Formation of the avian primitive streak from spatially restricted blastoderm: evidence for polarized cell division in the elongating streak

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SUMMARY

Gastrulation in the amniote begins with the formation of a primitive streak through which precursors of definitive mesoderm and endoderm ingress and migrate to their embryonic destinations. This organizing center for amniote gastrulation is induced by signal(s) from the posterior margin of the blastodisc. The mode of action of these inductive signal(s) remains unresolved, since various origins and developmental pathways of the primitive streak have been proposed. In the present study, the fate of chicken blastodermal cells was traced for the first time in ovo from prestreak stages XI-XII through HH stage 3, when the primitive streak is initially established and prior to the migration of mesoderm. Using replication-defective retrovirus-mediated gene transfer and vital dye labeling, precursor cells of the stage 3 primitive streak were mapped predominantly to a specific region where the embryonic midline crosses the posterior margin of the epiblast. No significant contribution to the early primitive streak was seen from the anterolateral epiblast. Instead, the precursor cells generated daughter cells that underwent a polarized cell division oriented perpendicular to the anteroposterior embryonic axis. The resulting daughter cell population was arranged in a longitudinal array extending the complete length of the primitive streak. Furthermore, expression of cVg1, a posterior margin-derived signal, at the anterior marginal zone induced adjacent epiblast cells, but not those lateral to or distant from the signal, to form an ectopic primitive streak. The cVg1-induced epiblast cells also exhibited polarized cell divisions during ectopic primitive streak formation. These results suggest that blastoderm cells located immediately anterior to the posterior marginal zone, which secretes an inductive signal, undergo spatially directed cytokineses during early primitive streak formation.

Key words: Primitive streak, Gastrulation, Fate map, Retrovirus, DiI, Chick, Epiblast, Cytokinesis

INTRODUCTION

Gastrulation is the morphogenetic event through which the three definitive germ layers, ectoderm, mesoderm and endoderm, are established. Coordinated behaviors of embryonic cells during gastrulation have been carefully studied in non-amniotes, particularly frog embryos (reviewed in Keller, 1986). In the frog, animal hemisphere blastomeres involute through the dorsal blastopore lip, undergo convergent-extension and move as a sheet on the inner surface of the blastocoel toward the animal pole of the embryo. Embryos of higher amniotes, such as birds and mammals, do not utilize blastopore-mediated involution as the mechanism of gastrulation. Instead, they initially develop a primitive streak consisting of bottle-shaped mesenchymal cells along the posterior half of the midline of the epithelioid epiblast. Once the primitive streak is established, mesenchymal cells begin an anterolateral migration to differentiate into embryonic mesoderm and endoderm.

In the chicken embryo, blastoderm cells of the area pellucida first differentiate into a single layer of epithelioid cells called the epiblast by stage X (Eyal-Giladi and Kochav, 1976; Kochav et al., 1980). The hypoblast underlying the epiblast begins to grow anteriorly from the posterior margin of the area pellucida at stage XI and covers the entire ventral surface of the area pellucida by stage XIII. Primitive streak formation is first detected as a cluster of cells in the posterior margin of the area pellucida along the embryonic midline at HH stage 2 (Hamburger and Hamilton, 1951) and is completed by HH stage 3. Mesodermal cell migration begins by HH stage 4 as Hensen’s node and groove are established.

Recent studies have identified several transcriptional factors and growth factor signals involved in primitive streak formation and/or gastrulation (Stern et al., 1990; Knezevic et al., 1995, 1997; Boncinelli and Mallamaci, 1995; Wilson et al., 1995; Winnier et al., 1995; Seleiro et al., 1996). It has also been shown that epiblast cells of the area pellucida are competent to respond to inductive signaling and can be induced to form an ectopic primitive streak (Khaner and Eyal-Giladi, 1986; Stern, 1990; Mitran and Shimon, 1990; Mitran et al., 1990; Ziv et al., 1992; Callebaut and Van Nueten, 1994; Bachvarova et al., 1998). The role(s) of these
factors in inducing the primitive streak is presently unclear, partly due to uncertainty in our understanding of the origin and development of the authentic primitive streak. The pattern of primitive streak development might be critical for considering the mechanisms of inductive signaling. At least three distinct models have been proposed to describe primitive streak formation, based on migration patterns of epiblast cells in cultured blastodiscs (Fig. 1). The first model hypothesizes that epiblast cells migrate from lateral regions towards the midline when they ingress to form the primitive streak (reviewed in Balinsky, 1975). Another model presumes that HNK-1-positive cells, sparsely distributed within the epiblast, individually ingress into the blastocoel, migrate to the posterior midline and form the initial primitive streak (reviewed in Stern, 1992). The third model predicts that posterolateral epiblast cells sequentially migrate to the midline and then move anteriorly along the midline (Eyal-Giladi et al., 1992).

In the present study, an in ovo protocol was established for tagging chicken blastoderm cells at prestreak stages (XI-XII) in order to trace primitive streak formation. Data are presented that show that the majority of precursor cells giving rise to the HH stage 3 primitive streak are restricted to a midline area within the posterior margin of the blastodisc. During early primitive streak formation, the precursor cells undergo polarized cytokinesis oriented along the midline. Their daughter cells are then longitudinally arrayed along the primitive streak at HH stage 3.

MATERIALS AND METHODS

Virus

A replication-defective variant of spleen necrosis retrovirus (Dougherty and Temin, 1986) encoding bacterial β-galactosidase (β-gal) was generated from a packaging cell line, D17.2G, as described (Mikawa et al., 1992a). The virus particles were concentrated to titers of ~10^7-10^8 virions/ml (Mima et al., 1995) just before injection. Propagation of the β-gal viral vector in vitro and proof of helper virus-free stocks have been presented elsewhere (Mikawa et al., 1992b; Mikawa and Fischman, 1992).

In vivo labeling

Fertilized chicken eggs were obtained from an outbred flock (Truslow Farms, MD). A small window of ~20 mm diameter was opened in the shell at the lateral side of the unincubated egg and the underlying shell membrane removed. Embryos were illuminated by cool light through a blue filter and monitored through a color videocamera (Hitachi VKC150) connected to a trinocular stereomicroscope. The monitored image of embryos was examined to identify Koller’s sickle characteristic of the posterior margin of stages XI-XIII preskeleton embryos (Eyal-Giladi and Kochav, 1976). Embryos lacking an obvious Koller’s sickle were not used. Dil (1,1'-dioctadecyl 3,3',3'-tetramethyl indocarbocyanine perchlorate; Molecular Probes, Inc.), at 0.25 % in dimethyl formamide or concentrated viral suspensions containing 100 μg/ml polybrene, were pressure injected into ovo (Mikawa and Gourdie, 1996) into one of five selected sites (Fig. 1) under the control of a Pikospritzer II (General Valve Co., NJ). To map these sites within the blastodisc, the embryonic midline was first drawn on the monitor as the line intersecting the midpoint of Koller’s sickle. A complete circle was then superimposed from the arc. Site 1 was demarcated as the point where the midline meets Koller’s sickle, site 2 was designated as the region anterior to site 1 by 1/5 the diameter of the circle, site 3 was defined as the spot immediately inside the circle along a diagonal 135° from the apex of the AP axis, site 4 was the midpoint between sites 2 and 3, and site 5 was defined as the region posterior to the midpoint of Koller’s sickle. Other general procedures for microsurgery, injection of virus and incubation of infected embryos have been described previously (Mikawa et al., 1992a,b; Itoh et al., 1996).

Histology

All embryos were fixed by injecting 2% paraformaldehyde in PBS underneath the embryonic discs. The fixed discs were then removed from the eggs and processed for morphological examination. Virally tagged embryos were stained with X-gal for detection of β-gal in whole mount as described (Mikawa et al., 1992a). Dil in some labeled embryos was photoconverted, according to Ruiz i Altaba et al. (1993). Embryos exhibiting β-gal-positive cells or photoconverted Dil precipitates were embedded in Epon or paraffin, serially sectioned at 4-7 μm thickness and examined by bright-field, phase or Hoffman Modulation Contrast optics. Actin and chromosomes of stages XI, XII, 2+ and 3 were stained with fluorescein-conjugated phalloidin and DAPI (4’, 6-diamidino-2-phylindole, Molecular Probe), respectively.

BrdU labeling

S-phase nuclei during primitive streak formation were labeled with a thymidine analogue, BrdU (5'-bromo-2'-deoxyuridine, Amersham). At various times of incubation, 500 μg of BrdU per egg were introduced onto embryos through a small window in the egg shell and shell membrane. Eggs were reincubated for further development until stages 3~ to 3. All embryos were then fixed and removed from the eggs as above. After removal of unincorporated BrdU by washing with PBS, embryos were immunolabeled in whole mount with anti-BrdU antibodies (Amersham) followed by detection with the ABC kit (Vector lab) with a peroxidase-based substrate. BrdU-negative nuclei were counterstained with DAPI (1 μg/ml).

Ectopic induction of primitive streak

Ectopic primitive streaks were induced by implanting COS cells expressing a chick Vg1 (cVg1, gifts from Drs Claudio Stern and Jane Dodd, Columbia College of Physicians and Surgeons, NY), according to Shah et al. (1997). After 7-9 hours of incubation, the embryos were fixed with 2% paraformaldehyde in PBS and those exhibiting an ectopic primitive streak were further processed for DAPI staining or counterstained with DAPI (1 μg/ml).

In situ hybridization

Fixed embryos were hybridized with 1 μg/ml of DIG-labelled antisense or sense RNA probe for chicken Brachyury (provided by Dr Raymond B. Runyan, University of Arizona), according to Henrique et al. (1995) with the slight modification of substituting proteinase K digestion with three 30 minute incubations with RIPA-buffer (50 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0). Immunodetection of the RNA probes and cell development were carried out according to Henrique et al. (1995).

Photography and digital imaging

All images of embryos and histological sections were photographed using Kodak Ektachrome 400 film or captured directly by a digital camera, DKC-5000 (SONY, Japan). Color slides were scanned into Adobe Photoshop 3.0 (Adobe Systems, Inc., Mountain View, CA) using a Nikon film scanner LS-1000 (Nikon, Japan). All images were adjusted for brightness and contrast, and then cropped. To normalize distribution of β-gal-positive cells, digital images from six embryos were superimposed after each was adjusted to 16% opacity.
RESULTS

Tagging of blastodiscs in ovo

To identify the origin of the early primitive streak in ovo, five sites of unincubated, prestreak-stage embryos were tagged and the fate of labeled cells was examined at HH stage 3 prior to anterolateral migration of mesoderm (Fig. 1B). These sites were specifically selected to test the three models proposed for the genesis of the primitive streak in the in vitro fate map studies (Fig. 1A). The predicted pattern of labeled cell distribution in each model is summarized in Fig. 1C.

In the previous fate map studies on cultured embryos, Koller’s sickle representing the future posterior of the embryo has been a reliable landmark for detecting the embryonic anteroposterior (AP) axis, while the boundary between the area pellucida and area opaca has been used to detect the embryonic and extraembryonic boundaries. We first examined whether these landmarks, which are clearly identifiable in vitro, were detectable in ovo within the prestreak embryo. Fortunately, an arc- or crescent-shaped cluster of cells characteristic of Koller’s sickle was evident in ovo (Fig. 2A). To confirm that the arc-shaped cluster of cells is Koller’s sickle demarcating the posterior margin of epiblast, the midpoint of the arc was labeled with DiI (Fig. 2A). Immediately after DiI labeling, embryos were photographed (Fig. 2A), fixed and removed from the egg (Fig. 2B). The DiI-labeled region was identified as the site where the embryonic AP axis intersected the midpoint of Koller’s sickle (Fig. 2B). Histological inspection of these embryos further proved that DiI was localized in Koller’s sickle, a cluster of cells protruding toward the underlying hypoblast (Fig. 2C,D). Sagittal sections of such prestreak embryos revealed that the anterior portion of epiblast was composed of a simple cuboidal epithelium, while the more posterior region was more stratified. This gradient in epithelial aspect of the epiblast was used to verify the AP axis in the subsequent studies. Koller’s sickle was also used as a landmark for tagging individual sites of prestreak blastodiscs in ovo.

Migration pattern of labeled cells during primitive streak formation

The fate of tagged cells was examined at HH stage 3 (Fig. 3). In the first set of experiments, blastoderm cells were tagged with DiI (Fig. 3A). DiI-positive (DiI+) cells from the posterior end of the midline (site 1) were predominantly localized to the primitive streak (Fig. 3A). The results indicated that DiI-labeled embryos developed normally and the migration pattern of the embryonic cells could be examined in ovo. The results also suggested that cells of the HH stage 3 primitive streak predominantly arose from a small region within the posterior margin of the embryonic midline. However, since the DiI signal fades after cycles of several cell division, there might have been a population of cells that lost DiI but had migrated into the primitive streak. In addition, the DiI signal seen in HH stage 3 embryos might have arisen from cells labeled at the time of injection and from a second population that later passed through the DiI-deposit, since in several instances DiI crystals remained undissolved at the injection site.

To clarify those uncertainties of DiI-based cell tagging and tracing, cells of prestreak blastodiscs were genetically tagged with retrovirally encoded β-galactosidase (Fig. 3B). Marking of site 1 (n=82) gave rise to a β-galactosidase-positive (β-gal+) primitive streak in 75.1% of the tagged embryos (Fig. 3B). In the remainder of the embryos, β-gal+ cells were localized to areas adjacent to the primitive streak, either anteriorly (3.9%), laterally (20.7%) or posteriorly (1.3%). Of the embryos with primitive streak labeling, 35% exhibited β-gal+ cells exclusively in the primitive streak (Fig. 3C), while 65% contained β-gal+ cells both in the primitive streak and in an area juxtaposed to the primitive streak. Histological examination of these embryos (Fig. 3C) confirmed that tagging of site 1 gave rise to the primitive streak which was richly populated with β-gal+ cells. Tagging of other regions rarely resulted in significant numbers of DiI+ or β-gal+ cells within the primitive streak (Fig. 3A). Instead, cells from site 2 (n=8) were mainly found in the anterior region of the primitive streak (Fig. 4A,E) and those from site 3 (n=22) were mostly distributed in the area lateral to the primitive streak (Fig. 4B,F). Marking of cells at site 4 (n=24) and site 5 (n=8) gave rise to β-gal+ cells in the area anterolateral to the primitive streak (Fig. 4C,G) and in the extraembryonic region posterior to the primitive streak (Fig. 4D,H), respectively.

To normalize for variation in cell migration and targeting accuracy, the distribution patterns of tagged cells from 6 embryos were superimposed (Fig. 5). The data clearly demonstrated that in ovo the majority of cells forming the HH stage 3 primitive streak were derived from the posterior region of the blastodisc, particularly from the midline portion (site 1). Little contribution was evident from other areas surrounding site 1. Neither migration of precursor cells from the lateral epiblast and subsequent ingestion along the midline, nor ingestion of precursor cells sparsely distributed in the epiblast and followed by accumulation to the posterior region was detected as the main route for generating the HH stage 3 primitive streak. These results suggest that the majority of precursor cells giving rise to the HH stage 3 primitive streak are already localized in the posterior-midline region of stages XI-XII embryos.

Developmental process of HH stage 3 primitive streak

Although these results map the precursors of the HH stage 3 primitive streak predominantly to site 1 in the prestreak-stage embryo, it still does not explain how cells restricted to such a small area can generate the primitive streak, which expands to ~1 mm in length. Three potential hypotheses were tested (Fig. 6). The first, based on the model proposed by Eyal-Giladi et al. (1992), assumes that cells localized to the midline region of the precursor area first migrate cranially forming the most anterior domain of the primitive streak, followed by laterally localized precursors, which give rise to the more posterior sections of the streak (Fig. 6A). The second hypothesis predicts that daughter cells from different regions of the precursor area randomly migrate along the midline and intermingled within the resulting primitive streak (Fig. 6B). The third hypothesis assumes that individual cells within the precursor area migrate and/or proliferate unidirectionally toward the anterior tip of the primitive streak (Fig. 6C).

To test these possibilities, a subset of cells within the precursor region was labeled with β-gal and the distribution of β-gal+ cells in the resulting primitive streak was examined at HH stage 3. When a large number of precursor cells were tagged, there was no detectable pattern of β-gal+ cell distribution in the primitive streak (Fig. 7A,B), as seen in the
above experiment (Fig. 3). However, when the retroviral titer was reduced, a subdomain within the precursor area was tagged, giving rise to β-gal+ cells, which were arranged in a longitudinal strand that often extended the entire length of the HH stage 3 primitive streak (Fig. 7C-F). Tagging of smaller regions resulted in a narrower β-gal+ band. DiI-labeling gave rise to similar results (not shown). Histological sections through embryos bearing a single β-gal+ stripe revealed that β-gal+ cells formed arrays in either the dorsal epithelioid or middle mesenchymal layers (Fig. 7G,H). Importantly, when a few cells within the precursor area were tagged (Fig. 8A-C), β-gal+ daughter cells occupied only a portion of the entire longitudinal array.

Such a unique arrangement of daughter cells was observed only when parental cells were tagged within the precursor area, which extended anteriorly along the embryonic midline to 200-300 μm from the Koller’s sickle (Fig. 8C). These data are consistent only with the third model (Fig. 6C) in which individual precursor cells generate daughter cells arranged in a longitudinal array along the embryonic AP axis.

**Oriented cell division in the developing primitive streak**

This unique arrangement of daughter cells in a longitudinal array could be generated by elongation of individual cells and/or by oriented cell division along the midline. Cell shape and the orientation of cell division were both examined in the developing primitive streak and the non-gastrulating epiblast. Cell shapes were outlined by staining the actin-containing adherens belt with phalloidin, while the direction of cell division was predicted by the orientation of DAPI-stained metaphase chromosome plates. In the dorsal view of embryos stained with phalloidin, no elongation of cell shape along the AP axis was apparent in any area of the blastodisc. Instead, primitive streak cells were smaller (8.14±0.33 μm, n=96) than those in non-primitive streak epiblast layer (10.07±0.36 μm, n=96) (Fig. 9A,B).

But an alignment of metaphase chromosome plates was clearly evident in the developing primitive streak, especially when compared to other regions of the DAPI-stained embryos (Fig. 9C,D). The majority of primitive streak cells in metaphase exhibited metaphase plates aligned perpendicular to the AP axis (Fig. 9C). This oriented plane of the metaphase chromosome plates in the streak was distinct from the random orientation seen in non-primitive streak epiblast areas (Fig. 9D). Quantitative data (Fig. 9E) showed that in the primitive streak, about 50% of metaphase cells set up a plane of cell division perpendicularly to the AP axis, and an additional 30% of cells exhibited a plane tilted by more than 45° to the AP axis. Such polarized cell division was not evident once primitive streak elongation completed at stage 3+-4. These results suggest that, as the early primitive streak develops anteriorly along the midline, primitive streak cells undergo the oriented cytokinesis along the AP axis.

The increase in cell number along the AP axis and the consequence that this would have on the linear dimension of the early streak was established by counting the number of cells and measuring their diameter (Table 1). Based on the results of DiI and viral tagging, the anterior boundary of the precursor area extended 200-300 μm from the Koller’s sickle, while the

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**Fig. 1.** (A) Three distinct models proposed for genesis of the early primitive streak. Blue lines indicate migration or movement of primitive streak precursors. AP, anteroposterior axis. (B) Relative positions of five sites tagged with DiI or β-gal virus at pre-streak stages in vivo. (C) Predicted contribution of labeled cells (blue-filled circles) to the primitive streak by marking sites 1-5.

**Fig. 2.** (A) Dorsal view of a prestreak embryo immediately after DiI labeling (arrow) in ovo. Inset, higher magnification of DiI-labeled area. Note: DiI was introduced to the midpoint of the white arc (Koller’s sickle). (B) DiI-labeled embryos, fixed, isolated, photoconverted and viewed after removal of yolk. Inset: higher magnification of DiI-labeled (arrow) area. (C) Higher magnification of DiI-labeled area in D. (D) Montage of sagittal sections along the midline of DiI-labeled (arrow) prestreak embryo. a-p, anteroposterior axis; d-v, dorsal-ventral axis; e, epiblast; h, hypoblast, kc, Koller’s sickle; y, yolk.
The origin of early primitive streak

primitive streak length of 1.27 mm was obtained by direct measurement of HH stage 3 embryos under a dissecting microscope. These values were then divided by the average diameter of precursor and primitive streak cells, 8.8 μm and 8.1 μm, respectively, giving rise to estimated cell numbers of 23-34 cells in the precursor area along the AP axis and 127 cells in the single column of the primitive streak. Thus, a 6- to 7-fold increase in cell numbers along the AP axis was estimated during primitive streak extension between prestreak-stage XII and HH stage 3 (Table 1). This estimate is consistent with the data obtained independently by counting cells of isolated, trypsinized tissue fragments, in which 2.3×10^3 cells in the precursor area (n=4) and 1.4×10^4 cells in the early streak (n=3) were scored on average. These results suggest that precursor cells underwent three cell cycles on average during this 12 hour interval. This is consistent with the mitotic index (~2-4 hours, for cells of chicken gastrulae) which was independently obtained in earlier studies based on incorporation of a thymidine analogue (Sanders et al., 1993). However, since only a small number of cells were in the metaphase at the any given time point during primitive streak formation (Fig. 9C,D), it was uncertain whether primitive cells divided asynchronously and quickly completed M-phase or only a subpopulation of primitive streak entered an active cell cycle. These possibilities were tested by monitoring DNA synthesis of cells in developing primitive streak (Fig. 10). BrdU-positive nuclei were found throughout the primitive streak (Fig. 10A,B). Furthermore, the majority of primitive streak cells were labeled with BrdU within a few hours of incubation (Fig. 10C), indicating that the majority of cells in the developing primitive streak undergo an active, asynchronized cell cycle.

Vg1 induce adjacent epiblast cells to form an ectopic primitive streak

These results show that epiblast...
cells immediately anterior to the posterior marginal zone are the main precursors of stage 3 primitive streak. Therefore, it is likely that a posterior marginal zone-derived signal induces primitive streak by recruiting adjacent epiblast cells, rather than attracting either a lateral or anterior population to migrate towards the midline. To test this possibility, COS cells expressing cVg1 (cVg1/COS), one of the primitive-streak-inducing factors, were placed at the marginal zone in the anterior portion of prestreak-stage embryos. Within 12-14 hours after implantation, a primitive-streak-like cluster of cells formed at the site where cVg1/COS was implanted (Fig.

Table 1. Primitive streak extension between stages XII and 3

<table>
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<th>Factor</th>
<th>Score</th>
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<tr>
<td>Length along the AP axis (l)</td>
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<tr>
<td>Primitive streak (l_ps)</td>
<td>1.270±84 μm (n=8)</td>
</tr>
<tr>
<td>Precursor area (l_pa)</td>
<td>200 μm≤l_pa≤300 μm (n=18)</td>
</tr>
<tr>
<td>Cell diameter (Ø)</td>
<td></td>
</tr>
<tr>
<td>Primitive streak cells (Ø_ps)</td>
<td>8.14±0.33 μm (n=96)</td>
</tr>
<tr>
<td>Precursor area cells (Ø_pa)</td>
<td>8.8±0.36 μm (n=92)</td>
</tr>
<tr>
<td>Estimated cell numbers along the AP axis (l/Ø)</td>
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<tr>
<td>Primitive streak (l/Ø)_ps</td>
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<tr>
<td>Precursor area (l/Ø)_pa</td>
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<td>The maximal ps/pa ratio in</td>
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<tr>
<td>Cell number = log_2 (l/Ø)_ps/min (l/Ø)_pa</td>
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</tr>
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The maximal ps/pa ratio in Length = l_ps/min_l_pa was directly measured under a dissect microscope and presented as a mean value with 95% confidence interval, while (l_pa) was estimated based on the results of Fig. 8D. (Ø) was obtained by analysing Phalloidin-stained embryos as in Fig. 9.

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**Fig. 6.** Three distinct models proposed for the early primitive streak formation. See text.

**Fig. 7.** Development of the early primitive streak by unidirectional growth of precursors along the AP axis. The precursor region was labeled with β-gal virus either broadly (A,B) or discretely (C-F). Distribution of β-gal+ cell arrays (arrows) within the primitive streak was examined by transverse histological sections of discretely labeled embryos (G,H). Diagrams illustrate relative positions of labeled cells within the primitive streak.

**Fig. 8.** β-gal+ arrays oriented along the AP axis (A-C). A few cells in the precursor region were labeled with β-gal virus. (D) Frequency of primitive streak labeling (n=16) depends on distance (d in left diagram) of a tagging site from Koller’s sickle along the embryonic midline (arrow). Bar graph represents % of embryos exhibiting tagged cells only in primitive streak (filled bar), in both primitive streak and anterior epiblast (shaded bar), or only in anterior epiblast (open bar).
The origin of early primitive streak

11A,B) as shown by Shah et al. (1997). In situ hybridization of a primitive streak marker, Brachyury, identified that the cVg1-induced cluster of cells was indeed an ectopic primitive streak (Fig. 11C). To determine the source of cells forming cVg1-induced, ectopic primitive streak, epiblast cells at various positions relative to cVg1/COS were labeled with DiI and DiO (Fig. 11D-G). Marking of cells lateral or distant from cVg1/COS gave rise to an unlabeled primitive streak (Fig. 11E). By contrast, labeling of epiblast cells immediately adjacent to cVg1/COS frequently generated a DiI+ or DiO+ primitive streak (Fig. 11F,G). The plane of cell division was then examined in cVg1-induced primitive streak. Metaphase chromosome plates in cVg1-induced primitive streak were aligned perpendicularly to the ectopic streak axis (Fig. 11H), similarly to those seen in authentic primitive streaks (Fig. 9). The results indicate that only epiblast cells adjacent to an inductive signal undergo the primitive streak formation and these exhibit a polarized cell division.

DISCUSSION

Using both DiI and retroviral cell tagging procedures, the present study visualizes for the first time the in ovo development pattern of chicken blastoderm cells during formation of the early primitive streak. The data map early primitive streak precursors to a small area where the midline crosses the posterior margin (Koller’s sickle) of the prestreak blastodisc. Vg1-induced primitive streak formation strongly suggests that individual blastoderm cells adjacent to an inductive signal proliferate along the AP axis and exhibit polarized cell division during HH stage 3 primitive streak formation.

This study examined the fate of cells from five selected sites of prestreak embryos. DiI and retroviral tagging only of site 1 produced labeled cells throughout the primitive streak. A DiI+ or β-gal+ primitive streak was never generated by labelling the other four sites. Our results suggest that neither epiblast cell migration from lateral to the midline nor random ingression of epiblast cells followed by subsequent accumulation to the posterior region is the primary pathway for generating the HH stage 3 primitive streak in ovo. Rather, these results define a model in which primitive streak precursor cells are already localized to the posterior-midline region of stages XI-XII blastodiscs. This conclusion is further supported by our present results that exogenous Vg1 induces an ectopic primitive streak by recruiting cells adjacent to the signal but not those lateral or distal from it. These results also corroborate previous observations that the epiblast portion of the posterior marginal zone contributes to the primitive streak (Stern, 1990; Éyal-Giladi et al., 1992; Bachvarova, 1998).

These in ovo results differ from previously proposed models for the location and migration pathways of precursors.
generating the early primitive streak (reviewed in Stern, 1992; Eyal-Giladi et al., 1992). Although the reason for this variation is not fully understood, significantly different experimental approaches were used to analyze cell movement in these studies. Model-b (Fig. 1A) is based on the expression pattern of HNK-1, a sulfated form of glucuronic acid, and the fate of an internalized HNK1-antibody complex (Stern, 1992). Model-c (Fig. 1A) is based on the fate of a tissue fragment implanted into an isolated blastodisc (Eyal-Giladi et al., 1992) and on the distribution of diffusible vital dyes in ovo (Gräper, 1929). By contrast, the present study directly traced the fate of blastoderm cells using two non-invasive, non-diffusible cell tagging methods, DiI labeling and retroviral-mediated gene transfer. Importantly, these two independent in ovo approaches led to the same conclusion.

The posterior-midline epiblast, including the primitive precursor area, is stratified and not a simple epithelium. Our injection of higher viral titer into the precursor area, probably tagging both the surface and deeper layer of epiblast cells, results in β-gal+ cells distributed to both the surface and deeper layer of the primitive streak (Fig. 3C). In contrast, injection of lower viral titers to the area tagged a population of cells in the surface layer (Fig. 7G) or in the middle layer (Fig. 7H). Consistent with our observations, it has been shown that cells of both the surface and deeper layer of the posterior-midline epiblast generate a subpopulation of primitive streak cells (Bachvarova et al., 1998). The origin of these posterior-midline cells of the stage XI-XII embryos remains to be identified.

This study examined the location and developmental pattern of precursor cells that form the HH stage 3 primitive streak. At later stages, the primitive streak is composed of cells derived from the lateral epiblast. For example, at HH stage 4, cells that originally formed the HH stage 3 primitive streak migrate laterally and anteriorly and some of them differentiate into lateral mesoderm (Eyal-Giladi et al., 1992; Garcia-Martinez and Schoenwolf, 1993), while the HH stage 4 primitive streak consists of cells that were originally localized in the posterolateral region of the epiblast (Stern et al., 1995; Hatada and Stern, 1994). Thus, the origin and migration pattern of cells generating the early primitive streak are different from those constituting the later stage primitive streak.

Interestingly, the β-gal-labelled cells were arrayed longitudinally in the early primitive streak. Potential mechanisms by which progenitor cells at the posterior margin give rise to the extended streak include directed cell intercalation, directed cell migration, cell shape change and oriented cell division. Mitotic activity has been shown to be associated with primitive streak development (Sanders et al., 1993), and β-gal expression in cells forming the early primitive streak requires at least one cell cycle for integration and expression. This is clearly different from the non-mitotic convergent and extension mechanism underlying frog gastrulation (reviewed in Keller, 1986). Furthermore, cells of the developing avian primitive streak do not exhibit elongation perpendicular to the AP axis, the cell shape change predicted to occur with lateral-to-medial cell sliding during
convergent/extension (Keller, 1986). Our results do not support oriented cell intercalation as a major mechanism accounting for primitive streak development. Indeed, β-gal+ cells are arranged in a longitudinal array without significant interruption by β-gal-negative cells. A number of studies have demonstrated that migration of mesodermal cells is directed by a fibronectin cue (Harrison et al., 1993). However, fibronectin is absent from the primitive streak (Duband and Thiery, 1982). The hypothesis that the hypoblast underlying the epiblast may provide a cue for direction of primitive streak development is controversial (Azar and Eyal-Giladi, 1981; Khaner, 1995).

Polarized cell division plays a role in shaping the neural plate (Hamburger, 1948; Martin, 1967; Sausedo et al., 1997; Concha and Adams, 1998). Here we demonstrate that cells in the developing primitive streak have metaphase chromosome plates with a plane perpendicular to the AP axis. Cells in an ectopic primitive streak induced by Vg1 exhibit similar polarity in their cell division. The orientation of metaphase chromosome plates together with the observed longitudinal arrays of β-gal+ cells in the primitive streak suggest that directional cell division plays a role in a primitive streak elongation along the midline. This idea is consistent with the model illustrated in Fig. 12, where a local signal(s), such as epithelial scatter factor (Stern et al., 1990), activin (Mitrani et al., 1990; Ziv et al., 1992) and Vg1 (Seleiro et al., 1996), in a confined area of the posterior marginal zone, induces a blastoderm cell subpopulation that differentiates into the early primitive streak. During this process, primitive streak cells undergo cytokinesis with the plane perpendicular to the AP axis. Future studies should address mechanisms that orient the plane of cytokinesis in streak-forming cells. It remains to be established if oriented cell division is causal for primitive streak elongation or an associated phenomenon.

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