家禽誌 23(2) : 61~69(1996) Korean J. Poult. Sci. 23(2) : 61~69(1996)

Expression of *Lac Z* Gene in Young Chick Gonad by the Transfected Primordial Germ Cell Injection

Jae Y. Han, D. S. Seo, Y. H. Hong, D. K. Jeong, K. D. Choi and Y. S. Shin[1]

Department of Animal Science and Technology,
Seoul National University, Suweon, Korea 441-744

Lac Z 유전자가 전이된 원시생식세포 주입에 의한 병아리 생식기내 유전자 발현

한재용 · 서동삼 · 홍영호 · 정동기 · 최강덕 · 신영수[1]

서울대학교 농업생명과학대학 동물자원과학과

ABSTRACT

Primordial germ cells (PGCs) were manipulated as part of the system to produce transgenic chickens. PGCs were isolated from the germinal crescent of developmental stage 6 to 8 donor embryos of the Korean Native Ogol Chickens (KNOC). These PGCs were transfected with plasmid DNA containing the *lac Z* gene by liposome mediated transfection methods. The *lac Z* gene was transfected and expressed in the PGCs. These transfected PGCs were injected into the germinal crescent of White Leghorn embryos (stage 6 to 8). The injected transfected PGCs migrated via the circulatory system into the future gonad and expression observed in the gonads of 3 day old chick. Of the 47 embryos and 3 day old chickens, one positive PGCs gonad from sacrificed young chickens was detected by appearance of blue cells. Plasmid DNA with the foreign gene was incorporated into the population of germ cells in the gonad.

These results demonstrate that PGCs can be transfected and then transferred for colonization into the gonad, and show the potential to ultimately manipulate the genetic material of the chicken germline.

(Key words: primordial germ cells, *Lac Z*, germline chicken)

INTRODUCTION

In aves, PGCs arise from the epiblast and migrate into the hypoblast. During gastrulation, the PGCs migrate anteriorly via the hypoblast and reside in the extraembryonic area, the germinal crescent, located in the anterior region of the blastodisc (Eyal-Giladi et al., 1981; Urven et al., 1988). After a maturation period these cells migrate into gonadal ridge via the vascular network (Fujimoto et al., 1976) and settle down in the gonadal primordium at stage 20 to 24 where they rapidly proliferate to form germ

[1] 신구전문대 축산과(Department of Animal Science, Shingu Junior College, Sungnam, Korea, 462-743)

cells (Nakamura et al., 1988). The isolated PGCs are potentially suitable vehicles for manipulation of the chicken genome. Shuman (1981) reported that the isolated PGCs can be successfully transferred to the host embryo by using the microinjection technique. Donor PGCs colonized in the host gonad were identified by chromosomal markers. In addition, it was demonstrated that PGCs did not influence the sexual differentiation of the gonad. The successful production of transgenic chickens by infection with a retroviral vector was reported by several researchers (Bosselman et al., 1989; Reynaud, 1976; Salter et al., 1987; Shuman and Shoffner, 1986). More recently, PGCs were obtained from germinal crescent or blood vessel (dorsal arota) and transfected with defective retroviruses carrying the neo and *lac Z* genes. These transfected PGCs were microinjected into recipient embryos to form chimeras. The produced chimeras were grown to sexual maturity and produced offspring. Southern blot analysis of the DNA from the chimeric bird sperm and the offspring blood contained the foreign DNA (Vick et al., 1993). Simkiss et al. (1990) reported positive result from manipulated PGCs with a retroviral vector by amplification of DNA with polymerase chain reaction (PCR) following Southern blot analysis. This was identified by examination of 5 day embryos, 10 day urogenital regions, and 18 day gonads. But the use of retroviral vectors is questioned for its safety in the production of commercial chickens due to the pathogenic characteristics of retroviruses. Han et al. (1993, 1994) reported that PGCs were transfected in vitro by liposome mediated DNA transfection methods and expressed the exogenous plasmid DNA, indicating that PGCs are a possible vector for direct gene transfer into the germline. The purpose of this study is to determine if transfected PGCs with exogenous plasmid DNA can be located and proliferated in the gonad for production of germline chimeric chickens.

MATERIALS AND METHODS

1. Embryo preparation and PGCs isolation

The experimental stocks were the two inbred lines of White Leghorn (WL) and Korean Native Ogol Chicken (KNOC) which were maintained at Experimental Animal Farm, Seoul National University. Fertilized donor eggs were prepared from KNOC. The eggs were incubated to developmental stage 6 to 8 (incubation time 24~26hr) at 37.5℃ and 60 to 70% relative humidity. Following the incubation, the eggshell was wiped with 70% ethanol and then broken into a specimen dish containing chicken Ringer's solution (7.2g of NaCl, 0.37g of KCl and $CaCl_2 \cdot H_2O$ in one liter of double distilled, deionized water). The embryos were excised from the yolk with microdissecting scissors, removed with a sterile wide mouth pipette, and transferred to a 35 mm petri dish containing warmed sterile calcium and magnesium free PBS (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g of $KHPO_4$ in one liter of double distilled, deionized H_2O).

After 24 to 26 hr incubation (developmental stages 6 to 8), PGCs were isolated from the germinal crescent. The 15 to 20 germinal crescents were dissected with Vannus microdissecting scissors or scalpel under the dissecting microscope, and placed in a warmed, sterile calcium and magnesium free PBS. Excised germinal crescents were carefully washed with sterile calcium and magnesium free PBS to remove yolk granules. The 15 to 20 excised germinal crescents were pooled and gently aspirated with

micropipette to produce a single cell suspension. Periodic acid-Schiff (PAS) stain was used to determine presence of the glycogen filled PGCs which stain preferably a brilliant magenta.

Recipient fertilized eggs were prepared from White Leghorn (WL) females mated with White Leghorn (WL) males. The eggs were pre-warmed for several hours at room temperature, positioned on their sides and incubated to developmental stages 6 to 8 for 26 hr. Eggshells were cleaned with 70% ethanol and a 25mm^2 window was cut in the side of the egg with a dentist drill fitted with an abrasive stone drill bit.

2. Plasmid DNA

The RSVLTR/βG_2 plasmid was provided by Hua-Ming Wang, University of Utah. This plasmid contains the *lac Z* gene under the control of Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter (Han et al., 1993).

3. Plasmid DNA transfection into PGCs

The cell suspension containing PGCs were transfected by using liposome mediated DNA transfection method (Chen and Okayama, 1987; Felger and Holm, 1989; Han et al., 1994). To conduct liposome mediated DNA transfection into PGCs, 100μl cell suspension containing PGCs was mixed with liposome-DNA mixture (40μg liposome and 10μg plasmid DNA). These DNA-PGCs mixture was incubated for 4hr at 37℃. After incubation, DME/Ham's F-12 media with serum was added for additional 48hr at 37℃ to identify the transfected PGCs (Han et al., 1994).

After 48hr of incubation to determine transfection efficiency, the PGCs were fixed and rinsed with the fixer (50mM phosphate buffer (pH 7.4) containing 2% glutaraldehyde, 2% formaldehyde and 2mM $MgCl_2$) and rinser (50mM phosphate buffer (pH 7.4) containing 2mM $MgCl_2$ and 0.02% NP-40) solution, respectively. And the transfected PGCs were treated with X-gal at pH 7.4 for 4 to 8hr.

4. Microinjection of transfected PGCs

After 4 to 8 hr incubation of the cells containing PGCs in transfection cocktail including plasmid DNA, the transfected cells were injected into the host embryo. Recipient eggs (WL) were incubated and positioned on their sides. Eggshells were cleaned with 70% ethanol and 25mm2 window cut in the side of the egg with a dentist drill fitted with an abrasive stone drill bit. Transfected cell suspension including PGCs was injected into the area of the germinal crescent of recipient embryos which were incubated for 24 to 28hr. The windowed egg with the recipient embryo was positioned under a 100μl-syringe with a 31-gauge needle. After the germinal crescent was located under the window, 2μl (35±3 PGCs) and 5μl (100±7 PGCs) of the transfected PGCs were slowly injected into the germinal crescent, respectively. Once the injection was completed, the window was closed by sealing a piece of second eggshell and layered with paraffin. Next, the eggs were incubated with the "window" side up overnight at 37.5℃ and 60% to 70% relative humidified incubator which was not rotated. And next day, the eggs were moved and incubated at 37.5℃ and 60% to 70% relative humidity in an air forced incubator. The eggs were rotated 90° periodically during the incubation period.

5. Gonad screening

To screen the transfected and colonized PGCs, the embryonic and young chick gonads were removed at 12 to 21 day old embryos and 3 day old chicks gonad, respectively. After re-

moving the gonads, they were squashed on a slideglass. After fixation and rinsing, the gonads were treated with X-gal. The positive gonads were examined for blue colored cells. In this study, gonad screening for exogenous β-galactosidase expression was performed at pH 7.4 for 4 to 8 hr (Han et al., 1993).

RESULTS

PGCs isolated from germinal crescents were readily distinguished from other cells. The size range of PGCs in the germinal crescent were between 12μm to 18μm in diameter. Figure 1 shows collected PGCs in germinal crescent after 26hr incubation. The large eccentric nucleus was evident in PGCs. The PGCs were stained a brilliant magenta with Periodic acid- Schiff (PAS) due to abundant glycogen content in the cytoplasm. The total number of PGCs from the 10 germinal crescents was approximately 1900 (190 cells /embryo), determined with a standard hemocytometer under the light microscope. Transfected PGCs with blue color were identified and distinguishable from other somatic cells. The transfection efficiency of *lac Z* gene to PGCs was 2.37% (±0.78).

Table 1 shows a summary of hatchability of injected embryos after PGCs transfer into the blood vessel of chicken embryo (stage 6 to 8). A total of 276 embryos were injected with transfected cells. The 141 embryos injected with 5μl cell suspension did not survive until hatching. Fifteen young chicks of 135 embryos treated with 2μl of transfected cell suspension were hatched. From this group, 47 embryos and 15 three day old young chickens were sacrificed (Table 2). Gonads of 12 to 21 day old embryos and 3 day old birds were examined with X-gal staining to identify colonized and transfected PGCs. Only one positive PGCs gonad from sacrificed 3 day old chickens was detected by appearance of blue cells. The chimeric gonad with expression of exogenous *lac Z* gene was detected in a colony of several blue colored PGCs (Figure 2B). The transfected PGCs in the gonad were larger than other somatic cells. The results obtained here indicated that foreign PGCs with exogenous plasmid DNA might migrate into and colonized the embryonic gonad. The expression of the exogenous reporter gene

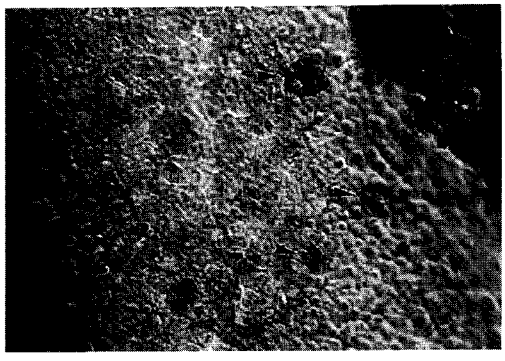

Figure 1. PAS positive stained PGCs (←) in germinal crescent (×500).

Table 1. Summary of the hatchability of manipulated eggs

Amount of injection	Number of eggs	Number of surival to 12 day incubation	Number of hatched
5μl	141	26	0
2μl	135	36	15
Total	276	62	15

Table 2. Number of embryos and young chicken expressed exogenous *lac Z* gene

	Embryos[a]	Chickens[b]	Total
No. of screening[c]	47	15	62
No. of positive[d]	0	1	1

[a] 12 to 21 day old incubation embryo gonads.

[b] Hatched 3 day old chicken gonads.

[c,d] Number of screening and positive gonads, respectively.

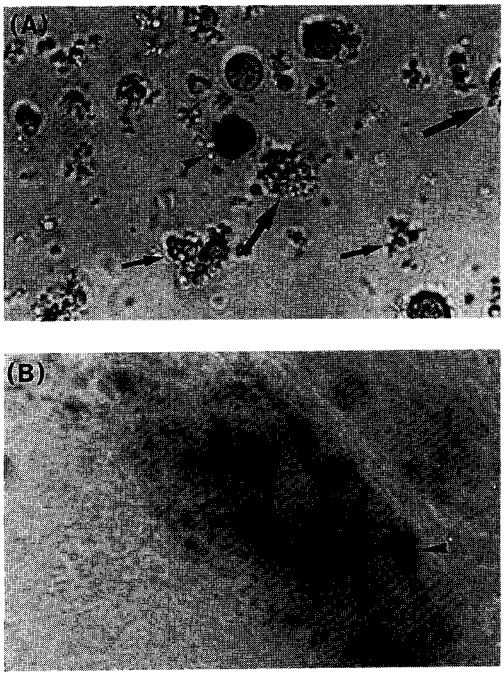

Figure 2. (A) Arrow heads (➤) indicate expressed exogenous *lac Z* gene from transfected PGCs (×400), bold arrows (➡) indicate disrupted PGCs and thin (→) arrows indicate other somatic cells and yolk cells. (B) Arrow heads (➤) indicate expressed exogenous *lac Z* gene from transfected PGCs in 3 day old chicken gonad (× 250).

in germ cells of 3 day old chick suggests that exogenous plasmid DNA can be incorporated into the genome of PGCs.

DISCUSSION

KNOC is an indigenous Korean black bone fowl, which has black pigment in most all parts, including feathers, beak, comb, shanks, bone, skin and meat. Ogol Chicken is very different phenotypically and has a different genetic background than White Leghorns. When KNOC was mated to WL, the feathers color of progenies were showed all white. So, this characteristic make it possible to confirm the germline chimeric chicken by mating of the manipulated chicks with control populations.

Only one chick gonad's PGCs, transfected with liposome, expressed the exogenous *lac Z* gene which were screened with X-gal *in vitro* (Table 2). And disrupted PGCs maybe resulted from centrifugation during cell collection and X-gal treatment because the PGCs were very fragible. The other somatic cells deplets and yolk cells are also isolated from germinal crescent (Figure 2A). Figure 2B shows expression of the exogenous *lac Z* gene in 3 day old chick gonad. It is suggested that the transfected PGCs are incorporated into the genome of PGCs and transfected microinjected PGCs are expressed in germ cells of 3 day old chick. Therefore the chicken PGCs can be used as a vectors for gene transfer to produce transgenic chickens.

About 26.7% of the embryo survivability during days 12 of incubation when the volume of the PGCs suspension injected was 2㎕ compared with 18.4% in 5㎕ PGCs suspension-injected egg cultured in the same way. When the 5㎕ PGCs suspension was injected into recipient embryos, the survivability was decreased at the 12 day incubation and number of hatched chicken was zero. It seems to be attributable to trauma of much more KNOC PGCs injected-suspension amount than 2㎕ KNOC PGCs injected-suspension. In recent, there are two categories to produce germline chimera chickens. The first method is using the chicken blastodermal cell at stage × and second is using the PGCs as a vectors. The stage × embryos have been used to obtain chicken chimeras because at this stage the cells are morphologically undifferentiated (Ginsburg and Eyal-Giladi, 1987). The injection of dispersed blastodermal cells from stage × embryos into recipients at the same stage of development has made possible the development of somatic and germline chimeras (Naito et al., 1991; Petitte et al., 1990). And, the use of this method provides only occasional germline chimerism (Petitte et al., 1990). It has been possible to increase the somatic and germline chimerism when the development of recipient embryos was compromised by exposure to γ irradiation (Carsience et al, 1993; Thoraval et al., 1994). Irradiation of the recipient embryo prior to the injection of donor cells consistently yielded somatic and germline chimeric chickens. Production of germline chimeric chickens using the blastodermal cells injection has many potential applications especially for the development of transgenic chickens.

In contrast, chicken PGCs have a unique pathway of migration in the early embryo. After PGCs settle down in the gonadal ridge, they differentiate into eggs or spermatozoa. Using these PGCs migratory characteristics in embryonic development and embryogenesis, Reynaud (1976) identified that PGCs derived from transplanted germinal crescent reached the host gonad and differentiated to germ cells there. Wentworth et al. (1989) have successed in the transfer of PGCs to vasculature in quails. Recently, germline chimeric chickens have been produced by transferring PGCs isolated from early embryonic blood (Tajima et al., 1993; Yasuda et al., 1992) or germinal crescents (Tsai and Wentworth, 1992), and these have given rise to viable offspring. In addition a high transmission rate of donor PGCs in the germline chimeras has successfully been achieved by increasing the number of injected PGCs and by partially sterilizing recipient embryos (Naito et al., 1994).

And, the first successful preservation of avian PGCs in liquid nitrogen to give rise to viable offspring was reported by Naito et al. (1994). It has become possible to conserve genetic resources in chickens by preserving PGCs in liquid nitrogen. Therefore, to enhance the efficiency of obtaining chicks derived from donor PGCs, Naito et al. (1994) eliminated endogenous PGCs by drawing off the blood from dorsal aorta in recipient embryos and injected the increased number of PGCs for transfer. Using the PGCs are most effective vectors for the production of germline chimera chicken.

The limitations of PGCs manipulation include the difficulty of isolating pure PGCs and low transfection efficiency. Recently, bovine PGCs derived cell lines maintained in long-term culture produced chimeric bovine embryos using PGC-derived cells (Cherny and Merei, 1994; Tajima et al., 1993). Tsai et al. (1992) suggested the possibility of long-term culture of

gested the possibility of long-term culture of chick PGCs either isolated from the blastodisc or germinal crescent. Accordingly, the first step will required creation of chicken PGCs derived cell lines followed by development of gene transfer methods such as electroporation or direct microinjection into PGCs along with development of percoll or ficoll density gradient centrifugation collection of pure PGCs from blood vessels or germinal crescent.

Because there is not accomplished the production of viable stem cell and PGCs cell-lines, the transfected PGCs injection method used in this study thought to be the most effective method in the production of transgenic chickens. Further technical refinements will undoubtly increase the frequency for incorporation of genetically modified PGCs into the recipient embryo. Transgenic chickens produced by transplanted primordial germ cells may be useful in the commercial poultry industry.

ACKNOWLEDGMENTS

This work was supported by grant from Korea Science and Engineering Foundation in 1995. The authors sincerely thank to Mr. Jae Kwon Han and all staff, Kang-Nam farm Co. (80, Kung-Ri, Go Deog-Myun, Pyung Taek-Si, Kyung Ki-Do, Korea) for financed support during the experiment.

적 요

본 연구는 닭의 원시생식세포를 형질전환 닭을 생산하기 위한 시스템의 한 방법으로서 사용코자 시행되었다. 원시생식세포는 오골계 수정란의 배 발달 단계 stage 6~8의 germinal crescent로부터 분리하여 사용하였다. 분리된 원시생식세포에 liposome을 이용하여 *lac Z* 유전자를 전이시켰으며, *lac Z* 유전자가 원시생식세포에서 안정되게 발현됨을 확인하였다. *lac Z* 유전자가 전이된 원시생식세포는 배 발달 단계 stage 6~8의 화이트 레그혼의 germinal crescent에 주입하였고, 원시생식세포는 혈관계를 따라 원시생식기에 도달하였으며, 3일령의 병아리 생식기에서 발현을 관찰하였다. 즉, 47개의 배자와 3일령 병아리 중 1마리의 병아리 생식기에서 X-gal screening에 의해 청색 colony를 확인하였다. 이러한 결과는 외래 유전자가 전이된 원시생식세포가 생식기의 생식세포에 도달하여 colony를 형성하였음을 증명하고 닭에 germline의 유전물질을 조작할 수 있다는 가능성을 제시한다고 할 수 있다.

(색인 : 원시생식세포, *Lac Z*, 생식선 카이메라)

Literature Cited

Bosselman RA, Hsu RY, Boggs T, Hu S, Bruszewski J, Ou S, Kozar L, Martin F, Green C, Jacobsen F, Nicolson M, Schultz JA, Semon K M, Rishell W, Stewart RG 1989 Germline transmission of exogenous genes in chicken. Sci 243: 533-535.

Carsience RS, Clark ME, Gibbins AMV, Etches RJ 1993 Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. Devel 117: 669-675.

Chen C, Okayama H 1987 High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7: 2475.

Cherny RA, Merei J 1994 Evidence for pluripotency of bovine primordial germ cell-derived cell lines maintained in long-term culture. Therio 41:175.

Eyal-Giladi H, Ginsburg M 1981 Farbarov A. Avian primordial germ cells are of epiblastic origin. J Embryol Exper Morph 65:139-147.

Felgner PL, Holm M 1989 Cationic liposome-mediated transfection. Bethesda Research Laboratory Focus 11: 21.

Fujimoto T, Ukeshima A, Kiyofuji B 1976 The origin, migration and morphology of the primordial germ cells in the chick embryo. Anat Rec 185: 139-154.

Ginsburg M, Eyal-Giliadi H 1987 Primordial germ cells of the young chick blastoderm originate from central zone of the area pellucida irrespective of the embryo-forming process. Devel 101: 209-219.

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

Han JY, Shin YS, Shoffner RN, Guise KS 1993 Stable transformation of cultured chicken cells. Asian-Austr J Anim Sci 6: 581-589.

Han JY, Shoffner RN, Guise KS 1994 Gene transfer by manipulation of primordial germ cells (PGCs) in the chicken. Asian-Austr J Anim Sci 7: 427-434.

Naito M, Agata K, Otsuka K, Kino K, Ohta M, Hirose K, Perry MM, Eguchi G 1991 Embryonic expression of β-actin-lacZ hybrid gene injected into the fertilized ovum of the domestic fowl. Int J Dev Biol 35: 69-75.

Naito, M, Tajima A, Tagami T, Yasuda Y, Kuwana T 1994 Preservation of chick primordial germ cells in liquid nitrogen and subsequent production of viable offspring. J Repro Fert 102: 321-325.

Naito M, Tajima A, Yasuda Y, Kuwana T 1994 Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells. Mol Repro Devel 39: 153-161.

Nakamura M, Kuwana T, Miyayama Y, Yoshinaga K, Fujimoto T 1988 Extragonadal distribution of primordial germ cells in the early chick embryo. Anat Rec 222: 90-94.

Petitte JN, Clark ME, Liu G, Verrinder-Gibbins AM, Etches RJ 1990 Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Devel 108: 185.

Reynaud G 1976 Capacities reproductrices et descendance de poulets ayant subi un transfert de cellules germinales primordiales durant la vie embryonnaire. Roux's Archi Devel Biol 179: 85-110.

Salter DW, Smith EJ, Hughes SH, Wright SE, Crittenden LB 1987 Transgenic chickens: insertion of retroviral genes into the chicken germ line. Virology 157: 236-240.

Shuman RM 1981 Primordial germ cell transfer in the chicken *Gallus domesticus*. M.S. Thesis, University of Minnesota.

Shuman RM, Shoffner RN 1986 Gene transfer by avian retroviruses. Poultry Sci 65: 1437-1444.

Simkiss K, Vick L, Luke G, Page N, Savva D 1990 Infection of primordial germ cells with defective retrovirus and their transfer to the developing embryo. Proc of the 4th World Cong on Genetics Applied to Livestock Prod XVI 111-113.

Stokes TM, Cherny RA, Brandon MR 1994 Production of chimaeric bovine embryos. Therio 41: 303.

Tajima A, Naito M, Yasuda Y, Kuwana T 1993 Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (*Gallus domesticus*). Therio 40: 509-519.

Thoraval P, Lasserre F, Coudert F, Dambrine G 1994 Somatic and germline chicken chimeras obtained fron brown and white leghorns by transfer of early blastodermal cells. Poultry Sci 73: 1897-1905.

Tsai H, Wentworth BC 1992 Long-term culture of chick primordial germ cells isolated form the blastodisc and germinal crescent. Poul-

try Sci Abst pp 72.

Urven LE, Erickson CA, Abbott UK, McCarrey RJ 1988 Analysis of germ line development in the chick embryo using an antimouse EC cell antibody. Devel 103: 299-304.

Vick L, Ying L, Simkiss K 1993 Transgenic birds from transformed primordial germ cells. Proc of the Royal Soc of Lond (B) 251: 179-182.

Wentworth BC, Tsai H, Hallett JH, Gonzales DS, Rajcic Spasojevic G 1988 Manipulation of avian primordial germ cell and gonadal differentiation. Poultry Sci 68: 999-1010.

Yasuda Y, Tajima A, Fujimoto T, Kuwana T 1992 A method to obtain avian germ-line chimaeras using isolated primordial germ cells. J Reprod Fert 96 : 521-528.