Migration Activity of Chicken Gonadal Primordial Germ Cells (gPGCs) and Post-transfer Localization of LacZ-transfected gPGCs in the Embryonic Gonads

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ABSTRACT : A powerful tool for chicken transgenesis could be established by employing a germline chimera production through primordial germ cell transplantation. This study was conducted to examine whether foreign gene-transfected gonadal primordial germ cells (gPGCs) have a migration activity into the gonad after transfer to recipient embryos. In Experiment 1, gPGCs of Korean Ogol Chicken were retrieved from 5.5-day-old embryos and subsequently transferred to the dorsal aorta of 2.5-day-old White Leghorn embryos after being labeled with PKH26 fluorescent dye. To confirm migration activity after transplantation, recipient embryos were sacrificed and examined on 3 days after transfer. Sex determination was concomitantly undertaken to examine whether sex of recipient embryos could affect the migration activity of gPGCs. All of embryonic gonads examined showed positive signals with PKH26 fluorescence and W-chromosome specific band by polymerase chain reaction (PCR) was detected in male embryos when gPGCs with ZW chromosome were transferred to recipient embryos. In Experiment 2, retrieved gPGCs were transfected with LacZ gene-containing cytomegalovirus promoter (pCMVβ) by electroporation and subsequently transferred to recipient embryos. LacZ gene expression was identified in the gonads of 6 or 10-day-old recipient embryos and hatched-chicks. A total of 20 embryos and 12 hatched-chicks were examined and 11 of them (10 embryos and one hatched chicken; 11/32=34.4%) expressed β-galactosidase, a marker substance of LacZ gene. The results of this study demonstrated that foreign gene-transfected gPGCs can migrate and settle down into the gonad after being transferred into the blood vessel of the recipient embryos. This established technique will contribute to developing a peer biotechnology for transgenic chicken. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 9 : 1227-1231)***

Key Words : Chicken, Gonadal Primordial Germ Cell, Transgenesis, Germline Chimera, LacZ, Electroporation

INTRODUCTION

Production of transgenic chicken has an unlimited value in various fields of animal biotechnology. The use of primordial germ cells (PGCs) is the most powerful tool for improving the efficacy of transgenic technology and PGCs of several types, which were retrieved from the germinal crescent, blood or gonad in embryos of different stages, have been used to date. Collection of PGC from embryonic gonad yielded a number of critical benefits for the production of germline chimera. Primordial germ cells can be easily and efficiently collected from the embryonic gonads, compared with those from the germinal crescent or embryonic blood. It was recently reported that gonadal primordial germ cells (gPGCs) cultured *in vitro* have an activity of embryonic germ cells (Park and Han, 2000) and that the transfer of cultured gPGCs into embryos can induce germline chimerism (Chang et al., 1997). Furthermore, gPGCs could maintain *in vitro* for up to 2 months without any morphological and functional alterations (Han et al., 2002). All of previous data strongly demonstrated the feasibility of gPGCs for improving chicken transgenic technology.

In this study, we consequently examined whether gPGCs could normally migrate into the gonad of recipient embryos even after being transfected with foreign genes. We employed an electroporation method for foreign gene (LacZ) transfection to gPGCs, since this method yielded improved efficiency of gene transfer in chicken primordial germ cells (Hong et al., 1998). Migration activity of gPGCs and the localization of LacZ gene expression in the gonad were confirmed by PKH26 fluorescent dye, X-gal staining for detecting β-galactosidase or polymerase chain reaction (PCR) using specific primers to W-chromosome and LacZ gene.

MATERIALS AND METHODS

Preparation of gPGCs and transfer to recipient embryos

Fertilized eggs were obtained from Korean Ogol Chicken (KOC) and White Leghorn (WL) stocks maintained in Experimental Animal Farm, Seoul National University, Korea. Eggs of WL and KOC were incubated for 2.5 (stage 17; Hamburg and Hamilton, 1951) and 5.5 days (stage 28; Hamburg and Hamilton, 1951) at 37.5°C in air atmosphere of 60-70% humidity, respectively. Cells in the gonadal ridges in KOC embryos were then retrieved as previously described (Park and Han, 2000), and gPGCs were isolated from the collected cells by a Ficoll-density-gradient separation (Chang et al., 1992). After *in vitro* manipulation according to the experimental design, gPGCs were transferred into WL recipient embryos of 2.5-day-old by our standard protocol. Recipient embryos were then

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incubated and embryos of 6 or 10-day-old or hatched chicks were provided for each experiment.

PKH26 labeling of gPGCs

gPGCs isolated from 5.5-day-old chick embryonic gonads were labeled with PKH26 fluorescent dye by a modified protocol of the supplier (ZYNAXIS Cell Science INC., Japan). PKH26-labeled gPGCs were then injected into the dorsal aorta of recipient embryos at the stage of 17. The recipient embryos were then incubated for 3 days. The embryos were removed, fixed with 1% (v/v) formaldehyde in PBS, and embedded in paraffin. Sections (10 μm thickness) were prepared using the Pika microtome (Seiko Ltd, Tokyo, Japan). To identify localization of donor gPGCs, gonadal tissue of the recipient was stained with Periodic Acid Schiff's (PAS) reaction.

Sex determination by PCR

To determine sex of a recipient embryo, blood (1-2 μl) was removed from 5-day-old embryo using microcapillary pipette. Collected blood was transferred into a fresh 1.5 ml tube and mixed with 100 μl ddH_2O. An aliquot of sample (10 μl) was transferred to a 0.5 ml tube and overlaid with 40 μl mineral oil. After boiling at 97°C and 55°C three times each, the sample was subjected to PCR. To identify donor-derived PGCs, gonads were isolated from recipient embryo on 6 days post-incubation and PCR was carried out in a solution containing 250 μM of dNTPs, 1X reaction buffer, 10 pmoles each of forward and reverse primers, and 2 unit of Taq polymerase. Amplification was carried out in a DNA thermal cycler (Perkin Elmer Cetus, USA). Each thermal cycle consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 40 sec was repeated 35 times. W-chromosome specific DNA fragment of 348 bp was amplified in the female embryo, but not in the male embryo. Female specific primers, forward primer of 5'-ACC TGT CTC CCA AAA ATT CTG C-3' and reverse primer of 5'-TGG GGT GAA ATG GGG TTG-3', were used for PCR.

Transfection of gPGCs

The reporter construct used for transfection was bacterial LacZ gene downstream of the CMV immediately/early promoter (pCMVβ). Plasmid DNA (20 μg) prepared by a large-scale matrix-based purification method according to manufacturer's recommendations (Qiagen, USA) was added to 1.6×10^5 gonadal cells containing gPGCs and stroma cells. Electroporation was then conducted in the presence of 1.25% (v/v) DMSO by our standard protocol using 250 V with a capacitance of 950 μF (Hong et al., 1998).

Analysis of transgene expression

Following transfection, approximately a total of 200 gPGCs were injected into the bloodstream through the dorsal aorta of a 2.5-day-old recipient embryo. To identify pCMV β sequences in the recipients, gonads were dissected from 6 or 10-day-old embryos and hatched chicks. Semen was collected from the manipulated male chickens after sexual maturation. Extracted DNA was then subjected to PCR of 30 cycles and chicken genomic DNA from uninjected embryos was used as a negative control. Primers were designed to amplify sequences of the LacZ and their nucleotide sequences as follows; 5'-AGA TGC ACG GTT ACG ATG C-3' and 5'-GGT CAA ATT CAG ACG GCA AAC G-3' (Love et al., 1994). PCR products (246 bp) were analyzed on 2% (v/v) agarose gels. Identity of PCR products was further confirmed by Southern blot hybridization using probes labeled with [α-^{32}P]dCTP. On the other hand, the expression of β-galactosidase in the embryonic or neonatal gonads was examined for LacZ gene expression at the cell level by the X-gal staining, which was previously described by Han et al. (1994).

Experimental design

In Experiment 1, migration of gPGCs into the embryonic gonad was determined by PKH26 labelling and sex of recipient embryo was examined for evaluating sex effect on gPGCs migration. In Experiment 2, gPGCs were transfected with LacZ gene-containing pCMVβ and migration of transfected gPGC and localization of LacZ gene were examined in the gonads of recipient embryos and hatched chicks by PCR and X-gal staining.

RESULTS

Experiment 1

Gonads were retrieved from recipient embryos at the stage 29 (6 days) and all examined gonads contained PKH26-positive gPGCs. PKH26-positive donor gPGCs, which were mixed with gPGCs of the recipients, were evenly distributed into the gonadal tissue. As shown in Figure 1, all gonadal tissues have positive signal against both PKH26 and PAS reaction, although some gPGCs were stained with PAS both exogenously and endogenously. In another set of Experiment 1, it was shown that such staining feature was found similarly in the recipient embryos having different sex chromosomes. Of male recipient embryos provided for examination, DNAs from the embryonic gonads had ZW chromosome as well as ZZ chromosome (Figure 2). It was further found that no amplification product was generated from DNAs of the mesenchymal tissues in recipient embryos such as blood cells and fibroblasts.

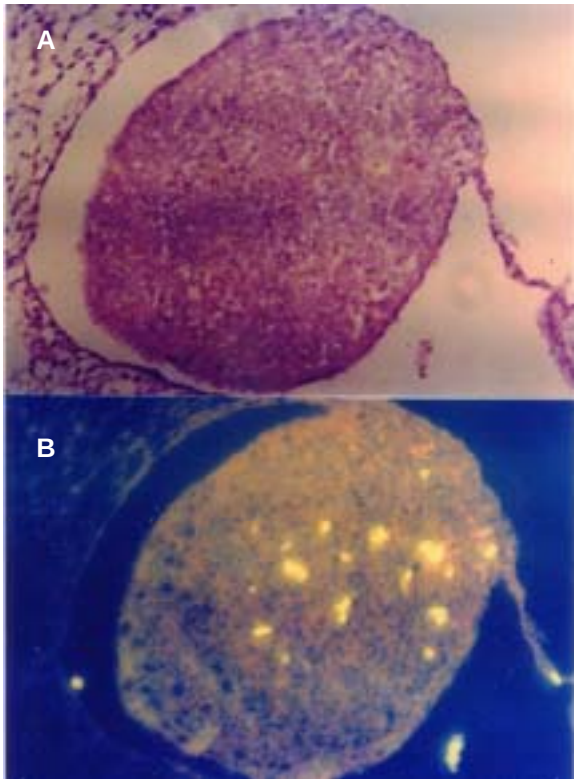

Figure 1. Identification of donor-derived gPGCs in the recipient gonad at 6 days post-incubation. (A) Both donor and recipient PGCs were stained with PAS reaction. (B) Donor-derived gPGCs are shown as PKH26-labeled cells in the recipient gonad.

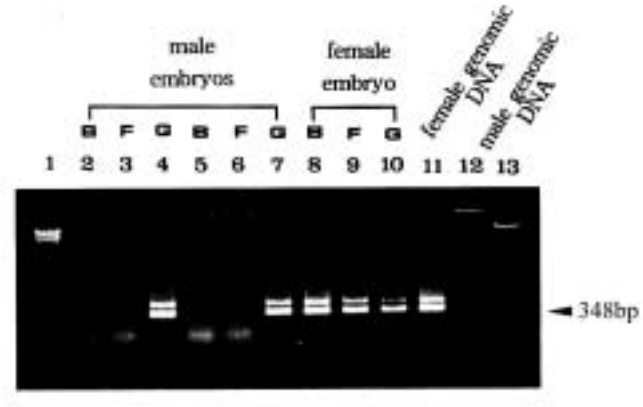

Figure 2. Identification of female donor-derived gPGCs in male recipient embryonic gonads at 6 days post-incubation. PCR primers for sex determination amplified 348 bp fragment on W-chromosome. Lanes 1, size marker (λDNA digested with Hind III); 2 and 5, blood of male embryos; 3 and 6, fibroblast cells of male embryos; 4 and 7, gonads of male embryos; 8, 9, 10 and 11, positive control; 12, negative control (male genomic DNA); 13, no template DNA.

Experiment 2

A total of 20 embryos and 12 hatched chicks were examined for the detection of LacZ expression, which was controlled with cytomegalovirus promoter. At the cellular level, expression of β-galactosidase, a marker substance of LacZ gene, was found in the gonads of recipient embryos at 6 (stage 29) and 10 days post-incubation (stage 36), as well as of newly hatched chicks. Of 32 recipients, 11 (11/32=34.4%) expressed β-galactosidase (Figure 3). As shown in Table 1, 6 (60%) and 4 (40%) gonads of recipient embryos at the stage 29 and 36, respectively, expressed β-galactosidase, while one hatched chick was detected.

As shown in Figures 4, LacZ gene was present in tested genomic DNAs retrieved from the gonads of embryos and hatched chicks. Such LacZ presence at the molecule level is independent of the expression of β-galactosidase in the gonadal cells. On the other hand, DNA was extracted from sperms of male individuals and subjected to PCR analysis

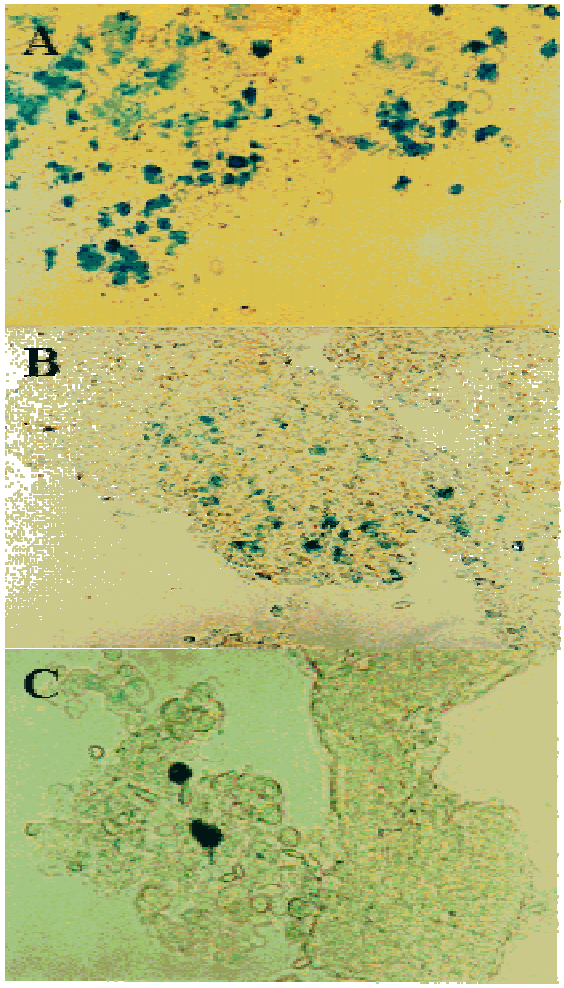

Figure 3. Expression of β-galactosidase in the recipient gonadal region. (A) Squashed view of 6-day-old embryo (stage 29). (B) Squashed view of 10-day-old embryo (stage 36). (C) newly hatched chick's gonad. (magnification (A) and (B) ×100, (c) ×200)

Table 1. Expression of β-galatosidase in the gonads of recipient embryos or newly hatched chicks

Sources of gonadal tissue	Stage or age of the samples	No. (%)[b] of recipient embryos	
		Examined samples	Expressed β-galatosidase
Embryos	Stage 29 (6-day-old)	10	6 (60)
	Stage 36 (10-day-old)	10	4 (40)
Hatched chicks	1 day[a]	12	1 (8.3)
Total	-	32	11 (34.4)

[a] Days after hatching.
[b] Percentage of the number of embryos or hatched chickens examined.

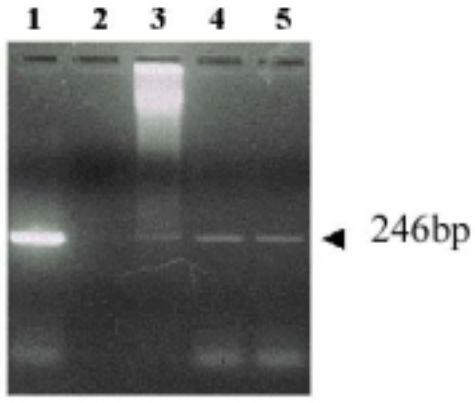

Figure 4. PCR analysis of DNA from the gonads of 6-day-old, 10-day-old embryo and hatched chick. PCR primers amplified Lac Z specific fragment of 246 bp. Lanes 1, positive control; 2, uninjected embryonic gonadal genomic DNA; 3, 6-day-old embryonic gonads (stage 29); 4, 10-day-old embryonic gonads (stage 36); and 5, newly hatched chick's gonads.

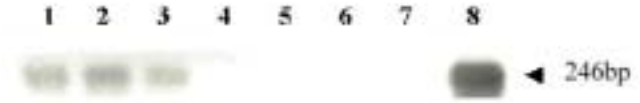

Figure 5. Southern blotting analysis of PCR products from sperm genomic DNA of the male recipient. Identity of PCR products were confirmed by hybridization blotting using probes labeled with [α-^{32}P] dCTP. Lanes 1, 2, 3 and 4, manipulated chickens; 5, female genomic DNA; 6,: male genomic DNA; 7, no template DNA; and 8, pCMVβDNA.

using primers for the LacZ sequences. Although amplification product was not definitely identifiable by ethidium bromide staining, Southern blot hybridization revealed the presence of LacZ sequence (Figure 5).

DISCUSSION

The results of this study demonstrated that gPGCs transfected with pCMVβ by electroporation can migrate and settle down into the gonads after being transferred into the blood vessel of the recipient embryos. Localization and expression of LacZ gene and its maker substance, β-galactosidase, were confirmed in the gonads retrieved from recipient embryos or hatched chicks developed from recipient embryos. Sex of recipient embryos did not affect the migration activity of transfected gPGCs and dornor gPGCs were evenly and randomly distributed into the recipient gonads.

Use of gPGCs might yield a number of benefits for transgenic chicken production, compared with the use of germinal crescent or circulatory PGCs. So, data on the migration and distribution of gPGC in recipients' gonads in this study strongly suggest that gPGCs are competent to migrate and normally settle down in the gonad even after transfer to the blood vessel of embryos. Furthermore, asynchronization between donor gPGCs from 5.5-day-old and 2.5-day-old recipient embryos might not affect the migration activity of donor gPGCs. This result is consistent with our previous finding showing the migration activity of gPGCs (Chang et al., 1997).

Transient transfection of target gene is one of major obstacles to effectively produce transgenic chicken. In our experiment, both recipient embryos of different ages and hatched chicks expressed LacZ gene or had LacZ gene-specific sequence. This result indicated that transfection of gPGCs with pCMVβ may be available for gene manipulation and electroporation method is also effective. Nevertheless, level of expression was decreased as development proceeded (Figure 4 and Table 1) and it might be due to dilution of foreign gene expression by extrachromosomal component. Further research will be required for stable expression and for maintaining foreign gene activity after being transfected into gPGCs.

It has been suggested that PGC can be used as a strong vehicle to produce transgenic chicken (Wentworth et al., 1989). In our previous report (Hong et al., 1998), foreign gene could be efficiently transferred to PGCs. On the other hand, we recently reported that gPGCs could maintain their specific characteristics as embryonic germ cells even after being cultured *in vitro* for up to 2 months (Han et al., 2002). By employing a number of technique from our previous results, *in vitro* gene manipulation using gPGCs become more feasible and all of our efforts in the field of transgenic biotechnology may contribute to effective production of transgenic chicken through germline transmission. We are

currently attempting to produce transgenic chicken from the progeny of germline chimera derived from the transfer of gPGCs.

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REFERENCES

Chang, I., A. Tajima, Y. Yasuda, T. Chikamune and T. Ohno. 1992. Simple method for isolation of primordial germ cells for chick embryos. Cell Biol. Int. Report 16:853-857.

Chang, I., D. K. Jeong, Y. H. Hong, T. S. Park, Y. K. Moon, T. Ohno and Y. J. Han. 1997. Production of germline chimeric chicken by transfer of cultured chick primordial germ cells. Cell Biol. Int. 21:495-499.

Hamburger, V. and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morph. 88:49-92.

Han, J. Y., R. N. Shoffner and K. S. Guise. 1994. Gene transfer by manipulation of primordial germ cell in the chicken. Asian-Aust. J. Anim. Sci. 7:427-434.

Han, J. Y., T. S. Park, Y. H. Hong, D. K. Jeong, J. N. Kim, K. D. Kim and J. M. Kim. 2002. Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained *in vitro* for an extended period. Theriogenology (in press)

Hong, Y. H., Y. H. Moon, D. K. Jeong and J. Y. Han. 1998. Improved transfection efficiency of chicken gonadal primordial germ cells for the production of transgenic poultry. Trans. Res. 7:247-252.

Love, J., C. Gribbin, C. Mather and H. Sang. 1994. Transgenic birds by DNA microinjection. Biotechnology 12:60-63.

Park, T. S. and J. Y. Han. 2000. Derivation and characterization of pluripotent embryonic germ cells in chicken. Mol. Reprod. Dev. 56:475-482.

Wentworth, B. C., H. Tsai, J. H. Lallett, D. S. Gonzales and G. Rajcic-Spasojevic. 1989. Manipulation of avian primordial germ cells and gonadal differentiation. Poultry Sci. 68:999-1010.