NOTE *Wildlife Science*

Migratory ability of gonadal germ cells (GGCs) isolated from *Ciconia boyciana* and *Geronticus eremita* embryos into the gonad of developing chicken embryos

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(Received 19 November 2015/Accepted 12 February 2016/Published online in J-STAGE 26 February 2016)

ABSTRACT. We conducted experiments to evaluate the ability of gonadal germ cells (GGCs), isolated from the embryonic gonads of *Ciconia boyciana* or *Geronticus eremita,* to migrate into the gonads of developing chicken embryos. Fluorescently labeled GGCs, isolated by the PBS (−) method, were transferred into the dorsal aorta of 2-day-old chicken embryos. Five days after transfer, fluorescent GGCs were detected in the gonads of recipient embryos. Our results indicate that GGCs from *Ciconia boyciana* and *Geronticus eremita* are capable of migrating into the gonads of developing chicken embryos.

KEY WORDS: chicken, *Ciconia boyciana*, *Geronticus eremita*, gonadal germ cells (GGCs), migratory ability

doi: 10.1292/jvms.15-0664; *J. Vet. Med. Sci.* 78(6): 1055–1058, 2016

The development of a system to conserve and propagate endangered wild animal is critical in order to maintain biodiversity. However, currently, no method exists for cryopreserving eggs from oviparous animals, such as avian species. The absence of such a method can be attributed primarily to the difficulty of retrieving viable embryos after cryopresevation, which is in turn due to the megalecithal nature of avian eggs. An alternative method of conserving avian genetic diversity has been developed for the domestic chicken (*Gallus gallus*): germline chimeras are produced by collecting, freezing and transferring primordial germ cells (PGCs) isolated from embryonic blood [16] or gonadal germ cells (GGCs) isolated from the gonads of developing embryos [15, 17].

Recently, efforts have been made to conserve endangered avian species through the production of interspecies germline chimeras: for example, interspecies blastodermal chimeras have been produced by transferring duck blastodermal cells into the blastoderms of male chicken embryos [6]. Interspecies germline chimeras have also been produced by transferring pheasant GGCs [5] or houbara bustard GGCs [18] into male chicken embryos, as well as by transferring chicken PGCs into male duck embryos [7].

These reports suggest that it may be possible to conserve endangered wild birds by sacrificing fertilized eggs in order to collect PGCs or GGCs. It was reported that chicken PGCs initially circulate in the bloodstreams of 2-day-old embryos for several hr [14] and subsequently escape from the vascular system, migrating into the developing gonads of the recipient embryos by 5 days after the start of incubation [8]. Assuming this sequence of PGC migration is present among wild birds, collecting circulating PGCs from the bloodstream is thought to be difficult owing to the practical difficulty of recovering eggs from nests at the precise time of incubation suitable for PGC collection. Thus, the recent development in the domestic chicken of the PBS (−) method of collecting GGCs from developing gonads [11] and subsequently producing germline chimera [10] may provide a promising alternative method for conserving avian genetic resources.

In the present study, we investigated the possibility of collecting GGCs from the developing gonads of *Ciconia boyciana* (*Cb*) and *Geronticus eremita* (*Ge*) using the PBS (−) method. We also examined the ability of GGCs to migrate into the gonads after being transferred into the bloodstream of 2-day-old chicken embryos.

Our experiments used fertilized eggs recovered from parental nests of *Cb* and *Ge* maintained at the Zoorasia Yokohama Zoological Gardens and the Preservation and Research Center in Yokohama, Japan, respectively. The date of oviposition in each species was estimated by careful visual

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observation of nesting behavior by experienced managers. Eggs in nests were recovered, placed in a Styrofoam box maintained at 37°C and transported by car to the University of Tsukuba in Tsukuba City (a journey of 4 −6 hr, depending on traffic). After arrival, eggs were incubated in an forced-air incubator (P-008; Showa Furanki Laboratory, Saitama, Japan) maintained at 37.8°C, 70% relative humidity. After incubation for 4 −6 days, the embryonic gonads of each species were surgically isolated under a dissection microscope. The developmental stage of each embryo was identified according to morphological criteria developed for chicken embryos [2].

Germ cells in developing gonads were recovered using the PBS (−) method [11]. Briefly, isolated gonads were placed in 1.5-m*l* centrifuge tubes containing 100 μl of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS (−), cat. no. 05913; Nissui, Tokyo, Japan). The centrifuge tubes were placed in an incubator maintained at 37.8°C for 1 hr to induce spontaneous discharge of GGCs in developing gonads into PBS (−). After removing gonadal tissues in centrifuge tubes using pipette, 20 μl cell suspension volumes were placed on 12-well Hard Teflon-Coated Slides (HTCSs, cat. no. 10–226-CC; Erie Scientific Co., Portsmouth, NH, U.S.A.), and the numbers of discharged GGCs in PBS (−) were counted. As a control, we also counted the number of GGCs from the gonads of White Leghorn (WL) embryos at the same developmental stages as *Cb* and *Ge.* The number of germ cells remaining in the gonads after applying the PBS (−) method was counted after digesting gonads in PBS (−) containing 0.05% trypsin at 37.8°C for 20 min. Polymerase chain reaction (PCR) was performed to determinate the sex of embryos using embryonic skin tissues according to the method described by Ito *et al.* [4], with slight modifications.

In both *Cb* and *Ge*, a discharge of GGCs from developing gonads was observed with the PBS (−) method (Fig. 1). The morphology and size of GGCs from *Cb* and *Ge* were similar to those of chicken GGCs. Both the number of discharged GGCs and the total number of GGCs were higher in left gonads than right gonads (Table 1). In most cases, the recovery rate of GGCs using the PBS (−) method was also higher from left gonads than right gonads. These results are consistent with observations reported for chickens by Nakajima *et al.* [11]. In an earlier study on the developing gonads of chicken embryos, we suggested that the discharge of GGCs into PBS (−) is affected by two main factors: Ca^{2+} concentration and incubation temperature [9]. Future research should examine the factors involved in spontaneous GGC discharge in *Cb* and *Ge.* Nevertheless, it was demonstrated that the PBS (−) method to isolate GGCs from embryonic gonads, first developed in chicken embryos, can also be applied to both *Cb* and *Ge*.

To examine cross-species migratory ability, GGCs were first isolated from *Cb* and *Ge* by the PBS (−) method as described by Nakajima *et al*. [11]. After recovery, GGCs from each species were labeled with PKH26 fluorescent dye (cat. No. Z-PKH26-GL; Zynaxis, Inc., Malvern, PA, U.S.A.). 30−200 fluorescently labeled GGCs (f-GGC) from each species were injected into the dorsal aorta of Rhode Island Red (RIR) embryos at stages 13−16 of development [2]. Fertilized RIR eggs were produced at the Agriculture and Forestry Research Center, University of Tsukuba, Japan. A cross-species GGC transfer was carried out using a fine glass pipette under a dissection microscope (S6E; Leica, Wetzlar, Germany). Recipient eggs were prepared 1 hr before injection to remove approximately 3 μl of blood through apertures in the eggshells approximately 1 cm in diameter. After injection, eggs were incubated at 37.8°C for 5 days. After incubation, left and right gonads, as well as mesonephroi, were collected from recipient embryos and placed in 1.5 m*l* centrifuge tubes containing 100 μl of 0.05% trypsin in PBS (−) and incubated at 37.8°C for 20 min. After incubation, the cells were placed on a 2-well HTCS, and the total number of fluorescently labeled cells recovered from each gonad was counted under a fluorescence microscope (IMT-2; Olympus, Tokyo, Japan) using a 546 nm excitation filter. The number of f-GGCs found in the recipient's gonad (G) or mesonephros (M) compared to the number of f-GGCs injected (ij) into the recipient embryo was denoted as the G-ratio (f-GGC[G]/f-GGC[ij] x 100) or M-ratio (f-GGC[M]/f-GGC[ij] x 100), respectively. G-ratio and M-ratio were calculated in the present study, since we have previously reported that the migratory ability of PGCs into gonadal areas was little influenced by the number of injected germ cells under cross species PGCs transfer conditions between chicken and quail [3].

Figure 2 shows the G-ratio and M-ratio for *Cb* and *Ge* in the gonads and mesonephroi of recipient chicken embryos at 5 days after transfer. As depicted in Fig. 2, f-GGCs from both *Cb* and *Ge* were observed in the gonads of recipient chicken embryos. This indicates that GGCs from the developing gonads of *Cb* and *Ge* are capable of migrating into the gonads of chicken embryos.

However, the G-ratios for both *Cb* and *Ge* in chicken gonads were lower than those of chicken GGCs. This effect was consistent across all developmental stages, except for stage 30 of *Cb* and chicken male embryos, where no significant difference in the M-ratio of *Cb* and *Ge* in chicken mesonephroi was observed compared with chickens (Fig. 2). Since it has been reported that PGCs escaped from dorsal aorta migrate into embryonic gonad via intermediate mesoderm including mesonephros [1, 12], these findings suggest that GGCs from *Cb* and *Ge* share a common migratory mechanism with GGCs from chickens to the mesonephros, whereas the mechanism of GGC migration from the mesonephos to gonad may be species-specific.

It was suggested that germ cell migration from the bloodstream to the intermediate mesoderm, including the gonadal region, is influenced by the development of the vascular system in chickens [1], as well as by a peptide chemokine (stromal cell-derived factor 1) secreted from the embryonic gonad and C-X-C chemokine receptor [13]. However, the mechanisms of germ cell migration into the gonads of embryos remain unclear. A future study should examine the ability of GGCs in recipient gonads to proliferate and differentiate, as well as the mechanisms of GGC migration into recipient gonads during interspecies GGC transfer.

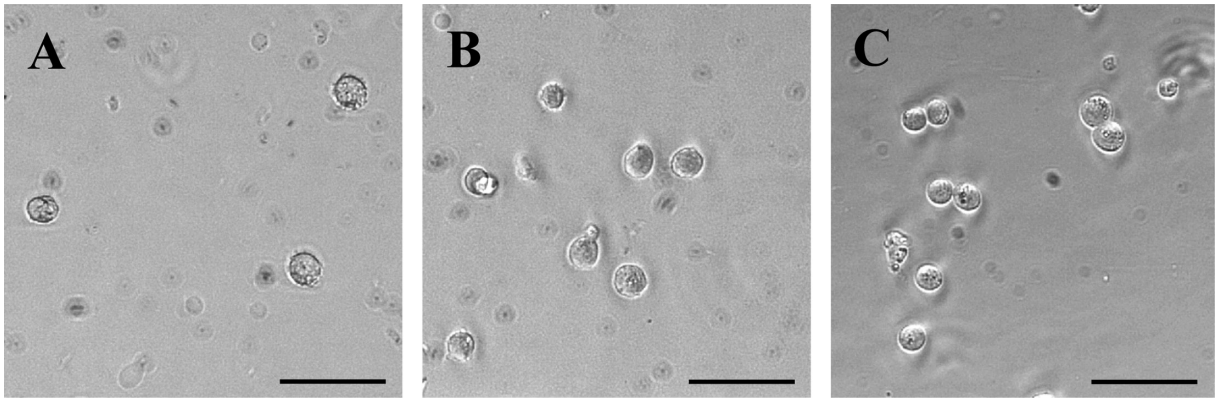

Fig. 1. Spontaneously discharged gonadal germ cells from embryonic gonads of *Ciconia boyciana* (A), *Geronticus eremita* (B) and chicken (*Gallus gallus*) (C). Bar=50 μm.

Table 1. The number of gonadal germ cells discharged from embryonic gonads after incubation in phosphate buffered saline without Ca^{2+} and Mg^{2+} for 1 hr

A) *Ciconia boyciana*

		Ciconia boyciana				Chicken (*Gallus gallus*) n≥4			
		Total num. of GGCs		PBS(–) 1 hr num. of GGCs		Total num. of GGCs		PBS(–) 1 hr num. of GGCs	
Dev. St.	Sex	Left	Right	Left	Right	Left	Right	Left	Right
28	Male	1,490	395	120	10	3,374 ± 1,222	873 ± 369	525 ± 172	150 ± 93
29	Male	550	240	270	20	1,930 ± 633	660 ± 146	604 ± 215	182 ± 55
30	Male	1,360	1,040	430	30	2,368 ± 473	856 ± 222	640 ± 73	252 ± 59
31	Male	2,545	1,195	600	90	3,137 ± 322	1,048 ± 244	745 ± 96	230 ± 102
	Male	3,260	860	60	60				
34	Male	6,420	1,080	260	40	4,637 ± 1,356	1,356 ± 231	1,045 ± 430	75 ± 28
36	Female	8,500	3,205	1,100	5	27,523 ± 4,127	2,475 ± 507	6,950 ± 1,058	70 ± 57
37	Male	260	420	60	20	9,907 ± 1,758	4,827 ± 2,725	150 ± 35	50 ± 11

B) *Geronticus eremita*

		Geronticus eremita				Chicken (*Gallus gallus*) n≥4			
		Total num. of GGCs		PBS(–) 1 hr num. of GGCs		Total num. of GGCs		PBS(–) 1 hr num. of GGCs	
Dev. St.	Sex	Left	Right	Left	Right	Left	Right	Left	Right
30	Female	1,115	178	160	20	5,140 ± 684	505 ± 139	1,325 ± 274	100 ± 39
	Female	530	270	180	10				
32	Female	5,030	820	1,800	120	4,263 ± 367	1,101 ± 150	2,385 ± 175	45 ± 19
32	Male	1,070	670	150	10	7,495 ± 1,301	2,090 ± 552	1,285 ± 285	230 ± 124
34	Female	4,680	1,610	2,490	120	5,482 ± 1,244	867 ± 193	1,830 ± 395	247 ± 54
45	Male	20,149	8,617	20	20	26,036 ± 7,789	14,384 ± 2,499	36 ± 22	24 ± 7
45	Female	192,165	5,440	6,600	420	165,065 ± 23,897	9,270 ± 1,610	3,208 ± 317	70 ± 50
	Female	146,674	4,520	9,760	20				

In conclusion, we have demonstrated that viable GGCs from *Ciconia boyciana* and *Geronticus eremita* can be collected using the PBS (−) method and that collected GGCs are capable of migrating into the gonads of developing chicken embryos.

ACKNOWLEDGMENTS. We acknowledge the staff of the Agricultural and Forestry Research Center (University of Tsukuba, Japan) for their technical support in maintaining the animals. This study was funded in parted by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant Number: 25 • 353) to YN and Grant-in-Aid for Scientific Research (B) from JSPS KAKENHI (Grant Number: 25290082) to MO.

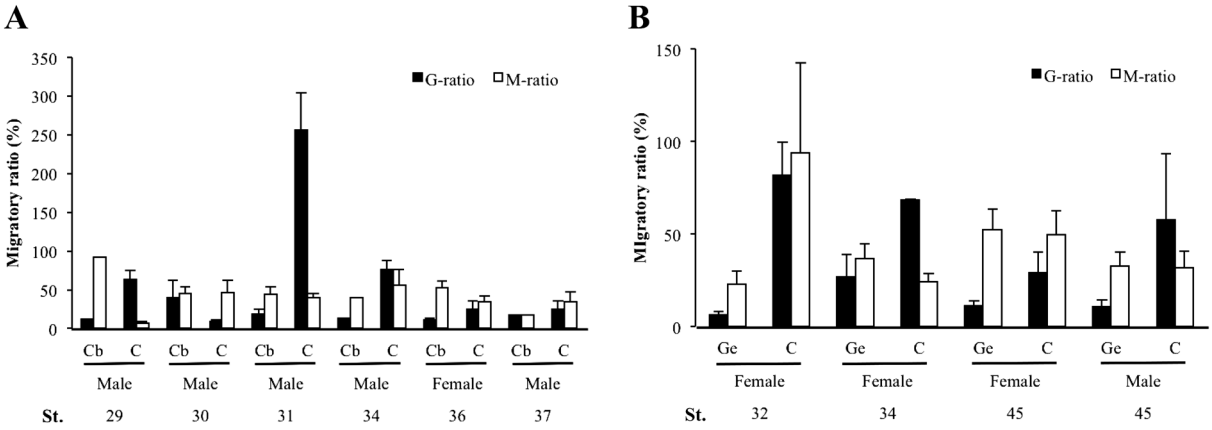

Fig. 2. The migratory ratio of transferred PKH26-positive gonadal germ cells into the gonad and the mesonephros of chicken recipients. A) *Ciconia boyciana* (*Cb*), B) *Geronticus eremita* (*Ge*) and chicken (C). G-ratio: number of fluorescent-labeled GGCs found in recipient gonad/ number of fluorescent-labeled GGCs injected into the recipient. M-ratio: number of fluorescent-labeled GGCs found in recipient mesonephros/ number of fluorescent-labeled GGCs injected into the recipient.

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