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Isolation and Culture of Mouse Primordial Germ Cells

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생쥐 원시생식세포의 분리와 체외배양

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적 요

원시생식세포(primordial germ cell; PGC)는 성성숙 이후에 기능을 갖는 생식세포의 근원이 되는 세포로서, 다능성을 갖고 있는 것으로 알려져 있다. 그러므로 chimera 및 유전자 변환동물 생산을 위해 널리 사용되어 온 배아주(embryonic stem; ES)세포를 대신할 다른 세포계라고 생각되어져 많은 연구가 진행되고 있다. 본 실험은 체외배양을 통하여 원시생식세포의 증식과 확립을 위해 배양조건을 구명하고, 또한 성장인자의 효과를 검증하기 위하여 실시되었다. 원시생식세포는 12.5일째의 ICR 생쥐태아의 원시생식선 융기조직으로부터 추출하였으며, DMEM + 20% FCS + nucleosides + antibiotics로 조성된 sDMEM 배양액을 사용하여 mitomycin C로 전처리한 되먹임세포단층(feeder layer)위에서 체외배양하였다. bFGF 및 LIF를 20, 40ng/ml 농도로 각각 또는 함께 첨가하여 성장인자의 효과를 검토하였다. 원시생식세포는 성에 따라 유의적인 colony 형성율을 보였고(♂:1.9 colonies /genital ridge, ♀:1.13 colonies/genital ridge), bFGF 및 LIF의 첨가 및 첨가농도에 따라서도 유의성 있는 결과를 보였다(0.3~1.9 colonies/genital ridge). 그러나 3회 이상 계대배양을 할 경우, 원시생식세포의 colony 형성율은 급격히 감소되었다. 원시생식세포의 colony를 4% paraformaldehyde로 20분간 고정한 후, tris-maleate buffer(pH 9.0)로 10분간 3회 세정하였다. Fast Red로 염색을 실시한 결과, 대부분의 colony가 염색반응을 보여 다능성을 갖는 원시생식세포의 colony임이 입증되었다. 그러나 대부분의 colony가 3회 이상의 계대배양시 생존율이 급격히 떨어지는 것을 감안하면, 또 다른 미지의 성장인자나 보다 적절한 배양조건이 요구된다고 생각된다.

INTRODUCTION

Primordial germ cells (PGCs) are the founder cells of the functional germ cells and have been known to have a partial pluripotency (Tsunoda *et al.*, 1989). Recently, pluripotent embryonic germ (EG) cells have been established from mouse PGCs (Matsui *et al.*, 1992). During mouse embryogenesis about 100 alkaline phosphatase positive PGCs can be detected at the 7 day post coitum (dpc). However, these cells continue proliferation and their number increases rapidly to around 25,000 cells at 13.5 dpc embryos (Tam and Snow, 1981). We initiated a study for the fate and behavior of PGCs cultured *in vitro* to establish PGC culture system. To improve survival and proliferation of PGCs, basic fibroblast

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growth factor (bFGF) and leukemia inhibiting factor (LIF) were included in the culture medium.

MATERIALS AND METHODS

1. Isolation of PGCs

PGCs were recovered from pregnant ICR mice at 12.5 dpc by pricking of the isolated embryonic genital ridges with a 26 gauge needle. Since male and female genital ridges can be distinguished by their morphology. Male and female PGCs were prepared separately by sexing the fetus.

2. Culture of PGCs

The isolated PGCs were cultured on mitomycin-C treated murine embryonic fibroblast cell monolayer in sDMEM ; DMEM + 20% FCS + 50 mM β-mercaptoethanol + nucleosides (8 mg/ml adenosine, 8.5 mg/ml guanosine, 7.3 mg/ml cytidine, 7.3 mg/ml uridine and 2.4 mg/ml thymidine) + antibiotics (50 IU/ml penicillin, 50 mg/ml streptomycin) as described previously (Park *et al.*, 1993, 1994). Complete medium was prepared several hours before the culture starts. Changes of the medium was made after initial 2 days of culture every 48h.

3. Addition of growth factors

bFGF and LIF were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). Either bFGF or LIF and both were added to the PGC culture giving final concentrations of 20 and 40 ng/ml bFGF and 20 and 40 ng/ml LIF, respectively or as combined to examine the effects on the PGC proliferation. The numbers of colonies formed were recorded to compare the effects of growth factors by counting the number of colonies per genital ridges.

4. Subculture of PGC colonies

PGCs colonies were taken and divided by 0.1%(v/w) trypsin + 0.1%(v/w) EDTA solution and the similar aliquots of dispersed cells were plated on freshly-made feeder layers.

5. Histochemistry for alkaline phosphatase (AP)

AP staining was carried out according to Cooke's method (Donovan *et al.*, 1986). Briefly, PGC colonies were fixed in 4% paraformaldehyde for 20 min and then washed three times in tris-maleate buffer (pH 9.0) for 10 min for adjusting pH 9.0. Reaction mixture consisted of 1 mg/ml fast red TR, 0.4 mg/ml naphtol AS-MX phosphate and 0.5 mM $MgSO_4$ tris-maleate buffer, pH 9.0.

RESULTS AND DISCUSSION

Genital ridges were differentially isolated from either male or female embryos. The morphology of genital ridges was distinct at 12.5 dpc the time we have chosen (Fig. 1a and b). From this genital ridges it was not difficult to obtain dispersed PGCs without somatic cell contamination (Fig. 1c). The gross morphology of PGC was different from somatic cells. The somatic cells tends to attach first to the dish bottom, thus separating PGC cells. To facilitate colony formation they were cultured in a small drop of medium containing feeder layer under oil. Colonies were found at day 3 afterwards. Overall efficiency of colony formation was slightly higher in male PGCs without no significant difference. The sex differences of PGC proliferation *in vitro* have not been well documented in the literature. However, female PGCs was shown to have similar viability at 1st day of culture of PGCs at 12.5 dpc, but slightly better viability

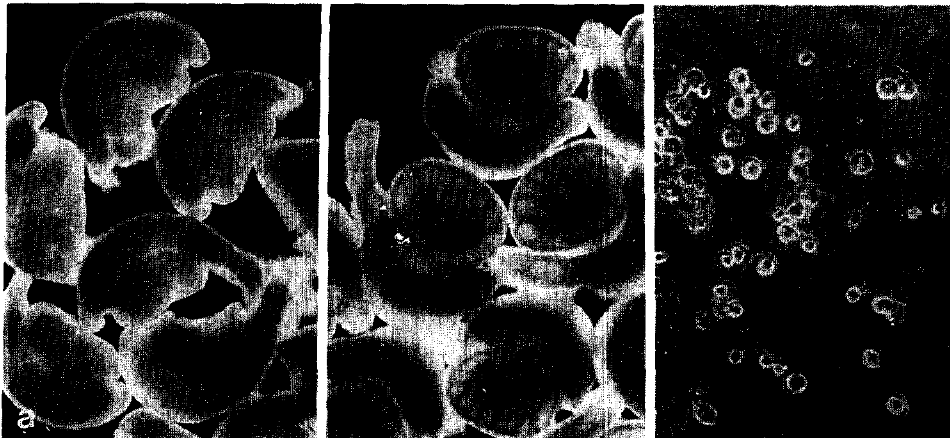

Fig. 1. Male and female genital ridges dissected from pregnant female mice at 12.5 dpc. Female genital ridges are smaller than male's, and spotty form (a). Male genital ridges showed tubular form (b). After taking off the mesonephric tissue (M) PGCs were squeezed out by pricking. The morphology is distinct for differentiation between female and male embryos after dissection. Associated PGCs (c) were cultured on the feeder layer.

Table 1. The colonization rates of male and female PGCs after 5 days culture *in vitro*

No. of experiments	Male PGC colonies /genital ridge	Female PGC colonies /genital ridge
1	10/8	4/8
2	15/8	7/8
3	20/8	15/8
4	16/8	10/8
5	15/8	–
Total	76/40 (1.90/genital ridge)	36/32 (1.13/genital ridge)

afterwards (De Felici and McLaren, 1983).

The behavior and fate of dispersed PGCs were examined during colony formation. The dispersed PGCs started to aggregate while proliferating probably at limited level (Fig. 2a). Small cell clumps became larger to form discrete margin (Fig. 2b). They become clear colonies by establishing tight cellular interactions becoming cellular masses. Most of single cells around the colonies showed typical morphology of cell death (Fig. 2c and d). It is well known that PGCs at 12.5~13.5 day stop proliferation *in vivo*. The PGCs in this study did not survive or maintain undifferentiated state *in vitro* after 10 days, too. PGC colonies either perished or differentiated to various extents during passages to freshly-made feeder layers (Table 2).

The feeder layer obtained from primary embryonic fibroblast have been known to secrete growth factors (Resnick *et al.*, 1992). We exam-

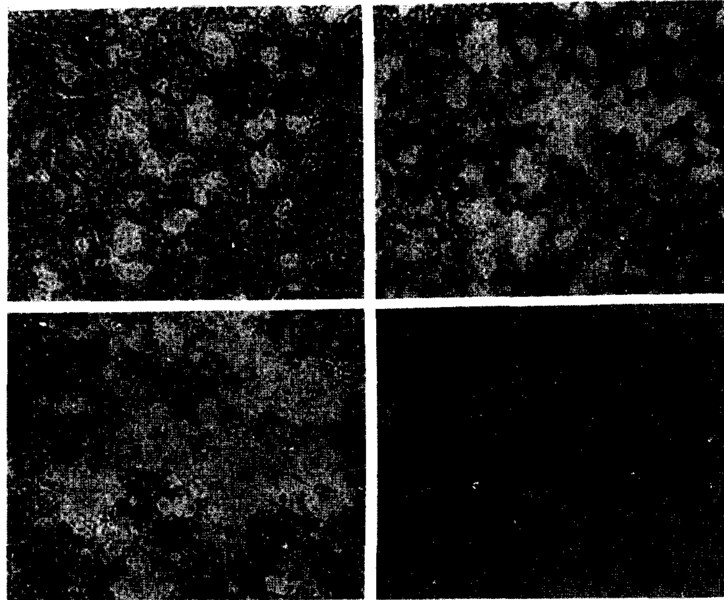

Fig. 2. Colonies of PGCs during culture *in vitro*. After 24h, PGCs aggregated each other (a). At day 2, the tightly aggregated PGCs formed clumps (b). At day 4, various sizes of PGC colonies are visible phase contrast (c) and bright field (d).

Table 2. The survival rates of subcultured PGC colonies

No. of experiments	No. of fetus used	No. of colonies primary culture	No. of survived / seed during subcultures (%)	
			1st subculture	2nd subculture
1	11	15	26 / 57 (45.6)	32 / 79 (40.1)
2	10	10	19 / 43 (44.2)	13 / 74 (17.6)
3	12	10	27 / 48 (56.3)	19 / 69 (27.5)
4	10	14	28 / 50 (56.0)	24 / 73 (32.9)
Total	43	49	100 / 198 (50.5)	88 / 295 (29.8)

ined the effects of exogenous growth factors by adding either bFGF or LIF and both to the dispersed PGC cell suspension. bFGF group, LIF group and bFGF+LIF group showed higher results than control group when the colonies formed were calculated per genital ridge in male PGCs. In Female PGCs 20 ng / ml bFGF was most effective for colony formation(Fig. 3). Although it may not be conclusive to say the establishment of an effective *in vitro* culture system from the result, it would be safe to suggest that 20 ng / ml bFGF facilitate formation of female PGC colonies when PGCs are recovered from embryos at 12.5 dpc. Furthermore, additional LIF did not appear to help female PGC colony formation, thus suggesting that endogenous LIF secreted from feeder layer and / or PGCs may be sufficient to maintain undifferentiated state of PGCs.

Finally, the PGC colonies were tested for AP

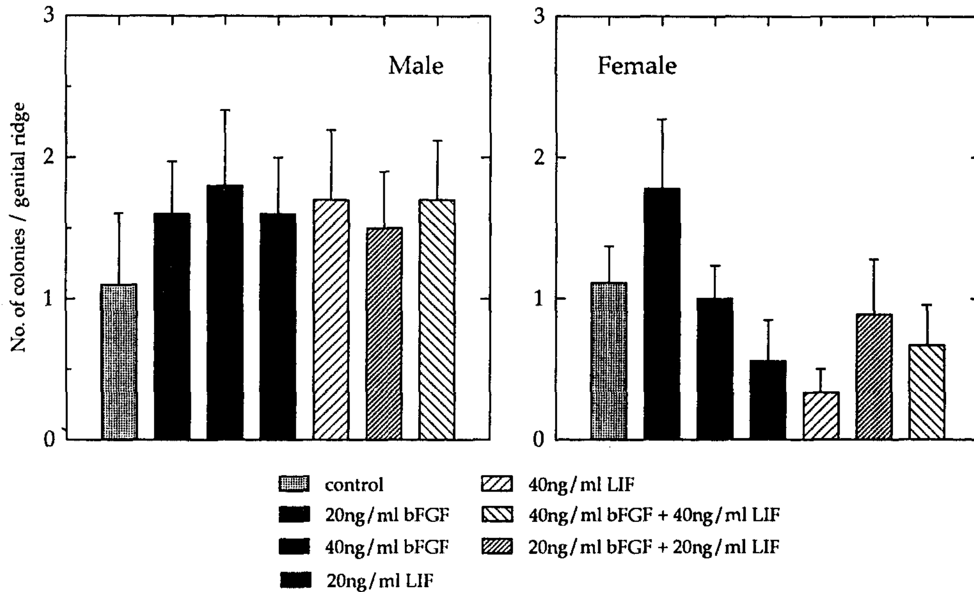

Fig. 3. The effects of additional bFGF and LIF on the colony formation during male and female PGC culture. Appropriate amount of growth factors were delivered into the culture drops to give the final concentrations.

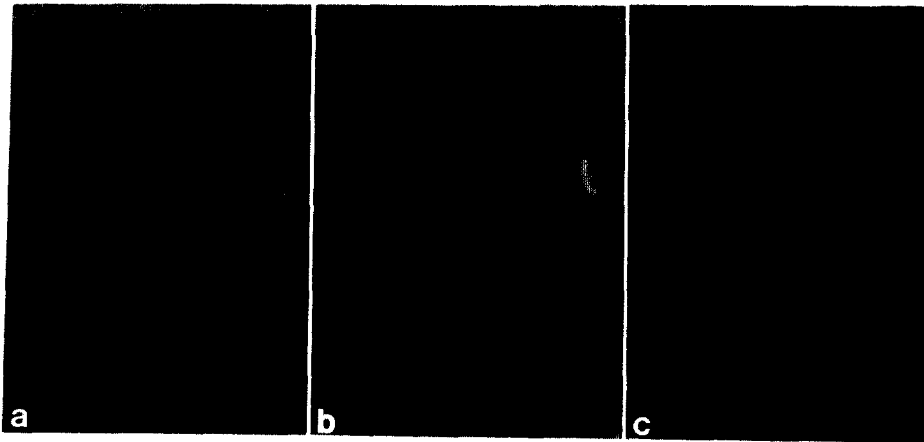

Fig. 4. Confirmation of true PGC colonies shown by histochemical staining for alkaline phosphatase. Positive colonies showing red precipitation, and discernable staining on dead or differentiated PGCs around the positive colonies (a). No staining is found in somatic cells, the feeder layer cells. In magnified view, note the clear staining around the membrane of single PGCs (b). Another healthy PGC colony shows massive precipitation and 2 derived small colonies also shows positive staining (c).

staining. Positive color precipitation was found in the presumptive PGC colonies maintained *in vitro* (Fig. 4). The AP activity found in the colonies was very specific showing clear boundary between PGCs and differentiated or somatic cells contaminated and feeder layers. In conclusion, the isolation and culture of murine PGCs have been established. The addition of growth factors to the culture may help in unhealthy feeder layer. However, preparation and maintenance of feeder layer should be considered critical for PGCs.

ABSTRACT

Primordial germ cells(PGC) are the founder cells of the functional germ line, and have been known to have a pluripotency. So they can be used for making chimeric /transgenic animal instead of stem cells. We examined the survival and proliferation of mouse PGCs under *in vitro* culture condition. PGCs were recovered from ICR mouse at 12.5 day post coitum(dpc) by pricking of the genital ridges. PGCs were cultured on mitomycin C - treated murine embryonic fibroblast cell monolayer in DMEM + 20% FCS + nucleosides + antibiotics (sDMEM). PGCs obtained from male genital ridges were more efficient to maker the colonies than that of female(1.19 : 1.13 colonies /genital ridges). But after 3rd subculture, colonies did not survive. Basic fibroblast growth factor and leukemia inhibitory factor were added to examine of the effection of exogenous growth factors. In Female PGCs, 20 ng /ml bFGF was most effective for colony formation, but generally colony formation of female PGCs was much lower than male's. To demonstarate alkaline phosphatase(AP) activity, colonies were fixed with 4% paraformaldehyde for 20 min, and adjusted pH for alkali by tris-maleate buffer(pH 9.0) for 10 min three times. Fast Red TR salt stained almost colonies. AP staining revealed that the presumptive cell colonies were PGCs judging by the positively stained color precipitation. However PGC colonies did not survive after 3rd subculture, thus suggesting inapprepriate culture condition.

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