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Efficiency of Exogenous Gene Introduction into Chicken Primordial Germ Cells for Producing Transgenic Chicken

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Abstract: It has been investigated that transgenic chicken can be produced by various methods by introducing an exogenous gene into chicken Primordial Germ Cells (PGCs). Blood containing PGCs was collected from the blood vessels of embryos at stages 12-15. An exogenous gene encoding Green Fluorescent Protein (GFP) was introduced into the PGCs by lipofection and electroporation. Electroporation was carried out under 200 V/25 μF, 200 V/200 μF and 200 V/950 μF. GFP expression was observed in PGCs. Efficiency of introduction into PGCs was low. Electroporation was superior to lipofection for the introduction of the exogenous gene into chicken PGCs. PGCs treated for GFP introduction by electroporation were injected into blood vessels of recipient embryos at stages 12-15. After incubation until stage 26, GFP bands were obtained from gonads of embryos, thereby demonstrating that treated PGCs had migrated to the gonad.

Key words: Transgenic chicken, PGCs, GFP, lipofection, electroporation

INTRODUCTION

Techniques for introducing exogenous genes have been studied by several methods. Transgenic mice are routinely produced by microinjection of exogenous genes and from chimeric animals using Embryonic Stem (ES) cells.

Avian Primordial Germ Cells (PGCs) are an important resource for transgenic chicken. Chicken PGCs have been reported to originate from the epiblast (Eyal-Giladi *et al*., 1981), appear in the hypoblast of the germinal crescent region and circulate in the blood vascular system at stages 11-15, finally migrating into the germinal ridge (Kuwana, 1993). PGCs can be easily isolated from the blood vascular system of embryo at stages 11-15. Germline chimeric chickens have been successfully produced by transferring PGCs (Naito *et al*., 1994; Furuta *et al*., 2001, 2007, 2008, 2009; Yamaguchi *et al*., 2000) and donor-derived offspring have been obtained from germline chimeric chickens (Naito *et al*., 1994; Furuta *et al*., 2001).

Exogenous gene introduction into chicken has been investigated in early chick embryos by lipofection and electroporation (Inada *et al*., 1997; Furuta *et al*., 2000, 2010; Furuta and Fujihara, 2000). Successful transfer of exogenous genes into chicken embryos was achieved by lipofection when the gene was introduced into blastodermal cells of fertilized embryos (Inada *et al*., 1997; Furuta *et al*., 2000). The expression of exogenous genes introduced into chicken embryos has been reported to vary depending on the electrical loading conditions during electroporation (Furuta *et al*., 2000; Furuta and Fujihara, 2000). These methods were aimed at introducing exogenous genes directly into the embryo. In general, the frequency of transgenic offspring is very low. Transgenes could be introduced into ES cells which could be evaluated to make chimeras and transgenic products. PGCs derived from ES cells may be used to make germline chimeric chickens (Van de Lavoir *et al*., 2006). However, there are few reports on electroporation conditions for introducing exogenous genes into PGCs.

The present experiments were performed to introduce an exogenous gene into PGCs isolated from embryos at stages 12-15 (Hamburger and Hamilton, 1951) by electroporation.

MATERIALS AND METHODS

Preparation of PGCs: Fertilized eggs were obtained from white Leghorn Maria line hens (GHEN Corporation, Gifu Japan). The eggs were incubated for 48-55 h and then cracked to collect blood using a fine glass micropipette (G-1, Narishige, Tokyo, Japan). At this time, PGCs appeared in blood vessels at stages 12-15 (Hamburger and Hamilton, 1951). The blood was suspended in 100 μL of 3.8% sodium citrate. The prepared

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blood samples were added to 900 μL of ACK lysis buffer containing 150 mM NH_4Cl, 1 mM $KHCO_3$ and 0.001 mM EDTA and incubated on ice for 30 min. The samples were centrifuged at 2000 rpm for 10 min at 4°C. The pellet was resuspended in 1000 μL of ACK lysis buffer, incubated on ice for 15 min and washed twice in PBS (Yamamoto *et al*., 2007). The PGCs were cultured in KAv-1 medium (Kuwana *et al*., 1996) at 37°C in 5% CO_2.

Experiment 1 (Transfer of exogenous gene)

Marker gene (*GFP* Green Fluorescent Protein): pEGFP-N1, Clontech Laboratories, Inc. CA, USA) was used as a marker gene.

Lipofection: The circular form of 0.8 or 2.0 μg GFP was mixed with lipofectamine 2000 (Invitrogen, CA, USA), opti-MEM (Gibco, BRL, USA) and approximately 2.0×10^3 PGCs.

Electroporation: The circular form of 6.25 μg GFP was mixed in opti-MEM (Gibco, BRL, USA) and approximately 2.0×10^3 PGCs and blood cells that contained a total of 1.0×10^6 cells. Electrical loading was conducted at 200 V/25 μF, 200 V/200 μF or 250 V/950 μF. After treatment, culture of the PGCs in KAv-1 medium was continued.

Detection of GFP: The PGCs were incubated for 24 h at 37°C and 5% CO_2 in air. Expression of GFP was examined in the PGCs using a fluorescence microscope.

Statistical analysis: Significant differences in GFC expression rates in PGCs were determined with the Mann-Whitney U-test.

Experiment 2

Transfer of PGCs: A window of approximately 1.0 mm diameter was opened at the small end of the egg. Approximately 200 PGCs treated by electroporation were injected into a blood vessel of the recipient embryo at stages 12-15. The small hole on the surface of the recipient embryo's vessel was closed with a drop of 199 medium with FCS. The windows in the recipient eggs were closed with tape and the eggs were incubated until stage 26. The gonads of embryos at stage 26 were removed and DNA was extracted. PCR was used to determine whether PGCs with GFP had settled in the gonad.

RESULTS AND DISCUSSION

Experiment 1

Lipofection: The median rates of introduction of the exogenous gene into chicken PGCs were 0.95% for 0.8 μg and 2.34% for 2.0 μg (Table 1).

Electroporation: The median rates of introduction were 0.85% for 200 V/25 μF, 2.34% for 200 V/200 μF and 0% for 250 V/950 μF (Table 2). GFP fluorescence was confirmed in PGCs (Fig. 1 and 2). The detection frequency of GFP at 200 V/200 μF was higher ($p<0.05$) than that in other trials.

Experiment 2: Approximately 200 PGCs treated by electroporation at 200 V/200 μF for introducing GFP were injected into the blood vessels of recipient embryos at stages 12-15. In stage 26, specific bands of GFP were detected in right and left gonads by PCR.

A transgenic mouse has been produced by the introduction of an exogenous gene into the germline using microinjection and ES cells. These skills are routine

Table 1: Expression of Green Fluorescent Protein (GFP) in Primordial Germ Cells (PGCs) by lipofection

DNA 0.8 μg (n = 19)			DNA 2.0 μg (n = 16)		
Median	------Percentile------		Median	----Percentile----	
0.95[a]	25	75.00	0.26[b]	25	75.00
	0.64	1.76		0.22	0.91
Data distribution	0.22-6.89			0-2.2	

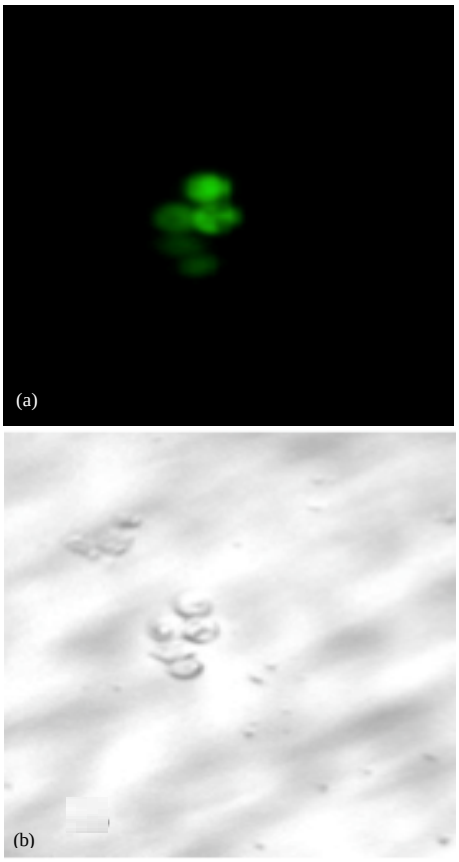

Fig. 1: GFP fluorescence was detected in PGCs with lipofection; a) Dark field; b) Light field

Table 2: Expression of Green Fluorescent Protein (GFP) in Primordial Germ Cells (PGCs) by electroporation

200 V/25 μF (n = 12)			200 V/200 μF (n = 26)			250 V/950 μF (n = 6)
Median	Percentile		Median	Percentile		Median
0.85[a]	25	75.00	2.43[b]	25	75.00	0
	0.53	1.92	-	1.55	3.41	-
Data distribution	0.0-2.95	-	-	0.32-10.25	-	-

[ab]$p<0.05$

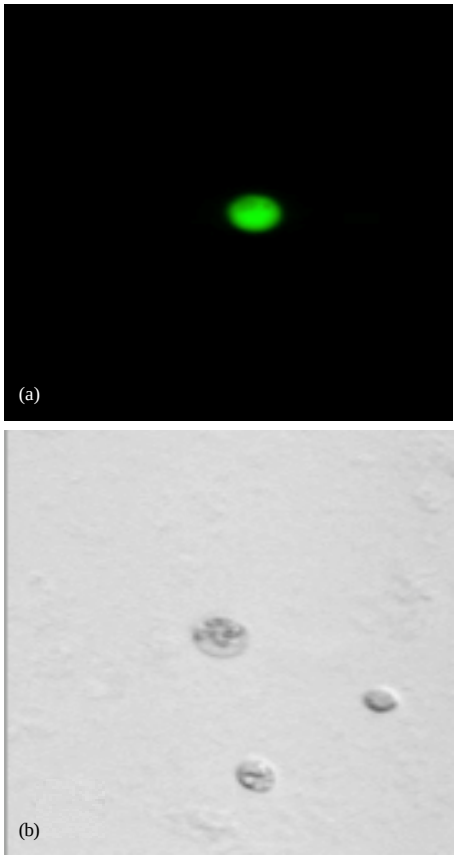

Fig. 2: GFP fluorescence was detected in PGCs with electroporation; a) Dark field b) Light field

used in mammalian biology. In avians, however, transgenic technology has been delayed compared with mammals because of the difficulty of obtaining single fertilized ova. Primordial Germ Cells (PGCs) differentiate into sperm and ovum in the gonads and are the only cells that transmit genomic information to offsprings. PGCs are useful tools for transgenic chicken. Germline chimeric chickens have been successfully produced using PGCs (Naito *et al*., 1994; Furuta *et al*., 2001, 2007, 2008, 2009; Yamaguchi *et al*., 2000). Exogenous genes could be introduced into PGCs for the production of transgenic chickens (Inada *et al*., 1997; Furuta *et al*., 2000; Furuta and Fujihara, 2000). Researchers focused on integrating an exogenous gene into PGCs, leading to the germline incorporation of the gene. However, the efficiency of introduction was very low.

CONCLUSION

In this study, an exogenous gene from PGCs migrated to germinal ridges following its introduction into a blood vessel at stages 12-15. It will be possible to generate transgenic chicken offspring from germline chimeric chickens.

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