Embryonic Fibroblast-Conditioned Medium Enhances Viability and Proliferation of Chick Circulating Primordial Germ Cells (cPGCs) in Suspension Culture

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Abstract. Primordial germ cells circulating in the embryonic vascular system (cPGCs) were isolated from 2.5-day-old chick embryos and cultured individually in suspension for 5 days in embryonic stem-cell medium (ESM) adjusted to pH 8.0 supplemented with fetal bovine serum. Most cells died within 5 days, during which time only a few cells (16–23%) underwent mitosis once, and only a very few cells (less than 3%) twice. The use of conditioned medium (CM) derived from embryonic fibroblasts enhanced the viability and proliferation of the cPGCs, resulting in survival of up to one-third of the cells for 5 days. A fraction of the cells (3–5%) even divided 3 times. The in vitro cultured cPGCs maintain the capacity to migrate into the gonadal area. The chick cPGCs cultured in vitro for up to 5 days in the CM produced germ-line chimeras upon injection into the bloodstream of 2-day-old quail embryos.

Key words: Circulating primordial germ cells, Embryonic fibroblast, Conditioned medium, Suspension culture, Chicken

Primordial germ cells (PGCs) in Aves originate in the epiblast and first appear in the hypoblast of the area pellucida, the so-called germinal crescent, on the first day of incubation [1, 2]. They enter the developing blood vessels, circulate in the bloodstream, where they are called circulating PGCs (cPGCs) [3], settle in the gonadal anlage and differentiate into either spermatogonia or oogonia [4–6]. The unique migration of avian PGCs through the circulatory system makes them easy to isolate and manipulate. cPGCs have been successfully employed to produce germ-line chimeras by means of intravascular transplantation [7–9].

The morphological aspects of avian PGCs have been thoroughly studied [10, 11]. Under the phase contrast microscope, cPGCs can be identified by their large size relative to the blood cells, their eccentrically located nuclei, and the presence of refractile vacuoles within their cytoplasm [12], but the cellular and molecular mechanisms of the proliferation of avian PGCs remain largely unknown.
One practical application of avian cPGCs may be their use as a vehicle to introduce foreign genes. For this purpose, these cells would need to be isolated and cultured in vitro, and then used as a target for the incorporation of foreign genes. Through the transfer of appropriately selected cells, germ-line chimeric chicks could be obtained, eventually producing transgenic animals. In order for this scheme to work, it is essential to develop a system in which cPGCs proliferate in vitro.

Avian cPGCs do undergo mitosis in situ at least sporadically [13, 14]. It has been reported, however, that cPGCs never proliferate in vitro in suspension culture, though they survive, and the viability is enhanced at a high pH (8.0) [15]. There may be some yet unknown factors necessary for the survival and proliferation of chick cPGCs in vivo and in vitro. Soluble factors produced by mouse fibroblast feeder layer cells have been shown to enhance the development of chick PGCs in culture [16].

In the present study, we first examined the survival and proliferation of chick cPGCs in suspension culture with the embryonic stem-cell medium (ESM) adjusted to pH 8.0 developed by Robertson [17]. The cells survived poorly and divided only very infrequently. We then examined the effect of conditioned medium (CM) from chick and mouse embryonic fibroblast monolayer cultures. The CM enhanced the viability of the cPGCs, and even resulted in some of the cells (3–5%) dividing 3 times. We also found that the cPGCs cultured in suspension for up to 5 days in the CM, upon transfer to quail embryos, successfully migrated into the gonads and produced chick-quail germ-line chimeras.

Materials and Methods

Embryos

Fertilized laid eggs of White Leghorn chickens (Gallus gallus domesticus) and Japanese quails (Coturnix coturnix japonica) were purchased from commercial sources, Shiroyama-Syukeijou, Kakogawa, and Tokai-Yuki, Toyohashi, Japan, respectively.

Isolation and culture of chick cPGCs

Embryonic blood was collected from the vitelline artery, heart and terminal sinus of 2.5-day-old chick embryos (stages 14 and 15) [18] with fine glass pipettes. The pipettes were made by pulling glass capillary tubing (50 µl; Drummond Scientific Co., PA, USA) through a small ring-shaped electric heater (Narishige, Tokyo, Japan), and by cutting the tips at a sharp angle. The inner diameter of the tip was adjusted to about 50 µm. The blood collected as described above was subjected to Ficoll density gradient centrifugation [7], and the cPGC-rich fraction was transferred to a plastic dish (35 × 10mm; Falcon, Becton Dickinson Co., Lincoln Park, NJ) containing 2 ml of Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, MD, USA) adjusted to pH 8.0 supplemented with 10% fetal bovine serum (FBS; Filtron Ltd., Victoria, Australia). This medium prevents cPGCs from attaching to the substrate or aggregating with blood cells at room temperature. cPGCs were then picked up with the fine glass pipettes described above under a dissection microscope, and were placed in the center of another dish. The cells in the dish were then examined under a phase contrast microscope. cPGCs could be distinguished from blood cells by their remarkably large size and cytoplasmic lipid droplets [10, 19]. cPGCs were individually transferred to wells of 96-well microtiter plates (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) and were cultured for 5 days at 38°C in an atmosphere of 5% CO2 in air, in embryonic stem-cell medium (ESM) [17] which consisted of DMEM supplemented with 1 × nonessential amino acids (Gibco-BRL, MD, USA), nucleosides (Sigma, MO, USA) (final concentration in µmol/l: adenosine 30; guanosine 30; cytidine 30; uridine 30; thymidine 10) and 20% FBS adjusted to pH 8.0 (pH 8.0 ESM).

Viability and proliferation of cPGCs

cPGCs were individually cultured in wells, and were examined morphologically on Days 0, 1, 3 and 5. The criteria used were the presence of bright lipid droplets distributed in their cytoplasm and large asymmetrically located nuclei [7]. The proliferation of cells was judged on Day 5 cultures as follows: no division in wells with single cells, 1 division with 2 cells, 2 divisions with 3–4 cells, and 3 divisions with 5–8 cells.

Preparation of conditioned medium

Chick embryonic fibroblasts (CEF) obtained from stage 27 embryos [18], and mouse embryonic fibroblasts (MEF) obtained from 14-day post
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coitum (dpc) stage embryos were used to prepare conditioned media. Essentially following the procedure described previously [15, 20], cells were washed in PBS, treated with PBS containing 0.05% trypsin (Gibco-BRL, MD, USA) and 1 mM EDTA (Nacalai tesque, Kyoto, Japan), and suspended in the culture medium (DMEM supplemented with 10% FBS). The suspension was centrifuged, the cells were resuspended in the culture medium, and 10 ml of the cell suspension at a density of $5 \times 10^5$ cells/ml was seeded in each culture dish (100 × 10 mm, Falcon, Becton Dickinson Co., Lincoln Park, NJ) precoated with a 0.1% (w/v) solution of gelatin (Sigma, MO, USA).

The cells were grown to confluence and the culture medium was then changed to pH 8.0 ESM, supplemented with 20% FBS, and the cells were further cultured for 24 h at 38°C in an atmosphere of 5% CO2 in air. The culture was then centrifuged at 200 × g for 10 min, and the supernatant was used as conditioned medium.

Migrating ability of cultured cPGCs

To examine the ability to migrate into the germinal epithelium and gonadal area, chick cPGCs were cultured as described above but en masse rather than singly, collected and injected intravascularly into the blood area of the yolk sac of 2-day-old Japanese quail embryos (stage 15), through a window (about 1.5 cm in diameter) opened at the tapered end of the quail egg shell. The window was then sealed with a clear film (Saran Wrap; Asahi Kasei Co., Tokyo, Japan) and the embryos were incubated for 1 more day at 38°C. Host embryos were then dissected out and fixed in Rossman’s fixative saturated with picric acid in absolute ethanol and neutralized formalin (9:1, v:v) at 4°C for more than 4 h, dehydrated through a series of graded ethanol, and embedded in paraffin. Serial sections were cut at 10 µm, and double-stained with PAS and hematoxylin for light microscopy.

Statistical analysis

Data were analyzed by the $\chi^2$ test and by Fisher's exact test to assess the statistical significance of differences. A probability of less than 0.05 was considered significant.

Results

Viability and proliferation of chick cPGCs in suspension culture

The cPGCs were separated from 2.5-day-old chick embryos and cultured individually in wells in suspension for 5 days in the ESM. We first examined the effect of the FBS concentration in the medium on the viability and proliferation of cPGCs. Viability was checked by morphological criteria, as reported by Yasuda et al. [7]. In a separate experiment, vital staining with trypan blue was carried out to confirm the results based on morphology. At all FBS concentrations examined (5, 10, 20 and 50%) the viability of cPGCs was significantly higher than that in the FBS-free medium, but no differences in the effect on viability were observed among the FBS concentrations examined (Fig. 1a). The viability decreased rapidly with culture time, and most cells, regardless of the presence or absence of FBS in the medium, died within 5 days. A small number of the cells (16–23%) did divide once. Only a very few cells (less than 3%) divided twice (Fig. 1b). The frequency of cell division was essentially the same in all the samples regardless of the FBS concentration.

Effects of conditioned media (CM) on viability and proliferation

With the aim of enhancing the viability and proliferation of chick cPGCs in suspension culture, two types of CM, CEF-CM and MEF-CM, were tested. Control cPGCs were cultured in ESM containing 20% FBS. Both types of CM were effective in promoting viability (Fig. 2a), but the CEF-CM was more effective than the MEF-CM throughout the culture period. Both types of conditioned medium were also effective in promoting cell proliferation (Fig. 2b), but in this regard MEF-CM was as effective as CEF-CM. About half of the cells that were individually examined did not divide. Of the remaining cells, more than half divided only once, which was twice as frequently as the control cells, whereas about one-quarter of the cells divided twice (25 times more frequently than the controls), and the rest 3 times (compared to none in the controls) (Fig. 3).

Production of chick-quail germ-line chimera

cPGCs were cultured in suspension en masse in
CEF-CM. The morphology of the cultured cPGCs changed, reflecting the viability, as the culture period increased (Fig. 4). Cells were collected, disregarding morphological differences, after 1, 3 and 5 days in culture, and introduced intravascularly into 2-day-old quail embryos. Thus the preparation included normal viable cells as well as abnormal nonviable cells. After incubation for 1 day, the host embryos were fixed, serial sections were prepared and the germinal epithelium and the gonadal area were examined for the presence of chick PGCs (PAS-positive) (Fig. 5). Quail PGCs, which also could be easily distinguished morphologically from other cell types, are PAS-negative. We observed that following the in vitro culture, the chick cPGCs migrated upon transfer into the quail germinal epithelium and gonadal area. The frequency of cells that successfully migrated decreased rather rapidly from 41 to 58%.

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**Fig. 1.** Effects of FBS concentration on viability (a) and proliferation (b) of cPGCs in suspension culture in ESM medium. cPGCs isolated from the bloodstream of 2-day-old chick embryos were individually seeded into wells of microtiter plates, and cultured for 5 days. The number of cell divisions was determined after 5 days.

**Fig. 2.** Effects of conditioned media derived from chick embryonic fibroblasts (CEF-CM) or from mouse embryonic fibroblasts (MEF-CM) on viability (a) and proliferation (b) of cPGCs in suspension culture.
in samples cultured for 1 day, to 13 to 29% in those cultured for 3 days, and finally to 0 to 3% in those cultured for 5 days (Table 1), most probably reflecting an increase in the frequency of nonviable cells transferred (Fig. 4c).

**Discussion**

The purpose of the present work was to develop culture conditions that promote improved survival and proliferation of cPGCs. High pH of the culture medium was reported to be essential for survival of *in vitro* cultured cPGCs, although the cells still failed to divide [15]. The present results showed that cPGCs cultured in suspension did undergo cell division, although very infrequently, and generally only once. The present results further showed that embryonic fibroblasts apparently provide a factor(s) that cause(s) embryonic fibroblast-derived...
CM to increase both cell survival and proliferation. We noted that the distinct factors appeared to be responsible for the survival and for the proliferation of cPGCs in culture, since for survival CEF-CM was more effective than MEF-CM, whereas for proliferation both CMs were equally

Table 1. Distribution and number of chick PGCs in quail embryos. Chick cPGCs were cultured in vitro and transferred into the bloodstream of quail embryos. The host embryos were incubated for another day, and sacrificed.

<table>
<thead>
<tr>
<th>Culture day</th>
<th>Experiment No.</th>
<th>PGCs injected</th>
<th>No. of chick PGCs in quail embryo (%)</th>
</tr>
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<tr>
<td></td>
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<td>germinal epithelium</td>
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| 0           | 1              | 45            | 8                          | 31          | 39 (87)
|             | 2              | 46            | 10                         | 34          | 44 (96)
| 1           | 3              | 55            | 5                          | 27          | 32 (58)
|             | 4              | 51            | 1                          | 20          | 21 (41)
| 3           | 5              | 45            | 6                          | 17          | 23 (29)
|             | 6              | 78            | 2                          | 4           | 6 (13)
| 5           | 7              | 73            | 0                          | 2           | 2 (3)
|             | 8              | 80            | 0                          | 0           | 0 (0)

1) The gonadal area is the region including the dorsal mesentery and dorsal part of the coelomic wall, and is limited to the region caudal to the vitelline artery, medial to the mesonephros, dorsal to the hind-gut, and ventral to the dorsal aorta. The caudal end of the gonadal area is set at the caudal terminus of the coelom. The coelomic epithelium in this region is designated as the germinal epithelium and is represented here in a separate column.

2) The values in parentheses are proportions (%) of the numbers of PGCs that migrated to the germinal epithelium and gonadal area relative to the number of PGCs injected.

a-d Values with different superscripts are significantly different (P<0.05).

Fig. 5. Photomicrographs of PAS and hematoxylin double-stained sections of the gonadal regions of host quail embryos. Sections were prepared 1 day after the injection of chick cPGCs cultured in suspension for 0 day (a, control), for 1 day (b) and for 3 days (c). Chick PGCs are PAS positive (arrows), whereas quail PGCs are PAS negative (arrowheads). Scale bars indicate 10 µm.
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Effective.

About half of the cPGCs individually cultured in suspension with CM divided up to 3 times in 5 days. Considering the difficulty previous workers have experienced in inducing cell divisions in cPGCs, our results may be taken as a good starting point upon which further improvement can be made. Avian cPGCs have hitherto been treated as anchorage-dependent cells, as have mouse PGCs, and have been studied in co-cultures with feeder cells. For example, chicken cPGCs were cultured with stroma cells from the genital ridge as feeder cells, and were shown to proliferate [21, 22]. The suspension culture method we employed included no feeder cells, and thus provides a direct way to identify the factors affecting the survival and proliferation of cPGCs. The present method should also provide an efficient system for producing germ-line chimeras.

In mice, previous studies have identified several factors that increase the proliferation and survival of PGCs: stem cell factor (SCF) [23–25], leukemia inhibitory factor (LIF) [25, 26], basic fibroblast growth factor (bFGF) [27, 28], tumor necrosis factor-α (TNF-α) [29] and forskolin [30]. Whether any of these factors can promote the proliferation of chick cPGCs remains to be examined.

The present results also showed that the chick cPGCs, separated and cultured in suspension for 5 days, still retain the characteristics of PGCs. The chick cPGCs cultured in suspension for 5 days, upon transfer to quail embryos, successfully migrated into the host gonadal areas, most probably divided there, and produced germ-line chimeras. Whether these chick cells in the chick-quail chimeras can actually differentiate into functional germ cells remains to be examined. But a chick chimera could have produced offspring derived from cultured cPGCs (unpublished data).

cPGCs have been thought to be promising as potential tools for the production of transgenic chickens [8, 9]. Avian zygotes, unlike those of mammals, are extremely difficult to manipulate. Transplantation of cPGCs is an effective way of producing germ-line chimeras. The present results seem to open a way for the production of germ-line chimeras by using cPGCs into which foreign genes have been introduced while in culture, and eventually producing transgenic animals. Another more direct application may be to use the present method to preserve the genes of individuals whose survival, for one reason or another, is in danger. The technique may also be used to augment the population of a given variety of chickens that is endangered under natural mating conditions [8].

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