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FULL PAPER

Differentiative potential of a mouse parthenogenetic embryonic stem cell line revealed by embryoid body formation *in vitro*

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Abstract

The *in vitro* differentiative potential of mouse parthenogenetic (PG) embryonic stem (PGES) cells were investigated in the formation of embryoid bodies (EBs). EBs derived from PGES cells retarded in growth and showed restricted differentiation compared to their fertilized counterpart. In chimeric EBs from the aggregation of PGES and fertilized ES cells, morphological examination revealed that PGES cells were reduced in their population and distributed in endodermal layer as culture periods proceeded. These findings were comparable to those in aggregation chimeras of fertilized and PG embryos, and suggest that the differentiation of PGES cells *in vitro* is restricted in the formation of EBs.

Key words : differentiation, embryoid bodies, embryonic stem cells, parthenogenesis,

Introduction

Mammalian parthenogenetic (PG) embryos do not survive until term[36,38)]. In the laboratory mouse, PG embryos are lost at various stages of development ; most of them are resorbed immediately, a majority of embryos surviving implantation are lost before 9 days of gestation with no or extremely underdeveloped extraembryonic structures, and even exceptionally well-grown parthenogenones die by midgestation, probably mainly due to poor differentiation of extraembryonic tissues[38)]. On the other hand, PG cells are capable of contributing to adult somatic cells as well as germ cells in chimeras produced by aggregation of PG and fertilized embryos[1,12-15,27,39)]. PG cells in chimeras distributed randomly early stages after aggregation[7,12)], but they are lost from the trophoblastic cell lineage substantially in early gastrulation stage[7)]. Elimination of the PG component from mesodermal tissues such as skeletal muscle became evident later[12-15)]. Through midgestation and post natal period, PG cells are restricted to tissues of mostly ectodermal origin[1,21)]. It is interesting to notice that cells of androgenone origin are mainly distributed in mesodermal tissues such as skeletal muscles and bones[22,23)]. This contrasting contribution of PG and androgenic (AG) cells in chimera may due to genomic imprinting[40,41)]. More than twenty endogenous imprinted genes have now been reported in human and mouse[10)],

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but no candidate gene involved in the differential elimination of AG and PG cells is identified.

Recently, embryonic stem (ES) cells bearing uniparental genomes are frequently used for studies related to imprinting[1,19,21,23,37,42]. ES cells are undifferentiated cultured cell lines established from preimplantation embryos[11,24]. Since ES cells were isolated first in the laboratory mouse more than a decade ago, they have been indispensable tools for dissecting early mammalian embryogenesis[2,4,30]. ES cells have been shown to maintain remarkable pluripotency in chimera formation with host embryos ; they can differentiate into functional gametes as well as various types of somatic cells[2,3]. Totipotency of certain ES cell lines has been demonstrated by the birth of mice consisting exclusively of ES derived cells[26,48]. In addition to these unique properties *in vivo*, ES cells undergo spontaneous differentiation under appropriate culture conditions[8,9]. When grown in suspension, ES cells stick one another to form large cell aggregates that differentiate into a structure called embryoid bodies (EBs) closely resembling embryonic region of the egg cylinder stage embryo[2,9]. There are various lines of evidence to indicate that EBs can be used as an *in vitro* model system for studying certain events of occurring in early embryogenesis[5,20,29].

ES cells are especially valuable for the study for the PG and AG cells because both types of embryos are hard to obtain routinely, although behavior of PG embryonic cells and that of parthenogenetic ES (PGES) cells in chimeric embryos are slightly different[1,37]. It is possible that certain changes in imprinting occurring during establishment and maintenance of ES cell lines are responsible for the differences found between them[25,37,42]. The aim of this study is to understand why PG conceptuses ultimately fail to survive in mammals. This study describes the distribution and behavior of PGES cells in EBs formation by using newly isolated cell line with *lac Z* gene as *in situ* maker.

Material and Methods

Cell lines

β-galactosidase-expressing ES cell line NR2 (a kind gift from Dr. M. Azim Surani, Wellcom/CRC, UK.) was derived from a male transgenic mice line bearing *E. coli lac Z* transgene[1]. TMA-24 and TMA-48P cells, which arose from a fertilized male and a PG blastocyst of 129/Sv background, respectively[35]. T48PZ4 cell line was isolated from TMA-48P cells transfected with *lac Z* reporter gene. Briefly, pENL containing *lac Z* gene under the control of elongation factor 1a promotor (a generous gift from Dr. Kazunori Hanaoka, Kitasato University) and pSTneo[18] (a kind gift from Drs. Kazuto Katoh and Hisato Kondoh, Osaka University) as a selection marker were linearized, and introduced into TMA-48P cells by Shimadzu GTE-1 electroporator. After electroporation, cells were grown in a selection medium containing 250 μg/ml Geneticin (G418, Sigma) for 10 days. T48PZ4 cells thus isolated was used for this study.

Culture conditions

ES cells used in this study were maintained in an undifferentiated state on mitomycin C (10 μg/ml) treated STO cell feeder layer in Dulbecco's Modified Eagle's Medium (DMEM, Dainippon pharmaceutical Co.) supplemented with 10% fetal bovine serum and 1×10^3 unit/ml of recombinant leukemia inhibitory factor (LIF, Amrad) at 37°C, in humidified air containing 5% CO_2[43]. ES cells were grown then in collagen coated plastic petri dishes (Iwaki) without STO cell feeder layer.

Embryoid bodies preparation

EBs were produced as described before[32,43]. About 1×10^7 ES cells grown without feeder layer were trypsinized and plated onto a bacteriological petri dish (Eiken) to which they do not adhere. Chimeric EBs were produced by aggregation of TMA-48P (*lac Z*-negative)/NR2

(*lac Z*-positive), T48PZ4 (*lac Z*-positive)/TMA-24 (*lac Z*-negative) and NR2/TMA-24 cell lines. About 5×10^6 cells from each cell line were seeded onto a bacteriological petri dish allowing free aggregation. EBs were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum without LIF. The medium was changed everyday thereafter. EBs were harvested for histological and morphological examination 3, 5, 7, 10 and 14 days after initiation of differentiation.

Histological examination of embryoid bodies

To compare the growth of EBs derived from NR2, TMA-48P and aggregation chimera of the two cell lines, EBs were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (0.1 M PBS, containing NaCl 8.0 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l and KH_2PO_4 0.24g/l), embedded in paraffin, sectioned serially at 3 μm in thickness, and stained with haematoxylin and eosin. The contribution and distribution of PGES cells in chimeric EBs were determined using X-gal histochemistry[16]. Briefly, chimeric EBs were fixed with 4% (w/v) paraformaldehyde and incubated in X-gal reaction mixture which contained 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.02% NP40 and 0.01% sodium deoxycholate, and 1 mg/ml X-gal in PBS, at 37°C overnight. After X-gal staining, specimens were dehydrated and embedded in paraffin, sectioned serially at 3 μm in thickness, and stained with haematoxylin and eosin.

Data Analysis

Numbers of total cells and lacZ-positive cells in each EB were counted using the preparation which gave the maximum area of the EB. Contribution of PGES cells was evaluated by calculating the percentage of the cells derived from PGES cells based on the total cell number. In older EBs, dead cells present at the central region of the section were omitted from counting. Data were compared using Student's t test.

Results

Growth of embryoid bodies

Most EBs derived from NR2 cells tended to be larger than those derived from TMA-48P cells in cell number (Table 1). In fact, the cell number in average-sized EBs in each culture was lower in PGES than fertilized ES cells for the first 3 days in culture ($P<0.05$). Cell number continued to increase slowly in PGES-derived EBs beyond day 5. Growth of chimeric and fertilized EBs was slightly better than PGES-derived EBs. Dead cells increased in the core region of fertilized and chimeric EBs thereafter, which made it difficult to count the cell number precisely. PGES-derived EBs scarcely developed into a cystic type, and the core region remained more or less homogeneous.

Morphology of embryoid bodies

Fertilized ES cells

NR2 and TMA-24 cells formed aggregates within 12 hr of suspension culture. Three days after initiation of differentiation, EBs were still simple cell aggregates encircled with endoderm-like cells (Fig. 1, A). By day 5, most of EBs were composed of embryonic ectoderm-like cells and cell debris surrounded by endoderm-like cell

Table 1. Cell numbers of embryoid bodies (EBs) counted at day 3 to day 10

EBs derived from	No. of cells in EBs (No. of EBs examined)			
	Day 3	Day 5	Day 7	Day 10
NR2	$151.9\pm7.5(10)^{a)}$	$208.6\pm7.6(10)^{a)}$	$382.7\pm7.3(7)^{a)}$	$469.6\pm9.1(5)^{a)}$
TMA-48P	$105.7\pm3.3(10)^{b)}$	$144.8\pm5.7(10)^{b)}$	$204.4\pm4.5(6)^{b)}$	$289.4\pm4.5(7)^{b)}$
NR2/TMA-48P	$156.4\pm8.4(10)^{a)}$	$282.9\pm4.9(10)^{a)}$	$293.1\pm8.0(5)^{a)}$	$459.8\pm4.1(5)^{a)}$

Values are mean±SEM.

a, b) Values with different superscript differ significantly ($P<0.05$).

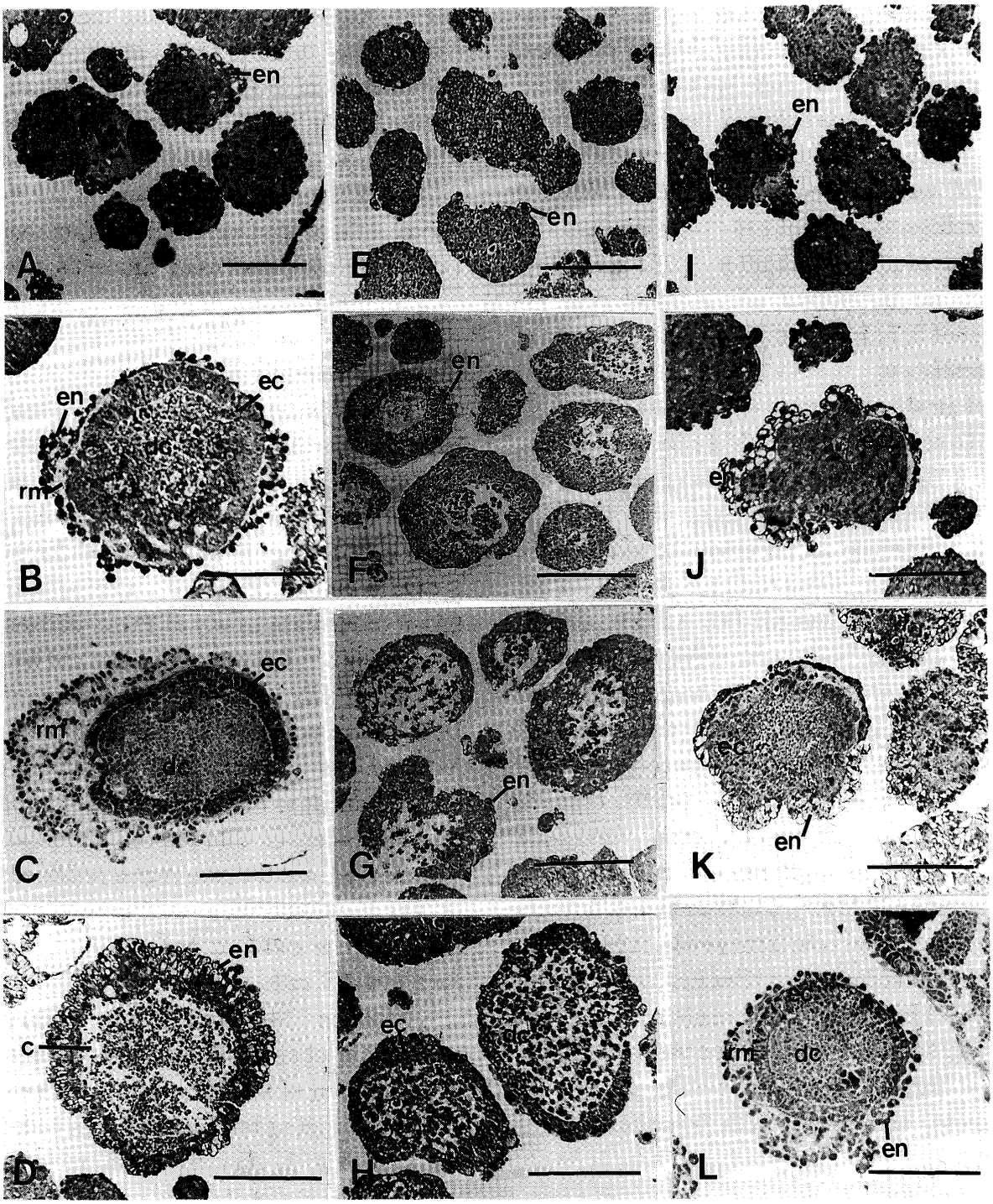

Figure 1. Histological sections of embryoid bodies (EBs) at day 3 (A, E and I), day 5 (B, F and J), day 7 (C, G and K), and day 10 (D, H and L). EBs derived from NR2 cell line (A-D) and chimeric EBs derived from TMA-24/T48PZ4 cell lines (I-L) showing well developed endoderm (en) with Reichert's membrane-like material (rm), embryonic ectoderm-like cells (ec), dead cells (dc) and cavity (c) formation. EBs derived from T48PZ4 cell line (E-H) showing flattened embryonic ectoderm-like cells with sparse endodermal layer and poorly organized core. Bar ; 0.5 mm. Haematoxylin and eosin staining.

layer developed fully on the surface of most of EBs (Fig. 1, B). They kept growing, embryonic ectoderm-like cells showed columnar arrangement and an eccentric cavity began to appear in the core. The cavities initiated to expand in most EBs on day 7 (Fig. 1, C). About half of EBs developed balloon-like cysts by day 10 (Fig. 1, D). Under a stereo microscope, beating muscle was first observed in these EBs. Over the next few days, a large proportion of the EBs developed in a similar manner. A few of EBs continued to enlarge and reached several millimeters in diameter. Development ceased at this stage.

Parthenogenetic ES cells

TMA-48P and T48PZ4 cells completed aggregation within 12 hr as in fertilized ES cell lines. Aggregations were homogeneous on day 3 (Fig. 1, E). Endoderm-like cells began to appear on the surface on day 5, but they developed poorly than fertilized counterparts (Fig. 1, F). Cell death began to be observed in core structure. By day 7, a small cavity appeared in only exceptional EBs. Endoderm-like cells in PG EBs were consistently more sparse in density than those in fertilized EBs of the same age (Fig. 1, G). Extensive cell death disclosed by the accumulation of a large amount of cell

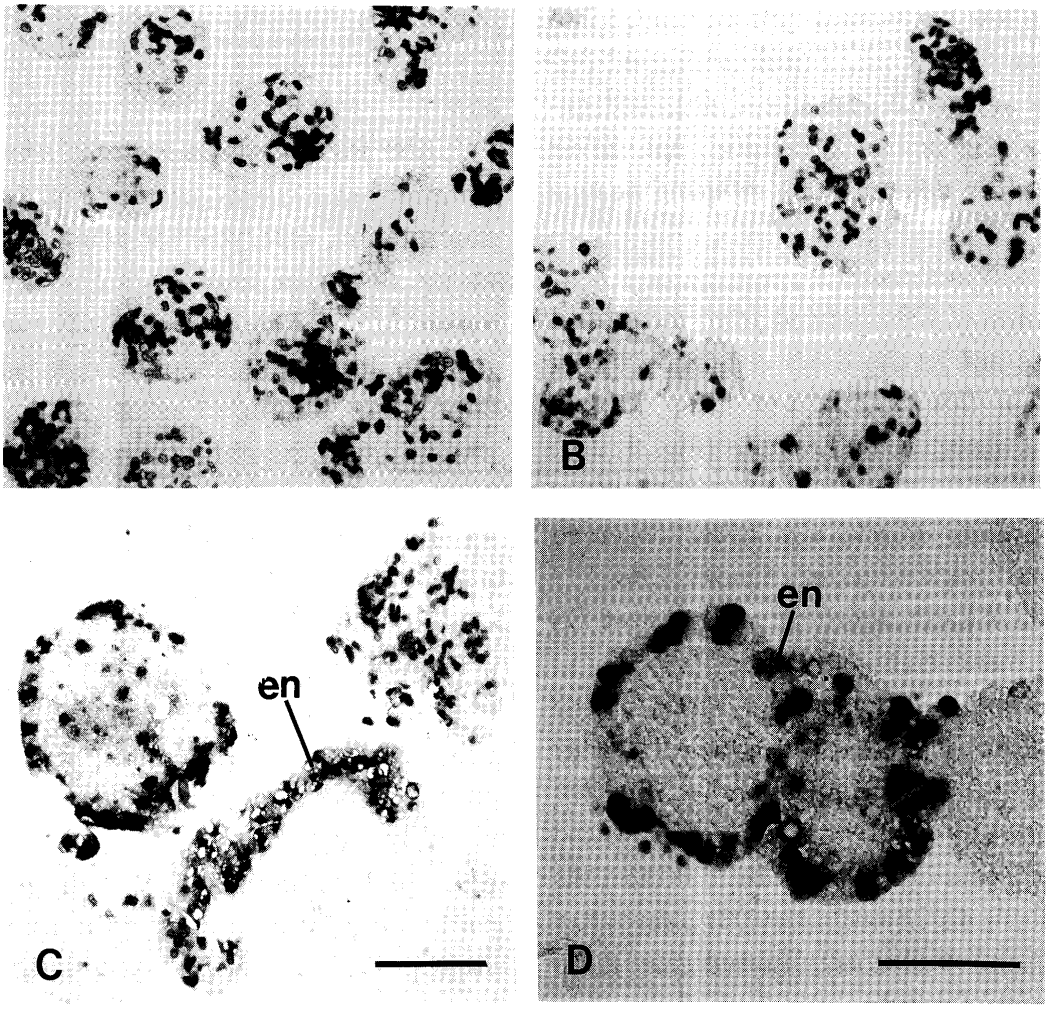

Figure 2. Morphology of chimeric embryoid bodies (EBs) derived from TMA-24 and T48PZ4 cell lines stained with X-gal at day 3 (A), day 5 (B), day 7 (C), and day 10 (D). Note that the distribution of T48PZ cells in endodermal layer (en). Bar ; 0.5 mm (A, B, and C in the same magnification), 0.2 mm (D).

Table 2. Contribution of parthenogenetic ES cells to chimeric embryoid bodies

	% of contribution (No. of EBs examined)			
	3 day	5 day	7 day	10 day
PGES[a]/FtES[b]				
T48PZ4/TMA-24	57.8±4.1(23)	29.3±3.8(12)[d]	18.1±3.1(21)[d]	4.0±2.1(46)[d]
TMA-48P/NR-2	49.5±4.1(16)	31.4±3.5(15)[d]	20.3±2.9(15)[d]	6.1±2.6(10)[d]
FtES/FtES[c]				
NR2/TMA-24	48.4±3.6(10)	49.8±4.1(10)[e]	55.2±3.4(10)[e]	57.0±4.0(5)[e]

Values are mean±SEM.

a) parthenogenetic ES cells. b) fertilized ES cells. c) contribution of NR2.

d, e) Values with different superscript differ significantly ($P<0.05$)

debris on the bottom of the petri dish should be responsible for the slower growth of PG EBs. At day 10, though EBs were larger in size, no orderly differentiated structure was observed, and only a few exceptional EBs developed into cystic form (Fig. 1, H). After prolonged culture, only a few EBs became cystic, and developed a balloon-like structure, but never showed further differentiation including beating muscles.

Chimeric embryoid bodies

The size of EBs derived from mixture of PGES cells and fertilized ES cells was slightly smaller than that of fertilized EBs. Overall growth and morphological feature (Fig. 1, I-L) were almost comparable to fertilized EBs, although beating muscle was never observed under stereo microscope in chimeric EBs.

Distribution of parthenogenetic and fertilized ES cells in chimeric embryoid bodies

While degree of chimerism varied among individual EBs, PGES cells decreased steadily throughout the entire culture period (Fig. 2). Table 2 shows the contribution of PGES cells and fertilized ES cells in chimeric EBs. PGES cells were randomly distributed in the EBs 3 days after differentiation. Cell count showed that there was no significant difference in the frequency and distribution of PGES and fertilized ES cells in chimeric EBs at this stage ($P<0.01$). By day 5, PGES cells had been lost from core structure considerably and remaining PGES scattered in the core as small colonies of a few cells. Loss of PGES cells from chimeric EBs continued thereafter, but PGES cells remained in the outer endoderm and the ectoderm layer adjacent to it in 7-day EBs.

Discussion

EBs from fertilized ES cells mimic certain developmental events occurring in early post-implantation embryos including proamniotic cavity formation and X chromosome inactivation[2,9,35,44]. The present study was initiated to understand the nature of PG cells using this *in vitro* model system. Findings obtained in the present study invariably show that PGES cells are deficient in undergoing differentiation via EB formation. PGES cells were capable of aggregation as fertilized ES cells, but subsequent poor differentiation of endodermal cells on the surface of the EBs and extensive cell death seemed major factors responsible for the defective EBs formation. Reduced differentiative potential into endoderm in PGES cells might reflect the abnormal endoderm differentiation in PG embryos *in vivo* reported by Sturm *et al.*[38]. Recent findings point to important inductive roles played by the visceral endoderm in implanting embryos[6,17,29]. It is possible that defects in endoderm formation is a crucial role trait that explains, at least partly, the abnormal development of PG embryos.

Contrary to the above findings, PGES cells persisted in the outer endodermal layer in

chimeric EBs consisting of fertilized ES and PGES cells. The cellular position in the cell aggregate is an important factor for determining the fate of each cell[34]. Thus, outside cells at the morula stage are destined to differentiate into trophectoderm cells, and inside cells remain undifferentiated as inner cell mass cells[31]. In the case of the EBs, it may be assumed that outside cells transform into endoderm cells. In view of the reduced differentiative potential into endoderm, various assumptions have to be made elucidate the prevalence of PGES cells in the endodermal layer of chimeric EBs. Most probably, PGES cells were forced to take the outside position due to slight differences in the cell surface structure between the two ES cell lines comprising the EBs. This consistent with finding reported by Rosenstraus and Levine[33] in EBs formed by nullipotent and pluripotent embryonal carcinoma (EC) cell lines. In this case, nullipotent cells were allocated at the center and pluripotent cells were allocated to outer region to form the endodermal layer. Interestingly, there was apparent correlation between the allocation of PGES cells in EBs and their size in the present study (data was not shown). Chimeric EBs tended to be smaller if PGES cells were present in the inner ectodermal portion. While they were bigger than fertilized EBs in size if PGES cells were present mainly in the outer endoderm layer. It was reported that EBs that developed into cystic stage had the outer endodermal layer composed mainly of visceral endoderm-like cells[9]. An intriguing possibility would be that the coexisting fertilized ES cells complemented the defective differentiative potential of the PGES cells into endoderm.

Another remarkable feature of EBs formed by PGES cells was inability of cavitation, and hence formation of neuroepithelial tissue lining the cavity, which may be attributed to either lack of inductive signal form the defective endoderm or failure of response by inner cells to exogenous signals. Previously Sado *et al.*[35] obtained evidence to show that fertilized ES cells helped differentiation of PGES cells and female embryonic germ (EG) cells in chimeric EBs. The frequency of PGES cells and female EG cells having an inactive X chromosome increased significantly in differentiating chimeric EBs than in EBs formed by either PGES cells or female EG cells alone. In agreement with this finding Coucouvanis and Martin[8] succeeded in inducing cavitation at the center of homogenous aggregates formed by nullipotent EC cells by enveloping with visceral endoderm layer. They proposed that the cavitation in early embryos and EBs is the result of the interaction of two signals, the dead signal from the outer endodermal layer and the survival signal from the basement membrane.

Gradual but consistent decrease of PGES cells from chimeric EBs hindered pursuit of the initial aim of the present study. This phenomenon resembles the exclusion of PG cells from PG/fertilized chimeric embryos *in vivo*[12-15,26], which could be elucidated by a low mitotic activity and/or precocious cell death in PG cells[28,44,46]. PG embryos are probably short of certain growth factors such as insulin-like growth factor-II coded by maternally imprinted gene at specific stages of development and EB formation[28,47]. However, LT-1 EC cell line established from PG ovarian teratocarcinoma differentiated into balloon-like cystic EBs[45]. It is likely that imprinting is more stable in ES cell lines than in EC cell lines.

Although *in vitro* model system have various limitations as shown in this study, further careful studies of differentiating PG and AG ES cells may throw a new light on the role of genomic imprinting in early mammalian embryogenesis.

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