Comparative Analysis for In Vitro Differentiation Potential of Induced Pluripotent Stem Cells, Embryonic Stem Cells, and Multipotent Spermatogonial Stem Cells into Germ-lineage Cells

Young Eun Go¹, Hyung Joon Kim², Junghyun Jo¹, Hyun Jung Lee², Jeong Tae Do¹, Jung Jae Ko¹ and Dong Ryul Lee¹,²,*

¹Dept. of Biomedical Science, College of Life Science, CHA University, Seoul 135-081, Korea
²Fertility Center of CHA Gangnam Medical Center, College of Medicine, CHA University, Seoul 135-081, Korea

ABSTRACT: In the present study, embryoid bodies (EBs) obtained from induced pluripotent stem cells (iPSCs) were induced to differentiate into germ lineage cells by treatment with bone morphogenetic protein 4 (BMP4) and retinoic acid (RA). The results were compared to the results for embryonic stem cells (ESCs) and multipotent spermatogonial stem cells (mSSCs) and quantified using immunocytochemical analysis of germ cell-specific markers (integrin-α6, GFR-α1, CD90/Thy1), fluorescence activating cell sorting (FACS), and real time-RT-PCR. We show that the highest levels of germ cell marker-expressing cells were obtained from groups treated with 10 ng/㎖ BMP4 or 0.01 μM RA. In the BMP4-treated group, GFR-α1 and CD90/Thy-1 were highly expressed in the EBs of iPSCs and ESCs compared to EBs of mSSCs. The expression of Nanog was much lower in iPSCs compared to ESCs and mSSCs. In the RA treated group, the level of GFR-α1 expression in the EBs of mSSCs was much higher than the levels found in the EBs of iPSCs and similar to the levels found in the EBs of ESCs. FACS analysis using integrin-α6, GFR-α1, CD90/Thy1 and immunocytochemistry using GFR-α1 antibody showed similar gene expression results. Therefore our results show that iPSC has the potential to differentiate into germ cells and suggest that a protocol optimizing germ cell induction from iPSC should be developed because of their potential usefulness in clinical applications requiring patient-specific cells.

Key words: Induced pluripotent stem cells, Mouse embryonic stem cells, Multipotent spermatogonial stem cells, Germ cell lineage, Differentiation potential.

INTRODUCTION

Spermatogenesis is the process by which a Type-A single spermatogonium, referred to as a spermatogonial stem cell (SSC), differentiates into fertile sperm through successive divisions and differentiation. This process is essential for the preservation of the species. Especially for humans, the study and understanding of spermatogenesis may improve the treatment of male infertility.

In mammalian species, SSCs reside in the testis in very small quantities (Tegelenbosch & de Rooij, 1993; Orwig et al., 2002). Despite the establishment of long-term culture systems for some species, the ability of SSCs to proliferate is low compared to other stem cells (Kanatsu-Shinohara et al., 2008b). To establish in vitro model systems for studying spermatogenesis, pluripotent or multipotent stem cells are differentiated into the germ cell lineage. Toyooka et al. used embryoid bodies (EBs) and testis transplantation methods to produce spermatogenic cells from mouse embryonic stem cells (ESCs) and successfully obtained functional spermatozoa as an end product (Toyooka et al., 2003). In this study, they suggest that bone morphogenetic protein 4 (BMP4), a well-known influencing factor in primordial germ cells (PGCs) differentiation, plays an important role in the in vitro differen-
rentiation into germ cells of ESCs (Lawson et al., 1999; Fujiwara et al., 2001; Pellegrini et al., 2003; Ohinata et al., 2009). Another study on germ cell differentiation from ESCs used retinoic acid (RA), the active derivative of vitamin A, and obtained functional primordial germ cells, even though meiosis was unsuccessful (Geijsen et al., 2004). Based on previous studies that demonstrate that RA is involved in controlling meiosis initiation (Anderson et al., 2008; Bowles and Koopman, 2007; Koubova et al., 2006; Lin et al., 2008), the Naryernia group induced germ cell differentiation using RA from human embryonic carcinoma cells (ECCs), mouse bone marrow-derived mesenchymal stem cells (MSCs), and mouse ESCs (Nayernia et al., 2004; Nayernia et al., 2006a; Nayernia et al., 2006b) and succeeded in differentiating them into germ cells.

Even though ESCs have advantageous properties, such as pluripotency and the ability to proliferate infinitely, the problem of autogenous origin still remains, which is limited in being used in clinical applications. Therefore, patient-specific pluripotent stem cells, such as induced pluripotent stem cells (iPSCs) that are derived from the in vitro reprogramming of differentiated cells are preferable for cell therapy (Takahashi & Yamanaka, 2006). Takahashi and Yamanaka inserted the genes Oct3/4, Sox2, c-Myc, and Klf4, which are referred to as the 4 factors, into fibroblasts using a virus transfection method and obtained pluripotent iPSCs (Takahashi & Yamanaka, 2006). This technology has been applied to primary fibroblasts obtained from various tissues and has resulted in the establishment of iPSCs with different memories and differentiation potential (Kim et al., 2010b; Lee et al., 2010c). Another Japanese group spontaneously produced pluripotent ESC-like cells, referred to as multipotent spermatogonial stem cells (mSSCs), from neonatal mouse SSCs during a long-term culture. They reported that these mSSCs can differentiate into cells of the three-germ layers in vitro, forming teratomas, chimera (Kanatsu-Shinohara et al., 2004). Several reports have confirmed that the mSSC is comparable to a pluripotent cell type suitable for customized cell therapy, despite its low efficiency results for spontaneous reprogramming (Guan et al., 2006; Kim et al., 2010a; Seandel et al., 2007). Unlike previous reports (Kanatsu-Shinohara et al., 2004; Kanatsu-Shinohara et al., 2008a), we found that mSSCs still contain a memory of the germ cell-specific gene expression profile in early passages (Kim et al., 2010a).

In the present study, we analyzed the differentiation potential of three types of pluripotent stem cells with different memories (ESC derived from the embryo, iPSC derived from the fibroblast, and mSSC derived from the germ cell) by inducing their differentiation into germ cell lineages with RA and/or BMP4 supplementation. We also suggest that future studies should focus on developing the most suitable protocol for germ cell differentiation from reprogrammed pluripotent stem cells.

**MATERIALS & METHODS**

1. Maintenance of ESCs, iPSCs and mSSCs

All animal experiments involving the handling of mice were approved (IACUC-100013 and IACUC-100020) by the Institutional Animals Care and Use Committee of CHA University. For this study, three types of pluripotent stem cells were maintained using following method. Undifferentiated mouse ESCs (129X1/SvJ, SCC#10, ATCC, Manassas, VA), iPSCs (generated by transduction of Oct3/4, Sox2, c-Myc, and Klf4 into fibroblasts, which originated from OG2+/−/ROSA26+/− embryos, using the retrovirus system, Fig. 1), and mSSCs (CHA-mSSC-1) (Kim et al., 2010a) were cultured on mouse (CF1 strain, Jackson Laboratory, Los Gotos, CA) embryonic fibroblasts (MEFs) treated with irradiation in 6-well plates coated with 0.1% gelatin. Subculturing was performed every 3-4 days onto new MEF-seeded dishes. All cells were maintained in ESC medium that consisted of Dulbecco's modified Eagle's medium (DMEM) with high glucose (Hyclone, Logan, UT) and supplemented with 20% Knockout serum replacement (KSR, GIBCO, Grand Island, NY), 1× non-essential amino
acid (GIBCO), 50 μM β-mercaptoethanol (GIBCO), 100 U/㎖ penicillin (Hyclone), 100 μg/㎖ streptomycin (Hyclone) and 10³ U/㎖ ESGRO (LIF, Chemicon, Temecula, CA). All cells were cultured at 37°C in 5% CO₂ and 100% humidity.

2. Differentiation into Mouse Male Germ Lineage Cells

Colonies of ESCs, iPSCs and mSSCs were dissociated in 0.05% trypsin-EDTA (Hyclone) and resuspended in medium containing all of the previously described supplements except for LIF to inactivate the trypsin. Cell pellets were obtained by precipitation through centrifugation at 1,300 rpm for 5 min. Cell pellets were resuspended in Hanks’ Balanced Salt Solution (HBSS, GIBCO) and then loaded into 40% of Percoll solution to remove the mixed MEF cells. After centrifugation at 1,000 rpm for 15 min, purified ESCs, iPSCs and mSSCs were transferred onto Petri dishes (F-1007, BD Biosciences, Franklin lakes, NJ) at a density of 1 to 2.5×10⁴ cells per cm² and cultured for 24 hr in a 20% SR-containing ESC medium without LIF to obtain EBs. For germ cell differentiation, EBs were formed and differentiated in ESC medium without LIF supplemented with 0.1-10 ng/㎖ mouse BMP4 (R&D systems, Minneapolis, MN) and 0.01-1.0 μM RA (Sigma-Aldrich, St. Louis, MO). For the efficient differentiation into germ lineage cells, cells were cultured for 3-9 days. The medium was changed every 24 hr.

3. Immunocytochemical Analysis of Germ Lineage Cells

Colonies of undifferentiated ESCs, iPSCs and mSSCs were fixed with 4% paraformaldehyde (PFA) for 10 min and washed with phosphate buffered saline (PBS) to characterize their stemness. Colonies were treated with proteinase K solution (Dako, Produktionsvej, Denmark) for 15 min and blocked with protein block solution (Dako) for 1 hour at room temperature. The cells were then incubated overnight at 4°C with a primary antibody against Nanog (1:50, rabbit polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Oct3/4 (1:100, mouse monoclonal, Santa Cruz Biotechnology), Sox2 (1:100, rabbit polyclonal, Santa Cruz Biotechnology), or GFR-α-1 (1:100, Rabbit polyclonal, Santa Cruz Biotechnology). Further incubation with the secondary antibody Alexa fluor® 555 goat anti-rabbit IgG (H+L) (1:400, Invitrogen, Eugene, OR), Alexa fluor® 546 goat anti-mouse IgG (H+L) (1:500, Invitrogen), or PE goat anti-rabbit IgG (H+L) (1:100, Abcam, Cambridge, MA) was performed for 60 min at room temperature. Cells with only secondary antibody staining served as negative controls. The nuclei were detected by DAPI (Chemicon, 1:500) staining. Additionally, the activity of alkaline phosphatase (AP; Sigma-Aldrich St Louis, MO, USA), another marker for pluripotency, was detected by staining. The stained cells were observed under a fluorescent microscope (NIKON Eclipse TE-2000 U, Nikon, Tokyo, Japan).

To analyze the potency of germ cell differentiation, the EBs of ESCs, iPSCs and mSSCs were dissociated after 3 days of incubation to single cells by treatment with 0.05% Trypsin-EDTA for 5 min. These cells were then fixed with 4% PFA for 10 min and attached to coated slides by cytospin. Slides with samples were stored in PBS at 4°C until needed. Additionally, slides of cells obtained from undifferentiated ESCs, iPSCs and mSSCs were fixed, stored in PBS, and used as negative controls. They were stained with GFR-α-1 (Santa Cruz Biotechnology) under aforementioned conditions. The stained cells were observed under the Carl Zeiss LSM 510 META (Zeiss, Jena, Germany).

4. Flow Cytometric Analysis of Germ Lineage Cells

The EBs of ESCs, iPSCs and mSSCs were dissociated after 3 days of incubation to single cells by treatment with 0.05% Trypsin-EDTA for 5 min. These cells were then fixed with 4% PFA for 10 min and analyzed by flow cytometrical analysis using SSC-specific markers (Integrin-α6, CD90/Thy-1, and GFR-α1). Fixed cells from con-
trol and experimental groups were treated with APC Conjugated rat monoclonal IgG anti-Integrin-α 6 (1:200, R&D), PE/Cy5 conjugated Rat monoclonal anti-CD90/Thy-1 (1:100, Abcam), or Rabbit polyclonal anti-GFR-α 1 (1:200, Santa Cruz Biotechnology) at room temperature for 30 min, and then washed three times with Ca\(^{2+}\), Mg\(^{2+}\)-free PBS. PE goat anti-rabbit IgG (H+L)(1:100, Abcam) was used as the secondary antibody, and cells were incubated in the secondary antibody under the same conditions used for primary antibody incubation. The cells were then washed twice with PBS, resuspended in fluorescence-activated cell sorting (FACS) buffer (1× PBS, 2% fetal bovine serum), and filtered through a 5 ㎖poly-styrene round-bottom tube with a cell-strainer cap (12×75 mm style, BD Biosciences). Flow cytometrical analysis was performed using a FACS Calibur Flow Cytometer and Cell Quest Pro software (BD Bioscience).

5. Reverse Transcription-polymerase Chain Reaction (RT-PCR) and Quantitative-RT-PCR Analysis

Differentiated EB cells obtained from ESCs, iPSCs and mSSCs, mouse embryonic fibroblast (MEF), mSSCs, mESCs, and miPSCs were placed in Trizol (Invitrogen) and stored at −20°C. Total RNAs were prepared according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using a PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Shiga, Japan). PCR was performed with Accupower RT premix (Bioneer, Daejeon, South Korea). The cycle conditions were as follows: 94°C for 5 min, followed by 28-30 cycles of a 94°C denaturation period for 30 sec, a 59°C (GFR-α 1 and Esrrb) or 61°C (CD90/Thy-1, Sox2, Oct3/4, Klf4, c-Myc, and Nanog) annealing period for 30 sec, and a 72°C elongation period for 40 sec, with a final elongation period at 72°C for 10 min. Quantitative-RT-PCR was performed using the DyNAamo HS SYBR Green qPCR kit (Finnzymes, Espoo, Finland) on a Bio-Rad IQ5 Real-time RCR machine. The PCR reaction consisted of 10 ㎕ of SYBR green PCR Master Mix, 1 ㎕ of 10 ㎕ M forward and reverse primers, 7 ㎕ water, and 1 ㎕ template cDNA in a total volume of 20 ㎕. The results of qPCR were analyzed using the delta-delta Ct method and using the 18S as a normalization control. qPCR results were collected after 35-40 cycles, which, depending on the primer sets, consisted of the following: 94°C, 30 sec; 58.2°C (GFR-α 1)/61°C (CD90/Thy-1, Nanog), 30 sec; 72°C, 40 sec. The PCR primer sequences were as follows: GFR-α 1: F: 5'-AGA AGC AGT TTC ACC CAG-3' and R: 5'-ATC ATC ACC ACC ACC ATC-3'; CD90/Thy1: F: 5'-AGC CAA CTT CAC CAC CAA GGA TGA-3' and R: 5'-AAA TGA AGT CCA GGG CTT GTA GGA-3'; Nanog: F: 5'-AGG GTC TGC TAC TGA CCA-3' and R: 5'-GGA ATA CAA GAA GG-3'; 18S: F: 5'-TTG ACG GAA GGG CAC VAV CA-3' and R: 5'-GCA CCA CCA CCC ACG GAA TC-3'.

6. Statistical Analysis

All data presented are representative of at least three independent experiments unless otherwise indicated. The results are expressed as the mean±SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA and followed by a t-test if necessary. A value was considered statistically significant with p<0.05.

RESULTS

Prior to culturing for differentiation into mouse male germ cells, we first checked the pluripotency of mouse ESCs, iPSCs, and mSSCs with the pluripotent stem cell-related markers (gene expression of Nanog, Oct3/4, and Sox2 and AP activity) test. High levels of AP activity as well as Nanog, Oct4, and Sox2 gene expression were observed in all ESCs, iPSCs, and mSSCs by immunocytochemistry. The expression of GFR-α 1, however, which is a specific marker for SSCs, was not observed (Fig. 1).

To compare the potency of germ cell differentiation of each stem cell with different memories, different combinations of BMP4 and/or RA were used to induce the differentiation of cultured cells in this study, and flow cytometric
Fig. 1. Characterization of mouse ESCs, iPSCs and mSSCs. (A) Positive alkaline phosphatase (AP) signal (B) and embryoid body (EB) formation also were observed in three pluripotent cells (ESC, iPSC, and mSSC). Three cell lines expressed pluripotent markers; (C) Nanog, (D) Oct3/4, and (E) Sox2. (F) But GFR-α1 (spermatogonial specific marker) was not detected in all cell lines. BF means bright field. Scale bars represented 200 μm.

Fig. S1. Characterization of mouse iPSCs. (A) Karyotype analysis of iPSCs lines using the G-band method. The karyotype of iPSCs line was examined at passage 21 and was found to be a normal karyotype (2n = 40, XY). (B) RT-PCR analysis of RNAs extracted from mouse embryonic fibroblast (MEF), mSSC, ESC and iPSC with pluripotency gene primers.

analysis was performed on ESCs, iPSCs, and mSSCs. We determined the optimal concentration of BMP4 and RA for inducing the germ cell differentiation of mSSCs and ESCs before comparing the differentiation efficiency between these cells. In mSSCs and ESCs, we found that the highest expression level of the specific marker GFR-α1 was obtained from groups treated with 10 ng/ml BMP4 and 0.01 μM RA (Fig. S2). We also determined the optimal incubation time for BMP4 and RA-induced differentiation and found that the highest expression level of SSC-specific markers was obtained in EBs cultured for 3 days (Fig. S3).

Fig. S2. Comparison of GFR-α1 expression level in embryoid bodies of mSSCs and ESCs cultured in different concentration of RA and BMP4 by flow cytometric analysis. In the culture conditions of 0.01 μM RA or 10.0 μg/ml BMP4 for 3 days, EBs of mSSC and ESC showed the best level of expression ratio. Three independent experiments were performed.

Fig. S3. Comparison of GFR-α1 expression level in embryoid bodies of mSSCs cultured for the different culture period by flow cytometric analysis. The 3 day cultured EBs showed the best level in Integrin-α6, GFR-α1 and Thy-1 expression. Black line indicates control (undifferentiated mSSCs) and red line indicates the expression of Integrin-α6 and yellow and green line is GFR-α1 and Thy-1, respectively. ‘D’ means the culture day.
Based on this result, culturing EB for 3 days was used as the differentiation method for this study, and it proved to be an effective method for differentiating pluripotent mouse stem cells into germ cells.

In the next round of experiments, we determined the optimal differentiation conditions necessary for germ cell differentiation of our different cell types. Fig. 2 shows a quantitative comparison of integrin-α6, GFR-α1, and CD90/Thy-1 expression in each stem cell when cultured in different combinations of BMP4 and RA. Treatment with BMP4 or RA increased the expression of GFR-α1 and CD90/Thy-1 in iPSCs and mSSCs, respectively. There was no difference in the expression levels of germ cell markers in ESCs no matter what the treatment was. From these results, we chose the groups treated with BMP4 and RA for a direct comparative analysis among the three types of mouse pluripotent stem cells. Unlike previous reports (Shinohara et al., 1999), we found that the expression of

![Flow cytometric analysis of differentiation into male germ lineage cells of mouse ESCs, iPSCs, and mSSCs.](image)

Fig. 2. Flow cytometric analysis of differentiation into male germ lineage cells of mouse ESCs, iPSCs, and mSSCs. The differentiated ESCs, iPSCs, and mSSCs expressed SSC specific markers (Integrin-α6, GFR-α1, and Thy-1). Each bar represents the mean of three cytometric analysis data, with SEM shown by vertical lines. White, grey and black bars represent RA, BMP4 and co-treated EB groups cultured for 3 days, respectively. Statistically significant differences between groups were denoted by different letters. RA is retinoic acid and BMP4 is bone morphogenetic protein 4, and R+B indicates RA+BMP4.
integrin-α6 was not a specific marker of germ cell differentiation in our system because of the high sensitivity to other markers. Therefore, in our next direct comparison study, we used GFR-α1 and CD90/Thy-1 for real-time RT-PCR analysis, GFR-α1 antibody for immuno-cytochemistry, and integrin-α6 and GFR-α1 antibodies for dual FACS analysis.

Based on the results shown in Fig. 2, we selected the groups treated with BMP4 or RA only and compared the expression levels of GFR-α1 and CD90/Thy-1 and stem cell marker, Nanog in the three different pluripotent stem cells through quantitative real-time RT-PCR (Fig. 3A). We extracted RNA from each type of stem cell in either their undifferentiated or their differentiated states and analyzed the expression pattern of germ-lineage cell (GFR-α1 and CD90/Thy-1) and stem cell markers (Nanog). BMP4 treatment significantly increased the expression of GFR-α1 and CD90/Thy-1 in EBs from iPSCs and ESCs compared to EBs from mSSCs (p<0.05). The expression of Nanog (a pluripotent marker) was much lower in iPSCs than ESCs.

![Graph A](image1)

![Graph B](image2)

**Fig. 3.** Comparison of the potential for differentiation into mouse male germ lineage cell by qRT-PCR and RT-PCR. (A) For qRT-PCR analysis, three independent experiments were performed and each experiment with ESCs, iPSCs and mSSCs are performed at the same time to make a comparison among stem cell lines dependably. The white and black bars represented the each group of the undifferentiated-control and -sample, respectively. Statistically significant differences among groups are denoted by different letters. (B) The same expression pattern was shown in RT-PCR analysis. ‘D’ means the culture day. RA is retinoic acid and BMP4 is bone morphogenetic protein 4.
Fig. 4. Analysis of GFR-α1 expression in mouse male germ lineage cells differentiated from ESCs, iPSCs, and mSSCs by immunocytochemistry.

The spermatogonia stem cells (SSCs), positive control, expressed GFR-α1 (red fluorescence). The undifferentiated ESCs, iPSCs and mSSCs did not express GFR-α1. RA and BMP4 treated ESCs EB group cultured for 3 days exhibited positive staining for GFR-α1. The iPSC and mSSCs EB group treated with RA and BMP4 also exhibited positive staining for GFR-α1. The DAPI staining was detected as blue fluorescence in all cell lines. ‘D’ means the culture day. RA is retinoic acid and BMP4 is bone morphogenetic protein. Scale bars represented 10 μm.

Fig. S4. Dual flow cytometric analysis for confirmation of differentiation into male germ lineage cells of mouse ESCs, iPSCs, and mSSCs with dot plot.

EB cell groups of ESC, iPSC and mSSC cultured in different combination of RA and/or BMP4 for 3 days were stained with antibodies against Integrin-α6, GFR-α1. The percent of Integrin-α6 and GFR-α1-expressed cells was high in cells obtained from ESCs treated RA, iPSCs treated BMP4, and mSSCs treated RA.

and mSSCs. RA treatment, however, significantly increased the expression of GFR-α1 and CD90/Thy-1 (markers for differentiated SSC) in EBs from mSSCs and ESCs, and these levels were higher than the levels in iPSCs (p<0.05). The expression of Nanog decreased in all cell types.

DISCUSSION

To generate patient-specific stem cells for stem cell therapy, various types of fully differentiated cells or uni-
potent stem cells have been induced or spontaneously reprogrammed, and then several types of pluripotent stem cells were generated (Kanatsu-Shinohara et al., 2004; Lee et al., 2010c; Park et al., 2008; Takahashi & Yamanaka, 2006). Pluripotent or multipotent stem cells from various types of embryonic and fetal tissues are also valuable candidates for stem cell therapy (De Coppi et al., 2007; Parolini et al., 2008; Lee et al., 2010b; Matthay et al., 2010), as are ESCs from somatic cell nuclear transfer or stem cell banks (Drukker, 2008; Lee et al., 2010a). Therefore, it is very important that we determine which type of stem cells will be the most useful for specific types of cell therapy and which methods may be used to induce germ cell differentiation in clinical trials. In our previous study, we found that mSSCs have a memory of the germ cell-specific gene expression profile in early passages (Kim et al., 2010a) and may have a higher potential for germ cell differentiation compared to ESCs (unpublished data). Additionally, iPSCs generated from human aortic vascular smooth muscle cells (HASMC) are pluripotent and can differentiate into functional SMC. Furthermore, these iPS-derived SMCs have similar gene expression patterns to their parent SMC (Lee et al., 2010c). Kim et al. (Kim et al., 2010b) reported that low-passage iPSCs contain residual DNA methylation signatures that are characteristic of their somatic tissue of origin, and this ‘epigenetic memory’ may favor their differentiation along the lineages related to the donor cells. In the present study, we analyzed the potency of three types of pluripotent stem cells with different memories for differentiating into germ cells. As shown in Fig. 1, ESCs, iPSC, and mSSCs express all of the pluripotent stem cell markers while they are being maintained. In our preliminary results, the EB method was favorable to germ cell differentiation compared to the attachment culture method (data not shown), and this result was similar to a previous report (Wei et al., 2008). Before comparing the differentiation efficiency of the cells, we determined the optimal concentrations of, and incubation period in, BMP4 and/or RA for mSSCs and ESCs differentiation (Fig. S2, S3). By flow cytometric analysis, we found that treatment with either BMP4 or RA alone was more effective than treatment with BMP4 and RA (Fig. 2). So, we used this protocol for the differentiation of iPSCs, mSSCs and ESCs. BMP4 is produced by Sertoli cells during the very early postnatal stages and is successively down regulated in peri-pubertal Sertoli cells. It has been reported that exposing undifferentiated spermatogonia to BMP4 in vitro increases the expression of BMP4 receptors, R-Smad, Smad5 and Alk3 and exerts a mitogenic and differentiation effect (Pellegrini et al., 2003). Additionally, it is believed that RA, the active derivative of vitamin A, is one of the factors that controls meiosis around 13.5 days post coitum (dpc) in the ovary and controls sperm formation by meiosis in the testis (Bowles & Koopman, 2007). Furthermore, RA is recognized as a signal that induces meiosis by activating intrinsic factors, such as mouse RNA-binding protein DAZL, that are expressed in the gonocyte (Lin et al., 2008).

It has been reported that mouse spermatogonial stem cells, which express integrin-α6 on their surface, obtain the ability to colonize at the recipient testes. This finding contradicts the expression pattern of c-kit, which is specifically expressed in differentiated spermatogonia (A1-A4 spermatogonia) (Shinohara et al., 1999). Another report demonstrates that CD90/Thy-1 can be used as a marker for proliferating undifferentiated spermatogonia by culturing Thy-1 positive cells obtained from undifferentiated spermatogonia separately (Reding et al., 2010). GFR-α1, the most specific marker used to compare the differen-
tiation efficiency in our study, is a part of the GDNF receptor complex that controls the proliferation and differentiation of SSCs (He et al., 2007). In Fig. 2, lower levels of GFR-α1 were seen in all of the cell types compared to the other two markers. In this study, we may confirm that GFR-α1 is a more specific maker for SSCs and/or differentiated germ cells.

In the present study, we found that iPSCs, which are pluripotent like ESC, have a high and low differentiation efficiency for generating germ cells under the influence of BMP4 and RA, respectively. We also found that pluripotent mSSCs have a low and high differentiation efficiency for generating germ cells under the influence of BMP4 and RA, respectively. We have shown that ESCs have a high differentiation potential in both BMP4 and RA treatments. Therefore, we suggest that pluripotent iPSCs from an autogenous origin may differentiate into germ lineage cells if favorable conditions are applied. In fact, it was reported that differentiation of germ cells from iPSCs was less effective compared to the study using ESCs and it was improved by modifying differentiation-supporting activity (Imamura et al., 2010). They may even be a candidate cell source of cell therapy for germ cell, and their potential are comparable to other pluripotent stem cells such as ESCs and mSSCs, although they are reprogrammed from differentiated fibroblast cells that have a different memory. We also suggest that future studies should focus on developing an optimal protocol for differentiating iPSCs into advanced germ cells so that they may be used in applications requiring customized patient-specific pluripotent stem cells and for the study of reproduction.

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