

The retention of primary hypoblastic cells underneath the developing primitive streak allows for their prolonged inductive influence

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SUMMARY

An experimental study was made of the distribution of the primary hypoblastic cells in the lower layer of the avian blastoderm throughout primitive streak formation and until stage 10 (Hamburger & Hamilton, 1951). The primary hypoblast of stage XIII (Eyal-Giladi & Kochav, 1976) chick blastoderms was exchanged for either an [³H]thymidine-labelled similar chick hypoblast, or a quail primary hypoblast. During the entire period of primitive streak formation, the lower layer proved to be a mosaic of labelled hypoblastic and non-labelled entodermal cells (chick cells of epiblastic origin). The persistence of hypoblastic cells underneath the developing primitive streak is regarded by us as a possible way to prolong the inductive influence of the hypoblast upon the forming streak.

INTRODUCTION

The cellular composition of the lower (hypoblastic) layer of a chick blastoderm from stage XIII E.G & K (Eyal-Giladi & Kochav, 1976) onwards is complex and has a major impact on embryonic development. A stage XIII primary hypoblast comprises two different cell populations: one derived from poly-invaginated cells shed from the epiblastic layer downwards and another composed of cells derived from the marginal zone which grow as a fairly continuous shelf from the posterior end of the blastoderm anteriorly. The two populations merge to form a single sheet – the primary hypoblast (Eyal-Giladi & Kochav, 1976; Kochav, Ginsburg & Eyal-Giladi, 1980). Although we do not yet know the exact distribution of the two different cell populations within the primary hypoblast, we were able to show by ablation experiments that only cells of marginal origin possess the ability to induce a primitive streak (PS) in the epiblast (Azar & Eyal-Giladi, 1979). The maturation of a fully grown PS, followed by normal embryonic development requires the continuous presence of the lower layer underneath the epiblast, until the forming PS reaches about half of its final length (Eyal-Giladi, 1970; Eyal-Giladi & Rangini, unpublished). The growing PS is known to be the source

* Part of the doctoral thesis of Y.A.

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of both the mesoblastic layer and the definitive entodermal component of the lower layer. The definitive entodermal cells have been shown by many authors to infiltrate during the early stages of PS formation into the central area of the primary hypoblast and to push the latter's cells to the periphery of the lower layer (Bellairs, 1953*a,b*; Modak, 1966; Nicolet, 1970, 1971; Rosenquist, 1966, 1971, 1972; Vakaet, 1970; Fontaine & Le Douarin, 1977; Wolk & Eyal-Giladi, 1977; Stern & Ireland, 1981).

It seemed difficult to comprehend how the lower layer could continue to exert an inductive influence on the forming PS, while the inductive cells were believed to be pushed away to the periphery. We therefore decided to study the distribution pattern of the primary hypoblastic cells during the different stages of PS formation.

Stage XIII E.G & K chick blastoderms were used, the primary hypoblast of which was exchanged either for an [H^3]thymidine-labelled stage XIII chick hypoblast or for a similar quail hypoblast. The development of the experimental blastoderms was interrupted at various stages of development, and the location of the primary hypoblastic cells (recognized either by radioactive or quail nuclei) was determined.

MATERIALS AND METHODS

Fresh eggs laid by hybrid New Hampshire \times Leghorn hens were incubated at 38°C for about 12 h. Only blastoderms which were at the time of the operation exactly at stage XIII E.G & K (Eyal-Giladi & Kochav, 1976) were used in this study as recipients of a labelled primary hypoblast. Two parallel series of experiments were performed:

(1) Freshly laid eggs were injected with 0.1 ml Ringer's solution containing 125 mCi/ml [H^3]thymidine after which incubation proceeded for 12 h to reach stage XIII E.G & K. Blastoderms were removed from unincubated eggs and grown in culture according to New's technique (1966), on a drop of 0.1 ml fluid albumin containing 12.5 mCi/ml [H^3]thymidine, until they reached stage XIII E.G & K. Almost all the cells of the donors were found to be heavily labelled at the time of transplantation.

The recipient non-labelled blastoderms were removed from the yolk together with a large piece of vitelline membrane which was stretched over a glass ring in a Petri dish containing Ringer's solution. The primary hypoblast was removed and replaced by a thoroughly rinsed, [H^3]thymidine-labelled, primary hypoblast. Attention was paid to the anterioposterior orientation of the transplant so that it would match the orientation of the recipient epiblast. After the operation the fluid was sucked out of the ring, which was then transferred into a watch glass containing fluid albumin and further incubated in a humid atmosphere to the desired stage (New, 1966). At the end of the experiment the glass ring, upon which the vitelline membrane carrying the incubated blastoderm was stretched,

was transferred to a dry dish to which cold Carnoy fixative was carefully added. After 15 min of fixation at 4 °C it was transferred into 100 % ethanol, where the blastoderm was separated from the vitelline membrane, and rinsed again in 100 % ethanol, followed by three changes of 5 min each in amyl acetate and three 10 min changes of benzene. The blastoderm was then transferred into a mixture of paraffin: benzene at 37 °C for 30 min, followed by three changes of 30 min in paraffin at 60 °C, after which it was embedded in paraffin and sectioned serially at 8 µm. The sections were deparaffinized and immersed in distilled water. Further treatment was done in a dark room illuminated by a 15 V bulb protected by a No. 2 Wratten safe light. The slides were coated with diluted Kodak NTB₂ emulsion at 45 °C (three dips), dried for 1–2 h in a stream of air at room temperature while standing vertically on a sheet of filter paper, then put into a sealed box containing silica gel and incubated for 7 days at 4 °C. The slides were then developed in Kodak D19 high-contrast negative developer for 6 min at 18 °C, rinsed in distilled water and transferred into 18 °C Kodak fixer for 15 min, and then stained with nuclear fast red.

(2) A parallel series of chimaeric blastoderms was performed by combining stage XIII E.G & K chick epiblasts with quail hypoblasts of an equal developmental stage, the cells of which were known to contain a natural nuclear marker (Le Douarin, 1973). The chimaeric blastoderms were incubated to the desired stage, fixed in Carnoy and stained by Feulgen's method, using Schiff's reagent prepared according to de Tomasi (Pearse, 1961, pp. 822–823).

All slides were mounted with Picolyte (Turtox).

RESULTS

Controls for [³H]thymidine-labelling efficiency

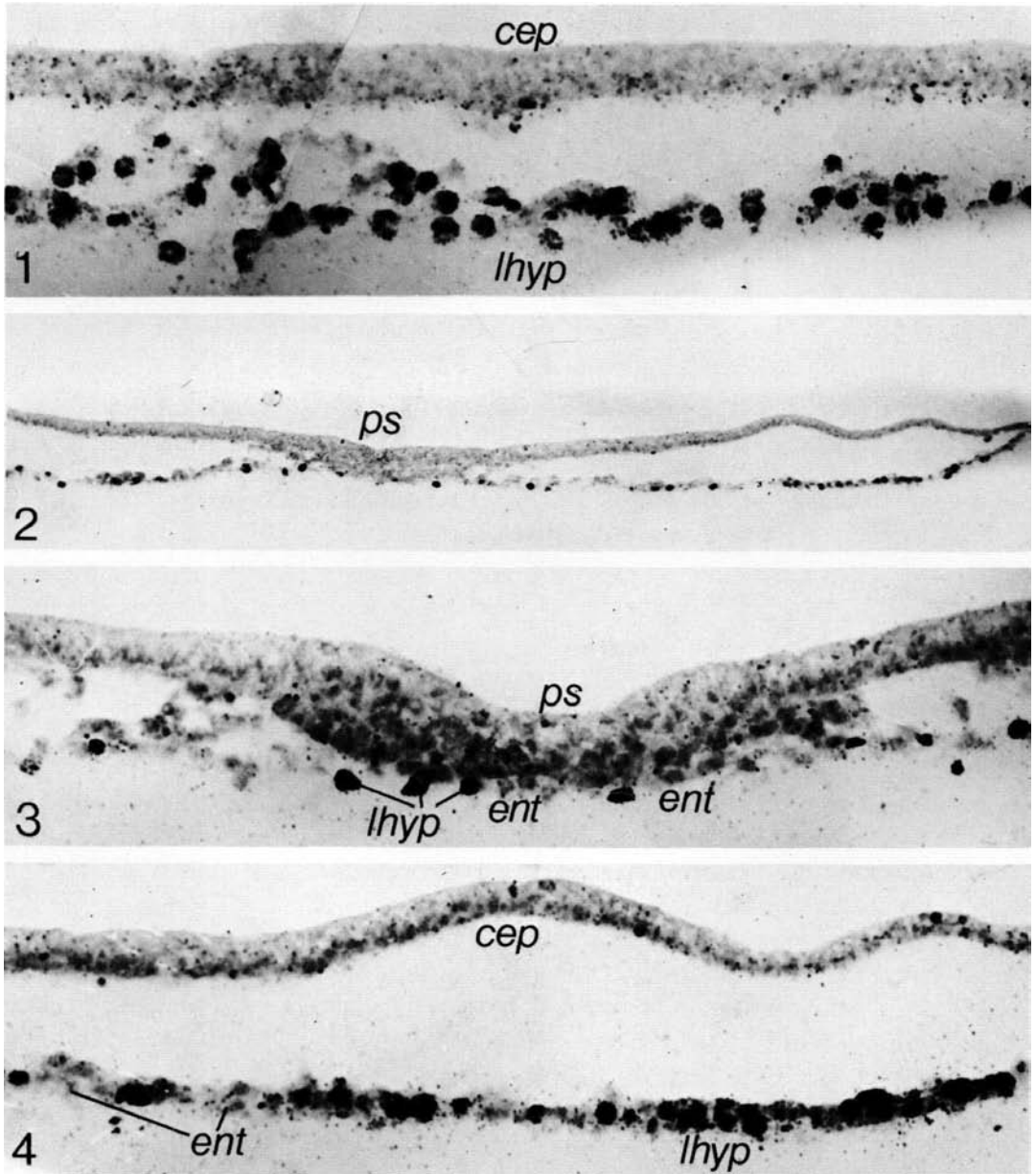
Three unincubated eggs were injected with [³H]thymidine and incubated for 10 h, after which the blastoderms were excised from the yolk together with a large circular piece of the vitelline membrane. They were rinsed several times with fresh Ringer's solution after which they were cultured on non-labelled liquid albumin until they reached stage 8 H & H (Hamburger & Hamilton, 1951), which was the latest studied in the experimental series.

Three other blastoderms, recovered from unincubated eggs, were cultured *in vitro* for 12 h on an [³H]thymidine-labelled culture medium. They were then removed from the medium, washed by several changes of fresh Ringer's solution, transferred to a fresh non-labelled medium for further incubation until they also reached stage 8 H & H.

All six blastoderms were fixed, serially sectioned and treated according to the above procedure for autoradiography. All their nuclei were found to be labelled even after the relatively long period of incubation on a non-labelled medium (Fig. 5).

The experimental series

The group of blastoderms with a non-labelled chick epiblast and an [H^3]-thymidine-labelled chick hypoblast consisted of 17 blastoderms, while the group of chimaeric blastoderms, which comprised a chick epiblast and a quail hypoblast, contained nine specimens.



Figs 1-4

The above blastoderms were incubated after the operation for varying lengths of time, and eventually reached final developmental stages ranging between 2–8 H & H. One blastoderm only was incubated to stage 10 H & H. The results of both experimental groups proved to be identical and were therefore pooled, treated as a single experiment and summarized in Fig. 8. Between stages 2–4 H & H the primitive streak gradually evolves from a posteriorly located, vaguely outlined, short and broad thickening, into the well-defined and mature primitive streak. During this entire period, cells of epiblastic origin migrated downwards into both the middle and lower layer. However, an appreciable number of scattered labelled primary hypoblastic cells was found throughout this entire period, underneath the PS. The central area of the lower layer thus turned into a mosaic of intermingled labelled primary hypoblastic cells and non-labelled definitive entodermal cells (Figs 2, 3). The peripheral belt of the lower layer contained primary hypoblastic cells only (Figs 1, 2, 4).

At stages 5–7 H & H the primary hypoblastic cells have disappeared from the central area of the lower layer and form a peripheral belt (Figs 6, 7).

The central area of the lower layer, including the area underneath the PS, is occupied by non-labelled definitive entodermal cells. At stage 10 H & H the primary hypoblastic cells disappear from the posterior side and are condensed into a sickle-shaped area at the anteriolateral margins of the lower layer (Fig. 8).

DISCUSSION

Several investigators have studied the migration of the entodermal cells from the epiblast, through the primitive streak, into the lower layer. Their main purpose was to map the different epiblastic and primitive streak regions which contribute to the formation of the various parts of the embryonic gut. These studies included vital staining and particle marking (Hunt, 1937; Spratt & Haas, 1960; Vakaet, 1962; Modak, 1966), transplantation of [H^3]thymidine-labelled primitive streak fragments into intact non-labelled primitive streaks, and determination of the position of the labelled cells after a relatively long incubation

Fig. 1. Transverse section through anterior part of a stage-2⁺ H & H blastoderm composed of stage XIII E.G & K non-labelled chick epiblast (cep) and a stage XIII [H^3]thymidine-labelled hypoblast (lhyp). Epiblastic nuclei are not labelled whereas hypoblastic nuclei are intensely labelled. Mag. $\times 360$.

Fig. 2. Same blastoderm as in Fig. 1. An overall view of a more posterior level. Section passing through the forming primitive streak (ps). Mag. $\times 100$.

Fig. 3. Central part of Fig. 2. Epiblastic nuclei are not labelled (ent). In the lower layer labelled primary hypoblastic nuclei (lhyp) are situated among non-labelled nuclei of epiblastic origin. Note four labelled nuclei underneath the ps. Mag. $\times 360$.

Fig. 4. The right lateral part of Fig. 2. Nuclei of the peripheral belt of lower layer are labelled (lhyp). Towards the centre (arrow) a few non-labelled nuclei (ent) can be seen among the labelled ones. cep = epiblast. Mag. $\times 360$.

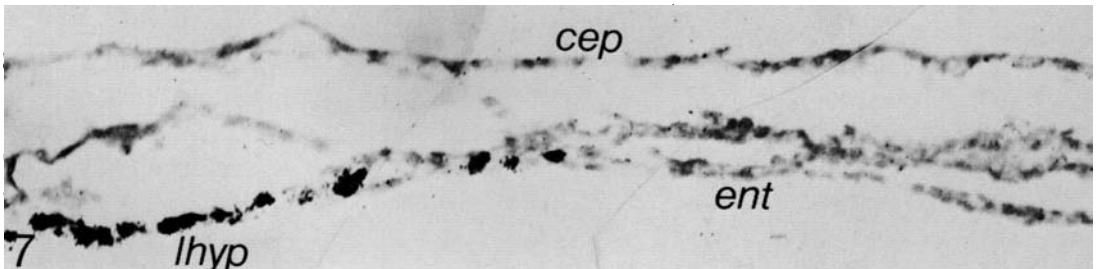
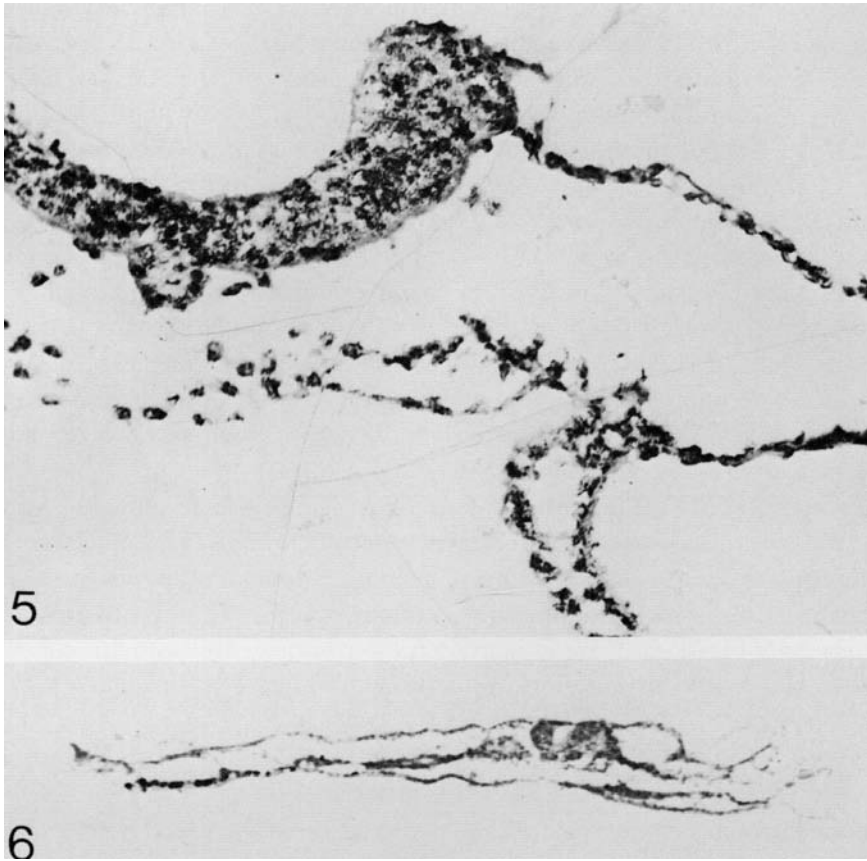


Fig. 5. Transverse section of a control blastoderm (stage 6 H & H) to show efficiency of labelling. The blastoderm was incubated with $[H^3]$ thymidine until stage XIII E.G & K, thoroughly rinsed and further incubated *in vitro* without label for 24 h. All nuclei appear to contain the radioactive label, although its intensity is less than when grown for a shorter period. Mag. $\times 360$.

Fig. 6. Transverse section of a stage-6 H & H experimental embryo. Mag. $\times 100$.

Fig. 7. Same as Fig. 6. Labelled primary hypoblastic nuclei (lhyp) concentrated at the periphery on the left side. More centrally located lower layer nuclei (ent) are not labelled. cep = epiblast. Mag. $\times 360$.

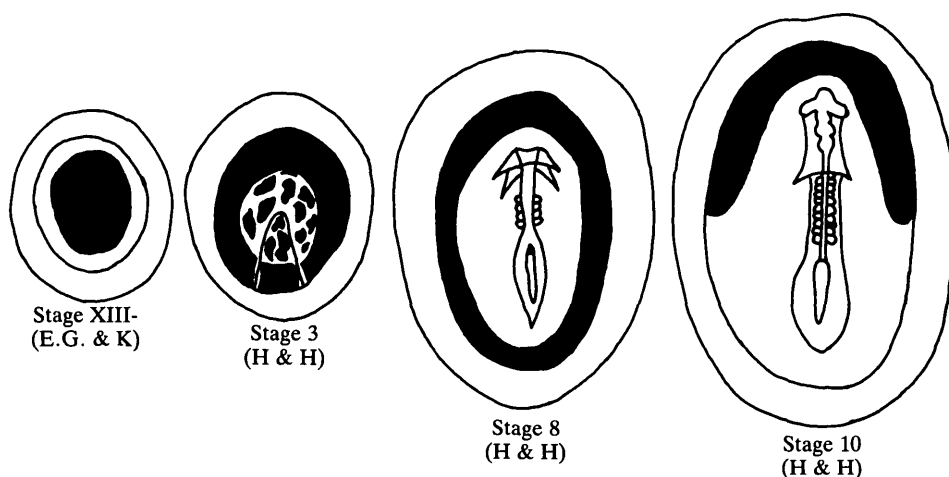


Fig. 8. A diagram showing the positions of the stage XIII (E.G. & K) primary hypoblastic cells (black) during incubation through stages 3-10 (H & H).

period (Modak, 1966; Rosenquist, 1966, 1970, 1971; Nicolet, 1970, 1971). A different method used for the same purpose was that of chick-quail chimaeric embryos (Fontaine & Le Douarin, 1977). All the above authors agree that the cells constituting the gut entoderm in the chick move down from the epiblast via the primitive streak into the centre of the lower layer and spread there anteriorly, laterally and posteriorly. Vakaet (1970), relying on his cinematographic studies, proposed a scheme for the distribution of the different components constituting the lower layer, according to which at stage 3⁺ H & H the anterior part of the PS was underlain by a coherent entodermal sheath.

The induction of the PS from the epiblast was shown to be an action of the primary hypoblast starting at prestreak stages (Eyal-Giladi & Wolk, 1970; Azar & Eyal-Giladi, 1979, 1981). However, in order to achieve the formation of a mature PS, capable of proceeding with normal differentiation and formation of axial mesodermal organs, the interaction between the epiblast and the inductive hypoblast has to continue at least until the PS reaches half of its final length (Eyal-Giladi, 1970; Eyal-Giladi & Rangini, unpublished). There seems to be a contradiction between the two kinds of information mentioned above. If the inductive influence of the primary hypoblast underneath the growing PS is indispensable, how can this be reconciled with the hitherto commonly accepted notion that, during the above stages, the invagination of the pregut entoderm already takes place via the PS and the inductive primary hypoblastic cells are pushed to the periphery? We therefore designed our experiment in such a way that the invaginating non-labelled cells would penetrate into a 100 %-labelled primary hypoblastic layer. By interrupting this process during the different stages of PS formation, we hoped to be able to determine the distribution of the labelled hypoblastic cells within the lower layer at any given time. The findings,

summarized in Fig. 8, show that at least until stage 4 H & H the central area of the lower layer is a mosaic of hypoblastic and entodermal cells, whereas the peripheral ring of the lower layer, including its most posterior region underlying the posterior part of the PS, comprises primary hypoblastic cells. These findings agree very well with the *in vitro* results of Sanders, Bellairs & Portch (1978) who studied the interaction of the following explant pairs in culture: hypoblastic explant versus another hypoblastic explant, entodermal explant versus another entodermal explant and an hypoblastic explant versus an entodermal explant. These authors found that in the first and last combinations, the cells from both cultures intermingle readily, while entodermal cells confronted with their own kind are contact inhibited. In an intermingled culture of hypoblast-entoderm, the entodermal cells tended to remain in place, while the hypoblastic cells tended to move around the former until complete sorting out was achieved, with the hypoblastic cells forming a belt around the entodermal cells. This is exactly what we found to happen in normal development. Despite the invagination of the entodermal cells into the lower layer, during the early PS stages, many hypoblastic cells still remain temporarily underneath the growing PS and particularly under its most posterior part, and the hypoblastic cells probably continue to exert their inductive influence on the forming PS. These cells move out of the central area of the lower layer very gradually, probably when their inductive and supportive action is no longer required. They first withdraw from an oval central area, to form a peripheral hypoblastic belt, and somewhat later this belt splits at its posterior end into two lateral flanks which start shifting in an anterior direction, to form at stage 10 H & H an anterior crescent-like area, resembling the germinal crescent.

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(Accepted 31 May 1983)

