Expression of GFP Gene in Cultured PGCs Isolated from Embryonic Blood and Incorporation into Gonads of Recipient Embryos

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The present study was conducted to develop a technique for generating primordial germ cells expressing GFP gene and introducing them into gonads of recipient embryos. Primordial germ cells isolated from embryonic blood were cultured on feeder cells for more than 40 days. They proliferated, occasionally formed cell colonies, and showed the characteristics of germline cells as detected by anti-CVH antibody. The cultured PGCs were transferred to the stage X blastoderm, bloodstream of stages 14–15 embryos, and the coelomic epithelium of stages 17–19 embryos, and examined to determine whether they could migrate to the gonads of recipient embryos. As a result, they successfully entered the gonads of recipient embryos when they were transferred to the coelomic epithelium, although they failed to migrate to the gonads of recipient embryos when they were transferred to the stage X blastoderm or bloodstream. The cultured PGCs were then transfected with GFP gene by nucleofection and selected for PGCs expressing GFP gene in the presence of G418. Cultured PGCs expressing GFP gene proliferated slowly, forming cell colonies, and successfully entering the gonads by transferring into the coelomic epithelium of recipient embryos. Those results suggest that gene transfer into the chicken germline is possible via cultured PGCs, and that the PGC culture system thus holds enormous possibilities for avian embryo manipulation.

Key words: chicken embryo, germline chimaera, GFP gene, primordial germ cell

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Introduction

Development of a gene transfer system in chickens is very useful for genetic modification of chickens, and for the analysis of the functions of cloned genes. So far, various attempts have been made to produce transgenic chickens by either the retroviral vector method or the germline cell-mediated method (Naito, 2003; Han, 2009; Sang, 2009; Song *et al.*, 2010). Although the former method is effective for introducing exogenous genes into chickens, it has disadvantages: it restricts transgene size and poses safety problems. Therefore, manipulation of germline cells is currently considered to be the more useful method for gene transfer into chickens (Naito *et al.*, 2010). Since primordial

germ cells (PGCs) are progenitor cells of ova and spermatozoa, germline chimaeric chickens can be produced by transfer of PGCs between embryos (Tajima *et al.*, 1993; Naito *et al.*, 1994a, 1994b, 1998, 1999). An *in vitro* culture of PGCs makes it possible to incorporate stably exogenous genes into PGCs. A long-term culture of chicken PGCs was reported by successful, so that the GFP gene was successfully introduced into PGCs, resulting in the production of transgenic chickens (van de Lavoie *et al.*, 2006; Leighton *et al.*, 2008; Macdonald *et al.*, 2010). However, in that culture system, the authors used mouse or rat feeder cells for culturing chicken PGCs *in vitro*. To avoid the risk of a cross-transfer of animal pathogens from other animal cells, it is preferable not to use cells of other species for culturing chicken PGCs.

In our previous study, PGCs isolated from the blood of 2.5-day incubated embryos were cultured on feeder cells derived from the gonads of 7-day incubated chicken embryos (Naito *et al.*, 2010). The cultured PGCs proliferated even

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after transfecting GFP gene, and produced a germline chimaeric chicken after transferring them into the bloodstream of recipient embryos. The efficiency of producing germline chimaeric chickens by such a transfer of cultured PGCs, however, proved disappointingly low, and most of the cultured PGCs lost their migratory ability to the gonads of recipient embryos. A PGC culture system should, therefore, be improved so as to preserve the undifferentiated state of PGCs. On the other hand, it is thought that cultured PGCs may be introduced by devising a novel transfer method to recipient embryos. When cultured gonocytes obtained from 19-day incubated chicken embryos were transferred to recipient embryos, they successfully entered the gonads of those embryos by transferring them into the coelomic epithelium corresponding to the future gonadal region of the recipient embryos (Naito *et al.*, 2011). Thus, it may be expected that this novel transfer system will be applied to the cultured PGCs whose migratory ability was lost during the culture period.

In the present study, PGCs isolated from embryonic blood were cultured on feeder cells derived from chicken gonadal stroma cells and were transfected with GFP gene. The successfully transfected PGCs expressing GFP gene were then transferred to recipient embryos to determine whether they could be incorporated into the germline of recipient embryos.

Materials and Methods

Fertilised Eggs and Animal Care

Fertilised eggs of White Leghorn (WL) and Barred Plymouth Rock (BPR) chickens were obtained by artificial insemination. WL and BPR populations are maintained at the National Institute of Livestock and Grassland Science. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (National Institute of Agrobiological Sciences, Animal Care Committee).

Experimental Design

In experiment 1, the method for incorporating the cultured PGCs into the gonads of recipient embryos was examined. PGCs were cultured *in vitro* and then transferred into the blastoderm, bloodstream or coelomic epithelium of recipient embryos. Transferred PGCs were then examined for incorporation into the gonads of 7-day and 20-day incubated recipient embryos. In experiment 2, introduction of GFP gene into the cultured PGCs and incorporation of the GFP-positive PGCs into the gonads of recipient embryos were examined according to the results of experiment 1. Cultured PGCs were transfected with GFP gene and then selected for GFP-expressing cells. GFP-positive PGCs were transferred into the coelomic epithelium of recipient embryos and then examined for incorporation in the gonads of 6-day incubated recipient embryos.

PGC Culture in vitro

Fertilised eggs of BPR were incubated at 38°C for about 53 in a forced-air incubator (P-008B Bio-type, Showa Furanki, Saitama, Japan). Blood was collected from the dorsal aorta of embryos at stages 13–15 (Hamburger and Hamilton, 1951) using a fine glass micropipette. The

collected blood thus pooled was dispersed in a KAv-1 culture medium (Kuwana *et al.*, 1996) supplemented with a 10 ng/ml human leukemia inhibitory factor (hLIF) (LIF1010, Chemicon, Temecula, CA, USA), a 10 ng/ml human basic fibroblast growth factor (bFGF) (Upstate, Temecula, CA, USA), and a 10 ng/ml human stem cell factor (SCF) (R&D Systems, Minneapolis, MN, USA), and then cultured on feeder cells derived from gonads of 20-day incubated WL embryos.

Identification of Cultured PGCs by Immunohistochemistry

Cultured PGCs were identified by immunostaining. The cultured cells were fixed with 4% paraformaldehyde (163-20145, Wako Pure Chemical, Osaka, Japan) for 1 h. After washing with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS(–)) (Cat. No. 28-103-05 FN, Dainippon Pharmaceutical, Osaka, Japan), blocking was carried out with Blocking One (03953-95, Nacalai Tesque, Kyoto, Japan) for 1 h. The cells were then incubated with chicken *vasa* homologue (CVH) antibodies (1:4,000 dilution, Tsunekawa *et al.*, 2000) for 1 h. After a second washing with DPBS(–), the cells were incubated with alkaline phosphatase-labelled goat anti-rabbit immunoglobulin (1:200 dilution, SAB1005, Open Biosystems, Huntsville, AL, USA) for 30 min. The cells were then washed with DPBS(–), incubated with 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tertazolum chloride substrate (K0598, Dako Cytomation, Glostrup, Denmark) for several minutes, and then washed with distilled water. The treated cells were observed using an inverted microscope (DMIRE2, Leica Microsystems, Tokyo, Japan).

Fluorescent Labelling of Cultured PGCs

PGCs cultured for 19 days were collected by trypsin treatment (T4049, Sigma-Aldrich, St. Louis, MO, USA) and washed with DPBS(–). They were then labelled with fluorescent dye (Vybrant CFDA Cell Tracer Kit, Cat. No. V12863, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The fluorescent-labelled cultured PGCs were dispersed in a fresh KAv-1 medium.

Transfection of Cultured PGCs and Selection of GFP-Positive Cells

When the transfection of PGCs was conducted, the collected blood was cultured in suspension for 5 days in a 12-well plate (CS2002, CellSeed, Tokyo, Japan), and the cultured cells were then recovered for transfection. The transfection of PGCs involved a new electroporation-based technique known as “nucleofection”. The collected PGCs were dispersed in a 100 μl Nucleofector solution (solution V, Amaxa GmbH, Köln, Germany) containing 5 μg of linearised pbAEGFP plasmid (GFP gene under the control of chicken β -actin gene promoter). The solution was transferred into a kit-provided cuvette and inserted into a Nucleofector device, and the transfection was accomplished using the nucleofection programme A-033. Subsequently, 500 μl of culture medium was added to the cuvette. The recovered cells containing PGCs were cultured for 5 days on feeder cells fixed with 2.5% glutaraldehyde (17052-36, Nacalai Tesque, Kyoto, Japan). The culture medium was then exchanged for a selection medium containing 300 $\mu\text{g}/\text{ml}$ G418

disulfate (108321-42-2, Nacalai Tesque, Kyoto, Japan) and cultured for 14 days. The selection medium was subsequently replaced with fresh culture medium and cultured for a further 14 days or more.

Transfer of Cultured PGCs into Recipient Embryos

PGC colonies in the culture were isolated from a feeder layer, then treated with trypsin, dispersed in a 100 μ l fresh KAV-1 medium, and placed in a plastic dish (Cat. No. 3001, Becton Dickinson, Franklin Lakes, NJ, USA). Freshly laid WL eggs were broken to remove the thick albumen capsule, and the yolk was put in a glass vessel placing the blastoderm on top of the yolk. The egg was placed in the chamber of a soft X-ray apparatus (E3, Softex Inc., Tokyo, Japan), and the embryos were irradiated (15 kVP, 5 mA, 0.083 nm) for 60 sec (0.8 Gy) (Nakamichi *et al.*, 2006). When transferring cells into the stage X blastoderm (Eyal-Giladi and Kochav, 1976), 200 cultured PGCs were picked up with a fine glass micropipette and injected into the subgerminal cavity of the blastoderm (Naito *et al.*, 1991). The manipulated embryos (eggs) were cultured in host eggshells (systems II and III) as described by Perry (1988) and Naito *et al.* (1990). When transferring the cultured PGCs into the bloodstream or the coelomic epithelium, recipient embryos were cultured in host eggshells (system II) at 38°C for about 53–70 hours. Once the embryos reached stages 14–15, 200 cultured PGCs were picked up and injected into the bloodstream of recipient embryos (Naito *et al.*, 1994a). When the cultured PGCs were transferred into the coelomic epithelium of recipient embryos, 200 cultured PGCs were picked up and injected into the coelomic epithelium corresponding to the presumptive gonadal region of recipient embryos at stages 17–19. The manipulated embryos were cultured in host eggshells (system III) until analysis.

Detection of Donor-Derived Cells in Recipient Gonads

The presence of donor-derived cells in the recipient gonads was detected by PCR. Gonads were obtained from the manipulated embryos, and their DNA was extracted using a DNA extraction kit (SepaGene, Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions. The extracted DNA was dissolved in distilled water at a concentration of 100 ng/ μ l, and PCR analysis was then performed to detect the presence of donor-derived cells (BPR). Detection of WL- and BPR-specific sequences in *PMEL17* gene (Kerje *et al.*, 2004) was carried out by the method of Choi *et al.* (2007) with some modifications. The primer sequences were 5'-CTG CCT CAA CGT CTC GTT GGC-3' and 5'-AGC AGC GGC GAT GAG CGG TG-3' for detecting WL, and 5'-CTG CCT CAA CGT CTC GTT GGC-3' and 5'-AGC AGC GGC GAT GAG CAG CA-3' for detecting BPR. A PCR mixture was prepared using the Takara PrimeSTAR GXL kit (R050A, Takara Bio Inc. Tokyo, Japan), and the reaction was carried out in 25 μ l reactions containing 50 ng genomic DNA, 0.5 μ M primers, 0.2 mM dNTPs, and 0.25U DNA polymerase, using the GenAmp PCR system 9700 (Applied Biosystems, Tokyo, Japan). After an initial denaturation step of 94°C for 5 min, 40 cycles were conducted; 30 seconds of denaturation at 94°C, 30 seconds of annealing and extension

at 72°C, and a final 5 minute extension at 72°C. After amplification, the PCR products were separated on a 2% agarose gel, with the bands (WL: 222 bp, BPR: 213 bp) visualised under UV light after ethidium bromide staining.

The incorporation of donor-derived cells in the gonads of recipient embryos was also confirmed by detecting fluorescent labelling cells or GFP-positive cells in the gonads of recipient embryos. Embryos cultured for 3–4 days (stage 28–30) after injection of the cultured PGCs were removed from the yolk, washed with DPBS(–), and the gonads were exposed. Fluorescently labelled cells or GFP-positive cells in the gonads of manipulated embryos were examined under a fluorescent microscope (MZFL-III, Leica Microsystems, Tokyo, Japan).

Results

Experiment 1

PGCs Cultured in vitro

Collected blood cells containing PGCs were cultured on feeder cells derived from gonads of 20-day incubated embryos. Most of the blood cells disappeared during the first 10–14 days of culture, after which PGCs were detected (Fig. 1A) that occasionally formed cell colonies, and were loosely attached to the feeder layer. Once passages were carried out, PGCs were collected and dissociated by trypsin treatment, and cultured again on freshly prepared feeder cells (Fig. 1B). The cultured PGCs were analysed to clarify whether they exhibited characteristics of germline cells by staining with anti-CVH antibody. As shown in Fig. 1C, most of the cultured PGCs were vasa-positive.

PCR Analysis and Sensitivity of Detecting WL and BPR

WL and BPR DNA were clearly distinguished by a modified PCR analysis (Fig. 2). WL genomic DNA was serially diluted with BPR genomic DNA, while BPR genomic DNA was serially diluted with WL genomic DNA. PCR analysis was carried out to detect the presence of WL or BPR DNA (results are shown in Fig. 2). Both WL and BPR DNA were clearly detected at a low concentration (1:1,000), but no clear bands were detected following a further dilution (1:10,000).

Migration of Cultured PGCs into Gonads of Recipient Embryos

Cultured PGCs were collected and dissociated by trypsin treatment, then transferred to the blastoderm, bloodstream or coelomic epithelium of recipient embryos. The manipulated embryos were then cultured in host eggshells until 20-day of incubation. Gonads were obtained from embryos and DNA was extracted. The presence of donor-derived cells in recipient gonads was analysed by PCR (Fig. 3). When cultured PGCs were transferred into the stage X blastoderm or bloodstream of stages 14–15 embryos, no donor-derived DNA (BPR) was detected in the gonads of 20-day incubated embryos. On the other hand, when cultured PGCs were transferred into the coelomic epithelium of stages 17–19 embryos, donor-derived DNA (BPR) was detected in the gonads of 8 (8.4%) out of 95 embryos analysed (Table 1).

To further confirm the migration of cultured PGCs into the gonads of recipient embryos by injection into the coelomic

Fig. 1. **PGCs cultured *in vitro*.** PGCs isolated from embryonic blood were cultured on feeder cells. Cultured PGCs proliferated and occasionally formed cell colonies. PGCs cultured for 16 days (A), PGCs cultured for 41 days, just after passage (B), and PGCs cultured for 44 days and stained with anti-CVH antibody (C). Bars indicate 20 μ m.

Fig. 2. **Identification of chicken breeds (WL and BPR) by PCR analysis of *PMEL17* gene.** WL DNA was serially diluted with BPR DNA, which was serially diluted in turn with WL DNA. Upper lanes: BPR detection, Lower lanes: WL detection. Lane 1: size marker, Lane 2: WL (control, 50 ng), Lane 3: BPR (control, 50 ng), Lane 4: negative control (DW), Lane 5: (1:10), Lane 6: (1:100), Lane 7: (1:1,000), Lane 8 (1:10,000).

epithelium, the 19-day cultured PGCs were labelled with fluorescent dye and transferred into the coelomic epithelium of recipient embryos. After 4 days of incubation, the manipulated embryos were analysed for the presence of transferred donor PGC-derived cells in the gonads of recipient embryos. Fluorescent labelled cells were detected in the gonads of 4 (50.0%) out of 8 embryos examined (Table 2), and were distributed in the left and/or right gonads of recipient embryos (Fig. 4).

Experiment 2

Generation of Cultured PGCs Expressing GFP Gene

The collected blood containing PGCs was cultured for 5 days in suspension. During this period, PGCs proliferated several times. Collected PGCs were successfully transfected with GFP gene by nucleofection. The proportion of PGCs

Fig. 3. **Detection of donor-derived cells in the gonads of 20-day cultured recipient embryos.** Cultured BPR PGCs were transferred into the coelomic epithelium of WL recipient embryos at stages 17–19 and cultured until just before hatching. Gonads were obtained from the manipulated embryos and analysed for the presence of the donor-derived cells. Upper lanes: BPR detection, Lower lanes: WL detection (*PMEL17* gene), Lane 1: size marker, Lane 2: positive control (WL), Lane 3: positive control (BPR), Lane 4: negative control (DW), Lane 5: negative sample, Lanes 6–9: positive samples.

expressing GFP gene was about 50% after 2 days of transfection treatment. The manipulated PGCs were then cultured on feeder cells and selected for successfully transfected PGCs expressing GFP gene. Cultured PGCs expressing GFP gene were further cultured; they proliferated slowly, formed cell colonies, and the size of the cell colonies gradually increased (Fig. 5).

Migration of GFP-Positive PGCs into Gonads of Recipient Embryos

PGCs expressing GFP gene cultured for 40–56 days were isolated from feeder cells, dissociated, and transferred into the coelomic epithelium of recipient embryos. Manipulated embryos were cultured for further 3 days and analysed for the

Table 1. Detection of cultured PGCs (BPR)-derived cells in gonads by PCR after transfer into recipient embryos (WL)

Cultured PGCs transferred	Number of embryos treated at stage X	Number (%) of embryos surviving at day 3 of incubation	Number (%) of embryos surviving at day 20 of incubation	Number (%) of BPR-positive embryos/examined
Stage X blastoderm	72	53 (73.6)	22 (30.6)	0 (0.0)
Bloodstream at stages 14–15	72	71 (98.6)	21 (29.2)	0 (0.0)
Coelomic epithelium at stages 17–19	476	434 (91.2)	95 (20.0)	8 (8.4)

Table 2. Detection of fluorescent labelled cells in gonads of recipient embryos

Cultured PGCs transferred	Number of embryos treated at stage X	Number (%) of embryos surviving at day 3 of incubation	Number (%) of embryos surviving at day 7 of incubation	Number (%) of embryos with fluorescent labelled-positive cells in gonads/examined
Coelomic epithelium at stages 17–19	18	17 (94.4)	8 (44.4)	4 (50.0)

Fig. 4. Introduction of cultured PGCs into gonads of recipient embryos. PGCs cultured for 19 days were labelled with fluorescent dye and then transferred into the coelomic epithelium of recipient embryos. Manipulated embryos were then cultured for a further 4 days and examined for the presence of donor-derived cells in the recipient embryos. Fluorescence-labelled cells were detected in the left and/or right gonads of recipient embryos (A, B). Bars indicate 0.5 mm.

presence of GFP-positive cells in the gonads. GFP-positive gonads were detected in 46 (22.9%) out of 201 embryos examined (Table 3). GFP-positive cells were distributed in the left and/or right gonads of recipient embryos (Fig. 6).

Discussion

The present study shows that PGCs isolated from embryonic blood were cultured *in vitro* and that the PGCs cultured for more than 40 days retained the characteristics of germline cells as detected with anti-CVH antibody. Cultured

PGCs could enter the gonads of recipient embryos by transferring them into the coelomic epithelium, which corresponds to the future gonadal region as confirmed by fluorescent labelling of the PGCs. Incorporation of cultured PGCs into recipient gonads was also confirmed by PCR analysis. Transfection of the cultured PGCs with GFP gene was performed, and successfully transfected PGCs were selected in the presence of G418. The cultured PGCs expressing GFP gene proliferated slowly and formed cell colonies. Isolated cultured PGCs expressing GFP gene could

Fig. 5. PGCs expressing GFP gene cultured for 40 days. PGCs were obtained from embryonic blood and cultured for 5 days in suspension. Cultured PGCs were then transfected with GFP gene and selected for GFP-positive cells. Cultured PGCs expressing GFP gene proliferated and formed cell colonies (A: PGC colony, B: Expression of GFP gene). Bars indicate 20 μ m.

Fig. 6. Introduction of cultured PGCs expressing GFP gene into gonads of recipient embryos. Cultured PGCs expressing GFP gene were transferred into the coelomic epithelium of recipient embryos at stages 17–19. Manipulated embryos were cultured for a further 3 days and examined for the presence of GFP-positive cells in the gonads. Cultured PGCs expressing GFP gene were successfully introduced into the gonads of left and/or right gonads of recipient embryos. Bars indicate 0.5 mm.

enter the recipient gonads by transferring them into the coelomic epithelium. Thus, GFP gene was successfully introduced into PGCs, and those expressing GFP gene were incorporated into the germline of recipient embryos.

PGCs cultured on feeder cells proliferated slowly, occasionally forming cell colonies. Although they retained the characteristics of germline cells, it was difficult to maintain an undifferentiated state during the culture period similar to those from our previous results (Naito *et al.*, 2010). bFGF plays an important role in maintaining the undifferentiated state of cultured PGCs (van de Lavoie *et al.*, 2006; Choi *et al.*, 2010; Macdonald *et al.*, 2010), and also the foetal bovine serum also affected the proliferation and maintenance of an undifferentiated state of cultured PGCs (Macdonald *et al.*, 2010). Chicken leukemia inhibitory factor (cLIF) was also shown to be important in preserving the pluripotency of chicken embryonic stem cells (Horiuchi *et al.*, 2004; Nakano *et al.*, 2011). Despite the presence of bFGF in the culture medium, cultured PGCs occasionally differentiated during the culture period when they attached to the feeder layer (van de Lavoie *et al.*, 2006). Thus, PGC culture conditions still require improvement. For maintaining an undifferentiated

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Table 3. Detection of GFP-positive cells in gonads of recipient embryos

Cultured PGCs transferred	Number of embryos treated at stage X	Number (%) of embryos surviving at day 3 of incubation	Number (%) of embryos surviving at day 6 of incubation	Number (%) of embryos with GFP-positive cells in gonads/examined
Coelomic epithelium at stages 17–19	255	234 (91.8)	201 (78.8)	46 (22.9)

state of cultured PGCs, it is important to prepare feeder cells secreting factors for the proliferation and maintenance of an undifferentiated state of PGCs.

When the cultured PGCs were transferred to the stage X blastoderm or to the bloodstream of recipient embryos, they failed to migrate to the germinal ridges. Freshly collected PGCs usually migrate to the recipient gonads by transferring them into the stage X blastoderm (Naito *et al.*, 2004) or bloodstream (Yasuda *et al.*, 1992; Tajima *et al.*, 1993; Naito *et al.*, 1994a, 1994b, 2010). On the other hand, when cultured PGCs were transferred to the coelomic epithelium of recipient embryos, they were able to enter the gonads, as in our previous report (Naito *et al.*, 2011). This transfer method of donor PGCs to recipient embryos may become a novel system for entering the donor PGCs that have lost their migratory ability to germinal ridges into recipient embryos. As the next step, one should be determined whether cultured PGCs entering the recipient gonads could differentiate into functional gametes.

Germline chimaeric chickens were produced by the transfer of 3,000 (van de Lavoie *et al.*, 2006) or 100–500 (Macdonald *et al.*, 2010) cultured PGCs. In the present study, 200 cultured PGCs were transferred to the recipient embryos to avoid the haemorrhage when they were transferred to the bloodstream. Usually, 200 PGCs can be transferred to the bloodstream of stage 14–15 embryos without haemorrhage (Naito *et al.*, 1994a). By improving the PGC culture conditions, especially for maintaining the undifferentiated state, a large number of cultured PGCs can be transferred to recipient embryos to enhance the germline contribution of donor PGCs in recipient embryos.

Cultured PGCs were transfected with GFP gene by nucleofection and were subsequently selected for successfully transfected PGCs. The electroporation and selection conditions were the same as those of Leighton *et al.* (2008). GFP gene was efficiently introduced into cultured PGCs, and those selected were continuously expressed GFP gene for long periods without silencing transgenes. However, the proliferation rate of the PGCs expressing GFP gene was low, making it difficult to obtain a large number of PGCs expressing GFP gene. Nevertheless, the cultured PGCs expressing GFP gene could enter the gonads of recipient embryos. Those transferred PGCs expressing GFP gene could be expected to differentiate normally.

It is preferable to develop a simple system for culturing PGCs under the conditions of maintaining an undifferentiated state of PGCs. In that regard, the culture method for PGCs developed in the present study should be further im-

proved. The PGC culture system will contribute to the propagation of endangered bird species (Kang *et al.*, 2008; Wernery *et al.*, 2010) as well as to produce transgenic chickens (van de Lavoie *et al.*, 2006; Leighton *et al.*, 2008; Macdonald *et al.*, 2010). Thus, the PGC culture system holds enormous possibilities for embryo manipulation in avian species.

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