Temporal and spatial aspects of the gradual migration of primordial germ cells from the epiblast into the germinal crescent in the avian embryo

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SUMMARY

The migration of the PGCs from a stage XIII epiblast into the germinal crescent of a stage 10 chick blastoderm was experimentally investigated. Considerable numbers of PGCs start to come down from the epiblastic layer at stage XII-XIII and continue to do so in relatively smaller numbers until stage 6. The earliest PGCs land on the primary hypoblast and probably are carried by it into the GC. The PGCs that descend at relatively later stages land on the mesodermal wings and are either carried by them passively, or move actively into the GC. After the removal of the lower layer from stage 4 and older blastoderms, there is no regeneration of a GC in the blastoderm. Other consequences of this operation are that the mesodermal wings do not spread anteriorly as in normal controls and the PGCs that are still in the mesodermal domain are trapped. PGCs were found in explants of the lower layer and of the mesoderm. No identifiable PGCs were encountered in explants of the PS.

INTRODUCTION

To this day it is not clear whether the Primordial Germ Cells (PGCs) of the different vertebrate groups are determined according to the same developmental rules.

The origin of PGCs in the anuran embryo is believed to be clarified and understood and to support the germ-plasm theory which is also valid for many invertebrates. In the anuran oocyte a special cytoplasmic fraction has been detected near the vegetal pole, which can be traced in the zygote and the cleaving embryo (Bounoure, 1939; Blackler, 1958, 1966; Whittington & Dixon, 1975). This cytoplasm is found later within a small number of cells, identified as PGCs, localized in the centre of the endoderm of the blastula. Successive translocation of the PGCs into the genital ridges at later stages is mainly due to their active amoeboidal migration (Wylie & Roos, 1976).

As for other vertebrate groups, no distinct germ plasm has ever been detected either in the zygote or in specific blastomeres.

* Part of doctoral thesis of M. Ginsburg.

Key words: primordial germ cells, epiblast, germinal crescent, migration, chick blastoderm, quail blastoderm, blastoderm.
In urodeles, Sutasurya & Nieuwkoop (1974) claim that PGCs arise by way of induction during the morula-blastula stages. According to them the yolk endoderm induces PGC formation in the ectoderm as one of the mesodermal components of the marginal zone.

In mammals, a body of indirect evidence, supports the idea that the PGCs are of epiblastic origin (Mintz, Cronmiller & Custer, 1978; Jacob, 1977; Gardner & Rossant, 1976). The only report on a direct identification of relatively early mammalian PGCs is by Ożdżenski (1967) who claims to have detected them, in presomite stages of mice, in the caudal region of the primitive streak.

In reptiles there is no information about PGCs in stages earlier than those in which they are encountered in the extraembryonic germinal crescent.

In the avian embryo, PGCs can be easily identified in the germinal crescent, according to established morphocytologic criteria and PAS-positive stainability. Their dislocation from the germinal crescent via the blood circulation and into the genital ridges has been clarified. However, their previous developmental history and the paths of migration into the germinal crescent have been an enigma.

According to Swift (1914), cells originating from the germ wall, which have been described by Dantschakoff (1908) as entodermal ‘wander-cells’, are actually PGCs. They first appear during primitive streak (PS) stages and continue to do so until at least a stage of three pairs of somites (stage 8 of Hamburger & Hamilton, 1951), when they populate the germinal crescent (GC). Dubois (1967a,b), although unable to identify the PGCs microscopically at stages prior to PS formation, was able to show by an indirect experimental procedure that the cells destined to become PGCs are carried by the lower layer into the GC. This led him to the conclusion that they also originate from the lower layer, called by him the ‘vitelline endoblast’ (Dubois, 1969). Eyal-Giladi, Ginsburg & Farbarov (1981), by forming stage XIII (Eyal-Giladi & Kochav, 1976) chick-quail chimaerae, demonstrated that at this early stage, the cells that are to become PGCs are of epiblastic and not hypoblastic origin. Unpublished results of Azar and of Rangini from our laboratory, indicate that many PGCs do appear in isolated central epiblastic fragments, from which the marginal zone and area opaca have been removed, while there is no sign of embryo formation. Sutasurya, Yasugi & Mizuno (1983) separated the lower layer from the rest of the blastoderm at stages XIII to 4, cultured each fragment separately, wrapped in a piece of vitelline membrane, and checked for the appearance of PGCs. They conclude that the PGCs begin to translocate gradually into the lower layer during PS formation, but that many are still included in the upper layer at stage 4.

The goal of the present study was to accurately check the temporal, spatial and quantitative aspects of PGC migration from the epiblast into the GC from stage XII to stage 10. Special attention was paid to the question of whether the lower layer is the only carrier of PGCs into the GC, or whether mesodermal elements and the PS are also involved in the process.
MATERIALS AND METHODS

Both chick and quail blastoderms were used for series 1, and only chick was used for series 2. Blastoderms, both intact and after operation, were cultured on solid albumen (New, 1966), until they reached the desired stage. The blastoderms of series 1 were fixed in Brodski's fluid (1960), embedded in paraffin, serially sectioned at 7 μm and stained with Harris haematoxylin–eosin. PGCs were identified in the sections according to the following cytologic criteria: large size, relatively big nucleus usually eccentric, and clearly stained nuclear membrane. The PGCs were marked and counted. Whole mounts of series 2 blastoderms were prepared by spreading each blastoderm on a gelatine-coated glass slide, after which it was fixed with Rossman's fluid and stained with periodic-acid–Schiff (PAS) (Meyer, 1960). The transparent whole mounts were viewed with a light microscope and both the quantity and location of the conspicuous PGCs, were assessed. Explants were wrapped in a piece of vitelline membrane and cultured on solid albumen (Wolf & Haffen, 1952) for the same period as the blastodermic fragment from which they were separated. The explants were fixed either in Brodski's (series 1) or Rossman's fluid (series 2) embedded in paraffin, serially sectioned at 7 μm and stained with either Harris haematoxylin–eosin (series 1) or PAS (series 2), and then screened for PGCs.

RESULTS

Series 1

The aim of this series was to record possible changes in the distribution pattern of PGCs, between the main blastodermic part (epiblast plus mesoderm) and the lower layer, from stage XII to stage 4. The difficulty was that there is no specific marker for PGCs at stages younger than 4. Therefore we performed the separation experiments at the desired early stages and cultured the fragments long enough to allow the PGCs to differentiate to a degree at which they would be identifiable by either one of the staining methods used in this study.

The lower layer was separated from the blastoderms and both components belonging to the same blastoderm were incubated until the blastodermic part reached stages 7–8. The two components were then simultaneously fixed, embedded and serially sectioned. The PGCs at these relatively late stages could be reliably identified and counted. The total numbers of PGCs (Table 1) in the different blastoderms represent the added numbers of PGCs counted in the two separated components of the same blastoderm. The numbers of PGCs involved are in the same order of magnitude despite the different stages at which the separation was performed, because in all cases the blastoderms were further incubated to the same final stage.

The blastodermic fragments (the blastoderms denuded of their lower layers)

In all blastoderms there seemed to be regeneration of a lower layer but the older the blastoderm was at the time of the operation, the poorer was its regeneration and morphogenesis. In blastoderms operated upon earlier than stage 3, a germinal crescent (GC) always formed, whereas in blastoderms operated upon at stage 4, the extraembryonic region anterior to the head, where the GC usually forms, remained homogeneously transparent.
The lower layer fragments

Explants from stage XII and XIII blastoderms did not differentiate and their cells remained large, isolated and loaded with yolk.

Explants from blastoderms older than stage XIII always contained at the end of the incubation period some entodermal tissue and vascular elements, the quality of which depended on the initial stage of culturing, improving with age of the donor. The PGCs within the explants (Fig. 6) showed a clear affinity for either cavities or vascular elements. Occasionally some amoeboidal PGCs were encountered on the outer side of the explant close to the vitelline membrane, or even embedded in it.

The distribution of PGCs between the two fragments (Table 1)

Only in one blastoderm which was operated upon at stage XIII were all the PGCs found in the blastodermic fragment after it reached the stage of three somites, while none were found in the lower layer fragment removed from it and

Table 1. Changing distribution of PGCs between the lower layer and rest of blastodermic fragment at progressive developmental stages

<table>
<thead>
<tr>
<th>Stage at operation</th>
<th>Quail or chick</th>
<th>Stage of blastoderm at end of experiment</th>
<th>Blastodermic fragment</th>
<th>Expiant</th>
<th>Total amount of PGCs (blastodermic fragment plus explant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XII-XIII</td>
<td>Q</td>
<td>3 somites</td>
<td>69</td>
<td>8</td>
<td>77</td>
</tr>
<tr>
<td>EG&amp;K</td>
<td>Q, C</td>
<td>3 somites</td>
<td>99</td>
<td>20</td>
<td>119</td>
</tr>
<tr>
<td>2 H&amp;H (1/3 PS)</td>
<td>C</td>
<td>3 somites</td>
<td>47</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td>3–H&amp;H (1/2 PS)</td>
<td>Q, C</td>
<td>2 somites</td>
<td>74</td>
<td>25</td>
<td>99</td>
</tr>
<tr>
<td>3+H&amp;H (2/3 PS)</td>
<td>Q</td>
<td>3 somites</td>
<td>24</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>4 H&amp;H (full PS)</td>
<td>C</td>
<td>2 somites</td>
<td>43</td>
<td>133</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3 somites</td>
<td>50</td>
<td>92</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3 somites</td>
<td>50</td>
<td>92</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3 somites</td>
<td>35-2</td>
<td>64-8</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3 somites</td>
<td>35-2</td>
<td>64-8</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3 somites</td>
<td>35-2</td>
<td>64-8</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3 somites</td>
<td>35-2</td>
<td>64-8</td>
<td>142</td>
</tr>
</tbody>
</table>

* Tissue lost during histologic procedure.
cultured for the same length of time. In all the other blastoderms of this series, both fragments contained PGCs at the end of the incubation. However, the relative distribution of the PGCs between the two fragments of each blastoderm was found to be dependent upon the stage of the blastoderm at the time of the operation.

In most of the blastoderms treated as early as stage XIII, the bulk of PGCs was confined to the blastodermic fragment, with only a few in the lower layer explant. When the separation between the two components took place at stage 3 (1/2 PS), the number of PGCs in each of the two fragments was about equal. At a full PS (stage 4) most of the PGCs were found in the lower layer explant and only a few were found in the blastodermic fragment. The results of both chick and quail experiments showed the same distribution with time.

*The distribution of PGCs within the blastodermic fragment* (Table 2)

The spatial distribution of the PGCs within the blastodermic fragments listed in Table 1, was carefully studied. In addition to the stage 4 H&H blastoderms which were included in the original experimental group and were incubated after the removal of the lower layer to reach stage 7–8, three stage 4 blastoderms were similarly treated and incubated to reach stage 9 (six pairs of somites). A distinction was made between PGCs situated within the limits of the GC and those found lateral to the body axis.

The PGCs in the GC were found to be either attached to the regenerated lower layer, or scattered in the space between the upper and lower layers. The PGCs situated on both sides of the body axis were mainly found within the mesodermal tissue, lateral to the somites and usually associated with developing blood islands. Some of the latter were found in the space separating the mesodermal layer and the newly regenerated lower layer, while others were found in the space separating the mesoderm from the epiblast. In unoperated controls or in blastodermic fragments separated from their lower layer at stage XIII (with the exception of 27E) most of the PGCs were found at the end of the incubation period within the GC. Only relatively few PGCs were found in or near the mesoderm of the body region. When the lower layer was removed at stage 3, only about half of the PGCs of the blastodermic fragments were found in the GC, while the rest were associated with the lateral mesoderm. Relatively few PGCs were found in blastodermic fragments separated from their lower layer as late as stage 4. Most of them were within the mesodermal domain, while only a few were anterior to the head. When such blastoderms were cultured to stage 9, the total number of PGCs increased, probably as a result of cell division. However the increase mainly involved the PGCs attached to the lateral mesoderm, and not those in the GC.

*Series 2*

The above observations on the distribution pattern of PGCs within series 1 blastoderms, the lower layer of which was removed prior to further incubation,
Table 2. Distribution of PGCs within the blastodermic fragment after removal of the lower layer and further incubation

<table>
<thead>
<tr>
<th>Stage at operation</th>
<th>Blastoderm No.</th>
<th>Stage of blastoderm at the end of experiment</th>
<th>Total amount of PGCs in blastodermic fragment</th>
<th>Amount of PGCs in GC area</th>
<th>Amount of PGCs lateral to body axis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated control blastoderms</td>
<td>6E,HE (Q)</td>
<td>7–8 H&amp;H</td>
<td>67</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td>XII+ EG&amp;K</td>
<td>25E,HE (Q)</td>
<td>7–8 H&amp;H</td>
<td>69</td>
<td>62</td>
<td>7</td>
</tr>
<tr>
<td>XIII+ EG&amp;K</td>
<td>C6E,HE (C)</td>
<td>,,</td>
<td>47</td>
<td>29</td>
<td>18</td>
</tr>
<tr>
<td>XIII+ EG&amp;K</td>
<td>27E,HE (Q)</td>
<td>,,</td>
<td>99</td>
<td>18</td>
<td>81</td>
</tr>
<tr>
<td>2 H&amp;H</td>
<td>1P (C)</td>
<td>7–8 H&amp;H</td>
<td>53</td>
<td>53</td>
<td>0</td>
</tr>
<tr>
<td>23E,HE (Q)</td>
<td>,,</td>
<td>74</td>
<td>40</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>3 H&amp;H</td>
<td>12E,HE (Q)</td>
<td>7–8 H&amp;H</td>
<td>104</td>
<td>64</td>
<td>40</td>
</tr>
<tr>
<td>17E,HE (Q)</td>
<td>,,</td>
<td>68</td>
<td>33</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>24E,HE (Q)</td>
<td>,,</td>
<td>50</td>
<td>29</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>4 H&amp;H</td>
<td>C20E,HE (C)</td>
<td>7–8 H&amp;H</td>
<td>33</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>C21E,HE (C)</td>
<td>,,</td>
<td>23</td>
<td>9</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>C22E,HE (C)</td>
<td>,,</td>
<td>26</td>
<td>5</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>C24E,HE (C)</td>
<td>,,</td>
<td>20</td>
<td>8</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>4 H&amp;H</td>
<td>C25E (C)</td>
<td>9 H&amp;H</td>
<td>96</td>
<td>9</td>
<td>87</td>
</tr>
<tr>
<td>C3E (C)</td>
<td>,,</td>
<td>65</td>
<td>10</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>C4E (C)</td>
<td>,,</td>
<td>129</td>
<td>16</td>
<td>113</td>
<td></td>
</tr>
</tbody>
</table>

indicated a possible involvement of the mesoderm in the process of PGC translocation. Series 2 was therefore planned to study the distribution of PGCs between the lower layer, the lateral mesodermal wings and the GC at stages 4 to 6 during which the main invagination of the mesodermal layer takes place. Here a different technique was applied and the unoperated control blastoderms as well as the

Figs 1–8. Abbreviations: gc, germinal crescent (GC); mw, mesodermal wing; pgc, primordial germ cell; ps, primitive streak (PS).

Fig. 1. Whole mount of normal stage 4 H&H chick blastoderm, PAS staining. ×15.

Fig. 2. Whole mount of normal stage 10 H&H chick blastoderm, PAS staining. ×15.

Fig. 3. Whole mount of normal stage 5 H&H chick blastoderm after removal of the lower layer. PAS staining. ×15.

Fig. 4. Whole mount of stage 10 H&H chick blastoderm the lower layer of which was removed at stage 4 H&H. PAS staining. ×15.

Fig. 5. Whole mount of a stage 10 H&H chick blastoderm both lower and middle layer of which were removed at stage 4 H&H. PAS staining. ×15.

Fig. 6. 7 μm section of a lower layer explant removed from a stage 4 H&H chick blastoderm and cultured until its complementary blastodermic fragment reached stage 10 H&H, stained with haematoxylin–eosin. Contains characteristic PGCs. ×600.

Fig. 7. 7 μm section of a middle layer (mesoderm) explant removed from a stage 4 H&H chick blastoderm and cultured until its complementary blastodermic fragment reached stage 10 H&H. Stained with PAS and haematoxylin. ×600.

Fig. 8. 7 μm section of a stage 4 H&H PS cultured for 24 h. Cells have a characteristic amoeboidal appearance. No recognizable PGCs were detected. Haematoxylin–eosin staining. ×600.
operated ones (either after the removal of the lower layer alone, or of both lower layer and lateral mesoderm) were whole mounted and PAS stained. This enabled us to get a good view of the entire blastoderm and of the clearly visible PAS-positive PGCs within its boundaries.

The lateral mesodermal tissue removed from the above blastoderms, and stage 3 primitive streaks removed from others, were cultured individually beneath a vitelline membrane. In each case, thick, opaque lumps were formed which were fixed and serially sectioned for further study.

Series 2 therefore contained the following categories, each subdivided into groups:

**Whole mounts** (Figs 12, 17)
- **Group I**: Stage 4, 5, 6 and 10 blastoderms serving as controls (unoperated nonincubated).
- **Group II**: Stage 4, 5 and 6 blastoderms the lower layer of which was removed, and which were fixed and stained.
- **Group III**: Stage 4, 5 and 6 blastoderms the lower layer of which was removed and which were further incubated until they reached stage 10 (10 pairs of somites).
- **Group IV**: Stage 4, 5 and 6 blastoderms both the lower layer and mesodermal wings of which have been removed and which were incubated to stage 10.

**Serial sections**
- **Group V**: The mesodermal fragments removed from the blastoderms of group IV were cultured beneath a vitelline membrane for the same length of time as the blastoderms from which they were removed, after which they were fixed and sectioned.
- **Group VI**: Stage 3 primitive streaks were dissected out of blastoderms after the removal of the latters’ lower and middle layers and cultured beneath a vitelline membrane for 17–24 h, and then fixed and sectioned.

**Group I: Unoperated nonincubated control blastoderms** (Fig. 12A–C)

**Stage 4** (Figs 1, 12A): Already at this early stage, many large cells with big nuclei could be identified within a relatively wide anterolateral crescent-shaped band (Fig. 9). These cells are regarded by us as PGCs because of their size, shape,
Fig. 12. A schematic distribution pattern of PGCs based on the study of whole mounts stained with PAS viewed from the ventral side. (A–C) represent normal controls of stages 4, 5 and 6 respectively. (D–F) represent blastodermic fragments of stages 4, 5 and 6 respectively, following the removal of the entire lower layer. (G–I) represent blastodermic fragments of stages 4, 5 and 6 respectively, the entire lower layer of which was removed and the fragments incubated until they reached stage 10 H&H. Viewed from the ventral side. The dots represent PGCs. The etched area represents the mesodermal wings. gc, germinal crescent (GC); pgc, primordial germ cells (PGC); ps, primitive streak (PS); so, somites.
PAS-positive cytoplasm and the fact that they were packed with large yolk granules. The outer limit of the PGC-containing area coincided with the border between the area opaca and area pellucida. However, it stretched back much further than the posterior corner of the already recognizable GC. The distribution of the PGCs within the above band was consistently asymmetric, being more abundant on the left than on the right side. The anterior PGCs appeared more PAS positive than the lateral ones.

Stage 5 (Fig. 12B): The distribution pattern of PGCs was very similar to stage 4.

Stage 6 (Fig. 12C): The shape of the PGC-containing band remained essentially the same as in earlier stages, but its posteriorly pointing arms contracted in an anterior direction towards the GC. Most of the PGCs were round and strongly PAS positive and were located in the clearly demarcated GC. The PGCs lagging behind, were found on both sides of the blastoderm at the level of the head process, again unevenly distributed with a preference for the left. They appeared amoeboidal and were less PAS positive than the anterior ones.

Stage 10 (Fig. 2): PGCs were concentrated in the GC (Fig. 11). They were found in clusters and many seemed to have just undergone mitosis. Only a few small, faintly stained amoeboid PGCs were encountered in the body region, again unequally distributed between left and right.

Group II: Blastoderms without lower layer (Fig. 12D–F)

Stage 4 (Fig. 12D): Only a few PGCs were found in the blastoderm after the removal of the lower layer, most of them attached to the lateral mesoderm, and some found in the anterior part of the blastoderm (Fig. 10).

Stage 5 (Figs 3, 12E): Results were very similar to stage 4 but there was an increase in the number of anterior PGCs.

Stage 6 (Fig. 3F): The total number of PGCs increased, most of them in the area of the GC and only very few were found in the lateral wings of the mesoderm.

Group III: Blastoderms without lower layer incubated to stage 10 (Fig. 12G–I)

In neither of these operated blastoderms did an identifiable GC ever regenerate, and the area pellucida, anterior to the head of the embryo, remained homogeneously transparent up to the border of the area opaca.

Stage 4 (Figs 4, 12G): Only a few PGCs were found after incubation to stage 10 in the ‘would be’ GC area. Their number did not differ significantly from those in the same area at stage 4 immediately after the removal of the lower layer. This probably indicates that the PGCs present in the mesodermal domain did not move
anteriorly during incubation from stage 4 to 10, and that the anterior PGCs did not undergo cell division after the operation. On the other hand the number of PGCs in the mesodermal domain did increase during the incubation from stage 4 to 10. They were found to be mainly associated with blood islands (Figs 14, 16) in the most anterior section of the mesodermal wings (Fig. 14) which did not spread anteriorly as did the mesoderm of the control blastoderms (Fig. 12). These PGCs were intensely PAS positive, usually round and frequently paired, indicating that they had recently undergone mitosis. Some of the PGCs in this area appeared amoeboidal with finely granulated cytoplasm, but they did not contain the large yolk granules characteristic of PGCs in non-incubated stage 4 blastoderms. The distribution of PGCs was asymmetric with a left-sided bias.

Stage 5 (Fig. 12H): The results after incubation to stage 10 were similar to blastoderms operated upon at stage 4 and similarly incubated. The main difference between the two groups was that relatively more PGCs were found here anterior to the head. The PGCs in the mesodermal area were again associated with developing blood vessels (Fig. 15).

Stage 6 (Fig. 12I): The general appearance of the blastodermic area anterior to the head was similar to blastoderms operated upon at an earlier stage, but here the number of anterior PGCs was still higher. The main difference from blastoderms operated at earlier stages concerned the more posterior regions, where both the lateral mesodermal wings and blood islands were almost entirely free of PGCs. When operating on stage 6, it was difficult to neatly separate the lower layer of the anterior region of the GC from the tissue above it, so that fragments of the lower layer sometimes remained attached to the operated blastoderm. These tissue fragments could be easily identified at the end of the incubation period, and after staining were shown to be associated with clusters of PGCs.

Group IV: Blastoderms with both lower layer and mesodermal wings removed; incubated to stage 10 (Fig. 17)

As in group III, here too no GC developed during incubation. On the other hand, there was some development of mesodermal wings due to the postoperative

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Figs 13–16. Micrographs of selected areas of blastoderms. Figs 13–15, ×200; Fig. 16, ×600. pgc, primordial germ cells (PGC); vv, vitelline vein.

Fig. 13. Anterior tip of the right mesodermal wing of the blastoderm in Fig. 3 showing the PGCs in this region that were not removed with the lower layer. PAS staining.

Fig. 14. Anterior tip of the right mesodermal wing of the blastoderm in Fig. 4. PGCs are strongly PAS positive, but their amount and position did not change remarkably during incubation from stage 4 to 10 H&H.

Fig. 15. Stage 10 H&H chick blastodermic fragment the lower layer of which was removed at stage 5 H&H, stained with PAS. The PGCs are lined up on the forming vitelline vein (vv).

Fig. 16. 7 μm section in a blood island area of a stage 10 H&H chick blastoderm the lower layer of which was removed at stage 4 H&H, stained with haematoxylin–eosin.
continued invagination *via* the PS. The development of the mesoderm became progressively poorer, the older the blastoderm was at the time of the operation.

**Stage 4** (Figs 5, 17A): After incubation to stage 10 only very few PGCs could be found anterior to the head. Many PGCs were concentrated at the anterolateral borders of the narrow mesodermal wings, and again remarkably more on the left than on the right side.

**Stage 5** (Fig. 17B): Very similar to stage 4, but relatively more PGCs were located in the anterior region whereas fewer PGCs were found in the lateral mesoderm.

**Stage 6** (Fig. 17C): Despite the absence of a morphologically defined GC, many PGCs were found anteriorly, where the GC would have formed. Only very few PGCs were associated with the mesodermal wings lateral to the somites.

**Group V: Mesodermal explants**

The mesodermal fragments that had been removed from the blastoderms of group IV and cultured as explants for the same length of time as the donor blastoderm, were poorly differentiated. However, at their periphery a few isolated PAS-positive PGCs could always be found (Fig. 7).

**Group VI: PS explants**

As a control to the mesodermal fragments, primitive streaks were cut out of stage 3 (nonexperimental) blastoderms and cultured under a vitelline membrane for 17–24 h. The PSs generally retained their original shape and the histologic sections did not reveal any conspicuous differentiation.

Fig. 17. A schematic distribution pattern of PGCs in blastodermic fragments, the lower layer as well as the mesodermal layer of which have been removed, and the fragment incubated until it reached stage 10 H&H, viewed from the ventral side. The dots represent PGCs. The etched area represents the mesodermal wings. A–C were operated upon at stage 4, 5 and 6 respectively.
There was no indication of the existence of PGCs and there were no PAS-positive cells (Fig. 8), although occasionally the shape of some isolated cells at the periphery of the explant remotely resembled PGCs.

**DISCUSSION**

The paper by Sutasurya *et al.* (1983) is the only one so far, that has the same approach as ours to the question of the appearance of PGCs in young avian blastoderms. Their study on the translocation of the PGCs into the lower layer was based on two points: (a) the demonstration by Eyal-Giladi *et al.* (1981) that avian PGCs are of an epiblastic origin; (b) the common assumption that the lower layer is involved in PGC formation either as a source (Dubois, 1969), a carrier (Dubois, 1967a,b) or an inducer. One of their main conclusions, that ‘the PGCs translocate gradually to the lower layer during the early stages of primitive streak formation, though substantial numbers of presumptive PGCs remain in the upper layer’ can be generally accepted as correct. However, in their experiments which are parallel to part of our series 1, no effort was made to deal with each blastoderm separately and to compare the PGCs’ presence in the upper and lower layers of individual blastoderms. Moreover, the PGCs in the above experiments were not counted but only roughly estimated. Sutasurya *et al.* (1983) also claim that in many of the fragments of either upper or lower layer, no PGCs were found whatsoever. In our study PGCs were invariably found in all the operated blastoderms as well as in the lower layer explants. Nevertheless, we did find a significant stage dependant difference in the relative distribution of PGCs between the blastoderm and the lower layer. In those cases when the lower layer was separated from the blastoderm at stage XIII (and each was incubated separately), the majority of the PGCs was found in the blastoderm and only a few in the cultured lower layer. This proportion changed gradually until stage 4, when most of the PGCs were found associated with the lower layer fragment.

An important point not raised by Sutasurya *et al.* was the fact that the ‘upper layer’ at the stages studied, included in most cases also the mesodermal sheath, which had already invaginated via the PS. In our study this reservation was taken into consideration, thus: (1) explanted lower layer fragments were always compared with their complementary blastoderm (series 1); (2) PGCs were carefully counted (series 1); (3) a group of blastoderms, of which the lower layer was removed, were further separated into epiblastic and mesodermal components (series 2), cultured separately and the number of PGCs in each was checked. We believe that our results indicate (Table 1) that the PGCs start to translocate from the epiblast at stage XII–XIII. At stage 4 already about 85% of the PGCs have established some contact with the lower layer and were therefore removed with it.

The regional distribution of PGCs within the operated blastoderms of series 1 was studied at the end of the incubation period (stage 7–8). In all cases PGCs were found both in the GC and lateral to the body axis (Table 2), but their relative distribution between the two areas depended on the stage of the blastoderm at the
time of the operation. The younger the blastoderms were at the time of the operation, the fewer PGCs were found in the area lateral to the body axis and more in the GC. However, the above result does not provide a dynamic picture of the pathways of PGC migration from the epiblast until they reach the GC. To this purpose we planned to interrupt the development of normal and operated blastoderms, following different periods of incubation in order to get the overall picture of PGC distribution at all stages between 4 and 10. PAS staining of whole-mount blastoderms was done, in order to clearly visualize the contour of the blastoderm, the embryo, the shape of the extraembryonic mesodermal elements and of the GC. Each PGC could be easily identified, its shape assessed and the intensity of its PAS staining could be compared with that of the other PGCs of the same blastoderm. Series 2 is complementary to series 1 as it deals with older stages, only stage 4 overlapping.

The results presented in Figs 12 and 17 provide a clear picture of the gradual translocation of the PGCs and their association with the different germ layers during the stages studied. In normal controls, fixed as early as stage 4 (Fig. 12A), many PGCs were already visible, the majority being located in the GC (see also Swift, 1914; Fujimoto, Ukeshima & Kiyofuji, 1976). In addition, posterolateral concentrations of trailing PGCs were found on both sides of the PS (usually more on the left). At stage 5 (Fig. 12B) the PGCs’ distribution pattern was similar to stage 4 while at stage 6 (Fig. 12C) the lateroposterior PGCs have already shifted anteriorly towards the GC. At stage 10 (Fig. 2) almost all of the PGCs were found within the limits of the GC.

The immediate picture seen after the removal of the lower layer at stage 4 (Fig. 12) confirms the results of series 1. Only a few PGCs remained attached to the blastoderm (in series 1 about 15%), distributed between both the GC and the lateral areas. At stages 5 and 6, following the removal of the lower layer (Fig. 12E, F), increasing numbers of PGCs remained attached to the blastoderm in the region anterior to the head, whereas the number of PGCs in the lateral areas decreased. When any of the blastoderms, either without a lower layer or without both a lower layer and lateral mesoderm, was incubated to stage 10, the number of PGCs in the anterior region remained as it was at the time of the operation. This means that no PGCs were added to the GC during incubation either by migration or cell division (compare: Fig. 12D with 12G and 17A; Fig. 12E with 12H and 17B; Fig. 12F with 12I and 17C). As for the lateral PGCs in blastoderms without lower layer (Fig. 16), their number did increase after incubation to stage 10 (especially in the blastoderms operated at stages 4 and 5), probably as a result of some mitotic activity as well as postoperative arrivals from the epiblast. This conclusion is supported by the data in Fig. 17, where after the removal of both lower layer and mesoderm at stages 4, 5 and 6 and incubation to stage 10, some new lateral mesoderm developed in which PGCs could be identified (Fig. 17A–C). There seems to be a slowing down of PGC translocation from the epiblast after stage 5, as reflected by the reduced numbers in the lateral mesoderm of (B) and (C) as compared to (A) (Fig. 17). The PGCs in the mesodermal domain seemed to be
unable to translocate into the GC, in blastoderms without lower layer, incubated to stage 10. This is in contrast to nonoperated stage 10 control blastoderms in which almost all PGCs are already concentrated in the GC. As PGCs were never found in the somites and have not been found in explants of the PS, they probably translocate vertically from the epiblast, lateral to the PS and enter the more laterally situated mesoderm.

The answer to the question as to why PGCs in blastoderms without lower layer cannot move anteriorly, may shed light on the mechanism of PGC migration in the early blastoderm. Nicolet (1971), discussing the regeneration of a new lower layer after the removal of the original one at different early developmental stages, states that the regeneration process at stages earlier than 3+ is complete, while from stage 3+ and onwards regeneration is defective in the sense that no GC is formed. We confirm the above observation and add that whenever a GC is reformed the migration of PGCs into it is normal, and that PGCs which were at the time of the operation either in the lateral mesoderm, or on their way down from the epiblast towards it, do succeed in finding their way into the anteriorly situated GC. Blastoderms in which no regeneration of a GC takes place after the removal of their lower layer, are probably defective in that they lack the primary hypoblastic component of the original lower layer and are able only to regenerate the central entodermal component. In such blastoderms the PGCs remain trapped in the region which they occupied at the time of the operation. The effect of the absence of the GC, namely the defective distribution of PGCs in the above blastoderms, may be explained by one, or by a combination of the following assumptions.

1) The primary hypoblast, but no other lower layer component has the ability to bind PGCs descending from the epiblast and carry them passively by the former's morphogenetic movements, into the GC.

2) The primary hypoblast attracts PGCs from a distance and guides them into the already formed GC by way of amoeboidal movement.

3) The primary hypoblast is the only suitable substrate to facilitate the anteriorly directed spreading of the lateral mesodermal wings which contain the youngest PGCs. In normal development these mesodermal wings, while growing anteriorly, carry the PGCs into the GC. In the case of a defective postoperative regeneration of the primary hypoblast, the mesodermal wings cannot spread and the PGCs remain trapped within them.

We therefore suggest the following scenario for the gradual translocation of the PGCs from the epiblast into the GC during normal development. The PGCs that begin to descend from the epiblast at stages XII–XIII land directly on the hypoblast and establish a close contact with it. At later stages an intermediate layer of mesoderm spreads centrifugally from the PS and invades the space between the epiblast and the hypoblast. The PGCs leaving the epiblast at later stages therefore have to land on the dorsal side of the lateral mesoderm, from where they have three possible pathways into the GC.

1) PGCs reach the GC by amoeboidal movement either on or within the lateral mesoderm.
(2) They may be carried into the GC passively by the mesodermal wings which are spreading anteriorly in concert with the hypoblast.

(3) PGCs may cross the mesodermal layer vertically, to reach the hypoblast and join the earlier PGCs anchored to it. From there the PGCs are carried passively by the anteriorly shifting hypoblast until they reach the GC area. They would then be gradually released into the space above the hypoblast (Fig. 12 compare D,E and F), round up and start to divide (Fig. 11). In other words, the lower layer at stages XIII and onwards, probably until stage 10, might furnish anchorage to the PGCs and function as their carrier into the GC.

The latter hypothesis is indirectly supported by the fact that at stages 4, 5, 6 and 10 (Figs 2, 12A–C) the distribution patterns of PGCs seem to overlap the territory occupied by the primary hypoblast (Azar & Eyal-Giladi, 1983). We can therefore speculate that the PGCs have an affinity for the hypoblastic component of the lower layer, but not for the entodermal cells which later invade it and occupy the centre of the lower layer. At stage 10 (Azar & Eyal-Giladi, 1983) the primary hypoblast has completed its anteriorly directed movement and occupies the same position as the GC. We therefore assume that the primary hypoblast is in effect the main component of the GC (Vakaet, 1962). However, after releasing the PGCs into the space above it, the primary hypoblastic area continues its centrifugal movement into the yolk sac.

REFERENCES


Migration of avian primordial germ cells


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