The active migration of *Drosophila* primordial germ cells

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SUMMARY

We describe our analysis of primordial germ cell migration in *Drosophila* wild-type and mutant embryos using high resolution microscopy and primary culture in vitro. During migratory events the germ cells form transient interactions with each other and surrounding somatic cells. Both in vivo and in vitro they extend pseudopodia and the accompanying changes in the cytoskeleton suggest that actin polymerization drives these movements.

These cellular events occur from the end of the blastoderm stage and are regulated by environmental cues. We show that the vital transepithelial migration allowing exit from the gut primordium and passage into the interior of the embryo is facilitated by changes in the structure of this epithelium.

Migrating germ cells extend processes in different directions. This phenomenon also occurs in primary culture where the cells move in an unoriented fashion at substrate concentration-dependent rates. In vivo this migration is oriented leading germ cells to the gonadal mesoderm. We suggest that this guidance involves stabilization of states of an intrinsic cellular oscillator resulting in cell polarization and oriented movement.

Key words: primordial germ cells, cell migration, gonadogenesis, *Drosophila*

INTRODUCTION

In many invertebrate and vertebrate animals, the formation of primordial germ cells (PGCs) is spatially and temporarily separated from the somatic gonad (Nieuwkoop and Sutasurya, 1979, 1981). Often PGCs form early during development and display elaborate behavior including an active search for the somatic gonad. In *Drosophila melanogaster* PGCs form at the posterior pole of the embryo in a process driven by interaction of the centrosome, actin and myosin (reviewed in Schejter and Wieschaus, 1993). Initially, PGCs lie outside the blastoderm and during gastrulation are included in the amnio-proctodeal invagination. This invagination elongates during germ band extension to form the primordia of both the hindgut and posterior midgut (PMG), which develops at the distal, blind end of the invagination (reviewed in Skaer, 1993). By early embryonic stage 10, PGCs lie packed at the end of the PMG. Subsequently, they leave its lumen and associate with the somatic gonad.

These events were first described by Rabinowitz (1941) and Sonnenblick (1941). More recently further studies of PGC migration have used specific markers for the PGCs and vital dyes in living embryos (Hay et al., 1988; Jaglarz and Howard, 1994; Warrior, 1994). These studies show that the somatic tissue regulates the time of exit of the PGCs (reviewed in Wei and Mahowald, 1994).

Earlier morphological studies of this process have suggested either that exit occurs by formation of a discontinuity in the gut tissue or by PGCs insinuating themselves through the gut epithelium (Campos-Ortega and Hartenstein, 1985 and references therein). Here we resolve this question showing that there is a novel and specific interaction between the PGCs and the cells of the PMG and that the PGC transendodermal migration is permitted by changes in the structure of the PMG.

MATERIALS AND METHODS

For light and electron microscopy (EM), embryos were collected at 25°C on apple juice plates and staged under oil. After chorion removal in 50% commercial bleach (Clorox), the embryos were extensively washed with distilled water and fixed at the interface of 50% glutaraldehyde and heptane for 10 minutes, transferred to sticky tape and the vitelline membranes removed manually with a tungsten needle under 2.5% glutaraldehyde in either 0.1 M phosphate or cacodylate buffer, pH 7.2. The embryos were fixed for 2 hours in glutaraldehyde and after several washes postfixed in 1% OsO₄ in the same buffer. After dehydration in a graded series of ethanol and acetone the embryos were embedded in Polybed 812 (Polysciences). Sections were stained with 1% methylene blue in 1% borax for light microscopy or contrasted with uranyl acetate and lead citrate, and examined in a JEM 1200 EX, electron microscope at 80 kV.

A total of 75 wild-type and mutant embryos at stages 9-11 were fixed and 5 per stage were picked for sectioning. They were examined first as semithin then ultrathin sections. In areas of interest, serial ultrathin sections were examined and interpreted in conjunction with data from light and confocal microscopes. PGCs are easy to recognize in the EM by lipid-deficient cytoplasm and the presence of polar granules (Allis et al., 1979).

For phalloidin (specific recognition of F-actin, Wulf et al., 1979) and immunostaining, the embryos were collected and staged as described above and fixed with 8% paraformaldehyde. After hand-peeling, the embryos were stained with anti-vasa antibody and...
fluorescein-conjugated secondary reagents (Jackson Immuno Res.). Following several washes in PBS/0.1% Triton X-100, the embryos were stained with rhodamine-labeled phalloidin (Molecular Probes) for 20 minutes, dehydrated in ethanol, mounted in methyl salicylate and examined with a Bio-Rad MRC 600 confocal microscope, equipped with a Krypton-Argon laser. At least 10 embryos were optically sectioned for each embryonic stage (stages 5-16).

**In vitro experiments**

Extracellular matrix proteins (EMPs) were obtained from Collaborative Biomedical Products and prepared according to the manufacturer’s recommendations. PGCs were removed from embryos at stages 4-7 with glass transplantation needles and placed on a coverslip (coated with the EMPs at different concentrations, Table 1) in a drop of Schneider’s medium (Sigma) supplemented with 10% bovine serum. The coverslip was then carefully transferred to a culture dish and more medium added. In the case of Matrigel, both the thin coating and the thick gel method were used. In the latter, PGCs were cultured inside the Matrigel. The cells were observed at 18°C and the images recorded using video equipment.

**Stocks**

hkb^A/TM3^b-gal; hkb homozygous embryos were identified by virtue of their lack of expression of β-gal and for EM examination by lack of the stomodeum and anterior midgut invaginations (Reuter and Leptin, 1994); lam A^6-26 A101/TM6β-gal; stg^TM3/TM3; osk^301/TM3. OreR was used as the wild-type stock.

**RESULTS**

Initially PGCs are almost perfectly spherical with a well-developed layer of cortical F-actin (Fig. 1A). Extensive changes in shape are first seen at the onset of gastrulation when PGCs become elongated and extend broad pseudopodia (Fig. 1B,C). These changes are accompanied by rearrangements of the actin cytoskeleton. The cortical layer of F-actin is pronounced in the body of the PGCs but thins around the pseudopodia (Fig. 1C). In the cytoplasm of both the pseudopodia and the body of the cells, there are punctate focal accumulations of F-actin. As they enter the lumen of the gut primordium during germ band elongation, PGCs are in close contact with each other and their pseudopodia are extended in different directions (Fig. 1D). As germ band elongation proceeds, they pack into the blind end of the PMG.

At this time both the hindgut and posterior midgut primordia form a single-layered epithelium, with cells closely apposed to each other (Figs 1D, 2A). In stages 9 and early 10 in the apical region of this epithelium, two types of specialized contact are
Migration of Drosophila primordial germ cells observed: adherens junctions (in the form of zonula adherens) and gap junctions (Fig. 2D). There are also distinct surface specializations of this epithelium. The luminal surface of the cells of the hindgut primordium are smooth or have only very short projections. In contrast, the luminal surface of the cells of the PMG is characterized by the presence of up to 2 μm long cytoplasmic protrusions which contact PGCs (Figs 2B, 3A,B). These cytoplasmic ‘arms’ contain axial microtubules (Fig. 3B). Fine intercellular spaces between endodermal cells are observed. (Figs 2B, 3A).

At the extended germ band stage, PGCs are found inside a cup-shaped formation of endodermal cells in the distal part of the PMG and are separated from each other by cytoplasmic extensions of the somatic cells (Fig. 3C). At this time the PGCs show a characteristic ‘tear drop’ form, with the side of the PGCs in close contact with the surrounding somatic cells being broad and rounded.

Following this stage, there is a drop in the F-actin level in the apical region of the endodermal cells and the distribution of cellular junctions changes (Fig. 1E). Intercellular spaces between neighboring cells become more pronounced (Fig. 2C) and adherens junctions no longer form continuous belts around the cells apices (Fig. 2E).

The transendodermal migration occurs in several places in the blind region of the PMG (Fig. 1E). During this transit, cytoplasmic processes of the PGCs are found in contact with each other (Fig. 1F,G). F-actin is variably distributed in the PGCs in the form of rather inconspicuous fibers, which are best seen when several optical sections are stacked together (Fig. 1G).

PGCs maintain numerous contacts with the surrounding endodermal cells as they pass between them (Fig. 4A). In the areas of close membrane apposition, no intercellular space is visible (Fig. 4B). In 4 out of 5 cases, the endodermal cells and their nuclei in the immediate vicinity of a PGC pseudopodium assume a concave shape (Fig. 4A).

After their exit, PGCs migrate on the endoderm and enter the mesoderm. During migration both on the endoderm and mesoderm, about 20% of PGCs are found with two or three pseudopodia extended in different directions (Fig. 5A-C). In every examined embryo (stage 10-12), we find 3 or 4 PGCs with F-actin arranged in a ring of dots at the base of the pseudopodia and a similar number of cells with aggregates of F-actin inside the processes (Fig. 5D,E).

Finally, as the PGCs meet the gonadal mesoderm and condense into a gonad, they once again show a spherical form and pseudopodia are no longer observed (Fig. 5F). Interestingly, PGCs not included in the forming gonads retain pseudopodia (Fig. 5F).

**Mutants studies**

The beginning of the migration of PGCs through the PMG coincides with changes in the F-actin distribution and the ultrastructural organization of the endodermal epithelium. To ask if the migrating PGCs induce these changes, we examined two mutants: *oskar*301 (*osk*) and *huckebein* (*hkb*). In the first mutation, PGCs are absent (Lehmann and Nüsslein-Volhard, 1986); in the second, changes in the gut primordium prevent exit (Jaglarz and Howard, 1994; Warrior, 1994).

Initially, the PMG of *osk* embryos shows a similar pattern of phalloidin staining to wild type: the apical region of the...
epithelium is intensely stained (not shown). However, as germ band elongation proceeds this staining is reduced in the most distal region of the PMG, although it remains strong in the hindgut primordium and proximal part of the PMG (Fig. 6A). In addition, cells in the blind region of the PMG extend cytoplasmic processes similar to those in wild-type embryos (Fig. 7A). Adherens junctions no longer form continuous belts around the apical regions of all the endodermal cells and intercellular spaces are observed between cells (Fig. 7A).

In hkb embryos, PGCs do not leave the gut primordium at stage 10 and remain inside after germ band retraction (Fig. 6B, C). In contrast to wild-type, the apical regions of the cells of the gut primordium are intensely stained with phalloidin throughout germ band extension (Fig. 6B) and retraction (Fig. 6C). Zonula adherens are well developed between these cells (Fig. 7B). Only fine cytoplasmic processes, which never exceed 400 nm, are observed on the apical surfaces. Interestingly, PGCs in the lumen of the mutant gut primordium are in contact with each other but they never show elaborate interactions with the somatic cells (Fig. 7B). In neither osk nor hkb mutants are the cup-shaped cells present.

Prior to PGC exit cells of the PMG undergo the 14th cycle of mitotic divisions (Foe, 1989). If this is necessary for the proper differentiation of the gut primordium the PGCs will not exit until after this division. To investigate this possibility, we examined string (stg) mutants, which never undergo division 14 (Edgar and O’Farrell, 1989). In stg embryos PGCs leave the PMG at the same time as in the wild-type and no significant differences in their migration to the gonadal mesoderm were observed. We conclude that the cell cycle clock does not drive the changes necessary for germ cell exit from the PMG.

Fig. 3. (A) At the onset of penetration of the PMG epithelium, both the body and a cytoplasmic projection (small star) of a PGC are found in contact (arrows) with the cytoplasmic ‘arms’ of somatic cells. Arrowhead, adherens junction; m, mitochondria; n, PGC nucleus; large star, intercellular space between endodermal cells. Bar, 1 μm. (B) Higher magnification of a PGC in contact (arrows) with the cytoplasmic extension of an endodermal cell. Arrowheads, microtubules; m, mitochondria. Bar, 400 nm. (C) Different steps of the PGC interaction with endodermal epithelium. Note a concave shape of the somatic cells and cytoplasmic processes (arrows) separating PGCs. Asterisks, PGCs; curved arrows, areas of contact between PGCs and the endodermal cells. Bar, 2 μm.
Behavior of PGCs in primary culture

In order to determine if PGCs could be motile outside the developing embryo, they were removed prior to gastrulation, placed in a simple primary culture and their behavior examined using video. These studies follow earlier work of Allis et al. (1979) who showed that PGCs can be successfully cultured in vitro for a short period of time and after transplantation into embryos are able to give rise to functional germ cells. Here we demonstrate that these cells not only adhere to all substrata they were presented with (Table 1), but also extend cytoplasmic processes and translocate significant distances. In contrast, blastoderm cells taken from the mid-dorsal region of a stage 7 embryo and cultured under the same conditions do not translocate. They do however attach to the surface and extend processes (not shown).

The behavior of the PGCs in culture depends primarily on the nature of the substratum and its concentration. There is a threshold of concentration above which PGCs adhere too strongly to the substratum and remain immobile (Table 1). Although PGCs are motile on all substrata, only laminin and Matrigel produced morphology similar to that in fixed embryos (Fig. 8). The migration on all substrata was unoriented. If migrating cells contact each other, they crawl over each other and continue their migration as cell clusters (Fig. 8D-F). Apparently these cells have higher affinity to each other than to the substratum.

The migration of PGCs in vitro consists of four steps. First, there is an initial quiescent period that is extended in cells removed from early stage 4 embryos, which undergo divisions before displaying motility. Subsequently, PGCs randomly extend

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The images were recorded every 5 seconds and the mean velocity of migrating PGCs was measured for three PGCs from three repetitions of each experiment.
Fig. 5. Optical sections through embryos stained with anti-vasa antibody (green) and Rh-phalloidin (red). (A-C) PGCs migrating on the endoderm. Differences in F-actin distribution coincide with variable morphological forms of the PGCs. Arrows, local accumulations of F-actin. (D) Stack of four optical sections (0.5 μm apart) showing aggregates (arrowhead) of F-actin arranged in a ring around the base of the pseudopodium. Two of the sequential sections (red channel) shown at right. (E) PGCs migrating in the mesoderm. Arrows, different localizations of F-actin. (F) Gonad condensation. Only PGCs incorporated into the forming gonad (asterisk) become round, the remaining cells continue to show pseudopodia (arrows). Bars, 10 μm; bar in C, 5 μm.

Fig. 6. Optical sections through mutant embryos stained with anti-vasa antibody (green) and Rh-phalloidin (red). (A) The gut primordium of an oskar301 embryo at stage 10. Note reduced F-actin staining in the distal part of the PMG, although the staining in the hindgut primordium (hp) and proximal part of the PMG (arrowheads) remains strong. Bar, 20 μm. (B,C) The gut primordium of a huckebein embryo at stage 10(A) and 13 (B). Intense F-actin staining in the apical region of the cells of the gut primordium (arrowheads) continues throughout different stages of development. Arrows, aggregates of F-actin. Bars, 10 μm.

Fig. 7. (A) The PMG of an oskar301 embryo at early stage 10. Note the lack of the PGCs in the lumen of the PMG. The apical regions of the most distal endodermal cells are extended into cytoplasmic projections (stars, some projections in cross section) similar to those observed in wild-type embryos. Arrowheads, adherens junctions; asterisk, intercellular bridge; open stars, intercellular space; l, lipid droplets; m, mitochondria. (B) The gut primordium of a huckebein embryo at late stage 10. In contrast to wild-type embryos at this stage, well developed zonula adherens (arrowheads) continue to be present between the closely apposed somatic cells. PGCs are in close contact with each other and do not interact with the somatic cells. m, mitochondria; n, PGC nucleus. Bars, 1 μm.
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small projections some of which attach to the substratum. This is followed either by rolling or cytoplasmic flow into one broad pseudopodium, resulting in polarization of the cell (Fig. 8). PGCs differed significantly in their migratory behavior. For instance cells 5 and 6 did not translocate. Interestingly, these cells were in contact with cellular debris (star). In contrast, cell 1 migrated a long distance (compare its position in A, C and F) and came in contact with cell 2 (D). After that encounter these two cells crawled over each other and continued to migrate together (E,F). Bar, 20 μm.

Fig. 8. (A-F) Frames from a time-lapse analysis of PGC behavior on laminin (3.0 μg/cm²), in vitro. Numbers in the upper right hand corner, time (hours : minutes) from the beginning of the experiment; numbers on the cells, the same cell over the course of the experiment. For about an hour the PGCs remained spherical and a cell divided and produced cells 6 and 7 (A). Over the next 6 hours PGCs extended pseudopodia (e.g. cells 1,2,4,7 in B and 1,3 in C) and translocated variable distances. Since PGCs migrated back and forth the cell position does not reflect the actual distance of migration. PGCs differed significantly in their migratory behavior. For instance cells 5 and 6 did not translocate.

PGCs frequently change the direction of migration and move in a random fashion. They are capable of translocating in that way for relatively long distances (100 μm or more) with variable speed (for instance 0.2 μm/minute on poly-D-lysine or 2.4 μm/minute on laminin, see Table 1). When cultured inside Matrigel, PGCs that are embedded in groups crawl over each other and will exchange places. However they do not penetrate the gel or disperse.

The possibility that laminin may be necessary for migration in vivo was investigated by examining the zygotic phenotype of a null mutant for chain A of Drosophila laminin (for review of Drosophila extracellular matrix proteins see Hortsch and Goodman, 1991). In these mutants, an increased number of PGCs were not incorporated into the gonads and remained dispersed throughout the embryo (Fig. 9). The defect in PGC migration in the laminin mutant occurs late during germ band retraction and this late effect may be a consequence of the persistence of maternal laminin.

DISCUSSION

Our data allow us to conclude three things about the migration of Drosophila PGCs. First, that these cells are capable of

Fig. 9. A dorsal view of a stage 14 laminin A mutant embryo stained with anti-vasa antibody. Only some PGCs are included in the gonad (arrow), the remaining cells are dispersed in the posterior part of the embryo. Bar, 50 μm.

Fig. 10. Schematic representation of PGC (red) migration through the endodermal epithelium of the PMG. Changes occur from left to right. Arrow, adherens junction.
moving on their own, that they are likely to do this in vivo and that the underlying cytoplasmic events are associated with actin polymerization. Secondly, that the orientation of the PGC migration is likely to be achieved by stabilization of particular quasi randomly generated cytoplasmic extensions and not simply by instructive cues driving de novo formation of these extensions. Finally, we show that the transendodermal migration from the extraembryonic gut lumen to the interior of the embryo is mediated by remodeling of the endodermal epithelium and is not driven simply by an activation of the PGCs motile machinery, which has by this time been active for several hours.

**Drosophila germ cells move actively soon after their formation**

Our culture experiments show that the PGCs can migrate by the extension of pseudopodia and respond to different substrata with different degrees of activity. Furthermore, this migration is specific in the sense that blastoderm cells do not move under the same conditions. Two pieces of evidence strongly argue that these migratory properties are also manifested in vivo. First, blastoderm cells do not migrate after transplantation to the PMG whilst germ cells do (Jaglarz and Howard, 1994). Secondly, similar migratory morphology is seen in fixed embryos. Interestingly, this migratory activity begins at the blastoderm stage, well before their exit from the PMG (see also Counce, 1963; Jura, 1964; Rabinowitz, 1941; Somenblick, 1941) and it is not the onset of migratory activity that initiates the transendodermal movement.

Although PGCs show migratory morphology from late blastoderm stage on and this activity could help these cells to move into the developing gut, we do not propose that this activity is solely responsible for this movement. In fact, blastoderm cells transplanted before gastrulation at the posterior pole of the embryo are included in the PMG as can inanimate objects like fragments of the transplantation needle (Jaglarz and Howard, 1994; Rabinowitz, 1941). However, in all of these cases PGCs were also present and it is possible that their activity pushed the transplants inside.

**Changes in the actin cytoskeleton**

Actin polymerization is involved in the formation of pseudopodia and movement of many cell types (reviewed in Condeelis, 1993; Stossel, 1993). Association of F-actin with adherens junctions is also well established (Fristrom, 1988). We have described extensive rearrangements of the actin cytoskeleton during *Drosophila* PGC migration. These modifications are quite variable in fixed material at any one stage of development, a feature that we attribute to the dynamic nature of these processes. There are also stage-specific changes in these cells, which we suggest reflect their interactions with different environments as they navigate to the gonad. The aggregates of phalloidin-stained material are of particular interest and punctate distributions of F-actin, very similar to those that we see in *Drosophila* embryos, have been reported in yeast, hyphae of filamentous fungi, murine macrophages and embryonic cells (Adams and Pringle, 1984; Amato et al., 1983; Lehtonen and Badley, 1980). Interestingly, aggregates of actin are usually associated with those parts of the cell that undergo elongation. In yeast dots of F-actin are concentrated in areas of incipient bud formation and elongation. Moreover, a ring of actin spots was found at the base of the forming buds (Adams and Pringle, 1984).

The randomly distributed dots and patches of F-actin may mark actin nucleation sites involved in driving the formation of pseudopodia. However, some of the actin patches that we describe may correspond to sites of close plasma membrane apposition between migrating PGCs and their environment.

**Interactions between endoderm and the germ cells**

We propose that PGCs in contact with each other in the developing gut actively search their environment by extending pseudopodia that eventually come into contact with cytoplasmic extensions of the somatic cells. These contain microtubules and may provide a rigid substratum that would guide PGCs centripetally. This initial movement however, would not result in transepithelial exit due to the presence of intact adherens junctions. These junctions are rearranged at the time of exit and intercellular spaces appear between endodermal cells. We suggest that this results in the formation of low-resistance paths that permit exit. Similar changes in the adherens junction distribution in the wild-type PMG have been reported by Tepass and Hartenstein (1994) and are most likely to represent the initial steps in the transition of the PMG epithelium into mesenchyme. These observations are schematically represented in Fig. 10.

There is a possibility that PGCs initiate the changes in the PMG. However, examination of *osk* mutants shows that they occur even when PGCs are absent. Note that, despite the loosening of the epithelium, PGCs do not penetrate the PMG freely and leave as single cells that are interconnected by cytoplasmic extensions. This interaction of migrating *Drosophila* PGCs with each other has not been previously reported and is reminiscent of the behavior of mouse PGCs where migrating cells link to each other by means of long cytoplasmic processes (Gomperts et al., 1994).

This transendodermal migration bears some similarity to the migration of leukocytes during inflammatory responses. The initial weak binding of the leukocytes to the surface of the endothelium is followed by sustained attachment and activation of the cells and only then the migration through the intercellular spaces of the endothelium can take place (reviewed in Springer, 1994).

PGCs always leave the gut primordium in a restricted area of the PMG. The behavior of PGCs in *hkb* embryos indicates that this area is defined by *hkb*, which encodes a transcription factor specifying the endodermal cells (Brömer et al., 1994). Lack of *hkb* product causes transformation of these cells into hindgut-like cells, which retain their epithelial structure. We propose that both the preferential adhesion of germ cells to the *hkb*-dependent epithelium and the changes seen as it develops are caused by *hkb* target genes, which directly modify cell structure and surface properties and have yet to be identified, at least in this context.

The regulation of PGC behavior by the endodermal tissue is not peculiar to *Drosophila*. In a frog (*Rana pipiens*), it has been shown that, although PGCs have inherent migratory properties, they do not leave endoderm until changes occur in their somatic environment (Subtelny and Penkala, 1984).

**The orientation of migration**

We have suggested that *Drosophila* PGCs extend processes in
a relatively random fashion. Consequently, the direction of migration is determined by which processes mature, something that is regulated by environmental factors. During migration, membranes of PGCs are found in close apposition with membranes of other cells. These contact zones lack any particular specializations suggesting that they are transient attachment points allowing the cells to explore their environment, making new contacts and releasing old ones as they propel themselves. During transendodermal migration orientation is clearly provided by the structure of the PMG. However, in the other movements of these cells, our ultrastructural studies do not identify guidance cues, which are likely to be evident only at the molecular level.

*Drosophila* PGCs in culture do not show contact paralysis of movement, a feature typical for many motile cells in vitro (Abercrombie, 1980). In this respect they behave similarly to other movements of these cells, our ultrastructural studies do not identify guidance cues, which are likely to be evident only at the molecular level.

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