Microtubule arrays of the zebrafish yolk cell: organization and function during epiboly

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SUMMARY

In zebrafish (*Danio rerio*), meroblastic cleavages generate an embryo in which blastomeres cover the animal pole of a large yolk cell. At the 500-1000 cell stage, the marginal blastomeres fuse with the yolk cell forming the yolk syncytial layer. During epiboly the blastoderm and the yolk syncytial layer spread toward the vegetal pole. We have studied developmental changes in organization and function during epiboly of two distinct microtubule arrays located in the cortical cytoplasm of the yolk cell. In the anuclear yolk cytoplasmic layer, an array of microtubules extends along the animal-vegetal axis to the vegetal pole. In the early blastula the yolk cytoplasmic layer microtubules appear to originate from the marginal blastomeres. Once formed, the yolk syncytial layer exhibits its own network of intercrossing mitotic or interphase microtubules. The microtubules of the yolk cytoplasmic layer emanate from the microtubule network of the syncytial layer.

At the onset of epiboly, the external yolk syncytial layer narrows, the syncytial nuclei become tightly packed and the network of intercrossing microtubules surrounding them becomes denser. Soon after, there is a vegetal expansion of the blastoderm and of the yolk syncytial layer with its network of intercrossing microtubules. Concomitantly, the yolk cytoplasmic layer diminishes and its set of animal-vegetal microtubules becomes shorter.

We investigated the involvement of microtubules in epiboly using the microtubule depolymerizing agent nocodazole and a stabilizing agent taxol. In embryos treated with nocodazole, microtubules were absent and epibolic movements of the yolk syncytial nuclei were blocked. In contrast, the vegetal expansion of the enveloping layer and deep cells was only partially inhibited. The process of endocytosis, proposed to play a major role in epiboly of the yolk syncytial layer (Betchaku, T. and Trinkaus, J. P. (1986) *Am. Zool.* 26, 193-199), was still observed in nocodazole-treated embryos. Treatment of embryos with taxol led to a delay in all epibolic movements.

We propose that the yolk cell microtubules contribute either directly or indirectly to all epibolic movements. However, the epibolic movements of the yolk syncytial layer nuclei and of the blastoderm are not coupled, and only movements of the yolk syncytial nuclei are absolutely dependent on microtubules. We hypothesize that the microtubule network of the syncytial layer and the animal-vegetal set of the yolk cytoplasmic layer contribute differently to various aspects of epiboly. Models that address the mechanisms by which the two microtubule arrays might function during epiboly are discussed.

Key words: teleost, yolk syncytial layer, morphogenetic movements, nocodazole, taxol

INTRODUCTION

During gastrulation of a vertebrate embryo, various cell movements including involution and convergence lead to formation of a gastrula with the three classically defined germ layers and a blueprint of the body plan. In amphibians and fishes, gastrulation is preceded and accompanied by epiboly - a process of vegetal expansion of the blastoderm (Keller, 1980; Trinkaus, 1951). The mechanisms that determine directions of cell migrations and forces that drive epiboly and gastrulation movements are still poorly understood. Several features make teleost embryos attractive for studies of these processes (Trinkaus et al., 1992; Kimmel, 1989). First, due to optical clarity of certain teleost embryos, cell movements and behavior can be observed in intact embryos (Trinkaus et al., 1991; Warga and Kimmel, 1990). Second, the relatively large size of *Fundulus heteroclitus* or zebrafish (*Danio rerio*) embryos allows for easy experimental manipulations (Ho, 1992). Finally, zebrafish is amenable to genetic analysis (Streisinger et al., 1981).

At the onset of epiboly in teleosts with meroblastic early cleavages, a syncytial yolk cell is capped by the blastoderm (Fig. 1A). A superficial, single-cell-thick enveloping layer (EVL) is attached by its vegetal rim to the yolk cell, and covers a mass of deep cells. Three compartments of continuous cortical cytoplasm can be distinguished in the yolk cell (Trinkaus, 1992, 1993; Long, 1984). A thin, anuclear yolk cytoplasmic layer (YCL) surrounds the bulk of the yolk mass with the vegetal pole. The external yolk syncytial layer (YSL), located between the YCL and the blastoderm rim, is a rela-
The YSN nuclei are now visible in front of the blastoderm and lead the epibolic movement. The YSN are often stretched along the animal-vegetal microtubules is visible vegetally to the blastoderm. (D) 60% epiboly (6.5h). Deep cells cover 60% of the yolk cell.

taking on a dome shape. The external YSL has contracted and exhibits densely packed YSN and a dense network of microtubules. The external YSL is partially covered by the expanding vegetally blastoderm. (C) 50% epiboly (5.2h). The blastoderm arrives at 50% of the yolk cell taking on a dome shape. The external YSL has contracted and exhibits densely packed YSN and a dense network of microtubules. The organization of microtubule networks in the YSL and YCL observed in this study is indicated by thin lines. Only part of the blastoderm is shown to reveal the morphology of the yolk cell. The relative sizes of elements are not proportional. (A) The late blastula just before the onset of epiboly (sphere stage, 4.0 h). The blastoderm, composed of the internal deep cells and the superficial enveloping layer (EVL), is positioned atop of the syncytial yolk cell. The animal surface of the yolk cell underlying the blastoderm is flat. Most of the yolk syncytial nuclei (YSN) are in the external yolk syncytial layer (external YSL) vegetal to the blastoderm. The microtubules of the external YSL form a network. The organization of microtubules in the internal YSL at this stage of development is not known. The microtubules of the anuclear yolk cytoplasmic layer (YCL) radiate from the organizing centers associated with the vegetal-most YSN and are aligned along the animal-vegetal axis. (B) 30% epiboly (4.7h). The blastoderm covers 30% of the yolk cell that bulged toward the animal pole.
tively thick belt of cytoplasm populated by the yolk syncytial nuclei (YSN). Beneath the blastoderm is the internal YSL, comprising a thinner cytoplasm also populated by the YSN. Epiboly in Fundulus begins with a contraction of the external YSL that becomes covered by the expanding blastoderm. Subsequently, the YSL and blastoderm expand vegetally, while the YCL progressively disappears (Trinkaus, 1993; Warga and Kimmel, 1990). Studies in Fundulus indicated that the epiboly of the YSL is autonomous, i.e. it can occur in the absence of the blastoderm (Betchaku and Trinkaus, 1978; Trinkaus, 1951). Moreover, vegetal expansion of the EVL during epiboly may be driven by the epibolic movements of the YSL, to which the EVL is tightly linked at its margin (Betchaku and Trinkaus, 1978). The molecular basis for the expansion of the YSL is not understood. It has been suggested that microfilaments may play a role in this process (Trinkaus, 1984). Recently, it has been proposed that epiboly is also driven by a pulling force dependent on microtubules present in the yolk cell (Strähle and Jesuthasan, 1993). However, developmental changes of the yolk cell microtubule organization and their involvement in distinct aspects of epiboly were not studied.

Here, we demonstrate that two distinct microtubule arrays exist in the cortical cytoplasm of the zebrafish yolk cell. The YCL contains an array of microtubules aligned in the direction of epiboly, extending toward the vegetal pole. Once formed, the YSL exhibits its own network of intercrossing interphase or mitotic microtubules. Our studies demonstrate that the changes in the configuration of the yolk cell microtubules are strictly correlated with epibolic movements. Analysis of epiboly in embryos treated with nocodazole and taxol indicates that microtubules are necessary and actively involved in epibolic movements of the syncytial nuclei, and only partially required for the remaining aspects of epiboly.

**MATERIALS AND METHODS**

**Reagents**

Reagents were obtained from Sigma Chemical Co. (St Louis, MO), with the following exceptions. Formaldehyde (EM grade) and Ladder-olac from LADD (Burlington, VT), glutaraldehyde from Ted Pella (Redding, CA), taxol from Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, of the National Cancer Institute (Bethesda, MD), and Hoechst 33258 from Molecular Probes (Eugene, OR). Antibodies: KMX-1, a mouse IgG monoclonal antibody from Boehringer Mannheim (Germany); DM1A a mouse IgG antibody (ICN Immunobiologicals, Costa Mesa, CA); alkaline phosphatase-conjugated anti-mouse IgG serum and Texas Red-conjugated anti-mouse IgG antibody Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Vectastain ABC Elite Mouse IgG kit was from Vector Laboratories, Inc. (Burlington, CA). The sample of a purified mouse tubulin was a generous gift of Dr B. Weinstein (MGH, Boston, MA).

**Treatment of embryos**

Wild-type AB strain zebrafish embryos were obtained by single pair crosses. Embryos were collected within the first hour after fertilization and maintained at 28.5-29.5°C in ‘egg water’ (0.03% Instant Ocean salt mix in Millipore MilipLQplus deionized water) (Westertield, 1993). From each egg lay 8-cell embryos were selected, ensuring that they differed less than 15 minutes with respect to the time of fertilization. Staging of embryos was performed as described by Kimmel et al. (1993). Developmental times are given in hours after fertilization.

Nocodazole (Methyl-5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)-carbamate was prepared as a stock solution of 10 mg/ml in 50% DMSO and diluted to concentrations of 0.5-20 µg/ml. Taxol was prepared as a 10 mM stock solution in DMSO and diluted to concentrations of 10-100 µM. In the case of the 50 and 100 µM dilutions, precipitation was observed. Therefore the actual concentrations of taxol were probably lower. Culture of control embryos in corresponding concentrations of DMSO in egg water did not have any adverse effect on development.

**Whole-mount antibody stainings**

The best preservation of microtubules and structure of the embryo were achieved with the formaldehyde-glutaraldehyde-taxol fix (FGT fix) developed for Xenopus oocytes (Gard, 1991). Embryos were fixed at room temperature or at 28.5°C for 2-4 hours. As in Xenopus (Gard, 1991), microtubules were also preserved when taxol was omitted from the FGT-fix (data not shown). Staining with the KMX-1 and DM1A antibodies was performed as described by Gard (1991) with the following modifications. Incubations with antibodies were carried out overnight in a cold room. Washes were performed at room temperature: 1× 5 minutes and 3× 30 minutes. Embryos stained with the secondary antibody conjugated to Texas Red were dehydrated and mounted in benzyl benzoate-benzyl alcohol (Gard, 1991), in bridge slides described by Warga and Kimmel (1990), and sealed with Ladder-olac. Alternatively, detection of the antigen was carried out using the Vectastain Avidin/Biotin/Horseradish peroxidase ABC Elite System according to standard procedures (Roth et al., 1989). Embryos stained with the Vectastain ABC detection system were kept in 100% glycerol. To visualize DNA, embryos stained with antibodies were washed 4-5 times for 5 to 10 minutes with deionized water, and then stained for 10 minutes with 0.2 µg/ml solutions of DAPI (4′,6-diamidino-2-phenylindole) or Hoechst 33258. Embryos stained with DNA dyes were washed once with PBS and transferred to 1% w/v p-phenylenediamine, 90% v/v glycerol, 0.1 M Tris-HCl, pH 7.5.

**Microscopy**

Observation of living embryos was performed either with a dissecting microscope Wild, or with a Zeiss Axioscop microscope using Nomarski optics. Embryos stained with fluorescent antibodies or DNA dyes were observed under epifluorescent illumination using Neofluar objectives on the Axioskop microscope, or using confocal imaging (Bio-Rad MRC 600 attached to a Zeiss Axioscop).

**RESULTS**

The origin and organization of the yolk cell microtubules during cleavage stages

Two anti-tubulin monoclonal antibodies, DM1A and KMX-1, were examined for their ability to detect zebrafish tubulins by...
western blotting (see Materials and Methods) and by whole-mount immunocytochemistry. In early cleavage-stage embryos the marginal blastomeres contacting the yolk cell maintain the cytoplasmic bridges with the YCL (Kimmel and Law, 1985a). At these stages, we observed an array of microtubules that appeared to emerge from the marginal blastomeres into the yolk cell, and extended along the animal-vegetal axis towards the vegetal pole. The YCL microtubules persisted even during synchronous mitotic divisions of the blastoderm cells (Fig. 2A,B, and data not shown).

At the 500-1000 cell stage (3h) the marginal blastomeres fuse with the yolk cell (Kimmel and Law, 1985b) forming the YSL. At this stage the syncytial nuclei were organized in a single row in the narrow external YSL (Fig. 3A) (Trinkaus, 1993; Wilson, 1889). In the late blastula embryos (3.7-4h) the enlarged external YSL usually took shape of a syncytial corona (Long, 1980; Ballard, 1973), with single nuclei in each of the vegetal protrusions of the corona (Fig. 4A). Within individual blastomeres of the late blastula embryos, microtubule arrays typical of animal cells were observed: mitotic spindles, midbodies and interphase arrays emanating from centers adjacent to cell nuclei (Fig. 3B). The yolk cell exhibited two distinct types of microtubule organization. In the external YSL microtubules radiated from centers associated with the syncytial nuclei both during mitosis (prometaphase in Fig. 3B,C) and interphase (see below) forming a network. During metasynchronous mitotic divisions of the YSN (Kane et al., 1992), astral microtubules of adjacent mitotic arrays formed in the YSL appeared to interdigitate (Fig. 3C). The YSL network of intercrossing or mitotic microtubules usually filled the entire syncytial corona (Fig. 3B,D). The YCL exhibited a distinct set of intercrossing or mitotic microtubules that extended vegetally from the centers organizing the adjacent YSN mitotic arrays interdigitate. (B,D) The YSL microtubules fill the entire external YSL, which takes the shape of a syncytial corona. (D,E) The YCL microtubules originate from the organizing centers associated with the most vegetal YSN. (F) An array of the YCL microtubules extending in the animal-vegetal direction on the lateral side of the yolk cell of the sphere-stage embryo. (G) View of the vegetal pole (vp) of the sphere stage embryo. Microtubules of the YCL converge at the vegetal pole in a concentric fashion.
Changes in the organization of the yolk cell microtubules during epiboly

Epiboly in zebrafish and Fundulus starts in the late blastula, after cessation of mitotic divisions of the YSN (Kane et al., 1992; Trinkaus, 1993). At the onset of epiboly, in Fundulus, the external YSL contracts and the YSN become densely packed (Trinkaus, 1984; 1993). Similarly in zebrafish, starting at the sphere/dome stage, the wide belt of the external YSL narrowed in the animal-vegetal direction and the syncytial nuclei became increasingly crowded. When the blastoderm...
expanding vegetally, reached 30% of yolk sphere latitude (30% epiboly), the nuclei of the external YSL concentrated near and were partially covered by the rim of the blastoderm (Fig. 4A-B'). Simultaneously, the microtubule network of the external YSL became denser (Figs 4C, 1B). By 40% epiboly usually most of the YSN were covered by the blastoderm and only a narrow belt of the external YSL with a dense microtubule network was visible below the blastoderm rim (Fig. 4D). At 50% epiboly, the blastoderm margin arrives at the equator of the embryo and deep cells engage in the gastrulation movements of involution and convergence (Kimmel and Warga, 1990). At this stage blastoderm cells almost completely covered the YSN and the associated microtubule network. The YCL microtubules emerged from below the rim of EVL cells (Figs 5E,F, 1C).

The initial stages of gastrulation are correlated with a transient cessation of epiboly by deep cells (Warga and Kimmel, 1990). Consequently, after formation of the embryonic shield, when the EVL covered approximately 65% of the yolk cell, the deep cells lagged in epibolic movements (Fig. 5A,C). At this stage, the YSN, surrounded by their microtubule network, reemerged from below the EVL rim around the circumference of the embryo and populated up to 70% of the yolk cytoplasm (Figs 5A, 1D). Some of the nuclei of the external YSL exhibited variable, elongated shapes (Fig. 5A,B). The YSL microtubules surrounded the nuclei forming basket-like arrangements (Fig. 5B,C). The expansion of the YSN and their associated microtubule network, and the apparent shortening of the YCL microtubules continued until the YSL and the blastoderm covered the entire yolk cell (data not shown).

**Organization of microtubules in the internal YSL**

The analysis described above revealed changes in the organization of microtubule arrays in the external YSL and in the YCL. To determine the microtubule configuration in the internal YSL, the blastoderm cap was removed manually from fixed embryos. Uncapped yolk cells were stained with the anti-tubulin antibody. Fig. 6 shows the microtubule configuration of the yolk cell from an embryo at 60% epiboly. As in the external YSL, microtubules of the internal YSL form a dense network surrounding the YSN. Such an organization of the internal YSL microtubules was observed from the dome stage, until the completion of epiboly.
Microtubule drugs reveal functions of the yolk cell microtubules during epiboly

To investigate a potential role of microtubules in epibolic movements, we studied the effects of the microtubule depolymerizing agent nocodazole (Lee et al., 1980), and the microtubule stabilizing agent taxol (Schiff et al., 1979), on epiboly of the YSN, YSL and blastoderm. Embryos were cultured in the presence of drugs starting at the late blastula (3.7-4 h, Fig. 3D), or 40% epiboly stages. The progress of development was monitored in treated and control embryos for the next several hours until the completion of epiboly in control embryos. First, at the time when control embryos reached 30 or 40% epiboly, treated embryos were inspected with Nomarski optics to determine whether the thick external YSL became narrow and the YSN densely packed (Trinkaus, 1993). Second, the epibolic movements during the later stages of epiboly were monitored by determining the vegetal extent of the YSN, the rim of EVL and of deep cells in live and fixed embryos. The distribution of YSN and the organization of microtubules were also assessed in embryos.

Fig. 5. Organization of yolk cell microtubules during late stages of epiboly. In all figures the animal pole is at the top. The margin of deep cells is indicated by large arrowheads, the margin of the EVL by arrows, and the vegetal extent of the external YSL by small arrowheads. Fluorescent (A,B) and Nomarski (C) images of fixed embryos with anti-tubulin and Hoechst 33258 DNA stainings. After the formation of the embryonic shield, (60% epiboly; 6.5 hours), deep cells approach 60% of the yolk sphere latitude, and the EVL rim is ahead of the deep cells margin (A,C). The YSN and their associated microtubules are again visible in front of the blastoderm (B,C). At this stage some YSN are extended along the animal-vegetal axis (A,B). The YCL microtubules appear to be continuous with the YSN microtubules (B,C). Bars, 100 µm.

Fig. 6. Organization of microtubules of the internal-YSL in embryos at 60% epiboly. To expose the internal YSL, the blastoderm was removed manually from fixed embryos which were subsequently stained with anti-tubulin antibodies and a DNA dye. The animal pole is at the top. Bars, 100 µm. (A) Fluorescent image of an embryo with anti-tubulin and Hoechst 33258 staining. Lateral view showing the rim of EVL which was not completely removed (arrow). The YSN are present in both internal YSL, and also in front of the EVL rim in the external YSL. (B) Bright-field image of a fixed embryo with anti-tubulin staining. Magnified view of a border between the internal and external YSL (i-ysl and e-ysl, respectively) which is demarcated by the residues of the EVL rim (arrow). Microtubules form a dense network in both layers. (C) Confocal image of microtubules of the internal YSL at the animal pole. A dense network of microtubules surrounds the YSN (n).
fixed and stained with anti-tubulin antibodies and a DNA dye.

(i) Nocodazole inhibits contraction of the YSL and crowding of the YSN

Treatment of the late blastula stage embryos with 5-20 µg/ml nocodazole dramatically affected the organization of microtubules, cell divisions, epiboly and gastrulation. In embryos fixed 30 minutes after addition of 10 µg/ml nocodazole, microtubule arrays of the blastoderm and yolk cells were completely disorganized (compare with Fig. 3B). (B,C) Effect of different concentrations of nocodazole on the initial phase of epiboly. In embryos treated at the sphere stage with 0.5 µg/ml nocodazole crowding of the YSN is reduced but not totally inhibited (B). The crowding of the YSN appears blocked in embryos treated with 10 µg/ml nocodazole (C).

(D) Effect of taxol on organization of microtubules and the initial phase of epiboly. 30 minutes after application of 100 µM taxol to sphere-stage embryos, no YSN are visible in front of the blastoderm rim, indicating that the crowding of the YSN took place. A very dense microtubule network is visible vegetal to the blastoderm. (E) Effect of taxol on the organization of yolk cell microtubules, 8 hours after beginning of treatment (12h of development). Big gaps and bundles of microtubules (black arrow) are apparent in the microtubule array of the YCL.

Throughout the experiment, indicating that cell divisions were inhibited (Fig. 8). Neither germ ring nor embryonic shield were detected, indicating that both involution and convergence towards the dorsal side (Warga and Kimmel, 1990) were either greatly repressed or completely stopped.

Distinct aspects of epiboly were variably impaired in the absence of microtubules. The external YSL remained a wide belt below the blastoderm ring and no crowding of the YSN was observed (Fig. 7C). Embryos treated at the sphere stage (4h) for 1h with 0.5 µg/ml nocodazole exhibited numerous long microtubules of the yolk cell (data not shown). In these embryos, in contrast to the embryos treated with higher concentrations of nocodazole, the YSN concentrated close to the blastoderm rim (Fig. 7B versus C). These observations are con-
consistent with a function of the yolk cell microtubules in the compaction of YSN and contraction of the YSL at the beginning of epiboly.

(i) Nocodazole blocks vegetal migration of the YSN but only partially inhibits epiboly of the EVL and deep cells

In the later stages of epiboly, the movements of the YSN towards the vegetal pole appeared blocked in the nocodazole-treated embryos, while the vegetal expansion of EVL and deep blastoderm cells were only partially inhibited. At 75% epiboly in control embryos, YSN covered up to 80% of the yolk sphere (Fig. 8A,D). At the same time in treated embryos, the YSN reached only 40% of the yolk sphere latitude and did not exhibit elongated shapes, characteristic to this stage of epiboly (Fig. 8F). The border between the YSL and the YCL was not easily visible in the nocodazole-treated embryos. Therefore, we could not determine whether movements of other components of the YSL were also inhibited. The margin of deep cells...
was uneven, and reached only to 40-50% of the yolk sphere latitude, almost completely covering the YSN (Fig. 8B,F). The EVL rim arrived, however, at the 55-60% of the yolk sphere latitude, and was vegetal to both the deep cells and the YSN (Figs 8B,E, 1E). The EVL and deep cells of treated embryos moved very slowly toward the vegetal pole. When control embryos were at the tail bud stage (9.5h), the EVL rim of the treated embryos was about 70% of the yolk sphere latitude, and treated embryos started to degenerate (data not shown).

(iii) Localized endocytosis can occur in the absence of microtubules
Thus, in the nocodazole-treated embryos all epibolic movements of the YSN were either severely inhibited or completely blocked. In contrast, epiboly of EVL was only partially repressed. It has been proposed that epiboly of the EVL is passive and is driven by epiboly of the surface of the YSL to which the EVL is tightly linked (Betchaku and Trinkaus, 1978). Epiboly of the surface of the YSL has been explained in part by the process of endocytosis localized during epiboly to the narrow ring of the external YSL, vegetally to the EVL rim (Betchaku and Trinkaus, 1986). To test whether the process of endocytosis is still occurring in embryos in which microtubules have been disrupted, embryos treated for 1 or 2 hours with 5 or 10 µg/ml nocodazole and control embryos were incubated in a solution of Lucifer Yellow (Betchaku and Trinkaus, 1986). Fluorescent microscopy revealed a ring of endocytic vesicles localized vegetally to the blastoderm margin in control embryos (Fig. 8G-I), as well as in nocodazole-treated embryos (Fig. 8J-L). This observation suggests that the disruption of microtubules does not equally affect all the aspects of epiboly in zebrafish embryos, but rather primarily impairs movements of the YSN.

(iv) Taxol delays all epibolic movements toward the vegetal pole
30 minutes after incubation of sphere-stage embryos (4h) in 100 µM taxol, the YSN were covered by the blastoderm, and only a belt of a dense network of the YSL microtubules was visible vegetal to the blastoderm rim (Fig. 7D). Thus the contraction of the YSL was not inhibited. In taxol-treated embryos both the YSL, and the YCL microtubule arrays had a denser appearance than in control embryos (Fig. 7D versus Fig. 4D), covered the vegetal pole completely and were more resistant to nocodazole (data not shown). The movements of the YSN, EVL and deep cells towards the vegetal pole in the later stages of epiboly were delayed. In contrast to nocodazole treatment, in embryos treated with taxol, epibolic movements of YSN, EVL and deep cells were affected to a similar extent. When control embryos reached 60% epiboly (s.d.=±6%, 48 embryos examined), treated embryos exhibited only 48% epiboly (s.d.=±6%, 46 embryos examined). At the completion of epiboly in control embryos (Fig. 9A, 9h; 96±5%, 23 embryos examined), the blastoderm of treated embryos arrived at 82±7% of the yolk sphere latitude (35 embryos examined; Fig. 9B,C). However, when control embryos were at the 2-somite stage (10.5h), the embryos treated with taxol also exhibited two somites and a forming notochord (Fig. 9D,E). This indicated that taxol treatment affected only epiboly, but not gastrulation or morphogenesis. However, most of the taxol-treated embryos have not completed epiboly at this stage. Instead, they formed abnormally shaped gastrulae, with a portion of the yolk cell protruding from contracted blastopore lips (Fig. 9D). Embryos continued development in the taxol solution for the next several days.
although up to 30% of them exhibited tail defects (data not shown).

Examination of the tubulin staining pattern of taxol-treated embryos delayed in epiboly revealed bundles of microtubules and large gaps in the microtubule arrays of the YCL and YSL (Fig. 7E). Examination of live taxol-treated embryos with Nomarski optics indicated that the yolk cortical cytoplasm exhibited apparent discontinuities or thinner regions between thicker cytoplasmic regions (Fig. 9C,F). The apparent discontinuities of the YCL observed in live embryos most likely corresponded to gaps in the microtubule array, with thick cytoplasmic regions corresponding to bundles of microtubules.

**DISCUSSION**

**Two distinct microtubule arrays of the yolk cell**

This work demonstrates that the yolk cell of the zebrafish embryo is equipped with two distinct microtubule arrays. The YSL exhibits a network of mitotic or interphase microtubules, while an array of microtubules aligned along the animal-vegetal axis exists in the YCL (Fig. 1A). The developmental changes in the organization of these microtubule arrays correlate with epibolic movements (Fig. 1A-E). We provide evidence that these microtubule arrays are actively involved in the epibolic movements of the YSN, and contribute to other aspects of epiboly.

**Yolk cell microtubules during cleavage and blastula stages**

During early cleavage stages the YCL microtubules appear to emerge from the marginal blastomeres. Most likely microtubules extend through large cytoplasmic bridges connecting these blastomeres with the YCL (Kimmel and Law, 1986a). After formation of the YSL, microtubules of the YCL appear to emanate from the centers associated with the syncytial nuclei. Since these centers also radiate mitotic spindle microtubules during the mitotic divisions of the YSN, they probably correspond to true microtubule organizing centers. We presume that when the marginal blastomeres completely fuse with the yolk cell, generating the YSL (Kimmel and Law, 1985b), both nuclei and microtubule organizing centers are introduced into the yolk cell. Additionally, the animal-vegetal YCL microtubules are likely to exhibit uniform polarity: the minus ends in the blastomeres or in the YSL with plus ends pointing toward the vegetal pole.

During the period of mitotic divisions of the YSN, the external YSL expands and the YSN spread towards the vegetal pole while maintaining a very regular distribution (Trinkaus, 1993 and this work). We found that the astral microtubules of neighboring spindles in the YSL overlap and interdigitate. A similar configuration of interdigitating astral microtubules has been reported for syncytial nuclei undergoing cortical migration during the 8th and 9th division cycles in the Drosophila embryo (Baker et al., 1993). These authors propose that microtubule-dependent forces generated by plus-end directed microtubule motors act between anti-parallel astral microtubules of adjacent spindles to push nuclei apart. These putative repulsive forces drive nuclei toward the surface (Baker et al., 1993). A similar array of forces could explain both the spreading of the YSN and their regular distribution during mitotic cycles.

**Yolk cell microtubules during epiboly**

Our analysis revealed that the changes in the organization of the yolk cell microtubules correlate with both the process of crowding of the YSN at the beginning of epiboly, and with their subsequent vegetal movements (Fig. 1). These correlations could result from the action of a yet different cellular component, such as a microfilament network (Betchaku and Trinkaus, 1978), acting on both the YSN and the microtubules, or on the entire YSL. Alternatively, the correlations could indicate that microtubule activity is required for epibolic movements. This alternative is supported by analysis of embryos treated with nocodazole. In the absence of microtubules, the YSL did not contract and the YSN did not become densely packed. Further, YSN movement toward the vegetal pole in the later stages of epiboly was greatly inhibited. Additionally, the YSN of the nocodazole-treated embryos did not exhibit elongated shapes observed during epiboly in control embryos (Fig. 1D,E). This indicated that the tension that stretches the nuclei along the animal-vegetal axis during epiboly is reduced or absent from the nocodazole-treated embryos.

Several observations argue that the inhibition of nuclear movements in the nocodazole-treated embryos results specifically from the loss of microtubule function. First, some treatments with nocodazole that led to inhibition of the nuclear movements were initiated at the sphere or at later stages of epiboly, after the cessation of mitotic divisions of the syncytial nuclei (Kane et al., 1992). Thus, observed effects are rather unlikely to be an indirect consequence of interference with proliferation of the YSN. Second, when sphere-stage embryos were treated with a low concentration of nocodazole such that disruption of microtubules was delayed, then the YSN became densely packed near the blastoderm rim. Finally, epiboly of blastoderm proceeded farther than epiboly of the YSN. We cannot exclude, however, that some aspects of inhibition of epiboly in nocodazole-treated embryos were secondary to the disruption of microtubules.

Microtubule depolymerizing drugs are known to inhibit directional movements of treated cells (Vasiliev, 1991). Loss of directional movements of deep cells could both explain the impairment of gastrulation movements (Winklbauer and Nagel, 1991), and account for the observed slow vegetal expansion of deep cells in the embryos treated with nocodazole. Epiboly of the EVL was the least affected in the absence of microtubules. It has been proposed that epiboly of the EVL is accompanied and driven in part by a process of endocytosis localized vegetally to the blastoderm rim (Betchaku and Trinkaus, 1986). We have demonstrated that localized endocytosis does occur in embryos treated with nocodazole and, thus, it could account for the observed expansion of EVL. This observation underscores the requirement for microtubule function specifically in YSN movements.

Taxol treatment experiments also support the involvement of microtubules in epiboly. In embryos treated with taxol, the vegetal movements of both the YSN and blastoderm were delayed to the same degree. The inhibition of epiboly was specific, since gastrulation and morphogenesis seemed to progress normally. In treated embryos, microtubules were
longer than those of untreated embryos, more resistant to nocodazole, and often microtubule bundles and gaps in microtubule arrays were observed. Thus, inhibition of epiboly is most likely mediated by the effect of taxol on microtubules of the yolk cell. Interphase PtK2 cells treated with taxol form long microtubule bundles. This is accompanied by the loss of centrosome-nucleated microtubules (DeBrabander et al., 1981). Therefore, the changes in the microtubule organization in the yolk cell of taxol-treated embryos are consistent with effects of taxol on interphase cells.

How might taxol delay epiboly? Stabilization of microtubules by taxol has been proposed to explain the inhibition of pronuclear movement in sea urchin embryos (Schatten et al., 1982). Since YCL microtubules become progressively shorter during epiboly, taxol stabilization could interfere with this process. Alternatively, impairment of epiboly could be only a secondary consequence of the increased microtubule stability.

**Toward a model of epiboly**

The YSL provides the major force in the vegetal spreading of the overlying blastoderm (Trinkaus, 1951; Betchaku and Trinkaus, 1978). The following mechanisms were proposed to be involved in the epiboly of the YSL by Trinkaus and his colleagues (Trinkaus, 1984). Microfilaments forming a concentric ring in the external YSL were proposed to generate a contractive force that would drive expansion of the YSL. Epiboly of the surface of the yolk cell involves expansion of the surface of the YSL with simultaneous diminishing of the surface of the YCL. The expanding surface of the YSL would tag along the EVL attached to it. The diminishing of the surface of the YCL is explained by a process of programmed endocytosis taking place in the area of the external YSL in front of the EVL. Concurrent expansion of the YSL surface would be achieved by the release of the excess of membrane stored in the internal YSL in a form of membranous microvilli.

Recently, it has been proposed that an array of YCL microtubules would aid epiboly by pulling the blastoderm toward the vegetal pole (Strähle and Jesuthasan, 1993). The taxol-induced general delay in epiboly that we observed is reminiscent of the phenotype described for embryos treated with UV or short pulses of nocodazole, when the YCL microtubules are present but somewhat disorganized (Strähle and Jesuthasan, 1993). The above treatments indicate that microtubules contribute to general epibolic movements. However, the observation of a block of YSN movements, with only partial inhibition of epiboly of the blastoderm and an unaffected localized endocytosis in the absence of microtubules, points to a more complex role of microtubules in epiboly. Therefore, we propose that the epibolic movements of the yolk syncytial nuclei are not coupled to the process of endocytosis and to epiboly of the blastoderm. Further, epibolic movements of the YSN but not of the blastoderm are absolutely dependent on microtubules.

We hypothesize that both the YSL network and the YCL microtubules contribute differently to various aspects of epiboly, and that they are primarily involved in movements of the YSN. Microtubules have been implicated in nuclear movements in a variety of organisms, based on either morphological criteria (Picket-Heaps, 1991; Lloyd et al., 1987), correlation between the organization of microtubules and nuclear movements (Baker et al., 1993), sensitivity of nuclear movements to anti-microtubule drugs (Picket-Heaps, 1991; Lutz and Inouye, 1982; Oakley and Morris, 1981), or on a genetic evidence (Huffaker et al., 1988; Morris, 1976). One possible mechanism for microtubule-mediated nuclear migration involves molecules that interact between the microtubules and a nucleus generating force to move it along microtubules (e.g. Osmani et al., 1990). Such a mechanism, involving the YSL and YCL microtubules and a plus end-directed microtubule motor, could explain epibolic movements of the YSN. Alternatively, motor molecules working between interdigitating microtubules in the YSL network would generate a pushing force similar to that used in anaphase B of mitosis, effectively expanding the YSL and spreading YSN (e.g. Nislow et al., 1992; reviewed in Saunders, 1993).

The YCL animal-vegetal microtubules would act in the expansion of the margin of the YSL and possibly in transport of cellular components localized in the external YSL. Since the cellular components of the external YSL, like microfilaments, may also be involved in epiboly; interference with the YCL microtubules could affect all epibolic movements. Motor molecules, similar to those involved in pulling the spindle pole by astral microtubules (Saunders, 1993), would pull their organizing centers located in the YSL toward the vegetal pole. Coordinate action of the YSL and YCL arrays would result in the expansion of the whole YSL, whereas action of the YSL network alone at the beginning of epiboly could account for crowding of the YSN.

Distinguishing between these models will require identification of the involved motor molecules and their functional analysis during epiboly. Additionally, it is important to understand the relationship between the microtubule and microfilament systems in this process. Since the large syncytial yolk cell proves very accessible to experimental analysis, further studies of microtubule arrays of the yolk cell in zebrafish can contribute to our understanding of the role of the cytoskeleton in vertebrate development.

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