F-3.4 Study on the dynamics and cell biology of circulating primordial germ cells in early chick embryos

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Abstract

We conducted a series of experiments assuming that production of the germline chimeras by collecting, conserving and transferring primordial germ cells (PGCs) would fulfill our goal. In the present study, 8 experiments were performed, those are 1) change in the PGC concentration during early embryonic development, 2) effect of storage on the PGC concentration, 3) effects of female on the concentration of PGCs, 4) change on the concentration of circulating PGCs within an embryo, 5) total number of circulating PGCs in an embryo, 6) a new method of collecting circulating PGCs, and 7) production of germ-line chimeras by transferring cryopreserved gonadal PGCs.

Introduction

Development of the technique to conserve genetic resources in avian species is beneficial not only for poultry industry (especially for the primary breeders) but also for preserving endangered avian varieties. However, due to the inability of cryopreserving avian embryos, primarily because of its large size and yolk laden structure, only available means to conserve genetic resources in avian species has been through freezing spermatozoa. Since it is not possible to conserve genetic resources at complete form only by storing spermatozoa, it is apparently important to develop an alternative means in avian species, which is equivalent to embryo transfer in mammalian species. We, therefore, conducted a series of experiments assuming that production of the germline chimeras by collecting, conserving and transferring primordial germ cells (PGCs) would fulfill our goal.

PGCs, which are predecessor of spermatogonia or oogonia, arise from epiblast and translocate gradually to lower layer and locate in the germinal crescent by stage 4. Upon development of the vascular system, the PGCs circulate temporarily through the blood stream, migrate into the gonadal anlage. Migrated PGCs (referred as gPGCs) are known to proliferate and finally differentiate to either spermatogonia in the testis or oogonia in the ovary. Therefore, PGCs can be collected from germinal crescent or blood and gPGCs can be collected from gonadal anlage, respectively.

However, it is our constant experience that the numbers of cPGCs that can be collected from pooled blood sample differ considerably among replicate or experiments. It was considered that the basic information

on the concentration of cPGCs in an early chick embryo is essential for the efficient production of germ-line chimeras in the domestic chicken.

Therefore, experiments were designed to elucidate the factor that influence the concentration of cPGCs in the two-day old chick embryos using a different blood collection protocols.

Research Objective

Development of the technique to conserve genetic resources in avian species is beneficial not only for poultry industry (especially for the primary breeders) but also for preserving endangered avian varieties. However, due to the inability of cryopreserving avian embryos, primarily because of its large size and yolk laden structure, only available means to conserve genetic resources in avian species has been through freezing spermatozoa. Since it is not possible to conserve genetic resources at complete form only by storing spermatozoa, it is apparently important to develop an alternative means in avian species, which is equivalent to embryo transfer in mammalian species. We, therefore, conducted a series of experiments assuming that production of the germline chimeras by collecting, conserving and transferring primordial germ cells (PGCs) would fulfill our goal.

Research Methods

Experiment 1 Change in the PGC concentration during early embryonic development

Fertilized eggs were incubated at 38 oC for 48 to 69 hours to enable the embryos to reach stages 13 through 18 (Hamburger and Hamilton, 51) of development. Eggs were broken into plastic dish and approximately 1μl of blood was collected from the dorsal aorta of the embryos by using a fine glass pipette. A fine glass pipette was prepared by pulling a 50 1μl glass pipette (Drummond Sci. CN, USA) with a micro-pipette puller (Narishige PA-81-8811, Japan). Collected blood was mixed with 200 1μl of phosphate buffed saline without Mg and Ca (PBS (-)) which contain 3.7 % formaldehyde. Diluted blood sample was placed on the Heavy Teflon Coating Slide (HTCS, Bokusui Brown, USA). An HTCS is originally designed to culture cells on a glass slide. Samples were coated with 0.05 % celloidin and stained with Periodic Acid Schiff (PAS). Ten to 12 embryos per stage were used in the Experiment 1. Number of cPGCs as well as blood cells (BCs) in 1/1μl of blood were counted under inverted microscope (IMT-2, Olympus, Japan). Finally, concentration of PGCs/1μl and BCs/1μl was obtained by calculation.

Experiment 2 Effect of storage on the PGC concentration

Fertilized eggs were stored for 1, 2, or 3 week in a refrigerator maintained at 15 oC. After storage, eggs were incubated at 38 oC until reached stages 14 through 16 of development and embryonic blood was collected. Collected blood was processed and observed as described in Experiment 1.

Experiment 3 Effects of female on the concentration of PGCs

Four roosters and nine hens were used in Experiment 3. Semen was collected individually from roosters and each female was inseminated

once a week with semen produced by fixed males. Eggs were incubated and blood samples were collected from embryos that had reached stage s 14, 15 or 16 of development. Collected blood was processed and obse rved as described in Experiment 1. Between 10 to 12 embryos per stag e per female were used in the Experiment 3.

Experiment 4 Change on the concentration of circulating PGCs within a n embryo

Fertilized WR eggs were incubated at 38 oC for approximately 50 ho urs to enable the embryos to reach stage 14 of the development. An i ncubated egg was broken into the plastic dish and approximately 0.5 μl of blood was collected from the dorsal aorta of an embryo by usi ng a fine glass pipette. A second and third blood collection were pe rformed from the same embryo after allowing the embryo to develop co ntinuously in an incubator maintained at 38 oC for four hours and ei ght hours, respectively. Finest care was taken to avoid blood leak u pon each blood collection. Collected blood was mixed with 100 μl of PBS(-) which contain 3.7 % formaldehyde. Number of PGCs/μl blood was obtained as described in Experiment 1. Forty-five embryos were u sed in Experiment 4.

Experiment 5 Total number of circulating PGCs in an embryo

Fertilized WR eggs were incubated at 38 oC for approximately 60-64 hours to enable the embryos to reach stage 18 of the development. A pproximately 45 μl of blood was collected from the dorsal aorta of a n embryo by using a fine glass pipette. Collected blood was placed i nto a centrifuge tube and centrifuged at 400 g for 5 minutes. After centrifuge, supernatant was removed and blood cells were fluorescent labeled with PKH2 (Zynaxis, Cell Science). After staining, blood sa mple was washed 3 times with PBS(-) followed by diluting blood sampl e with 15 μl of PBS(-). In order to prevent evaporation, fluorescent labeled blood cell sample (fBCs) was covered with mineral oil until used. A concentration of the fBCs was measured using an hemocytomet er.

Fertilized WR eggs were incubated at 38 oC for approximately 48-55 hours to enable the embryos to reach either stage 14 or 15 of the d evelopment. An incubated egg was broken into the plastic dish and 0.7 μl of the fBCs was injected into dorsal aorta of the embryo. A fin est care was taken to avoid blood leaking upon injection of fBCs int o dorsal aorta. Injected embryos were placed into an incubator maint ained at 38 oC for 30 minutes. After incubation, 1.5 μl of the blood sample was collected from dorsal aorta and placed into a centrifuge tube containing 200 μl PBS (-). Diluted blood sample was further di vided into 6 equal fractions of 7 μl and each fraction was placed in the hole of the HTCS and allowed to dry at room temperature. Dried samples were observed under fluorescent microscope (excitation 490nm, emission 504 nm) and the number of cells emitting fluorescent was c ounted. From the dilution rate of the fBCs, blood volume of the embr yos was calculated. Total number of cPGCs was obtained by calculatio n.

Experiment 6 Filtration: a new method of collecting circulating PGCs

Eggs were incubated at 38 oC until reached stages 14 through 16 o f development and embryonic blood was collected and filtered immedia

telty. After filtration, the filter was placed into a 1.7 ml capacity centrifuge tube containing 50 μl of MEM-F. The centrifuge tube containing a filter was mixed gently. After mixing, the filter mesh was placed to the area close to the cap of the centrifuge tube, so that the mesh does not touch the fluid surface, and centrifuged.

Experiment 7 Production of germ-line chimeras using PGCs recovered by using filtration method.

In preparation

Experiment 8 Production of germ-line chimeras by transferring cryopreserved gonadal PGCs.

Gonadal PGCs were collected from germinal ridge of 5-day old chick embryos. Collected gPGCs were frozen/thawed using conventional method. After thawing, gPGCs were injected into recipient embryos at stage 15-16. Hatched chicks were raised until sexually mature and progeny test was performed.

Results

Experiment 1 . Change in the PGC concentration during early embryonic development

Number of cPGCs per 1 μl of circulating blood for each developmental stage is shown in Fig. 1. The number of cPGCs per 1 μl of embryonic blood were 58.83.8, 66.923.2, 54.111.4, 28.15.6, 9.94.1, and 11.95.5 for stages 13, 14, 15, 16, 17 and 18, respectively. The average number of cPGCs at stages 17 and 18 were significantly smaller ($P < 0.05$) than the stages 13 through 15 (t-test).

Experiment 2 Effect of storage on the PGC concentration.

Concentration of circulating PGCs in early chick embryos stored for 0, 1, and 2 weeks is shown in Fig. 2. It was revealed that concentration of circulating PGCs decreased significantly in eggs stored for more than one week.

Experiment 3 Effects of female on the concentration of PGCs

The concentration of cPGCs per 1 μl in circulating blood for each developmental stage after fixed male-female insemination is shown in Fig. 3. The concentration of cPGCs (means.e.) in female no. 1, 3, 4, 5, 6, 7, 9, 11, and 12 at stage 14 were 37.425.55, 29.436.97, 54.418.00, 44.4210.11, 90.60 14.96, 28.044.89, 43.584.64, 55.5810.29, and 47.6311.15, at stage 15 were 23.764.06, 21.754.93, 45.426.20, 28.634.40, 96.1110.40, 17.532.63, 45.086.71, 47.824.82, 40.76 3.85, and at stage 16 were 22.155.30, 15.253.19, 32.484.24, 27.034.67, 87.8710.63, 15.782.55, 33.984.16, 38.523.90, and 37.595.72, respectively

Experiment 4 Change on the concentration of circulating PGCs within an embryo

The average concentration of cPGCs in the blood collected 3 times at 4 hours interval from a single embryo was shown in the Fig. 4. Average concentration of cPGCs at 0, 4, and 8 hours after initial blood collection at stage 14 was 54.77.2, 38.84.5, and 28.03.0, respectively. Average concentration of cPGCs were highest at initial blood collection and decreased significantly thereafter ($P<0.05$), similar t

o the pattern observed in Experiment 1.
However, several different patterns were observed in the dynamic c
hange of the cPGC concentration among individual embryo. Among forty
-five embryos observed in the Experiment 2, the result of 3 represen
tative embryos, embryo no. 1, no. 27, and no. 32 was shown in the Fig.
3.

Experiment 5 Total number of circulating PGCs in an embryo
Blood volume is shown in Table 1. Blood volume were estimated to b
e 25.6 ｱ 2.8 and 30.5 ｱ 4.1 μl for stages 14 and 15 embryo, respect
ively. No significant differences were observed between blood volume
at stage 14 and stage 15 (t-test).
Based on the results obtained in all the 3 experiments, average to
tal number of cPGCs at stage 14 and 15 in Experiment 1 are estimated
to be 807.4 ± 137 and 857.0 ± 239, respectively.

Experiment 6 Filtration: a new method of collecting circulating PGCs
Result on the recovery of circulating PGCs is shown in Table 2.
It was shown that filtration method could be an efficient way of c
ollecting circulating PGCs.

Experiment 7 Production of germ-line chimeras using PGCs recov ered by using filtration method.
In preparation

Experiment 8 Production of germ-line chimeras by transferring cryopreserved gonadal PGCs.
The results of the progeny testing are shown in Table 3. It was re
vealed that cryopreserved gonadal PGCs could produce the germ-line c
himeras.

Discussion

In the present study, the histological features of chick germ-line c
ells were analyzed experimentally. The number of chick PGCs per 1 μl
of embryonic blood reached a peak at stage 13 through 15. According
to the result of experiment 5, the total number of PGCs did not increa
se so rapidly comparing to that of the blood cells. These results sug
gest that stage 13 to 14 might be a suitable stage to collect the circ
ulating PGCs. Furthermore, it might be suggested that the number of t
otal PGCs have a tendency to belong to an individual female not to mal
es.
In the other hand, the effect of storage on concentration did not ob
served clearly. In this experiment, further study might be required i
n future.
When the gonadal PGCs (gPGCs) transplanted into the bloodstream of a
nother line embryo, we can get offspring originated from transplanted
gPGCs. This result suggests the possibility that we may have a new ve
hicle to make a germ-line chimera. In the case of gPGCs, we can colle
ct lot of them compared to the case of the PGCs. Moreover, we can als
o get an offspring originated from injected g PGCs from germ-line chim
era, which was produced by transplantation of cryopreserved gPGCs.
Finally, we developed a new method to isolate the circulating PGCs f
rom embryonic blood using a micro-pore filter. Though the efficiency

of PGCs isolation was not now so high comparing to the ficoll method, the rate of purification was higher than that of other methods.