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Culture Conditions for Maintain Propagation, Long-term Survival and Germline Transmission of Chicken Primordial Germ Cell-Like Cells

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Transplantation of primordial germ cells (PGCs), which are the progenitor cells of gametes, is a powerful tool for generation of transgenic chickens. However, the frequencies of transgene integration into the genome of purified PGCs still remain low. An *in vitro* culture system enabling chicken PGCs to propagate efficiently would be useful for efficient transgenesis of PGCs. In the present study, we optimized the culture conditions for chicken PGCs to enhance the proliferation and evaluated the germline transmission of cultured PGCs that proliferated for long periods of time. PGC-like cells (PGC-LCs), that have remarkably similar morphological characteristics to intact PGCs, could be derived by cultivation of blood containing PGCs obtained from 2.5-day-old chicken embryos according to the protocol of van de Lavoir *et al*. (2006). We determined which feeder cells and which growth factors were required to improve proliferation of PGC-LCs. Male PGC-LCs survival and proliferation were enhanced during culture in the basic medium containing either basic fibroblast growth factor (bFGF) alone or both bFGF and stem cell factor (SCF) on a feeder of buffalo rat liver (BRL) cells. Male PGC-LCs could be propagated in defined culture condition for extended periods. These cells expressed the germline-specific protein Vasa and undifferentiated cell marker stage-specific embryonic antigen-1 (SSEA-1) and pluripotency genes *Nanog* and *PouV*. Furthermore, Male PGC-LCs cultured for 225 d could migrate toward and colonize within recipient gonads and transmit to the next generation following transplantation. We succeeded in produce 3 offspring originating from long-term cultured PGC-LCs from a germline chimeric rooster (6%). The present study represents valuable steps toward defining a culture condition enabling PGC-LCs to propagate efficiently for long periods *in vitr*o with maintenance of their commitment to the germline.

Key words: chicken, germline chimera, PGCs, PGC-LCs

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Introduction

Primordial germ cells (PGCs) are the only cells in developing embryos with the potential to transmit genetic information to the next generation. In the chicken, about 30−150 PGCs (or their precursors) are initially localized in the central zone of the blastoderm (Tsunekawa *et al*., 2000, Nakamura *et al*., 2007). Following the formation of primitive streak, PGCs are carried anteriorly to an extraembryonic region termed the germinal crescent, then enter the blood vessels from anterior vitelline veins (Nakamura *et al*., 2007, De Melo Bernardo *et al*., 2012). From stage 11 (St. 11; Hamburger and Hamilton, 1951), PGCs circulate temporally in bloodstream, and migrate to genital ridge at St. 15−19. At 15.5−16.5 days of incubation (St. 41−42), primary meiotic germ cells in female embryonic gonads found to be differentiated into oocyte. In contrast, male germ cells retained undifferentiated state with inhibition signals by *CYP26b1* derived from gonadal somatic cells (Smith *et al*., 2008). Finally, female and male germ cells were differentiated into ova and spermatozoa at sexually maturation, respectively.

Several techniques for transplantation of PGCs have been established in chicken and quail. PGC-transplantation prom-

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ised to be an efficient tool for generation of transgenic birds. Recently, transgenic chickens and quails were produced by viral transduction of PGCs (Motono *et al*., 2010; Shin *et al*., 2008); however, foreign genes were integrated randomly into the genome, and make it difficult to manipulate the PGC genome by gene targeting. A robust *in vitro* maintenance and proliferation system of chicken PGCs that can be transmit to next generation provides the opportunity to manipulate the genome and select transfected clones in a manner similar to mice embryonic stem (ES) cells. van de Lavoir *et al*. (2006) reported that chicken PGC cultures (PGC-like cells: PGC-LCs) could be derived from embryonic blood and proliferated in culture for several months. It was also demonstrated that KAv-1 medium (Kuwana *et al*., 1996) could maintain chicken PGC-LCs for long-term *in vitro* (Naito *et al*., 2010, 2012). Unlike in mice ES cells, chicken PGC-LCs proliferated more slowly *in vitro*. Enhancement of chicken PGC-LCs proliferation for long periods without losing germline competence will provide an opportunity to manipulate the genome of PGC-LCs routinely. Therefore, improvement and simplification of culture protocol for chicken PGC-LCs is urgently needed. In addition, few reports regarding on male and female PGC-LCs *in vitro* proliferative and the combination of various growth factors to self-renewal of PGC-LCs.

In the present study, we report on the optimization of culture conditions that support the survival and proliferation of chicken PGC-LCs with maintenance of their commitment to the germ cell lineage *in vitro*. First, we determined which feeder cells and which growth factors were required to improve growth of PGC-LCs. Second, we demonstrated whether the defined culture condition can propagate the number of PGC-LCs for long periods. Third, we characterized long-term cultured PGC-LCs by immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR). Finally, we performed transplantation of PGC-LCs after 225 d or 232 d culture to ascertain whether they could maintain their ability to colonize within recipient gonads and transmit to next generation.

Materials and Methods

Experimental Animals and Animal Care

Fertilized eggs were obtained from White Leghorn (WL) and Barred Plymouth Rock (BPR). All procedures described here were reviewed and approved by the Animal Care and Use Committee of Shinshu University, and were performed in accordance with the Guiding Principle for the Care and Use of Laboratory Animals.

Establishment and Maintenance of PGC-like cells (PGC-LCs)

Whole blood cells containing PGCs were collected from WL or BPR embryos at St. 13−15, and then were seeded on mitomycin C treated Buffalo Rat Liver (BRL) cells. The culture medium used was essentially as described previous works (van de Lavoir *et al*., 2006, Oishi, 2010), with some modifications. Briefly, the basal medium was used BRL cells conditioned KnockOut DMEM (KO-DMEM; Gibco, CA, USA), supplemented with 7.5% FBS ES-Qualified (Gibco), 2.5% chicken serum (CS; Biowest, Nuaillei, France), 2 mM GlutaMAX (Gibco), 1 mM Sodium Pyruvate (Sigma), 1×Nuleosides (Millipore, MA, USA), 1×Nonessential Amino Acids (Gibco), 0.5 mM Monothioglycerol (Wako Pure Chemicals, Osaka, Japan), 5 ng/mL human basic fibroblast growth factor (bFGF; Wako), 4 ng/mL human stem cell factor (SCF; Wako), and 2 ng/mL human leukemia inhibitory factor (LIF; Sigma). About 10 d after incubation of blood cells, PGC-like cells (PGC-LCs) were identified followed by morphological characteristics; diameter larger than 10 μm, observed large nucleus and many lipid in cytoplasm that were very similar to intact chicken PGCs. Cells were cultured on 48-well tissue culture plate (Sumitomo Bakelite, Tokyo, Japan), and amplified for culture scale followed PGC-LCs growth, the high limit culture scales of 6-well plate. Culture media was replaced with half volume in each day and passaged in every 10 d.

Preparation of Feeder Cells

In order to compare the proliferation of PGC-LCs by feeder cells, three kinds of cells were prepared. BRL cells, Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) cells, and chicken embryonic fibroblast primary cultured cells (CEF) obtained from St. 30−32 embryos were used as feeder cells. The culture medias for BRL or STO cells, Dullbecco's modified eagle's medium (DMEM; Sigma, MO, USA) containing 10% fatal bovine serum (FBS; Biowest, Nuaillel, France) and CEF culture media containing 10% FBS, 2.5% CS in DMEM were used, respectively. All cells were cultured on T-75 tissue culture flasks and incubated at 37℃ /5% CO_2.

Evaluation of Culture Conditions for PGC-LCs

PGC-LCs from WL and BPR cultured for 50 d according to the protocol of previous works (van de Lavoir *et al*., 2006, Oishi, 2010) were used for definition of culture conditions. To evaluate which feeder cells support growth of PGC-LCs from WL and BPR, they were cultured in basic media on the either BRL, STO or CEF. To identify the essential growth factors for propagation of PGC-LCs from WL and BPR, they were culture under various combinations of growth factors such as bFGF, SCF and LIF. The primary concentration of PGC-LCs in each culture conditions was adjusted to 1.0×10^4 cells/mL. Growth of PGC-LCs in each culture condition was evaluated after 30 d.

Sexing of Samples

Blood samples were subjected for sexing by polymerase chain reaction (PCR). Approximately 0.5−1.0 μL of collected blood samples were transferred into micro tubes and mixed 15 μL DNA extraction buffer (100 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA) with 10 μL Proteinase K solution (Wako), incubated for 15−30 min at 56℃. After that, 1 μL digested samples were provided for PCR. DNA samples were amplified of W-chromosome *XhoI* repetitive sequence (WCS) and 18S-rebosomal gene sequence (RGS) followed previously (Clinton *et al*., 2001). The reaction buffers were mixed with Quick Taq HS Dyemix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The fol-

lowing reactions were carried out; 94℃ for 2 min, followed by 25 cycles of 94℃ for 30 sec, 56℃ for 30 sec, 68℃ for 30 sec, and a final extension of 68℃ for 5 min. Samples were resolved on a 1.2% TAE agarose gel.

RT-PCR

Total RNA was extracted from the cultured PGC-like cells (PGC-LCs) using RNeasy Plus Micro kit (Qiagen, CA, USA) according to the manufacturer's instructions. RNA samples were reversely transcribed using ReverTra Ace qPCR RT master mix (TOYOBO). PCR reaction mixture was prepared using Takara Ex Taq Hot start version (TaKaRa Bio, Shiga, Japan). The following reactions were carried out; 95℃ for 2 min, followed by 40 cycles of 95℃ for 30 sec, 55℃ for 30 sec, 72℃ for 30 sec, and a final extension of 72℃ for 5 min. Set of the primers used for PCR were shown in Table 1.

Immunofluorescence

Cultured cells were re-suspended by vigorous pipetting and centrifugation, pasted on Teflon-printed multi well slide glass. The cells were fixed with 4% paraformaldehyde (PFA) for 5 min at room temperature (RT). After washing with Ca^{2+} and Mg^{2+} -free Dulbecco's Phosphate Buffered Saline (PBS [-]), the specimens were incubated with 5% goat serum/PBS (-) for 15 min at RT and subsequently incubated with primary antibodies at 4℃ overnight. The cells were washed with PBS (-) and then incubated with secondary antibodies at 4℃ 2 h. Samples were washed with PBS (-) and counter stained with 1 μg/mL DAPI. The specimens were examined using a fluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with an ORCA-ER CCD camera system (Hamamatsu Photonics, Shizuoka, Japan).

Primary antibodies were used at the following dilutions: rat IgG anti chicken vasa homolog (CVH; 1:5,000; Nakamura *et al*., 2007), mouse IgM anti stage-specific embryonic antigen-1 (SSEA-1; 1:50; DSHB, IA, USA). All secondary antibodies were used at a 1:2,000 dilution; anti-rat IgG conjugated with Alexa-488 and anti-mouse IgM conjugated with Alexa-546 (Molecular Probes, CA, USA).

Gonadal Migration Assay

Fertilized eggs of WL were incubated for 53−56 h to obtain St. 13−15 embryos. PGC-LCs of BPR were used until cultured for 250 d. Circulating PGCs (cPGCs) purified from St. 13−14 BPR embryonic blood by Nycodentz density gradient centrifugation (Zhao and Kuwana, 2003, Nakamura *et al*., 2010) were used as control group. Cultured PGC-LCs or cPGCs were labeled with PKH26 GL dye (Sigma) and 100 of the cells were microinjected into bloodstream through the dorsal aorta of the recipient WL embryos (Tajima *et al*., 1993). The manipulated embryos were incubated until St. 30−32, picked out whole gonads, and counted the PKH26 positive cells in left or right gonads under the fluorescence microscope.

Progeny Test

Hatched presumptive chimeric chickens with initially microinjected 1,000 of PGC-LCs from BPR were raised until sexually maturation. Male putative chimeric chickens were crossed with female BPR (*i*/*i*) by artificial insemination, and the feather color of their offspring was examined. Black offspring (*i*/*i*) indicated that the offspring was derived from BPR donor PGC-LCs, whereas white offspring with small patches of black pigmentation (*I*/*i*) indicated that the offspring was derived from recipient WL PGCs. The proportions of BPR progeny were expressed as germline transmission rates.

Statistical Analysis

Statistical analysis was used by Statcel 3 Microsoft Excel add-in program (Yanai, 2010). The proliferation rate of PGC-LCs in each culture condition was analyzed by one-way ANOVA. If model effect was significant, differences between mean values for each treatment were then evaluated using the Bonferroni test. Difference of migration ability was compared between PGC-LCs and cPGCs using Student's *t*-test. All data was presented mean±SEM.

Table 1. **PCR primer sets**

Gene	Seqence	Annealing Temp.	Product Size (bp)	Accession No. or Ref.
Nanog F	CAGCAGACCTCTCCTTGACC	55℃	435	NM_001146142
Nanog R	CCAGATACGCAGCTTGATGA			
PouV F	CTCAGAGGAGCTGGAGCAGT	55℃	588	NM_001110178
PouV R	TTGTGGAAAGGTGGCATGTA			
Cvh F	GCATGCTCGATATGGGTTT	55℃	551	NM_204708
Cvh R	ACGACCAGTTCGTCCAATTC			
Dazl F	GTCAACAACCTGCCAAGGAT	55℃	519	NM_204218
Dazl R	CACTGTGGTGGAGCCTGATA			
B-actin F	ACTGGATTTCGAGCAGGAGA	55℃	581	NM_205518
B-actin R	AGTCAAGCGCCAAAAGAAAA			
WCS F	CCCAAATATAACACGCTTCACT	56℃	416	Clinton *et al*., 2001
WCS R	GAAATGAATTATTTTCTGGCGAC			
RGS F	AGCTCTTTCTCGATTCCGTG		256	
RGS R	GGGTAGACACAAGCTGAGCC			

Results

PGC-LCs Proliferation in vitro

After 10 d culture of the cells obtained from embryonic blood according to the protocol of van de Lavoir *et al*. (2006), PGC-LCs were confirmed by their morphological feature. PGC-LCs contain many lipid and large nucleus in cytoplasm with diameter approximately 10−20 μm, and these morphological characteristics were very similar to cPGCs (Fig. 1). Male PGC-LCs were exponentially proliferated and the cell numbers after 40 d of culture were $1.85\pm0.73\times10^3$ cells/mL (WL) and $8.43\pm2.80\times10^3$ cells/mL (BPR), respectively (Fig. 2A, B). In contrast, few female PGC-LCs proliferated neither in WL or BPR.

Proliferation of the PGC-LCs was further investigated using various conditions with modified feeder cells or additional growth factors. First, we examined proliferative effects of various feeder cells in PGC-LCs. As a result, BRL as feeder layer was most effective in proliferation of PGC-LCs; the number of cultured cells reached to $8.70\pm0.66\times10^6$ cells/mL (WL) and $9.72\pm0.82\times10^6$ cells/mL (BPR), respectively (Fig.3A). No significant difference ($P>0.05$) was observed in proliferation of PGC-LCs between STO cells ($2.99\pm0.67\times10^6$ cells/mL [WL]; $2.56\pm0.12\times10^6$ cells/mL [BPR]) and CEF ($5.94\pm1.16\times10^6$ cells/mL [WL]; $2.89\pm0.30\times10^6$ cells/mL [BPR]).

Subsequently, we investigated the effects of various combinations of growth factors on the proliferation of PGC-LCs. Most effective growth factor combination(s) for PGC-

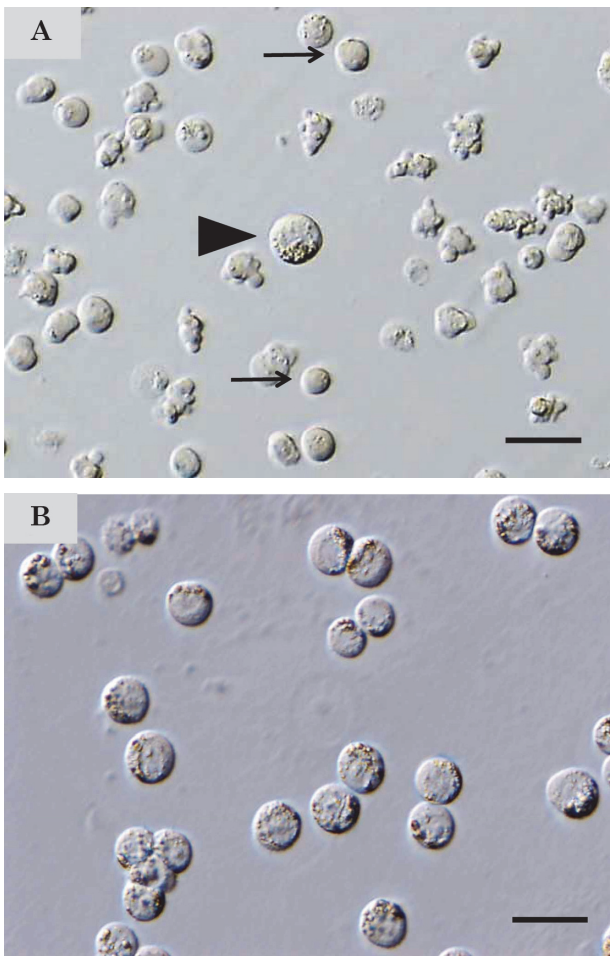

Fig. 1. **Morphological characteristics of the cPGCs and PGC-LCs**
(A) Intact cPGCs in the blood cells isolated from St. 14 BPR embryo. (B) Male PGC-LCs derived from BPR embryonic blood after 247 d cultured on BRL feeder layer. PGC-LCs contains large nucleus and many lipids in cytoplasm, the morphological characteristics is similarly to cPGCs. An arrow head indicate one of the PGCs, and arrows indicate blood cells. Bars＝20 μm.

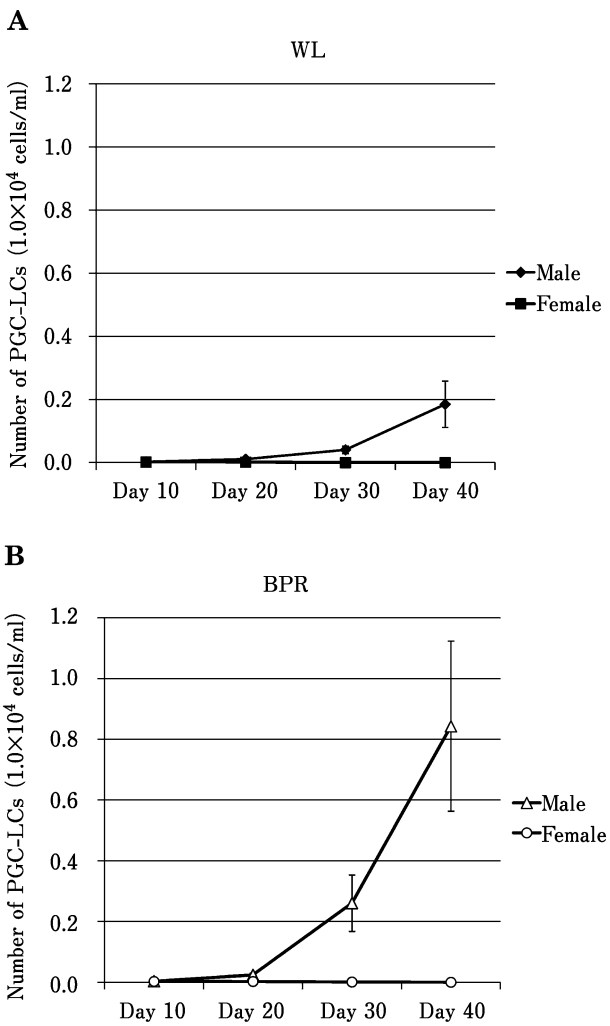

Fig. 2. **Growth curves of primary PGC-LCs from 10 days after culture started**
(A) WL derived PGC-LCs (male, $n=20$; female, $n=20$). (B) BPR derived PGC-LCs (male, $n=20$; female, $n=20$). All data was presented mean±SEM.

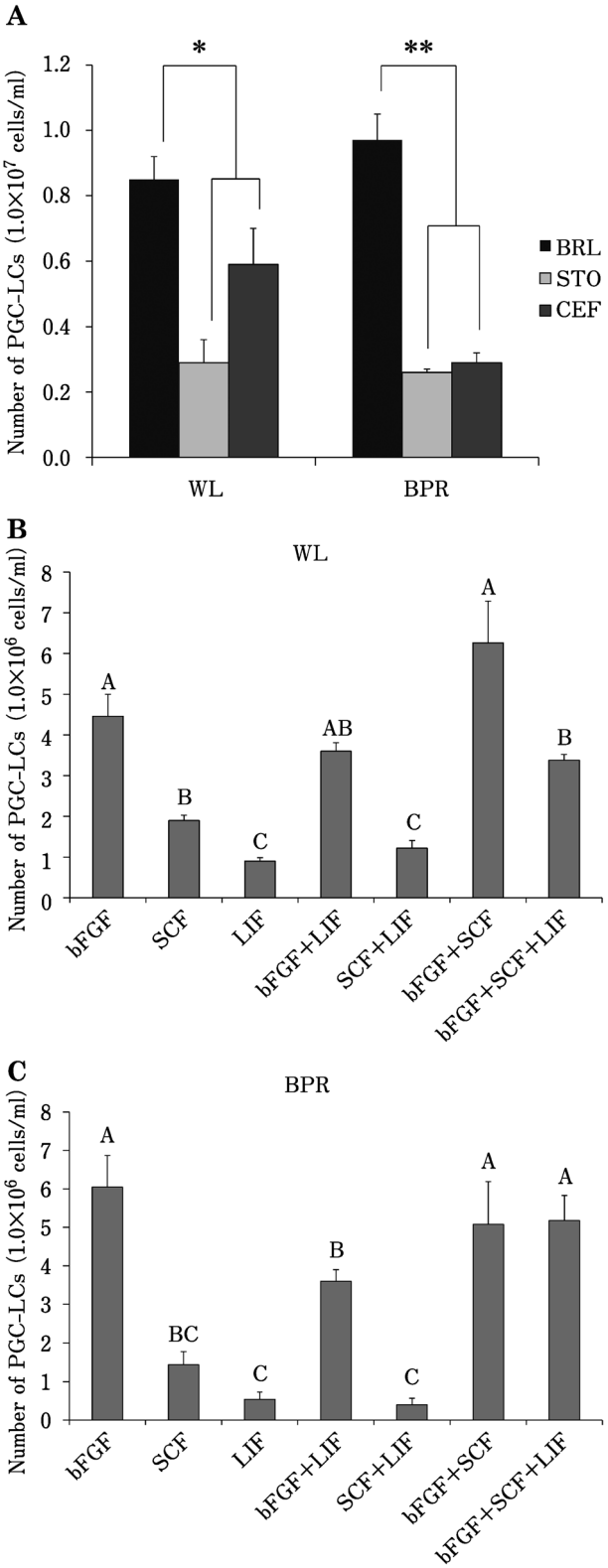

Fig. 3. **Validation of PGC-LCs culture condition**
(A) Effects of various feeder layer in PGC-LCs culture (*: $P<0.05$, **: $P<0.01$). (B) Effects of various growth factors in WL and (C) BPR. Different letters were indicated significantly difference ($n=4$, mean±SEM, $P<0.05$).

LCs proliferative was supplementation of bFGF alone or both bFGF and SCF ($P<0.05$; Fig. 3B, C). PGC-LCs numbers of supplementation bFGF alone, $4.46\pm0.54\times10^6$ cells/mL (WL) and $6.05\pm0.82\times10^6$ cells/mL (BPR), both bFGF and SCF supplementation, $6.26\pm1.03\times10^6$ cells/mL (WL) and $5.08\pm1.08\times10^6$ cells/mL (BPR), respectively. One of the most efficient culture conditions for PGC-LCs from male embryo was determined as usage of BRL feeder layer with BRL conditioned KO-DMEM supplementation with bFGF alone or both bFGF and SCF.

Immunofluorescence and Molecular Characteristic of PGC-LCs

Cultured PGC-LCs were stained with CVH and SSEA-1 antibodies. Cells were co-expressed CVH and SSEA-1; CVH protein strong expressed in a cytoplasm, and SSEA-1 was detected on plasma membrane (Fig. 4A–D).

The *Nanog*, *PouV* (pluripotent cell marker), and *Cvh*, *Dazl* (germ cell marker) expression were detected in PGC-LCs from WL and BPR (Fig. 4E). These expression patterns in PGC-LCs were similar to those of the cPGCs.

Germline Transmission of Long-term Cultured PGC-LCs

To examine migration abilities in long-term cultured PGC-LCs, 100 of PGC-LCs cultured until 250 d or purified cPGCs were labeled with PKH26 dye, and microinjected into St. 13–15 recipient dorsal aorta. The PKH26 positive cells were observed in all of the manipulated embryonic gonads (Fig. 5A), the results demonstrated that long-term cultured PGC-LCs have migration ability into recipient gonads. However, the migrated PGC-LCs numbers were significantly decreased compared to cPGCs in the both gonads, these cell numbers in the left gonad, 31.83 ± 4.23 (control) and 18.67 ± 1.45 (PGC-LCs, $P<0.05$), in the right gonad, 22.50 ± 2.14 (control) and 10.83 ± 1.25 (PGC-LCs, $P<0.01$), respectively (Fig. 5B).

Subsequently, 1,000 of the cultured PGC-LCs were transferred into recipient embryos. The 8 germline chimeric chickens were sexually matured among manipulated embryos (5 males and 3 females), and carried out progeny test in male chimeric chickens. One male chimera (ID; 325) produced by microinjection of the 225 d cultured PGC-LCs, generated three donor derived offspring (6%; 3/50) (Table 2, Fig. 5C). Thus, the cultured PGC-LCs found to be differentiated into fertilizable gamete. However, live-offspring derived from PGC-LCs were not obtained.

Discussion

In the present studies, long-term *in vitro* culture of chicken PGC-LCs could be achieved. One of the most efficient culture conditions was determined as usage of BRL feeder layer and BRL conditioned KO-DMEM supplemented with bFGF alone or bFGF and SCF. BRL cells are known to secrete of differentiation inhibitor such as LIF and SCF that important to pluripotent ES cell culture (Smith *et al*., 1988, Zsedo *et al*., 1990). It was considered that the PGC-LCs *in vitro* should be propagated by various growth factors secreted from BRL cells. Especially, the bFGF is considered as an important factor of the *in vitro* survival and proliferation of chicken PGCs. This factor effects to the cell pro-

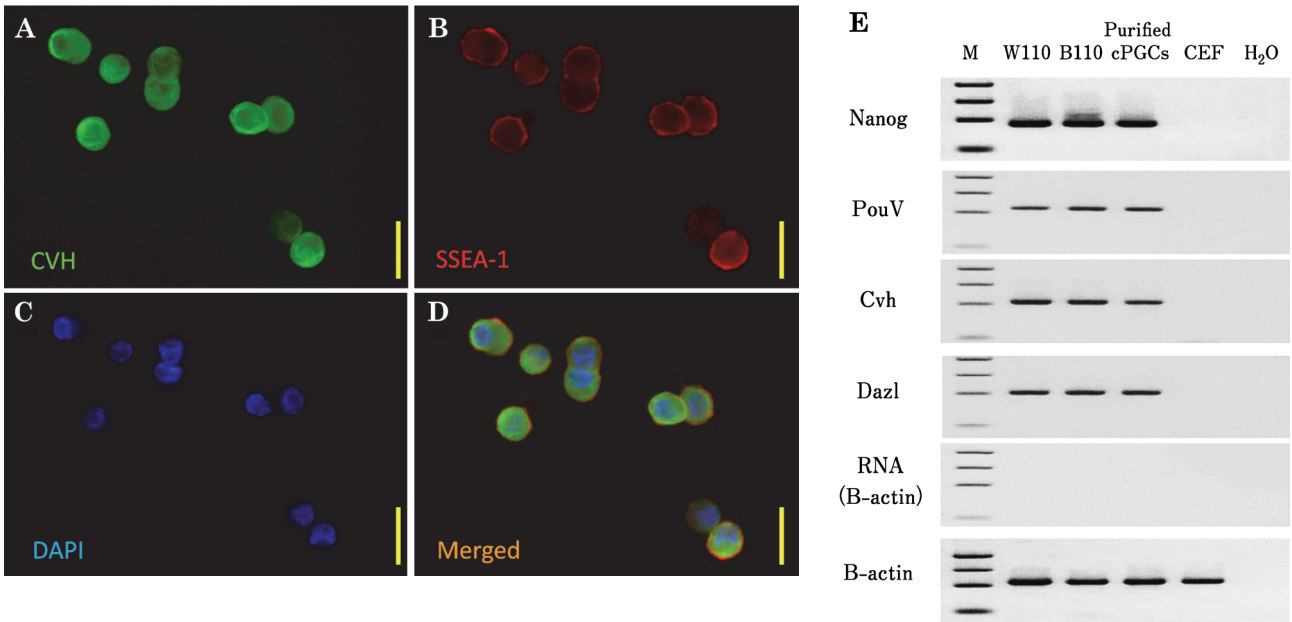

Fig. 4. **Immunohistochemistry and molecular characterization of the PGC-LCs**
Immunofluorescence used CVH and SSEA-1 antibodies, (A) CVH (Green), (B) SSEA-1 (Red), (C) Nucleus (Blue), (D) Merged image. (E) RT-PCR analyses were carried out PGC-LC lines, purified cPGCs and CEF. M; DNA size marker, W110; WL derived PGC-LC line, B110; BPR derived PGC-LC line, H_2O; no-cDNA negative control, RNA; isolated RNA (RT-) derived B110. Bars＝20 μm.

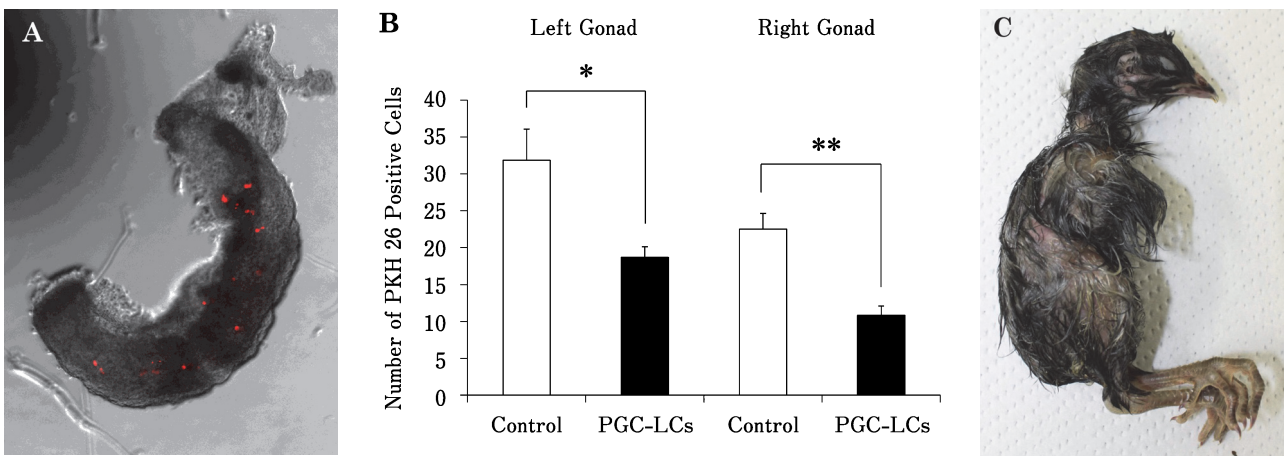

Fig. 5. **Germline transmission of long-term cultured PGC-LCs**
(A) PKH26 positive cells were observed in recipient left gonads. (B) Number of PKH26 positive cells in St. 30−32 left or right gonads (n＝6, *: P＜0.05, **: P＜0.01, mean±SEM). (C) Offspring derived from PGC-LCs of cultured 225 d.

liferation by activation of MEK/ERK signaling pathway (Choi *et al*., 2010). On the other hand, SCF is the c-Kit ligand, known as an activation of PI3K/Akt signaling pathway. In a previous study, administration of MEK/ERK or PI3K/Akt inhibitor for the culture media resulted to apoptosis or proliferative inhibition in PGCs culture (Macdonald *et al*., 2010). However, addition of SCF alone, did not effect for PGC-LCs proliferative (Fig. 3B, C). The result suggested

Table 2. **Progeny tests of male chimeric chickens generated with long-term cultured PGC-LCs**

ID	Days of PGC-LCs Culture	Number of		
		White (*I*/*i*) Offspring	Black (*i*/*i*) Offspring	Donor Derived Offspring (%)
325	225	50	3	6
326	225	36	0	0
328	232	35	0	0
330	232	35	0	0
331	232	50	0	0

that addition of SCF into the media might lead small affection for the PI3K/Akt signal activation. Choi *et al*. (2010) and Macdonald *et al*. (2010) demonstrated that the bFGF could activate not only MEK/ERK but also PI3K/Akt signaling pathway. Therefore, it was suggested that the phosphorylation MEK/ERK and PI3K/Akt signaling was activated by bFGF addition. Our data supported these findings. However, female PGC-LCs could not be cultured in the same condition. Therefore, our culture condition could be effective only in male PGC-LCs culture.

Kagami *et al*. (1995, 1997) were demonstrated that genetically male and female PGCs derived from blastoderm could differentiate into functional gametes in the mixed-sex chimeric chicken gonads. Meanwhile, female cPGCs derived from St. 15 embryonic blood could not differentiate into functional spermatozoa in the male chimeric chicken testis; a frequency of W-bearing spermatozoa in the ejaculated semen was 0.2% (Tagami *et al*., 2007). These results were suggested that the plasticity on sexual differentiation of the PGCs disappeared between blastodermal stage and St. 15. These results suggested that cPGCs has already started sex differentiation, and it was expected that female PGC has a different self-renewal system compared to male PGCs. Thus, it is necessary to establish the novel culture system that adapted to female PGCs.

In addition, the present culture system showed the inter-strain difference of the *in vitro* propagation ability between WL and BPR PGC-LCs (Fig. 2A, B). Naito *et al*. (1994) and Nakamura *et al*. (2011) were reported that PGCs propagation abilities *in vivo* are varied between chicken breeds, and *in vitro* proliferative of PGC-LCs also might be different between WL and BPR.

PGC-LCs were positively detected with CVH (germ cells marker) and SSEA-1 (undifferentiated cells marker; Fig. 4A-D). The *Nanog*, *PouV*, *Cvh*, *Dazl* expression were also detected by the RT-PCR (Fig. 4E). CVH protein is the germ cell specific protein, detected from PGCs until round spermatid or primary oocyte in germline (Tsunekawa *et al*., 2000). Chicken *Nanog* and *PouV* homolog were expressed in undifferentiated cells such as chicken embryonic stem cell-like cells and PGCs. It was assumed that these genes regulate critical role for maintenance as undifferentiate (Lavial *et al*., 2007). Thus, it was suggested that the cultured cells were maintained undifferentiated state by *Nanog* and *PouV* expression.

When the cultured PGC-LCs for 250 d were labeled with PKH26 dye and transferred into recipient embryos, migration of the labeled PGC-LCs into recipient gonad was confirmed. However, the detected donor cell number in chimeric gonads has been decreased as compared to control and initially microinjected cell numbers (Fig. 5B). These results suggested that long-term cultured PGC-LCs have been decreased migration abilities. Chemokine C-X-C motif ligand 12 (CXCL 12) was considered one of the candidate chemoattractants of migration chicken PGCs (Stebler *et al*., 2004).

CXCL12 is ligand of the Chemokine C-X-C motif receptor 4 (CXCR4) that is expressed on the surface of PGCs. The migration ability of PGC-LCs might be decreased by down regulation of *Cxcr4* expression.

In the present study, donor derived offspring were obtained from cultured for 225 d PGC-LCs. Therefore, the long-term cultured PGC-LCs could differentiate into fertilizable spermatozoa. Thus, the culture system would be a useful tool for avian genetic modification with both research and industrial applications. The previous studies reported efficient transgenesis methods using long-term cultured transgenic PGCs mediated by *piggyBac* or *Tol2* transposon (Macdonald *et al*., 2012, Park and Han, 2012). However, in this study, germline transmission rate of the long-term cultured PGC-LCs were low (6%; 3/50). Thus, sterilization of the recipient should be essential for improvement of donor-germline transmission by use of irradiation (Carsience *et al*., 1993, Atsumi *et al*., 2009, Macdonald *et al*., 2010, Nakamura *et al*., 2012), busulfan treatment (Song *et al*., 2005, Nakamura *et al*., 2008, 2010). On the other hand, gametic differentiation ability of PGC-LCs might be decreased during long-term culture, this system should be progress so as to improve the ability of gametic differentiation in PGC-LCs.

In conclusion, we elucidated of most efficient PGC-LCs culture condition as usage of BRL feeder layer and BRL conditioned KO-DMEM supplemented with bFGF alone or bFGF and SCF. The cells cultured in the most efficient condition could differentiate into fertilizable spermatozoa. Our developed culture system should be one of the useful tools for generation of transgenic chicken.

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