Cell Populations and Morphogenetic Movements Underlying Formation of the Avian Primitive Streak and Organizer

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Summary: The cell populations and morphogenetic movements that contribute to the formation of the avian primitive streak and organizer—Hensen’s node—are poorly understood. We labeled selected groups of cells with fluorescent dyes and then followed them over time during formation and progression of the primitive streak and formation of Hensen’s node. We show that (1) the primitive streak arises from a localized population of epiblast cells spanning the caudal midline of Koller’s sickle, with the mid-dorsal cells of the primitive streak arising from the midline of the epiblast overlying Koller’s sickle and the deeper and more lateral primitive streak cells arising more laterally within the epiblast overlying the sickle, from an arch subtending about 30°; (2) convergent extension movements of cells in the epiblast overlying Koller’s sickle contribute to formation of the initial primitive streak; and (3) Hensen’s node is derived from a mixture of cells originating both from the epiblast just rostral to the incipient (stage 2) primitive streak and later from the epiblast just rostral to the elongating (stage 3a/b) primitive streak, as well as from the rostral tip of the progressing streak itself. Collectively, these results provide new information on the formation of the avian primitive streak and organizer, increasing our understanding of these important events of early development of amniotes. * Correspondence to: Gary C. Schoenwolf, Department of Neurobiology and Anatomy, University of Utah School of Medicine, 50 N. Medical Drive, Salt Lake City, UT 84132. E-mail: Schoenwolf@med.utah.edu

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INTRODUCTION

Gastrulation is a key event of early animal development. It is characterized by extensive and highly coordinated morphogenetic movements that result in the formation of the three primary germ layers: the ectoderm, mesoderm, and endoderm. The hallmark of gastrulation in higher vertebrates is the formation of the primitive streak, a thickening of the epiblast which in avian embryos, appears suddenly at stage 2 (Hamburger and Hamilton, 1951) in the caudal third of the area pellucida. The nascent primitive streak is triangular in shape when viewed from its dorsal or ventral surface, and its appearance overtly defines the midline of the embryo and the rostrocaudal axis. Soon after its appearance, the primitive streak undergoes progression, during which it rapidly elongates rostrocaudally and assumes a linear profile. At about this time, the rostral end of the primitive streak becomes swollen, marking the location of the organizer (called Hensen’s node in avian embryos). During progression of the primitive streak, epiblast cells programmed to form the mesoderm and endoderm begin to ingress through the streak, thereby establishing the germ layers. The final change that occurs in the primitive streak is regression, a rostral-to-caudal shortening of the streak that continues until Hensen’s node and a short persisting remnant of the primitive streak is incorporated into the tail bud (Schoenwolf, 1979; for reviews of avian gastrulation, see Bellairs, 1986; Lemaire and Kessel, 1997; Schoenwolf and Smith, 2000; Stern and Canning, 1988).

Several studies have focused on the induction of the primitive streak and considerable evidence, based mainly on tissue transplantation, suggests that the inductive signal is provided by the posterior (caudal) marginal zone (PMZ; i.e., the posterior/caudal blastoderm ring that forms an interface between the area pellucida and area opaca) (Bachvarova et al., 1998; Eyal-Giladi and Khaner, 1989; Eyal-Giladi et al., 1992; Khaner, 1998; Eyal-Giladi et al., 1992, 1998; Khaner and Eyal-Giladi, 1986, 1989; Khaner et al., 1985). Additional evidence implicating the PMZ in induction of the primitive streak comes from the demonstration that secreted proteins, such as Vg1 (Shah et al., 1997), chordin (Streit et al., 1998), and Wnt-8c (Hume and Dodd, 1993), are expressed in the PMZ, and some of these (i.e.,...
Vg1 and cbordin) are capable of inducing primitive streaks when expressed ectopically. Existing data on how the primitive streak forms are contradictory. Current ideas can be summarized with two extreme models. The first, based on the assumption that primitive streak precursor cells reside randomly throughout the epiblast, proposes that formation of the primitive streak is achieved by a long-range caudomedial migration of precursor cells, followed by their aggregation in the caudal midline (Stern and Canning, 1990). The second, based on the assumption that the primitive streak precursor cells reside in the PMZ, proposes that the formation of the primitive streak is achieved by the short-range rostral displacement of streak precursor cells from the midline of the PMZ (Eyal-Giladi et al., 1992; Vakaet, 1970; Wei and Mikawa, 2000). Both of these models fail to account for the important fact that the initial primitive streak has a triangular profile. Moreover, a recent descriptive study using scanning electron microscopy suggested that the primitive streak does not arise from an extensive movement of cells from more rostral or peripheral areas of the epiblast. Instead, epiblast cells that are already in the vicinity of the forming primitive streak at stage XIV (Eyal-Giladi and Kochav, 1976) seem to become transformed locally into primitive streak cells by stage 2 (Lawson and Schoenwolf, 2001). However, the precise origin and identity of cells that respond to the inductive influence of the PMZ, as well as the behaviors that such precursor cells exhibit to generate the incipient primitive streak, remain largely unknown.

Hensen’s node, the organizer of the avian embryo, first can be identified morphologically during the linear primitive streak stage. Fate mapping studies demonstrated that Hensen’s node at the fully elongated streak stage is fated to form midline ectodermal, mesodermal, and endodermal derivatives (Garcia-Martinez et al., 1993; Schoenwolf et al., 1992; Selleck and Stern, 1991). Reports based largely on fate mapping and the expression pattern of the homeobox gene, goosecoid, indicate that Hensen’s node precursor cells exist as two populations prior to the appearance of the primitive streak (Hatada and Stern, 1994; Izpisúa-Belmonte et al., 1993; Streit et al., 2000). Both populations seem to migrate to the center of the embryo, where they come together to colonize Hensen’s node (Streit et al., 2000). However, details of their positions and movement in relation to progression of the primitive streak are yet to be clarified.

This study had two aims. First, we determined by fate mapping the origin of cells that contribute to the incipient primitive streak. By labeling epiblast cells overlying the mediolateral extent of Koller’s (Rauber’s) sickle (the latter is defined as a sickle-shaped ventralward projection from the epiblast located within the caudal area pellucida, just rostral to the posterior marginal zone; Khaner, 1993) with fluorescent dyes at stages XI–XIII, and following their subsequent movement over time, we show that the cells that contribute to the incipient primitive streak reside in the middle two-thirds of the epiblast overlying the rostral part of Koller’s sickle. Additionally, our results suggest that these precursor cells undergo a convergent-extension movement along Koller’s sickle during their rostral displacement, thereby establishing the incipient (triangularly shaped) primitive streak. Second, we labeled cells in areas “c” and “a” (i.e., at the rostral end of the primitive streak, and the epiblast just rostral to the streak, respectively; Garcia-Martinez et al., 1993) at stages 2 and 3a/b (Hamburger and Hamilton, 1951; with stage 3 subdivided as described by Schoenwolf et al., 1992; also see Darnell et al., 1999), to determine their relative positions during progression of the primitive streak, as well as their contributions to Hensen’s node. Our results demonstrate that during progression of the primitive streak, cells from area “c” move rostrally to co-mingle with those from area “a,” and together they form Hensen’s node. Thus, the term Hensen’s node should be used to designate the rostral end of the primitive streak only at stages near the end of primitive streak progression (when these two populations of precursor cells become intermixed) and later.

RESULTS

Mapping Primitive Streak Precursor Cells

To determine the origin of primitive streak precursor cells, injections were made into the epiblast at the following sites: overlying the midline of Koller’s sickle, at the 3, 9, and 12 o’clock positions of the epiblast and at equidistantly spaced intervals (unilaterally or bilaterally) along the mediolateral extent of the epiblast overlying Koller’s sickle, either along its rostral or caudal borders (Fig. 1). Our results are summarized in Figure 8a–d. Injections placed in the midline of the epiblast overlying Koller’s sickle (labeling essentially its entire rostro-caudal extent) labeled the midline dorsal surface cells of the primitive streak (for a description of these cells, see Lawson and Schoenwolf, 2001), together with the underlying hypoblast and mesodermal cells adjacent to the hypoblast (Fig. 2a–c). Additionally, such injections labeled the midline of the caudal area opaca. It is important to note that these so-called midline injections labeled clusters of cells that spanned the midline rather than being restricted to the midline. It is also important to note that by the stage illustrated in Figure 2b, labeled cells were already ingressing through the primitive streak and moving laterally. Finally, not all cells in the dorsoventral extent of the primitive streak are labeled (Fig. 2c); thus, more lateral cells moved into the primitive streak during its formation, supporting the idea that formation of the primitive streak involves convergent extension (among other morphogenetic movements).

Injections placed in the epiblast moved during the next few hours either centrifugally or centripetally depending on their position: 12 o’clock injections moved directly rostrally (centrifugally) with progression of the primitive streak, whereas 3 and 9 o’clock injections moved medially (centripetally) toward the progressing streak. Such epiblast injections never contributed la-
FIG. 1. Scheme showing the locations of the injection sites in embryos at stages XI–XIII. The outer ring indicates the area opaca, the inner disc, the area pellucida and the curvature at the bottom (i.e., caudal end) of the area pellucida, Koller’s sickle. The marginal zone is not shown. Koller’s sickle lies within the area pellucida just rostral to the posterior (caudal) marginal zone. In one set of experiments on prestreak embryos, injections were placed in the epiblast overlying the midline of Koller’s sickle (large white X), and in the epiblast of the area pellucida at the 3, 9, and 12 o’clock positions (small black Xs). In another set of experiments on prestreak embryos, injections were placed in the epiblast overlying the entire mediolateral extent of Koller’s sickle, either positioned along its rostral border or its caudal border. The oblique lines indicate the angle (approximately 30°) subtended by the mediolateral extent of the portion of the epiblast overlying Koller’s sickle that contributed to the formation of the primitive streak.

FIG. 2. Injections in the midline of the epiblast overlying Koller’s sickle and in the epiblast at 3, 9, and 12 o’clock positions in an embryo at stage XIII. Whole-mounts (a, b) and a transverse paraffin section after peroxidase immunocytochemistry (c). (a) Locations of the injected dye at time zero; the arrowhead marks the midline of the epiblast overlying Koller’s sickle. (b) Locations of the injected dye at 4 h of reincubation. Labeled cells in the epiblast overlying the rostral border of the midline of Koller’s sickle moved rostrally during the 4 h of reincubation to populate much of the rostrocaudal extent of the primitive streak. Labeled epiblast cells at the 3 and 9 o’clock positions moved medially, whereas those at the 12 o’clock position moved rostrally. (c) A transverse section through level c in b. The midline dorsal surface cells of the primitive streak (arrowhead), the hypoblast and a few mesodermal cells adjacent to the hypoblast are labeled. Scale bar, 250 \( \mu \text{m} \) in a and b; 50 \( \mu \text{m} \) in c.

FIG. 3. Formation of the primitive streak in embryos in which the epiblast overlying the rostral border of Koller’s sickle was labeled with Dil/CRSE at stage XII. Following reincubation, embryos were processed for peroxidase immunocytochemistry. (a) Within 6 h of reincubation, labeled cells had migrated rostrally to form the triangularly-shaped, initial primitive streak characteristic of embryos at stage 2 (the initial primitive streak stage). Inset: a similar embryo at time zero showing three injections in the epiblast overlying Koller’s sickle. (b) Labeled cells from the epiblast overlying the rostral border of Koller’s sickle participated in the progression of the primitive streak. Injections were placed only on one side of the midline in this embryo. (c, d) Transverse sections through levels c and d, respectively, in b. Labeled cells were present on only the injected side of the midline (arrow marks the midline) throughout the rostrocaudal extent of the stage 3c primitive streak, except at its most rostral end (d) where Hensen’s node was just beginning to form. Scale bar, 250 \( \mu \text{m} \) in a and b; 50 \( \mu \text{m} \) in c and d.
beled cells to the primitive streak during its formation and early progression, demonstrating conclusively that the incipient primitive streak does not arise from a random delamination of epiblast cells followed by a wide-scale caudomediial migration to the forming streak.

Injections placed along the mediolateral extent of the epiblast overlying Koller’s sickle were localized either to its rostral or caudal border. Those placed along its rostral border, labeled cells that populated the entire incipient primitive streak (Fig. 3a, inset), with those placed unilaterally remaining on that side of the midline as the primitive streak formed and underwent progression (Fig. 3b, c). Bilateral injections placed as far laterally in the epiblast overlying Koller’s sickle so as to subtend an angle of about 30° (Fig. 1), contributed to the primitive streak, but more lateral injections did not (rather, they marked the caudal epiblast of the area pellucida). Moreover, examination of embryos at stage 3c that contained injections placed along the mediolateral extent of the epiblast overlying Koller’s sickle showed that the extreme rostral end of the elongating primitive streak was not labeled (Fig. 3d), suggesting that this portion of the primitive streak receives a contribution of cells from outside of the region of the epiblast overlying Koller’s sickle (see following section).

Inj ection of the epiblast overlying Koller’s sickle of the same embryo with two types of markers that fluoresced at different wavelengths, revealed that the primitive streak precursor cells intermixed mediolaterally, providing direct evidence that they underwent a convergent extension movement during their rostral displacement (Fig. 4a, b, inset). The latter occurred in an orderly fashion with precursor cells located laterally along Koller’s sickle following an oblique course, contributing to outer and rostral portions of the incipient primitive streak, whereas those closer to the midline following a less oblique course, giving rise to inner and caudal portions of the incipient primitive streak. This orderly movement is sufficient to account for the triangular shape of the incipient primitive streak. Thus, the incipient primitive streak was formed from precursor cells located both within the midline and lateral portions of the epiblast overlying the rostral border of Koller’s sickle.

In contrast to injections placed along the rostral border of the epiblast overlying Koller’s sickle, injections placed along its caudal border moved into area opaca, without contributing to the primitive streak (Fig. 5a, b). Sections revealed that such injections labeled the epiblast in this area (data not shown).

Mapping Hensen’s Node Precursor Cells

To determine the origin and migratory routes of Hensen’s node precursor cells during primitive streak formation and progression, injections were made at the tip of the incipient primitive streak (area “c,” Fig. 7a), and also, in the epiblast immediately rostral to it (area “a,” Figs. 6a, 7a) in stage 2, 3a/b, and 3c embryos. Our results are summarized in Figure 8c–d.

Following about 4 h of reincubation, labeled cells in area “a” had moved rostrally, concomitant with progression of the primitive streak, but now these cells populated the rostral end of the streak, contributing not only epiblast cells but essentially the entire population of area “c” cells at this stage (Fig 6b). Upon further incubation (15–24 h), such labeled cells colonized Hensen’s node (Fig. 6c), from which they subsequently contributed to the prechordal plate and rostral extraembryonic endoderm and midline axial structures (García-Martínez et al., 1993). Because area “a” epiblast cells incorporated into the rostral end of the primitive streak between stages 2 and 3a/b, to determine whether additional epiblast cells contributed to Hensen’s node during subsequent stages, we labeled cells in both areas “a” and “c” in the same embryo at stage 3a/b with two types of markers that fluoresced at different wavelengths. Such double labeling revealed conclusively that within 4 h of reincubation, labeled cells from area “c” had moved rostrally at a greater rate than area “a” cells, overlapping those from area “a” (Fig. 7b). These results support the view, suggested from previous descriptive studies using electron microscopy (Lawson and Schoenwolf, 2001), that late progression of the primitive streak and forma-

FIG. 4. A stage XI embryo with DiI/CRSE (red) and CFSE (green) injections in the epiblast overlying the rostral border of Koller’s sickle. The oblique lines mark the approximate midline of the blastoderm and incipient primitive streak. (a) The numbers 1–7 indicate the locations of the injected dye at time zero. (b) Within 4 h of reincubation, the new positions of the injected dyes (i.e., 1–7) show that labeled cells underwent convergent extension movement to initiate formation of the primitive streak (note: some injection sites split into multiple sites). Inset: 8 h of reincubation. The figure has been rotated so that the primitive streak (arrow) extends vertically down the midline. Scale bar, 400 μm in a and b.

FIG. 5. A stage XIII embryo with DiI/CRSE injections in the epiblast overlying the caudal border of Koller’s sickle. (a) Injections at time zero (because of the intense fluorescence, many individual injections appear fused as a single one). Arrows indicate the unlabeled epiblast overlying the rostral border of Koller’s sickle. (b) After 4 h of reincubation, labeled cells had migrated caudally into the area opaca, without contributing to the primitive streak (lateral borders of the primitive streak are indicated by vertical lines). Scale bar, 400 μm in a and b.

FIG. 6. DiI/CRSE injection of area “a” (epiblast just rostral to the incipient primitive streak) of a stage 2 embryo. White lines indicate the lateral sides of primitive streak, which converge just caudal to the injection site. (a) Injection at time zero. (b) Following 4 h of reincubation, labeled cells occupy the tip of the primitive streak. (c) By 24 h of reincubation (by stage 6), labeled cells had populated Hensen’s node (arrowhead) and contributed to the prechordal plate and rostral extraembryonic endoderm (asterisk) and the midline axis. Scale bar, 400 μm in a–c.

FIG. 7. Double labeling of areas “a” (green) and “c” (red) at stage 3a/b. (a) Injection at time zero. (b) Cells from areas “a” and “c” migrated rostrally, with those of area “c” overlapping those from level “a” (yellow) within 4 h of reincubation; arrowhead, incipient Hensen’s node. (c) By 15 h of reincubation, both populations of precursor cells had populated Hensen’s node (arrow) and contributed to prechordal plate and rostral extraembryonic endoderm and the midline axis (the red spot to the right of the green head process at about the 1 o’clock position is artifactual i.e., dye that leaked between the epiblast and underlying vitelline membrane). Scale bar, 400 μm in a–c.
tion of Hensen’s node occurs both by the accretion of newly delaminated epiblast cells to its rostral end and a rostral movement of cells from its elongating tip. During subsequent development, cells in Hensen’s node moved rostrally, contributing to midline cells. The red cells contributed to the midline near the rostral end of the neural plate, and the green cells contributed to the midline from about the mesencephalon level caudalward (Fig. 7c). Although molecular labels were not used in this study, based on their characteristic position, the green cells would be expected to form the midline cells that express Sonic hedgehog; further studies are underway to characterize in more detail the populations of cells derived from areas “a” and “c”).

DISCUSSION

There are three principal findings of the present study to discuss: (1) primitive streak precursor cells originate from the epiblast overlying the rostral border of Koller’s sickle, with the midline dorsal surface cells of the primitive streak arising from the midline of the epiblast overlying Koller’s sickle and the deeper and more lateral primitive streak cells arising more laterally within the epiblast overlying the sickle, from an arch subtending about 30°; (2) the incipient primitive streak is formed in part through the convergent extension of its precursor cells; and (3) during progression of the primitive streak, Hensen’s node continuously receives contributions from both epiblast cells located rostral to the primitive streak (i.e., from area “a” as defined at stage 2 and at stage 3a/b) and cells moving rostrally within the rostral end of the elongating streak (i.e., from area “c”).

Origin of the Primitive Streak

Our labeling studies of the epiblast overlying Koller’s sickle and the epiblast at the 3, 9, and 12 o’clock positions demonstrate that prior to the appearance of the initial primitive streak, its precursor cells are localized within the epiblast overlying the middle two-thirds of Koller’s sickle along its rostral border. Neither more rostral epiblast cells, at the 3, 9, and 12 o’clock positions, nor more caudal epiblast cells, overlying the caudal border of Koller’s sickle, contribute to the formation of the initial primitive streak. Thus, our findings are in contrast with the idea that the initial primitive streak arises from the aggregation of cells originally distributed randomly throughout the epiblast (Stern 1992; Stern and Canning, 1990). Instead, they support the labeling studies of Bachvarova and co-workers (1998), which suggested a localized origin of the primitive streak from the caudal midline of the area pellucida, as well as our previous labeling and descriptive studies (Lawson and Schoenwolf, 2001). Moreover, our results do not support the view that the primitive streak is formed exclusively from epiblast cells localized in the caudal midline (Vakaet 1970; Wei and Mikawa, 2000). Instead, we show that the epiblast overlying Koller’s sickle contributes to the entire dorsoventral thickness of the primitive streak. (c’–d’) Fate maps of areas “a” (i.e., epiblast just rostral to the primitive streak) and “c” (i.e., rostral tip of the primitive streak) showing the origins of Hensen’s node cells at stage 3a (c’) and stage 3c (d’) and their locations at stages 3c and 3d/4, respectively. Note that area “c” cells migrate rostrally to intermix with area “a” cells, forming by stage 3d/4 Hensen’s node.

FIG. 8. Diagram summarizing the results of our labeling experiments. The outer ring indicates the area opaca, the inner disc, the area pellucida, and the curvature at the bottom (i.e., caudal end) of the area pellucida, Koller’s sickle. The marginal zone is not shown. Koller’s sickle lies within the area pellucida just rostral to the caudal (posterior) marginal zone. (a–d) Fate maps of the epiblast overlying Koller’s sickle showing the origins of primitive-streak cells at stage XII (a), and their locations at stages 2 (b), 3a (c), and 3c (d). With the exception of the epiblast overlying the midline of Koller’s sickle, which contributes to the mid-dorsal surface cells of the primitive streak, the epiblast overlying Koller’s sickle contributes cells to the entire dorsoventral thickness of the primitive streak. (c’–d’) Fate maps of areas “a” (i.e., epiblast just rostral to the primitive streak) and “c” (i.e., rostral tip of the primitive streak) showing the origins of Hensen’s node cells at stage 3a (c’) and stage 3c (d’) and their locations at stages 3c and 3d/4, respectively. Note that area “c” cells migrate rostrally to intermix with area “a” cells, forming by stage 3d/4 Hensen’s node.

In summary, our present findings provide a more accurate fate map (Figure 8a-d) of the distribution of the
epiblast cells overlying Koller’s sickle that contribute to the formation of the initial primitive streak, advancing our understanding of the earliest events of formation of the primitive streak.

**Role of Convergent Extension in Formation of the Primitive Streak**

By tagging the primitive streak precursor cells and following their movement over time, we present evidence that formation of the triangularly shaped, initial (stage 2) primitive streak involves a convergent-extension movement of its precursor cells. Convergent extension is defined as the narrowing of one dimension (e.g., the width or transverse extent) of a column of cells and its concomitant elongation in a second dimension (e.g., the length or rostrocaudal extent) perpendicular to the narrowing dimension. Over an 8-h period, labeled groups of cells arranged mediolaterally at the outset of the experiment become interdigitated along the midline of the forming primitive streak (Figs. 4a, b, inset). This pattern of cell labeling is supported by studies using in situ hybridization to detect gene expression in the forming primitive streak (e.g., Lawson et al., 2001), providing further evidence of convergent extension during formation of the primitive streak. We show here that cells from lateral portions of the precursor area of the epiblast overlying Koller’s sickle follow an oblique course to contribute to more rostral portions of the streak, whereas those closer to the midline contribute to more caudal portions of the streak. The movement occurring during formation of the initial primitive streak is similar to that occurring during neurulation (particularly, shaping of the neural plate; Smith and Schoenwolf, 1997) and primitive streak progression (Lawson and Schoenwolf, 2001). In the latter case, measurements of the length and width of the essentially rectangular primitive streak during its progression show clearly that coordinated narrowing and lengthening occur. Such a relationship is more difficult to quantify with an epiblast area that begins as sickle-shaped and then transforms to triangular. Nevertheless, such shape changes are consistent with a process of convergent extension. Thus, collectively our results support the idea that formation of the primitive streak is driven at least in part by convergent extension movements.

Although our results support the view of Eyal-Giladi and co-workers (1992) on the origin of primitive streak precursor cells, they are not in agreement with their model on how the precursor cells move rostrally to establish the incipient streak. According to their model, the primitive streak forms in two stages. In the first stage of their model, cells located in the caudal midline move rostrally to occupy the most rostral portion of the primitive streak, with precursor cells located more laterally moving to a midline position to replace the midline cells that have moved rostrally. In the second stage of their model, the new midline cells move rostrally to populate the caudal portion of the streak. Their model, however, would predict the formation of a linear incipient primitive streak, rather than a triangular one (that is, the actual shape of the incipient streak).

This study also demonstrates that subsequent to the establishment of the initial primitive streak, the precursor cells also participate in its progression until the definitive (stage 4) primitive streak is formed. This underscores the complexity of the process, which as suggested in previous studies, involves the accretion of cells to the growing rostral end of the primitive streak (Lawson and Schoenwolf, 2001), convergent-extension movements mediated by cell–cell intercalation (Lawson and Schoenwolf, 2001) and rostrocaudally oriented cell division (Wei and Mikawa, 2000).

The expression pattern of what we have described as group 1A gastrulation genes, namely Wnt 8c, Slug, Vg1, and Nodal (Lawson et al., 2001), seems to correlate with our current map of the origin of the primitive streak precursor cells and their rostral movement to establish the incipient streak. We have defined such genes as those that are expressed in the epiblast overlying Koller’s sickle and subsequently throughout the progressing primitive streak, except at its extreme rostral end (Vg1 is an exception to this exception, as it is expressed in the most rostral end of the progressing primitive streak). Our results thus provide additional evidence to support the suggestion that group 1A genes act in establishing the form and/or functional activities of the primitive streak (Lawson et al., 2001). An upregulation of the zinc-finger gene, Slug, along Koller’s sickle, for example, may initiate the transformation of epiblast cells in the precursor region to primitive streak precursor cells. Further evidence in support of the role of group 1A genes in primitive streak formation comes from studies that show that an ectopic expression of some of these genes, notably Vg1 (Shah et al., 1997) and ebordin (Streit et al., 1998), induces the formation of a primitive streak. The results of the this study provide an important foundation upon which future studies on the molecular mechanisms underlying primitive streak formation can be built.

**Origin and Definition of Hensen’s Node, the Organizer of the Avian Embryo**

It is well known that the rostral end of the avian primitive streak functions as an organizer when transplanted to ectopic sites (Smith and Schoenwolf, 1998). Previous studies have shown that Hensen’s node, the spherical swelling present at the rostral end of the fully elongated (stage 3d/4) primitive streak, arises from two populations of progenitor cells called central and posterior cells (Streit et al., 2000). We have expanded these findings to show that the posterior progenitor cells arise from only the midline of the epiblast overlying Koller’s sickle, and not from more lateral sickle cells, and that the central progenitor cells arise continuously during progression of the primitive streak (i.e., during stages 2–3c) from the epiblast just rostral to the elongating tip of the primitive streak (i.e., from the area we designate at each stage as area “a”). Additionally, we have tracked the two
populations of precursor cells throughout the process of primitive streak progression. Our results confirm the data of Streit and co-workers (2000) by directly demonstrating that the two populations of progenitor cells intermix to form the node during late progression, and we agree with their suggestion that the term Hensen’s node should be reserved for the structure formed by such intermixing.

MATERIALS AND METHODS

Fertilized chicken eggs were incubated at 38°C in humidified incubators to obtain embryos at stages XI–XIII (Eyal-Giladi and Kochav, 1976) or stages 2–3a (Hamburger and Hamilton, 1951; substaging of stage 3 embryos as described by Schoenwolf et al., 1992; also see Darnell et al., 1999). Culture dishes and embryos were prepared as described by Darnell and Schoenwolf (2000) for modified New (1955) culture. Embryos were injected with a mixture of 5-carboxytetramethylrhodamine, succinimidyl ester (CRSE, Molecular Probes Inc., Eugene, OR, USA) and 1,1′-diododecyl-3, 3′,tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes Inc.), as described previously (Darnell et al., 2000). The dye solution was injected with the aid of a Picospritzer II (General Valve Corp., Fairfield, NJ, USA) and a micromanipulator. All embryos were examined immediately after injection with fluorescence microscopy, and embryos in which injections were too large or too small, or were misplaced, were discarded.

Mapping Primitive Streak Precursor Cells

For mapping primitive streak precursor cells, two sets of experiments were done in embryos at stages XI–XIII. The first set (involving 55 embryos) was designed to assess the contribution of cells located in the midline of the epiblast overlying Koller’s sickle to the incipient primitive streak, and also to test the hypothesis that cells scattered randomly throughout the epiblast contribute to primitive streak formation. Small quantities of DiI/CRSE were injected at four sites, namely, the midline of the epiblast overlying Koller’s sickle, and the epiblast of the area pellucida at the 3, 9, and 12 o’clock positions (Fig. 1). Embryos were then reincubated for 5 h.

The second set (involving 65 embryos) of experiments was designed to determine whether the epiblast of Koller’s sickle contributes cells to the incipient primitive streak. The epiblast overlying Koller’s sickle was divided into rostral and caudal halves along its mediolateral extent. Three to eight injections of DiI/CRSE were placed in the epiblast overlying the caudal border of Koller’s sickle, additional injections with 5-, 6-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes Inc.) were placed in the epiblast overlying Koller’s sickle between the DiI/CRSE injection sites (Fig. 4a). In six additional embryos, DiI/CRSE injections were placed in the epiblast overlying the rostral border of Koller’s sickle.

Mapping Hensen’s Node Precursor Cells

For mapping Hensen’s node precursor cells, two sets of experiments were done in embryos at stages 2–3c. A total of 65 embryos was used for these experiments. In the first set, a mixture of DiI/CRSE was injected at area “a” (the epiblast just rostral to the primitive streak; Garcia-Martinez et al., 1993), whereas in the second set, the DiI/CRSE was injected at area “c” (the rostral end of the primitive streak; Garcia-Martinez et al., 1993) and an additional injection of CSFE was placed at area “a.” Embryos were reincubated for periods ranging from 4 to 15 h prior to fixation.

Immunocytochemistry

All embryos labeled with the DiI/CRSE solution were processed for immunocytochemistry as described by Patel and co-workers (1989), except that they were fixed with 4% paraformaldehyde in PBS and the peroxidase reaction was enhanced by the addition of 2% CoCl2 and Ni(NH4)2(SO4) per ml of DAB-PBT. The antibodies used were anti-rhodamine (rabbit IgG polyclonal, primary; Molecular Probes Inc.) and horseradish peroxidase-conjugated goat anti-rabbit IgG (secondary antibody; Boehringer Mannheim Corp., Indianapolis, IN, USA). The embryos were first examined as whole-mounts; selected ones were then processed for paraffin histology as described in the following section.

Paraffin Histology

Embryos for histology were dehydrated with an ascending graded series of ethanol up to 100% ethanol. They were taken through two 5-min changes of Histosol, infiltrated with Paraplast and embedded in fresh Paraplast. Sectioning was done at 10 μm.

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