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Isolation and Characterization of Parthenogenetic Embryonic Stem (pES) Cells Containing Genetic Background of the Kunming Mouse Strain*

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ABSTRACT : Parthenogenetic embryonic stem (pES) cells could provide a valuable model for research into genomic imprinting and X-linked diseases. In this study, pES cell lines were established from oocytes of hybrid offspring of Kunming and 129/Sv mice, and pluripotency of pES cells was evaluated. The pES cells maintained in the undifferentiated state for more than 50 passages had normal karyotypes with XX sex chromosomes and exhibited high activities of alkaline phosphatase (AKP) and telomerase. Meanwhile, these cells expressed ES cell molecular markers SSEA-1, Oct-4, Nanog, and GDF3 but not SSEA-3 detected by immunohistochemistry and RT-PCR. The pES cells could be differentiated into various types of cells from three germ layers *in vitro* by analysis of embryoid bodies (EBs) with immunohistochemistry and RT-PCR, and *in vivo* by observation of pES cell-derived teratoma sections. Therefore, the established pES cell lines contained all features of mouse ES cells. This work provides a new strategy for isolating pES cells from Kunming mice, and the pES cell lines could be applied as the cell model in research into genomic imprinting and epigenetic regulation of Kunming mice. (**Key Words :** Mouse, Parthenogenetical Activation, Parthenogenetic Embryonic Stem (pES) Cells, Kunming Mouse (*Mus musculus* Km), Genetic Background)

INTRODUCTION

Embryonic stem (ES) cells, which are pluripotent stem cells derived from inner cell mass (ICM) of pre-implantation embryos, are capable of proliferating permanently in the undifferentiated state and differentiating into various types of cells from three germ layers *in vitro* or *in vivo* (Evans and Kaufman, 1981; Thomson et al., 1998). ES cells could not only provide the unique cell model for many issues of developmental biology and human diseases, but also provide promising sources for cell transplantation and gene therapy (Drukker, 2008). Immune rejection, resulting from expression of the major histocompatibility complex (MHC) genes in ES cell-derived cells, has become one of the main obstacles to application of ES cells in clinical therapy. Parthenogenetically activated oocytes could easily develop to blastocysts which only contain a duplication of maternal genome. ES cells derived from parthenogenetic embryos (pES cells) are either uniformly homozygous or include minimal crossover-associated heterozygosity. So there exists a greater likelihood of obtaining a match between the pES cell derivative and the recipient than that from fertilized embryos. Kim et al. (2007) demonstrated that selected pES cells could serve as a source of histocompatible tissues. Therefore, it would be a feasible alternative to establish pES cell lines for autologous transplantation in females, instead of the ES cell lines from embryos created by somatic cell nuclear transfer with limitations of low success and ethical controversies. Moreover, pES cells could provide a cell model for gene imprinting and X-linked diseases (Allen et al., 1994; Cibelli

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et al., 2002; Jiang et al., 2007). In recent decades, many pES cell lines had been continuously established from oocytes of monkeys (Cibelli et al., 2002), rabbits (Fang et al., 2006), rats (Sritanaudomchai et al., 2007) and human (Lin et al., 2007; Mai et al., 2007; Revazova et al., 2007).

Kaufman et al. (1983) first established pES cell lines from oocytes of 129/SvE female mice and hybrid female mice (C57BL×CBA), and evaluated pluripotency of the pES cells. Other researchers demonstrated that mouse pES cells could undergo extensive differentiation *in vitro* (Lin et al., 2003), and contribute to germline chimeras (Jiang et al., 2007). However, there were some discrepancies regarding capacities of proliferation and differentiation of mouse pES cells (Jagerbauer et al., 1992; Newman-Smith and Werb, 1995; Park et al., 1998). Up to now, most mouse pES cell lines were derived from oocytes of the 129 mice or other hybrid mice. The Kunming mouse strain (*Mus musculus* Km) is one of the house-breeding albino mouse strains widely used as the laboratory animal in China. This mouse strain exhibits significant advantages over such inbred strains as 129, C57BL/J, BALB/c, DBA and CBA in respect of environmental resistance, reproduction and breeding, etc. Attempts had been made to isolate ES cells from inbred Kunming mice but few successes were achieved, and especially there are no reports regarding establishment of pES cell lines from them. Kunming mice were generally believed to be unsuitable for isolation of ES cells. 129 mice are acknowledged to be the most conducive for isolation of ES cells among many mouse strains, and ES cells could be easily isolated from hybrid embryos containing genetic background of 129 mice (Kress et al., 1998; Brook et al., 2003). Therefore, we hypothesized that oocytes of hybrid offspring of Kunming and 129 mice would acquire higher developmental competence after parthenogenetic activation, increasing the chances of isolating the pES cells. Here, we acquired pES cell lines derived from hybrid offspring of Kunming and 129/Sv mice, and these cells maintained the undifferentiated state *in vitro* for a long term and could differentiate into various types of cells from three germ layers *in vitro* or *in vivo*.

MATERIAL AND METHODS

Collection, parthenogenetic activation and development *in vitro* of mouse MII oocytes

Oocytes of hybrid female offspring produced by Kunming female mice (*Mus musculus* Km) (the Laboratory Animal Centre, the Fourth Military-Medicine University in R. P. China) and 129/Sv male mice (the Laboratory Animal Centre, Peking University) were used to isolate pES cells. Collection, parthenogenetic activation and development *in vitro* of mouse MII oocytes were performed as described previously (Nagy et al., 2003). Briefly, the cumulus-oocyte complexes (COCs) were collected from oviducts of female mice 15-17 hours after injection of human chorionic gonadotropin (hCG). The cumulus cells surrounding oocytes were removed by hyaluronidase (Sigma) (1 mg/ml). Then, oocytes were activated with Ionomycin Calcium (Sigma) (2.5 μg/ml) followed by 6-DMAP (Sigma) (2.5 mM) and cultured with droplets of KSOM medium (Chemicon) in a CO_2 incubator (Thermo, Forma 311) at 37.5°C for 4-4.5 days. The blastocysts were harvested to isolate the pES cells.

Isolation and maintenance of the pES cells

The harvested blastocysts were cultured on inactivated mouse embryonic fibroblasts (MEF) with mitomycin C (Sigma) (10 μg/ml). ES cell medium was composed of Knockout™ high glucose DMEM (Invitrogen) supplemented with 11.25% KSR (vol/vol) (Invitrogen), 3.75% FBS (vol/vol) (Hyclone), 20 ng/ml leukemia inhibitory factor (LIF) (Chemicon), 1mM nonessential amino acids (NEAA) (Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma). Within 4-6 days, embryos attached to the feeder layers and formed ICM outgrowths with prominent nucleoli and dense morphology. Subsequently, ICM outgrowths were mechanically dissected into small clumps after treatment with trypsin (Invitrogen) (0.5 mg/ml)/EDTA (Sigma) (0.4 mg/ml) solution for 2-3 min, and individually plated on the MEF feeder layers. The fresh ES cell medium was fed every 2-3 days, and the ES cell-like colonies usually appeared within 3-7 days. At the initial stage, the pES cells were subcultured by trypsinization in combination with mechanical dissection. When a large number of ES cell colonies appeared in the plates with 3.5 cm diameter, pES cell lines were affirmed to be established. The putative pES cells were subcultured or frozen every 60-72 h.

Karyotype analysis of of the pES cells

Karyotype analysis of pES cells was performed as described previously (Nagy et al., 2003). Briefly, after culture in feeder-free conditions for 48 h, pES cells were exposed to colchicine (Sigma) (10 μg/ml) for 1-2 h. Subsequently, these cells were trypsinized into a single-cell suspension followed by suspension for 15 min in 0.075 mM KCl solution. Cells were repeatedly fixed in methanol/ acetic acid mixture, then spread over slides and stained with Giemsa (Sigma) solution. The karyotypes were observed and imaged under a Digital Microscopic Imaging System (Leica, DMIRB).

Analysis of antigens and genes of the pES cells

After culture for 48 h, pES cells were fixed with 4% formaldehyde and stained by alkaline phosphatase (AKP) or immunohistochemical methods. AKP staining was carried

out as described previously (Nagy et al., 2003), and the AKP-positive cells were stained as red or brown-red. Immunohistochemical staining was performed according to instructions of the SP-9000 General Immunohistochemical Kit (Zhongshan Jinqiao Co. Ltd). Briefly, cells were blocked with 10% goat serum plus 0.2% Triton X-100, then incubated at 4°C overnight with primary antibody against Oct-4 (1:100), SSEA-1 (1:100), SSEA-3 (1:100), Nanog (1:100) and tolemerase (1:100) (all antibodies purchased from Chemicon Co. Ltd), respectively. Subsequently, cells were exposed to TRITC-conjugated secondary antibodies (goat anti-mouse IgG) and dyed as red-brown or yellow-brown if positive.

RT-PCR was performed to examine the expression of *Oct4*, *nanog* and *gdf3* genes in pES cells. Total RNA was extracted according to instructions of the RNeasy Mini kit (Qiagen Co. Ltd) and reversely transcribed into cDNA using RevertAid^{TM} First Strand Kits (Fermentas Co. Ltd). PCR reaction were carried out as follows: 2 min denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, and a final 10 min extension at 72°C. PCR products were separated by electrophoresis and examined by ethidium bromide staining under the Gel Image System (Syngene Co. Ltd). PCR primers for amplification of the sequences of *Oct4* gene (forward: 5'-TTCAGACTTCGCCTCCTCACCC-3', reverse: 5'-TTGTCGGCTTCCTCCACCCAC TT-3'), *nanog* gene (forward: 5'-TGGTGTCTTGCTCTTTCTGTGGG-3', reverse: 5'-GC ACTTCATCCTTTGGTTTTG-3'), *gdf3* gene (forward: 5'-CCTTATCAACGGCTTCTG GCGC-3', 5'-CTCTAAGTGTAAGTCCAAGT-3') and *GAPDH* gene (forward: 5'-CGGT GCTGAGTATGTCGTG-3', reverse: 5'-AGGTGGAAGAGTGGGAGTT-3') were designed according to the corresponding gene sequences published in GenBank.

Differentiation experiments of the pES cells

Embryoid bodies (EBs) were prepared as described previously (Nagy et al., 2003). Briefly, ES cells were cultured in ES cell medium without the feeder layers for 24-28 h, then the clumps were mechanically separated and cultured in suspension in LIF-free medium containing 15% FBS for 5-10 days until EBs formed. Subsequently, EBs or their single cells were cultured in a gelatin-pretreated plate with LIF-free medium for 5-7 days until various types of differentiated cells appeared. These differentiated cells were immunohistochemically stained with primary antibodies against the antigens specific for different germ layers (α-fetoprotein (1:100) (AFP) for endoderm cells, α-actin (1:100) for mesoderm cells, Nestin (1:100), β-Tubulin III (1:100) and GFAP (1:100) for ectoderm cells) (all antibodies purchased from Chemicon Co. Ltd).

Moreover, EBs were harvested to examine the expression of *fgf5* and *nf68* specific for ectoderm, *BraT* specific for mesoderm, and *Afp* and *TTR* specific for endoderm by RT-PCR. PCR primers for amplification of the sequences of *fgf5* (forward: 5'-CCTTGCTCTTCC TCATCTTCTGC-3', reverse: 5'-GAGCCATTGACTTTG CCATCC G-3'), *nf68* (forward: 5'-TTCTCCCCCGTTCTT CTCTCTAG-3', reverse: 5'-CTTCTCG TTAGTGGCGT CTTCC-3'), *BraT* (forward: 5'-AAGGTGGCTGTTGGGT AGGGAGT-3', reverse: 5'- ATTGGGCGAGTCTGGGTGG ATGT-3'), *TTR* (forward: 5'-ACTCTTCCTC CTTTGCCTC GCTG-3', reverse: 5'-GCAGGGGAGAAAAATGAGGAA AT-3'), *Afp* (forward: 5'-ATCCTCCTGCTACATTTCGC TGC-3', reverse: 5'-TGAGCAGCCAAGGA CAGAATG-3'), and *GAPDH* (forward: 5'-CGGTGCTGAGTATGTCGTG-3', reverse: 5'AGG TGGAAGAGTGGGAGTT3') were designed according to the corresponding gene sequences published in GenBank.

pES cells (about 5×10^5 cells/mouse) were subcutaneously injected into nude mice. After 4-6 weeks, the harvested teratomas were fixed in 4% formaldehyde to prepare paraffin sections. Sections were stained by hematoxylin/eosin (HE) and observed under a Digital Microscopic Imaging System.

Statistical analysis

Statistical analyses were performed using SPSS 11.5.0 standard version (SPSS Inc., Illinois, USA). An independent simple T-test was employed to analyze differences of developmental potential to blastocyst (No. of blastocysts/No. of activated oocytes), attached rate (No. of attached blastocysts/No. of blastocysts cultured), ICM outgrowth rate (No. of ICM outgrowth/No. of blastocysts cultured) and establishing rate (No. of the established pES cell lines/No. of blastocysts cultured) between hybrid and inbred groups. For all tests, statistical significance was taken as $p<0.05$.

RESULTS

Establishment and characterization of mouse pES cells

The MII oocytes developed *in vitro* to the blastocysts 4-4.5 days after parthogenetic activation, which showed that developmental competence of oocytes from hybrid mice (129♂×KM♀) was significantly higher than that from inbred Kunming mice ($p<0.05$) (Figure 1A) (Table 1). Moreover, blastocysts from inbred Kunming mice were morphologically inferior to those from hybrid mice. The blastocysts cultured on the feeder layers successively attached and formed ICM outgrowths with prominent nucleoli (Figure 1B), which showed significantly higher rates of ICM outgrowth formation than those from inbred Kunming mice ($p<0.05$) (Table 1). The four ICM-derived cell clumps from hybrid mice formed ES cell colonies at the

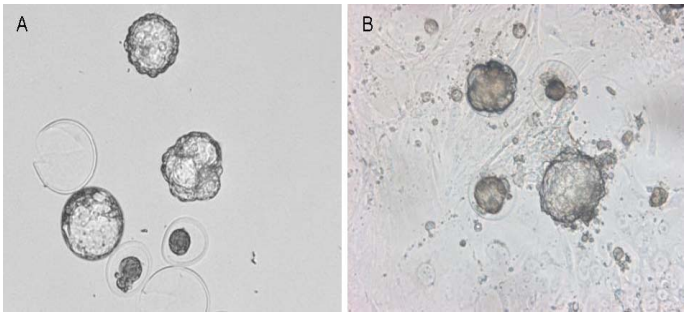

Figure 1. Parthenogenetic blastocysts (A, 100×) and their ICM outgrowths (B, 100×) from oocytes of the hybrid offspring of Kunming and 129/Sv mice.

Table 1. *In vitro* development of MII mouse oocytes after parthenogenetic activation

Hybrid group		No. of oocytes activated	No. of development to blastocysts
129♂×KM♀	1	19	7
	2	23	15
	3	21	13
	Total	63	35(56.1%)[a]
KM♂×KM♀	1	41	6
	2	37	6
	3	47	8
	Total	125	20(16.0%)[b]

Statistically significant differences between the data marked by the different letters.

first passage. However, no pES cell clone appeared from inbred Kunming mice. Three putative pES cell lines were established from oocytes of hybrid mice, and one cell line maintained the undifferentiated state for more than 50 passages.

Karyotype analysis of pES cells at the 14th and 23rd passage revealed that most cells held 20 pairs of chromosomes with a XX chromosome (83.3% at 14th passage vs. 70% at 23rd passage) (Figure 2). During extended culture, pES cells exhibited high activities of AKP, and were immunohistochemically positive for SSEA-1, Nanog, Oct-4 and telomerase but negative for SSEA-3 (Figure 2). They also expressed such genes as *Oct-4*, *nanog* and *gdf3* by RT-PCR (Figure 3A). Therefore, the pES cells exhibited the same characteristic enzyme activities, antigen profiles and gene expression as mouse ES cells. These

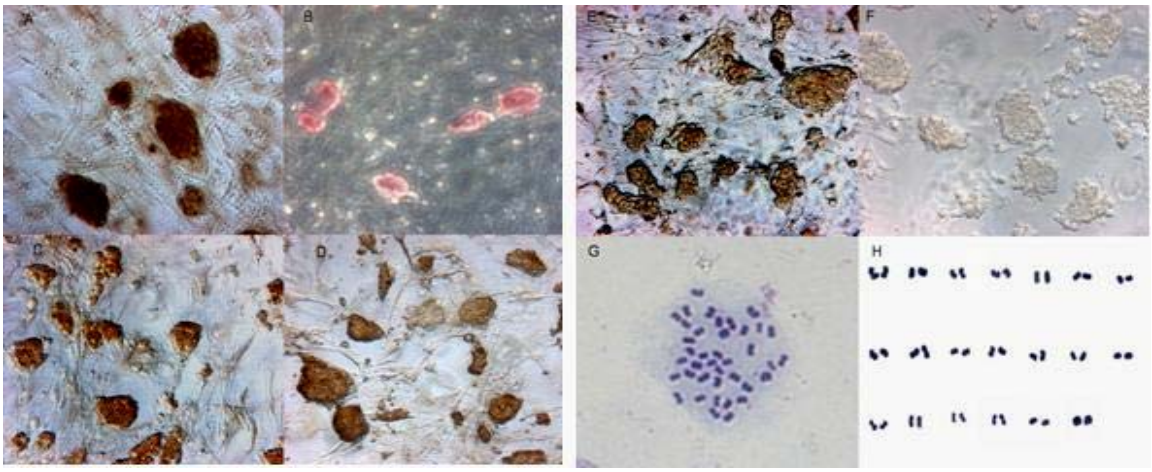

Figure 2. Karyotype and immunohistochemical analysis of the pES cells. The pES cells at the 42th passage were positive for AKP (B, 200×), and positive for antibodies against telomerase (A, 200×), Nanog (C, 100×), Oct-4 (D, 200×), SSEA-1 (E, 200×), but negative for antibody against SSEA-3 (F, 200×). The pES cells at the 23rd passage had 20 pairs of chromosomes with XX sex chromosomes (G and H).

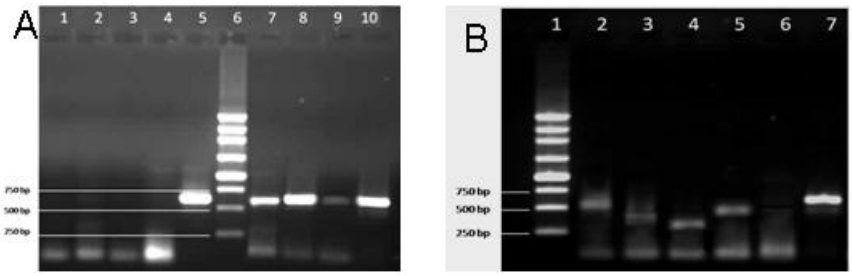

Figure 3. Detection of the ES cell marker genes in pES cells and genes of three germ layers in the pES cell-derived EBs by RT-PCR. A shows that the pES cells at the 45th passage expressed *nanog* (Lane 7), *Oct-4* (Lane 8), *gdf3* (Lane 9), but MEF did not express *nanog* (Lane 2), *Oct-4* (Lane 3), *gdf3* (Lane 4). *GADPH* (Lane 5 and 10) were expressed in MEF and the pES cells as positive control. B shows that the EBs expressed *nf68* (Lane 2) and *fgf5* (Lane 4) for ectoderm, *BraT* (Lane 3) for mesoderm, *Afp* (Lane 5) for endoderm, and *GADPH* (Lane 7) as positive control, but did not express *TTR* (Lane 6) for endoderm.

results suggested that the pES cells could be permanently retained in the undifferentiated state.

Pluripotency of mouse pES cells

Some different sizes of EBs continuously appeared 5-10 days after culture in suspension under LIF- and feeder-free conditions (Figure 4). RT-PCR results demonstrated that EBs expressed the specific genes for three germ layers, *fgf5* and *nf-68* (for ectoderm), *BraT* (for mesoderm), and *Afp* (for endoderm) (Figure 3B); meanwhile these EB-derived cells immunohistochemically expressed the specific antigens α-Actin (for mesoderm), AFP (for endoderm), GFAP, β-Tubulin III and Nestin (for neuroectoderm) (Figure 4). Moreover, teratoma sections demonstrated tissue structure resembling connective tissue, blood cells, blood tube, epithelial cells, muscle, lymphatic tissues, skin and endocrine gland (Figure 5). These results suggested that the pES cells could differentiate into various types of cells from three germ layers *in vitro* or *in vivo*.

DISCUSSION

In this work, we established pES cell lines from parthenogentically activated MII oocytes of hybrid offspring of Kunming and 129/Sv mice, which shared all features of mouse ES cells.

Oct-4 and *nanog* genes, essential for maintenance of pluripotency in ICM cells and ES cells *in vitro* (Nichols et al., 1998; Chambers et al., 2003; Mitsui et al., 2003), were generally employed as the molecular markers for characterization of ES cells. *Gdf3* is known to be a BMP4 inhibitor and to regulate stem cells to retain the undifferentiated state and differentiate into the full spectrum of cell types, which was also expressed in ES cells *in vitro* (Levine and Brivanlou, 2005). In this work, the pES cells expressed *Oct-4* and *nanog* in high levels but *gdf3* in low levels, which was in accordance with the view that decreased expression of the *gdf3* gene permits BMP4 to support maintenance of pluripotency in mouse ES cells

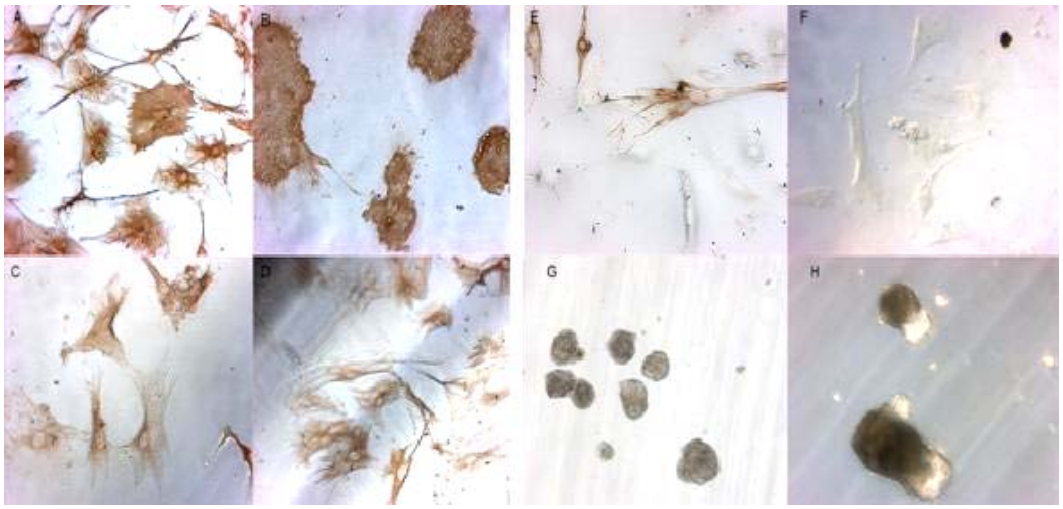

Figure 4. Analysis of Differentiated potential of the pES cells *in vitro* by Immuno- histochemistry. The pES cell aggregates suspended in LIF- and feeder-free conditions formed the EBs (G, 50× and H, 100×), and their differentiated cells held phenotypes against these antibodies: α-actin for mesoderm cells (A, 200×), AFP for endoderm cells (B, 200×), GFAP (C, 200×), β-tubulin III (D, 200×) and Nestin (E, 200×) for ectoderm cells. F (200×) did not dye as negative control.

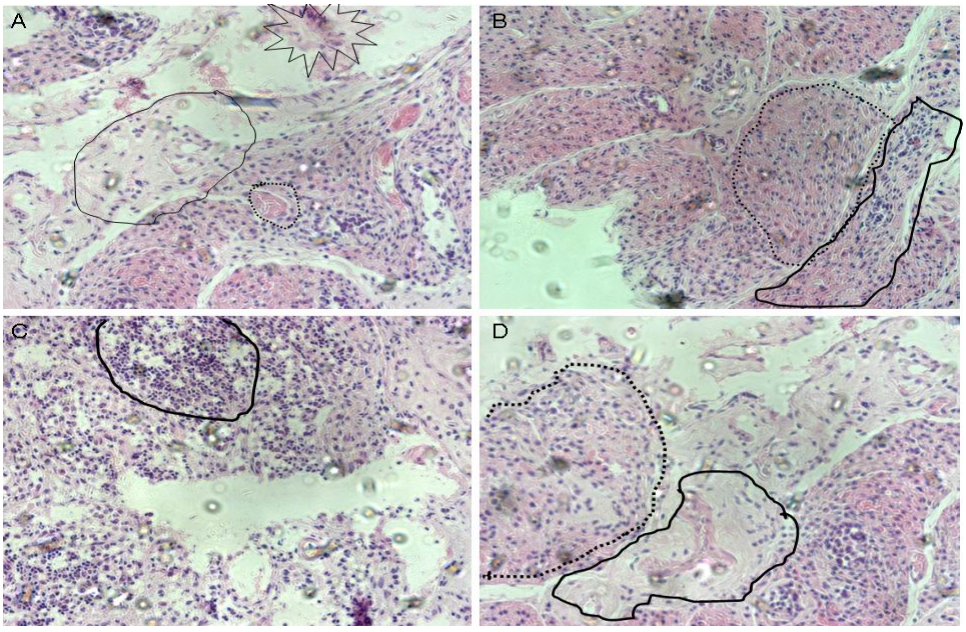

Figure 5. Histological sections of teratomas derived from pES cells (H/E staining, 200×). Teratoma sections showed various types of tissue structures resembling connective tissue (the solid line-surrounding region in A), blood cells and blood tube (the dotted line-surrounding region in A), columnar epithelium (the starlike-marked regions in A), muscle (the marked region in B), lymphatic tissues (the marked region in C), skin keratinous layers (the solid line-surrounding region in D) and endocrine gland (the dotted line-surrounding region in D).

(Levine and Brivanlou, 2005). These results suggested that the established pES cell lines could maintain the undifferentiated state *in vitro* for a long time.

Genetic background of mouse was known to influence isolation of ES cells, and there exhibited some differences among different mouse strains in the conditions required for isolation of ES cells (Kawase et al., 1994; Suzuki et al., 1999; Baharvand and Matthaei, 2004). We attempted to isolate pES cells from oocytes of inbred Kunming mice, but the oocytes exhibited significantly lower developmental competence to blastocysts *in vitro* after parthenogenetic activation. The harvested blastocysts were also morphologically inferior to those from hybrid mice. All these factors constrained isolation of pES cells. Previous reports indicated that rapid loss of *Oct-4* gene in ICM cells *in vitro* was the constraint for isolation of ES cells (Buehr and Smith, 2003; Buehr et al., 2003; Tielens et al., 2006). Similarly, we noticed that ICM outgrowths derived from parthenogenetic embryos of inbred Kunming mice, expressed *Oct-4* at even lower level or unexpressed *in vitro,* as determined by immunohistochemical methods (not presented here). On the contrary, *Oct-4* was steadily expressed in ICM outgrowths and pES cells from hybrid mice (not presented). Therefore, we inferred that rapid loss of *Oct-4* in ICM outgrowths and pES cells *in vitro* constrained establishment of the pES cell line from inbred Kunming mice. These results implied that introduction of 129/sv mouse genetic background could significantly improve isolation of pES cells, which was also supported by the findings of Kress et al. (1998) and Brook et al. (2003). Jiang et al. (2007) demonstrated that disruption of genomic methylation and activation of some paternally-

Table 2. Isolation of the pES cells from parthenogenetic embryos

Hybrid group		No. of blastocysts cultured	No. of attached embryos	No. of ICM outgrowths	No. of ES cell clonies at passage one	No. of the established pES cell lines
129♂×KM♀	1	5	1	1	0	0
	2	12	6	4	2	1
	3	13	8	6	4	2
	Total	30	15(50%) [a]	11(36.7%) [a]	4(13.3%)	3
KM♂×KM♀	1	3	0	0	0	0
	2	4	2	2	0	0
	3	6	3	2	0	0
	Total	13	5(38.4%) [b]	4(30.8%) [a]	0	0

expressed imprinting genes might improve developmental potential of parthenogenetically-activated oocytes and enhance the proliferation and pluripotency of pES cells *in vitro*. However, further study is necessary to explore the constraints in isolating pES cells from inbred Kunming female mice by investigating genomic methylation and expression patterns of paternal imprinting genes in parthenogenetic embryos and their ICM outgrowths.

Overall, this work may provide a new strategy for establishment of pES cell lines from Kunming female mice, and also be a valuable model for investigating differences of epigenetic regulation and gene imprinting between Kunming and other mouse strains.

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