Differential behavior of centrosomes in unequally dividing blastomeres during fourth cleavage of sea urchin embryos

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Summary

The fourth cleavage in sea urchin embryos is unequal and represents an initial step in cell differentiation: vegetal blastomeres divide to produce micromeres, which are precursors of the skeletogenic mesenchyme, and macromeres. The mitotic spindles of these unequally dividing cells are peripherally located and lie orthogonal to the vegetal pole plasma membrane; the aster of the micromere pole is closely apposed to the plasmalemma and presents a characteristic flattened profile. In order to investigate the role of centrosomes in the generation of the asymmetric vegetal spindle at fourth cleavage, structural dynamics of centrosomes in both equally and unequally dividing blastomeres were compared using immunofluorescence methods. Quantitation of immunofluorescence and three-dimensional reconstruction techniques demonstrate that micromere centrosomes differ from macromere centrosomes in two respects: (1) micromere spindle poles contain less centrosomal material than macromere poles, and (2) micromere centrosomes undergo a specific filiform elongation during late anaphase and telophase. The behavior of micromere centrosomes suggests that a unique spindle pole event, involving interactions of the microtubular cytoskeleton, centrosome and cell cortex, occurs during the process of unequal cleavage of vegetal blastomeres.

Key words: micromere, macromere, mitosis, immunofluorescence, three-dimensional computer reconstruction.

Introduction

Cell division usually results in the production of two daughter cells similar in size and appearance. Instances of unequal cleavage, however, are relatively rare, with the exception that they commonly occur during embryonic differentiation in many animals. Examples of unequal division in early development include polar body formation during meiotic maturation of eggs (Wolpert, 1960), the first divisions of nematode (Albertson, 1984; Strome and Hill, 1988), oligochaete (Shimuzu, 1982) and mollusc (Allen, 1953; Dan and Ito, 1984) embryos, division of grasshopper neuroblasts (Carlson, 1977) and fourth division of sea urchin vegetal blastomeres (Okazaki, 1975; Dan, 1979). Unequal cleavage during embryogenesis is important in that it represents an initial event in cell differentiation. For example, sea urchin vegetal blastomeres divide unequally to produce micromeres, which are precursors of the skeletogenic mesenchyme, and macromeres.

Unequal division results from an eccentric positioning of the cleavage furrow, and the location of the cleavage furrow has been shown to be determined by the location of the spindle (Conklin, 1917; Harvey, 1935; Kawamura, 1960, 1977) or the spindle asters (Rappaport, 1969; Hiramoto, 1971). In sea urchin vegetal blastomeres, the spindle is assembled in an eccentric location, subsequent to a migration of the nucleus toward the vegetal pole (Dan, 1979). The peripheral spindle pole becomes closely associated with the plasmalemma; in fact, the peripheral aster is flattened and truncated (Dan and Nakajima, 1956), presumably due to the physical proximity of the plasma membrane. Dan and Ito (1984) suggested that the physical interaction of a spindle pole with the plasma membrane is an essential component of unequal division. In addition, the fact that sea urchin vegetal blastomeres possess dissimilar spindle poles raises the possibility that differential spindle pole behavior during mitosis and cytokinesis is also an integral part of unequal cleavage. Because centrosomes organize the spindle (Sluder and Rieder, 1985a), they may represent the organelles that engineer spindle pole behavior during unequal cleavage. To test this possibility, centrosomal dynamics during fourth cleavage of sea urchin embryos were examined by immunofluorescence methods in conjunction with laser scanning confocal microscopy and three-dimensional computer reconstruction techniques in order to correlate centrosomal morphology with the structural events characterizing this unequal cell division.

Materials and methods

Eggs and sperm from the sea urchins Lytechinus pictus and Strongylocentrotus purpuratus were obtained by injecting 0.5 M KCl into the coelomic cavity; eggs were shed into artificial seawater (AFSW) and sperm were collected 'dry' on ice. Fertilization envelopes were removed by inseminating eggs in
Immunofluorescent labeling

Specimens of *L. pictus* were transferred to Ca\(^{2+}\)-free AFSW shortly after third cleavage (about 190 min post-insemination). Because of the tendency of sea urchin embryos to dissociate in Ca\(^{2+}\)-free AFSW, numerous isolated blastomeres, blastomere pairs and blastomere tetrads were obtained. At various time-points from about 200–250 min post-insemination, blastomeres were pipetted onto polylysine-coated coverslips and allowed to adhere for only 1 min or less in order to avoid cytoskeletal breakdown and strong signal exhibited by this antibody.

Centrosomes were labeled with one of two monoclonal antibodies, Ah6 (described previously by Schatten et al. 1987) or 4D2. Monoclonal antibody 4D2 was raised against purified centrosomal material from *S. purpuratus* (Thompson-Coffe et al. unpublished) and was found to produce staining patterns similar to those obtained with the anti-centrosomal antibodies Ah6 and 3651 (Calarco-Gillam et al. 1983, Schatten et al. 1986, 1987). All of the immunofluorescent centrosomal images depicted in this report were taken from 4D2-labeled specimens because of the low background and strong signal exhibited by this antibody. Methanol-fixed or extracted and fixed embryos were incubated in undiluted 4D2 hybridoma culture medium for 1 h at 37°C. Following fixation, specimens were incubated for 1 h at 37°C with goat anti-mouse polyvalent antibodies (anti-IgG, -IgA and -IgM) conjugated to fluorescein (Zymed Laboratories, Inc., San Francisco, CA) diluted 1:100. Embryos were counterstained with 5 μg/ml Hoechst 33258 (Polysciences, Inc., Warrington, PA) to label DNA, washed in PBS and mounted in 90% glycerol and 10% glycerol and 1.24,4-triazole (Sigma Chemical Co., St Louis, MO) and subsequently passing zygotes or embryos through 54–74 μm mesh nylon membranes (Showman and Foerder, 1979). Embryos were cultured in suspension in artificial seawater with stirring until fourth cleavage, at which time they were prepared for either fluorescent labeling or spindle isolation.

**Spindle isolation**

At approximately 30 min prior to the onset of fourth cleavage, cultured *S. purpuratus* embryos were treated with 0.6% trypsin in Ca\(^{2+}\)-free AFSW for 10 min at 12°C. Embryos were then washed with 0.1 mg/ml soy bean trypsin inhibitor, 5 mM benzamidine and 100 μM PMSF (phenylmethylsulfonyl fluoride) in Ca\(^{2+}\)-free AFSW for 10 min at 12°C. When most embryos appeared to reach metaphase of fourth cleavage, they were gently pelleted with a hand centrifuge and suspended in lysis medium consisting of 1 mM glycerol, 25 mM Pipes, 0.5 mM EGTA and 100 mM PMSF. Spindles were collected from lysed cells by low-speed centrifugation and resuspended and stored at 4°C in lysis medium containing 25% glycerol.

**Results**

Although animal and vegetal blastomeres are about the same size in eight-cell *S. purpuratus* embryos, vegetal blastomeres are slightly smaller than animal blastomeres at the same stage in *L. pictus* (Fig. 1A). In both dissociated and intact embryos, blastomere divisions are already somewhat asynchronous by fourth cleavage. By prophase of fourth cleavage, vegetal blastomere nuclei have migrated close to the vegetal pole, while animal blastomere nuclei remain positioned near the cell center (Fig. 1A,F). In early prophase, centrosomal material circumscribes the nucleus (Fig. 1B). As prophase progresses, bipolarization of centrosomal material occurs: the thin, diffuse nuclear blanket of centrosomal material condenses to form two aggregations on opposite sides of the nucleus (Fig. 1G). Bipolarization originates in an orientation predictive of the ensuing cleavage pattern of the animal and vegetal blastomeres (Fig. 1D,E).

Shortly after bipolarization, the nuclear envelope breaks down and spindle formation occurs. Spindles form centrally within animal blastomeres but are assembled in an eccentric position close to the vegetal pole in vegetal blastomeres (Fig. 2A). During prometaphase, centrosomal material continues to condense and eventually forms compact spheres at late metaphase (Fig. 2B). Isolated spindles from animal and vegetal blastomeres display distinct differences with respect to astral organization. Animal blastomere spindles possess symmetrical, radiate asters resembling those formed during the first three equal cleavages. By contrast, the aster adjacent to the vegetal pole plasmalemma in vegetal blastomeres, i.e. the micromere aster, is highly flattened (Fig. 2C,D).

A total of 60 maximally compacted centrosomes from both animal and vegetal blastomeres were measured for fluorescence intensity (mean pixel value) and volume. The product of the volume and mean fluorescence intensity was used as a quantitative measure of centrosomal material present within each spindle pole; by this method.
measure, spindle poles segregating to micromeres contained significantly less 4D2-immunoreactive centrosomal material than did the macromere spindle poles (Fig. 3).

Additionally, the sum of the mean values for macromere and micromere poles was slightly less than twice the sum of the mean animal centrosomal value (Table 1). This indicates that micromere spindle poles contain less immunoreactive centrosomal material than macromere spindle poles (36% and 64% of total vegetal blastomere immunoreactive centrosomal material, respectively), and that the total amount of immunoreactive centrosomal material within vegetal blastomeres is somewhat less than the amount within animal blastomeres (47% and 53% of combined amount of vegetal and animal blastomere immunoreactive centrosomal material, respectively) in L. pictus.

As anaphase progresses, micromere spindle pole centrosomes begin to elongate, while macromere pole centrosomes remain spherical (Fig. 4B). The micromere centrosome continues to elongate throughout late anaphase and telophase and eventually resembles a long thread (Fig. 4E,H,K). Three-dimensional reconstruction indicates that the initial elongation of micromere centrosomes results in the formation of a thin oval plate that progressively becomes more filiform as elongation continues (Fig. 5). By telophase, macromere centrosomes flatten slightly, giving rise to a thick disk-like structure (Fig. 5).

Immunofluorescent labeling of microtubules indicates that the microtubule organizing centers of both macromeres and micromeres split by late telophase (Fig. 4J). By early interphase following fourth cleavage, centrosomal material decondenses and begins to spread out and cover the nuclear surface once again.

Unlike the differential behavior of micromere and macromere centrosomes throughout anaphase and telophase, both spindle pole centrosomes of equally dividing animal blastomeres follow a common pattern of structural reorganization. During anaphase, the previously spherical centrosomes elongate slightly and generally form oval disks (Fig. 4L); these disks lengthen somewhat through-out telophase and eventually decondense to envelope the nucleus by early interphase following fourth cleavage in a manner similar to that of the vegetal centrosomes.

Discussion

The products of the unequal fourth cleavage of sea urchin embryos are macromeres and micromeres (reviewed by Okazaki, 1975; Horstadius, 1975; Dan, 1984; Davidson,
Fig. 2. (A) Extracted S. purpuratus blastomeres at late metaphase/early anaphase of fourth cleavage, differential interference contrast microscopy. The spindle of the animal blastomere (upper cell) is centrally located, whereas that of the vegetal blastomere (lower cell) is eccentrically positioned next to the vegetal pole. Bar, 25 μm. (B) Methanol-fixed animal (upper cell) and vegetal (lower cell) blastomeres of L. pictus at a stage similar to A, i.e. late metaphase. Double exposure showing compacted centrosomes flanking chromosomes aligned on the metaphase plate (arrows). Note that the micromere (mi) centrosome is smaller than the macromere (ma) centrosome. Same magnification as A. (C) and (D) Metaphase and anaphase spindles, respectively, from S. purpuratus vegetal blastomeres at fourth cleavage. The micromere aster (mi) is flattened, whereas the macromere aster (ma) is radiate. Bar, 10 μm.

1989). Micromere progeny differentiate to form the primary, skeletogenic mesenchyme, and unequal division is thought to constitute the initial step in this differentiation (Dan et al. 1983). Tanaka (1976) found that when fourth cleavage of Hemicentrotus pulcherrimus, Temnopleurus torematicus and Pseudocentrotus depressus embryos were made equal by detergent treatment, deficiencies in primary mesenchyme formation occurred. Some species of sea urchins, however, are still able to produce skeletal structures following equalization of the fourth division by detergent treatment (Langelan and Whitley, 1985), presumably by the conversion of secondary mesenchyme cells into skeletogenic mesenchyme (Ettensohn and McClay, 1988).

Spindles are peripherally located in unequally dividing cells

A hallmark of unequally dividing cells is a peripherally positioned spindle that closely adjoins the plasma membrane. Specific mechanical linkages between the spindle and plasma membrane have been demonstrated for a number of unequally dividing cell types (Conklin, 1917; Harvey, 1935; Lutz et al. 1988), and Dan and Ito (1984) suggested that spindle–membrane interaction constitutes a fundamental prerequisite for unequal division. It appears that a spindle can attain an eccentric location within the cell by two distinct mechanisms: (1) peripheral migration of a centrally formed spindle, and (2) peripheral migration of the nucleus prior to spindle assembly.

Centrally formed spindles migrate peripherally prior to cytokinesis during meiotic maturation or early development in the eggs and embryos of nematodes (Albertson, 1984), annelids (Shimizu, 1982; Hamaguchi et al. 1983; Lutz et al. 1988) clams (Longo and Anderson, 1970; Dan and Ito, 1984) and mice (Longo and Chen, 1985). In contrast, the fourth division of sea urchin vegetal blastomeres begins with migration of the nucleus toward the vegetal pole (Dan, 1979, 1984; Lutz and Inoue, 1982; Schroeder, 1987). In Strongylocentrotus purpuratus, nuclear migration is well advanced by late prophase, at which time one centrosome (the macromere centrosome) is centrally located while the other (micromere centrosome) lies subjacent to the vegetal pole plasma membrane (Schroeder, 1987). The mitotic spindle at fourth cleavage in sea urchins is therefore assembled in an eccentric location.

Peripheral spindles may possess dissimilar asters

In many types of unequally dividing cells, peripheral spindles are oriented orthogonal to the plasma membrane. In contrast, mouse oocytes display anastral spindles that lie tangential to the plasma membrane. During polar body formation, however, mouse oocyte spindles rotate to an orthogonal position (e.g. see Sato and Blandau, 1979). In orthogonal spindles, the spindle pole adjoining the plasmalemma (the peripheral pole) frequently displays a truncated or flattened aster, and it has been suggested that differential astral growth or disassembly represents the mechanism by which these spindles attain their eccentric location (Jahizaka, 1969; Czihak, 1973; Kawamura, 1977). Dan and Ito (1984) argue, however, that, at least in Spisula oocytes, both asters are similar, one merely becoming deformed as a result of its close apposition to the plasma membrane. The asters of Chaetopterus sp. eggs also appear to be identical: the flattened peripheral aster is able to regain a radiate shape when mechanically displaced from the membrane by micromanipulation methods, and either aster is capable of
forming a mechanical linkage with the plasmalemma (Lutz et al. 1988).

Quantitative differences in the amount of immunoreactive centrosomal material between the macromere and micromere poles at metaphase suggest that spindle poles of sea urchin vegetal blastomeres at fourth cleavage are distinct. Furthermore, micromere asters remain flattened after removal from the cell, indicating that
either specific structural elements are present within the aster that help maintain its flattened shape, or the micromere aster is intrinsically radiate in only two dimensions. The centrosome model advanced by Mazia (1984) could conceivably produce such a two-dimensionally radiate aster if the microtubule nucleating subunits were appropriately arranged. Interaction of the micromere pole with the plasmalemma appears to be required for the assembly of a two-dimensionally radiate aster, because the detergent treatment used to equalize fourth cleavage produces centrally formed spindles that display asters similar in size and shape (Tanaka, 1976).

The dissimilar asters of vegetal blastomeres contain dissimilar centrosomes

Two differences were noted between the peripheral (micromere) and central (macromere) spindle pole centrosomes in sea urchin vegetal blastomeres at fourth cleavage: (1) micromere centrosomes contain less 4D2-immunoreactive material than macromere centrosomes at metaphase, and (2) micromere centrosomes undergo a unique filiform elongation during late anaphase and telophase. Quantitation of 4D2 immunofluorescence shows that the fourth cleavage of vegetal blastomeres is unequal not only in the division of cytoplasm, but also in the partitioning of centrosomal material. Although the centrosomal component carrying the 4D2-binding epitope has not been identified biochemically, the labeling pattern produced by this monoclonal antibody resembles that obtained with the well-characterized anti-centrosomal antibodies 5051 and Ah6 (Calarco-Gillam et al. 1983; Schatten et al. 1986, 1987, 1988). The labeling pattern produced with all three of these antibodies appears to correspond to the distribution of peri-centriolar material; for this discussion, we will simply refer to this as 'centrosomal material'. Notwithstanding the lack of biochemical information regarding the 4D2-binding epitope, this antibody has revealed significant behavioral differences between the centrosomes of sea urchin blastomeres at fourth cleavage.

The difference in the amount of centrosomal material present within each metaphase spindle pole primarily reflects differences in the volumes of the spherical centrosomes, because the mean pixel value, i.e. intensity of fluorescence, is closely comparable between animal, macromere and micromere centrosomes (Fig. 3). It might be argued that micromere centrosomes possibly contain the same amount of material as macromere centrosomes but are more condensed, resulting in steric hindrance during immunolabeling and artifically low pixel values. This appears not to be the case for three reasons: (1) by this argument, a correlation should exist between centrosomal spherical volume (i.e. degree of condensation of centro-

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**Table 1. Relative amounts of centrosomal material in animal and vegetal blastomeres**

<table>
<thead>
<tr>
<th></th>
<th>Mean pixel value × volume*</th>
<th>Animal versus vegetal blastomere† (%)</th>
<th>Macromere versus micromere centrosome (%)</th>
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</thead>
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<tr>
<td>Animal blastomere</td>
<td>Animal spindle pole</td>
<td>14375</td>
<td>53</td>
</tr>
<tr>
<td>Vegetal blastomere</td>
<td>Macromere spindle pole</td>
<td>16566</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Micromere spindle pole</td>
<td>9221</td>
<td>36</td>
</tr>
</tbody>
</table>

*Mean pixel value × volume derived from measurements of 32 animal blastomere centrosomes, 24 macromere centrosomes and 24 micromere centrosomes. Possible pixel values ranged from 0 to 256; volume expressed in μm³ (see Fig. 3).
†Total amount of centrosomal material in animal blastomeres estimated by multiplying mean value of one spindle pole by 2; total centrosomal material in vegetal blastomeres obtained by adding macromere and micromere poles.
Fig. 4. Elongation of micromere centrosome throughout anaphase and telophase, methanol-fixed *L. pictus* vegetal blastomeres. 

(A)-(C) Late anaphase; (A) phase contrast, (B) centrosome immunofluorescence, (C) DNA fluorescence. Micromere centrosomes (mi) are in an early stage of elongation. 

(D)-(F) Telophase; (D) phase contrast, (E) centrosome immunofluorescence, (F) DNA fluorescence. Micromere centrosomes (mi) are distinctly elongate, but macromere centrosomes (ma) remain spherical. 

(G)-(J) Late telophase; (G) phase contrast, (H) centrosome immunofluorescence, (I) DNA fluorescence. Micromere centrosome (mi) appears thread-like. (J) Tubulin immunofluorescence of a different, extracted blastomere at similar stage to G; microtubule organizing centers have split at both macromere (black arrows) and micromere (white arrows) poles. 

(A)-(I) Same magnification; bar in A, 26 /μm; bar in I, 10 /μm. 

(K) and (L) Comparison of equal and unequal cleavage at telophase, double exposure showing centrosome and DNA fluorescence in vegetal (K) and animal (L) blastomeres. Note the unique elongation of the micromere (mi) centrosome. ma, macromere centrosome; ch, chromosomes. Bar, 10 /μm.
Fig. 5. Computer reconstruction of vegetal centrosomes at late anaphase (A) and late telophase (C). (B) Same centrosome pair in A, rotated 80 degrees. The two angles, (A) and (B), show that the micromere centrosome (mi) is flattened and oval at early anaphase. (D) Same centrosome pair in C, rotated 80 degrees; angles (C) and (D) demonstrate that by late telophase, the micromere centrosome (mi) has become long and thread-like, whereas the macromere centrosome (ma) has flattened slightly. (A) and (C) Same magnification; bar, 5 \( \mu m \). (B) and (D) Same magnification; bar, 5 \( \mu m \).

The micromere spindle pole centrosome is different from the other centrosomes of the embryo at fourth cleavage in that it undergoes a striking filiform elongation during late anaphase and telophase. This elongation may be microtubule-dependent, because colchicine has been shown to affect centrosomal shape during the first cell cycle (Schatten et al. 1988). In this study, however, obvious differences between the microtubule organizing centers of the macromere and micromere poles have not been observed at telophase, with the important exception that the micromere microtubule organizing-center is closely apposed to the vegetal plasmalemma (Fig. 4J). It is possible that elongation of the micromere centrosome is mediated by the combined influences of the microtubular cytoskeleton and the cortical cytoplasm, including the plasma membrane.

Functional implications of specialized centrosomes in unequal cleavage

During the first three cleavage divisions, all centrosomes undergo similar structural rearrangements following bipolarization (see Boveri, 1901; Paweletz et al. 1984, 1987a,b; Schatten et al. 1986, 1987, 1988). During the fourth division, by contrast, micromere centrosomes behave differently from macromere and animal blastomere centrosomes. It therefore appears that in sea urchin embryos equal cleavages are characterized by equivalent centrosomes, whereas unequal cleavage involves specialized, divergent centrosomal behavior. The striking elongation exhibited by the micromere centrosome at late anaphase and telophase suggests that a unique event is occurring at this pole. It is tempting to presume that this event involves a specific interaction of this spindle pole with the plasmalemma, and it is intriguing that centrosomal elongation begins at about the same time as cytokinesis. Thus, although models of cytokinesis that rely upon forces produced by the aster have been largely supplanted, unequal division may represent a special case where microtubule dynamics are closely involved in the physical division of cytoplasm.

Quantitation of immunofluorescence indicates that vegetal centrosomes are already distinct from each other by metaphase, and it is possible that these quantitative differences arise during bipolarization. Unequal centrosomal inheritance may serve to maintain the proportionality of the centrosome-to-cytoplasm ratio in unequally dividing cells. It is interesting that centrosomal material is controlled in this fashion, because the essential reproductive capacity of sea urchin centrosomes has been shown to be correlated with the presence of centrioles, and not peri-centriolar material (Sluder and Rieder, 1985). This suggests that, in addition to receiving the necessary elements for centrosomal replication, the total amount of centrosomal material a daughter cell receives is also important. It is particularly intriguing that, in addition to the inequality of macromere/micromere centrosomal inheritance, vegetal blastomeres, which are slightly smaller than animal blastomeres in _L. pictus_, also receive slightly less centrosomal material (Table 1). It may be that the amount of centrosomal material a daughter cell inherits is closely correlated with the amount of cytoplasm it receives.

In summary, micromere spindle pole centrosomes exhibit quantitative and structural differences from macromere and animal pole centrosomes at fourth cleavage. The behavior of micromere centrosomes indicates that a unique reorganization of the micromere spindle pole and centrosome occurs within the vegetal pole cortex during fourth division. Centrosomes may therefore play a fundamental role in unequal cell division during fourth cleavage; such a role is of considerable developmental
importance because unequal division in sea urchin embryos constitutes a prelude to cell differentiation.

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