◀Research Note▶

A Simple Culture Method of Chicken Blastodermal Cells for Germline Transmission

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Introduction of genes into blastodermal cells is one of the procedures for chicken genetic modification in combination with construction of germline chimera. Currently, *in vitro* cultures of chicken blastodermal cells require feeder cells. In the present study, we employed a simple culture method of chicken blastodermal cells for their germline transmission without using feeder cells. The present study was undertaken to determine whether cultured blastodermal cells of Barred Plymouth Rock (*i/i*) contained periodic acid-schiff (PAS)-positive materials, alkaline phosphatase (ALP), SSEA-1, and EMA-1, which are the markers of pluripotent cells including chicken embryonic stem cells (cESCs). The examination revealed that not all but a large portion of cultured cells contained those markers. Then, the cultured cells (*i/i*) were injected into White Leghorn recipients (*I/I*) to generate chimeric chickens. The chimeric chickens thus obtained were crossed with Barred Plymouth Rock, and the cross produced chicks with black feathers (*i/i*). This observation demonstrated that cultured blastodermal cells were transmitted to germ cells in a chimera. In conclusion, the present culture system provided a tool for genetic modification of chicken.

Key words: blastodermal cells, chicken, chimera, germline transmission, simple culture method

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Introduction

The chicken embryo development initiated in the oviduct is divided into 14 stages, which were numbered in Roman numerals (stages I to XIV) by Eyal-Giladi and Kochav (1976). The fertilized cell undergoes division in oviduct to be laid as an “egg” at stage X 20–23 h after fertilization, which contains 20,000–50,000 blastodermal cells. The blastoderm of the stage X embryo is divided into area opaca and area pellucida consisting of a central disc and marginal zone.

In 1990, Petitte *et al.* first demonstrated that chicken blastoderm at stage X contains cells having the ability to contribute to somatic and germinal tissues when injected into a recipient embryo. Subsequently, Pain *et al.* (1996) explored the cultures of chicken blastodermal cells (cBCs)

containing chicken embryonic stem cells (cESCs) derived from stage-X embryos on a feeder of a mouse embryonic fibroblast cell line (STO). As an alternative way to culture cBCs, various cytokines, *i.e.*, recombinant leukemia inhibitory factor (LIF), Interleukin-6 (IL-6), Interleukin-11 (IL-11), stem cell factor (SCF), insulin growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF) were added to culture medium. Recently, it has been shown that the maintenance of cESCs can be solely dependent on the presence of avian LIF and serum (Horiuchi *et al.*, 2004). However, it has not been established that this cytokine is able to provide cESC culture competent for either somatic and/or germline contribution, because no chimera experiments have been reported. In the present study, we report the findings that, when the cBCs cultured by a simple method were injected into chicken embryos at stage X, some of the injected cells were transmitted to germ cells.

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Materials and Methods

Animals and Cell Culture

Barred Plymouth Rock (BPR; homozygous recessive at *I* locus, *i/i*) donors and White Leghorn (WL; homozygous dominant at *I* locus, *I/I*) recipients kept in the National Institute of Livestock and Grassland Science were provided by the institute. Fertilized eggs of BPR at stage X were obtained, and blastoderms were excised from the eggs. Some blastoderms thus excised were further separated into three different parts, *i.e.*, central disc, marginal zone, and area opaca. The whole or parts of blastoderms thus obtained were cultured in wells of 96-plate using a Dulbecco's Modified Eagle Medium supplemented with ingredients described in Table 1.

We examined periodically whether the cultured cells contained periodic acid-schiff (PAS)-positive materials, alkaline phosphatase (ALP) activity, stage specific embryonic antigen-1 (SSEA-1), and EMA-1, which were shown to be markers for cESCs (Urven *et al.*, 1989; Pain *et al.*, 1996).

All animal experiments in this study were carried out according to the Guidelines of the National Institute of Agrobiological Sciences for the care and use of experimental animals.

Generation of Chimeric Chicken

The cultured cBCs of BPR (*i/i*) at days 2 and 5 of culture were dissociated with 0.025% trypsin and 0.02% EDTA. Approximately 500 cultured cBCs were injected into the subgerminal cavity of recipient WL embryos (*I/I*), from which a cluster of ca. 700 cells at the center of the area pellucida had been removed by the method described by Kagami *et al.* (1997). The resulting embryos were cultured *ex vivo*, following the procedure described by Perry and Naito (Perry, 1988; Naito and Perry, 1989). The chimerism of hatched chicken was judged based on

the color of feathers.

Histological Analysis

The cultured cells were first fixed with 4% cold paraformaldehyde for 10 min, and then rinsed with phosphate-buffered saline. The fixed cells were subjected to PAS staining, using PAS staining kit (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) following the procedure recommended by the manufacturer. ALP in cells was detected with the NBT/BCIP system (Roche Diagnostics, Tokyo, Japan), using cells fixed in cold methanol for 10 min. SSEA-1 and EMA-1 in cells were detected immunologically using antibodies against SSEA-1 and EMA-1, which were purchased from DSHB (Developmental Studies Hybridoma Bank at the University of Iowa, IA, USA), following the procedures recommended by DSHB.

The cells thus treated with the above procedures were photographed using an Olympus microscope (Olympus Co., Tokyo, Japan).

Examination of Germline Transmission

The chimeric chickens thus obtained were crossed with BPR (*i/i*) to examine whether offspring obtained from the cross had black feathers. When some of the offspring have black feathers, BPR (*i/i*) cells in the chimeric chickens are considered to contribute to germ cells.

Results and Discussion

Culture of Chicken Blastodermal Cells

Blastoderm was isolated from stage X embryos, and subjected to culture in 96-well plates, as described in the Materials and Methods. The initial number of cells plated in a well was 5×10^4 in average. The cells were proliferated until 9-fold the initial cell number at day 3 of culture, and then the cell number decreased gradually to 1×10^5 at the end of culture (Fig. 1). When the blastoderm was separated into 3 parts, *i.e.*, central disc, marginal zone, and area opaca, and each of them was cultured; cells of each part were proliferated to 6–7 folds of the initial cell numbers at day 3 of the culture (Fig. 1). This observation together with the fact that the initial number of cells plated in a well showed no significant difference among the cultures led us to infer that interaction of cells from different parts of blastoderm was partly involved in cBC proliferation.

We then examined whether the cells at culture days 1 to 5 of culture contained PAS-positive materials and ALP activity, which are reported to be the marker of cESCs (Fig. 2). The examination demonstrated as follows: Large portions of cultured cells were found to contain PAS-positive materials at all the time cultured and comparable numbers of cultured cells were found to harbor ALP activity from the culture days 2 to 5 (Fig. 2). These findings indicated that the cultured cells might contain cESCs. In order to obtain further evidence that the cultured cells contained cESCs, the cultured cells were subjected to immunostaining for SSEA-1 and EMA-1. As shown in Fig. 2, comparable numbers of cultured cells contained SSEA-1 and EMA-1 at least from days 2 to 5.

Table 1. Medium composition

DMEM	90 ml
FBS	10 ml
chicken serum	2 ml
2-ME	0.1 ml
Sodium pyruvate	10 μ M
Non-essential amino acid	500 μ M
Nucleoside	
Adenosine, Guanosine	30 μ M
Cytidine, Uridine	30 μ M
Thymidine	10 μ M
Cytokines	
mLIF 10^3 units/ml hIL11	10 ng/ml
HbFGF 10 ng/ml hSCF	5 ng/ml
hIGF-I 20 ng/ml	
Antibiotics	
Streptomycin	100 ng/ml
Penicillin G	100 units/ml

These observations taken together strongly indicated that the cells cultured from days 2 to 5 contained cESCs.

Generation of Chimera

The cultured cells at day 0, day 2, and day 5 were injected into WL recipient embryos (*I/I*) at stage X. The embryos that had received cultured cells were incubated, following the procedure described in the Materials and Methods. As shown in Table 2, 80–86 injections into the recipients were performed using cells from each culture, giving 4, 5, and 8 hatched chickens for the culture cells at

day 0, day 2, and day 5, respectively. More than half the chickens thus hatched in each culture day survived over 5 months. The two chickens of culture day 2 and one chicken of culture day 5 which survived over 5 months were judged as chimera chickens based on the criteria described in the Materials and Methods (Table 2). One female chimeric chicken of culture day 2 and one male chicken of culture day 5 were mated with BPR (*i/i*) to generate offspring. Three chickens with black feathers (*i/i*) were found among 108 offspring from the male chicken,

Fig. 1. **Proliferation of chicken blastodermal cells cultured *in vitro*.** Blastoderms were excised from chicken embryos at stage X. Whole blastoderms and parts of blastoderms, *i.e.*, central disc, marginal zone, and area opaca, were cultured *in vitro* as described in Materials and Methods. Approximately 5×10^4 cBCs were plated in a well of 96-well culture-plate and cultured up to 10 day after plating. Culture cells were harvested after periods of time indicated in the figure and dissociated with 0.025% trypsin- 0.02% EDTA solution to count viable cells using trypan blue staining. Viable cell counting at each time point was performed using 3 or more culture wells.

Open circles indicate whole blastoderm; closed circles; central disk; open triangles; marginal zone; and closed triangles; area opaca.

Fig. 2. **Characterization of cultured cells of whole blastoderm.** The cultured cells of whole blastoderm at culture day from 1 to 5 were subjected to examination for PAS-positive materials, ALP activity, SSEA-1; EMA-1, as described in Materials and Methods.

Table 2. **Production of chimeric chickens by injection of cultured blastodermal cells**

Cells cultured	Recipients	Hatched chicks	Chickens over five months
for 0 day (Stage X)	86	4 (1)	2 (0)
for 2 days	80	5 (2)	4 (2)
for 5 days	86	8 (1)	4 (1)

Number of chickens in parentheses means chickens with black feathers.

Table 3. **Mating of the chimera with Barred Plymouth Rock (*i/i*)**

Chicken	Days of culture	Offspring	Offspring from donor cells
No. 119 ♀	2	—	—
No. 121 ♀	2	181	0
No. 115 ♂	5	108	3

No. 119 died in 5 months after hatching.

Fig. 3. **Chimeric chickens obtained in the present study.** Panel A shows two female chimeric chickens bearing black feathers constructed using two-days whole blastoderm culture; and panel B, a chimeric male chicken bearing black feathers (indicated by arrowheads) generated using 5-day whole blastoderm cultures.

whereas no chickens with black feathers (*i/i*) were found in 181 offspring from the female (Table 3). This result demonstrated that cells derived from donor-cBCs were

Fig. 4. **Three offspring with black feather obtained from mating between the male chimeric chicken and Barred Plymouth Rock (*i/i*).**

transmitted to germ cells through the injection of the cultured cells into recipient chicken embryos, though the transmission rate was very low (Table 3).

When one introduces gene(s) in cells of the blastodermal culture employed in the present study, it is possible to obtain chickens bearing transgene(s) in germ cells. In conclusion, the present study is the first, to our knowledge, to provide a simple culture procedure applicable for generation of transgenic chicken.

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