특강 II

Studies on the EfficientSeparation of Primordial Germ Cells and Introduction of Foreign DNA in the Chicken

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닭에서 원시생식세포의 효율적 분리 및 외래 유전자 전이에 관한 연구

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Abstract

Efficient separation of primordial germ cells from chick germinal crescent, blood and gonad

This study was conducted to determine the embryonic stages for the isolation of the highest number of PGCs and to improve PGCs enrichment method. The primordial germ cells (PGCs) from different sources of chick embryos were isolated. The embryonic stage having the highest number of PGCs from each sources was selected; 1-day-old embryos for germinal crescent (stage 6-8), 2.5-day-old embryos for blood (stage 17-18) and 5.5-day-old embryos for gonad (stage 27-28). The number of PGCs from one embryonic germinal crescent, blood and gonad was about 87±1.8, 103±4.0, and 932±10.9, respectively. The viability of PGCs after Ficoll from each sources was similar, showing approximately 70%. The PGCs enrichment method was improved using Ficoll density gradient centrifugation. After this step, the purity of PGCs from germinal crescent, blood, and gonad was 45±9.10%, 85±1.18%, and 86±0.19%, respectively. Also, PGCs were picked up by mouth pipette to improve the purity. This

improved method for the separation of PGCs from different sources will serve as a useful tool to preserve the foundation stocks of poultry and to produce germline chimeras.

Identification and introduction of foreign DNA in the recipient chicks

Expression of a foreign gene in embryos and embryonic germ cell as a gonadal PGCs would show the possibility to produce the transgenic chicken. However, transgenic chicken cannot be obtained using the transient transfection method by expression of early embryonic stage. And also, this studies must confirm the migration activity of foreign DNA transfected PGCs. Therefore, two methods of migration activity analysis were compared for the detection of exogenous PGCs. The labelled PGCs injected into 2.5-day embryos and then incubated until they reached stage 28, fluorescence was observed in the embryonic gonad of the embryos and W-specific DNA were not detected in blood and fibroblast cell of male embryos but embryonic gonad. Through this fact, gPGCs can be used as a genetic vector. In this experiment, foreign DNA was transfected into gPGCs using electroporation by Hong et al (1998). And these cells were injected into recipient embryos and estimated the efficiency of gene expression to the developmental stage, 6-day-old embryo, 10-day-old embryo and hatched chick. gPGCs transfected foreign gene producted lacZ gene were transferred sperm after sex maturation. This result indicated that gPGCs had characteristics of circulating PGCs and were useful genetic vector for the production of transgenic chicken.
In conclusion, these results indicate that the improved method for the separation of PGCs from different sources will serve as a useful tool to preserve the foundation stocks of poultry and to produce germline chimeras.

INTRODUCTION

Primordial germ cells (PGCs) have all the genomic information of an individual species and the unique cellular and molecular mechanisms with

which to pass this information from generation to generation. Avian PGCs originate in the epiblast (Eyal-Giladi et al., 1981) and translocate into the hypoblast layer of the germinal crescents (Swift, 1914; Clawson and Domm, 1969). In normal chick embryo, most of the PGCs migrate into gonadal ridge via the blood vascular system (Urven et al., 1988; Ukeshima and Fujimoto, 1984) and settle down in the gonadal primordium (Meyer, 1964; Nieuwkoop and Sutasurya, 1979) where they rapidly differentiate into either spermatogonia in males or oogonia in females (Nakamura et al., 1988). Development in molecular biology has allowed us to assess the genetic characteristics to elucidate the genome structure and to accelerate the genetic improvement of animal. By producing the transgenic livestocks, we can artificially manipulate the growth of animal sources and breed the disease resistant line. Also, useful protein can be produced from transgenic animal as a bioreactor. However, no efficient techniques have been developed for the production of transgenic birds; eggs of birds are large and contain lots of yolk, making them very difficult to be handled. The primordial germ cells (PGCs) have been considered as a vector for the production of transgenic chicken (Simkiss et al., 1989; Savva et al., 1991; Han et al., 1994). The genetic modification can be transferred by the manipulation of the PGCs. In most studies, PGCs have been isolated from blood for the production of germ-line chimera. Recently, Chang et al. (1995) separated PGCs from gonad which has relatively large number of PGCs compared to the number of PGCs from blood. And a number of techniques have been developed for purification of PGCs from several developmental sources. These include differential adhesion, simple disaggregation by EDTA (Allioli et al., 1994), Ficoll gradient centrifugation (Yasuda et al., 1992), and fluorescence-activated cell sorting (Wentworth et al., 1989). In FACS and MACS cases, PGCs were identified using monoclonal antibodies (EMA-1 or TG-1, respectively) which recognize epitopes expressed by PGCs during embryonic development. Although these techiques enabled to isolate of highly purified PGCs during embryonic development that antibody is expressed by PGCs. For the production of transgenic chicken,

several techniques have been used to introduce foreign DNA into the avian genome. These have included coating the sperm with DNA before fertilization (Gavora et al., 1991; Gruenbaum et al., 1991), attempting microinjection into the male pronucleus of the fertilized egg (Love et al., 1992; Sang and Perry, 1989). The transfer of exogenous DNA into a chicken germline has been achived by replication-competent or replication-defective retroviruses in developing embryos as vectors (Shuman and Lee, 1988; Bosselman et al., 1986; Briskin et al., 1991; Salter et al., 1986, 1987). Although retrovirus vectors made efficient gene transfer possible in chicken, these have a possibility of infectious. Primordial germ cells (PGCs) have therefore been considered as a gene transfer vector for production of transgenic chicken (Han et al., 1996, 1994; Li et al., 1995; Vick et al., 1993; Watanabe et al., 1994; Allioli et al., 1994).

The purposes of this study are 1) to improve the method of PGCs separation method using Ficoll density gradient centrifugation and acquire large number of PGCs from germinal crescent, blood and gonad, 2) to identify that cultured and proliferated PGCs *in vitro* keep the characteristics of germ cells during embryonic development, 3) to obtain the function gene expressed adult chicken.

Matrials and Methods

1. Experimental stocks

The experimental stocks were inbred lines of Korean Ogol Chicken (KOC) and White Leghorn (WL) which have been maintained at Experimental Animal Farm, Seoul National University, Korea.

2. Fertilized egg preparation

Fertile eggs were produced from KOC or WL female artificially inseminated weekely with pooled semen from males. Eggs prewarmed to room temperature, were incubated at 37.5℃ and 60-70% relatively humidity in incubator. The eggs were rotated 180°, 12 times per day. The fertilized eggs from KOC or WL were incubated for 1-day(stage

6-8), 2.5-day(stage 17-18), or 5.5-day(stage 27-28) at 37.5℃ (Hamburger and Hamilton, 1951).

3. Isolation of Primodial Germ Cells

From Germinal crescent

The shell, cleaned with 70% ethanol, was broken and albumin and yolk were separated. The albumin was discarded and the yolk was placed into a specimen dish containing chicken Ringer's solution prewarmed to 38℃. The embryo was cut away from the yolk with a microdissecting scissors, removed with a sterile wide mouth pipette and transferred to a 60 mm^2 petri dish containing warmed Ringer's solution. The germinal crescent was excised with the aid of a fine point forceps with a scalpel under the stereo microscope (Nikon, SMZ-1B, Japan), and placed in a warmed modified Ringer's solution composed of normal chick Ringer's without Ca^{++} plus additional sodium citrate and glucose. The cells were easily dissociated in this media and contained PGCs (Clawson and Domm, 1969; Fujimoto et al., 1976).

From embryonic blood vessels

The blood was collected from a vitelline artery and terminal sinus of 2.5 day old chicken embryos with a fine glass pipette with an inner diameter at the tip of about 30-40μm. The 2.5-day-old embryo blood contained circulating PGCs in the blood vessels. The blood (1~5$\mu\ell$) was collected from an embryo and was dispersed into the DMEM containing 10% fetal bovine serum.

From embryonic gonad

Whole embryo was carefully rinsed twice with phosphate buffered saline (PBS) to remove yolk and blood, and then transfered to 60 mm^2 glass petri dish coated with dental wax (Miles, USA). The embryo's head, wing, bud and tail regions were fixed with pins and embryonic gonads were dissected and carefully rinsed with PBS. The gonadal PGCs were released by pipetting up and down with 30-gauge needle

(mechanical treatment) or by treating with 0.5% trypsin-0.2% EDTA for 10 min at 37℃ (enzyme treatment) (Allioli et al., 1994).

4. Identification of PGCs

Identification of PGCs were stained by periodic acid-schiff (PAS) reaction(Allioli et al.,1994). PGCs were fixed with 10%(w/v) formaldehyde solution in PBS for 10 min and dried at room temperature and fixed with absolute methanol. The fixed PGCs were treated with 500$\mu\ell$ of periodic acid for 10 min and rinsed with distilled water. Then the cells were stained with 1 ml of Shiff's reagent(Sigma) for 15 min and washed with distilled water three times. In constrast to unreactive somatic cells, the cytoplasm of PGCs was stained in magenta.

PCR for sexing of embryonic gonadal PGCs

1-2$\mu\ell$ blood from 5-day-old embryo blood vessel was extracted using microcapillary pipette and transferred into fresh 1.5ml tube with 100$\mu\ell$ ddH_2O. Before PCR, for the release of genomic DNA, each 10$\mu\ell$ blood sample was mixed with 20$\mu\ell$ ddH_2O in 0.5ml PCR tube, laid 40$\mu\ell$ mineral oil and boiled at 97℃ for 3minutes and 55℃ for 3minutes five cycles. And then, 20$\mu\ell$ PCR reaction containing 4$\mu\ell$ of dNTP mixture, 5$\mu\ell$ of reaction buffer, 2$\mu\ell$ of forward primer, 2$\mu\ell$ of reverse primer, 0.4$\mu\ell$ of Taq polymerase and 34.6$\mu\ell$ of ddH_2O was mixed into boiled blood sample through mineral oil layer. Amplification was conducted in a Perkin Elmer Cetus DNA Thermal Cycler. PCR was followed by 30 cycles of denaturation for 30 sec at 94℃, annealing for 30 sec at 60℃, and extension for 30 sec at 72℃. PCR produced a W-chromosome specific DNA fragment in female embryo, not in male embryo. These primer were; forward 5′-CCC AAA TAT AAC ACG CTT CAC T-3′ and reverse 5′-AAA TGA ATT ATT TTC TGG CGA C-3′.

PKH26 labelling of gonadal PGCs

Collected PGCs from 5.5-day-old chick embryos were labelled with a PKH26 fluorescent staining kit A (ZYNAXIS Cell Science INC.). The

method of fluorescent staining(Horan et al., 1990) described in the manual of the kit was strictly followed except that volumes of the reagents in each staining step were all reduced proportionally to 1/16 in 1.5ml tubes. Samples of labeled PGCs were injected into blood vessel of 2.5-day-old chick embryos. The recipient embryos were then incubated for 3 days at 37.5℃. After incubation, the embryos were removed from the dish, fixed with formaldehyde in PBS, and embedded sequentially and then the sample of embryos were molded with paraffin and trimmed in Pika (Seiko, Ltd) microtome(10㎛ sections were obtained).

Identification of migrated PGCs using W chromosome specific DNA

The fertilized eggs for the recipient WL embryo were incubated at 37.5℃ for 2.5 days. The sharp end of these eggs were windowed. Approximately 200 female sexed PGCs from KOC were injected into the bloodstream through the dorsal aorta of recipient embryos. The egg window of recipient embryos was sealed with paraffin film twice. Then the sealed eggs were incubated at 37.5℃ for 3.5 days. Gonad were dissected from 6-day-old embryo and minced with scissors. The DNA from gonad of the recipient embryo was extracted by the boiling methods (1994) and subjected to 30 cycles of polymerase chain reaction (PCR) as the same method for sexing of embryonic gonadal PGCs. Chicken genomic DNA and gonad DNA from uninjected embryo were used as negative control.

5. Ficoll density gradient centrifugation

PGCs were concentrated according to the method of Chang et al. (1992) with little modification. PGCs from germinal crescent, blood or gonads were mixed with 300 μl of 10% Ficoll solution and gently added over 900 ㎕ of 16% Ficoll solution in microcentrifuge. After centrifugation at 800 x g for 30 min, the PGC-rich fraction located at the border layer between 10% and 16% Ficoll solution was collected. This PGCs-rich fraction was diluted with medium 199 and centrifuged at 400 x g for 5

min.

6. PGCs picking for the high purity

This washing step was repeated. PGCs from germinal crescent were picked up under inverted microscope by mouth pipette to improve the purity. The number of PGCs was counted using hematocytometer. The viability of PGCs was determined using trypan blue exclusion method. The purity of PGCs was measured by the ratio of PGCs in the total cell population.

7. *In vivo* expression of β-galactosidase

Plasmid DNA transfection

The reporter construct used for transfection was pCMVβ which contains the bacterial lac Z gene expressed by a CMV promoter. Plasmid DNA was prepared using large scale matrix-based purification according to manufacturer's recommendatins(Qiagen). The conditions for electroporation were essentially the same as reported by Hong et al.,(1997). Briefly, 1.6×10^5 gonadal cells as a mixture of gPGCs and stromal cells were mixed with 20㎍ of plasmid DNA in 400㎕ of Opti-MEM (Gibco) for 1min at room temperature. Electroporation conditions were optimized in PGCs. 250V was chosen with a capacitance of 950㎌ for PGCs. Electroporation was done with DMSO (1.25% v/v). After electroporation gonadal cells were cultured in DMEM with 10% FBS, 10 ng/ml insulin-like growth factor-1 (Sigma), 10 ng/ml bFGF (Sigma), and 10 unit/ml of murine LIF (Sigma) for 24 hours.

DNA extraction and PCR analysis

The approximately 200 PGCs transfected with pCMVβ were injected into the bloodstream through the dorsal aorta of the 2.5-day-old recipient embryos. The egg window of recipient embryos was sealed with paraffin film. Then the sealed eggs were incubated at 37.5℃ for 3.5 days. Gonad were dissected from 6, 10-day old embryo and hatched chick and minced with scissors. The DNA from gonad of the recipient embryo was

extracted by boiling extraction methods and subjected to 30cycles of polymerase chain reaction (PCR). The three steps involved denaturation at 95℃ for 1min, primer annealing at 65℃ for 1min and extension at 72℃ for 40s. Chicken genomic DNA and gonad DNA from uninjected embryo were used as negative control. Primers derived from LacZ were 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 were analyzed on 2% agarose gels.

X-galactosidase expression

To screen the transfected and colonized PGCs, the embryonic and young chick gonads were removed at 5 to 10-day-old embryos and one-day-old chicks gonad, respectively. After fixation and rinsing, the gonads were treated with X-gal, they were squashed on a slideglass. The positive gonads were examined for blue colored cells. In ths study, gonad screening for exogenous β-galactosidase expression was performed at pH 7.4 for 4 to 8 hours.

Histochemicaly detection

Embryos were employed in the bouin fixative solution for 2 days, and then dehydrated in graded series of ethyl alcohol (50, 70, 80, 90, 95 and 100%) for 60min and transfer to xylene: ethyl alcohol mixture (1:3, 1:1, 3:1), xylene for each 60min at room temperature. Embryos were embedded in paraffin (melting point 60℃): xylene mixture (1:3, 1:1, 3:1), paraffin for each 60min. And then sample were molded and trimmed in Pika (Seiko, Ltd) microtome where 8㎛ sections were obtained. They were loosened in 50-70% ethyl alcohol and hot water (40-50℃), and mounted on the slide glass, dryed on the hot plate (30℃) for 3 hours. For X-gal staining, gonad sections were fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS) for 5min, and washed twice with PBS, and then stained at 37℃.

Southern Hybridization

To confirm PCR products, Southern hybridization was performed. Amplified DNA samples were electrophoresed in 1.0% agarose gel. After electrophoresis, agarose gel was denaturated with alkaline buffer (0.4 M NaOH, 0.6 M NaCl) for 50 minutes under the gentle rocking. And then the gel was washed with water two times and neutralized in 0.5 M Tri-Cl (pH 7.4), 1.5 M NaCl solution. The DNA in a gel was transfered to nylon membrane (Boehringer Mannheim Biochemica) by capillary transfer. The membrane was fixed by ultraviolate for 3 minutes. PCR product of LacZ plasmid DNA was used as probe of hybridization. Probe labeling was proceeded according to the procedures of random primed labeling kit (Boehringer Mannheim Biochemica) and ^{32}P radioisotope was used for labeling of probe.

Hybridization was proceeded at 63℃. Blotted nylon membrane was prehybridized with hybridization solution (0.3 M Na-phosphate buffer, 7% SDS, 1mM EDTA, 1% BSA) at 63℃ for 1 hour. After discard the prehybridization solution, the membrane was hybridized with hybridization solution containing probe for 18 hours under the gentle rocking. Membrane was washed twice with 2 X SSC, 0.1% (w/v) SDS for 5 minutes at room temperature and washed again twice with 0.1 X SSC, 0.1% (w/v) SDS at 63℃. The membrane was exposed to a Kodak BioMax film for 12 hours at -80℃ in a deep freezer and developed.

RESULTS AND DISCUSSION

Efficient separation of primordial germ cells from chick germinal crescent, blood and gonad

PGCs are the embryonic cells which are antecedent to mature sex cells. Various attempts have been made to produce germline chimeras by transfer of PGCs into the host embryos in Aves (Naito et al., 1994). This study was conducted to isolate and increase the purity of PGCs. The purity of PGCs from different sources, i.e., germinal crescents, blood, and gonads was compared (Table 1). The PGCs originated from epiblast and migrate into gonadal ridge via the blood vessel. Thus, the embryonic

stages having the highest number of PGCs were selected; stage 6 to 7 of germinal crescent, stage 17-18 of embryonic blood, and stage 27-28 of embryonic gonads. The percentage of PGCs in the total cell population was approximately 0.2-6% (Table 1). The purity of PGCs from blood and gonad were reported as 0.3% and 5%, respectively (Yasuda et al., 1992; Allioli et al., 1994). Matsumura and England(1993) reported the presence of PGCs from stage 4-8 embryo germinal crescent. However, there had been no report about isolation of PGCs from germinal crescent. This study reports the isolation of PGCs from germinal crescent with Ficoll gradient centrifugation.

Chang et al.(1992) concentrated blood PGCs using Ficoll density centrifugations. They dispersed blood cells into medium 199 and mixed with 16% Ficoll solution, which overlaid with 6.3% Ficoll solution. Suspension cells were mixed with 10% Ficoll solution and added over 16% Ficoll solution, exchanging the Ficoll layer. This modification makes it easy to collect the PGCs. After Ficoll gradient centrifugation, the purity of PGCs from germinal crescent, blood, and gonads was 45%, 85%, and 86%, respectively(Table 1). The purity of PGCs was increased 15-800 fold by Ficoll gradient centrifugation.

Table 1. Purity of PGCs from embryonic germinal crescent, blood and gonad.

Sources (embryonic age)	Purity of PGCs(%)[1]	
	Treatment I[2]	Treatment II
Germinal Crescent (1 day)	0.174±0.005	45.1±9.10
Embryonic Blood (2.5 day)	0.076±0.010	85.4±1.18
Embryonic Gonad (5.5 day)[3]		
Mechanical	4.174±0.011	80.9±0.08
Enzyme	6.431±0.062	86.1±0.19

[1] *The purity of PGCs was the ratio of PGCs in the total cell population. In 10 repeated experiments on 1-, 2.5-, or 5.5-day-old embryos, the average purity of PGCs±SD was obtained.*

[2] *Treatment I; before Ficoll treatment, Treatment II; after Ficoll treatment*

[3] *Gonadal PGCs were released by mechanical treatment (pipetting with 30-gauge needle) or enzyme treatment (0.5% Trypsin-0.2% EDTA for 10 min at 37℃).*

Table 2. Number and viability of PGCs from germinal crescent, embryonic blood and gonad

Sources(embryonic age)	Number[1] (Means±SD)	Viability(%)
Germinal Crescent (1 day)	87.5±1.8	67.3
Embryonic Blood (2.5 day)	103.2±4.0	75.7
Embryonic Gonad (5.5 day)	931.8±10.9	79.4

[1] *The number of PGCs from one embryo was counted. In 10 repeated experiments on 1-, 2.5-, or 5.5-day-old embryos, the average number of PGCs±SD was obtained.*

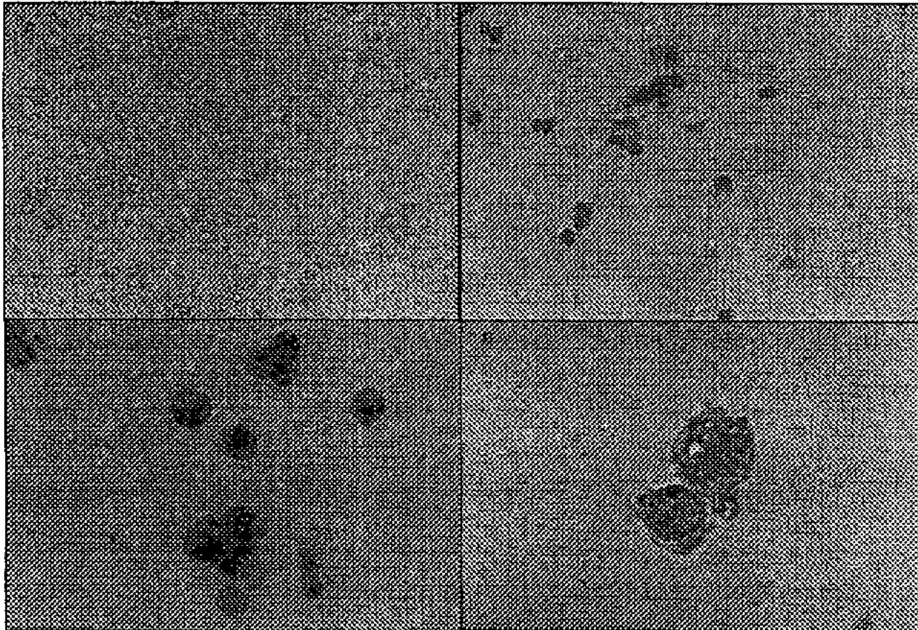


Figure 1. Concentrated PGCs from germinal crescent (x125).
(upper) *(A) before Ficoll (B) after Ficoll. PGCs from germinal crescent of stage 4-8 embryos were separated by Ficoll density gradient centrifugation and PAS stained.*
(lower) *High concentrated PGCs by picking up under inverted microscope using mouth pipette (A) x400 (B) x1000. PGCs were stained with PAS.*

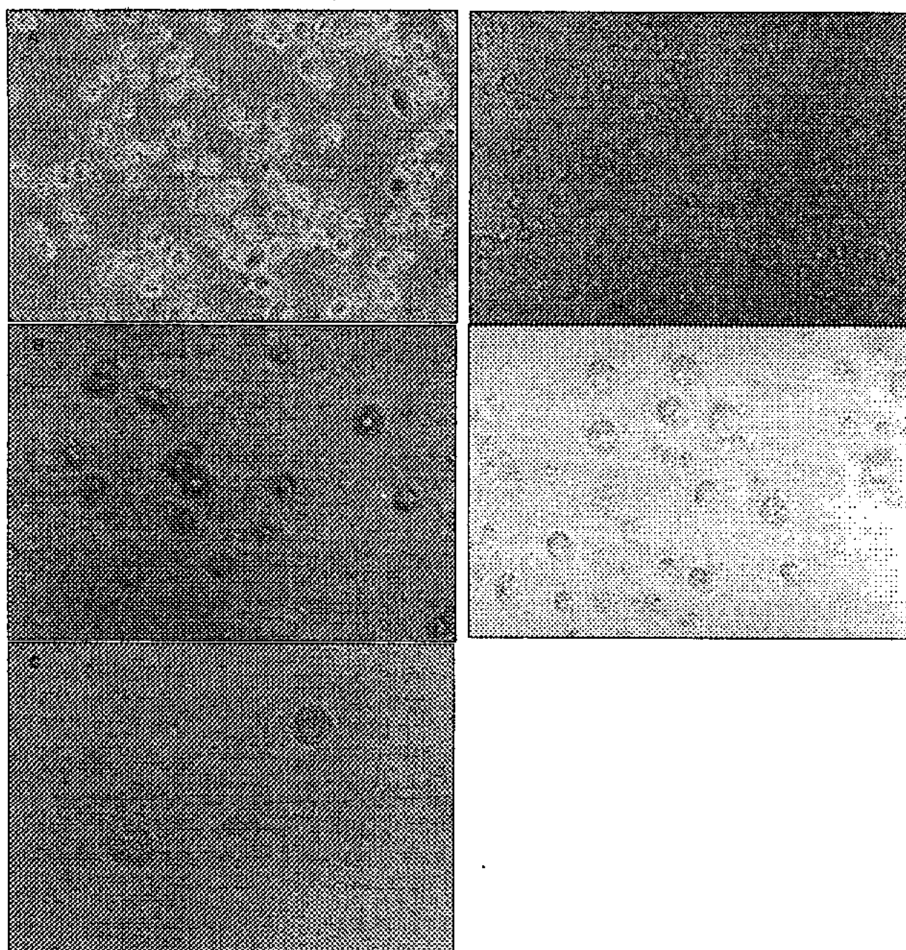


Figure 2. (left)Concentrated PGCs from 2.5-day-old embryonic blood vessel. (A) blood before Ficoll (x250) (B) PGCs after Ficoll (x250) (C) PGCs of x1000 magnification. PGCs from blood of stage 18-19 embryos were separated by Ficoll gradient centrifugation.
***(right)** Concentrated PGCs from 5.5-day-old embryonic gonad.*
(upper) *gonadal cell suspension before Ficoll (x250);* (lower) *PGCs after Ficoll. PGCs from embryonic gonad of stage 28 embryos were separated by Ficoll gradient centrifugation (x400).*

The purity of PGCs from germinal crescent was lower compared to other sources. This might be due to that PGCs from germinal crescent were not clearly isolated in yolk cell and other cells (Figure 1A, 1B; upper). Also, we picked up PGCs under inverted microscope by mouth pipette to improve the purity of PGCs and could isolate pure PGCs (Figure 1A, 1B; lower). There was no difference in the purity of gPGCs

between mechanical and enzyme treatment (Table 1). Table 2. showed the number and viability of PGCs isolated from different sources.

Routinely 25-30 viable PGCs were obtained from blood of one embryo (Yasuda et al., 1992). This result showed that the number of PGCs isolated from one embryonic germinal crescent, blood and gonad were about 87, 103, and 932, respectively. The number of PGCs isolated from one embryonic gonads was about 10-fold higher than those of germinal crescent or blood (Table 2). Figure 2. showed the PGCs isolated from 2.5-day-old embryonic blood(left) and 5.5 day old embryonic gonad(right) 2.5-day-old embryonic blood (Figure 2A), PGCs after Ficoll gradient centrifugation(Figure 2B) and PGCs of 1000× magnified(Figure 2C). Figure 2(C). showed the PGCs in the blood vessels, which appeared round or oval shape. Fujimoto et al.(1976) reported that in blood smear, their diameter range from 11-22㎛ with an average of 14㎛. The nucleus is large, 8-10㎛ in diameter, and its eccentric placement distinguishes the PGCs from somatic tissue. Figure 2 (right). showed the gonadal PGCs. Gonad seems ideal source for mass collection of PGCs. Gonadal PGCs (gPGCs) offer advantages over two other sources, ie., germinal crescent or blood; the proportion of PGCs is the highest, ie., 5% in gonad, 1.5% in germinal crescent and 0.003% in embryonic blood, PGCs in gonad activity proliferate once they reach at gonads (Wentworth et al., 1989; Allioli et al., 1994). The viable PGCs were determined using trypan blue exclusion method. There was no differences in the viability of PGCs among different sources, showing approximately 70%. This improved method for the separation of PGCs from different sources will serve as a powerful tool to preserve the foundation stocks of poultry and to produce germline chimeras.

Identification and introduction of foreign DNA in the recipient chicks

Expression of a foreign gene in embryos and embryonic germ cells as gonadal PGCs would show the possibility of transgenic chicken production. However, transgenic chicken cannot be obtained using the

transient transfection method by the expression of early embryonic stage. And also, these studies must confirm the migration activity of foreign DNA transfected PGCs. Therefore, two methods of migration activity analysis were compared for the detection of exogenous PGCs. First, PGCs collected from 5.5-day-old embryonic gonad were labelled with a PKH26 fluorescent staining kit A. The labelled PGCs injected into 2.5-day-old embryos and then incubated until they reached stage 28, fluorescence was observed in the embryonic gonad of the embryos (Figure 3(left), 3(right)). Figure 3(left). showed PAS staining of gonad under non-fluorescent microscope. Some PGCs were observed to stain both exogenous and endogenous. The PKH26 positive PGCs in the gonad were identified as exogenous PGCs from 5.5-day-old embryonic gonad (Figure 3(right). Chang et al. (1992) suggested that PGCs might have differentiated and loose the ability to migrate and settle in the germinal ridge. However, they reported that donor PGCs derived from 5-day-old embryo were able to re-enter recipient gonad. Present study has the same result. Sometimes, PKH26 positive cells lead false positive. Therefore, Genetic analysis was tried to gain correct result. Generally, gender of hetero chromosome in female is opposite to that of mammalian.

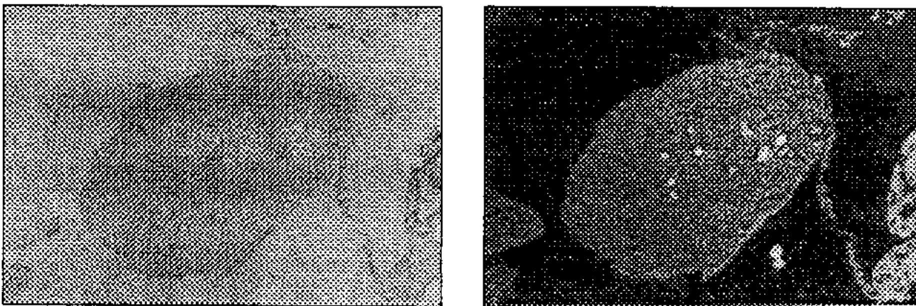


Figure 3. Donor PGCs were found in the recipient embryo. Donor PGCs were observed in the gonadal region of recipient embryo. (left) Both donor and recipient PGCs were stained with PAS. (right) Recipient embryo(3-day-old) was observed by the fluorescence microscope three days after transferred of PKH26 labelled donor PGCs.

PCR using W-specific DNA sequence in female could determine sexes. Therefore, using these methods, sex of female gPGCs injected into 2.5-day-old male embryos was determined. After injecting female gPGCs

into male recipient embryos, W-specific DNA was detected using PCR (Figure 4). Figure 4. showed that W-specific DNA was not detected in blood and fibroblast cell of male embryos but embryonic gonad. W-specific DNA in female was all detected in blood, fibroblast cell and embryonic gonad. gPGCs were able to migrate as PGCs in blood and germinal crescent. In other words, PKH26 labelled cells in embryonic gonad (Figure 3(right)) they were not false positive. Through this fact, gPGCs could be used as a genetic vector. In this experiment, foreign DNA was transfected into gPGCs using electroporation by Hong et al (1997). Figure 5 (upper left). showed that the lacZ was expressed in the gPGCs of transfection by X-gal staining. And these cells were injected into recipient embryos and were estimated the efficiency of gene expression to the developmental stage, 6-day-old embryo, 10-day-old embryo and hatched chick (Figure 5A, 5B, 5C). Figure 5A. showed the expression of 5-day-old embryonic gonad using squashed method. After lacZ was transfected, very many PGCs were expressed by X-gal.

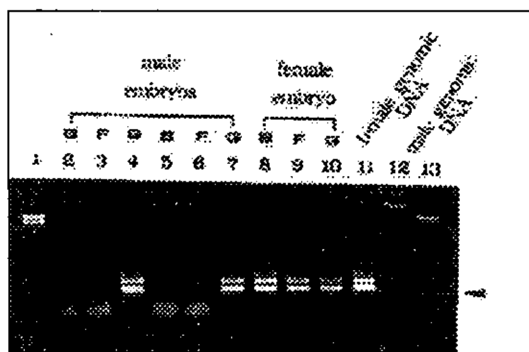


Figure 4. PCR product of female specific DNA primer in male embryos. PCR product was produced only gonad in male injected female PGCs. Lane 1 : size marker, λ DNA digested with Hind III, lane 2, 5: blood of male embryos, lane 3, 6 : fibroblast cell of male embryos, lane 4, 7 : gonad of male embryos, lane 8, 9, 10, 11 : positive control, lane 12 : negative control; male genomic DNA.

Figure 5B. showed the expression of 10-day-old embryonic gonad was expressed lower than that of 5-day-old embryonic gonads. Figure 5C. showed the expression of chick gonad by paraffin embedding section method. LacZ gene was expressed in chick gonad but had low efficiency

(Table 3). Generally, PGCs migrate into gonad and differentiate from 7 days to chick embryonic developmental stage. Because of this fact, efficiency of gene expression was decreased by transient transfection.

Figure 5. showed lacZ gene was expressed by X-gal after 10-day-old embryo which was introduced the production of transgenic chicken by embryonic gonadal PGCs. To identify this result, PGCs were isolated from each developmental stage, DNA was extracted and lacZ gene was screened using PCR.

Figure 6. showed PCR product by previous method. P is positive control, pCMVβlacZ plasmid DNA and N is negative control. Lane 1, 2, 3 are DNA extracted embryonic gonad of 5-day-old, 10-day-old and hatched chick gonad, which were all detected in lacZ gene. These testified that gene was not disappeared and expressed stablely.

Transfected PGCs were injected into recipient embryos, were bred hatched chick until sex maturity, were extracted DNA from male sperm and were analyzed lacZ gene (Figure 7(left)). PCR product were prepared for Southern blots and were probed with a ^{32}P-labelled lacZ plasmid fragment. Figure 7 (left). showed that lane 1, 2, 3, 4 were sperm DNA of experimental stocks, lane 5, 6 were negative control and lane 7 were positive pCMVβlacZ plasmid DNA. Lane 1, 2, 3 were identified lacZ gene. These mean that PGCs transfected foreign lacZ gene produced sperm even after sexual maturation.

Figure 7(right). showed the analysis of the sperm DNA produced by lacZ gene transfection using gPGCs cultured for 2 months. This result showed gPGCs had the characteristics of PGCs and useful genetic vector although cultured for 2 months. Because germline chimeric chicken was not produced, transgenic offspring couldn't be found.

Production of transgenic chicken using PGCs must have high efficiency of gene transfection and germline transmission. Genetic manipulation of chicken production traits such as disease resistance, growth and reproduction may be possible in the longer term. Relatively short generation time (less than six months) and the high production capabilities of chicken, make it possible to produce the production of

transgenic chicken and expand into productive flocks faster than other farm species.

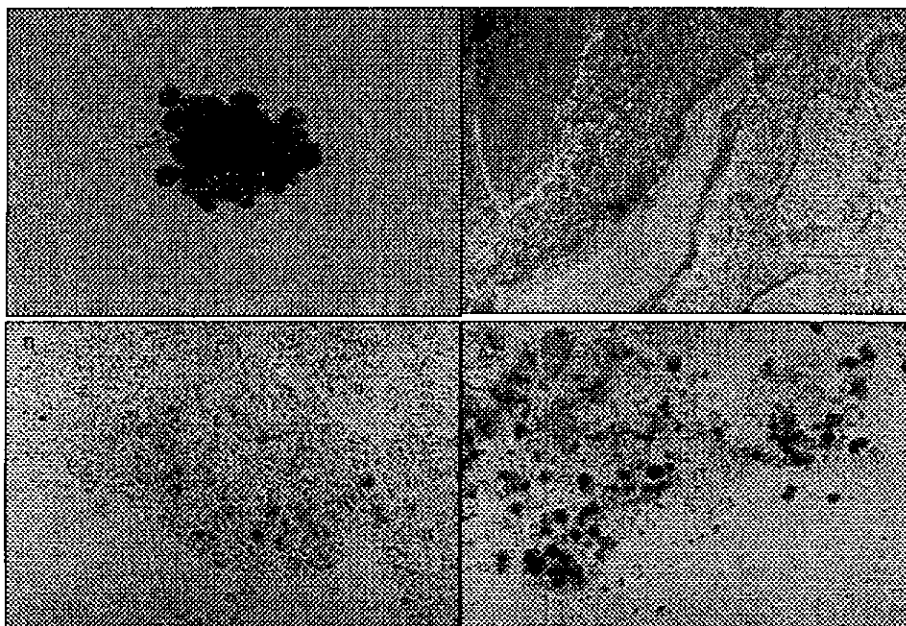


Figure 5. Detection of lacZ expression in the gonadal region of a transgenic chick embryo. Embryos were injected with electroporated lacZ plasmid DNA and stained with X-gal for β-gal activity.
(Upper left) β-galactosidase was expressed in PGCs after transfection of pCMVβlacZ by the electroporation (A) squashed view of embryo at stage 29 (B) squashed view of embryo at stage 36 (C) a section of gonad of a hatched chick.

Table 3. Number of embryos and young chicken expressed exogenous lacZ gene.

	Embryos		chicken[a]	Total
	5 days	10 days		
No. of screening	10	10	12	32
Positive No.	6	4	1	11

[a] hatched 1 day old chicken gonad

Figure 6. PCR analysis of DNA from the gonads 5-day-old, 10-day-old and hatched chick embryo. P: positive control; N: uninjected embryonic gonadal genomic DNA; lane 1 : 5-day-old embryonic gonad; lane 2 : 10-day-old embryonic gonad; lane 3 : hatched chick's gonad

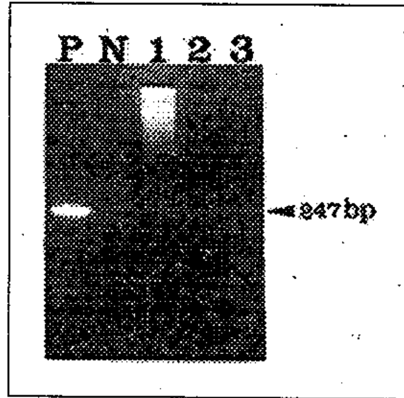
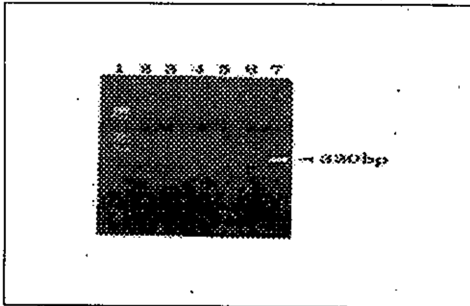
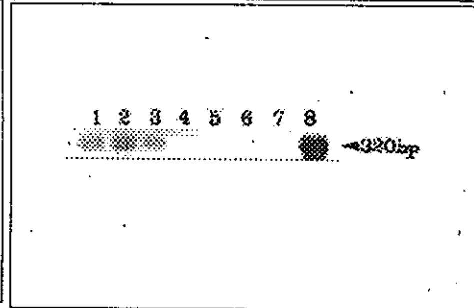


Figure 7. (left) Southern blotting analysis of PCR product from lacZ transferred to sperm DNA. PCR product of pCMV β lacZ DNA was used as probe and labelled with ^{32}P. Lane 1, 2, 3, 4 : manipulated chickens; lane 5 : female genomic DNA; lane 6 : male genomic DNA; lane 7 : no template





DNA; lane 8 : pCMV β lacZ DNA.
***(right)** PCR analysis of DNA from the sperm of male chicken was injected gPGCs cultured for 2 months.*
Lane 1 : size marker; 1kb ladder; lane 2, 3 : candidate stocks; lane 4 : female genomic DNA; lane 5 : male genomic DNA; lane 6 : no template; lane 7 : pCMV β lacZ DNA

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적 요

닭의 생식반월, 혈액, 그리고 원시생식기로부터 원시생식세포의 효율적 분리

본 연구는 다양한 발달단계로부터 원시생식세포를 분리하였다. 원시생식세포가 가장 많이 분포하고 있는 일령을 선택하였다. 1일령 배자의 생식반월, 2.5일령 배자의 혈액 그리고 5.5일령 배자의 원시생식기를 이용하였다.

원시생식세포 분리 개수는 생식반월, 혈액, 그리고 원시생식기 각각 87±1.8, 103±4.0, 932±10.9개였다. 각 발달단계별 원시생식세포의 생존율은 약 70%로 비슷하였다. Ficoll방법의 개선으로 원시생식세포 분리 방법은 개선되어, 분리율은 생식반월, 혈액, 그리고 원시생식기가 각각 45±9.10%, 85±1.18%, 그리고 86±0.19% 였다. 또한, 원시생식세포는 분리율을 높이기 위하여 마우스파이펫을 이용하여 개체로 분리하였다. 각기 다른 배발달 단계에 따라 분리한 원시생시세포 분리 방법은 종계의 보존 및 생식선 카이메라 생

산에 유용할 것이다.

<u>원시생식기 유래 원시생식세포 주입에 의한 외래 생식세포 확인 및 유전자 전이</u> 형질전환 닭 생산은 유전자의 발현 및 기능을 연구하는데 매우 유용한 도구이다. 그러나 이러한 목적을 위하여 개발된 방법은 조류에 적용하기가 어렵다. 조류는 포유류와 달리 형태학적 발생학적 차이로 인하여 미세주입과 같은 방법으로 형질전환 닭을 생산하기가 매우 어렵다. 원시생식기 유래 원시생식세포의 발현과 성세포 발달은 형질전환 닭 생산에 가능성을 보여준다. 그러나 대부분의 유전자 전이가 초기 배자에서 일시적으로 발현되기 때문에 어렵다. 그리고 또한, 본 연구에서는 이렇게 유전자 전이 또는 외부에서 처리한 원시생식세포의 이동능력에 대하여 확인을 할 필요가 있었다. 그러므로, 외래 원시생식세포에 형광염색하여 주입하거나 수컷배자에 암컷 원시생식세포를 주입하여 DNA 검색하는 방법을 이용하여 외래 원시생식세포가 유전자 전이체로 사용할 수 있음을 확인하였다. 그리고 또한 전기 충격법을 사용하여 유전자를 전이 시킨 후 이를 주입하여 배자 발달단계에 따라 유전자 발현 유무를 확인하였다. 결론적으로 각기 다른 배발달 단계에서 분리된 원시생식세포 멸종 조류의 보존 및 생식선 카이메라 생산에 유용한 도구임을 확인하였고 궁극적으로 성숙된 닭의 정액에서 외래 유전자가 전이된 닭을 생산 할 수 있었다.