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Birth of Germline Chimeras by Transfer of Chicken Embryonic Germ (EG) Cells Into Recipient Embryos

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ABSTRACT This study reports for the first time the production of chicken germline chimeras by transfer of embryonic germ (EG) cells into recipient embryos of different strain. EG cells were established by the subculture of gonadal tissue cells retrieved from stage 28 White Leghorn (WL) embryos with I/I gene. During primary culture (P_0), gonadal primordial germ cells (gPGCs) in the stromal cells began to form colonies after 7 days in culture with significant ($P < 0.0001$) increase in cell population. Colonized gPGCs were then subcultured with chicken embryonic fibroblast monolayer for EG cell preparation. Prepared EG cells or gPGCs at P_0 were transferred to stage 17 Korean Ogol chicken (KOC) embryos with i/i gene. The recipient chickens were raised for 6 months to sexual maturity, then a testcross analysis by artificial insemination was conducted for evaluating germline chimerism. As results, transfer of EG cells and gPGCs yielded total 17 germline chimeras; 2 out of 15 (13.3%) and 15 of 176 sexually matured chickens (8.5%), respectively. The efficiency of germline transmission in the chimeras was 1.5–14.6% in EG cells, while 1.3–27.6% in gPGCs. In conclusion, chicken germline chimeras could be produced by the transfer of EG cells, as well as gPGCs, which might enormously contribute to establishing various innovative technologies in the field of avian transgenic research for bioreactor production. *Mol. Reprod. Dev. 65: 389–395, 2003.* © 2003 Wiley-Liss, Inc.

Key Words: chicken; embryonic germ (EG) cell; germline chimera; transgenic aves

INTRODUCTION

Research on manipulating pluripotent stem cells derived from embryos and fetal tissues has a great impact on developing innovative technologies in various fields of life science including medicine, pharmaceutics, agriculture, and biotechnology. Since the first isolation in the mouse embryos (Evans and Kaufman, 1981), stem cells or stem cell-like colonies have been continuously established in the mouse of different strain (Martin, 1981), hamster (Doetschman et al., 1988), rabbit (Giles et al., 1993), bovine (First et al., 1994), porcine (Wheeler, 1994), and human (Thomson et al., 1998). However, full-term development originated from established pluripotent cells, which is an absolute criterion for proving cell plasticity and differentiation capacity, were only reported in the mouse (Bradley et al., 1984). Due to technical difficulties, no further progress has been made in the establishment of animal embryonic stem (ES) cells. The use of embryonic germ (EG) cells is alternative for establishing stem cells especially in the livestock. The finding of Labosky et al. (1994) on germline chimera development after transfer to embryos clearly proved the pluripotency of EG cells and their similar characteristics with ES cells in the mouse.

In the chicken, it has been suggested that EG cells could be established by subculture of primordial germ cells (PGCs) retrieved from the germinal crescent and the embryonic blood. However, only limited cell population could be collected from such tissues and it became one of obstacles to efficiently establish EG cells for germline chimera production. It was recently suggested to use gonadal PGCs (gPGCs) for overcoming such limitation (Chang et al., 1997; Park and Han, 2000). Technical feasibility and applicability of gPGCs for preparing chicken EG cells were confirmed in our previous study (Park and Han, 2000). Apparently, additional research was necessary for examining whether established EG cells could induce germline transmission after transfer to recipient embryos. The objectives of this study was therefore to examine the capacity of chicken EG cells established by in vitro culture of gPGCs to induce germline chimerism.

MATERIALS AND METHODS

Culture of Chicken gPGCs and EG Cells

For this study, two inbred chicken strains were maintained at the University Animal Farm; White

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Leghorn (WL) and Korean Ogol chicken (KOC), Seoul National University, Korea. gPGCs were retrieved from the gonads of WL embryos at the stage 28 (5.5 days of incubation) (Hamburger and Hamilton, 1951). After embryos were collected from the fertilized eggs, they were rinsed with Ca^{2+}- and Mg^{2+}-free PBS (−) for removing the yolk. The abdomen of the embryos was carefully dissected under a stereomicroscope and the gonads were collected with sharp tweezers. Gonadal tissues were dissociated by gentle pipetting in 0.05% (v/v) trypsin solution supplemented with 0.53 mM EDTA. After being centrifuged at 200*g* for 5 min, gPGCs in the gonadal tissue were seeded onto a 24-well culture plate according to the method of Park and Han (2000), and cultured in Dulbecco's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 2% (v/v) chicken serum (Gibco), 1 mM sodium pyruvate (Sigma, St. Louis, MO), 55 μM β-mercaptoethanol (Sigma), 20 ng/ml conalbumin (Sigma), 10 mM HEPES (Gibco), 1× antibiotics-antimycotics (Sigma), 5 ng/ml human stem cell factor (hSCF; Sigma), 5 U/ml murine leukemia inhibitory factor (mLIF; Sigma), 10 ng/ml bovine basic fibroblast growth factor (bFGF; Sigma), 0.04 ng/ml human interleukin-11 (hIL-11; Sigma), and 10 ng/ml human insulin-like growth factor-I (hIGF-I; Sigma). gPGCs were then maintained in a CO_2 incubator at 37°C until colonies were formed and the medium was changed every 5 days. For subculture, the colonies of chicken EG cells were agitated by gentle pipetting without trypsin–EDTA treatment and harvested from the plate. These cells were centrifuged at 200*g* for 5 min and allotted into a fresh 24-well plate containing chicken embryonic fibroblast (CEF) feeder cell monolayer. The colonies of EG cells seeded onto the feeder monolayer were then subcultured at intervals of 7–10 days (Park and Han, 2000) and provided for each experiment.

Periodic Acid Schiff's (PAS) Staining and Anti-SSEA-1 Antibody Screening

The chicken EG cells were fixed to the plate in 1% (v/v) glutaraldehyde for 5 min and rinsed with 1× PBS twice. EG cells were immersed in PAS solution (Sigma) for 5 min at the room temperature (20–25°C). After washing with PBS, fixed EG cells were immersed in Shiff's Solution (Sigma) for 15 min at the room temperature. After washing with PBS twice, PAS-stained EG cells were observed under an inverted microscope. On the other hand, gPGCs were stained daily with anti-SSEA-1 antibody from the onset of culture and the positive gPGCs number was counted under the inverted microscope for evaluating the proliferation profiles during the culture period. Anti-SSEA-1 antibody developed by Solter and Knowles (1978) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Development of Biological Science. Chicken EG cells were fixed with 1% (v/v) glutaraldehyde for 5 min and rinsed with PBS twice. The anti-SSEA-1 ascites fluid diluted 1:1,000 in PBS was added and subsequent steps were carried out using DAKO universal LSAB® kit, Peroxidase (DAKO, Carpenteria, CA) according to the manufacturer's instruction.

Labeling of Chicken EG Cells With PKH26 Fluorescent Dye

For monitoring gonadal migration after transfer, chicken EG cells were labeled with PKH26 fluorescent dye by a modified protocol of the supplier (ZYNAXIS Cell Science, Inc., Japan). After being centrifuged at 200*g* for 5 min, EG cells were suspended and gently pipetted in serum-free medium containing PKH26 fluorescent dye at the concentration of 4×10^{-3} mM. The labeling reaction was finally stopped with the addition of 125 μl FBS after 5 min. After the injection to recipient embryo and then further incubation of 3 days, the whole gonads of recipient embryos were dissected and the localization of donor EG cells was monitored under a microscope equipped with fluorescent apparatus (Olympus, IX70, Tokyo, Japan).

Production of Germline Chimeras and Testcross Analysis

To examine a potential to differentiate into the germ cell lineage in vivo, EG cells or gPGCs were injected into the dorsal aorta of KOC recipient embryos at the stage 17 (2.5 days of incubation) (Hamburger and Hamilton, 1951). For the transplantation, a small window was made on the sharp end of recipient egg and approximately 2–3 μl of cell suspension containing 150–200 gPGCs or EG cells was injected into the dorsal aorta of recipient embryo using a micropipette. The egg window of the recipient embryo was sealed twice with paraffin film and then laid down with the sharp end at the bottom until hatching. Hatched chickens were maintained for up to 6 months with our standard management program. Sexual maturity of hatched chickens was then monitored by the criterion whether each flock could produce egg or semen. All sexually matured chickens were then provided for testcross analysis using artificial insemination with adult KOCs. The white-feathered progenies indicated these chickens are derived from donor EG cells of WL, while the black-feathered progenies from endogenous KOC recipient. It was considered that autosomal I gene, a pigmentation inhibitor, governs the genes of melanin pigment stimulation by epistatic effect. Accordingly, the genotype of homozygous I/I in WL results in white feather color, while that of homozygous i/i in KOC results in black feather color. Hatched hybrid progenies between WL and KOC from testcross analysis have the heterozygous I/i that results in white feather color, which represents complete germline transmission of transplanted EG cells through the chimeras. Therefore, the chickens produced the progenies with white color feather in the testcross analysis were considered as a germline chimera. This also shows normal proliferation and differentiation of transplanted EG cells of WL in the recipient KOC embryos.

Experimental Scheme and Statistical Analysis

Based on our standard experimental procedures (Chang et al., 1997), we asynchronously transplanted EG cells or gPGCs of different types into the recipient embryos for germline chimera production. Transfer of stage 28 chicken gPGCs or EG cells derived from cultured gPGCs to the blood vessel of stage 17 embryos. WL and KOC embryos were employed as a cell donor and an embryo recipient, respectively. EG cells were characterized by PAS staining and anti-SSEA-1 antibody screening, and their localization into embryonic gonads after the transfer was monitored by PKH26 fluorescent dye. A testcross analysis was conducted for screening germline transmission of sexually matured chickens after transplantation. Experimental comparison was made with the data on germline chimera production after transfer of EG cells or gPGCs. Basically, two types of gPGCs (before or after colonization 2 and 10–15 days after culture, respectively) and of EG cells (at the first and second subculture). However, the group of gPGCs before colonizing was excluded in cell characterization and EG cells retrieved at the first and the second subculture were concomitantly provided for the detection of gonadal migration. Values in each experimental parameter were subjected to ANOVA using the general linear model (PROC-GLM) in SAS program (1992). When model effect was significant in each experimental parameter, treatment effects were subsequently compared by the least square method. Significant difference among treatments was determined where the P value was less than 0.05.

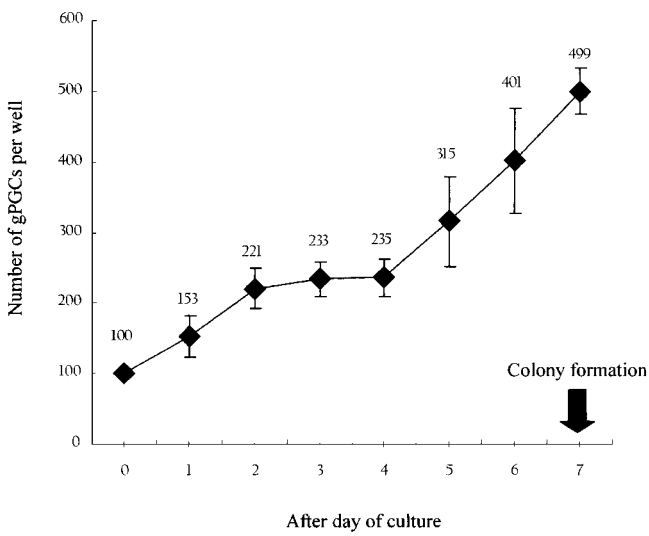

Fig. 1. Cell population of cultured gonadal primordial germ cells (gPGCs) on a gonadal stroma cells. A significant ($P = 0.0001$) model effect was found and the number of gPGC greatly increased from 2 days after culture compared with that at the onset of culture. Data obtained from five times replicates.

RESULTS

Culture of gPGCs

As shown in Figure 1, gPGCs could proliferate on the gonadal stromal cells without differentiation and formed colonies 7 days after primary culture. When cell number of cultured gPGCs was counted daily until colony formation (up to 7 days of culture), a significant ($P = 0.0001$) model effect was found. Compared with the number on day 0 of culture, gPGC number was significantly increased from 2 days after culture (221 vs. 100). As shown in Figure 1, average number of gPGCs at the time of colony formation was 499 and gPGC colonies fully developed until 10–15 days after primary culture.

Establishment and Characterization of EG Cells

For EG cell establishment, subculture of fully colonized gPGCs on day 10–15 of culture was conducted at intervals of 7–10 days. Maximum culture duration from gPGC collection to the end of the second passage therefore became within the range of 30–35 days. When cell characterization was done at the time of full colonization before subculture (P_0 stage) and first (P_1 stage) or second (P_2 stage) passages, all examined colonies were positively stained with PAS and expressed SSEA-1 epitope (Fig. 2). These specific EG cell characteristics were maintained throughout subculture.

Detection of gPGCs and EG Cells After Transfer to Recipient Embryos

Three types of cells, 2 or 10 day-cultured gPGCs and EG cells, were labeled with PKH26 fluorescent dye and 150–200 labeled cells were transferred to the recipient embryos. When fluorescent signal was monitored in the gonads of recipient embryos 3 days after transplantation, 16 out of 18 (88.9%) embryos transplanted 2 day-cultured gPGCs had positive signals, while 4 out of 12 (33.3%) in 10 day-cultured gPGC group. In contrast, 18 of 26 embryos (69.2%) received PKH26-labeled EG cells had positive signals in their gonads (Fig. 3).

Production of Germline Chimeras and Germline Transmission in Produced Chimeras

After gPGC or EG cell transfer, a total of 296 embryos hatched and 191 chickens were sexually matured (64.5%). Of those sexually matured progenies, 17 (8.9%) were a germline chimera. In the case of gPGC transfer, percentage of germline chimera production was 6.2% (7/113) and 12.7% (8/63) in the 2 day-cultured and colonized, respectively (Table 1). In EG cell transfer, 2 out of 15 (13.3%) sexually matured chickens were germline chimeras (Fig. 4); each from EG cells of subcultured once or twice.

As shown in Table 2, the efficacy of germline transmission after EG or gPGCs transfer was within the range of 1.3–27.6%. In the case of the EG cell, germline chimeras derived from the first and the second passage hatched 131 and 157 progenies, respectively, and

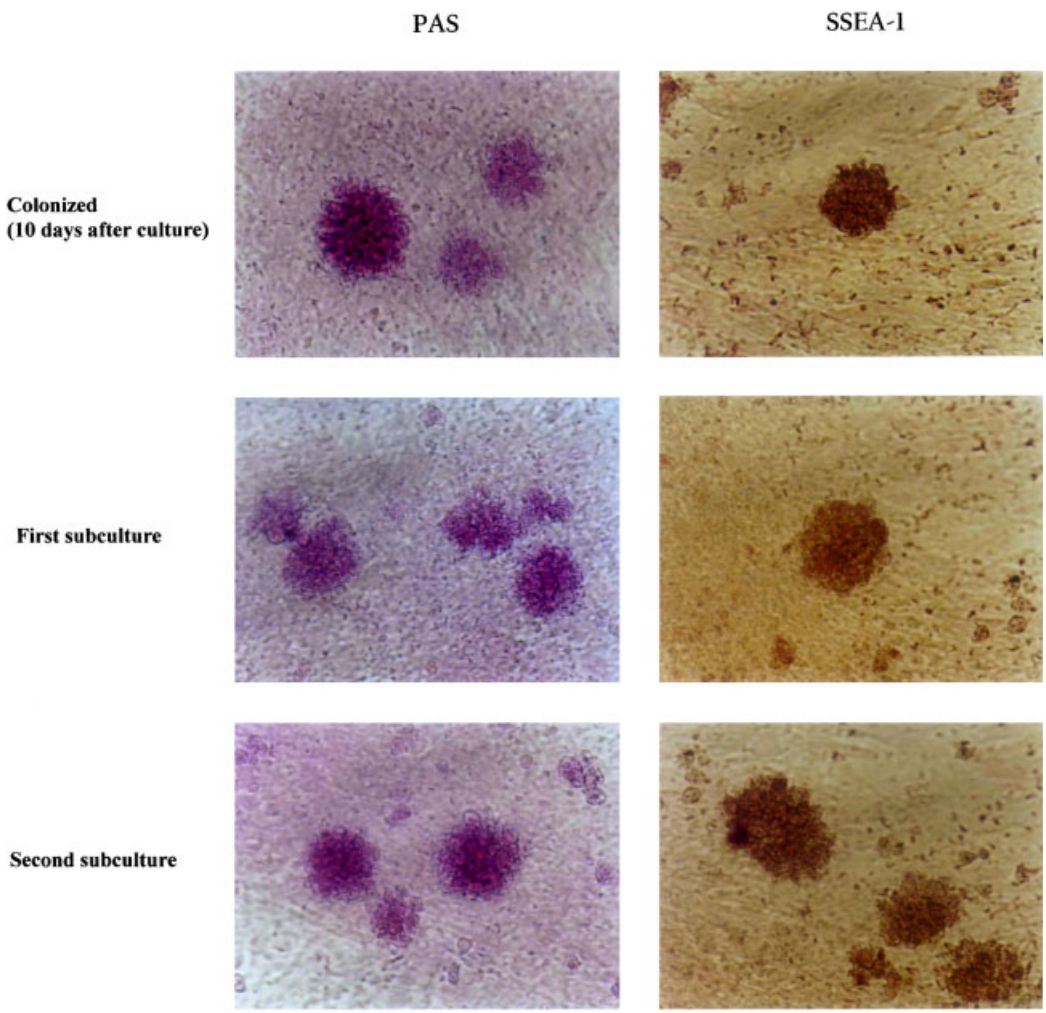

Fig. 2. Colonized gPGCs (10 days after culture) on gonadal stroma cells and chicken embryonic germ (EG) cells on a confluent chicken embryonic fibroblast monolayer, and its characterization by staining with periodic acid Schiff or anti-stage specific embryonic antigen-1 antibody during EG cell preparation (10 days after initial culture, the first and the second subculture) (×300 magnification).

the efficiency of individual germline transmission was 1.5% (2/131) and 14.6% (23/157). Such value was lower than that obtained from colonized gPGCs (27.6% = 261/944), but higher than gPGCs cultured for 2 days (1.3% = 31/2,332).

DISCUSSION

This is the first report to produce germline chimeras from gPGC-derived EG cells in nonmammalian species. Our experiments further demonstrated that chicken EG cells can be established from the culture of gPGCs and that these cells can induce germline transmission after transfer to recipient embryos. gPGC can be easily efficiently collected from the embryonic gonads compared with from the germinal crescent or embryonic blood. Transfer of EG cells or gPGCs to the recipient embryos yielded total 17 germline chimeras; 2 out of 15 sexually matured chickens in EG cell (13.3%) and 15 of 176 in gPGC (8.5%).

To standardize general procedure of EG cell establishment, cell culture dynamics during in vitro culture of gPGCs was monitored and characterization of cultured cells was subsequently made. In the presence of β-mercaptoethanol and growth factors in the medium, gPGCs continuously proliferated up to 7 days of culture without morphological differentiation. Considering extremely small portion less than 1% of seeded cells was gPGCs, increase in the population of gPGCs in the initial cell population might contribute to optimizing the efficacy of gPGC culture for EG cell establishment. On the other hand, development of a specific culture medium for chicken gPGCs on the basis of elucidating various factors regulating cell proliferation can also enhance the efficacy of a EG cell preparation system.

For the subculture of gPGCs for EG cell establishment, CEF without mitosis inactivation was used as a feeder cell. In our previous results (Park and Han, 2000), the use of mitotically inactivated CEF or STO (a SIM mouse embryo-derived, Thioguanine- and Ouabain-resistant fibrobalst cell line) monolayer by X-ray irradiation or mitomycin treatment was not effective for establishing and maintaining EG cells. Probably, the

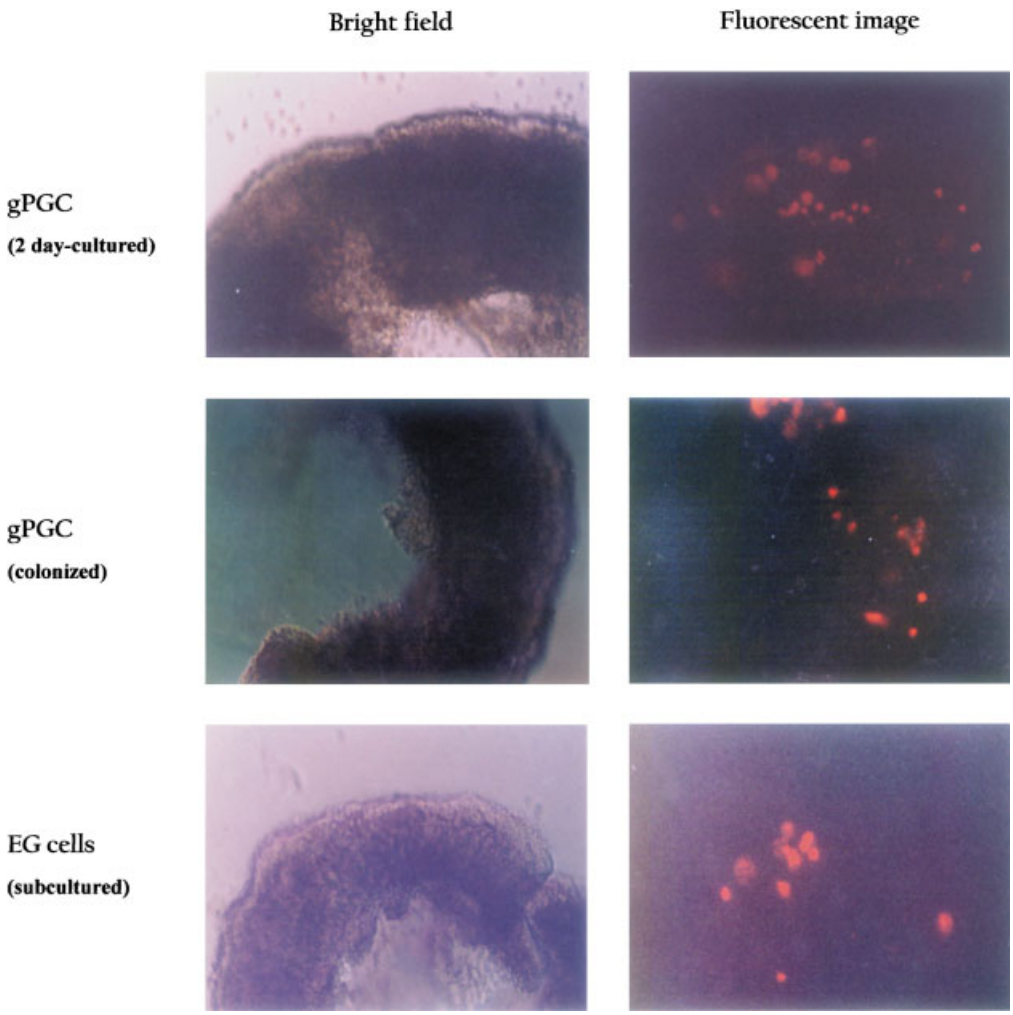

Fig. 3. Gonadal migration of chicken gPGCs and in vitro-established chicken EG cells after being transplanted into the dorsal aorta of the recipient embryos at the stage 17 (2.5 days after incubation). Fluorescent staining with PKH26 detected migration and localization of transplanted cells into the embryonic gonad. The gonads were collected from recipient embryos cultured further for 3 days after transplantation. gPGCs cultured in vitro for 2 days or fully colonized gPGCs were provided for transplantation, while EG cells at the first and the second passage were used.

properties of chicken pluripotent cells for cell undifferentiation and dedifferentiation are quite different from the cells of other species. Otherwise, the inactivation treatment might block signal generation in the feeder cells, which is required for regaining cell pluripotency.

In EG cell study, cell pluripotency has been proven by morphology, differentiation into somatic tissue cells

TABLE 1. Birth of Germline Chimeras by Transplantation of Chicken Gonadal Primordial Germ Cells (gPGCs) Cultured In Vitro for Different Durations or Chicken Embryonic Germ (EG) Cells Prepared From Subculture

Transferred cell types	Number of progenies undertaken testcross analysis[a]	Number (%)[b] of germline chimeras produced
gPGCs		
Cultured for 2 days	113	7 (6.2)
Colonized (P_0)	63	8 (12.7)
Subcultured EG cells		
First passage (P_1)	11	1 (9.1)
Second passage (P_2)	4	1 (25.0)

[a]Only sexually matured progenies provided for testcross analysis.
[b]Percentage of the number of germline chimeric chickens undertaken testcross analysis.

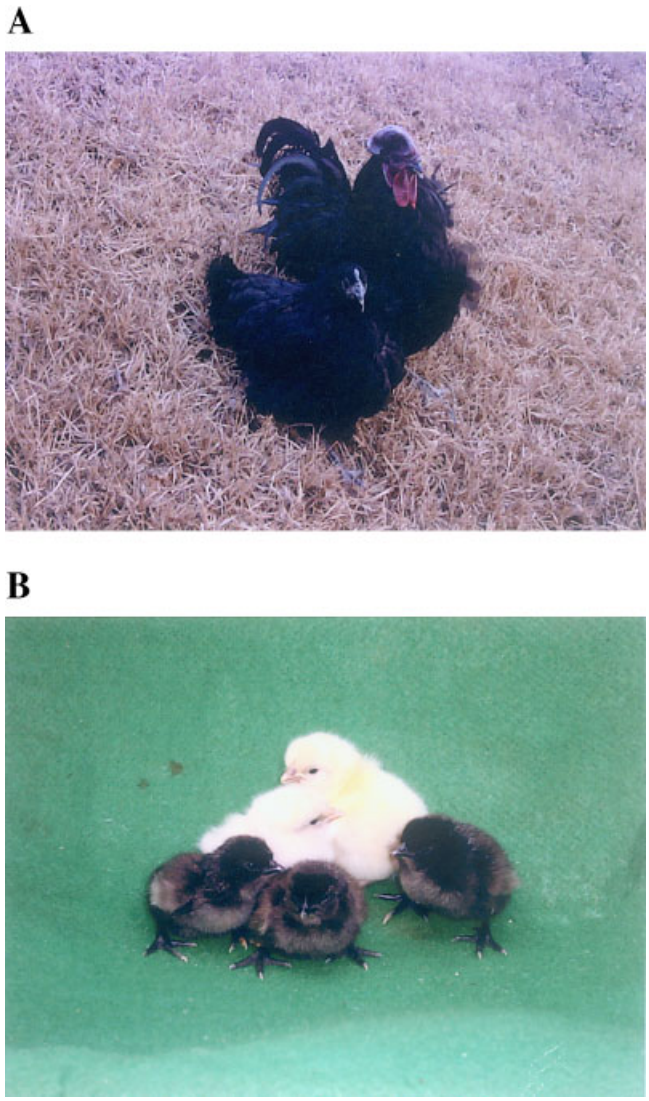

Fig. 4. Sexually matured chicken provided for testcross analysis using artificial insemination with adult Korean Ogol chicken (KOC). **A**: The male germline chimera produced by transplantation of EG cells subcultured twice. EG cells were derived from gPGCs of 5.5-day-old White Leghorn (WL) embryos and transplanted into the recipient embryos of 2.5-day-old KOC. **B**: Production of WL progenies from germline chimera between WL and KOC.

in vitro, somatic tissue incorporation after transplantation, intracellular alkaline phosphatase activity, and cell surface-specific markers such as stage specific embryonic antigens. In the avian research, induction of in vitro differentiation to three germinal tissue cells, gonadal and somatic tissue incorporation, and germline transmission have been used for manifesting EG cell properties. As the preliminary step of our research (Park and Han, 2000), we confirmed EG cell characteristics by in vitro differentiation and cell specific markers. In vivo somatic tissue incorporation after transfer to the blastoderm at stage X was also validated. In this study using the same system, we further confirmed gonadal migration and germline transmission activity of prepared EG cells.

In this series of study, we did not confirm somatic tissue incorporation activity after transplantation of chicken EG cells into the blood vessel, since it is extremely difficult to detect the somatic migration due to transferring low number of EG cells (less than 200) in our system. To improve the efficiency of EG cell transfer system, we have attempted to increase EG cell population at transfer and newly proposed system definitely contribute to manifesting the activity of EG cells to migrate embryonic somatic tissue after transfer. On the other hand, the use of cell surface-specific markers and staining dyes could be used for further confirmation of EG cell properties. Unfortunately, only PAS and SSEA-1 have been used for positive cell marker on both gPGC and EG cells and no other specific markers have been developed for the birds yet. Further research is necessary for developing avian EG cell specific markers to additionally confirm cell properties.

All examined colonies during subculture were positively stained with PAS and anti-SSEA-1 antibody of a pluripotent cell marker and these characteristics were maintained throughout subculture. These results imply that established EG cell colonies derived from chicken gPGCs are pluripotent. Colonized cells could migrate into the gonads and subsequently induced germline transmission after being transferred to recipient embryos of heterologous strains (WL cells to KOC embryos). Direct evidence on this hypothesis was shown from the results on germline chimera production, but the results of our previous study indicating established

TABLE 2. Transmission Efficacy in Germline Chimeras Produced by Transplantation of Chicken gPGCs Cultured In Vitro for 2 or 10 Days (Colonized) and Chicken EG Cells Prepared From Subculture

Origin of germline chimeras	Number of chickens proved as a germline chimera	Number of chickens hatched	Number (%)[a] of donor-derived white hybrid chicks produced
gPGCs			
Cultured for 2 days	7	2,332	31 (1.3)
Colonized (P_0)	8	944	261 (27.6)
Subcultured EG cells			
First passage (P_1)	1	131	2 (1.5)
Second passage (P_2)	1	157	23 (14.6)

[a]Percentage of the number of hatched chickens, which indicate the donor-derived progenies.

EG cells from gPGCs could differentiate into embryoid body or various types of somatic tissue cells in vitro (Park and Han, 2000), which were not shown in gPGCs, indirectly supported our suggestion.

Although there was no statistical significance due to low sample number, variations in the efficiency of germ cell migration after transfer and germline chimera production were found. Apparently, gPGCs collected from 5.5-day-old embryos and EG cells derived from cultured gPGCs can recover migration capacity, chemotatic movement, and the ability to colonize in the recipient gonadal ridges after in vitro culture. However, germ cell migration activity was the highest in 2 day-cultured gPGCs, while these cells yielded the lowest percentage of germline chimera production. These results indicated that, unless quantitative analysis is made, the migration detection by PKH26 is inadequate for expecting the induction efficacy of germline transmission by transferred cells.

Transgenic research of avian pluripotent cells has focused to bioreactor production, which can yield enormous pharmaceutical and neutroceutical gains (Sang, 1994). Bioreactor production in the birds is very much accessible, since in vitro manipulated germ cells can be easily transferred to the blood vessel of embryos for germline transmission. Acquired knowledge on the establishment of EG cell derived from gPGCs and confirmation of their pluripotency in this study greatly contributes to accelerating transgenic research for bioreactor production, as well as pluripotent germ cell studies. However, the rate of germline transmission was still limited, and EG cell characterization with the use of various cell surface markers and the optimization of culture system were further required for establishing highly potentiated EG cell line.

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