Developmental Biology 320 (2008) 175–184

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Mesendoderm cells induce oriented cell division and invagination in the marine shrimp *Sicyonia ingentis*

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ARTICLE INFO

Article history:
Received for publication 3 January 2008
Revised 20 April 2008
Accepted 2 May 2008
Available online 20 May 2008

Keywords:
Sicyonia
Decapoda
Crustacea
Mitotic spindle
Spindle orientation
Gastrulation
Archenteron elongation

ABSTRACT

The mesendoderm (ME) cells are the two most vegetal blastomeres in the early developing embryo of the marine shrimp *Sicyonia ingentis*. These two cells enter mitotic arrest for three cycles after the 5th cell cycle (32-cell stage) and ingress into the blastocoel at the 6th cycle (62-cell stage). Circumjacent to the ingressing ME cells are nine presumptive naupliar mesoderm (PNM) cells that exhibit a predictable pattern of spindle orientation into the blastopore, followed by invagination. We examined the role of ME cells and PNM cells in gastrulation using blastomere recombinations and confocal microscopy. Removal of ME progenitors prevented gastrulation. Removal of any other blastomeres, including PNM progenitors, did not interfere with normal invagination. Altered spindle orientations occurred in blastomeres that had direct contact with one of the ME cells; one spindle pole localized to the cytoplasmic region closest to ME cell contact. In recombined embryos, this resulted in an extension of the region of ME-embryo contact. Our results show that ME cells direct the spindle orientations of their adjacent cells and are consistent with a mechanism of oriented cell division being a responsible force for archenteron elongation.

Introduction

Gastrulation is usually initiated by the invagination or ingression of blastula cells and proceeds by other morphogenetic movements such as involution, convergence and extension, and epiboly with the elongation of the archenteron or expansion of endo-mesodermal germ layers (reviewed by Keller and Davidson, 2004). Several cellular mechanisms have been proposed to account for invagination movements, including apical constriction (Lewis, 1947), cell tractoring (Burke et al., 1991), apical contractile ring model (Davidson et al., 1995), apical–basal shortening (Gustafson and Wolpert, 1995), and gel-swelling (Lane et al., 1993; reviewed by Ettensohn, 1985; Keller and Davidson, 2004). Coordinated cell shape changes are believed to be responsible for several morphogenetic events during gastrulation in *Drosophila* (Leptin and Grunewald, 1990; Leptin, 1991; Kam et al., 1991; Costa et al., 1994). Controlled changes of cell adhesion properties are responsible for ingression and migration of primary mesenchyme cells in sea urchin embryos and other embryos (reviewed by McClay, 1991). Convergent-extension during gastrulation in *Xenopus* and in sea urchins is clearly due to cell rearrangements (Keller, 1986; Wilson and Keller, 1991; Keller and Winklbauer, 1992; Hardin and Cheng, 1986).

During the latter part of the 19th century His (1874) proposed that oriented cell division functions as a force for gastrulation. Holtfreter (1943), however, discounted this hypothesis based on the observations that cell division is greatly reduced, if not discontinued, during gastrulation in several animals. Subsequent studies showed that in sea urchins, archenteron elongation was not altered in embryos treated with drugs inhibiting both karyokinesis and cytokinesis (Burke, 1980; Stephens et al., 1986; Hardin, 1987). Elongation of structures by oriented cell division is common in plants and is also found in the teloblastic cell divisions of leeches and in the posterior growth zone of crustaceans and short germ band insects (reviewed by Sander, 1976; Scholtz and Dohle, 1996; Shankland and Savage, 1997). For example, in the amphipod crustacean *Parhyale hawaiensis*, ectoteloblast and mesoteloblast stem cells undergo anterior–posterior cell divisions as growth occurs at the posterior (Browne et al., 2005). However, the molecular and biophysical details of how force is transmitted by oriented cell division are largely unknown (Keller, 2006).

Cleavage and gastrulation in the crustaceans occurs through a diversity of mechanisms (reviewed by Gerberding and Patel, 2004). Cleavage can be complete or superficial, in random or fixed patterns. In the non-Malacostracan crustaceans, gastrulation occurs by invagination and ingression or by epiboly. In the Malacostracan crustaceans, gastrulation occurs by ingression and invagination. Some levels of cell fate commitment have been demonstrated prior to or during gastrulation in crustaceans. For example, in the amphipod *P. hawaiensis*, the germ layer is likely to be specified by cytoplasmic determinants

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doi:10.1016/j.ydbio.2008.05.525

(Extavour, 2005). In the penaeoidean *Sicyonia ingentis*, vegetal determinants likely specify the mesendoderm cells, which in turn may induce the invagination of naupliar mesoderm cells and organize the anterior–posterior axes of the embryo (Hertzler et al., 1994). Different rates of cell division also correlate with different cell types in *S. ingentis*. Gastrulation begins when two mesendoderm (ME) cells arrest and ingress between the 32–62 cell stage. In the next phase, the presumptive naupliar mesoderm (PNM) cells orient their spindles into the blastopore and invaginate from the 62–122 cell stage. The PNM cells are retarded in their rate of cell division relative to the ectoderm, and it is possible the oriented cell division provides the force for invagination and extension of the PNM. A similar pattern of oriented and retarded cell division occurs in krill, where the mesodermal crown cells undergo oriented division during invagination (Taube, 1909; Alwes and Scholtz, 2004).

Using blastomere recombinations and confocal microscopy, we have attempted to address the necessity of ME cells and the original PNM cells for the initiation of invagination during shrimp gastrulation. In addition, we present data consistent with the hypothesis that oriented cell division is a contributory force for gastrulation, especially for later archenteron elongation. In normal unmanipulated embryos the cells surrounding the ME cells are prospective post-naupliar mesoderm cells, mostly of CD origin (Hertzler and Clark, 1992). In the manipulated embryos, the ancestry of cells surrounding the ME cells is altered, and to distinguish this, the immediate neighbors of ME cells in the manipulated embryos are termed as ME-circumjacent-cells or ME-adjacent-cells. We conclude that ME cells can induce cells of non-CD descent to orient their spindles while invaginating, suggesting that such cells are competent to respond to contact with ME cells and regulate their fate.

Materials and methods

Experimental materials

S. ingentis were collected off the coast of Southern California at a depth of approximately 100 m. Gravid females were transported to the Bodega Marine Laboratory and maintained in 3785-L aquaria with running sea water. Spawning was induced as described by Pillai et al. (1988). Spawning females were held over 11-cm finger bowls containing approximately 200 ml artificial sea water (ASW), prepared according to the formula of Cavanaugh (1956). The finger bowls were gently swirled for approximately 1 min post spawning to prevent eggs from aggregating or adhering to the bowls. Hatching envelopes (HE) were removed using a technique modified from Lynn et al. (1993). At 5 min post spawning, eggs were transferred to ASW containing 0.5 mM 3-amino-1,2,4-triazole (ATA-ASW) to prevent hardening of the HE. The eggs remained in ATA-ASW for approximately 50 min and were filtered through 200 μm mesh Nitex screen to remove the HE. Eggs were washed three times with ASW subsequent to HE removal.

Blastomere isolation and recombination

Zygotes lacking HE were transferred to agarose-coated (Sigma type III agarose, 0.9% in ASW) disposable 60 mm petri dishes, washed three times with ASW, and allowed to settle and develop at room temperature (20–23 °C). Blastomeres were mechanically dissociated and re-associated with a hand held capillary glass needle. ME cells were obtained by the method of Hertzler et al. (1994). For each recombination experiment, ~30 healthy looking embryos were selected using a mouth pipette at early 2-cell stage immediate before the completion of anaphase. Timing was important because cleavage process might be perturbed if the embryos were picked up too early and cells were easily dissociated within the pipette if they were picked up too late. Usually 10~20% of the embryos became unusable because one of the blastomeres was destroyed during the manipulation process. Conservatively, each experiment, with 5~6 repetitions, would have at least 20 interpretable embryos at the end point. For assessing gastrulation, 50% of remaining embryos were fixed for confocal analysis at the time when gastrulation was obvious (depending on the room temperature). The other 50% were allowed to develop into either amorphous or swimming larvae (usually overnight). For assessing spindle orientation of ME-circumjacent cells, samples were fixed immediately after ME ingression. Only samples containing distinct mitotic spindles in the PNM cells or ME-circumjacent cells were used. We were unable to determine a specific pattern of centrosome movement prior to mitotic spindle formation. However, we did notice that the centrosomes of each ME-adjacent cell were not located close to the ME contact prior to prophase.

Immunofluorescent staining and images

Samples were fixed on ice for 1 h in 90% methanol containing 50 mM EGTA and 10 mM HEPES at pH 6.0 (modified from Harris, 1987). Fixed samples were infiltrated with 10 mM Tris-buffered saline (TBS) for 30 min and embedded in 1% agarose (Sigma, type IX; in TBS). Agarose containing samples were cut into small cubes, reimmersed in cold methanol, and stored at 4 °C.

For immunofluorescent staining, stored samples were washed three times with TBS, treated with 1% bovine serum albumin (BSA) in TBS for 1.5 h, incubated with anti-α-tubulin antibody (Accurate Chem. Co., MAS077B; diluted five times with BSA-TBS) for 1.5 h. Samples were washed three times with TBS, treated with BSA-TBS for 1.5 h, and incubated for 2 h with either rhodamine or fluorescein-conjugated anti-rat IgG antibodies (Jackson Immuno Research) diluted 50 times with BSA-TBS. Finally, samples were washed three times with TBS, dehydrated in an ethanol series, and cleared with methyl salicylate (Summers et al., 1991). Samples were observed, and images collected, using an Olympus IMT-2 inverted microscope coupled to a BioRad MRC 600 laser scanning confocal system equipped with a krypton/argon laser at the Bodega Marine Laboratory. Digital images were assembled into plates using Adobe Photoshop CS2. In some cases existing figure panel labels were erased and replaced with ones of different font and size, but only if they appeared over the background.

Results

S. ingentis development

Fig. 1 shows normal development of *S. ingentis* through hatching of the nauplius larva (Hertzler and Clark, 1992; Hertzler et al., 1994). The fertilized egg enters first mitosis at about 55 min post-spawning (Fig. 1A), and first cleavage produces two blastomeres designated AB and CD (Fig. 1B). Blastomere AB is closer to the polar bodies and is slightly, but noticeably, smaller than CD. The second cleavage produces A, B, C, and D blastomeres; D is slightly larger than the others while A, B, and C are indistinguishable in size (Figs. 1C, D). After three more cleavage cycles (Fig. 1C–H) the embryo becomes a hollow blastula containing 32 blastomeres (Figs. 1I, J). At this stage, a pair of D descendants, the mesendoderm (ME) cells, enter mitotic arrest resulting in a blastula which contains 62 blastomeres (Figs. 1K, L). At the end of the 62-cell stage, the mitotically-arrested ME cells start to ingress. Surrounding the ME cells are 9 (or 8 in other penaeoidean species) presumptive naupliar mesoderm (PNM) cells (Kajishima, 1951; Zilch, 1979; Hertzler and Clark, 1992; Hertzler, 2005). Hertzler and Clark (1992) demonstrated, in *S. ingentis*, that in a majority of cases, four of the PNM cells are descended from the C blastomere and five from the D. The PNM cells exhibit retarded mitosis and altered orientations of their mitotic spindles; one pole of each spindle becomes situated in the cortical region proximal to one of the ME cells. As a consequence, PNM spindles are aligned radially around the blastopore circumference and are directed inward toward the ingressed ME cells (Fig. 1K; Hertzler and Clark, 1992; Hertzler, 2005). The orientation of PNM spindles dictates a common cleavage plane in the PNM cells that is perpendicular to the long axis of the future archenteron. The ME cells resume division after a three cell cycle delay (Fig. 1N). The embryo completes germ layer formation, segmentation and organogenesis (Fig. 1O), hatching as a nauplius larva in about one day (Fig. 1P).

4-cell stage blastomere rearrangements

If a normal spatial arrangement of blastomere contacts at the 4-cell stage is necessary for ME cell ingression or PNM cell invagination, then rearrangement into other patterns may block these processes. The effects of early blastomere arrangement on ME ingression and PNM spindle orientations were examined by dissociating blastomeres at the 2-cell stage, allowing each to divide, and re-associating them at the 4-cell stage into three different conformations. First, A/B blastomeres were positioned to contact the C blastomere of a C/D pair (see Figs. 1C, D for blastomere designations) so that the axis of the A/B division was perpendicular to that of the C/D division, forming a T shaped conformation (Fig. 2A). In this case, neither the A nor B blastomere was in direct contact with D. Second, A/B blastomeres were rotated 180° and re-associated with C/D blastomeres (Fig. 2B). In