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Original Paper

Differentiation of migratory-stage cultured primordial germ cells into oocytes

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ABSTRACT

Previous studies have shown that several growth factors stimulate proliferation or survival of mouse primordial germ cells (PGCs) in culture. However, these growth factors alone could not support the reappearance of PGC proliferation *in vivo*. Moreover, the preceding research has mainly reported the number of PGCs, though cultured PGCs may lose their inherent character and/or transform into EG cells. There we showed that PGCs obtained at migratory stage proliferated in culture at a rate comparable to that *in vivo*. In the present study, we made reaggregates of these cultured PGCs and transplanted them into ovariectomized adult females. After three weeks, the oocytes differentiated in

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aggregates with or without embryonic ovarian somatic cells. This result suggests that PGCs in culture still maintain PGC characteristics and interaction between germ cells and ovarian somatic cells was not essential for the initial meiotic development of PGCs into oocyte.

Key Words: *migratory PGC, proliferation, differentiation, oocyte, mouse*

INTRODUCTION

Primordial germ cells (PGCs) first appear in the posterior extraembryonic region of the primitive streak-stage mouse embryo around 7 days post coitum (dpc) as a cluster of alkaline phosphatase-positive cells [1]. PGCs migrate from the allantois along the hind gut and reach the genital ridge by 11.5 dpc. PGCs rapidly proliferate from 30 - 50 cells at 7.5 dpc to around 20,000 - 25,000 at 13.5 dpc [1-4]. After this proliferative stage, PGCs begin to cease cell division at around 13.5 dpc and enter into either meiosis in the ovary or mitotic arrest in the testis [5]. Previous studies have shown that several growth factors, such as stem cell factor (SCF, [6-8]), leukemia inhibitory factor (LIF, [8,9]) and basic fibroblast growth factor (bFGF, [10,11]), increase proliferation and survival of mouse PGCs (reviewed in, [12-14]). Other cytokines such as tumor necrosis factor- α (TNF- α) [15] and interleukin-4 (IL-4) [16] stimulate PGC proliferation or survival in culture and are expressed in the embryo when PGC are present. Even after addition of these growth factors to culture medium, however, it is still not possible to get PGCs to proliferate at a rate similar to that *in vivo*. Moreover, it is not understood whether PGCs maintain their inherent character after addition of growth factors in culture. Under certain conditions, PGCs continue cell growth and transform into pluripotent embryonic stem (ES) like cells, termed with embryonic germ (EG) cells [10,11]. Recently, we show that PGCs have an autocrine mechanism of proliferation by FGFs stimulation and that growth inhibitors may be important for PGC proliferation *in vivo* [17]. TGF

(transforming growth factor) β 1 and soluble SCF are thought candidates for such an inhibitory mechanism [17,18].

We previously showed that the conditioned medium made from Buffalo rat liver cells (BRL-CM) promoted proliferation and/or survival of PGCs isolated from embryos at 7.5 to 11.5 dpc. Moreover, a combination of BRL-CM and forskolin with Sl/Sl4-m220 (m220) feeder cells, which express the membrane-bound form of SCF, stimulated rapid proliferation of PGCs in culture [19]. BRL-CM contains both growth factors (e.g. LIF) and growth inhibitors (e.g. TGF β 1 and soluble SCF) for PGCs [20-22]. Our previous observation did not detect that PGCs transformed into EG cells Under this culture condition [19]. However, it was not clear whether these cultured PGCs had their inherent character and the PGCs could develop into mature germ cells.

Here, we investigated the developmental potentiality of migratory-stage cultured PGCs into oocytes using transplantation of reaggregates including the PGCs into female mice. As a result, we had found that the cultured PGCs in reaggregates could develop into oocytes without embryonic gonadal somatic cells.

MATERIALS AND METHODS

Preparation and culture of mouse PGCs

C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Preparation and culture of mouse PGCs derived from 8.5 dpc embryos were carried out as previously described [19]. Such multiple fragments were dissociated into single cells with incubation in 0.05% trypsin (Gibco-BRL, MD, USA) and 1mM EDTA in Ca^{2+}- and Mg^{2+}- free Dulbecco's phosphate buffered saline. These dissociated cells were seeded onto mitomycin C-treated m220 feeder cells. The culture medium was PGC basal medium (PGC-BM) with 20% fetal calf serum (FCS) and 20 μ M forskolin (Sigma, MO, USA). The PGC-BM consisted of Dulbecco's modified Eagle's medium supplemented with 1x

nonessential amino acids (Gibco-BRL, MD, USA), nucleosides (final concentration; 30 μ M adenosine, 30 μ M guanosine, 30 μ M cytidine, 30 μ M uridine and 10 μ M thymidine, Sigma, MO, USA), BRL-CM (final concentration 50%) and antibiotics. The culture medium was changed every day.

Preparation of reaggregates and their transplantation

PGCs in primary culture were trypsinized, dissociated into single cells in the culture medium and filtered by cell strainers (Falcon, Becton Dickinson and Company, NJ, USA) to remove aggregates. The dissociated cells from five embryos were incubated on a gelatin-coated dish for 20 min to remove feeder cells. PGCs were recovered as unattached cells. In some experiments, the PGCs were mixed with embryonic gonadal somatic cells isolated from ten 12.5 dpc fetal ovaries as previously described [23,24]. The gonadal somatic cells were determined to be < 5% contaminated with germ cells as identified by alkaline phosphatase staining (data not shown).

Making reaggregates and their transplantation were carried out in a manner similar to that previously described [25]. The reaggregates were made of PGCs proliferated in primary culture, using Ehrlenmeyer flasks (5 ml) siliconized their concave bottoms which filled with PGC-BM supplemented with 10% FCS . The flasks were sealed with Milliwrap (Millipore, MA, USA) and were mounted on a gyratory shaker (New Brunswick Scientific, NJ, USA) which gave a circular movement at 60 rpm inside a incubator for 1-2 days.

Reaggregates were inserted into the ovarian capsule of 2-month-old C57BL/6 female mice, from which both host ovaries were partially (80 - 90%) removed. Then, a small piece of denatured collagen (Spongel;Yamanouchi Co., Tokyo. Japan) was also inserted for hemostasis. Three weeks after, transplants were recovered andfixed with Bouin's solution, embedded in paraffin and sectioned seriously at 6 μ

m. Sections were stained with hematoxilin, eosin and alcian blue.

RESULTS

We had previously reported that BRL-CM promoted proliferation of and/or survival of PGCs and accelerated their proliferation [19]. BRL-CM acted as both a mitogen and a survival factor by BrdU incorporation (data not shown).

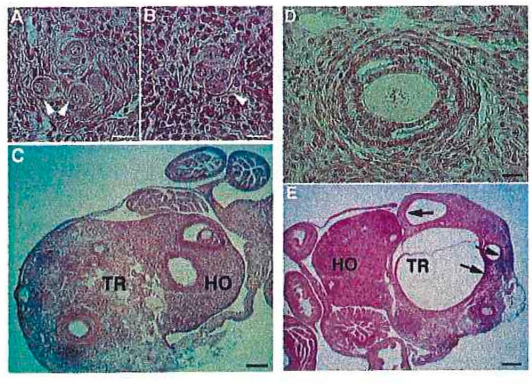

Fig.1. Primordial follicles in transplanted aggregates without (a) and with (b) embryonic gonadal somatic cells. (c, e) Transplanted aggregates into ovary capsules. (d) High magnification of (c), primary follicles into transplanted aggregates. TR; transplant, HO; remained host ovary. Arrowheads and arrows showed primordial follicles and ovarian follicular cysts, respectively. Bars; (a, b, d) 20 μ m, (c, e) 200 μ m.

The use of m200 feeder cells and the addition of BRL-CM and forskolin stimulated PGCs obtained from 8.5 dpc embryos in culture to proliferate rapidly. Under these conditions, however, we could not detect whether PGCs would enter meiosis. We had already succeeded in obtaining mouse offspring derived from reaggregates of fetal ovaries cultured and transplanted into adult females [25]. On the other hand, when we transplanted aggregates of mouse germline-transmissible ES cell or EG cells in mice, the ES or EG cells had differentiated into tumors (data not shown). Thus, this technique can lead whether cultured PGCs maintain PGC character. Using this method, we reaggregated cultured PGCs isolated from 8.5 dpc embryos, transplanted the aggregates under the ovary capsule, and examined their oogenesis development.

Such reaggragates included not only PGCs but also other somatic cells except feeder cells. Therefore, their transplants were different and were easily

distinguishable from the rest of the host ovaries. Moreover, we carefully studied them by seriously sectioning to determine whether they were transplants or host ovaries. In two out of five transplants without gonadal somatic cells, PGCs developed into primordial follicle stage (Table 1, Fig.1a). In 23 out of 24 transplants with embryonic gonadal somatic cells, PGCs developed into primordial follicle stage (Table 1, Fig. 1b).

Table 1. Transplantation of reaggregates from migratory-stage cultured PGCs* in mouse

	No. of transplants	No. of transplants recovered	No. of transplants developed into follicles		
			Primordial	Primary	Secondary - Graafian
Cultured PGCs	**5**	**5**	**2**	**0**	**0**
Cultured PGCs + Gonadal somatic cells	**24**	**24**	**23**	**6**	**0**

● PGCs were obtained from 8.5 dpc embryos and cultured for 4 - 6 days.

Interestingly, most germ cells formed clusters in the aggregates (Fig. 1a, b). The aggregates without embryonic gonadal somatic cells could not develop beyond the primordial follicle stage. On the other hand, six transplants aggregates mixed with embryonic somatic cells from gonads formed primary follicles were observed (Table 1, Fig.1c, d). We often found ovarian follicular cysts in transplants (Fig. 1e).

DISCUSSION

Although some stages of PGC were identified by some marker expressions, for example, alkaline phosphatase, 4C9, SSEA-1 or EMA-1 (see Table 1 in [26]), these markers were also detected in ES or EG cells. Thus, it was poorly understood

whether proliferated PGCs cultured *in vitro* maintain PGC characteristics.

Here, we showed that in 40% transplants with gonadal somatic cells, cultured PGCs developed into primordial follicle stage. We also found that in reaggregates with gonadal somatic cells promote further development into primordial follicle stage. Similarly, it was recently found that PGCs from 10.5-13.5 dpc female embryos can develop into growing oocytes in aggregates mixed with embryonic lung cells [27]. These results suggest that gonadal somatic cells may not be essential for early oogenesis (development from PGCs to primordial follicle stage), although they promote the early development of germ cells.

Most germ cells formed clusters in transplants. PGCs may interact with each other during their migration [28] and proliferation [17]. In the mouse ovaries of 11.5 to 17.5 dpc, germ cells are formed from clusters which are connected and divide synchronously [29]. Taken together, we lead to a hypothesis that early oogenesis may be fundamentally regulated by the germ cells themselves.

On the other hand, no cultured PGCs developed into later oogenesis (primary to Graaffian stage) in transplants without gonadal somatic cells. Thus, later development of cultured PGCs may require direct interactions between the germ cells and the embryonic gonadal somatic cells. We could detect no follicles at the secondary to Graafian stage in both types of aggregates. Since the aggregates still included non-gonadal somatic cells (feeder cells), the interaction between PGCs and gonadal somatic cells might be prevented in their transplants. If we purified PGCs in culture, cultured PGCs could interact well with gonadal somatic cells and developed into Graafian stage with a high efficiency.

We found the cysts inside transplants (Fig. 1e) . They are often seen with hypergonadotropin, especially increased levels of luteinzing hormone (LH) [30] . The cysts observed in the present study may be induced by ovariectomy used for

the transplantation because it induces high expression of follistatin, follicle-stimulating hormone and LH [31,32].

In conclusion, migratory-stage cultured PGCs have maintained inherent PGC character under our method (the combination of growth factors and inhibitors in media). Early oogenesis from cultured PGCs might be regulated without support from gonadal somatic cells.

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