Interaction of epiblast and hypoblast in the formation of the primitive streak and the embryonic axis in chick, as revealed by hypoblast-rotation experiments

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SUMMARY

Three types of experiments were performed to determine the interaction between the epiblast and hypoblast for primitive streak formation: (1) Hypoblasts of blastoderms from stages XIII E.G & K to 3 H & H were separated from the epiblasts and rotated by 90° counterclockwise; (2) hypoblasts from stages XIII E.G & K to 3 H & H blastoderms were rotated by 180°; (3) hypoblasts were exchanged between blastoderms of different developmental stages and placed at 90° counterclockwise to the axis of the recipient epiblast. In all blastoderms studied only a single PS developed. After rotation of the hypoblast by 90°, the direction of the PS was according to the orientation of the hypoblast at stage XIII, whereas at older stages it gradually shifted towards the axis of the epiblast. At stage 3 H & H the PS is already imprinted in the epiblast and cannot be shifted. After rotation of the hypoblast by 180° the PS originated at the point near the marginal zone at which the inductive part of the hypoblast interacted with a competent epiblast. Conclusions are drawn about the dynamics of the inductiveness of the hypoblast and the competence of the epiblast for the PS formation and orientation.

INTRODUCTION

Waddington (1932) was the first to indicate that the lower layer, which he called endoderm, of a chick blastoderm with a young primitive streak (PS) influences the PS to deflect towards the anterior end of the endoderm when placed at right angles to the epiblast. According to Waddington, three factors were important for reliable results: the early stage of the blastoderm, a good healing of the wound and a central position of the endoderm underneath the turned epiblast, which was rather difficult to achieve.

In the experiments described above, there was no definite conclusion ‘whether the endoderm merely guided movements once they had started, or whether it was also able to initiate them’. Therefore, Waddington (1933) performed another series of experiments in which he turned the endoderm by 180°, thinking that ‘if the endoderm is capable of inducing a new set of tissue movements there

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would be a greater likelihood for the formation of a new (second) PS at 180°
to the original one'. From his results which were variable and included a spectrum
of embryos developed according to the epiblastic orientation, curved embryos,
reversed embryos and head to head embryos, it was concluded that the endoderm
induces tissue movements by virtue of a quality which is spread throughout the
tissue, namely, a gradient system.

Since the thirties during which the above pioneering investigations were done,
additional information has been accumulated on the cellular composition of the
lower layer. Presently, the lower layer prior to PS formation is regarded as the
primary hypoblast and is known to contribute only to extraembryonic structures
such as yolk-sac endoderm, later in development (Rosenquist, 1972; Wolk &
Eyal-Giladi, 1977). At the advancing PS stages the lower layer is constantly
changing its cellular composition, the primary hypoblastic cells are moving to
the periphery while the central region is being occupied by cells of PS origin
which will form the embryonic endoderm (Vakaet, 1962, 1970). The primary
hypoblast was found capable of inducing a PS in a competent epiblast when
separated from it by a milipore filter (Eyal-Giladi & Wolk, 1970). The hypoblast
was also found to support and stabilize the PS during the initial steps of its
formation (Eyal-Giladi, 1970; Azar & Eyal-Giladi, 1979). It was also found by
Azar & Eyal-Giladi (1979) that cells derived from the marginal zone constitute
the inductive element of the primary hypoblast and that they probably move
from the posterior marginal zone anteriorly during the gradual process of the
formation of the hypoblast (Eyal-Giladi & Kochav, 1976; Kochav, Ginsburg &
Eyal-Giladi, 1980). This is in agreement with Spratt’s (1946) observations which
show that the PS is formed in the direction of the hypoblast’s movement and
that inhibition of this movement (Spratt & Haas, 1960b) inhibits the formation
of the streak.

The operation and culture techniques have been improved since Waddington’s
experiments and the blastoderms are now being treated while placed dorsal side
down. This facilitates the handling of the hypoblast and enables one to place it
in the right position on the ventral side of the epiblast.

We therefore thought that it was worthwhile to re-examine more carefully
Waddington’s experiments while paying attention to the stage of the epiblasts
and hypoblasts involved. We also wanted to see whether it was a mere stationary
inductive process which determined the direction of the PS and the embryonic
axis, or whether tissue movements were also involved.

**MATERIALS AND METHODS**

Fresh eggs laid by hybrid New Hampshire x Leghorn hens were incubated at
38 °C for 11-13 h. A spectrum of stages between XIII E.G & K (Eyal-Giladi &
Kochav, 1976) and 3 H & H (Hamburger & Hamilton, 1951) were acquired.
Blastoderms were removed from the yolk together with a big piece of vitelline
membrane which was stretched over a glass ring, and were operated in Ringer’s
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solution. Before the operation the posterior side of the area opaca was marked by a longitudinal line of small carbon particles. During the time of operation, the marking was crucial for not losing the orientation of stage XIII E.G. & K blastoderms while in older blastoderms the already existing PS formed a reference point. At the end of the experiment the carbon particles were used in all cases to determine the original posterior epiblastic end. The posterior side of the hypoblast was not marked by carbon but the orientation was preserved during the time of the operation according to distinct morphologic criteria such as differences in shape and the distribution of attached yolk-rich cells.

Three kinds of experiments were performed which involved the rotation of the hypoblast by either 90°, 180° or the transplantation of a heteroplastic hypoblast from a donor at a different developmental stage onto an epiblast and its horizontal rotation by 90°. Care was taken that the hypoblast be placed after turning on the centre of the recipient epiblast. Blastoderms were then incubated for 20–24 h in vitro according to New (1955), but instead of liquid albumin, a piece of the viscous albumin was left attached to the vitelline membrane as a substrate.

At the end of the experiment, the blastoderms were observed under a stereoscopic microscope, and the orientation of the PS as referred to the original epiblastic axis was recorded with 15° precision. The reason for recording the orientation of the PS and not of the embryonic axis was that in some cases a distinct angle (other than 180°) developed between the PS and the embryonic axis, which was also recorded.

RESULTS

Experiment I. The hypoblast was separated from the epiblast and rotated 90° counterclockwise. After 20–24 h incubation, both the direction of the PS axis and the embryonic axis when different from the direction of the streak, were recorded. Fifty blastoderms were divided into four groups according to their developmental stage at the time of operation, and the results were recorded in Table 1. When the operation is performed at the full primary hypoblast stage (XIII E.G & K), the axis of the formed primitive streak (PS) deviates by 88.9° from the epiblastic axis. The older the operated blastoderm, the smaller the deviation of the PS axis from the epiblastic axis, until stage 3~ H & H when the direction of the PS coincides with the epiblastic axis. In all blastoderms operated on from stage 2 H & H and older, which displayed a deviation of the PS, the original, already existing rudiment of the PS disappeared.

In most blastoderms of the above experimental group the PS and its resulting embryo anteriorly, formed a straight line. Thus the deviation of the PS was also reflected in the deviation of the embryonic axis from the epiblastic axis (Fig. 1 a, b).

However, in some embryos the deviation of the axis turned to be a complex one, and there was an angle between the PS axis and the axis of the embryo which developed from it (Fig. 1 c). Such an additional angle always increased
Table 1

<table>
<thead>
<tr>
<th>Deviation of PS from epiblastic axis</th>
<th>Stage at operation</th>
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<tbody>
<tr>
<td></td>
<td>XIII E.G &amp; K</td>
</tr>
<tr>
<td>0°</td>
<td>—</td>
</tr>
<tr>
<td>15°</td>
<td>—</td>
</tr>
<tr>
<td>30°</td>
<td>—</td>
</tr>
<tr>
<td>45°</td>
<td>—</td>
</tr>
<tr>
<td>60°</td>
<td>—</td>
</tr>
<tr>
<td>75°</td>
<td>1</td>
</tr>
<tr>
<td>90°</td>
<td>13</td>
</tr>
<tr>
<td>Total no. of blastoderms</td>
<td>14</td>
</tr>
<tr>
<td>Mean deviation of axis</td>
<td>88.9° ± 11°</td>
</tr>
<tr>
<td>Significance (t test)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Degree of deviation of the PS from the epiblastic axis following the rotation of the hypoblast by 90°. Significance of mean deviation angle was checked for every two neighbouring stage groups by t test.

Table 2

<table>
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<th>Stage at operation</th>
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<tbody>
<tr>
<td>XIII E.G &amp; K</td>
</tr>
<tr>
<td>Total number of blastoderms</td>
</tr>
<tr>
<td>Number of blastoderms with an angle</td>
</tr>
</tbody>
</table>

Cases of experiment I in which an angle is formed between the PS and the embryonic axis. The difference between stages XIII E.G&K and 2 H&H is highly significant (0.005 according to $\chi^2$ test), and the difference between 2+ H&H and 3− to 3 is significant (0.03). All the other differences, namely between 2 H & H and 2+ H & H as well as between XIII E.G&K and 3− to 3 H & H are non-significant.

The deviation of the embryonic axis from the epiblastic axis and brought it closer to the hypoblastic axis. A graphic representation of the epiblastic, embryonic and PS axes of typical blastoderms (1a, b, c) of Experiment I is shown in Fig. 1.

The additional deviation is characteristic and significant for blastoderms operated on at stages 2−2+ H & H with a relatively small PS (Table 2), in which the deviation of the newly formed PS from the first one did not exceed 45°. From the 11 cases in which such a deviation was observed, in five the PS coincided with the epiblastic axis, in two it deviated from it by 15°, in two by 30° and in two other cases the PS deviated by 45° from the epiblastic axis.

Experiment II. The hypoblast was separated from the epiblast and rotated 180° counterclockwise. The 40 blastoderms of this experiment were divided into
Fig. 1. Photographs of three embryos (a, b, c) from experimental group I after 20–24 h in culture. The PS and embryonic axes' deviations from the epiblastic axis represented graphically in the lower right-hand corner. In blastoderms (a) and (b) the PS and embryo axes are on a straight line. In (a) there is an angle of 15°, while in (b) an angle of 45° between the epiblastic axis (as indicated by the straight line originating from the carbon mark) and the PS-embryonic axis. In (c) the PS axis coincides with the epiblastic axis, while the embryonic axis deviates by 30° from the epiblastic towards the posterior end of the hypoblastic axis which is at 90° to the right. cm, carbon mark; em, embryonic axis (embryo), ep, epiblastic axis; ps, primitive streak axis (primitive streak).
Degree of deviation of PS from epiblastic axis in blastoderms in which the hypoblast was rotated by 180°. The differences between the consequent age groups was proven highly significant by t test (< 0.001).

Table 3

<table>
<thead>
<tr>
<th>Deviation of PS from epiblastic axis</th>
<th>Stage at operation</th>
<th>2-2+ H &amp; H</th>
<th>3~ to 3 H &amp; H</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°</td>
<td>XIII E.G &amp; K</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>15°</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>30°</td>
<td>7</td>
<td>1</td>
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<tr>
<td>45°</td>
<td>6</td>
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</tr>
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<td>60°</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>75°</td>
<td></td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>90°</td>
<td>12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>105°</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°</td>
<td></td>
<td>1</td>
<td></td>
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<td>135°</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>150°</td>
<td>1</td>
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<tr>
<td>165°</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>180°</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of blastoderms</td>
<td>17</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Mean deviation of axis</td>
<td>108.5° ± 8.0°</td>
<td>40.7° ± 4.3°</td>
<td>1.7° ± 1.7°</td>
</tr>
<tr>
<td>+ s.e.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of right-hand PS</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

three age groups according to their developmental stage at the time of the operation. The results (Table 3) indicate that at stage XIII E.G & K, only in one of the 17 blastoderms did the PS develop according to the polarity of the 180° turned hypoblast. In four additional blastoderms the PS developed at positions between 180° and 90° to the epiblastic orientation, while in most blastoderms (12) the PS developed at exactly 90° to either the original epiblastic or hypoblastic axis namely acquired an intermediate position between the two. From the 16 streaks with an angle other than 180°, ten developed from the right side of the blastoderm and six from the left.

Rotations performed at stages 2-2+ H & H resulted in a deviation of the majority of the streaks by 30°-45° from the epiblastic axis. In 9 out of the 14 blastoderms studied, the PS developed from the right side of the blastoderm. Rotations at stages 3~ to 3 H & H did not change the orientation of the already existing primitive streak which continued to develop according to the epiblastic axis.

An angle between the PS and the embryonic axis was observed only in three blastoderms of stage XIII E.G & K in which the PS deviated by 90° from the epiblastic axis which means that the deviation of the embryonic axis from the epiblastic axis in these cases was more than 90°.

Experiment III. In contrast to the procedure in Experiment I, the hypoblasts
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Table 4

<table>
<thead>
<tr>
<th>Stage of hypoblast</th>
<th>Stage of epiblast</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>XIII E.G &amp; K</td>
</tr>
<tr>
<td>XIII E.G &amp; K</td>
<td>(1)88°9' ± 1°10'</td>
</tr>
<tr>
<td></td>
<td>n = 14</td>
</tr>
<tr>
<td>2–2+ H &amp; H</td>
<td>(4)57°5' ± 8°4'</td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
</tr>
<tr>
<td>3– to 3 H &amp; H</td>
<td>(5)15°0' ± 5°0'</td>
</tr>
<tr>
<td></td>
<td>n = 10</td>
</tr>
</tbody>
</table>

Deviation of PS axis from the epiblastic axis in epiblast-hypoblast combinations of different developmental stages. The hypoblastic axis is at 90° to the epiblastic axis.

n = number of blastoderms.

Significance of results according to \( t \) test was checked for the following pairs: (1)–(2) < 0.001; (2)–(3) = 0.002; (1)–(4) = 0.001; (4)–(5) < 0.001; (2)–(4) = 0.01; (3)–(5) = 0.002.

were exchanged between blastoderms of different developmental stages and placed at 90° counterclockwise to the axis of the recipient epiblast (Table 4). Fifty-eight blastoderms were divided into groups representing different hypoblast–epiblast stage combinations.

A hypoblast of stage XIII E.G & K placed on an epiblast of the same stage causes a shift of the PS axis by 90° from the axis of the epiblast. A hypoblast of a stage 2–2+ H & H blastoderm when transplanted into a stage XIII E.G & K epiblast causes a deviation of the forming PS axis by about 57°5' from the epiblastic axis toward the posterior side of the hypoblastic axis. Even a stage 3– to 3 H & H hypoblast is capable of slightly influencing the orientation of the forming PS although the deviation is of about 15° only.

In a reverse experiment, in which stage XIII hypoblasts were placed at 90° on epiblasts of older stages, the hypoblast changed the orientation of a stage 2–2+ H & H epiblast with an already started PS by 25°. However, a stage XIII hypoblast cannot change the polarity of a stage 3– to 3 H & H epiblast.

DISCUSSION

Three groups of experiments were performed, I and II being a more precise repetition of Waddington's hypoblast rotation experiments (1932, 1933) in which we recorded the exact developmental stage of the blastoderms involved and III being a variation of experiment I in which epiblasts were combined with 90°-rotated hypoblasts of a different stage. Analysis of these experiments, especially experiment III, enabled us to distinguish between the inductivity of the hypoblast and the competence of the epiblast, to assess the intensity and distribution of these qualities and their influence on the polarity of the PS throughout the stages studied.

Our criterion for inductivity was the angle by which a 90° hypoblast was able
to shift towards its own posterior side the position of the PS in a stage XIII E.G & K epiblast. The competence was determined by the angle by which a stage XIII E.G & K hypoblast rotated by 90° was able to shift towards its posterior end the PS in an epiblast of a specific stage (Table 4).

On the basis of the 90°-rotation experiments the following conclusions concerning PS induction can be drawn:

At stage XIII E.G. & K the hypoblast which does not contain any entodermal elements yet (Vakaet, 1962, 1967, 1970; Nicolet, 1970) is at the top of its inductiveness. The inductive cellular component which originates from the posterior marginal zone (Azar & Eyal-Giladi, 1979) is the apex of an anteriorly and laterally declining induction-gradient field. The competence of the epiblast, also maximal at this stage, is similarly a gradient field with a maximum at the posterior end. In normal development the apices of the two overlapping gradient fields interact to form the rudiment of the PS as an anterior continuity of the posterior marginal zone. When the hypoblast is rotated by 90° and the apices of the two fields do not overlap, the induction field is dominant and determines the location of the PS (Table 1).

The inductivity of the hypoblast although declining remains quite high throughout stages 2–2+ H & H and still exists even at stages 3–3+ H & H (Table 4). The competence of the epiblast is weak at stage 2–2+ H & H and disappears completely at stage 3 H & H thus showing a narrower temporal range.

The determination by induction of the PS during stage XIII E.G & K is still labile and can be entirely erased from the epiblast by the rotation of the hypoblast and the initiation of an ectopic induction process. However, at stage 2 H & H and later, the original induction has already some impact on the final location of the PS which will materialize at the epiblastic area which received the maximal cumulative inductive influences.

The 180°-rotation experiments have shed additional light on the pattern of both the inductiveness in the hypoblast and the competence in the epiblast. At stage XIII E.G & K only, is the rotation of the hypoblast by 180° capable of causing in a few cases a larger than 90° deviation of the PS (Table 3). This indicates that the competence of the epiblast rapidly regresses in a posterior direction during stage XIII E.G & K (Fig. 2, column II), and by stage 2 H & H it is already limited to the posterior half.

The posteriorly located inductive component of the hypoblast participates in the anteriorly directed ‘fountain-like movement’ of the lower layer (Spratt & Haas, 1960b) and in this manner, perhaps, causes a similar shift of the induction gradient field (Fig. 2, column III). A normal PS gradually forms from the most posterior side of the epiblast anteriorly, occupying about two-thirds of the epiblast’s diameter, but never reaching the most anterior side. This strengthens our interpretation expressed above and suggests that the anterior end of the PS is determined by the most anterior point at which a still inductive hypoblast interacts with a still competent area of the regressing epiblastic field.
Fig. 2. Changes in competence of epiblast (vertical column II) and inductivity of hypoblast (vertical column III) during stage XIII of the chick. The interaction of epiblast and hypoblast resulting in PS formation is shown for normal position in vertical column I, for a 90° rotated hypoblast in column IV, and for 180° rotated hypoblast in column V. Density of lines - intensity of competence; density of dots - intensity of inductivity; arrow with continuous line - the only possible or preferable position for PS; arrow with broken line - an alternative position for PS with lesser probability.
In the 180°-rotation experiments the number of right side PSs exceeded that of the left side ones (Table 3). This can be explained by assuming that both competence and inductivity are asymmetric, being more pronounced on the left side of the gradient fields concerned (Fig. 2, columns II and III). Because the 180° rotation of the hypoblast probably took place (except for one case) after the onset of the posteriorly directed regression of epiblastic competence, there were in every blastoderm two anterior (left and right) still competent epiblastic regions in close proximity to the marginal zone (Spratt & Haas, 1960a) interacting with a relatively strong hypoblastic inductive area (Fig. 2, Vb and Vc arrows).

The presence of a well-developed centrally positioned hypoblast seems to permit the formation of only one PS. The choice had therefore to be made between the right side of the blastoderm, the competence of its epiblast being lower but the inductivity of the apposed turned hypoblast higher, and the left side in which a reverse situation operated. The fact that there were more right side PSs indicates that, for PS determination, the strength of inductivity is more important than the strength of competence. The concept of asymmetry is in line with previous reports on the left side dominance of various developmental trends in both young (Eyal-Giladi & Spratt, 1964, 1965; Eyal-Giladi, 1969, 1970) and older (Rudnick, 1932; Rawles, 1936, 1943; Mulherkar, 1958) blastoderms.

Waddington (1932) described the phenomenon of head-to-head twins after a 180° rotation of the hypoblast. This was never observed in our experiments, perhaps due to differences in technique. Waddington cultured the blastoderms ventral side down by placing the epiblast on top of the small, curling hypoblast, thus rendering any further manipulation impossible. We cultured blastoderms ventral side up and were able to move the hypoblast until it occupied a proper central position. The relative position of the hypoblast proved important in another study (Eyal-Giladi, unpublished) in which the hypoblast was placed eccentrically so that part of the area pellucida remained uncovered. Twin embryos developed in this case, one at the epiblastic area covered by the original hypoblast, the other in the originally naked area covered later by a regenerative hypoblast. The hypoblastic-gradient-field thus seems to have a regulatory influence which insures the induction of a single PS. The development of twins is possible only when two independent hypoblasts and induction fields develop side by side, which seems to be the case in Waddington's experiments.

A separate point which needs clarification is the fact that in some of the blastoderms the hypoblast of which was rotated by 90° and the PS showed deviation only by 45° or less from the epiblastic axis, there was an angle between the PS and embryo proper, the latter tending to approach the axis of the hypoblast. It seems as if during axis formation the migration of the mesodermal layer from the streak, was influenced by the ‘fountain-like movement’ inside the hypoblastic sheet, probably proceeding according to the hypoblast's original
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polarity, which formed an angle of 45° or more to the migratory direction of the mesoderm.

The induction and stabilization of the antero-posterior polarity in the chick blastoderm is a stepwise process starting with the effect of gravity (Kochav & Eyal-Giladi, 1971) which manifests itself by the orderly formation of the area pellucida (Eyal-Giladi & Kochav, 1976; Eyal-Giladi & Fabian, 1980; Kochav, Ginsburg & Eyal-Giladi, 1980). The formation of the hypoblast during the first hours of incubation follows the same orientation and respectively determines through a polar induction process which starts at stage XIII E.G & K the orientation of the PS. The inductive component of the hypoblast is of marginal zone origin (Azar & Eyal-Giladi, 1979) is initially located at the posterior end and moves during the inductive period anteriorly. It creates in the hypoblast an asymmetric gradient field with an apex at the posterior side and a dominance of the left side. In the epiblast there is a similar gradient field of competence which regresses rapidly in a posterior direction during the inductive period. The inductiveness of the hypoblast as well as the competence of the epiblast are maximal at stage XIII E.G & K. The competence decreases rapidly and is lost already at stage 3 H & H while inductivity is still partially retained.

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