—Full Paper—

Isolation and Culture of Rabbit Primordial Germ Cells

Ryo KAKEGAWA¹⁾, Takeshi TERAMURA^{2,3)}, Toshiyuki TAKEHARA¹⁾, Masayuki ANZAI⁴⁾,
Tasuku MITANI⁴⁾, Kazuya MATSUMOTO¹⁾, Kazuhiro SAEKI¹⁾, Norimasa SAGAWA²⁾,
Kanji FUKUDA^{3,5)} and Yoshihiko HOSOI¹⁾

¹⁾Graduate School of Biology-Oriented Science and Technology, Kinki University, Wakayama 649-6493, ²⁾Department of Obstetrics and Gynecology, Graduate School of Medicine, Mie University, Mie 514-8507, ³⁾Institute of Advanced Clinical Medicine, Kinki University School of Medicine, Osaka 589-8511, ⁴⁾Institute of Advanced Technology, Kinki University, Wakayama 642-0017 and ⁵⁾Department of Orthopaedic Surgery, Kinki University School of Medicine, Osaka 589-8511, Japan

Abstract. Primordial germ cells (PGCs) are embryonic precursors of the gametes of adult animals and are considered stem cells of the germline. 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 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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PGCs are embryonic cells that migrate through the genital ridge, where they ultimately give rise to gametes. PGCs carry forward the phenotype of each individual, including all of its defects, into future generations, and characterization and detailed description of their germ cell differentiation will lead to better understanding of fertility, childhood diseases, germ-cell tumors and methods of contraception.

In mice, primitive germ cells were first identified at embryonic day 6.25 in the most proximal layer of the epiblast [1] and were observed throughout PGC migration from somatic tissues by expression of tissue non-specific alkaline phosphatase (TNAP) activity [2]. During migration, PGCs actively proliferate and increase in number by about 300-fold until initiation of sex differentiation of the fetal gonads [3–5]. Then, PGCs mitotically arrest or enter the first meiotic division to form a gamete [6]. Interestingly, they also show a limited period of proliferation *in vitro* and enter growth arrest, which correlates well with their developmental changes *in vivo* [7]. Furthermore, when isolated mouse PGCs were cultured, electron microscopy and enzymatic analyses indicated that a large proportion of the PGCs died as a result of apoptosis, probably because some fetal germ cells die as a result of apoptosis during migration and differentiation in the developing fetal gonad

[8, 9]. To maintain the PGCs in culture, supplementation of growth factors is essential. Treatment with leukemia inhibitory factor (LIF) and stem cell factor (SCF) increases the survival of mouse PGCs by reducing apoptosis [10, 11].

In the presence of multiple growth signals, PGCs can restart rapid proliferation and sometimes transform into pluripotent EG cells [12, 13]. EG cells share several important characteristics with ES cells, including their morphology, cell surface markers such as stage-specific embryonic antigen-1 (SSEA-1) and transcription factors such as Oct-4 [5, 12]. More importantly, EG cells can also contribute to the germ line of chimeras when injected into blastocysts [14].

Because of their potential use for gene manipulation, isolation of EG cells in various species might give rise to numerous agricultural and biomedical applications; however, establishment of EG cells in species other than mice [12] and humans [15] has proven difficult.

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 uteri. 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) at final concentration 20% (v/v) L-glutamine, sodium pyruvate, MEM nonessential amino acids and 2-mercaptoethanol. The PGC culture medium was supplemented with or without leukemia inhibitory factor (LIF) at 1,000 IU/ml (ESGRO; Chemicon International, Temecula, CA, USA) and recombinant basic FGF (bFGF; Upstate, Charlottesville, VA, USA) at 0, 10, 20 or 30 ng/mL. Furthermore, the effect of forskolin (Sigma-Aldrich) was also examined by adding it at 0, 20 or 40 μ M to PGC culture medium containing 1000 IU/ml LIF and 20 ng/ml bFGF. The efficiency of PGC colony formation was evaluated at primary culture.

To determine the period in which PGCs can be obtained by culture, genital ridges were collected from the embryos at 8.5, 9.5, 10.5 and 11.5 dpc. The collected tissues were dispersed and cultured on feeder cells in PGC culture medium supplemented with 1000 IU LIF, 20 ng/ml bFGF and 20 μ M forskolin. The cultured PGCs were passaged at 4- to 7-day intervals. Plates containing colonies and feeder cells were dissociated with 0.04% (w/v) Trypsin-0.25% (w/v) EDTA, centrifuged and seeded onto a fresh feeder layer in a 4-well multidish.

The following experiments to evaluate pluripotency and differentiation properties were performed using cultured PGCs obtained from 9.5 dpc embryos.

Comparison experiments related to the suitable embryonic days for obtaining EG-like colonies or their characterizations were performed using mouse fetuses collected from female mice of the hybrid strain BDF1 (CLEA Japan, Tokyo, Japan) that were mated with male BDF1. Mouse PGCs were cultured under the same conditions as the rabbit PGCs.

Evaluation of alkaline phosphatase (ALP) activity

PGCs were identified by evaluating the alkaline phosphatase (ALP) activities of cells collected from the genital ridges and cultured cells. For evaluation of the collected cells, digested tissues were spread on a slide glass, air-dried and used for ALP assays. Furthermore, the ALP-assay (ALP histochemistry kit; Sigma-Aldrich) was also performed for undifferentiated EG-like cells. Cultured cells were fixed with 90% (v/v) ethanol and used for this

assay.

Evaluation of differentiation properties

To induce differentiation *in vitro*, EG-like cell colonies were gently dissociated from the plate with the aid of a micropipette and cultured in 10% (v/v) FCS-DMEM on a 35-mm non-adhesive petri dish. Embryoid bodies (EB) were maintained in suspension culture, and the medium was changed every other day. After culture for a week, the EBs were plated on gelatin coated dishes and allowed to further differentiate. Differentiation properties were also observed by transplanting the cells into immunodeficient mice (SCID) and inducing teratoma formation. Briefly, approximately 3×10^6 cultured PGCs were injected into the kidney capsule of an SCID mouse. Twenty days after transplantation, the kidneys were recovered, fixed with neutral formalin and processed with a cryomicrotome. The sections were evaluated following Hematoxylin-Eosin staining.

Immunocytochemistry for characterization of PGCs and differentiated cells

Cultured PGCs were fixed by 4% (w/v) paraformaldehyde in PBS (4% PFA; pH 7.4). For 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, Santa Cruz, CA, USA) diluted 1/100 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 Horse radish peroxidase (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 β -tubulin mouse monoclonal IgG antibody (TuJ; Abcam, Tokyo, Japan) 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-ATTTGGTCTA, TGTGCAAAGATGGAG-TCCTC for *Vasa*; ACAGCGAAAAGCCATTACAG, TCTGGAATTCTCAGC-TTGCA for *Scp-1*; TCTAGAATTGTTTCAGAGCCAGAG, CAGAATAACATGGATTGAAGAGAC for *Scp-3*; TTTGCA-GATGAGGAAGAGAG, ATGCTCTGACTCCTCCAAG for *Nestin*; AGCAGGAGATGATGGAATAC, TCCAGCAGCTTC-CGGTAGG for *Desmin*; CCTCAAAGCCATCATCTTC, GAAGAGCTTGATGAA-CTGGA for *Hnf4a*; GGAGCC-AAACGGGTCATCATCTC, GAGGGGCCATCCACAGTCT-TCT 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). Under the conditions of LIF, bFGF and forskolin supplementation, rEG-like colonies were found if the rPGCs were isolated from the embryos at 9.5, 10.5 and 11.5 dpc. However, these colonies were not observed when 8.5 dpc embryos were used (Fig. 2).

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

Table 1. Effects of LIF, bFGF and forskolin supplementation on colony formation in primary culture

(a) The number of ALP-positive colonies cultured in bFGF at variable concentrations with or without LIF (P<0.05, n=3)

LIF	bFGF (ng/ml)			
	0	10	20	30
–	1.5 ± 0.5 ^a	3.5 ± 1.5 ^{a,b}	3.5 ± 1.5 ^{a,b}	3 ± 0 ^{a,b}
+	1.5 ± 1.5 ^a	4 ± 2 ^{a,b}	6 ± 1 ^b	6 ± 1 ^b

(b) The number of ALP-positive colonies cultured in the presence of forskolin at 0, 20 and 40 μM

Forskolin (μM)		
0	20	40
10.5 ± 5.5 ^a	50 ± 5 ^b	47 ± 3 ^b

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).

Fig. 1. ALP stained rPGCs in the digested genital ridge of an embryo at 9.5 dpc. Scale bar=100 μ m.

A

	8.5 dpc	9.5 dpc	10.5 dpc	11.5 dpc
Mouse	+	+	+	+
Rabbit	-	+	+	+

B

Fig. 2. Establishment of rEG-like cells at various developmental stages. (A) Summary of embryonic days and rEG-like cell foundation after primary culture. (B) Embryos of mice and rabbits on each embryonic day. Scale bars=1 mm.

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

Fig. 3. Characteristics of rEG-like cell colonies. Alkaline phosphatase activity assay (upper left, scale bar=50 μ m). Immunostaining of cell colonies with anti-Oct-4 (upper middle) and anti-SSEA-1 (upper right). The rEG-like cells formed an EB-like structure (middle panel, scale bar=100 μ m). Neural cell differentiation (lower panel). Neuraxis-like structures were immunofluorescently stained using TuJ antibody (scale bar=50 μ m).

Fig. 4. Pluripotent mouse EG cells. The mEG cells were established as a control using the same culture conditions as for the rEG-like cells. A chimera mouse was obtained by injection of EG cells derived from an 8.5 dpc embryo into an ICR blastocyst.

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

Fig. 5. RT-PCR-based evaluation of lineage specific gene expressions. (A) Examination of pluripotent cell markers or germ cell-related genes, *Oct-4*, *Sox-2*, *Vasa*, *Scp-1* and *Scp-3*. NC=negative control (rabbit dermis-derived fibroblast cells) and PC=positive control (rabbit testis). (B) Differentiated cell marker expressions were detected in EBs. Mesodermal (*Desmin*), ectodermal (*Nestin*) and endodermal (*Hnf4a*) cell markers were observed. Pc, positive control (fetal heart was used for *Desmin*, fetal brain was used for *Nestin* and fetal liver was used for *Hnf4a*); Nc, negative control (adult lung); Eg, rabbit EG-like cells; Df, differentiated EBs; Br, fetal brain; Ht, fetal heart; Lv, fetal liver; aL, adult lung.

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 formed dome-shaped colonies in the same manner as mEG cells. These colonies expressed one of the pluripotent cell markers, ALP (Fig. 3).

We also isolated rPGCs from embryos at various developmental stages from 8.5 to 11.5 dpc. The rPGCs could form rEG-like cell colonies when collected from 9.5 to 11.5 dpc, but no colonies were observed when we used earlier stage rPGCs (Fig. 2). In mice, pluripotent EG cells could be established from embryos at all stages from 8.5 to 11.5 dpc (Figs. 2 and 4). The mEG cells obtained from early stages such as 7.5 to 8.5 dpc have high pluripotency and germ-line transmission capability [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 also 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].

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 due to activation of the Rho kinase pathway that induces apoptosis [28]. Alternatively, mES cells can proliferate from single cells. It is possible that rabbit cells might have a pathway that is similar to that in human ES cells and that treatment with a Rho-associated kinase inhibitor might be effective for cell survival.

In summary, we performed rabbit PGC culture and observed the formation of rEG-like colonies. These cells expressed pluripotent markers Oct-4, Sox-2, SSEA-1 and ALP. Furthermore, we demonstrated the synergistic effects of LIF and bFGF and the further effect of forskolin in cell culture; however, these cells possessed restricted differentiation capacity and could not expand past four passages. Although some issues, such as the term of culture, suitable stages of embryos and species-specific characteristics, remain to be clarified, studies using PGCs of various species might give rise to important information related to understanding the mechanisms of mammalian germ cell development.

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