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Induction of a second neural axis by the mouse node

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SUMMARY

The anterior aspect of the mouse primitive streak resembles the organizer of *Xenopus* and chick in terms of its developmental fate, ability to alter pattern in the chick limb bud and with respect to the repertoire of genes that its constituent cells express. However, until now there has been no direct evidence that the mouse node organizes pattern during gastrulation, nor that the exceptionally small mouse embryonic egg cylinder can be induced to form a second axis. Grafts of transgenically marked mid-gastrulation mouse node, or node labelled with DiI, to a posterolateral location in a host embryo of the same developmental stage results in the induction of a second neural axis and the formation of ectopic somites. The graft gives rise predominantly to notochord and endoderm tissue whereas the neurectoderm and somites are mainly of host origin. The ectopic notochord formed is derived solely from the donor node which suggests that the node can serve as a 'stem cell' source of axial mesoderm. This is corroborated by the observation that labelling in situ the population of cells on the outer surface of the mid-gastrulation node with DiI results in continuous labelling of the notochord. DiI-labelled cells are present throughout the notochord from a rostral boundary in the cranial region to its most caudal extreme and the node itself always remains labelled.

Key words: organizer, mouse, node, gastrulation

INTRODUCTION

The body plan of vertebrates is thought to be established by a sequence of tissue interactions between cells in different parts of the embryo. These interactions are best understood in *Xenopus* (Melton, 1991), where explantation and transplantation experiments have demonstrated the existence of signals emanating from the vegetal pole of the early embryo that induce cells in the equatorial region to form mesoderm. As a consequence of these early events, a region at the dorsal aspect of the equator, the dorsal blastopore lip, with special organizing properties is induced. Transplantation of this region to a ventral location can induce the surrounding tissue to develop into a second axis (Spemann and Mangold, 1924). The equivalent region in avian embryos, Hensen's node, is also capable of organizing a secondary axis when transplanted to a lateral site in a host embryo (Waddington, 1932). The predominant fate of cells in both the dorsal blastopore lip and Hensen's node is to form notochord (Selleck and Stern, 1991; Smith and Slack, 1983). In mammalian embryos, the anterior aspect of the primitive streak, the node, resembles such an organizing region in terms of its developmental fate and the repertoire of genes that it expresses (Beddington, 1981a; Beddington and Smith, 1993; Blum et al., 1992; Hornbruch et al., 1979; Lawson et al., 1991; Selleck and Stern, 1991; Smith et al., 1991; Storey et al., 1992; Waddington, 1932). In addition, the anterior part of the mouse primitive streak, like Hensen's node in the chick (Hornbruch and Wolpert, 1986), can cause digit duplication when grafted into avian limb buds (Hogan et al., 1992) and induce anterior structures in *Xenopus* gastrulae (Blum et al., 1992; Kintner and Dodd, 1991). There is, however, no direct evidence in the mouse for the occurrence of a region with organizer properties during gastrulation. Nor is there evidence that mouse epiblast is competent to respond to organiser activity. The experiments described here demonstrate, for the first time, that the mammalian node is indeed functionally equivalent to an organizer in that, if transplanted heterotopically, it can induce a secondary axis during gastrulation in the mouse embryo. In addition, both grafting experiments and in situ labelling of the ventral surface of the node indicate that the node may serve as a stem cell population supplying nascent notochord tissue.

MATERIALS AND METHODS

Recovery of and culture of embryos

All host embryos and embryos to be labelled *in situ* with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate; Molecular Probes) were recovered on the morning of the 8th day of gestation from matings between outbred MF1 mice, maintained on a regime of 10 hours dark: 14 hours light, the mid-point of the dark period being 12.00 a.m. Embryos used as a source of donor tissue were recovered at the same developmental stage either from MF1 mice or from a transgenic line of mice expressing *E. coli lacZ* in all embryonic tissues of the mid-gestation embryo (Beddington and Martin, 1989). All dissections were carried out in PB1 medium (Whittingham and Wales, 1969) containing 10% fetal calf serum instead of bovine serum albumen. Both host and donor embryos were carefully selected for developmental stage and only those that had a full-length primitive streak but no distinct allantoic bud were used. The preparation of embryos for culture and the culture conditions used were as described previously (Beddington, 1987). Cultures were terminated at two time points: 20 hours and 30 hours of culture.

Preparation of graft tissue

The location of tissue used for node grafts and for control anterior epiblast grafts is shown in Fig. 1. The mouse node is not a distinctive structure at mid-gastrulation, so proteolytic digestion of the embryonic region using 0.5% trypsin, 2.5% pancreatin in Ca^{2+}-, Mg^{2+}-free Tyrodes at 4°C (Beddington, 1987), which is sufficient to separate the germ layers, was used to identify precisely the location of the node. The node is the source of nascent definitive endoderm and notochord and, because these tissues intercalate directly into the outer visceral endoderm layer, the node corresponds to the small area near the tip of the egg cylinder where enzyme treatment fails to separate endoderm from epiblast. Isolation of this region by mechanical dissection with siliconized glass needles and removal of all adherent endoderm cells yields node grafts approximately 60 μm × 60 μm, comprising approximately 100 cells. Anterior epiblast grafts were of the same size and dissected from the midline of the embryo at a point halfway between the distal tip of the cylinder and the embryonic/extraembryonic junction.

Graft tissue dissected from transgenic embryos was transplanted immediately, whereas potential node grafts from wild-type embryos were labelled for 1 minutes in 0.3 M sucrose solution containing 0.05% DiI (Serbedzija et al., 1990) and subsequently rinsed 3 times in PB1 + 10% FCS before transplantation. Grafts were performed by hand using a glass micropipette with a flame-polished tip of internal diameter 50 μm (Beddington, 1987). The pipette was braked with paraffin oil (Boots UK Ltd). The host embryo was braced between the arms of a pair of watchmakers' forceps and the mouth of the pipette positioned against the endoderm of the posterolateral region of the embryonic region. Gentle aspiration was used to hold the pipette in place. Using a rapid jabbing movement, the pipette was pushed through the germ layers into the amniotic cavity. This is sufficient to punch out a small circle of tissue, slightly smaller than the circumference of the donor node, in the side of the cylinder. The pipette was withdrawn, the circle of host tissue ejected and the donor node sucked into the mouth of the pipette followed by a small volume of medium. No attempt was made to preserve the orientation of the node. The pipette was re-inserted into the amniotic cavity through the hole in the side of cylinder and then slowly withdrawn while aspirating first medium and then graft. The node was released as the pipette passed through the germ layers of the host embryonic region and the slight pressure created from blowing medium into the amniotic cavity, and the fact that the graft was rather bigger than the hole created to put it in, was sufficient to keep the graft in position.

DiI labelling of the node in situ

The ventral surface of the node was labelled with DiI by apposing a micropipette (internal diameter 50 μm) containing a 0.05% solution of DiI in 0.3 M sucrose to the appropriate region at the anterior aspect of the streak. Due to the viscosity of the labelling solution gentle aspiration, produced a localised bolus of DiI solution approximately the same size as the node itself. The embryo was kept in contact with this bolus of label for about 10 seconds and was then transferred immediately to fresh PB1 + 10% FCS. Subsequently, labelled embryos were cultured for 24 hours before fixation in 4% paraformaldehyde and inspection with rhodamine filter epifluorescence optics in a compound microscope (Vanox, Olympus). Embryos were photographed using Kodak 1600P film.

Analysis of grafted embryos

The colonisation pattern of donor cells was visualized after staining for bacterial β-galactosidase activity or epifluorescent identification and photobleaching of DiI-labelled donor cells. Embryos that had received a transgenic graft were rinsed in PBS before fixing and staining for *E. coli* β-galactosidase activity as described elsewhere (Beddington et al., 1989). Embryos were post-fixed in 0.2% glutaraldehyde in 0.1 M phosphate buffer containing 2 mM $MgCl_2$ and 5 mM EGTA. Embryos that had received a DiI-labelled graft were fixed for 20 minutes in 4% paraformaldehyde and viewed with rhodamine filter epifluorescence optics in a compound microscope (Vanox, Olympus), photographed (Kodak 1600P film) and then transferred to a 0.05% solution of 3.3′-diaminobenzidine in 0.1 M Tris, pH 7.4 to produce permanent photooxidised staining of DiI-labelled cells (Selleck and Stern, 1991). All embryos were dehydrated through a series of alcohols, cleared in Histoclear (National Diagnostics), and finally infiltrated, orientated and embedded in Histoplast paraffin wax (Shandon). Serial sections were cut at 8 μm and subsequently stained with haemotoxylin and/or alcoholic eosin.

RESULTS

The fate of ventral node cells

10 embryos were labelled with DiI in the ventral region of the node (i.e. the outer surface of the node). The intention was to label a significant proportion of cells in the ventral node in order to observe the maximum variety of tissues to which this population would contribute. Fixation and inspection of 4 embryos immediately after labelling demonstrated that, despite the relatively crude method of labelling, fluorescence was limited to a discrete patch on the ventral surface of the distal tip of the egg cylinder (data not shown). After 24 hours in culture, the remaining six embryos all showed a similar distribution of fluorescent cells and a representative example is given in Fig. 3A. The notochord was the predominant tissue labelled although some fluorescent cells were also observed in adjacent endodermal epithelium and in particular in the foregut region. Occasional labelled cells were seen on the surface of the developing heart. The most notable feature about the distribution of fluorescent cells was that labelling of the notochord was continuous along its length, extending uninterrupted from the node to the most cephalic limit of DiI labelling. The node itself always retained a population of DiI-labelled cells (Fig. 3A).

Differentiation of grafted tissue and axis induction

Node tissue, either derived from a transgenic line of mice expressing *E. coli lacZ* or labelled with DiI, was transplanted to a heterotopic posterolateral site (Fig. 1) in full-length streak-stage embryos (stage OB; Downs and Davies, 1993). This region normally gives rise to lateral mesoderm but, because the mouse epiblast is cylindrical, it immediately abuts prospective surface ectoderm and cranial neurectoderm tissue (Lawson and Pedersen, 1992; Tam, 1989; Tam and Beddington, 1987). The inductive potential of grafted node tissue was compared with that of anterior epiblast grafts of a similar size. Embryos that received a graft were cultured for 20-30 hours before their morphology and the colonisation pattern of donor cells were assessed (Fig. 1). 13 embryos (three experiments) received an ectopic transgenic node and 7 embryos (two experiments) transgenic anterior ectoderm grafts. The results of these experiments and four others (22 embryos) in which DiI-labelled, rather than transgenic, node tissue was used are shown in Table 1.

The progeny of anterior ectoderm grafts either formed a small coherent patch of cells in the vicinity of the primitive streak or colonised rostral neurectoderm, including one embryo containing marked neural crest cells (Table 2). The exclusive colonisation of cranial regions by some anterior ectoderm

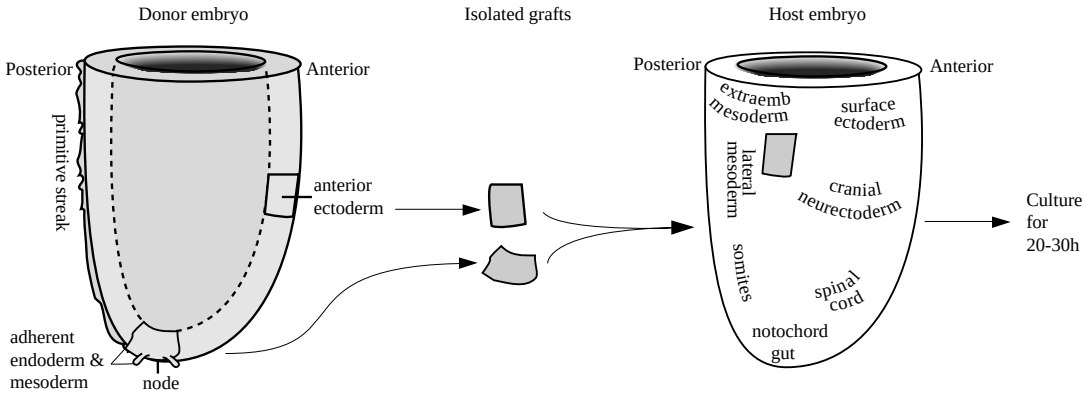

Fig. 1. A diagram illustrating the origin and site of transplantation of grafts. An approximate fate map of the epiblast is depicted on the host embryo (from Beddington, 1981b; Beddington, 1982; Lawson et al., 1991; Tam, 1989; Tam et al., 1987).

Table 1. Transplantation of 7.5 day node or anterior ectoderm to posterolateral region of 7.5 day embryo

Type of graft	No. retained/ No. grafted†	No. posterior ectoderm	No. anterior self-organized	No. anterior neural	No. showing axial extension*	Duplicated axis§
Tg node	10/13 76.9%	2 20%	2 20%	0 0%	6 60%	4 40%
DiI node	14/22 63.6%	3 21.4%	2 14.3%	0 0%	9 64.3%	5 35.7%
Total	24/35 68.6%	5 20.8%	4 16.7%	0 0%	15 62.5%	9 37.5%
Tg anterior ectoderm	6/7 85.7%	4 66.7%	0 0%	2 33.3%	0 0%	0 0%

†Excluding unincorporated lumps in the amniotic cavity.
*Axial extension of graft tissue anteriorwards from the primitive streak region.
§All embryos with duplicated axes, except 1 with an additional cranial neural fold, showed axial extension of graft tissue.

Table 2. Tissue distribution of donor cells in heterotopic grafts

Embryo	Notochord	Endoderm	Streak region	Neurect. surface ect.	Lat. mes. somites	Length of 2° axis§
(A) Node duplicated axes						
1	+++	+++	++	+	++	385 μm
2	+++	+++	+++	+	+	330 μm
3	+++	+++	++	+	+	312 μm
4	+++	+++	++	+	+	354 μm
5	++	+++	+++	–	–	230 μm
6	+++	++	++	–	+	376 μm
7	+++	+	+	+	+	336 μm#
8	+++	++	+	–	++	420 μm
9‡	+++	++	+	+	–	Nd
(B) Anterior ectoderm						
1	–	–	+†	+++	–	
2	–	–	+†	+++	–	
3	–	–	+†	+++	–	
4	–	–	+†	+++	–	
5	–	–	–	+++	–	
6	–	–	–	+++*	–	

+++ >30% of graft cells
++ Approximately 10-30% of graft cells
+ <10% of graft cells
– No donor cells detected

§The average total length of the rostocaudal axis is approximately 1 mm.
‡Embryo with additional cranial neural fold but no axial extension of graft tissue.
*Including cells in cranial neural crest.
†Coherent ectoderm patch without evidence of ingressing cells.
#Sum of ventral protrusion and axis parallel to that of the host (see Fig. 2F).

grafts (2/6) must result from grafts being placed slightly too far anteriorly at the time of transplantation. In all cases, anterior ectoderm descendants were restricted to the ectoderm germ layer (Fig. 2G,H), a sign of reduced developmental lability which has been noted previously (Beddington, 1982), and all grafted embryos appeared morphologically normal, the graft cells apparently having no effect on adjacent host tissue.

In contrast, descendants of heterotopic nodes showed a pronounced tendency to colonise the embryo in a narrow rostrocaudal strip, either incorporated into the host midline (Fig. 2A) or located laterally and running parallel to it (Figs 2A,C, 3B,C; Table 1). In all these embryos, some of which contained donor cells located as far rostrally as the level of the developing hindbrain, some graft-derived cells remained in the primitive streak region. The predominant tissues formed by the grafts were endoderm and notochord (Fig. 2D-F) with some cells populating adjacent mesodermal structures (somites and lateral mesoderm). With the exception of two node grafts that formed self-organized structures (see below), descendants of the node were seldom observed in ectodermal tissues (Table 2). The repertoire of tissues generated by node grafts is largely consistent with the normal fate of cells in the node of both mouse and chick (Beddington, 1981a; Lawson et al., 1991; Selleck and Stern, 1991). In transgenic grafts, all cells in the ectopic notochords were donor in origin (Fig. 2C). This was not true of notochords derived from DiI-labelled node viewed after photoconversion of diaminobenzidine, but the occurrence of unstained cells in these ectopic notochords is probably due to incomplete photooxidation, rather than to recruitment of host cells. Therefore, in general, the colonisation pattern of donor cells in grafted embryos is entirely consistent with the distribution of fluorescent cells after DiI labelling the outer surface of the node in situ.

The occurrence of two self-organized grafts (2/14) adhering to the cranial region may result from a failure of the graft to incorporate into the host embryo. These structures were composed wholly of donor tissue and both contained prominent neurectoderm vesicles. Therefore, the tendency for

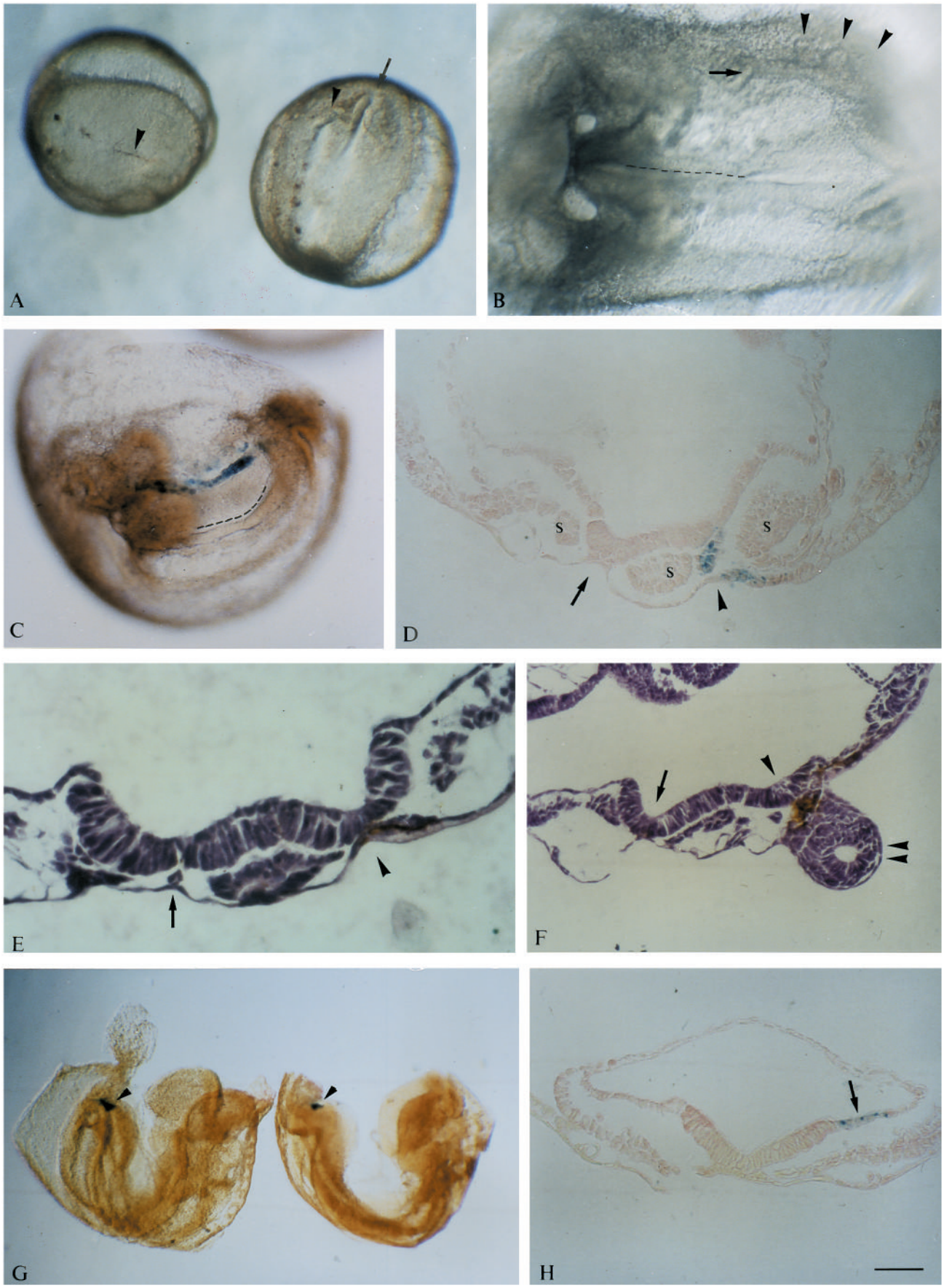
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Fig. 2. Axial duplication in embryos receiving heterotopic node grafts. The embryo number corresponds to the number shown in Table 2. (A) Ventral view of head-fold-stage embryos grafted with DiI-labelled node and cultured from mid-streak stage for 20 hours. The embryo on the left has brown staining donor cells (arrowhead) incorporated into the host midline (anterior to the right). The embryo on the right (embryo 5) has a duplicated axis (arrowhead) to the left of the host axis (arrow). The anterior aspect of the axis is at the top. Bar, 150 μm. (B) Ventral view of mid-streak-stage embryo (embryo 1) grafted with DiI-labelled cells cultured for 30 hours to the 7-somite stage. Anterior is to the left and the host midline is indicated by a dotted line. Ectopic neural folds (arrow) and somites (arrowheads) are evident. Bar, 70 μm. (C) Dorsal view of embryo 2 grafted with transgenic node. The host midline is indicated by a dotted line and anterior is to the left. The host axis has developed 7 pairs of somites. Bar, 150 μm. (D) Transverse section of embryo 2 showing transgenic cells in the notochord and endoderm of the secondary axis (arrowhead). Three blocks of condensed mesoderm (somites, s) can be distinguished, including a single, enlarged somite situated between the two axes. The host axis is indicated by the arrow. Bar, 80 μm. (E) Transverse section of embryo 6 (6-somite stage). The host midline and notochord are indicated by the arrow. The secondary axis and a midline DAB-stained (brown) donor cell (notochord) overlie the arrowhead. Bar, 20 μm. (F) Transverse section of embryo 7 (6-somite stage) showing a more complex induction involving both an additional neural fold (arrowhead) adjacent to the host neural axis (arrow) and an apparent induction of axial tissue, including a neurectoderm vesicle, emanating from the ventral aspect of the host embryo (double arrowhead). This protuberance is considerably larger than any self-organized growth developing from node grafts and is composed largely of host cells, containing only a few fluorescent DiI-labelled or photooxidised DAB-stained cells. Bar, 50 μm. (G) Lateral view of two control embryos grafted with transgenic anterior ectoderm. The grafts remain as a compact patch lateral to the primitive streak (arrowheads). Anterior is to the right. Bar, 175 μm. (H) Transverse section through one of the embryos shown in G. Transgenic cells (arrow) are restricted to surface ectoderm tissue. Bar, 80 μm.

the node to differentiate into ectodermal tissues appears to be enhanced in the absence of surrounding embryonic tissue. The failure of these grafts to integrate into the host embryo may be fortuitous or it may reflect subtle differences in the developmental stage of the grafted node. In the chick, such behaviour has been shown to increase with age: nodes from more advanced embryos form self-organized growths at a higher frequency (Gallera, 1971; Dias and Schoenwolf, 1990; Storey et al., 1992).

In all the embryos showing axial extension of donor tissue lateral to the host midline (Table 1), ectopic axial structures, such as additional neural folds (thickened, darkly staining columnar epithelium) or ectopic somites (discreet blocks of condensed mesoderm), could be distinguished (Fig. 2B,D-F), and these were composed almost entirely of host cells. The relative elevation of the induced neural folds, viewed in transverse histological sections, indicated that the secondary axis contained spinal cord precursor rather than more rostral elements of the CNS. In all cases, the induced second axis converged with the host axis caudally, a consistent feature of induced axes in chick (Hornbruch et al., 1979) and in *Xenopus* when the second axis does not form exactly opposite the natural one (Cooke, 1972). This phenomenon is most likely due to convergent extension movements gradually decreasing the distance between grafted node and host primitive streak.

DISCUSSION

These results demonstrate that the anterior extreme of the mouse primitive streak can differentiate autonomously and exhibit organizing properties during gastrulation equivalent to those of the dorsal blastopore lip of *Xenopus* and Hensen's node in chick. When grafted heterotopically, all three regions show a marked propensity to differentiate into notochordal tissue, as they would if left in situ (Beddington, 1982; Garcia-Martinez and Schoenwolf, 1992; Gimlich and Cooke, 1983; Lawson et al., 1991; Schoenwolf et al., 1992; Selleck and Stern, 1991). This appears to be sufficient to induce a second neural axis and, in some cases, ectopic somites, by altering the developmental fate of adjacent host tissue. Thus, these experiments confirm the homologous function ascribed to the mouse node, which previously could only be inferred on the basis of developmental fate and gene expression patterns.

The origin of the notochord

DiI labelling of the outer surface of the node demonstrates that this region represents the major source of notochord tissue (Fig. 3A). The injection of single head process cells with horseradish peroxidase at a similar stage has shown that descendant clones are mostly restricted to notochord and trunk endoderm tissues (Lawson et al., 1986). In addition, clonal analysis of the fate of cells in the node region showed that progeny could be widely dispersed along the rostrocaudal extent of the notochord but that frequently one or more descendants remained in the node (Lawson and Pedersen, 1991). A similar distribution of labelled progeny is seen when DiI is used to label the population of ventral node cells. The pattern of cell labelling observed is not consistent with the outer surface of the node comprising only a transitory population of cells produced by the continuous efflux of epiblast from the inside of the embryo to the outside. If this were the case one would expect a stretch of labelled notochord followed caudally by an unlabelled region and no residual labelled cells remaining in the node. Instead, the distribution of label in the notochord and node, using either clonal analysis or population labelling, argues that much of the notochord, at the full-length primitive streak stage, may be provided by a resident proliferating population in the ventral part of the node. The distribution of donor cells in transgenic node grafts where invariably donor notochord tissue was continuous with an undifferentiated caudal population of donor cells also suggests that the node may serve as a stem cell source of axial mesoderm cells.

One surprising aspect of such a 'stem cell' supply of nascent notochord tissue, from a relatively small population of cells, is that when primitive-streak-stage embryos are labelled with [^{3}H]thymidine using conditions that label all epiblast and embryonic endoderm nuclei, both notochord and dorsal gut endoderm retain particularly high levels of nuclear labelling when analysed at the 15-20 somite stage (Beddington, 1981b). This suggests that the formation of notochord and gut endoderm does not require extensive cell proliferation. The probable explanation for this apparent paradox is that the mouse notochord is not composed of many cells (eventually it amounts to little more than a single cell in transverse section) and its extensive axial elongation is probably effected largely by cell rearrangements and relative shearing movements rather

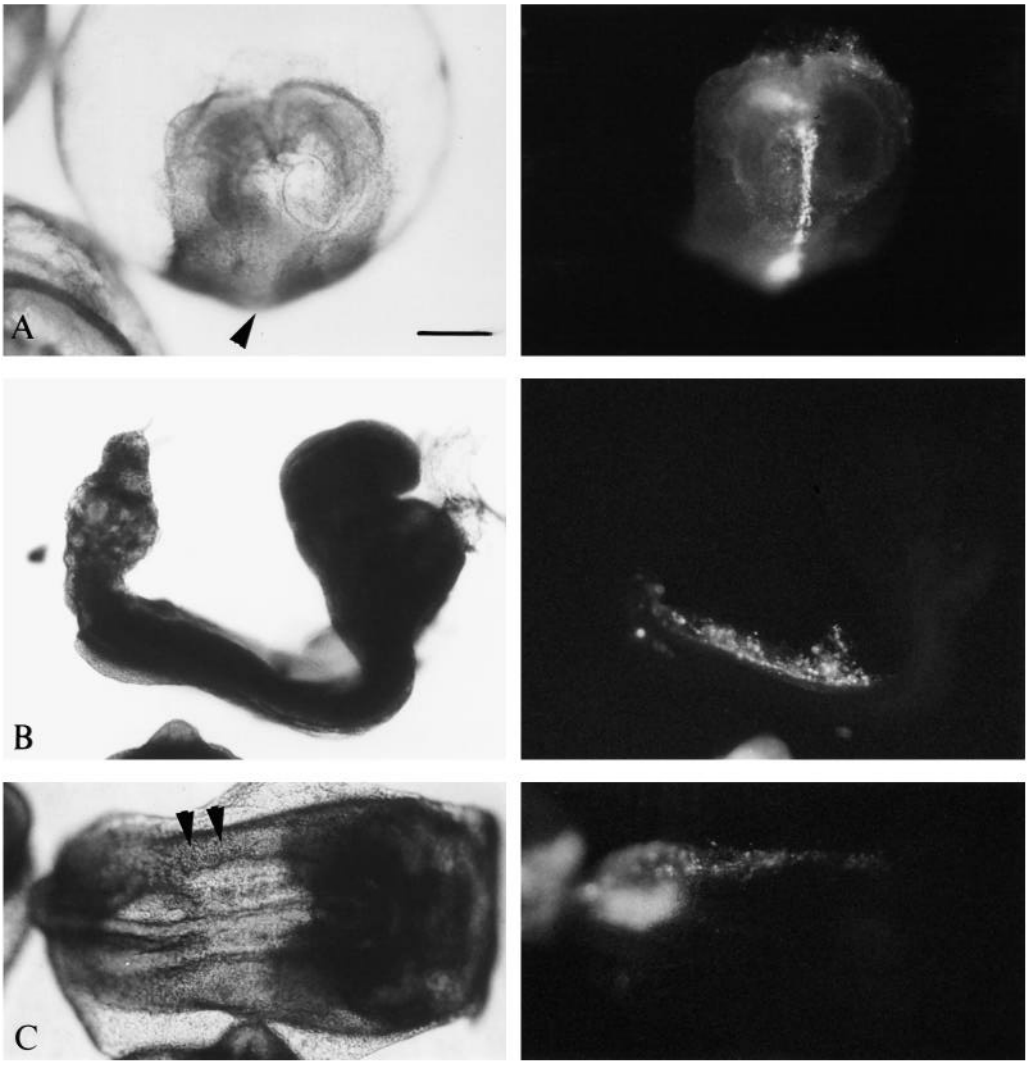

Fig. 3. Bright-field (left) and fluorescent (right) photomicrographs of DiI-labelled cells. (A) Mid-streak stage embryo labelled on the ventral surface of the node with DiI and cultured for 20 hours to the 3-somite stage. DiI label is mainly restricted to the notochord with a few scattered labelled cells in adjacent endoderm tissue. Labelling persists in the node (arrowhead). Bar, 170 μm. (B) Lateral view of an embryo that received a heterotopic graft of DiI-labelled node after 30 hours in culture having reached the 9 somite stage. Progeny of the graft are incorporated into the host midline and paraxial mesoderm. Bar, 140 μm. (C) Dorsal view of embryo 3 showing a duplicated axis including formation of ectopic somites (arrowheads). Bar, 175 μm.

than extensive cell recruitment (Bancroft and Bellairs, 1976; Jurand, 1962; Woo Youn et al., 1980).

Induction of secondary axes

Ectopic neural axes always occurred immediately dorsal to axially extended donor notochord, except for one embryo where an additional cranial neural fold was seen overlying a compact patch of graft tissue. As in the chick (Hornbruch et al., 1979), no ectopic somites were seen in the absence of donor notochord tissue. However, these results do not discriminate between the induction of host tissue being mediated by differentiating notochord or by signals emanating from the node itself. When chick Hensen's nodes are recombined with *Xenopus* animal caps at 22°C, circumstances that prevent the chick node forming notochord, neurectoderm (but not paraxial mesoderm) is induced and the animal caps undergo axial elongation (Kintner and Dodd, 1991). This suggests that the node can influence adjacent host tissue directly to induce neural differentiation. In contrast, no additional host neurectoderm was detected when mouse node grafts failed to form patent notochord and remained as coherent patches of donor tissue within the ectoderm germ layer (5/24 grafts; Table 1).

In certain respects, the results presented here appear to conflict with the reported developmental lability of 8th day distal ectoderm (recovered from a region corresponding to the location of the node) when grafted heterotopically in the embryonic egg cylinder (Beddington, 1982). In these experiments, grafted node tissue showed no tendency to self-organise or to differentiate according to its normal fate when moved to a new site in the embryo: instead, the grafts always differentiated in accordance with their new position (Beddington, 1981b, 1982). However, fewer cells were transplanted (approximately 20 as opposed to about 100 cells in node grafts) and the outer layer of the node was removed enzymatically prior to grafting. Thus, the exceptional organizing properties of the node must require its structural integrity. In particular, for induction to occur, the outer population of cells must be present, since the in situ DiI-labelling experiments point to these cells being the major source of notochord.

An assay for potential inducers

In addition to confirming the homology of the mouse node to the organizer of lower vertebrates, these experiments demonstrate that pattern formation can be directly and precisely manipulated in the gastrulating mouse embryo. Therefore, in spite of the small size of the mouse embryonic egg cylinder

(primitive streak length of 400 μm as opposed to 1.9 mm in chick), the molecular nature of factors affecting axial pattern can be studied directly in the mouse embryo. This is important because mammalian gastrulation exhibits several unique characteristics not seen in lower vertebrates. In particular, pattern formation coincides with a phase of extremely rapid growth (Snow, 1978). In addition, establishing the basic body pattern of the embryo is unlikely to be regulated by any residual maternal factors laid down in the egg: gastrulation commences almost a week after fertilisation whereas activation of the zygotic genome begins after the first cleavage division. Furthermore, spontaneous monoamniotic twins arise during mammalian gastrulation (Bulmer, 1970) and they can be induced by the systemic administration of drugs that disrupt microtubules during the first 24 hours of mouse gastrulation (Kaufman and O'Shea, 1978; Kaufman, 1992). In *Xenopus*, the equivalent category of twins can only be induced during early cleavage, all later axial duplications requiring precisely localised transplantation of cells or introduction of molecules (Cooke, 1985). Therefore, despite the circumstantial evidence for conservation of patterning mechanisms during vertebrate evolution, it is important to test the function of genes and signalling molecules in the mouse itself rather than to rely solely on extrapolation from other organisms. In order to do this, there must be a convincing assay for the ability of candidate molecules to influence pattern in the intact mouse embryo. The demonstration that secondary axes can be induced provides one such assay.

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