Original Article

Comparative Characterization of Four Mouse Parthenogenetic Embryonic Stem (pES) Cell Lines

Sasitorn Rungarunlert[1,2] Ruttachuk Rungsiwiwut[3] Sirilada Suphankong[4]
Sasithorn Panasophonkul[1] Kamthorn Pruksananonda[3] Pramuan Virutamasen[3]
Melinda K Pirity[2] Andras Dinnyes[2,5] Theerawat Tharasanit[1] Mongkol Techakumphu[1*]

Abstract

Derivation of embryonic stem (ES) cells from parthenogenetic embryos represents a possible alternative approach to create histocompatible cells for regenerative medicine. The objectives of this study were to establish mouse parthenogenetic ES (pES) cell lines from parthenogenetically-derived blastocysts as a model system for human and animal research and to examine pluripotency differences among the pES cell lines. We are able to report the successful establishment of four pluripotent pES cell lines from blastocysts of parthenogenetic origin (22% efficiency of pES cell line establishment). Four pES cell lines (pES#1-4) exhibited a typical ES cell morphology and expression of key pluripotency markers (ALP, Oct4, Nanog and SSEA-1). Three of the four pES cell lines have shown a high percentage of normal karyotype during long-term culture. Variability in the *in vitro* differentiation potential into cell types of the 3 germ layers was observed among the different pES cell lines. Three of these (pES#1-3) exhibited a higher efficiency towards endo-mesoderm differentiation, strongly expressed differentiation markers towards endo-mesoderm lineage (α-fetoprotein; Flk-1; PECAM and collagen IV) than pES#4. Differentiation towards cardiac cells resulted in all cell lines 33-100% of spontaneous beating cell clusters/well. Furthermore, following injection into blastocysts pES#1 cells differentiated successfully *in vivo* into chimeric mice with an efficiency of 75% (three chimeras of four newborns). In conclusion, our results have demonstrated that there are major differences among pES lines in their differentiation ability *in vitro* and that it was possible to generate chimera forming pES cell lines in mouse.

Keywords: differentiation, embryonic stem cells, mouse, parthenogenetic activation, pluripotency

[1]*Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*BioTalentum Ltd, Gödöllö, Hungary*
[3]*Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*
[4]*Department of Medical Science, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*
[5]*Molecular Animal Biotechnology Laboratory, Szent Istvan University, Gödöllö, Hungary*
Corresponding author E-mail: *tmongkol@chula.ac.th*

บทคัดย่อ

การเปรียบเทียบลักษณะคุณสมบัติของเซลล์ต้นกำเนิดจากตัวอ่อนหนูเม้าส์ที่ผลิตโดยวิธีการกระตุ้นด้วยกระแสไฟฟ้าจำนวน 4 เซลล์ไลน์

ศศิธร รุ่งอรุณเลิศ [1,2] รัฐจักร รังสิวิวัฒน์ [3] สิริลดา สุพรรณคง [4]
ศศิธร พนโสภณกล [1] กำธร พฤกษานานนท์ [3] ประมวล วีรุตมเสน [3]
มิลินดา เค พิริตี [2] แอนดาส ดินนเยส [2,5] ธีรวัฒน์ ธาราศานิต [1] มงคล เตชะกำพุ [1]

การสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตโดยวิธีการกระตุ้นด้วยสารเคมีเป็นทางเลือกหนึ่งสำหรับผลิตเซลล์ที่เหมาะสมเฉพาะบุคคลในการรักษาโรค การศึกษานี้มีจุดประสงค์เพื่อการสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตโดย วิธีการกระตุ้นด้วยสารเคมีเพื่อเป็นตัวแทนสำหรับการศึกษาวิจัยในมนุษย์และสัตว์ รวมทั้งศึกษาความแตกต่างลักษณะคุณสมบัติของแต่ละเซลล์ไลน์ การสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตโดยวิธีการกระตุ้นด้วยสารเคมีได้ประสบความสำเร็จจำนวน 4 เซลล์ไลน์ มีประสิทธิภาพในการสร้างเซลล์ต้นกำเนิดจากตัวอ่อนเป็นร้อยละ 22 เซลล์ต้นกำเนิดจากตัวอ่อนทั้งหมด แสดงคุณสมบัติ รูปร่างเหมือนเซลล์ต้นกำเนิดจากตัวอ่อน และให้ผลบวกเมื่อทำปฏิกิริยากับอัคคาไลน์ฟอสฟาเตส และ แอนติบอดีที่จำเพาะต่อ Oct4 Nanog และ SSEA-1 เซลล์ต้นกำเนิดจากตัวอ่อนจำนวน 3 เซลล์ไลน์ มีจำนวนคาริโอไทป์ที่ปกติระหว่างการเพาะเลี้ยง เซลล์ต้นกำเนิดจากตัวอ่อนแสดงคุณสมบัติหลากหลายเมื่อมีการเปลี่ยนแปลงเซลล์ต้นกำเนิดจากตัวอ่อนเป็นเซลล์ชนิดต่างๆ เซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตจากการกระตุ้นด้วยสารเคมี จำนวน 3 เซลล์ไลน์ (pES#1-3) มีประสิทธิภาพในการเปลี่ยนแปลงเป็นเซลล์เนื้อเยื่อชั้นในและชั้นกลางของตัวอ่อน และ ให้ผลบวกเมื่อทำปฏิกิริยากับแอนติบอดีที่จำเพาะต่อเซลล์เนื้อเยื่อชั้นในและชั้นกลาง เช่น เช่น α-fetoprotein, Flk-1, PECAM และ collagen IV มากกว่าอีกหนึ่งเซลล์ไลน์ (pES#4) การเปลี่ยนแปลงเซลล์ต้นกำเนิดจากตัวอ่อนเป็นเซลล์กล้ามเนื้อหัวใจมีประสิทธิภาพร้อยละ 33-100 ของจำนวนการเต้นของหัวใจต่อจำนวนตัวอย่าง การฉีดเซลล์ต้นกำเนิดจากตัวอ่อนไปในตัวอ่อนระยะ บลาสโตซิสทำให้เซลล์ต้นกำเนิดจากตัวอ่อนสามารถเปลี่ยนแปลงไปเป็นเนื้อเยื่อชนิดต่างในหนูเม้าส์โดยมีประสิทธิภาพร้อยละ 75 ได้หนูไครเมราจำนวนสามตัวจากหนูเม้าส์สี่ตัว ผลการทดลองนี้สรุปได้ว่าเซลล์ต้นกำเนิดจากตัวอ่อนมีประสิทธิภาพในการเปลี่ยนแปลงไปเป็นเซลล์ชนิดต่างๆต่างกัน

คำสำคัญ: การเปลี่ยนแปลง เซลล์ต้นกำเนิดจากตัวอ่อน หนูเม้าส์ การกระตุ้นด้วยสารเคมี พลูริโพเท็นซี

[1]*ภาควิชา สูติศาสตร์ เธนุเวชวิทยา และ วิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330*
[2]*BioTalentum Ltd, Godollo, Hungary*
[3]*หน่วยชีววิทยาการเจริญพันธุ์ ภาควิชาสูติศาสตร์-นรีเวชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330*
[4]*ภาควิชาสาขาวิทยาศาสตร์การแพทย์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330*
[5]*Molecular Animal Biotechnology Laboratory, Szent Istvan University, Godollo, Hungary*
***ผู้รับผิดชอบบทความ E-mail:** tmongkol@chula.ac.th*

Introduction

To date, stem cells have been intensively studied in cell replacement therapy for treating several incurable diseases, such as Alzheimer's, Parkinson's and heart diseases. With the creation of embryonic stem (ES) cells, there is resource with potential to differentiate into all tissues of the body. Interestingly, at the present time this technology is a fast-developing area in not only human but also in companion animals (dogs, cats and horses) because many of the diseases such as diabetes and musculoskeletal injuries of companion animals are similar to those found in human (Schneider et al., 2008, 2010). Furthermore, the establishment of companion animal ES cell lines and the availability of those cell lines for *in vitro* differentiation have already been developed and investigated (Guest and Allen, 2007; Wilcox et al., 2009; Gómez et al., 2010). If the therapeutic possibilities in terms of tissue repair in organs with a limited capacity for functional regeneration or for generating tissues for reconstructive surgery in human and companion animals can be exploited, the field of regenerative medicine in human and animal will expand very

rapidly. These properties make pluripotent cells an attractive candidate for the cell replacement therapy of various degenerative diseases that are associated with a loss of functional cells. This pluripotent cell technology thus opens the 'window' of cell replacement therapy for both human and veterinary medicines (Tecirlioglu and Trounson, 2007).

Generally, ES cells are derived from the inner cell mass (ICM) of fertilized embryos. These cells, however, may induce Graft Versus Host Disease (GVHD) after allogenic transplantation (Lerou and Daley, 2005). To solve this problem, the establishment of ES cell lines from patient derived somatic cell nuclear transferred (SCNT) embryos or from female patient's parthenogenetic embryos has recently been developed (Kim et al., 2007) as a potential source of genetically and immunologically "matching" stem cell derivation. The parthenogenetic embryos have the additional advantage of being ethically more acceptable, as such embryos are produced by parthenogenetic activation without fertilization and these embryos cannot develop up to term after transfer into a surrogate female (Strum et al., 1994). We must emphasize that generating human embryos intentionally for research purposes is an ethically debated issue, and in many countries legally unacceptable at the moment. Such research will not be financed by the European Union Framework programme. Technically it is also challenging to generate patient-specific ES cells by SCNT in human as human oocytes needed for this inefficient process are hard to obtain.

Currently, an alternative method known as "induced pluripotent stem (iPS) cells technology" is gaining popularity, which is based on the derivation of pluripotent stem cells by genetic reprogramming of somatic cell types. This was achieved by retroviral transduction of a combination of four transcription factors, Oct4, Sox2, C-*myc* and Klf4 into somatic cells first in mouse (Takahashi et al., 2006) and soon after that in human (Takahashi et al., 2007). Since then the technology has evolved rapidly (Sun and Liu, 2011). However, there are many questions which need to be clarified on the genetic and functional equivalence of these cells compared to human ES cells (Lister et al., 2011), and on the safety evaluation of the iPS technology prior to further applications in human therapy.

Compared to the iPS cells, parthenogenetic ES (pES) cell lines derived from parthenogenetic embryos have several advantages as they are more close to the "natural" pluripotent cell types (Brevini et al., 2008). The derivation efficiency for pES cell lines is much higher than that for human nuclear transfer ES cell lines where it has never been achieved. Thus, the prime method for derivation of major histocompatibility complex (MHC) matched ES cell lines might be the pES cell line based approach (Revazova et al., 2007). Although an ultimate objective of research in ES cells is undoubtedly for use in human therapy, the religious and ethical considerations of using human embryos have restricted the use of human as a potential model for studying and producing ES cells (Snyder et al., 2006). Therefore, animals such as mouse and rat (Iannaccone et al., 1994) have been used as models for the procedures.

To fully harness the pES method, it is necessary to understand their biology in depth and to clarify technological elements. Here we report the successful establishment of four mouse pES cell lines. We also investigated them by karyotyping for normal chromosomal counts, and by *in vitro* and *in vivo* differentiation assays. The variability among the different pES cell lines in mouse was considerably high and this is an ideal model for human and companion animal research. The potential of *in vitro* differentiation of pES cells into cell types of the three germ layers and into cardiomyocytes have been compared, and chimera embryos have also been created.

Materials and Methods

Chemicals: All chemicals were purchased from Sigma (http://www.sigmaaldrich.com) and culture reagents were purchased from Invitrogen (http://www.invitrogen.com) unless otherwise specified.

In vitro cell culture: Cells were cultured at 37°C in a humidified atmosphere containing 5%CO_2. The medium was changed daily for mouse pES cell cultures and every two days during differentiation.

Animals: Male DBA2, female C57BL/6 and outbred ICR mice were purchased from the National Laboratory Animal Centre, Mahidol University which were raised at the laboratory of the Faculty of Veterinary Science, Chulalongkorn University under controlled lighting (14:10 hour light-dark cycle), 20-22°C, and 40-60% of humidity. F1 mice used in our experiment were derived by mating male DBA2 with female C57BL/6 mice. All procedures for animal management, breeding, and surgery followed the standard operation protocols of Chulalongkorn University under ethical consideration. Appropriate management of experimental samples and the quality control of the laboratory facilities and equipment were maintained.

Production of embryos: F1 female mice (8-10 weeks old) were superovulated by 7.5 IU pregnant mare serum gonadotropin (PMSG; FolligonR, Intervet) i.p., followed 48 hours later by 7.5 IU human chorionic gonadotropin (hCG Chorulon, Intervet) i.p.. The cumulus-oocyte complexes (COCs) were recovered from oviducts 15-16 hours post-hCG injection in M2 medium, and cumulus cells were removed by incubation in 150 IU/ml bovine testicular hyaluronidase in M2 medium. After washing three times in M2 medium, matured oocytes (MII) with clear cytoplasm with uniform texture, homogeneous fine granularity, and intact first polar body were selected. Subsequently, the MII oocytes were activated by using 10 mM/mL Sr^{2+} plus 5 µg/ml Cytochalasin B (CB) in Ca^{2+}-free CZB medium for 6 hours (Kishigami et al., 2006). Afterward, oocytes were

considered activated when two pronuclei (2PN) developed. Activated oocytes were cultured for four days in KSOM-AA medium (Speciality Media, Phillipsburg, NJ) at 37.5°C under a humidified atmosphere with 5% CO_2 in air until they developed to expanded or hatched blastocysts.

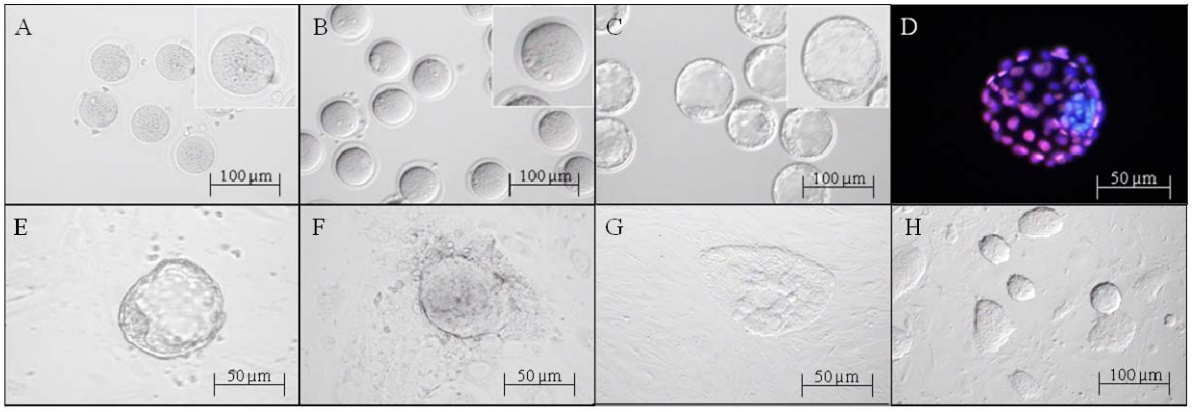

Figure 1 Establishment of mouse pES cells. The procedure of deriving pluripotent stem cells from parthenogenetic activation is shown. A: MII oocytes exhibit an intact first polar body and homogeneously fine granular and light-colored ooplasm. B: Oocytes with 2PN and one polar body. C: Expanded blastocyst with the ICM. D: Differential staining of blastocyst-stage embryos. E: The attachment of a hatched blastocyst to feeder layers. F: ICM-outgrowths; G: ES-like colony. H: ES cell-like cell line (1st passage). Scale bars correspond to 100 µm (20x magnification: A-C and H) and 50 µm (40x magnification: D-G). Abbreviations: pES, parthenogenetic embryonic stem; MII, metaphase II; 2PN, two pronuclei; ICM, inner cell mass; ES, embryonic stem.

Differential staining of blastocysts: Differential staining of the ICM and the trophectoderm (TE) cells was performed as described earlier by Thouas et al. (2001) with a slight modification. Briefly, intact expanded blastocysts (approximately 96 hours after treating with parthenogenetic agent) were incubated with 1% (v/v) Triton X-100, 0.01% polyvinylpyrrolidone (PVP) and 100 µg/ml propidium iodide (Molecular Probes, Invitrogen, Oregon, USA) in PBS for up to 15 sec. Subsequently, the blastocysts were transferred into a fixative solution of 100% ethanol with 25 µg/ml bisbenzimide (Hoechst 33342, Molecular Probes) and maintained in this fixative at room temperature (RT) for 10 min. After that, embryos were mounted onto a glass microscopic slide in a droplet of glycerol and sealed with a coverslip. The embryos were counted for ICM and TE cell numbers using an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan). The ICM was classified as a group of embryonic cells stained with only Hoechst 33342 (blue), whilst TE cells were positive to both Hoechst 33342 and propidium iodide. Hence, trophoblast cells appeared pink, whereas ICM cells appeared blue.

Establishment and culture of pES cells: Intact expanded blastocysts (approximately 96-102 hours after parthenogenetic activation) were cultured on feeder layers of mitomycin C-inactivated confluent mouse embryonic fibroblasts (MEFs) which were obtained from E13.5 (E; the day of embryonic development) mouse embryos as described earlier (Nagy et al., 2003). The blastocysts were observed every 24 hours for hatching and attachment of trophoblasts to a single MEF layer, while ICM size was also monitored. On day 5 or 6 after plating, ICM outgrowths were separated and dissociated from trophoblasts using a glass pipette, followed by trypsinization using 0.25% (w/v) trypsin with EDTA to promote cell dispersion. After re-plating, the ES cell-like colonies were observed, trypsinized and propagated by passaging every 2 to 3 days until freezing or characterization. These cells were maintained in ES medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 20% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT) supplemented with 2,000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon Int., Temecula, CA, USA), 0.1 mM nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME) and 50 U/ml penicillin, 50 µg/ml streptomycin. The medium was changed daily.

Characterizations of pES cells

ES cell morphology: Pluripotent mouse pES cells were maintained in an undifferentiated state on MEFs. The pES cells were examined by phase-contrast light microscopy whether their gross morphology correspond to the typical ES cell morphology with round shape, compact, and shiny colonies.

Alkaline phosphatase (ALP) activity and immunohistochemistry (IHC): The pES cells and embryoid bodies (EBs: as described below) were cultured on 1% gelatin-coated coverslips and were fixed with 4% PFA in PBS for 15 min. ALP expression was determined using the standard methodology. Samples were permeabilized with 0.25% Triton-X100 for 10 min. Blocking was performed in 1% bovine serum albumin (BSA), after which samples were incubated with a primary antibody overnight at 4°C. Primary antibodies for pES cells include goat polyclonal against Oct4 (sc9081, diluted 1:100; Santa Cruz Biotechnology, Germany), rabbit polyclonal Nanog (sc 33760, diluted 1:100 dilution, Santa Cruz Biotechnology, Germany) and mouse monoclonal against stage-specific embryonic antigen 1 (SSEA-1, MC-480, diluted 1:200 dilution; Abcam, UK) and

primary antibodies for cardiac differentiation include mouse monoclonal against cardiac troponin T (cTnT, ab33589, diluted 1:200; Abcam, UK). After extensive washing, EBs were exposed to Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 594 goat anti-rabbit, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG-conjugated secondary antibody (A-11055, A-11012, A-11008 and A11005, respectively, diluted 1:2,000; Invitrogen, USA) at RT for 60 min. The nuclei of cells were counter-stained with 4'-6- Diamidino-2-phenylindole (DAPI) and visualized by fluorescent microscopy.

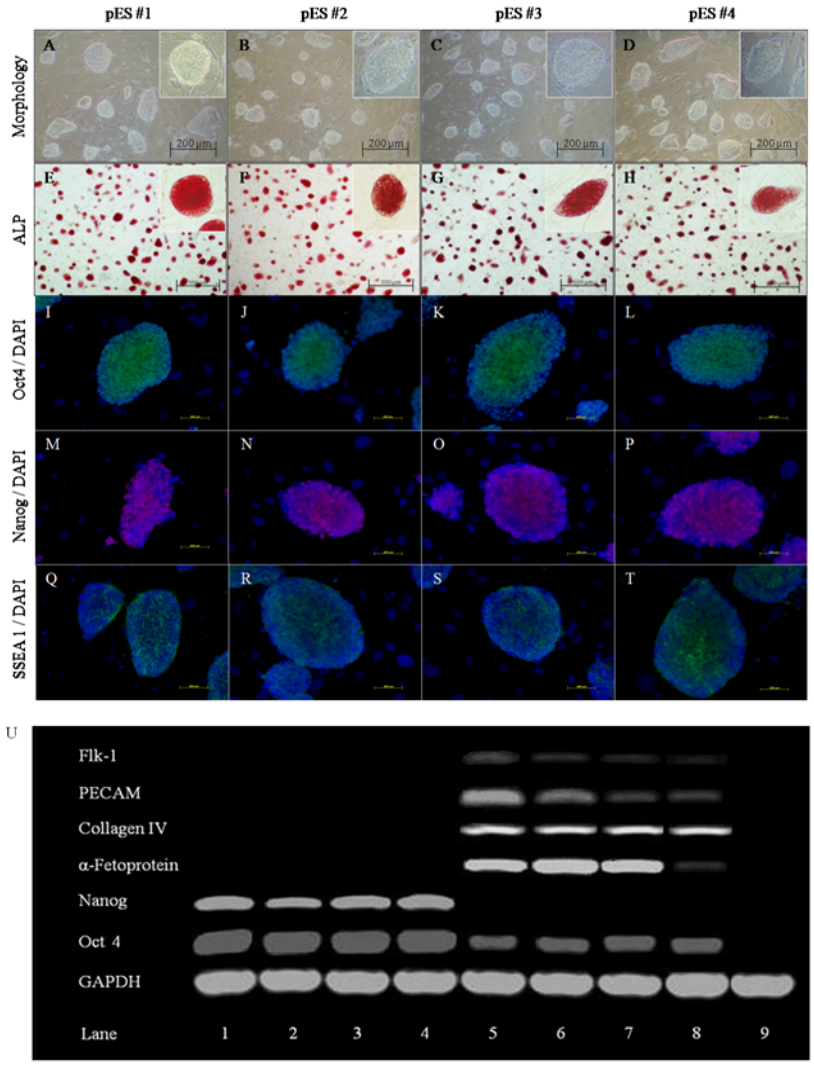

Figure 2 Characterization of mouse pES cell lines (pES #1-4). (A-D) all pES cell lines exhibit a typical gross morphology of mouse ES cells with high nuclear/cytoplasmic ratio and round shape. The cell lines strongly expressed ES cell markers (ALP: E-H, Oct4: I-L, Nanog: M-P and SSEA-1: Q-T counterstaining with or without DAPI). (U) RT-PCR analysis for ES cell marker and differentiation marker gene expression in pES cells; 4 different pES cell lines and their EBs (day 15). ES cell markers: Oct4 and Nanog; endoderm lineage marker: α-fetoprotein and mesoderm lineage markers: Flk-1, PECAM and collagen IV). RT (-) indicates that the reverse transcriptase is omitted in the cDNA synthesis step, and is subjected to PCR in the same manner with primer sets for GAPDH. Lane 1-4: pES #1-4: Lane 5-8: pEBs (day 15) #1-4: Lane 9: feeder layer. Scale bars 200 µm (A-D: 10x magnification and I-T: 20x magnification) and 500 µm (E-H: 4x magnification). Abbreviations: pES, parthenogenetic embryonic stem; DAPI, 4,6'-diamidino-2-phenylindole; pEBs, parthenogenetic embryoid bodies. RT-PCR reactions to detect pluripotent (Oct4 and Nanog) and endo-mesoderm differentiation (entoderm lineage: α-fetoprotein and mesoderm lineage: Flk-1, PECAM and collagen IV).

In vitro differentiation of pES cells into cells of the three germ layers and cardiomyocytes: EBs were prepared by using the hanging drop (HD) method (adapted from Mummery et al., 2007). In brief, pES cells were dissociated with a 0.05% trypsin-EDTA solution to get a single cell suspension and seeded as $4x10^5$ cells/ml (resulting in 8,000 cells/drop) suspension in differentiation medium (ES medium without LIF). Three days later, EBs were transferred to a 100-mm bacteriological Petri-dish in 10 ml of differentiation medium. For differentiation into cell types of the three germ layers, EBs were continuously cultured in suspension for 15 days. EBs were collected, washed 3 times with PBS, fixed overnight in 10% neutral-buffered formalin, dehydrated in a series of alcohol gradients (70-100%), embedded in paraffin and examined for general histological analysis. Sections of 5 µm were stained with hematoxylin and eosin (H&E) for visually assessing morphology. For

spontaneous cardiac differentiation, individual EBs on the 5th day were placed in gelatin coated 24-well plates for an additional 15 days and observed daily under a phase-contrast microscope. Twenty-four EBs were counted for each group and the rate of beating EBs was evaluated as a percentage of the total number of EBs plated. At the end point of culturing (twenty-day EBs) EBs were fixed, permeabilized and blocked as described above for immunohistochemistry.

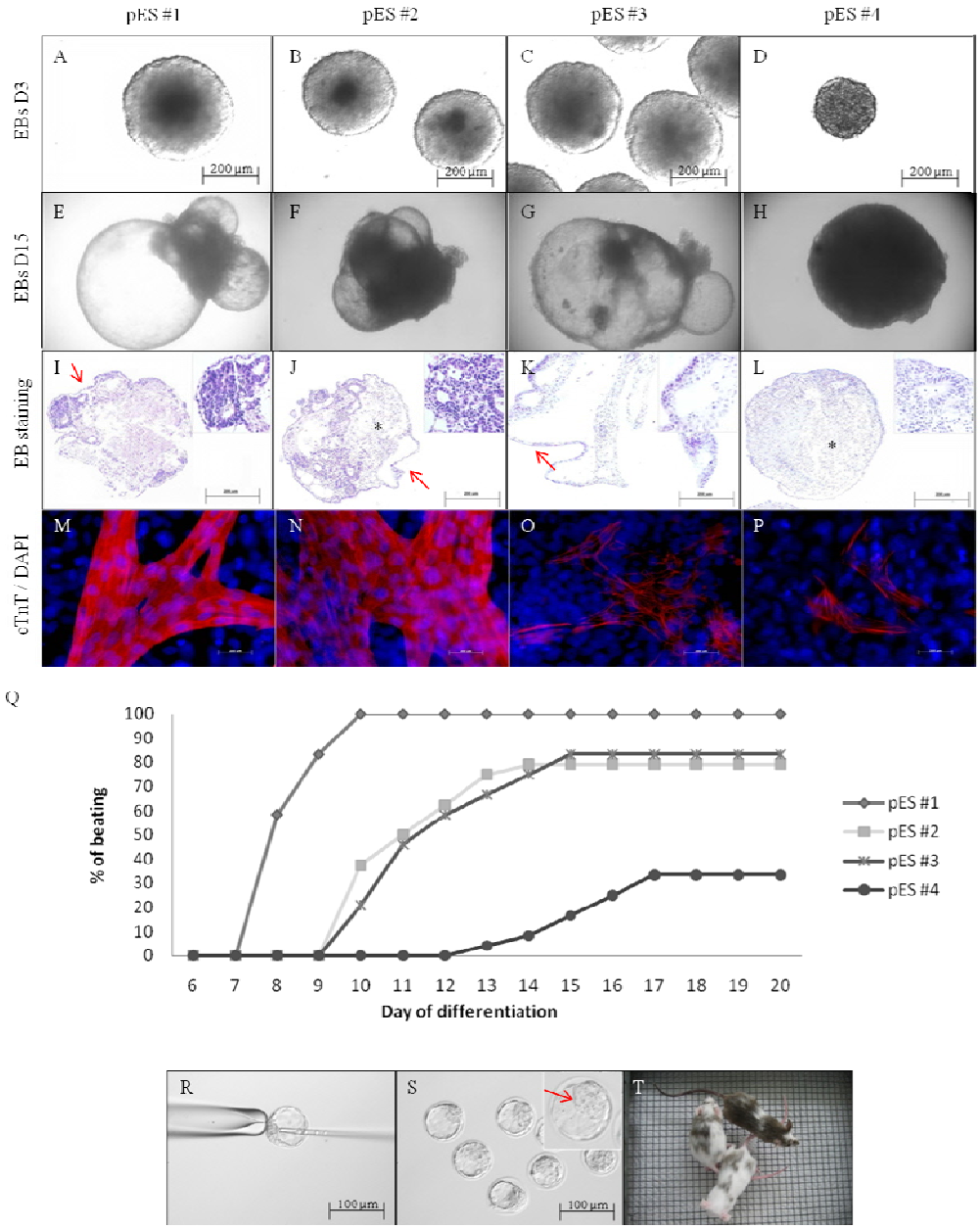

Figure 3 Comparison of *in vitro* and *in vivo* differentiation of mouse pES cells into cell types of the 3 germ layers. (A-D) Day 3 EB culture: gross morphology of simple EBs. (E-G) Day 15 EB culture: gross morphology of typical cystic EBs with visceral yolk-sac-like structures (pES#1-3). (H) EBs Day 15 EB culture: gross morphology of EBs without cavitation structure (pES#4)(I-L). Histological analysis by hematoxylin and eosin (H&E) staining of EBs cultured on day 15. Note that I-J and L insert: neural rosette formations (ectoderm lineage); J and L (*): connective tissue (mesoderm lineage) and I-K (red arrow): simple squamous epithelium in the periphery and epithelial cells surrounding a lumen in the center, resemble the yolk sac structure (endoderm lineage). (M-P) Detection of typical cardiomyocyte proteins differentiation cultures by immunofluorescence staining on day 21 of differentiation. Cells are stained with cTnT (red) and nuclei are stained with DAPI (blue). (Q) Percentage of contracting EBs formed from 4 different 4 pES cell lines (R) Blastocyst injection is carried out by injecting ES cells into an ICR blastocyst under microscope attached with micromanipulator. (S) Chimeric blastocysts obtained by blastocyst injection of pES#1. (T) Chimeric offspring from pES #1 cells (mouse with gray coat).

In vivo differentiation of pES cells into chimeric offsprings: The ability of pES cells to colonize the germline of a host embryo was tested by injection of pES cells after the 5th passage into host blastocysts and implantation of these chimeric embryos into pseudopregnant foster mothers as described by Nagy et al. (2003). Briefly, blastocyst injection was carried out using day 3.5 blastocysts collected from the uteri

of superovulated ICR females by flushing with M2 medium. The collected blastocysts were washed and cultivated at 37°C in KSOM-AA medium under humidified 5% CO_2 in air before being used as recipient blastocysts. The pES cells were dissociated with a 0.05% trypsin-EDTA solution and were resuspended in ES medium. Blastocyst injection was carried out by injecting five pES cells into an ICR blastocyst under microscope equipped with a Narishige -micromanipulator system. After injection, 7-8 chimeric blastocysts were transferred into both uterine horns of a day 2.5 pseudopregnant ICR female mice, which had previously been mated with vasectomized males. The coat-color difference of the B6D2 pES cell line genetics (dark) in the ICR blastocysts (white) allowed an estimation of chimerism rate (i.e., contribution of ES cell genome to chimeric offspring). Germline transmission of pES cell genome was then tested by crossing high-rate chimeras with ICR mice.

Karyotype analysis: Chromosomal numbers of established pES cell lines were performed either every 5th passage of subcultures or before chimera production as described by Nagy et al. (2003) with minor modifications. Briefly, mouse pES cell lines were treated with colcemid in ES medium for 1.5 hours at 37°C in an atmosphere of 5% CO_2 in air. Then, the treated pES cell lines were harvested and resuspended for 15 min in 0.075 M KCl at 37°C. The pES cells were placed in hypotonic solution and subsequently fixed in 3:1 mixture of methanol and acetic acid. Chromosome spreads were performed by dropping cell suspension onto slides and stained by the trypsin-Giemsa banding technique. At least 50 metaphases from each cell line were examined in order to count their chromosome numbers.

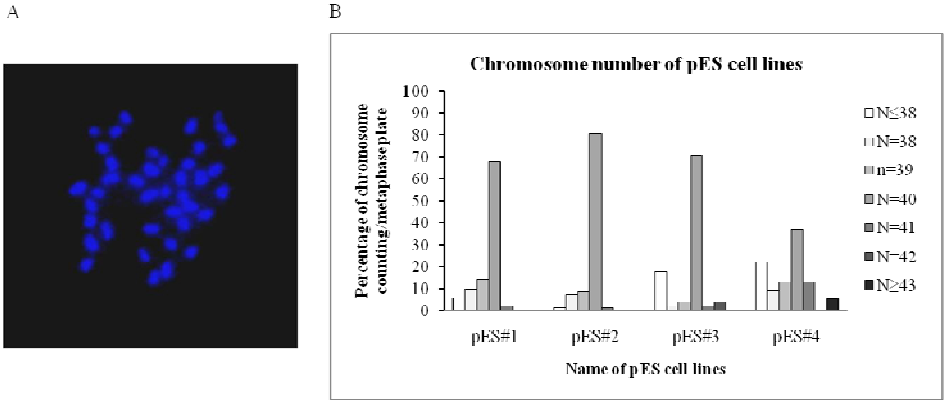

Figure 4 Karyotyping of pES cells. (A) Chromosome complements observed by fluorescent microscopy stained with DAPI. (B) The ploidy ratios of the pES cell lines, pES#1-3 cells exhibit a mostly (above 60%) normal 40 XX chromosomes ploidy after 10 passages, however pES#4 cells show higher ratio of cells with aneuploid chromosomes. Abbreviations: DAPI, 4, 6'-diamidino-2-phenylindole.

Reverse Transcription (RT)-PCR analysis of pluripotent and differentiation expression: RT-PCR reactions to detect pluripotent (Oct4 and Nanog) and endo-mesoderm differentiation (endoderm lineage: α-fetoprotein and mesoderm lineage: Flk-1, PECAM and collagen IV) transcripts were performed according to Kishigami et al. (2006) with slight changes in the cDNA preparation and the use of Taq DNA polymerase. Total RNA from pES cells, EBs and fibroblast cells was prepared using the RNeasy Protect Mini Kit (Qiagen) and was reversely transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Ex Taq Hot Start Version (Takara, Shiga, Japan) was used in the PCR reaction (Table 1).

Table 1. Properties of the oligonucleotide primers used in RT-PCR reaction

Primer	Size (bp)	Annealing (˚C)	Sequence	
Oct4	293	58	(Forward)	5'-GGCGTTCTCTTTGGAAAGGTGTTC-3'
			(Reverse)	5'-CTCGAACCACATCCTTCTCT-3'
Nanog	449	58	(Forward)	5'- TGAGAT–GCTCTGCACAGAGG-3'
			(Reverse)	5'-CAGATGCGTTCACCAGATAG -3'
Flk-1	599	58	(Forward)	5'-CCTGGTCAAACAGCTCATCA-3'
			(Reverse)	5'-AAGCGTCTGCCTCAATCACT-3'
PECAM	588	58	(Forward)	5'-AGACTTTAACCAAGGGCGGT-3'
			(Reverse)	5'-TAGCCAGGTTGCGAAGAACT-3'
Collagen IV	463	58	(Forward)	5'-CAAGCATAGTGGTCCGAGTC-3'
			(Reverse)	5'-AGGCAGGTCAAGTTCTAGCG-3'
α-Fetoprotein	494	58	(Forward)	5'AGTGCGTGACGGAGAAGAAT-3'
			(Reverse)	5'-TGTCTGGAAGCACTCCTCCT-3'
GAPDH	27	58	(Forward)	5'-ACCTCAACTACATGGTCTAC-3'
			(Reverse)	5'-TTGTCATTGAGAGCAATGCC -3'

Experimental design: After the establishment of pES cell, the new pES cell lines were characterized by morphology of ES cells, chromosome number analysis, IHC for pluripotency markers, and pluripotency *in vitro* by differentiation into the 3 germ layers (via EB formation and cardiomyocytes then IHC and RT-PCR) and pluripotency *in vivo* by chimera progeny production (see materials and methods). Three individual replicates of each test were performed and evaluated.

Statistical analyses: Data are expressed as mean ± SEM of at least three independent experiments.

Results

Parthenogenetic activation: A total of 130 oocytes were collected (Fig. 1A) and activated parthenogenetically as described above. 91.5±6.06% was activated successfully based on the presence of 2PN and one polar body (Fig. 1B). 87.7±4.02 and 83.9±5.52 of activated oocytes developed into 2-cell stage embryos and blastocytes, respectively (Fig. 1C). In this experiment, we found that the total cell number, ICM cell number and ICM: trophectoderm ratios of parthenogenetic blastocysts were 66.8±2.88, 8.1±0.63 and 1:7.24, respectively (Fig. 1D).

Derivation of pES cell lines: Pictures of the blastocysts derived from parthenogenetic activation and plated onto mitomycin-inactivated MEFs (Fig. 1C); the hatched blastocysts attached to MEFs (Fig. 1E); and the primary ES cell-like colonies 4 to 5 days after trypsinization (Fig. 1G) are presented. Four pES cell lines were successfully derived from 18 parthenogenetic blastocysts (22% efficiency, Fig. 1H).

The effect of different pES cell lines on morphology and proliferation: Four pES cell lines were maintained in culture until passage 20. These cell lines displayed typical morphology of mouse ES cells, including round shape colonies, a high nuclear:cytoplasmic ratio and exhibited vigorous growth rate during long-term culture and were usually passaged every 2 days during culture (Fig. 2A-D; pES#1-4). The morphology and proliferation rates were very similar among the 4 pES cell lines and all of them remained pluripotent following long term expansion.

Detection of pluripotency markers in different pES cell lines: Subsequently, we examined if pES cells following long term expansion retain the expression of pluripotency markers. Expression of pluripotency markers was constant during the culture based on ALP activity, IHC (Oct4, Nanog and SSEA-1) and RT-PCR (Oct4 and Nanog) examinations. Four pES cells were cultured on MEF forming colonies morphologically similar to that of undifferentiated ES cells and were strongly positive for ALP, Oct4, Nanog and SSEA-1 by IHC (Fig. 2A-T) and confirmed by RT-PCR analysis. Our results showed that all pES cell lines expressed both pluripotency markers (Oct4 and Nanog) and no differences in gene expressions were observed among the pES cell lines (Fig. 2U). The expression of differentiation-related genes (α-fetoprotein, Flk-1, PECAM and collagen IV) tested was not detected in the pES cell lines, thus confirming the undifferentiated state (Fig. 2U). Our findings showed that different pES cell lines were able to maintain in long term culture without differentiation and expression (or lack of it) of all pluripotency, and differentiation markers examined was not different among the cell lines.

In vitro differentiation of pES cell lines into various cell types: Culturing pES cells in suspension culture without LIF by using the HD method, these cells aggregated, spontaneously differentiated and formed EBs. After day 3 of culture, a distinctive outer layer of endodermal cells appeared in each droplets containing a spherical EB. In this study, we determined the efficiency of EB formation in HD culture by measuring a percentage of single EB produced from one drop. Our results showed that pES#4 exhibited lower efficiency of EB formation (pES#4; 35% of EB formed versus pES#1-3 where 100% of EB formed) and also produced smaller EBs compared to the other pES cell lines (Fig. 3A-D). Subsequent culture of EBs resulted in EBs displaying foci of pulsatile contractions typical of cardiomyocyte differentiation on day 9 in suspension culture and stopped beating on day 12 only in pES#1-2. There was no beating EBs in pES#3-4. PES#1 formed EBs early which became cystic structures by day 12 compared to pES#2-3 which formed cystic structures 1 day later (day 13) (Fig. 3E-G). However, pES#4 did not form cystic EBs at all (Fig. 3H). This study also examined the generation of tissues in the differentiating EBs by histology. Overall the pES cell lines were able to differentiate into cell types of all 3 germ layers containing neural rosette formations (ectoderm lineage, Fig. 3I-J and 3L, insert), connective tissue (mesoderm lineage, Fig. 3J-L, *) and simple squamous epithelium in the periphery and epithelial cells surrounding a lumen in the center, similar to yolk sac structures (endoderm lineage, Fig. 3K, red arrow). However, there were differences in the proportion of the cell types formed among the different cell lines. For example pES#4 formed EBs with low proportion of cells of endodermal lineage and differentiated mainly into neural lineage direction (Fig. 3L).

Furthermore, we also evaluated endo-mesoderm differentiation in EBs on day 15 by the expression of α-fetoprotein which was initially expressed in the primitive endoderm during early post-implantation stages and was maintained in the visceral and parietal endoderm of the yolk sac during gastrulation. Interestingly, the expression of α-fetoprotein was strongly expressed in pES#1-3 but weakly in pES#4 EBs. Expressions of the vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1), platelet endothelial cellular adhesion molecule (PECAM) and collagen IV, all referred as mesoderm specific, were differentially regulated as well. Flk-1 expression, marker of progenitors with vascular potential, was low in all four lines. PECAM, a member of immunoglobulin superfamily expressed by endothelial cells and a subset of hematopoietic cells, was strongly expressed in pES#1-2 EBs and at a much lower level in pES#3-4. The level of collagen IV expression, a cartilage component, was high is all 4 lines. The expression of pluripotency markers was not

different among cell lines: Oct4 gradually decreased during differentiation with a still detectable expression level and Nanog expression almost entirely disappeared in EBs on day 15 (Fig. 2U). These results strongly suggest that although all 4 pES cell lines were capable of differentiation into cell types of the 3 germ layers, major variation exists among different pES cell lines in their quantitative and qualitative capacity of differentiation by the EB-method.

In vitro differentiation of pES cell lines into cardiomyocytes: Spontaneous cardiomyocyte differentiation was observed in EBs which were plated from day 5 onto gelatin coated dishes and cultured until day 20 significantly varied among the cell lines. The efficiency of forming contracting EBs after 20 days varied from 33% to 100% of the EBs having contracting regions. Spontaneously beating EBs started to appear at day 8 (pES#1), day 10 (pES#2-3) and day 13 (pES#4). PES#1 cells showed the greatest efficiency, reaching a maximum of 100% of EBs, in contrast to the pES#2, 3 and 4, which reached 79%, 83% and 33%, respectively (Fig. 3Q). All contracting EBs kept continuously beating during the long term culture. The above results confirmed that there was not only observable difference in the time course for the development of contraction but also the efficiency of forming contracting EBs varied for the different pES cell lines.

We have evaluated pES cell-derived cardiomyocytes by IHC for cTnT, which is a highly cardiac-specific myofilament protein (Fig. 3M-P). PES#1-2 formed an extensive and well-organized cTnT network and showed higher amounts of cTnT positive areas than that in pES#3-4 cell-derived cultures. Although, pES#2-3 gave a similar ratio of beating EBs, the level of organisation of the cTnT network and the positive areas were different. These results suggested that besides the observable differences in the ratio of cardiac beating, it is important to examine the organization of sarcomeric structures which might reveal important differences among the pES cell-derived cardiomyocytes.

In vivo developmental potential of pES cell line-derived chimeric embryos: Finally, we chose one pES cell line (pES#1) based on its superior potential of differentiation *in vitro* (EB formation and cardiac differentiation) to test the pluripotency of pES cell lines for *in vivo* differentiation. Figure 3R displays chimeric blastocysts produced by injecting the B6D2 pES cell into ICR blastocysts. Our result demonstrated that the pES cells were incorporated in the recipient ICM (Fig. 3S: insert box with red arrow) and developed further. Chimeric mice with a donor genotype of dark, brown and gray coat color were obtained from the pES cells with an efficiency of 75% (3 chimeric pups from 4 newborns) (Fig. 3T). Unfortunately, only male chimeras have been obtained and they did not give germ-line chimeras. Our result demonstrated that pES cell line could differentiate *in vivo* by contributing to the body formation of the progeny.

Ploidy analyses of the pES cell lines: The 4 pES cell lines were analyzed every 5 passages during long term culture with at least 50 metaphases from each cell line examined for their chromosome numbers (Fig. 4A). Our results showed that in 3 pES cell lines the majority of the cells (above 60-70%) possessed a normal diploid chromosomal set of 40 (68, 81 and 71% of the cells in pES#1, 2, and 3, respectively), but in pES#4 the majority (63%) of the cells were aneuploid (Fig. 4B).

Discussion

Establishment of pluripotent stem cells derived from somatic cells via SCNT or iPS cells represents an important model for biomedical research and will provide unlimited resources for cell replacement therapies. On the other hand, the overall efficiency of the SCNT is still very low and has not been achieved in human, and the equivalency and safety issues of iPS cells remain a major concern including activation of potential oncogenes (Hao et al., 2009). Pluripotent stem cells generated from parthenogenetic blastocysts are good candidates as a source of histocompatible cells for transplantation (Drukker, 2008). Parthenogenetic ES cells have a relatively high efficiency for cell line establishment (around 14%) and the procedures are relatively simple compared to SCNT (Müller and Lengerke, 2009) in human. The pES cells can provide patients with cells for allogeneic (i.e., between genetically unrelated individuals) and even autologous (i.e., within the same individual) transplantation following MHC compatibility profiling and cell banking (De Sousa and Wilmut, 2007). Studies on human pES cells have revealed that these cells are very similar to the human ES cells derived from either *in vitro* fertilized or *in vivo* produced blastocysts in their gene expressions and other characteristics (Hao et al., 2009). However, the full differentiation and development potential of these human pES cells have to be further investigated before clinical research and therapeutic interventions.

In this study, four mouse pES cell lines (pES#1-4) were established from parthenogenetically activated blastocysts. Subsequently, we have evaluated the differences among individual pES cell lines in mouse as a model for human research. Our result demonstrated that the profile for undifferentiated state of the pES cell lines matched that of other undifferentiated mouse pES cell colonies in previous studies (Shao et al., 2007; Lee et al., 2008). There were no differences detected among the 4 pES cell lines regarding typical mouse ES morphology, the strongly positive ALP staining, expression of transcription factors (Oct4 and Nanog), and cell surface antigens (SSEA-1). Our results are in accordance with a study on 59 human ES cell lines from 17 laboratories worldwide where in spite of diverse genotypes and different techniques used for establishment and maintenance, all ES cell lines exhibited indistinguishable expression patterns for several markers of human ES cells. However, those cell lines were not identical as differences in expression of several lineage markers were evident (Adewumi et al., 2007). Additionally, different gene

expression profiles were contributed to different genetic backgrounds of mouse ES cell lines and not the methods (SCNT versus *in vivo* fertilization) used to produce those (Brambrink et al., 2006). In contrast, more detailed microarray analyses can reveal differences among long-term cultured mouse ES cell lines with essentially the same genetic background (Mamo et al,. 2010).

Even though the formation of EBs in a suspension culture has been the most popular method to differentiate ES cells derived from cloned and fertilized blastocysts into a wide range of cells (Kurosawa et al., 2007), not much is known about the characteristics of EBs derived from pES cells. In this study, we compared the EB formation in suspension culture among the 4 pES cell lines. Three of the pES cell lines (pES#1-3) showed higher ratio and size of EBs and the presence of cystic EBs formed compared to that in pES#4. This demonstrates that to some extent the simple test of EB formation is already a useful tool to compare the quality of cell lines, as the low-performance cell line was also identified as mostly aneuploid (see below).

Cardiomyocyte differentiation potential, by forming beating EBs in suspension, revealed that only pES#1-2 produced such EBs, despite that all pES cell lines were able to differentiate into cell types of all 3 germ layers, containing neural rosette formations (ectoderm lineage), connective tissue (mesoderm lineage) and simple squamous epithelium in the periphery and epithelial cells surrounding a lumen in the center, similar to yolk sac structures (endoderm lineage). These later results were similar to that observed with fertilized ES cell lines and EB formation in other studies (Mogi et al., 2009).

Although the functional potential of pES cells to differentiate into ectoderm lineages including dopaminergic and serotonergic neurons were well known and characterized (Sanchez-Pernaute et al., 2009), there have been little reporting regarding the differentiation towards endo-mesodermal lineages. When we investigated the potential of endo-mesoderm differentiation in EBs the results suggested that 3 cell lines (pES#1-3) were able to differentiate into endoderm lineages and form cystic EBs with a simple squamous epithelium surrounding a lumen in the center by histological analysis and strongly expressing α-fetoprotein. However, pES#4 demonstrated low efficiency of endoderm differentiation without forming cystic EBa and a weak expression of α-fetoprotein. The good results with pES#1-3 endoderm differentiation were an interesting as in contrast in parthenogenetic embryos differentiation and proliferation of trophectoderm and primitive endoderm were abnormal (Strum et al., 1994). Our pES cell lines were also capable of differentiation into mesoderm lineages, formed connective tissues in EBs and expressed Flk-1, PECAM and collagen IV which are similar to the patterns expected in fertilized ES cells (Choi et al., 2005). Our results suggested that the lack of paternal imprinting on certain genes (Igf2) did not affect mesoderm differentiation in this case, in contrast with a previous report (Morali et al., 2000).

Recently, pES cell lines demonstrated low efficiency of myogenic differentiation: exclusion of pES cells from the myogenic lineage *in vivo* (Clarke et al., 1988) or delays of myogenin expression *in vitro* (McKarney et al., 1997) because of the impairment in expression of the paternal allele expressed gene Igf2. In that regard, Igf2 is a strong promoting factor of myogenesis thus loss of Igf2 significantly decreased the proliferative capacity of the myoblast population. However, there was no impairment in cardiogenesis *in vivo* (Allen et al., 1994). Most interestingly, our results showed a significant variability in the efficiency of cardiac differentiation among mouse pES cell lines. The efficiency cardiac beating/well derived from pES#1-3 cell lines were 80-100%, however, a well-organized cTnT network and more positive areas for cTnT were found in pES#1-2 cell lines but not in pES#3. This suggested that most of our pES cell lines were able to differentiate into cardiomyocytes similarly as reported (Koh et al., 2009) and the presence of only maternal genomes did not interfere with stem cell function and differentiation (Onodera et al., 2010). Our results showed that differences exist among the individual cell lines for their "favored pathways" and capacity of spontaneous differentiation, which has also been observed in human ES cell lines.

It was demonstrated that prolonged passage in culture decreased the potential for germline transmission in mouse ES cell lines (Nagy et al., 2003). Euploid ES cell lines cultured *in vitro* for more than 20th passages become mostly aneuploid and the ability of these lines to contribute to the germ line was mostly lost when the proportion of euploid cells dropped under 50% (Longo et al., 1997). Abnormal chromosome numbers might result in lower efficiency of *in vitro* differentiation, as well. The karyotyping of our pES cell lines revealed that pES#4 cells were mostly abnormal which possibly resulted in low efficiency of *in vitro* differentiation, especially into cardiomyocytes. However, it is encouraging that 2/3 of our pES cell lines maintained a mostly euploid karyotype during long-term culture demonstrating a good genetic stability of pES cell lines.

Finally, one of the pES cell lines was tested positively to differentiate *in vivo* into chimeric mice. This is encouraging, despite the lack of germline transmission demonstrated. After all, this later feature would not be important for human medical application of the cells. Our findings were in harmony with an earlier study on the poor potential of pES cells in chimera studies, which was improved by using a nuclear transfer step (Hikichi et al., 2007). Another report suggested that germline-competent pES cell lines could be produced by using *in vitro* maturation of immature oocytes from adult mouse ovaries and activation instead of using *in vivo* matured MII oocytes (Liu et al., 2011).

Table 2. Comparative mouse ES cells characteristics among the pES cell lines studied

ES cell lines	Pluripotent of ES cells				*In vitro* differentiation into cell types of the 3 germ layers							*In vivo* differentiation (Chimera production)	Normal chromosome number (40xx: %)
	ES cell markers				EB formation		Differentiation			Cardiac differentiation			
	ALP	Oct4	Nanog	SSEA1	Simple EBs	Cystic EBs	Ectoderm	Mesoderm	Endoderm	% of beating	cTnT		
pES#1	++	++	++	++	++	++	++	++	++	100	++	+	68
pES#2	++	++	++	++	++	++	++	++	++	79	++	not tested	81
pES#3	++	++	++	++	++	++	-	+	++	83	+	not tested	71
pES#4	++	++	++	++	+	-	+	+	+	33	+	not tested	37

Conclusion

In conclusion, according to our results among the 4 pES cell lines studied all showed similar pluripotency characteristics compared to that of ES cells. The pES cell lines were able to differentiate *in vitro* into cell types of all 3 germ layers, especially into cardiomyocytes; however, differences in the differentiation process outcomes were revealed (table 2). The reason for this variability is not fully understood and may be related to subtle differences in epigenetic state or chromosomal abnormalities presented in a proportion of the cells. Nevertheless, one of the pES cell lines was tested successfully to contribute to *in vivo* chimeric development of pups, generating medium to strong coat-color chimeras. The establishment of pluripotent pES cell lines derived from parthenogenetic embryos demonstrated the feasibility of this approach and its potential to generate patient-specific cell lines. In the mean time the differences observed among pES cell lines make it clear that further basic research and continuous quality control is needed prior to any practical applications in medicine.

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