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Gonadal Germ Cell Migration and Proliferation after Transfer in Developing Chicken Embryos

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A germline chimera is a useful model for developing and differentiating germ cells *in vivo*. Gonadal germ cells (GGCs) collected from chicken embryonic gonads may be used to produce germline chimeras as donor cells. However, the migratory and proliferative abilities of GGCs after transfer into recipient embryos are unclear. Here, the migratory and proliferative abilities of GGCs collected from 7-day-old White Leghorn embryos and fluorescently labeled were analyzed following transfer into the dorsal aorta of 2.5-day-old Rhode Island Red (RIR) embryos. Five days after transfer, the numbers of male and female GGCs were significantly higher in the RIR gonads than those in non-gonadal RIR organs when 50 GGCs were transferred per embryo. To analyze the temporal migration of GGCs in intermediate mesoderm, 50 GGCs were again transferred. The numbers of male and female GGCs in RIR gonads increased significantly from days 3 to 6 after transfer. To analyze GGC migration and proliferation in the gonads, a single GGC was transferred into 100 male and 100 female embryos. Five days after transfer, the frequencies of settled and proliferated GGCs were 37% (37/100) and 24% (24/100) in males, and 23% (23/100) and 8% (8/100) in females, respectively. Thus, GGCs are a heterogeneous cell population that may or may not have migratory and proliferative abilities. The heterogeneity of GGCs may be greater in females than that in males. When 50 GGCs were transplanted, almost all those present in embryos had settled and proliferated in the gonads and mesonephros. The migratory and proliferative abilities of GGCs in recipient gonads were considerably diverse in individual GGCs or between donor sexes.

Key words: 7-day-old embryo, chicken, gonadal germ cells (GGCs), migration, proliferation

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Introduction

Manipulation of germ cells to produce germline chimeras is a useful model to analyze the developmental potential of germ cells, including their migration, proliferation, and differentiation *in vivo*. This model has been applied in studies of transgenic production and genetic resource conservation. Primordial germ cells (PGCs), the progenitor cells of gametes, have been widely used in developmental engineering research in poultry science. PGCs circulate temporarily through the bloodstream and then migrate to the intermediate mesoderm region, which differentiates into the gonadal ridge[1]. Migrating PGCs in the bloodstream are often called circulating PGCs (cPGCs). After migration to the gonadal ridge, PGCs are termed gonadal germ cells (GGCs); they ultimately differentiate into spermatogonia in the testes or oogonia in the ovary. The sexual differentiation of germ cells depends on the sexual differentiation of gonadal somatic cells. Several factors involved in the sexual determination of gonads, including *Drosophila* Doublesex, Mab-3 related transcription factor 1 (DMRT1), and anti-Müllerian hormone (AMH), are expressed at different levels between the sexes in 5.5-day-old chicken embryos[2,3]. Histological differentiation of the gonads into the ovary is initiated in 6.5-day-old chicken embryos by the expression of the P450 aromatase gene[4]. More recently, female-specific gene expression has been observed in female PGCs recovered from 6.5-day-old embryos using RNA sequencing[5]. Expression of the retinoic acid (RA)-degrading enzyme, cytochrome P450, family 26, subfamily B, member 1 (Cyp26B1), which induces meiosis, decreases after 10.5 days in female embryos[6]. Follow-

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ing Cyp26B1 downregulation, expression of its downstream factor stimulated by RA 8 (Stra8) increases, and germ cells proceed to meiosis[6]. In contrast, high-level expression of Cyp26B1 is maintained in the gonads, and germ cells proliferate until the onset of meiosis in male embryos[6,7].

When GGCs that have migrated to the gonads are transferred into the blood vessels of 2-day-old recipient embryos, they circulate with the endogenous cPGCs in the blood vessels and reestablish in the recipient's gonads[8–10]. Like endogenous PGCs, transferred GGCs differentiate into gametes through germline chimeras produced by transferring GGCs collected from 5-, 7-, and 9-day-old embryonic gonads[10–12].

GGC manipulation obtains more cells for longer developmental periods than that of cPGC manipulation. However, the efficiency of obtaining donor-derived progeny from a germline chimera varies considerably depending on the recipients[10,13]. Additionally, male GGCs may differentiate into either sperm or ova, depending on the sex of the recipient. However, female GGCs cannot differentiate into fertilizable sperm in male recipients[10,13]. Therefore, GGCs may already be differentiated between males and females in embryonic gonads. The migratory and proliferative abilities of GGCs are lower than those of PGCs, as they have migrated to the gonads and proliferated[8]. The migration of transferred GGCs and the proliferation of migrated GGCs are independent phenomena that have not been investigated in detail. Therefore, differences in the competencies of GGCs to migrate and proliferate between sexes were investigated using multiple- or single-GGC transfer.

Materials and Methods

Animals

Fertilized White Leghorn (WL) and Rhode Island Red (RIR) eggs produced at the Agriculture and Forestry Research Center, University of Tsukuba, Japan, and the National Agriculture and Food Research Organization (NARO) Institute of Livestock and Grassland Science were used. The measurement of the migratory and proliferative abilities of GGCs into gonadal and non-gonadal organs was performed following the standards of the Committee for the Care and Use of Experimental Animals at the NARO (approval number: 1811B099). The animal care and handling procedures of the remaining experiments were performed following the standards of the University of Tsukuba (approval number: 14-68).

Recovery of GGCs

Fertilized WL eggs were incubated for 7 days in an air-forced incubator (P-008; Showa Furanki Laboratory, Saitama, Japan) at 37.8 °C. After incubation, left gonads were isolated from 7-day-old embryos at developmental stage 31[14]. The isolated left gonads were placed in 1.5 mL centrifuge tubes containing 100 µL of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS[–], cat. no. 05913; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The centrifuge tubes were placed in an incubator and maintained at 37.8 °C for approximately 1−3 h. When viewed under an inverted microscope (IMT-2; Olympus, Tokyo, Japan), the morphological characteristics of the isolated GGCs were identical to those of PGCs, i.e., large granulated round cells with large nuclei[15]. After recovery, the GGCs were labeled with PKH26 fluorescent dye (cat. no. Z-PKH26-GL; Zynaxis, Inc., Malvern, PA, USA). Polymerase chain reaction (PCR) was performed to determine the sex of the GGC donors using embryonic skin tissues, as described by Nakajima et al.[10].

Migratory and proliferative abilities of GGCs into gonadal and non-gonadal organs

Fifty GGCs from the left gonad of a 7-day-old WL embryo were injected into the dorsal aorta of 20 RIR embryos at developmental stages 13–16 for all four possible sex combinations determined by donor and recipient embryos. Throughout the study, GGC transfer was conducted using a fine glass pipette under a dissection microscope. Recipient eggs were prepared 1 h before injection to remove approximately 3 µL of blood through a small window (approximately 1 cm in diameter) in the eggshell. After injection, the window was sealed with plastic tape, and the egg was incubated at 37.8 °C for 5 days. The brain, heart, mesonephros, mesentery, and gonads were collected from recipient embryos and placed in a 48-well plate containing 50–100 µL 0.05% trypsin in PBS(–) and incubated at 37.8 °C for 20 min. After incubation, an equal volume of fetal bovine serum was added. The number of fluorescently labeled cells recovered from each organ was counted under a fluorescence microscope (IMT-2; Olympus) using a 546-nm excitation filter. PCR was performed to determine the sex of the recipients. A one-way analysis of variance (ANOVA) was conducted, with each combination repeated five times.

Migratory and proliferative abilities of GGCs in the intermediate mesoderm

Fifty GGCs from the left gonad of a 7-day-old WL embryo were injected into the dorsal aorta of 44 RIR embryos at developmental stages 13–16 for all four possible sex combinations. After injection, the window was sealed with plastic tape, and the egg was incubated at 37.8 °C for 3 days (5.5-day-old embryo) or 6 days (8.5-day-old embryo). The right and left gonads and mesonephros were collected from recipient embryos, placed in 1.5 mL centrifuge tubes containing 100 µL 0.05% trypsin in PBS(–), and incubated at 37.8 °C for 20 min. After incubation, the cell suspensions were placed onto a two-well fluororesin-coated glass slide (cat. no. TF0215; Matsunami Glass Ind. Ltd., Osaka, Japan), and the total number of fluorescently labeled GGCs recovered from each gonad was counted under a fluorescence microscope using a 546-nm excitation filter. PCR was performed to determine the sex of the recipients. A paired *t*-test and one-way ANOVA were conducted, with each donor sex repeated greater than seven times.

Migratory and proliferative abilities of GGCs following single-cell injection

The diameters of the GGCs were determined before injection using a micrometer under a microscope. A single GGC from the left gonad of a 7-day-old WL embryo was injected into the dorsal aorta of an RIR embryo at developmental stages 13–16 for

all four possible sex combinations (200 embryos). After injection, the window was sealed with plastic tape, and the egg was incubated at 37.8 °C for 5 days. The right and left gonads were collected from the recipient embryos, placed in 1.5 mL centrifuge tubes containing 50 µL 0.05% trypsin in PBS(–), and incubated at 37.8 °C for 20 min. After incubation, the cell suspensions were placed onto a two-well fluororesin-coated glass slide, and the total number of fluorescently labeled GGCs recovered from each gonad was counted under a fluorescence microscope using a 546-nm excitation filter. In this manuscript, settled GGCs were defined as fluorescently labeled GGCs observed in the recipient gonads 5 days after transfer. Settled GGCs were considered to have migrated and survived after transfer. The presence or absence of settled GGCs in a recipient gonad was confirmed by fluorescence-positive GGCs. The migratory ratio was measured based on the number of fluorescence-positive GGCs in the gonads of the recipient embryo.

Eight male and nine female donor embryos were used in the experiment; GGCs were injected into at least 10 recipients per donor. The injection of GGCs collected from males and females was repeated 100 times. PCR was performed to determine the sex of the recipients. A chi-square contingency analysis and one- and two-way ANOVAs were conducted.

Results

Migration of GGCs to the gonadal and extragonadal organs

Fig. 1 shows the migratory and proliferative abilities of GGCs in recipient organs after GGCs were transferred into recipient blood vessels. Due to the fluorescent labeling before injection, the migrated and proliferated GGCs in recipient organs were distinguished from endogenous GGCs as fluorescence-positive GGCs. Almost all fluorescence-positive GGCs detected inside embryos had migrated into the gonads and mesonephros. The number of fluorescence-positive GGCs detected in these organs was higher than the number of GGCs injected (Figs. 1 and S1A). The proportion of fluorescently labeled GGCs was significantly higher in the gonads than that in other organs for all sex combinations ($P < 0.05$). A large variation was observed in the GGC numbers in the gonads among donors and/or recipients, with no significant differences observed among all sex combinations (Fig. S1B) ($P > 0.05$). However, GGC numbers in the gonads tended to be higher in male recipients than those in female recipients, although no significant differences were observed (Figs. 1 and S1B) ($P > 0.05$). When male GGCs were transferred to both male and female recipients, the number and proportion of fluorescently labeled male GGCs in the mesonephros tended to be higher than those of female GGCs. However, there was no significant difference observed (Figs. 1 and S1A) ($P > 0.05$).

GGC migratory and proliferative abilities in the intermediate mesoderm region of recipient embryos

To examine serial changes in GGC migration and proliferation in the intermediate mesoderm region after injection, the numbers of fluorescence-positive GGCs in the gonads and mesonephros of recipient embryos were analyzed at 3 days (5.5-day-old embryo) and 6 days (8.5-day-old embryo) after transfer (Fig. 2). Since there was no trend in the GGC number in sexual combinations between donor and recipient, the difference between embryonic days are shown in donor sex. The GGC number in the gonads and mesonephros differed significantly between 3 and 6 days after transfer, regardless of the GGC donor's and recipient's sex ($P < 0.05$). GGCs in the gonads increased significantly between 3 and 6 days after transfer. In contrast, the numbers in the mesonephros decreased significantly, regardless of the sex of the GGC donor

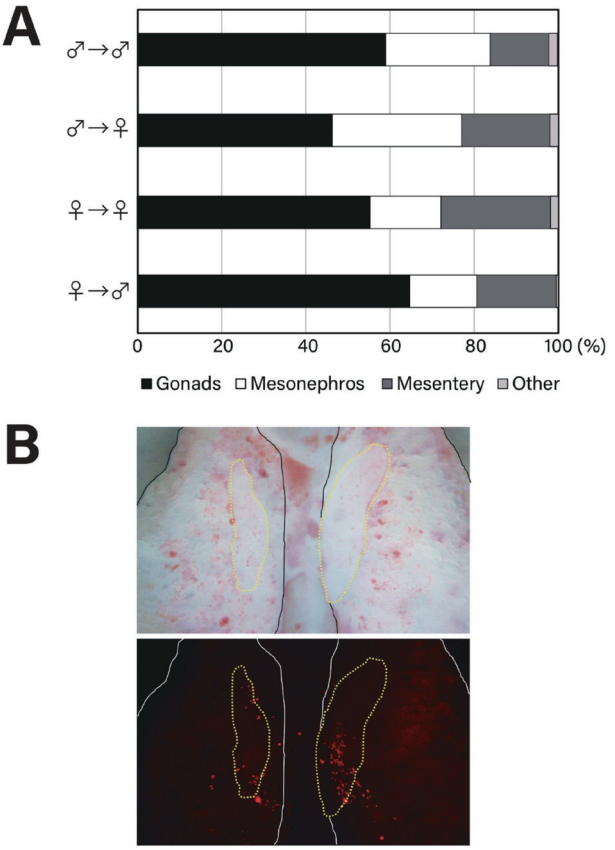

Fig. 1. **Distribution of PKH26-positive 7-day-old White Leghorn (WL) gonadal germ cells (GGCs) in the gonads and extragonadal organs of a Rhode Island Red (RIR) embryo 5 days after transfer.** A, Proportion of PKH26-positive GGCs in the gonads, mesonephros, mesentery, brain, and heart of male and female RIR embryos (n = 5). B, Distribution of PKH26-positive male GGCs in the gonads and mesonephros of a male RIR embryo. Upper image: Bright field microscopy. Lower image: Fluorescence microscopy.

Notations: ♀, Female; ♂, Male. Example: ♀→♂ indicates that a male RIR recipient received female WL GGCs. Other: head and brain

Dotted line: surrounds the gonads; solid line: surrounds the mesonephros

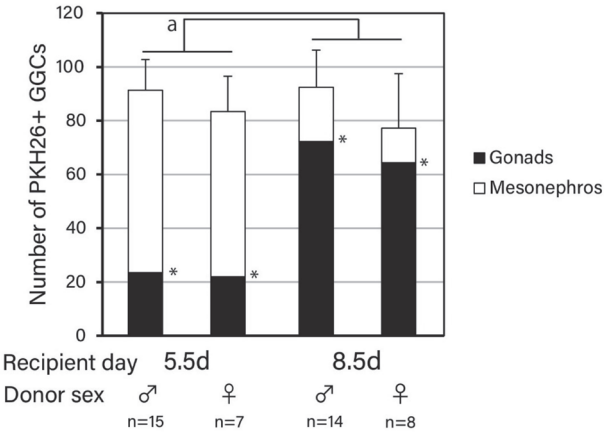

Fig. 2. **Number of PKH26-positive 7-day-old White Leghorn gonadal germ cells (GGCs) in the gonads and mesonephros of 5.5- and 8.5-day-old male and female Rhode Island Red embryos (mean ± standard error (SE) of the total number).**

*: The numbers of PKH26-positive GGCs in the gonads differed significantly from those of PKH26-positive GGCs in the mesonephros ($P < 0.05$).

a: The number of PKH26-positive GGCs in the mesonephros of a 5.5-day-old embryo differed significantly from that of an 8.5-day-old embryo ($P < 0.05$).

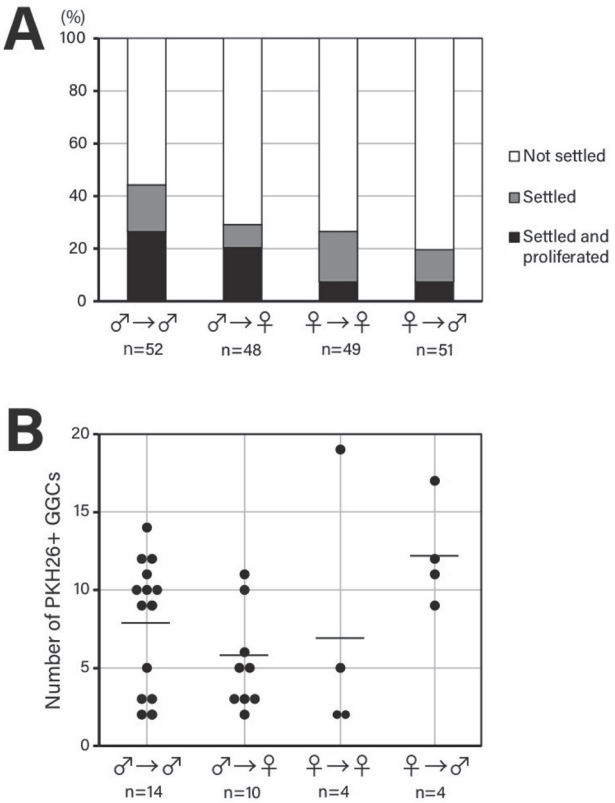

Fig. 3. **Migratory and proliferative abilities of 7-day-old White Leghorn (WL) single gonadal germ cells (GGCs) in the gonads of male and female Rhode Island Red (RIR) embryos 5 days after transfer.** A, Ratios of settled and proliferated single GGCs in the gonads to GGCs transferred into RIR embryos. B, Dot plot of the number of PKH26-positive GGCs in the gonads of RIR embryos. Bars represent the mean.

Notations: ♀, Female; ♂, Male. Example: ♀→♂ indicates that a male RIR recipient received a female WL GGC

and recipient ($P < 0.05$). When the developmental stages of recipient embryos were identical, no significant difference between donor sexes in the numbers of GGCs in recipient gonads was observed. Furthermore, the total numbers of GGCs in the gonads and mesonephros did not differ significantly with donor sex, recipient sex, or the developmental stage of recipient embryos.

Migration and proliferation of a single GGC in recipient gonads

To determine the variation in GGC diameters recovered from 7-day-old embryonic gonads, GGC diameter was measured before the injection of a single GGC. The average GGC diameters isolated from male and female 7-day-old embryos were 13.07 ± 0.18 μm (9.6–16.8 μm) and 13.02 ± 0.17 μm (9.6–16.8 μm), respectively. Settled GGCs were determined from survived and migrated GGCs in the gonads of RIR embryos 5 days after transfer. No significant interactions between settling or proliferative abilities and diameter were observed. Fig. 3 shows the number of GGCs that maintained their settling and proliferative abilities after transferring male and female 7-day-old GGCs into the gonads of male and female embryos. In total, 100 male GGCs were injected into 52 male recipients (MM) and 48 female recipients (MF), and 100 female GGCs were injected into 49 female recipients (FF) and 51 male recipients (FM). The presence-to-absence ratios of settled GGCs in MM, MF, FF, and FM were 44.2% (23/52), 29.1% (14/48), 26.5% (13/49), and 19.6% (10/51), respectively. No significant differences were observed either in settling or proliferative abilities among the different sex combinations of donors and recipients. The presence-to-absence ratio of settled GGCs tended to be higher in male GGCs than that in female GGCs. However, no significant differences were apparent (Fig. 3A). The proportion of settled GGCs tended to be higher under same-sex transfer conditions than that under cross-sex transfer conditions (Fig. 3A). The presence-to-absence ratios of proliferative ability in MM, MF, FF, and FM were 26.9% (14/52), 20.8% (10/48), 8.2% (4/49), and 7.8% (4/51), respectively. Proliferative ability was significantly higher in male GGCs than that in female GGCs ($P < 0.05$) (Fig. 3A). Fig. 3B shows the average and range of the proliferative rate of male and female GGCs transferred into recipients, which were 7.1 ± 0.8 fold (2–12 fold) and 9.6 ± 2.3 fold (2–19 fold), respectively. No significant interaction was observed between the sex of the donor and that of the recipient regarding the presence or absence of proliferative ability and the proliferative rate. This indicates the possibility of

diversity in individual GGCs.

Discussion

The production of germline chimeric embryos was analyzed to investigate the migratory and proliferative abilities of GGCs in male and female 7-day-old embryos. Although GGCs had migrated into the gonads and proliferated, 90% of donor-derived fluorescent GGCs that migrated to the recipient embryo were present in the gonads, mesonephros, and mesentery when GGCs were transferred in a group (Fig. 1). Thus, most GGCs appeared to maintain their ability to migrate into the intermediate mesoderm and rarely proliferated in the extragonadal region, although GGCs proliferated in the gonads of recipient embryos after transfer. These results are similar to those of a previous observation in which 80% of PGCs were present in the gonadal region at developmental stages 20−22 after PGC migration[16]. To our knowledge, no reports have quantitatively examined the number and ratio of migrated GGCs in the gonadal and extragonadal regions. It was hypothesized that the migratory ability of GGCs into the gonadal region would be lower than that of cPGCs. However, when transplanted as a population, approximately 60% of migrated GGCs were observed in the gonads 5 days after transfer. GGCs retained their migratory ability into gonads and had a proliferative ability similar to that of PGCs. These results implied that the migratory and proliferative abilities of GGCs 5 days after transfer did not differ between the sexes of donor GGCs recovered from 7-day-old embryonic gonads. However, gametogenesis and/or ontogenesis are not efficient or difficult when germline chimeras are produced by the cross-sex injection of both cPGCs and GGCs[10,13,17,18]. No significant differences were observed when the migratory and proliferative abilities of GGCs were averaged, and similar trends were observed in the donor and recipient sexes (Figs. 1 and S1). However, because GGC migration into the gonads and GGC proliferation in recipient gonads varied in each recipient embryo, it was speculated that the migratory and proliferative abilities of GGCs into recipient gonads were affected by individual differences in each GGC (Fig. S1B). Therefore, the migration and proliferation of individual GGCs were examined.

Monitoring GGC behavior following transfer to recipient embryos provides fundamental information on germ cell differentiation and the production of germline chimeras. The gonadal ridge, which is a progenitor tissue of the gonads, is derived from the intermediate mesoderm[7]. During early gonadogenesis, undifferentiated embryonic gonads differentiate into ovaries or testes while maintaining adherence to the mesonephros[7]. Furthermore, the development of gonads and sexual maturity fail in chickens when the mesonephros are removed at the early embryonic developmental stage[19]. Therefore, the mesonephros are important for gonadogenesis and ensuring normal reproductive function in chicken embryos[19]. When examining migration into the intermediate mesoderm region of recipient embryos, a serial change in GGC migration from the intermediate mesoderm to the gonads during development was confirmed. A negative correlation was observed between the number of fluorescently labeled GGCs in the gonad and that in the mesonephros during development. After injection of single GGCs, 20–40% of injected GGCs were detected in the gonad, and 64.9% (24/37) of the male GGCs and 34.8% (8/23) of the female GGCs detected in the gonad proliferated. Fluorescently labeled GGCs were detected using a stereomicroscope and inverted microscope in both the gonad and mesonephros of only two out of 100 RIR embryos 5 days after single-GGC transfer. Additionally, three fluorescent-positive GGCs were not detected in the gonads; however, they were detected in the right mesonephros of an embryo 5 days after the single-GGC injection (data not shown). These findings imply that transferred GGCs migrated from blood vessels into the intermediate mesoderm and then migrated into the gonads through the mesonephros. Therefore, GGCs that remained in the mesonephros within 3 days after transfer either migrated to the gonads or died, whereas those that migrated to the gonads within 3 days after transfer settled and/or proliferated. In males, the medulla of the testis originates from the mesonephros, and the primitive gonads contain common tissue with the mesonephros during embryonic development[20]. Additionally, mesonephric cell migration from the mesonephros to the gonads before sexual differentiation of the gonads has been demonstrated in male chicken embryos[14,21,22], female sheep[23], mice[24], and cattle[25]. Therefore, it is possible that the phenomenon of GGCs migrating from the mesonephros to the gonads 3–6 days after GGC transfer occurred as a result of mesonephric cells and germ cells migrating into the gonads, in addition to the active migration of GGCs.

GGCs are likely the cell population most affected by the various modifications that occur during gonadal development. To analyze the characteristics of individual GGCs, there is a need to distinguish between migration into the gonadal region and proliferation in the gonads. The migratory and proliferative abilities of GGCs in recipient gonads were examined using single-GGC injections to segregate the settling and proliferative abilities of individual GGCs. When chicken single cPGCs were transferred into quail embryos, approximately 60% of the chicken cPGCs could migrate to the quail gonadal region at developmental stages 20–21[26]. In this study, only 20–40% of transferred single GGCs settled and/or proliferated in the gonads. Hence, GGCs that did not settle to the gonads may have remained in the extragonadal region, including extraembryonic blood vessels. Alternatively, a few of the GGCs that migrated into the gonads may have died within 4 days after transfer. These results indicate that the GGCs that migrated into the gonads and proliferated may have had a reduced migratory ability compared with that of cPGCs. In this study, migratory and proliferative abilities were analyzed in 7.5-day-old recipient embryos simultaneously, which is the mitotic proliferative stage. However, in the future, cPGC and GGC migration should be analyzed a short time after GGC transfer. Additionally, the physiological function of cPGCs and GGCs should be examined by serial analysis of survival and proliferative abilities using germ cells induced by reporter genes, e.g., GFP.

The ratios of settled and proliferated GGCs in the gonads compared to the total numbers of GGCs transferred were higher in male GGCs than those in female GGCs (Fig. 3A). When the GGC donor and recipient were the same sex, settling of GGCs was higher than under cross-sex GGC transfer conditions (Fig. 3A). In contrast, a large proportion of proliferative GGCs were present in 7-day-old female embryos; the proportion was higher in males than that in females (Fig. 3). The settling and proliferation of GGCs were more homogeneous in males. At the same time, females may have more diversity (Fig. 3). This result suggested that female GGCs become differentiated by the sexual differentiation of the gonads. In this study, the GGCs isolated from the gonads of 7-day-old embryos were classified into three types, according to the presence and/or absence of GGC settling and proliferative abilities: type 1) settling present and proliferative ability absent; type 2) both settling and proliferative abilities present; and type 3) both settling and proliferative abilities absent, including death until 4 days after transfer. Furthermore, type 2 GGCs could be classified according to their high or low proliferative rates in recipient gonads. Based on these results, it is speculated that the diversity of the migratory and proliferative abilities of GGC populations differs between males and females. This diversity depends on the proportion of each GGC type. The suggested sexual differences of GGCs in this study may have been affected by the histological and molecular sexual differentiation of GGCs and developing gonads, which started before collection in 7-day-old embryos. To characterize GGCs morphologically in terms of migration and proliferation, all GGC diameters were measured before transfer; however, a relationship between GGC diameter and migratory and proliferative abilities was not observed. Discrimination of highly proliferative GGCs in GGC populations would benefit reproductive approaches such as culturing germ cells and producing germline chimeras. In the future, an analysis of gene expression and the differentiation of GGCs into gametes in single cells should be performed.

In conclusion, in this study, the migratory and proliferative abilities of GGCs differed between the sexes and individual GGCs, demonstrating that GGCs are a heterogeneous cell population. Furthermore, GGCs may migrate from the intermediate mesoderm to the gonads through the mesonephros during gonadogenesis. We speculate that the efficiency of germline chimera production may be influenced by the proportion of a specific GGC type in the overall GGC population. Although GGCs were useful donors for the production of germline chimeras, it is necessary to transfer as many GGCs as possible for reliable germline chimera production.

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Author Contributions

Yuki Nakajima designed and performed all the experiments and wrote the manuscript; Takahiro Tagami and Atsushi Tajima discussed and wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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