Development 130, 3491-3501

doi:10.1242/dev.00579


# Epiblast and primitive-streak origins of the endoderm in the gastrulating chick embryo

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*Accepted 2 May 2003*

## SUMMARY

**Gastrulation is characterized by the extensive movements of cells. Fate mapping is used to follow such cell movements as they occur over time, and prospective fate maps have been constructed for several stages of the model organisms used in modern studies in developmental biology. In chick embryos, detailed fate maps have been constructed for both prospective mesodermal and ectodermal cells. However, the origin and displacement of the prospective endodermal cells during crucial periods in gastrulation remain unclear. This study had three aims. First, we determined the primitive-streak origin of the endoderm using supravital fluorescent markers, and followed the movement of the prospective endodermal cells as they dispersed to generate the definitive endodermal layer. We show that between stages 3a/b and 4, the intraembryonic definitive endoderm receives contributions mainly from the rostral half of the primitive streak, and that endodermal movements parallel those of ingressing adjacent mesodermal subdivisions. Second, the question of the epiblast origin of the endodermal layer was addressed by precisely labeling epiblast cells in a region known to give rise to prospective somitic cells, and following their movement as they underwent ingression through the primitive streak. We show that the epiblast clearly contributes prospective endodermal cells to the primitive streak, and subsequently to definitive endoderm of the area pellucida. Finally, the relationship between the hypoblast and the definitive endoderm was defined by following labeled rostral primitive-streak cells over a short period of time as they contributed to the definitive endoderm, and combining this with in situ hybridization with a riboprobe for *Crescent*, a marker of the hypoblast. We show that as the definitive endodermal layer is laid down, there is cell-cell intercalation at its interface with the displaced hypoblast cells. These data were used to construct detailed prospective fate maps of the endoderm in the chick embryo, delineating the origins and migrations of endodermal cells in various rostrocaudal levels of the primitive streak during key periods in early development.**

Key words: Avian embryos, Cell migration, Gastrulation, Germ layers, Hypoblast, Ingression, Morphogenetic movements

## INTRODUCTION

Gastrulation is characterized by the extensive movements of cells. Such morphogenetic movements culminate in vertebrate embryos in the formation of the three primary germ layers: the ectoderm, mesoderm and endoderm. In mammalian and avian embryos, gastrulation is centered around the primitive streak, a transient midline structure that serves as a conduit through which epiblast cells fated to become mesoderm and endoderm ingress to establish the germ layers. The primitive streak may also function in specifying cell fate, based on the range of genes it expresses during its development (Lawson et al., 2001).

Identification of precursor cells that ingress through the primitive streak, and tracking of their subsequent movement to various destinations in the developing embryo, have been the subjects of intense research. Not only do the prospective fate maps generated from these studies suggest mechanisms underlying cell commitment and patterning of the early vertebrate embryo, they also provide insight into morphogenesis as a whole. The primitive-streak origin of mesodermal precursor cells in the chick embryo has been well-defined in previous studies (Pasteels, 1937; Spratt, 1942; Rosenquist, 1966; Rosenquist and DeHaan, 1966; Nicolet, 1971; Vakaet, 1984; Vakaet, 1985; Selleck and Stern, 1991; Schoenwolf et al., 1992; Garcia Martinez and Schoenwolf, 1993; Garcia-Martinez et al., 1993; Psychoyos and Stern, 1996; Lopez-Sanchez et al., 2001). For example, prospective cardiogenic cells occupy most of the rostral half of the primitive streak at its early stages of development (Garcia-Martinez and Schoenwolf, 1993). By mid-primitive-streak stages, when the prospective cardiogenic cells have undergone ingression, their position within the primitive streak becomes occupied by ingressing prospective somitic and lateral plate mesoderm cells (Schoenwolf et al., 1992; Garcia Martinez and Schoenwolf, 1993). Even prior to the movement of the prospective cardiogenic cells into the primitive streak, their precise epiblast origin is known: they reside in the epiblast lateral to the primitive streak and caudal to the neural plate (Lopez-Sanchez et al., 2001). These findings provide a cellular basis for elucidating the molecular mechanisms involved in the morphogenesis of the cardiovascular system of the avian embryo.

By contrast, the cellular mechanisms that underlie the formation of the endoderm in the avian embryo have not been fully elucidated, and the existing studies partially contradict one another. For example, several studies reported that the endoderm receives contributions from different sources, including the epiblast, the caudolateral region of the blastoderm and the yolky cells derived from the germ wall (i.e. the lower layer of the area opaca near its border with the area pellucida) (Hunt, 1937; Pasteels, 1937; Bellairs, 1953; Nicolet, 1971; Rosenquist, 1966; Vakaet, 1962; Stern and Ireland, 1981), whereas a study by Spratt and Haas (Spratt and Haas, 1965) concluded that endodermal precursors did not have an epiblast origin. Moreover, a model based largely on timelapse observations (Vakaet, 1970) suggests that three waves of cell movements occur during formation of the endoderm. First, cells were proposed to move centrifugally (i.e. towards the center of the blastoderm) from the area pellucida-area opaca border to give rise to the junctional endoblast (i.e. the endoderm at the periphery of the area pellucida). Second, cells from the region of Koller's sickle (i.e. at the posterior marginal zone) were proposed to move rostrally to generate the sickle endoblast [also called endoblast; see figure 1 by Foley et al. (Foley et al., 2000)]. Finally, cells from the epiblast were proposed to ingress through the primitive streak to generate the definitive endoderm. A detailed study by Rosenquist (Rosenquist, 1972) is noteworthy here. He reported that the majority of cells that formed the endodermal layer originated from the 'endodermal center' within the primitive streak, and that following ingression through the streak, they occupied an oval area, which surrounded the rostral half of the streak. Furthermore, he reported that these cells underwent a convergent-extension movement to generate the definitive endoderm.

The disparity in the results of the early studies on endoderm formation is due largely to technical limitations. For example, the resolution of timelapse cinematography is inadequate to follow movements of individual cells, and movements in one germ layer cannot be readily distinguished from those in deeper or more superficial layers. Grafting of radiolabeled cells and autoradiography are laborious approaches, necessitating small sample sizes and incomplete cell labeling. Grafted cells that fail to heal into the primitive streak or epiblast can end up in ectopic locations, leading to misinterpretation of the results. In addition, the assessment of the endodermal layer in ovo, the deepest of the germ layers, can be fraught with difficulties. With the development of new and improved techniques for labeling cells and for tracking flattened and widely dispersed cells such as the endodermal cells in vitro, there is a need for a systematic study of endoderm formation that clearly defines the relative positions of the precursor cells in the primitive streak and epiblast at precise stages of their development, and follows their movements as they disperse to generate the endodermal layer.

In the avian embryo, the spatial and temporal relationships between the hypoblast and the developing definitive endoderm also are not fully understood. Formation of the hypoblast layer is complex. It begins with polyingression of cells from the epiblast (Weinberger and Brick, 1982a; Weinberger and Brick, 1982b; Penner and Brick, 1984; Weinberger et al., 1984; Stern and Canning, 1990; Harrisson et al., 1991; Lawson and Schoenwolf, 2001a) and is supplemented by cells moving rostrally from Koller's sickle and the posterior marginal zone (Vakaet, 1970; Stern and Canning, 1990; Callebaut et al., 1999). By the incipient primitive streak stage of development, the hypoblast consists of a complete layer beneath the epiblast. Its subsequent movement to the germ cell crescent can be tracked by using hypoblast markers such as *Crescent* (Pfeffer et al., 1997) and *Goosecoid* (Izpisúa-Belmonte et al., 1993). Although there is evidence that when hypoblast cells are confronted with endodermal cells in culture they tend to be displaced peripherally, thereby surrounding the endodermal cells (Sanders et al., 1978), it is unknown whether similar interactions occur in the intact embryo.

The present study had three aims. First, we determined the primitive-streak origin of the endoderm using supravital fluorescent markers, and followed the movement of the prospective endodermal cells as they dispersed to generate the definitive endodermal layer. The results demonstrate that between stages 3a/b and 4, the intra-embryonic definitive endoderm receives contributions mainly from the rostral half of the primitive streak. Second, the question of the epiblast origin of the endodermal layer was addressed by labeling epiblast cells in a region known to give rise to prospective somitic cells, and following their movement as they underwent ingression through the primitive streak. The results demonstrate that the epiblast clearly contributes prospective endodermal cells to the primitive streak, and subsequently to the definitive endoderm of the area pellucida. Finally, the relationship between the hypoblast and the definitive endoderm was defined by following labeled rostral primitive-streak cells over a short period of time as they contributed to the endoderm, and combining this with in situ hybridization with a riboprobe for *Crescent*. The results show that as the definitive endodermal layer is laid down, there is cell-cell intercalation at its interface with the displaced hypoblast cells, which results in the intermingling of the two populations of cells.

## MATERIALS AND METHODS

### Whole embryo culture and staging

Fertilized eggs were incubated at 38°C to obtain embryos at stages ranging from 2 to 4 (Hamburger and Hamilton, 1951). Sub-stages of HH stage 3 were as described previously (Schoenwolf et al., 1992), with embryos from stages 3a and 3b grouped together as stage 3a/b. Briefly, embryos at 3a/b contain short primitive streaks that have not yet extended rostrally up to the widest level of the oval- to pear-shaped area pellucida. Embryos at stage 3c contain primitive streaks whose rostral end has extended forward to lie at the widest level of the area pellucida. Embryos at stage 3d contain primitive streaks whose rostral end has extended beyond the widest level. And embryos at stage 4, like those at stage 3d, contain primitive streaks whose rostral end has extended beyond the widest level, but their Hensen's node is now capped by a triangular ingression of cells, as pictured in Hamburger and Hamilton's stage series for stage 4. Culture dishes and embryos were prepared as described by Darnell and Schoenwolf (Darnell and Schoenwolf, 2000) for modified New (New, 1955) culture.

### Fate mapping experiments

#### Injections of the primitive streak

Chick embryos at stages 3a/b, 3c, 3d and 4 ($n$=230; $n$ here and subsequently refers to the number of embryos successfully labeled and studied) were selected for injections of the primitive streak. The primitive streak was injected with supravital fluorescent dyes at one of three levels along its length (Fig. 1): rostral, mid and caudal. For each level, two dyes were injected in tandem, with the centers of the two injections being separated rostrocaudally by ~125 μm: a mixture of 5-carboxytetramethylrhodamine, succinimidyl ester (CRSE; Molecular Probes, Eugene, OR) and 1,1′-dioctadecyl-3,3,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes), and 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes). Each injection was made across the width of the primitive streak. Embryos were immediately examined with a fluorescence microscope to confirm the size and site of each injection, and they were then reincubated for periods up to 24 hours, during which they were examined at regular intervals. At the end of culture, all embryos were fixed in 4% paraformaldehyde in PBS, and then processed either for cryostat sectioning or for immunocytochemistry, as described below.

#### Injections of the parastreak epiblast

For injections of the parastreak epiblast, embryos at stages 3a/b and 3c ($n$=15) were cultured dorsal side upwards in Spratt culture (Spratt, 1947), modified as described by Schoenwolf (Schoenwolf, 1988). The epiblast was injected lateral to the rostral end of the primitive streak (Fig. 1) at a site known to contribute cells to the somites (site $b_L$) (see Lopez-Sanchez et al., 2001) with a mixture of DiI/CRSE, and the embryos were reincubated for up to 24 hours (a subset was collected immediately after injection without reincubation to determine the precise location and size of the injection). They were then fixed in 4% paraformaldehyde in PBS and processed for immunocytochemistry, as described below.

#### Mapping the hypoblast and definitive endoderm

For mapping the hypoblast and definitive endoderm, the primitive streaks of embryos at stages 2-3a/b ($n$=18) were first injected at the most-rostral end of the primitive streak (Fig. 1; to mark the most rostrally ingressing endoderm) with a mixture of DiI/CRSE and reincubated for 4 hours. They were then fixed in 4% paraformaldehyde, followed by in situ hybridization as described below. Immunocytochemistry was subsequently carried out, also as described below.

### Immunocytochemistry

Embryos labeled with fluorescent markers were processed for whole-mount immunocytochemistry as described previously by Patel et al. (Patel et al., 1989), with the following modification: the embryos were fixed with 4% paraformaldehyde in PBS. For embryos labeled with both DiI/CRSE and CFSE, fluorescein immunocytochemistry was carried out first as follows. The embryos were incubated in PBT containing 3 mM levamisole for 30 minutes at room temperature to block phosphatase activity. This was followed by treatment with the same solution containing 5% sheep serum for a further 30 minutes. The embryos were then incubated overnight at 4°C with alkaline phosphatase conjugated anti-fluorescein antibody (Roche Diagnostics, Indianapolis, IN) at a dilution of 1:600. The secondary antibody used was alkaline phosphatase conjugated anti-digoxigenin antibody (Roche Diagnostics). For DiI/CRSE labeling, we used anti-rhodamine (rabbit IgG polyclonal, primary; Molecular Probes) and horseradish peroxidase-conjugated goat anti-rabbit IgG (secondary antibody; Roche, Indianapolis). The embryos were examined and photographed as whole mounts, and then processed for paraffin histology as described below.

### In situ hybridization

In situ hybridization was carried out with *Crescent* RNA probe (kindly provided by C. Stern) as described by Nieto and colleagues (Nieto et al., 1996), except that the proteinase K, hydrogen peroxide, and RNase A steps were omitted.

### Paraffin histology

Embryos selected for histology were passed through an ascending series of ethanol up to 100% ethanol. They were then cleared with two changes of Histosol, infiltrated with Paraplast and embedded in fresh Paraplast. Sectioning was carried out at 12 μm, and the sections were examined with Hoffman modulation contrast optics.

### Cryostat sectioning

Embryos selected for cryostat sectioning were passed through an ascending series of sucrose-PBS solutions up to 30% sucrose in PBS. They were finally embedded in OCT compound and sectioned at 30 μm. The sections were then examined with a fluorescence microscope.

## RESULTS

To determine the primitive-streak origin of cells that contribute to the endodermal layer in embryos at stages 3a/b to stage 4, three levels along the primitive streak (rostral, mid and caudal) were injected with vital fluorescent dyes (Fig. 1). Two dyes were used, a DiI/CRSE mixture and CFSE, and both dyes were injected into each embryo but at different rostrocaudal sites about 125 μm apart, allowing the relative displacement of prospective endodermal cells residing in adjacent levels of the primitive streak to be followed as they migrated to new locations in the forming endodermal layer. Below, we emphasize only the contributions to the endodermal layer, as the mesodermal contributions have been described in detail previously in other studies (e.g. Garcia-Martinez et al., 1993; Lopez-Sanchez et al., 2001).

### Fate mapping the rostral primitive streak

After injections of the rostral level of the primitive streak at stage 3a/b (Fig. 2A), both the DiI/rhodamine and fluorescein labeled cells migrated rostrally where they contributed to the rostral endoderm and mesoderm within 6 hours (Fig. 2B,C). With further rostral migration by 9 hours of reincubation, the labeled cells from the more caudal injection (green fluorescence) slightly overlapped those from the more rostral injection (red fluorescence) (Fig. 2D). After 18-24 hours of reincubation, the embryos had developed to stages ranging from 7-8. At these stages, cells from the more rostral injection (red fluorescence; brown after immunocytochemistry) populated most of the intraembryonic endodermal layer, which extended from the head fold to the caudal end of the primitive streak (Fig. 2E,F). In addition, this endoderm delineated a pear-shaped zone, extending the full width of the area pellucida rostrally and tapering caudally to the width of the intraembryonic region (Fig. 2E). By contrast, there was minimal displacement of labeled cells from the more caudal site [green fluorescence; purple after immunocytochemistry; the latter cells occupied only a small region of the endodermal layer at the caudal end of the primitive streak (Fig. 2E) and overlap between the two populations was minimal or non-existent]. Thus, the location of these cells was largely caudal to that of endodermal cells derived from the more rostral site.

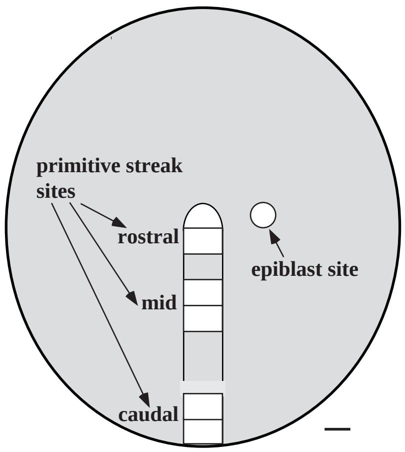


**Fig. 1.** Schematic diagram based on details from Lopez-Sanchez et al. (Lopez-Sanchez et al., 2001) showing the two rostral (in rostral-to-caudal sequence, sites $c_r$ and $c_{0.5}$ by Lopez-Sanchez et al.), two mid (in rostral-to-caudal sequence, approximately sites $c_{1.5}$ and $c_2$ by Lopez-Sanchez et al.), and two caudal primitive streak sites labeled in this study, as well as the one epiblast site (site $b_L$ by Lopez-Sanchez et al.). Scale bar: 125 μm.

In conclusion, the rostral tip of the primitive streak at stage 3a/b contains cells fated to form most of the rostrocaudal extent of the future dorsal and ventral endoderm of the gut. A similar fate was observed when cells from the rostral tip of the primitive streak were labeled at stage 2 (data not shown).

The migration of labeled cells during the first 9 hours of reincubation from the rostral level of the primitive streak (Fig. 2G) of embryos injected at stage 3c largely paralleled that of rostral injections at stage 3a/b (compare Fig. 2H,I with Fig. 2B,D), except that cells originating from the more rostral site were more rostrally restricted in embryos injected at stage 3c. From 18 hours of reincubation onwards, differences between the two developmental stages became more apparent. Labeled cells from the more rostral site in embryos injected at stage 3c now broadly populated the region of the endodermal layer principally rostral to the cranial intestinal portal (Fig. 2J), whereas labeled cells from the more caudal site c incorporated into the endodermal layer between the cranial intestinal portal and the caudal end of the primitive streak; at the cranial intestinal portal, these cells intermixed with cells derived from the more rostral site (Fig. 2J,K). Within the midline (Fig. 2J,K), endodermal cells originated from both sites, with more rostral midline cells tending to be derived from the more rostral site and more caudal midline cells deriving from the more caudal site. Thus, the midline endoderm extends caudally with regression of the rostral end of the primitive streak and formation of the head process.

The contribution of labeled cells from the rostral level of the primitive streak (Fig. 2L) in embryos injected at stage 3d was similar to that at stage 3c (compare Fig. 2H-J with Fig. 2M-O), except that in embryos injected at stage 3d all labeled cells were restricted to the region of endoderm rostral to Hensen's node (Fig. 2O).

By stage 4, significant differences were apparent as compared to younger stages in the migration of prospective endodermal cells from the rostral level of the primitive streak (Fig. 2P) and their subsequent contribution to the developing endodermal layer. Unlike the pattern in the younger embryos described above, the migration of labeled cells at stage 4 from the most rostral level was virtually restricted to the midline (Fig. 2Q,R). Cells from this rostral level, therefore, contributed to the floor plate of the neural tube, notochord and the mid-dorsal endoderm, whereas those from the more caudal site contributed to the somites and underlying endoderm (Fig. 2S,T).

In summary, the rostral level of the primitive streak contributes to most of the rostrocaudal and mediolateral extent of the definitive endoderm. In progressively older embryos, contributions of the rostral primitive streak becomes correspondingly restricted toward the midline.

## Fate mapping the mid-primitive streak

Injections of DiI/CRSE and CFSE into the mid-primitive streak level in embryos at stage 3a/b (Fig. 3A) resulted in the labeling of both endodermal and mesodermal cells of the primitive streak and adjacent parts of the blastoderm by 6 hours of reincubation (Fig. 3B,C). Subsequently (i.e. by 9 hours), both the DiI/CRSE- and CFSE-labeled cells within the endodermal and mesodermal layers migrated rostrolaterally, following v-shaped streams towards the lateral margin of the area pellucida (Fig. 3D). Between 18 and 24 hours of reincubation, the labeled prospective endodermal and mesodermal cells at the leading edge migrated further rostrally and laterally to occupy the cardiogenic areas of the blastoderm (i.e. the rostrolateral margins of the area pellucida; Fig. 3E,F). Caudally, labeled cells were found in the endoderm of the area pellucida and the overlying mesoderm (Fig. 3E,G). Mid-primitive streak cells labeled at stages 3c and 3d (Fig. 3H,M) followed similar routes to populate corresponding areas of the endoderm and mesoderm (Fig. 3I-L,N-P). Between stages 3a/b and 3d, therefore, labeled mid-primitive streak cells populated rostrally the endoderm of the heart-forming region, the cardiogenic mesoderm, and the lining of the pericardial cavity, whereas caudally, they populated the endoderm of the lateral area pellucida (i.e. the future ventral gut and extraembryonic endoderm) and its overlying mesoderm. Labeled mid-primitive streak cells at stage 4 (Fig. 3Q) followed one of two different routes to contribute to two different subpopulations of endodermal and mesodermal cells. Whereas the rostromost subpopulation remained closer to the midline, eventually contributing to the intermediate mesoderm and its underlying endoderm (Fig. 3R,S,U), the more caudal subpopulation of labeled cells migrated more laterally to contribute to the lateral plate mesoderm and its underlying endoderm (Fig. 3T,V,W).

## Fate mapping the caudal primitive streak

The movement of labeled cells after injections of the caudal primitive streak at stages ranging from 3a/b to 4 (Fig. 4A,F,L) followed a similar pattern, being restricted to the caudal end of the area pellucida within the first 9 hours (Fig. 4B,C,G,I,M), where they contributed to both endodermal and mesodermal layers (Fig. 4H). Generally, cells labeled in older embryos tended to be displaced more caudally. Thereafter, labeled cells from both layers in embryos injected at stage 3a/b moved rostrally along the area pellucida-area opaca border by 18-24 hours (Fig. 4D,E), whereas cells labeled from injections performed at stage 3c to stage 4 ended up in the deep and middle layers of the area opaca (Fig. 4J,K,N).

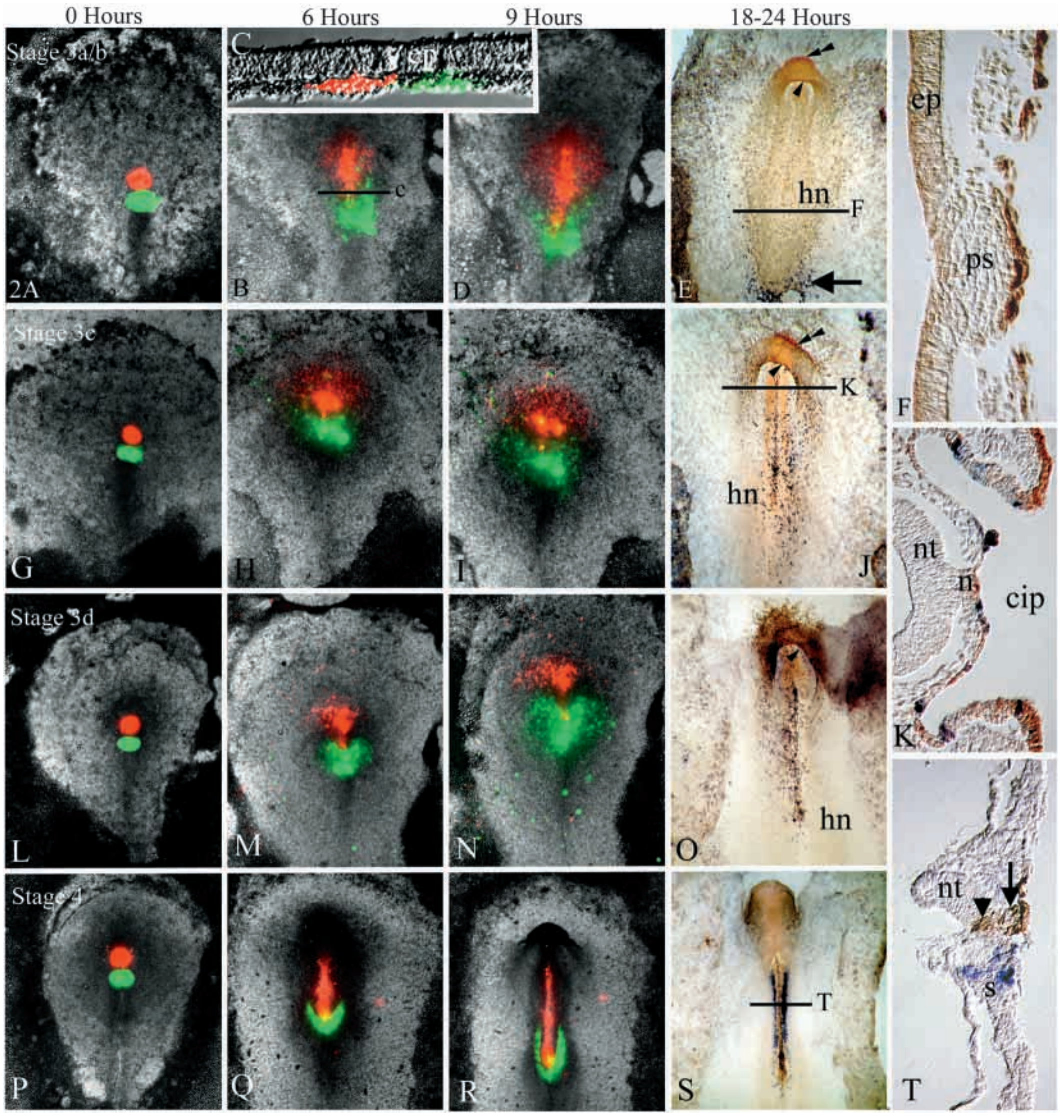


**Fig. 2.** Fate mapping the rostral primitive streak. DiI/CRSE (red) and CFSE (green) injections at sites $c_r$ and $c_{0.5}$ at stages 3a/b (A-F), 3c (G-K), 3d (L-O), and 4 (P-T), respectively. (A,G,L,P) *t*=0 hours of reincubation. (B,C,H,M,Q) *t*=6 hours of reincubation. (D,I,N,R) *t*=9 hours of reincubation. (E,F,J,K,O,S,T) *t*=18-24 hours of reincubation. Lines in B,E,J,S indicate levels of sections shown in C,F,K,T, respectively. (E,F,J,K,O,S,T) Immunocytochemistry with anti-rhodamine (brown) and anti-fluorescein (purple) antibodies. In T, the more caudal injection site contributed to the somites (s; purple cells) and underlying endoderm, whereas the more rostral site contributed to the mid-dorsal endoderm and notochord (arrow), as well as the floor plate (arrowhead) of the neural tube (nt). cip, cranial intestinal portal; ep, epiblast; hn, level of Hensen's node; n, notochord; nt, neural tube, ps, primitive streak. Arrow in E indicates fluorescein-labeled (purple) cells; single arrowheads in E,J,O indicate the cranial intestinal portal; double arrowheads in E and J indicate the rostral border of the head.

### Generation of endoderm from the epiblast

To address the question of whether the epiblast contributed cells to the endodermal layer, we fate mapped in embryos at stage 3a/b and 3c a lateral epiblast site (Fig. 1, Fig. 5A,B) known to contribute cells to the somites (see Lopez-Sanchez et al., 2001), and followed the movement of the labeled cells over time. Sections of embryos fixed immediately after injection revealed that the injection was typically confined to the epiblast layer, rather than extending into deeper layers (Fig. 5B). Between 3 and 6 hours of reincubation, the labeled cells had moved to the primitive streak and were undergoing ingression (Fig. 5C,D). By 22 hours of reincubation, the labeled cells had incorporated into the somites and the underlying endodermal layer (Fig. 5E,F). These results clearly demonstrate that the definitive endoderm is generated from cells of lateral epiblast origin that move toward the primitive streak and ingress through it, and not just from cells originally within the primitive streak per se.

### Displacement of the hypoblast by the definitive endoderm

Next, we addressed the issue of the spatial and temporal relationships between the hypoblast and the forming definitive endodermal layer. The rostral end of the primitive streak was marked with DiI/CRSE in stage 2-3a/b embryos (Fig. 1), which were reincubated for a period of 4 hours. Then, embryos were

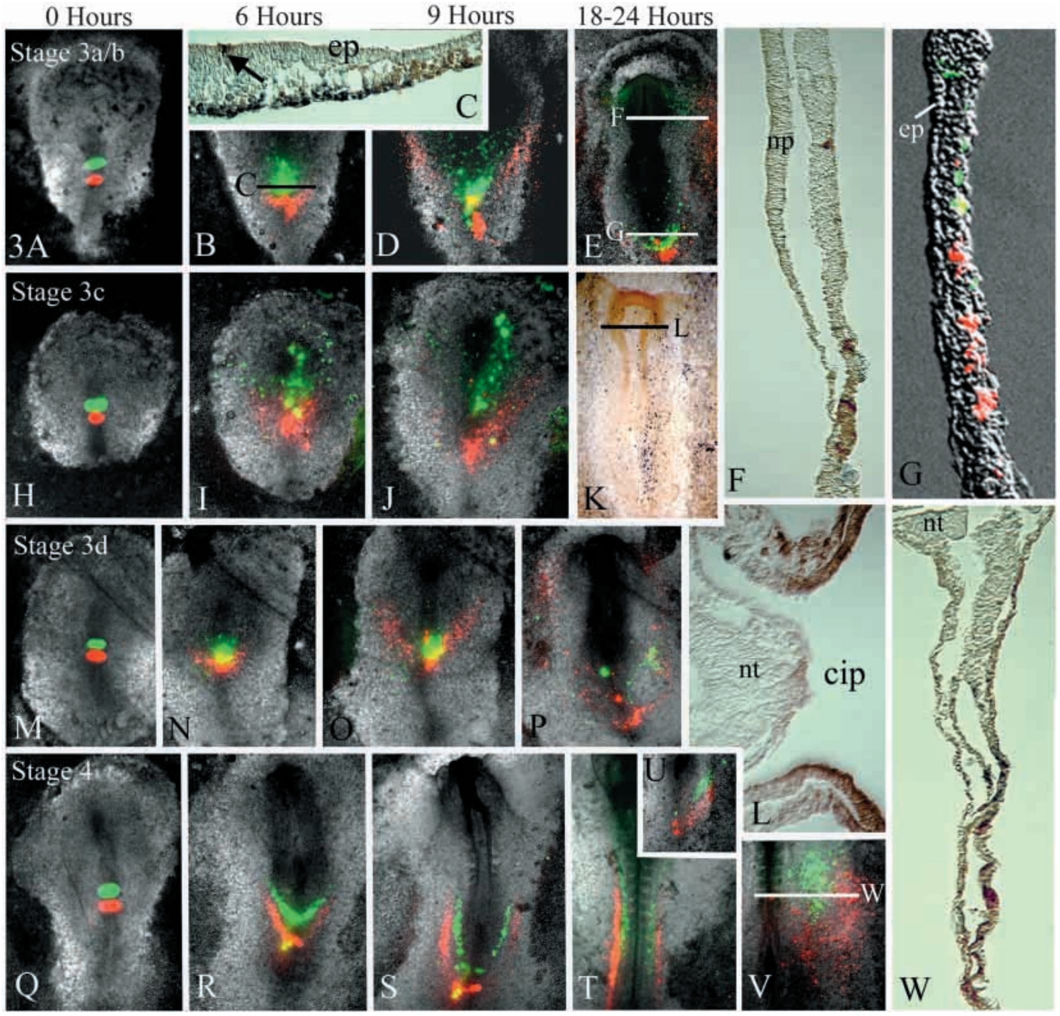


**Fig. 3.** Fate mapping the mid-primitive streak. DiI/CRSE (red) and CFSE (green) injections at mid-primitive streak level (red) and an adjacent rostral level (green) at stages 3a/b (A-G), 3c (H-L), 3d (M-P) and 4 (Q-W), respectively. (A,H,M,Q) *t*=0 hours of reincubation. (B,C,I,N,R) *t*=6 hours of reincubation. (D,J,O,S,U) *t*=9 hours of reincubation. (E-G,K,L,P,T,V,W) *t*=18-24 hours of reincubation. (Note that two stage 4 embryos are shown at 9 hours (S,U) and subsequently at 18-24 hours (T and V,W, respectively). Lines in B,E,K,V indicate levels of sections shown in C,F,G,L,W. (F,K,L,W) Immunocytochemistry with anti-rhodamine (brown) and anti-fluorescein (purple) antibodies. In C, arrow indicates the midline dorsal surface cells of the primitive streak (see Lawson and Schoenwolf, 2001a; Lawson and Schoenwolf, 2001b). cip, cranial intestinal portal; ep, epiblast; np, neural plate; nt, neural tube.

labeled with in situ hybridization using *Crescent*, a hypoblast marker (Fig. 6A). As the rhodamine-labeled primitive streak cells contributed to the rostral endodermal layer, the *Crescent*-labeled hypoblast cells formed a cap at their leading edge (Fig. 6B). At the interface between the two, the two subpopulations of cells intercalated with each other (Fig. 6C). Ultimately, the hypoblast cells were displaced rostrally to the germ cell crescent (not shown), and a distinct salt-and-pepper relationship (owing to such intercalation) develops between the hypoblast and definitive endoderm as revealed by detailed in situ hybridization studies [see right column of figure 2 by Chapman et al. (Chapman et al., 2002)].

## DISCUSSION

The key findings of the present study are that: (1) the intra-embryonic region of the endodermal layer is generated mainly by cells from the rostral half of the primitive streak between stages 3a/b and 4; (2) more peripheral regions of the endodermal layer (extending peripherally to the area pellucida-area opaca border and beyond) are derived from principally more caudal levels of the primitive streak (with the exception of the rostral peripheral endoderm, which derives from the rostral end of the primitive streak); (3) epiblast cells fated to become endoderm undergo ingression through the primitive streak to contribute to the definitive endodermal layer of the area pellucida; and (4) displacement of the hypoblast layer by the forming endoderm is accompanied by cell-cell intercalation between the two populations.

On the basis of our mapping experiments, we constructed prospective fate maps of the definitive endoderm (Fig. 7). As most of the intra-embryonic endoderm arises from the rostral end of the primitive streak as the latter forms and undergoes

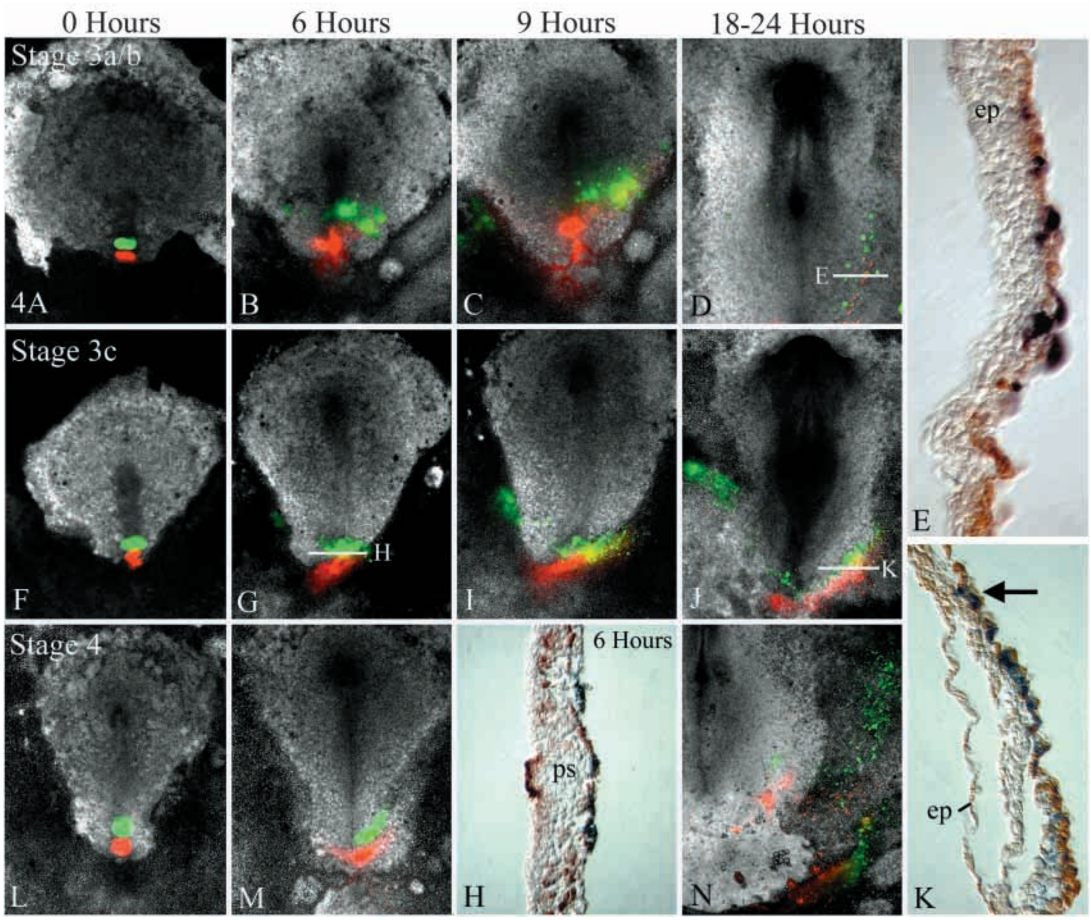


**Fig. 4.** Fate mapping the caudal primitive streak. DiI/CRSE (red) and CFSE (green) injections at the caudal end of primitive streak at stages 3a/b (A-E), 3c (F-K) and 4 (L-N), respectively. (A,F,L) *t*=0 hours of reincubation. (B,G,H,M) *t*=6 hours of reincubation. (C,I) *t*=9 hours of reincubation. (D,E,J,K,N) *t*=18-24 hours of reincubation. Lines in D,G,J indicate levels of sections shown in E,H,K. (E,H,K) Immunocytochemistry with anti-rhodamine (brown) and anti-fluorescein (purple) antibodies. ep, epiblast; ps, primitive streak; arrow in K indicates the junction between the area pellucida (above) and area opaca (below).

progression, we constructed prospective fate maps that show the rostrocaudal subdivisions of the definitive endoderm originating from the rostral end of the primitive streak (Fig. 7, to the left of the broken vertical line). In addition, as rostrocaudal position within the primitive streak ultimately translate into mediolateral position within the definitive endoderm, we constructed a prospective fate map that shows the mediolateral subdivisions of the definitive endoderm originating from the rostral, mid and caudal levels of the primitive streak (Fig. 7, to the right of the broken vertical line and above the broken horizontal line). Moreover, as mesodermal and endodermal regions arise in concert within the primitive streak and migrate in parallel after ingression, we constructed a prospective fate map comparing mesodermal and endodermal subdivisions (Fig. 7, to the right of the broken vertical line and below the broken horizontal line).

## The early phase of endoderm formation

This phase of endoderm formation coincides with the pre- and incipient stages of primitive streak formation. During this period of development, cells fated to contribute to the endodermal layer are generally believed to originate from two sources. First, cells residing in the germ wall (i.e. the lower layer of the area opaca near its border with the area pellucida) are presumed to undergo a centrifugal movement to give rise to the junctional endoblast, defined as the endodermal layer at the periphery of the area pellucida (Vakaet, 1970; Stern and Ireland, 1981; Stern, 1990). Experimental evidence in support of the above mechanism of formation of the junctional endoblast comes from a study in which the original lower layer was ablated in pre-and incipient primitive streak embryos (Stern and Ireland, 1981). It was noted that after ablation, cells migrated from the germ wall to adjacent parts of the area pellucida. The observations of the present study on the origin of the junctional endoblast, however, are not in accordance with the idea that a junctional endoblast arises from the germ wall during normal development in the intact embryo; rather, they demonstrate that the junctional endoblast in the intact embryo receives contributions from the primitive streak (and largely from its caudal region), instead of from the germ wall (Fig. 7: origins of mediolateral endodermal subdivisions). The migration of cells from the germ wall in Stern and Ireland's experiment (Stern and Ireland, 1981) is, therefore, likely to represent a response to an experimental manipulation of the lower layer, rather than an event that occurs normally during development. The second source of endodermal cells is

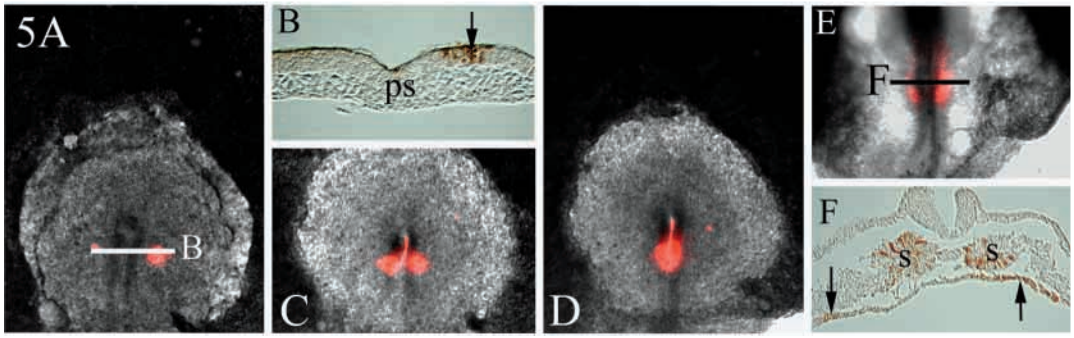


**Fig. 5.** Fate mapping the epiblast. DiI/CRSE (red) injections of epiblast adjacent to the primitive streak at stage 3c. (A,B) *t*=0 hours of reincubation. (C) *t*=3 hours of reincubation. (D) *t*=6 hours of reincubation. (E,F) *t*=22 h of reincubation. Lines in A,E represent levels of sections shown in B,F. (B,F) Immunocytochemistry with anti-rhodamine antibody (brown). Note in B that the injection is confined to the epiblast (arrow). ps, primitive streak. Note in F that the epiblast injection labeled the mesodermal cells of the somites (s) and the underlying endoderm (arrows).

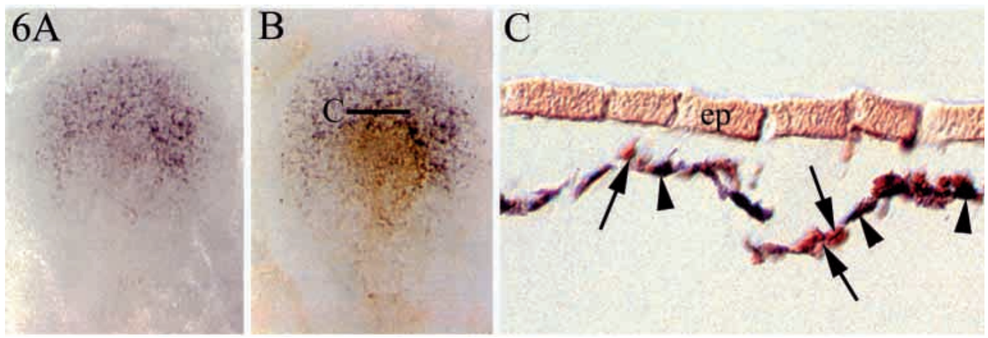


**Fig. 6.** Labeling the hypoblast and the rostral primitive streak. In situ hybridization with a *Crescent* RNA probe (purple), and immunocytochemistry with anti-rhodamine antibody (brown), 4 hours after injection of DiI/CRSE site $c_r$ at stage 2. (A) *Crescent*-labeled hypoblast in rostral half of area pellucida. (B) *Crescent*-labeled hypoblast as in a (purple) and rostral endoderm (brown). Line in B indicates the level of section shown in C. ep, epiblast. Note the intermixing of cells in the lower layer at the interface between the hypoblast (purple; arrowheads) and definitive endoderm (brown; arrows).

believed to be the lower layer underlying Koller's sickle (Pasteels, 1937; Bellairs, 1953; Malan, 1953; Vakaet, 1970; Bachvarova et al., 1998). Studies suggest that cells from this location migrate rostrally in tandem with cells from the epiblast overlying Koller's sickle to contribute to the sickle endoblast and the primitive streak, respectively (Vakaet, 1970; Stern and Ireland, 1981; Callebaut and Van Nueten, 1994; Bachvarova et al., 1998; Foley et al., 2000; Lawson and Schoenwolf, 2001b). Both components of the early endoderm (the junctional and sickle endoblasts) are believed to be fated to become extra-embryonic (Rosenquist, 1971; Rosenquist, 1972; Fontaine and Le Douarin, 1977), not contributing to the definitive structure of the embryo (Callebaut et al., 2000).

## The intermediate phase of endoderm formation

This stage of endoderm formation takes place as the primitive streak lengthens and cells ingress through it. It is characterized by the formation of the definitive endodermal layer of the area pellucida. The demonstration in the present study that the primitive streak serves as a source of cells for the definitive endodermal layer confirms the findings of several earlier studies (Vakaet, 1970; Nicolet, 1971; Rosenquist, 1971; Rosenquist, 1972; Fontaine and Le Douarin, 1977; Stern and Ireland, 1981; Garcia-Martinez et al., 1993; Lopez-Sanchez et al., 2001; Bachvarova et al., 1998; Foley et al., 2000). By following the movements of labeled cells at different levels of the primitive streak between stages 3a/b and 3d, we demonstrated that the rostral part of the primitive streak generates most of the intraembryonic endoderm (Fig. 7: origins of rostrocaudal endodermal subdivisions). This confirms the findings of Rosenquist (Rosenquist, 1971), who used grafts labeled with tritiated thymidine followed by autoradiography. Additionally, age-related differences were observed in the contribution of labeled cells from the rostral end of the primitive streak to the definitive endodermal layer. Whereas at stage 3a/b labeled cells from the rostral streak populated most of the definitive endodermal layer, at stage 3d the contribution from the rostral primitive streak was restricted only to the region of the definitive endoderm rostral to Hensen's node. These findings, while demonstrating that formation of the definitive endoderm is spatially and temporally regulated, also provide insight into the nature of cell movement taking place at the rostral end of the primitive streak during its progression.

## The late phase of endoderm formation

The late phase of endoderm formation occurs at stage 4 when the primitive streak has attained its maximum length and Hensen's node has formed. With the formation of Hensen's node, contributions of cells from the rostral streak were virtually restricted to the midline, probably because Hensen's node generates midline structures such as floor plate, notochord and dorsal midline endoderm (as well as the ventral midline endoderm of the foregut and outflow tract of the heart) (Kirby et al., 2003). Cells from the rostral end of the primitive streak also generate the prechordal plate, important for regionalizing the forebrain (Pera and Kessel, 1997), and the ventral midline endoderm of the foregut, a region well situated to form a ventral axis of the head (Kirby et al., 2003).

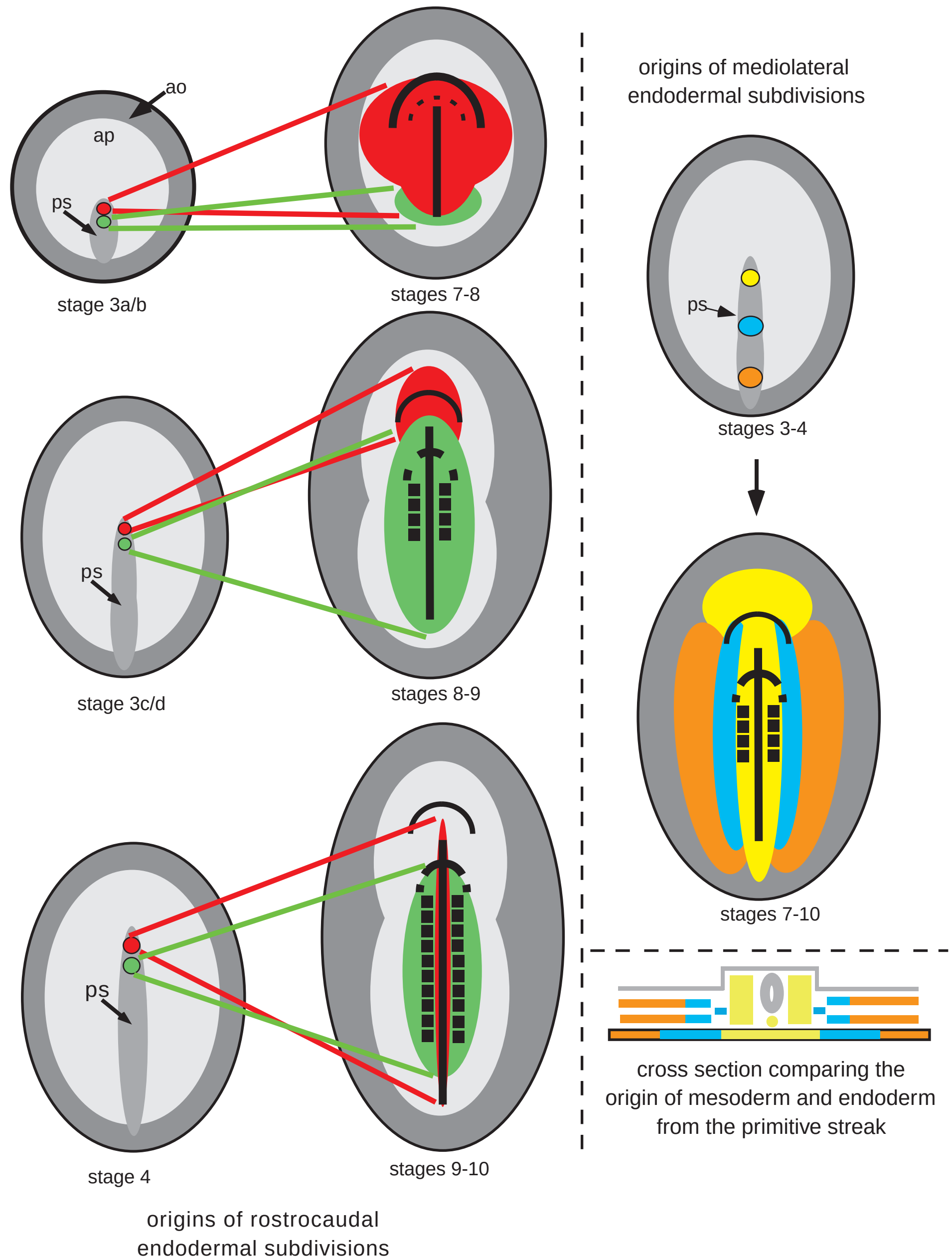


**Fig. 7.** Prospective fate maps showing the primitive-streak origins of the definitive endoderm. The origins of the rostrocaudal subdivisions of the definitive endoderm are shown to the left of the broken vertical line in embryos at three pairs of stages: stage 3a/b to stages 7-8, stage 3c/d to stages 8-9, and stage 4 to stages 9-10. Only injections at the rostral end of the streak are shown, as more caudal injections (mid-streak and caudal streak) contributed endoderm only to the most caudal end of the embryo in a u-shaped configuration centered on the midline (more rostral injections labeled slightly more rostral cells, whereas more caudal injections labeled slightly more caudal cells). The origins of the mediolateral subdivisions of the definitive endoderm are summarized to the right of the broken vertical line, above the broken horizontal line. More rostral injections label more medial definitive endoderm, whereas more caudal injections label more lateral definitive endoderm. A comparison of the mesodermal and endodermal origins from the primitive streak is summarized in a diagrammatic cross-section to the right of the broken vertical line, below the broken horizontal line. Ectoderm is indicated in gray. Endoderm is indicated by different colors in the mediolateral plane and outlined in black; it consists of only the lower layer. Mesoderm consists of the tissue below the ectoderm and above the endoderm and is also indicated by different colors in the mediolateral plane. The comparison of the origin of the mesoderm and endoderm from the primitive streak is based on the data in the current study, as well as those published previously (Garcia-Martinez et al., 1993; Lopez-Sanchez et al., 2001). Note: (1) that for both mesoderm and endoderm, rostrocaudal position within the primitive streak translates after ingression to mediolateral position within the blastoderm (that is, more rostral sites contribute to more medial tissues and more caudal sites contribute to more lateral tissues); and (2) endoderm contributions from a particular rostrocaudal site tend to be spread more laterally than do mesodermal contributions from the same site. ao, area opaca; ap, area pellucida; ps, primitive streak; solid inverted u-shaped line, rostral border of neural plate/head; broken inverted u-shaped line, location of the cranial intestinal portal; vertical line in midline, notochord; squares, somites.

## Comparison of the origin of the mesoderm and endoderm during avian gastrulation

Previous fate mapping studies from our laboratory have revealed the detailed origins and fates of prospective mesodermal cells in both the primitive streak and epiblast at the full range of elongating primitive streak stages (Garcia-Martinez and Schoenwolf, 1993; Lopez-Sanchez et al., 2001). Although contributions of the primitive streak and the epiblast to the definitive endoderm were observed, they were not highlighted in these studies. In one study (Garcia-Martinez and Schoenwolf, 1993), epiblast plugs of quail cells were transplanted isochronically and homotopically to chick hosts. Quail cells were detected in resulting chimeras by using Feulgen staining to label the quail nucleolus. However, this method is unreliable in our hands for detecting quail cells in highly squamous sheets of cells like the endoderm or endothelium; thus, contributions to such layers are grossly underestimated. In the second study (Lopez-Sanchez et al., 2001), also based on the transplantation of quail cells to chick hosts, an anti-quail antibody was used to detect quail cells. This method is very effective in detecting quail cells regardless of their histological structure. However, our small epiblast grafts contained only a couple of hundred cells, and as the vast majority (certainly more than three-quarters) of epiblast cells contribute to mesoderm rather than endoderm, only small patches of endodermal cells were detected in the published study. By contrast, with the use of dyes, sites (especially within the primitive streak) contain reservoirs of label, such that labeled cells are generated within the site as cells move through it throughout the course of the experiment. Thus, many more cells are labeled, and the contributions of such sites to the endoderm can be analyzed readily. Although data on contributions to the endoderm are limited in our previous studies, when their results are compared with those of the present study it is clear that the results are consistent. Two general findings arise from these results (Fig. 7: cross section comparing the origin of mesoderm and endoderm from the primitive streak): contributions to the mesoderm and endoderm occur in concert from any particular site (i.e. primitive streak or epiblast), and contributions to the endoderm from any particular site tend to spread more laterally than those to the mesoderm from the same site. As reported previously, more rostral sites within the primitive streak or epiblast tend to contribute to more medial regions of the mesoderm, and more caudal sites tend to contribute to more lateral regions of the mesoderm. We show here that this same relationship holds for the endoderm, with more rostral sites tending to form more medial regions of the definitive endoderm and more caudal sites tending to form more lateral sites (see Fig. 7: origins of mediolateral endodermal subdivisions).

The striking relationship in the origins of prospective mesodermal and endodermal cells implies that signals for mesodermal patterning may be similar to those of the underlying endodermal layer, or that both signals may occur concurrently. Alternatively, each may be involved in the patterning of the other. Indeed, there is evidence to suggest that interactions between endoderm and adjacent mesodermal tissues allow the endoderm to acquire some positional identity and morphogenetic information (Cleaver et al., 2000; Cleaver and Krieg, 2001). In addition, specification of the prechordal mesoderm may occur via rostral endoderm-derived TGFβ family signaling (Vesque et al., 2000).

These studies were supported by NIH grant nos. NS 18112 and DC 04185.

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