A New Method for Isolating Viable Gonadal Germ Cells from 7-day-old Chick Embryos

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A novel and simple method for isolating viable gonadal germ cells (GGCs) was developed in the domestic chicken, *Gallus domesticus*. Developing gonads were isolated from 7-day-old chick embryos and cultured for up to 24 hours at 37.8°C in phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS [–]). A discharge of GGCs from the gonad was observed within 30 minutes after introducing the embryonic gonad into PBS [–], and the number of discharged GGCs increased until 12 hours of incubation. The purity of the GGCs (number of discharged GGCs/total number of discharged cells) was approximately 50% for the initial 1.5 hours of incubation and decreased thereafter. The number of discharged GGCs from the left gonad was significantly higher than that from the right gonads in females. Fifty discharged GGCs in PBS [–] were injected into the blood stream of 2-day-old chick embryos after staining with PHK26 fluorescent dye. GGCs exhibiting a fluorescent signal were detected after incubating recipient embryos for 5 days.

These results indicate that high-purity, viable GGCs can be collected by simply introducing isolated developing gonads into PBS [–].

Key words: chicken, gonadal germ cells, isolation, PBS [–]

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Introduction

Recent technological progress in producing germline chimeras has provided an important means of studying germline development, genetic resource conservation, and transgenics production. In avian species, primordial germ cells (PGCs) originate from the central zone of the area pellucida (Ginsburg and Eyal-Giladi, 1987; Kagami *et al.*, 1997). At the primitive streak stage, PGCs are found at the extra-embryonic region called the germinal crescent (England and Matsumura, 1993). PGCs circulate temporarily through the bloodstream, followed by migration to the gonadal ridge (Kuwana, 1993; Fujimoto *et al.*, 1976). After migration to the gonadal ridge, PGCs are called gonadal germ cells (GGCs). Gonadal germ cells in the gonadal ridge ultimately differentiate into spermatogonia in the testes or oogonia in the ovaries.

Germline chimeras can be produced in domestic chicken by transferring circulating PGCs (Tajima *et al.*, 1993) or by transferring GGCs collected from the embryonic

gonad (Tajima *et al.*, 1998; Park *et al.*, 2003; Tajima *et al.*, 2004) into the bloodstream of 2-day-old recipient embryos. GGCs have been isolated commonly by digesting embryonic gonads using proteinases such as trypsin (Tajima *et al.*, 1998). However, it is difficult to purify GGCs from the overwhelming number of somatic cells after digesting the gonad using enzymes.

Therefore, we conducted the present experiments to develop a novel and simple method for isolating GGCs from the gonads of 7-day-old chick embryos.

Materials and Methods

Experiment 1: Isolation of Gonadal Germ Cells

Fertilized White Leghorn (WL) and Rhode Island Red (RIR) eggs produced at the Agriculture and Forestry Research Center, University of Tsukuba, Japan were used in the experiments. Fertilized WL eggs were incubated for 7 days in an air-forced incubator (Showa Furanki Laboratory, Saitama, Japan) maintained at 37.8°C. After incubation, both right and left gonads were isolated from 7-day-old embryos. Isolated gonads were placed in a 1.5 mL centrifuge tube containing 500 µL of phosphate buffered saline without Mg²⁺ and Ca²⁺ (PBS [–]). The centrifuge tube was placed in an incubator maintained at 37.8°C for 0.5, 1, 1.5, 2, 3, 4, 5, 6, 9, 12, 18, or 24 hours. Cultured

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gonads were pipetted gently, and 100 μ L of cell suspension was placed on 2-well Heavy Teflon Coated Slides (HTCS, Erie Scientific Co., Portsmouth, NH, USA). The number, survival rate, and purity of discharged GGCs were observed. The purity of discharged GGCs was defined as the ratio between the number of discharged GGCs and the total number of discharged cells expressed as a percentage. The survival rate of isolated GGCs was determined using trypan blue staining. For a control, the same parameters were measured after digesting embryonic gonad using 0.05% trypsin for 20 minutes in an incubator maintained at 37.8°C. The morphological characteristics of the GGCs were identical to the PGCs, i.e., large granulated round cells, 14–19 μ m in diameter with large nuclei (Minematsu *et al.*, 2004), under an inverted microscope (Olympus, Tokyo, Japan). To confirm the histochemical properties of the discharged GGCs, discharged cells were stained with two staining methods, periodic acid-Schiff (PAS) (Tajima *et al.*, 1999) and anti-chicken *vasa* homologue (CVH) staining, according to the method described by Tsunekawa *et al.* (2000). For PAS staining, the cell suspension was placed on HTCS, fixed with 10% formalin in PBS [–], and coated with celloidin, and the number of GGCs on each HTCS was determined after the PAS reaction. For CVH staining, the recovered cell suspension was placed on 24-well plates (Falcon, Franklin Lakes, NJ, USA) and fixed with 10% formalin in PBS [–]. After blocking and permeabilization using Blocking One (Nacalai Tesque, Kyoto, Japan), the cells were incubated with rabbit anti-CVH antibodies and treated with alkaline phosphatase-conjugated goat anti-rabbit IgG, IgM, and IgA (H & L, 1: 200 dilution; Open Biosystems, Huntsville, AL, USA) as secondary antibodies. Alkaline phosphatase staining was conducted using the BCIP/NBT Substrate System (DakoCytomation, Glostrup, Denmark). The number of GGCs in each plate was assessed after alkaline phosphatase staining. Each experiment was repeated more than five times.

A one-way analysis of variance was conducted using the generalized linear model (GLM; SAS/STAT, SAS Institute Inc., Cary, NC, USA) followed by Tukey's Honesty Significant Difference (HSD) test.

Experiment 2: Sexual Differences in the GGC Numbers from the Right and Left Gonads

Right and left gonads recovered from 7-day-old embryos were placed separately in a 24-well plate containing 1 mL PBS [–] and incubated for 30, 60 and 90 minutes. The number of discharged GGCs was counted after CVH staining. A polymerase chain reaction (PCR) was conducted to determinate the gender of the germ cell donors. Each experiment was repeated more than five times.

PCR for Sexing

Embryonic skin tissue was collected from the leg of each embryo. The sample was placed in DNA extraction buffer (Minematsu *et al.*, 2004), and a 276 bp fragment of the 717 bp W chromosome-specific XhoI repetitive sequence was amplified according to the methods of Petitte

and Kegelmeyer (1995) using the following primer sets: 5'-CGTGAGAAAAGTGGTAGTT-3' and 3'-CTCT-GTCCACCATAAAAAACC-5'.

The PCR was conducted with an initial denaturation step at 96°C for 2 minutes, followed by 25 cycles at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes in a Takara PCR Thermal Cycler SP (TP-400; Takara Shuzo, Kyoto, Japan). The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized under UV light after staining with ethidium bromide. Only samples that were obtained from females were expected to express the 276 bp PCR product.

A split-plot design was used to conduct the experiment, and the data were analyzed using the GLM procedure, followed by Tukey's HSD test using SAS/STAT.

Experiment 3: Migratory Ability of the GGCs

Right and left gonads from each embryos were placed in 1.5 mL centrifuge tubes and incubated in 100 μ L PBS [–] for 1 hour. Discharged GGCs were labeled with PKH26 fluorescent dye (Zynaxis, Inc., Malvern, PA, USA) and 50 GGCs were injected into the dorsal aorta of RIR embryos at stages 13–16 of development (Hamburger and Hamilton, 1951) using a fine glass pipette under a dissection microscope. Recipient eggs were prepared 1 hour before injection to remove approximately 5 μ L of blood through a small window (about 1 cm diameter) in the eggshell using a fine glass pipette under a dissection microscope. After the injection, the window was sealed with plastic tape, and the egg was incubated at 37.8°C for 5 days. Right and left gonads were collected and placed in 1.5 mL centrifuge tubes containing 50 μ L of 0.05% trypsin in PBS [–] and incubated at 37.8°C for 20 minutes. After the incubation, the cells were placed on HTCS, and the total number of fluorescently labeled cells recovered from each gonad were counted under a fluorescence microscope (IMT-2, Olympus, Tokyo, Japan) using a 546-nm excitation filter.

All animal care and handling procedures were performed in accordance with the standards of the University of Tsukuba.

Results

A discharge of GGCs from gonad of 7-day-old chick embryos after a 1-hour incubation in PBS [–] at 37.8°C was shown in Fig. 1 and discharged cells stained with either PAS or anti-CVH was shown in Fig. 2. The proportion of PAS positive morphological GGCs was 95% until 8 hours after the introduction of the gonads into PBS [–], and the proportion decreased to 90% at 24 hours (data not shown). The proportion of anti-CVH-positive morphological GGCs was > 93% at 24 hours of incubation (data not shown).

The temporal change in the number, survival rate, and purity of GGCs discharged from incubated embryonic gonads in PBS [–] during 24 hours is shown in Fig. 3. The number of discharged GGCs at 30 minutes and 12

hours after incubation were 402 ± 55.6 and $2,438 \pm 190.4$, respectively ($P < 0.05$). However, no further increase in the number of discharged GGCs was observed between 12 and 24 hours of incubation. The GGC purity, defined as the proportion of GGCs vs. the total number of observed cells, at 0.5, 1, and 1.5 hours after the incubation was $46.9 \pm 5.9\%$, $48.2 \pm 6.3\%$, and $51.3 \pm 6.6\%$, respectively ($P > 0.05$), but the value decreased significantly thereafter and reached 20% at 24 hours. For comparison, the number of dissociated GGCs and the GGC purity were $1,052 \pm 239$ and $4.26 \pm 0.49\%$, respectively, when trypsin was used to dissociate the gonadal tissue isolated from 7-day-old chick embryos. The survival rate of discharged GGCs remained

$>80\%$ for the first 9 hours of gonadal incubation ($P > 0.05$), followed by a significant decrease thereafter, and reached 40% at 24 hours ($P < 0.05$).

Fig. 1. Discharge of gonadal germ cells (GGCs) from chick embryonic gonads after a 1-hour incubation in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS [–]) at 37.8°C . (A) Gonad of 7-day-old chick embryo. Bar = $200\mu\text{m}$ (B) Bar = $50\mu\text{m}$.

Fig. 2. Discharged cells after incubating 7-day-old chick embryos in (A) phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS[–]) for 1 hour, (B) trypsin treatment, (C) periodic acid-Schiff (PAS) staining, and (D) anti-chicken *vasa* homologue (anti-CVH) staining. *: gonadal germ cells (GGCs), Bar = $30\mu\text{m}$.

Fig. 3. Number, survival rate and purity of gonadal germ cells (GGCs) discharged from embryonic gonad. *: First observation of significant difference compared with the value at 30 minutes after incubation ($P < 0.05$)

Fig. 4. Sexual differences in the number of discharged gonadal germ cells (GGCs) after incubating right and left embryonic gonads in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS [–]). A: Significantly different from other treatments within each incubation time ($P < 0.05$) *: First observation of significant difference compared with the value at 30 minutes after incubation ($P < 0.05$).

The number of GGCs discharged into PBS [–] from the right and left gonad by gender of the embryo is shown in Fig. 4. In both male and female gonads, the number of GGCs discharged into PBS [–] after the incubation was significantly higher from the left gonad than from the right ($P < 0.05$).

The migratory ability of the GGCs collected by the PBS [–] method is shown in Table 1. When 50 fluorescently labeled GGCs were transferred into recipient embryos, 78.7 ± 11.5 fluorescently labeled GGCs were detected, among which 45.2 ± 5.3 and 33.6 ± 7.3 were detected from the left and right gonads, respectively ($P < 0.05$).

Discussion

In the present study, highly pure (approximately 50% of total cells) viable GGCs that retained migratory ability toward the developing gonad were recovered efficiently by simply culturing embryonic gonads in PBS [–] at 37.8°C for 0.5–1.5 hours (Fig. 3).

Although the underlying mechanism for the predominant discharge of GGCs from the developing gonad into PBS [–] for the first 1.5 hours after the introduction of embryonic gonad is not understood at present, this rapid and simple method of isolating GGCs from developing gonads will considerably facilitate the study of germline chimera production. The predominant initial discharge of GGCs into the medium may indicate a weak cellular association between discharged GGCs and gonadal somatic cells in the embryonic gonad of 7-day-old chick embryos.

A significant increase in the number of discharged GGCs was observed between 9 and 12 hours after introducing the embryonic gonads into PBS [–] (Fig. 3). However, the GGC purity did not change during the same period. It was speculated from these results that spontaneous gonadal dissociation was accelerated by 9 hours after incubating the embryonic gonads in PBS [–]. Alternatively, the present result may indicate a depletion of unassociated free GGCs in the embryonic gonad. The significant decrease in the survival rate of GGCs over 9 hours of incubation could be attributed to depletion of nutrients essential for maintaining the cellular metabolism of discharged GGCs.

Although a morphological analysis of embryonic gonads has been reported (Drews *et al.*, 1988; Civinini *et al.*, 1994), few reports are available on the cellular association between germ cells and somatic cells in the gonads. It is generally accepted that the gonadal somatic cells support maturation and differentiation of germ cells in the gonad (Johnson, 2000; Kirby and Froman, 2000). Further study should be conducted to understand the development and differentiation of germ cells in relation to the mode of cellular association between GGCs and gonadal somatic cells.

It was observed that the GGCs were released mainly from the edge of the gonad where the gonad was previously attached to the kidney (Fig. 1). It was, therefore, hypothesized that the developing medullary cords in male or cortical cords in females may play a role in the discharge of GGCs.

Table 1. Number of PKH26 positive gonadal germ cells (GGCs) in the gonad of recipient embryos that were transferred 50 GGCs. (N=38)

	Left Gonad ¹⁾	Right Gonad ¹⁾	Total
The number of PKH26 positive GGCs	45.2±5.3	33.6±7.3	78.7±11.5

¹⁾ The number of PKH26 positive GGCs differs significantly between right and left gonad using a paired t-test ($P<0.05$).

A histological study to compare the differences in gonadal structure before and after incubation in PBS [–] needs to be conducted to elucidate the GGC discharge mechanism.

Furthermore, the chemical and physical factors affecting GGC discharge into the medium, i.e., medium composition, incubation temperature, and gas environments need to be studied.

The concentration of circulating PGCs in the blood is highest at either stage 14 or 15 in chick embryos (Tajima *et al.*, 1999). However, the incubation time required to reach developmental stages 14–15 varies considerably among individual embryos (Tajima *et al.*, 1999), and this can only be observed after exposing the embryo from the egg shell. Furthermore, the proportion of PGCs against blood cells is reported to be 0.02% (Yasuda *et al.*, 1992). Therefore, multiple eggs must be sacrificed to ensure the collection of circulating PGCs from embryos at stages 14–15 to produce germline chimeras. The scarcity of circulating PGCs has been a major limiting factor in research on germline development. In this respect, the method of collecting viable GGCs from developing gonads, as described in the present study, has a clear advantage of recovering viable germ cells when the availability of fertilized embryos is limited, such as the case with low-fertility strains or wild birds.

It was observed that the intensity of PAS staining against GGCs collected from 7-day-old embryos was weaker after a 24-hour incubation than with GGCs collected from embryos incubated for less than 24 hours (data not shown). Because the Schiff reagent used in PAS staining reacts with the aldehyde residue produced by treating samples with periodic acid, which dissociates the covalent bonds between the second and third carbon residue of the glycopyranose, it was speculated that there was a depletion of glycogen in the cytoplasm of the GGCs.

A significantly higher number of GGCs was discharged from the left gonad in females in this study (Fig. 4). The same tendency was observed in male gonads, although no statistically significant difference was observed. This observation might simply be due to an asymmetrical migration of PGCs in favor of the left gonad over the right gonad, which has been reported previously (Zaccanti *et al.*, 1990; Nakamura *et al.*, 2007; Naito *et al.*, 2009). Alternatively, the post-migratory proliferation of GGCs may have been higher in the left than in the right gonad. Reasons for the higher number of discharged GGCs from

the left gonad of females need to be studied.

Fluorescently labeled GGCs were found in gonads from recipient embryos using the method of discharging the GGCs into PBS [–], indicating that the GGCs discharged into PBS [–] retained their migratory ability in the developing gonad (Table. 1). A progeny test should be conducted by transferring GGCs collected with the PBS [–] method.

Seven-day-old chick embryos were used in the present study. Although germline chimeras have been produced by transferring GGCs collected from 5-day (Tajima *et al.*, 1998) as well as 7- and 9-day-old embryos (Tajima *et al.*, 2004), the biological nature of GGCs is currently not fully understood. Future study should be directed toward extending the present method to the entire period of embryonic development.

In conclusion, viable GGCs were collected by simply culturing 7-day-old gonads from chick embryos. The results provide an alternative means for producing germline chimeras to conserve genetic resources and to produce transgenic avian species. Future experiments should examine the discharge of GGCs from other than 7-day-old embryos.

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