F-3.2.2 Development of an exprimental system for the evaluation of manipulated primordial germ cells for the ability to develop into functional gametes.

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Abstract Transplantation of primordial germ cells (PGCs) of endangered avian species into embryos of other birds is expected to be a feasible manner to preserve and multiply the endangered wild life. The final goal of this study was to develop an experimental system to test the quail PGCs which had been manipulated in vitro for the ability to differentiate into fuctional gametes and thus to give rise to progeny. Toward this goal, the conditions for efficient cryopreservation of quail PGCs were established. and a method for culture of the quail embryos into which exogeneous PGCs were injected was developed. By using these techniques, PGCs obtained from the gonadal anlage of wild-type and F1 (AMRP x SBPN) embryos were stored in liquid nitrogen for up to 5 months, and transferred to quail embryos of the other strain. Upon mating of the chimeras with F1 quail, the progenies derived from injected PGCs were obtained, indicating the cryopreserved PGCs retained the ability to differentiate into functional gametes. This result also indicates that the methods developed in this study provide a feasible experimental system for the evaluation of the abitily of quail PGCs to become functional gametes.

Key Words avian, primordial germ cells, endangered wildlife, cryopreservation, progeny test

1. Introduction

In chickens, the production of germline chimeras upon translpantation of primordial germ cells (PGCs) taken from the blood of embryos, and of viable progenies derived from the transplanted PGCs has been reported[1, 2]. In addition, the cryopreservation of PGCs and subsequent production of germline chimeras by transfer of the cryopreserved PGCs have been performed[3, 4]. Therefore, transplantation of PGCs of endangered avian species into other birds is expected to a feasible manner to preserve and multiply the wild life. However, as shown with chickens, the number of PGCs in the blood of an avian embryo is small [5]. Thus, if avian PGCs could proliferate in vitro, the preservation of endangered birds by the transplantation of PGCs would be greatly facilitated. In a study done by us entittled 'Study on the mechanism of growth and differentiation of primordial germ cells' (F3.3.1), we have

examined the in vitro growth of quail and chicken PGCs. However, there is a possibility that the PGCs which have grown in vitro could lose the ability to differentiate into functional gametes. Therefore, an experimental system for the evaluation of the PGCs which are manipulated in vitro under various conditions for the ability to develop into functional gametes had to be constructed. Such a system would consist of methods for purification of PGCs, for identification of PGCs, for injection of PGCs into embryos and subsequent culture of the embryos until hatching, and for cryopreservation of PGCs. As mentioned above, these techniques have been developed in chickens[1,2,3,4,5]. However, the sexual maturation period of chickens is long (5 months), making it inappropriate to use them for the evaluation of PGCs manipulated under various in vitro conditions for the ability to give rise to functional gametes. Quail is a more appropritate model species because sexual maturation period of quail (1.5 months) is much shorter than that of chickens. In this study, methods for cryopreservation of quail PGCs and for transplantation of PGCs into quail embryos have been developed. By using these techniques, the ability of the quail PGCs which had been cryopreserved in liquid nitrogen to differentiate into functional gametes was examined.

2. Research objectives

(1) To establish the conditions under which quail PGCs were efficiently cryopreserved.
(2) To develop methods for injection of PGCs into quail embryos and for subsequent culture of the embryos until hatching.
(3) To examine the ability of the quail PGCs which had been stored in liquid nitrogen to differentiate into functional gametes, and thus to give rise to their progenies.

3. Research methods

(1) Strains of quail used in this study

In this study, wild-type (WT) Japanese quail and F1(AMRP[6] xSBPN[6]) quail, referred to as 'panda' quail below, were used. The panda quail have white feathers with a little of black ones, and this feather color is controlled by a recessive gene s[7]. Fertilized eggs were incubated at 38° C and used in the experiments.

(2) Preparation of quail PGCs

For the preparation of PGCs from the blood of quail embryos at 2.5 days of incubation, the blood of the embryos was collected with a fine glass pipette. Then, PGCs in the blood were purified by Ficoll density gradient centrifugation as described by us in 'Study on the growth and differentiation of primordial germ cells' (F-3.2.1).

The preparation of PGCs from the gonadal anlage of quail embryos at 5 days of incubation was done according to Chang et al.[8]. The gonadal anlages of embryos were cut out, and then digested with 0.05% trypsin/0.53 mM EDTA. The resulting cell suspensions were cultured in a 3.5 cm plastic culture dish with 5 ml of M119 medium containing 20% fetal calf serum (FCS) for 12 - 16 h. Then, PGCs, which were landing on the layer of adherent cells, were collected by gentle pipetting. The number of PGCs were counted under a phase-contrast microscope.

(3) Cryopreservation of quai PGCs

To seek for the conditions under which quail PGCs were efficiently stored in

liquid nitrogen, the purified PGCs from the blood of embryos at 2.5 days of incubation (200 - 1400 per one experiment) were suspended in 40μl of Medium A [DME medium/ 10% FCS/ 10% DMSO/ 25 mM Hepes (pH7.4)] or Medium B [M199 medium(Hanks)/ 20% FCS/ 10% DMSO].

The suspensions were stored in liquid nitrogen for up to 4 months, according to the method described by Naito et al[3]. After storage, the cells were thawed and the viability of the frozen-thawed cells was examined under a phase-contrast microscope by use of 0.25% trypan blue dye.

(4) Window culture of quail embryos

Fertilized eggs of WT quail were incubated at 38° C until the developmental stages of the embryos reach to stages 17 - 19[9]. At these stages, a window with a diameter of 5 - 8 mm was made on the sharp or blunt end of each of the eggs so that the embryos in the eggs were able to be seen from outside. Then the windows were sealed with Parafilm, and the eggs were incubated until hatching.

(5) Transplantation of cryopreserved PGCs into quai embryos

Fertilized eggs of WT and panda quail with windows at their blunt ends were prepared as described in Research methods (4). The PGCs prepared from the gonadal anlages of WT and panda quail were stored in liquid nitrogen as described in Reserch methods (2) for up to 5 months. After thawing, the cells were suspended in M199 containing 20% FCS at concentrations of approximately 8 x 10^4/ml. Two μl of the suspension were injected into the dorsal aorta of the embryo with a fine glass pipette though the window, so that 150 PGCs were injected to each embryo. WT quail injected with PGCs derived from panda quail, and panda quail injected with PGCs derived from WT quail are referred to as WT(P) and P(WT), respectively.

(6) Progeny test

Chimeras, WT(P) and P(WT), were mated with panda quail. Each male chimera was mated with one or two female panda quail, while each female chimera was mated with one male panda quail. The fertilized eggs obtained were incubated at 38° C until hatching, and the feather color of the progenies was examined. Progenies with panda feather color indicate that the progenies were derived from the donor PGCs (panda) when the parent was WT(P), while progenies with WT feather color indicate that the progenies were derived from the donor PGCs (WT) when the parent was P(WT).

4. Results

(1) Preparation of PGCs.

Approximately 30 PGCs per embryo were obtained from the blood of 2.5-day embryos, and the purity was 20% to 50%. Meanwhile, 500 - 800 PGCs per embryo were obtained from the gonadal anlages of 5-day embryos, and the purity was 10% to 20%. The PGCs obtained from the gonadal anlage were morphologically similar to PGCs from the blood.

(2) Cryopreservation of quail PGCs

The viability of cryopreserved PGCs upon thawing was higher than 80% when either of the media (Medium A and Medium B) was used for storage. Results of representative experiments are shown in Table 1. The frozen-thawed PGCs were morphologically indistinguishable from fresh PGCs.

Table 1 Cryopreservation of PGCs

Exp. No.	Medium[a)]	No. of PGCs frozon	Viability (%)
1	A	846	70.8
2	A	495	91.2
3	A	432	78.4
4	A	455	91.3
5	A	1420	81.9
			(82.7 on average)
6	B	198	86.7
7	B	525	83.3
8	B	600	82.8
9	B	480	87.5
10	B	460	79.4
			(85.1 on average)

[a)] Recipis of Medium A and B are shown in Research methods (3)

(3) Window culture

Hatching rate of the eggs when a window was made on the blunt end of the eggshell was higher than that when a window was made on the sharp end (34.4% versus 16.6%).

(4) Transplantation of cryopreseved PGCs into embryos

The PGCs prepared from panda quail were injected into 55 WT embryos,and 16 quail (29.1%) hatched. Meanwhile, 7 of P(WT) (8.3%) hatched out of 84 panda embryos injected with WT PGCs.

(5) Progeny test

Ten of WT(P) and two of P(WT) that reached sexual maturity were mated with panda quail, and the feather color of the progenies was examined (Fig. 2). Progenies derived from injected PGCs were obtained from one of WT(P) quail (No.23) and one of P(WT) (No.27), indicating that the cryopreserved quail PGCs retained the ability to differentiate into functional gametes. However, the proportion of progenies derived from the injected PGCs was relatively low (2.4% for No.23, and 2.5 % for No.17).

5. Discussion

(1) Cryopreservation of quail PGCs

The viability of cryopreserved quail PGCs upon thawing was higher than 80% when either of Medium A or Medium B was used for storage. This viability is comparable to that reported on cryopreserved chicken PGCs[3)] (Table 1). From this result, the conditions for the cryopreservation of quail PGCs have been established.

Table 2 Results of progeny test

Quail No.	No. of eggs	No. of progenies hatched	No. of progenies derived from injected PGCs	Progenies derived from injected PGCs (%)
WT(P)				
male				
No.8	104	81	0	0
No.11	116	103	0	0
No.19	57	40	0	0
No.20	17	8	0	0
No.22	64	40	0	0
female				
No.9	93	77	0	0
No.10	76	68	0	0
No.12	118	101	0	0
No.16	72	57	0	0
No.23	75	42	1[a)]	2.4
P(WT)				
male				
No.25	91	55	0	0
No.27	124	81	2[b)]	2.5

[a)] This progeny died within several days before hatch.
[b)] One progeny hatched, while the other died within several days before hatch.

(2) Window culture

The hatching rate of embryos when a window was made on the blunt end of eggshell was high (34.4%). And, even when the injection of cryopreserved PGCs was done, a comparable level of hatching rate was obtained (29.1%) (see Results (4)). Thus, this culture method would be used as a feasible manner for the production of quail germline chimeras.

(3) Production of gerlime chimeras by transfer of cryopreserved PGCs

In the progent test, progenies derived from the injected PGCs which had been stored in liquid nitrogen were obtained, indicating that the cryopreserved quail PGCs retained the ability to differentiate into functional gametes.

In this study, PGCs from the gonadal anlage of embryos instead of those from the blood were used as donor cells, because a relatively large number of PGCs from the gonads were prepared more readily as compared to PGCs from the blood. So far, production of germline chimeras by transfer of the chicken gonadal PGCs which had been cultured in vitro[10)] or cryopreserved[4)] has been reported. Thus the result in the

present study also indicates that quail gonadal PGCs, as well as chicken gonadal PGCs, retain the ability to give rise to functional gametes after they are transferred into other embryos.

In the present study, the proportion of progenies derived from injected PGCs was relatively low. In the production of chicken germline chimeras[2,3,4], the blood of recipient embryos was withdrawn prior to injection of PGCs so that the number of endogeneous PGCs in the embryos would be reduced. However, in the present study, the blood of the recipients was not withdrawn prior to injection. Moreover, in the production of chicken germline chimeras mentioned above[2,3,4], PGCs were transferred to embryos at stages 14-15[9]. However, in the present study, PGCs were injected into embryos at stages 17 - 19. These relatively late stages of injection would have reduced the efficiency of incorporation of the injected PGCs into the gonads. Improvements at these points would make the methods described in this study a more efficient experimental system for the evaluation of PGCs for the ability to develop in to functional gametes.

6. References

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