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Relationships between Mesoderm Induction and Formation of the Embryonic Axis in the Chick Embryo

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When a young chick embryo (blastoerm) is cut into several portions, each portion is capable of regulation, that is, it can give rise to a complete, albeit smaller embryo (see Spratt and Haas, 1960; Khaner et al., 1985; Nieuwkoop et al., 1985; Bellairs, 1986). There are, in theory, two possible interpretations of this phenomenon: The first is that cells in the embryo are pluripotent, becoming specified to their ultimate fates by cell interactions. This first interpretation explains regulation by supposing that when a fragment is separated from the embryo, cells in the fragment interact to generate the correct proportions of different cell types. The second interpretation of the phenomenon of regulation requires the embryo to consist of a fairly random mix of cells already specified to their ultimate fates, which later sort to their correct destinations. According to this, isolation of a fragment of embryo leads to sorting of the cell types to newly defined sites, but the cells do not change fates.

One problem with the second interpretation is that it does not account for our ability to construct fate maps. If embryos consist of completely random mixtures of cells of different types, there should
not be discrete regions with defined fates. However, it is possible that cells sort rather soon after becoming specified, and the mosaic arrangement of two or more cell types explains rather well the ill-defined boundaries between different regions that characterise the fate maps of most vertebrates. Nevertheless, the first interpretation is the one usually accepted; it is generally assumed that cells become determined as a result of inductive interactions with other cells, so that the correct cell types are generated at least near their ultimate locations.

GERM LAYERS AND CELL MOVEMENT PATTERNS DURING PRIMITIVE STREAK FORMATION IN THE CHICK EMBRYO

At the time of egg laying, the chick blastoderm consists of a disc, some 2 mm in diameter, comprising an inner, translucent area pellucida and an outer, more opaque area opaca. The latter region only contributes to extraembryonic structures. The first of the germ layers to be present as such is the epiblast, which is continuous over both areae opaca and pellucida. It is a one-cell-thick epithelium which soon becomes pseudostratified and columnar, the apices of its cells facing the albumen. From the center of this initial layer arises a second layer of cells, the primary hypoblast (for an explanation of the terminology see Stern, 1990). At this time, the hypoblast is no more than several unconnected islands of about 5–20 cells. However, by about 6 hr of incubation, more cells are added to it (the secondary hypoblast) and it becomes a loose but continuous epithelium. The source of these secondary hypoblast cells is the deep (endodermal) portion of a crescent-shaped region, the marginal zone, which separates areae pellucida and opaca at the future posterior (caudal) end of the embryo.

The embryo now consists of two layers: the epiblast proper, facing the albumen, from which will arise all of the embryonic tissues, and the hypoblast (primary and secondary), facing the yolk, which will give rise only to extraembryonic tissues (mainly the yolk sac stalk) although it may also contain some primordial germ cells (see Bellairs, 1986; Ginsburg and Eyal-Giladi, 1987). As the hypoblast continues to spread as a layer from posterior to anterior parts of the blastodisc, cells appear between the other two layers. These are the first cells of the mesoderm (Vakact, 1984). As more of these accumulate, they coalesce to form the first axial structure of the embryo, the primitive
streak, which makes its appearance at the posterior margin of the area pellucida after about 10 hr of incubation (see Bellairs, 1986 for review). Later, more mesodermal cells are recruited into the primitive streak as this structure elongates along the anteroposterior axis of the embryo (Vakaet, 1984). All the mesoderm eventually migrates out of the streak to give rise to the lateral plates, intermediate mesoderm, paraxial (somitic) mesoderm, and the axial notochord.

Soon after the primitive streak forms, some of its cells begin to insert into the hypoblast, displacing it toward the edges of the area pellucida. These primitive streak-derived, endodermal cells form the definitive endoderm, which will give rise to the lining of the gut. The elongation of the primitive streak and the expansion of the blastodisc, together with further recruitment of cells derived from the posterior marginal zone (this contribution now forming the junctional endoblast, which is also extraembryonic), confine the original hypoblast to a crescent-shaped region underlying the anterior portion of the area pellucida. This region is known as the germinal crescent because it contains, transiently, the primordial germ cells, which will later migrate into the gonads (see Bellairs, 1986; Ginsburg and Eyal-Giladi, 1987).

A Note on the Staging System

Stages of development after the appearance of the primitive streak are classified according to the system of Hamburger and Hamilton (1951) and use Arabic numerals. The primitive streak appears at stage 2 and reaches its full length at stage 4. Before the appearance of the primitive streak, the staging system of Eyal-Giladi and Kochav (1976) is used, in Roman numerals. The last stage in this system before the appearance of the streak is stage XIV, characterized by a full hypoblast. The hypoblast begins to form as islands at stage IX and the contribution of posterior marginal zone cells to it begins at about stage XI.

ESTABLISHMENT OF CELL DIVERSITY: EXPRESSION OF CELL-TYPE-SPECIFIC MARKERS DURING PRIMITIVE STREAK FORMATION

The apparent complexity of the processes that lead to germ layer formation and of the subsequent movements that rearrange cells leads us to address the questions: when do different embryonic cells

become committed to their fates, and what are the mechanisms responsible?

To determine the time during embryonic development at which cell diversity becomes established, it is advantageous to possess cell-type-specific markers that allow different cell types to be identified as soon as possible after their appearance. Until recently, no such markers were available in the avian embryo; different cell types could only be recognized by their morphology and their position. As a result of an exhaustive search, several molecular markers for different tissues or regions of the primitive streak-stage embryo have been reported (Stern and Canning, 1988).

In our recent studies, we have concentrated mainly on the expression of immunoreactivity with monoclonal antibody HNK-1. This antibody was originally raised by Abo and Balch (1981) against a cell surface determinant on human natural killer cells. Since then, it has been used extensively as a marker for cells of the developing peripheral nervous system of avian embryos, particularly neural crest cells (e.g. Tucker et al., 1984, 1988; Rickmann et al., 1985; Bronner-Fraser, 1986; Stern et al., 1986). At early stages of chick development, HNK-1 recognizes the cells of the hypoblast from the time they ingress from the overlying epiblast to form the islands of the primary hypoblast. It also recognizes the cells of the hypoblast and their precursors in the posterior marginal zone (Canning and Stern, 1988; Stern, 1990). The cells of the hypoblast remain HNK-1-positive even after they have become confined to the anterior germinal crescent.

As the primitive streak makes its appearance, the antibody recognizes all of the middle layer cells; these cells continue to express immunoreactivity until the end of primitive streak formation (stage 4), when some of them leave the streak region. However, just before streak formation (stages XII–XIV), a mosaic of HNK-1-positive and -negative cells is found in the epiblast; positive cells gradually become concentrated at the posterior end of the blastodisc and eventually disappear from the epiblast as the primitive streak forms. This finding led to the suggestion (Canning and Stern, 1988; Stern and Canning, 1990), which will be discussed in detail below, that the HNK-1-positive cells of the epiblast prior to streak formation are the progenitors of the (also HNK-1-positive) cells of the streak itself, which will give rise to the mesoderm and endoderm of the embryo.

At later stages of development, the notochord, splanchnopleural mesoderm (especially in cranial regions), neural crest cells, motor axons, and associated glial cells express the epitope (see Lim et al., 1987; Lunn et al., 1987; Canning and Stern, 1988; Tucker et al.,

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In addition, other structures, like the posterior half of the otic vesicle (Lim et al., 1987; Kuratani, 1991) and portions of the developing retina and lens and of the aortic endothelium, also express immunoreactivity (unpublished observations). It has been suggested (Canning and Stern, 1988) that HNK-1 immunoreactivity characterizes cells involved in inductive interactions during development.

NATURE OF MACROMOLECULES CARRYING THE L2/ HNK-1 EPITOPE AND THEIR POSSIBLE INVOLVEMENT IN GASTRULATION

The epitope recognized by the HNK-1 antibody has been studied extensively. It is a complex, sulfated carbohydrate structure (Chou et al., 1986) known as L2/HNK-1 and is common to many molecules thought to play a role in cell–cell or cell–substrate adhesion (Kruse et al., 1984, 1985; Keilhauer et al., 1985; Bollensen and Schachner, 1987; Cole and Schachner, 1987; Hoffman and Edelman, 1987; Pesheva et al., 1987; Hoffman et al., 1988; Küнемund et al., 1988). They include neural cell adhesion molecule (NCAM), neural–glial cell adhesion molecule (NG-CAM) (also known as G4 in the chick and L1 in the mouse), a chondroitin sulfate proteoglycan receptor for cytotactin (cytotactin-binding proteoglycan; CTB-proteoglycan), the adhesion molecule on glia (AMOG), and the laminin/fibronectin receptor, integrin. It is also present on other molecules expressed in association with the nervous system such as the glycoprotein P0, myelin-associated glycoprotein (MAG), myelin basic protein (MBP), and the enzyme acetylcholinesterase (Bon et al., 1987). The epitope has also been reported to form part of certain complex glycolipids (e.g., Chou et al., 1986; Dennis et al., 1988).

The promiscuity of the L2/HNK-1 epitope among molecules with putative roles in adhesion has led several investigators (Keilhauer et al., 1985; Bronner-Fraser, 1987; Küнемund et al., 1988) to suggest that it may play a role in modulating the adhesive properties of its host molecules. There is, in fact, some direct evidence supporting this view: sulfated oligosaccharides contained within the epitope can disrupt cell–substrate adhesion of certain cultured cells (Küнемund et al., 1988). However, how this modulation is achieved is not yet understood. The epitope may also be involved in conferring specificity to cell recognition because the bonds between the component sugar groups may display some variation, as they do in blood-group-specific oligosaccharides (T. Rademacher, personal communication).
Because of this complexity, it becomes important to determine the nature of the molecules bearing the L2/HNK-1 epitope during early development of the chick embryo. Initial investigations (Canning and Stern, 1988; Canning, 1989, and unpublished) used affinity purification of detergent-solubilized proteins followed by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and immunoblotting with antibodies against glycoproteins known to express the epitope.

Several bands were seen in gels run from eluted material. The most prominent is a triplet of bands at about 70 kDa, which can be obtained from samples of all germ layers. Other bands are more tissue-specific; the epiblast from stage XIV embryos shows a major unique band in excess of 300 kDa. This polypeptide is recognized by antibodies against J1/tenascin and against chondroitin sulfate (Canning, 1989, and unpublished observations). This suggests that it is related or identical to the CTB-proteoglycan, which is known to contain the L2/HNK-1 epitope (Hoffman and Edelman, 1987; Hoffman et al., 1988). Antibodies directed to J1/tenascin or to chondroitin sulfate only stain the basal lamina underlying the epiblast from stage XIV. Samples of hypoblast show a more minor specific component of about 140 kDa. This reacts with mono- and polyclonal antibodies directed to G4 (the chick equivalent of L1, or NG-CAM), which is also known to carry the L2/HNK-1 epitope (see Fushiki and Schachner, 1986). In frozen sections, antibodies to G4 stain the hypoblast and the basal lamina of the epiblast as well as cells in the posterior marginal zone. Samples of primitive streak are characterized by a band of 32 kDa, which does not change in mobility upon reduction and which is not recognized by antibodies to any of the known HNK-1-related molecules. However, antiserum raised against purified 32 kDa protein eluted from SDS–PAGE gels does not show any tissue specificity (Canning, 1989, and unpublished observations).

**FATE OF HNK-1-POSITIVE CELLS**

Although we do not yet know the precise functions of the L2/HNK-1 epitope, some recent experiments have used this antibody for following the fates of HNK-1-positive cells. Expression of region-specific markers is not a sufficient criterion to decide whether cells that differ in their expression of antibody immunoreactivity are developmentally different from one another. For this reason, two simple experiments were performed to establish whether the HNK-1-
positive cells found in the epiblast prior to primitive streak formation are in fact the precursors of the (HNK-1-positive) cells of the primitive streak, which in turn give rise to the mesoderm and embryonic endoderm (Stern and Canning, 1990).

Fate of HNK-1-Positive Cells of the Epiblast

In the first experiment, the HNK-1 antibody was coupled directly to colloidal gold particles. Living embryos (stage XIII) from which the hypoblast had been removed were incubated briefly in this reagent, during which time the HNK-1-positive cells become labeled; during subsequent incubation at 37°C these labeled cells endocytose the antibody–gold complex and subsequently can be recognized by the presence of gold particles in their cytoplasm. After further incubation, the fates of the cells that were HNK-1-positive at the time of labeling can be identified. It was found that gold-labeled cells are found in the primitive streak and its mesodermal and endodermal derivatives, but not in the epiblast. This result suggests that the HNK-1-positive cells of the epiblast found prior to primitive streak-derived mesendoderm.

The second experiment of Stern and Canning (1990) was designed to test whether the HNK-1-negative cells of the epiblast have the ability to contribute to the primitive streak in the absence of HNK-1-positive cells. Embryos at stage XIII (before streak formation) without their hypoblasts were incubated in a mixture of the HNK-1 antibody and guinea pig complement (Stern et al., 1986) to kill the epiblast HNK-1-positive cells. Subsequent incubation of the embryo led to normal expansion of the treated embryo, but no axial structures formed. However, such embryos could be rescued by a graft of quail primitive streak cells. In this case, a normal axis formed, and the mesoderm and endoderm of such chimeric embryos was composed entirely of quail cells. This result suggests that the HNK-1-positive cells of the epiblast at stage XIII are necessary for the formation of a normal primitive streak.

Fate of the HNK-1-Positive Cells of the Deep Posterior Marginal Zone

In another series of experiments (Stern, 1990), the fate of the HNK-1-positive cells of the deep posterior marginal zone was followed using the HNK-1–gold technique in combination with grafting. A donor embryo was incubated in the HNK-1–gold reagent and its deep
posterior marginal zone grafted into an unlabeled recipient at the same stage of development (stage XII). The gold-labeled cells contributed to the hypoblast, but not to other tissues. When the HNK-1-positive cells of the posterior margin were ablated with a mixture of HNK-1 and complement, the grafted margin did not contribute to the hypoblast. These experiments suggest that the deep posterior marginal zone contains special, HNK-1-positive cells which are precursors of the hypoblast and that the remaining (HNK-1-negative) cells are unable to contribute to this germ layer.

MARGINAL ZONE: ORGANIZER OF THE EARLY EMBRYO

In 1933, C. H. Waddington (see also Azar and Eyal-Giladi, 1981) rotated the hypoblast of a prestreak chick embryo to reverse its anteroposterior orientation. He found that the primitive streak now formed from the opposite (anterior) margin of the blastodisc and proposed that the hypoblast induces the primitive streak.

Spratt and Haas (1960) demonstrated the importance of the posterior marginal zone in the formation of the embryonic axis, based on a study of the regulative ability of isolated pieces of blastoderm. They postulated the existence of a gradient in embryo-forming potential along the circumference of the marginal zone ring, with its highest point at the posterior margin. Since then, Eyal-Giladi and colleagues have expanded on this suggestion (Eyal-Giladi and Spratt, 1965; Azar and Eyal-Giladi, 1979; Mitrani et al., 1983; Khaner et al., 1985; Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989); they found that rotation of the marginal zone about its anteroposterior axis resulted in reversal of the orientation of the streak, as Waddington had found after hypoblast rotation. They also suggested that the posterior marginal zone acts both to induce the primitive streak and to prevent the formation of secondary axes (Eyal-Giladi and Khaner, 1989).

However, the epiblast of the early chick embryo can give rise to mesoderm cells even in the absence of both hypoblast and marginal zone (Mitrani and Shimoni, 1990; Stern, 1990). It therefore seems unlikely that the interaction between marginal zone or hypoblast and epiblast represents an induction of mesoderm by these tissues as defined by Gurdon (1987). It is clear, nevertheless, that the marginal zone is required both for the formation of a primitive streak with normal morphology which undergoes normal elongation and to

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prevent the formation of secondary axes (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989), but not for the differentiation of mesodermal cells. These observations emphasize the importance of distinguishing between induction of the mesoderm and induction of the primitive streak, a distinction that has not always been made in the literature of either amniotes or amphibians.

INDUCTION OF THE PRIMITIVE STREAK: A REVISED VIEW

To accommodate the above findings, I suggest a new scheme for the formation of the primitive streak in the chick embryo. The following experimental conclusions have to be accounted for in this model: (a) the epiblast at stage XII–XIII will give rise to mesoderm cells in the absence of hypoblast or marginal zone; (b) rotation of the marginal zone or of the hypoblast results in reversal of the anteroposterior orientation of the primitive streak and resulting embryo; (c) HNK-1-positive cells are the precursors of cells of the primitive streak, at least at early stages of development, and are required for the formation of this structure; (d) the deep posterior marginal zone contributes to the hypoblast and junctional endoblast from the HNK-1-positive cells of its deep layer and to the epiblast portion of the streak from the epiblast layer; (e) ingress of presumptive mesodermal cells into the primitive streak occurs in two stages: first as a distributed polyvinson in a large region of the blastoderm and then as part of localized invagination of epiblast at the primitive streak (see Vakaet, 1984).

Based on these premises, I suggest that primitive streak formation occurs in three stages, and that this axial structure contains cells derived from three different sources. First, HNK-1-positive cells, which appear as a randomly distributed population in the epiblast, ingress individually or in small groups into the interior of the blastoderm, starting at stages XII–XIII. In the second stage (stages XIV–X), these cells accumulate at the posterior margin of the blastoderm, where they interact with the overlying epiblast. I propose that this interaction is required in order to allow the cells of the epiblast of this region to undergo a process analogous to the convergent extension of the amphibian marginal zone cells (Keller and Danilchik, 1988) which leads to elongation of the primitive streak. When the cells of the epiblast portion of the streak, derived from the posterior margin, have reached maximum elongation along the future anteroposterior
axis (stage 3*), the third and final stage begins: a groove develops in the center of this structure and this is accompanied by the dissolution of the basement membrane underlying this region of epiblast. This allows further cells (many of which must be HNK-1-negative, because at this stage almost no positive cells remain in the epiblast) to be recruited into the primitive streak, where they will mix with the earlier HNK-1-positive cells and will contribute, together, to the embryonic mesoderm and definitive endoderm.

However, the above model does not explain three features: first, it fails to provide a mechanism for the convergence of the early HNK-1-positive epiblast cells toward the posterior marginal zone. Second, it does not account for the effects of hypoblast rotation on the orientation of the primitive streak. Finally, it does not explain why or how some cells are allocated as HNK-1-positive, early streak precursors while others are not. The following discussion addresses these three issues.

**HNK-1-Positive Cells Find the Posterior Marginal Zone: Evidence for Chemotaxis**

Given that the epiblast contains a seemingly random mixture of HNK-1-positive and -negative cells, in which the former are the precursors of the primitive streak-derived mesoderm and endoderm, how do these primitive streak cells find their destination in the embryo? One possibility is that one of the roles of the posterior marginal zone is to direct the migration of the HNK-1-positive cells to its proximity, where the primitive streak starts to form. To test this hypothesis, a collagen gel assay was used to find out if chemotaxis is involved (Stern and Jephcott, unpublished results).

A small explant of central epiblast cells was placed in the center of a collagen gel matrix and confronted on one side with an explant of posterior marginal tissue and on the other with a piece of anterior marginal tissue. It was found that cells of the central epiblast migrated toward the posterior marginal explant, suggesting that the posterior marginal zone emits a chemoattractant.

It is important to determine that it is the HNK-1-positive cells and not others that respond to this chemotactic signal. To address this, we prelabeled the central epiblast explant using HNK-1-gold complex as described above and examined the cultures using a confocal scanning laser microscope. It was found that HNK-1-positive cells dis-
played chemoattraction to the posterior margin, while HNK-1-negative cells spread evenly away from the explant.

In an attempt to establish the molecular nature of the chemotactic signal, we incorporated heparin immobilized on agarose beads into the collagen gel. This did not prevent the migration of cells from the epiblast explant, but migration was no longer directed toward the posterior margin explant. This result suggests that the chemoattractant(s) are heparin-binding molecules; it also provides an explanation for the results of Mitrani and colleagues (Mitrani and Shimoni, 1990; Mitrani et al., 1990), in which it was suggested that heparin-binding molecules (e.g., basic-FGF, activin A) play a role in the inductive effects of the posterior marginal zone.

Role of the Hypoblast in Anteroposterior Polarity of the Embryo

The hypoblast may play a role in the process discussed above and contribute to the guidance of HNK-1-positive premesendodermal cells to the posterior marginal zone. It is possible, for example, that the chemoattractant binds to its extracellular matrix, as several heparin-binding factors are known to do. Rotation of the hypoblast, which will include the gradient of attractant, will therefore, result in migration of HNK-1-positive cells in reversed direction. Thus, rotation of the hypoblast (gradient) and rotation of the marginal zone (source of these chemicals) will have the same effects.

The Origins of Cell Diversity

The experiments that have been conducted to date on the early chick embryo cannot address the question of how the HNK-1-positive cells of the epiblast become different from their HNK-1-negative counterparts, because once they can be recognized they have already diversified. However, we might speculate on the type of mechanism that could lead to a constant proportion of cells, within a large population, to become different from the rest irrespective of their position in the embryo. In principle, there are two main ways in which this could be achieved: by local cell interactions, or by some intrinsic, preexisting diversity between the cells.

Local cell interactions could lead to diversification, if, for example, random cells within the population became different at some particu-
lar time in development, and then these cells inhibited their neighbors from undergoing a similar change. By limiting the range of such an inhibition, the proportions of the two cell types can be controlled. This mechanism will also prevent cells of the divergent cell type from forming large clusters and would distribute them evenly in the embryo.

For the second mechanism we have to assume some parameter that differs between different cells of the population at the time of diversification. An obvious candidate is the cell cycle. Suppose, for example, that at some time in development, a signal is produced (perhaps by the marginal zone, or by the hypoblast, or even by all cells) which is an inductor of mesoderm and which diffuses easily all over the embryo. Activin might be such an inducer (Mitrani et al., 1990; Cooke and Wong, 1991). However, competence to respond at any one time is restricted to a subpopulation of cells, for example, to those that are in one particular phase of the cell cycle.

Within a large population of cells (the early blastoderm contains about 20,000 cells), both of the mechanisms proposed above will regulate perfectly for the size of the embryo, even within very large limits. I suggest that most vertebrate embryos will be found to contain restrictions to the competence of cells for inductive signals and that it is these restrictions that are primarily responsible for embryonic size regulation during mesoderm induction.

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REFERENCES


