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Blastodisc morphology during first cleavage of the *Oryzias latipes* (medaka) fish egg

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Abstract The objectives of the study were a) to monitor changes in the morphology of the blastodisc of the fertilized medaka egg during the first cleavage, and b) to correlate these changes temporally to mitotic events. The morphology of the blastodisc was monitored by time-lapse video microscopy and an image analysis system. To see the boundary between the blastodisc and the yolk vacuole more clearly, we microinjected FITC-dextran into the yolk vacuole. To monitor mitotic events, we either microinjected Hoechst 33342 into living eggs or stained fixed eggs with Hoechst 33258; these eggs were monitored via a SIT camera and video monitor. We found that the first sign of cytokinesis is the formation of an internal furrow that forms at the surface between the blastodisc and the yolk vacuole. At 25°C, this furrow begins to form 3 minutes after the metaphase-anaphase transition and 7 minutes before the external furrow begins to form.

Introduction

In his magnum opus on mitosis, Mazia (1961, p. 314) wrote that cytokinesis is "governed by the mitotic apparatus, both in time and place": Cytokinesis begins only after the metaphase-anaphase transition, and the plane of cytokinesis is normally perpendicular to the midpoint of the mitotic spindle. However, the causal links between mitotic and cytokinetic events have eluded cell biologists and were among the central questions addressed at a recent conference on cytokinesis (Conrad and Schroeder, 1990; Inuoé, 1990).

We are pursuing these causal links in the medaka egg. This large (diameter ≈ 1000 μm) clear egg can be obtained year-round (Yamamoto, 1967) and is particularly suitable for light microscopic studies. For example, this egg was used recently to visualize slow calcium waves that accompany cytokinesis (Fluck *et al.*, 1991). The cytoplasm of the egg is bounded by two membranes: a plasma membrane at its outer (external) surface, which separates the cytoplasm from the perivitelline space; and a yolk membrane at its inner (internal) surface, which separates the cytoplasm from a large, central yolk vacuole. During the first hour after fertilization, most of the cytoplasm streams toward the animal pole of the egg and forms a blastodisc (Abraham *et al.*, 1993). Then meroblastic cleavage begins.

Changes in the morphology of the blastodisc of fish eggs during the first cleavage have been described in several reports. Agassiz and Whitman (1884) found in four species of pelagic fish that "the first cleavage begins, not by a groove on the external surface of the blastodisc, but by one on the internal face," that is the interface between the blastodisc and the yolk vacuole; while Kamito (1928), studying the medaka, reported that the "cleavage furrow appears first as a cleft extending from the interior towards the exterior of the germinal disc." (Other images of this peculiar structure can be seen in Fig. 2b of Plate 1 in Yamamoto (1975) and in Fig. 4 in Iwamatsu (1976)). None of these studies determined whether the formation of the internal groove begins after the metaphase-anaphase transition (and is thus a cytokinetic event) or gave a detailed description of the timing of the formation of the internal and the external grooves. The purpose of the present study was to determine the timing of these events in the medaka egg.

Materials and Methods

We removed gonads from breeding medaka (Yamamoto, 1967; Kirchen and West, 1976; Abraham *et al.*, 1993) and placed them in a balanced saline solution (BSS: 111 mM NaCl; 5.37 mM KCl: 1.0 mM $CaCl_2$: 0.6 mM $MgSO_4$; 5 mM HEPES, pH 7.3). Eggs were removed from the ovary, the long chorionic fibers at the vegetal pole were removed with scissors, and the eggs were fertilized in BSS by mincing testis to release sperm (Yamamoto, 1967). For microscopic observation of fertilized eggs, they were transferred to a microscope slide on which a cover glass was supported by four pillars of petroleum jelly. The cover

glass was then pressed gently against the chorion to flatten a small region of the egg. Such flattening facilitated optical studies and also enabled us to roll the egg to achieve the desired orientation (Abraham *et al.*, 1993). All procedures were performed at room temperature (23–26°C); in this temperature range, the first cell division begins about 70 min after fertilization. Because the rate of development varies with temperature, we have reported the timing of events not only as "minutes after fertilization" but also as "normalized time" (t_n), where t_n is defined as 1.0 when cytokinesis begins.

Relation between mitotic and cytokinetic events

We used four protocols to determine the temporal relationship between mitotic and cytokinetic events. In the first protocol, seven eggs from one female were fertilized simultaneously. Each egg was placed on a microscope slide and observed via an inverted microscope (Nikon Diaphot). Measurements of three morphological parameters of the blastodisc (Fig. 1) were made at frequent intervals (4–6 min) with commercial image analysis software (Microcomp Planar Morphometry, Southern Micro Instruments, Atlanta, Georgia). At specific times before and during cytokinesis, individual eggs were fixed in 3.7% formaldehyde in BSS for at least 36 hr. We then mechanically dechorionated the eggs and stained the nuclei with Hoechst 33258 (10 μg ml^{-1} in BSS containing 1% (w/v) Triton X-100). The eggs were then viewed with epifluorescence optics via a Nikon Optiphot microscope coupled to a SIT camera and video monitor. Internuclear distances were measured on the screen of the monitor, and appropriate scale conversions were made.

In the second protocol, we used eight eggs from three females. The eggs were fertilized one at a time and monitored until the completion of cytokinesis. This protocol enabled us to measure the blastodisc of each egg at more frequent intervals (1–2 min).

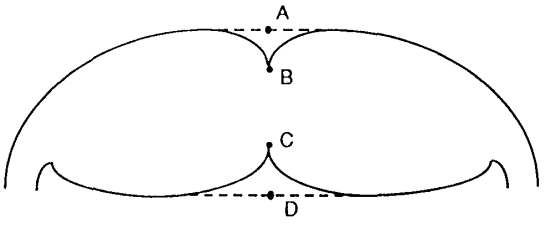

Fig. 1. Morphological parameters measured on the blastodisc. All measurements were made along the aminal-vegetal axis (pole-to-pole) of the egg while viewing the blastodisc from the side. AD, thickness of the blastodisc; AB, depth of external furrow; CD, depth of the internal furrow.

In the third protocol, we used 22 eggs from two females and divided them into three batches of 6–8 eggs each. After fertilizing a batch of eggs, we measured the blastodisc of one of the eggs at very frequent intervals and fixed the others at 4-min intervals just before and during cytokinesis. Internuclear distances were measured as above.

In the fourth protocol, we used 22 eggs from three females. Immediately after fertilizing them, we injected Hoechst 33342 (1.5 nl of 100 μg ml^{-1} dissolved in 50 mM K_2SO_4 and 10 mM HEPES, pH 7.2) into the cytoplasm to stain the nuclei (Abraham *et al.*, 1993). After incubating the eggs in the dark for 45 min to minimize photodamage, we viewed them intermittently (0.5–5 min intervals) with either epifluorescence optics to see the nuclei or brightfield optics to see the blastodisc.

Injection of FITC-dextran into the yolk vacuole

To see the boundary between the blastodisc and the yolk vacuole more clearly, we injected 10 nl of FITC-dextran (mol. wt. = 17,200 daltons; 10 μg ml^{-1} dissolved in 50 mM K_2SO_4 and 10 mM HEPES, pH 7.2) into the yolk vacuole of just-fertilized eggs and observed them with epifluorescence optics.

Results

Changes in the morphology of the blastodisc before and during cleavage

By about 1 hr after fertilization ($t_n \approx 0.8$), most of the ooplasm has streamed to the animal pole of the egg and formed a blastodisc; when viewed from the side, the blastodisc is biconvex at this time (Fig. 2a). Soon thereafter, the internal surface of the blastodisc begins to flatten, changing the shape of the blastodisc to plano-convex (Fig. 2b); this flattening is a sign that cytokinesis will soon begin. The first sign of cytokinesis is the formation of a furrow at the internal surface of the blastodisc (Fig. 2c). About 7 min later, a furrow forms at the external surface (Fig. 2d); this furrow forms in the same plane as the internal one. The external furrow then deepens, cutting the blastodisc in half, while the internal furrow simultaneously recedes. Finally, the surfaces of the two blastomeres "zip" together (Fig. 2f).

That the formation of the internal furrow was accompanied by movement of the yolk membrane —and was not just a localized accumulation of vesicles near the center of the internal surface of

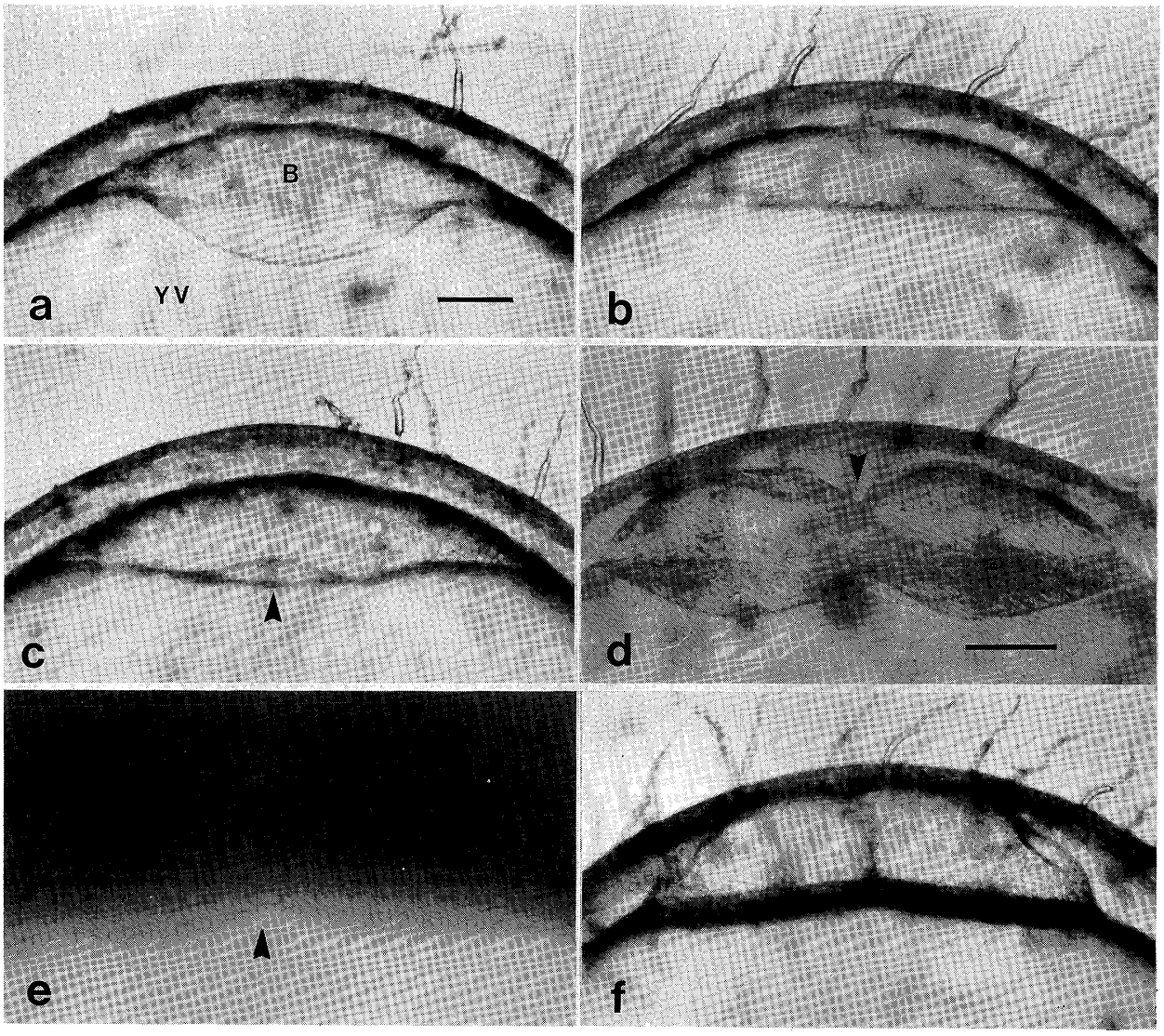

Fig. 2. Morphology of the blastodisc before and during cytokinesis. The fertilized eggs were grown at 25°C. a) 51 min after fertilization; $t_n \approx 0.8$. The interface between the blastodisc (B) and the yolk vacuole (YV) is convex; the overall shape of the blastodisc is biconvex. b) 58 min after fertilization; $t_n \approx 0.9$. The interface between the blastodisc and yolk vacuole has flattened, a sign of impending cytokinesis. c) 64 min after fertilization; $t_n = 1.0$ by definition. The central portion of the internal surface of the blastodisc (arrowhead) has begun to rise toward the plasma membrane; this is the internal furrow. d) 71 min after fertilization; $t_n \approx 1.1$. A furrow has begun to form at the external surface of the blastodisc (arrowhead). e) This is the same egg as the one shown in Fig. 2d. To determine whether the yolk membrane was moving toward the plasma membrane, we injected FITC-dextran into the yolk vacuole and then photographed the same microscopic field with brightfield (d) and epifluorescence optics (e). Note how closely the fluorescent image of the yolk vacuole conforms to the internal surface of the blastodisc, including the internal furrow (arrowhead). f) 89 min after fertilization; $t_n \approx 1.4$. The interface between the blastodisc and yolk vacuole has flattened, and the blastomeres have zipped up against each other. Scale bars, 100 μm. Figure 2a, 2b, 2c, and 2f are printed at the same magnification and Figures 2d and 2e at another.

the blastodisc—was shown by injecting FITC-dextran into the yolk vacuole. In such eggs, a clear delineation could be seen between the yolk vacuole and the blastodisc, and it could clearly be seen that a furrow had been formed along the internal surface of the blastodisc (Fig. 2e).

These events are summarized quantitatively in Fig. 3, in which changes in the thickness of the blastodisc and the depths of the internal and external furrows in a single egg are shown to occur in the following sequence: 1) The thickness of the blastodisc increased to about 135 μm and then decreased to about 110 μm; 2) the internal furrow began to form; 3) the external furrow began to form. This temporal sequence was confirmed when data from a number of eggs were combined (Table 1).

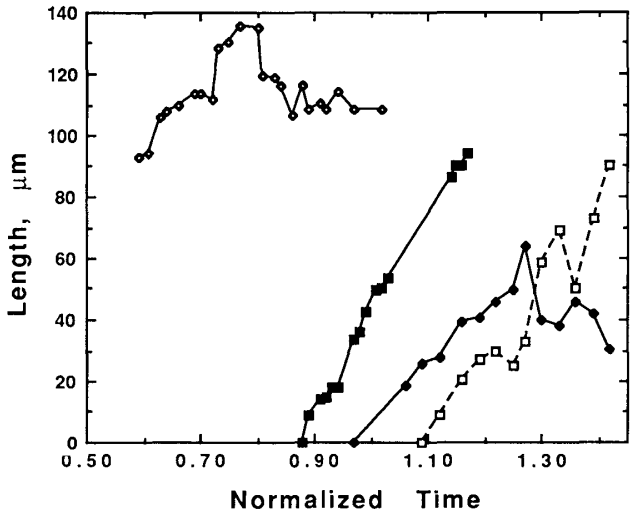

Fig. 3. Changes in the thickness of the blastodisc, the lengths of the internal and external furrows, and internuclear distance during mitosis and cytokinesis. All measurements of the blastodisc were made on one egg, and those of internuclear distances on another; both eggs were grown at 25°C. The thickness of the blastodisc (◇—◇, AD in Fig. 1) increased to 135 µm by $t_n \approx 0.8$ and then decreased to 110 µm by $t_n \approx 0.9$. The metaphase-anaphase transition, as measured by an increase in internuclear distance (■—■) began at $t_n \approx 0.9$. The internal furrow began to form at $t_n \approx 1.0$ (◆—◆, CD in Fig. 1) and the external furrow to form at $t_n \approx 1.10$ (□—□, AB in Fig. 1). The depth of the internal furrow increased to 65 µm and then began to decrease.

Table 1. Timing of mitotic and cytokinetic events

Event	Number of eggs	Minutes after fertilization[a] X±S.D.	t_n X±S.D.
Metaphase-anaphase transition	9	61.0 ± 6.4	0.89 ± 0.06
Internal furrow begins to form	17	64.4 ± 2.7	1.00[b]
External furrow begins to form	13	71.2 ± 2.9	1.11 ± 0.03

[a]Eggs were grown at 25°C.
[b]The beginning of this event for each egg was defined as $t_n = 1.0$.

The internal furrow forms after the metaphase-anaphase transition

When we microinjected Hoechst 33342 into living eggs and simultaneously monitored both mitosis (Fig. 4) and changes in themorphology of the blastodisc in individual eggs, we found in every egg (N = 6) that the metaphase-anaphase transition occurred before the internal furrow began to form. Data from other eggs are summarized in Fig. 3 and Table 1.

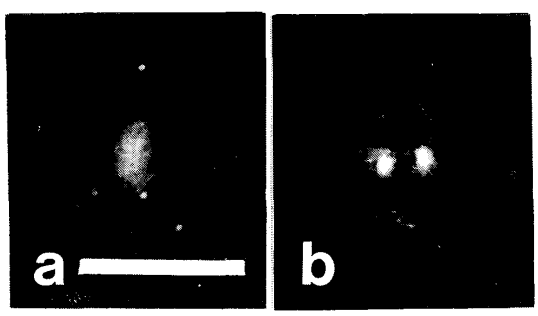

Fig. 4. Metaphase and anaphase in living eggs stained with Hoechst 33342. a) metaphase; b) anaphase. Scale bar, 50 µm.

Discussion

The internal furrow a) formed in the same plane as the external furrow and b) appeared after the metaphase-anaphase transition. Thus we conclude that the internal furrow is, in fact, a cytokinetic structure in the medaka egg. We have seen this internal furrow during the second and third cleavages as well (data not shown), and it thus appears to be a feature of at least the early cleavages in the medaka egg.

The identification of the internal furrow as a cytokinetic structure raises several questions about the generation and maintenance of the cleavage furrow in the medaka egg. For example, is the formation of the internal furrow caused by the generation and contraction of a contractile band/arc of microfilaments (Schroeder, 1990)? What is the structural relationship between the internal and external furrows, both of which originate near the center of the blastodisc? Do two contractile bands of microfilaments form—one at the internal surface and another at the external surface—and then meet at the lateral margins of the blastodisc? During cytokinesis, the margin of the blastodisc,

where these two bands would be expected to meet, appears to be under tension and is the site of persistent elevated cytosolic [Ca^{2+}] (Fluck *et al.*, 1991). We have seen a faint band of microfilaments in eggs that were fixed when the internal furrow was forming and that were subsequently stained with BODIPY-phallacidin (Fluck and Webb, unpublished observations); however, given the thickness of the blastodisc and the distortion caused by fixation, resolution of this question will likely require the injection of fluorescent derivatives of phalloidin into living eggs and analysis via a confocal microscope and/or an image processor (Cao and Wang, 1990). Another question raised by this study is whether a slow wave of elevated calcium accompanies the generation of the internal furrow as previously reported for the formation and "zipping" of the external furrow (Fluck *et al.*, 1991).

The formation of the internal furrow before the external one may reflect the position of the mitotic apparatus vis-a-vis the two respective surfaces of the egg. In sand dollar eggs, there is evidence for the movement of a cleavage stimulus from the mitotic apparatus to the cell surface. Thus the time interval between the appearance of furrows at two sites on opposite sides of the egg is a function of the proximity of the mitotic apparatus to the two surfaces (Rappaport, 1973). We are pursuing this question in our laboratory.

The medaka egg also appears to be an excellent system in which to pursue the question of the formation of a telophase disc during cytokinesis. This organelle, which has been described in HeLa cells and human lymphoma cells, forms in the midplane of the mitotic spindle during anaphase, grows toward the cell surface, and has been proposed to initiate cytokinesis (Andreassen *et al.*, 1991; Margolis and Andreassen, 1993). The disc contains myosin II and proteins that were attached to the centromeres of chromosomes until mid-anaphase. In terms of its shape and the timing of its appearance, this disc is reminiscent of the diastema, which has been defined as "an outward extension of the metaphase plate ... toward the cell surface" (Selman, 1982; see also Zotin, 1964, and Sawai and Yomota, 1990) which foreshadows the future cleavage plane.

Acknowledgements

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