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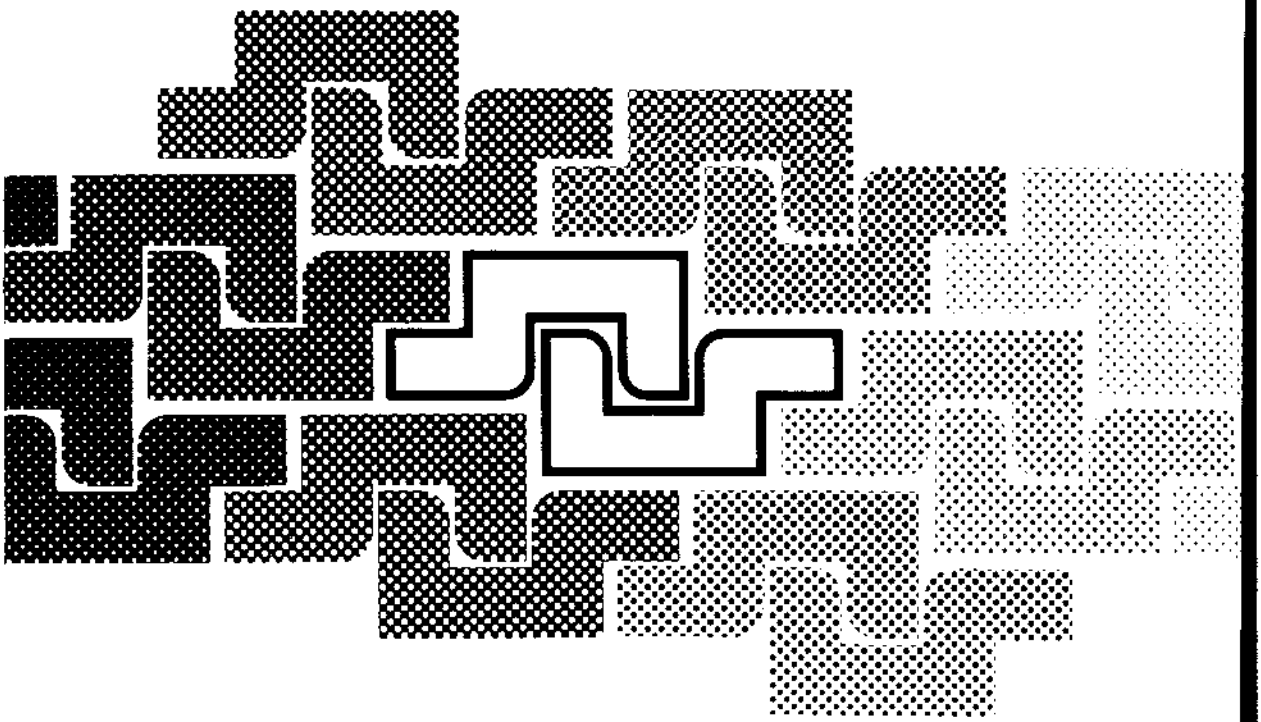

Formation and Differentiation of Early Embryonic Mesoderm

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EVIDENCE FOR STEM CELLS IN THE MESODERM OF HENSEN'S NODE AND THEIR ROLE IN EMBRYONIC PATTERN FORMATION

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In the chick embryo, both mesoderm and endoderm are derived from a single germ layer, the epiblast, through the primitive streak, which is the first axial structure to develop. The streak arises after about 8 hours' incubation, at stage 2 (Hamburger and Hamilton, 1951), as a thickening at the future caudal end of the blastoderm, just beneath the epiblast. It elongates cranially over the next 9 hours, while a groove appears along its length in the midline; cells migrate laterally from it to form the early mesoderm and the definitive (gut) endoderm. At the definitive streak stage (16-18 h of incubation; stage 4), the streak extends about three-quarters of the way across the area pellucida, with the primitive groove terminating rostrally in a pit. Anterior to this pit lies a mass of cells, termed Hensen's node, after Viktor Hensen who first described it in 1876.

Many lines of evidence suggested Hensen's node as the "organiser" of the amniote embryo, since it can induce the formation of a second embryonic axis if grafted into a host embryo, much like the dorsal lip of the blastopore in the amphibian (for reviews see Leikola, 1976; Hara, 1978; Nieuwkoop et al., 1985; Slack, 1991). Wetzel (1924) found that damage to Hensen's node prevented correct somitogenesis. His chorioallantoic grafts of portions of the early blastoderm showed that head or trunk structures developed only when the fragment of the blastoderm cultured contained Hensen's node. Furthermore, when a node is grown on the chorioallantoic membrane, its cells can autonomously differentiate into a number of cell types (Hunt, 1931; Willier and Rawles, 1931; Viswanath and Mulherkar, 1972; Leikola, 1975, 1978; Veini and Hara, 1975). Direct evidence that Hensen's node is the organiser of the avian embryo came from Waddington (1932; 1933) and Waddington and Schmidt (1933) who grafted Hensen's node from a donor embryo to the lateral margin of a host embryo and found that a second neural tube was induced from the host ectoderm. This result compares directly with the findings of Spemann and Mangold in their original organiser experiment, and has since been confirmed by many workers (Vakaet, 1965; Gallera, 1971; McCallion and Shinde, 1973; Dias and Schoenwolf, 1990). Furthermore, when the node is grafted into the anterior margin of the developing limb bud, it can induce supernumerary digits (Hornbruch and Wolpert, 1986; Stocker and Carlson, 1990).

The node has been studied by various fate mapping techniques such as marking with carbon particles, vital stains or tritiated thyimidine (Spratt, 1955; Rosenquist, 1983). However, these techniques are not always reliable because, for instance, one cannot be sure that the marker remains with those cells originally labelled. Furthermore, the resolution of these techniques is poor since only large groups of cells can be labelled. We have used new fate mapping techniques to generate more refined fate maps (Selleck and Stern, 1991). The carbocyanine dye, DiI, was used to label groups of cells in Hensen's node and the lineages of single cells within the node were also studied by intracellular iontophoretic injection of Lysine-Rhodamine-Dextran (LRD). The findings showed that at the definitive streak stage

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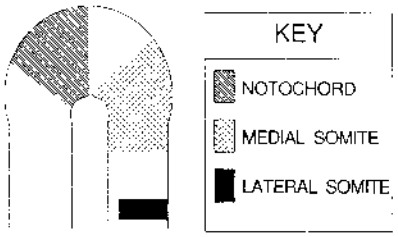

Fig. 1. Diagram illustrating the fate of mesoderm cells within different regions of Hensen's node and rostral primitive streak at the definitive primitive streak stage. In a ∇-shaped midline region of the node, mesoderm cells will contribute only to notochord, while in more lateral regions, cells will contribute to the medial halves of somites. The dorsal part of the lateral node sector also contains presumptive notochord cells (not shown). The lateral parts of somites are derived from more caudal regions of the primitive streak.

(stage 4), prospective notochord cells are contained within a ∇-shaped medial sector, and in the dorsal parts of lateral sectors (Fig. 1). The mesoderm of the lateral sectors contains prospective medial somite cells, while the lateral halves of somites are derived from a region of rostral primitive streak situated behind the node (Fig. 1). The results obtained from injection of LRD into single cells suggest that the regions intervening between the medial and lateral sectors contain 'intermediate' cells, whose progeny can contribute to both notochord and medial half somite. At stages 3-3+, the node also contains prospective endoderm cells. At later stages (5-9), the medial ∇-sector becomes narrower as presumptive notochord cells are lost from the node, and the proportion of prospective somite cells within it increases.

Commitment of node mesoderm cells to their fates

To test the commitment of cells to their fates, it is necessary to challenge the fates of the cells in different environments. We have performed experiments to investigate whether the prospective notochord and somite cells within Hensen's node are committed by heterotopic grafting between various sectors of the node and rostral primitive streak (Selleck and Stern, 1992). The cells of the graft were labelled with DiI to follow their incorporation into different tissues; notochord was identified using a specific antibody (Not1; Yamada et al., 1991). At the definitive streak stage, prospective notochord cells appear to be committed to their fates because cells in the medial node sector always contribute to notochord, or form Not1-positive notochord-like structures when grafted into different positions in the embryo. In contrast, the presumptive somite cells are able to contribute to notochord and stain with Not1, showing that they are not committed to a somitic fate.

We were unable to determine the time at which notochord cells first become committed. It is difficult to perform grafting experiments to investigate this at stage 3 or earlier, since the notochord cells are spread throughtout the node, intermingled with other prospective cell types. However, three lines of evidence suggest that at least some prospective notochord cells become committed to their fate in Hensen's node. The first is that some individual cells in the epiblast of the node can populate both the notochord and the neural tube as late as stage 4, showing that they cannot yet be committed to either fate (Selleck and Stern, 1991). Second, the progeny of single cells lying between the medial and lateral node sectors contribute to both notochord and somite (Selleck and Stern, 1991). The third piece of evidence is that prospective somite cells in the lateral node sectors can become notochord cells when grafted into the medial sector, which suggests that mechanisms that specify cells as notochord are still operating at this stage (Selleck and Stern, 1992).

It seems likely that presumptive notochord cells become committed when they enter the medial sector of the node. What could be the mechanism? Amphibian embryos could provide part of the answer. In *Xenopus*, factors related to TGFβ, especially the activins, can

induce ectoderm cells to become notochord (Green and Smith, 1990; Smith et al., 1990; Van den Eijnden-Van Raaij et al., 1990; Green and Smith, 1991). They operate in a concentration-dependent manner: the highest concentrations give rise to notochord (Green and Smith, 1990; Smith et al., 1991) and to cells that display 'organiser' activity when mixed with untreated cells (Green and Smith, 1990). It has been suggested (Smith et al., 1985; Stewart and Gerhart, 1991) that organiser cells are responsible for the induction of notochord. If the notochord is specified in a similar way in the chick, then one might expect that cells with organiser activity will be located in Hensen's node.

If 'organiser' cells are present in the node, and serve to commit notochord cells to their fate, where do they come from? Both fate maps and specification maps of the epiblast at pre-streak stages place the presumptive notochord cells at the centre of the blastoderm (Rudnick, 1948; Waddington, 1952; Balinsky, 1975), which may indicate that these cells are already committed to notochord. However, this finding is also consistent with the view that this region contains organiser cells. When isolated, some cells in this region would form notochord, under the influence of the organiser cells. This conclusion suggests that the tip of the elongating streak between stages 2 and 3 does not yet contain organiser cells, and therefore no prospective notochord cells. These cells can only be recruited when the tip of the streak reaches the centre of the blastoderm, at about stage 3. This agrees with the findings of Nicolet (1970), who found that the tip of the streak at stage 2 does not contribute to the notochord.

The organiser cells found at the centre of the blastoderm may originate from the posterior end of the embryo, at or near the posterior marginal zone, which also appears to have 'organiser' qualities such as controlling the orientation of the primitive streak (Eyal-Giladi and Spratt, 1965; Azar and Eyal-Giladi, 1979; 1981; Mitrani et al., 1983; Khaner et al., 1985; Khaner and Eyal-Giladi, 1986; 1989; Eyal-Giladi and Khaner, 1989; Stern, 1990). Carbon particle marking experiments (Spratt, 1946) show that before the primitive streak appears, cells at the posterior end of the blastoderm migrate rostrally. This finding is compatible with a movement of organiser cells in the epiblast towards the centre of the blastoderm prior to streak formation, and may indicate that organiser cells play different controlling roles at different stages of development.

HENSEN'S NODE AND THE GENERATION OF PATTERN

Periodic contribution of node-derived cells to notochord and somite

One striking finding obtained from our single cell lineage experiments (Selleck and Stern, 1991) is that in some cases, the progeny of single LRD-labelled cells were arranged in a periodic fashion in the notochord and somites (Fig. 2a). Several groups of labelled cells could be found along the length of the notochord, each separated by about 2 somite intervals. In specimens where single cells in Hensen's node populated the somites, about 2-3 consecutive somites were labelled (Fig. 2d), with a periodicity of about 5-6 somites. A similar finding was made when DiI labelled lateral node sectors were placed in the medial sector (Selleck and Stern, 1992; Fig. 2b,c): labelled cells populated the notochord in a periodic fashion. These findings are consistent with the idea that Hensen's node may contain presumptive notochord and somite founder cells, with stem-cell-like, self-renewing ability.

Founder cells and stem cells in the node?

Further evidence that the node contains stem-cell-like cells that can give rise to notochord is the finding (Selleck and Stern, 1991) of a group of cells that can populate the entire length of the notochord, lying between the medial and lateral node sectors. These observations suggest that the node contains a population of 'notochord founder cells' (FC_N; Fig. 3), with stem cell properties: at each division of one of these cells, one daughter remains in the node and becomes the progenitor of subsequent groups of cells, whilst the other daughter gives rise to a single, more anterior group of cells. At each division of a founder cell, therefore, the ejected cell and its progeny occupy successively more posterior levels of the notochord, with clusters separated by about 2-3 somite lengths (400μm). The distance between successive clusters would be determined by the rate of division of the founder cell (FC_N), while the length of each cluster depends on the rate of division of its daughters.

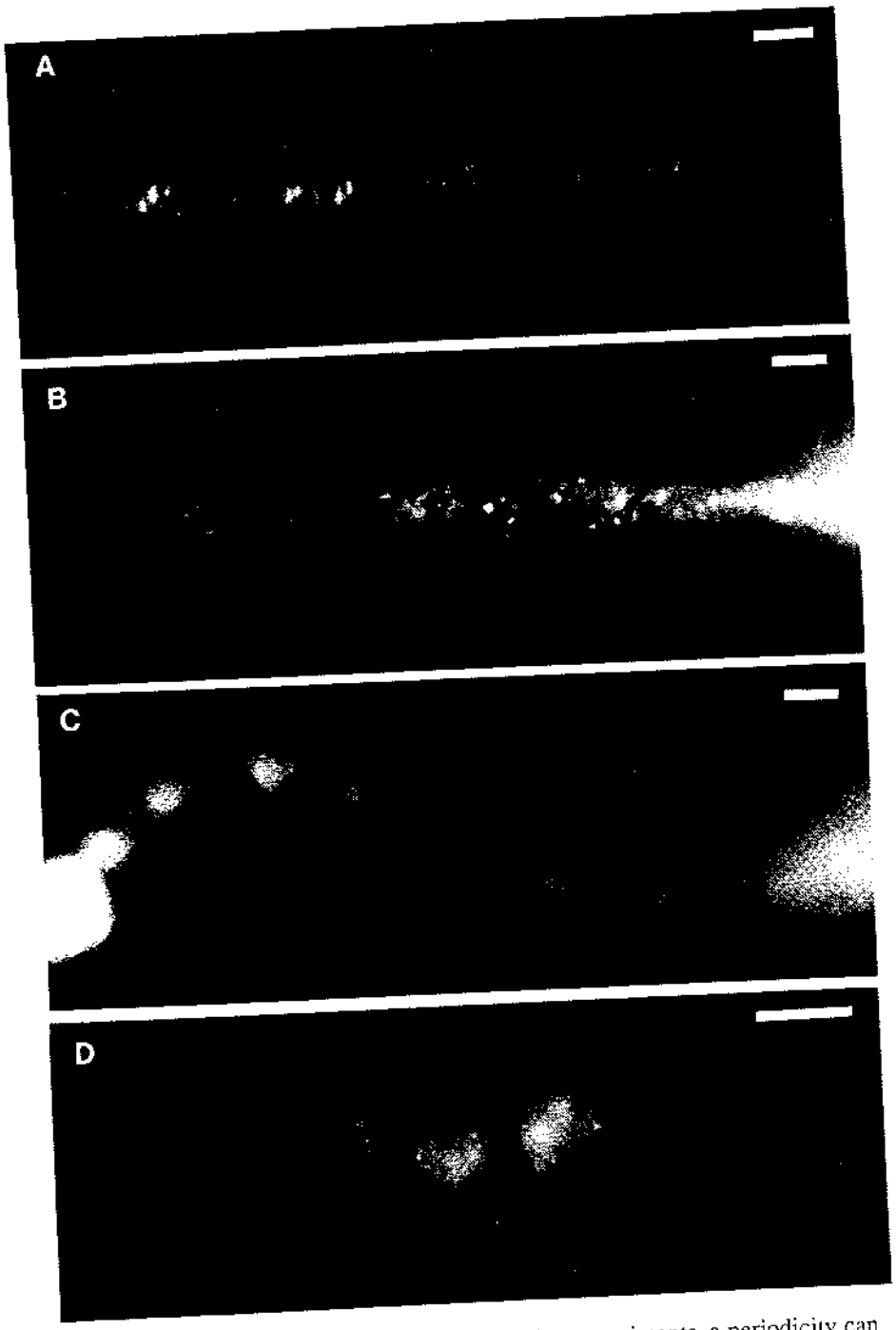

Fig. 2. In some of the fate mapping and grafting experiments, a periodicity can be seen in the allocation of cells to notochord and somite. A. Labelled progeny derived from a single node cell that has been injected with LRD can be found in several groups along the notochord. The groups are separated by about 2 somite-lengths. B, C. The same periodicity of fluorescent cells can be found when DiI-labelled medial sectors are placed into the lateral sector (B) or when lateral sectors are placed into the medial portion of Hensen's node (C). D. Single cells in the lateral node sectors typically populate 2-3 consecutive somites. Rostral to the right. Space bars= 100μm.

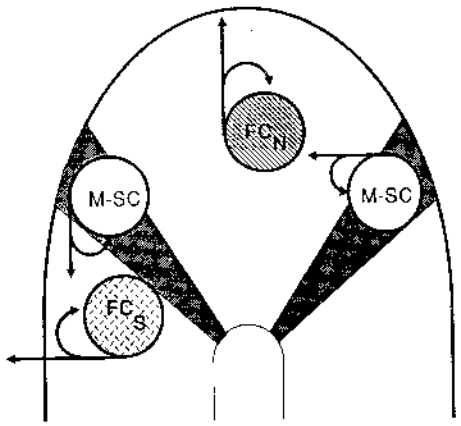

Fig. 3. Diagram illustrating a stem cell and founder cell mechanism that may control patterning of the notochord and somites. Stem cells (M-SC) lying between the medial and lateral node sectors give rise to daughter cells that populate both areas. These daughter cells are founders for prospective notochord or somite cells (FC_N and FC_S respectively). The cell cycle time of the notochord founder cells is different from that of somite founder cells. The cell cycle time of the founder cells will control the periodicty in allocation of cells to their mesoderm structures.

Medial somite cells also seem to be derived from founder cells in Hensen's node (*FC_S*; Fig. 3). Single labelled cells give rise to progeny that are similarly arranged into clusters in the medial halves of the somites, each cluster being some 2-3 somites long (about 300-500μm; Fig. 2d) and with a spacing of about 5-6 somites (800μm). The difference in spacing between clusters in notochord and somites indicates that each kind of founder cell has a characteristic mitotic rate.

Single cells lying between the medial and lateral node sectors are able to populate both notochord and somites. Therefore, these may represent a population of multipotent stem cells (*M-SC*; Fig. 3), which gives rise to the founder cells of both tissues (FC_N and FC_S). The rate of division of the daughters of M-SC may be set according to whether they are specified as notochord (FC_N) or somite (FC_S) founder cells.

Hensen's node and the control of segmentation

Many workers have investigated the role of Hensen's node in somitogenesis, but have reached conflicting conclusions as to its importance (see Bellairs, 1963; Nicolet, 1971; Stern and Bellairs, 1984; Bellairs, 1986). This may be due to the absence of detailed information about the cellular contents of the node. Moreover, some workers have assumed that the node comprises only presumptive notochord cells.

The finding (Selleck and Stern, 1991; Ordahl and Le Douarin, 1992) that somites are subdivided into medial and lateral halves, derived from two separate sources (lateral node and anterior primitive streak, respectively) suggests that this dual origin may play an important role in some aspect of somite development. However, despite a difference in fate of medial and lateral somite cells in muscle formation, they appear to be equivalent in their commitment (Ordahl and Le Douarin, 1992). Perhaps the medial/lateral subdivision of somites reflects a property required for the generation of a metameric pattern rather than for the subsequent differentiation of different cell types. Bellairs and Veini (1984; see also Bellairs, 1986) have suggested that "somitogenic clusters" might be present in the embryo, which recruit cells from unsegmented paraxial mesoderm to form somites. They propose that the number of such clusters is correlated with the number of somites that will form, and that the recruited cells make up cell numbers so that sufficient cells gather to form individual somites.

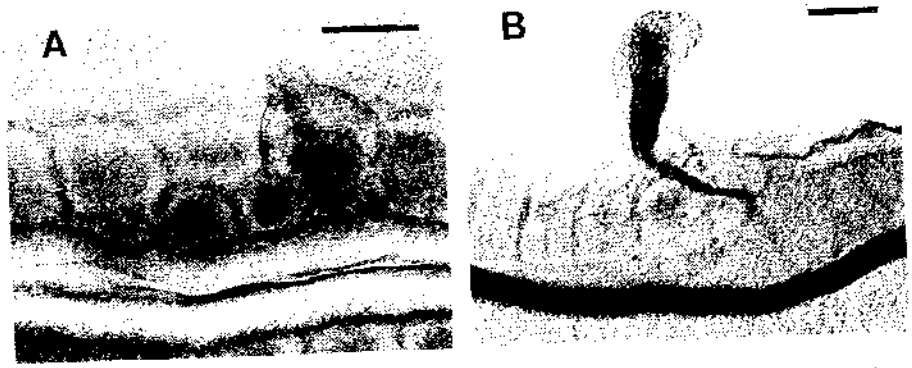

Fig. 4. When grafted into the segmental plate of a host embryo, medial and lateral node sectors behave very differently. A. Grafts of lateral node sector differentiate into small epithelial spheroids adjacent to, but not aligned with, the host somites. These spheres are like miniature epithelial somites. In contrast (B), medial node sectors autonomously differentiate into rod-like structures that stain with Not1, an antibody specific for notochord. Scale bars= 100μm.

Could the medial somite cells derived from the lateral sectors of the node contain these somitogenic clusters, and the presumptive lateral somite cells in the anterior primitive streak constitute the source of recruited cells? Transplantation experiments (Selleck and Stern, 1992) suggest that the cells of the lateral node sector are the ones that determine the spacing of the metameric pattern, because when transplanted into the segmental plate, they often form somite-like epithelial spheroids (Fig. 4a). In contrast, grafts of primitive streak or medial node sector (Fig. 4b) do not give rise to such spheres.

It therefore seems possible that the somitogenic cells in Hensen's node, which will contribute to the medial halves of the somites, are required for setting up a metameric pattern and the spacing of somites. What is the mechanism that sets up such a spacing pattern? If somite-stage embryos are subjected to brief heat-shock, periodic anomalies, separated by intervals of 5-8 somites, are seen in the somites that form after the shock (Primmett et al. 1988). Given that somites form at a rate of about one pair every 100 minutes (Menkes et al., 1961), this period corresponds to about 10 hours. Primmett et al. (1989) showed that this same period corresponds to the duration of the cell cycle of somite precursor cells. From these observations, they put forward a model to account for the periodicity of somitogenesis, based on a subdivision of the cell cycle into 7 periods, during each of which the cells destined for a somite are allocated. The last anomalies produced by a single heat shock in Primmett et al.'s (1988) experiments affect cells that segment 4-5 cell cycles after the time of the shock. Some of these cells may have been in Hensen's node (medial somite) at the time of the shock.

Thus, the medial somite precursor cells in Hensen's node may be responsible for setting up metameric pattern in the paraxial mesoderm, and the periodicity of this pattern may depend upon cell-autonomous timing mechanisms in these cells. This conclusion agrees with the above proposal that the node contains stem-cell-like founder cells (FC_S; Fig. 3). These arguments suggest that the somitic founder cells in the lateral node sectors (FC_S) are those whose cycle time is affected by heat shock, thereby affecting the periodicity of segmentation.

The conclusion that the lateral sectors of the node contain somitic founder cells equivalent to Bellairs and Veini's (1984) somitogenic clusters could also account for another result: Hornbruch et al. (1979) found that when quail Hensen's nodes were transplanted into the area pellucida of a chick host, some host cells formed somites. In contrast, grafts into the area opaca do not generate somites from the host, although small donor-derived somites develop. This finding can be explained by assuming that the area opaca, which does not contain mesoderm cells at this stage, cannot replace the cells normally derived from the anterior primitive streak (presumptive lateral half somite).

REGULATION FOLLOWING NODE EXTIRPATION

The preceding discussion has suggested that Hensen's node contains organiser cells and stem cells retained within it as it regresses, rather than being merely a passageway for ingressing cells.

One problem with this view is that node extirpation or rostral primitive streak rotation at the definitive streak stage allows more or less normal development, provided that the wound heals (Waddington, 1932; Abercrombie, 1950; Spratt, 1955; Grabowski, 1956; Gallera, 1965, 1972, 1974a,b; Gallera and Nicolet, 1974; Hara, 1978). Under appropriate conditions, such embryos form a notochord and somites. In all of these experiments, the embryo shows a remarkable ability to compensate for the loss of Hensen's node or for a large disturbance in its position or orientation. If there are special cells with organiser and stem cell properties in the node, then these cells and properties must be maintained as a result of interactions with the neighbouring cells, and these interactions must continue at least until the definitive streak stage.

CONCLUSIONS

Hensen's node appears to contain a population of organiser cells responsible for the commitment of notochord cells to their fates, and a population of stem/founder cells that contribute to somites and to the notochord. Somitic founder cells appear to be responsible for controlling the spacing of the segmental pattern of somites.

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