Migratory Ability of Chick Primordial Germ Cells Transferred into Quail Embryos

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Abstract. The migratory ability of chick primordial germ cells (PGCs) transferred into quail embryos was investigated. One, ten, twenty, fifty or one hundred chick PGCs were transferred into the dorsal aorta of 2.5-day-old quail embryos. One day later, the embryos were isolated, and serial sections were prepared after embedding in paraffin. The sections were then double-stained with periodic acid-Schiff (PAS) and hematoxylin, and the numbers of PAS-positive chick PGCs were counted. Approximately 70% of the transferred PGCs were detected in the embryos 1 day after transfer, with roughly 60% in the gonadal region and 10% in the extragonadal region. This ratio was consistent regardless of the number of PGCs transferred into the embryos. These data suggest that migration of chick PGCs into the gonadal and extragonadal regions of the quail embryo occurs probabilistically regardless of the number of chick PGCs transferred into quail embryos.

Key words: Chicken, Chimera, Embryo, Migration rate, Primordial germ cells, Quail

Migration of primordial germ cells (PGCs) toward the developing gonads during embryonic development provides a unique model of cellular migration in vertebrates. In birds, PGCs originate from the central zone of the area pellucida [1, 2]. At the primitive streak stage, PGCs are found in an extraembrionic region called the germinal crescent [3]. They then circulate temporarily through the bloodstream [4] before migrating toward the gonadal ridge [4, 5]. PGCs ultimately differentiate into spermatogonia in the testes or oogonia in the ovaries.

Circulating PGCs can be recovered from embryonic blood at stages 13–15 [6] and concentrated by Ficoll density gradient centrifugation [7], filtration [8] or Nycodenz density gradient centrifugation [9]. Intraspesies germline chimeras can be produced in the domestic chickens by transferring PGCs into the bloodstream of 2-day-old recipient embryos [10]. Recently, interspecies germ-line chimeras have been produced by transferring PGCs colleted from pheasants (Phasianus colchicus) into the bloodstream of 2.5-day-old chicken (Gallus gallus domesticus) embryos [11].

Understanding the interspecies migratory ability of PGCs during gonadal development is important not only for improving the production efficiency of interspecies germline chimeras but also for elucidating the mechanism of PGC migration toward the developing gonads. Yasuda et al. [7] reported that 63 of 100 chick PGCs transferred into quail embryos were present in the gonadal ridge. Furthermore, Ono et al. [12–14] reported migration of chick and quail PGCs following transfer of embryonic blood containing circulating PGCs.

It has been considered that the migratory ability of the PGC toward the gonadal ridge could be influenced by possible interaction among exogenous chick PGCs in the bloodstream of quail embryos. In the present study, therefore, the migratory ability of PGCs was evaluated by transferring various numbers of chick PGCs into quail embryos under interspecies PGC transfer conditions.

Materials and Methods

Animals

Fertilized eggs were obtained from Rhode Island Red chickens (Gallus gallus domesticus) maintained at the Agricultural and Forestry Research Center, University of Tsukuba (Tsukuba, Japan). All animal care and handling procedures were performed in accordance with the standards of the University of Tsukuba. Fertilized Japanese quail (Coturnix coturnix japonica) eggs were purchased from Tokai-Yuki (Toyohashi, Japan).

Preparation of the donor chick PGCs

Fertilized chicken eggs were incubated at 37.8 C for 2.5 days. Blood was collected from the dorsal aorta of the developing embryos at stages 13–15 [6] using a fine glass pipette prepared from a 50 μl capillary micropipette (2-000-050; Drummond Scientific, Broomall, PA, USA) using a micropipette puller (PA-81-8811, Narishige, Tokyo, Japan). PGCs were isolated from the blood by Ficoll density gradient centrifugation as described by Yasuda et al. [7]. Briefly, Ficoll (F-9378; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in Dulbecco’s modified Eagle’s medium (DMEM; 31600-034; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (26140-0790; Gibco BRL) to produce 16% (w/v) and 6.3% (w/v) Ficoll solutions. Each
Validation of PGC transfer
A validation experiment was conducted to ensure transfer of a fixed number of PGCs into the recipient embryos. One, ten, twenty, fifty or one hundred chick PGCs were aspirated into a fine glass pipette and discharged onto a Heavy Teflon Coated Slide (HTCS; 10-226-CC; Erie Scientific, Portsmouth, NH, USA). The PGCs on the slides were then fixed with 10% formalin in PBS(–), coated with celloidin, and stained with periodic acid–Schiff (PAS) as described by Tajima et al. [16]. The number of PGCs on each HTCS was determined before and after PAS reaction. The experiment was repeated five times. In addition, 50 putative chick PGCs were immunostained using antibodies against chicken vasal homolog (Cvh) as reported by Tsunekawa et al. [17]. Briefly, 50 PGCs were placed on an HTCS, fixed with 10% formalin in PBS(–) and coated with celloidin. The slide was then blocked and permeabilized using Blocking One (Nacalai Tesque, Kyoto, Japan), incubated with rabbit anti-Cvh antibodies [17] and treated with alkaline phosphatase-conjugated goat anti-rabbit IgG, IgM and IgA (H&L; 1:200 dilution; SAB1005, Open Biosystems, Huntsville, AL, USA) as secondary antibodies. Alkaline phosphatase staining was carried out using the BCIP/NBT Substrate System (K0598; DakoCytomation, Glostrup, Denmark). The number of PGCs on each HTCS was assessed after alkaline phosphatase staining. The experiment was repeated seven times.

Transfer of chick PGCs into the bloodstream of quail embryos
Fertilized quail eggs were incubated at 37.8 °C for 2.5 days to obtain stages 13–15 embryos, which were then used as chick PGC recipients. The eggshells were cleaned with 70% ethanol, and a window approximately 1.5 cm in diameter was made on the pointed end of the egg. Approximately 5 μl of blood were removed from the recipient embryos 1–2 h before PGC transfer primarily to reduce the blood pressure of the recipient embryos. One, ten, twenty, fifty or one hundred chick PGCs were then aspirated into a glass pipette, and 1–2 μl of medium containing the PGCs were then transferred into the dorsal aorta of the recipient embryo. Following transfer, the window on each eggshell was sealed with cling-wrap using chicken thin albumen as a glue. The recipient embryos were subsequently incubated for 1 day to allow them to reach stages 20–21 [6]. The whole embryos were then fixed in Rossman’s fluid (mixture of 9 parts saturated picric acid in absolute ethanol and 1 part neutralized formalin) [7] for 2 h at 4.0 °C, dehydrated and then embedded in paraffin. Serial sections of whole embryos (14 μm thick) were then prepared for histological observation. Five recipient quail embryos were used for preparation of serial sections for each treatment except for the treatment in which a single PGC was transferred into the recipient; ten embryos were used in this case.

Histological identification of chick PGCs in the embryos
The sections were double-stained with PAS and hematoxylin for light microscopy. Chick PGCs were identified in the quail embryos based on differences in the staining characteristics of chick and quail PGCs: chick PGCs appear red in response to PAS reaction [18], while quail somatic and germ cells remain unstained [19].

The locations and numbers of chick PGCs transferred into the quail embryos were recorded as described by Yasuda et al. [7]. Briefly, the gonadal area was defined as the region including the dorsal mesentery and dorsal part of the coelomic wall and limited as follows: the region caudal to the vitelline artery, medial to the mesonephros, dorsal to the hindgut and ventral to the dorsal aorta. The caudal end of the gonadal area was considered to be the caudal terminus of the coelom. The coelomic epithelium in this region was designated as the germinal epithelium. In the present study, the gonadal region was defined as the combination of the germinal epithelium and the gonadal area.

In addition, a serial section of the gonadal region of one quail embryo, into which 50 chick PGCs were transferred, was immunostained with anti-Cvh antibodies using a method as described previously [17].

Statistical analysis
The data were subjected to arcsine-square root transformation, and one-way analysis of variance was conducted using SAS/STAT [20].

Results
Validation of PGC transfer
One, ten, twenty, fifty or one hundred PGCs, identified based on their morphological characteristics, were aspirated and discharged onto HTCSs and then stained with PAS. Of the discharged cells, 100.0, 100.0, 98.0, 99.1 and 98.6% were stained with PAS (Fig. 1). Furthermore, 99.1% of 50 putative PGCs were stained with anti-Cvh antibodies (Fig. 1). The histological section of the gonadal region of the one quail embryo in which 50 chick PGCs were transferred was stained with anti-Cvh antibodies (Fig. 2A).

Histological identification of chick PGCs in quail embryos
The PAS-positive chick PGCs located in the gonadal region, head area and trunk area of the quail embryo are shown in Fig. 2B, 2C and 2D, respectively.

As shown in Table 1, the proportions of chick PGCs that migrated into the germinal epithelium, gonadal area, head area and trunk area of the quail embryo were 12.0 ± 4.3%, 50.7 ± 8.6%, 6.2 ± 5.5% and 2.5 ± 2.3%, respectively. A significantly higher proportion of PGCs was observed in the gonadal area compared with the other three areas (P<0.05).
Discussion

Although germline chimeras have been produced by the transfer of PGCs in the domestic chickens [10, 21], the underlying mechanism of PGC migration in birds is not fully understood. It has been postulated that PGCs are chemically attracted by unknown substances released from the developing gonads [22]. Components of the extracellular matrix such as fibronectin have also been suggested to be involved in guiding migrating PGCs from the vascular system toward the developing gonads [23]. Previous research has suggested that chicken-quail germline chimeras are valuable models for studying the mechanism of germline establishment in amniotes [24]. Therefore, the present experiment was designed to elucidate the migratory ability of PGCs by transferring various numbers of chick PGCs into the bloodstream of developing quail embryos.

Assuming that chick PGCs neither proliferate nor die in quail embryos within 24 h after PGC transfer, the average proportions of chick PGCs that migrated to the gonadal region and extragonadal region of the quail embryos were 62.7% and 8.7%, respectively, regardless of the number of chick PGCs transferred into the quail embryos. The present results are in agreement with those of Yasuda et al. [7], who transferred 100 chick PGCs into the marginal sinus of 2.5-day-old quail embryos and found that approximately 63 and 13% of the PGCs were in the gonadal and extragonadal regions of the quail embryos 24 h after transfer, respectively. Nakamura et al. [25] reported that the proportion of extragonadal PGCs in the normal developing 3-day-old chick embryo at stage 19 [6] is 15.5%. These results indicate that extragonadal migration of chick PGCs occurs probabilistically in normal

### Table 1. Migratory ability of chick circulating PGCs transferred into quail embryos (Means ± SE)

<table>
<thead>
<tr>
<th>No. of chick PGCs transferred into quail embryos</th>
<th>No. of chick PGCs in germinai epithelium</th>
<th>No. of chick PGCs in gonadal area</th>
<th>No. of chick PGCs in head area</th>
<th>No. of chick PGCs in trunk area</th>
<th>Total no. of chick PGCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.6 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>1.2 ± 0.3</td>
<td>5.0 ± 0.7</td>
<td>0.6 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td>7.0 ± 0.8</td>
</tr>
<tr>
<td>20</td>
<td>4.2 ± 0.9</td>
<td>9.2 ± 0.8</td>
<td>0.2 ± 0.2</td>
<td>1.0 ± 0.5</td>
<td>14.6 ± 0.5</td>
</tr>
<tr>
<td>50</td>
<td>10.0 ± 2.6</td>
<td>20.4 ± 4.6</td>
<td>3.0 ± 1.2</td>
<td>2.2 ± 0.5</td>
<td>35.6 ± 2.7</td>
</tr>
<tr>
<td>100</td>
<td>18.8 ± 3.2</td>
<td>47.2 ± 4.9</td>
<td>4.2 ± 1.4</td>
<td>3.6 ± 0.7</td>
<td>73.8 ± 6.0</td>
</tr>
</tbody>
</table>
| Overall                                       | 12.0 ± 4.3%                           | 50.7 ± 8.6%                      | 6.2 ± 5.5%                   | 2.5 ± 2.3%                   | 73.8 ± 6.0

1) The numbers in parentheses indicate the proportion of chick PGCs in the respective area against the number of transferred PGCs. * Values with same superscript in the same column do not differ significantly (P>0.05). ** Values with different superscripts in the same row indicate significant differences (P<0.05).
embryos and under interspecies PGC transfer conditions. In mice, testicular teratomas, or embryonic carcinoma cells, are generated from exognadal PGCs [26]. The ultimate fate of these exognadal PGCs in avian species remains to be studied.

In the present study, the proportions of chick PGCs observed in the germinal epithelium and gonadal area were 12.0 ± 4.3% and 50.7 ± 8.6%, respectively. It is possible that the efficiency of producing germline chimeras is influenced by the proportion and distribution of the PGCs that migrated into the gonadal region of the recipient embryo. In this respect, development of a methodology to evaluate the migratory ability of PGCs toward the gonadal area is likely necessary.

In conclusion, the proportions of chick PGCs that migrated into the gonadal and extragonadal regions of developing quail embryos were approximately 62.7 and 8.7%, respectively, regardless of the number of chick PGCs transferred into the bloodstream of the quail embryo. By using this technology, the migratory ability of PGCs can be evaluated by characterizing individual PGCs prior to transfer. Future study should be directed toward sorting PGCs based on migratory ability toward the developing gonad of the recipient embryo.

Acknowledgments

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References