GENE TRANSFER BY MANIPULATION OF PRIMORDIAL GERM CELLS IN THE CHICKEN

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Summary

The primordial germ cells (PGCs) were transfected *in vitro* and expressed the exogenous RSVLTR/βG2 plasmid, suggesting that PGC is a possible vector for direct gene transfer into the germ line. Transfection efficiency of cell suspensions containing PGCs was 1.5% by liposome mediated DNA transfection. By microinjection of the transfected PGCs into the host germinal crescent, PGCs migrated via blood vessel to the future gonad and these transfected PGCs resulted in the RSVLTR/βG2 expression in the gonad. The results from the seeding of PGCs on the chorioallantoic membrane were insufficient to test the hypothesis that PGCs can penetrate or invade the chorioallantoic membrane for transport via the circulatory system.

(Key Words : Transgenic Chicken, Primordial Germ Cells, Transfection, Microinjection, Plasmid)

Introduction

Transfection with plasmid vectors has been achieved in species where it is possible to directly inject plasmid constructs into either the cytoplasm or the nucleus of recipient stem cells. Production of transgenic chickens by direct injection of DNA into the pronucleus is extremely difficult due to the morphological and developmental characteristics of the avian embryo. Retroviral vectors have been used successfully but have limitations for production of commercial chicks because of the pathogenic characteristics of retroviruses (Bosselman et al., 1990). Recently, gene transfer into chickens by direct microinjection into the fertilized ova has successfully been achieved (Love et al., 1994).

The stem cells most readily available as vehicles for genetic manipulation in avian species are the primordial germ cells (PGCs). The PGCs originate in the epiblast, migrate and concentrate in the germinal crescent at stage 4-8 (Hamburger and Hamilton, 1951). After a period of about 30 hr, they begin to migrate via the circulatory system to the future gonadal region at stages 9-10 (30-38 hr of incubation) (Eyal-Giladi et al., 1981; Ginsburg and Eyal-Giladi, 1986, 1987; Urven et al., 1988). PGCs begin to arrive at the region of the future gonad at stage 15 (50-55 hr of incubation) (Meyer, 1964) and settle down in the gonadal primordium at stages 20-24 where they rapidly proliferate to form germ cells (Nakamura et al., 1988). The optimum stage to intercept the PGCs during migration is when the PGCs concentrate in the germinal crescent at stages 4-8 (18-28 hr of incubation). While isolated PGCs are potentially suitable vehicles, direct microinjection attempts failed because of collapse of the turgid glycogen filled cells (Shoffner, 1991, personal communication). Consequently, in this study attempts were made to transfect plasmids into the PGCs via $CaPO_4$ or liposomes to mediate the entry of DNA. In theory, after introduction of foreign genes into PGCs, they can be transferred to the recipient embryo, to continue their journey to produce a chimeric embryonic gonad. The objective of this investigation was to develop PGCs transfer methodology including the transfection of PGCs *in vitro* and their introduction into the gonadal germ line.

Materials and Methods

Isolation of the PGCs

Donor eggs were prepared from White Leghorn females artificially inseminated weekly with semen from Rhode Island Red males, which were maintained at the University of Minnesota poultry facility. Fertilized donor eggs were incubated to

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developmental stages 6-8 (Hamburger and Hamilton, 1951), head-fold through 4 somites (approximately 26 hr of incubation). These stages are optimal for obtaining PGCs population within the germinal crescent. Following the incubation period, the shell was cleaned with 70% ethanol and then broken into a specimen dish containing chick Ringer's solution (7.2 g of NaCl, 0.37 g of KCl and $CaCl_2 \cdot H_2O$ in one liter of double distilled, deionized water). The embryo was 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 (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of $KHPO_4$ in one liter of distilled, deionized H_2O).

The germinal crescents shown in figure 1 were dissected with the aid of fine point forceps 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 two times to remove yolk granules. For each trial, ten to twenty germinal crescents were pooled and gently aspirated together in 5 ml to 10 ml of prewarmed, sterile calcium and magnesium free PBS to produce a single cell suspension. The cells were concentrated by centrifugation at 1,200 rpm for 10 min. The total number of cells from the ten germinal crescents were approximately 10^5 to 10^6 cells, determined with a standard hemocytometer. The PGCs were separated from the somatic cells by lack of attachment (Shuman, 1981). Periodic acid-Schiff (PAS) stain was used to determine presence of the glycogen filled PGCs which stain preferably a brilliant magenta.

Transfection of PGCs in culture

The plasmid RSVLTR/βG2 was provided by Hua-Ming Wang, University of Utha. This plasmid contains the lacZ gene under the control of Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter (Han et al., 1993). The lacZ gene for β-galactosidase originated from plasmid pGA307 (An et al., 1982). This plasmid contains the tufB--lacZ fusion in which the distal part of tufB, a gene for elongation factor EF-Tu, was replaced with bacterial lacZ structural gene. The promoter RSVLTR originated from the LTR/tk plasmid and is a strong promoter, as well as an enhancer, when introduced into a variety of eukaryotic cells (Gorman et al., 1982). The pGA307 was completely digested with Hind Ⅲ and Bam HI to produce the DNA fragment containing tufB-lacZ gene. To remove DNA fragments containing tk genes, LTR/tk was also thoroughly digested with Hind Ⅲ and Bam HI. These two fragments were ligated to produce RSVLTR/βG2 plasmid. The cell suspension containing PGCs was transfected by using lipofection (Bethesda Research Lab.) and $CaPO_4$ mediated DNA transfection (Stratagene) methods (Felgner and Holm, 1989; Chen and Okayama, 1987).

Lipofectin mediated DNA transfection into PGCs

The procedure for liposome-mediated transfection was followed from the methods for Felgner and Holm (1989). Lipofectin is a preformed cationic liposome which is thought to facilitate DNA transfection by forming a complex with nucleic acid. Subsequently, the complex fuses with the cell membrane and delivers DNA to the cell.

Following concentration of the cell suspension containing PGCs to 500 μl, the pellet was resuspended in 2.5 ml Opti-MEM media (Gibco) without serum. Prepared lipofectin (40 μg/50 μl) and circular DNA (10 μg/50 μl) RSVLTR/βG2 mixture (total 100 μl) was applied to transfect PGCs (Felgner and Holm, 1989). A second trial was conducted by mixing approximately 100-200 μl cell suspension containing PGCs and 100 μl lipofectin-DNA mixture. These PGCs and DNA were incubated for 4 hr at 37℃. After incubation, DME/Ham's F-12 media with serum was added for additional 48 hr at 41℃ to assay transfection efficiency.

The lipofectin-DNA mixture was not removed from the cell suspension. Because PGCs are fragile (Wentworth et al., 1989), the step of centrifugation was minimized. After 48 hr of incubation to determine transfection efficiency, media was aspirated carefully with a Pasteur pipette, and the adherent cells were fixed and rinsed with methods as described in Han et al. (1993). Afterwards, the cells were treated with X-gal at pH 7.4 for 12-16 hr.

Calcium phosphate mediated PGCs transfection

The following procedure for plasmid DNA transfection is a modification of the method used by Chen and Okayama (1987, 1988). The modi-

fication consists of introducing $CaPO_4$ in a very gradual way. For the calcium phosphate mediated transfection trial, a DNA mixture, 10 μg of circular plasmid, RSVLTR/βG2 was diluted with 225 μl distilled, deionized water. Twenty five μl of 2.5 M $CaCl_2$ and 250 μl of 2X BBS (Stratagene) was added to obtain a 500 μl $CaPO_4$-DNA mixture for 500 μl cell suspension containing PGCs. Two different treatments were performed on these cells. One aliquot was kept with $CaPO_4$-DNA for 20 min at room temperature prior to culturing with 5 ml DME/Ham's F-12 media including serum in a 37℃, 2.5% CO_2 incubator for 4-8 hr. The second portion was placed in the incubator immediately in the same medium. After 4-8 hr incubation with DNA, all cells were transferred to a 15 ml centrifuge tube. Adherent chicken embryonic fibroblasts (CEFs) were removed by sequential treatment with 1 ml 1X Trypsin-EDTA and then 3 ml PBS and serum mixture (1:1). All cells were concentrated by centrifugation at 1,200 rpm for 10 min. Cultures were added with fresh media (DME/Ham's F-12 containing serum), for 48 hr in a 41℃ incubator for possible replication of transfected cells. After 48 hr of incubation, cells were screened with X-gal.

Host embryo preparation

Fertilized eggs were prepared from White Leghorn females crossed with Rhode Island Red males (University of Minnesota, USA). Host embryos were prewarmed for several hours at room temperature and incubated to developmental stages 6-8 (approximately 26 hr), positioned on their sides. Eggshells were cleaned and a 25 mm^2 window was cut in the side of the egg with a dentist drill fitted with an abrasive stone drill bit (Healthco-Krause).

PGCs transfer by microinjection into germinal crescent

After 4-8 hr of incubation, media was removed from the cell suspension and the pellet resuspended with 200 μl of calcium and magnesium free modified Ringer's solution (6.9 g of NaCl, 0.37 g of KCl, 0.18 g of sodium citrate and 1.0 g of glucose in one liter of distilled, deionized H_2O). In later experiments, 100 μl to 200 μl cell suspension including PGCs transfected with 100 μl lipofectin-DNA mixture was microinjected without washing with modified Ringer's solution. In early experiments, cells were transfected by $CaPO_4$-DNA copre cipitation for 8 hr with 20 min preincubation but later for 4 hr with no preincubation because of the high percentage of mortality. When these cells were stained with trypan blue, only 50% of the cells showed viability after 8 hr of transfection and 20 min preincubation with $CaPO_4$-DNA precipitates.

The windowed egg with the host embryo supported in a nest of sterile cotton was positioned under a 50 μl-syringe with a 30-gauge needle. After the germinal crescent was located under the window, 5-10 μl of 50-100 PGCs and fluid were slowly released into the germinal crescent (figure 1). The germinal crescent was first identified under the stereomicroscope, then PGCs were microinjected without the stereomicroscope. Identification of the germinal crescent was difficult even with the use of the stereomicroscope because the embryo is transparent and wrapped by the vitelline membrane. When the small end of the egg is located at the 3 o'clock direction, the head of the embryo is usually located in the 12 o'clock direction with the folds in front of the head. This is one way to identify the germinal crescent. The needle was inserted very slowly through the vitelline membrane and pulled back under the vitelline membrane and upper layer of cells (epiblast). When the fluid with PGCs was released, the embryo was easily damaged, therefore, injection was conducted very carefully. Once the injection was completed, the window was closed by heat sealing a piece of parafilm to the shell and layered by a combination of paraffin and beeswax, or Magic scotch tape (3M, St. Paul, MN). Next, the eggs were incubated with the "window" side up overnight. After injection the next day, the eggs were moved and incubated at 37.5℃ and 50% relative humidity in a Robbins forced air incubator. The eggs were rotated 180° periodically for the remainder of the 4-6 day incubation period.

Seeding of PGCs on chorioallantoic membrane (CAM)

The chorioallantoic membrance (CAM) is heavily vascularized, and the rationale for this procedure was focused on the potential for the PGCs to enter the circulatory system. The fertilized eggs were incubated on their sides for 2-2.5 days for seeding experiment purposes. At this time, the embryo had already formed the extraembryonic CAM adhering to the shell membrane. Eggs were cleaned with 70% ethanol. The embryos were

located by a candle lamp attached to a vacuum pump. The holes (1-2 mm in diameter) were made with an egg punch in the egg shell on the site of the CAM and the air cell. By vacuum force, the air cell in the blunt end of the egg was moved over the CAM of the embryo, making it possible to seed the PGCs on the chorioallantoic membrane. The 10 μl of transfected cell suspension containing PGCs was injected by 50 μl-syringe with a 30-gauge needle through the shell membrane. The hole was covered with Magic scotch tape. The eggs containing seeded PGCs were incubated on their sides, overnight and then rotated periodically for the remainder of the 3-5 day incubation period in a Robbins forced air incubator.

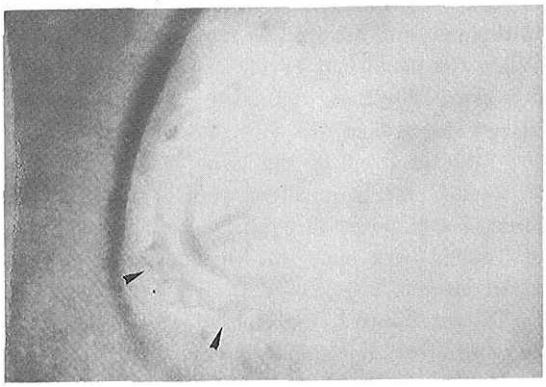

Figure 1. Germinal crescent (►) covering primordial germ cells anterior to the head fold in embryos incubated for 24 hr.

Gonad screening

The fate of the transfected, transferred PGCs was determined by screening the 5-7 day embryonic gonad. After removing both (left, right) gonads, they were squashed on a slide. After fixation and rinsing, the gonads were treated with X-gal, and the positive gonads were examined for blue coloring. Results showed faint background when incubated with X-gal at pH 7.4 after 12 hr. In this study, gonad screening for exogenous β-galactosidase was performed at pH 7.4 for 5-6 hr.

Results

Transfection of PGCs

The transfection efficiencies of cell suspensions containing PGCs are shown in table 1. When comparing the efficiencies, 100-200 μl of cell suspension in Opti MEM media transfected with 100 μl liposome-DNA mixture resulted in the much higher efficiency (approximately 1.5%) than the 3 ml of cell suspension (approximately 0.1%) in Opti-MEM media transfected with 100 μl liposome-DNA complexes. This transfection efficiency is that of adherent cells including CEFs incubated for 48 hr. The plate of 100 μl-200 μl of cell suspension from the ten to twenty germinal crescents in media transfected with liposome and DNA complexes showed transfected blue PGCs. There was no difference in cell viability of these two lipofectin methods (70-80% viability in trypan blue) after 4 hours of transfection.

TABLE 1. TRANSFECTION EFFICIENCIES OF CELL SUSPENSIONS CONTAINING PGCs

Methods	Treatment	Cell viability (%)	Transfection time (hr)	Efficiency (%)
Lipofection	3 ml cell suspension	70-80	4-8	0.096
Lipofection	100-200 μl cell suspension	70-80	4	1.51
$CaPO_4$	20 min preincubation	50	4-8	–
$CaPO_4$	no preincubation	70-80	4-8	0.11

To determine the transfection efficiency of the nonadhering cells after 48 hr of incubation, the cell suspension was concentrated by centrifugation. Nonadhering cells after 48 hr of incubation did not give positive results. Almost all cells were dead and PGCs were not detected on the slides. These results may be due to the loss or breakage of PGCs by centrifugation during fixer, rinser, or X-gal treatment because the PGCs were fragile. Also, PGCs in the cell suspension may not have survived 48 hr of incubation.

The $CaPO_4$ method for transfection of cell suspension $CaPO_4$ showed more toxicity when the cells were preincubated with a $CaPO_4$-DNA mixture. Transfected PGCs were found from the $CaPO_4$ method not preincubated with the $CaPO_4$-

DNA mixtrue. The transfection efficiency of cell suspension on $CaPO_4$-DNA mediated transfection without preincubation was 0.11%. Efficiency with preincubation was not determined (table 1).

Figure 2 shows the PGCs transfected with lipofectin-DNA. The large eccentric nucleus is evident in PGCs.

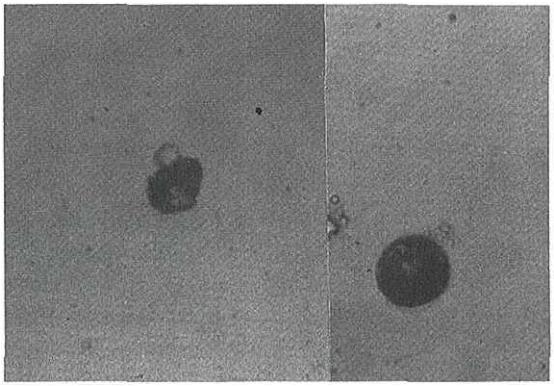

Figure 2. Expression of transfected PGCs in culture.

Transfer of PGCs into host germinal crescent

Table 2 is a summary of PGCs transfer into germinal crescent. A total of 389 embryos were microinjected with 5-10 μl of $CaPO_4$-DNA or lipofectin-DNA treated PGCs. Of the total, 47 embryos were sacrificed. Embryonic gonads were examined after PGCs settlement, but before sexual differentiation. One positive gonad from embryos injected with lipofectin-DNA treated PGCs was detected by appearance of a blue PGC. Figure 3 consists of photograph of positively identified gonad from the lipofectin transfection method. From this picture, the transfected PGCs appeared to be dividing. The PGCs in the gonad were larger than the other cells. These transfected PGCs were identified after 1 hr of incubation with a solution containing X-gal. A 12% survival rate of recipient eggs in PGCs transfer was lower than that of DNA transfection (24%) (Han et al., 1994).

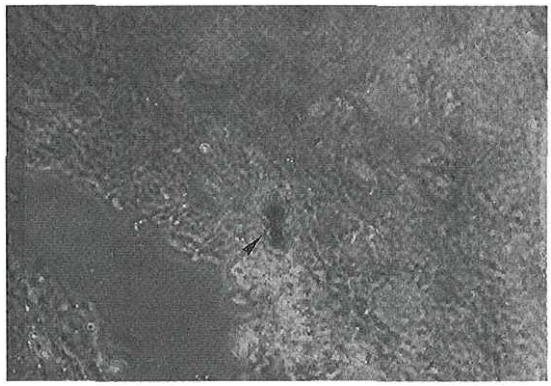

Figure 3. Expression of transfected PGCs (►) in gonad of 5 day embryo.

TABLE 2. SUMMARY OF TRANSFER OF PGCs INJECTED INTO GERMINAL CRESCENT

Transfection method	Number of eggs	Number of survived eggs (%)	Screened embryo age (day)
$CaPO_4$	268	28 (10.4)	5.0-7.5
Lipofectin	121	19 (15.7)	5.0-7.0
Total	389	47 (12.1)	5.0-7.5
Control*	34	8 (24.0)	7.0

* Injection of 2 μl H_2O.

Seeding of PGCs on CAM

Table 3 illustrates the results of the PGCs seeding to extraembryonic blood vessels. Unfortunately, positive gonads were not obtained from 87 tested embryos. The seeding process had no effect on embryo mortality. Although trial 2 in table 3 indicates high mortality when compared with the control, this was expected due to problems with the incubator. Excluding the results from trial 2, the survival rate of PGCs seeded embryos (95 %) was the same as the rate of the control (93%).

TABLE 3. SUMMARY OF THE RESULTS OF SEEDING PGCs ON THE CAM

Trial	Number of eggs	Transfection method	Number dead	Screened embryo age (days)
1	5	lipofection	0	6.5
2	33	lipofection	8	7.5
3	39	lipofection	2	7.0
4	21	lipofection	1	7.0
Total	98		11 (11%)	
Control	30	untreated	2 (7%)	7.0

Discussion

Transfection efficiency of the cell suspension differed from the results of the adherent CEF (Han et al., 1993); the efficiency of the cell suspension was lower than that of the adherent CEFs. These results correspond to the reports from Yen et al. (1988). The cell suspension preincubated with $CaPO_4$-DNA complex for 20 min showed high toxicity. However, a higher concentration of lipofectin-DNA complex in the cell suspension raised transfection efficiency without increasing cytotoxicity over the level of efficiency found in a 3 ml cell suspension in medium containing a lipofectin-DNA mixture.

In this study, PGCs were transfected and expressed successfully. These results indicate that PGCs can be manipulated *in vitro*. The transfection of PGCs provides the possibility of direct gene transfer into the germ line to overcome the limitation of retroviral vectors. The limitations of PGCs manipulation are: 1) Difficulty of isolating pure PGCs and their tendency to aggregate and form clumps. 2) Low transfection efficiency. The highest transient transfection efficiency found in previous study was 2.5-3% by the $CaPO_4$-DNA coprecipitation in CEF (Han et al., 1993) and in this study 1.5% by the liposome mediated transfection in cell suspension. Furthermore, efficiency of stable integration of DNA into chromosomes was very low, 5 to 7 $\times$ 10^{-4} in CEF (Han et al., 1993). 3) Apparent lack of PGCs replication *in vitro*. Successful culturing of chicken PGCs and replication in the culture have not been reported.

Successful PGCs transfer into host embryos to create germ line chimeras has been reported (Shuman, 1981; Wentworth et al., 1989; Vick et al., 1993). The data from this study demonstrate that PGCs can be transfected and transferred for colonization in the gonad. Unfortunately, the data in this study are not adequate to show evidence of such colonization. The low probability of colonization and proliferation of transfected PGCs in the gonad was explained by the following reasons: 1) Low transfection efficiency, 2) Most transfected cells showed transient expression (Han et al., 1993). Transfection efficiency of cell suspensions containing PGCs was 1.5% by liposome mediated DNA transfection. Gibbins et al. (1990) reported transfection efficiencies in the cell suspension of up to 4% by the lipofectin-mediated DNA transfection. However, almost all the DNA introduced into the chicken cell was degraded between 3-6 days of transfection (Han et al., 1993).

The survival rate of host eggs in PGCs transfer was lower than that of the control (table 2). The factors affecting the mortality of the embryos may be 1) The temperature of the eggs during PGCs transfer. A temperature drop experienced by the embryo during PGCs injection can cause stress to the embryo (Gonzales, 1989). 2) Injected air during PGCs injection. One characteristic of PGCs isolated from the germinal crescent is an inclination to aggregate (Wentworth et al., 1989). After culturing in media containing calcium and magnesium, the concentrated PGCs aggregated together or to other cells. Therefore, in comparison to the DNA injection method, it was important to dispense the PGCs fluid into the syringe without air bubbles. 3) Trauma and injury during injection. PGCs fluids damage the embryo. After 24 hr, an embryo has already formed a head. As a result, injury to the head was common during injection of PGCs into

the germinal crescent. The rolling of the egg during injection also damaged the embryo.

Although this study did not show positive results from the seeding of the PGCs on the CAM, this method for PGCs transfer does have potential. This experiment was undertaken with the following hypothesis. After PGCs start to migrate via blood vessels to the future gonad from stages 9-10 (30-38 hr of incubation), most PGCs were found in the blood stream at stage 12 (16 somites, 45 to 49 hr of incubation) (Lee et al., 1978; Fujimoto et al., 1976) and settled in the gonadal primordium at stage 20-24 (3-4 days of incubation) (Nakamura et al., 1988). Therefore, PGCs are circulating in the blood vessels from stage 10 to stage 24 (2-4 days of incubation). PGCs have migrating characteristics to penetrate blood vessels. Fujimoto et al. (1976) suggested that the PGCs possess pseudopodia and their amoeboid movement as a possible mechanism for migration. At the end of the circulation in the intravasculature, PGCs extravasate from the blood vessels by unknown mechanisms and invade the thickened coelomic epithelium (Ukeshima et al., 1987), indicating the active migration capabilities of PGCs. Therefore, seeding PGCs near the developing embryonic blood vessel makes it possible to penetrate or invade the blood vessel. With this background, the PGCs were seeded on the CAM of 2-2.5 day old embryos. However, this hypothesis was not proven in this experiment. One possibility for each of recovery of transfected cells in the gonad is that few or none of the seeded PGCs were in fact transfected. Other reports (Fujimoto et al., 1976; Lee et al., 1978; Ukeshima et al., 1987) describe the active migratory characteristics of PGCs but further research to study these characteristics such as the penetration of the CAM, the mechanism of extravasation into the epithelium, and the process of invasion of the PGCs must be conducted to prove this hypothesis.

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Literature Cited

An, G., Hidaka, K. and Siminovitch, L. 1982. Expression of bacterial β-galactosidase in animal cells. Mol. Cell Biol. 2:1628-1632.

Bosselman, R. A., R. Y. Hsu, T. Boggs, S. Hu, M. Nicolson and M. J. Briskin. 1990. Insertion and expression of model genes in the chicken germline using a replication-defective rev vector. Proceedings of the 4th World Congress on Genetic Applied to Livestock Production XVI: 94-96.

Chen, C. and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7:2745-2752.

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

Felgner, P. L. and M. Holm. 1989. Cationic liposome-mediated transfection. Betheda Res. Lab. Focus 11:21.

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

Gibbins, A. M., C. L. Brazolot, J. N. Petitte, G. Liu and R. J. Etches. 1990. Efficient transfection of chicken blastodermal cells and their incorporation into recipient embryos to produce chimeric chicks. Proceedings of the 4th World Congress on Genetics Applied to Livestock Production XVI: 119-121.

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

Ginsburg, M. and H. Eyal-Giladi. 1986. Temporal and sparial aspects of the gradual migration of primordial germ cells from the epiblast into the germinal crescent in the avian embryo. J. Embryo. Exp. Morph. 95:53-71.

German, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. and Howard, B. H. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA- mediated transfection. Proc. Natl. Acad. Sci. USA. 79:6777-6781.

Gonzales, D. S. 1989. Gonadal germ cell chimeras produced by microinjecting primordial germ cells into the vasculature of early chicken embryos. M. S. Thesis, University of Wisconsin-Madison, p. 65.

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

Han, J. Y., Y. S. Shin, R. N. Shoffner and K. S. Guise. 1993. Stable transformation of cultured chicken cells. Asian-Australasian Journal of Animal Sci. 6(4): 581-589.

Han, J. Y., R. N. Shoffner and K. S. Guise. 1994. Microinjection and expression of marker genes in the early chicken embryo. Korean J. of Animal Science 36(3):244-251.

Lee, M., N. Karasanyi and R. G. Jr. Nagele. 1978. The role of the cell surface in the migration of primordial germ cells in early chick embryos: effects of concа-

ravalin. A. J. Embryol. Exp. Morph. 46:5-20.

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

Meyer, D. B. 1964. The migration of primordial germ cells in the chick embryo. Dev. Biol. 10:154-190.

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

Shuman, R. M. 1981. Primordial germ cell transfer in the chicken *Gallus domesticus*. M.S. Thesis, University of Minnesota, p. 54.

Ukeshima, A., M. Kudo and T. Fujimoto. 1987. Relationship between genital ridge formation and settlement site of primordial germ cells in chick embryos. Anat. Rec. 219:311-314.

Urven, L. E., C. A. Erickson, U. K. Abbott and J. R. McCarrey. 1988. Analysis of germ line development in the chick embryo using an antimouse EC cell antibody. Development 103:299-304.

Vick, L., Ying Li and K. Simkiss. 1993. Transgenic birds from transformed primordial germ cells. Proc. R. Soc. Lond. B. 251:179-182.

Wentworth, B. C., H. Tasi, J. H. Hallett, D. S. Gonzales and G. Ra;cic-Spasojevic. 1989. Manipulation of avian primordial germ cell and gonadal differentiation. Poultry Sci. 68:999-1010.

Yen, T. S. B., Y. F. Wang and E. Seto. 1988. DNA transfection into suspension cell lines with a modified calcium phosphate precipitate method. Bio/Technology 6:413-414.