In Vivo Transfer of Foreign DNA into Primordial Germ Cells (PGCs) of Chicken Embryos

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ABSTRACT : The present experiments were designed to examine whether exogenous DNA injected into the germinal crescent region (GCR) of early stage of developing embryos, which is considered to be the main place from which PGCs originate, can be transferred to recipient chicken embryos. In this experiment, Miw Z (DNA) dissolved in the transfection reagent (TR: Boehringer, Germany) was introduced into the GCR of donor embryos at stage 3-5 or 9-11, followed by continued incubation until the stage 13-15 of embryonic development. The PGCs collected from the embryonic blood vessels were examined for the incorporation of the injected DNA into the PGCs by the methods of X-gal staining and PCR analysis. As the results, the foreign DNA was successfully incorporated into the PGCs, leading to their transfer to the gonadal tissues. The present results, therefore, suggest that the early stage (3-5 or 9-11) of chicken embryonic development would be more successful than stage 13-15 in transferring exogenous genes to the recipient embryos, leading to the possibility of producing transgenic chicken medianting the PGCs. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 4 : 520-524*)

Key Words : Gene Transfer, PGCs, Chicken, Transgenic

INTRODUCTION

Primordial germ cells (PGCs), which are the progenitor of ova and spermatozoa, have been studied as useful tool for producing transgenic chicken by many researchers. At stage X (Eyal-Giladi et al., 1981), PGCs have been located in the epiblast (Eyal-Giladi et al., 1981; Karagenc et al., 1996; Kagami et al., 1997). Although their origin prior to stage X is obscure, it has been considered that they move to the hypoblast and appear in the germinal crescent reagent as PAS positive cells. They finally migrate into the germinal ridge (Kuwana, 1993) and differentiate to ova or spermatozoa, following circulation in the developing blood vascular system (Fujimoto et al., 1976).

Isolated PGCs have been transferred to recipient embryo to obtain germ-line chimeras which produce donor-derived offsprings (Wentworth et al., 1989; Petitte et al., 1991; Yasuda et al., 1992; Carsience et al., 1993; Vick et al., 1993; Tajima et al., 1993; Naito et al., 1994b; Kagami et al., 1995; Ono et al., 1996; Simkiss et al., 1996; Kagami et al., 1997; Tagami et al., 1997).

In this way, if the foreign DNA was introduced to donor PGCs, donor-derived offsprings will be transgenic chicken (Vick et al., 1993). Foreign DNA has also been directly injected into the blastodisc in order to produce transgenic chickens (Sang and Perry, 1989; Naito et al., 1991; Naito et al., 1994a; Inada et al., 1997). The expression of injected DNA was observed in PGCs collected from blood of the developing embryo (Inada et al., 1997) or in germ cells (Naito et al., 1994b).

In this study, therefore, foreign DNA was examined for injecting into the germinal crescent region of the embryos from which PGCs originate and migrate to the germinal anlage through embryonic blood vessels to develop to the gonads. This study aimed to develop more simple and easier techniques for introducing exogenous genes than the methods reported previously.

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MATERIALS AND METHODS

Birds and artificial insemination (AI)

Roosters and hens from Rhode Island or White Leghorn were maintained in individual cages, exposing to a 14-h photoperiod (lights on from 5:00 to 19:00 h). Feed and water were available *ad libitum*. Semen was collected by the method of lumbar massage (Wambeke and Fujihara, 1992). Collected semen was diluted twofold with PBS and subjected to artificial insemination (AI) into the hens corresponding strain.

Eggs were collected for 2 to 7 days after AI and incubated at 37.5℃ under relative humidity of 60-70%.

Preparation of DNA solution

The circular form Miw Z containing the *E. coli*-β -galactosidase gene (*lac Z*) under the control of RSV enhancer (Inada et al., 1997) was employed in the present experiments. In our preliminary works, the circulara form of Miw Z was much more successful compared with the linear one (Inada et al., 1997). The Miw Z DNA (6.25 μg) was mixed with 22.5 μl of transfection reagent (TR:Boehringer, Germany), and then diluted up to 50 μl with Hepes-buffered saline (HBS; 20 mM Hepes containing 150 mM NaCl, pH 7.4). A micropipette (G-1, Narishige, Tokyo, Japan), whose tip was developed down to the outside diameter of about 40 μm, was filled with the DNA solution prior to microinjection.

Microinjection of DNA into germinal crescent region

For comparing the effect of developmental stages of embryos, the eggs incubated for 20 h (stage 3-5, trial 1) or 40 h (stage 9-11, trial 2) were used as host embryos. Prior to microinjection, the window of 10 mm in diameter was opened at the sharp edge of the egg shell

(figure 1) according to Inada et al. (1997). The DNA solution (1.0 μl) was injected into both the anterior and posterior junction of the area pellucida and the area opaca in the primitive streak of the host embryos incubated for 20 h (trial 1). These two kinds of area (figure 2) have been considered to be germinal crescent region (GCR: Nieuwkoop and Sutasurya, 1979). To another embryos incubated for 40 h, 1.5 μl of DNA solution was injected into germinal crescent region (trial 2). The manipulated eggs were incubated for additional periods after the window was closed with clear tape. The embryos incubated for 3 days (52 h for stage 13-15, being 32 h after the injection for trials 1 and 2, respectively) and incubated for 6 days (stage 26-28; 5 and 4 days after injection for trials 1 and 2, respectively) were subjected to the detection of introduced DNA using PCR analysis.

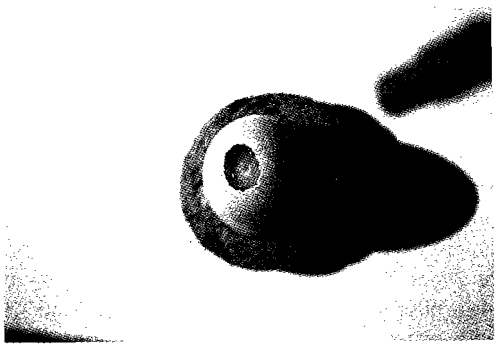

Figure 1. A recipient egg with an window on the sharp edge of the shell

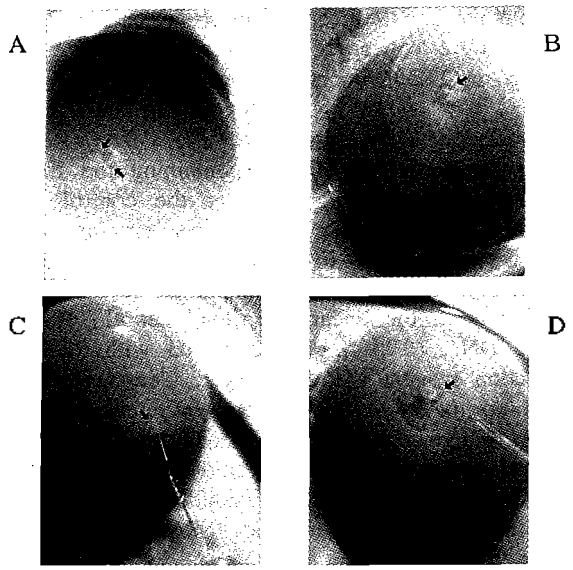

Figure 2. Germinal crescent region of chick embryos
A: stage 2-5, B: stage 9-11, C: PGCs injected at stage 3-5, D: PGCs injected at stage 9-11

Detection of Miw Z DNA in the embryos

The whole embryos and extra-embryonic tissues were removed from the yolk, immersed in PBS, and fixed with 1% glutaraldehyde in PBS. After washing twice with PBS, the embryos were stained with 0.05% 5-bromo-4chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma, USA), 1 mM $MgCl_2$, 0.1% Triton X-100, 3mM potassium ferrocyanide and 3 mM potassium ferricyanide in PBS at 4℃ (in a refrigerator). The times for fixing and staining were 15 min and 2.5 h for the embryos at stage 13-15, and 45 min and 5 h for the embryos at stage 26-28, respectively.

PCR analysis

The presence of Miw Z DNA in the blood collected from the embryos at stage 13-15 (including blood of embryos which were incubated to 6th day stage 26-28 after collection) and some pieces (head, arms and gonads) of the embryo at stage 26-28 was analyzed by PCR analysis. The total DNA was extracted from 2 μl of blood samples and 0.2 mg of homogenized pieces of the embryo by the phenol-chloroform method (Sambrook et al., 1989) and was subjected to PCR analysis. The primers for PCR analysis used here were as follows; 5 'GCGTTACCCAACTTAAT CG3' and 5'TGTGAGCGAT AACAACC3' (Stone et al., 1985). The products of each reaction were subjected to 1% agarose gel electrophoresis (Love et al., 1994).

RESULTS

Survivability and abnormality of DNA-injected embryos

Embryonic survivabilties in trial 1 and 2 were 65 and 73% for stage 13-15, and 61 and 57% for stage 26-28, respectively (table 1). These rates were not different from control groups (69% for stage 13-15 and 56% for stage 26-28). On the one hand, embryonic abnormalities in trial 1 were 2% for stage 13-15 and 30% for stage 26-28, respectively, and there were significant difference ($p<0.01$) between these stages. The embryonic abnormalities in trial 2 and controls were 4 and 0% for stage 13-15, and 13 and 21% for stage 26-28, respectively.

Table 1. Survival and abnormal embryos injected foreign DNA

Trials[1]	Stage of embryos	No. of embryos	Survival embryos (%)	Abnormal embryos (%)
Trial 1	stage 13-15	168	109 (65%)	2 (2%)
Trial 1	stage 26-28	65	33 (51%)	10 (30%)
Trial 2	stage 13-15	33	24 (73%)	1 (4%)
Trial 2	stage 26-28	68	39 (57%)	5 (13%)
Control 1	stage 13-15	16	11 (69%)	0 (0%)
Control 2	stage 26-28	25	14 (56%)	3 (21%)

[1] DNA solution was injected at stagge 3-5 (trial 1) and stage 9=11 (trial 2). Window was opened and closed without injection at stage 3-5 (control 1) and stage 9-11 (control 2).

Comparing these two stages each other, the survival rates significantly decreased ($p<0.05$), but contrarily the abnormal rates clearly increased ($p<0.01$).

Expression of introduced Miw Z DNA

The expression of injected Miw Z DNA in the gonads (figure 4) was observed in only 3 embryos out of 37 analyzed samples in the trial 2 when injected at stage 9-11 (table 2). Most of the expression of introduced Miw Z DNA was detected in extra-embryonic tissues while the embryonic expression was detected in a few embryos (figure 3). Regarding the expression sites of the injected MiwZ DNA, there some spot-like β -galactosidase activities were recognized in the heart, head and somatic segments of the embryos, along the vitelline artery and sinus terminalis of extra-embryonic tissues (table 2). The numbers of expression spots were fewer in trial 1 than trial 2. The expression of the MiwZ DNA in the gonad was in a mosaic manner along the blood vessels.

Table 2. Expression of Miw Z DNA in injected embryos

Trials	Stages of embryos	No. of analysed embryos	Expression of Miw Z (%)		
			Embryonic	Extra-embryonic	Gonad
Trial 1	stage 13-15	95	3 (3%)	38 (40%)	0 (0%)
Trial 1	stage 26-28	27	1 (4%)		
Trial 1	stage 13-15	22	2 (9%)	14 (64%)	3 (8%)
Trial 1	stage 26-28	37	1 (3%)		
Control 1	stage 13-15	5	0 (0%)	0 (0%)	0 (0%)
Control 2	stage 26-28	17	0 (0%)		

[1] See footnote of table 1.

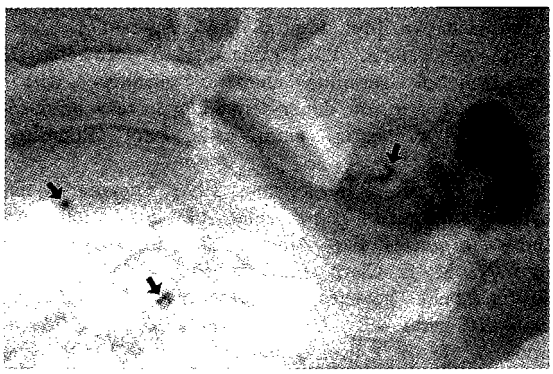

Figure 3. Expression of injected DNA (Miw Z) in the embryo (arrow)

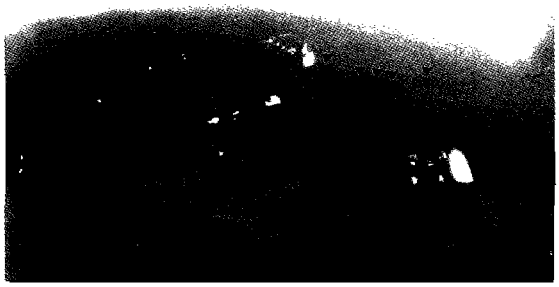

Figure 4. Introduced DNA expressed in the gonad (arrow head)

Detection of the injected Miw Z DNA by PCR analysis

The Miw Z DNA was much more clearly detected by PCR analysis than by β-galactosidase activity (table 4). Percentages of the detection in the gonad were very high, 83 and 82% for trial 1 and 2, respectively. On the one hand, the introduced DNA was also clearly detected, even in the blood, head and arms (39 to 86% for trial 1 and 2).

Tabel 3. Expession site of MiwZ DNA

Expression site	No. of detected embryos (%)	
	Trial 1[1]	Trial 2[1]
Extraembryonic	38 (84%)	14 (64%)
Heart	3 (4%)	2 (3%)
Head	2 (2%)	0 (0%)
Gonad	0 (0%)	3 (8%)
Others[2]	3 (2%)	1 (2%)

[1] See footnote of table 1.

[2] Embryonic tissues except heart, head, and gonad.

DISCUSSION

In the present study, we tried to introduce the foreign DNA (Miw Z) into the PGCs through the embryo by single injection. This method is free from the manipulations transferring donor PGCs to recipient embryos, increasing the rate of germ-line chimeras, and determining the sexes of donor and recipient embryos. The MiwZ DNA was injected to the site which will develop to the germinal crescent from at stage 3-5 (trial

Table 4. Detection of Miw Z DNA in embryos by PCR analysis

Trials[1]	Stages of embryos	Detection of MiwZ DNA			
		Blood	Head	Arm	Gonad
Trial 1	stage 13-15	39% (16/41)			
Trial 1	stage 13-15	86% (25/29)[2]	70% (16/23)	61% (14/23)	83% (19/23)
Trial 2	stage 13-15	63% (12/19)			
Trial 2	stage 13-15	76% (28/37)[2]	61% (20/33)	45% (15/33)	82% (27/33)
Ccontrols	stage 13-15 & stage 26-28	0% (0/13)[2]	0% (0/ 8)	0% (0/ 8)	0% (0/ 8)

[1] See footnote of table 1; [2] Samples of stage 13-15 and 26-28.

1) to the stage 9-11 (trial 2). The expression of the MiwZ DNA detected in a few embryos in both trial 1 and 2, but in the gonad only in trial 2. However, PCR analysis clearly revealed the presence of the MiwZ DNA in the gonads of more than 80% in the embryos from both trial 1 and 2. The reason why some differences were observed in the detection between X-gal staining and PCR analysis for was not clarified in this experiment. One of the reasons for this may be from the differences between DNA products (protein) for x-gal staining and DNA contents for PCR analysis.

From these results together with no difference in the rates of survival and abnormal embryos between two trials, the efficiency of introducing the foreign DNA into the PGCs was almost the same for injection at stage 3-5 and stage 9-11. Since the germinal crescent is clearly formed at stage 9-11, the manipulating embryos is more simple and easier than the prior stage at 3-5.

The question that the introduced DNA was incorporated into the nucleus or the remaining episomal part of the cells is still unsolved in this experiment. Including predictable decrease in survival rate and the increase in abnormality of embryos with development, prolonged investigation to hatch or post hatching is still remaining.

In the previous works, the foreign DNA injected into blastodermal cells of freshly oviposited fertile eggs was certainly detected in the PGCs obtained from circulating blood of developing embryos (Inada et al., 1997).

On the one hand, the transgened DNA (Miw Z) was successfully incorporated into the germline, producing offspring carrying introduced foreign gene (Ebara et al., 1998), suggesting the possibility of transferring exogenous gene to the next generation. Some evidences similar to this were also reported using chicken embryos, showing the incorporation of injected DNA into the PGCs circulating in blood stream, resulting in positive transgene to the embryonic gonads (Yamaguchi et al., 1996).

The present experiments, therefore, suggest that the introduction of foreign gene to the PGCs originating in the germinal crescent region is probably most successful for producing transgened chicken.

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