In vitro analysis of epiblast tissue potency for hematopoietic cell differentiation

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SUMMARY

In murine embryogenesis, all cells that will constitute the embryonic structures originate from the epiblast (primitive ectoderm) tissue, the epithelial cell sheet of the gastrulating embryo. The cells of this tissue are totipotent at the beginning of gastrulation, but at the end of this period are specified to particular cell lineages. Thus, it is likely that during murine gastrulation, the potency of epiblast cells that were originally totipotent becomes restricted as development progresses. However, the mechanisms of this process are unknown.

We have investigated this process in vitro, focusing on the hematopoietic cell lineage. To detect the hematogenic potency of the epiblast tissue, we established an in vitro culture system in which the hematopoietic cell differentiation of the epiblast tissue was supported by a stromal cell layer. With this culture system, we investigated the process by which this potency becomes spatially and temporally restricted during gastrulation. The results showed that hematogenic potency resides in the entire epiblast of the early- to mid-gastrulating embryo, but becomes restricted to the posterior half of the epiblast at the headfold stage. Furthermore, we showed that this process is altered by exogenous bone morphogenetic protein-4 (BMP-4) or activin A, which may be mesoderm inducers in Xenopus embryogenesis.

Key words: mouse embryo, epiblast tissue, analysis of potency, hematopoietic cell differentiation, in vitro culture, stromal cell line OP9

INTRODUCTION

During gastrulation, the simple pre-gastrulating embryo is organized into a complex structure consisting of three germ layers. In the mouse, the epiblast tissue, the epithelial cell sheet of the gastrulating embryo, is supposed to be the source of all future embryonic structures (Fig. 1A,B; Lawson et al., 1991; Tam, 1989; Lawson and Pedersen, 1992; Tam and Beddington, 1992; reviewed by Tam et al., 1993). Through proliferation and dynamic cell rearrangement, all three germ layers are generated from this tissue during gastrulation. Cells of the epiblast tissue are totipotent (Svajger et al., 1981; Beddington, 1983). In in vivo studies, when transplanted to ectopic sites such as under the renal or testicular capsules, this tissue developed into benign teratomas composed of multilineage structures. Usually, the tissue potencies of the individual parts of the epiblast were not restricted to their presumptive fates.

Embryogenesis is a process in which the differentiation potency of the originally totipotent cells becomes restricted according to their spatio-temporal position. It is likely that the epiblast tissue loses its potency as development proceeds. In this study, we investigated the process by which the epiblast tissue potency becomes spatially-temporally restricted during gastrulation.

We studied the potency of epiblast tissue for hematopoietic cell differentiation (hematogenic potency), for the following reasons. Firstly, mature hematopoietic cells originate as a derivative of mesodermal tissue within 48 hours of the onset of gastrulation. Secondly, a number of cell-surface markers are available for defining various stages of hematopoietic cell differentiation. Thirdly, various feeder cell layers that support hematopoietic cell growth and differentiation are available. Among them, the stromal cell line OP9, which was established from the newborn calvaria of the M-CSF deficient (C57BL/6xC3H) F2-op/op mouse, reportedly supports hematopoietic cell differentiation from an embryonic stem cell line (Nakano et al., 1994). This suggested that stromal cell lines could provide an environment that supports the hematogenic potency of epiblast tissue, and this could be effective for in vitro analysis.
To investigate the hematogenic potency of epiblast tissue in vitro, we established culture conditions in which the mouse epiblast tissue could differentiate into mature hematopoietic cells supported by a stromal OP9 cell layer. With this in vitro culture system, we investigated the regionalization of the hematogenic potency of epiblast tissue. In addition, we investigated whether or not this potency distribution is influenced by exogenous BMP-4 or activin A, both of which alter the developmental fate of mesoderm in Xenopus (reviewed by Slack, 1994). The results show that the hematogenic potency of epiblast tissue is lost earlier in the anterior region and that this process can be affected by BMP-4 and activin A.

MATERIALS AND METHODS

Isolation of epiblasts
ICR mice were purchased from Shimizu Co. (Japan). The midpoint of the dark cycle was considered the beginning of gestation. We dissected the females at 6.7-6.75 days post coitum (d.p.c.) to obtain pre-streak and early streak stage embryos, and at 7.7 d.p.c. to obtain allantoic-bud and headfold stage embryos.

The decidua were removed and embryos were dissected in α-minimum essential medium (α-MEM; Gibco) containing 5% calf serum. Reichert’s membrane and the extra-embryonic portion were removed with a tungsten needle. Germ layers were separated as described by Beddington (1994), except that we used Dispase (Boehringer Mannheim, grade II) instead of pancreatin/trypsin. Briefly, embryonic portions were cut off using tungsten needles and subsequently incubated in Dispase for 1 to 2 minutes at room temperature. Thereafter, the embryos were separated into germ layers by gentle pipetting two or three times.

Coculture of explants with OP9
The stromal cell line OP9 was maintained in α-MEM containing 20% calf serum. Explants were cultured in α-MEM containing 10% FBS (fetal bovine serum) and 5×10⁻⁵ M 2-ME (2-mercaptoethanol). Dissected explants were transferred onto confluent OP9 in 96-well plates or fibronectin-coated 96-well plates. The plates were coated with fibronectin (20 μg/ml) in PBS for 2 hours at 37°C.

The cultured explants were dissociated by incubation with 0.05% trypsin/EDTA for 3 minutes at 37°C, suspended in α-MEM supplemented with 10% FBS and 2-ME, and pipetted vigorously to make single-cell suspensions. These were seeded onto confluent OP9 cell layers in 24-well plates. Round hematopoietic cell colonies that eventually filled with epithelial structures and beating myocardium change morphologically after 3 days and the culture was eventually filled with epithelial structures and beating myocardium (Fig. 3B). However, hematopoietic cells were not detected either morphologically or immunohistochemically under these conditions.

RESULTS

Culture conditions required for hematopoietic cell production from epiblast
To develop an in vitro culture system that could detect the hematogenic potency of epiblast tissue, we established optimal conditions in which the hematopoietic cell differentiation of the epiblast tissue was supported by a stromal cell layer. We cultured explants of gastrulating mouse epiblasts with the stromal cell line, OP9. We placed the 6.7 d.p.c. epiblast explants directly on the confluent OP9 monolayer (Fig. 2A). Explants attached to the surface of OP9 and formed a monolayer of epithelial cells that was clearly distinct from the OP9 monolayer (Fig. 3A). The epiblast cell sheet started to change morphologically after 3 days and the culture was eventually filled with epithelial structures and beating myocardium (Fig. 3B).

Analysis of hematopoietic lineage marker and MHC class I antigen expressions by flow cytometry
The expression of hematopoietic lineage markers was investigated by two-color flow cytometry using a biotinylated or fluorescein isothiocyanate (FITC)-conjugated mAb against c-Kit (hybridoma ACK-4; Ogawa et al., 1991), B220 (hybridoma 6B2; Sander et al., 1988), TER119 (erythroid lineage-specific antigen; Ikuta et al., 1990) and Ogawa et al., 1991), B220 (hybridoma 6B2; Sander et al., 1988), TER119 (erythroid lineage-specific antigen; Ikuta et al., 1990) and cyanate (FITC)-conjugated mAb against c-Kit (hybridoma ACK-4; Sander et al., 1988)

To analyze MHC class I expression, we cultured the explants isolated from 7.7 d.p.c. (BALB/cJ[C3H/An]) F1 embryos. Because the developmental stage appeared to be 1 day behind that of the ICR strain, we dissected pregnant BALB/c females around 7.7 days of gestation to obtain pre- and early streak stage embryos. MHC class I expression was analyzed using an FITC-conjugated mAb against H-2D (hybridoma 34-5-8S; Ozato et al., 1982) and biotinylated mAb against H-2K (hybridoma 11-4.1; Oi et al., 1978).

Fig. 1. Schematic diagram of cell lineage and fate map of early mouse development. (A) Schematic diagram of cell lineage in early mouse embryo, based on a review by Nakatsuji (1992). (B) Fate map of the pre-streak/early streak (based on the work of Lawson and colleagues, 1991, 1992) and late streak stage mouse embryo (based on the work of Tam, 1989). ICM, inner cell mass; PS, pre-streak; ES, early streak; LS, late streak; fb, forebrain; hb, hindbrain; mb, midbrain; ne, neuroectoderm; ps, primitive streak; se, surface ectoderm; sp, spinal cord; x.meso, extra-embryonic mesoderm.
formed in B and D, but not in A and C.

Fig. 2. Coculture of epiblast cells with OP9. Colonies of round cells attached to fibronectin and showed an epithelial morphology characteristic of epithelial cells, in contrast to mesoderm, which had a fibroblast-like cell morphology similar to OP9 (Burdsal et al., 1993). These culture conditions were therefore used for further analyses.

We analyzed the expression of hematopoietic cell-specific markers on round cells generated under the stroma cell-free conditions. By passaging onto fresh OP9, we obtained enough cells to perform flow cytometry. Expression of c-Kit (Ogawa et al., 1991), TER119 (Ikuta et al., 1990), Mac-1 (Springer et al., 1978) and B220 (Sander et al., 1988) were used to identify the undifferentiated hematopoietic stem cells, erythrocytes, myeloid cells and lymphocytes, respectively. At day 10 from the onset of the culture, the TER119(+) population predominated (Fig. 4A), while c-Kit(+) and Mac-1(+) populations were also detected. Upon further incubation, the TER119(+) and the Mac-1(+) populations decreased and the B220(+) population increased (Fig. 4B). Thus, while there was a difference in the time schedules of generation of each cell lineage, all three cell lineages were eventually generated in this culture. The histology of the cells harvested from day-16 cultures demonstrated the presence of mature mast cells, macrophages, erythroblasts and lymphocytes (Fig. 4C). All the erythroid cells were of the nucleated cell type, and by RT-PCR (reverse-transcriptase-polymerase chain reaction) analysis, day-10 culture cells were detected to express fetal β-globin, but not the adult β-globin (data not shown). This suggested that primitive hematopoiesis was supported under these conditions.

Finally, to exclude the possibility that the hematopoietic cells in the culture had been derived from contaminating maternal blood cells, we investigated the expression of MHC class I by the round cells generated from the explants of (BALB/c x C3H/HeJ) F1 embryos dissected from BALB/c pregnant mice. The H-2 antigen is H-2 d/d in BALB/c and H-2 k/k in C3H/HeJ. On day 8, we detected an H-2 d/k population (34.8%) (54.5%) (78.1%) (100%) of fragments producing blood cells/number of explants (%).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Anterior</th>
<th>Distal</th>
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<td>Late headfold</td>
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<td>(0.0%)</td>
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Table 1. Frequency of blood cell formation from epiblast fragments

It was likely that under these conditions, the prolonged cell-cell contact between the epiblast cells would promote the growth of other cell lineages, making it difficult to detect the hematopoietic cells. Alternatively, the hematopoietic cell differentiation of the epiblast tissue might be inhibited. Despite this, it was possible that the dissociation of the epiblast tissue during the process would be effective. To investigate this possibility, we trypsinized the epiblast and OP9 cells after 2 days in culture, before other lineages started to predominate, and reseeded a single cell suspension onto a fresh OP9 layer (Fig. 2B). Colonies of round cells appeared on the OP9 monolayer immediately after reseeding. Histologically, these cells were of the hematopoietic lineage. Thus, it appeared that epiblast fragments needed to be dissociated to form colonies. However, when we dissociated the freshly dissected explants and cultured them directly on OP9 monolayer (Fig. 2C), colonies did not form. This suggested that the explants needed to be cultured initially as intact epiblasts.

We tested whether OP9 stromal cells are required for this initial 2-day process. Because epiblast explants could not survive on non-coated culture dishes, we substituted fibronectin for OP9 during this period. In 12 hours, epiblasts attached to fibronectin and showed an epithelial morphology (Fig. 3C). We trypsinized the whole culture at day 2 and reseeded onto OP9 monolayers (Fig. 2D). 1-2 days thereafter, round cell colonies formed (Fig. 3D). Histologically, the colonies appeared to consist of undifferentiated hematopoietic cells (Fig. 3E). There were no significant differences between the cultures with OP9 or fibronectin. Therefore OP9 was not essential for the initial process of epiblast tissue differentiation.

Culture without stroma cells defined the phase at which the stromal cell line was required. In addition, we confirmed that mesodermal cells did not contaminate the culture by checking the morphology of explants before seeding onto OP9. On fibronectin-coated dishes, the epiblast explants had the close-packed morphology characteristic of epithelial cells, in contrast to mesoderm, which had a fibroblast-like cell morphology similar to OP9 (Burdsal et al., 1993). These culture conditions were therefore used for further analyses.

Regional distribution of epiblast tissue potency for hematopoietic cell differentiation

To investigate the process by which the hematogenic potency of epiblast becomes spatially-temporally restricted during the gastrulation period, we used our culture system to analyse the hematogenic potency of various regions of the epiblast tissue. We dissected the anterior, distal and posterior fragments of epiblasts from various stage embryos between 6.7 and 7.7

![Diagram of epiblast differentiation](image1.png)

Fig. 3. Epiblast cells cultured initially as intact epiblasts. To investigate the process by which the hematogenic potency of various regions of the epiblast tissue might be inhibited. Despite this, it was possible that the dissociation of the epiblast tissue during the process would be effective. To investigate this possibility, we trypsinized the epiblast and OP9 cells after 2 days in culture, before other lineages started to predominate, and reseeded a single cell suspension onto a fresh OP9 layer (Fig. 2B). Colonies of round cells appeared on the OP9 monolayer immediately after reseeding. Histologically, these cells were of the hematopoietic lineage. Thus, it appeared that epiblast fragments needed to be dissociated to form colonies. However, when we dissociated the freshly dissected explants and cultured them directly on OP9 monolayer (Fig. 2C), colonies did not form. This suggested that the explants needed to be cultured initially as intact epiblasts.

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d.p.c. (Fig. 6A). Embryos were strictly staged (Downs and Davies, 1993) to select early streak (ES) stage embryos from 6.7 d.p.c. littermates, and early bud (EB)/late bud (LB) stage, early headfold (EHF) stage and late headfold (LHF) stage embryos from 7.6 to 7.7 d.p.c. littermates. Individual epiblast fragments from each embryo were cultured in wells of fibronectin-coated dishes. At day 2 of culture, they were dissociated and reseeded onto OP9 monolayers. We counted the number of wells that produced round cell colonies as positive in terms of hematogenic potency.

Fig. 6B and Table 1 show the distribution of the hematogenic potency. In the ES stage embryo, hematopoietic cells were produced at high frequency from the posterior epiblast (81.1%), which is supposed to be a source of future mesoderm. In addition, colonies were induced from the anterior (50.0%) and distal (15.8%) portions. Although the fate map analysis suggested that the progenitor mesodermal cells of hematopoietic lineage have already migrated to the extra-embryonic portion at the LB stage (Lawson et al., 1991), we detected colony formation from the posterior epiblast at high frequency (78.1%). Hematopoietic cell colonies were also induced from anterior (34.8%), presumptive ectoderm and distal (54.5%) regions, including the node. Even at the EHF stage, the potency persisted in the distal (23.1%) and posterior (33.3%) epiblast, whereas it was lost from the anterior headfold region (0.0%). These results suggested that the anterior epiblast had less hematogenic potency than the posterior epiblast, as the frequency of positive cells was lower in the anterior than in the posterior epiblast (Table 1). In addition, at the ES stage, there were more round cell colonies in the positive wells, and they appeared 1 day earlier (at day 3 from the onset of the culture) in the posterior than in anterior fragments (data not shown). Thus, our culture system detected regional differences in hematogenic potency.

Fig. 3. Appearance of epiblast cell layer and colonies of round hematopoietic cells. (A,B) Epiblast cells from an early streak stage embryo resembled an epithelial cell sheet on OP9 monolayer at day 2 (A). After an additional incubation, the epiblast fragment frequently changed into one of several other lineages such as beating myocardium (B). (C) Epithelial appearance of epiblast fragments attached to a fibronectin-coated dish at day 2. (D) Colonies of round cells in day 4 of the stroma-cell free culture. (E) May-Grunwald-Giemsa-stained cytospin specimen of round cells in day-4 culture. Scale bar, 100 μm (A,C,D); 50 μm (B); 25 μm (E).

Fig. 4. Flow cytometry of hematopoietic lineage marker expression. (A,B) At days 10 (A) and 18 (B), the undifferentiated hematopoietic stem cell marker c-Kit, the erythroid lineage marker TER119, the myeloid lineage marker Mac-1, and the B-cell lineage marker B220, were expressed. (C) Cytospin specimen of cells from a day-16 culture stained with May-Grunwald-Giemsa. Scale bar, 25 μm.
The results showed hematogenic potency in the anterior and distal epiblast fragment at the ES stage, and also in later stage epiblasts. According to a fate map analysis, the future hematopoietic cell-fated region is localized to the posterior-proximal portion of the epiblast of the early gastrulating embryo (Lawson et al., 1991; Tam, 1989). However, our system also detected hematogenic potency in other regions. Thus, the findings showed that hematogenic potency is initially distributed throughout the epiblast, irrespective of the presumptive fate map.

Effects of exogenous BMP-4 and activin A on epiblast tissue differentiation in vitro

In Xenopus, a number of inducing factors influence the developmental fate of the mesoderm. For example, transforming growth factor-β (TGFβ)-family member proteins such as activin A and BMP-4 induce mesoderm with dorsoanterior and ventroposterior features, respectively (reviewed by Slack, 1994). Here, we investigated the effect of these compounds (0.5-16.0 ng/ml) on the differentiation of epiblast tissue in vitro. The anterior headfold region of EHF stage epiblast did not differentiate into hematopoietic cells in our system. The result showed that BMP-4 and activin A induced hematopoietic cells from the anterior headfold region, which otherwise could not produce hematopoietic cells (Table 2). While increasing the BMP-4 dose had no substantial effect, the frequency of positive wells increased by increasing the dose of activin A.

DISCUSSION

Culture system for analyzing hematogenic potency

We have established an in vitro culture system that can detect the process of hematogenic potency restriction in epiblast tissue.

Most of the methods previously reported where embryonic stem cells were induced to become cells of various lineages, including hematopoietic cells, in vitro (Doetschman et al., 1985; Wiles and Keller, 1991; reviewed by Wiles, 1993), depended on the formation of a complex structure designated embryoid body, in which derivatives of all three germ layers differentiated in a coordinated manner. In contrast, Nakano et al. (1994) described a stromal cell-dependent method in which embryonic stem cells can differentiate into hematopoietic cells without forming an embryoid body, when supported by a stromal cell layer.

Table 2. Promoting effect of exogenous BMP-4 and activin A on hematopoietic differentiation of epiblast

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Results of each independent experiment are shown.
In vitro hematopoiesis from the embryonic stem cell is a powerful system for addressing the mechanisms of early hematopoietic cell differentiation. However, the differentiation of embryonic stem cells proceeds in a disorganized manner. In contrast, actual embryogenesis is a process in which the differentiation of the cells is spatial-temporally organized. Hence, to investigate how the potency of the epiblast tissue is regulated during actual embryogenesis, the epiblast tissue should be examined. To do so, an environment was required that could support epiblast tissue differentiation, because epiblasts cannot produce hematopoietic cells in culture. We considered that stromal cell layers would provide such a supportive environment. In this study, we used a stromal cell line OP9 because it supports the hematopoietic differentiation of embryonic stem cells, according to Nakano et al. (1994). Epiblast tissue is a descendant of the inner cell mass of the blastocyst, the source of the embryonic stem cell. We thought it likely that OP9 could also provide a supportive environment for the differentiation of epiblast tissue.

However, the results showed that the epiblast tissue never gave rise to hematopoietic cells when the tissue explants were simply placed on OP9, indicating that OP9 does not provide all the signals required for differentiation. We showed that the epiblast tissue needs to be dissociated to differentiate into hematopoietic cells during culture on OP9. In addition, the hematopoietic cells were not produced when the explants were dissociated immediately after dissection, but were efficiently produced when dissociated after culture for 2 days without OP9. This suggests that the initial phase of differentiation proceeds autonomously by cell-cell interaction within the epiblast cell layer. Thus, it is likely that the process of hematopoietic cell differentiation needs not only the support by stromal cell line OP9, but also the intrinsic differentiative capacity of the epiblast tissue. The results suggested that the initial phase of differentiation proceeds without OP9, but that further differentiation requires the support of OP9. Hence, the role of OP9 in this process may be to support the further differentiation of the cells after they have differentiated intrinsically within the epiblast tissue. In normal embryogenesis, the epiblast tissue is surrounded by the primitive endoderm (hypoblast). However, no hematopoietic cells were generated from 12 explants that were cultured without removing the primitive endoderm. In addition, when these explants were seeded on the OP9 after trypsinization at day 2 of culture, four out of five explants gave rise to hematopoietic cells. This suggested that the primitive endoderm could neither substitute for the OP9 layer, nor inhibit the hematopoietic differentiation of epiblast.

Our results suggested that the hematopoietic cell differentiation of the epiblast tissue might be inhibited by prolonged cell-cell contact. When we cultured the epiblast fragment continuously without dissociation, other cell lineages such as myocardial or epithelial were predominantly produced from the epiblast tissue, but hematopoietic cell production was not detected. Several reports have suggested a significant role for cell-cell interaction in the differentiation of various mesodermal lineages. In *Xenopus*, myoblast differentiation is dependent on cell-cell interaction between the induced mesodermal cells, and the phenomenon is called the ‘community effect’ (Gurdon et al., 1993; Cossu et al., 1995). It remains obscure as to whether or not a similar ‘community effect’ is also involved in mice, but our study suggested that an inducing signal from neighboring epiblast cells is involved in the differentiation process.

Aside from OP9, we also tried several other stromal cell lines, such as ST2 and PA6, but none of them were effective (data not shown). On the other hand, dissociated extra-embryonic tissue produces hematopoietic cell colonies on either ST2 (Ogawa et al., 1988) or PA6 (M. Kanatsu et al., unpublished observation) stromal cell lines. This suggests that the extra-embryonic mesoderm, that had become specified to the hematopoietic cell lineage, could form colonies on these cell lines from a single cell suspension. Thus, some inductive signal peculiar to OP9 appears to be required in the differentiation process between epiblast cells and the extra-embryonic mesoderm. The stromal cell line OP9 was established from an osteopetrosis op/op mouse that lacks functional M-CSF because of a mutation in the M-CSF gene (Yoshida et al., 1990). Nakano et al. (1994) have suggested that M-CSF inhibits early hematopoietic cell differentiation. However, hematopoietic cells were produced neither from the epiblast (M. Kanatsu et al., unpublished observation), nor from the embryonic stem cell line (T. Nakano, personal communication) on PA6 or ST2 stromal cell lines, even in the presence of a saturating amount of antagonistic mAb AFS98 (Sudo et al., 1995) against murine c-fms, the receptor for M-CSF. Thus, M-CSF may not be the sole factor that determines the abilities of these stromal cell lines. Nonetheless, a comparison between various stromal cell lines will be useful for elucidating the molecular requirements for hematopoietic cell differentiation.

**Potency of epiblast tissue analyzed in vitro**

Analyses of histogenetic potency of epiblast tissue have used tissue transplantation into ectopic sites, such as under the renal or testicular capsules (Svajger et al., 1981; Beddington, 1983). The environment of these ectopic sites appears to be permissive for inducing the differentiation of a full range of cell lineages from the totipotent epiblast tissue (reviewed by Tam, 1993), but it is difficult to dissect or control the differentiation process in vivo. On the other hand, although only a limited repertoire of cell lineages are inducible in vitro, this environment is advantageous for dissecting the process. Here, we have demonstrated that the histogenetic potency of the epiblast tissue can be efficiently analyzed in vitro.

Our results show that hematopoietic cells could be induced from any region of the epiblast tissue of the gastrulating embryo, including the presumptive ectoderm. Though the frequency of hematopoietic cell production was decreased along the developmental stages, the potency persisted in all regions of the late bud stage epiblast, and in the headfold stage everywhere except the anterior headfold region. This indicated that by this stage the potency of epiblast tissue was not yet restricted to a specific cell lineage, in contrast to the predictions of the prospective fate map, which shows that the future hematopoietic cell-fated region is localized in the posterior-proximal portion of the epiblast (Lawson et al., 1991). On the other hand, hematopoietic cells were not induced from the anterior epiblast of the headfold stage embryo. It is likely that the specification of the anterior epiblast to the ectoderm was completed around the moment of headfold formation.

However, exogenous BMP-4 and activin A altered the process of specification of the anterior headfold region that
otherwise does not give rise to hematopoietic cells. These factors play roles in the mesoderm induction of *Xenopus*. In particular, ventral-type mesoderm, such as blood cells, is induced from animal pole cells either by BMP-4 or by a low concentration of activin (Green et al., 1992; Graff et al., 1994). Moreover, Johansson and Wiles (1995) showed that the factors have a promoting effect on the hematopoietic differentiation of embryonic stem cells in the embryoid body. In agreement with these findings, our study showed that the anterior headfold region epiblast is reactive to these factors. In the mouse, BMP-4 expression is detected at low levels in the extra-embryonic region adjacent to the posterior epiblast of the gastrulating embryo (Jones et al., 1991). Null mutant mice, generated by targeted disruption of the BMP-4 gene, die between about 7.5 and 10.5 d.p.c. and show abnormalities in the extra-embryonic mesoderm of the yolk sac (Winnier et al., 1995), implicating BMP-4 in the differentiation of hematopoiesis. Our data also show that BMP-4 promoted the hematopoietic cell differentiation of the anterior headfold region, which otherwise has no capacity to do so. It could be that during normal embryogenesis, BMP-4 emanates from the extra-embryonic portion to act selectively on the posterior epiblast cells, although the anterior epiblast cells were also competent for induction by BMP-4. In contrast, the role of activin in embryogenesis remains unknown. Our results show that the epiblast tissue is reactive to activin A, and that its specification can be altered by the administration of this factor. However, the expression of inhibin-βA or inhibin-βB, the components of activin, are detected only in the decidua, the structure surrounding the embryo, and not in the gastrulating embryo itself (Albano et al., 1994). Furthermore, neither inhibin-βA- nor inhibin-βB-deficient mice show any defective phenotype around the gastrulation period (Matzuk et al., 1995). According to these reports, activin might not be involved in mesoderm induction in the mammalian embryo. Nevertheless, we cannot exclude the possibility that maternal activin acts as a mesoderm inducer in vivo.

It remains obscure how the specification state of the anterior headfold region is altered by these factors. It is possible that this region is in the process of specification to ectoderm, but is still reversible to mesoderm in the presence of potent inducers. Alternatively, this region might be composed of heterogeneous populations of specified and unspecified cells, of which in this study, hematopoietic cells were generated from the latter population by BMP-4 or activin A.

In conclusion, this study shows that the distribution of the hematogenic potency of epiblast tissue is quite different from the presumptive fate map, and that it is observed even in the prospective ectoderm region. Along the developmental stages, hematogenic potency was first lost from the anterior epiblast, but potent mesoderm inducers altered the process of specification. In this analysis, a stromal cell line provided an appropriate environment for the in vitro study of hematogenic potency of epiblast tissue. Our culture system was sensitive enough to detect the regional restriction of hematogenic potency of epiblasts along the developmental stages. Because the supporting environment provided by OP9 selectively enabled the detection of hematopoietic cell differentiation, we analyzed hematogenic potency. However, in the future, other cell lineages should be examined using different supporting environments. Stromal cell layers will also be useful for analyzing tissues derived from many mutant mice, which are so lethal early in the embryonic period that they are difficult to investigate. Further examinations with in vitro cultures will help in elucidating the process of hematopoietic cell differentiation, and also of mesoderm induction.

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