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Change in gene expression of mouse embryonic stem cells derived from parthenogenetic activation

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BACKGROUND: We previously established parthenogenetic mouse embryonic stem cells (ESCs) and this study was subsequently conducted for elucidating the influence of oocyte parthenogenesis on gene expression profile of ESCs.

METHODS: Gene expression of parthenogenetic ESC (pESC)-1 or pESC-2 was separately compared with that of two normally fertilized ESC (nfESC) lines (B6D2F1 and R1 strains), and quantification of mRNA expression was conducted for validating microarray data.

RESULTS: In two sets of comparison, reaction of 11 347 and 15 454 gene probes were altered by parthenogenesis, while strain difference changed the expression of 15 750 and 14 944 probes. Level of correlation coefficient was higher in the comparisons between normal fertilization and parthenogenesis (0.974–0.985) than in the comparisons between strains of nfESCs (0.97–0.971). Overall, the expression of 3276–3329 genes was changed after parthenogenesis, and 88% (96/109) of major functional genes differentially ($P < 0.01$) expressed in one comparison set showed the same change in the other. When we monitored imprinted genes, expression of nine paternal and eight maternal genes were altered after parthenogenesis and 88% (14/16) of these was confirmed by mRNA quantification.

CONCLUSIONS: The change in gene expression after parthenogenesis was similar to, or less than, the change induced by a strain difference under a certain genetic background. These results may suggest the clinical feasibility of parthenogenesis-derived, pluripotent cells.

Key words: mouse model / embryonic stem cell / normal fertilization / parthenogenesis / gene expression

Introduction

Successful cell replacement therapy requires either establishing immune-specific pluripotent cell lines or acquiring immune tolerance in heterogeneous stem cells. To eliminate the risk of cloning in autologous stem cell therapy and to efficiently establish immune-specific stem cells, various alternatives to somatic cell nuclear transfer have been suggested (Cowan *et al*., 2005; Chung *et al*., 2006; Meissner and Jaenisch, 2006; Okita *et al*., 2007; Wernig *et al*., 2007). Using a mouse model, we have previously established autologous embryonic stem cells (ESCs) by parthenogenetic activation of oocytes (Lee *et al*., 2008), and the physiological and genetic properties of the parthenogenetic ESC (pESC) lines have been characterized (Gong *et al*., 2008). To confirm clinical feasibility of pESCs established, it is absolutely necessary to evaluate alteration of genetic and cellular properties after parthenogenesis. Unfortunately, there was no report on evaluating difference in gene expression in pESCs.

In this study, we made two sets of comparison using pESC-1 and pESC-2 lines. In each comparison, we first compared the gene expression between normally fertilized ESCs (nfESCs) of B6D2F1 (C57BL/6 X DBA2) strain (established from our laboratory) and those of R1 (129X1 × 129S1) strain (commercially purchased from ATCC) in mice. Second, comparison was subsequently made between B6D2F1 pESCs (pESC-1 for the first and pESC-2 line for the second comparison) and nfESCs of the same strain, and between B6D2F1 pESC-1 or pESC-2 and nfESCs of R1 strain. Using a microarray Genechip technology and a gene network map, genes being up-regulated or down-regulated after parthenogenesis were identified. Gene ontogeny annotation was performed for assuming changes in cellular function after parthenogenesis and real-time

PCR analysis was conducted to verify the microarray results. As additional analyses, major regulatory hub genes were identified in the comparisons between pESCs and nfESCs.

Materials and Methods

Preparation of mouse blastocysts fertilized *in vivo*

All procedures for animal management, breeding and surgery followed the standard protocols of Seoul National University. Appropriate management of experimental samples and quality control of the laboratory facility and equipment were also conducted. The Institutional Animal Care and Use Committee, Seoul National University, approved our research proposal in April 2005 (approval number: SNU0050331-02). The B6D2F1 hybrid strain, produced by the mating of a female C57BL/6 with a male DBA2, was maintained under controlled lighting (14 h Light:10 h Dark), temperature (20–22°C) and humidity (40–60%). Naturally ovulated females in estrus were then mated naturally, and we collected blastocysts by uterine flushing 72 h after mating. The flushing medium was M2 medium consisting of 94.7 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 4.2 mM $NaHCO_3$, 20.9 mM HEPES, 23.3 mM sodium lactate, 0.3 mM sodium pyruvate, 5.6 mM glucose, 1% (v/v) penicillin/streptomycin solution and 4 mg/ml bovine serum albumin (BSA). All medium substrates were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA) unless otherwise stated.

Collection of mature oocytes and production of blastocysts after parthenogenetic activation

Ovulated oocytes were collected by oviduct flushing of naturally ovulated F1 females in estrus 15 h after mating with a vasectomized male. Maturation of the oocytes to the metaphase II stage was determined by extrusion of the first polar body and expansion of cumulus cells. Oocytes were freed from cumulus cells by placement in M2 medium supplemented with hyaluronidase (200 units/ml) for 5 min at 37°C. The oocytes were then activated by culture in Ca^{2+}-free KSOM medium supplemented with 10 mM $SrCl_2$ and 5 μg/ml cytochalasin B for 4 h. Modified Chatot, Ziomek and Bavister medium consisting of 81.6 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 1.7 mM $CaCl_2{\cdot}2H_2O$, 25.1 mM $NaHCO_3$, 31.3 mM sodium lactate, 0.3 mM sodium pyruvate, 1 mM glutamine, 0.1 mM EDTA and 5 mg/ml BSA, to which 0.001 mg/ml Hb (methemoglobin) and 5.5 μM β-mercaptoethanol (Gibco Invitrogen, Grand Island, NY, USA) were added, was used for culturing parthenogenetically activated oocytes. Activated oocytes were cultured in a 5-μl droplet of the medium overlaid with washed mineral oil at 37°C in an atmosphere containing 5% CO_2. Blastocysts obtained were used for ESC establishment.

Establishment and culture of ESCs

The zona pellucida of blastocysts derived from normal fertilization and parthenogenesis were removed using acid Tyrode's solution, and the zona-free blastocysts were subsequently cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) treated with 10 μg/ml mitomycin C (Chemicon, Temecula, CA, USA) for 3 h in gelatin-coated 4-well multi-dishes. Knock-out Dulbecco's minimal essential medium (KDMEM; Gibco Invitrogen) supplemented with 0.1 mM β-mercaptoethanol (Gibco Invitrogen), 1% (v/v) non-essential amino acids (Gibco Invitrogen), 2 mM L-glutamine, 1% (v/v) mixture of penicillin and streptomycin, 2000 units/ml mouse leukemia inhibitory factor (LIF; Chemicon), and a 3:1 mixture of fetal bovine serum (FBS) and knock-out serum replacement was used for initial culture of the blastocysts. On Day 4 of culture, inner cell mass cell-derived cell colonies were mechanically removed with a capillary pipette and replated on MEF feeder cells for further expansion. Expanded colonies were dissociated with 0.04% (w/v) trypsin–EDTA (Gibco Invitrogen) and subcultured on a 35-mm tissue culture dish in the presence of MEF feeder cells under a humidified atmosphere of 5% CO_2 at 37°C. After colony expansion, the LIF concentration in ESC culture media was reduced to 1000 units/ml, and subpassage was conducted at intervals of 4 days, when the cultured ESCs had reached 70–80% confluency. The medium was changed daily during subculture. R1 nfESCs purchased from ATCC were subpassaged with the standard protocols of our laboratory (Lee *et al.*, 2008).

Characterization of established ESCs

For characterization using stem cell-specific markers, ESCs collected at the 20th subpassage were fixed in 4% (v/v) formaldehyde (Sigma-Aldrich) at room temperature for 10 min. The reactivity of the ESCs to alkaline phosphatase was assessed with Fast Red TR/naphthol AS-MX phosphate (Sigma-Aldrich). Antibodies against Oct-4 (BD Biosciences, San Jose, CA, USA), stage-specific embryonic antigens (SSEA)-1 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), SSEA-3 (Developmental Studies Hybridoma Bank), SSEA-4 (Developmental Studies Hybridoma Bank), integrin α6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and integrin β1 (Santa Cruz Biotechnology) were provided for the marker staining. Localization of SSEA-1, SSEA-3, SSEA-4, Oct-4, integrin α6 and integrin β1 was performed using the Alexa Fluor 488-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR, USA), the Alexa Fluor 568-conjugated anti-mouse antibody (Molecular Probes), and the DakoCytomation kit (DakoCytomation, Carpinteria, CA, USA).

To confirm spontaneous differentiation *in vitro*, the ESCs were treated with 0.04% (v/v) trypsin–EDTA (Gibco Invitrogen), and the dissociated cells were subsequently transferred to 100-mm plastic Petri dishes that contained LIF-free DMEM (Gibco Invitrogen) that was supplemented with 10% (v/v) FBS. The cells were grown until the embryoid bodies (Ebs) formed. The EBs were seeded separately into 4-well culture plates and cultured for 10–14 days. The EBs were stained with the following specific markers for the three germ layers: nestin (Santa Cruz Biotechnology) and S-100 (Biodesign International, Saco, ME) for ectodermal cells; muscle actin (Biodesign International) and desmin (Santa Cruz Biotechnology) for mesodermal cells and α-fetoprotein (Biodesign International) and troma-1 (Developmental Studies Hybridoma Bank) for endodermal cells. Antibody localization was performed with the DakoCytomation kit (DakoCytomation). For monitoring ESC capacity to differentiate *in vivo*, 1×10^7 ESCs retrieved at the 20th subpassage were injected s.c. into adult NOD-SCID (non-obese diabetic/severely compromised immunodeficient) mice. Teratomas that formed in the subcutaneous region were collected 8 weeks post-transplantation and fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich). After embedding in paraffin blocks, the tissues were stained with hematoxylin and eosin for examination under a phase-contrast microscope (BX51TF; Olympus, Kogaku, Japan).

Generation of Affymetrix chip data

All analyses were performed in triplicate. The generation of Affymetrix data from the three different stem cell samples was performed by Seoulin Bioscience Cooperation (Seoul, Korea). About 4 μg of total RNA from the samples were used for labeling. Probe synthesis from total RNA samples, hybridization, detection and scanning were performed according to standard protocols from Affymetrix, Inc. (Santa Clara, CA, USA). Briefly, complementary DNA (cDNA) was synthesized using the One-Cycle cDNA Synthesis Kit (Affymetrix). Single-stranded cDNA was

synthesized using Superscript II reverse transcriptase and T7-oligo (dT) primers at 42°C for 1 h. Double-stranded (ds) cDNA was obtained using DNA ligase, DNA polymerase I and RNase H at 16°C for 2 h, followed by T4 DNA polymerase at 16°C for 5 min. After cleanup with a Sample Cleanup Module (Affymetrix), ds-cDNA was used for *in vitro* transcription (IVT). cDNA was transcribed using the Affymetrix GeneChip® IVT Labeling Kit in the presence of biotin-labeled CTP and UTP. The biotin-labeled IVT-RNA was then fragmented. Fragmented cRNA was hybridized to the Mouse Genome 430 2.0 Array, which has 45 000 gene probes for over 34 000 well-characterized mouse genes, at 45°C for 16 h according to the manufacturer's instructions. After hybridization, the arrays were washed in a GeneChip® Fluidics Station 450 with a non-stringent wash buffer at 25°C followed by a stringent wash buffer at 50°C. After washing, the arrays were stained with a streptavidin–phycoerythrin complex. After staining, intensities were determined with a GeneChip scanner, which was controlled by GeneChip® Operating Software (Affymetrix).

Analysis of DNA microarrays

The quality of the array image was assessed as described in the Affymetrix GeneChip expression analysis manual. All arrays were processed by robust multi-array average (RMA) using the R package Affy. Expression values were computed in detail from raw *CEL* files by applying the RMA model of the probe-specific correction of perfect-match probes. These corrected probe values were then normalized via quantile normalization, and a median polish was applied to compute one expression measure from all probe values. The resulting RMA expression values were $\log_2$-transformed. The individual gene expression levels were compared using an unpaired Welch *t*-test. The Benjamin–Hochberg correction for false discovery rate (FDR) was used for all probe-level normalized data. We selected differentially expressed genes (DEGs) that met a FDR-adjusted *P*-value of less than 0.05 (compatible with a 1.2- to 1.4-fold difference in gene expression) using an unpaired Welch *t*-test. The gene ontology annotation was conducted using the NetAffy tool (http://www.affymetrix.com) and DAVID bioinformatics resources (http://david.abcc.ncifcrf.gov).

Construction of reference gene network

Data set consisting of 180 Affymetrix GeneChip Murine Genome U74 Version 2 Set MG-U74A Array data that were derived from six different tissues (skeletal muscle, heart, fetal heart, myoblast, bone marrow, lung and skin) were used. Relative expression intensity ($\log_2$-transformed) for microarray data for all probes was estimated using the RMA algorithm within the 'affy' package in Bioconductor (Irizarry *et al*., 2003), which performs background correction, normalization, probe-specific correction and summary. The Pearson correlation coefficients obtained for all pairwise comparisons of the expression data were then converted into a coexpression matrix by defining a similarity matrix function, which is a signum function with a thresholding parameter τ.

$$a_{ij} = \text{signum}(s_{ij}, \tau) \equiv \begin{cases} 1 & \text{if } |s_{ij}| \geq \tau \\ 0 & \text{if } s_{ij} < \tau \end{cases}$$

Nodes and edges for the DEGs were identified using this signum function, where τ was 0.7. The gene-coexpression network was visualized using the Cytoscape spring-embedded layout algorithm (Shannon *et al*., 2003).

Quantitative real-time PCR

Primary3 software (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA, USA) was used to design all specific primers used in these experiments. All PCR primers were designed based on mouse cDNA sequences obtained from GenBank. The primer sequences and annealing temperatures are listed in Supplementary data, S1. Total RNA was extracted from each sample using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from ~1 μg of each RNA sample using the Reverse Transcription System (Promega, Madison, WI, USA). Subsequently, the expression of specific genes in each sample was quantified by real-time PCR using the DyNAmo HS SYBRGreen qPCR Kit (Finnzymes, Espoo, Finland). PCR amplification was performed in a final volume of 25 μl using the ABI PRISM 7700 sequence detection system (Applied Biosystems) with the following cycling parameters: 2 min at 50°C, 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 1 min at 72°C. A dissociation curve was recorded to check the specificity of the amplification. The final optimized concentration of each primer was 300 nM, and the absence of inter- and/or intra-molecular duplex formation between the primers was confirmed in a control real-time PCR run that lacked a template. The mRNA level of each gene in the samples was normalized to that of β-actin. The relative mRNA level was defined as $2^{-\Delta\Delta Ct}$, where Ct = the threshold cycle for target amplification, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal reference}}$ (β-actin) and $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$.

Results

Before analysis, the ESC lines used for this study were characterized with our standard protocols. The nfESC, pESC-1 and pESC-2 of B6D2F1 line were characterized by ESC-specific markers for alkaline phosphatase (+), and anti-SSEA-1 (+), SSEA-3 (−), SSEA-4 (−), Oct-4 (+), integrin α6 (+) and integrin β1 (+) antibodies, *in vitro*-differentiation into the EB, and *in vivo*-differentiation into teratomas (Fig. 1).

Analysis of microarray data

In the first set of comparisons, we monitored the number of genes (gene probes) differentially expressed between B6D2F1 nfESCs and R1 nfESCs, between B6D2F1 pESCs-1 and R1 nfESCs, and B6D2F1 pESCs-1 and B6D2F1 nfESCs (Fig. 2). Of total 45 000 gene probes monitored, the expression of 15 750, 14 187 and 11 347 probes changed, respectively, and a gradual decrease in the number was detected as variables (strain and origin) between nfESCs and pESCs were reduced (B6D2F1 nfESCs versus B6D2F1 pESCs <R1 nfESCs versus B6D2F1 pESCs). In all three comparisons, 4984 gene probes were simultaneously changed. In the second comparison consisting of the same procedure except for using of pESC-2, the expression of 14 944, 15 381 and 15 454 gene probes were different, respectively. Total 5391 gene probes were simultaneously changed in all comparisons. Level of correlation in each comparison was subsequently monitored to statistically evaluate the extent of the change after parthenogenesis. As shown in Fig. 3, overall levels in each replicate of comparisons were higher in the comparisons between normal fertilization (B6D2F1 nfESCs) and parthenogenesis (B6D2F1 pESCs) than in the comparisons between strains (R1 nfESCs and B6D2F1 nfESCs) (0.974–0.985 versus 0.97–0.971).

Consequently, for each probe set we determined the genes showing different expression between pESCs and nfESCs (Table 1). In the comparison of B6D2F1 pESCs-1 versus nfESCs of the same strain, 7776 genes showed significantly altered expression after parthenogenesis. Of those, 53% (4096 genes) were up-regulated in pESCs-1, whereas the rest were down-regulated. The number of genes that had

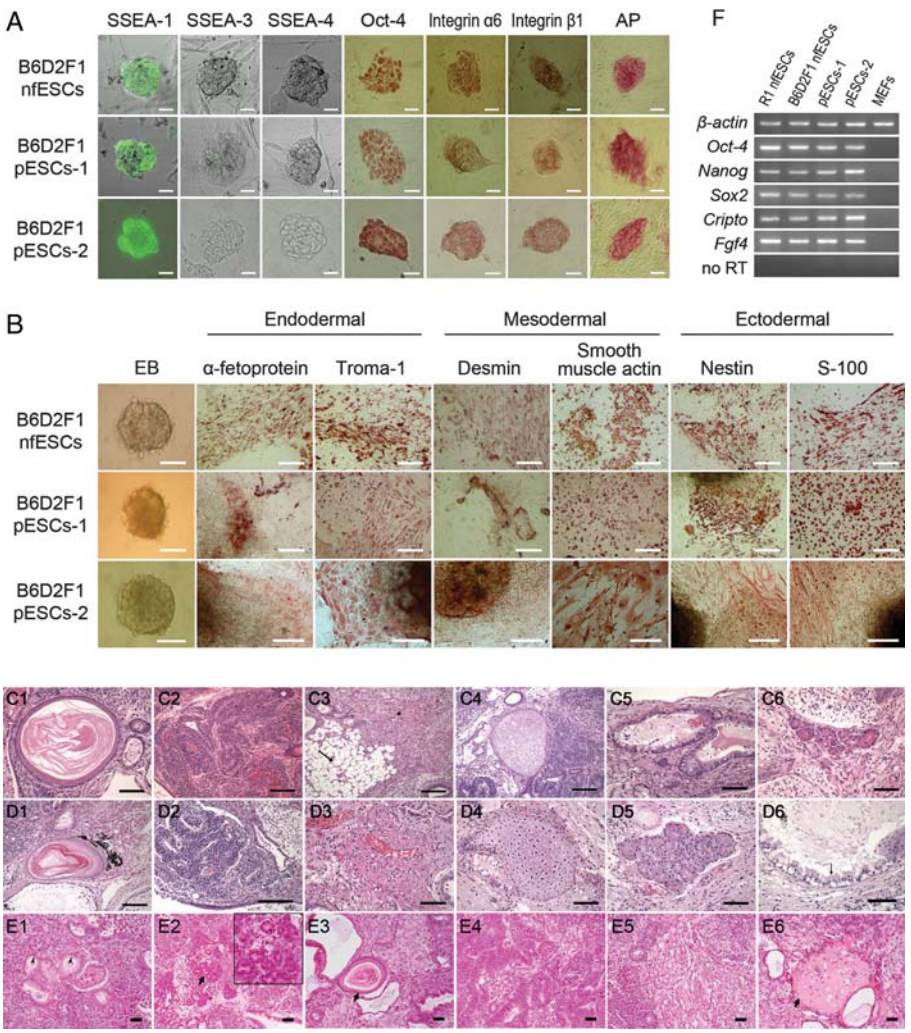

Figure 1 Characterization for two types of mouse ESC that are derived from normal fertilization (nfESCs) and parthenogenesis (pESCs-1 and pESCs-2) of B6D2F1 strain.

(**A**) Identification of ESC-specific markers by immunostaining. Bar = 50 μm. (**B**) *in vitro*-differentiation. Bar = 100 μm. (**C**–**E**) *in vivo* differentiation. (C1) Keratinized stratified squamous epithelial cells. Bar = 50 μm. (C2) Neuroepithelial cells. Bar = 100 μm. (C3) Adipose tissue (arrow) and muscle (arrow-head). Bar = 100 μm. (C4) Cartilage. Bar = 100 μm. (C5) Glandular epithelium with goblet cells. Bar = 50 μm. (C6) Pancreatic tissue. Bar = 50 μm. (D1) Pigmented and keratinized epithelial cells. Bar = 100 μm. (D2) Neuroepithelial cells. Bar = 100 μm. (D3) Muscle and blood vessels. Bar = 50 μm. (D4) Cartilage. Bar = 50 μm. (D5) Pancreatic tissue. Bar = 50 μm. (D6) Ciliated columnar epithelial cells (arrow). Bar = 50 μm. (E1) Glandular epithelium-Goblet cell-like (arrow head). (E2) Exocrine pancreas. (E3) Stratified squamous epithelium (arrow). (E4) Neuroepithelial rosettes. (E5) Skeletal muscle bundles. (E6) Bone tissue (arrow). All bars in (E) are 50 μm. (**F**) Identification of ESC-specific gene expression by RT–PCR. AP, alkaline phosphatase; EB, embryoid body; ESC, embryonic stem cells; MEF, mouse embryonic fibroblasts; SSEA, stage-specific embryonic antigens.

significantly different expression after parthenogenesis increased when pESCs-1 were compared with nfESCs of different strain (R1). In total, 8943 genes were expressed differently in pESCs-1 and R1 nfESCs; approximately half of the genes (4490/8943) were up-regulated after parthenogenesis. Only 3276 genes were expressed differently in both comparisons, which consisted of 1878 up-regulated and 1398 down-regulated genes. In addition, total 2766 genes were up-regulated in one comparison and down-regulated in another, which consisted of 1618 genes up-regulated in B6D2F1 nfESCs and down-regulated in R1 nfESCs, and 1148 genes up-regulated in R1 nfESCs and down-regulated B6D2F1 nfESCs. Thus total 6042 genes were changed after parthenogenesis, regardless of up- and down-regulation. In the second comparison employing pESCs-2, 7633 genes showed significantly different expression after parthenogenesis, while 9074 genes were expressed differently in pESCs-2 and R1 nfESCs. Total 3329 genes were expressed differently in both comparisons and 2029 genes were up-regulated in one comparison and down-regulated in another. Thus, total 5358 genes were changed after parthenogenesis, regardless of up- and down-regulation.

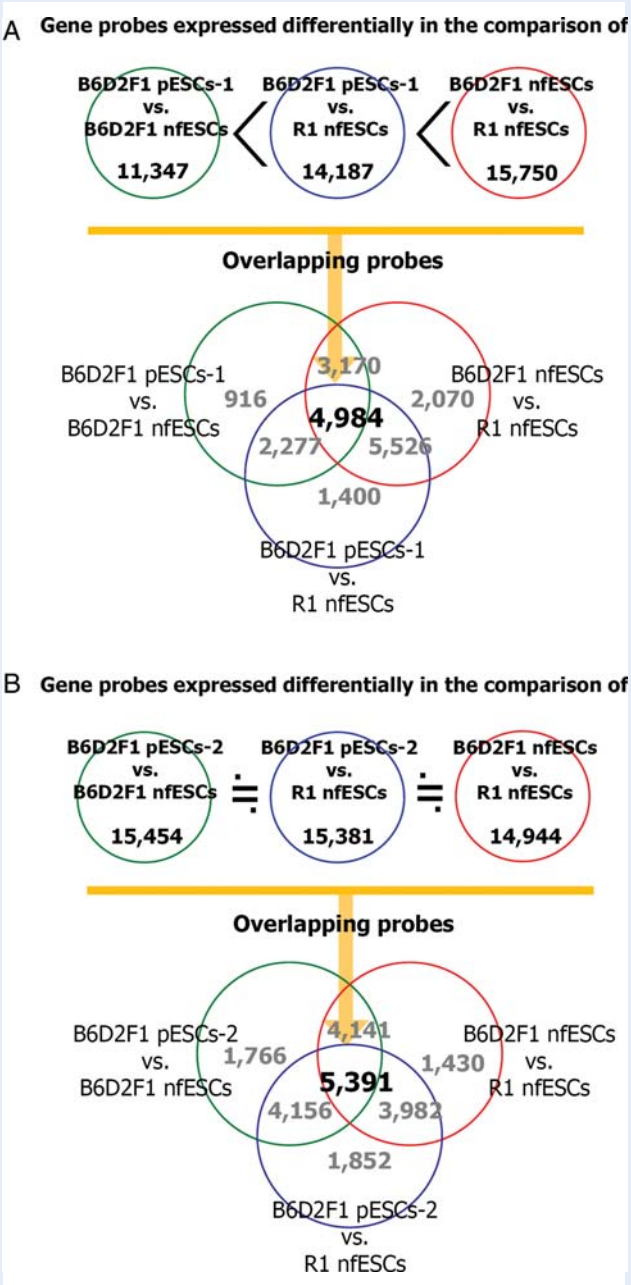

Figure 2 Sequential comparison of gene expression of ESCs derived from different origins.

Two sets (**A** and **B**) of comparison were attempted using two lines of pESCs [pESC-1 and pESC-2 for (A) and (B), respectively]. In each set, gene expression of nfESCs derived from R1 strain was first compared with that of nfESCs derived from B6D2F1 strain. Comparisons were subsequently made between pESCs and nfESCs of the same strain (B6D2F1) and between pESCs and nfESCs of different strain (R1). In the first comparison (A), the change in gene expression after parthenogenesis was less than the change induced by strain difference, while the number of genes with altered expression was similar among all comparisons in the second set (B).

Gene ontology annotation

The genes expressed differently in pESCs compared with both nfESCs were further analyzed by the percentage distribution of gene ontology. As listed in Table II, the DEGs fall into various functional categories when distributed into biological processes, molecular functions or cellular components in gene ontology term level 2. In two comparisons, an overall 88% (96/109) of the major functional genes differentially ($P < 0.01$) expressed in one comparison also showed the difference in the other. Eighty-nine percent (48/54) of the annotated genes listed in the first comparison set was repeatedly listed in the second comparison, while 87% (48/55) of the genes listed in the second set was listed in the first comparison.

Imprinted genes

Of those genes differentially expressed after parthenogenesis in the first comparison, 16 were imprinted genes consisting of eight paternally imprinted genes (*Mcts2*, *Mest*, *Zfp264*, *Snrpn*, *Nap1l5*, *Impact* and *Dlk1* were up-regulated, whereas *Igf2* was down-regulated) and eight maternally imprinted genes (*Pon2*, *Cdkn1c*, *Grb10*, *Rian*, *Igf2r*, *Gnas*, *Ube3a* and *Phlda2* were down-regulated; Table III). On the other hand, 15 imprinted genes consisting of eight paternally imprinted genes (*Mcts2*, *Mest*, *Zfp264*, *Snrpn*, *Impact*, *Dlk1* and *Ddc* were up-regulated, whereas *Igf2* was down-regulated) and seven maternally imprinted genes (*Pon2*, *Cdkn1c*, *Grb10*, *Igf2r*, *Gnas*, *Ube3a* and *Phlda2*, all were down-regulated) showed different expression after parthenogenesis in the second comparison. In two comparisons, however, parthenogenesis altered the expression of only nine paternally imprinting and eight maternally imprinting genes because of the similarity of the gene listing. Four imprinted genes (*Snrpn*, *Mest*, *Ndn* and *Phlda2*) which showed altered expression after parthenogenesis were randomly selected from the database, and their expression level was quantified by real-time PCR. As shown in Fig. 4, 88% (14/16) of the microarray results was consistent with the results of mRNA quantification by real-time PCR.

Analysis of 3276 genes that were expressed differently in pESCs by reference gene network

A gene-coexpression network using the data sets derived from six tissues was constructed. Using the reference gene network, the 3276 genes up-regulated or down-regulated after parthenogenesis in the first comparison using pESC-1 were determined (Supplementary data). The hub genes were subdivided into stimulatory or inhibitory based on their promotion or repression of networked gene expression, respectively. Up to 20 up-regulated and down-regulated hub genes were ranked according to the number of correlations among genes from the following four groups; genes that are up-regulated in pESCs relative to both nfESCs and have a stimulatory correlation with other genes, genes that are up-regulated in pESCs and have an inhibitory correlation with other genes, genes that are down-regulated in pESCs and have a stimulatory correlation with other genes and genes that are down-regulated in pESCs and have an inhibitory

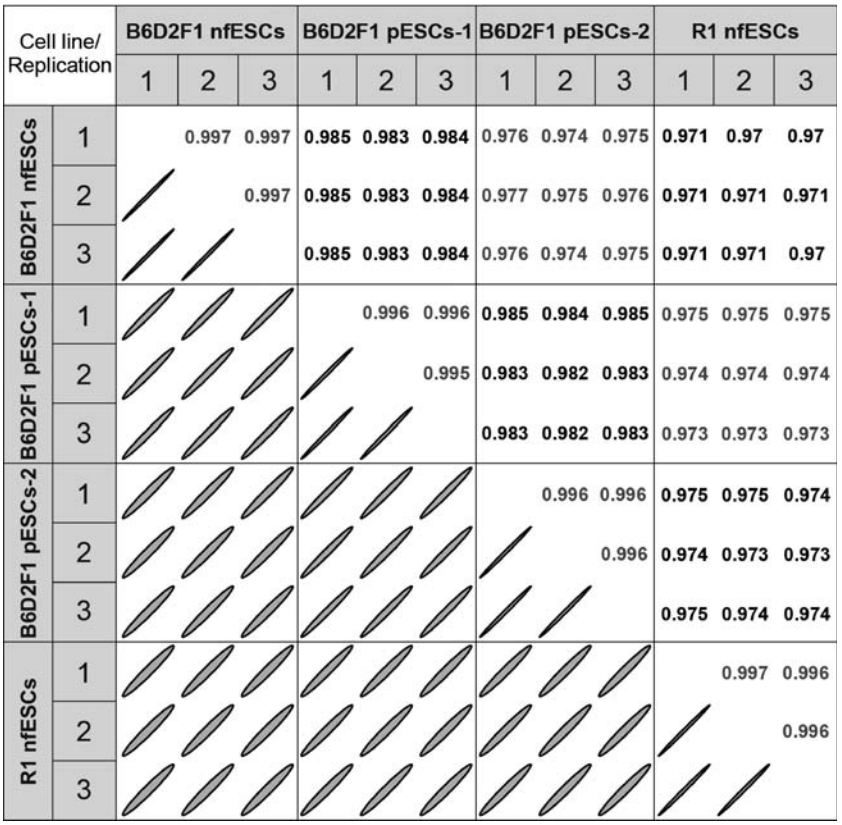

Figure 3 Similarity of gene expression in different lines of nfESC or pESC.
Similarity was evaluated by the comparison of correlation coefficients in each set of DNA microarray data and this figure subsequently shows bivariate normal ellipse and correlation coefficient between all pairs compared. High levels of correlation (more than 0.97) were detected between all cell lines, but correlation coefficients were higher in the comparisons between nfESCs and pESCs of the same strain than in the comparisons between nfESCs of different strains.

correlation with other genes. As shown in Supplementary data, S2, *Ndufb10*, *Ndufb11*, *Etfa*, *Atp5k*, *Idh3g*, *1810013D10Rik*, *Ndufa11*, *Acyp2*, *Pdha1*, *Slc25a20*, *Ankrd40*, *Art3*, *Slc25a4*, *Deb1*, *Cstf2*, *Smyd1*, *Pdk3*, *Sec61a1*, *Pxmp2* and *Lama2* were stimulatory hub genes, and *Dazl*, *5430432M24Rik*, *Skap2*, *Gltp*, *Slc10a3*, *Cggbp1*, *Sema5b*, *1110004F10Rik*, *Bcap31*, *Etfb*, *Dmrtb1*, *Sult4a1*, *Grid2*, *Sec61a1*,

Table 1 DEG from the comparisons between mouse B6D2F1 pESCs and B6D2F1 nfESCs, and between B6D2F1 pESCs and R1 nfESCs

Sets of comparison	No. of genes expressed differentially in pESCs		
	Up-regulated	Down-regulated	Total
First comparison using pESCs-1			
B6D2F1 pESCs-1 versus B6D2F1 nfESCs	4096	3680	7776
B6D2F1 pESCs-1 versus R1 nfESCs	4490	4453	8943
Both[a]	1878	1398	3276 (2766)[b]
Second comparison using pESCs-2			
B6D2F1 pESCs-2 versus B6D2F1 nfESCs	4094	3539	7633
B6D2F1 pESCs-2 versus R1 nfESCs	4599	4475	9074
Both[a]	1923	1406	3329 (2029)[c]

[a]Number of DEGs concomitantly in two comparisons of B6D2F1 pESCs versus B6D2F1 nfESCs and B6D2F1 pESCs versus R1 nfESCs; [b,c]Number in parenthesis indicates the number of the genes up-regulated and down-regulated in different comparisons (1618[b] and 1127[c] genes up-regulated in B6D2F1 nfESCs and down-regulated in R1 nfESCs, and 1148[b] and 902[c] genes up-regulated in R1 nfESCs and down-regulated in B6D2F1 nfESCs, respectively). DEG, differentially expressed genes; nfESCs, normally fertilized ESCs; pESCs, parthenogenetic embryonic stem cells.

Table II Gene ontology annotation of the common up-regulated or down-regulated DEGs in pESCs comparing with B6D2F1 and R1 ESCs derived from normal fertilization

Category	Function annotation of common DEGs in pESCs-1		Function annotation of common DEGs in pESCs-2	
	Up-regulated DEGs	Down-regulated DEGs	Up-regulated DEGs	Down-regulated DEGs
Biological process	Primary metabolic process/ cellular metabolic process/ macromolecule metabolic process/regulation of cellular process/cellular component organization and biogenesis/ regulation of biological process/regulation of metabolic process/ regulation of gene expression/transcription/ cell proliferation	Cellular developmental process/ primary metabolic process/cellular component organization and biogenesis/anatomical structure development/cellular metabolic process/regulation of biological process/cell development/ regulation of cellular process/ regulation of developmental process/macromolecule metabolic process/multicellular organismal development/cell cycle/anatomical structure morphogenesis/cell cycle process/death/cell motility/ localization of cell/regulation of a molecular function/cellular localization/establishment of cellular localization/catabolic process/ establishment of protein localization	Primary metabolic process/cellular metabolic process/macromolecule metabolic process/cellular component organization and biogenesis/regulation of cellular process/regulation of biological process/regulation of metabolic process/catabolic process/cell proliferation/transcription/cell development/localization establishment	Cellular developmental process/cell development/ anatomical structure development/cellular component organization and biogenesis/regulation of biological process/regulation of cellular process/regulation of developmental process/ death/multi-cellular organ development/anatomical structure morphogenesis/ primary metabolic process/cell cycle/cellular metabolic process/macromolecule metabolic process/cell cycle process/cell motility/ localization of cell/regulation of a molecular function/cellular localization/establishment of cellular localization/cell proliferation
First versus second	8/10 (80%) of identity	20/22 (91%) of identity	8/12 (67%) of identity	20/21 (95%) of identity
Molecular function	Protein binding/nucleic acid binding/transferase activity	Protein binding/nucleotide binding	Protein binding/nucleotide binding	Protein binding/nucleotide binding
First versus second	1/3 (33%) of identity	2/2 (100%) of identity	1/2 (50%) of identity	2/2 (100%) of identity
Cellular component	Intracellular/intracellular part/membrane-bound organelle/intracellular organelle/organelle part/ intracellular organelle part/ organelle envelope/protein complex/organelle membrane/organelle lumen	Intracellular part/intracellular/ intracellular organelle/ membrane-bound organelle/cell projection/cell part/leading edge	intracellular/intracellular part/ membrane-bound organelle/ intracellular organelle/organelle part/intracellular organelle part/ organelle envelope/protein complex/organelle membrane/ organelle lumen/ non-membrane-bound organelle	Intracellular part/intracellular/ intracellular organelle/ membrane-bound organelle/ cell projection/leading edge/ cell part
First versus second	10/10 (100%) of identity	7/7 (100%) of identity	10/11 (91%) of identity	7/7 (100%) of identity

This table shows only the terms satisfying *P*-value of <0.01 from the result of gene ontology term level 2 by DAVID annotation tool.

Nkiras1, *Cstf2*, *Pxmp2*, *Dnajb5*, *Art3* and *Sptlc2* were inhibitory hub genes, which networked with 67–96 genes and with 28–54 genes, respectively; these genes were listed as up-regulated and highly networked genes. In contrast, *Vegfb*, *Fh1*, *1500032D16Rik*, *Ogdh*, *Grsf1*, *Sdha*, *2410003P15Rik*, *Phyh*, *Coq5*, *Myl3*, *Acadl*, *Hrc*, *Golga4*, *Spag7*, *Higd2a*, *Gbe1*, *Foxh1*, *Src*, *Ndrg2* and *Hspb2* were stimulatory hub genes, and *Arl4c*, *Foxh1*, *Elovl1*, *Mlycd*, *Cdc42se1*, *Runx1*, *Klc2*, *Atp1a2*, *Hif3a*, *Iqgap1*, *9030612M13Rik*, *Hrc*, *Gnai3*, *Rbm39*, *Tuft1*, *Vegfb*, *Ndrg2*, *Litaf*, *Src* and *Zfp207* were inhibitory hub genes, which networked with 57– 88 genes and with 28– 64 genes, respectively; these genes were listed as down-regulated genes (Supplementary data, S3). The hub genes that ranked within the top 20 based on their networking mainly controlled protein transport, metabolism and ATP production, whereas several development-related genes including *Tuft1*, *Foxh1*, *Cdc42sel*, *Src*, *Ndrg2*, *Runx1* and *Vegfb* were either down-regulated or inhibitory to other networked genes in pESCs. Seven major genes that are considered as regulatory factors in pESCs-1 or nfESCs in the first comparison were randomly selected from the database, and their expression level was quantified. Of those genes selected, the patterns of 11 (79%) pairs from 14 total comparisons were consistent with results obtained from the DNA microarray (Supplementary data, S4). Lists of genes differentially expressed in each comparison are provided in Supplementary data, S5.

Discussion

Gene expression profiles of various pluripotent cells have been examined to reveal the molecular regulation of stem cells from somatic

Table III List of imprinted genes expressed differentially between pESCs and nfESCs of two strains

Sets of comparisons	Paternally expressed imprinted genes expressed differentially in pESCs		Maternally expressed imprinted genes expressed differentially in pESCs	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
First comparison using pESC-1				
B6D2F1 pESCs-1 versus B6D2F1 nfESCs	*Peg3*, *Usp29*, *Ndn*, *Mkrn3*	*Nnat*, *Peg10*, *Ins2*, *Slc38a4*	*Dlx5*, *Tssc4*, *Mirg*	*Copg2*, *H19*, *Gtl2*
B6D2F1 pESCs-1 versus R1 nfESCs	*Nnat*, *Slc38a4*	*Sgce*, *Ndn*, *Inpp5f*, *Igf2as*	*Gatm*, *H19*, *Gtl2*	*H13*, *Asb4*, *Atp10a*, *Cd81*, *Tssc4*
Both[a]	*Mcts2*, *Mest*, *Zfp264*, *Snrpn*, *Nap115*, *Impact*, *Dlk1*	*Igf2*	–	*Pon2*, *Cdkn1c*, *Grb10*, *Rian*, *Igf2r*, *Gnas*, *Ube3a*, *Phlda2*
Second comparison using pESC-2				
B6D2F1 pESCs-2 versus B6D2F1 nfESCs	*Peg3*, *Usp29*, *Ndn*, *Mkrn3*, *Peg12*	*Nnat*, *Peg10*, *Slc38a4*	*Tssc4*, *Mirg*	*Copg2*, *H19*, *Gtl2*, *Ascl2*, *Rian*
B6D2F1 pESCs-2 versus R1 nfESCs	*Nnat*, *Slc38a4*, *Ins1*, *Nespas*, *Nap115*	*Sgce*, *Ndn*, *Inpp5f*, *Igf2as*, *Peg12*	*Gatm*, *H19*	*H13*, *Asb4*, *Atp10a*, *Cd81*, *Tssc4*
Both[a]	*Mcts2*, *Mest*, *Zfp264*, *Snrpn*, *Impact*, *Dlk1*, *Ddc*	*Igf2*	–	*Pon2*, *Cdkn1c*, *Grb10*, *Igf2r*, *Gnas*, *Ube3a*, *phlda2*

[a]DEGs concomitantly in two comparisons of B6D2F1 pESCs versus B6D2F1 nfESCs and B6D2F1 pESCs versus R1 nfESCs.

tissue (Phillips *et al*., 2000; Ivanova *et al*., 2002; Park *et al*., 2002; Ramalho-Santos *et al*., 2002; Bhattacharya *et al*., 2004; Blanpain *et al*., 2004; Tumbar *et al*., 2004; Byrne *et al*., 2006). ESCs derived from parthenogenesis have a homozygous genome with minimal crossover-associated heterozygosity (Kim *et al*., 2007), so they can be used for autologous cell and tissue therapy. However, the molecular signature of pESCs has been poorly investigated to date.

In our results, differences in the origin of ESCs (normal fertilization with spermatozoa versus parthenogenetic activation in the same strain) significantly affected the gene expression profile of ESCs; of 34 000 genes evaluated by microarray, 22.6% (7776 genes) had altered expression in pESC-1. A concomitant change of strain (B6D2F1 versus R1) with alteration of ESC origin induced further change in gene expression profile (8943 genes; 26.5% of the genes tested). However, a change of ESC strain derived from normal fertilization affected gene expression more profoundly (15 750 gene probes; equivalent to 10 414 genes; 30.6% of the genes tested). In the second comparison using pESC-2, the change in gene expression after parthenogenesis was similar to that between strains. These results suggest that the alteration of gene expression induced by parthenogenesis is similar to, or quantitatively less, than that induced by strain differences. Parthenogenesis may therefore be less likely to affect the clinical feasibility of using ESCs for autologous cell therapy than genetic background. This suggestion was supported by statistical analysis to evaluate correlation coefficient. Our previous results showing that no significant difference in stem cell characteristics, including self-renewal and differentiation, was detected in pESCs when compared with nfESCs (Lee *et al*., 2008) also supported this hypothesis.

Parthenogenesis-derived ESCs are generated from homozygous embryos consisting only of a diploid female genome. It has been reported that the absence of paternal alleles in parthenotes results in the loss of paternally imprinted genes, which may restrict the development of parthenogenetic embryos (Kaufman *et al*., 1977; Barton *et al*., 1984). We, however, paradoxically detected expression of 17 paternally imprinted genes (*Mcts2*, *Mest*, *Nap115*, *Peg3*, *Usp29*, *Zfp264*, *Snrpn*, *Ndn*, *Mkrn3*, *Peg12*, *Impact*, *Dlk1*, *Nnat*, *Slc38a4*, *Ins1*, *Nespas* and *Ddc*) in pESCs. Our results are consistent with previous results (Jiang *et al*., 2007) finding expression of the paternally imprinted genes *Snrpn*, *U2af1-rs1*, *Peg3*, *Impact*, *Zfp127*, *Dlk1* and *Mest* in parthenogenesis-derived ESCs. pESCs in various species have been established to date (Allen *et al*., 1994; Cibelli *et al*., 2002; Fang *et al*., 2006; Revazova *et al*., 2007; Sritanaudomchai *et al*., 2007) and in some species, paternally imprinted genes are expressed in pESCs. From these results, aberrant expression of paternally imprinted genes after parthenogenesis may have little or no effect on ESC establishment and self-renewal in specific lines. Otherwise, transcription activity of paternally imprinted genes may be modified to adversely affect embryo development after parthenogenesis.

We utilized FDR levels to compare the change of gene expression after parthenogenesis, which has generally been used for statistical analyzing of microarray data. An FDR level <0.05 was equivalent to 1.2- to 1.4-fold difference in the strength of gene expression in our statistical analysis. Employing this system, we obtained 83% similarity between microarray and qRT–PCR, a level which was quite acceptable compared with other reports employing bioinformatics tool (Bhattacharya *et al*., 2005; Goossens *et al*., 2007; Ushizawa *et al*., 2007). The mRNA levels for *Dazl*, *Vegfb*,and *Foxh1* detected by qRT–PCR analysis did not match the microarray data, showing a 2.5-, 1.4- and 1.8-fold difference after parthenogenesis, respectively. If the significance level of the FDR is adjusted for detecting more than a 2-fold difference, the matching rate become significantly higher.

In this study, the two strains of nfESC were derived from blastocysts growing *in vivo*, while pESCs originated from an *in vitro*-culture system. Therefore, differences between *in vivo* and *in vitro* growth of embryos may interfere with determining the net effect of parthenogenesis on gene expression profile. Considering previous work reporting similar gene expression between *in vivo*-derived and *in vitro*-derived

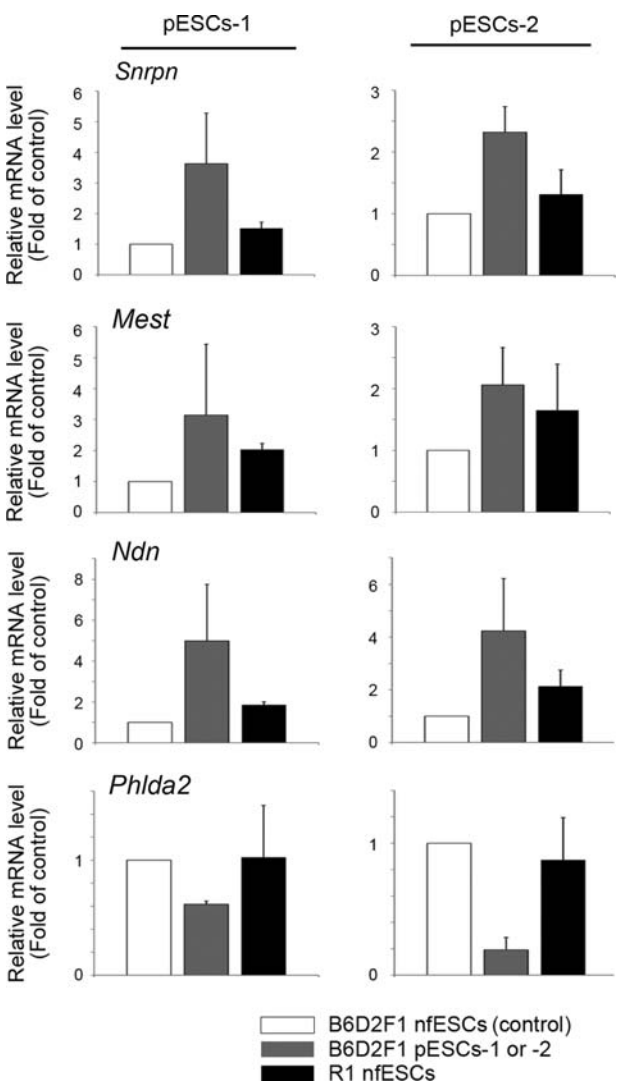

Figure 4 Quantification of mRNA expression of the imprinted genes either up-regulated or down-regulated in pESCs by RT–PCR. These genes were randomly selected from the lists of paternally-imprinted genes and both pESC-1 and pESC-2 were provided for this analysis. Triplicate PCRs were conducted for each gene and the relative value compared with two nfESCs is shown. In general, similar results from microarray Genechip data and real-time PCR were obtained from 14 (88%) of 16 comparison groups.

blastocysts (Whitworth *et al*., 2005), however, such a difference may only minimally affect the results obtained in this study. A report from Giritharan *et al*. (2007) demonstrated that the difference in gene expression between *in vivo*-growing and *in vitro*-growing blastocysts was not as great as that between *in vivo*-fertilized and *in vitro*-fertilized blastocysts. It is possible that the variation in expression of the hub genes found when comparing nfESCs of different strains is simply part of the natural variation between ESC lines. From a different viewpoint, it is possible that a greater change in gene expression may be induced by parthenogenesis under a different genetic background from B6D2F1.

We used two lines of ESC derived from parthenogenetic activation for DNA microarray analysis, and the use of only two lines limits the final conclusions from this study. Nevertheless, we carefully conclude that parthenogenesis consistently influences the expression of certain imprinted genes. This hypothesis is supported by the fact that the difference between gene expression of nfESCs and that of pESC was constant in all comparisons and that expression pattern of each pESC is very similar. It is possible that these imprinted genes, changing their expression after parthenogenesis, may be the key for elucidating the functional alteration of parthenogenesis-derived stem cells. The importance of imprinted genes for understanding characterization of parthenogenetic ESCs has been raised (Jiang *et al*., 2007).

In conclusion, we found alteration of gene expression in ESCs after parthenogenesis, but this was either similar to, or less than, that observed after a change of strain without altering the derivation of ESCs under a certain genetic environment. Accordingly, these results may imply that the same protocol utilized for nfESCs can be applied for pESCs. For evaluating the feasibility of use of pESCs, however, it is necessary to further examine the function of the imprinted genes with altered expression in every case of parthenogenesis. Large-scale experiments using multiple lines of pESC may also be required. The analytical system developed from this study may be useful for monitoring the clinical feasibility of various autologous stem cells, such as induced pluripotent stem cells (Revazova *et al*., 2007; Takahashi *et al*., 2007; Yu *et al*., 2007; Park *et al*., 2008), as well as pESCs.

Supplementary material

Supplementary material is available at *HUMREP* Journal online.

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