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# Electron microscopic studies on the structure of motile primordial germ cells of *Xenopus laevis in vitro*

By JANET HEASMAN[1] AND C. C. WYLIE[1]

*From the Department of Structural Biology, St George's Hospital Medical School, London*

SUMMARY

Primordial germ cells (PGCs) of *Xenopus laevis* have been isolated from early embryos and kept alive *in vitro*, in order to study the structural basis of their motility, using the transmission and scanning electron microscope. The culture conditions used mimicked as closely as possible the *in vivo* environment of migrating PGCs, in that isolated PGCs were seeded onto monolayers of amphibian mesentery cells.

In these conditions we have demonstrated that:

(*a*) No significant differences were found between the morphology of PGCs *in vitro* and *in vivo*.

(*b*) Structural features involved in PGC movement *in vitro* include (i) the presence of a filamentous substructure, (ii) filopodial and blunt cell processes, (iii) cell surface specializations. These features are also characteristic of migratory PGCs studied *in vivo*.

(*c*) PGCs *in vitro* have powers of invasion similar to those of migrating PGCs *in vivo*. They occasionally become completely surrounded by cells of the monolayer and, in this situation, bear striking resemblance to PGCs moving between mesentery cells to the site of the developing gonad in stage-44 tadpoles.

We conclude that as far as it is possible to assess, the behaviour of isolated PGCs in these *in vitro* conditions mimics their activities *in vivo*. This allows us to study the ultrastructural basis of their migration.

## INTRODUCTION

Cell movement and interaction are key features of early morphogenesis. The contractile and structural elements which underlie these phenomena have been studied here in the primordial germ cells (PGCs) of the anuran amphibian *Xenopus laevis*. At stage 43–44 of development (Nieuwkoop & Faber, 1956), these cells migrate individually from the embryonic gut to the root of the dorsal mesentery, and thence laterally across the dorsal abdominal wall to the site of formation of the gonadal ridge (Kalt & Gall, 1974; Whitington & Dixon, 1975; Wylie & Heasman, 1976). While several light and electron microscopic studies of this process have described the morphology of primordial germ cells (Mahowald & Hennen, 1971; Kalt, 1973; Wylie & Heasman, 1976),

[1] *Authors' address:* Department of Structural Biology, St George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE, U.K.

they have been restricted to observations on the characteristic organelles – 'nuage' material, mitochondrial clouds, multi-lobed nuclei, yolk platelets, Golgi apparatus, etc. As the morphology responsible for the migratory activity of germ cells is difficult to study *in vivo* (Heasman, Mohun & Wylie, 1977), we have used isolated germ cells, cultured *in vitro* by a method published elsewhere (Wylie & Roos, 1975). Germ cells have been shown to move *in vitro* on monolayers of amphibian cells, by a mechanism involving alternate phases of elongation and contraction and the extension of filopodial processes (Heasman *et al.* 1977).

In this paper we seek firstly to compare the ultrastructure of PGCs *in vitro* with that of PGCs in their natural environment in the dorsal mesentery. Secondly, a combination of scanning and transmission electron microscopy is used to describe the features of germ cells which form the structural basis of their movement over, and interaction with the cellular monolayer. Finally, evidence is presented that these features are also involved in the migration of germ cells along the dorsal mesentery during embryogenesis.

## MATERIALS AND METHODS

### 1. *Preparation of germ cells*

Fertilized *Xenopus laevis* eggs were obtained by hormonal stimulation of pairs of adult toads using chorionic gonadotrophin. Embryos were allowed to develop to stage 43–44 at which time PGCs were isolated from them by a method described previously (Heasman *et al.* 1977). Disaggregated germ cells were seeded individually onto monolayers of amphibian cells in 70% Liebovitz medium.

### 2. *Preparation of cellular substrates*

Monolayers of amphibian cells were obtained by the culture of mesentery dissected from adult *Xenopus laevis*. Small pieces of mesentery were held down with glass rings in tissue culture dishes ('Lux Permanox' dishes, suitable for electron microscopic processing), containing 70% Liebovitz medium, 20% distilled water, 10% foetal calf serum, gentamycin (50 $\mu$g/ml) and fungizone (2·5 $\mu$g/ml). Outgrowth from the tissue results in a monolayer of epithelial-type cells, which is a suitable cellular substrate for PGCs.

### 3. *Preparation of tissue for electron microscopy*

After seeding onto monolayers of cells, PGCs required 2–3 days before they attached and moved on these surfaces. Once their characteristic movement and appearance had been established (Heasman *et al.* 1977), PGCs were fixed using 2·5% glutaraldehyde in 0·2 M phosphate buffer, pH 7·2, at room temperature. Material was postfixed with 2% $O_sO_4$ in 0·2 M phosphate buffer, dehydrated and embedded in Araldite resin. Embedded germ cells were identified among the cells of the monolayer using a dissecting microscope, and their

position was marked for correct trimming of the Araldite blocks. Ultrathin sections were cut, using a Sorvall microtome, and these were stained with lead citrate and analysed with a Philips 301 electron microscope.

For scanning electron microscopy, PGCs were seeded onto amphibian monolayers grown on glass coverslips. After fixation and dehydration as above, the alcohol was substituted through a graded series of Freon 113, and the material was critical-point-dried from liquid $CO_2$ in a Polaron critical point drying apparatus. The specimens were mounted on aluminium stubs coated with gold in a sputter-coating unit, and examined with a Coates and Welter field-emission scanning electron microscope. While magnifications are included on scanning electron micrographs used in the text, they are intended as a guide-line only, as the three-dimensional nature of the photographs renders such measurements inaccurate.

For examination of the structure of germ cells *in vivo*, stage-43–44 *Xenopus laevis* tadpoles were fixed as above, dehydrated and embedded in Araldite resin. Gold sections of PGCs on the mesentery and posterior body wall of tadpoles were stained with lead citrate and examined with a Philips 301 electron microscope.

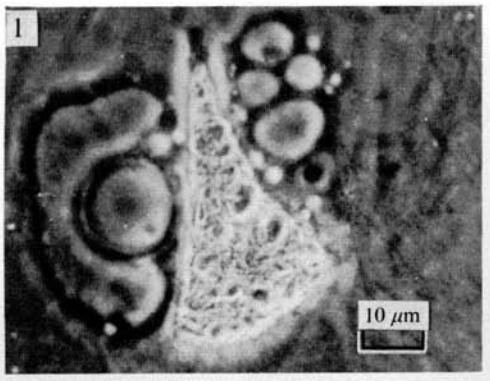
## RESULTS

Figures 1 and 2 are phase-contrast light micrographs of PGCs moving on a cellular substrate. Their elongation and distinct single filopodia can be seen. Figure 3 shows the same PGC photographed for Fig. 1, seen under the scanning electron microscope. The prominent bulges under the surface membrane, which make the PGCs easily recognizable, are due to the yolk platelets, seen clearly under phase-contrast (Fig. 1).

Figure 4 is a scanning electron micrograph of a PGC during the rounded-up phase of its activity. The cell bulges much more prominently from the monolayer (in this case a broken piece of Pasteur pipette overgrown by amphibian cells), and both yolk platelets (Y) and lipid droplets (L) can be clearly identified, protruding upwards underneath the cell membrane. Even in the rounded state, germ cells extend small cytoplasmic processes along the substrate (arrow).

### *The ultrastructure of PGCs on cellular substrates*

After the establishment of their normal motile behaviour on cellular substrates, germ cells were fixed in both the elongated and rounded state and processed for electron microscopy. Figure 5 is an electron micrograph of an elongated PGC, cut in longitudinal section, and lying on several layers of adult mesentery cells. PGCs *in vitro* have the same characteristic features which have been demonstrated previously in the germ cells of fixed stage-44 tadpoles (Wylie & Heasman, 1976). The cells are packed with yolk platelets and lipid droplets, and the intervening cytoplasm is considerably more electron dense than that of the underlying somatic cells (Fig. 5). This electron density is due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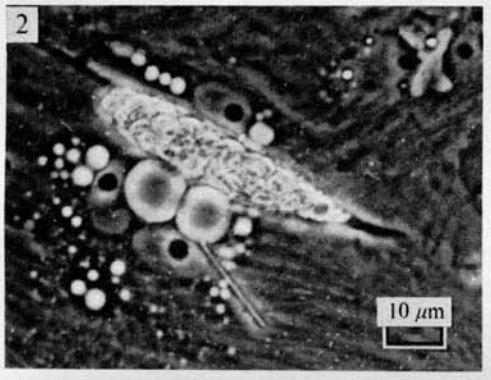
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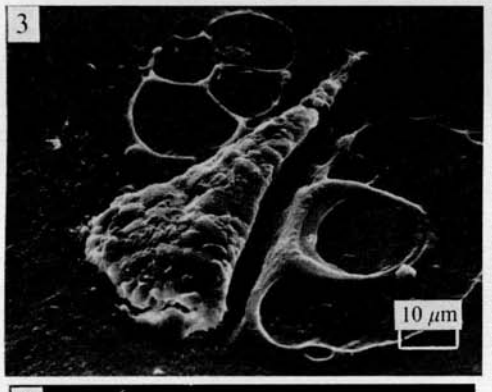
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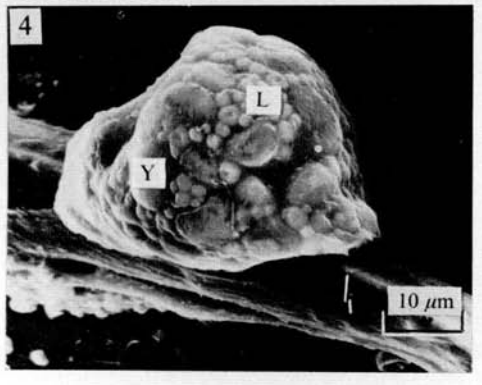
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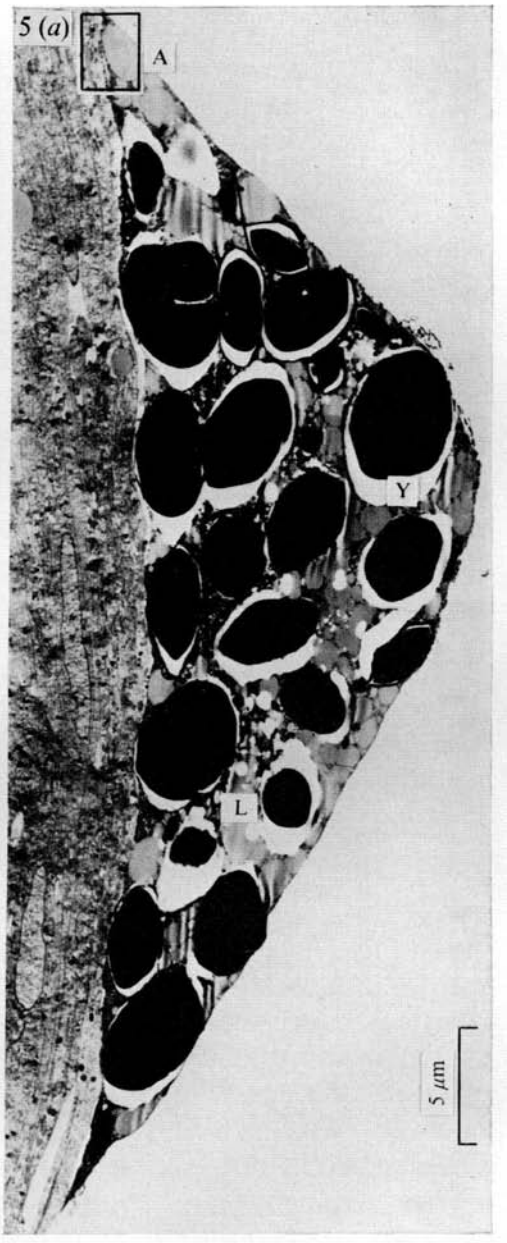
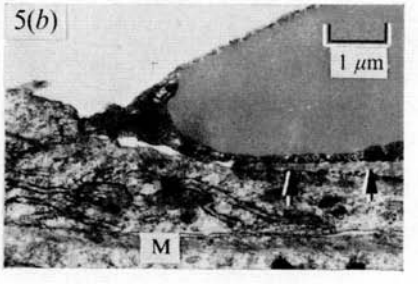
L
Y
10 μm


5 (a)
A
Y
L
5 μm


5(b)
1 μm
M

to an unusually large number of free ribosomes which are uniformally distributed throughout the cytoplasm, (see below). Other features which are seen in cultured PGCs are shown in Figs. 6 and 7. They include:

(*a*) clusters of mitochondria of a characteristic structure, which are often associated with vesicles of smooth endoplasmic reticulum (Fig. 6);

(*b*) a well-developed Golgi apparatus (Fig. 6);

(*c*) a multilobed nucleus (Fig. 6);

(*d*) microvilli (Fig. 7).

We have previously established that these are characteristic features of PGCs *in vivo* (Wylie & Heasman, 1976).

Thus it appears that isolation and culture of PGCs does not disrupt their ultrastructure, which is, in fact, often more successfully preserved in fixed, cultured material than in fixed whole tadpoles. Even after 7–10 days of culture conditions, no alteration of these features is seen (Fig. 14), suggesting that this artificial environment can support normal germ cell function for a considerable time.

### *Ultrastructural features involved in germ cell movement* in vitro

The structural basis of the movement of PGCs on cellular substrates has been studied using scanning and transmission electron microscopy. Particular attention has been paid to:

(*a*) the presence of a filamentous substructure;

(*b*) the mode of attachment and interaction with the substrate during locomotion;

(*c*) cell processes involved in movement.

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FIGURES 1–5

Fig. 1. Phase-contrast light micrograph of PGC moving on a cellular substrate. Note the single filopod extending from one pole, and refractile yolk platelets in the PGC cytoplasm.

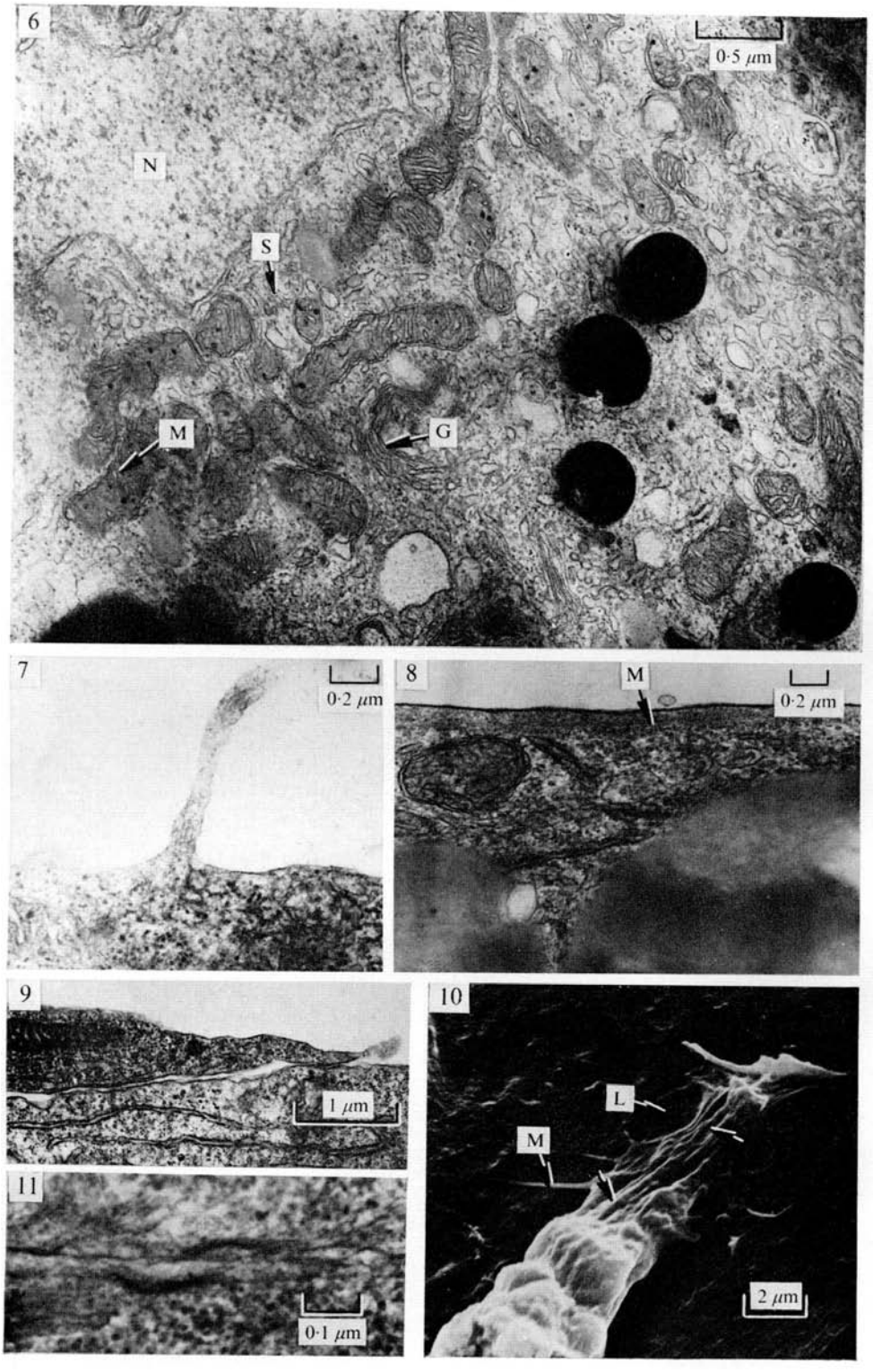
Fig. 2. Phase-contrast light micrograph of a PGC which has extended a long and well defined single filopod over the cellular substrate.

Fig. 3. Scanning electron micrograph of the same PGC as photographed for Fig. 1.

Fig. 4. Scanning electron micrograph of a PGC during the rounded up phase of its activity. This cell is attached to a broken piece of Pasteur pipette covered in cells. Yolk platelets (Y) and lipid droplets (L) protrude upwards underneath the cell membrane, and small cytoplasmic processes extend along the substrate (arrow).

Fig. 5. Electron micrograph of an elongated PGC, cut in longitudinal section, and lying on several layers of adult mesentery cells. Note the high electron density of PGC cytoplasm compared to that of the underlying cells. Y = yolk platelet, L = lipid droplet.

Fig. (5*a*). High power view of region A of Fig. 5 to show the close association of this pole of the cell with the underlying cell. The area of contact is stained with ruthenium red (arrow). Notice the well-defined bundle of microfilaments (M) in the cytoplasm of the substrate cell.


6
0·5 μm
N
S
M
G
7
0·2 μm
8
M
0·2 μm
9
1 μm
10
L
M
2 μm
11
0·1 μm

(*a*) *Filamentous substructure*

In contrast to the well-developed bundles of microfilaments commonly seen in the somatic cells of the substrate (Fig. 5*a*), PGCs do not possess a very obvious, organized microfilamentous substructure. However, examination of the germ cell surface reveals an underlying network of filaments (Fig. 8). By careful measurement using high power electron micrographs, these filaments are estimated to be approximately 6·5 nm in diameter. This network forms a continuous sheet over the upper surface, but is more difficult to define on the surface in contact with the substrate. Microfilaments are most frequently seen aligned longitudinally, when elongated germ cells are sectioned transversely, suggesting that they are running at right-angles to the long-axis of the cell. Cell processes and microvilli also contain microfilaments, which lie in the long axis of the protrusion (Figs. 7, 12*a*). The general organization of this filamentous network in PGCs *in vitro* mirrors very closely their arrangement in PGCs sectioned in whole tadpoles (Wylie & Heasman, 1976).

Microtubules have not been seen in the germ cell cytoplasm.

(*b*) *Attachment of germ cells to the substrate*

After 2–3 days in culture, germ cells cannot be removed from their substrate by washing, even though they are free to move over the underlying cells. This suggests that they perform a continual process of cell junction formation and breakage, which never leaves the cell unattached to the substrate. In scanned specimens, PGCs appear to be firmly attached both at their filopodial extremities and also at the opposite, posterior pole (Fig. 3). The lateral surfaces are rounded, overhanging and unattached. The structure of these attached sites has

FIGURES 6–11

Fig. 6. Electron micrograph to show characteristic cytoplasmic structures of PGC *in vitro*. Note the Golgi body (G), clusters of mitochondria (M), smooth endoplasmic reticulum (S) and a lobe of nucleus (N).

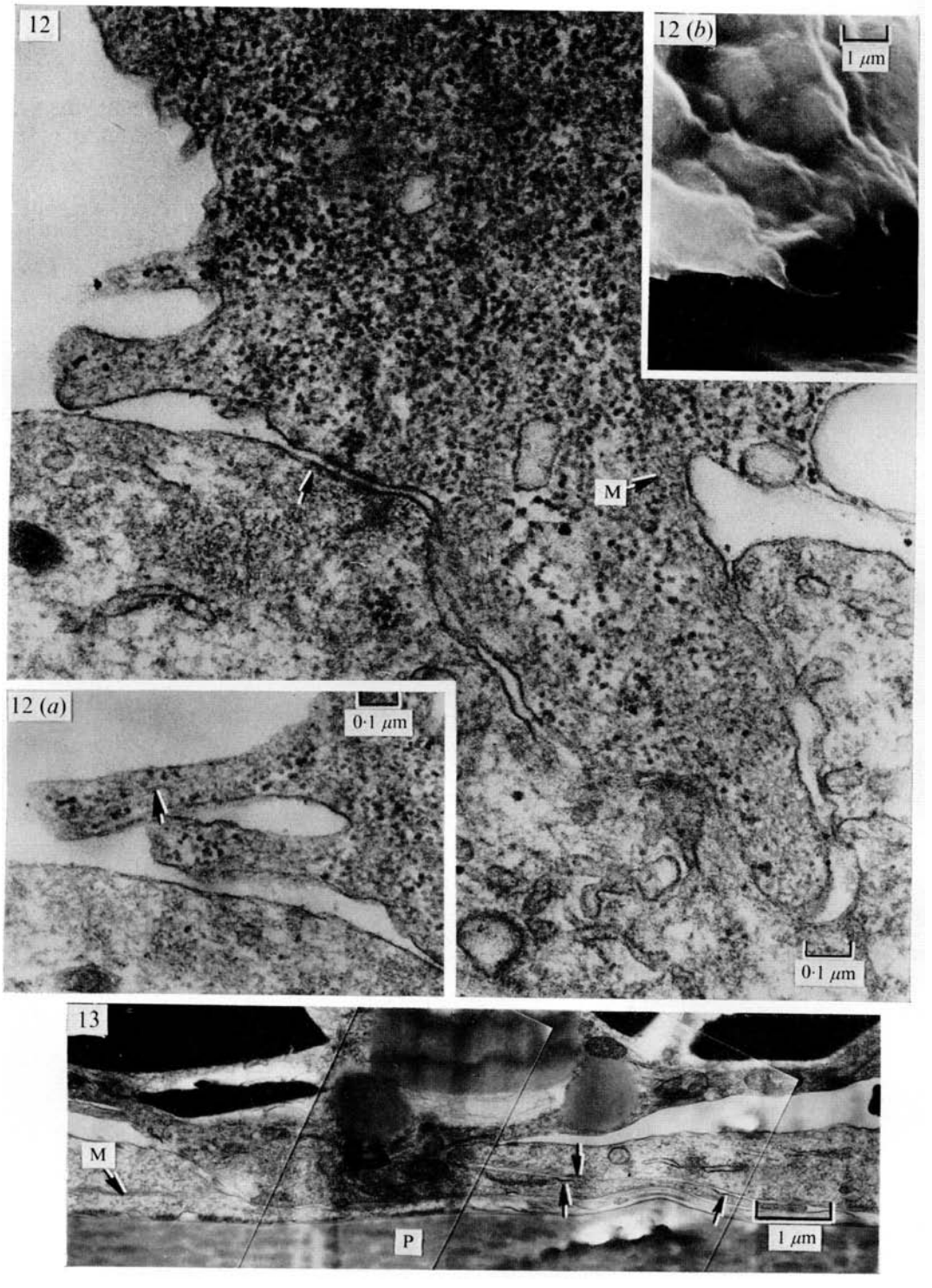
Fig. 7. A microvillus protruding from the surface of a PGC. The microfilamentous substructure is not well-defined here.

Fig. 8. The upper surface of a PGC to show the microfilamentous substructure (M) beneath the cell membrane. This area is not penetrated by cell organelles such as mitochondria, granular e.r., lipid droplets and yolk platelets.

Fig. 9. Electron micrograph of a filopod cut in longitudinal section. Mitochondria, ribosomes and agranular endoplasmic reticulum are found in the cytoplasm. Note the electron density of the cytoplasm compared to that of the underlying cell.

Fig. 10. High power view of filopod of PGC scanned in Fig. 3. The main filopodial trunk has microspikes (M) and lamella-like areas (L) protruding from it. Note the longitudinal ridges (arrows) in the upper surface of the filopod.

Fig. 11. High power electron micrograph to show a membrane specialization between a PGC (below) and somatic cell (above). Note the numerous free ribosomes in the PGC cytoplasm.


12
12 (b)
1 μm
M
12 (a)
0·1 μm
0·1 μm
13
M
P
1 μm

been examined by transmission electron microscopy of sectioned material. Figure 5(*a*) shows the posterior pole of a PGC in close association with the underlying cell; in this case the area is stained with ruthenium red. The filopodial tip, although not the main body of the filopod, is also in close contact with the substrate. It is difficult to analyse the degree of contact between germ cell and somatic cell, as fixation undoubtedly causes some cell retraction. However, in comparison with the close association between adjacent somatic cells, the body of the germ cell is only in close contact with the substrate over small distances (Fig. 5). These points of association remain intact even after considerable fixation contraction. Areas of attachment are often characterized by a 20 nm intercellular gap, an increase in the electron density of the adjacent cell membranes, and an increase in the amount of intercellular material in this site (Fig. 12, arrow). They vary considerably in length but the two opposed membranes are never in contact. A particular membrane specialization, which may be involved in adhesion, has been seen occasionally between germ cells and somatic cells (Fig. 11). This consists of an electron-dense concavity of approximately 120 nm in length, which is present on one or both of the opposed cell surfaces. They have never been seen on the free upper surface of PGCs *in vitro*, but are present between germ cells and mesentery cells in fixed, stage-43–44 tadpoles (Fig. 17).

(*c*) *Cell processes*

The movement of PGCs *in vitro* is associated with the extension of a variety of cell processes. The most obvious type of process is the single long filopod which is often extended from one pole of an elongated germ cell (Figs. 1–3). Cinemicrography of migrating PGCs indicates that such filopodia may be extended and withdrawn rapidly, or may persist for some hours as the pole to which the trailing cytoplasm is gradually drawn. They vary considerably in length, the maximum extension so far recorded being 10 $\mu$m (Fig. 2). Figure 9

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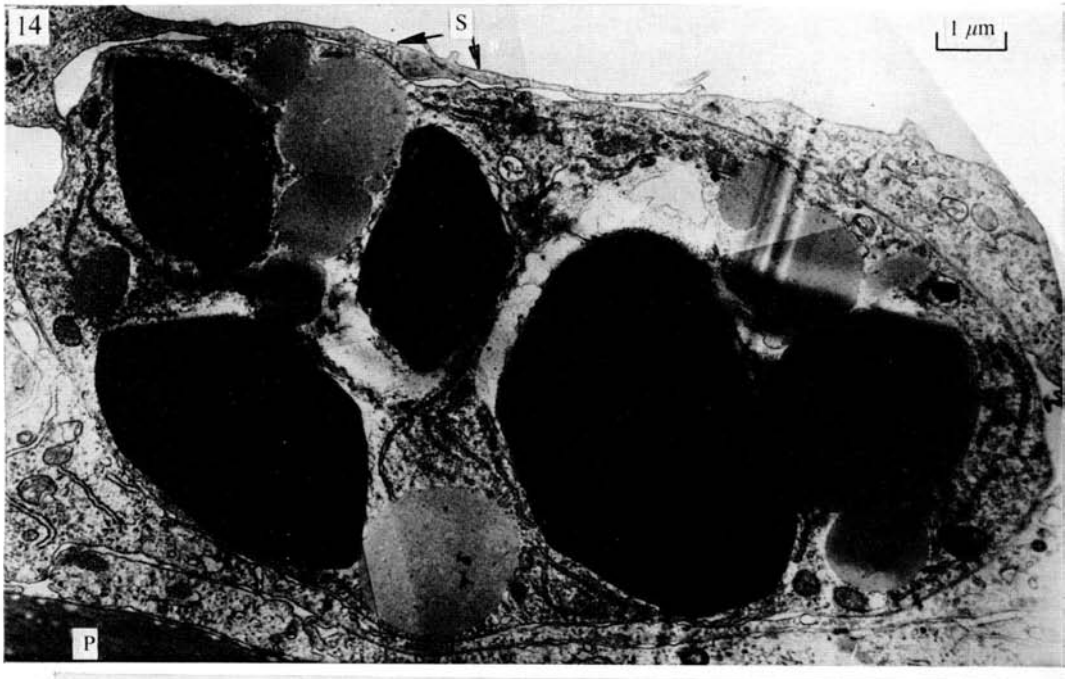
FIGURES 12 AND 13

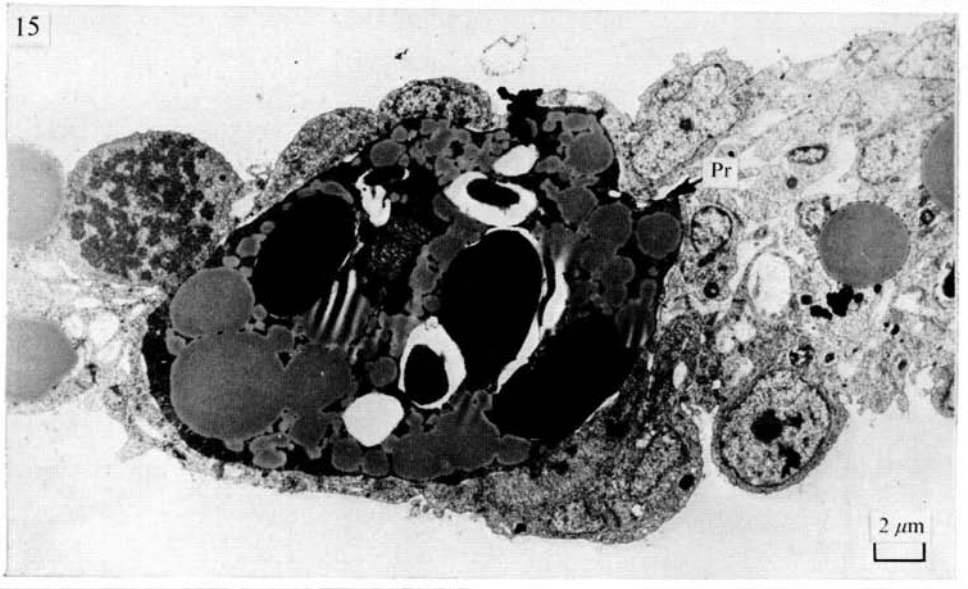
Fig. 12. A broad process of a PGC invaginating the surface of the underlying cell. The process has a rather indistinct substructure of microfilaments (M) and contains many free ribosomes. Note the cell surface specialization (arrow) between the PGC and somatic cell.

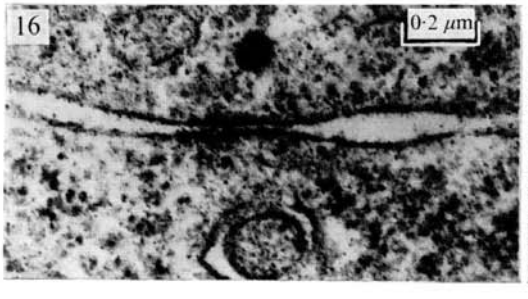
Fig. (12*a*). A high power view of the same blunt processes, as seen in Fig. 12, from a nearby section which cuts one process in the mid saggital plane. A microfilamentous substructure can be distinguished in the protrusions (arrow).

Fig. (12*b*). Scanning electron micrograph to show blunt processes protruding from a rounded PGC.

Fig. 13. A process extending from the undersurface of a PGC and penetrating between adjacent cells of the substrate. The cellular monolayer is attached to tissue culture plastic (P). Note microfilamentous bundles (M) in the somatic cell cytoplasm and cell surface specializations (arrows) between the PGC process and the adjacent cells.


14
S
1 μm
P


15
Pr
2 μm


16
0·2 μm


17
0·2 μm

is an electron micrograph of a filopod cut in longitudinal section. It contains no remarkable ultrastructural features, but appears to be a simple outpushing of the normal cytoplasmic material containing mitochondria, granular and agranular endoplasmic reticulum, and many free ribosomes. Yolk platelets, the nuclear membrane and Golgi apparatus do not extend into filopodia. A microfilamentous substructure is not easily distinguished.

Under the scanning electron microscope, filopodia are found to have a more complex structure than we formerly supposed (Fig. 10). They are seen as flattened, yolk-free cytoplasmic extensions with a number of subsidiary short spikes branching from the main body, extending for considerable distances over the substrate. Between microspikes there are membranous lamella-like areas (L). The upper surface of the filopod is thrown into parallel longitudinal ridges (arrows), which may correspond to the submembranous arrays of microfilaments seen occasionally in electron micrographs.

A second type of process which is characteristic of motile germ cells is shown in Fig. 12(*a*). Several such processes are often found protruding laterally from both rounded and elongated PGCs. They are shorter than filopodia, have rounded rather than flattened tips, and are not restricted to the substrate surface. They are distinct from microvilli, which are of a more constant diameter and length.

Processes of a comparable size and shape have been seen in scanned specimens (Fig. 12*b*), where they are particularly numerous at the free edges of rounded PGCs.

On several occasions we have observed a very interesting variety of cell process, which is extended from the undersurface of the PGC, and appears to invade the underlying monolayer. In one case (Fig. 12), this broad pseudopod invaginates the surface of one somatic cell, while on other occasions processes appear to force their way between and beneath adjacent cells (Fig. 13). Notice in Fig. 12 the large number of ribosomes characteristic of PGC cytoplasm, which are particularly well displayed here.

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FIGURES 14–17

Fig. 14. PGC which has been in culture for 10 days and is completely surrounded by somatic cells of the substrate. The cells are lying on tissue culture plastic (P). Note the overlapping somatic cell processes (S) covering the upper surface of the PGC.

Fig. 15. PGC fixed *in vivo* during its migration along the mesentery of stage-44 tadpole. Note the electron density of PGC cytoplasm *in vivo*, and the blunt process (Pr) extending from the leading end of this PGC.

Fig. 16. Cell surface specialization between a germ cell (above) and mesentery cell (below) fixed *in vivo* in a stage-44 tadpole. Its structure bears considerable resemblance to that seen *in vitro* in Fig. 11.

Fig. 17. Cell surface specialization between germ cell (below) and mesentery cell (above) fixed *in vivo* in a stage-44 tadpole. Compare with Fig. 11.

*Comparison of PGCs* in vitro *and* in vivo

Occasionally, germ cells which have been in culture for several days actually become incorporated into the monolayer (Fig. 14). This invasion bears striking resemblance to the appearance of germ cells migrating between mesentery cells *in vivo* (Fig. 15). In both cases, the somatic cells form a complete covering over the PGC, and are held together by specialized junctions resembling desmosomes. Cell surfaces specializations between PGCs and somatic cells are very similar n shape and size *in vivo* and *in vitro* (compare Figs. 16 and 17 with Figs. 11 and 12). Finally, both long filopodia (Wylie & Heasman, 1976) and broad blunt processes (Fig. 15) similar to those *in vitro* have been seen in germ cells fixed in the dorsal mesentery during their natural migratory phase. This suggests not only that the *in vitro* environment used here mimics the natural one, but also that the invasive, motile properties of PGCs which are manifest in culture are also responsible for their migration *in vivo*.

## DISCUSSION

The structural and contractile elements of embryonic cells which underlie the process of morphogenetic movement have been studied previously in a variety of ways, chiefly involving either whole embryo fixation and processing, e.g. Johnson (1977), or *in vitro* culture methods, e.g. Spooner & Conrad (1975). While tissue preservation in whole embryo fixation is often poor, the *in vitro* method suffers the disadvantage of subjecting cells to artificial surfaces. The PGC *in vitro* system used here is a compromise between the two techniques, and has the advantages of easy observation and fixation in culture conditions combined with the provision of the most 'natural' substrate possible, that of living cells originating from amphibian mesentery. By comparing the appearance of germ cells *in vitro* and *in vivo*, we have shown that isolation and culture of PGCs in this way does not disrupt their normal ultrastructure.

The structural features of PGCs which are concerned in cell movement include:

(*a*) a microfilamentous network;

(*b*) attachment sites;

(*c*) cell processes.

It is remarkable that such enormous cells (average 60 $\mu$m diameter), packed with dense yolk platelets, are capable of migration. It seems logical to assume that a considerable contractile skeleton is required to support such movement. However, the only evidence of such a system is the narrow sheet of microfilaments, which lines the cell surface and extends into all cell protrusions. The likelihood that poor fixation has destroyed a more extensive network is small, as the surrounding somatic cells always contain well preserved microfilament bundles. It is possible that the dense nature of PGC cytoplasm obscures a

cytoplasmic contractile system. Alternatively, the surface layer of approximately 6·5 nm microfilaments may be sufficient to account for the contractile properties of PGCs.

The role of microfilaments in cell movement is not clear, although they are recognized as an important organelle in motile cells. For example, the movement of epithelial cells by lamellipodial extension in culture has been shown to depend on bundles and networks of 4–8 nm microfilaments (Dipasquale, 1975). Thin filaments, similar to those in PGCs, have been reported in a variety of motile cell types including cultured fibroblasts (Ishikawa, Bischoff & Holtzer, 1969; Wessels, Spooner & Luduena, 1973), cultured nerve cells (Luduena & Wessels, 1973), embryonic epithelia (Spooner *et al.* 1973) and chondrogenic cells (Ishikawa *et al.* 1969). In all these cases the microfilaments have been identified as actin by the criterion of heavy meromyosin or myosin S1 binding. Work is continuing to verify that PGC 6·5 nm filaments are also composed of actin, and that actin filament-membrane complexes are associated with sites of active motility.

In elongated PGCs microfilaments are most frequently aligned at right-angles to the long axis, suggesting that they may act by squeezing cytoplasm forward by peristaltic-like contractions. This possibility is supported by time-lapse cinemicroscopy, where germ cells have been seen to undergo peristaltic waves (Heasman *et al.* 1977). Also germ cells fixed *in vivo* and *vitro* often have narrow, bilateral constrictions, which may correspond to sites of microfilament contraction.

The relative roles of the different types of cell process we have described, are difficult to assess. All three varieties of protrusions, single filopodia, short blunt filopodia and 'invasive' processes, have the same ultrastructural components. Both filopodial types are dynamic structures which we have seen rapidly extended and withdrawn, although single filopodia may remain for a number of hours. The complexity of this more permanent structure is revealed in scanning electron micrographs. Each long filopod is, in fact, a trunk from which smaller, smooth-surfaced and pointed microspikes branch, and from which membranous lamellae spread over the substrate. In some cases, the microspikes follow well-defined ridges in the substrate, possibly caused by bundles of microfilaments in the substrate cells. The overall picture is consistent with the hypothesis that each trunk with its radiating branches is exploring the substrate. A similar function has been suggested for the filopodia of 3T3 mouse cells in culture (Albrecht-Buehler, 1976). An alternative or complementary role is that filopodia act as selective anchors, which are themselves contractile, and pull up the rest of the germ cell body. Evidence for this hypothesis comes from cinemicrography of PGCs (Heasman, *et al.* 1977), and from the observation of a submembranous network of microfilaments, in filopodia. Contractile filopodia of this nature have been reported in the cells of the ascidian, *Botryllus schlosseri* (Izzard, 1974).

The ability of germ cells to extend broad processes from their undersurface, which penetrate between underlying cells, is, in itself, an unusual cell property. It has been demonstrated that both epithelial cells and fibroblasts will not adhere to, or move over, the upper surface of cells in culture (Dispasquale & Bell, 1974). Germ cells not only adhere and move, but appear to invade the underlying substrate. This ability is not, however, surprising, when the normal migratory path of PGCs in tadpoles, lies not on the surface of mesentery cells but sandwiched between layers of cells (Wylie & Heasman, 1975).

Careful examination of germ cells fixed *in vivo* during their migration up the dorsal mesentery, reveals cell processes which resemble in size and form all three types of protrusions which we have described *in vitro*. Occasional long filopodia extend from the leading pole, while broad-based processes are more commonly seen (Fig. 15). These may be the counterparts of the invasive processes from the undersurface of germ cells *in vitro*, or may simply represent lateral sections through single filopodia, or incompletely extended filopodia.

The mechanism by which germ cells seeded onto a monolayer occasionally become completely incorporated into it is unknown. Either the PGC actively migrates between cells, presumably using the invasive processes described, or the somatic cells grow over the upper surface of the germ cell. We are currently studying this problem by comparing the behaviour of PGCs on monolayers with that of PGCs seeded adjacent to blocks of explanted mesentery. Whatever the case, the phenomenon suggests unusual cell surface influences mediated by PGCs on the surrounding cells. We hope to determine the nature of the PGC surface coat using histochemical staining techniques, *in vivo* and *in vitro*.

We have established here that germ cells seeded and cultured on monolayers of cells, retain both their characteristic fine structure and their ability to move over and penetrate between somatic cells. The results described strongly support the hypothesis that the behaviour of PGCs in these *in vitro* conditions mimics closely their normal migratory function. One important difference in their comparative behaviour is that germ cells *in vivo* move in a specific direction, to the site of gonad formation, whereas motility *in vitro* is random. This model system now provides the exciting possibility of studying the cellular and molecular basis of germ cell guidance.

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