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Developmental Biology 289 (2006) 283 – 295
DEVELOPMENTAL BIOLOGY
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Fate and plasticity of the endoderm in the early chick embryo

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Received for publication 18 March 2005, revised 30 August 2005, accepted 6 September 2005
Available online 9 December 2005

Abstract

In vertebrates, the endoderm is established during gastrulation and gradually becomes regionalized into domains destined for different organs. Here, we present precise fate maps of the gastrulation stage chick endoderm, using a method designed to label cells specifically in the lower layer. We show that the first population of endodermal cells to enter the lower layer contributes only to the midgut and hindgut; the next cells to ingress contribute to the dorsal foregut and followed finally by the presumptive ventral foregut endoderm. Grafting experiments show that some migrating endodermal cells, including the presumptive ventral foregut, ingress from Hensen's node, not directly into the lower layer but rather after migrating some distance within the middle layer. Cell transplantation reveals that cells in the middle layer are already committed to mesoderm or endoderm, whereas cells in the primitive streak are plastic. Based on these results, we present a revised fate map of the locations and movements of prospective definitive endoderm cells during gastrulation.

Keywords: Endoderm; Fate map; Gastrulation; Cell movement; Chick embryo; Primitive streak; Hensen's node

Introduction

In vertebrates, the definitive endoderm, which gives rise to the epithelium of the digestive tract, arises from the epiblast during gastrulation. The endoderm starts to become regionalized along its anteroposterior and dorsoventral axes after gastrulation and finally subdivides to give rise to morphologically and functionally diversified regions and to the organs of the digestive and respiratory systems. Although there are many studies of the molecular mechanisms involved in the establishment of the endoderm during gastrulation (Stainier, 2002; Tam et al., 2003) and of the differentiation of certain digestive organs (Yasugi, 1994; Wells and Melton, 1999; Duncan, 2000; Grapin-Botton and Melton, 2000; Yasugi, 2000; Fukuda and Yasugi, 2002), little is known about when or how the endoderm segregates from the other germ layers and starts to become regionalized. To start to address these issues, fate maps of early stages showing both the location of endodermal progenitor cells and the origin of these cells that contribute to the various regions of the gut are essential.

Fate maps of the endoderm of the chick embryo during gastrulation have already been constructed by many authors using carbon particles (Bellairs, 1953a,b, 1955, 1957), ^{3}H-thymidine labeled grafts (Rosenquist, 1966, 1970a,b, 1971a,b, 1972), quail–chick transplantation (Fontaine and Le Douarin, 1977) and fluorescent dyes (Kirby et al., 2003; Lawson and Schoenwolf, 2003). All of these studies showed that the definitive endoderm forms during gastrulation from cells in the anterior primitive streak or Hensen's node, which ingress into the lower layer and replace the hypoblast, forcing the latter into an extraembryonic position. These fate maps also suggested that presumptive ventral gut endoderm ingresses into the lower layer earlier than dorsal endoderm (Rosenquist, 1971a). Nevertheless, these maps were made at very low resolution, and, in most cases, the middle layer was also labeled by these methods, which precluded precise distinction of endoderm and mesoderm cells.

Here, we present a detailed fate maps of the endoderm of the primitive streak stage (Hamburger and Hamilton, 1951; HH 2–5) chick embryo by a newly developed labeling method: very small focal injections of DiI placed exclusively in the lower layer. This enabled us to find hitherto undescribed behaviors of

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doi:10.1016/j.ydbio.2005.09.009

prospective endoderm cells during gastrulation. First, we reveal that the endodermal cells that appear first in the lower layer will contribute to the mid- and hindgut, later ingressing cells contribute to the dorsal foregut, followed finally by presumptive ventral foregut cells. Second, cell labeling and grafting experiments reveal that many migrating endodermal cells, including the prospective ventral foregut, ingress from the anterior primitive streak not directly into the lower layer but only after lateral migration in the middle layer, which was previously thought to contribute only to the mesoderm. Finally, we use grafting experiments to show that mesendoderm cells acquire their mesoderm or endoderm identity during gastrulation. These results reveal a more complex pattern of movements of endodermal cells than previously thought and provide a base to examine the molecular mechanisms responsible for endoderm specification.

Materials and methods

Method for focal labeling of lower layer cells

In this study, 499 embryos were labeled, of which 324 survived. Of these, 93 embryos had been appropriately labeled and were used for analysis.

To construct a detailed fate map of the lower layer of the chick embryo and to determine the exact timing of incorporation of endodermal cells into the lower layer during gastrulation, we devised a strategy to label very small groups of cells restricted to the lower layer. During gastrulation, the ventralmost layer of the embryo is very thin and fragile, and established methods of DiI labeling (pressure injection of a dye solution) tend to spill into the adjacent middle layer. After exploring several alternatives, we found that placing a "microcrystal" of DiI (see below) on the lower layer for 1 h before removing it carefully allowed us to label a very small group of cells exclusively within the lower layer (Fig. 1A).

Fertilized hens' (White Leghorn) eggs were incubated at 38°C for 12–24 h to obtain embryos from HH stages 2 to 5. Embryos were explanted in Pannett–Compton saline (Pannett and Compton, 1924) using a modified version of the New culture method (Stern and Ireland, 1981). Microcrystals of the carbocyanine dye DiI (1,1-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate) (DiI-C_{18}; Molecular Probes) were prepared as follows: DiI was first dissolved at 0.5% (w/v) in absolute ethanol and the solution diluted 1:1 in 50% sucrose in distilled water. A droplet of this was deposited into a large volume of Pannett–Compton saline, which generated a precipitate of very small DiI crystals (each approximately 5–30 μm in diameter). After 30 min, DiI crystals of appropriate size, some 10–15 μm in diameter, were selected for labeling.

An individual DiI crystal was placed directly onto the lower layer carefully to avoid injury. One hour later, the DiI crystal was carefully removed. Following marking with DiI, embryos were incubated at 38°C in a humid atmosphere until stage 11. Images of the labeled embryos were taken immediately after labeling and subsequently at stages 5 and 11, using a MZ FLIII fluorescence stereomicroscope and Image Manager (Leica). After incubation, some embryos were processed histologically to confirm the localization of labeled cells. For this, embryos were fixed in PBS containing 0.25% glutaraldehyde and 4% paraformaldehyde for 1 h then the fluorescence was photooxidized with 3-3′ diaminobenzidine (DAB) in 0.1 M Tris–Cl (pH 7.5) as previously described (Izpisúa-Belmonte et al., 1993). The embryos were then embedded in paraffin, serially sectioned at 10 μm, mounted on glass slides and dewaxed in xylene before being mounted in Entellan NEW (Merck).

Transplantation experiments

Cells in the middle layer just lateral to Hensen's node or lateral to the mid-primitive streak at stages 3^{+}–4 were labeled by applying a solution of DiI (0.5% DiI in ethanol, diluted 1:10 in 0.3M sucrose) using air pressure from a micropipet. A small group of these labeled cells (approximately 20 cells) was then excised and grafted homo- or heterotopically and homo- or heterochronically into host chick embryos in modified New culture. The host embryos were allowed to heal at room temperature for 30 min and then photographed. They were then cultured at 38°C, and the positions of DiI-labeled cells examined every 2–4 h. At the end of the incubation period (various times following the graft), embryos were fixed overnight in PBS containing 4% paraformaldehyde, embedded in paraffin and sectioned at 10 μm and examined by bright-field and fluorescence microscopy.

In situ hybridization

Embryos were fixed with 4% paraformaldehyde overnight, replaced with 30% sucrose in PBS at 4°C for 5 h and embedded in OCT compound (Sakura Finetechnical Co.). In situ hybridization with digoxigenin-labeled probes was performed on 12 μm frozen section as described by Ishii et al. (1998), after recording the DiI fluorescence photographically. *cSox2*, *cSox3* (Uwanogho et al., 1995), *CdxA* (Ishii et al., 1997), *HFH8* (Clevidence et al., 1994) and *cPax9* (Muller et al., 1996) were used as probes for in situ hybridization.

Results

Fate map of the lower layer at HH stages 2–3^{+}

Previous studies (Bellairs, 1953a,b, 1955, 1957; Vakaet, 1962, 1970, 1984; Nicolet, 1965, 1967, 1970; Rosenquist, 1966, 1970a,b, 1971a,b, 1972; Fontaine and Le Douarin, 1977; Selleck and Stern, 1991; Psychoyos and Stern, 1996; Kirby et al., 2003; Lawson and Schoenwolf, 2001, 2003) have established that the definitive (gut) endoderm arises from the epiblast via the anterior primitive streak prior to HH stage 4. In

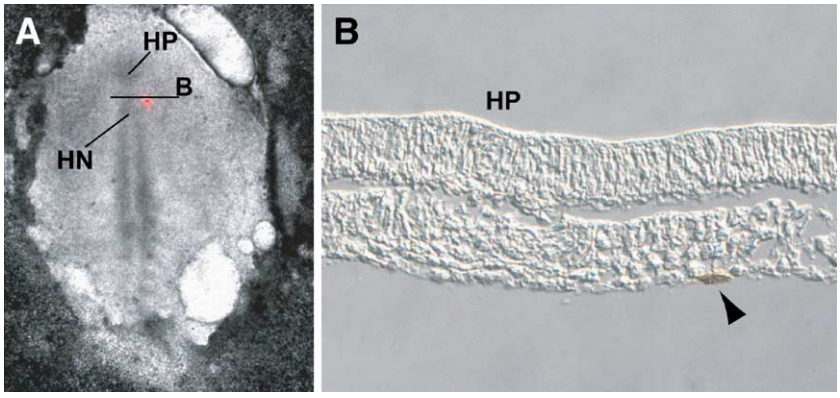

Fig. 1. Specific labeling of the lower layer with DiI in the embryo. (A) Embryo labeled at stage 5. (B) Transverse section through the embryo shown in panel A, at the level indicated by the transverse line. Photooxidized DiI was found exclusively in the lower layer. HN, Hensen's node; HP, head process.

a very short time, a large area becomes completely covered by new cells, arising from a very restricted region. How does this happen, and how does the embryo ensure that cells ingressing into the endoderm do not collide with mesoderm cells, which are ingressing at the same time and location?

We began by constructing a fate map of the lower layer of stage 2–3 embryos (early primitive streak). As summarized in Fig. 2, almost all cells in the lower layer around the anterior tip of the extending streak at stage 2–3 contributed to extraembryonic endoderm, and only a very limited region at the tip of the streak contributed to embryonic endoderm (gut endoderm), as previously reported (see references above). In addition, these few prospective endodermal cells contributed only to the mid/hindgut.

By stage 3^+ (mid-primitive streak stage), the regions that contributed to the gut endoderm had expanded caudally and laterally in the lower layer. Cells that contribute to the foregut start to be found at this stage in a restricted region of the lower layer adjacent to the tip of the primitive streak (Fig. 2).

Fate map of the lower layer at HH stages 4–5

Next, the fate of lower layer cells at stage 4 (definitive streak stage) was determined. The results obtained are summarized in Figs. 3A–C, and typical examples are shown in Figs. 3D–O. Compared to the fate map of stage 3^+ embryos, the presumptive gut endodermal region now extends laterally and caudally within the lower layer (Fig. 3A). The lateral border between gut and extraembryonic endoderm now coincides with the border of the adjacent middle layer. At the same time, the anterior border of the definitive endoderm still resides at the level of Hensen's node (Fig. 3A). By this stage, the presumptive foregut region has extended laterally but not along the rostrocaudal axis, forming a narrow horizontal band at the level of Hensen's node, while the mid- and hindgut extend caudolaterally from the node. Within the foregut territory, presumptive dorsal foregut is found around Hensen's node, while the prospective lateral foregut resides in a more peripheral area. No ventral foregut precursors were found in the lower layer at this stage.

To determine how cells contributing to these various regions of the gut reach their final destinations, we followed the movements of the descendants of the labeled cells and recorded them at stage 5 and stage 11 (Figs. 3B, C). At stage 5, the presumptive mid/hindgut region spreads caudally and laterally, now reaching the most caudal part of the embryo. On the other hand, the presumptive dorsal foregut region does not move significantly apart from some convergence towards the midline. The presumptive lateral foregut region has moved laterally along with the border of the middle layer (Fig. 3B). At stage 5, there appear to be no prospective endoderm cells outside the border of the middle layer (dashed line in Fig. 3B).

The resulting fate maps at stage 5 appear to have a gap devoid of labeled cells in the region just inside the edge of the middle layer (Fig. 3B, between points 15/18 and 10/11/17/14). To determine the fates of cells in this region, we placed DiI marks directly in this area at stage 5 (Fig. 4). The entire arc-shaped region contributed to the ventral foregut (Figs. 4A, C, yellow spots and D–I).

In summary, these fate maps show that the lower layer contains presumptive mid/hindgut cells at stage 3. The prospective dorsal foregut endoderm first appears at stage 3^+ followed finally by presumptive ventral foregut at stage 5. Since ventral foregut cells were never found until stage 5 and they first appear far away from the primitive streak (from which all endoderm cells arise), this result opens the question of what is the trajectory by which prospective ventral foregut cells enter the lower layer.

The anterior portion of the middle layer at stage 4 is a source of endoderm

A possible answer to the above question is that presumptive ventral foregut endoderm cells from Hensen's node may ingress

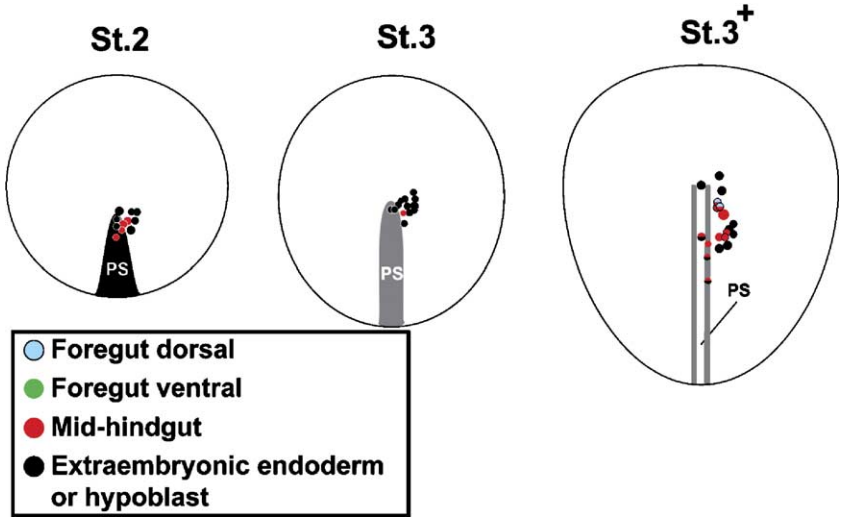

Fig. 2. Diagrams summarizing the contribution of different regions of the lower layer at stage 2–3^+ to different rostrocaudal positions in the gut. Each point represents one group of DiI-labeled cells in the lower layer in one embryo. The colors denote the fates of the progeny of the labeled cells. PS, primitive streak.

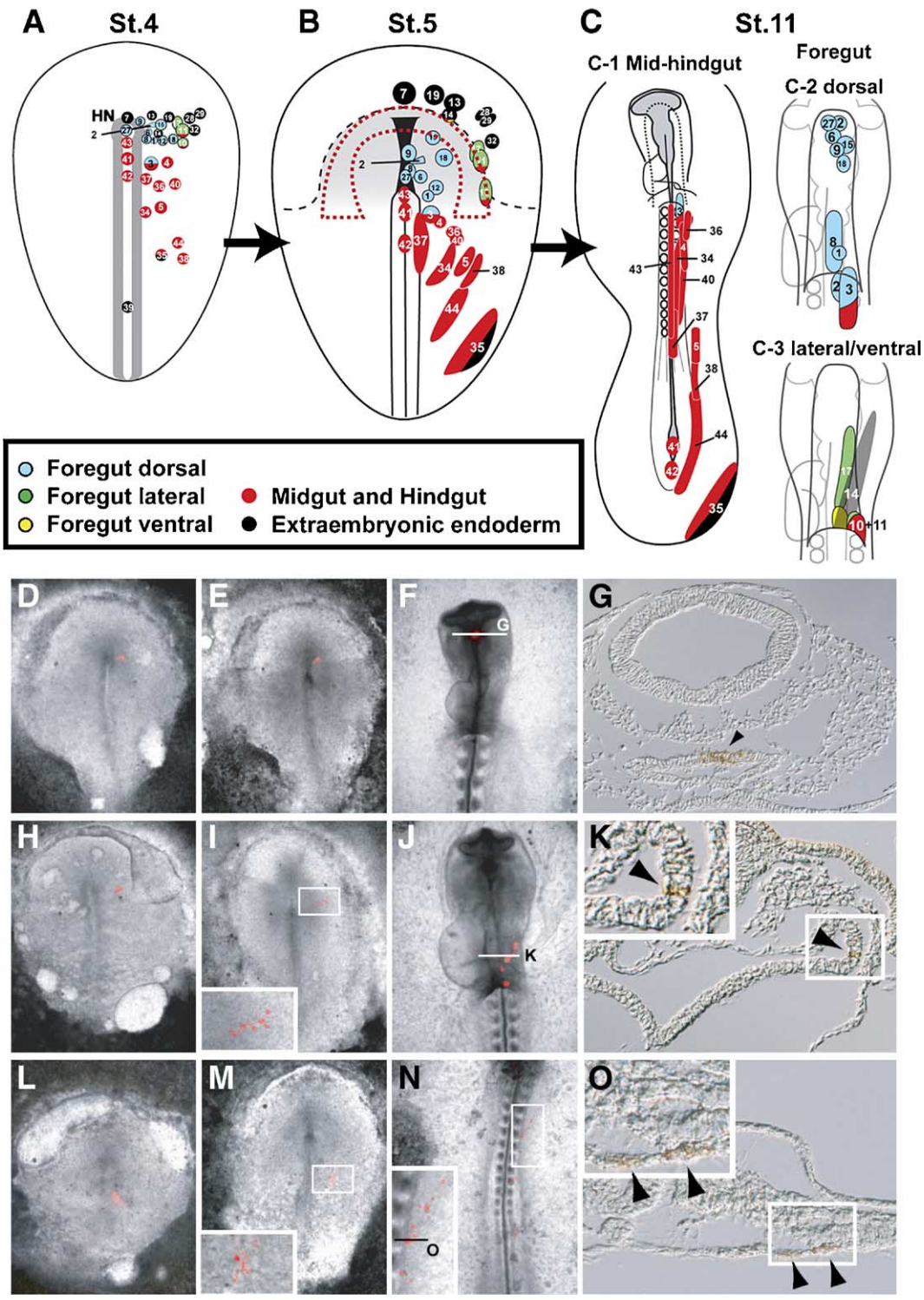

Fig. 3. (A–C) Diagrams summarizing the contribution of different regions of the lower layer at stage 4 to different rostrocaudal and dorsoventral positions in the gut. Each point represents a group of DiI-labeled cells in the lower layer in one embryo. The position of their descendants at stage 11 is represented in different colors. The size of the points is in proportion to the actual size of each label. (B) Distribution of the descendants of the labeled cells when embryos reached stage 5. (C) Distribution of labeled progeny when embryos reached stage 11. (C-1) Contribution to the mid- or hindgut endoderm. (C-2) Contribution to the dorsal foregut endoderm. (C-3) Contribution to the ventro-lateral foregut endoderm. (D–O) Examples of the results obtained from the DiI labeling experiment at stage 4. (D, H, L) The embryos were labeled at positions "2", "11" and "34" in panel A, respectively. (E, I, M) Embryos shown in panels D, H, L viewed at stage 5. (F, J, N) The same embryos viewed at stage 11. (G, K, O) Sections of the embryos in panels F, J, N after photooxidation. The labeled cells were found in anterior–dorsal foregut endoderm (G, arrowhead), lateral foregut endoderm (K, arrowhead) and mid/hindgut endoderm (O, arrowhead). Panels I, K, M–O are enlargements of the labeled regions.

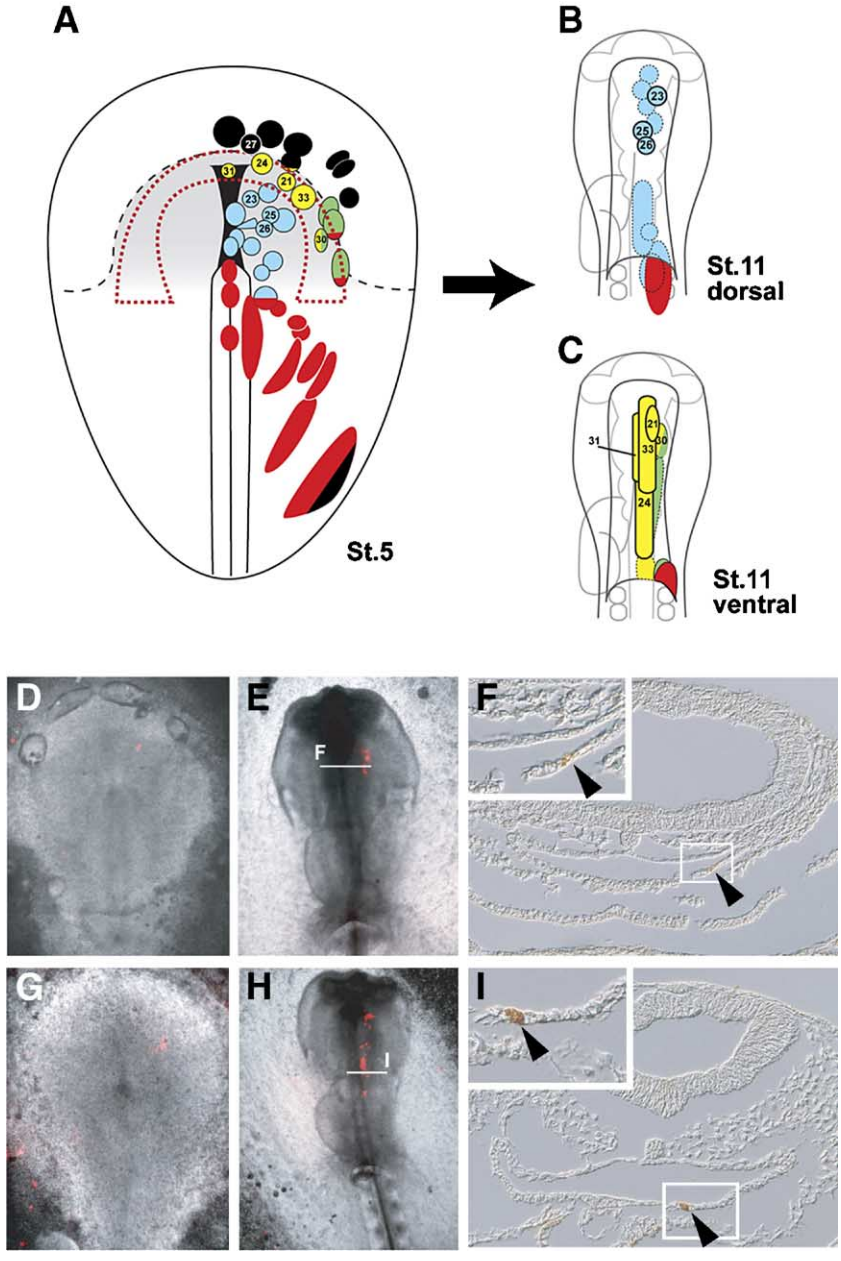

Fig. 4. (A–C) Diagram summarizing the contribution of different regions of the lower layer at stage 5 to different dorsoventral positions in the foregut. Each numbered point represents one group of DiI-labeled cells in the lower layer in one embryo at stage 5. Points without numbers represent the position (at stage 5) of descendants of cells labeled with DiI at stage 4 (see Figs. 3A, B). The position of their descendants at stage 11 is distinguished by different colors. (B–C) Distribution of labeled descendants at stage 11. (B) Contribution to the dorsal–foregut endoderm. (C) Contribution to the ventro-lateral foregut endoderm. Some examples of the results obtained from the DiI labeling experiment at stage 5: (D–F) Embryo labeled at position "21" (D) cultured until stage 11 (E) and after sectioning (F). The labeled cells were found in the rostro-ventral foregut endoderm (F, arrowhead). (G–I) Embryo labeled at position "33" (G) cultured until stage 11 (H) and after sectioning (I). The labeled cells were found in the caudal–ventral foregut endoderm (I, arrowhead). (F, I) Show enlargements of the labeled regions.

not directly, but only after some anterolateral migration within the middle layer before intercalating into the lower layer. To test this, we labeled cells in the middle layer at stage 4. Cells in the middle layer labeled at stage 4 (Supplemental Figs. 1A, C) contributed to the ventral foregut endoderm (Supplemental Figs. 1B, D). However, it is very difficult to label these cells directly without also marking the adjacent layers (data not shown). To follow the fate and movements of the anterior middle layer cells specifically, we resorted to homotopic and homochronic grafting of labeled cells from donor embryos.

First, to control for the possibility of indiscriminate transfer of the dye, DiI-labeled quail cells were transplanted into the middle layer of host chick embryos (Supplemental Figs. 2A–D). All DiI-positive cells were also QCPN-positive and therefore derived from the quail graft (Supplemental Figs. 2C, D, below).

Small groups of cells from the middle layer lateral to Hensen's node of a donor chick embryo at stage 4 were labeled with DiI then excised and grafted homotopically and homochronically into a recipient embryo (Figs. 5A, E) which was

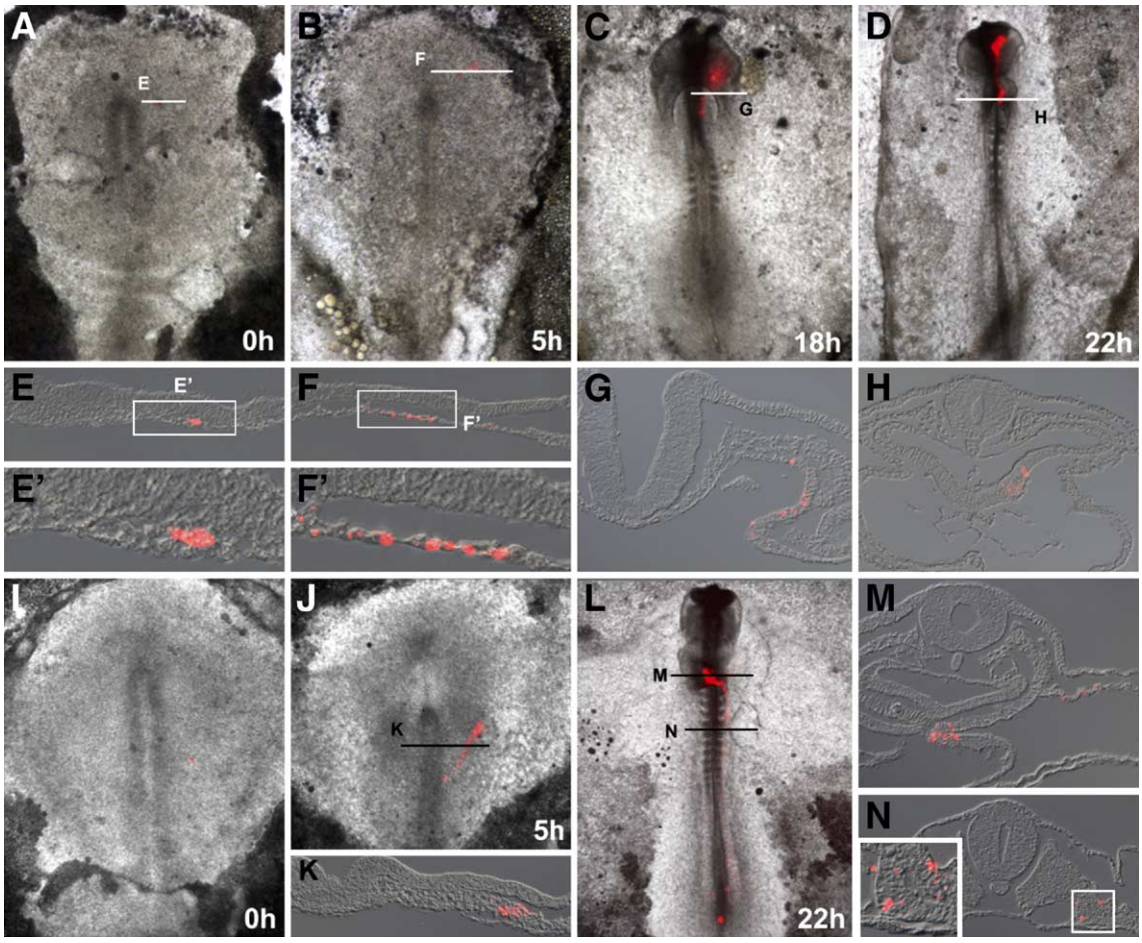

Fig. 5. Movement of cells in the middle layer at stage 4. (A) Embryo transplanted with DiI-labeled cells homotopically into the middle layer lateral to the Hensen's node. Embryos transplanted with labeled cells into the same position as (A) were incubated for 5 h (B), 18 h (C) and 22 h (D). The labeled cells moved anteriorly and laterally (B) and finally were found in the foregut (C, D). (E) Transverse section of the embryo in panel A. Descendants of the transplanted cells were found in the middle layer. (F) Transverse section of the embryo in panel B. Labeled cells were found only in the lower layer. (E′, F′) Higher magnification of boxed regions in panels E, F, respectively. (G) Transverse section of the embryo in panel C. Labeled cells were found in the ventral foregut endoderm. (H) Transverse section of the embryo in panel D. Labeled cells were found in the ventral foregut endoderm. (I) Embryo transplanted with labeled cells homotopically lateral to the mid-primitive streak. (J) Embryo transplanted with labeled cells into the same position as (I), viewed after 5 h (J) and 24 h (L) incubation. The labeled cells moved anteriorly and laterally (J) and were eventually found in caudal foregut, midgut and hindgut (L). (K) Transverse section at the level indicated by the transverse line in panel J. Labeled cells were found in the middle layer. (M, N) Transverse sections at the levels indicated by the transverse lines in panel L. Labeled cells were found only in the lateral plate mesoderm. (F, I) Show enlargements of the labeled regions.

then cultured for 22 h. The movements of the labeled cells were followed during this period. After 5 h, labeled cells had moved anteriorly and laterally toward the "arc-shaped region" defined above (Figs. 5A–B). Sections from these embryos obtained at various stages showed that the labeled cells had inserted themselves into the lower layer (Figs. 5E–F). Eventually (HH stages 8–10), the labeled cells contributed to the ventral foregut, and none of them was found in mesodermal tissues (Figs. 5C, D, G, H, 5/5). This result shows that cells in the middle layer lateral to Hensen's node at stage 4 contribute to the ventral foregut endoderm by stage 5. By contrast, homotopic grafts of labeled cells from the middle layer at the mid-primitive streak level moved caudally and laterally in the middle layer within 5 h of incubation (Figs. 5I–K) and eventually contributed only to lateral plate mesoderm (Figs. 5L–N). We also performed the transplantation experiments through the epiblast instead of through the lower layer to exclude the possibility that damage of the lower layer may artifactually increase the contribution to the endoderm. Grafted cells moved in a very similar way to when they were grafted through the lower layer (Fig. 5) and also contributed to the ventral foregut (Supplemental Fig. 3).

Do cells from the middle layer insert into the lower layer before migrating laterally, or do they migrate before inserting? To answer this, we followed the movement of labeled middle layer cells immediately adjacent to Hensen's node (Figs. 6A, I) from stage 3^+ to stage 11 using the approach described above. After 8 h (stage 4), labeled cells had moved rostrally and laterally (Figs. 6B–C). At stage $4^+/5^-$ (about 12 h incubation; Fig. 6D), labeled cells were found in both the middle (Fig. 6J, arrow) and lower (Fig. 6J, arrowheads) layers, at the level of the lateral border of the former. Eventually, the marked cells contributed to the ventral foregut (Figs. 6E–H, K, 13/13). This result shows that cells in the middle layer adjacent to Hensen's node at stage 3^+ move laterally within the middle layer up to stage 4 then intercalate into the lower layer as they reach the

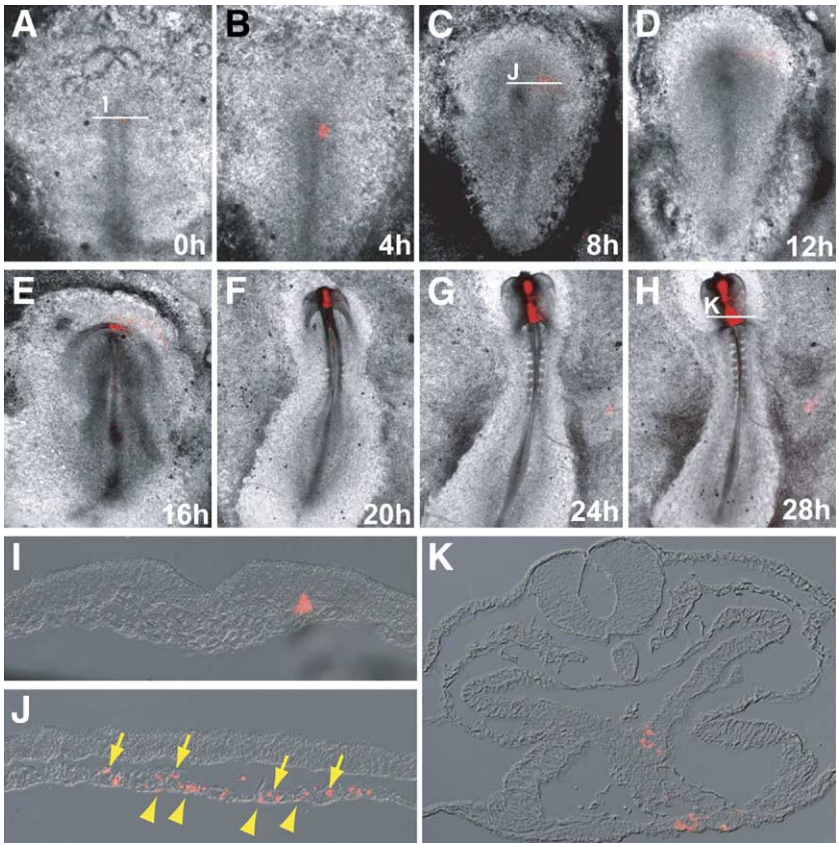

Fig. 6. Movement of cells within the middle layer at stage 3$^+$. (A) Embryo that received a transplant of DiI-labeled cells homotopically into the middle layer just lateral to the node. Embryos transplanted with labeled cells in the same position as (A) were incubated for 4 (B), 8 (C), 12 (D), 16 (E), 20 (F), 24 (G) and 28 h (H). The labeled cells moved anteriorly and laterally and were eventually found in the foregut. (I) Transverse section of the embryo in panel A. (J) Transverse section of the embryo in panel C. DiI-positive cells were found in both the lower layer (arrowheads) and middle layer (arrows). (K) Transverse section of the embryo in panel H. DiI-positive cells were found only in the ventral foregut endoderm.

lateral border of the middle layer (arc-shaped region) at stage 5 and finally contribute to the ventral foregut. We also examined whether the cells in the middle layer lateral to Hensen's node derive from the rostral part of the primitive streak, which is the source of endodermal cells (Lopez-Sanchez et al., 2001; Garcia-Martinez et al., 1993; Selleck and Stern, 1991; Psychoyos and Stern, 1996). Cells in the rostral part of the primitive streak at stage 3 (data not shown) and stage 3$^+$ (Supplemental Fig. 4) were found in the middle layer lateral to Hensen's node and in the lower layer at stage 4 and contributed to the ventral and lateral foregut, heart mesoderm and notochord. These results show that presumptive ventral foregut cells located in the middle layer at stage 4 arise from the rostral part of the primitive streak.

Endodermal cell fate determination during gastrulation

Next, we performed heterochronic and heterotopic grafting experiments to address when and where cells become committed to an endodermal identity. At stage 3$^+$, mesodermal/endodermal cells are restricted to the rostral tip of the primitive streak, whereas mesodermal cells are found all along the primitive streak (Selleck and Stern, 1991; Schoenwolf et al., 1992; Schoenwolf and Garcia-Martinez, 1995; Psychoyos and Stern, 1996; Lawson and Schoenwolf, 2003). We grafted cells from the rostral tip of the primitive streak (including the presumptive mesendodermal cells at stage 3$^+$) into the mid-primitive streak which contains only presumptive mesodermal cells (Fig. 7A). The progeny of the grafted cells expanded anteroposteriorly and laterally caudal to the anterior intestinal portal level (Fig. 7B), and almost all of them contributed to the lateral plate mesoderm, according to the new position of the grafted cells (Fig. 7B′, 9/10). We checked the expression of tissue specific genes in some embryos by in situ hybridization. Grafted cells found in the lateral plate mesoderm expressed *HFH8* (Figs. 7B′, B″, 2/2), which is specifically expressed in splanchnic mesoderm (Funayama et al., 1999). After the converse operation (grafts from the mid-primitive streak into the rostral tip of the primitive streak; Fig. 7C), the graft expanded within the foregut (Fig. 7D), and almost all of its cells contributed both to the foregut endoderm (Fig. 7D′, 11/11) and to the notochord (data not shown), also similar to the fates of the host cells surrounding the graft. Grafted cells found in the foregut endoderm express *cSox2* (Fig. 7D′, 2/2), which is expressed in the foregut endoderm and neural tube, but not *cSox3* (Fig. 7D″, 2/2), which is restricted to the neural tube. Thus, cells in the stage 3$^+$ primitive streak can change their fate according to their environment.

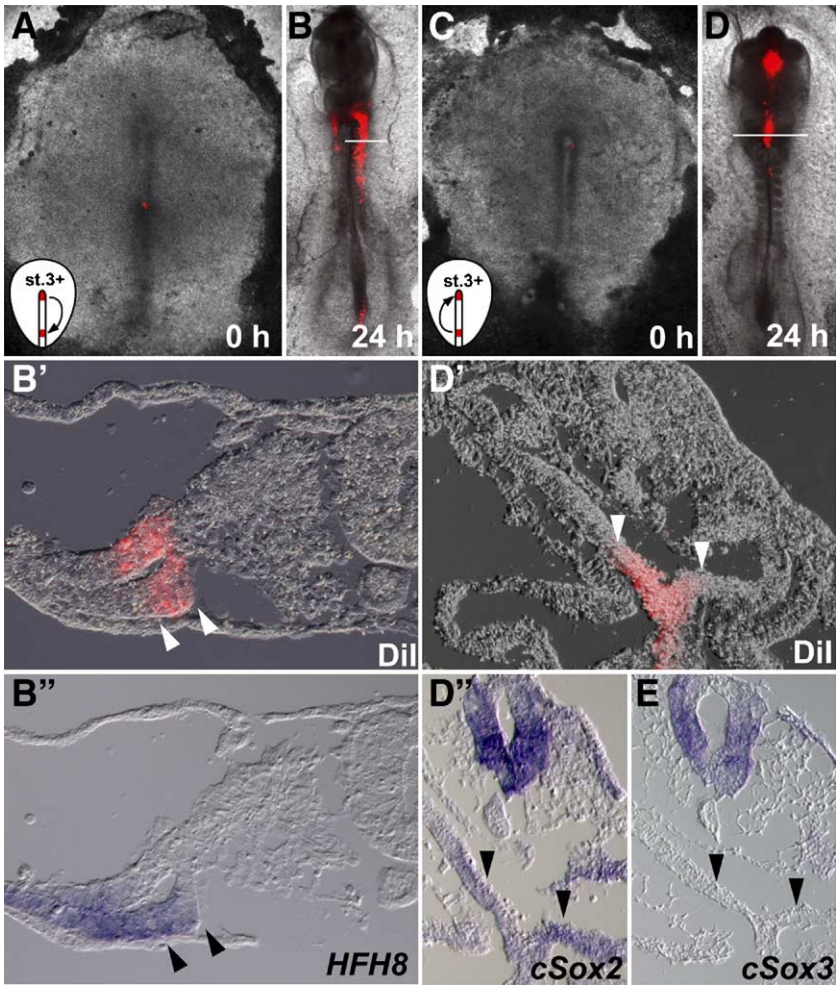

Fig. 7. Endodermal specification at stage 3^+. (A, C) Embryo transplanted with DiI-labeled cells from the rostral tip of the stage 3^+ primitive streak into the mid-primitive streak (A) and vice versa (C). (B, D) The embryos in panels A, C were cultured for 24 h. (B′, D′) Sections of the embryos in panels B, D at the levels indicated. (B″, D″) In situ hybridization for *HFH8* and *cSox2* on the same sections (B′, D′, respectively). (E) In situ hybridization for *cSox3* on a neighboring section to panel D′. Arrowheads indicate the border between transplant and host tissue.

Next, at stage 4, cells in the middle layer lateral to Hensen's node were grafted into the middle layer lateral to the mid-primitive streak, where cells normally contribute to the mesoderm (Fig. 8A). Grafted cells expanded anteroposteriorly and laterally (Fig. 8B), and almost all of them contributed to endodermal tissue (Fig. 8B′, 9/11). Grafted cells found in the endoderm expressed *CdxA* (Fig. 8B″, 2/2), which is expressed specifically in the mid- and hindgut endoderm at this stage (Ishii et al., 1997). Their original endodermal fate was therefore maintained after transplantation to the presumptive mesodermal region. On the other hand, the converse grafts of cells from the middle layer lateral to the mid-primitive streak into that lateral to the Hensen's node (Fig. 8C) moved to the foregut and the anterior intestinal portal (Fig. 8D), and all of them contributed to mesodermal tissues, such as notochord (Fig. 8D′, 7/8), head mesenchyme and paraxial mesoderm (data not shown). Grafted cells found in the notochord expressed the notochord marker *chordin* (Fig. 8D″, 2/2). Thus, their original mesodermal fate was also maintained after transfer to the presumptive endodermal region.

To confirm the above conclusion that commitment to endoderm and mesoderm is established by stage 4, cells in the middle layer lateral to Hensen's node at stage 4 were grafted into the middle layer lateral to Hensen's node at stage 4^+ (a region destined to form mesoderm) (Fig. 9A). Grafted cells expanded in the foregut, and almost all of them contributed to the foregut (Figs. 9B, B′, 8/10) and midgut endoderm (data not shown). Graft-derived cells expressed the pharyngeal endoderm marker *Pax9* (Fig. 9B″, 2/2, Muller et al., 1996). Cells in the middle layer lateral to Hensen's node at stage 4^+ were grafted into the middle layer lateral to Hensen's node at stage 4 (Fig. 9C). These grafted cells moved to the foregut, and almost all of them contributed to mesoderm-derived tissues, such as notochord (Figs. 8D, D′), head mesenchyme and paraxial mesoderm. When grafted cells contributed to the notochord, they expressed *chordin* (Fig. 9D″, 2/2). These data indicate that cells in the primitive streak at stage 3^+ can change their endodermal or mesodermal fates in response to their surrounding environment, while after their migration from the primitive streak to the middle layer at stage 4, they can no longer change their fates, suggesting that

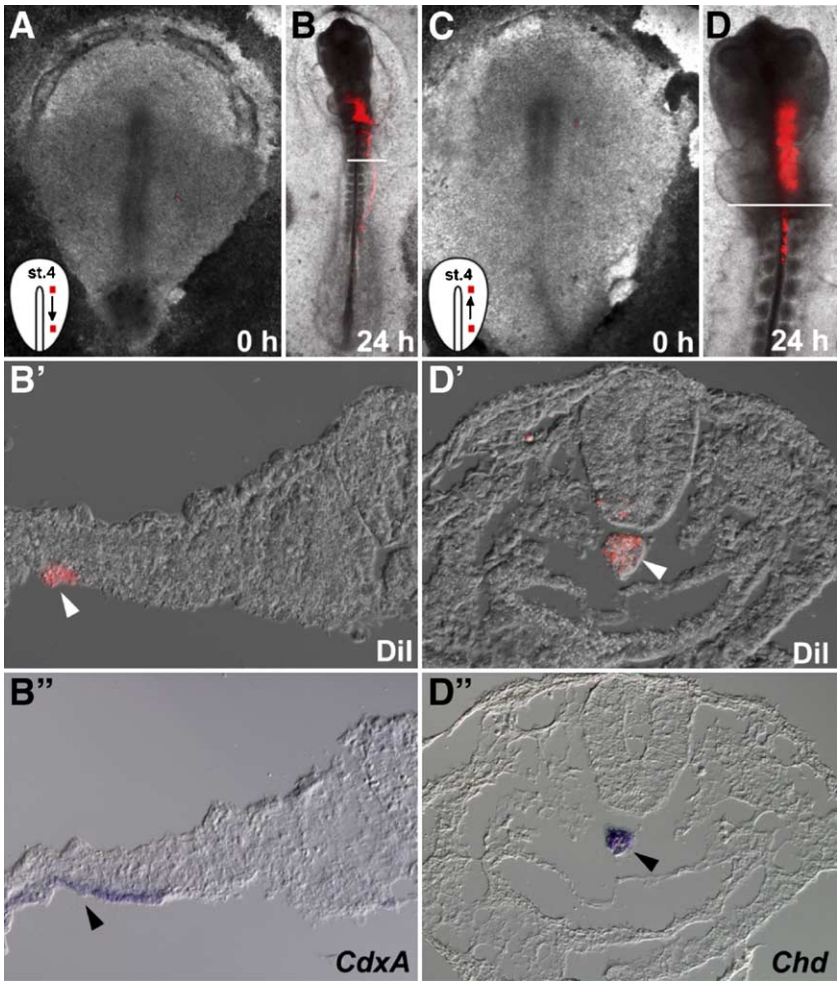

Fig. 8. Endodermal specification at stage 4. (A, C) Embryos transplanted with DiI-labeled cells at stage 4 from the middle layer lateral to Hensen's node into the middle layer lateral to the mid-primitive streak (A) and vice versa (C). (B, D) The embryos in panels A, C were cultured for 24 h. (B′, D′) Sections of the embryos in panels B, D at the levels indicated. (B″, D″) In situ hybridization for *CdxA* and *chordin* on the same sections (B′, D′ respectively). Arrowheads indicate DiI-labeled transplants.

commitment to an endodermal fate takes place within the primitive streak between stages 3^{+} and 4.

Discussion

Fate maps of the lower layer at different stages of development

In this study, we labeled cells in various positions of the lower layer of stages 2–5 chick embryos and traced their lineages. From the data presented in Figs. 2, 3 and 5, we constructed prospective fate maps of the lower layer (Fig. 10A). At stage 2, a very limited region under the rostral primitive streak contributes to gut endoderm as shown by Rosenquist (1966, 1971b, 1972). This first population of gut endoderm contributes only to the mid- and hindgut. Until stage 4, the forming gut endoderm expands laterally and caudally. Even at this stage, while the presumptive mid- and hindgut region expands laterally and caudally, the presumptive dorsal foregut region does not expand rostrocaudally, but only laterally. Meanwhile, the presumptive ventral foregut region does not emerge in the lower layer before stage 4. At stage 5, the presumptive ventral foregut region emerges into the lower layer at the lateral border of the middle layer. The gut endoderm starts ingressing from the epiblast at the onset of gastrulation; the earliest ingressing endodermal cells become mid- and hindgut. The next cells to enter colonize the dorsal foregut and the final cells to ingress into the lower layer contribute to the ventral foregut. In summary, our fate mapping experiments reveal: (1) a clear border between presumptive foregut and hindgut, as well as between prospective dorsal and ventral territories in the foregut, (2) the migratory route of each prospective region of the endoderm during gastrulation. These results are useful to analyze the timing and mechanisms of anterior/posterior regionalization of the endoderm.

Endoderm cell movements during gastrulation

Our fate maps, which show a spatial and temporal transition of each region of the gut endoderm, reveal new aspects of the migration of endodermal precursor cells. It has been reported that endodermal cells ingress from the epiblast into the lower layer through Hensen's node or the anterior primitive streak

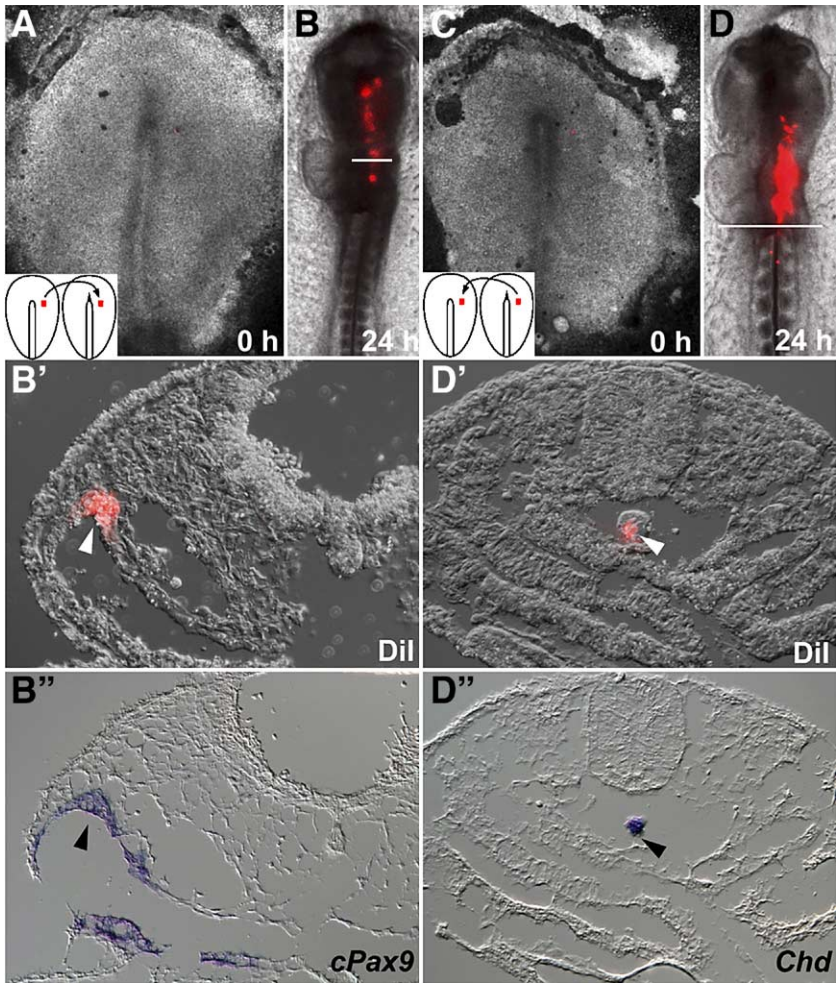

Fig. 9. Endodermal specification during gastrulation. (A, C) Embryos transplanted with DiI-labeled cells from the middle layer lateral to Hensen's node at stage 4 into the same position at stage 4^+ (A) and vice versa (C). (B, D) The embryos in panels A, C were cultured for 24 h. (B′, D′) Sections of the embryos in panels B, D at the levels indicated. (B″, D″) In situ hybridization for *cPax9* and *chordin* on the same sections (B′, D′ respectively). Arrowheads indicate DiI-labeled transplants.

and then spread out laterally and rostrocaudally in the lower layer (Rosenquist, 1966, 1972; Lawson and Schoenwolf, 2003). The movement of presumptive midgut, hindgut and dorsal foregut in our results supports this. However, unlike what is found for the earlier-ingressing midgut, hindgut and dorsal foregut, presumptive ventral foregut cells are not present in the lower layer adjacent to Hensen's node at any of the stages examined but can only be found more remotely, adjacent to emerged middle layer cells. How do these presumptive ventral foregut cells ingress into the lower layer? There are three possible explanations for how presumptive ventral foregut endoderm cells migrate:

1. At stage 4, the presumptive ventral foregut endoderm is still in the epiblast, which then migrates directly to the lateral lower layer at stage 5;
2. The presumptive ventral foregut endoderm ingresses into a very limited region in the lower layer at stage 4, but this region is too small to be targeted by our labeling procedure;
3. Presumptive ventral foregut endoderm does not ingress directly into the lower layer from the node but rather ingresses after some lateral migration within the middle layer before ingressing into the lower layer at stage 5.

Previous reports indicating that there are no foregut endodermal cells in the epiblast at stage 4 (Selleck and Stern, 1991; Garcia-Martinez et al., 1993; Psychoyos and Stern, 1996) make the first possibility very unlikely. We find that cells in the middle layer lateral to Hensen's node at stage 3^+–4 contribute to the ventral foregut endoderm (Figs. 5 and 6): cells in the middle layer adjacent to the primitive steak at stage 3^+ move laterally within the middle layer up to stage 4^+, and only then enter the lower layer. These results indicate that presumptive ventral foregut cells migrating out from Hensen's node arrive at the lower layer only after moving away from the midline within the middle layer. Tracing cells in the epiblast (Supplemental Fig. 4) give further support to this hypothesis: cells in the epiblast near Hensen's node move into the middle layer before reaching the lower layer.

Based on these observations, we propose a model for the movement of endodermal cells during gastrulation (Fig. 10B). At stage 2, gut endoderm precursor cells start ingressing from the

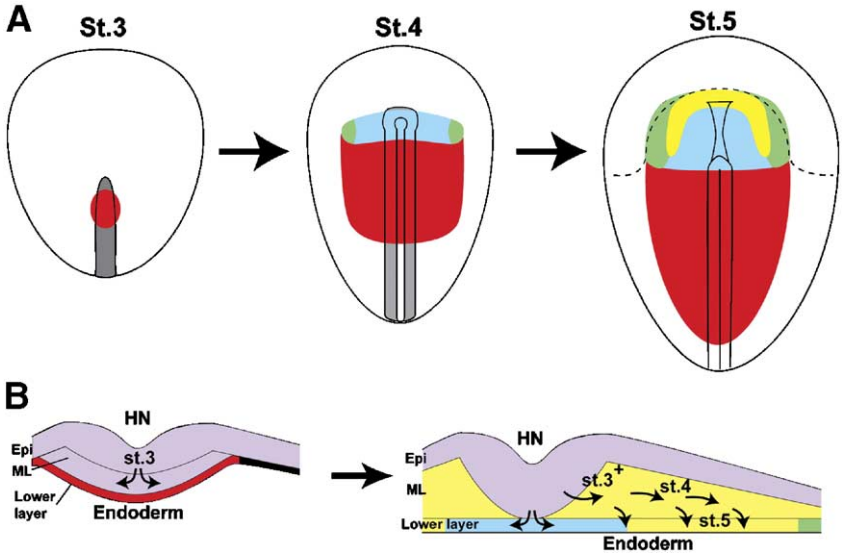

Fig. 10. Summary fate maps of the lower layer at stages 3–5. Cells that ingress early during gastrulation give rise to the mid/hindgut endoderm (red) followed by dorsal foregut endoderm (light blue) and lateral foregut endoderm (green); finally, the presumptive ventral foregut territory extends to the peripheral area of the head process (yellow). (B) Patterns of movement of endodermal precursor cells. At stage 3, cells that contribute to mid/hindgut endoderm ingress from Hensen's node directly into the lower layer (left). At stage 3^{+}, cells which contribute to dorsal foregut ingress from Hensen's node directly into the lower layer (right, light blue), while cells that contribute to ventral foregut ingress into the middle layer, migrate laterally and only then ingress into the lower layer (right, yellow) between dorsal foregut endoderm and lateral foregut endoderm (green). HN, Hensen's node; Epi, epiblast; ML, middle layer.

primitive streak; these cells are destined to contribute to the mid- and hindgut. At stage 3^{+}, presumptive dorsal foregut cells ingress from the primitive streak into the lower layer, at the same time as presumptive ventral foregut cells ingress into the middle layer. The latter cells then move laterally within the middle layer and only enter the lower layer when they reach the lateral border of the middle layer.

At stage 5, the presumptive ventral foregut lies medial to the presumptive lateral foregut. How are these positions rearranged during gut tube formation? Cells that are found between the presumptive dorsal and lateral areas move anteriorly until the embryo reaches stage 6 (data not shown). As the anterior intestinal portal (AIP) moves posteriorly, these cells accompany it and finally contribute to the ventral foregut endoderm (Fig. 5, "33", "24", "21"), consistent with observations by Kirby et al. (2003). On the other hand, cells found in the presumptive lateral foregut endoderm stay in place until the AIP reaches that position (data not shown). As gut tube closure proceeds, they also contribute to the foregut endoderm but do not move anteriorly or medially and become located in the lateral part of the caudal foregut (Fig. 4, "10", "11", "17").

Comparison with previous maps

To date, fate maps of the endoderm of early-stage vertebrate embryos have been constructed for the mouse (Lawson and Pedersen, 1987; Lawson et al., 1986, 1991), frog (Keller, 1975, 1976; Chalmers and Slack, 2000) and zebrafish (Warga and Nusslein-Volhard, 1999) as well as for the chick. Detailed maps for the chick embryo have been built using carbon particles (Bellairs, 1953a,b, 1955, 1957), grafting ^{3}H-thymidine-labeled cells (Rosenquist, 1966, 1970a,b, 1971a,b, 1972) and chick–quail transplantation (Fontaine and Le Douarin, 1977). According to these maps, the gut endoderm moves to the lower layer during extension of the primitive streak, then these endodermal cells gradually expand in the lower layer around the rostral tip of the primitive streak and occupy the lower layer of the embryo, pushing the hypoblast laterally. Our data support these conclusions. In addition, Rosenquist (1966, 1970b) reported that, in stage 4^{+}–5 embryos, ventral gut endoderm cells are located outside the dorsal endoderm as shown in Fig. 9A. However, our fate maps differ from previous ones in several respects. For example, Rosenquist (1972) and Lawson and Schoenwolf (2003) reported that, at stages 2–3, the anterior tip of the primitive streak contributes to a very large portion of the endoderm including the presumptive foregut and hindgut at stage 5. In addition, while presumptive ventral foregut endoderm cells were found in the region anterior and lateral to the Hensen's node at stage 4 in a previous report (Rosenquist, 1971a), our fate map shows that presumptive ventral foregut endodermal cells only appear in the lower layer from stages 4^{+}–5. These differences are probably due to the different methods of labeling. While our fate maps were obtained using targeted labeling of a small number of cells in the lower layer, previous studies labeled not only a large number of cells in the lower layer but also some in the adjacent middle layer (Rosenquist, 1971a). In another study, Kirby et al. (2003) showed that the rostral part of Hensen's node of stage 4 embryos and the prechordal plate of stage 5 embryos include presumptive ventralmost foregut endodermal cells. Together, with our results, these observations suggest that the ventral foregut endodermal cells may arise from two different sets of precursors, located respectively in (a) the rostral part of Hensen's node at stage 4 and in the prechordal

plate at stage 5, which contributes to the midline of the ventral foregut endoderm, and (b) the middle layer lateral to Hensen's node at stage 4 and an arc-shaped region in the lower layer at stage 5, which also contribute to the ventral foregut endoderm, but they become lateral to the midline (which arises from prechordal plate cells). Our fate maps suggest that cells in the lower layer and those in the middle layer adjacent to the lower layer may have different fates: the middle layer around the node contributes to the ventral foregut endoderm, whereas the lower layer adjacent to that area becomes dorsal foregut.

Endoderm and mesoderm fates are specified during gastrulation

Although the middle layer cells present during gastrulation have generally been assumed to be entirely mesodermal (Vakaet, 1970; Balinski, 1975), our fate maps and transplantation experiments show that they are destined for the endoderm. A recent study (Kirby et al., 2003) showed that anterior prechordal cells contribute to both the ventralmost endoderm of the foregut and to heart endothelial cells. Taken together, with our results, it is possible that during gastrulation the anterior middle layer includes presumptive ventral foregut cells. We examined whether the cells in the middle layer are already specified to become endoderm. Both anterior and posterior primitive streak cells, which include endoderm/mesoderm and mesoderm precursors, respectively (Selleck and Stern, 1991; Psychoyos and Stern, 1996), can change their fates when placed in a new environment with respect to both their contribution and tissue-specific marker gene expression (Fig. 7). However, anterior and posterior middle layer cells never change their fate regardless of where they are grafted, as assessed both by their locations and by marker gene expression (Figs. 8, 9). This result indicates that, while endodermal precursor cells in the primitive streak are not yet committed to become endoderm, they do become committed after emerging from the primitive streak/Hensen's node into the middle layer. On the other hand, middle layer cells (presumptive ventral foregut endoderm) contribute to the posterior gut endoderm when grafted into the posterior middle layer. These results suggest that the commitment of cells to endoderm and mesoderm precedes the commitment of prospective endoderm to a specific gut region; middle layer cells around the node are committed to endoderm but not committed to specific anteroposterior or medio-lateral fates (Figs. 8B′, B″, 9B′, B″).

Acknowledgments

We thank Dr. Funayama, N. and Dr. Takahashi, Y. for providing chicken *HFH8* and Dr. Christ, B. for providing *cPax9*. This work was supported in part by Grants-in-aid from the Ministry of Education, Technology, Science and Culture of Japan to K.F. and S.Y, and the Medical Research Council and BBSRC to C.D.S.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.09.009.

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