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Recolonization of Transfected Blastodermal Cells in Developing Embryos after Transferring into UV-irradiated Fertilized Hen's Egg

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UV-조사 수정란 내로 이식한 유전자 변환 배반엽 세포의 재구성

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ABSTRACT : Unfortunately, there is no technique which is stable and repetitive to produce transgenic chicken, although various ways of gene transfer including PGC- and embryonic cell-mediated gene transfer, DNA microinjection, virus inoculation and sperm cells have been employed. The aims of this study were to develop and establish such a stable, repetitive and efficient way of gene transfer giving a faithful gene expression during development after the reconstruction of embryo in an UV-irradiated egg. A dual reporter plasmid (pJJ9), a fusion gene containing *lacZ* and *GFP* driven by a CMV promoter was used to exploit either merits of both reporting markers, *lacZ* with strong signal or *GFP* with vital marking. Electroporated embryonic blastodermal cells (EBCs) in the presence of the pJJ9 DNA faithfully showed 377 bp PCR product and *lacZ* or *GFP* expressions in the identical cells *in vitro* or *in vivo*. Furthermore, analyses of expression patterns of the foreign DNA demonstrated that microinjected EBCs cells into the UV-irradiated recipient egg should participate in normal developmental process, for example, proliferation and differentiation into various tissues. Thirty percentages of the manipulated eggs showed *lacZ* expression in their tissues. These results together with the specific procedures used in this study should facilitate avian transgenesis.
(Key words : dual reporter, embryonic blastodermal cells, differentiation, reconstructed embryo)

INTRODUCTION

Gene transfer into the germline of chickens should provide new and revolutionary opportunities for both basic and applied researches (Lee, 1994). Many studies have been carried out to try to transfer genes that may provide commercial chickens with resistance to diseases and even to produce human peptides in hen's eggs. The development of gene transfer technology for avian species, however, has not progressed as rapidly as that in mammals. Progress in producing transgenic chickens has been hampered, in part, by the reproductive and embryonic developmental systems of the chicken.

Successful gene transfer involves the identification, isolation and characterization of genes of interest, appropriate modification, and preliminary testing in cultured cells (Crittenden and Salter, 1986; Freeman and Bumstead, 1987; Shuman, 1989). So far infection of embryonic cells with retrovirus vectors (Bosselman et al., 1989; Thoraval et al., 1995), transfected sperm cells (Gruenbaum et al., 1991; Nakanishi and Iritani, 1993; Lee et al., 1999), blastodermal cells (Pain et al., 1996; Lee et al., 2000), and primordial germ cells (Hong et al., 1998; Naito et al., 1998) have been used for the transgenic chicken. More recently, *Drosophila mariner* element has been proposed for a potential use for new vector development for avain transgenesis (Sherman et al., 1998). The aims of this study are to develop and establish an efficient stable and repetitive gene transfer, which avoids difficulties, high mortality, and complexity, rather than to develop a new variety of methodology.

MATERIALS AND METHODS

1. Culture Medium and Solutions

Fertilized hen's eggs were purchased from Pulmoowon Co. They were stored in a humid incubator at 15°C until use.

In vitro culture of embryonic blastodermal cells (EBCs) was carried out in ESA medium (Pain et al., 1996). ESA medium consists of Glasgow-MEM (Gibco, NY, USA) containing 10% fetal bovine serum, 20 ng/ml conalbumin (Sigma, MO, USA), 1 mM sodium pyruvate (Gibco), 1% non-essential amino acid (Gibco), 1 μM of each nucleotide adenosine, guanosine, cytidine, uridine, thymidine (Sigma), 50 mM Hepes, pH 7.6 (Sigma), 0.16 mM β-mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco). ESA medium was equilibrated in an atmosphere of 5 % CO_2 in air at 37.5°C. Medium had pH 7.4 after the equilibration.

2. Culture of the EBCs

To isolate EBCs, the eggshell was cleaned with 70% ethanol and then broken into a specimen dish containing warmed sterile Ca^{2+} · Mg^{2+}-free phosphate-buffered saline (PBS, pH 7.4). The blastodiscs were excised from the yolk with a pair of microdissecting scissors, and removed from the yolk with a sterile forceps. The blastodiscs were isolated with the aid of fine point forceps under a stereomicroscope. The isolated EBC masses were carefully washed with sterile PBS twice to remove the remaining yolk granules. The EBCs were seeded in a 110 mm culture dish containing ESA medium at a final concentration of 1 cell mass per 100 μl. The EBCs were maintained at 37.5 °C in 5% CO_2.

3. Alkaline Posphatase (AP) Reaction

To demonstrate ES cell properties in the proliferating EBC culture, alkaline phosphatase staining was performed (Pain et al., 1996). The EBCs were fixed in ethanol-acetic acid (7:1) mixture for 10~15 min. After several washings with distilled water, the fixed cells were incubated in a reaction mixture of 1 mg/ml fast red TR salt and 40 μl/ml napthol AS-MX phosphate (Sigma), 10 % $MgCl_2$ in Tris-Maleate buffer (pH 9) for 5~30 min. The reaction was stopped by rinsing the cells with PBS or distilled water. Coloured colonies were scored for analysing the potential of the EBC to form putative ES cell colonies under an inverted microscope (SZ-40 OLYMPUS, Japan).

4. Periodic Acid-Schiff (PAS) Staining

EBCs possess abundant cytoplasmic deposits of glycogen that can be stained by PAS reaction. Chicken embryonic germ cells were also identified as PAS-positive cells in many literature (Meyer, 1960). Therefore, cells were also processed for PAS staining during EBC culture.

The cells were fixed in 70 % ethanol previously cooled at -20°C for 30 min After the fixation, the cells were incubated in 1 % periodic acid in 90 % ethanol for 2 h, and stained in Schiff reagent containing 1 % basic fuchin, 1.9 % sodium metabisulfite ($Na_2S_2O_5$) and 0.15N HCl. A dish containing the reaction mixture was incubated at 37°C for 20 min. Color reaction would appear at about 5 min of the reaction. The resulting staining on colored colonies were scored under an inverted microscope.

5. The Plasmid DNAs

Two reporter genes, pEGFP-C1 (CLONTECH Laboratories, Palo Alto, CA; GenBank Accession No. U55763) or pJJ9 plasmids were used for gene transfer into EBCs to demonstrate expression of foreign gene and colonization of the transferred EBCs *in vivo*.

To construct pJJ9, pEGFP-C1 was modified to accept cloned whole *lacZ* gene from pMC1871 (Pharmacia Biotech, Uppsala, Sweden) to be expressed in frame with EGFP followed by β-galactosidase and *gfp* fusion gene, so that pJJ9 can be used as double reporter gene (Fig. 1: Joo, 1997).

Extraction and purification of the plasmid DNAs were performed according to alkaline lysis and polyethylene glycol purification (Sambrook et al., 1989). The purified circular type plasmid DNAs were used for transfection of EBCs.

6. Transfection of EBCs

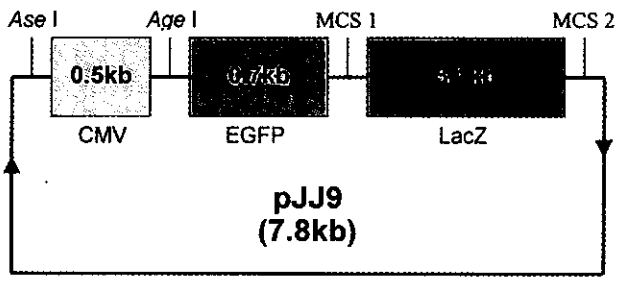

Fig. 1. The schematic plasmid map of a dual reporter pJJ9.

The EBCs were transfected by using electroporation method. The plasmid DNAs was added to the mixture of 20 μl of suspended EBC in PBS and 20 μl of 0.3 M sucrose in PBS in an electroporation chamber to give a final concentration of 300 μg DNA/ml. The mixture was placed between the platinum wires at a distance of 2 mm. The electroporation was initiated by pulsing 5 times at 0.1mA. After the electroporation, the treated EBCs were washed with ESA medium several times to equilibrate cells and subsequently seeded in ESA medium at a final concentration of 1×10^2 cell mass per 50 μl. The EBCs were maintained at 37.5 °C in 5 % CO_2 or used for microinjection into recipient embryos.

7. Microinjection of Cells into Recipient Eggs

Recipient hen's eggs were irradiated to cause maximal death of EBCs in the egg according to Aige-Gil et al. (1991). Briefly a small window was made on the lateral part of the egg by removing shell and other membranes. The windowed eggs were exposed to an ultraviolet light source (UV, GL20, Japan) at 15 cm distance for 30 min.

To demonstrate recolonization of the transfected EBCs, culture of the transfected EBCs were collected, and centrifuged at 1,500 rpm for 10 min and well dispersed by repeated pipettings in fresh 300 μl GMEM medium. After the UV-irradiated recipient eggs were located under a stereomicroscope, 2 μl of the transfected EBCs suspension was slowly injected into the subgerminal cavity of the chick embryo using a microinjector connected with a fine glass pipette. The number of injected EBC was about 100~200 cells. The injected eggs were sealed with a sealing film (Whatman, USA). The sealed eggs were incubated and laid aside in 37.5 °C, 65~75 % relative humidity incubator for 1 day. Next day, to help normal embryo development, the eggs were rotated 90° periodically during the incubation period.

8. Genomic DNA Preparation and PCR Analysis

Transfected EBCs were transferred to a 0.5 ml sterile Eppendorf tubes containing 10 μl DDW. After repeated freezing (- 196 °C) and thawing (37 °C), the genomic DNA of cells was prepared. The extracted DNA sample of EBCs was transferred into different PCR tube for PCR amplifications. The PCR amplification was carried out with 5 IU of Taq polymerase (Promega, USA) in a mixture containing 10×PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 150 mM $MgCl_2$, 1 % Triton-X 100), 200 μM dNTP, and 0.2 μM primers. Amplification was carried out using *gfp* and *lacZ* specific primers 5'-GAA CGG CAT CAA GGT GAA CT-3' and 5'-GTG CTG CAA GGC GAT TAA GT-3' with cell's genomic DNA as a template. Reaction was performed on a Delta Cycler ITM (ERICOMP Co.) during 30 cyclers. Parameters were 30 seconds at 94 °C for denaturation, 30 seconds at 60 °C for annealing and 1 min at 72 °C for elongation. The last step of elongation for 10 min. PCR products were analyzed on a 2% (w/v) agarose gel.

9. Fluorescent Microscopy for GFP Detection

Expression of the *gfp* was examined in the transfected EBC culture or the injected embryos under a Zeiss Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany). Photographs were taken on Kodak color film (ASA 400, Kodak, Australia) using an FITC filter block.

10. Histochemical Staining for β-Galactosidase

Culture of transfected EBCs and the reconstructed embryos after 5 days of incubation, embryos were washed twice with PBS and fixed for 1 h at 4 °C in 0.2 % (v/v) glutaraldehyde, 2 % (v/v) formaldehyde and 2 mM $MgCl_2$ in PBS. Fixed EBCs or whole embryos were rinsed three times in 2 mM $MgCl_2$, 0.02 % (v/v) NP-40 in PBS and incubated 5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 5 mM potassium ferrocyanide ($K_4Fe(CN)_6$) and 1 mg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml in PBS for detection of the expressed β-Gal at 37 °C for 1 h or overnight. After the staining, the solution was aspirated, EBCs and whole embryos were washed three times in PBS to remove excess substrate and background non-specific stained materials. Specific stainings in the EBCs and embryos were photographed on Kodak color film (ASA 100, Kodak, Australia) under a Zeiss Axioplan microscope with automatic exposures.

RESULTS AND DISCUSSION

1. Characterization of EBCs

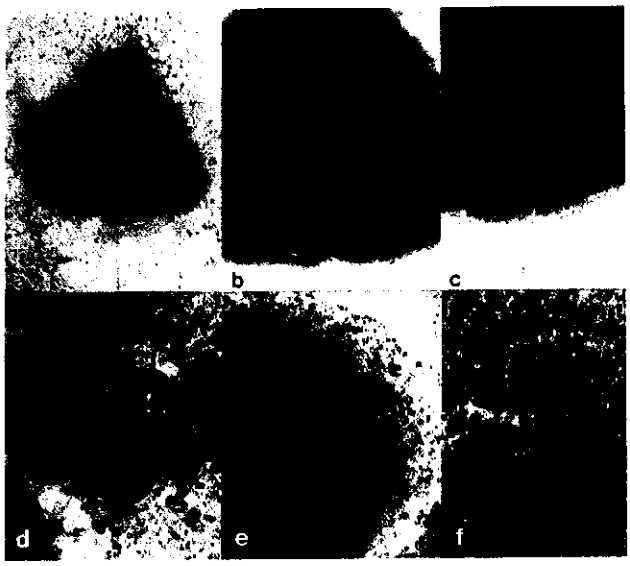

Fig. 2. Both alkaline phosphatase (AP) and periodic acid Schiff (PAS) positive embryonic colonies appear from day 3 of culture suggesting putative ES colonies (a, b, c). Chicken blastodermal cells were stained for AP activity (d, e, f). these cells were also PAS-positive.

One of the aims in this work to characterize putative chicken ES cells because EBCs isolated and cultured *in vitro* should show at least pluripotency when transplanted into the recipient eggs. Petitte et al. (1990) demonstrated that chicken early blastoderm contains cells able to contribute to both somatic and germinal tissue when injected into a recipient embryo. However, these cells were neither identified nor maintained *in vitro*. Pain et al. (1996) showed that chicken early blastoderm contains cells characterized as putative ES cells that can be maintained *in vitro* for long-term culture.

mES cells are characterized by their round and small shape, their large nucleus, the presence of one or two prominent nucleoli and their small cytoplasm amount (Robertson, 1987). In addition, mES cells possess strong endogenous alkaline phosphatase activity similar to the EC-derived cells (Strickland et al., 1980). As shown in our previous study, EBCs exhibit a strong endogenous AP activity that is lost as these cells differentiate in culture (Fig. 2a-c). This positive reaction with endogenous AP was used as one of the criteria to identify putative undifferentiated embryonic cells. Another way of showing pluripotent embryonic cells is to determine relatively intensive staining with PAS reagent due to the presence of glycogen granules in the PGCs or PGC-like cells. We also PAS-stained the colonies resulting from EBC culture *in vitro* at 48 h. Similar results were obtained as in those colonies recognized by AP staining (Fig. 2d-e).

2. Foreign Gene Introduction by Electroporation to EBCs

EBCs were transfected with pJJ9, a fusion gene of *gfp* and *lacZ*, by electroporation to determine the efficacy of the dual reporter gene in the proliferating EBCs *in vitro*. We tried to colocalize GFP and *lacZ* expressions in an identical EBC sample. First, GFP expression pattern was observed under an FITC-filter block in live cells and recorded. Similarly the identical sample was processed for X-gal satining. Fig. 3 shows such a result by double-screening of gfp and *lacZ* fusion gene. Colocalized expressions of *gfp* and *lacZ* gene were detected in the transfected EBCs. This result demonstrates a perfect coincidence of incorporation of *gfp* and *lacZ* gene, although *lacZ* gene expression was relatively more extensive that GFP.

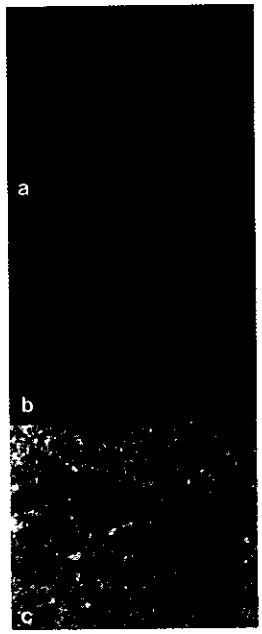

Fig. 3. Expression of co-localized *gfp* (a) and *lacZ* (c) gene in transfected blastodermal cell. This result demonstrates a perfect coincidence of incorporation of *gfp* and *lacZ* gene. Magnifications is × 400.

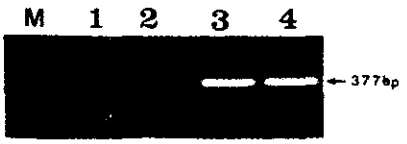

Fig. 4. PCR analysis of DNA from cultured blastodermal cells after transfection of pJJ9. M : PCR marker (1000, 750, 500, 300, 150 and 50bp respectively), lane 1, water control; lane 2, nagative control; lane 3, positive control (pJJ9-10 pg/(l) and lane 4, transfected blastodermal cells.

DNAs extracted from the transfected EBCs was also assayed by PCR to confirm the gene transfer by electroporation, to detect the *gfp* and *lacZ* genes. The amplification product corresponding to the expected 377 bp size for a fragment containing 3'-GFP and 5'-LacZ was also detected in the transfected EBCs (Fig. 4).

3. Microinjection into UV-irradiated Chicken Embryo

The exposure to γ-irradiation has previously been used to compromise the development of White Leghorn recipient embryos prior to injection with EBCs from a Barred Plymouth Rock donor (Carsience et al., 1993). This technique allowed an increase in somatic and germline chimerism. The same technique was used to produce chimeric chickens between Brown and White Leghorns. Prior to inoculation of cells the effects of γ-irradiation on the early development of embryos was firstly tested. The embryonic development was delayed proportionally to the doses of γ-irradiation from the cesium-137 source. This result is comparable with the data obtained by Carsience et al. (1993).

Host hen's eggs were exposed to UV-irradiation to compromise their development. Development of UV-irradiated chick embryos was checked after 3 days of incubation to determine proper doses of UV-irradiation sufficient contribution of donor cells in the reconstructed eggs. Embryonic development was delayed, and the extent of the delay was proportional to the durations of exposure to UV-irradiation (Table 1). The development of embryos exposed to 30 min was slightly delayed when compared to the development of that of unexposed embryos. Of the embryos exposed to 20, 30 and 60 min, 95, 90 and 100%, respectively, did not reach to stage 19 (72 h).

Table 1. *In vitro* development of UV irradiated eggs

UV irradiation	No. of eggs used	No. of eggs developed to Stages[1]			
		7	8~13	14~19	19
0	40	4	6	11	19
10	40	11	15	7	7
20	40	23	9	4	4
30	40	28	7	3	2
60	40	39	1	0	0

[1] Stages were judged by classification of Hamburger and Hamilton (1951).

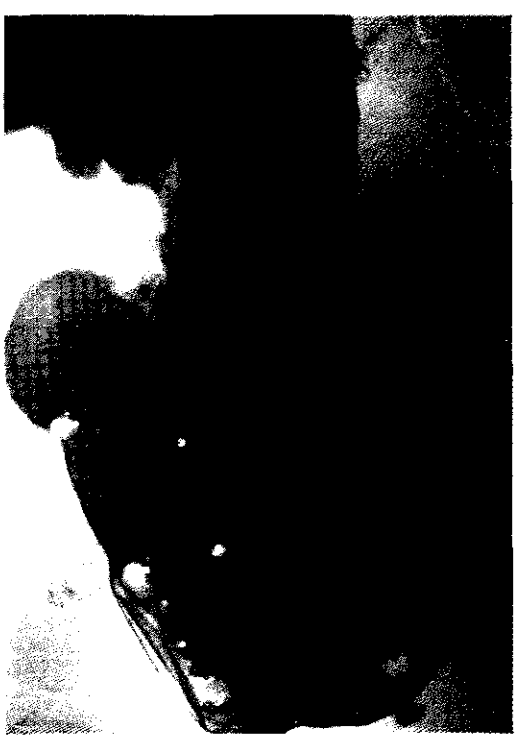

Fig. 5. Expression of *lacZ* gene in developing embryos after microinjection of transfected blastodermal cells. X-gal staining was performed with the whole mount of chicken embryo. The *lacZ* gene expression was found in the spinal cord (an arrow) and genital ridge (arrowheads). Magnificatian is ×40.

Among 60 host embryos exposed to 30 min of UV-irradiation and received approximately 100 donor cells, 18 (30%) survived past day 5 of incubation. The expression of the *lacZ* gene, as revealed by β-galactosidase activity, was detected in 8 (44%) of the survived embryos (Table 2). Most of them were detected only in the spinal cord and genital ridge (Fig. 5).

This technique for successive genetic operations should

Table 2. Survival and expression rates of the chick embryos injected with transfected blastodermal cells

	Groups	No. of eggs used	Injection vol[1]. (μl)	No. of embryos survived[2] (%)	No. of embryos expressing (%)	
					gfp	*lacZ*
Control	Untreated	13	–	12 (92)	0 (0)	0 (0)
	Window alone	16	–	10 (63)	0 (0)	0 (0)
	Sham alone[3]	24	2	10 (42)	0 (0)	0 (0)
	Exp.	60	2	18 (30)	0 (0)	8 (44)

[1] Injected volume of transfected blastodermal cells.

[2] Number of embryos survived upto 5 days.

[3] Sham-injection with G-MEM.

facilitate the investigation of embryogenesis in early chick embryos at the molecular level. However, two questions remain unanswered: the reason that many expressions of DNA were detected in spinal cord or genital ridge and the frequence that the DNA was integrated with the host chromosomes. In order to assure its chromosome integration, devises for introducing foreign DNA may be essential and an increased efficiency in the production of a germline chimera may be necessary for the production of transgenic chickens.

적 요

유전자변환 닭 생산기술을 개발하기 위해 PGC세포, 배세포 유전자 미세주입, virus 접종 및 정자세포 등이 이용되고 있으나 아직 안정되고 반복적인 유전자변환기술은 확립되어 있지 않다. 보다 안정되고 반복적인 단순한 유전자도입기술을 확립하기 위하여 이중 reporter유전자 (LacZ-GFP)를 도입한 배반엽세포가 정상적인 배 발생에 기여하는가를 조사하였다. 수정란의 배반엽세포를 electroporation에 의해 전이시킨 후 UV조사한 수정란에 이식하고 배 발생 중 이식한 세포의 운명과 유전자발현을 분석함으로써 그 가능성을 추정하였다. 이중 reporter유전자인 pJJ9은 전이된 배반엽세포에서 추출한 DNA의 *gfp*와 *lacZ*유전자를 PCR분석에 의해 377 bp 크기의 산물을 확인할 수 있었다. 또한 이들 세포를 30분 동안 자외선 조사한 60개의 수정란 내에 각각 약 100~200 세포를 이식하여 5일 동안 배양한 결과 18개 수정란 (30%)의 배아가 생존하였고, 그 중 8개의 수정란의 배아에서 *lacZ* 유전자가 확인되었다. 그들 대부분은 척수와 비뇨생식기원기에서 발현되었다. 이 같은 결과는 배반엽세포 내로 electroporation에 의해 외래유전자가 도입되었을 뿐만 아니라 이들 배반엽세포에서 *in vitro* 및 *in vivo*에서 도입유전자가 안정되게 발현되었으며 또한 증식과 분화에 의해서 수용체 수정란에서 정상적인 발생에 기여함을 보여준 것이다. 이 같은 효율적인 유전자 도입과 발현은 유전자변환 닭 생산을 가속화시킬 수 있을 것이다.

(색인어 : 이중 reporter, 배반엽세포, 분화, 재구축배)

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