Possible Abnormalities of Chimeric Chicken Caused by the Introduction of Exogenous Genes Into Chicken Embryos via Primordial Germ Cells (PGCs)

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ABSTRACT : In chicken, exogenous genes introduced into germinal crescent region (GCR) of the early developmental stage, where primordial germ cells (PGCs) were concentrated, were successfully transferred to the gonads via PGCs. The foreign genes were also confirmed to be successfully incorporated into F1 and F2 generations. We tried to incorporate the exogenous genes into PGCs by lipofection, then the DNA mixture was injected into GCR at stage 3-5 or 9-11 of embryonic development (Hamburger and Hamilton, 1951). The manipulated eggs were incubated, and hatched chicks were reared until sexual maturation. F1 generation was obtained from the DNA-treated chicken (DNA-chicken) mated with normal birds. Furthermore, F2 generation was also obtained from the F1 chicken mated with normal birds. The transfer of introduced foreign genes were confirmed by marker gene detection methods and PCR analysis in the hatched chicks, F1 and F2 generations. However, in our experiments, DNA-chickens showed abnormal characteristics such as low egg production rate, abnormal appearance and decreased number of spermatozoa. In the case of F1 chicken, low egg production and the deterioration of sperm capacity for insemination in male chicken were observed. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 11 : 1514-1517*)

Key Words : Exogenous Genes, Primordial Germ Cells, Germinal Crescent Region, Abnormality

INTRODUCTION

Many trials have been conducted to introduce exogenous gene into chicken embryos via primordial germ cells (PGCs) for the production for transgenic chicken (Han et al., 1994; Watanabe et al., 1994; Naito et al., 1998). In avian species, the PGCs temporarily concentrate into germinal crescent region (GCR) at the early stage of embryonic development (Hong et al., 1995; Ginsburg, 1997). In our previous studies, the exogenous gene (lacZ/MiwZ) introduced into germinal crescent region (GCR) was successfully transmitted to the gonads (Eguma et al., 1999; Ebara and Fujihara, 2000c), and the DNA was also successfully transferred to the offspring (Ebara and Fujihara, 1999). On the other hand, using another DNA containing lacZ and green fluorescent protein (GFP) as a marker gene (lacZ&GFP/pkkv4-lacZ), the DNA was also incorporated into offspring (Ebara and Fujihara, 2000a). Furthermore, the incorporation of the foreign DNA was verified in F2 generation (Ebara and Fujihara, 2000b).

In our experiments, however, the DNA-treated chicken (DNA-chicken) showed some abnormal features such as low egg production rate, low fertility and abnormal appearance. Therefore, we tried to examine whether the introduction of exogenous genes caused or not for development and reproductive functions.

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MATERIALS AND METHODS

Preparation of fertilized eggs

Fertilized eggs of Rhode Island Red were collected from 2 to 7 days after artificial insemination (AI).

Preparation of the DNA solution

In trial 1, the circular form MiwZ containing the *E. coli*-β-galactosidase (lacZ) gene under the control of β-action promoter (Suemori et al., 1990) was employed. In trial 2, the circular form pkkv4-lacZ containing the *E. coli*-β-galactosidase (lacZ) gene under the control of CMV promoter and the green fluorescent protein (GFP) gene under the control of RSV promoter (Ebara and Fujihara, 2000a) was used.

In trial 1, the MiwZ DNA (6.25 μg) was mixed with 22.5 μl of trasfection reagent (DOTAP/No1781995; Boehringer Mannheim, Germany), diluted up to 50 μl with Hepes-buffered saline (HBS; 20 mM Hepes containing 150 mM NaCl, pH 7.4) in a polystyrene tube. In trial 2, the pkkv4-lacZ DNA (5.0 μg) was mixed with 30 μl of transfection reagent (DOTAP), diluted up to 150 μl with HBS in a polystyrene tube. The transfection mixture was then incubated for 10-15 minutes at room temperature (22-25℃). A micropipet (G-1, Narishige, Tokyo, Japan), the tip of which was reformed to an outside diameter of about 40 μm, was filled with the DNA

solution prior to microinjection.

Microinjection of the DNA solution

Fertilized eggs were incubated at 38.5℃ under a relative humidity at 60-70%. A window with 10-15mm in a diameter was opened at the sharp edge of egg shell when embryos reached to the stage 9-11 (Hamburger and Hamilton, 1951) of the development. The DNA solution was injected into the GCR with the approximately 1.0 μl in total volume. After the injection, the window was closed by adhesive tape and the incubation was continued until hatching.

Preparation of fertilized eggs (F2) from F1 chicken

Hatched chicks were raised until the stage of sexual maturation. Female DNA-treated chicken was mated with normal males to gain the fertilized eggs. The eggs were incubated at 37.8℃ under a relative humidity of 60-70% until hatching. Hatched F1 chicks were raised until the stage of sexual maturation. Female F1 chickens were mated with normal males, and male F1 chickens were mated with normal females to obtain the fertilized eggs.

The results described hereinafter have been obtained from the experiments so far in our laboratory, then the readers should refer to the sections for materials and methods in the previous reports (Eguma et al., 1999; Ebara and Fujihara, 1999, 2000a, b, c).

RESULTS

In all DNA-chickens used for this study, No. 15, 17, 18, 57, 60, 394, 395 and 398 were obtained from trial 1, No. 54, 69, 89, 95, 397, 399 and 400 were obtained from trial 2. In all F1 chickens used for this study, No. 90, 97 and 98 were obtained from trial 1, No. 36, 51, 83, 84 and 85 were obtained from trial 2.

Egg production rate of DNA-chickens and F1 generation

Of DNA-chickens, bird No. 17 showed very low egg production rate, and No. 395, 399 and 18 also showed low rates rather than other chicken (table 1). On the other hand, of F1 generations, No. 97 and 83 also had low production rate (table 2).

Fertility of eggs obtained from DNA-chickens and F1 generation

Chicken No. 18 indicated lower fertility than the others. There was very little relationship between low egg production rate and fertility in this experiment. Normal fertility was obtained from the chickens with low egg production rates (table 3). On the other hand, in F1 generation, fertility was high, though there was no relationship between egg production rate and fertility (table 4).

Table 1. Egg production rate of DNA-chicken

DNA-treated chicken No.	Rate of egg production (%)
395 (♀)*	100/160 (62.5)
398 (♀)*	142/160 (88.8)
17 (♀)*	76/160 (47.5)
18 (♀)*	111/160 (69.4)
397 (♀)#	145/160 (90.6)
399 (♀)#	94/160 (58.8)
60 (♀)#	142/160 (88.8)
69 (♀)#	142/160 (88.8)

* lacZ/MiwZ introduced chicken in trial 1.

lacZ&GFP/pkkv4-lacZ introduced chicken in trial 2.

Table 2. Egg production rate of next generation (F1)

F1 chicken No.	Rate of egg production (%)
97 (♀)*	56/92 (60.9)
98 (♀)*	131/143 (91.6)
51 (♀)#	150/160 (93.8)
83 (♀)#	49/75 (65.3)
85 (♀)#	114/160 (71.3)

* lacZ/MiwZ introduced chicken in trial 1.

lacZ&GFP/pkkv4-lacZ introduced chicken in trial 2.

F1 chicken No. 97 and 98 were obtained from DNA-chicken No. 17. F1 chicken No. 51 and 83 were obtained from DNA-chicken No. 397. F1 chicken No. 85 was obtained from DNA chicken No. 399.

Table 3. Fertility of the eggs obtained from DNA-chicken

DNA-treated chicken No.	Fertility (%)
395 (♀)*	34/36 (94.4)
398 (♀)*	44/47 (93.6)
17 (♀)#	42/49 (85.7)
18 (♀)#	30/48 (62.5)
397 (♀)#	36/49 (73.5)
399 (♀)#	18/22 (81.8)
60 (♀)#	29/31 (93.5)
69 (♀)#	31/31 (100)

* lacZ/MiwZ introduced chicken in trial 1.

lacZ&GFP/pkkv4-lacZ introduced chicken in trial 2.

Number of spermatozoa from male DNA-chicken and F1 generation

In chicken No. 394, the number of spermatozoa was approximately five times as much as those obtained from normal males. The number of spermatozoa from chicken No. 54 and 57 was about one third of normal birds (table 5). In either case, however, no effects were observed upon artificial insemination (AI) with these spermatozoa. On the other hand, in F1 generation, the number of

Table 4. Fertility of the eggs obtained from generation (F1)

F1 chicken No.	Fertility (%)
97 (♀)*	13/15 (86.7)
98 (♀)*	15/17 (88.2)
51 (♀)#	16/20 (80.0)
83 (♀)#	-
85 (♀)#	15/15 (100)

* lacZ/MiwZ introduced chicken in trial 1.
# lacZ&GFP/pkkv4-lacZ introduced chicken in trial 2.
F1 chicken No. 97 and 98 were obtained from DNA-chicken No. 17. F1 chicken No. 51 and 83 were obtained from DNA-chicken No. 397. F1 chicken No. 85 was obtained from DNA chicken No. 399.

spermatozoa indicated normal value (table 6).

Fertilizing ability of spermatozoa obtained from F1 chicken

F1 male chicken produced semen containing normal number of spermatozoa. Semen obtained from F1 males were diluted to be 1.0×10^8/ml and subjected to AI with normal females. Fertilized eggs were collected from 2 to 7 day after AI. As a results, the eggs obtained from chicken No. 36 showed very low fertility, and other two birds (No. 90 and 84) also had low fertility (table 7).

DISCUSSION

In general hatchability of manipulated eggs was very low in this experiment. When exogenous gene (*lacZ/MiwZ*) was introduced, 18 chicks were hatched (18/248 : 7.26%). Of these hatched chicks, 6 chicks raised to the sexual maturation. On the other hand, with another DNA (*lacZ*&GFP/pkkv4-lacZ), 17 chicks were hatched (17/41 : 41.5%) and 9 chicks were reared until sexual maturation (data not shown). It has been reported that the hatchability of the DNA injected eggs was not significantly ($p>0.05$) different

Table 5. Number of spermatozoa in semen obtained from DNA-chicken

DNA-treated chicken No.	Number of spermatozoa (ml)
15 (♂)*	4.2×10^9
394 (♂)*	11×10^9
54 (♂)#	0.8×10^9
57 (♂)#	0.8×10^9
89 (♂)#	1.1×10^9
95 (♂)#	1.4×10^9
400 (♂)#	1.1×10^9
Control (♂)	1.6×10^9

* lacZ/MiwZ introduced chicken in trial 1.
# lacZ&GFP/pkkv4-lacZ introduced chicken in trial 2.

Table 6. Number of spermatozoa in semen obtained from F1-chicken

F1 chicken No.	Number of spermatozoa (ml)
90 (♂)*	3.4×10^9
36 (♂)#	1.9×10^9
84 (♂)#	2.6×10^9
Control (♂)	2.4×10^9

* lacZ/MiwZ introduced chicken in trial 1.
# lacZ&GFP/pkkv4-lacZ introduced chicken in trial 2.
F1 chicken No. 90 was obtained from DNA-chicken No. 18. F1 chicken No. 36 was obtained from DNA-chicken No. 399. F1 chicken No. 84 was obtained from DNA-chicken No. 69.

from that of the uninjected windowed eggs but was significantly lower than that observed for the nonwindowed eggs (Petitte et al., 1990). Therefore, in this method, windowed eggs might bring about low hatchability. For another reason, the big difference in hatchability might be caused by the technique of manipulation.

On the other hand, chicken No. 399 had abnormal appearance with sight loss of the left eye. The other chickens possessed the weak legs, being no standing. Bird No. 394, 83 and 395 died earlier than others after sexual maturation, such that No. 394 died nineteen months after hatching. For these birds, we carried out abdominal operation to observe internal organs. Most of the organs seemed normal in appearance, except that the right and left testes atrophied. Chicken No. 83 died nine months after hatching, leaving the ovary atrophied. Chicken No. 395 died eleven months after hatching, showing no abnormality in the organs. These abnormal gonads might relate to the injection of exogenous genes into the embryos.

As a conclusion, therefore, the introduction of foreign genes into chicken embryos might bring about

Table 7. Fertilizing capacity of spermatozoa obtained from F1 chicken

F1 chicken No.	Fertility (%)
90 (♂)*	4/7 (57.1)
36 (♂)#	1/7 (14.3)
84 (♂)#	4/7 (57.1)

* lacZ/MiwZ introduced chicken in trial 1.
# lacZ&GFP/pkkv4-lacZ introduced chicken in trial 2.
F1 chicken No. 90 was obtained from DNA-chicken No. 18. F1 chicken No. 36 was obtained from DNA-chicken No. 399. F1 chicken No. 84 was obtained from DNA-chicken No. 69.
Semen obtained from F1 chickens were diluted to 1.0×10^8/ml and artificial insemination (AI) was conducted to normal female chicken. Fertilized eggs were collected from 2 to 7 day after AI.

these abnormal characteristics, though the occurrence of deformed features was different from the sites of the birds, suggested the possibility of random incorporation of exogenous genes into the cellular components. Thus, these abnormal characteristics from transgened birds occurred in many site of the body of chicken after hatching.

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