In Vitro Induction of Potential Primordial Germ Cells from Mouse Embryonic Stem Cells by Culture with Undifferentiated Gonadal Cells

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Abstract: *In normal development, a limited number of cells in the proximal epiblast differentiate into germ cells. In this process, the interaction between primordial germ cells (PGCs) and surrounding somatic cells in the gonad is essential for germ cell development. In the present study, we demonstrated a novel method for inducing mouse embryonic stem cells (ESCs) to differentiate into PGCs using co-culture with undifferentiated gonadal cells or with their extracts. After embryoid bodies (EBs) formed, they were cultured for 10 days. Mvh and Dazla gene expressions increased to a peak, and also the number of Mvh-positive cells increased and were distributed in the peripheral region of EBs co-cultured with undifferentiated gonadal cells, whereas for EBs cultured in the medium supplemented with cell extracts, Mvh-positive cells were scattered within the EBs. Our results demonstrate that growth factors derived from undifferentiated gonadal cells may induce differentiation of ESCs into potential PGCs.*

Key words: *Embryonic stem cells (ESCs), Gonad, Primordial germ cells (PGCs)*

Introduction

Embryonic stem cells have the potential to be an important tool for establishing organogenesis as well as producing transgenic animals. Many trials attempting to induce ESCs to differentiate into somatic cells have been performed, and recent studies have reported successful differentiation of mouse ESCs into mature male and female gametes *in vitro* and *in vivo* [1–3]. It seems quite probable that ESCs differentiated in gametes via PGCs *in vitro*. After successive *in vitro* or *in vivo* culture, they occasionally form early spermatids or oogonia, the latter of which develop to follicles, ovarian-like structures or oocytes that subsequently develop to blastocyst-like structures, presumably due to parthenogenetic activation. Recently, immature sperm cells derived from murine ESCs in culture have generated live offspring [4, 5], although how such gamete-like cells are generated during stem cell culture remains unclear especially as *in vitro* conditions are ill-defined. Strategies for manipulating stem cell differentiation should be based on knowledge of the mechanisms by which lineage decisions are made during early embryogenesis. In normal development, egg and sperm (gamete cells) of the mouse are derived from a founder population of PGCs that arise in the proximal epiblast, a region of the early mouse embryo. Therefore, it seems to be necessary to first induce ESCs into EBs with tissue lineages typical of PGCs in order to produce gametes *in vitro* [4, 5]. Considering that there is no defined culture medium for germ cell proliferation and differentiation, a better way to induce differentiation of ESCs in PGCs *in vitro* may be to utilize a co-culture system with undifferentiated gonadal cells in which PGCs can proliferate. In the present study, we examined the competence of undifferentiated gonadal cells from mouse fetuses to differentiate ESCs into PGCs.

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Materials and Methods

Animals, feed and housing

The present study was approved by the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research of the Graduate School of Agricultural Science, Tohoku University. ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and bred in our laboratory. Animals were kept in polycarbonate cages (25 cm × 40 cm × 20 cm) with wood shavings under controlled conditions with lights on at 6:00 and off at 18:00, and given food and tap water *ad libitum*.

Preparation of undifferentiated gonadal cells from mouse fetuses

Undifferentiated gonads were isolated from mouse fetuses on day 11.5 of gestation. The samples were dissected in Dulbecco's phosphate buffered saline (PBS; Nissui, Tokyo, Japan) and minced with a surgical blade on a 35 mm culture dish. The minced gonadal tissues were dissociated and washed in PBS followed by incubation in PBS supplemented with 0.1% (w/v) trypsin (BD Bioscience, MD, USA) and 1 mM of EDTA (DOJINDO, Japan) for 30 min. After trypsinization, an equal amount of the culture medium, Dulbecco's Modified Eagle Medium (DMEM; GIBCO, USA) containing 10% (v/v) fetal bovine serum (FBS, Gemini Bio-Products, CA, USA) was added, and then the cells were washed once by centrifugation at 190 *g* for 5 min and seeded into 100 mm plastic culture dishes. After seeding, the cells were cultured for 6 to 8 days in DMEM supplemented with 10% (v/v) FBS and 50 μg/ml each of penicillin and streptomycin (Invitrogen, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The gonadal cells were subjected to each experiment.

Collection of cell extracts from undifferentiated gonads

The undifferentiated gonads of day 11.5 of gestation were collected and trypisinized by the same method as described above. The gonads prepared were washed twice in PBS and once in cell lysis buffer (pH 8.2) consisted of 20 mM Hepes, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol, and a cocktail of protease inhibitors (P8340; Sigma, USA). Sediment cells were resuspended in ice-cold cell lysis buffer and homogenized by sonication using an ultrasonic disruptor (TOMY, Japan). The lysate was centrifuged at 15,000 *g* for 15 min at 4°C, and the supernatant (cell extracts) was collected and stored at –80°C until use.

Cell culture and in vitro differentiation

ESCs derived from F1 mice crossbred between C57BL/6 and CBA were purchased from Invitrogen. ESCs were maintained on a feeder layer of mitomycin C-treated STO cells in 0.1% gelatin-coated tissue culture plates in stem cell medium (SCM; DMEM medium supplemented with 20% FBS, 100 μM non-essential amino acids (Invitrogen, USA), 0.1 mM β-mercaptoethanol (Sigma, USA), and 50 μg/ml each of penicillin and streptomycin), and were passaged every 3 days by 0.25% (w/v) trypsin and 5 mM of EDTA for dissociation. For preparation of STO and gonadal cells before use, each cell was cultured to confluence in the culture medium. To induce formation of EBs, ESCs were transferred to non-tissue culture grade 35-mm petri dishes (SUMILON, Japan) to prevent adherence to the surface of the culture dish and to allow proliferation and aggregation. ESCs were seeded at 2×10^4 cells per Petri dish and cultured in SCM. After 5 days, EBs formed were transferred on a cell culture insert with a polyethylene terephthalate (PET) track-etched membrane (pore size, 0.4 μm; BD Biosciences), and then, the cell culture insert was set up in a culture dish seeded with undifferentiated gonadal cells. In the case of treatment with cell extracts, EBs that formed were cultured in the culture medium supplemented with cell extracts in which the protein concentration was adjusted to 40 μg/ml.

RT-PCR for marker genes

Total mRNA was isolated from undifferentiated gonadal cells, ESCs and STO cells with the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA), and each genomic DNA was digested by using the RNase-Free DNase kit. Total RNA was treated with DNase I (Qiagen) on the spin column at room temperature for 15 min, and 20% of the resulting purified RNA was set aside for a non-RT control. The remaining RNA was reverse-transcribed into cDNA using SUPERSCRIPT II Reverse Transcriptase (Invitrogen). RNAguard (Amersham Biosciences) was used for protection from RNase. PCRs were performed with the Qiagen HotStarTaq DNA Polymerase kit with the relevant primers. Each reaction mixture contained cDNA solution, 0.2 μM of each primer, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 10% Triton-X, and 1 U of rTaq polymerase (Promega). All RT-PCRs were carried out at least three times for each individual RNA sample. To detect genomic DNA contamination, total RNA was subjected to RT-PCR without reverse transcriptase using each primer pair.

The RT-PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Primers used were: Ad4BP/SF-1(f) 5'-AGCAAGAGCAGAGTAACCGA-3', Ad4BP/SF-1(r) 5'-GAGGTTGTTGAACGCGACTT-3'; Lhx9(f) 5'-TGGCATCTTAGGTGCCTGAA-3', Lhx9(r) 5'-TTGCAAGAGGTTCTCGAAGT-3'; Wt-1(f) 5'-AACCACGGTATAGGGTACGA-3', Wt-1(r) 5'-GGTTTCTCACCAGTGTGCTT-3'; M33(f) 5'-CAGAGCTGAAGGATCCCATT-3', M33(r) 5'-GCGGTTCATGTAGTGTACGA-3'; Follistatin (Fst) (f) 5'-AGCAAGGAAG AGTGTTGCAG-3', Fst(r) 5'-CTCTTTGCATCTGGCCTTGA-3'; *Oct-3/4*(f) 5'-GGCGTTC TCTTTGGAAAGGTGTTC-3', *Oct-3/4*(r) 5'-CTCGAACCACATCCTTCTCT-3'; and β-actin(f) 5'-GGCCCAGCGCAAGAGAGGTATCC-3', β-actin(r) 5'-ACGCACGATTTCCC TCTCAGC-3'.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described in a previous report [6] using primers for mouse vasa-homolog (Mvh) (f) 5'-GGAAACCAGCAGCAAGTGAT-3', Mvh(r) 5'-TGGAG TCCTCATCCTCTGG-3', Dazla(f) 5'-GTGTGTCGAAGGGCTATGGAT-3', Dazla(r) 5'-AC AGGCAGCTGATATCCA GTG-3' and β-actin. For semi-quantitative PCR, their cycle numbers were optimized to ensure amplification of cDNA in the exponential phase of PCR. The cycle numbers used were as follows: 30 for Mvh, 30 for Dazla and 24 for β-actin. The amplified products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. For quantification, the intensity of the objective bands was quantified by densitometric scanning using NIH Image version 1.62 free software (NIH, Bethesda, MD). The respective values of Mvh and Dazla were normalized according to those of β-actin to evaluate in arbitrary units the relative abundance of the targets.

Immunocytochemical examination of Mvh

After being washed three times in PBS containing 0.1% polyvinyl alcohol (PBS-PVP; Sigma, USA), specimens such as EBs, ESCs and gonads were fixed in 2% paraformaldehyde (Wako, Tokyo, Japan) in PBS-PVP supplemented with 0.2% Triton X-100 (Wako) for 40 min. After fixation, the specimens were washed in PBS-PVP twice for 15 min each, and stored in PBS-PVP containing 1% bovine serum albumin (BSA-PBS-PVP; Sigma, USA) overnight. On the following day, the specimens were blocked with 10% FBS in BSA-PBS-PVP for 40 min, incubated in BSA-PBS-PVA containing a polyclonal anti-DDX4/VASA antibody (1:500 dilution; ABCAM) at 4°C overnight, washed in BSA-PBS-PVP 3 times for 15 min each, and incubated with molecular probe alexa fluor 488 conjugated goat anti-rabbit IgG (1:200 dilution; Molecular Probes) for 40 min at room temperature. After being washed in PBS-PVP 3 times for 15 min each, they were mounted onto slide glasses. Immunocytochemical examination was carried out using a confocal microscope (MRC-1024; Hercules, CA, USA, Bio-Rad). The Laser Sharp Processing software (Bio-Rad) was used to analyze the confocal images [7].

Results

Induction of potential PGCs in EBs by culture with undifferentiated gonadal cells

Before co-culture with undifferentiated gonadal cells, single ESCs were allowed to form EBs. After formation of EBs, they were co-cultured with undifferentiated gonadal cells by transferring them onto the insert membrane and setting it up in a culture dish seeded with gonadal cells. The expression of PGC markers, Mvh [8] and Dazla, was analyzed by semi-quantitative RT-PCR throughout co-culture of EBs from 2 to 20 days after the start of culture (Fig. 1). The transcripts of Mvh in EBs gradually increased and at Day 6 reached a level comparable to that of undifferentiated gonadal cells. At Day 10 of culture, both expressions of Mvh and Dazla reached a peak, increasing over two times compared to the early days of culture and also compared to Day 10 EBs cultured without the gonadal cells and undifferentiated ESCs.

To ascertain the extent of Mvh-positive cells, contribution to the increment of gene expression in EBs, we used immunohistochemistry to visualize Mvh-positive cells in Day 10 EBs co-cultured with undifferentiated gonadal cells (Fig. 2b). Mvh-positive cells existed in the peripheral part of EBs but not at the center of EBs, showing that the outer cells which were exposed to soluble factor(s) secreted from undifferentiated gonadal cells could be differentiated to potential PGCs. On the other hand, Mvh-positive cells did not exist in EBs cultured alone (Fig. 2g), and also in undifferentiated ESCs (Fig. 2h). Mvh-positive cells existed collectively in undifferentiated gonads (Fig. 2i).

Induction of potential PGCs in EBs by culture with cell extracts

In the first experiment using co-culture with undifferentiated gonadal cells, we showed that co-culture but not direct cell contact may help to induce

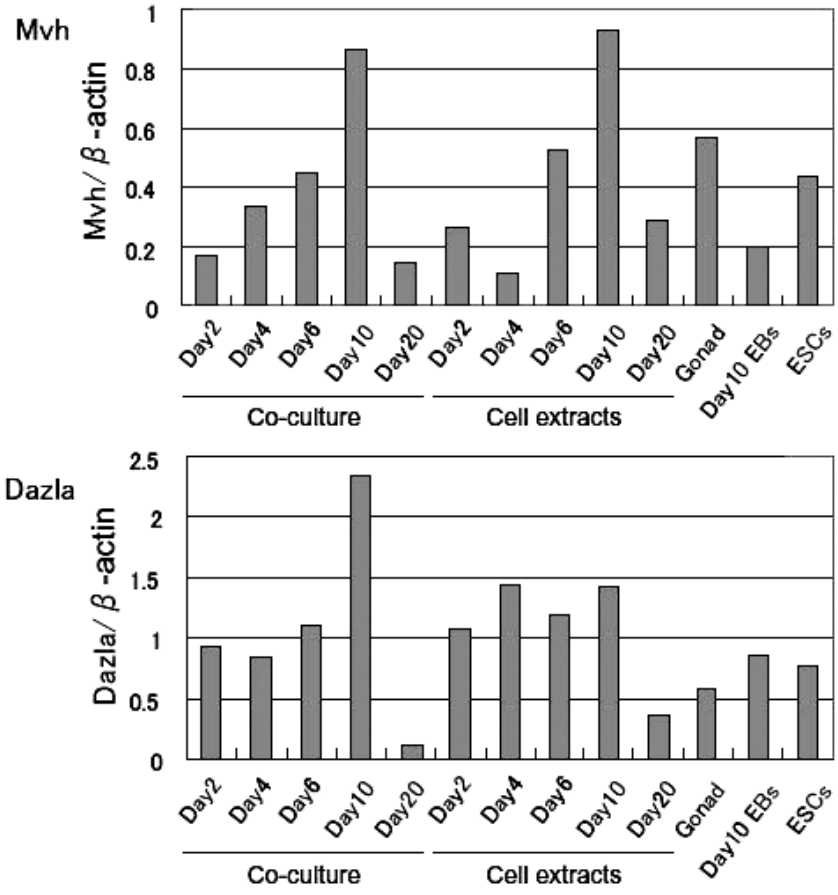

Fig. 1. Profiles of expressions of Mvh and Dazla genes in cultured EBs by semi-quantitative RT-PCR. mRNAs were prepared from EBs cultured for 2–20 days with either undifferentiated gonadal cells or cell extracts, undifferentiated gonads and EBs cultured alone for 10 days. The values were standardized to the level of β-actin expression which served as an internal marker.

potential PGCs in EBs. Thus, it is possible that the cell extracts from undifferentiated gonadal cells may contain effective factor(s) for differentiating EBs into PGCs. To examine this possibility, EBs were cultured in a medium supplemented with cell extracts.

The overall gene expression of Mvh was similar to that in EBs co-cultured with the gonadal cells (Fig. 1), showing a peak on Day 10 of culture, while the expression of Dazla at Day 10 did not show a remarkable increase. Interestingly, the localization of Mvh-positive cells was quite different from that in EBs co-cultured with gonadal cells (Fig. 2b); Mvh-positive cells were homogeneously distributed throughout the whole of the EBs (Fig. 2e).

Gene expressions in undifferentiated gonadal cells

To speculate what factor(s) would be effective for induction of potential PGCs from EBs, we analyzed typical gene expressions in STO cells, ESCs and undifferentiated gonadal cells. Total mRNA isolated from each cell was analyzed by RT-PCR to detect expression of genes related to germ cell development (Ad4BP/SF-1, Wt1, Lhx9, M33, Fst and BMP4) and ESCs pluripotency (the POU domain transcription factor Oct-3/4) [9–13]. All of these genes were expressed in undifferentiated gonadal cells, though Oct-3/4 transcript was only slightly detectable (Fig. 3). These expressions continued after several passages beyond Day 15. In contrast, STO cells expressed Lhx9, M33 and Fst genes, while ESCs expressed Ad4BP/SF-1, M33, BMP4 and Oct-3/4.

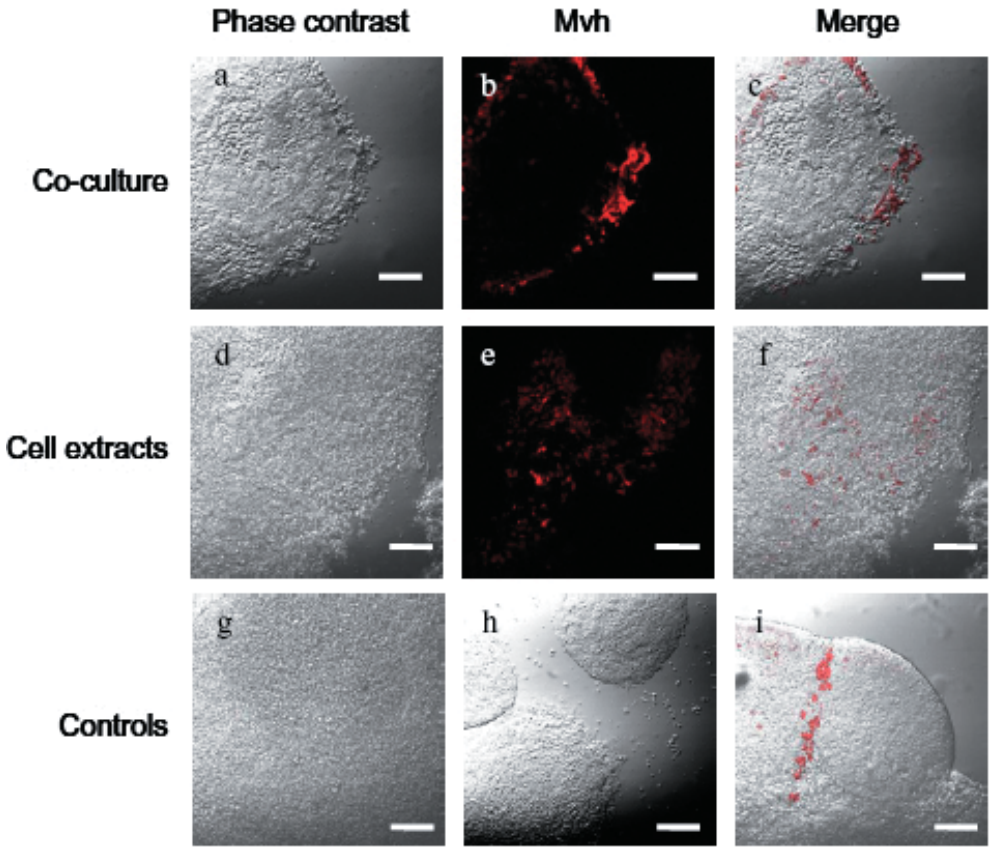

Fig. 2. Immunohistochemical localization of Mvh-positive cells in EBs co-cultured with either undifferentiated gonadal cells or cell extracts. Phase contrast, fluorescence and merged images of EBs co-cultured with undifferentiated gonadal cells (a–c), and with cell extracts (d–f), respectively. Merged images of Day 10 EBs cultured alone (g), undifferentiated ESCs (h) and undifferentiated gonad (i) are shown.

Discussion

The present study showed that by co-culture with undifferentiated gonadal cells EBs derived from ESCs effectively yield potential PGCs expressing Mvh and Dazla genes, both of which play crucial roles in germ cell differentiation. A mouse vasa homolog (*Mvh*), which encodes an ATP-dependent RNA helicase, is specific for differentiating germ cells from the late migration stage to the postmeiotic stage [8, 14], and loss of *Mvh* function causes a deficiency in the proliferation and differentiation of male germ cells [15], while a DAZ homolog, Dazla (DAZ-Like), found in diverse organisms including humans, is required for germ cell development in males and females [16].

In the mouse embryo, specification of tissue lineages requires cell-to-cell interactions that are influenced by coordinated cell migration. In the process of the formation of a gonad, a limited number of the cells in the proximal epiblast differentiate into germ cells, demonstrating that direct contact between PGCs and surrounding somatic cells in the gonad is essential for germ cell development. Mimicking the cellular interactions in the embryo by providing appropriate signaling molecules in culture has enabled the differentiation of ESCs to be directed predominately toward particular lineages. In this study, therefore, we used 11.5 day undifferentiated gonads, in which PGCs are proliferating. To avoid contamination with endogenous PGCs contained in the gonads, differentiated EBs were transferred onto an insert membrane set up in a culture dish seeded with undifferentiated gonadal cells. After culture, we found an increase in gene expressions of Mvh and Dazla and also in the number of Mvh-positive cells in Day 10 EBs. The increase of Mvh-positive cells was confirmed in a

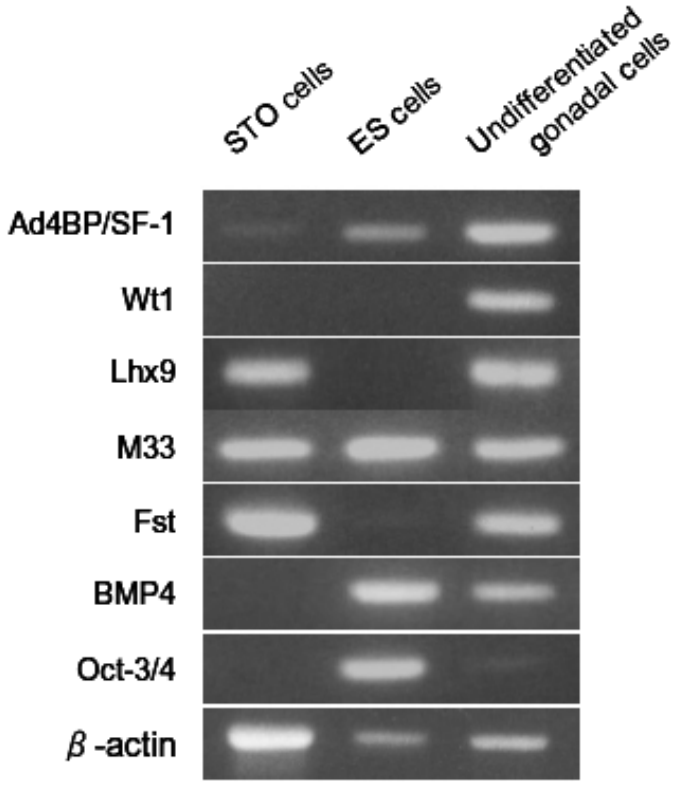

Fig. 3. RT–PCR detection of germ cell related genes in STO, ES and undifferentiated gonadal cells.

following experiment using cell extract of undifferentiated cells. These results are substantially consistent with a previous study [2], in which PGCs were induced from EBs cells cultured in the presence of retinoic acid. In a recent study [17], when EBs were cultured in a conditioning medium prepared from testicular tissues of mouse neonates, they developed into ovarian structures, which contained putative oocytes. Considering these results together, they may suggest that exposure of EBs to appropriate growth factors the germ cell precursor within the EBs can differentiate into gametes. As for effective factors, although they remain undetermined, growth factors such as bone morphogenetic protein 4 (BMP-4), SCF, LIF, beta-FGF and growth differentiation factor-9 (GDF-9) are possible candidates, because the testicular tissue is an abundant source of numerous factors including them [17]. In this study, we also confirmed typical gene expression in the undifferentiated gonads, which was different from the patterns of expression in STOs and ESCs.

Interestingly, the localization of Mvh-positive cells was different between co-culture with undifferentiated gonadal cells and cell extract. In EBs co-cultured with undifferentiated gonadal cells, Mvh-positive cells existed in marginal parts of EBs close to feeder cells. Dazla gene expression also differed between the two treatments. These differences might be caused by the concentration of effective factor(s) in each medium, or the use of secreted factor(s) or whole constituents in the cells.

In summary, we have demonstrated an alternative approach for inducing mouse ESCs to differentiate into potential PGCs using undifferentiated gonadal cells. This approach also offers an *in vitro* system for studying the interactions between PGCs and undifferentiated gonadal cells. In future study, it will be necessary to isolate Mvh-positive cells from EBs, and to continuously derive growing of germ cells. Together, the gene expressions of Mvh and Dazla, and the increase of the number of Mvh-positive cells, suggest the purification of Mvh-positive cells in EBs should be done on Day 10 of culture. Our attempt to derive germ cells from ESCs provides an accessible *in vitro* model system for studies of germline epigenetic modification and mammalian gametogenesis.

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