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New Insights Into Critical Events of Avian Gastrulation

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ABSTRACT

The formation and progression of the primitive streak are key events of avian gastrulation. We examine these processes in detail, using various morphological approaches. We show that formation of the primitive streak occurs locally at the caudal midline of the area pellucida, as cells in the caudal midline undergo an epithelial-to-mesenchymal transformation, and that extensive migration of delaminated cells arising from more rostral or peripheral areas of the blastoderm is not involved in streak formation. Instead, such delamination occurs earlier and is restricted to the process of hypoblast formation. Moreover, we provide evidence that progression of the primitive streak involves two processes: convergent-extension movements within the streak per se, and progressive delamination of midline epiblast cells in a caudal-to-rostral sequence. We have identified a subpopulation of primitive-streak cells located at its dorsal midline surface that undergoes extensive rostral displacement concomitant with streak progression. The fact that these cells are located only dorsally and do not elongate ventrally as do adjacent ingressing cells, suggests that these cells retain their residency within the primitive streak, at least until regression of the primitive streak occurs. Finally, by following labeled cells over time we establish the timing of movement of epiblast cells toward and into the primitive streak, providing direct evidence that cell–cell intercalation occurs within the primitive streak during its progression. Collectively, our results provide new insight into complex and central events of avian gastrulation. Anat Rec 262:238–252, 2001.

Key words: blastoderm; cell migration; epiblast; Hensen's node; hypoblast; induction; ingression; primitive streak

In birds and mammals, the most obvious feature that heralds gastrulation is the appearance of the primitive streak, a linear, midline thickening of the epiblast. Gastrulation is far more complex, however, involving a series of events such as the formation of the embryonic axes; subdivision of the blastoderm into embryonic and extraembryonic areas (in birds, the area pellucida and area opaca, respectively); formation of the bilaminar blastoderm, consisting of the epiblast and hypoblast; and formation and elongation of the primitive streak and movement of cells through it to form two of the three primary germ layers, the endoderm and mesoderm, with the remaining cells of the epiblast forming the third primary germ layer, the ectoderm (for reviews, see Bellairs, 1986; Sanders, 1986; Stern and Canning, 1988; Lemaire and Kessel, 1997; Schoenwolf and Smith, 2000).

Formation of the primitive streak is initiated at HH Stage 2 (Hamburger and Hamilton, 1951). Subsequently, the primitive streak undergoes three major changes. The first change, known as progression, consists of a rapid elongation of the primitive streak along its rostrocaudal axis, until the streak attains a maximum length of about

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1.8 mm at HH Stage 4 (Spratt, 1947). While progression is occurring, the second change, ingression, begins. During ingression, prospective mesodermal and endodermal cells leave the epiblast and move through the primitive streak. Finally, the primitive streak undergoes the third change, regression, during which its length gradually decreases until it is finally incorporated into the tail bud (Schoenwolf, 1979).

The mechanisms underlying formation of the primitive streak have interested investigators for many years. As revealed by time-lapse films, the primitive streak forms rather suddenly (Vakaet, 1970; Schoenwolf, 1997, and unpublished observations; also see Stern and Canning, 1988), suggesting that the primitive streak is induced locally from the epiblast, rather than forming by the mass, long-distance migration of precursor cells from other areas of the blastoderm and their subsequent aggregation along the midline. The portion of the blastoderm that could be responsible for such an inductive process has been the subject of considerable debate. In a pioneering experiment, Waddington (1933) reported that rotating the hypoblast relative to the epiblast, altered the orientation of the primitive streak relative to the original rostrocaudal axis of the blastoderm. Subsequent reports by Azar and Eyal-Giladi (1979, 1981) and Mitrani and Eyal-Giladi (1981) also favored induction by the hypoblast. The demonstration that secreted proteins such as *activin* (Mitrani et al., 1990; Cooke et al., 1994), *Vg1* (Saleiro et al., 1996; Shah et al., 1997), and *Wnt* (Hume and Dodd, 1993), all of which the hypoblast expresses, are capable of inducing ectopic primitive streaks provides further evidence implicating the hypoblast in formation of the primitive streak. Eyal-Giladi et al. (1992), in a model designed to define the role that the hypoblast plays in induction of the primitive streak, hypothesized that hypoblast cells interact with epiblast cells rostral to Köller's sickle and induce them to form the rostral tip of the primitive streak (Hensen's node). They further proposed that subsequently the induced cells migrated rostrally, with their previous position becoming occupied by epiblast cells originally positioned more laterally; subsequently these epiblast cells become induced to form more caudal segments of the primitive streak. Studies to date, however, have failed to provide direct evidence in support of the view that the hypoblast can induce the primitive streak. Results from other studies, moreover, do not lend support to the putative role ascribed to the hypoblast by Eyal-Giladi and others. These include a recent failure to reproduce the results of Waddington's rotation experiments (Khaner, 1995) and the reports that primitive streaks can be induced in the absence of the hypoblast (Stern, 1990; Bachvarova et al., 1998; Khaner, 1998). According to Stern and Canning (1990), the hypoblast probably determines the site of primitive streak formation rather than acting as its inducer. Thus, although the hypoblast may play an important role in the formation of the primitive streak, its precise role is, as yet, uncertain.

In contrast, several previous studies point to the inductive potential of the marginal zone (MZ) of the pre-primitive streak embryo in formation of the primitive streak (Gräper, 1929; Rudnick, 1935; Spratt and Haas, 1960a,b; Eyal-Giladi and Spratt, 1965; Vakaet, 1970; Azar and Eyal-Giladi, 1979, 1981; Mitrani et al., 1983; Khaner et al., 1985; Bellairs, 1986; Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989; Eyal-Giladi et al., 1992; Bachvarova et al., 1998; Khaner, 1998). The marginal zone is a thickened region that lies at the outer boundary of the area pellucida. In the caudal midline, it overlaps the area opaca and the caudal portion of Köller's sickle (see Bachvarova et al., 1998). Therefore, the caudal MZ consists of a superficial (epiblast) portion, continuous with the epiblast of the area pellucida and area opaca, and a deep (hypoblast or endodermal) portion. Evidence that the caudal MZ acts in formation of the primitive streak has come mainly from cell labeling and transplantation studies. For example, fragments of blastoderm containing the caudal MZ possess a greater capacity when cultured of producing complete embryos than those lacking the MZ (Spratt and Haas, 1960b). Also, transplantation of the caudal MZ to ectopic sites increases the frequency at which primitive streaks are generated (Azar and Eyal-Giladi, 1979; Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989; Eyal-Giladi et al., 1992; Bachvarova et al., 1998; Khaner, 1998). A major drawback of most of the transplantation and cell labeling studies, however, is that the graft often contributed cells to the primitive streak and the organizer. This problem has been addressed in recent studies in which cells from the caudal MZ (without Köller's sickle) have been carefully grafted to ectopic sites, and primitive streaks have been successfully induced from neighboring host epiblast cells without contributions from the graft (Bachvorava et al., 1998; Khaner, 1998).

Molecular markers expressed in the caudal MZ provide additional evidence in its favor as an inducer of the primitive streak. Notable among these are *Vg1* (Shah et al., 1997), *chordin* (Streit et al., 1998), and *Wnt-8c* (Hume and Dodd, 1993), which when expressed ectopically in the MZ are capable of initiating the formation of a primitive streak. Notwithstanding these pieces of evidence, we still do not know exactly how the primitive streak comes into being; nor the characteristics of the cells that are induced to form it. Also, it is uncertain whether induction by the caudal MZ is required for normal formation of the primitive streak or whether such induction acts in parallel with autonomous processes within the epiblast that are sufficient for streak formation (Bachvarova et al., 1998). The lack of a clear understanding of the precise sequence of events after the appearance of the primitive streak (such as progression and ingression) and the factors that control these events stems not only from the complexity of the events themselves, but also from the fact that there have been relatively few studies in this area. Additionally, the early primitive streak is a fragile, rather nondescript structure that develops rapidly, complicating its study.

The present study examines systematically the formation and progression of the primitive streak, as well as ingression of cells through the streak, using several morphological approaches. Our results provide new insight into critical events of avian gastrulation, setting the stage for future experimental studies.

MATERIALS AND METHODS

Whole Embryo Culture and Staging

Fertilized chicken eggs were incubated at 38°C to obtain embryos at stages ranging from X (Eyal-Giladi and Kochav, 1976) to 6 (Hamburger and Hamilton, 1951). Substages of HH Stage 3 were defined as described previously (Schoenwolf et al., 1992), with embryos from Stages 3a and 3b grouped together as Stage 3a/b, and those from Stages 3d and 4 grouped together as Stage 3d/4. Culture

dishes and embryos were prepared as described by Darnell and Schoenwolf (2000) for modified New (1955) culture.

Scanning and Transmission Electron Microscopy

Chick embryos were fixed overnight with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer and then washed briefly with 0.1 M phosphate buffer. After this, they were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer and dehydrated with an ascending graded ethanol series up to 100%. After this, embryos for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were separated. Those for SEM were passed through one 10-min change of 1:1 100% ethanol:hexamethyldisilazane (HMDS, Electron Microscopy Sciences, Fort Washington, PA), followed by two 10-min changes in HMDS, after which the HMDS was allowed to evaporate overnight, drying the samples. Dried samples were then mounted onto stubs, coated with gold/palladium, and examined with an Hitachi S2460N scanning electron microscope. Embryos for TEM were transferred to propylene oxide and embedded in Epon. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with an Hitachi H-7100 transmission electron microscope.

Many of the embryos collected for SEM were dissected or cut into slices. Dissection involved the removal of the hypoblast or definitive endodermal layer using fine cactus needles while the embryos were in buffer after primary fixation. Careful observations of embryos during this process suggested that such dissection had little or no adverse effect on the underlying mesoderm, with the possible exception that occasional mesodermal cells may have become detached with the endoderm. Similarly, embryos were transversely sliced using razor blades, again in buffer after primary fixation.

Fate Mapping Experiments

Injections of the primitive streak. Chick embryos at Stages 2, 3a/b, and 3c, cultured as described above, were selected for injections of the primitive streak. A mixture of 5-carboxytetramethylrhodamine, succinimidyl ester (CRSE; Molecular Probes, Inc., Eugene, OR) and 1,1′-dioctadecyl-3, 3, 3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Inc.) was injected as described by Darnell et al. (2000) into the primitive streak at three levels (i.e., rostral, middle, and caudal, designated as Sites 1, 2, and 3, respectively). Embryos were immediately examined with a fluorescence microscope, to ascertain the size and site of each injection, and then re-incubated for 4 hr. After fixation in 4% paraformaldehyde in PBS, they were processed for immunocytochemistry and subsequent paraffin histology as described below. Some embryos were fixed after the initial examination for Time 0 injections. All embryos were processed for immunocytochemistry and subsequent paraffin histology as described below. Control embryos were treated exactly as experimentals, except that they were not injected.

Injections of the parastreak epiblast. For injections of the parastreak epiblast, chick embryos were selected at Stages 2–3c and cultured as described above. A mixture of CRSE and DiI was made into the epiblast at three levels, rostral, middle, and caudal, also designated (like primitive-streak injections) as Sites 1, 2, and 3, respectively. Embryos were immediately examined with a fluorescent microscope and then re-incubated for 5, 7, and 24 hr before fixation. All embryos were processed for immunocytochemistry and subsequent paraffin histology as described below. Control embryos were treated exactly as experimentals, except that they were not injected.

Immunocytochemistry

All embryos labeled with the fluorescent markers CRSE and DiI were processed for whole-mount immunocytochemistry (ICC) as described previously by Patel et al. (1989), except that the embryos were fixed in 4% paraformaldehyde in PBS and the peroxidase reaction product was intensified by the addition of 2% $CoCl_2$ and 2% Ni $(NH_4)_2$ (SO_4) per ml of DAB-PBT. To label the rhodamine groups of CRSE-DiI labeled embryos with a permanent reaction product detectable in paraffin section, we used anti-rhodamine (rabbit IgG polyclonal, primary; Molecular Probes, Inc.) and horseradish peroxidase-conjugated goat anti-rabbit IgG (secondary antibody; Boehringer Mannheim, Germany). After their examination and photography as whole-mounts, embryos were processed for paraffin histology as described below.

Additional embryos were processed as whole mounts for ICC at stages XII-4 using anti-fibronectin and anti-laminin antibodies (Developmental Studies Hybridoma Bank) (secondary antibody for both consisted of horseradish peroxidase-conjugated goat anti-mouse IgG; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Before each primary antibody treatment, either the hypoblast or endoderm was removed to ensure antibody penetration. Subsequently, embryos were processed for paraffin histology as described below.

Paraffin Histology

Histological analysis was carried out on embryos previously labeled by whole-mount ICC. Embryos were dehydrated in an ascending ethanol series up to 100%. Those labeled for ICC were taken through two 5-min changes of Histosol. After infiltration in Paraplast, they were finally embedded in Paraplast and sectioned at 10 μm.

Measurements of the Primitive Streak

Chick embryos at Stages 2, 3a/b, and 3c (five for each stage) were used for measuring the length and width of the primitive streak in living embryos, cultured as described above. Embryos were selected carefully to ensure that only blastoderms with margins completely attached to the vitelline membrane were used; thus, we avoided measuring embryos with artifactually distorted primitive streaks. For each embryo, an eyepiece graticule mounted in the eyepiece of a dissecting microscope was used to measure the length and width of the primitive streak. The width of the primitive streak was measured at two levels: rostral and mid-streak. These initial measurements represented Time 0 values. Embryos were re-incubated further, and measurements were subsequently taken at 2-hr intervals up to 8 hours of re-incubation. The values obtained were analyzed by calculating the mean length and width of the primitive streak for each stage and time of re-incubation. The percentage increase in the length of the primitive streak, the percentage increase in the width of

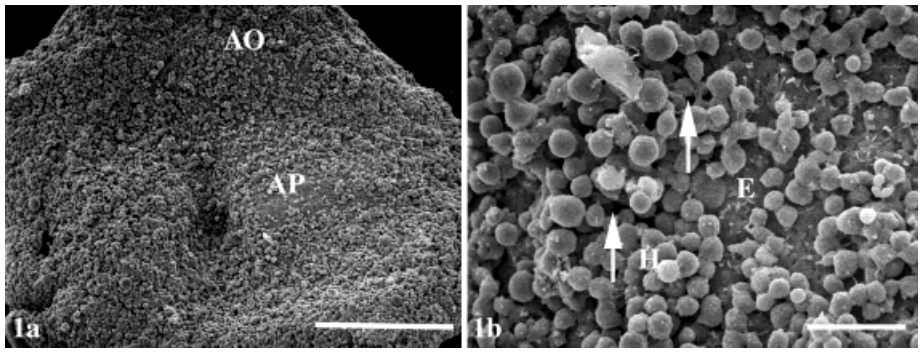


Fig. 1. **a:** SEM of the ventral surface of a stage XI/XII chick embryo. Area opaca (AO); area pellucida (AP). Scale bar = 400 μm. **b:** enlargement of the central part of a showing a non-epithelialized hypoblast layer (H) consisting of numerous rounded cells. Arrows point to gaps in the epiblast layer (E), through which the hypoblast cells (H) are polyingressing. Scale bar = 40 μm.

the rostral primitive streak, and the percentage decrease in the width of the mid-primitive streak was computed from the mean values obtained.

RESULTS

Scanning Electron Microscopy

Stages X–XII. The blastoderm during Stages X–XII consists of two parts: a central disc-like region, the area pellucida, and a peripheral ring-like area, the area opaca. In the area pellucida, the blastoderm comprises two layers, a superficial epiblast layer and a deep, incomplete hypoblast layer. The epiblast has a basal lamina on its ventral surface (not shown, but see below: "Distribution of fibronectin and laminin"), that masks cell morphology. The hypoblast at these stages is a non-epithelialized layer consisting of numerous globular cells scattered on the ventral surface of the epiblast (Fig. 1a,b). Most of the hypoblast cells are grouped into clusters. Previous studies have shown that they are derived from the epiblast and undergo polyingression (Weinberger and Brick, 1982a,b; Penner and Brick, 1984; Weinberger et al., 1984; Stern and Canning, 1990; Harrisson et al., 1991). Additional evidence of this is the presence of rounded holes in the ventral surface of the epiblast, with hypoblast cells exhibiting processes extending through the holes into the epiblast layer (Fig. 1b).

Stages XIII–XIV. By Stages XIII–XIV, the hypoblast consists of a complete layer of cells ventral to the epiblast throughout the entire area pellucida (Fig. 2a,b). Hypoblast cells are large and irregularly shaped, with some having bulging surfaces and others, flattened surfaces. Most hypoblast cells have small globular projections on their ventral surface. Epithelialization of the hypoblast seems to progress rostrally from the caudal end, as in some embryos at this stage of development, the rostral portion of the area pellucida is devoid of this layer and scattered non-epithelialized hypoblast cells are still present there. Surrounding the margin of the epithelialized hypoblast layer at the junction between the area pellucida and area opaca is a layer of cells that constitutes the marginal zone (MZ; Fig. 2a). An extracellular matrix covers the ventral surfaces of the MZ cells, giving them a flattened appearance (Fig. 2c).

The basal surface of the epiblast can be examined after removal of the epithelialized hypoblast layer. The position of the incipient primitive streak is often indicated in the caudal half of the basal surface of the epiblast by a midline ridge (Fig. 3a). Higher magnification of this ridge reveals a line of cells emerging from the ventral surface of the epiblast (Fig. 3b); this line constitutes the first crop of primitive streak cells (and perhaps a few attached hypoblast or endodermal cells). A transverse section through the caudal half of the area pellucida shows that the epiblast and hypoblast consist of simple columnar and flattened epithelial layers, respectively, and that the midline epiblast at the level of the forming primitive streak is thicker than the more lateral epiblast (Fig. 3c).

Stage 2. At Stage 2, the initial primitive-streak stage, the epithelialized hypoblast seems similar to that at Stages XIII–XIV. Overt formation of the primitive streak commences at this stage. The initial primitive streak is triangular in shape, with its apex pointing rostrally (Fig. 4a), and it occupies the caudal one-third of the length of the area pellucida. On its ventral surface, the basal lamina of the epiblast is disrupted in the entire region occupied by the primitive streak, thus exposing the surfaces of the cells forming the streak (Fig. 4b). Within the primitive streak, remnants of the disrupted basal lamina are identified in patches on some of the cells. Elsewhere on the epiblast, the basal lamina of the epiblast is intact (Fig. 4c). In comparison with the flanking epiblast, the primitive streak is about twice the thickness, with a greater cell density (Fig. 5a,b). Also, whereas the cells of the primitive streak are mostly globular in shape, those of the epiblast are columnar. Near the dorsal part of the primitive streak, a few bottle-shaped cells can be identified. At this stage, no apparent distinguishing features are present at the dorsal surface of the epiblast between the primitive streak cells and those of the rest of the epiblast.

Stages 3a/b. With formation of the linear primitive streak, the definitive (gut) endoderm begins to appear ventral to the primitive streak (not shown), where it is surrounded peripherally by the hypoblast. The definitive endoderm is derived from two sources: the rostral portion is from the epiblast; the caudal portion is from the deep

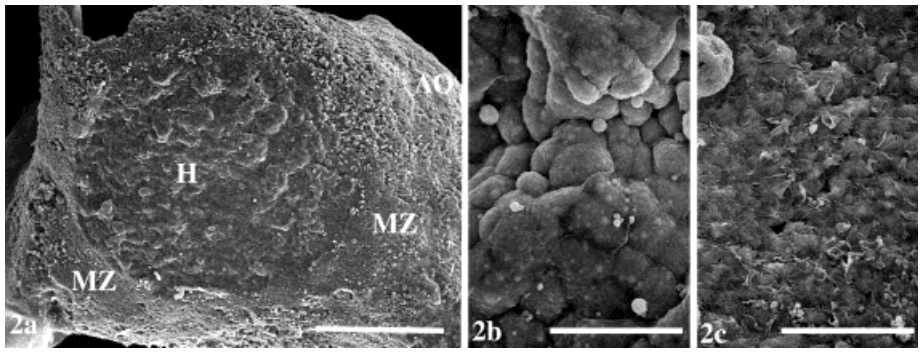


Fig. 2. **a:** SEM of the ventral surface of a stage XIII chick embryo showing an epithelialized hypoblast layer (H) and surrounding marginal zone (MZ); area opaca (AO). Scale bar = 500 μm. **b:** Enlargement the hypoblast layer showing both flattened cells and cells with bulging surfaces. Scale bar = 500 μm. **c:** Enlargement of the marginal zone showing cells with numerous lamellipodia. Scale bar = 40 μm.

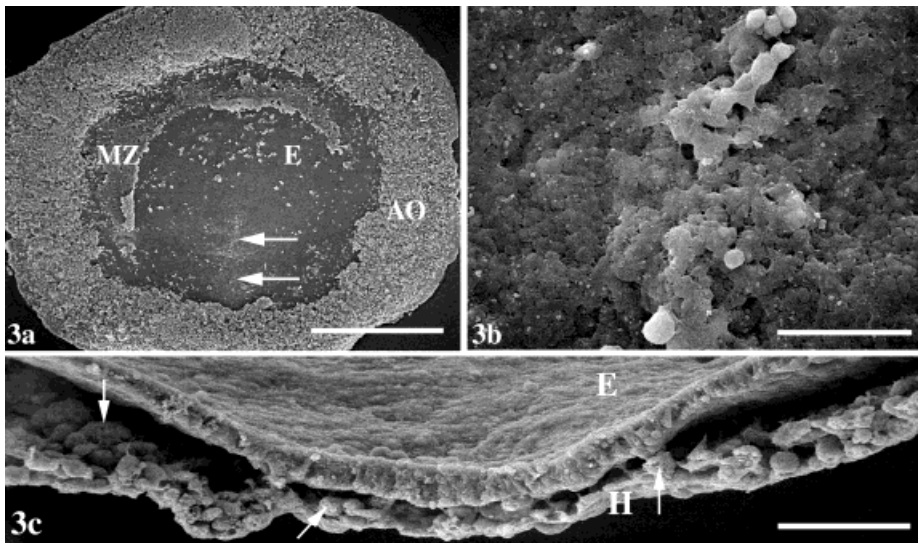


Fig. 3. **a:** SEM of a Stage XIV chick embryo in which the hypoblast was removed revealing the ventral (basal) surface of the epiblast (E). Arrows indicate the caudal midline of the epiblast where the primitive streak is forming. Area opaca (AO); marginal zone (MZ). Scale bar = 60 μm. **b:** Enlargement of the forming primitive streak showing cells emerging from the ventral (basal) surface of the epiblast. The lightest-appearing cells may represent attached hypoblast or endodermal cells. Scale bar = 40 μm. **c:** SEM of a transverse slice through the primitive streak-forming area of a stage XIV chick embryo. Note the increased thickness of the epiblast (E) in the midline. Arrows point to cells between the epiblast and the epithelialized hypoblast (H) layers; these cells are probably non-epithelialized hypoblast cells not yet integrated into the hypoblast. Scale bar = 100 μm.

layer of the caudal MZ (Vakaet, 1970; Stern and Ireland, 1981; Eyal-Giladi et al., 1992; Bachvarova et al., 1998). The primitive streak occupies the midline of about the caudal half of the area pellucida (Fig. 6a). It is more elongated than that at Stage 2, and its caudal end is usually broader than its middle and rostral portions (in early Stage 3a embryos, the caudal end of the primitive streak is often triangular in shape, as is seen in Stage 2 embryos). Along the entire length of the primitive streak, cell surfaces are covered by remnants of the basal lamina formerly underlying the epiblast (Fig. 6a–d). Rostral to the rostral end of the primitive streak, cells seem to emerge from the ventral surface of the epiblast (Fig. 6b). Because they are not in continuity with the rostral tip of the primitive streak, this gives the impression that lengthening of the primitive streak between Stages 2 and 3a/b probably occurs by successive rostral "induction" of the primitive streak from midline epiblast cells. Although what tissue might provide such a signal is currently unknown, two candidates seem likely: the primitive streak itself, and the hypoblast or ingressed endoderm underlying the epiblast. Migration of mesodermal cells away from the primitive streak is in progress at its lateral margins along its entire length (Fig. 6a–d). Such migrating cells are interconnected by their processes as they move on the ectodermal basal lamina. At the rostral end of the primitive streak, however, a rostral migration of mesodermal cells is not always apparent. Unlike the cells within the primitive streak, the surfaces of migrating mesoderm cells are devoid of basal lamina material. A transverse section through the middle portion of the primitive streak reveals a predominance of globular primitive streak cells, as in

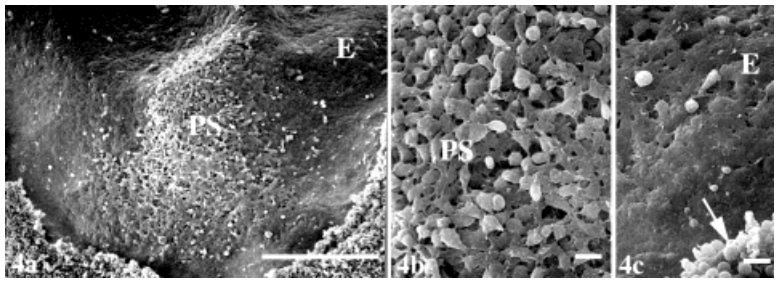


Fig. 4. **a:** SEM of a stage 2 chick embryo in which the hypoblast was removed revealing the ventral (basal) surface of the epiblast (E) and the initial primitive streak (PS), which has a triangular shape. Scale bar = 60 μm. **b:** Enlargement of the primitive streak (PS). A basal lamina is absent ventral to the primitive streak cells, allowing the identification of individual cells. Scale bar = 100 μm. **c:** Enlargement of the more lateral epiblast (E). A basal lamina is present ventral to the more lateral epiblast, obscuring cell borders. Arrow points to the area opaca. Scale bar = 100 μm.

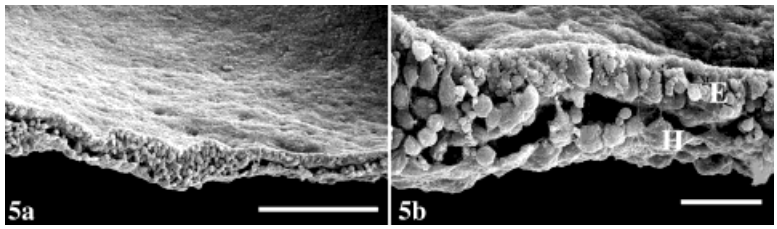


Fig. 5. **a:** SEM of a transverse slice through a Stage 2 chick embryo showing the primitive streak in the midline. Scale bar = 180 μm. **b:** Enlargement of the primitive streak and adjacent epiblast (E). The former consists of principally globular cells, whereas the latter, of columnar cells. Hypoblast (H). Scale bar = 20 μm.

Stage 2 embryos, with only a few columnar cells near the dorsal surface (Fig. 6e).

Stage 3c. The area pellucida is overtly pear shaped by Stage 3c, with a broader rostral portion and a narrower caudal end. The endodermal layer has spread to cover most of the area pellucida, displacing the hypoblast with which it is in contact, toward the germ cell crescent at the rostral end of the blastoderm. At Stage 3c, the primitive streak is more elongated than that at Stage 3a/b, with its rostral end reaching the level of the widest diameter of the area pellucida (Fig. 7a). It is also narrower transversely than that at Stage 3a/b. Its rostral portion consists of a "ball" of concentrically arranged cells, indicating the position of Hensen's node (Fig. 7b). Remnants of basal lamina material are often observed on the surfaces of the cells forming the ball. Disruption of the basal lamina of the epiblast is no longer apparent rostral to the ball of cells. Mesodermal cell migration from the lateral margins of the primitive streak is more advanced than at earlier stages (cf. Figs. 6a and 7a), reaching about midway between the midline and the lateral margin of the area pellucida. Rostrally, the lateral mesodermal front on each side of the streak is caudal to the rostral end of the streak (Fig. 7a). Another difference between the primitive streaks of embryos at Stages 3c and 3a/b is a marked increase in cell density at Stage 3c (cf. Figs. 6c and 7c). At Stage 3c, ingression of cells through the primitive streak is well underway as evidenced by the presence of a midline groove marking the midline of the primitive streak on the dorsal surface of the epiblast (Fig. 7d); additionally, many elongated cells are present within the primitive streak (Fig. 7e).

Stages 3d/4. During Stage 3d/4, the hypoblast is concentrated in the germ cell crescent (Fig. 8a), and the primitive streak has reached its maximum length (Fig. 8b); its rostral end is rostral to the level of the widest diameter of the area pellucida. A coating appears on the surfaces of the cells along the entire extent of the primitive streak from this stage onwards; it progressively obscures cellular outlines. This coating is also present on the migrating mesodermal cells caudally and laterally (Fig. 8c,d) and it is similar in appearance to the basal lamina of the epiblast. Laterally, the mesoderm cell front is at the level of the rostral end of the primitive streak, whereas in the midline, mesodermal cells extend beyond the rostral end of the primitive streak (beyond Hensen's node).

Stages 5 and 6. The primitive streak initiates regression during Stages 5 and 6. Laterally, the mesodermal front extends beyond the level of the rostral end of the streak, reaching almost the rostral end of the notochord by Stage 6 (Fig. 9, 10). Ingression of cells through the primitive streak continues as the primitive streak undergoes regression (Fig. 11).

Changes in the Shape of the Primitive Streak

The length of the primitive streak and its width at two rostrocaudal levels were calculated in three groups of living embryos over an 8-hr period. In the first group, measurements were made beginning at Stage 2, in the second group, beginning at Stage 3a/b, and in the third group, beginning at Stage 3c. By the end of the 8-hr period, embryos reached Stages 3c–4.

As shown in Table 1, the length of the primitive streak progressively increased in each of the three groups over

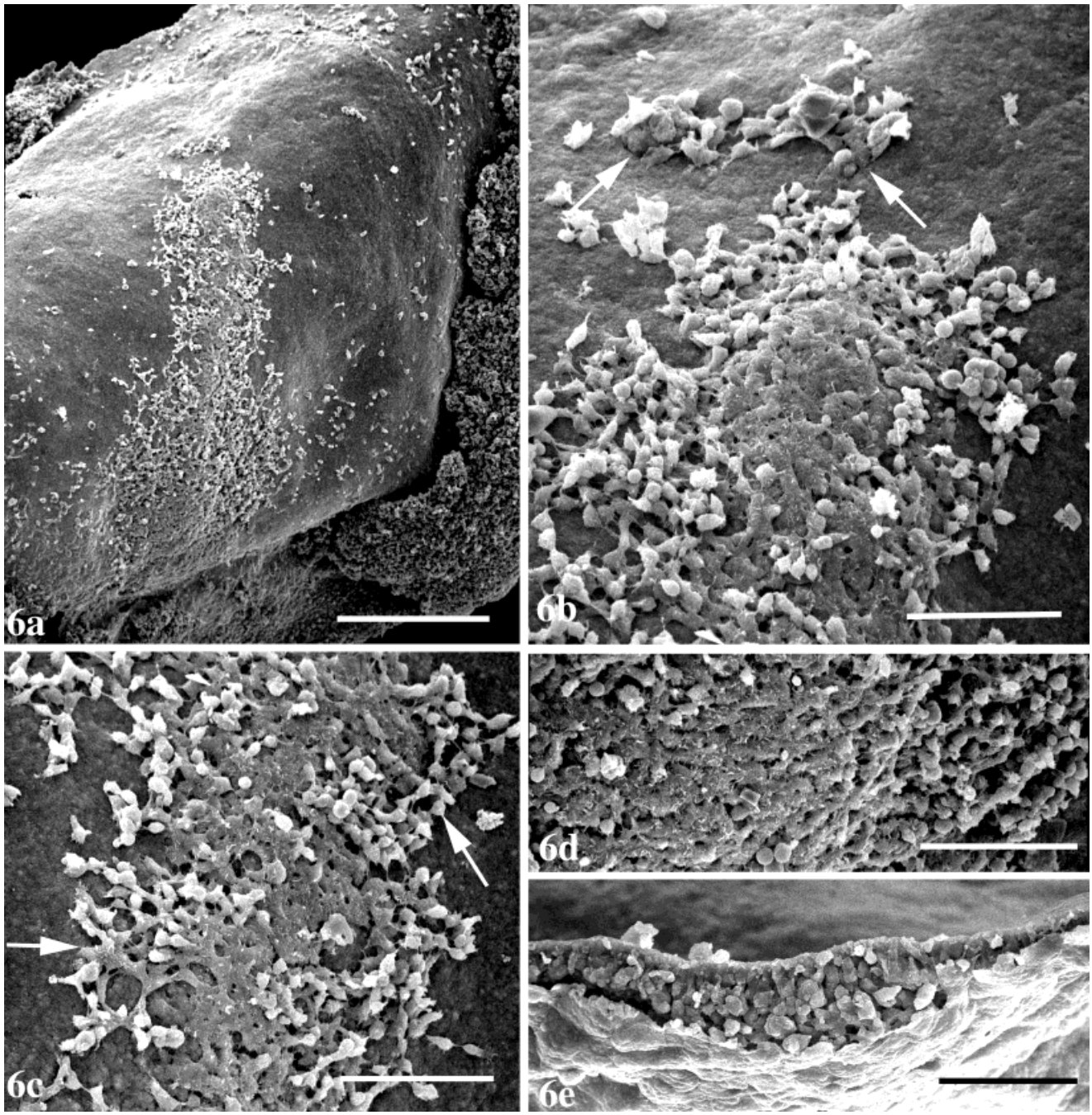
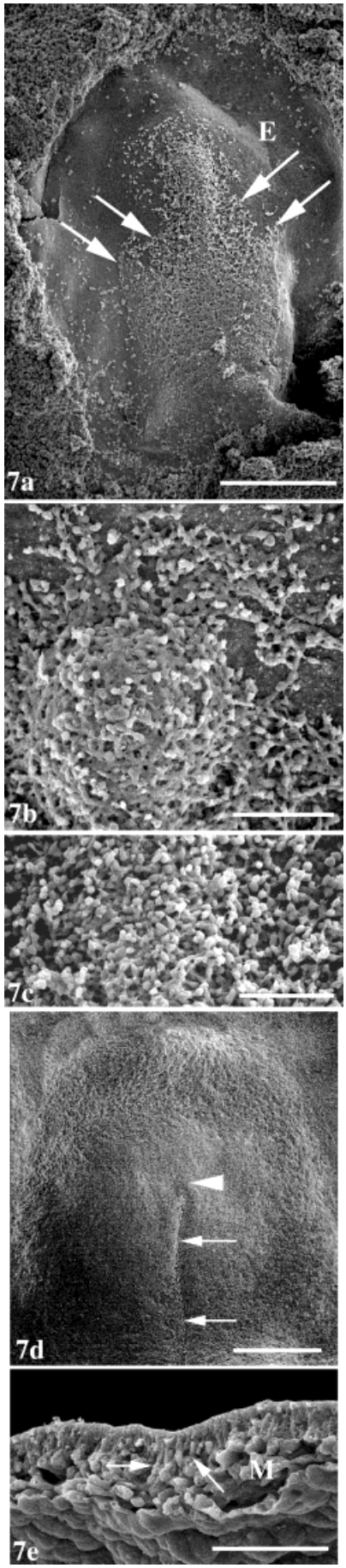


Fig. 6. **a:** SEM of the ventral surface of a Stage 3a/b chick embryo in which the hypoblast and definitive endoderm was removed, revealing the ventral (basal) surface of the epiblast and the linear primitive streak (the blastoderm curled during processing). Scale bar = 400 μm. **b:** Enlargement of the rostral end of the primitive streak. Cells (arrows) are emerging from the ventral (basal) surface of the epiblast. Scale bar = 100 μm. **c:** Enlargement of the mid-streak level. Mesoderm cells are migrating from the lateral margins of the primitive streak (arrows). Scale bar = 100 μm. **d:** Enlargement of the caudal end of the primitive streak. Primitive streak cells are densely packed at the caudal end of the primitive streak. Scale bar = 100 μm. **e:** SEM of a transverse slice through the mid-primitive streak of a Stage 3a/b chick embryo; primitive streak cells have predominantly globular shapes. Scale bar = 65 μm.

the 8-hr period, with embryos from the first group almost doubling the length of their primitive streaks. In addition, the width of the rostral primitive streak increased in the first two groups, with formation of Hensen's node. In contrast, as the length of the primitive streak increased, the width of the mid-streak decreased. In the first two groups, where the greatest change occurred in the length of the primitive streak, a concomitant decrease occurred in the



width of the mid-streak. In particular, note in the first group that as the length of the primitive streak approximately doubled (770–1475 μm), the width of the mid-streak approximately halved (325–145 μm). This relationship provides evidence that coordinated convergent-extension movements occur during elongation of the primitive streak.

Distribution of Fibronectin and Laminin

The distribution patterns of both fibronectin and laminin were similar in appearance and, therefore, are described together. Both glycoproteins are present in the basal lamina before the appearance of the primitive streak (Fig. 12a). With the appearance of the streak, however, fibronectin and laminin become depleted in the region it occupies (Fig. 12b,c). Elsewhere beneath the epiblast, the glycoproteins continue to be expressed (Fig. 12b,c). As ingression occurs, remnants of the disrupted glycoproteins are observed within the streak (Fig. 12d).

Fate Mapping Experiments

Injections of the primitive streak. To document early mesodermal and endodermal cell migration from the primitive streak and the timing of this migration in relation to progression of the primitive streak, dye was injected into the primitive streaks of embryos at Stages 2, 3a/b, and 3c at three levels (i.e., rostral, middle, and caudal, designated, respectively, as Sites 1, 2, and 3; Fig. 13a). At each injection site when examined immediately after injection, the dye labeled a small group of dorsal surface cells in the midline of the primitive streak together with ingressing cells in the midline (Fig. 13b). After 4 hours of re-incubation, Stage 2 embryos had developed to Stage 3a/b, and Stages 3a/b and 3c embryos, to Stages 3c and 3d/4, respectively. In embryos injected at Stage 2, cell migration from Sites 1, 2, and 3 was generally away from the midline in a lateral direction (Fig. 13c). Additionally, a few labeled cells had migrated rostrally from the rostral end of the streak (Fig. 13d). A majority of the mesodermal cells together with the underlying endoderm was labeled at all injection sites (Fig. 13e). Between injection sites, however, the mesodermal cells that were labeled were only those that were close to the endodermal layer (Fig. 13f). In the dorsal midline of the primitive streak, a small group of surface cells located mainly rostral and caudal to injection Site 2, as well as within injection Site 2, were labeled (Fig. 13c,e,f).

Injections in embryos at Stages 3a/b and 3c showed similar features, so they are described together. The la-

Fig. 7. **a:** SEM of the ventral surface of the blastoderm of a stage 3c chick embryo in which the definitive endoderm and hypoblast were removed revealing the ventral (basal) surface of the epiblast (E), primitive streak, and ingressing mesoderm. Arrows point to the mesodermal front. Scale bar = 500 μm. **b,c:** Enlargements of the rostral and mid-primitive streak, respectively. Note that cells in the rostral streak (i.e., Hensen's node) collectively form a ball-like structure. Scale bar = 100 μm. **d:** SEM of the dorsal surface of the epiblast of Stage 3c chick embryo. The primitive groove (arrows) has formed along the length of the primitive streak. The arrowhead points to Hensens node. Scale bar = 170 μm. **e:** SEM of a transverse slice through the level of the mid-streak of a Stage 3c chick embryo. Arrows point to ingressing cells. Newly formed mesoderm (M). Scale bar = 50 μm.

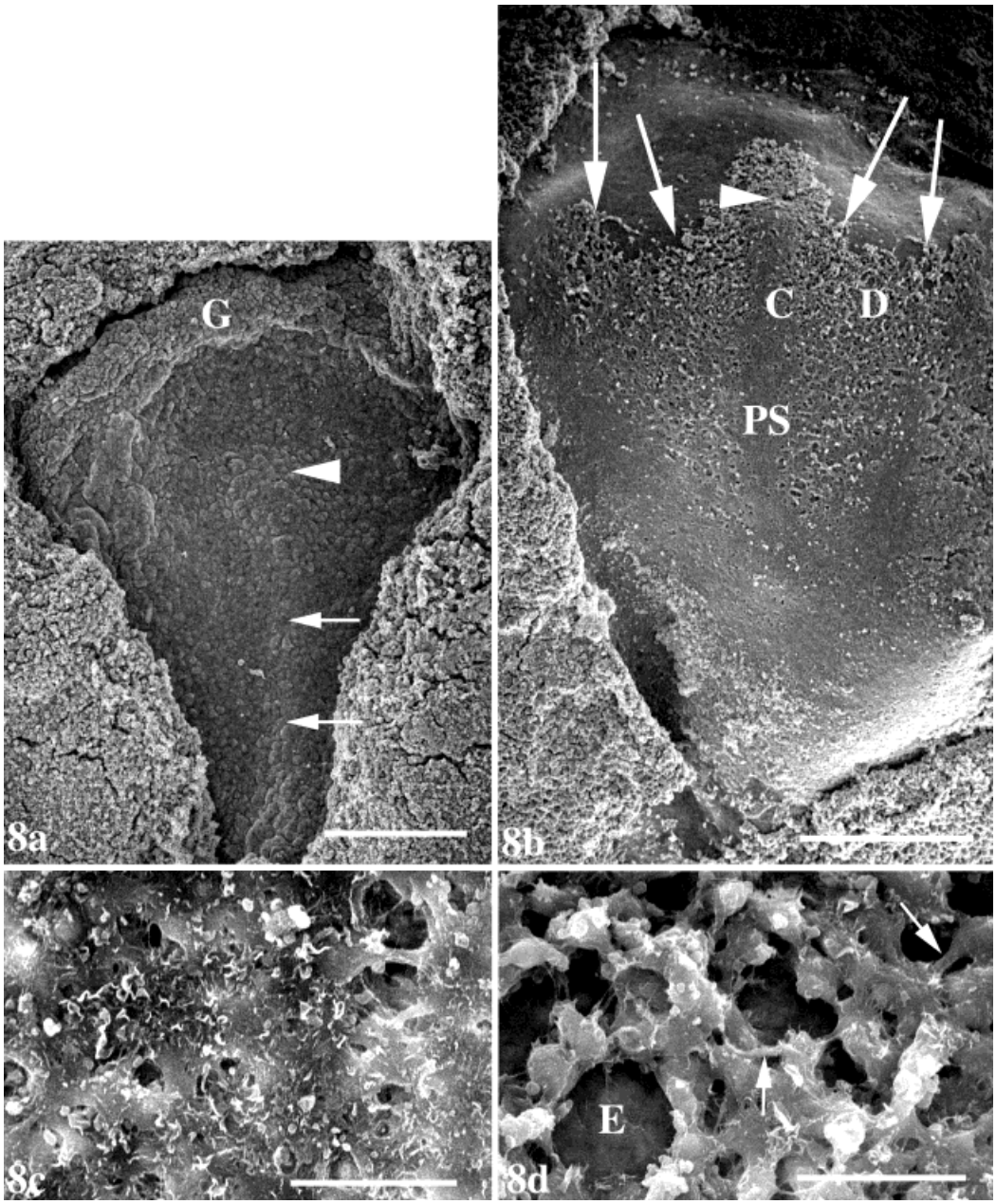


Fig. 8. **a:** SEM of the ventral surface of a Stage 3d chick embryo; the endoderm and hypoblast have been left in place; arrows point to the position of the primitive streak; arrowhead points to the position of Hensen's node. Germ cell crescent (G). Scale bar = 250 μm. **b:** SEM of the ventral surface of a stage 4 chick embryo in which the endoderm was removed. Arrows point to the mesodermal front; arrowhead points to Hensen's node; C and D mark the positions enlarged in 8c and d, respectively. Primitive streak (PS). Scale bar = 375 μm. **c:** Primitive streak cells from the area marked C in b. Cell boundaries are not easily discernible. Scale bar = 10 μm. **d:** Mesodermal cells from the area marked D in b. Unlike the primitive streak cells shown in c, individual cell outlines are clear. Arrows point to mesodermal cell processes. Epiblast (E). Scale bar = 20 μm.

beling characteristics of cells at the injection sites for 0 hours and 4 hours of reincubation were similar to those for Stage 2 embryos. Cell migration from Site 1 occurred in a rostral direction, but the ingressed cells spread laterally as they advanced (Fig. 14a). Most cells rostral to the node were labeled, apart from a few at the extreme lateral sides (Fig. 14b), which presumably were derived from more caudal levels of the primitive streak. Movement of cells from Site 2 was often similar to that at Stage 2, but sometimes, it resembled that at Site 3, which tended to occur rostrolaterally, with the cells forming v-shaped streams. Close to injection Sites 2 and 3, most of the mesodermal cells within and close to the primitive streak were labeled (Fig. 14c), but more rostral or caudal to these

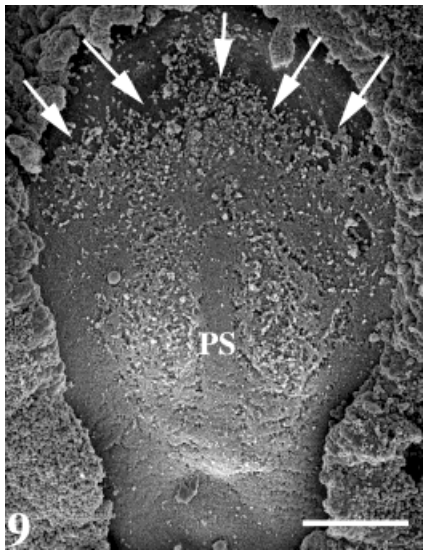


Fig. 9. SEM of the ventral surface of a Stage 5 chick embryo in which the endoderm was removed. The front of the ingressing mesoderm (arrows) has advanced rostrally as compared with earlier stages. Primitive streak (PS). Scale bar = 250 μm.

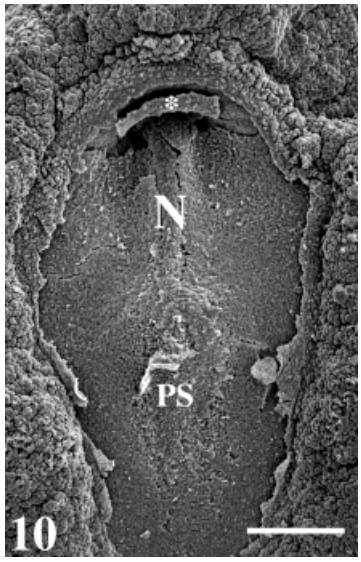


Fig. 10. SEM of the ventral surface of a Stage 6 chick embryo in which the endoderm was removed. Asterisk marks the head fold of the body and the incipient cranial intestinal portal; primitive streak (PS); notochord (N). Scale bar = 300 μm.

injection sites, only mesodermal cells close to the endoderm and those lateral to the streak were labeled (Fig. 14d). At these stages of development, movement of the midline group of dorsal surface cells in the primitive streak occurred both rostrally and caudally from Site 2, but mainly rostrally from Site 3; sometimes, these joined together in the dorsal midline (Fig. 14e).

To establish whether the labeling of the midline group of dorsal surface cells of the primitive streak was real or an artifact of endogenous peroxidase activity, control embryos lacking dye injections were processed in a manner identical to that used for injected embryos. No labeling was detected anywhere in the blastoderm (Fig. 15a,b), demonstrating the lack of endogenous peroxidase activity.

To determine whether the labeled midline group of primitive streak cells were complete cells or cell fragments, embryos at Stages 2–3c were examined with transmission electron microscopy. The results revealed that the cells in the dorsal midline of the primitive streak had nuclei (Fig. 16); consequently, such cells were not cell fragments, such as left-behind apical cytoplasmic processes of previously ingressed cells.

Injections of the parastreak epiblast. To determine the timing and pattern of ingression of epiblast cells through the primitive streak, dye was injected on each side of the primitive streak in embryos at Stages 2–3c of development. At 0 hr, the dye labeled the entire thickness of the epiblast at the injection site (Fig. 17a,b). After 5 hours of re-incubation, although the labeled cells in the epiblast had moved closer to the midline, none had ingressed through the streak. The migration of the labeled epiblast cells was in a rostromedial direction at mid and caudal levels of the streak, but caudomedially at rostral streak levels (Fig. 17c,d). By 7 hours of re-incubation, the two bilaterally labeled epiblast patches had merged into one overlapping patch in the midline primitive streak, and labeled cells were now present within the mesodermal layer (Fig. 17e,f). Additionally, by 7 hours of re-incubation primitive streak cells from injection site 2 had spread both rostrally and caudally within the primitive streak; such labeled cells occupied the entire thickness of the midline primitive streak, including much of its dorsal surface (Fig. 17e,f).

DISCUSSION

The principal findings of the study are: 1) the primitive streak forms locally at the caudal midline of the area pellucida, without contributions from more rostral or peripheral, extensively migrating cells; 2) progression of the primitive streak involves convergent-extension movements within the streak per se; 3) progression of the primitive streak involves the accretion of newly delaminated epiblast cells to its rostral end; and 4) a subpopulation of cells located at the midline dorsal surface of the primitive streak undergoes substantial rostral migration within the streak during its progression. Additionally, we establish the timing of epiblast cell migration toward and into the primitive streak, and we show that cells from bilateral sites undergo intercalation within the streak during ingression.

Formation of the Primitive Streak

The primitive streak has traditionally been known to appear suddenly at Stage 2 in the caudal one-third of the area pellucida, where it occupies a triangular area with its apex pointing rostrally. Although previous studies have demonstrated the ability of the caudal MZ to induce streak formation at ectopic sites (Khaner et al., 1985; Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989; Eyal-Giladi et al., 1992; Bachvarova et al., 1998; Khaner, 1998), and have also identified molecular markers that can mediate streak induction (Hume and Dodd, 1993; Shah et al., 1997; Streit et al., 1998), events in the epiblast that immediately precede overt appearance of

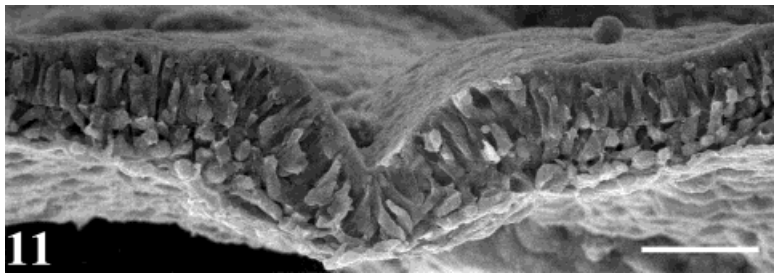


Fig. 11. SEM of a transverse slice of a Stage 5 chick embryo through the mid-streak level showing ingressing cells. Scale bar = 50 μm.

TABLE 1. Coordinated convergent-extension movements during elongation of the primitive streak*

Stage	0 Hr	2 Hr	4 Hr	6 Hr	8 Hr	% Increase or decrease
Length of primitive streak						
2	770 (44)	985 (109)	1170 (72)	1290 (68)	1475 (187)	91
3a/b	1075 (130)	1323 (129)	1465 (137)	1655 (89)	1815 (106)	69
3c	1235 (19)	1390 (21)	1600 (51)	1765 (77)	1995 (77)	62
Width of rostral primitive streak						
2	115 (6)	145 (6)	170 (12)	175 (18)	190 (22)	65
3a/b	193 (33)	235 (31)	235 (12)	233 (15)	230 (12)	19
3c	175 (28)	195 (22)	225 (12)	165 (24)	175 (7)	0
Width of mid-primitive streak						
2	325 (19)	295 (77)	200 (39)	145 (28)	145 (12)	−55
3a/b	233 (30)	190 (24)	148 (24)	120 (12)	135 (12)	−42
3c	130 (26)	130 (23)	115 (13)	110 (16)	120 (6)	−8

*Values represent mean lengths or widths (μm) for five embryos in each cell. The standard error of the mean is given in parentheses. Negative values are percentage decrease.

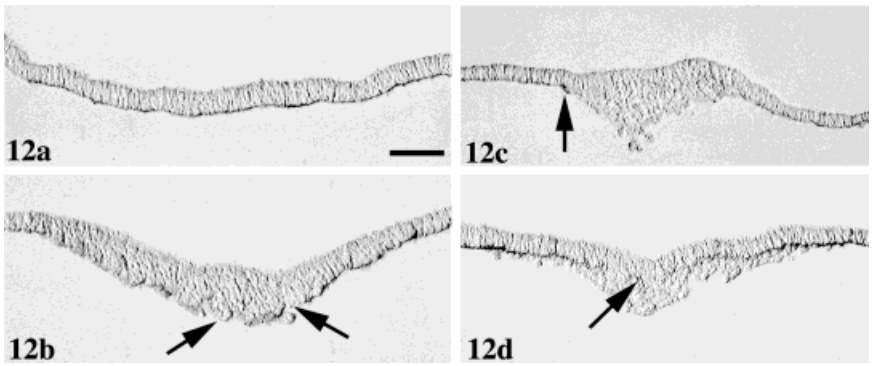


Fig. 12. Transverse sections of chick embryos labeled with anti-fibronectin (a, b, d) or anti-laminin (c) antibodies. The epithelialized hypoblast or endoderm have been removed to ensure antibody penetration. **a**: Stage XIII. The ventral surface of the entire pre-streak epiblast is labeled. **b**: Stage 2. Labeling of the ventral surface of the initial primitive streak is patchy. Arrows point to gaps in labeling. **c**: Stage 3a/b. As the primitive streak elongates, its entire ventral surface becomes devoid of label. Arrow points to the junction between epiblast and primitive streak on one side. Note that the ventral side of the epiblast is labeled in contrast to the ventral side of the primitive streak. **d**: Stage 3c. With ingression well underway, remnants of labeled basement membrane (arrow) can be identified at the ventral side of individual ingressing epiblast cells. Scale bar = 100 μm.

the primitive streak have not been elucidated. The results of the present study indicate that the formation of the primitive streak is initiated first by an increase in the height (thickness) of the cells in the primitive streak-forming part of the epiblast (i.e., the caudal midline of the area pellucida) and then, by the emergence of cells from the ventral midline of the caudal epiblast. The identification of this first crop of primitive streak cells in the caudal one-third of the ventral midline of the epiblast is significant, as these cells define the initial length of the primitive streak before its appearance. Subsequently, adjacent epiblast cells are triggered to transform from columnar to globular streak cells that occupy a triangular area in the caudal one-third of the area pellucida. The transformation

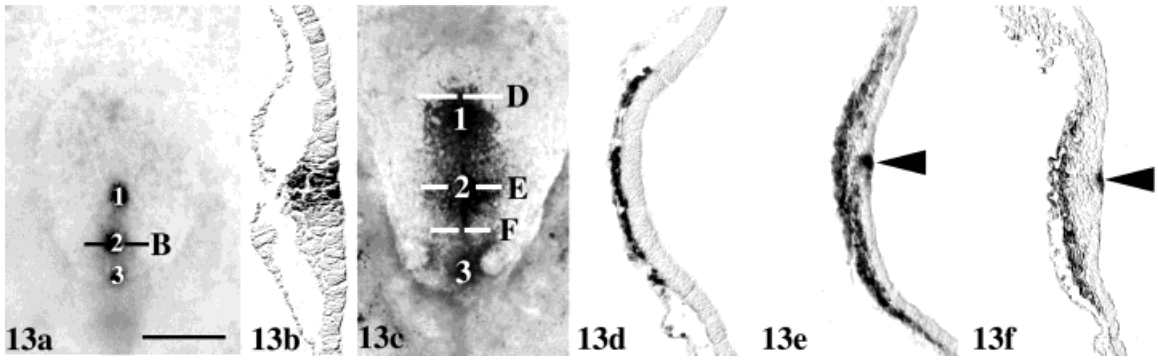


Fig. 13. Injections of the primitive streak at three levels (1, 2, 3) in chick embryos at Stage 2 (processed for peroxidase immunocytochemistry using anti-rhodamine primary antibody). **a:** Whole mount (viewed dorsally); at time zero, the dye is confined to cells at the three injection sites. Scale bar = 750 μm. **b:** A transverse section through level B (at the original injection Site 2) in a shows labeling of cells in the midline of the primitive streak throughout its entire dorsoventral extent. Scale bar (shown in a) = 100 μm. **c:** Whole mount (viewed dorsally) of an embryo similar to that shown in a after 4 hours of re-incubation after injection at three sites. Movement of labeled cells from the primitive streak has occurred at all three levels; such movement from all levels is bilateral, with some rostral migration also occurring from original injection Site 1. Scale bar (shown in a) = 500 μm. **d:** A transverse section at level D (rostral to original injection Site 1) in c shows that the mesendoderm is labeled at a level that is rostral to the labeling of Hensens node (hence, the epiblast is unlabeled at level D). Scale bar (shown in a) = 100 μm. **e,f:** Transverse sections at levels E (at original injection Site 2) and F (rostral to original injection Site 3), respectively, of c shows that the midline dorsal surface cells (arrowheads) are labeled, as are the deeper cells of the primitive streak, but not the intermediate cells. Scale bar (shown in a) = 100 μm.

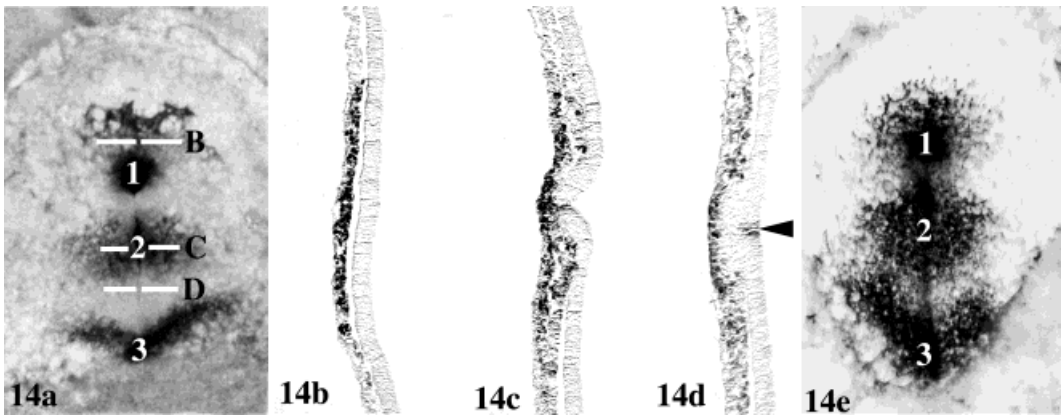


Fig. 14. Injections of the primitive streak at three levels (1, 2, 3) in a chick embryo at Stage 3c (processed for peroxidase immunocytochemistry using anti-rhodamine primary antibody). **a:** Whole mount (viewed ventrally) 4 hours postinjection. **e:** Whole mount (viewed dorsally) of a similar embryo as in a 4 hours postinjection. Note the spread of cells in the dorsal midline between original injection Sites 1 and 2, and 2 and 3. **b:** Ingressed mesoderm cells rostral to the node at level B (rostral to original injection Site 1) in a are labeled except for those at the extreme lateral edges. The definitive endoderm cells at this level are mostly unlabeled. **c:** At level C (at original injection Site 2) in a ingressed cells within and adjacent to the primitive streak are labeled. **d:** More caudally at level D (rostral to original injection Site 3 and just caudal to original injection Site 2) in a only the midline dorsal surface cells (arrowhead), deeper primitive-streak cells, and those ingressed cells lateral to the streak are labeled. Scale bar (shown in 13a) for a, e = 500 μm; b–d = 100 μm.

is accompanied by a breakdown in the basal lamina, as evidenced by our observations using SEM, and also by the absence of fibronectin and laminin (integral components of the basal lamina) beneath the globular cells. Collectively, our results demonstrate that the primitive streak does not arise from an extensive movement of cells from more rostral or peripheral areas of the epiblast. Rather, epiblast cells that are already in the primitive streak-forming area become locally transformed into primitive streak cells. Therefore, our results do not support the view that a linear primitive streak arises in the caudal midline at Stage 2 from a wide-scale convergence and rostral migration of epiblast cells (Vakaet, 1970; Eyal-Giladi et al., 1992), or from the aggregation of cells randomly delaminated throughout the epiblast (i.e., the HNK-1+ cells of Stern and Canning, 1990). Rather, our results are consistent with those of previous studies that suggested that cells from the caudal MZ contribute to the epiblast and hypoblast (Stern, 1990; Bachvarova et al., 1998; Khaner, 1998).

Progression of the Primitive Streak

Our measurements of the length and width of the primitive streak of living embryos provides direct evidence that progression of the primitive streak involves convergent-extension movements. Namely, increase in length of the primitive streak (i.e., doubling) is correlated with a concomitant decrease in its width (i.e., halving). This sug-

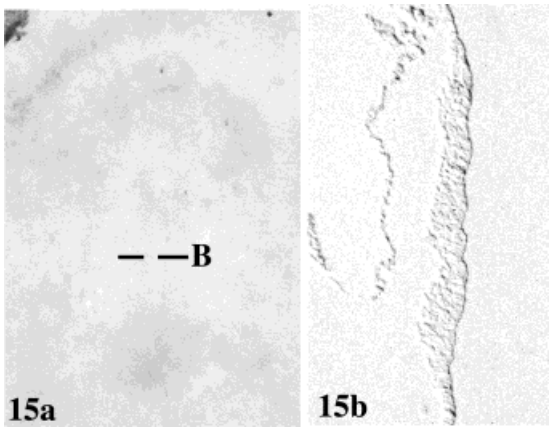


Fig. 15. A control Stage 3a/b chick embryo in which dye was not injected (processed for peroxidase immunocytochemistry using anti-rhodamine primary antibody). **a,b:** Background labeling, for example, due to endogenous peroxidase activity, is completely lacking in both whole mounts (a; viewed dorsally) and transverse sections (b; through level B in a). Note in particular that the existence of labeled midline dorsal surface cells in injected embryos cannot be explained by an artifact of immunocytochemistry. Scale bar (shown in 13a) for a = 500 μm; b = 100 μm.

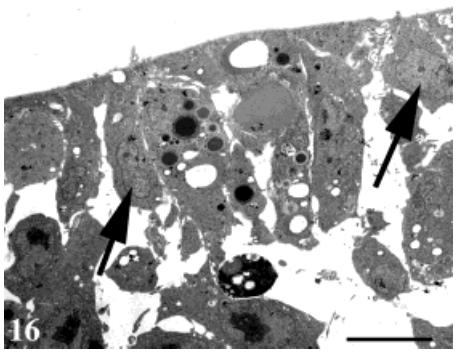


Fig. 16. TEM of a transverse section at the mid-primitive streak level showing the midline dorsal surface cells in a Stage 3a/b chick embryo. Arrows point to those midline dorsal surface cells whose nuclei fall within the plane of the section and are located near the dorsal surface of the epiblast, showing that the midline dorsal surface cells are truly complete cells and not merely cell processes. Mitotic figures also occur in the dorsal midline, suggesting that midline dorsal surface cells undergo mitosis. Scale bar = 6 μm.

gests that one cell behavior that plays a role in progression is mediolateral cell-cell intercalation, a process underlying virtually all convergent-extension movements (e.g., Gilbert, 2000). In birds and mammals, however, higher vertebrates in which growth accompanies morphogenesis, oriented cell division also plays a role in convergent-extension (e.g., Smith and Schoenwolf, 1997). Indeed, primitive streak cells undergo rapid division throughout gastrulation, with a greater rate of division occurring caudally than rostrally (Sanders et al., 1993), and a role for rostrocaudally oriented cell division in progression of the chick primitive streak has been established (Wei and Mikawa, 2000). Nevertheless, the fact that doubling and halving, respectively, of the length and width of the primitive streak occur simultaneous suggests that cell-cell intercalation likely also is involved (note: only one round of cell–cell intercalation would be necessary to halve the width of the primitive streak and double its length; owing to the small size of primitive streak cells and the fact that both labeled and unlabeled cells would be intercalating in the same direction, one round of intercalation would be difficult to detect along borders formed by labeled and unlabeled cells). Direct evidence for such intercalation is provided by our epiblast labeling studies, which show that bilateral patches of labeled epiblast cells merge together in the midline of the primitive streak, 7 hours post-injection (cf. Fig. 17d,f).

Additionally, our study has identified a subpopulation of primitive streak cells, located at its midline dorsal surface, which undergo extensive displacement rostrally during progression. The mechanism by which these cells undergo displacement is unclear: they could move rostrally owing to oriented cell division, cell–cell intercalation, or direct migration; further studies will be needed to resolve this issue. Here, we show that these cells exist and that they are indeed complete cells and not cell fragments. The fact that these are intact cells was evidenced by the presence of nuclei within them (as demonstrated with TEM), providing proof that they are not merely apical remnants of previously ingressed cells. By virtue of their midline location, coupled (as revealed by the dye labeling studies) with the fact that they migrate mainly in the midline along a rostrocaudal axis and fail to ingress during the period of study, we hypothesize that they have important regulative functions during development of the primitive streak. We propose that the midline dorsal surface cells might be responsible for initiating the formation of the primitive streak. Moreover, the midline dorsal surface cells might function during ingression in directing epiblast cells as they approach the midline so that they ingress ventrally, principally on the ipsilateral side. Their presence may account for the lack of cells crossing the midline within the epiblast (Levy and Khaner, 1998), and only a limited migration of cells across the midline within the mesodermal layer (Rosenquist, 1966; Vakaet, 1984; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Levy and Khaner, 1998).

Current views regarding the rostral progression of the primitive streak after its first appearance are divergent. The results of our SEM study favor one, first advanced by Spratt (1946), that states that the rostral progression of the primitive streak occurs by a successive addition of epiblast cells to its rostral end. We show here using SEM that cells progressively leave the epiblast in caudal-to-rostral sequence, ingressing (or delaminating) rostral to the streak in precise coordination with its progression. In addition, our conclusion that convergent-extension of the primitive streak occurs (based on measurement of the length and width of the streak during its progression) is consistent with other views suggesting that the primitive streak progresses rostrally through either a rostral migration of primitive streak cells (Wetzel, 1929; Pasteels, 1937; Eyal-Giladi et al., 1992) or stretching of the streak (Vakaet, 1970). Our data and that of others (Wei and Mikawa, 2000) suggest that progression is a complex process involving accretion of cells to its growing rostral end, convergent-extension mediated by cell–cell intercalation (ascertained by cell labeling), and oriented cell division. Thus, cell behaviors similar to those occurring in avian

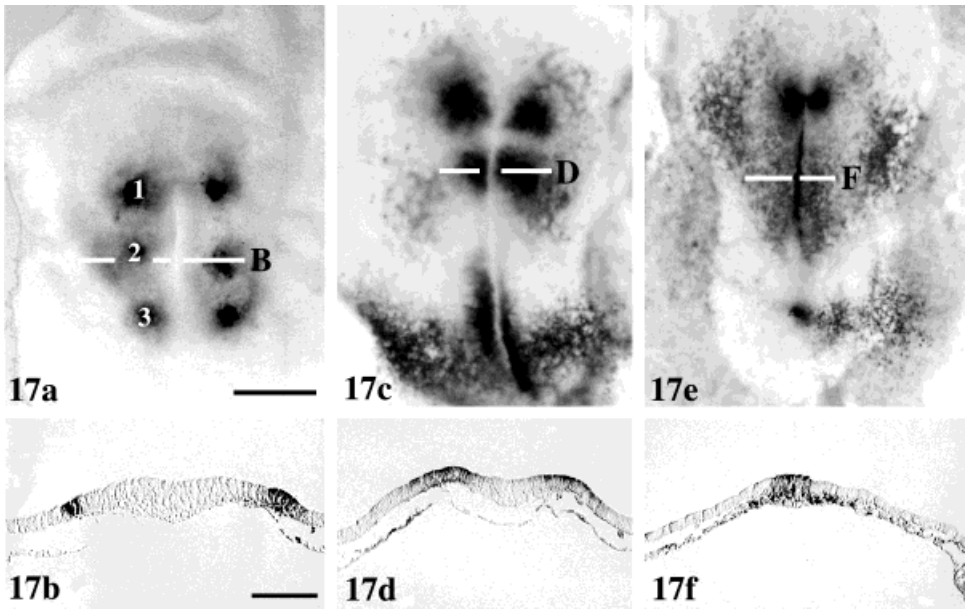


Fig. 17. Bilateral injections of the epiblast at three levels (1, 2, 3) in chick embryos at Stage 3a/b (processed for peroxidase immunocytochemistry using anti-rhodamine primary antibody). **a,c,e:** Whole mounts (viewed dorsally) at 0 hours, 5 hours, and 7 hours post-injection. **b,d,f:** Transverse sections through level B, D, F in a, c, e, respectively. By 5 hours post-injection, labeled epiblast cells have migrated medially toward the primitive streak but have not yet ingressed (c, d). Ingression commences about 7 hours post-injection (e, f). Scale bars (shown in a, b) for a, c, e = 500 μm; b, d, f = 50 μm.

embryos during shaping of the neural plate (reviewed by Smith and Schoenwolf, 1997) and extension of the notochord (Sausedo and Schoenwolf, 1993) also seem to underlie progression of the avian primitive streak.

Ingression of Cells Through the Primitive Streak

By definition, ingression is the process by which cells leave the epiblast, become part of the primitive streak, and then migrate away from the streak (Bellairs, 1986). Based on the results from both SEM and ectodermal injections, we propose a breakdown of the process into two overlapping phases: early and late. The early phase involves epiblast cells that are already located in the primitive streak-forming area before its formation. The main features of this phase are 1) an epithelial-to-mesenchymal transformation, manifested morphologically by a change in cell shape; 2) a breakdown of the basal lamina beneath the transformed cells, as evidenced by SEM and also by the absence of fibronectin and laminin in this area; and 3) the migration of the transformed cells out of the streak. As these cells leave the primitive streak, the late phase of ingression begins. This involves the transformation of laterally located epiblast cells as they move into the streak to replace the initial streak cells. Moreover, as these cells move medially they carry their basement membrane with them (Sanders, 1984; Van Hoof and Harrisson, 1986) so that it accumulates at the dorsolateral edges of the primitive streak (Sanders, 1984). The molecular factors that control these interesting events are largely unknown.

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