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Establishment of Bovine Trophoblast Stem-Like Cells from In Vitro-Produced Blastocyst-Stage Embryos Using Two Inhibitors

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The trophoblast (TR) is the first to differentiate during mammalian embryogenesis and play a pivotal role in the development of the placenta. We used a dual inhibitor system (PD0325901 and CHIR99021) with mixed feeders to successfully obtain bovine trophoblast stem-like (bTS) cells, which were similar in phenotype to mouse trophoblast stem cells (TSCs). The bTS cells that were generated using this system continually proliferated, displayed a normal diploid karyotype, and had no signs of altered morphology or differentiation even after 150 passages. These cells exhibited alkaline phosphatase (AP) activity and expressed pluripotency markers, such as *OCT4*, *NANOG*, *SOX2*, *SSEA-1*, *SSEA-4*, *TRA-1-60*, and *TRA-1-81*, and TR lineage markers such as *CDX2*, as determined by both immunofluorescence and reverse transcription-polymerase chain reaction (RT-PCR). Additionally, these cells generated dome-like structures, formed teratomas when injected into NOD-SCID mice, and differentiated into placenta TR cells in vitro. The microarray analysis of bTS cells showed high expression levels of many TR markers, such as *TEAD4*, *EOMES*, *GATA3*, *ETS2*, *TFAP2A*, *ELF5*, *SMARCA4* (*BRG1*), *CDH3*, *MASH2*, *HSD17B1*, *CYP11A1*, *PPARG*, *ID2*, *GCM1*, *HAND1*, *TDK*, *PAG*, *IFN-τ*, and *THAP11*. The expression of many pluripotency markers, such as *OCT4*, *SOX2*, *NANOG*, and *GDF3*, was lower in bTS cells compared with in vitro-produced blastocysts; however, compared with bovine fetal fibroblasts, the expression of these pluripotency markers was elevated in bTS cells. The DNA methylation status of the promoter regions of *OCT4*, *NANOG*, and *SOX2* was investigated, which were significantly higher in bTS cells (*OCT4* 23.90%, *NANOG* 74.40%, and *SOX2* 8.50%) compared with blastocysts (*OCT4* 8.90%, *NANOG* 34.4%, and *SOX2* 3.80%). In contrast, two promoter regions of *CDX2* were hypomethylated in bTS cells (13.80% and 3.90%) compared with blastocysts (18.80% and 9.10%). The TSC lines that were established in this study may be used either for basic research that is focused on peri-implantation and placenta development or as donor cells for transgenic animal production.

Introduction

Up to now embryonic stem cells (ESCs) have been successfully derived from mice, humans, monkeys, and rats, and trophoblast stem cells (TSCs) have been established from mice [1,2], humans [3,4], rabbits [5], rhesus monkeys [6], and common vole [7]. Despite these successes, it has been notoriously difficult to establish either ESCs or TSCs from ungulates. Although embryonic stem-like cells have been derived from goat [8], cattle [9], and pig [10], these cell lines are morphologically and functionally different from authentic ESCs, which are so-called primed ESCs. Several groups have attempted to harvest TSCs from goat, cattle, and pig, however, only the trophoblast (TR) cell lines have been reported to be obtained from these ungulates [7,11–17]. These TR cell lines continuously grow in culture and show high expression levels of TR cell marker genes. However, the pluripotency and stem cell characteristics of these cells have not been thoroughly examined. These cells likely represent a differentiation stage beyond that of TSCs.

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Efforts to establish naïve ESCs from nonrodent animals began over two decades ago, soon after the first studies that described ESCs that were derived from the inner cell mass (ICM) of mouse blastocysts [18].The extrinsic stimuli were thought to be dispensable for the derivation, propagation, and pluripotency of ES cells. However, self-renewal is enabled by the elimination of differentiation-inducing signaling from mitogen-activated protein kinase. Ying et al. developed a culture medium containing three inhibitors (3i): CHIR99021, PD184352, and SU5402. This 3i medium supports the efficient derivation and maintenance of ES cells from different strains of mice [19]. Li et al. established a rat germline competent ES cell line by adding two small molecule inhibitors (CHIR99021 and PD0325901) to standard medium; these authors also used mixed mouse embryonic fibroblast (MEF) and mouse subcutaneous connective tissue L Wnt-3A cells as feeder cells. This modified system worked not only on rats but also on the NOD strain of mice, which had previously failed to yield ESCs [20]. CHIR99021 is a highly selective small molecule inhibitor of glycogen synthase kinase 3 (GSK3) [21]. PD0325901 is an effective mitogen-activated protein kinase kinase (MEK) inhibitor [22]. Recent successes in the rat prompted researchers to test the efficacy of this modified culture system to generate ESCs from large animals. The double inhibition (2i) and 3i culture systems have been used to obtain ESCs from ungulates [23,24]. Porcine cells that were cultured in 3i medium without additional growth factors expressed the pluripotency markers *OCT4*, *NANOG*, *SOX2*, and *REX1* and preserved their pluripotent state over time [23]. Ovine ES-like cells can be efficiently derived and propagated in a semi-defined medium that contains N-2 Supplement, B-27 Serum-Free Supplement, CHIR99021, and basic fibroblast growth factor (bFGF). These ovine ES-like cells display a characteristic three-dimensional appearance, show a dependence on bFGF, express specific markers, such as alkaline phosphatase (AP), *OCT-4*, *SOX2*, and *NANOG*, and can be maintained for 30 passages [24].

In this study, we seeded whole bovine blastocysts with the zona pellucida removed and ICM after trophectoderm cells were stripped in 2i medium with mixed feeder cells (mixture of primary MEF cells and mouse subcutaneous connective tissue cell L Wnt-3A as feeder layers). Although none of the cells of the ICMs formed ESC colonies, 40 percent of all whole blastocysts generated colonies. The cell lines that were derived from the entire blastocysts were confirmed to be TR stem-like cells. In this study, we describe our strategy for deriving bovine TSC lines and propose that the 2i system might be a powerful tool to derive TSCs from other species.

Materials and Methods

Chemicals

All chemicals and reagents were purchased from Invitrogen, unless otherwise indicated.

Animal care and use

All experiments with mice (generation of embryonic fibroblasts and teratoma formation) were conducted in accordance with the Guide for Care and Use of Laboratory Research Involving Animals and were approved by Inner Mongolia University's Animal Care and Use Committee.

Bovine embryo culture and TS cell derivation

Bovine in vitro fertilization (IVF), embryo culture and quality evaluation. Bovine oocyte retrieval, in vitro maturation, IVF, and embryo culture were performed as previously described [25,26]. Cumulus oocyte complexes (COCs) were retrieved from 3- to 8-mm follicles of cow ovaries, which were collected at a local slaughterhouse. Oocytes with an intact cumulus oophorus were cultured in medium 199 (M199; Gibco) with 0.05 IU/mL recombinant human follicle stimulating hormone (Organon), 1 μg/mL estrodiol (E2), 1 IU/mL luteinizing hormone, and 1% (v/v) penicillin/streptomycin (Gibco). Matured oocytes were transferred to fertilization medium. Frozen-thawed semen from a fertile bull was centrifuged, and sperm was added to the COCs at a final concentration of 1×10^6 sperm/mL. After 6 h, presumptive zygotes were denuded and placed in synthetic oviduct fluid (SOF) medium. Incubation occurred at 38.5°C in a humidified atmosphere with 5% CO_2. After 48 h of culture, cleaved embryos were transferred to fresh SOF medium [containing 5% fetal bovine serum (FBS) Hyclone NWG0445] and cultured to day 7. The day 7 blastocysts were collected. Most of the blastocysts were used for bovine trophoblast stem-like cell (bTSC) derivation, and some of the blastocysts were used for ICM and trophectoderm (TE) differential staining.

For the differential staining, the expanded day 7 blastocysts were treated with pronase to remove the zona pellucida, which was followed by exposure to rabbit anti-bovine whole serum (B3759; Sigma) at a 1:4 dilution in Hepes-buffered SOF (HSOF) for 45 min. Then, the blastocysts were rinsed in HSOF with 0.1 mg/mL cold soluble polyvinyl alcohol (HSOF-PVA, molecular weight: 10,000–30,000) and placed into a 1:4 dilution in HSOF of guinea pig complement (S1639; Sigma), which contained 5 μg/mL propidium iodide and 40 μg/mL Hoechst 33342 for 15 min. The blastocysts were rinsed in HSOF-PVA and mounted on glass slides, which was followed by observation using an epifluorescence microscope (A1; Nikon). Blue and pink cells were designated as ICM and TE cells, respectively.

Feeder cell preparation. MEF cells that were used as feeders were derived from E13.5 embryos. Cell suspension were prepared by mincing fetal tissue and culturing those in high glucose Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS in 10 cm flasks. A monolayer of cells was harvested by trypsinization after 3 days in culture (passage 1). L Wnt-3A cells (CRL-2647; American Type Culture Collection) were grown in DMEM: F12 (1:1), which was supplemented with 10% FBS (Hyclone NWG0445). MEFs and L Wnt-3A cells were mixed at a ratio of 1:1 and were treated with mitomycin C (17 μg/mL; Haizheng) for 2.5–3 h at 37°C. Cells were then washed thrice with phosphate buffered saline (PBS) and frozen in FBS with 10% DMSO. Feeder cells were seeded at 10^5/well in four-well culture plates (Nunc, Thermo) before bTSCs were added.

bTSC derivation. In vitro-produced day 7 embryos and ICM after TE removal were cultured on feeder layers in bovine TS cell medium, which consisted of DMEM: F12 (1:1) and Neurobasal™ Medium, with 0.5 μM PD0325901, 3 μM CHIR99021, B-27 Supplement (50×), N-2 Supplement (100×), and Glutamax (100×) [27]. Approximately 1 week later, cells began to grow out from the blastocysts. These cells were mechanically collected and maintained by cutting under

a microscope. The medium was changed every day, and cells were subcultured onto fresh feeder cells every 7 days.

Characterization of bTSCs for the expression of pluripotency and TSC markers

AP activity. For AP staining, bTSC cells were cultured on feeder layers for 2–3 days before staining. The 2i medium was removed from the plates, and cells were washed with PBS. The bTSC cells were fixed with 4% paraformaldehyde (PFA) for 2 min at room temperature in the dark. Fixed cells were washed thrice with TBST (25 mM Tris–HCl, 0.14 M NaCl, 2.7 mM KCl, and 0.1% Tween-20) and stained using a 5-Bromo-4-Chloro-3-IndolylPhosphate (BCIP)/nitrotetrazolium blue chloride (NBT) Color Development Substrate Kit (Promega) for 90 min at room temperature in the dark. Cells were washed with TBST to terminate the staining reaction. Stained cells were maintained in PBS.

Immunofluorescence analysis. BTS cells and blastocysts were briefly washed in PBS and fixed in 4% PFA for 15 min at room temperature. After washing in PBS, bTSCs and embryos were permeabilized in PBS with 0.8% Triton X-100 at room temperature for 15 or 60 min, respectively. Then, bTSCs and embryos were incubated in PBS with 10% goat or donkey serum for 1 h at room temperature to block nonspecific binding. This step was followed by overnight incubation with primary antibodies, which were diluted in PBS at concentrations of 1:100 with 10% goat serum; all primary antibody incubations were performed at 4°C. For SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 staining, the permeabilization step was omitted. Primary antibodies that were used were rabbit anti-Oct-3/4 (H-134) (Santa Cruz Biotechnology), rabbit anti-Nanog (Abcam, Inc.), mouse Ab SOX2 (4900S; Cell signaling), mouse anti-Stage-Specific Embryonic Antigen-1 (SSEA-1; Millipore), mouse anti-Stage-Specific Embryonic Antigen-4 (SSEA-4; Millipore), mouse anti-TRA-1-60 (Millipore), mouse anti-TRA-1-81 (Millipore), and mouse anti-CDX2 (Biogenex). The bTSCs and embryos were then washed in PBS and transferred to PBS that contained appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen) at a dilution of 1:300 and were incubated at room temperature for 1 h. Then, bTSCs and embryos were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma). BFFs and feeder cells served as negative control. Negative controls with only secondary antibodies to stain the cells were done as well. Two confocal laser-scanning microscopes (Olympus and Nikon) were used for visualizing fluorescent signals. Images were further analyzed using the FV10-ASW 2.1 Viewer software. The image acquisition, analysis, and processing were standardized within each experiment.

Reverse transcript PCR and real-time PCR. Cells and blastocysts were lysed in TRIzol Reagent (Life Technologies). Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen), which was followed by treatment with DNase I (Ambion) according to the manufacturer's protocol. RNA quality and quantity were determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). For reverse transcription, 2 μg of total RNA was used in a 25 μL reaction, which contained 0.5 μg of oligo(dT)12–18 primer, reverse transcription (RT) buffer, 10 mM dithiothreitol, 0.5 mM dNTP, 5 U RNase inhibitor, and 10 U SuperScript II Reverse transcriptase (Invitrogen). Reverse transcription was performed at 42°C for 1 h. Polymerase chain reaction (PCR) amplification was performed using Applied Biosystems ThermoCycler. The primer sequences are provided in Table 1. PCR reactions were performed by initially denaturing cDNA at 94°C for 3 min, which was followed by 35 cycles of denaturing at 94°C for 30 s, annealing for 30 s at the temperature that was specified above for each primer pair, and extension at 72°C for 20 s, with a final 10 min extension step. For semi-quantitative reverse transcription PCR, equal amount of cDNA was added into the reaction. PCR products were resolved on a 1.5% agarose gel and imaged using a ChemiDoc XRSÞ Molecular Imager (Bio-Rad).

Chromosomal analysis. Bovine TS cells that were grown in 2i medium at passages 35, 70, and 140 were incubated for 3 h in culture medium that contained 0.2 μg/mL colcemid (KaryoMax). The cells were harvested by mechanical disruption, collected in a 15 mL tube, washed twice in PBS, and resuspended in 0.075 M KCl for 30 min at 37°C. Briefly, the cells were fixed twice in cold Carnoy's fixative (3:1

Table 1. Primer Sets for Reverse Transcription Polymerase Chain Reactions (RT-PCRs)

Gene	*Forward primer*	*Reverse primer*
OCT3/4	GGTTCTCTTTGGAAAGGTGTTC	ACACTCGGACCACGTCTTTC
NANOG	TTCCCTCCTCCATGGATCTG	ATTTGCTGGAGACTGAGGTA
CDX2	CCTGTGCGAGTGGATGCGGAAG	CCTTTGCTCTGCGGTTCT
SOX2	CATCCACAGCAAATGACAGC	TTTCTGCAAAGCTCCTACCG
GATA3	GAAGGCATCCAGACCAGAAA	CAGCATGTGGCTGGAGTG
ETS2	CGACAAGAACATCATCCACAAG	GATGGCGTGCAGTTCCTC
ELF5	GCTTGAAAACAAGTGGCATC	TCTTCCTTTGTCCCCACATC
SMARCA4	CTGACCTGTGAGGAGGAGGA	GCCTTGAGCCACTGCTTCT
IFND	CCTGTACTCACATTACCTCATGAGC	GTTCCTACCTGCAGGATGCAG
CDH3	GTGCTGCCTGGCACTTCAGTGA	AACATGAGGTCGTGCGGGTCCT
bPL-I	CAACCTACTAGTCCATCTCCCCA TCAGCAGCAGT	CATACAAAGCGGCCGCGAGACC CATTACACCCAAACAT
HAND1	ACATCGCCTACCTGATGGAC	TAACTCCAGCGCCCAGACT
GCM1	*GACATCTACTATCCAGCCTATC*	*CTTTGAAACCCGTCTTCTAAG*
MASH2	GTGCCGCACCAGAACTCGTA	CAGCTTCTTGTTGGCGCCGC
PAG	ACCTCAAGTGGGTGCCCC	CAGGCCAATCCTGTTCTGTCC
Acrogranin	CACTGGAAAGTATGGCTGCT	GCTCACCTCCATGTCGCACTT
ERR2	CCAACGGTCTGGACTCGCC	GCACACCTTCCTTCAGCAT

methanol/glacial acetic acid), attached to glass slides, and stained with Giemsa stain.

Teratoma formation and real-time PCR and immunohistochemical analysis. BTS-1 cells (10^7) at passage 44 from three 35-mm culture dishes were subcutaneously injected into the dorsal flanks of three NOD-SCID mice. At 8 to 10 weeks after injection, tumors were cut into pieces and fixed in Bouin's solution, which included saturated picric acid, formaldehyde, and glacial acetic acid. Tumors were then paraffin-embedded, sectioned, and stained using hematoxylin and eosin. To confirm the teratoma sections are bovine origin, real-time PCR and immunohistochemical analysis was performed on the embedded teratoma sections. DNA extraction from paraffin-embedded tissue was performed following the instruction of TaKaRa DEXPAT™ Easy Kit. The real-time PCR to detect bovine DNA of paraffin-embedded sections was performed following the instruction of TAKARA Real-Time PCR Bovine DNA Detection kit. For immunohistochemical analysis, the sections were dewaxed first, following dewaxing of paraffin sections, heat-mediated antigen retrieval was performed by microwaving sections for 20 min in 10 mM Sodium Citrate, pH 6.0. Sections were allowed to cool for 15 min, followed by a brief wash in deionized water, and rinsed twice in PBS. Sections were incubated for 30 min in 5% normal goat serum in PBS containing 0.1% Tween and 0.5% BSA. The sections were incubated overnight at 4°C with primary antibody Rabbit anti-Bovine IgM Antibody (NB753; Novus Biologicals) at the appropriate dilution overnight at 4°C. An Alexa Fluor-conjugated donkey-anti-rabbit secondary antibody (Invitrogen) was used to detect IgM staining.

Transcriptional profiling by microarray

An RNeasy Mini Kit (QIAGEN) was used to extract RNA from BTS-1 P81 cells, BTS-2 P68 cells, BTS-3 P69 cells, bovine fetal fibroblasts (BFFs), and 360 in vitro-produced day 7 blastocysts. An Ambion® WT Expression Kit (4411973) was used to prepare aRNA samples for Affymetrix whole transcription microarray analysis, and an Affymetrix GeneChip® WT Terminal Labeling Kit (PN 900671) was used to label the RNAs. The fragmented and labeled samples were hybridized to Affymetrix GeneChip Bovine Genome Arrays, which are based on the Bovine UniGene Build 57 (March 24, 2004) and GenBank® mRNAs. The Bovine Array contains 24,072 probe sets that represent over 23,000 transcripts, and 19,000 UniGene clusters. The data were normalized by mas5. The selected genes were processed by Cluster 3.0 and the euclidean distance was used as the cluster method for heatmap-making. The two-fold change in gene expression was taken as cutoff level for data analysis.

Methylation analysis

The methylation status of the coding strands of the promoter regions of *OCT4*, *SOX2*, *NANOG*, and *CDX2* from both P139 BTS-1 cells and in vitro-derived day 7 bovine embryos was analyzed. An AxyPrep Genomic DNA Mini Kit (AxyPrep) was used for the preparation of genomic DNA and a MethylCode™ Bisulfite Conversion Kit (Invitrogen) was used for the bisulfite conversion of genomic DNA. The PCR primers to amplify the promoter regions of the four genes that were mentioned above are provided in Table 2. The amplification of bisulfite-modified DNA was performed using Invitrogen Platinum DNA Polymerase and a dNTP mix at the following conditions: 95°C for 5 min, followed by 40 three-step cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were separated on 1.5% agarose gels and purified. The products were cloned into the pMD18-T vector (Takara Bio); clones were sequenced and analyzed for methylation status. At least nine clones were sequenced and analyzed for each sample.

Differentiation of bTSC cells in vitro

We used two different systems to test whether BTS-1 cells that were cultured in the presence of two inhibitors with mixed feeders could differentiate into TR lineages. The first system was a culture of BTS-1 cells in a medium that contained high glucose DMEM supplement 10% FBS (Hyclone NWG0445) in the absence of PD0325901, CHIR99021, and mixed feeders. The second system was a culture of BTS-1 cells in the 2i culture system but without mixed feeders, PD0325901, and CHIR99021. Undifferentiated bovine TS cells that were mechanically cut under a microscope were cultured in the conditions that were mentioned above on 0.2% gelatin-coated dishes. The cells were incubated at 38.5°C for 4–5 days, and the medium was changed every 48 h. In addition, on days 6 and 12, total RNA was isolated from cells using an RNeasy Mini kit (Qiagen), which was followed by treatment with DNase I (Ambion) according to the manufacturer's protocol. Semi-quantitative PCR was performed to detect the gene expression differences between cells cultured in different media.

Lentivirus production and infection

293T cells were plated at 6×10^6 cells per 100 mm dish and incubated overnight. The characterization of doxycycline (Dox)-controlled Tet-on-inducible lentivirus expressing green fluorescent protein (GFP) has been previously described [28]. 293T cells were transfected with a mixture of viral plasmid, packaging constructs that expressed viral packaging functions and the VSV-G protein using Lipofectamine 2000 (Invitrogen). At 24 and 48 h after transfection, the viral supernatant was harvested. In total, 30 mL of supernatant was typically harvested per virus. The viral supernatant was filtered through a 0.45 mm syringe filter (Millipore) and loaded into an Amcion Ultra-15 Centrifugal Filter (Millipore) for concentration.

Table 2. Primer Sets for Polymerase Chain Reactions of Methylation Analysis

Gene	*Forward primer*	*Reverse primer*
OCT4	TGGGTCGGGAGGGTTAGAGT	CAACAACTCACTCGCCTCCTC
SOX2	GCGTTTTTTTTTTTATTTTAGTAGT	ACTTTCCCCCTTTTACAAACA
NANOG	AGGGATTGAAGGTTATTTGTTTT	TATCCAAACATCCAAAAATTAAAA
CDX2-1	TGTTCGGAGATGAGGAGAAG	CCCAAAATTTTATAACCCTAAAT
CDX2-2	TGGAGGGGCGTAGGGTTTA	ACTCCTACGCCGACGAACAA

The viral supernatant was concentrated by centrifugation at 1,000 rpm for 5 min. Infections were performed in four-well culture plates in 1 mL of medium that contained 5 mg/mL polybrene (Sigma) with 5–10 μL of each viral concentrate. BTS-1 cells were infected at a density of 10^5 cells/well, and the medium was replaced 24 h after infection. All the cell culture medium was subsequently replaced by 2i medium that was supplemented with 2 mg/mL Dox.

Results

Bovine embryo culture and TS cell derivation

The blastocyst development rate, which was based on original oocytes that were co-cultured with sperm was 30.90% (212/686) (four replicates). Total fifteen day 7 blastocysts from four IVF replicates were treated for differential staining. The average total cell number of day 7 blastocyst was 115 ± 6 (ICM: 43 ± 2;TE:72 ± 4). The ratio of ICM to total cells was 0.38 ± 0.01.

Most day 7 bovine blastocysts (Fig. 1A) attached to feeders after 3 days in culture; however, some blastocysts remained in suspension, and continued to expand (Fig. 1B). Flat outgrowths appeared from the attached blastocysts after another 3–4 days in culture (Fig. 1C); the cells formed distinct colonies at day 7 (Fig. 1D). The ratio of outgrowth was 40%. The outgrowths were mechanically cut and transferred to new culture dishes at approximately day 10 (Fig. 1E, F); cells were then passaged every weeks. The border and nuclei of the cells were visible. In some colonies, liquid accumulated under monolayer cells and formed a dome-shaped structure (Fig. 1F). The dome continued to grow until these structures detached from the colony to form floating spheres. Although these floating spheres morphologically resembled blastocysts, these structures did not possess an ICM. Three cell lines were obtained from this whole blastocyst attachment method. One of the cell lines was cultured for 36 months for over 150 passages; this line was named BTS-1 (Fig. 1F), the other two cell lines named BTS-2 and BTS-3, respectively. Outgrowths were also derived from somatic cell nuclear transfer derived-blastocysts; these cells were cultured for over 50 passages. The separated ICMs were also cultured in the identical conditions. Although few outgrowths were observed, these outgrowths could not survive mechanical passaging.

Expression of pluripotency markers in bovine TS cells

BTS cells cultured and passaged in vitro over 36 months, displayed a normal morphology and anormal karyotype (Fig. 2D) and stained positively for pluripotency markers, such as *OCT-4* (*POU5F1*), *SOX2*, *SSEA-1*, *SSEA-4*, *TRA-1-60*, and *TRA-1-81* (Fig. 2A). *SOX2* and *OCT-4* (*POU5F1*) staining was localized to nuclei, which resembled the staining of d7 blastocysts (Supplementary Fig. S1, the negative controls for SOX2 staining were shown in Supplementary Fig. S2; Supplementary Data are available online at www.liebertpub.com/scd). Staining for SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 was clearly detected on the cell membrane (Fig. 2A and Supplementary Fig. S3). RNA transcripts of *POU5F1 (OCT-4)*, *NANOG*, and *SOX2* were detected in BTS-1 cells by RT-PCR (Fig. 2B). BTS-1 cells at passage 16 and 157 displayed positive AP activities (Fig. 2C), and the negative control for AP staining was shown in Supplementary Fig. S5.

We performed a microarray analysis on passage 81 BTS-1 cells, passage 68 BTS-2, passage 69 BTS-3, BFFs, and in

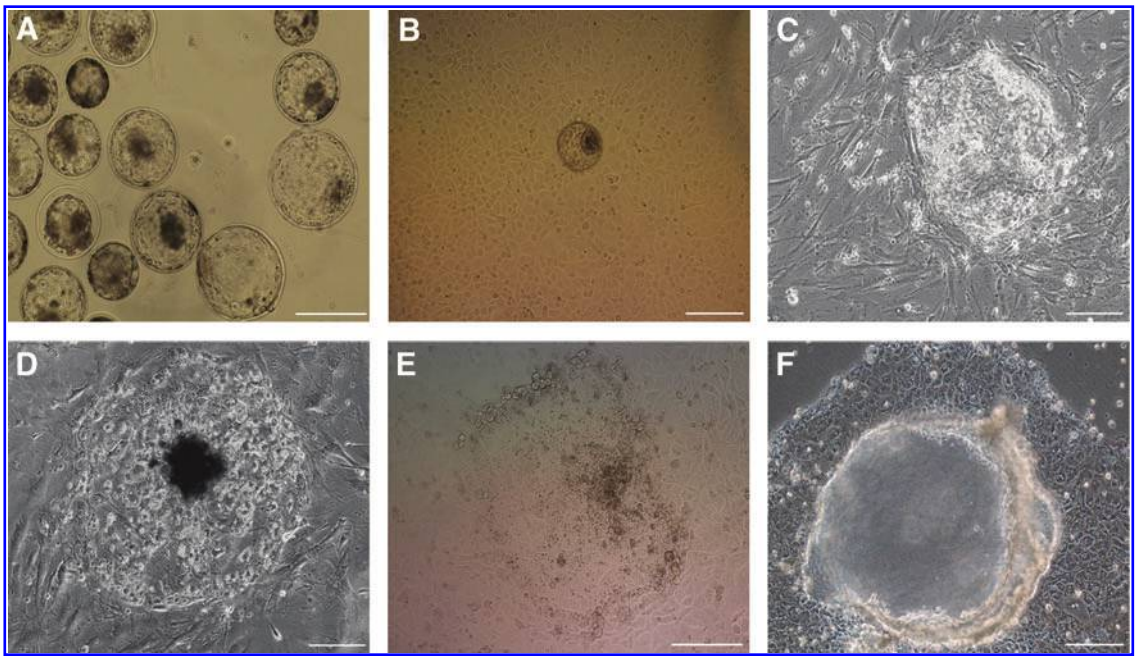

FIG. 1. Derivation and morphology of bovine trophoblast stem-like (bTS) cells. **(A)** In vitro fertilized blastocysts at day 7. **(B)** Day 3 attached blastocyst. **(C)** The outgrowth of a blastocyst after 7 days of culture. **(D)** Expanded outgrowth. **(E)** The morphology of a typical BTS cell colony (BTS-1). **(F)** The dome structure of the colony. The scale bar represents 200 μm. BTS-1, bovine trophoblast stem cell line 1.

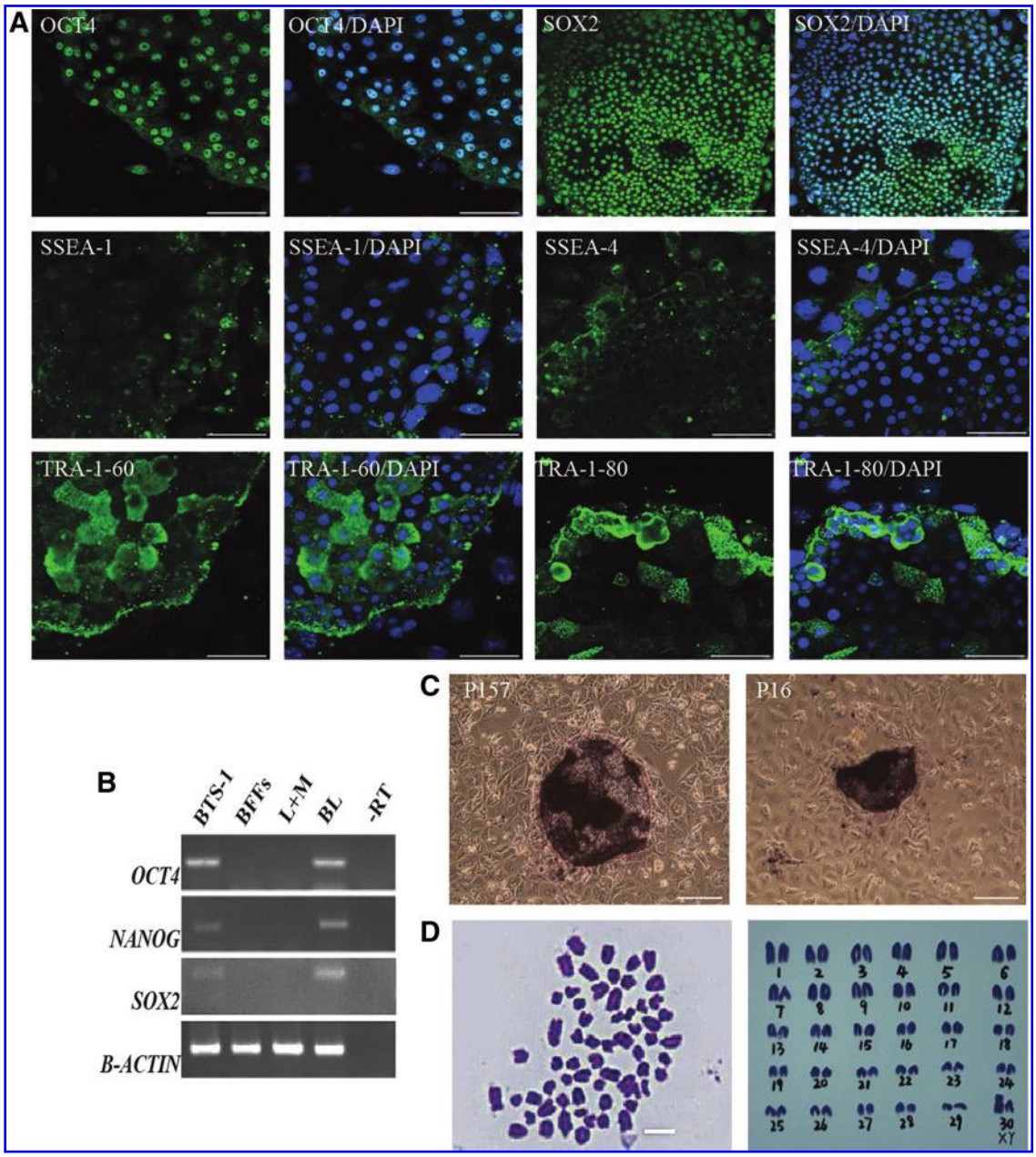

FIG. 2. Pluripotency gene expression analysis of bTS cells. **(A)** Immunofluorescence staining of pluripotency markers. BTS cells are *OCT4* (BTS-1 P99), *SOX2* (BTS-2 P73), *SSEA-1* (BTS-1 P94), *SSEA-4* (BTS-1 P94), *TRA-1-60* (BTS-1 P94), and *TRA-1-81* (BTS-1 P94) positive, and there is no difference between passage numbers. The scale bars for SOX2 staining represent 100 μm, and scale bars for other staining represent 50 μm. **(B)** RT-PCR demonstrated BTS-1 cells expression of *OCT4*, *NANOG*, and *SOX2*. BFF, bovine fetal fibroblast; L + M, L cell, and mouse embryonic fibroblast feeder cells; BL, blastocysts. **(C)** Alkaline phosphatase staining of P157 BTS-1 and P16 BTS-2 cells. The scale bar represents 200 μm. **(D)** Karyotype analysis of BTS-1 cells. The scale bar represents 5 μm. RT-PCR, reverse transcription-polymerase chain reaction; bTSC; bovine trophoblast stem-like cell.

vitro-fertilized blastocysts, the spearman correlation coefficient between same cell lines and different cell lines is over 90% and the original data for heatmap analysis is shown in Supplementary Table S1. Heat maps of selected data that were generated from Affymetrix gene chip analysis are shown in Fig. 3A. In addition, the log2-fold changes in expression levels of pluripotency, TSC-, and TR-specific genes in BTS-1 cells compared with blastocysts and BFFs are provided (Figs. 3B and 4C). Some pluripotency genes were expressed in bTS cells but at lower levels compared with those levels in blastocysts. Examples of genes that fall into this category include *OCT-4* (*POU5F1*), *SOX2*, *GDF3*, *KLF4*, *KLF5*, *SFRP2*, *SMAD3*, *SPARC*, *STAT3*, *TBX3*, and *THAP11*. However, the expression of most of these genes was higher in bTS cells compared with their expression in BFFs. *TDGF*, *MYCN*, *CCR4*, *ALPL*, *SMAD2*, and *SUZ12* were expressed at similar levels in bTS cells compared with blastocysts; but significantly higher than BFFs. The expression of other well-known pluripotency genes, including *NANOG* and *REX1*, could not be assessed by microarray,

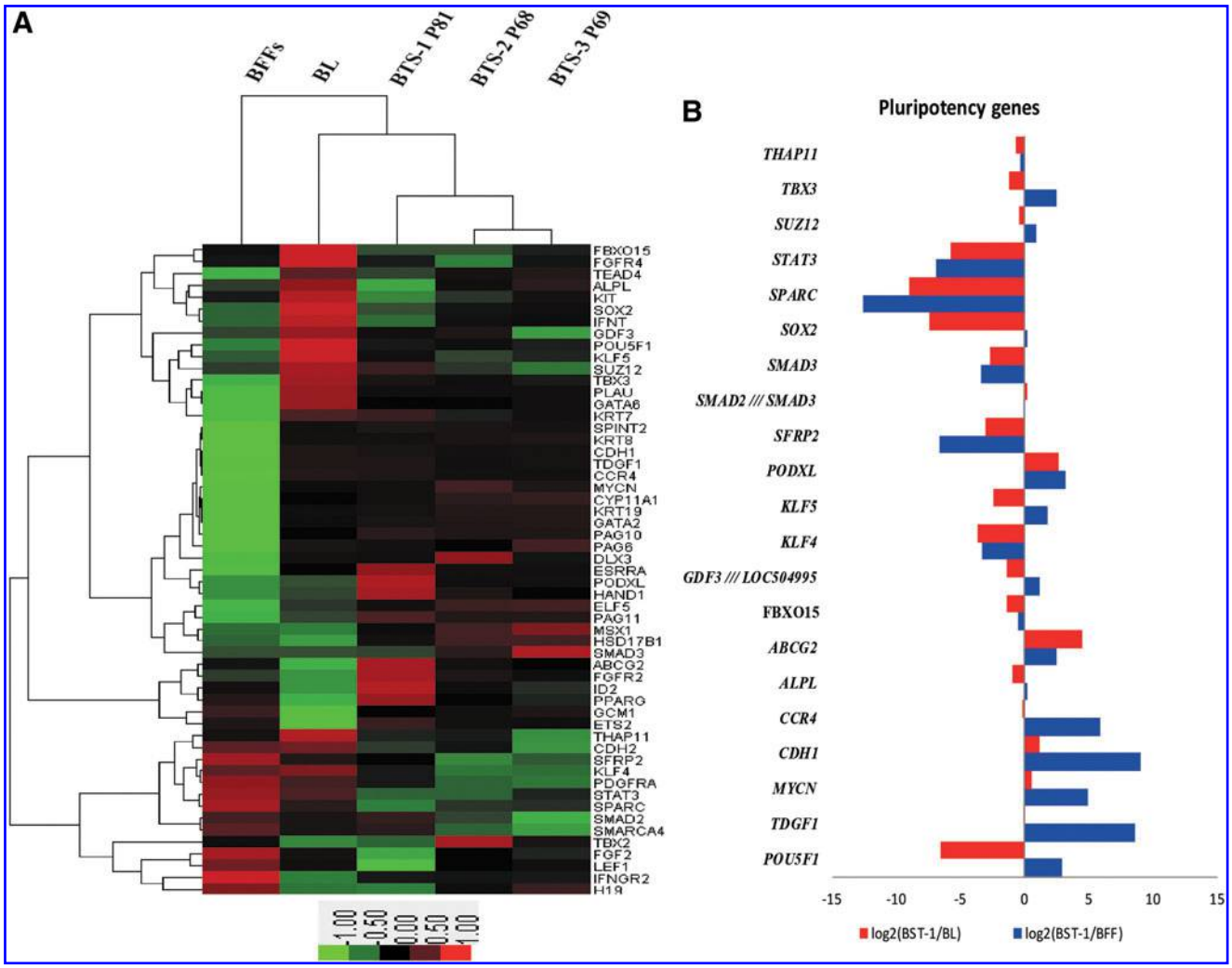

FIG. 3. Gene expression profiling of bTS cells using Affymetrix microarrays. **(A)** Two-color heat map representation of selected data that derived from Affymetrix gene chip analysis of BTS-1 P81, BTS-2 P68, BTS-3 P69, BFFs, and bovine in vitro-fertilized blastocysts. Genes that were included were upregulated more than twofold in all pairwise comparisons of each of the independently derived samples. Heat map represents log2 transformed data with normalized averages. Hierarchical clustering of the samples was performed using Pearson correlation with average linkage. **(B)** Log2-fold change in pluripotency gene expression in bTS compared with in vitro fertilized d7 blastocysts (*red bars*) and BFFs (*blue bars*).

either because of inadequate annotation or because appropriate diagnostic probes were absent from the arrays.

Expression of TSC and TR markers in bovine TS cells

The typical TSC marker *CDX2* was detected in the nuclei in bTS cells by immunofluorescence (Fig. 4A) and coexpressed with *OCT4* in these cells (Fig. 4A and Supplementary Fig. S4). These cells also stained positively for KRT18, particularly near the edges of the colonies. *KRT* gene transcription was detected at extremely high levels by the microarray analysis in both bTS cells and blastocysts. Transcription factors that were associated with TSCs (eg, *TEAD4*, *GATA3*, *ELF5*, *SMARCA4* (*BRG1*), *ETS2*, and *ESRRB*) and genes that were associated with differentiated TR (eg, *CDH3*, *IFN-τ*, and *ACROGRANIN*) were detected by reverse transcription PCR in both bTS cells and blastocysts. *EOMES* was only weakly expressed in BTS-1 cells and not in blastocysts. *HAND1*, *GCM1*, and *MASH2* were also only weakly detected in BTS-1 cells (data not shown). The log2-fold change comparison of BTS-1 cells/Blastocysts and of BTS-1 cells/BFF for both TSC-specific and TR-specific genes that were identified by the microarray analysis are shown in Figure 4C. Typical TSC and TR genes were highly expressed in bTS cells; these genes included *TFAP2A*,*ELF5*, *CYP11A1*,*PPARG*,*ID2*,*GCM1*,*HAND1*, and *THAP11*. IFN-τ is the primary signal for bovine pregnancy at early stages [29]. The expression level of IFN-τ is significantly higher in blastocysts than in BTS-1 cells, which indicates that the 2i medium may inhibit IFN-τ expression. Markers of bovine TR, such as *TDK* and *PAG*, were strongly expressed in BTS-1 cells. *TKDP1*, *TKDP3*, and *TDKP4* were expressed at extremely high levels in bTS cells. The pregnancy-associated glycoproteins, *PAG2*, *PAG10*, *PAG11*, and *PAG12*, were slightly more elevated in bTS cells than in blastocysts. Of the cytokeratins, *KRT7*, which is generally regarded as a diagnostic TR marker, was not detected

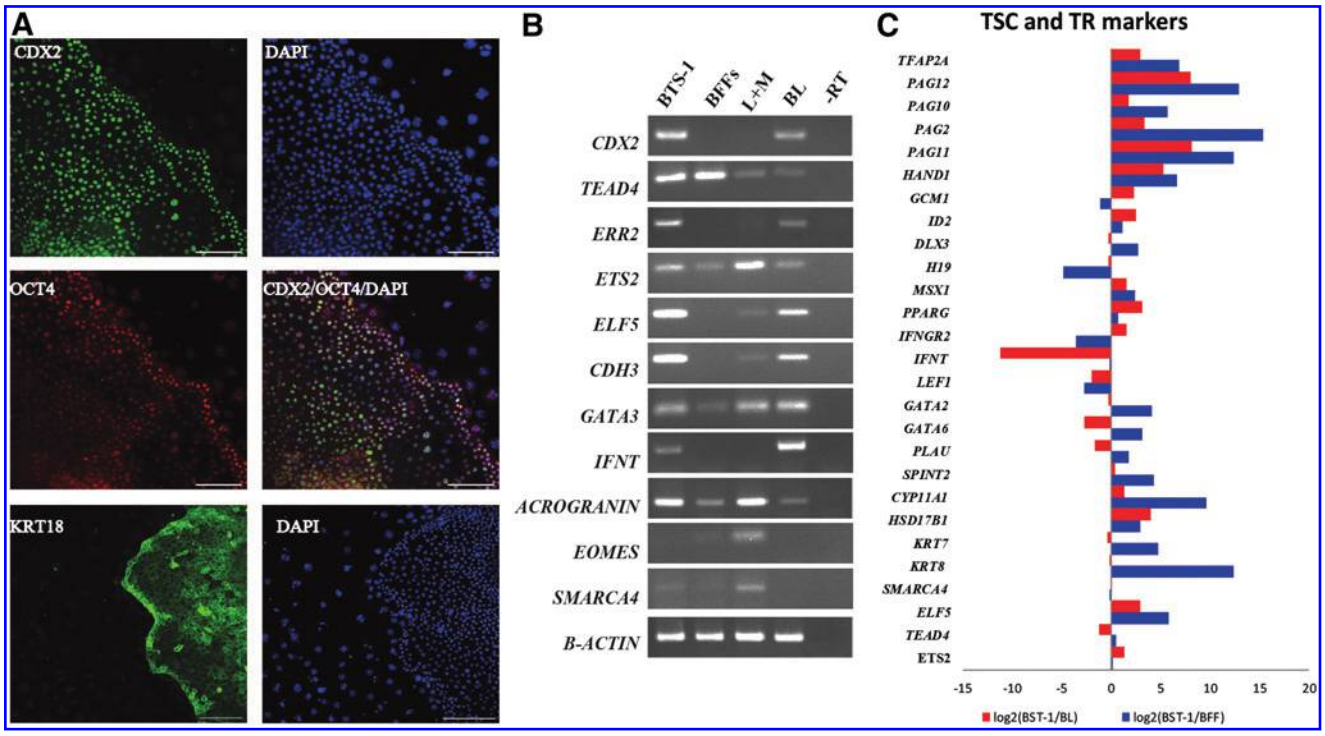

FIG. 4. TSC and TR gene expression analysis of bTS cells. **(A)** Immunofluorescence staining of CDX2, OCT4 (scale bar = 100 μm), and cytokeratin 18 (KRT18) for passage 141 BTS-1 (scale bar = 200 μm) cells. CDX2 clearly showed nuclear staining in BTS-1 cells and coexpression with OCT4. **(B)** RT-PCR showed TSC and TR marker expression in BTS-1 and blastocysts. **(C)** Log2-fold change in TSC and TR gene expression in BTS-1 cells compared with in vitro fertilized d7 blastocysts (*red bars*) and BFFs (*blue bars*). L + M, mixture of mouse subcutaneous connective tissue cell L Wnt-3A, and primary mouse embryonic fibroblast cells; BL, blastocysts; -RT, minus reserve transcription; TR, trophoblast; TSC, trophoblast stem cell.

in either BFFs or blastocysts. In contrast, *KRT19*, *KRT18*, and *KRT8* were overexpressed in bTS cells compared with BFFs; these three cytokeratin genes showed similar expression between bTS cells and blastocysts (Supplementary Fig. S5).

Subcutaneous injection of bovine TS cells causes teratoma formation

To analyze in vivo differentiation properties of bovine TS cells, we subcutaneously injected 3.3×10^6 undifferentiated BTS-1 cells into three NOD-SCID nude mice. Teratomas were observed in all mice, and tumors were clearly apparent after 45 days. Teratoma diameters measured 1.5 cm at the time of dissection from the mice at day 60 (Fig. 5A, B). The formation of a clear capsule was not observed at the junction zone between the teratoma and the host tissue. The results of real-time PCR and immunosatining confirmed that the teratoma sections contained different types of bovine cells (Supplementary Fig. S4). The histological analysis of the teratoma sections revealed that the teratoma consisted largely of packed layers of what appeared to be fibroblast-like cells and necrotic areas (Fig. 5C–E). The hemorrhagic structures that contained large blood-filled lacunae are similar to those structures that emerged from mouse and vole TSCs [7,30] (Fig. 5C–G). Differentiated structures of identifiable tissue types were rare; however, islands of neuronal rosettes and striated muscle could be observed (Fig. 5F, H), particularly near the margins of the tumors that were close to site of attachment to the body wall. A similar observation was reported in pig iTR [31]. The detailed histological analysis also showed that the tumors contained both viable and dying TR cells. Some areas of the teratoma contained actively proliferating cells, which were composed of giant TR cells at different stages of differentiation. TR cells with two nuclei were also observed, and these cells represent typical TR cells of ungulates (Fig. 5I).

Pluripotency genes were hypermethylated, and CDX2 was demethylated in BTS-1 cells

Sodium bisulfite sequencing was used to assess the DNA methylation status of the promoter regions of *OCT4*, *NANOG*, *SOX2*, and *CDX2*. The methylation of *OCT4*, *NANOG*, and *SOX2* was 168% ($P < 0.001$), 120% ($P < 0.05$), and 116% ($P < 0.001$) higher in BST-1 cells compared with blastocysts, respectively. In contrast, two promoter regions of *CDX2* showed less methylation (27%; $P < 0.5$ and 57.7%; $P < 0.001$) in BTS-1 cells compared with blastocysts (Fig. 6).

BTS cells have the ability to differentiate into mature TR cells

BTS cells that were cultured in a medium without 2i or in DMEM that was supplemented with 10% FBS formed spheres (Fig. 7A, B) and differentiated into mature TR cells, which were marked by the disappearance of *OCT4*, *NANOG*, and *SOX2*, and by the appearance of *HAND1* (TR giant cells), *MASH2*, and *PL-I* (Fig. 7E). Some cells with two nuclei (putative TR giant cells) were observed following culture in DMEM with FBS for 12 days (Fig. 7C).

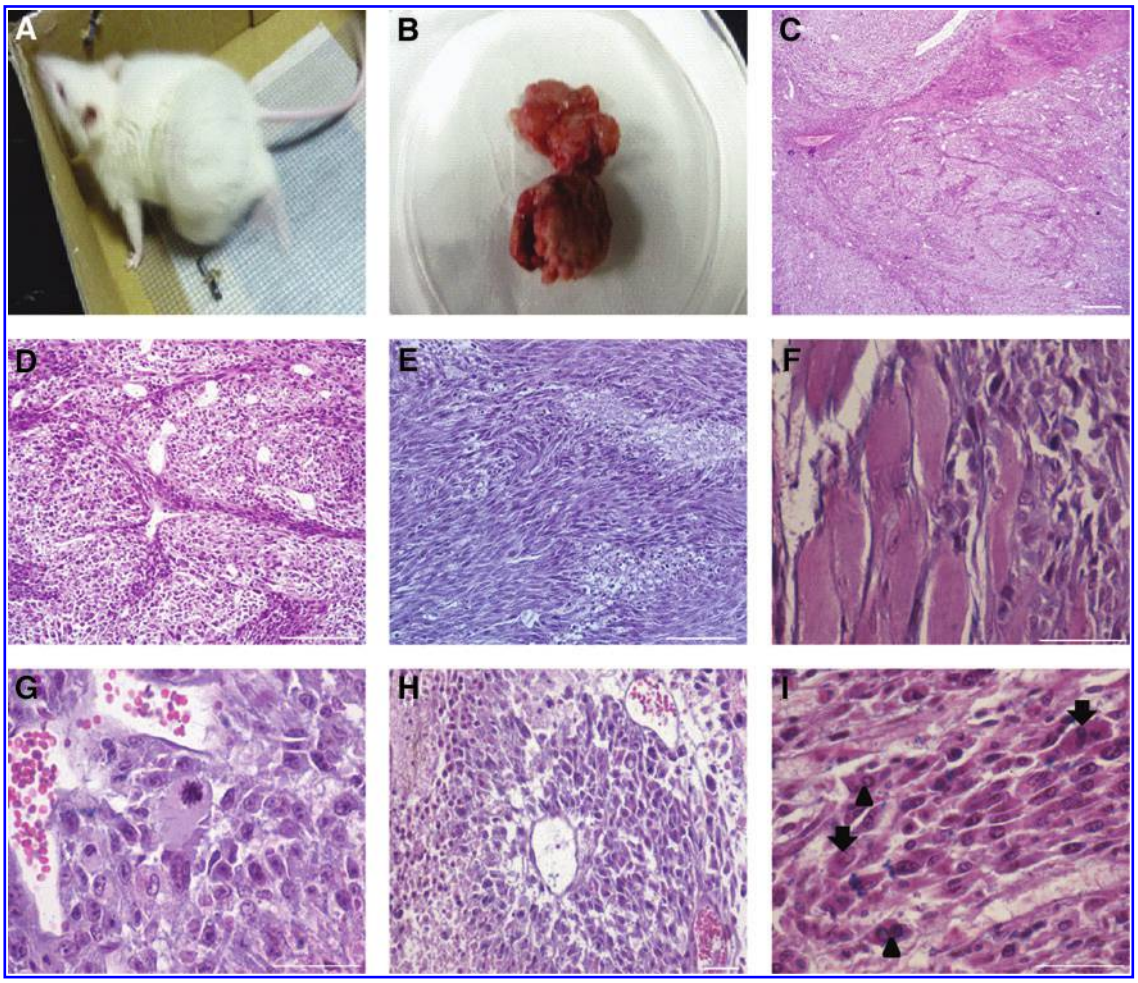

FIG. 5. Teratoma formation from injected BTS-1 cells is shown. **(A)** NOD-SCID mice with tumor formed after BTS-1 cell were injected. **(B)** Tumors removed from mice after 60 days injection. **(C–E)** Packed layers of cells and necrosis area. **(F)** Islands of striated muscle were observed near the margins of the tumor. **(G)** Area with blood vessels and giant cells. **(H)** Neural rosettes structure. **(I)** Area containing double nuclei cells and trophoblast giant cells. The *arrow* indicates the trophoblast giant cells and the *arrowhead* indicates the double nuclei trophoblast cells. The scale bars of **(C, D, E, H)** represent 200 μm. The scale bars of **(F, G, I)** represent 50 μm.

However, *GCM1* expression was not detected, and the unsuitable differentiation condition to other TR lineages might be the reason for this lack of *GCM1* expression.

BTS cells could be infected by lentivirus expressing GFP and induced by Dox

After BTS cells were infected with a Tet-on-inducible GFP-expressing lentivirus and induced by Dox, GFP was observed in over 14.81% of cells (116/783), and this GFP expression is shown in Figure 7D. Importantly, these cells maintained a normal morphology.

Discussion

Double inhibition is a culture system that supports the maintenance of pluripotent cell lines [32,33]. Unlike other potent inhibitors of GSK3, CHIR99021 does not exhibit the cross-reactivity against cyclin-dependent kinases (CDKs); in fact, CHIR99021 is ~350 times more selective toward GSK-3β than toward CDKs [34]. In addition to blocking differentiation signals by MAPKs, CHIR99021 blocks the activity of GSK-3β to promote the self-renewal of ESCs [19]. PD0325901, which is a derivative of the MEK inhibitor CI-1040, selectively binds to and inhibits MEK [35]. Since Li et al. obtained germline-competent ESCs from rat blastocysts in 2008 [27], 2i and 3i culture systems have been used to obtain embryonic stem-like cells from ungulates and the expression of pluripotency markers such as *OCT4*, *NANOG*, and *SOX2,* was observed [23,24]. Bovine embryos cultured in the presence of the MEK inhibitor PD0325901 alone contained a significantly higher percentage of NANOG-positive cells than those cultured in the control condition [36]. Inhibition of GSK3 with CT99021 (3 μM) resulted in a significant increase in the percentage and quality of blastocysts [37]. Another study demonstrated that dual kinase inhibition promoted bovine blastocyst development, increased ICM and TR cell numbers by 30% and 27%, respectively, and sustained ICMs to express the epiblast-specific pluripotency markers *SOX2* and *NANOG*, whereas repressing the hypoblast marker *GATA4* [38]. ICMs were immunosurgically isolated from in vitro fertilized bovine

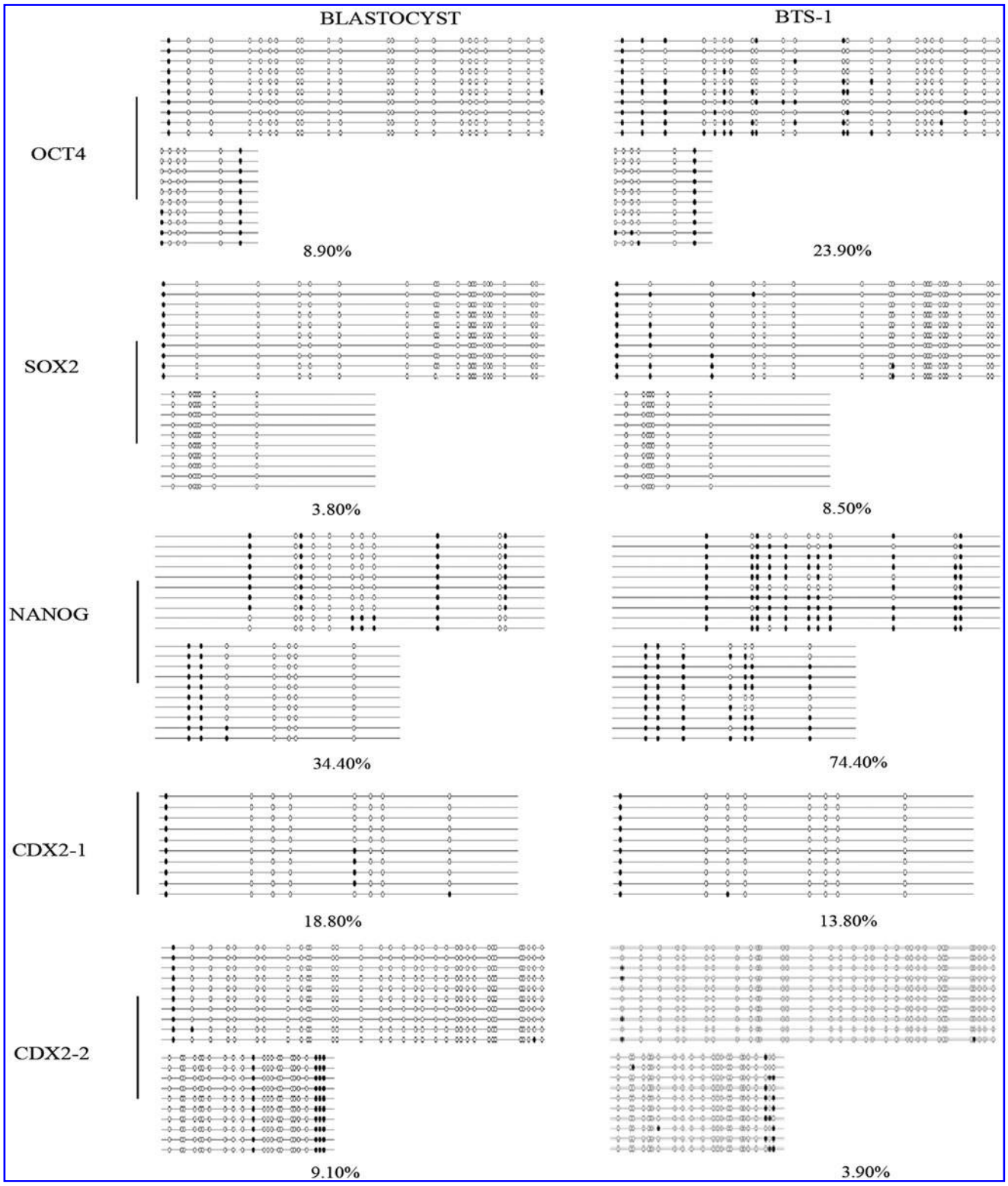

FIG. 6. The methylation status in the promoter regions of OCT4, SOX2, NANOG, and CDX2 by bisulfate sequencing. The open circles indicate unmethylated CpGs, and the closed circles indicate methylated CpGs.

blastocysts and cultured feeder-free in the 2i medium. Following mechanical passage, the 2i culture system supported limited proliferation for several weeks. Continuously cultured cell lines expressed discriminatory markers of naïve pluripotency and primordial germ cells, but not of primed epiblast stem cells [39]. Our attempt to use this system to obtain bovine ESCs from IVP bovine blastocysts was also not successful, even after we removed TR cells from the ICM. However, we successfully obtained bovine blastocysts outgrowths and bovine trophoblastic stem-like cell lines

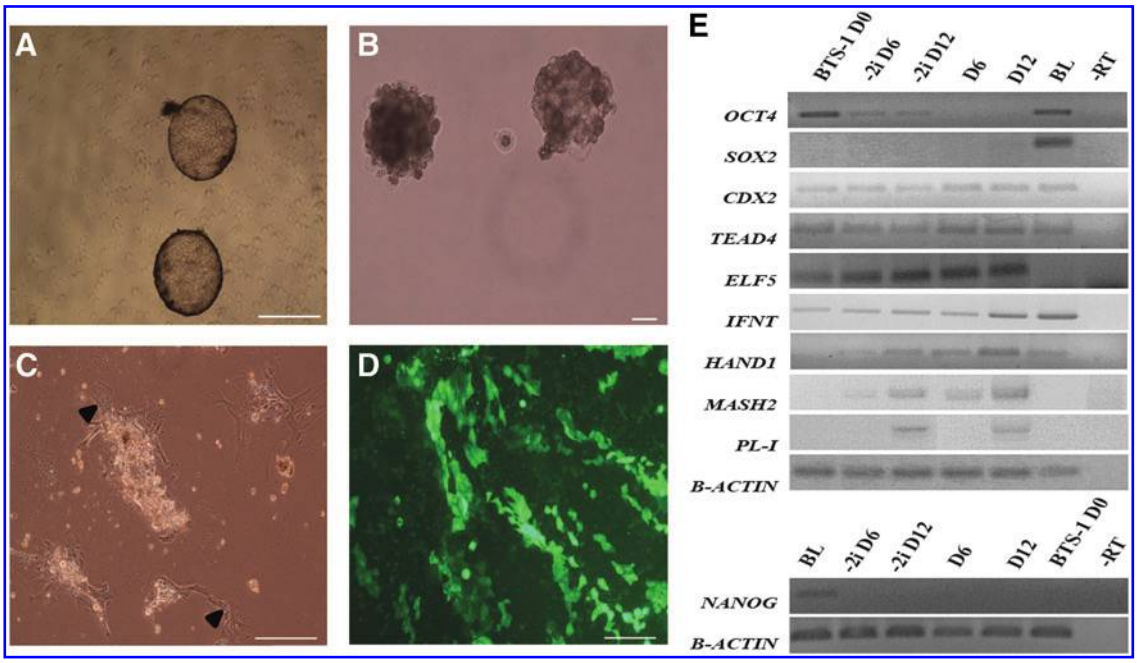

FIG. 7. Differentiation and transfection of BTS-1 cells in vitro. **(A)** Spheres formed when double inhibition was removed from the medium after 12 days. **(B)** Spheres formed in DMEM, which was supplemented with 10% FBS, at day 12. **(C)** Adherent cells in DMEM, which was supplemented with 10% FBS, at day 12. Double nuclei cells were indicated by an *arrowhead*. **(D)** GFP-expressing cells after BTS-1 cells were infected by Tet-on-inducible GFP expressing lentiviruses and induced by doxycycline. The scale bars of **(A, C, D)** represent 200 μm. The scale bar of **B** represent 100 μm. **(E)** Semi-quantitative reverse transcription PCR for differentiated BTS-1 cells at day 6 and at day 12. DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescent protein.

using this system with WNT 3a-secreting L cells and MEF as feeders.

The TR stem-like cells that were derived in this study have the basic features of stem cells. These cells showed a normal diploid karyotype and proliferate long term in culture in an undifferentiated state. The bTS cells expressed known pluripotency markers, including *OCT-4* (*POU5F1), NANOG, SOX2, TRA-1-60*, *TRA-1-81*, *SSEA-1*, and *SSEA-4*. Interestingly, some of the pluripotency markers, such as *OCT4*, *SOX2*, and *NANOG* were expressed at lower levels in bTS cells compared with blastocysts; this lower level expression is consistent with their elevated levels of methylation. The partial inactivation of *OCT4*, which is possibly mediated by DNA methylation, is important for TE formation, which is an observation that has been supported by others [40,41]. The expression of *SOX2* in bTS cells was less surprising, because *SOX2* is known to be necessary for the establishment of TR lineages in mouse embryos [42]. *SOX2* was also found highly expressed in TSCs [43,44], where the expression level was even higher in TSCs than in ESCs [44].These observations suggest that there may be cohorts of factors that potentiate the stemness of both ESCs and TSCs [45]. In cattle, the expression of genes that are important for ICM commitment, such as *POU5F1* and *SALL4* was detected in both ICM and TE cells separated from d7 IVP blastocysts. *NANOG* and *SOX2* were significantly higher for ICM than TE cells; whereas the expression *POU5F1* and *SALL4* did not significantly differ between ICM and TE cells [46].When these cells differentiated, the above-mentioned pluripotency markers dramatically decreased. This result indicates that the expression of pluripotency markers in bovine TSCs may be lower than in ESCs but higher than in differentiated cells. This basic feature of bovine TSCs is different from mouse TSCs, which do not express pluripotency genes, but consistent with porcine iTR, which expresses some pluripotency markers, such as *OCT4* and *SOX2* [31]. Also our immunofluorescence results showed these two markers were expressed in TE cells of bovine d7 blastocysts (Supplementary Fig. S4). It is unclear whether the bTS cells first passed through a transient state, that was absent of pluripotency or whether the conversion to bTS with pluripotency was more direct and without such an intermediate step.

Several genes, including *CDX2*, *TEAD4*, *EOMES*, *ELF5*, *GATA3*, *SMARCA4* (*BRG1*), *ETS2*, and *ERR2,* have been used to define TSC self-renewal and multipotency [45]. In this study, we demonstrated that all of these genes are expressed in bTS cells. These results confirmed that the cells that we have cultured from bovine IVF blastocysts have characteristics of TSCs. *CDX2* is a specific marker that is capable of distinguishing between TE and ICM cells in mice and in other animals, which include bovine [47–50]. *CDX2* is expressed in bTS cells at both the transcription and protein levels (Fig. 4). Interestingly, the expression of *CDX2* in bTS cells resembles its expression in mouse undifferentiated TSCs [50]. Furthermore, the promoter region of *CDX2* is hypomethylated in BTS-1 cells (13.80% and 3.9%) compared with blastocysts (18.80% and 9.1%), but the demethylation level is not as obvious as the methylation increase of the promoter regions of *OCT4*, *SOX2*, and *NANOG*. At the same time, the results of reverse transcription PCR and real-time PCR (data not shown) did not show obvious difference of the *CDX2* expression between bTS cells and blastocysts. This result also agrees with others report that TR-enriched *CDX2*

mRNA concentration was not affected in 2i-cultured bovine blastocysts [38]. The *CDX2* expression level also did not decrease upon differentiation, which implied that *CDX2* might be continuously expressed during bovine TR development. *EOMES*, which is another TSC marker, was only weakly detected in bTS cells. Its role in bovine TSCs requires further investigation. BTS cells also express markers of a differentiated TR lineage, such as *HAND1*, *CDH3*, *MASH2*, *GCM1*, *IFN-τ*, *TDK*, and *PAG*. However, the cells did not express the differentiation gene *PL-I*. We found that bTS cells that were continuously cultivated in the 2i and mixed feeders system express high levels of IFN-τ, which is a major product of ovine and bovine concepetuses prior to TR attachment to the uterine wall [29]. However, the expression of IFN-τ was lower in bTS cells than that in blastocysts.

The BTS-1 cell line has been stably maintained for more than 150 passages over a period of 36 months, with no apparent change in morphology or viability in the presence of PD0325901 and CHIR99021. Removing double inhibitors from the culture medium or culturing in DMEM that was supplemented with 10% FBS allowed the cells to differentiate into spheres, which are morphologically similar to bovine ES cells differentiation [51]. The expression levels of the TR differentiation markers *HAND1*, *MASH2*, and *PL-I* was upregulated upon differentiation, and TR cells with two nuclei could also be observed. In the absence of double inhibitors, some BTS-1 cells differentiated into cells that exhibited neuron-like morphology (data not shown). The differentiation potential of bTS cells in vivo was also examined. Teratoma formation is commonly used as a measure of ESC pluripotency. Similar to teratomas that were formed from human and mouse ESCs, BTS cells generated solid, relatively uniform tumors in NOD-SCID mice. These teratomas consisted of a range of differentiated tissues, including those tissues of both ectodermal and mesodermal origins. The islands of striated muscle tissue that were observed in the teratomas, although unexpected, are consistent with the observation that human chorionic villi that were transplanted into SCID/mdx mice can form striated muscle [52] and provide further evidence that the bTS cells that were generated by the 2i system are pluripotent. To our knowledge, the current study is the first report of teratoma formation in TS cells that were derived from fertilized farm animal embryos. The embryoid bodies and teratoma formation provide evidence that BTS-1 cells have many features that are typical of stem cells.

The proliferation of mouse TSCs was stimulated by Fgf4 [1], but reduced by 2i or Mapk-inhibition alone [53]. TSCs from other species, such as the rhesus monkey [6] and the common vole [7], were obtained without FGF4. Bovine TR cell lines did not require FGF to survive, proliferate, and prevent differentiation [17,54]. It remains to be determined whether other FGF effects, such as an increase of TR migratory or chemotactic activity [55,56], are impaired by 2i culture. We successfully derived bovine TS cells by the 2i of MEK and GSK3 with feeders secreting Wnt3a, and we also found when WNT3a-secreting feeders were replaced by BFFs, exogenous WNT3a is needed for bTS outgrowth (data not shown). The results suggested that, in contrast to studies of mice [1], other pathways such as WNT and not the FGF might be the dominant pathway that is required for the maintenance of the proliferative undifferentiated state of bovine TSCs. The WNT signaling system plays important roles in directing developmental processes, which include the maintenance of pluripotency, cell migration during gastrulation and neurulation, and axis formation [57]. Evidence from the mouse indicates that the Wnt system is present and activated as early as the two-cell stage [58–61]. However, inhibition of Wnt signaling does not compromise development to the blastocyst stage [60] and, therefore, activation of this signaling system may not be a requirement for preimplantation development. Bovine preimplantation embryo also possesses a functional WNT signaling system [38]. In bovine IVF embryos, CHIR99021 decreased phosphorylated β-catenin at the two-cell stage and improved blastocyst rate and quality [37]. 2i accelerated development and increased blastocyst quality [37]. This may again be a result of stimulating anabolic processes via GSK3 inhibition because CHIR99021 alone was sufficient to increase cell numbers in the TR and ICM. The Wnt signaling pathway has been indicated as an important regulator of embryo implantation and placental development in mice, sheep, and humans [62,63]. In 2012, Kohan-Ghadr et al. [64] reported that E-cadherin and β-catenin play critical roles in bovine TE formation and function, but little is known about the function of Wnt signaling in bovine TR differentiation. The bTS cells we have obtained will be a useful tool to study the function of WNT and FGF signaling in bovine TR development.

The system that we have described here may provide new possibilities for studying the functions of genes and signaling pathways that are involved in the development of bovine TR lineages. Because these cells have a normal karyotype and can be transfected with lentivirus expressing GFP, these cells may also be a candidate for generating transgenic farm animals.

Acknowledgments

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Author Disclosure Statement

The authors have no conflict of interest.

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