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Characterization and Multilineage Differentiation of Embryonic Stem Cells Derived From a Buffalo Parthenogenetic Embryo

HATHAITIP SRITANAUDOMCHAI,[1,3] KANOK PAVASUTHIPAISIT,[2,3†] YINDEE KITIYANANT,[2,3] PIENGCHAI KUPRADINUN,[4] SHOUKHRAT MITALIPOV,[5] AND THANIT KUSAMRAN[1]*

[1]*Department of Biochemistry, Faculty of science, Mahidol University, Bangkok, Thailand*
[2]*Department of Anatomy, Faculty of science, Mahidol University, Bangkok, Thailand*
[3]*Research Center on Reproductive Biology and Economically Valuable Animals, Institute of Science and Technology for Research and Development, Nakornpratom, Thailand*
[4]*Research Division, National Cancer Institute, Bangkok, Thailand*
[5]*Division of Reproductive Sciences, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, Oregon*

ABSTRACT Embryonic stem (ES) cells derived from mammalian embryos have the ability to form any terminally differentiated cell of the body. We herein describe production of parthenogenetic buffalo (*Bubalus Bubalis*) blastocysts and subsequent isolation of an ES cell line. Established parthenogenetic ES (PGES) cells exhibited diploid karyotype and high telomerase activity. PGES cells showed remarkable long-term proliferative capacity providing the possibility for unlimited expansion in culture. Furthermore, these cells expressed key ES cell-specific markers defined for primate species including stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antigen-1-81 (TRA-1-81), and octamer-binding transcription factor 4 (Oct-4). In vitro, in the absence of a feeder layer, cells readily formed embryoid bodies (EBs). When cultured for an extended period of time, EBs spontaneously differentiated into derivatives of three embryonic germ layers as detected by PCR for ectodermal (*nestin*, *oligodendrocytes*, and *tubulin*), mesodermal (*scleraxis*, *α-skeletal actin*, *collagen II*, and *osteocalcin*) and endodermal markers (*insulin* and *α-fetoprotein*). Differentiation of PGES cells toward chondrocyte lineage was directed by supplementing serum-containing media with ascorbic acid, β-glycerophosphate, and dexamethasone. Moreover, when PGES cells were injected into nude mice, teratomas with derivatives representing all three embryonic germ layers were produced. Our results suggest that the cell line isolated from a parthenogenetic blastocyst holds properties of ES cells, and can be used as an in vitro model to study the effects of imprinting on cell differentiation and as an a invaluable material for extensive molecular studies on imprinted genes. *Mol. Reprod. Dev. 74: 1295–1302, 2007.*

Key Words: embryonic stem cells; parthenogenesis; buffalo; pluripotent

INTRODUCTION

Pluripotent cells exist transiently in the early embryo and have the capacity to give rise to differentiated progeny representative of all three embryonic germ layers. Under certain conditions, these pluripotent cells can be adapted to in vitro culture as ES cell line and propagated indefinitely in undifferentiated state. ES cells are routinely derived from the inner cell mass (ICM) of preinplantation blastocysts. These cells are immortal and pluripotent, that is, they are capable of proliferating indefinitely and differentiating into a wide variety of cell types both in vitro and in vivo (Call et al., 2000; Buttery et al., 2001). ES cells from farm animals serve as a model for application in regenerative medicine.

Stable ES cell lines have been established in several species including mouse (Evans and Kaufman, 1981; Martin, 1981), hamsters (Doetschman et al., 1988), mink (Sukoyan et al., 1993), pigs (Wheeler, 1994), rhesus monkeys (Thomson et al., 1995), chicken (Pain et al., 1996), common marmoset (Thomson et al., 1996), humans (Thomson et al., 1998), cattle (Mitalipova et al., 2001), and horse (Saito et al., 2002). There are currently two types of embryos used as a source for derivation ES cells: precompacted stage embryos and blastocysts produced by in vivo or in vitro fertilization (IVF) and parthenogenetic embryos (PG) (Thomson et al., 1996; Mitalipova et al., 2001; Cibelli et al., 2002; Nakatsuji and Suemori, 2002). The use of human embryos for

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*Correspondence to: Thanit Kusamran, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand, 10400. E-mail: sctks@mahidol.ac.th

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derivation of ES cells is associated with bioethical concerns. However, utilization of parthenogenetically produced embryos on the other hand may circumvent this problem. PGES cells can be derived without destroying a viable embryo since parthenotes have limited developmental potential in vivo (Boediono et al., 1991). During mouse parthenogenetic embryo development, the dramatic consequences of genomic imprinting have been clearly demonstrated resulting in failure to develop to term and by the limited capacities of cells with uniparental genotypes to participate in development in chimeras (Surani et al., 1990). Considering the dynamic pattern of epigenetic changes during early development, it is of particular interest to examine the stability of imprinting in ES cells. The properties of parental imprints could be examined by comparing ES cells derived from PG and normal blastocysts (Allen et al., 1994; Zvetkova et al., 2005).

We report here isolation and characterization of ES cells from buffalo parthenogenetic blastocysts. We show unique pluripotent properties of these cells that closely resemble primate ES cells. We also studied the ability of these ES cells to differentiate in vitro and in vivo into derivatives of embryonic ectoderm, mesoderm, and endoderm.

MATERIALS AND METHODS

Production of Parthenogenic Buffalo Blastocysts

Buffalo oocytes were collected and matured in vitro as previously described (Kitiyanant et al., 2001). Briefly, cumulus oocyte complexes (COCs) from abattoir ovaries were collected by aspiration of antral follicles (2–6 mm in diameter) using a 18-guage needle. After rinsing with TALP-HEPES, COCs were morphologically assessed under a stereomicroscope and oocytes with compact and homogeneous cytoplasm were selected for in vitro maturation (IVM).Unless indicated otherwise, all reagents were from Sigma-Aldrich Co. (St. Louis, MO). Selected COCs were cultured in 50 μl drops of TCM 199 maturation medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 0.2 mM pyruvate and 5 μg/ml FSH in humidified atmosphere with 5% CO_2 at 39°C. After culturing for 24 hr, mature oocytes were subjected to parthenogenetic activation (PA) as described previously (Saikhun et al., 2004a). Buffalo oocytes were denuded by pipetting in TALP-HEPES containing 0.2% hyaluronidase. Oocytes with first polar body (MII) were activated by exposure to 7% ethanol for 5 min followed by incubation in 2 mM 6-DMAP for 4 hr. The PA oocytes were co-cultured with buffalo rat liver (BRL, American Type Culture Collection, Rockville, MD) cells in 50 μl of TCM 199 supplemented with 10% FBS in 5% CO_2 at 39°C for 6–7 days.

Isolation of ES Cells

Zona-free parthenogenetic blastocysts were individually cultured in 50 μl drops of mitotically-inactivated mouse embryonic fibroblasts (mEF) in medium containing 80% DMEM/F-12 (Invitrogen), 15% FBS, 1 mM glutamine, 0.1 mM 2-mercaptoethanol (Invitrogen) and BME amino acids in 5% CO_2 in air at 37°C. ICMs of blastocyst that attached to the feeder layer were mechanically separated from trophectodermal cells by 29-gauge needle and replated onto 50 μl drops of a fresh feeder layer and cultured under the same conditions. After 10–12 days, cells with ES-like morphology (Robertson, 1987) were manually picked up and expanded mechanically into a 35 mm tissue culture dish (Nunc, Naperville, IL) with a fresh feeder layer (passage 1 or P1 cells). P1 cells were then subjected to repeated passages at 6- to 7-day intervals.

Characterization of Stem Cells

Cells were fixed in 4% paraformaldehyde for 30 min and washed two to three times with PBS. After permeabilization with 0.1% Triton X-100 for 10 min, nonspecific reactions were blocked with 1% bovine serum albumin for 30 min. The cells were then incubated for 60 min in primary monoclonal antibodies (1:10 dilution) for SSEA-1, SSEA-3, SSEA-4 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), TRA-1-60, and TRA-1-81 (a gift from Peter Andrews, University of Sheffield, England) and polyclonal antibody raised against recombinant protein corresponding to amino acids 1–134 of human Oct-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing in PBS, cells were exposed to fluorescein isothiocyanate (FITC)-labeled secondary antibodies for 60 min and washed three times with PBS buffer.

Telomerase Activity Assay

Telomerase activity was measured by telomere repeat amplification protocol (TRAP) using Telomerase PCR ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) as described (Saikhun et al., 2004b). Extracts from mouse ES cells, mEF, buffalo ES cells, buffalo embryoid bodies were normalized by protein concentration. Heat inactivated extracts were boiled for 3 min before the assay. The level of telomerase activity in each sample was determined by comparing the signal from the sample to the signal obtained using a known amount of a control template. Relative telomerase activity (RTA) of each sample was calculated using the following formula:

$$\mathrm{RTA} = \frac{(A_S - A_{S0}/A_{S,IS})}{(A_{TS8} - A_{TS8,0})/A_{TS8,IS}}$$

where A_S is the absorbance of sample; A_{S0}, absorbance of sample of RNase treated sample; $A_{S,IS}$, absorbance of IS of the sample; A_{TS8}, absorbance of control template (TS8); $A_{TS8,0}$, absorbance of lysis buffer and $A_{TS8,IS}$, absorbance of internal standard (IS) of the control template (TS8).

Embryoid Body Formation and Chondrocyte Differentiation

To induce formation of EBs, ES cell clumps were cultured in "hanging drops" of DMEM supplemented

with 10% FBS as described previously (Kramer et al., 2000). After 5 days, EBs were collected for further experiments.

For chondrocyte differentiation, gently dissociated feeder-free ES cells were cultured in DMEM supplemented with 10% FBS, 50 μg/ml ascorbic acid, 50 mM β-glycerophosphate, and 1 μM dexamethasone for 30 days. The composition and concentrations of supplements described are based on conditions established for the growth and differentiation of primary chondrocytes (Buttery et al., 2001). Differentiated cells were analyzed for expression of cartilage-specific sulfated proteoglycans using alcian blue dye (Kimmel and Trammell, 1981) and collagen II using mouse collagen-specific antibody (Neomarkers, Fremont, CA) and FITC-conjugated secondary antibody. RT-PCR analysis for the expression of collagen II, Sox-9, and scleraxis transcription factor was carried out as described below.

In Vivo Differentiation

After culture for 14 or more passages, approximately, 1×10^7 ES cells were injected subcutaneously into right front leg or intraperitonially into 6–8-week-old nude female mice. Fourteen to 16 weeks after injection resulting teratomas were isolated and fixed in neutral buffered 10% formalin, embedded in paraffin, and examined histologically using hematoxylin and eosin stainings.

RT-PCR Analysis

Total RNA was isolated from EBs at day 5, 10, and 20 or undifferentiated ES cells using the NucleoSpin® RNAII Kit (Macherey-Nagel, MN), according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed by using oligo-dT primer and Superscript III reverse transcriptase (Invitrogen).

Aliquots of 1 μl from the reverse transcriptase reactions were used for amplification of transcripts using primers specific for the analyzed genes. PCR reactions were denatured for 5 min at 95°C, followed by 30–40 cycles of 40 sec denaturation at 95°C, 40 sec annealing at the primer-specific temperature (see below) and 50 sec elongation at 72°C. Expression of the following genes was studied (oligonucleotide sequences are given in brackets in the order antisense-, sense-primer followed by the annealing temperature used for PCR, expected length of the amplified fragment and a reference or blank (our designed primer)): genes encoding nestin (5′-AGTGTGAAGGCAAAGATAGC-3′, 5′-TCTGTCAGGATTGGGATGGG-3′, 58°C, 317 bp (Cai et al., 2002)), oligodendrocyte (5′-AAGTAGTTGGCCACAGCAAG-3′, 5′-CAGAGCGGCTGTCTCTTC-3′, 54°C, 600 bp (Hegert et al., 2002)), tubulin (5′-TGCGTGTGTACAGGTGAATGC-3′, 5′-AGGCTGCATAGTCATTTCCAAG-3′, 54°C, 250 bp (Cai et al., 2002)), scleraxis (5′-GTGGACCCTCCTCCTTCTAATTCG-3′, 5′-GACCGCA CCAACAGCGTGAA-3′, 63°C, 375 bp (Metsaranta et al., 1991)), α-skeletal actin (5′-GGCATCATCACCAACTGGGA-3′, 5′-TTGCCGATGGTGATGACCTG-3′, 57°C, 536 bp), collagen II (5′-AGGGGTACCAGGTTCTCCATC-3′, 5′-CTGCTCATCGCCGCGGTCCTA-3′, 60°C, 432 bp (Metsaranta et al., 1991)), Sox-9 (5′-TCTTTCTTGTGCTGCACGCGC-3′, 5′-TGGCAGACCAGTACCCGCATCT-3′; 57°C; 135 bp (Metsaranta et al., 1991)), osteocalcin (5′-ATGCTACTGGACGCTGGAGGGT-3′, 5′-GCGGTCTTCAAGCCATACTGGTC-3′, 64°C, 330 bp (Hegert et al., 2002)) insulin II (5′-ATGG CCCTGTGGATCCGCTT-3′, nested 5′-CCTGCTC ATCC TCTGGGAGCC-3′, 5′-TGCCAAGGTCTGAAGGTCAC-3′, 58°C, 209 bp (Hori et al., 2002)), AFP (5′-ACATCSAGGAGAGCCARGCA-3′, 5′-CCCTGAGCTTGGCACAGATC-3′, 58°C, 415 bp (Ohneda et al., 2001)), Oct-4 (5′-GGTTCTCTTTGGAAAGGTGTTC-3′, nested 5′-TGGAGGAAGCTGACAACAACGA-3′, 5′-ACACTCGG ACCACGTCTTTC-3′, 58°C, 207 bp), and the 'housekeeping' gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH, 5′-ATCACCATCTTCCAGGAGCG-3′, 5′-TAGGAACACGGAAGGCCATG-3′, 58°C, 495 bp (McKinney and Robbins, 1992)). Electrophoretic separation of PCR products was carried out on 2% agarose gels. Distilled water was always included as a negative control.

Karyotyping

Cells prepared for cytogenetic analysis were incubated in growth medium supplemented with 0.2 μg/ml chochicine for 2 hr at 37°C, 5%CO_2. After washing, cells were disaggregated with trypsin/EDTA solution for 10 min, and resuspended in 75 mM KCl. Cells were fixed in series of cool methanol:acetic acid (3:1, 2:1, and 3:1, respectively) and drops of cell suspension were spread on clean microscope slides. The chromosomes were stained with 5% Giemsa for 40 min and examined at magnification 1,000×.

RESULTS

Derivation of ES Cells From Buffalo Parthenogenetic Embryos

For parthenote production, 40 COCs were isolated from buffalo ovaries and placed in maturation medium for 24 hr. Twenty-seven oocytes reached metaphase II stage and were subsequently artificially activated. Six embryos developed to the blastocyst stage within 6–7 days in culture (Fig. 1A). Blastocysts were then freed of zona pellucida and transferred onto mEF feeder layer in ES medium and cultured for an additional 5–7 days (Fig. 1B). Three blastocysts attached to the feeder and their ICMs formed colonies composed of tightly packed compact cells. These colonies were mechanically dislodged and isolated from outer trophectodermal cells and replated onto fresh feeder. Subsequently, one stable cell line (PGES) (Fig. 1C) was established and propagated for an extended period of time (>6 months). Established PGES cells had a high nucleus/cytoplasm ratio and prominent nucleoli characteristic of ES cells from other species (Fig. 1D). Cytogenetic analysis of the cell line revealed diploid 46 + 2 (XX) set of chromosomes (Fig. 1E), as previously described for *Bubalus bubalis* [Kitiyanant et al., 2001; Saikhun et al., 2004a]. These

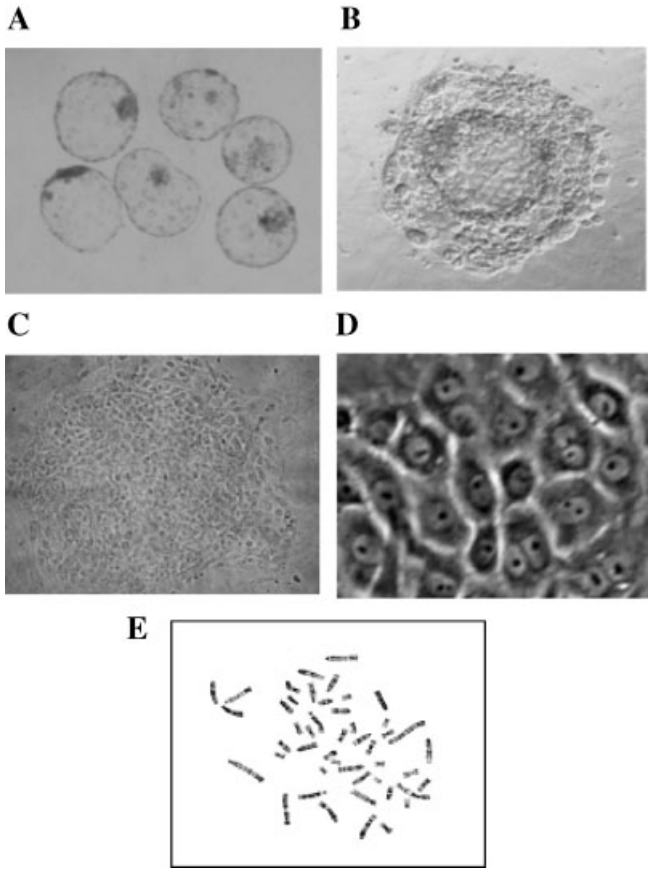

Fig. 1. Isolation of buffalo PGES cells. **A**: Parthenogenetic buffalo blastocysts. **B**: ICM outgrowth after 6 days in culture. **C**, **D**: Morphology of buffalo PGES cells. **E**: A metaphase spread of PGES cells. Magnification: A, 100×; B and C, 200×; D, 400×; E, 1,000×.

cells also displayed high levels of telomerase activity comparable to mouse ES cells as detected by TRAP assay (Fig. 2). In contrast, differentiated ES cells (EBs) displayed diminished telomerase activity levels and no telomerase activity was detected in mEF (Fig. 2).

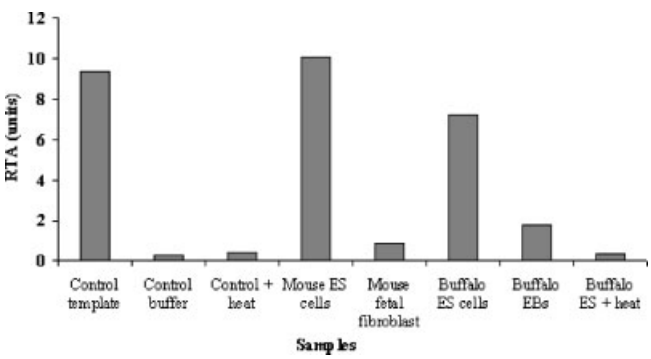

Fig. 2. Relative telomerase activity (RTA) in undifferentiated buffalo PGES cells. Cells were analyzed for telomerase activity by the TRAP assay. Buffalo PGES cells (passage 14), maintained in the undifferentiated state on mEF layers, expressed high telomerase activity that diminished upon differentiation into EBs. No telomerase activity was detected in mEF.

Expression of ES Cell Markers by Immunocytochemistry

Established buffalo PGES cells strongly reacted with SSEA-4, TRA-1-81 and Oct-4 antibodies. However, the signal was weak for SSEA-1 and TRA-1-60 and no signal was detected for SSEA-3 (Fig. 3A–F). Mouse embryonic fibroblasts were used as a negative control.

In Vitro Differentiation

Developmental potential of buffalo PGES cells was determined by EB formation and further differentiation into chondrocytes. In absence of feeder cells, PGES cells readily formed EBs within 5 days (5+) of suspension culture (Fig. 4A–C). EBs formed tight aggregates that increased rapidly in size with time in culture (Fig. 4C, see largest EB). RT-PCR analysis of the EBs harvested on day $5+5$, $5+10$, and $5+20$ of the cultures demonstrated the presence of variety of differentiated cell types including derivatives of all three embryonic germ layers (Fig. 4D). As neuron-specific representatives of the ectodermal tissue, expression of *nestin*, *oligodendrocyte* and *tubulin* genes was studied. *Nestin*, is expressed in all precursor cells throughout the embryonic nervous system that can give rise to neurons or to supporting cells of the brain such as glia and oligodendrocytes. In EBs, *Nestin* expression was first detected at day $5+5$ and continued until day $5+20$, whereas *tubulin*, expression was first detected at day $5+10$ and continued until day $5+20$. A mesodermal marker, *scleraxis*, encoding transcription factor normally expressed at high levels in mesenchymal cells was first detected in EBs at day $5+5$, $5+10$, and $5+20$. Another mesodermal marker, *α-skeletal actin* that is expressed during muscle cell development was detected in EBs in similar to *scleraxis* pattern. Mesodermal derivative *collagen II*, encoding transcription factor involved in extracellular matrix proteins of cartilage tissue, was expressed in EBs at day $5+10$ and day $5+20$. *Osteocalcin*, the most abundant noncollagenous protein in bones was expressed in EBs on day $5+20$. A marker for extra-embryonic yolk sac and embryonic (fetal liver and intestines) endoderm, *α-fetoprotein* was expressed in EBs harvested on day $5+20$. *Insulin*, encoding transcription factor in pancreatic beta cells, was also detected in $5+20$ days old EBs.

To examine the ability to differentiate into chondrocytes, we subjected buffalo PGES cells to protocols previously developed for the growth and differentiation of primary chondrocytes (Buttery et al., 2001). Buffalo PGES cells were cultured in medium that contained a mixture of growth factors as described in Materials and Methods. Approximately 30 days after induction of the differentiation, aggregates consisting of small round cells were noted. These cells were positive for Alcian blue (Fig. 5A) and immunoreactive for collagen II antibody (Fig. 5B). Additionally, transcripts of *scleraxis*, *collagen II*, and *sox-9* were detected by RT-PCR (Fig. 5C).

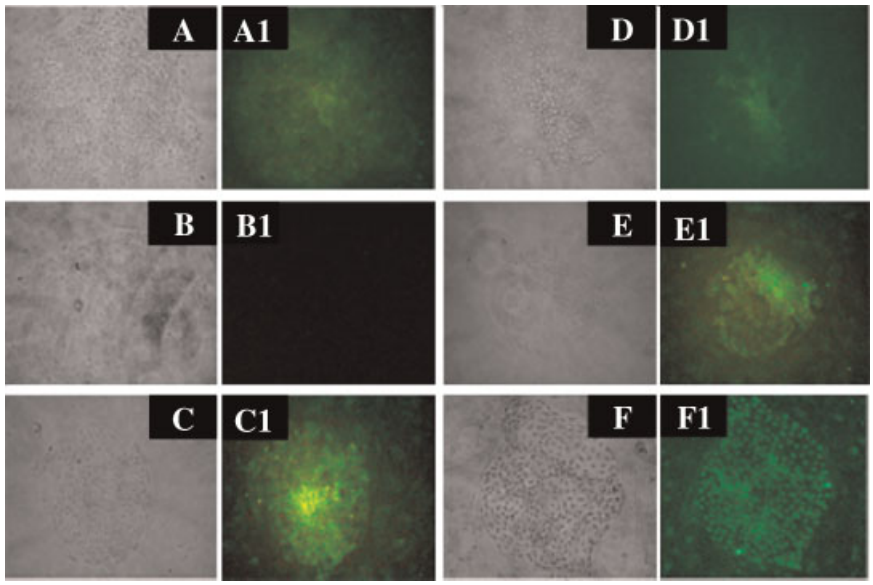

Fig. 3. Expression of cell surface markers in undifferentiated buffalo PGES cells (passage 4). (**A**, **A1**) SSEA-1. (**B**, **B1**) SSEA-3. (**C**, **C1**) SSEA-4. (**D**, **D1**) TRA-1–60. (**E**, **E1**) TRA-1-81. (**F**, **F1**) Oct-4. Magnification: A–E, 200×; F, 100×.[See color version online at www.interscience.wiley.com.]

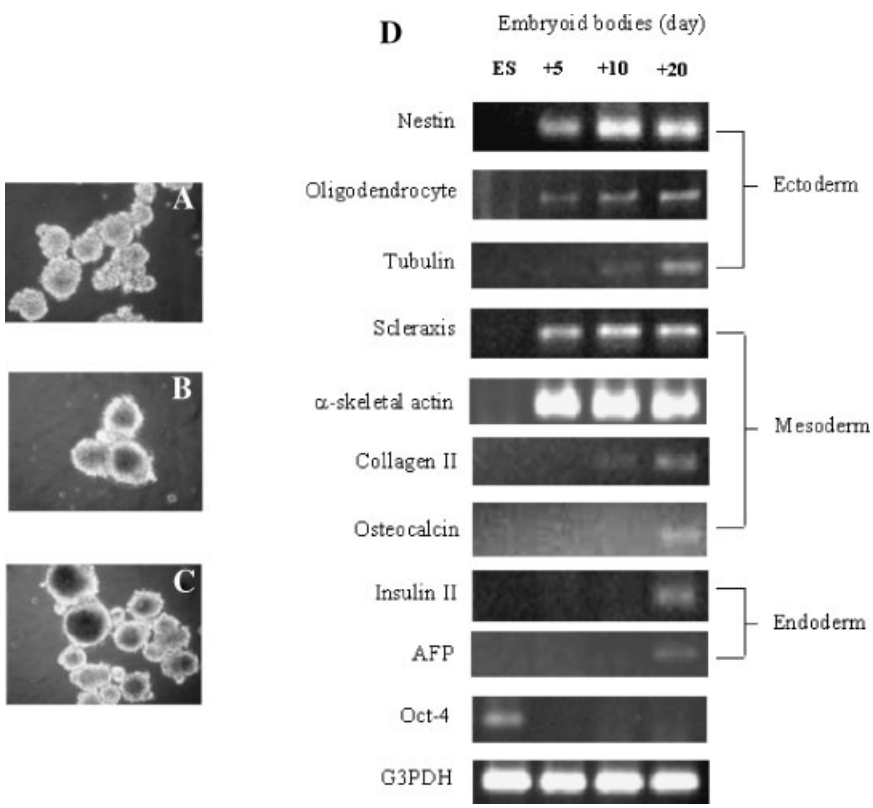

Fig. 4. Differential expression of specific genes during buffalo PGES cell differentiation in vitro. Phase contrast micrographs of EBs at (**A**) day 5. (**B**) day 10. (**C**) day 20. Magnification: A–C, ×100. (**D**) Genes encoding transcription factors, markers of germ layers; ectoderm (*Nestin, Oligodendrocyte*, and *Tubulin*), mesoderm (*Scleraxis,α-skeletal actin, Collagen II*, and *Osteocalcin*), and endoderm (*Insulin II* and *AFP*) expressed in EBs at various stages of development from 5 to 20 day.

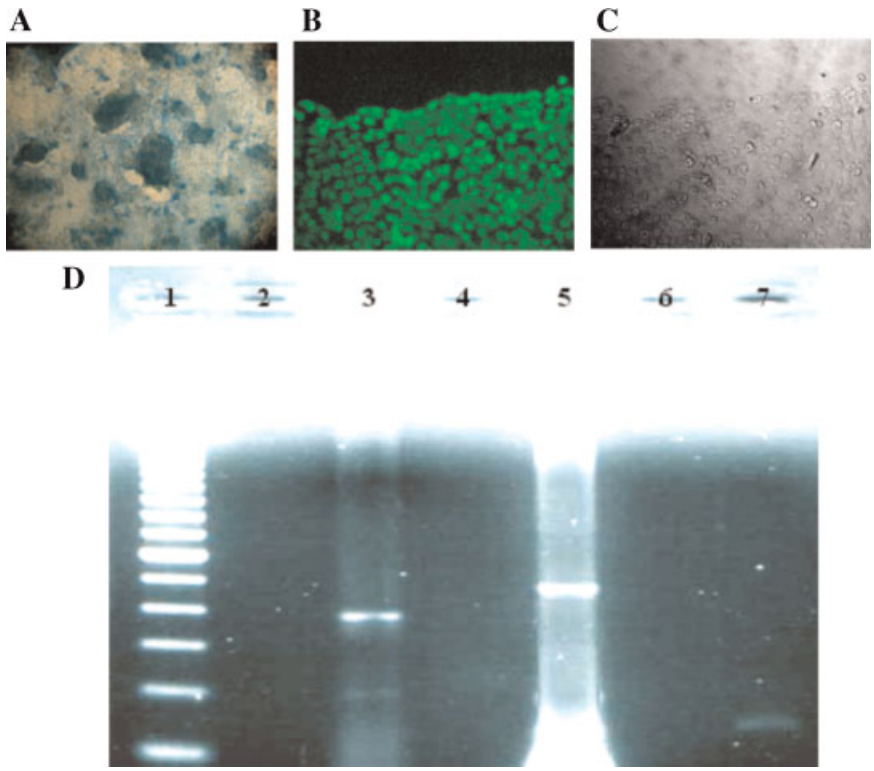

Fig. 5. Chondrocyte differentiation of buffalo PGES cells in vitro. (**A**) Determination of proteoglycans in EBs with alcian blue following differentiation for 30 days in presence of β-glycerophosphate, ascorbic acid and dexamethasone (phase contrast). (**B**) Immunostaining of differentiated cells with collagen II-specific antibody. (**C**) Phase contrast micrograph of chondrocyte differentiated cells. (**D**) Expression of scleraxis, collagen II, and Sox-9 in buffalo PGES cells and differentiated cells. Lane 1, 100 bp DNA ladder; lane 2, scleraxis in PGES cells; lane 3, scleraxis in differentiated cells; lane 4, collagen II in PGES cells; lane 5, collagen II in differentiatied cells; lane 6, Sox-9 in PGES cells; lane 7, sox-9 in differentiated cells. Scleraxis band is 375 bp, collagen II band is 432 bp, and Sox-9 band is 135 bp. [See color version online at www.interscience.wiley.com.]

In Vivo Differentiation of Parthenogenetic Stem Cells

When undifferentiated PGES cells were injected subcutaneously or intraperitoneally into nude mice, 80% (8/10) of mice developed tumors. Teratomas were isolated 14–16 weeks after injection and analyzed histologically. Upon autopsy, lesions consisting of cystic masses filled with pale fluid and areas of solid tissue were observed (Fig. 6A). There was no gross evidence of metastic spreading of PGES cell derivatives to other sites. Tumors contained tissues representing all three germlayers as judged by detection of ganglionic structures (ectoderm), muscle, cartilage (mesoderm), adrenal gland and glandular structures (endoderm) (Fig. 6B–G).

DISCUSSION

In this study, we report an establishment of PGES cell line from buffalo parthenogenetic blastocyst. Our subsequent analysis confirmed that these cells posses key properties of ES cells. Buffalo PGES cells morphologically closely resembled primate ES cells (Thomson et al., 1995), and showed indefinite proliferation capacity in vitro (>12 months), while maintaining their potential to differentiate into derivatives of all three embryonic germ layers. Mouse and horse ES cells grow as multilayer colonies with a distinctive margin and high nuclear to cytoplasmic ratio (Evans and Kaufman, 1981; Martin, 1981; Saito et al., 2002). Buffalo PGES cells also displayed a high nuclear/cytoplasmic ratio, but grew as a monolayer colony similar to that reported for bovine, monkey, and human ES cells (Thomson et al., 1995; Reubinoff et al., 2000; Mitalipova et al., 2001). High telomerase activity is often correlated with replicative immortality and is typically expressed in germ cells, cancer cells, and variety of stem cells, including ES cells, but absent in most somatic cell types (Amit et al., 2000; Kim and Hruszkewycz, 2001; Armstrong et al., 2004). Undifferentiated buffalo PGES cells expressed telomerase activity consistent with their indefinite lifespan property. Buffalo PGES cells weakly expressed SSEA-1, a marker of undifferentiated mouse ES cells, but indicator of differentiated primate ES cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1995). Similar to primate ES cells, buffalo PGES cells strongly expressed SSEA-4 and TRA-1-81 epitopes but did not express SSEA-3 (Cibelli et al., 2002). Oct-4 is a transcription factor specific for pluripotent cells of early mouse, monkey, and human embryos as well as ES and primordial germ cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1995; Reubinoff et al., 2000; Pesce and Scholer, 2001; Boiani et al., 2002;

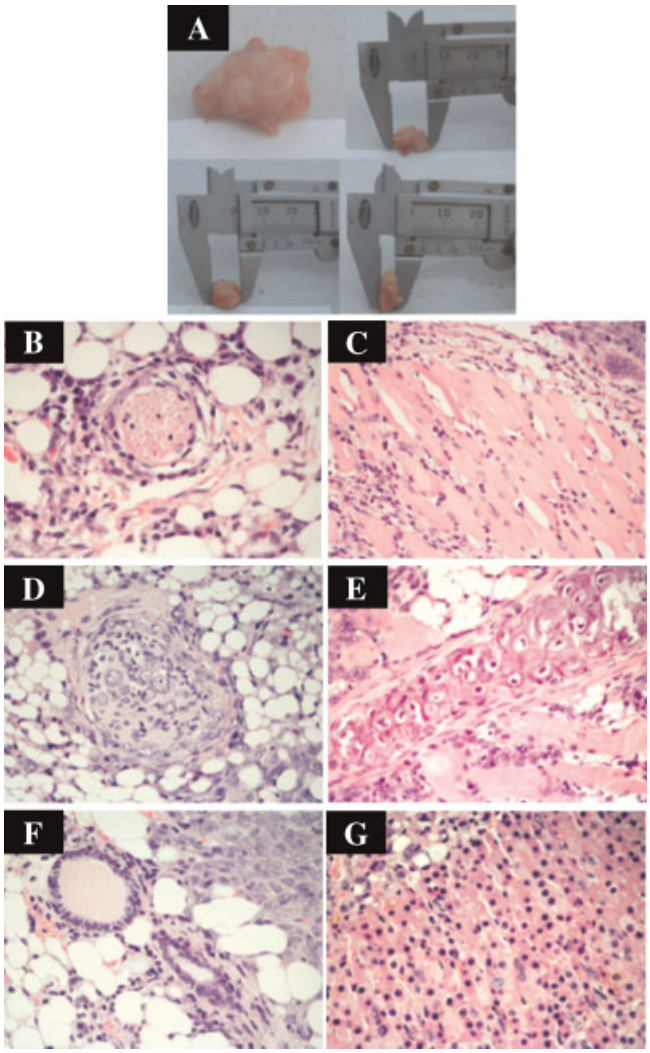

Fig. 6. In vivo differentiation of parthenogenetic buffalo PGES cells. Cells were injected s.c. and i.p. in nude mice. Fourteen to 16 weeks after injection, teratomas (**A**) were isolated, fixed with 10% formalin, and paraffin-embedded. Sections were stained with hematoxylin/eosin. Present within these teratomas are advanced derivatives of ectoderm, such as **B**) ganglionic structure, of mesoderm, such as **C**) striated muscle, **D**) cartilage (cross section), and **E**) cartilage (long section), and of endoderm, such as **F**) glandular structure, and **G**) adrenal gland. Magnification: B–E, ×400, F, ×600. All photomicrographs are of hematoxylin- and eosin-stained sections. [See color version online at www.interscience.wiley.com.]

Mitalipov et al., 2003; Turnpenny et al., 2003; Cauffman et al., 2005). Interestingly, in bovine and porcine blastocysts Oct-4 protein expression is not restricted to the pluripotent lineages, but rather is detected in both the ICM and trophectoderm of fully expanded day 8 blastocysts (Kirchhof et al., 2000). However, recent report conducted by in situ hybridization demonstrated that *Oct-4* transcript is restricted to the ICM, indicating that in contrast to protein distribution, pattern of *Oct-4* mRNA expression is similar to that observed in the mouse and other mammals (Kurosaka et al., 2004). Here we demonstrate strong Oct-4 expression in undifferentiated buffalo PGES cells both by immunocytochemistry and RT-PCR.

The most remarkable property of ES cells is their ability to differentiate into ectodermal, mesodermal, and endodermal derivatives. Similar to mouse, monkey, human, and bovine ES cells (Evans and Kaufman, 1981; Thomson et al., 1995, 1998; Mitalipova et al., 2001), buffalo PGES cells easily formed EBs in suspension culture. EBs are typically the starting material for differentiation protocols into neuronal (Saito et al., 2002), cartilage, osteoblast (Metsaranta et al., 1991; Hegert et al., 2002), muscle (Cserjesi et al., 1995), or endodermal progenitors (Ohneda et al., 2001; Hori et al., 2002). We identified derivatives of all three embryonic germ layers in buffalo PGES cell-derived Ebs. Moreover, directed in vitro differentiation to chondrocyte-like cells was evident when previously developed protocols were applied to buffalo PGES cells.

In vivo differentiation capability following injection into a host blastocyst or nude mice has been used as an indicator of pluripotent properties of ES cells (Bradley et al., 1984). In this study, we further investigated developmental potential of buffalo PGES cells in vivo by injection into nude mice. High proportion of injected mice formed teratomas, with advanced differentiation into ganglionic structures (ectoderm), muscle, cartilage (mesoderm), adrenal gland and glandular structures (endoderm) suggesting that pluripotent nature of PGES cells.

Stem cells derived from human parthenogenetic embryos could alleviate some ethical concerns over embryo and stem cell research. Moreover, PGES cells provide a valuable in vitro model to study effect of imprinting on cell differentiation.

In conclusion, we report here for the first time isolation and detailed in vitro and in vivo characterization of buffalo PGES cells. These cells express key stem cell markers and capable of differentiating in vitro and in vivo into variety of cell types.

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