Inter embryonic (homo- or hetero-sexual) transfer of primordial germ cells between chicken embryos

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Abstract

Chicken primordial germ cells (PGCs) collected from the circulating blood in embryonic vessels at stage 13–15 were inter-embryonically, homo- or hetero-sexually, transferred to the blood vessels of recipient embryos at the same stage of development. Approximately 30% of the embryos treated with hetero-sexual transfer of PGCs had abnormal gonads, showing ovotestis like organs. In this case, some of these reversed gonads were considered to be dependent upon the ratio of the number of PGCs from donor to recipient embryos. One of the treated embryos possessed completely reversed organs. Therefore, the introduction of exogenous embryonic vessels was thought to be also useful for producing transgened gonads.

Abbreviations: PGCs, primordial germ cells.

Introduction

In avian species, transgenic birds have so far been reported by many investigators employing the method of introduction of foreign genes into host birds (Tajima et al., 1993) and into germinal disc of fertilized eggs (Inada et al., 1996, 1997).

On the other hand, in our previous experiments, avian primordial germ cells (PGCs) have been shown to be very useful tools for producing transgenic birds (Yamaguchi et al., 1996; Ebara et al., 1998; Ebara and Fujihara, 1999a, b, c; Eguma et al., 1998; Furuta et al., 1998, 1999a, b, c; Fujihara et al., 1998; Fujihara, 1999a, b). Similar experimental results have been reported by a lot of researchers (Yasuda et al., 1992; Tajima et al., 1993; Kuwana et al., 1996; Naito et al., 1998). Since the PGCs are the precursor of the germ cells, the cells might be the best way to use for creating transgened animals.

Avian PGCs have been characterized by the cells that originate in the epiblast and appear in the germinal

crescent region of primitive streak (stage 9–10), and then the cells circulate through the blood vascular system (stage 13–15) and finally migrate into the gonadal ridges where the PGCs differentiate into the ovary and testis (Fujimoto et al., 1976; Fujihara, 1998b, c, 1999).

In the previous reports, we demonstrated the possibility of very successful proliferation and growth of avian PGCs outside the body (Yang and Fujihara, 1998, 1999a, b). In this case, some of the growth factors revealed remarkable effect on the proliferation of PGCs cultured *in vitro* (Yang and Fujihara, 1998, 1999a, b).

These reports suggest that the PGCs may play a critical role for determining the gonadal development of chicken. In the present experiments, the growth of embryos and sexual characters of chicks were determined following the hetero-sexual transfer of the PGCs to examine the ability of PGCs to affect sexual differentiation.

On the other hand, we examined the possibility of introduction a foreign gene into PGCs by the

method of microinjection of DNA into embryonic blood vessels.

Materials and methods

Experiment 1: Transfer of PGCs into developing embryos

Preparation of donor PGCs

Freshly laid fertilized eggs were incubated at 38.5 °C for around 72 h in an incubator under a relative humidity of 60–70%, until developmental stage at 13–14 (Hamberger and Hamilton, 1951).

Eggs were cracked to collect blood samples from dorsal aorta and vitelline vein of embryos by a glass micropipette (ca. 4 µm in outer diameter) which contained bubbles of MEM supplemented with 10% calf serum (CS), to close the hole of blood vessels. Approximately 1–2 µl of the blood samples were stored at –20 °C for the determination of the sex by PCR analysis.

Preparation of recipient embryos

Fertilized eggs were incubated at the same conditions as mentioned above until the same stage of embryonic development. The sharp edge of an egg was carefully broken to make a small window (ca. 1.0 cm in diameter), and the window was closed simply with scotch tape until the transfer of donor's PGCs.

Transfer of PGCs into recipient embryos

Prior to the transfer of PGCs, a considerable amount of blood was taken out from blood vessels of the recipient embryos to make place and keep the high ratio of donor-PGCs transferred. At the same time, approximately 1–2 µl of the blood samples, both donor and recipient embryos, was stored for sexing by PCR analysis.

DNA extraction for PCR analysis

The DNA analysis was conducted for both, donor and recipient embryos, using Micro Extraction Kit (Stratagene, Canada). After this treatment, PCR analysis was carried out for the presence of repeated sequences on the W-chromosome of females. At first, under standard procedure, W-repeat reaction was performed on DNA samples. Following amplification of DNA, the products of reaction were combined and subjected to 1.0% agarose gel electrophoresis (Love et al., 1994) (Figure 1).

The sequences of primers used in this experiment were W-5, 5'CCCAAATATACACGCTTCAST3', W-3, 5'GAAATGATTATTTTCTGGCGAAC3' (Clinton, 1994).

Viability and abnormality of manipulated eggs

Manipulated embryos were cultured to check hatching rate, paying special attention to hatching rate for treated eggs between homo-sexual transfer and hetero-sexual one. All the hatched chicks were sacrificed within 3 days after hatching. The hatched chicks and embryos dead after 17th day of incubation were autopsied to examine gonadal development. The gonads having abnormal appearance were fixed with Bouin's solution for further histological examination.

Histological examination of gonads

The gonads with abnormal features of treated embryos and normal ones from non-manipulated embryos were fixed with Bouin's solution, processed for routine work for histological examination (Bancroft et al., 1990).

Experiment 2: Microinjection of foreign DNA into developing embryos

Preparation of foreign DNA

Circular form of Miw Z DNA (6.25 µg) was mixed with 22.5 µg of transfection reagent (Boehringer, Germany), and further diluted with Hepes-buffered saline to 50 µl in total. The DNA solution was aspirated by a glass micropipette together with a tiny amount of bubbles of MEM containing 10% fetal calf serum (FCS).

The preparation of recipient embryos

Fertilized eggs were incubated until the stage 13–15. On the sharp edge of an egg, an window with a diameter of about 1.0 cm was opened to transfer foreign DNA into the embryos.

Microinjection of exogenous DNA into embryos

Prior to the injection of DNA, a given amount of blood was taken out from the recipient embryos, and then exogenous DNA was introduced to the same blood vessel as mentioned above through the window. The window was closed with scotch tape and incubated for additional 6 days. Following 6 days of incubation, the eggs were cracked to check the viability.

Figure 1. PCR products with W-chromosome-specific primer. (m: male, f: female, M: marker gene).

Detection of introduced foreign DNA

After checking the viability, embryos were removed from the yolk on the sixth day and rinsed in phosphate-buffered saline (PBS), fixed with 1.0% glutaraldehyde in PBS for 30 min at 4 °C. Then, the embryos were washed with PBS, stained with 0.05% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) for 3 h at room temperature (22–25 °C) and checked the expression of introduced DNA in the whole of embryos and gonads.

On the other hand, some tissues from each embryo were examined for DNA extraction and PCR analysis. The primers used in this experiment were Lac Z: 5'GCGTTACCCATTAATCG3, 5'TCTGATAACAACC3' and GAPDH: 5'CAGATCAGTTTCTATCAGC3' and 5'TGTAGACTTCAATGGTGACA3' (Stone et al., 1985).

Results

Experiment 1: Transfer of PGCs into developing embryos

The development of treated embryos was not significantly ($P > 0.05$) different between homo- and hetero-sexual transfers of PGCs (Table 1). However, approximately 30% of the embryos following hetero-sexual transfer of PGCs showed abnormal gonads when ob-

served with the naked eye (Table 2). These abnormal gonads were considered to be ovotestis like organs (Figures 2a and b), indicating that some of the embryos with hetero-sexual transfer of PGCs showed no gonads *in situ*. Furthermore, these gonads also seemed to be from the donor PGCs, when examined by histological observations (Figures 3a–d). One of the treated embryos had completely reversed gonads from hetero-sexual transfer of PGCs (Figure 4).

Experiment 2: Microinjection of exogenous DNA into embryos

The survival rate of DNA-microinjected embryos was around 75% on day 6 of the incubation. Almost no significant ($P > 0.05$) difference was found in embryonic viability between treated embryos and the control ones. The expression of Miw Z DNA by a histochemical staining method of X-gal was around 67% (Table 3). In this case, expression sites for injected DNA were in the head, heart and gonads, but some of the gonads clearly showed existence of introduced DNA when examined by PCR analysis.

Discussion

Recent experiments have demonstrated that the PGCs originate in the central part of the area pellucida and

Figure 2. Embryos with normal and abnormal gonads. (A) Embryo with hetero-sexual transfer, male for donor embryo and female for recipient ones. (B) Normal gonad of a male embryo.

Table 1. Survival and hatching rates of chicken embryos after inter-embryonic transfer of PGCs

Transfer	No. of embryos manipulated	No. of survived embryos	Hatching rate (%)
Homo-sexual transfer	76	43	57
Hetero-sexual transfer	47	27	61
Total ^a	144	70	49

^a Embryos which did not grow for 2 days after PGCs transfer were excluded.

Table 2. Gonadal development of chicken embryos following hetero-sexual transfer of PGCs

Sex of		No. of embryos manipulated	No. of gonads investigated ^a	No. of gonads showing abnormal appearance
Donor	Recipient			
Male	Female	24	16	4 ^b
Female	Male	23	18	8
Total		47	34	11

^a Some of the embryos having hetero-sexual transfer of PGCs had the gonads with ovotestis-like organs.

^b One of two abnormal gonads was not observed with the naked eyes.

that the developmental fate to PGCs has been destined at stage X blastoderm in chickens (Kagami et al., 1997).

In the previous reports, female PGCs could differentiate into spermatozoa in the male gonads of the chicken (Tagami et al., 1997). In this case, the ratio of produced W chromosome-bearing spermatozoa was substantially below expectations, suggesting that most of the W-bearing PGCs could not differentiate into spermatozoa due to the restricted spermatogenesis (Tagami and Kagami, 1998).

Most recent reports suggested that W-specific sequence in mature testis germline cells were detected except spermatozoa by *in situ* hybridization (Tagami et al., 1998). Therefore, it is possible that the differentiation of almost all germ cells derived from female PGCs were restricted at the spermiogenesis in the testis (Tagami et al., 1998).

The present experiments demonstrated that some of the embryos having hetero-sexually transferred PGCs possessed abnormal gonads in appearance compared with the embryos with homo-sexual transfer of PGCs and normally developing embryos.

So far the PGCs have been considered not to play any role for determining the sex of embryos and

the gonadal development would be controlled by the sex of the body, not by the PGCs (Nieuwkoop and Sutasurya, 1979).

In the present experiments, exogenous DNA, introduced into the blood vessels of developing embryos, was detected in the embryos when examined by PCR analysis. Some of the embryonic gonads showed clearly positive expression of introduced DNA through the examination by PCR analysis. This result suggested the possibility of the introduction of foreign DNA into PGCs by the method of microinjection, resulting in the detection of the gene in the developing gonads of embryos.

The germline chimeras have been reported by transferring blood-born PGCs from White Leghorn embryos into Barred Plymouth Rock recipients (Petitte et al., 1991). It also has been demonstrated that the PGCs, taken out from the chicken, were transferred to the quail embryos at the same stage of development, resulting in the incorporation of chicken PGCs into the quail germinal epithelium (Yasuda et al., 1992).

These results clearly suggest that PGCs transferred between different species or between different genera may be able to proliferate, differentiate into gonads which may have some function of the germ cells. The

Figure 3. Histological features of gonads obtained from hetero-sexual transfer of PGCS. (a) Gonad having complex organ from male of donor PGCs and from female of the recipient ones. (b) Normal gonad of female embryo. (c) Gonad of mixed PGCs, showing donor PGCs from male. (d) Normal gonad of male embryo.

Table 3. The expression of introduced Miw Z DNA in the chicken embryos^a

Trial	No. of embryos manipulated	Survival rate (%)	Rate (%) of expression	
			Embryos	Gonads
Trial 1	5	80	60	— ^b
Trial 2	11	73	38	50
Total	16	75 (average)	67 (8/2)	

^a The expression of Miw Z DNA was detected by histochemical staining by X-gal.

^b The expression of gonads was not exactly determined due to insufficient staining.

Figure 4. Gonads showing completely reversed gonads, from donor to recipient embryos. (a) Donor PGCs were female. (b) Donor PGCs were male.

present findings together with the previous ones suggest the possibility of altering gonadal development of recipient embryos via transferring PGCs from donor embryos into recipient embryos. Another finding also suggests that PGCs containing foreign DNA may be a useful tool for introducing exogenous genes into the gonads.

Judging from the present experiments, it may be possible that the birds having hetero-sexual transfer might grow to reach sexual maturity in which a large amount of donor PGCs are transferred into recipient embryos even in hetero-sexual transfer.

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