig. 8. biPSCs cultured in the absence of Dox (- Dox).

(A) Phase-contrast image of pnbiPSCs cultured in the absence of Dox for 4 days. (B) Phase-contrast image of pbiPSCs cultured in the absence of Dox for 7 days. (C) Endogenous and exogenous gene expression in biPSCs cultured in the absence of Dox. (A), (B), scale bars = 500 μm.

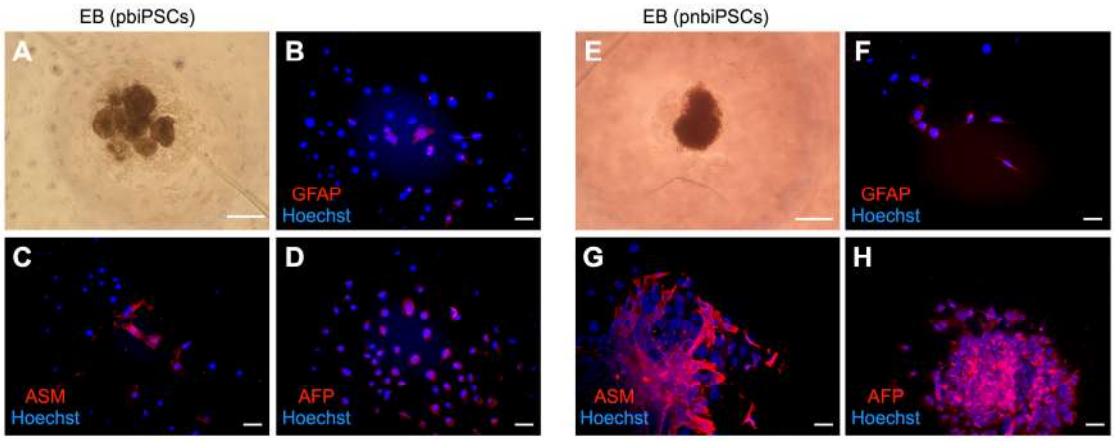

Fig. 9. Differentiation potential of biPSCs in culture.

(A) Embryoid body formation of pbiPSCs grown for 6 days in low cell-adhesion dishes. Immunocytochemical staining for markers for the three germ-layer in differentiated cells derived from pbiPSCs. α-fetoprotein (B, endoderm), actin smooth muscle (C, mesoderm), and glial fibrillary acidic protein (D, ectoderm) were used as markers. (E) Embryoid body formation by pnbiPSCs. Immunocytochemical staining for α-fetoprotein (F), actin smooth muscle (G), glial fibrillary acidic protein (H). (A), (E), scale bars = 500 μm. (B)-(D), (F)-(H), scale bars = 100 μm.

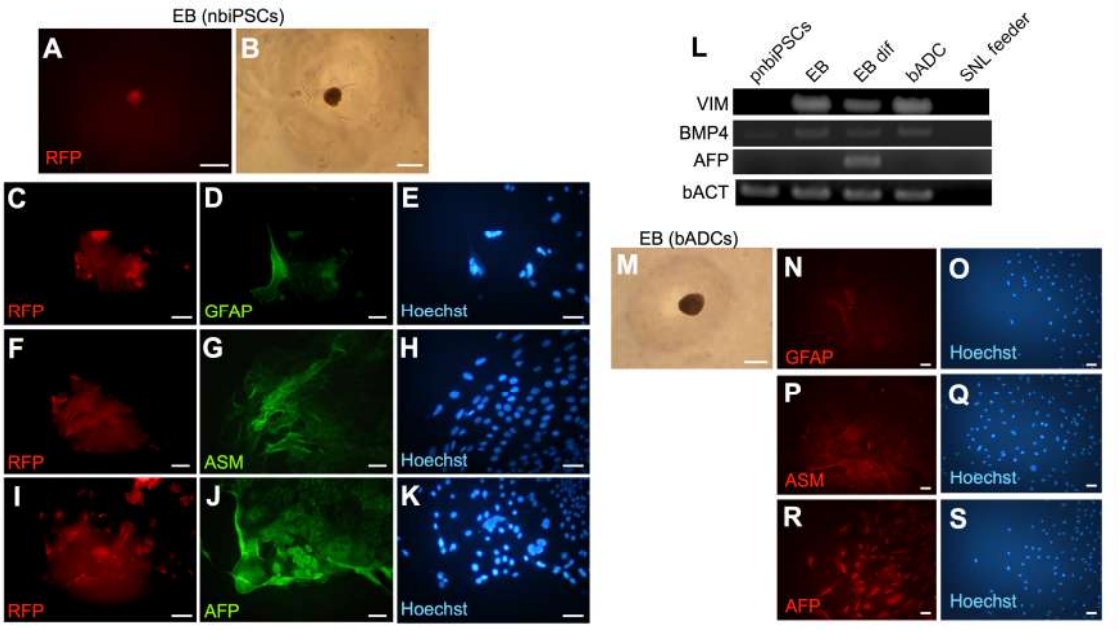

Fig. 10. Differentiation potential of nbiPSCs expressing RFP.

(A) Embryoid body formation of nbiPSCs grown in low cell-adhesion dishes for 6 days. Glial fibrillary acidic protein (C, RFP-positive image; D, glial fibrillary acidic protein; E, Hoechst staining), actin smooth muscle (F, RFP-positive image; G, actin smooth muscle; H, Hoechst staining) and α -fetoprotein (I, RFP-positive image; J, α -fetoprotein; K, Hoechst staining) were used for the markers. (L) Gene-expression profile of pnbiPSCs after embryoid body differentiation. EB, Embryoid body; EB dif, EB-derived cells cultured for an additional 6 days on a gelatin-coated dish; VIM, VIMENTIN. (A), (B), scale bars = 500 μm. (C)–(K) scale bars = 70 μm.

To test whether naïve-type biPSCs had the potential to associate with the normal development of bovine embryos, we generated naïve-type biPSCs expressing Tag-RFP and aggregated them with 8- to 16-cell stage embryos. In preliminary experiments, pbiPSCs were allowed to aggregate with 8- to 16-cell stage embryos by two different ways of cell dissociation. When we trypsinized pbiPSCs and aggregated them with embryos, the cells were scattered around and didn't aggregate with embryonic cells. On the other hand, when we mechanically split pbiPSCs and aggregated with embryos, cell clumps were formed outside embryos, however, didn't make aggregation. Meanwhile, naïve-type biPSCs developed normally into blastocysts, to the same degree as aggregation performed only with 8- to 16-cell stage embryos (Table 2). Judging from RFP fluorescence, pnbiPSCs and nbiPSCs were successfully incorporated into the ICM region of blastocyst stage embryos (Fig. 11A). In addition, we sometimes observed incorporation into both the ICM and TE regions (13.9% and 34.4% in the case of pnbiPSCs and nbiPSCs, respectively; Table 2). By contrast, we did not observe incorporation of bADCs expressing RFP into the embryos (Table 2).

Chimeric embryos formed by aggregation of 8- to 16-cell bovine embryos and pnbiPSCs were subsequently transferred to the uteruses of a surrogate mother in order to trace their developmental capacity *in vivo*. Three chimeric embryos were transferred into each uterine horn, and the resultant fetuses were recovered at 90 days after transplantation (Fig. 11B). All of the fetuses developed normally. The fetuses were then isolated and separated by individual organs/tissues representing ectoderm (brain, skin), endoderm (lung, liver, stomach, small intestine, large intestine), mesoderm (heart, kidney, spleen, muscle), germline (gonad), fetal membrane (amnion) and trophectoderm (placenta). PCR analysis of integrated Oct3/4-2A-Klf4 sequences were performed in each fetal tissue. Eleven out of 14 tissues (brain, skin, lung, stomach, small intestine, large intestine, spleen, muscle, gonad, amnion, and placenta) showed evidence of a pnbiPSC contribution (Fig. 11C).

Table 2. Aggregation of pnbiPSCs into *in vitro* fertilized embryos, and their development *in vitro*.

			Chimeric blastocysts (%)		
Donor cells	No. of aggregated embryos	No. of blastcysts developed (%)	No. contributed to ICM	No. contributed to both ICM and TE	No. contributed to TE
pnbiPSCs	60	38 (63.3)	31 (86.1)	5 (13.9)	0 (0)
nbiPSCs	60	35 (58.3)	21 (65.6)	11 (34.4)	0 (0)
bADCs*	60	35 (58.3)	0 (0)	0 (0)	0 (0)
Embryo only	60	34 (56.7)	0 (0)	0 (0)	0 (0)

*RFP vector was introduced

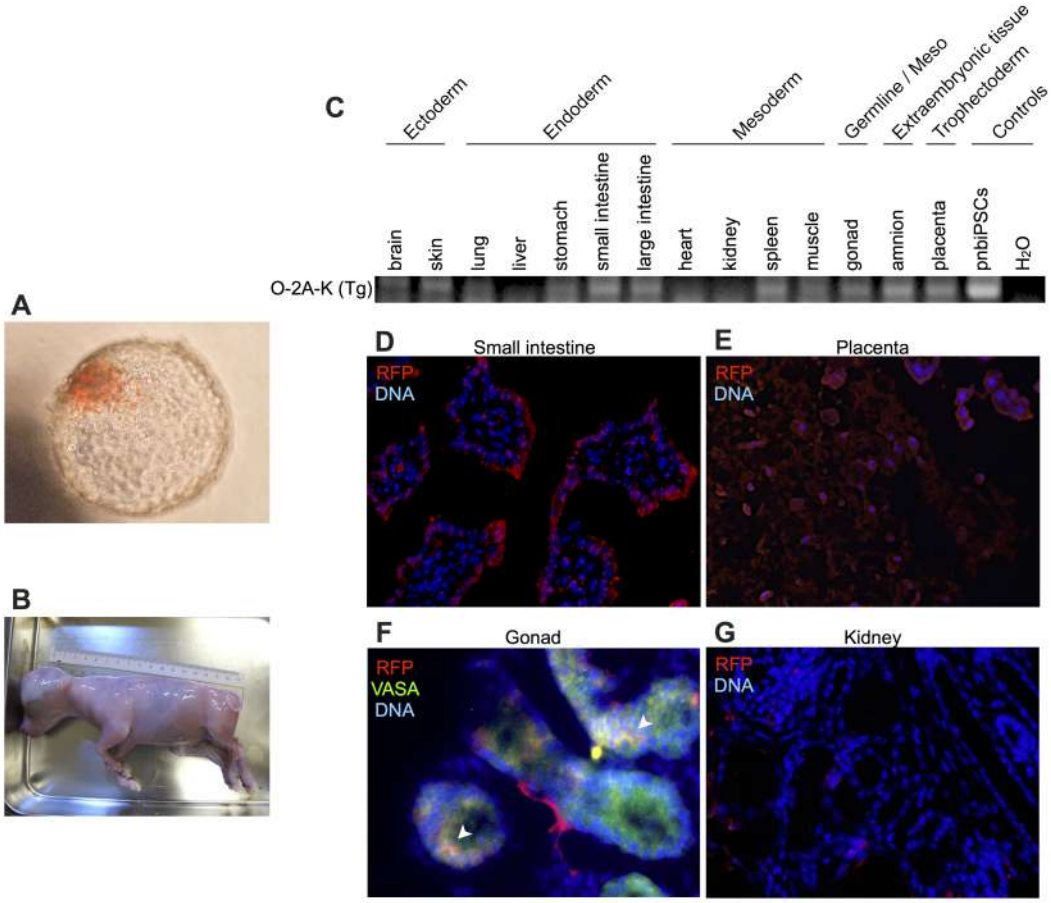

Fig. 11. Production of chimeric fetuses from bovine embryos using the aggregation method.

(A) Naïve-type biPSCs expressing Tag-RFP were aggregated with host at the 8- to 16-cell stage of *in vitro* fertilized embryos. (B) Chimeric fetuses at day 90 of gestation derived from aggregated embryos. (C) PCR analysis using transgene-specific primers for genomically integrated Oct3/4-2A-Klf4 sequences in 14 tissues. Genomic DNA isolated from pnbiPSCs was used as a positive control. H_2O was used as a negative control (buffer alone for RT-PCR). Immunofluorescence analysis showing the distribution of pnbiPSC-derived cells (RFP-positive with red signals) in the small intestine (D), placenta (E), gonad (F, VASA-positive cells with green signals; arrowheads indicate the portion that is double-positive for RFP and VASA), and kidney (G) of the chimeric fetus. Nuclei were stained with DAPI (blue).

PCR analysis revealed the contribution of pnbiPSCs to chimeric fetuses. In addition, we performed immunofluorescence analysis on frozen tissue sections of small intestine, kidney, placenta, and gonad. Red fluorescence from TagRFP-expressing vector was observed in the small intestine, placenta, and gonad (Fig. 11D, E, F). By contrast, fluorescence was faint or absent in the kidney (Fig. 11G). These immunofluorescence results were coincident with those of PCR analysis. Moreover, in the gonad, some of the cells were double-positive for RFP (red) and VASA (green), a primordial germ cell marker (Fig. 11F, arrowheads).

Discussion

Since the first generation of iPSCs in mouse (Takahashi & Yamanaka, 2006), a large number of studies of iPSCs have been performed in both mouse and human. Despite numerous attempts, however, very few studies have reported the generation of iPSCs from somatic cells or ESCs from preimplantation embryos in other species. In cattle, Cao et al (2009), Ozawa et al (2012), and Furusawa et al (2013) reported the generation of bovine embryonic stem cell–like cells, and Sumer et al (2011) reported the generation of primed biPSCs, but also described the difficulty of derivation and maintenance of these cells. Most iPSCs reported in non-rodent species were primed iPSCs (Esteban et al., 2009, Ezashi et al., 2009, Honda et al., 2010, Liu et al., 2008, Montserrat et al., 2011, Sumer et al., 2011, Tomioka et al., 2010, Wu et al., 2009), which have a limited capacity to produce chimeras relative to naïve iPSCs (Hanna et al., 2010b, Nichols & Smith, 2009, Tesar et al., 2007). Here, we report the generation of two different types (i.e., primed and naïve) of biPSCs established from bADCs by introducing Dox-inducible PB vectors. Both types had characteristics of pluripotent stem cells; in particular, the naïve-type biPSCs exhibited several characteristics of pluripotent cells, comparable to those of naïve mouse iPSCs, and contributed to the ICM of bovine host blastocysts and chimeric fetuses.

Sumer et al (2011) reported previously that generation of biPSCs by retroviral delivery and the ectopic expression of *POU5F1*, *SOX2*, *KLF4*, *c-MYC*, and *NANOG*, and demonstrated that ectopic expression of *NANOG* is necessary for the generation and maintenance of biPSCs from bovine fetal fibroblasts. In this study, we were able to generate biPSCs without ectopic expression of *NANOG*, possibly because the bADCs used in this study expressed intrinsic endogenous *NANOG* (Fig. 6). Therefore, ectopic expression of *NANOG* may not be necessary for production and maintenance of

biPSCs. In addition, studies on human and mouse iPSCs have suggested that reprogramming via introduction of transcription factors in ADCs is more efficient and faster than in fibroblasts (Li et al., 2009, Nagata et al., 2009, Zhao et al., 2010) probably because ADCs in mouse and human express high endogenous levels of *Klf4*, *c-Myc*, and *Ronin*, which support proliferation and self-renewal of iPSCs (Nagata et al., 2009). When we used bovine embryonic fibroblast cells and transfected them with the same combination of transcription factors, iPSC-like colonies appeared, but no stable iPSC lines were established (data not shown). Moreover, when we cultured bADCs in low-adhesion culture dishes for 6 days, they formed EB-like cell masses and differentiated a part of cell types representing each germ layer after culturing on gelatin-coated dishes for another 6 days. Previous reports showed that a subpopulation of human ADCs exhibited stem cell-like characteristics such as an expression of pluripotency markers and an ability to differentiate into various cell types representing each germ layer (Miki et al., 2005). In our study, bADCs also exhibited the expression of *NANOG* as well as several lineage specification markers such as *VIM* (ectoderm marker) and *BMP4* (mesoderm marker). Thus, bADCs may have stem cell-like characteristics and which may make them an appropriate cell source for generation of iPSCs in cattle.

The choice of reprogramming components, and the order of these factors in the vector, also affect the efficiency of reprogramming of somatic cells and the generation of iPSCs. Okita et al (2008) reported that expression of Oct3/4, Klf4, and then Sox2 (OKS) in that order in polycistronic vectors improves efficiency of reprogramming and facilitates the generation of murine iPSCs. Tsukiyama et al (2011) reported that cell reprogramming induced by the combination of OKS and c-Myc (M) vectors (OKS + M) was more efficient than that induced by polycistronic MKOS vectors. When we used MKOS vectors instead of OKS + M vectors, no iPSC-like colonies appeared (data not shown), whereas OKS + M vectors could generate iPSC-like colonies with an efficiency of 0.01%.

Therefore, the OKS + M vector combination is also useful for generation of iPSCs in cattle.

The biPSCs reported by Sumer et al (2011) appeared in the primed state, as judged by their morphology and the use of a culture medium containing FGF. In this study, when the primed-type biPSCs were passaged and maintained in the niPSCs medium containing bLIF, 2i, and forskolin, naïve-type biPSCs appeared. These cells exhibited several hallmarks of naïve PSCs, such as the expression of naïve marker genes (including the STAT3 target *SOCS3*) (van Oosten et al., 2012) LIF-dependent proliferation (Hanna et al., 2010b, Nichols & Smith, 2009), and reactivation of the X chromosome (X_aX_a) (Hanna et al., 2010a, Hanna et al., 2010b, Nichols & Smith, 2009). In humans, primed iPSCs can be converted into naïve iPSCs by the addition of 2i and forskolin to the culture medium (Hanna et al., 2010a). In our experiment, once naïve-type cells appeared, only the addition of bLIF + GSK3 β inhibitor could maintain them in this state (Fig. 3C.), indicating that the continuous expression of transcription factors in naïve-type cells could substitute for the effects of Mek/Erk inhibitor and forskolin. On the other hand, our primed-type biPSCs exhibited a flattened morphology, LIF-independent proliferation, inactivation of the X chromosome (X_aX_i), and the expression of primed marker genes such as *FGF5* (Fig. 6); however, they also expressed naïve marker genes such as *REX1*, *ESRR* β, *STELLA*, *LIFR*, and *SOCS3* (Fig. 6). Recent reports describe intermediate states of cells of murine ESCs or iPSCs that share characteristics with the primed- and naïve-cell states (Ozawa et al., 2014, Tsukiyama & Ohinata, 2014), even in primed culture conditions (Ozawa et al., 2014). These intermediate cells express both primed marker genes, such as *Fgf5*, and naïve marker genes such as *Rex1*, *Esrr* β, and *Stella*. Tsukiyama et al. (Tsukiyama & Ohinata, 2014) also showed that intermediate cells do not depend on the LIF-Jak/Stat pathway for proliferation. These data are in agreement with our findings; hence, our primed-type biPSCs should be considered as similar to intermediate cells.

In the absence of Dox, biPSCs readily changed their morphology and no longer expressed exogenous transgenes and endogenous pluripotent genes. Instead, they began to express endogenous *c-MYC* (Fig. 8C.). These results indicated that transgene expression could be controlled by the removal of Dox, and that continuous transgene expression is necessary to maintain biPSCs in a pluripotent state. Optimal culture conditions for the establishment and maintenance of ESCs vary among species (Pease et al., 1990, Suemori & Nakatsuji, 2006, Xu et al., 2005). Most of the iPSC lines established in non-rodent species depend on continuous transgene expression to maintain their pluripotency (Esteban et al., 2009, Ezashi et al., 2009, Shimada et al., 2010, Sumer et al., 2011, Wu et al., 2009). More recently, naïve human ESCs or iPSCs can be established without transgene expression in the presence of LIF, FGF2, and TGF β 1 (a member of the TGF- β superfamily, and the same as activin A) and inhibitors against four signaling pathways (ERK1/2, GSK3 β , JNK, and p38) for stable cell propagation (Gafni et al., 2013). Therefore, further studies are required in order to determine the optimal culture conditions for maintaining established biPSCs in the absence of Dox. This study provides a model for generating authentic naïve-type biPSCs under the control of transgenes.

The biPSCs we established exhibited many features of pluripotency; however, they did not form teratomas in nude mice (BALB/c nu/nu, data not shown). The difficulty of developing mature teratomas from naïve-like human iPSCs has been described previously (Buecker et al., 2010, Hirano et al., 2012, Nishishita et al., 2012), and the specific strain of immunodeficient mouse affects teratoma formation (Drukker et al., 2006). On the other hand, biPSCs could form embryoid bodies and differentiate into all three germ layers *in vitro*. In addition, they were incorporated into ICM, and sometimes into TE, after aggregation with 8- to 16-cell stage embryos. These observations led us to examine the potential of biPSCs to contribute to chimeric fetuses. Although primed-type biPSCs

could propagate stably for more than 70 passages, naïve-type biPSCs were somewhat difficult to maintain, and could be propagated for only 10 to 15 passages; therefore, pnbiPSCs (derived from pbiPSCs) were employed for aggregation and transplantation. Aggregated embryos transplanted into the uteruses of surrogate mothers successfully produced three chimeric fetuses. Although the contribution of pnbiPSCs to some tissues was faint or absent, these cells had the potential to differentiate into all three germ layers, trophectoderm, and potentially germline *in vivo*. Incorporation into the trophectoderm has not been observed in mice, but has been frequently observed in pigs (Fujishiro et al., 2013). This difference is possibly due to interspecies diversity in *OCT3/4* regulation, resulting in changes in Cdx2:Oct4 ratios and the relative timing of trophectoderm commitment observed not only in cattle but also in human, pig, and rabbit blastocysts (Berg et al., 2011). Our study provides the first demonstration that biPSCs can contribute to chimeric fetuses and differentiate into all tissues, including extraembryonic tissues. The next step to be achieved is the production of adult chimeras and offspring.

In summary, we generated two different types of biPSCs from bADCs using Dox-inducible PB vectors. Our results show for the first time that biPSCs meet the criteria for naïve iPSCs and have the capacity to contribute to chimeric fetuses, including the germ cell lineage. These cell lines should facilitate the optimization of culture conditions for generation and and maintenance of bovine iPSCs that have the potential to generate chimeric offspring.

Chapter 3

Derivation of induced trophoblast cell lines in cattle by doxycycline-inducible piggyBac vectors

Abstract

Trophectoderm lineage specification is one of the earliest differentiation events in mammalian development. The trophoblast lineage, which is derived from the trophectoderm, mediates implantation and placental formation. However, the processes involved in trophoblastic differentiation and placental formation in cattle remain unclear due to interspecies differences when compared with other model systems and the small repertoire of available trophoblast cell lines. Here, we describe the generation of trophoblast cell lines (biTBCs) from bovine amnion-derived cells (bADCs) using an induced pluripotent stem cell technique. bADCs were introduced with piggyBac vectors containing doxycycline (Dox)-inducible transcription factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*). Colonies that appeared showed a flattened epithelial-like morphology similar to cobblestones, had a more definite cell boundary between cells, and frequently formed balloon-like spheroids similar to trophoblastic vesicles (TVs). biTBCs were propagated for over 60 passages and expressed trophoblast-related (*CDX2*, *ELF5*, *ERR*β, and *IFN-*τ) and pluripotency-related genes (endogenous *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*). Furthermore, when biTBCs were induced to differentiate by removing Dox from culture, they formed binucleate cells and began to express pregnancy-related genes (*PL*, *PRP1*, and *PAG1*). This is the first report demonstrating that the induction of pluripotency in bovine amniotic cells allows the generation of trophoblastic cell lines that possess trophoblast stem cell-like characteristics and have the potential to differentiate into the extra-embryonic cell lineage. These cell

lines can be a new cell source as a model for studying trophoblast cell lineages and implantation processes in cattle.

Introduction

Mammalian blastocysts are composed of two distinct cell types: the inner cell mass and the trophectoderm. The trophectoderm is the first cell type that differentiates from pre-implantation embryos at the blastocyst stage. The trophectoderm cell lineage plays an important role in implantation and placental formation (Pfeffer & Pearton, 2012). However, the processes trophoblastic differentiation and placental formation in cattle are poorly understood due to interspecies differences compared with mice and humans. In cattle, a blastocyst hatches in the uterus at day 9 of pregnancy and initiates the rapid elongation of the trophectoderm at around day 12 (Sakurai et al., 2012). The extraembryonic membrane extends throughout the entire uterine horns by day 24 and subsequently attaches to endometrial cells (Degrelle et al., 2005). At the beginning of implantation and throughout pregnancy, trophoblastic binucleate cells are differentiated from mononuclear cells (Shimada et al., 2001). During this peri-implantation period, trophoblastic cells produce a number of molecules such as interferon-tau (IFN-τ), placental lactogen (PL), prolactin-related proteins (PRPs) and pregnancy-associated glycoproteins (PAGs). IFN- τ is a cytokine that mononuclear trophoblast cells of ruminant conceptuses secrete (Kimura et al., 2004, Roberts et al., 1992, Sakurai et al., 2012, Suzuki et al., 2011), and the signals support the recognition of pregnancy (Imakawa et al., 1987, Roberts et al., 1992). IFN-τ is detected on days 7-8, increases on day 14, peaks on days 19–20 and declines soon after the conceptus attaches to the uterine epithelium (Guillomot et al., 1990, Imakawa et al., 2006). In contrast, PL, PRPs, and PAGs are hormones that are secreted by binucleate cells and play a main role in the fetal-maternal interface (Shimada et al., 2001).

Recently, a decrease in the conception rate of cattle has become a serious problem for many countries. Dunne (Dunne et al., 2000) reported that most embryonic losses in heifers have occurred

before day 14 of pregnancy. Given that this period is closely related to the time of elongation of trophoblasts, it is likely that the development and differentiation of trophoblast cells affect conception ability in cattle. In rodents, trophoblast stem cells (TSCs) have been derived from the polar trophectoderm of blastocysts and retain the capacity to differentiate into all trophoblast derivatives of the later placenta *in vitro* (Roberts & Fisher, 2011, Tanaka et al., 1998), and these cells have been used to investigate their role in the placenta (Sahgal et al., 2006). In contrast, authenticated TSCs have not been generated from ungulate species, although primary trophoblast cell lines have been produced from conceptuses from sheep and goat (Miyazaki et al., 2002), pig (Fléchon et al., 1995, Ka et al., 2001, Ramsoondar et al., 1993), and cattle (Bai et al., 2009, Hashizume et al., 2006, Talbot et al., 2000). Many of these cell lines grow continuously in culture without apparent senescence and display characteristics expressed in trophoblast cells, but they likely represent a differentiation state beyond TSCs in terms of morphology, the presence of binucleate cells in colonies and gene expression related to binucleate cells.

Since the first generation of induced pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006), the technique for inducing pluripotency by ectopic expression of transcription factors in somatic cells has allowed the generation and maintenance of iPSCs in species including cattle (Sumer et al., 2011) in which it has been difficult to isolate and culture embryonic stem cells (Ezashi et al., 2009, Fujishiro et al., 2013, Honda et al., 2010). Recently, the iPS cell technique has also allowed the generation of trophoblast cell lines from somatic cells in pigs (Ezashi et al., 2011) and in humans (Chen et al., 2013). This cell lineage also showed trophoblast-like characteristics such as an epithelial-type morphology, the expression of trophoblast-related genes and the formation of trophoblastic vesicles (TVs). However, to date, there are no reports regarding the generation of a trophoblast stem cell line in cattle.

In this study, to provide cattle trophoblast stem cell lines, we attempted to establish induced trophoblast cells (biTBCs) from bovine amnion-derived cells (bADCs) and estimate the cellular characteristics and potential to differentiate into the trophoblast cell lineage.

Materials and Methods

Ethics statements

All cattle were fed grass silage-based diet *ad libitum*. All procedures involving the care and use of animals were approved by the Animal Research Committee of NARO institute of Livestock and Grassland Science.

Isolation of bovine amnion-derived cells (bADCs) and fetal liver-derived cells (bFLCs)

A bovine amnion layer was harvested from a female Japanese black cattle fetus after 50 days of gestation at the National Institute of Livestock and Grassland Science, Japan. The amnion was mechanically peeled away from the chorion and allantois, divided into small pieces with fine surgical scissors, and dissociated by incubating for 2 hours at 37°C with 0.3% collagenase (Wako, Osaka, Japan) in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA). After collagenase digestion, the cell suspension was maintained at room temperature for 5 min and then poured through a cell strainer; the filtered suspension was then centrifuged at 200 *g* for 5 min. The precipitated cells were cultured in DMEM containing 10% FBS, penicillin (Sigma-Aldrich, St. Louis, MO, USA), and streptomycin (Sigma-Aldrich). When the cells reached confluence, they were cryopreserved in liquid nitrogen until use.

Bovine liver tissue was isolated from a female Japanese black cattle fetus at 68 days of gestation at the National Institute of Livestock and Grassland Science, Japan. The liver was divided into small pieces with fine surgical scissors, and dissociated by incubating for 2 hours at 37°C with

0.1% collagenase in DMEM. After collagenase digestion, the cell suspension was diluted with DMEM containing 10% FBS and then poured through a cell strainer; the filtered suspension was then centrifuged at 200 *g* for 5 min. The precipitated cells were cultured in DMEM containing 10% FBS, penicillin, streptomycin, and primocin (InvivoGen, San Diego, CA, USA). When the cells reached confluence, they were cryopreserved in liquid nitrogen until use.

Cell culture

bADCs and bFLCs were maintained on collagen-coated (Nitta Gelatin, Osaka, Japan) dishes in somatic cell medium consisting of DMEM containing 10% FBS, 50 ng/ml epidermal growth factor (EGF, Calbiochem, San Diego, CA, USA), penicillin, and streptomycin. The cells were dissociated enzymatically with TrypLE Select (Invitrogen) for further propagation.

biTBCs were generated by introducing Dox-inducible PB vectors into bADCs or bFLCs as described below. biTBCs were first generated in iPS medium consisting of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Invitrogen) containing 20% Knockout Serum Replacement (KSR, Invitrogen), 2 mM L-glutamine (MP Biomedicals, Santa Ana, CA, USA), 1 × MEM nonessential amino acids (NEAA, Invitrogen), 0.1 mM 2-mercaptoethanol (2-ME, Wako), penicillin and streptomycin supplemented with 2.0 μg/mL doxycycline (Dox, Sigma-Aldrich) and 5 ng/mL human basic fibroblast growth factor (bFGF, Wako or ReproCELL, Kanagawa, Japan). In subsequent experiments, biTBCs were generated and maintained in trophoblast medium (TBM) consisting of DMEM/F12 containing 10% FBS, 2 mM L-glutamine, 1 × NEAA, 0.1 mM 2-ME, penicillin and streptomycin supplemented with 2.0 μg/mL Dox. Culture medium was changed every other day. Cells were subcultured every 7 days by physically splitting the cells into clumps using a pulled Pasteur pipette and maintaining them on 35 mm diameter cell culture dishes (IWAKI, Tokyo,

Japan) on a feeder layer of 3–5 × 10^5 cells SNL cells (McMahon & Bradley, 1990, Okita et al., 2007) inactivated with 10 μg /ml mitomycin C (Sigma-Aldrich).

For feeder-free culture, biTBCs were split into cell clumps, and the clumps were transferred onto collagen-coated dishes. Cells were maintained in TBM and subcultured every 7 days.

All cultures were maintained in a humidified incubator at 38.5°C with 5% CO_2 in air.

Generation of biTBCs using Dox-inducible PB vectors

bADCs were plated at 1.25 × 10^5 cells per 35-mm dish in the culture medium without antibiotics and incubated overnight. Cells were then transfected using Lipofectamine LTX (Invitrogen). Briefly, equal amounts (0.4 μg) of hyPBase vectors (pCAG-hyPBase) (Yusa et al., 2011), PB vectors with reprogramming factors (PB-TET-OKS and pPB-TET-c-Myc) (Okita et al., 2008, Tsukiyama et al., 2011, Woltjen et al., 2009), the rtTA PB vector (PB-CAG-rtTA Adv, Addgene), and/or the TagRFP PB vector (pPBCAG-TagRFP-IH) (Tsukiyama et al., 2011), 2 μL of Plus reagent (Invitrogen) and 10 μL of Lipofectamine LTX transfection reagent were diluted and mixed in 400 μL Opti-MEM medium (Invitrogen). The DNA-lipid complexes were then added to the culture dish. The culture medium was changed 6 hours after transfection. One day after transfection, the culture was supplemented with 2.0 μg /mL Dox. Four days after the Dox addition, cells were dissociated with TrypLE Select, 1 × 10^5 cells were reseeded on a SNL feeder layer, and the medium was replaced with iPS medum or TBM. Fourteen days after the addition of Dox, primary colonies were mechanically collected and transferred onto a SNL feeder layer in 4-well plates. The medium was changed every day or every other day, depending on cell growth (Fig. 12).

The original vectors including PB-TET-MKOS (a tetracycline inducible polycistronic vector containing transcription factors in the following order: *c-Myc*, *Klf4, Oct3/4*, and *Sox2*), PB-CAG-rtTA

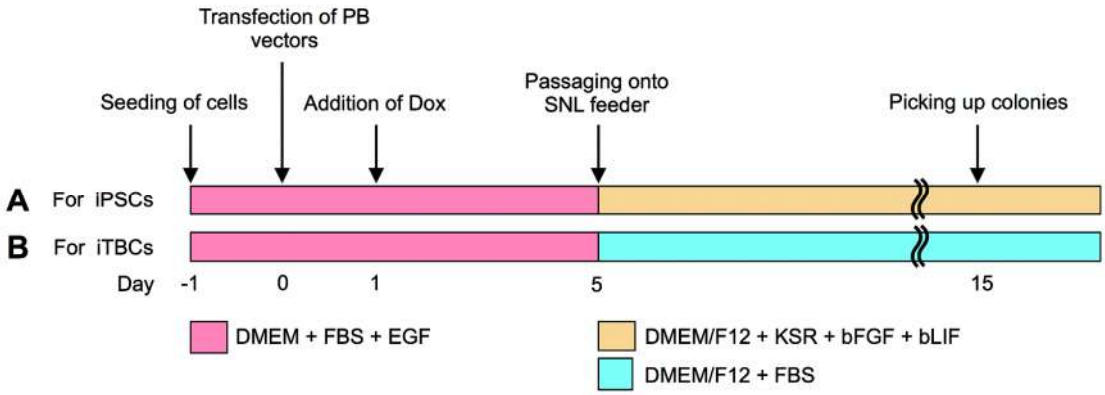

Fig. 12. Generation of bovine-induced trophoblastic cells (biTBCs) using Dox-inducible PB vectors.

(A) Timeline for the establishment of biTBCs in culture medium used for human iPSCs. (B) Timeline for the establishment of biTBCs in culture medium used for bovine trophoblast cells (TBM).

Adv and pCX-OKS-2A (a polycistronic retroviral vector containing transcription factors in the following order: *Oct3/4*, *Klf4*, and *Sox2*) were obtained from Addgene (plasmids 20959, 20910, and 19771, respectively) (Okita et al., 2008, Woltjen et al., 2009). The empty PB vector and the c-Myc PB vector (pPB-TET-c-Myc) were kind gifts from Dr. Hitoshi Niwa at the RIKEN Center for Developmental Biology. The hyPBase vector (pCMV-hyPBase) was a kind gift from Dr. Keisuke Yusa at the Sanger Institute. To generate pCAG-hyPBase, pCMV-hyPBase was inserted into the pCAGGS vector (Niwa et al., 1991).

Alkaline phosphatase and immunofluorescence assays

Alkaline phosphatase activity in biTBCs was measured using the Vector Alkaline Phosphatase Substrate kit (Vector, Burlingame, CA, USA). For immunofluorescence analysis, cells were fixed with PBS containing 3.7% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were blocked with 5% bovine serum albumin (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) for 45 min at room temperature and then incubated overnight at 4°C with primary antibodies directed against OCT3/4 (1:50, SC-9081, Santa Cruz, Dallas, TX, USA), NANOG (1:250, AB5731, Millipore, Billerica, MA, USA), or CDX2 (1:100, MU392A-UC, BioGenex, Fremont, CA, USA). Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen) or Alexa Fluor 488 goat anti-rabbit IgG (1:500, Invitrogen) were used as secondary antibodies. Nuclei were stained with 1 μg⁄mL Hoechst 33342 (Sigma-Aldrich).

Reverse transcription PCR

Total RNAs of cells were prepared using the TRIzol reagent (Invitrogen). DNase I (Takara, Shiga, Japan) was added to preparations to avoid genomic DNA contamination. For reverse

transcription, ReverTra Ace (Toyobo, Osaka, Japan) and Random Primer (Invitrogen) were used. PCR was performed with ExTaq (Takara). PCR reactions were set up as follows: 94°C for 2 min followed by 25-40 amplification cycles (94°C for 20 s, 60°C for 30 s, and 72°C for 30-45 s). The reactions included a final elongation step at 72°C for 7 min. Primer sequences are shown in Table 3.

Trophoblastic vesicle formation and differentiation of biTBCs

To assess the differentiation potential of biTBCs, these cells were cultured in low-adhesion culture dishes to induce differentiation similar to the method for inducing embryoid body formation from iPSCs. biTBCs were physically split into cell clumps and transferred to MPC-treated round-bottom dishes (Nunc, Roskilde, Denmark) in TBM supplemented with 2.0 μg/mL Dox. After 3 days of culture, the medium was changed to fresh medium without Dox and cultured for another 3 days.

The differentiation potential of biTBCs was further examined by culturing the cells without Dox for a long period. biTBCs cultured in TBM supplemented with Dox were physically split into cell clumps and transferred to collagen-coated dishes in TBM in the absence of Dox for 20 to 30 days. The attached cell colonies were subcultured approximately every 10 days. After the culture, total RNA from cells was collected using the TRIzol reagent, and PCR analysis was performed as described above. Immunofluorescence analyses were also performed as described above.

Table 3. Primers for RT-PCR

Gene	Forward	Reverce
O-2A-K (Tg)	GCTCTCCCATGCATTCAAA	ACGCAGTGTCTTCTCCCTTC
c-Myc (Tg)	CTGTCCATTCAAGCAGACGA	ATCAGCCTCGACTGTGCCT
OCT3/4 (Endo)	AGCCAAACGACTATCTGCCG	ACACTCGGACCACGTCTTTC
SOX2 (Endo)	TTACCTCTTCTTCCCACTCC	TTCTTGCTGTCCTCCATTTC
KLF4 (Endo)	CTGCTCACGACTTTCCCTTG	GCAAACTTCCACCCACAACC
c-MYC (Endo)	AGCGACTCTGAGGAAGAACA	CAAGAGTTCCGTATCTGTTCAAG
NANOG	GACACCCTCGACACGGACAC	CTTGACCGGGACCGTCTCTT
CDX2	GCCACCATGTACGTGAGCTAC	ACATGGTATCCGCCGTAGTC
ELF5	TAAATCAGAAGCCCTGGCGAAGA	ACATGAGCTGGATGATGGAGCA
IFN-τ	TCCCCATGGCCTTCGTGCTCTCTCT	CTCAAAGTGAGTTCAGATCTCCACC
ERRβ	CCAACGGTCTGGACTCGCC	GCACACCTTCCTTCAGCAT
EOMES	ACTGGTTCCCACTGGATGAG	CACAGCAATGAACTGCGTTT
PL	CTGCTGGTGGTGTCAAATCTAC	TGGTTGGGTTAATTGTGGGC
PRP1	CGGTCCTGACGTGTTTGTCT	TGTGGCGCTTGATAGGAAGG
PAG1	TCCACTTTCCGGCTTACCAA	CCTTTCATTCTCCCAGATCCAT
bACT	GCGGCATTCACGAAACTACC	CGGACTCATCGTACTCCTGC

Results

Generation of biTBCs using Dox-inducible piggyBac vectors

bADCs isolated from a female Japanese black cattle fetus at 50 days of gestation originally exhibited a heterogeneous population consisting of epithelial and fibroblastic cells in culture (Fig. 13A). During trials for the generation of bovine-induced pluripotent stem cells (biPSCs) from bADCs using Dox-inducible PB vectors in culture medium used for human iPSCs, a small portion of the colonies appeared in a similar morphology as human iPSCs (Fig. 13B). However, approximately 30% of the colonies showed relatively flattened epithelial-like morphology similar to cobblestones and had more definite cell boundaries between cells (Fig. 13C). When the transfected cells were cultured in medium used for culturing bovine trophoblast cells (TBM) (Hashizume et al., 2006), the cobblestone-like colonies also appeared at day 8, and approximately 70% of the colonies exhibited trophoblast-like morphology at day 14 (Fig. 13D). The colonies were then mechanically picked up and transferred onto a fresh SNL feeder layer and subcultured (Fig. 13E). To examine whether trophoblastic types of colonies can be obtained not only from the extra-embryonic cell lineage but also from the embryonic cell lineage, bovine fetal liver-derived cells (bFLCs) were used. Introduction of Dox-inducible PB vectors into bFLCs also resulted in the appearance of trophoblast-like colonies (Fig.13F), and these colonies were propagated at least 5 passages under the same culture conditions.

Approximately 20 cell lines (biTBCs) were generated from 1×10^5 transfected bADCs, and two (iTB#1 and iTB#2) were further examined to determine cell characteristics. Both cell lines were subcultured every 7 days and stably propagated for over 60 passages. During the culture of these cell lines, they began to form a balloon-like morphology similar to trophoblastic vesicles (TVs), and some detached into the medium (Fig. 13G). The TVs frequently occupied the central areas of the colonies. At

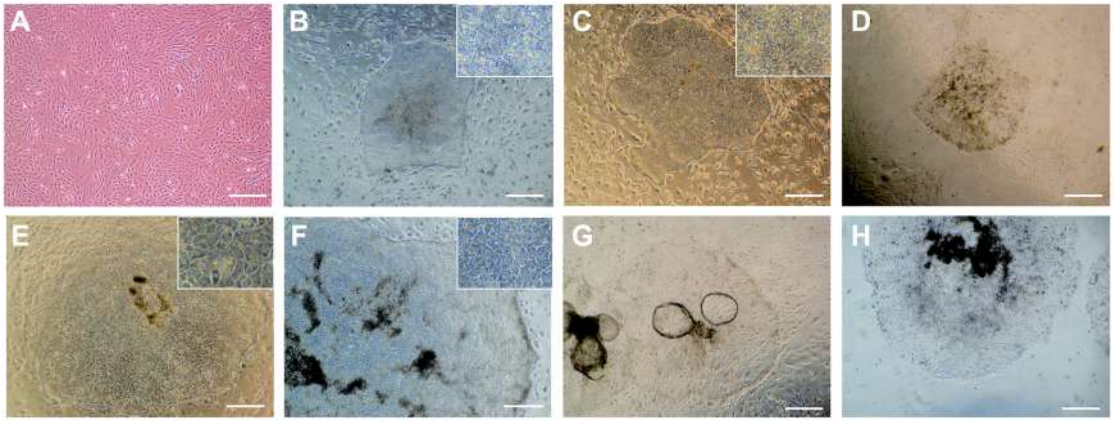

Fig. 13. Phase-contrast images of bovine-induced trophoblastic cells (biTBCs).

(A) Bovine amniotic cells (bADCs). (B) iPSC-like colonies appearing in the culture medium used for human iPSCs. (C) Trophoblast-like colonies appearing in the culture medium used for human iPSCs. (D) Primary trophoblast-like colonies appearing in the trophoblast cell medium at day 14. (E) Established biTBCs derived from bADCs. (F) Established biTBCs derived from bovine fetal liver cells (bFLCs). (G) Formation of trophoblastic vesicles (TVs) in central areas of colonies during culture. (H) Established biTBCs cultured on collagen-coated dishes. (A)-(H) scale bars = 500 μm.

the time of subculture, biTBCs were split into small cell clumps and transferred onto a fresh SNL layer. The cell clumps easily transformed into TVs and approximately half of the cells newly outgrew but the other half remained suspended. Thus, medium changes were performed with care not to remove floating cell clumps.

biTBCs that were cultured on a SNL feeder layer were split into cell clumps, and then the clumps were transferred onto collagen-coated dishes. The cell clumps also formed TVs and produced new extended outgrowths of TVs in a similar manner and morphology as that of biTBCs cultured on a SNL feeder layer (Fig. 13H). The cells could be stably passaged on collagen-coated dishes at least 10 times.

Characterization of biTBCs

To elucidate the differences between biTBCs and biPSCs, biTBCs were measured for their alkaline phosphatase (AP) activity, a well-known pluripotent marker. The two established biTBC lines were only partially positive for AP activity (Fig. 14A). To further examine the characteristics of these cells, the expression of pluripotent-related and trophoblast-related genes in biTBCs was assessed. Immunocytochemical analysis showed that the cells expressed the caudal-related homeobox 2 transcription factor (CDX2) required for TSC specification and maintenance (Fig. 14B). On the one hand, the cells expressed OCT3/4 (Fig. 14C) but did not express or only weakly expressed NANOG (Fig. 14D), which are well-known transcription factors required for pluripotent stem cell self-renewal. RT-PCR analysis showed that they expressed trophoblast-related genes including *CDX2*, *ELF5*, *ERR* β, and *IFN-* τ, although the expression of *IFN-* τ in iTB#2 was relatively low (Fig. 15). These cells also expressed pluripotency-related genes including endogenous *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*

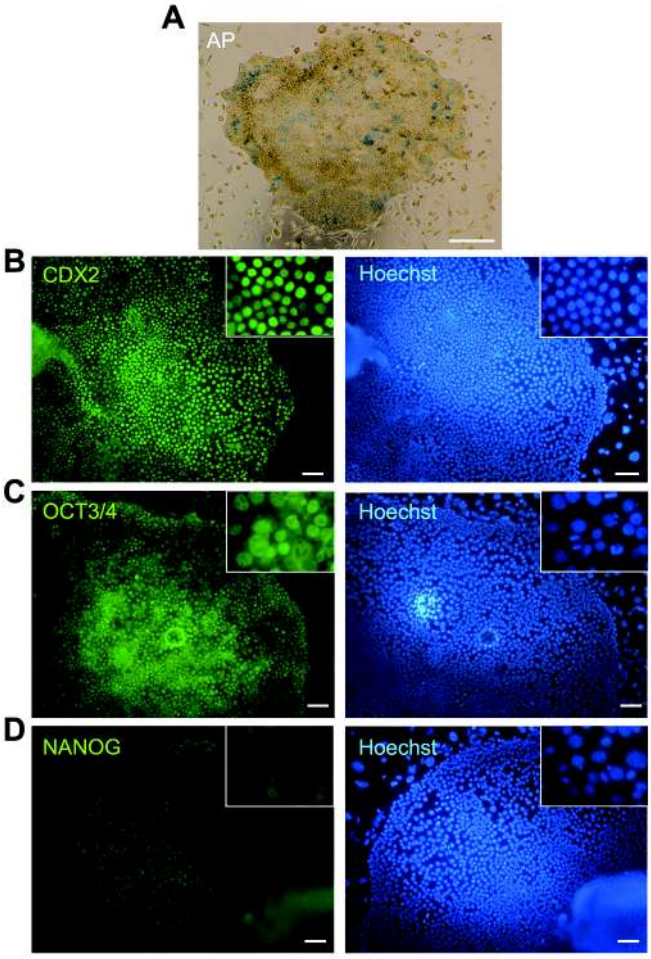

Fig. 14. Characterization of bovine-induced trophoblastic cells (biTBCs).

(A) Alkaline phosphatase activity in biTBCs. (B) CDX2 expression in biTBCs. (C) OCT3/4 expression in biTBCs. (D) NANOG expression in biTBCs. (A) scale bars = 500 μm. (B)-(D) scale bars = 100 μm.

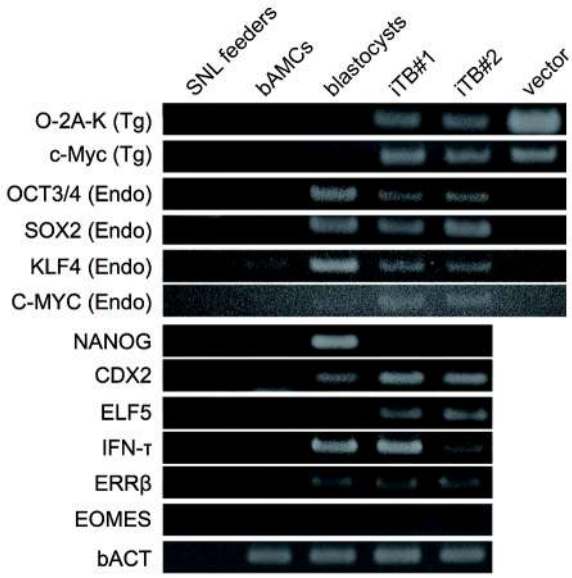

Fig. 15. Endogenous and exogenous expression of genes related to pluripotent stem cells or trophoblast cells in bovine-induced trophoblastic cells (biTBCs).

mRNA expression was evaluated by reverse-transcription polymerase chain reaction (RT-PCR). SNL feeder, SNL feeder cells; bADCs, bovine amnion-derived cells; iTB#1, biTBC line #1; iTB#2, biTBC line #2; vector, plasmid DNA of PB vectors.

(Fig. 15). Transgene expression was also detected because the cells were maintained in culture containing Dox (Fig. 15).

Differentiation potential of biTBCs

To examine the effects of Dox on the cellular characteristics of biTBCs, Dox was removed from culture. For 2 passages after culturing in the absence of Dox, the cells readily changed their cobblestone-like morphology into a more epithelial-like appearance (Fig. 16A) and lost the ability to form TVs. These observations ware also found even when FGF4 and heparin, which support the derivation and proliferation of murine TSCs (Tanaka et al., 1998), were added in culture (Fig. 16B).

To assess the differentiation potential of biTBCs, the cells were cultured in low-adhesion culture dishes to induce differentiation similar to the method for inducing embryoid body formation from iPSCs. Even after the cells were cultured for 6 days, biTBCs did not form embryoid bodies but produced TVs similar to those formed during their culture (Fig. 16C). When iTB#2 cells were cultured in the absence of Dox for over 30 days on collagen-coated dishes, they lost their original morphology and some of the differentiated cells formed binucleate cells (Fig. 16D). RT-PCR analysis showed that differentiation-induced iTB#2 no longer expressed transgenes but began to express pregnancy-related genes generally expressed in binucleate cells such as *PL*, *PRP1*, and *PAG1* after 30 days of culture in the absence of Dox (Fig. 16E).

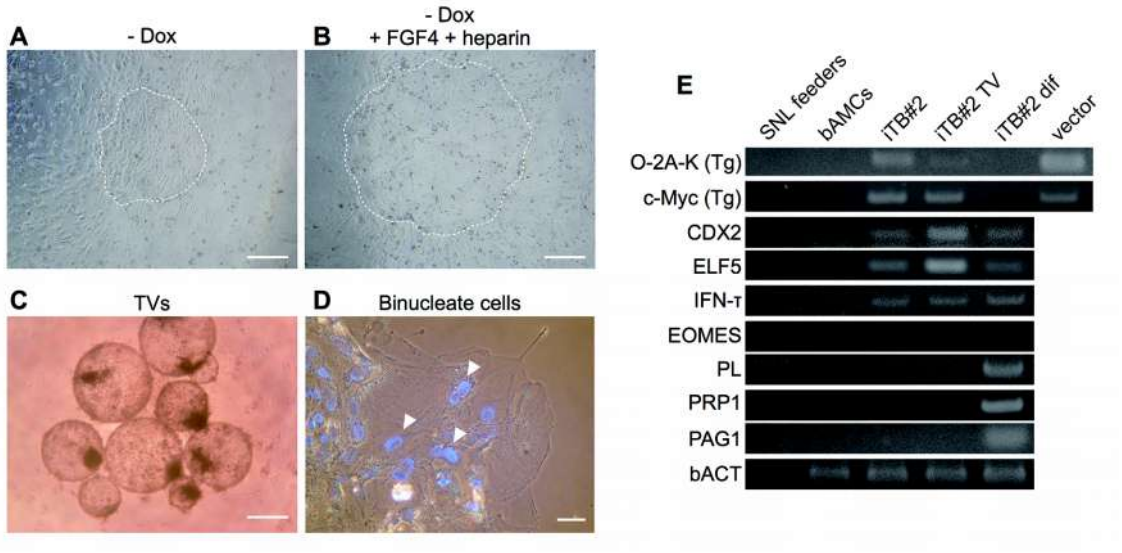

Fig. 16. Differentiation potential of bovine-induced trophoblastic cells (biTBCs).

(A) Phase-contrast image of biTBCs cultured in the absence of Dox (- Dox) for 7 days. The circled dotted area indicates the colony. (B) Phase-contrast image of biTBCs cultured in the absence of Dox and in the presence of FGF4 and heparin for 7 days. The circled dotted area indicates the colony. (C) Phase-contrast image of TVs generated from biTBCs. (D) Phase-contrast image of differentiated biTBCs. Nuclei were stained with Hoechst. Arrowheads indicate binucleated cells. (E) Endogenous and exogenous gene expression in differentiated biTBCs. iTB#2: Induced trophoblast cell line #2; iTB#2 TV: TVs generated from biTBC line #2 by culturing in low-adhesion culture dishes for 6 days; iTB#2 dif, biTBC line #2 after culturing in the absence of Dox for over 30 days. (A)-(C) scale bars = 500 µm. (D) scale bars = 70 µm.

Discussion

Trophoblastic cell functions and cell lineages are characterized based on a large number of trophoblast cell lines including TSCs in mice (Roberts & Fisher, 2011, Tanaka et al., 1998). However, studies regarding trophoblast cell lines in cattle are limited, and the cellular characteristics are poorly understood because of the lack of an *in vitro* culture system for trophoblast cells and their differentiation in culture. Here, we report the generation of new trophoblast cell lines derived from bovine somatic cells by introducing transcription factors used for the generation of iPS cells. Our established trophoblast cell lines (biTBCs) exhibited several criteria of trophoblast stem cells regarding their morphology, cellular behavior, gene expression, and differentiation potential.

After introducing transcription factors by piggyBac vectors, morphologically different colonies from iPS cells appeared even in medium consisting of KSR and FGF2, which are usually used for the generation and maintenance of human iPSCs (Gafni et al., 2013). These colonies were readily distinguishable from iPSC-like colonies according to their morphology because the colonies exhibited relatively flattened epithelial-like morphology similar to cobblestones and had a more definite cell boundary between the cells. Judging from the morphology, we assumed that the cells were trophoblast cells. Therefore, KSR and FGF2 were replaced by FBS in the culture medium (TBM) because previously reported bovine trophoblast cell lines were usually maintained in FBS-containing medium (Hashizume et al., 2006). As a result, TBM facilitated the emergence of colonies with the trophoblast cell morphology, suggesting that the FBS prompted the induction of stem cell colony differentiation into the trophoblastic cells. Interestingly, trophoblastic cell lines were established not only from amnion-derived cells but also from fetal liver-derived cells, suggesting that biTBCs could be generated not only from extra-embryonic but also from embryonic cell lineages. The

timing for the appearance of iPSC-like colonies and biTBCs was approximately the same at around day 8. However, it is not clear if the derivation of biTBCs is provided through a transient and metastable iPS cell state or a direct conversion to biTBCs without such an intermediate cell state. Human embryonic stem cells (hESCs) tend to spontaneously convert to trophoblastic cells under standard culture conditions (Ezashi et al., 2005) and rapidly differentiate into trophoblast cells upon exposure to BMP4 and related growth factors (Amita et al., 2013, Xu et al., 2002). Therefore, it is likely that bovine somatic cells underwent cellular reprogramming by the introduction of pluripotency-related transcription factors and readily settled into a trophoblastic cell state.

During the culture of biTBCs, cells frequently formed TVs. This phenomenon is also observed in trophoblast cell lines previously reported in cattle (Hashizume et al., 2006). In addition, when biTBCs were transferred and maintained on collagen-coated dishes, they could also stably propagate for over 10 passages, indicating that our generated biTBCs have similar cellular characteristics in culture as previously established trophoblast cell lines from bovine embryos (Hashizume et al., 2006).

biTBCs exhibited partial AP activity, which is known as a pluripotent stem cell marker. This activity has also been observed in embryo-derived TSCs in mice and rats and TBCs generated from porcine somatic cells through the introduction of transcription factors (iTR cells) (Ezashi et al., 2011). Thus, a portion of the population of trophoblast cell lines maintains AP activity; however, the significance of this cell population is obscure in the trophoblastic cell lineage. Moreover, RT-PCR showed that these cells express trophoblast-specific genes, such as *ELF5*, *CDX2*, and *SOX2*, which were also expressed in mouse TSCs (Kubaczka et al., 2014, Ohinata & Tsukiyama, 2014). In addition, biTBCs express *IFN-* τ, an antiluteolytic factor responsible for preventing the regression of the maternal corpus luteum and supporting the sustainability of pregnancy for cattle uteri (Kimura et al.,

2004, Nakano et al., 2002). IFN-τ together with CDX2 is thought to be expressed from mononuclear trophoblast cells or undifferentiated trophoblast cells in ruminants (Nakano et al., 2002, Suzuki et al., 2011). These results indicate that biTBCs have a heterogeneous population of trophoblast cells and possibly possess cellular characteristics such as those of trophoblast stem cells.

biTBCs expressed endogenous *OCT3/4*, a well-known pluripotent marker. In mouse pluripotent stem cells, OCT3/4 antagonizes CDX2 function and reciprocally regulates their expression, and OCT3/4 expression decreases as trophoblasts differentiate (Chen et al., 2009, Kinoshita, 2014, Strumpf et al., 2005). However, co-expression of OCT3/4 and CDX2 has been reported in the trophectoderm of bovine blastocysts, and OCT3/4 expression continues in the trophectoderm (TE) of late blastocysts although CDX2 is still expressed in this tissue (Berg et al., 2011). The endogenous CDX2 expression in the TE does not cause a decrease in OCT4 expression, suggesting that bovine OCT4 expression is not simply repressed by CDX2 at the late blastocyst stage (Berg et al., 2011). These species-specific differences in *OCT3/4* expression between mice and cattle have been caused by a deficiency in AP2 (TCFAP2) binding sites, which are regulatory regions of the OCT3/4 promoter (Ushizawa et al., 2007). These repressive sequences should be unique in mouse OCT3/4 regulatory regions and absent in other species including human, rabbit, and pig (Berg et al., 2011). In fact, *OCT3/4* expression in the TE at the blastocyst stage is not downregulated in these species (Cauffman et al., 2005, Kobolak et al., 2009, Kuijk et al., 2008). Moreover, the species differences in the relationship between OCT3/4 and CDX2 can be attributed to the difficulty in generating biTBCs even by controlling the ectopic expression of transcription factors in the presence of Dox.

When the established biTBCs were cultured in the absence of Dox, they readily changed their morphology and lost the ability to form TVs even in the presence of FGF4 and heparin in culture.

A potential solution for achieving the cultivation of iTBCs in the absence of Dox may be to optimize the culture conditions. However, even in the case of mouse TSCs in culture, culture conditions remain complex and require feeder cells or conditioned medium and fetal bovine serum (FBS), fibroblast growth factor 4 (FGF4) and heparin (Bai et al., 2009, Hashizume et al., 2006, Talbot et al., 2000). Recently, more defined culture conditions for the derivation and maintenance of murine TSCs have been reported (Kubaczka et al., 2014) in which TSCs proliferate well with their stem cell characteristics and differentiation ability in media in the absence of FBS supplemented with insulin, transferrin, and low levels of the cytokines FGF4 and TGF-ß1. In addition, a number of fibroblast growth factors such as FGF1, FGF2, and FGF10 are shown to be involved in development of bovine embryos and expression of IFN-τ in cattle (Cooke et al., 2009, Fields et al., 2011, Ozawa et al., 2013). This study provides a model for screening for optimal culture conditions for generating authentic bovine TSCs.

biTBCs failed to form EBs after they were cultured in low adhesive culture dishes, but they alternatively formed TVs. Furthermore, when biTBCs were induced to differentiate by culturing in the absence of Dox for over 30 days, they formed binucleate cells and began to express pregnancy-related genes such as PL1, PAG1, and PRP1. The formation of binucleate cells differentiated from mononuclear cells is a characteristic of trophoblast cells in cattle (Bai et al., 2011, Nakano et al., 2002), and PL1, PAG1 and PRP1 are genes expressed from binucleate cells (Nakano et al., 2002, Suzuki et al., 2011). Therefore, the biTBCs in this study have the potential to be differentiated into later stages of the trophoblast cell lineage.

In conclusion, this study reports for the first time that the induction of pluripotency in bovine cells allows for the generation of trophoblastic cells that have trophoblast stem cell-like characteristics and the potential to become differentiated into an extra-embryonic cell lineage. The established cell lines

can be a new cell source as a model for studying the trophoblast cell lineage and implantation processes in cattle.

Chapter 4

General Summary

Since the first generation of iPSCs in mouse (Takahashi & Yamanaka, 2006), a large number of studies of iPSCs have been performed in both mouse and human. iPSCs in human are regarded as reproductive medicine and in large animals and iPSCs in large domestic animals could be applied to biomedical and reproductive biotechnology research. Despite numerous attempts, however, very few studies have reported the generation of iPSCs from somatic cells or ESCs from pre-implantation embryos in other species. Cao et al (2009), Ozawa et al (2012), and Furusawa et al (2013) reported the generation of bovine embryonic stem cell–like cells, and Sumer et al (2011) reported the generation of primed biPSCs, but also described the difficulty of derivation and maintenance of these cells. Most iPSCs reported in non-rodent species were primed iPSCs (Esteban et al., 2009, Ezashi et al., 2009, Honda et al., 2010, Liu et al., 2008, Montserrat et al., 2011, Sumer et al., 2011, Tomioka et al., 2010, Wu et al., 2009), which have a limited capacity to produce chimeras relative to naïve iPSCs (Hanna et al., 2010b, Nichols & Smith, 2009, Tesar et al., 2007). For the application of iPSCs in large domestic aminals, production of naïve iPSCs are needed in terms of their potantials in homologous recombination and in chimeric production. Here, we attempted to establish naïve-type biPSCs from bovine amnion-derived cells by introducing Dox-inducible PB vectors expressing the mouse reprogramming factors (*Oct3/4*, *Klf4*, *Sox2*, and *c-Myc*).

Firstly, we attempted to generate primed-type biPSCs in piPSC medium containing bFGF. The emerging colonies (pbiPSCs) were passaged and cultured over 70 passages. pbIPSCs exhibited

several characteristics of pluripotent stem cells such as strong alkaline phosphatase activity, expression of pluripotent markers (*OCT3/4*, *NANOG*, *REX1*, *ESRR β*, *STELLA*, and *SOCS3*) as well as primed markers (*FGF5* and *OTX2*), and formation of embryoid bodies that gave rise to differentiated cells from all three embryonic germ layers. Although previous report regarding the generation of biPSCs (Sumer et al., 2011) demonstrated that ectopic expression of *NANOG* is necessary for the generation and maintenance of biPSCs from bovine fetal fibroblasts, we were able to generate biPSCs without ectopic expression of *NANOG*. It is possibly because the bADCs used in this study expressed intrinsic endogenous *NANOG*. Therefore, ectopic expression of *NANOG* may not be necessary for production and maintenance of biPSCs. In addition, studies on human and mouse iPSCs have suggested that reprogramming via introduction of transcription factors in ADCs is more efficient and faster than in fibroblasts (Li et al., 2009, Nagata et al., 2009, Zhao et al., 2010) probably because ADCs in mouse and human express high endogenous levels of *Klf4*, *c-Myc*, and *Ronin*, which support proliferation and self-renewal of iPSCs (Nagata et al., 2009). Thus, bADCs have stem cell-like characteristics and represent an appropriate cell source for generation of iPSCs in cattle.

Secondly, we attempted to generate naïve-type biPSCs. Addition of 2i and forskolin to culture medium can support naïve characteristics of human iPSCs (Hanna et al., 2010a). In this study, pbiPSCs were dissociated enzymatically and transferred to niPSC medium containing KSR, bLIF, 2i, and forskolin. (niPSC medium) These cells proliferated and formed mouse ES cell-like colonies with 3-dimensional morphology. Cells converted from the primed to the naïve state (pnbiPSCs) were maintained in niPSC medium by at least 10 rounds of trypsinization and single-cell dissociation. Furthermore, when the transfected cells were directly cultured in niPSC medium from 8 days after Dox addition, when primary colonies appeared, nbiPSCs colonies with dome-shape and compact morphology emerged at day 14. After passage of nbiPSCs by trypsinization and reseeding onto a

fresh SNL feeder layer, cells could be maintained for at least 10 passages by single-cell dissociation every 4 days. Both pnbiPSCs and nbiPSCs also exhibited strong alkaline phosphatase activity, expressed pluripotent markers (*OCT3/4*, *NANOG*, *REX1*, *ESRR β*, *STELLA*, and *SOCS3*), and formed embryoid bodies that gave rise to differentiated cells from all three embryonic germ layers. Only naïve-type biPSCs showed the hallmarks of naïve mouse PSCs, such as LIF-dependent proliferation, lack of *FGF5* expression, and active *XIST* expression with two active X chromosomes.

The naïve-type biPSCs exhibited many features of pluripotency. Furthermore, they were incorporated into ICM, and sometimes into TE, after aggregation with 8- to 16-cell stage embryos. Although primed-type biPSCs could propagate stably for more than 70 passages, naïve-type biPSCs were somewhat difficult to maintain, and could be propagated for only 10 to 15 passages; therefore, pnbiPSCs (derived from pbiPSCs) were employed for further aggregation and transplantation experiments. Aggregated embryos transplanted into the uteruses of surrogate mothers successfully produced three chimeric fetuses. Although the contribution of pnbiPSCs to some tissues was faint or absent, these cells had the potential to differentiate into all three germ layers, trophectoderm, and potentially germline in vivo. This is the first report of generation of authentic naïve-type biPSCs with several characteristics similar to those of naïve mouse PSCs and a demonstrated potential to contribute to chimeras including their germline.

Lastly, during trials for the generation of biPSCs from bADCs using Dox-inducible PB vectors in piPSC medium, morphologically different colonies from iPS cells appeared. These colonies were readily distinguishable from iPSC-like colonies according to their morphology because the colonies exhibited relatively flattened epithelial-like morphology similar to cobblestones and had a more definite cell boundary between the cells. The cells (biTBCs) were then mechanically picked up and transferred onto a fresh SNL feeder layer and subcultured for over 60 passages.

Although, it is not clear if the derivation of biTBCs is provided through a transient and metastable iPS cell state or a direct conversion to biTBCs without such an intermediate cell state. Human embryonic stem cells (hESCs) tend to spontaneously convert to trophoblastic cells under standard culture conditions (Ezashi et al., 2005) and rapidly differentiate into trophoblast cells upon exposure to BMP4 and related growth factors (Amita et al., 2013, Xu et al., 2002). Therefore, it is likely that bovine somatic cells underwent cellular reprogramming by the introduction of pluripotency-related transcription factors and readily settled into a trophoblastic cell state.

biTBCs exhibited several characteristics of trophoblast cells such as formoation of TVs, and expression of trophoblast-specific genes (*ELF5*, *CDX2*, *IFN-* τ, and *SOX2*). Moreover, biTBCs also exhibited several characteristics of pliripotent stem cells such as partial AP activity and expression of endogenous *OCT3/4*. AP activity has also been observed in embryo-derived TSCs in mice and rats and TBCs generated from porcine somatic cells through the introduction of transcription factors (iTR cells) (Ezashi et al., 2011). Co-expression of OCT3/4 and CDX2 has been reported in the trophectoderm of bovine blastocysts, and OCT3/4 expression continues in the trophectoderm (TE) of late blastocysts although CDX2 is still expressed in this tissue (Berg et al., 2011). The endogenous CDX2 expression in the TE does not cause a decrease in OCT4 expression, suggesting that bovine OCT4 expression is not simply repressed by CDX2 at the late blastocyst stage (Berg et al., 2011). These results indicate that biTBCs have a heterogeneous population of trophoblast cells and possibly possess cellular characteristics such as those of trophoblast stem cells.

When biTBCs were induced to differentiate by culturing in the absence of Dox for over 30 days, they formed binucleate cells and began to express pregnancy-related genes such as PL1, PAG1, and PRP1. The formation of binucleate cells differentiated from mononuclear cells is a characteristic of trophoblast cells in cattle (Bai et al., 2011, Nakano et al., 2002), and PL1, PAG1 and PRP1 are

genes expressed from binucleate cells (Nakano et al., 2002, Suzuki et al., 2011). Therefore, the biTBCs in this study have the potential to be differentiated into later stages of the trophoblast cell lineage. This study reports for the first time that the induction of pluripotency in bovine cells allows for the generation of trophoblastic cells that have trophoblast stem cell-like characteristics and the potential to become differentiated into an extra-embryonic cell lineage.

In summary, we generated two different types of biPSCs from bADCs using Dox-inducible PB vectors. Our results show for the first time that biPSCs meet the criteria for naïve iPSCs and have the capacity to contribute to chimeric fetuses, including the germ cell lineage. These cell lines may facilitate the optimization of culture conditions for generation and maintenance of bovine iPSCs that have the potential to generate chimeric offspring. On the other hand, trophoblastic cell functions and cell lineages are characterized based on a large number of trophoblast cell lines including TSCs in mice (Roberts & Fisher, 2011, Tanaka et al., 1998). However, studies regarding trophoblast cell lines in cattle are limited, and the cellular characteristics are poorly understood because of the lack of an *in vitro* culture system for trophoblast cells and their differentiation in culture. The cell lines established in this study can provide a new cell source as a model for studying the trophoblast cell lineage and implantation processes in cattle.

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