Stage-specific tissue and cell interactions play key roles in mouse germ cell specification

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SUMMARY

Primordial germ cells (PGCs) in mice have been recognized histologically as alkaline phosphatase (AP) activity-positive cells at 7.2 days post coitum (dpc) in the extra-embryonic mesoderm. However, mechanisms regulating PGC formation are unknown, and an appropriate in vitro system to study the mechanisms has not been established. Therefore, we have developed a primary culture of explanted embryos at pre- and early-streak stages, and have studied roles of cell and/or tissue interactions in PGC formation. The emergence of PGCs from 5.5 dpc epiblasts was observed only when they were co-cultured with extra-embryonic ectoderm, which may induce the conditions required for PGC formation within epiblasts. From 6.0 dpc onwards, PGCs emerged from whole epiblasts as did a fragment of proximal epiblast that corresponds to the area containing presumptive PGC precursors without neighboring extra-embryonic ectoderm and visceral endoderm. Dissociated epiblasts at these stages, however, did not give rise to PGCs, indicating that interactions among a cluster of a specific number of proximal epiblast cells is needed for PGC differentiation. In contrast, we observed that dissociated epiblast cells from a 6.5-b (6.5+15-16 hours) to 6.75 dpc embryo that had undergone gastrulation gave rise to PGCs. Our results demonstrate that stage-dependent tissue and cell interactions play key roles in PGC determination.

Key words: Primordial germ cell, Epiblast, Gastrulation, Mouse

INTRODUCTION

In such organisms as Drosophila melanogaster, Caenorhabditis elegans and Xenopus laevis, germ plasm (polished plasm) containing germ cell determinants is maternally accumulated and localized in eggs, and only blastomeres that inherit germ plasm develop into germ cells. In contrast, in early mammalian embryos, germ plasm and even the Nuage-like structure that is characteristic of germ plasm (Eddy, 1975) have not been found (Snow and Monk, 1983), and the restriction of the germ and somatic cell lineage is thought to occur after a mid-gastrula stage of development.

During mouse embryogenesis, primordial germ cells (PGCs) are first identified histologically within the posterior extra-embryonic mesoderm at 7.2 dpc as a cluster of cells expressing tissue-nonspecific alkaline phosphatase (AP) (Chiquoine, 1954; Mintz and Russell, 1957; Ginsburg et al., 1990). Thereafter, PGCs undergo migration from the base of the allantois through the dorsal mesentery, and reach the genital ridges by 11 dpc. Throughout these developmental stages, PGCs continue to display AP activity.

Several experiments have been carried out to determine the timing of germ cell and somatic cell segregation in mouse embryos. By genetically marking a blastomere of a four-cell embryo (Kelly, 1977) or by marking cells of the inner cell mass (ICM) of a blastocyst with retroviruses (Soriano and Jaenisch, 1985), single cells at these developmental stages have been shown to contribute to both germ cells and somatic cells. A single cell in the ICM, however, has been observed to contribute only to germ cells in just a few cases, suggesting that the germ cell lineage can occasionally be set aside in ICM (Soriano and Jaenisch, 1986).

A perigastrula-stage mouse embryo consists of three major tissues: the epiblast, the extra-embryonic ectoderm and the visceral endoderm. Extra-embryonic ectoderm and visceral endoderm originate from the trophectoderm and the most parietal ICM, respectively, and they give rise to placenta and other extra-embryonic tissues. In contrast, cup-shaped epiblast originates from ICM and differentiates into all types of cells found throughout the entire embryo, including germ cells. The developmental potential of epiblast cells has been examined by injecting those cells into blastocysts (Gardner and Rossant, 1979). The contribution of the donor epiblast cells in chimeric mice was judged either by the presence of distinct GPI markers...
or by coat colors. The results indicated that at least two cells in 4.5 dpc epiblast cells can give rise to both germ cells and somatic cells (Gardner and Rossant, 1979).

The fates of later epiblast cells have been examined by clonal analysis (Lawson and Hage, 1994). The results showed that presumptive precursors of PGCs are scattered in a ring in the most proximal region of the epiblast, adjacent to the extra-embryonic ectoderm at 6.0-6.5 dpc. In these experiments, all cells that gave rise to PGCs also differentiated into extra-embryonic mesoderm, indicating that the allocation to PGCs had not occurred at 6.5 dpc, which is the start of gastrulation. Clonal analysis has also suggested that allocation to the germ cell lineage occurs at 7.2 dpc, with the founding population of PGCs consisting of 45 cells (Lawson and Hage, 1994). In addition, the results of a heterotopic transplantation of epiblast cells (Tam and Zhou, 1996) have demonstrated that even distal epiblast cells have the ability to give rise to PGCs as proximal cells when they are transplanted to a proximal region. This observation suggests the existence of putative local cues in the proximal regions of epiblasts that specify the germ cell lineage.

After the onset of gastrulation at 6.5 dpc, totipotent epiblast cells are destined for each somatic cell lineage by passing through the primitive streak during gastrulation (Hogan et al., 1994). The presumptive PGC precursors located within the proximal epiblast may also pass through the posterior end of the primitive streak and reside in the extra-embryonic mesoderm. Although it has been suggested that cell movement accompanied by gastrulation can lead to determination of the germ line fate in the new environment, probably at the posterior primitive streak (Tam and Zhou, 1996), the requirement of gastrulation for PGC formation is still unclear. Microsurgical grafting experiments have indicated that posterior primitive streak cells of 7 dpc embryos grafted orthotopically to the same site in other embryos yield PGCs (Copp et al., 1986). In contrast, lateral ectoderm/mesoderm cells yield somatic derivatives (but not PGCs) after heterotopic grafting to the posterior primitive streak site, indicating that the lateral ectoderm/mesoderm at the early allantoic bud stage no longer has the potential to give rise to PGCs.

As described above, the outline of PGC formation from epiblasts has been reported, but the mechanisms that regulate allocation to germ cell lineage are unknown. Although only little evidence is available, it is thought that interactions between tissues and/or cells play a critical role in the formation of mammalian germ cells in relation to the differentiation and patterning of somatic tissues. We have therefore designed a primary culture of 5.5-6.75 dpc embryos to assay the conditions required for PGC formation from epiblasts. The results demonstrate the role of cell-cell communication within proximal epiblasts and of the extra-embryonic ectoderm in PGC formation.

MATERIALS AND METHODS

Source, recovery and staging of concepti

Concepti used throughout this study were obtained from female mice of an outbred strain (CD-1) that were ICR-mated with male mice of BDF1 (C57/B6j × DBA2). Those mice were exposed to light daily between 08:00 and 20:00 hours. Taking the time of mating as the mid-point of the dark period (noon on the day of plug is 0.5 days postcoitum; dpc); pregnant females were sacrificed at 5.5 (5 days + 12 hours), 6.5-a (6 days + 12 hours), and 6.5-b (6 days + 15-16 hours) dpc, and their uteri were placed in PB-1 medium (Quinn et al., 1982) for isolating decidua. Concepti were staged by visual inspection, according to the scheme shown in Fig. 1.

Isolation of explants is shown in Fig. 2. Concepti were dissected out from decidua (Fig. 2A-a), and then Reichert’s membrane and visceral endoderm were mechanically removed (Fig. 2A-b) with fine forceps and a tungsten needle. Thereafter, extra-embryonic ectoderm (Fig. 2A-c) and epiblast (Fig. 2A-d) were separated by a fine tungsten needle. The boundary between extra-embryonic ectoderm and epiblast was identified based on the circumferential constriction (Downs and Davies, 1993).

Isolated epiblasts were further processed for each experiment as follows. (1) Epiblasts were divided into sub-fragments using tungsten needles and fine forceps (Fig. 2Af-h). (2) Epiblasts were trypsinized for 2.5-3.5 minutes at room temperature and subsequently dispersed by pipetting using a capillary micropipette (Fig. 2Ai). (3) For recombining epiblasts and extra-embryonic ectoderm at different developmental stages (asynchronous recombination), two female mice, mated 24 hours apart, were sacrificed at the same time (i.e. 5.5 and 6.5-a dpc), and extra-embryonic ectoderm and distal epiblast were then separated (Fig. 2Bf-d) and used for culture (Fig. 2Be).

Explant culture and examination of PGC emergence

Isolated tissues were cultured on mitomycin C-treated STO...
fibroblast (Donovan et al., 1986) or Sl/Sl4 (Matsui et al., 1991) cells plated at a density of 1 × 10^5 or 2 × 10^5 cells per well, respectively, in 24-well culture plates (Falcon) with DMEM medium (15% FCS, 0.22 mg/ml sodium pyruvate, 1.8 mg/ml L-glutamine, 100 units/ml Penicillin, 100 μg/ml Streptomycin) at 37°C in the gas phase of 5.0% CO2 in air. In some cases, isolated epiblasts were cultured in fibronectin-coated 24-well plates (CBP Bioproducts). The explants were allowed to develop until the time corresponding to 9.5 dpc (approximately 96 hours for 5.5 dpc, 84 hours for 6.0 dpc and 72 hours for 6.5-a and 6.5-b dpc).

For the recombination culture, a small hole was made in the bottom of a four-well culture plate (Nunc) by means of pressure from a Hungarian needle (Fig. 2Be; Ang and Rossant, 1993), and the culture wells with holes were covered with a feeder layer of STO cells. Thereafter, an extra-embryonic ectoderm and a distal epiblast were placed together in the hole.

To examine the emergence of PGCs, alkaline-phosphatase (AP) staining was carried out as described (Matsui et al., 1992). Each experiment was repeated at least three times. PGCs were identified based on high AP activity, 4C9 and Oct3/4 expression.

**Immunohistochemistry for 4C9 and Oct3/4**

Explants were fixed in 4% paraformaldehyde and were stained by each antibody as described (Davis, 1993). After incubation with 4C9 monoclonal antibody (a kind gift from Dr T. Muramatsu; Yoshinaga et al., 1991) or affinity-purified polyclonal antibody against Oct3/4 (a kind gift from Dr H. Hamada; Shimazaki et al., 1993), explants were incubated with FITC-conjugated anti-rat IgM (Zymed) or rhodamine-conjugated anti-rabbit IgG (Reinco technology), respectively. Thereafter, the same explants were stained for AP activity.

**Immunohistochemistry for mesoderm markers**

Explants were fixed in 4% paraformaldehyde, followed by AP staining. Explants that contained PGCs were subsequently incubated with monoclonal antibody against Flk-1 (Kdr; a kind gift from Dr S.-I. Nishikawa; Kataoka et al., 1997), or with affinity-purified polyclonal antibody against HNF-3b (Foxa2: a kind gift from Dr H. Sasaki; Yasui et al., 1997) or Brachyury (a kind gift from Drs B. Hermann and H. Koseki; Hermann, 1991), followed by incubation with HRP-conjugated anti-rat IgG (Flk-1) or HRP-conjugated anti-rabbit IgG (HNF-3) and Brachyury).
RESULTS

Establishment of explant culture of epiblast at pre- and early-streak stages

We first established a primary culture system for mouse epiblasts at pre- and early-streak (corresponding to 6.25 or 6.5-a dpc in Fig. 1C,D, respectively) stages, in which the PGCs developed. Staging of the embryos used in this study is described in Fig. 1. Typical pictures of each embryo stage are shown, and the staging is based on both morphological and morphometrical features. Epiblasts were isolated free of visceral endoderm and extra-embryonic ectoderm and were opened up to form a flat sheet. Epiblast fragments before culture are shown in Fig. 2, confirming complete removal of extra-embryonic tissues. They were then cultured on a feeder layer of STO fibroblasts until the time corresponding to 9.5 dpc, i.e. approximately 78 hours for 6.25 dpc embryos and 72 hours for 6.5-a dpc embryos.

After culture, AP-positive PGC-like cells were scattered around outgrowing epiblast (Fig. 3A). In case of 6.25 dpc explants, none of these cells were recognized after 24 hours in culture, but by 48 hours, migratory PGC-like cells were observed (data not shown), and the timing of PGC-like cell emergence in culture corresponded well with that in vivo. To judge whether the AP-positive PGC-like cells (Fig. 3A,B,D) were PGCs, we examined the expression of markers such as 4C9 (Fig. 3C, Yoshinaga et al., 1991), Oct3 (Fig. 3E, Okamoto et al., 1990) Oct4 (Schöler et al., 1990) by immunostaining. As shown in Fig. 3, all AP-positive PGC-like cells also expressed 4C9 (Fig. 3B,C shows double staining of an explant with AP and 4C9, respectively) and Oct3/4 (Fig. 3D,E shows double staining of an explant with AP and Oct3/4). In addition, when we cultured whole epiblasts, those marker positive cells exhibited the typical morphology of migrating PGCs (Fig. 3C; Donovan et al., 1986). However, only a part of the marker-positive cells from fragments of epiblasts or from dissociated epiblast cells showed the morphology of migrating PGCs and none of marker-positive cells was migratory when epiblasts were cultured on fibronectin or a feeder layer of Sl/S14 cells. We also cultured epiblasts of the Oct3/4 (GOF18/deltaPE)/GFP transgenic mice (Yoshimizu et al., 1999), which express green fluorescent protein (GFP) only in PGCs but not in epiblasts. We found that AP-positive cells also expressed GFP (data not shown). Thus, from all the available evidence, the cells were judged to be PGCs. In contrast, no AP positive cells were seen when extra-embryonic ectoderm was cultured in the same condition.

Emergence of PGCs in response to culturing isolated whole epiblasts at 6.0-6.5 dpc on STO feeder cells or on fibronectin

Whole epiblasts obtained from 6.0-6.5-a dpc embryos were cultured on either an STO feeder layer (Donovan et al., 1986) or on a fibronectin (FN)-coated plate. Table 1 shows that approximately half of the explants of 6.25 and 6.5-a dpc epiblasts gave rise to PGC, both on STO cells and on FN. On an STO feeder layer, they formed a packed cluster of cells (Fig. 3A), whereas only a small cluster was found on FN (data not shown). However, 6.0 dpc epiblasts formed a cluster and gave rise to PGCs at a similar frequency only when they were cultured on fibronectin or a feeder layer of SI/S14 cells. We also cultured epiblasts of the Oct3/4 (GOF18/deltaPE)/GFP transgenic mice (Yoshimizu et al., 1999), which express green fluorescent protein (GFP) only in PGCs but not in epiblasts. We found that AP-positive cells also expressed GFP (data not shown). Thus, from all the available evidence, the cells were judged to be PGCs. In contrast, no AP positive cells were seen when extra-embryonic ectoderm was cultured in the same condition.

<table>
<thead>
<tr>
<th>Culture method</th>
<th>Stage (dpc)</th>
<th>Explants with PGCs/ total explants (%)</th>
<th>Range of PGC numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>STO</td>
<td>5.5</td>
<td>0/15 (0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>6/17 (35)</td>
<td>2-16</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>13/21 (62)</td>
<td>3-27</td>
</tr>
<tr>
<td></td>
<td>6.5-a</td>
<td>10/20 (50)</td>
<td>4-37</td>
</tr>
<tr>
<td>FN</td>
<td>6.0</td>
<td>0/21 (0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>21/49 (43)</td>
<td>1-30</td>
</tr>
<tr>
<td></td>
<td>6.5-a</td>
<td>10/21 (48)</td>
<td>3-34</td>
</tr>
</tbody>
</table>

Fig. 3. Emergence of primordial germ cells (PGCs) from early-streak epiblasts in primary culture. (A) An explant with AP-positive cells (arrows). A whole epiblast isolated from a 6.5-a dpc embryo was cultured on an STO feeder layer for 72 hours, and stained for AP activity. AP-positive cells were also positive for other markers for PGCs, 4C9 (C) and Oct3/4(E, arrowheads), and showed the typical morphology of migrating PGCs (B,C). B and D show counterstaining by AP staining for C and E, respectively. AP expression within a cell mass of an explanted epiblast was downregulated at the time of staining, but some Oct3/4-positive cells remained (upper left in E). Scale bar; A, 200 μm; B,C, 30 μm; D, E, 60 μm.
It seems likely that this explant culture also supports the development of cells of other lineages, and for this reason the expression of mesodermal markers in the cultured explants of proximal epiblasts at 6.0 and 6.25 dpc was examined immunohistologically. We have examined Flk-1, which, in vivo, is first expressed in the allantois and later in endothelial cell precursors (Yamaguchi et al., 1993) that share common precursor cells with PGCs. We have also tested Brachyury, which is expressed in the proximal region of prestreak-stage epiblasts and later in the gastrulating primitive streak (Herrmann, 1991), and HNF-3β, which is expressed in the prechordal mesoderm and their derivatives, the node and head process (Sasaki and Hogan, 1993). As shown in Fig. 4E,G,H explants were found to be Flk-1, Brachyury and HNF-3β negative, even when PGCs were observed (Fig. 4A,C,D). Although Flk-1 expression was sometimes detected (Fig. 4F), other markers were not. These results suggest that mesoderm cell differentiation does not progress well in this explant culture, even when PGCs are differentiated.

**PGCs specifically emerge from a fragment of proximal epiblast consisting of a specific number of cells**

A heterotopic transplantation experiment (Tam and Zhou, 1996) has revealed that the fate of epiblast cells depends on their position in the epiblast, i.e. localization in the proximal

**Table 2. Derivation of PGCs**

A From fragments of epiblast cultured on a feeder layer of STO cells

<table>
<thead>
<tr>
<th>Region of explant</th>
<th>6.0 dpc</th>
<th>6.25 dpc</th>
<th>6.5-a dpc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td>4/11 (36)</td>
<td>13/21 (62)</td>
<td>10/22 (46)</td>
</tr>
<tr>
<td>Distal</td>
<td>0/11 (0)</td>
<td>0/21 (0)</td>
<td>0/22 (0)</td>
</tr>
<tr>
<td>Proximal explants with PGCs/total explants (%)</td>
<td>40</td>
<td>62</td>
<td>46</td>
</tr>
<tr>
<td>Range of PGC numbers</td>
<td>2-5</td>
<td>1-45</td>
<td>2-34</td>
</tr>
</tbody>
</table>

B From subdivided proximal epiblasts on a feeder layer of S1/S14 cells

<table>
<thead>
<tr>
<th>No. of cells in a subdivided fragment</th>
<th>6.0 dpc</th>
<th>6.25 dpc</th>
<th>Whole proximal epiblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-60</td>
<td>5/15 (33)</td>
<td>2/7 (29)</td>
<td>7/20 (35)</td>
</tr>
<tr>
<td>20-40</td>
<td>4/13 (8)</td>
<td>2/4 (0)</td>
<td>7/20 (35)</td>
</tr>
<tr>
<td>10-20</td>
<td>1/11 (1)</td>
<td>0/1 (0)</td>
<td>4-7 (0)</td>
</tr>
<tr>
<td>Whole proximal epiblast</td>
<td>7/20</td>
<td>7/20</td>
<td>7/20</td>
</tr>
</tbody>
</table>
region is necessary for them to differentiate into PGCs. To explicate local effects on PGC determination further, an epiblast was cut into proximal and distal fragments, and each fragment was cultured on an STO feeder layer. As shown in Table 2A, in all stages tested, 36-62% of the proximal fragments gave rise to PGCs (Fig. 5A), but no distal fragments did so (Fig. 5B). These results indicate that the proximal fragment of an epiblast is sufficient for PGC formation in culture, and that the region-specific competence of epiblast cells that is required for PGC determination is already established at 6.0 dpc. Proximal and distal explants were also distinct with regard to their morphology after culture, i.e. proximal explants formed a packed cluster of cells (Fig. 5A), while distal explants spread out into a monolayer (Fig. 5B). The cluster of cells may provide the environmental interaction required for PGC differentiation.

We next cultured proximal epiblast fragments cut into up to four pieces (Fig. 2A,f,h shows an example of four pieces) on a feeder layer of Sl/Sl4 cells (Matsui et al., 1991), which we had previously shown do not support proliferation of PGCs. As shown in Table 2B, a proximal fragment (up to one fifth of a proximal fragment) of 6.25 dpc epiblast cells could yield PGCs at a similar frequency to those raised on an STO feeder layer. The number of cells within these small subfragments of epiblast were easily counted under an inverted microscope, and only 20-40 cells were counted. However epiblasts dissociated by trypsinization failed to give rise to PGCs. After trypsinization, the cell suspensions contained single cells as well as a small number of aggregates consisting of less than 10 cells. With 6.0 dpc epiblasts, one third of the fragments (found in 40-60 cells) gave rise to PGCs, but the frequency of PGC formation from smaller fragments was decreased from 40% to 8%. These results indicate that PGC formation requires a rather small mass of epiblast cells, consisting of 40-60 cells at the start of the culture period, and that close cell interactions appear to be essential. In addition, these results suggest that normal gastrulation is not required for PGC determination.

Emergence of PGCs is independent of cell-cell interaction after the onset of gastrulation

We further tested the requirement for cell interactions among epiblast cells for PGC differentiation in later embryos (Table 3). After trypsinization, we cultured cell suspensions obtained from 6.5-a, 6.5-b and 6.75 dpc embryos on an STO feeder layer for 66-72 hours. As shown in Table 3, PGCs were never formed from dissociated 6.5-a dpc epiblast, but thereafter the frequency of PGC emergence gradually rose, with 29% and 70%, respectively, of dissociated epiblasts at 6.5-b and 6.75 dpc giving rise to PGCs. AP-positive cells (Fig. 5C) that emerged in the cultures were also positive for 4C9 (Fig. 5D,E). These results indicate that cell interactions among epiblast cells becomes less important for PGC differentiation shortly after the onset of gastrulation.

<table>
<thead>
<tr>
<th>Stage (dpc)</th>
<th>Explants with PGCs/total explants (%)</th>
<th>Range of PGC numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5-a</td>
<td>0/20 (0)</td>
<td>0</td>
</tr>
<tr>
<td>6.5-b</td>
<td>5/17 (29)</td>
<td>13-30</td>
</tr>
<tr>
<td>6.75</td>
<td>14/22 (70)</td>
<td>9-34</td>
</tr>
</tbody>
</table>
The extra-embryonic ectoderm induces the proximal epiblast-specific competence for PGC formation before 6.0 dpc.

Isolated 5.5 dpc epiblasts free of extra-embryonic tissues never gave rise to PGCs in culture (Table 1). In order to investigate the effect of the extra-embryonic ectoderm on germline development, 5.5 dpc epiblasts with or without extra-embryonic ectoderm were isolated free of visceral endoderm and cultured on an STO feeder layer. Explants were fixed after 90-96 hours in culture, and the emergence of PGCs was examined by staining for AP activity (Fig. 6A,B). The emergence of PGCs as well as overall cell proliferation and the subsequent formation of packed cluster of cells appeared to depend completely on the existence of the extra-embryonic ectoderm (Fig. 6A,B; Tables 1, 4).

Distal epiblasts at early-streak stages respond to putative inducing signals from the extra-embryonic ectoderm

As described above, we demonstrated that 5.5 dpc epiblasts can respond to the extra-embryonic ectoderm, thereby acquiring the ability to differentiate into PGCs. We next examined whether even later epiblasts had the competence to respond to the extra-embryonic ectoderm and whether even later extra-embryonic ectoderm could induce competence in epiblasts. To answer these questions, we isolated fragments of distal epiblast at 5.5, 6.5-b and 6.75 dpc that could not alone give rise to PGCs and recombined them with extra-embryonic ectoderm at the same developmental stages (synchronous recombination). We found PGC differentiation in all of these cultures (Table 4), indicating that distal epiblast cells and extra-embryonic ectoderm at 6.5-b and 6.75 dpc maintain competence and induction ability, respectively. We next examined whether recombination of distal epiblast and extra-embryonic ectoderm at different developmental stages (asynchronous recombination) could give rise to PGCs. PGC differentiation was also observed in these cases (Table 4; Fig. 6C,D).

DISCUSSION

These results demonstrate that primordial germ cell (PGC) differentiation from epiblast cells in mouse embryos is accomplished by stage-specific cell or tissue interactions. At the beginning of this study, we tried to culture isolated epiblasts without any extra-embryonic tissue at an early-streak stage (6.5-a dpc) on a feeder layer of STO cells known to support proliferation of 8.5 dpc PGCs in primary culture (Donovan et al, 1986). As shown in Fig. 3A, AP-positive cells crawling out from an explant were observed. These AP-positive cells were also positive for 4C9 (Fig. 3B,C) and Oct3/4 (Fig. 3D,E,
arrested cell proliferation that may be necessary for subsequent PGC differentiation. These observations suggest that the feeder layer may provide a critical function independent of the support of a feeder layer and that epiblast tissues, including hind gut endoderm, in which PGCs normally give rise to neuroectoderm, have the ability to differentiate into PGCs, but that the proximal localization of tissues is required (Tam and Zhou, 1996). Our result also showed that earlier differentiated tissues, including hind gut endoderm, along with a morphogenetic expansion of embryonic tissues. Therefore, one possibility is that tissues, including hind gut endoderm, in which PGCs differentiate to become migratory, do not develop properly from fragmented epiblasts either on a feeder layer of Sl/Sl4 or under dissociated culture conditions.

We initially used a feeder layer for culturing isolated epiblast, which raised the question of whether feeder cells provide factors that support the emergence of PGCs. To clarify this point, we cultured epiblast cells on fibronectin (FN)-coated plates (Table 1). We found PGC differentiation on FN was of a similar frequency to that on a feeder layer of STO cells when epiblasts at 6.25 dpc onwards were cultured. The same result was also obtained by culturing epiblast on biologically non-active poly-D-lysine-coated plates (data not shown). These observations indicate that the emergence of PGCs from isolated epiblasts at 6.25 dpc onwards is independent of the support of a feeder layer and that epiblast can establish all conditions required for PGC formation by themselves. In contrast, PGC differentiation from 6.0 dpc epiblast appears to depend on the presence of a feeder layer. We also found that the explants always formed packed clusters of cells when PGCs emerge (Fig. 5A, B and data not shown). These observations suggest that the feeder layer may provide external signals that could help 6.0 dpc epiblasts to maintain the three-dimensional tissue organization and/or simply the cell proliferation that may be necessary for subsequent PGC differentiation.

Our results also indicated that Steel factor, which is known to be essential for PGC growth and survival (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991), is not necessary for PGC determination because a feeder layer of Sl/Sl4 cells lacking the Steel factor gene also supported PGC formation in culture (Table 2B). Although the frequencies of explants with PGCs on Sl/Sl4 and on STO feeder layers were similar, the number of PGCs on STO cells was more than twice that on Sl/Sl4 cells (Tables 1, 2). In culture, Steel factor may support the growth of newly formed PGCs. This observation is consistent with the phenotype of Sl or W mutant mice, which are deficient in Steel factor or its receptor gene c-kit, respectively (Williams et al., 1992). In these mutant mice, a normal number of PGCs is observed in early embryos, but these PGCs fail to increase in number (Mintz and Russell, 1957).

We observed PGC differentiation from proximal fragments of epiblasts after 6.0 dpc, but distal fragments never gave rise to PGCs (Table 2A), suggesting that cell populations in proximal and distal regions have distinct character with regard to PGC formation. In other words, the specific local cues or competence required for differentiation into PGCs may be established within the proximal region. In addition, the differentiation of PGCs was not observed from trypsinized epiblasts. PGCs arose from a small subfragment consisting of a specific number of cells or of the two most proximal rows of epiblast cells (Table 2B and data not shown). The minimum number of cells required was found to be 40 and 20 at 6.0 and 6.25 dpc, respectively. Taken together, these observations suggest that presumptive local cues within proximal epiblast are maintained among a small but specific number of cells.

We have shown that the functions of the extra-embryonic ectoderm are essential for PGC determination, and a study by Lawson et al. has demonstrated that BMP4 expressed in the extra-embryonic ectoderm may play a key role (Lawson et al., 1999). A 5.5 dpc epiblast required extra-embryonic ectoderm to form PGCs, while isolated proximal epiblasts without extra-embryonic ectoderm at 6.0 dpc did give rise to PGCs (Tables 1, 2 and 4). These results indicate that the functions of the extra-embryonic ectoderm before 6.0 dpc are critical for establishing putative local cues and subsequent PGC formation, and that BMP4 could be involved in this process. BMP4 expression in the extra-embryonic ectoderm is, however, observed even at later stages (Lawson et al., 1999), suggesting that it has additional and/or alternative functions for PGC formation at later stages. Consistent with this hypothesis, we found that extra-embryonic ectoderm at 6.5-b and 6.75 dpc is fully active in inducing PGCs (Table 4). The extra-embryonic ectoderm stimulates the growth of epiblast in culture (Fig. 6 and data not shown), which might consequently support differentiation of the putative progenitors of PGCs in the proximal region at later stages.

Previous experiments involving heterotopic transplantation of epiblast cells have indicated that even distal epiblast cells, which normally gave rise to neuroectoderm, have the ability to differentiate into PGCs, but that the proximal localization of cells is required (Tam and Zhou, 1996). Our result also showed that isolated distal epiblast fails to give rise to PGCs, but in the presence of extra-embryonic ectoderm, PGCs are formed (Table 4). Together these observations suggest that the proximal localization of cells is necessary for receiving signals from extra-embryonic ectoderm, which may induce putative local competence or cues within a population of totipotent
epiblast cells. The recombination experiment also indicated that distal epiblast after the onset of gastrulation (6.75 dpc) still maintains the ability to respond to extra-embryonic ectoderm and to give rise to PGCs (Table 4, Fig. 6) suggesting a remarkable plasticity of gastrulating epiblast cells with regard to the restriction of cell lineage, including germ line lineage.

Snow found that fragments of different parts of epiblast at 7.0 dpc follow the same fates in culture as in vivo, and that cells differentiate autonomously into PGCs as well as into other cell lineages, including mesoderm (Snow, 1981). In contrast, pre- and early-streak epiblasts failed to express mesoderm markers such as Brachyury, Flk-1, and HNF-3β in our culture (Fig. 4). The development of cells in 7.0 dpc epiblast may progress with regard to the lineage restriction of somatic cells, which may explain why they can follow various somatic cell fates, even in culture. In contrast, the culture of pre- or early-streak epiblast may not be able to fully reproduce gastrulation, which may result in a failure to express mesodermal markers in culture. In particular, recent studies have implicated Brachyury (Wilson et al., 1993; Wilson et al., 1995) and FGF (Ciruna et al., 1997; Sun et al., 1999) signaling in the control of migration of epiblast cells through the primitive streak. If this is so then the failure to express Brachyury further suggests that gastrulation does not occur in our explant culture. Even if gastrulation did not occur properly, however, PGCs did emerge in our culture, which is consistent with observations of the emergence of AP-positive cells in eed mutant embryos that fail to undergo gastrulation (Faust et al., 1995). Taken together, these observations suggest that PGC differentiation need not be accompanied by gastrulation. The expression of Flk-1 should also be noted. Flk-1 is normally expressed over the nascent extra-embryonic mesoderm area after passing through the primitive streak (Katoaka et al., 1997). Based on fate-map analysis, PGCs and Flk-1-expressing mesoderm cells should be derived from common precursors (Lawson and Hage, 1994). Therefore, if the differentiation of PGCs and mesoderm cells is concomitantly regulated, Flk-1 must always be expressed when PGCs emerge in culture. This is not, however, the case.

Our explant culture described here provided novel approaches to studying the mechanisms of mouse germline formation. This culture system may also be useful for identifying and/or analyzing the functions of molecules involved in germline formation in future experiments.

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