Primordial germ cells in the mouse embryo during gastrulation

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Summary

With the aid of a whole-mount technique, we have detected a small cluster of alkaline phosphatase (ALP)-positive cells in whole mounts of mid-primitive-streak-stage embryos, 7–7½ days post coitum (dpc). Within the cluster, about 8 cells contain a small cytoplasmic spot, intensely stained for ALP activity and possibly associated with an active Golgi complex. The cluster lies just posterior to the definitive primitive streak in the extraembryonic mesoderm, separated from the embryo by the amniotic fold. Towards the end of gastrulation, the number of cells containing the ALP-positive spot rises to between 50 and 80. Thereafter the number of cells in the extraembryonic cluster declines, and similar cells start to be seen in the mesoderm of the primitive streak and then in the endoderm. At 8 dpc, about 125 ALP-stained cells are found, mainly in the hindgut endoderm and also at the base of the allantois, their appearance and location at this stage agreeing closely with previous reports on primordial germ cells (PGCs). Embryos from which the cluster area has been removed at the 7-day stage are devoid of PGCs after culture for 48 h, whereas the excised tissue is rich in PGCs. We argue that the cells in the cluster are indeed primordial germ cells, at a stage significantly earlier than any reported previously. This would indicate that the PGC lineage in the mouse is set aside at least as early as 7 dpc, possibly as one of the first 'mesodermal' cell types to emerge, and that its differentiation, as expressed by ALP activity, is gradual.

Key words: primordial germ cells, mouse embryo, alkaline phosphatase staining.

Introduction

Chiquoine (1954) identified a population of cells in the posterior part of the mouse embryo 8½ days post coitum (dpc), subsequently to be found along the wall of the invaginating hind gut and in the genital ridges. These putative primordial germ cells were characterized by high activity of alkaline phosphatase (ALP; EC3.1.3.1). The number of such ALP-positive cells was found to be greatly reduced in two sterile mouse mutants known to lack germ cells in the gonads, White-spotting (Mintz and Russell, 1957) and Steel (McCoshen and McCallion, 1975).

Further evidence that these were indeed primordial germ cells came from light and electron microscope studies (Spiegelman and Bennett, 1973; Clark and Eddy, 1975). Ozdzenski (1967) was able to identify the ALP-positive cells as early as 8 dpc, at the base of the allantois, while Tam and Snow (1981) made counts from 8½ dpc (100 germ cells per embryo) to 13½ dpc (25 000 germ cells per embryo).

Attempts to identify primordial germ cells before 8 dpc were unsuccessful because the histochemical methods used either failed to detect any cells with high ALP activity, or gave too high a level of background staining. Snow (1981) adopted an alternative approach. By excising small pieces of 7- and 7½-day embryos, and culturing both the pieces and the donor embryos for 24–36 h, he was able to show that the progenitors of the ALP-positive cells were at that time located in a single small region in the posterior part of the embryo, i.e. in approximately the location in which they had first been identified.

All previous studies of ALP activity in mouse primordial germ cells have used sectioned material. With the aid of a whole-mount technique devised for studies of chick primordial germ cells (Ginsburg and Eyal-Giladi, 1986) and a modified method of detecting ALP activity, we have been able to identify germ cells in early 7-day mouse embryos, before the formation of the allantois and before the completion of the amniotic fold.

Materials and methods

Embryos were obtained on days 7 and 8 of gestation (day 0= day of finding a copulation plug) from Q-strain mice. 27 embryos were used for sectioning and 30 for whole-mount preparations. For sectioning, embryos were left in deciduae
but for whole-mount preparations they were dissected from the decidua and both Reichert's membrane and the ectoplacental cone were removed.

Intact deciduae and embryos were fixed in absolute ethanol:glacial acetic acid (7:1) at 4°C for 1 h, followed by two changes of absolute ethanol at 4°C, for 24 h each.

The deciduae containing the embryos were passed through two changes of chloroform at 4°C, 1 h each, and then were transferred to wax at 54°C. Serial transverse sections, thickness 7 μm, were stained to demonstrate ALP activity using the following staining solution freshly made up:

- Sodium α-naphthyl phosphate (Sigma) 5 mg
- 5% borax solution 5 ml
- Distilled water 44.6 ml
- 10% MgCl₂ solution 0.3 ml
- Diazonium salt (Fast Red TR salt) 25 mg

The pH of the staining solution was 9.2. Staining time was 15–30 min; the slides were checked at intervals for the development of the reaction. Coverslips were mounted with u.v.-inert aqueous mounting medium (Sigma). Whole mounts were cleared in xylene to make the tissue more transparent, hydrated and stained for ALP using the same protocol as for the sections. Whole mounts and sections were photographed using an Olympus BH-2 photomicroscope.

Control sections of day-9 and day-10 embryos, in which the distribution of ALP-positive germ cells is well-known, were stained in the same way to confirm that we were indeed staining for ALP.

Some early day-7 embryos were surgically manipulated in PB1 solution. The trophoblast and Reichert's membrane were removed as well as the ectoplacental cone. Tungsten needles were used to cut out a small piece of the posterior wall of the egg cylinder just above the amnion (Fig. 1). No attempt was made to separate the germ layers in these pieces. The operated embryo and the piece isolated from it were cultured simultaneously under liquid paraffin in drops of about 50 μl of Dulbecco's modified Eagle's medium supplemented with 50% inactivated rat serum, glutamine (4 mM), penicillin and streptomycin, in tissue-culture grade 5 ml plastic Petri dishes. The cultures were kept at 37°C under 5% CO₂ for 48 h, followed by fixation and staining for ALP activity as described above.

Results

(A) 7-day embryos

(1) Developmental criteria

Heterogeneity in respect to developmental stage was particularly marked during the early part of day 7, even among embryos belonging to the same litter. Rather than using the time of day as a reference for our analysis, we established developmental criteria for defining the various stages at which the spatial and temporal pattern of ALP activity was to be assessed. Many of these criteria (though not all) can be identified in whole egg cylinders: this enables staging to be done using the dissecting microscope.

Four developmental stages were distinguished (Fig. 2).

7-I (mid primitive streak: 7 day 1 h according to Green, 1941). A prominent posterior amniotic fold bulges into the proamniotic cavity. The area between the ectoderm and endoderm in that fold is filled with extraembryonic
mesoderm. A small exocoelomic cavity appears in the mesoderm at the dorsal side of the fold and elongates lateroanteriorly. No allantois has yet been formed.

7-II (full primitive streak: 7 day 6 h according to Green, 1941). The amniotic folds have met and the embryonic part of the egg cylinder is separated from the extraembryonic part by the thin amnion. The exocoelomic cavity has enlarged, but mainly at the posterior side. The anterior end of the chorion is therefore still situated very close to the amnion. No allantois has yet been formed.

7-III. Owing to the enlargement of the exocoelom, the anterior side of the chorion has been pushed further from the embryo, towards the ectoplacental cone. A small allantois, 50–70 μm in length, is present. A neural plate can be seen in the anterior part of the embryo.

7-IV (7½ days according to Green, 1941). The embryo has progressed in organogenesis, with well-developed neural folds and a neural groove in the anterior part. The allantois is larger.

(2) ALP activity in early embryos
When whole mounts of late 6-day embryos were stained for ALP activity, a faint reaction was seen in the epiblast, mainly in the area adjacent to the extraembryonic ectoderm, and in sections a stronger reaction was present in the interior third of the decidua, i.e. that immediately surrounding the embryonic sac. No localized reaction could be detected (data not shown). Stage 7-1 embryos processed in a similar way showed a weak localized reaction, which appeared as a faint area situated at the posterior amniotic fold close to the endoderm (Fig. 3A). The positive reaction was confirmed in serial transverse sections (Fig. 4). The

Fig. 3. (A) Stage 7-I embryo stained as whole mount for ALP activity. (B) Stage 7-II embryo stained as whole mount for ALP activity. af, amniotic fold; a, amnion; ec, exocoelom; APc, ALP-positive cells. Bar, 0.1 mm.

Fig. 4. Six consecutive transverse sections of the area of the amniotic fold of a stage 7-1 embryo, stained for ALP activity. A is 10–15 μm posterior to the primitive streak and the subsequent sections are serially more extraembryonic. In B, C, D and E, the section passes through the two distally projecting lateral diverticula of the exocoelom. en, endoderm; ec, exocoelom; ee, extraembryonic ectoderm; APc, ALP-positive cells; em, extraembryonic mesoderm; pc, proamniotic cavity. Bar, 50 μm.
staining began to appear in the second or third section (i.e. 10–15 μm) posterior to the primitive streak in the bulge of extraembryonic mesoderm, and was confined to a closely packed cluster of cells. The cluster was situated between the endoderm and the ventral area of the ectoderm fold; it was separated from the mesoderm dorsally and laterally by the cavity of the exocoelom. The cluster had a diameter of about 50 μm. Except for the weak ALP-positive reaction, the cells could not be distinguished from the other mesoderm cells in the vicinity. All other embryonic tissues were ALP-negative but there was the usual strong reaction in decidual cells, and some activity in the extraembryonic ectoderm.

Of the six stage 7-I embryos that were serially sectioned and stained for ALP, one showed no activity in the mesoderm, although the extraembryonic ectoderm and the decidual cells were positive. In another, the ALP reaction was homogeneously expressed in all the clustered cells, while in the other four embryos a stronger reaction was seen, in the form of a few coarse spots inside some of the cells within the cluster.

At stage 7-II, the embryonic part of the egg cylinder is separated from the extraembryonic part by the amnion. The ALP-positive cluster of cells was clearly located above the amnion (Fig. 3B). In whole-mount embryos, the cluster was located opposite the point at which the amnion and chorion are closely approximated on the anterior side. All other embryonic tissues were ALP-negative. The extraembryonic ectoderm is positive, which assists staging of whole-mount embryos. The apparent faint reaction in the epiblast is an artefact due to the optical thickness of the whole mounts. These observations were confirmed in serial sections. Dorsal to the posterior side of the amnion, the exocoelom occupies most of the space except for a bulge of mesoderm cells attached to the visceral endoderm. Embedded in this bulge and separated from the exocoelom by several layers of mesoderm cells, the ALP-positive cluster was very prominent (Fig. 5), showing stronger ALP activity than in the previous stage. There were also more cells showing an ALP-positive spot, mainly those cells that had separated from the cluster at its periphery. The spots were coarse but quite distinct. Not more than 50 cells per embryo contained spots (see Table 1). It proved impossible to make any accurate counts of the ALP-positive cells without spots, but rough estimation suggested that they made up about half the total number of cells in the cluster.

At stage 7-III, the location of the ALP-positive cluster was unchanged. The small allantois appeared to be growing from the extraembryonic mesoderm that surrounded the cluster, leaving it situated in a pocket-like structure (Fig. 6). The cluster was still rather compact but more cells with ALP-positive spots were seen isolated on the periphery. For the first time, a few cells (not more than 4–6) with spots were observed in the mesoderm of the primitive streak area. At the same time, there was lateral and distal distribution of cells away from the cluster. The number of cells that

<table>
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<th>Stage</th>
<th>No. of embryos</th>
<th>Mean number of cells containing spots (range)</th>
</tr>
</thead>
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<tr>
<td>7-I</td>
<td>6</td>
<td>8.0 (0–25)</td>
</tr>
<tr>
<td>7-II</td>
<td>7</td>
<td>35.9 (27–49)</td>
</tr>
<tr>
<td>7-III</td>
<td>6</td>
<td>54.2 (43–70)</td>
</tr>
<tr>
<td>7-IV</td>
<td>4</td>
<td>78.5 (61–88)</td>
</tr>
<tr>
<td>early 8</td>
<td>4</td>
<td>124.3 (93–193)</td>
</tr>
</tbody>
</table>

Fig. 5. 4 consecutive transverse sections stained for ALP activity, showing the posterior wall of a stage 7-II embryo on the extraembryonic side of the amnion. D is further from the embryo than A. ve, visceral endoderm; em, extraembryonic mesoderm; ec, exocoelom; APc, ALP-positive cells. Bar, 50 μm.
Fig. 7. (A) Stage 7-IV embryo stained as whole-mount for ALP activity. (B) Higher power view of the posterior part of the embryo shown in A. The ALP-positive cells are now more widely dispersed. a, amnion; al, allantois; ec, exocoelom; APc, ALP-positive cells. Bars, 0.1 mm.
PGCs in the early mouse embryo

525

Fig. 6. 2. consecutive transverse sections stained for ALP activity, at the base of the allantois of a stage 7-III embryo, showing the pocket-like structure at the base of the allantois. B is further from the embryo than A. ve, visceral endoderm; em, extraembryonic mesoderm; ec, exocoelom; APc, ALP-positive cells; al, allantois. Bar, 50 µm.

contained spots had increased somewhat (Table 1), but there were still some ALP-positive cells in the cluster that lacked spots.

The whole-mounts corresponding to stage 7-IV were almost twice the size of those at stage 7-II. The cells with ALP-positive spots were seen dispersed at the base of the allantois and in the posterior area of the embryo (Fig. 7A,B). Serial sections still showed a concentration of ALP-positive cells in a pocket-like mesodermal structure at the base of the allantois. Most of the cells now contained a very well defined ALP spot, although some positive cells still appeared spotless. Of the 80–85 cells per embryo that contained a spot (Table 1), most were located in the extraembryonic and posterior embryonic mesoderm. Some were seen between the mesoderm and the endoderm (Fig. 8A,B), and a few were located within the endoderm.

(B) Early 8-day embryos

Although both the location and number of PGCs on day 8 have been documented by previous workers, we examined some embryos at this stage, both as an additional control for the specificity of our staining procedure and to follow the pattern of ALP staining into the period of somitogenesis.

In the whole-mount staining of presomite to 4-somite embryos, a dense population of ALP-positive cells was demonstrated in the caudal part of the body and the base of the allantois (Fig. 9A). The ALP activity was very strongly expressed as a spot inside all the cells. While the population seemed to be concentrated at the caudal tip of the embryo, individual cells and aggregates were found more anteriorly, leaving behind isolated cells still embedded in the allantois.

Because the ALP activity was stronger at this stage than on day 7, and the ALP-positive spot was very distinct, it was possible to establish on sectioned material that the spot was located in the cytoplasm (Fig. 9C). Of the 100–200 cells per embryo counted on serial sections (Table 1), about a quarter were in the mesoderm, and the rest were in the endoderm of the hindgut (Table 2). Stray ALP-positive cells were very frequently found in pairs, suggesting that they are the products of a cell division and therefore that these presumptive PGCs are mitotically active at this stage.

(C) Isolation of the posterior piece from early 7-day embryos

Embryos at stages 7-II and 7-III were used for this

Fig. 8. (A,B) 2 consecutive transverse sections, at the base of the allantois of a stage 7-IV embryo, stained for ALP activity. Cells with an ALP-rich spot are shown leaving the cluster laterally, and towards the visceral endoderm. (B is further from the embryo than A). al, allantois; ec, exocoelom; APc, ALP-positive cells; em, extraembryonic mesoderm; ve, visceral endoderm. Bar, 50 µm.
Fig. 9. (A) Caudal part of early 8 dpc embryo stained for ALP activity as a whole-mount. (B) A posterior extraembryonic piece dissected from a stage 7-II embryo and cultured for 48 h, stained for ALP activity as a whole mount. (C) A section at the base of the allantois of an early 8-dpc embryo stained for ALP activity and counterstained with hematoxylin to demonstrate the cytoplasmic ALP-rich spots. al, allantois; APc, ALP-positive cells. Bars, 50 μm.

Table 2. Location of cells containing ALP-rich spots at different developmental stages

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of embryos</th>
<th>No. of cells counted</th>
<th>Extraembryonic mesoderm (%)</th>
<th>Visceral endoderm (%)</th>
<th>Embryonic mesoderm (%)</th>
<th>Hindgut endoderm (%)</th>
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<tr>
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<td>100</td>
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<td>8.1</td>
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<td>75.4</td>
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experiment. As it is often difficult to distinguish between these two stages in living embryos, some were defined as 7-II/7-III.

After 48 h of incubation, most of the operated embryos had a beating heart and a gut. No attempt was made to assess their precise degree of development. Of the eight operated embryos, six totally lacked ALP-positive cells in the caudal part. Of the remaining two embryos, both operated at stage 7-III, one contained 40 and the other 45 ALP-positive cells containing a spot. Some of these cells were in pairs.

Of the six excised pieces that survived to be stained at the end of the 48 h culture period, all were found to be packed with very strongly ALP-positive cells with spots. In some the cells were all clustered together; in others a few individual cells or pairs were distributed around the central cluster (Fig. 9B). All the pieces except one remained as an intact lump of tissue. In the one piece
that started to spread on the plastic plate, some of the ALP-positive cells had acquired an amoeboid form and failed to show a spot.

**Discussion**

Our observations on early 8-day embryos are in accord with those of previous investigators (Mintz and Russell, 1957; Ozdzenski, 1967; Tam and Snow, 1981). The cells with the ALP-positive spot that we detected in our whole-mounts and sections clearly represent the PGC population at this stage, since they are similar in number and location to those reported previously. We are therefore confident that the group of ALP-positive cells whose spatial and temporal pattern was followed in the 7-day embryos represents the same population of PGCs at an earlier stage of development.

The biological role of ALP in PGCs has not been defined, but it is not unique to germ cells (Johnson et al. 1977). The enzyme is active in embryos at all developmental stages, but different stages show characteristic changes in the multiple forms of the enzyme (Kim et al. 1989). We do not know why our staining procedure revealed such a distinct expression of ALP activity in the PGCs, the extraembryonic ectoderm and the decidual cells. Perhaps the combination of the acidic fixative and the very alkaline staining solution abolished non-specific reactions and/or detected only one form of ALP. Kim et al. (1989) report a sudden change in 7-day embryos, when the forms present earlier were replaced by a single form that persisted up to day 13 of gestation.

It is well established that the PGCs in mammals originate in the epiblast (Gardner, 1978; Falconer and Avery, 1978; McMahon, 1981). The appearance of ALP activity at the 7-day stage in our embryos is presumably indicative of a maturation process. At stage 7-I, the expression of ALP in the packed cluster of cells in the extraembryonic mesoderm of the posterior amniotic fold is very weak and seems to be dispersed throughout the cytoplasm. Individual cells, especially those on the periphery of the cluster, then start to express the enzyme activity in a restricted area of the cell. With time, more and more cells come to contain this spot, which changes from a coarse appearance at the earlier stages to become more defined later, and can be shown to be located in the cytoplasm. Jeon and Kennedy (1973) demonstrated by electron microscopy a localized ALP activity in the conspicuous Golgi complex of mouse PGCs between 84 and 11 dpc. Other cells in the treated tissues, and other intracellular membranes in the PGCs, showed no ALP activity. Golgi-type membranes forming a large spherical mass were also found in guinea pig oocytes (Anderson and Beam, 1960). In agreement with these observations, we detected ALP-rich spots in migratory PGCs at later stages and in germ cells in fetal gonads (unpublished data). However, we cannot exclude the possibility that the spots represent some kind of ‘nuage’-like material (Snow and Monk, 1983). It may be relevant that, in chick germ cells at early stages and during migration, certain lectins have been reported to bind to a perinuclear structure associated with the Golgi complex, giving a bright fluorescent spot (Didier, E., Didier, P., Fargeix, N., Guillot, J. and Thiery, J.-P., personal communication).

Early investigators suggested that PGCs originate within the yolk sac endoderm (Chiquoine, 1954; Mintz and Russell, 1957), but Ozdzenski (1967) indicated that they come from adjacent mesoderm and enter the endoderm secondarily. It has been reported that PGCs more nearly resemble mesoderm cells at the light (Spiegelman and Bennett, 1973) and electron microscope levels (Clark and Eddy, 1975). Our study demonstrates conclusively that the PGC cluster of the very early 7-day embryo is part of the extraembryonic mesoderm and only later in development do the cells move back to the embryo, to the mesoderm of the primitive streak and then to the endoderm (visceral and hindgut). Although no detailed study has been done concerning the formation of the extraembryonic mesoderm, it is considered that this tissue is amongst the first to be formed at the onset of gastrulation. The progenitors of the germ cells presumably formed part of the mesoderm as it moved to its extraembryonic site, and only when gastrulation terminated did the cells move back into the embryo. The same phenomenon has been observed in chick and quail embryos, where PGCs are translocated from the epiblast to the extraembryonic germinal crescent, and are brought back into the embryo through the blood circulation only after gastrulation (Ginsburg and Eyal-Giladi, 1986, 1987). Indeed, among vertebrates it is only in Urodeles that PGCs seem not to occupy an extraembryonic position in early development (Nieuwkoop and Sutasurya, 1979).

Snow (1981) demonstrated the appearance of PGCs in pieces that had been isolated from a particular region of 7- and 7½-day embryos and cultured for a further 24 h. The operated embryos, also cultured for 24 h, lacked germ cells. He described the critical location as being at the posterior end of the primitive streak, but the similar experiment that we carried out makes it likely that the pieces were dissected posterior to the amnion, i.e. in an extraembryonic region. Since the amnion is more distally located at the posterior than at the anterior side and is hard to see in the unfixed embryo, Snow probably misjudged the position of the amnion and hence considered the piece as part of the primitive streak rather than as extraembryonic. Copp et al. (1986) orthotopically grafted labelled pieces from the posterior regions of early 7-day embryos (equivalent to our stage 7-II) into host embryos, but found that labelled PGCs constituted only a small percentage (0.4–13.3 %) of the total PGC population in the host embryos on subsequent examination. They state that small pieces from the area of the primitive streak and allantois were dissected, and the cells that remained as coherent clumps were used for grafting. If the specific area in which we detected PGC progenitors did not stick together as a coherent clump, it may have escaped injection. The low number of PGCs that they recovered
were perhaps the descendants of PGCs that had already left the cluster and moved into the primitive streak.

Earlier studies have clearly shown mouse PGCs to move into the hind-gut endoderm of 8½-dpc embryos. Our observation of ALP-positive cells in the endoderm of 7-III stage embryos (Fig. 6A) may indicate that this definitive endoderm has already been laid down at this earlier time. A proper hind-gut invagination is not formed until around the 6-somite stage, at 8½ dpc.

In the Q strain of mice used for this study, the doubling time of the PGC population between 8½ and 13½ days is quite uniform, at about 16 h (Tam and Snow, 1981). Extrapolation back in time suggests that, with the same rate of proliferation, about 50 PGCs would be found at 7½ days and 25–30 at 7 days (Snow and Monk, 1983). These figures tally with our counts of cells with spots (Table 2). However, although the PGCs at 8 days showed evidence of mitosis, no such evidence was seen at 7 days. If, as our observations suggest, the cluster of ALP-positive cells detected at the base of the 7-day posterior amniotic fold represents the progenitors of the PGC population, a rough estimate obtained by dividing the volume of the cluster (50 μm diameter) by the volume of the cells (10 μm diameter) gives about 125 cells/cluster. Differentiation of all the cells of the cluster to form mature PGCs would provide the number observed at 8 days, without need for mitosis between 7 and 8 days.

Thus although our staining procedure has distinguished the germ cell lineage earlier in development than has been possible previously, our observations throw no new light on the origin of the lineage. In particular, they do not discriminate between an initial pool of a very small number of cells (e.g. <10) in the pregastrulation embryo which increases by mitotic proliferation, and a much larger initial pool of cells (e.g. >100) that progressively differentiate to give the observed increase in number of mature germ cells. (For a summary of evidence for and against these two models, see Snow and Monk, 1983). During the period when the putative PGC cluster is sequestered in the extraembryonic region, the cells with the ALP-rich spot increase in number at the expense of those showing diffuse ALP staining. There is also a striking increase in the intensity of the staining reaction during this period, suggesting that differentiation of the primordial germ cell population is gradual. At what point the germ cell lineage is determined remains unknown.

References


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