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| Original Article |

Promoted Expression of IGF-1, DNMT3a and OCT-4 in the Parthenogenetic Murine Blastocysts Developed in an Oil-Free Microtube Culture System may Support Stem Cell Generation

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Abstract : Oil-free culture system was implemented to generate parthenogenetic embryonic stem cell (PESC) lines in mice. The oocytes at metaphase II stage were activated and the parthenogenetic embryos were cultured in two different culture systems: The oil-free microtube culture method (MTC), and the traditional micro-drop culture method (Drop). The gene expression of the blastocysts in each culture system was analyzed by real-time PCR, and the efficiency of stem cell generation of these two culture methods was compared. The blastocyst outgrowth rate was not significantly different between the MTC (75.0% and 42.9%) and the Drop culture group (65.4% and 48.0%) in both C57BL6 x DBA2 F1 hybrid (B6D2F1) and C57BL/6 inbred strains respectively, and after seeding on the feeder layers, outgrowing inner cell masses were found in both groups. However, three PESC lines (two from B6D2F1 and one from C57BL/6 strains) were only established from the MTC system after passed until ESC colonies were formed while no PESC line was obtained from the blastocysts cultured in Drop. Gene expression levels of IGF-1, DNMT3a and OCT-4 were also higher for MTC derived blastocysts. The PESCs in MTC maintained normal murine ESC morphology and were positive to pluripotent markers such as ALP, OCT-4 and NANOG. In conclusions, Our MTC system, involving oil-free microtube culture method, was effective for the generation of PESC lines in either F1-hybrid (B6D2F1) or C57BL/6 mice, and this may be due to the promoted expression of developmentally important genes, such as IGF-1, DNMT3a and OCT-4.

Key words: *parthenogenesis, embryonic stem cells, oil-free culture*

1. Introduction

Several properties of parthenogenetic embryonic stem cells (PESCs) make them appealing as an alternative stem cell source. In addition, these cells are essential for understanding the differentiation process of mono-parental cells. Reportedly, when a parthenogenetic embryo is used to make embryonic stem cells (ESCs) and those cells are used to make chimera, chimeric offspring having tissues from PESCs can be obtained.[1] This result suggests that the PESCs can contribute to the variable tissues of a developing embryo. Moreover, the use of PESCs can avoid ethical concerns surrounding human ESC research because the parthenogenetic embryo itself cannot develop to term. These PESCs may improve organ transplantation efficiency by lessening the risk of major histocompatibility complex mismatch.[2,3] Due to these reasons, many groups harvested PESCs from various animal species, such as: mice,[2-4] monkeys,[5-7] rabbits,[8] buffalos,[9] and humans.[10-13] In order to obtain PESCs, artificial oocyte activation and *in vitro* culture (IVC) processes are required. Many groups have developed IVC systems for culturing fertilized oocytes in mice, pigs, and cows by altering physical conditions and introduced culture devices.[14-17] From those reports they were able to produce blastocysts efficiently. Typically, the starting material for generating mouse ESCs can be either a Day 3.5 expanded blastocyst or a further stage flushed from the uterine horns. In that system, the IVC process is not required to obtain the blastocyst. However, Liu et al.[18] used IVC-derived blastocysts

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as the starting material to obtain murine ESCs for the first time and showed that IVC systems for producing the blastocysts can influence the efficiency of ESC generation. Since ESCs are derived from the inner cell masses (ICMs) of the blastocysts, the quality of the blastocyst can determine the rate of successful ESC establishment.[18,19] For successful ESC generation, quality blastocysts should be provided efficiently. Previously, we reported that the microtube culture (MTC) system enhanced the developmental speed of embryos, blastocyst formation rate, and total cell number in blastocysts when compared with the conventional drop culture (Drop) system in the parthenogenetic murine embryo culture.[20] Because the embryos are placed together in the small area (< 2 mm diameter) of the U-shaped microtube bottom, the MTC system provides embryos more opportunities to affect each other through potential paracrine actions than a conventional drop culture system.[20]

In the present study, in order to increase the efficiency of mouse PESC isolation, we applied our MTC culture system to obtain more quality parthenogenetic blastocysts. The parthenogenetic blastocysts cultured either by Drop or MTC were seeded onto feeder layers, and their ICM outgrowth and PESC line establishment rates were compared. In addition, the gene expression levels of IGF-1, FGF-2, and EGF [for the cell growth],[21-23] IGF-2 [for imprinting status],[24] DNMT3a and DNMT3b [for methylation levels],[25] and NANOG and OCT-4 [for the level of pluripotency][26,27] in the blastocysts obtained by MTC and Drop were also analyzed by quantitative RT-PCR to compare their molecular potentials for generating PESC.

2. Materials and Methods

2.1 Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated. All media used were based on CZB and KSOM.[28,29] The mineral oil used in this study was an embryo-tested grade (cat. no. M-8410, Sigma).

2.2 Recovery of Oocytes, Parthenogenetic Activation, and IVC

All animal procedures were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-061023-1). Five- to seven-week-old female C57BL6 × DBA2 F1 hybrid mice (B6D2F1) and C57BL6 were superovulated with 5 IU equine chorionic gonadotropin followed by a second injection of 7.5 IU human chorionic gonadotropin (hCG) 48 h later. Oviducts were excised 15 h after hCG injection, and an average of 40 oocytes per mouse were obtained. Hyaluronidase (1 mg/ml) was used to remove cumulus cells, and oocytes were washed with Hepes-buffered CZB and exposed to an activation medium, consisting of 10 mM $SrCl_2$ with 5 μg/ml cytochalasin B in calcium-free CZB for 5 h. The activated oocytes were developed to the blastocyst stage in KSOM under two different culture conditions, which were described in our previous work.[20] Briefly, the activated oocytes were cultured either in a micro-droplet on a 35-mm cell culture dish (cat. no. 353002, Falcon; BD Biosciences, San Jose, CA, USA) of oil covering (Drop) or in a 250 μl microtube (the type commonly used for PCR; cat. no. PCR-02-C; Axygen, Union City, CA, USA) at the same embryo density (MTC). The oocytes were equally distributed to either MTC or Drop in a volume of 10 μl KSOM and then incubated for 5 days at 37.5°C under 5% CO_2 in air. After IVC, zona pellucida of the expanding blastocyst was removed by washing the embryo with acid-Tyrode solution.

2.3 Establishment of PESC Lines from Parthenogenetic Murine Embryos

To generate PESC, zona-free blastocysts were transferred onto a STO feeder layer in gelatinized 4-well tissue culture plates (Nunc, Rochester, NY, USA) in ESC medium consisting of Dulbecco's modified Eagle's medium (DMEM; high-glucose formation; Invitrogen, Carlsbad, USA) supplemented with 15% fetal bovine serum (Hyclone, Logan, USA), 1% nonessential amino acid stock (Invitrogen), 0.1 mM β-mercaptoethanol, and 1,000 units/ml leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, USA). After 6 or 7 days of culture, the outgrown clumps derived from the ICM were counted under the stereomicroscope. Once the ICM outgrowth was evaluated, the cell clumps were further trypsinized with 0.05% trypsin-EDTA and seeded onto gelatinized new 4-well dishes with fresh ESC medium in order to generate PESC. When the colonies appeared, they were considered passage 0 PESCs and propagated gradually under stringent culture conditions with careful monitoring and medium changing to maintain an undifferentiated state.

2.4 Characterization of Mouse PESC Lines

The PESCs after 20 or more passages were characterized by their expression of pluripotency markers. The PESCs that were grown on the cover slip coated with 0.1% gelatin were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed three times. After blocking with 1% bovine serum albumin in PBS for 30 min, cells were incubated with the first antibody. Antibodies used in

our experiments were: mouse monoclonal OCT-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 to 1:400), mouse monoclonal stage-specific embryonic antigen (SSEA)-1 and SSEA-4 (1:100). After washes with PBS, primary antibodies were applied using FITC-conjugated goat anti-mouse (Jackson Immunoresearch, West Grove, USA; 1:200). Alkaline phosphatase (ALP) staining was processed by manufacturer's instruction (Chemicon; SCR 001). The PESCs were fixed with 4% paraformaldehyde for 2 min and rinsed for 5 min with rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.05% Tween-20). During the rinse, reagents for ALP staining were prepared, as follows: Fast Red Violet with Naphthol AS-BI phosphate solution and water in a 2:1:1 ratio. After the stain solution was added, the cells were incubated in the dark for 15 min and washed with PBS, then observed.

2.5 *In Vivo* Differentiation

Early passage PESCs (2×10^6 cells per mouse) were injected subcutaneously into the rear thigh of a nude mouse (Balb/c, male). The ESCs from fertilized embryos (J1, 129Sv origin) were used as control. After 4 to 6 weeks, the mice with teratomas of 1-2 cm diameter were killed, and then the teratoma tissues were excised, fixed in 10% buffered formalin phosphate (Fisher, Loughborough, UK; SF 100-4), embedded in paraffin, and sectioned for histological analysis.

2.6 *In Vitro* Differentiation

PESCs were differentiated *in vitro* in a suspension culture without LIF. Floating ESC aggregates, called embryoid bodies (EBs), were formed in a bacteriological dish. On the second day of culture, EBs were transferred to a 15-ml tube and allowed to stand for 5 min until the EBs settled to the bottom of the tube. Then, the EBs were transferred to a new bacteriological dish and cultured for another 2 days. After 4 days in suspension culture, the gene expression levels representing 3 germ layers, AFP (endoderm), BRACHURY-T (mesoderm), and NESTIN (ectoderm), were measured by RT-PCR and real-time RT-PCR.

2.7 RT-PCR

At an early passage, trypsin-digested PESC and ESC cells were incubated in the bacteriological dish for 20 min to allow feeder cells to attach to the plates; then, PESCs and ESCs were harvested carefully. This step was repeated three times to remove feeder cells from the ESCs. RNA was extracted from undifferentiated PESCs/ESCs and EB using TRI reagent (Invitrogen). Reverse transcription for complementary DNA synthesis was performed with 6 μg RNA per sample using MMLV reverse transcriptase (Promega, Fitchburg, USA).

2.8 Real-Time PCR

Expression levels of developmentally important genes, such as IGF-1, FGF-2 and EGF (for growth), IGF-2 (for imprinting), DNMT3a and DNMT3b (for methylation), and NANOG and OCT-4 (for pluripotency) in the blastocysts (MTC vs. Drop) and AFP (for endoderm), NESTIN (for ectoderm) and BRACHURY-T (for mesoderm) in ESCs or PESCs were measured by real-time RT-PCR. For optimal quantification, primers were designed using Primer Express software (Applied Biosystems, Foster, USA). Real-time RT-PCR was performed using the ABI PRISM 7500 system and SYBR Green PCR Master Mix (Applied Biosystems). All points of the standard curve and all samples were run in triplets as technical replicates. The standard curves were calculated using the verified DNA as template for porcine GAPDH. In each run, 1 μl cDNA was

Table 1. Primer sequences (5'-3') used in real-time PCR

Gene name	Forward primer	Reversed primer
OCT-4	CCGTGTGAGGTGGAGTCTGGAG	GCGATGTGAGTGATCTGCTGTAGG
NANOG	GAAATCCCTTCCCTCGCCATC	CTCAGTAGCAGACCCTTGTAAGC
Alpha-fetoprotein (AFP)	CACACCCGCTTCCCTCATCC	TTCTTCTCCGTCACGCACTGG
BRACHURY-T	CACACCACTGACGCACAC	GAGGCTATGAGGAGGCTTTG
NESTIN	GGAGAAGCAGGGTCTACAG	AGCCACTTCCAGACTAAGG
DNMT3a	GATGTTCTTTGCCAATAACC	CAGGAGCCCTGTAGAATC
DNMT3b	CCTGCCCGCAAAGGTTTAT	GGCCACAACATTCTCGAACA
FGF-2	ATGGCGTCCGCGAGAAG	AGGTACCGGTTGGCACACA
EGF	GGTCCTGCTGCTCGTCTTG	TCCGCTTGGCTCATCACAA
IGF-1	GACAGGCATTGTGGATGAGT	GATAGAGCGGGCTGCTTTTG
IGF-2	CCCAGGTGTTTGCCTCAACT	ATTAGGTTTGCGAGCGTTAA
Gapdh	GCATGGCCTTCCGTGTTCCTA	CTTCAGTGGGCCCTCAGATGC

used as a template added to 5 µl double-distilled water, 2 µl forward and reverse primers (20 pmol/ml), and 10 µl SYBR Green PCR Master Mix. The following amplification procedure was employed: denaturation stage (95°C for 10 min), amplification and quantification repeated 40 times (94°C for 15 sec, 60°C for 1 min with single fluorescence measurement), dissociation curve stage (temperature increments of 0.1°C per 30 sec starting from 60 to 95°C with fluorescence measurements). Data was analyzed with 7500 System Sequence Detection software (Applied Biosystems), which, for all samples, calculated the starting quantities of all candidate reference genes, based on the standard curves for these genes. Primer sequences were described in Table 1.

2.9 Statistical Analysis

Outgrowth rates were analyzed using the Chi-square test of SPSS (Version 12.0; SPSS Inc., Chicago, IL, USA). Mean gene expression values were analyzed using the *t*-test to compare parameters between the different study groups. Difference at $P<0.05$ was considered significant.

3. Results

3.1 ICM Outgrowth Rate of Blastocysts Derived Either from MTC or Drop Methods

The blastocyst outgrowth rate was not different significantly between the MTC (75.0% and 42.9%) and the Drop culture group (65.4% and 48.0%) in both strains (B6D2F1 and C57BL/6, respectively; Table 2). Although outgrowing inner cell masses were found in both groups after seeding on the feeder layers, three PESC lines had been only generated from the MTC system (two lines from B6D2F1, and one line from C57BL/6) while no PESC line was generated from the blastocysts cultured via the Drop method.

Table 2. The outgrowth and PESC[1] generation from the blastocysts of B6D2F1[2] or C57BL/6 strain mice

Mouse strain	Group[3]	Blastocysts	Outgrowth on the feeder layer (%)	PESC line
B6D2F1	Drop	26	17 (65.4%)	0
	MTC	32	24 (75.0%)	2
C57BL/6	Drop	25	12 (48.0%)	0
	MTC	35	15 (42.9%)	1

Three replicates.
[1]Parthenogenetic embryonic stem cell line.
[2]C57BL/6 × DBA2 F1-hybrid.
[3]Drop: conventional micro-drop culture, MTC: microtube culture.

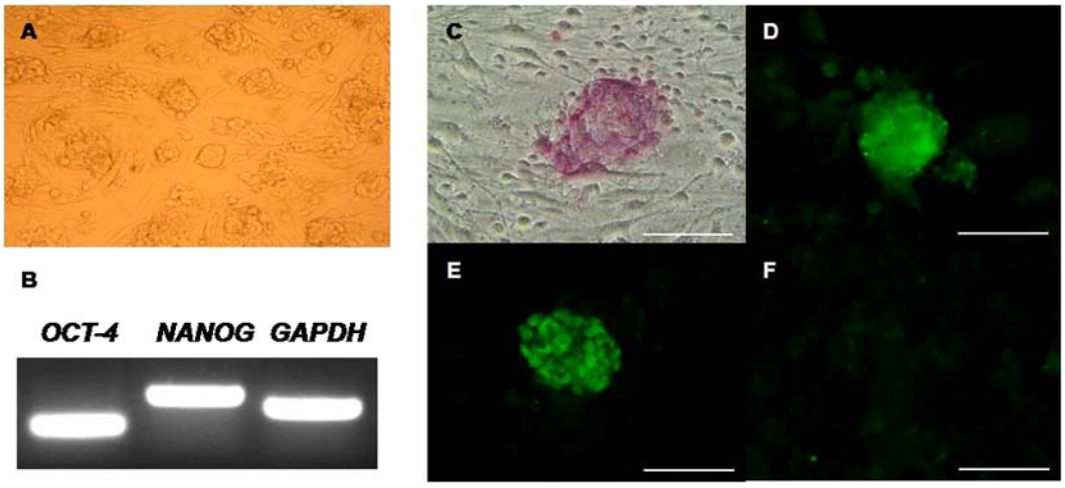

Figure 1. Parthenogenetic embryonic stem cells (PESCs) derived from the mouse parthenogenetic blastocysts (C57BL/6 × DBA2 F1-hybrid: B6D2F1) produced in a microtube culture system (MTC). The figure is from the first PESC cell line derived from the B6D2F1 blastocyst (BDF-PESC1). (A) Outgrown cell colonies at passage 4. (B) The PESCs showed the expression of the pluripotency marker genes, OCT-4 and NANOG, by RT-PCR. (C-F) The expression of pluripotent stem cell-specific markers was confirmed by immunostaining. The PESCs were positive for alkaline phosphatase (C), stage specific embryonic antigen-1 (D), and OCT-4 (E) while negative for stage-specific embryonic antigen-4 (F). Scale bar=100 µm.

3.2 Characterization of PESCs

The PESCs derived via MTC showed typical mouse ESC morphology (stacked like a dome or an oval with clear boundaries), and the cells adhered tightly to each other, making it impossible to visualize individual cells in the colonies. Immunochemical analysis showed that the cell lines are positive for pluripotent mouse stem cell markers, including OCT-4, SSEA-1, and ALP (Fig 1). In addition, RT-PCR analysis revealed that pluripotency genes OCT-4 and NANOG are expressed in PESCs. The figures presented here (Fig 1 to 3) were from the first PESC cell line derived from the B6D2F1 blastocyst (BDF-PESC1). The second PESC from B6D2F1 and the PESC from C57BL/6 also showed similar characteristics (data not shown).

3.3 *In Vitro* Differentiation: EB

The differentiating potential of EB was analyzed using RT-PCR. After 4 days in suspension culture, PESC-derived EBs only expressed genes representing mesoderm (BRACHURY-T) and ectoderm (NESTIN), not endoderm (AFP; Fig 2) while ESC-derived EBs expressed genes representing three germ layers.

3.4 *In Vivo* Differentiation

Teratomas derived from PESCs showed only ectodermal and mesodermal lineage differentiation (Fig 3). Rosettes of neural epithelium (Fig 3A), gut-like epithelium (Fig 3B), stratified squamous cells (Fig 3C), and fibrous tissues (Fig 3D) are shown in ESC-derived teratomas, while immature neural tissues (Fig 3E), gut-like epithelium (Fig 3E), neural tube structure (Fig 3F), blastemal tissue (Fig 3G), and glandular cells (Fig 3H) were shown in PESC-derived teratomas. No endodermal tissue was observed in PESC-derived teratoma samples.

3.5 Gene Expression Analysis in Parthenogenetic Murine Blastocysts

Gene expression levels of IGF-1, DNMT3a, and OCT-4 were significantly (P<0.01) higher in MTC-derived blastocysts than Drop culture-derived blastocysts (Fig 4). IGF-1 and OCT-4 gene expression in MTC-derived blastocysts were 4-fold higher than in Drop culture-derived blastocysts. Gene expression levels of other genes, such as FGF-2, IGF-2, DNMT3b, NANOG and EGF, were not significantly different between the two groups.

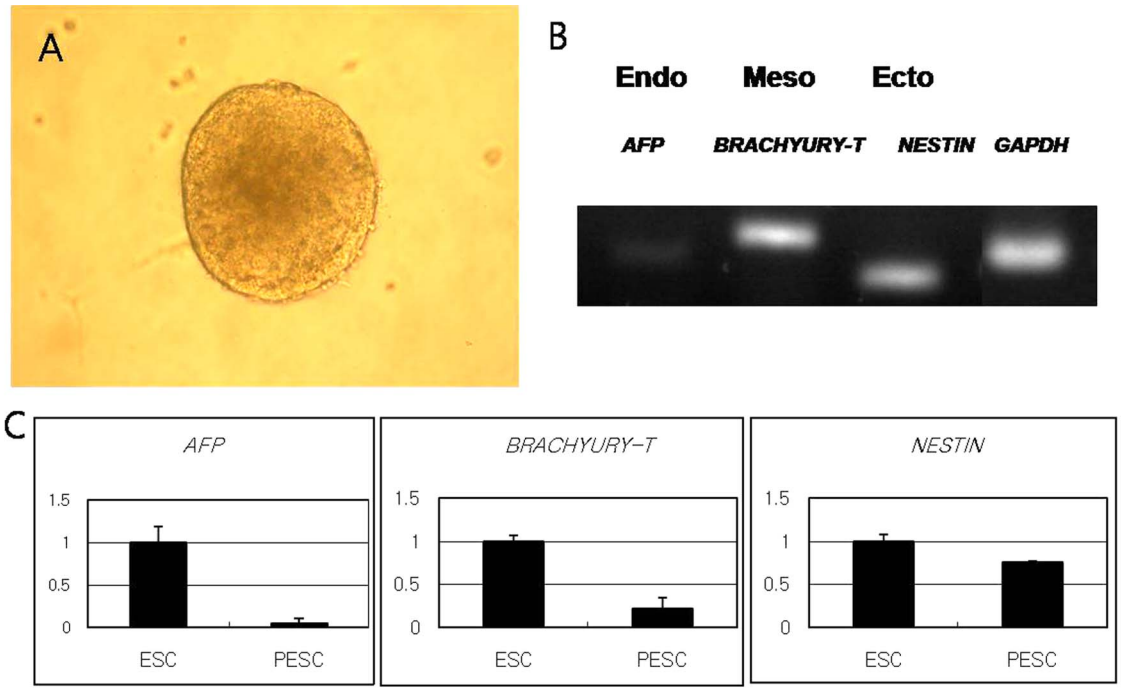

Figure 2. Differentiating potential of the embryoid body (EB) derived from the PESC: (A) Spherical EB was formed from the PESC (BDF-PESC1). (B) Expression of endodermal (AFP), mesodermal (BRACHYURY-T), and ectodermal (NESTIN) markers in EB were analyzed by RT-PCR. The EB from PESC does not show endodermal differentiation potential. (C) The graphs show gene expression level of differentiating EB derived from PESC and ESC measured by real time RT-PCR. Expression of endoderm-specific marker gene (AFP) is not detected in PESC.

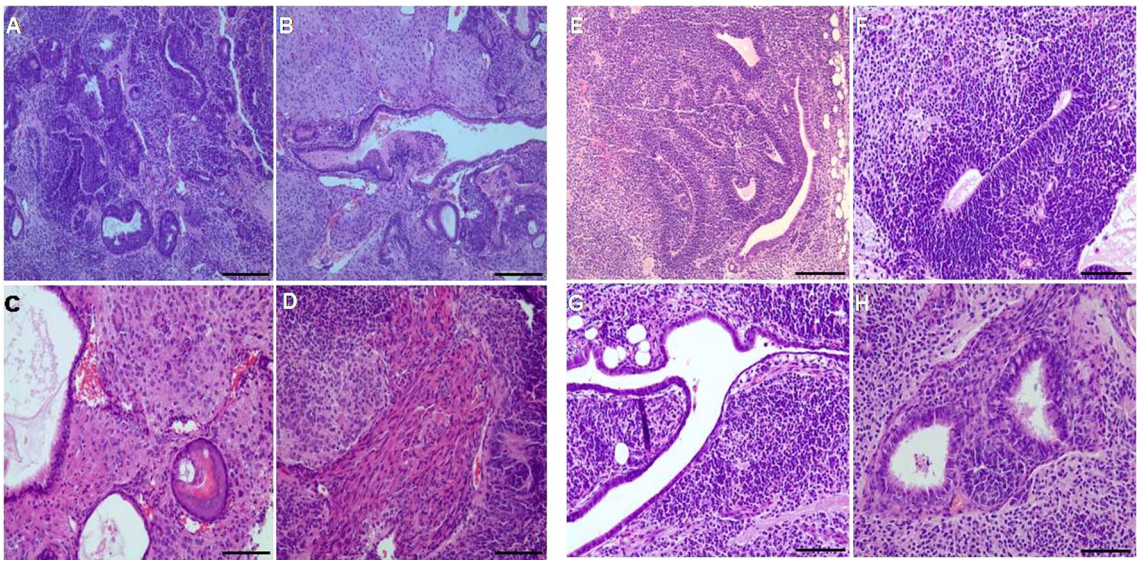

Figure 3. Transplantation of ESCs or PESCs from B6D2F1 embryos under the skin of the nude mouse. (A-D) Histological analysis of the ESC-derived teratoma. Rosettes of neural epithelium (A), gut-like epithelium (B), stratified squamous cells (C), and fibrous tissues (D) are shown in ESC-derived teratomas. (E-H) Histological analysis of the PESC-derived teratomas. Immature neural tissue (middle and left) and gut-like epithelium (right) (E), neural tube structure and blastemal tissue (F), gut-like epithelium (G), and glandular cells (H) are shown in PESC-derived teratomas. Scale bar=200 μm for (A, B, E) and 100 μm for (C, D, F, G, H).

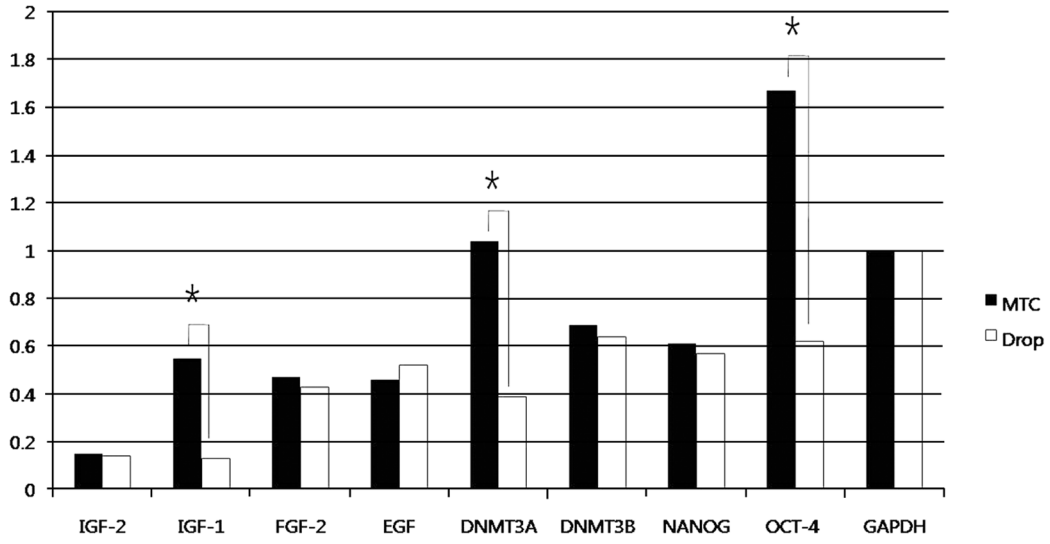

Figure 4. The graph represents the expression levels of developmentally important genes, such as IGF-1, IGF-2, FGF-2, EGF, DNMT3a, DNMT3b, NANOG and OCT-4, in parthenogenetic B6D2F1 blastocysts derived from either MTC or Drop, as measured by real time RT-PCR. Gene expression levels of IGF-1, DNMT3a, and OCT-4 are significantly higher (*P<0.01) in MTC-derived blastocysts than Drop culture-derived ones.

4. Discussion

In the previous report, we presented a new oil-free embryo culture system, called MTC.[20] This new culture system provides an oil-free culture environment and is easy to handle, as well. In addition, the MTC system enhanced the development of pre-implantation stage murine embryos. More embryos in MTC reached the blastocyst stage with a larger number of trophectodermal cells and developed faster than those in conventional micro-drop culture. Here, as the next step,

we established the ESC lines from parthenogenetic murine blastocysts produced *in vitro* and found that the embryo culture system can influence the efficiency of ESC generation.

The aim of the present study was to introduce the MTC system as a more effective way of generating PESC lines than the traditional oil-covered micro-droplet culture. Another aim was to generate a PESC line from the parthenotes of the C57BL/6 inbred mouse strain, which is more difficult than generating a cell line from 129Sv or F1-hybrid, such as B6D2F1 or C57BL/6 x CBA.[30-32] After the first derivation of ESC lines from the mouse blastocysts, several standard and modified protocols were introduced. However, these protocols produce ESCs with only low efficiency in the most outbred/inbred strains and require specialized tools for embryo handling.[31-33] During the long process of ESC derivation, IVC of the embryos can be a critical step because obtaining qualified blastocysts can result in successful ESC production.[34] Since the ICM of the blastocysts is the source of ESC, the ICM's condition is one of the important and conclusive factors for generating the ESC line. Although our result here did not show a significantly higher ICM outgrowth rate in MTC-derived parthenotes, we had shown that MTC system supports more blastocyst formation and more cells in the blastocyst in the previous report[20] and this might resulted in the generation of MTC-derived PESC lines in the present work.

To investigate the PESC generation potential of the MTC-derived blastocysts at molecular level, we compared the expression levels of genes related with embryonic cell growth (IGF-1, IGF-2, FGF-2, and EGF), methylation status (DNMT3a and DNMT3b), and pluripotency (OCT-4 and NANOG), in the blastocysts from either the MTC or Drop culture systems to investigate the differences of their ESC potential at the molecular level. The IGF family is important for the early embryogenesis in mammals.[22] The addition of IGFs to the culture medium increased the blastocyst rate and ICM cell number in the embryos produced *in vitro*.[35] Other growth factors, FGF and EGF, also have important roles during the early embryogenesis.[21,36,37] Among the growth factor genes we analyzed, the expression levels of IGF-2 were the same in both MTC and Drop. Since IGF-2 is a paternally expressed imprinting gene, this result indicates that the MTC system does not affect the imprinting status in the parthenogenetic blastocysts. However, expression of IGF-1 is significantly higher in the MTC blastocyst, and this may have resulted in higher blastocyst development and ICM cell number in the embryos as previously reported.[20] DNA methylation is implicated in controlling imprinting gene expression, X chromosome silencing, and embryonic development. It is also believed that methylation protects the genome from parasitic elements, such as transposons and viruses.[25] The murine DNMT3 family consists of two genes, DNMT3a and DNMT3b, which are essential for *de novo* methylation in murine ESC and early murine embryos but down-regulated after differentiation and expressed at low levels in adult somatic tissues.[38,39] Abnormal or weak expression of both DNMT3a and DNMT3b interrupts de novo DNA methylation in ESC or genome-wide de novo methylation during early mammalian development.[40] Therefore, highly expressed genes of the DNMT3 family are essential in generating ESCs.[25] DNMT3b is also expressed in mouse hematopoietic progenitor cells, spermatogonia, and during neural cell development in the murine embryo.[41,42] Here, we found that expression of DNMT3a increases significantly in the MTC group, whereas DNMT3b does not. This result suggests that our MTC system for embryo culture may affect the methylation status of *in vitro*-produced parthenogenetic embryos. This may be caused by *de novo* DNA methylation (a role of DNMT3a) in ESCs. The genes related to cell pluripotency, such as OCT-4 and NANOG, affect ICM quality and ESC line establishment.[26,27] Those genes also affect the expression of up- and down-stream genes, as well as embryonic growth and development during early embryogenesis.[27] The parthenogenetic blastocysts in MTC showed higher OCT-4 expression than those in Drop although the number of ICM cells was not increased in MTC.[20] On the other hand, no difference was found in NANOG expression between the two groups. As OCT-4 (POU transcription factor, also known as Oct-3 or Oct-3/4) is known as an ICM-related gene,[26] higher expression of this gene may correlate with an increased ICM number in MTC-derived blastocysts. Although OCT-4 expression is known to follow FGF expression in the pre-implantation embryos,[26] an increased OCT-4 level in the MTC system may not be due to FGF because there is no difference in the FGF expression level between MTC and Drop systems. Promoted expression of developmentally important genes in the MTC-derived blastocysts, such as OCT-4, IGF-1, and DNMT3a might result in more blastocysts, faster embryonic development, and larger cell numbers in the MTC blastocysts[20] and these should support generation of pluripotent stem cells from the parthenogenetic murine embryos.

In vivo and *in vitro* differentiation experiments showed interesting results. In general, the teratoma from ESC forms three germ layer lineage cells in the immune-deficient mouse, and this proves ESC pluripotency *in vivo*. However, in our experiment, PESC showed only 2-germ layer (mesoderm, ectoderm) differentiation in the teratoma (Fig 3). No endoderm tissue was observed in PESC-derived teratoma samples in our

experiments. No endoderm-specific gene expression was observed in PESC-derived EBs, as well (Fig 2). This phenomenon may due to abnormal imprinting gene expression in PESCs. Although other reports also claim that PESC shows restricted tissue distribution,[1,43] a recent study shows no contribution restriction in PESC chimeras.[44] Difference of the cell-lines used in each study is may be one of the explanation as well as the other environmental factors.

One murine PESC line was established from MTC-derived C57BL/6 blastocysts, whereas no cell line was founded from Drop-derived ones. This is the first report of ESC line establishment from parthenogenetic blastocysts of the C57BL/6 mouse strain, although our present data shows lower establishment rate of PESC in B6D2F1 than other groups reported.[1,45] During the manipulation of mammalian embryos, such as *in vitro* production, intracytoplasmic sperm injection, or nuclear transfer, the oocytes or embryos exposed to an artificial or unnatural environment show poorer developmental competence than *in vivo* ones.[46] This phenomenon occurs more often in inbred or outbred strains than in F1-hybrids in the production of *in vitro* embryos and ESC line establishment.[47,48] Since the generation of an ESC line from some inbred mice, such as C57BL/6, is rarely successful, our MTC system may provide a tool for efficiently generating ESCs (or PESCs) from inbred murine species, especially after *in vitro* manipulation of the oocytes or embryos of those strains.

After the first derivation of ESC lines from blastocysts, several standard and modified protocols were introduced. These protocols produce ESC with low efficiency in most outbred strains and require specialized tools for embryo handling.[33,49,50] Specifically conditioned medium,[31,47] genetic modification of the embryo,[51] and microdissection of the blastocyst[51] improve mouse ESC generation. Such modifications improve ESC efficiency but require specialized techniques. Because obtaining qualified blastocysts is a pre-requisite for successful ESC generation and, although many other laboratories have focused on the post-blastocyst seeding step to enhance ESC generation efficiency, our MTC system that increases the number of qualified blastocysts might result in efficient ESC generation.

In conclusion, the MTC system, involving oil-free microtube culture method, is an effective embryo culture method for generating PESC lines in either F1-hybrid (C57BL/6×DBA2) or C57BL/6 mice, and this may be due to the promoted expression of developmentally important genes, such as OCT-4, IGF-1, and DNMT3a.

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