Long Term in vitro Culture of Chicken Primordial Germ Cells Isolated from Embryonic Blood and Incorporation into Germline of Recipient Embryo

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The present study was carried out to develop a long-term in vitro culture system for chicken primordial germ cells (PGCs). PGCs were obtained from the blood of 2.5-day incubated embryos. GFP gene was transferred into the collected PGCs and then cultured on feeder cells derived from the gonads of 7-day incubated embryos. The GFP-positive cells attached loosely to the feeder cells, and their morphology varied from round to fibroblast-like in shape. They proliferated slowly and occasionally formed colonies. The PGCs cultured for 23–61 days were transferred into the bloodstream of recipient embryos, and we examined their incorporation into the germline of chimaeric chickens. Test mating was carried out, and one germline chimaeric chicken was detected out of putative chimaeric chickens. That one chicken was generated by transferring 58-day cultured PGCs and it produced one donor-derived offspring out of 270 examined. Thus, a small percentage of the cultured PGCs retained the ability to migrate to the germinal ridges, thus giving rise to functional gametes, although most of the cultured PGCs differentiated during the culture period. In conclusion, a germline chimaeric chicken was generated by transferring PGCs cultured in vitro for about 2 months; the culture system for PGCs developed in the present study will contribute to the germline manipulation in chickens.

Key words: chicken, culture, embryo, germline chimaera, primordial germ cell


Introduction

The generation of transgenic chickens is very useful for producing pharmaceutical materials in eggs or for the genetic manipulation of chickens. Primordial germ cells (PGCs) are the progenitor cells of ova or spermatozoa, and in chickens they circulate in the bloodstream before migrating to the germinal ridges, enabling PGC manipulation in vitro and their transfer between embryos (Kuwana, 1993). PGCs are, therefore, one of the most effective vehicles for introducing exogenous DNA into the germline of chickens (Tajima, 2002; Naito, 2003ab). The method for generating germline chimaeric chickens by transferring PGCs has already been established, and has also achieved the generation of germline chimaeric chickens that produce donor-derived offspring efficiently (Tajima et al., 1993; Naito et al., 1994ab, 1998b, 1999).

For the purpose of a stable incorporation of exogenous DNA into PGCs, however, a method for an in vitro culture system for PGCs must be developed.

The development of an in vitro culture of PGCs isolated from embryonic blood has already been carried out, but a long-term in vitro culture of PGCs has proven difficult to achieve. Recently, van de Lavoir et al. (2006a) have succeeded in culturing PGCs in vitro on the feeder cells of mouse STO fibroblast cells or buffalo rat liver (BRL) cells, using culture medium, containing stem cell factor and fibroblast growth factor, conditioned on BRL cells. Using this method, cultured PGCs have proliferated rapidly and retained their ability to migrate to the germinal ridges after transfer into the bloodstream of recipient embryos, and thus producing functional gametes efficiently. This culture method enables the detailed genetic manipulation of PGCs (Leighton et al., 2008). However, since that culture system uses xeno-animal cells, the system may run the risk of a cross-transfer of animal pathogens from other animal cells. It is, therefore, recommended to use chicken cells as feeder cells for culturing chicken PGCs.

Chicken PGCs isolated from embryonic blood could be
cultured on feeder cells derived from gonadal stroma cells of 5–7 day incubated embryos. Although cultured PGCs have proliferated several times during the first 4–5 days of culture (Chang et al., 1995; Yang and Fujihara, 1999a; Naito et al., 2001) and 29 times during 17 days of culture (Yang and Fujihara, 1999b), whether the subsequent proliferation of the cultured PGCs and their ability to migrate to the germlinal ridges after transfer into the bloodstream of recipient embryos remains to be established.

In the present study, the long-term culture of PGCs isolated from embryonic blood was attempted, and whether or not they retain their migratory ability to the germlinal ridges of recipient embryos and give rise to functional gametes after transfer into recipient embryos has been analysed.

Materials and Methods

Fertilised Eggs and Animal Care

Fertilised eggs of White Leghorn (WL) and Barred Plymouth Rock (BPR) chickens were obtained by artificial insemination. WL and BPR populations are maintained at the National Institute of Livestock and Grassland Science. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (National Institute of Agrobiological Sciences, Animal Care Committee).

Preparation of PGCs

Fertilised eggs of WL or BPR were incubated at 38°C for about 53 h in a forced-air incubator (P-0088 Bio-type; Showa Furanki, Saitama, Japan). Blood was collected from the dorsal aorta of embryos at stages 13–15 (Ham- burgen and Hamilton, 1951) using a fine glass micropipette. PGCs were concentrated by the Nycodenz density gradient centrifugation method (Zhao and Kuwana, 2003; Naito et al., 2004). Briefly, the collected blood was pooled, washed and dispersed in a 400µl KAv-1 medium (Kuwana et al., 1996). Five millilitres of 11% Nycodenz solution was placed in a 50 ml tube (Cat. No. 2070, Becton Dickinson, Franklin Lakes, NJ, USA), and 5 ml of 5.5% Nycodenz solution followed by 400 µl of blood solution were overlaid. The tube was centrifuged at 400 g for 15 min, and 10 ml of the PGC-rich solution was recovered from the top and washed with KAv-1 medium. A second purification was then carried out in the same manner.

Transfection and Culture of PGCs

The transfection of PGCs was carried out by a new electroporation-based technique known as “nucleofection”. The collected PGCs were dispersed in a 100 µl Nucleofector solution (solution V, Amaza GmBH, Köln, Germany) containing 2 µg of linearised pBEGFP plasmid (GFP gene under the control of chicken β-actin gene promoter). The solution was transferred into a kit-provided cuvette and inserted into a Nucleofector device. The transfection was carried out using the nucleofection programme T-01. Subsequently, 500 µl of KAv-1 medium was added to the cuvette. The recovered PGCs were cultured in vitro.

Feeder cells for culturing PGCs were prepared as follows. Gonads of 7-day incubated embryos of WL or BPR were collected, and cells were dissociated by trypsin treatment (T4049, Sigma-Aldrich, St. Louis, MO, USA). The collected cells were dispersed in a 5 ml fresh KAv-1 medium and cultured in a 6 cm dish (RepCell CS1014, CellSeed, Tokyo, Japan) for about 30 days. During time, the cells had grown confluent and became tightly attached to the culture dish. The transfected WL PGCs were cultured on BPR feeder cells, while the transfected BPR PGCs were cultured on WL feeder cells. The medium for culturing PGCs was KAv-1 supplemented with 10 ng/ml human leukemia inhibitory factor (hLIF) (LIF1010, Chemicon, Temecula, CA, USA). Expression of the GFP gene in cultured PGCs was detected under a fluorescent microscope (MZFL-III, Leica Microsystems, Tokyo, Japan).

Detection of PGC-Derived D-Loop Mitochondrial DNA in Cultured Cells

In order to detect the PGC-derived cells in the cultured cell populations, the cultured cells were analysed for the presence of the donor-derived D-loop region of the mitochondrial DNA. Cultured cells were collected by trypsin treatment, and DNA was extracted using a DNA extraction kit (SepaGene, Sanko Junyaku, Tokyo, Japan) according to the manufacturer’s instructions. The extracted DNA was dissolved in distilled water at a concentration of 100 ng/µl, and PCR analysis was then carried out on 200 ng DNA samples to detect the presence of the PGC-derived D-loop region of the mitochondrial DNA.

In the DNA sequences of chicken mitochondrial DNA of the D-loop region (DNA database accession number: AB091008), the 686th base is fixed as “A” in the WL and as “G” in the BPR used in this study (Harumi et al., 2004). In order to detect this SNP by PCR, mismatch-containing primers were designed. The sequences of the primers were 5′- TGG GCC TTC TTC ACA GGT CA-3′, 5′- CGA CAA GCA TTC ACT AAT TAG CAC C-3′ for detecting the WL, and 5′- CGG CAC CCG CAC TGT GAA GGC C-3′, 5′- CCA TTT GGT TAT GCT CGC GTG ATC-3′ for detecting the BPR (Harumi et al., 2004). PCR reaction mixture was prepared using Takara Ex Taq kit (PR001, Takara Bio Inc., Shiga, Japan), and the reaction was carried out using the GenAmp PCR system 9700 (Applied Biosystems Japan, Tokyo, Japan). After an initial denaturation step of 94°C for 2 min, 40 cycles were carried out; DNA was denatured at 94°C for 30 sec, annealed at 62°C for 30 sec, and extended at 72°C for 1 min. The reactions were then incubated at 72°C for 5 min. After amplification, 5 µl of the PCR products was separated on a 2% agarose gel, and the bands (WL: 224 bp, BPR: 334 bp) were visualised under UV light after ethidium bromide staining.

Detection of GFP Gene in Donor-Derived Offspring by PCR

Gonads were removed from the donor-derived offspring (embryo) at 14 days of incubation and washed with Dulbecco’s phosphate buffered saline without Ca^2+ and
Mg^{2+} (Cat. No. 28–103–05 FN, Dainippon Pharmaceuti-
cal, Osaka, Japan). DNA was extracted from the gonads,
and the extracted DNA was dissolved in distilled water.
PCR analysis was carried out on a 200 ng DNA sample to
detect the presence of GFP gene. The primers for detect-
ing GFP gene were as follows: 5′-TTC AAG TCC GCC
ATG CCC GAA-3′, 5′-ATG GGG GTG TTC TGC
tion step at 94°C for 5 min, 40 cycles of amplifica-
were performed; DNA was denatured at 94°C for 30 sec,
annealed at 60°C for 30 sec, and extended at 72°C for 30
sec. The reactions were then incubated at 72°C for 5 min.
For internal control, a sequence of the endogenous
glycereraldehyde-3-phosphate dehydrogenase (GAPDH)
gene was amplified using the following primers; 5′-CAG
ATC AGT TTC TAT CAG C-3′, 5′-TGT GAC TTC
GFP-negative cells were a mixture of cultured PGCs and
slowly. Proliferation of GFP-negative PGCs could not be
observed very clearly due to the difficulty of identification
based on their morphology during the culture period.

Cells isolated by trypsin treatment comprised a mixed
population of GFP-positive and GFP-negative cells (Fig.
2, A, B). GFP-positive cells were cultured PGCs, while
GFP-negative cells were a mixture of cultured PGCs and
feeder cells. GFP-positive cells tended to be larger than
GFP-negative cells (Fig. 2, A, B), and the large nucleus
of these cells was eccentrically placed (Fig. 2, C, D).

Detection of PGC-Derived D-Loop Mitochondrial DNA in
Cultured Cells
To further confirm the presence of PGCs in the cultured
cell population, DNA from the cultured cells was analysed
for the presence of cultured PGC-derived mitochondrial
DNA. When WL PGCs were cultured on BPR feeder
cells, WL-derived as well as BPR-derived mitochondrial
dNA was detected (Fig. 3). When BPR PGCs were
cultured on WL feeder cells, both BPR-derived and WL-
derived mitochondrial DNA was detected.

Production of Chimaeric Chicken
Cultured PGCs (23–61 days) were collected by trypsin
treatment, dispersed in a 100 μl fresh KAv-1 medium,
and placed in a plastic dish (Cat. No. 3001, Becton Dickin-
son, Franklin Lakes, NJ, USA). Recipient embryos of WL or
BPR were cultured in host eggshells at 38°C for about 53
h as described by Naito et al. (1990). When the embryos
reached stages 14–15, a population of five hundred cul-
tured PGCs containing feeder cells was picked up by a fine
glass micropipette and injected into the bloodstream of
recipient embryos. The manipulated embryos were cul-
inated in host eggshells at 37.8°C for an additional 18 days
until hatching (Perry, 1988; Naito et al., 1990).

Test Mating
The hatched chicks were raised until sexual maturity in
which cultured PGCs had been transferred at the embry-
onic stage. Both mature male and female putative chi-
maeric chickens were mated with BPR using artificial
insensation, the fertilised eggs were incubated for 14
days and the feather colour of the developing embryos was
then examined. BPR are homozygous recessive (i/i) at
the autosomal pigment inhibitor gene, and the chick feath-
ers are black, while WL are homozygous dominant (I/I),
and their feathers are white. Black offspring (i/i) indicate
that the embryos were derived from the BPR, while white
offspring (I/i) confirm their derivation from the WL.

Results
Proliferation of Cultured PGCs
Cultured PGCs attached loosely to the feeder cells, and
their morphology varied from round to fibroblast-like in
shape. Transfection efficiency of the GFP gene was about
10% judged by their expression in the cultured PGCs, and
some of the GFP-positive cell population started to slowly
proliferate. Fig. 1 (A-D) shows BPR and WL PGCs
cultured on WL and BPR feeder cells, respectively. GFP-
positive cells proliferated, and the cell population spread
slowly. Fig. 1 (E-H) shows BPR and WL PGCs cultured
on WL and BPR feeder cells, respectively, and GFP-
positive cells proliferating and occasionally forming col-
ones. These GFP-positive cell colonies also spread
slowly. Proliferation of GFP-negative PGCs could not be

Discussion
The importance of generating transgenic chickens that
can produce pharmaceutical materials in eggs is increas-
ing, since chickens can cost-effectively produce large
amounts of proteins as egg white compared with a system
using large animals. Although transgenic chickens have

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Production of Chimaeric Chicken

Test Mating
PGCs were isolated from embryonic blood and transfected GFP gene with nucleofection. The manipulated PGCs were cultured in vitro on feeder cells. BPR and WL PGCs were cultured for 47 and 39 days (A, C) and GFP gene expression was observed under a fluorescent microscope (B, D), respectively. BPR and WL PGCs forming colony were cultured for 38 and 27 days (E, G) and GFP gene expression was observed under a fluorescent microscope (F, H). Scale bars represent 30 μm.

WL PGCs cultured for days were collected by trypsin treatment (A). GFP gene expression in the collected cells containing PGCs and feeder cells was observed under a fluorescent microscope (B). Typical cultured PGCs (C) were observed under a fluorescent microscope (D). Scale bars represent 20 μm.
been generated by the retroviral vector method, non-viral methods are preferable for safety reasons. As for non-viral methods of gene transfer in chickens, various approaches have been attempted, such as the microinjection of DNA into the fertilized ovum (Love et al., 1994; Naito et al., 1994c; Sherman et al., 1998) or transfection of germline cells; blastodermal cells (Blazolot et al., 1991; Fraser et al., 1993), PGCs isolated from embryonic blood (Watanabe et al., 1994; Naito et al., 1998a, 2007a), gonadal PGCs (Park et al., 2003b; Shiue et al., 2009), gonocytes (Naito et al., 2007b), or spermatogonia (Lee et al., 2006; Jung et al., 2007). For the detailed manipulation of germline cells, embryonic stem (ES) cells (Zhu et al., 2005; van de Lavoir et al., 2006b) or embryonic germ (EG) cells (Park and Han, 2000; Park et al., 2003a) have been established. Although EG cells are able to incorporate into the germline of chickens after transfer into recipient embryos, ES cells are not. This is probably due to the presence of the germplasm determining the germline in chickens (Tsunekawa et al., 2000). Among many attempts, though the manipulation of cultured PGCs isolated from embryonic blood seems to be the most promising method for detailed germline manipulation in chickens, the current method uses mammalian cells as feeder cells (van de Lavoir, 2006a). To avoid the cross-contamination of pathogens, we have tried to use chicken cells as feeder cells for culturing PGCs isolated from embryonic blood.

In the present study, PGCs isolated from embryonic blood were cultured on feeder cells derived from the gonadal stroma cells of 7-day incubated chicken embryos. Among the cultured PGCs that proliferated, some retained the ability to migrate to the germinal ridges, giving rise to functional gametes after about 2 months of culture. Most of the other cultured PGCs, however, lost their ability to incorporate into the germline of recipient embryos during the culture period. Feeder cells play an important role in supporting PGC proliferation, and chicken gonadal cells can support the proliferation of PGCs as feeder cells over a long period. In our experiment, feeder cells were not treated with mitomycin C for inactivation, since such a treatment to the feeder cells derived from embryonic gonads suppresses the proliferation of PGCs isolated from embryonic blood (Naito et al., 2001). It has also been observed that no proliferation of gonadal PGCs cultured on feeder cells derived from embryonic gonads treated with mitomycin C occurred, even when some growth factors were added to the culture medium (Park and Han, 2000; Shiue et al., 2009). Feeder cells derived from embryonic gonads seem to secrete various kinds of growth factors for maintaining and proliferating PGCs, but the secretion of these factors from the feeder cells may be suppressed by treating with mitomycin C. Instead, feeder cells were tightly attached to the culture dish in the present study, and PGCs were successfully cultured on such feeder cells over a long period.

GFP gene was transferred into the PGCs to identify the cultured PGCs, and also to observe the proliferation of the cultured PGCs over a lengthy period. Some of the cultured PGCs were successfully transferred GFP gene, and were readily observable as GFP-positive cells. The presence of cultured PGCs was also confirmed by detecting the donor PGC-derived mitochondrial DNA in the collected cell populations. The GFP-positive cells proliferated over a long period though the proliferation rate was low. The proliferated GFP-positive cells occasionally
formed colonies that grew gradually. The morphology of GFP-positive cells revealed PGC characteristics, namely, large cells having eccentrically placed large nucleus. It has been reported that undifferentiated cultured PGCs were not attached to the feeder cells, maintained a round morphology, and were grown on feeder cells (van de Lavoir et al., 2006a). The GFP-positive cells in the present study derived from GFP-negative PGC, or even if the offspring were attached loosely to the feeder cells and presented various shapes as observed in previous reports (Chang et al., 1995; Yang and Fujihara, 1999ab; Naito et al., 2001), suggesting that most of the cultured PGCs differentiated.

As already noted, to analyse the germline transmission of the cultured PGCs, we carried out a test mating of the putative chimaeric chickens. As a result, the single germ-line chimaeric chicken (male) was detected and this germ-line chimaeric chicken produced one donor-derived offspring (BPR) out of seven offspring examined. Such low production rates (3.6%, 1/28) together with germline transmission of donor-derived cells in the germ-line chimaeric chicken (0.37%, 1/270) suggest that most of the cultured PGCs differentiated and lost their ability to migrate to the germinal ridges. Even under those culture conditions, however a small percentage of the PGC population was maintained in an undifferentiated state during the culture period, and was able to migrate to the germinal ridges, giving rise to functional gametes after their transfer to recipient embryos. The donor-derived offspring produced was GFP-negative. The offspring was probably derived from GFP-negative PGC, or even if the offspring was derived from GFP-positive cell, GFP gene was disappeared during the course of development and differentiation due to the presence in episomally.

The present study demonstrated that in vitro culture of PGCs isolated from embryonic blood is possible. A germ-line chimaeric chicken was generated by transferring the PGCs cultured for about 2 months, although only a small part of the cultured PGCs retained the ability to enter the germine of recipient embryos. van de Lavoir et al. (2006a) cultured PGCs using a culture medium conditioned on BRL cells (which are known to produce LIF), and the LIF probably acted to maintain the PGCs in an undifferentiated state. In the present experiment, although hLIF was added to the culture medium, it was not enough to maintain the PGCs in an undifferentiated state,
probably due to the species difference between chicken LIF and hLIF or to an insufficient amount of hLIF added to the culture medium. The present culture method should be improved so as to maintain the ability of PGCs to incorporate into the germline of recipient embryos after transfer into recipient embryos.

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