MICROTUBULES AND FILAMENTS IN THE
FILOPODIA OF THE SECONDARY
MESENCHYME CELLS OF ARBACIA
PUNCTULATA AND ECHINARACHNIUS PARMA

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SUMMARY

In an attempt to understand the mechanism of contraction of the filopodia of the secondary mesenchyme cells and thus secondary invagination of the archenteron, the fine structure of these processes was examined. Whereas microtubules are commonly encountered in the cell body and at the base of the filopodia, very few (one or two) are present near the tip of the filopodia. Instead the slender processes are filled with 50-Å filaments. Colchicine and hydrostatic pressure were applied to the embryos to elucidate the action of these different fibrous elements. Both agents cause cessation of archenteron movement and the disassembly of the microtubules. Hydrostatic pressure causes the disappearance of the filaments as well. Because of the small numbers of microtubules in the slender filopodia and the fact that in no other system is there any evidence for contraction of these elements, it was concluded that they do not function in the contraction process, but are probably involved in the formation of these cell extensions: hence the effect of colchicine on archenteron movement. The 50-Å filaments, on the other hand, are likely candidates for the contraction process.

INTRODUCTION

Gastrulation in echinoderms can be separated into two phases (Dan & Okazaki, 1956; Kinnander & Gustafson, 1960). In the first phase, primary invagination, the ectoderm at the vegetal pole begins to form the archenteron by a process of infolding. When almost one third the distance across the blastocoel has been traversed by the developing archenteron, the phase of secondary invagination begins. This is characterized by the appearance on the roof of the archenteron of secondary mesenchyme cells which extend long pseudopodial processes (often referred to as filopodia because of their small diameter) that make contact with the animal pole. Contraction of these filopodia appears to be responsible for pulling the archenteron the rest of the distance across the blastocoel. The evidence for this comes from the observations that the archenteron is unable to move across the blastocoel and even begins to retract if these filopodia break or disappear, either naturally (Gustafson, 1964) or as the result of

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treatment of the embryos with low calcium, pancreatin, or hypertonic media (Dan & Okazaki, 1956). Furthermore, Kinnander & Gustafson (1960) and Dan & Okazaki (1956) have described 'cones of attachment' where specific parts of the basal end of the ectodermal cells appear to be pulled into the blastocoel locally by the action of the filopodia. When the archenteron has nearly reached the animal pole its movement is redirected by filopodia towards the region of oral contact. Thus, the roof of the archenteron is guided to the specific point of oral contact in the ventral ectoderm, the site of the future mouth (Gustafson & Kinnander, 1960).

In an attempt to understand the mechanism of the contraction of the filopodia, we examined thin sections of these processes for a structure or structures which might be responsible. In particular we were interested in determining what role, if any, the microtubules play in filopodial contraction, since in an earlier study (Gibbins & Tilney, 1965) we had found small numbers of these elements in the filopodia. Therefore, we applied to the embryos agents known to affect the integrity of microtubules, namely colchicine and hydrostatic pressure (see Tilney & Gibbins, 1966). The action of these agents was analysed in relation to their effect on the filopodia and on the movement of the archenteron during secondary invagination. We also examined regions of presumed oral contact to try to obtain some information on the specific region of contact between the filopodia and the ventral ectoderm.

**MATERIALS AND METHODS**

Eggs, obtained from *Arbacia punctulata* by the voltage method (Harvey, 1952) and from *Echinarachnius parma* by injection with 0.5 M KCl, were fertilized and allowed to develop in finger bowls surrounded by running sea water. Over 95% fertilization was achieved. Some embryos from every batch were allowed to develop to the pluteus stage to ensure that development was normal. For the examination of normal morphology, embryos at the required stage in gastrulation were transferred to a centrifuge tube. The embryos were fixed in the centrifuge tube by the addition of glutaraldehyde to the sea water. The glutaraldehyde was first treated with BaCO₃ and the pH adjusted to 7.6 with 0.1 N NaOH. For fixation of *A. punctulata* we used 6% glutaraldehyde, for *E. parma* we used 1%. Fixation was carried out for 30 min. The embryos were washed in sea water and post-fixed in either 1% OsO₄ in 0.1 M phosphate buffer at pH 7.4 to which CaCl₂ had been added or in 1% OsO₄ in sea water. The embryos were dehydrated and embedded in Epon 812 in the usual fashion. Sections, cut with a diamond knife on a Servall Porter-Blum II ultramicrotome, were stained with uranyl acetate and lead citrate and examined with a Philips 200, a Siemens Elmiskop I or a Hitachi 11C electron microscope.

The secondary mesenchyme cells of living embryos were observed by phase-contrast microscopy (oil immersion).

In experiments with colchicine, gastrulae which had just completed primary invagination were transferred to a solution of colchicine dissolved in sea water. Concentrations of 5 × 10⁻⁵ to 5 × 10⁻⁴M were used. Some of the embryos in each batch were observed by phase-contrast microscopy for analysis of filopodial and archenteron movements; others were fixed for examination of fine structure.
Microtubules and filaments in filopodia

The equipment used in experiments with hydrostatic pressure has been described by Marsland (1950). We modified the chamber somewhat from the original design by using an ‘O’ ring as the pressure seal. The chamber consisted of two parts, each of which contained a clear sapphire window. When these parts were bolted together and thus sealed by the ‘O’ ring, the windows lay above one another so that light could pass through the chamber. The specimens could then be viewed by means of an inverted microscope.

The apparatus used for fixation during compression has been described by Landau & Thibodeau (1962). Fixation was carried out as described above.

RESULTS

General observations

Since the embryos of *A. punctulata* are not transparent, most of the observations made on living embryos were carried out on the clear embryos of *E. parma*. On the other hand, our fine-structural analysis was confined largely to *A. punctulata*, for fixation of *E. parma* proved difficult. Nevertheless we have examined the fine structure of untreated *E. parma* and have compared it with *A. punctulata*. No differences were noted. We have included electron micrographs of both organisms.

The secondary mesenchyme, derived from the ectoderm lining the roof of the archenteron, becomes evident towards the end of the primary invagination of the archenteron. The arrangement of the secondary mesenchyme cells on the roof of the archenteron is variable. In some instances they form a single layer of loosely connected cells: in others they tend to pile up as if giving rise to a double layer. The shape of individual cells appears to be largely dependent upon the size and location of the filopodia. The nucleus of the cell is generally found near that margin of the cell which makes closest contact with the archenteron proper.

Soon after their formation the secondary mesenchyme cells send out long filopodia with which they explore the wall of the blastocoel (Figs. 1, 2). The filopodia are straight, bristle-like cytoplasmic extensions nearly 2 /μm in diameter at their bases and tapering to less than the diameter of a cilium (0.2 /μm) near their connexions with the ectoderm. They frequently branch (Fig. 2).

Fine structure

The cell body. A centriole, and at right angles to it a basal body (Fig. 3), identified as such by the presence of a short ciliary rootlet (see inset to Fig. 3), are found near the centre of favourably sectioned secondary mesenchyme cells. Microtubules, which are commonly encountered, appear to radiate from these bodies. When the microtubules are observed near the free surface of the cell, they are generally present in pairs, separated by a minimum of 70 Å (Fig. 3). The mitochondria, although dispersed throughout the cytoplasm, frequently appear to be arranged along radii which extend from the centrosphere to the filopodia.

The filopodia. Within the broad filopodial base are mitochondria, yolk granules, short segments of endoplasmic reticulum, vesicles and vacuoles of a variety of sizes,
ribosomes and microtubules (Fig. 4). The latter are generally found in pairs. Towards the tips of the filopodia, identified in section by their slender diameter, are ribosomes, an occasional vesicle, one or two microtubules and a bundle of filaments, each filament being about 50 Å in diameter (Figs. 5–10). Filaments, although the most prominent constituent of the distal ends of the filopodia, are infrequently encountered in their bases. Both the microtubules and filaments are aligned parallel to the long axis of the filopodium. No specific arrangement between them could be determined when they occurred together (Figs. 7, 8).

In the blastocoel we often encountered droplets of cytoplasm each about 1 μm in diameter. Since these droplets invariably appear round in thin sections, we feel confident that they are not the result of grazing sections through the filopodia. They are membrane limited and contain small numbers of ribosomes and vesicles, no microtubules or filaments. We suspect that these bodies correspond to the structures which result from the fragmentation of certain of the retracting secondary mesenchyme filopodia described in detail by Kinnander & Gustafson (1960).

Connexions between the secondary mesenchyme filopodia and the ectoderm

Three types of connexions between the filopodia and the ectodermal cells are seen. The first and most common occurs when a filopodium makes contact with, but does not penetrate, the basement membrane underlying the ectoderm. The points of closest association do not display any specialized structures such as desmosomal contacts or tight junctions.*

A second type of connexion between the ectoderm and the mesenchyme is illustrated in Fig. 11. From the base of an ectodermal cell situated just at the border between the animal plate and the lateral ectoderm, presumably in the region of oral contact, a projection extends into the blastocoel, perforating the basement membrane. Within this projection and extending into the cell proper are a number of microtubules reminiscent of the arrangement of the microtubules in longitudinal sections of cilia. The tubules are anchored at the tip of the projection in a small mass of dense material. This dense material does not take the form of a basal body. A mesenchymal process has made contact with the ectodermal cells at this point. In two other embryos cross-sections of cilia within the cytoplasm of ectodermal cells in the region of presumed oral contact have been found as well.

In the third type of connexion between the ectoderm and mesenchyme, the mesenchyme cells or portions of them have penetrated the basement membrane underlying the ectoderm and lie between adjacent ectodermal cells.

*While this paper was in the last stages of preparation Dr J. P. Revel informed us that he had found tight junctions between the ectodermal cells of Arbacia blastulae. These junctions were present in embryos fixed in 1 % acrolein, but absent in those fixed in 6 % acrolein. In our studies, where most of the embryos were fixed in 6 % glutaraldehyde, no tight junctions were seen between the ectodermal cells of the blastulae. We cannot rule out the possibility that with our fixative tight junctions between the secondary mesenchyme processes and the ectodermal cells may not have been preserved.
Microtubules and filaments in filopodia

The effect of colchicine and hydrostatic pressure on the microtubules in the filopodia and on archenteron movement

In all cases archenteron movement was arrested by these agents. The agents were applied following the primary invagination of the archenteron.

In the case of colchicine, a time lag exists between the time of application of this drug and the time at which cessation of archenteron movement is observed. This lag is concentration dependent. With $5 \times 10^{-3} \text{M}$ colchicine an effect is seen within 30–45 min, while with $5 \times 10^{-5} \text{M}$ the effect takes several hours to become visible. Although there are individual variations, in most cases the number of filopodia is reduced. In some organisms the archenteron collapses around the developing anal region.

In concentrations of $5 \times 10^{-3} \text{M}$ to $5 \times 10^{-5} \text{M}$ the microtubules are absent from the cells. Filaments in the filopodia, however, do not appear to be affected (Fig. 12).

Because of limitations in the apparatus (the necessity of using thick windows) sufficient magnification could not be achieved to check for the presence of filopodia in living organisms subjected to hydrostatic pressure. We relied, therefore, on sections cut through cells fixed during the application of pressure.

Fixation of embryos 45 min after the initiation of treatment with hydrostatic pressure (44820–48260 kN m$^{-2}$ (6500–7000 psi)) showed an increase in the number of cells in the blastocoel, and a reduction in archenteron height. Filopodia could not be found. Microtubules and filaments were absent in all cell types, not just in the secondary mesenchyme filopodia. Micrographs depicting these results have been included in a separate communication (Tilney & Gibbins, 1969). Since they illustrate the absence of filopodia, microtubules and filaments, we have not felt it necessary to include illustrations here.

Upon release of pressure some of the embryos recover and develop into normal plutei.

DISCUSSION

The role of microtubules in archenteron movement

We have demonstrated that in the filopodia of the secondary mesenchyme cells there are two fibrous elements, microtubules and filaments. These two structures parallel the long axis of the filopodia and appear to be likely candidates for the contractile force developed by the filopodia during secondary invagination. In attempts to demonstrate a connexion between these elements and filopodial behaviour, we applied two agents known to affect the microtubules, colchicine and hydrostatic pressure. In both cases secondary invagination did not continue and development was arrested.

From these observations, therefore, it seems likely that the microtubules are involved in archenteron movement.

Yet it seems unlikely that the microtubules themselves generate the force necessary for archenteron movement. Our reasoning here is based on the following points. First, there is no evidence in the literature to support the contention that microtubules contract. Instead, the microtubules are thought to function in the production of cell
shape (see the reviews of Porter, 1966; Tilney, Hiramoto & Marsland, 1966; Tilney & Porter, 1967; Tilney, 1968). Even in motile systems there is no evidence of contraction; that is, active shortening of the microtubules: rather they appear to function by sliding past one another as in cilia (Satir, 1965) or in the contraction of *Stentor* (Bannister & Tatchell, 1968). Also relevant here may be the investigation of McIntosh & Porter (1967), in which the decrease in volume of the nucleus of the chick spermatid appears to result from the sliding of tubules past one another. Secondly, we have demonstrated that although small numbers of microtubules are present in the bases of the filopodia, towards the tip very few of these elements are present, in most cases only a single pair or even a single microtubule. Such small numbers of microtubules make any relation between the microtubules and shortening of the filopodia unlikely, even for models based upon the depolymerization of tubules.

Active contraction of the filopodia through interactions between tubules and filaments appears unlikely as well for two reasons. First, the microtubules are present in such small numbers that the filaments outnumber the tubules by more than 15 to 1. Secondly, there is a distinct lack of any organizational pattern between these structures. Thus specific interactions between fibrous elements as in skeletal muscle are improbable.

We believe, therefore, that the microtubules do not play an important role in filopodial contraction, but instead, as will be discussed in the next section, this function is carried out by the filaments. Yet if they are not active in filopodial contraction, what is the function of the microtubules and why do colchicine and hydrostatic pressure arrest archenteron movement?

From studies on the protozoan, *Actinosphaerium nucleofilum* (Tilney et al. 1966; Tilney & Porter, 1967; Tilney, 1968) and on the primary mesenchyme cells of *Arbacia* (Tilney & Gibbins, 1966) it now seems reasonably clear that the microtubules are intimately involved in the production of cell shape. It seems reasonable to expect that these elements may likewise play an important role in the formation and elongation of the filopodia of the secondary mesenchyme cells. Furthermore, we would suggest that the microtubules assemble first and by their orientation in the filopodia provide a direction for the assembly of other fibrous elements, namely the 50-A filaments. Since more than one filopodium is involved in archenteron movement, and since the filopodia frequently break connexion with the ectoderm, retract, and elongate again to make new connexions (see the description of Gustafson & Wolpert, 1963; Kinnander & Gustafson, 1960), the assembly of microtubules must be a frequent occurrence. Thus when the embryos are treated with colchicine or hydrostatic pressure which cause the disassembly of the microtubules and prevent their reassembly, the archenteron movement should cease, and indeed it does.

Yet we should point out that hydrostatic pressure also causes the disappearance of the 50-A filaments. Colchicine, on the other hand, does not appear to affect the filaments, or at least their morphological integrity, but does cause the complete disassembly of the microtubules. It may be significant that the effect of colchicine on archenteron movement is rather slow, compared to that of hydrostatic pressure which seems to take place instantaneously. Thus we have some experimental data which we would interpret
as indicating that the microtubules are involved in the formation and elongation of the filopodia.

**Role of the filaments in archenteron movement**

In a variety of other situations in which portions of a cell undergo active contraction, filaments of approximately 50 Å in diameter have been demonstrated oriented as if they could provide the active force; for example: during shortening of the ascidian tadpole tail (Cloney, 1966) and formation of the neural tube (Baker & Schroeder, 1967), during blastopore formation (Baker, 1965), during furrow formation in cleaving sea urchin eggs (L. G. Tilney & D. Marsland, in preparation), during retraction of the pseudopodia of *Difflugia* (Wohlman & Allen, 1968) and in the contractile stalks of the ciliates, *Stentor*, *Vorticella* and *Spirostomum* (Randall & Jackson, 1958; Yaqui & Shigenaka, 1963; Bannister & Tatchell, 1968; Favard & Carasso, 1965). Not only are there morphological similarities between the filaments in the above-mentioned systems and the filaments in the secondary mesenchyme cells, but also the rates of contraction are not dissimilar, as for example during blastopore formation or during furrow formation. The numbers of filaments in the secondary mesenchyme pseudopodia are also not unlike the numbers present in the retracting pseudopodia of *Difflugia*. Furthermore, in almost all the above-mentioned examples no microtubules are present in the contracting region, so that possibilities of functional confusion between these two types of fibrous structures are obviated.

By analogy to these other systems and from the fact that the filaments are present in sufficient numbers and in the proper orientation in the secondary mesenchyme filopodia, we conclude that the filaments are likely to be the basis of filopodial contraction. Furthermore, hydrostatic pressure, which breaks down the filaments, immediately arrests archenteron movement; in fact, the archenteron tends to collapse. Colchicine, on the other hand, which does not affect the filaments, at least morphologically, takes a considerably longer period to cause archenteron arrest. We interpret the latter as we did in the preceding section, as indicating the necessity for the production of new filopodia, the old ones continuing to shorten.

**Oral contact**

Kinnander & Gustafson (1960) have demonstrated that during secondary invagination the filopodia appear to make random contact with the animal pole. These points of contact are unstable, for they are frequently broken, new ones being formed. When the archenteron has migrated almost the entire way across the blastocoel, there is a delay until the filopodia make stable contact with the future mouth region. The filopodia then contract and oral contact is made. From this description it is clear that a specific type of junction must occur between the filopodia and the future mouth region during the stage when a stable contact is made.

Unfortunately in thin sections it is difficult to be sure that we have located the region of future oral contact. We can locate without difficulty the animal pole, identified by its long cilia in the apical tuft and the large, closely packed ectodermal cells, and thus we can identify a region which encircles the embryo, where at one point oral
contact will be made, but identifying this single point is difficult. Nevertheless, we have found short protuberances of characteristic structure which extend from the basal surface of one of the ectodermal cells in the region of presumed oral contact. It is possible that these are the sites which the filopodia recognize and to which they adhere.

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REFERENCES


Microtubules and filaments in filopodia


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Fig. 1. Light micrograph of a living gastrula of *Echinarchaeum*. On the tip of the archenteron are secondary mesenchyme cells (see arrow). Phase-contrast microscopy. × 125.

Fig. 2. The filopodia of a single secondary mesenchyme cell in a living *Echinarchaeum*. Note that the filopodia branch. Phase-contrast microscopy. × 1200.

Fig. 3. Thin section cut through the cell body of a secondary mesenchyme cell of *Arbacia*. In the centre of the cell is a centriole and at a right angle to it is a basal body of a cilium (see inset). The latter can be identified as such by its attached ciliary rootlet. Among the other organelles and inclusions are the Golgi apparatus (go), yolk granules (y), a cytolysome (cy) and microtubules. These tubules are commonly encountered around the basal body-centriole, but are also found near the cell surface. In this position they are frequently present in pairs (see inset). × 8500; inset × 48 000.
Fig. 4. Transverse section through the base of a filopodium of *Arbacia*. The arrows indicate microtubules. A mitochondrion (m) and numerous vacuoles are present as well. × 50,000.

Figs. 5, 6. Longitudinal sections through several filopodia of *Arbacia*. Of greatest interest are the microtubules which parallel the long axes of these processes. × 55,000.
Fig. 7. Transverse section of a filopodium of *Echinarchnium* near its tip. Within this slender cell extension is a microtubule in cross-section. Near the tubule are cross-sections of filaments (see arrows). × 100000.

Fig. 8. Transverse section of a filopodium of *Arbacia*. Within this filopodium is a pair of microtubules and near the tubules are filaments cut in transverse section (see arrow). × 60000.

Fig. 9. Transverse section of a filopodium of *Arbacia*. Two pairs of microtubules (mt) can be recognized on opposite sides of a yolk granule (y). × 65000.

Fig. 10. Longitudinal section of a filopodium of *Arbacia*. Within the centre of this filopodium are filaments. × 70000.
Fig. 11. Thin section of the base of an ectodermal cell of Arbacia in the region of presumptive oral contact. (bm, basement membrane.) \( \times 60000 \).

Fig. 12. Longitudinal section of a portion of a filopodium of Arbacia. This embryo was treated with colchicine for 2 h. Filaments are present within the filopodium. \( \times 95000 \).