Isolation and Culture of Rabbit Primordial Germ Cells

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Abstract. Primordial germ cells (PGCs) are embryonic precursors of the gametes of adult animals and are considered stem cells of the germ line. Since their proliferation in vitro correlates well with the schedule of developmental changes in vivo, they might be interesting research tools for genomic imprinting, germ-cell tumors and fertility. Furthermore, once primordial germ cells are separated and placed on a feeder layer with cytokines, they become cultured pluripotent cell lines called embryonic germ (EG) cells. EG cells share several important characteristics with embryonic stem (ES) cells as they can also contribute to the germ line of chimeras. To investigate the characteristics of PGCs and establish rabbit EG (rEG) cells, we cultured rabbit PGCs (rPGCs) in vitro with various combinations of leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and forskolin on inactivated mouse embryonic fibroblast (MEF) feeder layers. The present study found PGC proliferation in early cultures and induction of rEG-like colonies. These cells expressed pluripotent markers, such as alkaline phosphatase activity, OCT-4, Sox-2 and SSEA-1, in the undifferentiated state; however, the cells did not develop into a teratoma when injected into the kidney capsules of SCID mice, although the restricted differentiation potentials to neural cells were determined via embryoid body formation. From these characteristics and further characterization of the germ stem cell markers Vasa, SCP-1 and SCP-3, we suggested that the restricted differentiation potentials to neural cells were determined via embryoid body formation. From these characteristics and further characterization of the germ stem cell markers Vasa, SCP-1 and SCP-3, we suggested that these were hybrid cells with characteristics somewhere between PGC and EG cells.

Key words: Differentiation, Embryonic germ cells (EG cells), Pluripotency, Primordial germ cells (PGCs), Rabbit

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In the present study, we collected PGCs from rabbit fetuses at various embryonic stages and cultured them under various conditions. Furthermore, we characterized pluripotent cell markers of the cultured cells.

Materials and Methods

Collection of rPGCs

The embryo donors were New Zealand White female rabbits
(approximately 6 months of age) crossbred with males of the same strain. The animals were sacrificed on Day 8 to 11 of gestation, and embryos were dissected from the uterus. The genital ridges were dissected from the embryos, and the dorsal mesentery was removed. The isolated tissues were washed once with PBS and incubated in 0.04% (w/v) Trypsin-0.25% (w/v) EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature. After incubation, the PGCs were dissociated by gentle disruption of the tissues using a glass capillary tube.

These animal experiments were conducted in accordance with the Guidelines of Kinki University for the Care and Use of Laboratory animals.

Culture of rPGCs in various culture conditions

The 9.5 day post coitum (dpc) embryos were sacrificed in order to determine suitable PGC culture conditions. The dissociated cells were washed and cultured on mitotically inactivated MEF feeder layers in PGC culture medium composed of knockout DMEM (Invitrogen, Carlsbad, CA, USA), Knockout SR (Invitrogen) for 1000 IU LIF, 20 ng/ml bFGF and 20 \( \mu \)M forskolin. The cultured cells were permeabilized in 0.5% (v/v) Triton-X 100 and fixed with 4% paraformaldehyde in PBS (pH 7.4). Immunocytochemical staining of OCT-4, fixed cells were permeabilized in 0.5% (v/v) Triton-X (Sigma-Aldrich) diluted in PBS (0.5% PBT) for 5 min, blocked by incubation in 5% (w/v) skim milk (Sigma-Aldrich) diluted in PBS for 1 h and then incubated with the primary antibody overnight at 4°C. The antibodies were anti-OCT-4 rabbit IgG polyclonal antibody (Santa Cruz Biotechnology), anti-Sox-2 mouse monoclonal IgG antibody (Abcam, Tokyo, Japan) diluted 1/1,000 in 0.1% (v/v) PBT, for immunostaining of SSEA-1, fixed cells were washed with 0.5% (w/v) Bovine serum albumin (BSA) (Sigma-Aldrich) in PBS (0.5% BSA) and blocked by incubation in 5% skim milk for 1 h. The antibody was anti-SSEA-1 mouse monoclonal IgM antibody (Santa Cruz Biotechnology) diluted 1/100 in 0.5% BSA. For microscopic observation of immunostained samples, sections were incubated for 1 h at room temperature with IHC reagents (HRP-conjugated secondary antibody (all purchased from Santa Cruz Biotechnology) diluted 1/1,000 in 0.1% PBT or 0.5% BSA). For evaluation of neural cell differentiation, fixed cells were washed with 0.5% BSA, blocked with 5% skim milk for 1 h and immunized with anti-Neuronal class III \( \beta \)-tubulin (Abcam, Cambridge, U.K.) overnight at 4°C. The samples were then observed after reaction with FITC conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology).

RNA isolation, reverse transcription and quantitative PCR

The colonies or EBs were retrieved using a glass capillary pipette and were stored in liquid nitrogen for analysis. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. Single-strand cDNA was prepared from total RNA using an oligo-dT primer under standard conditions with Superscript III reverse transcriptase (Invitrogen). The cDNA from undifferentiated EG-like colonies were diluted and used for an RT-PCR-based assay for Oct4, Sox-2, Vasa, SCP-1, SCP-3 and Gapdh. Differentiation properties were observed by analysis for ectodermal and neural cell marker Nestin, mesodermal marker Desmin and
endodermal marker Hnf4a (hepatocyte nuclear factor 4a) using cDNA from EBs.

RT-PCR with total cDNA was performed using Platinum Taq PCRx DNA polymerase (Invitrogen) and the following primer sets (listed 5’ to 3’ in the order of forward and then, reverse primer):

- AGTCACTGCTTGATCGTTTG and AAGAACATGTGT-AAGCTGCG for Oct-4; AGCATGATGCAGGAGCAG, GGAGTGGGAGGAAGAGGT for Sox-2; CCGTGGAGG-ATTTGCTTA, TGTGCAAAATGGAG-TCCCTC for Vasa; ACAGCGAAAGCCATCCAGG, TCTGGAATTCTCAGC-TTGCA for Scp-1; TCTAGAATTGTTCAGAGCCAGAG, CAGAATAACATGGATTGAGAGAGAG, ATGCTCTGACTCCTCAGG for Desmin; CCTCAAAGCCATCATCTTC, GAAGAGCTTGATGAA-CTGGA for Hnf4a; GGAGGACGGGTCATCATCTC, GAGGGGCCATCCACAGTTCT for Gapdh.

PCR amplifications were performed at 94°C for 2 min followed by 35 cycles of 94°C for 20 sec, 56°C for 20 sec and 72°C for 20 sec. Reactions were replicated three times.

Statistical analysis

Statistical analyses of the data were performed with a one-way ANOVA followed by the Tukey-Kramer honestly significant difference (HSD) test for the three sets of results. A P-value of less than 0.05 was considered significant. Statistical analyses were done with a JMP Statistical Discovery Software (SAS Institute, Cary, NC, USA).

Results

Growth factor supplementation increased rPGC colonies

We isolated rPGCs from the germinal tissues of rabbit fetuses (Fig. 1). The rPGCs could be maintained under conditions with or without LIF supplementation of the culture medium. The number of rEG-like cell colonies increased with bFGF supplementation. LIF was not indispensable for colony formation, but the co-addition of LIF and bFGF increased the number of colonies (Table 1a). On the other hand, forskolin treatments dramatically affected the expansion and colony formation of rPGCs, increasing by approximately 8 times more than the non-treated controls (Table 1b).

Expression of pluripotent markers in EG-like colonies formed by rPGCs

The rPGCs formed colonies resembling mouse EG (mEG) cells in primary culture (i.e., dome-shaped colonies containing cells containing a large nucleus with prominent nucleoli and a relatively small amount of cytoplasm). These cells expressed ALP activity and pluripotent cell markers SSEA-1 and Oct-4 (Fig. 3) in the same manner as mEG cells (Fig. 4). The pluripotency of the cells was also determined by RT-PCR-based analysis (Fig. 5) for Oct-4 and Sox-2. However, most of the colonies were lost prior to completion of four passages, and no teratomas developed in the kidney capsules of the recipient mice that received transplants up to the end of the 20-day experimental period.

In regard to germ cell markers expressed in PGCs, SCP-1 expression was observed, although Vasa and SCP-3 were not detected (Fig. 5). ALP expression rapidly reduced with the differentiation of rEG-like cells.

In vitro differentiation

When rEG-like cells differentiated in vitro, they rapidly lost the expressions of pluripotent cell markers (data not shown). After approximately 7 days in suspension culture, the rEG-like cells formed simple embryoid bodies (EBs), occasionally containing several cell types, as judged by morphology. RT-PCR analysis revealed that the differentiation markers Desmin, Nestin and Hnf4a were expressed in the EBs. After adherent culture of the EBs, neuraxis-like structures developed (Fig. 3), and the neural cell marker TuJ was observed (Fig. 3).

Discussion

In the present study, we isolated, cultured and determined the characteristics of rPGCs in early passage. In mice, it has been

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<th>Table 1. Effects of LIF, bFGF and forskolin supplementation on colony formation in primary culture</th>
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<td>(a) The number of ALP-positive colonies cultured in bFGF at variable concentrations with or without LIF (P&lt;0.05, n=3)</td>
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<td>(b) The number of ALP-positive colonies cultured in the presence of forskolin at 0, 20 and 40 μM</td>
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<td>Forskolin (μM)</td>
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The rPGCs were isolated from 9.5 dpc genital ridges and seeded onto mitotically inactivated MEF. The scores are the mean values for three wells of a 4-well multidish (P<0.001, n=4). Different superscripts indicate significant differences (P<0.05).
reported that co-culture with MEF and addition of LIF and bFGF promotes PGC proliferation and gives rise to EG cells [12]. The hematopoietic cytokine bFGF stimulates stromal and stem cell growth and is a potent mitogenic factor in stimulating cell proliferation of multiple cell types [16]. LIF is a soluble glycoprotein of the interleukin (IL)-6 family of cytokines and is required to maintain pluripotency and permit self-renewal of mouse ES (mES) cells [17, 18]. Furthermore, it has been reported that supplementation with forskolin also improves cell proliferation. Forskolin is a mediator that enhances intracellular cAMP levels by stimulating adenylate cyclase (protein kinase A), and it has also been reported to significantly promote PGC proliferation [19, 20]. In the present study, we first demonstrated the synergistic effects of LIF and bFGF and the further effect of forskolin supplementation on rPGC proliferation. Supplementation of LIF improved rPGC survival in culture, and interestingly, bFGF promoted rPGC proliferation and colony formation in a dose-dependent manner only under the LIF-added condition. In addition, a further increase in the number of
colonies was observed with the combination of the three factors, namely, LIF, bFGF and forskolin (Table 1). It has been reported that bFGF and forskolin activate independent signaling pathways and that they can work additively for mEG cell formation [21]. The rPGCs cultured in the above conditions proliferated on feeder layers and that they retained PGC characteristics. Male and female PGCs show cell-autonomous entry into meiosis if they are taken out of the gonadal environment, and it has been reported that PGCs obtained from embryos at 8.5 dpc increase in number for 3 days and then start to decrease on day 4, while proliferation of 11.5 dpc PGCs stops immediately in vitro [22], if they do not develop EG cell characteristics. It has been reported that migrated PGCs express early germinal marker Vasa and meiotic markers such as Scp-1, Scp-3 and Dmc-1 [24–26]. Therefore, we examined the gene expressions of Scp-1, Scp-3 and Vasa in rEG-like cells obtained from 9.5 dpc embryos by RT-PCR analysis. Interestingly, no expression of Vasa or Scp-3 was detected; only Scp-1 expression was observed (Fig. 5). This suggested that the present rEG-like cells might be in an incomplete state of transformation to EG cells. Continuing culture or treatment with other cytokines that induce transformation, such as oncostatin-M, might be effective to obtain more complete cell lines [27].

On the other hand, we may need to consider species-specific characteristics such as vulnerability to enzymatic processing and ability to survive after single cell digestion. It has been reported that the viability of human ES cells is affected by single cell culture [14], and the epigenetic status of these stages resemble mES cells more than later stages [22]. Furthermore, in our experiment, the contributions of mEG cells established from embryos at 8.5 dpc were observed in chimera mice. The developmental schedule of the rabbit is delayed compared with that of the mouse when judged morphologically; in our observations, the 9.5 dpc rabbit fetus resembled the 8.5 dpc mouse fetus (Fig. 2). It is possible that there are very few rPGCs before 9.5 dpc, and this might cause the loss of colonies; therefore, we mainly collected rPGCs from 9.5 dpc that morphologically corresponded to 8.5 dpc embryos in mice.

The rEG-like cells established from embryos at 9.5 dpc expressed pluripotent cell markers Oct-4, Sox-2 and SSEA-1 (Figs. 3 and 5); however, the rEG-like cells could not survive past three passages, and they differentiated rapidly or degenerated after seeding without adherence to the feeder layer. These cells could differentiate to embryoid body-like structures, and they expressed differentiation markers. Interestingly, faint expressions of Desmin and Nestin were detected in rEG-like cells. Desmin and Nestin were early differentiation markers expressed in premature cells; the rEG-like cell cultures were not homogeneous and might have included the cells at various differentiation stages. On the other hand, the endodermal lineage specific gene Hnf4a was only detected after differentiation. Hnf4a has been implicated in liver development and hepatocyte differentiation. Furthermore, the EBs produced Tuj-positive neural cells when they attached to the culture dish. Although the rEG-like cells possessed multiple differentiation properties in vitro, they did not form a teratoma when injected into the kidney capsules of SCID mice (data not shown). Based on this restricted proliferation potency and differentiation diversity, we surmised that the cells were not EG cells and that they retained PGC characteristics. Male and female PGCs show cell-autonomous entry into meiosis if they are taken out of the gonadal environment, and it has been reported that PGCs obtained from embryos at 8.5 dpc increase in number for 3 days and then start to decrease on day 4, while proliferation of 11.5 dpc PGCs stops immediately in vitro [22], if they do not develop EG cell characteristics. It has been reported that migrated PGCs express early germinal marker Vasa and meiotic markers such as Scp-1, Scp-3 and Dmc-1 [24–26]. Therefore, we examined the gene expressions of Scp-1, Scp-3 and Vasa in rEG-like cells obtained from 9.5 dpc embryos by RT-PCR analysis. Interestingly, no expression of Vasa or Scp-3 was detected; only Scp-1 expression was observed (Fig. 5). This suggested that the present rEG-like cells might be in an incomplete state of transformation to EG cells. Continuing culture or treatment with other cytokines that induce transformation, such as oncostatin-M, might be effective to obtain more complete cell lines [27].

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