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≪**Research Note**≫

Population of Circulating Primordial Germ Cells in Early Japanese Quail Embryos

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Avian primordial germ cells (PGCs) show a unique migration pathway during early development. As soon as the blood vessels have formed, PGCs enter the circulatory system, and migrate to the gonadal primordium. We measured the concentration and the size of PGCs and erythrocytes from the wild-type plumage and the sex-linked brown strains of Japanese quail embryos when PGCs are in circulation at Hamburger and Hamiltons stages 13–19. PGCs were high in concentration and large in size at stage 13, and it showed a significant decrease as the function of embryonic development. Erythrocytes showed a low concentration at stage 13 and a significant increase as the function of embryonic development but the size was constant during stages 13–19. No strain difference was observed in these characters between the two strains of quail embryos.

Key words : primordial germ cells, circulation, erythrocytes, quail, embryo

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Introduction

Avian primordial germ cells (PGCs) display a unique migration pathway toward their target organ, gonadal anlage, during early development. PGCs are the first identifiable precursor cells for gametes. These cells first arise from the epiblast in the central zone of the area pellucida and gradually translocate to the hypoblast during early stages of primitive streak formation (Sutasurya *et al.*, 1983 ; Eyal-Giladi *et al.*, 1981 ; Ginsburg and Eyal-Giladi, 1986 ; 1987 ; Tajima *et al.*, 1999). During gastrulation, the PGCs migrate anteriorly via the hypoblast and reside in the extraembryonic germinal crescent (Eyal-Giladi *et al.*, 1981 ; Urven *et al.*, 1988 ; Han *et al.*, 1994). As soon as the blood vessels form at Hamburger and Hamilton (1951) stage 10, PGCs from the germinal crescent start to circulate temporarily through the bloodstream, and by stages 20–24 these PGCs have migrated into the gonadal anlage where they rapidly proliferate and differentiate into either spermatogonia in the testis or oogonia in the ovary (Mayer, 1964 ; Fujimoto *et al.*, 1976 ; Nakamura *et al.*, 1988 ; Kuwana, 1993 ; Fujihara, 1999). Migration features of PGCs facilitate their isolation and transfer in early developing avian embryos (Fujimoto *et al.*, 1976 ; Chang *et al.*, 1992 ; Yasuda *et al.*, 1992 ; Ono *et al.*, 1999).

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Avian genetic stocks as a manner of cryopreserved PGCs will be highly useful for the preservation of foundation stocks and endangered species (Tajima *et al.*, 1993 ; Fujihara, 1999). A number of attempts have been made to produce germline chimeras and donor-derived offspring by the transfer of PGCs in chickens (Naito *et al.*, 1994a ; Tajima *et al.*, 1993 ; Vick *et al.*, 1993), and Japanese quail (Wentworth *et al.*, 1989 ; Ono *et al.*, 1998). Offspring were produced from frozen/thawed donor-derived PGCs (Naito *et al.*, 1994b ; Chang *et al.*, 1998). It is also possible for PGCs to serve as a vector for introducing and expressing exogenous genes to produce transgenic birds (Allioli *et al.*, 1994 ; Naito *et al.*, 1998 ; Ebara amd Fujihara, 2000). For avian transgenesis, Japanese quail may serve as an excellent animal model because of its small body size and fast growth rate. They require 16 days to hatch and 6 weeks to mature to an adult body weight less than about 150 g.

Basic information is available on the concentration of circulating PGCs in early chick embryos at stages 13–18 (Tajima *et al.*, 1999). For quail embryos, limited information on the concentration of PGCs is available at stages 14–16 (Ono and Machida, 1999) and it is necessary to obtain more information to utilize quail as a pilot animal. Wild-type plumage and sex-linked brown quail are widely used because auto-sexing is available with the cross between wild-type females (Z^+/W) and sex-linked brown males (Z^b/Z^b), with the resulting male and female offspring being wild-type (Z^b/Z^+) and brown (Z^b/W), respectively. The present study, therefore, is concerned with the concentration and size of PGCs and erythrocytes of the wild-type plumage and the sex-linked brown quail from stage 13, the earliest stage at which blood sampling can practically be performed, to stage 19, when most PGCs are disappearing from the circulation.

Materials and Methods

Fertilized eggs of wild-type plumage (WP) and sex-linked brown (BR) strains of Japanese quail (*Coturnix japonica*, Somes, 1988 ; Cheng and Kimura, 1990), maintained in our laboratory, were collected daily and kept at 12℃ for not more than 3 days. Development of quail embryos was determined according to Hamburger and Hamilton's standard (1951). The eggs were then incubated in an egg incubator (P-03, Showa Furanki Inst., Yono) at 37.5℃ and 70% relative humidity with tilting to a 90° angle at 30-min intervals until the embryos reached stages 13–19 (about 45–68 hr of incubation).

The micropipettes used for blood collection from embryos were made from siliconized glass capillary tubes (G-1, Narishige, Tokyo) drawn to an outer diameter of 50–70 μm with a micropipette puller (PB-7, Narishige), then the tips were beveled to 25° with a pipette grinder (EG-4, Narishige). The micropipette was calibrated in microliters by sucking up a calibrated drop of water and drawing a mark on the pipette.

Quail embryos at stages 13–19 were cracked open and transferred into small plastic cups. Two μl of blood was collected from the dorsal aorta or the marginal vein of the embryo with the calibrated micropipette connected to an aspirator tube assembly (Drummond, Broomall, PA). The blood was then dispersed in 18 μl of Leibovitz's L-

15 medium (Sigma Chemical Co., St. Louis, MO) containing 10% fetal calf serum (FCS, Nippon Bio-Test Lab. Inc., Tokyo). A well dispersed blood sample was placed on an improved Neubauer blood corpuscle counter (Kayagaki Rika, Tokyo). The mean of duplicate measurements was represented as the cell number of each sample. Sizes of PGCs and erythrocytes were measured with an ocular micrometer. Data were analyzed statistically with Sheffé's test and/or ANOVA (Snedecor and Cochran, 1989). Differences were regarded as significant at $P<0.05$.

Results and Discussion

Only PGCs and erythrocytes were observed in the bloodstream during stages 13 and 19. PGCs were large roundish cells with conspicuously large nuclei and easily distinguished from erythrocytes when observed under a phase contrast microscope. PGCs circulate temporarily in the developing bloodstream in avian embryos, and this unique migratory pathway makes PGC collection easy. Basic characteristics of quail PGCs were similar to those of chick embryos which were described previously (Mayer, 1964 ; Yang and Fujihara, 1999).

Tables 1 and 2 show concentrations and size of PGCs and erythrocytes, and proportion of PGCs to all the blood cells (PGCs and erythrocytes) in the bloodstream of embryos from WP and BR strains at stages 13–19, respectively. The concentration and the size of PGCs showed significant decrease as the function of embryonic development in both WP and BR strains (ANOVA) and there was no strain difference observed. The concentration of erythrocytes, by contrast, showed a significant increase as the function of embryonic development in both strains, but the cell size remained constant during stages 13 and 19. There was no strain difference observed in

Table 1. Concentrations and diameters of PGCs and erythrocytes in the bloodstream of WP strain quail embryos at stages 13–19

Stage	No. of repeated analyses	No. (μl^{-1}) of	Diameter (μm) of	No. ($\times 10^3 \mu l^{-1}$) of	Diameter (μm) of	Proportion of PGCs* ($\times 10^6$)
		PGCs		Erythtrocytes		
13	10	137.3±19.7 a	15.4±0.3 a	222.5±13.5 a	10.1±0.2	619±78 a
14	9	111.1±17.2 ab	14.5±0.3 ab	236.8±11.5 a	11.0±0.5	456±50 a
15	16	71.9±6.0 bc	14.7±0.4 ab	253.1±17.1 a	10.0±0.2	294±27 b
16	13	67.3±7.7 bc	13.5±0.4 ab	305.2±14.5 a	10.6±0.2	221±24 b
17	10	27.5±6.9 c	13.1±0.5 ab	427.0±7.5 b	10.5±0.3	64±16 c
18	10	15.0±4.1 c	12.8±0.4 ab	546.3±21.6 c	10.8±0.5	29±8 cd
19	11	9.1±3.8 c	11.5±0.5 b	596.1±20.7 c	10.5±0.4	16±7 d

Results show mean±S.E.M. Statistical analysis of the data was based on Scheffé's test, and significantly differnet data within the same strain are indicated by different letters.

* Proportion of PGCs to all the blood cells (PGCs and erythrocytes) in the bloodstream.

Table 2. Concentrations and diameters of PGCs and erythrocytes in the bloodstream of BR strain quail embryos at stages 13–19

Stage	No. of repeated analyses	No. (μl^{-1}) of PGCs	Diameter (μm) of PGCs	No. ($\times 10^3 \mu l^{-1}$) of Erythtrocytes	Diameter (μm) of Erythtrocytes	Proportion of PGCs* ($\times 10^6$)
13	13	126.9±14.3 a	16.1±0.5 a	215.1±11.9 a	9.5±0.4	615±79 a
14	12	95.0±15.0 ab	15.1±0.4 ab	250.3±14.9 a	10.4±1.0	386±52 ab
15	10	70.0±9.0 ab	13.7±0.5 abc	301.1±16.9 ab	9.8±0.5	237±30 bc
16	12	50.0±4.4 b	12.4±0.5 bc	315.0±7.8 ab	9.8±0.7	159±13 bc
17	12	27.1±5.7 c	11.7±0.9 c	362.1±12.4 b	11.1±0.6	77±19 c
18	10	15.0±5.5 cd	10.4±0.4 c	424.1±20.7 c	10.7±0.6	36±14 c
19	11	6.8±3.5 d	10.3±0.5 c	602.5±15.2 d	11.4±0.6	12±6 c

Results show mean±S.E.M. Statistical analysis of the data was based on Scheffé's test, and significantly differnet data within the same strain are indicated by different letters.

* Proportion of PGCs to all the blood cells (PGCs and erythrocytes) in the bloodstream.

the concentration and size of erythrocytes. Proportion of PGCs showed a significant decrease as the function of embryonic development in both strains, but there was no strain difference observed.

In our previous report we measured the concentration of circulating PGCs and erythrocytes in WP strain of quail embryos at stages 14–16 (Ono and Machida, 1999). Concentrations of PGCs and erythrocytes, and the proportion of PGCs were coincidental with those of the present study. Tajima *et al.* (1999) reported the concentration of circulating PGCs in chick embryos at stages 13–18. The concentration of chick PGCs was reported to be highest at stage 14 and decreased thereafter, and that of quail PGCs shown in the present study was observed to be highest at stage 13 and decreased thereafter. Quail embryos were observed to have a higher concentration of circulating PGCs than chick embryos, and especially at stage 13 quail embryos were observed to have twice as many circulating PGCs as chick embryos. There is no information available concerning the concentration of circulating PGCs before stage 13. Such information whould be important and Kuwana *et al.* (1996) succeeded in collecting blood from the stage 12 chick and quail embryos, although it requires more refined techniques to obtain blood from such young embryos. Tajima *et al.* (1999) reported that there is considerable egg to egg variations in the concentration of PGCs. In the present study although we did not check the individual egg's parents, there were sample-to-sample variations in PGC concentration expressed as the standard error of the mean.

In the present studies, we have obtained some basic information about the concentration and proportion of circulating PGCs in the bloodstream at early embryonic stages that the concentration of circulating PGCs is high at stage 13 and decreased thereafter. This information will be useful for future studies not only for the efficient

production of germline chimeras but also for preserving PGCs.

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