Production of Transgenic Chimeric Chickens Using Blastodermal Cells*

Haifeng Yan, Chaeyoung Lee[1,] **, Bingnan Xiao, Pavel Trefil[2], Shixun Liu, Younyoung Kim[1] and Xiaolin Wu
Lab for Molecular Biology Applied to Animal Production, Hunan Institute of Animal and Veterinary Science
Changsha 410131, P. R. China

ABSTRACT : A practical approach was proposed to produce transgenic chimeric chickens using blastodermal cells (BCs). The chicken BCs were mechanically dissociated and transferred into the recipient eggs that had been exposed to 500 rads irradiation of ^{60}Co and windowed on the equatorial plane. Chimeric chickens were generated using two models: the crosses (MXL) from Black Minors (*ii,EE,b/b*) ♂×Barred Leghorns (*ii,ee,B/-*) ♀ as donors and White Leghorns (WL, II) as acceptors (Model 1), or the Black Heifengs (BH, *ii,EE,bb*) as donors and Hua-xing white (HW, II) as recipients (Model 2). The treated eggs were incubated in their original shells in normal conditions until hatching. Green fluorescent protein (GFP) gene was transferred into the BCs derived from MXL and BH via lipofectamine and the pEGFP-C1, and transfection efficiency into the BCs was examined under a fluorescent microscope. Potential transgenic chimeras were selected based on the proposed methods in this study. Using the fresh BCs, the best rate of phenotypic chimeras was 6.7% and 26.0% in model-1 groups, and model-2 groups, respectively. We also described the optimized conditions for transfection. Although 30% of the BCs transfected *in vitro* emitted green light under an inverted fluorescent microscope, no embryos injected with the transfected BCs expressed foreign GFP gene at 3-4 days. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 2 : 158-164)***

Key Words : Chimeric Chickens, Blastodermal Cells, Transfection, GFP

INTRODUCTION

Gene manipulation in cells in culture, followed by nuclear transfer, has opened up new possibilities in the genetic modification of farm mammals. However, nuclear transfer in birds is particularly difficult and inefficient. Instead, production of chimeras using blastodermal cells (BCs) is currently one of the main approaches in poultry. The chicken BCs have been cultured *in vitro* for a long time as putative chicken stem cells (Pain et al., 1996) and transfected *in vitro* (Brazolot et al., 1991; Speksnijder et al., 1999; Jeong and Han, 2002). The chicken BCs transfected and sorted *in vitro* also contributed to somatic and germline chimeras (Speksnijder et al., 1999; Lee and Piedrahita, 2003).

At present time, there are a couple of obstacles that hold back the further study on transgenic chimeric chickens (Yang et al., 2004). First, the previous procedures of embryo operations were complicated and laborious and the survival rate of the treated embryos was low (Petitte et al., 1990; Thoraval et al., 1994; Speksnijder et al., 1999; Naito et al., 2001). While surrogate eggshells (Speksnijder et al., 1999) and windowing on the blunt end of eggs (Bednarczyk et al., 2000) were previously used to improve the hatchability of treated eggs, it was very laborious and costly to prepare the surrogate eggshells and transfer the embryos. In addition, windowing on blunt of eggs is also inconvenient because of the destruction of the natural air-chamber of the eggs and the relative long distance of operating the embryos. Second, methods are in need that not only allows stably transfection of BCs but also maintains the abilities of BCs for contributing to somatic and germline chimeras. With previous methods, the gene transferred into chicken pluripotent cells appeared to persist episomally but was gradually lost during embryonic development (Brazolot et al., 1991; Naito et al., 1998; Speksnijder et al., 1999).

In the present research, we made a few modificaltions to improve current methods. In making chicken chimeras we dissociated donor blastoderms mechanically, windowed on equatorial plane of recipient eggs, transferred donor BCs with simple injection system and incubated the embryos in their original shells, instead of transferring them to surrogate eggshells. We transfected chicken BCs using pEGFP-C1 plasmids and liposome and the efficiency of transfection was detected using a fluorescent microscope. Green fluorescent protein (GFP) has been used widely in transgenic biology as a selectable marker and a reporter to study long-term transgene integration and expression, but few reports are available in poultry. There are several advantages for using GFP in contrast with using report genes: i) It is easy to detect the transfected cells with fluorescent microscope or exciting light. ii) It is possible to observe the living results without any pretreatment. iii) There is no need of any reacting substrates or other assistant factors. iv) False positive results will not occur since there

* This study was supported in parts by Hunan Nature Sciences Foundation, Sino-Czech Science and Technology Collaboration Foundation, and Hallym University, Korea.
** Corresponding Author: C. Lee. Tel: +82-31-380-1630, Fax: +82-31-388-3427, E-mail: clee@hallym.ac.kr
[1] Ilsong Institute of Life Science, Hallym University, Anyang, Kyonggi-do 431-060, Korea.
[2] Research Institute of Biopharmacy and Veterinary Drugs, Jilove 25449, Czech Republic.
Received April 9, 2004; Accepted September 6, 2004

is no GFP in poultry. Finally, we examined the possibility of transfecting donor BCs into recipients.

MATERIALS AND METHODS

Experimental birds

Birds used in the present experiment included Black Heifengs (*ii,EE,bb*) and White Huaxings (II), which were bred in the Poultry Breeds Center, Hunan Animal and Veterinary Science, and Black Minor (*ii,EE,b/b*), Barred Leghorn (*ii,ee,B/-*) and White Leghorn (II), which were bred and provided by the Research Institute of Biopharmacy and Veterinary Drugs, Czech Republic.

Plasmids and liposome

The pEGFP-C1 C-Terminal Protein Fusion Vector encodes a red-shifted variant of wild-type GFP, which has been optimized for brighter fluorescence and higher expression in mammalian cells (Excitation maxima=488 nm; emission maxima=507 nm). A neomycin-resistance cassetle (neo^r), consisting of the SV40 early promoter, the neomycin/ kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex thymidine kinase gene, allowed stably transfected eukaryotic cells to be selected using G418 Sulfate antibiotic. The plasmids were amplified in *E. coli* HB_{101}, extracted by an alkaline lysis procedure.

LIPOFECTAMINE Reagent is a 3:1 (w/w) liposome formulation of the polycationic lipid (DOSPA), and the neutral lipid (DOPE) in membrane filtered water.

Preparation of the BCs

The freshly oviposited eggs from Black Heifengs were sterilized with 70% alcohol and laid horizontally for about 30 minutes. The egg was cracked into petridish making the blastoderm appearing to the top. The thick egg white above the blastoderm was sniped and moved till the dry vitelline membrane appeared. The blastoderm was covered with a filter paper ring, and then cut and picked out into a petri dish containing M199. After the yolks were removed with a hair ring, the blastoderms were transferred into a tube containing growth M199 (10% FBS, 2% chicken serum, pyruvic acid and Gentamicin sulfate), dissociated slowly by a pipette, and then stored at 15-18°C.

Producing chimeras with fresh BCs

We used two models for examining chimeras phenotypically. In model 1, the crosses from Black Minor ♂×Barred Leghorn ♀ were used as donors, and White Leghorns were used as recipients. In model 2, the donors were Black Heifengs and the recipients were White Huaxings.

The amount of 5 μl fresh BCs from the center disk of the donor blastoderm (the concentration was 20 μl/embryo) were transferred into the subgerminal cavity of the acceptors at the same stage, which were exposed to 500 rads from a ^{60}Co and windowed equatorially using an improved injection and sealing system. The eggs transferred with donor BCs were incubated in an experimental incubator at 37.8°C, 70% relative humidity. Then the conditions were changed to 37.0°C and 75% of humidity in the last two days of incubation.

Prepartion for transfection

Coverglasses were pretreated as described by Etches et al. (1999) and put in a 35 mm petridish containing 2 ml growth M199. An appropriate volume of cells (about 4 embryos for each petridish) was added directly onto the coverglass in the petridish. The BCs were cultivated in an incubator (37°C, 5% CO_2) for about 4 h. Each 35 mm petridish was used as one transfection unit.

Diluted 1-2 μg plasmid DNA into a tube containing 100 μl M199 and 4-8 μl LIPOFECTAMINETM Reagent (1 mg/ml, Gibco BRL, Crewe, UK) into another tube containing 100 μl M199 for each transfection. Combined the two solutions, mixed gently and incubated at room temperature for 30 min to form the complex of DNA-liposome. While complexes formed, rinsed gently the BCs prepared before with 1 ml serum-free M199. For each transfection unit, added 0.8 ml M199 into the tube containing the complexes. Mixed gently and overlay the diluted complex solution onto the rinsed BCs. Then continue to incubate the cells for 5-12 h with the complexes, and added 1 ml M199 (containing twice normal concentration of serum) and continue to incubate for 12 h, replaced the medium in petridish with fresh complete medium and continue to incubate for 24 h after replacing. At the same time, the controls were prepared as the similar procedures described above, only without plasmid DNA.

Screening conditions for *in vitro* transfection

The eggs of hybridization (Black Minor ♂×Barred Leghorn ♀) and the 4-well petridishes were used to screen the conditions of cell culture, the amount of DNA, the duration of transfection, the duration of adding serum as well as the duration of replacing with fresh growth medium. One 4-well petridish was used as one transfection unit (the amount of BCs was 4 embryos) and 2 wells were used as one group.

Setting fluorescent microscope

For the fluorescent microscope (BH2-RFL, Olympus, Tokyo, Japan), the B (BP-490) exciter filter, EY-475 supplement exciter filter and B (DM-500+O-515) dichroic mirror were used in accordance with the spectrum features of GFP for examining the fluorescence (exciter region 475-490 nm, emission maxima 507 nm) emitted by the transfected cells. The coverglasses were fetched out and put

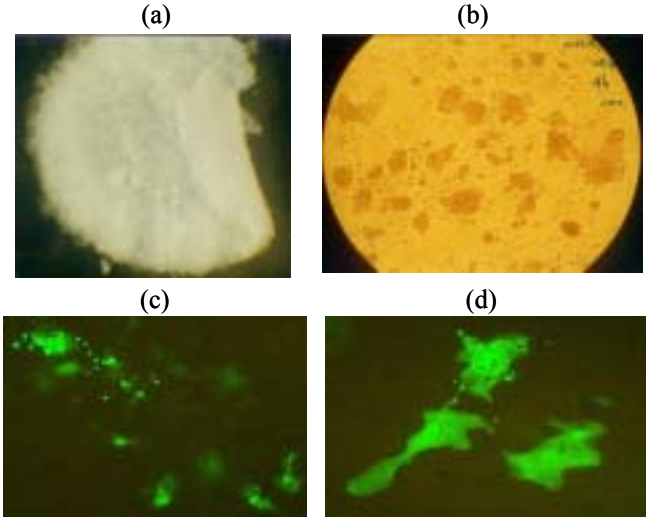

Figure 1. (a) Whole embryos seperated at stage X, (b) attached chicken blastodermal cells, (c) chicken BCs efficient expression foreign gene (GFP), (d) different BCs expressing foreign gene (GFP).

on the clean slide glass under the microscope.

For the invert fluorescent microscope (IFM) (CK40-RFL, Olympus), the CX-DMB-2 mirror cube, DM500 dichroic mirror and BP460-490 exciter filter were used. The petridish with transfected cells was checked directly under the microscope.

Culturing transfected BCs

Continue culturing the BCs for 2-3 d after the transfection procedure was finished. Then, discarded the medium, cultured the BCs with PBS containing 0.02% (w/v) EDTA for 12 min. Replaced with 0.05% (w/v) trypsin/0.02% (w/v) EDTA and continued incubation for 2 min at 37°C. Added them into growth medium, recovered BCs, centrifuged at 3,000 rpm for 5 min, and discarded the supernatant. Finally the dissociated cells were seeded in new cultures.

Transferring GFP to chicken embryos

The donor BCs, which were transfected with GFP and checked under IFM, were transferred to 199 recipiant eggs in 7 groups. The first 3 groups were done following model-1 and the rest 4 were done following model-2. The treated eggs were incubated for about 3 days and the embryos were transferred to the petridish containing medium using filter paper rings. The expression of GFP gene in the embryos was checked under IFM.

RESULTS AND DISCUSSION

***In vitro* cell culture and transfection**

While most previous researchers have dissociated the blastoderms with enzyme for making chimeras (Petitte et al., 1990; Etches et al., 1996; Kino et al., 1997; Speksnijder et al., 1999; Speksnijder and Ivarie, 2000), we dissociated them mechanically and the size of cell clusters depended on the times of aspirating and blowing. Although we were unable to get single cells, our method maintained a high activity. As seen in Figure 1a, the intact blastoderms were separated completely. Both the area pallucida and the area opaca were seen clearly and the loss of the blastoderm was also very low. In previous methods, however, in order to achieve a single cell suspension with no pretreatment, the attached cells had to be incubated in 0.05% (w/v) trypsin/0.02% (w/v) EDTA solution at 37°C for 15 min. By doing so, it did disperse the cells, but they could not be cultured later (Speksnijder et al., 1999). As getting a single cell was a necessary step, we preferred to disperse chicken blastoderm mechanically during culture of chicken embryonic stem cells.

Most cells attached on the coverglass in the petridish after 4 h culture and began to communicate each other (Figure 1b). But there were still some cell clusters that had the similar appearance to initial cells after 46 h incubation. Perhaps it was due to the concentration of cells that improved the condition of *in vitro* culture, since most cells of the four blastoderms were added onto the coverglass. More cells in the same volume would help together.

Transfection could be started when BCs cultured for about 4 h. Having been incubated with complexes of DNA-liposome for 6 h (10 h cultivation from start), most of the cells became monolayers and the profiles looked like to be fusiform. The cells were honeycombing when the whole procedure was finished (46 h cultivation from start). There were a few small droplets especially in controls, which seemed to be lipids inside cells.

The transfection conditions for BCs from Black Heifengs were optimized as: 1.5 μg plasmid DNA, 5 μl lipofectamine (1 mg/ml), 30 min to form the complexes of DNA-liposome, 6 h to incubate the cells with the complexes. Approximately, 30% transfected cells emitted green light under the IFM after being incubated for 20 h. Judging by the growth condition of BCs and intension of the green light when the volume of liposome varying from 4 μl to 8 μl, the optimal volume of liposome was 5 μl. Although the green light with 8 μl liposome was stronger than that of 5 μl, the extent of cell differentiation with 8 μl liposome was more obvious. The shape and intensity of the BCs expressed GFP, was asymmetric, and they were globate, lumpish, netted and shuttle (Figures 1c and 1d).

Different transfection conditions that we used were shown in Table 1. Though reducing the volume of DNA (group 7) did not dramatically change the percentage of positive cells, the green light in groupt 7 was weaker than that in group 6. Changing the time of the liposome-DNA complexes co-incubation with the BCs remarkably affected the efficiency of transfection (groups 1, 2 and 3), but the

Table 1. Adjustment of partial conditions in transfection

Groups	DNA (μg)	Lipofection duration	Duration of adding complete Medium	Cultured duration with growth medium (h)	Expressing spots
1	1.25	1 h in Tube at 37°C	Plated, 12 h, 37°C	10	10
2	1.25	2 h in tube at RT	Plated, 12 h, 37°C	9	15
3	1.25	2 h in tube at RT, 4 h in dish at 37°C	12 h, 37°C	7	30
4	1.25	1 h in tube at 37°C, 5 h in dish at 37°C	10 h, 37°C	7	30
5	1.25	1 h in tube at 37°C, 5 h in dish at 37°C	10 h, 37°C	2	30
6	1.25	6 h in dish at 37°C	10 h, 37°C	2	30
7	0.75	6 h in dish at 37°C	10 h, 37°C	2	20
8	1.25	6 h in dish at 37°C	10 h, 37°C	12	30
9	1.25	1 h in tube at RT Plated, 8 h at 37°C	12 h, 37°C	12	35
10	1.25	6 h in tube at RT	Plated, 12 h, 37°C	12	15

RT: room temperature.

Table 2. Survival embryos of treated eggs

Groups	Number	Survival embryos during hatching					
		6 d	12 d	18 d	20 d	hatched	Chimeras
1	30	13 (43%)	10 (33%)	5 (17%)	2 (6%)	0	2
2	20	9 (45%)	6 (30%)	1 (5%)	0		1
3	39	15 (39%)	8 (21%)	2 (5%)	1 (3%)	0	2
4	34	18 (53%)	15 (44%)	11 (32%)		9 (27%)	1 (3%)
5	69	39 (56%)	31 (45%)	26 (38%)	22 (32%)	15 (22%)	10 (15%)
6	73	53 (73%)	43 (59%)	39 (53%)		29 (40%)	13 (19%)

Figure 2. A phenotypic chimeric bird (feather, tibia, digiti, and crown).

difference was slight when exceeding 6 h of transfection (group 9). Seen under a IFM after 6 h of transfection, there was no green light in group 10, and the signal was weak in group 8. No increment of green cells was found when the BCs were cultured for additional 12 h with serum (groups 4, 5, 6 and 8). In groups 5 through 8, 8% cells that were cultured for 72 h emitted green light.

The present research demonstrated that using the method of detecting transfected cells using GFP was convenient. Previously, the positive cells were detected by showing exogenous β-gal activity using X-gal or C_{12}FDG as substrate (Speksnijder et al., 1999). Although Chloroguine was used as a inhibitor of endogenous lysozomal β-gal activity, the increase of the fluorescence signal with the time made it difficult to compare results among different experiments (Speksnijder et al., 1999). In addition, we found that the efficiency of transfection using liposomes was better than that using electroporation for chicken BCs (data was not shown). A similar report was available that the lipofection method was better than that of electroporation in terms of higher efficiency of transfection and more convenience in use (Qian and Xiao, 1999).

Production of chicken chimeras with fresh BCs

The result of producing chimeras was shown in Table 2. While trying to have as many as possible phenotypic markers, we chose native breed HB as donors, which have ten unique characteristics. In the experiment, only 5 chimeric embryos were obtained in model 1 (the first 3 groups). Among them one chimeric embryo derived almost dominantly from donor BCs judging by feather color, but no chicken were successfully hatched. There were 110 eggs

(a)

(b)

Figure 3. Phenotypic chimeric birds: (a) the whole body was black except the white head, (b) phenotypic half-chimerism.

used in model 2. One chimeric rooster was obtained in group 4 (no irradiation) with 25% feathers being black. In groups 5, the ratio of total chimeras (including dead embryos) was 23% and that of living chimeras was 17%. There were 2 chimeras that exhibited 50% of the phenotypic characters of the donor and another 2 chimeras that exhibited more than 90% of the phenotypic characters of donor. Figure 2 showed a chimeric bird whose the right body was black and the left body was white. A similar result was previously reported by Trefil (1997). We obtained 6

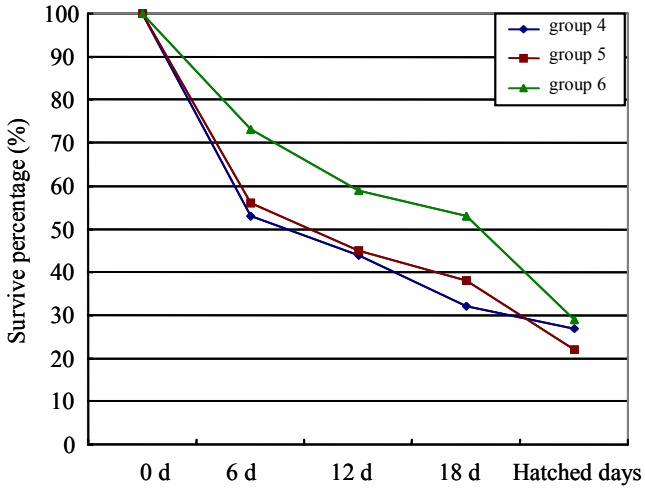

Figure 4. Survival rate of embryos decreased with age of hatchery.

chimeras in this group that exhibited more than 50% of the donor's phenotypic characters. Figure 3a showed a hen whose entire body was black except that the head was white. The chimerism was seen with mixed corona that showed donor phenotype (black moruloid corona) at the base and recipient phenotype (red leaf-comb) on the top, and seen with mix phenotypes in legs and toes. In figure 3b there was a bird that showed phenotypic half-chimerism. In group 6, the ratios of total and living chimeras was 26% and 18%, respectively, and the hatchability rate was 40%.

With our method, it was easy for two persons to finish at least 10 eggs per hour and the results were good. The curve of survive embryos in the last 3 groups showed that the falling range in unit time (FRUT) was greater during 0-6 d than that during 6-18 d. The FRUT of irradiated groups was higher than that of no irradiated group from 18 d to hatching. The result of group 6 was the best; the main reason might be due to the quality of the eggs (Figure 4). Several approaches were demonstrated to try to get high hatchability of treated eggs and high chimerism chimeras. The hatchability was 9.8% (14/143) via equatorially windowing and 41.0% (43/105) by opening a hole on blunt (Bednarczyk et al., 2000), where the egg airchamber was damaged and the distance was longer than that of windowing on equator when injection was performed. The hatchability of 33.7% was got with a modified shell windowing, which a volume of PBS was placed on the shell hole prior to piercing the shell membrane so that a droplet formed above and around the hole, allowing PBS, not air, to be drawn into the egg interior. The reason for the rescuing effect was unknown, but it was proposed that introduction of on artificial air space over the blastoderm caused mechanical stress on the embryo during the windowing procedure, which often led to death of treated embryos (Speksnijder and Ivarie, 2000). However, we got much

Table 3. Conditions for transferring transfected donor BCs into recipient embryos

Groups	Transfection				Number	Results (checked under a IFM)
	CC	Regent (U)	Pt (h)	Lt (h)		
1, 500 rad	8 E/800 μl	2	1	1	30	33% embryos alive after 3 d, no positive.
2, 500 rad	4 E/200 μl	1	0.5	2	32	13% embryos alive after 3 d, no positive.
3, 500 rad	4 E/200 μl	1	2	5	19	42% embryos alive after 3 d, no positive.
4*	6 E/200 μl	1	1	6	14	29% embryos alive after 3 d, only one positive dot. Most BCs emitting weak green light before transferring.
					13	one hatched and one chimeras (18 d).
5*	6 E/220 μl	1	1	5	25	40% embryos alive after 4 d, no positive. Most BCs emitting weak yellow light before transferring.
6*	6 E/220 μl	1	0.5	20	36	63.9% embryos alive after 3 d, no positive. Most BCs emitting strong green light before transferring.
7*, 500 rad	7 E/200 μl	1	0.5	6	30	50.0% embryos alive after 3 d, no positive. 2% BCs emitting strong green light before transferring.

* Model 2, E: Embryos, CC: cell concentration, 1 U: 5 μl liposome, 1.5 μg DNA.
Pt: pre-incubation time; Lt: lipofection time.

better results without any pretreatment. If the mechanical method was used to compromise the recipient embryos instead of irradiation, the hatchability was rarely low 6.6% (105/1,594) (Naito et al., 2001). It seemed that irradiation remarkably improved the ratio of chimeras and the percent of chimerism. In model 2, the rate of chimerism of group 4 (no irradiation), group 5 and group 6 were 3.0%, 15% and 19% respectively. The result of last 3 groups was much better than that of first 3 group, possibly due to differences among these groups in chicken breeds, the qualities of eggs and the contamination controlling.

Potential transgenic chimeric chicken embryos

The transfected BCs were transferred to 199 recipients using 2 models, but no embryos emitted green light was found when seen under IFM. The survival rate of potential chimeric embryos from transfected BCs was also lower than that of fresh BCs (Table 3). Further work is on the way to investigate the reasons for the failure and to increase the integration of the foreign DNA into chicken chromosomes.

There is much evidence that, particularly in circular form, DNA introduced into the cytoplasm of cells often exists episomally and is eventually lost by degradation or dilution as the embryo grows. Introduction of linear sequences can increase the frequency of integration of exogenous DNA into a chromosome. Speksnijder et al. (1999) showed that they were able to obtain 20-30% transfection rate on a routine basis (using circular or linear DNA sequences), but that only 1 in 10 of these events using linear DNA represents stable integration into the genome. At present time the BCs can be lipofected with foreign DNA, cultured overnight, selected by fluorescence activated cell sorting based on expression of that DNA and incorporated into the germline of chimeric embryos, but the foreign DNA detected by PCR in F1 generation of chimeras is rarely, non positive result was found by southern blot.

In conclusion, we demonstrated a model of producing chimeric chickens using BCs with a few modifications. In addition to be applied in transgenic chicken research, it also will fascinate the research of conservation of avian genetic resource. The present method was efficient in transfection of chicken BCs by lipofection and convenient to detect the transfected cells. These modifications are helpful toward stably transfecting of BCs and high rate of chimerism and hatchability. However, efforts are still in need to increase the chance of integration of the foreign DNA into chicken chromosomes.

ACKNOWLEDGMENTS

This research was supported in parts by Hunan Nature Sciences Foundation, Sino-Czech Science and Technology Collaboration Foundation, and Hallym University, Korea. The authors wish to thank Prof. B. Pain in France and Prof. J. Zhang in China for their valuable comments.

REFERENCE

Bednarczyk, M., P. Lakota and M. Siwek. 2000. Improvement of hatchability of chicken eggs injected by blastoderm cells. Poult. Sci. 79:1823-1828.

Brazolot, C. L., J. N. Petitte, R. J. Etches and A. M. Gibbins. 1991. Efficient transfection of chicken cells by lipofection, and introduction of transfected blastodermal cells into the embryo. Mol. Reprod. Dev. 30:304-312.

Etches, R. J., M. E. Clark, A. Toner, G. Liu and A. M. Gibbins. 1996. Contributions to somatic and germline lineages of chicken blastodermal cells maintained in culture. Mol. Reprod. Dev. 45:291-298.

Jeong, D. K. and J. Y. Han. 2002. Migration activity of chicken gonadal primordial germ cells (gPGCs) and post-transfer localization of LacZ-transfected gPGCs in the embryonic gonads. Asian-Aust. J. Anim. Sci. 15:1227-1231.

Kino, K., B. Pain, S. P. Leibo, M. Cochran, M. E. Clark and R. J. Etches. 1997. Production of chicken chimeras from injection of frozen-thawed Blastodermal cells. Poult. Sci. 76:753-761.

Lee, C. K. and J. A. Piedrahita. 2003. Transgenesis and germ cell engineering in domestic animals. Asian-Aust. J. Anim. Sci. 16:910-927.

Naito, M., M. Sakurai and T. Kuwana. 1998. Expressing of exogenous DNA in the gonads of chimeric chicken embryos produced by transfer of primordial germ cells transfected *in vitro* and subsequent fate of the introduced DNA. J. Reprod. Fertil. 113:137-143.

Naito, M., A. Sano, Y. Matsubara, T. Harumi, T. Tagami, M. Sakurai and T. Kuwana. 2001. Localization of primordial germ cells or their precursors in stage×blastoderm of chickens and their ability to differentiate into functional gametes in opposite-sex recipient gonads. Reproduction 121:547-552.

Qian, F. and C. Z. Xiao. 1999. Comparison of Lipofection and Electroporation Gene Transfer into Mammalian Cells. Prog. Biochem. Biophys. 26:289-291.

Pain, B., M. E. Clark, M. Shen, H. Nakazawa, M. Sakurai, J. Samarut and R. J. Etches. 1996. Long-term *in vitro* culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. Development 122:2339-2348.

Petitte, J. N., M. E. Clark, G. Liu, A. M. Verrinder Gibbins and R. J. Etches. 1990. Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development 108:185-189.

Speksnijder, G., R. J. Etches and A. M. Verrinder Gibbins. 1999. Germline chimeric chickens from FACS- sorted donor cells. Mol. Reprd. Dev. 52:33-42.

Speksnijder, G. and R. Ivarie. 2000. A modified method of shell windowing for producing somatic or germline chimeras in fertilized chicken eggs. Poult. Sci. 79:1430-1433.

Thoraval, P., F. Lasserre, F. Coudert and G. Dambrine. 1994. Somatic and germline chicken chimeras obtained from Brown and White Leghorns by transfer of early blastodermal cells. Poult. Sci. 73:1897-1905.

Trefil, P. 1997. Chicken somatic and germline chimeras. World Poultry-Misset 13:10.

Yang, C. C., H. S. Chang, C. J. Lin, C. C. Hsu, J. I. Cheung, L. Hwu and W. T. K. Cheng. 2004. Cock spermatozoa serve as the gene vector for generation of transgenic chicken (*Gallus gallus*). Asian-Aust. J. Anim. Sci. 17:885-891.