The role of secondary mesenchyme cells during sea urchin gastrulation studied by laser ablation

JEFF HARDIN*

Biophysics Group and Department of Zoology, University of California, Berkeley, CA 94720, USA
* Current address: Department of Zoology, Duke University, Durham, NC 27706, USA

Summary

It has long been thought that traction exerted by filopodia of secondary mesenchyme cells (SMCs) is a sufficient mechanism to account for elongation of the archenteron during sea urchin gastrulation. The filopodial traction hypothesis has been directly tested here by laser ablation of SMCs in gastrulae of the sea urchin, *Lytechinus pictus*. When SMCs are ablated at the onset of secondary invagination, the archenteron doubles in length at the normal rate of elongation, but advance of the tip of the archenteron stops at the $\frac{1}{4}$ gastrula stage. In contrast, when all SMCs are ablated at or following the $\frac{3}{4}$ gastrula stage, further elongation does not occur. However, if a few SMCs are allowed to remain in $\frac{3}{4}$-$\frac{1}{4}$ gastrulae, elongation continues, although more slowly than in controls. The final length of archenterons in embryos ablated at the $\frac{1}{4}$-$\frac{3}{4}$ gastrula stage is virtually identical to the final length of everted archenterons in LiCl-induced exogastrulae; since filopodial traction is not exerted in either case, an alternate, common mechanism of elongation probably operates in both cases. These results suggest that archenteron elongation involves two processes: (1) active, filopodia-independent elongation, which depends on active cell rearrangement and (2) filopodia-dependent elongation, which depends on mechanical tension exerted by the filopodia.

Key words: filopodia, sea urchin, gastrulation, secondary mesenchyme, laser ablation, *Lytechinus pictus*.

Introduction

The migration of mesenchymal cells and the invagination of epithelial sheets are ubiquitous phenomena during gastrulation (Gustafson & Wolpert, 1963, 1967; Holtfreter, 1943, 1944). For 30 years, the sea urchin embryo has been a useful system for studying the interaction between these two fundamental cell behaviours. Gastrulation in the sea urchin embryo occurs in two phases (Dan & Okazaki, 1956; Gustafson & Kinnander, 1956). **Primary invagination** involves the inward bending of the vegetal plate to form the archenteron, which is roughly cylindrical in shape and extends one third of the way across the blastocoel. During **secondary invagination** the archenteron elongates and this phase is marked by the extension of long filopodial protrusions towards the animal region of the embryo by secondary mesenchyme cells (SMCs) at the tip of the gut rudiment.

In 1956, two landmark studies independently suggested that traction exerted by filopodia could account for secondary invagination. Dan & Okazaki (1956) found a correlation between the presence and proper extension of filopodia and the completion of gastrulation under a variety of circumstances. Treatments or agents that disrupt filopodia or their contact with the animal ectoderm, including pancreatin, osmotic swelling and low calcium, are correlated with failure of the archenteron to elongate fully. In addition, the lateral margin of the tip of the archenteron appears to be under tension, reminiscent of a suspended fabric (Dan & Okazaki, 1956). Gustafson & Kinnander (1956) noticed a reproducible pause in the progress of invagination prior to secondary invagination, followed by a resumption of invagination at a distinctly more-rapid pace after the onset of filopodial activity. The lateral wall of the archenteron, in contrast, exhibits little, if any, obvious protrusive activity (Kinnander & Gustafson, 1960). This lack of protrusive activity led Gustafson & Kinnander to conclude that filopodial traction is the most-important mechanism for elongating the gut rudiment.
These ideas have been discussed in two reviews by Gustafson & Wolpert (1963, 1967).

The simplicity and economy of the filopodial traction hypothesis, coupled with the strong circumstantial evidence supporting it, have made it the accepted model of secondary invagination. However, several questions can be raised regarding its adequacy. First, the evidence supporting it is largely based on observation and it has proven difficult to devise an experimental treatment that can be demonstrated to affect SMCs specifically (reviewed in Hardin & Cheng, 1985). Second, the mechanical consequences of filopodial traction are unclear; mechanical simulations suggest that filopodial pulling alone cannot account for the shape of the embryo at the end of secondary invagination (Hardin & Cheng, 1985). Third, cell rearrangement has been demonstrated to occur in the wall of the gut rudiment as it elongates (Ettenson, 1985; Hardin & Cheng, 1986), whether this cell rearrangement is normally due to filopodial traction, to active forces within the archenteron, or to a combination of the two is not known. Finally, the findings that autonomous thinning of the archenteron wall (Dan & Okazaki, 1956; Dan & Inaba, 1968) and epithelial cell rearrangement (Hardin & Cheng, 1986) occur in exogastrulae strongly suggest that in this case archenteron elongation involves an active component which is independent of filopodial pulling, since the filopodia in exogastrulae cannot exert traction. In view of these facts, the relationship between filopodial traction and cell rearrangement should be clarified.

Laser microsurgery (reviewed by Berns et al., 1981) permits selective alteration or destruction of regions of a cell or embryo without disruption of neighboring tissues. These advantages are afforded by a combination of high energy intensity at the site of the focused laser spot, combined with rapid attenuation of energy intensity away from the focal plane of the spot (Berns et al., 1981). The transparency of the sea urchin embryo makes it a good candidate for the application of the laser microbeam and this technique now allows the role of filopodial traction in archenteron elongation to be examined directly for the first time through laser ablation of SMCs and their filopodia. The results indicate that significant, autonomous extension of the archenteron can occur without filopodial traction, although the filopodia have an important role during the final one third of gastrulation.

Materials and methods

Procurement and measurement of embryos
Gametes of *L. pictus* (Marinus, Westchester, CA) were obtained, fertilized and cultured as described by Hardin & Cheng (1986). Exogastrulae were produced using LiCl as described by Hardin & Cheng (1986). Archenteron length was defined as the distance from the vegetal edge of the blastopore lip to the middle of the archenteron roof, as measured from differential interference contrast (DIC) micrographs or video images. Some micrographs were obtained by photographing the video screen using a Polaroid CU-5 Land camera and Polaroid 611 positive film.

Laser ablation of secondary mesenchyme cells

Laser ablations were performed at the Beckman Laser Institute of the University of California, Irvine, CA. The basic apparatus and techniques involved are described in Berns et al. (1981). The chambers used for irradiating embryos were constructed using poly-1-lysine-coated coverslips mounted on microscope slides as described in Hardin & Cheng (1986). Irradiations were performed using a Zeiss Axiomat inverted microscope equipped with DIC optics and infrared and orange glass thermal filters connected to a Dage/MTI Newicon videocamera and a Sony three-quarter inch time-lapse video recorder. The second harmonic wavelength (532 nm) of a Quantel neodymium-YAG 481 laser was used to irradiate the specimens through a ×25 DIC objective with individual pulses of 10–15 nanoseconds, with an average energy per pulse of 8.4 mJ and an attenuation of 15–17 dB. Embryos were irradiated by directing one to several pulses at either the filopodia or cell bodies of visible secondary mesenchyme cells. Irradiated embryos were then followed individually, using time-lapse videomicroscopy or sequential 35 mm still micrography and visually inspected at 15 min intervals. Embryos showing evidence of additional incipient filopodia were reirradiated to ensure that all secondary mesenchyme cells and their filopodia were ablated.

Results

Microsurgery using the laser microbeam

Secondary mesenchyme cells or their filopodia can be ablated with the laser microbeam by focusing at various optical planes of section with a ×25 DIC objective. Unfortunately, for several reasons it was not technically possible to ablate all filopodia (as opposed to the cell bodies of SMCs) in a single embryo simultaneously. The diameter of the embryo (~120 µm) requires the use of an objective that provides substantial depth of field, resulting in a compromise in magnification and optical resolution which in turn limits the spatial resolution of the laser. Moreover, the sheer number of filopodia and an inability to visualize all of them with total confidence makes their complete ablation difficult. Finally, because filopodia are continually extended and retracted by SMCs throughout secondary invagination, it is necessary to ablate not only individual filopodia, but the cell bodies of SMCs as well, if the concerted action of many filopodia over time is to be prevented.
Indeed, SMCs in which individual filopodia were ablated invariably extended new filopodia within 1–3 min after the ablation (data not shown). Therefore most experiments involved the irradiation of the cell bodies of SMCs. The archenterons of control embryos in which other cell populations were irradiated (e.g. primary mesenchyme cells, lateral ectoderm) displayed no detectable alterations in elongation compared to unirradiated controls.

Cells often lyse upon irradiation; ablated SMCs sometimes liberate microplasts, which move through the blastocoel. If the cell does not lyse, it retracts or ceases extension of its protrusions. In both cases, it is apparent when an irradiated SMC has suffered permanent damage; inactivated or lysed cells are identified in real time by their damaged cell membrane and cellular debris, or by their subsequent quiescence and cessation of motility within 1–2 min. The results of laser ablation are even more dramatic when viewed in time lapse; the rapid ‘boiling’ protrusive activity of the basal surfaces of SMCs prior to irradiation abruptly ceases following irradiation with the micro-beam. Because the SMCs are clustered together so closely at the tip of the archenteron, it is not always immediately clear that all SMCs have been ablated following the first series of laser pulses. Therefore it was impossible to irradiate large numbers of embryos and to score them later for their ability to gastrulate in a batch-wise fashion. Instead, periodic inspections of individual irradiated embryos were performed at 10–15 min intervals; if new filopodia appeared, the protrusive cells were ablated. The experiments reported below represent time-lapse and real-time observations of five to ten individual embryos over the period of interest for each experimental series.

**Mechanical consequences of ablation of filopodia**

If an individual filopodium is severed, the base of the filopodium may either be resorbed or remain extended but inactive. In either case, little retraction of the archenteron tip occurs (Fig. 1A–C). When individual SMCs are ablated at any stage of elongation, a local retraction (~1 μm) of a small region of the tip of the archenteron usually occurs (Fig. 3A,B), but little or no retraction of the archenteron as a whole takes place. When a group of SMCs are ablated simultaneously, a small, immediate retraction of the tip of the archenteron occurs. However, even when the entire tip of the gut rudiment is rapidly irradiated, the amount of retraction is ~4 μm. Archenterons ablated at the 1/4–1/2 gastrula stage retract 1-7 ± 0-3 μm (mean ± S.E.M.; n = 6), while those ablated at the 3/4 gastrula stage retract 3-0 ± 0-6 μm (n = 3), which is at best a slight difference (0-1 < P < 0-05). After complete destruction of SMCs at the 3/4 gastrula stage, the archenterons of 20% of the embryos show a small degree of lateral buckling near their bases (Fig. 1D), suggesting that some axial tension is present in the gut rudiment at this time. However, such buckling was not observed when the ablation was performed prior to the 3/4 gastrula stage. If the filopodia on the uppermost portion of the archenteron tip are ablated at any time during secondary invagination, the archenteron is deflected towards the remaining laterally located filopodia, with the direction depending on which filopodia are left intact (Fig. 1E). This result provides experimental evidence that lateral movement of the tip of the gut rudiment can be guided by an asymmetric distribution of filopodia.

**Ablation of filopodia at the onset of archenteron elongation**

When individual embryos are followed using time-lapse videomicroscopy after laser ablation of SMCs, a consistent pattern emerges. If the tip of the archenteron is irradiated at the 1/4 gastrula stage, the archenteron elongates until it reaches two thirds of the way across the blastocoel (diamonds, Fig. 2). Within the first hour after ablation, the rate of advance of irradiated archenterons is identical to controls (~0-4 μm min^-1), but slows asymptotically, finally approaching zero after 2–2.5 h (Fig. 2). In contrast, control embryos continue elongating at a constant rate of ~0-4 μm min^-1 (dotted line, Fig. 2). Thus the elongation of archenterons ablated at the 1/4 gastrula stage occurs despite the absence of all motile SMCs and represents up to a 100% increase in the length of the archenteron compared to its length at the end of primary invagination. In time-lapse studies, archenteron elongation occurs without any obvious pulsatile or protrusive activity on the part of endoderm cells after ablation of SMCs, suggesting that pulsatile activity may be a misleading guide as to which cells in the archenteron are actively involved in morphogenetic movements and suggesting that a thorough reexamination of motile activity in the archenteron during its elongation is in order.

In addition to changes in the length of ablated archenterons, their blastopores close by 10–20%, depending on the initial length of the gut rudiment, to a final diameter of 33-0 ± 0-4 μm (mean ± S.E.M., n = 4). Similarly, the walls of the gut rudiment thin by 15–40%, to a final thickness of 4-4 ± 0-4 μm. In both cases, the final dimensions agree closely with previously determined values for normal archenterons at a comparable stage of elongation (Hardin & Cheng, 1986; Hardin, 1986). The contours of the archenteron often change as well, from slightly dome-shaped or nearly cylindrical with straight walls to a shape in which the central region of the gut rudiment is slightly narrower than the base or tip regions (Fig. 3). In 35% of the cases, the tip of the gut rudiment bends to one
Fig. 1. Immediate effects of laser ablation of filopodia. (A) A midgastrula following ablation of a single filopodium (small arrow). An intact filopodium is also visible (large arrow). (B) The same embryo following ablation of the intact filopodium. Little retraction of the archenteron occurs. (C) The same embryo following ablation of all SMCs. (D) A gastrula following rapid ablation of all SMCs. The original outline of the archenteron tip is shown as a dotted line and the outline after ablation is indicated by a solid line. A slight retraction of the archenteron occurs, accompanied by buckling in the thinnest region of the gut rudiment (arrows). (E) A gastrula in which all secondary mesenchyme cells were ablated except for a single prominent cell (arrow). Note the deflection of the archenteron tip in the direction of the active cell. Bar, 20 μm.

side slightly and the archenteron tip, which appears to be somewhat sticky, makes contact with the ectodermal epithelium somewhere in the animal-most one third of the blastocoel wall. In other cases, the archenteron simply stops without significant deflection. Other developmental processes appear unaffected by this treatment; primary mesenchyme cells continue aggregation, syncytium formation and the early phases of spicule formation in irradiated embryos in synchrony with unirradiated controls in the
Ablation of filopodia in late gastrulae

When SMCs are ablated after the tip of the archenteron has advanced two thirds of the way across the blastocoel, no further extension of the gut rudiment occurs (open square, Fig. 2). In all cases, advance ceases immediately upon complete ablation of all SMCs. If a few (≤3) SMCs are left intact, advance of the archenteron continues, albeit more slowly than usual (Figs 2, 4A,B). The rate of advance appears to be proportional to the number of remaining SMCs, and is significantly slower than in unirradiated controls. For example, in an embryo in which three intact SMCs remained, the rate of advance of the archenteron tip was ~0.15 μm min⁻¹ (filled square, Fig. 2), as compared to the control rate of ~0.4 μm min⁻¹. This result again indicates that general cytotoxic effects of the ablation procedure are not significant, since SMCs immediately adjacent to ablated cells show completely normal protrusive activity. Moreover, it suggests that the traction of at least a few filopodia is necessary for continued elongation of the gut rudiment at or beyond the ½ gastrula stage.

same culture chamber (Fig. 3). Unirradiated controls in the same chamber complete gastrulation normally (data not shown).

Fig. 2. Advance of the tip of the archenteron over time in ablated and normal embryos. Archenteron length (μm) was measured from time-lapse video images and plotted against time after onset of secondary invagination (min). Corresponding stages of elongation are shown schematically for reference. Time points for archenterons ablated at the ½-⅔ gastrula stage (mean ± s.e.m. both axes; n = 4 except for the final time point, where n = 2) are shown (diamonds). A representative control embryo followed from the 4 gastrula stage onward is provided for comparison (dotted line). Two records of archenterons ablated at the ½ gastrula stage are also shown (squares). One embryo (filled squares) retained three active SMCs, while the other had no intact filopodia (open squares).

Fig. 3. Results of ablation of secondary mesenchyme cells at the onset of archenteron elongation (A). Video image of ½ gastrula just prior to ablation. Note the prominent secondary mesenchyme cell (arrow). (B) Immediately after ablation. The remnants of the SMC are still visible (arrow). (C) 2 h 17 min after ablation. Significant elongation has occurred. Note the normal aggregation of primary mesenchyme cells into ventrolateral clusters (large arrow). Remnants of severed filopodia ablated after the first series of laser pulses are still visible (small arrows). Bar, 20 μm.
Fig. 4. Results of ablation of secondary mesenchyme cells at the $\frac{1}{2}$ gastrula stage. (A) Video image of a $\frac{1}{2}$ gastrula just prior to ablation. Many secondary mesenchyme cells are visible (arrows). (B) The same embryo following ablation of all but three secondary mesenchyme cells. Little retraction of the archenteron has occurred. (C) The same embryo 45 min after ablation; the archenteron has advanced noticeably. Protrusive cells are marked by arrows. Bar, 20 $\mu$m.

Fig. 5. Comparison of final lengths (mean $\pm$ s.d.) of exogastrulated archenterons ($n = 6$), archenterons ablated at the $\frac{1}{2}-\frac{1}{2}$ gastrula stage ($n = 4$), and normal archenterons ($n = 11$).

Comparison of ablated embryos and exogastrulae
It has been noted by several workers that the archenterons of exogastrulated embryos are intermediate in length between those of normal embryos at the onset and end of secondary invagination (Dan & Okazaki, 1956; Hörstadius, 1973; Ettenson, 1985). In addition, the gut rudiments of exogastrulae undergo autonomous cell rearrangement as they elongate, though to a lesser extent than normal embryos (Hardin & Cheng, 1986). Therefore it is of interest to compare the final lengths of exogastrulated archenterons and gut rudiments ablated at the onset of secondary invagination, since in both types of embryos elongation must result from processes independent of filopodial traction. As shown in Fig. 5, the final lengths of archenterons in the two types of embryos are virtually identical. For exogastrulae, the average final length is $67.8 \pm 1.5 \mu$m (mean $\pm$ s.d.); for ablated archenterons the average length is $68.5 \pm 2.8 \mu$m, which is not a significant difference ($P > 0.5$). The final average length of the archenteron in both cases is 73–74% of the final length of normal gastrulae (Fig. 5).

Discussion

Archenteron elongation involves two processes
The results presented here are the first direct test of the filopodial traction hypothesis of gastrulation in the sea urchin embryo. The results of laser ablation of secondary mesenchyme cells (SMCs) at the tip of the gut rudiment suggest that secondary invagination itself actually involves two processes: active extension and filopodia-dependent elongation. The first process is capable of extending the archenteron to two thirds of its final length and is independent of filopodia, as evidenced by the ability of archenterons to double in length when SMCs are ablated at the $\frac{1}{2}$ gastrula stage. This lengthening presumably involves autonomous cell rearrangement in the archenteron wall, since secondary invagination is accompanied by cell rearrangement at all stages (Ettenson, 1985; Hardin & Cheng, 1986).

The extension accomplished by embryos ablated at the $\frac{1}{2}-\frac{1}{2}$ gastrula stage corresponds very closely to the extent of elongation achieved by LiCl-induced exogastrulae. In the case of exogastrulae, the walls of the archenteron thin, the 'blastopore' region decreases in circumference, and the archenteron lengthens to two thirds of the length of normal gastrulae by active cell rearrangement (Hardin & Cheng, 1986). The striking
similarities between these two cases suggest a common mechanism: a filopodia-independent extension resulting in sufficient cell rearrangement and cell shape changes to achieve a doubling of archenteron length. In both cases, filopodia cannot extend properly following this partial extension and further lengthening of the gut rudiment is prevented. Additional evidence for autonomous extension during secondary invagination comes from observations of filopodial activity in Lytechinus variegatus gastrulae. In this species, filopodia are directed either laterally or even vegetally until the \( \frac{1}{2} \) gastrula stage, but extension and thinning of the archenteron nonetheless occur (Trinkaus, 1984, pp. 438–439; Morrill & Santos, 1985). Since the upward (animalward) force component due to filopodial traction would appear to be negligible in these embryos until the \( \frac{1}{2} \) gastrula stage, autonomous extension could account for the initial elongation.

**Mechanisms of action of secondary mesenchyme filopodia**

Successful completion of gastrulation requires intact filopodia, since ablation of all SMCs after the \( \frac{1}{2} \) gastrula stage results in cessation of further extension, whereas the presence of a small number of active SMCs allows elongation to continue. The experiments presented here do not address the precise mechanism of action of the filopodia. The most-likely possibility is that filopodial traction is necessary to achieve the final elongation of the archenteron by stretching the gut rudiment. Several lines of evidence suggest that filopodial traction may be more substantial late in gastrulation. The lateral buckling observed in some \( \frac{1}{2}-\frac{1}{3} \) gastrulae following ablation may reflect increased axial stress within the gut rudiment late in gastrulation. In *Lytechinus pictus*, there is a transient rise in the length/width ratio along the animal–vegetal axis of cells in the archenteron at the \( \frac{1}{2} \) gastrula stage (Hardin, 1986), again suggesting that considerable tension is being exerted on the gut rudiment at this time. The reversion to a more isodiametric shape by the end of gastrulation (Hardin & Cheng, 1986; Hardin, 1986) suggests that the archenteron responds to this tension by cell rearrangement, thereby relieving stress generated at the \( \frac{1}{2} \) gastrula stage. Alternatively, SMCs may transmit some sort of signal to the cells of the archenteron wall which they require to continue active extension beyond the \( \frac{1}{2} \) gastrula stage. This signal could presumably be mediated by mechanical, chemical, cell contact or other cues. A final possibility that cannot be ruled out is that by ablating cells at the archenteron tip, enough cells were damaged so that the animalmost region of the archenteron wall was unable to participate in cell rearrangement late in gastrulation. Further experiments are needed to distinguish between these possibilities, although *prima facie* a direct role for mechanical tension seems to be most plausible.

Whatever the mechanism is by which SMCs facilitate elongation of the gut rudiment, individual filopodia do not contribute significantly to the steady-state extension of the archenteron. The absence of appreciable retraction of the archenteron tip following local ablation of SMCs or their filopodia, or even following ablation of all active SMCs at once, strongly suggests that the deformation of the archenteron is permanent at intermediate stages of elongation. This is perhaps not surprising in light of the fact that cell rearrangement occurs at all stages of elongation (Hardin & Cheng, 1986).

**The role of filopodial traction in guiding and stabilizing the archenteron**

In addition to their role in elongating the archenteron, the filopodia are probably important for several other reasons. First, it is likely that filopodial traction lends lateral stability to the elongating gut rudiment. In ablated embryos, the tip of the archenteron sometimes leans to one side, or buckles slightly, suggesting that filopodial tension normally helps to keep it erect. Second, filopodia appear to guide the archenteron to the site of the oral primordium. The observations of Gustafson & Kinnander (1960) provide strong evidence for such a guidance mechanism. In *Psammechinus miliaris*, once the archenteron has spanned the blastocoel, filopodia are sent out laterally to the site of the stomodeal invagination, apparently pulling the tip of the gut rudiment to the correct site for fusion with the oral ectoderm. Based on the ablation studies presented here, filopodia can guide the archenteron from side to side if filopodial tension is asymmetrically distributed around the archenteron; in the normal embryo, such asymmetry could be provided by the preferential attachment of filopodia to specific adhesion molecules expressed in the vicinity of the oral ectoderm (Gustafson & Wolpert, 1963; McClay & Ettensohn, 1987). However, further experiments designed to determine the specific molecular basis of filopodial attachment and guidance are needed if the role of filopodia in sea urchin gastrulation is to be more thoroughly understood.

I thank Dr Saul Zackson for suggesting the use of high-energy lasers to study sea urchin gastrulation and Dr Bruce Nicklas for the use of his Polaroid camera and three-quarter inch video recorder. The tremendous helpfulness of the staff of the Beckman Laser Institute was greatly appreciated, especially that of Jeff Andrews, Glenn Profeta and Tao Wen. I am especially grateful to Drs Ray Keller and Fred Witt for their constant encouragement during my graduate studies. Discussions with Drs Steve Black and
Dave McClay concerning the manuscript were extremely helpful. This work was supported by a NSF predoctoral fellowship and by NIH grants HD14503 to F. Wilt and HD18979 to R. Keller.

References


(Accepted 11 March 1988)