Studies on the Migratory Ability of Primordial Germ Cells from Embryonic Gonads at Different Developmental Stages in Quail

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메추리의 발달 중 배자 생식선에서 분리한 원시생식세포의 이동능에 대한 연구

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ABSTRACT : Avian primordial germ cells (PGCs) originate from the epiblast and appear in the germinal crescent. These PGCs enter the developing blood vessels during stage 10~12 (H&H), circulate in the blood stream, migrate into the developing gonadal anlage and differentiate into germ cells. However, it is not clear until when the migratory ability of PGC is maintained. This study was conducted to examine whether migratory ability is present in PGCs from the gonad at later embryonic developmental stages. In the present study, gonads were dissected from 5−, 6− and 10−day old quail embryos and treated with trypsin−EDTA. Gonadal PGCs (gPGCs) were purified by Ficoll− density−gradient−centrifugation and labeled with PKH26 fluorescent dye. The PKH26−labeled gPGCs were microinjected into the blood vessel of the recipient quail embryo. Manipulated recipients were incubated for 3 days, embedded in paraffin and sectioned. The foreign gPGCs were detected by fluorescent and confocal laser microscopy. As a result, quail gPGCs, from 10, 6 and 5 day old embryos could migrate through the recipient blood stream at early stage and settle in the gonads. Thus, results suggest that gPGCs from upto 10−day old embryos keep properties seen in circulating PGC. Therefore, the PGCs of 10−day old embryonic gonads can be used for the tools of genetic manipulation.

(Key words : quail, gonad, PGCs, PKH26, Ficoll)

INTRODUCTION

Primordial germ cells (PGCs) are the founder cells of the gametes. Avian PGCs originate from the epiblast (Eyal−Giladi, 1981) and appear in the germinal cres− cent (Ginsburg and Eyal−Giladi, 1986). These PGCs enter the developing blood vessels at stages 10 to 12 (Hamburger and Hamilton, 1951) and migrate via the blood vascular system to colonize the gonadal anlagen in which they differentiate into germ cells. Generally, it has been known that the PGCs differentiate after 6 days of incubation, but the factors influencing on their differentiation and migration are still unclear. Factors affecting the differentiation of germ cells has been suggested to reside in the cortex of the chick gonad and the germ cells divide mitotically between 3 and 7 days of incubation (Erickson, 1974). Premeiotic DNA synthesis occurs in some germ cells between 15 and 16 days of incubation (Callebaut, 1967). Maeda et al. (1994) examined the 1− to 17−day chicken embryos by immunohistochemistry using the chicken PGC− positive 2C9 monoclonal antibodies. As a result, 2C9−

reactive PGCs were increased in number beyond the migrating stage. From this stage to the sexually differentiating stage (7 days of incubation), the 2C9 antibody reactivity was found in all over the cytoplasm of PGCs in both sexes. In the female gonads, the reactivity disappeared at 8 days of incubation, whereas the reactivity was gradually decreased and disappeared until 14 days of incubation in the male.

To date, attempts to produce germline chimeric birds using PGCs have focused on the PGCs from early embryonic stage. The PGCs arrived in the gonad proliferate into numerous cells through mitosis. Therefore, if these cells in the gonad maintain the characteristics as PGCs, a large number of PGCs can be easily isolated for the production of germline chimera.

Although the characteristics of PGCs have been described in chicken, little is known about in other avian species. The Japanese quail (Coturnix coturnix japonica) has the advantage of a short incubation period for hatching and sexual maturation period comparing to chicken. Thus, it would be an animal model to study experimental embryology, reproductive biology, and biotechnology. Quail PGCs show a specific nucleolus which has various amount of condensed chromatin closely associated with the nucleolar substance (Yoshinaga et al., 1993). Besides, no glycogen granules could be found in quail PGCs (Nakamura et al., 1992). However, these studies are limited to structural and hitochemical characteristics.

In the present study, we isolated the PGCs from gonads at later embryonic developmental stages and examined their migratory ability to the embryonic gonad of the recipient after microinjection.

MATERIALS AND METHODS

1. Preparation of Fertilized Embryos

The fertilized eggs were obtained from the Japanese black quail maintained at Experimental Animal Farm, Seoul National University, Korea and incubated at 37.5 ℃ and 60~70% relative humidity for 5 days, 6 days

and 10 days for the donor of PGCs and 2.5 days for the recipient respectively.

2. Isolation and Purification of Gonadal Primordial Germ Cells (gPGC)

For the preparation of 5-, 6-day old gonadal primordial germ cells (gPGCs), and 10-day old germ cells, gonads were dissected from the donor embryos and rended in small pieces. Tissue was treated with 0.25 % trypsin/ 0.05 % EDTA and the Ficoll-density-gradient centrifugation was performed to purify the gPGCs.

3. PKH26 Labelling of gPGCs

Cells were labeled using the PKH26 fluorescent staining kit C (Zynazis Cell Science Inc. Japan). Sections of recipient embryonic gonad were examined by fluorescent microscopy to identify the PKH26 labelled PGCs in the host gonad. The samples were observed by confocal laser microscopy with excitation wave length of 551 nm.

4. Microinjection of PGCs

The eggs of Japanese black quail incubated for 2 days were prepared and used as recipients. PKH26 labeled gPGCs were injected into the blood vessel through the dorsal aorta of the recipient embryo. After manipulation, the window was sealed with parafilm. The recipient embryos were then incubated for three days at 37.5 ℃.

5. Tissue Staining

The 5.5-day old embryos were fixed and paraffin-embedded. Embryos were sectioned using the microtome (Nippon Optical workers Co., Japan) to obtain 5-μm-thick sections. Sections were stained with hematoxylin and eosin.

RESULTS

The gPGCs were concentrated using the Ficoll-density-gradient-centrifugation method. The number

Table 1. Purity of quail gPGCs isolated by Ficoll−density−gradient−centrifugation

Trial	Purity[1] of 5−day old gPGCs (%)	Purity of 6−day old gPGCs (%)	Purity of 10−day old gPGCs (%)
1	76.5	72.6	85.7
2	75.6	73.8	88.9
3	79.7	69.5	80.0
4	83.3	79.7	76.3
5	80.0	70.0	79.2
Means	78.7	73.1	82.5
(±SD)	(±6.2)	(±8.2)	(±10.3)

[1], Purity of gPGCs = $\dfrac{\text{number of PGCs}}{\text{number of total cells}} \times 100$ (%)

of cells was counted under the microscope. The purity of gPGCs after Ficoll−density−gradient−centrifugation was 73.1~82.5 %. No difference was found in purity of cells from embryos at different stages (Table 1). Results also showed that the proportion of PGCs in gonads was higher than that in blood. It was also shown that there was no change in appearance and size of PGCs after Ficoll density centrifugation. In this study, change in the shape of PGCs was also not observed.

Injected gPGCs in the gonadal region of the recipient embryo were determined by fluorescence. Fig. 1 shows the recipient gonad following injection of gPGCs from 5−day embryos. Fluorescent labeled PGCs were observed with an intact cell shape in the gonadal region. Magnification made it clear of the characteristics of gPGC − large size, round shape and eccentric nucleus (Fig. 1B−1C).

Confocal laser microscopy was carried out to confirm these results. PKH26 is a red fluorochrome and has excitation (551 nm) and emission (567 nm) characteristics compatible with rhodamine or phycoerythrin detection system. Fig. 1D shows the fluorescent labelled donor gPGCs in the recipient gonad by the confocal microscopy.

The recipient embryos which were injected with gPGCs of 6−day old embryos were also analysed. Exogenous gPGCs were observed in the recipient gonad (Fig. 2A−2B). PKH26 labeled PGCs became visible inside of the recipient gonad.

The specimen injected with gPGCs of 10−day embryos was observed by fluorescent and confocal laser microscopy (Fig. 3A~D). The large, round cells that labeled with PKH26 were distributed in the area of the gonad.

DISCUSSION

Avian PGCs have been used in the production of germline chimeric birds. However, the concentration of PGCs has been found to be essential for the efficient production of germline chimera. It is a general notion that greater number of PGCs are present in the gondal analge than other tissues of earlier embryonic development. It has also been demonstrated that gPGCs maintain their migratory activity in the recipient embryo as evidenced by production of chimeric offsprings (Chang et al., 1997).

Yasuda et al. (1992) described that blood PGCs from 2 day old chick embryos were concentrated by Ficoll−density−centrifugation, then the concentrated fraction containing 3.9% PGCs in blood. In this study, quail gPGCs could be isolated with a relatively high concentration using the same method. Therefore, it is reasonable to assume that quail PGCs may have physical properties not much different from chicken PGCs.

Somatic and germline chimera have been produced by injection of chicken blastodermal cells into recipient embryos (Petitte et al., 1993; Naito et al., 1994; Seo et
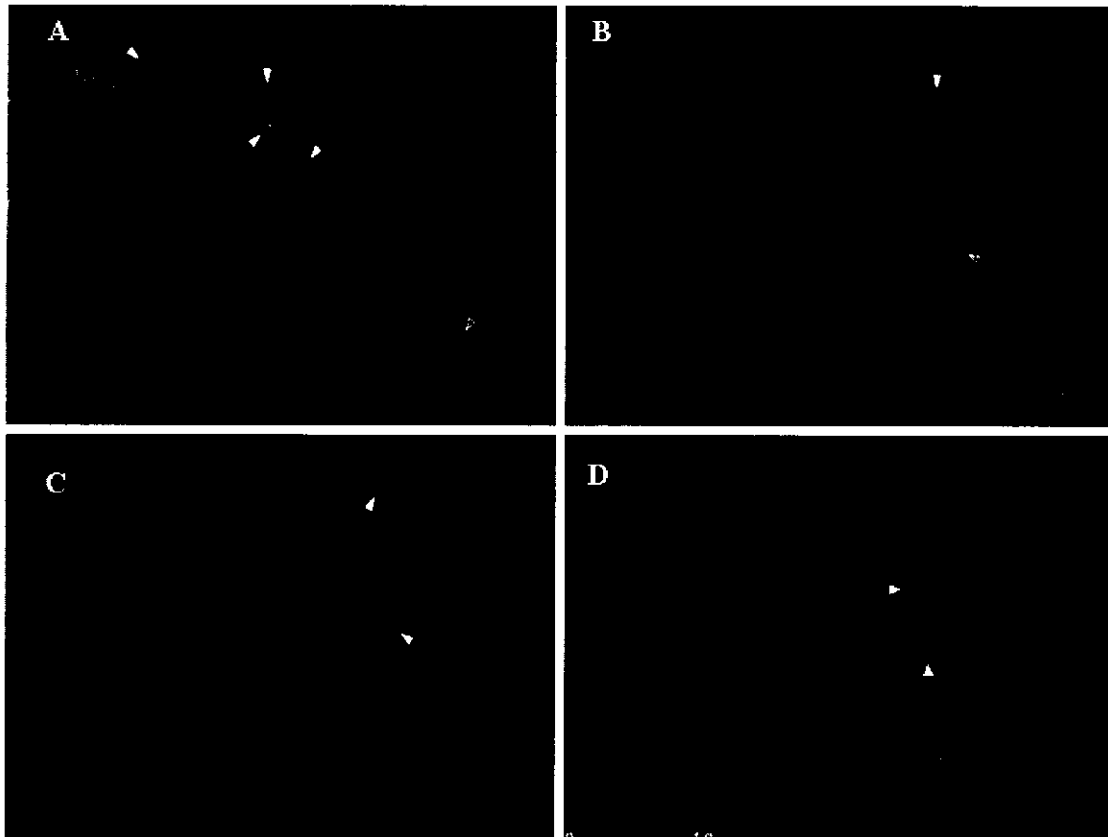
Fig. 1. Fluorescent donor gPGCs from the gonad of the 5-day old embryo found in the recipient embryo. Fluorescence-labeled gPGCs were observed in an embryonic gonad section at three days after microinjection (arrowheads) (A). Observation under the fluorescent microscope (B) and under the light microscope (C). The specimen was observed by confocal laser microscopy with 551 nm excitation and 567 nm emission (D). Magnifications : ×250 (A), ×1000 (B, C and D), respectively.
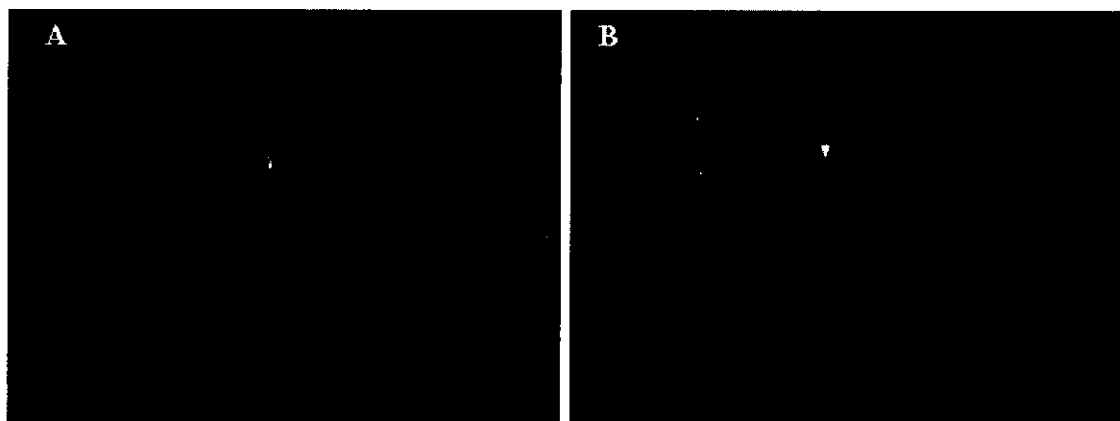
Fig. 2. Fluorescent donor gPGCs from the gonad of the 5-day old embryo found in the recipient embryo. Fluorescence-labeled gPGCs were present in the embryonic gonad section at three days after microinjection (arrowheads). Observation under the fluorescent microscope (A) and the light microscope (B), respectively.
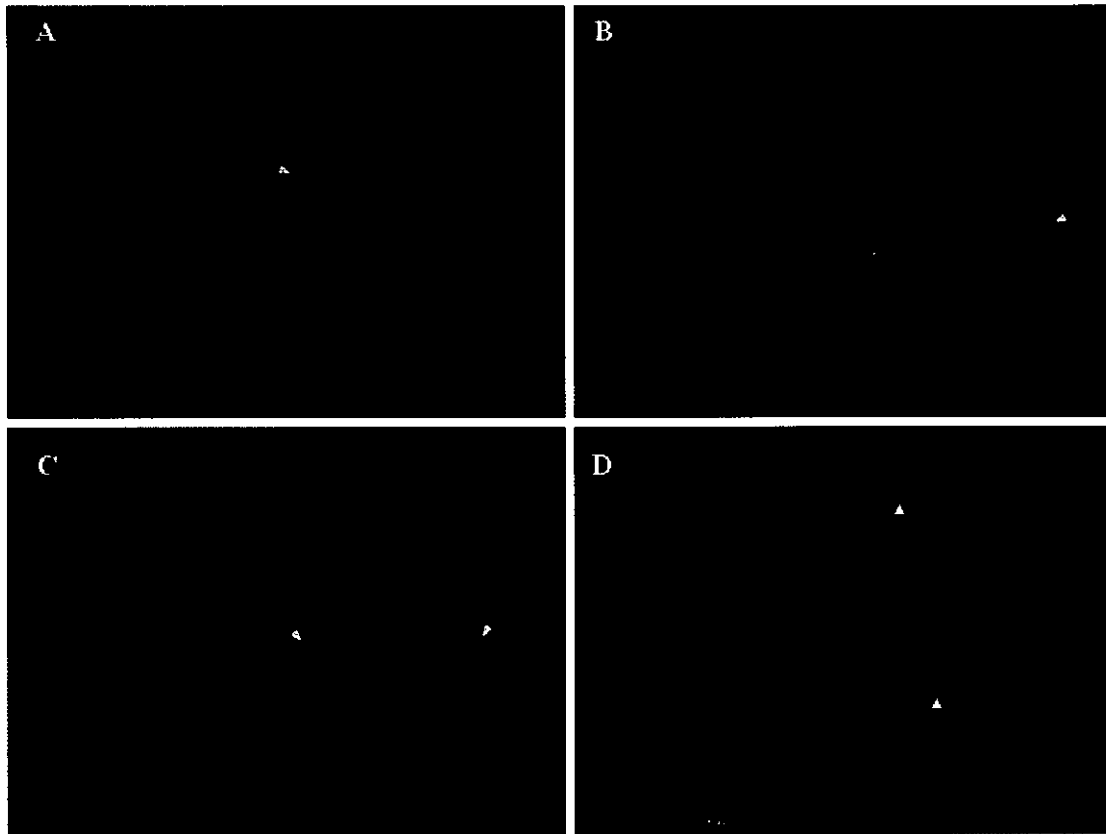
Fig. 3. Fluorescent gPGCs from the gonad of the 10-day old embryo found in the recipient embryo. Fluorescence-labeled gPGCs were present in embryonic gonad section at three days after microinjection (arrowheads) (A). Observation under the fluorescent microscope (B) and the light microscope (C). The specimen was observed by confocal laser microscopy with 551 nm excitation and 567 nm emission (arrowheads) (D). Magnifications : ×400 (A), ×1000 (B, C and D), respectively.

al., 1995). PGCs from germinal crescent were transferred into recipient chick embryos (Reynaud, 1976; Vick et al., 1993). PGCs from embryonic blood (Simkiss et al., 1989; Petitte et al., 1991; Tajima et al., 1993) and embryonic gonads (Tajima et al., 1996) were used to produce chimeric birds. The cultured gPGCs could also produce germline chimeric chickens successfully (Chang et al., 1997). In quail, Ono et al. (1994) produced a germline chimera by microinjection of dispersed blastoderm cells taken from the unincubated quail eggs. Wentworth et al. (1989) produced germline chimeric quail by transferring PGCs obtained from the germinal crescent into sterile recipient embryos. Ono et al. (1996) obtained PGCs from blood of the marginal vein on quail embryo and microinjected

into the chick embryo, and then identified donor PGCs by immunohistochemical assay using QCR1.

Several studies have been carried out to investigate the mechanisms involved in PGC migration in chicken. Classically, there are two factors involving in the migration of PGCs in the blood stream: chemotactic and mechanical factors. PGCs are attracted to developing gonads by a chemotactic-like agent possibly secreted from the gonad and that this agent might be steroid-like substance (Swartz, 1975). During migration, it has been suggested that regulated interactions occur between the glycoconjugates of the moving germ cells and the physicochemical characteristics of the extracellular matrices acting as substrates for the movement (Forgeix et al, 1980). Besides, the alter-

ation of cell surface properties using Con A inhibited migration of PGCs in early chick embryos (Lee et al, 1978). Incubation of eggs at reduced temperature leads to a decline in the rate of migration of PGCs (Al-Thani et al, 1992). It has also been suggested that one of the mechanisms of PGC migration is an autonomous trapping of PGCs by the capillary network quite close to the germinal epithelium and passive translocation (Kuwana et al., 1999).

Although a few studies have been carried out to investigate each factors and mechanisms in quail, it may be resonable to assume that situation is analogous to chicken. Therefore, our results suggest that quail gPGCs of upto 10-day embryos may migrate to the embryonic gonad.

The number of PGCs from the gonad is greater than that from the blastoderm or blood because of the mitotic proliferation during migration and after settlement in the gonad. The embryos of the later stage have the more PGCs. If the cells maintain the characteristics as PGCs in the later stage, we can obtain enormous PGCs from the gonads effectively.

This study shows that 10-day-old quail gPGCs could migrate and settle in the gonads suggesting that the gPGCs of later stage have the activity of migration into the gonads in the blood stream of recipient embryo. Therefore, the PGCs from up to 10-day-old embryonic gonads could be used for the tools of genetic manipulation. However, the migratory efficient of 10-day-old gPGCs was lower than that of gPGCs from earlier stage. However, it has to be learned whether the PGCs integrated could differentiate into germ cells.

In conclusion, quail gPGCs can be isolated by Ficoll density gradient centrifugation with high purity. Therefore this method can be used to obtain the PGCs for the production of autogenic quail.

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적 요

조류의 원시생식세포는 외배엽에서 생성되어 생식반월 부위에 모였다가, 혈관이 생성되는 배발달단계 10~12 (H&H)부터 혈류를 따라 순환하기 시작하여 발달 중인 원시생식기에 도달한 후 생식 세포로 분화하게 된다. 그러나 원시생식세포의 이동 능력이 언제까지 유지되는 지는 명확하지 않다. 본 연구는 메추리의 후기 배자 발달단계의 원시생식기에서 분리한 원시생식세포의 이동 능력을 검증하기 위해 수행되었다. 본 실험에서는 메추리의 원시생식기를 5일령, 6일령과 10일령의 배자에서 각각 분리한 후 trypsin-EDTA로 처리하였다. 원시생식기의 원시생식세포는 Ficoll-density-gradient -centrifugation 방법을 이용하여 순수하게 분리한 후 형광 염료인 PKH26로 염색하여 표지 한 후에 수용체 메추리 배자의 혈관 내로 미세 주입하였다. 조작된 수용체는 3일간 더 배양한 후 파라핀에 침잠시켜 굳힌 후 박편을 만들었다. 외부에서 주입된 원시생식세포는 형광 현미경과 공초점 레이저 주사현미경으로 관찰되었다. 결과에서 10일령의 원시생식기에서 분리한 원시생식세포도 5일령, 6일령의 원시생식세포와 같이 수용체의 혈관을 통해 이동하여 원시생식기에 도달하는 것이 관찰되었다. 이는 10일령까지의 메추리 원시생식기에서 분리한 원시생식세포가 초기 원시생식세포의 이동성을 유지하고 있음을 나타낸다. 따라서 10일령 원시생식기의 원시생식세포도 유전적 조작의 도구로 이용될 수 있는 가능성을 보여준다.

(색인어 : 메추리, 원시생식기, 원시생식세포, PKH26, Ficoll)

REFERRENCES

Al-Thani R, Simkiss K 1992 Effects of temperature on the migration of primordial germ cells in the chick embryo. Br Poult Sci 33:735-739.

Callebaut M 1967 Premeiosis and premeiotic DNA synthesis in the left ovary of the female chick embryo. J Embryol Exp Morphol 18:299-304.

Chang IK, Jeong DK, Hong YH, Park TS, Moon YK, Ohno T, Han JY 1997 Production of germline

chimeric chickens by transfer of cultured primordial germ cells. Cell Biol Int 21:495−499.

Erickson GF 1974 The control of the differentiation of female embryonic germ cells in the bird. Dev Biol 36:113−129.

Eyal−Giladi H, Ginsburg M, Fabarov AA 1981 Avian primordial germ cells are of epiblastic origin. J Embryol Exp Morphol 65:139−147.

Forgeix N, Didier E, Lutz H 1980 Germ cells of the bird embryo: early localization, migration and colonization of gonadal anlagen. Bull Assoc Anat 64:143−172.

Ginsburg M, Eyal−Giladi H 1986 Temporal and spatial aspects of the tradual migration of primordial germ cells from the epiblast into the germ cells from the epiblast into the germinal crescent in the avian embryo. J Embryol Exp Morphol 95:53−71.

Hamburger V, Hamilton HL 1951 A series of normal stage in the development of the chick embryo. J Morphol 88:49−92.

Kuwana T, Rogulska T 1999 Migratory mechanisms of chick primordial germ cells toward gonadal anlage. Cell Mol Biol 45:725−36.

Lee H, Karasanyi N, Nagele RGJr 1978 The role of the cell surface in the migration of primordial germ cells in early chick embryos: effects of concanavalin A. J Embryol Exp Morphol 1978 46:5−20.

Maeda S, Ohsako S, Kurohmaru M, Hayashi Y, Nishida T 1994 Analysis for the stage specific antigen of the primordial germ cells in the chick embryo. J Vet Med Sci 56:315−320.

Naito M, Sasaki E, Othtaki M, Sakurai M 1994b Introduction of exogenous DNA into somatic and germ cells of chickens by microinjection into the germinal disc of fertilized Ova. Mol Reprod Dev 37:167−171.

Nakamura M, Yoshinaga K, Fujimoto T 1992 Histochemical identification and behavior of quail primordial germ cells injected into chick embryos by the intravascular route. J Exp Zool 261:479−83.

Ono T, Murakami T, Mochii M, Agata K, Kino K, Otsuka K, Ohta M, Mizutan M, Yoshida M, Eguch G

1994 A complete culture system for avian transgenesis, supporting quail embryos from the single−cell stage to hatching. Dev Biol 161:126−130.

Ono T, Yokoi R, Aoyama H 1996 Transfer of male or female primordial germ cells of quail into chick embryonic gonads. Exp Anim 45:347−352.

Petitte JN, Brazolot CL, Clark ME, Liu G, Verrinder Gibbins AM, Etches RJ 1993 Accessing the genome of the chicken using germline chimeras. In manipulation of the Avian Genome. CRC Press USA pp 81−102.

Petitte JN, Clark ME, Etches RJ 1991 Assessment of functional gametes in chickens after transfer of primordial germ cells. J Reprod Fertil 92:225−229.

Reynaud G 1976 Capacites reproductrices et descendance de poulets ayant subi un transfert de cellules germinales primordiales durant la vie embryonnaire. Roux's Arch Dev Biol 179:85−110.

Seo DS, Hong YH, Choi D, Yang MH, Shin YS, Han JY 1995 Production of chimeric chickens (White Leghorn ↔ Korean Native Ogol) by blastodermal cell transfer. Kor J Anim Sci 37:27−32.

Simkiss K, Rowlett K, Bumstead N, Freeman BM 1989 transfer of primordial germ cell DNA between embryos. Protoplasma 151:164−166.

Swartz WJ 1975 Effect of steroids on definitive localization of primordial germ cells in the chick embryo. Am J Anat 142:499−513.

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

Tajima A, Naito M, Yasuda Y, Kuwana T 1996 Production of germline chimeras by transfer of cryopreserved gonadal primordial germ cells (gPGCs) in chicken. XX World's Poult Cong Vol(1):385−388.

Vick L, Luke G, Simkiss K 1993 Germ−line chimeras can produce both strains of fowl with high efficiency after partial sterilization. J Reprod Fertil 98:637−641.

Wentworth BC, Tasi H, Lallett JH, Gonzales DS, Rajcic−Spasojevic G 1989 Manipulation of avian primordial germ cells and gonadal differentiation. Poult

Sci 68:999−1010.

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

Yoshinaga K, Nakamura M, Ukeshima A 1993 Ultra−structural characteristics of primordial germ cells in the quail embryo. Anat Rec 236:547−52.